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**INTERACTION OF HUMAN BLOOD PLATELETS,
LYMPHOCYTES AND MONOCYTES WITH
VASCULAR LAMININ ISOFORMS**

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Cover picture. Confocal microscopy picture of laminin $\alpha 5$ chain expression in basement membrane of high endothelial venuels (HEV).

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This work is dedicated to Miserach Kebede, Samuel,
Edlawit, Yohannes Gezahegn, Muluwork Hundie,
Gorfu Tolla, Kebede Belachew and Bogalech H/Gebrihael.

ABSTRACT

Inflammatory and immune responses play a fundamental role in both health and disease, and leukocytes are important actors in these processes. Migration of the leukocytes to sites of injury or inflammation is a crucial component of innate and adaptive immunity. It is currently accepted that leukocyte extravasation is a multistep process. However, a later step in this cascade, namely the interaction of leukocytes with components of extracellular matrices (ECM), such as the vascular endothelial basement membrane (BM) and the interstitium ECM, is poorly understood. There is also limited information concerning the role of vascular BM proteins in hemostasis and/or thrombosis. In this thesis, the interaction of blood platelets, lymphocytes and monocytes with vascular BM components, particularly the endothelial laminin isoforms, has been studied.

Laminins (Lms), major components of all BMs, are a family of heterotrimeric molecules, each composed of α -, β -, and γ -chains. To date, five α -, three β -, and three γ -chains have been identified that associate to form at least 15 Lm isoforms. Lms regulate various cellular functions, such as adhesion, motility, differentiation and proliferation through various integrin and nonintegrin receptors. Lm-411 ($\alpha 4\beta 1\gamma 1$, laminin-8) and Lm-511 ($\alpha 5\beta 1\gamma 1$, laminin-10) are major Lm isoforms of vascular endothelial BMs. These BM components may participate in leukocyte extravasation and, following vascular injury, contribute to hemostasis and/or thrombosis when exposed to circulating platelets.

First, commercially available placenta laminin preparations, often used in functional studies, were characterized. These preparations differed from one another and consisted of highly fragmented proteins, a mixture of laminin isoforms, and/or contaminating fibronectin. They also exhibited major functional differences between batches. In a following study, megakaryocytic cells were found to synthesize and platelets to secrete heterotrimeric $\alpha 5$ -Lms. Lm-511 strongly promoted platelet adhesion, but not activation, *via* $\alpha 6\beta 1$ integrin. Thereafter, the pivotal role of $\alpha 5$ -Lm(s), expressed by high endothelial venules, in promoting adhesion and migration of blood lymphocytes *via* $\alpha 6\beta 1$ integrin was demonstrated. Lm-511 was also able to co-stimulated T cell proliferation, and stimulated blood lymphocytes secreted both $\alpha 4$ - and $\alpha 5$ -laminins. The lymph node cell number in Lm $\alpha 4$ -deficient mice compared to wild type did not differ significantly. Finally, Lm-411 and Lm-511 were found to mediate adhesion and chemokine-induced migration of monocytes *via* $\alpha M\beta 2$ and $\alpha X\beta 2$ integrins. Isolated Lm $\gamma 1$, but not Lm $\beta 1$, chain reproduced the effect of the Lm heterotrimers. Moreover, endogenous $\alpha 4$ -Lm(s) mediated chemokine-induced, $\alpha M\beta 2$ - and $\alpha X\beta 2$ -integrin-dependent monocyte migration on an albumin substrate.

Altogether, the present studies illustrate the differential effects of laminin isoforms in the biology of platelets, lymphocytes and monocytes, and their potential contribution to hemostasis, and to the generation of immune and inflammatory responses.

Key words: Vascular laminin isoforms, extracellular matrix, integrins, cell adhesion, cell migration, high endothelial venules, lymphocytes, monocytes, platelets, leukocyte extravasation

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals in the text:

- I. Zenebech Wondimu, **Gezahegn Gorfu**, Tomoyuki Kawataki, Sergei Smirnov, Peter Yurchenco, Karl Tryggvason and Manuel Patarroyo.
Characterization of commercial laminin preparations from human placenta in comparison to recombinant laminins 2 ($\alpha 2\beta 1\gamma 1$), 8 ($\alpha 4\beta 1\gamma 1$) and 10 ($\alpha 5\beta 1\gamma 1$).
Matrix Biol. 25:89-93 (2006).
- II. Ayele Nigatu, Wondossen Sime, **Gezahegn Gorfu**, Tarekegn Geberhiwot, Ingegerd Andurén, Sulev Ingerpuu, Masayuki Doi, Karl Tryggvason, Paul Hjemdahl and Manuel Patarroyo.
Megakaryocytic cells synthesize and platelets secrete $\alpha 5$ -laminins, and the endothelial laminin isoform laminin 10 ($\alpha 5\beta 1\gamma 1$) strongly promotes adhesion but not activation of platelets.
Thromb. Haemost. 95:85-93 (2006).
- III. **Gezahegn Gorfu**, Ismo Virtanen, Mika Hukkanen, Veli-Pekka Lehto, Patricia Rousselle, Ellinor Kenne, Lennart Lindbom, Sergei Smirnov, Peter Yurchenco, Randall Kramer, Karl Tryggvason and Manuel Patarroyo.
Laminin isoforms of high endothelial venules and reticular fibers of lymphoid tissue and predominant role of $\alpha 5$ -laminin (s) in adhesion, migration and co-stimulation of blood lymphocytes.
Manuscript.
- IV. **Gezahegn Gorfu**, Zenebech Wondimu, Patricia Rousselle, Sirpa Salo, Timo Pikkarainen, Anna Domogatskaya, Karl Tryggvason and Manuel Patarroyo.
Vascular laminin isoforms Lm-411 (laminin 8) and Lm-511 (laminin 10) and their Lm γ 1 chain promote migration of blood monocytes *via* $\alpha M\beta 2$ and $\alpha X\beta 2$ integrins, and Lm-332 (laminin 5) inhibits the cell motility.
Manuscript.

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LIST OF ABBREVIATIONS

Ag	Antigen
APC	Antigen presenting cell
BM	Basement membrane
CC	Chemokine
CD	Cluster of differentiation antigen
DC	Dendritic cells
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra acetic acid
EHS	Engelbreth-Holm Swarm
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FN	Fibronectin
HEV	High endothelial venule
HSA	Human serum albumin
ICAM	Intercellular adhesion molecule
Int	Integrin
kDa	kilodalton
KO	Knockout
LFA-1	Lymphocyte function associated antigen-1
Lm	Laminin
LN	Lymph nodes
mAbs	Monoclonal antibodies
MAdCAM-1	Mucosal addressin cell adhesion molecule
NK	Natural killer
PBMC	Peripheral blood mononuclear cells
pFN	Plasma fibronectin
PMN	Polymorphonuclear leukocyte
PNAd	Peripheral node addressins
PSGL	P-selectin glycoprotein ligand
RF	Reticular fiber
RGD	Arg-Gly-Asp
rh	Recombinant human
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfated polyacrylamide gel electrophoresis
TCR	T cell receptor
TPA	Tetradecanoylphorbol acetate
VLA	Very late antigen
VCAM	Vascular cell adhesion molecule
WB	Western blotting

1 INTRODUCTION

The immune system is an organization of cells, tissues and molecules whose primary function is to protect the body from foreign entities or antigens. The major players in this defense system are the leukocytes. Leukocytes are a heterogeneous population of cells consisting of granulocytes (neutrophils, eosinophils, basophils) and monocytes/macrophages, which play active roles in antigen nonspecific innate immunity, as well as lymphocytes, which are involved in the antigen-specific branch of the immune system. Cells of the innate immune system constitute an antigen nonspecific pool and respond to inflammation-induced traffic cues. They are the first effector cells to arrive to the inflammatory sites in large numbers in unidirectional migration from blood into tissues, and provide early defense until the activated lymphocytes of the adaptive immune response can play their role. Naïve lymphocytes are poorly responsive to inflammatory signals and are largely excluded from extralymphoid tissues. They rather migrate to secondary lymphoid tissues in search of their cognate peptide-MHC complex. The recirculation of naive lymphocytes from blood to the secondary lymphoid organs, and then, through lymph, back to blood increases the chance that rare antigen-specific cells meet their specific antigen. Once activated, effector lymphocytes localize to extralymphoid tissues where they help to coordinate immune and inflammatory responses in the periphery.

Migration of leukocytes to sites of injury or inflammation is thus a crucial component of innate and adaptive immunity. As a result of these defense mechanisms, diseased tissues may heal and continue their normal function. On the other hand, uncontrolled or dysregulated leukocyte recruitment can lead to excessive inflammation which can itself cause tissue damage and disease. It is therefore quite essential to understand the migration patterns of leukocytes and the molecular interactions that lead them to their target. This is not only for a better understanding of leukocyte biology but, perhaps even more importantly, because it provides insights for the development of novel therapies for inhibiting leukocyte trafficking in inflammatory diseases.

It is currently accepted that leukocyte extravasation is a multistep process. While a great deal has been learned about the early steps involving leukocyte interactions with the vascular endothelial cells, little is known about interaction of leukocytes with the underlying extracellular matrix (ECM) such as the vascular basement membrane (BM) and the interstitial ECM. There is also limited evidence about the role of ECM proteins of the blood vessel BM during hemostasis and/or thrombosis, as opposed to interstitial ECM proteins like fibrillar collagen. An interesting concept frequently ignored is that blood vessel BMs are quite different in their molecular composition compared to widely investigated interstitial ECM components and may have differential influence in leukocyte and platelet phenotype and responsiveness both within physiological and pathological scenarios. Recent work from our research group regarding molecular characterization and functional implications of BM proteins has focused on Lm-411 (Laminin-8, $\alpha4\beta1\gamma1$) in platelets, monocytes, lymphocytes and neutrophils. This thesis intends to further extend and explore the expression, recognition and usage of Lm-511 (Laminin-10, $\alpha5\beta1\gamma1$) in platelets, lymphocytes and monocytes. Lm-411 and Lm-511 are major Lm isoforms of vascular endothelial BMs. These BM components could participate in leukocyte extravasation and, following vascular injury, may contribute to hemostasis and/or thrombosis when exposed to circulating platelets.

In this thesis, I will outline the main aspects of interaction of blood lymphocytes, monocytes and platelets with subendothelial basement membrane components, particularly vascular laminin isoforms, and their potential contribution to the generation of immune and inflammatory responses and hemostasis/thrombosis.

1.1 EXTRACELLULAR MATRIX

The ECM is a supramolecular network that is usually present throughout the extracellular space within tissues, and is assembled from collagens, glycoproteins and proteoglycans synthesized and secreted by cells in tissues. At the ultrastructural level two basic domains of the ECM are identified: the basement membrane and the interstitial matrix. The former contains collagen type IV, laminins, perlecan, and nidogens and the latter contains mainly collagen types I and III, fibronectin, vitronectin, tenascins, dermatan sulphate and chondroitin sulphate (Timpl 1996; Bosman and Stamenkovic 2003). As a consequence of their molecular composition these two domains of ECM have different three-dimensional architecture. While the BM has condensed supramolecular structure, the interstitial matrix has a loose and fibrillar network structure.

ECM not only provides structural support, organization and orientation to tissues but also provide instructive signals that influences many cell behaviours such as cell growth, migration, proliferation, adhesion and differentiation. These effects are mediated *via* integrins, dystroglycans, cell surface proteoglycans and discoidin domain receptors. The interaction of these receptors with the ECM leads to activation of intracellular signaling pathways and rearrangement of the cytoskeleton followed by changes of cell behaviours. The ECM, particularly the glycosaminoglycan side chains of proteoglycans, has an additional way to elicit signals on the cell surface by acting as a binding, storing and presenting site of growth factors, cytokine and chemokines (Timpl 1996; Gustafsson and Fassler 2000; Bosman and Stamenkovic 2003). The ECM is remodeled constantly by family of matrix metalloproteases and release cleaved products and stored factors (Kalluri 2003), thereby regulating cell biological functions.

1.2 BASEMENT MEMBRANES

Basement membranes (BMs) are cell-associated, thin, sheet-like highly specialized extracellular matrices covering the basal structures of all epithelia and endothelial cells and surrounding muscles, fat, and peripheral nerve cells (Timpl 1996; Timpl and Brown 1996; Kalluri 2003). All BMs contain laminins, type IV collagen, perlecan, and nidogens as major components. Additional minor components include type XV and XVIII collagen, agrin, fibulins, and growth factors, which are present in tissue-specific basement membranes. The current model of BM structures postulates that the basic framework of BM is formed from two independent and distinct networks of type IV collagen and laminins. The interaction between these two networks is facilitated by nidogen/entactin. Nidogen also interacts with perlecan, which in turn binds to the C-terminal portion of the laminin and to type IV collagen, thereby stabilizing the basement membrane network. In addition, fibulins have been shown to incorporate into BM, which is mediated by its strong binding interaction with laminins and nidogens. They have also binding sites for tropoelastin, fibrillin, fibronectin and proteoglycans. Fibulins may thus participate in diverse supramolecular structures, involving cross-linking of basement membrane components and the interaction of the basement membrane with the underlying interstitial ECM (Timpl and Brown 1996; Miosge 2001; Kalluri 2003; Timpl et al. 2003; Sasaki et al. 2004). The first step in the assembly of a BM is the polymerization

of the laminin component just after their secretion from adjacent cells. The formation of Lm polymerization is guided by anchorage through cell membrane lipid leaflet (sulfated glycolipids), and its signal transduction through integrin and DG receptor interactions. Then, the polymerized laminin binds to entactin/nidogen, the latter serving as a link to the collagen IV polymer (Miner and Yurchenco 2004).

When visualized with light microscopy, matrix molecules in the region of epithelial BM or so-called BM zone have been localized as a linear staining pattern. However, structural details of basement membranes require an electron microscopic approach. Thus, it is only an ultrastructural method, eg. immunogold histochemistry, which can precisely confirm whether a given matrix molecule is a true basement membrane component (Miosge 2001). The ultrastructure of BM varies with their locations, where the typical BM (gastrointestinal tract or skin BM) displays three layers: lamina densa, which is electron-dense sheet of about 15-125 nm thick sandwiched between lamina lucida, which is adjacent to cells and lamina fibroreticularis, which fixes to the connective tissue. There are also double (alveolar) and multilayered (Reichert) BMs (Inoue 1989; Miosge 2001; Quondamatteo 2002).

As thin condensed matrices, BM provides structural support for cells and divides the cells of the parenchymal tissues from the interstitial matrix. The constitutive proteins, particularly laminins, also provide distinct spatial and molecular information that control cell behaviors including adhesion, polarization, differentiation, migration and survival as well as expression of specific genes. BM also provides the cells with biological information indirectly by virtue of its highly glycosylated nature of components such as laminin and heparin sulphate proteoglycans, which in turn serves as a reservoir of signaling molecules, including growth factors, cytokines, chemokines and proteases (Aumailley and Smyth 1998).

1.3 THE LAMININS

Laminins (Lms), one of the major components of basement membranes, are a growing family of large and multidomain heterotrimeric glycoprotein composed of α , β , and γ subunits. To date, 5 α chains (α 1- α 5), 3 β chains (β 1- β 3), and 3 γ chains (γ 1- γ 3) have been identified and sequenced, assembling to form at least 15 different laminin isoforms. The Lm α chains are expressed in a cell- and tissue-specific manner and possess several receptor binding sites, and are differentially recognized by cells (Libby et al. 2000; Tunggal et al. 2000; Patarroyo et al. 2002). Lms regulate various cellular functions, such as adhesion, motility, growth, differentiation, proliferation and apoptosis, through a variety of integrin and nonintegrin receptors.

1.3.1 Laminin nomenclature

The name laminin was originally given to a large glycoprotein, now called Lm-111, purified from the components of the stroma of the Engelbreth-Holm Swarm (EHS) tumor (Timpl et al. 1979) and the extracellular deposits of murine parietal endoderm PYS cells (Chung et al. 1977; Chung et al. 1979). This name was initially intended to designate a single laminin and it was also the only laminin isoform known for several years, but it now refers to a family of proteins. Originally the subunits of Lm-111/Lm-1 were referred to as A1, B1, and B2. Since then, several homologs of these chains were isolated, and a unifying systematic nomenclature was introduced in 1994 (Burgeson et al. 1994). The A1 subunit was then called α 1, and the B1 subunit termed β 1, and the B2 termed γ 1. Laminin isoforms got then a given Arabic number according to their order of discovery (eg, laminin-5, α 3 β 3 γ 2). This nomenclature was rational and widely used. However, now with 16 Lm trimers from mouse and human including the major ones and their variants, it proved difficult to memorize the chain

composition of a laminin isoform. As a result, a new simplified nomenclature was proposed in 2005 (Aumailley et al. 2005), in which laminin heterotrimers are named based on their chain composition, i.e. numbering of their α , β and γ chains (Table 1). Thus, the new nomenclature given for instance for laminin-10 is Lm-511, showing straightforward its chain composition of $\alpha 5$, $\beta 1$, and $\gamma 1$. The new nomenclature has been used throughout this thesis.

Chain composition	Previous names (Burgeson et al., 1994)	Current names (Aumailley, et al., 2005)	References
$\alpha 1\beta 1\gamma 1$	Laminin 1	Lm-111	Chung et al., 1979; Timpl et al., 1979
$\alpha 2\beta 1\gamma 1$	Laminin 2	Lm-211	Engvall et al., 1990
$\alpha 1\beta 2\gamma 1$	Laminin 3	Lm-121	Green et al., 1992
$\alpha 2\beta 2\gamma 1$	Laminin 4	Lm-221	Engvall et al., 1990
$\alpha 3\beta 3\gamma 2$	Laminin 5	Lm-332	Carter et al., 1991; Rousselle et al., 1991; Verrando et al., 1991
$\alpha 3\beta 1\gamma 1$	Laminin 6	Lm-311	Marinkovich et al., 1992
$\alpha 3\beta 2\gamma 1$	Laminin 7	Lm-321	Champlaud et al., 1996
$\alpha 4\beta 1\gamma 1$	Laminin 8	Lm-411	Miner et al., 1997
$\alpha 4\beta 2\gamma 1$	Laminin 9	Lm-421	Miner et al., 1997
$\alpha 5\beta 1\gamma 1$	Laminin 10	Lm-511	Miner et al., 1997
$\alpha 5\beta 2\gamma 1$	Laminin 11	Lm-521	Miner et al., 1997
$\alpha 2\beta 1\gamma 3$	Laminin 12	Lm-213	Koch et al. 1999
$\alpha 3\beta 2\gamma 3$	Laminin 13	Lm-323	Liby et al., 2000
$\alpha 4\beta 1\gamma 3$	Laminin 14	Lm-413	Liby et al., 2000
$\alpha 5\beta 2\gamma 3$	Laminin 15	Lm-523	Liby et al., 2000

Table 1: Nomenclature of Laminins

1.3.2 Laminin structure and domains

The individual polypeptide chains are distinct gene products differing in their sequence and modular organization, which evolved from duplication and reshuffling of an ancestral gene during evolution. Additional laminin variants also occur because of alternative splicing of some gene transcripts and proteolytic processing (Aumailley and Smyth 1998). The α chain genes of laminins show greater sequence divergence between chains and between species than either the β or γ chain genes. For example, comparison of the mouse $\alpha 1$, $\alpha 4$ and $\beta 1$ chain sequence with that of a human sequence revealed a sequence identity of 76%, 88.1% and 93% respectively (Tryggvason 1993; Iivanainen, Kortessmaa et al. 1997).

Lms are large (400 to 900 KDa) heterotrimeric glycoproteins whose α , β and γ chains associate by forming a triple-helical coiled-coil and are linked by disulfide bridges. Lm α chains can be full-length ($\alpha 1$, $\alpha 2$, $\alpha 3B$ and $\alpha 5$, 400 kDa) or truncated

($\alpha3A$ and $\alpha4$, 200 kDa) and their various domains have 20-60% amino acid sequence identity. The overall structure of Lms is a cruciform, T or Y-shaped molecules with two or three short arms and one long arm as visualized by electron microscopy. Short arms are composed of parts of one chain, whereas the long arm is formed by parts of each of the three chains (Fig. 1). Lm α , β , and γ chains share homologous structures that include globular domains (domains IV-called L4 in α and γ chains, LF in β chain- and VI (LN)), rod-like domains containing EGF-like repeats known as laminin-like EGF or LE repeats (domains III/LEb and V/LEa), and a 600 - residue domain II/I (LCC) that oligomerizes into a rod-like coiled-coil structure forming the long arm of laminin (Aumailley et al. 2005). Depending on the type of associated chains and on the structural differences in the short arms, laminins are divided into classical laminins, which have full complements of domain in each of the short arms; long laminins, which have elongated α short arm and are larger than the classical full sized laminins; topless laminins, which have extensive truncations in the α subunit short arms, but complete β and γ -subunit short arms; and truncated laminin, with truncations in all three short arms. Laminin $\alpha5$ has an exceptionally long N-terminus, due to the presence of a larger than usual IVb domain, and additional EGF repeats in domains V, IIIb, and IIIa. Sequence analysis reveals that this chain is more similar in domain structure to the only known *Drosophila* laminin α chain than it is to mammalian $\alpha1-4$ (Miner et al. 1995), suggesting that the $\alpha5$ chain is most likely to be the ancestral chain of all vertebrates α chains. All α chains share a unique C-terminal globular (G) domain, which consists of five tandem Lm G domain-like modules (LG1-5). The modules LG1-LG3 are connected to the LG4-LG5 moduels by a linker domain and are clearly visible in rotary shadowed images of isolated molecules.

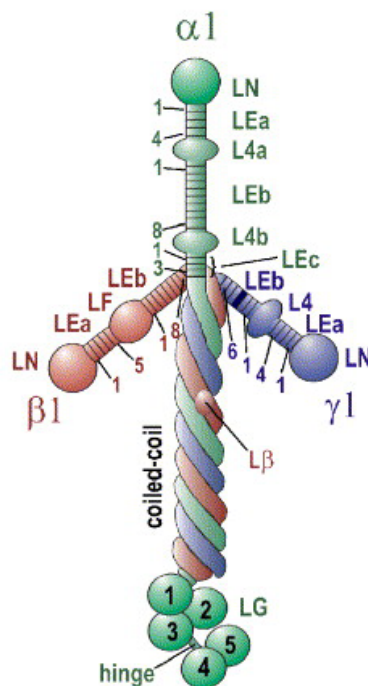


Fig. 1. Lm-111, as a schematic representation of laminin with the three chains and domain organization (Adapted from Aumailley et al., 2005).

The biologically relevant functions of Lms are thought to derive from coordinated contributions from different domains of all three subunits (Colognato and Yurchenco 2000). For instance, most N-terminal LN modules (IV and VI) of Lm chains have been shown to be essential for self-polymerization into large non-covalent networks, and therefore for the integrity of basement membranes. Domain III in the $\gamma1$ chain

harbors the binding site for nidogen, which in turn binds type IV collagen. The major cell binding domains, including for integrins, are located in the C-terminal G domain of the α chains (Colognato and Yurchenco 2000). Most of these modules are also found in several other ECM proteins, such as agrin, perlecan and proteoglycans, and thus for a ECM protein to be categorized as a laminin chain, sequence data of α , β or γ chain are necessary. In addition, α -helical coiled-coil domain is decisive in defining as Lm family (Ekblom and Timpl 1996).

1.3.3 Biosynthesis of laminins

Lms are synthesized and secreted by numerous cell types of solid tissues to which BM are attached as well as by hematopoietic and blood cells. The biosynthesis of Lm isoforms, as most other secreted glycoprotein destined for export out of the cell, ensues the general pathway of synthesis described as follows: after synthesis occurs in the endoplasmic reticulum, the vesicles containing Lm molecule are transferred through the cytoplasm up to the cell membrane, then fusion occurs, followed by the opening of the vesicle toward the exterior of the cell which leads to the expulsion of its content into the extracellular area and thereby the formation of a Lm mesh ensues (Aumailley and Smyth 1998; Geberhiwot et al. 1999).

