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Towards determining the importance of bone marrow-derived factors in supporting human B acute lymphoblastic leukaemia growth *in vivo*

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica de Koen Schepers, Ph.D. (University Medical Center Utrecht, Holanda), de Ester Rieter (University Medical Center Utrecht, Holanda) e do Professor Paul Coffer, Ph.D. (University Medical Center Utrecht, Holanda)

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Sometimes things aren't clear right away. That's where you need to be patient and persevere and see where things lead.

Mary Pierce

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The present work enclosures the work I performed during a year that I had to work hard, but at the same time was amazing, during which I took my first steps into my scientific career. Though this path wasn't always easy, I had wonderful people helping and encouraging me. They not only made it less difficult but also fantastic and full with great memories.

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Abbreviations

AML	acute myeloid leukaemia
B-ALL	B acute lymphoblastic leukaemia
BCP	biphasic calcium phosphate
BM	bone marrow
BMSCs	bone marrow-derived stromal factors
CALL4	MHH-CALL4
CAR cells	CXCL12-abundant reticular cells
CLP	common lymphoid progenitor
CML	chronic myeloid leukaemia
CMP	common myeloid progenitor
CRISPR	clustered regularly interspaced short palindromic repeats
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
Ct	Constitutive
DOX	Doxycycline
EC	endothelial cell
ECM	extracellular matrix
ELISA	enzyme-linked imunoabsorbent assay
FBS	fetal bovine serum
FLT3L	FLT3 ligand
GAL1	galectin 1
H&E	hematoxylin and eosin
HC	heavy chain
HDR	homology directed repair
HSCs	hematopoietic stem cells
IL-7	interleukin-7

Ind	inducible
ITS	insulin, transferrin and sodium selenite
KD	knock down
KO	knock out
LC	light chain
LMPP	lymphoid primed multipotent progenitor
LPC	lymphoid progenitor cell
MM	multiple myeloma
MPP	multipotent progenitor
NHEJ	non-homologous end joining
NK	natural killers
NSG mice	NOD. Cg-Prkdc^{scid}Il2rγ^{tm1Wjl}/SzJ mice
OB	Osteoblast
PBS	phosphate-buffered saline
PEI	poly(ethylenimine)
PL	platelet lysate
qRT PCR	quantitative real time-polymerase chain reaction
RT	room temperature
SCID	severe combined immunodeficient mouse
Scr	Scrambled
TNFα	tumor necrosis factor alpha
TSLP	thymic stromal lymphopoietin
VCAM-1	vascular cell adhesion molecule 1
VLA-4	integrin alpha4beta1or very late antigen-4
WP	well plate

Abstract

In the bone marrow (BM), stromal cells create special microenvironments or niches indispensable for the maintenance, proliferation and differentiation of haematopoietic stem cells (HSCs) and their derivatives, such as B cells. Moreover, evidence suggests that the BM microenvironment promotes malignant growth, including acute B lymphoblastic leukaemia (B-ALL). Currently, most of the knowledge on the interactions occurring in this malignant niche has been provided by *in vitro* systems and *in vivo* models unable to fully simulate the human BM. We set out to determine if C-X-C motif chemokine 12 (CXCL12), vascular cell adhesion molecule 1 (VCAM-1) and interleukin 7 (IL-7), stroma-derived factors, play a crucial role in B-ALL growth using a novel mouse model with subcutaneously implanted ectopic human BM niches downregulated for these factors. *In vitro* preliminary results not only demonstrated the biological importance of BM mesenchymal stromal cells (MSCs) and of these factors on B-ALL, but also allowed the development of functional assays to test the genetically manipulated MSCs produced. As constitutive knock down (KD) of these factors led in most cases to loss of *in vivo* bone forming, we generated human inducible KD MSCs for VCAM-1, IL-7 and CXCL12, and validated successful KD of VCAM-1 and IL-7 at mRNA and/or protein levels. Furthermore, to test complete ablation of our targets, we developed human knock out (KO) MSCs for CXCL12, VCAM-1 and CD44 using the CRISPR/Cas system. A decrease at the protein level of CXCL12 and VCAM-1 was observed in KO MSCs, which seemed to result in the reduction of adhesion of B-ALL cells to VCAM-1 KO MSCs. Taken together, we generated tools that allow the *in vivo* confirmation of our *in vitro* data which indicates the importance of CXCL12, VCAM-1, IL-7 for B-ALL migration, adhesion and growth. Further understanding on the communication between B-ALL cells and the BM cellular niches will contribute to the improvement of current as well as to the development of novel therapies.

KEY WORDS: bone marrow microenvironment · stromal-cell derived factors · acute lymphoblastic leukaemia · humanized mouse model

Resumo

As células do estroma presentes na medula óssea (MO) formam microambientes ou nichos que são indispensáveis à manutenção, proliferação e diferenciação de células estaminais hematopoiéticas, assim como de células que se diferenciam a partir destas, como os linfócitos B. O microambiente da MO também favorece e promove o crescimento maligno de células cancerígenas, tal como de leucemia linfóide aguda de linfócitos B (LLA-B). A maioria das evidências experimentais atuais acerca das interações existentes neste nicho provêm de experiências *in vitro* e de modelos animais incapazes de simular totalmente a MO humana. O principal objetivo deste trabalho foi determinar se C-X-C motif chemokine 12 (CXCL12), vascular cell adhesion molecule 1 (VCAM-1) and interleukin 7 (IL-7), fatores derivados do estroma da MO, desempenham um papel importante no crescimento de LLA-B, utilizando um novo modelo semelhante ao sistema humano. Este modelo consiste na implantação subcutânea em murganhos imunodeficientes de nichos de MO que expressam níveis reduzidos de CXCL12, VCAM-1 e IL-7. Resultados preliminares *in vitro* demonstraram a importância biológica das células mesenquimais do estroma (CME) da MO e dos fatores estudados em LLA-B, e permitiram desenvolver ensaios funcionais para testar o efeito de CME geneticamente manipuladas. Como a supressão (KD) permanente destes fatores levou à perda da capacidade de formar osso *in vivo,* gerámos CME cujos níveis de VCAM-1, IL-7 e CXCL12 podem ser suprimidos de uma forma controlada e validámos a supressão de VCAM-1 e IL-7 ao nível do mRNA e da proteína. Para testar o efeito da supressão total destes fatores, desenvolvemos CME deficientes em VCAM-1, CXCL12 e CD44 utilizando o sistema CRISPR/Cas. Foi observado um decréscimo na expressão de CXCL12 e VCAM-1 ao nível da proteína e, sob o ponto de vista funcional, a supressão dos níveis de VCAM-1 resultou na redução da adesão de células de LLA-B. Em resumo, neste trabalho desenvolvemos ferramentas que permitem a confirmação *in vivo* dos nossos resultados obtidos *in vitro* que sugerem a importância dos fatores estromais

VCAM-1, IL-7 e CXCL12 para a migração, adesão e crescimento de LLA-B. Uma melhor compreensão da comunicação entre células de LLA-B e os nichos celulares da MO permitirá a melhoria das terapias atuais e ainda o desenvolvimento de novos alvos terapêuticos.

PALAVRAS-CHAVE: microambiente da medula óssea · fatores derivados de células do estroma · leucemia linfóide aguda · modelo de murganho humanizado

Chapter 1 Introduction

1. Introduction

Haematopoietic Stem Cells (HSCs) and their more mature derivatives rely on the cellular niches existing in the bone marrow (BM) for support, survival, differentiation and proliferation. This complex BM niche comprises many different types of cells, some of which include osteoblasts, endothelial cells and mesenchymal cells. These BM-derived stromal cells (BMSCs) regulate blood cell behaviour by secreting various molecules and by establishing cell-cell contact with them. However, many factors remain unknown and/or their function are not fully understood yet in humans (Kaur-Bollinger et al., 2012). In addition to this physiological role in haematopoietic differentiation, BMSCs are crucial in the creation of an abnormal niche which serves as sanctuary for tumour cells, as occurs in B-acute lymphoblastic leukaemia (B-ALL), the most common paediatric cancer (Nagasawa, 2006). In this hematologic malignancy, immature B cells accumulate in the BM due to their exacerbated and uncontrolled growth, resulting in normal haematopoiesis impairment and in the inability to mount a normal immune response. Further understanding on the interactions between these leukemic cells and BMSCs would enable the development of novel therapies. Targeting them would inhibit leukemic cell growth and prevent relapse. So far, most studies were conducted using *in vitro* systems and *in vivo* animal models that don't fully resemble the human BM niche. Therefore, new animal models have been developed to more closely simulate the human BM microenvironment. By making use of such sophisticated models, we may increase our knowledge on the stromal factors present in BM and their relative importance in supporting B-ALL cell growth, thus contributing to the development and progress of therapeutical options.

1.1. Niches in the Bone Marrow

1.1.1. The Bone Marrow

The BM is a tissue that occupies the medullary cavities, which are the large central cavities of thick and hollow cylinders of compact bone of bone shafts of typical long bones (such as the femur or the humerus). Regarding their role in the human organism, the BM is a primary lymphoid tissue and the major haematopoietic organ, i.e. the principal place responsible for the active production and development of blood cells (erythrocytes, granulocytes, monocytes, lymphocytes and platelets) from HSCs, for which you can call this their home (Travlos, 2006).

In terms of spatial composition, the main constituents of BM are the bone and the vascular tissues. The endosteum is the inner surface of the BM cavity, consisting of cortical and trabecular bone types. Cortical bone is located in the diaphyseal region of bone, is thick and dense (80–90% of the bone is calcified) and has mainly mechanical and protective functions. On the other hand, trabecular bone is found in the metaphyseal region of bone, is less dense (15–25% is calcified) and has a metabolic role. Most studies refer to the trabecular region as the preferable anatomical location for HSCs (Calvi et al., 2003a; Fujisaki et al., 2011; Zhang et al., 2003). Trabecular surfaces are thought to establish a connection between the skeletal system and the haematopoietic system. The other component of BM is a dense network of thin vessels that allows the passage of a large number of blood cells through their endothelium, known as the medullary vascular sinuses. Blood cells and their precursors are located in the proximity of these vascular sinuses (Lichtman, 1981).

Besides HSCs and the diverse lineages that arise from them, the BM also contains non-haematopoietic cells, the stromal cells, which are adherent cells that create special microenvironments or niches. The niches give support to the HSCs and blood cells, maintaining their viability and supplying them with factors necessary for their survival and

differentiation. This concept and the properties of these niches will be reviewed in the following sections.

1.1.2. Haematopoiesis

As previously mentioned, the cellular components of the blood are produced in the BM. These blood cells originate by proliferation and differentiation from HSCs, a subset of BM cells. Haematopoiesis, the process of blood cell formation, is possible because of the characteristics of HSCs which, as stem cells, are multipotent, i.e. are able to give rise to multiple differentiated cell types, and are self-renewing, possessing almost unlimited ability to expand themselves without differentiating. Thus, HSCs are a long-term self-renewing cell population that gives rise to all mature blood and immune cells (Spangrude et al., 1988), thereby maintaining the homeostasis of the haematopoietic system, important for providing a constant supply of mature blood elements. This continuous provision of blood cells is critical as the body daily requires a considerable amount of blood cells. (Purton and Scadden, 2008). In adult mammals, this process occurs mainly within the BM but it is initiated during the third week of gestation in the yolk sac and only by the eleventh week does it shift to BM (Tavian and Peault, 2005). It has been suggested that during development, HSCs might migrate from the inner bone surface towards the central region of the BM cavity (Hermans et al., 1989; Jacobsen and Osmond, 1990; Lord et al., 1975). However, recent reports contradict this view, as they describe the existence of dormant HSCs in areas away from the bone surface and close to the vasculature (Kunisaki et al., 2013).

When HSCs lose self-renewal ability they mature into multipotent progenitors (MPPs) and become increasingly more differentiated as they become common myeloid progenitors (CMPs) or lymphoid-primed multipotent progenitors (LMPPs). CMPs will differentiate into erythrocytes and megakaryocytes, whereas LMPPs become common

lymphoid progenitors (CLPs) (Igarashi et al., 2002) which are able to differentiate into B and T lymphocytes as well as natural killer (NK) cells (Cobaleda and Busslinger, 2008; Kondo et al., 1997).

In order to distinguish and identify all these types of cells, one can characterize them in terms of changes in their phenotype by using monoclonal antibodies that are reactive against various cell surface antigens expressed on particular lineages of terminally differentiated cells. For instance, to identify T-lineage cells, CD3, CD4 and CD8 are used, whereas B-lineage cells are identified with markers such as B220/CD45RA, CD19 and CD20.

Overall, individual HSCs, but also more mature haematopoietic cells, can have many destinies: quiescence, apoptosis, migration and cell cycle in order to self-renew or to differentiate into their more mature progeny. These outcomes are influenced by combinations of both cell intrinsic and extrinsic factors, these last ones being specific of local microenvironments in which haematopoietic cells reside. A more complete knowledge of the effect of these factors on haematopoiesis may help understanding the pathology of certain hematologic diseases and may provide new and better therapies.

1.1.3. B cell development

To understand the role of the BM microenvironment in B-ALL, it is important to first understand how HSCs develop into mature B cells during normal haematopoiesis which involves the rearrangement and expression of immunoglobulin genes, crucial to make antibodies against a wide range of pathogens, which coincides with changes in the expression of cell-surface and intracellular molecules.

With CLPs as intermediate, HSCs give rise to mature B cells that produce only one heavy chain (HC) and one light chain (LC) through the sequential differentiation of lymphoid

progenitor cells (LPCs). Once committed to the lymphoid lineage, further sequential differentiation steps, defined by the sequential rearrangement and expression of HC and LC immunoglobulin genes, lead to the progressive formation of early B cell precursors, immature B cells, and, after the mature B cell state, the terminally differentiated plasma cells (Figure 1). In order for this progression to occur, the cells have to activate in each stage the specific genetic program of the new intermediate stage alongside with the suppression of the genetic program of the previous stage. In the first step, CLPs differentiate into the early-B cells, a cellular state where the DJH rearrangements begin and B-lineage specific proteins such as VpreB and Igα (CD79a) are expressed. There are two main B cell precursors of immature cells: the pro-B cells and the cells that develop from these, the pre-B cells. Pro-B cells are progenitor cells with limited self-renewal capacity. They express cell-surface CD19, CD34 and CD10 (Loken et al., 1987), but lack the expression of cytoplasmic or cell-surface μ heavy chains (HCs) and V-to-DJH rearrangements occur (Davi et al., 1997), whereas pre-B cells besides expressing CD19 also express cytoplasmic μHCs, and variably express the pre-B cell receptor (pre-BCR). The pre-BCR is a complex of proteins consisting of μHC, ψLC, and the Igα/Igβ signal transducing heterodimer (Benschop and Cambier, 1999) appearing at a critical initial checkpoint. Immature cells are no longer considered B cell precursors since they, in addition to expressing CD19, have on their cell-surface μHCs associated with κ or λ LCs, i.e. the B cell receptor (BCR). So, when B-lineage cells start expressing the BCR, they are no longer classified as B cell precursors (LeBien, 2000). Until this point, all development takes place in the BM and is independent of the presence of antigen. At this stage, immature B cells are selected for self-tolerance and ability to survive in the periphery. If they are able to do so, those B cells undergo further differentiation giving rise to naive mature B cells which express both IgG and IgM. These B cells circulate

through secondary lymphoid tissues where, if encountering their specific antigen, may be activated and are called mature cells.

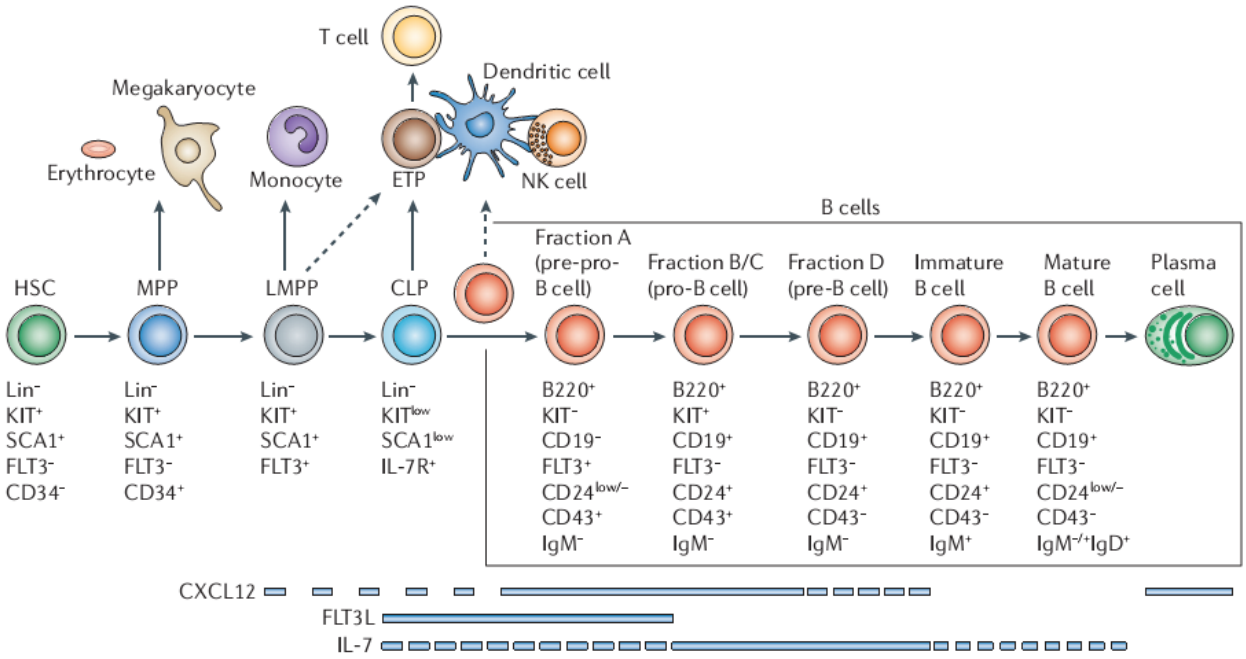


Figure 1 - Stages in human B-cell development (adapted from Nagasawa, 2006).

Many external factors play a role in B cell lymphopoiesis. BMSC-derived molecules can stimulate survival/growth, differentiation, or chemotaxis in cells in different stages of differentiation. Such factors include Interleukin-7 (IL-7), CXC-chemokine ligand 12 (CXCL12 or SDF1α and PBSF) which is a chemokine, a large family of structurally related chemoattractive cytokines, that primarily binds to CXC-chemokine receptor 4 (CXCR4) as well as FLT3 ligand (FLT3L) (Nagasawa, 2006), and thymic stromal lymphopoietin (TSLP) (LeBien, 2000). The role of these and other stromal factors in B cell development will be discussed in more detail below.

1.1.4. Cellular niches in the Bone Marrow

HSCs and haematopoietic cells are anatomically located and tightly regulated in the specialized microenvironment or niches in the BM (Fuchs et al., 2004). The microenvironment provides them with essential conditions for HSC maintenance by giving them cues to expand and differentiate, keeping a proper balance of the right amount of various immune cells, as well as creating a safe house that protects them from certain insults.

The concept of haematopoietic microenvironments was first proposed forty years ago by Dr. John Trentin (Trentin, 1971). Trentin demonstrated that stromal cells had an active role in the regulation of the differentiation of HSCs into all blood cell lineage types and that marrow stroma is geographically segmented into microenvironments, each of which directs differentiation along a specific lineage. Schofield further expanded on these ideas and introduced the 'niche' concept to describe the specialized domains in the BM where HSCs reside and which contribute to the maintenance of their stemness (Schofield, 1978). Moreover, he argued that once the HSCs leave the niche, they initiate maturation. Despite following studies (Dexter et al., 1973, 1977; Lichtman, 1981), the precise anatomical location of the BM niche and the exact identity of the cell(s) that the niche contained remained relatively unknown until the osteoblasts appeared as a critical component of the HSC niche (Calvi et al., 2003a, 2003b; Zhang et al., 2003), as well as the sinusoidal endothelial cells (Kiel et al., 2005).

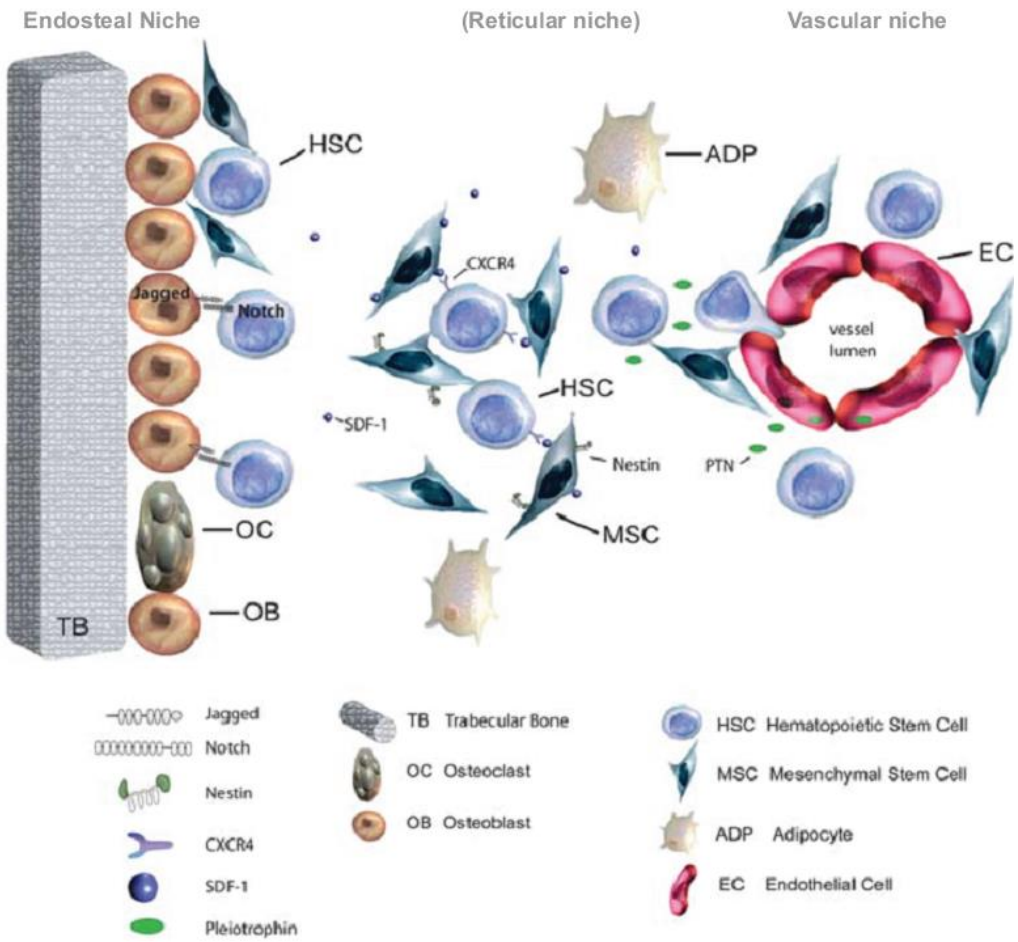


Figure 2 - Schematic representation of the cellular niches for haematopoietic stem cells in the bone marrow (adapted from Doan and Chute, 2012)

Nowadays it is accepted that HSCs are enriched within specialized areas of the BM microenvironment (Figure 2), namely the osteoblastic (or endosteal) niche (Nilsson et al., 2001; Zhang et al., 2003) and the vascular niche (Kiel et al., 2005), both of which are the major components responsible for supporting the maintenance, proliferation and differentiation of HSCs. Some authors also consider the existence of a third niche: the reticular niche (Purizaca et al., 2012). These reticular cells that have been found to be important for supporting haematopoiesis, might in fact be MSCs with the ability to differentiate towards the osteoblastic, adipocytic and chondrocytic lineage as well as expressing various factors important for supporting HSCs, such as CXCL12 and SCF (Frenette et al., 2013). However, since these reticular MSC-like cells surround the

vasculature and as the endosteal niche is highly vascularized, it is hard to distinguish between the endosteal, vascular and reticular niches. Altogether, this strengthens the concept that the BM niche should not be looked at as clear and defined structural compartments but rather viewed as an active interaction between different cellular components (Garrett and Emerson, 2009). Recently, also cells from the nervous system appeared as players in the BM niche, as glia cells maintain HSC hibernation by regulating the activation of latent TGF-β (Bruckner, 2011; Yamazaki et al., 2011). In addition the extracellular matrix (ECM) also plays essential roles. The ECM is a complex composition of collagens, proteoglycans, glycosaminoglycans, and glycoproteins such as fibronectin, osteopontin, laminins and thrombospondins (Klein, 1995). As such, HSC niches are quite complex, containing a broad range of BM cells which not only include bone lining cells (osteoblasts and osteoclasts), MSCs, sinusoidal endothelium and perivascular stromal cells, but also other cells such as adipocytes and mature immune cells. Elucidating the functions of these niches is crucial to understand the behaviour of HSC and hence exploit this knowledge for clinical applications.

1.1.4.1. Stromal Bone Marrow Niche Cells Supporting HSCs

The osteoblastic niche, thought to maintain HSC quiescence over the long term and located in the endosteum, contains both multi-cellular and acellular components. Growth factors and ECM constitute the acellular portion of BM. The main BM endosteal cells are multiple subsets of osteoblasts (OB) and mesenchymal progenitor cells (Nakamura et al., 2010). OBs, which differentiate from MSCs, lie at the inner surface of trabecular bone. Besides their role in providing mineralization during bone development and in replacing lost bone tissues in adults, since the early 1970s, studies have provided clues that OBs might also play an essential role in haematopoiesis (Gong, 1978; Lord and Hendry, 1972) and thereby play an important function as niche cells. In 2003, Li's and Scaden's groups

reported, in the same issue of the scientific journal Nature, experiments describing an important role for OBs in supporting HSCs *in vivo* (Calvi et al., 2003a; Zhang et al., 2003). Furthermore, defects in OB differentiation due to lack of Runx2, which plays a pivotal role in osteogenesis, lead to defective BM haematopoiesis (Deguchi et al., 1999; Wang et al., 2010). Endochondral ossification was demonstrated to be necessary for niche formation, as suppression of factors involved in the endochondral ossification inhibited niche generation (Chan et al., 2009). These studies indicate that osteoblastic cells represent a regulatory component of the BM microenvironment and suggested that HSC pool size is directly affecting OB numbers within the BM. OBs express factors such as cytokines, chemokines and adhesion molecules that have been specified for HSC regulation and adherence to OB. These include the ligand Angiopoetin-1 (Arai et al., 2004), the non-canonical Wnt signalling (Sugimura et al., 2012) and thrombopoietin (Qian et al., 2007; Yoshihara et al., 2007) which play an essential role in maintaining HSCs in a quiescent state. CXCL12 is a chemokine abundantly expressed by OBs that has crucial roles in chemotaxis, homing, survival of HSC as well as in the retention of HSCs in the BM (Broxmeyer et al., 2005). Although it was initially believed that, when HSCs are associated with OBs they are in a quiescent state, more recent studies suggest that this role could rather be played by MSCs that also localize in the endosteal niche (see below).

In addition to OBs, various cells that reside in the endosteal region of the BM are also able to either directly or indirectly regulate haematopoiesis. These include osteoclasts (Cho et al., 2010; Kollet et al., 2006; Lymperi et al., 2011; Mansour et al., 2012), macrophages (Christopher et al., 2011) and a specialized group of macrophages, the osteomacs (Winkler et al., 2010a).

Evidence that HSCs may occupy multiple niches besides the osteoblastic niche has been accumulating, such as the vascular niche localized in the proximity of the sinusoidal vascular endothelium (Kiel et al., 2005). The vascular bed of the BM is predominantly

composed of sinusoids. As these vessels are thin, constituted by a single layer of endothelial cells (ECs), the marrow cavity is able to communicate with the blood circulation. In addition to ECs, this vascular niche comprises other types of cells (reticular cells, perivascular stromal cells, MSCs, and neurons) creating an elaborate niche that supports and regulates HSCs. The population of HSCs that locate in this niche has been suggested to be maintained over a shorter term, actively cycling, and replace circulating cells (Kopp et al., 2005; Winkler et al., 2010b). As such, the vascular niche would function as a gateway for HSC migration, circulation and anchoring (Doan and Chute, 2012; Sipkins et al., 2005a). However, a new view of the vascular niche has been proposed in which the existence of multiple vascular niches playing opposite roles in the maintenance of HSCs quiescence was demonstrated. According to this recent perspective, the sinusoidal vascular niche maintains cycling HSCs, whereas the quiescent HSCs are located in the arteriolar niche (Kunisaki et al., 2013).

Moreover, the perivascular region also contains MSCs that are either identified as: CXCL-12-abundant reticular cells (CAR cells) or Nestin^{+} cells. CAR cells possess mesenchymal cell properties and are different from ECs and OBs (Greenbaum et al., 2013). In the vascular niche, they are located closely to the sinusoids in the BM (Tokoyoda et al., 2004) but they can also be present in the endosteal niche (Sugiyama et al., 2006), where they also associate with HSCs and as such probably establish a bridge between these niches. Ablation of CAR cells *in vivo* decreased the ability of stromal cells to differentiate into adipogenic and osteogenic lineages and to produce SCF and CXCL12. It also results in a reduction in the number and size of HSCs (Omatsu et al., 2010). Frennete's lab has demonstrated that MSCs can also be identified by Nestin expression since Nestin^{+} cells differentiate into mesenchymal lineages (osteoblastic, adipocytic and chondrocytic). These Nestin+ cells constitute a key component in the BM microenvironment, with their presence being necessary for homing and maintenance of

HSCs (Méndez-Ferrer et al., 2010; Muguruma et al., 2006). MSCs express molecules important for HSC control, including c-kit ligand, angiopoietin-1, IL-7 and osteopontin.

The existence of MSCs leads to a convergent idea and perhaps a unifying niche theory regarding the cellular components of the HSC niche (Muguruma et al., 2006). Despite the vascular and osteoblastic niches can usually be physically and functionally distinguished, in some cases they can be overlapping. It was demonstrated that blood vessels may be present on the endosteal surfaces, establishing connections and regulating each other (Lo Celso et al., 2009; Kanczler and Oreffo, 2008; Xie et al., 2009). For instance, interaction between angiopoetin-1 produced by OBs and ECs expressing its receptor Tie-2 promotes angiogenesis and reduces vascular permeability (Fukuhara et al., 2008; Saharinen et al., 2008).

In conclusion, the BM niche is an intricate microenvironment, which we are now little by little starting to understand. However, many questions remain unanswered. Considering the critical function of HSCs in the human body and its absolute dependence on communication with BM stromal cells, there is a huge need to fully understand the BM architecture and to address the molecular pathways by which these cells interact thereby regulating HSC state and function.

1.1.4.2. Stromal BM Niche Cells Supporting B cell development

Along with specific transcription factors (Ikaros, transcription factor E2A, early B cell factor and PAX5) which activities clearly determine B cell fate, it has long been known that microenvironmental cues, via surface bound as well as soluble molecules (Murti et al., 1996) are involved in regulating this differentiation process. I will therefore, after a brief introduction of the main factors involved in B cell development (IL-7, CXCL12 and galectin-1 or GAL1), describe the cells that express these factors: CXCL12-expressing

OBs, CXCL12-expressing MSCs, IL-7-expressing cells and GAL1-expressing cells. In addition, factors such as FLT3L, receptor activator of nuclear factor-κB ligand (RANKL), very-late antigen-4 (VLA-4) and vascular cell adhesion molecule-1 (VCAM) also appear to be crucial for B cell development (Jarvis et al., 1997; Nagasawa, 2006).

In addition to being important for haematopoiesis in general, a crucial role of the CXCL12-CXCR4 axis in B cell development has been established. By generating mutant mice with a targeted disruption of the gene encoding CXCL12 it was demonstrated that this chemokine is essential for B cell lymphopoiesis (Nagasawa et al., 1996). Moreover, CXCL12 was identified as the first soluble factor essential for the earliest identifiable B cell precursor (Egawa et al., 2001) and inactivation of CXCR4 in B cells in mice proved that CXCR4 is required for retention of B cell precursors in the BM (Nie et al., 2004).

ECs, OBs and MSCs are important stromal cell populations that have been suggested to express this chemokine. OBs appear to be able to support developmental transitions in B lymphopoiesis by locally secreting the stromal factors CXCL12 and IL-7, reviewed below, in addition to inductive signals that include VCAM-1–mediated adhesion. In accordance with this, selective elimination of OBs highly impaired both pre-pro-B and pro-B cells from BM (Zhu et al., 2007).

Despite the existence of these various CXCL12 expressing cells, the specific role for each individual CXCL12 producing cell type in supporting HSC maintenance and retention, and in the generation of certain lymphoid progenitors only recently became elucidated. For this purpose, scientists selectively deleted CXCL12 from candidate niche cell populations known to express CXCL12. To target OBs, the Morrison's lab used Col2.3-cre mice, and were able to reach the conclusion that CXCL12 impairment in OBs resulted in loss of B-lymphoid progenitors and B cell reconstitution (Ding and Morrison, 2013). Deletion of CXCL12 in osterix-expressing cells, which included CAR cells and immature OBs, reduces the number of B lymphoid progenitors. This is in agreement with

previous studies in which CAR cells were found to be crucial for B lymphoid commitment (Omatsu et al., 2010). Deletion of CXCL12 from ECs, although neither depleting myeloid or lymphoid progenitors, did deplete HSCs and it was suggested to have a crucial role in HSC maintenance. The BM of Prx1-cre;Cxcl12$^{fl/fl}$ mice, having CXCL12 deletion in multipotent mesenchymal progenitors and perivascular stromal cells, had fewer committed B cell progenitors. In conclusion, both Morrison's and Link's groups demonstrated that HSCs are supported by a perivascular niche created by ECs and Lepr-cre or Prx1-cre cells. On the other hand, some early B-lymphoid progenitors occupy an endosteal niche comprised of OBs (Ding and Morrison, 2013) /osterix-expressing cells (Greenbaum et al., 2013), with commited B-lineage progenitors depending on Prx1-cre cells that create a distinct perivascular niche.

