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

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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.

1. Introduction

The formation of all blood cell types from a single hematopoietic stem cell is named haematopoiesis (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], showing patient engraft capability and the ability to support their haematopoietic needs[2]. However limitations in faithfully recreating the *in vivo* haematopoietic process in *ex vivo* systems hinder the study of the biological mechanisms behind, leaving the full therapeutic potential of ex vivo expanded CB HSCs untapped[2, 3].

In vivo, the primary site for adult haematopoiesis is the BM, and as such, much of the cues controlling this 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[3, 4], definable 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[4, 5]. 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. Cytokines are also quite important to haematopoiesis, usually being small peptides that ultimately affect several HSCs processes, such as quiescence, apoptosis, proliferation, mobilization and differentiation[6], being an important part of the HIM.

Usually only low number of HSCs 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[7], 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[8]. 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[4]. Bioreactors were then proposed as a solution to the above mentioned problems and aalthough somewhat successful, these systems still require the addition of significant quantities of exogenous cytokines, greatly surpassing the *in vivo* levels, in order to achieve cell proliferation, generally failing 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[9-11]. Nevertheless this approach increases the complexity of the culture, hindering any scale-up potential and fails to replace the cytokine requirements.

More physiologic-like models of haematopoiesis were required, 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[3]. Professor Athanasios Mantalaris research group was responsible for the development of a 3D *ex vivo* biomimicry of the BM made out of polyurethane (PU) 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)[12]. This scaffold proved capable of expanding cells without the addition of exogenous cytokines, supporting stroma and in serum-free conditions. 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 devised to elucidate said production, by replicating the conditions of the experiment where stem cell expansion was achieved. CB MNCs were seeded into the 3D model previously developed within the research laboratory[13] and grown in three different conditions, with no addition of exogenous erythropoietin (EPO) and with two near physiological concentrations of EPO, 0.2 U/ml[14] and 1.875 U/ml[15] 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. Materials and methods

2.1. 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, with Dioxane (99.8% pure, Sigma-Aldrich, Dorset, UK) as the solvent for the polymer solution of PU (5% wt). 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%[16]. The wafers were cut to cubes of a standard 5x5x5 mm dimensions.

2.2. Collagen coating and scaffold sterilization

The scaffolds were dipped in ethanol at 70% for 1 min followed by rinsing in phosphate buffered saline (PBS) for 5 min. 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. 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[13]. The cubes were subsequently distributed into individual wells in 24 well plates under sterile conditions. Sterilization was then achieved by means of UV light exposure for 8 min followed by immersion in 70% ethanol for 2 h. The scaffold cubes were subsequently washed twice with PBS for 5 min and 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°C and 5% CO_2. 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.

2.3. Cell source and isolation

The cells used in main cell culture experiment were isolated from human umbilical cord blood units. The units are acquired in cryopreserved form and kept in liquid N_2. Cell isolation prior to the culture starts with the thawing of the blood 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. 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.

2.4. Mononuclear cell seeding and culture

An excess of 322 scaffolds were prepared 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 $2.5x10^6$ cells per scaffold. After seeding the plates were incubated for 15 min at 37ºC and 5% CO_2 in order to improve seeding efficiency. Each scaffold was covered with 30% FBS IMDM supplemented with 1% Pen/Strep and incubated at 37ºC 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 to achieve the three main culture conditions; without addition of EPO (No EPO), with 0.2 U/ml[14] of EPO (Low EPO) and with 1.875 U/ml[15] (High EPO).

2.5. Cell viability analysis

A sample of the extracted cells was stained with Erythrosin B (ATCC®, Manassas, VA) and counted using a standard haemocytometer, enabling the determination of the viability at that time point.

2.6. Cytospins

Media extracted from scaffolds was subjected to a cell count, centrifuged and resuspended in order to achieve the desired concentration of about $1x10^5$ to $5x10^6$ cells per 125 µl of suspension. The apparatus is assembled and secured into position and 125 µl of cell suspension are loaded with the help of a micro pipette. The device is centrifuged at 740 rpm for 2 min and then disassembled in order to leave the slide to dry. 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. 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).

2.7. Flow cytometry analysis

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. The necessary cells were extracted and counted, generating a cell suspension with a know concentration of cells. 8 tubes were prepared with roughly $1x10^6$ cells per tube, the cells were washed with PBS and 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 labelled 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. 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).

2.8. 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). For the *in situ* analysis a novel protocol was established based on a cryogenic grinding operation. A scaffold is dropped into the N_2 inside a mortar and a pestle is used to deliver swift blows to shatter the scaffold into smaller pieces. These pieces are then ground into fine powder which is transferred 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.

