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"The role of 67 kDa laminin receptor in G-CSF-induced hematopoietic stem cell mobilization"

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§ I CHAPTER

INTRODUCTION

I.1 67LR: isolation and structure

In 1983, three independent laboratories isolated a non-integrin 67kDa protein, designated the 67kDa laminin receptor (67LR), by affinity chromatography on laminin-Sepharose (1-3). To date, the structure of this molecule has not yet been fully elucidated, and only the cDNA encoding a cytoplasmic 37kDa precursor (37LRP) has been identified (4). Pulse-chase experiments performed on melanoma cells demonstrated that this 37kDa polypeptide is the precursor of the 67kDa form (5), and the post-translational mechanism by which the 67LR is synthesized from the precursor has been shown to consist in the acylation of the precursor and formation of homo-or heterodimers (6, 7). Based on the amino acid sequence of the 37LRP, a transmembrane domain (residues 86-101) and several laminin binding sites have been identified, consisting of a palindromic sequence, LMWWML, known as peptide G (8), a predicted helical domain corresponding to residues 205-229, and TEDWS-containing C-terminal repeats (9).
The specific site of 67LR interaction with laminin has been also identified; it resides in the sequence YIGSR, located on the short arm of laminin (10).

1.2 67LR: gene cloning

The human 37LRP gene has been mapped by fluorescence in situ hybridization to 3p21.3, a chromosomal locus frequently involved in karyotypic rearrangements associated with cancers (11). 37LRP gene contains seven exons and six introns with multiple transcription start sites. The promoter area does not bear a TATA box but contains four Sp1 sites. The first intron is also GC rich, containing five Sp1 sites. Intron 4 contains the full sequence of the small nuclear RNA E2, and two Alu sequences are found in intron 3. 37LRP gene shows a close correspondence with genes encoding the ribosomal protein p40 (12), which is a component of the translational machinery associated with the 40S ribosomal subunit. The amino acid sequence corresponding to the 37LRP cDNA is extremely conserved during evolution (13). 37LRP homologs among mammalian species (e.g., bovine, rat, mouse, human and hamster) all have an intact laminin binding domain, located in the carboxy-terminal part of the molecule, and share 99% homology (14). It is an intriguing feature that the homology of all the non mammalian 37LRP/p40 homologs with the
mammalian polypeptides is limited to the aminoterminal part of the molecule, due to a lack of the carboxy-terminal laminin-binding domain. Therefore, it has been proposed that, during evolution, the 37LRP/p40 gene gained in vertebrates a sequence encoding a laminin binding domain that is responsible for the appearance of a new function such as cell-matrix interaction (15).

I.3  Relationship between the 67LR, integrins, and laminin

A few years after the initial description of the 67LR as a laminin receptor, integrins were identified as primary mediators of cell adhesion and signal transduction to the nucleus. Actually, many studies have addressed the issue of the respective roles of these two types of receptors in mediating cell-cell or cell-matrix interactions. It has been shown that 67LR and VLA6 are co-expressed, co-regulated, and physically associated on the cell surface, suggesting their mutual involvement in laminin binding (16, 17). In fact, in A431 epidermoid carcinoma cells α6 antisense oligonucleotides, which downmodulate integrin expression on the membrane, also proportionally reduced 67LR membrane expression. This regulation occurred at the level of protein translocation from the cytoplasm to the membrane. Moreover, treatment with laminin, which increases 67LR expression, also increased the
membrane expression of laminin-specific integrins. On the cell membrane, the 67LR and the α6β4 receptor are physically associated, as demonstrated by coprecipitation experiments (17), thus suggesting a possible synergistic role of the two receptors in laminin recognition. In fact, peptide G, which contains the laminin-binding sequence of 67LR (8), increases and stabilizes laminin binding to tumor cells, possibly by changing laminin conformation to favor integrin binding (18).

I.4 Regulation of 67LR expression

Laminin receptor expression was found to be down-regulated during differentiation (19, 20) and up-regulated by cytokines and inflammatory agents (21). Up-regulation of the 67LR was also detected after interaction of cells with extracellular matrix proteins; human cell lines derived from breast cancer have increased levels of 37LRP mRNA after exposure to extracellular matrix proteins such as laminin and fibronectin (22). Moreover, a positive feed-back loop links the presence of laminin with increased 67LR expression (23). Steroid hormones have also been found to up-modulate 67LR expression in hormone-receptor positive cell lines, whereas hormone-receptor negative lines constitutively express high levels of 67LR (24).
It has been recently reported that the 67LR gene is overexpressed during transformation induced by anti-benzo[a]pyrene-7,8-diol-9,10-epoxide (25).

I.5 67LR in tumor progression

The strong correlation observed between increased 67LR expression and the metastatic potential of tumor cells suggests that this receptor plays a key role in development of the metastatic phenotype (26, 27). 67LR is expressed in a variety of human carcinomas (for example, breast, colon, lung, thyroid) and its over-expression is widely recognized as a molecular marker of metastatic aggressiveness (28). As its expression level correlates with the risk of invasion and metastasis and with malignant dissemination to the BM, 67LR is now considered to be an independent prognostic marker in breast carcinomas (29).

Given its prominent role in tumor metastasis, new therapies that utilize antagonists of 67LR have been tested in mouse (30) and anti-67LR immunotherapies have been experienced in metastatic human renal carcinoma (31) and proposed for hematological malignancies (32).

Concerning hematopoietic malignancies, laminin acts as a chemoattractant for multiple myeloma (MM) cells by interaction with the 67LR and this interaction is important for the extravasation and homing to BM of
circulating MM cells (33). The 67LR is also expressed in monocytic acute myeloid leukemias (34) and in T cell lymphomas (35).

The role of 67LR in tumor progression is based on the adhesion properties mediated by this receptor. The recent view of tumors as a functional tissue interconnected with the microenvironment suggests that the remodelling of the extracellular matrix (ECM) around the tumors is important in tumor invasion and dissemination to distant sites (36). In fact, a key step in the metastatic process is the attachment of tumor cells to laminin, the major component basement membranes. Interactions between tumor cells and basement membranes are mediated by specific membrane receptors, including the 67LR. During intra-vasation and extra-vasation, cancer cells need to come into contact with and to degrade host basement membranes, before passing through. Proteolytic degradation of basement membrane components such as proteoglycans, collagen type IV, laminin-1, and laminin-5 occurs through the action of specific proteases secreted by tumor and stromal cells. This proteolytic cleavage removes physical barriers to cell migration and converts ECM components in substrates suitable for migration (37). The invasive behaviour of metastatic tumor cells correlates with the expression of many enzymes with hydrolytic activity, such as
cysteine proteinases, cathepsin B, aspartic proteinases, cathepsin D, serine proteinases, and elastase (38, 39).

67LR binding to laminin enhances tumor-cell motility (40, 41) by determining a conformational modification of laminin, which increases its degradation rate and the release of chemotactic fragments (42). In addition, 67LR increases cancer cells invasion by up-regulating the expression and the activity of proteolytic enzymes able to degrade the extracellular matrix, such as membrane type 1 matrix metalloproteinase (MT1-MMP), stromelysin 3, cathepsin L, and the matrix metalloproteinase MMP-2 (43).

I.6 The hematopoietic stem cell

Stem cells are generally defined as cells capable of both self-renewal and mega- and multilineage differentiation. Self-renewal is the unique ability to produce daughter cells that retain stem-cell properties (44). The science of embryology has distinguished two type of stem cell: embryonic stem cell and adult stem cell (45). The term embryonic stem cell (ES) indicates a population obtained from embryos and pluripotent in that they can produce an embryo. Adult stem cell, instead, has been thought to give rise to cells committed to specific tissues (46). They have a limited ability to self-renewal with the exception of hematopoietic stem cells. Recently, studies
have suggested the existence of stem cells in many adult tissues such as the central nervous system (47, 48), lung (49, 50), liver (51), pancreas (52), and vascular system (53), in which the self-renewing is necessary after trauma, disease or aging (54). Adult stem cells also present the plasticity, as the ability of a cell to cross over its identity from one organ to another (55).

Mature blood cells are produced continuously by less-differentiated precursors that, in turn, descend from more primitive progenitors and, originally, from hematopoietic stem cells. Hematopoietic stem cells can be divided into a long-term subset, capable of indefinite self-renewal, as well as a short-term subset that self-renews for a defined interval. During late embryonic development, hematopoietic stem cells migrate towards blood circulation from the fetal liver to the bone marrow, where they are maintained throughout life.

