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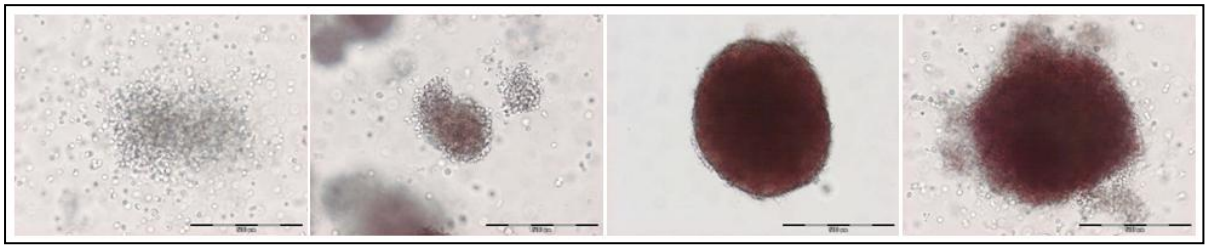

Elucidation of endogenous haematopoietic cytokines production in a three-dimensional biomimicry of human bone marrow

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Resumo

A recriação da hematopoiese *ex vivo* é importante para se obter um melhor entendimento dos mecanismos que a regulam bem como para o desenvolvimento das suas possíveis aplicações clínicas.

Previamente foi descrito um modelo 3D da medula óssea na forma de uma matriz de poliuretano revestida com colagénio do tipo I, capaz de expandir células mononucleadas do cordão umbilical em condições livres de adição de citocinas, soro animal e co-cultura com células do estroma. Foi avançada a hipótese que existia a produção de citocinas pelas células cultivadas, sendo o projecto em questão desenhado para pesquisar essa produção.

Estudos de viabilidade mostraram a capacidade do sistema para expandir as células durante quatro semanas. A análise dos cytospins mostrou a manutenção da capacidade de gerar múltiplas linhagens, além da preferência pelos eritrócitos nas culturas com suplemento de eritropoietina, resultados confirmados pela citometria de fluxo que registou um aumento dos marcadores eritropoiéticos combinados com uma perda de expressão do CD38, um marcador de linhagem mielóide. Nos eritrócitos notou-se um aumento em células imaturas com a queda do número de glóbulos vermelhos.

A expressão genómica confirma os resultados, com a activação de genes como o EPOR sugerindo o destino eritropoiético e com a expressão de TNF e TGF surgindo como uma explicação para a manutenção das células num estado primitivo. Descobriu-se ainda uma aparente pausa no mecanismo de transição de hemoglobina.

No futuro o uso de testes quantitativos de expressão genómica bem como análises proteómicas poderá ajudar a caracterizar o sistema mais pormenorizadamente.

Palavras chave: Hematopoiese, biomimética da medula óssea, células estaminais do cordão umbilical, cultura sem soro animal, cultura sem estroma, níveis fisiológicos de citocinas.

Abstract

Ex vivo recreation of the haematopoiesis process is of significant importance for the better understanding of the mechanisms behind it *in vivo* and for its clinical applications in cellular therapies and transplantations.

A 3D bone marrow biomimicry in the form of polyurethane scaffolds coated with type I collagen capable of expanding cord blood mononuclear cells under cytokine, serum and stroma-free conditions was previously developed. The hypothesis of endogenous production of haematopoietic cytokines responsible for providing the cues for cellular maintenance and differentiation was formulated, with this project envisioned to elucidate said production.

Viability results showed the capability to expand the cells for at least four weeks. Cytospin analysis reported the presence of multilineage capable progenitor cells with a preference for the erythroid fate in the cultures supplemented with erythropoietin, results confirmed by the flow cytometry data, where an increase of erythroid markers was accompanied by the drop in CD38, a myeloid marker. Within the erythroid lineage there was an increase in immature cells, with a drop in erythrocytes represented by the CD235a marker.

Gene expression analysis confirmed the results, with the activation of genes such as EPOR hinting towards the erythropoietic lineage and the expression of TNF along with TGF suggesting an explanation for the shift towards maintenance of earlier progenitor cells. An interesting arrest in the globin transition period was also detected.

Future work should focus on performing quantitative gene expression analysis as well as proteomic studies to characterize the *ex vivo* mimicry system and the mechanisms regulating it.

Keywords: Haematopoiesis, bone marrow biomimicry, cord blood stem cells, serum-free, stroma-free, physiological cytokine levels.

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Glossary

ACTB	Beta actin, a gene usually used as housekeeping control.
AKT	Protein kinase B, a multirole serine/threonine protein kinase.
AML	Acute myeloid leukemia, a haematological disorder.
ANOVA	Analysis of variance.
BFU-E	Burst forming units – erythroid, an early stage of erythroid development.
BM	Bone marrow, the tissue found in the interior of certain bones where adult haematopoiesis occurs.
CB	Cord blood, the blood that is recovered from the umbilical cord after childbirth because of its high concentration of stem cells used in research of haematopoietic disorder treatments.
CD	Cluster of differentiation, cell surface molecules used as targets for immunophenotyping, allowing the identification of cells.
cDNA	Complementary DNA, DNA synthesized from messenger RNA.
CFU	Colony forming units, used also to refer to the related assay.
CFU-E	Colony forming unit - erythroid, a middle stage of erythroid development.
CFU-GEMM	Colony forming unit – granulocyte/erythrocyte/monocyte/megakaryocyte, an early stage of haematopoietic development, representing the stemness of the culture.
CFU-GM	Colony forming unit – granulocyte/macrophage, the precursor of monoblasts and myeloblasts.
c-Kit	SCF receptor, highly expressed on early T-cell progenitors.
c-Mpl	TPO receptor, expressed by the megakaryocyte lineage.
CSF	Colony stimulating factors, glycoproteins responsible for stimulating certain cell lineages proliferation and differentiation.
CSF2	Colony stimulating factor 2 granulocyte-macrophage, a white cell growth factor.
DNA	Deoxyribonucleic acid.
ECM	Extracellular matrix, the part of animal tissue that provides structural support to cells.
EPO	Erythropoietin, a protein known to control erythropoiesis.
EPOR	Erythropoietin receptor, a membrane protein whose function is to bind erythropoietin, activating the related mechanisms and pathways.
FACS	Fluorescence-activated cell sorting, a type of flow cytometry that also can sort the cells based on their fluorescent characteristics.
FBS	Fetal bovine serum, the plasma portion of fetal blood, used as a growth factor containing complement for cellular culture.
FITC	Fluorescein isothiocynate, a derivative of the organic compound fluorescein used in flow cytometry.
FL	FMS-like tyrosine kinase 3 ligand, a small molecule involved in progenitor stem cell proliferation and mobilization.
flt3	FL receptor, known to be expressed in haematopoietic cells.

G-CSF	Granulocyte CSF, stimulates the growth of granulocytes.
GM-CSF	Granulocyte macrophage CSF, same as CSF2.
GMP	Good medical practices, a group of practices that must be adopted for clinical validation of the procedures and system.
GVHD	Graft versus host disease, a common complication with allogenic transplants.
HBB	Beta globin, a protein that along with alpha globin makes up adult haemoglobin.
HBG	Gamma globin, a protein that along with alpha globin makes up fetal haemoglobin.
HCA	Hydroxycarbonate apatite.
HepG2	Human liver carcinoma cell line.
HIF-1	Hypoxia-inducible factor-1, transcription factors that respond to hypoxia conditions.
High EPO	A defined culture condition with a 1.875 U/ml concentration of erythropoietin.
HIM	Haematopoietic inductive microenvironment, the conjugation of stromal, cellular and humoral cues responsible for HSC behavior *in vivo*.
HK	Housekeeping, a gene required for the basic function of the cells, therefore being consistently expressed.
HLA	Human leukocyte antigen, used to check donor compatibility for transplantation procedures.
HSC	Haematopoietic stem cell, multipotent stem cells that give rise to all blood cell types.
IL10	Interleukin 10, an anti-inflammatory cytokine.
IL11	Interleukin 11, known to stimulate megakaryocyte maturation.
IL1B	Interleukin 1 beta, a cytokine that acts as mediator in the inflammatory response.
IL6	Interleukin 6, a cytokine with both pro and anti-inflammatory effect.
IMDM	Iscove's modified Dulbecco's medium, a synthetic culture media suited for high-density cell cultures.
JAK2	Janus family tyrosine protein kinase 2, plays a role on certain signaling pathways.
K562	Leukemic cell line.
Low EPO	A defined culture condition with a 0.2 U/ml concentration of erythropoietin.
LPS	Lipopolysaccharides, large molecules from the outer membrane of Gram-negative bacteria, responsible for inducing immune responses in animal cells.
mAbs	Monoclonal antibodies.
MACS	Magnetic-activated cell sorting, uses immune-magnetic beads and magnets to sort the cells.
MAPK	Mitogen-activated protein kinases, responds to extracellular stimuli regulating various cellular activities.
MNC	Mononuclear cells, cells that exhibit a single, usually large nucleus. Includes HSCs.
mRNA	Messenger RNA, the transcription from the DNA template, its translation by ribosomes generates proteins.
MSC	Mesenchymal stromal cells, important for haematopoiesis and seemingly capable of suppressing alloresponses.

MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), also use to denote the cellular proliferation assay that uses this compound.
NHS	United Kingdom's National Health Service.
No EPO	A defined culture condition where there was no addition of exogenous erythropoietin.
OB	Osteoblasts, specialized fibroblasts responsible for bone formation.
PBS	Phosphate buffered saline, a water based, salt containing buffer solution used in biological research.
PCR	Polymerase chain reaction, a molecular biology technique used to amplify DNA fragments generating millions of copies of said fragment.
PE	Phycoerythrin, a red protein used as a fluorochrome in flow cytometry.
PE-Cy5	Phycoerythrin-Cy5, a tandem system combining phycoerythrin with a cyanine dye used in flow cytometry.
Pen/Strep	Penicillin and streptomycin, a combination of antibiotics used to avoid culture contamination.
PI3K	Phosphatidylinositol 3-kinase, intracellular signal transducer enzyme.
Project EPO	The study regarding the application of EPO as an exogenous cytokine to the previously developed bone marrow mimicry.
Project N	The study where the cytokine used to influence the bone marrow mimicry is stem cell factor.
Project R	In this work a cytokine cocktail composed of stem cell factor, fms-like tyrosine kinase 3 and thrombopoietin was used with the 3D PU scaffolds, evaluating its action on the system.
PU	Polyurethane, a polymer used in several industrial applications, composed of organic units joined by urethane links, chosen as the 3D scaffolds material due to its superiority in supporting and expanding cord blood mononuclear cells.
qPCR	Quantitative PCR, a designation for real-time PCR due to its quantification capabilities.
RCF	Relative centrifugal force, a relative acceleration unit used in centrifuges.
RNA	Ribonucleic acid.
RT	Reverse transcription, the biological process of transcribing RNA into cDNA
RT-PCR	A variation of PCR which includes RT as a first step.
SCF	Stem cell factor, a cytokine with established role in haematopoiesis.
SEM	Scanning electron microscopy, a type of microscopy using electrons to reach very high magnification values.
STAT5	Signal transducer and activator of transcription, mediates the cellular response to several ligands.
TBE	Tris/Borate/EDTA buffer, usually used on electrophoresis.
TGF	Transforming growth factor beta 1, a multifunctional cytokine.

TIPS Thermally-induced phase separation procedure, tissue engineering tool that uses phase separation procedures to generate porous scaffolds.

TNC Total nucleated cells, usually measured to assess the transplant success probability.

TNF Tumor necrosis factor alpha, a cytokine involved in the inflammatory response.

TPO Thrombopoietin, a cytokine produced in the liver, it stimulates megakaryopoiesis.

List of studied clusters of differentiation (CD)

Below is displayed a list of the CDs used in this work with a short description of the type of marked cells.

CD 33: Early myeloid progenitor cells.

CD 34: Multipotent haematopoietic stem cells and haematopoietic progenitor cells.

CD 38: Myeloid and lymphoid progenitors as well as mature lymphocytes and monocytes.

CD 45: Mature haematopoietic cells, excluding erythrocytes and platelets.

CD 68: Monocytes.

CD 71: Early erythroid progenitors.

CD 105: Endothelial cells.

CD 235a: Mature erythrocytes.

EPOR: Erythroid lineage cells in the intermediate stages of maturation.

1. Introduction

The formation of all blood cell types from a single multipotent progenitor is named haematopoiesis, a lifelong process that ensures the organism receives its daily needs for new cells through the expansion and differentiation of haematopoietic stem cells (HSC). These cells are known to be able to self-renew, undergo multilineage differentiation and to repopulate myeloablated hosts, making them interesting subjects for cellular therapies. Cord blood (CB) was shown to be a prime source for HSC over 20 years ago, with the recording of the first transplant using CB derived HSCs[1]. When transplanted into patients whose own bone marrow (BM) was depleted as a therapeutic step to fight some malignancies, such as leukaemias and genetic haemoglobin disorders, CB derived HSCs were shown to engraft to the patient and support their haematopoietic needs[2], with some clinical trial showing very promising results[3]. However limitations in faithfully recreating the *in vivo* haematopoietic process in *ex vivo* systems hinder the study of the biological mechanisms behind it, as well as the development of translational steps to apply the findings into clinical use, leaving the full therapeutic potential of ex vivo expanded CB HSCs untapped[2, 4].

In vivo, the primary site for adult haematopoiesis is the BM, and as such, many of the cues responsible for controlling the blood formation process are provided by this site. When defining the haematopoietic role of BM it is of critical importance to firstly acknowledge the notion of stem cell niches, entities found all throughout the characteristic 3D BM structure[4, 5]. Regarding haematopoiesis, the stem cell niches can be defined as the physical spot within the BM where HSC settle, coming into contact with the adhesion molecules and paracrine factors that provide the cells with the necessary stimulus for the activation of the survival and proliferation related pathways[5, 6]. The conditions required for the maintenance of HSCs function are aggregated under the denomination “haematopoietic inductive microenvironment” (HIM), which is composed by the combination of stromal, humoral and cellular components responsible for the control over HSCs self-renewal, proliferation and differentiation. The niches are mostly responsible for providing the structural cues in the HIM, acting along the stromal BM cells and some significant haematopoietic cytokines to regulate blood production within the BM[5]. These cytokines or growth factors are usually small peptides that can be delivered through endocrine or paracrine systems or be presented to the target cells attached to the membrane of the cell that produces them. They ultimately affect several HSCs processes, such as quiescence, apoptosis, proliferation, mobilization and differentiation[7], being an important part of the HIM. Despite the significant influence over haematopoiesis their production is normally fickle, with low circulation levels of short life span molecules[8]. Osteoblasts are an example of a type of cell usually found in the HSCs niches that produces some of these factors, however other hormones, such as erythropoietin (EPO) and thrombopoietin (TPO), are produced in the kidney and liver, relying on the circulatory system to deliver these biological messengers to the targeted cells.

With all the proliferation and differentiation required for the daily blood production, the most primitive stem cells must maintain the capability to self-renew, generating more multipotent HSCs so that the stem cell pool is not depleted. This process is also desirable when culturing the cell *in vitro*,

as unless the objective is to obtain a differentiated mature cell type, it allows the maintenance of the culture for longer time periods, practically expanding the original cell several times fold.

One of the reasons for the development of *ex vivo* cultures capable of mimicking the physiological BM conditions in order to expand the cells more efficiently comes from low number of HSCs that can be derived from a single CB unit, making them unsuitable for adult transplantation[2], leading to the development of novel clinical strategies, such as infusing a single patient with multiple units[9], and to the search for a viable expansion procedure.

The first attempts at *in vitro* cellular expansion and differentiation of HSCs was recorded in 1976 with a 2D static culture system[10]. Other configurations followed, namely well-plates, T-flasks and unmixed Teflon bags, however all of them were limited by their 2D configuration and its innate shortcomings, as the limited area available for cellular growth, the mass transfer difficulties and the scale-up issues[5]. Bioreactors were then proposed as a solution to the above mentioned problems, with the added bonus of the ability to constantly supply fresh media while removing the metabolites, as well as to monitor other critical culture parameters as pH and O_2 concentration. The first proposed bioreactor for HSCs culture was a continuous perfusion reactor[11], with other design proposals such as flatbed, grooved bed, airlift, hollow-fiber setups and rotating wall vessels being used as well.

Although successful, with a report of a rotating wall vessel achieving 435-fold expansion[12], these systems still require the addition of significant quantities of exogenous cytokines, greatly surpassing the *in vivo* levels, in order to achieve cell proliferation. These high concentrations fail to replicate the physiological environment, hindering the accurate mimic of the HIM. Also these cytokines are quite expensive and their extensive use turns a scale-up procedure cost prohibitive, affecting future applications for the system developed.

Co-culture with a stromal layer is also a popular strategy for HSCs expansion, as it mimics the stromal contribution to the HIM, a resemblance reflected on significant expansion values[13-15]. Nevertheless this approach increases the complexity of the culture, hindering any scale-up potential. It also doesn't replace the cytokine requirements and in the cases of xeno stromal cells usage, it presents an additional obstacle if the final goal is to translate the technology into the clinical field.

More physiological models of haematopoiesis were sought after, a need answered by the emergence of 3D systems specifically designed to mimic the physical and biochemical properties of the BM in an *ex vivo* environment[4]. These systems consisted of scaffolds made of several materials such as a tantalum-coated porous biomaterial[16] or a 3D porous biomatrix called Cellfoam[17]. The support shape is also quite variable, with some groups designing porous microspheres[18] while others focused on 3D cubes made out of polyurethane[19]. Professor Athanasios Mantalaris research group was responsible for the latter, a 3D *ex vivo* biomimicry of the BM made out of polyurethane scaffolds coated with type I collagen, designed first as a model for leukaemia studies, but later on successfully used in the expansion of CB mononuclear cells (MNC)[20]. This scaffold proved capable of expanding cells without the addition of exogenous cytokines and supporting stroma, and in later iterations, in the absence of animal serum as well. The hypothesis of endogenous haematopoietic cytokine production by the cultured cells was devised, regarding these molecules responsible for the achieved maintenance and proliferation of HSCs.

The present study was planned to elucidate said production, by replicating the serum and stroma-free culture conditions of the experiment where stem cell expansion was achieved. CB MNCs were seeded into the 3D model previously developed within the research laboratory[19] and grown in three different conditions, with no addition of exogenous EPO and with two near physiological concentrations of EPO, 0.2 U/ml[21] and 1.875 U/ml[18] to try to steer the culture into an erythroid fate. The culture was maintained for 28 days and samples were taken from time points every 7 days with PCR being performed on every sample as a gene expression assessment. Flow cytometry and cytospin analysis was also conducted to better characterize the system at hand.

2. Literature review

On average, a healthy adult has a daily production of 10^{11} to 10^{12} blood cells, replenishing the number of erythrocytes (red blood cells), leukocytes (white blood cells) and thrombocytes (platelets) lost due to cellular turnover. In an effort to maintain homeostasis, the production rate can be tuned in response to stresses such as injury or disease, ramping up in case of blood loss[22], for example.

The process through which these cells differentiate, mature and are released into circulation is called haematopoiesis, where all blood cell types are derived from a common progenitor, the HSC. This multilineage differentiation capability is complemented with the ability to self-renew, guaranteeing the maintenance of the stem cell pool while fulfilling the cellular needs of the haematopoietic system. Figure 2.1 displays the several different blood cell types that originate from the progenitor HSCs.

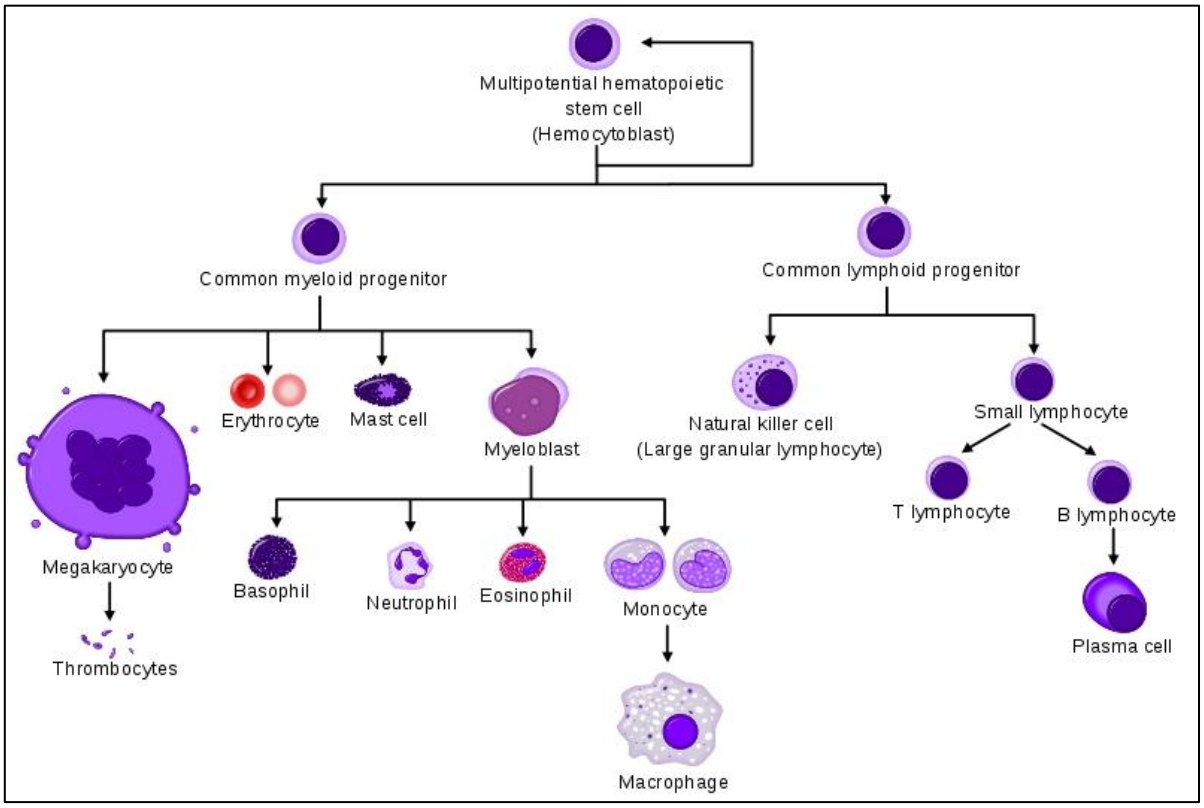

Figure 2.1. Haematopoiesis simplified overview. Generation of all blood cells from a common progenitor. Courtesy of A. Rad under CC BY-SA 3.0 license.

Haematopoiesis in humans occurs in different organs according to the development period, such as the yolk sac in the embryo and the liver and spleen in the fetus[23]. Prior to birth there is a decrease of HSC presence in those organs and the establishment of the bone marrow as the primary haematopoiesis site[24]. This is the final adult site for the maintenance of the larger populations of HSCs (Figure 2.2) with the lymph nodes serving as maturation and proliferation spots for lymphoid cells. There is also a change in the type of bones whose marrow supports this process, with the long

bones such as the tibia and femur loosing importance with the end of childhood and beginning of adult life. At this later stage the main sites remaining are the vertebrae, sternum, pelvis and cranium[25].

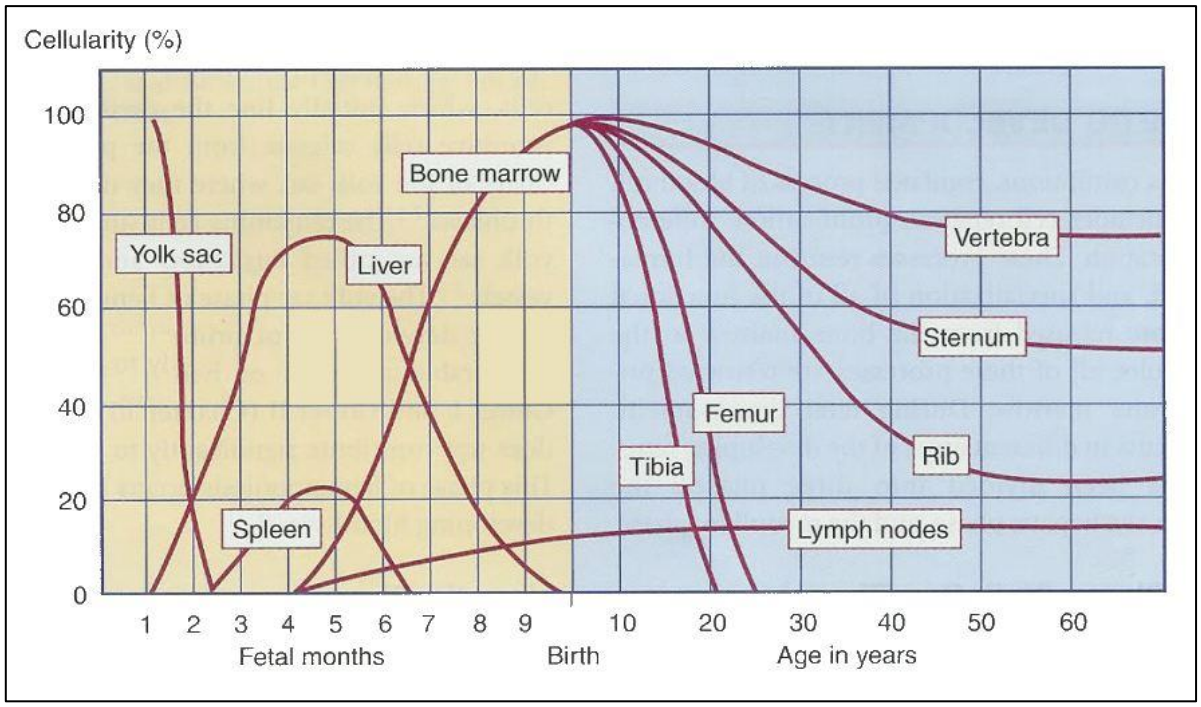

Figure 2.2. Sites of haematopoiesis. Graphic displaying the various sites for blood cell production along the various developmental stages of human life. Adapted from Rodak, B. F. *et al,* (2007)[26].

Clinically, HSCs are highly regarded due to their ability to reconstitute the haematopoietic and consequently the immune system of a patient whose bone marrow (BM) had to be ablated due to haematological diseases, hereditary disorders of the metabolism, congenital immunodeficiency, central nervous system diseases, malignant solid tumors or lower extremity ischemic disease[2, 27]. Nowadays HSCs transplant is still the main stem cell therapy available clinically with the first recorded bone marrow transplant dating back to 1959[28]. Technology evolved ever since, with the painful and very intrusive bone marrow aspiration techniques to retrieve the donor material being replaced by colony stimulating factor injections that mobilize HSCs into the peripheral blood[29], which is filtered through aphaeresis, saving the desired stem cells and returning the remaining components of the blood to the donor.

The previously described procedure is possible due to advances in the determination and classification of HSCs. Generally these cells are described as round non-adherent cells that express the surface glycoprotein CD34 and have low or non-existent levels of lineage specific markers such as CD33, CD38 and CD71[30]. A common and generally accepted phenotype that characterizes these progenitor cells is CD34+/CD38-[31], allowing the development of protocols and machinery, such as fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS), that isolates the HSCs for clinical applications or applied studies. Beyond the advantages of having a more clearly defined culture, this stem cell enrichment is also a way to remove immunogenic cells from allogenic donor samples and also other undesired contaminating cells[32].

Traditionally HSCs were collected from the bone marrow through the methods described previously[33]. Nowadays other sources of these prized cells are known, namely the peripheral blood[34], which after the cytokine treatment displays significant levels of HSCs, making their extraction viable through aphaeresis[35]. Another quite important technique used to obtain reasonable amounts of stem cells is from the cord blood gathered upon birth. This usually discarded remnant of the development and birthing process was found to be richer in both HSCs and committed progenitors of haematopoietic development than the peripheral blood[36].

2.1. The cord blood as a source of HSCs

With the first recorded case of using CB derived HSCs for transplantation dating back to 1989[1], CB has since established itself as one of the main sources for patients requiring bone marrow transplantations, as some studies show that HSCs isolated from different origins exhibit dissimilar reconstitutive capabilities, with cells displaying the same immunophenotype leading to different results. Research work then concluded on the advantages of CB isolated HSCs over the ones isolated from BM, with, for example, Hao *et al*[37] showing that CD34+/CD38- cells from CB proliferated more quickly, showed a higher cloning efficiency and generated more progeny than the similarly marked cells from BM when stimulated with interleukin-3 (IL3), interleukin-6 (IL6) and stem cell factor (SCF). Other studies showed a similar advantage when cultured with interleukin-11 (IL11)[38] or other cytokine cocktails[39] with the differences in the expansion factor as high as 8 fold. This apparent contradiction where identical cells behave dissimilarly most certainly comes from the cellular disparities between the samples, namely the proportions in early multipotent progenitor and late lineage committed cells as well as the other non-HSCs cells dragged along when the sample is extracted. A usually cited reason for this phenomenon is the biological younger age of the CB cells when compared with the adult counterparts, implying a longer telomere length as the explanation of the increased capacities[40].

CB HSCs are also credited with being less immunogenic, therefore less prone to cause complications such as graft versus host disease (GVHD) when used in clinical therapies[41]. The fact that blood production and the immune system are still immature in the newborn helps to account for this decreased immunoreactivity[42], making it theoretically more suitable for transplants than the adult peripheral blood or bone marrow.

More practical advantages of using CB as a source of HSCs for therapies include their very quick availability due to the storage of the CB units in blood banks, in contrast with the bone marrow and peripheral blood donations, which require a waiting time for the collection procedure to happen and the risk-free collection procedure for the donor, a disparity when compared to the brutal bone marrow collection technique. When a patient fails to find an appropriate donor in the existing bone marrow registries, CB is revealing itself to be an increasingly popular alternative source.

However there are some shortcomings inherent to the usage of CB. The primary concern is the low total progenitor cell number, especially when comparing to the dose required for a successful engraftment. This limited the use of single unit transplantation procedures to children under 20 kg, severely restraining the applicability of the procedure.

The main advantages and limitations of using CB in unrelated donor transplants when compared to traditional BM procedures are listed on Table 2.1, with the inclusion of the ability to overlook human leukocyte antigen (HLA) disparities not feasible before, greatly increasing the chance for a patient to find a suitable donor. From the disadvantages both the delayed engraftment and T-cell immune reconstitution are thought to derive from the stemness or primitiveness of the cell being used, as HSCs progenitors are responsible for the long-term engraftment capability.

This prompted the development of novel strategies, such as the use of multiple CB units for a single patient[9], lowering the engraftment time and therefore improving on the previous technique; the co-infusion of other cell types with the HSCs, with the immunomodulatory capabilities of mesenchymal stem cells lowering the GVHD probability[43], increasing engraftment success; the *ex vivo* expansion of CB HSCs through cellular culture[44], augmenting the number of available stem cells.

Since it was determined to be a viable source of HSCs about 20 years ago, CB shifted from being discarded as waste to become treasured as the building block in therapies for several malignancies. The observable trend nowadays is an attempt to translate previous knowledge into the regenerative medicine field, pioneering the use of CB HSCs in non-haematopoietic therapies, with studies occurring on liver injury and regeneration[45], stroke treatment[46] and type 1 diabetes[47] to name a few.

Table 2.1. Evaluation of cord blood as a cell source for transplantation. These advantages and disadvantages are when compared with adult donor stem cell transplantation. Both are referred to unrelated donors. HLA stands for human leukocyte antigen. Adapted from Liao, Y. *et al*, (2011)[48].