Lm α , β and γ chains are distinct gene products. Once the polypeptide chains are translated, the three chains are destined to form a homogenous trimer associate in the correct proportions. Biosynthetic studies with Lm-111 have shown that the process of trimer formation is subdivided into two steps: the cells first form a stable coiled-coil intermediate dimer comprising of one β and one γ chain, and subsequently this dimer interact with the α chain to form a triple coiled-coil (Aumailley and Smyth 1998). The α chain is transcribed separately from the other chains. Moreover, the α chain mRNA is translated at a slower rate than the β 1 and γ 1 chains, and is the rate-limiting step for the association and secretion of the trimeric molecules. This suggests that the secretion of the β 1 and γ 1-chains requires the presence of all three chains, while the α 1 chain can be secreted as a monomer subunit and drives secretion of the heterotrimers (Yurchenco et al. 1997). Similarly, $\alpha\beta\gamma$ trimers could be immunoprecipitated from the cell lysates of platelets (Geberhiwot et al. 1999). It is likely that the same scenario is true for other Lm isoform formation in which the addition of α chains is the rate-limiting step of Lm isoform biosynthesis, because in those cases only heterotrimers can be secreted.

All Lm with the exception of α 1-Lms are physiologically cleaved in tissues by proteolysis, often in the G-domain or the short arms due to posttranslational and possibly to postsecretion, modifications (Colognato and Yurchenco 2000). Interestingly, while some cleavages are accompanied by the release of the fragment from the 'parent' molecule as the case of Lm-332 (Aumailley et al. 2003) and Lm α 4 (Talts, Sasaki et al. 2000), others do not, for example in Lm-211 the 300 and 80 KDa cleaved components remain together by virtue of its non-covalent association (Smirnov et al. 2002). Alternative splicing also occurs in a few Lm chains (α 3 and α 4) but this has not been shown for Lm-111 chains (Colognato and Yurchenco 2000; Hayashi et al. 2002). The α 4 chain can be also modified by attachment of glycosaminoglycans (Sasaki et al. 2001; Kortessmaa et al. 2002), but the biological significance of this glycosylation is largely unknown.

1.3.4 Laminin isoforms and tissue distribution

The distribution patterns of Lm isoforms in BMs are complex. Lm isoforms show tissue- and/or developmental stage-specific expression and play important roles in the

structures and functions of BMs (Miner and Yurchenco 2004). From immunohistochemical distribution of laminins in tissues, some general conclusions can be made. Lms are mainly, but not exclusively, found in BMs, and all BMs contain at least one and some contain two or three distinct α chains. The main patterns of α chain expression are established embryonically, but some individual BMs change in α chain composition as development proceeds, as demonstrated in developing kidney (Miner et al. 1997). Lm α 1 and α 5 chains are crucial for early embryonic development and organogenesis, whereas Lm α 2, α 3, and α 4 chains are essential for postnatal development. While α 4 and α 5 chains exhibit broadest tissue distribution, with α 5 being the most widely distributed α chain in adult BMs, α 1 show most restricted expression (Miner and Yurchenco 2004). Mesenchymal cells produce Lm α 2 and α 4, epithelial cells produce Lm α 3 and α 5, and both epithelial and mesenchymal cells produce Lm α 1 (Ekblom et al., 1998). While β 1 chain is more widely spread, β 2-chain has more restricted distribution and occurs in the alveolar BM, glomerular mesangium and neuromuscular junction. While synaptic BM contains β 2 chain, extrasynaptic BM contains β 1. Patton et al. further demonstrated that, whereas motor nerves grow freely in Lm-111 and -211, their growth stops in Lm-521, suggesting differential response of motor axons to β 1 and β 2 chains (Patton et al. 1997). β 3- and γ 2 chains almost always occur together and are found in Lm-332, which is present in the BMs of stratified epithelia as part of the hemidesmosomal complex. The γ 1 chain is the most ubiquitous Lm chain, being present in 10 out of 15 known Lm isoforms. Lm γ 2 chain has been shown to generate two splice variants, termed γ 2 and γ 2*. The latter form has a more restricted expression pattern than the former one (Airenne et al. 2000). Lm- γ 3 is the most recently described Lm chain (Koch et al., 1999) and is a component of Lms 213/323/423/523. It is the only Lm chain reported not to be found in basement membranes in most epithelia tissues examined, but instead present at the apical surface (Koch et al. 1999; Libby et al. 2000). However, this assumption has been challenged recently by Gersdorff and co-workers (Gersdorff et al. 2005) reporting that Lm γ 3 chain is expressed exclusively in the basement membranes. More recently, Yan and Cheng indicated a "shifting" of γ 3 chain from the basal compartment to the apical surface during development (Yan and Cheng 2006), suggesting spatial and temporal change in the expression pattern of laminin chain in a given tissue during development.

The cellular expression pattern and developmental changes of Lm chains may reflect the functions of the different Lm isoforms, making it essential to know the cellular origin of the laminin chains whether it is epithelial, mesenchymal or both. Moreover, understanding the profile of Lm isoforms present in a given BM entails whether compensation or coregulation of specific Lm chains occurs in mutant mice (Miner and Yurchenco 2004). For instance, whereas Lm α 4 chain is upregulated in the Lm α 2 chain deficient muscle (Patton et al. 1997), Lm β 1 chain is upregulated in the glomerular BM of Lm β 2 chain deficient kidneys (Noakes et al. 1995). In another scenario, ectopic deposition of other Lm chains is reported in some BM, like in α 5 deficient mice BM (Miner et al. 1997; Miner et al. 1998). Whether such a compensation/coregulation holds functional *in vivo* is poorly known. Recent work, however, has shown that Lm α 1 chain transgene functionally compensate for Lm α 2 chain deficiency (Gawlik et al. 2004), thereby reducing muscular dystrophy. In the following section I briefly describe Lm isoforms based on their α subunits.

α 1 containing Laminins (Lm-111, -121)

Lm-111 is also termed the 'classical', 'prototype' or EHS laminin. It was the first identified laminin and is the first laminin appearing during early embryogenesis (Ekblom et al. 2003). It is expressed mainly by developing epithelial cells during organogenesis in mouse and human embryos (Ekblom et al. 1998; Colognato and Yurchenco 2000). It is not detected, or present minimally, in blood vessels, muscle, fat or nerves of embryos and adults. In adult tissues of mouse and human, remarkably similar 'restricted' expression patterns in some epithelial basement membranes have been demonstrated (Falk et al. 1999; Virtanen et al. 2000; Ekblom et al. 2003). Lm-111 has been extensively studied because of its availability after isolation from the mouse transplantable EHS tumor. Lm-121, also known as s-laminin, is present in the crypt region of the intestine. It has also been found in human placenta (Champlaud et al. 2000).

α 2 containing laminins (Lm-211, -221, -213)

Lm-211, also known as merosin, is predominantly expressed in tissues derived from mesoderm such as in basement membrane of skeletal and cardiac muscles, and it is also expressed in placental membranes and peripheral nerves (Ehrig et al. 1990). Lm-211 is also one of the major Lms during muscle formation and it is shown to participate in the development of muscle from the early stages (Tiger et al. 1997; Gullberg et al. 1999). Lm-221, or s-merosin, is expressed in the synaptic cleft of NMJ, and Lm-211/221 induce clustering of the acetylcholine receptors (Burkin et al. 2000). Lm-213 is reported not to be found in basement membranes in most epithelia tissues examined, but is rather present at the apical surface. It was first purified from human placental chorionic villi (Koch et al. 1999).

α 3 containing laminins (Lm-332, -311, -321)

Lm-332, previously termed kallinin, nicein (or BM 600), epiligrin and ladsin, has a unique function and structure as compared with other Lms: it is the major adhesive component of the epidermal BM. All α 3, β 3, and γ 2 chains are truncated in the short arms (N-terminal) and the β 3 and γ 2 chains are found mainly in Lm-332. The alternative splicing of the α 3 leads to the synthesis of two variants of Lm-332: a truncated-variant (Lm-3A32) and a full-sized variant (Lm-3B32). The α 3 and γ 2 chains of Lm-332 are also proteolytically processed after secretion. The 190-kDa, precursor α 3 chain is cleaved to the 160-kDa mature form, and partly to the 145-kDa form. The γ 2 chain is processed from the 150-kDa precursor form to the 105-kDa mature form. Lm-332 is secreted into the BM by basal cells of stratified and translational epithelia, and by certain glandular epithelia with predominantly secretory or protective functions. It forms the anchoring filaments of the hemidesmosome and is therefore involved in the adhesion of epithelial cells to the basement membrane (Rousselle et al. 1991). Laminins-3A32 and -3B32 along with Lm-311 and Lm-321 are isoforms specifically found in the BM of the dermal-epidermal junction (DEJ) (Aumailley et al. 2003). This site also contains LM-511 or Lm-521. Lm-332 exists not only in the BM of the skin but it is also expressed at a high level in other epithelial tissues, including adult lung, placenta and fetal kidney, and moderately in thymus, spleen, testis and ovary (Mizushima et al. 1996; Mizushima, Koshikawa et al. 1998). It has been implicated in migration of tumor cells in various types of carcinomas. Because of its overexpression in invasive lesions, γ 2 chain has been suggested as sensitive marker for the detection of early invasive carcinoma lesions (Miyazaki 2006). The expression of the Lm γ 2 chain monomer has been also associated with invasiveness of tumor cells (Kagesato et al. 2001). Lm-311 or k-laminin, has been found in the cultures of keratinocytes and

squamous carcinoma, and in tissue extracts from the skin and the amnion. Its physiological roles are mostly unknown. Lm-321 or ks-laminin, has been extracted from human amnion, and is concentrated with Lm-332 in dermal-epidermal junctional BM (Champlaud et al. 1996). Lm-323 is still poorly understood.

α 4 containing laminins (Lm-411, -421, -413)

Lm-411 is a major vascular endothelial basement membrane component. It is synthesized by cells of mesodermal origin, such as endothelial cells, adipocytes, muscle cells and others (Miner et al. 1997; Petajaniemi et al. 2002). Developmentally regulated expression of α 4-laminins occurs in skeletal muscle, walls of vessels and intestinal crypts. It is also relatively abundant in bone marrow (Siler et al. 2000; Gu et al., 1999; Gu et al., 2003). Lm-411 has an important function in vascular and blood cell biology, where it is shown to play an important role in cell migration during development, wound healing, and angiogenesis (Miner et al. 1997; Gonzalez et al. 2002; Patarroyo et al. 2002; Thyboll et al. 2002), as well as for blood monocyte, lymphocyte and neutrophil adhesion, migration and extravasation (Pedraza et al. 2000; Geberhiwot et al. 2001; Wondimu et al. 2004). Over-expression of Lm-411 is also detected in glioma (Fujita et al. 2006). Proteolytic processing of the LG region of Lm α 4 chain is reflected in absence of α 4LG4-5 from tissues in vivo (Talts et al. 2000). This is evident in cultured endothelia cells and in Lm-411 purified from platelets which contain a 180-200 kDa α 4 chain (Geberhiwot et al. 1999; Petajaniemi et al. 2002). Alternative splicing of α 4 transcripts also cause additional heterogeneity of α 4 chains of Lm-411 (Hayashi et al. 2002). Lm-421 is present in the synaptic BM but absent in the majority of muscle fiber BM and has been shown to be critical for synaptic differentiation as demonstrated in Lm β 2 deficient mice (Patton et al. 2001). Lm-411/421 is synthesized by rat glomerular mesangial cells and Lm-421 is required for their migration (Berfield et al. 2006). Lm-423 is present together with Lm-513 in the retinal matrix and CNS (Libby et al. 2000).

α 5 containing laminins (Lm-511, -521, 513)

Lm-511 is a major laminin isoform widely expressed in adult tissues including placenta, heart, lung, blood vessels, skeletal muscle, kidney, and pancreas. It constitutes Lm isoforms of DEJ and mouse hippocampus (Aumailley and Rousselle 1999; Indyk et al. 2003). Lm-511 displays a predominant cell adhesive property for hematopoietic cell lines, encephalitogenic T-cell lines, malignant lymphoid cells, a subpopulation of thymocytes, and bone marrow progenitor cells (Siler et al. 2000; Sixt et al. 2001; Kutlesa et al. 2002; Gu et al. 2003; Spessotto et al. 2003). It also shows a predominant migration promoting activity for bone marrow progenitor cells and malignant lymphoid cells (Gu et al. 2003; Spessotto et al. 2003). Lm-511 also seems to be essential for hair morphogenesis (Li et al. 2003). In addition, it is ubiquitously expressed in carcinoma (Tani et al. 1999). The potential involvement of Lm-511 in tumor invasion and metastasis has been recently suggested (Zamurs et al. 2003), with the identification of this laminin in the leading invasion and autocrine secretion products of some colon cancers. Lm-521 is found in the renal glomerular BM in kidney, in the neuromuscular synaptic cleft in skeletal muscle and in other tissues such as placenta and lung (Miner and Patton 1999; Champlaud et al. 2000). Lm-523 is present together with Lm-423 in the retinal matrix and CNS and expressed at sites of photoreceptor development where they play roles in photoreceptor production, stability, and synaptic organization (Libby et al. 2000).

It is worth mentioning that the recent reevaluation of mAb 4C7, which was previously thought to recognize the Lm α 1 chain, as Lm α 5 specific paved the way to unravel the expression and function of Lm-511 *in vivo* (Tiger et al. 1997). The human

and mouse Lm α 5 chain migrate as 350 kDa band in SDS-PAGE compared to the 400 kDa band of Lm α 1 chain. Despite the 50 amino acid residue longer peptide in the α 5 chain compared to the α 1 chain, as deduced from the complete human cDNA sequence, its apparent molecular weight appears lower to the α 1 chain. It is currently unknown whether this indicates that either alternative splicing or proteolytic processing accounts for the apparent shortening of the α 5 chain (Champlaud et al. 2000).

1.3.5 Vascular laminin isoforms

The studies of vascular BM Lm chain distributions in cultured mammalian endothelial cells, murine bone marrow, fetal and adult human blood vessels and developing murine endothelia have identified that vascular endothelium expresses two laminin α chains, α 4 and α 5 (Sorokin et al. 1994; Frieser et al. 1997; Miner et al. 1997). These chains have been discovered relatively recently and were first identified as cDNAs by molecular cloning (Richards et al. 1994; Iivanainen et al. 1995; Miner et al. 1995). By generating specific antibodies, Miner et al. identified in 1997 four novel laminin heterotrimers: Lm-411, Lm-421, Lm-511, and Lm-521 (Miner et al. 1997). Vascular endothelial BM consists primarily of Lm-411 and Lm-511, with some additional contributions of Lm-421 and Lm-521 (Sorokin et al. 1994; Frieser et al. 1997; Miner et al. 1997; Sixt et al. 2001). The former two isoforms are the major vascular endothelial laminin isoforms expressed at sites of vascular injury and leukocyte extravasation *in vivo* (Sixt et al. 2001). Whereas Lm-511 is abundantly synthesized by ECs in adult tissues and constitutes the major self-polymerizing Lm, Lm-411 appears preferentially synthesized by ECs in embryonic development and is predominant over Lm-511 during this time. Both Lm isoform expression also overlap in some vascular beds during development. Because of its topless structure, Lm-411 fails to self-polymerize to form BM (Hallmann et al. 2005). The phenotype of Lm- α 4 chain knockout mice exhibits hemorrhages around many vessels in embryos (Thyboll et al. 2002), but this phenotype is rescued by Lm- α 5 expression in vascular BM within 2-3 wk postnatally. These knockout mice in a lung tumor model show increased metastasis (Zhou et al. 2004). Lm α 5 genetic deletion leads to death late in embryogenesis that does not progress beyond embryonic day 16.5, before Lm α 5 appears in endothelial BM (Miner et al. 1998), which is probably due to defects in the placental as well as to the fetal vasculature.

The α 3-containing Lms are not localized to most adult vascular compartments. However, they may be found in fetal blood vessels and in capillaries and larger vessels of lymph nodes, tonsils, thymus and spleen (Kallunki et al. 1992; Gerecke et al. 1994; Ljubimov et al. 1995; Jaspars et al. 1996; Miner et al. 1997; Mizushima et al. 1998). α 2-containing Lms (Lm-211 and Lm-221) have a weak expression in blood vessels, but rather they accumulate in the surrounding pericyte and smooth muscle layer of larger arteries (Glukhova et al. 1993; Durbeej et al. 1996). α 1-containing isoforms are scarcely expressed during embryonic vasculogenesis (Glukhova, Koteliansky et al. 1993; Durbeej, Fecker et al. 1996; Sorokin, Pausch et al. 1997), whereas in the adult vascular tissues they are expressed in capillaries and smaller vessels in brain but are absent from the vascular endothelial BMs (Falk et al. 1999; Virtanen et al. 2000).

1.3.6 Biological functions of laminins

The interaction of Lms with cells are essential for basement membrane formation and for other diverse biological activities such as cell adhesion, migration,

differentiation, cell polarity, survival, neurite outgrowth, tumor metastasis, and angiogenesis (Kleinman et al. 2001; Patarroyo et al. 2002). Lm is the only BM component that is shown to be critical for BM formation, suggesting that laminins precisely and dynamically regulate development, tissue formation and maintenance. Five Lm α chains are tissue –and /or developmental stage specifically expressed and the specificity of the α chains critically contributes to Lm isoform-specific functions (Miner and Yurchenco 2004).

Mouse knockouts and human disease gene studies have demonstrated that Lm chains have specific functions *in vivo* in accordance with their tissue distribution. Thus, deletion of mouse Lm $\alpha 1$ chain results in premature death (Miner and Yurchenco 2004). Deleting mouse Lm $\alpha 2$ chain can be lethal soon after birth, with evidence of muscular dystrophy and peripheral neuropathy (Li et al., 2003). In the human, mutations of the gene encoding this chain induce congenital muscular dystrophy (McGowan and Marinkovich 2000). Deleting mouse Lm $\alpha 3$ can be lethal and induces epidermolysis bullosa (Li et al. 2003). A mutation in any of the three chains present in Lm-332 in human cause junctional epidermolysis bullosa gravis (McGowan and Marinkovich 2000), often with a fatal consequence. Mice and humans lacking Lm $\beta 2$ exhibit congenital nephrotic syndrome, as well as severe developmental defects at the neuromuscular junction (Miner et al. 2006). Deletion of Lm $\gamma 1$ chain results in very early embryonic lethality through an arrest of blastocyst differentiation (Smyth et al. 1999), because the absence of Lm $\gamma 1$ chain precludes the formation of Lm trimers and of a BM, which are crucial for early development of embryos.

1.4 CELLULAR RECEPTORS FOR LAMININS

The interaction of cells with Lms, which leads to different cellular responses, are mediated by cellular receptors specifically recognizing laminins such as integrins, α -dystroglycan, syndecans, and lutheran blood group glycoproteins, Table 2 (Patarroyo et al. 2002).

α chains	Cellular receptors
$\alpha 1$	Integrin $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$, α -dystroglycan; syndecan
$\alpha 2$	Integrin $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$, α -dystroglycan; syndecan
$\alpha 3$	Integrin $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$; syndecan
$\alpha 4$	Integrin $\alpha 6\beta 1$; $\alpha 7\beta 1$; syndecan
$\alpha 5$	Integrin $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$, $\alpha 7\beta 1$, $\alpha v\beta 3$; α -dystroglycan; syndecan; lutheran

Table 2: Receptors for Laminin α chains

1.4.1 Integrins

Integrins are type I transmembrane adhesion molecules containing noncovalently associated α and β subunits. They are implicated in various functions such as cell to

cell interactions, and as linkers of extracellular matrix to cytoskeleton. So far 18 different integrin α subunits and eight different β subunits have been reported, forming at 24 $\alpha\beta$ heterodimers. These integrins have distinct functions depending on which cells they are expressed by, the extracellular matrix /ligand they interact with, the mechanism of their activation upon ligand binding, the type of cytoskeletal components to which they bind to, and the signalling pathways they activate within cells (Hynes 1992). All integrins share a common heterodimeric structural organization. Half of all α subunits (α L, α M, α X, α D, α 1, α 2, α 10, α 11 and α E) contain a distal N-terminal extracellular additional "inserted" (I) or A domain of approximately 200 amino acids residues, which serves as the principal ligand-binding site. Integrin recognition sequences have been also identified from many ECM proteins, such as the RGD (Arg-Gly-Asp) sequence. Integrins containing the α 4, α 5, α 8, α IIb or α V subunits can bind to ECM components containing RGD motifs, such as fibronectin and vitronectin (Ruoslahti and Pierschbacher 1987; Ruoslahti 2003). Laminins also contain functional and cryptic/inaccessible RGD sequences in Lm α 5 and Lm α 1 chains respectively (Aumailley, Gerl et al. 1990; Sasaki and Timpl 2001).

Integrins are divided into subfamilies based on their distinct β subunits, as β 1, β 2, β 3 and so on. Each subfamily has distinct structural and functional characteristics. The β 1 integrins, or very late antigens (VLA), are mainly involved in mediating cell adhesion to ECM components. The β 2 integrins are exclusively restricted to leucocytes, and are mainly involved in cell to cell interactions. The four member of β 2 integrin family includes α L β 2 (CD11a/CD18, LFA-1), α M β 2 (CD11b/CD18, Mac-1), α X β 2 (CD11c/CD18, p150.95) and α D β 2 (CD11d/CD18). α L β 2 is expressed on all leukocytes and mainly used for lymphocyte adhesion and migration. α M β 2 is restricted to mature neutrophils, monocytes, macrophages, DCs and NK cells, and is involved in migration of these cells. α X β 2 is also expressed on neutrophils, monocytes, NK cells, DCs, tissue macrophages, and subsets of of chronically activated lymphocytes (Hynes 1992). α D β 2 is selectively expressed by a macrophage subpopulation.

Integrins are usually found in an inactive state favouring that cells do not bind inappropriately. They need to be activated in order to bind to their ligands by either avidity modulation that clusters the integrins in the plane of membrane increasing their density or affinity modulation that lead to a conformational change in the integrin chains. Different intercellular signals, such as lipids, antigens, cytokines and chemokines can lead to a conformational change in the integrin structure (inside-out-signalling), thereby assuming an open active structure and ligand-binding. Alternatively, engagement of integrins with specific ligands or cross-linking of integrins can delivers outside-in signals that triggers intracellular transduction cascades (outside-in-signalling), leading to different cellular responses such as leukocyte activation upon adhesion, leukocyte differentiation, actin polymerization and eosinophil degranulation (Ley and Reutershan 2006; Luo and Springer 2006).

1.4.2 Laminin-binding integrins

Integrins, the most characterized Lm receptors, are involved in the various biological activities of Lm. To date, at least 10 integrins (α 1 β 1, α 2 β 1, α 3 β 1, α 6 β 1, α 7 β 1, α 9 β 1, α 6 β 4, α V β 3, α V β 5, α V β 8) have been implicated in binding to various Lm isoforms (Belkin and Stepp 2000; Patarroyo et al. 2002). Several of these Lm-binding integrins mediate attachment to additional ligands; α 1 β 1 and α 2 β 1 are primarily collagen receptors; whereas α v-containing integrins and α 9 β 1 mediate attachment of cells to other matrix molecules. However α 3 β 1, α 6 β 1, α 7 β 1 and α 6 β 4

have been shown to be the major Lm-binding integrins, often called ‘classical’ or ‘laminin-specific’ integrins (Belkin and Stepp 2000). Lms are the only known ligand for $\alpha7\beta1$ and $\alpha6\beta4$. All the three α subunits of these Lm-specific integrins are the most closely related integrin α subunits, are posttranslationally cleaved into light and heavy chains held together by disulfide bonds and lack I (inserted) domain. The $\alpha6$ and $\alpha7$ subunits are more homologous to each other than to the $\alpha3$ subunit and have splice variants, $\alpha6X1\beta1$ and $\alpha6X1X2\beta1$, and $\alpha7X1\beta1$ and $\alpha7X2\beta1$ (Brown 2000).

In an elegant series of studies, Sekiguchi and coworkers used either intact or recombinant integrins combined with purified Lm isoforms and demonstrated that the isoforms of both integrins and Lms differ in their binding specificities and affinities (Kikkawa et al. 2000; Nishiuchi et al. 2003; Nishiuchi et al. 2006), summarized in table 3.

