Expression, Recognition and Usage of Laminin-8 (α4β1γ1, Lm-411) by Monocytes and Neutrophils

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Stockholm, 2004

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In memory of my brother, Solomon, my sisters Kebebush, Tegest, and Roman, my niece Hana, my brother in law Zekarias, and my friend Yitbarek Elias

Abstract

Laminins (LNs) are a family of large αβγ heterotrimeric glycoproteins found in all basement membranes (BMs). They are expressed in a tissue- and developmental stage-specific manner and implicated in vital cellular functions, including cell adhesion, migration and signaling. So far, eleven laminin chains (5α, 3β and 3γ) that assemble into 15 LN isoforms (LN-1 to 15, Lm-111 to Lm-523) have been identified. LN-8 (α4β1γ1, Lm-411) is a major LN isoform of vascular endothelial BM. Its expression and functional importance in blood platelets and lymphocytes have been documented. However, expression, recognition and utilization of LNs by blood monocytes and neutrophils are poorly understood. Monocytes and neutrophils originate from a common myeloid progenitor cell and are crucial cellular elements in both innate and adaptive immunity. In response to inflammatory signals, these leukocytes extravasate and migrate to the affected tissue. Leukocyte extravasation is a multi-step process involving sequential participation of various adhesion molecules. While the initial steps, such as leukocyte interaction with endothelium, are well characterized, the subsequent steps of leukocyte extravasation, such as migration through the vascular BM, are not well defined. In this thesis, efforts have been made to detect, isolate and functionally characterize LN-8 in monocytes and neutrophils, and to define the role of α4 LNs in leukocyte migration and extravasation.

First, we determined the chain specificity of 16 commonly used monoclonal antibodies (mAbs) to human LN by ELISA, Western blotting, and immunoprecipitation using recombinant (r) LNβ1 and LNγ1 chains. In addition, eight novel mAbs to LNα4 chain were generated and characterized. By immunohistochemistry, differential distribution of LNα4 chain in developing and adult human tissues was found. LNα4 was mainly localized in tissues of mesodermal origin, such as endothelial BM. By indirect immunofluorescence, LN-8 chains were detected in permeabilized monocytes and neutrophils, and intact LN-8 was isolated from these cells. This LN isoform was synthesized by monoblastoid cells and secreted by stimulated neutrophils. mAbs to LNα4 chain inhibited neutrophil migration through human serum albumin coated inserts, suggesting participation of the endogenous LN-8 in the cell migration. Monoblastoid JOSK-I cells adhered constitutively to rhLN-8 via α6β1 and, to a lower extent, β2 integrins, whereas stimulated neutrophils adhered to rhLN-8, rhLN-10 (α5β1γ1, Lm-511), and mouse LN-1 (α1β1γ1, Lm-111) via αMβ2 integrin. rhLN-8 strongly promoted monocyte and neutrophil migration both in the absence and presence of chemoattractants, and the migration-promoting activity on neutrophils was mediated by αMβ2 and, to a lower extent, β1 integrins. Compared to rhLN-8, several commercial LN preparations isolated from human placenta displayed lower migration-promoting activity on neutrophils, in general. These preparations contained fragmented LN chains, a mixture of LN isoforms, and/or contaminating fibronectin. In LNα4 deficient mice, neutrophil recruitment to inflamed tissue was significantly impaired. rhLN-8 also protected neutrophils against spontaneous apoptosis.

Altogether, the results from these studies indicate that both monocytes and neutrophils express LN-8, and that this laminin isoform can be secreted by the cells. In addition, LN-8 plays a major role in the physiology of myeloid cells, including their adhesion, migration, extravasation and survival. The results also indicate that αMβ2 integrin may constitute a novel receptor for LN-8 and other LN isoforms.

Key words: Basement membrane, laminins, myeloid cells, extravasation, migration.

List of publications

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

I Geberhiwot, T.,* **Wondimu, Z.,*** Salo, S., Pikkarainen, T., Kortesmaa, J., Tryggvason, K., Virtanen, I., and Patarroyo, M. (2000). Chain specificity assignment of monoclonal antibodies to human laminins by using recombinant laminin β1 and γ1 chains. *Matrix Biol*. 19:163-167.

II Petäjäniemi, N., Korhonen, M., Kortesmaa, J., Tryggvason, K., Sekiguchi, K., Fujiwara, H., Sorokin, L., Thornell, LE., **Wondimu, Z.,** Assefa, D., Patarroyo, M., and Virtanen, I. (2002). Localization of laminin α4-chain in developing and adult human tissues. *J. Histochem. Cytochem*. 50:1113-1130.

III Pedraza, C., Geberhiwot, T., Ingerpuu, S., Assefa, D., **Wondimu, Z.**, Kortesmaa, J., Tryggvason, K., Virtanen, I., and Patarroyo, M. (2000). Monocytic cells synthesize, adhere to, and migrate on laminin-8 (α4β1γ1). *J. Immunol*. 165:5831-5838.

IV **Wondimu, Z.**, Geberhiwot, T., Ingerpuu, I., Juronen, E., Xie, X., Lindbom, L., Doi, M., Kortesmaa, J., Thyboll, J., Tryggvason, K., Fadeel, B., and Patarroyo, M. (2004). An endothelial laminin isoform, laminin 8 (α4β1γ1), is secreted by blood neutrophils, promotes neutrophil migration and extravasation, and protects neutrophils from apoptosis. *Blood* 104:1859-1866.

V **Wondimu, Z.,** Gorfu, G., Tryggvason, K., and Patarroyo, M. Immunochemical and functional characterization of commercial laminin preparations from human placenta in comparison to recombinant laminins 8 (α4β1γ1) and 10 (α5β1γ1). *Manuscript.*

* Equal contribution

Other relevant publication by the author

Geberhiwot, T., Assefa, D., Kortesmaa, J., Ingerpuu, S., Claudio, P., **Wondimu, Z**., Charo, J., Kiessling, R., Virtanen, I., Tryggvason, K., and Patarroyo, M. (2001). Laminin-8 (α4β1γ1) is synthesized by lymphoid cells, promotes lymphocyte migration and costimulates T cell proliferation. *J. Cell Sci*.114:423-433.

Contents

List of abbreviations

BM:	Basement membrane
Col IV:	Collagen type IV
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
ECM:	Extracellular matrix
EDTA:	Ethylenediamine tetra acetic acid
FBS	Fetal bovine serum
ELISA:	Enzyme linked immuno sorbent assay
FcR:	Fc receptor
FITC:	Fluoreiscin isothiocyanate
fMLP:	Formyl methionyl leucyl phenylalanine
HSA:	Human serum albumin
HSC:	Haematopoietic stem cell
ICAM:	Intercellular adhesion molecule
Ig:	Immunoglobulin
IL:	Interleukin
kDa:	Kilodalton
KO:	Knockout
LN:	Laminin
LT:	Leukotrine
mAb:	Monoclonal antibody
NK:	Natural killer
pAb:	Polyclonal antibody
PBMC:	Peripheral blood mononuclear cells
PBS:	Phosphate buffered saline
PMSF:	Phenylmethylsulfonyl fluoride
PMN:	Polymorphonuclear leukocyte
rh:	Recombinant human
RNA:	Ribonucleic acid
RT:	Room temperature
RT-PCR:	Revese transcriptase polymerase chain reaction
SDS-PAGE:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TPA:	Tetradecanoylphorbol acetate
WB:	Western blotting

1 Introduction

For quite a long time, laminins (LNs) were believed to be the exclusive product of solid tissue-forming cells, like epithelial, endothelial and muscle cells. Recent report on expression and usage of LN-8 (α4β1γ1, Lm-411) by blood platelets and lymphocytes, particularly its effect on cell adhesion and migration, was both an inspiration and a challenge for the work of this thesis. LN-8, as a major LN isoform of vascular endothelial basement membrane (BM), together with LN-10 (α5β1γ1, Lm-511), may interact with blood cells during hemostasis and inflammation. Neutrophils and monoytes are leukocyte populations that migrate to the site of tissue injury during acute inflammatory responses to perform phagocytosis and other effector functions. These cells are highly migratory and patrol the tissues constantly. On their way from blood stream to the injured tissue, leukocytes encounter LN-8 in the endothelial BM. Besides investigation on LN expression by monocytes and neutrophils, this study took into account if LNs contribute to the extravasation and migration of leukocytes. Different valuable tools and techniques, such as monoclonal antibodies (mAbs), recombinant human (rh) laminins, knock out (KO) mice, affinity chromatography, and functional assays, such as cell adherence and migration assays were employed to answer these questions.

1.1 Basement membrane

Basement membranes (BMs), also known as basal lamina, are specialized forms of extracellular matrices that are found in almost all vertebrate tissues. BMs are very thin (20-300nM), and are found in close association with cells, either surrounding them completely as is the case of muscle, fat, and nerve axons, or separating them from the underlying stroma (epithelium, endothelium). BMs are formed early during embryogenesis and perform diverse functions ranging from tissue architecture and mechanical support to cell signaling and have unique molecular composition (Ekblom and Timpl, 1996; Timpl and Brown, 1996; Aumailley and Smyth, 1998; Colognato and Yurchenco, 2000).

Laminins (LNs), together with collagen type IV (Col IV), perlecan and nidogen (entactin) constitute prominent components of all BMs. Other molecules, like chondroitin sulfateproteoglycan, bamacan, fibulin-1 and -2 and BM-40 are present only in some BMs. (Ekblom and Timpl, 1996; Aumailley and Smyth, 1998; Colognato and Yurchenco, 2000). The differences in macromolecular composition together with spatial and temporal distribution of the molecules give BMs their inherent functional and structural heterogeneity (Yurchenco and O'Rear, 1994; Timpl and Brown, 1996). The macromolecules of BM possess different domains involved in different types of interactions. Col IV and LN self assemble to form networks. The networks are bound together by nidogen and other proteins. LNs also contain cell-binding sites to interact with different cellular receptors. These interactions are important in the control of cell behavior (Aumailley and Smyth, 1998; Colognato and Yurchenco, 2000). According to the generally accepted old model, BM assembly and integrity is provided by multiple component interactions consisting of polymerization, intercomponent binding and cell-surface interactions. Recent technical advancements provided evidence that laminins are essential for the formation of an initial polymer scaffold (Yurchenco et al., 2004).

1.2 The laminins

Laminins (LNs) are a family of large (400 – 900 kDa) heterotrimeric glycoproteins composed of three genetically distinct but homologous chains termed as α, β and γ (Beck et al., 1990; Engel, 1992; Burgeson et al., 1994; Aumailley and Krieg, 1996; Aumailley et al., 2004). They constitute major structural components of all BMs throughout the vertebrate body, and are known to mediate diverse cellular functions ranging from cell-adhesion to signaling through their interaction with cellular receptors such as integrins (Timpl and Brown, 1994; Aumailley and Krieg, 1996; Ekblom and Timpl, 1996; Colognato and Yurchenco, 2000; Tunggal et al., 2000; Patarroyo 2002). At present 11 LN chains (α1–5, β1–3, γ1–3) that assemble to form at least 15 LN isoforms (LN-1 to -15, Lm-111 to Lm–523) (Colognato and Yurchenco, 2000; Libby et al., 2000; Tunggal et al., 2000) (Aumailley et al., 2004) have been identified. These LN isoforms are expressed in a tissue- and developmental stage-specific manner (Aumailley and Krieg, 1996; Ekblom et al., 1998). LN-8 is expressed by endothelial and smooth muscle cells (Aumailley and Smyth, 1998), blood platelets and lymphocytes, and promotes adhesion and migration of the leukocytes *in vitro* (Geberhiwot et al., 1999; Geberhiwot et al., 2001). At present, the expression, recognition and use of LNs by blood monocytes and neutrophils are still not well understood.

1.2.1 Structure and domains

Each LN is formed by disulphide-linkage of one α, one β and one γ chain (Beck et al., 1993). All LN chains share a common structural and domain homology, even though they vary in their amino acid sequence (Fig 1). The chains can be full length or truncated. Those isoforms composed of the three full length chains (LN-1/Lm-111, LN-2/Lm-211, LN-3/Lm-121, LN-4/Lm-221, LN-10/Lm-511, LN-11/Lm-521, LN-12/Lm-213, and LN-15/Lm-523) adopt non-asymmetrical cross-shape with one long arm and three short arms. The long arm is a common structural domain to all isoforms where trimerization takes place (Beck et al., 1990). Isoforms with one truncated short arm (LN-8/Lm-411, LN-9/Lm-421, LN-13/Lm-323, and LN-14/Lm-423) adopt T or Y shape. In certain situation where all the three short arms are truncated, the laminin can take dumbbell shape (LN-5/Lm-332). The three structural features, common to all the three chains are the small globular domains (domain IV and VI), rod like domains, rich in cysteine (domain III and V) and the α-helical heptad repeats (domain I and II). Domain I and II, located at the carboxy-terminus (C-terminus) of each chain are involved in chain assembly, forming the α-helical coiled-coil trimmer (Beck et al., 1990). The full length α chains contain additional domains IIIa and IVa in the N-terminus region. The large globular domain (G domain), composed of five homologous repeats G1-G5, at the most C-terminus region is a common structural feature to all α chains (Beck et al., 1990). A 40 amino acid long sequence between domain I and II looped out of the long arm is unique to the β chain and termed as domain α or β-knob (Engvall and Wewer, 1996; Aumailley et al., 2004).

LNs possess different binding sites that are involved in self-assembly, and in the interaction with other BM macromolecules and cell-surface receptors (Aumailley and Smyth, 1998). These interactions are central for structural integrity and tissue organization as well as signaling. Domain III on γ1 chain contains a binding site for nidogen. As nidogen has also a binding site for Col IV, it acts as a bridge between LN

and Col IV. The five homologous regions, G1-G5, constituting the long-arm globule harbor binding sites for cell-surface receptors such as integrins and dystroglycans, and other ECM proteins such as heparan sulfate proteoglycans. LNs have many active sites, and some of them are cryptic, that become available after proteolytic processing, increasing the functional diversity of LN isoforms (Kleinman et al., 2003).