Advantages of CB for transplantation	Disadvantages of CB for transplantation
	Difficult to achieve sufficient total nucleated cell dose in larger recipients using single cord blood unit
Decreased risk to donor	Delayed engraftment and increased risk of graft failure
Faster procurement	
Lower incidence of grade II_IV acute GVHD	Delayed T-cell immune reconstitution
Enhanced ability to cross donor-recipient HLA disparities	Increased risk of transplant-related mortality
	Increased costs of hospitalization
	No obvious cell source for post-transplant donor lymphocyte infusions

2.2. Bone marrow structure

After determining the main cell source for the work at hand it is imperative to delve deeper into the haematopoietic process and the structure where said process occurs during human adulthood, the BM. This tissue is defined as being formed of cells, both haematopoietic and non-haematopoietic, organic matter as the extracellular matrix (ECM) proteins and inorganic matter, mainly hydroxycarbonate apatite (HCA) existing as a deposit within the existing structure[5]. These components make the elastic material found inside the trabecular bone, an osseous tissue type also referred as spongy bone usually present at the end of long bones and vertebrae. This tissue is served by a complex network of blood vessels, accounting for the high vascularisation profile of the BM

BM cells exist alongside stromal cells, with the latter playing important roles in the regulation of the haematopoietic function of the former. Endosteal, adipocytes and cells from the vasculature are the main stromal cell types, being responsible for the production of ECM proteins such as collagen, structural macromolecules as proteoglycans and other proteins relevant for cell attachment procedures, as fibronectin, haemonectin and laminin[49]. These stromal elements along with BM components and humoral cues compose the structure identifiable as the haematopoietic inductive microenvironment (HIM), the entity behind HSC self-renewal, proliferation and differentiation regulation.

Physically, BM usually has two major interfaces, the one with the inside of the bones and the other with the vascular endothelium of the blood vessels scattered through the marrow. The bony tissue is lined with connective tissue layers, the periosteum on the outside and the endosteum separating BM from bone[50]. In this inner boundary it is possible to find layers of osteoblastic stromal cells. Osteoblasts (OB) are cells of mesenchymal origin that have the important function of forming new bone, a necessary work due to the action of osteoclasts in removing bone tissue, a phenomena called bone resorption. Along with the secretion of proteins necessary for bone remodelling, osteoblasts are known to regulate osteoclast differentiation from their haematopoietic origin[51] as well as influencing the HIM[52], a discovery hinted by the previously known capability to support haematopoietic cultures *in vitro* as well as *in vivo*[53].

As for endothelial cells, these line every blood vessel existent, and as such, inside the bone cavity they serve as the frontier between BM and the circulating blood. This imbues these interface cells with critical relevance to haematopoiesis, as it is through this barrier that HSCs derived mature blood cells enter the circulation[54]. BM is immersed in a network of blood vessels, namely sinuses, which are capillary-like structures but with increased pores (fenestrations) and inter-cellular gaps, greatly increasing their permeability[5]. This augmented capability comes in hand for the high volume cellular trafficking related to haematopoietic production. Studies also show that BM sinusoidal endothelial cells display different properties than their non-BM counterparts, being capable of supporting HSCs culture *in vitro*[55].

2.2.1. HSCs niches

BM has a complex 3D structure and the site where HSCs settle within that structure is referred to as a niche. However these are more than just convenient resting places for the stem cells to hang out in, they provide the cells with the necessary cues for HSCs survival and proliferation through adhesion molecules and paracrine factors[5]. In fact these niches are the physical locations that provide the HIM to the HSCs, therefore being critical for the correct behaviour of the BM.

Recent evidence points to the existence of two niches for the HSCs inside the bone marrow, each with specific characteristics. Figure 2.3 illustrates both niches inside the BM.

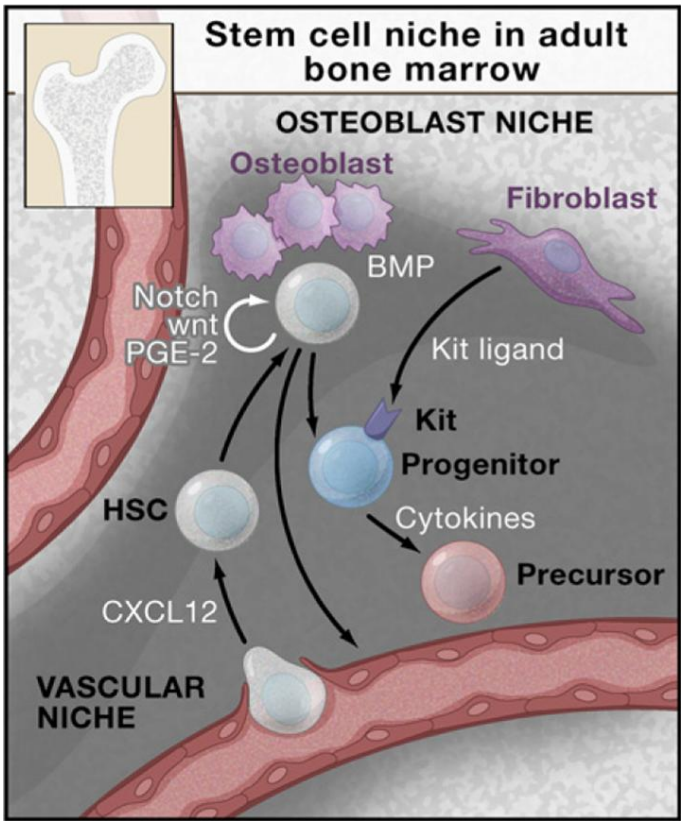

Figure 2.3. Haematopoietic stem cell niches in bone marrow. This illustration provides a clear picture on both the osteoblastic and vascular niches organization. It serves as a good example of the haematopoietic inductive microenvironment. Orkin, S. *et al*, (2008)[27].

The OB niche, also known as endosteal or as storage niche represents a location where HSCs are maintained adherent to the OBs lining the endosteum in an undifferentiated and quiescent state[56]. This cellular arrest at the G_0 step of the cell cycle was shown to be mediated through the interaction of certain molecules present on the surface of OBs and their respective receptors expressed by HSCs, namely N-cadherin, angiopoietin-1 and thrombopoietin[25]. Studies confirm the importance of these bone producing cells to the HIM, with the discovery of a direct relation between an increase in OBs and the numbers of progenitor HSCs[52].

Contrastingly, the vascular niche appears to cater to more active stem cells, with their adhesion to the vasculature endothelial cells allowing them to mobilize more rapidly in response to stresses or stimulus[5]. Although this quick response capability is present, there are still some debates on if this is an actual niche where a stable population of HSCs grows and differentiates in response to the HIM, or if it is just a place every cell has to go through on its way to reach the circulation.

2.3. Haematopoietic cytokines

Cytokines are defined as being small peptides that when secreted influence several important processes such as immunity, inflammation response and haematopoiesis. Their role in blood cell production turns them into an integral part of the HIM, regulating cell quiescence, apoptosis, proliferation, mobilization and differentiation[7]. Even though they regulate such important procedures, they are quite ephemeral molecules, present only for a short time span and in very low

concentrations, in the pico to nanogram per millilitre range[8], and as such their action range is short. Unsurprisingly some of these proteins are produced in the BM by cells of the previously discussed stem cell niches, the osteoblasts, the endothelial cells lining the blood vessels and other stromal cells existent on the bone cavity, such as fibroblasts[7]. However not all haematopoietic factors follow this paracrine system, others such as EPO are produced in the kidneys and must be delivered to the HSCs in the BM through the circulatory system, an endocrine process. This production varies according to the needs of the organism, the presence of stress or trauma situations causes the basal levels to quickly modify, sometimes increasing up to 1000 fold, inducing the required response of the haematopoietic system. Using EPO as an example, when the body detects an anemia or hypoxia situation, the kidney increases the production and secretion of EPO, stimulating the formation of new blood cells. As such, haematopoietic cytokine production does not occur in a single organ or tissue, in fact, for colony stimulating factors (CSF) multiple organs were found to be able express them, sometimes with a single organ producing multiple CSF types simultaneously[57].

In addition to the paracrine and endocrine system delivery, some of these molecules, with stem cell factor (SCF) as an example, were documented being displayed in an active form anchored to the membrane[58].

The conversion of cytokine presence into cellular responses is mediated through the adhesion of these molecules to the specialized receptors present on the surface of HSCs. Each receptor has a cytoplasmic domain responsible for the initiation of the signalling cascades or processes required for the response it mediates. Studies have classified the types of receptors into three distinct groups: firstly those with a tyrosine kinase domain in the cytoplasmic region, for molecules such as fibroblast growth factor, endothelial growth factor, colony stimulating factor-1, tyrosine kinase and c-kit receptor; secondly the receptors with a serine/threonine kinase domain, complements of molecules such as transforming growth factor beta (TGF); and thirdly the type I cytokine receptors, for commonly referred haematopoietic molecules such as the interleukins, various colony stimulating factors and hormones[5].

Some of these cytokines directly affect the physical interactions between HSCs and the surrounding microenvironment[59], affecting the adhesive capabilities of the cells through the activation of integrins. This role is played by stem cell factor (SCF), granulocyte monocyte colony-stimulating factor (GM-CSF) and interleukin 3 (IL3)[60]. The last two cytokines are also known to induce cellular proliferation[61] while SCF along with flt-3 helps averting the apoptosis mechanism[62]. TGF and tumor necrosis factor (TNF) were found to be fundamental for engraftment phenomenon, modulating cellular cycle activity[63]. Granulocyte colony-stimulating factor (G-CSF) and TNF are examples of polyfunctional cytokines, actuating on multiple cellular systems. TNF also modulates its effect according to its concentration *in vivo*, shifting from an inhibitory role to the exact opposite, potentiating the related physiologic processes[64].

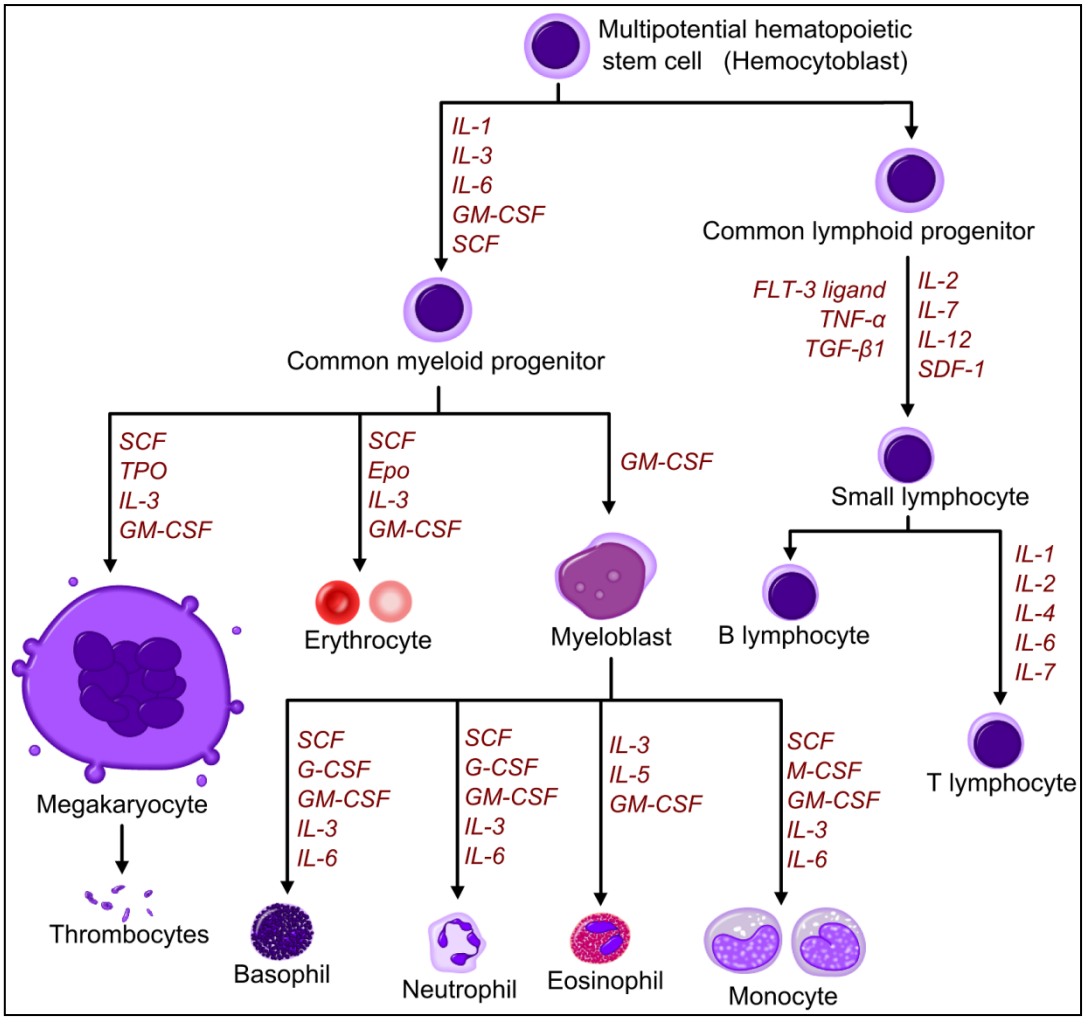

Figure 2.4. Haematopoietic cytokines. An illustration of the various haematopoietic cytokines and the final blood cells they are related to. Stem Cell Factor (SCF), Thrombopoietin (TPO), Interleukin (IL), Granulocyte Macrophage-colony stimulating factor (GM-CSF), Erythropoietin (EPO), Macrophage-colony stimulating factor (M-CSF), Granulocyte-colony stimulating factor (G-CSF), Stromal cell-derived factor-1 (SDF-1), ligand= FMS-like tyrosine kinase 3 ligand (FLT-3), Tumour necrosis factor-alpha (TNF), Transforming growth factor beta (TGF). Courtesy of A. Rad under CC BY-SA 3.0 license

Each cytokine can be specific for a determined haematopoietic lineage, with, for example GM-CSF for the myeloid lineage and thrombopoietin for megakaryocytes and the derived platelets. There are also cases of cytokines that exert effects on several different lineages, as IL3 with different myeloid fates, and some cell types that require the concerted action of multiple cytokines to generate the required responses[5].

A more detailed description of the cytokine used in this project, EPO, follows, as well as a short reference to the ones used in the parallel projects being developed in the laboratory, SCF, FMS-like tyrosine kinase 3 ligand (FL) and TPO.

2.3.1. Erythropoietin

Human EPO consists of a 165 amino acids long protein with three N-linked and one O-linked glycans. Both glycosylations seem to be redundant, with the O-linked glycan missing in other organisms and the N-linked showing little effect on *in vitro* activity of EPO. However the latter seem to

be important for a correct production, secretion and interaction with EPO receptor (EPOR) of the molecule[65].

This hormone is mainly produced by the liver in the fetal development stage and by the peritubular interstitial cells in the kidneys in adults, and stands as a major regulator of erythropoiesis, stimulating the proliferation and differentiation of burst forming unit erythroid cells (BFU-E), early progenitor erythroid lineage committed cells, and colony forming unit erythroid cells (CFU-E), more developed erythroid progenitors, closer to erythroblast development stage[66]. The major stimulus responsible for EPO production in the adult seems to be oxygen concentration, namely by augmenting production under hypoxia conditions.

An increase in EPO levels is achieved through the induction of the respective gene expression, in this case due to the accumulation of the hypoxia-inducible factor-1 (HIF-1) transcriptional factor[67]. The EPO gene contains, as most of the O_2 sensitive genes, a sequence called hypoxia-response element (5' RCGTG 3'), a HIF-1 binding site, responsible for activating gene transcription. HIF-1 transcription is not O_2 dependant, being stable regardless of oxygen concentration, however its mRNA is degraded quickly due to the action of the O_2 requiring proline hydroxylase. Under hypoxia the proline hydroxylases are inactivated due to the inexistence of O_2 therefore stabilizing HIF-1 mRNA and consequently activating the EPO gene. EPO mRNA is also stabilized by the lack of O_2, contributing towards final EPO production levels[68].

The boosted red cell production will eventually improve O_2 supply, returning normoxia conditions and ending the HIF-1 mediated stimulus to EPO production with the reestablishment of homeostasis.

As a hormone, after production EPO is secreted, travelling from the kidneys into the bone marrow with the plasma in the circulatory system. After arriving to the final destination, EPO binds to its respective receptor present in the surface of the target progenitor cells of erythroid origin, EPOR. This receptor is characteristic of erythroid lineage cells in the middle development stages, both early multipotent progenitor cells as well as more mature erythrocytes are devoid of such surface molecules[69]. The binding triggers a conformational change on EPOR, physically approximating it to Janus family tyrosine protein kinase 2 (JAK2) molecules, activating them through transphosphorylation[70]. JAK2 then phosphorylates eight tyrosine residues on the EPOR domain in contact with cellular cytoplasm, which activate various Src homology 2-domain-containing signaling peptides through tyrosine phosphorylation[71]. One of these proteins is described as a signal transducer and activator of transcription (STAT5), that after activation dimerizes and translocates to the nucleus, activating several genes[71]. Bcl-x is one of said genes[72], being an apoptosis inhibitor, and in that role important for erythropoiesis.

Other activated proteins through the JAK2 molecules include, for example, Shc, which may play a role on erythroid proliferation, phosphatidylinositol 3-kinase (PI3K), helping erythroid cell survival and phospholipase C-γ1 believed to have a role also in cellular proliferation[73-75].

Recent studies seem to suggest that EPO has other roles than the originally determined haematopoietic one. Since said roles deviated from the focus of this work, only a brief summary of the discovered production sites and possible functions will be provided with Table 2.2. These results are

quite interesting, as they are quite different from the original role established for EPO, showing its production on tissues quite distinct from both liver and kidney.

Table 2.2. Proposed non-haematopoietic roles of erythropoietin. Several different production sites are listed along the theoretical stimulus required to activate EPO production. Sasaki, R. (2003)[76].

Production sites	Possible functions	Stimuli of EPO production
Fetal liver	Erythropoiesis	Retinoic acid
Kidney	Erythropoiesis	Hypoxia
Central nervous system	Neuroprotection & neurogenesis	Hypoxia
Retina	Neuroprotection	Hypoxia
Uterus	Angiogenesis	Estrogen & hypoxia*
Oviduct	?	Estrogen & hypoxia
Testis	Steroidogenesis	Hypoxia
Epididymis	?	Hypoxia
Mammary gland	Gut maturation	?

*Estrogen is required for hypoxic induction.

2.3.2. Stem cell factor

This cytokine is also known as steel factor or kit-ligand, due to its affinity towards the membrane receptor c-Kit (CD117). It exists under two different configurations originated through alternate DNA splicing, as an extracellular soluble protein with a 165 amino acids long chain and as a membrane anchored peptide, 220 amino acids long[77]. The shorter soluble form results of a proteolytic cleavage, while the longer membrane bound counterpart lacks said cleavage site encoded in exon 6[78], a relevant difference as studies show that both isoforms induce different biological responses[79]. These two forms are expressed in various tissues, the rate varying from the tissue type and the developmental stage. BM stromal cells, namely fibroblasts are a good example of a cell type with recorded expression of this cytokine[80]. Its production seems to be constitutive, with no known evidence of positive or negative regulation mechanisms, the lack of which account for relatively high concentration values in the serum under regular steady state conditions[81].

To understand SCF effects on haematopoiesis it is important to refer to the cells that express its receptor, c-Kit, as those will be the ones susceptible to the cytokine presence. Earlier studies determined the presence of this receptor on 1 to 5% of all bone marrow cells, with a focus on primitive progenitors (CD34+/CD38-), megakaryocytes and their derivates, platelets[82]. Later research confirmed expression on HSCs and later myeloid progenitor cells, as the stages preceding dendritic cells, B and T cells[83]. The last two are known to lose c-Kit expression upon maturation. Other non-haematopoietic cells are also known to produce this ligand, reflecting its important role in other key biological functions as spermatogenesis and melanin formation[84]. There is an agreement on the role of this system of SCF and c-Kit on early haematopoiesis, as most CD34+ cells seem to express this membrane receptor[85].

When a SCF molecule binds with the respective c-Kit receptor, the latter homodimerization induces the phosphorylation of tyrosine residues in the receptor, allowing the docking of Src homology 2-domain-containing molecules, which serve as signal transducers for various pathways[86]. One of said pathways is the PI3-kinase, whose p85 regulatory subunit interacts specifically with the c-Kit's phosphorylated tyrosine residue 719, ultimately activating protein kinase B (AKT) through phosphorylation[87]. Activated AKT is known to enhance survival and proliferation, as well as indirectly inhibiting pro-apoptotic molecules, therefore being important for the targeted cells development.

This cytokine was shown to act in combination with others to induce biological responses, with TPO and SCF displaying an integrated role in HSC proliferation[88] and the G-CSF with SCF combination being important in mediating HSC and derived cells mobilization[89]. Under hypoxia conditions, SCF was shown to act along with EPO, regulating the stress response erythropoiesis[90], with the association of both molecules receptors.

2.3.3. FMS-like tyrosine kinase 3 ligand

Expression of this small cytokine was found in various tissues, both of haematopoietic and non-haematopoietic origin, with brain, heart, kidney, testis and lung as examples of the latter[91]. Despite the seemingly wide general distribution of FL mRNA, the respective protein production was only registered in BM stromal fibroblasts and by T lymphocytes[81]. As with SCF, FL has both a soluble and a membrane bound isoform, but unlike the previously described cytokine both seem to play the same role when bounding to the FL receptor, flt3.

FL concentration *in vivo* is kept in check through a unique process, controlling the cytokine's supply to the organism by regulating its release from the cytoplasm of FL producing cells. In fact during normal haematopoietic conditions the FL gene is constitutively expressed, but actual protein levels are quite low both in circulation and bound to the cell surface due to the retention of the preformed FL protein inside the cells. In T cells and fibroblasts this withholding occurs along with proteins from the Golgi and trans-Golgi compartments. This whole process suggests the existence of a yet unknown control mechanism restricting the release of FL during steady state haematopoiesis conditions. BM failure situations where there is a decrease in HSCs up-regulate FL expression, suggesting the important role of this cytokine in early haematopoiesis required for the HSCs niche recovery. FL concentrations return to normal values following the restoration of the stem cell regular quorum.

Both the growth and maturation of HSCs and of T, B and natural killer cells was shown to be related to FL action. Studies also determined the capability of this cytokine to positively affect the number of mature dendritic cells in the organism[92]. Despite these findings numerous studies demonstrated that the primary function of FL was to act along with other cytokines in triggering and enhancing the respective responses. When combined with SCF and TPO, this molecule cocktail was shown to induce CD34+ cells expansion in stroma-free cultures. Its usage with GM-CSF and G-CSF increased the number of generated colonies and when present along interleukins such as IL7, IL3 or IL11 the respective biological effects were enhanced[81].

FL's receptor belongs to the same family as the previously mentioned c-Kit, the class III tyrosine receptors[62], the both being structurally related. It is expressed mainly on early haematopoietic progenitors, reinforcing the notion that this cytokine is important for HSCs and the primitive haematopoiesis steps. The difference between flt3 and c-Kit lies on the fact that the latter is found on more basophilic lineage cells. Eventually there is an overlap on both receptors expression and consequent biological effect, however studies show that these play additive complementary roles, not redundant[81].

2.3.4. Thrombopoietin

This cytokine, also referred to as c-Mpl ligand, is best known for its haematopoietic role, more specifically as a stimulator of megakaryocyte proliferation and maturation into platelets. In fact, TPO was determined to be the most important thrombopoietic growth factor[93].

Structurally TPO is a 332 amino acids protein, highly glycosylated and with two distinct functional sections. The N-terminal shows similarity to EPO, being sufficient for the induction of the related biological responses. The C-terminal contains several O-linked glycosylation sites, being quite distinct in between species, and conferring stability and potency to the TPO molecule while improving its biosynthesis and secretion[94].

TPO production occurs mainly in the liver and kidneys, respectively in the parenchymal and sinusoidal endothelial cells[95, 96]. The liver is where most of TPO originates, however, studies shown the existence of TPO expression and translation into proteins in other cell types, with BM stromal cells as an example[97]. Most of the developed work seems to indicate that TPO is constitutively expressed and translated, as its mRNA levels show no relation with platelet in circulation count. Nevertheless the TPO levels in the plasma show an inverse relation to the same count, suggesting that the platelet mass affects TPO concentration, and consequently its biological effects by binding the circulating cytokines to the respective c-Mpl receptors[98]. Other studies suggest that megakaryocytes also play a role on this regulation of TPO levels, pointing out that there seems to be no sensing and regulatory system for TPO gene expression[99].

Other than the already established importance for development of megakaryocytes and platelets, being crucial for the full development of these cells, TPO is also known to act in synergy with other haematopoietic factors in order to stimulate blood cell production[100]. With FL, SCF and IL3 studies show the capability to induce cellular proliferation of HSCs, and along with only FL and SCF the maintenance of multipotent progenitors in culture is achieved. In fact TPO seems to play a role in CD34+/CD38- cells survival and proliferation[101].

TPO's receptor, c-Mpl regularly exists as a homodimer, with two tails on the cytoplasmic domain that are brought to close proximity by a conformational change that occurs when TPO binds to the extracellular domain. The JAK2 molecules on the tails trans-autophosphorylate due to the close proximity between both of them, becoming biologically active. Through the activation of signaling peptides several downstream pathways are induced, with STAT, MAPK and PI3K as an example.

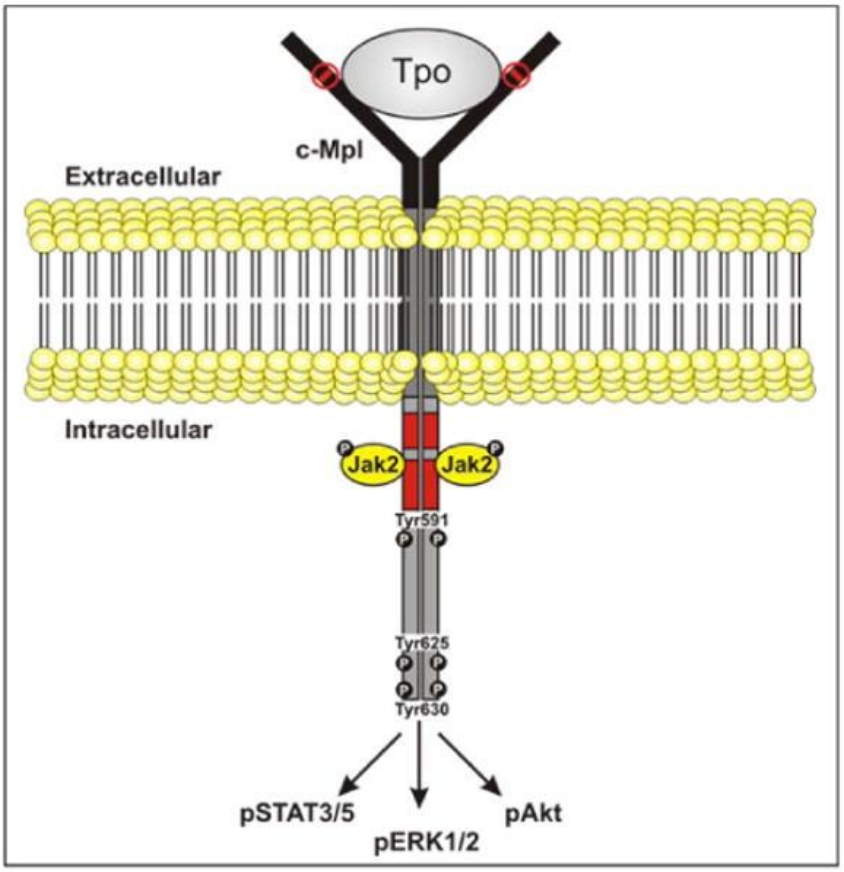

Figure 2.5. TPO signalling illustration. There are several similarities with the pathways of the previously described cytokines, as the activation of PI3K as with EPO and the phosphorylation of JAK2 molecules as an intermediate step on the translation of cytokine presence to biological response. Geddis, A. E. (2010)[102].

2.4. Haematopoietic stem cell culturing

As discussed before, CB is an excellent source of HSC for clinical therapies and research efforts, it only falls short of perfection due to the relatively low numbers of progenitor stem cells that can be isolated from a single CB unit. In an attempt to overcome this significant hurdle scientists dedicated themselves to developing culture systems capable of expand the initial HSCs. These systems could be directed towards maintaining multipotent early progenitor cells or differentiating them towards a certain lineage, as required for the final application. When applied to blood transplant the objective is to achieve a necessary amount of cell for an adult patient through a relatively simple protocol that could be scaled-up for industrial production.

Beyond the easily discernible benefits of developing a method to multiply the number of sorely sought after HSCs, the recreation *ex vivo* of the haematopoietic process would prove to be an invaluable tool for the better understanding the *in vivo* equivalent as well as its host tissue. Due to the importance of both in several relevant malignancies the discovery of an accurate model of the HIM has incalculable value to translational science.

2.4.1. Traditional 2D culture methods

The history of HSCs culture to achieve relevant expansion and differentiation began with Dexter and Testa in 1976 with the development of a static culture system[10]. This 2D system that used culture flasks was quickly followed by other configurations according to the laboratory responsible for the protocol with records of cultures using well-plates, T-flasks and unmixed gas-permeable blood bags[103]. These supports suffer from the usual limitations of 2D culture systems, namely the limited area available for the cells to develop to, the hurdles for mass transfer, resulting in poor O_2, nutrients and metabolites distribution that create non-homogeneous culture conditions throughout the support structure and the difficulties in scaling up the experiments.

To overcome most of these issues that inherently plague 2D cultures, research groups proposed the use of bioreactors, this way exerting a higher control on culture conditions including nutrient supply and metabolite removal. The first recorded automated bioreactor culture system for haematopoietic cells was a continuous perfusion reactor[11] that supplied fresh culture medium through a syringe pump. This setup best result was achieving a 30-fold expansion of colony forming units – granulocyte/macrophage (CFU-GM) progenitor cells, with other progenitor types not as successfully expanded. Other types of reactors are also available and were used in HSCs culture, such as flatbed, grooved bed, airlift, hollow-fiber setups and rotating wall vessels. An example of this last type application is provided by Liu *et al*[12] where a total cell expansion of 435-fold was achieved. While these numbers are quite impressive, when counting only CD34+ cells representing the more primitive progenitors essential for long-term engraftment capabilities the tally was only increased 30 times, a more modest result dimmed by the ones achieved by more simple applications such as the previously mentioned gas-permeable Teflon bag[103], where an expansion of 113-fold was recorded.

These systems are similar on their requirements for combinations of exogenous cytokines to potentiate HSCs expansion. Earlier setups could not abdicate either from the use of fetal bovine serum or equivalents, as this is an excellent source of nutrients, hormones, growth factors, adhesion factors and all sorts of beneficial proteins for cell survival, greatly enhancing proliferation and expansion. However being of animal origin, this product is undefined in terms of its contents, introducing variability to the culture setup and the possibility of contamination from bacterial, mycoplasma and viral origins[104]. The unknown presence of inhibitory or enhancing cytokines along the mixture may also shift the culture away from the intended cellular results. An effort to expand HSCs using serum-free conditions is underway, as a way to circumvent the referred issues and to implement good medical practices (GMP) conditions, required for clinical validation of the developed protocols. However this renders the culture highly dependent of the chosen cytokine cocktail.