	$\alpha3\beta1$	$\alpha6\beta1$	$\alpha6\beta4$	$\alpha7X1\beta1$	$\alpha7X2\beta1$
Lm-111	-	++	-	+	++++
Lm-211/221	+	+	-	++	+++
Lm-332	++	+++	++	-	-
Lm-411	-	+	-	+	-
Lm-511	+++	++++	++	++++	+

-, No specific binding; +, mild avidity; ++, moderate avidity; +++, higher binding avidity; +++++, highest avidity

Table 3. Integrin-laminin binding specificity (summarized based on Nishiuchi et al., 2006).

1.4.3 Active sites in laminins

A number of biologically active sites (cellular receptor binding domains) in the Lm isoforms have been identified by using proteolytic fragments, recombinant proteins, and synthetic peptides. The Lm α chains are considered to be the functionally active portion of the heterotrimers as they carry the major active peptides/cellular receptor binding domains. These domains are localized in the G domain and the VI domain of the laminin α chains and play a critical role in binding to cell surface receptors in a peptide- and cell type-specific manner (Timpl et al. 2000; Suzuki et al. 2005). In the $\alpha1$ chain, the major cell binding domain corresponds to the two proteolytic fragments, E8 and E3. E8 consists of LG1-3 module of the $\alpha1$ chain and the adjacent C-terminal part of the triple-stranded helix, whereas E3 consists of LG4-5 module respectively. While E8 binds to integrins $\alpha6\beta1$, $\alpha7\beta1$ and, maybe, $\alpha6\beta4$ (Sonnenberg et al. 1990; Kramer et al. 1991; Lee et al. 1992; Suzuki et al. 2005), E3 binds to heparin/syndecan and α -dystroglycan, and the LG4 module contains both binding activities (Hoffman, Nomizu et al. 1998; Talts, Andac et al. 1999). In the $\alpha2$ chain G domain, the integrins $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha7\beta1$ bind to the LG1-3 module (Talts et al. 1999; Smirnov et al. 2002). Heparin and α -dystroglycan interact with both the LG1-3 and LG4-5 modules of the $\alpha2$ chain (Talts et al. 1998; Smirnov et al. 2002; Wizemann et al. 2003). In the $\alpha3$ chain G domain, integrins $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$ bind to the LG1-3 module (Hirosaki et al. 2000; Shang et al. 2001). On the contrary, heparin and syndecan-1, -2, and -4 binds to the LG4 module (Utani et al. 2001)(Utani et al., 2001; Okamoto et al., 2003). In the $\alpha4$ chain G

domain, the integrin $\alpha 6\beta 1$ binds to LG1–3 module (Talts et al. 2000), and heparin and syndecan-2 and -4 bind to the LG4 module (Matsuura et al. 2004; Yamashita et al. 2004). In the $\alpha 5$ chain G domain, the integrins $\alpha 3\beta 1$ and $\alpha 6\beta 1$, and lutheran, another laminin $\alpha 5$ chain specific receptor, bind to the LG1–3 module (Kikkawa et al. 2002; Yu and Talts 2003), and heparin and α -dystroglycan bind to the LG4 module (Nielsen et al. 2000; Yu and Talts 2003; Ido et al. 2004). The other major active site on Lm α chains, Domain VI, is found within the N-terminus of four α chains, $\alpha 1$, $\alpha 2$, $\alpha 3B$, and $\alpha 5$. Whereas the former two α chains bind to $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (Cognato et al. 1997), the latter two binds to $\alpha 3\beta 1$ integrin (Nielsen and Yamada 2001; Kariya et al. 2004). In addition, all the four chains contain heparin-binding activity (Cognato et al. 1997; Nielsen and Yamada 2001; Kariya et al. 2004). Lm $\alpha 5$ carries exposed RGD-cell binding sites in the domain IVa and adjacent EGF-like repeats of the NH₂-terminal portion of the chain, and bind to the integrins $\alpha v\beta 3$ -, $\alpha v\beta 5$ -, and $\alpha 5\beta 1$ (Sasaki and Timpl 2001; Hallmann et al. 2005). Mouse Lm $\alpha 1$ chain also contains a cryptic binding site with a RGD sequence, potentially exposed during proteolysis, but this does not represent a major cell binding site (Aumailley et al. 1990).

The complexity in the structural basis for integrin recognition of Lms have been highlighted by the fact that a cell binding site located within the LG modules requires correct folding of the LG and, in addition, the C-terminus of the coiled coil, as suggested above for E8 fragment of Lm-111 (Aumailley and Smyth 1998; Cognato and Yurchenco 2000). Recently, Ido et al. mapped the epitope of the function-blocking mAb 4C7, which inhibits binding of integrin $\alpha 6\beta 1$, to the LG1 module of the $\alpha 5$ chain (Ido et al. 2006). They demonstrated that this putative integrin recognition site requires the LG2–3 modules and the preceding coiled-coil domain to stabilize the active conformation. These findings explain the difficulties to ascribe the cell-binding site at the aminoacid level.

In spite of this fact, the $\alpha 1$ chain of Lm-111 has been most extensively characterized and contains several sites at the peptide level that mediate various biological responses (Aumailley et al. 1990; Kanemoto et al. 1990; Kibbey et al. 1994; Corcoran et al. 1995; Khan and Falcone 1997; Nomizu et al. 1998; Khan and Falcone 2000). It also contains cryptic domains with different biological activity from the intact Lm-111 molecule (Khan and Falcone 2000). The cryptic SIKVAV sequence of $\alpha 1$ chain from the linker region, which is between the coiled-coil region and the globular domain, unlike its intact Lm-111, induces expression of gelatinase B (gelB/MMP-9) by monocytes/macrophages (Corcoran et al. 1995; Khan and Falcone 2000). However the role of this cryptic domain *in vivo* is unclear because of restricted epithelial expression of Lm-111. In an effort to identify biologically active sites from other Lm α chains, Adair-Kirk et al recently demonstrated that a synthetic homologue laminin $\alpha 5$ chain peptide, AQARSAASKVKVSMKF, has the capacity to induce MMP-9 release by neutrophils and that it is chemotactic for inflammatory cells *in vitro* and *in vivo* (Adair-Kirk et al. 2003), unlike its intact chain and the corresponding $\alpha 3$ peptide. These authors further demonstrated a role for this $\alpha 5$ chain synthetic peptide in orchestrating immune response by inducing expression of several cytokines, including TNF- α at injury sites (Adair-Kirk et al. 2005). This $\alpha 5$ peptide is derived from the corresponding region of Lm $\alpha 1$ that contains the cryptic SIKVAV sequence.

The proteolytic processing of LG modules by either endogenous or external proteolysis modulates laminin function in a cell- and tissue specific manner. For example, furin cleavage of LG3 of the $\alpha 2$ chain G domain, without (Nguyen, Gil et al. 2000) releasing the fragment, is required for for clustering of acetylcholine

receptor, which contributes to neuromuscular junction formation together with agrin (Smirnov et al. 2002), whereas the $\alpha 3$ and $\alpha 4$ chains G domain are processed in the linker regions between LG3 and LG4, releasing LG4-5 from the parent molecule. Plasmin promotes the cleavage of the LG4-5 fragment of the $\alpha 3$ chain (Goldfinger et al. 2000), converting the precursor LN-332 from a migration ligand at wound edges to an anchorage ligand in hemidesmosomes by interacting with integrin $\alpha 6\beta 4$ (Nguyen et al. 2000).

1.5 LAMININ PURIFICATION

Lms have been previously isolated from EHS murine sarcoma (Timpl et al. 1979), mouse embryonal carcinoma cultures (Chung et al. 1977; Chung et al. 1979), Lewis rat yolk sac tumor (Wewer et al. 1983), *Drosophila melanogaster* (Fessler et al. 1987; Montell and Goodman 1988), sea urchin (McCarthy et al. 1987) and human placenta (Ohno et al. 1983; Wewer et al. 1983; Ohno et al. 1986).

Biochemical and structure-function studies with Lms depend on the availability of sufficient quantities of highly purified materials. However, Lms represent only a small portion of the total protein in tissues, have low solubilities and show tendency to aggregate. These characteristics have made it difficult to handle with the traditional biochemical methods and have hindered studies related to function of Lm isoforms. Most *in vitro* studies on structure and function of Lms were performed on Lm-111, which could be readily isolated from Engelbreth-Holm-Swarm murine tumor cells (Timpl et al. 1979). However, no human homologue of the mouse tumor is presently available for large-scale purification of Lm-111 or other Lm isoforms. Instead, human Lms are frequently prepared from placenta by various methods, such as using proteolytic digestion or EDTA and/or salt extraction and subsequent affinity chromatography (Wewer et al. 1983; Ehrig et al. 1990). Lm isoforms have been also purified from cell culture conditioned media using an immunoaffinity step along with size-exclusion chromatography (Church and Aplin 1998; Amano et al. 1999; Kikkawa et al. 2000; Pouliot et al. 2000; Zamurs et al. 2003). It is important to note that such a strategy using a particular Lm chain-specific mAb in immunoaffinity does not necessarily mean that the final purified product will be a single Lm isoform. Moreover, these purification methods from placenta and cell culture are accompanied with low recovery and purity problems.

The recent development in recombinant technology has enabled a source of pure Lms in larger quantities, despite their large subunit size and disulfide-bonded structure. Of greater advantage was the establishment of mammalian expression systems to produce recombinant human Lms (rhLm), such as rhLm-411, rhLm-511, rhLm-332 and rhLm-211 (Kortessmaa et al. 2000; Doi et al. 2002; Kariya et al. 2002; Smirnov et al. 2002) and, more recently, recombinant mouse Lm-111 and Lm-121 (Mascarenhas et al. 2005). rhLm preparations provide intact, pure and native trimeric proteins to study the biological role of intact laminin molecules, and also facilitates studies related to Lm domain and epitope analyses. Furthermore, the rLms are indistinguishable from the natural Lms in their protein composition and biological activity. The use of recombinant laminins, rather than the commercial placental laminin preparations, have circumvented the problems associated with the latter one which are highly truncated, a mixture of laminin isoforms and contaminated with other matrix protein (Sixt et al. 2001; Wondimu et al. 2006). Moreover, exposure of masked receptor binding sites caused by pepsin proteolytic digestion in the latter preparation can lead to erroneous results, which are not fully comparable to native Lms (Sixt et al. 2001; Vainionpaa et al. 2006).

1.6 PLATELETS

1.6.1 Platelet function

Platelets are discoid, anucleate cytoplasmic fragments derived from megakaryocytes in the bone marrow, which are released into the circulation, where they remain for 10 days. They are either consumed in thrombus formation or destroyed in the spleen. Platelets provide a first line of defence following endothelial damage, forming platelet-rich thrombus that seals off the vessel wall thereby playing an important role in haemostasis. However inappropriate formation of thrombi in pathological conditions may precipitate to thrombosis, myocardial infarction and stroke. Platelets also have additional roles in wound healing and repair, activation of inflammatory and immune responses and metastasis (Andrews and Berndt 2004; Weyrich and Zimmerman 2004; Zarbock et al. 2006). Recently, platelets have been recognized to retain a small but functionally significant amount of constitutive megakaryocyte-derived mRNA as well as the proteins and molecular machinery necessary for translation. Thus, they can synthesize biologically relevant proteins in response to physiological stimuli (Weyrich and Zimmerman 2004).

1.6.2 Platelet secretion

Platelets secrete biologically active molecules from their granules that influence widely different biological functions such as cell adhesion, cell aggregation, chemotaxis, cell survival and proliferation, coagulation and proteolysis. Platelets contain three types of secretory organelles—lysosomes, alpha and dense granules. While alpha and dense granules are found only in megakaryocytes and platelets, lysosomes are ubiquitous. Alpha granules are the largest and most abundant secretory granules in platelets containing adhesion molecules, coagulation factors, cellular mitogens, growth factors, chemokines, protease inhibitors and other proteins. These molecules include platelet-specific (β -thromboglobulin and platelet factor IV), platelet-selective molecules (coagulation factor V, thrombospondin, P-selectin, and von Willebrand factor) and molecules not synthesized by platelets (fibrinogen). Dense granules contain small non-protein cell activating agents such as adenosine diphosphate (ADP), serotonin, Ca^{2+} , and pyrophosphate. Lysosomes contain glycosidase, proteases, and cationic proteins with bactericidal activity (Rendu and Brohard-Bohn 2001).

1.6.3 Platelet interactions with vascular ECM

Damage to vascular integrity results in adhesion of platelets to exposed subendothelial ECM, triggering platelet activation and thrombus formation. The current model of this process suggests that fibrillar collagen is the most thrombogenic constituent of subendothelial ECM as it acts as both adhesion substrate and strong activator of platelets. Platelet adhesion to circulating vWf through GP Ib-V-IX receptor mediates platelet tethering at arterial rates of flow. This is followed by platelet activation mediated by interaction of collagen with GPVI receptor leading to activation of $\alpha_2\beta_1$ (collagen receptor) and $\alpha_{IIb}\beta_3$ (GPIIb/IIIa, fibrinogen receptor) integrins, resulting in stable adhesion to the respective ligands (Table 4). Subsequently, platelets spread and further strengthen the adhesion, forming a platelet monolayer surface for the recruitment of additional platelets (Ruggeri 1997; Nieswandt and Watson 2003; Andrews and Berndt 2004). Stimuli originating from the initial adhesive interaction with exposed collagen and from agonists released or generated at the site of injury, such as thrombin, ADP, epinephrine, and thromboxane A₂, activate the platelets and lead to a change in platelet morphology. This platelet

activation enhances the adhesive and procoagulant properties of platelets tethered to a lesion or circulating in the proximity by providing stimulus for release of platelet-derived growth factors, adhesion molecules (vWF and P-selectin) and coagulation factors from intracellular granules, activation of adjacent platelets, and conformational changes in the platelet $\alpha_{IIb}\beta_3$ integrin. The interaction of activated platelet $\alpha_{IIb}\beta_3$ receptor with its soluble adhesive ligands such as fibrinogen and vWf mediates the formation of platelet aggregation, resulting in the formation of a platelet-rich thrombus (Ruggeri 2002; Steinhubl and Moliterno 2005).

Phase	Substrate/ligand	Receptor
Platelet tethering and adhesion	vWF	GP Ib-V-IX
	Collagen	$\alpha_2\beta_1$ (GP Ia/IIa)
	Fibrinogen/fibrin	$\alpha_{IIb}\beta_3$ (GP IIb/IIIa)
	Fibronectin	$\alpha_5\beta_1$
	Laminin	$\alpha_6\beta_1$
Platelet activation	Thrombin	PAR1, PAR4 GPIb-V-IX
	ADP	P2Y ₁ , P2Y ₁₂
	TXA ₂	TP
	Epinephrine	α_2 -Adrenoreceptor
Platelet aggregation	Fibrinogen, vWF, fibronectin	$\alpha_{IIb}\beta_3$ (activated)

ADP = adenosine diphosphate; **GP** = glycoprotein; **PAR** = protease-activated receptor; **TP** = TXA₂ receptor; **TXA₂** = thromboxane A₂; **vWF** = von Willebrand factor

Table 4. Receptors and ligands involved in platelet thrombus formation (Adapted from Steinhubl and Moliterno 2005).

Depending on the depth of injury, platelets can be exposed to non-fibrillar collagen ECM constituents, such as Lm, type IV collagen, fibronectin, fibulins and perlecan. Furthermore, platelets express $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_v\beta_3$ integrins that support platelet adhesion to fibronectin, laminin, as well as osteopontin and vitronectin, respectively (Bennett 2005). However, there is limited evidence about the role of these ECM constituents during hemostasis and/or thrombosis, as opposed to fibrillar collagen. This thesis work addresses the interaction of platelets with vascular endothelial Lm isoforms, Lm-411 and Lm-511, which are exposed during superficial disruption of vascular integrity.

1.7 LYMPHOCYTES

Lymphocytes are at the center of the adaptive immune response and immunological memory. The major types of lymphocytes include bursa (bone marrow)-derived lymphocytes (B-cells), thymus-derived lymphocytes (T-cells) and natural killer (NK) cells. They develop from lymphoid progenitors in the bone marrow where B cells mature, whereas T-cell precursors emigrate to the thymus for selection and maturation. The antigen specificity of lymphocytes occurs early in the development of the cells, before exposure to antigen, and is the result of somatic

DNA recombination, a mechanism known as gene rearrangement. The repertoire of lymphocytes produced by this mechanism consists of 10^8 T-cell receptors and of 10^{10} antibody specificities. After release from their primary lymphoid organs, naïve lymphocytes circulate between bloodstream and lymphatic system, and the secondary lymphoid tissues. Human blood lymphocytes constitute 5-15% B cells, 70% T cells and 15% NK cells. Two morphological types of resting lymphocytes can be distinguished: B cells and the majority of T cells are small lymphocytes with a thin rim of cytoplasm surrounding the nucleus, whereas NK cells, $\gamma\delta$ T cells and about 50% of CD8+ T cells are larger, have more cytoplasm and distinct cytoplasmic granules and are known as large granular lymphocytes (Delves and Roitt 2000).

1.7.1 B lymphocytes

The main function of a B cell is to secrete soluble recognition molecules called antibodies (also known as immunoglobulins) and are thus responsible for the humoral mediated immune response of adaptive immunity. The response of mature B cells to foreign antigen occurs in the secondary lymphoid tissues, in a specialized region called germinal center. Interaction of mature B cells with antigen leads to activation of B cells, which consist of size change (B cell blast), proliferation and differentiation into plasma cells and memory cells. Plasma cells synthesize and secrete antibody of the same antigenic specificity as the naïve B cell membrane receptors that was initially triggered by antigen. During B cell proliferation in GC, the genes encoding the BCR undergo two types of changes. First, they change their antigen-binding properties by a process called somatic hypermutation, which fine-tune the recognition of antigen by BCR and determine the strength of binding (affinity) of the antibody. Second, under the influence of the interaction of CD40L expressed on activated T cells and CD40 expressed on B cell and different cytokines (IFN- γ and IL-4) secreted by helper T cells, B cells change their immunoglobulin class, a process called heavy chain class (isotype) switching. This change includes a switch of heavy chain class which is initially IgM and IgD, to produce antibodies of different heavy chain class (IgG, IgA or IgE), which mediate different biological effector functions and are specialized to combat different types of microbes (Delves and Roitt 2000).

1.7.2 T lymphocytes

T cells are responsible for the cell mediated immune response of adaptive immunity by exerting their effects on other cells, either regulating the activity of cells of the immune system or killing cells that are infected or malignant. Most T cells have surface antigen receptors composed of α and β polypeptides that recognize only the peptide-MHC complexes presented on the surface of antigen presenting cells (dendritic cells, macrophages and B cells). There are various categories of T cells based on their antigen receptor polypeptide chains that make up the TCR ($\alpha\beta$, $\gamma\delta$), coreceptor molecule (CD4, CD8), function (helper, effector, regulatory) and profile of cytokines released (T helper cells T_H0 , T_H1 , T_H2 , T_H3). For practical reasons, I discuss the two subpopulations of T lymphocytes depending on their surface markers and function: CD4+ T helper (T_H) cells and CD8+ T cytotoxic (T_C) cells.

1.7.3 CD4+ T lymphocytes

T_H cells express CD4 and recognize peptide-MHC II complex displayed on the surfaces of antigen-presenting cells. The main function of these lymphocyte subsets is to help other cells of the immune system to mediate immune responses. For example, T_H cells help B cells to proliferate and differentiate into plasma cells, or secrete cytokines that activate B cells, T c cells and macrophages. T_H cells are subdivided by

the pattern of cytokines produced, generating different types of immune response. T_H1 cells secrete IL-2, which induce T cell proliferation and also stimulates CD8+ T cell division and cytotoxicity. The other major cytokine produced by T_H1 is interferon γ , which activate macrophages, inducing NK cells to cytotoxicity. They also stimulate production of antibody isotypes, principally of subclasses IgG1, IgG2 and IgG3 that promotes the phagocytosis of microbes. Thus T_H1 cells play a crucial role in cell-mediated immunity. T_H2 cells produce IL-4 and IL-10 that primarily stimulates antibody production by B cells, inducing the production of IgG1–4, IgA and IgE. T_H2 cells also produce IL-5 that activates eosinophils. Thus T_H2 cells stimulate eosinophil-mediated immunity, which is particularly effective against helminthic parasites. T_H3 cells produce transforming growth factor β , which is immunosuppressive. A population of about 5–10% of circulating CD4+ T cells regulates the activity of other cells and is necessary to maintain peripheral tolerance to self-antigens. They constitutively express CD25, α chain of the IL-2 receptor, and are known as CD4+CD25+ T-Reg cells (Delves and Roitt 2000).

1.7.4 CD8+ T lymphocytes

T_C cells express CD8 and kill virally infected cells or malignant cells that have been presented as peptide-MHC I complex on APC. Once the TC cells have bound to the infected cells, they have several mechanisms by which they can kill their targets. The TC cells secrete proteins called cytotoxins, stored in granules in their cytoplasm. These include perforins that polymerize to form pores in the target cell membrane, and granzymes pass into the target cell through the perforin pores and activate caspase enzymes involved in apoptosis. Alternatively, target cell killing occurs via interaction of a surface molecule called Fas ligand on CD8+ cell with Fas on the target cells and induction of apoptosis. The specificity of cytotoxic T lymphocytes (CTLs) response is increased by the ‘help’ from CD4 T cells, otherwise are potentially dangerous to all cells in the body (Delves and Roitt 2000).

1.7.5 Lymph nodes

The complexity of the cellular interactions that occur during adaptive immune responses requires specialized environmental fields in which the relevant cells collaborate efficiently. Secondary lymphoid tissues, including lymph nodes (LNs), spleen and Peyer's patches, are the kind of professional specialized microenvironments that allow lymphocytes to encounter antigen, become activated and proliferate, and induce adaptive immune responses. In particular, LNs are strategically located at the interface of the blood and lymphatic systems, filtering and monitoring the tissue fluid exudate drained from peripheral tissues and carrying cell-associated and soluble antigens from those tissues (Cyster 1999; von Andrian and Mempel 2003). The functions of the LNs are carried out via a unique microarchitecture composed of distinct cellular compartments and structures (Fig. 2). The two main regions in LNs are the cortex and the medulla. Naïve lymphocytes enter these organs through high endothelial venules (HEV) and subsequently B and T lymphocytes are clearly compartmentalized at the cortical region. B cells are organized into either primary follicles within the outer cortex (B zone) of naïve B cells or secondary follicles of activated B cells forming germinal centers (GCs) after antigen challenge. A majority of T cells reside in the paracortex (T zone), which is outside follicle. Dendritic cells (DCs) carrying information about peripheral tissues function as messengers that arrive at the subcapsular sinus (SCS) through afferent lymphatics and further migrate into the paracortex (T zone) of the LN, where they prime antigen-specific responses. The medulla contains many plasma cells, several

macrophage subsets and memory T cells (Cyster 1999; von Andrian and Mempel 2003).

There are two mesenchymal origin stromal cells that are critical for generating the microarchitecture of LN by acting as a structural backbone to guide distribution and movement within the LN. Follicular DCs are a common type of lymphoid stromal cells that are distributed in B cell follicles or germinal centers. They play a critical role in activating B cells by retaining antigens for a long period of time in the context of intact Ag-Ab complexes. Moreover, these cells are the main producers of CXCL13 and BAFF (B cell activating factor), which are implicated in the attraction of B cells to follicles and B cell maturation respectively (von Andrian and Mempel 2003; Mempel et al. 2006). Fibroblastic reticular cells (FRCs) are common in the T cell area, and are responsible for formation of a complex reticular network (RN) by producing various extracellular matrix components and interweaving them to make reticular fibers (RFs). The RN has a core of collagen fibers surrounded by other ECM molecules, a basement membrane-like structure, and the FRCs. The space between collagen fibers and the BM serves as a conduit system for transport of chemokines and other low molecular weight molecules, which can be channeled from the SCS to perivascular space around HEVs, Fig. 2 (Gretz et al. 2000; Kaldjian et al. 2001). FRC also provides an adhesive substrate for conduit-associated DC (Sixt et al. 2005). The ECM content of RN may also serve as adhesive functional areas in LNs by localizing a particular population of cells exhibiting the appropriate receptor. For instance, a recent work has identified a novel functional DC population expressing $\alpha 1\beta 1$ and $\alpha E\beta 7$ integrins and localizing in LNs (Pribila et al. 2004).