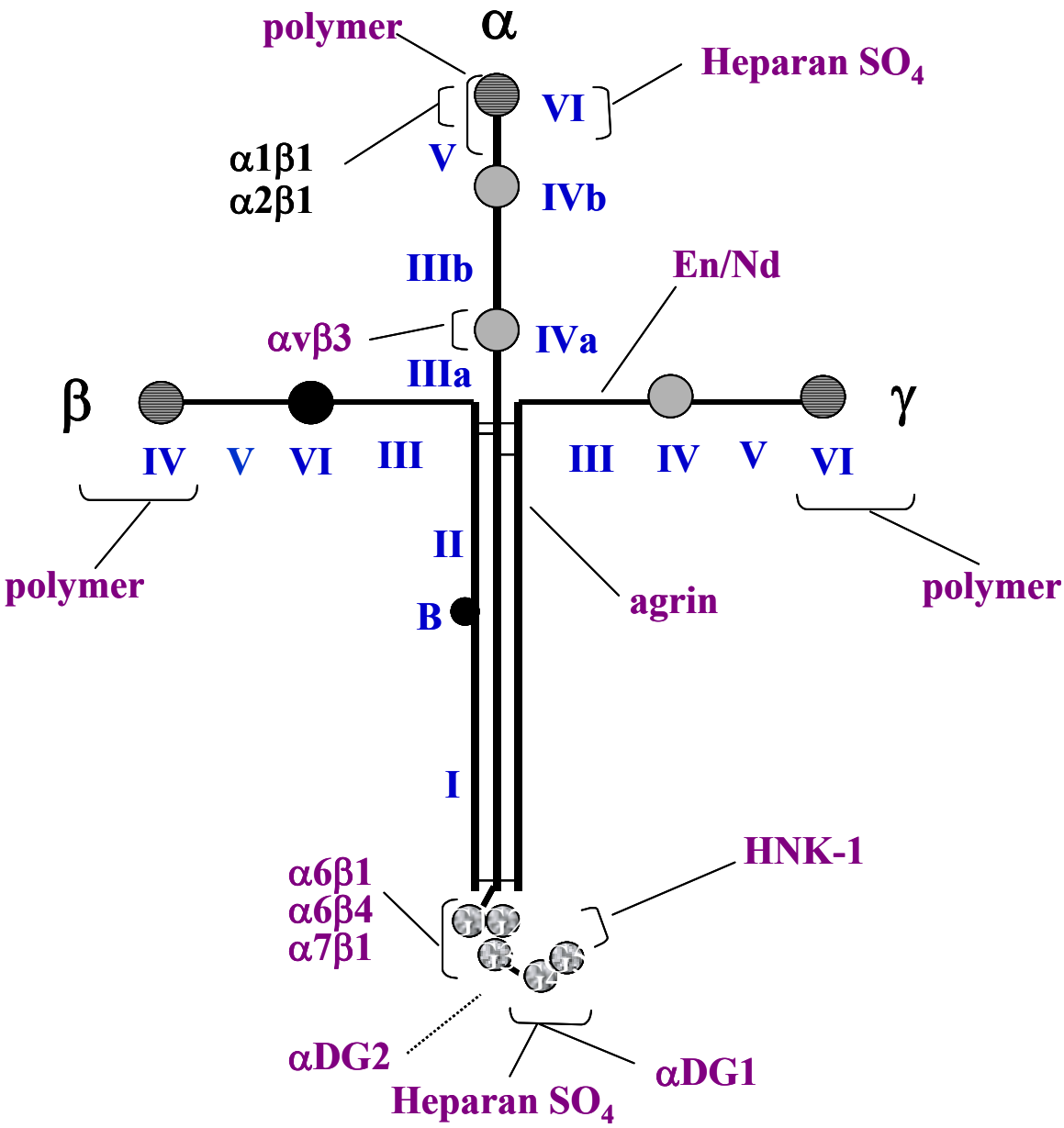

Figure 1. Schematic representation of structure and domain organization of classical laminin isoform

1.2.2 Isoforms and tissue distribution

The first member of the LN family was isolated 25 years ago from the Engelbreth-Holm Swarm tumor (EHS) (Timpl et al., 1979), and from the culture supernatant of a mouse embryonic carcinoma cell line (Chung et al., 1979), and named as laminin. It consists of 400 (α1), 220 (β1) and 200 (γ1) kDa chains (Beck et al., 1990). Though initially it was considered to be the only LN to exist, additional LN chains and isoforms were found later (Wewer et al., 1983; Martin and Timpl, 1987; Hunter et al., 1989; Ehrig et al., 1990; Kallunki et al., 1992; Burgeson, 1993; Paulsson et al., 1993). This necessitated for a convenient nomenclature. According to the present nomenclature, the first identified LN was called as laminin-1 (LN-1, α1β1γ1) (Burgeson et al., 1994). Other isoforms were named similarly according to the order of their discovery, as LN-2 (α2β1γ1), LN-3 (α1β2γ1), etc. At present, eleven genetically distinct laminin chains (α1-5, β1-3, γ1-3) that assemble into at least 15 laminin isoforms (LN1-15; Lm111-523; Fig. 2) have been identified. Classification of the chains as α, β and γ is based on their sequence identity and domain organization. The different LN isoforms can differ from each other by one, two, or all the three chains (Fig 2). Recently, a simplified LN nomenclature based on the chain composition has been proposed (Aumailley et al., 2004, submitted). Thus, LN-1 (α1β1γ1) will be named as Lm-111, LN-2 (α2β1γ1) as Lm-211, etc. This nomenclature avoids the necessity for memorizing the chain composition.

Systematic classification of laminins has not been an easy task. Since the α chain diversity has been implicated in functional diversity of LN isoforms, it seems reasonable to classify LNs based on their α chains. However, it is worth mentioning that some function and expression overlaps exist. It is also important to mention some general understandings concerning all the three (α, β and γ) LN chains. All α chains (α1-5) are expressed in both embryos and adults, but with distinct pattern of expression (Miner et al., 1997). In adult stage α4 and α5 exhibit the broadest tissue distribution (Miner et al., 1997), while α1 show the most restricted expression (Miner et al., 1997; Virtanen et al., 2000). All developing and adult BMs contain at least one α chain, all α chains are present in multiple BMs, and some BMs contain two or three α chains (Miner et al., 1997). Among the β chains, the β1 and β2 chains are constituents of most LN isoforms, being found in 7/15 and 8/15 LN-isoforms respectively. Among the γ chains, γ1 is so far the most widely expressed (in 10/15 isoforms) (Patarroyo, 2002). If still novel isoforms are to be discovered, this proportion might be altered. On the other hand, the expression of β3 and γ2 chains is much peculiar. So far these two chains are known to assemble only as LN-5 (α3β3γ2) leading to a speculation that β3 can associate only with γ2 chain.

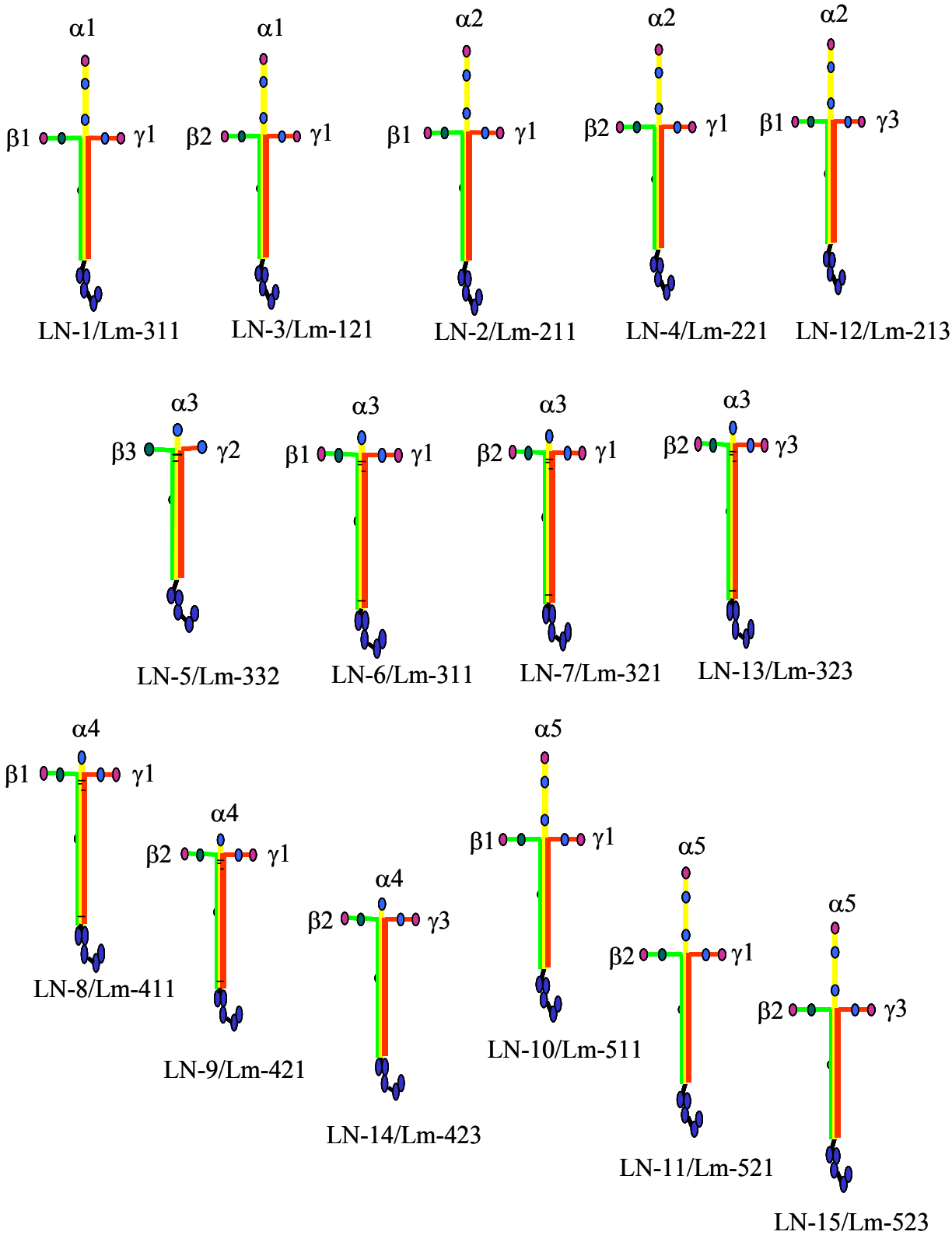

Figure 2. Schematic representation of laminin isoforms

1.2.2.1 α1 containing LNs

So far, two LN isoforms, LN-1 (α1β1γ1/Lm-111), and LN-3 (α1β2γ1/Lm-121) are known to consist of α1 chain. LN-1 (Timpl et al., 1979), also known as EHS LN, is termed as "classical" or "prototype" LN. It is the most biochemically and functionally characterized LN isoform. However, its expression is restricted to distinct epithelial BMs of adult human tissues (Virtanen et al., 2000). It is detected early in development but disappears from many sites with maturation (Miner et al., 1997). During myogenesis the LNα1 chain is present around the epithelial somite, but later, in forming muscle, its expression is restricted to the myotendinous junction (Gullberg et al., 1999). The LNα1 expression is restricted to BMs of most proximal tubules in kidneys and the seminiferous epithelium in testis in both fetal and adults (Virtanen et al., 2000). It is not detected in BMs of blood vessels, except in capillary walls of both fetal and adult central nervous system. Similar pattern of expression to that of humans was observed in the tissues analyzed in mice. LNα1 is present in BMs of renal proximal tubule (Horikoshi et al., 1988; Sorokin et al., 1992; Miner et al., 1997) and glomerular mesangium (Horikoshi et al., 1988; Miner et al., 1997) in the adult stage. However, it is absent from glomerular BM and BMs of arteries, veins and capillaries (Miner et al., 1997).

1.2.2.2 α2 containing LNs

LNα2 is predominantly the product of cells of mesodermal origin. Three LN isoforms are known to contain LNα2 chain, LN-2 (α2β1γ1/Lm-211), LN-4 (α2β2γ1/Lm-221) and LN-12 (α2β1γ3/Lm213). LNα2 chain (also known as merosin or M component) (Ehrig et al., 1990) has similar size to LNα1, but is usually cleaved into 300 and 80 kDa components that remain non-covalently associated. It is found in BMs surrounding the skeletal and cardiac muscles (Leivo and Engvall, 1988; Paulsson et al., 1991), as well as in peripheral nerves and placenta (Paulsson et al., 1991). LNα2 expression is restricted to certain regions in kidney in humans (Sanes et al., 1990; Virtanen et al., 1995), and it is also found in cardiomyocyte BMs in mice (Miner et al., 1997). LNα2 chain is one of the major LNα chains in addition to LNα4 and LNα5 chains in the muscle BM during muscle formation (Gullberg et al., 1999).

1.2.2.3 α3 containing LNs

LNα3 is expressed in most epithelial BMs, with the exception of some simple epithelia. It is the component of LN-5 (α3β3γ2/Lm-332), -6 (α3β1γ1/Lm-311), LN-7 (α3β2γ1/Lm-321) and LN-13 (α3β2γ3/Lm-323). Two variants, a full length, α3B and a truncated, α3A chains have been described (Ryan et al., 1994; Galliano et al., 1995; Ferrigno et al., 1997). The truncated variant is expressed as LN-5 and -6 in the skin, bladder and oesophagus (Miner et al., 1997) and as LN-7 in placenta (Champliaud et al., 1996). Differentiating ameloblasts express LNα3 chain at the time they begin to secret enamel (Salmivirta and Ekblom, 1998). LN-5, previously identified as kalinin, epiligrin or nicein (Burgeson, 1993; Marchisio et al., 1993), is significantly smaller and all the three short arms are truncated as observed in electron micrographs (Burgeson, 1993). LNα3 is present in the kidney papillary and lung alveolar BMs, while absent

from glomeruli, tubules and vasculature of renal cortex in adult mice (Miner et al., 1997).