Normally each laboratory supports its own “recipe” of used cytokine cocktails, nevertheless there are some popular cytokines for stem cell culture. The formerly mentioned FL, SCF and TPO stand among the usual choices for at least one of the exogenous molecules to add to the system, with for example, the use of SCF, TPO and G-CSF over 10 days culture resulting in a 56-fold expansion of total nucleated cells (TNC)[105]. A similar combination but with the addition of megakaryocyte growth and development factor reported a 400-fold expansion over 14 days[106]. Others used a cocktail of IL3, IL6, SCF and EPO achieving a 2990.2±1936.1-fold expansion after 14 days[107] and using similar

conditions but changing the cytokine combination to FL, TPO, IL6 and IL 11 an 600-fold increase of TNC was recorded[108]. To all the above mentioned it is worth to add IL3 due to its extensive use as well. Overall, SCF has been used for longer, although early reports seem to flag FL as more effective than SCF[109]. As described previously FL presence seems to amplify cellular response to other cytokines, enhancing their effects, a characteristic shared by IL6 as well, whose sole presence is reported to be ineffective[110]. This synergistic role makes them no less important for cellular culture as increasing the SCF early haematopoiesis beneficial effects is regarded as a quite relevant effect.

Other cytokines are used, all reaching for the goal of expanding HSCs while controlling their differentiation, as some cellular fates are undesirable. One of such mature cell populations to avoid is the T-cells, as they may compromise clinical engraftment due to increased immunological response by the host. Although suppliers nowadays carry a great choice of recombinant cytokines ready to use in culture they are still a very expensive product and its current usage philosophy requires very high concentrations in order to get a cellular response from the system. This hinders the understanding of the mechanisms present in the BM *in vivo* as it exposes the cells to abnormally high levels, resulting in a failure to accurately mimic the HIM.

A different strategy regarding cell culture under investigation is the use of a feeder layer in a co-culture system. This approach exchanges the easier maintenance and more accurate definition and standardization of stroma-free cultures for a system that resembles the HIM more accurately, as stromal cells are a very important part of the niches required for HSCs maintenance and expansion *in vivo*. Early reports show the effectiveness of using a feeder layer of porcine microvascular endothelial cells for *ex vivo* expansion of CB cells[15], with an increase of 5-fold in CD34+ expansion capability and 241-fold in CD34+/CD38- cells over stroma-free, cytokine containing culture system[14]. Later on a shift towards the use of human stroma was taken as part of an effort to eliminate animal products from cellular cultures in order to more accurately define them and eliminate the xeno contamination sources, complying with GMP conditions. Da Silva *et al*[13] successfully expanded CB derived CD34+ cells using serum-free medium complemented with SCF, b-fibroblast growth factor, leukaemia inhibitory factor and FL over a 26 day culture, achieving a 35-fold increase. In another laboratory the expansion of CD34+ progenitor cells and their complete differentiation into erythrocytes was achieved through complementary use of SCF, IL3 and EPO along with co-culture on stromal cells, resulting in a number of red blood cells 1.95×10^6 times higher than the originally seeded cells. Research into these stromal layers shown an interesting interaction between the seeded HSCs and the mesenchymal stromal cells (MSC) used in the co-culture, where HSC migrate underneath the MSC layer effectively forming two separate sites, the one below the layer mimicking the niche of more immature progenitor cells and the one above a more active niche for haematopoietic proliferation[111] (Figure 2.6).

This approach has some limitations, the presence of stromal cells alone is not sufficient for the sustenance of HSCs *in vitro*, requiring exogenous cytokines with the inherent issues. Also the usage of xeno origin stromal layers is unadvisable if the final goal is to translate the knowledge into clinical practice. Finally, the complexity inherent to this type of culture hinders any scale-up development of this system.

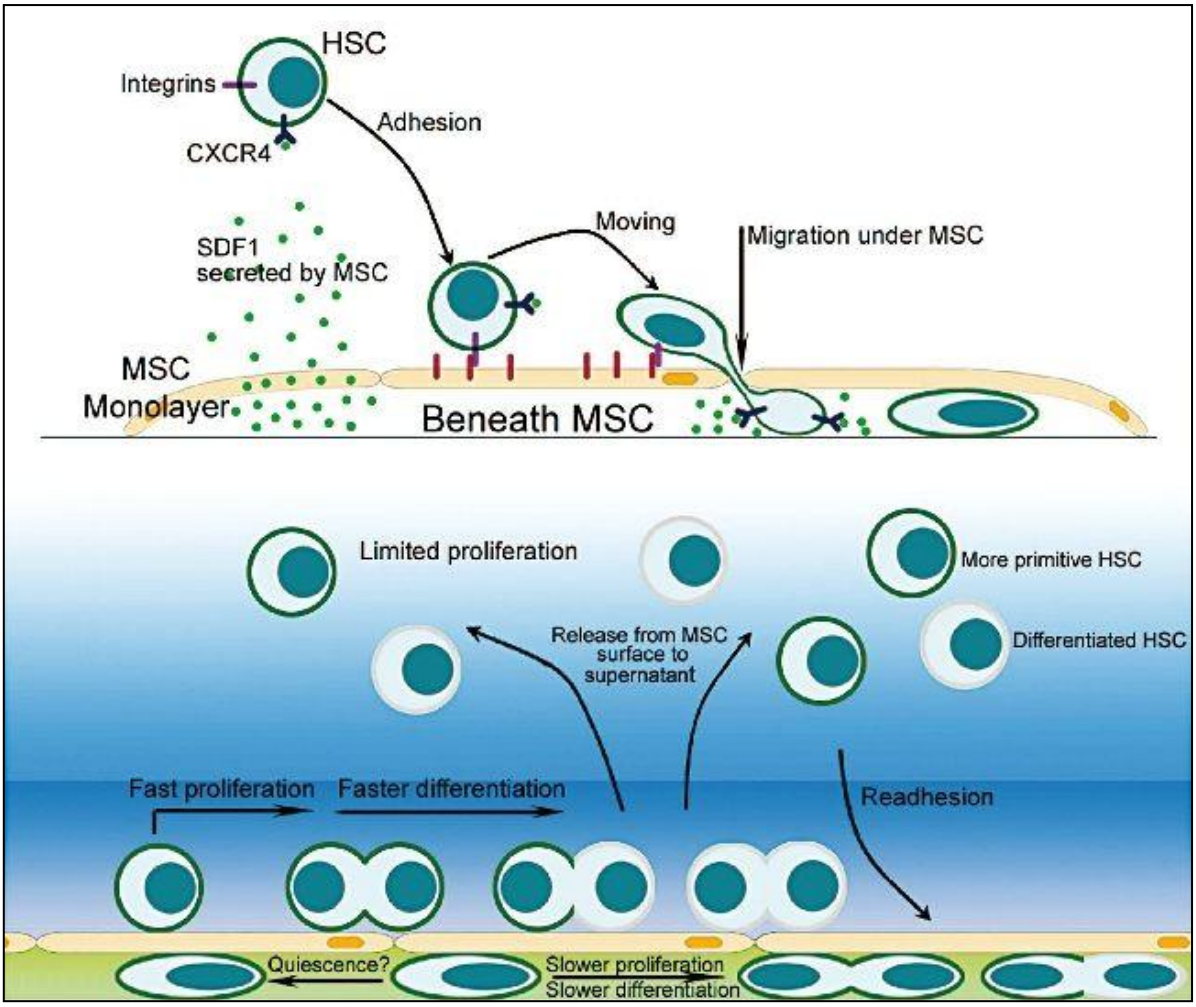

Figure 2.6. Co-culture system with mesenchymal stromal cell (MSC) layer. Top image shows the migration to and retention of HSCs by the MSC layer, while the bottom one illustrates the creation of a niche like site for HSCs proliferation, creating a boundary between two distinct expansion sites. Adapted from Jing, D. *et al*, (2010)[111].

The research into more efficient strategies in ongoing, and in result, the proposal for using the Notch signaling pathway to increase HSCs expansion efficiency was advanced by some research teams. This gene was known to be expressed by CD34+ progenitor haematopoietic cells[112] and its use in the culture of HSCs showed significant results, namely the 100-fold expansion of CD34+ cells. Follow up tests to the produced cells demonstrated their ability to successfully engraft animal models, reconstituting their BM[113]. Along with a combination of SCF, FL, IL6, TPO and IL3, Notch ligand complemented culture resulted in a 600-fold expansion over 16 days[114]. These results are quite recent and as such there are ongoing clinical trials whose results are yet to be disclosed. Despite the bright panorama some caution is advised when approaching this issue, as the protein currently used to induce this pathway, Delta 1 Notch ligand, is also known to promote cellular apoptosis[115]. A detailed cytotoxic profile, along with the listing of the conditions that trigger the nefarious effects on the cells by this ligand is then in order for the complete determination of the systems tapping into the Notch pathway.

In addition to the biological molecules previously addressed, some chemical compounds were found to be capable of enhancing HSCs expansion. Theoretically having a chemically induced stem cell expansion system could prove advantageous, as chemicals are usually cheaper and fully defined. Nowadays, that is still not possible, with the discovered compounds still being dependant on exogenous cytokines to successfully maintain the culture with all the associated issues. Examples of some of these chemicals are an aryl hydrocarbon receptor antagonist identified through high-throughput screening[116] and the use of tetraethylenepentamine, a copper chelator along with SCF, FL, IL6 and TPO, expanding the TNC 219-fold over 21 days. The latter molecule was already known for its effect on HSCs survival and expansion[117].

Table 2.3 is a review of the topics discussed in this chapter, presenting the main conclusions of the advantages and disadvantages of every described culture complement.

Table 2.3. Summary of traditional 2D culture systems supplements. Summing up the literature review on the state of traditional 2D culture of HSCs. Adapted from Kita, K. et al, (2011)[118].

Supplements	Advantages	Disadvantages
FL, SCF, ILs & TPO	Used in many studies, with serum-free systems available.	Expensive when considering scale-up cultures. Too many non-standardized combinations. Cocktails hinder the pinpoint of molecular mechanisms. Some may even be unnecessary.
Feeder cell layer	Better HIM mimicking, reflected on expansion levels.	Complicated culture systems
Notch ligand	Effective through known mechanisms.	Requires immobilization of the not yet fully understood ligand.
Chemical compounds	Strictly defined cultures. Cheaper than cytokines.	Quite recent, requiring further studies

Every system described so far failed to accurately portray the HIM *in vitro*, with the stromal cell culture being the closest to the niche that HSCs require to thrive inside the BM. The fact is that these niches derive from the intricate BM inner structure, something a 2D culture could never replicate due to the intrinsically 3D nature of every biological system. As such a well designed three dimensional system will almost always be a more accurate representation of the respective in vivo microenvironment it pretends to emulate, by providing a more faithful mimicry of cell to cell and cell to substrate interactions.

2.4.2. 3D haematopoietic culture systems

These developments appear as an attempt to overcome the 2D shortcomings, designing the 3D systems to mimic as accurately as possible the physical properties of the HIM. By recreating the *in vivo* microenvironment researchers hope to maintain and differentiate HSCs without the need for abnormally high concentrations of exogenous factors, with all the issues it raises.

A non-exhaustive list of proposed 3D systems includes the use of a tantalum-coated porous biomaterial[16], which achieved the maintenance and expansion of HSCs cells in the absence of cytokines and co-culture with BM stroma. The system used fetal bovine serum added as a complement to the medium, thereby making it unsuitable if GMPs are to be enforced. Others reported the use of porous microspheres to culture BM cells with a complement of EPO, SCF, IL3, GM-CSF and insulin-like growth factor-1, achieving sustained erythropoiesis for a 5 week period[18]. Direct comparisons to 2D cultures under the same conditions reinforced the 3D system advantage over the traditional flask culture. Banu *et al*[17] used a previously described 3D porous biomatrix called Cellfoam in an effort to increase its efficiency. For that they used a combination of SCF, IL3 and FL to achieve HSCs proliferation while maintaining the multipotent capacity of the culture.

All of the described above fail to deliver on the 3D system promise of an accurate *in vitro* mimicry as they are still heavily dependent of cytokines or animal serum to achieve expansion. The ideal BM model does include cytokines, as they are present *in vivo*, but it does so at physiological levels.

2.4.3. In-house developed bone marrow biomimicry

In an effort to generate a practical model to aid the study of primary acute myeloid leukemia (AML), the research group of Professor Athanasios Mantalaris dedicated a team lead by Dr. Teresa Mortera-Blanco to the development of an *ex vivo* mimicry designed to facilitate the acquirement of knowledge on AML and on which to investigate possible therapies.

To better mimic the BM microenvironment the choice was made to use a highly porous scaffold, constructed of an ideal polymer previously selected from a range of tested biomedical materials. This work lead to the determination of polyurethane (PU) as the best of all the six tested biodegradable and non-biodegradable materials when used with a coating of type I collagen, one of the main BM extracellular matrix components. The rationale behind the coating is to achieve a HIM like structure without co-culture and its complexities[19].

The scaffold structure was achieved through the thermally induced phase separation (TIPS)[119] technique using dioxane as the solvent, generating a foam like material with pores sizes ranging from 100 to 250 μm and a porosity of circa 95%[120]. The resulting wafers were cut into 5x5x5 mm cubes and the cells were seeded onto them after the collagen coating.

This system was proven capable of sustaining leukemic cell lines without the addition of exogenous cytokines[19]. AML is characterized for developing in BM niches which protect the leukemic stem cells, therefore the hypothesis being tested next was if the mimicry developed could support CB MNCs as well, due to the similar *in vivo* microenvironments of both cell types. Experimental work confirmed the theory and CB cells were expanded without exogenous cytokines for over four weeks[20].

Although promising, these results were based in protocols requiring fetal bovine serum, a breach of GMP. Further work was then performed, studying the applicability of the developed 3D scaffolds to a serum-free and cytokine-free culture of CB MNCs. This project will be referred to as “Project EPO”, since its aim was also to try to coax the culture into an erythroid fate through the addition of near-physiological levels of EPO. As such “Project EPO” consisted of three different

culture conditions, one where no EPO was added and another two with different EPO concentrations to better assess this cytokine effect. The results from this work are yet unpublished, but due to their significance to the work at hand they are displayed on the results section of this manuscript. These results show the capability of the novel 3D scaffolds to maintain and expand CB MNCs for a period over four weeks with no addition of serum or cytokines, a unique achievement in the HSCs expansion panorama. This bolsters the system's claim as a useful tool for haematopoiesis studies and the clinical application of HSCs proliferation and differentiation protocols.

Two other projects were developed along with the work that originated this thesis, both using the novel 3D system but with different cytokines cocktails in order to determine their effects on the *in vitro* mimicry. "Project N" was developed by Mr. Nayer Youakim who used SCF in two different concentrations along with a cytokine-free condition over a 35 day culture timeframe. "Project R" consisted of a cocktail of physiological levels of SCF, FL and TPO again for 35 days. This was Mr. Remus Winn responsibility, with both projects being supervised by Dr. Teresa Mortera-Blanco. "Project N" showed that even the small concentrations of SCF used were capable of influencing the system while "Project R" confirmed the beneficial effects of adding exogenous cytokines even when using very low physiological levels.

3. Materials and methods

3.1. Scaffold creation

In order to fabricate the scaffold with the desired mechanical properties such as pore size, shape and distribution, the thermally-induced phase separation procedure (TIPS) was used. Dioxane (99.8% pure, Sigma-Aldrich, Dorset, UK) was used as the solvent for the polymer solution of PU (5% wt) and ultimately removed by sublimation. More specifically the PU solution was cooled to -86^{o}C and kept at that temperature for a period of 2 h. Afterwards the dioxane was removed by freeze-drying for 3 days at -15^{o}C in an ethylene glycol bath[119]. The resulting wafers were spongy in texture and were previously characterized as having pore sizes from 150 to 250 μm and a porosity of roughly 90%[120]. In order to achieve the minimum variability possible in the culture conditions the wafers were cut to cubes of a standard 5x5x5 mm dimensions by freezing them in liquid nitrogen and quickly slicing the desired pieces with razor blades discarding the outside edges of the wafer.

3.2. Collagen coating and scaffold sterilization

Due to the high hydrophobicity of the PU scaffold it was necessary to wet the structure by dipping it in ethanol at 70% for 1 min followed by rinsing in phosphate buffered saline (PBS) for 5 min to remove most of the ethanol. The cubes were then transferred to a centrifuged tube filled with PBS and subjected to a centrifugation step of 10 min at 2500 rpm. Meanwhile a collagen solution made of calf skin-derived collagen type I (Sigma-Aldrich, Gillingham, UK) dissolved in acetic acid 0.1 M (Fisher Scientific, UK) with a concentration of 62.5 μg/ml was prepared from a concentrated stock by dissolving in deionised water from a NANOpure (Barnstead, Duque, IA, conductivity 1.83 m Ω/cm). After decanting, the tubes were filled with the collagen solution previously mentioned and further centrifuged for 20 min at 2000 rpm. Following another decantation, PBS was added and the scaffolds centrifuged for 10 min at 1500 rpm, clearing the pores of the surface from obstructions that could hinder cell migration into the structure[19]. The cubes were subsequently distributed into individual wells in 24 well plates under sterile conditions for further treatments. Sterilization was then achieved by means of UV light exposure (230 V, 50 Hz, 0.14 A, Kendro Laboratory Products UK) for 8 min followed by immersion in 70% ethanol for 2 h. The scaffold cubes were subsequently washed twice with PBS for 5 min each time in order to remove most traces of ethanol. Later on the wells were filled with 30% Fetal Bovine Serum-containing (FBS; GIBCO Invitrogen, Paisley, UK) Iscove's Modified Dulbecco's Medium (IMDM; GIBCO Invitrogen, Paisley, UK) supplemented with 1% Penicillin and Streptomycin (Pen/Strep; Gibco, Invitrogen Ltd) and the plates incubated for 48 h in a humidified environment at 37^{o}C and 5% CO_2 to assure a successful sterilization. If the media looks clear and nothing has grown after the incubation time the scaffolds are deemed safe to use in the subsequent cell culture.

3.3. Cell source and isolation

The cells used in main cell culture experiment were isolated from human umbilical cord blood units acquired from the United Kingdom National Health Service (NHS) Blood and Transplant

authority's UK Cord Blood Bank (Bristol, UK), all in accordance to the regulatory guidelines currently in practice (Harrow Research Ethics Committee 05/Q0405/20). The units were acquired in cryopreserved form and kept in liquid N_2. Cell isolation prior to the culture starts with the thawing of the blood units in a water bath at 37ºC and adding its contents into three 50 ml centrifuge tubes which are then topped up to 40 ml with 30% FBS IMDM and centrifuged for 10 min at 260 RCF with a break level of 4. A decanting step ensues, making sure the pellet stays undisturbed. The contents of one vial are then distributed among the others and the topping up, centrifuge and decanting processes repeated with one less vial each time. All centrifugation steps last for 10 min at 180 RCF and feature a break level of 9.

If necessary 100 µl of DNase can be added at any point to counter eventual coagulation phenomenon. When a single vial remains with a cell pellet it is topped up with 2% FBS IMDM and used for the standard Ficoll-Paque density separation (Ficoll-Paque PLUS, Amersham Biosciences) where the mononuclear cells (MNCs) contained in the buffy coat layer are extracted and washed once with sterile PBS. After doing a cell count and viability check, the cells are centrifuged and resuspended according to the cell count results and concentration required for the cell seeding.

3.3.1. Ficoll-Paque separation

Using a syringe, 10 ml of Ficoll-Paque are added very slowly to the bottom of the tube containing the blood. A centrifugation step ensues, at 450 RCF during 35 min. Brake level is set to 0 in order to make sure that the formed layers are not disturbed. These layers are clearly visible in Figure 3.1. Mononuclear cells are extracted by gathering the buffy coat layer with a syringe.

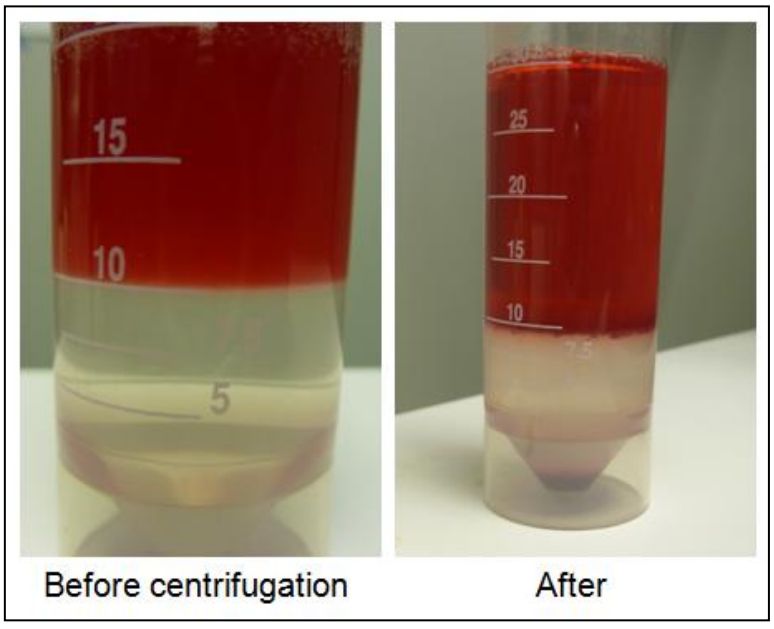

Figure 3.1. Ilustration of the Ficoll-Paque procedure. Notice the clearer top layer (when compared to before the centrifugation), the rough looking buffy-coat layer and the cell pellet at the bottom of the tube. Courtesy of Mr. Nayer Youakim.

3.4. Mononuclear cell seeding and culture

An excess of 322 scaffolds were prepared according to the protocols described previously in order to account for possible losses during the experiment and distributed into 24 well plates (Costar®, Corning®, New York). Of the 322, 24 (a whole plate) were used as control, therefore remaining unseeded, with the remaining cubes being seeded with 100 µl of cell suspension at a $25x10^6$ cells/ml concentration, achieving a final number of $2.5x10^6$ cells per scaffold. After seeding the plates were incubated for 15 min at 37°C and 5% CO_2 in order to improve seeding efficiency. Each scaffold was covered with 30% FBS IMDM supplemented with 1% Pen/Strep by adding 1.5 ml to each well and incubated at 37°C and 5% CO_2. The media was refreshed every other day via half-medium exchange until day 7, where all the media was extracted in order to introduce the serum-free StemSpan® (STEMCELL Technologies Inc., Grenoble, France) supplemented with 1% Pen/Strep as the growth media for the remainder of the culture. This new media was also refreshed every other day until the end of the experiment at day 28. Also from the day 7 onward, erythropoietin (EPO; R&D systems, Inc., Minneapolis, MN) was added with every culture media exchange in calculated dosages to achieve the three main culture conditions; without addition of EPO (No EPO), with 0.2 U/ml[21] of EPO (Low EPO) and with 1.875 U/ml[18] (High EPO). After harvesting the scaffolds required for all the analysis to be performed at the day 7 time point, each condition described above was assigned 89 scaffolds.

As previously stated, the cell culture was maintained for 28 days, with time points for data gathering being set at days 0, 7, 14, 21 and 28, where PU cubes were sacrificed to provide the cells required leading to a gradual decrease of the available scaffolds throughout the experiment. At data points 0, 7, 14 and 21 the cells extracted were used for cytospins, polymerase chain reaction (PCR) and flow cytometry analysis and scaffolds were frozen directly for *in situ* PCR. For the final day of the experiment multiphoton fluorescence microscopy was performed as well. In all time points a sample of the extracted cells was used for cell counting and viability check.

3.5. Cell extraction

As the cells were cultured inside a scaffold an extraction procedure was established in order to remove them for further processing and studies. The technique consisted of removing the cube from its corresponding well with sterile tweezers and setting them in a sterile petri dish. There the cells are aspirated with a micro pipette, using the tweezers later on to remove all the media inside by means of gentle squeezing. All the media containing the cells is then transferred to an eppendorf awaiting further treatment.

3.6. Cell viability analysis

A sample of the extracted cells was stained with Erythrosin B (ATCC®, Manassas, VA) and counted using a haemocytometer (improved Neubauer haemocytometer; Marienfeld Superior, Lauda-Königshofen, Germany). This stain allows the distinction between live and dead cells, enabling the determination of the viability at that time point. The total number of cells is extrapolated from the direct

count through a formula specific to the haemocytometer in use along with the knowledge of the dilution steps used in the preparation of the sample.

3.7. Cytospins

In order to perform a histological examination of the cells at the various time points considered, cytospins were produced and later on studied by microscopy techniques. Media extracted from scaffolds was subjected to a cell count, centrifuged and resuspended in a calculated volume of PBS in order to achieve the desired concentration of about $1x10^5$ to $5x10^6$ cells per 125 µl of suspension. The glass slides (Polysine slides; Thermo Scientific; Langenselbold, Germany) are labelled and loaded into a holder (Hettich Zentrifugen; Tuttlingen, Germany), along with a sheet of filter paper (Hettich Zentrifugen; Tuttlingen, Germany). The funnel is then attached and secured into position and 125 µl of cell suspension loaded with the help of a micro pipette. The apparatus is centrifuged at 740 rpm for 2 min and the device disassembled in order to leave the slide to dry overnight at room temperature. In preparation for microscopy, the slides are fixed by immersing onto a solution of 95% ethanol and 5% acetic acid for 3 min and stained for 5 s by dipping in Wright-Giemsa (Sigma-Aldrich, Dorset, UK) stain, rinsed and left to dry. If the cells look understained the dipping protocol can be repeated until a desired colour is achieved. Slides are then visualized in an optical microscope (Olympus BX51; Olympus, Essex, UK) using a 50x oil immersion lens and images representative of the cell morphology and different populations are recorded through a digital camera (Olympus DP50; Olympus, Essex, UK) and processed by an image analysis program (analySIS^D; Olympus, Essex, UK).

3.8. Flow cytometry analysis

By studying the surface markers of a given population derived from haematopoietic stem cells it is possible to infer their lineage and their state of commitment to said lineage, helping us determine the effects of the proposed scaffold and culture methods on the stem cell development. The method chosen to perform this immunophenotyping study was flow cytometry, where several clusters of differentiation (CD) were investigated to define the populations present at the various time points throughout the experiment. The fluorochromes in use were the phycoerythrin (PE), phycoerythrin-Cy5 (PE-Cy5) or fluorescein isothiocynate (FITC), each conjugated to a mouse monoclonal antibody (mAbs) (BD Biosciences, San Jose, California, USA) specific to a certain CD. Along with the isotype controls, CD45 specific mAbs conjugated with either FITC, PE or PE-Cy5, the mAbs-fluorochrome combinations in use were: CD45-FITC to identify most of the mature haematopoietic populations; CD34-PE to assess the early haematopoietic lineage progenitors, giving us a measurement of the stem like capabilities of the cells analysed; CD38-PE-Cy5 as a determination of the amount of cells exhibiting an early myeloid specificity and mature monocytical or lymphocytical nature; CD33-FITC as a similar early progenitor marker specific for the myeloid lineage and at last CD71-PE, CD235a-PE-Cy5 and EPOR-FITC to characterize cells belonging to the erythroid lineage. Listing these last three from an earlier stage to a more developed one we have EPOR signalling roughly the earlier CFU-E

stage, CD71 the later erythroblast stage and finally CD235a as a marker of red blood cells, the end point of erythrocyte development.

The necessary cells were extracted from circa 8 scaffolds per timepoint and condition, following the protocol established and counted, generating a cell suspension with a known concentration of cells. From this solution the necessary calculations were made to achieve a number of about $1x10^6$ cells per eppendorf tube, bearing in mind that 8 tubes were needed for the analysis at hand. The suspension in each eppendorf was them topped up to 1 ml with PBS to wash the cells, followed by a centrifugation at 2500 rpm for 5 min to gather the cell pellet, discarding the supernatant. This pellet was then resuspended in 20 µl of FBS along with 80 µl of previously prepared flow cytometry buffer (97.9% PBS, 2% Fetal Bovine Serum, 0.1% Sodium Azide) and 10 µl of each fluorochrome conjugated mAbs used. One eppendorf was left without any fluorochome to form the autofluorescence control while three others were prepared to be the positive isotype controls by labelling them with CD45-FITC, CD45-PE or CD45-PE-Cy5. The remaining tubes were set up as combinations of the mAbs at our disposal, allowing a study of the populations represented by the expression of one or multiple markers, namely: CD34 + CD45 + CD38, CD33 + CD38 + CD34, CD71 + CD45 + CD235a and CD71 + CD235a + EPOR. Each combination was designed to avoid the overlapping of fluorochromes, always attributing a different flow cytometer channel to the three populations studied. After labelling, the samples were incubated for 45 min at 4ºC and then centrifuged and washed with 1 ml of PBS, preparing them for a final fixing step consisting of resuspension in 1 ml of 2% paraformaldehyde (DH Industries Ltd, India), storing them at this stage at 4ºC for a maximum of 48 h awaiting flow cytometer availability. The machine used for this analysis was an EPICS ALTRA flow cytometer (Beckman Coulter Inc, CA, USA), calibrated with flow-check fluorspheres (Flow-Check™, Beckman Coulter Inc, CA, USA). Raw data was then saved and processed using Winlist 5.0 software (Verity Software House, Maine, USA), using this program to handle all the data treatment, compensation and plotting.

3.9. Cell sample storage

In each time point scaffolds were taken from the culturing well plates and frozen directly for the *in situ* study. Other cells were then extracted from further scaffolds, pooled together, counted and checked for viability. After distributing $1x10^6$ cells per eppendorf tube (1.5 ml), sterile PBS (Gibco, UK) was used to top up each tube. These were then centrifuged for 5 min at 2000 rpm and the supernatant was discarded. The resulting cell pellet was frozen at -80^oC. For this experiment 6 scaffolds were saved per condition per time point for the *in situ* extraction protocol and 7 had their cells extracted according to the method described above. The option to freeze the samples was chosen in order to process them all at the same time, reducing the variability in their handling.

3.10. RNA extraction

Total RNA was extracted from the pelleted cells ($1x10^6$) using an extraction kit (RNeasy Plus Mini, Qiagen, West Sussex, UK) complemented with an homogenization kit (QIAshredder, Qiagen, West Sussex, UK). RNA quantification and quality assessment were performed in a photometer

(BioPhotometer, Eppendorf, Cambridge, UK) using specially designed plastic couvettes (UVette, Eppendorf, Cambridge, UK). In order to get the most accurate reading of the $A_{260\ nm}/A_{280\ nm}$ ratio, a 10 mM tris-Cl (VWR International, Poole, UK) buffer at pH 7.5 was used as blank and to dilute the samples prior to measurings, following recommendations of the RNA extraction protocol. For the *in situ* analysis a novel protocol was established based on a cryogenic grinding operation. Due to the relative small scale of the extraction and the scaffolds to be milled, simple equipment was used. The process is started by cooling the ceramic mortar (8 cm in diameter) and pestle with liquid N_2 and holding it tight in an appropriate support, as the mortar quickly becomes too cold to hold manually. A scaffold is then dropped into the N_2 inside the mortar and the pestle is used to deliver swift blows to shatter the scaffold into smaller pieces. These pieces are then ground into fine powder, always with care not to run out of liquid N_2. After reaching a fine enough powder the N_2 is allowed to evaporate completely and the ground scaffold carried into 1.5 ml eppendorfs with the help of a spatula. From this step onwards the powder is treated as a regular sample and the RNA extraction protocol applies with no change required. Between milling procedures the mortar must be cleaned with ethanol 70% and a cloth, as water just freezes instantly when in contact with the very cold ceramic. The processing steps are summarized in the images from Figure 3.2.

