# Investigating the Support Mechanisms Provided by Macrophages in Human Erythropoiesis




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**To my mother, father and sister.**

47,248 Words

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# Contributions

I declare that all the work in this thesis is my own and has only been presented to the University of Oxford for the award of a Doctor of Philosophy.

All experiments and data presented in this thesis were designed, performed and analysed by myself. There are only a few exceptions to this, as outlined in the following.

Pat Tsang (Blood Research Lab, NDCLS) helped with $CD34^+$ cell isolations on day 0. After culturing and harvesting erythroblasts or macrophages, Prof. David Ferguson (NDCLS) performed the electron microscopy on these cells and provided me with the images. Lastly, Dr. Dominic Waithe (WIMM) wrote the script that compared the fluorescence intensities of FOXO3 in the nucleus versus the cytoplasm of individual erythroblasts that had previously been imaged using the widefield microscope. This allowed me to compare the subcellular distribution of FOXO3 in erythroblasts following co-culture with macrophages under the different treatment conditions.

# Abstract

Definitive erythropoiesis takes place within the bone marrow in erythroblastic islands, where the central macrophage plays a role in the expansion and terminal differentiation of surrounding erythroid cells. Central macrophages have been shown to promote erythropoiesis, although the precise mechanisms of how they regulate erythroblast development are still largely undefined. Furthermore, clinical and experimental studies suggest that macrophages treated with dexamethasone or IL10 may have an enhanced ability to support erythropoiesis. Therefore, this thesis investigates the effects of growing autologous human peripheral blood mononuclear cell-derived macrophages and erythroblasts in direct contact using an adapted *in vitro* culture system and the mechanisms of enhanced erythropoiesis during erythroblast co-culture with dexamethasone or IL10 treated macrophages. Supplementation of MCSF differentiated macrophages for four days with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) induced M2c phenotypes ($CD163^{+}CD169^{+}CD16^{+}HLA\text{-}DR^{-}$) but promoted differential expression of surface markers CD14, VCAM1, CD64, CD86 and CD80.

These dexamethasone or IL10 modulated macrophages substantially improved erythroblast expansion after 48 hours (1.73 ±SEM 0.39-fold; p=0.0401 and 1.68 ±SEM 0.35-fold; p=0.0397, respectively, compared to co-culture with untreated macrophages). The enhanced erythroid expansion was found to be a result of increased proliferation as opposed to heightened survival. However, erythroblast maturation, as defined by the appearance of a $CD71^{+}$glycophorin A $(gpA)^{+}$ population, was reduced after co-culture with dexamethasone treated macrophages

(25 ±SEM 3.73% $CD71^{+}gpA^{+}$ erythroblasts) but unaffected by the culture with IL10 polarised cells (42 ±SEM 3.01% $CD71^{+}gpA^{+}$ erythroblasts).

The thesis discovers that macrophages promote erythroblast expansion via inactivation of the transcription factor FOXO3. The presence of either untreated or dexamethasone or IL10 treated macrophages lowered total cellular FOXO3 protein levels in erythroblasts by almost half (0.63 ±SEM 0.02-fold; p=0.0013) while dexamethasone or IL10 modulated macrophages simultaneously increased levels of inactivated phosphorylated-FOXO3 in erythroblasts by 1.39 ±SEM 0.05-fold (p=0.0500) and 1.41 ±SEM 0.04-fold (p=0.0498), respectively. Dexamethasone treated macrophages also elevated levels of the adaptor protein 14-3-3ζ in erythroblasts, suggesting increased export of phosphorylated-FOXO3 to the cytoplasm. However, IL10 treated macrophages did not alter 14-3-3ζ levels, which may indicate that these macrophages did not induce export of phosphorylated FOXO3.

The precise role of the macrophage adhesion molecule CD163 was also examined and it was found that siRNA targeted knockdown of this receptor approximately doubled both the total cellular FOXO3 levels and nuclear FOXO3 abundance in erythroblasts (1.81 ±SEM 0.07-fold; p=0.0047 and 2.09 ±SEM 0.21-fold; p=0.0109, respectively, compared to co-cultures involving macrophages treated with the control siRNA). The levels of phospho-FOXO3 in contrast were not affected in erythroblasts grown with CD163 deficient macrophages.

Analysis of co-cultured supernatants revealed that dexamethasone or IL10 stimulated macrophages had a senescence-associated secretory profile that included

CCL2, IL8, Serpin E1, IL1ra and GROα. The addition of IL8 (3,600 pg/ml), Serpin E1 (100 pg/ml) or GROα (110 pg/ml) to erythroblasts cultured alone without macrophages augmented expansion via FOXO3 phosphorylation while GROα simultaneously improved erythroid maturation in a FOXO3-independent manner. The addition to erythroblasts only of all five senescence-associated molecules together at concentrations found in the dexamethasone or IL10 treated macrophage supernatants also elevated erythroid expansion, while the IL10 treated macrophage molecule combinations simultaneously augmented the proportion of mature ($CD71^{+}gpA^{+}$) erythroid cells by 8 ±SEM 4.18% ($p=0.0412$).

In summary, different *in vitro* models of macrophage-assisted enhanced erythroblast expansion are provided that either block or preserve erythroid maturation. Furthermore, the contributions of erythroblast-macrophage cell-cell contacts and secreted factors are highlighted and an important role for FOXO3 inactivation in erythroid cell expansion is identified. More specifically, the repression of FOXO3 activity is multifactorial, involving both the inhibition of total cellular levels as well as phosphorylation of FOXO3.

# Abbreviations

**BFU-E**: Burst forming unit erythroid

**BSA**: Bovine serum albumin

**CCL2**: Chemokine (C-C motif) ligand 2 (also known as MCP1: monocyte chemoattractant protein 1, or small inducible cytokine A2)

**CD71**: Cluster of differentiation 71 (also known as the transferrin receptor)

**CFU-E**: colony forming unit erythroid

**EPO**: Erythropoietin

**FBS**: Foetal bovine serum

**FOXO3**: Forkhead box 03 (also known as FOXO3a or FKHRL1)

**GM-CSF**: Granulocyte macrophage colony-stimulating factor

**gpA**: glycophorin A (also known as CD235a: cluster of differentiation 235a)

**GROα**: growth-related oncogene-α (also known as CXCL1: chemokine (C-X-C motif) ligand 1, or previously as NAP-3: neutrophil-activating protein 3, or MSGA-α: melanoma growth stimulating activity-α)

**IL10**: Interleukin-10

**IL1ra**: Interleukin-1 receptor antagonist (previously known as IL1 inhibitor)

**IL3**: Interleukin-3

**IL8**: Interleukin-8

**MCSF**: Macrophage colony-stimulating factor

**PBMC**: Peripheral blood monocyte derived

**PKB**: Protein Kinase B (also known as AKT)

**ROS**: Reactive oxygen species

**SCF**: Stem cell factor

**Serpin E1**: Serpin Family E Member 1 (also known as PAI-1: Plasminogen Activator Inhibitor-1)

**siRNA**: small (or short) interfering RNA

**TNFα**: Tumour necrosis factor-α

**VCAM1**: Vascular cell adhesion molecule 1 (also known as CD106: cluster of differentiation 106)

# Chapter 1: Introduction

## 1.1 Haematopoiesis in Mammals

The creation of various types of mature blood cells, termed haematopoiesis, arises from the proliferation and gradual differentiation of an initially multipotent haematopoietic stem cell (HSC) (**Figure 1.1**) (Zon, 1995). These maturep blood cells comprise the erythroid, myeloid and lymphoid lineages which, in general, have a role in transporting oxygen, in innate immunity or in the adaptive immune response, respectively.

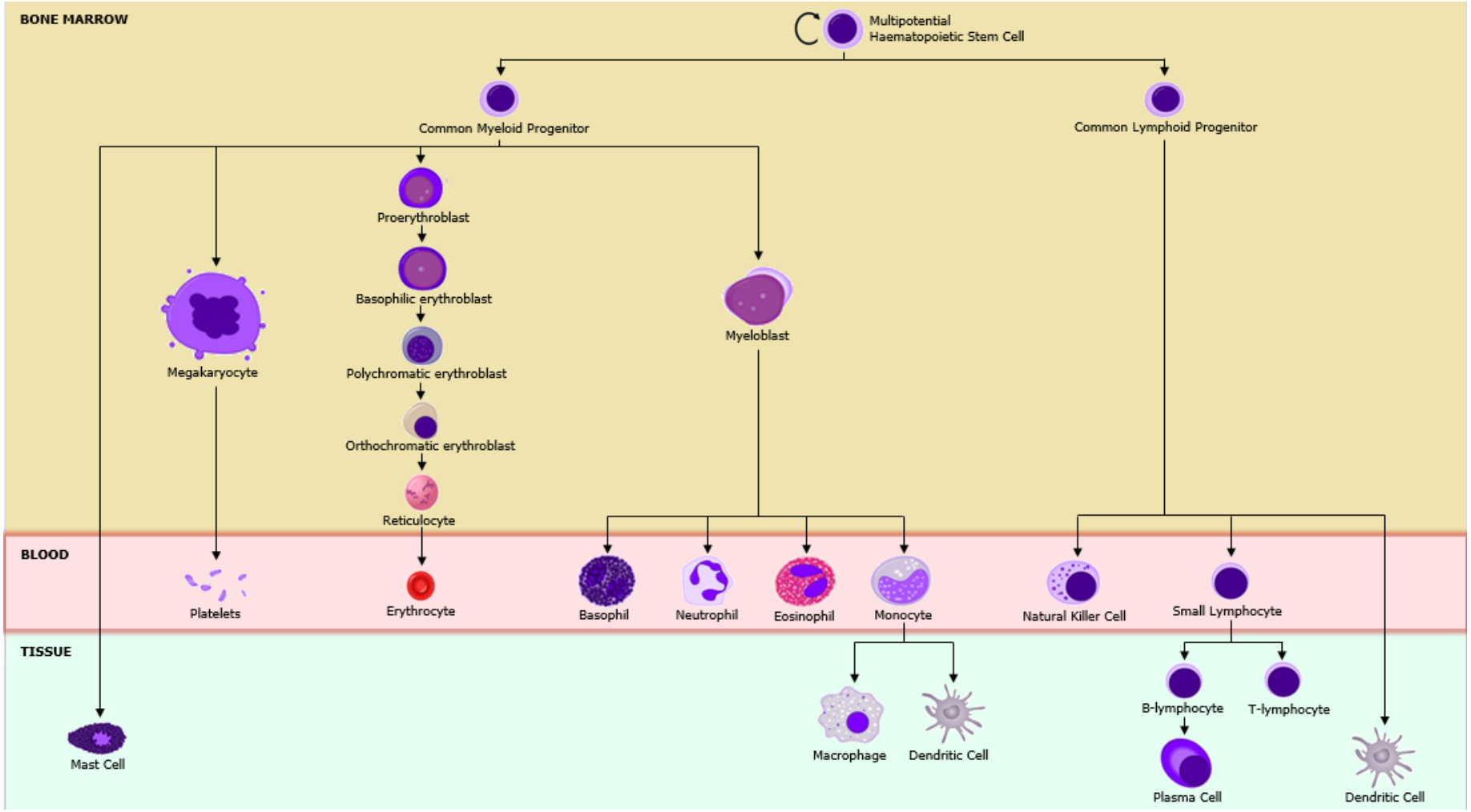
Ontogenesis determines the kind of haematopoiesis in a vertebrate, with primitive haematopoiesis occurring in the early embryo but definitive haematopoiesis taking over during the later stages of development. During primitive haematopoiesis, only erythroid cells are produced, whereas definitive haematopoiesis involves the generation of all erythroid, myeloid and lymphoid cells (Hess and Boehm, 2016; Qiu et al., 2016). This pattern of development is due at least in part to myelopoiesis and lymphopoiesis both requiring the proper bone marrow microenvironment, which is only fully developed in the late embryo.

In the deterministic theory of haematopoiesis, haematopoietic stem cells and undifferentiated progenitor cells are believed to differentiate into specific cell types under the influence of key factors in the microenvironment (Kimmel, 2014). For instance, IL1, IL3, IL6 and GM-CSF promote the generation of the common myeloid progenitor, whereas IL2, IL12 and FLT-3 ligand, among others, trigger the generation of small lymphocytes from common lymphoid progenitors. Contrastingly,

the stochastic theory of haematopoiesis states that the differentiation of cells is unpredictable and random (Kimmel, 2014). This is important for providing an element of variation in the types of cells that are produced in the bone marrow under certain conditions as well as for altering the proportions of cells undergoing self-renewal or apoptosis (Alenzi et al., 2009; Birbrair and Frenette, 2016; Chang et al., 2008).

The classical dichotomous model depicted in **Figure 1.1** has been challenged by the more recent proposal that HSCs diverge in a more myeloid-prone fashion (Kawamoto et al., 2010). In this myeloid-based model, HSCs divide to produce a common myeloid progenitor and a common myelo-lymphoid progenitor instead of the common lymphoid progenitor. This means that B- and T-lymphocytes are produced by bipotential progenitor cells that have capabilities of generating these particular cell types as well as myeloid cells. Hence, this new theory postulates that even following their isolation, erythroid, B- and T-cell lineages all have the ability to produce myeloid cells. Although more detailed investigations are necessary, several pieces of evidence have already suggested that HSCs may in fact diverge at a later stage in haematopoiesis than was formerly considered (reviewed in Kawamoto et al., 2010).

**Figure 1.1: The production of the different types of cells from the haematopoietic stem cell and the locations where the mature cells have their effects.**



## 1.2 Primitive versus Definitive Erythropoiesis in Mammals

Erythropoiesis is the process by which erythrocytes are developed, releasing approximately two million cells per second into the circulation in a human adult. It was first documented that the adult mammalian bloodstream contained erythrocytes without a nucleus, which differed from mature erythrocytes in fish, amphibians and birds that contained a pyknotic nucleus (Gulliver, 1840). Erythroid cells in embryonic mammals mature as they circulate in the bloodstream and it is believed they can subsequently enter the foetal liver, where they rest in close proximity to liver macrophages, to undergo ordered enucleation (McGrath et al., 2008b). In fact, the co-culture of late primitive erythroblasts with macrophages *in vitro* is essential for enucleation. Mature, enucleated erythrocytes are produced in the bone marrow of mammals at the later stages of gestation (Palis, 2008).

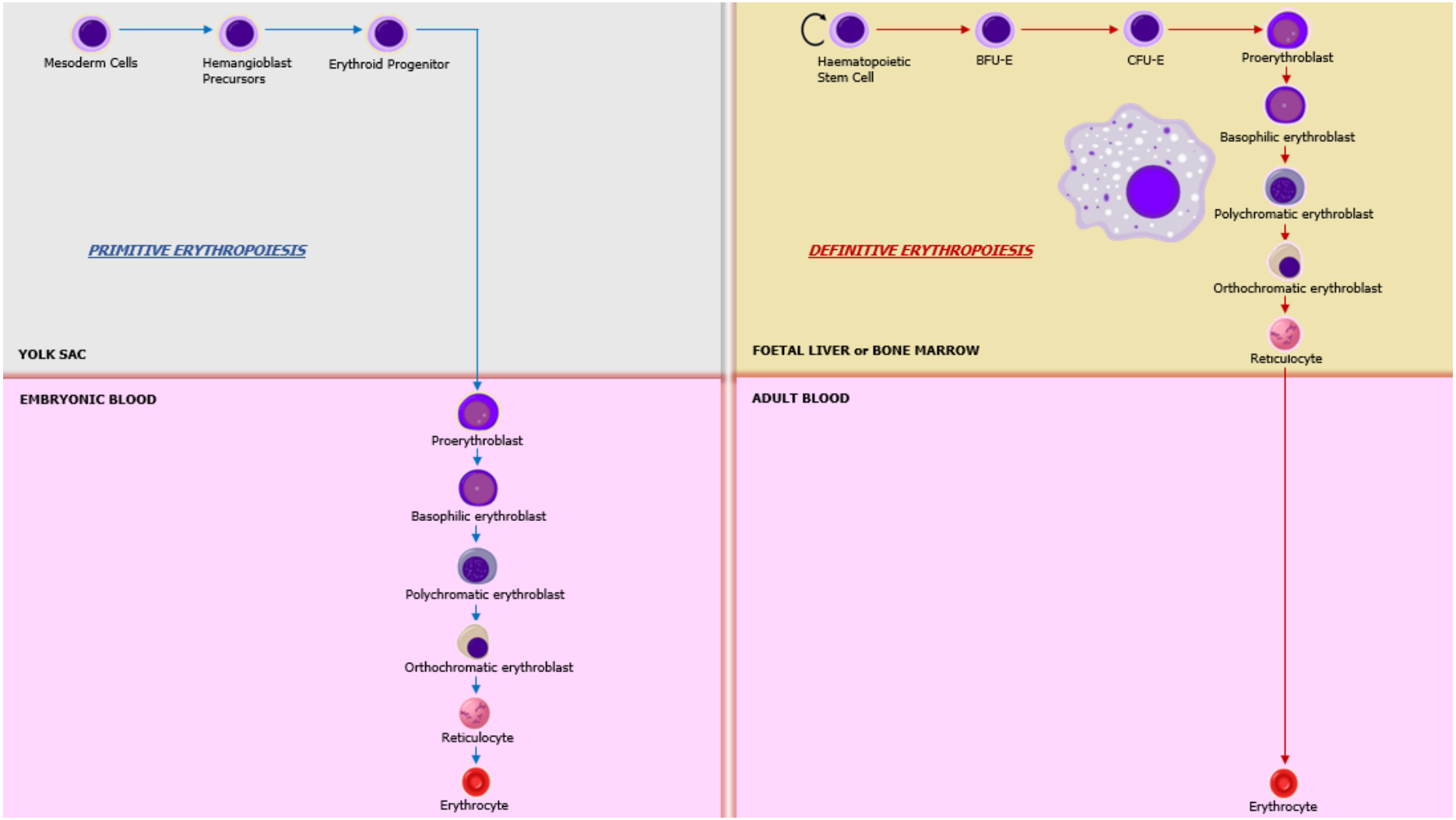
Therefore, mammalian erythropoiesis can be defined in terms of primitive erythropoiesis and definitive erythropoiesis, with the precise location of erythroid maturation being one discriminating feature (Palis, 2014) (**Figure 1.2**). Primitive erythropoiesis produces large erythroblasts with an intact nucleus in the yolk sac, which subsequently mature in the embryonic bloodstream. In contrast, definitive erythropoiesis generates reticulocytes in the liver of the foetus or in the bone marrow later in foetal life or after birth, with the final development into mature erythrocytes occurring in the bloodstream. Thus, there is a developmental transition during definitive erythropoiesis that comprises temporary generation of erythroid cell generation in the foetal liver of the human embryo, which is subsequently replaced by a permanent production of erythroblasts from hematopoietic stem cells in the

adult bone marrow. However, further work is necessary to clarify the temporal regulation of this developmental switch in humans.

Another distinctive characteristic of definitive erythropoiesis is that erythroid cells mature asynchronously in contact with macrophages in the foetal liver or adult bone marrow, whereas primitive erythroblasts grow in an almost synchronised fashion and only interact with macrophages in the placenta (Van Handel et al., 2010). Additionally, globin switching ensures that the embryonic globin genes expressed in primitive erythrocytes are replaced with foetal globins by the end of the first trimester and with adult globins following birth in definitive erythrocytes (Bauer et al., 2012; Brownlie et al., 2003).

In the bone marrow, erythroid progenitors develop from lineage-committed stem cells. These progenitor cells consist of immature erythroid progenitors (BFU-E: burst-forming unit-erythroid) that differentiate via mature erythroid progenitors (CFU-E: colony-forming unit-erythroid) to give the distinct erythroblasts that eventually enucleate and form reticulocytes (Ney, 2006; Ubukawa et al., 2012). The gradual process of erythroid cell maturation culminates in the eventual enucleation of orthochromatic erythroblasts and final autophagic clearance of residual organelles in reticulocytes to form the mature erythrocytes (Konstantinidis et al., 2012; McGrath et al., 2008a; Peslak et al., 2012; Peslak et al., 2011). These biconcave erythrocytes can then carry oxygen around the human body for 120 days and their lack of mitochondria means they produce energy entirely by anaerobic glycolysis (Yoshida and Shevkoplyas, 2010).

**Figure 1.2: The development and production of erythrocytes during primitive or definitive erythropoiesis.** *(Adapted from Palis, 2014).*



A number of major signalling pathways such as the p38, JAK-STAT and JNK pathways are activated by erythropoietin (EPO) to induce proliferation, survival and differentiation of primitive erythroid cells (Jacobs-Helber et al., 2000; Nagata et al., 1997; Richmond et al., 2005; Witthuhn et al., 1993). In addition to its role in regulating homeostatic erythropoiesis, EPO controls CFU-E proliferation as well as erythroid enucleation under acute stress conditions such as anaemia (Flygare et al., 2011; Peslak et al., 2012; Schultze et al., 2012). However, the cytokine promotes only the survival of erythroid progenitors during definitive erythropoiesis. EPO receptors start to appear on immature erythroid progenitors (BFU-E) and reach peak numbers in more mature erythroid progenitors (CFU-E) (Sawada et al., 1990). Erythroblasts rely on EPO in these earlier stages of maturation for survival but become EPO-independent once they develop into polychromatic erythroblasts (Wickrema et al., 1992; Wu et al., 1995b).

Transcription factors common to both primitive and definitive erythropoiesis are GATA1 and KLF1, whereas BCL11A, MYB and SOX6 are differentially expressed in the two separate developmental lineages of erythroid cells (Miller and Bieker, 1993; Xu et al., 2010; Xu et al., 2012; Yi et al., 2006). Furthermore, human primitive and definitive erythropoiesis involve many of the same cytoskeletal proteins that provide cellular structural integrity, including band 3, spectrin and Ankyrin (Ponnusamy et al., 2012). Therefore, there are various similarities between human primitive and definitive erythropoiesis but recent genomic and bioinformatic analyses have been pivotal for highlighting the major differences in the transcriptional control of the two processes (Kingsley et al., 2013; Merryweather-Clarke et al., 2011).

## 1.3 Erythro-Myeloid Progenitors and Embryonic Macrophages

The early mammalian embryo lacks HSCs and therefore requires other sources of transient haematopoietic progenitor cells that can generate an efficient haematopoietic system until birth (Frame et al., 2013; Gomez Perdiguero et al., 2015; Kasaai et al., 2017). This HSC-independent haematopoiesis must have the capacity to produce very large numbers of precursor cells to promote the growth and development of the embryo. Primitive haematopoiesis in the murine embryo is supported by a second wave of haematopoiesis originating in the yolk sac that consists of definitive erythro-myeloid progenitors (EMPs) (Frame et al., 2013). These EMPs give rise to erythroid, myeloid, megakaryocyte and multipotent progenitor cells and quickly differentiate in the foetal liver at a time when HSCs are not yet functional (Chen et al., 2011; McGrath et al., 2015). In fact, the crucial dependence on EMPs prior to birth was demonstrated by a study showing that transplantable HSCs could not rescue murine embryos deficient in EMPs from lethality by E13.5 (Chen et al., 2011).

EMPs are not only limited to their functions in the embryo but can also temporarily produce erythrocytes in adults, albeit with some embryonic βh1-globin expression in addition to the adult β1- and β2-globins (McGrath et al., 2011). However, EMPs transplanted in immunocompromised adults only have a short term potential. Adult definitive haematopoietic cells and EMPs share common transcription factors and surface markers but their precise combinations means the two progenitor types can be distinguished from one another (Iwasaki et al., 2006; Orkin and Zon, 2008).

Tissue-resident macrophages develop mainly from EMPs and they display a wide range of phenotypes that depend on the microenvironmental niche in which they are present (Davies et al., 2013). They include lung alveolar macrophages, brain microglia, spleen red pulp macrophages and F4/80$^{hi}$ kidney, pancreatic and cardiac macrophages. Their precise phenotype corresponds to a specific function, such as fighting an infection or clearing cellular debris. The 'mononuclear phagocyte system' model has stipulated that bone marrow HSC-derived monocytes in the peripheral blood migrate into tissues where they differentiate to maintain the local population of resident macrophages (Ochando et al., 2016). However, an alternative model has recently been proposed which states that embryonic macrophages are preserved by both the recruitment of bone marrow derived monocytes as well as by the proliferation of tissue macrophages themselves (Gomez Perdiguero et al., 2015; Takata et al., 2017). Several conditions, including inflammation, infection or ageing, trigger the expansion of fully differentiated tissue macrophages and while the exact mechanisms are still to be elucidated, it is believed that the Maf family of transcription factors probably play a role in their proliferation (Aziz et al., 2009). However, future studies are necessary to establish whether the repopulation of local tissue by resident macrophages is always due to their mitotic division or whether a population of native progenitors also exists.

## 1.4 Steady-State versus Stress Erythropoiesis

Homeostatic, or steady-state, erythropoiesis involves a careful balance between reticulocyte release into the circulation and removal of senescent erythrocytes (Bennett and Kay, 1981). The incorporation of diverse signals from the neighbouring

microenvironment is vital to this regulation and any disruption to the process can lead to a substantially altered erythrocyte number. An increased rate of erythrocyte development is known as stress erythropoiesis. Several growth factors have been shown to coordinate steady-state erythropoiesis (Cerdan et al., 2004; Miyagawa et al., 2000; Wu et al., 1995a) but EPO is one of the major regulators (Sathyanarayana et al., 2008; Wojchowski et al., 2006).

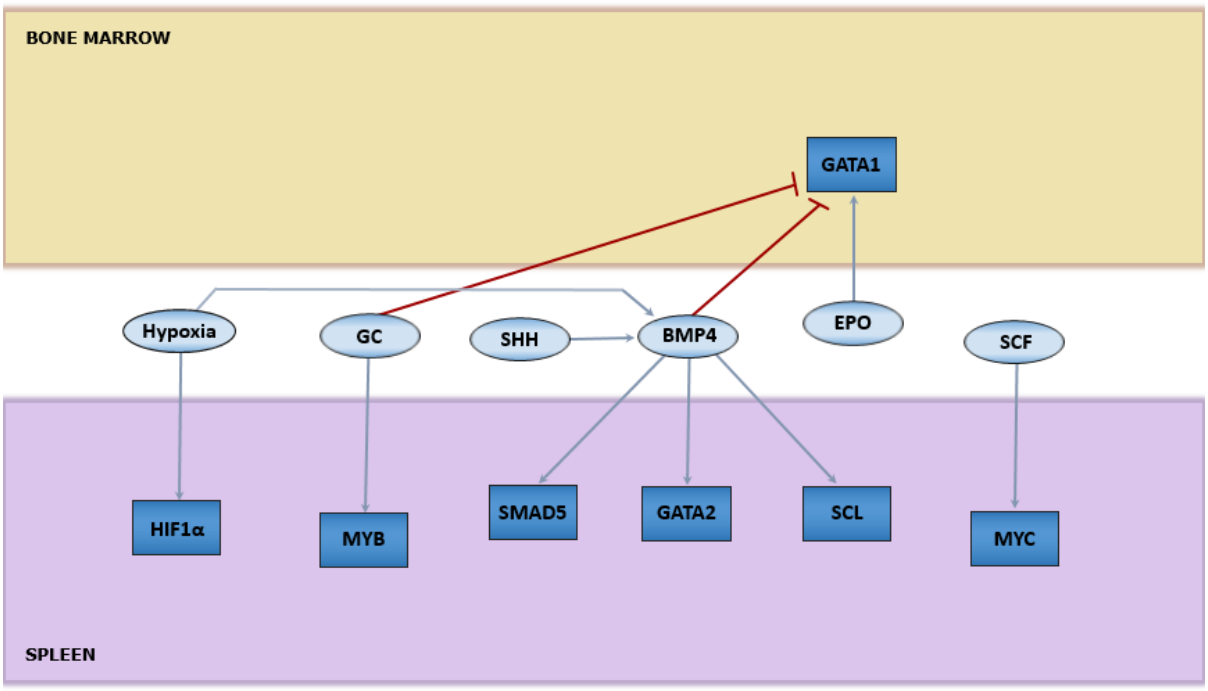
The inherited and acquired genetic diseases β-thalassaemia and polycythaemia vera, respectively, drastically affect erythroid homeostasis and trigger ineffective erythropoiesis and anaemia or highly viscous blood, respectively. Both conditions lead to hyperactivation of the JAK2 protein, either as a result of heightened serum EPO levels in patients with β-thalassaemia or because of the $JAK2^{V617F}$ mutation during polycythaemia vera (Ginzburg and Rivella, 2011; James et al., 2005; Mullally et al., 2010; Rivella, 2009). These situations lead to expansion of erythroid progenitors in the bone marrow and in extramedullary sites, for example the spleen, which becomes enlarged in a process known as splenomegaly (Libani et al., 2008; Mullally et al., 2010; Tiedt et al., 2008). However, β-thalassaemia is characterised by a reduced synthesis of β-globin chains that causes anaemia, whereas polycythaemia vera has massively augmented erythrocytosis. Nonetheless, these disorders resemble states of chronic stress erythropoiesis, since the biological mechanisms that drive reticulocyte production are pushed beyond the limits of steady-state (Paulson et al., 2011).

Other circumstances that can severely impair steady-state erythropoiesis and potentially induce a stressed erythropoietic response are bone marrow transplantation or chemotherapy, as well as the exposure to radiation or infection

with the malarial parasite, Plasmodium falciparum (de Vetten et al., 2001; Pathak and Ghosh, 2016; Ushakov et al., 1998). Furthermore, embryogenesis also triggers stress erythropoiesis via enhanced BMP4/SMAD5 signalling in order to meet the demands of the rapidly growing embryo (Porayette and Paulson, 2008). More precisely, the BMP4/SMAD5 axis induces expansion of stress erythroid progenitors that resemble those found in the adult spleen and hypoxia (1% $CO_2$) strengthens this response (Paulson et al., 2011).

A stressed erythropoietic response combines signalling cascades such as those triggered by EPO, BMP4 and integrins (Menon et al., 2006; Porayette and Paulson, 2008; Socolovsky, 2007; Vemula et al., 2010). Murine, chicken and human erythroblasts all exhibit amplified proliferation but blocked maturation *in vitro* following stimulation with glucocorticoids that provoke cooperation of the glucocorticoid receptor with the EPO and SCF receptors (EPOR and c-Kit, respectively) (Reichardt and Schutz, 1998; von Lindern et al., 1999; Wessely et al., 1999). Thus, numerous different factors trigger stress erythropoiesis by activating transcription factors such as SMAD5, GATA2 and MYB. This is summarised in **Figure 1.3**.

**Figure 1.3**: **Extrinsic stimuli that induce stress erythropoiesis via the activation of different transcription factors.** *GATA1 is a transcription factor that promotes erythroid differentiation. GC, glucocorticoid; SHH, Sonic Hedgehog; BMP4, Bone Morphogenetic Protein 4; EPO, Erythropoietin; SCF, Stem Cell Factor; MYB, Myeloblastosis family of transcription factors; SMAD5, Small Mothers Against Decapentaplegic 5; SCL, Stem Cell Leukemia; MYC, Myelocytomatosis. Note that the bottom row of transcription factors are also active in the bone marrow as well as in the spleen (Adapted from Hattangadi et al., 2011).*




## 1.5 β-Thalassaemia

B-thalassaemia is an inherited genetic disease that causes enhanced, abnormal and ineffective erythropoiesis. It is caused by HBB gene mutations on chromosome 11 that result in reduced synthesis of the β-globin chain of haemoglobin. It is

characterised by microcytic hypochromic anaemia, nucleated erythrocytes in the peripheral blood in severe cases and sometimes a decreased abundance of adult haemoglobin (HbA) but increased foetal haemoglobin (HbF) (Origa, 1993). Patients can present with β-thalassaemia minor, intermedia or major depending on the type of mutation and whether one or both alleles are affected and this determines the severity of the disease. B-thalassaemia major ($\beta^0/\beta^0$) is defined by the complete absence of β-globin chains, which induces early erythrocyte death and leads to hepatosplenomegaly in an attempt to replace the lost cells (Sun et al., 2017). However, the increased proliferation of immature erythroblasts is followed by a minimal production of mature erythrocytes, a process called ineffective erythropoiesis, and means that affected patients are dependent on regular blood transfusions. Not only do these individuals present with severe symptoms, including facial bone deformities and slow growth, but their continued transfusion program leads to chronic iron overload, which in turn demands regular iron chelation therapy (Weatherall, 2010). Patients with β-thalassaemia intermedia have a milder anaemia but also tend to present with iron overload. Currently, the only cure for β-thalassaemia is bone marrow transplantation from a matched donor or otherwise cord blood transplantation from a related donor. There is hope that in the future gene therapy will provide a cure that permanently corrects the faulty gene(s) in an individual's own HSCs.

## 1.6 Anti-Band 3 Antibodies on β-Thalassaemic RBCs

In β-thalassaemia, the free α-globins lead to unstable haemoglobin molecules that precipitate and form haemichromes (Pantaleo et al., 2008). These haemichromes

oxidise band 3 proteins on the RBC plasma membrane and induce disulphide linkages between adjacent cytoplasmic domains of band 3 (Mannu et al., 1995). The oxidative stress of β-thalassaemic RBCs also activates tyrosine kinases, which phosphorylate band 3 and trigger their clustering (Bordin et al., 2002; Pantaleo et al., 2008). This in turn inhibits their attachment to the cytoskeleton via ankyrin and causes membrane blebbing and the release of microparticles (Ferru et al., 2011; Mannu et al., 1995). Furthermore, clustered band 3 proteins are unable to adequately arrange glycolytic enzymes on the cell surface, resulting in dysregulated breakdown of glucose via glycolysis as opposed to the pentose phosphate pathway (PPP) (Harrison et al., 1991). The subsequent reduction in NADPH, which is generated by the PPP, leads to a decrease in antioxidant processes within the RBC and intensifies the oxidative burden (Beutler, 1972). Additionally, naturally occurring antibodies (NAbs), which are thought to act both physiologically and pathologically, bind to the band 3 clusters and activate complement, which induces the removal of β-thalassaemic RBCs by macrophages (Lutz et al., 1993; Mannu et al., 1995).

## 1.7 Erythroblast Maturation

The maturation of erythroblasts involves a synchronised regulation of various signalling pathways that together bring about key phenotypic changes. As erythroblasts mature they become smaller in size, alter their cell surface antigen expression, produce haemoglobin, condense the chromatin and repress transcription of most genes (Keerthivasan et al., 2011).

The condensation of chromosomes is triggered by the action of several histone modifying proteins that promote the formation of dense heterochromatin. Histone

deacetylases (HDAC) induce the deacetylation of histones so that the chromatin can easily condense (Migliaccio et al., 2008). HDAC interact with protein kinases and transcription factors in order to control their activity and DNA binding capacities. There are a number of HDAC isoforms with distinct functions during erythropoiesis: HDAC1 plays a part in cell proliferation, HDAC2 affects chromatin condensation and HDAC3 promotes adult haemoglobin expression (Ji et al., 2010; Mankidy et al., 2006; Miccio et al., 2010). In murine erythroblasts, deacetylation of histone H4(K12) as well as demethylation of H3(K9) have been implicated in nuclear condensation (Popova et al., 2009).

MicroRNAs (miRNAs) can influence the post-translational modifications of histone proteins, for example miR-191 that inhibits histone deacetylation. As erythroblasts differentiate, miR-191 is downregulated, which leads to the activation of Mxi1 and therefore the condensation of chromatin (Zhang et al., 2011).

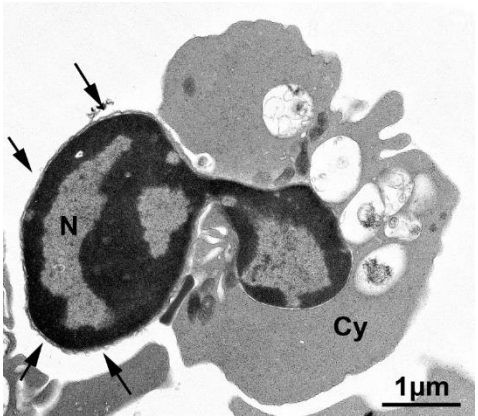
## 1.8 Erythroid Cell Enucleation

Once the chromatin has condensed, actin and microtubules act in concert to polarise the erythroid nucleus to one side of the cell prior to enucleation (Le Clainche et al., 2007; Merrifield et al., 2005). The process of enucleation requires the intricate coordination of signalling cascades that normally induce vesicle trafficking, apoptosis and cytokinesis. The final stage of erythroid enucleation involving the separation of the pyrenocyte from the rest of the cell is completed in 10 minutes *in vitro* and may be induced by soluble molecules derived from erythroblasts or stromal cells.

Subsequent to nuclear polarisation, plasma membrane is added by lipid rafts that traffic vesicles near to the nucleus (Konstantinidis et al., 2012). The orthochromatic erythroblast and incipient pyrenocyte can then separate after the actomyosin ring has assembled at the cleavage furrow and contracts following the phosphorylation of myosin regulatory light-chain (MRLC). The localisation of lipid rafts and the organisation of the actomyosin ring are both regulated by Rac GTPases (Konstantinidis et al., 2012).

The final stage and eventual expulsion of the nucleus occurs in a coordinated and controlled manner and is illustrated by the plasma membrane that surrounds the pyrenocyte (**Figure 1.4**). When the pyrenocyte partitions from the rest of the cell, it eliminates certain proteins together with the nucleus while essential membrane and cytoskeletal proteins are specifically sorted to the reticulocyte (Bell et al., 2013). α4-integrin, for example, segregates to the pyrenocyte cell surface membrane whereas glycophorin A is retained on the reticulocyte plasma membrane and this occurs during both primitive and definitive erythropoiesis (Fraser et al., 2007). In humans, a considerable amount of the surface band 3 protein is restricted to the pyrenocyte but this is not the case in mice (van den Akker et al., 2010). The precise separation of proteins contributes to the function of reticulocytes in disengaging from the bone marrow microenvironment and circulating in the bloodstream and of pyrenocytes in being recognised and phagocytosed by neighbouring macrophages. Moreover, patients with defects in cytoskeletal proteins including hereditary elliptocytosis and spherocytosis present with haemolytic anaemias (Gallagher, 2013).

**Figure 1.4**: **Electron micrograph of an enucleating erythroblast in culture**. *The nucleus (N) is in the process of being expelled from the rest of the cell cytoplasm (Cy). A thin rim of cytoplasm enclosed by the plasma membrane can be seen surrounding the nascent pyrenocyte (arrows), supporting the hypothesis that the nucleus is released from the cytoplasm in a controlled manner that does not resist forces exerted by cytoskeletal proteins. (Cells cultured by myself; EM and image supplied by David Ferguson, University of Oxford).*




## 1.9 Support of Erythropoiesis by Stromal Cells in the Bone Marrow

Stromal cells within the bone marrow parenchyma are thought to support erythropoiesis (Chasis and Mohandas, 2008). Macrophages form part of this supporting stroma and it is likely that they more closely resemble alternatively activated tissue macrophages. The differentiation and classification of these cells as well as their functional relationship with erythropoiesis are described in the following sections.

## 1.10 Monocyte Phenotypic and Functional Heterogeneity

Monocytes belong to the mononuclear phagocyte system (MPS), which also comprises macrophages and dendritic cells (Taylor and Gordon, 2003). Monocytes have several functions in common with macrophages for example in pathogen clearance and the inflammatory response but tissue resident macrophages also play crucial roles in resolving inflammation and promoting cellular homeostasis. Circulating monocytes have traditionally been thought of as precursors for resident macrophages, being recruited into tissues via chemotactic factors (e.g. CCL2) and differentiating to replace the local macrophage population (van Furth and Cohn, 1968). However, this dogma is now being challenged due to more recent discoveries that tissue resident macrophages are also present in the embryo and that they possess self-renewal capabilities (Sieweke and Allen, 2013). Further support for this monocyte-independent resident macrophage self-renewal ability is provided by the identification of yolk sac derived EMPs (described in section **1.3**).

Approximately 10% of the total leukocytes in humans are monocytes and peripheral blood monocytes are known to be a heterogeneous population. These can be grouped into three different subsets based on varying phenotypic markers and function (**Table 1.1**). The main differences are the classical monocytes, which upregulate CD14 but lack CD16 surface expression ($Ly6C^+CCR2^+CD43^+$ equivalent in mice) and that are the most abundant in the blood. In contrast, non-classical monocytes express both CD14 and CD16 ($Ly6C^-CX3CR1^+CD62L^+$ in mice) and are present at a much lower frequency in the circulation. However, an intermediate monocyte population has also been described and these monocytes are likely to be derived from the non-classical cells due to their similar gene expression profiles,

although this is yet to be corroborated (Wong et al., 2011). Therefore, it is evident that monocytes are not simply precursors to macrophages but that they also have important effector functions.