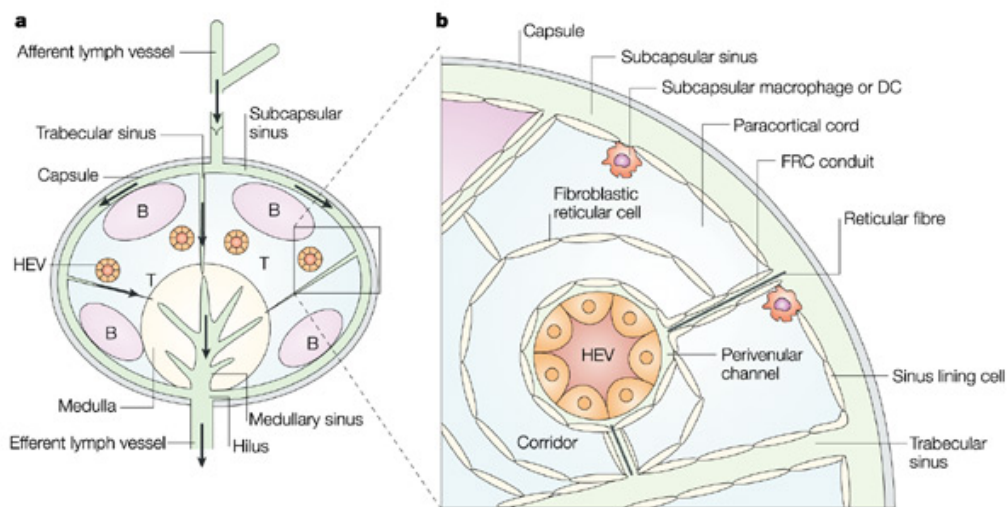


Fig. 2 Lymph-node architecture (adapted from Von Andrian and Mempel, 2003)

1.7.6 High endothelial venuels

To enter LNs, naïve lymphocytes selectively adhere to and migrate through specialized sites of the vasculature, the so-called high endothelial venuels (Girard and Springer 1995; Miyasaka and Tanaka 2004). These microvessels are postcapillary venules located mainly in the T- cell zones, such as the paracortical areas of lymph nodes and the interfollicular areas of Peyer's patches. HEVs are found in all secondary lymphoid tissues with the exception of the spleen, and in these tissues also support the extensive trafficking of naïve lymphocytes. HEVs have morphological

and physiological features distinct from postcapillary venules located at most other sites. They have a tall plump, almost cuboidal appearance with a prominent perivascular sheath, thick BM and discontinuous 'spot-welded' junctions. Ultrastructurally, HEVs have abundant cytoplasm, pale nuclei and a prominent dense nucleus, and prominent Golgi complex, abundant polyribosomes and rough endoplasmic reticulum. This is a sign of active biosynthesis and secretion activity not found in normal flat walled endothelium. HEVs express highly glycosylated and sulphated forms of sialomucins termed peripheral node addressins (PNAd), which includes CD34, podocalyxin, sgp200 and GlyCAM-1, and mucosal addressin cell adhesion molecule (MAdCAM) in LNs and PPs, respectively. Sialomucins constitute, after carbohydrate-based posttranslational modification, ligands for L-selectin. The principal structure involved in L-selectin binding is the fucosylated, sialylated and sulfated tetrasaccharide LewisX, which is recognized by mAb MECA-79 (Kraal and Mebius 1997; Ley and Kansas 2004). HEVs express high levels of lymphoid chemokines, whereas normal venules do not. These chemokines include CCL21 and CCL19, which support constitutive trafficking of lymphocytes. Whereas CCL21 is produced by the endothelial cells of HEVs, CCL19 is produced by stromal cells in the surrounding HEVs and transported to the luminal surface of HEVs (Miyasaka and Tanaka 2004). The expression of adhesion molecules and the specialized features of HEVs are highly dependent on the lymphoid microenvironment and/or factors present in the afferent lymph coming into lymphoid tissues.

It has been known that HEV-like vessels, possessing plump endothelial cells and other features of normal HEVs in secondary lymphoid organs, are induced along the vasculature in tertiary extralymphoid sites of chronic inflammation. In human diseases, such HEV-like vessels have been observed in rheumatoid arthritis, Crohn's disease, ulcerative colitis, Graves' disease, Hashimoto's thyroiditis, diabetes mellitus, bronchial asthma, rheumatoid arthritis (RA), heart allografts, kidney allografts, and gastritis induced by *Helicobacter pylori* infection. These HEV-like regions appear to act as a major site of lymphocyte extravasation from the blood into chronically inflamed tissues (Rosen 1999; Rosen 2004). Thus controlling the development of these regions holds promising therapeutic approaches to treat chronic inflammation.

1.7.7 Lymphocyte-HEV interaction

Lymphocyte recruitment is a highly controlled process that is critical in immune system regulation. Lymphocyte trafficking from blood into lymphoid tissues across HEVs is mediated by consecutive molecular interactions, termed as the multistep adhesion cascade (Fig.3) (Butcher and Picker 1996). These cascade reactions begin with lymphocyte tethering and rolling mediated by T cell expressed L-selectin and $\alpha 4\beta 7$ which binds to PNAd and MAdCAM displayed on HEVs. This is followed by a triggering of pertussis toxin-sensitive G-protein-coupled receptors by chemoattractants. HEVs chemoattractants such as CCL21 and CCL19 bind to CCR7 on naïve T cells and, in turn, induce intracellular signals for the activation-dependent firm adherence of rolling lymphocytes, in which the leukocyte integrin $\alpha L\beta 2$ (CD11a/CD18, LFA-1) binds to ICAM-1 (CD54) and -2 (CD102) on HEV (von Andrian and Mempel 2003; Miyasaka and Tanaka 2004; Tanaka et al. 2004). Activation by CXCL12 (SDF-1), the ligand for CXCR4, has also been shown to promote transendothelial migration of T cells across HEVs (Phillips and Ager 2002). Interestingly, mice genetically deficient in CCR7 or mice harboring a spontaneous mutation (plt; paucity of LN T cells), which have defective production of CCL19 and CCL21, demonstrate greatly reduced number of naïve T cells in LNs (Nakano et al. 1997) (Nakano et al., 1997; (Warnock et al. 2000). These observations show that the

CCR7 and its ligand chemokines are critical for triggering lymphocyte firm adhesion to HEVs, which is a prerequisite to transmigrate across HEVs into LNs.

The molecular mechanism involved in the final step to gain access to the parenchyma of the lymphoid tissue, lymphocytes transendothelial migration and penetration of the underlying basement membrane, are poorly understood. In this thesis we attempted to unravel this step by studying the expression of laminin isoforms in HEVs BM of LNs and their relevance to lymphocyte adhesion and transmigration.

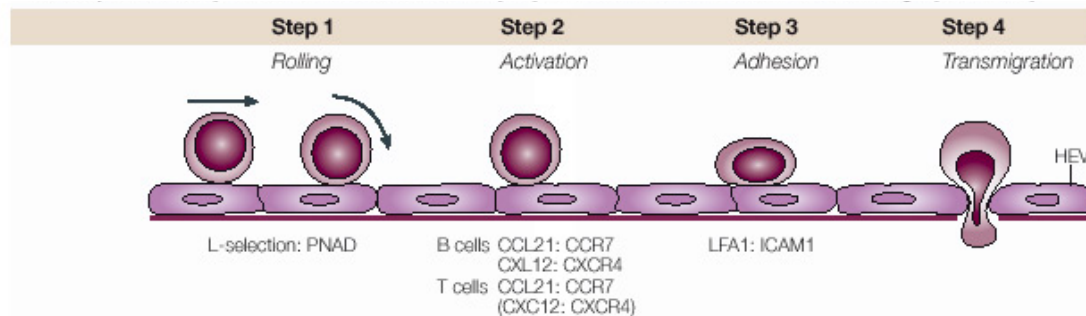


Fig 3. Multiple adhesion cascades in lymphocyte-HEV interaction (Adapted from Von Andrian and Mempel, 2003).

1.7.8 Lymphocyte trafficking in inflammation

On antigen stimulation in the secondary lymphoid tissues, naïve T cells proliferate by clonal expansion and differentiate into effector cells. Some CD4⁺ effector cells migrate towards the B cell follicles and provide help in a process that is crucial for germinal center formation. Others, together with CD8⁺ effector T cells, must immediately home to peripheral tissues that contain cognate antigen, which elicit local inflammation by stimulating innate immune cells (Fig. 4). The antigenic stimulation reprograms the trafficking properties of naïve T cells and B cells so that different effector cells home to tissue specific sites of inflammation. Such tissue tropism is governed by upregulation of receptors on effector cells for inflammation induced adhesion molecules and inflammatory chemokines. In addition, these cells down regulate homing receptors, thereby preventing them from recirculation and homing back to the peripheral lymph nodes (Butcher and Picker 1996; von Andrian and Mackay 2000; Luster, Alon et al. 2005).

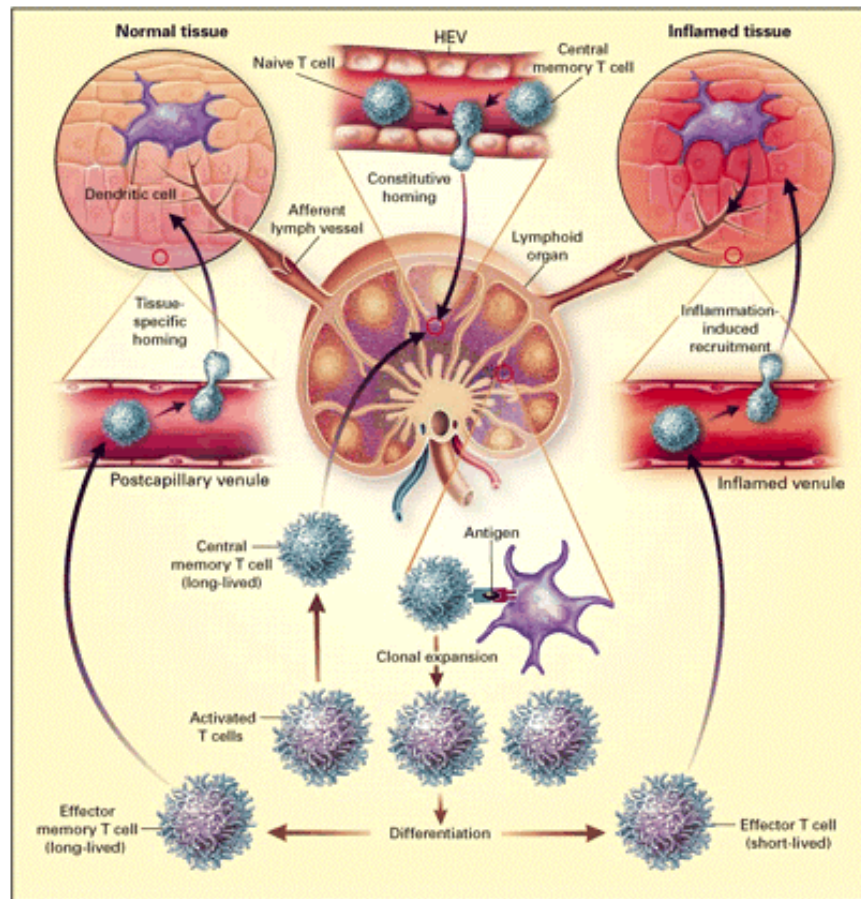


Fig. 4 Migratory routes of T cells (Adapted from Von Andrian and Mackay, 2000).

The determinants for induction of tissue-homing receptors during T cell-priming are the unique microenvironments of the secondary lymphoid tissues and selective tissue-specific DC imprinting (Campbell and Butcher 2000; Mora et al. 2003; Agace 2006). For instance, skin-tropic lymphocytes preferentially express functional PSGL-1 that binds E- and P-selectin on inflamed endothelium. In contrast, brain infiltrating T_H1 cells express $\alpha4\beta1$ integrin that binds VCAM-1, and gut tropic T cells express high levels of $\alpha4\beta7$ integrin that binds MadCAM-1 displayed on postcapillary venules in the intestine, whose interaction mediates both the rolling and the firm adhesion of lymphocytes. In addition to adhesion molecules, T_H1 and T_H2 cells express characteristic chemokine receptors and obey different traffic signals. CCR5 and CXCR3 are preferentially expressed on T_H1 cells, whereas CRTh2, CCR3 and CCR4 are expressed on T_H2 cells. In rheumatoid arthritis and multiple sclerosis, both diseases associated with type 1 responses, virtually all T cells in the lesion express CCR5 and CXCR3. Eotaxin, a ligand of CCR3, has been shown to be crucial in recruitment of eosinophils to airspace in asthma and in tissues where allergic and anti-parasitic responses occur (von Andrian and Mackay 2000; Olson and Ley 2002; Luster et al. 2005).

The recruitment of effector cells into extralymphoid organs and into sites of inflammation follows the same paradigm of cascades mentioned above for lymphocyte-HEV interaction: lymphocyte rolling, chemokine-mediated activation and subsequent firm adhesion, followed by lymphocyte transendothelial migration across perivascular BM. Thus this thesis works on expression of laminins in HEVs of LNs and their role in adhesion and migration may also have implication in inflammatory conditions.

1.7.9 Lymphocyte-ECM interaction

In recent years the interaction between lymphocytes and ECM components, such as fibronectin, collagens and laminins, has been shown to modulate/regulate several functions of lymphocytes. In the following section, those functions of ECM that may have similar implications to my thesis work on laminins are discussed.

During the final step in lymphocyte extravasation, lymphocytes migrate through the underlying BM and interstitial ECM. T-cells express numerous receptors to interact with components of ECM. Resting CD4⁺ T-cells express the $\alpha4\beta1$, $\alpha5\beta1$ as well as $\alpha6\beta1$ integrins, which have been shown to mediate binding of these cells to FN and Lms respectively (Shimizu et al. 1990; Geberhiwot et al. 2001). Activated T cells also exhibit upregulated expression of the $\alpha2\beta1$ integrin (VLA-2), which subsequently mediates binding to collagen (Goldman et al. 1992).

Besides its function as specific adhesive substrate, ECM components also mediate T cell migration. Hauenberger et al have shown both haptotactic and chemotactic migration of several T cell line and PMA-stimulated T cells on FN, collagen type IV, and laminin in Boyden chambers assay (Hauenberger et al. 1994), which is mediated via $\alpha4\beta1$ or $\alpha5\beta1$ on FN. In another study in 3D collagen matrix, locomotion of T cells is accompanied by alterations of cell adhesion molecules expression, such as upregulation of $\alpha2\beta1$ integrin and downregulation of CD44 and L-selectin (Friedl et al. 1995), suggesting differentiation-induced expression of adhesion molecules. T cell adhesion can be also regulated by proteoglycan-immobilized chemokines (Tanaka et al. 1993). Such triggers cause functional activation of integrins, which subsequently facilitate T cell migration. In the basal lamina of HEVs, laminins could specifically bind chemokines and present them to receptor expressing lymphocytes (Pelletier et al. 2000; Tanaka et al. 2004).

T cells produce ECM-degrading enzymes whose production is regulated by integrin mediated cell-ECM interaction. These enzymes have been proposed to play a role in T cell migration across endothelial BM and into inflamed tissues. Regulated production by T cells of gelatinase A and B which degrade type IV collagen and type V collagen, respectively, has been documented (Leppert et al. 1995)(Leppert et al., 1995). In addition, the use of metalloproteinase (MMP) inhibitor in Matrigel locomotion assay blocked the T cell migration (Leppert et al., 1995). Ivanoff et al. also demonstrated MMP dependent infiltrative capacity of T leukemia cell lines in Matrigel locomotion assay (Ivanoff et al. 1999). Recent work, however, has shown a $\beta1$ -integrin- and protease-independent mode of lymphocyte migration in interstitial matrices (Wolf et al. 2003). Agrawal et al. has further demonstrated non-protease associated leukocyte transmigration across endothelial BM in a EAE model (Agrawal et al. 2006).

ECM also regulates T cell migration within tissues. T cells interact with different ECM components in peripheral and lymphoid tissues. While peripheral tissues are rich in BM components like IV collagen and laminins, the intercellular space in tissues is largely made of of mostly type I collagen fibrils (Dustin and de Fougères 2001). Thus T cells entering and migrating through peripheral tissues are constantly in contact with collagen, which stimulates T cells to migrate and form only transient interaction with DC and other cells (Gunzer et al. 2000). This favors the effector or memory T cells that can be rapidly activated, while discouraging the activation of naïve T cells that require prolonged interaction with MHC and costimulation to be activated. On the other hand, as discussed previously, it has been suggested that the ECM in lymphoid tissues are ensheathed by FRC with a little accessibility of about 10% for cellular components of LN (Hayakawa et al. 1988; Gretz et al. 2000). Thus,

T cells are likely free to interact with dendritic cells for the prolonged time, which is required for T cell activation (Dustin and de Fougères 2001). In a recent study by de Fougères and co-workers they used antibody blockade and genetic inactivation in several models of inflammation including cutaneous hypersensitivity, experimental arthritis and colitis, and suggested a role for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrin-mediated retention of T cells within the interstitium (de Fougères et al. 2000; Fiorucci et al. 2002; Krieglstein et al. 2002). In addition, in two models of mouse influenza A infections, the interaction of $\alpha 1\beta 1$ integrin (VLA-1) on memory CD8⁺T cells with collagen have been shown to enhance both survival and the long term retention of CD8 memory T cells in the lung (Andreasen et al. 2003; Ray et al. 2004), which could be reduced by anti- $\alpha 1\beta 1$ mAb administration.

ECM components have been shown to play a role in T cell activation by facilitating adhesion and signal transduction in T cells. T cell activation in response to recognition of foreign antigen involves interaction with cell surface receptors of APC which leads to T cell proliferation and to the generation of an appropriate effector function, such as cytokine secretion or cytotoxicity. Insights about the potential role of ECM in T cell activation has come from studies showing a 'co-stimulatory' signal provided by various ECM proteins to induce T cell proliferation in combination with the 'primary' signal CD3/TCR cross-linking. Conversely, the immobilized protein or CD3/TCR cross-linking by its own fails to induce proliferation (Shimizu and Shaw 1991). Costimulation of T cell proliferation by FN, Lm and collagen via integrins has been reported (Shimizu et al. 1990; Rao et al. 2000; Geberhiwot et al. 2001) (Paper III).

ECM may also serve as a storage depot for cytokines and degradative enzymes released by migrating lymphocytes or surrounding cells, such as fibroblasts, epithelial cells, and keratinocytes. These soluble mediators may exhibit multiple regulatory effects as chemoattractants and as regulators of lymphocyte biology and thereby influence the outcome of inflammatory/ immune responses (Vaday and Lider 2000). In another scenario, ECM itself initiate cytokine production like TNF- α and immobilizes on it the secreted cytokine at high local concentration thereby enhancing the adhesion of activated T cells to surrounding ECM, which in turn ensures a continued cytokine production in regulatory loop fashion (Alon et al. 1994).

1.8 MONOCYTES

1.8.1 Monocyte development and function

Monocytes are a population of mononuclear leukocytes that originate from pluripotent haematopoietic stem cells in the bone marrow. These stem cells differentiate into myeloid or lymphoid precursor cells. The former precursors differentiate to granulocyte-monocyte progenitor. Under the influence of hematopoietic factors such as stem cell factor, IL-3, M-CSF and GM-CSF, these precursor cells become proliferating monoblasts, then promonocytes that finally differentiate into monocytes. Newly produced monocytes are released into the blood where they circulate for 1 to 3 days before entering tissues where they differentiate into macrophages or myeloid dendritic cells (Taylor and Gordon 2003).

Macrophages can be found in all tissues whose phenotypic and functional heterogeneity are greatly influenced by the local environment at distinct anatomical sites. Though circulating blood monocytes continuously supply for most tissue macrophages, there is evidence that macrophages in several different organs self-renew themselves without input from blood precursors (Gordon and Taylor 2005). Macrophages have a large amount of lysozyme and various hydrolytic enzymes, such

as phosphatases, lysozymes and nonspecific esterase. The major roles of macrophages include phagocytosis and subsequent elimination of senescent or apoptotic cells, tumor cells, virus infected cells and other broad range pathogens, the secretion of cytokines, chemokines, complement proteins and other soluble mediators, and antigen processing and presentation of peptide-MHC complex on their surface to T lymphocytes. Whereas some of these functions are constitutively expressed, in most cases macrophages should be activated by exogenous signals (delivered by cytokines, interaction with T cells or certain ECM components) to enhance their competence in tumor cell killing, destruction of intracellular pathogens or antigen presentation. Macrophages are also central in tissue remodeling and tissue repair (Gordon and Taylor 2005).

Dendritic cells constitutively express high levels of both MHC class II molecules and members of costimulatory B7 molecules and thus are the most potent antigen presenting cells compared to other professional APC. In the peripheral tissues immature DCs act as sentinels for signs of pathogen invasion, and acquire antigen by phagocytosis, macropinocytosis and receptor-mediated endocytosis. The internalized antigens are processed into peptides and presented to cell surface as peptide-MHC complex and finally mature into potent APCs after stimulation by cytokines and microbial products (pathogen-associated molecular patterns). The stimulation of Toll-like receptors by microbial products triggers DC maturation and results in the increased induction of costimulatory molecules and inflammatory molecules (Steinman et al. 1995; Janeway and Medzhitov 2002). Upon maturation, DC downregulate their receptors for inflammatory chemokines (CCR6) and upregulate the expression of receptors for lymphoid chemokines (CCR7), allowing mature DCs to migrate to T zone of secondary lymphoid tissues. Moreover, while in transit, they lose antigen-capturing capacity and acquire T cell sensitizing capacity and begin to produce chemokines that make them attractive to T cells waiting for them in lymph nodes (von Andrian and Mempel 2003). Then in T cell area they form clusters with T and B cells and stimulate antigen-specific immune responses. Immature DCs in low activation state while presenting self-antigens may also induce and maintain tolerance by preventing T cell reactivity against self-antigen, preventing autoimmune reactions in the lymphoid organs (Moser 2003).

1.8.2 Human monocyte subsets

In recent years, the phenotypical and functional heterogeneity of macrophages, dendritic cells and blood monocytes have been recognized. Monocytes constitute about 5-15% of peripheral blood leukocytes in human and are defined as blood mononuclear cells with variability in size, nuclear morphology and degree of granularity. The first clue of the heterogeneity in human monocyte was provided by a study of Passlick et al. who demonstrated that human monocyte could be divided into two major subsets depending on the differential expression of CD14 (part of the receptor for lipopolysaccharide) and CD16 (FcγRIII): the so called 'classical' CD14⁺CD16⁻ monocytes and 'non-classical' CD14⁺CD16⁺ monocytes, representing about 95% and 5% of the monocytes in a health individuals respectively (Passlick et al. 1989). These subsets differ in expression of chemokine receptors and adhesion molecules. While CD14⁺CD16⁻ express CCR2, CD62L and CD64 (FcγRI), CD14⁺CD16⁺ do not express CCR2 and have higher amounts of MHC class II and CD32 (FcγRII), which resembles mature tissue macrophages (Ziegler-Heitbrock et al. 1993; Weber et al. 2000). The latter also has an enhanced capacity for transendothelial migration (Randolph et al. 2002). Both subsets are able to differentiate into dendritic cells in culture (Sallusto and

Lanzavecchia 1994). With the recent identification of mouse monocyte heterogeneity with distinct chemokine receptors and adhesion molecules ('CX3CR1^{high} CCR2⁻CD62L⁻ vs 'CX3CR1^{low} CCR2⁺CD62L⁺) and differential recruitment to inflammatory lesions or normal tissues ('inflammatory' vs 'steady state' monocytes), there is great hope in understanding the role of human monocyte in immune response and in the pathogenesis of diseases, such as atherosclerosis, rheumatoid arthritis and AIDS (Geissmann et al. 2003).