The current stem cell model identifies two main stem cell populations with distinct progenies housed within the bone marrow, named hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). The HSCs include all lymphoid and myeloid lineages that produce blood circulating cells and organ-resident cells of the immune response, and are regulated by the BM microenvironment. This microenvironment is composed of osteoblasts, adipocytes, endothelial and vascular cells (56, 57).
HSCs are largely absent from peripheral blood (PB), except for a small pool of circulating HSCs whose physiological role in steady-state homeostasis is still unknown. One HSC can restore the entire lympho-hematopoietic system. Therefore, transplantation of marrow HSCs has been largely employed in a wide variety of acquired neoplastic and non-malignant disorders to reconstitute the hematopoietic system after chemotherapy and/or radiation.

I.7 Stem cell mobilization

The first source of HSCs for transplantation purposes was the bone marrow, obtained by repeated aspiration of the posterior iliac crests while the donor is under general or local anesthesia. This procedure has not serious side effects and the discomforts are rare.

HSCs can also be collected from the peripheral blood. Under steady-state conditions the number of HSCs is much lower in peripheral blood than in bone marrow. Chemotherapy and/or cytokine administration may lead to a concentration of HSCs in the peripheral blood that equals or exceeds the concentration in bone marrow. This process mimics the enhancement of the physiological release of stem and progenitor cells from the bone marrow reservoir in response to stress signals during inflammation (58) and is
termed “mobilization”. Currently, mobilized HSCs are the preferable and major source of stem cells harvested for autologous and allogenic transplantations, because of faster engraftment and decreased procedural risks.

Generally, peripheral blood stem cells are obtained by apheresis via a peripheral venous access. These HSCs, or better progenitor cells, are mostly expressed as the percentage of cells that react with CD34 antibodies or that form colonies in a semi-solid medium. A single apheresis procedure can bear to 3.7 times more CD34⁺ cells than the standard bone marrow harvest. Clinical studies have shown that infusion of at least 2x10⁶ CD34⁺ cells/kg recipient body weight results in reliable engraftment after 14 days from transplant (59). Normally, mobilized HSCs represent a subpopulation of all CD34⁺ cells found in the circulation; these very primitive cells do not express CD38 and lineage-specific markers, but a small subset can express the Thy-1 antigen. This CD34⁺/CD38⁻/Lin⁻/Thy⁺1 population is characterized by high repopulating activity in NOD/SCID mice and is enriched in cells initiating long-term cultures (LTC-IC) in vitro (60), representing the best surrogate of BM hematopoiesis in vivo.

The road to stem cell mobilization began in the 1960s, with publication of a few reports documenting the presence of HSCs in the peripheral blood of
mice, dogs, and monkeys, followed by reports revealing low levels of progenitors in the human circulation during steady-state homeostasis. A transient increase in circulating progenitors was documented in dogs treated with dextran sulfate and humans treated with endotoxin or other stress-inducing agents. These results were followed by preliminary reports in the late 1970s documenting increased levels of progenitors in the circulation of patients after chemotherapy treatment with cyclophosphamide and other drugs. Technical improvements, including in vitro colony assays and the availability of large-scale harvesting of human progenitors by continuous-flow leukapheresis, enabled closer examination of chemotherapy/drug induced mobilization in treated patients. Clinical studies of To and his group were the first to document the beneficial faster repopulation in patients transplanted with autologous mobilized peripheral blood HSCs (61). Initially, the mobilization protocols were based on chemotherapy alone. After the discovery of human granulocyte colony stimulating factor (G-CSF) by Welte et al, mobilization protocols began to include G-CSF, which today is the standard mobilizing agent (62). This is because it has been shown to both mobilize more CD34+ cells and have less toxicity in comparison with other single agents. G-CSF is commonly administered daily at a dose of 5-10 µg/kg for 5-10 days, alone or after chemotherapy. Sometimes the kinetics
of mobilization identify the peak of CD34+ cell in circulation in 3-6 hours after each dose (46). Alternative agent used instead of G-CSF is sometimes granulocyte-monocyte colony stimulating factor (GM-CSF), but it is used less than G-CSF, because it mobilizes less well and because of a higher incidence of mild and severe effects (63). Stem cell factor (SCF) is another excellent mobilizing agent, especially used in combination with G-CSF (64). A new mobilizing agent is AMD3100, a small reversible inhibitor of the binding of stromal derived factor (SDF-1α) to its receptor, CXCR4 (65, 66).

**1.8 Factors influencing stem cells mobilization**

The localization, as well as survival and proliferation/differentiation, of adult hematopoietic cells within bone marrow are vested on their relationships with microenvironmental cells and extracellular matrix. At steady state, proliferating hematopoietic cells in various stages of differentiation are confined within specialized BM "niches," whereas terminally differentiated, mature cells leave the BM and migrate into blood. In addition, a small proportion of morphologically unrecognizable primitive stem cells regularly escapes the BM and circulates throughout life. More importantly, the circulating pool of stem cells can increase in significant numbers by their enforced migration from BM using several interventions. Enforced
The emigration of hematopoietic cells or "mobilization" has been observed in all species examined up to now, suggesting preservation of similar mechanisms of stem cell trafficking across species. Despite its significance and great clinical impact, the mechanisms of mobilization have not been fully elucidated yet.

It appears that during mobilization the bone marrow becomes the playground of a complex interplay between cytokines/chemokines and their receptors, potent proteases, and adhesion molecules (67).

**The role of proteases.** Direct in vivo studies have shown that, after G-CSF treatment, neutrophils release the contents of either specific (i.e., matrix metalloproteinase 9 [MMP-9] or lactoferrin) or azurophilic (elastase, cathepsin G, or proteinase 3) granules (68). Lévesque et al showed that following G-CSF mobilization, serine proteases, especially neutrophil elastase, accumulate within the bone marrow environment, and their substrates included molecules implicated in mobilization, such as vascular cell adhesion molecule 1 (VCAM-1), c-kit, CXCR4 and its ligand SDF-1, as well as many components of ECM (69, 70, 71). In addition to serine proteases, metalloproteases or dipeptidyl peptidase IV (DPPIV/CD26) are also known to cleave some of the same target molecules, including SDF-1 (72).

Several subsequent studies were performed with mouse models deficient in
serine proteases, such as MMP-9, or DPP1, with mice bearing combined elastase and cathepsin-G deficiency, or with mice deficient in DPP1 and lacking functional activation of many neutrophil proteases. Each of these mice responded to G-CSF–induced mobilization to the same extent as control mice (73). To test whether other overlapping protease activities contributed to the response, MMP-9–deficient mice, or DPP1–deficient mice were treated with a broad spectrum of MMP inhibitors and G-CSF. Despite the expectation that combinations of deficiencies would greatly attenuate the response, G-CSF–induced mobilization was not impaired (73). Compensation by other proteases in these deficient animals was offered as an explanation.

A common characteristic of all the protease-deficient mice responding to G-CSF was a reduction of SDF-1 protein levels within the bone marrow, suggesting that reduction in SDF-1 may be the common denominator in G-CSF–mediated response. The emerging picture from the studies with protease-deficient mice is that the contribution of a single protease may not be critical; a coalition of proteases within bone marrow seems to be required (68).

- The role of chemokines. Mobilization has been achieved thus far with the use of several chemokines, such as IL-8, monocyte chemoattractant protein-
1 macrophage inflammatory protein 1α or 1β, Groβ, SDF-1, or others (74). Mechanisms of chemokine mobilization have been explored only with the use of IL-8, Groβ or SDF-1. The IL-8 and Gro responses appear to be dependent on adequate numbers and normal function of circulating neutrophils, and they are associated with increase of MMP-9, presumably released by mature neutrophils (75). In fact, the use of antineutrophil antibodies or of MMP-9 inhibitors greatly attenuated the response (76).