**Table 1.1**: **The classification of human and murine monocytes.** **Inducible MHCII surface expression in the mouse. Ly6C, lymphocyte antigen 6 complex; CCR2, chemokine (C-C motif) receptor 2; CX3CR1, CX3C-chemokine receptor 1. (Adapted from Italiani and Boraschi, 2014).*

| Species | Subgroup | Surface Markers | Other Surface Markers | Chemokine Receptors | Functions |
|---|---|---|---|---|---|
| Human | Classical | $CD14^{++}CD16^{-}$ | $CD163^{+}MHCII^{+}CD64^{-}$ $CD62L^{+}$ | $CCR2^{+}CX3CR1^{-}$ | Inflammation, Phagocytosis |
| | Intermediate | $CD14^{++}CD16^{+}$ | $CD163^{+}MHCII^{++}CD64^{-}$ $CD62L^{+}$ | $CCR2^{-}CX3CR1^{+}$ | Inflammation |
| | Non-classical | $CD14^{+}CD16^{++}$ | $CD163^{-}MHCII^{++}CD64^{+}$ $CD62L^{-}$ | $CCR2^{-}CX3CR1^{+}$ | Tissue homeostasis, Antiviral defence |
| Mouse | Inflammatory | $Ly6C^{+}CD11b^{+}CD115^{+}$ | $F4/80^{+}CD43^{+}CD62L^{-}$ MHCII* | $CCR2^{+}CX3CR1^{-}$ | Inflammation, Phagocytosis |
| | Alternative | $Ly6C^{-}CD11b^{+}CD115^{+}$ | $F4/80^{+}CD43^{-}$ $CD62L^{+}MHCII$* | $CCR2^{-}CX3CR1^{+}$ | Tissue homeostasis |

## 1.11 Classically and Alternatively Activated Macrophages

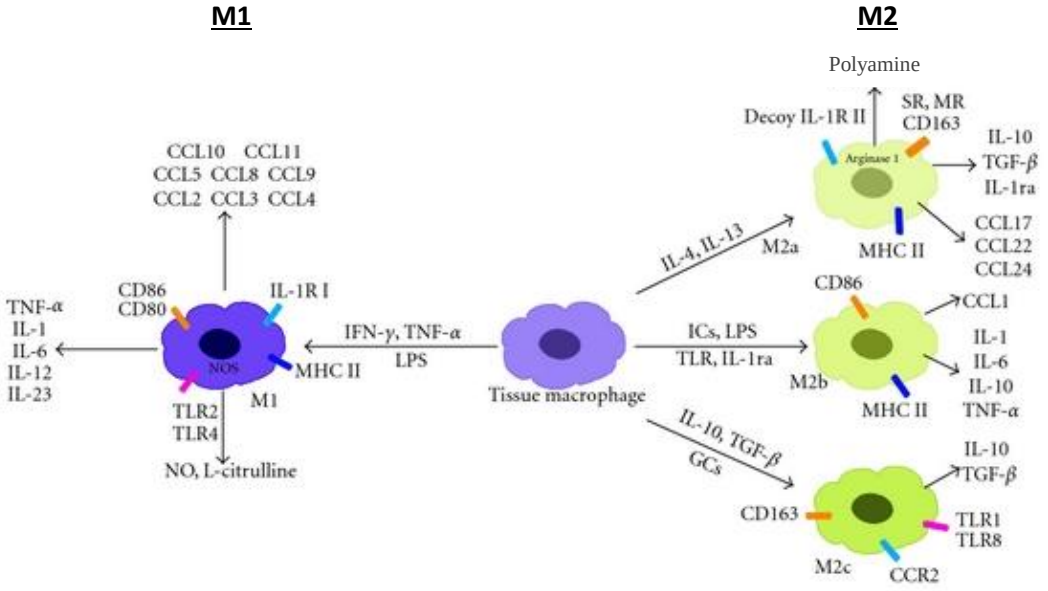
The experimental work in this thesis involves mature macrophages derived from monocytes. Therefore, this section describes the alternative phenotypes and functions of macrophages. Macrophages are large cells (approximately 14µm in diameter in mice and 21µm in humans) derived from monocytes with diverse

functions that depend on their specialised phenotype and location within an organism (Martinez and Gordon, 2014). As the surrounding microenvironment changes, macrophages can adapt accordingly – a property called 'plasticity'. Thus, numerous subpopulations of macrophages are produced as a result of different stimuli but they have widely been classified into two broad groups that are distinguished based on a specific set of surface proteins expressed, cytokines and enzymes released, and transcription factors that are activated (Classen et al., 2009). These two groups are known as the classically, or M1, activated macrophages or alternatively, or M2, activated macrophages. This general classification also discriminates between the basic functions of the various groups of macrophages.

M1 macrophages are cells that secrete pro-inflammatory cytokines and microbicidal factors, and exhibit strong phagocytic properties to ingest invading pathogens or cellular debris (Martinez and Gordon, 2014). Stimuli that induce M1 polarisation are for example IFN-γ (released by other cells such as $T_H1$ cells or cytotoxic T cells) and lipopolysaccharide (LPS) (found on the outer membrane of Gram-negative bacteria) (Billiau and Matthys, 2009; Guha and Mackman, 2001). These stimuli induce STAT1 phosphorylation and activation in M1 macrophages and trigger activity of the inducible nitric oxide synthase (iNOS) enzyme that generates NO, which functions as a microbicidal agent (Nairz et al., 2013; Podinovskaia et al., 2013). Additionally, these macrophages express high levels of the costimulatory molecules CD80 and CD86 that play a crucial part in the adaptive immune response, as well as the major histocompatibility complex class II (MHC II), which is essential for antigen presentation. They also release a range of cytokines, including TNFα, IL6 and IL12 among many others that aid them in performing their adapted roles.

In contrast, M2 macrophages secrete anti-inflammatory cytokines and do not display the same phagocytic abilities of M1 cells. Thus, M2 polarised macrophages are adapted for their functions in tissue remodelling, wound healing and angiogenesis (Jenkins et al., 2013; Martinez et al., 2008). Several stimuli divide alternatively activated cells further into subgroups- namely M2a macrophages (induced by IL4 or IL13), M2b macrophages (triggered by immune complexes or IL1ra) and M2c macrophages (polarised by IL10, glucocorticoids and TGF-β) (**Figure 1.5**). Unlike M1 activated macrophages, M2-like cells have an elevated activity of the STAT3 transcription factor and M2a macrophages also metabolise L-arginine via the action of the arginase 1 enzyme. Whereas both M2a and M2c macrophages highly express the scavenger receptor CD163, M2b cells have an augmented surface expression of CD86 and MHC II (Hao et al., 2012). Moreover, a common characteristic of all classes of M2 macrophages is their secretion of large amounts of IL10 but low levels of IL12.

**Figure 1.5: The different factors that induce a tissue macrophage to polarise towards an M1 or the various M2 phenotypes.** *Molecules such as IFN-γ, TNFα or lipopolysaccharide (LPS) trigger the formation of M1-like macrophages. M1-type cells commonly express surface co-stimulatory (CD80 and CD86) or antigen presenting (MHC II) molecules, and also secrete a number of cytokines such as IL6, IL12 or CCL2. On the other hand, M2-like macrophages can be sub-divided into different groups depending on the factors in the microenvironment, specifically M2a (IL4, IL13), M2b (ICs, IL1ra, LPS, TLR) or M2c (IL10, TGF-β, GCs) macrophages. These subgroups of M2 macrophages have distinct characteristics, including CD163 (M2a, M2c) or MHC II (M2a, M2b) surface expression and IL1- (M2a, M2b, M2c), IL1ra (M2a) or TGF-β (M2a, M2c) release. MHC II, major histocompatibility complex II; TLR, Toll like receptor; NO, nitric oxide; SR, scavenger receptor; MR, mannose receptor; ICs, immune complexes; GCs, glucocorticoids. (Taken from Hao et al., 2012).*



While macrophages have been extensively characterised into classically or alternatively activated cells, their polarisation is usually not as bipolar and as simple as this and instead they usually encompass a wide range of skewed phenotypes that fall somewhere in between these two extremes. This presents huge problems when trying to define subsets of macrophages *in vitro* or *in vivo* and can obscure the interpretation of experimental data. Murray and colleagues describe some of the main issues associated with this and emphasise the need for a common consensus when relating to macrophage activation (Murray et al., 2014). The typical characteristics and functions of different subtypes of macrophages have been reviewed and a new classification has been put forward (**Figure 1.6**). This includes the original M1 and M2-like macrophages, but also involves their additional grouping into $CD169^+$ macrophages, tumour associated macrophages (TAMs) or T-cell receptor ($TCR^+$) macrophages. Although the origin and features of these novel sets of macrophages are still to be fully elucidated, they each display distinct functions and contributions to human pathologies.

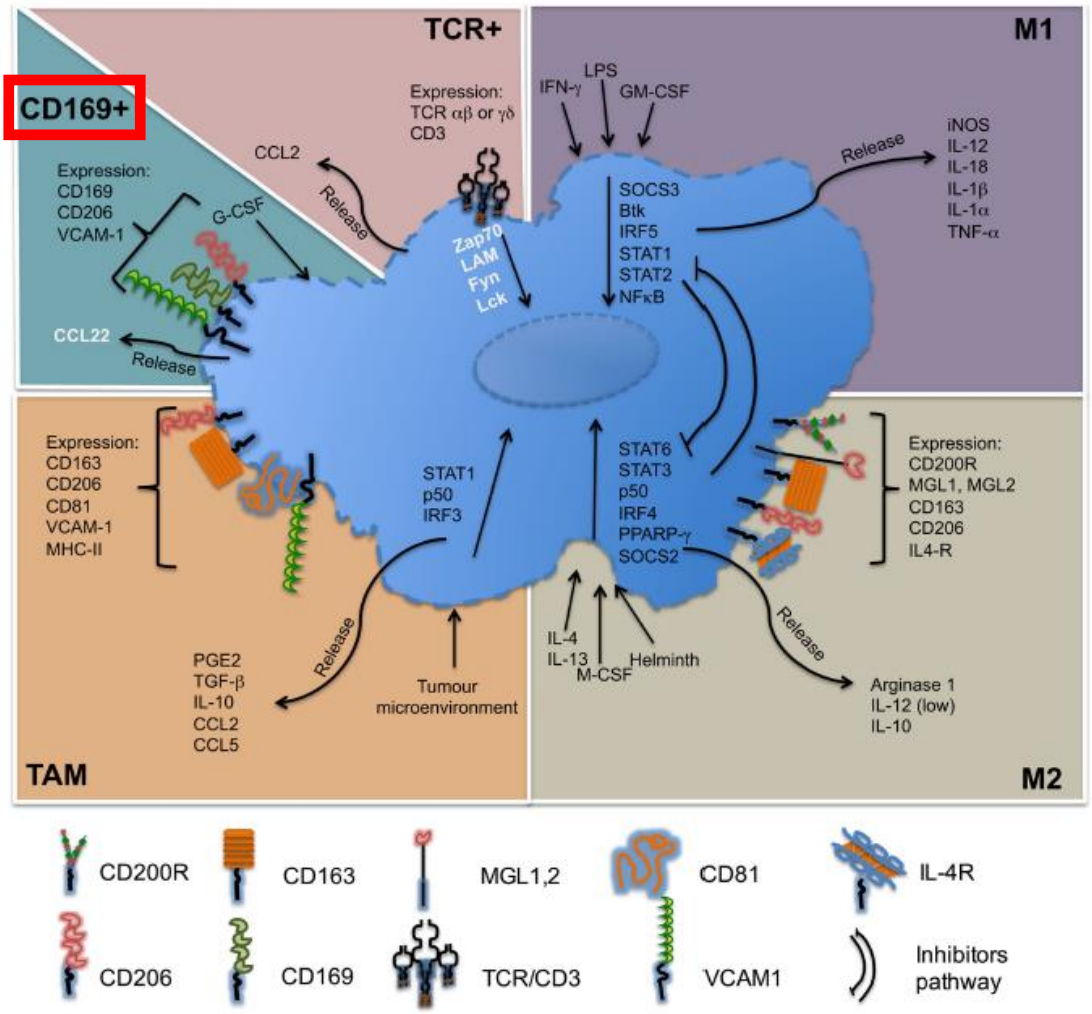
While CD169 is upregulated after stimulation with interferons and is thus often considered an M1 marker, $CD169^+$ macrophages have also been described in the context of erythrocyte development and show features characteristic of central macrophages within erythroblastic islands, with contributions to polycythaemia vera and β-thalassaemia (Chow et al., 2013; Falchi et al., 2014). These $CD169^+$ macrophages are an additional grouping separate from M1 or M2 macrophages because they provide distinct contributions to human pathologies and cannot simply be grouped into either the M1 or M2 classes. The type of macrophages associated with the stimulation of erythropoiesis are $CD169^+CD163^+VCAM1^+CD16^+$ cells. The

experimental work in this thesis shows that human monocyte-derived macrophages with a similar phenotype support erythroid cell expansion (see **chapter 4**).

Tumour associated macrophages (TAMs) are found in many tumours with roles in angiogenesis and metastasis and while they have often been described as possessing qualities resembling M2-type macrophages, they also share M1 signatures (Allavena et al., 2008; Mantovani et al., 2006; Sica et al., 2006). On the other hand, $TCR^{+}$ macrophages are associated with the progression of atherosclerosis and involve the expression of TCRαβ on the macrophage surface, which can be modified by either TNF or cholesterol (Beham et al., 2011; Fuchs et al., 2015).

The advances in modern technologies such as proteomics, microarray and deep sequencing now portray the vast heterogeneity of macrophages. As a consequence, it is likely that a new paradigm for describing macrophage activation will emerge in the years to come to replace the original and outdated model. This will, in turn, hopefully advance the field of drug discovery with respect to diseases that involve differentially polarised macrophages.

**Figure 1.6: Several subsets of macrophages as defined by characteristic surface and secreted molecules.** *A recent classification of macrophages has been proposed that divides these cells into the further subtypes TAM, CD169⁺ and TCR⁺ macrophages other than just the traditional M1 or M2 polarised cells. The molecules in white illustrate those that are found at more variable concentrations within the cell. The red box indicates that CD169⁺ macrophages are the ideal type for the stimulation of erythropoiesis. TAM, tumour associated macrophage; TCR, T-cell receptor; iNOS, inducible nitric oxide synthase; IRF, interferon regulatory factor; LAM, lipoarabinomannan; MGL, macrophage galactose lectin; PGE2, prostaglandin E2. (Taken from Chávez-Galán et al., 2015).*



## 1.12 Types of Phagocytosis

Phagocytosis is the process of particle uptake by phagocytes such as macrophages. There are several types of phagocytotic mechanisms that depend on factors including the size of the particle, the ligand-receptor dynamics and the type of cytoskeletal interactions. The process is initiated by plasma membrane receptors that are generally categorised as opsonic, FcR or complement receptors (Flannagan et al., 2012). FcR receptors (e.g. FcγRIIa or FcγRIIb) bind IgG antibodies and can either activate or inhibit phagocytosis, while complement receptors (e.g. CR3) bind products of complement cleavage fragments. A non-opsonic receptor commonly expressed by phagocytic cells is the sialylated glycan binding protein CD169 (Canton et al., 2013; Klaas and Crocker, 2012). On the other hand, toll-like receptors (TLRs) do not themselves induce phagocytosis but they can interact with non-opsonic receptors to induce cellular uptake. Crucially, FcR-mediated uptake of erythrocytes involves the formation of cytoplasmic extensions whereas phagocytosis by complement does not.

Phosphatidyl serine on the outer surface of apoptotic cells acts as an 'eat me' signal for phagocytes. There are numerous phagocytic receptors that recognise apoptotic cells, for instance the scavenger receptors A (SR-A), CD3, MARCO and CD36 (Penberthy and Ravichandran, 2016). Other important receptors that have been identified are the TAM family of protein tyrosine kinases– Tyro3, Axl and Mer-TK (Rothlin and Lemke, 2010).

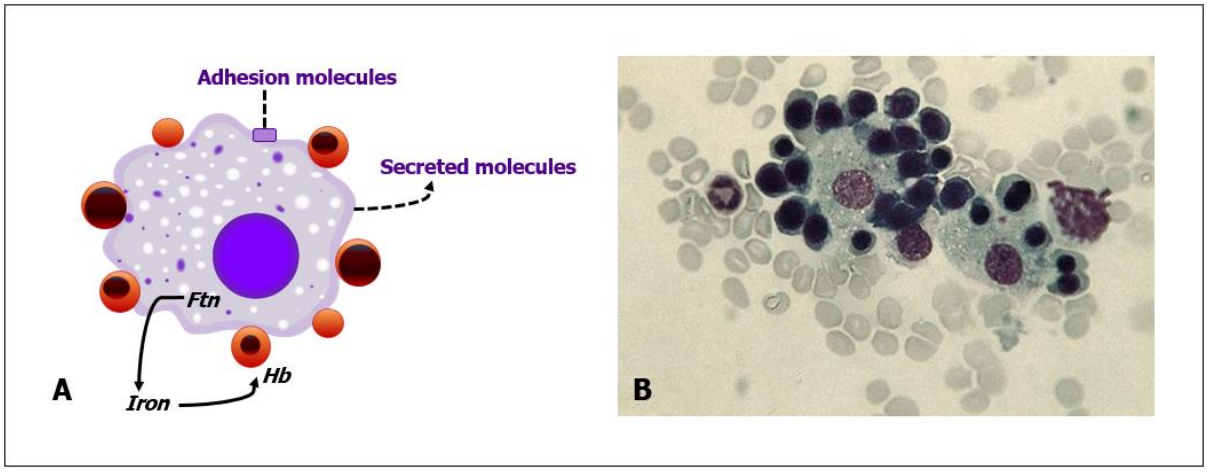
Different types of tissue resident macrophages display varying degrees of phagocytic capabilities (Taylor et al., 2005). F4/80$^{-}$CD169$^{+}$ macrophages in the rodent splenic

marginal zone are weakly phagocytic, whereas $MARCO^{+}SignR1^{+}$ macrophages in the outer marginal zone can efficiently take up bacteria. In contrast, lymph node $F4/80^{-}$ $CD169^{+}$ macrophages are able to capture viruses (Gray and Cyster, 2012). Central M2-like macrophages of erythroblastic islands phagocytose pyrenocytes expelled by orthochromatic erythroblasts. However, PU-1 knockout mice have diminished macrophage numbers, which leads to nucleated erythrocytes and as a consequence die shortly after birth (Gilmour et al., 2014). Red pulp macrophages found in the spleen also have phagocytic functions but distinctly play a key role in removing senescent erythrocytes.

## 1.13 Macrophages and Erythroblastic Islands

*In vivo*, the terminal differentiation of erythroid cells, including enucleation, can occur in erythroblastic islands. The erythroblastic island is a multicellular arrangement of erythroblasts at different stages of maturity that surround a central macrophage (Bessis, 1958; Mohandas, 1991; Palis, 2008) (**Figure 1.7**). The erythroblasts proliferate, differentiate and enucleate, and it is thought the central macrophage plays an essential role in supplying iron for the synthesis of heme (the non-protein component of haemoglobin) as well as for phagocytosing expelled nuclei (An and Mohandas, 2011).

**Figure 1.7: The erythroblastic island.** *Erythroblastic islands are distributed throughout the bone marrow and consist of a central macrophage surrounded by erythroid cells at various stages of differentiation. Central macrophages secrete factors and bind erythroid cells via specific adhesion molecules. However, these macrophages also recycle iron to enable erythroblasts to synthesise haemoglobin as they mature. Ftn, ferritin; Hb, haemoglobin. (Image in 'B' taken from Wickramasinghe et al., 2015).*




There is evidence that supports a requirement for direct contact between erythroblasts and macrophages to improve erythroid expansion and differentiation (Hanspal and Hanspal, 1994; Rhodes et al., 2008). Erythroblast macrophage protein (EMP), the first receptor on both erythroblasts and macrophages to be identified, connects erythroblasts to macrophages and it also promotes the survival of maturing erythroblasts (Hanspal et al., 1998). Furthermore, both cell-cell and cell-extracellular matrix (ECM) adhesions are known to be vital *in vivo* for erythroid cell differentiation (Sakamoto et al., 2004). **Table 1.2** highlights different adhesion molecules found on erythroblasts and macrophages and describes their proposed functions within erythroblastic islands.

**Table 1.2**: **Cell surface receptors and ligands mediating interactions between macrophages and erythroblasts.** *ECs, Erythroid cells; ICAM-4, Intercellular adhesion molecule-4; DLC-1, Deleted in liver cancer-1; VCAM1, Vascular cell adhesion molecule-1; Emp, Erythroblast macrophage protein.*

| Macrophage receptor | Erythroblast ligand | Function | Reference |
|---|---|---|---|
| CD163 | Unknown | • Scavenger receptor for the hemoglobin-haptoglobin complex<br>• Clearance of free haemoglobin via domain 3 of CD163<br>• Binds erythroblasts via domain 2 of CD163 and promotes their expansion *in vitro* | (Fabriek et al., 2007) |
| CD169 (sialoadhesin) | Unknown | • Binds sialic acid molecules on erythroblasts<br>• Maintains ECs within erythroblastic islands | (Crocker et al., 1990) |
| EMP | EMP | • Erythroblast-erythroblast and erythroblast-macrophage interactions<br>• Prevents apoptosis of erythroblasts<br>• Promotes actin organisation for nucleus separation<br>• Macrophages bind extruded nuclei | (Hanspal et al., 1998)<br>(Soni et al., 2006)<br>(Bala et al., 2006) |
| Ephrin-B2 | EphB4 | • Activation of EphB4 induces erythroid differentiation | (Inada et al., 1997)<br>(Suenobu et al., 2002) |
| $\alpha_v$ | ICAM-4 | • Erythroblastic island formation<br>• DLC-1, a Rho-GTPase-activating protein on ECs, interacts with ICAM-4 | (Lee et al., 2006)<br>(Choi et al., 2013) |
| VCAM1 | $\alpha_4\beta_1$ | • Terminal erythropoiesis<br>• Tetraspanins CD81 and CD82 facilitate $\alpha_4\beta_1$-mediated adhesion of human erythroblasts to VCAM1 and the extracellular matrix | (McGrath et al., 2008b)<br>(Spring et al., 2013) |

In addition to cell-cell and cell-ECM interactions, there is much evidence to suggest that local secreted factors also regulate erythropoiesis. Macrophages and erythroid cells are both positively and negatively controlled by these secreted factors, some of which are outlined in **Tables 1.3** and **1.4**. The regulation of erythropoiesis by macrophages is, thus, very complex (Chazaud, 2014).

**Table 1.3**: **Soluble factors, produced by erythroblasts, acting as positive regulators of erythropoiesis in erythroblastic islands.** *ICAM-4, Intercellular adhesion molecule-4; VEGF-A, Vascular endothelial growth factor-A; PGF-A, Placental growth factor-A; Flt-1, Fms-like tyrosine kinase-1; Gas6, Growth arrest specific 6.*

| Secreted protein | Ligand | Function | Reference |
|---|---|---|---|
| ICAM-4S | ICAM-4 on macrophages | • Possibly blocks ICAM-4 mediated interactions to allow reticulocyte detachment | (Lee et al., 2003)<br>(Choi et al., 2013) |
| VEGF-A | VEGF receptor on macrophages | • Attracts macrophages to erythroblasts | (Tordjman et al., 2001) |
| | | | |
| Gas6 | Unknown receptor on erythroblasts | • Enhances EPO receptor signalling in erythroblasts | (Angelillo-Scherrer et al., 2008) |
| PGF-A | Flt-1 on erythroblasts | • Down-regulates activity of inflammatory cytokines e.g. IFN-γ | (Tordjman et al., 2001)<br>(Dallalio and Means, 2008) |

**Table 1.4**: **Soluble factors acting as negative regulators of erythropoiesis in erythroblastic islands.** *RCAS1, Receptor binding cancer antigen expressed in SiSo cells; IFN-γ, Interferon-γ; TNFα, Tumour necrosis factor α; TGFβ, Transforming growth factor β. Fas and RCAS1 are produced by erythroblasts, while IL6, IFN-γ, TNFα and TGFβ are secreted by macrophages.*

| Secreted protein | Ligand | Function | Reference |
|---|---|---|---|
| Fas | FasL on late erythroblasts | • Self-regulation when mature cells accumulate with FasL, preventing erythroblast expansion. EPO suppresses Fas-FasL coexpression. | (De Maria et al., 1999)<br>(Liu et al., 2006) |
| IFN-γ | TRAILR | • Activates ERK/MAPK pathway<br>• Causes secretion of soluble TRAIL, which induces apoptosis | Reviewed in (Chasis and Mohandas, 2008) |
| IL6 | IL6 R | • Promotes hepcidin secretion<br>• Inhibits iron export by macrophages | (Verga Falzacappa et al., 2007) |
| RCAS1 | RCAS1 receptor on erythroblasts | • Induces apoptosis in CFU-Es | (Suehiro et al., 2005) |
| TGFβ | TGFβ-R | • Reduces erythroid progenitors in cycle<br>• Activates Rho and Rac GTPases, causing reorganisation of cytoskeleton | Reviewed in (Chasis and Mohandas, 2008) |
| TNFα | TNFR1 and 2 | • Activates PI3K pathway<br>• Pro-apoptotic | Reviewed in (Chasis and Mohandas, 2008) |

Erythroblasts are capable of completing definitive erythropoiesis when cultured without supporting macrophages *in vitro* (Kang et al., 2008; Miharada et al., 2006). Therefore, macrophages seem to have only a limited role in regulating erythroid homeostasis and appear to be most important during steady state erythropoiesis for recycling iron from senescent erythrocytes as well as for phagocytosing extruding nuclei during enucleation (Chasis and Mohandas, 2008; Palis, 2008; Richmond et al., 2005). Macrophages instead play a more crucial part during stress erythropoiesis, with a positive influence during acute stress but a pathological impact during chronic stress erythropoiesis (Chow et al., 2013; Ramos et al., 2013) (This is discussed in more detail in the following section **1.14**).

Following formation of the pyrenocyte, phosphatidylserine expression on its surface leads to phagocytosis by macrophages (Yoshida et al., 2005). This is followed by degradation of nuclear DNA by DNase II in a lysosomal compartment of the macrophage (Kawane et al., 2001). Studies by Iavarone et al. have shown the contribution of macrophages in definitive erythropoiesis by demonstrating a requirement for the retinoblastoma (Rb) protein in macrophages for cell-cell contact with erythroblasts and for promoting erythroblast enucleation. Knock-out of the Rb gene results in embryonic death. The authors reason that Rb is required to block Id2, an inhibitor of macrophage differentiation. Furthermore, in the absence of Rb, defective erythroblasts are unable to enucleate (Iavarone et al., 2004). Thus, the Rb protein appears to be required in both macrophages and erythroblasts for effective terminal maturation and enucleation under normal and stress conditions.

## 1.14 The Contribution of Macrophages to Elevated Erythropoiesis

Several studies in mice have proved that macrophages play vital roles in generating erythrocytes, especially during stressful conditions. In 2013, two independent groups demonstrated that central macrophages in erythroblastic islands produce erythrocytes under both normal and abnormal conditions (Chow et al., 2013; Ramos et al., 2013). Their findings were of fundamental importance to the field because they demonstrated for the first time that macrophages significantly participate in the pathophysiology of polycythaemia vera and β-thalassaemia, which are both considered disorders of chronic enhanced erythropoiesis. While Ramos and colleagues identified bone marrow resident macrophages via surface F4/80 and vascular cell adhesion molecule-1 (VCAM1) expression, Chow's group used plasma membrane bound CD169, which was first identified in 1990 (Crocker et al., 1990). Since macrophages are heterogeneous cells with varied functions dependent on the environment in which they are found, it was imperative to discriminate the central macrophages of erythroblastic islands from the various other types that exist in an organism. Nonetheless, both studies demonstrated that depletion of the central macrophage resulted in fewer erythroblasts in the bone marrow and that these macrophages had dual roles in normal and aberrant erythrocyte development. For example, acute blood loss, myeloablation and some anaemias appeared to depend on central macrophages for recovery whereas in murine models of polycythaemia vera or β-thalassaemia the disease outcome was improved when central macrophages were depleted. Both groups also show that while central macrophages in the bone marrow played crucial functions in producing reticulocytes, macrophages resident in the liver or spleen specifically acted to remove old erythrocytes from the

circulation. The exact mechanisms of action of central macrophages are still unclear but Chow and his colleagues have suggested that the macrophage ligand VCAM1 and bone marrow protein BMP4 may be involved.

It was recently shown that VCAM1$^{-/-}$ mice had approximately one-fifth the number of native F4/80$^{+}$CD11b$^{lo}$ macrophages compared to controls, but despite this diminished macrophage expansion the erythropoietic response was similar to that in normal mice (Ulyanova et al., 2016). The authors suggest that these VCAM1$^{-/-}$ mice, with a reduced resident macrophage abundance, experienced a type of stress that activated macrophage-independent signalling pathways, in turn elevating erythrocyte numbers to normal levels. This might imply that the number of resident macrophages is irrelevant or that these native macrophages themselves are not important during stress erythropoiesis. It is also possible that VCAM1$^{-/-}$ macrophages had an altered phenotype with augmented pro-inflammatory secretions that induced the enhanced erythropoiesis. In the same study, the group also found that normal mice subjected to either haemolytic- or EPO-induced stress had substantially increased proliferation of native F4/80$^{+}$CD11b$^{lo}$ macrophages in the spleen but not of recruited monocyte-derived F4/80$^{+}$CD11b$^{hi}$ macrophages (Ulyanova et al., 2016).

Gupta and colleagues investigated the gene expression signatures of macrophages from murine models of acute stress (induced by phlebotomy) or chronic stress (caused by the JAK2$^{V617F}$ mutation of polycythaemia vera) (Gupta et al., 2014). It was found that both acute and chronic stress altered gene expression profiles to a greater extent in macrophages in the spleen than in the bone marrow, but the patterns were similar in both the acute and chronically stressed splenic

macrophages. Another study also using mice identified a critical requirement for a special subset of macrophages ($F4/80^+VCAM1^+CD169^+ER\text{-}HR3^+Ly6G^+$) in the bone marrow to sustain erythropoiesis (Jacobsen et al., 2014).

It is evident that the mouse has become the pre-eminent model for studying enhanced erythropoiesis and that the correct formation of erythroblastic islands is crucial during this response (Angelillo-Scherrer et al., 2008; Liu et al., 2007; Millot et al., 2010; Ulyanova et al., 2011). Accumulating evidence is highlighting the key contribution of central macrophages in erythroid production, particularly during stressful conditions, but more work involving human macrophages and erythroid cells is necessary to corroborate the significance of recent findings for human physiology and pathology. Understanding the precise functions of central macrophages in definitive erythropoiesis would draw us closer to developing new therapies for disorders with abnormal erythropoiesis, including polycythaemia vera, myelodysplasia and β-thalassaemia. It would also improve our understanding of patient recovery following chemotherapy-induced anaemia or bone marrow transplantation.

## 1.15 The Effects of Glucocorticoids on Macrophage Polarisation

This thesis involves modulating the macrophage phenotype with the glucocorticoid dexamethasone to induce and M2c-like phenotype that was supportive of enhanced erythroblast expansion. Glucocorticoids are a class of molecule that not only act to suppress the immune system but also modulate macrophage development and function. They are produced naturally in the body to counter the effects of inflammation which, if left unresolved, can cause damage to host tissue (Asadullah

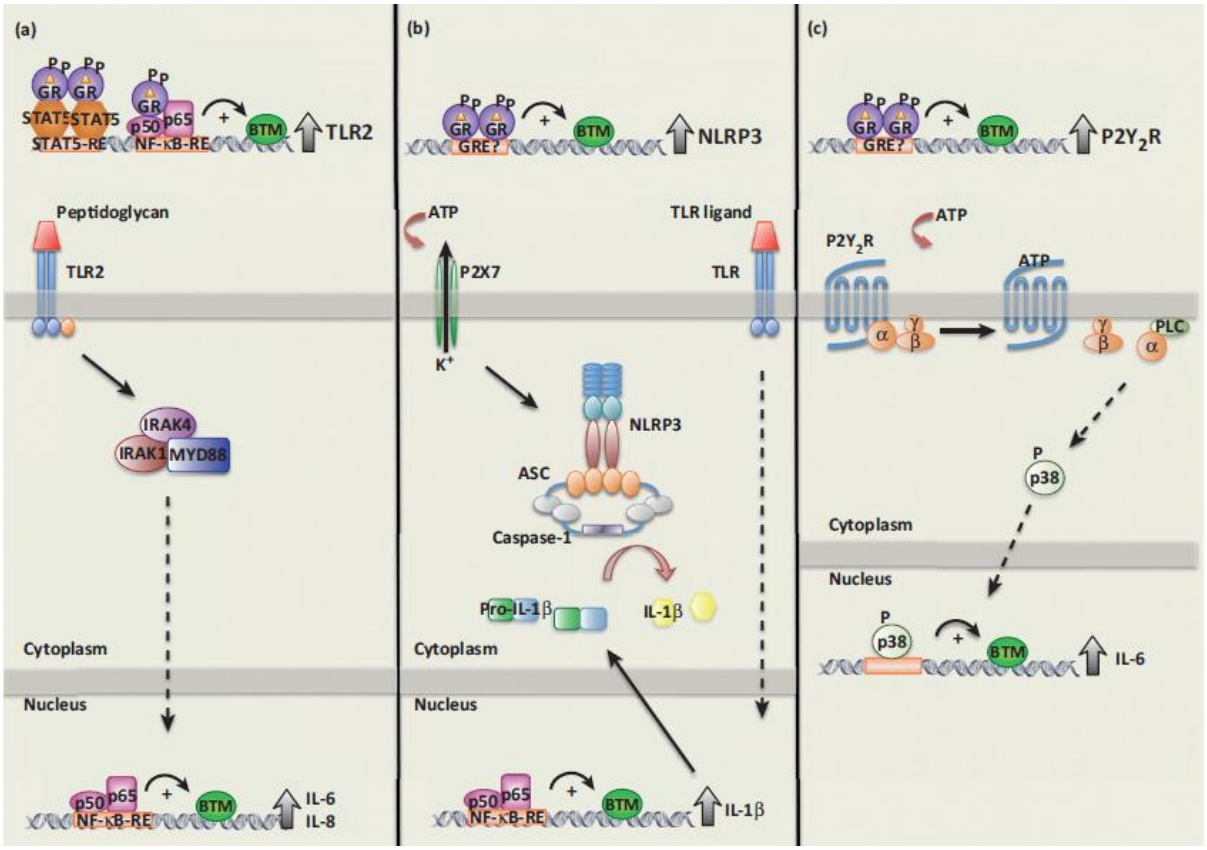
et al., 2002). While endogenous glucocorticoids are steroid hormones made in the adrenal cortex, synthetic glucocorticoid drugs include prednisone, dexamethasone, budesonide and hydrocortisone that have similar powerful anti-inflammatory effects. Although these drugs have been used for over 50 years to treat various diseases of chronic inflammation such as rheumatoid arthritis, multiple sclerosis and chronic obstructive pulmonary disease (COPD) (Murray and Wynn, 2011), their prolonged use can cause adverse side effects ranging from skin changes and weight gain to psychological changes and the development of diabetes (Dagogo-Jack et al., 2000).

Glucocorticoid ligand binding to the glucocorticoid receptor on a cell triggers a conformational change, activation and translocation to the nucleus where the nuclear receptor can act as a transcription factor to modulate inflammatory networks (Hazra et al., 2007). Specifically, the glucocorticoid receptor can interfere with p65-interferon regulatory factor (IRF) complexes to inhibit the expression of numerous pro-inflammatory cytokines in macrophages normally induced following TLR4 or TLR9 engagement (Ogawa et al., 2005). The adaptor protein MyD88 plays a crucial role in this signalling and STAT3 functions as an important co-activator that induces the expression of anti-inflammatory pathways (Ogawa et al., 2005). Furthermore, the glucocorticoid nuclear receptor is known to activate MAP kinase phosphatase-1 (MKP-1) gene expression, which in turn selectively represses p38 MAPK and downstream signalling for cytokine production (Bhattacharyya et al., 2007). TLR-dependent genes are also regulated via the integration of signals from other nuclear receptors such as peroxisome-proliferator-activated receptors (PPARs) (Jiang et al., 1998; Ricote et al., 2004) and liver-X receptors (LXRs) (Joseph et al., 2004; Valledor et al., 2004). However, the specific mechanisms whereby these nuclear

receptors cooperate to transrepress distinct inflammatory gene responses still remains to be determined. Furthermore, the established anti-inflammatory roles of glucocorticoids is juxtaposed, paradoxically, with their additional pro-inflammatory functions which serve to 'prime' the innate immune system for a quick response against a microbial infection or to tissue damage (**Figure 1.8**).

Numerous studies suggest that glucocorticoid drugs are in fact not as effective in conditions where macrophages play the predominant role, for instance in COPD. COPD involves inflammation of the respiratory tract that prevents airflow to the lungs, as in bronchitis and emphysema, and is often caused by smoking. Culpitt and colleagues show that bronchoalveolar lavage macrophages from patients with COPD produce roughly five times as much IL8 than that produced by alveolar macrophages from people who smoke, and that this release can be increased via stimulation with either IL-1β or cigarette smoke media (CSM) (Culpitt et al., 2003). They found that while dexamethasone was capable of reducing both IL8 and GM-CSF basal or stimulated release from smoker-derived macrophages in a dose-dependent manner, this glucocorticoid was unable to reduce IL8 levels from alveolar macrophages of COPD patients. While asthma patients tend to respond well to glucocorticoid treatment (Barnes, 2011), the authors hypothesise that people suffering from COPD might not experience the same beneficial effects possibly because their respiratory tract macrophages are no longer sensitive to these drugs. However, there are several limitations to their work and to this conclusion.

**Figure 1.8: Glucocorticoids prime macrophages for an adequate response against microbial products or cellular damage.** *(a) Glucocorticoids, together with STAT5 and NF-κB, promote TLR2 expression. When the bacterial component peptidoglycan binds to TLR2, it triggers release of both IL6 and IL8. (b) Glucocorticoids induce the intracellular pattern-recognition receptor NLRP3, which enables macrophages to produce IL-1β in response to cellular damage and TLR activation. (c) Glucocorticoids stimulate expression of the G-protein-coupled receptor $P2Y_2R$. This receptor binds extracellular ATP, which is a signal for cellular damage, and promotes p38 activation and subsequent IL6 release by macrophages. (Taken from Busillo and Cidlowski, 2013).*



Culpitt and co-workers only investigated the effects of dexamethasone on IL8 and GM-CSF release into culture supernatants but it is likely that the steroid also affects levels of other cytokines, chemokines, proteases and the generation of reactive oxygen species (ROS) by alveolar macrophages, which are all known to play a part in airway inflammation (Fantone and Ward, 1982; Lim et al., 2000; Lohmann-Matthes et al., 1994; Mio et al., 1997). In fact, van de Garde et al. recently showed that human peripheral monocyte-derived macrophages treated with the normally inhaled glucocorticoid fluticasone propionate (FP) for 7 days actually had increased gene expression of IL8, CCL7 and CCL2 but reduced CCL22 expression compared to untreated cells; and these macrophages also exhibited partial plasticity through altered gene expression profiles after simultaneous FP and acute LPS or chronic INF-γ treatment (van de Garde et al., 2014). In addition, they show that treatment of mice with COPD-like inflammation with budesonide five times a week for 4 months led to higher, rather than lower, levels of TNFα, IL6, CCL2, GROα, CXCL10, CCL3, CCL5 and G-CSF in the lung tissue, as well as increased macrophage and B-lymphocyte infiltration.

This is in contrast to an earlier study in 2007 which found that mice with knockout of the glucocorticoid receptor (MGRKO) had elevated secretion of TNFα, IL6 and COX-2 compared to controls following treatment with either LPS alone or in combination with dexamethasone for up to 12 hours (Bhattacharyya et al., 2007). Therefore, while different glucocorticoids were used in the various studies, it nevertheless highlights the complex and multiphasic effects of glucocorticoid drugs when added over long versus short periods of time. Moreover, conclusions that glucocorticoids suppress macrophage inflammatory gene expression have to be made with caution

as this depends on the physiological context and time points, and persisting complications are frequently related to the continued use of these drugs (Barnes, 1998; Calverley et al., 2007; Ek et al., 1999; Hakim et al., 2012; Lin et al., 1994; Rabe and Wedzicha, 2011). Van de Garde and colleagues more precisely argue that long-term use of glucocorticoids induces a weaker adaptive immune response in macrophages (with reduced antigen presenting MHC class II, cell adhesion and costimulatory protein expression), but intensifies their innate immune capabilities via an increased production of chemoattractants involved in phagocytic cell recruitment (van de Garde et al., 2014).

## 1.16 The Effects of IL10 on Macrophage Polarisation

Similarly to the use of dexamethasone, the thesis also investigates how modulating the macrophage phenotype with IL10 promoted erythroblast expansion. IL10 is a cytokine with an immunosuppressive role, which, when secreted by Th2 cell clones, was shown to inhibit production of IFN-γ by Th1 cells (Fiorentino et al., 1989). IL10 is released by many immune cells but macrophages are the main producers and targets. Apart from its function in suppressing inflammatory pathways, this cytokine plays fundamental roles in modulating the differentiation of other immune cells including B-lymphocytes, T-lymphocytes and antigen-presenting cells. Therefore, it is imperative that one does not simply think of IL10 as immunosuppressive but rather as an immunoregulatory cytokine. In fact, some studies even point to IL10 having immunostimulatory effects to eliminate foreign substances in situations where there is little inflammation, further highlighting the complex activity of this cytokine (Ding et al., 2000; Duramad et al., 2003).

IL10 is associated with several pathophysiological conditions for example during tumour development in melanomas and lymphomas where IL10 overexpression aids in progression of the disease, compared to various inflammatory and autoimmune diseases including rheumatoid arthritis, psoriasis, inflammatory bowel syndrome and multiple sclerosis where IL10 is deficient. The release of host IL10 by macrophages is a property that is exploited by some viruses such as parainfluenza virus 3 and respiratory syncytial virus, whereas other viral pathogens encode their own IL10 homologues for instance herpes viruses and pox viruses. In both situations, IL10 production leads to inflammatory response suppression which allows for the viruses to infect the host (Moore et al., 2001; Redpath et al., 2001; Spencer et al., 2002).