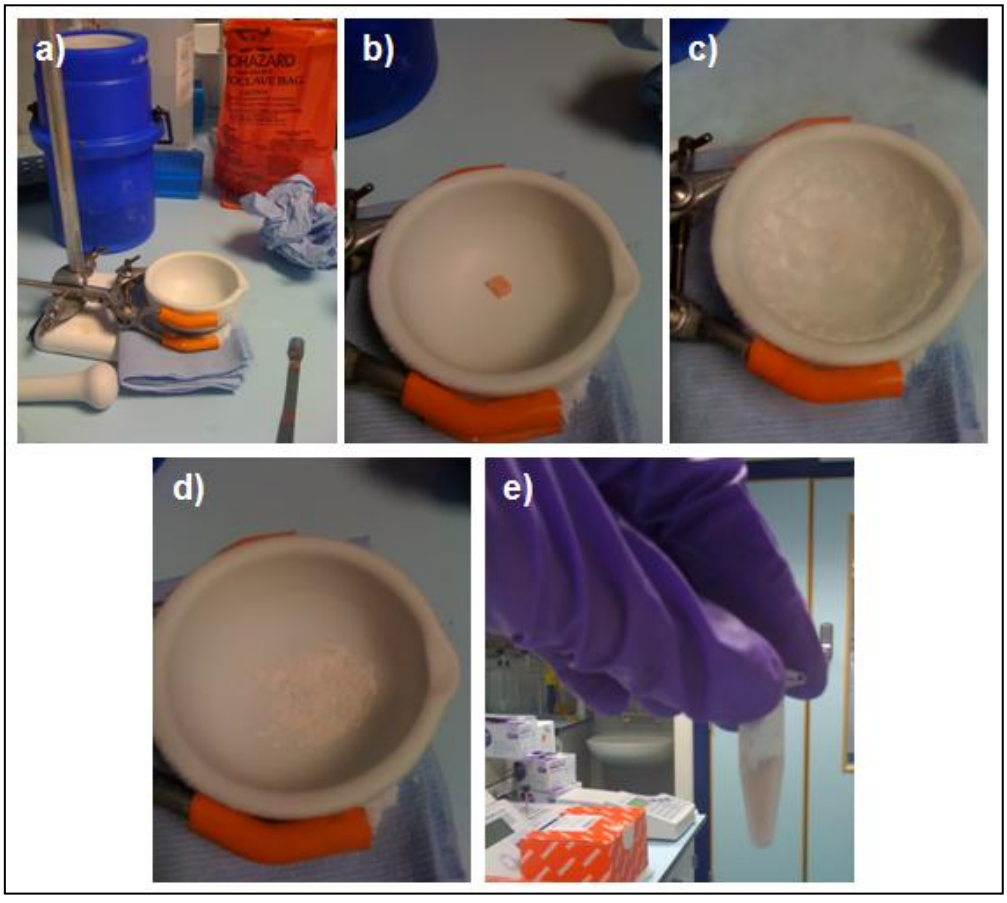

Figure 3.2. Ilustration of the cryomilling process for *in situ* extraction. In a) we have the overview of the setup, with mortar, pestle, metalic claw, spatula and liquid N_2 container. The scaffold is dropped into the mortar (b), which is filled with liquid N_2 (c). A few sharp blows from the pestle, followed by a grinding motion reduce the scaffold to a fine powder (d), that is transferred into an eppendorf tube (e) with the help of a spatula. The sample now can be processed through a regular RNA extraction kit.

3.11. Reverse transcriptase reaction

First strand cDNA synthesis was achieved through the use of a reverse transcription kit (GoScript™ Reverse Transcription System, Promega, Southampton, UK). In order to study the mRNA present in the cells of interest, oligo$(dT)_{15}$ were used as primers for the reaction. Care was taken not to exceed the maximum amount of experimental RNA allowed by the reaction system. $MgCl_2$ was used at a final concentration of 3.25 mM that proved adequate and required no further optimization. For a 20 µl reaction the components were as follows: 4 µl of experimental RNA extracted from $1x10^6$ cells or one whole scaffold for the *in situ* study, with care being taken not to exceed the recommended maximum concentration, 1 µl of Oligo$(dT)_{15}$ primer (for a total of 0.5 µg in each reaction), 3.8 µl of $MgCl_2$ for the final concentration stated above, 4 µl of GoScript™ 5x reaction buffer, 1 µl of PCR Nucleotide Mix (for a final concentration of 0.5 mM of each dNTP), 0.5 µl of Recombinant RNasin® Ribonuclease Inhibitor (final concentration of 20 units), 1 µl of GoScript™ Reverse Transcriptase and 4.7 µl of Nuclease-Free Water to bring the total volume up to 20 µl.

The primers were incubated with the RNA for 5 min at 70°C and then chilled in ice until the reaction mix was ready. The mix was prepared on ice and combined with the RNA and primer mix. The reaction consists of an annealing step for 5 min at 25°C followed by an extension step for 1 h at 42°C and finalized by the inactivation of the reverse transcriptase enzyme for 15 min at 70°C. Afterwards the products were stored at -20°C, following the recommendations by the manufacturer, until required for the Polymerase Chain Reaction (PCR) protocols.

3.12. Polymerase chain reaction experiments

Primers for the PCR experiments were chosen from commercially available sets, namely the QuantiTect Primer Assays (Qiagen, West Sussex, UK) as these follow the general guidelines for a good primer design, such as crossing exon-exon boundaries to guarantee the amplification of only the mRNA. The size of the fragments generated by the primers for the identification of the presence of the correspondent mRNA are as follows: erythropoietin receptor (EPOR, 81 bp), thrombopoietin (TPO, 87 bp), tumor necrosis factor alpha (TNF 104 bp), beta globin (HBB, 105 bp), interleukin 1 beta (IL1B, 117 bp), interleukin 6 (IL6, 107 bp), interleukin 10 (IL10, 113 bp), colony stimulating factor 2 granulocyte-macrophage (CSF2, 106 bp), gamma globin (HBG, 60 bp), transforming growth factor beta 1 (TGF, 108 bp) and finally beta actin (ACTB, 104 bp) as a house keeping gene control.

For the reagents and enzymes a commercial kit was used, the Taq PCR Core Kit (Qiagen, West Sussex, UK) with a PCR composition for a 50 µl reaction of 5 µl of CoralLoad PCR Buffer (1x final concentration) , 10 µl of Q-Solution (1x final concentration), 1 µl of dNTP mix (200 µM final concentration for each dNTP), 5 µl of QuantiTect Primer solution (1x final concentration), 0.5 µl of Taq DNA polymerase (2.5 units final concentration), 2 µl of cDNA synthetized from the RNA extracted previously and 26.5 µl of H_2O. These quantities found to be the optimal for the experiment at hand, with no further $MgCl_2$ being added as the CoralLoad PCR Buffer already contains 15 mM and the use of the optional Q-Solution usually removes the need for further $MgCl_2$. For each gene studied, a suitable positive control, housekeeping gene and negative controls were made in order to validate the results. The housekeeping gene chosen was beta-actin, a very common choice for this role[121]. For the

negative control, experiments were run without the cDNA template in order to check for possible contaminations of the reagents used. Positive controls were chosen by searching the existing studies and publications for documented evidence of the gene expression in a given tissue or cell line. As such K562 served as positive control for EPOR, HBB, HBG and TGF[122-125], HepG2 cell line was chosen as a control for TPO, TNF, IL10 and CSF2[126-129] and the reverse transcribed product of all the mRNA extracted from brain tissue for IL1B and IL6[130, 131].

All PCR amplification reactions were performed in a G-STORM GS1 thermocycler (G-Storm Ltd, Surrey, UK) equipped with a 96 well block for 0.2 ml PCR tubes. After several optimization steps the best conditions were determined to be a 3 min initial denaturation step at 94^{o}C, 30 cycles of a 45 s denaturation step at 94^{o}C with a 45 s annealing step at 60^{o}C and a final 1 min extension step at 72^{o}C, a final extension for 10 min at 72^{o}C after the 30 cycles and an unlimited storage step at 4^{o}C to keep the reactions until pickup and further handling.

For the visualization of the bands a 3.5% agarose (UltraPure^{TM} Agarose, Invitrogen, Paisley, UK) gel was prepared with 1x TBE buffer (made from UltraPure^{TM} 10x TBE Buffer, Invitrogen, Paisley, UK) and 10 µl of each sample was loaded directly onto the gel, as the CoralLoad PCR Buffer contains a gel loading buffer with tracking dyes. The gel was stained using a precast protocol with ethidium bromide (supplied at 500 µg/ml, Sigma-Aldrich, Dorset, UK) by diluting the main solution into the molten agarose at a 1:20000 ratio. To get a good resolution of the small bands that are amplified a 25 bp DNA step ladder (Promega, Southampton, UK) was used at 5 µl per lane (plus 1 µl of the 6x loading dye supplied with the ladder). The gels were then run for 40 min at 80 V and then for another 40 min at 100 V (Consort Electrophoresis Power Supply, Sigma-Aldrich, Dorset, UK) and visualized and captured by a GeneFlash gel documentation system (Syngene, Cambridge, UK) equipped with a PULNIX RM-300 CCD camera and a Computar H6Z0812 8-48 mm lens. All gels were saved as digital copies for further image treatment necessary for presentation.

3.13. Techniques used for parallel projects

These are protocols regularly used for the complete determination of the system being studied. However since the main project, concerning the addition of EPO as an exogenous cytokine to the novel 3D biomimicry of the bone marrow microenvironment, had a high amount of data from these techniques from previous iterations, the strategic choice was made to focus the efforts on gathering new data for parallel projects that studied the effect of other cytokine cocktails on the same platform. As such the protocols were learned and performed, gathering crucial data for the definition of effects of the new cytokines in study, familiarizing the user with these lab procedures and achieving an efficient management of collaborators time and laboratory resources.

3.13.1. Colony Forming Units (CFU) assays

This assay allows the quantification and functional analysis of lineage-specific progenitors. The formulation used in the chosen assay is carefully designed for the haematopoietic lineage, providing an assessment of the development and commitment status of the cells expanded in the scaffolds. In order to expedite the seeding procedure the specialized growth media, MethoCult®

(StemCell Technologies, USA) was previously aliquoted into 3 ml volumes and stored at -20ºC. This preparation and the following seeding procedures are done in sterile conditions. The cells are extracted from the scaffolds and washed with PBS, adjusting the volumes of cell suspension to achieve a total of $4x10^5$ cells. As the original media would interfere with the assay the cells are centrifuged and ressuspended in 300 µl of IMDM supplemented with 2% FBS and 1% Penicillin and Streptomycin. This suspension is then added to the MethoCult® aliquot, mixed thoroughly by using the vortex and left to rest for a while to lose some of the bubbles formed.

For the assay setup three small plastic sterile petri dishes (35 mm Ø, Corning®, The Netherlands) are placed inside one big one (100 mm Ø, Corning®, The Netherlands) and the cells and culture media mixture is split equally between two of the small dishes with a syringe and needle, this way achieving the cell density recommended by the assay manufacturer, $2x10^5$ cells per dish. The third small petri dish is filled with sterile water in order to keep the culture hydrated during the culture period of 14 days at 37ºC and 5% CO_2. After the required incubation time the number of different haematopoietic progenitor colonies (BFU-Es, CFU-Es, CFU-GMs and CFU-GEMMs) are counted manually using an inverted microscope (Leica DM microscope, Milton Keynes, UK) according to existing protocols[132], the classification into each category decided by colour, morphology and cell number analysis of each colony. Besides serving as a backup result if one of the plates gets contaminated, the two culture plates assure a duplicate culture, providing more data in order to validate the results. Each plate is counted once by two experienced laboratory members, taking care not to bias each other's result.

3.13.2. Cell proliferation assay (MTS)

MTS is a tetrazolium compound, more specifically, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium][133], used in a colorimetric assay that quantifies the activity of certain enzymes through the degradation of the MTS dye into a purple coloured molecule, this way inferring the cell proliferation in a certain culture by monitoring the changes in the cellular metabolic activity at different time points. For each condition and time point two whole scaffolds were extracted along with a control un-seeded cube and transferred into a new well plate. Every well was then topped with 1 ml of IMDM supplemented with 30% FBS and 1% Penicillin and Streptomycin along with 200 µl of previously thawed MTS solution (MTS, Promega, CellTiter96® Aqueous Solution Cell Proliferation Assay, Southampton, UK) and incubated for 3 hours at 37ºC and 5% CO_2. After the incubation period the scaffold is discarded and the remaining liquid samples are loaded into 96 well plates, the media from each scaffold divided into a column of wells for replications of the measurements and therefore a minimization of the reading errors. The compound formed by the reduction of the MTS by the dehydrogenases expressed by the cells, formazan, is purple in colour and therefore its quantification is done in an absorbance microplate reader (ELx808™ Absorbance Microplate Reader, BioTek Instruments, Potton, UK) reading at the 490 nm wavelength linked to a laptop featuring the appropriate software. The absorbance readings of the whole plate are then exported in a spreadsheet format for processing and analysis.

3.13.3. Scanning electron microscopy (SEM)

Desiring to better understand the cell organization inside the scaffolds, these were prepared for visualization with scanning electron microscopy. This preparation consisted of removing the scaffolds from the culture media and fixing the cells inside by immersing them in a 2.5% PBS buffered glutaraldehyde fixing solution (Fluka BioChemika, Switzerland) and incubating for 60 min at 4ºC, with care to wrap the incubating vessel in foil as the glutaraldehyde solution is light sensitive. As with the MTS assay, for each time point two scaffolds were used per condition along with one un-seeded control. After washing twice with PBS each scaffold underwent a dehydration procedure comprised of a series of 10 min dippings in ethanol of increasing concentration (25%, 50%, 70%, 80%, 90% and 100%) followed by a thorough drying achieved by leaving the samples in an aseptic environment overnight.

In order to attain a reasonably flat surface for a better visualization and to be able to see the behaviour of the cells in relation to the depth of the scaffold, the cubes were sectioned in half and according to the necessary protocol for SEM, sputter-coated in gold for 2 min in an argon atmosphere. Using an acceleration voltage of 15 kV the images captured by the microscope (JEOL JSM-840A, JEOL Ltd., Welwyn Garden City, U.K.) were then saved for posterior analysis.

3.13.4. Immunostaining for confocal and multiphoton microscopy

This technique was used to study the specimens in greater depth than by SEM, as it generates images of the several planes along the height of the sample, allowing the construction of a digital 3D model expressing the structure and the fluorophores associated to the desired markers on the cells. This allows for cell type identification and study of their preferred niches. The scaffolds were fixed according to a novel process using ethanol vapour overnight and then dried and stored at -20ºC. After harvesting all the desired time points, all the desired scaffolds were prepared for the immunostaining protocol by wetting them in PBS. Following a optimized protocol developed in the laboratory, the scaffolds were blocked with an incubation in 10% FBS for 30 min at room temperature and incubated at 4ºC overnight kept away from the light with the following primary antibody combinations: goat anti-CD68 with mouse anti-CD45, goat anti-CD68 with mouse anti-CD71 and goat anti-CD68 with mouse anti-CD105. The blocking helps to minimize non-specific binding and each antibody was used after a 1:50 dilution in 10% FBS. The cubes were the washed thrice with PBS and then incubated with the secondary antibodies, Alexa Fluor 568, conjugated to anti-goat antibodies, and Alexa Fluor 488 conjugated to anti-mouse antibodies, both used at a 6 µl/ml concentration. Previous to microscopic visualization, the scaffolds were again washed three times with PBS to remove non-binded fluorophores and then observed with a confocal microscope (Leica SP5, Leica Microsystems, Wetzlar, Germany). For the excitation of the fluorophores used a 576 nm pulsatile laser was used along with a x63 water emission lens, with the images being taken at 1 µm intervals through a depth of 50 µm. These images were later treated and analysed recurring to specialized software, Volocity 5.3.2 software (PerkinElmer, Waltham, USA).

3.13.5. Histochemistry (thin sections)

For each time point and condition considered, two scaffolds were taken as samples and fixed through the use of ethanol vapour for 8 h. Afterwards they were embedded in a xylene replacement (Paraclear™; Polyscience Inc, Warrington, PA) to give them the necessary rigidity for sectioning using a Shandon Finesse® ME Microtome (Shandon, Pittsburgh, Pennsylvania, USA), generating 5 mm thick slices. A xylene replacement had to be used as the compound dissolved the polymeric scaffold. After dewaxing, the sectioned slides were immersed fro 30 s in haematoxylin (Sigma-Aldrich, Dorset, UK), thoroughly rinsed with water and stained again with 1% v/v eosin (Sigma-Aldrich, Dorset, UK) for 2 min.

3.14. Statistical analysis

Every time it was deemed necessary the statistical significance of the results achieved were assessed by linear regression GraphPad Prism 5 (GraphPad software, La Jolla, CA, USA) using one way analysis of variance (ANOVA) with a level of significance $p<0.05$.

4. Results

It is important to note that this project evolved as a follow up study of previous work (previously described “Project EPO”), as an attempt to further elucidate the mechanisms responsible for behaviour exhibited by the cells when grown in the novel 3D scaffolds[20]. In that sense some of the tests executed served as a complement to the data previously gathered, helping its validation through a high number of repetitions, while other procedures necessary for the overall determination of the culture weren’t executed due to the abundance of data already collected.

The cytospins and flow cytometry procedures served as a test to determine if the culture performed as the previous experiments, therefore establishing a bridge through which the novel PCR experiments data, could be linked to the results achieved in the earlier work (section 4.6) of developing a platform to derive *ex-vivo*, long-term and serum-free culture of CB MNCs towards erythropoiesis. This effort was centred on a 3D PU scaffold functionalized with collagen type I, a major ECM component, helping the cells attachment to the structure and subsequent maintenance by providing structural support. The effect of near physiological levels of EPO are then studied through three separate experiments, one featuring 0.2 U/ml of EPO added from day 7 onwards on each media change every other day called Low EPO, a second one with 1.875 U/ml of EPO also from day 7 called High EPO and a control with no addition of EPO all throughout the experiment, against which the results are compared. In order to achieve this a culture was maintained EPO free until day 7 and then split into the 3 desired groups, adding the necessary amount of cytokine to both the Low and High EPO experiments.

4.1. Viability

All the three different culture conditions on the 3D scaffolds maintained the cells viable during the experimental time span of 28 days, a feat unaccomplished by 2D control cultures, where the cells die out in less than 7 days[20]. The viability of the cells extracted from the scaffolds was determined in all experimental time points, namely at days 0, 7, 14, 21 and 28, and plotted over time (Figure 4.1). On day 0 the alive and dead cells were counted after the Ficoll-Paque extraction prior to scaffold seeding, with the viability determined to be around 90%. Afterwards it is possible to notice a steady decrease of all measured values up until day 21 when the angle of all three curves becomes steeper, plunging from approximately 80% to circa 25%. The behavior of all three culture conditions is very similar, and even though it seems that the higher EPO concentration favors a higher viability there is no statistically significant difference ($p<0.05$) between every measurement for a given time point.

From these results it is possible to infer that the platform used for the cellular culture and expansion, the 3D scaffolds, is adequate to maintain viable cultures for at least 4 weeks, even without the addition of exogenous cytokines or animal derived serum.

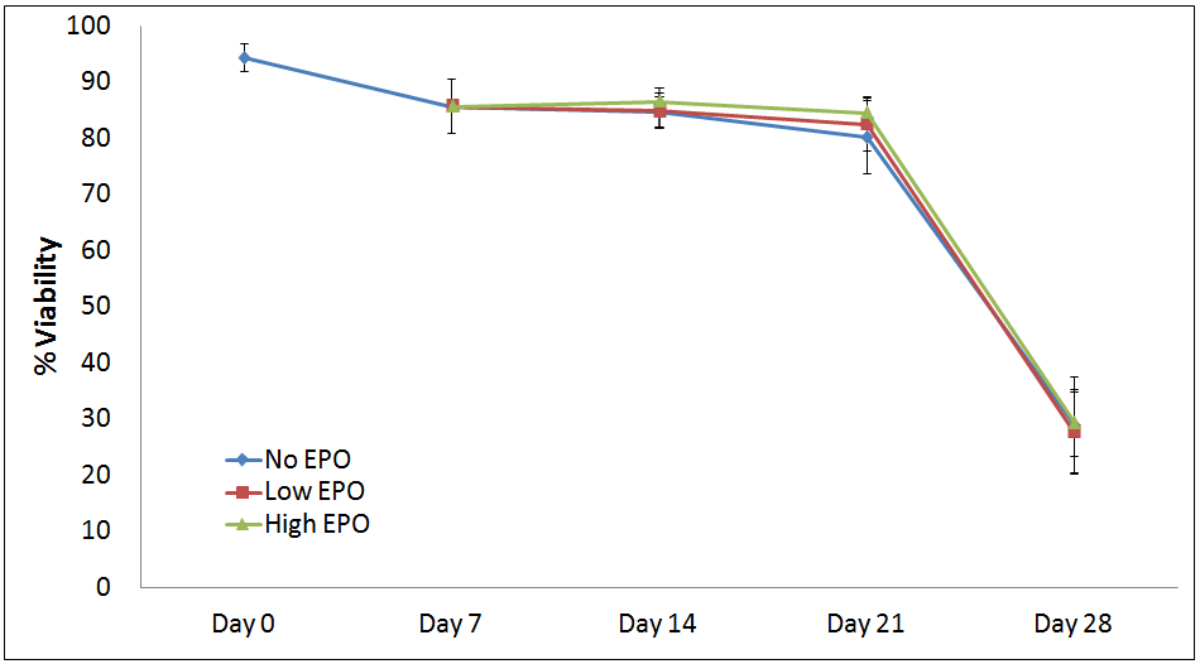

Figure 4.1. Viability on every time point established through haemocytometer count of the cells stained with Erythrosin B. There is no significant difference between the 3 studied culture conditions, all of them displaying a gentle drop until day 21, followed by a steep plunge to day 28.

4.2. Cytospins

An assessment of the differentiation capability of the cultured cells was taken in the form of cytospin analysis where after preparing the slides according to the protocol, these were visualized at x50 magnification, and the resulting images recorded (Figure 4.2). In this composed picture it is possible to identify cells of multiple lineages at distinct states of maturation. There is a predominance of erythroid cells in the first days of culture and it's possible to identify this lineage on all time points and conditions. From day 7 onwards the presence of cells from myeloid lineage, both early progenitors as well as committed ones is apparent and even though in very small numbers. Later on, it is possible to discern the presence of lymphoid cells, therefore stressing the multilineage differentiation capability of the all the cultures.

Higher EPO culture seems to develop a higher number of erythroid cells, as the reticulocytes and mature looking erythroids with a reddish tint. This is as expected since EPO is known to directly control erythropoiesis and while it suggests the steering of cellular fate towards an erythroid lineage it is also perceptible that the culture maintains the capability of generating other types of cells.

Technically there was an increasing difficulty in extracting the cells from the scaffolds as the experiment proceeded, prompting an extra effort on the cell recovery protocols, which along the decreasing viability can justify the reduced cell density and noticeable presence of structural debris characteristic of later time point images.

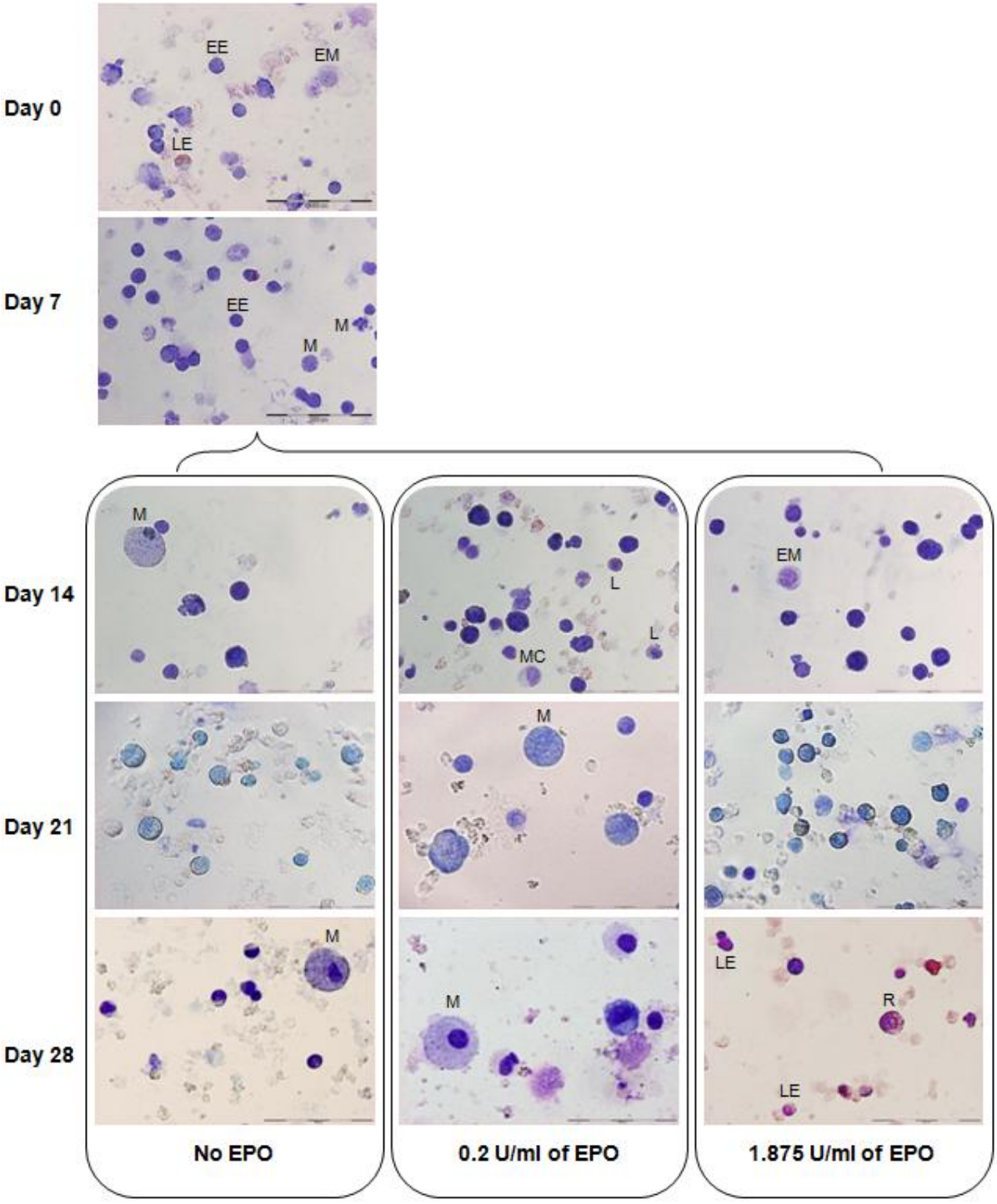

Figure 4.2. Cytospins of the various time points and conditions captured at x50 magnification. Cells were coloured using Wright stain. The images illustrate the existence of multilineage precursors and mature cells all along the duration of the experiments, as well as across the various culture conditions. In earlier time points there is a predominance of cells from the erythroid lineage, with the appearance of myeloid cells along with some of lymphoid origin on later cytospins. There is a noticeable increase in debris in later time points along with reduced cell density, demonstrating the increasing difficulty in extracting the cells from the scaffolds as time goes by. Legend: LE = late erythroid, EM = early myeloid, EE = early erythroid, M = of myeloid lineage, MC = monocyte, L = lymphoid lineage, R = reticulocyte.

4.3. Flow cytometry

To further characterize the cultured cells, flow cytometry was used to process samples from all experimental time points, allowing the study of the chronological evolution of the number of cells displaying certain lineage specific markers.

This immunophenotyping study produced the raw results displayed in Figure 4.3 to Figure 4.5 below. It is important to note that all the day 0 and 7 analysis were done under no exogenous EPO addition, the definition of 3 different culture conditions was only from day 7 onwards.

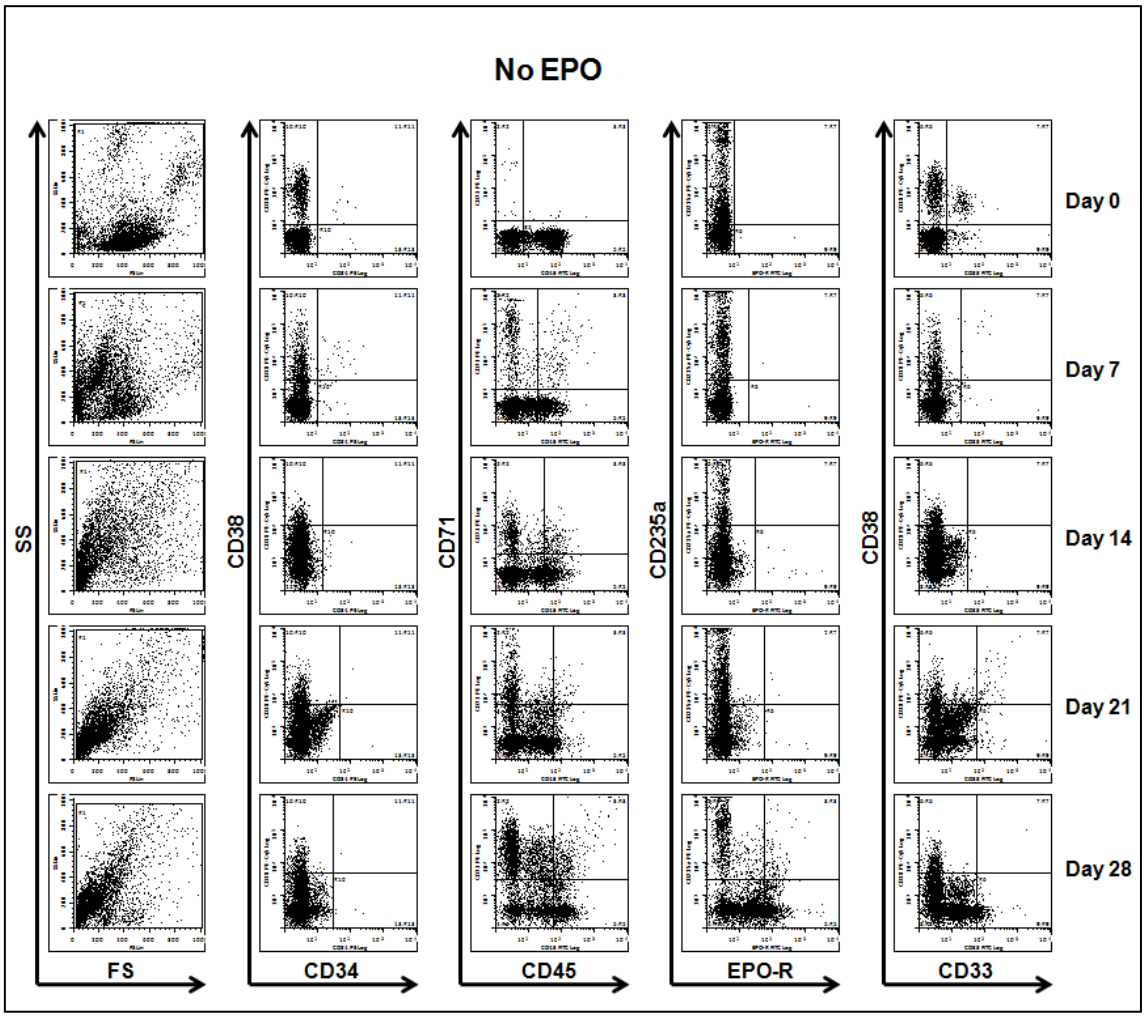

Figure 4.3. Flow cytometry data for the no EPO culture condition. It is possible to notice the increase in CD71 positive cells. Forward and side scatter, correlated respectively to cell size and granularity, are displayed on the leftmost side of the figure, allowing a preliminary identification of certain cell populations.