1.2.2.4 α4 containing LNs

LNα4 is the constituent of three LN isoforms, LN-8 (α4β1γ1/Lm–411), LN-9 (α4β2γ1/Lm-421) (Miner et al., 1997) and LN-14 (α4β2γ3/Lm-423) (Libby et al., 2000). It is expressed in all adult and fetal endothelial BM except in glomerular BM. Its expression is high in aorta (Frieser et al., 1997). LNα4 is expressed by cells of mesenchymal origin such as skeletal and cardiac muscle (Liu and Mayne, 1996; Lefebvre et al., 1999) and adipose cells (Niimi et al., 1997). It is present in most capillaries of the renal medulla and heart, in alveolar BM in lung, but absent from renal cortex (Miner et al., 1997). LNα4 is also expressed in post capillary venules (Sixt et al., 2001), which is the site of exit of inflammatory cells. Its expression in non-BM locations includes bone marrow stroma cells (Gu et al., 1999; Siler et al., 2000), and blood platelets and lymphocytes (Geberhiwot et al., 1999; Geberhiwot et al., 2001). Its presence in BM zones of blood vessels and fibroblast-like cells in the skin has been documented (Matsuura et al., 2004). Splice variants of LNα4 (α4A, α4B) that could assemble as two types of LN-8, α4Aβ1γ1 and α4Bβ1γ1, have been detected in human cell lines (Hayashi et al., 2002).

1.2.2.5 α5 containing LNs

LNα5 chain is a component of three LN-isoforms, LN-10 (α5β1γ1/Lm-511), LN-11 (α5β2γ1/Lm-521) (Miner et al., 1997), and LN-15 (α5β2γ3/Lm-523) (Libby et al., 2000), and is the most abundantly expressed α chain. It appears later in embryonic development, and is retained in adult stage (Miner et al., 1995; Sorokin et al., 1997a; Ekblom et al., 1998). It is expressed in epithelial and certain non-epithelial tissues such as endothelial, skeletal and smooth muscle BMs (Sorokin et al., 1997b). In skeletal muscle, its expression is reduced with maturation, and in adults it is primarily seen at the neuromuscular junction. It is found in all BMs (glomerular, tubular and arterial) in kidney, in heart blood vessels, and some cardiomyocyte BMs and alveolar BM in lung (Miner et al., 1997).

1.2.3 Biological activities and associated pathologies

Diverse biological activities have been attributed to LNs. They influence processes like cell adhesion, migration, growth, morphology, differentiation, as well as the assembly of the extracellular matrix (ECM) and hence tissue integrity. It was originally thought that LNs primarily affected cells of epithelial origin (Kleinman et al., 1985). However, the vast amount of data accumulated over the last decade show their role in all types of tissues, including blood cells. LNs play major role in ECM architecture, cell adhesion, spreading and migration (Ryan et al., 1996). They are required for organization of BM components, ECM deposition and stability that is crucial for survival (Yurchenco and Cheng, 1994; Smyth et al., 1999). They promote myogenesis in skeletal muscle (Gullberg et al., 1999), mesenchymal to epithelial transition in kidney (Ekblom et al., 1990), epithelial morphogenesis (Ekblom et al., 1998; Ekblom et al., 2003), and neurite outgrowth from peripheral and central neurons (Dziadek et al., 1986; Suzuki et al., 2003)). LN-8 and LN-10/11 have been shown to

promote adhesion and migration of human bone marrow hematopoietic progenitor and more differentiated cells (Gu et al., 2003). In co-operation with other ECM proteins and cell-surface receptors, they promote cell proliferation, differentiation, maintenance of the differentiated tissues, and tissue repair after injury (Kleinman et al., 2003). The roles LNs are not only limited to physiological functions, as aberrant expression can lead to specific pathologies.

Since LNs influence diverse cellular activities, it is expected that deletion or mutation of the genes encoding for LN chains can cause alteration or loss of function, unless functional compensation occur. Analysis of naturally occurring mutations in humans and mice and/or gene knock out (KO) experiments in mice provided valuable information in this regard. LNγ1 chain is the first ECM molecule to be synthesized at the two-cell stage and serves as initial matrix scaffolding and it is the component of most LN isoforms (Ekblom et al., 2003). Loss of function by deletion of the *LAMC*1 gene encoding for LNγ1 chain results in early embryonic death in mice because of failure to organize BM. Mutations or deletions of the gene encoding for the LNα2 chain (*LAMA2*) result in muscular dystrophies in human and muscular dystrophy-like phenotype in mice (Wewer and Engvall, 1996; McGowan and Marinkovich, 2000; Patton, 2000). Mutations of each gene encoding for LN-5 (*LAMA*3, *LAMB*3, *LAMG*2) lead to a skin blistering disease known as epidermolysis bullosa (Wewer and Engvall, 1996). Deletion of *LAMA4* gene encoding for LNα4 in mice resulted in transient hemorrhage at birth as a result of defective BMs of capillaries and subtle motor impairment as adult (Thyboll et al., 2002). In a recent study in a murine tumor model, deletion of LNα4 chain resulted in hyperneovascularization and promotion of tumor growth and metastasis (Zhou et al., 2004).

1.3 Laminin receptors

Different cell-surface receptors, including integrins, membrane bound proteoglycans and dystroglycans interact with laminins (Timpl and Brown, 1996; Aumailley and Smyth, 1998). Both dystroglycan and integrins can distinguish amongst different LNα chains indicating that the α chain diversity is functionally significant (Belkin and Stepp, 2000). For convenience and practical reasons I will focus only on integrin receptors.

Integrins

Integrins, named so (Hynes et al., 1987) because of their function in integrating the cell's extracellular environment with the cell's interior (cytoskeleton), are a family of heterodimeric cell-surface adhesive receptors, formed by a non-covalent linkage of one α and one β subunits (Hynes, 1992). They are expressed by different types of cells and most cells express more than one type of integrin. Nineteen different α and 8 different β integrin subunits have been reported in vertebrate (Humphries, 2000a; Hynes, 2002), that assemble into 25 heterodimers. Each of these appear to have distinct and non-redundant functions (Hynes, 2002), and mediate dynamic linkage between extracellular adhesion molecules and the intracellular actin cytoskeleton (Humphries, 2000a).

All integrins are type I transmembrane integral proteins and posses three major structural domains, the large extracellular domain, the transmembrane region and the small cytoplasmic domain. In addition, the extracellular domain of half of integrin α-

subunits contain an extra insertion of 200 amino acids (I/A domain), in which the metal ion dependent adhesion site (MIDAS) is located. This domain is critical for ligand binding (Kanazashi et al., 1997; Plow et al., 2000). The ligand-binding site is located in the globular head domain contributed by both the α and β subunits. Some integrins recognize the tri peptide RGD (Arg-Lys-Asp) sequence on their ligands (Pierschbacher and Ruoslahti, 1984; Aumailley et al., 1990; Ruoslahti, 1996; Humphries et al., 2000b).

Integrins are classified into subfamilies, based on the common β subunit, as β1 (CD29), β2 (CD18), etc. The β1 integrins are expressed by all types of cells except erythrocytes. The β2 integrins are exclusively expressed by leukocytes and mediate intercellular adhesion by interacting with their counter receptors (immunoglobulin (Ig) superfamily molecules such as ICAMs) on the adjacent cells (Patarroyo et al., 1990; Springer, 1990; Gahmberg et al., 1997). The four β2 integrins known to date are αLβ2 (CD11a/CD18, LFA-1), αMβ2 (CD11b/CD18, MAC-1), αXβ2 (CD11c/CD18, p150,95) and αDβ2 (CD11d/CD18). They show characteristic distribution among the leukocyte subpopulation. CD11a/CD18 is predominantly expressed by lymphocyte, CD11b/CD18 by granulocytes, CD11c/CD18 and CD11d/CD18 by monocytes. It should be noted that often individual leukocytes express more than one β2 integrin (Gahmberg 1997).

Integrins on "resting" leukocytes stay in inactive or "off" state and are rapidly activated when the cells are stimulated (Humphries et al., 2000b; Hynes, 2002). The same is true for platelet integrin (αIIbβ3). This is specifically important for circulating blood cells, which otherwise could have devastating pathological consequences. Affinity of integrins for their ligands can be modified by conformational changes. Integrins can transduce signals in two directions, referred to as inside-out and outside-in signaling (Hynes, 1992). In inside-out signaling, the activation of non-integrin receptors (e.g. chemokine receptor) induces cytoplasmic signals that alter the conformation of integrin cytoplasmic domain, rendering the cell capable of recognizing the ligand. This is evident for leukocyte and platelet integrins that need activation by agonists. When ECM proteins such as LNs bind to integrins, the signal will be transduced to the interior of the cell (outside-in signaling) (Clark and Hynes, 1997; Yamada and Geiger, 1997). These signals are central for diverse cellular functions. Integrins are known to activate specific protein kinases (e.g. focal adhesion kinase, FAK) that are important in signal transduction (Giancotti, 2000).

Most integrins bind to ECM components (cell-ECM), others to their counter-receptors on other cells (cell-cell, cell-pathogen interaction). Through such interactions, integrins control vital cellular processes such as cell growth, proliferation, differentiation, survival, development and immune and inflammatory responses. Integrin affinity for their ligands may vary depending on the cell type in which they are expressed (Diamond and Springer, 1994).

Integrins as laminin receptors

A total of ten integrins (α1β1, α2β1 α3β1, α6β1, α7β1, α9β1, αvβ3, αvβ5, αvβ8 and α6β4) have been reported to be receptors for LN (Belkin and Stepp, 2000; Patarroyo, 2002) (Table I). Some of these integrins also recognize other ligands. However, α3β1, α6β1, α7β1 and α6β4 are considered to be classical LN receptors and all bind to the G domain at the C-terminus region of LNα chains. All the three α chains (α3, α6, α7) of these integrins are posttranslationally cleaved into light and heavy chains that are held together by disulfide bonds, do not posses I domain, and are closely

related. Domain VI of the short arms of LN chains is reported to be recognized by α1β1 and α2β1integrins. It seems LNs harbor two major binding sites for integrins, one in the LG1-3 modules of LNα chains and a second in the N-terminal region of the short arms. Accordingly, the four classical LN receptors recognize the α1 LG1-3 modules in association with the C-terminal end of the LNβ1 and LNγ1 chains (Aumailley and Krieg, 1996). Integrins α1β1 and α2β1 bind to the domain VI of α1 and α2 and α2β1 to the N-terminus of β1 and γ1 LN chains. α3β1 integrin binds to LNα3G2 (Mizushima et al., 1997). Using inhibitory Abs, LNα2G1-3 has been identified to be the binding site for α3β1 and α6β1 integrin (Talts and Timpl, 1999). Later, α6β1 integrin was identified to be a receptor for LN-8 (Geberhiwot et al., 1999; Kortesmaa et al., 2000)

Table I. Laminins and their integrin receptors

Current nomenclature	Chain composition	Proposed nomenclature	Integrin receptors
Laminin-1	α1β1γ1	Lm-111	α1β1, α2β1, α6β1, α7β1, α6β4
Laminin-2	α2β1γ1	Lm-211	α1β1, α2β1, α3β1,α6β1, α7β1, α6β4
Laminin-3	α1β2γ1	Lm-121	
Laminin-4	α2β2γ1	Lm-221	
Laminin-5	α3β3γ2	Lm-331	α3β1,α6β1, α6β4
Laminin-6	α3β1γ1	Lm-311	
Laminin-7	α3β2γ1	Lm-321	
Laminin-8	α4β1γ1	Lm-411	α3β1,α6β1, α7β1
Laminin-9	α4β2γ1	Lm-421	
Laminin-10	α5β1γ1	Lm-511	α3β1,α6β1, α7β1, α6β4, αvβ3
Laminin-11	α5β2γ1	Lm-521	α3β1,α6β1
Laminin-12	α2β1γ3	Lm-211	
Laminin-13	α3β2γ3	Lm-323	
Laminin-14	α4β1γ3	Lm-413	
Laminin-15	α5β2γ3	Lm-523	

Recognition of LNs by integrins is known to elicit a wide range of biological responses. For example recognition of LN-8 by α6β1 has been shown to promote cell adhesion (Talts et al., 2000).

The binding specificity for different laminin isoforms has been determined for a few integrins. Thus, α3β1 binds more strongly to LN-5 (α3β3γ2, Lm-332), LN-10 (α5β1γ, Lm-5111) and LN-11 (α5β2γ1, Lm-521) than to LN-1 (α1β1γ1, Lm-111) or LN-2 (α2β1γ1, Lm-211); α7β1 binds more strongly to LN-2 (α2β1γ1) and LN-4 (α2β2γ1) than LN-5 (α3β3γ2, Lm332) (Belkin and Stepp, 2000). Important information regarding the functions of integrins and the consequences of aberrant expression of the relevant genes have been obtained by using combinations of different techniques, such as inhibiting and activating Abs, and gene transfection.

1.4 Myeloid cells

All blood cells, originate from a small common pool of haematopoietic stem cells (HSC) in bone marrow by haematopoiesis (the process of blood cell formation). HSC, besides self-renewal gives rise to two lineage restricted progenitors, the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). CLP give rise to T- and B- lymphocytes and NK cells, and CMP give rise to granulocytes, monocytes, erythrocytes and platelets (Fig. 3). These cells are quite heterogeneous and differ from each other not only morphologically but also functionally.