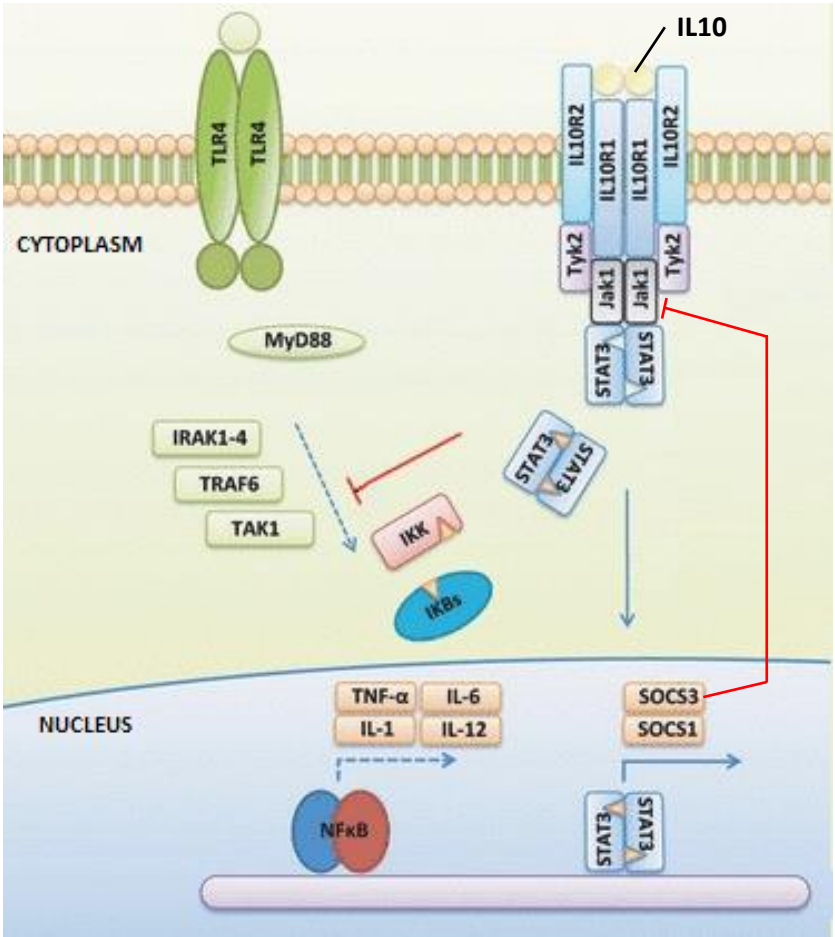
Macrophages are the immune cell type with the most abundant number of distinct response elements within the IL10 promoter for transcription factors such as NF-κB, C/EBPβ, CREB/ATF1 and SP1/SP3 (Platzer et al., 1994). This means that IL10 transcription in macrophages is enhanced by various molecules for example TNFα and endotoxin, both of which signal via NF-κB, or catecholamines and cAMP-inducing compounds, which signal via CREB-1/ATF-1 (Meisel et al., 1996; Platzer et al., 2000; Riese et al., 2000). The receptors on the macrophage surface responsible for inducing IL10 expression are TLR2 and TLR4. Active SP1 controls IL10 transcription in humans via p38 MAPK, indicating overlap with glucocorticoid receptor signalling pathways (Ma et al., 2001). Moreover, the p38 MAPK pathway also has an important function in stimulating production of the heat shock protein haem oxygenase-1 (HO-1), which converts haem into carbon monoxide, free iron and bilirubin, in response to IL10 (Lee and Chau, 2002).

The binding of IL10 to the IL10 receptor (IL10R) is an intricate process that requires both the IL10 receptor-1 (IL10R1) and IL10 receptor-2 (IL10R2) proteins to be present on the cell (Spencer et al., 1998). It appears that only once the ligand has bound to the IL10R1 dimer can this complex interact with IL10R2 proteins for complete ligand-receptor cooperation and downstream intracellular signalling (**Figure 1.9**). Glucocorticoids and vitamin $D_3$ have the ability to considerably augment IL10R expression (Michel et al., 1997). While IL10 ligand-receptor complexes tend to upregulate the expression of many genes, they target a significantly smaller subset of genes for transcriptional repression. IL10 leads to recruitment and activation of the tyrosine kinase JAK1, which subsequently phosphorylates transcription factor STAT3 and triggers its nuclear translocation, facilitating transcriptional control of IL10 target genes. On the other hand, STAT3 activity is negatively regulated by suppressor of cytokine synthesis-3 (SOCS3) following binding of specific cytokines, such as IL10, IL6 and IFN-γ, to the gp130 receptor (Traum et al., 2012). Thus, SOCS3 plays a crucial role as a modulator of homeostasis that can control various signalling pathways. It is evident, therefore, that IL10 is able to both positively and negatively regulate STAT3 activity, with SOCS3 playing an important role as a negative feedback regulator of STAT3 activation when SOCS3 levels accumulate (Berlato et al., 2002). SOCS3 inhibits this signalling through the binding of JAK proteins. IL10 is also able to block NF-κB activity either through inhibition of nuclear translocation or via inhibition of DNA-binding of this transcription factor (Al-Hanbali et al., 2009; Wehinger et al., 1996).

STAT1, which is phosphorylated and activated by IFN-γ to trigger antiviral gene expression programmes, relies on the endogenous production of TNFα and this

process is impeded by IL10 (Moore et al., 2001; Oswald et al., 1992). However, there is competition between the IFN-γ and IL10 mediated pathways, meaning that an elevation in IFN-γ levels can counteract the blockade by IL10. Additionally, it has been shown that STAT1 can be further regulated through the action of the proteins SOCS-2 and SOCS-3 (Shen et al., 2000).

**Figure 1.9: IL10 receptor mediated signalling in macrophages.** *The IL10 receptor consists of two copies of IL10R1 and IL10R2 and upon IL10 binding the intracellular Janus kinases Jak1 and Tyk2 are recruited to this receptor complex. This in turn induces binding of STAT transcription factors, for example STAT3. Phosphorylation and activation of STAT3 triggers its translocation to the nucleus, where it can promote expression of genes such as SOCS1 and SOCS3. SOCS3 can bind to Jak1 and negatively regulate STAT3 downstream signalling. STAT3 also inhibits MyD88 signalling following TLR engagement and blocks NF-κB dependent pro-inflammatory cytokine production via the degradation of the signalling molecules IRAK1-4, TRAF6 or TAK1. (Taken from Paul et al., 2012).*



Simply administering IL10 in situations with heightened inflammation or neutralising the cytokine during tumorigenesis or after major surgery does not seem to yield promising results. The reason for this is likely to be due to the multifaceted effects of IL10 on macrophages as well as various other immune cells. In fact, while treatment with IL10 in patients with rheumatoid arthritis does not seem to cause any major clinical benefits, administering the cytokine to prevent psoriasis relapse appears to yield considerably improved responses and this is presumably because of the abilities of the molecule to modulate chronic inflammation (Narula, 2000). Therefore, it might be more suitable to combine synthetic glucocorticoid drugs or anti-TNF antibodies with the long-term use of IL10 for treatment of these diseases (Maini and Taylor, 2000; Moore et al., 2001).

## 1.17 Reactive Oxygen Species in Macrophages

Reactive oxygen species (ROS) are produced during the metabolism of oxygen and the enzyme NADPH oxidase is responsible for producing superoxide and hydrogen peroxide. ROS can act as second messengers that induce a range of cellular responses such as proliferation or apoptosis, or they can play a key role in macrophages during inflammation, bacterial killing and tissue injury (Forman and Torres, 2001). The oxidant load in a cell determines the biological response, ranging from hypoxic or redox signalling during low levels of oxidative stress to apoptosis or necrosis when the oxidative burden is high and damaging. While redox signalling is important in macrophages for differentiation and polarisation, the excessive surface membrane oxidative stress in erythrocytes contributes to the defects observed during β-thalassaemia (De Franceschi et al., 2013; Tan et al., 2016).

A disruption in the homeostatic redox balance that triggers chronic oxidative stress can lead to certain diseases for example diabetes, cancer and atherosclerosis (Tan et al, 2016). A major contribution to these pathological conditions is due to the effects of ROS on macrophage polarisation, with primarily M1 activated cells in diabetes and atherosclerosis but mostly M2 polarised macrophages during cancer. While ROS from apoptotic cells initiates phagocytosis, intracellular ROS production within macrophages, induced by the NADPH oxidase 2 (Nox2) gene, is also crucial for enabling the phagocytic process (Lo et al., 2013). The bactericidal activity of macrophages is further enhanced through the action of immunoresponsive gene 1 (IRG1), which increases mitochondrial ROS (mROS) production following β-oxidation of fatty acids (Hall et al., 2013). Since excessive ROS in the macrophage is damaging, these cells express resolvin D1 (RvD1) following phagocytosis to inactivate Nox2 and prevent oxidative stress-mediated macrophage death (Lee and Surh, 2013). Whereas the uptake of apoptotic cells triggers this mechanism in order to prevent persistent ROS release, necrotic cells on the other hand induce further ROS production in a Nox-2-dependent fashion (Lim et al., 2010; Moore and MacKenzie, 2009).

Intracellular ROS in macrophages are also generated by circulating heme, which provokes programmed necrosis through the synergistic action of TNF and ROS (Fortes et al., 2012). Other factors causing elevated ROS levels leading to death of macrophages are oxidatively modified high density lipoproteins (Soumyarani and Jayakumari, 2012), free cholesterol (Ha et al., 2012), cytokines (Lombardo et al., 2007) and endoplasmic reticular stress (Tavakoli and Asmis, 2012).

In addition to their functions in regulating phagocytosis and programmed cell death, ROS have vital roles in monocyte recruitment, differentiation and macrophage polarisation. Zebrafish larvae have been found to release hydrogen peroxide at the site of a wound for rapidly attracting monocytes (Niethammer et al., 2009). Furthermore, THP1 human monocytes with overexpression of uncoupling protein 2 (UCP2), which lowered intracellular ROS levels, blocked their adhesion and migration (Ryu et al., 2004). Metabolic stress is believed to play a role in this augmented THP1 monocyte chemotaxis via upregulation of Nox4 (Ullevig et al., 2012).

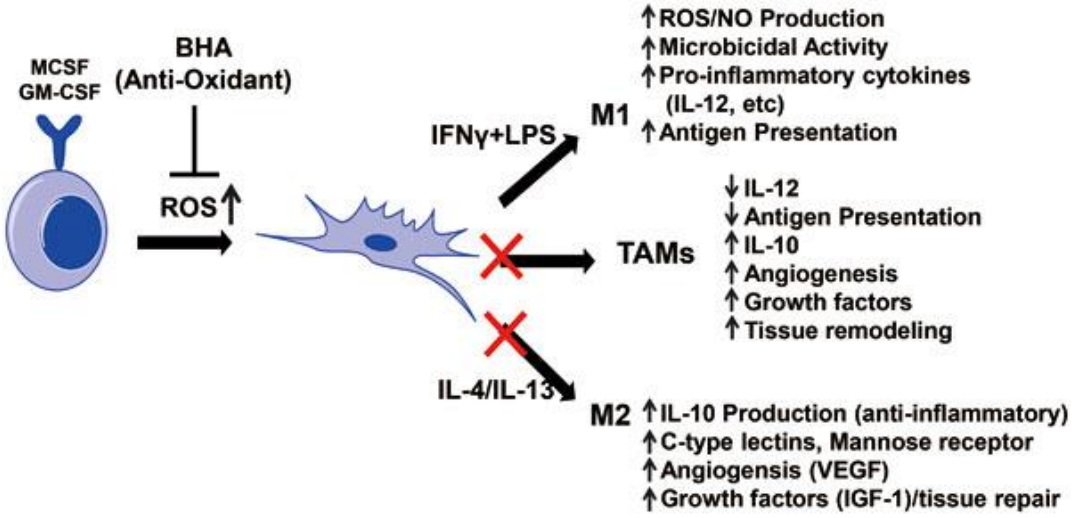
Monocyte to macrophage 1α,25-dihydroxyvitamin D3-induced differentiation is associated with ROS production and heightened ROS levels are required to activate the p38 MAPK signalling pathway (Stixova et al., 2009). The pro-inflammatory gene cyclooxygenase-2 (Cox-2) is also induced during monocytic differentiation and its expression is regulated by Nox in a ROS-dependent manner (Barbieri et al., 2003). 12-O-tetradecanoylphorbol-13-acetate (TPA) provokes Nox-derived ROS production and has been shown to downregulate endogenous expression of the antioxidant catalase in the U937 macrophage cell line (Motohashi et al., 2010). The central role of ROS in the differentiation process is highlighted by the addition of catalase to TPA-treated monocytes which inhibits both ROS generation and maturation into macrophages. It was recently discovered that the nuclear factor erythroid 2-related factor 2 (Nrf2) regulator of antioxidant enzymes has vital implications in reducing phorbol-12-myristate-13-acetate (PMA)-induced ROS levels in U937 cells and consequently inhibiting pro-inflammatory cytokine secretion (Song et al., 2015). Moreover, lipid metabolism contributes to macrophage polarisation towards an M1-like phenotype with elevated pro-inflammatory cytokine production during PMA-

stimulated maturation when monocytes are pre-treated with saturated fatty acids. This is due to inhibition by ceramide of the oxidative stress quenching nuclear receptor PPARγ, which leads to high levels of intracellular ROS (Pararasa et al., 2014; Pararasa et al., 2016). The fatty acid palmitate, together with LPS, causes the synthesis of ceramide in the endoplasmic reticulum within macrophages and increases TNFα and IL-1β secretion (Schilling et al., 2013).

ROS have different implications when macrophages are polarised towards M1 or M2 activated states. While classically activated macrophages can be generated with or without ROS being present prior to differentiation, alternatively activated cells are dependent on ROS levels during monocytic differentiation for their subsequent polarisation and appropriate function. This was demonstrated by Zhang and colleagues who illustrated that treatment of monocytes with the antioxidant butylated hydroxyanisole (BHA) for 1 hour before the addition of MCSF or GM-CSF to cultures for 6 days prevented M2 but not M1 macrophage polarisation (Zhang et al., 2013) (**Figure 1.10**). They also show that BHA suppresses tumorigenesis by affecting tumour associated macrophage (TAM) polarisation. Additionally, knockdown of the plasma membrane-associated Nox component RAC1 inhibited macrophage differentiation into an M2 polarised state (Zhang et al., 2013). Although BHA evidently has crucial effects on TAMs, one cannot exclude the possibility that it also disrupts other cell types, especially given that ROS influence numerous cellular activities. Another study showed that IL4 stimulated murine macrophages induce MCP-1-induced protein (MCPIP)-mediated ROS production via the transcription factors STAT6 and KLF4 to promote M2 polarisation. Specifically, MCPIP produces its effects through dual deubiquitinase and RNase capabilities (Kapoor et al., 2015).

Therefore, by targeting TAMs, various studies point to the potential use of antioxidants as a useful treatment during cancer development and progression as well as in other diseases. However, antioxidants have a long history of therapeutic failures.

**Figure 1.10: Treatment of monocytes with the antioxidant BHA inhibits M2 polarisation and TAM formation.** *Treatment of monocytes with BHA for 1 hour prior to their differentiation with MCSF of GM-CSF blocked M2 and TAM polarisation, but had no effect on M1 macrophages. Furthermore, these effects can be overcome by the addition of LPS + IFN-γ. BHA, butylated hydroxyanisole; TAM, tumour associated macrophage. (Taken from Covarrubias et al., 2013).*

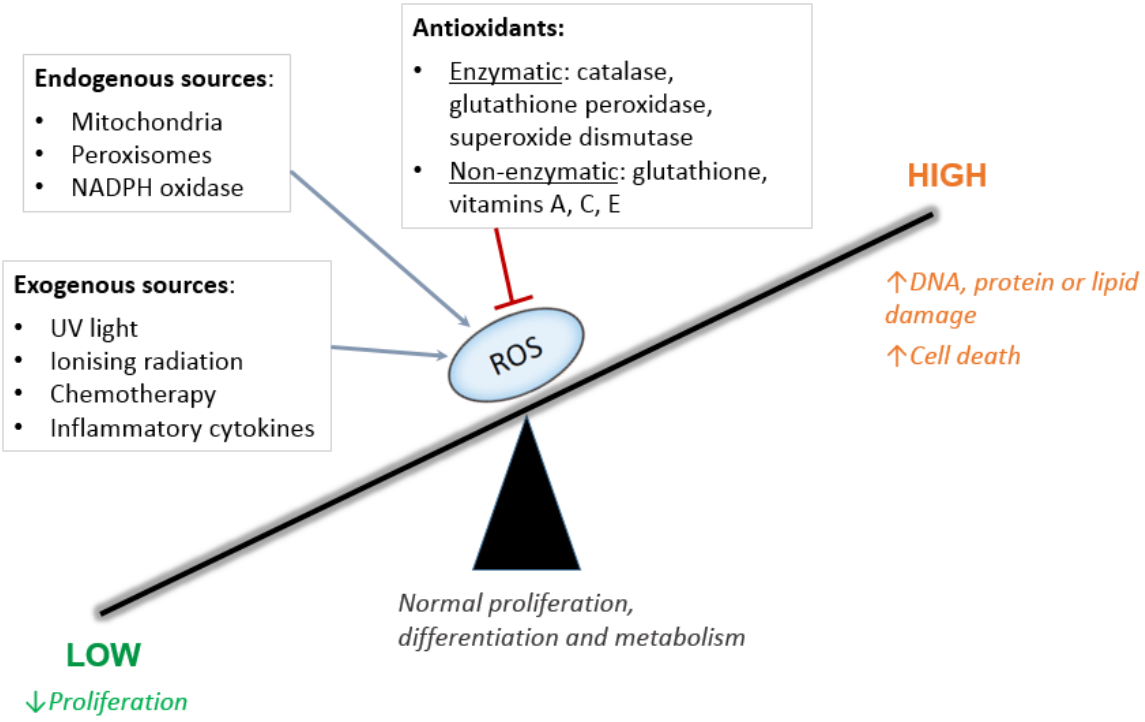



## 1.18 Reactive Oxygen Species in Erythroid Cells

Oxidative stress plays dual roles in erythroid cells depending on maturational stages but when ROS levels are exceedingly high and disrupt cellular homeostasis, this can lead to abnormalities as observed in β-thalassaemia and malaria or can even cause cellular death (**Figure 1.11**). Moreover, the essential blood transfusions during β-thalassaemia, myelodysplastic syndromes or bone marrow failure as well as hereditary haemochromatosis often lead to iron overload, which itself elevates the ROS levels in haematopoietic stem and progenitor cells (Chai et al., 2015). While

ROS act as second messengers in the ERK signalling cascade and trigger proliferation of EPO-dependent erythroblasts (Elorza et al., 2008), mature erythrocytes are much more susceptible to oxidant-induced haemolysis following the accumulation of haemichromes (Marinkovic et al., 2007). In fact, red blood cells experience some of the greatest oxidative pressures in the body. Therefore, erythroid cells must tightly control their intracellular ROS levels as they differentiate and antioxidant enzymes, for instance catalase, glutathione peroxidase or superoxide dismutase, help to lower the oxidant burden (Nishikawa et al., 2009).

**Figure 1.11: A balance in the intracellular ROS levels in erythroid cells is essential for their adequate function and survival.** *When ROS levels are too low in erythroid cells this can prevent their proper proliferation and when intracellular ROS is too high this causes damage to the cell and induces cellular death. There are numerous sources of ROS, either exogenous (for example UV light) or endogenous (for instance from the NADPH oxidase enzyme). Various enzymatic and non-enzymatic factors act as effective antioxidants.*




The anaemia of β-thalassaemia is characterised by ineffective erythropoiesis and decreased red cell survival, where abnormal cells have a very high membrane oxidative stress caused by the free iron and α-chains (De Franceschi et al., 2013). The effects of the severe oxidative stress in these cells are three-fold. Firstly, the

membrane oxidant load diminishes $K^+$ levels within the cell due to an impaired K-Cl co-transport system (Olivieri et al., 1994). Secondly, macrophages recognise phosphatidylserine as well as anti-band 3 antibodies bound to the β-thalassaemic red cell surface and initiate erythrophagocytosis (Cappellini et al., 1999). Finally, elevated ROS levels induce cytoprotective mechanisms, for instance activation of α haemoglobin-stabilising protein (AHSP-α) that binds denatured α-globin chains to prevent their precipitation (Khandros et al., 2012; Lai et al., 2006), as well as heightened expression of the antioxidant peroxiredoxin-2 (PRDX2), which targets both heme and ROS levels (Jang et al., 2004), and heme-regulated inhibitor of protein translation (HRI) as an important inhibitor of globin chain synthesis and activator of antioxidant gene expression (Lu and Chiang, 2001). Thus, antioxidants are emerging as attractive drug candidates for β-thalassaemia intermedia and major owing to their multitarget effects, with vitamin E and resveratrol already showing some promising results in murine and human studies (Fibach and Rachmilewitz, 2010; Santos et al., 2013; Tesoriere et al., 2001).

In fact, resveratrol improves the ineffective erythropoiesis of murine β-thalassaemic cells through a reduction of intracellular ROS levels via the action of transcription factor FOXO3 (Santos et al., 2013). FOXO3 is the main FOXO Forkhead family protein with vital functions during erythropoiesis and is negatively regulated by the serine/threonine protein kinase PKB (Greer and Brunet, 2005). During erythroid cell maturation, FOXO3 translocates to the nucleus where its activity triggers transcription of many genes that confer resistance to oxidative stress and is a crucial protective step against the potentially hazardous haemichrome accumulation. Another molecule with essential roles during erythroid development is miR-451,

which was found to repress oxidant damage in mice and zebrafish by blocking 14-3-3ζ production and consequently preventing re-localisation of FOXO3 to the cytosol (Yu et al., 2010).

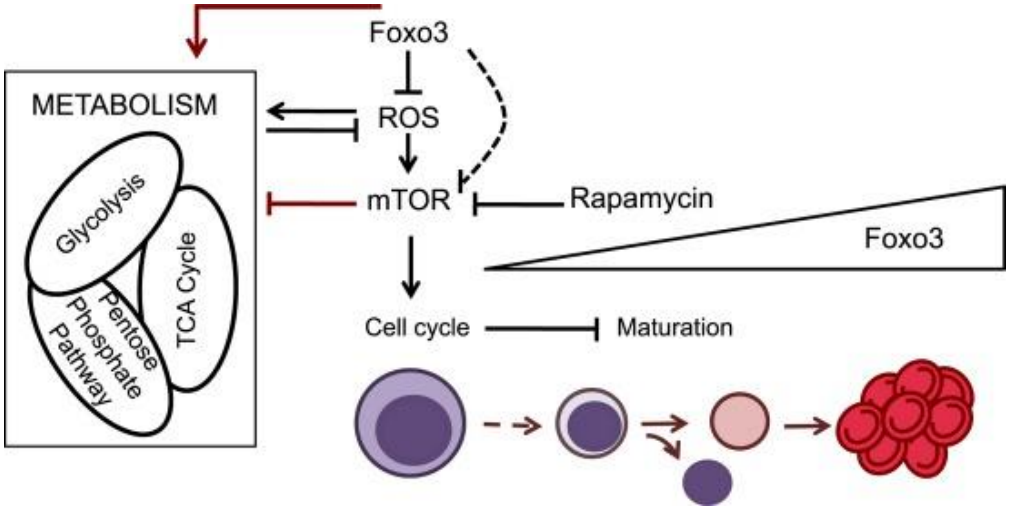
## 1.19 The FOXO3 Transcription Factor in Erythroid Cells

The latter parts of this thesis investigate the FOXO3 pathway in erythroid cells as a possible mechanism implicated during the elevated macrophage-mediated expansion (**chapter 5**). The family of forkhead box O (FOXO) transcription factors have crucial roles in diverse biological processes that regulate tumour suppression and longevity via induction of apoptosis, cell cycle arrest, reduction of intracellular ROS and metabolic control (Mammucari et al., 2007; Medema et al., 2000). Mammals contain various FOXO members, namely FOXO1, 3, 4 and 6, which have comparable structures and functions (Anderson et al., 1998). However, FOXO3, located on chromosome 6, is the main FOXO factor involved during erythroid cell development (Marinkovic et al., 2007). The activity of FOXO3 is determined primarily through its subcellular localisation, which is phosphorylation dependent at residues T32 and S253 that enables binding by the chaperone protein 14-3-3ζ and subsequent export of the transcription factor from the nucleus to the cytoplasm (Biggs et al., 1999; Rinner et al., 2007). The DNA-binding capacity of FOXO3 provides another level of regulation of its activity, with acetylation blocking binding to DNA. Furthermore, changes in total FOXO3 protein levels, either via regulation of protein stability or basal transcription, also alter FOXO3 activity (Hu et al., 2004).

Early studies identified PKB as a major negative regulator of FOXO3-dependent gene expression networks via the specific phosphorylation and nuclear exclusion of FOXO3 (Brunet et al., 1999).

The FOXO3 pathway is involved in maintaining erythroid cell homeostasis and in regulating various aspects of erythropoiesis, including the expression of differentiation-promoting genes such as B cell translocation gene 1 (BTG1), inhibition of proliferation, and protecting against oxidant stress and apoptosis (Liang et al., 2015; Wang et al., 2015; Zhang et al., 2014). Numerous studies have outlined the essential role of FOXO3 as a transcriptional regulator during erythroid maturation in various organisms, whereby its knockdown blocks the ordered progression of erythroid development (Wang et al., 2005). It was shown more recently that primary bone marrow erythroblasts with a loss of FOXO3 function had ROS-mediated hyperactivation of mammalian target of rapamycin (mTOR) signalling, which is normally essential for proliferation and cell growth, and a simultaneous inhibition of maturation (Zhang et al., 2014). The same group also demonstrated that ineffective erythropoiesis could be improved *in vivo* in a model of β-thalassaemia through the inhibition of the protein kinase mTOR using rapamycin, which enhanced production of functional mature erythrocytes. They found that mTOR signalling is related to altered glycolytic enzyme activities and other metabolic abnormalities that lead to cell cycle defects and the reduced maturation (Zhang et al., 2014). (**Figure 1.12**).

**Figure 1.12: The cooperation between FOXO3 signalling, mTOR and metabolic pathways.** *Active FOXO3 in the nucleus of erythroblasts promotes expression of antioxidant enzymes and metabolic enzymes, which keeps intracellular ROS levels in check and promotes erythroid differentiation. The drug rapamycin inhibits mTOR signalling and as a result alters glycolytic enzyme expression. (Taken from Zhang et al., 2014).*




The addition of the polyphenolic-stilbene resveratrol to β-thalassaemic mice recovered haemoglobin levels as well as erythrocyte survival, while decreasing the reticulocyte count (Franco et al., 2014). Similarly, treatment of $CD34^+$ cells derived from β-thalassaemic patients with 5μM resveratrol triggered resistance to intracellular oxidative damage via a reduced PKB activity and augmented FOXO3 activation, thereby allowing these cells to continue to mature (Franco et al., 2014). Liang and colleagues more specifically demonstrated that the FOXO3-induced genetic program plays important roles in several processes during terminal erythroid maturation, including directing cell polarity and nucleosome formation, which

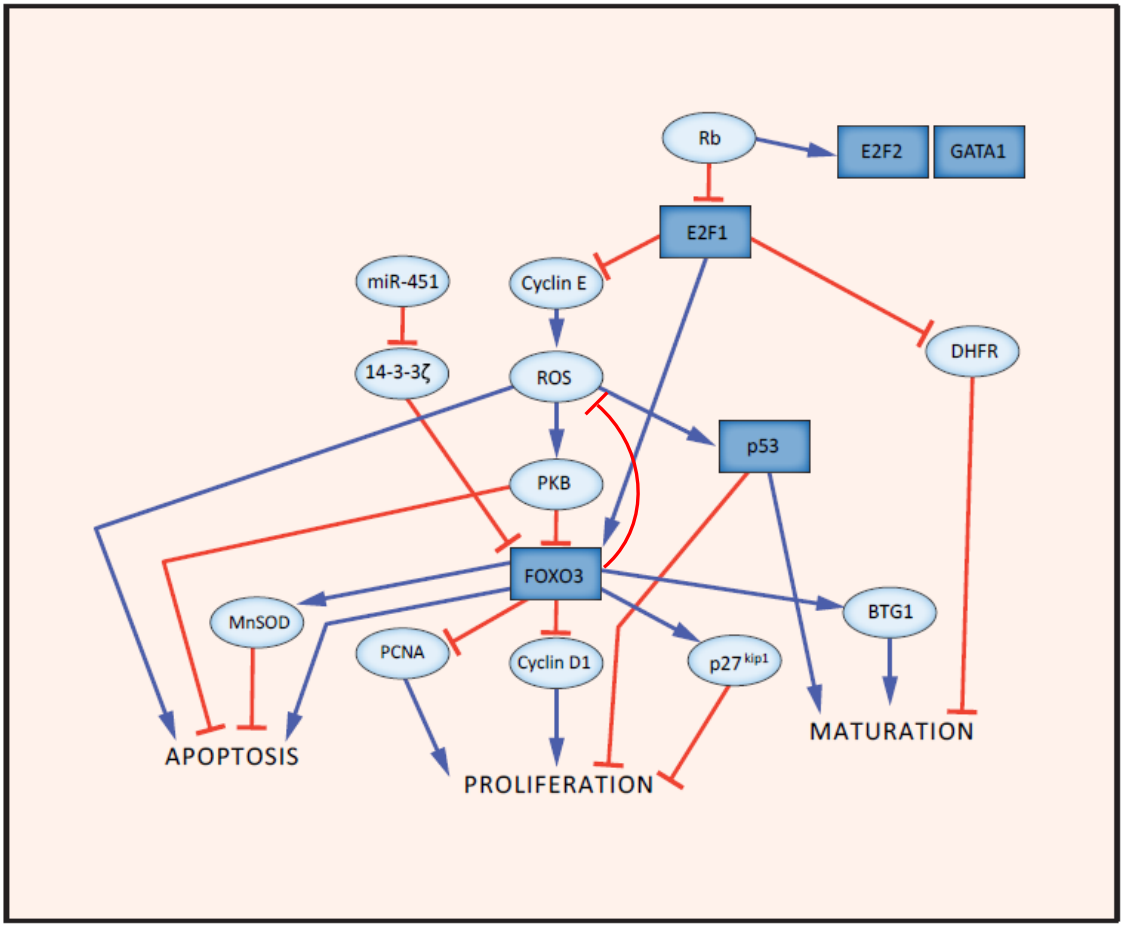
together promote enucleation and autophagy (Liang et al., 2015). Ectopic expression of the FOXO3 transcription factor ameliorated both enucleation and maturation in wild type erythroblasts (Liang et al., 2015), suggesting targeting FOXO3 might be a valuable treatment option in diseases involving faulty erythroid differentiation, such as β-thalassaemia.

Proper erythroid maturation must be accompanied by the synchronised control of oxidant stress. Although its physiological functions have not yet been fully elucidated, the micro RNA miR-451 is known to be implicated during erythroid cell differentiation (Yu et al., 2010). Its knockdown in mice or zebrafish induces erythrocyte instability and anaemia due to the increased susceptibility to oxidant damage. miR-451 acts by repressing 14-3-3ζ mRNA, thus retaining FOXO3 in the nucleus and allowing for enhanced expression of antioxidant genes such as catalase and glutathione peroxidase.

Whereas numerous studies have highlighted the fundamental role of FOXO3 in ROS resistance and erythroid maturation, there is a more limited range of evidence for the significance of this transcription factor during erythroblast proliferation. A study by Bakker and colleagues compared the differential regulation of FOXO3-dependent gene expression networks during erythroblast renewal versus differentiation by EPO and stem cell factor (SCF) (Bakker et al., 2007). Several common signalling cascades are induced by both EPO and SCF, for example the phosphorylation and activation of PKB, and cooperation between the two cytokines is necessary for erythroid proliferation, whilst only EPO is essential during maturation (Broudy et al., 1996; Dolznig et al., 2001; von Lindern et al., 2001). Since Bakker and colleagues identified only a very small subset of FOXO3-induced genes to be downregulated

following EPO or SCF stimulation, it highlights the complex nature of the signalling pathways promoting erythroid development and suggests that FOXO3 must cooperate with other transcription factors activated by various stimuli (Bakker et al., 2007). More precisely, they found that EPO alone induces FOXO3 binding to STAT5 in the nucleus, upregulating the expression of Cited2. However, the same cytokine appears to promote the association of FOXO3 with Creb family proteins for downregulation of B-cell translocation gene-1 (Btg1). Therefore, progression in erythropoiesis is determined by the integration of several gene expression networks controlled by FOXO3. A summary of the upstream and downstream regulators of FOXO3 are shown in **Figure 1.13**.

**Figure 1.13**: **The FOXO3 transcription factor and its regulation of multiple cellular processes**. *Several key proteins are involved upstream of FOXO3, for instance Rb and PKB. FOXO3 regulates expression of various proteins involved in erythroid apoptosis, proliferation or maturation. FOXO3 also positively regulates expression of antioxidants such as catalase, which inhibit ROS accumulation and lower the oxidative burden of the cell. The proteins shown in dark blue squares are transcription factors. Rb, retinoblastoma; DHFR, dihydrofolate reductase; PKB, protein kinase B (also known as AKT); MnSOD, manganese superoxide dismutase; PCNA, proliferating cell nuclear antigen; BTG1, B-cell translocation gene 1. (Image supplied by Gerry Black, University of Oxford).*



## 1.20 Relevance to the Thesis

The aim of the thesis was to establish an *in vitro* model system for enhanced erythroblast expansion mediated by IL10 modulated human macrophages. Dexamethasone ($10^{-6}$M) had already been shown to stimulate expansion of erythroid cells in cultures containing a small number (approximately 3%) of macrophages (Falchi et al., 2015). Thus, it was hypothesised that treating macrophages with IL10 would support increased expansion of autologous erythroblasts. This is because both dexamethasone and IL10 are known to polarise macrophages towards an M2c phenotype, with upregulated surface CD163 expression (Zizzo and Cohen, 2015; Zizzo et al., 2012). Moreover, CD163 is an important macrophage receptor that binds erythroblasts through 'loose' or low avidity interactions to support their proliferation (Fabriek et al., 2007; Falchi et al., 2015). However, the signalling pathways that are activated in erythroid cells when they adhere to the CD163 glycoprotein are yet to be identified.

This thesis investigates the functions that human monocyte-derived macrophages have in erythropoiesis. Rather than examining the effects of altered macrophage numbers (which is studied in the first part of **chapter 3**), the bulk of the thesis explores how altered macrophage phenotypes, achieved through their treatment with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml), affected expansion during the early stages of human erythroid development when the majority of cells were still at the proerythroblast and basophilic erythroblast stage (**chapter 4**). Several senescence-associated molecules (CCL2, IL8, Serpin E1, IL1ra and GROα) derived from dexamethasone or IL10 treated macrophages were identified, which were found to play a significant part in the elevated erythroblast expansion (**chapter 5**).

Additionally, substantial changes in the total cellular as well as the nuclear levels of FOXO3 in erythroblasts suggest that macrophages promoted expansion through the inactivation of FOXO3 in co-cultured erythroblasts (**chapter 5**). The thesis finds that the macrophage CD163 glycoprotein reduces these total FOXO3 levels in erythroblasts, while it appears that IL8, Serpin E1 and GROα could further inhibit FOXO3 activity through its phosphorylation (**chapter 5**).

## 1.21 Significance Statement

FOXO3 induces numerous signalling pathways that ultimately affect erythroid proliferation, maturation and/or apoptosis (see **Figure 1.13**). Most studies investigating the FOXO3 transcription factor have focused on more mature erythroblasts nearing the terminal maturation stage or erythrocytes. They have shown that reduced or loss of FOXO3 activity blocks erythroid maturation or induces oxidative stress in these cells (Liang et al., 2015; Marinkovic et al., 2007; Wang et al., 2005; Wang et al., 2015; Zhang et al., 2014). In β-thalassaemic mice, a lack of FOXO3 decreases erythroblast apoptosis and improves RBC production (Liang et al., 2015). Therefore, the maturational stage of erythroid cells and whether homeostatic or pathological conditions are involved determine the precise effects of FOXO3 activity on erythropoiesis.

Moreover, erythropoiesis is a multi-step process that involves expansion of erythroblasts followed by their ordered maturation into erythrocytes. Thus, the novelty of the findings in this thesis are several-fold:

1. It identifies that the FOXO3 transcription factor plays a role in human erythroblast expansion during the early stages of erythropoiesis (more precisely, that FOXO3 inactivation enhances erythroblast expansion).
2. This reduction in FOXO3 activity is induced by autologous macrophages.
3. Untreated or dexamethasone/IL10 treated macrophages lower erythroid FOXO3 activity via a reduction in the total cellular FOXO3 levels (by approximately half compared to erythroblasts cultured without macrophages) and these effects are induced by the CD163 macrophage receptor.
4. The senescence-associated molecules IL8, Serpin E1 and GROα that are secreted in greater quantities by dexamethasone or IL10 modulated macrophages further inactivate FOXO3 through increased phosphorylation.

# Chapter 2: Methods

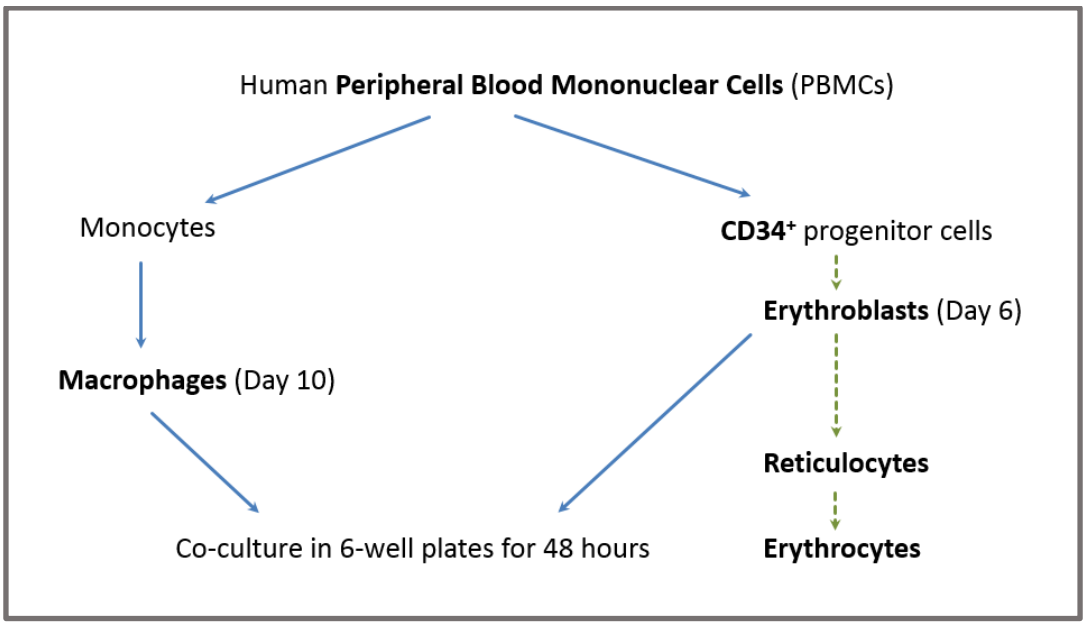
All tissue culture grade reagents were obtained from Sigma Aldrich unless stated otherwise.

## 2.1 Cell cultures

### 2.1.1 Peripheral blood mononuclear cell (PBMC) isolation

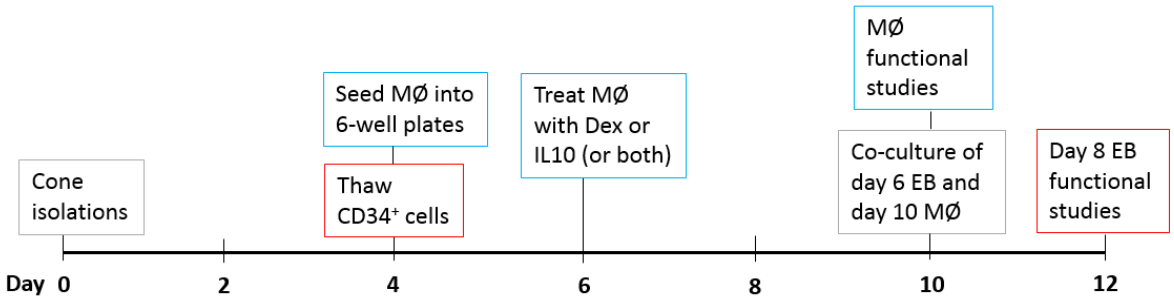
Human PBMCs were isolated from fresh cones, derived from filtration of whole blood from regular blood donors (provided by NHS Blood & Transplant, Oxford). The volume of fresh peripheral blood from each cone was approximately 8ml. This was diluted 1 in 4 (vol/vol) in Dulbecco's Phosphate Buffered Saline (DPBS) medium and the diluted blood was layered onto 15ml of polysucrose 5.7g/dl and sodium ditriazoate 9.0g/dl (Ficoll-paque-1.077, GE Healthcare) and centrifuged at 400 x g for 30 mins with the brake off. The interface that formed between the DPBS and Ficoll-Paque was collected and washed twice in DPBS as this contained the mononuclear cells. Cell number and viability were determined by the dye exclusion method using 0.4% trypan blue solution in the Vi-Cell counter (Beckman Coulter). Approximately 1 in 4 of the total mononuclear fraction was used to obtain $CD34^+$ cells, while the remainder of the cells were used for isolating monocytes. Isolation of autologous monocytes and $CD34^+$ progenitor cells allowed for their differentiation into macrophages and erythroblasts, respectively, and enabled the study of interactions between the two cell types during *in vitro* co-culture (**Figure 2.1**).