2.9. 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. $MgCl_2$ 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, 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. This 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.

2.10. Polymerase chain reaction experiments

Primers for the PCR experiments were chosen from the QuantiTect Primer Assays (Qiagen, West Sussex, UK). The size of the fragments is 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. The housekeeping gene chosen was beta-actin, a very common choice for this role[17]. 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[18-21], HepG2 cell line was chosen as a control for TPO, TNF, IL10 and CSF2[22-25] and the reverse transcribed product of all the mRNA extracted from brain tissue for IL1B and IL6[26, 27].

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°C, 30 cycles of a 45 s denaturation step at 94°C with a 45 s annealing step at 60°C and a final 1 min extension step at 72°C, a final extension for 10 min at 72°C after the 30 cycles.

For the visualization of the bands a 3.5% agarose (UltraPure™ Agarose, Invitrogen, Paisley, UK) gel was prepared with 1x TBE buffer (made from UltraPure™ 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. 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.

2.11. 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 $p<0.05$.

3. Results

The effect of near physiological levels of EPO were 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.

3.1. Viability

All culture conditions on the 3D scaffolds maintained cell viability 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[12]. The viability of the cells extracted from the scaffolds was determined in all experimental time points and plotted over time (Figure 3.1).

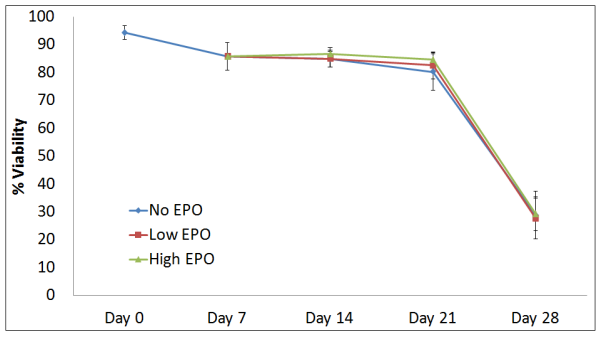

Figure 3.1. Viability established through haemocytometer count of the cells stained with Erythrosin B. There is no significant difference between the 3 studied culture conditions.

On day 0 the viability was 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 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.

3.2. Cytospins

An assessment of the differentiation capability of the cultured cells was taken in the form of cytospin analysis (Figure 3.2).

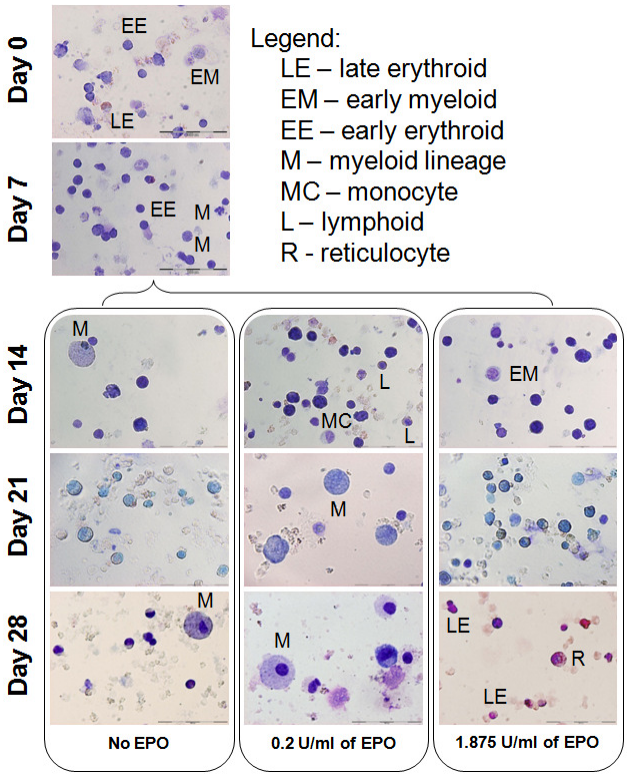

Figure 3.2. Cytospins of the various samples. The images illustrate the existence of multilineage precursors and mature cells all along the duration of the experiments.

In this composed picture it is possible to identify cells of multiple lineages at distinct states of maturation. It is possible to identify erythroid cells 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. 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.

3.3. Flow cytometry

To further study of the chronological evolution of the number of cells displaying certain lineage specific markers, flow cytometry was used.

After gathering all the percentages of gated cells in each quadrant of the flow cytometry raw graphs the fraction of cells positive for each marker was determined and plotted on Figure 3.3.