Monocytes and granulocytes develop from a common progenitor (CMP) in bone marrow by myelopoiesis, a tightly regulated process. Both monocyte and neutrophil precursors pass through a series of developmental stages to differentiate and mature.

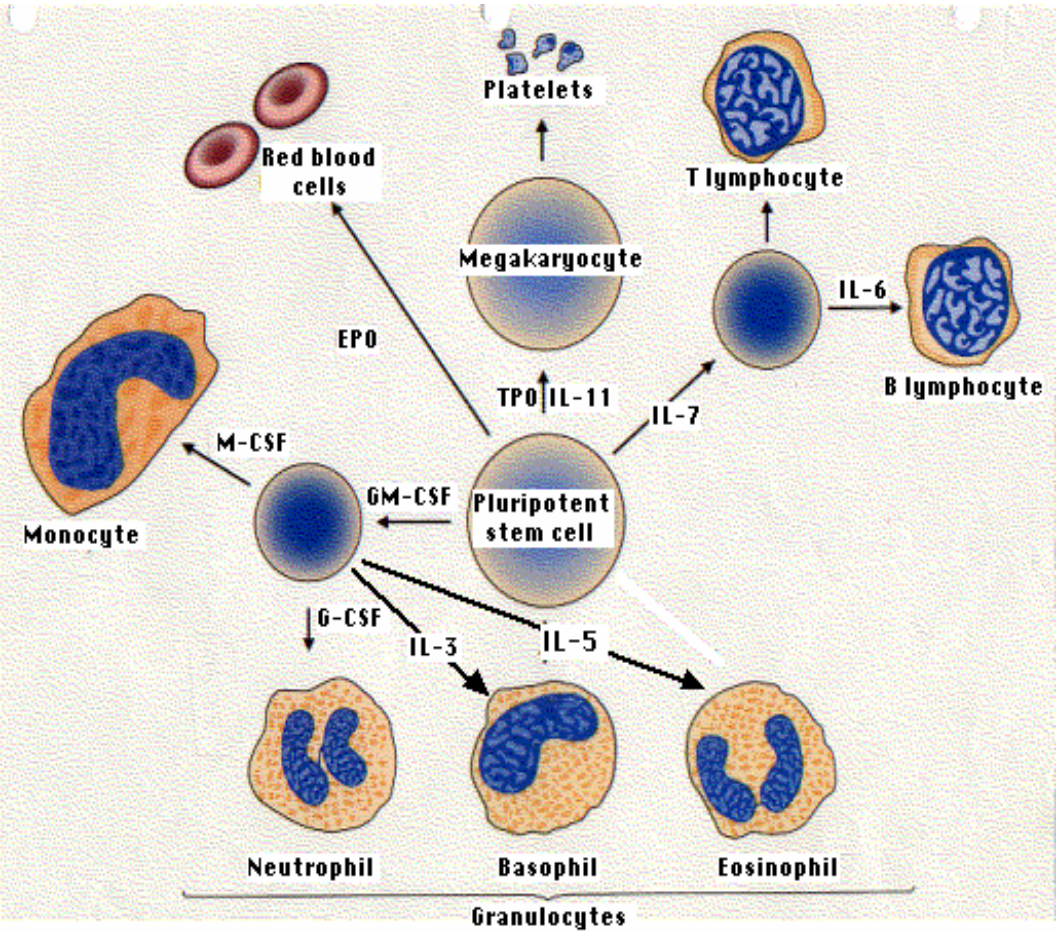

Figure 3. Schematic representation of myeloid cell development

1.4.1 Monocytes

Monocytes play an essential role in both adaptive and innate immune responses. The first committed cell along the monocyte development tract is the monoblast, a cell type similar in morphology to the myeloblast of the granulocytic series. Monoblasts develop into promonocytes, large cells with a slightly indented nucleus. Promonocytes develop into mature monocytes. The mature monocytes are released into circulation

and from blood they migrate to various tissues to generate dendritic cells and tissue resident macrophage (Johnston, 1988; Greaves and Gordon, 2002). Macrophages play essential roles in disposal of foreign agents and cellular debris, initiation and mediation of immune and inflammatory responses as well as repair process following tissue injury.

1.4.2 Granulocytes

Granulocytes are recognizable by their multi-lobed nuclei and numerous cytoplasmic granules, for which they have got their name. They play important roles in inflammation and innate immunity, and function to eliminate infectious agents. Like macrophages, they are stimulated by T cell-derived cytokines, phagocytose opsonized particles and serve important effector functions in immune responses as well. Based on the staining characteristic of their prominent granules they are classified as basophils, eosinophils and neutrophils (Ehrlich and Lazarius, 1900; Witko-Sarsat et al., 2000)

Neutrophils, also known as polymorphonuclear leukocytes (PMNs), are the most abundant subset of granulocytes and represent 50-60% of total circulating leukocytes. Once an inflammatory signal is initiated, neutrophils are the first cells to be recruited to sites of infection or injury. As phagocytes, these cells constitute first line of defense against infectious agents that penetrate the bodies' physical barriers. Together with macrophages they are called 'professional phagocytes'. They spend less than 24 hr in circulation, then they migrate into tissues under the influence of chemotactic stimuli and adhesion molecules, where they phagocytose materials and die eventually by apoptosis.

1.4.2.1 Neutrophil development

Neutrophil development in bone marrow takes about 2 weeks. During maturation they pass through 6 stages (myeloblast, promyelocyte, myelocyte, metamyelocyte, band and mature PMN) that differ by size, morphology and granular content (Bainton et al., 1971). Bone marrow of normal healthy adult produces about 10^{11} neutrophils per day under physiological state and 10^{12} in settings of acute inflammation. When they are released from bone marrow into circulation neutrophils are in resting state. In blood, they have a half-life of 6-10 h, before marginating and entering tissue pools, where they survive for 1 to 2 days. Neutrophil exist in dormant (resting), primed (intermediate) and activated state. By priming, 'resting' neutrophils acquire a state of pre-activation that enables a more powerful response to be generated once microbial activity is initiated. (Borregaard and Cowland, 1997b).

1.4.2.2 Neutrophil granules

Neutrophil granules are of major importance for neutrophil function and are generated for storage during differentiation. On the basis of function and enzyme content, three main types of neutrophil granules are known, namely azurophil, specific, and storage granules (Bainton et al., 1971; Borregaard, 1997a). Their function is to provide enzymes for hydrolytic substrate degradation, to kill ingested bacteria and to secrete their contents to regulate various physiological and pathological processes during inflammation.

Neutrophil granules contain anti-microbial (e.g. defensins, perforins) or cytotoxic substances, neutral proteinases, acid hydrolases and a pool of cytoplasmic membrane

receptors including integrins. Among the azurophil granule constituents, myeloperoxidase is a critical enzyme in the conversion of hydrogen peroxide to hydrochlorous acid. Together with H_2O_2 and a halide cofactor, it forms the most effective microbicidal and cytotoxic mechanism of leukocytes, termed as the myeloperoxidase system.

1.4.2.3 Functions

The major function of neutrophils is phagocytosis and destruction of infectious agents, especially bacteria. Neutrophils are the first cells to be recruited to inflammatory loci, providing the first line of host defense. They have all the machinery ready for prompt attack of the intruder. The other important function of neutrophils is cytokine synthesis (Cassatella, 1999). Neutrophils are both target and source of proinflammatory cytokines (e.g. IL-1 and TNF-α), chemokines (IL-8) and growth factors (GM-CSF, G-CSF). Both cytokines and chemokines act as priming agents and amplify several functions of neutrophils such as neutrophil-endothelial cell adhesion, and production of reactive oxygen species. Chemokines such as IL-8 act as potent chemoattractants and guide the neutrophils to migrate directionally towards the source of inflammation. The globally accepted notion is that neutrophils are terminally differentiated end cells, devoid of transcriptional activity with no or little protein synthesis capacity. This view has been repeatedly challenged by several researchers. It has also been shown that neutrophils either constitutively or in an inducible manner can synthesize, and release a wide range of pro- and anti-inflammatory cytokines, as well as other cytokines, but at lower degree than mononuclear phagocytes (Witko-Sarsat et al., 2000).

Neutrophils express diverse receptors, including receptors for the Fc portion of immunoglobulin (Ig) (FcR), and complement (CR) that facilitate the uptake of opsonized particles. They also express multiple receptors for chemoattractants, platelet activating factor (PAF), leukotrien B4 (LB4), formyl-Methionyl-Leucyl-Phenylalanin (fMLP), interleukin 8 (IL-8), tumor necrosis factor α (TNF-α) that trigger adhesion, migration, degranulation and oxidative response.

1.4.3 Neutrophil extravasation

The cardinal feature of innate immunity is recruitment and activation of leukocytes at the site of infection to eliminate and/or contain invading pathogens. The same process if occurs inappropriately leads to chronic inflammatory disorders, such as rheumatoid arthritis, psoriasis, allergies, etc.

Similar to most cells in immune system, neutrophils are not static within a particular compartment, but are mobile and patrol the body. They circulate freely in the blood stream under a steady state. In response to proinflammatory signals generated by tissue injury or infection, a series of events resulting in activation of both leukocytes and endothelial cells take place that enables the extravasation of leukocytes (monocytes and neutrophils). Leukocyte homing to the loci of injury is a multi-step process and involves sequential interaction of adhesion molecules (selectins, integrins, ICAMs) and proinflammatory mediators and chemoattractants (IL-8, fMLP). A generally accepted model for extravasation consists of four sequential steps, namely rolling, activation/signaling, firm adhesion/arrest and transedothelial migration (Fig. 4), (Witko-Sarsat et al., 2000).

The initial event is expression of P -and E-selectins in response to inflammatory signals (LB4, histamine, TNF-α) on local endothelium (Hattori et al., 1989; Geng et al., 1990). The first step, rolling, is mediated by the interaction of selectins with the counter receptor sialyl-Lewis^x and is of low avidity, allowing leukocytes to adhere reversibly to the vessel wall. This enables neutrophils to tether and roll along the vessel wall to make more stable interaction.

The second step of activation/signaling involves the binding of chemokine IL-8, or other chemoattractants, such as PAF, complement fragment products (C5a, C3a) or N-formyl peptides of bacterial product, to cell-surface receptors. The chemokines can be derived from endothelial cells or the injured tissue either diffusing into the vessel or exposed on the luminal side of endothelial cells. This binding activates neutrophil integrins by transducing signals via the G protein coupled receptors resulting in conformational changes and increased affinity for their counter receptor. This results in activation of neutrophils and high avidity interaction between leukocyte integrins (LFA-1, CD11a/CD18; Mac-1, CD11b/CD18) and the counter receptors on endothelial cells, immunoglobulin (Ig) superfamily cell adhesion molecules (CAM). This interaction results in arrest of the rolling neutrophils and their firm adhesion to the endothelium.

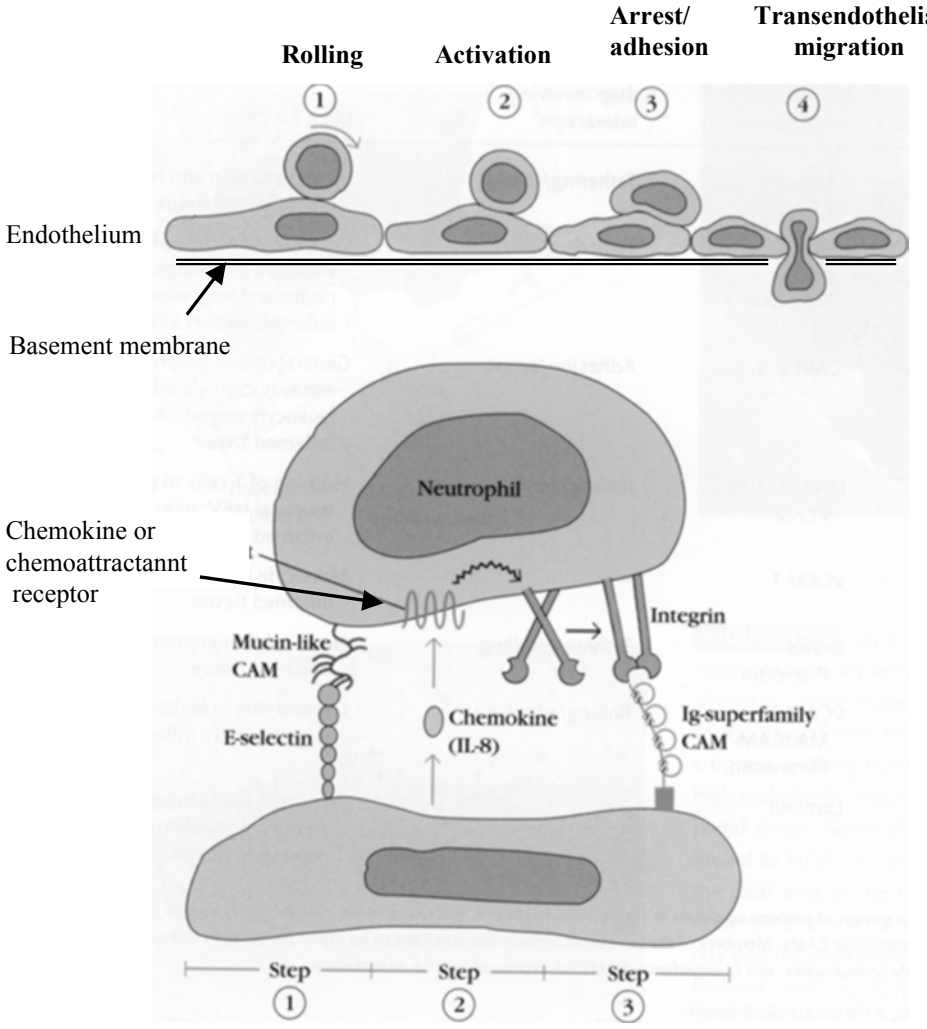

Figure 4. Schematic representation of leukocyte extravasation

In the subsequent step, the firmly adhered neutrophil squeezes itself between the adjacent endothelial cells (diapedesis), and crosses the underlying BM, where it encounters LNs. The endothelial BM is rich in LN-8 ($\alpha4\beta1\gamma1$, Lm-411) and -10 ($\alpha5\beta1\gamma1$, Lm-511). This last step of neutrophil extravasation is not fully characterized. Whether LNs could contribute to leukocyte extravasation is still an open question.