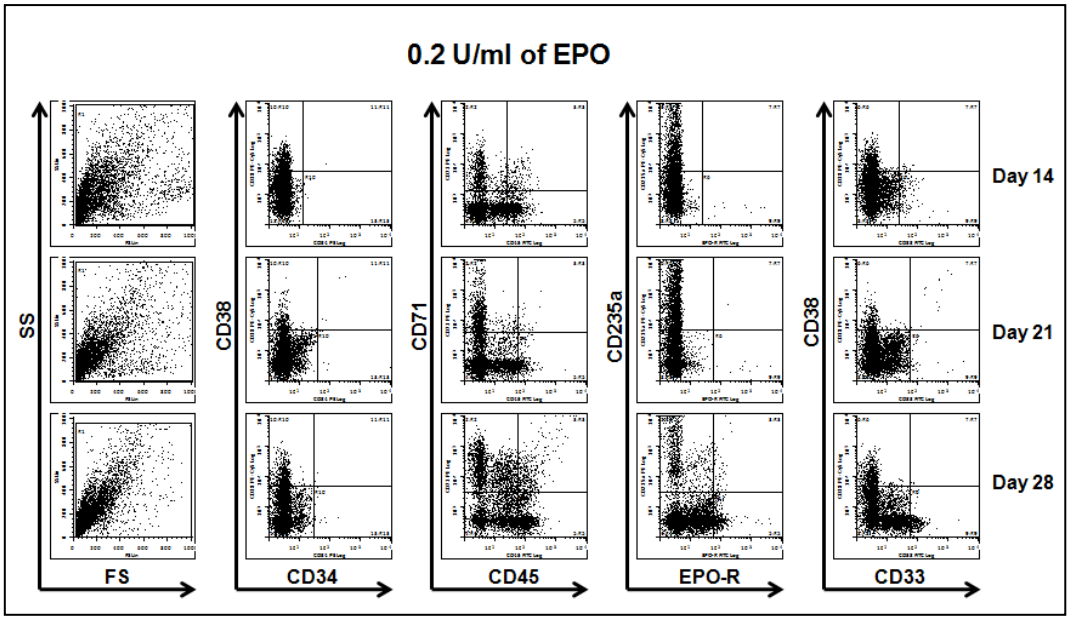

Figure 4.4. Flow cytometry raw data for the Low EPO condition. Day 0 and day 7 are no longer displayed as exogenous EPO addition only begun from the day 7 time point.

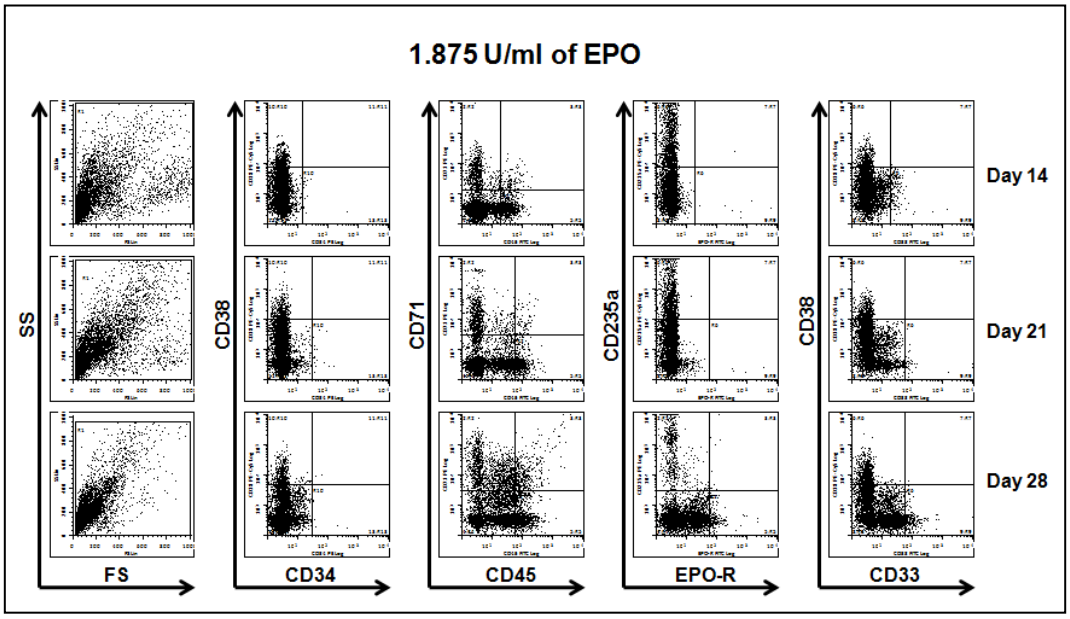

Figure 4.5. Flow cytometry data for the High EPO condition.

From the analysis of the flow cytometry graphs above it is possible to detect some changes on the populations studied, as for example the increase in CD71 positive cells in every condition as time goes by. However this change is easy to see only due to its scale, more subtle variations require a treatment of the data, presenting it in a format that facilitates the comparison between conditions and time points.

After gathering all the percentages of gated cells in each quadrant of the flow cytometry graphs the fraction of cells positive for each marker was determined and plotted on Figure 4.6.

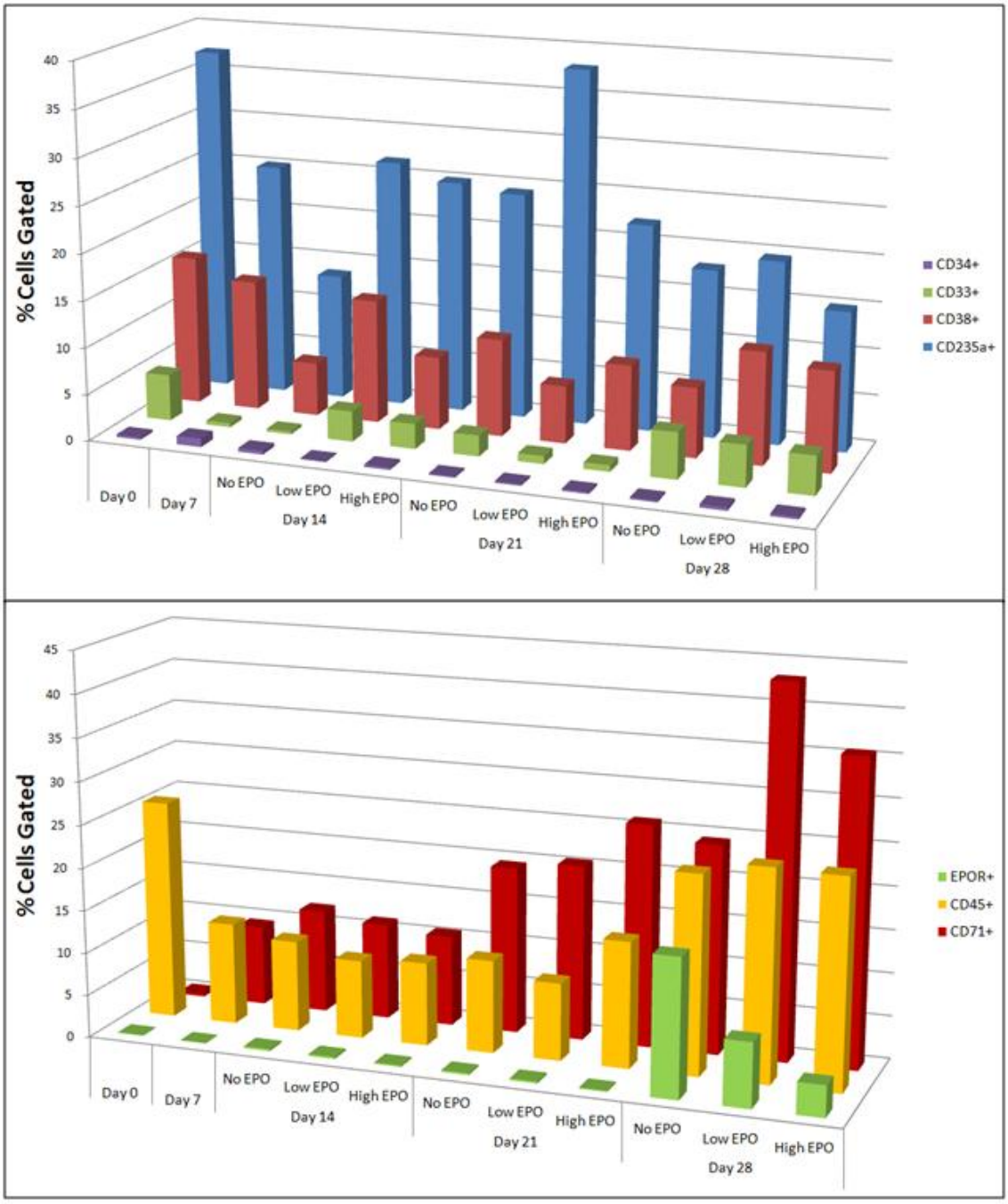

Figure 4.6. Graphical analysis of the flow cytometry data. The data was divided into two different plots in order to improve the displaying of all the values. Here it is easier to discern the tendencies of the populations, the increase in CD71 positive cells being confirmed along side with other interesting phenomena as the decrease of CD235a positive cells or the late spike in EPOR positive cells values.

The improved visualization of the percentage of cells positive for the markers being analysed allows a better understanding of the effects of the novel 3D scaffolds on CB MNC culture, along with the assessment of the outcome of EPO addition. There are very low numbers of CD34 positive cells throughout the experiments and although the data hints at their presence, the low percentage (always under 1.5%) invalidates any comparison between conditions as any disparity could be easily attributed to errors on data acquisition and its subsequent treatment such as the compensation procedure.

Further analysis of the graphed data shows that CD33 positive cells also stand out as having low numbers for every time point and condition. Although higher than the CD34+ events, with a maximum of circa 5%, these numbers are also hardly higher than what can be attributed to experimental variation due to handling and interpretation errors, and as such the inter-condition variation can't be accurately assessed. It is noted that the recorded values are somewhat constant, so for the record these results show a general presence of CD33+ cells at a higher frequency than the CD34+ cells. Both CD33+ and CD34+ are used as stem cell markers, with the latter being the earliest[134] and the former being specific for the myeloid lineage[135], and as such the low values are expected. Even in CB, where the concentration of stem cells is higher than usual, the actual number of progenitor cells is quite small, as demonstrated by the day 0 values.

At first glance the CD45+ cells data seems very interesting, with a high percentage of gated cells all along the experiments and a "U" shaped behaviour of the bars on the graph, suggesting perhaps an initial shock and subsequent adjustment phase, however a statistical analysis of the gathered data shows that there is no significant difference between the values displayed. This can be attributed to the high variation on the readings for each time point, generating a significant standard deviation and invalidating any comparison between the conditions being studied. As such the information gathered from said data is that there is a significant amount of CD45+ cells throughout the experiment and as a marker of most differentiated haematopoietic cells[136] this is an expected result.

The graphs of the cells positive for the remainder markers exhibit some interesting behaviours that prompt a more detailed statistical analysis. On Figure 4.7 it is possible to visualize more intricate details of the flow cytometry data collected previously. Along with a better resolution that the one presented in the general 3D graphs, it is also possible to see the groups that exhibit a statistically significant difference to one another, therefore enabling some conclusions about the behaviour of the cultures at hand.

On the top left graph of Figure 4.7 it is possible to notice a steady overall decrease of the number of CD38+ cells with time and on day 28 the percentage is perceptibly lower than when the experiment started (*, $p<0.05$), dropping from about 16% to between 8% and 12%. On the final time point it is also worthy of note the difference between the cultures supplemented with exogenous cytokines and the No EPO condition, as the latter displays less CD38+ cells (**, $p<0.05$). These results seem to indicate a shift in cellular fate away from the myeloid lineage[137], with the added cytokine providing a small boost to the cells marked by CD38.

CD71 is known to be expressed by erythroblasts and erythroid progenitors in cord blood[138], therefore being used as way to determine an early commitment to the erythroid lineage. In the top rightmost graph the evolution of CD71+ cells is shown to be a significant boost from nearly inexistent numbers to 40% plus on the final experimental point. It is safe to state that there was an increase on the percentage of gated CD71+ cells from day 0 to day 28 (*, $p<0.05$), however there is no statistically significant difference between the three different conditions on the last day, even though it appears that a higher concentration of EPO prompts a lower number of CD71+ cells. When combined with the CD38 results these seem to suggest that the shift away from the myeloid lineage was directed towards the erythroid lineage.

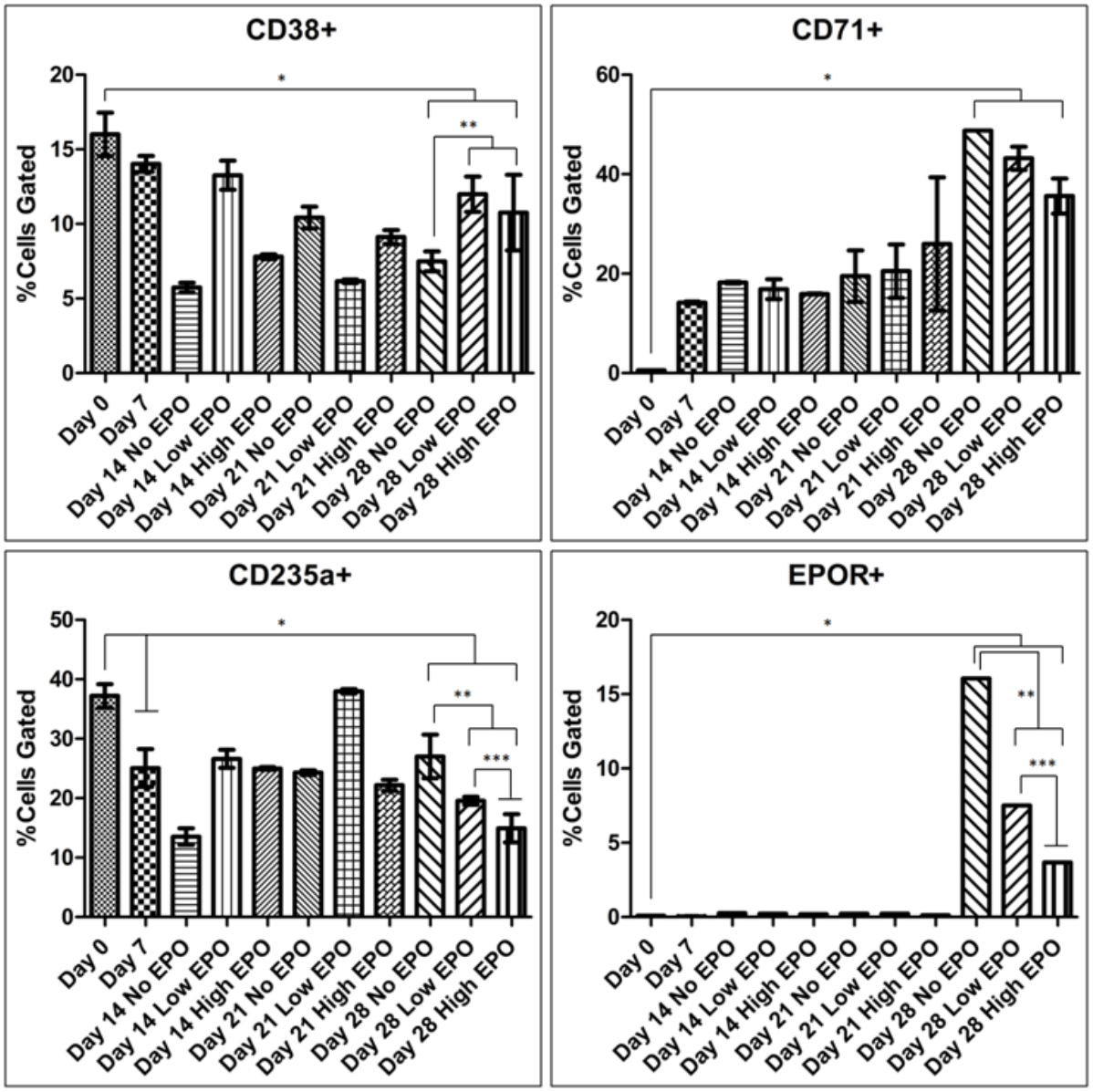

Figure 4.7. Detailed view of flow cytometry data of selected markers. Statistical significance was determined through one-way analysis of variance (ANOVA) tests using Tukey post test with a significance level of 0.05 ($p<0.05$, 95% confidence intervals).

In order to provide another tool for the determination of the differentiation status and lineage commitment of the cultured cells, CD235a was chosen as a way to reveal the erythrocytes[139] present in the media samples extracted from the scaffolds. After starting with a high percentage (approximately 48%) of gated cells in day 0 there is a noticeable drop at day 7 to a lower level (circa 25%) further decreasing softly through the rest of the culture until day 28 (*, $p<0.05$). At the last day there is a conspicuous relation between a higher concentration of exogenous EPO added to the culture and a lower value of CD235a+ cells (**,***, $p<0.05$). With the previously analysed increase in CD71+ this could mean that the culture is becoming more immature, allowing the expansion of cells with greater differentiation ability.

A final erythroid marker closes the roundup chosen to be part of this study. EPOR stands for erythropoietin receptor and is characterized by being present in the middle stages of erythropoietic maturation[140]. The flow cytometry analysis displays some interesting results regarding this marker, as after a barely perceptible increase from day 0 to day 21, the recorded values soar on the last experimental time point, again with an inverse relationship between added EPO concentration and percentage of gated positive cells. Conjugated with the previous results this data implies that the cultures are in the middle maturation stages.

4.4. Extraction protocol and data validation

The *in situ* extraction procedure used was specifically devised for the work at hand and as such it was necessary to validate it, assuring the quality of the RNA obtained. Pure RNA is known to have an absorbance at 260 nm/absorbance at 280 nm (A_{260}/A_{280}) ratio in the range of 1.9 to 2.1, so optical spectroscopy was used to determine said ratio of samples that underwent the novel extraction protocol. Four different readings were taken, averaging $A_{260}/A_{280} = 2.06 \pm 0.12$, ensuring the purity of the samples and efficiency of the process.

Afterwards the samples taken from each time point and condition were also submitted to the above mentioned extraction procedure, with one scaffold used per event, the data gathered in that experimental step being displayed in Table 4.1.

Table 4.1. RNA extraction results. Samples of the extracted RNA were diluted 12 times for the absorbance reading. Knowing that an absorbance value of 1 corresponds to 40 µg/ml, and taking the dilution factor into account, RNA final concentration was determined. The amount of RNA extracted is derived from the concentration and the remaining extracted RNA volume, 45 µl.

		$A_{260\ nm}$	A_{260}/A_{280}	RNA concentration (µg/ml)	RNA extracted (µg)
Day 0		0.168	1.92	80.6	3.6
Day 7		0.06	1.88	28.8	1.3
Day 14	**No EPO**	0.058	1.85	27.8	1.3
	Low EPO	0.02	2	9.6	0.4
	High EPO	0.054	1.87	25.9	1.2
Day 21	**No EPO**	0.07	2.13	33.6	1.5
	Low EPO	0.078	1.83	37.4	1.7
	High EPO	0.077	1.74	37.0	1.7
Day 28	**No EPO**	0.034	1.84	16.3	0.7
	Low EPO	0.072	1.99	34.6	1.6
	High EPO	0.047	1.88	22.6	1.0

From the table analysis it's possible to comment on the good overall quality of the RNA extracted, as the A_{260}/A_{280} ratios are near the optimum range, guaranteeing the accuracy of the subsequent RT-PCR protocols. The absorbance at 260 nm ($A_{260\ nm}$) is somewhat low and in a future

works a lower dilution factor should be used to avoid a drop beneath the 0.1 value that denotes the suggested lower limit to ensure measurement significance. In this work it wasn't possible to follow the suggested course due to scarcity of the volume of extracted RNA and the time constraints forbidding further cell cultures to obtain more time point samples. This low value reflects an overall low concentration of RNA obtained through the novel extraction procedure however the amount gathered was sufficient for the work developed. In case of higher demands for purified nucleic acids, more scaffolds can be used per condition to meet the required concentration.

Knowing the amount of RNA available allowed the optimization of the reverse transcriptase (RT) reaction in order not to exceed the enzyme capacity as recommended by the RT kit manufacturer.

4.5. Polymerase chain reaction

Previous results have shown that by using the novel 3D scaffold it is possible to maintain and expand CB cells for at least 4 weeks without the addition of exogenous cytokines[20], a feat unaccomplished by traditional culture methods (e.g. 2D plate cultures). This prompted a search for the mechanisms behind the properties displayed by the bone-marrow mimicry engineered previously, with reverse transcription PCR (RT-PCR) being used as a mean to elucidate endogenous cytokine production that could be accounted for success of 3D scaffolds when culturing CB MNC.

Due to the rather small fragments amplified by the chosen primer sets, ranging from 60 to 136 bp, an adequate (25 bp step) DNA ladder had to be procured and the agarose gel concentration had to be changed from the regular 1.5%. An overview of the optimization procedure is displayed on Figure 4.8 below.

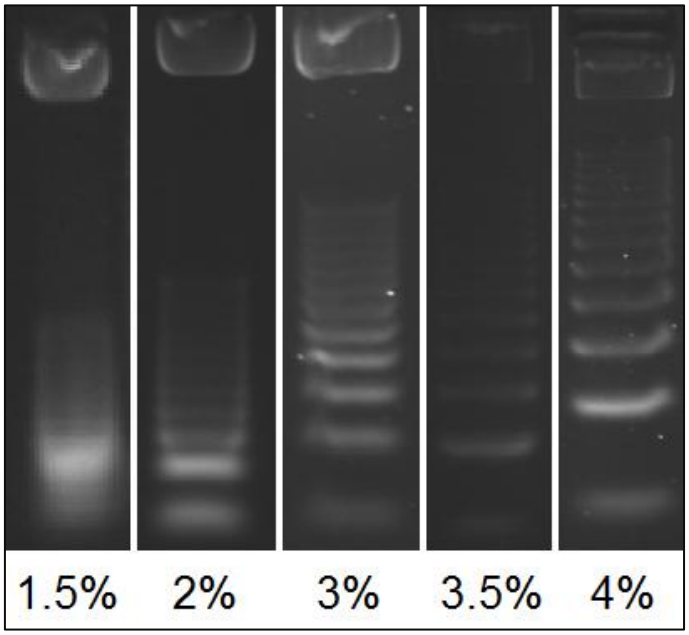

Figure 4.8. Agarose gel concentration optimization tests. At concentrations of 3.5% and above the handling of the gel become increasingly difficult, with an increase in complexity of the mixing, melting and casting steps. As such 4% was deemed as ideal for the clear separation of the bands to be visualized.

With the increase in agarose concentration a more clear separation of the DNA ladder bands was achieved, however it became more difficult to handle and cast the molten gel. The higher experimented concentration, 4%, displayed a very satisfactory resolution of the ladder zone where the PCR fragments fall in and generated a gel that required some special attention but was still easy enough to reproduce the several necessary times. 4% was then chosen as the standard concentration for all the agarose gels for the visualization of the PCR experiments.

Optimization of the PCR conditions such as cycle number, the temperature of critical steps and concentration of the reagents was also thoroughly pursued to ensure the quality of the results achieved, with the final ideal values being presented in sub-chapter 3.12 of materials and methods.

The set of images displayed below represent the agarose gels for the various cytokines studied. Every gel is subtitled to provide a better understanding of the results and a brief discussion of the data in each picture is below the respective image.

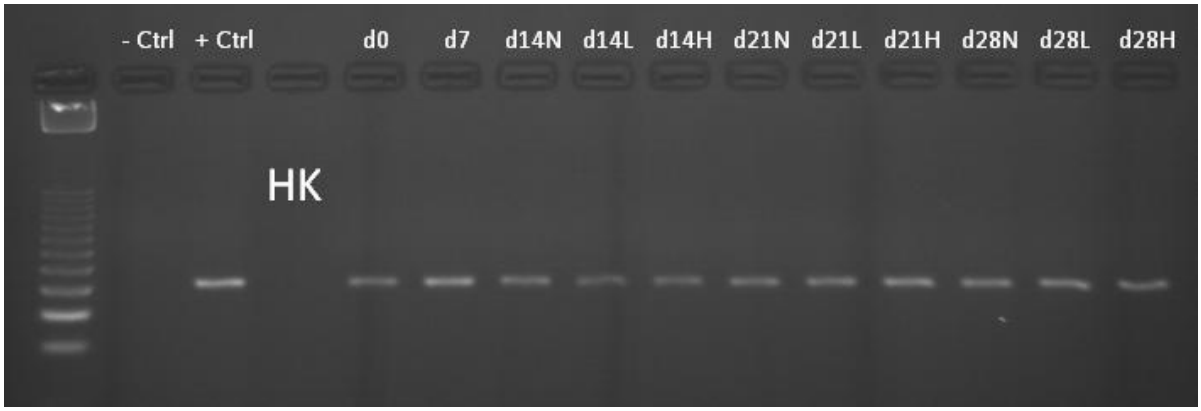

Figure 4.9. PCR results for the housekeeping gene (HK). The chosen gene was beta-actin (ACTB) that with the chosen primers amplifies a 104 bp fragment. The ladder has a 25 bp step, with the first band being too faint is this particular gel. Therefore the first visible band is the 50 bp one.

The HK gene analysis serves as a type of positive control, ensuring the presence of genetic material on the samples extracted from each condition. Along with the regular positive control, that assesses if the primers do detect the desired molecules, HK testing ensures that the negative results are truly a reflection of the inexistence of the cytokine of study transcripts and not the result of faulty primers or no genetic material in a given sample.

Beta-actin was chosen as the HK gene as it is known to have a stable expression even with changing culture conditions[121], being well established as a good reference for gene expression assays. In this study, the fragment amplified through PCR had a length of 104 bp and the results show the presence of the gene transcrips in all time points and conditions, as was expected. RNA extracted from K562 cells was used as a positive control, since due to the studied subject being a HK gene, theoretically any cell would be suitable as a control. Also K562 cells are fairly easy to grow and were used also as positive controls for other PCR in this work, therefore being readily available.

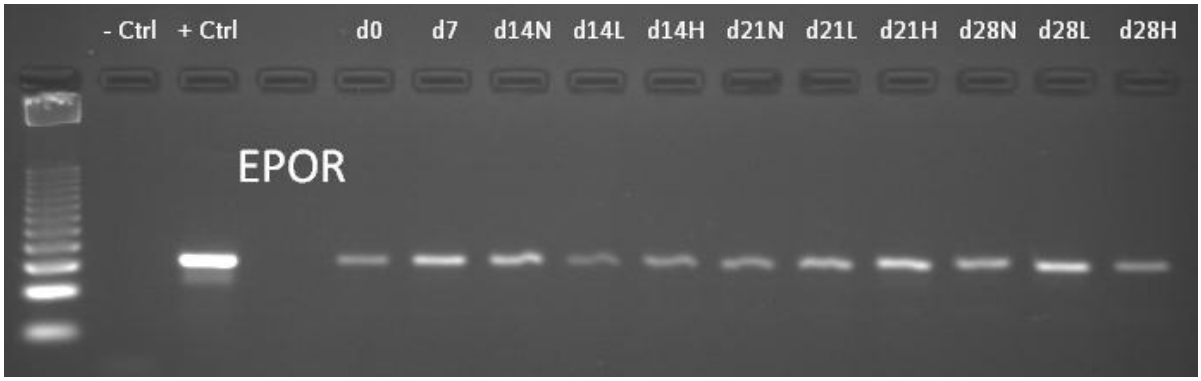

Figure 4.10. PCR results for erythropoietin receptor (EPOR). Fragment size was 81 bp, with the total RNA extracted from a culture of K562 cell line being used as positive control.

In this image, the DNA ladder is fully visible through its entire length, with the lowest band corresponding to the 25 bp mark. As such it is possible to verify the results bands position at circa 80 bp, as it should be, given the disclosed fragment size generated by the primers used. For the positive control an extract from K562 cell line culture was used, as it is well documented the expression of EPOR by that cancer cell line[122]. While not directly translatable to actual protein concentration, the expression of the gene was recorded as present in all situations. It is generally accepted that regarding erythropoiesis, EPOR is expressed in the middle stages of erythroid maturation, the more immature progenitors (early BFU-E) and the stages after erythroblasts are not affected by EPO and do not display the EPOR protein[140]. This suggests the maintenance of not fully differentiated erythroid lineage commited cells throughout the culture.

Although EPOR production has been documented in non-erythroid tissues[141], the nature of the seeding, CB MNC, make it highly unlikely the presence of such cells in our culture.

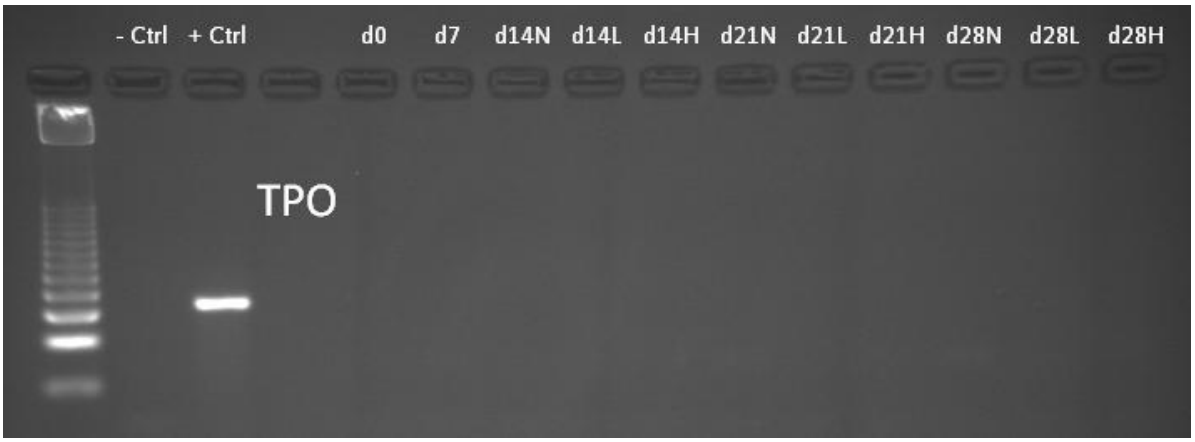

Figure 4.11. PCR results for thrombopoietin (TPO). With a fragment 87 bp long and HepG2 cells as positive control.

The gel above is quite easy to analyse, there is no recorded expression of TPO throughout the entire experimental timeframe. HepG2 is a liver carcinoma cell line known to express TPO[126], therefore proving to be suitable for the positive control role. These results are expected since TPO is expressed mainly by liver cells in all human life stages[142], however as the main cytokine responsible

for megakaryopoiesis its presence would be an indicative of the development of the culture towards megakaryocytes and platelets, which doesn't seem to be the case.

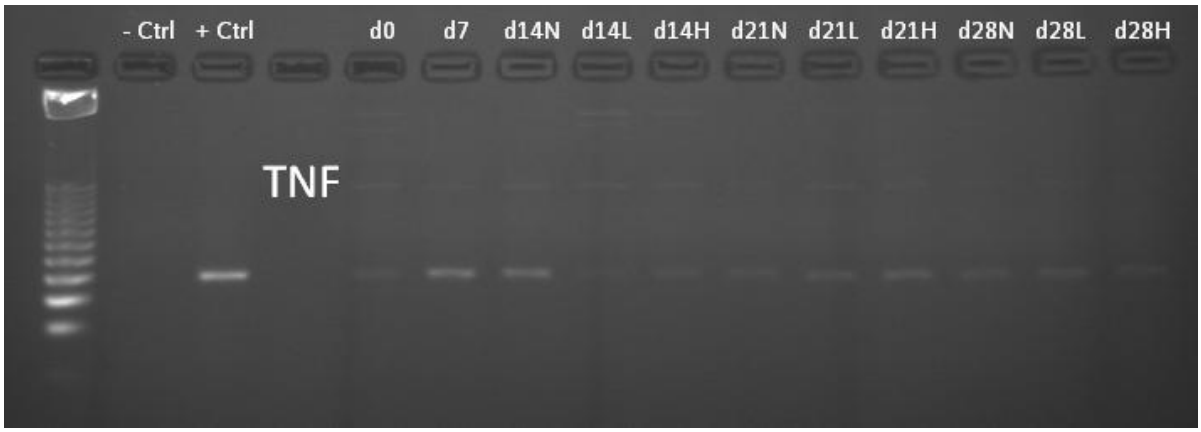

Figure 4.12. PCR results for tumor necrosis factor alpha (TNF). 104 bp fragment with a HepG2 positive control.