**FIGURE 2.1. Schematic depicting the isolation of monocytes and CD34⁺ progenitor cells from human peripheral blood mononuclear cells (PBMCs) for subsequent studies involving macrophage and erythroblast co-cultures.** *PBMCs were isolated from cones prepared from human blood donors.*




The following figure summarises the timeline for the general experimental framework, which adds up to a total of 12 days of cell culture.

**FIGURE 2.2. Timeline summarising the methods leading up to and after erythroblast and macrophage co-culture.** *MØ, macrophages; Dex, $10^{-6}$M dexamethasone; IL10, 10ng/ml IL10; EB, erythroblasts.*



### 2.1.2 Monocyte isolation

Monocytes were allowed to adhere onto plastic in RPMI-1640 (Lonza) supplemented with 10% FBS (Seralab) in addition to 2mM L-glutamine, 100U/ml penicillin and 100ug/ml streptomycin (RP-10). For each donor, four T150 flasks (Becton Dickinson) were used to obtain monocytes. After 1 hour, any non-adherent cells were removed and 20ml fresh RP-10 supplemented with 25ng/ml MCSF (R&D) (RP-10 + MCSF) was added per flask. The medium was then completely changed every 48 hours. This method of monocyte adherence was chosen over other techniques such as magnetic-activated cell sorting or counterflow centrifugal elutriation because it is simple, relatively inexpensive and facilitated a consistent and high yield of monocytes per donor (typically at least 9 x $10^6$ monocytes per donor). Although negative bead selection or counterflow centrifugal elutriation may prevent activation of cells during isolation, the purity of M2-polarised macrophages determined by flow cytometry 18 hours after monocyte adherence was consistently 96-98%.

### 2.1.3 Macrophage culture and differentiation

Macrophages were transferred from flasks and were seeded onto 6-well plates (Nunc) on day 4 of culture as follows. The growth medium was removed and adherent cells were washed twice with 10ml of DPBS. 6ml of cell dissociation buffer (Gibco) was then added per T150 flask and incubated at 37ºC for 8 mins. The sides of each flask were tapped to dislodge cells and any still adhered were gently detached using a cell scraper (Nunc). The cells were diluted in 30ml DPBS and centrifuged at 300 x g for 10 mins at 4ºC. Pellets were diluted in fresh RP-10 + MCSF medium and seeded at 1.2 x $10^5$ cells/well (for cells to be treated with $10^{-10}$, $10^{-8}$ or $10^{-6}$M dexamethasone), 0.8, 0.6 or 0.4 x $10^5$ cells/well (for cultures to be

treated with 2, 10 or 50ng/ml IL10) or at 1 x $10^5$ cells/well (for macrophages to be left untreated). The different seeding densities on day 4 were necessary depending on the subsequent treatment conditions to be used between macrophage days 6 – 10 because addition of IL10 to macrophages induced their proliferation in a dose-dependent manner while dexamethasone inhibited proliferation. Furthermore, while the optimal seeding density on day 4 for macrophages to be left untreated was determined as 1 x $10^5$ cells/well (**chapter 3**), this resulted in some proliferation of the cells so that they reached a final macrophage density of 1.2 x $10^5$ cells/well by the day of co-culture with erythroblasts (macrophage day 10). Therefore, seeding macrophages at these different initial densities on day 4 depending on the treatments to follow allowed for comparable final macrophage densities (1.2 x $10^5$ cells/well) and viabilities (91.0 ± 6.8%) to be reached by day 10 of macrophage culture (when erythroblasts where added in co-culture). To prevent depletion of the medium and of cytokines in between medium replacement, the total volume used per well was 3ml. The purity of macrophages on day 10 of macrophage culture was 100%, as measured by CD14 expression.

The final density of macrophages on the day of co-culture (macrophage day 10) was determined by collecting and counting cells from spare wells involving each treatment condition. Any macrophages that were not at 1.2 ± 0.15 x $10^5$ cells/well were discarded. To ensure that the final macrophage densities were always similar under the different conditions, spare control wells for each treatment were checked for macrophage number in the same manner in all experiments.

After seeding macrophages on day 4 at the appropriate densities, they were cultured for another six days in RP-10 + MCSF medium and fully differentiated into mature

M2-like macrophages. For treatment conditions that involved dexamethasone ($10^{-10}$, $10^{-8}$ or $10^{-6}$M) or IL10 (2, 10 or 50ng/ml) (R&D), macrophages were further supplemented with these respective molecules from day 6 for the last four days and changed together with the culture medium every 24 hours. For conditions consisting of simultaneous dexamethasone ($10^{-6}$M) and IL10 (10ng/ml) (dexamethasone + IL10) treatment, cells were seeded at 1 x $10^5$ cells/well and on day 6 treated with IL10 initially, followed by the addition of dexamethasone 6 hours later; on subsequent days both molecules were added at the same time during media replacement. This was done because dexamethasone appeared to have more potent effects than IL10 and thus would not mask the effects of IL10 when added in this manner. The addition of the different concentrations of dexamethasone ($10^{-10}$, $10^{-8}$ or $10^{-6}$M) did not affect final macrophage numbers or viabilities; the difference was in the proportion of cells that became rounded up, with approximately 98% of rounded macrophages for the highest concentration of dexamethasone ($10^{-6}$M). An even higher dose of dexamethasone ($10^{-4}$M) was tested, but this was toxic to macrophages. The addition of the different concentrations of IL10 (2, 10 or 50ng/ml) or the simultaneous addition of dexamethasone and IL10 also did not substantially alter the final macrophage densities or viabilities. Therefore, by controlling for macrophage numbers and viabilities, any subsequent functional effects observed in co-cultured erythroblasts were concluded to be as a result of the altered macrophage phenotypes.

### 2.1.4 $CD34^+$ progenitor cell isolation

$CD34^+$ cells were isolated using immunomagnetic separation (Miltenyi Biotec). 100µl of human Ig FcR blocking reagent (Miltenyi Biotec) and 100µl of CD34 beads

(Miltenty Biotec) were added for every $10^8$ cells in MACS buffer (PBS + 2mM EDTA + 0.5% BSA) and incubated at 4ºC for 30 mins, gently mixing every 10 mins. Cells were washed in DPBS with centrifugation at 300 x g for 10 mins. The pellet was resuspended and cells loaded into the filter placed on an LS positive selection column (Miltenyi Biotec). The column was washed three times with 3ml cold MACS buffer and subsequently removed from the magnetic field to elute cells in 5ml MACS buffer. Following centrifugation at 300 x g for 10 mins, the cells were resuspended in 4ml cold DPBS and the total number of cells calculated via haemocytometer cell counts. The cells were again centrifuged at 300 x g for 10 mins, the DPBS completely aspirated and the cells were subsequently frozen by slowly adding 500µl freezing medium (10% DMSO in FBS) per 1 x $10^7$cells/ml into appropriate cryovial tubes and placed in a Mr Frosty™ freezing container at -80ºC for optimal cooling at -1ºC/minute. All columns, filters and tubes were cooled at 4ºC prior to use. All steps for isolation of $CD34^+$ cells were also performed using equipment cooled to 4ºC. The purity of cells following $CD34^+$ bead isolation was at least 98%, as assessed by CD34 surface expression.

### 2.1.5 Erythroblast culture

Human PBMC-derived $CD34^+$ cells were thawed by placing cryovials directly from -80ºC into a 37ºC water bath. Once thawed, DMSO was diluted by gently using 10ml Iscove's Modified Dulbecco's Medium (IMDM) per tube. This was done as quickly as possible to minimise cell death. The viability of thawed cells was typically 97% ± 2.4 SEM. Cells were centrifuged at 300 x g for 10 mins and washed again in 20ml IMDM. They were subsequently cultured in a T25 low adherence flask (Corning) in stage I erythroblast medium (**Tables 2.1** and **2.2**) that was based on

the protocol by Douay (Douay, 2009), which enabled $CD34^+$ cells to massively proliferate and grow into erythroblasts. Flasks were checked every other day for confluency and kept below 1 x $10^6$ cells/ml at all times to maintain them in a proliferative state and prevent their maturation. Day 6 erythroblasts were washed twice with IMDM and subsequently cultured in 6-well plates (Nunc) at 6 x $10^3$ cells/well in stage I co-culture medium (described in section **2.1.6** and **Table 2.3**) with or without autologous macrophages.

The *in vitro* growth strategy of erythroid cells requires different media at various stages for their proper expansion and subsequent differentiation into mature erythrocytes. The stage I medium contains dexamethasone because, in combination with EPO and SCF, it allows for the massive proliferation of immature erythroblasts before they are able to terminally differentiate. This optimal outgrowth is triggered by the simultaneous activation of nuclear hormone receptors and tyrosine kinase receptors on immature erythroid cells. Thus, dexamethasone is crucial for the self-renewal capacity of erythroblasts and allows for the expansion of erythroid cells for use in autologous co-cultures with macrophages. Moreover, *in vitro* and *ex vivo* cultures of erythroid cells consist of similar media at the different stages and always contain EPO, SCF and a glucocorticoid as their first stage medium. The use of medium containing EPO but deficient in dexamethasone results in earlier terminal maturation and enucleation of erythroid cells with a reduced phase of expansion.

**TABLE 2.1. The basal medium components that make up the basic constituents of the erythroblast or co-culture medium.** *Gln, Glutamine; Pen/Strep, Penicillin/Streptomycin; FBS, Foetal Bovine Serum (batch tested to determine the best support of erythroid cultures); BSA, Bovine Serum Albumin.*

| **Basal medium component** | **Final concentration** |
|---|---|
| IMDM + Gln (1/10), Pen/Strep (1/10) | Stock concentration |
| Folic Acid | 10µg/ml |
| Myo-inositol | 40µg/ml |
| Transferrin | 200µg/ml |
| Insulin | 10µg/ml |
| Monothioglycerol | $1.6 \times 10^{-4}$M |
| FBS | 2% v/v |
| BSA | 1% v/v |

**TABLE 2.2. The components of Stage I erythroblast (EB) medium used from day 0 to day 6 of erythroid culture.** *This culture medium consists of all the basal medium components described in Table 2.1, plus those indicated in this table. EPO, Erythropoietin; SCF, Stem Cell Factor.*

| **Stage I EB medium** | **Final concentration** |
|---|---|
| Basal medium | (See **Table 2.1**) |
| EPO | 3U/ml |
| SCF | 100ng/ml |
| IL3 | 1ng/ml |
| Dexamethasone | $1 \times 10^{-6}$M |

IMDM, glutamine and penicillin/streptomycin, folic acid, myo-inositol, holo-transferrin, human insulin, monothioglycerol and dexamethasone were all from Sigma Aldrich. FBS was obtained from Sera Lab and BSA from Stem Cell Technologies. Recombinant human EPO, SCF and IL3 were purchased from R&D. These lyophilised cytokines were dissolved in cold PBS containing 0.1% BSA and stored in 100µl aliquots at -80ºC.

### 2.1.6 Co-culture of macrophages and erythroblasts

Autologous day 6 erythroblasts were resuspended in 4ml of stage I co-culture medium and added at 6 x $10^3$ cells/well to day 10 MCSF-differentiated macrophages (at a final density of 1 x $10^5$ macrophages/well). Macrophages were previously left untreated or stimulated with dexamethasone, IL10, or dexamethasone + IL10. Prior to co-culture, each well containing macrophages was gently washed once with IMDM and the culture medium completely aspirated to ensure all previous medium with stimulating agents had been removed. The components of stage I co-culture medium are described in **Table 2.3**. Note that they differ to the stage I EB medium described above. Functional studies on erythroblasts were performed on specified days after co-culture depending on the experiment.

**TABLE 2.3. The components of Stage I co-culture medium used for the co-culture of day 6 erythroblasts with macrophages.** *This culture medium consists of all the basal medium components described in Table 2.1, plus the others indicated in this table. EPO, Erythropoietin; SCF, Stem Cell Factor; IL3, Interleukin 3.*

| **Basal medium component** | **Final concentration** |
|---|---|
| Basal medium, without transferrin | 30% FBS used instead (See Table 2.1) |
| EPO | 3U/ml |
| SCF | 100ng/ml |
| IL3 | 1ng/ml |

## 2.2 Flow cytometry for characterisation of macrophages and erythroblasts

### 2.2.1 Immunophenotype of macrophages

The protein expression of cell surface markers was measured via direct immunofluorescence staining. Cells from each experimental condition were collected into 15ml Falcon tubes as described in section **2.1.3**. 10ml of DPBS was added to each Falcon tube and subsequently centrifuged at 300 x g for 10 mins at 4ºC. The pellets were washed twice in PBS with 0.03% w.v sodium azide (PBS azide) at 300 x g for 5 mins. Cells were resuspended in 100µl of PBS azide and incubated with the manufacturer's recommended concentration of the respective directly conjugated monoclonal antibody for 30 mins at 4ºC in the dark. Cells were washed twice as before and stained with 1µg/ml of DAPI for 15 mins at room temperature in the dark before running through the flow cytometer (LSR II, Becton Dickinson). All steps

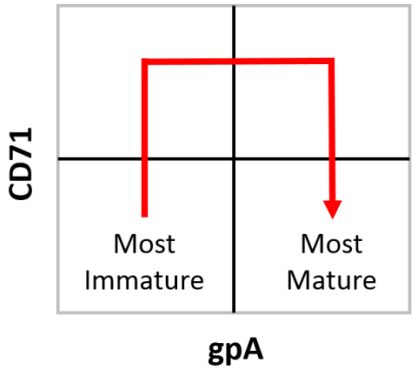
between incubations were performed on ice. Dead cells were excluded from analyses and the DAPI negative cells used as the live macrophages for phenotypic characterisation based on appropriate forward (FSC) and side (SSC) scatters as indicated. The antibodies that were used for staining macrophage cell surface receptors were CD163 PE, CD14 FITC, CD169 PE, CD16 PE, CD80 (B7-1) PE, CD64 FITC, HLA-DR PerCP-Cy5.5 (eBioscience), CD86 PerCP-Cy5.5, CD83 APC, CD106 (VCAM1) APC (BioLegend). Spectral compensation was performed as described in section 2.2.6.

### 2.2.2 Immunophenotype of erythroblast maturity

Direct immunofluorescence staining was used to measure the expression of cell surface markers on erythroblasts. Cells were collected from each well and centrifuged at 300 x g for 10 mins. Pellets were washed twice in PBS azide and resuspended in a total volume of 100µl per test. The density of cells ranged between $10^5$ and $10^6$ per test. Cells were incubated with respective antibodies for 30 mins at 4ºC in the dark and were then washed in PBS azide (as before). Cells were resuspended in 300µl PBS azide ready to be run on the flow cytometer. All steps between incubations were performed on ice. Dead cells were excluded from analyses by including only DAPI negative cells, which represent the live cells, when immunophenotyping erythroblasts based on appropriate forward (FSC) and side (SSC) scatters. The surface antigens used for the evaluation of erythroid maturation were the transferrin receptor (CD71) and glycophorin A (gpA). Early erythroid progenitors from days 0-3 do not express CD71, but as they differentiate CD71 expression increases and is at its highest between days 6-8. This is followed by expression of CD71 and gpA together from days 8-10, after which surface levels of

CD71 are gradually decreased until only gpA is expressed and CD71 expression is lost (from day 14 onwards) (**Figure 2.3**). The antibodies used to monitor erythroblast differentiation were CD71 FITC (Dako) and gpA PE (Dako), or CD71 APC (eBioscience) and gpA FITC (Dako), depending on the other set of markers used in the experiment.

**FIGURE 2.3. Schematic illustrating erythroid cell surface expression of CD71 and glycophorin A as cells mature.** *As erythroblasts mature, they first express CD71 on their surface ($CD71^+gpA^-$) followed by glycophorin A (gpA) expression ($CD71^+gpA^+$), until CD71 is lost on the plasma membrane of the most mature erythroid cells ($CD71^-gpA^+$).*

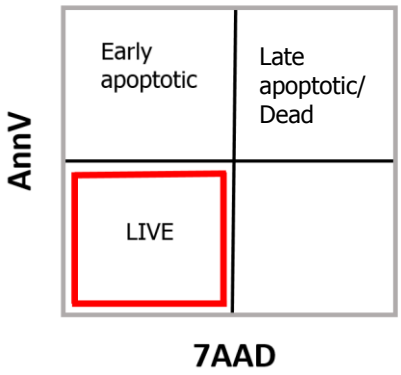



### 2.2.3 Apoptosis in macrophages or erythroblasts

The proportion of apoptotic macrophages or erythroblasts was determined via staining with 5µl annexin V (AnnV) (Invitrogen) for 15 mins in the dark at room temperature and in AnnV binding buffer (10mM Hepes/NaOH pH7.4, 140mM NaCl, 2.5mM $CaCl_2$). It was essential to use the AnnV binding buffer in this case because the presence of calcium is necessary for binding of AnnV to phosphatidyl serine on the outer surface of cells. A subsequent wash step in AnnV binding buffer followed

by addition of 5µl 7-aminoactinomycin D (7AAD) for 10 mins in the dark at room temperature allowed for the further distinction between early and late/dead apoptotic cells (**Figure 2.4**). Cells were not washed after this final step to prevent washing away the 7AAD signal and then were directly acquired on the flow cytometer as soon as possible. Unlike with the cell surface staining procedures, cells were not stained with DAPI for dead cell exclusion but were gated simply on forward (FSC) and side (SSC) scatters. The percentage of apoptotic cells includes both early ($AnnV^+7AAD^-$) and late apoptotic/dead ($AnnV^+7AAD^+$) events.

**FIGURE 2.4. Schematic showing the detection of apoptotic cells using AnnV and 7AAD.** *Live cells are $AnnV^-7AAD^-$, early apoptotic are $AnnV^+7AAD^-$ and late apoptotic or dead are $AnnV^+7AAD^+$.*




### 2.2.4 Catalase detection

Erythroblasts were fixed with 80% ice-cold methanol for 5 mins and, after washing with 0.5% BSA in PBS, were permeabilised with 0.1% PBS-Tween for 20 mins at room temperature. PBS containing 10% normal goat serum and 0.3M glycine was then added to the cells and incubated for 5 mins at room temperature to block any non-specific protein interactions. This was followed by the addition of the anti-catalase antibody (ab125688, abcam) at a dilution of 1/100 and incubated for

30 mins at 20ºC. Cells were then washed in FACS buffer. Alexa Fluor 488 goat anti-mouse IgG (H+L) (Invitrogen) was used as the secondary antibody and added at a dilution of 1/2000 for 30 mins at 20ºC. The erythroblasts were washed twice with 0.5% BSA in PBS and the samples were kept on ice until ready to be acquired by flow cytometry. Cells were passed through a 20mW Argon ion laser (488nm) with a 525/30 bandpass filter and at least 8,000 total events were collected.

### 2.2.5 Immunophenotype controls

An aliquot of unstained cells was used in each experiment, consisting of a sample of cells without the addition of any antibodies. Another aliquot of cells was stained with the respective isotype control (described below for each experiment) either at 5µl per 100µl of cells or at 1µg per 1 x $10^6$ cells as corresponded to the sample stained with the antibody of interest and under the same conditions.

For the macrophage surface markers, the negative isotype controls used for all PE, FITC or APC conjugated monoclonal antibodies were isotypes IgG1-PE (eBioscience), IgG1-FITC (eBioscience) and IgG1-APC (BioLegend), respectively. Isotype IgG2b-PerCP-Cyµ.5 was used as the isotype for the CD86 and HLA-DR PerCP-Cy5.5 monoclonal antibodies.

For the erythroid surface markers, the isotype controls used for PE, FITC and APC were IgG1-PE (Dako), IgG1-FITC (Dako) and IgG1-APC (eBioscience). The isotype control for catalase protein measurements in erythroblasts was mouse IgG1 [ICIGG1] (ab91353, Abcam).

### 2.2.6 Spectral compensation

It is crucial to apply spectral compensation during dual colour immunofluorescence analyses because otherwise spectral overlap of emission spectrums with detectors of other fluorochromes can lead to false positives. Spectral compensation was carried out by electronically subtracting the inappropriate signal of another fluorochrome from the corresponding fluorochrome signal. Single stain compensation tubes were prepared using compensation beads conjugated with antibodies specific to the isotype of the conjugated antibody. Briefly, a drop each of the negative beads (no antibody) and of the positive beads (anti-IgG1 antibody) (Becton Dickinson) into 100µl PBS azide (or corresponding buffer used in sample conditions containing cells). Each compensation tube contained beads that were stained separately with a single fluorochrome. Here the corresponding conjugated antibody used for staining the sample of cells was also incubated with the compensation beads at the same concentration on the day of the experiment. The compensation tubes were then washed with PBS azide and centrifuged at 300 x g for 10mins. Cells and compensation beads were resuspended in 300µl PBS azide before acquisition on the flow cytometer. As with the usual staining of cells, all steps between incubations were performed on ice. The precise amount of compensation between the various fluorochromes was determined for each experiment, to account for slight differences in the conditions and staining procedures between different experiments that could affect the signal for each colour.

### 2.2.7 Flow cytometric analyses

As cells were passed through the flow cytometer, the instrument was able to measure the fluorescence intensity of each fluorochrome that was conjugated to a

specific cell-bound antibody and appropriate software was used to analyse these signals (Weasel 3.3 or FlowJo). The proportion of positively stained cells was calculated by taking into account and subtracting the amount of fluorescence from isotype controls that resemble the background signal as a result of non-specific Fc receptor-mediated binding. This proportion of positive signal was represented as the median fluorescence intensity (MFI), which was calculated as:

$$\text{MFI} = \text{MFI (sample)} - \text{MFI (isotype)}$$

In the case of macrophage surface marker expression, the values are presented as the MFI relative to the isotype for ease of presentation and to allow several markers to be shown on the same graph. The MFI (relative to isotype) was calculated as:

$$\text{MFI (Relative to Isotype)} = \frac{\text{MFI (sample)} - \text{MFI (isotype)}}{\text{MFI (isotype)}}$$

## 2.3 Other erythroid functional studies

### 2.3.1 Erythroblast expansion

Erythroid expansion was calculated as the fold increase in cell number compared to the initial number of erythroblasts added into the well on the day of co-culture (6 x $10^3$ cells, unless otherwise specified). Viable cells were determined via haemocytometer cell counts, using the trypan blue exclusion method for indicating dead cells in blue. An aliquot of 50µl of cells was diluted 1:1 with trypan blue and mixed by pipetting up and down approximately 10 times. 11µl was loaded onto the haemocytometer. At least 100 events were counted per sample to ensure data was reliable. All cell counts were done as quickly as possible after addition of trypan blue

to prevent false negatives from being excluded from the counts as a result of the cells dying when not kept on ice.

## 2.4 Other studies in macrophages and erythroblasts

### 2.4.1 Measurement of intracellular Reactive Oxygen Species

All reagents were equilibrated to room temperature before their use. Intracellular reactive oxygen species (ROS) were detected in macrophages on day 10 just prior to their co-culture with erythroblasts, whereas ROS levels were measured in day 8 erythroblasts after 48 hours of co-culture with macrophages. Macrophages or erythroblasts were harvested using the appropriate method for adherent or suspension cells, respectively, as described in sections **2.1.3** and **2.1.5** above. Cells were gently pipetted up and down several times to dislodge any clumps of cells and the total number of cells collected for each cell type and treatment condition were a minimum of 1.5 x $10^4$ cells. Cells were stained using 20μM of the cell permeant 2',7'-dichlorofluorescin diacetate (DCFDA) (ab113851, Abcam) in culture media for 30 mins at 37ºC to detect intracellular ROS levels including hydroxyl and peroxyl activity. Cells exposed to each condition were subsequently analysed by using the flow cytometer without washing after the staining step. The 488nm laser was used for excitation of any 2',7'-dichlorofluorescein (DCF) produced in the cell following the oxidation by ROS of deacetylated DCFDA, which could then be detected at a wavelength of 535nm. At least 5,000 viable cells were analysed for each treatment condition and run through the machine at a density not greater than 1 x $10^6$ cells/ml. Results were analysed using appropriate software (Weasel 3.3 or FlowJo) and cellular debris and aggregates were omitted from the forward and side scatter gates,

while gating on the appropriate cell population. Aggregates were omitted by doublet discrimination based on the scatter plots for FSC-Height against FSC-Area, with singlets determined as the population with the larger FSC-Height for the same FSC-Area. The fluorescence intensity was shown as the measure of intracellular ROS.

### 2.4.2 Electron microscopy

Macrophages or erythroblasts were fixed in 4% glutaraldehyde/0.1M phosphate buffer, pH7.2. Samples were post-fixed in osmium tetroxide prior to subsequent dehydration and embedding in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate prior to examination in a Joel 1200EX transmission electron microscope. Samples were processed and imaged by Prof. David Ferguson (Nuffield Division of Clinical Laboratory Sciences, John Radcliffe Hospital, Oxford).

## 2.5 Statistical methods

The student's t-test was used to compare the means from only two samples. The one-way ANOVA with Tukey's post-test correction was used when comparing the means of parametric variables from more than two samples (with one dependent variable for every independent variable), while the two-way ANOVA with Sidak's multiple comparisons correction was used to compare the means from more than two samples (where there were at least two dependent variables for each independent variable). The type of test used is indicated for each situation. In most cases and unless otherwise indicated, erythroblasts co-cultured with the differentially modulated macrophages are compared to erythroblasts cultured with untreated macrophages as well as to erythroblasts cultured on their own.

# Chapter 3: Macrophage enhancement of erythropoiesis

## 3.1 Introduction

Macrophages have roles in the innate and adaptive immune response, wound healing, tissue homeostasis and erythropoiesis (Amit et al., 2017; Boniakowski et al., 2017; Chen et al., 2015; Ulyanova et al., 2016; Zuo et al., 2012). These diverse roles demonstrate how a variety of macrophage functions can be induced following their response to the surrounding microenvironment. One mechanism that may underlie this critical plasticity is differential gene expression, which can lead to changes in specific protein levels and ultimately an altered macrophage phenotype.

Several studies have demonstrated that macrophages in the bone marrow play an important role in the regulation of erythroid cell development in both mice and humans (Hangoc et al., 1991; Manakova and Kapina, 1991). Bone marrow macrophages have been described as M2-polarised tissue supportive cells present in erythroid niches termed 'erythroblastic islands' and they promote erythroblast proliferation and maturation in an EPO-independent manner (Rhodes et al., 2008). Although the precise mechanisms of how bone marrow macrophages aid in erythroid development and erythrocyte production have not yet been identified, the scavenger receptor CD163 is known to play a crucial role in binding erythroblasts and enhancing their proliferative rates (Fabriek et al., 2007).

In this chapter, human peripheral blood monocyte-derived macrophages are seeded at a range of densities (0.25 – 4 x $10^5$ cells/well) and grown *in vitro* using MCSF to

induce an M2 phenotype. The aim was to determine the optimal macrophage seeding conditions to be able to study the significance of macrophages in the control of erythropoiesis. It was hypothesised that the phenotype of human macrophages would be affected by their culture at various concentrations, as has previously been shown with murine bone marrow-derived macrophages (Lee and Hu, 2013). Lee and Hu found that plating murine macrophages at 4 x $10^5$ cells per 10cm dish produced mainly $CD11b^+F4/80^+Ly6C^-Ly6G^-cFms^+$ cells, whereas seeding them at 5 x $10^6$ cells per 10cm dish resulted in mostly $CD11b^+F4/80^+CD11c^+Ly6C^-Ly6G^+$ cells. The lower seeding density also resulted in more phagocytic macrophages that secreted greater amounts of pro-inflammatory cytokines, such as IL6 and TNFα.

Identifying the optimal seeding density for macrophages to promote erythropoiesis would allow for subsequent studies that aim to investigate whether adhesive interactions between erythroblasts and macrophages or secreted factors derived from the macrophage are more important during macrophage-mediated erythropoiesis. Given that the macrophage surface receptor CD163 promotes erythroblast proliferation (Fabriek et al., 2007), it was expected that the co-culture of erythroid cells with autologous macrophages in direct contact would be more effective at enhancing erythropoiesis than when the two cell types are physically separated. Thus, by establishing this co-culture model of erythropoiesis it may identify some insights into the ways in which human macrophages may enhance steady-state erythropoiesis.

## 3.2 Specific Methods

Most of the methods are described in the general methods section (**chapter 2**). However, experimental work relevant to this chapter only is described below.

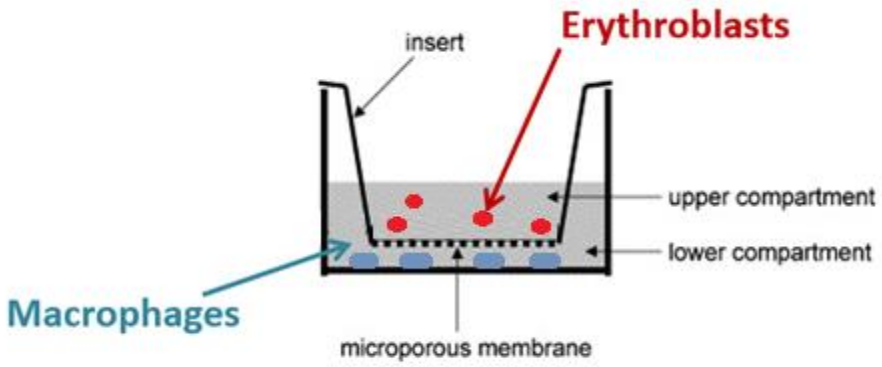
### 3.2.1 Seeding macrophages at various densities

Macrophages were seeded on day 4 from T150 flasks (Becton Dickinson) into 6-well plates (Nunc) at a range of densities (0.25, 0.5, 1, 2, 4 x $10^5$ cells/well). Macrophages were harvested from T150 flasks as described in section **2.1.3** of **chapter 2** ('Macrophage culture and differentiation') and the same protocol was also used for the culture of these cells after seeding them on day 4.

### 3.2.2 Macrophages and erythroblasts co-cultured in contact or separation

To study the effects of adhesion molecules versus secreted factors, macrophages and erythroblasts were either co-cultured in direct contact or in physical separation, respectively. For studies involving the two cell types in contact, day 6 erythroblasts were added into the co-culture medium of a well containing macrophages, allowing erythroblasts to settle on top of macrophages that were adhered to the bottom surface of the well. However, to separate the two different cell types, a transwell insert (Appleton Woods) was placed into each well so that erythroblasts could be added into the upper compartment away from the macrophages underneath (**Figure 3.1**). To control for any indirect effects that may be caused by the transwell insert, one was also placed into cultures of macrophages and erythroblasts in contact as well as into wells containing erythroblasts only. No significant differences in erythroid development were found by the addition of the transwell insert compared to the absence of the transwell during culture (data not shown).

**FIGURE 3.1. Schematic of the transwell insert placed into a well to physically separate erythroblasts from macrophages during co-culture.**




### 3.2.3 Long-term culture of macrophages and erythroblasts

Macrophages were co-cultured with day 6 erythroblasts in stage I co-culture medium as described previously in section **2.1.6** (**chapter 2**). However, to allow for the progressive differentiation of erythroblasts, it was necessary to change the components of the co-culture medium. Thus, after 48 hours of co-culture, the medium was changed to stage II co-culture medium (from erythroblast day 8 – 11) and then to stage III co-culture medium (from erythroblast day 11 and onwards). The components of each of the different media are described in **Table 3.1**. In each case, EPO was used at a final concentration of 3U/ml, SCF at 100ng/ml and IL3 at 1ng/ml. The basal medium was made up as described in **Table 2.1** (**chapter 2**) but with FBS at 30% and without any holo-transferrin. From erythroblast day 11 onwards, the culture medium was replaced with 4ml fresh stage III co-culture medium every three days.

**TABLE 3.1. The components of Stage I, Stage II and Stage III co-culture media.** *This consists of all the basal medium components described in Table 2.1 (chapter 2), plus the others indicated in this table and was used for the co-culture of erythroblasts and macrophages together after erythroblast day 6. EPO, Erythropoietin; SCF, Stem Cell Factor.*

| Co-culture Medium | EB Day | Components |
|---|---|---|
| Stage I | 6-8 | Basal* + EPO, SCF, IL3 |
| Stage II | 8-11 | Basal* + EPO, SCF |
| Stage III | 11-onwards | Basal* + EPO |

*Basal medium contained 30% FBS and lacked transferrin.

### 3.2.4 Erythroblast enucleation

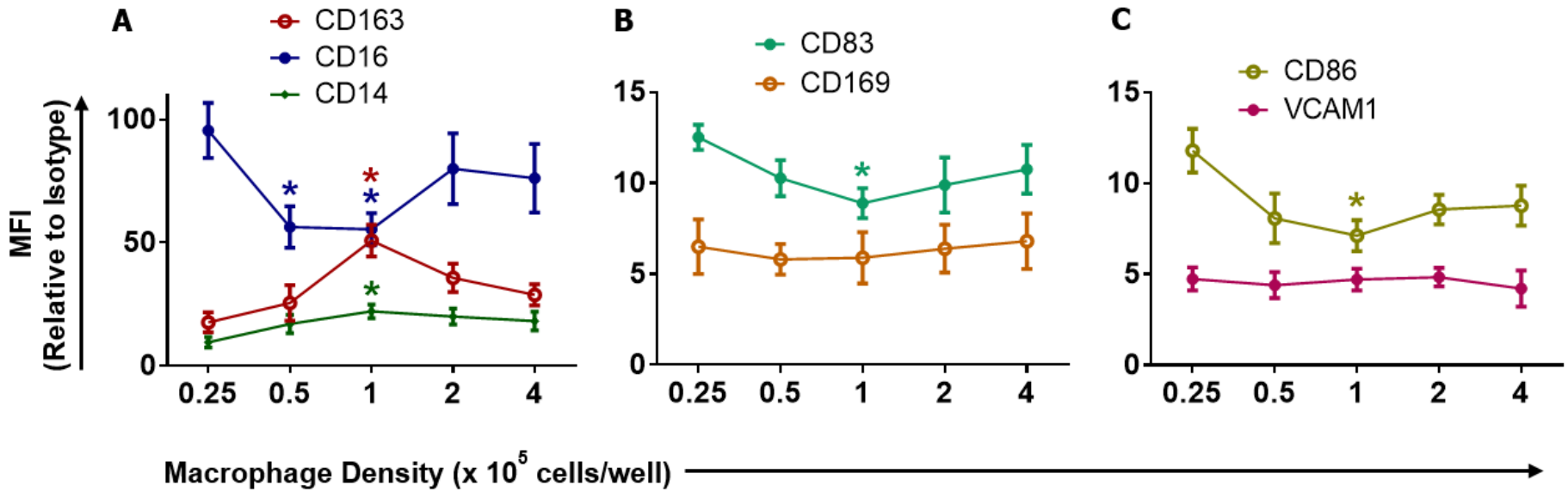
The proportion of enucleated erythroid cells was determined by staining for both surface glycophorin A (gpA) and for DNA using Hoechst 33342 (HO). Cells were stained for gpA first at 4ºC for 25 mins and after washing in 1ml of PBS azide they were subsequently stained with HO at room temperature for 20 mins. Erythroblasts were then passed through the flow cytometer (FACScan LSR II, Becton Dickinson) and enucleated cells were represented as the $gpA^{+}HO^{-}$ population.

## 3.3 Results

### 3.3.1 Macrophage Seeding Density effects on Surface Marker Expression and Viability

To establish the optimal density of macrophages in culture, a range of seeding concentrations (0.25, 0.5, 1, 2, 4 x $10^5$ cells/well) were tested. Macrophages found in the human bone marrow are $CD163^+CD169^+VCAM1^+$ and so it was important to determine a cell density where the surface marker profile represented this phenotype as closely as possible. However, it was imperative that these macrophages also had good viabilities that would not detrimentally affect erythroblasts when added into co-culture. Hence, the effects of the various macrophage concentrations on their cell surface expression as well as on their viability were investigated.

**FIGURE 3.2. Effect of seeding macrophages at a range of concentrations on expression of cell surface markers on day 10 of macrophage culture.** *Macrophages were seeded at 0.25, 0.5, 1, 2 and 4 x $10^5$ cells/well and the expression of various surface antigens measured by flow cytometry using directly labelled antibodies. The change in CD163, CD16, CD14 (A), CD83, CD169 (B), CD86 and VCAM1 (C) at each macrophage density are shown. Data represent the mean and SEM of 3–5 biological repeats. *P<0.05, **P<0.01 compared to the lowest seeding density (0.25 x $10^5$ cells/well) (one-way ANOVA with Tukey's post-test correction for each marker).*



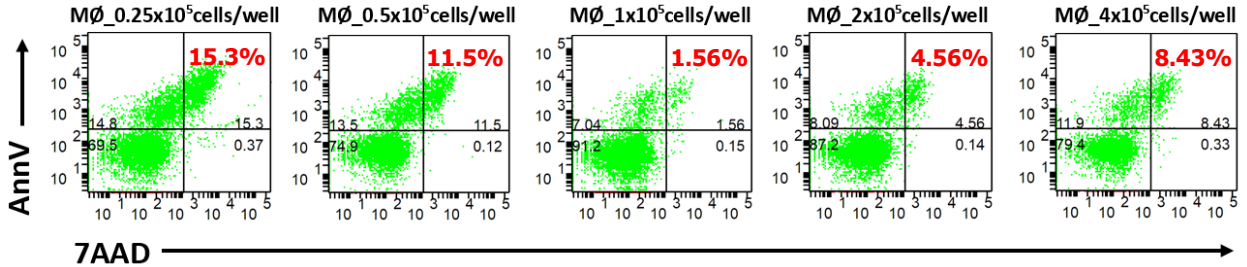
Seeding macrophages at 1 x $10^5$ cells/well resulted in the highest CD163 and CD14 surface protein expression, and lowest CD16, CD86 and CD83 expression. However, the protein levels of CD169 and VCAM1 on the macrophage surface remained constant at the different seeding concentrations. Conversely, seeding macrophages at 0.25 x $10^5$ cells/well produced cells with only 17% CD163 expression and 33% CD14 expression relative to that at 1 x $10^5$ cells/well, while CD16, CD86 an CD83 surface levels were 7.8-, 1.5- and 4.0-fold greater, respectively, than at 1 x $10^5$ cells/well. All the markers measured were found to have similar surface expression at the lowest (0.25 x $10^5$ cells/well) and highest (4 x $10^5$ cells/well) plating concentrations, with the exception of CD163, which was 2.7-fold greater at 4 x $10^5$ cells/well than at 0.25 x $10^5$ cells/well.

The proportion of macrophages that co-express markers may be important and so these data are also represented as the percentages of various combinations of surface receptors (**Table 3.2**). Furthermore, the effects on viability of plating macrophages at different densities are demonstrated (**Table 3.3**).