The recruited neutrophils phagocytose the infectious agent. The ultimate goal of leukocyte recruitment is killing of the microorganism by either oxygen-dependent or oxygen-independent mechanism. Oxygen-dependent response (respiratory burst) involves the sequential reduction of oxygen by NADPH oxidase, leading to production of toxic oxygen metabolites (H_2O_2, OH, singlet oxygen). Oxygen-independent response uses the highly toxic cationic proteins and enzymes (e.g. myeloperoxidase and lyzozyme) contained within the neutrophil cytoplasmic granules. Ingestion and killing of organisms is 100-fold more effective if the particles are first opsonized with specific antibody or complement. The interaction between the opsonized particle and the phagocyte (via FcR or CR) increases the adhesion between the leukocytes and the pathogen, by priming the cells for activation. The destruction of the phagocytosed organisms results in death of the neutrophils by apoptosis followed by clearance of the apoptotic bodies by macrophages.

1.4.4 Laminins in leukocyte function

Since LNs have been implicated in different cellular responses, including adhesion and migration, efforts were made to identify their role in leukocyte functions. Most of the earlier studies have been conducted using mLN-1 (EHS LN). Although the information is valuable, the restricted tissue distribution of LN-1 makes its relevance to leukocyte physiology questionable. LN has been reported to promote neutrophil chemotaxis, to increase dispase-treated attachment of neutrophil to collagen type IV coated or uncoated plastic (Terranova et al., 1986), and to increase TNF-α stimulated adherence of human neutrophil via CD11bCD18 (Thompson and Matsushima, 1992). Stimulation of neutrophils resulted in increased surface expression of non-integrin LN-receptor (Yoon et al., 1987). A peptide from LN$\alpha1$ chain long arm, SIKVAV, induced angiogenesis in a murine model ((Kibbey and Mazurkiewicz, 1992), and contributed to neutrophil migration into matrigel plugs (Kibbey et al., 1994). In another study, LN of unknown isoform stimulated lysozyme release and superoxide production by neutrophils in response to fMLP (Pike et al., 1989). Adherence of TNF-α stimulated neutrophils to LN-1 induced respiratory burst via $\alpha M\beta2$ not via $\alpha L\beta2$ integrin (Decleva et al., 2002). Despite the fact that LN-1 may not be directly implicated in leukocyte physiology, the results from these studies have shown the ability of a LN isoform to induce important leukocyte responses. Since LN-1 possess active sites both unique and common to different isoforms, it might be possible to get similar cellular responses with other LN isoforms, such as LN-8 and -10, which are present in vascular endothelial BM. In fact, work at our laboratory demonstrated the expression of LN-8 by lymphocytes and its role in promoting adhesion, migration, and proliferation of these cells (Geberhiwot et al., 2001)

LNs possess different active/functional sites. Engagements of these sites have been shown to promote/induce different responses in leukocytes. Much effort has been made to map the functional sites and several sites have been identified on LN$\alpha1$ chain. A peptide containing SIKVAV derived from LN$\alpha1$ chain has been shown to promote

tumor invasion, metastasis and angiogenesis (Corcaran et al., 1995; Kanemoto et al., et al., 1990; Khan and Falcone, 1997; Kibbey et al., 1994). It has also been shown to up-regulate macrophage matrix metaloproteinase (MMP) 9 expression (Kanemoto et al., 1990; Kibbey et al., 1994; Corcoran et al., 1995; Khan and Falcone, 1997). SIKVAV is a linker between the coiled-coil and G domain and fairly conserved among different LNα chains. Adair-Kirk et al. (2003) analyzed the in vitro and in vivo effect of peptide sequences derived from the corresponding regions from LNα3 and LNα5 chains. The sequence derived from the LNα5 chain increased MMP-9 and -14 production by macrophages, induced MMP-9 release by neutrophils, and promoted macrophage and neutrophil chemotactic migration both in vitro and in vivo.

2 Aims of the study

Laminins are implicated in diverse cellular functions, including promotion of cell adhesion and migration. Adhesion and migration are central activities in leukocyte functions, such as extravasation, phagocytosis and oxidative burst. The primary objective of this study was to identify the role of laminin isoforms in myeloid cell physiology. To achieve this objective, the following specific aims were set:

- To characterize commonly used monoclonal antibodies (mAbs) to laminins and generate novel mAbs to LNα4 chain to be used as tools.

- To investigate the expression of LN-8 (α4β1γ1, Lm-411) and other laminin isoforms by monocytes and neutrophils.

- To determine the role of LN-8 and other laminin isoforms in leukocyte adhesion, migration, extravasation and survival.

- To identify integrin receptors for LN-8 and other laminin isoforms in myeloid cells.

- To characterize commercial laminin preparations isolated from human placenta, which are widely used in functional studies, and to analyze their effect on leukocyte migration in comparison to rhLN-8 and -10.

3 Materials and methods

3.1 Production and characterization of antibodies to LN chains (papers, I, II, IV)

The chain specificity of 16 commonly used mAbs to human laminin chains was determined by enzyme linked immunosorbent assay (ELISA) by comparing their reactivity with recombinant (r) β1 (rβ1) and rγ1 LN chains (Pikkarainen T, 1992) and purified human placenta LN. Briefly, 96-well plates (Maxi-Sorp, Nunc, Roskilde, Denmark) were coated overnight at 4ºC (or 3 h at 37ºC) with either human serum albumin (HSA) (Sigma), rhLN-8 or rhLN-10 at 1μg/ml. The non-coated space of the wells was blocked with 10 mg/ml of HSA for 1 h at room temperature (RT). Then, 50 μl of each Ab under test was added at 0.8 μg/ml to the identified wells and incubated for 2 hr at 4ºC. After three washes with 0.1% Tween-20 in phosphate buffered saline (PBS), bound antibodies were detected using goat antibodies to mouse Ig conjugated to horseradish peroxidase (HRP) (Dakopatts, Copenhagen, Denmark) and the enzyme activity was measured using orthophenylenediamine (OPD) as substrate (Sigma, St. Louis, MO) in a plate reader

The Abs were further characterized by their reactivity against rβ1 and rγ1 LN chains by Western blot analysis under reducing and non-reducing conditions. Briefly, the purified proteins were boiled separately in sodium dodecyl sulphate (SDS) sample loading buffer containing β-mercaptoethanol as reducing agent for 5 min and run in 6% acrylamide gel. The separated proteins were transferred to nitrocellulose membrane, blocked with 5% fat-free milk/1% tween-20 in PBS, and the membrane strips were incubated with the Abs under test. The bound antibodies were detected with goat antibodies to mouse Ig conjugated to HRP (Dakopatts, Copenhagen, Denmark) and developed with ECL (Amersham, UK). The capacity of the mAbs to precipitate β1 and γ1 LN chains in associated state was analyzed by immunoprecipitation from platelet lysate.

In addition to the commercially available mAbs, eight mAbs to LNα4 chain were generated and characterized similarly to the commercial Abs above. LN-8 (α4β1γ1) purified from platelets by immunoaffinity chromatography was used to immunize Balb/c mice. Seven novel mAbs to human LNα4 chain were generated by hybridoma technology as described elsewhere. The specificity of the Abs was determined by ELISA by comparing their reactivity with both rhLN-8 (α4β1γ1) (Kortesmaa et al., 2000) and -10 (α5β1γ1) (Doi et al., 2002). Further characterization of the Abs was done by comparing their reactivity against platelet LN-8 by Western blot analysis under reducing and non-reducing conditions. The capacity of the Abs to immunoprecipitate LN-8 from platelet lysate was also determined. Isotyping was done with Iso-2 kit (Sigma).

The proteins and the mAbs to LN and integrin chains used in the studies are listed in table II.

3.2 Monocyte and neutrophil isolation and cell culture (III, IV, V)

Neutrophils and monocytes were isolated from citrate anticoagulated blood obtained from healthy donors (Blood Center at Karolinska Hospital, Huddinge

Hospital, Söder Hospital and Kringeltappen). Neutrophils were isolated by discontinuous Percoll gradient centrifugation (Pharmacia, Uppsala Sweden). The initial separation step was carried out at RT and subsequent washings were done at 4ºC with cold PBS (Dulbecos without Ca^{+2} and Mg^{+2}). Contaminant erythrocyte were lysed by hypotonic shock for 20 sec. Monocytes were separated from Ficoll-Hypaque gradient isolated peripheral blood mononuclear cells (PBMCs) by adherence to tissue culture flask. Cell viability was determined by trypan blue exclusion. Purity of the cells was determined morphologically and by immunostaining with mAbs C3D-1 to CD15 (a neutrophil marker), TUK4 to CD 14 (a monocyte marker) and SZ.22 to CD41 (a platelet marker). Human monoblastoid cell lines, THP-1, U-937 (Prieto et al., 1994), and JOSK-I (Ohta et al., 1986) were maintained in culture, in humidified atmosphere, with 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin, and L-glutamine. Mono Mac 6 cells (Ziegler-Heitbrock et al., 1988) were maintained similarly,but the medium was supplemented with sodium pyruvate, nonessential amino acids, oxaloacetic acid and bovine insulin.

3.3 Detection, isolation and characterization of laminins

3.3.1 Immunofluoresce flow cytometry (FACS) (III, IV)

Analysis of cell surface and intracellular expression of LN chains was done by indirect immunofluorescence flow cytometry. For intracellular staining, isolated neutrophils and monocytes were permeabilized with IntraStain Kit (DAKO) as recommended by the manufacturer. Fc-receptors (FcR)were blocked by incubating $20x10^6$ cells with 2 mg/ml of heat aggregated hIgG (Sigma). The cells were washed once, resuspended in cold PBS and incubated with 20 μg/ml of mAbs to LNα4 (5D8A), -β1 (2G6) ,-γ1 (CAF2P) or -γ1 (LN-41) chains in microtiter plate (at 0.2 - $1x10^6$/well). The bound Abs were detected with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments of rabbit anti-mouse Ig,, (1:20) (DAKO). All the incubations were done for 30 min at 4ºC. After four washes as above, cells were fixed with 1% parafolmaldehyde, resuspended in PBS and analyzed in a FACScan flow cytometer (BD Biosciences, San Jose, CA). Neutrophils and monocytes were identified according to the forward and side scatter properties and by expression of their immunological markers. Ten thousand events were collected and analyzed using CellQuest software. The results were presented as histograms of mean fluorescence versus cell count.

3.3.2 RNA extraction and RT-PCR (III)

Total ribonucleic acid (RNA) was extracted from cultured THP-1 cells using RNazol B (AMS Biotechnology) as recommended by the manufacturer. cDNA synthesis and polymerase chain reaction (PCR) was carried out using the advantage RT-for-PCR kit (Clontech laboratories) according to the instructions of the manufacturer. First strand cDNA synthesis was done in 20 μl final volume reaction mixture containing the RNA sample, $oligo(dT)_{18}$ primer, M-MLV reverse transcriptase, recombinant RNAse inhibitor and each of dNTP. The conditions for reverse transcription (RT) were 60 min at 42ºC followed by 5 min at 94ºC. Amplification of the resulting cDNA was done in a reaction mixture containing $MgCl_2$, dNTP mix and each primer in 20 μl final volume using Ampli Taq DNA polymerase (Perkin Elmer/Roche Molecular systems, Inc., Braunchburg, NJ). The conditions for PCR were 1 min at 94º;

5 min at 60ºC; 1 min at 72ºC for 35 cycles. The reaction was carried out using paired primers for PCR of human laminin (Geberhiwot et al., 2000b). For amplification of LNα4 chain, nested PCR was done, in which two rounds of amplifications were carried out. The amplified product from the first PCR was used as a template in the second amplification. PCR products were analysed by electrophoresis in 2% agarose gel.

3.3.3 Metabolic labeling (III)

THP-1 cells maintained in culture were washed with PBS, resuspended in methionine and cysteine free RPMI 1640 medium containing 10% dialyzed fetal FBS and incubated for 30 min at 37ºC. Afterwards cells were labeled with 0.20 mCi/ml Trans ^{35}S-label (ICN Radiochemical Inc.) for 4 h at 37ºC. After three washes with cold PBS, cells were lysed in 1% Triton X-100 containing protease inhibitors. The soluble fraction (cell lysate) was used for immunoprecipitation as will be described below.

3.3.4 Immunoprecipitation and SDS-PAGE/WB (I, III, IV, V)

Cell lysate was prepared from 100-200 x 10^6 leukocytes per ml in 1% Triton X-100 lysis buffer containing protease inhibitors (aprotinin, leupeptin, pepstatin) (Amersham Pharmacia Biotech), phenylmethylsulfonyl fluoride (PMSF) (Sigma) and ethylenediamine tetra acetic acid (EDTA). The lysate was pre-cleared by incubating with protein G Sepharose for 1 h. Aliquots of pre-cleared cell lysate were incubated with mAbs to LN chains for 1 h. In parallel, the secondary Ab, rabbit anti-mouse Ig was incubated with protein G Sepharose. Then excess secondary Ab was washed off with lysis buffer and the immune complexes were added to the beads to which the secondary Ab was bound and incubated for additional 2 h. All the incubations were done at 4ºC under continuous mixing. Finally, the unbound complex was washed off and the precipitated protein was eluted by incubating at 100ºC for 5 min in SDS sample buffer containing β mercaptoethanol. The immunoprecipitate was separated by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) in 6% polyacrylamide gel and electroblotted to nitrocellulose membrane. The blots were incubated with primary Abs to LN chains and detected with HRP-conjugated antibodies (DAKO). ECL (Amersham Pharmacia Biotech) was used as developer.