Tumor necrosis factor alpha (TNF) is one of several cytokines produced and released by macrophages when activated by the presence of immunological stimuli such as the bacterial lipopolysaccharides (LPS)[143]. TNF has multiple functions, being important for homeostasis and the immune responses such as inflammation, apoptosis, tumor necrosis, sepsis and the inhibition of viral replication.

Although studies indicate that TNF is expressed by other cell types[144], the main source is from activated monocytes, and as such its occurrence in culture suggest the presence of differentiated cells of myeloid lineage. The gel bands seem to start faint in day 0 and increase a bit in day 7, decreasing again for the rest of the culture, however the differences are minute and therefore no conclusion can be attained other then relatively low existence of TNF transcripts all along the experiment. For the positive control HepG2 cells were used as their expression of the desired cytokine is described in published literature[127].

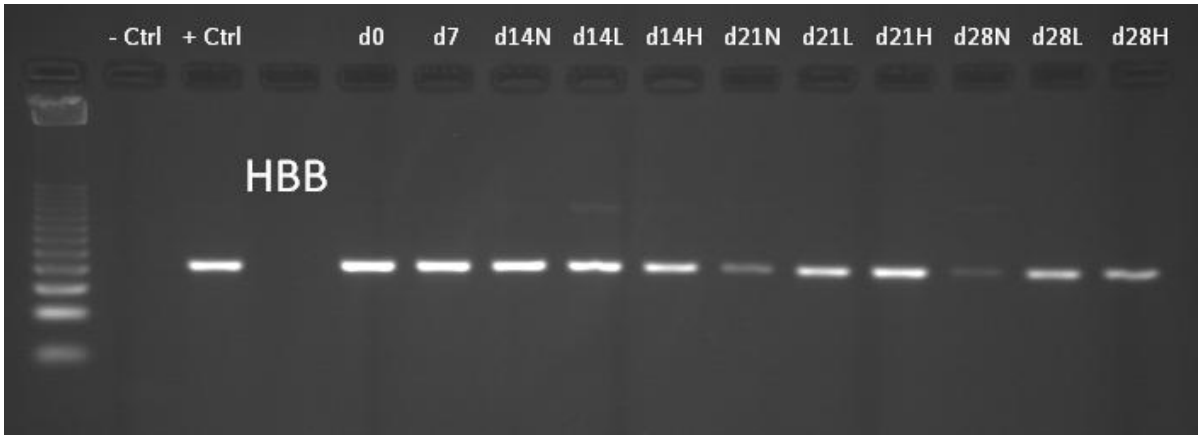

Figure 4.13. PCR results for beta globin (HBB). For this transcript, the final fragment size was of 105 bp. As for the positive control, K562 cells were used.

According to some studies, K562 cells express HBB[123], therefore being suitable to act as a positive control for the PCR experiment. After ensuring that the size of the fragment displayed corresponds to the one advertised by the primers manufacturers, a more detailed analysis of the gel picture revealed some interesting features. There seems to be expression on all samples tested, however the intensity of the bands appears to diminish slightly as the culture time increases. Also the bands corresponding to the No EPO condition are visibly fainter than the ones from other conditions, implying that the exogenous EPO added allowed the maintenance of HBB expression. This effect is expected since EPO is a known erythropoiesis agent and HBB is a critical protein for mature red blood cells, it makes for 2 of the 4 monomers that form the adult haemoglobin.

Since PCR analyses the gene expression profile it is wrong to directly correlate HBB levels to the number of mature red blood cells in culture, as erythrocytes, being enucleated, do not possess the cellular machinery to generate gene transcripts. Perhaps it is more accurate to connect the higher HBB expression to a more active middle stage of erythropoiesis and the lower values to a maturation of the erythroid culture or the redirecting of cellular fate towards a different lineage.

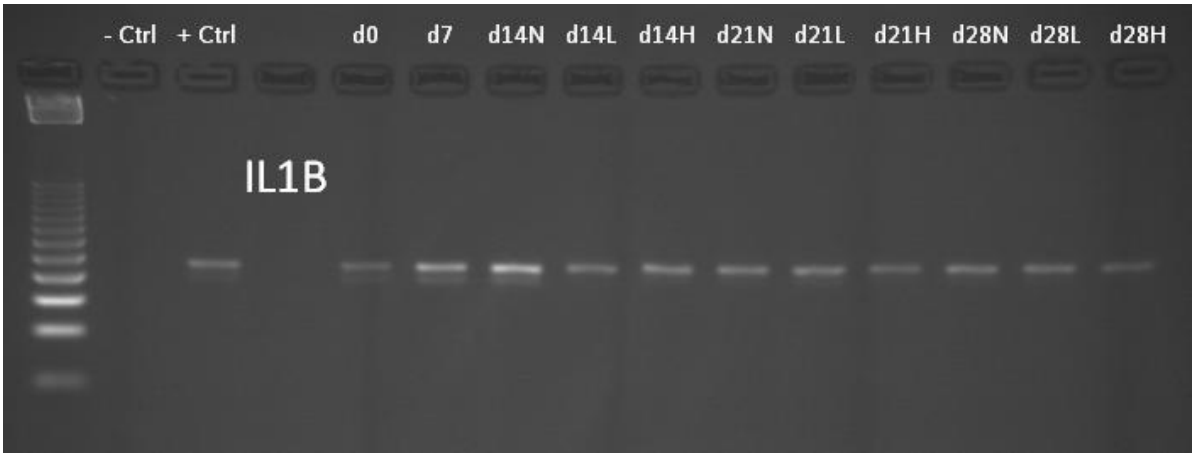

Figure 4.14. PCR results of interleukine-1 beta (IL1B). The chosen positive control was the reverse transcribed product of RNA extraction from brain cells. Fragment size is documented as 117 bp.

Interleukine-1 beta is a cytokine similar to TNF, being produced by activated macrophages in response to immunological stimulus, playing a role in triggering the inflammatory reactions. In fact IL1B is known to induce TNF[145] along with other pro-inflammatory factors, so deficiencies on its regulation mechanisms can lead to several crippling inflammatory diseases.

Although monocytes and macrophages are the main cell types that produce this protein, IL1B expression has been detected in several other cells and tissues, such as T cells, fibroblasts, neurons and glial cells[130, 146]. The documented expression by cells in the brain prompted the choice of the positive control; DNA transcribed from all the mRNA extracted from general brain tissue samples.

Due to the similarities with TNF is not surprising that the general expression profile in culture is also similar, with the presence of transcripts registered all throughout the duration of the experiment, suggesting once again the presence of developed cells derived of monocytopoiesis.

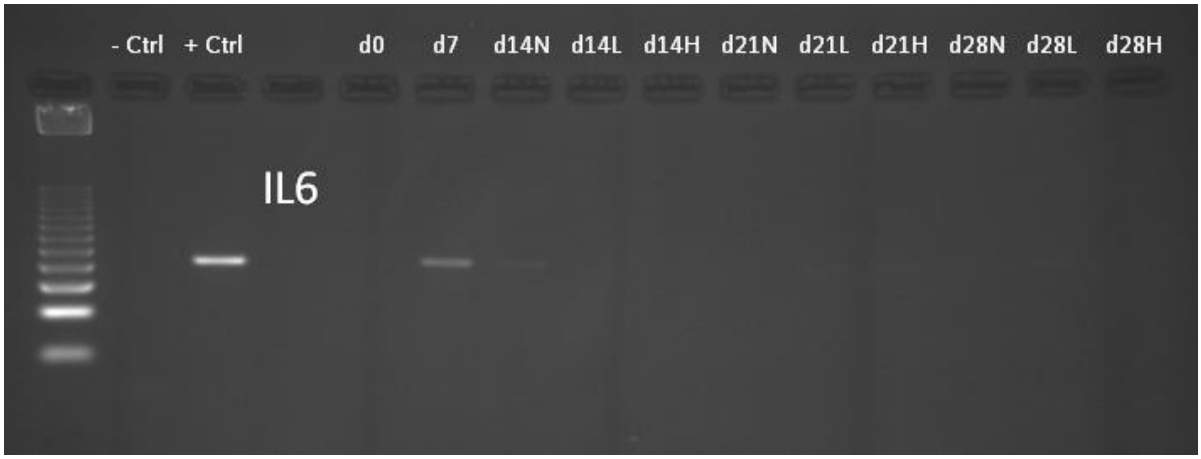

Figure 4.15. PCR results for interleukine 6 (IL6). As with IL1B, the positive control consisted of the brain cells extract. The fragment is theoretically 107 bp, the length being confirmed by the 25 bp step DNA ladder.

In response to infection, interleukine-6 works along side with IL1B and TNF as the main cytokines responsible for systemic acute-phase response. IL6 is classified as a pleiotropic cytokine and the mechanisms of its production are tightly regulated since IL6 over expression is credited with being involved in certain diseases.

There is data supporting the expression of IL6 in several different types of cells, monocytes and macrophages as IL1B and TNF but also osteoblasts, fibroblasts, astrocytes and microglial cells[131]. The last two types validate the choice of the positive control as the brain cells DNA. Regarding haematopoiesis IL6 is known to induce the maturation of B cells and along with IL3 to improve the proliferation of haematopoietic progenitors.

By analysing the electrophoresis gel image, it is possible to discern a peak in IL6 expression, as there are no bands clearly visible save for the day 7 one. A plausible explanation for this phenomenon is the regulation of IL6 production by external factors such as stress, as the one caused by the seeding procedure and the adaptation to the new environment.

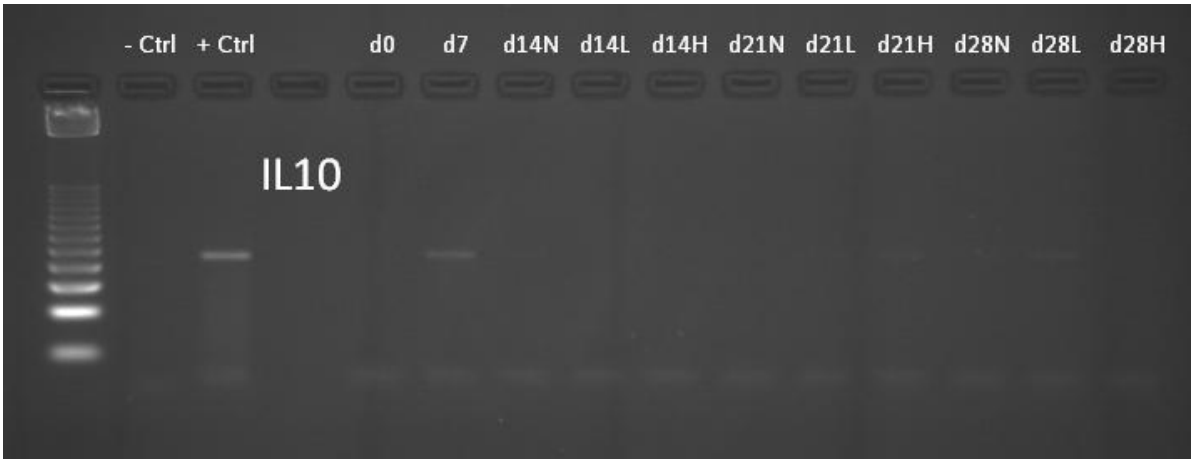

Figure 4.16. PCR results concerning interleukine 10 (IL10). 113 bp long fragment alongside a HepG2 positive control.

IL10 clearly follows the same expression profile as IL6, with no production of the transcript being clearly recorded except for the day 7 sample. Multiple cell types express this cytokine, monocytes, macrophages and T cells being some of the more notable sources, alongside other ones such as epithelial, tumor and liver cells[128], the latter justifying the choice of HepG2 for control.

This cytokine is classified as having anti-inflammatory properties even repressing the 3 pro-inflammatory ones mentioned above, IL1B, IL6 and TNF. Regarding haematopoiesis, IL10 can have an inhibitory effect on the differentiation of monocytes into dendritic cells, hindering the maturation step and favouring the macrophage fate[147].

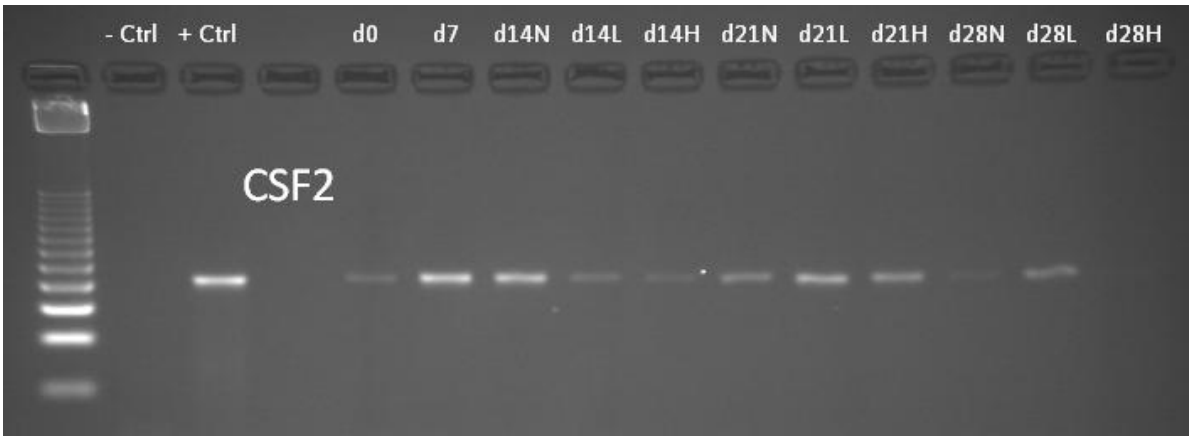

Figure 4.17. PCR results for colony stimulating factor 2 - granulocyte macrophage (CSF2). Primer selection lead to a 106 bp long fragment. Positive control is done through the use of HepG2 cells.

Also known as granulocyte-macrophage colony stimulating factor (GM-CSF or CSF2), this cytokine is notorious for the proliferation and differentiation of haematopoietic stem cells, hence its importance to the study at hand. More specifically CSF2 steers cellular fate towards the myeloid lineage, with the formation of granulocytes and monocytes[148]. Besides this ability, CSF2 also plays a role in stimulating the mature myeloid lineage cells in response to immunological stimulus[129], being expressed in these conditions by T-cells, fibroblasts, macrophages and endothelial cells.

The results of the gene expression analysis for this cytokine show a presence of the amplified fragment through all the time points except for the High EPO condition at day 28. These bands have an irregular intensity, suggesting perhaps a fluctuation in the expression levels. However since RT-PCR is simply a qualitative analysis method is hard to compare between samples except for a simple enquiry on the existence or not of expression. The presence of CSF2 in culture can justify at least a partial commitment towards the myeloid lineage, explaining the eventual presence of mature monocytes derived from an initial CB MNC culture.

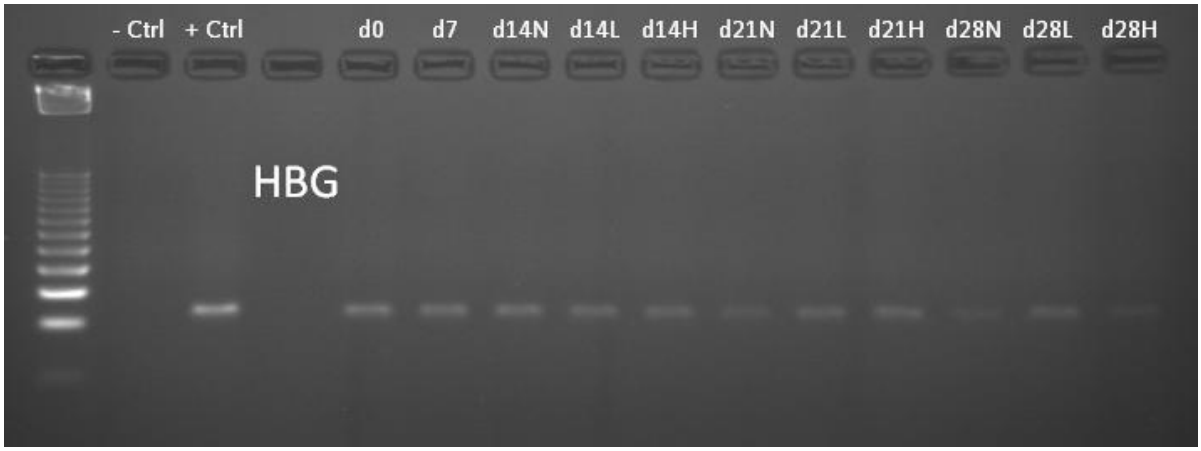

Figure 4.18. PCR results of gamma globin (HBG). With 60 bp, this is by far the smallest fragment amplified according to the experimental setup. K562 cells were used as positive control.

Haemoglobin is critical for the gas transport function of the red blood cells, being slightly different according to the development stage of the organism. In its composition there are 2 α chains and two other subunits; ε chains during the first 6 weeks of embryonic development, γ chains for the fetal period and β chains for the adult life. Between every stage there is a transition period where the expression of the new type of globin steadily represses the previous type[149].

Featuring the smallest amplified fragment of the chosen set of genes, HBG expression is seen in all lanes, although the bands seem fainter in later time points. The presence of these transcripts hints at an erythroid presence in culture, the haemoglobin being necessary for red blood cells maturation.

It is interesting to note that HBG expression occurs alongside HBB expression during culture. This is expected since at the time of CB MNC collection the organism is going through the last switch from γ to β globin, changing the main haematopoiesis site from the fetal liver to the bone marrow, and during this change there is an overlap in both globins expression. This parallel expression is maintained throughout the culture, suggesting an arrest in the interchange period, as *in vivo* the expected behavior would be the decrease of HBG until it is no longer produced while sustaining HBB production.

For the positive control K562 cells were chosen as they are normally used in studies regarding the various globin genes expression[124].

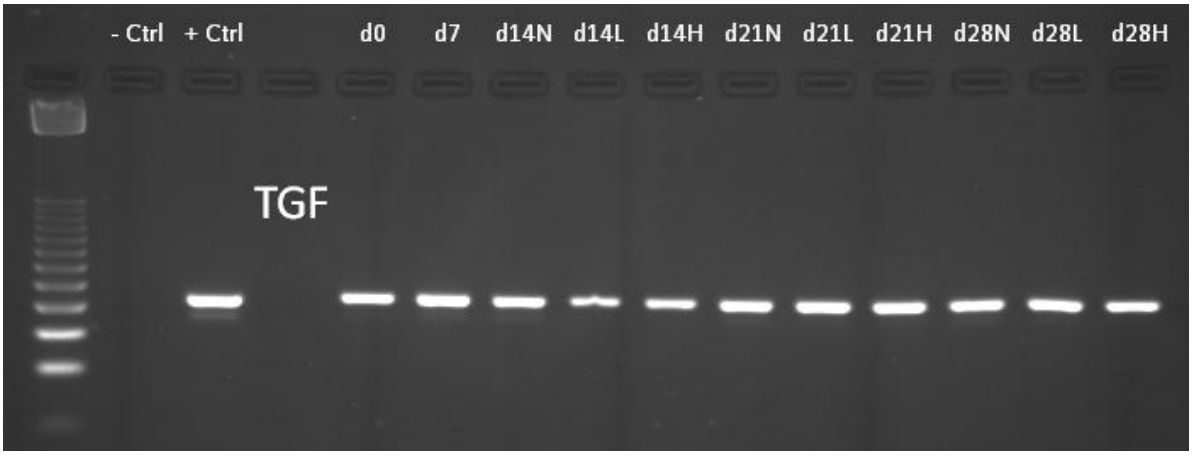

Figure 4.19. PCR results for transforming growth factor beta 1 (TGF). PCR amplified fragment 108 bp long. K562 cells used as positive control.

Yet another pleiotropic cytokine, transforming growth factor beta 1 (TGF) is known for having effect over several key processes such as haematopoiesis, embryogenesis, angiogenesis and immune modulation. It is expressed by tissues such as platelets, bone, lung and kidney[125]. Specifically, previous work demonstrated the importance of TGF in maintaining CD34+ progenitor haematopoietic cells in an undifferentiated state[150].

In the cell culture samples from each time point and condition it is possible to identify expression of TGF through the presence of intense bands in the agarose gel. The production of this cytokine could justify the upholding of multipotency by the cellular culture, allowing for the expansion of the progenitor cells without committing them all to a certain lineage.

4.6. Unpublished results from "Project EPO"

As previously stated in the materials and methods section, the complete test portfolio used to fully define the cultures within the novel biomimicry of the human bone marrow includes CFU and MTS assays used to determine, respectively, the capacity for multipotency and the proliferation of the culture and SEM along with Multiphoton to gather data on cell types and their physical organization inside the 3D cubes.

The existing quantity and quality of previous gathered data from these procedures, regarding the system using exogenous EPO addition, allowed the elaboration of a solid description of the cellular fate, growth and spatial arrangement within the scaffolds, with the results being presented below. As such and taking into account the time and resources constraints, since some of the tests are quite onerous, with the SEM, for example, taking several days of preparation and proper examination in order to produce sharp and meaningful images, the strategic decision was made to perform these four tests only for the novel projects where no previous data was available. This plan allowed for a detailed study of the new conditions in detriment of a new set of data for an already well defined system, a bargain deemed acceptable.

As a consequence there are no new results for MTS, CFU, SEM and Multiphoton for the experiment with the addition of exogenous EPO as it is assumed the culture behaves as in the

previous studies. This makes imperative to present said studies results in order to fully understand the system and to draw some conclusions on the novel procedures being undertaken.

These results were achieved by Dr. Teresa Mortera-Blanco, and their presentation is deemed necessary due to the fact that they are yet unpublished and therefore unavailable for the general public.

4.6.1. Cellular viability and proliferation

Using a standard haemocytometer, viability was assessed for every time point of the experiment, namely from day 0 to day 28 at 7 days intervals (Figure 4.20).

Noticeable right away is the collapse of the 2D control culture before the first time point. Also with decreasing values, although with a gentler slope, is the No EPO condition, before settling at around 50% towards the end of the considered timeframe. The cultures featuring addition of EPO have higher viability than the No EPO one (*, $p<0.05$), with the higher concentration achieving also higher alive to total cell number ratio. All conditions were successful in maintaining and expanding CB MNCs through the 4 week timeframe.

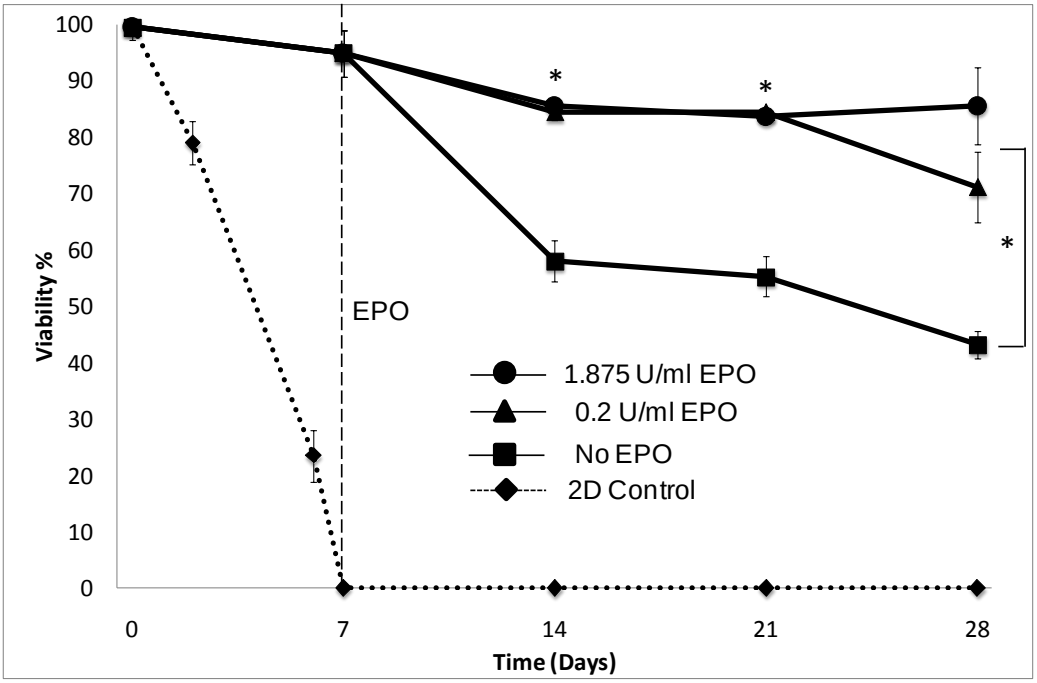

Figure 4.20. Cellular viability for every time point and discerning the 3 culture conditions. The 2D control culture died out before the first time point assessment. There is a noticeable higher viability in the cultures supplemented with exogenous EPO (*, $p<0.05$), with a stabilization around the 80% value, versus the final 50% for No EPO conditions. Dots show means ± SD, n = 2, N = 3. Courtesy of Dr. Teresa Mortera-Blanco.

Regarding cell proliferation, a MTS assay was used, translating the metabolic rates into absorbance values directly related to proliferation.

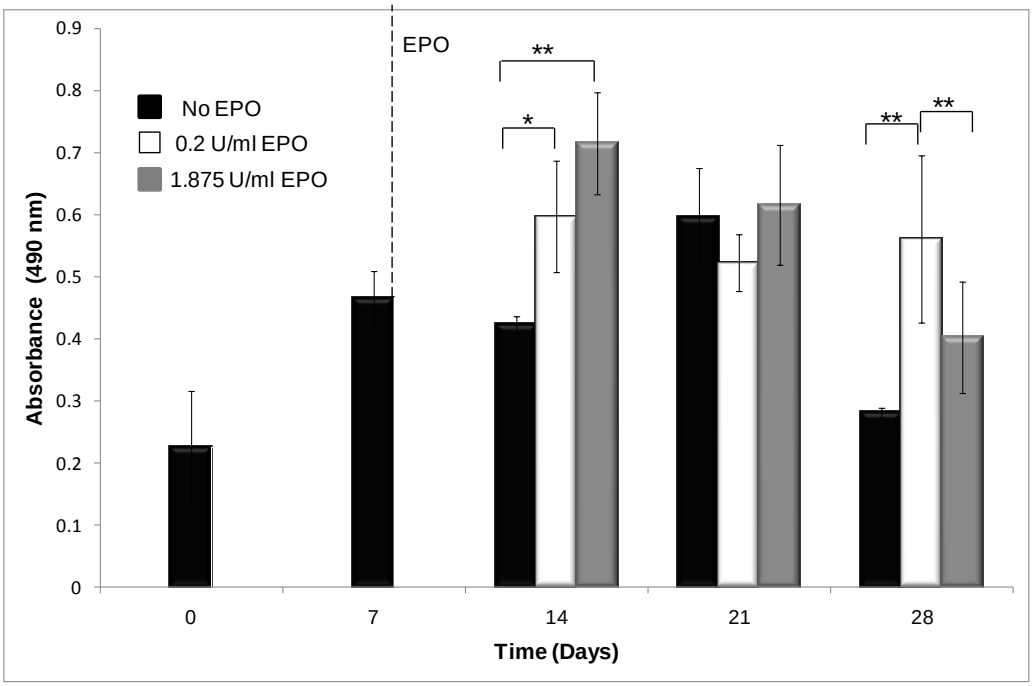

Figure 4.21. Cell proliferation assay (MTS). Higher columns translate to higher growth of cells over time. The dotted line marks the beginning of the addition of EPO to the cultures. Higher EPO concentrations lead to a peak at day 14 that slowly decreased into lower values. In the lower EPO concentration a constant level was achieved. Columns show means ± SD, n = 2, N = 3. (*, $p<0.05$; **, $p<0.001$). Courtesy of Dr. Teresa Mortera-Blanco.

Cellular proliferation increased from its initial value, almost doubling it, from day 0 to day 7. Afterwards there was the addition of EPO to the culture which increased the proliferation, as at day 14 the No EPO value is lower than the one from the lower EPO concentration (*, $p<0.05$) and way lower than the higher concentration (**, $p<0.001$). From this point onwards, the High EPO values decreased consistently, while the Low EPO ones maintained a plateau at the original value. In day 35 both conditions had again higher proliferation values than the No EPO culture, however, this time the Low EPO condition had the superior level in relation to both remaining cultures (**, $p<0.001$).

These results hint at the fact that EPO enhances cell proliferation, however it also induces differentiation, therefore speeding the High EPO condition kinectics into maturation of the cells, hence the lowering of the proliferation values. The lower EPO concentration was successful in striking a balance between the proliferative capacity and cellular differentiation, the reason behind the value plateau observed.

Conjugating both viability and proliferation tests results it is safe to conclude that the developed system is capable of long-term maintenance and expansion of CB MNCs under serum-free and cytokine-free conditions, with near-physiological levels of EPO as a supplement.

4.6.2. Scanning electron microscopy

In order to get an idea on the topography of the scaffolds, as well as the cellular organization on their interior, SEM was performed in sectioned halves of the 3D cubes (Figure 4.22). There is a visually noticeable increase in cellular number, with the expanding cells covering the nearby walls by day 14. Enucleated red blood cells were noted first as contaminants of the seeded MNCs and later on becoming more obvious after the addition of EPO to the studied cultures.

The establishment of cells in the niches of the scaffolds implies the formation of colonies as well as validating the collagen coating as cell-wall contact facilitator.