Biologic responses to SDF-1 have been also extensively studied (77). Studies with SDF-1 or CXCR4 knock-out animals revealed important roles of the CXCR4/SDF-1 pathway in the active retention of hematopoietic cells within bone marrow. Furthermore, a number of subsequent studies suggested important roles in homing and stem cell mobilization, and SDF-1 has emerged as the most potent chemoattractant of hematopoietic stem/progenitor cells (78). Mobilization was seen using Met SDF-1 (an SDF-1 analog), which is refractory to cleavage/degradation and results in prolonged desensitization of CXCR4 (79). On the other hand, adenovirus-driven SDF-1 with release of high circulating levels of SDF-1 or administration of a peptide-agonist of SDF-1 (80) resulted in mobilization, presumably because of a change in SDF-1 gradient between bone marrow and peripheral blood (81). Further observations showed that AMD3100, a
small molecule inhibitor of CXCR4 which inhibits its binding to SDF-1 and disrupts the SDF-1–dependent signaling, induced significant mobilization both in mice and humans (82).

Mobilization through the use of Met SDF-1 (83), or CXCR4 antagonists, or following Pertussis toxin (Ptx) treatment (84), is likely mediated through down-regulation of the CXCR4 receptor and/or disruption of its signaling on hematopoietic cells. Further, truncation of SDF-1 by serine proteases or MMP-9, as demonstrated during G-CSF-induced mobilization, also inhibits SDF-1–dependent signaling in hematopoietic cells, rather than change the bone marrow to peripheral blood SDF-1 gradient.

The preponderance of evidence thus suggests that inhibition of the CXCR4/SDF-1 signaling is a dominant pathway in G-CSF–induced mobilization.

-The role of adhesion molecules. The most consistent difference between mobilized progenitor cells and steady-state BM cells is the absence of cycling cells and a decreased expression of very late activation antigen 4 (VLA-4) and kit in mobilized cells (61). More important than the expression level, however, is the reduced functional state of VLA-4 receptors on mobilized HSCs (85). As the same functional change was also observed in bone marrow HSCs after G-CSF treatment, it invited the speculation that
this phenotype promotes transmigration. Whatever the predominant mechanism is, it is well accepted that migratory responses are mediated through integrin participation. Thus, one could envision that integrin (functional) down-regulation is a common step at the final stages of transmigration through the endothelial sinuses. Such a proposition is in line with VLA-4 involvement in firm retention of normal (86) or leukemic cells within the bone marrow. The fact that inducible ablation of alpha4 integrins in adults leads to hematopoietic progenitor egress in mice (87) further reinforces this concept. Like the down-regulation of alpha4 integrin, additional hallmarks of transmigrated cells are the down-regulation of CXCR4 receptor (88) and hyporesponsiveness to SDF-1. This could suggest that transmigration can be efficiently achieved only in cells that have down-regulated either their alpha4 integrin or CXCR4/SDF-1 function. It is important to emphasize that down-regulation of VLA-4 or CXCR4 receptor responses by themselves may not be sufficient to induce mobilization in all cells and additional complex interactions and cooperative signaling with other pathways involved in transendothelial migration (ie, small GTPases) may be necessary for the cells to exit the BM.

In conclusion, mobilization sets in motion an intramarrow proteolytic machinery with participation of diverse cell-bound or free proteases, largely
liberated by mature cells, and acting on multiple target molecules located on hematopoietic or stromal cells and on their matrix (adhesion receptors, chemokines and their receptors, or signaling molecules, etc). Depending on the stimulus applied, several distinct pathways can initiate mobilization. However, there is a broad interdependency between the pathways initiating and/or amplifying mobilization. For example, proteases may affect each other’s function (elastase affecting MMP-9 or MMP-9 affecting SDF-1, and vice versa), may target cytokine/chemokines receptors (CXCR4, kit) as well as their ligands (SDF-1, kit-ligand), or may additionally modulate integrins and endothelial structural components. It is evident, therefore, that there are large avenues open for future exploration. Unveiling the regulatory pathways in mobilization will not only reap clinical benefits, but greatly enhance our basic understanding of the concept of stem cell "niche", pivotal in the retention and development of hematopoietic cells within bone marrow.
§ II CHAPTER

OBJECTIVE

Mobilized hematopoietic stem cells (HSCs) are rapidly replacing traditional bone marrow (BM) harvesting as a source of stem cells for transplantation purposes. Mobilization of HSCs from BM into the circulation is obtained by stimulation with growth factors and chemokines, administered either alone or in combination with chemotherapy. Over the past 5-10 years, granulocyte colony-stimulating factor (G-CSF) has emerged as the most widely used mobilizing agent because of its potency and safety.

HSC mobilization is a multistep process that depend on a complex interplay between chemokines, growth factors, proteolytic enzymes and adhesion molecules. HSC express several adhesion molecules that are responsible for cell-cell and cell-matrix interactions. The 67 kDa laminin receptor (67LR) is a non-integrin cell-surface receptor with high affinity for laminin, which plays a key role in tumor invasion and metastasis.

The aim of this project is to investigate the involvement of 67LR in G-CSF-induced HSC mobilization. To this end, we analyzed 67LR expression and
function in G-CSF-mobilized HSCs from healthy donors, as compared to untreated BM HSCs. We also investigated the ability of neutralizing anti-67LR antibodies to affect G-CSF-induced HSC mobilization in mice.
§ III CHAPTER

MATERIALS AND METHODS

III.1 Reagents

Horseradish peroxidase-conjugated anti-rabbit IgG were from BIORAD (Richmond, CO, USA); FITC-labelled goat anti-rabbit IgG were from Jackson Lab (West Grove, PA, USA). Protease inhibitors, Ficoll-Hypaque (specific gravity 1077), bovine serum albumin (BSA) and hydrocortisone sodium hemisuccinate were from Sigma Chemical Co (St. Louis, MO, USA). Human placental laminin (LM) was from Chemicon (Temecula, CA, USA). The enhanced chemioluminescence (ECL) detection kit was from Amersham International (Amersham, England), polyvinylidene fluoride (PVDF) filters from Millipore (Windsor, MA, USA). RPMI 1640 medium, Medium 199, FN-coated culture flasks, heat-inactivated Fetal Calf Serum (FCS), LipofectAMINE, and Geneticin were from Life Technologies (Gaithersburg, MD, USA). 96-well microtiter plates and Transwell plates were from Costar (Cambridge, MA, USA). Chemotaxis PVPF filters were purchased from Corning (NY, USA). The stromal derived factor
1α (SDF1) was purchased from PEPROTECH (London, England). Human specific fluorescein isothiocyanate (FITC)-, peridinin chlorophyll (PerCP)- and phycoerythrin (PE)-labeled monoclonal antibodies (mAb) were purchased from Becton Dickinson (Mountain View, CA, USA). Methylcellulose supplemented with a specific mouse cytokine cocktail was from Stem Cell Technologies (Vancouver, British Columbia, Canada). Interleukin-3 (IL-3), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF) and erythropoietin (EPO) were from Amgen (Thousand Oaks, CA, USA). Recombinant human G-CSF (rhG-CSF, lenograstim) was purchased from Italfarmaco (Milan, Italy). 67LR cDNA was cloned into the pcDNA3 vector and the resulting plasmid was named 67LR-pcDNA3. The rabbit anti-67LR polyclonal antibody ab711, recognizing both human and rodent 67LR was from Abcam (Cambridge, UK); the monoclonal anti-67LR antibody MLuC5 were kindly provided by Dr. S. Menard and Dr E. Tagliabue (National Cancer Institute, Milan, Italy). Goat polyclonal anti-α6 integrin subunit antibody, anti phospo-ERKs and anti-ERK2 polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The ELISA kit for quantitative determination of laminin in human sera was from TAKARA (Otsu, Shiga, Japan).
III.2 Sample collection

Heparinized blood samples were obtained after informed consent (according to the procedures outlined by the ethical committee of our institution) before, during and after the mobilizing procedure, from 35 healthy adults (19 males and 16 females, range 20-55). The donors received glycosylated rhG-CSF administered subcutaneously, at 10 µg/kg/day, in two divided doses, for 5 days, to mobilize and collect CD34+ cells. Heparinized bone marrow specimens were obtained by aspiration from the posterior iliac crest from healthy young donors (8 males and 7 females, range 25-45).