In addition to CXCL12-expressing cells, several other cell types have been implicated in providing the specific factors necessary for B cell development (Figure 3).

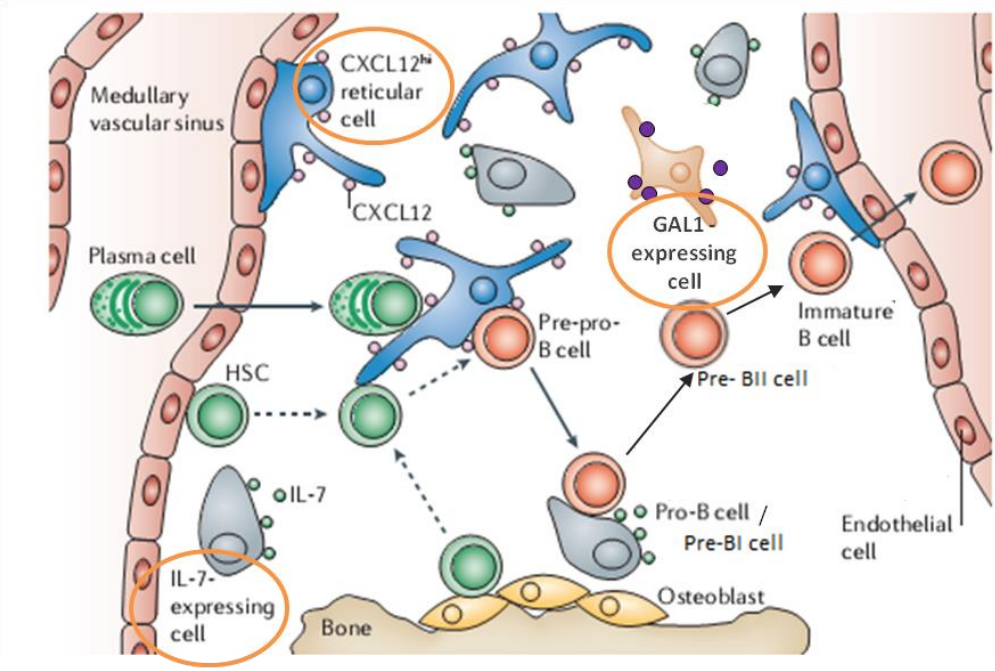


Figure 3 - Possible cellular niches involved in B-cell development and model of the movement of multipotent hematopoietic progenitors, B cells and their precursors in the bone marrow, throughout development (adapted from Nagasawa, 2006)

IL-7 was the first identified environmental factor to be able to induce proliferation of B cell precursors (Namen et al., 1988), having a unique and nonredundant role in the formation of both B and T cell lineages (von Freeden-Jeffry et al., 1995; Peschon et al., 1994). The receptor of IL-7 includes two subunits: the IL-7Rα chain, shared with TSLP, and the cytokine-receptor common γ chain (γ_c). IL-7 has been shown to be essential at the pro-B cell stage as mice deficient in either IL-7 or IL-7Rα had normal numbers of pre-pro-B cells but a significant impairment in the number of pro-B cells, pre-B cells and B cells in a more differentiated stage (von Freeden-Jeffry et al., 1995; Peschon et al., 1994). Contradicting these results, by the analysis of IL-7-deficient mice, Dias et al demonstrated that IL-7 appears to have an important role as early as the CLP stage during B cell development (Dias et al., 2005). However, it appears that in humans there is no such dependency on IL-7 as people who lack the expression of IL-7Rα don't have impaired B cell development (Puel et al., 1998).

A population of fibroblast-like cells scattered throughout the BM expresses IL-7, along with VCAM-1 (Funk et al., 1995; Tokoyoda et al., 2004). IL-7 production by stromal cells is induced by the IL-7-dependent B cells (Sudo et al., 1989). The niche provided by these cells is thought to contribute to clonal expansion of B cell precursors and also plays a role in the earliest stages of B cell development. Even though both IL-7 and CXCL12 play crucial roles in B cell development and frequently may have additive or synergistic functions, B cell precursors are present distinct stromal-cell subsets as they are located differently in the BM and CAR cells don't express IL-7 (Tokoyoda et al., 2004).

Recently, a specific stromal cell microenvironment was identified for pre-BII cells (Mourcin et al., 2011), one subtype of human pre-B cells that express a functional pre-BCR and, in opposition to pre-BI cell which are large proliferating cells, are small postmitotic cells (Ghia et al., 1996). This niche is comprised of GAL1-expressing stromal cells. Mourcin and colleagues provided evidence supporting early B cell migration from IL-7$^+$ to GAL1$^+$ niches during their development.

In summary, the stage-specific cellular niches, which secrete factors that regulate the mobilization and stromal cell interactions of haematopoietic precursors, are required throughout the development of B cells. B cell precursors and plasma cells are localized in their specific niches, migrating between them as development proceeds (Nagasawa, 2006). Early B cell precursors require CXCL12 and IL-7 (Egawa et al., 2001; von Freeden-Jeffry et al., 1995; Nagasawa, 2006) with the cellular candidates for CXCL12 expression being CAR cells and/or OBs (Greenbaum et al., 2013). Pre-pro-B cells require CXCL12 associating with CAR cells close to the vasculature. As IL-7 is essential for pro-B cells/pre-BI cells, they migrate from CAR cells in direction to IL-7 expressing cells. These cells may induce an expansion division, considering the characteristics of pro-B cells/pre-BI cells (mitotically active and requirement of IL-7 for proliferation) (von Freeden-Jeffry et al., 1995). At the next step, the pre-BII cell stage, the pre-BCR is formed and expressed at the surface. At this stage, cells leave IL-7 expressing cells and move to the $GAL1^{+}$ niche during their differentiation (Mourcin et al., 2011). Newly generated immature B cells which express IgM leave the BM and enter the blood to reach the spleen. There they may encounter their specific antigen leading to maturation into peripheral mature B cells. End-state cells reside once again in close proximity of CAR cells in the BM. The majority of long-lived plasma cells reside in the BM, homing that fails upon deletion of CXCR4 (Tokoyoda et al., 2004). CXCL12 likely supports homing thereby contributing to the survival of plasma cells as they consequently localize in the niches of BM.

1.1.5. Acute lymphoblastic Leukaemia

Uncontrolled malignant growth of blood cells is characteristic of leukaemia, which comprises two main classes: acute, commonly found in children, being its incidence peaks between 2 and 5 years of age, and chronic. The major difference between them

is that in the acute form there is a rapid accumulation of malignant cells in the BM and bloodstream due to loss of regulation of haematopoiesis at the very initial steps, while in the chronic form the progression is slower and malignant cells are relatively differentiated and potentially not fully functional. Acute leukaemias can be divided into lymphoblastic (ALL) and myeloid (AML) cell malignancies, according to the affected cell lineage. ALL is a malignant neoplasm of the lymphocyte precursor cells or lymphoblasts, i.e., of the cells having lymphoid origin. These leukemic lymphoblasts have an exacerbated and uncontrolled growth that results in the clonal accumulation of immature blood cells in the BM. These abnormal cells are arrested in the lymphoblast stage of the normal maturation pathway so they are unable to mount a normal immune response. They may also lead to an impaired production of normal BM cells since normal haematopoiesis is suppressed, consequently depleting the number of circulating erythrocytes, platelets and leukocytes other than lymphocytes that may cause anaemia, thrombocytopenia or neutropenia, respectively. The most common symptoms are fatigue and spontaneous bleeding, and often present, malaise, lethargy, weight loss, fevers as well as bone and joint pain. ALL represents approximately less than 1% of adult cancers, but accounts for 25% of all childhood cancers and 75% to 85% of all childhood leukaemias (Miller et al., 1995; Perez-Saldivar et al., 2011). Despite less than half of all adult leukaemia patients survive 5 years after diagnosis, almost two-thirds of all adult ALL cases survive at least 5 years after diagnosis. In children the survival is much better with more than three-quarters of all children with leukaemias and more than four-fifths of ALL cases surviving at least 5 years past diagnosis (Ries et al., 2006), now approaching 90% of survival (Hunger et al., 2012).

In terms of aetiology, the pathology of ALL is associated with genetic abnormalities in more than 75% of the cases (Pui et al., 2008) that can be chromosomal translocations, chromosome copy number alterations or gene-specific mutations. In most cases, these abnormalities are acquired rather than inherited. A hallmark of acute leukaemias is the

presence of aberrant fusion genes that, even though alone are unable to induce the disease, increase the predisposition of the carriers to the disease. The disease onset may also require acquisition of additional genetic or epigenetic alterations. Translocations are especially common in childhood ALL and the ones frequently found in ALL cases include the TEL-AML1 translocation - the most common reciprocal translocation which occurs in about 25% of cases (Shurtleff et al., 1995), MLL translocations, MLL-AF4gene fusion, t(9,11)(p22;q23) and t(11, 19), and CDK6-MLL. Modifications in the function of certain transcription factors including Ikaros, E2A, EBF and Pax5 are thought to contribute as well to leukaemogenesis (Pérez-Vera et al., 2011). Furthermore, physical and chemical risk factors may also make a contribution to this complex and heterogeneous disease.

Despite the advances of the therapeutic options and the prolonged survival in recent years thanks to them, devastating developmental effects may arise due to high dose chemotherapy as well as an increase on the risk of secondary cancers (Haddy et al., 2009). Moreover, ALL is still far from being eradicated because of the recurrence after the treatment in many cases (Yang et al., 2008). The BM niche is thought be a critical component in relapse.

1.1.5.1. B Acute Lymphoblastic Leukaemia

From the ALL-cases in children, around 80-85% are B- cell ALL (B-ALL) (Perez-Saldivar et al., 2011). Also in adults, B-lineage leukaemias dominate within the lymphoid leukaemia patient group (>75%) (Hjalgrim et al., 2003). B-ALL is characterized by malignant and uncontrolled growth of B-lymphoid precursor cells in BM. The accumulation of blast B cells leads to a decrease in normal haematopoiesis besides invasion of extramedullary regions by these cells.

Being a subtype of ALL, a set of gene fusions created by chromosomal translocations are involved in the genesis of B-ALL: TEL-AML1, MLL rearrangements, BCR-ABL and E2A-PBX1. The frequency of chromosome translocations varies between children and adults. TEL-AML$^+$ leukaemias are most prevalent in children (22% vs. 2% in adults), whereas BCR-ABL$^+$ are most prevalent in adults (25% vs. 3% in children). Although it is difficult to determine in which stage of the B-lymphoid lineage differentiation process the translocation occurred, considering their known biological properties some origins have been proposed: if occurring in an early progenitor, the BCR-ABL and MLL translocations may be involved, while TEL-AML1- and E2A-PBX1 appear in a more advanced state of differentiation (Cobaleda and Sanchez-Garcia, 2009). These primary oncogenic events seem to be insufficient to cause a completely malignant cell, a process which requires other supportive changes. Somatic mutations leading to the constitutive activation of certain signalling pathways contribute to proliferation and survival of B lymphoblasts, including abnormal IL-7 and TSLP receptors that are constitutively activated (Tal et al., 2013). Besides this, as a cancer supportive effect, interaction of B precursor cells with cells in their surroundings influences leukaemia progression.

1.1.6. Contribution of the bone microenvironment to leukaemia

The BM niches are also important in malignancy, as it has been established that alterations in cell composition and function in the microenvironment regulates stem cell activity. BM is the most common site of disease in hematologic malignancies, as its microenvironment is a fertile location for leukemic cells growth and tumour dormancy, i.e. cells that are not proliferative but can later enter a proliferative state. This happens mostly because the BM provides cues which support leukemic cells homing, adhesion and survival. When comparing BM-derived MSCs from normal and leukemic patients, B-ALL cells survive more and better *in vitro* when placed in the leukemic environment

(Nwabo Kamdje et al., 2011; Rodríguez-Pardo and Vernot, 2013). Of note, the influence appears to be in both directions, with cells from different types of leukemia creating abnormal niches and altering stromal cell function and these stromal cells in return contributing to the survival and proliferation of tumour cells (Ayala et al., 2009). Some hypotheses have been proposed regarding the mechanisms by which the niche contributes to leukemogenesis (Purizaca et al., 2012): (1) competition of leukemic cells for the BM niche leading to dysfunctional microenvironments for normal HSCs (Colmone and Sipkins, 2008); (2) the capability of certain stromal cells to facilitate tumour progression and/or (3) disruption of cross-talk between HSC and microenvironment. The group of Emmanuelle Passegué recently obtained proof for these concepts in a mouse model for chronic myeloid leukaemia (CML) (Schepers et al., 2013). This study described in detail the cellular and molecular cross-talk between leukemic cells and the microenvironment that result in a self-reinforcing leukemic niche. In addition, interaction of leukaemia cells with the BM stroma has been proposed as a mechanism for chemotherapy resistance (Ayala et al., 2009; Azab et al., 2009; Mudry et al., 2000; Tesfai et al., 2012), as stromal interactions protect leukemic cells from the effects of chemotherapeutic agents, and cancer recurrence by up-regulating anti-apoptotic proteins (Frolova et al., 2012).

In particular, a number of alterations have been suggested to occur in the BM microenvironment in B-ALL and other types of leukemia, a few of which I briefly mention here and others that I will describe in more detail in the following section. Leukaemia-associated genetic aberrations in MSCs have been detected which indicates that different cell types are involved in the pathogenesis and pathophysiology of leukaemia and suggests that MSC may be involved in the generation of relapse (Menendez et al., 2009; Shalapour et al., 2010). Deleting *Dicer1,* an RNase III enzyme, from mouse osteoprogenitors causes haematopoietic malignancies (Raaijmakers et al., 2010), emphasizing the relevance of external cues provided by stromal cells.

Neoangiogenesis, the formation of new blood vessels, is a hallmark of cancer, and contributes contributing to cancer progression. Even though ALL is not a solid malignancy, various proangiogenic factors are abnormally expressed in B-ALL patients and angiogenesis in ALL is of utmost importance in leukemogenesis, leukemic progression and treatment (Aref et al., 2007; Perez-Atayde et al., 1997; Veiga et al., 2006; Zhou et al., 2005). B-ALL cells thus seem to stimulate ECs, and these in a reciprocate manner induce B-ALL survival.

In addition to genetic modifications and increased vascularization, over-activation of some signalling pathways due to intrinsic activation of these pathways by genetic changes or extrinsic factors may promote leukemogenesis (Chung et al., 2002). Alterations in ECM components may also lead to haematological malignancies. For instance, reticulin fiber density was shown to be a useful prognostic marker in pediatric ALL (Noren-Nystrom et al., 2008). Moreover, in the ALL malignant niche there is a prevalence of pro-inflamatory cytokines: TNF-α and IL-6, thus creating an abnormally inflammatory niche (Espinoza-Hernandez et al., 2001).

Recently, another type of interaction between leukemic cells and the microenvironment was identified. AML cells were shown to secrete core histones associated with fragmented DNA which can enter the nuclei of BM cells, integrate into host cell DNA and lead to death of stromal cells (Dvořáková et al., 2012).

All these further stress the importance of the BM stroma in leukaemia.

1.2. Role of stromal-derived molecules in B-ALL growth

As discussed above, the BM niche is a main contributor to cancer progression and resistance to treatment. The growth and survival of leukemic B cell progenitors is dependent on intimate contact with BM stromal layers (Kumagai et al., 1996; Manabe et

al., 1992). In this section I will focus on and describe how BM-derived stromal factors may lead to B-ALL. I will give special attention to VCAM-1, CXCL12 and IL-7 which have been demonstrated to be critically involved in leukaemia-microenvironment cross-talk. All the interactions described here are summarized in Figure 4.

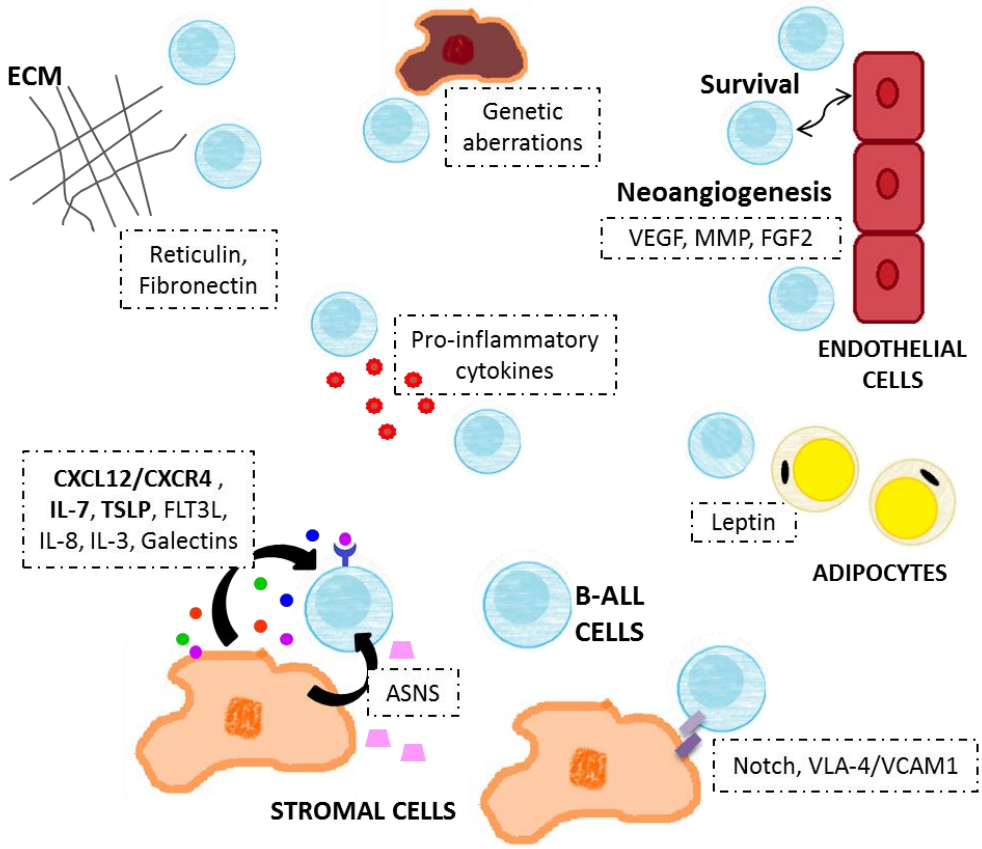


Figure 4 - B-ALL microenvironment: interactions between stromal cells in the malignant niche and leukemic cells.

1.2.1. *In vitro* murine/human studies

Following treatment of B-ALL, the origin of recurring malignant cells appears to be the BM. Consequently, it has been proposed that the BM creates niches that protect leukemic cells from apoptosis. Research has been performed trying to understand the biologic architecture of this host microenvironment since this has huge implications for treating tumors. The BM niche has also been implicated in B-ALL growth. One approach

that may facilitate identification of BM stromal cell factors essential for both survival and expansion of human leukemic B cell precursors is the *in vitro* co-culture of human BM stromal cells with leukemic cells. First of all, *in vitro* studies have shown that culturing primary B-ALL lymphoblasts in the presence of B-ALL-patient derived BM MSCs leads to changes in the cytokine expression of the leukemic cells, which may explain BM MSCs support of B-ALL cells (Wu et al., 2005) and close association of ALL to BM niche cells results in an important ALL survival effect *in vitro* (Murti et al., 1996). Nevertheless, this BM microenvironment dependency may vary depending on the leukemic subclone as it was observed that distinct B-ALL subclones present different requirements from the BM niche (Shah et al., 2001).

By monitoring apoptosis, direct contact with stroma has been established as a survival requirement for normal B lymphoblasts (Kumagai et al., 1996; Manabe et al., 1994). A crucial adhesion pathway through which precursor-B ALL cells bind to BM stroma has been demonstrated to be via the $beta_1$ integrins VLA-4 and VLA-5 and this adhesion is partly mediated by VCAM-1 on stromal cells (Makrynikola and Bradstock, 1993; Miyake et al., 1992). BM stromal cells constitutively expressing VCAM-1 increase the survival of B-ALL cells (Hall et al., 2004a). Notch signalling also plays a role in the survival of B-ALL cells, as Notch neutralizing antibodies decreased the survival of primary B-ALL cells when co-cultured in the presence of BM-derived MSCs (Nwabo Kamdje et al., 2011). Furthermore, both pathways were shown to be important in conferring resistance to certain chemotherapies (Hall et al., 2004a; Nwajei and Konopleva, 2013).

Stromal cells secrete selective chemotactic factors which attract and concentrate leukaemia cells in their selective local microenvironment, being therefore important for both migration and homing. As mentioned above, CXCL12 was demonstrated to be a potent chemokine for normal B precursor cells. Moreover, CXCL12 has also been implicated in malignant B cell development as Bradstock and colleagues showed precursor-B ALL cells preferentially localize close to the BM-derived cells *in vitro* in a

process dependent on CXCL12 binding to its receptor CXCR4 on leukemic cells (Bradstock et al., 2000).

It has long been known that IL-7 is able to induce a proliferative response in both B cell precursor-acute lymphoblastic leukaemia and immature T-ALL cells (Eder et al., 1990; Touw et al., 1990). When studying the mechanism of action of an mTOR inhibitor in malignant progenitor B cells, IL-7 played a critical role in stimulating the proliferation of leukemic cells (Brown et al., 2003). Findings further support a role for the IL-7/IL-7R axis in B cell oncogenesis given the upregulation of IL-7 receptor (IL-7Rα) by B-ALL cells (Sasson et al., 2010). This suggests an increased activation of this pathway in B cells in the BM niche. On the other hand, overactivation of IL-7 signalling pathway may occur due to gain-of-function mutations in IL-7Rα (Shochat et al., 2011). An elegant experiment using primary T-ALL cells cultured *in vitro* with either human BM stromal or a murine fibroblast-like cell line of BM stromal origin, resulted in enhanced survival of T-ALL cells mediated by BM stroma in both cases and requiring the IL-7/IL-7R interaction. When blocking IL-7/IL-7R, the survival was only partially inhibited, suggesting the existence of cooperation by other mechanisms (Scupoli et al., 2007). In addition to a supportive effect mediated by IL-7 as mentioned above, IL-7 has also been shown to enhance the survival mediated by BM stroma in B-ALL cells when in the presence of CXCL12 and IL-3 (Juarez et al., 2007a).

TSLP, not only shares one of its receptor's chains, IL-7Rα, with IL-7, but also has similar biological functions to IL-7 (Sims et al., 2000). Brown and colleagues have shown that, similar to IL-7, TSLP stimulates B-ALL cell proliferation, in a dose-dependent manner (Brown et al., 2007). Furthermore, TSLP contributed to chemoresistance by decreasing the growth inhibition and pro-apoptotic effect of rapamycin. Since in this study they treated the cells directly with TSLP, it remains unknown whether BMSCs could be a potential source of this factor.

Co-culture of patient-derived B-ALL blasts with B-ALL BM stromal cells leads to up-regulation of a group of BM stromal genes encoding secreted proteins, including CCL2 and IL-8/CXCL8 which were able to increase the adhesion of ALL cells to BM stromal cells and to promote survival and proliferation of BM stromal cells (de Vasconcellos et al., 2011). Therefore this may represent a molecular mechanism by which tumor cells contribute to the establishment of a malignant BM microenvironment.

Using an animal model of both obesity and leukaemia, a link between obesity and leukemogenesis. Leptin, IL-6 and insulin were in higher levels in obese mice when compared to control mice and may accelerate the progression of B-ALL (Yun et al., 2010). Leptin is of particular interest since B-ALL lymphoblasts may express leptin receptors, thus suggesting a possible involvement of adipocytes in leukemogenesis.

Additional factors that support B-ALL cell growth are factors such as FLT3 ligand (Eder et al., 1996), Interleukin-3 (IL-3) and low-molecular-weight B cell growth factor (Duyn et al., 1999; Wormann et al., 1987), that all stimulate B-ALL cell growth *in vitro.*

In conclusion, leukemic cells are highly reliant on the BM microenvironment for their survival and proliferation *in vitro*. Many cytokines, chemokines and other stromal cell derived factors have been examined for their contribution, but none is able to fully substitute BM stromal support, suggesting that combinations and synergistic interactions of these and other factors still not identified may be required for optimal *in vitro* growth and survival. Actually, Juarez and colleagues have demonstrated that there is a complex pattern of interaction between the signalling pathways initiated by CXCL12, IL-7 and IL-3, with additive or synergistic interactions between CXCL12 and IL-7 or IL-3 which promote the proliferation of ALL cells in some cases (Juarez et al., 2007a), highlighting the necessity to study such complex interactions in further detail.

1.2.2. *In vivo* mouse studies

Although interaction of B-ALL cells with the BM microenvironment has been extensively studied *in vitro* thereby shedding some light on BM biology and its role in leukemic cell homing and progression, *in vitro* techniques are quite limited in their ability to mimic the whole physiology of BM architecture. *In vivo* studies appear as better models to study the role of BM in leukemogenesis. In this section, I will review various animal models that have been used to study B-ALL. Most *in vivo* studies performed so far are based on transplantation of cell lines into immunodeficient mice and/or rely on genetically manipulated mice, in which the expression of a certain gene was ablated or increased.

In vivo studies have confirmed the role that adhesion molecules such as integrins, VLA4 being the most prevalent one, play in the homing of B-ALL cells. B-ALL cells lacking expression of VLA-4 were generated from the pre-B-ALL cells line NALM-6 and intravenously injected into immunodeficient mice. In comparison to mice injected with wild-type NALM-6 cells, mice injected with NALM-6 cells lacking expression of VLA-4 had a prolonged survival and leukemic cells engrafted less efficiently in the mice (Filshie et al., 1998). In another study, there was a significant decrease in the homing of B-ALL cells in the BM of NOD/SCID mice when these were treated with neutralizing VLA4 antibodies (Spiegel et al., 2004). Recently, it was demonstrated that pharmacological blocking of integrin alpha4, a constituent of VLA-4, in mice as well as deletion of integrin alpha4 in mice make B-ALL cells more sensitive to chemotherapy (Hsieh et al., 2013).

Using dynamic *in vivo* confocal imaging, Sipkins and colleagues revealed that NALM-6 cells specifically localized in a unique and spatially-restricted vascular domain which are CXCL12 and E-selectin positive. They demonstrated that the BM expressed specific vascular cell surface adhesion molecules and chemoattractants necessary for malignant cell homing to these areas, as when inhibiting CXCL12 signalling the homing was decreased by almost 80% (Sipkins et al., 2005a). This was in accordance to previous

studies in which it had been demonstrated that CXCR4 and CXCL12 play a significant role in the *in vivo* engraftment and homing of NALM-6 cells to the BM of NOD/SCID mice (Shen et al., 2001). Moreover, others have used CXCR4 antagonists in NOD/SCID mice administered with human leukemic cells showing that this resulted in the inhibition of the growth and dissemination of patient ALL cells (Juarez et al., 2009).

Mice expressing IL-7 under control of an immunoglobulin heavy chain promoter and enhancer, which leads to overexpression of IL-7 in hematopoietic cells in the BM, lymph nodes, spleen, thymus and skin, developed both B and T lymphomas (Rich et al., 1993). Although use of mice overexpressing IL-7 in hematopoietic cells allows the study of the role of IL-7 as an oncogene, i.e. as an initial promoter of leukaemia, this does not allow studying whether stromal cell-derived IL-7 is also involved in the support of *in vivo* growth of leukemic cells. Other studies that report on the role of IL-7 *in vivo* focus mainly on T-ALL and not B-ALL (Zenatti et al., 2011). IL7R mutations promote tumor formation in T-ALL. In another study, in which the aim was to directly assess the role of stromal cell-derived IL-7 in leukaemia growth, the ability of human T-ALL cell lines to engraft and expand in an immunodeficent mouse was significantly reduced if the mouse that lacked IL-7 (Silva et al., 2011).

Leukemic dormant cells are able to evade death from cytotoxic chemotherapy since this mostly targets cells in proliferation. Boyerinas et al. have provided proof that osteopontin, a protein secreted by OBs, anchors NALM-6 cells and regulates their dormancy in xenografted mice. Targeting this interaction therefore increased the sensitivity of leukemic cells to chemotherapy (Boyerinas et al., 2013). Once again, the importance of the communication between tumor cells and the microenvironment was highlighted, suggesting that targeting this interaction may improve the efficacy of B-ALL treatment.

1.3. Microenvironmental targets for B-ALL treatment

As described previously, evidence indicates that the BM microenvironment plays a crucial role in the survival and proliferation of B-ALL cells, supporting their growth. Because of the sanctuary for leukemic cells that the (malignant) niche creates, stroma-derived factors are thought to contribute to the decrease in the sensitivity, and thus efficacy of chemotherapy, as well as to the relapse of the disease. Consequently, the disruption of such communication appears as a promising approach to treat cancer in combination with chemotherapy and prevent its relapse. A scarce number of patients is able to survive long-term due to leukaemia recurrence (Pui and Evans, 2006; Stock, 2010). Some molecular pathways have been suggested to be critical for the interactions between leukemic cells and the BM niche thus emerging as possible therapeutical targets.

Currently, the main approaches consist of directly inhibiting the molecules which are responsible for the activation of the proliferative/anti-apoptotic pathways or, as alternative, targeting the molecules that contribute to the homing process. Regarding the first approach which is directed at the proliferative compartment, the most common targets are specific signalling pathways, such the PI3K/Akt, JAK/STAT and Notch1 pathways, that are key in regulating B-ALL growth and survival, and inhibiting the activity of anti-apoptotic proteins (Brown et al., 2008; Fowler and Oki, 2013; Konopleva et al., 2009). B-ALL studies are still in a preclinical stage but presenting interesting results. For instance, Akt pathway appears to be a promising target as its activation has been demonstrated to play an important role in chemotherapy, and is associated with poor prognosis and relapse (Morishita et al., 2012). Other targets such as angiogenesis inhibitors are also being studied (Konopleva et al., 2009).

In order for leukemic cells to successfully home to the BM microenvironment, they have to go through three steps: migration, adhesion and secretion of metalloproteinases to

clear their path so that they are able to reach their destination. Therefore, efficient targeting of these stages has appeared as an exciting option for therapy, either alone or in combination with the above mentioned approaches. Given the large amount of evidence demonstrating its crucial effect in the attraction and homing of leukemic cells, targeting the CXCL12/CXCR4 axis by CXCR4 antagonists has been particularly well studied. The importance of leukemic migration has been confirmed since as a result of CXCR4 inhibition, human B-ALL cells were more responsive to chemotherapy in NSG mice (Yu et al., 2011). Furthermore, the combination of CXCR4 antagonists with the conventional chemotherapy drugs improved the survival and reduced the presence of human pre-B-ALL cells in NSG mice (Parameswaran et al., 2011). In addition to migration, adhesion molecules are crucial. Studies focusing on interfering with adhesion molecules function, specially targeting integrins (VLA4/VCAM-1 interaction), are being developed (Konopleva et al., 2009).

Finally, other strategies to modulate the BM niche have been developed. A particular attention has been given to hypoxia mediators because long-term HSCs may be located in regions in the BM with lower oxygen content (Parmar et al., 2007) and because hypoxia stimulates angiogenesis. Importantly, in pre-B ALL co-cultured with BM-derived MSCs HIF-1α-mediated signalling has also been demonstrated to be part of the mechanism of chemoresistance in hypoxic conditions (Frolova et al., 2012).

In addition to these, many other factors might still be unidentified and/or their complete function remains unknown. The identification of the synergistic and additive interactions between them is imperative in order to be able to fully manipulate the BM microenvironment and thereby improve B-ALL therapy.

Despite the approaches used so far, the role of various stromal factors in B-ALL growth and survival remains not fully known, mainly due to the lack of appropriate models that simulate human haematopoiesis and its interaction with the BM in an *in vivo* setting.

However, with recent advances in xenograft models we start to get closer to being able to simulate the human BM niche and to study the role of stromal cell-derived factors in human B-ALL growth and survival. The novel and sophisticated animal models will play a crucial role as they are becoming closer to simulating the human BM niche.

1.4. Xenograft models of ALL

In vivo disease models present some advantages in comparison to *in vitro* models: they are more representative of the physiological human situation and increase our knowledge in a clinically more relevant setting. Regarding haematological diseases, the preclinical models most commonly used are the genetically engineered mouse models and human tumour xenografts in immunocompromised mice. In spite of all the development that has been put into animal models of ALL, progress has been partially limited due to lack of knowledge of the microenvironmental factors needed for successful engraftment. In addition, we must be cautious when interpreting the results obtained as whereas the leukemic cells in these xenograft models are human, the microenvironment is murine thereby not fully mirroring the human/patient condition (Lee et al., 2007).

In this section I will briefly mention the various xenograft models that have thus far been used for studying ALL, giving thereafter emphasis on recently developed *in vivo* xenograft models that have the potential to really move forward the study of the role of the BM microenvironment in human B-ALL growth.

1.4.1. Human tumour xenografts models

Xenograft models show a high degree of consistency and predictability for disease development, having provided an important tool to study various aspects of leukaemia

biology. Furthermore, if properly used and interpreted they can be quite useful. However, it is always good to consider that they probably don't completely predict the behaviour of the disease in humans and the conditions for engraftment in the host may exert a distinct pressure for selection of subclones. Consequently, the results obtained may vary with the experimental setup.