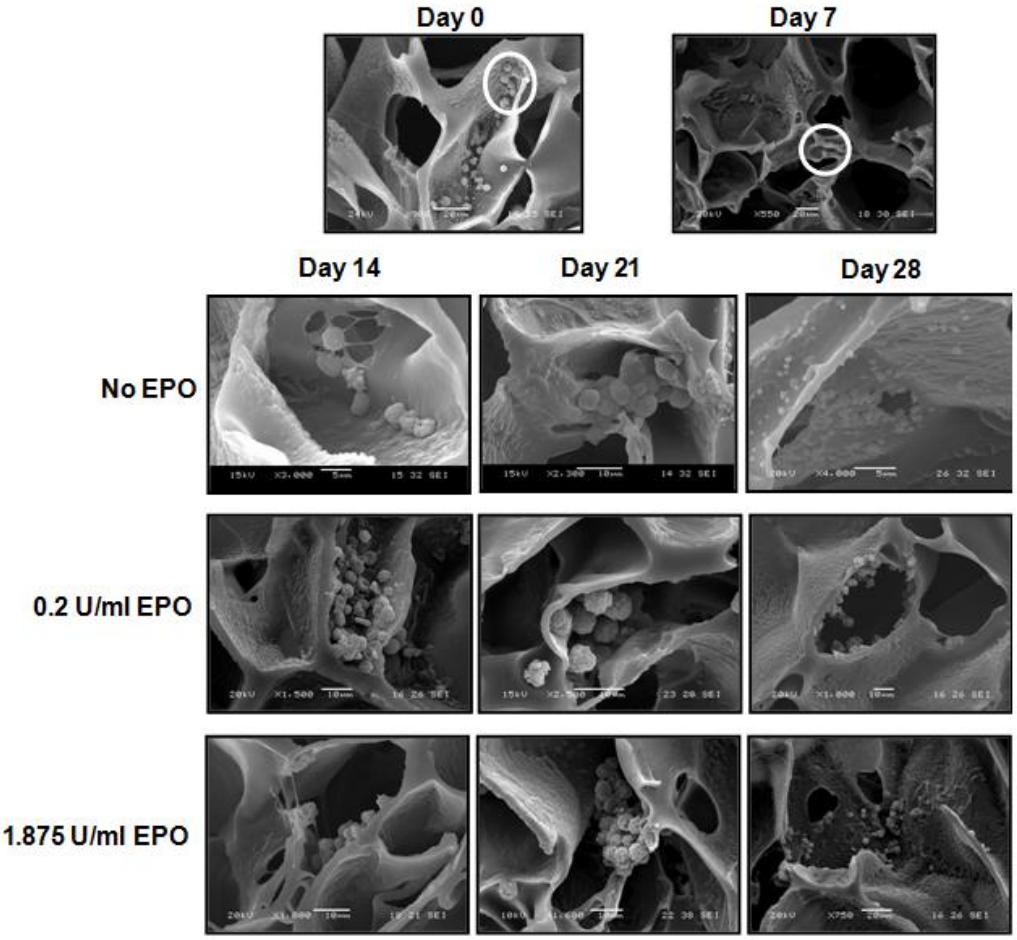

Figure 4.22. Scaffold topography and CB MNCs morphology through SEM imaging. It is possible to discern enucleated red blood cells (encircled) along with the seeded MNCs. Day 14 images show an expansion of the cells covering the scaffold walls. At day 28 cells are shown growing inwards from the walls, with red blood cells visible in the EPO containing cultures. Courtesy of Dr. Teresa Mortera-Blanco.

4.6.3. Histological examination through thin sections and cytospins

Thin-sections were taken from the mid-section region of the scaffolds, in order to take a meaningful analysis of the phenomena happening inside of the 3D cubes. Samples were then stained with haematoxylin & eosin, which showed cluster of cells in central niches of the scaffolds, as with the previous SEM results (Figure 4.23).

Cytospin analysis was used to detect the multilineage capability of the culture at hand (Figure 4.23). Myeloid and erythroid cells at different maturation stages were found, with mature red blood cells being identified at day 14 in the EPO supplemented studies. Evidences of cells from myeloid lineage exist even at day 28, suggesting that although the culture seems to be favouring the erythroid fate, it is still capable of multilineage differentiation.

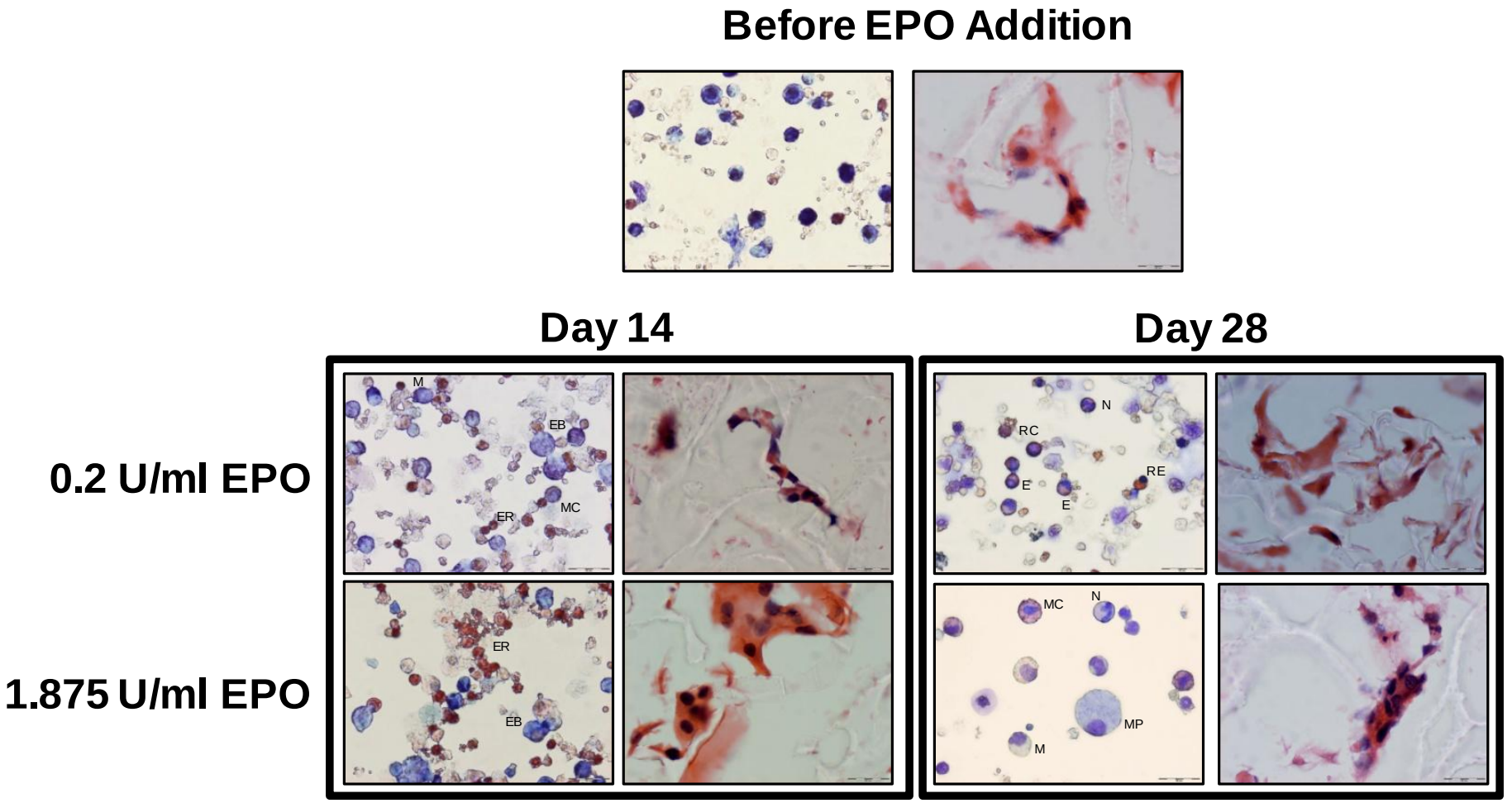

Figure 4.23. Histochemical (H&E) and Wright-Giemsa staining of cytospins. As with SEM samples, thin-sections were taken from the core of the scaffolds, showing cell aggregates. Cytospins showed cell maturation towards erythropoiesis with increased enucleated concentration of red blood cells at day 14. E: eosinophil; EB: erythroblast; ER: erythrocyte; M: myeloblast; MC: myelocyte; MP: macrophage; N: neutrophils; PM: promyelocyte; RC: reticulocyte. Courtesy of Dr. Teresa Mortera-Blanco.

4.6.4. Colony forming units assay

This test complements the cytospin data, evaluating the multilineage capability of the cultured cells by counting the different colonies types occurrence in the assay plates. Figure 4.24 displays the progenitor colonies assessable by the chosen assay. Data was gathered from every considered time point and compared with the day 0 values, establishing a tendency (Figure 4.25). At day 28 it is possible to discern the capability to generate forming BFU-Es, CFU-Es and CFU-GMs, although the CFU-GEMMs, representing the stemness of the culture, couldn't be identified. Day 21 records quite a drop in the counts for every colony type (**, $p<0.001$) in every condition, with no distinction between the EPO supplemented cultures and the No EPO control. This decrease in colony formation capability is described by MTS data that shows a decrease in absorbance at the same time, indicating cell maturation and loss of potency.

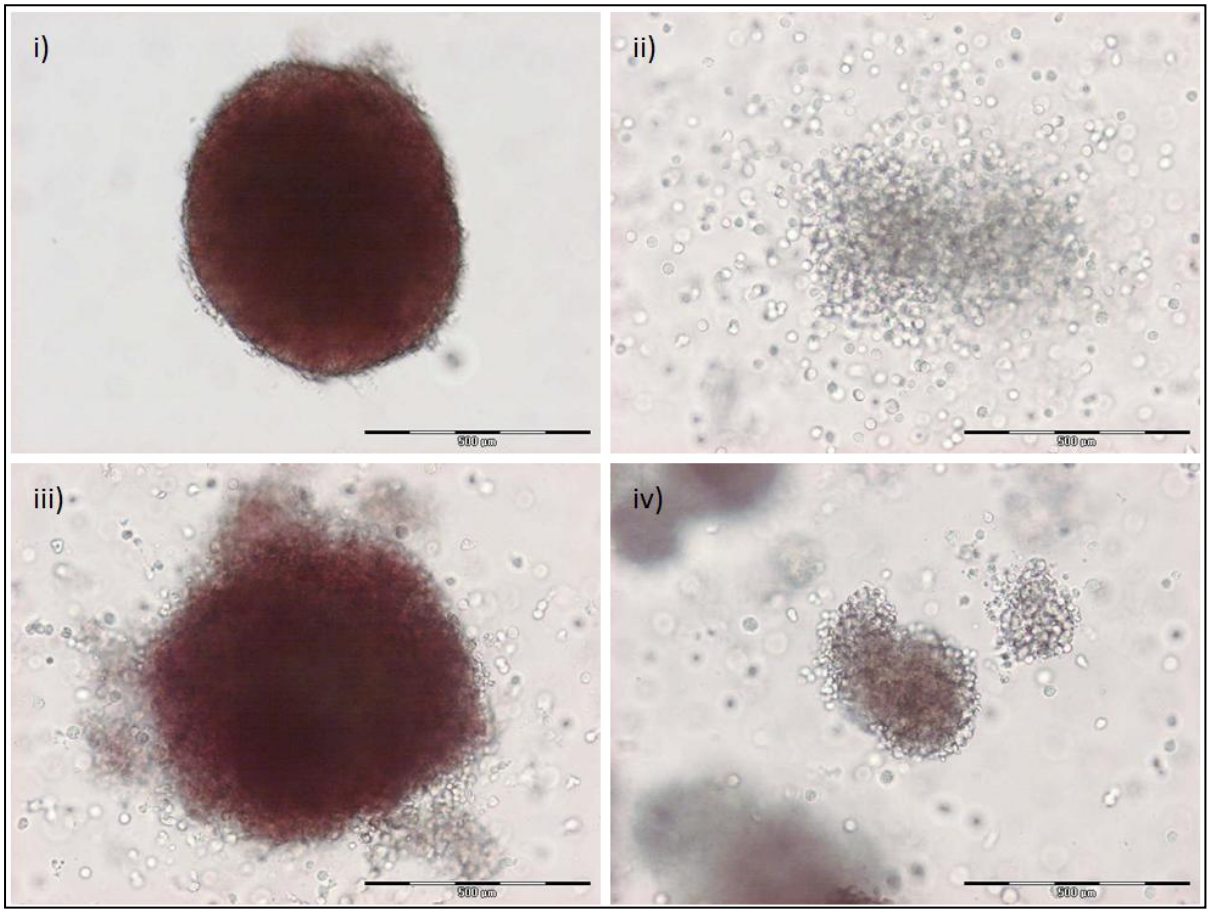

Figure 4.24. Types of colonies identifiable when using the CFU assay. On the top left corner of the image, listed under i) we have an example of an erythroid burst-forming units (BFU-E) colony, composed of primitive erythroid progenitor cells. ii) is a CFU-GM, where GM stands for granulocyte-monocyte, therefore denoting the progenitors of monoblasts and myeloblasts. CFU-GEMMs are represented by colony iii). These are myeloid early progenitors, with GEMM being the acronym for granulocyte, erythrocyte, monocyte and megakaryocyte. Finally at the bottom right image and marked with iv) we have CFU-E, colonies derived from CFU-GEMMs and representing more developed erythroid progenitor cells than BFU-E. Regards to Mr. Remus Winn and Mr. Nayer Youakim for the assistance on retrieving this data.

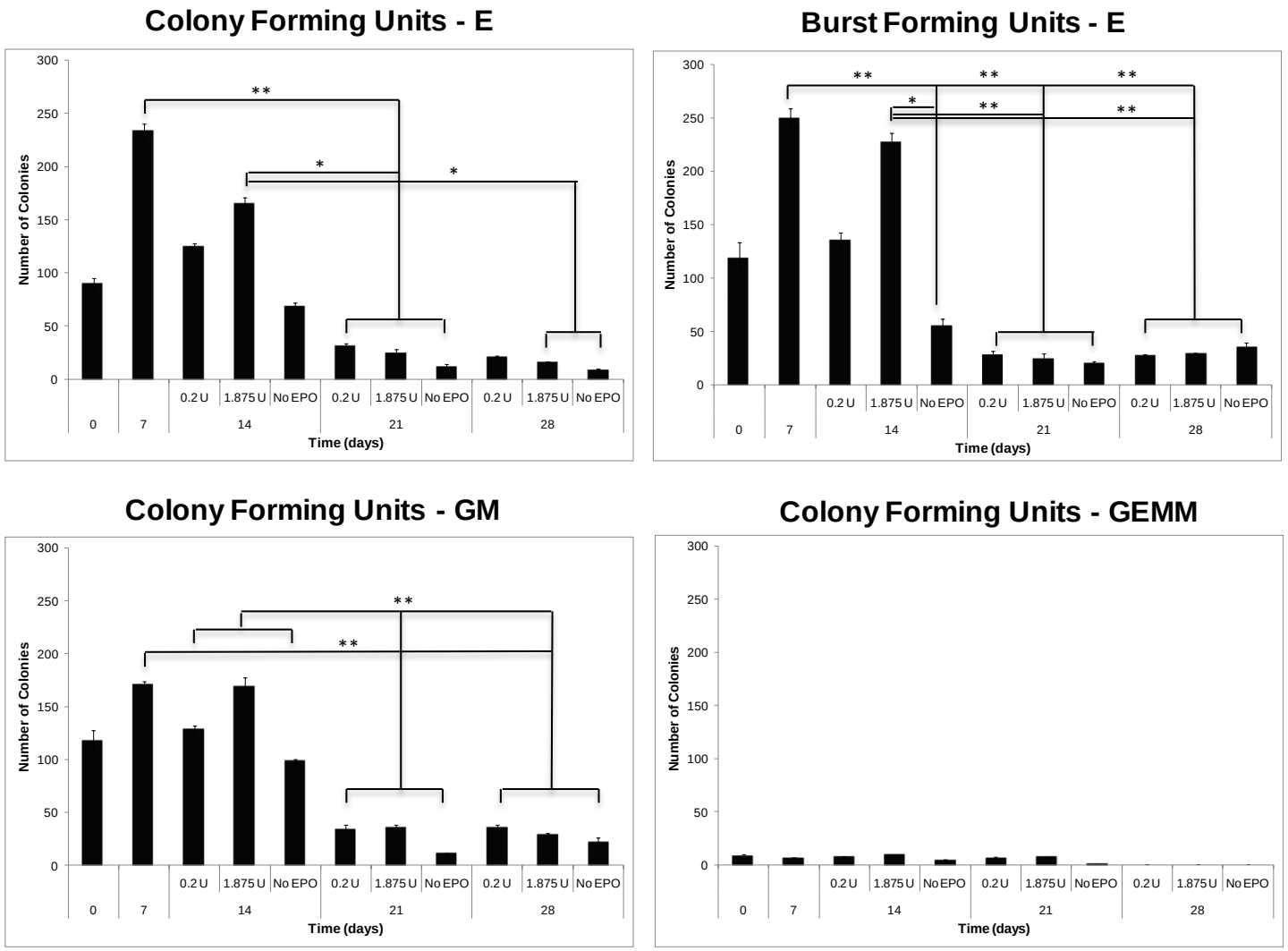

Figure 4.25. Clonogenic capacity of the CB MNCs assessed by colony forming units assay. Colony forming units were counted using a standard methylcellulose assay; numbers were expressed as colony forming units erythroid (CFU-E), burst forming units erythroid (BFU-E), colony forming units granulocyte-macrophage (CFU-GM) and colony forming units granulocyte-erythroid-monocyte-macrophage (CFU-GEMM). Numbers of colonies decreased over time ($p < 0.001$) which is an indication of culture maturation and differentiation. Results are means ± SD, n = 2, N = 3, (*, p<0.05; **, p<0.001). Courtesy of Dr. Teresa Mortera-Blanco.

4.6.5. Flow cytometry analysis

Immunophenotyping through flow cytometry (Figure 4.26) was used to further characterize the cells extracted from the scaffolds. The plotted data shows a move towards the erythroid fate after the addition of EPO at day 7, ultimately decreasing the usually high expression of CD45 positive cells at day 28, while increasing both CD71 and CD235a positive cells, an early and late erythroid marker, respectively.

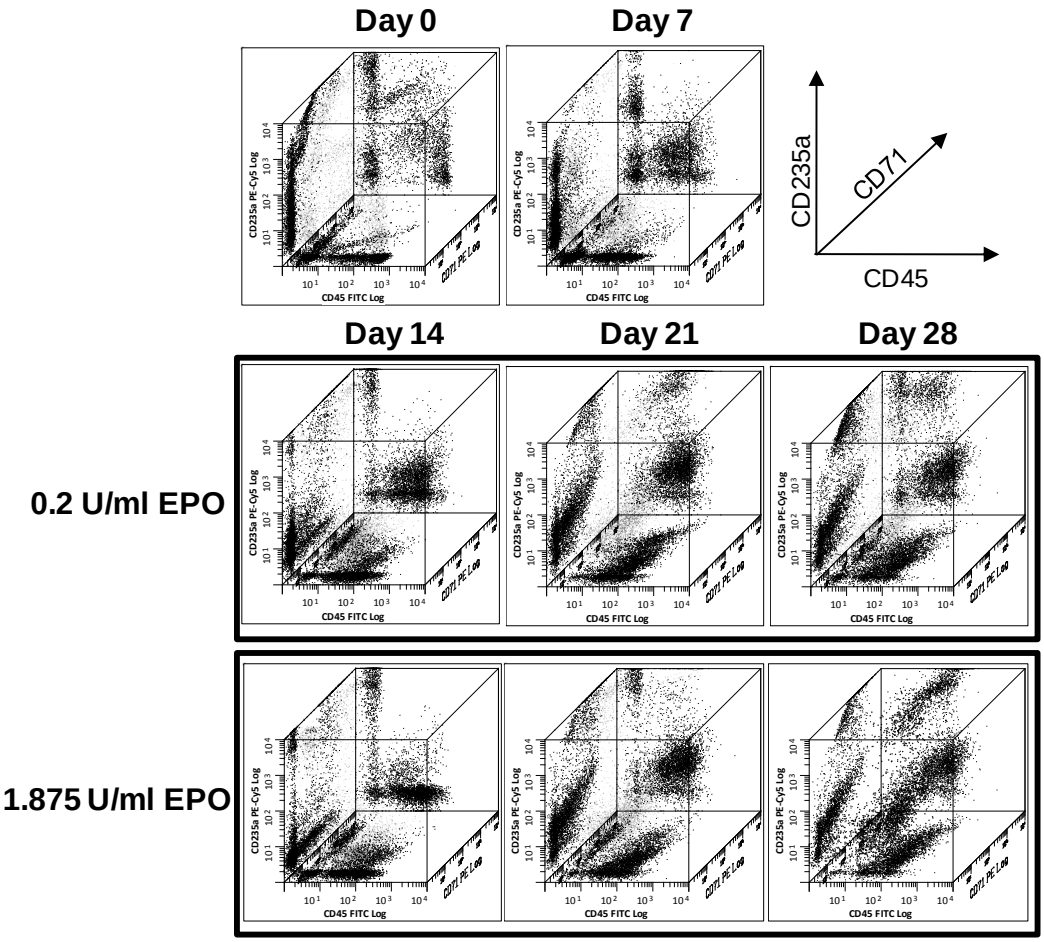

Figure 4.26. Immunophenotype of CB MNCs extracted from PU scaffolds assessed by flow cytometry. Expression of CD235a, CD71 and CD45 was analysed by flow cytometry using directly labelled antibodies. Analysis of the graph shows differentiation of the culture towards an erythroid lineage, through the increase in expression from CD235a. Courtesy of Dr. Teresa Mortera-Blanco.

4.6.6. Multiphoton microscopy

Used as a tool to complement the SEM results on scaffold topography as well as cellular morphology and physical organization inside the 3D cubes, multiphoton microscopy was performed on a High EPO condition scaffold at the last time point, day 28 (Figure 4.27). Erythroblasts were stained through the targeting of CD71, showing up as bright dots clustered in the scaffolds pores, as described earlier through SEM and thin sections. The 3D intern structure of the scaffold is also observable, demonstrating its convoluted nature.

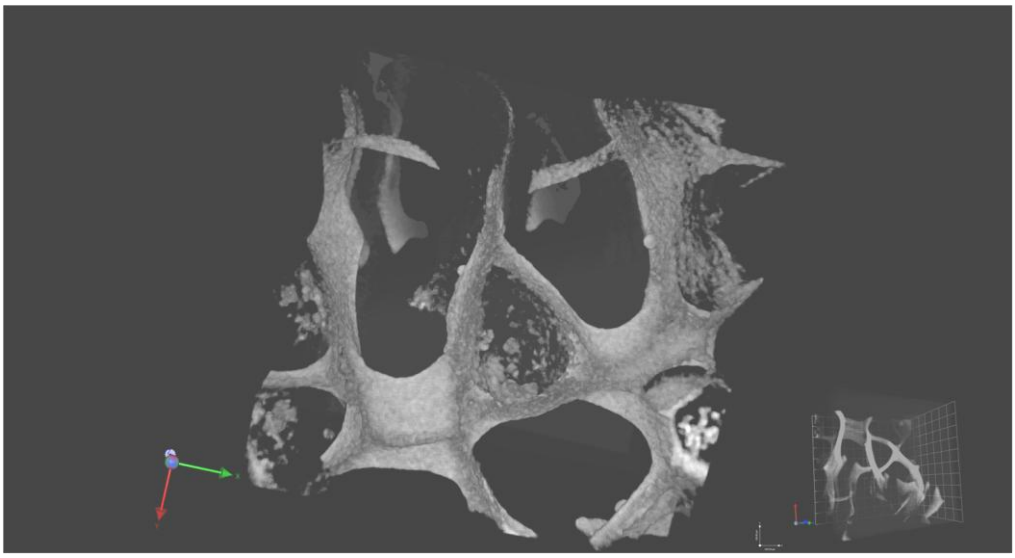

Figure 4.27. Multiphoton 3D image at day 28 of one High EPO condition scaffold. Cells positive for CD71 were immunostained, showing up as bright dots. On the bottom right there is a 3D image of a control unseeded scaffold. Courtesy of Dr. Teresa Mortera-Blanco.

5. Discussion

This work occurs as a follow up to several successful experiments performed within the hosting laboratory under the leadership of Professor Athanasios Mantalaris, namely the developing of a 3D PU scaffold that serves as a biomimicry of the bone marrow[19] and its application to culturing CB MNC, expanding them with the addition of only physiological levels of EPO (Project EPO, results displayed in section 4.6). While the system is fairly well defined in terms of the effects induced in the cellular cultures there are few clues regarding the reasons behind the behaviour observed. So after establishing that the 3D scaffolds coated with collagen and used in a culture supplemented with exogenous EPO could expand CB MNCs without the addition of any other cytokine and in serum-free conditions (section 4.6), the search for the reasons enabling such properties was deemed necessary, prompting the planning and execution of the work at hand.

As such, a cellular culture was performed using similar conditions to the previous work developed, with the exception of safeguarding samples for the expression analysis procedures. Some routine tests such as the CFU and the MTS proliferation assay were disregarded due to the abundance of data derived from various previous repetitions of the experiment, while other more complex ones such as SEM and immunofluorescence assisted confocal and multiphoton microscopy could not be performed due to time and manpower constraints. However due to the importance of understanding the complete panel of tests to fully define a culture, the missing protocols were practiced on similar parallel experiments (Results from parallel projects section in annex), providing the experience and know-how to successfully tackle an analogous situation in the future.

In the culture experiment developed for this work there is a hint towards lower viability values for the No EPO condition (Figure 4.1), however, a detailed statistical analysis determined no significant difference in the values between the various conditions through the time points. Previous work (Figure 4.20) determined the No EPO condition to have a noticeable lower viability value and the EPO containing branches to maintain the live to total cell ratio around 85%, with a slight advantage to the higher EPO concentration. Our experiment replicated the circa 85% values for the EPO conditions, lowering slightly until day 21, where the values plummet to low records for the last samples in day 28. The final steep drop seems to be an odd behaviour provoked by unknown reasons as the culture parameters were kept unaltered from the previous work, where no abnormality was noted.

Practically, it was more difficult to extract the cells from the scaffolds the longer these were in culture, yielding notably less cells and more cellular and structural debris. This characteristic has been reported previously[20] and alongside with the fact that during the last stages of the culture some of the research group's incubators were suffering from contaminations may help explain the anomalously low values. It is important to point out that the contamination problems happened in other projects, no evidence of sterilization problems were found in the cultures performed for this work.

The culture maintained a significant viability value (higher than 20%) in all time point samples, implying the ability of the developed system to sustain viable cells for 4 weeks and possibly further. Data from previous proliferation assays (Figure 4.21) support this conclusion, even determining that

the EPO addition induced cell proliferation while enhancing the differentiation as well. Nowadays no 2D culture under the same serum-free conditions can claim similar longevity (Figure 4.20), establishing right away one major advantage of the novel 3D biomimicry scaffolds over the traditional culture methods. This higher performance can be attributed to the relative success of the PU scaffolds in replicating the complex haematopoietic microenvironment structure, the bone marrow. Also important in said mimicry are the presented surfaces characteristics as the wettability in the regulation of cellular adhesion[151], as well as the mechanical contact itself between the scaffold and the cells. Collagen coating proves to be a key step in achieving the desired features[20, 152], providing the cells with a microenvironment similar to the *in vivo* counterpart, which is critical for HSC survival and self-renewal[5].

As for the study of the effects on the cellular fate, some techniques were used to determine the cells differentiation capacity, with the cytospin results confirming the maintenance of multilineage progenitor and developed cells. Figure 4.2 illustrates the previous statement, being a composed image of samples for each time point chosen for their relevance for the discussion at hand. It is possible to discern early and late erythroid, myeloid and lymphoid cells, the latter ones in very small numbers and more towards the end of the experiment. Earlier work displayed similar results (Figure 4.23), corroborating the ability of the developed culture systems to support multilineal differentiation, an important characteristic for a complete mimicry of the bone marrow, useful for the validation of the scaffolds as valid model of that human structure, serving as a research tool to better elucidate the mechanisms behind stem cell differentiation and lineage specification.

In between the various cell types it is possible to see an early predominance of erythroid cells, as expected since the seeded samples were MNCs extracted from CB. Later on, that predominance is maintained in the cultures supplemented with exogenous EPO, with an emphasis on the higher concentration study. The effects of EPO on erythropoiesis are long known[153], and as such the results are of no surprise, and were confirmed in similar previous experiments, namely through the analysis of the MTS assay and cytospin results (sections 4.6.1 and 4.6.3).

Also regarding cellular fate, the samples for each time point and cytokine concentration were subjected to flow cytometry analysis in order to determine the percentage of cells bearing a given marker, that way classifying the culture through specific marker expression. A clear depiction of the results is shown on Figure 4.6, a more simple presentation of the graphed raw data.

Using CD34 as a marker for HSCs[134] it is possible to identify their presence throughout the experiment in every condition, although at a very low percentage and with no significant difference between the samples with different cytokine concentration. Although Figure 4.6 displays only the total positive cells for a certain marker, it is possible to see from the raw 2D plots in Figure 4.3 to Figure 4.5 that the CD34+/CD38- cells, regarded as the primitive undifferentiated form[154], comprise most, when not all, of the CD34+ cells. CD33 arises as a similar early progenitor marker, but in this case, specific for the myeloid lineage[135]. As before this marker was found to be expressed in quite small percentages, although higher than CD34, and somewhat consistently through the timeframe considered. Once again there could not be established a significant difference between conditions, as the perceived errors greatly outweigh the measured differences, and as such the major conclusion

drawn is the presence of CD33 positive cells in culture at all times regardless of EPO concentration. This hints at the culture system capability to support HSC maintenance due to the scaffold properties only, as EPO addition had no noticeable effect. Corroborating these results are the cytospin images were early progenitors are shown in most of the slides, belonging to both erythroid and myeloid lineages, along with the CFU results gathered previously (Figure 4.25) where it is possible to see a small number of CFU-GEMMs throughout the experimental timeframe, save for the last day. These cells represent the stemness of the culture and no difference in colony formation was recorded between the various EPO concentrations.

The data concerning CD45 is deceiving at first glance, as the seemingly odd behaviour displayed by the bar graphic is, after statistical analysis, deemed non significant, leaving only the possibility to state that there is a proportional high percentage of gated CD45+ cells in all samples. Since this is a haematopoietic mature cell marker, excluding mature erythrocytes and platelets[136], these were the predicted results, due to the haematopoietic nature of the cells seeded and the microenvironment being mimicked. This certainty of the presence CD45+ cells in culture is exposed by the choice of this marker for the isotype controls in the flow cytometry protocol.

The first of the four markers that showed statistical significant results between the established conditions was CD38, a glycoprotein known to be expressed by myeloid and lymphoid progenitors[137] and later mature lymphocytes and monocytes[155]. The noticeable drop in the registered values provides insight into general cellular fate as it hints a deviation from the myeloid and lymphoid lineages. Interestingly EPO seems to positively impact the percentage of CD38+ cells, an unexpected outcome due to its established role in erythropoiesis.

Of the seven markers mentioned previously, the remaining three are related to the erythroid lineage. CD71 or transferrin receptor protein 1 represents the early progenitors, as its expression dwindles from previously high values when the cell reaches the normoblast and reticulocyte phase[138, 156], CD235a is also known as glycophorin A, a major protein of the mature red blood cell membrane[139], therefore used to measure mature erythrocyte levels and finally EPOR as middle ground between the two stages previously mentioned[140], being expressed by cells in the intermediate stages of maturation. This complicity in the marked cells lineage prompts a simultaneous analysis of all three markers, generating an overview of erythropoiesis in our system. CD71 positive cells percentage grows steadily from near nonexistence into very high values towards the end of the experiment and while it appears that higher EPO concentrations lead to lower percentages, that difference is not statistically significant, meaning that no conclusion can be derived on the cytokine effect for this marker. Analogously CD235a expression drops as time elapses although, once again, the final time point is characterized by a higher expression in the No EPO condition, lowering as the EPO concentration increases. EPOR positive cells percentage follows a more intriguing pattern, as after displaying almost no expression until day 21, its values jump into life, again with High EPO as the lowest percentage.

In conclusion, analysing the erythroid lineage, there seems to be a regression in terms of cell maturity, displayed by the drop in CD235a and the increase in EPOR and CD71. Conjugating these results with the ones from CD38 there is a clear hint that the previously mentioned shift away from the

myeloid lineage is connected to the noticeable convergence towards a middle stage erythroid fate. Previous work yielded similar conclusions (section 4.6), while it did not record the lower percentage values for the erythroid markers in High EPO conditions. As an erythropoietic factor it was supposed to enhance the appearance of related markers, however looking at EPOR expression it is possible to see that the recorded values are very faint until day 28. Cells without EPOR cannot be affected by EPO so the late meaningful expression of the receptor might explain why the expected effects of EPO are not recorded experimentally. Overall these results attest the capability of the developed 3D biomimicry to expand both progenitor and mature multilineage haematopoietic cells, with some emphasis on the middle maturation stage of the erythroid lineage.