3.3.5 Immunoaffinity chromatography, SDS-PAGE/WB (III, IV)

Neutrophil lysate was prepared from 10^9 cells as for immunoprecipitation above. Purification of LNs was done as described else where by using LN β1 mAb (DG10) column. Briefly, the lysate was pre-adsorbed with Sepharose CL-4B (1:1) under continuous mixing at 4ºC overnight. The LN was captured by passing the pre-cleared lysate through the column twice at a rate of 0.05 to 0.1 ml/min. The bound protein was eluted after extensive washing of the column and analyzed by SDS-PAGE and western blotting as in 3.3.4, above.

3.4 Cell adhesion assay (III, IV)

To analyze the role of exogenous laminin isoforms in monocyte and neutrophil adhesion, 96 well flat bottomed polystyrene plates (BD Biosciences, Heidelberg, Germany) were coated with 50 µl/well of PBS (Dulbecos with Ca and Mg) or 50 µl/well in PBS of either HSA (Sigma), mLN-1, rhLN-8 or rhLN-10 at 20 µg/ml at

37ºC for 3 hr or (4ºC overnight) prior to plating the isolated cells. After rinsing the wells with PBS non-coated space was blocked with 2% polyvinylpyrrolidone (PVP, molecular weight 360 kD, Sigma) or 0.5 % HSA for 1 h at RT. Cultured JOSK-I cells or isolated neutrophils were resuspended at $2x10^6$ cells/ml in RPMI-1640 and pre-incubated with 20 μg/ml of either mIgG (isotype control), mAbs IB4 (to integrin β2 chain, CD18) , 60.1 (to integrin αM chain, CD11b), 6S6 (to integrin β1 chain, CD29), 13 (to integrin β1 chain, CD29) or GoH3 (to integrin α6 chain, CD49f) for 20 min at RT. The plate was washed twice with PBS and once with RPMI 1640 and the cells were plated in the identified wells and pre-warmed for 10 min at 37ºC before stimulation with 200 nM tetradecanoyl phorbol acetate (TPA) for 30 min. The non-adherent cells were removed with 4 washes of RPMI 1640 equilibrated at 37ºC. The adherent cells were fixed with 4% formaldehyde in PBS for 15 min at RT and stained overnight with 0.5% toluidine blue in PBS (Sigma) at RT. Excess dye was washed off with copious amounts of distilled water and adherent cells were quantified in a plate reader (Multiskan MS, Labsystems, Helsinki, Finland) at 620 nm by releasing the blue dye with 100 μl of 2% SDS (Bio-Rad Laboratories, Richmond, CA). The result was expressed as % absorbance considering the absorbance of mIgG treated cells as 100%.

3.5 Cell migration assay (III, IV, V)

To investigate the effect of exogenous LN isoforms on monocyte and neutrophil migration, polycarbonate Transwell culture inserts with 3- or 5-μm pore size and 6.5 mm diameter (Costar, Cambridge, MA) were coated and blocked as for the cell adhesion assay. Isolated PBMCs ($500x10^3$) or neutrophils ($250x10^3$) in 100 μl of RPMI 1640 medium were added to the coated inserts and 600 μl of medium in the absence (spontaneous migration) or presence (chemoattractant-stimulated migration) to the cluster wells. The cells were then incubated for 3 h (monocytes) and 1:5 h (neutrophils) at 37ºC. Stroma derived factor 1α (SDF-1α, 500 ng/ml), and formyl-methionine-leucyl-phenylalanin (fMLP, 10 nm), or 100 ng/ml interleukin (IL) 8, were used as chemoattractants for monocytes and neutrophils respectively. In migration inhibition assay cells were pre-incubated with mAbs to integrin chains as in 3.4. To assess the role of endogenous laminins, inserts were coated with 20 μg/ml of HSA and blocked with 0.5% (W/V) HSA, and cells were treated with mIgG or mAbs to integrin or laminin chains either separately or in combination. mAb G46-2.6 to a common frame epitope of major histocompatibility complex (MHC) class I molecules (BD Biosciences), and mAbs ASC-3 and ASC-8 to integrin β4 (CD104) (Chemicon), which is not expressed by neutrophils, were also included as controls. To efficiently remove all transmigrated cells from the lower chamber, a final concentration of 10 mM EDTA was added to each well and cells were vigorously resuspended and collected. Thereafter, the cells were fixed with 1% formaldehyde and counted microscopically using a haemocytometer at 400x magnification or by flow cytometry.

3.6 Leukocyte recruitment assay (IV)

LNα4 KO mice generated by gene targeting in embryonic stem cells (Thybol, 2002) were used as acute inflammatory model of peritonitis to analyze the role of α4 LNs (LN-8, -9, -14) in leukocyte extravasation. The LNα4 deficient mice were viable, fertile, but displayed transient hemorrhage at birth and subtle motor impairment as adult (Thyboll et al., 2002). Wild-type (WT, +/+) and knockout (KO, -/-) adult male mice with similar genetic background were used to analyze leukocyte

recruitment into the peritoneal cavity. The animals were temporarily anesthetized with Isoflurane, and 3 ml of either PBS or 3% proteose peptone (PP) (Unipath Ltd., Basingstoke, United Kingdom) was injected intraperitoneally (i.p.). After 6 h, the mice were sacrificed, and 4 ml of cold PBS containing 0.25% bovine serum albumin and 2 mM EDTA was injected i.p. The peritoneal exudates were collected after gentle abdominal massage. Cells from 0.5ml samples were centrifuged and stained with 10 μg/ml ethidium bromide to gate out nucleus-free debris. After three washes the cells were re-suspended in 0.5 ml PBS and analyzed by flow cytometry in a FACSort (BD Biosciences). Forward and side scatter properties were used to identify the neutrophils.

3.7 Apoptosis detection assays (IV)

To assess the role of LN isoforms on neutrophil survival, 24 well-flat bottom culture plates (Costar) were coated with 20 μg/ml of either HSA, mLN-1, rhLN-8 or rhLN-10 at 4^{o}C overnight. Two million neutrophils at 1 x10^{6} cells/ml in RPMI 1640 (Sigma) were plated in each well and incubated for 12 h or longer at 37^{o}C in a humidified atmosphere containing 5% CO_2. Thereafter, cell samples were collected for annexin V staining (flow cytometry) and caspase enzyme activity (fluorometric assay), respectively, as previously described (Fadeel et al., 1998).

3.8 Characterization of commercial LN preparations (V)

Four commercial preparations, human merosin (Chemicon), Me/C; human LN (Takara), LN/T; human LN (Sigma), LN/S and human LN (Chemicon), LN/C, all purified from human placenta by different methods were analysed in comparison to rhLN-8, -10 and mLN-1 (BD. Bioscince), (Tabel 2). The chain composition of the preparations was first analysed by ELISA as described above under.3.1. Coating of the wells was done with 0.25 to 3 μg/ml of different LNs. Detection of the bound protein was done using mAbs to all LN chains including mAbs to other ECM proteins that are expected to be potential contaminants of the LN preparations. The rest was done as described above under 3.1.

For further characterization, protein samples were run in SDS-PAGE using 6% polyacrylamide gel under reducing condition. The proteins thus separated were analysed either by silver staining or western blotting as described in 3.1 above.
The effect of the LN preparations on transmigration of lymphocytes and neutrophils was tested using 3 μm Transwell culture inserts coated with various LN preparations as described above in 3.5. Isolated PBMCs (3 h) and neutrophils (1:5 h) were allowed to migrate in the presence of chemoattractant as described above. Counting of the migrated leukocyte was done microscopically using haemocytometer. The percentage of migrated lymphocyte was determined by flow cytometry based on side and forward scatter properties and computing from the starting cell population.

Table IIa. Laminin preparations used in this thesis.

Laminin preparations	Method of purification	Supplier/Reference
Human merosin (Me/C)	From placenta by EDTA and salt extraction, and size exclusion and ion exchange chromatography	Chemicon
Human laminin (LN/T)	From placenta by salt extraction and unspecified mAb-affinity chromatography	Takara
Human laminin (LN/S)	From placenta by mild digestion and unspecified immunoaffinity chromatography	Sigma
Human laminin (LN/C)	From placenta by mild pepsin digestion and mAb 4C7 (LNα5)-affinity chromatography	Chemicon
Mouse laminin-1 (α1β1γ1)	From Engelbreth-Holm-Swarm tumor	BD Biosciences
Recombinat human laminin 8 (α4β1γ1)	From a mammalian expression system by ion exchange and affinity chromatography	Kortesmaa et al., 2002
Recombinant human laminin 10 (α5β1γ1)	From a mammalian expression system by ion exchange and affinity chromatography	Doi et al., 2002

Table IIb. Monoclonal antibodies (mAbs) to laminin chains used in this thesis.

mobs	Specificity	Source/supplier	Reference
EB7	LNα1	Prof. IsmoVirtanen	Patarroyo et al., 2002
5H2	LNα2	Chemicon	
4H8	LNα2	Sigma	
P3H9-2	LNα3	Chemicon	
3D7	LNα4	Prof. Manuel Patarroyo	Paper IV
3H2	LNα4	" "	" "
5D8	LNα4	" "	" "
6A12	LNα4	" "	" "
6C3	LNα4	" "	" "
8C10	LNα4	" "	" "
9B2	LNα4	" "	" "
FC10	LNα4	Prof. Ismo Virtanen	" II
4C7	LNα5	Dako	Engvall et al., 1990
11D5	LNα5	Prof Eva Engvall	Engvall et al., 1990
15H5	LNα5	Prof Kiyotoshi Sekiguchi	Kikkawa et al., 1998
DG10	LNβ1	Prof IsmoVirtanen	Paper I
LAM-89	LNβ1	Sigma	Paper I
2G6	LNβ1	Sera-Lab	
3E5	LNβ1	Prof Eva Engvall	Paper I
C4	LNβ2	Neomarkers	
17	LNβ3	BD Biosciences	
CAF-2	LNγ1	Prof Harold P. Erickson	Paper I
LN-41	LNγ1	Takara	Paper I
2E8	LNγ1	Prof Eva Engvall	
22	LNγ1	BD Biosciences	Paper I
D4B5	LNγ2	Chemicon	

Table IIc. Monoclonal antibodies (mAbs) to integrin chains used in this thesis.

mAbs	Specificity	Source/Supplier
H12	INTαL, CD11a	Prof. Hans Wigzel
60.1	INTαM, CDCD11b	Patrick Beatty
2LPM	INTαM, CD11b	Dako
GoH3	INTα6, CD49f	BD Biosciences; Immunothech
6S6	INTβ1, CD29	Chemicon
13	INTβ1, CD29	Prof. Kenneth Yamada
IB4	INTβ2, CD18	Dr. Claes Lundberg
ASC-3	INTβ4, CD104	Chemicon
ASC-8	INTβ4, CD104	Chemicon

Statistics

Results were presented as mean ± standard deviation (SD). Statistical significance was assessed by paired Student's *t* test for paired groups (*in vitro* studies) and by unpaired Student's *t* test for unpaired groups (*in vivo* studies). Differences between means were considered significant when $p<0.05$.

4 Results and discussion

4.1 Chain specificity assignment and production of mAbs to human LN chains (P I, II, IV)

Mononclonal antibodies (mAbs) first produce by (Milstein et al., 1980) are essential complementary tools in characterization of proteins. In particular, functional and molecular characterization of multi-chain and multi-domain proteins like LN requires highly specific mAbs that recognize various functional epitopes and are effective in different assay systems. Several mAbs to LN chains have been produced by different groups (Engvall et al., 1986; Ljubimov et al., 1986; Lissitzky et al., 1988; LeMosy et al., 1996; Virtanen et al., 1997), and some are commercially available. However, since most of these antibodies were produced using LN heterotrimers isolated from tissues as immunogen, their chain specificity is often indistinct. When the specificities were reported, deductions were often made based on immunostaining of various tissues. Considering such problems, the molecular complexity of laminin isoforms, and the indispensable value of mAbs in structural and functional studies, we first determined the specificity of 16 commonly used mAbs to human laminin using rLN β1 and γ1chains. Later, we produced and characterized eight novel mAbs to LNα4 chain as these reagents were crucial for the characterization of α4 containing LNs, such as LN-8 (α4β1γ1). These mAbs were used as tools in the exploration and characterization of LNs in blood cells in general and neutrophils and monocytes in particular. Different methods were employed to characterize these mAbs.

By ELISA, all the 16 mAbs tested reacted with purified human placenta laminin, confirming their LN specificity. Most mAbs reacted either with rLNβ1 or rLNγ1 (Fig. 1A, Paper I). The LNβ1 and γ1 specificity of the mAbs was further confirmed by their reactivity with the respective chains in western blotting under non-reducing condition. Under reducing condition, however, only four mAbs were reactive (Fig. 1B, Paper I), suggesting that the corresponding epitopes are conformational and dependent on intrachain disulfide bonds. Since disulfide bonds are abundant in the LE modules of domain III and V (Ekblom and Timpl, 1996) these mAbs may recognize either of these domains. All the mAbs to LNβ1 and LNγ1 chains, except 22, were capable of immunoprecipiting associated LNβ1 and LNγ1 chains from platelet lysate (Fig 2A, B, Paper I). The β1 and γ1 chains have been shown to associate to LNα4 chain in platelets (Geberhiwot et al., 1999) forming LN-8. mAbs 4E10, 4G12 and 4C7 did not react with either rLNβ1or rLNγ chains. The placenta LN preparation used in this study contains LN-10 (α5β1γ1/Lm-511) and LN-11 (α5β2γ1/Lm-521) (Ferletta and Ekblom, 1999). As expected, mAb 4C7 to LNα5 chain (Tiger et al., 1997) reacted with the placenta LN preparation. However, lack of reactivity of mAb 4E10 that has been reported to be LNβ1 chain specific, is intriguing. The epitope may be formed by association of LNβ1 with other LN chains.