Xenograft leukemia models can be divided in two main subtypes: ectopic or subcutaneous models and orthotopic models. The first one is unable to represent the physiology of the disease due to its subcutaneous localization (Kerbel, 2003). When the implanted cells are grown in the tissue of the host where they normally reside, the model is known as orthotopic. Clinically relevant targets are better represented by this tumour system as it better simulates the morphology, microenvironment, growth and metastatic patterns of the human disease (Bibby, 2004).

Xenotransplantation of human tumour explants and cell lines has been made possible with the development of immunocompromised animals. Immunodeficient mice have been generated aiming at creating animals with the least residual immunity. Athymic mice, which inherit an autosomal recessive gene (*nu*/*nu*) and lack functional T cells, were the first type of such animals used in xenograft experiments. When chronic myelogenous leukemic (CML) cells were transplanted into these mice, they developed tumours resembling those of the patient (Lozzio et al., 1976). The engraftment of leukaemia cells improved with the development of the severe combined immunodeficient (SCID) mouse (Bosma et al., 1983; McCune et al., 1988) and the bg/nu/xid (BNX) mouse (Kamel-Reid and Dick, 1988). The BNX mouse model has three crucial mutations: the *nude*(nu) that leaves the animal athymic, the *beige*(bg) which reduces the number of NK cells and the *xid* that limits the number of lymphokine activated killer cells (LAK). In addition to the BNX mouse, NOD (non obese diabetic)/SCID mice are the most common models, which has proven to be an accurate and reliable model to study ALL (Baersch et al., 1997; Lock et al., 2002). Even so, there were still some barriers that limited engraftment of leukemic

cells. Taking that into consideration, McKenzie et al. improved it by using an anti-CD122 antibody (McKenzie et al., 2005). This antibody targets the interleukin-2 receptor beta (IL-2Rβ) therefore affecting many haematopoietic populations including NK cells and macrophages (Shultz et al., 2003). The levels of engraftment were higher when anti-CD122 antibody was injected in NOD (Shultz et al., 2003) and NOD/SCID mice (McKenzie et al., 2005). Other strains have been developed with impaired innate immunity, stressing the importance of lack of macrophage activity, for human engraftement as well as absent NK activity. This was achieved by introducing into NOD/SCID mice additional defects for instance the lack of the the common cytokine receptor γ chain (γ_c) (i.e. creating the NSG strain) (Ohbo et al., 1996; Shultz et al., 2005). Another mouse strain frequently used is a genetic cross of a recombinase activating gene 2 (RAG2)-deficient strain, which lacks all T, B and NK cells, with mice deficient for γ_c ($RAG2^{-/-}\gamma_c^{-/-}$).

Besides the mouse strain used, the biology and clinical course of leukaemia in the xenograft model also depends on many other factors that may increase or decrease the success of the engraftment, including: (a) the source and processing of leukaemia cells; (b) the route of administration and conditioning of the host; and (c) the method selected to characterize the manifestation of leukaemia (Meyer and Debatin, 2011). Furthermore, the environment that the leukemic cells encounter in the recipient affects their survival and homing not only because of the potential presence of residual immunity but also due to cross-species reactivity of homing molecules and growth factors (Manz, 2007). Aiming at overcoming this, Rongvaux et al. developed a humanized transgenic mouse model in which the gene encoding mouse thrombopoietin (TPO), which has been demonstrated to be an essential cytokine in the maintenance and self-renewal of HSCs, was replaces by its human form in mice with a $Rag2^{-/-}\gamma_c^{-/-}$ background. This humanized model represents a valuable model to study human haematopoiesis *in vivo* as it has higher levels of human engraftment in the BM of the hosts as compared to regular $Rag2^{-/-}\gamma_c^{-/-}$

mice (Rongvaux et al., 2011). Others have also reported that improved engraftment of AML xenografts engraft significantly better in β2-microglobulin-deficient NOD/SCID and NOD/SCID mice which were transgenic for the human growth factors genes Steel factor (SF), IL-3 and granulocyte macrophage-colony-stimulating factor, which are elevated in the serum of these mice (Feuring-Buske et al., 2003).

In summary, by increased immunodeficiency and generation of transgenic mice expressing human cytokines, the models have obtained a better ability to engraft human cells. Nonetheless, for a long time human cancer xenografts have not been simulating the appropriate cancer stroma environment in immune-compromised mice. Consequently, they may be more sensitive to drug treatment (Olive et al., 2009) and may not fully mirror inflammatory responses (Quintana et al., 2008). Trying to overcome this, xenograft approaches are currently under study in order to better model the tumour microenvironment. These methods include the transplantation of human leukemic cells into mice that have been “humanized” by the incorporation of implants that simulate the human BM microenvironment, as will be discussed below.

1.4.1.1. *In vivo* models to study the BM microenvironment

One of the major limitations of the previously mentioned xenograft models is that the human cells engraft in a murine BM environment that may not reflect the interactions between the hematopoietic cells and the BM microenvironment in humans. Trying to overcome this issue, MSCs have been used as scaffolds for the formation of humanized stem cell niches in mice as they demonstrate both positive and negative regulatory effects on the self-renewal, proliferation, and differentiation of HSCs (Chen et al., 2008; Pittenger et al., 1999) and are able to differentiate into various other stromal cells normally present in the BM, such as adipocytes and osteoblasts. Nevertheless, the engraftment of transplanted human MSCs at murine sites of haematopoiesis is not very

efficient, maybe due to competition with a functional murine microenvironment. It was only until the development of human 3-dimensional bone-tissue-like structures using scaffolds that revolutionized these efforts (Nichols et al., 2009; Song et al., 2010). Since Polyurethane, 3D poly-e-caprolac-tone polymeric and high-performance titanium-based scaffolds coated with human MSCs result in human bone formation (Calimeri et al., 2011; Lai et al., 2013) were shown to effectively allow engraftment of human cancer cells thus allowing creation of ectopic human niches (Calimeri et al., 2011; Vaiselbuh et al., 2010).

Along the same line, the Martens lab has recently also developed a novel humanized mouse model that allows the study of the *in vivo* interactions between BM stromal cells and human haematopoietic and leukemic cells (Groen et al., 2012). This model implements a scaffold-based technology in which culture-expanded BM-derived stromal cells are loaded on biphasic calciumphosphate (BCP) particles which are then implanted subcutaneously on the dorsal side of RAG2$^{-/-}\gamma_c^{-/-}$ mice (Figure 5A). Eight weeks after implantation, human OBs grow out on top of a layer of human bone in connection with vessels capable of supporting haematopoiesis thus generating a human BM environment (Figure 5B). Inoculation of these mice with umbilical cord blood-derived CD34$^+$ results in their engraftment and differentiation into multiple blood cell lineages (Figure 5C). This humanized model has been tested for the engraftment and growth of a multiple myeloma (MM) cell line and for patient-derived MM (pMM) cells, and showed successful support of both types of cells. We have shown that these BM ectopic niches support the growth of primary human B-ALL cells (data not shown). Therefore, this novel mouse model allows the study of the human haematopoietic niche in the context of normal and malignant haematopoiesis. Since this model is based on *in vitro* cultured human MSCs, this provides the opportunity for genetic manipulation of the BM microenvironment, either by overexpressing, silencing or deletion of specific genes.

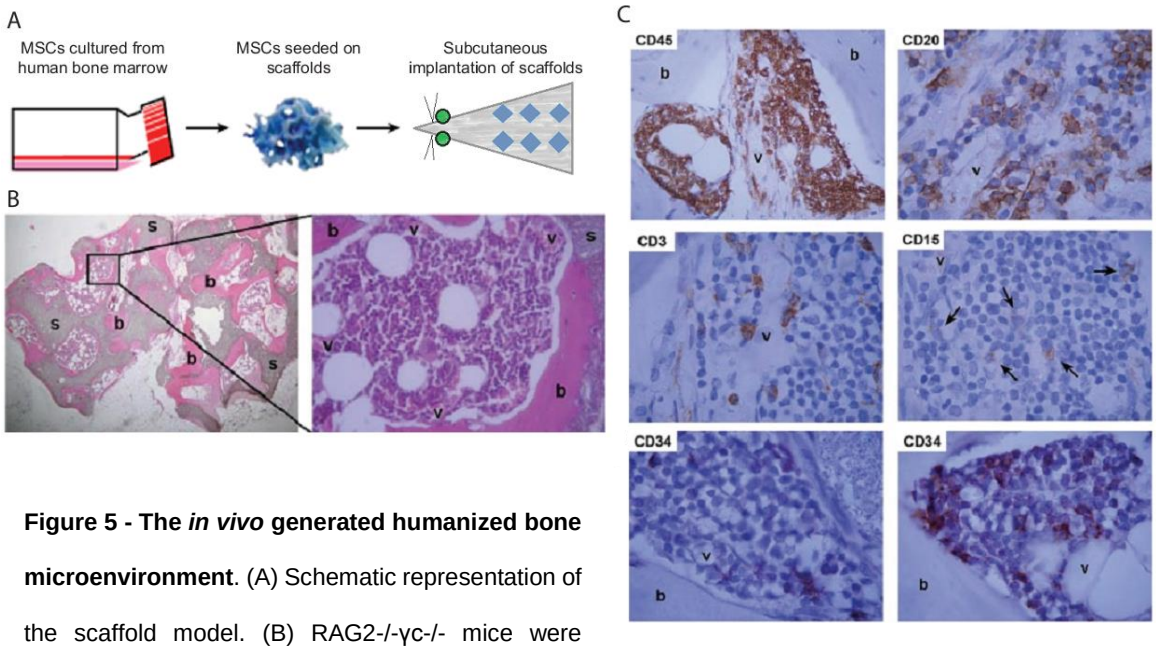


Figure 5 - The *in vivo* generated humanized bone microenvironment. (A) Schematic representation of the scaffold model. (B) RAG2-/-γc-/- mice were implanted with human MSC-loaded BCP scaffolds (s). Eight weeks after implantation, this led to the formation of human bone (b) in the vascularized (v) open spaces that was capable of supporting mouse hematopoiesis. (C) CD34+ cells engraft and differentiate in humanized ossicles. At week 16 ossicles with human CD45+ cells (CD45) were revealed. These included B cells (CD20), T cells (CD3), and myeloid cells (CD15). Undifferentiated CD34+ cells (CD34) were detected adjacent to both bone (b) and vessels (v). (Adapted from Groen et al., 2012)

Recently, Chen et al. have developed an *in vivo* extramedullary bone model in NOD/SCID/IL-2rγ^{null} mice by subcutaneously injecting them with a matrigel scaffold seeded with human BM-derived MSCs mixed with endothelial colony-forming cells (EPCs) (Chen et al., 2012). This is the first model in which both the stroma and the endothelium are humanized, besides showing the usefulness of the humanized BM niche as a tool to study the role of certain factors by genetically manipulating these niches.

In summary, the humanized mouse models mentioned here are state of the art technology to study the interactions of tumour cells with the BM microenvironment, including studying the influence of BMSCs-derived factors on the growth of human B-ALL *in vivo*. Thereby these open up a new field of research into the *in vivo* role of the BM

niche in human B-ALL growth, in addition to other leukaemias and normal haematopoiesis.

1.4.1.1.1. *The CRISPR/Cas System*

One way to establish the importance of one particular stroma-derived factor in the BM microenvironment is to reduce or ablate its expression. In addition to the commonly used shRNA approach that allows down regulation of genes of interest, so called knock down, recent advances have been made in genome editing that even allow the generation of human knock out cells. Recently, an efficient technique that allows genome editing has been developed based on a system that bacteria and archae use in their immune defence (Wiedenheft et al., 2012), termed Clustered Regularly Interspaced Short Palindromic Repeats or CRISPR/Cas system (Cong et al., 2013; Mali et al., 2013). This system relies on a single-guide RNA (gRNA) that directs Cas9, an endonuclease, to its complementary DNA sequence in the genome. After binding, Cas9 cleaves the DNA in both strands therefore creating a double strand break (DSB). The cell activates mechanisms to repair these DSBs. If they are repaired by error-prone non-homologous end-joining (NHEJ), this can result in the introduction of deletions or insertions, which may generate frameshift mutations. Therefore, this cellular repair mechanism can lead to a knock out of the gene targeted with the gRNA. Alternatively, another DNA repair mechanism can be activated: the homology-directed repair (HDR), in which case it is possible to either add or correct a gene since it requires a homologous DNA segment for the process (Figure 6) (Charpentier and Doudna, 2013; Gaj et al., 2013; Sander and Joung, 2014).

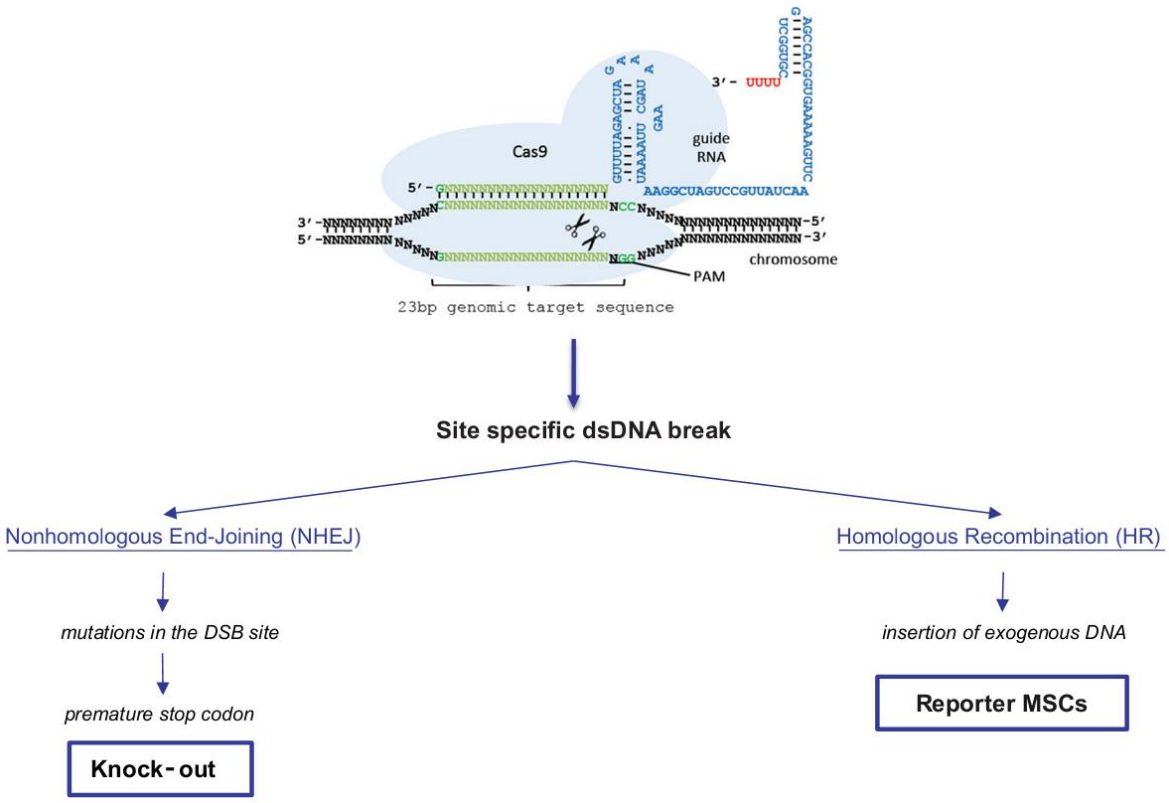


Figure 6 - Targeted genome editing in human cells using CRISPR/Cas system. (adapted from Mali et.al, 2013)

Due to its enormous potential, the CRISPR/Cas system is being widely used and exciting breakthroughs have been made. For instance, a dominant mutation that causes a genetic disease has been corrected in mice by HDR (Wu et al., 2013). Even more impressive, monkeys genetically-modified in a precise target gene have been recently generated (Niu et al., 2014). This genome engineering technique can have different applications as well, such as in human genetic screening (Wang et al., 2014).

In summary, the CRISPR/Cas system is certainly going to transform biological research and impulse the development of novel and more successful therapies. Particularly, this technique in combination with the scaffold model can be used to study the role of stromal factors in haematopoiesis/leukemia. As it allows the genomic edition of human MSCs,

we can either totally ablate a target gene, thus determining its importance for B-ALL, or label it, which should allow the tracking of (cells expressing) these factors.

1.5. Scope and aims of this thesis

The main goal of the present study was to setup techniques to study the role of various stromal-derived factors in supporting human B-ALL growth in *vivo*. For this purpose, we aimed at generating ectopic humanized niches in immunocompromised mice that upon induction lose the expression of the studied factors. In addition, we wanted to study the effect of total ablation of the selected stromal factors. The importance of these factors in leukaemia can be addressed by analysis of the engraftment and growth of primary human B-ALL samples in such BM niches.

The specific aims of this project were:

a. To confirm the *in vitro* capacity of BM MSCs to support the growth of B-ALL cells;
b. To determine the biological function of the studied stromal factors in B-ALL *in vitro* and to develop assays that allow functional validation of the genetic manipulated MSCs;
c. To generate and validate inducible knock down MSCs for CXCL12, VCAM-1 and IL-7 and to investigate their biological effect on B-ALL cells *in vitro*;
d. To generate knock out MSCs for CXCL12 and VCAM-1 using the CRISPR/Cas system followed by validation at the protein and functional level;
e. To test if the genetically manipulated MSCs are able to form ectopic human bone marrow niches *in vivo.*

Chapter 2 Materials and Methods

2. Materials and Methods

2.1. Materials

The plasmids pH1tet-flex and pFH1t(INSR)UTP were obtained from Taconic Artemis and pRP-418 was kindly provided by Robert Jan Lebbink (Emmanuel Wiertz lab, UMC Utrecht). The oligonucleotides were purchased from Sigma Aldrich.

The human B cell precursor leukaemia cell lines, REH, NALM-6, MHH-CALL4 (CALL4), NALM-6 lentiviral transduced with Luc-mCherry-Puromycin (Puro), and REH with Luc-mCherry-Puro, were provided by the group of Monique den Boer (EMC Rotterdam). NALM-6 cells were lentiviral transduced with Luciferase (Luc)-Green Fluorescent Protein (GFP) by Anne van der Leun.

The human recombinant IL-7, TSLP and CXCL12 were obtained from Immunoblots. The FC Receptor Block (anti-CD16/32), CXCR4-PE (anti-CD184) and IL-7-Rα-PE (anti-CD127) antibodies were purchased from BD Bioscience and the TSLPR-PerCP and VCAM-1-PerCP (anti-CD106) antibodies from eBioscience. Tra-1-85-FITC (R&D systems) was used. Poly(ethylenimine) was purchased from Polysciences.

AMD3100 (Sigma) was kindly provided by Debby Gawlitta (Wouter J.A. Dhert lab, UMC Utrecht). TNFα was acquired from Immunotools. Doxycycline hyclate (DOX) and Puromycin were obtained from Sigma Aldrich.

2.2. Animals

The animal experiments were performed according to the Dutch guidelines on animal experimentation and ethically approved by the local Animal Experimentation Committee.

2.3. Cell culture

After written informed consent and approval by the Institutional Medical Ethical Committee to use the material for research purposes only, extracts from the BM of healthy donors were obtained. MSCs were obtained from the BM of the healthy donors, hereafter mentioned as Donor 99 (D99) or Donor 06 (D06) cells, and cultured at 37ºC 5% CO2 in MSC medium consisting of MEM alpha medium (Gibco) supplemented with 10U/ml Heparin (Leo Pharma), 1% Penicillin/Streptomycin (Invitrogen) and 5% platelet lysate (PL), which was provided by the Bloodbank Sanquin Northwest (Utrecht, The Netherlands) and consisted of a pool of PL from 5 different donors (MSC medium with PL). NALM-6-Luc-GFP, NALM-6-Luc-mCherry-Puro and REH-Luc-mCherry-Puro cells were cultured at 37ºC 5% CO2 in RPMI-1640, GlutaMax (Gibco) supplemented with 10% heat inactivated HyClone Fetal Bovine Serum (FBS) (GEHealthcare) and 1% Penicillin/Streptomycin (Invitrogen). CALL4 cells were cultured at 37ºC 5% CO2 in RPMI-1640, GlutaMax (Gibco) supplemented with 20% FBS (GEHealthcare) and 1% Penicillin/Streptomycin (Invitrogen). Inducible (ind) knock down (KD) BM MSCs were cultured in Tet-free medium (DOX free medium) consisting of MEM alpha medium (Gibco) supplemented with 1% Penicillin/Streptomycin (Invitrogen), 10% FBS provided by the Patrick Derksen group (UMC Utrecht) that had been tested to not induce downregulation of genes in cells that were transduced with inducible shRNAs, L-glutamine (2 mM, Gibco), fibroblast growth factor (b-FGF, 1 ng/ml, Invitrogen) and L-ascorbic acid 2-phosphate (0.2 mM, Sigma Aldrich). To test if there was leakiness in the inducible system and to determine which was the optimal medium for culturing the indKD MSCs, these MSCs were cultured as well in an MSC medium similar to DOX free medium, only differing on the supplemented FBS, which was HyClone Fetal Bovine Serum (MSC medium with FBS, GEHealthcare).

2.4. Co-culture of B-ALL cells and BM MSCs and Luciferase Activity Measurement

In order to assess expansion of B-ALL cells NALM-6-Luc-mCherry-Puro and REH-Luc-mCherry-Puro cells were co-cultured in the presence of MSCs in a 1:1 mixture of RPMI-1640, GlutaMax (Gibco) supplemented with 5 µg/ml insulin, transferrin and sodium selenite (ITS, Invitrogen) and 1% Penicillin/Streptomycin (Invitrogen), and MEM alpha medium (Gibco) supplemented with 1% Penicillin/Streptomycin (Invitrogen) and 5µg/ml ITS (Invitrogen). In order to reach the best ratio MSCs:B-ALL cells, different numbers of MSCs (825, 1650, 3300 and 6600) were seeded in a white polystyrene 96 wells assay plate (Costar). After 3-4h, B-ALL cells were added in three different concentrations (9,38x10^3 cells/ml, 1,88x10^4 cells/ml and 3,75x10^4 cells/ml) in a total volume of 100 µl per well. The cells were incubated at 37ºC 5% CO2 for 3 days. To determine the relative degree of B-ALL cells expansion, we measured the luciferase activity in each respective well by adding 4 µl of Luciferine (Promega) and by measuring the bioluminescence at 550 nm (Ugarova, 1989) using a Centro LB 960 Microplate Luminometer (Berthold Technologies). The optimized numbers of MSCs and B-ALL cells used in the follow up experiments were 1650, 3300 or 6600 cells and 3,75x10^4 cells/ml, respectively. Expansion of NALM-6-Luc-mCherry-Puro cells (3,75x10^4 cells/ml) when co-cultured with VCAM-1 constitutive KD MSCs (6600 MSCs) in the presence or absence of 20 ng/ml Tumor Necrosis Factor alpha (TNFα, Immunotools) was analysed. To test the effect of IL-7 on the expansion of co-cultured B-ALL cells, NALM-6-Luc-mCherry-Puro cells (3,75x10^4 cells/ml) were cultured in the presence of 1650 or 3300 BM MSCs, IL-7 (100ng/ml) (Immunoblots) and 0.25 ng/ml DOX (Sigma Aldrich). After three days, Luciferase Activity was measured.

2.5. Migration Assays

To test whether B-ALL cells migrate in response to CXCL12, NALM-6-Luc-GFP cells (5x10^6 cells/ml) were incubated for 30min at 4ºC in the presence or absence of AMD3100 (10µg/ml, Sigma). MSC medium containing CXCL12 (100ng/ml, Immunotools) +/- AMD3100 (10µg/ml) was added to the bottom chamber of Corning Transwell polycarbonate membrane cell culture inserts (Sigma Aldrich). NALM-6-Luc-GFP cells (5x10^5 cells) were added to the top chamber. After 3h incubation at 37ºC 5%CO2, the presence of B-ALL cells in the bottom chamber was determined by measuring the Luciferase Activity as described above.

2.6. Presto Blue Cell Viability Assay

To determine CALL4 growth, cells (6x10^5 cells/ml in 100 µl, 96 well plate round bottom) were incubated with 10ng/ml or 100ng/ml of human recombinant TSLP (Immunoblots) in RPMI 10%FBS medium for 3 days at 37ºC 5%CO2. PrestoBlue® Cell Viability Reagent (Invitrogen) was added (10µL/well) and cells were incubated at 37ºC 5%CO2 for 30-45 minutes. Fluorescence was read at 560nm (excitation)/590nm (emission) using a Spectramax Plus384.

2.7. Quantitative Real Time-Polymerase Chain Reaction for mRNA (qRT-PCR)

To validate the inducible knock down of the selected factors in ind KD MSCs, these cells were incubated for either 16h (400,000 cells), 24h (200,000 cells), 48h (180,000 cells) or 5 days (18,000 cells) in the presence of DOX (Sigma Aldrich) (0.25µg/ml), added every two days. The RNA was isolated using RNeasy Mini Kit (Qiagen) following the

manufacturer's instructions. cDNA was synthesized using 5x iScript Reaction mix (Biorad) and iScript Reverse Transcriptase (Biorad) according to the manufacturer's instruction and incubating at the following temperatures: 25ºC 5min, 42ºC 45min, 85ºC 5 min, 4ºC. cDNA was diluted 1:10 with RNase-free water. For the qRT-PCR reaction, the samples were added to a mix of forward and reverse primers (10 µM, see table) and Sybr® Green Super mix in a total volume of 15µl per sample. The reactions were performed at the following temperatures: 95ºC 3min (1 cycle); 95ºC 30s, 60ºC 30s, 72ºC 30s, 95ºC 1min (40 cycles); 65ºC 1min, 65ºC 10s (30 cycles), 4ºC. The quantification analysis was based on the detection of a fluorescence signal obtained when SYBR® Green intercalates in the DNA double strand, which is proportional to the amount of amplified DNA. All reactions were performed in triplicate. The expression of Beta-2-microglobulin (B2M) was used to normalize the expression of the target DNA.

Table 1 - qRT-PCR primers

Gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
hCXCL12	ATTCTCAACACTCCAAACTGTGC	ACTTTAGCTTCGGGTCAATGC
hIL-7	TCTAATGGTCAGCATCGATCA	GTGGAGATCAAAATCACCAGT
hVCAM-1	GGACCACATCTACGCTGACA	TTGACTGTGATCGGCTTCCC
hB2M	ATGAGTATGCCTGCCGTGTGA	GGCATCTTCAAACCTCCATG

2.8. Enzyme-linked Immunoabsorbent assay (ELISA)

Quantitative analysis of the presence of CXCL12 in the medium of CXCL12 indKD MSCs, CXCL12 KO MSCs and their respective Scrambled (Scr) MSCs was performed using Quantikine ELISA (R&D Systems) according to the manufacturer's

recommendations. This assay was performed with conditioned medium that was collected after 3 days of cell culture (35000 cells per well in a 24WP) and stored at -80ºC. IndKD MSCs had been previously cultured for 7 days in medium with PL in the presence of 0.25 ng/ml DOX, added every two days. Briefly, 100µl conditioned medium was added to the 96 well plate (R&D Systems) already containing Assay Diluent and incubated for 2h at room temperature (RT) on a horizontal orbital microplate shaker set at 500±50 rpm. After washing the wells, CXCL12 conjugate was added to each well and the plate was incubated at RT as mentioned above. The substrate solution was added and the plate was incubated for 30 min on the bench top. The optical density was determined using Spectramax Plus384 set to 450nm. Optical corrections were made by measuring the optical density at 540nm as well.

2.9. Adhesion Assays

With the aim of determining if knocking out VCAM-1 in BM-MSCs affected the adhesion of B-ALL cells to the stromal cells, NALM-6-Luc-mCherry-Puro cells (3,75x10^4 cells/ml) were cultured in the presence of VCAM-1 KO MSCs (3300 or 6600 MSCs per well) and ± 20 ng/ml TNFα (Immunotools), as described previously. After 24h incubation at 37ºC 5% CO2, the plate was placed for 5 min in a horizontal orbital microplate shaker set at 450±50 rpm. The medium was collected (aproximatelly 100 µl per well) and transferred to a new well. Phosphate-buffered saline (PBS) was added (150 µl per well) to the remaining cells in the bottom and the plate was placed for 5 min in a horizontal orbital microplate shaker set at 450±50 rpm. The PBS was removed and the previous step was repeated. After aspiration of the PBS, new medium was added (100 µl). Luciferase Activity was determined.

2.10. Vectors Construction

Aiming at obtaining BM MSCs with inducible knock down of certain stromal derived factors, we used the DOX-inducible system from TaconicArtemis in which we cloned shRNAs into the pH1tet-flex/FH1t(INSR)UTP vector system.

After vector amplification through *Escherichia coli* (DH5α cells) transformation by heat shock and plasmid DNA isolation (Genopure Buffer Set, Roche) following the manufacturer's instructions, 1 µl Bovine Serum Albumine (BSA, New England BioLabs), 5 µl buffer 2 (New England BioLabs) and 30 µl deionized water, and the pFH1t(INSR)UTP vector (1.3 µg/µl, 12 µl) with 1 µl *PacI* (New England BioLabs), 1 µl Bovine Serum Albumine (BSA, New England BioLabs), 5 µl buffer 1 (New England BioLabs) and 31 µl deionized water, were digested both at 37ºC during 2,5h. The pFH1t(INSR)UTP vector (44 µl) was dephosphorylated with 1 µl Phosphatase Alcaline (Roche) and 5 µl 10x dephosphorylation buffer (Roche) during 1h at 37ºC, and then purified using High Pure PCR Product Purification Kit (Roche) according to manufacturer's instructions. Oligonucleotides containing the shRNA sequence (see Table 2) were annealed – 10 µl top strand, 10 µl bottom strand and 80 µl Annealing Buffer (10 mM Tris pH 7.5, 1 mM EDTA and 50 mM NaCl). Then, the annealed oligonucleotides were diluted (1:100) and cloned into the digested pH1tet-flex vector. For the ligation reaction, 2 µl pH1tet-flex vector (20.3 ng/ml), 2 µl annealed oligonucleotides, 13 µl deionized water, 2 µl T4 DNA ligase buffer (Roche) and 1 µl T4 DNA ligase were incubated O/N at 16ºC. Following bacteria transformation with the ligated vectors, colonies were picked and plasmid DNA was isolated. Positive colonies were sequenced. Plasmids containing the correct DNA sequence were amplified though PCR and digested with *PacI.* Then the inserts (4,64 ng) were cloned in a 1:3 ratio into *PacI*-digested pFH1t(INSR)UTP (3,3 µl, 50 ng), 12 µl deionized water, 2 µl T4 DNA ligase buffer (Roche), 1 µl T4 DNA ligase and 1 µl ATP (10 mM), O/N at 16ºC. The end product was also sequenced.

Table 2 - DNA sequence of shRNAs

Target	Sense	Anti-sense
CXCL12	tcccCGTCAGCCTGAGCTACAGATTCAAG AGATCTGTAGCTCAGGCTGACGttttttc	TCGAgaaaaaCGTCAGCCTGAGCTACAGAT CTCTTGAATCTGTAGCTCAGGCTGACG
IL-7	tcccAAGGTATCAGTTGCAATAATTCAAG AGATTATTGCAACTGATACCTTttttttc	TCGAgaaaaaAAGGTATCAGTTGCAATAATC TCTTGAATTATTGCAACTGATACCTT
TSLP	tcccCTATTCGGAAACTCAGATATTCAAG AGATATCTGAGTTTCCGAATAGttttttc	TCGAgaaaaaCTATTCGGAAACTCAGATATC TCTTGAATATCTGAGTTTCCGAATAG
VCAM-1	tcccTGGAGATAGACTTACTGAATTCAAG AGATTCAGTAAGTCTATCTCCAttttttc	TCGAgaaaaaTGGAGATAGACTTACTGAAT CTCTTGAATTCAGTAAGTCTATCTCCA

To generate BM MSCs knocked out for the factors of interest, the CRISPR/Cas9 system was used. The backbone vector pRP-418 was digested with *EspEI* (Thermo scientific) at 37ºC O/N by Ester Rieter (UMC Utrecht). Oligonucleotides containing the target sequence (see Table 3) were annealed as described above. Then, annealed oligonucleotides (0.52ng, 1:1000 dilution) were cloned in a 1:5 ration into the digested pRP-418 vector (28,7 ng/µl, 2 µl), 12 µl deionized water, 2 µl T4 DNA ligase buffer (Roche), 1 µl T4 DNA ligase and 1 µl ATP (10 mM), O/N at 16ºC. Following bacteria transformation with the ligated vectors, colonies were picked and plasmid DNA was isolated. Positive colonies were sequenced.

Table 3 - DNA sequence of CRISPR oligonucleotides

Target	Oligonucleotide
CXCL12_1	accgACCTTGGCGTTCATGGCGC
CXCL12_2	accgAGATGCTTGACGTTGGCTC
VCAM-1_1	accgGGAAGATGGTCGTGATCCT
VCAM-1_2	accgACCAAGACGGTTGTATCTC
VCAM-1_3	accgAGGCTGTAGCTCCCCGTTA
CD44_1*	TGGAAAGGACGAAACACCGCGCCAGGCTCAGCGGCACGGTTTTAGAGCTAGAA
CD44_2*	TGGAAAGGACGAAACACCGGCACTCACCGATCTGCGCCGTTTTAGAGCTAGAA
CD44_3*	TGGAAAGGACGAAACACCGGGACTGTCTTCGTCTGGGAGTTTTAGAGCTAGAA

** CD44 CRISPR lentivirus media were kindly provided by Robert Jan Lebbink (Emmanuel Wiertz lab, UMCU) and a different method for generating the KO vectors was used (Robert Jan Lebbink, unpublished).*

2.11. Production of Lentivirus

HEK293T cells (1,5x10^6 cells per 9cm culture dish) were seeded in DMEM (Invitrogen) with 10% FBS, 1% Penicillin/Streptomycin (Invitrogen). Next day, the medium was replaced and cells were transfected with a mixture of 2µg shRNA previously generated, 1,2 µg pHDM-HgpM2, 1,2 µg pRC-CMV-Rev1b, 1,2 µg pHDM-tat1b and 2,4 µg pHDMG-G with Poly(ethylenimine) (6µg/ml, PEI, Polysciences), which had been incubated for 15min at RT. After 24h the medium was refreshed. At 2 and 3 days post transfection the medium was collected, filtered and then stored at -80ºC (lentivirus medium).