1.8.3 Monocyte extravasation

Monocytes constitutively emigrate into tissues in physiological condition and their emigration is increased during inflammation at the level of postcapillary venuels. Monocyte extravasation is a multistep process which can be divided into four steps: 1) rolling, mediated by selectins; 2) activation by chemoattractant stimulus; 3) arrest and firm adhesion to the endothelium, mediated by integrin binding to Ig-family members; 4) migration between endothelial cells (transendothelial migration or diapedesis) and penetration through the basement membrane into the ECM of the tissues.

Monocytes must first loosely adhere to the vascular endothelium to initiate transendothelial migration. The rolling slows down the monocytes and allows physical contact with the vascular endothelium. These initial capturing interactions are mediated by selectins and their glycosylated ligands bearing the tetrasaccharide sialyl-Lewis X motif. Under physiological condition, L-selectin on the monocyte can bind to proteins such as the sialomucin CD34 on the endothelium in order to initiate monocyte adhesion. Increases in shear force at the vascular wall can actually enhance PSGL-1 on monocyte adhesion with L-selectin forming catch bonds. During acute inflammation, rapid exocytosis of P-selectin from intracellular Weibel-Palade bodies of endothelial cells and alpha granules of platelets is stimulated by histamine and thrombin respectively. E-selectin is not expressed by resting endothelium. Its expression is induced by activation of endothelium by inflammatory mediators, such as IL-1 β and TNF, which involves *de novo* gene transcription and expression of endothelial E-selectin, thereby enhancing monocyte adhesion (Muller 2002; Maslin et al. 2005).

The process of tethering and rolling of monocytes are reversible, and may be followed by dissociation from the vessel surface and release back into the blood stream. Alternatively, high affinity adhesion follows between integrin of monocytes and their ligands or counter receptor on endothelium. Such high affinity interaction requires activation of monocyte β 1 and β 2 integrins by stimuli such as intrinsic or proteoglycan-immobilized chemokines (eg. MCP-1, MIP-1 α/β) presented to the monocytes on the endothelial surface. The α 4 β 1 integrin can mediate both loose and firm adhesion of monocytes to the endothelium via L-selectin and selectin ligands as well as VCAM-1 respectively. β 2 integrins such as α L β 2, α M β 2 and α X β 2 further enhance the firm adhesion via high affinity binding to the ICAM-1 and ICAM-2 (Muller 2002; Maslin et al. 2005).

The next step is transendothelial migration between adjacent endothelium. Thus, after monocytes firmly bound to endothelium, they display remarkable cytoskeletal remodeling to extend filopodia and migrate towards an intercellular junction via β 2 integrin/ICAM interactions. Homophilic interaction between adhesion molecules, including PECAM-1 (CD31) and CD99, on the leukocyte and the same molecule concentrated at endothelial junction contributes to monocyte diapedesis. Interestingly, these two adhesion molecules mediate separate steps in the diapedesis. CD99 controls

a step distal to the one controlled by PECAM-1. Both of them are constitutively expressed by monocytes and at EC junctions. Other junctional adhesion molecules such as endothelial JAM-A and adherens junction proteins VE-cadherin and catenins have been also reported to play a role in diapedesis (Muller 2002; Maslin et al. 2005). In addition to a widely recognized route of leukocyte extravasation between adjacent endothelial cells (paracellular), leukocyte can migrate through an individual EC (transcellular). However, the significance of the latter pathway is poorly known.

The molecular mechanism involved in the final step of monocyte extravasation into the tissue, which involves passage across the subendothelial BM and ECM of the interstitium, are poorly understood. Monocytes express $\beta 1$ and $\beta 2$ integrins, which have been implicated in the migration via these ECM components. Domain six of PECAM-1 has a role in mediating migration of monocytes across BM *via* yet unidentified ligand, unlike its role in diapedesis, which is mediated via the first molecular domain. In addition, heparin and heparin sulfate also participate in passage across the BM (Muller 2002; Maslin et al. 2005). In this thesis work, the potential interaction of $\beta 2$ integrins of monocytes, particularly $\alpha M\beta 2$ and $\alpha X\beta 2$ with vascular endothelial Lm isoforms, Lm-411 and 511 is addressed (Paper IV).

1.8.4 Monocyte trafficking to non-inflamed and inflamed tissues

Monocytes constitutively traffic to peripheral tissues differentiating into tissue macrophages and dendritic cells. This constitutive or steady state efflux of monocytes from blood is mediated by selective responses of monocytes to chemokines and adhesion molecules expressed by cells of tissues without any premature stimulation or genetically programmed effector function (Muller 2001; Imhof and Aurrand-Lions 2004). Among the chemokines that are constitutively expressed and act specifically on circulating monocytes, CXCL14 (breast and kidney expressed chemokine, BRAK) has been proposed to be involved in the constitutive trafficking of monocytes (Kurth et al. 2001). BRAK mRNA is expressed constitutively by a variety of epithelia including the basal keratinocytes and dermal fibroblasts of skin, and cells in the lamina propria of gut, suggesting a homeostatic rather than an inflammatory role, which is possibly related to macrophage development than inflammatory role. The contribution of adhesion molecules to the constitutive migration of monocyte to differentiate to DC is highlighted by studies of mice deficient for $\beta 2$ integrins, which show a marked decrease in number of DCs in the lungs, unlike ICAM-1 or E-selectin or P-selectin deficiency. This result indicates that $\beta 2$ integrin interacts with non-ICAM-1 ligands on the lung vasculature (Schneeberger et al. 2000). DCs also constitutively migrate from tissues to LNs in the absence of inflammatory stimuli to transport apoptotic cells or self-antigens, participating in the maintenance of antigen-mediated peripheral tolerance (Moser 2003).

During inflammation, tissues express various chemokines which influence recruitment of monocytes expressing the chemokine receptors, such as CCR2, the receptor for CCL2 (also known as monocyte chemoattractant protein 1, MCP1). This chemokine is responsible for recruitment of inflammatory CD14⁺ monocytes, unlike CD16⁺ monocytes (Gerszten et al. 1999). The role of various adhesion molecules, such as CD62L and $\alpha L\beta 2$, $\alpha M\beta 2$ and $\alpha 4\beta 1$ integrins has also been suggested for recruitment of inflammatory monocytes using knockout mice for these molecules and inflammatory models (Rosen and Gordon 1990; Issekutz and Issekutz 1995; Meerschaert and Furie 1995; Tedder, Steeber et al. 1995; Henderson, Hobbs et al. 2003).

The fate of monocytes in inflammatory sites differs depending on whether they differentiate into macrophages or DCs. While macrophages are engaged in local

phagocytic activity and do not further migrate to draining LNs, DC migrate to draining lymph nodes (Muller 2001; Imhof and Aurrand-Lions 2004). This process can be demonstrated *in vivo* in mice by monitoring emigrating monocyte/macrophages after peritoneal injection of thioglycolate or injection of latex beads into the skin. In the former model of peritonitis, $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins mediates the adhesion of monocytes to mesothelial cells, facilitating their exit from the peritoneum through lymphatic vessels (Bellingan et al. 2002). After intradermal injection of fluorescent latex beads, MCP-1 is produced and thereby likely to stimulate migration of phagocytic murine $CD11b^+F4/80^+$ monocytes into the draining lymph nodes (Palframan et al. 2001). Randolph et al (Randolph et al. 1998) used culture system mimicking the migration of monocytes from the tissue to bloodstream or lymphatics. In this model, monocytes cross a layer of endothelial cells and thereafter enter a subendothelial collagen matrix. A proportion of these monocyte-derived cells then "reverse transmigrate," mimicking migration of DCs out of the tissues into lymph. Interestingly, while those cells that remain in the "tissue" become macrophages, the reverse transmigrated ones become DCs. Furthermore, the migration of Langerhans cells from the epidermis to the draining lymph nodes involves both $\alpha L\beta 2$ (Ma et al. 1994) and $\alpha 6$ integrins (Price et al. 1997). In the latter study association of the $\alpha 6$ -integrin chain with an as yet uncharacterized β -integrin subunit mediates DC migration from the epidermis, probably through interacting with laminin in the epithelial BM.

Monocytes can also be recruited to inflamed LNs directly from bloodstream. They bind to HEVs of inflamed LNs, but not HEVs draining non-inflamed LNs, suggesting that monocytes can be recruited across HEVs *in vivo*. Thus, recruitment of monocytes across HEVs to LN cortex, where monocytes and macrophages are normally scarce, could provide a rapidly mobilized source of effector monocytes and a potential source of immature DCs. Insights into the recruitment of monocytes into LNs draining sites of inflammation have also come from recent evidence that chemokines and other small solutes arrive to draining LNs via conduits that link the SCS with the basal surface of HEVs and are presented on HEVs (Gretz et al. 2000). Once chemokines are bound and presented on the apical surface of HEVs, they can activate firm adhesion and recruit lymphocytes and also monocytes. Recently, two studies demonstrated how inflammatory chemokines originating in distant sites and from LNs draining inflamed tissues can stimulate the influx of circulating blood monocytes into the draining LNs via HEVs. These studies showed that the CCL2 (MCP-1) produced at inflamed skin and the CXCL9 (also known as monokine induced by interferon- γ , MIG) secreted by LNs draining inflamed tissues can be displayed on HEVs and recruit monocytes selectively to these HEVs (Janatpour et al. 2001; Palframan et al. 2001).

The works of this thesis can be relevant in understanding monocyte trafficking where monocytes can encounter laminins. As discussed above, monocytes can encounter Lms during constitutive and inflammation-induced extravasation, during reverse transmigration of monocytes from tissues to lymphatics and, finally, during recruitment to LNs across HEVs and for *in situ* localization within LNs.

1.8.5 Monocyte-ECM interaction

Following extravasation, monocytes adhere to ECM constituents, such as fibronectin, collagens and laminins through integrins. The major integrins expressed on monocytes includes $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (Collagen), $\alpha 4\beta 1$ and $\alpha 5\beta 1$ (VCAM-1 and fibronectin), as well as $\beta 2$ integrins ($\alpha L\beta 2$, $\alpha M\beta 2$, $\alpha X\beta 2$). While $\alpha 4\beta 1$, $\alpha 5\beta 1$ and $\beta 2$ integrins are known to be abundant and constitutively expressed on monocytes, $\alpha 2\beta 1$

is expressed in low amount, and $\alpha 1\beta 1$ is expressed only on activated monocytes (Hemler 1990). Constitutive expression of $\alpha 6\beta 1$ integrin on monocytes has been previously reported (Hemler 1990). This assumption, however, has been challenged by this thesis work (paper IV). Moreover, the role of $\beta 2$ integrins expressed on monocytes with regard to ECM interactions is not well defined. $\alpha M\beta 2$ integrins are abundant on the cell surface and their expression also increases, originating from an intracellular pool, upon stimulation. They have been reported to exhibit promiscuous ligand specificity, as they non-specifically stick to many protein and nonprotein ligands (Lishko et al. 2003). This assumption lead to questioning the role of this integrin in leukocyte migration through subendothelial BM and interstitial ECM (Sixt et al. 2001). However, our group has recently shown a specific role of $\alpha M\beta 2$ integrin in mediating adhesion and migration of neutrophils on Lm-411 (Wondimu et al. 2004). In the present thesis, we have extended this role of $\alpha M\beta 2$ integrin for the adhesion and migration of monocytes on Lm-411 and Lm-511, as well as on Lmy1 monomer (paper IV).

The integrin-ECM interactions have been shown to regulate various phenotypic and functional changes of monocytes, such as differentiation to macrophages, migration and phagocytosis. Furthermore, recent work has shown that such interactions could lead to induction of genes encoding transcription factors, chemokines, cytokines, and lipid metabolism, stressing their significance in immune and inflammatory responses (de Fougerolles and Koteliansky 2002; Nourshargh and Marelli-Berg 2005)

1.9 INTERACTIONS OF PLATELETS AND LEUKOCYTES WITH VASCULAR LAMININ ISOFORMS

In response to an inflammatory stimulus/injury and immunosurveillance, the endothelial barrier function can be breached, resulting in recruitment of platelets and leukocytes into the extravascular tissues. During these processes, leukocyte and platelet interact with endothelium and underlying BM, and interstitial ECM. Relatively little is known of the interaction of leukocytes with the underlying extracellular matrix proteins of the blood vessel BM and of the interstitium. There is also limited evidence about the role of ECM proteins of the blood vessel BM during hemostasis and/or thrombosis, as opposed to interstitial ECM proteins like fibrillar collagens. Notably, these two domains of ECM are biochemically distinct and may confer a differential influence in leukocyte and platelet phenotype and responsiveness both in physiological and pathological scenarios. As discussed in previous sections, most of the earlier studies regarding leukocyte and platelet interaction with ECM involved mainly components of interstitial stroma, such as collagen type I, fibronectin, vitronectin, thrombospondin and tenascin, which are readily isolated by traditional biochemical methods from tissues or plasma.

In this thesis work I focused on the interactions of platelets, lymphocytes and monocytes with subendothelial BM components, particularly Lm-411 and -511 isoforms, which are the major endothelial laminin isoforms expressed at sites of vascular injury and leukocyte extravasations *in vivo*. In an effort to address these interactions, several of the early studies used Lm-111, Matrigel and commercial placental laminin preparations. As mentioned in a previous section, Lm-111 has an expression in adult tissues which is highly restricted to some epithelia, and is absent from most blood vessels, suggesting that such studies may have no *in vivo* relevance. Similarly, Matrigel, a reconstituted BM from the EHS tumor, is significantly less cross-linked and contains substantial quantities of growth factors, which is different from normal basement membranes *in vivo*. Moreover, commercial Lm preparations

isolated from placenta are often highly fragmented, a mixture of Lm isoforms and/or contaminating proteins, exhibiting major functional difference between batches. Thanks to the present availability of pure and intact human recombinant Lm preparations, molecularly characterized natural and commercial placental Lm preparations, mAbs to Lm α 4 and α 5 chains and *in vivo* inflammatory models, relevant studies included in this thesis work have been performed to address the role of vascular endothelial laminin isoforms in platelet adhesion and activation, as well as in leukocyte extravasation.

1.9.1 Platelet adhesion and activation (cell aggregation and P-selectin expression)

Following vascular injury, BM components are exposed to circulating platelets, which express α 6 β 1 integrin, a Lm receptor. However, it is unclear whether platelet-Lm interaction has a (patho)physiological relevance with regard to hemostasis and thrombosis. Recently, our group has performed molecular characterization of platelet Lms, and demonstrated that platelets contain, secrete, and adhere to Lm-411 (Geberhiwot et al. 1999), and that α 6 β 1 integrin mediates adhesion to Lm-411. More recently, three independent studies have further investigated the role of Lms in the sequence of events related to platelet adhesion, spreading and activation. In this regard our study demonstrated that endothelial Lm isoform Lm-511 strongly promotes adhesion *via* α 6 β 1 integrin but not activation of platelets (Nigatu et al. 2006). Moreover, we have demonstrated synthesis/expression of heterotrimeric α 5-Lms in megakaryocytic/platelets. Watson's group has further shown stimulation of α 6 β 1 integrin-mediated platelet spreading by a placental laminin, as demonstrated by formation of filopodia and lamellipodia (Inoue et al. 2006). They also identified GPVI as a novel receptor for laminin and speculated that laminin contributes to platelet spreading *in vivo* through a direct interaction with GPVI. In the other study, interaction of platelet α 6 β 1 integrin with laminin has been shown to induce active formation of filopodia and novel actin-filament pattern changes (Chang et al. 2005), which are very different from the morphological characteristics of platelets stimulated *via* integrin α _{IIB} β 3. This study demonstrated that the signals induced by integrin α 6 β 1 modulate the level of PI3K and Cdc42 activity to allow platelets to actively form filopodia.

Altogether, the current model of platelet-laminin interactions indicate that platelets contain, secrete and adhere *via* α 6 β 1 integrin to vascular endothelial laminin isoforms, Lm-411 and Lm-511. Moreover, Lm-511 has been shown to effectively mediate platelet adhesion but no aggregation. Thus, these observations demonstrate that laminin is able to support both platelet adhesion and platelet spreading at sites of vascular injury, thereby contributing to the hemostatic process by the formation of platelet monolayers without ensuing thrombus formation.

1.9.2 Leukocyte extravasation

A recent study revealed that homophilic interaction between platelet endothelial cell adhesion molecule-1 (PECAM-1) on neutrophil and endothelium induces upregulation of the laminin binding α 6 β 1-integrin by transmigrating neutrophils, which in turn is required for neutrophil migration through the perivascular basement membrane (Dangerfield et al. 2002). Interestingly, such PECAM-1 homophilic interaction appears to activate Rap-1 GTPase on transmigrating leukocytes, resulting in increased avidity of specific β 1 and β 2 integrin receptors for ECM constituents (Reedquist et al. 2000). However, this sequence of events does not hold a general

phenomenon, because granulocytes could still extravasate in response to tumour necrosis factor α independently of PECAM-1 and $\alpha 6\beta 1$ integrin (Dangerfield et al. 2005).

With the identification of Lm-411 and Lm-511 as major vascular endothelial Lm isoforms (Frieser et al. 1997), several data from our laboratory and others have proved that these Lm isoforms have differential activity (adhesive and/or promigratory) on different leukocyte types. We reported constitutive adhesion of monocytoïd cells to Lm-411 and -511 via $\alpha M\beta 2$ and $\alpha 6\beta 1$ integrins, and strong promotion of blood monocyte and neutrophils migration by these Lm isoforms *via* $\alpha M\beta 2$ integrin (Pedraza et al. 2000; Wondimu et al. 2004) (paper IV). We have also shown Lm-511 and -411 to promote strong adhesion, migration and co-stimulation of lymphocyte activation *via* $\alpha 6\beta 1$ integrin (Geberhiwot et al. 2001) (paper III). Furthermore, we have proven that lymphocytes and PMNs can secrete Lm-411 and -511 (Geberhiwot et al. 2001; Wondimu et al. 2004) (paper III), suggesting that endogenous laminins might also contribute to the process of leukocyte migration. Recently, Wondimu et al. showed the relevance of Lm-411 in extravasation of PMNs by demonstrating impairment of PMN recruitment in laminin $\alpha 4$ knockout mice (Wondimu et al. 2004). The study suggested that this defect is most likely due to the absence of laminin $\alpha 4$ in the vascular basement membrane because this ECM is a richer source of Lm-411 than PMNs. Similarly, it has been reported that in *in vivo*, using a model of experimental autoimmune encephalomyelitis (EAE), T-cell transmigration occurs exclusively at sites defined by the presence of laminin $\alpha 4$ and the absence of laminin $\alpha 5$, suggesting that laminin $\alpha 4$ is permissive for transmigration and laminin $\alpha 5$ is inhibitory (Sixt et al. 2001). However, a more recent study by Horssen et al. has challenged this observation, showing that in both active and chronic multiple sclerosis lesions, leukocytes accumulate around endothelial BMs that contain both laminin $\alpha 5$ and $\alpha 4$ chains (van Horssen et al. 2005). These authors found no differential regulation of leukocyte migration post-interaction with these laminin chains, nor that formation of perivascular cuffs was associated with the presence or absence of distinct laminin isoforms, unlike that reported in murine EAE (Sixt et al., 2001). In support to the latter study, our present results (paper III) show that Lm-511 preferentially promotes migration of lymphocytes, and that both $\alpha 4$ and $\alpha 5$ Lm chains are expressed in HEVs. In addition to providing stimulatory adhesive substrates, basement membrane constituents could regulate leukocyte transmigration through their cleavage products. For example, a cryptic peptide, AQARSAASKVKVSMKF, in the α -chain of Lm-511, but not the intact molecule, has been shown to induce T-lymphocyte, macrophages, and PMNs chemotaxis *in vitro* and migration *in vivo*, and to induce MMP9 mRNA expression (Adair-Kirk et al. 2003). The vascular basement membrane component density along postcapillary venules may also have an impact on the exit site of transmigrating leukocytes. Wang et al. (Wang et al. 2006) have observed regions within the walls of murine cremasteric venules with low expression of Lm-511, collagen IV and nidogen-2 colocalizing with gaps between pericytes underneath endothelial junctions, which are preferred sites for neutrophils migration in cytokine-activated endothelium. Whether the same mechanism holds for monocytes and lymphocytes is presently unknown.

2 PRESENT INVESTIGATION

Aims of the study

- Immunological, biochemical and functional characterization of commercial human placenta Lm preparations, including their α chains.
- Isolation and characterization of $\alpha 4$ - and $\alpha 5$ -Lms expressed by megakaryocytic/platelet, lymphocyte and monocytes.
- Mapping of the distribution of Lm chains in lymphoid tissues, particularly in high endothelial venules and reticular fibers.
- Investigation of the role of endothelial Lm isoforms Lm-411 and Lm-511 in platelet adhesion and activation, as well as in lymphocyte and monocyte adhesion, migration, and proliferation.
- Investigation of the effect of Lm $\beta 1$ and Lm $\gamma 1$ monomers on monocyte adhesion and migration.
- Determining the number of lymphocytes in lymph nodes of Lm $\alpha 4$ knockout mice.
- Identification of the integrin receptors mediating adhesion and migration of platelet, lymphocyte and monocytes on vascular laminins.

3 MATERIALS AND METHODS

3.1 CELLS (PAPER I, II, III, IV)

Human leukemia cell lines HEL and DAMI (Martin and Papayannopoulou 1982; Greenberg et al. 1988), and human KG1C glioma cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Sigma-Aldrich) and antibiotics. Human platelets were isolated from citrated blood from healthy donors (Karolinska Hospital at Huddinge). Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors after Ficoll-Hypaque gradient centrifugation (Amersham Pharmacia AB, Uppsala, Sweden). Lymphocytes were subsequently isolated either by removing tissue culture flask-adherent cells or by Percoll gradients (see below). CD4⁺ T-cells were obtained from PBMC by positive selection using magnetic beads coated with anti-CD4 antibody according to instructions from the manufacturer (Dynal AS, Oslo, Norway). In order to isolate monocytes, PBMC were extensively washed to remove contaminating platelets and monocytes were subsequently isolated by Percoll gradient (see below). To study the effect of platelet contamination in the expression of $\alpha 6\beta 1$ integrin on the surface of monocytes, PBMC were washed in two different ways. While the 'clean' monocytes were obtained by washing twice with 10 mM EDTA and thereafter 5-10 times with phosphate buffered saline (PBS), the 'contaminated' monocytes were obtained after only a couple of washes with PBS without 10mM EDTA, as commonly used in several protocols. Specific markers were used to identify particular cell types, including contaminating ones.

3.1.1 Lymphocyte and monocyte isolation by Percoll gradient (Paper III, IV)

During isolation of monocyte and lymphocyte from blood, several factors were taken into account, such as purity of the isolated population, activation status of the cells, tendency of possible subpopulation selection, influence of serum components, length of time and cost. Accordingly, we found Percoll density gradient separation as a good alternative because it provided relatively pure, functional and viable cells, good recovery, and it was rather fast and inexpensive.

Initially lymphocytes and monocytes were obtained as PBMC using Ficoll-Hypaque gradient centrifugation. Monocytes and lymphocytes were subsequently purified by centrifugation on a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden), with modifications of a previously described method (Kouwenhoven et al. 2001). Briefly, 20×10^6 PBMC were suspended in complete medium (10% FCS in RPMI) and centrifuged at $500 \times g$ for 10 min at 4°C. Supernatant was discarded and cells were carefully suspended in 60% (v/v) Percoll in complete medium. Subsequently, 47.5% and 34% Percoll solutions in complete medium were layered upon the cell suspension (Fig. 5). Cells were centrifuged for 40 min at $1700 \times g$. The low-density fraction, 47.5-34% interface, and the high-density fraction, 60-47.5% interface representing monocytes and lymphocytes, respectively, were collected and washed twice with complete medium. The cell purity was established by FACS analysis using forward side scattering and mAb TUK4 against the monocyte marker CD14 (Dakopatts, Glostrup, Denmark) and mAb 9.6 against the lymphocyte marker CD2, and found to be >94% and >97% respectively. Viability was consistently high (>95%). This isolation technique has been used particularly for functional studies of laminin interactions with lymphocytes and monocytes.