In the work already developed on “Project EPO”, thin sections, SEM and multiphoton microscopy served as topographical analysis of the scaffold structure and the arrangement of the cells inside said structure. SEM imaging (Figure 4.22) recorded the clustering of cells in niches, along with the presence of mature erythroblasts, confirming the erythroid fate of the culture hinted by previous tests. The thin sections results confirmed the clustering of cells in scaffold niches (Figure 4.23), while multiphoton microscopy revealed the erythroblastic nature of some of those aggregates (Figure 4.27).

With the reported results resembling the data gathered in previous iterations of the system under study (section 4.6), it was possible to ensure that the results from the novel procedures concerning gene expression analysis can accurately complement the knowledge already gathered about the 3D PU scaffolds. These protocols were chosen to shed some light into the system characteristics and consist of RT-PCR analysis.

Due to the novelty of the *in situ* extraction procedure inside the research group, this was quickly validated, at the same time ensuring the good quality of the extracted mRNA (Table 4.1) to certify the following protocols. An optimization step was required to find the optimal agarose concentration to correctly visualize the desired bands (Figure 4.8), with a compromise achieved between easiness of gel preparation and casting and separation capability.

It is known that achieved results are only as good as the controls, negative and positive, that ensure their validation, and as such a great care was taken into the designing of these controls for the experiment at hand, their identity and the literature consulted for their selection being listed in chapter 3.12. Beta actin was the HK gene and with Figure 4.9 the presence of cDNA is detected for all samples, therefore ensuring that if a result appears negative is due to the lack of the studied gene expression, not the lack of cDNA template.

EPOR is the first analysed gene, showing expression in all time points and conditions (Figure 4.10). Although the bands in the gel show different intensities, no meaningful conclusion can be drawn on expression levels, as RT-PCR results are qualitative, not quantitative. As such, this procedure is best used in pioneering studies, such as the one at hand, where the question is if there exists or not expression of a given gene, with the accurate measuring and comparing of expression levels left for more adequate follow up procedures, such as quantitative real time PCR (qPCR), after identifying the interesting genes to be scrutinized. From the gel it is possible establish the existence of EPOR expression throughout the experimental timeframe, a result seemingly in conflict with the gated

cells percentage from flow cytometry analysis of EPOR+ cells, which is almost non-existent up until the last time point. This conflict is not real due to the difference between gene and actual protein expression, other factors may have played the part on impeding the translation of the noticed mRNA.

Production of EPOR is traditionally attributed to the middle stages if erythroid maturation[140] although more recent studies detected the expression of the gene in other tissues[141]. Nevertheless, due to the haematopoietic nature of the seeded and cultured cells, it is safe to assume that the results imply the presence in culture of cells belonging to the erythroid lineage, as confirmed in previous tests.

The gel image for TPO (Figure 4.11) shows no expression at all for the gene in question. As a major cytokine for megakaryopoiesis[157] the recorded presence of it in culture would hint for a stimulus in the formation of platelets. However its absence isn't surprising as the major recorded production of TPO is by hepatocytes[142], and there is no recorded presence of such and similar cells in the culture system developed.

Of the genes selected for the study, TNF, IL1B, IL6 and IL10 are known to play related roles on the systemic immunological response, more specifically regarding inflammatory response[158-161], therefore being produced mainly by activated monocytes or macrophages[128, 131, 144, 146]. Both TNF and IL1B show similar expression patterns, with the presence of faint bands all throughout the experimental activity. Their presence implies the existence in culture of the cells that produce said cytokines, therefore being a testament to the late monocytic cells in culture. Interestingly IL1B has a stimulating effect on TNF expression[145], somewhat justifying the similarity between the gel profiles. TNF is known to inhibit early haematopoiesis, having a potentiating effect on early haematopoietic progenitors expansion and proliferation[162]. As such its presence may explain the maintenance of multilineage differentiation capacity through the experiment reported before through cytospins and flow cytometry analysis. The inhibitory effect might be negligent due to the seemingly low expression of TNF, as it is possible to discern clues of haematopoietic development through the other tests performed.

Along with the TNF stimulation, IL1B has a role in IL6 expression induction[145], however the latter's expression profile behaves more interestingly than the two previously analysed, with a single peak of expression in day 7, with no surrounding bands whatsoever (Figure 4.15), similar to IL10 gel image (Figure 4.16). This seems to be a response of the cells the seeding procedure, perhaps reacting to the new environment with the activation of related pathways and returning to the initial setting once the steady-state condition ensues.

IL6 is known to act along side with IL3 stimulating haematopoietic progenitors[163] and to have an important role in the maturation of B cells, and as such a link can be established between its expression reported on day 7 and the leap on CD71+ cells from day 0 to day 7 as noticed by flow cytometry. IL10 shares a similar expression profile, but its effects are quite the opposite, being regarded as an anti-inflammatory cytokine, playing a role in the repression of the previously mentioned three[164]. As for its effects on haematopoiesis, it mainly directs monocytes into a macrophage fate, away from dendritic cells[147], its other effects are through indirect stimulation, by inducing or repressing other cytokines and factors such as TNF[165]. However the recorded expression

is both faint and ephemeral, making it harder to attribute any phenotypic alteration to the action of this gene in the described culture system.

Another two genes that are similar in function are HBB and HBG, differing only in the fact that HBG is expressed before birth and HBB after and through all adult life. The recorded results show an expression of HBB that grows fainter as time goes by, being notably stimulated by the addition of exogenous EPO (Figure 4.13). Since EPO induces erythropoiesis[153], these results are expected. HBG on the other hand presents faint expression bands in every sample, staying seemingly unaffected by the EPO (Figure 4.18). Both result sets point towards the presence of cells from the erythroid lineage, as haemoglobin is a critical protein of mature red blood cells. Since those are enucleated, prohibiting any gene expression, the erythroid cells contemplated here must be late progenitors, although the presence of mature erythrocytes is also hinted mainly from SEM (section 4.6.2) and cytospins (sections 4.2 and 4.6.3). Figure 5.1 shows the normal expression of the genes involved in the formation of haemoglobin, namely the profiles of ε, γ and β globin.

Interestingly the expression of both genes in our experiment is overlapped, as it is expected at the time of birth, where HBG hasn't still been completely replaced by HBB[149]. While this is normal for the day 0 samples, since CB is frozen after collection from the newborn child, it was expected for the system to evolve as *in vivo*, by performing the previously cited replacement illustrated in Figure 5.1. That doesn't happen, suggesting an arrest of the whole culture in the post-natal change period, when concerning the globin expression.

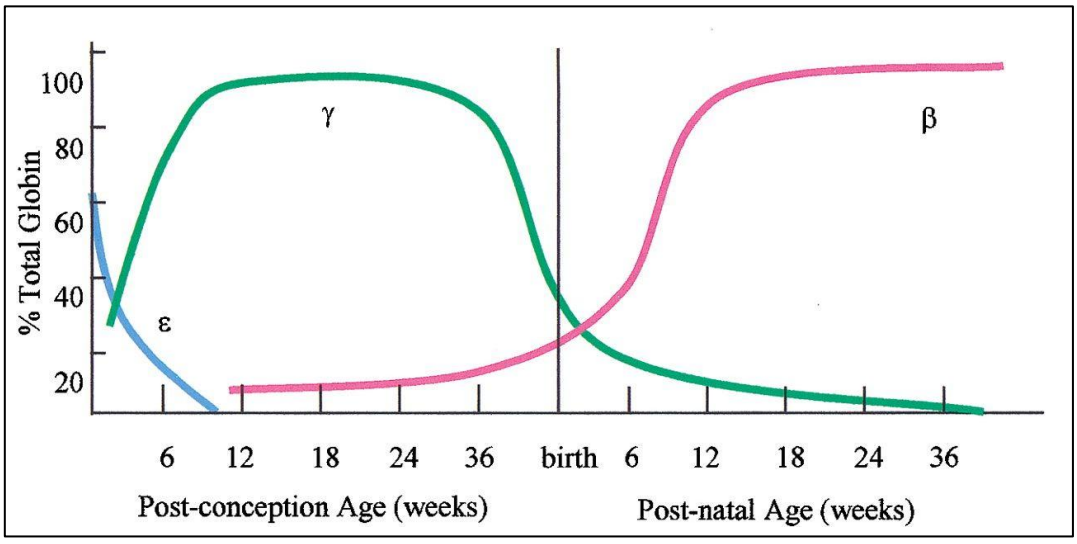

Figure 5.1. The various globin types expression through human development. It is possible to see the various exchanges between haemoglobins along the timeframe. During these switches there is a window where both forms are expressed, co-existing in the organism. Adapted from Harju, S. et al, (2002)[149].

CSF2 shows an irregular expression pattern along the timeline (Figure 4.17), with a distinct lack of band in the last time point for the highest EPO concentration. Scientific reports indicate the role of CSF2 in directing cells towards the myeloid lineage[148], however the erratic distribution of band intensity dismisses any link with other data in order to elucidate what is happening inside the scaffolds. A proteomic analysis could translate the expression data into actual cytokine concentration,

therefore establishing the presence of CSF2 in culture, determining if this constitutes or not a biochemical nudge into the myeloid lineage.

The last studied gene was TGF, which demonstrated a quite intense string of bands in all samples. This cytokine is recorded as being important for maintaining progenitor haematopoietic stem cells at an undifferentiated state[150], inhibiting early haematopoiesis[162], helping the culture system to maintain undifferentiated early haematopoietic CD34+/CD38- cells, providing multilineage differentiation capability as described in cytospins and CFU assays from both previous (section 4.6) and presently developed work.

6. Conclusion

In summary, this work derived from the earlier project where using a novel 3D collagen coated PU scaffold mimicking the bone marrow microenvironment, it was possible to maintain and expand CB MNCs for at least 4 weeks in a serum and cytokine free environment. In an effort to enhance erythropoiesis, the developed system was tested with the addition of near physiological levels of EPO, achieving cellular expansion, with clear hints pointing at a tendency of the maturation towards the erythroid fate.

Contrary to the belief that serum-free media is generally unsatisfactory for culturing primitive HSC[166], all culture conditions were capable of maintaining CD34+ cells throughout the timeframe of the experiment, demonstrating as well multilineage differentiation capacity in all samples collected from the established time points. So far no 2D culture under the same culture conditions can claim similar performances, with the traditional method for CB CD34+ cells culture involving the addition of various cytokine cocktails to support the expansion[105-108, 114, 117, 167], or with the further use of stromal layers in co-culture with the desired cells[13, 168]. Although displaying promising results, the published works fail to reach a consensus on the optimal cytokine cocktail composition, requiring as well high cytokine concentrations that limit their applicability in scale-up projects as well as hindering the accurate mimic of the physiological microenvironment and haematopoietic process, a problem avoided in the proposed novel BM biomimicry.

Elucidation on the presence of mRNAs hinting towards erythropoietic lineage favouring was achieved through the gene expression analysis (EPOR, HBB and HBG). Also expressed were some genes that explain the ability of the culture to maintain undifferentiated progenitor cells (TNF and TGF) and more importantly, an arrest in the post-natal globin transition period was noted through HBB and HBG transcript study.

6.1. Critical evaluation and thoughts on future research

One noticed shortcoming of the developed system was the extraction method used to gather the cells prior to their processing and usage in several tests. It was noted an increased practical difficulty in the mentioned protocol as time in culture passes by, being unclear whether all cells can detach easily enough from the scaffold, being therefore extracted. The question of how many and which type of cells remain in the scaffold and if the extracted sample is representative of the general population remains unanswered, being the subject of ongoing studies.

Regarding gene expression, the full validation of the achieved results leads through proteomic assays, in order to determine if the expression is fully translated into protein levels or not. Also, regarding the most interesting cases, the application of a quantitative assay, such as qPCR, might improve the conclusions to be drawn on the mechanisms behind the exhibited qualities of 3D culturing of CB MNCs. An example of a pair of genes that could benefit from said upgrade in test capability is the HBB/HBG pair, as it would be interesting to verify the effects on the whole system of the arrest in the transition period.

6.2. Concluding remarks

The developed 3D bone marrow biomimicry can be a useful tool when applied to the study of haematopoiesis *ex vivo*, being also applicable to the expansion of human haematopoietic stem cells for clinical applications such as cellular therapies and transplantation.

Overall the collaboration experience with Professor Athanasios Mantalaris research group was a very positive one, leading to the elaboration of significant work while enriching the author's personal and professional experience.

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Annex

Results from parallel projects

Alongside with the main work developed towards the elucidation of cytokine presence in the culture using the novel 3D scaffolds designed to mimic the bone marrow environment, other projects were being pursued within the research group using the same culture apparatus but with the addition other exogenous cytokines at day 7.

As such, in order to learn and to be aware of all the techniques involved in characterizing the cultured cells, there was an active participation on the parallel projects concerning the protocols for MTS, CFU, SEM and Multiphoton. The data generated is presented below with a brief critique of the meaning of the results within the project it concerns.

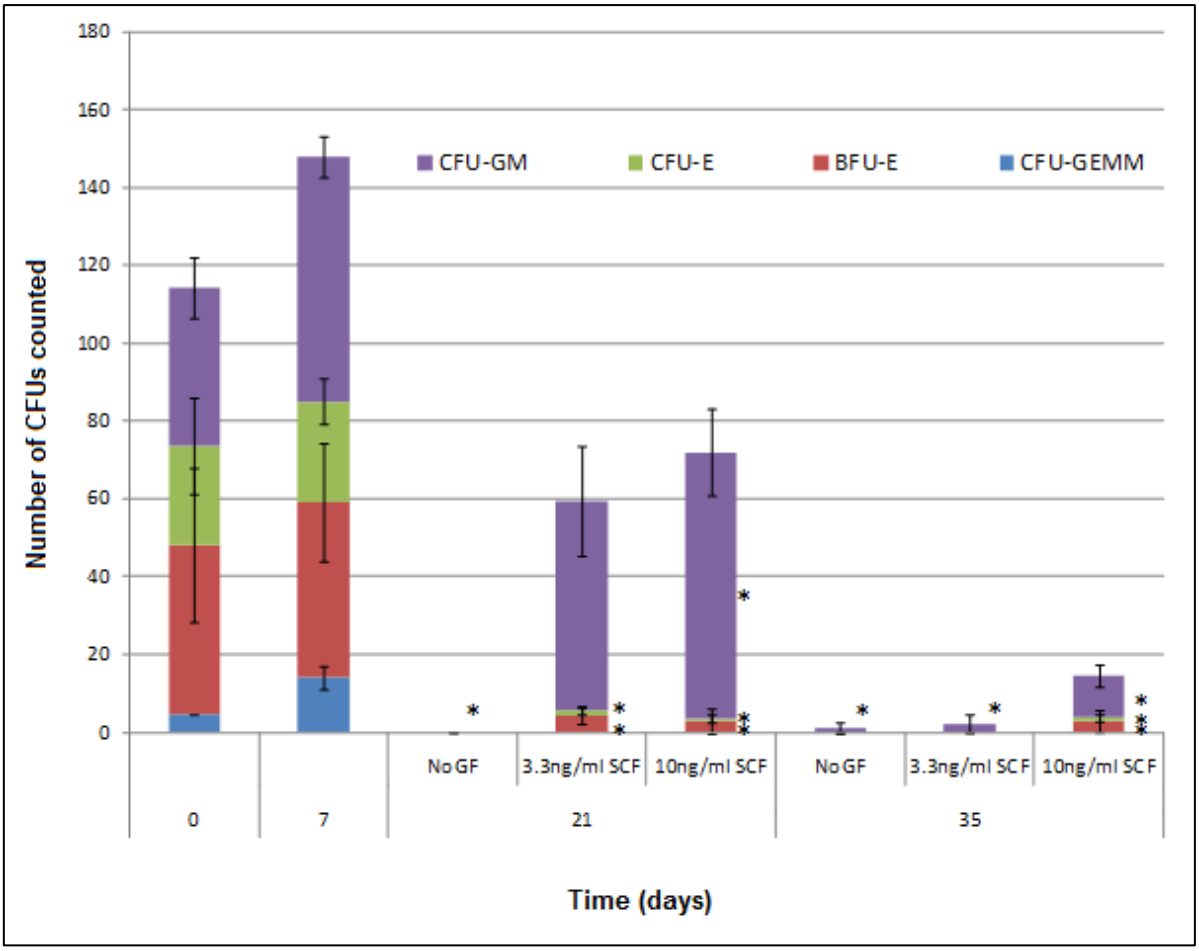
In total, 2 new projects were under way and data was gathered for both of them. However, since its inclusion here is mainly to illustrate the able learning, executing and discussing of the obtained results, only one of them will be referred, namely the “Project N”, where two near physiological concentrations of SCF were used as exogenous cytokines to influence the culture fate.

Colony forming units (CFU) assay

This test is performed on every time point selected to extract samples from the culture, quantifying the ability of the cells to differentiate into multilineal colonies. The expansion media used, MethoCult®, is specific for progenitor haematopoietic cells, and as such, the main types of colonies derived are presented in Figure 4.24.

Through the use of an inverted microscope it was possible to identify colonies similar to the ones pictured above and to count them in every time point and condition. Using two different collaborators to do separate counts and then averaging the results helped to eliminate any bias in classifying the colonies.

Figure 4.24 back in the results chapter displays the distribution of the different types throughout the experiment, bearing in mind that “Project N” established an addition of SCF to the culture in two concentrations, 3.3 ng/ml and 10 ng/ml, from day 7 onwards. Along with the control where no exogenous cytokine is added, this makes the 3 different conditions studied.



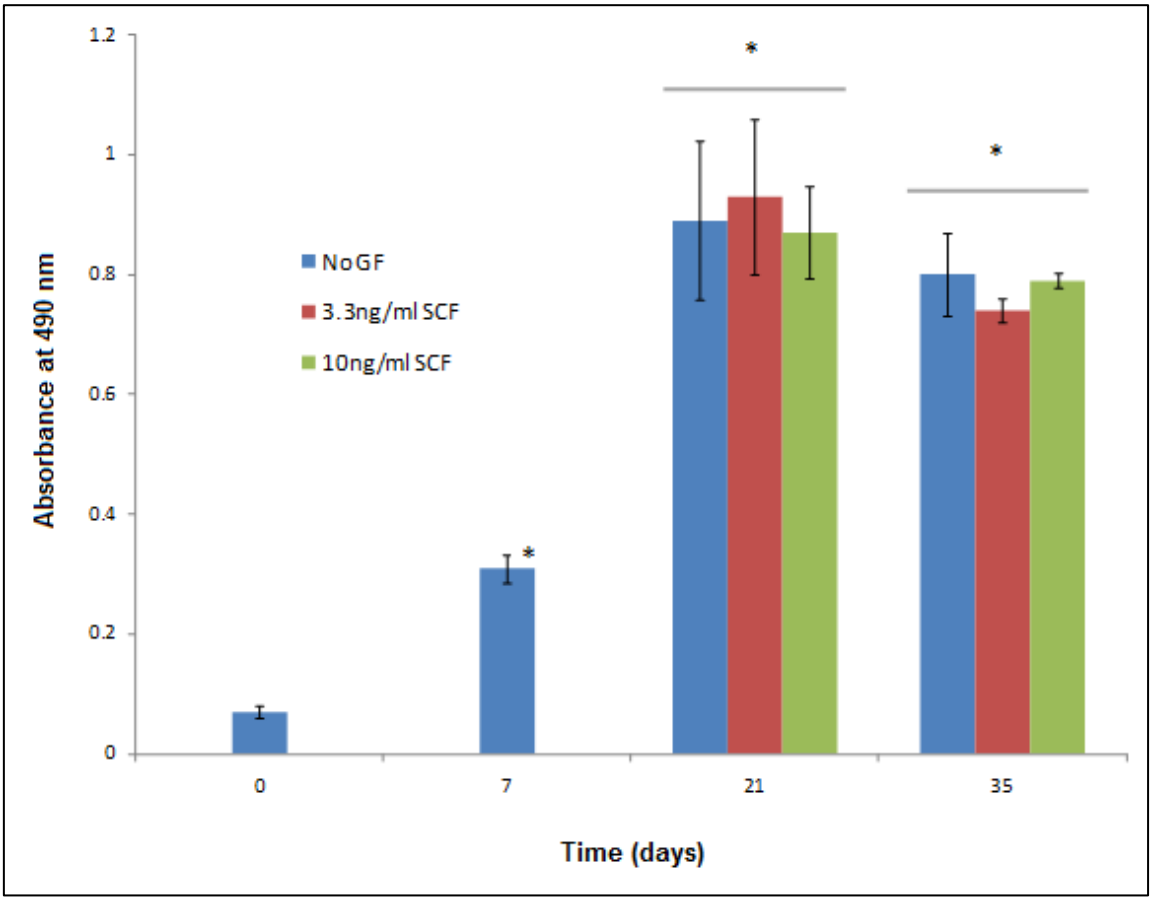
Annex Figure I. Ilustration of the CFU countings for "Project N". SCF was added to the culture since day 7, establishing 3 different culture conditions. Each colour refers to a different type of colony, as depicted in the legend. The error bars for each colony type count is displayed on the top of the column concerning said type. The asterisks denote a statistical significant difference between the marked value and the day 0 counterpart. Columns display means ± SD, n = 2, N = 1. Courtesy of Mr. Nayer Youakim.

Analyzing the graphed values, it is possible to discern an increase from day 0 to day 7, followed by a steady decrease from the latter until the end of the experiment at day 35. Also noticeable is the predominance of CFU-GM in all time points with increased numbers of colonies in the SCF complemented cultures on the later stages. It is important to point out the subjectivity of the colony identifying procedure, which allied to the novelty of the work for the researcher involved, lead to the significant standard deviations noticeable on the first samples.

The decrease in CFUs can be attributed to the fact that with culture, cells differentiate and therefore leave fewer progenitors capable of generating multilineal colonies. It is known that SCF increases the number of BFU-E, CFU-GM and CFU-GEMMs when added to the culture media[89], therefore justifying the results showing a higher number of colonies in SCF containing conditions. These results confirm that SCF is useful in maintaining the capacity for the culture to differentiate into distinct lineages when used with the novel 3D scaffold developed.

Cell proliferation assay (MTS)

An indirect measure of cell proliferation is achieved through the monitoring of the metabolic activity of the cells by the use of MTS dye. After the required preparation and incubation steps the absorbance at 490 nm is read in a microplate reader and the raw data is saved in a spreadsheet format. Further processing allows the construction of graphs that better elucidate the peculiarities of each measurement.



Annex Figure II. MTS assay results for "Project N". Here we have the absorbance values for each experimental sample. Bigger bars come from greater metabolic activity and therefore imply greater proliferation. There is a statistical significant difference between day 0 and all the other days (*, $p<0.05$), however the same cannot be said of the values between conditions in days 21 and 35. Columns display means ± SD, n = 2, N = 1. Courtesy of Mr. Nayer Youakim.

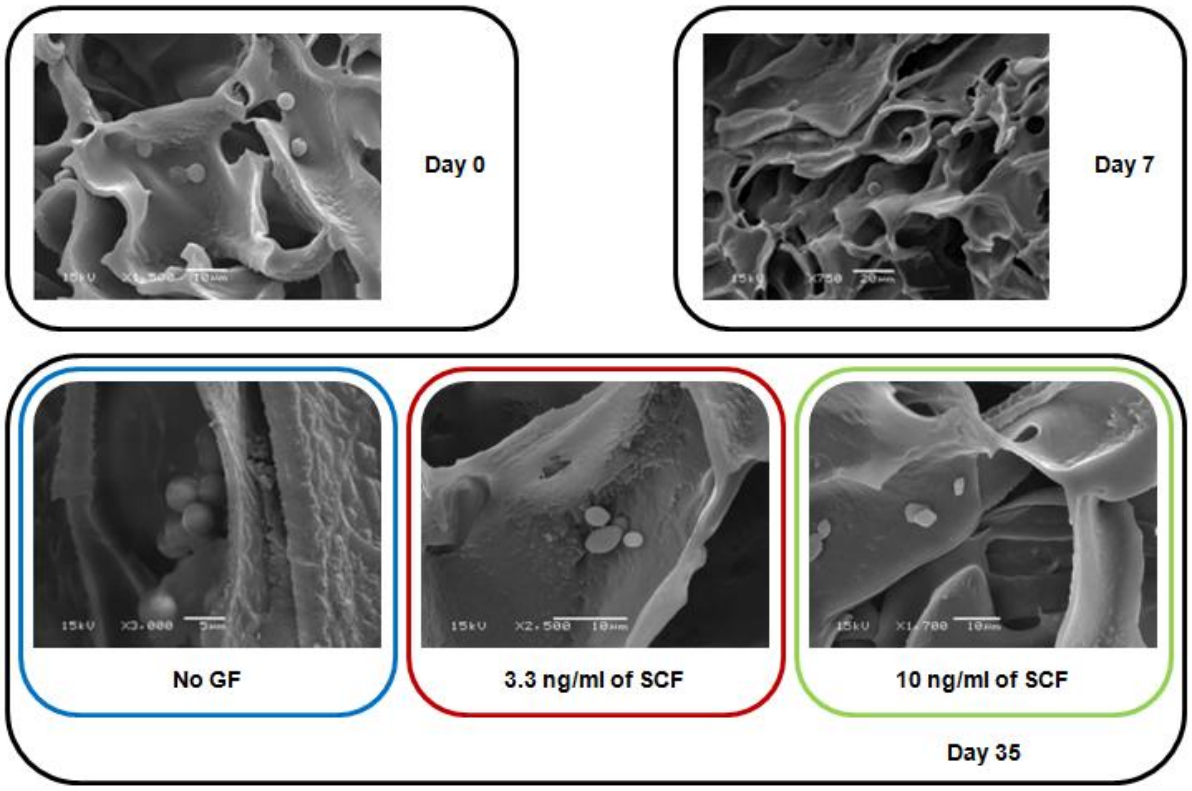
This test shows an increase in cellular proliferation from day 0 until day 21, where it peaks and drops very slightly to the day 35 results, a well documented behavior[19]. These results were expected as the longer time in culture allows the cellular number to increase and therefore resulting in a greater metabolic activity. The slight decrease in the last time point may suggest the culture reaches a steady state, not being able to support a greater number of viable cells. In general there is an over 10 fold increase in the absorbance from the beginning of the experiment.

Even though a familiar behavior was noted, no novel conclusions could be drawn from this assay, as the difference between the studied conditions is not statistical significant, therefore

invalidating any affirmation on the effects of SCF on the cellular proliferation capacity when applied to the novel 3D scaffolds.

Scanning electron microscopy (SEM)

In order to get a closer look at cell morphology and cellular physical arrangement inside the scaffolds the samples were coated in gold and visualized in a scanning electron microscope. A sample of the resulting images is presented in Annex Figure III.



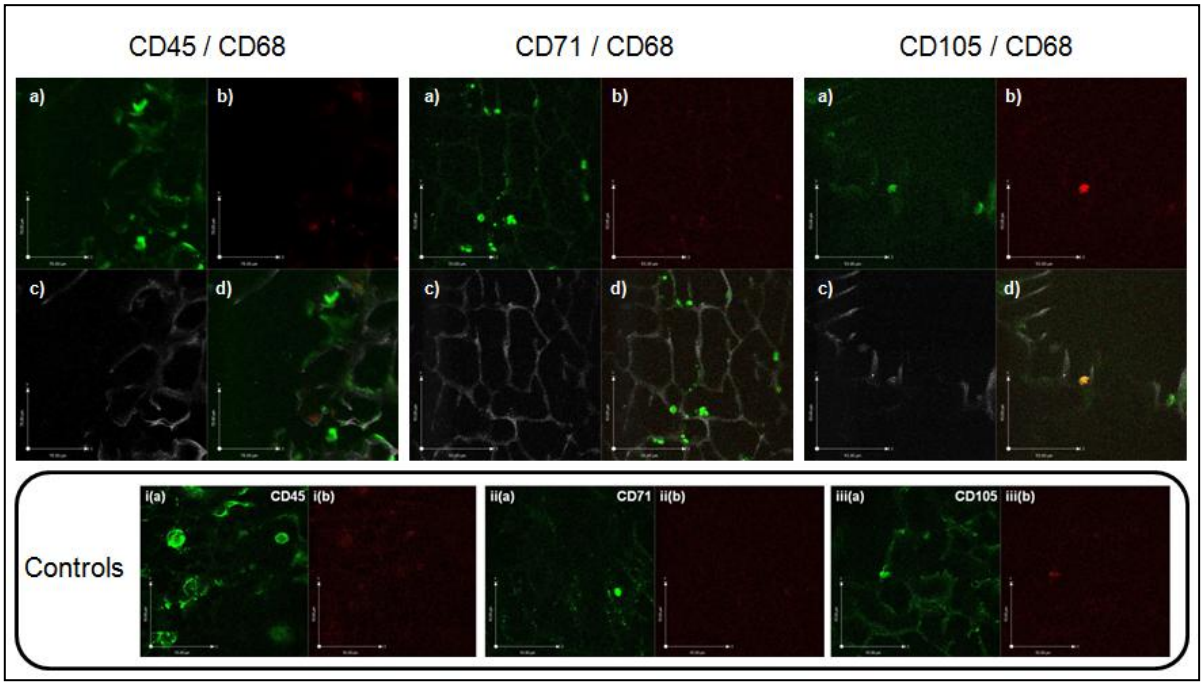
Annex Figure III. SEM images of sectioned scaffolds on several time points. The gold coated surfaces are observed using an acceleration voltage of 15 kV. The scaffold porous nature and convoluted topography is observable, especially on day 7, where a lower magnification was used. Regards to Mr. Remus Winn and Mr. Nayer Youakim for the assistance on retrieving this data.

The images obtained using this technique hold interesting information regarding the novel PU scaffolds and the cells interaction with it. SEM revealed the complex architecture of the scaffolds and the cells within, with a healthy looking spherical shape and sometimes an association in clusters, interactions that are analogous to the ones that take place in the bone marrow.

Although no difference was detected between the cultures with added cytokines and the control, this protocol revealed the similarities of the developed structures to the microenvironment it pretends to mimic, generating fit cells.

Immunostaining for confocal and multiphoton microscopy

This experimental protocol was used as yet another microscopy technique that coupled with previous immunostaining treatment allowed the identification of cell types and their organization inside the studied structure.



Annex Figure IV. Multiphoton imaging of the scaffolds cultured with 10 ng/ml of SCF at day 35. In all images, the square marked as a) corresponds to the binding of the fluorochrome Alexa 488 to the cluster of differentiation on the left in the title. A mouse antibody versus the wanted CD is targeted by the anti-mouse secondary antibody conjugated to the Alexa 488. On the sub-image b) we have always CD68 bound to Alexa 568. Panel c) is a multiphoton image of the scaffold topography and d) is a composite image of the previous 3. As controls we have samples that were only labelled with the antibody versus the specified CD. Regards to Mr. Remus Winn and Mr. Nayer Youakim for the assistance on retrieving this data.

Using immunostaining to specifically detect the desired CDs, it was possible to construct images that showed the presence of the marked cells inside the 3D scaffolds. The CDs under scrutiny were CD45, a marker of haematopoietic cells, CD71 for erythroblasts, CD68 to hint the presence of monocytes or macrophages and finally CD105 for endothelial cells.

The presence of CD45 positive cells is not surprising, given the nature of the seeding cells and the fact that there are CD71 positive cells as well suggests that the developed system, using SCF as the exogenous cytokine, can expand and differentiate CB MNCs towards the erythroid fate. It is interesting to note the combined expression of CD68 with CD105, implying the presence of an activated macrophage, as those cells are known to express both markers[169, 170]. Through the multiphoton panels is possible to perceive the complex structure of the scaffold, and the composite image noted as a powerful tool to identify clusters of cells and their preference in spatial organization.