2.12. Transduction of MSCs with Lentivirus

Low passage D06 BM MSCs were plated in a 6 well plate (25000 cells or 50000 cells per well). After 24h, the medium was replaced with 2 ml MSC medium, 1 ml of lentivirus medium and Polybrene (6µg/ml, Sigma Aldrich). The next day the medium was refreshed. Three days after transduction the medium was refreshed again and Puromycin (Sigma) was added (2µg/ml). Six days post transduction, the medium was refreshed.

2.13. Transfection of MSCs using Neon Electroporation

Transfection of D06 with a small vector (4,700bp) encoding GFP (sGFP), a big (9,289bp) GFP vector (bGFP), VCAM-1 tg1 and tg3 and CD44 tg2 CRISPR vectors was performed using the Neon® Transfection System (Life Technologies) according to the manufacturer's instructions. Briefly, 0.5x10^6 cells were resuspended in 100µl Neon® Buffer R (Life Technologies) and 1µg of the plasmid was added. The samples were aspirated into the Neon® Pipette. Cells were electroporated in Neon® 100µl tips using the following settings: (A) 1600 V, 20 ms, 1 pulses; (B) 1000 V, 40 ms, 1 pulse or (C) 1400 V, 10 ms, 2 pulses. Immediately after electroporation, cells were plated in MEM alpha medium supplemented with 10% FBS without antibiotics. After 24h, the medium was replaced with MEM alpha medium supplemented with 10% FBS and 1% Penicillin/Streptomycin (Invitrogen). Two days after transfection, Puromycin (1mg/ml) was added to select the cells that had been transfected with CRISPR vectors. After two days the medium was replaced with regular MSC medium (with PL).

2.14. Generation of humanized bone in Mice

The *in vivo* generation of human bone from MSCs seeded on BCP scaffolds was performed as described previously (Groen et al., 2012). Briefly, to test the ability of constitutive KD MSCs to form bone, 3 BCP scaffolds per mouse were loaded with CXCL12, IL-7 or TSLP constitutive KD BM MSCs (generated by Anne van der Leun) and implanted subcutaneously into 3 RAG2$^{-/-}\gamma_c^{-/-}$ mice. As control, one scaffold was loaded with wild-type (wt) human MSCs and another with MSCs KO with scrambled gene target, per mouse. Eight weeks after implantation, the mice were euthanized and scaffolds were isolated from the skin of the mice and fixed in 4% formaldehyde for 24h. After one week incubation in 12.5% EDTA 1,25% NaOH pH7 to decalcify and overnight (O/N) incubation in 70% EtOH, the scaffolds were embedded in paraffin and placed into blocks. To test if the indKD MSCs were able to form bone, scaffolds seeded with Scr1, VCAM-1 or CXCL12 indKD MSCs were implanted in 6 mice. Six weeks after implantation, three of the mice were injected intraperitoneally twice per week with DOX (125 µg/mouse) and DOX (1mg/ml) was also added to their water, containing 5% sucrose (Reijmers et al., 2010). Two weeks after, the mice were sacrificed. Half of the scaffolds were isolated and embedded in paraffin as described above. The other half were treated with Collagenase D (Roche), stained with Tra-1-85-FITC and FACS sorted according to the expression of human TRA-1-85 antigen, a pan-human antigen. Total RNA was extracted from these cells. In addition, VCAM-1 expression in VCAM-1 indKD cells was determined by flow cytometry. All the *in vivo* experiments were performed together with Anita Govers, Paul Coffer's group, UMC Utrecht.

2.15. Hematoxilin and Eosin Staining of Mice Scaffolds

Sections were cut and stained with Hematoxilin and Eosin (H&E) (Klinipath). Briefly, following xylene and decreasingly graded alcohol solutions, sections were

counterstained with hematoxylin, washed, incubated in eosin. They were subsequently dehydrated through graded alcohol, cleared in xylene, and covered with a coverslip.

2.16. Flow Cytometry and Cell Sorting

In order to analyse receptor downregulation, CALL4 cells were incubated O/N at 37ºC 5% CO2 with the respective ligand and then analysed using antibodies against human CXCR4 (CXCR4-PE, BD Bioscience), IL-7Rα (IL-7R-PE, BD Bioscience) or TSLPR (TSLPR-PerCP, eBioscience). Sytox Blue Dead Cell Stain (Invitrogen) was used to exclude dead cells. Analysis of the expression of VCAM-1 in both indKD and KO MSCs (40,000 cells) cultured during 3 to 7 days was performed after 12-16h incubation at 37ºC 5% CO2 with TNFα (10ng/ml) using antibody directed against VCAM-1 (VCAM-1-Pe, eBioscience). To determine if contact with B-ALL cells leads to VCAM-1 upregulation on MSCs, Scr1 and VCAM-1 ind KD MSCs (320,000 cells) that had been cultured for 3 days in the presence of DOX (0.25 ng/ml), were co-cultured (3300 MSCs) with NALM-6-Luc-mCherry-Puro (3,75x10^4 cells/ml) and then analysed using VCAM-1-Pe (eBioscience). In addition, MSCs were cultured in conditioned medium from B-ALL cells that was harvested from NALM-6-Luc-mCherry-Puro (0.6x10^6 cells/ml) that had been cultured for one day in RPMI medium supplemented with 1% Penicillin/Streptomycin (Invitrogen) and 5µg/ml ITS (Invitrogen). All acquisitions were performed on a FACSCanto II or a FACS LSRII flow cytometers with FACSDiva Version 6.1.3 software (BD Biosciences). The data obtained was analysed by FlowJo.

With the aim of obtaining single clones from BM MSCs, wt MSCs (D06 and D99) or Scr1 and VCAM-1 KO MSCs that had been incubated during 12-16h at 37ºC 5% CO2 with TNFα (10ng/ml), when indicated, were sorted into 96 well plates (1 cell per well, 48 or 95 wells per cell type) using a FACS Aria II cytometer with FACSDiva Version 6.1.3 software (BD Biosciences). The sorting of VCAM-1 KO MSCs was based on their VCAM-

1 expression which was analysed using antibody directed against VCAM-1(VCAM-1-Pe, eBioscience).

2.17. Statistical Analysis

Data is presented as mean ± standard deviation (SD) when two or three independent experiments were performed. As indicated, in some cases experiments were only performed once, in which case the data is presented as mean ± SD of duplicates or triplicates. Non-parametric Mann-Whitney two-tailed test was used when three independent experiments were performed. GraphPad Prism 5 was used for these tests/calculations.

Chapter 3 Results

3. Results

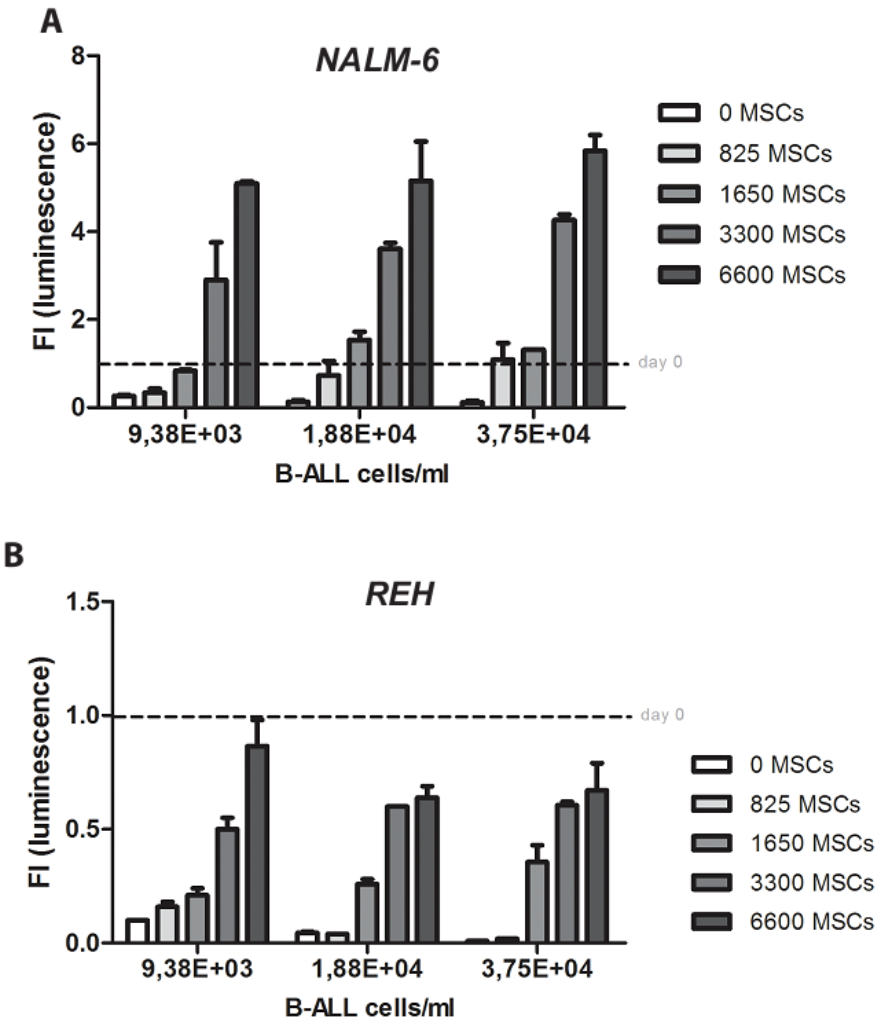
3.1. The role of specific stromal-derived factors in the growth and survival of B-ALL cells *in vitro* and *in vivo*

As discussed, the crucial role of BM stromal cells in the growth and survival of B-ALL cells has been demonstrated (Bradstock et al., 1996; Gluck et al., 1989; Umiel et al., 1986), particularly when these cells are maintained in direct contact (Manabe et al., 1994). This supports the idea that there may be certain molecules such as cytokines, chemokines and adhesion factors, which are responsible for this enhanced survival and proliferation of cancer cells. Therefore, a better understanding of which stroma-derived factors are responsible for this effect and hence a better knowledge of the biology of leukemic BM can lead to a considerable improvement in therapeutics.

3.1.1. BM MSCs support expansion of B-ALL cell line NALM-6

First, we wanted to determine if the BM stromal cells that we had obtained from a healthy donor (D06) were able to support the proliferation of B-ALL cell lines. In order to do so, the luciferase-containing B-ALL cell lines NALM-6-Luc-mCherry-Puro and REH-Luc-mCherry-Puro were cultured in the presence of different numbers of MSCs. The expansion of the B-ALL cells was monitored by adding luciferine upon which luciferase converts it in a reaction that emits light. By quantifying the luminescent signal obtained after three days and comparing it to the signal of the first day, we calculated the relative growth (fold induction) and observed that NALM-6 cells expanded when in co-culture with BM MSCs (Figure 7a). Under the conditions used, this B-ALL cell line was unable to survive in the absence of MSCs. With the lowest amount of MSCs, MSCs induced survival of NALM-6 rather than expansion. The expansion effect due to the contact with MSCs was enhanced with increasing numbers of MSCs, reaching up to four (1650

MSCs) or six (3300) times the initial number of NALM-6 cells. On the other hand, the amount of NALM-6 cells had only slight effects on their survival/expansion, especially in the presence of no or low amounts of MSCs. In contrast to NALM-6 cells, REH cells were not able to proliferate even in the presence of MSCs (Figure 7B). However, MSCs improved the survival of these B-ALL cells, especially when higher amounts of MSCs were used in the co-cultures. Furthermore, for both cells lines, the support of BM MSCs was lost not only when higher amounts of either MSCs or leukemic cells were cultured but also if other MSCs:B-ALL cells ratios were used (data not shown). Together these results, confirm the ability of BM MSCs to induce the survival and, in case of NALM-6 cell line, also the expansion of B-ALL cells.



(previous page) **Figure 7 - BM MSCs support expansion of B-ALL cell line NALM-6 *in vitro*.** NALM-6-Luc-mCherry-Puro cells (NALM-6) (A) and REH-Luc-mCherry-Puro (REH) (B) were cultured for 3 days at the indicated concentrations on top of different numbers of D06 BM MSCs. Fold induction (FI) of the luminescence as compared to the luminescence obtained on day 0 for each concentration of B-ALL cells. Results represent two independent experiments.

3.1.2. Responsiveness of B-ALL cell lines to CXCL12 and IL-7, but not to TSLP

Many BM-derived stromal factors have been described to play a role in B-ALL growth (Tabe and Konopleva, 2014). However, these conclusions were mainly drawn based on *in vitro* studies, which have many limitations, or *in vivo* experiments where the human BM microenvironment was not properly simulated. The overall aim of our study is to determine if certain stromal factors are indeed important for B-ALL growth using a novel *in vivo* model that allows the generation of an ectopic human niche in immunodeficient mice. We have focused on studying the role of CXCL12, IL-7 and VCAM-1, factors that have been demonstrated to be critical in the interaction between BM MSCs and B-ALL cells (Bradstock et al., 2000; Filshie et al., 1998; Hall et al., 2004a; Juarez et al., 2007a; Rich et al., 1993; Sipkins et al., 2005b). In addition, due to the similar biological function as IL-7 and lack of appropriate studies on the role of TSLP in B-ALL growth, we decided to also take this cytokine along (Brown et al., 2007). Our first goal was to confirm previous literature that *in vitro* B-ALL cell lines are responsive to the selected factors and that these factors affect the growth (IL-7 and TSLP) or chemotaxis (CXCL12) of B-ALL cells. In addition, these assays were also developed to functionally validate the genetically manipulated MSCs (see below).

As a first test of responsiveness, we used flow cytometry to analyse the levels of expression of the receptors for the ligands of interest in the B-ALL cell lines CALL4 and NALM-6-Luc-mCherry-Puro upon overnight exposure to the respective ligands. Since

receptors are internalized upon interaction with their respective ligand (Henriques et al., 2010; Neel et al., 2005), this allows us to determine the responsiveness of B-ALL cells for IL-7, CXCL12 and TSLP by quantifying the expression levels of their receptors, IL-7Rα, CXCR4 and TSLPRα (TSLPR), respectively. The expression of IL-7Rα and CXCR4 in CALL4 cells decreased after overnight treatment with IL-7 and CXCL12, respectively, confirming their sensitivity to these stromal factors (Figure 8). The heterodimeric TSLP receptor is comprised of TSLPRα (here mentioned as TSLPR) and IL-7Rα (He and Geha, 2010). Overnight incubation with TSLP induced downregulation of IL-7Rα in CALL4, but not of TSLPR. Similar results were obtained with overnight stimulation of the NALM-6 cell line (data not shown).

To determine whether the induction of receptor downregulation also led to a biological significant response, we analysed B-ALL growth when cells were treated with IL-7 or TSLP and migration of B-ALL cells towards CXCL12. CXCL12-induced leukemic cell expansion was analysed, but CXCL12 didn't stimulate expansion of B-ALL cells (data not shown). The chemotaxis effect of CXCL12 was determined using a transwell assay in which migration of NALM-6-Luc-mCherry-Puro cells was analysed using luciferine-induced luminescence. The results are represented as percentage of luminescence relative to luminescence of the initial number of B-ALL cells added. CXCL12 in the lower chamber induced an increase of approximately 14% in the migration of B-ALL cells, in comparison to the cells that already migrated towards MSC medium (2,3%, Figure 9A). This effect was abrogated when AMD3100, an inhibitor of CXCR4 signalling, was added in combination with CXCL12, indicating that the chemotaxis observed is dependent on CXCL12. Quantification of migrated cells by flow cytometry confirmed these results (data not shown).

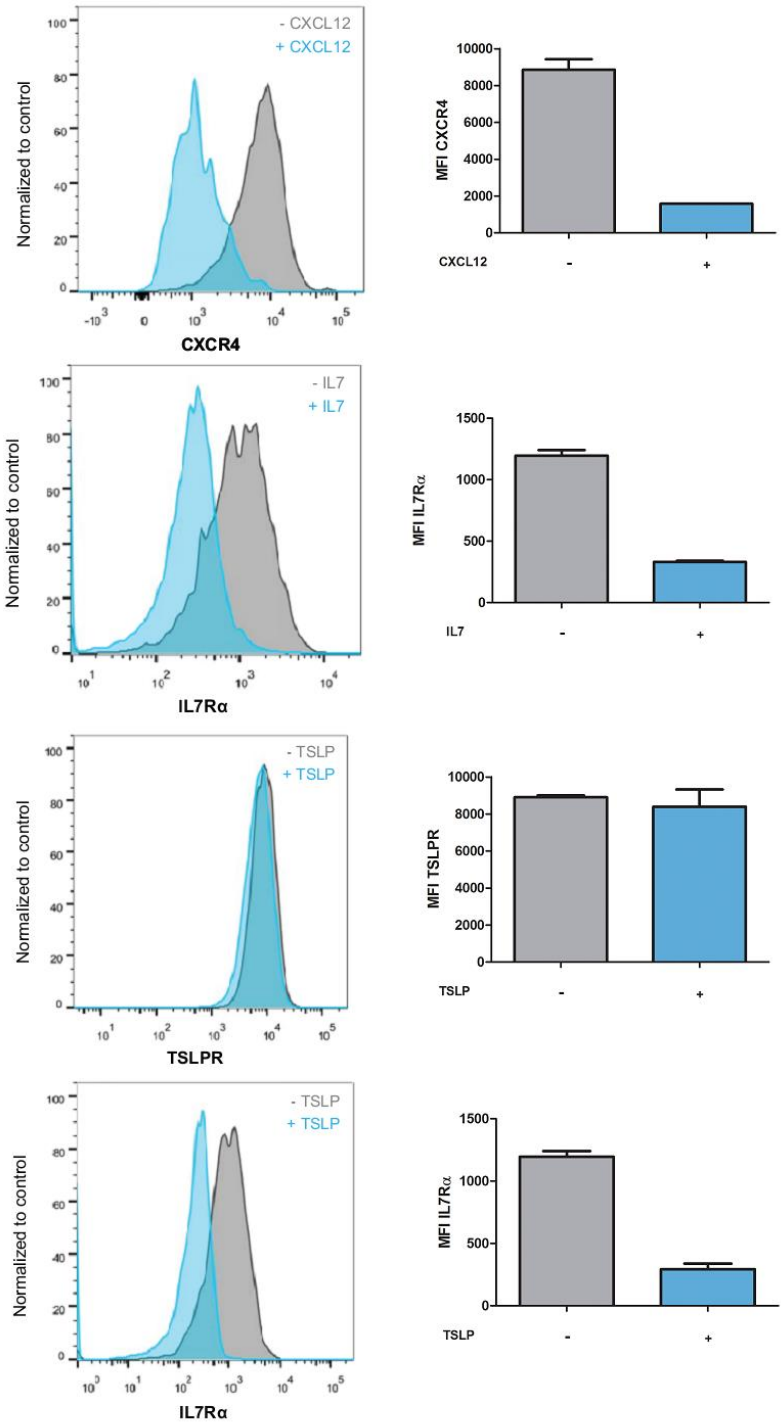


Figure 8 – B-ALL cell line CALL4 respond to CXCL12, IL-7 and TSLP stimulation. B-ALL cell line MHH-CALL4 (CALL4, 6x10^5 cells/ml) was treated overnight with 100 ng/ml IL-7, CXCL12 or TSLP and the expression of the respective receptors (IL-7Ralpha, CXCR4 and TSLPR) was analysed by flow cytometry. Untreated cells are shown in grey and ligand-treated cells in blue. Graphs represent the mean fluorescence intensity (MFI) of duplicates of the same experiment. Results are representative of two independent experiments.

For testing the effect of IL-7 and TSLP on B-ALL growth, we used NALM-6-Luc-mCherry-Puro and CALL4 cells. In line with the results in Figure 7A, at the cell concentrations used NALM-6 cells that were cultured in the absence MSCs or without addition of a growth factor were not able to survive (Figure 9B). However, addition of IL-7 (100ng/ml) led to their survival and caused some slight expansion (2.3 ± 1.8 fold). This effect of IL-7 was even enhanced when the B-ALL cells were co-cultured with BM MSCs (11.5 fold, 3300 MSCs). Similar results were obtained with another B-ALL cell line (REH-Luc-mCherry-Puro, data not shown). To determine the effect of TSLP on the growth of B-ALL cells we used a B-ALL cell line CALL4 that previously had been described to be sensitive for TSLP (Tasian et al., 2012). Since these cells, in contrast to NALM-6 and REH, had not been transduced with Luciferase, we used Presto Blue® as a means to determine the amount of viable cells. In contrast to Tasian and colleagues, who observed a significant increase in the number of CALL4 even upon addition of only 10 ng/ml of TSLP (Tasian et al., 2012), we did not observe significant expansion of CALL-4 cells upon stimulation with TSLP (Figure 9C and data not shown). Even when using different initial amounts of B-ALL cells (0.3; 0.6 and 0.9x10^6 cells/ml), RPMI medium with different percentages of FBS (0, 10 and 20%), other B-ALL cell lines (NALM-6-Luc-mCherry-Puro and RS4;11) and different time points (3 and 6 days) (data not shown) we did not observe any expansion of B-ALL cells upon adding either 10 or 100 ng/ml of TSLP.

Although we were not able to demonstrate that TSLP induces growth of CALL4 cells and some of assays above were only performed once, these data confirm previous reports that B-ALL cells are responsive to both IL-7 and CXCL12 (Juarez et al., 2003, 2007a; Mowafi et al., 2008; Sbaa-Ketata et al., 2001).

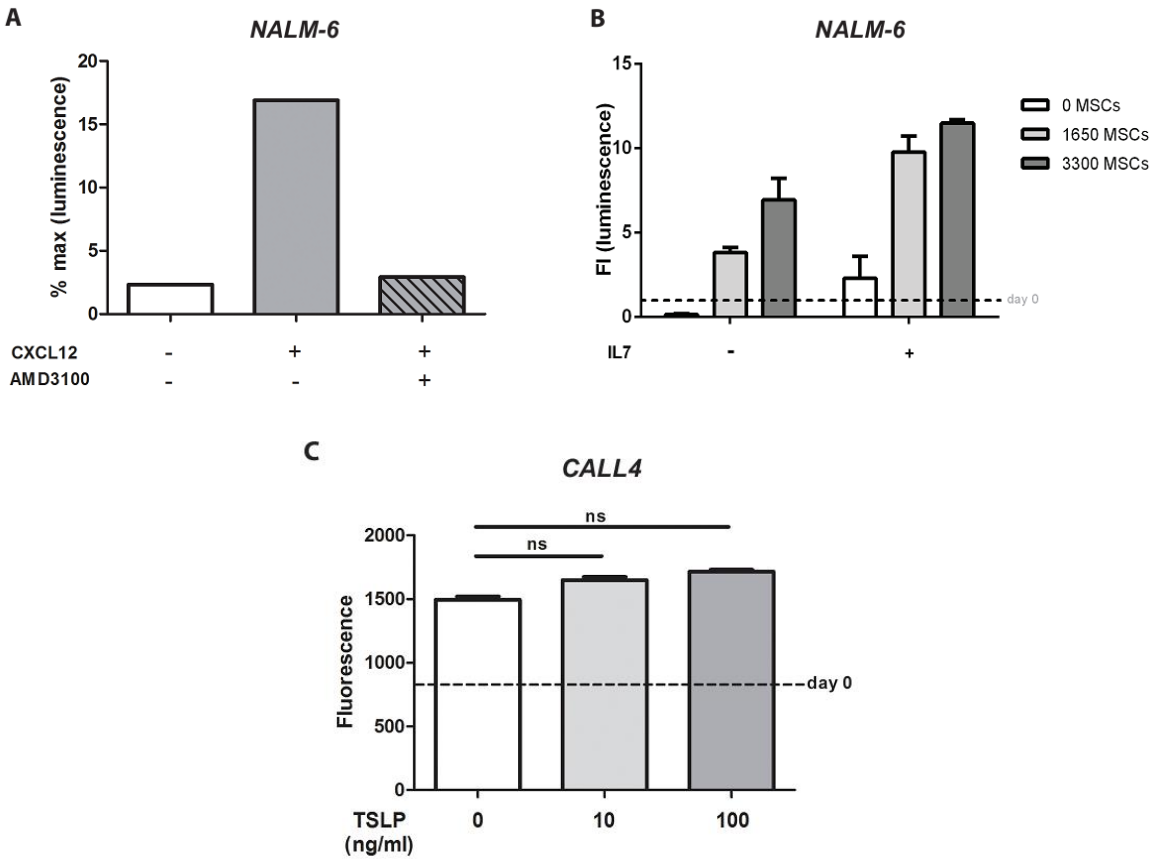
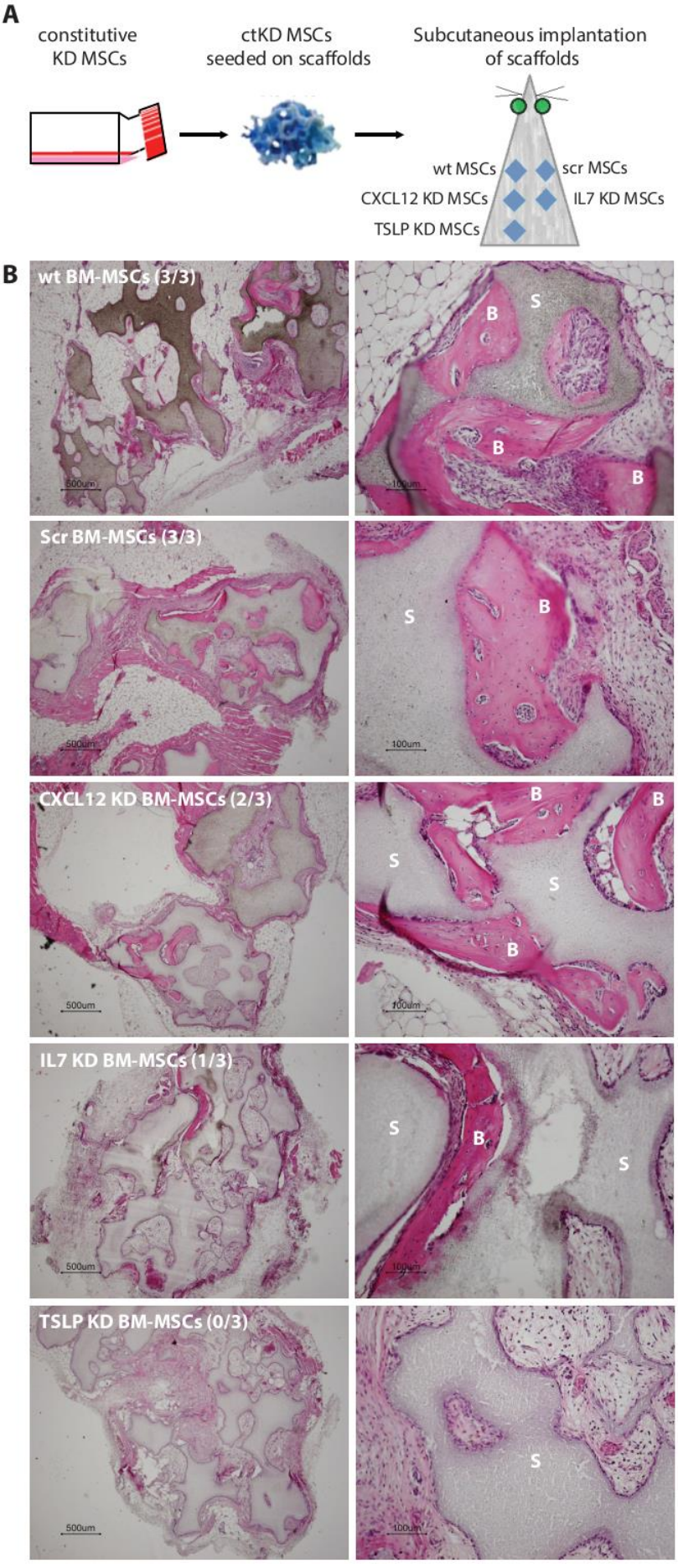


Figure 9 - B-ALL cell lines are functionally responsive to IL-7 and CXCL12, but not to TSLP. (A) *Migration of NALM-6-Luc-GFP cells (NALM-6) in response to CXCL12 (100 ng/ml) within 3h at 37°C incubation*. The results are normalized to luminescence of a well in which the cells (5x10^5 cells) were directly placed in the lower well (i.e. 100% migration). AMD3100 (10 µg/ml) is a drug that blocks CXCR4 signalling. Values represent one independent experiment. (B) *Expansion of B-ALL cell line NALM-6-Luc-mCherry-Puro (NALM-6) after treatment with IL-7*. B-ALL cell line NALM-6-Luc-mCherry-Puro (3X10^4 cells/ml) was cultured in the presence of different amounts of BM MSCs, with or without IL-7 (100 ng/ml) during three days. Luminescence was analysed and fold induction (FI) in relation to luminescence on day 0 was calculated. Results represent duplicates of one experiment. (C) *Growth of CALL4 cells (6x10^5 cells/ml) after treatment with different concentrations of TSLP for 3 days.* The amount of viable cells was determined by measuring the fluorescence obtained using PrestoBlue® Cell Viability Reagent. ns = non-significant ($p > .05$), Mann-Whitney two-tailed test. Results represent the average of three independent experiments.

3.1.3. Constitutive KD CXCL12 BM-MSCs are able to form bone *in vivo*

As the factors of our interest are produced by BM MSCs, we could nicely and more accurately determine their biological importance by decreasing or ablating their gene expression in BM MSCs. Therefore, we previously generated constitutive (ct) knock down (KD) BM MSCs for CXCL12, IL-7 and TSLP and validated successful knock down using qRT PCR (for all three factors) and ELISA (for CXCL12) (Anne van der Leun, unpublished). A prerequisite for use of these cells to allow study of the *in vivo* role of these factors in the homing, engraftment and growth of B-ALL cells is that they should allow generation of an ectopic human BM microenvironment under the skin of immunodeficient mice.

In order to test that, ctKD MSCs were seeded onto scaffolds, implanted into mice and tested for bone forming capacity by staining paraffin embedded tissue sections with Haematoxylin & Eosin (H&E) staining (Figure 10A). Not only wild type (wt) MSCs were able to form bone, but also Scr ctKD MSCs maintained this ability. In two out of three scaffolds seeded with CXCL12 ctKD MSCs, MSCs were still able to form bone to a similar extent as the controls. The IL-7 ctKD MSCs showed only modest bone formation in one out of the three mice, and TSLP KD MSCs were unable to form bone in any of the mice. (Figure 10B). Constitutive VCAM-1 KD BM MSCs were also unable to form bone *in vivo* (data not shown; by Jessica Sigmans and Regina de Jong). Taken together, these results show that Scr and CXCL12 KD MSCs were able to form bone *in vivo,* indicating that on itself lentiviral infection does not affect the bone forming capacity of MSCs. Unfortunately TSLP, VCAM-1 or IL-7 KD MSCs lack *in vivo* bone forming ability, indicating that depending on the gene that is being knocked down, MSCs will either keep or lose their bone forming ability.


A
constitutive
KD MSCs
ctKD MSCs
seeded on scaffolds
Subcutaneous implantation
of scaffolds
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CXCL12 KD MSCs
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TSLP KD MSCs
B
wt BM-MSCs (3/3)
Scr BM-MSCs (3/3)
CXCL12 KD BM-MSCs (2/3)
IL7 KD BM-MSCs (1/3)
TSLP KD BM-MSCs (0/3)
B
S
500um
100um

(previous page) **Figure 10 – CXCL12 constitutive KD MSCs are able to form bone *in vivo*.** (A) Schematic representation of the scaffold model. RAG2$^{-/-}$γc$^{-/-}$ mice were implanted with wild type (wt), Scrambled (Scr) or CXCL12, IL-7 or TSLP constitutive (ct) KD MSC-loaded BCP scaffolds. (B) Representative images of Hematoxylin & Eosin staining of the scaffolds at eight weeks post implantation. Results are representative of three scaffolds each implanted in different mice. S = scaffold; B = bone.