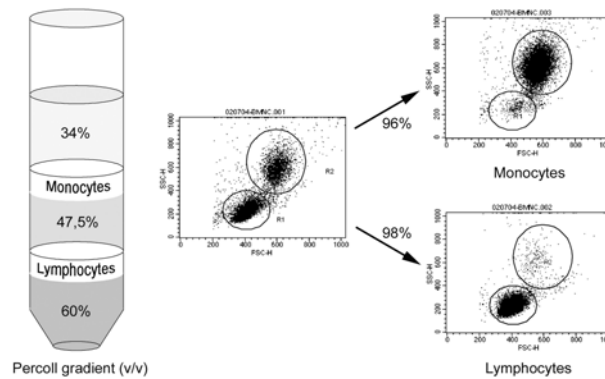


Fig 5. Lymphocyte and monocyte purification by Percoll gradient (Starting population of 74% lymphocytes and 26% monocytes).

3.2 EXPRESSION ANALYSIS

3.2.1 Immunofluorescence flow cytometry (Paper II, III, IV)

Isolated PBMC and platelets were analyzed for cell surface (non-permeabilized) and intracellular (permeabilized) expression of Lm chains as well as for cell surface expression of integrins by immunofluorescence flow cytometry. Cells were permeabilized by fixation and permeabilization with IntraStain kit (Dakopatts, Glostrup, Denmark). Indirect immunofluorescence was performed by incubating the cells, after blocking with 2 mg/ml of heat aggregated human IgG (Sigma-Aldrich), for 30 min at 4°C with saturating amounts (1 µg IgG to 1×10^6 cells) of a panel of integrin and Lms chain specific mAbs, followed by fluorescein-conjugated F(ab')₂ fragments of rabbit anti-mouse Ig (Dakopatts) at 1:20 dilution. mIgG and specific mAbs against markers for platelets, lymphocytes and monocytes were also included as negative and positive controls, respectively. After staining, cells were gated, according to forward and side scatter, as well as expression of their specific immunological markers and analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

3.2.2 Immunohistochemistry and confocal microscopy (Paper III)

A panel of mAbs to all Lm chains, except $\gamma 3$, were used to label BMs and associated structures in human lymph nodes. For double immunolabelling, mAb 3G4 against type III collagen (mouse IgM, Gibco) and mAb MECA-79 against sulphation-dependent high-endothelial venule-associated carbohydrate (mouse IgM, BD Bioscience, Stockholm, Sweden) were used. Tetramethylrhodamine-isothiocyanate-(TRITC)-coupled Ulex Europaeus I-lectin was also used in double immunostainings to identify blood vessel endothelia. For double immunolabelling the specimens were reactive with primary mAb for 30 min and washed in phosphate buffered saline (PBS). Thereafter, the specimens were exposed to Alexa Fluor 488 goat anti-mouse-IgG, -IgM or rat-IgG (Molecular Probes, Eugene, OR) followed by secondary antibody, and then Alexa Fluor 594 goat anti-mouse,-rat or rabbit-IgG, and were then embedded for confocal laser scanning microscopy (CLSM) in glycerol/PBS mixture (90%/10%). CLSM was carried out using Leica TCS SP2 microscopy system with

argon excitation line 488 nm and HCX BL APO CS 63 x 1 x 40 NA oil immersion objective.

3.2.3 RNA preparation and RT-PCR (Paper II, III)

Total RNA was extracted from cultured HEL and DAMI cells, and purified lymphocytes by using Total RNA Extraction kit (Amersham Bioscience, Sweden) and RibopureTM-WBC kit (Ambion Europe Ltd, UK), respectively. RNA was transcribed to cDNA using the Advantage RT-for-PCR kit (Clontech laboratories). After oligo (dT) 18 priming, mRNA was reverse-transcribed into cDNA by incubating for 60 minutes at 42°C with 200 units of M-MLV reverse transcriptase (BD Biosciences, Palo Alto, CA) in 20 µl of reaction buffer containing 20 units of recombinant RNase inhibitor, and 0.5 mM of each dNTP. The reactions were terminated by heating the samples at 94°C for 5 minutes and the mixture was diluted to 100 µl with DEPC-treated water. Single-stranded cDNAs in 20 µl of reaction buffer containing 1.5 mM MgCl₂, 0.5 µM of each primer of Lm chains and dNTP mix (200 µM each) were used for PCR using 1.25 units of Ampli Taq DNA polymerase (Perkin Elmer/Roche Molecular Systems, Inc., Branchburg, NJ, USA). The conditions for PCR were: initial denaturation for 2 minutes at 94°C, then 30 cycles at 94°C, 45 seconds; 60°C, 1 minute; 72°C, 1½ minute, followed by a final extension of 72°C for 7 minutes. A reaction without reverse transcriptase product was performed as a negative control to exclude the possibility of contaminating genomic DNA. PCR products were resolved by electrophoresis using 2% agarose gels.

3.2.4 Metabolic labelling (Paper II)

HEL cells from exponentially growing cultures were washed with PBS and resuspended in methionine- and cysteine-free medium containing 10% dialyzed fetal bovine serum. Following incubation at 37°C for 0.5 h, cells were labelled with 0.20 mCi/ml Trans³⁵S label (ICN Radiochemical Inc) for 4 h at 37°C. After removing the medium, the cells were washed three times with cold PBS and then lysed with lysis buffer, 1% Triton X-100 in PBS containing 1 µg/ml of aprotinin, 2 µM leupeptin, 2 µM pepstatin, 1 mM PMSF, and 2 mM EDTA as protease inhibitors. The soluble fraction (cell lysate) was used for immunoprecipitation as described below.

3.2.5 Immunoprecipitation and Western blotting (Paper II)

Cell lysate was prepared by lysing 3 x 10⁸ cells (HEL/DAMI) or 1 x 10⁹ platelets with 1 ml of lysis buffer containing protease inhibitors. The soluble fractions and concentrated material secreted by platelets were precleared with protein G-Sepharose beads for 1 hr at 4°C with gentle agitation (Amersham Pharmacia AB, Uppsala, Sweden). Aliquots of these materials were immunoprecipitated by incubating with primary Lm mAb(s). In parallel, secondary rabbit anti-mouse Ig (Dakopatts, Copenhagen, Denmark) was incubated with protein G-Sepharose beads for 2 hrs at 4°C. Pellets containing the secondary antibody was washed with lysis buffer and incubated with aliquots containing the primary antibody for additional 2 hrs at 4°C. Finally, the immune complexes were washed extensively with various buffers and eluted by boiling the samples with SDS-containing sample buffer with β-mercaptoethanol for 5 min at 100°C. For Western blotting, immunoprecipitates were separated by 5% SDS-PAGE, and electroblotted to a nitrocellulose membrane. Then, filters were blocked with 0.1% Tween 20/5% dry skimmed milk in PBS and incubated with primary Mabs to Lm chains. The bound antibody was detected with HRP-conjugated anti-mouse Ig (Dakopatts) and developed with ECL (Amersham Pharmacia AB, Uppsala, Sweden).

3.2.6 Immunoaffinity chromatography and Western blotting (paper III)

Lymphocyte lysate was prepared by lysing 1×10^9 blood lymphocytes isolated as mentioned above. The soluble fractions were precleared with Sepharose CL-4B beads (Amersham Pharmacia AB, Uppsala, Sweden), and laminin was purified by applying the cell lysate through a mAb 4C7-(Lm α 5 specific) Sepharose column, which had been equilibrated with lysis buffer, and cycled twice. Following extensive washing, the protein bound to the column was eluted using high pH and the samples were collected in neutralizing buffer and analyzed by SDS-PAGE and Western blotting as mentioned above.

3.3 FUNCTIONAL ASSAYS

3.3.1 Cell adhesion assay (paper II, III, IV)

Cell adhesion on plastic surfaces coated with Lm isoforms and Lm chains was carried out as described in a previous study (Wondimu et al. 2004). Briefly, 96-well flat bottom polystyrene plates (BD Biosciences) were coated with 50 μ L/well of PBS or of 20 μ g/ml human serum albumin (HSA), Lm-111, Lm-211, Lm-332, Lm-411, rLm-511, rhLm β 1, rhLm γ 1 or pFN in PBS at 37°C for 3 hrs. After rinsing with PBS, free sites were blocked with 2% polyvinylpyrrolidone (PVP; molecular weight 360 kDa; Sigma) for 1 hr at room temperature and, after further washing, $100/200 \times 10^3$ cells/ well (100 μ l) of monocytes and lymphocytes isolated by Percoll were incubated in presence of 200 nM tetradecanoyl phorbol acetate (TPA, also known as PMA, Sigma) for 1 hour at 37°C. Following five washes with plain medium, adherent cells were fixed by adding 50 μ l of fixative solution (paraformaldehyde 40g/1NaH₂PO₄.H₂O, 16.97g/l; NaOH, 3.86g/l; and D-glucose, 5.4g/l; prepared at 65°C, pH 7.4) for 15 min and thereafter 50 μ l of filtered Toluidine blue dye (Sigma, 0.5% W/V in PBS) was added overnight at room temperature. The plate was then washed with copious amounts of distilled water. Adherent cells were quantified in a microplate reader (Multiskan MS; Labsystems, Helsinki, Finland) at 620 nm by releasing the blue dye with 100 μ L of 2% SDS (Bio-Rad Laboratories, Richmond, CA). Constitutive platelet adhesion to laminins was also performed in a similar way with minor modifications such as incubating the plate with proteins at 4°C overnight and blocking with 0.1% HSA in PBS for 1 h at 37°C and having platelet suspension (100 μ l per well of 3×10^8 cells/ml) in RPMI.

To identify the participating integrin receptors in cell adhesion assays, isolated cell suspension were incubated separately with 20 μ g/ml of either mIgG (negative control), or function-blocking integrin mAbs at room temperature for 20 min before adding the the cell suspension to laminin-coated wells.

3.3.2 Cell migration assay (paper I, III, IV)

Transmigration of lymphocytes and monocytes through protein-coated filters was measured microscopically and by flow cytometry as described in previous study (Wondimu, Geberhiwot et al. 2004). Briefly, cells were added to the top of polycarbonate Transwell culture inserts coated with the proteins of interest and incubated at 37°C for 3 hr in the presence or absence of chemoattractant in the lower chamber. After incubation, the number of transmigrated cells was determined microscopically and the percentage of lymphocytes and monocytes in the cell population was established by flow cytometry. For migration of human KG1C glioma cells, the cells were incubated for 18 hours, and the number of attached cells to the lower side of the filter was determined microscopically after staining. To identify the

participating integrin receptors in the migration assays, the cell suspensions were pretreated with the antibodies (20 µg/ml) at room temperature for 20 min before the mixture was added to the laminin-coated filters.

3.3.3 Cell proliferation assay (paper IV)

Cell proliferation, measured as ³H-thymidine incorporation, was performed in triplicate in 96-well microtiter plates (Costar Corp., Cambridge, MA, USA) coated overnight with 0.5µg/ml of mAb UCHT1 to CD3 (BD Biosciences). Following three washes with PBS, wells were coated with 2.5µg/ml of either HSA or various Lm isoforms for 4 hours at 37°C. Thereafter, purified CD4 T cells resuspended in AIM-V serum-free medium (Life Technologies, Inc.) were cultured in the plates (4x10⁴ cells/well) for 4 days at 37°C in 5% CO₂ humidified air, and pulsed with 1 µCi/well of (methyl-³H) thymidine (Amersham, UK) for the final 12 hours. Cells were then harvested onto filter paper using a semiautomatic cell harvester (Tomtec Inc., Orange, CT) and ³H-thymidine incorporation was measured in a liquid scintillation counter (Wallac Sverige AB, Stockholm, Sweden).

3.3.4 Lymph node cell number in Lmα4-deficient mice (paper IV)

Lmα4 KO mice were generated by gene targeting in embryonic stem cells, as previously described (Thyboll et al. 2002). Wild-type (WT, +/+) and knock-out (KO, -/-) adult male mice were used to determine the cell number in peripheral lymph nodes. Cells were isolated from inguinal lymph nodes by using a mesh iron screen. Total isolated cells resuspended in 0.5 ml PBS were quantified by flow cytometry in a FACSort (BD Biosciences, San Jose).

3.3.5 Platelet P-selectin expression and cell aggregation (paper II)

P-selectin expression on the cell surface of platelets was measured by using Coulter EPICS XL-MCL flow cytometer as described previously (Hjemdahl et al. 1994). Briefly, within three min of collection, 5 µl of blood was added to 45 µl of HEPES-buffered saline (150 mM NaCl, 5 mM KCl, 1 mM Mg SO₄, 10 mM HEPES, pH 7.4) containing FITC-conjugated mAb AC1.2 to P-selectin (CD62P) (BD Biosciences, Stockholm) and different concentrations of adenosine diphosphate (ADP) (Sigma-Aldrich) in the presence or absence of either rhLm-411 and rhm-511, and incubated for 20 min at room temperature. Labeled, fixed and diluted samples were then analyzed by flow cytometry. After gating of the platelets, the percentage of P-selectin positive cells and their mean fluorescence intensity was determined in the platelet population.

Platelet aggregation studies were performed with a four-channel platelet aggregation profiler (PAP-4, Bio/Data Corporation, Hatboro, PA, USA) using 200 µl of platelet rich plasma (PRP) as described previously (Wallen et al. 1993). Briefly, the individual's sensitivity to ADP was tested by an initial dose-response procedure to establish the ADP concentration eliciting 80% aggregation (to allow the detection of both inhibition and further stimulation by another agent). After adding PBS, rhLm-411 or rhLm-511 to the PRP, the change in light transmission without and with ADP (ED₈₀ concentration) was measured during four min to study the effect of laminins. In both functional assays, rhLms were tested at 2 nM and 20 nM, which correspond to approximately 1.5 µg/ml and 15 µg/ml. For most collagen types, a concentration of 2 mg/ml is sufficient to induce platelet responses such as cell aggregation (Tryggvason et al. 1981).

Name of mAbs	Specificity	Source	Papers
EB7	Lm α 1	I.V	I
5H2	Lm α 2	Chemicon	I, III
4H8	Lm α 2	Sigma	I
BM-2	Lm α 3	I.V	III
P3H9-2	Lm α 3	Chemicon	I, IV
8C10	Lm α 4	M.P	I
168FC10	Lm α 4	I.V	III
6A12	Lm α 4	M.P	IV
6C3	Lma4	M.P	II, III
3H2	Lm α 4	M.P	II
4C7	Lm α 5	E.E, Dako	I, III
11D5	Lm α 5	E.E	I, II
15H5	Lm α 5	K.S	I, III, IV
DG10	Lm β 1	Sigma	I, II, III
LAM-89	Lm β 1	Sigma	I
3E5	Lm β 1	E.E	I
2G6	Lm β 1	E.E	II, IV
LN-26	Lm β 1	T.S	III
C4	Lm β 2	Neomarkers	I, II, III, IV
S5F11	Lm β 2	I.V	III
17	Lm β 3	BD Biosciences	I
CAF-2	Lm β 3	H.P.E	I
GF12	Lm β 3	I.V	III
LN-41	Lm γ 1	Takara	I, II
LN-82	Lm γ 1	Takara	III
22	Lm γ 1	BD Biosciences	I, II, III
2E8	Lm γ 1	E.E	IV
113BC7	Lm γ 1	I.V	III
D4B5	Lm γ 2	Chemicon	I, III
FB12	Int α 1	Chemicon	III, IV
P1E6	Int α 2	Chemicon	II, III, IV
P1B5	Int α 3	Chemicon	III, IV
BQ16	Int α 6	Ancell	III, IV
GoH3	Int α 6	BD Bioscience	II, III, IV
9.1	Int α 7	R.K	III, IV
69.6.5	Int α V	Beckman Coulter	III, IV
IB4	Int β 2	S.W	III, IV
25.3.1	Int α L	Beckman	IV
H12	Int α L	H.W	III, IV
2LPM	Int α M	Dakopatts	III, IV
60.1	Int α M	P.B	III, IV
3.9	Int α X	Harlan Sera-Lab	III, IV
13	Int β 1	BD Bioscience	IV
6S6	Int β 1	Chemicon	III, IV
P4C10	Int β 1	Chemicon	II

SZ.21	Int β 3	Beckman Coulter	III, IV
SZ.22	IntIIb	Beckman Coulter	III, IV
LM609	Int α V β 3	Chemicon	II, III, IV
ASC-9	Int β 4	Chemicon	III, IV
P1F6	Int α V β 5	Chemicon	III, IV

E.E, Eva Engvall, **H.P.E**, Harold P. Erickson, **H.W**, Hans Wigzell; **I.V**, Ismo Virtanen
K.S, Kiyotoshi Sekiguchi; **M. P**, Manuel Patarroyo; **P.B**, Patrick Beatty ; **R.K**, Randall Kramer; **S.W**, Samuel Wright

Table 5. List of mAbs used in this thesis.

Name	Purification	Source
Human merosin (Me/C)	From placenta by EDTA and salt extraction, and size exclusion and ion exchange chromatography	Chemicon Cat. No. CC085
Human merosin (Me/L)	From placenta by EDTA and salt extraction and size exclusion and ion exchange chromatography	Life Technologies Cat. No. 12162-012
Human laminin (LN/T)	From placenta by salt extraction and unspecified mAb-affinity chromatography	Takara Cat. No. T016
Human laminin (LN/S)	From placenta by mild pepsin digestion and immunoaffinity chromatography	Sigma Cat. No. L-6274
Human laminin (LN/C)	From placenta by mild pepsin digestion and immunoaffinity chromatography	Chemicon Cat. No. AG56P
Mouse Lm-111	From Engelbreth–Holm–Swarm tumor	BD Biosciences Cat. No. 354239
Purified human Lm-332	From conditioned medium of SCC25 cells by Immunopurification	Rousselle et al. 1994.
Recombinant Lm β 1	From a baculovirus expression system by chromatography	Pikkarainen et al. 1992
Recombinant Lm γ 1	From a baculovirus expression system by chromatography	Pikkarainen et al. 1992
Recombinant human Lm-211	From a mammalian expression system by affinity chromatography (furin-processed)	Smirnov et al. 2002
Recombinant human Lm-411	From a mammalian expression system by ion exchange and affinity chromatography	Kortesmaa et al. 2002
Recombinant human Lm-511	From a mammalian expression system by ion exchange and affinity chromatography	Doi et al. 2002

Table 6. List of proteins used in this thesis.

4 RESULTS AND DISCUSSION

4.1 MOLECULAR CHARACTERIZATION OF PLACENTA LAMININ PREPARATIONS (PAPER I).

While many groups have previously used commercial laminin preparations isolated from human placenta in cell functional studies, the biochemical and structural characteristics of these preparations are largely unknown. In addition, our observation of opposing effects of various placental 'Lm-511' in monocyte migration assays prompted us to carry out a comprehensive study of these preparations. Accordingly, we characterized Me/C, LN/T, LN/S and LN/C placental Lm preparations by ELISA, silver staining and Western blotting, in comparison to mouse Lm-111, and rhLm-211, rhLm-411 and rhLm-511. We also tested the cell migration-promoting activity of different batches on human KG1C glioma cells.

By ELISA, all Lm preparations except for LN/S, were highly reactive with mAbs to Lm β 1 and Lm γ 1 chains. LN/S reacted with the former, but not with the latter, mAb (Fig. 1A, paper I). However, recognition of all preparations by mAbs to Lm α 1, Lm α 3, Lm β 3 and Lm γ 2 was negligible. Me/C and LN/T reacted weakly with mAb to Lm β 2. Contamination with FN was also observed. As expected, the recombinant Lms reacted with mAbs to their respective Lm α chains. By silver staining and Western blotting, polypeptides of Lm chains comprising small and large fragments, a mixture of Lm isoforms and contamination with other ECM protein (FN) were observed. Two batches of the same products (LN/C) and different Lm preparations (Me/C and Me/L) also reacted differently by ELISA and WB (Fig. 2A, B Paper I). A major contamination of α 2-Lm with α 4-Lm was observed in a "merosin" product purchased from Life Technologies (Me/L) (Fig. 2A, Paper I). Similar Lm preparations, which have been previously characterized, also revealed a mixture of Lms, such as Lm-511 and Lm-521 (Ferletta and Ekblom 1999; Geberhiwot et al. 1999; Spessotto et al. 2003). In functional studies, major differences between various laminin preparations, and between different batches of the same preparation were demonstrated, indicating molecular heterogeneity (Fig. 2A, B, Paper I). Compared to recombinant Lms, several commercial placental Lm preparations are poor promoters of leukocyte migration (Gorfu et al., paper IV, Gorfu et al., paper III). The major isoform of each preparation, though often proteolytically degraded and/or contaminated with fibronectin, was: LN-2 (α 2 β 1 γ 1) in Me/C, Lm-211/411? in LN/T, and LN-10 (α 5 β 1 γ 1) in LN/S and LN/C. A schematic representation of the laminins was proposed (Fig. 2C).

The characterization of commercial placenta laminins revealed major molecular differences among the preparations, which may be attributed to the different purification protocols. Accordingly, EDTA and/or salt extraction seem to be more selective for α 2- and α 4-laminins, whereas mild pepsin digestion seems to be necessary for isolation of α 5-laminins. In the latter preparation, the coiled-coil region of laminin is preserved but the short arms and/or the globular domain may be eventually removed by the proteolysis. Moreover, the particular laminin chain-specific mAb used in immunoaffinity chromatography is another decisive determinant. Considering the heterogeneity of the placenta laminin preparations, as well as their proteolysis and contamination with other matrix proteins, previous data obtained in functional studies using these preparations need to be interpreted with caution and may require revision. Future functional studies demand prior molecular characterization of the laminins, particularly their α -chain.

4.2 EXPRESSION, RECOGNITION AND FUNCTION OF LAMININ ISOFORMS IN MEGAKARYOCYTIC CELLS AND PLATELETS.

4.2.1 Erythromegakaryocytic cell lines synthesize Lm-511 and -521 (Paper II).

Megakaryocytes, the platelet precursors, could synthesize the platelet Lms, and/or secrete them for their own use as adhesive substrates. To determine Lm synthesis by megakaryocytic cells, HEL and DAMI cell lines were used, as they strongly express $\beta 3$ integrin gpIIb/IIIa and other markers of the megakaryocytic lineage (Martin and Papayannopoulou 1982; Greenberg et al. 1988). Previous clues of Lm $\beta 2$ transcript in these cells (Geberhiwot et al. 1999; Geberhiwot et al. 2000) together with the immunohistochemical reactivity of a mAb to Lm $\beta 2$ with megakaryocytes in bone marrow (Vogel et al. 1999) have suggested the existence of Lm isoform(s) additional to Lm-411 in these cells. Our RT-PCR studies in HEL cells reproduced the detection of transcripts for Lm $\beta 2$ and the Lm-411 chains and demonstrated, for the first time, the presence of Lm $\alpha 5$ transcripts (Fig. 1A).

To further analyze Lm synthesis in HEL cells, metabolic labeling was performed, followed by immunoprecipitation of the cell lysate with Lm chain specific mAbs. After gel electrophoresis of immunoprecipitates, specific bands of expected size were revealed corresponding to Lm $\alpha 5$, Lm $\beta 1$ and Lm $\gamma 1$.

To unambiguously demonstrate the identity of Lm chains and their association, mAb 4C7 (Lm $\alpha 5$)-immunoprecipitate from non-radioactive HEL cell lysate was analyzed for Western blotting (Fig. 1C). In accordance with the metabolic labeling studies, specific bands corresponding to Lm $\alpha 5$, Lm $\beta 1$ and Lm $\gamma 1$ were detected.

Altogether, the results from RT-PCR, metabolic labeling and immunoprecipitation/Western blotting demonstrated synthesis of $\alpha 5$ -Lm by erythromegakaryocytic HEL and DAMI cells. These results strongly suggest that platelet $\alpha 5$ -Lms, as Lm-411, are synthesized by the platelet precursors in bone marrow, like other large adhesive proteins present in platelets, such as vWF and FN. On the other hand, fibrinogen is synthesized by other cells and taken up by megakaryocyte/platelets into granules through endocytosis.