**TABLE 3.2. Co-expression of markers on macrophages seeded at various concentrations.** *The percentages of certain combinations of surface markers for macrophages at a range of seeding densities (0.25, 0.5, 1, 2 and 4 x $10^5$ cells/well) are shown. Data are the mean and SEM from 3 different donors. *P<0.05 compared to 0.25 x $10^5$ cells/well (one-way ANOVA with Tukey's post-test correction).*

| **MØ Density** (x$10^5$ cells/well) | **CD169$^+$VCAM1$^-$** | **CD169$^+$VCAM1$^+$** | **CD163$^+$** | **CD163$^+$CD14$^+$** | **CD86$^+$CD83$^-$** | **CD86$^+$CD83$^+$** | **CD14$^+$CD16$^+$** | **CD16$^-$** |
|---|---|---|---|---|---|---|---|---|
| **0.25** | 44.6 (±5.9) | 55.4 (±7.0) | 99.2 (±0.5) | 98.7 (±0.2) | 62.6 (±7.6) | 36.8 (±5.2) | 3.9 (±1.5) | 96.5 (±1.4) |
| **0.5** | 60.6 (±7.3) | 39.4 (±6.5) | 99.7 (±0.2) | 98.5 (±0.1) | 84.6 (±7.7) | 15.3 (±3.3) | 6.7 (±2.9) | 92.8 (±2.8) |
| **1.0** | 21.8 (±4.6) | 78.0 (±8.6)* | 100 (±0.0) | 99.9 (±0.1) | 90.1 (±7.3)* | 10.2 (±3.3) | 11.3 (±4.0)* | 86.7 (±2.9) |
| **2.0** | 58.7 (±6.3) | 41.3 (±6.4) | 99.4 (±0.5) | 98.9 (±0.1) | 89.6 (±8.3) | 9.8 (±4.7) | 9.8 (±4.8) | 90.9 (±3.4) |
| **4.0** | 62.8 (±7.2) | 37.1 (±5.6) | 99.3 (±0.6) | 98.6 (±0.3) | 64.9 (±6.5) | 34.2 (±5.8) | 3.6 (±1.9) | 97.4 (±0.9) |

**TABLE 3.3. The percentages of viable and apoptotic/dead macrophages on day 10 of macrophage culture when seeded at different concentrations.** *The percentages of viable (AnnV$^-$7AAD$^-$), early apoptotic (AnnV$^+$7AAD$^-$), late apoptotic or dead (AnnV$^+$7AAD$^+$, red figures), and all AnnV$^+$ macrophages are shown for the various plating densities (0.25, 0.5, 1, 2 and 4 x 10$^5$ cells/well). Representative FACS plots from one donor are illustrated. Data are the mean and SEM from 3 different donors. *P<0.05 compared to 0.25 x 10$^5$ cells/well (one-way ANOVA with Tukey's post-test correction).*




| **MØ Density** (x10$^5$ cells/well) | **Viable** (AnnV$^-$7AAD$^-$) | **AnnV$^+$7AAD$^-$** | **AnnV$^+$7AAD$^+$** | **All AnnV$^+$** (7AAD$^-$ and 7AAD$^+$) |
|---|---|---|---|---|
| **0.25** | 65.2 (±8.8) | 18.5 (±3.8) | 15.8 (±1.9) | 34.3 (±5.7) |
| **0.5** | 74.7 (±9.0) | 14.7 (±2.7) | 10.2 (±1.5) | 24.9 (±4.2) |
| **1.0** | 91.9 (±7.1)* | 6.4 (±1.1)* | 1.6 (±0.4)* | 8.0 (±1.5)* |
| **2.0** | 85.5 (±7.7) | 8.9 (±2.0) | 5.6 (±1.7) | 14.5 (±3.7) |
| **4.0** | 77.1 (±9.0) | 13.5 (±3.3) | 9.4 (±2.6) | 22.9 (±5.9) |

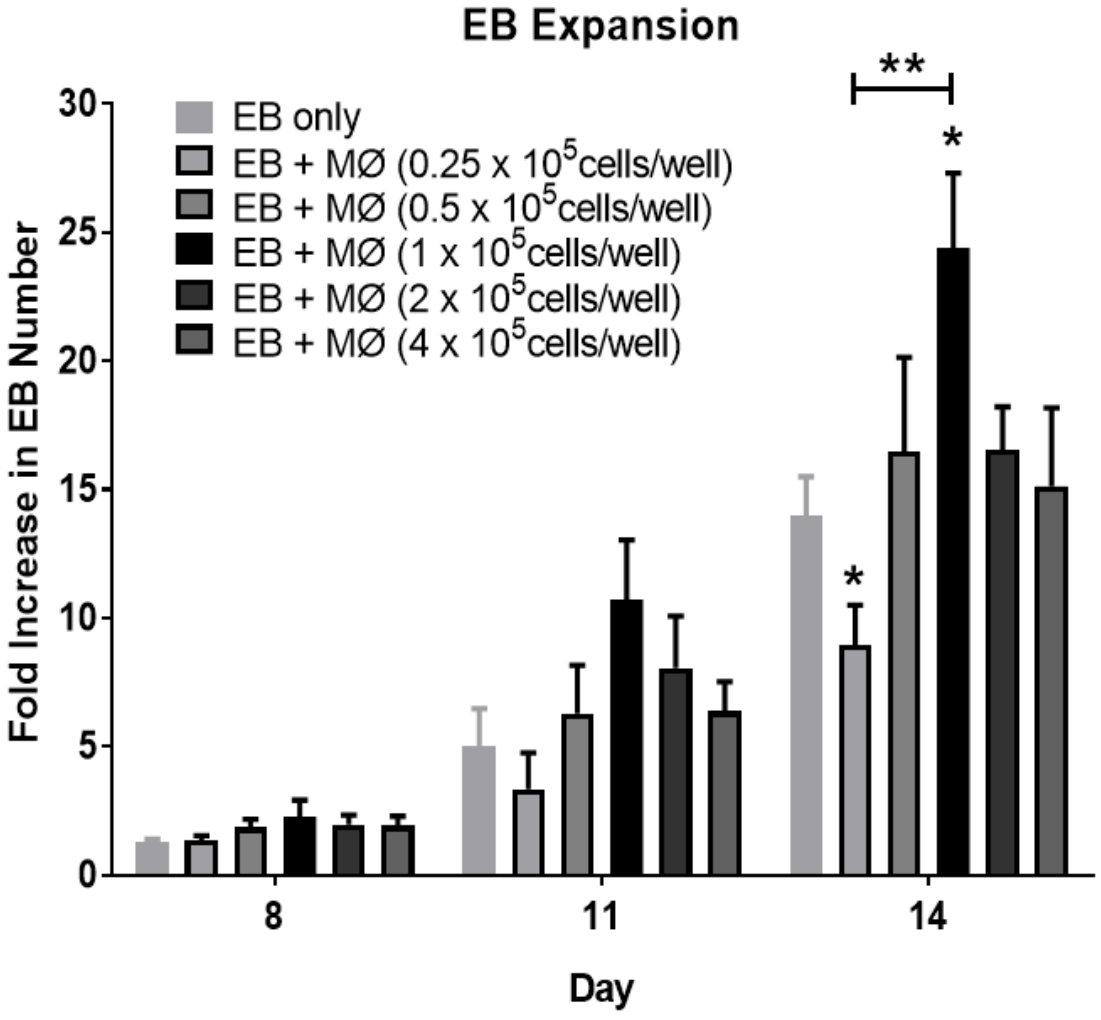
Over 98.7% of macrophages had CD163 surface expression at all the plating densities tested, indicating that although the levels of this receptor varied depending on the macrophage concentration, they were nonetheless always positive in expression for this glycoprotein (**Table 3.2**). However, 1 x $10^5$ cells/well produced the optimal macrophage phenotype because cells at this density were all $CD163^+$ and a substantial proportion (78.0 ±SEM 8.6%) were also positive for both CD169 and VCAM1. Thus, macrophages seeded at 1 x $10^5$ cells/well most closely resembled bone marrow macrophages that are known to be $CD163^+CD169^+VCAM1^+$ (Chow et al., 2013; Ramos et al., 2013).

Plating macrophages at 1 x $10^5$ cells/well also led to the greatest viability ($AnnV^-$ $7AAD^-$) and lowest levels of $AnnV^+$ cells, with this density resulting in 26% fewer total apoptotic cells than macrophages seeded at 0.25 x $10^5$ cells/well (**Table 3.3**). Therefore, the data from **Figure 3.2**, **Table 3.2** and **Table 3.3** together illustrate that the phenotype and viability of primary peripheral blood monocyte-derived macrophages varied when plated at different densities during differentiation but that 1 x $10^5$ cells/well induced a phenotype that was representative of macrophages found in the human bone marrow.

### 3.3.2 Macrophage Seeding Density effects on Erythroid Development

The effects of the different macrophage seeding densities on co-cultured erythroblasts were subsequently investigated to determine whether the altered macrophage phenotypes and viabilities affected erythropoiesis. Erythroid expansion, maturation and viability were measured.

**FIGURE 3.3. Erythroblast expansion on days 8, 11 and 14 following co-culture with increasing macrophage densities.** *Erythroblasts were added at 6 x $10^3$ cells/well to cultures containing macrophages at different densities (0.25, 0.5, 1, 2 and 4 x $10^5$ cells/well). Erythroblast numbers were determined via haemocytometer cell counts after co-culture with macrophages for 2 days (day 8), 5 days (day 11) and 8 days (day 14). Expansion is determined as the erythroid cell number compared to the initial number of cells added to co-cultures. Data represent the mean and SEM of 3 biological repeats. *P<0.05, **P<0.01 compared to EB only unless indicated otherwise (one-way ANOVA with Tukey's post-test correction at each time point).*



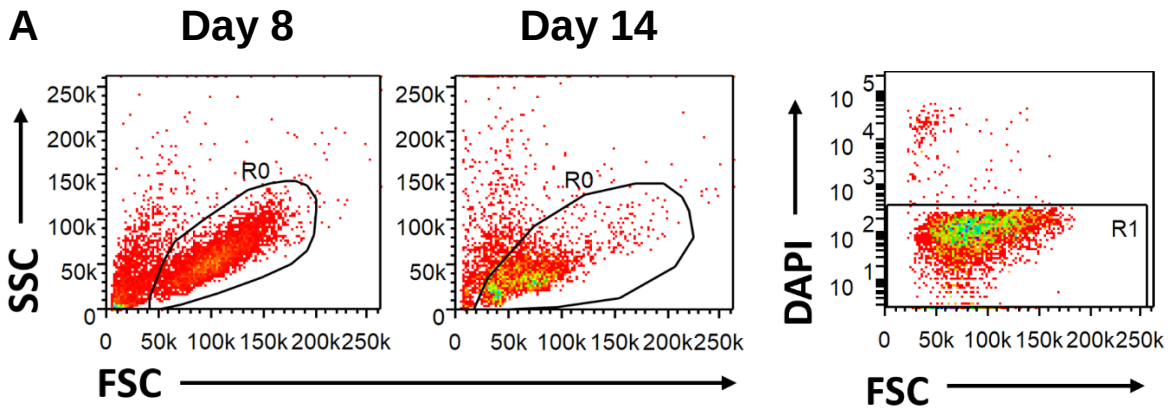
Erythroblasts co-cultured with macrophages at 1 x $10^5$ cells/well resulted in improved erythroblast expansion by day 14 (1.7- and 2.7-fold compared to erythroblasts only or co-cultures containing 0.25 x $10^5$ macrophages/well, respectively). However, these positive effects on erythroid expansion were not found at earlier time points on day 8 or day 11 of culture. In contrast, erythroblasts co-cultured with macrophages at 0.25 x $10^5$ cells/well had reduced erythroid expansion by day 14, suggesting that macrophages at this density had an inhibitory effect. This is most likely due to the lowest viability of macrophages at this seeding density (**Table 3.3**), that could have led to an accumulation of inhibitory factors secreted by the apoptotic and dead/dying macrophages that in turn blocked erythroblast proliferation and/or survival. The growth of erythroblasts with macrophages at all other densities (0.5, 2 or 4 x $10^5$ cells/well) had no effect on erythroblast expansion on days 8, 11 or 14.

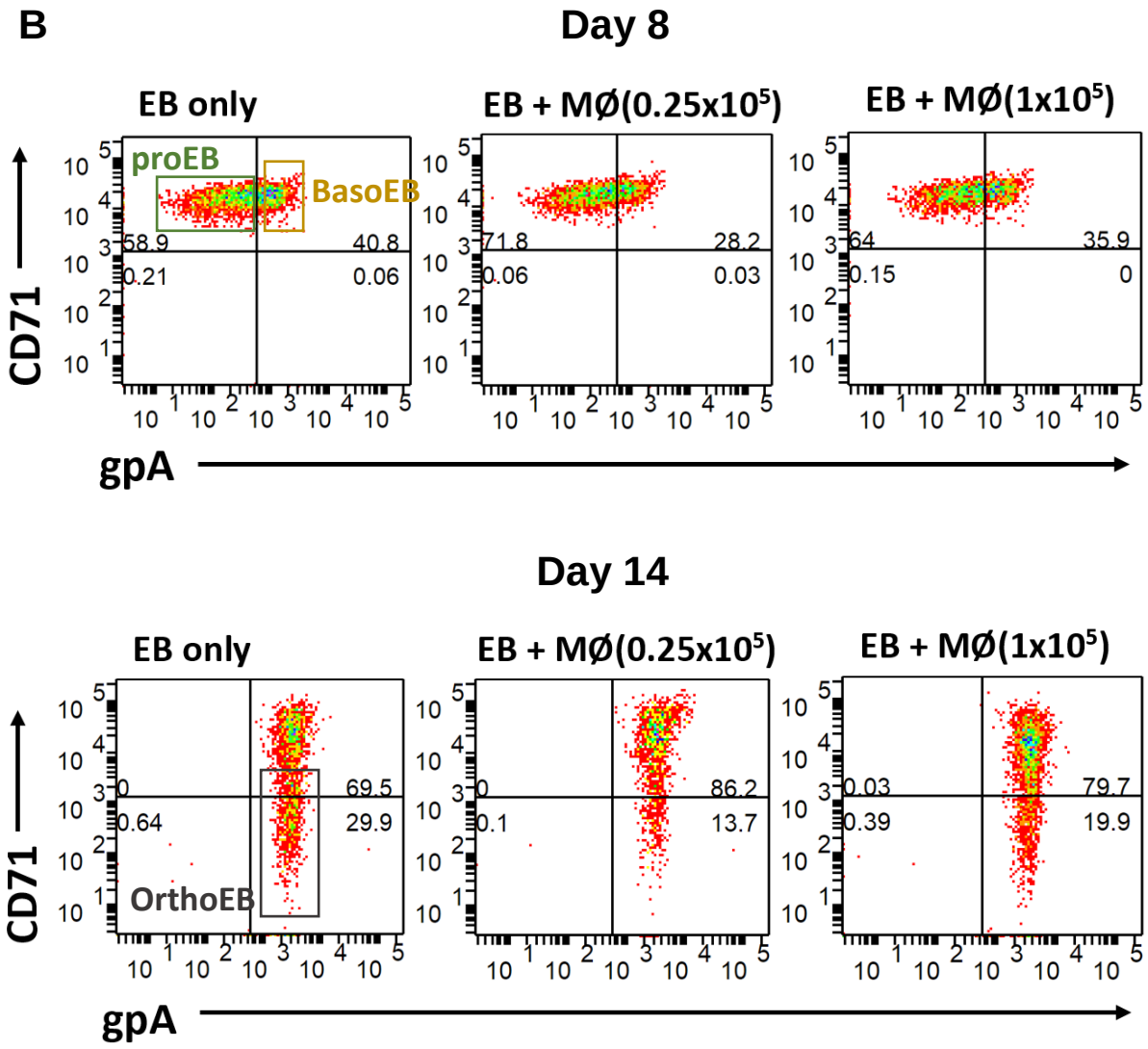
These results indicate that the various macrophage seeding densities, which alter both the macrophage phenotype in terms of cell surface marker expression (**Figure 3.2 and Table 3.2**) as well as their viability (**Table 3.3**), can have different effects on erythroblast expansion during co-culture. However, the similar pattern between macrophage viability and erythroblast expansion suggests that the survival of macrophages was also crucial to support erythroid growth. More precisely, macrophages improved erythroblast expansion only when seeded at 1 x $10^5$ cells/well possibly because this density produced the greatest proportion of viable macrophages (91.9 ±SEM 7.1%) with the appropriate phenotype, whilst the lowest plating density (0.25 x $10^5$ cells/well) diminished erythroid expansion probably due

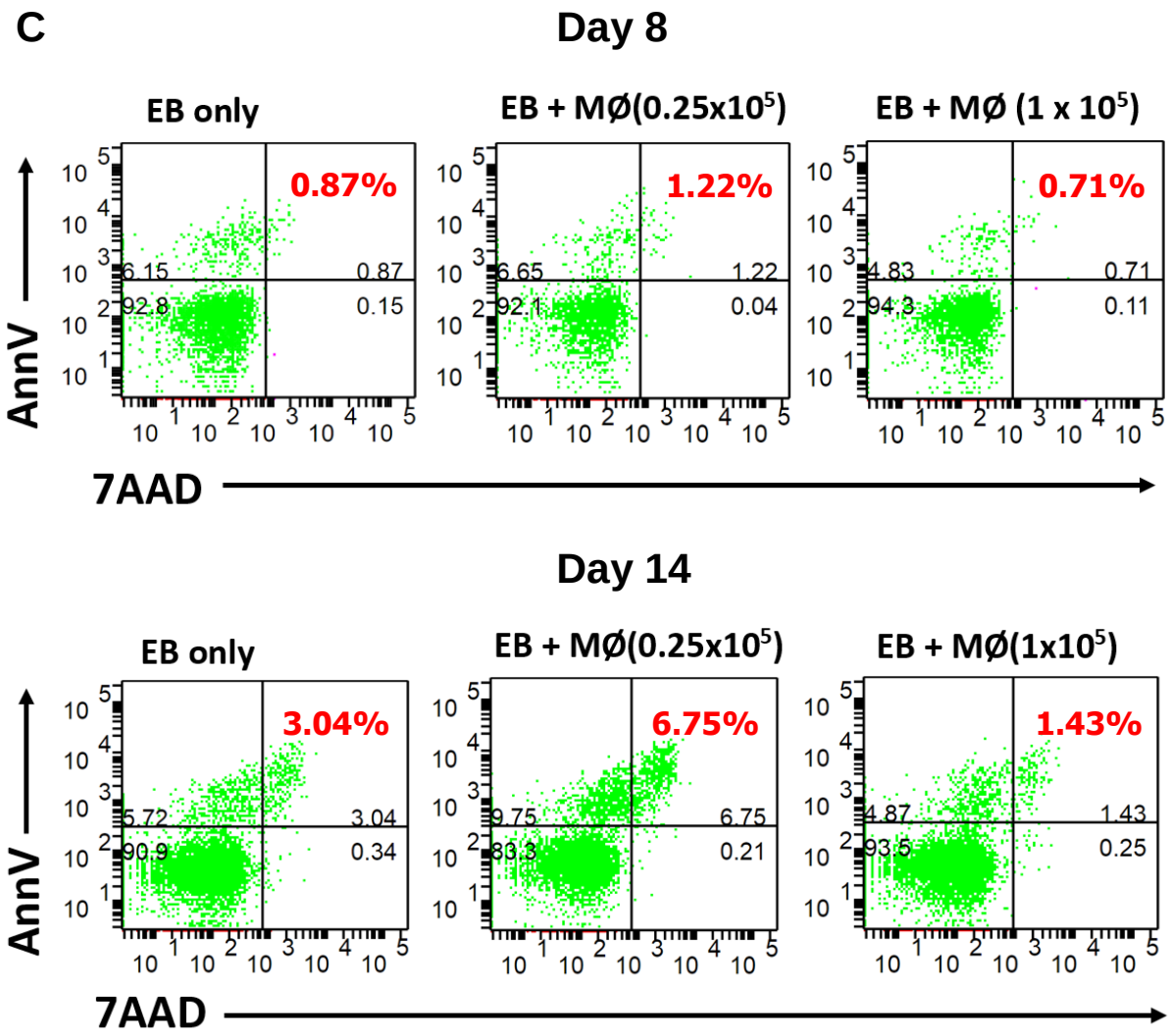
to the reduced macrophage viability (65.2 ±SEM 8.8%) and increased apoptotic death that would have released inhibitory molecules into the culture medium.

In addition to expansion, erythroblast maturation and survival were also affected during erythroid development and therefore these were investigated next.

**FIGURE 3.4. Erythroblast maturation and viability on days 8 and 14 following co-culture with different macrophage densities.** *Erythroblasts were gated based on size (FSC) and granularity (SSC) (R0 gate) and viable cells ($DAPI^-$) (R1 gate) were also selected when measuring maturation (A). Erythroblast maturation for cells cultured alone or with macrophages at 0.25 or 1 x $10^5$ cells/well was determined by dual surface staining of CD71 and gpA of the R0 and R1 gates on days 8 and 14 (B). Pro- ($CD71^+gpA^-$), basophilic ($CD71^+gpA^+$) and orthochromatic ($CD71^{low/-}gpA^+$) erythroblasts are indicated by green, yellow and dark grey boxes, respectively. Erythroid viability was measured by staining for both 7AAD and AnnV of the R0 gate on days 8 and 14 (C). In 'B' and 'C' the percentages of cells in each quadrant are shown and the proportion of late apoptotic/dead ($AnnV^+7AAD^+$) erythroblasts are shown in red figures in 'C'. In all cases, representative FACS plots from 1 donor for erythroblasts cultured either alone or with macrophages at 0.25 or 1 x $10^5$ cells/well are shown.*



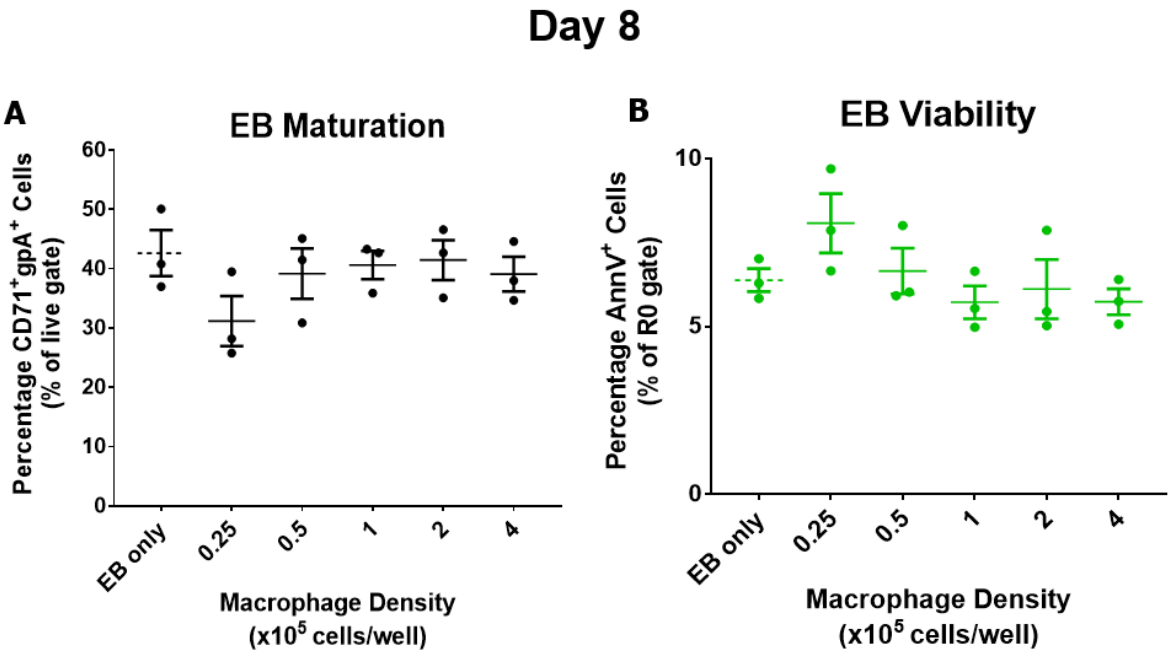

B
Day 8
EB only
EB + MØ(0.25x10⁵)
EB + MØ(1x10⁵)
proEB
BasoEB
58.9
40.8
0.21
0.06
71.8
28.2
0.06
0.03
64
35.9
0.15
0
CD71
gpA
Day 14
EB only
EB + MØ(0.25x10⁵)
EB + MØ(1x10⁵)
0
69.5
0.64
29.9
OrthoEB
0
86.2
0.1
13.7
0.03
79.7
0.39
19.9
CD71
gpA




The FACS plots show that the majority of cells on day 8 consisted of proerythroblasts and basophilic erythroblasts, while those on day 14 involved mainly basophilic and orthochromatic erythroblasts (**Figure 3.4B**). The viability of erythroblasts was high under all culture conditions on day 8, at approximately 93 ±SEM 6.6%, but was substantially lower on day 14 for erythroblasts grown with 0.25 x $10^5$ macrophages/well, at 83.3 ±SEM 6.4% (**Figure 3.4C**).

The following graphs illustrate erythroid maturation and viability from three separate donors for erythroblasts co-cultured with macrophages at the various densities.

**FIGURE 3.5. Erythroblast maturation and viability on day 8 following co-culture with increasing macrophage densities.** *Erythroblasts were added at 6 x $10^3$ cells/well to cultures containing macrophages at the different densities shown. The proportion of basophilic erythroblasts ($CD71^+gpA^+$) based on the R0 gate and DAPI negativity (A) and the percentage of $AnnV^+$ ($7AAD^-$ and $7AAD^+$) erythroblasts in the R0 gate (B) on day 8 were determined by direct staining and detection on a flow cytometer. Data are the mean and SEM from 3 donors. There are no significant differences between different conditions.*



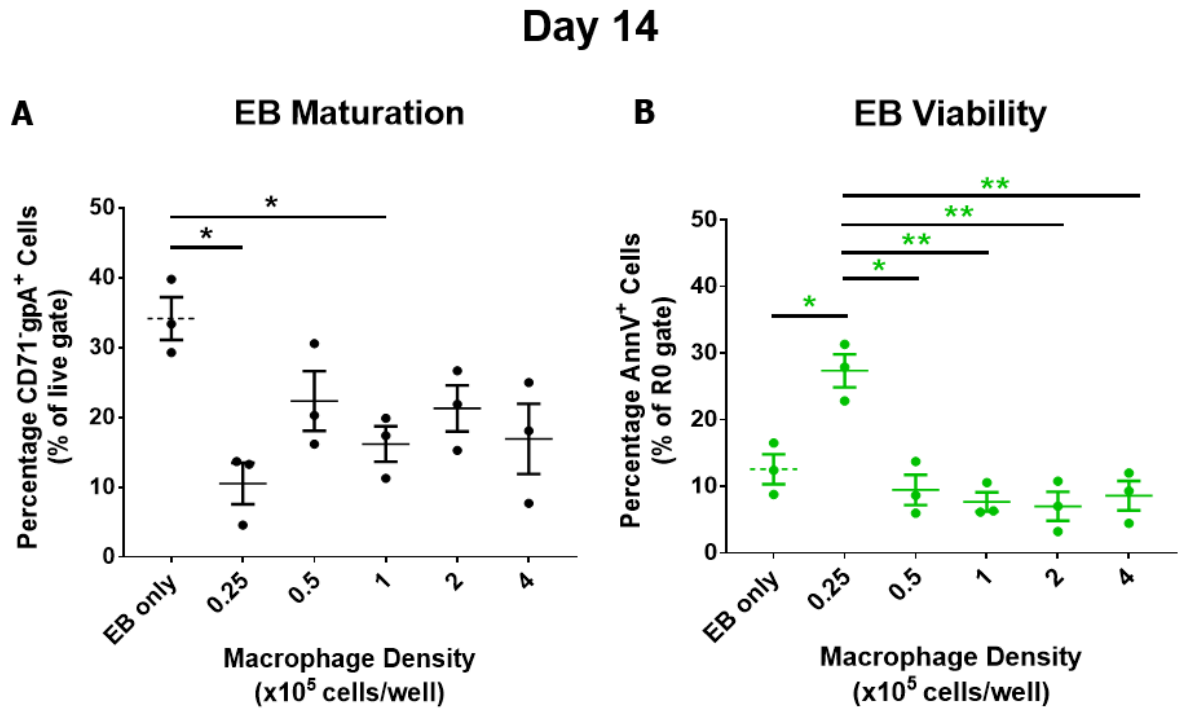

These data illustrate that on day 8 there were no differences in maturation when erythroblasts were cultured alone or with macrophages at any density, with approximately 40 ±SEM 4.7% $CD71^+gpA^+$ erythroid cells (**Figure 3.5A**). Erythroblasts grown with macrophages seeded at 0.25 x $10^5$ cells/well had 9.5% and 11.5% fewer cells at the same erythroid stage ($CD71^+gpA^+$) compared to

erythroblasts cultured with 1 x $10^5$ macrophages/well or to erythroid cells grown alone, respectively, but these differences were not statistically significant.

Erythroblast viabilities were also unchanged by the culture of erythroblasts alone or with macrophages at the various concentrations, with approximately 6.0 ±SEM 1.7% $AnnV^+$ cells on day 8 (**Figure 3.5B**). Percentages for the total $AnnV^+$ erythroblasts, which includes both the early apoptotic ($AnnV^+7AAD^-$) and late apoptotic/dead ($AnnV^+7AAD^+$) cells, are shown because this more clearly indicates any changes in erythroid viabilities under the various culture conditions. Although not significant, there was a slight increase in the proportion of $AnnV^+$ cells co-cultured with 0.25 x $10^5$ macrophages/well (1.4- and 1.2-fold compared to erythroid cells cultured with 1 x $10^5$ macrophages/well or without macrophages, respectively).

Thus, together these data indicate that macrophages, regardless of their seeding density, did not alter erythroid development in terms of expansion, maturation or viability on day 8 of culture. However, erythroblasts grown with macrophages at 0.25 x $10^5$ cells/well showed a trend towards reduced maturation and viability.

**FIGURE 3.6. Erythroblast maturation and viability on day 14 following co-culture with increasing macrophage densities.** *Erythroblasts were added at 6 x $10^3$ cells/well to cultures containing macrophages at the different densities shown. The proportion of orthochromatic erythroblasts ($CD71^-gpA^+$) based on the R0 gate and DAPI negativity (A) and the percentage of $AnnV^+$ ($7AAD^-$ and $7AAD^+$) erythroblasts in the R0 gate (B) on day 14 were determined by direct staining and detection on a flow cytometer. Data are the mean and SEM from 3 donors. *P<0.05, **P<0.01 compared to EB only unless indicated otherwise (one-way ANOVA with Tukey's post-test correction).*




On day 14, erythroblasts had decreased maturation when cultured in the presence of macrophages than when grown alone, especially with 0.25 x $10^5$ macrophages/well or 1 x $10^5$ macrophages/well that reduced the percentage of $CD71^-gpA^+$ erythroblasts by 22.5% and 16.8%, respectively, compared to erythroblasts only

(**Figure 3.6A**). The culture of erythroblasts with macrophages at 0.25 x $10^5$ cells/well also reduced erythroid viability, increasing the proportion of $AnnV^+$ erythroid cells by 2.0- and 3.3-fold compared to erythroblasts only or to those grown with 1 x $10^5$ macrophages/well (**Figure 3.6B**).

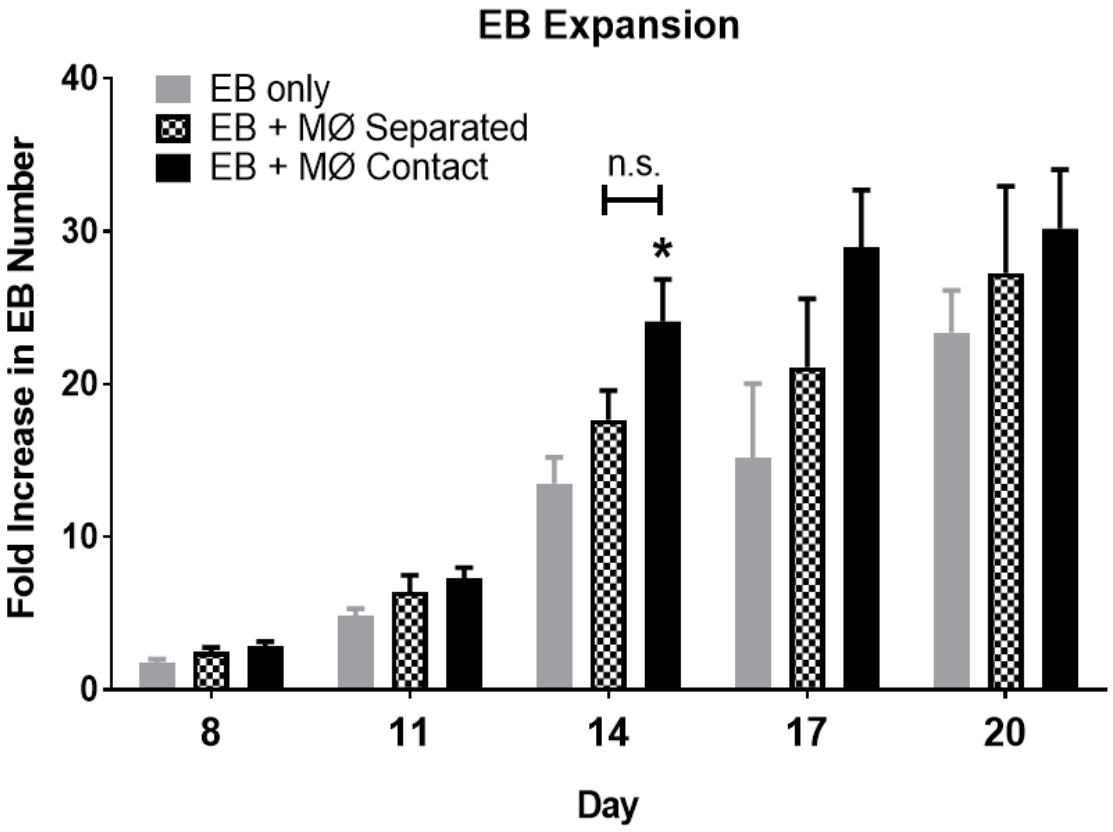
Therefore, erythroblasts cultured with 0.25 x $10^5$ macrophages/well showed diminished expansion, maturation and viability by day 14, which indicates that macrophages at this density had an inhibitory effect on erythropoiesis. This is in contrast to erythroblasts co-cultured with 1 x $10^5$ macrophages/well, which displayed improved erythroid expansion albeit with inhibited maturation by day 14. Given that there was a trend for reduced erythroblast maturation on day 14 at all macrophage concentrations, it suggests macrophages have an inherent property for blocking erythroid differentiation as opposed to this being caused by any negative effects of the co-culture conditions. Consequently, the macrophage seeding density of 1 x $10^5$ cells/well was the optimal concentration to support erythroblast expansion and was chosen as the plating density in all future investigations.

### 3.3.3 The effects of Macrophages in Contact versus Separation on Erythroid Development

Having already determined the optimal macrophage seeding density, it was of interest to subsequently investigate whether co-culturing erythroblasts in direct contact or in physical separation had different effects on their development. This would also provide some insight into whether adhesive factors or soluble molecules derived from macrophages are more important in erythropoiesis. Again erythroid expansion and maturation were measured, but by maintaining cultures until day 20

the longer term effects of macrophages on terminal erythropoiesis, including enucleation rates, could also be examined. Measuring erythroid enucleation is key when studying terminal erythroid differentiation as it is one of the final stages towards the production of mature erythrocytes.

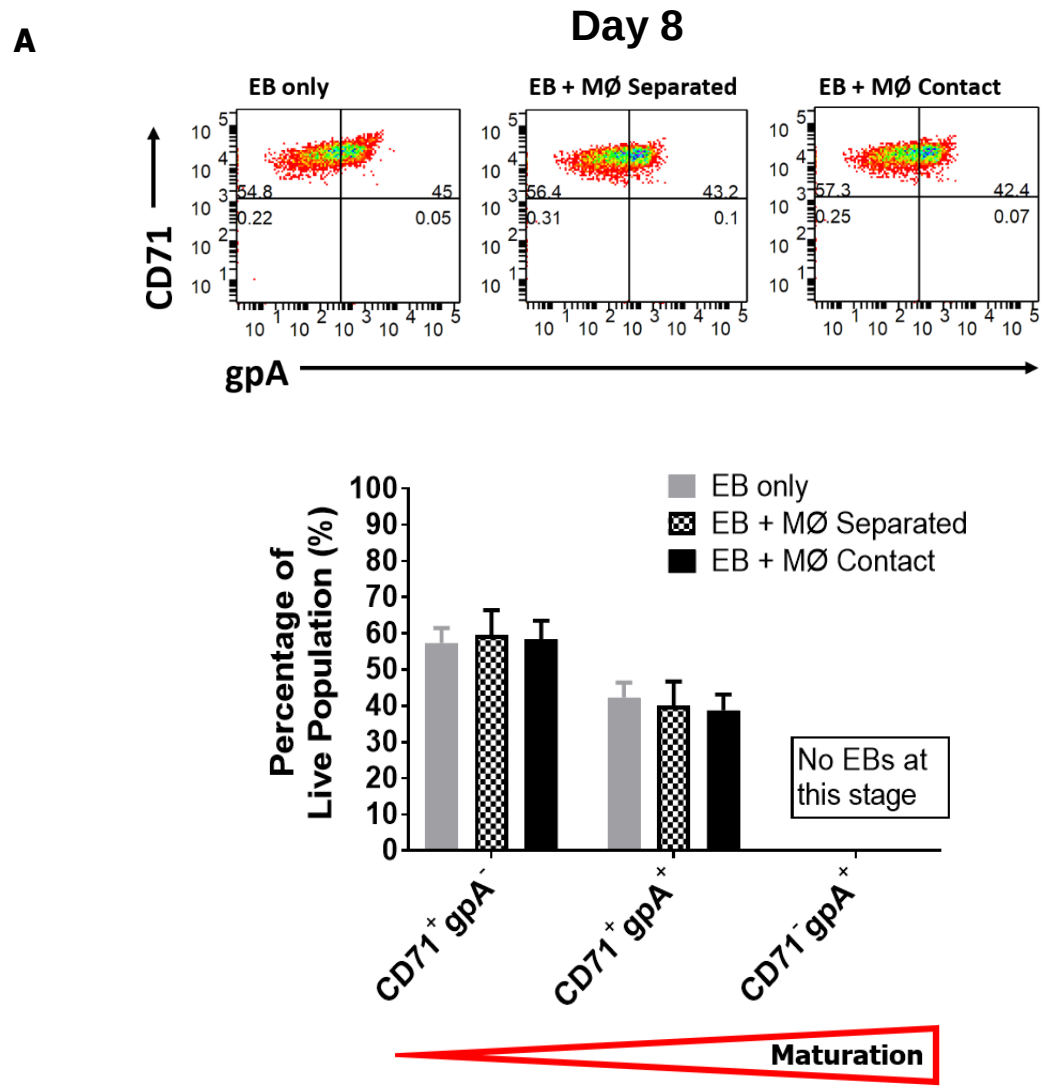
**FIGURE 3.7. Erythroid expansion following co-culture with macrophages in contact or in separation.** *Erythroblasts were added to macrophages either in direct contact (black bars) or in separation (chequered bars), or grown without any macrophages (grey bars). In co-cultures involving the separation of erythroid cells from macrophages, erythroblasts were added into the upper compartment of a transwell insert. Erythroblast numbers were determined via haemocytometer cell counts (with trypan blue exclusion of dead cells) after culture with macrophages for 2 days (day 8), 5 days (day 11), 8 days (day 14), 11 days (day 17) or 14 days (day 20). Data are the mean and SEM of 6 biological repeats. *P<0.05 compared to EB only, unless otherwise indicated (one-way ANOVA with Tukey's post-test correction at each time point; paired t-test to compare separated and contact). n.s. stands for not statistically significant.*

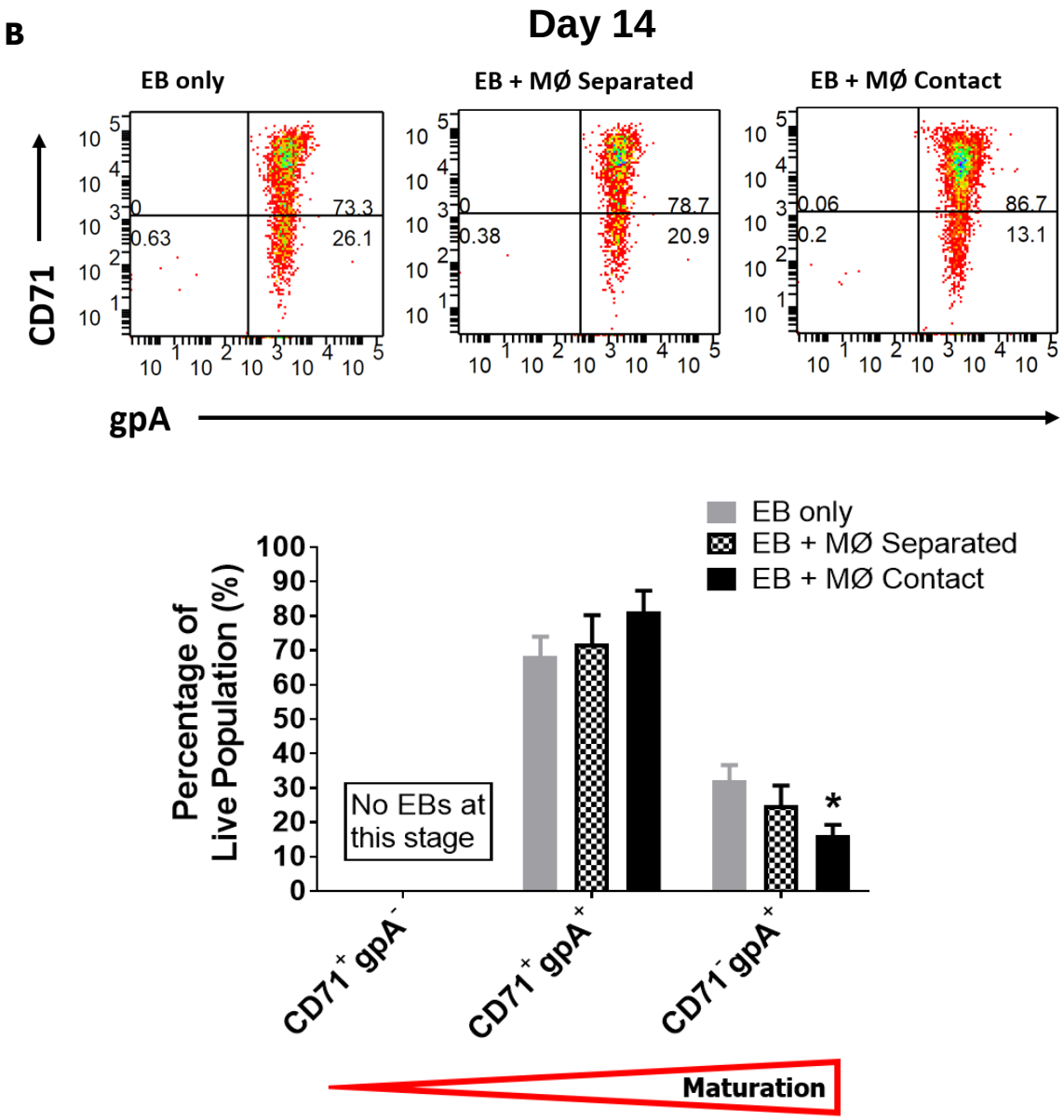


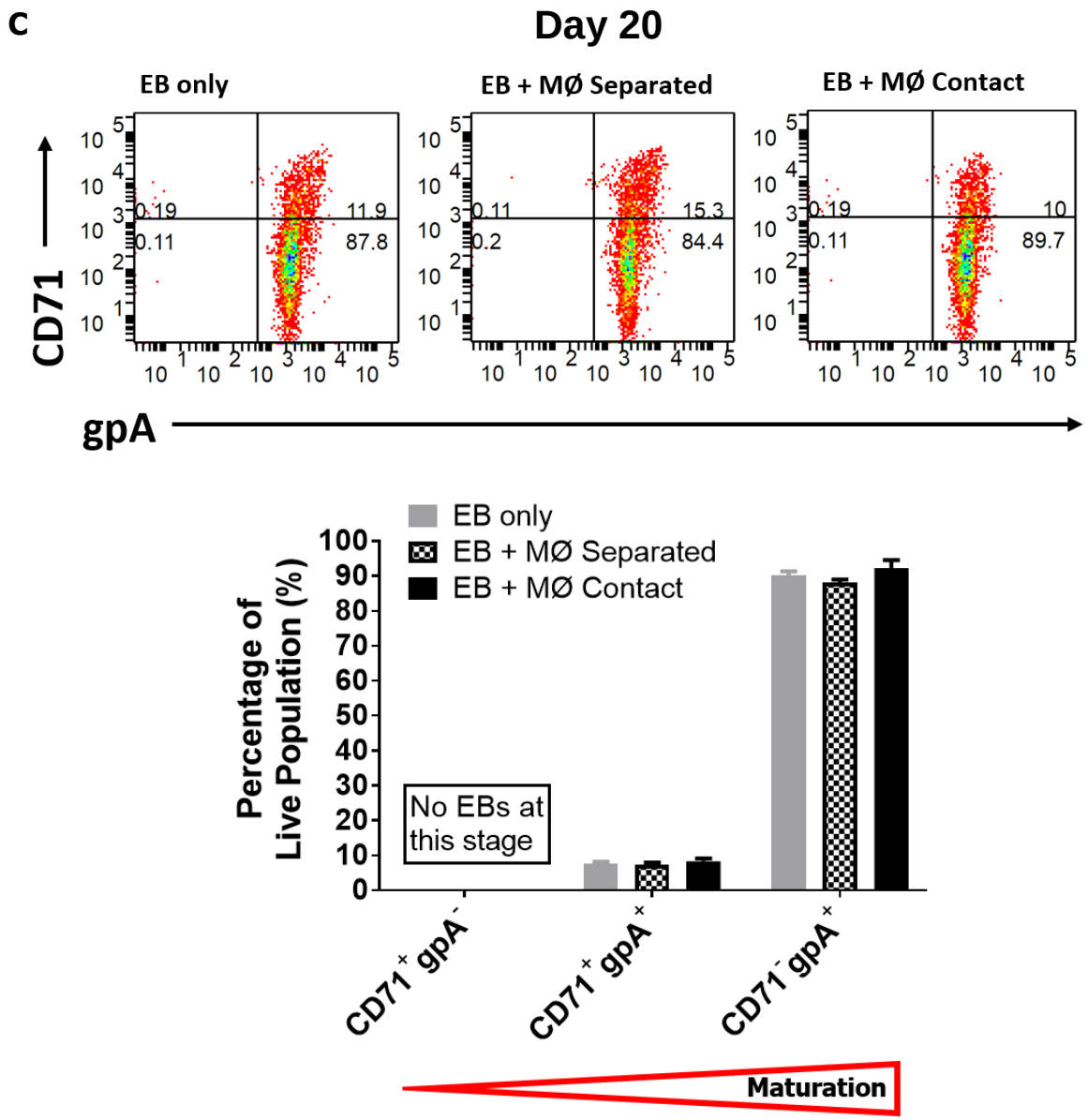
In line with previous observations, it was found that on day 14 the growth of erythroid cells with macrophages in direct contact augmented expansion when compared to erythroblasts only, but the culture of the two cell types in contact was not significantly better compared to their co-culture in separation (**Figure 3.7**). While erythroblasts in contact with macrophages had 1.8-fold improved expansion on day 14 compared to erythroid cells alone, the effects of culture in direct contact appeared to plateau after day 14. Thus, these results suggest that cell-cell contacts between erythroblasts and macrophages together with secreted molecules played important roles during erythroid expansion. However, given that the co-culture of erythroblasts with macrophages in separation did not lead to a significant increase in erythroid expansion at any time point, it implies that secreted factors on their own did not have a significant effect on erythroblast expansion. Instead, as suggested by the greatest improvement with co-cultures in direct contact than erythroid cells grown alone, it seems that macrophage-derived adhesive molecules in combination with soluble factors were crucial for heightened erythroid cell expansion on day 14.