3.1.4. Generation of inducible KD MSCs

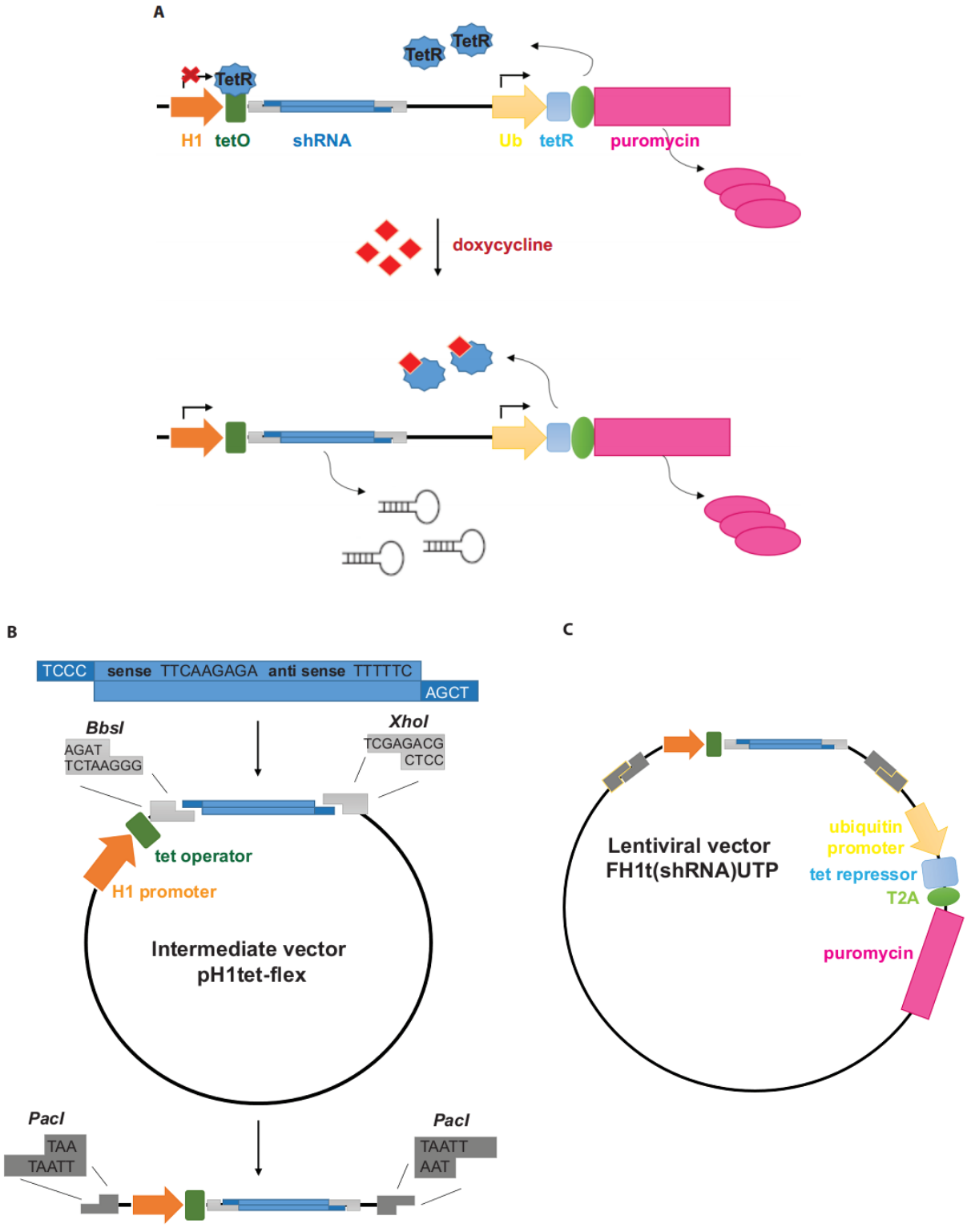
One possible reason for the inability of the constitutive KD MSCs to form bone might be that the factors that are knocked down are crucial for the process of bone formation. To overcome this problem we used a system to knock down these factors in an inducible way, which should allow us to first form bone and only then induce knock down of the respective genes.

3.1.4.1. Cloning of the inducible KD vectors

We have used the pH1tet-flex/FH1t(INSR)UTP vector system that allows inducible knock down of gene expression by short hairpin RNAs (shRNAs). In this system, the promoter that leads to the transcription of the shRNAs is regulated by DOX using a H1 promoter (H1)-driven tetracycline (tet) operator (tetO) and a Ubiquitin promoter(Ub)-driven tet resistance operon repressor (tetR, Figure 11).

The final lentiviral vector pFH1t(INSR)UTP does neither contain the H1 promoter nor tetO, so we had to clone those sequences, as well as the gene specific shRNAs, into the final vector. For this purpose, we used an intermediary vector pH1tet-flex which contains H1, tetO and cloning sites (BbsI/XhoI) for the insertion of the shRNA-oligos. For the design of the shRNA-oligos we inserted a BbsI overhang and a XhoI recognition site flanking the sense, loop and anti-sense sequences taken from (previously validated)

shRNA vectors of the Mission TRC shRNA library from Sigma (Fig 11B). The H1tet-shRNA cassette was amplified by PCR and digested with PacI to be inserted into PacI-digested pFH1t(INSR)UTP.



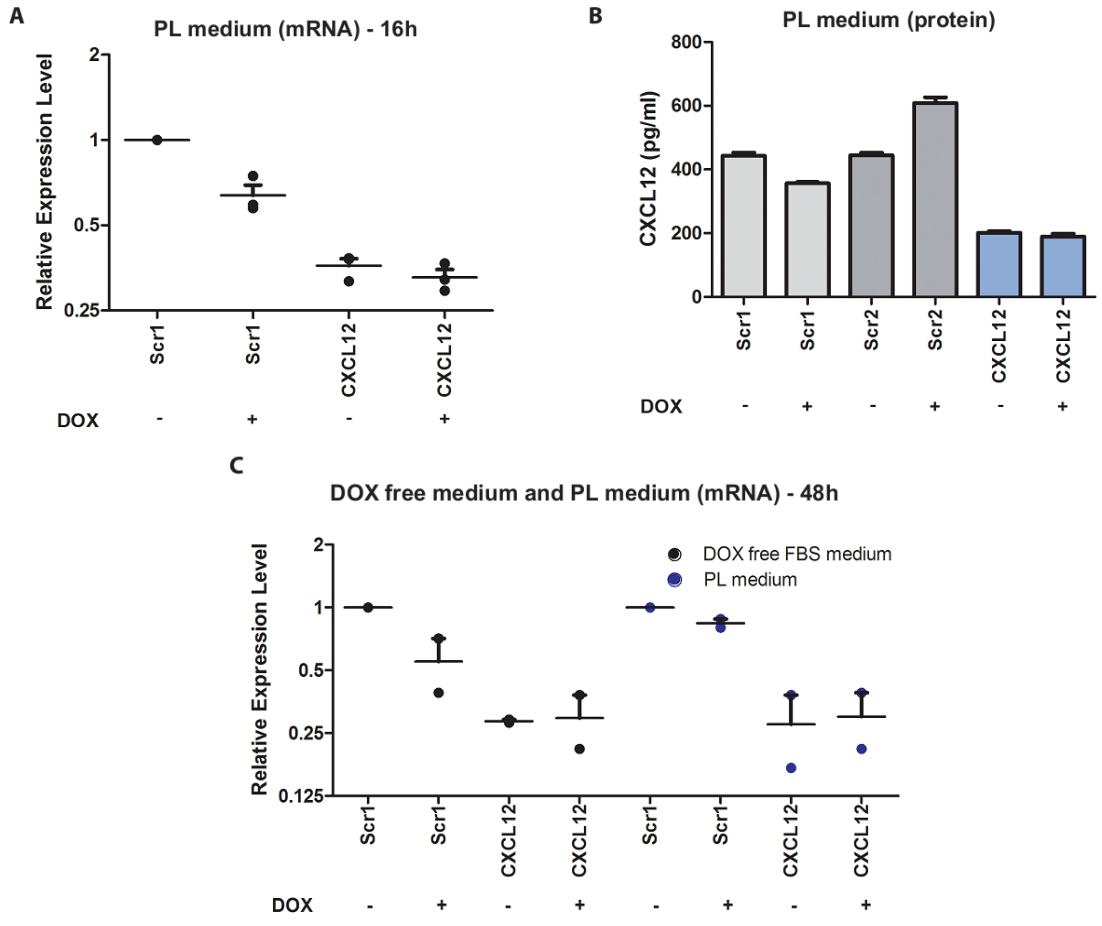
(previous page) **Figure 11 - Schematics and cloning strategy of the inducible knock down system.** (A) Schematic representation of the mechanism of action of the lentiviral vector. Briefly, the human H1 promoter of RNA polymerase III (H1) with a tetracycline (tet) operator (tetO) sequence are upstream to the shRNA. The tetracycline resistance operon repressor (tetR) and puromycin are constitutively transcribed as they are downstream the Ubiquitin promoter (Ub). When tetR binds to tetO, the tetR represses the transcription of the shRNA. Upon addition of the inducer doxycycline (DOX), DOX binds to tetO competing away tetR and leading to transcription of the shRNA (Seibler et al., 2007; van de Wetering et al., 2003). (B) Schematic representation of the cloning strategy to generate inducible KD shRNAs. (C) Main components of the final lentiviral vector FH1t(shRNA)UTP.

3.1.4.2. Validation of CXCL12 inducible KD in BM MSCs

With the generated vectors, we produced lentivirus and transduced BM MSCs that were then selected with Puromycin (CXCL12, VCAM-1 and IL-7 indKD MSCs) or based on GFP expression (Scr KD MSCs).

To evaluate the efficiency of the knock down in CXCL12 indKD MSCs, we performed qRT-PCR to determine the CXCL12 mRNA expression levels and, since CXCL12 is secreted by BM MSCs, ELISA to quantify protein levels. The expression of CXCL12 mRNA in CXCL12 indKD MSCs was substantially decreased in the KD cells in the presence of DOX (approximately 50% in comparison to indScr1 with DOX) (Figure 12A). However, the low expression of CXCL12 was already observed in CXCL12 indKD MSCs without DOX addition. Similar to the mRNA level, the secretion of CXCL12 protein in one of the non-targeting vectors (Scr1) was reduced upon treatment with DOX (20% reduction), whereas the other (Scr2) MSCs showed a higher expression of CXCL12 after DOX treatment (37% increase, not tested at the mRNA level, Figure 12B). Nevertheless, when no DOX was added, both Scr1 and Scr2 had a similar expression of CXCL12 (approximately 440 pg/ml), suggesting that this effect is dependent on DOX and that these cells behave in a similar way before addition of DOX. Since we already observed

CXCL12 downregulation in CXCL12 indKD MSCs without DOX addition (almost 55% reduction when compared to CXCL12 protein levels in Scr1 and Scr2 MSCs in the absence of DOX) similarly to the CXCL12 mRNA levels in the same condition, we hypothesized that there could potentially be leakiness in the inducible system, i.e. that there is a substance present in the culture medium (MSC medium with PL) that already inhibits the binding of tetR to tetO, resulting in the transcription of the shRNAs. To investigate if there was leakiness in the system, we analysed the CXCL12 mRNA expression when the cells were cultured in a medium containing a type of FBS that had been shown by Patrick Derksen's group (UMC Utrecht) to be free of DOX-like substances (DOX free medium). Surprisingly, in this DOX free medium the levels of CXCL12 mRNA were already decreased in untreated CXCL12 indKD MSCs (Figure 12C), similar to the CXCL12 mRNA levels in PL containing medium. No clear differences in CXCL12 mRNA levels were observed for Scr1 indKD MSCs in between the two culture media.



(previous page) **Figure 12 – Validation of BM MSCs with inducible KD of CXCL12.** (A) Scrambled1 (Scr1) and CXCL12 inducible KD MSCs (D06) were cultured for 16h in medium with Platelet Lysate (PL) in the presence of 0.25 ng/ml Doxycycline (DOX). Total RNA was extracted from each condition and CXCL12 mRNA levels were quantified by qRT-PCR. Results are expressed as relative expression to Scr1 without DOX. The results represent one experiment (lines) performed in triplicate (dots). (B) Scr1, Scr2 and inducible KD CXCL12 MSCs were cultured for 7 days in medium with PL in the presence of 0.25 ng/ml DOX. DOX was added every two days. Cells were then cultured (35,000 cells per well in a 24 well plate) for 3 days in the presence of DOX. Cultured medium was collected and CXCL12 expression was quantified by ELISA. The results represent the average of one experiment performed in duplicate. (C) After 48h in the presence of DOX (0.25 ng/ml) in MSC medium with DOX free FBS or PL, total RNA was extracted and CXCL12 mRNA expression was quantified by qRT-PCR. Results are expressed as relative expression to Scr1 without DOX and represent the average (lines) of two independent experiments (dots) each performed in triplicate.

In conclusion, in cells that are lentivirally transduced with shRNAs vectors targeting CXCL12 there is a DOX-independent reduction of CXCL12 both at the mRNA and protein level suggesting that either the DOX free medium is still leaky or that there is an error in the lentiviral vector used for knocking down CXCL12. However, since other inducible shRNA vectors did not show any sign of leakiness in DOX free medium (Derksen et al. unpublished, and see below), an error in the CXCL12 indKD vector is the most plausible explanation.

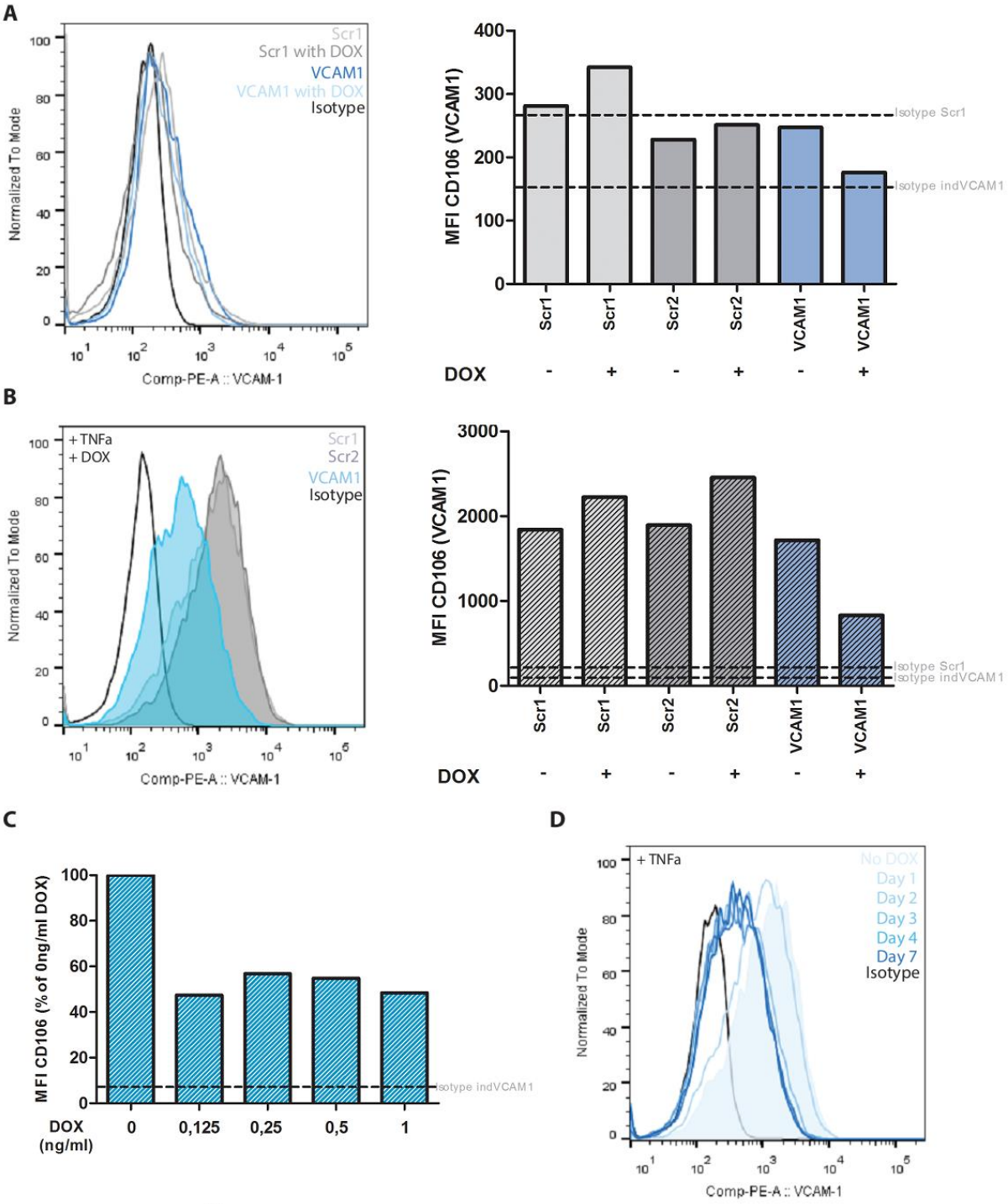
3.1.4.3. Validation of inducible VCAM-1 KD in BM MSCs

To determine if the BM MSCs lentivirally transduced with shRNA targeting VCAM-1 had inducible downregulation of the expression of VCAM-1 protein, we used flow cytometry. We cultured Scr1, Scr2 and VCAM-1 indKD MSCs in the presence of DOX (1 ng/ml) for 3 days, after which we stained the cells with PerCP labelled human anti-CD106 (VCAM-1). In standard culture conditions, the expression of VCAM-1 was very low, being only slightly higher than in the isotype controls (Scr1 and VCAM-1 indKD) or even below

(Scr2) (Figure 13A). However, it was promising to already observe a decrease in the expression of VCAM-1 in VCAM-1 indKD MSCs upon DOX treatment (29% reduction in comparison to untreated VCAM-1 indKD MSCs). To more definitely show DOX-induced VCAM-1 down-regulation, we tested DOX-induced VCAM-1 downregulation in VCAM-1 indKD MSCs that were stimulated with Tumor Necrosis Factor-alpha (TNFα), a cytokine that via the NF-κB signalling pathway induces the up-regulation of VCAM-1 (Uchibori et al., 2012; Xiao et al., 2012). We started by optimizing the concentration of TNFα needed to obtain a maximal upregulation of VCAM-1 by treating wild type MSCs (D06) for 24h with different concentrations of TNFα. The maximum expression of VCAM-1 was already achieved with a dose of 5 ng/ml TNFα and higher concentrations of TNFα (10 ng/ml and 20 ng/ml) induced a similar VCAM-1 up-regulation (data not shown). To assure that the maximal upregulation would always be achieved, we decided to hereafter treat the MSCs with 10 ng/ml TNFα. In addition, we decreased the incubation time, as the maximal upregulation was already observed with 16 to 18 hours of TNFα treatment (data not shown). When Scr1, Scr2 and VCAM-1 indKD were treated with TNFα, addition of DOX (1 ng/ml) resulted in a dramatic reduction (almost 70%) of VCAM-1 expression on VCAM-1 indKD MSCs in comparison to both non-targeting MSCs (Figure 13B). To determine if we could achieve the same reduction of VCAM-1 expression with lower concentrations of DOX and therefore avoid potential secondary effects of DOX addition, we performed a titration of DOX. As depicted in Figure 13C, the lowest concentration of DOX (0.125ng/ml) already induced the maximal downregulation of VCAM-1. In the follow up experiments we used 0.25 ng/ml of DOX to be sure that the maximal decrease of VCAM-1 expression would always be reached. To determine how long it takes to achieve the maximal downregulation of VCAM-1 after DOX addition, we treated both Scr1 and VCAM-1 indKD MSCs with DOX for different periods of time (Figure 13D and 13E). To assure a relatively constant and sufficient level of DOX, additional DOX was added every second day. In contrast to the two previous experiments, the expression of VCAM-1 in DOX-treated Scr1 was slightly decreased (21% reduction). The maximal downregulation

of VCAM-1 expression in VCAM-1 indKD MSCs (65% downregulation) was reached after two days of treatment with DOX and thereafter the expression of VCAM-1 remained stable over time.

To confirm successful VCAM-1 KD as based on flow cytometry, we also analysed the effect of DOX on VCAM-1 mRNA expression levels in VCAM-1 indKD MSCs. Taking into consideration the results obtained with the CXCL12 indKD MSCs (Figure 12), we also wanted to test the optimal medium for culturing the indKD MSCs. Upon DOX treatment, the VCAM-1 mRNA expression was reduced when the MSCs were cultured in either PL-containing MSC medium or in medium with DOX free FBS (Figures 14A and 14B). The DOX-induced downregulation of VCAM-1 in VCAM-1 indKD MSCs was approximately 25 % more efficient when the cells were cultured in DOX free medium than in PL medium (75% vs 50% reduction), especially after 5 days treatment (Figure 14A). These results suggest that there is no leakiness of the inducible system in either DOX free FBS or PL media. Though this experiment was only performed once, culture of VCAM-1 indKD MSCs in MSC medium containing our regular FBS (standard), which had not been previously tested for potential DOX-like activity, there was already knock down of VCAM-1 in the absence of DOX suggesting that a substance that induces the downregulation of VCAM-1 is already present in this FBS. To confirm the effect of different culture media on VCAM-1 protein levels, we used flow cytometry to analyse the VCAM-1 expression on VCAM-1 indKD MSCs that were cultured for 3 days in the different media. In line with what was observed at the mRNA level, there was already downregulation of VCAM-1 in MSCs cultured in MSC medium with standard FBS without DOX addition. Although, this reduction at the protein level was not as dramatic as the downregulation at the mRNA level and the experiment was only performed once, these results suggest that standard FBS-containing medium is probably not the best medium to culture the indKD MSCs cells in. As for the mRNA levels, in the absence of DOX no difference in protein


A
Scr1
Scr1 with DOX
VCAM1
VCAM1 with DOX
Isotype
Normalized To Mode
Comp-PE-A :: VCAM-1
MFI CD106 (VCAM1)
Isotype Scr1
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Scr1
Scr1
Scr2
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+ TNFa
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Isotype Scr1
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0
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D
+ TNFa
No DOX
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Day 2
Day 3
Day 4
Day 7
Isotype
Normalized To Mode
Comp-PE-A :: VCAM-1
E
21% reduction
65% reduction
Scr1
VCAM1
MFI CD106 (VCAM1)
Isotype Scr1
Isotype indVCAM1
DOX (days)

(previous page) **Figure 13 - Validation of BM MSCs with inducible KD for VCAM-1.** Scrambled1 (Scr1), Scrambled2 (Scr2) and VCAM-1 inducible KD MSCs (D06) were cultured in medium with Platelet Lysate (PL) in the presence of the indicated concentrations of Doxycycline (DOX). After three days, cells were collected and expression of VCAM-1 protein on the cell surface was determined by flow cytometry using an anti-human CD106 (VCAM-1) antibody. Cells were treated with TNF-α (10 ng/ml) to induce the VCAM-1 expression when indicated. Graphs represent one experiment. Representative histograms of untreated (A) and TNF-α-treated cells (B) that were treated with 1 ng/ml of Dox. (A) and (B) Graphs represent VCAM-1 mean fluorescence intensities (MFI) of the indicated cells/conditions. Results represent one experiment. (C) VCAM-1 inducible KD MSCs were cultured in the presence of different concentrations of DOX and treated with TNF-α (10 ng/ml). Graphs represent relative VCAM-1 expression on VCAM-1 indKD MSCs. Results represent one experiment. (D) and (E) Kinetics of VCAM-1 expression in VCAM-1 indKD MSCs as above. Cells were cultured for 1-7 days in the presence of DOX (0.25 ng/ml). New DOX was added every two days. Graphs represent histograms (D) and MFIs of one experiment (E).

expression in DOX free medium and PL medium was observed, supporting the idea that there is no leakiness in either. In addition, the knock down of VCAM-1 was more efficient in DOX free medium similar to what we previously observed at the mRNA level.

Taken together, the DOX free medium is the best medium for culturing the VCAM-1 indKD cells since this medium prevents leakiness of the system and allows the highest level of mRNA/protein down-regulation.

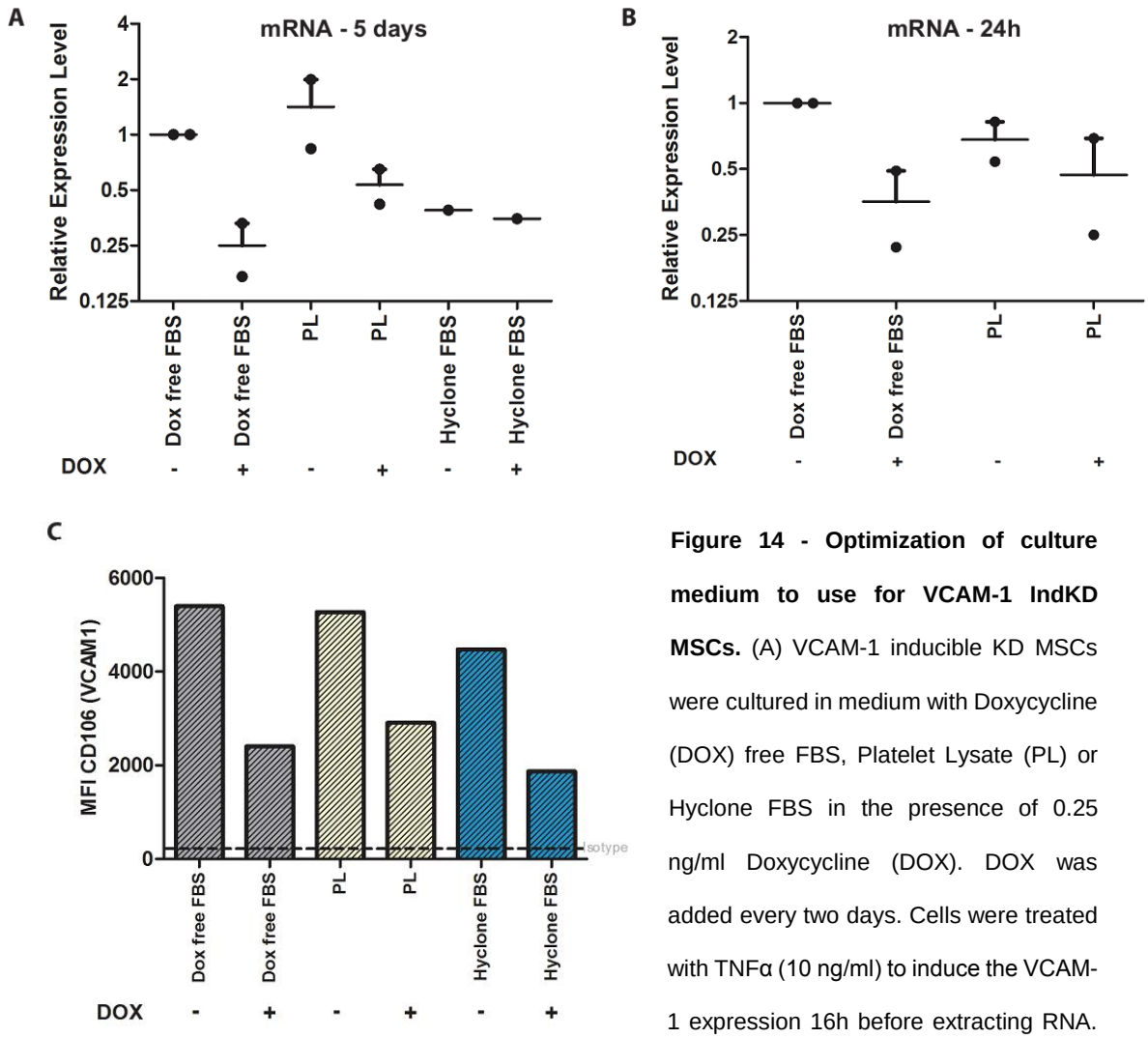


Figure 14 - Optimization of culture medium to use for VCAM-1 IndKD MSCs. (A) VCAM-1 inducible KD MSCs were cultured in medium with Doxycycline (DOX) free FBS, Platelet Lysate (PL) or Hyclone FBS in the presence of 0.25 ng/ml Doxycycline (DOX). DOX was added every two days. Cells were treated with TNFα (10 ng/ml) to induce the VCAM-1 expression 16h before extracting RNA. After five days, total RNA was extracted from each condition and VCAM-1 mRNA levels were quantified by qRT-PCR. Results are expressed as relative expression to DOX free FBS without DOX. Results represent the average (lines) of two experiments (dots) performed in triplicate (for Dox free FBS and PL medium) or of triplicates of one experiment (Hyclone FBS medium). (B) After 24h DOX exposure (0.25 ng/ml) in MSC medium with DOX free FBS or PL, total RNA was extracted and VCAM-1 mRNA expression was quantified by qRT-PCR. TNFα (10 ng/ml) was added 16h before RNA extraction. Results are expressed as relative expression as compared to DOX free FBS without DOX and represent the average (lines) of two independent experiments (dots) performed in triplicate. (C) VCAM-1 indKD cells were cultured in the presence of DOX (0.25 ng/ml) for 3 days in the same conditions as described in (A). Cells were collected and expression of VCAM-1 protein on the cell surface was determined by flow cytometry using an anti-human CD106 (VCAM-1) antibody. The graph represents the mean fluorescence intensity (MFI) of one experiment.

3.1.4.4. Functional validation of inducible VCAM-1 KD in BM MSCs

After validating the VCAM-1 indKD MSCs at the mRNA and protein level, we aimed at analysing the effect of VCAM-1 knock down in a biological context. Recently, it was demonstrated that co-culture of B-ALL cells with MSCs leads to approximately 2.5 fold upregulation of VCAM-1 in the BM MCSs (Jacamo et al., 2014). Furthermore, this signalling pathway was shown to be necessary to induce NF-kB activation and to trigger stroma-mediated chemoresistance. Based on this, we conducted a similar co-culture experiment to determine if there was a difference in B-ALL-induced upregulation of VCAM-1 between VCAM-1 indKD MSCs and Scr indKD MSCs. Scr1 and VCAM-1 indKD MSCs were cultured for 3 days in either the absence or presence of DOX (0.25 ng/ml), after which MSCs were plated in a 6 well plate in the presence or absence of DOX. As positive control, TNFα (20 ng/ml) was added. NALM-6-Luc-mCherry-Puro cells or 24h conditioned medium of this B-ALL cell line were added to the MSCs. 3 days later, the expression of VCAM-1 was analysed using flow cytometry. In line with the results described above, TNFα treatment of both Scr1 and VCAM-1 indKD MSCs cells cultured in DOX resulted in an up to 12 fold increase of the expression of VCAM-1 in the Scr1 MSCs, whereas the VCAM-1 indKD MSCs only showed an 3 fold TNFα-mediated VCAM-1 increase (Figure 15A and 15B). In all conditions and even with both control MSCs, DOX addition slightly decreased the expression of VCAM-1. B-ALL cells induced a slight increase of the expression of VCAM-1, an effect more evident in the absence of DOX. When DOX was added, the expression of VCAM-1 in MSCs co-cultured with B-ALL cells was higher in Scr1 MSCs than in VCAM-1 indKD MSCs (1.62 fold versus 1.09 fold), suggesting that the KD also prevents upregulation of VCAM-1 in conditions in which MSCs are exposed to B-ALL cells. Furthermore, we observed a similar trend upon addition of B-ALL conditioned medium, albeit to a lesser extent, suggesting that there may be certain soluble factors responsible for this upregulation but that cell-cell contact

is also required. In conclusion, though we only performed a pilot experiment and thus it should be repeated, co-culturing B-ALL cells with VCAM1 indKD MSCs to investigate the biological importance of VCAM1 KD appears to be promising. Albeit B-ALL cells do not induce much VCAM-1 expression, we can observe some differences in B-ALL cells-dependent VCAM1 upregulation between Scr1 and indKD VCAM1 MSCs. Moreover, this B-ALL-MSCs interaction may as well influence leukemic cells. Thus, we decided to look as well at the effect of VCAM-1 on B-ALL cells.