III.3 CD34+ hematopoietic stem cell separation

Peripheral blood (PB) and bone marrow (BM) mononuclear cells (MNC) were isolated by Ficoll-Hypaque centrifugation. CD34+ cells were highly purified by MiniMacs high-gradient magnetic separation columns (Miltenyi Biotec, Auburn, CA, USA). CD34+ cells reached 90% purity by 2 sequential selections through the magnetic cell separator. Purity of the positively selected CD34+ cells was checked by flow cytometry. For in vitro cultures purified CD34+ cells were resuspended in RPMI medium supplemented with 5% FCS. For CD34+/CD38- enrichment, 5x10^7 PBMNC/mL were incubated for 30 minutes in ice with a mixture of antiglycophorin A, -CD3, -CD2,
CD14, -CD16,-CD19, -CD24, -CD56 and -CD66b tetrameric antibody complexes (StemCell Technologies). After three washings, cells were incubated for 30 minutes with 60 µl/mL magnetic colloidal iron/dextran particles, and finally processed through the StemSepTM device for depletion of targeted cells (StemCell Technologies). At the end of the procedure, the amount of CD34⁺ cell was 60% to 80% and purity in the range 70-95%.

III.4 Flow cytometry analysis

Enumeration and immunophenotyping of CD34⁺ cells were performed by 2- and 3-color flow cytometry, respectively, in which CD34⁺ were gated by a CD45-gating method. Briefly, whole blood containing approximately 1x10⁶ cells was incubated for 20 minutes at 4°C with the following directly conjugated monoclonal antibodies: 20µL of both PerCP-labeled anti-CD45 antibody and PE-conjugated anti-CD34 antibody. The sample was treated with red blood cell lysis buffer (Becton-Dickinson) and the cells were washed with PBS containing 1% human serum albumin and 0.1% sodium azide. After treatment with 2% formaldehyde cell fixation buffer (Becton-Dickinson) for 10 minutes at 37°C, the cells were washed and stained first with 1µL of anti-67LR polyclonal antibody ab711 for 30 minutes at 4°C and then with a FITC-conjugated anti-rabbit antibody for 30 minutes at 4°C.
The cells were analyzed immediately on a FACScan flow cytometer (Becton- Dickinson). At least 5.10x10^5 total events were acquired in each sample using the Cellquest software (Becton-Dickinson). A mononuclear gate was created, based on CD45 expression and side light scatter; the total number of CD34^+ cells was calculated on the basis of the relative percentage of CD34+ cells in the total number of nucleated cells. The expression of 67LR on enriched CD34^+/CD38^- cells was assessed by triple staining using an anti-CD34-PerCP, an anti-CD38-PE and the polyclonal anti-67LR antibody ab711 detected by a FITC-conjugated anti-rabbit antibody. Equivalent gating on isotype-matched negative controls was used for background subtraction in all assays.

III.5 Cell cultures

CD34^+ KG1 cell were grown in RPMI supplemented with 5% heat-inactivated FCS, 300 µg/ml glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. 5x10^6 cells were transfected with 10 µg of 67LR-pcDNA3 or control vector pcDNA3 and 60 µL of Lipofectamine for 5h at 37°C (5% CO2). Transfected cells were selected by geneticin at 0.4 mg/mL; the resulting clones were pooled and cultured in the presence of 0.2 mg/mL Geneticin. Clonogenic human progenitors were measured in
methylocellulose, as previously described. Clonogenic mouse progenitors were also grown in methylcellulose in the presence of a recombinant mouse growth factor cocktail. The human bone marrow-derived endothelial cell line HBMEC was a gift of Dr C. Ellen van der Schoot (Amsterdam, Netherlands). Cells were cultured in FN-coated culture flasks in Medium 199 supplemented with 10% pooled, heat-inactivated human serum, 10% FCS, 1 ng/mL basic fibroblast factor (Boehringer-Mannheim, Mannheim, Germany), 5 U/mL heparin, 300 µg/mL glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. In all experiments, HBMEC monolayers were pretreated with IL-1β for 4 h (Peprotech, Rocky Hill, NJ, USA).

III.6 Mobilization of mouse CD34+ cells

Male Balb/c mice with age of 8-9 weeks were purchased from Charles River Laboratory (Lecco, Italy). All the experiments were approved by the animal care committee of our institute. Mice received intraperitoneally a daily dose of 300 µg/Kg G-CSF, for 4 days. Some mice also received intraperitoneal injections of the neutralizing anti-67LR antibody MLuC5 (100 µg in 200 µL saline) on day 3 and 4, immediately after G-CSF treatment. Four hours after the last injection of G-CSF, mice were sacrificed, BM and PB were harvested and analyzed by 2-color flow cytometric analysis, using a mouse-specific
PE-conjugated anti-CD34 antibody (Becton-Dickinson) and the anti-67LR polyclonal antibody ab711, revealed by a FITC-conjugated anti-rabbit secondary antibody, as described above.

### III.7 Western Blot

CD34⁺ KG1 cells, PBMNC, purified PB or BM CD34⁺ cells were lysed in 1% Triton X-100/PBS with proteases inhibitors. 100 µg of protein was electrophoresed on a 12% SDS-PAGE, under reducing conditions, and transferred onto a PVDF membrane. The membrane was blocked and probed with 1 µg/mL of the anti-67LR polyclonal antibody ab711. Finally, washed filters were incubated with horseradish peroxidase-conjugated anti-rabbit antibodies and detected by ECL. For MAPK activation, KG1 cells, highly purified BM CD34⁺ cells and highly purified G-CSF-mobilized PB CD34⁺ cells were plated in 35 mm wells previously coated overnight at 4°C with LM (100 µg/well) or heat-denatured BSA, as a negative control. Cells were allowed to adhere for 16 h at 37°C, lysed and subjected to Western blot with anti-phospho-ERKs and anti-ERK 2 polyclonal antibodies, as described above.
III.8 Adhesion assay

96-well microtiter plates were coated overnight at 4°C with LM (5 µg/well) or heat-denatured BSA, as a negative control. Plates were incubated for 16 h at 37°C with 1x10^5 CD34+ KG1 cells, BM CD34+ cells or G-CSF-mobilized PB CD34+ cells in 100 µL RPMI medium supplemented with 5% FCS. Attached cells were fixed with 3% paraformaldehyde, permeabilized by 2% methanol and stained with 0.5% crystal violet in 20% methanol. The staining was eluted with a solution of 0.1 M sodium citrate, pH 4.2, in 50% ethanol, and the absorbance measured at 540 nm. In some experiments, cells were pre-incubated with 1µg of anti-67LR polyclonal antibody ab711 or with non-immune rabbit Igs, as a negative control, and then plated on LM. All the experiments were performed in triplicate. Results are reported as a percentage of control; 100% values represent cell adhesion to heat-inactivated BSA.

III.9 Cell migration assay

Cell migration assays were performed in Boyden chambers, using uncoated 5 µm pore size PVPF polycarbonate filters. 2x10^5 CD34+ KG1 cells or G-CSF mobilized PB CD34+ cells were plated in the upper chamber in serum-
free medium; 25 µg/mL LM or serum-free medium was added in the lower chamber. Cells were allowed to migrate for 90 min at 37°C, 5% CO2. Cells on the lower surface of the filter were then fixed in ethanol, stained with hematoxylin and counted at 200x magnification (10 random fields/filter). In some experiments, cells were pre-incubated for 1 h at 37°C with 1 µg/mL anti-67LR polyclonal antibody ab711 or with non-immune rabbit IgGs and then plated. All the experiments were performed in triplicate.

**III.10 Transendothelial migration assay**

Migration assays were performed on FN-coated filters in Transwell plates of 6.5 mm diameter with 5 µm pore filters. Endothelial cells were plated at 2-3x10⁴ cells/Transwell to obtain confluent endothelial monolayers. Monolayers of endothelial cells were pre-treated for 4 h with IL-1β and washed with assay medium (DMEM with 0.25% BSA). 1x10⁴ G-CSF-stimulated BM CD34⁺ cells were added to the upper compartment in 0.1 mL assay medium; 0.6 mL assay medium, with or without SDF-1 (100 ng/mL), was added to the lower compartment. After 4 h incubation at 37°C, 5% CO2, cells migrating into the lower compartment as well as non-migrating cells into the upper compartment were collected and analyzed for their CFC content. In blocking experiments, G-CSF-stimulated BM CD34⁺ cells were
pre-incubated for 30 min at 37°C with 1 µg/mL anti-67LR polyclonal antibody ab711 or with non-immune rabbit Ig.