The observed benefits of culturing erythroid cells with macrophages in contact on day 14 led to the hypothesis that the co-culture of erythroblasts with macrophages in contact would also differentially affect erythroid maturation.

**FIGURE 3.8. Erythroid maturation on days 8, 14 and 20 after co-culture with macrophages in contact versus in separation.** *Erythroblasts were added to macrophages either in contact (black bars), in separation (chequered bars), or alone without macrophages (grey bars). The percentage of live (DAPI negative) erythroid cells at the distinct differentiation stages ($CD71^+gpA^-$, $CD71^+gpA^+$ or $CD71^-gpA^+$) were determined on days 8 (A), 14 (B) and 20 (C). This was determined by surface staining and subsequent detection on a flow cytometer. Representative FACS plots are shown at each time point. Data are the mean and SEM of 4 different donors. *P<0.05 compared to EB only (one-way ANOVA with Tukey's post-test correction for each erythroid stage).*




B
Day 14
EB only
EB + MØ Separated
EB + MØ Contact
CD71
gpA
0
0.63
73.3
26.1
0
0.38
78.7
20.9
0.06
0.2
86.7
13.1
EB only
EB + MØ Separated
EB + MØ Contact
Percentage of Live Population (%)
No EBs at this stage
CD71+ gpA-
CD71+ gpA+
CD71- gpA+
*
Maturation

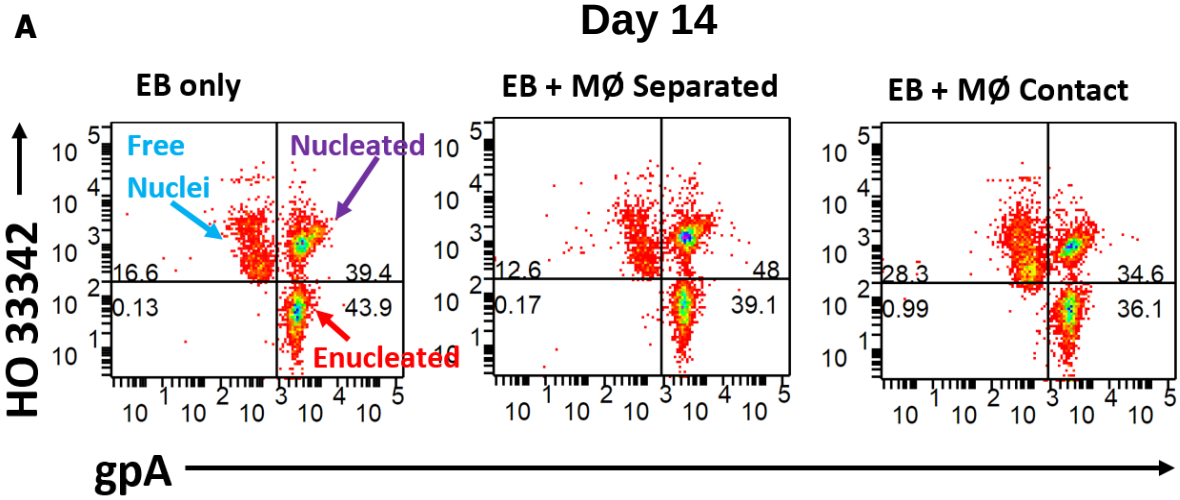



These data illustrate that there were no significant gdifferences in erythroid maturation on days 8 or 20 when erythroblasts were cultured either in direct contact or in physical separation with macrophages, with approximately 40 ±SEM 4.5% $CD71^{+}gpA^{+}$ cells on day 8 and 90 ±SEM 3.6% $CD71^{-}gpA^{+}$ erythroblasts by day 20 (**Figure 3.8A** and **C**). Although on day 14 erythroblasts cultured with macrophages in contact had 50% fewer cells at the $CD71^{-}gpA^{+}$ stage than erythroid cells grown

alone, this was not significantly different to erythroblasts co-cultured in separation (**Figure 3.8B**). However, erythroblasts grown with macrophages in separation consistently induced intermediate effects on erythroid maturation and although this was not statistically significant, it could suggest that secreted factors also played a part in the blocked erythroid maturation, even if their effects were small. Nevertheless, the effects appeared to be lost by day 20, where the proportions of erythroblasts at the distinct maturational stages were comparable under all culture conditions.

Finally, the effects on enucleation of co-culturing erythroid cells with macrophages in contact or separation were compared.

**FIGURE 3.9. Erythroid enucleation on days 14 and 20 following co-culture with macrophages in contact or in separation.** *Erythroblasts were added to macrophages either in direct contact, separation, or grown alone without macrophages. The percentage of enucleated cells was determined by dual staining for gpA and Hoechst 33342 (HO), with the gpA$^+$HO$^-$ population representing enucleated cells. 'A' shows representative FACS plots on day 14 from one donor. The three populations that represent enucleated cells (red arrow), nucleated cells (purple arrow) and free nuclei (blue arrow) are indicated. 'B' shows the mean proportion of enucleated erythroid cells on days 14 and 20 following culture alone (grey bars) or with macrophages either in contact (black bars) or in separation (chequered bars). Data shown are the mean and SEM of 4 biological repeats. *P<0.05 compared to EB only (one-way ANOVA with Tukey's post-test correction at each time point). n.s. means not statistically significant.*



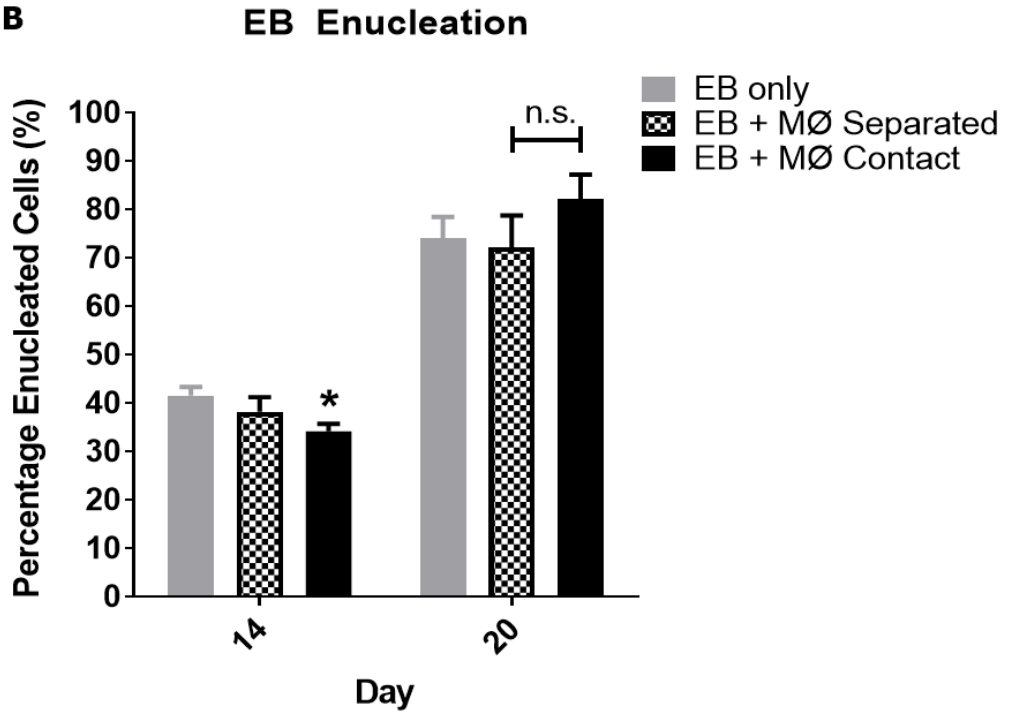
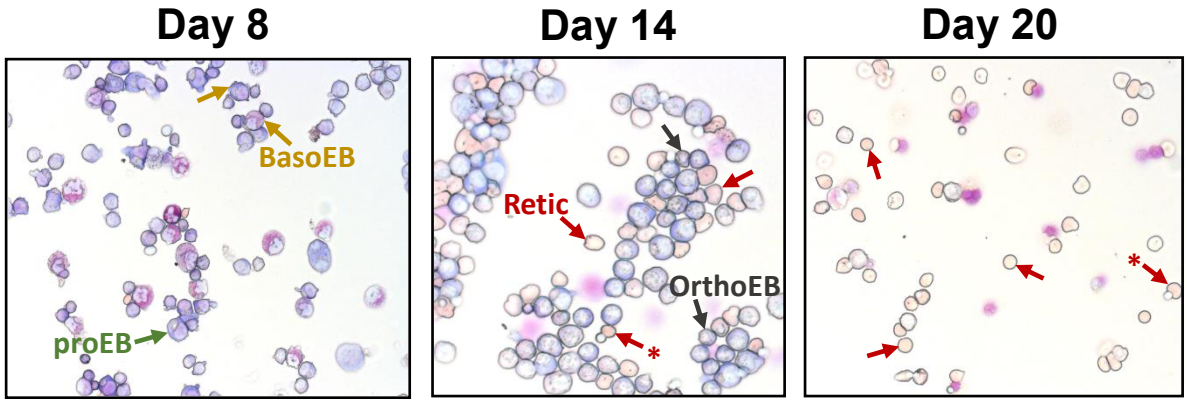



**Figure 3.9** shows that on day 14 erythroid cells cultured on their own had the greatest enucleation rates at approximately 41 ±SEM 1.4% enucleated cells (1.2-fold compared to erythroid cells grown with macrophages in contact). This followed a similar trend to erythroid maturation at the same time point (**Figure 3.8B**). However, by day 20 there were no differences in the percentages of enucleated cells under any of the culture conditions. Since erythroid maturation on day 20 was also unchanged by the various culture conditions (**Figure 3.8C**), it suggests that any inhibitory effects caused by macrophages on terminal erythroid differentiation when in direct contact were lost by day 20.

**FIGURE 3.10. Cytospins of erythroblasts on days 8, 14 and 20 following co-culture with macrophages in contact.** *10,000 erythroblasts after culture in direct contact with macrophages for 2 days (day 8), 8 days (day 14) and 14 days (day 20) were collected, cytospun and stained using Leishman's stain. Proerythroblasts (green arrow), basophilic erythroblasts (yellow arrows), orthochromatic erythroblasts (grey arrow) and reticulocytes (red arrows) are shown. The red arrows with an asterisk (*) indicate reticulocytes undergoing the process of reticulocyte maturation, which is evident by the white vesicle protruding from the pink cell.*




The cytospins show how the morphology of erythroblasts altered on days 8, 14 and 20 during erythroid differentiation following their culture with macrophages in contact. Proerythroblasts (green arrow) became smaller with more condensed chromatin as they differentiated into basophilic erythroblasts (yellow arrows) and ultimately orthochromatic erythroblasts (grey arrow), which eventually extruded their nucleus to form the enucleated reticulocytes (red arrows). These reticulocytes could then mature by eliminating remaining organelles such as the endoplasmic reticulum (red arrows with asterisk) to form erythrocytes. In humans, most of reticulocyte maturation in the first 24 hours occurs in the spleen.

## 3.4 Discussion

An *in vitro* culture system for the co-culture of human erythroblasts with autologous macrophages was optimised to study the role of macrophages in erythropoiesis. It involved establishing a macrophage seeding density that would enable these cells to mature into an M2 phenotype when grown in MCSF media for 10 days. By using the optimal macrophage density (1 x $10^5$ cells/well) established in these initial studies in all subsequent work, it would avoid any potential significant variabilities in the macrophage phenotype and viability. This ensured consistency between all experiments. Furthermore, it means that any differences in erythroid development observed following co-culture with macrophages could be attributed to some intrinsic properties of the macrophages themselves.

Chow *et al*. identified a population of $CD15^-CD163^+VCAM1^+CD169^+$ macrophages from bone marrow aspirates of healthy humans that share a phenotype similar to the murine $VCAM1^+CD169^+$ macrophages shown to play a pivotal role in promoting erythropoiesis (Chow et al., 2013). Therefore, to resemble these human resident bone marrow macrophages as closely as possible, it was important to polarise human peripheral blood monocyte-derived macrophages towards an M2 phenotype *in vitro* using RPMI-1640 + MCSF media (RPMI-1640, 10% FBS, 25ng/ml MCSF). The ideal seeding density was found to be 1 x $10^5$ cells/well, which yielded $CD163^{hi}CD14^{hi}CD169^{hi}CD16^{low}CD86^{low}CD83^{low}$ macrophages with the lowest levels of apoptosis (8.0 ±SEM 1.5% $AnnV^+7AAD^-$ and $AnnV^+7AAD^+$ cells), and hence this seeding density was used in all subsequent studies.

However, one limitation is that there were inevitably going to be some differences in macrophage numbers between donors even when seeding at the same density. This is because cells derived from one person might inherently be more adherent to plastic than cells from another person, or they might be more viable, which would ultimately result in more macrophages remaining on the well after several days. This could be important because as shown in **Figure 3.2** and **Table 3.2**, surface marker expression varies depending on the seeding concentration of macrophages during differentiation. **Table 3.3** also shows how plating cells at different densities can adversely affect their viabilities, with the lowest macrophage concentration of 0.25 x $10^5$ cells/well having reduced the percentage of $AnnV^-7AAD^-$ cells by 26.7%. One would then expect macrophages with poor viabilities to have detrimental effects on erythropoiesis during their co-culture with erythroid cells.

The effects observed of altered cell surface marker expression during different cell densities is illustrated by Lee and Hu who demonstrated how two separate populations of murine bone marrow-derived macrophages (BMDMs) were produced when cells were plated at either 4 x $10^5$ cells or 5 x $10^6$ cells per 10cm and grown for 7 days in 20% L-cell conditioned media (Lee and Hu, 2013). Plating at the lower concentration gave rise to more mature $Ly6C^-Ly6G^-CD11b^+F4/80^+c\text{-}Fms^+$ macrophages, while the higher plating density yielded $Ly6C^-Ly6G^+CD11b^+F4/80^+CD11c^+$ cells. The authors concluded that the population generated following the higher plating concentration might have been more immature (also suggested by their smaller and rounder morphology) and these cells had lower pro-inflammatory cytokine secretions, were less phagocytic and had a greater expression of M2 polarisation state related genes following IL4 treatment.

Since different macrophage densities are associated with altered viabilities and surface marker profiles, it was imperative to ensure consistency in the plating densities between various donors during my investigations to prevent any drastic changes in the macrophage phenotype that could subsequently affect the outcome of an experiment. Therefore, to check the final concentration of macrophages, spare wells containing cells at the desired macrophage density were gently removed 2 hours after their initial seeding and counted using a haemocytometer with trypan blue exclusion of the dead cells. When macrophages were not at their desired density, the corresponding wells were discarded.

The effects on erythroblast development of modified macrophage phenotypes and viabilities, caused by varied plating densities, after several days of co-culture are demonstrated in **Figures 3.3 – 3.6**. While macrophages at the optimal density ($1 \times 10^5$ cells/well) did not affect erythroblast expansion, maturity or viability on day 8, they had a substantial effect on improving erythroid expansion but diminishing maturation by day 14. In contrast, macrophages plated at $0.25 \times 10^5$ cells/well reduced both expansion and maturity while at the same time increasing the apoptotic death of erythroblasts by day 14. Therefore, the differential effects on erythroid development of macrophages seeded at $0.25 \times 10^5$ cells/well versus $1 \times 10^5$ cells/well can be attributed mainly to the differences in macrophage viabilities as opposed to the changes in cell surface markers because the lowest density produced 27% fewer viable macrophages than those seeded at the optimal concentration. Apoptotic macrophages could also have secreted inhibitory molecules into the culture medium that adversely affected erythropoiesis in terms of reduced erythroid expansion, maturation and viability, which could be tested through further

experiments. These negative effects became most evident during the longer term culture (day 14) when the proportion of apoptotic and dead macrophages increased, which may have resulted in the accumulation of inhibitory molecules. Furthermore, although macrophages at 0.25 x $10^5$ cells/well strongly prevented erythroid cells from differentiating, producing only 10% $CD71^-gpA^+$ erythroblasts on day 14, macrophages at 1 x $10^5$ cells/well also inhibited erythroid maturation compared to erythroblasts cultured alone, albeit to a lesser extent (**Figure 3.6A**). This suggests that macrophages have an intrinsic ability to block the maturation of erythroblasts even when simultaneously supporting their expansion. The finding of reduced erythroid enucleation on day 14 (**Figure 3.9**) adds weight to the argument that macrophages prevent erythroblast maturation and extends this inhibitory function of macrophages to the terminal differentiation of erythroid cells. It also suggests that erythroblasts that fail to differentiate normally will not enucleate. However, whether macrophages indirectly affect enucleation through the inhibition of erythroid differentiation or directly block the molecular process of enucleation itself would need to be determined through further work. A recent study by Liang and colleagues highlighted a crucial involvement of the FOXO3 transcription factor in erythroblast enucleation (Liang et al., 2015) and thus it would be worthwhile investigating whether macrophages might be affecting the signalling pathways implicated with FOXO3. It would also be valuable to determine through further experiments whether macrophages differentially affect enucleation rates beyond day 20 and if at any of these time points they are able to support, rather than block, the terminal differentiation of erythroid cells.

Although only the effects on macrophage surface markers were investigated when studying the macrophage phenotype, it is possible that there were also differences in secreted molecules, including cytokines and microRNAs. It would therefore be interesting to know whether the macrophages seeded at 1 x $10^5$ cells/well had further functional differences compared to macrophages at the other plating densities that were beneficial to erythropoiesis, such as lower pro-inflammatory cytokine secretions, less phagocytic capabilities, and altered transcription factor and enzyme expression. However, it would be sensible to study this at the densities where there were comparable macrophage viabilities (i.e. at 1 x $10^5$ and 2 x $10^5$ cells/well) to minimise any differences caused by the apoptotic macrophages. This might also shed some light into the main driving forces behind the distinct expansion and maturation observed in erythroblasts following co-culture with autologous macrophages. It could potentially elucidate whether, for instance, adhesive factors play more important roles in erythroblast expansion while secreted molecules mostly alter erythroid maturation, or vice versa. Studying the effects of macrophages in direct contact versus separation provided some clarification into their precise contributions to erythroid expansion and maturation.

Central macrophages and erythroblasts interact closely in erythroblastic islands and as such it was of interest to explore the effects that co-culturing in contact or in separation had on erythroblast development. As shown in **Figure 3.7**, the addition of macrophages in culture improves erythroid expansion by day 14, but it is when the two cell types are in direct physical contact that erythroblast numbers are significantly increased. This suggests that the combination of adhesive interactions between macrophages and erythroblasts and secreted factors are both crucial for

enhancing erythroid expansion on day 14. Furthermore, these adhesive molecules and soluble factors together may have additive or synergistic effects on the erythroid cell number. In contrast, secreted molecules alone were not sufficient to improve expansion at any time point, as demonstrated by the co-culture of erythroblasts with macrophages in separation.

Several adhesion molecules have already been shown to have pivotal roles in the formation of erythroblastic islands in the bone marrow and one of these is CD163, which is highly expressed on central macrophages (Fabriek et al., 2007). Fabriek and colleagues were able to discriminate between the contrasting functions of different scavenger receptor domains in CD163: while domain 3 is involved in binding haemoglobin-haptoglobin complexes, domain 2 is crucial for adherence of erythroblasts and augments erythroid expansion *in vitro* (Fabriek et al., 2007). Thus, the increased effects observed on erythroblast expansion during co-culture in physical contact may be explained, at least in part, by the high levels of surface CD163 on the macrophage. However, one cannot exclude the possibility of other surface molecules contributing to this effect, for instance Emp or even other as yet unidentified cell surface proteins. It would be valuable to distinguish the precise involvement that CD163 and other macrophage surface molecules have on erythroblast development and if when expressed in combination these proteins have additive or synergistic effects.

Differences were also observed in erythroblast maturation and enucleation following co-culture with human macrophages in contact or separation (**Figures 3.8** and **3.9**). There appeared to be an inverse association on day 14 between erythroblast expansion and maturation, where erythroid cells co-cultured with macrophages in

contact displayed heightened expansion but diminished maturation. However, this was only statistically significant when compared to erythroblasts cultured alone but not compared to co-cultures in separation. Nonetheless, given that erythroblasts grown with macrophages in separation consistently displayed an expansion and maturation that was intermediate to the culture in contact or alone without macrophages, it suggests that macrophage-derived secreted molecules on their own have a small effect on erythroid development. More detailed studies would be required to determine if the observed effects on expansion and maturation on day 14 are in any way related. In other words, do the signalling cascades for improved erythroid expansion consequently inhibit erythroid maturation, or are the two processes affected by pathways that are independent of one another? In the former case, this would imply that the main pathways involved in expansion and maturation in erythroblasts are mutually exclusive on day 14. Another possibility is that the adhesion of erythroblasts to macrophages might induce some changes to the macrophage phenotype that in turn could hinder erythroid differentiation. By day 20, however, the maturation and enucleation of erythroid cells cultured in contact was no different to that of cells grown in separation or to the culture of erythroblasts alone. Since the expansion of cells in contact appeared to have plateaued by day 20, it might suggest that these erythroblasts reached a certain density by this time point that caused them to stop expanding and instead switched to signalling for terminal erythroid differentiation. Therefore, erythroblasts at this time point may have become independent of the inhibitory effects of macrophages on maturation and enucleation, or another possibility is that the macrophages themselves might have altered their functions on day 20 to no longer block the terminal maturation of

erythroblasts. Future work involving studies beyond day 20 could help to clarify this and, if altered macrophage functions would appear to be the case, might even show a positive influence of macrophages on erythroid enucleation after day 20.

In conclusion, the work carried out in this chapter highlights how erythroblast development can be modified in various ways by macrophages. An altered macrophage viability and phenotype, caused by changing the plating density, was found to influence erythroid expansion, maturation and viability. Controlling for macrophage viability and phenotype, by only seeding at the optimal density of $1 \times 10^5$ macrophages/well, allowed for more informative investigations into the potential contributions of adhesive and secreted factors on erythropoiesis. It was determined that the presence of soluble molecules and cell-cell interactions between macrophages and erythroblasts together were crucial for the simultaneous increase in erythroid expansion and decrease in maturation.

These initial studies therefore set the framework for all future experimental work and demonstrate the importance of the macrophage phenotype and especially viability for subsequent functional studies in erythroblasts. They also provide insights into the role that human macrophages play in erythropoiesis and identify that cell-cell contacts between macrophages and erythroblasts and soluble factors secreted by macrophages affect erythroid development.

## 3.5 Summary of Findings

- Macrophage viability and cell surface marker expression varied with different seeding densities.

- 1 x $10^5$ macrophages/well was the optimal seeding concentration, because:

    - Resulted in the greatest macrophage viability (91.9 ±SEM 7.1% $AnnV^-7AAD^-$)

    - Induced the strongest M2-like polarisation ($CD163^{hi}CD14^{hi}CD16^{low}CD86^{low}CD83^{low}$)

    - Caused the greatest erythroblast expansion on day 14 after co-culture

- Erythroblasts in direct physical contact with macrophages had greater expansion on day 14 compared to erythroblasts grown on their own, but not compared to erythroblasts grown with macrophages in separation.
- Erythroblasts in contact with macrophages had reduced maturation (percentage of $CD71^-gpA^+$ erythroid cells) on day 14 compared to erythroblasts only, but this difference was lost by day 20.
- Erythroblasts in contact with macrophages had a significantly reduced percentage of enucleated cells on day 14 than erythroid cells cultured alone, but enucleation rates were similar under all culture conditions on day 20.

# Chapter 4: Modulation of macrophages that support elevated erythropoiesis

## 4.1 Introduction

Macrophages provide significant contributions to enhance erythropoiesis. During homeostasis, macrophages play an important part in erythropoiesis by recycling iron from damaged or aged erythrocytes to be used as a substrate for haemoglobin synthesis by developing erythroblasts (Ganz and Nemeth, 2012; Theurl et al., 2016). In contrast, during enhanced physiological or pathological erythropoiesis macrophages complement EPOR/Jak2/Stat signalling in erythroblasts to augment erythroid expansion and reticulocytosis (Chow et al., 2013; Ramos et al., 2013). This elevated erythroid output is beneficial for the recovery from acute anaemia induced, for instance, by acute blood loss but contributes to the pathology of diseases such as polycythaemia vera and β-thalassaemia. Much of what has been learnt about enhanced or aberrant erythropoiesis is owed to some key studies in mice (Chow et al., 2013; Ramos et al., 2013; Paulson et al., 2011).

Falchi and colleagues recently found that human $CD34^+$ cell cultures grown with $10^{-6}$M dexamethasone generated a small number (3%) of mature $CD169^+CD163^+CD16^+$ macrophages that interacted loosely with proerythroblasts and induced their cell cycle progression (Falchi et al., 2015). However, the work did not clarify whether this erythroid promoting capacity was mainly due to the effects of the glucocorticoid in modifying the macrophage phenotype that subsequently supported erythroblast proliferation or because dexamethasone induced its effects directly on erythroblasts themselves. Therefore, this chapter of the thesis aimed to

explain how dexamethasone directly alters the macrophage phenotype and the resultant effect of these dexamethasone treated macrophages on erythropoiesis. The experimental plan involved adding dexamethasone for four days to human macrophages prior to their co-culture with erythroid cells. The key difference between the studies of this thesis and the work by Falchi and colleagues is that here dexamethasone was targeted directly to macrophages but the glucocorticoid stimulation was absent during subsequent *in vitro* co-culture with erythroblasts.

Similarly, this chapter investigates how supplementing macrophages with IL10 modified their erythropoiesis-enhancing capacities. As with dexamethasone, it was hypothesised that IL10 would alter the macrophage phenotype to elevate erythroid expansion independently of any effects caused by the cytokine on erythroblasts themselves. Dexamethasone and IL10 are both known to induce an M2c-like polarisation of macrophages, which upregulate CD163 surface expression, and the macrophage CD163 receptor is known to adhere to erythroblasts and promote their proliferation (Fabriek et al., 2007; Zizzo and Cohen, 2015; Zizzo et al., 2012; Zizzo et al., 2013). Thus, it seemed logical to predict an enhancement in erythroid expansion when CD163 levels are increased. Moreover, dexamethasone and IL10 were chosen because both are associated with treatment of haematological disorders – dexamethasone is often used to treat Diamond-Blackfan anaemia (where the bone marrow fails to make enough red blood cells) while IL10 has been associated with a better outcome in severe malaria (Boeuf et al., 2012; Kugelberg, 2014; Niikura et al., 2011; Sjogren et al., 2015).

## 4.2 Specific Methods

For detailed procedures of commonly used techniques, see **chapter 2**. This section describes the methods that were specifically used in this chapter only. All tissue culture grade reagents were obtained from Sigma Aldrich unless stated otherwise.

### 4.2.1 Immunofluorescence microscopy of macrophages

Macrophages were grown on glass coverslips in a 6-well plate and on day 10 were washed twice with PBS, followed by fixation with 4% paraformaldehyde (Electron Microscopic Sciences) for 5mins at room temperature. To block non-specific protein-protein interactions and permeabilise the cells, macrophages were subsequently incubated with 1% BSA + 10% normal goat serum + 0.3M glycine in 0.1% PBS-Tween for 1 hour at room temperature. 5µg/ml of the CD163 antibody (ab87099, Abcam) was added to the cells and incubated at 4ºC overnight. Cells were washed twice gently with PBS and then stained with the secondary goat anti-rabbit IgG (H+L) Alexa Fluor 488 (1/2000 dilution) for 30mins at room temperature. The cells were washed twice again as before and a drop of VectorShield Hard Set mounting solution with DAPI (Vector Lab) was added to each coverslip to stain nuclei before inverting onto a glass slide. Coverslips were left to dry for 24 hours at room temperature in the dark before being imaged using a wide-field microscope (DeltaVision Elite, MerckMillipore).

### 4.2.2 BrdU cell cycle analysis of erythroblasts

The BrdU staining kit was used to determine the proportion of proliferating cells by flow cytometry. Approximately 1 x $10^5$ erythroblasts were taken from the different conditions 48 hours post co-culture and labelled with 10µM BrdU solution

(eBioscience) in culture medium for 1 hour. Cells were then washed twice with FACS buffer (PBS + 0.5% BSA). 1µl of Fixable Viability Dye eFluor 455UV (FVD, eBioscience) was added per 1ml of cells, vortexed immediately and incubated for 30 mins at 4ºC in the dark. This step was included in order to label any dead cells prior to fixation. The cells were washed twice in FACS buffer and gently resuspended by pulse-vortexing once. 1ml of 1X BrdU staining buffer (eBioscience) working solution was added to the cells, mixed and incubated for 15 mins at room temperature in the dark. The cells were then washed twice in FACS buffer before adding 100µl of DNase I (eBioscience) working solution and incubated for 1 hour at 4ºC in the dark. Cell were again washed twice in FACS buffer and stained with 5µl anti-BrdU FITC-conjugated antibody (eBioscience) for 30 mins at room temperature in the dark. Erythroblasts were then washed twice in FACS buffer before acquiring data on the flow cytometer.

### 4.2.3 Human cytokine array to detect cytokines in co-culture supernatants

All reagents of the proteome profiler human cytokine array kit (ARY005, R&D) were brought to room temperature before use. Each well of the 4-well Multi-dish was filled with 2ml of array buffer 4, a membrane placed into each well and incubated for 1 hour on a rocking platform shaker. Meanwhile, the samples were diluted with array buffers 4 and 5 as instructed in the manufacturer's protocol and 15µl of reconstituted Human Cytokine Array Detection Antibody Cocktail was added each sample. This mixture was incubated for 1 hour at room temperature. The buffer from the wells of the dish was aspirated and each sample was then added into a separate well and left to incubate on a rocking shaker overnight at 4ºC. Each membrane was washed for 10 mins in 20ml of 1X wash buffer three times. 1µg/ml

of IRDye® 800CW Streptavidin (LI-COR) diluted in array buffer 5 was subsequently added to each sample and incubated for 30 mins at room temperature on a rocking shaker. The membranes were then imaged on the Odyssey® CLx instrument (LI-COR).

### 4.2.4 ELISAs for measuring specific co-culture cytokines

Co-culture supernatants after the *in vitro* culture for 48 hours of macrophages and erythroblasts together were collected and stored at -80ºC. Enzyme-linked immunosorbent assays (ELISAs) were performed in triplicate wells according to the manufacturer's instructions for human chemokine (C-C motif) ligand 2 (CCL2), interleukin 8 (IL8) (ELISA Ready-SET-Go!®, eBioscience), interleukin 1 receptor antagonist (IL1ra), chemokine (C-X-C motif) ligand 1 (CXCL1/GROα) (Platinum ELISA, eBioscience), and plasminogen activator inhibitor-1 (PAI-1/Serpin E1, R&D). The basic protocol for the measurement of each cytokine involved coating the plate with the capture antibody (if not already coated) at 4ºC overnight. After washing the wells with $dH_2O$, non-specific binding was blocked by incubating with 1% BSA in PBS for 30-60 mins at room temperature. After washing the wells with $dH_2O$, 100µl of the supernatants (diluted at 1:10, 1:5, 1:2 and neat) and 100µl of previously prepared protein standards were added to corresponding wells of the plate and incubated for 1 hour at room temperature. Wells were again washed as before and subsequently 100µl of the secondary antibody conjugated to Horseradish Peroxidase (HRP) added for 1 hour. Wells were washed in $dH_2O$. Following the addition of 100µl of the substrate, the reaction was stopped with 50µl of stopping solution once a strong yellow colour had started to develop. The plates could then immediately be read on a microplate reader (Bio-Rad) at 450nm with correction at 570nm.

### 4.2.5 Cytospins and Leishman's staining of erythroblast morphology

Approximately 1 x $10^4$ erythroblasts were washed with cold FACS buffer and then diluted in 100µl FACS buffer. The slides and filters were prepared and the diluted cells placed into a well of the cytospin (Shandon Cytospin 3 Cytocentrifuge). The erythroblasts were spun at 800 rpm for 3 mins and the slides subsequently left to dry at room temperature for a minimum of 30 mins. Once dry, cells were stained using Leishman's staining to study their morphology and determine their stage of differentiation. This involved adding 1ml of Leishman's staining solution Gurr® (350224L, VWR) directly on top of the slide for 2 mins. This was followed by carefully adding 2ml of Sorensen's phosphate buffer, pH6.8 (consisting of 0.2M $NaH_2PO_4$ and 0.2M $Na_2HPO_4$ in equal quantities) and homogenously spreading with the Gurr® by pipetting up and down slowly a few times. The mixture was left for 7 mins and then rinsed with distilled water ($dH_2O$) and subsequently with Sorensen's phosphate buffer. The cells were then covered with fresh Sorensen's phosphate buffer and left for exactly 2 mins. After rinsing again with the buffer and leaving the slides to dry for at least an hour, erythroblasts could be imaged under a normal Brightfield microscope (VanGuard 1220CM-BP).

### 4.2.6 IncuCyte ZOOM® Phagocytosis Assay

Day 10 macrophages that had previously been treated under the different conditions were plated in duplicate in 96-well flat clear bottom microplates (Corning) and left for 2 hours to adhere to the plastic. 200µg/ml green *E.coli* bioparticles were added into each well and incubated for 1 hour at 37ºC in the IncuCyte ZOOM platform (Essen Bioscience). Images using a 20x objective lens were taken of selected wells within the plate with an acquisition time of 400 ms. Images were then analysed with

the IncuCyte$^{TM}$ Basic Software, with any objects smaller than 50μm$^2$ not included. The green fluorescence signal was determined per cell and this was a measure of the proportion of *E.coli* bioparticles that were taken up by macrophages. An attractive feature of this technique is that the bioparticles only fluoresce when in the acidic environment of the phagolysosome and hence is a reliable indication of phagocytosis.

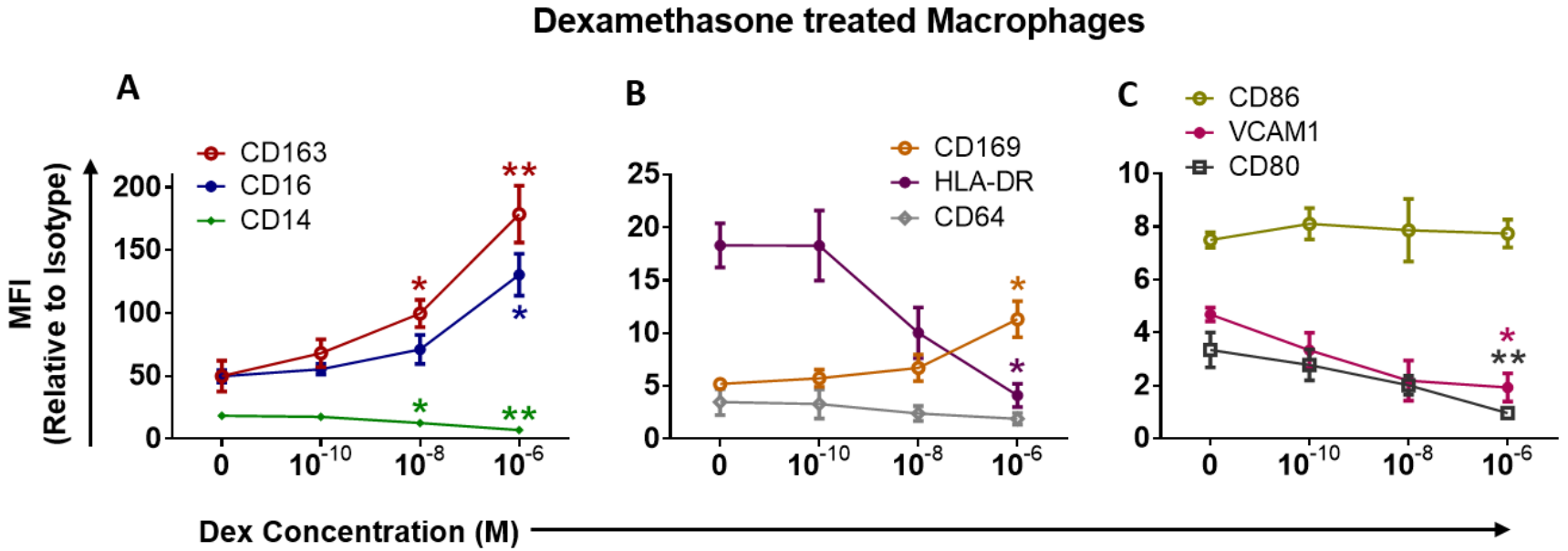
## 4.3 Results

Human macrophages were treated with dexamethasone or IL10 and the effect on expression of various cell surface markers was investigated as an indication of modified macrophage phenotypes that could potentially affect erythroblast development. Dexamethasone and IL10 are both known to upregulate CD163 expression on macrophages (Buechler et al., 2000; Sulahian et al., 2000; van de Garde et al., 2014), which is an important receptor that supports erythroid expansion (Fabriek et al., 2007; Falchi et al., 2015). However, in this thesis dexamethasone and IL10 were added to macrophages, with the molecules replaced daily along with the media for a total of four days, because this was observed to subsequently induce the greatest expansion of co-cultured erythroblasts.

### 4.3.1 Surface marker expression of modulated M2 macrophages

This section explores how stimulating macrophages with a range of concentrations of dexamethasone ($10^{-10}$, $10^{-8}$, $10^{-6}$M) or IL10 (2, 10, 50ng/ml) altered the phenotype in terms of cell surface marker expression. Given that the seeding of macrophages at different densities was previously found to affect both their surface marker levels and viabilities (**chapter 3**), it was imperative to establish whether treating macrophages with dexamethasone or IL10 compromised their viability, especially as this would likely have subsequent detrimental effects on co-cultured erythroblasts.