4.2.2 Blood platelets contain and, following stimulation, secrete Lm-511 and -521 (Paper II).

To study the localization of Lm-511 and -521 in blood platelets, immunofluorescence flow cytometry was carried out in isolated intact and permeabilized platelets. Intact cells were minimally reactive, whereas practically all platelets reacted with mAbs to Lm $\alpha 5$, Lm $\beta 2$ and Lm $\gamma 1$ and, to a lower extent, LN $\beta 1$ chains, following permeabilization. We went further and determined the identity of Lm chains and their association by using mAb 4C7 (Lm $\alpha 5$)-immunoprecipitate from platelet cell lysate followed by Western blotting. Specific bands corresponding to Lm $\beta 1$, Lm $\beta 2$, Lm $\gamma 1$ and Lm $\alpha 5$ were detected (Fig. 3A). The 190 kDa band of Lm $\beta 2$ was stronger than the 230 kDa band of Lm $\beta 1$, suggesting that Lm-521 was more abundant than Lm-511 in platelets. Since Lm-411 had been previously found in these cells, we compared in parallel this $\alpha 4$ -laminin with the novel $\alpha 5$ -laminins by immunoprecipitation with the respective mAbs to their α chains (Fig. 3B). Western blot analysis of the mAb 3H2 (LN $\alpha 4$)-immunoprecipitate demonstrated specific bands

corresponding to Lm β 1, Lm γ 1 and Lm α 4, as well as a weak 190 kDa band corresponding to Lm β 2, indicating small amounts of Lm-421 (Fig. 3B). Roughly, the amounts of α 4- and α 5-laminins in the platelet lysate were similar, as determined by reactivity with mAb 22 to their common Lm γ 1 chain, but Lm α 4 and Lm α 5 chains preferentially associated to Lm β 1 and Lm β 2 chains, respectively. Thus, the results indicated intracellular presence of Lm-411 and Lm-521 in blood platelets, as well as smaller amounts of Lm-421 and Lm-511. Though both HEL/DAMI cells and platelets contained Lm-511 and Lm-521, the proportion between these two α 5-laminins differed between the cell types. The dominant presence of Lm-521 over Lm-511 in fresh platelets may be more physiologically relevant, considering the fact that HEL/DAMI cells are of malignant origin and have been cultured for several years. The molecular mechanisms responsible for the preferential association of Lm α 5 with Lm β 2, and of Lm α 4 with Lm β 1 in platelets are presently unknown. Moreover, natural processing of α 5 chain in platelets may account for the reactivity of a major 300 kDa form of Lm α 5 in platelets compared to the 350 kDa of HEL/DAMI cells and rhLm-511.

Platelet Lms may be biologically relevant once in the extracellular microenvironment, following secretion from stimulated cells. A previous study has demonstrated thrombin- and PMA-induced secretion of Lm γ 1-containing laminin(s) from platelets within a few minutes, indicating that both physiological and pharmacological stimuli are able to induce secretion of Lms, possibly of both α 4- and α 5-laminins. To address the potential secretion of α 5-laminins by platelets, the supernatant of TPA-stimulated platelets was analyzed by Western blotting following Lm α 5-immunoprecipitation (Fig. 4A). As in the platelet lysate, both Lm-511 and Lm-521, with a more intense Lm β 2 band than the Lm β 1 band, were detected in the secreted material. Occasionally, a lower band around 225 kDa was observed, which may correspond to either a large fragment or a shorter form of Lm α 5. As expected, Lm-411 was also detected in the supernatant of activated platelets, together with minor amounts of Lm-421 (Fig. 4B). These results indicated secretion of both α 4- and α 5-laminins by activated platelets, identifying these Lms as novel platelet secretory products, and confirming the preferential association of LN α 5 and LN α 4 chains with LN β 2 and LN β 1 chains, respectively, as observed in the platelet lysate.

Lms may be localized in platelet α granules, like fibronectin and other large adhesive structures (Rendu and Brohard-Bohn 2001). However, this remains to be proven. By releasing Lms and other adhesive proteins, activated platelets may promptly provide a provisional extracellular matrix and hence contribute to early vascular repair and wound healing after vascular/tissue injury. Platelets themselves, vascular endothelial cells, pericytes or other cell types could use these Lms.

4.2.3 Lm-511 is highly adhesive for platelets via α 6 β 1 integrin (Paper II)

Lms are implicated in several cellular functions. In order to analyze the interaction of platelets with vascular endothelial Lm isoforms, a static adhesion assay was employed where isolated platelets were incubated with immobilized rhLm-411 and rhLm-511. In addition, mLm-111 and HSA were also tested. Constitutive platelet adhesion to all three Lm isoforms was higher than to HSA. However, the level of cell adhesion to Lm-511 was by far higher than to the other Lms and, when compared to HSA, highly statistically significant. Though both rhLm-411 and rhLm-511 were more adhesive than HSA for isolated platelets, the level of cell adhesion on rhLm-511 was higher than that on rhLm-411. These results indicate that Lm α 5 is more platelet-adhesive than Lm α 4, as both Lm isoforms share Lm β 1 and Lm γ 1 chains and were

produced and purified in a similar way. Recently, a stronger cell adhesive ability of $\alpha 5$ -laminins over that of other Lm isoforms has also been described for epithelial cells, hematopoietic stem cells and vascular endothelial cells (Ferletta and Ekblom 1999; Doi et al. 2002; Gu et al. 2003). In the present adhesion assay, we employed rhLms preparations unlike previous studies that used commercial placental Lm preparations. The cell adhesion was largely inhibited by the chelating agent EDTA, indicating the requirement of extracellular divalent cations, which in turn suggests participation of integrin receptors. Among the laminin-binding integrins, platelets are known to express $\alpha 2\beta 1$, $\alpha 6\beta 1$ and $\alpha V\beta 3$, but lack $\alpha 3\beta 1$ and $\alpha 6\beta 4$ (Shattil and Newman 2004). To identify the adhesive receptor(s), function-blocking blocking mAbs to integrins, $\beta 1$, $\alpha 2$, $\alpha 6$ and $\alpha V\beta 3$ were tested on platelet adhesion to Lms-111, -411 and -511 (Fig 5B). The results indicated the predominant role of $\alpha 6\beta 1$ integrin as a receptor for all three Lm isoforms, including Lm-511. The antibody inhibition was highly statistically significant ($p < 0.001$). No effect was observed with mAb to $\text{Int}\alpha 2$, whereas mAb to $\text{Int}\alpha V\beta 3$ exerted a minimal, but not significant, inhibition on Lm-511. In other cell types, integrins $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$ and $\alpha V\beta 3$ are known to bind $\alpha 5$ -Lms (Kikkawa et al. 2000; Patarroyo et al. 2002).

4.2.4 Lm-511 induces neither platelet P-selectin expression nor platelet aggregation (Paper II)

Extracellular matrix components of extravascular sites, such as collagens, are known to be potent inducers of platelet adhesion and activation, and hence to be thrombogenic, when exposed to circulating platelets following vascular injury (Farndale et al., 2004). During de-endothelization and vascular injury, subendothelial Lms, particularly Lm-411 and Lm-511, are exposed to circulating platelets. Here, we further investigated the biological role of rhLm-411 and rhLm-511 on platelet activation, as measured by P-selectin expression and cell aggregation in PRP under conditions of fluid phase (Fig. 6). Lm-511, at two different concentrations (2 nM and 20 nM), was incapable to induce P-selectin expression in platelets. Moreover, this Lm isoform did not affect either platelet P-selectin expression induced by different concentrations of adenosine diphosphate (ADP) (Fig. 6A). ADP induced a rapid and concentration-dependent aggregation in PRP. In contrast, neither rhLm-411 nor rhLm-511 at 20 nM elicited platelet aggregation within five minutes (longer incubation times were also tested). A representative experiment is shown in Fig. 6B. Moreover, laminins had no effect on the platelet responsiveness to ADP. Reproducible results were obtained in the two assays with platelets from five different donors. Altogether, the results indicate that Lm-511 is highly adhesive for platelets, and that endothelial Lm isoforms Lm-411 and Lm-511 are unable to induce platelet activation. In accordance, other BM components, such as Lm-111, type IV collagen and perlecan (Tryggvason et al. 1981) have been shown not to induce platelet aggregation. Similar results have been obtained with commercial placental Lm preparation in most donors (Willette et al. 1994).

Elegant *in vivo* studies have demonstrated that the method used for vascular injury determines the type of platelet response (Buchanan et al. 1987). Thus, de-endothelization of rabbit carotid arteries by air perfusion did not lead to platelet accumulation onto the subendothelium, whereas vascular injury by balloon catheter stripping resulted in marked platelet accumulation. The authors concluded that the endothelial BM was not thrombogenic, and that a deeper injury in the vessel wall was required to induce a thrombogenic response. Adhesion to Lms may thus be of

importance when platelet monolayers are formed without ensuing thrombus formation.

4.3 EXPRESSION, RECOGNITION AND FUNCTION OF LAMININ ISOFORMS IN LYMPHOID TISSUES AND LYMPHOCYTES

4.3.1 Expression of Lms in HEVs (Paper III).

During lymphocyte recirculation, lymphocytes migrate through the vascular BM of HEV to lymphoid tissues. Early morphological studies reported a thick, dense and strongly negatively charged BM, distinct from that of flat endothelium (Freemont and Jones 1983; Freemont et al. 1986; Sunami-Kataoka et al. 2001). Migrating lymphocytes were often found in intimate contact with the HEV BM, either traversing or surrounding it. However the role and composition of HEV BM are poorly understood. We studied the presence of laminin chains in HEVs of the lymph node by confocal laser scanning microscopy (Fig. 1). Lm γ 1 chain immunoreactivity (B) was broad in the basal aspect of HEVs, visualized with mAb MECA-79 (A), as demonstrated by a variable yellow-red color in the merged figure (C). Immunoreactivity for Lm γ 1 chain was also found in BM of ordinary vessels (B), and in fibril-like structures outside the vessels. Merged figures for HEV and Lm α 1 (E) and Lm α 2 (F) chains, respectively, showed that these components were not found in HEVs or other vessels, but that Lm α 2 chain was present in stromal fibers. Moreover, immunoreactivity for Lm α 3 (F), Lm α 4 (G), Lm α 5 (H), Lm β 1 (I) and Lm β 2 (J) chains located variably to the BM region of practically all HEVs and other blood vessels, and to stromal fibers, suggesting expression of Lm-311/321, Lm-411/421 and Lm-511/521 by the HEV BM. Neither Lm-332 nor α 1- or α 2-Lms were detected in these vessels. Instead, immunoreactivities for Lm β 3 (K) and Lm γ 2 (L) chains were confined solely to capillaries in the germinal center area (GC). Under the present experimental conditions, neither apical staining nor double-layered BM could be observed in HEVs with the laminin antibodies, unlike previous reports (Girard and Springer 1995). Presence of lymphocytes and/or scanty pericytes within the thick BM may give such an impression. Since all HEVs expressed the same Lm chains, each HEV BM should contain simultaneously at least three different Lm isoforms. These Lms may be synthesized by either vascular endothelial cells or pericytes, or both cell types.

As other vascular BMs, the HEV BM may function as a barrier and a scaffold that regulates endothelial cell adhesion and differentiation (Kalluri 2003). For the lymphocytes, it may provide an adhesive and migration-promoting substrate, as demonstrated for Lms in the present study. Moreover, the Lms and other BM components may also be able to bind and present chemokines and other cytokines (Miyasaka and Tanaka 2004). Glycoaminoglycans and proteoglycans are thought to exert this function. A chondroitin sulfate proteoglycan, which may account for strong negative charges, has been reported to be selectively expressed by HEV BM (Sunami-Kataoka et al. 2001). We have previously described that Lm-411, a major endothelial Lm isoform, can display a chondroitin sulfate modification in its α 4-chain (Kortesmaa et al. 2002). It is possible that this form is found in HEV BM and is able to present chemokines to migrating lymphocytes. In contrast to α 4- and α 5-Lms, α 3-Lm is rarely found in blood vessels, as only vasculature of lymphoid and a few other tissues express this Lm chain (Wayner et al. 1993; Sime et al., to be published). Among the lymphoid vessels, selective staining of the capillaries in the lymphoid follicles by antibodies to β 3 and γ 2 Lm chains indicated presence of Lm-332 in this

vasculature, but not in HEVs. Similar results have been reported by Jaspars et al. (Jaspars et al. 1996). This BM Lm may be a ligand for the endothelial cells, which express the receptor $\alpha 6\beta 4$ integrin (Jaspars et al. 1996). Expression of $\alpha 5$ -Lm by LN vessels, including HEV, has been described in mice by Sorokin et al. (Sorokin et al. 1997), and broad staining of human lymph node vasculature, without specifying HEVs, with antibodies to $\alpha 3$ -, $\alpha 4$ - and $\alpha 5$ -laminins has been recently reported by Määttä et al. (Maatta et al. 2004).

4.3.2 Expression of Lms in RFs (Paper III).

As major components of the reticular network, RFs connects the subcapsular sinus to HEVs in lymphoid tissues, allowing the transport of small molecules from the afferent lymph in a conduit system (Gretz et al. 2000; Kaldjian et al. 2001; Sixt et al. 2005). Here, we studied the localization of Lm chains in RFs of the lymph node by using double immunoreaction for laminin chains and type III collagen, a major component of these stromal fibers. Figure 2 shows that immunoreactivity for type III collagen was prominent in the walls of vessels and in fiber-like structures outside them (A). Immunoreactivity for Lm $\gamma 1$ chain showed a rather similar localization (B) and the merged figure (C) showed an extensive co-localization for these proteins in the fiber-like structures. Immunoreactivity of Lm $\alpha 1$ chain was not found in the lymph node (D), while immunoreactivities for Lm $\alpha 2$ (E), Lm $\alpha 3$ (F), Lm $\alpha 4$ (G), Lm $\alpha 5$ (H), Lm $\beta 1$ (I) and Lm $\beta 2$ (J) chains showed an extensive co-localization with that of type III collagen, suggesting expression of Lm-211/221, Lm-311/321, Lm-411/421 and Lm-511/521. Immunoreactivities for Lm $\beta 3$ (K) and Lm $\gamma 2$ (L) chains were found only in the germinal center area. Lack of co-reactivity with mAbs to $\beta 3$ and $\gamma 2$ Lm chains indicated that RFs in LNs, in contrast to thymus (Drumea-Mirancea et al., 2006), do not express Lm-332. Interestingly, the punctuated pattern for $\beta 3$ and $\gamma 2$ Lm chains detected in the lymphoid follicles by us and others (Jaspars et al. 1996) has been reported to colocalize with $\alpha 6\beta 4$ integrin on follicular dendritic cells (Jaspars et al. 1996). Expression of $\alpha 2$, $\alpha 3$, $\alpha 4$, and $\alpha 5$, but not $\alpha 1$, Lms by LN RFs has also been reported by Kaldjian et al. (Kaldjian al. 2001), Määttä et al. (Maatta et al. 2004) and Sixt et al. (Sixt et al. 2005).

With a core of collagens type I and III and surrounded by BM components, RFs are largely, but not fully, ensheathed by fibroblastic reticular cells (FRCs) (Kramer et al. 1988; Kaldjian et al. 2001; Sixt et al. 2005). Nearly 10% of the surface of these interfollicular fibers appears to be accessible to migratory cells, allowing physical interaction with lymphocytes, dendritic cells and macrophages (Hayakawa et al. 1988). In addition to reticular fibroblastic cells, which probably synthesize the RFs, resident dendritic cells also appear to interact with the fibers, trapping soluble antigens from the conduit systems (Sixt et al. 2005). Lms and other outer components of the RFs may function not only as cell-adhesive substrates and *in situ* specific localization sites, but also as migration-promoting proteins for lymphocytes and other migratory immune cells (Pribila et al. 2004). These functions are crucial to the generation of immune responses.

4.3.3 Expression of Lm-binding integrins in blood lymphocytes (Paper III)

As an initial approach to study the functional relevance of those Lm chains expressed in LN HEV BM, we studied first expression of laminin-binding integrins (INTs) in lymphocytes from blood mononuclear cells by immunofluorescence flow cytometry (Fig. 3). Lymphocytes, gated by forward and side scatter, displayed

marked immunoreactivity for integrin $\alpha 6$, $\beta 1$, αL , αM and $\beta 2$ chains. A low expression of integrin $\alpha 1$, $\alpha 3$ and $\alpha V\beta 3$ was also noted. Integrin $\alpha 2$, $\alpha 7$, IIb, $\beta 4$ and $\alpha V\beta 5$ were not detected. Among Lm-binding INTs expressed by blood lymphocytes, $\alpha 6\beta 1$ was the most abundant, followed by $\alpha 1\beta 1$, $\alpha 3\beta 1$ and $\alpha V\beta 3$ at much lower levels. $\alpha M\beta 2$ INT, which has been proposed to be a Lm receptor (Wondimu et al. 2004), was detected in half of the population.

4.3.4 Lm-511 is highly adhesive for blood lymphocytes via $\alpha 6\beta 1$ integrin (Paper III)

In order to analyze the interaction of lymphocytes with Lm-511, a major vascular endothelial BM Lm isoform, during extravasation, a cell adhesion assay under static conditions was first performed. Previous studies have demonstrated the lymphocyte adhesive property of Lm-411, another endothelial BM isoform (Geberhiwot et al. 2001). Here, immobilized recombinant Lm-511 and Lm-411 were compared in cell adhesion assays with isolated blood lymphocytes. mLm-111, pFN and HSA were also used. The constitutive cell adhesion to all proteins was minimal, if any (data not shown). However, following stimulation of the cells with phorbol ester, the Lm isoforms were adhesive, Lm-511 being more active than Lm-411, followed by Lm-111 ($p < 0.01$, Fig. 4A). Lm-211 and Lm-332 were less adhesive than Lm-111, and hence poorly active (data not shown). To identify the adhesive receptor (s), function blocking blocking mAbs to integrins, $\beta 2$, $\beta 1$, and $\alpha 6$ were tested on lymphocyte adhesion to Lm-111, Lm-411, Lm-511 and pFN. Statistically significant inhibition ($p < 0.01$, Fig. 4B) was obtained with mAbs to $\alpha 6$ and $\beta 1$ integrins, but not to $\beta 2$ integrin, on Lm-411 and Lm-511 only, indicating a role of $\alpha 6\beta 1$ integrin as a receptor for both vascular laminin isoforms. Altogether, $\alpha 5$ -Lm was the most active one, and both vascular $\alpha 4$ - and $\alpha 5$ -Lm isoforms used $\alpha 6\beta 1$ INT as the primary receptor, with some contribution of $(\alpha M)\beta 2$ INT. In a previous study concerning the effect of $\alpha 4$ -Lm on blood lymphocytes, we observed in addition a higher $\alpha 6\beta 1$ INT-dependent cell adhesion to a commercial Lm-511/521 preparation (Geberhiwot et al. 2001).

4.3.5 Lm-511 predominantly promotes blood lymphocyte migration via $\alpha 6\beta 1$ integrin (Paper III).

To further analyze interactions between lymphocytes and laminin isoforms, lymphocyte migration on Lm-111, Lm-211, Lm-332, Lm-411 and Lm-511 (both recombinant, rLm-511, and from placenta, pLm-511) was studied in cell transmigration assays, and participation of integrins receptors was determined by using function-blocking mAbs. Compared to HSA, rLm-411 and rLm-511 significantly promoted lymphocyte migration in the presence of SDF-1 α as chemoattractant (Fig. 5A, $p < 0.01$ and $p < 0.001$, respectively). The level of migration on Lm-511 was by far higher than that on Lm-111, rLm-411 and pFN, and the difference with rLm-411, the other major endothelial Lm isoforms, was highly significant ($p < 0.001$). In a second group of experiments, pLm-511 was tested and compared to Lm-111, rLm-211, Lm-332 and rLm-411. This native Lm-511 was also found to strongly promote migration of blood lymphocytes (Fig. 5B, $p < 0.001$), and to a higher level than the other Lm isoforms. In decreasing order, the migration-promoting activity of the various laminin isoforms could be summarized as follows: Lm-511 > Lm-332 > Lm-411 > Lm-111 > Lm-221, indicating that Lm-511 predominantly promotes migration of blood lymphocytes compared to other Lm isoforms. Interestingly, Lm-511 was more migration-promoting than pFN, which has

been widely used in lymphocyte migration studies. The effect of function-blocking mAbs to integrins indicated that migration of blood lymphocytes on Lm-511 was largely mediated by $\alpha 6\beta 1$ integrin (Fig. 5C). On Lm-411, mAb to $\text{Int}\alpha 6$ was also inhibitory. In contrast, mAb to $\beta 2$ integrin exerted partial inhibition on both laminin isoforms but the effect was not statistically significant. On Lm-332, mAbs to $\text{Int}\alpha 3$ and $\text{Int}\beta 1$, but not to $\text{Int}\alpha 6$, inhibited significantly, as well as mAbs to $\text{Int}\alpha M$ and $\text{Int}\beta 2$, indicating participation of both $\alpha 3\beta 1$ and $\alpha M\beta 2$ integrins in the cell migration (Fig. 5D). Lm-332 has been reported to promote thymocyte migration *via* $\alpha 3\beta 1$ Int, and to be poorly adhesive for blood lymphocytes, which expressed minimal amounts of the latter INT (Wayner et al. 1993; Vivinus-Nebot et al. 2004). Similarly in the present study, Lm-332 was poorly adhesive for blood lymphocytes but significantly promoted migration of these cells *via* $\alpha 3\beta 1$ and $\alpha M\beta 2$ Ints. It should be noted that we used Lm-332 as a source of $\alpha 3$ -Lm, since Lm-311/321 were not available. $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ INTs are known to bind the LG tandem of $\text{Lm}\alpha 3$ chain, which is common to Lm-311, Lm-321 and Lm-332 (Patarroyo et al. 2002). $\alpha 3$ -Lms may be similarly important for the interaction of newly extravasated lymphocytes with epithelial BMs (Wayner et al. 1993).

In a previous study from our group, a commercial Lm-511/521 preparation did not promote blood lymphocytes migration (Geberhiwot et al. 2001). This discrepancy may be explained by molecular heterogeneity of these preparations, including batch-to-batch differences of the same product, as recently reported (Wondimu et al. 2006). In our present study, the migration-promoting activity of $\alpha 5$ -Lm on blood lymphocytes was superior to that of $\alpha 1$ -, $\alpha 2$ -, $\alpha 3$ - and $\alpha 4$ -Lms, as well as to that of FN, probably the most extensively used ECM protein in lymphocyte studies. The role of Lms in lymphocyte migration *in vivo* is indirectly supported by studies with mice lacking $\alpha 6A$ INT, the Lm-binding INT splice variant expressed by the immune cells (Gimond et al. 1998). In these $\alpha 6A$ INT KO mice, lymphocytes display decreased migration on an $\alpha 1$ -Lm substrate, and a reduction in the number of T-cells isolated from peripheral LNs. As a “classical” Lm receptor, $\alpha 6\beta 1$ INT binds Lms covering all α chains, but with some preference for $\alpha 5$ -Lm (Nishiuchi et al. 2006).

A study by Sixt et al. (Sixt et al. 2001) showing colocalization of extravasating mononuclear leukocytes with $\alpha 4$ -Lm, but not $\alpha 5$ -Lm, expressing brain blood vessels in experimental autoimmune encephalitis in mice has led to the assumption that the $\alpha 4$ -Lm promotes cell extravasation, whereas $\alpha 5$ -Lm is non-permissive, and even inhibitory. A more recent study of normal and pathological (multiple sclerosis) human brain has not confirmed this exclusive expression of either $\alpha 4$ - or $\alpha 5$ -Lm by brain vessels, but demonstrated instead simultaneous expression of the two isoforms by the vasculature and accumulation of leukocytes around vessels expressing $\text{Lm}\alpha 5$ (van Horssen et al. 2005). Whereas the role of vascular Lm isoforms may differ in inflammatory and homeostatic lymphocyte extravasation, our results do not support the concept of $\alpha 5$ -Lm as a non-permissive or inhibitory substrate for blood lymphocyte migration. On the contrary, functional studies, preferential $\alpha 6\beta 1$ Int recognition, and expression by HEVs strongly suggest a predominant role of this Lm isoform in lymphocyte extravasation.