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.

Further analysis of the graphed data shows that CD33 positive cells also stand out as having low numbers for every time point and condition. 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 stem cell markers, with the latter being the earliest[28] and the former being specific for the myeloid lineage[29].

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, however a statistical analysis of the gathered data shows that there is no significant difference between the values displayed. As such the information gathered from said data is that there is a significant amount of CD45 expression throughout the experiment, a marker of most differentiated haematopoietic cells[30].

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 ($p<0.05$). 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[31], 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[32], therefore being used

as way to determine an early commitment to the erythroid lineage. The evolution of CD71+ cells displays a significant boost from nearly inexistent numbers to 40% plus on the final experimental point. However there is no statistically significant difference between the three different conditions on the last day.

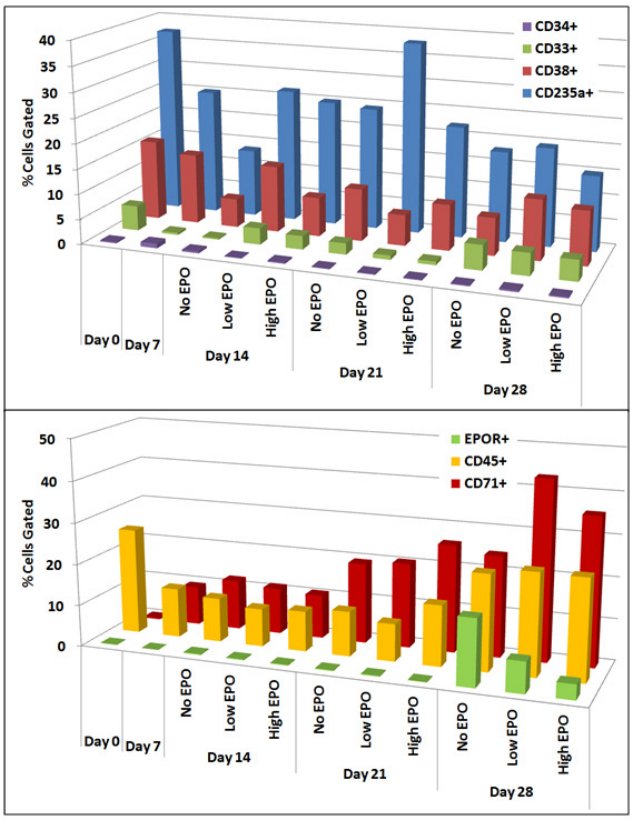

Figure 3.3. Graphical analysis of the flow cytometry data. The data was plotted as such to make it easier to analyze.

CD235a was chosen as a way to reveal the erythrocytes[33] 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 ($p<0.05$).

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[34]. 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.

3.4. Polymerase chain reaction

Reverse transcription PCR (RT-PCR) was used as a tool in the elucidation of endogenous cytokine production that could be accounted for success of 3D scaffolds when culturing CB MNC.

Optimization of the PCR conditions such as cycle number, the temperature of critical steps and concentration of the reagents was pursued to ensure the quality of the results achieved, with the final ideal values being presented in sub-chapter 2.10 of materials and methods.

The image displayed below (Figure 3.4) represents the agarose gels for the various cytokines studied. Every row is subtitled to provide a better understanding of the results.

Beta-actin was chosen as the HK gene as it is known to be a good reference for gene expression assays[17].

While not directly translatable to actual protein concentration, the expression of EPOR was recorded as present in all situations. There is no recorded expression of TPO throughout the entire experimental timeframe, its presence would be an indicative of the development of the culture towards megakaryocytes and platelets, which doesn't seem to be the case.

Tumor necrosis factor alpha (TNF) is one of several cytokines produced and released by macrophages when activated by the presence of immunological stimuli. Its occurrence in culture suggests 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.

In the HBB gel 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. Interleukine-1 beta (IL1B) is a cytokine similar to TNF, playing a role in triggering the inflammatory reactions. Due to these similarities 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.

In response to infection, interleukine-6 (IL6) works along side with IL1B and TNF as the main cytokines responsible for systemic acute-phase response. 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.