**III.11 Statistical analysis**

Results of *in vivo* and *in vitro* studies were expressed as a mean ± standard error of the mean (SEM) or standard deviation (SD), as required. Differences between groups were evaluated using the Student’s t-test. Correlation between variables was assessed using the Pearson’ linear regression.
§ IV CHAPTER

RESULTS

IV.1 67LR expression in human G-CSF mobilized CD34⁺ peripheral blood stem cells

We first analyzed circulating CD34⁺ cells of normal donors after G-CSF treatment to investigate whether there was a modulation of 67LR expression. 35 healthy donors were treated with G-CSF to induce mobilization and their PBMNCs were analyzed at steady-state (day=0) and at various time-points (days 3-5). Phenotypic analysis of 67LR expressing CD34⁺ cells was determined by 3-color flow cytometry on a mononuclear gate of CD45⁺ cells. G-CSF administration increased 67LR expression in circulating CD34⁺ cells; by contrast, unstimulated BM CD34⁺ cells showed very low levels of 67LR (Fig 1A, B). The mean percentage ± SEM of 67LR positive unstimulated BM CD34⁺ cells from 15 normal subjects was 5.1±1.1% (range 1-13%) (Fig.1B).

Using as cut off a percentage of 67LR positive circulating CD34⁺ cells higher than 20%, 67LR expression was increased in 31/35 donors, after G-
CSF administration. The mean percentage ± SEM of 67LR positive circulating CD34\(^+\) cells was 1.86±0.2% (range 0.5-7%) before G-CSF administration and 46.3±4.1% (range 23-86%) on the day of cell harvesting (day 4 or 5 of G-CSF administration) (p <0.0001) (Fig. 1C). 67LR expression on CD34\(^+\) cells was rapidly reduced in all G-CSF-treated donors (Fig. 1D) after G-CSF withdrawal. Noteworthy, 4 of 5 donors not showing 67LR increase on circulating CD34\(^+\) cells after G-CSF treatment can be considered poor mobilizers (Fig. 2A and B: donor 5, 11, 19 and 23); indeed, they obtained a peak of less than 20 CD34\(^+\) cells/\(\mu\)L and did not achieve the target CD34\(^+\) cell yield =2x 10\(^6\) CD34\(^+\) cells/kg in one apheresis procedure after 5 days of G-CSF administration. Accordingly, statistic analysis in basis of linear regression showed that both numbers and percentages of 67LR positive circulating CD34\(^+\) cells after G-CSF administration directly correlated with CD34\(^+\) cell peak values on the day of collection (r=0.7, p=0.001 and r=0.5, p=0.002, respectively) (Fig. 2C).

We also investigated 67LR expression by Western blot with an anti-67LR polyclonal antibody on highly purified G-CSF-mobilized CD34\(^+\) cells and on PBMNC from the corresponding donors, collected before and at various time points during G-CSF stimulation. Western blot analysis showed increased 67LR expression in PBMNC during G-CSF treatment and
confirmed that G-CSF mobilized CD34+ cells expressed high levels of 67LR at the time of cell harvesting by apheresis, as compared to unstimulated BM HSC (Fig. 3A).

We also investigated 67LR expression in G-CSF-mobilized CD34+/CD38- cells, a population enriched in stem cells and characterized by high repopulating activity in NOD/SCID mice (60). Phenotypic analysis of 67LR expression in enriched CD34+/CD38- cells, as determined by flow cytometry on PBMNC from five G-CSF treated donors, showed that the mean ± SEM of 67LR expressing CD34+/CD38- cells at day 5 of G-CSF stimulation was 43±3% (range 36-60%), whereas it was 7.5±2% (range 4-10%), before G-CSF administration (Fig. 3C).

III.2 G-CSF modulation of CD34+ cell adhesion to laminin

We also studied 67LR involvement in CD34+ cell adhesion to laminin by in vitro cell adhesion assays. For this purpose we used CD34+ KG1 leukemic cells, because of CD34+ antigen, highly purified unstimulated normal BM CD34+ cells and highly purified G-CSF mobilized PB CD34+ cells. All these were able to adhere to laminin (Fig. 4A). Different receptors were involved in KG1 cell adhesion to laminin; indeed, pre-incubation with anti-67LR and anti-α6 integrin antibodies caused a 53% and a 56% reduction of
cell attachment to laminin, respectively. Preincubation with anti-67LR antibodies did not affect the adhesion to laminin of normal unstimulated BM CD34^+ cells, expressing low levels of 67LR, whereas anti-α6 integrin antibodies caused a 67% reduction of their laminin-dependent cell adhesion. Interestingly, G-CSF-mobilized PB CD34^+ cells adhered to laminin to the same extent as BM CD34^+ cells; however, such cell adhesion to laminin was almost completely inhibited by anti-67LR antibodies. Noteworthy, anti-α6 integrin antibodies did not affect PB CD34^+ cell adhesion to laminin, since α6 integrin expression was strongly down-regulated during G-CSF-induced HSC mobilization. Indeed, flow cytometry analysis performed on BMMNCs obtained from BM of 10 healthy donors and on PBMNCs obtained from 10 healthy donors after G-CSF stimulation showed that the mean percentage ± SEM of α6 expressing CD34^+ cells was 27.3±3.2%, (range 9.6-36.3%) in unstimulated BM CD34^+ cells and 4±1.1% (range 0-10%) in G-CSF mobilized PB CD34^+ cells.

Therefore, 67LR upregulation during G-CSF-induced HSC mobilization does not increase CD34^+ cell adhesion to laminin. However, unstimulated BM CD34^+ cell adhesion to laminin is mostly mediated by α6 integrin receptors, whereas G-CSF-mobilized CD34^+ cell adhesion to the same
substrate is selectively mediated by 67LR, because $\alpha_6$ integrins are downregulated during mobilization and replaced by 67LR.

A laminin promoting effect on KG1 and BM CD34$^+$ cell adhesion and migration, largely mediated by $\alpha_6$ integrin receptors, has been already reported. Laminin also exerts mitogenic activity on the same cell types. We found that 67LR and $\alpha_6$ transduce different signals. Indeed, we showed increased MAPK phosphorylation after KG1 and BM CD34$^+$ cell adhesion to laminin (Fig.4B); on the contrary, PB CD34$^+$ cell adhesion to laminin, mostly mediated by 67LR engagement, did not increase MAPK activation.

III.3 67LR dependent migration of KG1 and G-CSF mobilized CD34$^+$ cells

We also investigated by in vitro chemotaxis assays whether 67LR upregulation could be implicated in increased laminin-dependent CD34$^+$ cell migration. For this purpose, leukemic CD34$^+$ KG1 cells were transfected with 67LR cDNA; transfected cells showed a 3.8 fold increase in 67LR expression (mean fluorescence index: 170.4 and 634.96 before and after transfection, respectively). We showed that 67LR-transfected KG1 cells showed increased migration toward laminin, as compared to wild-type cells (Fig.4C). 67LR overexpression in transfected KG1 cells did not increase
their adhesion to laminin (not shown), demonstrating that 67LR is mainly involved in mediating CD34$^+$ cell migration rather than adhesion. Then, we investigated by *in vitro* chemotaxis assays whether 67LR could mediate G-CSF-mobilized CD34$^+$ cell migration to laminin; BM CD34$^+$ cells were not analyzed being 67LR expression was not significant (Fig.1 and 3A). Highly purified PB CD34$^+$ cells from three G-CSF-treated donors migrated toward human laminin and cell pre-incubation with anti-67LR antibodies strongly reduced the migratory response to laminin (Fig.4D). Therefore, 67LR engagement is mainly implicated in laminin dependent CD34$^+$ cell migration after G-CSF stimulation.

**III.4 Laminin concentrations in human sera during G-CSF-induced mobilization**

We investigated whether laminin serum concentrations were increased in donors after G-CSF administration, thus creating a chemotactic signalling toward peripheral blood. ELISA assays of 15 donor sera obtained before (day 0) and after (day 4 or 5) G-CSF-induced CD34$^+$ cell mobilization showed that laminin concentration in sera were not modified by the G-CSF treatment, being the mean ± SEM of serum laminin concentration 256±78 ng/mL and 268.2±66 ng/mL, before and after G-CSF administration,
respectively. Thus, it seems that increased 67LR expression in circulating CD34+ cells during GCSF-induced HSC mobilization participates in their migration toward laminin, even though a laminin gradient between BM and peripheral blood is not created.