Thus, we could identify the LN chain specificity of three mAbs (LAM-89, 2G6, and LN-26) and confirm that of three others (DG10, 3E5 and 1928). Similarly, two mAbs (LN-41 and 4C12) were identified and five mAbs (2E8, 22, CAF2, LN-41, BC7) were confirmed as specific for LNγ1 chain. We could not either confirm or identify the LN chain specificity of 4E10 and 4G12. Based on our present results and data from the

literature, a tentative epitope mapping of LN β1 and γ1 chains was proposed (Fig. 2C, Paper I). The knowledge of chain specificity of mAbs facilitates the identification and characterization of the increasing number of LN isoforms. It also helps to localize the functional sites in laminins.

To facilitate the characterization of α4 LNs, eight novel mAbs to LNα4 chains were generated by hybridoma technique. The mAbs 3D7, 3H2, 5D8, 6A12, 6C3, 8C10 9B2, and FC10 were first analysed by ELISA and were reactive only with rhLN-8 but not with rhLN-10 (Fig. 1A, Paper IV; Fig. 1A; Paper II). rhLN-8 and -10 share laminin β1 and γ1 chains but differ in their α chain, the former contains α4 and the latter α5. Reactivity of the mAbs with only rhLN-8 defined their α4 specificity. In contrast to the novel antibodies, mAb 4C7 reacted with rhLN-10, but not with rhLN-8, confirming the LN α5 specificity of this antibody. Further analysis of the mAbs was done by Western blotting. All of the mAbs, except 3H2, 5D8 and FC10 were reactive under reducing condition against platelet lysate recognizing bands of 180 and 200 kDa (Fig 1B, Paper IV). Under non-reducing condition all except 3D7 reacted with a band of 630 kDa corresponding to human platelet LN-8 (Fig. C, Paper IV). Previous microsequencing and immunoblotting with a polyclonal antibody against a recombinat LNα4 domain have demonstrated that, in platelets under reducing conditions, LNα4 chain consists of a major polypeptide of 180 kDa, and a minor one of 200 kDa (Geberhiwot 1999). The reactivity of the mAbs with these bands further confirms their α4 specificity. LN β1 and γ1 chains have an apparent mol. wt. of 230 and 220 kDa, respectively. The control mAbs DG10 (to LNβ1 chain) and 22 (to LNγ1 chain) recognized the respective bands. The 630 kDa band was also detected with mAbs DG10 and 2E8 (to LNγ1 chain), indicating the disulfied linkage of laminin α4, β1 and γ1 chains in the heterotrimer. Finally, the mAbs were tested in immunoprecipitation. Western blot analysis of the precipitate showed that mAbs 3H2, 5D8, and 9B2 precipitated α4 LNs (mainly LN-8) from platelet lysate (Fig. 1D, Paper IV). In contrast, mAb 6C3, which works very well in Western blotting, was itself a poor immunoprecipitating antibody.

4.2 Laminin α4 chain is primarily localized in tissues of mesodermal origin in human (P II)

One of the novel mAbs to LNα4 chain, FC10, was used for immunohistochemistry in developing and adult human tissues. LNα4 chain was localized in tissues of mesodermal origin (BM of endothelia, adipocytes, and skeletal, smooth and cardiac muscle cells), in some epithelial BM (epidermis, salivary glands, pancreas, esophageal and gastric glands, intestinal crypts and some renal medullar tubules). Developmental stage differences in the distribution of LNα4 chain were observed in skeletal muscle, vessel walls and intestinal crypt. Co-localization of LNα4 and LNα2 chains were detected in BMs of fetal skeletal muscle cells and some epithelial BMs (gastric glands, and acini of pancreas). Two forms of LNα4 chain (Mr 180,000 and 200,000) were detected in cultured human pulmonary alveolar endothelial cells (HPAE). In cell-free extracellular matrices of human kidney and lung, LNα4 chain was found as Mr 180,000 protein.

4.3 Monocyte precursors synthesize and monocytes express laminin-8 (P III)

To investigate synthesis of LN-8 by myeloid cells, expression of the transcripts for LN-8 chains was assessed in monoblastic THP-1, Mono Mac 6 and U-937 cells by RT-PCR. In THP-1 cells, the mRNAs for all the three chains α4, β1 and γ1 of LN-8, were detected. For the detection of LNα4 mRNA, it was necessary to do nested PCR. In Mono Mac 6 cells, similar results were obtained. In U-937 cells however, we could detect only the γ1 transcript.

Once the transcripts were detected, synthesis and expression of the corresponding polypeptides was assessed in THP-1 cells by metabolic labeling with ^{32}S-methionine/cysteine followed by immunoprecipitation with mAbs to LN chains. A polypeptide of 200 kDa was detected with mAbs to LNγ1, but no band was detected with mAb to LNβ1. In Western blot analysis of total THP-1 cell lysate, bands of 200 kDa and 220 kDa were detected with mAbs to LNβ1 and LNγ1 chains respectively under reducing condition. Under non-reducing condition however, a strong band of 200 kDa and a faint band of about 420 kDa were detected with mAbs to LNγ1 chain. A high MW polypeptide was also detected by mAbs to LNβ1. This high MW polypeptide that was detected with both the β1 and γ1 mAbs corresponds most likely to the disulfide linked β1γ1 heterodimer and the smaller polypeptide to the γ1 monomer. In addition, LNβ1 and LNγ1 chains were detected in the THP-1 lysate prepared from cells grown in serum-free culture medium. All these results indicate the synthesis of LNβ1 and γ1 chains by these cells. At the time that this study was conducted, the only available reagent against LNα4 chain was a polyclonal Ab that reacts with different bands in the total cell lysate. To overcome this problem, laminin was purified from the lysate of the three monoblasatic cells by immunoaffinity purification through a LNβ1 mAb column. In Western blot analysis of the purified material, bands of 220 (β1) and 200 (γ1) and 200, 180, 130 (α4) were detected with the corresponding Abs under reducing condition, indicating the physical association of the three chains for LN-8. The detection of only the γ1 chain in metabolically labeled THP-1 cells and the necessity of nested PCR for the detection of the α4 chain may indicate low turnover of β1 and α4 LN chains.

THP-1, Mono Mac 6 and U-937 represent monoblast/promonocytes, monocyte precursor in bone marrow. Since LN-8 has been detected in the precursor cells, we investigated its expression in mature monocytes. By indirect immunofluorescence, all the tested monocytes were reactive with mAbs to LN-γ1 chains after cell permeabilization. In a few experiments intact monocytes were also reactive, indicating the surface expression of LN epitopes. To analyze the expression of LN-8, monocytes were isolated from PBMCs by adherence to tissue culture flasks. Intact LN-8 heterotrimer was isolated from monocyte lysate in a similar way as for monoblastoid THP-1 cells. Bands of the expected molecular mass were detected with the corresponding Abs. Thus, the reactivity of the permeabilized monocytes indicates the intracellular localization of the laminin in these cells. Altogether, the results showed that monoblastic cells synthesize and mature monocytes contain intact LN-8 (α4β1γ1, Lm-411).

4.4 LN-8 promotes monocytic cell adhesion and migration (P III)

LN-8 has been shown to mediate adhesion of blood platelets (Geberhiwot et al., 1999), and adhesion and migration of lymphocyte *in vitro* (Geberhiwot et al., 2000). Cell adhesion and migration are highly interdependent cell activities and are central to most leukocyte functions *in vivo*. Monocytic cell functions, such as migration, phagocytosis, and induction of immune and inflammatory responses, all involve adhesion-de-adhesion steps. Identification of molecules involved in adhesion and migration of these cells contributes to a better understanding of the molecular mechanisms lying behind their functions.

Theoretically, cells could encounter laminins in different ways. Cells may interact with endogenous LNs exposed on the cell surface or secreted in the surrounding, or with exogenous LN. In an attempt to identify the role of LNs in monocytic cell functions, exogenous LN isoforms were analysed for cell adhesion and migration promoting effects, and the integrin receptors mediating these effects were also assessed. All the three LN isoforms (rhLN-8, -10 and mLN-1) tested were adhesive for monoblastic JOSK-I cells. Nevertheless, only LN-8 and LN-10/11 promoted statistically significant constitutive adhesion of JOSK-1 cells, although LN-10/11 was more adhesive. Adhesion to LN-8 was mediated predominantly by $\alpha6\beta1$ integrin (Fig.6B, paper III) and to some extent by $\beta2$ integrins. In contrast, $\alpha6\beta1$ integrin had only minor role on the JOSK-I cell adherence to LN-10/11, and rather insignificant role on LN-1. The results show preferential recognition of different LNα chains by different integrin receptors. From immunofluorescence studies, JOSK-I cells have been shown to express CD29 ($\beta1$), CD49f ($\alpha6$) and CD18 ($\beta2$) integrin chains.

The effect of exogenous LN isoforms on monocyte migration was analyzed using PBMCs in the presence or absence of chemoattractant. Both LN-1 and -8 promoted significant migration of monocytes under both conditions. Interestingly, LN-8 promoted much more efficient migration than LN-1, and cell migration was more pronounced under the influence of chemoattractant (Fig.7, paper III). The LN-10/11preparation, on the contrary, was inhibitory to monocyte migration.

4.5 Neutrophils contain and secrete intact LN-8 ($\alpha4\beta1\gamma1$) (P IV)

4.5.1 Neutrophils contain LN $\alpha4$, $\beta1$ and $\gamma1$ chains intracellularly.

Molecular and functional characterization of LN-8 is highly facilitated by the availability of mAbs to LN$\alpha4$ chain. Hence, the initial steps in this study were production and characterization of mAbs to this chain. The specificity and reactivity of the mAbs were determined by ELISA, Western blotting and immunoprecipitation using rhLN-8 and -10 (Fig. 1 Paper IV). These mAbs together with the previously characterized ones (Table 1, Paper I), rhLN-8 and -10 and LN$\alpha4$ KO mice were used to do the painstaking exploration and characterization of LN-8 in neutrophils.

Intact and permeabilized neutrophils were investigated for expression of LN chains by indirect immunofluorescence flow cytometry. Cell identity and purity was confirmed morphologically and by reactivity with mAb C3D-1 to CD15, a neutrophil marker. Cell permeabilization was controlled by staining with mAb to myeloperoxidase, an intracellular marker of myelomonocytic cells. mAbs 5D8A (LN$\alpha4$), 2G6 (LN$\beta1$) and CAF2P (LN$\gamma1$) were reactive with practically all

permeabilized neutrophils (Fig.2A, Paper IV) indicating intracellular localization of the chains. Though these results demonstrated the presence of the individual chains, at this point, it was not possible to tell if the cells were able to assemble the heterotrimeric LN-8 as the other blood cells.

4.5.2 Neutrophils contain intact LN-8 and secret this LN when stimulated.

Once we knew that neutrophils contain the chains for LN-8 intracellularly, we attempted to purify the protein from neutrophils total lysate using mAb DG10 (LN β1) affinity column. Analysis of the purified protein by SDS-PAGE and western blotting revealed the presence of a 230, 220 and 180 kDa polypeptides reactive with mAbs, DG10 (LNβ1), 22 (LNγ1) and 6C3 (LNα4) respectively (Fig.2B, Paper IV). Similar results were obtained from the supernatant of TPA stimulated neutrophils (Fig. 2B, Paper IV). These results indicate the presence of intact LN-8 in neutrophils that can be secreted when the cells are stimulated. The next question was, do neutrophils utilize this LN? What about the effect of exogenous LNs? Which is the receptor? During inflammatory responses neutrophils traverse the vascular BM, which is rich in LN-8 and LN-10. What is the role of endothelial LNs on neutrophil functions?

4.5.3 LN-8 (α4β1γ1) promotes neutrophil adhesion and migration via αMβ2 integrin (P IV)

Once we knew that neutrophils express and secrete LN-8 we tried to understand if the endogenous LN has some role on the transmigration of the cells. Secreted LN-8 could be used by neutrophils or other cells. In previous studies, fMLP-induced migration of rabbit peritoneal exudate neutrophils through uncoated filters was blocked by Abs to mLN-1 (Terranova et al., 1986). Deposited endogenous LN-5 (α3β3γ2) was also shown to promote keratinocyte motility (Zhang and Kramer, 1996). We used mAbs to LNα4 chains to analyze the role of endogenous LN-8. fLMP-induced migration of neutrophils through inserts coated and blocked with HSA was inhibited significantly by mAbs 6A12 and 8C10 to LNα4 chain (Fig. 5, paper IV). Further inhibition was attained when each of the mAbs was combined with mAb P3H9-2 to LNα3 chain, indicating the participation of at least two LN isoforms. Participation of αMβ2 integrin was also detected, suggesting the recognition of endogenous LN isoforms (LN-8 and α3 LNs) by this integrin. In unpublished studies (Patarroyo et al.) both α3 and α5 LNs have been detected in blood cells. These results suggested that neutrophil migration involves the participation of endogenous LNs, including LN-8 and an α3 LN.