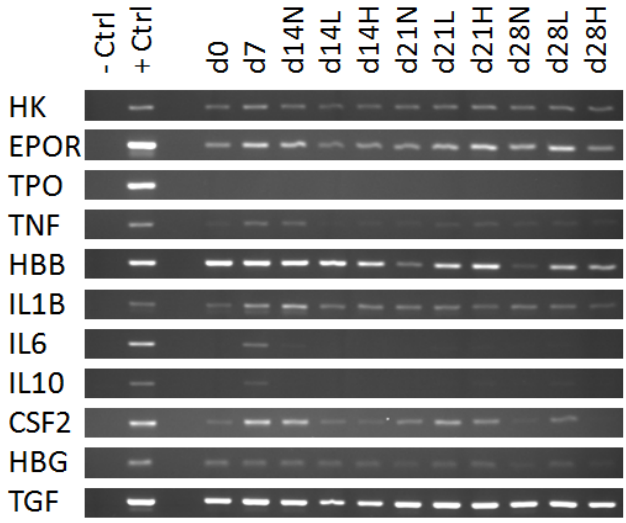

Figure 3.4. PCR results for the considered genes.

IL10 clearly follows the same expression profile as IL6, with no production of the transcript being clearly recorded except for the day 7 sample. 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[35].

Also known as granulocyte-macrophage colony stimulating factor (GM-CSF or CSF2), this cytokine is notorious for the proliferation and differentiation of myeloid haematopoietic stem cells, hence its importance to the study at hand. 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. 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.

HBG expression is seen in all lanes, although the bands seem fainter in later time points. 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.

Yet another pleiotropic cytokine, transforming growth factor beta 1 (TGF) is known maintaining CD34+ progenitor haematopoietic cells in an undifferentiated state[36].

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.

4. Discussion

A cellular culture was performed using similar conditions to the previous work developed. Some routine tests such as the CFU and the MTS proliferation assay were disregarded due to the abundance of data, 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 experiment, 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 3.1), however, a detailed statistical analysis determined no significant difference in the values between the various conditions through the time points. Previous work determined the No EPO condition to have a noticeable lower viability value with the higher EPO concentration responsible for the highest value. Our experiment replicated the circa 85% values for the EPO conditions until day 21, where the values plummet to low records for the last samples in day 28.

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[12] 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. Despite this, no evidence of

sterilization problems were found in the cultures performed for this work.

The cytospin results confirmed the maintenance of multilineage progenitor and developed cells. 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[37], and as such the results are of no surprise, and were confirmed in similar previous experiments.

Also regarding cellular fate, the samples for each time point and cytokine concentration were subjected to flow cytometry analysis. Using CD34 as a marker for HSCs[28] 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. CD33 arises as a similar early progenitor marker, but in this case, specific for the myeloid lineage[29]. Once again 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. The data concerning CD45 shows a proportionally high percentage of gated cells in all samples. Since this is a haematopoietic mature cell marker, excluding mature erythrocytes and platelets[30], these were the predicted results.

The noticeable drop in the registered CD38 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 represents the early progenitors[38], CD235a is used to measure mature erythrocyte levels[33], and finally EPOR as middle ground between the two stages previously mentioned[34]. CD71 positive cells percentage grows steadily from near nonexistence into very high values towards the end of the experiment. Analogously CD235a expression drops as time elapses. EPOR positive cells percentage follows a more intriguing pattern, as after displaying almost no expression until day 21, its values jump into life, 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. 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.

Gene expression analysis through RT-PCR was used in the elucidation of the mechanisms behind the displayed cellular behaviours. 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.

Of the genes selected for the study, TNF, IL1B, IL6 and IL10 are known to play related roles on the systemic immunological response, therefore being produced mainly by activated monocytes or macrophages[24, 27, 39, 40]. Both TNF and IL1B show similar expression patterns, with the presence of faint bands all throughout the experimental activity. Their presence implies the existence of late monocytic cells in culture. Interestingly IL1B has a stimulating effect on TNF expression[41], 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[42]. As such its presence may explain the maintenance of multilineage differentiation capacity through the experiment reported before through cytospins and flow cytometry analysis. Along with the TNF stimulation, IL1B has a role in IL6 expression induction[41], 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, similar to IL10 gel image. 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[43] 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.

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. HBG on the other hand presents faint expression bands in every sample. Both result sets point towards the presence of cells from the erythroid lineage. 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[44]. While this is normal for the day 0 samples, it was expected for the system to evolve as *in vivo*, by performing the previously cited replacement. That doesn't happen, suggesting an arrest of the whole culture in the post-natal change period, when concerning the globin expression.

CSF2 shows an irregular expression pattern along the timeline, with a distinct lack of band in the last time point for the highest EPO concentration. 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[36], inhibiting early haematopoiesis[42], 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 and the presently work.

5. 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[45], 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 cytokine, serum and stroma-free culture conditions can claim similar performances, 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.

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.

One noticed shortcoming of the developed system was the extraction method used to gather the cells. 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.

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.

6. References

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