**III.5 67LR expression in BM CD34+ cells after in vitro G-CSF treatment and its involvement in trans-endothelial migration**

We investigated by *in vitro* experiments whether G-CSF was able to directly increase 67LR expression in normal BM CD34+ cells. Three-color flow cytometric analysis of BM CD34+ cells from three normal donors, cultured for 24 and 48 h in medium with or without 200 ng/mL G-CSF, showed increased 67LR expression after *in vitro* G-CSF treatment (mean percentages ± SEM of 67LR expressing CD34+ cells: 8±4% vs 25±3% and 33±4% without and with G-CSF at 24 and 48h, respectively; both p: <0.0001) (Fig.5A). By contrast, α6 integrin expression decreased after *in vitro* G-CSF treatment (mean percentages ± SEM of α6 integrin expressing CD34+ cells: 33.3±3.1% vs 14.7± 4% and 7.1± 2% without and with G-CSF at 24h and 48 h, respectively; both p: <0.0001) (Fig.5B). Interestingly, *in vitro* G-CSF-treated BM CD34+ cells, expressing high levels of 67LR, showed a further increase of this receptor after contact with BM derived
endothelial cell layers (mean percentages ± SD of 67LR expressing G-CSF-treated CD34<sup>+</sup> cells: 25±4% and 45±5% before and after co-culture with endothelial cells, respectively; p: <0.0001) (Fig.5B), suggesting that marrow CD34<sup>+</sup> cells 67LR up-regulation in response to G-CSF can be further increased by adhesion to endothelium. Thus, G-CSF-induced 67LR expression in BM CD34<sup>+</sup> cells could participate in their egress from BM by mediating adhesion and trans-migration through laminin, as formerly documented for T lymphocytes and cancer cells. Therefore, we investigated whether 67LR could promote trans-endothelial migration of in vitro G-CSF-stimulated BM CD34<sup>+</sup> cells, expressing high levels of 67LR (Fig.5A). SDF-1-dependent trans-endothelial migration of G-CSF-stimulated BM CD34<sup>+</sup> cells was strongly affected by pre-incubation with polyclonal anti-67LR antibodies (Fig.5C), thus suggesting that G-CSF-mediated 67LR up-regulation contributes to CD34<sup>+</sup> cell migration across the BM endothelium, an important step in CD34<sup>+</sup> cell trafficking from and to BM.

**III.6 67LR expression in mouse BM CD34<sup>+</sup> cells after G-CSF-induced HSC mobilization**

We investigated whether 67LR up-regulation in response to G-CSF could occur in BM CD34<sup>+</sup> cells also in vivo, thus possibly playing a role in their
egress from BM. Male Balb/c mice were treated daily for 4 days with intra-peritoneal injections of 300 µg/Kg G-CSF or saline, as a control; four hours after the last injection, PB and BM were analyzed by flow cytometry. Murine mobilization experiments were performed four times, each group being composed of 4 mice. As already observed in human CD34\(^+\) cell mobilization (Fig.1), G-CSF treatment induced 67LR up-regulation in mouse PB CD34\(^+\) cells (mean percentage ± SEM of 67LR positive PB CD34\(^+\) cells before and after G-CSF treatment: 11.5±2% vs 47.3±8.2%, respectively; p: <0.0001). Interestingly, G-CSF increased 67LR expression also in mouse BM CD34\(^+\) cells (mean percentage ± SEM of 67LR positive BM CD34\(^+\) cells before and after G-CSF treatment: 12.5±0.3% vs 62±4.1%, respectively; p <0.0001) (Fig.6A). These findings demonstrate that G-CSF administration increases 67LR expression in BM CD34\(^+\) cells, confirming what observed by in vitro stimulation experiments.

III.7 Effects of 67LR inhibition on G-CSF-induced HSC mobilization

We then investigated whether the increased 67LR expression in BM CD34\(^+\) cells could be involved in their egress from BM, during G-CSF-induced mobilization. To interfere with 67LR function, we injected a neutralizing
anti-67LR antibody into Balb/c mice on days 3 and 4 of mobilization, immediately after each G-CSF stimulation, and examined its effect on mobilization. We observed a significantly reduced number of mobilized CD34+ cells (Fig.7B) and of circulating progenitor cells, evaluated as colony forming cells (CFC) (Fig.7C). In a control group of G-CSF-treated mice, nonimmune antibodies did not significantly affect the number of circulating CD34+ cell and progenitor cells (Fig.7A and B). Total BM cellularity did not change in MLuC5-treated mice, as compared to controls. In addition, percentages of 67LR positive CD34+ cells in the BM of MLuC5- and non immune Ab-treated mice were similar, demonstrating that the inhibition of CD34+ cell mobilization occurred without affecting BM-resident CD34+ cells (Fig.7C). The attenuation of HSC mobilization in mice was obtained by using the neutralizing anti-67LR antibody MLuC5, which is an IgM; therefore, we had to exclude that circulating 67LR positive CD34+ cells could have been removed by complement-mediated lysis. Our results were confirmed in the C5 deficient Mba/2J strain, in which we also observed a significant reduction of CD34+ cell mobilization after 67LR inhibition. The efficiency of mobilization in this strain was evaluated by comparing the mean ± SEM of PB CD34+ cells in saline-treated mice (20.7/µL±3.3%) to that of G-CSF-treated mice (99.8/µL±24.2%): p< 0.05. MLuC5 treatment
strongly decreased mobilization; indeed, the mean ± SEM of PB CD34+ cells in mice treated with G-CSF plus control antibody was 67.1/µL±11.2% whereas it was 39.2/µL±7.6% in mice treated with G-CSF plus MLuC5: p<0.05. Non-immune antibody did not affect the efficiency of G-CSF induced mobilization, since the slight decrease in mobilized CD34+ cells, as compared to G-CSF alone (67.1/µL vs 99.8/µL), was not statistically significant (p=0.134). In C5 deficient mice, C3-mediated clearance of 67LR positive CD34+ cells might still occur. However, WBC counts in PB were not significantly modified by MLuC5 antibody treatment, as compared to controls (not shown). Since many types of circulating WBC, such as monocytes, T lymphocytes and neutrophils, are 67LR positive, a non-specific C3-mediated cell clearance can be excluded.
In vivo G-CSF administration increases 67LR expression in circulating CD34+ cells as compared to normal unstimulated marrow CD34+ cells.

**Panel A:** 67LR expression in circulating CD34+ cells during G-CSF-induced stem cell mobilization in 2 representative donors (n. 1 and 20 in figure 2A and 2B). Immunophenotyping of 67LR expressing CD34+ cells was performed by 3-color flow cytometry on mononuclear cells gated for side light scatter (SSC-H) and CD45+, with an anti-67LR polyclonal antibody (Ab) detected by a FITC-conjugated anti-rabbit Ab. Peripheral blood mononuclear cells (PBMNC) were collected before (day 0) or at various time-points (day 3 and 5) during G-CSF administration.

**Panel B:** Immunophenotyping of 67LR expressing CD34+ cells on BMMNC from 3 representative normal subjects.

**Panel C:** Percentage (%) of 67LR expressing CD34+ cells in bone marrow mononuclear cells (BMMNC) from 15 normal subjects and in PBMNC collected from 35 healthy donors before G-CSF administration (day 0) or at the time of cell harvesting (day 5).

**Panel D:** Immunophenotyping of 67LR expressing CD34+ cells performed by 3-color flow cytometry on mononuclear cells gated for side light scatter (SSC-H) and CD45+, with an anti-67LR polyclonal Ab on PBMNC collected before (day 0), during (day 3 and 5) G-CSF administration and at G-CSF withdrawal (day 6 and 9). Increased 67LR expression in circulating CD34+ cells is strictly dependent on G-CSF administration.
Increased 67LR expression in circulating CD34⁺ cells after GCSF administration is significantly correlated with mobilization efficiency.

**Panel A:** Percentage (%) of 67LR expressing CD34⁺ cells in PBMNC collected from 35 healthy donors before G-CSF administration (day 0, □) or at the time of cell harvesting (day 5, □). Mean percentages ± SEM of 67LR positive circulating CD34⁺ cells were 2.36±0.4% before G-CSF administration and 42.4±4.1% on the day of cell harvesting (p<0.00001).

**Panel B:** 67LR expressing CD34⁺ cells/μL in PBMNC collected from 35 healthy donors at the time of cell harvesting (day 5 of G-CSF administration, □) and the corresponding peak value of CD34⁺ cells / μL ( ). Mean ± SEM of 67LR positive circulating CD34⁺ cells was 0.11±0.02/μL before G-CSF administration and 29.6±4.2/μL on the day of cell harvesting (p<0.00001). 4/5 poor mobilizing donors (*) did not show 67LR increase in circulating CD34⁺ cells.