4.3.6 Lm-511 co-stimulates T cell proliferation (Paper III).

Antigen-specific activation of T-cells is known to require not only CD3/TCR engagement by Ag/MHC, but also additional costimuli (Shimizu and Shaw 1991). In a previous study, our group showed a synergistic effect of Lm-411 together with mAb to CD3 to drive CD4 T-cells to proliferate (Geberhiwot et al. 2001), in line with

previous reports (Shimizu et al. 1990). The co-stimulatory signal was transduced via $\alpha 6\beta 1$ Int, the same receptor that mediated cell adhesion and migration. In the present study, we further investigated whether additional Lm isoforms expressed by monocytic, lymphoid B, and endothelial cells (Pedraza et al. 2000; Geberhiwot et al. 2001) were able to provide a similar costimulatory signal. We demonstrated extensive cell proliferation induced by both recombinant and placenta Lm-511 co-immobilized with mAb to CD3 (Fig. 5E), as measured by ^3H -thymidine incorporation. Lm-511 or mAb to CD3 on their own failed to induce any significant cell proliferation (Fig. 5E). Thus Lm-511 exhibits a stronger synergistic effect. Inasmuch as T cells normally recognize antigen present on cells (with MHC molecules) rather than on ECMs, it is unlikely that Lms may exert such an effect as ECM components. Instead, Lms may be co-stimulatory to T cells when exposed on the surface of monocytes and other antigen-presenting cells. In accordance, we recently found presence of $\alpha 4$ - and $\alpha 5$ -Lms in monocytes, and some of these moieties were localized on the cell surface (Pedraza et al. 2000) (paper IV). In contrast to other Lms, Lm-332, which is expressed by the thymus, has been described not to be comitogenic to thymocytes (Vivinus-Nebot et al. 1999).

4.3.7 Lymph node cell number in Lm $\alpha 4$ -deficient mice compared to wild type does not differ significantly (Paper III).

One approach for addressing the *in vivo* role of laminin is to produce Lm chain deficient mice and to analyze their phenotype. Recently, Thyboll et al (Thyboll et al. 2002) produced Lm $\alpha 4$ KO mice and demonstrated that these mice were normal and survived postnatally, in spite of transient hemorrhages at birth, which reflect impaired microvessel maturation. Our group used these mice and reported defective recruitment of neutrophils in a peritonitis model (Wondimu et al. 2004). In the present study, these mice have been used to further investigate the role of laminins in lymphocyte biology *in vivo*, by comparing the cell number of inguinal lymph nodes in Lm $\alpha 4$ -deficient and wild type mice (Fig. 6). A slightly higher cell number was found in the wild type animals, but this difference was not statistically significant ($p=0.67$), suggesting no significant alteration in the LN cell number. Given that lymphocyte trafficking (homing and egress), proliferation and survival in the lymphoid tissue may all affect the LN cell number, this result does not support a pivotal role of $\alpha 4$ -Lms in these lymphocyte activities, despite its role in lymphocyte adhesion and migration in *in vitro* studies. It may, instead, suggest a major functional compensation by $\alpha 5$ -Lms. Lm $\alpha 5$ genetic deletion leads to death late in embryogenesis (Miner et al. 1998). Thus, production of Lm $\alpha 5$ conditional- knockout mice where the Lm $\alpha 5$ deficiency is restricted to blood vessels may delineate the role of Lm $\alpha 5$ in lymphocyte trafficking *in vivo*. Interestingly, mice deficient in $\alpha 2$ -Lms, which survive and develop a neuromuscular disorder, show reduction in thymus size and number of thymocytes compared to littermates (Magner et al. 2000). However, this Lm chain is irrelevant to blood lymphocyte extravasation as it is not expressed by HEVs. No immunological abnormalities have been reported in Lm $\alpha 3$ KO mice, which die around birth due to defects in skin and other epithelia (Ryan et al. 1999).

4.3.8 Blood lymphocytes contain and, following stimulation, secrete Lm- 511 and Lm-521 (Paper III)

We have previously described synthesis and secretion of $\alpha 4$ -Lm, as Lm-411, by lymphoid cells (Geberhiwot et al. 2001). In the present study we further investigated synthesis and secretion of $\alpha 5$ -containing Lms in blood lymphocytes. Initially, we

searched for expression of $\alpha 5$ -laminins by blood lymphocytes, detecting the presence of mRNAs encoding for Lm-511 and Lm-521 chains by RT-PCR using pairs of primers based on the reported cDNA sequences of human laminin $\alpha 5$, $\beta 1$, $\beta 2$ and $\gamma 1$ chains. Amplified products with the expected size were detected with primers for $\alpha 5$ (655), $\beta 2$ (632) and $\gamma 1$ (687) after 30 cycles of PCR with reverse transcripts from blood lymphocytes (Fig. 7A), suggesting synthesis of Lm-521. Results for $\beta 1$ transcripts were inconclusive. Moreover, the weak Lm bands obtained may reflect low amount of transcripts present in these resting cells. To further address the presence of $\alpha 5$ -laminins in blood lymphocytes, immunofluorescence flow cytometry was performed. Lymphocytes, gated by forward and side scatter from PBMC, displayed reactivity with mAbs to CD2, a marker of most lymphocytes, as well as to Lm $\alpha 5$, $\beta 1$, $\beta 2$ and $\gamma 1$ chains. These mAbs did not react with intact lymphocytes, whereas practically all cells were stained with the antibodies following cell permeabilization (Fig. 7B), showing intracellular expression of Lms in lymphocytes. In early studies, cell surface reactivity of polyclonal anti-Lm-111 antibodies with mouse, rat and human NK cells was demonstrated (Geberhiwot, Assefa et al. 2001). We occasionally observed a cell surface reactivity of mAbs to $\alpha 4$ -, $\alpha 5$ -, $\beta 1$ - and $\gamma 1$ -Lm chains with a minority of blood lymphocytes from a few, but not most, donors. This difference may be due to variability in the percentage of NK cells and/or their cell activation state among donors, and indicated that $\alpha 4$ - and/or $\alpha 5$ -Lms may correspond to the Lm moieties detected in the early studies.

To further investigate expression of $\alpha 5$ -laminins, the cell lysate of isolated blood lymphocytes was immunopurified on a mAb 4C7 (Lm $\alpha 5$)-Sepharose column, and the purified material was then analyzed by Western blotting. Under reducing conditions, specific bands for Lm $\beta 1$, Lm $\beta 2$, Lm $\gamma 1$ and Lm $\alpha 5$ were recognized as polypeptides of 230, 190, 220 and 350 kDa, respectively (Fig. 7C, upper panel). The intensity of Lm $\beta 1$ and $\beta 2$ bands suggested that Lm-521 was more abundant than Lm-511. In the unfractionated supernatant of blood lymphocytes stimulated with phorbol ester for 20 min, Lm $\beta 1$, $\beta 2$ and $\gamma 1$ were also detected, in addition to Lm $\alpha 4$ chain (Fig. 7C, lower panel), and Lm $\alpha 5$ appeared as smaller 300/280 kDa bands (Fig. 7C, lower panel). This is the first report in leukocytes demonstrating synthesis and secretion of $\alpha 5$ -Lms, mainly as Lm-521 with some Lm-511, similar to a recent report on platelets, where $\alpha 5$ -Lm chain associated more to $\beta 2$ - than to $\beta 1$ -Lm chains (Nigatu et al. 2006).

4.4 EXPRESSION, RECOGNITION AND FUNCTION OF LAMININ

ISOFORMS IN MONOCYTES

4.4.1 Vascular laminin isoforms Lm-411 and Lm-511 mediate adhesion and strongly promote migration of blood monocytes (paper IV).

During extravasation to tissues, monocytes encounter vascular endothelial Lm isoforms. In order to address the role of these Lm isoforms, we first studied the ability of endothelial Lm isoforms Lm-411 and Lm-511 to mediate adhesion of blood monocytes (Fig. 1A). Lm-111 and pFN were tested for comparison. When compared to PBS (uncoated plastic), all three Lm isoforms were significantly adhesive and to a similar extent, but less than pFN. Under the present experimental conditions, no major differences in monocyte adhesion were observed in absence or presence of phorbol ester, and the cells adhered also to HSA (data not shown). The adhesion to Lms was significantly higher than to uncoated surfaces, but lower than adhesion on HSA or pFN. Using a different isolation protocol which yields a lower degree of

monocyte purity, Jiang et al. (Jiang et al. 1994) reported minimal adhesion of monocytes to Lm-111-coated surfaces and stimulation of the cell attachment by treatment with the chemokine MCP-1. The relatively high constitutive adhesion found in our present study may reflect activation of the cells during isolation. In cell migration assays, Lm-111, Lm-411 and Lm-511 strongly promoted monocyte migration in presence of SDF-1 α (Fig. 1B). The effect of all three Lms was similar, highly significant when compared to HSA, and, in contrast to the cell adhesion assay, higher than that of pFN. In absence of chemokine, monocyte migration on the various substrata was low (data not shown). Similarly, by using MCP-1 and fMLP as chemoattractants, Penberthy et al., (Penberthy et al. 1995) demonstrated that monocytes migrated more effectively on Lm-111 compared to other ECM proteins, including FN, type I collagen and type IV collagen.

Monocytes account for 5-20% of the mononuclear population in blood (Jones et al. 1989). It is essential to note that technical difficulties associated with monocyte isolation and purification has previously constituted major problems to study monocyte-ECM interactions. As a result, our group used in an early study the monocytic cell line JOSK (Pedraza et al. 2000), and demonstrated that constitutive adhesion of these cells on Lm-411 was mediated by $\alpha 6\beta 1$ and $\beta 2$ integrins. Moreover, the activation status of monocytes after purification may account for some of the discrepancy between different studies.

4.4.2 Monocytes do not, or only minimally, express the “classical” Lm-binding integrins (Paper IV).

Among the 24 integrin heterodimers, nearly 10 are known to bind distinct Lm isoforms. To identify receptors mediating the monocyte interactions with the Lms, we determined first expression of laminin-binding integrins on the cells by immunofluorescence flow cytometry (Fig. 2). Since platelets and platelet particles are known to bind strongly to the monocyte surface, particular caution was taken to avoid platelet contamination. Surprisingly, under the present experimental conditions, “classical” Lm-binding integrins $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$ and $\alpha 6\beta 4$ were absent or minimally expressed on blood monocytes (Fig. 2A). Moreover, other integrins such as $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha V\beta 3$ and $\alpha V\beta 5$, previously reported in a few studies to interact with Lms, were similarly undetectable or expressed at very low levels. Minimal expression of $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins by fresh monocytes has also been reported by Ammon et al. (Ammon et al. 2000). Interestingly, upregulation of the latter integrins may occur during differentiation towards macrophages, but not towards dendritic cells (Prieto et al. 1994; Ammon et al. 2000). As expected, $\beta 2$ integrins were strongly expressed by practically all monocytes, and the levels of $\alpha L\beta 2$ and $\alpha M\beta 2$ were higher than those of $\alpha X\beta 2$. These leukocyte-specific adhesion proteins are not widely recognized as Lm-receptors.

It is often stated in literature that blood monocytes express considerable amounts of Int $\alpha 6$ (CD49f) (Shang and Issekutz 1999). Fig. 2B illustrates, in two different blood donors, that a reduction of platelet marker CD41 reactivity on monocytes by extensive washing paralleled a decrease in Int $\alpha 6$ expression. It also shows that blood monocytes, gated by forward and side scatter, positive for CD14 and minimally reactive with CD41 mAbs (“clean” monocytes of donor 1), exhibited negligible reactivity with mAbs to Int $\alpha 6$. In platelets, Int $\alpha 6$ was highly expressed, whereas Int $\alpha 3$, Int $\alpha 7$ and Int $\beta 4$ were not detectable (Fig. 2B and data not shown). Our present results indicated that reduction of expression of CD41 in blood monocytes by extensive washing decreased in parallel Int $\alpha 6$ reactivity, suggesting that most, if not

all, of the $\text{Int}\alpha 6$ expression in monocytes may be due to contaminating platelet particles (Prieto et al. 1994; Shang and Issekutz 1999). The degree of expression of CD41, and thereafter of $\text{Int}\alpha 6$, by monocytes may vary from donor to donor probably due to the activation state of both monocytes and platelets.

4.4.3 $\alpha\text{M}\beta 2$ and $\alpha\text{X}\beta 2$ integrins mediate monocyte adhesion and migration promoted by Lm-411 and Lm-511 (Paper IV).

Once we screened the integrin receptors profiles on monocytes, we identified receptors mediating the interactions of monocytes with the Lms by using function-blocking mAbs to integrins (Fig. 3). Monocyte adhesion to Lm-111, Lm-411 and Lm-511, but not to pFN, was largely and significantly inhibited by mAbs to $\text{Int}\beta 2$ and $\text{Int}\alpha\text{M}$ chains (Fig. 3A). In contrast, mAbs to $\text{Int}\beta 1$ and $\text{Int}\alpha 6$ were without effect, except for a minor inhibition of the latter mAb on the Lm-511-mediated adhesion. These results indicated $\alpha\text{M}\beta 2$ as the primary integrin mediating monocyte adhesion to all three Lm isoforms but not to pFN. $\beta 1$ integrins, including $\alpha 6$, had minor contributions, if any. Our results agreed with the inhibition of MCP-1-induced monocyte adhesion to Lm-111 by mAbs to $\alpha\text{M}\beta 2$ integrin previously reported by Jiang et al. (Jiang et al. 1994), and expanded these results to the biologically relevant vascular isoforms Lm-411 and Lm-511. Interestingly, Jiang et al. (Jiang et al. 1994) demonstrated the inhibitory effect of intact antibodies and also of Fab' fragments, excluding contribution of possible artefacts mediated by Fc receptors or cross-linking of cell surface molecules. Participation of unspecified $\beta 2$ integrins in monocyte adhesion to Lm-111 has also been reported by Bauvois et al (Bauvois et al. 1996).

Thereafter, the effect of function-blocking mAbs to integrins was tested on the monocyte migration promoted by Lm-411 and Lm-511 (Fig. 3B). Similarly to the results obtained in the adhesion assay, mAbs to $\text{Int}\beta 2$, but not to $\text{Int}\beta 1$ and $\text{Int}\alpha 6$, were highly inhibitory on both vascular Lm isoforms. Mixture of mAbs to $\text{Int}\beta 1$ and $\text{Int}\beta 2$ resulted in a inhibition slightly higher than that with mAb to $\text{Int}\beta 2$ alone. Within members of the $\beta 2$ integrin subfamily, mAb to $\text{Int}\alpha\text{M}$ exerted the highest inhibitory effect, followed by mAb to $\text{Int}\alpha\text{X}$, whose inhibitory effect reached statistical significance on Lm-411. In contrast, mAb to $\text{Int}\alpha\text{L}$ exerted no significant effect. Thus, monocyte migration promoted by vascular Lm isoforms Lm-411 and Lm-511 is mediated by $\alpha\text{M}\beta 2$ and, to a lower extent, $\alpha\text{X}\beta 2$, but not $\alpha\text{L}\beta 2$ and $\beta 1$ integrins.

4.4.4 Isolated $\text{Lm}\gamma 1$ monomer mediates adhesion and promotes migration of blood monocytes via $\alpha\text{M}\beta 2$ and $\alpha\text{X}\beta 2$ integrins (Paper IV).

The laminin α chains are often considered to be the functionally active portion of the heterotrimers as they carry the major cellular receptor binding domains, G domain and VI domain. However, the similar monocyte responses to Lm-111, Lm-411 and Lm-511 in adhesion and migration assays and the participation of identical integrins suggested recognition of a common determinant in all three Lm isoforms. Lm-111, Lm-411 and Lm-511 share $\beta 1$ and $\gamma 1$ chains, and differ in their α chain. To identify the active Lm chain, isolated recombinant Lm $\beta 1$ and $\gamma 1$ monomers were tested in functional assays (Fig. 4). Both chains were adhesive for monocytes, but only adhesion to the $\text{Lm}\gamma 1$ monomer could be significantly inhibited by mAb to $\text{Int}\beta 2$

(Fig. 4A) as well as by mAb to Int α M and, to a lower extent, Int α X, and the combination of these two antibodies was as effective as the mAb to Int β 2 (Fig. 4B).

In cell migration assays, Lm γ 1, but not Lm β 1, monomers significantly promoted monocyte migration when compared to HSA (Fig. 4C) and, by itself, it could reproduce the effect of the Lm heterotrimers. It also reached statistical significance when compared to Lm β 1. Similarly to the migration promoted by Lm heterotrimers, the Lm γ 1-promoted monocyte migration was mediated by α M β 2 and, to a lower extent, α X β 2 integrins (Fig. 4D and E). These results indicated that Lm γ 1 chain by itself was able to mediate adhesion and to promote migration of blood monocytes, and that both activities were mediated *via* α M β 2 and α X β 2 integrins. As eleven Lm chains are presently known, we cannot exclude additional recognition of Lm chains other than γ 1. α M β 2 and α X β 2 integrins are mainly expressed by myeloid cells, including monocytes, macrophages, dendritic cells and neutrophils, as well as by NK cells. These integrins share fibrinogen and iC3b as ligands, and α M β 2 has also been reported to recognize ICAM-1 and other proteins (Ley and Reutershan 2006). Though both integrins have been considered to be rather promiscuous in terms of ligand-binding, particular recognition sequences have been identified in fibrinogen (Ugarova and Yakubenko 2001).

4.4.5 Lm-332 inhibits blood monocyte migration promoted by Lm-411 (Paper IV).

Given that BMs often contain more than one Lm isoform (Miner and Yurchenco, 2004), we tested the effects of Lms covering all α chains on monocyte migration (Fig. 5). In contrast to other Lm isoforms, Lm-332 inhibited monocyte migration, when compared to HSA, and this effect was statistically significant. The inhibitory activity of Lm-332 predominated over the promoting effect of Lm-411 when the two proteins were mixed in a 1:1 ratio (Fig. 5B). Lm-332 contains Lm γ 2 chain and is preferentially expressed by epithelial BMs, but can also be expressed by blood vessels (Wayner et al. 1993; Patarroyo et al. 2002) (Paper III). With opposite effect compared to other Lm isoforms, Lm-332 may provide migration-inhibitory signals leading to adhesion, retention and tissue localization of certain leukocyte subpopulations. Since Lm-332 is commonly found in epithelial BMs together with Lm-511, it may impose migration-inhibition due to its dominant effect.

4.4.6 Blood monocytes contain α 4- and α 5-Lms and express α 4-Lm on the cell surface (Paper IV).

Our research group has previously reported presence of Lm-411 in blood monocyte cell lysate, and reactivity of mAbs to Lm γ 1 chain with these cells following permeabilization (Pedraza et al. 2000). In the present study, immunofluorescence flow cytometry analysis of permeabilized monocytes indicated presence of Lm α 4 and Lm α 5 chains, in addition to Lm β 1, Lm β 2 and Lm γ 1 (Fig. 6). However, only Lm α 4 chain was reproducibly detected on the cell-surface, most likely as Lm-411. Occasionally Lm β 1 and Lm γ 1 were also detected on intact cells (data not shown). Following permeabilization, as confirmed by myeloperoxidase reactivity, all the Lm chains were detected, including Lm α 5. As described for platelets and lymphocytes, the Lm α 5 chain detected in monocytes may constitute Lm-521 and, together with Lm-411, could be secreted following stimulation of the cells (Nigatu et al. 2006). Moreover, Lm-411 and other Lm isoforms, when expressed on the cell surface of monocytes and other antigen-presenting cells but not as ECM components, may

provide co-stimulatory signals for T cell proliferation (Geberhiwot et al. 2001). As described for platelets and lymphocytes, the Lm α 5 chain detected in monocytes may constitute Lm-521 and, together with Lm-411, could be secreted following stimulation of the cells (Nigatu et al. 2006).

4.4.7 Endogenous α 4-Lm mediates blood monocyte migration through albumin-coated filters via α M β 2 and α X β 2 integrins (Paper IV).

Monocyte migration may be dependent on endogenously secreted laminins in addition to exogenous ligands. As an initial approach to determine the role of endogenous α 4-Lm in blood monocytes, the effect of several mAbs to this chain in chemokine-induced migration of the cells through albumin-coated filters was investigated (Fig. 7A), as recently done for neutrophils (Wondimu et al. 2004). Monocyte migration on HSA, though much lower than on exogenous Lm-411, was significantly inhibited by mAbs to Lm α 4, strongly suggesting participation of endogenous Lm-411 in the cell motility. α 3-Lms appeared to contribute to a lower extent. Similar results have been obtained with neutrophils (Wondimu et al. 2004). Interestingly, as on exogenous Lm-411, the monocyte migration required both α M β 2 and α X β 2 integrins. Thus, monocytes and other leukocytes appear to be able to deposit endogenous Lm-411, and probably other Lm isoforms, and to use them as substrata for migration *via* integrin receptors. Similarly, keratinocyte motility is promoted by deposition of endogenous Lm-332 (Zhang and Kramer 1996).

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The biological role of the interaction of platelets and leukocytes with vascular laminin isoforms is beginning to emerge. With the recent identification of the major components of vascular BM, we addressed in this thesis work the role of different vascular Lm isoforms in thrombus formation and leukocyte extravasation. Based on these results the following conclusions can be drawn.

- Megakaryocytes and platelets synthesize/express heterotrimeric $\alpha 5$ -Lms, and stimulated platelets secrete $\alpha 5$ -Lms. Endothelial Lm isoform Lm-511 strongly promotes platelet adhesion, but not activation, *via* $\alpha 6\beta 1$ integrin.
- $\alpha 5$ -Lms (Lm-511), expressed by HEVs, are the most adhesive and migration-promoting isoforms for blood lymphocytes, followed by $\alpha 3$ - (Lm-332) and $\alpha 4$ - (Lm-411) laminins, and use $\alpha 6\beta 1$ integrin as a primary receptor. Moreover, $\alpha 5$ -laminins strongly co-stimulate T cell proliferation, and stimulated blood lymphocytes secrete both $\alpha 4$ - and $\alpha 5$ -laminins. Analysis of LN cell number in Lm $\alpha 4$ -deficient mice compared to wild type does not reveal any significant difference.
- Endothelial Lm isoforms Lm-411 and Lm-511 mediate adhesion and promote chemokine-induced migration of blood monocytes *via* $\alpha M\beta 2$ and $\alpha X\beta 2$ integrins. Isolated rhLm $\gamma 1$, but not rhLm $\beta 1$, monomer reproduces the effect of the Lm heterotrimers. In contrast, Lm-332 inhibits monocyte motility, including that promoted by other Lm isoforms. Monocytes contain both $\alpha 4$ - and $\alpha 5$ -laminins, and the former laminin, which is expressed on the cell surface, contributes to chemokine-induced, $\alpha M\beta 2$ and $\alpha X\beta 2$ integrin-dependent monocyte migration on albumin.

These results can explain the differential effect of laminin isoforms in the biology of platelets, lymphocytes and monocytes, and their potential contribution to hemostasis, and the generation of immune and inflammatory responses. Moreover, these findings can provide a basis for analyzing the Lm isoforms expressed in HEV BMs that could specifically bind chemokines to be presented to receptor-expressing lymphocytes. Future studies aiming to determine whether Lms expressed in HEV-like vessels mediate sustained lymphocyte recruitment leading to amplification and maintenance of chronic inflammation, would be of great interest. The generation of Lm $\alpha 5$ conditional-knockout mice will be useful to address the role of this Lm chain in lymphocyte homing to LNs. Defining the recognition sites in the Lm $\alpha 5$ chain that mediate $\alpha 6\beta 1$ integrin-dependent lymphocyte adhesion and migration may have a therapeutic potential. Determining the interaction of isolated $\alpha M\beta 2$ integrins and their ligand-binding I domain with recombinant domains of Lm $\gamma 1$ and of other Lm chains is pending further study.

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