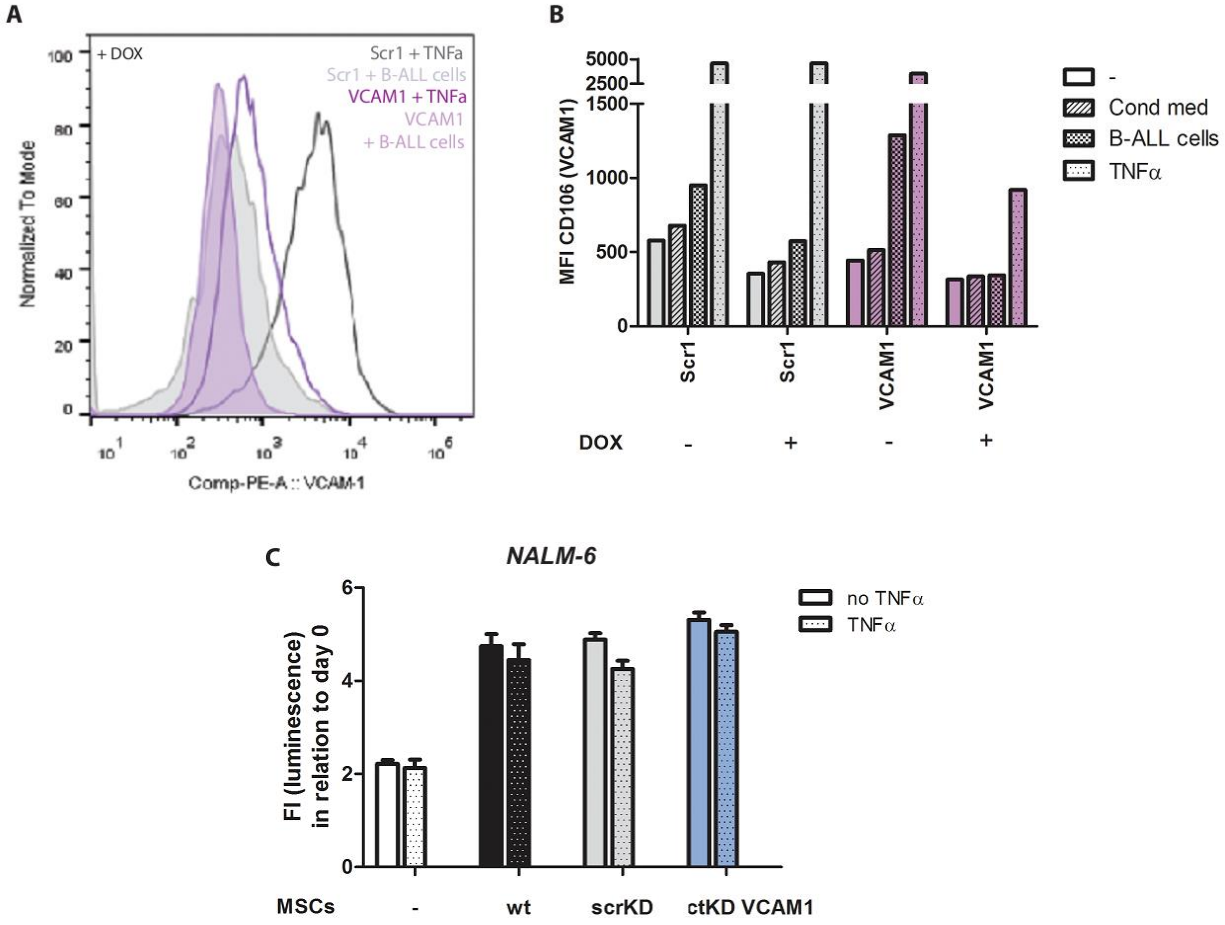


Figure 15 – B-ALL cells induce upregulation of VCAM-1 in BM MSCs. (A) and (B) Scrambled1 (Scr1) and VCAM-1 inducible KD VCAM-1 MSCs were cultured for 3 days in Doxycycline (DOX) free medium in the presence of 0.25 ng/ml DOX. Cells were harvested and plated in a 6 well plate (100000 cells/well) (-), in the presence of TNFα (20ng/ml) (TNFα), B-ALL cell line NALM-6-Luc-mCherry-Puro (57500 cells/well) (B-ALL cells) or with conditioned medium from NALM-6-Luc-mCherry-Puro cells (0.6x10^6 cells/ml) cultured during 24h (Cond med). Cells were also plated in the same conditions in the presence of DOX (0.5ng/ml). After 3 days, cells were collected and expression of VCAM-1 protein on the cell surface was determined by

flow cytometry using an anti-human CD106 (VCAM-1) antibody. Graphs represent one experiment. (A) Representative histograms of TNFα-treated cells and MSCs cultured in the presence of B-ALL cells that were treated with 0.5 ng/ml of DOX. (B) VCAM-1 mean fluorescence intensities (MFI) of the indicated cells/conditions. (C) NALM-6-Luc-mCherry-Puro cells (3,75x10^4 cells/ml) were cultured on top of wild type (wt), Scr and VCAM-1 constitutive KD MSCs. Cells were treated with TNFα (20 ng/ml) to induce the VCAM-1 expression when indicated. Fold induction (FI) of the luminescence as compared to the luminescence obtained on day 0. Results represent duplicates of one independent experiment.

Althogh VCAM-1 has not shown to affect the viability of B-ALL cells (Hsieh et al., 2013), we used a co-culture assay to determine if VCAM-1 had any effect on the expansion of B-ALL cells. As a pilot experiment, we cultured the B-ALL cell line NALM-6-Luc-mCherry-Puro in the presence of VCAM-1 ctKD MSCs (these cells were generated by Jessica Sigmans, Anton Marten's lab, and show a 52% KD at the protein level, data not shown). We used ctKD instead of indKD because the indKD MSCs require the constant presence of DOX in the medium and we first wanted to analyse the effect of VCAM-1 downregulation only. Briefly, NALM-6 cells were co-cultured with wt MSCs, Scr KD MSCs and VCAM-1 ctKD MSCs for 3 days. In some conditions TNFα was added to induce the expression of VCAM-1 and therefore potentially more clearly determine possible differences between the different MSCs. Expansion of B-ALL cells was analysed by adding luciferine and quantifying the luminescence. As previously observed, B-ALL cells expanded more in the presence of MSCs than without the stromal support (Figure 15C). However, there was no obvious difference between the expansion of B-ALL cells when cultured with the different MSCs. In addition, TNFα treatment appeared to have a negative effect in B-ALL expansion. This results suggest that VCAM-1 has no effect on B-ALL growth. Thus, a better read-out will probably be an adhesion assay, as VCAM-1 in an adhesion molecule, which will be described later.

3.1.4.5. Validation of inducible IL-7 KD in BM MSCs

Lastly, we also generated inducible IL-7 KD MSCs. To determine if the DOX-inducible system also allows inducible downregulation of IL-7, IL-7 mRNA expression in IL-7 indKD MSCs was analysed by qRT-PCR. Whereas the IL-7 mRNA levels in Scr1 MSCs didn't vary in the presence of DOX (Figure 16), IL-7 indKD MSCs already presented some downregulation of IL-7 without addition of DOX (approximately 50% downregulation), suggesting some level of leakiness. Importantly, upon addition of DOX these IL-7 indKD MSCs showed a strong decrease in IL-7 expression, which was almost 90% lower than the expression in Scr1 MSCs that were not treated with DOX. Comparing the IL-7 mRNA expression in these MSCs upon culture in MSC medium with PL or with DOX free medium showed a similar level of leakiness and, in contrast to VCAM-1, no improved IL-7 mRNA downregulation in the DOX free medium, suggesting that for these IL-7 IndKD MSCs it did not matter which medium was used.

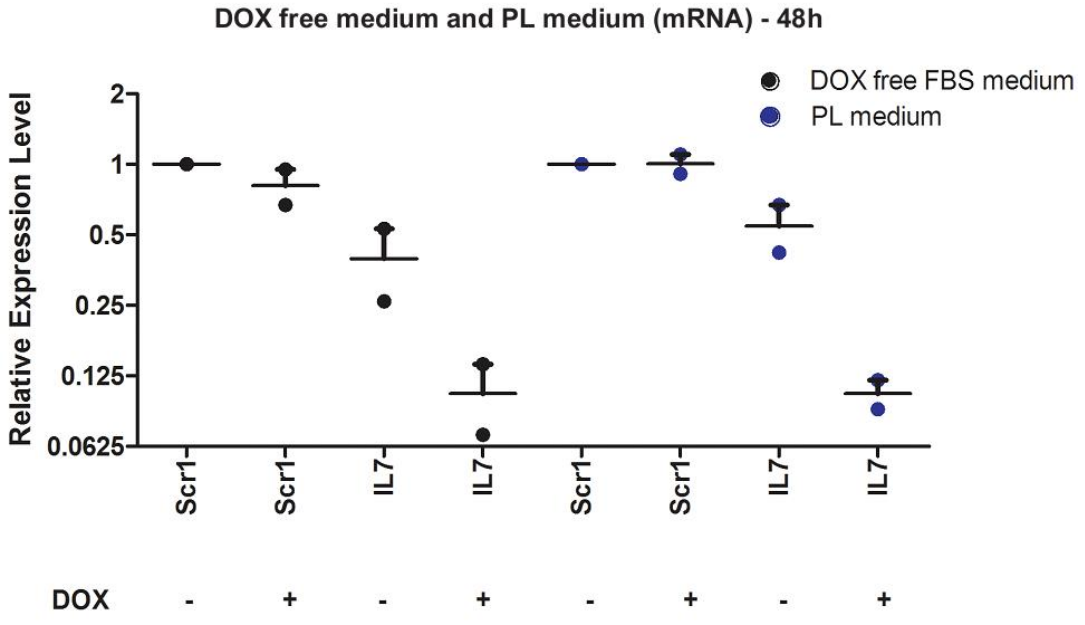


Figure 16 – Validation of BM MSCs with inducible KD of IL-7. Scramble (Scr1) and IL-7 inducible (ind) KD MSCs (D06) were cultured for 48h in medium with Doxycycline (DOX) free FBS or in Platelet Lysate (PL) in the presence of 0.25 ng/ml DOX. Total RNA was extracted from each condition and IL-7 mRNA levels were quantified by qRT-PCR. Results are expressed as relative expression as compared to Scr1 without DOX. Results represent the average (lines) of two independent experiments (dots) performed in triplicate.

As already indicated in Figure 9B in which B-ALL cells expanded more when cultured in the presence of the combination of MSCs and IL-7 than with either alone, and as demonstrated by others (Juarez et al., 2007b), IL-7 has been shown to be a growth factor that stimulates expansion of B-ALL cells. This co-culture system is thus ideal to functionally validate our IL-7 indKD MSCs. For this purpose, we co-cultured B-ALL cell line NALM-6-Luc-mCherry-Puro with wt, Scr1 or IL-7 indKD MSCs, in either the absence or presence of DOX (0.5 ng/ml) and/or IL-7 (100 ng/ml) and after 3 days we measured the relative B-ALL expansion using luciferine. In agreement with the data presented above, B-ALL cells expanded more in the presence of MSCs, an effect which is enhanced in combination with IL-7 (Figure 17). No clear differences were observed between the growth of NALM-6 cell line co-cultured with wt MSCs and with IL-7 indKD MSCs in the absence of DOX (Figure 17A). The presence of DOX had a negative effect on B-ALL growth in all conditions that contained MSCs. In MSCs treated with DOX, only upon addition of IL-7 could we observe an effect of IL-7 indKD MSCs in B-ALL cells' expansion when compared to wt MSCs, as leukemic cells didn't proliferate as much with the first (Figure 17B). When comparing the support of IL-7 indKD MSCs upon addition of DOX, this had a negative effect on the growth of B-ALL cells, slightly overcome by the addition of IL-7 (Figure 17C). However, as a negative effect of DOX was already observed in wt MSCs (Figure 17A) we cannot conclude whether the effect observed in IL-7 indKD MSCs upon DOX addition is solely due to IL-7 downregulation. This experiment should thus be repeated to study whether IL-7 downregulation is relevant for the expansion of leukemic cells *in vitro,* since with this pilot experiment we were not able observe any considerable difference in the abiltity of IL-7 indKD MSCs to support B-ALL cells.

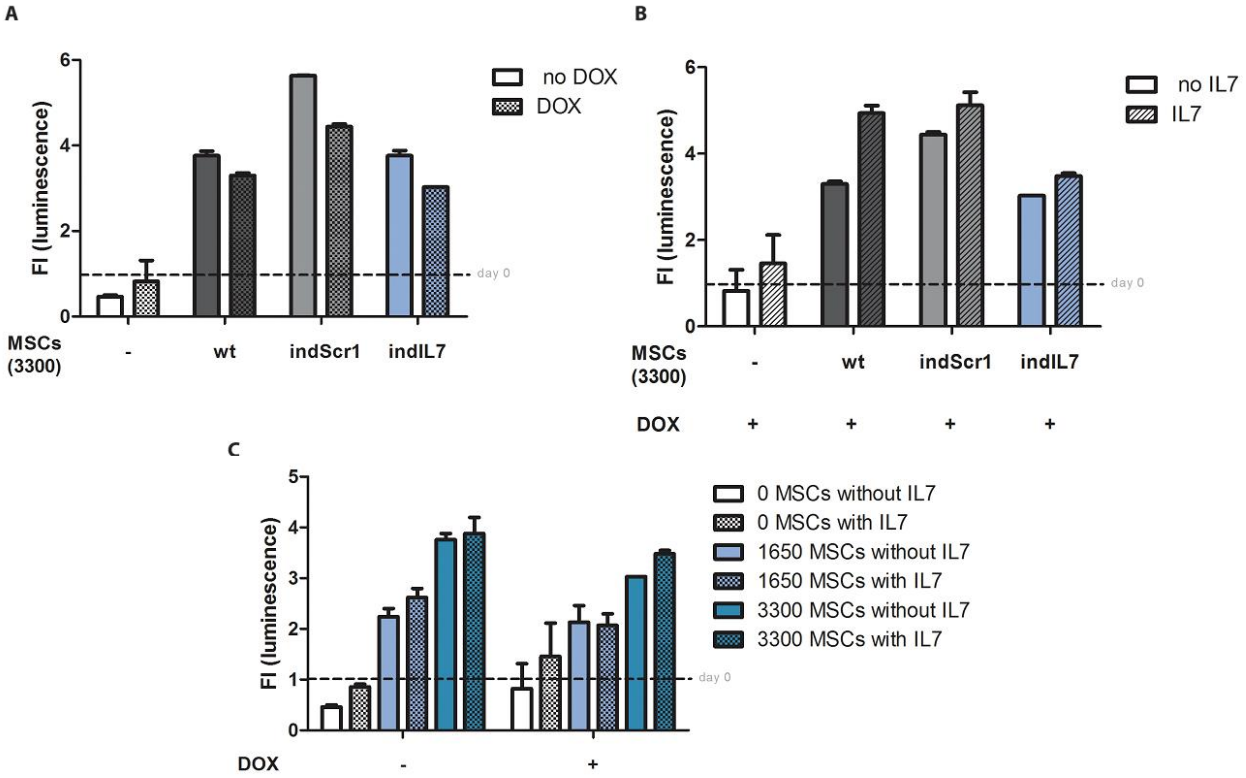


Figure 17 - IL-7 inducible KD MSCs are still able to support expansion of B-ALL cell line NALM-6 *in vitro*. B-ALL cell line NALM-6-Luc-mCherry-Puro (3,75x10^4 cells/ml) was cultured in the presence of different amounts of wild-type (wt), Scr1 or IL-7 indKD MSCs; with or without IL-7 (100 ng/ml) and in the presence or absence of DOX (0.5 ng/ml) for three days. Luminescence was measured and fold induction (FI) in relation to luminescence on day 0 was calculated. Graphs represent the effect of DOX (A), IL-7 in DOX-treated conditions (B) and the effect of IL-7, DOX and different amounts of IL-7 indKD MSCs (C) on B-ALL expansion. Results represent one independent experiment performed in duplicate.

3.1.5. *In vivo* bone formation capacity of inducible KD MSCs

Now that we have generated MSCs with inducible KD for VCAM-1 and IL-7 and validated successful KD, it is necessary to test if they didn't lose their ability to differentiate towards osteoblastic lineage *in vivo* after the genetic manipulation. If they are able to form bone *in vivo* we can use them to answer our main question: whether these stromal factors are crucial for the growth of B-ALL in a humanized niche *in vivo*.

To determine if the ind KD BM MSCs maintained their capacity to form bone *in vivo*, Scr1, VCAM-1 and CXCL12 indKD MSCs were seeded into scaffolds. IL-7 indKD MSCs were not taken along because their knock down hadn't been validated yet when we started the experiment. On the other hand, scaffolds seeded with CXCL12 indKD MSCs were implanted in mice since by the time we began this experiment we were thinking that the KD was working and the reason why CXCL12 was already downregulated without DOX addition was because of leakiness in the medium in which cells were cultured.

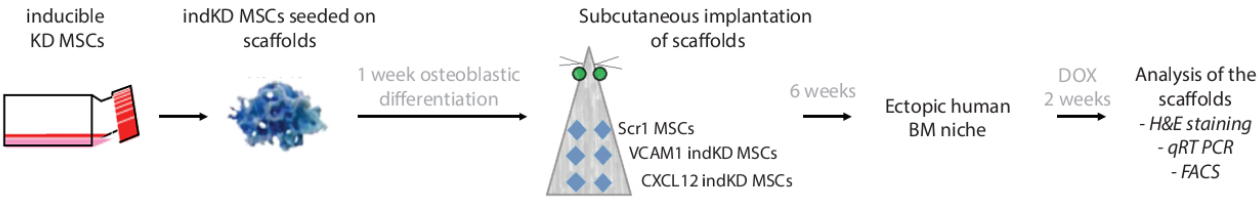


Figure 18 – *In vivo* bone formation by inducible VCAM-1 and CXCL12 MSCs. Schematic representation of the scaffold model. RAG2$^{-/-}$γc$^{-/-}$ mice were implanted with Scrambled1 (Scr1) or CXCL12 or VCAM-1 indKD MSC-loaded BCP scaffolds. Six weeks after implantation, half of the total mice (3/6) were injected intraperitoneally twice per week with 125 µg Doxycycline (DOX) and DOX (1 mg/ml) was added to their water containing 5% sucrose. Eight weeks after implantation, the formation of bone will be analyzed by Hematoxylin & Eosin staining of the scaffolds. Expression of CXCL12 and VCAM-1 mRNA will be analysed by qRT-PCR and expression of VCAM-1 by Flow Cytometry.

Six mice were subcutaneously implanted in their dorsal side with six scaffolds seeded with Scr1, VCAM-1 and CXCL12 indKD MSCs, two per each type of MSCs (Figure 18). Bone formation usually takes six to eight weeks to occur. Therefore, we assumed that after 6 weeks the MSCs, if able to differentiate towards osteoblastic lineage, should have form bone so we added DOX to half of the mice (3/6) to induce the KD of the stromal factors. DOX (125 µg) was intraperitoneally injected twice per week and DOX (1mg/ml) was also added to the water, containing 5% sucrose. Eight weeks after the implantation, mice will be sacrificied and the scaffolds will be collected. We are planning on fixing half

of the scaffolds in 4% formaldehyde to assess through H&E staining the ability of these MSCs to form bone *in vivo*. We will also isolate human cells from the remaining scaffolds by flow cytometry using an antibody that recognizes a pan-human antigen (Tra-1-85). For VCAM-1 indKD scaffolds, we will analyse the expression of VCAM-1 protein in mice treated with DOX and compare it to untreated mice by flow cytometry. Total RNA will be extracted from all cells to quantify the mRNA expression of CXCL12 and VCAM-1.

3.2. Generation of Knock out (KO) MSCs using the CRISPR/Cas system

The use of inducible shRNAs that knock down the expression of our target genes has many advantages, as previously described. However, this system has also some limitations such as the off-target effects on other mRNAs (Kaelin, 2012) and the inability to totally delete the expression of the target gene. Thus, a system which would involve genome editing and ablation of a certain gene would allow a clearer study of the functional effect of that gene. Recently, a powerful tool for genome engineering became available: the CRISPR/Cas system. This is a precise and efficient genome targeting technology that is based on guiding an endonuclease (Cas9) to the target DNA sequence through a short RNA sequence complementary to this genome region. After the creation of double strand breaks by Cas9, naturally occurring cellular DNA repair mechanisms are activated which may result in knock out of the gene. Therefore, we here used CRISPR/Cas9 vectors targeting CXCL12 and VCAM-1 in order to obtain a full knock out of these stromal factors in BM MSCs.

3.2.1. Cloning of the CRISPR/Cas9 vectors

We have used the lentiviral vector pRP-418 generated and kindly provided by Robert Jan Lebbink (Emmanuel Wiertz lab, UMCU). This vector contains a gRNA scaffold and human codon-optimized Cas9. In addition, it contains the cloning sites *EspEI* that allow the insertion of double strand (ds) oligonucleotides which are complementary to the target DNA sequence in the genome. This insert is localized downstream to the U6 promoter and upstream to the gRNA scaffold (Figure 19). Briefly, the cloning strategy consisted of ligating the annealed ds oligonucleotides inserts into *EspEI* digested pRP-418 vector (see also material and methods).

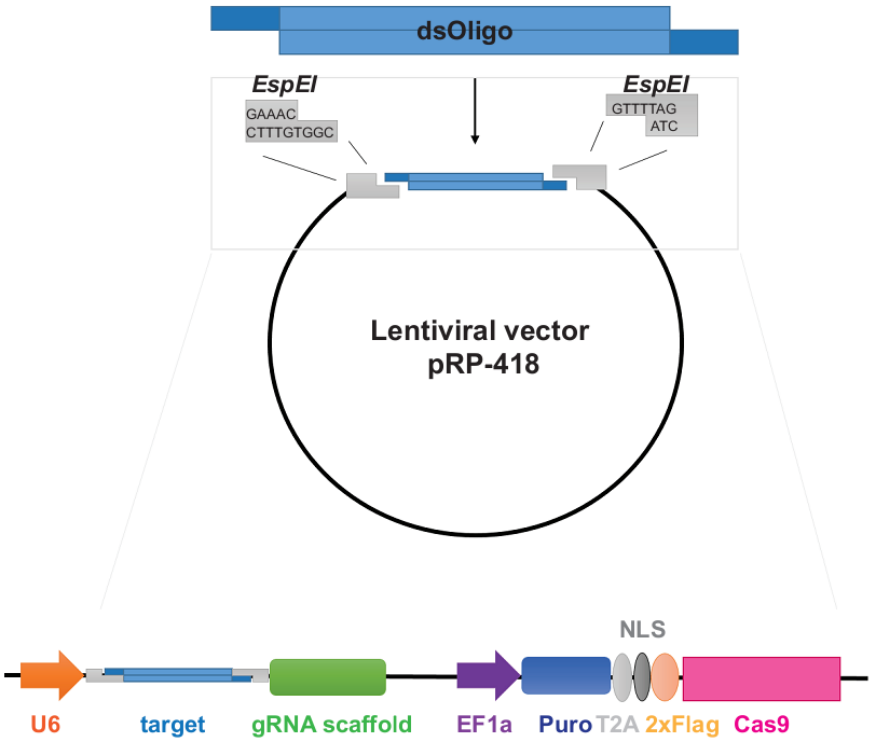


Figure 19 – Cloning strategy of CRIPR vectors for generating knock out MSCs. Schematic representation of the cloning strategy to generate CRISPR vectors.

3.2.2. Lentivirus-mediated CRISPering

With the generated vectors, we produced lentivirus, transduced BM MSC and selected successfully transduced BM MSCs with Puromycin. In addition, we also transduced BM MSCs with CD44 CRISPR vectors using lentivirus medium kindly provided by Robert Jan Lebbink (UMC Utrecht).

For all studied genes, we generated CRISPR vectors with RNA sequences targeting different regions of the target gene. To determine if the knock out was successful, we analysed CD44 and VCAM-1 expression by flow cytometry and CXCL12 secretion through ELISA. Two populations of BM MSCs were observed based on their CD44 protein expression. Most of BM MSCs transduced with CD44 lentivirus vector were CD44 negative, ranging from 50% to 74% depending on the guide RNA (Fig 20A). VCAM-1 expression in BM MSCs transduced with VCAM-1 CRISPR vectors was determined after treatment with TNFα to stimulate the expression of VCAM-1 protein. Once again, the KO was successful and 60% to 82% of MSCs were VCAM-1 negative. To determine if CXCL12 KO was effective, CXCL12 KO MSCs were cultured for 3 days after which the medium was collected and the secretion of CXCL12 was quantified by ELISA. Lentivirally transduced cells secreted approximately 83% less CXCL12 than Scr BM MSCs. Starting with lower cell numbers during the transduction gave similar results (data not shown). Even though the experiments were only performed once (with the exception of VCAM-1 KO MSCs in which VCAM-1 protein expression using flow cytometry was analysed twice) and therefore should be repeated to obtain full confirmation of the KO of the target genes, these data suggests that we were able to successfully generate CD44, VCAM-1 and CXCL12 KO BM MSCs.

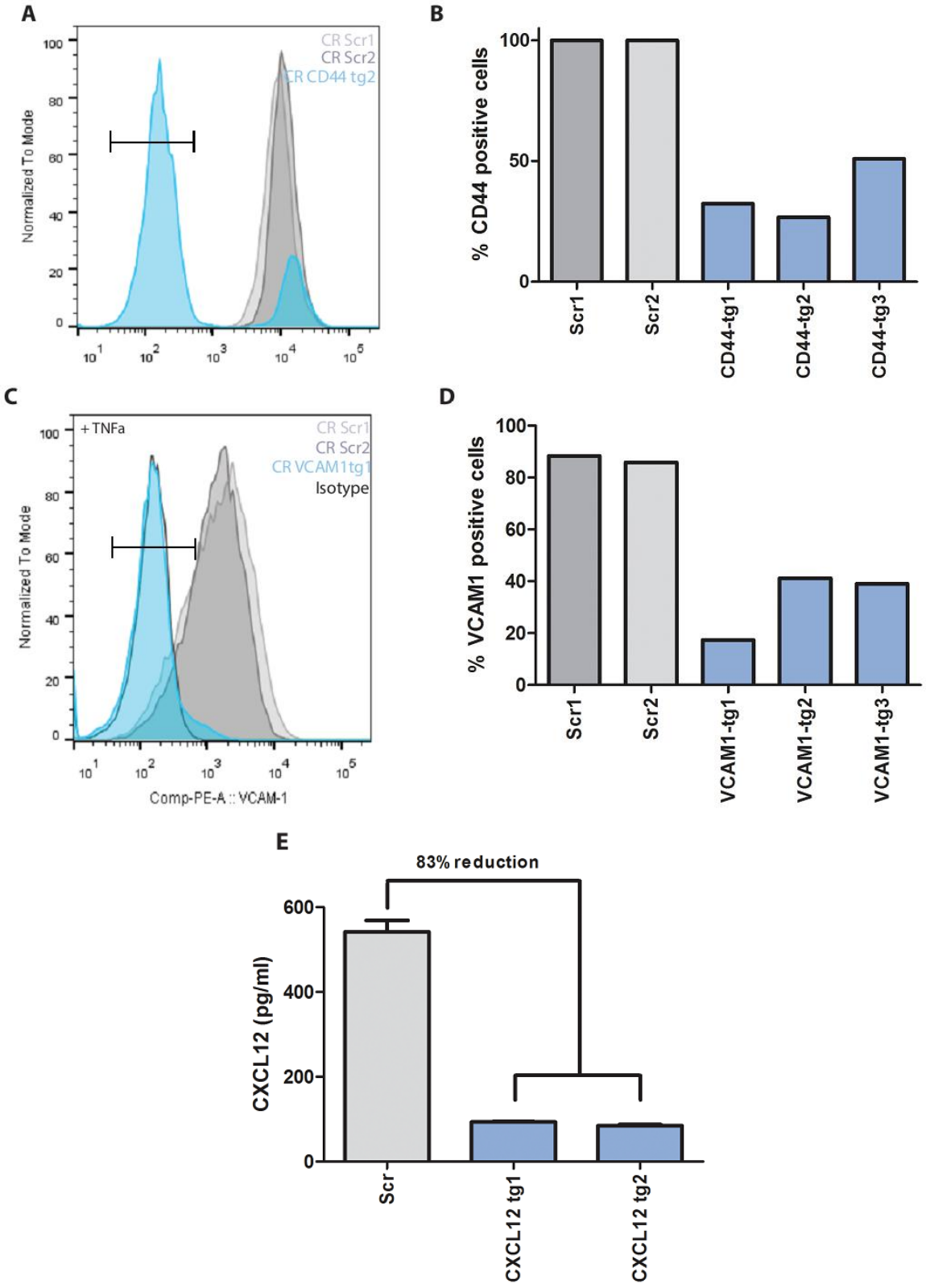


Figure 20 – Successful generation of KO MSCs for CD44, VCAM-1 and CXCL12. (A) CD44 KO MSCs (D06) were generated by lentivirus-mediated CRISPering using two different gRNA (tg1 and tg2). Expression of CD44 was analysed by flow cytometry using an anti-human CD44 antibody. Results represent one

independent experiment. (B) Percentage of CD44 negative cells as determined in (A). (C) Generation of VCAM-1 KO MSCs by lentivirus-mediated CRISPering. The KO obtained using three different gRNA (tg1, tg2 and tg3) is represented by analysis of the expression of VCAM-1 after 16h of TNFα treatment (10 ng/ml). Results are representative of two experiments. (D) Percentage of VCAM-1 negative cells obtained after CRISPering using three different target sequences. These results were obtained by gating the graphs as indicated in (C). (E) CXCL12 KO MSCs were generated by lentivirus transduction with two different gRNA (tg1 and tg2). After transduction and Puromycin selection, cells were cultured (35,000 cells per well in a 24 well plate) for 3 days. Culture medium was collected and CXCL12 expression was quantified by ELISA. Results represent one experiment performed in duplicate.

Since assessing B-ALL expansion upon co-culture of BM MSCs with decreased VCAM-1 expression did not allow functional validation of VCAM-1 indKD MSCs (Figure 15C) we performed a pilot experiment in which we used the newly generated VCAM-1 KO MSCs in an adhesion assay to 1) functionally validate these VCAM-1 KO MSCs and 2) to develop an assay that allows as well the functional validation of the VCAM-1 indKD MSCs. For this purpose, we incubated B-ALL cells with wt MSCs and VCAM-1 KO MSCs in the absence or presence of TNFα. After 24h, we determined the amount of B-ALL cells that remained adherent to MSCs by quantifying luminescence after addition of luciferine. As expected, the adhesion of leukemic cells was higher when these were cultured with MSCs than without stromal cells (Figure 21). Less B-ALL cells adhered when TNFα was added. We observed that B-ALL cells in the absence of TNFα adhered approximately 24% less to VCAM-1 KO MSCs (CR VCAM-1) than to wt MSCs. The same trend was observed when a higher number of MSCs was used (data not shown). Upon addition of TNFα, such differences are attenuated as there is not an obvious difference between the MSCs. Though this experiment should be repeated to verify if this effect is reproducible, we were already able to observe a reduction on the adhesion of B-ALL cells to VCAM-1 KO MSCs in comparison to B-ALL cells co-cultured with wt MSCs, in the absence of TNFα.

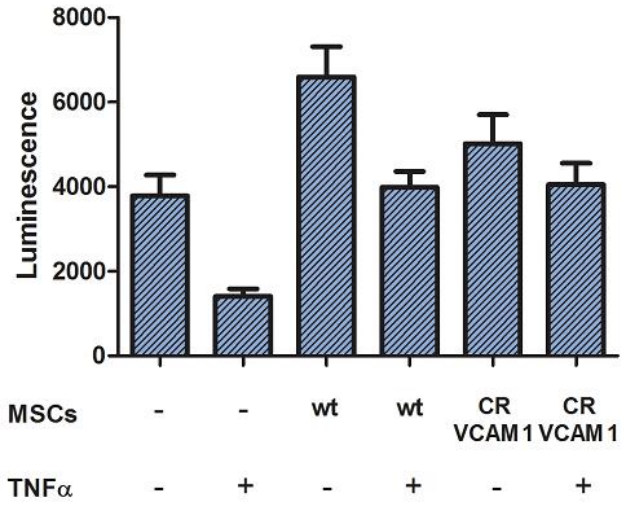


Figure 21 – Reduced NALM-6 adhesion to VCAM-1 KO BM MSCs. NALM-6-Luc-mCherry-Puro cells (3,75x10^4 cells/ml) were cultured on top of wild type (wt) and VCAM-1 KO MSCs in the presence of 10 ng/ml TNFα when indicated. To wash away non adherent cells, every wells were washed twice with PBS. New medium was added to these cells and luminescence was analysed. The results represent the duplicates of one experiment.

3.2.3. Transfection of MSCs using Neon Electroporation

Although we were able to efficiently and easily generate BM MSCs knocked out for CD44, VCAM-1 and CXCL12 through transduction with lentiviral vectors, this method of generating KO MSCs has a few drawbacks. First, since we transduced the MSCs with lentivirus, the vector was randomly inserted into the cell's genome and we cannot predict the side effects of this integration. Moreover, Cas9 is being constitutively transcribed leading to long and high presence of this endonuclease in the cell, which may lead to off-target effects (Cho et al., 2014; Cradick et al., 2013). Therefore, a system in which the vector wouldn't be integrated into the genome and with only transient expression of Cas9 would overcome the drawbacks of lentiviral transduction. We hence tried to transfect MSCs with the CRISPR vectors we had generated.

Different methods of transfection were tried. In all cases we first transfected the MSCs with either a small vector (4,700bp) encoding GFP (sGFP) or with a big (9,289bp) GFP vector (bGFP) to have a straightforward read out for the efficiency of transfection by analysing GFP expression using microscopy and/or flow cytometry. We used vectors with two different lengths because we expected that the shorter one would be more easily transfected into MSCs and the longer one is of a similar size as the CRISPR vector.

For the first transfection, we used a Gene Pulser MXcell™ Electroporation System (BioRad). Briefly, MSCs were placed in a sterile 2mm electroporation cuvette and were electroporated with 600V, 100 microseconds. Three days after electroporation, the GFP expression was analysed using a flow cytometer. Only the MSCs transfected with the sGFP vector expressed GFP (4-6%, data not shown). To improve the efficiency, we then tried a different method for transfection. As we had been successful in generating lentivirus using HEK293T and Poly(ethylenimine) (PEI), which establishes ionic interactions with the phosphate backbone of nucleic acids creating compact complexes that are taken up by cells via endocytosis (Godbey et al., 1999), we tested PEI transfection on MSCs. However, neither an adapted protocol from the one used to transfect HEK293T cells nor a protocol available online (http://www.cytographica.com/lab/PEItransfect.html) led to GFP expression upon transfection of MSCs with either sGFP or bGFP (data not shown). Lastly, we used a microcapillary system, the Neon® Transfection System, in which cells are electroporated in a pipette tip chamber instead of a cuvette, which allows application of a more uniform electric field overcoming the problems faced in standard electroporations, such as low efficiency in gene transferring and high cell mortality (Lim et al., 2010; Madeira et al., 2011; Nishikawa and Huang, 2001). With this method, it was possible to achieve GFP expression in MSCs transfected with sGFP one day after microporation in all settings tested (Figure 22A). Two days post transfection, we quantified GFP expression in transfected MSCs using a flow cytometer. We got the highest level of transfection in

setting C (70% of MSCs transfected with sGFP were GFP positive), which was a setting in which we gave two short electrical pulses instead of one longer pulse as in settings A and B, suggesting that the number of pulses is important in determining the transfection efficiency. However, MSCs transfected with bGFP had no GFP expression (data not shown). As this was also observed in the standard electroporation, we hypothesized that there could be some problem with the bGFP vector, as the vector was kindly given to us and we didn't test it to confirm that it was functional. As the transfection of MSCs with sGFP had been highly successful, we decided to microporate low passage wt MSCs (D06) with VCAM-1 tg1, VCAM-1 tg3 and CD44 tg2 CRISPR vectors using setting C. Two days after transfection, Puromycin was added to select transfected MSCs. Two days after Puromycin treatment, the medium was replaced with regular MSC medium. Nearly all cells died in all conditions, suggesting that the transfection of MSCs with the Puromycin-resistant vectors hadn't been successful. Even so, we kept the remaining cells in culture. Thirty days post transfection, VCAM-1 and CD44 expression was analysed by flow cytometry in MSCs transfected with VCAM-1 or CD44 CRISPR vectors, respectively. All MSCs transfected with CD44 tg2 CRISPR vector were CD44 positive. Likewise, VCAM-1 expression in TNFα-treated MSCs transfected with VCAM-1 CRISPR vectors was as high as in the control condition. Taken together, Neon® Transfection is a successful transfection method, however, optimization is needed to use this technique for CRISPRering.