**Panel C:** Pearson’s linear regression analysis between percentages of 67LR expressing PB CD34⁺ cells/μL after G-CSF administration and PB CD34⁺ cell peak values on the day of collection (r=0.5, p=0.002). Percentages of 67LR positive circulating CD34⁺ cells are significantly correlated with the degree of mobilization.
67LR expression increases in circulating CD34+ and CD34+/CD38- cells during G-CSF administration.

Panel A: Western blot analysis with an anti-67LR polyclonal Ab of PBMNC collected from 2 representative donors before (day 0) or at different time-points (day 3 and 5) of G-CSF administration, of highly purified circulating CD34+ cells collected from the same donors at day 5 of G-CSF administration and of highly purified BM CD34+ cells collected two different donors. Treatment by G-CSF increases 67LR production, especially in CD34+ cells.

Panel B: 67LR expression in CD34+/CD38- progenitor cells during G-CSF-induced stem cell mobilization from a representative case. Immunophenotyping of 67LR expressing cells was performed on enriched CD34+/CD38- cells (see material and methods) collected at day 0 (upper panel) and 5 (lower panel) of G-CSF administration on a mononuclear gate by triple staining with a PeRCP-conjugated anti-CD34, a PE-conjugated anti-CD38 and an anti-67LR polyclonal Ab detected by a FITC-conjugated anti-rabbit Ab. CD34+/CD38- cells expressing 67LR were evaluated within the gate of CD34+/CD38- cells (circled). Treatment by G-CSF increases 67LR production even in CD34+/CD38- cells.
67LR mediates *in vitro* cell adhesion and migration to laminin of G-CSF mobilized CD34+ cells and affects MAPK phosphorylation in these cells.

**Panel A:** *In vitro* cell adhesion to laminin of KG1, unstimulated BM and G-CSF-mobilized PB CD34+ cells. KG1 cells, highly purified BM CD34+ cells from three donors and highly purified GCSF-mobilized PB CD34+ cells from three donors were plated in wells coated with human placental laminin (5 ?g/well), after pre-incubation with non immune rabbit antibodies ( ), an anti 67LR polyclonal Ab ( ), an anti ? Ab ( ), and allowed to adhere for 16 h at 37°C. As negative controls, heat-denatured BSA was used for coating. Attached cells were fixed and stained with crystal violet; the absorbance (OD) of the eluted stain was measured by a spectrophotometer at 540 nm. All experiments were performed in triplicate and reported as a percentage of total cell input. Results are presented as mean ± SD. 67LR supports cell adhesion to laminin of KG1 and G-CSF mobilized CD34+ cells, while adhesion to laminin of unstimulated marrow CD34+ cells is 67LR-independent and mostly mediated by ? integrins.

**Panel B:** MAPK activation in CD34+ cell adhered to laminin. KG1 cells, highly purified unstimulated BM CD34+ cells and highly purified GCSF-mobilized PB CD34+ cells were plated in 35 mm wells coated with human placental laminin and allowed to adhere for 16 h at 37°C. As negative controls, heat-denatured BSA was used for coating. Cells were then lysed and subjected to Western blot with anti-phospho-ERKs and anti-ERK 2 (as a loading control) polyclonal antibodies. Adhesion to laminin increased MAPK phosphorylation in both KG1 and BM CD34+ cells whereas PB CD34+ cell adhesion to laminin, mostly mediated by 67LR engagement, did not increase MAPK activation.

**Panel C:** *In vitro* cell migration to laminin of wild type ( ) and 67LR-transfected ( ) KG1 cells. With incubation with non immune Ab (-) and a polyclonal anti-67LR Ab (pAb anti-67LR), KG1 cells were plated in Boyden chambers and allowed to migrate toward human placental laminin (25 ?g/mL). 100% values represent cell migration in the absence of chemoattractant. Values are the mean ± SD of three experiments performed in triplicate. Laminin-induced migration is increased in 67LR-transfected KG1 cell and is strongly reduced by blocking 67LR.

**Panel D:** *In vitro* cell migration to laminin of G-CSF-mobilized PB CD34+ cells ( ). After incubation with non immune Ab (-) and a polyclonal anti 67LR Ab (pAb anti-67LR), highly purified GCSF mobilized CD34+ cells were plated in Boyden chambers and allowed to migrate toward human placental laminin. 100% values represent cell migration in the absence of chemoattractant. Values are the mean ± SD of three experiments performed in triplicate. Laminin-induced migration of G-CSF-mobilized PB CD34+ cells is almost totally abolished by blocking 67LR.
67LR is up-regulated by G-CSF and by exposure to BM-derived endothelial cells in human BM CD34+ cells and is involved in their trans-endothelial migration.

**Panel A:** 67LR and α6 integrin expression on BM CD34+ cells after *in vitro* G-CSF treatment, in one representative experiment. BMMNCs from normal donors were cultured for 24 and 48 h with medium alone or in presence of 200 ng/mL G-CSF and analyzed by 3-color flow cytometry on mononuclear cells gated for side light scatter (SSC-H) and CD45+ with anti-67LR and anti-α6 polyclonal Abs or non-immune polyclonal Abs, as a negative control, and detected by a FITC-conjugated anti-rabbit Ab. 67LR expression on human BM CD34+ cells is increased by *in vitro* G-CSF treatment, whereas α6 integrin expression decreases.

**Panel B:** 67LR expression on BM CD34+ cells after *in vitro* G-CSF treatment and exposure to BM-derived endothelial cells, in one representative experiment. BMMNC from normal donors were cultured for 24 h with medium alone, in the presence of 200 ng/mL GCSF, or on confluent endothelial cell layers in the presence of 200 ng/mL GCSF, and analyzed by 3-color flow cytometry, as above described. 67LR expression of human G-CSF-stimulated BM CD34+ cells is further increased by exposure to BM-derived endothelial cells.

**Panel C:** Transendothelial migration of G-CSF-stimulated BM CD34+ cells. After incubation with a polyclonal anti-67LR Ab or a non-immune Ab, CD34+ cells, stimulated for 72 h with 200 ng/mL G-CSF, were plated in Transwell plates coated with confluent endothelial monolayers and allowed to migrate toward 200 ng/mL SDF1 (+ or medium alone for 4 h). Cells migrating into the lower compartment were collected and analyzed for their colony-forming cells (CFC) content by methyl-cellulose colony assay. Data are the mean ± SEM of three separate experiments, *** = p < 0.0001. G-CSF-stimulated BM CD34+ cell trans-endothelial migration is significantly inhibited by anti-67LR antibodies.
G-CSF increases 67LR expression in mouse BM and PB CD34⁺ cells.

67LR expression on PB CD34⁺ cells and on BM CD34⁺ cells before (central panels) and after G-CSF administration (right panels) from one representative mouse. BALB/c mice were treated daily for five days with 300 μg/Kg G-CSF or with saline, as a control. Four hours after the last injection, PB and BM CD34⁺ cells were analyzed by flow cytometry, with a mouse-specific anti-CD34-PE Ab and with an anti-67LR polyclonal Ab, detected by a FITC-conjugated antirabbit Ab.
Increased 67LR expression in BM CD34+ cells is involved in G-CSF-induced mobilization of murine HSCs.

**Panel A:** G-CSF-mobilized CD34+ cells after treatment with anti-67LR antibodies. Balb/c mice were treated daily for four days with 300 μg/Kg G-CSF (●) or saline (□), as a control. Non-immune (🙂 or the MLuC5 neutralizing anti-67LR monoclonal Abs (◆) were injected into G-CSF-treated mice on days 3 and 4 of mobilization, immediately after each G-CSF stimulation. Four hours after the last injection, PB CD34+ cells were evaluated by flow cytometry, with a mouse-specific anti-CD34-PE antibody. Data are mean ± SEM of three separate experiments, each group being composed of 4 mice; **= p < 0.001

**Panel B:** G-CSF-mobilized progenitors after treatment with anti-67LR antibodies. Balb/c mice were treated daily for four days with 300 μg/Kg G-CSF (●) or saline (□), as a control. G-CSF-treated mice were injected with non-immune (🙂 or MLuC5 neutralizing anti-67LR Abs (◆) on days 3 and 4 of mobilization, immediately after each G-CSF stimulation. Four hours after the last injection, the number of progenitors mobilized into the circulation was evaluated by colony assays and reported as the number of CFC x 10^3/mL. Data are mean ± SEM of three separate experiments, each group being composed of 4 mice; *** = p < 0.0001. Mobilization of murine progenitor cells, evaluated both as circulating CD34+ cells and as CFC, is significantly inhibited by anti-67LR antibodies.