**FIGURE 4.1. Dose response effect of dexamethasone treatment on macrophage surface marker expression.** *MCSF differentiated macrophages were treated for 4 days with a range of concentrations of dexamethasone ($10^{-10}$, $10^{-8}$ or $10^{-6}$M). $10^{-4}$M dexamethasone was toxic to cells. The final cell density by macrophage day 10 was 1.2 x $10^5$ macrophages/well and cell surface expression was measured on this day by staining macrophages with directly conjugated antibodies against the glycoproteins CD163, CD16, CD14 (A), CD169, HLA-DR, CD64 (B), CD86, VCAM1 and CD80 (C) with fluorescence detected on a flow cytometer. The MFI was based on the appropriate FSC/SSC gate and DAPI negativity for viable cells. The mean and SEM of 4-6 independent biological repeats from separate donors are depicted. *P<0.05, **P<0.01 compared to 0M (one-way ANOVA with Tukey's post-test correction for each marker).*



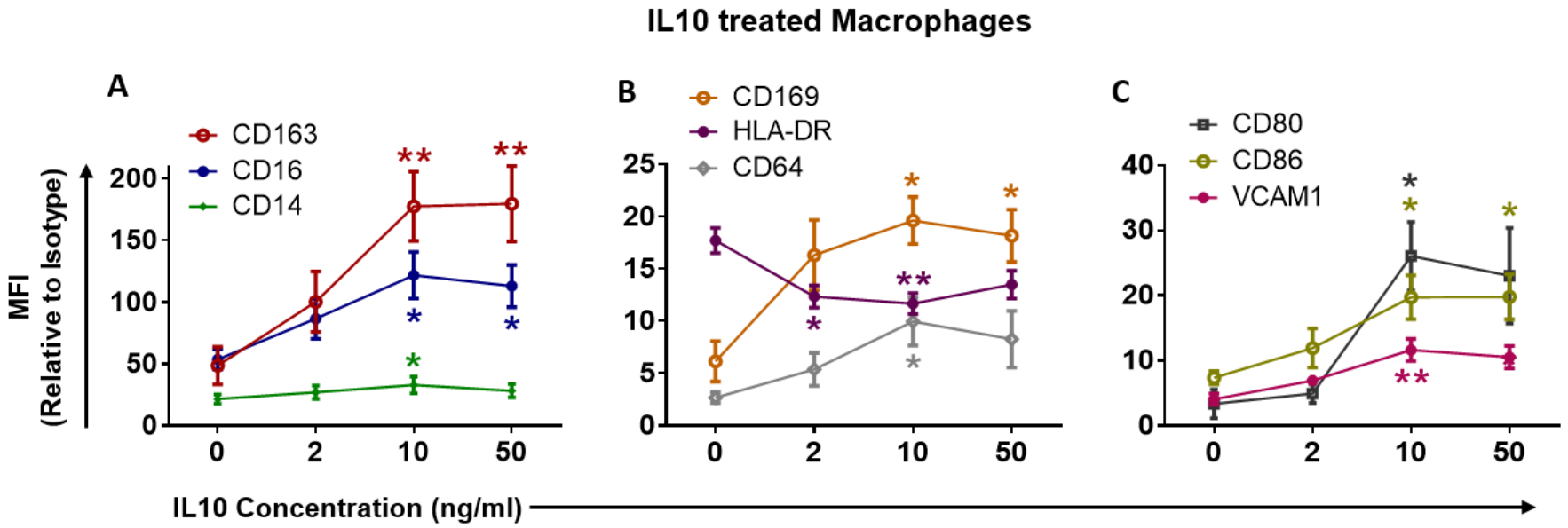
Dexamethasone altered the macrophage phenotype in a dose-dependent manner, with maximal effects at $10^{-6}$M, changing the expression of all surface markers except the co-stimulatory molecule CD86, which was not affected by any concentration of the glucocorticoid (**Figure 4.1**). The addition of $10^{-6}$M dexamethasone for four days to macrophages grown in MCSF increased CD163, CD169 and CD16 receptor expression while decreasing CD14, VCAM1, HLA-DR and CD80 surface levels compared to untreated macrophages grown only with MCSF (0M). The Fc receptor CD64 showed a trend towards decreasing surface levels with increasing concentrations of dexamethasone but this was not statistically significant.

While dexamethasone treatment induced substantial changes in surface marker levels, none of the doses affected the viability of macrophages, which was maintained at around 89.4 ±SEM 7.0% $AnnV^{-}7AAD^{-}$ cells (**Table 4.1**).

**TABLE 4.1. Effect of dexamethasone treatment on macrophage viability.** *The percentage of viable ($AnnV^{-}7AAD^{-}$) macrophages following treatment with various doses of dexamethasone ($10^{-10}$, $10^{-8}$ or $10^{-6}$M) was measured by dual staining for AnnexinV and 7AAD and subsequent detection on a flow cytometer. The mean and SEM of 4 biological repeats are shown. There is no significant difference in viability for any dose of dexamethasone; P > 0.05 in each case.*

| **Dexamethasone concentration (M)** | **$AnnV^{-}7AAD^{-}$ macrophages (%)** |
|---|---|
| 0 | 92.1 (± 6.6) |
| $10^{-10}$ | 90.4 (± 6.1) |
| $10^{-8}$ | 87.9 (± 7.6) |
| $10^{-6}$ | 89.8 (± 7.2) |

**FIGURE 4.2. Dose response effect of IL10 treatment on macrophage surface marker expression.** *Human MCSF differentiated macrophages were treated for 4 days with a range of concentrations of IL10 (2, 10 or 50ng/ml). The final cell density by macrophage day 10 was 1.2 x $10^5$ macrophages/well and cell surface expression was then measured by staining macrophages with directly conjugated antibodies against the glycoproteins CD163, CD16, CD14 (A), CD169, HLA-DR, CD64 (B), CD80, CD86 and VCAM1 (C) and fluorescence was detected on a flow cytometer. The MFI was based on the appropriate FSC/SSC gate and DAPI negativity for viable cells. See* ***Appendix 4.1*** *for representative raw histograms of the altered surface proteins following L10 treatment. The mean and SEM of 4-6 independent repeats from separate donors are shown. *P<0.05, **P<0.01 compared to 0ng/ml (one-way ANOVA with Tukey's post-test correction for each marker).*



IL10 altered the macrophage phenotype in a dose-dependent manner, with maximal effects at 10ng/ml, after which the effects appeared to plateau and the addition of 50ng/ml IL10 caused no further change (**Figure 4.2**). Supplementation of MCSF differentiated macrophages with 10ng/ml IL10 augmented surface expression of CD163, CD169, CD14, CD16, VCAM1, CD64, CD80 and CD86 but reduced HLA-DR plasma membrane levels compared to untreated cells (0ng/ml).

While significantly altering surface glycoprotein expression, IL10 treatment did not affect the macrophage viability (**Table 4.2**).

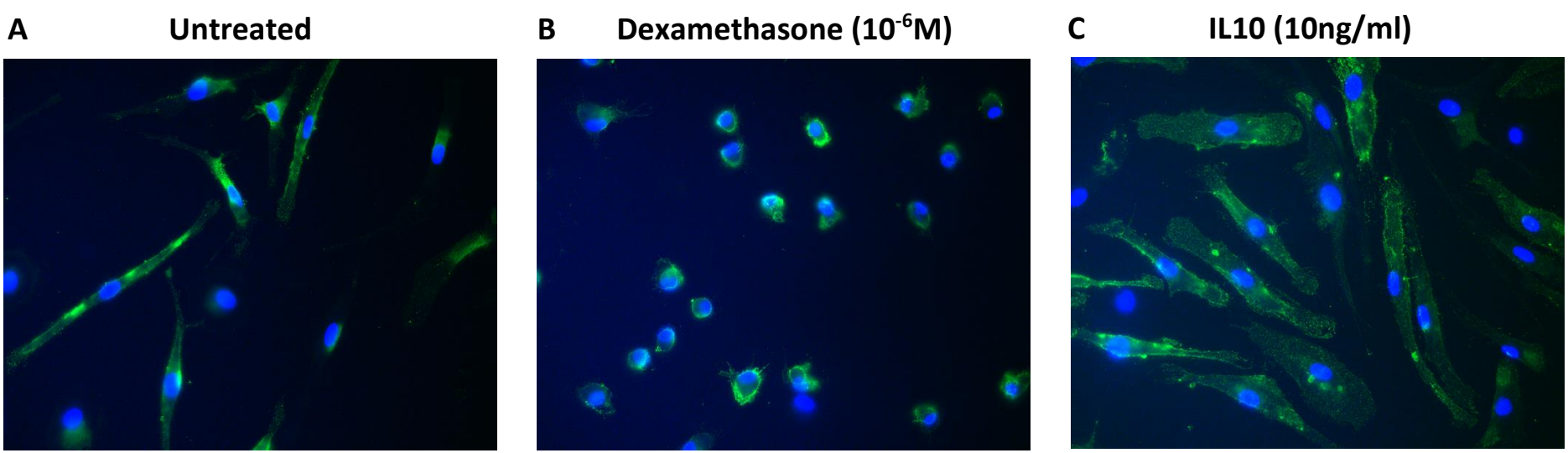
**TABLE 4.2. Effect of IL10 treatment on macrophage viability.** *The percentage of viable (AnnV$^-$7AAD$^-$) macrophages following treatment with various doses of IL10 (2, 10 or 50ng/ml) was measured by dual staining for Annexin V and 7AAD and subsequent detection on a flow cytometer. The mean and SEM of 4 biological repeats are shown. There is no significant difference in viability for any dose of IL10 compared to untreated macrophages; P > 0.05 in each case.*

| **IL10 concentration (ng/ml)** | **AnnV$^-$7AAD$^-$ macrophages (%)** |
|---|---|
| 0 | 92.1 (± 6.6) |
| 2 | 93.5 (± 5.8) |
| 10 | 90.4 (± 7.5) |
| 50 | 88.8 (± 6.9) |

### 4.3.2 Morphology of dexamethasone or IL10 modulated macrophages

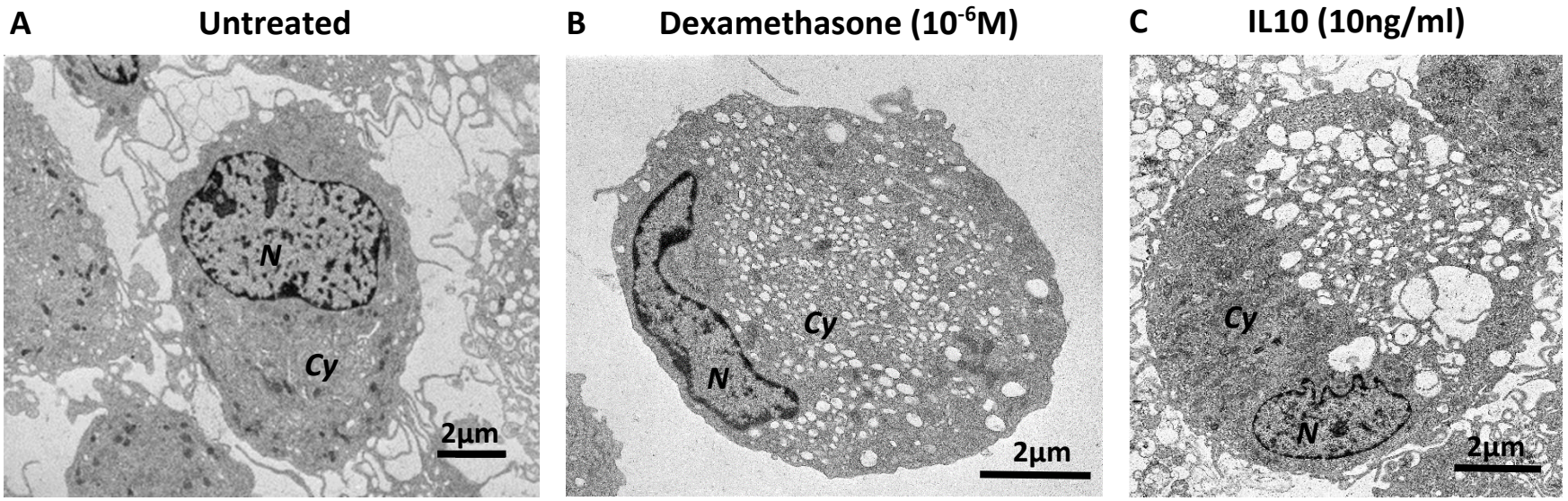
Having found significant differences in surface marker expressions following treatment of MCSF differentiated macrophages with dexamethasone or IL10, it was of interest to examine if this was associated with changes in the structure of these cells. Thus, immunofluorescence and electron microscopy were used to study the morphologies of macrophages treated with either $10^{-6}$M dexamethasone or 10ng/ml IL10, which induced the strongest changes in receptor levels, and compared to untreated macrophages.

**FIGURE 4.3. Morphology and CD163 receptor expression on human macrophages following treatment with dexamethasone or IL10.** *CD163 distribution (green) and DAPI nuclear staining (blue) are shown in macrophages grown in MCSF only (A) or after treatment for 4 days with $10^{-6}$M dexamethasone (B) or 10ng/ml IL10 (C). The representative images are of macrophages from the same donor, but differentially treated.*



Supplementing macrophages with $10^{-6}$M dexamethasone caused a rounding up of cells, whereas the addition of 10ng/ml IL10 induced a long and wider cell shape. The rounding up of macrophages following dexamethasone stimulation has been previously reported as a specific effect of the glucocorticoid (Giles et al., 2001) and this change in morphology did not represent a deterioration in the viability of cells (**Table 4.1**), which also remained firmly attached to the plastic wells. The distribution of CD163 within macrophages under the various treatment conditions was also different. CD163 appeared to be at the cell surface in dexamethasone supplemented macrophages but after IL10 treatment, CD163 concentrated within the cell as well as on the plasma membrane.

**FIGURE 4.4. Electron microscopy images of macrophages after treatment with dexamethasone or IL10.** *The morphology of macrophages left untreated (A) or treated with $10^{-6}$M dexamethasone (B) or 10ng/ml IL10 (C) for 4 days. The nucleus (N) and cytoplasm (Cy) are indicated in each cell. Condensed electron dense heterochromatin is shown in black, whereas euchromatin is represented by the more lucent areas in the nucleus.*



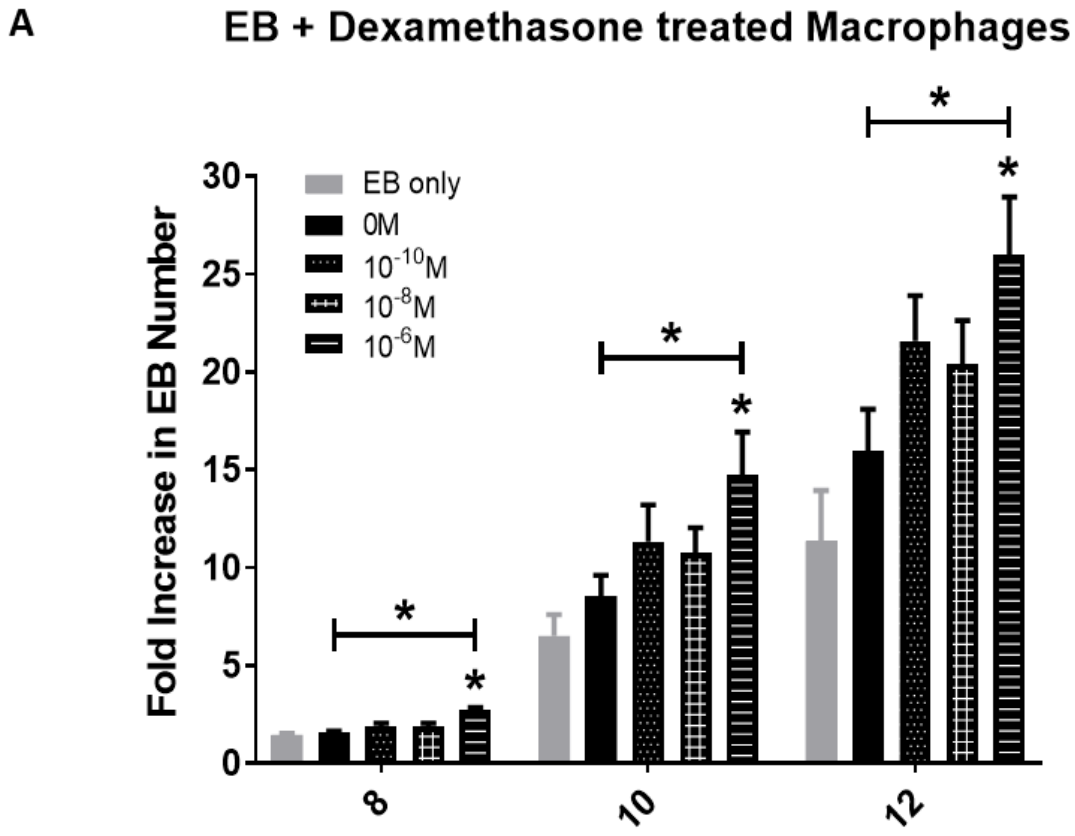
The electron micrographs show some ultrastructural differences in the nucleus and cytoplasm between the different treatments, such as reduced numbers of cytoplasmic extrusions in dexamethasone treated macrophages, which probably contribute to their rounding up in shape during *in vitro* culture. The most striking difference, however, is the increased cytoplasmic vacuolation in dexamethasone or IL10 modulated macrophages that indicate cellular stress and may represent swelling of the rough endoplasmic reticulum but without showing advanced apoptotic or necrotic changes. The vast majority (approximately 80-90%) of dexamethasone or IL10 treated macrophages display these changes in vacuolation. Moreover, there is a greater abundance of euchromatin (grey) compared to heterochromatin (black) under each treatment condition, suggesting the cells are all transcriptionally active and, as there is no observable dramatic difference in the relative amount of euchromatin, also suggests there might be comparable transcriptional activities between the various conditions.

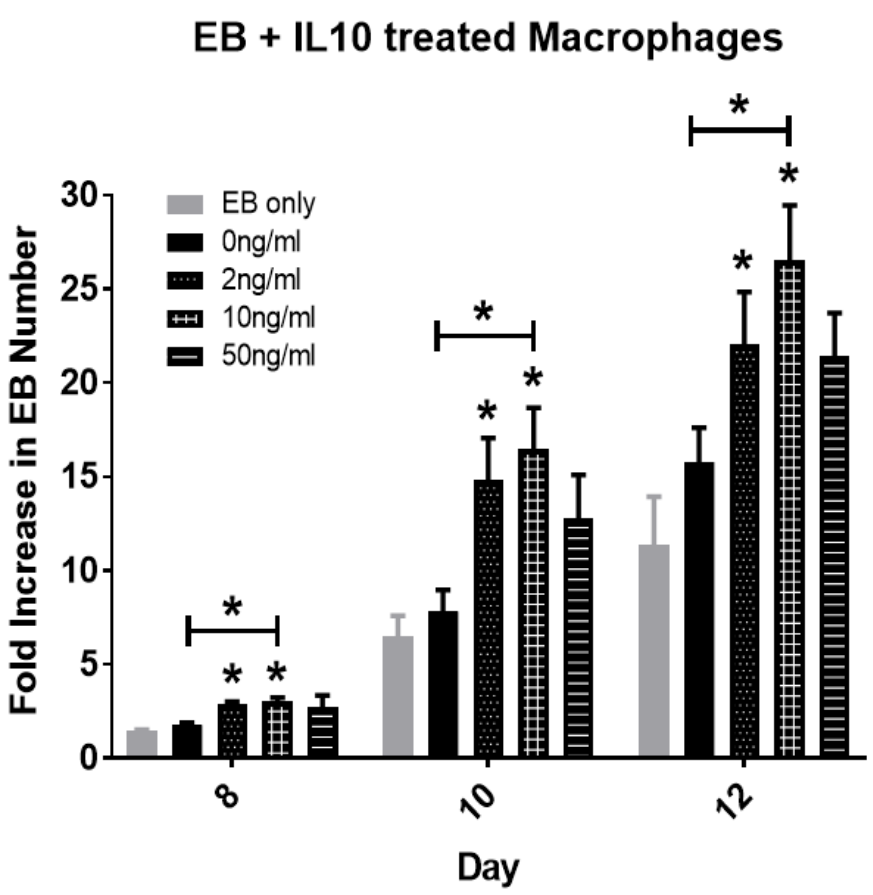
### 4.3.3 Erythroblast expansion following co-culture with dexamethasone or IL10 stimulated macrophages

Having examined how targeting dexamethasone or IL10 directly to human macrophages altered their phenotype, the effect these modulated cells had on erythroid development during *in vitro* co-culture were investigated next. Only one previous study (Falchi et al., 2015) has explored the use of dexamethasone supplementation to cultures of human $CD34^+$ cells, generating both erythroid cells and some macrophages, as a model of enhanced erythropoiesis. However, there have been no publications on the role of IL10 stimulated macrophages on

erythropoiesis. Therefore, the subsequent studies are novel in that they investigate how macrophages directly modified by dexamethasone or IL10 themselves affect erythropoiesis.

**FIGURE 4.5. Erythroblast expansion on days 8, 10 and 12 following co-culture with dexamethasone or IL10 treated macrophages.** *Fold increase in erythroblast number was determined on days 8, 10 and 12 after the co-culture with dexamethasone (A) or IL10 (B) modulated macrophages via haemocytometer counts of trypan blue excluded live cells. The fold increase in number was calculated as the cell number compared to the initial erythroblast number on day 6 (6 x $10^3$ cells/well). Various doses of dexamethasone ($10^{-10}$, $10^{-8}$ or $10^{-6}$M) or IL10 (2, 10 or 50ng/ml) were used to treat macrophages prior to co-culture. The mean and SEM of 9 biological repeats are shown. *P<0.05, **P<0.01, ***P<0.001 compared to EB only or as indicated otherwise (one-way ANOVA with Tukey's post-test correction at each time point).*






The differentially modulated macrophages significantly augmented erythroblast expansion on days 8, 10 and 12 when macrophages were previously treated with $10^{-6}$M dexamethasone or with either 2 or 10ng/ml IL10 (**Figure 4.5**). $10^{-6}$M dexamethasone stimulated macrophages elevated erythroid expansion by 1.7-, 1.7- and 1.6-fold on days 8, 10 and 12 compared to untreated macrophages, while 10ng/ml IL10 supplemented macrophages heightened expansion by 1.7-, 2.1- and 1.7-fold on the same days. Moreover, comparable fold increases in erythroblast number (approximately 26.0-fold) compared to the initial density of erythroid cells were reached by day 12 with $10^{-6}$M dexamethasone and 10ng/ml IL10 treated macrophages. All other doses of dexamethasone ($10^{-10}$ or $10^{-8}$M) or IL10 (50ng/ml)

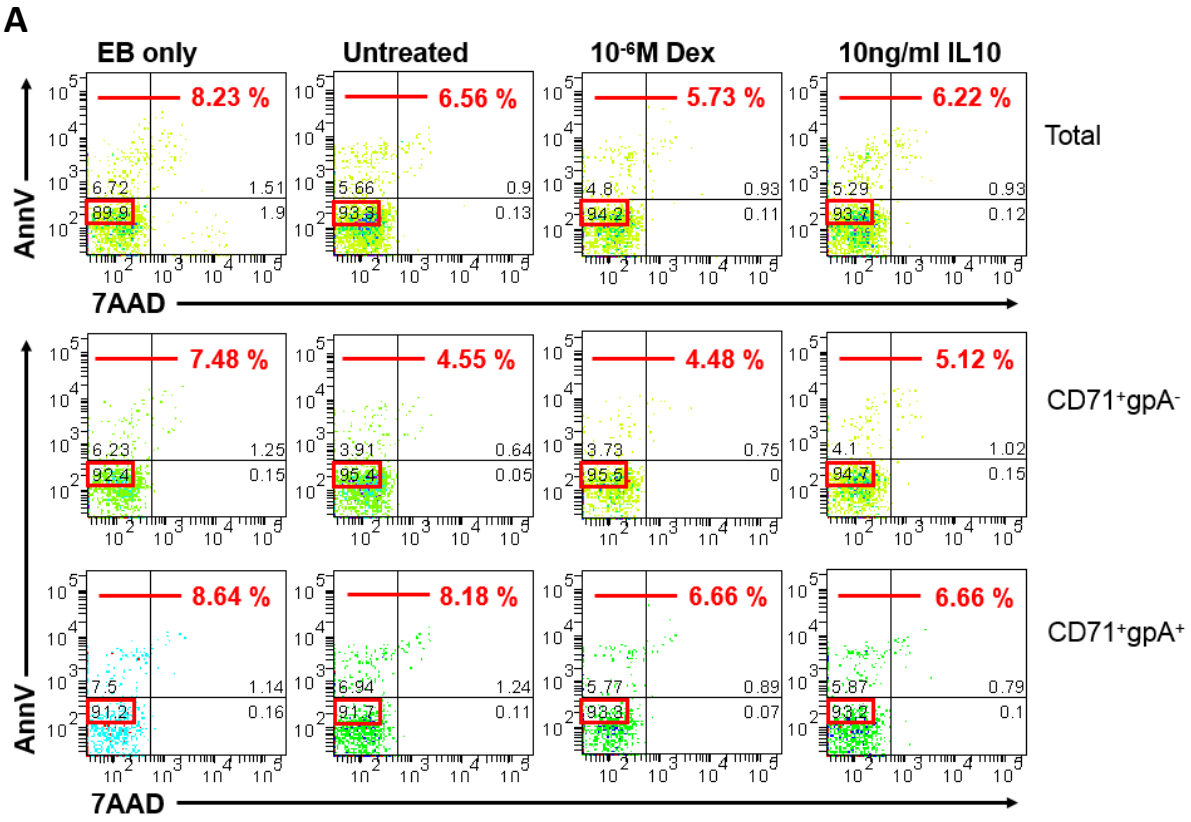
gave a trend towards somewhat improved erythroid expansion but this was not significant at any time point.

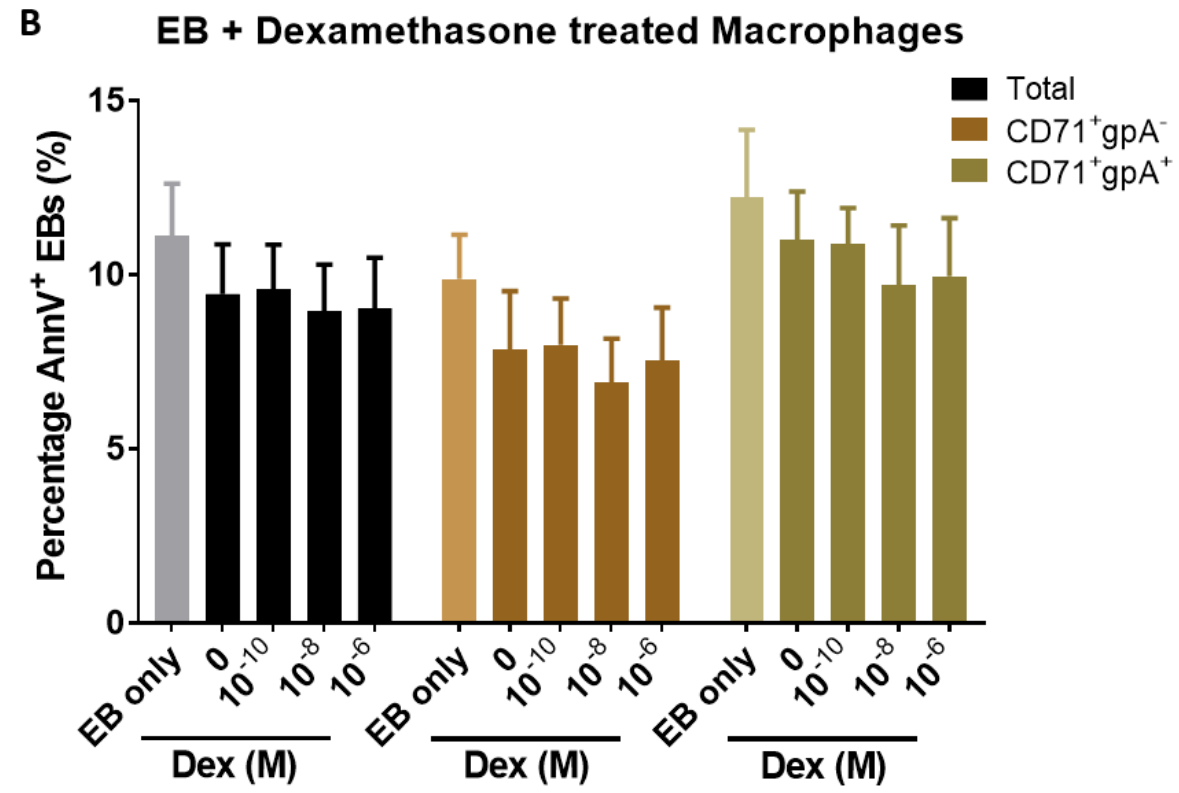
### 4.3.4 Erythroblast survival following co-culture with dexamethasone or IL10 treated macrophages

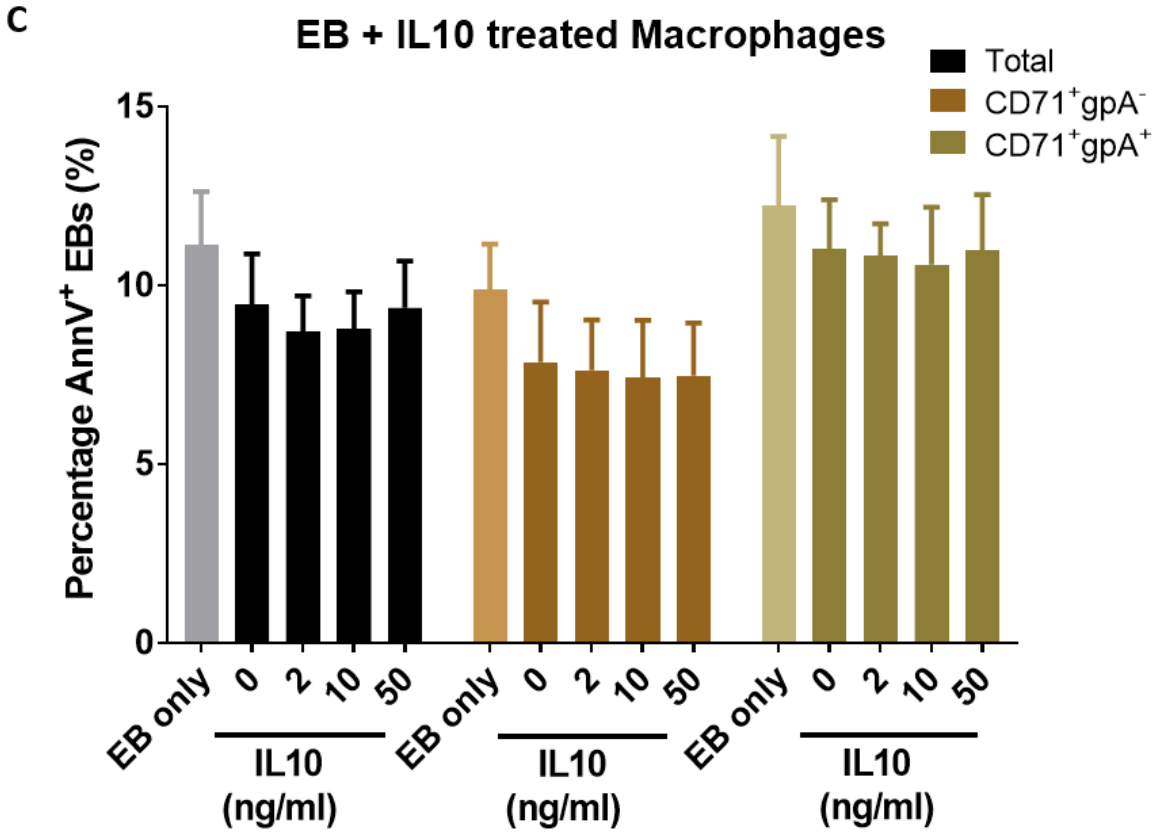
Erythroblast expansion is a function of both proliferation and survival. Therefore, the elevated expansion observed during the culture with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) treated macrophages could be as a result of either increased erythroid proliferation or survival, or both. Thus, the viability and percentage of apoptotic erythroblasts following co-culture with the differentially modulated macrophages were measured to determine whether this might be contributing towards the enhanced expansion.

The functional effects of modulated macrophages on erythroblasts were determined on day 8 in all subsequent work, given that significant increases in expansion were already observed on this day. It also meant that any direct effects caused by the macrophages themselves could be investigated more easily as opposed to possible indirect effects caused by long-term culture or alterations in the macrophage phenotype over time.

**FIGURE 4.6. Proportion of apoptotic erythroid cells following co-culture with the differentially treated macrophages.** *The percentage of total apoptotic (AnnV$^+$7AAD$^-$ and AnnV$^+$7AAD$^+$) erythroblasts was determined by dual staining with Annexin V and 7AAD and subsequent detection on a flow cytometer. The amount of apoptosis and death was determined for cultures of erythroblasts alone (EB only) or co-cultures involving untreated, dexamethasone ($10^{-10}$, $10^{-8}$, $10^{-6}$M) treated or IL10 (2, 10, 50ng/ml) modulated macrophages. 'A' shows representative FACS plots from one donor of the total, CD71$^+$gpA$^-$ and CD71$^+$gpA$^+$ erythroid populations stained dually for Annexin V and 7AAD. 'B' and 'C' show the percentage of AnnV$^+$ erythroblasts for each population after co-culture with macrophages previously treated with a range of doses of dexamethasone or IL10, respectively. Data are the mean and SEM of 4 biological repeats. There were no statistically significant differences in any case.*




B
EB + Dexamethasone treated Macrophages
Total
CD71+gpA-
CD71+gpA+
Percentage AnnV+ EBs (%)
15
10
5
0
EB only
0
10-10
10-8
10-6
Dex (M)

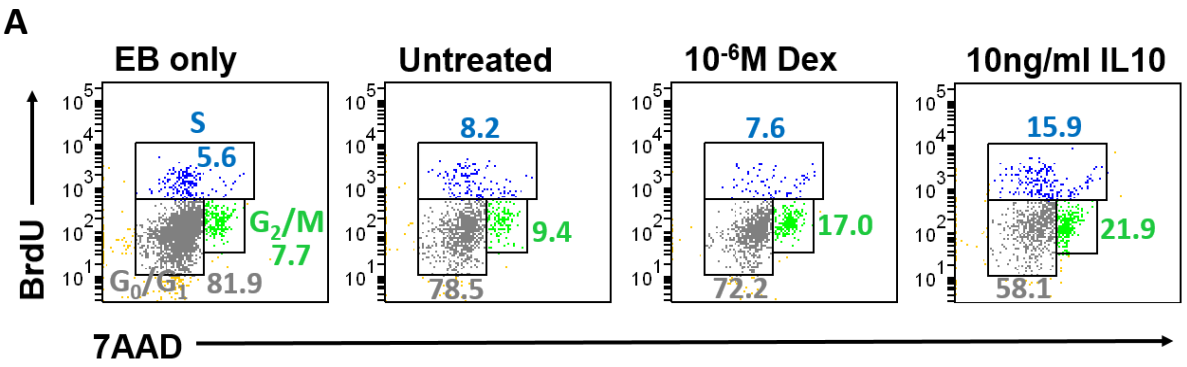

C
EB + IL10 treated Macrophages
Total
CD71+gpA-
CD71+gpA+
Percentage AnnV+ EBs (%)
15
10
5
0
EB only
0
2
10
50
IL10
(ng/ml)

There were no significant differences in the percentages of early apoptotic ($AnnV^+7AAD^-$) and dead ($AnnV^+7AAD^+$) erythroblasts for any of the erythroid populations (total, $CD71^+gpA^-$ or $CD71^+gpA^+$) when co-cultured with macrophages treated with dexamethasone or IL10 at any dose (**Figure 4.6**). The only slight difference was for erythroblasts cultured on their own, which consistently showed a trend towards somewhat greater percentages of $AnnV^+$ cells for all erythroid populations compared to any of the co-culture conditions. The likely explanation for this is due to the ability of macrophages to phagocytose apoptotic and dead cells. However, in each case there was a small difference of not more than 2.7% in the proportion of $AnnV^+$ erythroblasts. Hence, one can conclude from these data that the observed improvements in erythroblast expansion following culture with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) treated macrophages (**Figure 4.5**) were likely not as a result of heightened erythroid survival. However, any phagocytosis of apoptotic erythroid cells would make these data difficult to interpret.

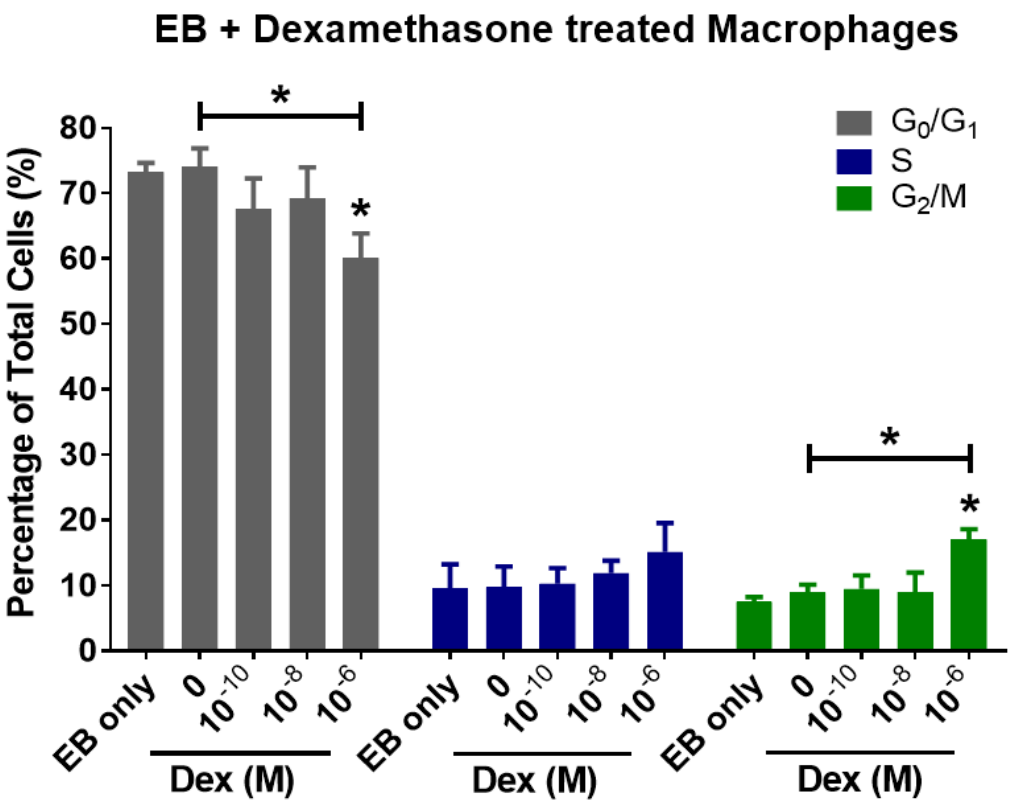
### 4.3.5 Erythroid cell cycle transition after culture with dexamethasone or IL10 treated macrophages

The contribution of erythroid proliferation to the overall expansion rate was investigated in line with the previous findings for unchanged erythroblast survival. BrdU incorporation allows one to measure the proportion of cells in the $G_0/G_1$, S or $G_2$/M stages and thus provides an indication for the amount of proliferation.