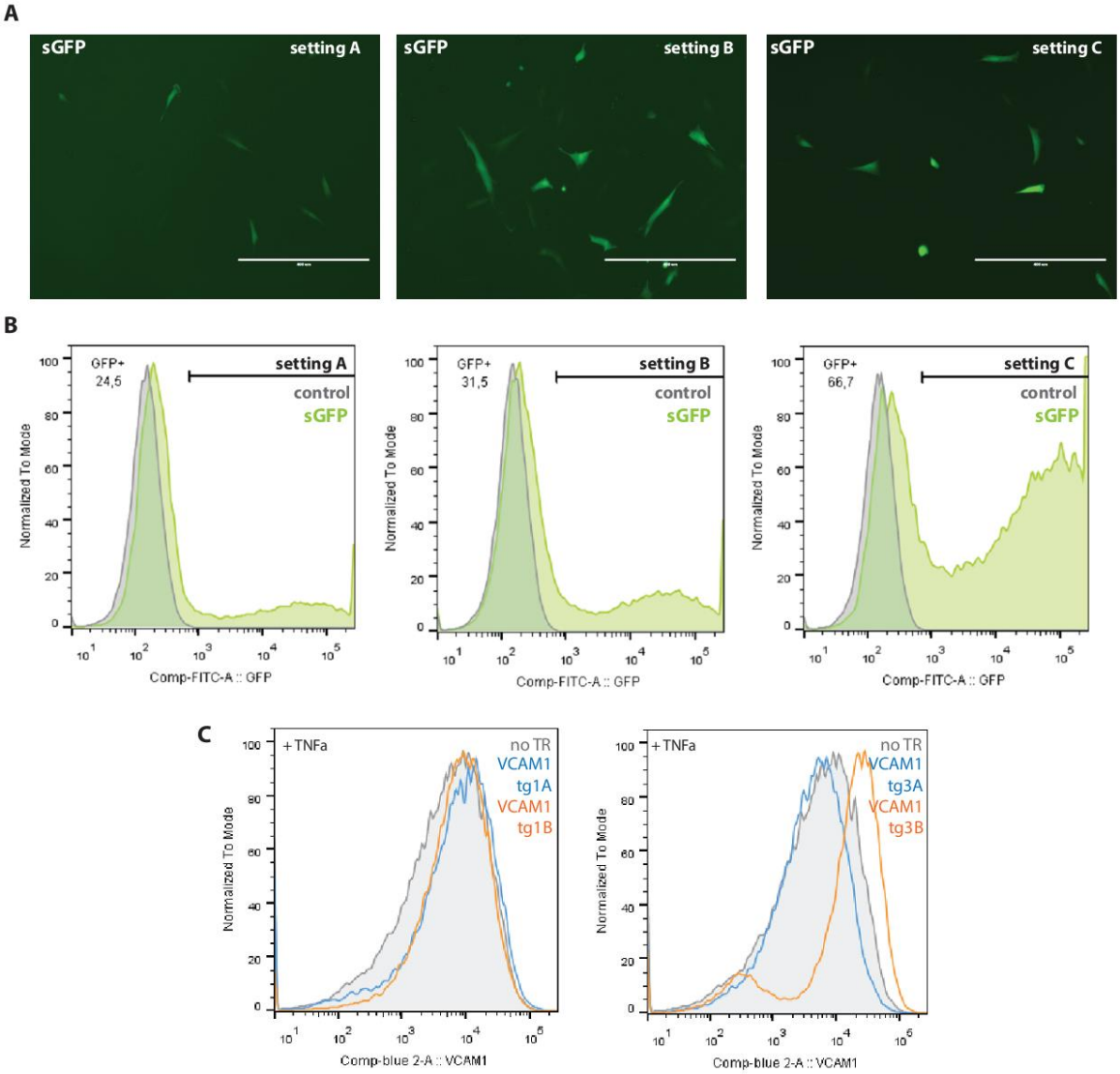


Figure 22 - Successful transfection with GFP, but no successful transfection-mediated CRISPering of BM MSCs. (A) and (B) A 4,700bp vector encoding GFP (sGFP) vector was transfected into BM MSCs using the Neon Transfection System. Three different electroporation settings were used: A - 1600 V, 20 ms, 1 pulses; B - 1000 V, 40 ms, 1 pulse; C - 1400 V, 10 ms, 2 pulses. One day after the transfection, microscopy images were taken (A). Expression of GFP on sGFP transfected MSCs and on electroporated wild-type (wt) MSCs (control) was analysed 48h after transfection by flow cytometry (B). Results represent one independent experiment. (C) BM MSCs were transfected with VCAM-1 target (tg) 1 and tg 3 CRISPR vectors using setting C. Two days after transfection, Puromycin (1 mg/ml) was added for two days to select for transfected cells. 30 days after Puromycin treatment, the expression of VCAM-1 was analysed by flow cytometry using an anti-human CD106 antibody. Cells were treated with TNFα (10 ng/ml) 16h before the harvesting to increase the expression of VCAM-1. Results represent one independent experiment performed in duplicate (A and B).

3.3. BM MSCs capacity of forming single clones

The ultimate goal of CRISPR-mediated generation of KO cells was to generate a pool of MSCs in which all cells are knock out for a certain protein. In the case of surface molecules, such as VCAM-1, we can easily sort VCAM-1 negative cells by flow cytometry. However, this is not the case for CXCL12 KO MSCs or MSCs knock out for other soluble MSC-derived factors. There are different methods to measure CXCL12 but none allows the detection of CXCL12 at the single cell level while allowing to keep the cells alive. First, Western Blot analysis requires cell lysis and CXCL12 is not analysed at single cell level. Intracellular FACS allows single cell analysis and even sorting of cells, but we would have to permeabilise the cells which would kill them. Furthermore, we don't have a good anti-CXCL12 antibody, since the antibody tested did not show lower expression of CXCL12 in CXCL12 ctKD MSCs, which by ELISA were confirmed to express lower levels of CXCL12 protein (data not shown). ELISA would overcome the problem of killing the cells, but it doesn't quantify CXCL12 secretion at the single cell level. Therefore, the potential solution would be to sort single cells into a 96 well plate thus creating single clones of MSCs and expand these. We could then do a screen on the clones for CXCL12 expression by ELISA, and then either use the individual clones or pool the CXCL12 negative clones, hence generating a polyclonal CXCL12 knock out MSC population (see also in the discussion).

For this strategy to work, we first had to investigate whether BM MSCs were able to survive and expand as single cells. To determine their capacity to grow as single clones, BM MSCs from two donors (D06 and D99) cells were single sorted into a 96 well plate and their growth was followed over time by microscopy. Both types of BM MSCs were able to form colonies, but more single cells grew out from D99 cells than from D06 cells (Figure 23A, 27 to 40% and 12 to 27%, respectively). Then, to determine if the process of lentiviral-mediated VCAM-1 ablation, in VCAM-1 KO MSCs, had any effect on the colony forming ability of MSCs, Scr KO MSCs and VCAM-1 KO MSCs were treated with

TNFα and single cells were sorted into 96 well plates. For TNFα-treated VCAM-1 KO MSCs, we separately sorted VCAM-1 positive and VCAM-1 negative cells (Figure 23B). In comparison to wt MSCs, less transduced cells grew out, which could be related to the fact that transduced MSCs were of a higher passage than wt MSCs. TNFα treatment had no major effect on colony forming ability of VCAM-1 KO MSCs. Importantly, VCAM-1 negative MSCs showed a slightly improved colony forming ability, indicating that VCAM-1 negativity does not inhibit the ability of MSCs to grow (Figure 23C). The MSC clones that formed big colonies were harvested and VCAM-1 expression was analysed again to determine whether these colonies expressed VCAM-1 levels that can be expected based on how the cells had been sorted. All VCAM-1 KO MSCs that hadn't been treated with TNFα before sorting didn't express VCAM-1. As expected, TNFα-treated VCAM-1 negative KO MSCs remained VCAM-1 negative. In one TNFα-treated VCAM-1 negative MSC colony we were able to observe two populations of MSCs, one VCAM-1 negative and the other VCAM-1 positive, suggesting that two cells instead of one cell were sorted. Furthermore, none of VCAM-1 positive cells survived after harvesting from 96 well plate and plating in T25 flask when a big MSC colony was observed. These data suggests that single MSC clones are able to expand and that neither lentiviral transduction nor TNFα treatment negatively affected the colony forming ability of MSCs.

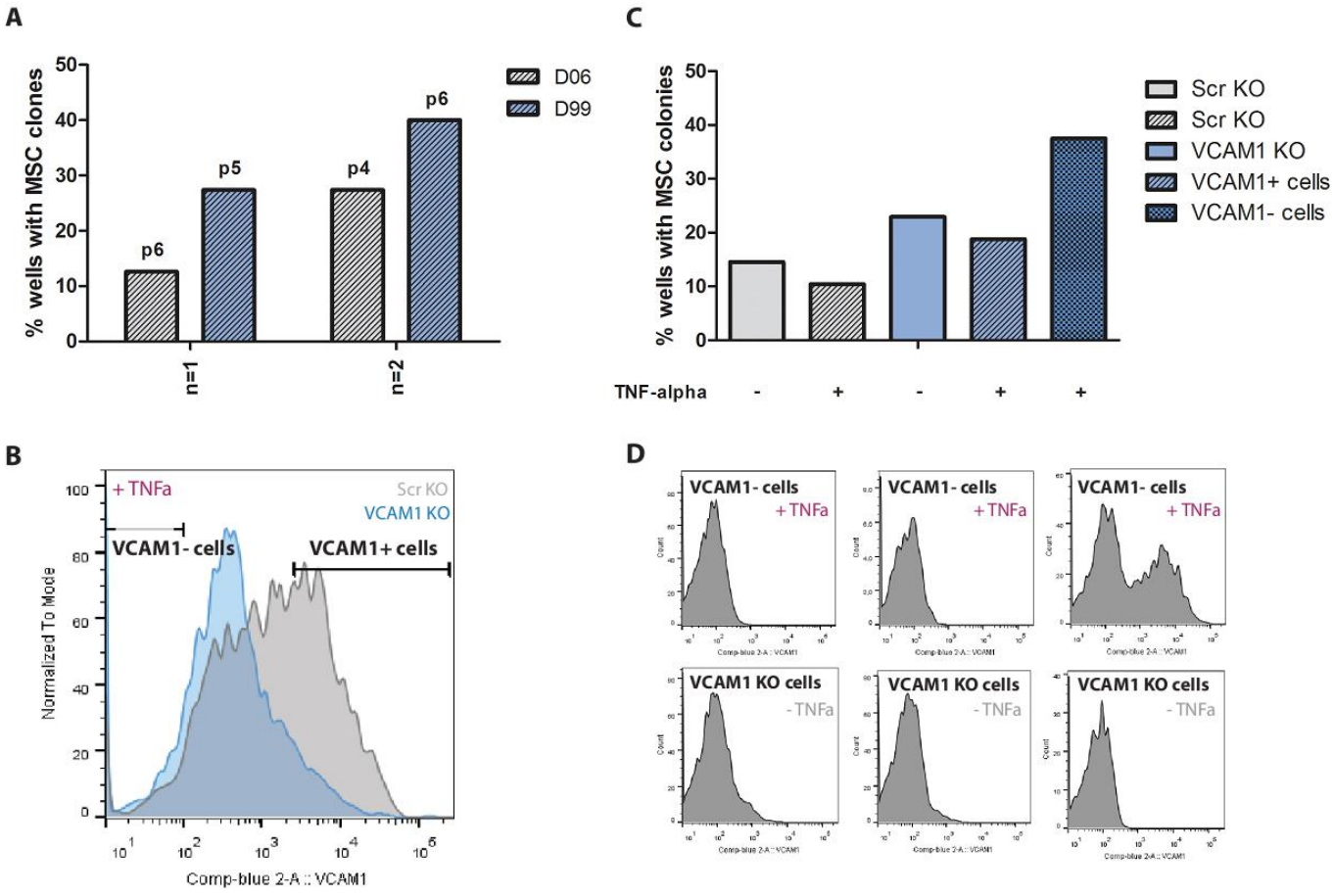


Figure 23 - BM MSCs are able to grow as single clones and are not negatively affected by VCAM-1 deletion or TNFα treatment. (A) Different passages (as indicated) of BM MSCs from two donors (D06 and D99) were sorted into a 96 well plate, (1 cell per well, 95 wells per cell type). The formation of colonies was followed by bright field microscopy. The graph presents the percentage of wells with MSC colonies 8-10 days after the sorting. (B) and (C) BM MSCs that had been lentivirally transduced with scrambled CRISPR (Scr) or VCAM-1 CR (VCAM-1 KO MSCs) were treated for 16h with TNFα (10 ng/ml) to induce the expression of VCAM-1. VCAM-1 KO MSCs cells were sorted into 96 well plates (1 cell per well, 48 wells per cell type) according to their VCAM-1 expression (B). The growth of cells was followed by bright field microscopy. The graph presents the percentage of wells with MSC colonies 10 days after the sorting (C). Results are representative one experiment. (D) MSC colonies were harvested and cultured for one week. Cells were treated with TNFα (10 ng/ml) to induce the VCAM-1 expression 16h before being collected. Expression of VCAM-1 was determined by flow cytometry using anti-human CD106 (VCAM-1). The results represent one experiment.

Chapter 4 Discussion

4. Discussion

The importance of the BM microenvironment in leukaemia has been extensively studied and it has been accepted that the BM plays a crucial role in leukaemia growth, chemoresistance and relapse (Tabe and Konopleva, 2014). Nevertheless, further understanding is needed and can be provided by novel *in vivo* models that create BM niches that resemble the human BM. Previously, using an human ectopic BM niche model in which BCP scaffolds are seeded with MSCs (Groen et al., 2012), we showed that primary human B-ALL cells can grow in human ectopic BM niches subcutaneously implanted in immunodeficient mice (unpublished data). Taking that into consideration, we aimed at demonstrating the importance of CXCL12, IL-7 and VCAM-1 in B-ALL growth using the state of the art system that best resembles the human BM niche. With this goal in mind we here developed tools to test the role of these stromal factors by generating genetically manipulated MSCs in which CXCL12, IL-7 and VCAM-1 are downregulated or ablated, that can be used to create ectopic human BM niches. Aiming at confirming literature describing the role of these factors on B-ALL *in vitro* and, mainly, at developing *in vitro* assays to functionally validate the genetically modified MSCs that were generated in this study, we used pilot studies to demonstrate that CXCL12 and IL-7 induce migration and expansion of B-ALL cells, respectively, and that MSCs are able to support survival and expansion of B-ALL cells. When MSCs constitutively knocked down for CXCL12, IL-7, VCAM-1 and TSLP previously generated and validated at mRNA and/or protein level were seeded into scaffolds and implanted in mice to test *in vivo* bone formation capacity, only control and CXCL12 ctKD MSCs were able to form bone. Trying to overcome the loss of the ability to form bone, which could be caused by the role of the downregulated factors in bone formation, inducible KD MSCs for CXCL12, VCAM-1 and IL-7 were generated. Successful inducible KD was confirmed at the mRNA and/or protein levels for VCAM-1 and IL-7 indKD MSCs and pilot experiments were performed to investigate the functional impact of the KD. Inducible KD of IL-7 didn't substantially

influence the expansion of B-ALL cells. To test the full ablation of VCAM-1 and CXCL12, KO MSCs were generated using the CRISPR/Cas system and KO was analysed at the protein level. A pilot experiment in which leukemic cells where co-cultured with VCAM-1 KO MSCs showed that VCAM-1 knock out decreased the adhesion of B-ALL cells to the stroma. Testing *in vivo* bone formation capacity of the genetically manipulated MSCs will determine if these MSCs are able to form human ectopic niches in immunodeficient mice. Injection of primary B-ALL cells or B-ALL cell lines into mice implanted with scaffolds seeded with CXCL12 and VCAM-1 KO MSCs, and/or with IL-7 and VCAM-1 indKD MSCs will allow us to determine the importance of the presence of these factors in the human BM on leukaemia.

4.1. Response of B-ALL cells to BM stromal cells and stromal factors *in vitro*

First, we aimed at verifying the importance of BM MSCs in supporting B-ALL cells using an *in vitro* co-culture system, to later also use this to validate the genetically modified MSCs. Manabe and colleagues were one of the firsts to demonstrate that BM MSCs positively influence B-ALL cell survival (Manabe et al., 1992). Apoptosis of B-ALL cells was prevented when these were co-cultured with MSCs. Others have also demonstrated a positive influence of stromal support on ALL cell viability (Juarez et al., 2007a). Using the cell lines NALM-6 and REH as a model system for B-ALL, we here demonstrated a similar effect, as when low numbers of B-ALL cells are cultured in the absence of MSCs for 3 days, both B-ALL cells lines decrease in numbers. (Figure 7). This is not a general phenomenon, since we are able to culture B-ALL cell lines over long periods of time in the absence of stromal cells, as long as leukemic cells are cultured at high density (at least 0.3x10^6 cells/ml). While NALM-6 cells expanded in co-culture, addition of BM-MSCs to REH cells only led to their survival. This discrepancy is in line with previous studies that reported that the survival length and stroma contact requirement differs

between blast cells from 10 cases of precursor-B-ALL cells (Bradstock et al., 1996). We observed that the support of BM MSCs was lost when higher amounts of both MSCs and leukemic cells were plated and different MSCs:B-ALL cells ratios were used (data not shown). Even though this data may appear contradictory, nutrient exhaustion in the co-culture system may have contributed to B-ALL cells death when high amounts of MSCs were plated or when a very high concentration of B-ALL cells were cultured, whereas low numbers of MSCs are insufficient to provide the B-ALL support. Together, these data confirm the previously described role of BM MSCs in supporting *in vitro* expansion and survival of B-ALL cell lines.

Aiming at determining which factors produced by MSCs could be responsible or most important for the support of leukemic cells, either in our *in vitro* co-culture system, or, more importantly, in human B-ALL growth *in vivo*, we initially selected CXCL12, IL-7, TSLP and VCAM-1 which have been described to play a role in B-ALL migration, growth and survival (Filshie et al., 1998; Juarez et al., 2003, 2007a; Mowafi et al., 2008; Sbaa-Ketata et al., 2001; Tasian et al., 2012). Our first goal was to determine the specific role of these factors on B-ALL cells *in vitro* by setting up assays to analyse ligand-induced receptor downregulation, expansion, adhesion, and migration. Importantly, these assays were mainly developed to functionally validate the generated genetically manipulated MSCs with decreased expression or ablation of these factors, as will be described below.

The observation that both NALM-6 and CALL4 down-regulated IL-7Rα upon IL-7 stimulation was a first indication that these cell lines are intrinsically able to respond to IL-7 (Figure 8 and data not shown). Similarly CXCL12-induced CXCR4 downregulation showed that CALL4 is also responsive to CXCL12.

TSLP treatment of CALL4 only led to downregulation of the IL-7Rα subunit of the TSLP receptor and not of TSLPR. CALL4 cells express a low to intermediate level of IL-7Rα, but a high expression of TSLPR (Chapiro et al., 2010; Tasian et al., 2012), which has

been suggested to be a consequence of a TSLPR rearrangement in CALL4. Therefore, even if TSLP induces downregulation of TSLPR, the expression levels of this receptor subunit are kept very high due to a mechanism independent of the presence of the ligand, thus justifying the absence of a noticeable effect of TSLP on TSLPR expression. Looking at the functional level, we only observed a minimal CALL4 expansion upon TSLP exposure (up to 1.14 fold). It has been previously reported that CALL4 cells show an up to 1.4 fold expansion when exposed to TSLP, but we were not able to reproduce this even when using similar conditions such as incubation times and TSLP concentrations (Tasian et al., 2012) as well as varying concentrations of FBS. We hypothesize that the high cell numbers used in our assay explain the lack of effect of TSLP on CALL4 growth, as when grown at higher concentrations B-ALL cells seem to be less dependent on growth factors. For instance, we also didn't observe any effect of IL-7 on NALM-6 expansion when the same high numbers of leukemic cells were used. Only when we decreased the amount of NALM-6 cells cultured in the presence of IL-7, an IL-7-dependent effect on NALM-6 growth was verified (Figure 9B). We therefore should test the effect of TSLP on CALL4 in an assay in which lower numbers of CALL4 cells are used. To determine the role of TSLP on B-ALL growth *in vivo* we should then generate TSLP indKD MSCs that can be used in our *in vivo* model.

In line with previous reports, IL-7 treatment alone also didn't cause a substantial expansion of the B-ALL cell line REH (Brown et al., 2003; Juarez et al., 2007a). IL-7 only induced expansion when leukemic cells were exposed to IL-7 in the presence of MSCs. This suggests that there may be some factors expressed by BM MSCs that act synergistically with IL-7 increasing its effect. In fact, synergistic interactions of IL-7 with stem cell factor (Funk et al., 1993; Mcniece et al., 1991) and FLT3L (Hirayama et al., 1995; Veiby et al., 1996), for instance, result in an increase on the proliferation of B cell progenitors and/or B-cell commitment. Mechanisms explaining this synergy have been proposed. IL-7 and FL3TL activate parallel but separate proliferation signalling pathways

(Åhsberg et al., 2010), and stem cell factor is able to phosphorylate both subunits of the IL-7 receptor (γ_c and IL-7Rα), the first events of IL-7–mediated signalling. Furthermore, the combination of IL-7 and CXCL12 leads to optimal growth and survival of leukemic cells *in vitro* (Juarez et al., 2007a). Alternatively, IL-7 may act on MSCs inducing changes on the stroma that lead to an increase of B-ALL support, which is supported by the observation that MSCs, besides producing IL-7, also express IL-7Rα (Deans and Moseley, 2000). Along similar lines, it might be that TSLP, which on its own did not significantly impact on B-ALL growth *in vitro*, could enhance B-ALL growth in combination with other MSC-derived factors; something we could actually test if we would perform a MSC - B-ALL co-culture experiment in the presence of TSLP. However, for such co-culture experiment we would first need to Luciferase-label the CALL4 cells, since Presto Blue ® cannot distinguish between B-ALL cells and MSCs.

Though CXCL12 is a chemokine, it has been described to also stimulate proliferation of B-ALL cells (Juarez et al., 2007a; Mowafi et al., 2008). We tested this hypothesis using two different B-ALL cell lines, NALM-6 and CALL4, of which the first has been widely described to be sensitive to CXCL12 (Bradstock et al., 2000). However, we couldn't observe any effect of CXCL12 on leukemic cell expansion in either cell lines (data not shown). In line with chemotaxis being CXCL12's most well-known and accepted function (Sipkins et al., 2005a), we observed in a pilot experiment that NALM-6 cells can effectively migrate towards CXCL12 (Figure 9A). Although the extent of migration (± 14%) was lower than previously reported (almost 60%) (Bradstock et al., 2000), this could potentially be optimized in future experiments, especially considering that the experiment settings were not the same and that we have performed this experiment only once and without duplicates. Cell starvation and increasing the time of incubation may lead to greater degree of migration of NALM-6 (Bradstock et al., 2000; Hidalgo et al., 2001). In line with previous reports, AMD3100 addition inhibited the CXCL12 chemotactic effect. Though the level of inhibition (almost 80%) was higher than previously described,

such differences may be attributed to the type of B-ALL cells that were used in the assay, since Parameswaran et. al observed that, depending on the B-ALL cell type, a different level of inhibition was achieved (Parameswaran et al., 2011).

Taken together, we showed in pilot *in vitro* assays that leukemic cells respond to CXCL12 and IL-7 which we can use to validate our genetically manipulated MSCs. Furthermore, this preliminary data suggests that these factors may be crucial for B-ALL growth, as described in literature.

4.2. Bone forming ability of constitutive KD MSCs

To confirm the importance of BM-derived factors in a model that more closely resembles the human BM, we have generated MSCs lacking these factors as well as VCAM-1, which has been described by others to play a role in B-ALL as well (Filshie et al., 1998; Hall et al., 2004b), using three different approaches: constitutive knock down, inducible knock down and knock out. Use of such MSCs will not only allow *in vitro* validation, but can also be used to address their *in vivo* role by implantation into mice to form human ectopic BM niches. We first tested the bone forming ability of constitutive KD MSCs (generated by Anne van der Leun), but only CXCL12 ctKD MSCs had this capacity (Figure 10). As previously discussed, ctKD MSCs may have lost the ability to form bone because the factors that have been downregulated may be important for osteoblastic differentiation. Indeed, in ctKD MSCs, osteoblastic differentiation *in vitro* was compromised (Anne van der Leun, unpublished results). In line with that, Anne van der Leun analysed the mRNA levels of IL-7 and TSLP throughout 14 days of osteoblastic differentiation of wt MSCs. IL-7 expression reached a peak (4 fold) at day 7 after which the levels remained high (2 fold). Likewise, the levels of TSLP increased throughout osteoblastic differentiation. This correlation of increased expression of IL-7 and TSLP

upon osteoblastic differentiation might suggest that IL-7 and TSLP could be important for differentiation of MSCs towards osteoblasts.

To confirm that the bone formed in the CXCL12 KD MSC-seeded scaffolds showed lower CXCL12 expression than the control scaffolds, we tried to develop an immunohistochemistry-based detection of CXCL12. However, since the CXCL12 KO MSCs that showed an 80% decrease in expression of CXCL12 based on ELISA, showed all equally positive for CXCL12 with the anti-CXCL12 antibody used, we thus far have no trustable anti-CXCL12 antibody. Although we still need to confirm the CXCL12 knock down *in vivo* in the future using a different immunohistochemistry antibody, the observations that CXCL12 ctKD MSCs can form bone *in vivo* and that CXCL12 is stably downregulated *in vitro*, now allow us to investigate the *in vivo* role of CXCL12 in the migration of B-ALL cells towards the BM niche (see future plans).

4.3. Inducible KD MSCs for stromal factors to study their importance for B-ALL growth

To overcome potential loss of bone forming capacity upon lentivirus-induced knock down and to be able to regulate the induction of KD over time, which also would allow us to investigate the impact of downregulating stromal factors in different stages of B-ALL development, we here used a previously described inducible KD system (Schackmann et al., 2011) to target VCAM1, IL-7 and CXCL12 in BM MSCs.

To allow validation of the VCAM-1 indKD MSCs, we used TNFα treatment to induce upregulation of VCAM-1, as without addition of TNFα, VCAM-1 protein expression was only slightly higher than the isotype control. As an alternative, we could have differentiated the MSCs towards osteoblastic lineage as osteoblasts express high levels of VCAM-1 (Zhu et al., 2007). We opted for the TNFα treatment because the osteoblastic

differentiation process is technically more laborious and longer (14 days) and because MSCs would lose their multipotency. We managed to validate the inducible knock down system for VCAM1 indKD MSCs at both mRNA and protein level (Figure 13 and 14). VCAM-1 was downregulated at the mRNA level 24h after DOX addition. We saw a nice time-dependent decrease of VCAM-1 protein expression by flow cytometry, which reached its maximal level of downregulation in 3 days. This DOX-induced decrease was an all or nothing event, at least with the doses we tested.

For the IL-7 indKD MSCs, we confirmed by qRT-PCR that there is downregulation of IL-7 upon DOX treatment (Figure 16). To further validate the knock down in the IL-7 indKD MSCs, we should in the future analyse IL-7 protein expression by techniques such as ELISA and Western Blot.

For the CXCL12 indKD MSCs, there was a DOX-independent reduction of CXCL12 both at the mRNA and protein level (Figure 12). We excluded that the medium in which indKD MSCs were being cultured was leaky as we compared mRNA and/or protein expression of all indKD MSCs cultured in DOX free medium and PL medium and no clear differences were observed. Therefore, the most likely reason for this DOX-independent induced downregulation is that there is an error in the sequence of the vector. To test this hypothesis we can sequence both the original lentiviral vector used to transduce the MSCs and the one integrated into the MSCs to check if there is a mistake in the tet repressor and/or tet operator. We had planned to functionally validate the CXCL12 indKD MSCs by analysing the migration of B-ALL cells toward CXCL12 indKD MSCs conditioned medium. However, as we cannot regulate the CXCL12 KD in these MSCs, we are not planning on using these CXCL12 indKD MSCs in the future.

Although our main objective is to use the validated VCAM-1 and IL-7 indKD MSCs to test the effect of the respective knock downs in the support of B-ALL cells *in vivo*, we first need to functionally validate the indKD MSCs *in vitro* and to test their ability to form bone

in vivo. In a pilot experiment, aiming at investigating if B-ALL cells interaction with MSCs would induce VCAM-1 expression in MSCs, we used flow cytometry to demonstrate that Scr1 MSCs co-cultured with B-ALL cells had a slight increase (1.6 fold) in VCAM-1 expression when compared to Scr1 MSCs cultured in the absence of leukemic cells (Figure 15A and 15B). This increase was lower than the previously reported VCAM-1 upregulation by Jacamo et al. who observed a 2.5 fold increase in VCAM-1 expression on B-ALL cell upon co-culture with B-ALL cells. (Jacamo et al., 2014). However, this discrepancy might lie in the fact that we analysed VCAM-1 protein expression by flow cytometry whereas they quantified the VCAM-1 expression at the mRNA level by qRT-PCR. Importantly, the effect of the presence of B-ALL cells was not observed in VCAM-1 indKD MSCs after DOX addition, reconfirming that we have generated MSCs with inducible VCAM-1 downregulation. Similarly, we also observed a slight VCAM-1 upregulation when B-ALL conditioned medium was added to the MSCs, except on DOX-treated VCAM-1 indKD MSCs, suggesting that one or more substances secreted by leukemic cells induce VCAM-1 expression in MSCs. Still, VCAM-1 expression was not as high as in the co-culture conditions, suggesting that cell-cell contact is required as well. Although addition of exogenous TNFα, IL-1β and IL-4 has been demonstrated to induce VCAM-1 expression in BM MSCs (Dittel et al., 1993), it remains undetermined which B-ALL derived factor(s) are able to mediate VCAM-1 upregulation in MSCs.

As we observed that interaction between leukemic cells and MSCs leads to an increase of VCAM-1 expression, we next analysed if VCAM-1 knock down in MSCs had any effect on support of B-ALL cell both in the absence and presence of TNFα. No considerable difference on B-ALL expansion between wt MSCs and VCAM-1 ctKD MSCs was observed (Figure 15A), a phenomenon not necessarily unexpected since VCAM-1 signalling blockade either using anti-functional integrin alpha-4 antibody or anti-human VCAM-1 antibody has previously been shown not to inhibit B-ALL growth in a B-ALL – MSC co-culture (Hsieh et al., 2013; Mudry et al., 2000). Performing this assay we also

observed that TNFα had a negative impact on the expansion of B-ALL cells when in co-culture, suggesting that TNFα has an inhibitory effect on leukemic cell growth. In fact, TNFα has also been shown to inhibit myeloid leukaemia cells expansion (Munker, 1987). and TNFα was shown to act synergistically with chemotherapeutic agents increasing their anti-tumoral activity (Veen, 2000). Besides, AML cells cultured in medium containing, amongst others, TNFα differentiate into dendritic cells which stimulate antileukemic cytotoxicity (Choudhury et al., 1999). Together these data suggest that also for B-ALL cells TNFα either alone or in combination with factors expressed by MSCs might inhibit B-ALL growth. As this TNFα negative impact on leukemic cells was lessened when B-ALL cells were cultured in the absence of MSCs, TNFα may instead, or in addition, induce some change in the stroma which leads to its decrease on the ability to support B-ALL cells. To overcome this issue for the validation of the VCAM-1 indKD MSCs, we could instead of TNFα use MSCs which have been stimulated to differentiate along the osteoblastic lineage, which also leads to VCAM-1 upregulation.

Although it has been described in literature that VCAM1 signalling blockade doesn't affect B-ALL expansion, disruption of VCAM-1 binding was shown to decrease stromal-induced leukemia cell protection during chemotherapy (Mudry et al., 2000). A nice way to investigate the effect of VCAM-1 knock down would thus be to test the stromal-induced B-ALL support when in the presence of chemotherapeutic agents. In addition, considering that VCAM-1 is an adhesion molecule, we could also determine the impact of VCAM-1 knock down by analysing the adhesion of leukemic cells to wild type MSCs and compare it to the genetically manipulated MSCs. For this method we actually performed a first pilot with the newly generated VCAM-1 KO MSCs, as will be discussed below.