**Panel C:** 67LR expression BM CD34+ cells in a representative mouse treated with non immune (left panels) and MLuC5 (right panels) Abs. Total BM cellularity (upper panels) as well as the percentage of 67LR positive BM CD34+ cells (lower panels) in non immune Ab and MLuC5-treated mice were similar, thus excluding both a toxic effect of the antibody and a selective antibody-mediated removal of 67LR positive CD34+ cells.
§ V CHAPTER

DISCUSSION

Mobilized hematopoietic stem cells (HSCs) obtained after cytokines treatment are rapidly replacing traditional bone marrow harvest as a source of stem cells for transplantation purposes (89). Several observations suggest that the HSCs mobilization is a mechanism similar to the enhancement of the physiological stem cell release after stress signal (60).

During stem cell mobilization, the adhesion profile of HSCs changes facilitating their egress from bone marrow (65, 90). Adhesion molecules establish interactions between HSCs and cellular and matrix components in the BM environment. These adhesive interactions play an important role in the retention of HSCs within the BM microenvironment (91). Direct in vivo studies showed that HSC release from bone marrow also bears to activation of a protease’s coalition. (92, 65). Therefore, HSC mobilization resembles leukocyte recruitment to inflammatory sites and cancer-cell migration; all these phenomena likely share common biochemical mechanisms.

Inflammatory cells, metastasizing cells, and mobilized HSCs have to migrate through the blood-vessel wall; subendothelial basement membrane proteins, such as laminin, fibronectin, and collagen, regulate cell migration and
responsiveness to cytokines by interacting with cell-surface adhesion receptors. We investigated a possible role of 67LR, a non-integrin cell-surface receptor for laminin that plays a key role in tumor invasion and metastasis (8), during G-CSF-induced CD34+ HSC mobilization. We showed that after G-CSF stimulation 67LR expression increases in circulating CD34+ cells, as compared with unstimulated BM CD34+ cells. Numbers and percentages of 67LR positive circulating CD34+ cells after G-CSF administration significantly correlated with the efficiency of CD34+ cell mobilization; indeed, poor mobilizing donors did not show 67LR increase in circulating CD34+ cells. We investigated whether during G-CSF administration 67LR could be involved in CD34+ cell adhesion to laminin, as reported for lymphocytes, leukemic cells, cancer cells and endothelial cells. Unexpectedly, unstimulated BM adhered to laminin to the same extent as G-CSF mobilized CD34+ cells; even though the latter expressed a higher level of 67LR. However, different receptors were involved in transducing laminin effects. Adhesion to laminin of unstimulated BM CD34+ cells was regulated via α6 integrins, whereas G-CSF-mobilized CD34+ cells adhered to laminin exclusively through 67LR. It seems that BM is the first anchoring site for unstimulated CD34+ cells, where anchor to laminin and move to migration and proliferation during steady-state conditions are
mediated by integrin receptors. After G-CSF stimulation, CD34\(^+\) cell release from BM into the circulation requires the expression of 67LR, which replaces downregulated \(\alpha_6\) integrins in mediating CD34\(^+\) cell attachment to laminin. In fact, we demonstrated that 67LR upregulation in transfected KG1 cells did not modify cell adhesion to laminin but increased their migration toward the same substrate. We also found \(\alpha_6\) integrin down-regulation in circulating CD34\(^+\) cells, as compared to steady-state BM. Our observations are in agreement with other reports that showed decreased expression and activity of other integrins, such as \(\alpha_4\) and \(\alpha_5\), during HSC mobilization. In addition, \(\alpha_6\) integrins are not involved in HSC mobilization whereas they promotes progenitor cell homing and engraftment to BM of lethally irradiated mice (93).

In our hypothesis 67LR could be involved in HSC mobilization through a mechanism recently proposed for tumor cells. After binding to laminin, 67LR induce a conformational modification of laminin which increases its degradation rate and the release of chemotactic fragments. It seems that, 67LR overexpression in cancer cells increases their invasiveness by up-regulating the expression and the activity of proteolytic enzymes able to degrade the extracellular matrix, such as membrane type 1 matrix metalloproteinase (MT1-MMP), stromelysin 3, cathepsin L and the matrix
metalloproteinase MMP-2. Proteolytic degradation of matrix became a key step to facilitate cancer cell migration and HSC mobilization. G-CSF induced 67LR up-regulation could generate changes in BM CD34\(^+\) cells similar to those acquired by tumor cells; indeed, matrix metallo-proteases, such as MT1-MMP and MMP-2, are expressed by human CD34\(^+\) progenitors and are needed for \textit{in vivo} mobilization (94, 95); they also could be secreted by circulating but not BM CD34\(^+\) cells (96). Interestingly, circulating CD34\(^+\) cells are mostly quiescent and in the G0/G1 phase of the cell cycle, even during G-CSF administration (97). G-CSF-induced 67LR upregulation in BM CD34\(^+\) cells could be involved in transducing migratory signals upon binding to laminin, without inducing cell proliferation, unlike integrins. Indeed, we found that adhesion to laminin determined a reduced activation of MAPK in PB CD34\(^+\) cells, as compared to KG1 and BM CD34\(^+\) cells; a phenomenon that could be related to 67LR-mediated activation of dual specific phosphatases, as already reported in tumor cells (40) and by 67LR \textit{in vitro} interaction with protein phosphatase-1 (98).

We also provide evidence that G-CSF-induced 67LR up-regulation on CD34\(^+\) cell mediates their migration toward laminin. Although we could not document the creation of a laminin gradient from BM to blood during mobilization, we cannot exclude the possibility that it may be generated
locally and/or transiently by laminin degradation, due to the increased proteolytic enzyme production in the BM, after G-CSF administration. *In vitro* exposure to G-CSF increased 67LR expression in normal human BM CD34+ cells, confirming GCSF direct involvement in the modulation of 67LR expression during *in vivo* administration. We also found that G-CSF-treated human BM CD34+ cells, expressing high levels of 67LR, showed a further increase of such a receptor after adhesion to BM-derived endothelial cell layers, thus suggesting a possible role of 67LR in BM CD34+ cell intra-vasation, in response to G-CSF. Indeed, 67LR regulated G-CSF-stimulated human BM CD34+ cell transendothelial migration toward SDF-1, a key chemokine in HSC trafficking from and to BM.

These observations led us to investigate the involvement of 67LR in the mobilization process by injection of G-CSF and anti-67LR antibodies into BALB/c mice. After G-CSF administration, 67LR expression was increased in circulating CD34+ cells, as observed in humans. In addition, 67LR was also up-regulated in BM CD34+ cells and strongly contributed to their migration into the circulation; indeed, anti-67LR antibodies significantly reduced G-CSF-induced CD34+ and progenitor cell mobilization. These data provide the first *in vivo* evidence that 67LR plays an important role in stem cell egress from BM in response to G-CSF.
All together, our data document that 67LR expression is increased in G-CSF mobilized CD34⁺ cells as compared with unstimulated BM CD34⁺ cells. Noteworthy, the level of 67LR expression in circulating CD34⁺ cells significantly correlates with the mobilization efficiency. Up-regulated 67LR, which replaces α6 integrin receptors after G-CSF stimulation, is required for an efficient HSC mobilization. Indeed, G-CSF administration increases 67LR expression in BM HSCs and contributes to their migration into the circulation most probably by mediating their trans-endothelial migration. Interestingly, 67LR overexpression, which is peculiar of metastatic cancer cells, occurs also in BM and circulating CD34⁺ stem cells after GCSF stimulation, as well as in BM and circulating CD34⁺ leukemic cells (unpublished observation). Thus, an intriguing parallel can be drawn between cytokine-stimulated hematopoietic stem cells, leukemic cells and metastatic cells from solid tumors (99); indeed, a similar signalling pathway, led by 67LR activation, is involved in cell adhesion, motility and dissemination in all these different cell types. These findings further support a model where HSC mobilization could represent a physiologic counterpart of leukemic and metastatic cell spread.
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