The *in vitro* effect of exogenous LN-8 on neutrophils adhesion and migration was analysed in parallel and compared to other LN isoforms. HSA coating or just PBS treatment were included as control. LN-1 is irrelevant to vascular endothelium whereas LN-8, together with LN-10, is one of the major laminins of the vascular endothelial BM. Neutrophils were allowed to adhere to the coated wells or to migrate through coated culture insert in absence or presence of stimuli. All the three LN isoforms tested including HSA promoted adhesion of TPA stimulated neutrophils. However, non-coated plates did not promote neutrophil adhesion even after cell stimulation. Neutrophil adhesion to all the three LN isoforms was blocked to the baseline level with

mAbs 60.1 and IB4 directed to αM and β2 integrin chains, respectively, but not with mAbs 6S6 to β1 integrin (CD29) or GoH3 to α6 integrin chain (Fig.4, Paper IV). In contrast to our results, lack of adhesion of neutrophils from WT and β2 integrin KO mice to mLN-1 and mLN-8 has been reported (Sixt et al., 2001). The basis of this discrepancy is unknown at present. Different experimental design and different reagents and/or species may be explanations for the differences.

In our *in vitro* migration assay, all the three LNs promoted chemoattractant (fMLP or IL-8) induced migration of neutrophils. In contrast to its effect on neutrophil adherence, HSA has minimal effect on the transmigration the cells. Although LN-1 and -10 promoted migration of neutrophils at a comparable level to that of rhLN-8, only rhLN-8 promoted spontaneous migration at a statistically significant level (Fig. 5A, Paper IV). As for cell adhesion, neutrophil migration was blocked with mAbs 60.1 and IB4, but only to a minimal degree with mAb 6S6 (Fig.5B, Paper IV).). Sixt and colleagues (Sixt et al., 2001) reported that LN-8 compared to LN-10 was poor promoter of migration for β2 integrin deficient neutrophils and that the effect was partially α6β1 integrin dependent. Our results deviate from this observation in that both LN-8 and -10 promote migration of normal neutrophils, and that αMβ2 integrin is the major mediator of the response. In our study, partial inhibition of neutrophil migration on LN-8 and -10 by mAb 6S6 to β1 integrin supports participation of α6β1 integrin. On the other hand, the results reported by Sixt and colleagues indirectly suggest a major contribution of β2 integrin(s) to neutrophil migration on LN-8. In support of our *in vitro* data, neutrophil transmigration through blood vessel of αL integrin deficient mice has been reported to be inhibited by blocking mAb to αM integrin (Henderson et al., 2001). Furthermore, MCP-1 stimulated migration of monocytes, and TNF-α-induced respiratory burst of neutrophil on mLN-1substrate require β2 integrins (Nathan et al., 1989; Penberthy et al., 1995). These results indicate that LN-8 promotes adhesion and migration of neutrophils *in vitro*, and these responses are αMβ2 integrin mediated.

4.5.4 α4-LN is necessary for extravasation of neutrophils to inflamed peritoneum (P IV).

Neutrophils are the first leucocytes to extravasate in acute inflammatory responses. Our *in vitro* migration data suggested the role of LN-8 in extravasation of neutrophils, as this LN isoform is a major constituent of vascular endothelial BM. To assess the role of α4-LNs in the extravasation of neutrophils, acute peritonitis was compared between LN α4 KO mice (-/-) and WT mice (+/+). Cells collected from peritoneal exudates were analyzed by flow cytometry. Differential leukocyte count was performed, and the results were expressed as mean cell count. The result showed that the neutrophil count was decreased significantly in LNα4 null mice (mean ± SD; $p<0.01$) (Fig.6, Paper IV), indicating the critical role of α4-LN in neutrophil extravasation. Besides, preliminary intravital microscopy examination showed impaired leukocyte migration through the vessel wall in LNα4 deficient mice (Lindbom et al., unpublished data). Since both WT and KO mice had similar number of blood neutrophil count (Wondimu et al., unpublished data), neutropenia can not explain the observed difference. As LN-8 content is much higher in vascular endothelial BM than in neutrophils, the most likely explanation is that lack of this LN isoform in the vessel wall accounts for the impaired extravasation. In support of this belief, similar adhesion of blood neutrophils from WT and KO mice to rhLN-8 was detected after TPA

stimulation (Wondimu et al., unpublished data). In experimental autoimmune encephalomyelitis, inflammatory cuffs of T cells accumulated exclusively around endothelial BMs containing LN-8, but not LN-10 (Sixt et al., 2001). Neutrophil extravasation through perivascular BM has been repoted to be inhibited by mAb to α6 integrin (Dangerfield et al., 2002). Transendothelial migration appears to upregulate α6β1 integrin expression on neutrophils by homophilic interaction of platelet-endothelial cell adhesion molecule (PECAM)-1 (Roussel and Gingras, 1997; Dangerfield et al., 2002).

4.5.5 LN-8 (α4β1γ1, Lm-411) protects neutrophil against spontaneous apoptosis (P IV).

Human neutrophils undergo spontaneous apoptosis both *in vitro* and *in vivo* (Payne et al., 1994; Fadeel and Kagan, 2003) and various mediators are known to either delay or enhance neutrophil apoptosis (Colotta, 1992). Interestingly, extravasation is known to delay neutrophil apoptosis indicating that the vessel wall provides a survival signal (Watson, 1997). Since LN-8 is a major endothelial LN that neutrophils encounter during extravasation, we studied its effect on neutrophil apoptosis. After 12 h of neutrophil incubation on wells coated with rhLN-8, rhLN-10, mouse LN-1 or HSA, apoptotic cells were detected by analyzing posphatedylserine (PS) exposure by flow cytometry, and by measurement of caspase-3 like activity. Marked delay in apoptosis was observed in neutrophils plated on rhLN-8 coated wells compared to cells plated on HSA (Fig.7, Paper IV). A significant decrease in both PS exposure and caspase-3 like activity was detected, as measured by FACS and DEVD-AMC cleavage, respectively (Fig.7, Paper IV). These results indicated that LN-8 prolonged neutrophils survival. Prolonged survival might be of physiological relevance. Though β2 integrins have been shown to either delay or enhance apoptosis depending on the activation state of the integrin and the proapoptotic stimuli (Whitlock, 2000), clustering of αMβ2 integrin by immobilized LN-8 may mediate the observed protective effect in our study. Neutrophils are essential components of acute inflammatory responses. They are short-lived cells and die spontaneously even at the site of development. Their role as phagocytes comprise two complementary components. The primary component is killing the detainee without mercy. If this action fails, the next equally important component is to contain and prevent dissemination of the infection until the adaptive immunity is ready to take over the job. Agents which provide survival signal are thus of physiological importance.

4.6 High fragmentation, mixture of isoforms and functional differences in placenta LN preparations (P V)

Cell culture supernatant or human placenta is commonly used for large scale purification of human LNs (Wewer et al., 1983; Ehrig et al., 1990; Kikkawa et al., 1998). Though placenta laminin preparations have been used in functional studies, they have not been fully characterized structurally and functionally. Moreover, we have observed opposing effects of different LN-10 preparations on monocyte migration. In our previous studies, before rhLN-10 was made available, one of commercial LN-10 preparations inhibited monocyte transmigration (Fig. 7, Paper III). In contrast, rhLN-10

strongly promotes monocyte migration (Gorfu et al., unpublished data).These observations together with the complex nature of the laminin family and the wide application of these preparations in research, attracted our attention to carry out this study. We then characterized four commercial laminin preparations, M/C, LN/T, LN/S and LN/C, purified from human placenta for chain composition and their effect on lymphocyte and neutrophil migration in comparison to mLN-1, rhLN-8 and -10 (Kortesmaa et al., 2000; Doi et al., 2002). By ELISA, all laminin preparations, except LN/S, were highly reactive with mAbs LN-41 to LNγ1 chain, one of the most widely distributed laminin chains (Fig. 1A, Paper V). In contrast, no or negligible reactivity was detected with mAbs to LNα1, LNα3, LNβ3 and LNγ2 chains. Me/C and LN/T were reactive weakly but reproducible with mAb C4 to LNβ2 chain. As expected, rhLN-8 (α4β1γ1/Lm-411) reacted with mAb to LNα4, and rhLN-10 (α5β1γ1/Lm-511) with mAbs to LNα5 chains. In silver staining and Western blot analysis, polypeptides of 230 (LNα4), 230 (LNβ1) and 220 (LNγ1) kDa in rhLN-8, and 350 (LNα5), 230 (LNβ1) and 220 (LNγ1) kDa in rhLN-10 were detected (Fig. 1B and C, Paper V,).

Me/C strongly reacted with mAb 5H2 to LNα2 by ELISA (Fig. 1A, Paper V) and weakly with mAb 4H8 that recognizes the N-terminal region of the same chain (data not shown). Polypeptides of 300 (LNα2), 230 (LNβ1), 220 (LNγ1), 190 (LNβ2) and 80/65 (LNα2 C-terminal fragments) kDa were detected (Fig. 1B and C, Paper V,). Weak contamination with fibronectin was also observed. In contrast to Me/C, other merosin preparations purchased earlier from other company strongly reacted with mAb 8C10, indicating contamination with α4 LNs (data not shown).

LN/T, slightly reacted with mAbs 5H2 (LNα2) and 8C10 (LNα4), but only by ELISA (Fig. 1A, Paper V,). Polypeptides of approximately 200 KDa corresponding to intact LNβ1 and LNγ1 and 190 KDa corresponding to LNβ2 chains were detected, but their fragments were also noted. This preparation was highly contaminated with fibronectin (Fig. 1A and C, Paper V,).

LN/S reacted with mAb 11D5 to LNα5, but was not recognized by mAbs 4C7 or 15H5, which recognize the globular domain and short arm of the same chain, respectively (Fig. 1A and C, Paper V). Polypeptides of 160 KDa were detected as major components by mAbs DG10 (LNβ1) and CAF-2 (LNγ1), indicating substantial proteolysis of this preparation. These fragments correspond to the C-terminal region of LNβ1 and LNγ1 chains, as determined by reactivity with mAb 3E5 that recognizes the C-terminus of LNβ1 and lack of reactivity with mAbs LN-41 and 22 that recognize the short arm of LNγ1.

LN/C was similar to LN/S, but showed weaker reactivity with mAbs 11D5 (LNα5) and contained some intact LNγ1 chain. Surprisingly, other batches of LN/C and LN/S exhibited strong reactivity with mAbs 4C7 and 11D5, indicating batch to batch differences of the same preparations (data not shown). A LN preparation similar to LN/C has been previously characterized by others as LN10/11 (α5β1/β2γ1, Lm-511/521) (Ferletta and Ekblom, 1999; Spessotto et al., 2003) and also in this thesis (Fig. 1A, paper I). It should be noted that LN/C used in our study (Paper V) was not, however, reactive with mAbs to LNβ2.

When the placenta LN preparations were tested for their effect on cell migration, all except LN/S were rather poor promoters of chemoattractant-induced migration of neutrophils (Fig. 2A, Paper V) and lymphocytes (Fig. 2B, Paper V). This is not in agreement with the effect of rhLN-8 and -10, which significantly enhance the migration of both cell types (Paper IV, Fig. 7; Gorfu et al., unpublished data). Altogether, the

results showed large fragmentation of the proteins, a mixture of laminin isoforms, and contamination with fibronectin among the isolated placenta laminins. Functional differences on leukocyte migration were also observed. The purification protocol employed could contribute for the observed differences among the preparations. Accordingly, EDTA and/or salt extraction seems more selective for α2- and α4-laminins. Mild pepsin digestion seems to be necessary for isolation of α5-laminins. By this method, the coiled-coil region has been preserved but the short arms and/or the globular domain might be lost by proteolysis.

According to the present results, the major isoforms of each preparation were: LN-2 (α2β1γ1, Lm-211) in Me/C; LN-2/8? (α2/α4β1γ1, Lm-211) in LN/T and LN-10 (α5β1γ1, Lm-511) in LN/S and LN/C. Based on these results, a schematic representation of these isoforms is shown in (Fig. 2C, Paper V). Considering the heterogeneity of the placenta laminin preparations, as well as their proteolysis and contamination with other matrix proteins, data obtained in functional studies using these laminins need to be interpreted with caution.

5 Conclusions

The work of this thesis has revealed the expression and functions of LN-8 in mononcytes and neutrophils. These results may be summarized as follows:

- The synthesis of LN-8 (α4β1γ1, Lm-411) by precursor cells and expression of intact LN-8 by mature blood monocytes and neutrophils have been documented.
- Laminin 8 has been shown to promote adhesion and migration of myeloid cells.
- The requirement of α4 LNs (most likely LN-8) for neutrophil recruitment to inflammatory locus has been evidenced.
- The role of LN-8 in prolonging neutrophil survival has been shown.
- The role of α6β1 integrin on myeloid cell adhesion to LN-8, and that of αMβ2 integrin in neutrophil adhesion and migration was determined.

Interestingly, the adhesive responses of lymphocytes (Geberhiwot et al. 2001) and neutrophils were mediated by different integrin subfamilies. Utilization of LN-8 by lymphocytes was primarily β1 integrin (α6β1) dependent. However, neutrophils response to LN-8 was primarily β2 integrin (αMβ2) dependent. This preferential recognition of the same ligand by different integrin receptors on different leukocyte subpopulation may generate different signaling/cellular responses.

The results of these studies hopefully will contribute to a better understanding of the role of LNs on myeloid cell physiology in general and neutrophil and monocyte physiology in particular.

Although important information could be documented, much remains for future investigation. For example myeloid cells express LN-8. One may then ponder the question, which is the sub-cellular localization of LN-8? Since epitopes for LNs could sometimes be detected on the cell-surface, one may also ask what could be the relevance in myeloid cell physiology? LN-8 has also been shown to be protective for neutrophils. Which receptor(s) mediate this effect? Could β2 integrins be involved in this effect also? The requirement for α4 LNs for extravasation of neutrophil has further been demonstrated; could α4 LNs have similar role in extravasation of other leukocytes? What is the role of endogenous LN-8 in the migration of neutrophils in tissues? Most of these questions have been treated during the course of this study, but conclusive data could not be generated. Hopefully, these questions will be answered in the future.

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