Using the assay we had developed in which NALM-6-Luc-mCherry-Puro cells were co-cultured with MSCs in the absence or presence of IL-7, we showed that IL-7 indKD MSCs were still able to support expansion of leukemic cells (Figure 17). Even though the

downregulation of IL-7 was of almost 90% (Figure 16), we only observed a slightly reduced B-ALL support by IL-7 indKD MSCs upon addition of DOX. However, since DOX also induced a certain decrease in B-ALL support when added to co-cultures containing either wt or Scr MSCs, it is unclear whether the reduced support by DOX-induced IL-7 indKD MSCs is either related to IL-7 downregulation or DOX treatment itself. This might be related to the fact that IL-7 is secreted in the range of pictograms by MSCs whereas induction of B-ALL growth by exogenous addition of IL-7 required a concentration of IL-7 that was in the nanograms range. Although we expected that adding IL-7 to either DOX-treated or untreated IL-7 indKD MSCs would restore their supporting capacity to a similar extent as wtMSCs, IL-7 addition to IL-7 indKD MSCs as well as to Scr MSCs showed a lower induction of B-ALL expansion upon exogenous IL-7 than wt MSCs. This suggests that the lentiviral transduction process and subsequent process required to introduce the IL-7 knocking down may have affected other signalling pathways in the stromal cells that act synergistically with IL-7. As this experiment was only performed once due to time limitations, we plan to repeat it to determine whether it can be used to *in vitro* validate the indKD MSCs and to confirm the *in vitro* relevance of IL-7 on B-ALL growth. For this *in vitro* validation we also would like to use other B-ALL cell lines, such as SEM and NAG8/7 that have been previously reported to be responsive to IL-7 (Friend et al., 1994; Greil et al., 1994). To further confirm the role of IL-7, we can treat the MSCs with blocking anti-IL-7 antibody, which would be our control to determine the effect of blocking IL-7.

As we aim at using the indKD MSCs to generate human ectopic BM niches to test the role of BM-derived factors in the support of B-ALL cell growth, it is crucial that the genetically manipulated MSCs are able to form a bone-containing BM microenvironment *in vivo*. To test the ability of indKD MSCs to form bone, we started to test them in our *in vivo* model. Unfortunately, since we will only sacrifice the mice on 10th June 2014, we have not been able to report on the results in this thesis. We will not only test the bone

forming capacity of the indKD MSCs by H&E staining on paraffin embedded scaffold sections, but also determine if there is knock down of the niche factors upon *in vivo* administration of DOX (Reijmers et al., 2010) by qRT PCR and flow cytometry analysis.

Although we thus far did not succeed in generating CXCL12 indKD MSCs, we successfully generated and validated inducible KD MSCs for VCAM-1 and IL-7. We want to solve the problem encountered with the CXCL12 indKD MSCs either by regenerating these MSCs after checking the sequence of the used vector and/or using a vector containing another CXCL12-targeting shRNA. If the on-going experiment will show us that indKD MSCs are able to form bone and we can down-regulate the stroma-derived factors upon DOX addition *in vivo*, these will be the ideal tools to test the role of B-ALL growth *in vivo* (see also the conclusions and future perspective section).

4.4. Generation of MSCs KO for VCAM-1 and CXCL12 using the CRISPR/Cas system

These inducible system has many advantages, as stated above, but there are some limitations of the shRNA knock down system of which the most important one is the incomplete ablation of the gene expression. Therefore, we set out to develop a method to generate MSCs which are knock out for a certain gene for which we used the recently developed CRISPR/Cas system. Here we show that by using this system we are able to generate CXCL12, VCAM-1 and CD44 knock out MSCs (Figure 20). The knock out reached was very high for both for VCAM-1 and CD44 (up to 80%), determined by analysis of protein expression by flow cytometry. Although we selected transduced cells based on puromycin resistance, the DSB created by the Cas 9 does not always lead to an incorrect DNA repair that would lead to abrogation of expression of the gene. Hence we did not expect a 100% efficiency of the KO. Since we have showed by ELISA that CXCL12 KO MSCs secrete approximately 80% less CXCL12 than wt MSCs, we also

assume we successfully created CXCL12 KO MSCs. In the future we plan to use the Surveyor Assay Kit or sequencing of single KO MSC clones to test whether we can confirm the successful genetic manipulation using CRISPR at the genomic DNA level (Cong et al., 2013; Gennequin et al., 2013). Using the Surveyor Assay Kit, the target genome sequence is amplified, denaturated and re-annealed with the same amplified sequence in non CRISPR cells. As short insertions or deletions are created during the repair of DSB in CRISPR cells, the alignment won't be totally correct. Surveyor is an endonuclease that cleaves misaligned single-stranded DNA. If we then run the genomic DNA in an electrophoresis gel we can detect the presence of DNA fragments with different sizes which indicate the existence of a mutation.

To functionally validate the VCAM-1 KO MSCs we performed a pilot experiment in which we showed that VCAM-1 ablation in MSCs results in decreased B-ALL adherence to these cells (Figure 21). Although the reduction was not dramatic and the experiment should be repeated not only to confirm the reproducibility of the assay, but also to take along the proper control cells (Scr MSCs rather than wt MSCs), our data indicate that VCAM-1 deletion affects the ability of B-ALL cells to adhere. This suggests that the low level of B-ALL cell-induced VCAM-1 expression, as we have shown above by co-culturing B-ALL and MSCs after which we analysed VCAM-1 protein expression on MSCs by flow cytometry, is sufficient to have a functional impact on B-ALL cells adhesion. Moreover, as VCAM1 constitutive KD didn't influence B-ALL cells expansion, the lower number of leukemic cells in the adherence assay was likely not due to a decrease in the ability of B-ALL cells to grow. An experiment has been performed by others in which they co-cultured the same human B-ALL cell line we used but with a murine stromal cell line (Filshie et al., 1998). Though they reached a higher decrease in adhesion with the addition of the VCAM-1 antibody blocking than we did with VCAM-1 KO MSCs (44% versus 23%), we cannot directly compare the experiments as not only the method to inhibit VCAM-1 mediated adhesion was different but also the stromal cells

had another origin. Still, this experiment showed that B-ALL adhesion can be significantly reduced by addition of a VCAM-1 blocking antibody which we can use in future experiments as our positive control for decreasing VCAM-1 adhesion. TNFα was added to induce VCAM-1 expression and thereby more clearly allow determination of the impact of VCAM-1 ablation. In accordance to what was observed for B-ALL expansion, TNFα also negatively influenced B-ALL cells adhesion. Also in this adhesion assay, we could try the use of VCAM-1 KO MSCs that have been differentiated along the osteoblastic lineage, since that also is known to upregulate VCAM-1 expression. In the future, *in vivo* experiments where we implant mice with scaffolds seeded with VCAM-1 KO or VCAM-1 MSCs, osteoblasts as well as other potential MSC derivatives will be present, thus allowing a better and more relevant assessment of the role of stromal-derived VCAM-1 on B-ALL growth and adhesion (see also below). Together, our data obtained from this pilot experiment suggests that this adhesion assay is an elegant method to functionally test the effect of deletion or down-regulation of VCAM-1 on B-ALL cells *in vitro*.

To functionally validate the effect of CXCL12 ablation on B-ALL cells, we plan to test the migration of leukemic cells towards conditioned medium of CXCL12 KO MSCs, using the chemotaxis assay in which we showed in a pilot experiment that we can demonstrate CXCL12-dependent migration of B-ALL. For this purpose we already collected conditioned medium from these MSCs, but due to time limitations we haven't performed the experiment. Since these KO MSCs secrete only 20% of CXCL12 when compared to wt MSCs, we expect to obtain a decrease on B-ALL cells migration.

4.5. Transfection of MSCs aiming at generating KO MSCs

Though we were able to successfully generate KO MSCs by lentiviral-mediated transduction of MSCs with CRISPR/Cas vectors, this system has some limitations, including the (previously mentioned) lentivirus-mediated random genomic insertion and

the constitutive expression of Cas9, increasing the likelihood of the reported off-target effects (Cho et al., 2014; Cradick et al., 2013). To overcome such issues we aimed at introducing the CRISPR/Cas vector by transfection of MSCs. Despite the use of different transfection methods, none has so far allowed the successful generation of KO MSCs. The most promising technique was the Neon® Transfection, as both Lim et al. and Madeira et al. have demonstrated that it was possible to transfect MSCs with high efficiency using Microporation. Using a similar GFP vector and the same Microporation settings (settings A and B, respectively), we didn't obtain transfection efficiencies as high as in literature (Figure 22B), which could be due to electroporating a slightly higher number of cells, adding a lower amount of DNA (1 µg vs 2 µg in Lim et al.) and/or the MSCs being isolated from different tissues (BM vs umbilical cord blood in Lim et al.). The highest transfection efficiency we obtained was with setting C that was optimized in our lab to transfect IPS cells by Ester Rieter, Paul Coffer lab, UMC Utrecht. The biggest difference between setting C and the other settings is the duration and number of pulses, suggesting that shorter, but repeated, stimuli increases the efficiency of transfection. Nevertheless, transfecting MSCs with CRISPR vectors did not result in decreased expression of the respective target genes (Figure 22C). Since the experimental setup was similar to the GFP transfection's, the most likely reason for the poor outcome was the vector used for transfecting the MSCs, which was almost 3 times bigger than the sGFP vector. Taking into consideration our transfection data and what has been described in literature, the microporation efficiency can potentially be optimized in future experiments if we increase the amount of DNA and perhaps the number of pulses. We could also try different methods of transfection that have been demonstrated to be successful such as cationic liposomes (Madeira et al., 2010; Marquez-Curtis et al., 2013), PEI conjugated with amphipathic bile acids (Moon et al., 2014) or with alginate (He et al., 2012). Alternatively, we could also use non-integrating lentiviral vectors (Banasik and McCray, 2010; Sarkis et al., 2008) or adenoviruses (Meinel et al., 2006) for introduction of the CRISPR/Cas system.

4.6. MSCs colony forming ability

The KO efficiency obtained by lentiviral transduction with CRISPR/Cas vectors is quite high, but it would be more accurate to create a population of MSCs with 100% ablation of the target genes. For VCAM-1 KO MSCs, this can be achieved by sorting VCAM-1 negative cells. However, for the detection of VCAM-1 expression, MSCs need to be treated with TNFα, which may affect their MSC properties. As a first test, we investigated whether TNFα addition negatively influenced colony formation capacity by MSCs. By testing in a pilot experiment the ability of TNFα-treated MSCs to survive and expand as single cells, our data showed that TNFα treatment had no sizeable effect on MSCs colony forming ability (Figure 23C). We further analysed VCAM-1 expression of the populations obtained from single clone expansion to confirm that VCAM-1 was ablated in these single sorted MSCs, thus checking the cell sorting process based on VCAM-1 expression and investigating if the cells remained with similar VCAM-1 protein expression as before the sorting. We observed that TNFα-treated VCAM-1 negative MSCs remained VCAM-1 negative, indicating that it may be possible to generate a pure VCAM-1 knock out population of MSCs using this method. We could have drawn a stronger conclusion if we had been able to analyse the VCAM-1 expression on VCAM-1 positive cells to determine if the observed reduction of VCAM-1 expression is not due to single MSC clones expansion. Unfortunately, this was not possible as these cells died during the expansion process, for thus far unexplained reasons. We can argue whether the reason for cells dying was the upregulation of VCAM-1, but since we had similar outcomes from two unrelated experiments with different MSCs, we think this was not linked to VCAM-1 being highly expressed in these MSCs. All non TNFα-treated single clones of VCAM-1 KO MSCs which were able to expand were VCAM-1 negative. As 80% of VCAM-1 KO MSCs are VCAM-1 negative, it is not surprising that after TNFα treatment non TNFα-treated single clones of VCAM-1 KO MSCs don’t express VCAM-1 as this goes in accordance to probability. In addition, it indicates once again that VCAM-

1 deletion doesn't negatively influence the proliferation of MSCs. Besides repeating this experiment in the future to confirm the ability of VCAM-1 negative KO MSCs to grow as single clones, more tests should be done to verify that these MSCs maintain their characteristics after TNFα treatment. Ability to differentiate towards adipocytic and osteoblastic lineage, expression of characteristic surface and intracellular markers, proliferation capacity and, most importantly, the ability to create an ectopic BM niche *in vivo* are assays that could be performed to characterize these MSCs (Bühring et al., 2009; Romanov et al., 2005).

On the other hand, so far we haven't been able to develop a method in which all MSCs are KO for CXCL12. Our data, in which MSCs are able to grow as single clones, supports the idea that it may be possible to grow CXCL12 KO MSCs as single clones as well. By determining CXCL12 secretion of single clone-derived MSCs populations by ELISA, we may be able to generate pure MSC populations with total ablation of CXCL12. To create a less homogenous population of CXCL12 KO MSCs, we can pool different populations of CXCL12 negative MSCs. It is important to have a more heterogeneous MSC population as it was demonstrated that single colony-derived clonal BM MSCs frequently don't possess *in vivo* bone forming capacity (Prins et al., 2013).

4.7. Final remarks

In summary, the principal objective of this study is to use genetically manipulated MSCs which are knock down or knock out for stroma-derived factors to study their role in B-ALL growth, survival and migration using our state of the art *in vivo* model. Through the implantation of scaffolds seeded with the inducible KD MSCs into immunodeficient mice injected with B-ALL cells, we may be able to answer our main question (see also in conclusion and future perspective). Moreover, the importance of the stromal factors could be further confirmed if we would use a genetic manipulation system that would

enable the total deletion of the stroma-derived factor. Therefore, KO MSCs for CXCL12 and VCAM-1 using the CRISPR/Cas system were generated, as well as IL-7 KO MSCs. So far, we haven't been able to test if IL-7 is successfully knock out in these MSCs, which we are planning to do in the future by ELISA. As we confirmed that the CRISPR/Cas vectors we lentivirally transduced into MSCs are functioning, our next step is to generate KO MSCs using the same system but by transfection of MSCs. Until this date, we haven't been successful in generating transfected KO MSCs thus we will optimize transfection protocols and try different techniques. If succeeding on the generation of such MSCs and after isolation of KO MSCs to create a pure KO MSC population, we intend to implant as well scaffolds seeded with these MSCs in immunodeficient mice to first test bone formation ability *in vivo.* If this capacity is maintained, we would like to investigate the impact of ablation of these stromal factors in B-ALL growth. These studies will contribute to further understanding the role of B-ALL cells and stroma interactions for B-ALL growth and thereby open doors to new and more effective therapeutic approaches.

Chapter 5 Conclusions and Future Perspectives

5. Conclusions and Future Perspectives

Given the importance of BM stromal cells in providing factors to B-ALL cells which contribute to the perpetuation and development of the malignancies, it is vital to examine which stromal factors are most important in this support. Gaining insight will allow the generation of new and innovative therapies targeting the complex and intricate communication amongst them, also taking into account the physiological interactions between HSCs and derivatives with stromal cells, leaving these untouched. In order to achieve this knowledge, the development of more sophisticated models which resemble the human BM niche is required. Key findings in such models will certainly increase not only the survival rates but also decrease the cancer relapse, therefore expanding the life of B-ALL patients.

The combination of our state of the art humanized *in vivo* model with genetically manipulated MSCs which are knock down or knock out for stromal factors to determine the importance of certain stroma-derived factors in B-ALL growth is very promising. Further studies using the tools we have generated are likely to increase the current knowledge of the interactions in the B-ALL malignant niche and therefore improve existing therapies.

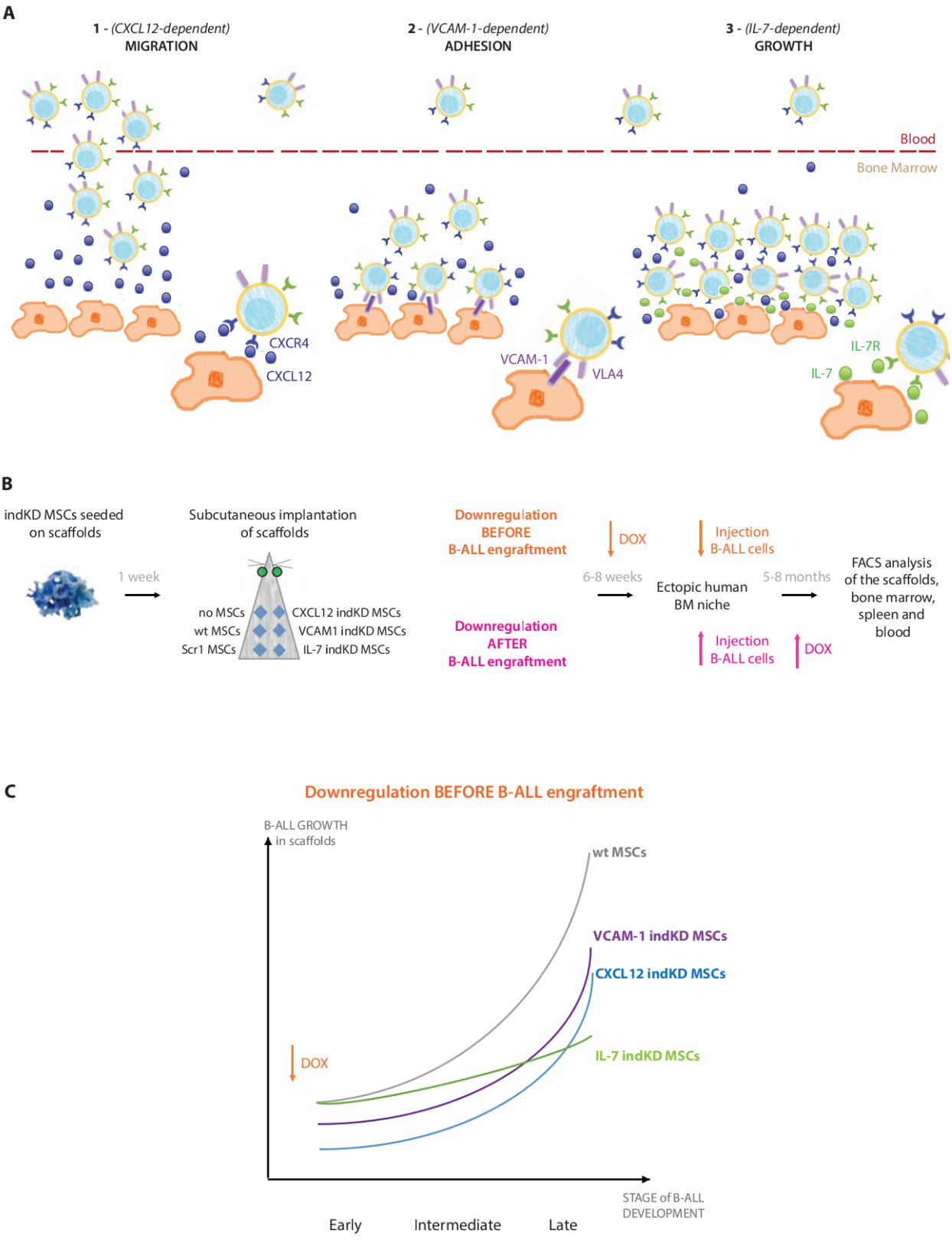
First, we should have in mind that there are many types of B-ALL that have different characteristics (Juárez-Velázquez, 2013). Therefore, the interactions of the B-ALL cells with the stromal cells and the dependence of leukemic cells to certain stromal factors may vary between them (Civini et al., 2013). Thus, depending on the donor of primary B-ALL cells, the outcome and relative importance of the studied stromal factors will probably be different. A more complete understanding of the role of these stroma-derived factors in B-ALL could be provided if we would test the leukaemia growth of cells derived from various patient derived B-ALL cells in the ectopic niches seeded with the genetically manipulated MSCs.

Using the generated indKD MSCs, we could investigate the *in vivo* role of these factors in B-ALL growth, adhesion and migration (Figure 24A). In a first experiment we would like to start KD induction after bone formation and before mice would be injected with either a B-ALL cell line luciferase labelled or with primary B-ALL cells (Figure 24B). The use of luciferase labelled B-ALL cell lines allows the tracking of the cells, but they are a homogeneous population thus they are not fully representative of the disease. Primary B-ALL cells are heterogeneous but the transduction of these leukemic cells is not as efficient, though some promising methods have been developed (Frecha et al., 2010; Mock et al., 2012). Migration of leukemic cells towards the human BM niches would be determined by bioluminescence as B-ALL cells would be transduced with luciferase (Figure 24C). This effect would be studied in the first phases of B-ALL development and analysed based on the localization and intensity of the luminescent signal.

Going from the assumption that we use a B-ALL cell line in which all B-ALL cells are equally sensitive for CXCL12, IL-7 and VCAM-1, the CXCL12 indKD MSCs-derived niche is predicted to showed a decreased level of luminenscence than wt or Scr MSCs, as it was previously observed by Juarez et al. that the chemotaxis and homing of NALM-6 cells in femoral BM of NOD/SCID mice was dependent on CXCL12 binding to CXCR4 (Juarez et al., 2009). Similarly we predict lower luminescence signals in VCAM-1 indKD MSCs, since leukemic cells are believed to adhere less efficiently to VCAM-1 indKD MSCs-derived ectopic niches (Filshie et al., 1998). In contrast, leukemic cells are still expected to engraft in IL-7 indKD MSCs-derived ectopic niches, thus the bioluminescence signal should initially be higher than the other indKD MSCs and more similar to control MSCs. However leukemic cells' growth should be compromised as IL-7 is an important growth factor (Duyn et al., 1999; Eder et al., 1990, 1996; Juarez et al., 2007), and thereby the signal intensity in later stages would be less intense than in the control MSCs. In addition to analysing bioluminescence, we plan to collect the scaffolds,

spleen, bone marrow and blood from the mice to confirm the findings observed by bioluminescence by determining the presence of hCD19+ cells by flow cytometry.

As we can induce the knock down, it would also be interesting to only down-regulate the factors after leukemic cells have engrafted in the BM niche to test if the decrease of expression of stromal factors would affect the growth of B-ALL cells. Again luciferase activity and flow cytometry analysis of scaffolds, spleen, bone marrow and blood would be used as our read-out. Though the leukemic cells would be allowed to engraft in the ectopic niches, knock down of factors responsible for migration and/or adhesion should still have a high impact on B-ALL development as CXCR4 and VLA-4 are crucial for the retention of B-ALL cells in the BM (Rettig et al., 2012). Consequently, it is expected that B-ALL cells which were present in the scaffold before DOX administration will be mobilized to the blood (Figure 24D). Downregulation of growth factors, such as IL-7, will likely also have an impact on B-ALL development, but not as evident and immediate. This approach, especially when using primary human B-ALL cells, would be the closest to the clinical situation as chemotherapeutic agents which block the action of these stromal factors, here mimicked by inducible downregulation, are administered after the prognosis of leukaemia, thus, after B-ALL cells engraftment and development. This experiment would be the first to demonstrate the importance of these stromal factors in a system that resembles the human BM microenvironment, thus making a huge contribution to furthering our understanding of the cell-cell interactions occurring in the malignant niche.


A
1 - (CXCL12-dependent)
MIGRATION
2 - (VCAM-1-dependent)
ADHESION
3 - (IL-7-dependent)
GROWTH
Blood
Bone Marrow
CXCR4
CXCL12
VCAM-1
VLA4
IL-7R
IL-7
B
indKD MSCs seeded
on scaffolds
1 week
Subcutaneous implantation
of scaffolds
no MSCs
wt MSCs
Scr1 MSCs
CXCL12 indKD MSCs
VCAM1 indKD MSCs
IL-7 indKD MSCs
Downregulation
BEFORE
B-ALL engraftment
DOX
Injection
B-ALL cells
6-8 weeks
Ectopic human
BM niche
5-8 months
FACS analysis
of the scaffolds,
bone marrow,
spleen and
blood
Downregulation
AFTER
B-ALL engraftment
Injection
B-ALL cells
DOX
C
Downregulation BEFORE B-ALL engraftment
B-ALL GROWTH
in scaffolds
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CXCL12 indKD MSCs
IL-7 indKD MSCs
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STAGE of B-ALL
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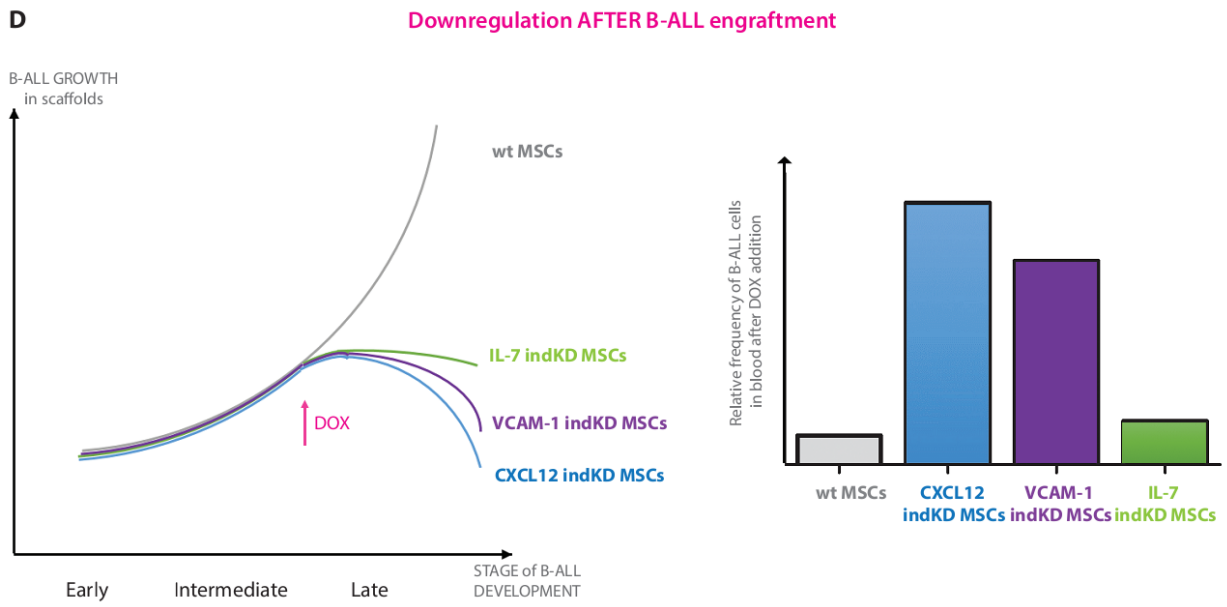

***Figure* 24– Model for B-ALL development in inducible CXCL12, VCAM-1 and IL-7 MSCs.** (A) Schematic representation of the role of CXCL12, VCAM-1 and IL-7 on migration, adhesion and growth of B-ALL. (B) Schematic representation of the mouse model. RAG2$^{-/-}$γc$^{-/-}$ mice implanted with wild-type (wt), Scrambled (Scr), CXCL12, VCAM-1 or IL-7 indKD MSC-loaded BCP scaffolds. Two approaches are represented: administration of Doxycycline (DOX) before or after injection of B-ALL cells. (C) and (D) Schematic representation of the predicted levels of B-ALL cells present in the scaffolds during the stages of B-ALL development on the different humanized ectopic niches when DOX is added either before (C) or after (D) B-ALL engraftment, and comparison between wt, CXCL12, VCAM-1 and IL-7 indKD MSCs. Relative frequency of B-ALL cells present in the blood shortly after DOX addition (D).

In subsequent experiments we could also try to ablate more factors simultaneously, which in theory could have a bigger negative effect on the growth of B-ALL cells. Accordingly, it has been reported that *in vitro* the CXCL12 chemotactic effect is enhanced by the presence of fibronectin, a component of the ECM to which VLA4 can also bind (Sbaa-Ketata et al., 2001). The influence of fibronectin in leukemia is poorly studied, but it appears as an interesting target of research as it has been shown to promote proliferation of pre-B cell lines in a mechanism dependent on VLA4/VLA5 (Harima et al., 2008) and VLA4 in turn is modulated by CXCL12 (Hidalgo et al., 2001). Thus, a possibly

important and yet not fully understood interaction involving fibronectin, VLA4 and CXCL12 (the last two already considered to be relevant in leukaemia and in the malignant niche) in ALL may exist and its modulation can be a promising therapeutic target. We could use our humanized *in vivo* model to study this interaction. For this purpose, scaffolds seeded with CXCL12 or VCAM-1 indKD or KO MSCs would be implanted into mice which would be later injected with B-ALL cells. To test the effect of the silencing of both pathways, the mice implanted with CXCL12 indKD or KO MSCs could be injected with anti-Integrin alpha4 antibody, Natalizumab, (Hsieh et al., 2013) or with anti-VCAM-1 antibody (Filshie et al., 1998). On the other hand, AMD3100, an CXCR4 blocking drug, could be administered to mice implanted with VCAM-1 indKD or KO MSCs (Yu et al., 2011).

In addition to studying the effect of simultaneously silencing two or more stromal factors, we could investigate the effect of combining stromal factors downregulation or ablation with chemotherapy on B-ALL growth. It has been demonstrated that combining antagonists or inhibitors of certain stroma-derived factors with chemotherapeutic drugs increases the survival of mice transplanted with B-ALL cells (Macanas-Pirard et al., 2012; Parameswaran et al., 2011; Yu et al., 2011). Our model is an elegant system to test the usefulness of combination treatment with ablation of stromal factors, as the ectopic BM would lack the expression of such factors and mice could be injected with chemotherapeutic drugs. Importantly, we would be able to test this for the first time in a humanized BM environment. Therefore, we could determine which combination would be more promising in B-ALL treatment.

So far we have investigated the negative effect of the stromal derived factors by down-regulating or ablating them in human MSCs. We hypothesize that if a certain factor is crucial for leukaemia growth, its decrease or deletion will have a negative impact on cancer proliferation. To complement this study we also could generate MSCs overexpressing such factors which would confirm its role on leukaemia expansion.

Besides, this is more representative of the clinical situation as in the malignant niche several factors have been described that have been upregulated rather than downregulated.

CXCL12, VCAM-1 and IL-7 have also been shown to be also implicated in crucial steps of haematopoietic and, in particular, B cell development (Ding and Morrison, 2013; Greenbaum et al., 2013; Namen et al., 1988; Zhu et al., 2007), but so far all the studies performed *in vivo* analysed murine rather than human haematopoiesis. Using our newly generated tools we could also expand our studies into determining the role of these stromal factors in human haematopoiesis. The experimental procedure is similar, the main difference being that we would inject mice with human CD34+ cells instead of leukemic cells, which previously have previously been shown to nicely engraft in the humanized scaffolds (Groen et al., 2012). To analyse haematopoietic support, we would then stain sections from paraffin embedded scaffolds with antibodies against markers specific of the different haematopoietic lineages. In addition, we could quantify the different populations by flow cytometry based approaches.

We have focused our study on CXCL12, IL-7, VCAM-1 and TSLP. Besides these stromal factors, many others have been described in literature. Indeed, it was recently reported that a new factor may also contribute to the creation of this special niche for leukemic cells: galectin-3 (Fei et al., 2013). This protein is expressed at significantly higher levels in BM and peripheral blood of ALL patients as compared to controls. Stromal cells can secrete galectin-3, which can later be detected in ALL cells. Furthermore, they showed that galectin-3 overexpression promotes chemoresistance. This is an exciting and relevant discovery of which the *in vivo* relevance still needs to be addressed, something for which our *in vivo* model is very well suited.

Moreover, until now, we have focused on stroma-derived factors that contribute to the progression of leukaemia. However, it is important to mention that there are other factors

which are able to induce the opposite effect. Interleukin-4 is one of those, in which both *in vitro* and *in vivo* studies have provided evidence that this cytokine inhibits the proliferation of B-ALL cells (Mitchell et al., 1996; Pandrau et al., 1992). Assessing the role of IL-4 using our model would be very advantageous as we would be able to confirm the importance of IL-4 in a setting more close to the human. These are some examples of additional promising factors that we could focus on in the future. In addition to these known factors, it is quite likely that new ones will be identified in the forthcoming future, for which we can then use our model to study their role *in vivo*. To determine their importance we would generate inducible KD and KO MSCs for these, besides developing overexpression systems, all of which would create human ectopic niches ideal to determine their role in B-ALL.

Each system of genetically manipulation we have used has its own advantages: the inducible KD system allows the control of the down-regulation, and the CRISPR/Cas system, the total deletion of a certain gene. Especially since the KO MSCs, similar to the constitutive KD MSCs, may be unable to form bone *in vivo,* the ultimate system would be one that would combine both, thus, an inducible KO CRISPR/Cas system. Several approaches could be taken to develop such inducible system. We could for instance design a plasmid carrying Cas9 which transcription would be regulated by a Tet operator sequence upstream to it, similar to the strategy of the inducible knock down system described in this thesis. We would therefore transduce the MSCs with a guideRNA-tetO-Cas9 plasmid carrying a puromycin-resistant gene. As this vector would be quite big, we could alternatively generate two vectors, one with the Cas9 inducible system and the other with the guideRNA. In this case, Cas9 would only be expressed upon DOX addition, since it is the constant presence of the endonuclease and not of the gRNA that leads to off target effects.

Finally, though this *in vivo* model allows the study of the human haematopoietic niche in the context of normal and malignant haematopoiesis, there is still room for improvement.

The BM consists of two niches: the endosteal and the vascular. In this model we are able to generate a human endosteal niche but the vascular components rise from murine endothelial cells. Thus, the generation of an ectopic BM with both human-derived MSCs and endothelial cells would be a more truthful representation of the human BM microenvironment. In theory, this could be achieved if we would seed the ceramic scaffolds not only with human BM MSCs but also with human endothelial progenitor cells. Chen et. al have developed a human extramedullary BM using human BM MSCs and human peripheral blood–derived endothelial colony-forming cells in Matrigel, indicating that we may be able to generate such ectopic human BM in our *in vivo* model as well (Chen et al., 2012). Use of our ceramic scaffolds would be more advantageous over their system as the cells would be better spatially organized as they wouldn't be encapsulated by matrigel and therefore they would more closely resemble the human BM.

As described, this humanized *in vivo* model has a lot of potential and allows answering many questions which will contribute to furthering understanding of B-ALL and other haematopoietic malignancies, as well as normal haematopoiesis. In this study we laid the groundwork for such investigation that ultimately will contribute to therapeutic advancements.

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