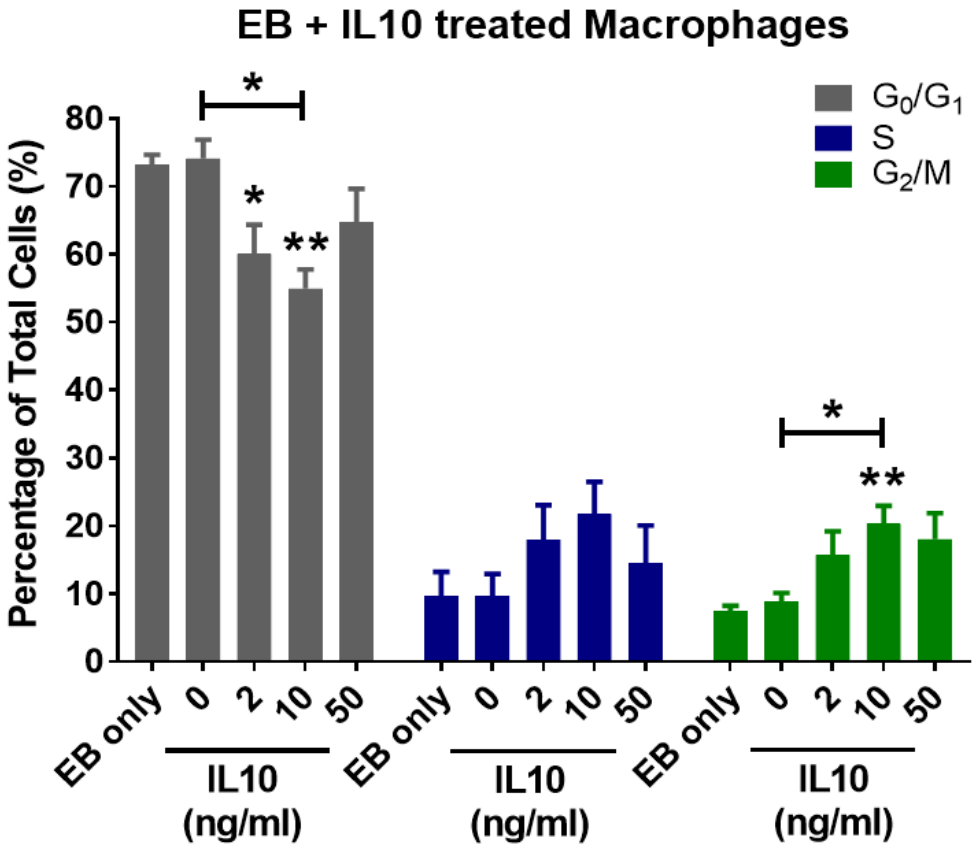
**FIGURE 4.7. Cell cycle progression of erythroblasts co-cultured with the differentially stimulated macrophages.** *'A' shows the gating strategy that was employed to determine the proportion of erythroblasts in the $G_0/G_1$, S or $G_2/M$ stages of the cell cycle, following BrdU staining of fixed viable cells. The percentages at each stage were measured after 48 hours for erythroid cells grown alone or in culture with untreated macrophages or with macrophages supplemented with either dexamethasone (B) or IL10 (C) at the range of concentrations shown. Data are the mean and SEM of 4 independent biological repeats from separate donors. *P<0.05, **P<0.01 compared to EB only unless indicated otherwise (one-way ANOVA with Tukey's post-test correction for each cell cycle stage).*



B


EB + Dexamethasone treated Macrophages
Percentage of Total Cells (%)
G0/G1
S
G2/M
EB only
0
10-10
10-8
10-6
Dex (M)

C


EB + IL10 treated Macrophages
Percentage of Total Cells (%)
G0/G1
S
G2/M
EB only
0
2
10
50
IL10 (ng/ml)

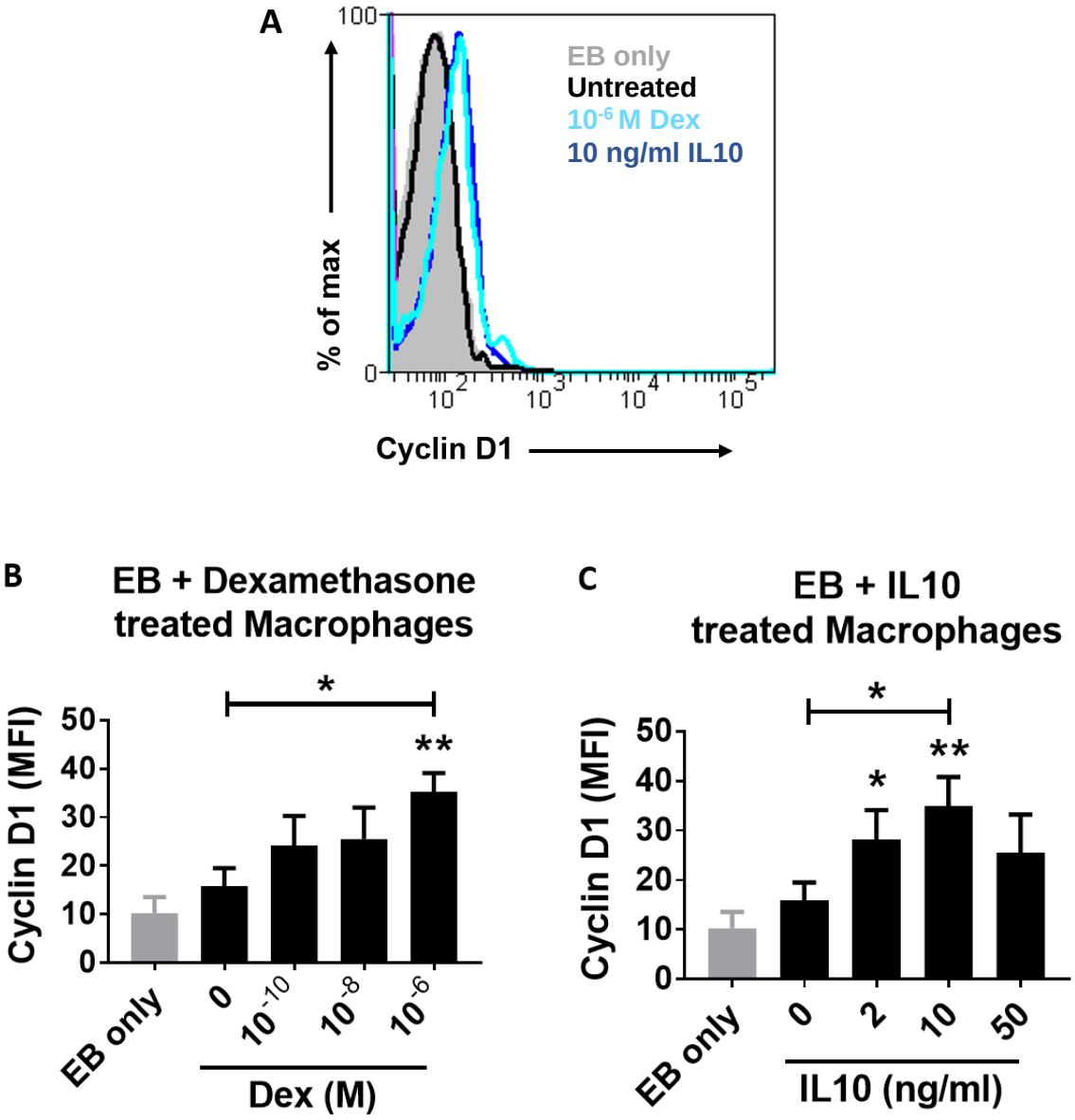
Dexamethasone ($10^{-6}$M) stimulated macrophages significantly reduced the percentage of erythroblasts at the $G_0/G_1$ stage, while at the same time augmenting the proportion of cells in $G_2$/M by 1.9-fold compared to co-cultures involving untreated macrophages (**Figure 4.7B**). Similarly, IL10 (10ng/ml) modulated macrophages diminished the fraction of erythroid cells at $G_0/G_1$ and promoted their transition to the $G_2$/M phase of the cell cycle, increasing the percentage of erythroblasts at this stage by 2.3-fold compared to co-cultures consisting of untreated macrophages (**Figure 4.7C**). Therefore, this provides evidence that dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) modulated macrophages improve erythroid cell cycle transitioning. Furthermore and together with the data showing an unaltered erythroblast survival (**Figure 4.6**), this suggests that the elevated expansion supported by the differentially modulated macrophages (**Figure 4.5**) is solely due to increased erythroid cell proliferation.

### 4.3.6 Cyclin D1 protein levels in erythroblasts cultured with dexamethasone or IL10 supplemented macrophages

The cyclin D1 protein interacts with the cyclin-dependent kinases CDK4 and CDK6 to promote transition through the $G_1$/S checkpoint and induce DNA synthesis, but cyclin D1 levels must also be high during the $G_2$ stage of the cell cycle to trigger proliferation (Yang et al., 2006). Overexpression, amplification and mutations of the cyclin D1 gene are commonly found in several types of tumours (Arnold and Papanikolaou, 2005; Moreno-Bueno et al., 2003; Musgrove et al., 2011; Shan et al., 2017).

Having found that erythroblasts cultured with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) treated macrophages transitioned faster through the cell cycle (**Figure 4.7**), it was hypothesised that there was dysregulated cyclin protein expression under these conditions. Here, the altered levels of cyclin D1 under the various culture conditions are shown.

**FIGURE 4.8. Cyclin D1 protein expression in erythroblasts co-cultured with the dexamethasone or IL10 modulated macrophages.** *'A' shows the shift in cyclin D1 as detected by direct antibody staining of fixed viable cells. 'B' and 'C' show cyclin D1 protein levels in erythroblasts after culture with macrophages treated with differing concentrations of dexamethasone or IL10, respectively. Data are the mean and SEM of 4 independent biological repeats from separate donors. *P<0.05, **P<0.01 compared to EB only unless indicated otherwise (one-way ANOVA with Tukey's post-test correction).*

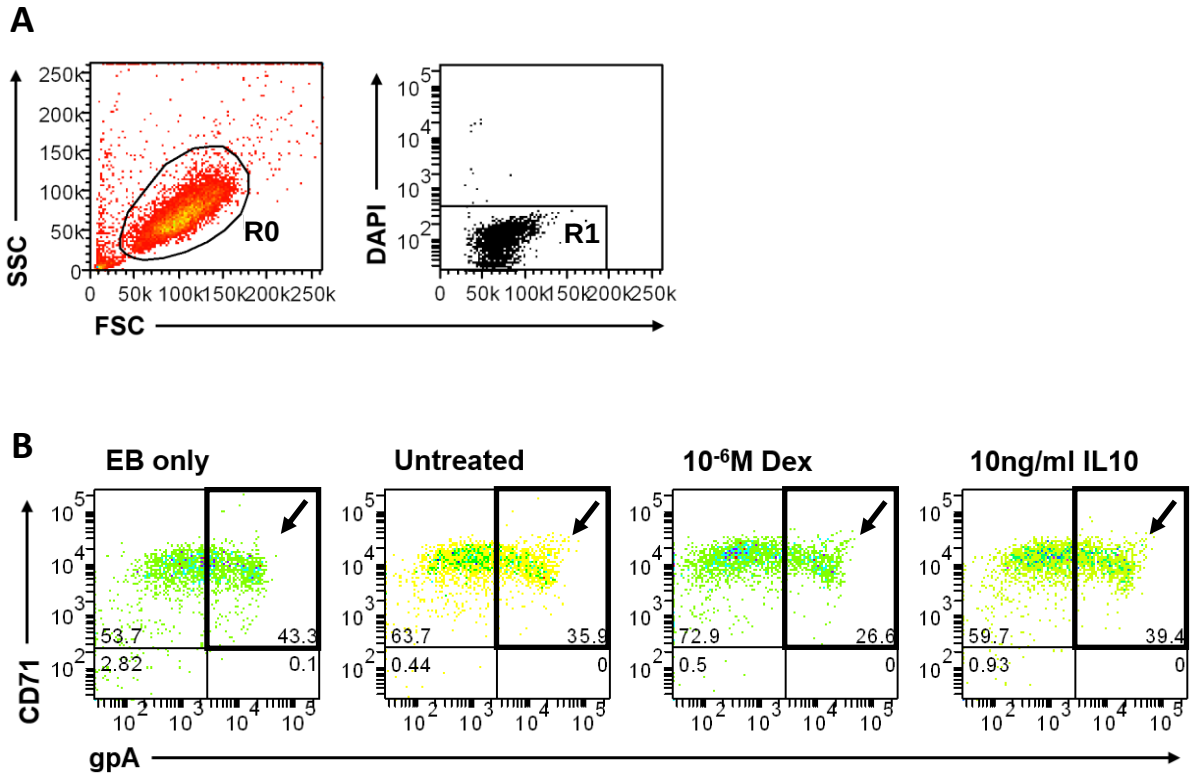


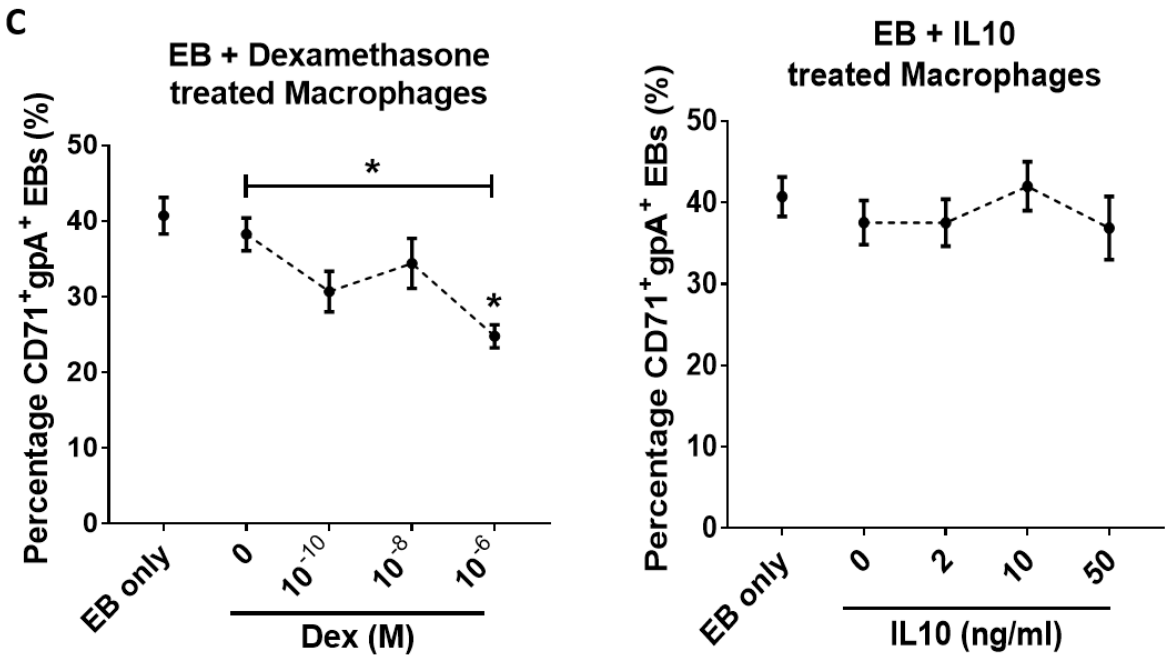
Consistent with the previous findings for heightened erythroblast expansion and cell cycle progression (**Figures 4.5** and **4.7**), there was a similar trend for cyclin D1 protein expression in erythroid cells grown with macrophages modulated by the different doses of dexamethasone or IL10 (**Figure 4.8**). Macrophages stimulated with the optimal concentration of dexamethasone ($10^{-6}$M) or IL10 (10/ml) augmented cyclin D1 levels in erythroblasts by 2.2-fold. Thus, this provides further evidence that the differentially modulated macrophages enhance erythroid expansion by elevating their proliferative rates.

### 4.3.7 Erythroid maturation following co-culture with dexamethasone or IL10 treated macrophages

Other than expansion, another feature that defines erythroid development is maturation. Steady-state erythropoiesis under homeostatic conditions involves signalling in erythroid cells for synchronised proliferation and maturation. However, erythropoietic stress tends to shift this balance, elevating erythroid proliferation while compromising maturation. Therefore, to fully characterise the development of erythroblasts during macrophage-mediated erythropoiesis, the maturation of erythroid cells was evaluated by measuring the surface expression of both CD71 and gpA.

**FIGURE 4.9. Erythroid maturation following co-culture with dexamethasone or IL10 treated macrophages.** *Erythroblasts grown under the various culture conditions were collected after 48hours and stained for CD71, gpA and DAPI. Erythroid cells were gated on forward and side scatter (R0) as well as on DAPI negativity (R1) for selection of viable cells only (A). The most mature erythroblasts at this time point (day 8) were those dually positive for CD71 and gpA and so the percentage of CD71$^{+}$gpA$^{+}$ erythroblasts gated on R0 and R1 (indicated by black boxes with arrow) was used as a measure for erythroid maturation (B). The proportion of mature erythroid cells after growth with macrophages modulated by the range of dexamethasone or IL10 doses is shown (C). The mean and SEM from 6-7 different donors is shown. *P<0.05 compared to EB only or as indicated (one-way ANOVA with Tukey's post-test correction).*



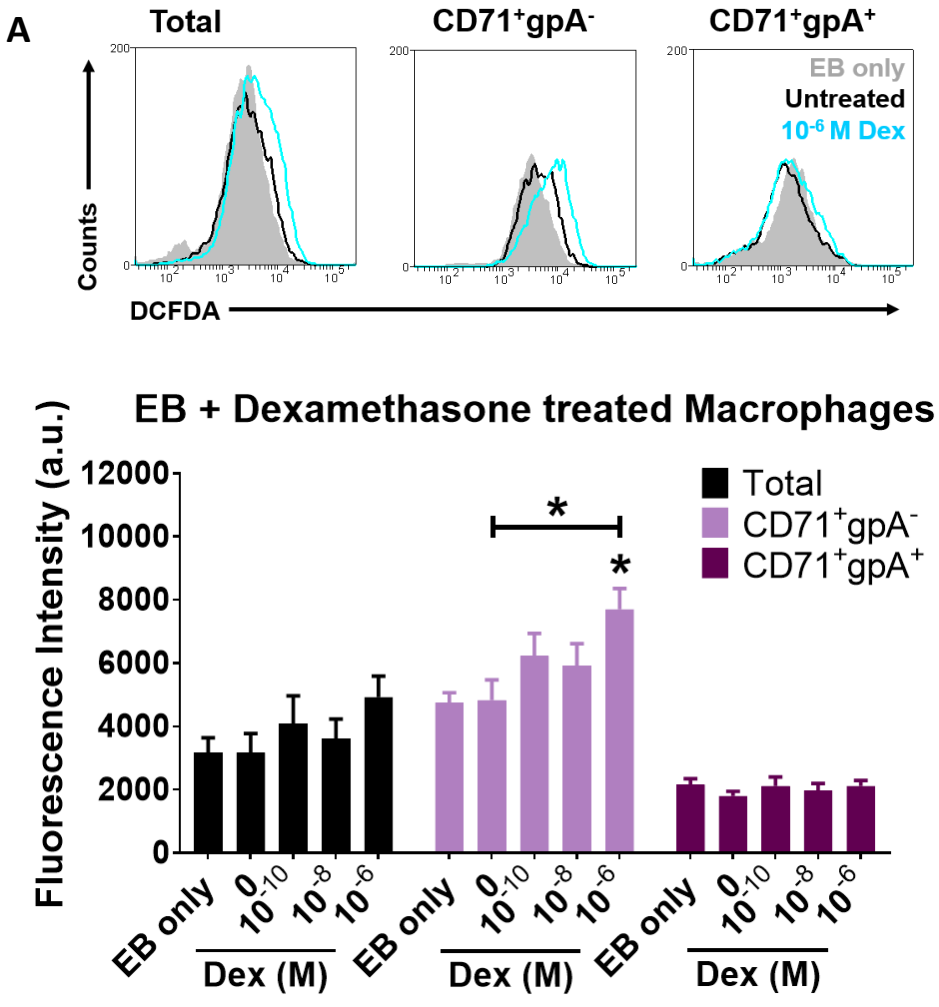


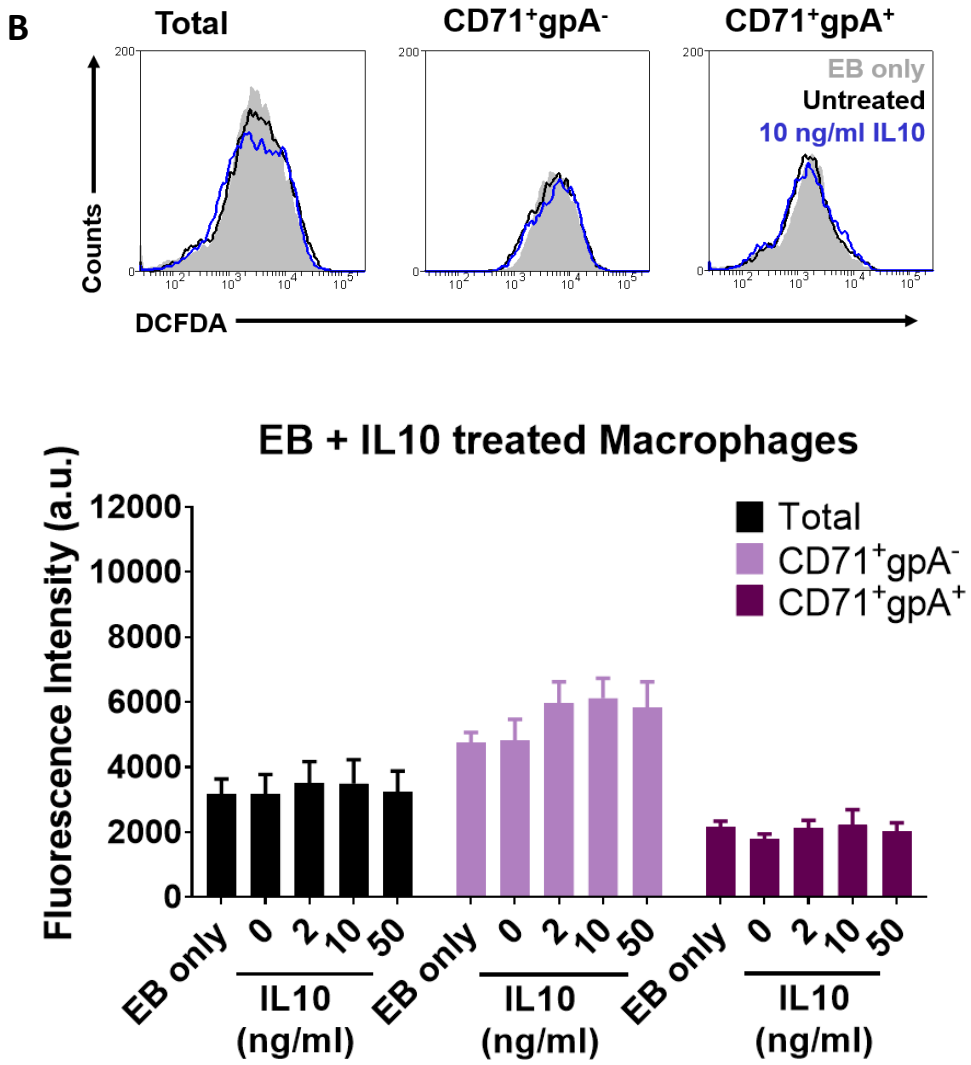

Whereas dexamethasone treatment of macrophages at a dose of $10^{-6}$M significantly reduced the percentage of $CD71^{+}gpA^{+}$ erythroblasts by 13.5% compared to erythroblasts cultured with unmodulated macrophages, none of the concentrations of IL10 used to stimulate macrophages prior to co-culture had any subsequent effect on the proportion of mature erythroid cells (**Figure 4.9**). This suggests that the dexamethasone ($10^{-6}$M) modulated, but not the IL10 (10ng/ml) treated, macrophages inhibited erythroblast maturation. Moreover, the maturity of erythroblasts co-cultured with dexamethasone ($10^{-6}$M) supplemented macrophages showed an inverse association to their expansion (**Figure 4.5A**). Hence, this implies that while dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) treatment induced macrophage phenotypes that supported enhanced erythroblast expansion, only IL10 provided a system where erythroid maturation was simultaneously maintained whereas dexamethasone modulated macrophages in contrast hindered maturation.

### 4.3.8 ROS levels in erythroblasts cultured with differentially supplemented macrophages

Intracellular ROS are implicated in various signalling cascades during the EPO-dependent stages of erythropoiesis. They play key roles in promoting erythroblast proliferation, for instance via the phosphorylation of ERK (Elorza et al., 2008). Hence, to uncover whether oxidative stress contributes towards the elevated erythroid expansion observed during macrophage-mediated erythropoiesis, free radical levels were measured in erythroblasts cultured under the various conditions.

**FIGURE 4.10. Reactive oxygen species in erythroblasts after culture with dexamethasone or IL10 modulated macrophages.** *Intracellular ROS levels in erythroblasts were measured via DCFDA staining and DCF excitation by flow cytometry of DAPI negative viable cells that were also stained for surface CD71 and gpA. 'A' and 'B' show ROS levels in the different erythroid populations (total, CD71⁺gpA⁻, CD71⁺gpA⁺) following culture alone or with macrophages stimulated with various doses of either dexamethasone or IL10, respectively. Representative histograms from one donor are presented in the upper panels. Approximately 8,000-10,000 events were collected and analysed. Data are the mean and SEM of 6 separate donors. *P<0.05 compared to EB only or as indicated otherwise (one-way ANOVA with Tukey's post-test correction for each erythroid population).*





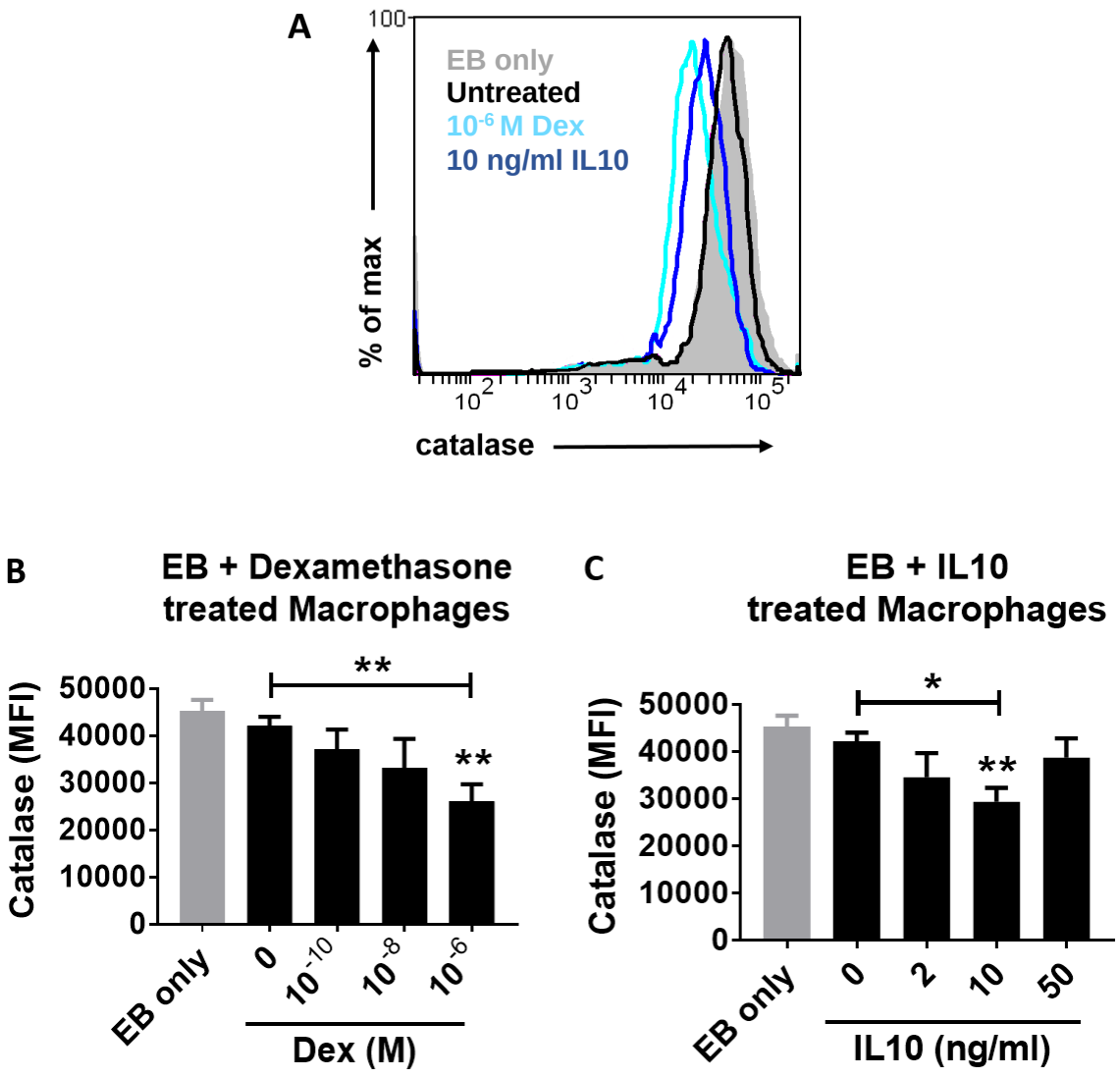

These data show, firstly, that intracellular ROS was reduced as erythroblasts matured from $CD71^{+}gpA^{-}$ into $CD71^{+}gpA^{+}$ cells, suggesting that free radicals were removed from these cells as they matured. This supports the established notion for the need to diminish the oxidative burden in mature erythroid cells that accumulate haemoglobin as they differentiate. Secondly, it was found that macrophages treated with $10^{-6}$M dexamethasone augmented ROS levels in immature ($CD71^{+}gpA^{-}$) erythroblasts by 1.6-fold compared to cells grown with untreated macrophages, while not affecting free radical levels in the more mature ($CD71^{+}gpA^{+}$) erythroid

population (**Figure 4.10A**). Contrastingly, macrophages stimulated with any concentration of IL10 did not significantly affect the intracellular ROS in co-cultured erythroblasts at either erythroid stage (**Figure 4.10B**). These results indicate that only dexamethasone ($10^{-6}$M) modulated macrophages triggered heightened free radical release in immature ($CD71^{+}gpA^{-}$) but not in more mature ($CD71^{+}gpA^{+}$) erythroblasts. Hence, the differences in intracellular ROS suggest there may be altered signalling pathways in immature erythroblasts that might be activated downstream of the signalling cascades induced for improved expansion, and thus possibly not contributing towards the elevated proliferative rates themselves.

### 4.3.9 Catalase levels in erythroblasts after co-culture with differentially stimulated macrophages

Since clear differences in intracellular ROS were found in immature erythroblasts co-cultured with the differentially modulated macrophages, it was speculated that there might also be changes in antioxidant systems either as a cause or consequence of the ROS levels. For this reason, protein expression of the antioxidant catalase, which is directly regulated by the FOXO3 transcription factor, was measured under the various co-culture conditions.

**FIGURE 4.11. Catalase levels in erythroblasts after culture with dexamethasone or IL10 supplemented macrophages.** *'A' shows the shift in protein levels of the antioxidant catalase as detected by indirect staining and subsequent detection on a flow cytometer. 'B' and 'C' show catalase levels in erythroblasts after culture with macrophages treated with various concentrations of dexamethasone or IL10, respectively. The mean and SEM for 4 independent biological repeats from different donors are shown. *P<0.05, **P<0.01 compared to EB only or as indicated otherwise (one-way ANOVA with Tukey's post-test correction).*



A significant reduction in catalase levels was found in erythroblasts that were co-cultured with either $10^{-6}$M dexamethasone treated or 10ng/ml IL10 stimulated macrophages (61% and 70%, respectively, of the catalase levels in erythroblasts grown with untreated macrophages) (**Figure 4.11**). This inhibited catalase expression was associated with elevated intracellular ROS in $CD71^{+}gpA^{-}$ erythroblasts (**Figure 4.10**) only during culture with dexamethasone ($10^{-6}$M) but not IL10 (10ng/ml) treated macrophages. This suggests IL10 modulated macrophages may induce alternative processes within erythroid cells that either directly or indirectly lower the oxidative stress that would build as a result of the diminished antioxidant enzyme levels. However, the similar inverse trends in catalase expression and erythroid expansion (**Figure 4.5**) for co-cultures involving either dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) modulated macrophages suggests a potential link in the pathways that regulate both catalase levels and proliferation.

### 4.3.10 Soluble molecules in co-cultures involving dexamethasone or IL10 modulated macrophages

Other than surface marker expression, another feature that defines the macrophage phenotype is the secretory profile. Therefore, the soluble factors that were present in supernatants following co-culture of erythroblasts with the differentially treated macrophages were investigated.

**FIGURE 4.12. Human cytokine array of culture supernatants.** *The levels of several soluble molecules present in culture supernatants of erythroblasts grown alone or with untreated, dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) treated macrophages is shown. The orange arrows point to the senescence-associated molecules CCL2, IL8, GROα, IL1ra and Serpin E1 that were subsequently measured by ELISA. The relative signal of another 23 molecules is presented in* ***Appendix 4.2****, but all these levels were lower than for the molecules shown here. The data is the mean and SD from one donor in duplicate.*

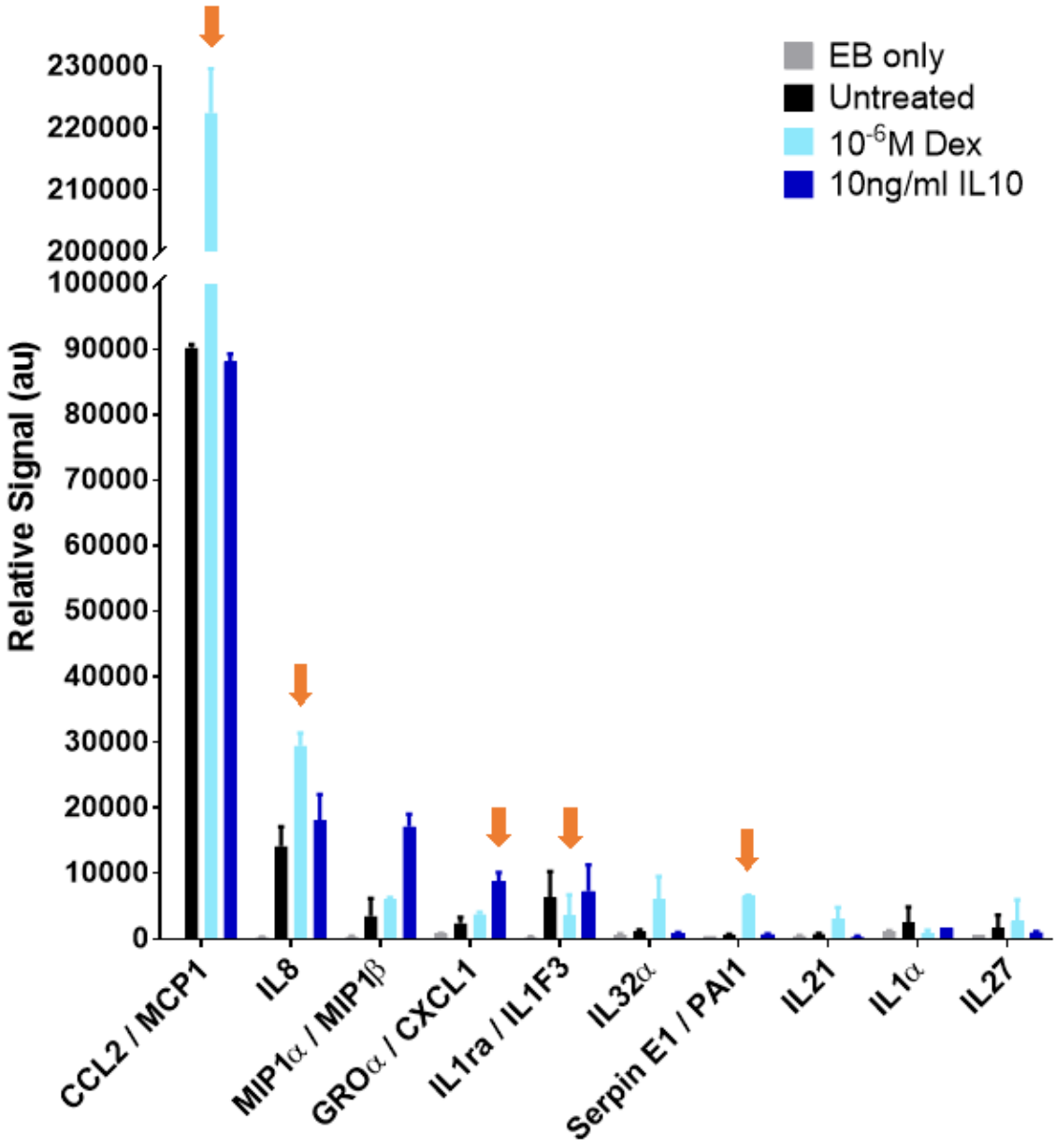


**Figure 4.12** shows that CCL2 was present at extremely high levels in co-cultures involving dexamethasone treated macrophages and IL8 was also found at high levels under these culture conditions. However, CCL2 and IL8 were detected at lower and comparable levels in co-cultures including either untreated or IL10 modulated macrophages. Other molecules that appeared to be differentially expressed under the different treatment conditions were Serpin E1 and IL32α for cultures including dexamethasone stimulated macrophages, but MIP1α/MIP1β and GROα for co-cultures with IL10 treated macrophages. Most of these cytokines are known to be secreted by macrophages during an inflammatory response to serve as chemoattractants (**Appendix 4.3**) and the undetectable levels in cultures involving erythroblasts only suggests they were indeed released by the macrophages and not erythroid cells. Serpin E1 has a unique role as a serine protease inhibitor that prevents the breakdown of blood clots whereas IL1ra has anti-inflammatory properties and modulates IL1 inflammatory responses.

Remarkably, several of the factors induced most prominently are related to cellular senescence, including CCL2, IL8, GROα, IL1ra and Serpin E1 (Acosta et al., 2008; Acosta et al., 2013; Marthandan et al., 2016; Uekawa et al., 2004). IL1ra, being the only anti-inflammatory molecule, may have a function in dampening down the senescence-associated inflammation.

To obtain accurate values, the five senescence-associated molecules indicated by orange arrows in **Figure 4.12** were measured by ELISA.

**TABLE 4.3. The detection of soluble molecules in co-culture supernatants.**

*The levels of senescence-associated secreted molecules were measured in cultures involving erythroblasts only or in co-cultures including macrophages previously untreated or treated with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml). The abundance of each molecule is shown as pg/ml and in bold typeface for the condition where it is differentially detected. Values are represented as the mean ± SEM from 3 different donors. U.D. means undetected. *P<0.05 compared to untreated macrophages; #P<0.05 compared to IL10 treated macrophages (one-way ANOVA with Tukey's post-test correction).*

| | **GROα** | **IL8** | **CCL2** | **IL1ra** | **SerpinE1** |
|---|---|---|---|---|---|
| EB only | U.D. | U.D. | U.D. | U.D. | U.D. |
| Untreated | 46 (±9) | 2471 (±503) | 16193 (±2951) | 220 (±43) | U.D. |
| Dex | 57 (±12)# | **3621 (±725)*#** | **31022 (±6005)*#** | **143 (±29)*#** | **100 (±19)*#** |
| IL10 | **109 (±19)*** | 2690 (±518) | 12305 (±2317) | 226 (±42) | 55 (±14)* |

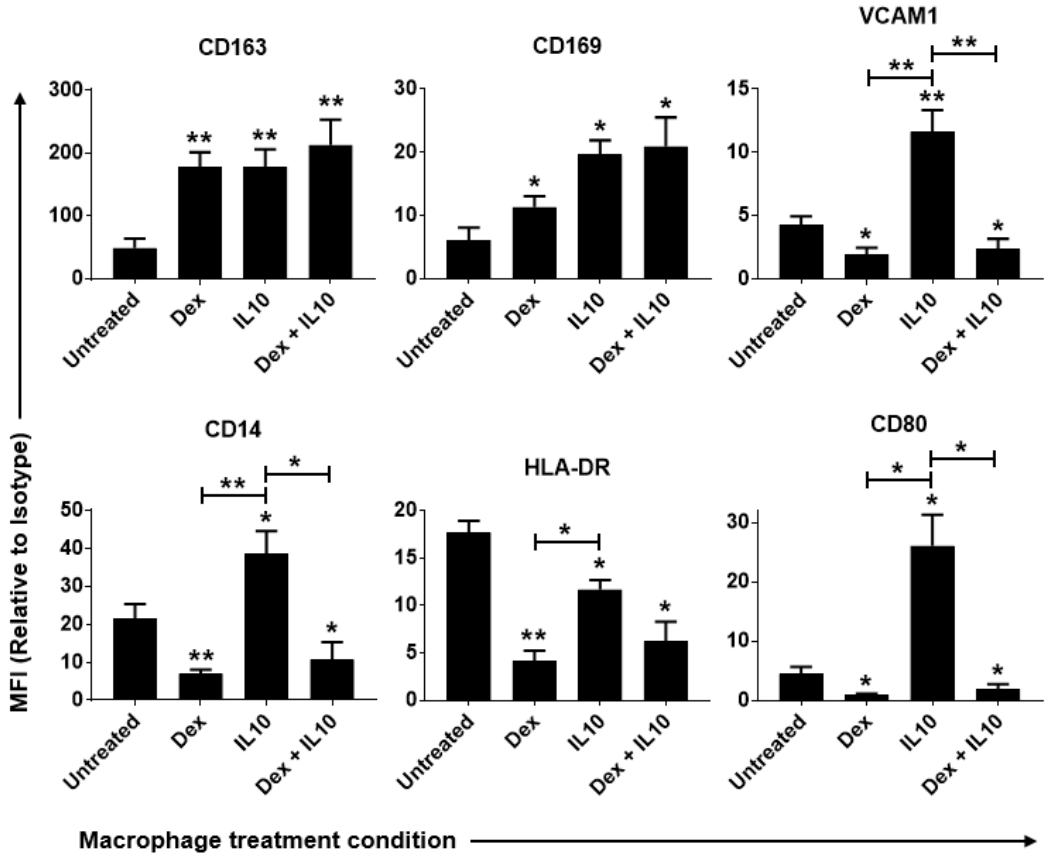
The ELISAs for the different molecules confirm that CCL2 was consistently present in greatest abundance in all cultures that contained macrophages. However, its levels in co-culture supernatants involving dexamethasone treated macrophages were 1.9- and 2.5-fold compared to conditions involving untreated or IL10 modulated macrophages, respectively. IL8 was another molecule detected at high levels in all supernatants except for erythroblasts cultured alone, with the greatest level again for the condition involving dexamethasone supplemented macrophages (1.5- and 1.3-fold compared to untreated and IL10 treated macrophage conditions, respectively). Dexamethasone stimulated macrophages secreted almost twice the

amount of Serpin E1 that was produced by IL10 modulated macrophages. On the other hand, IL1ra was expressed more weakly by dexamethasone treated cells while IL10 modulated macrophages released 1.9-fold greater levels or GROα. Therefore, it appears that the treatment of macrophages with IL10 and especially with dexamethasone triggered a senescence-associated secretory phenotype that may have affected erythropoiesis.

### 4.3.11 Surface marker expression on macrophages following simultaneous treatment with dexamethasone and IL10

A study in 1999 highlighted the role of IL10 in increasing human monocyte sensitivity to dexamethasone, suggesting a potential function of the cytokine in regulating the glucocorticoid receptor (Franchimont et al., 1999). Thus, examining whether simultaneous dexamethasone and IL10 treatment of human macrophages had any further effect on the phenotype than just treating the cells with either molecule alone proved of interest. In all situations, $10^{-6}$M dexamethasone and 10ng/ml IL10 were used when adding both molecules to macrophages since, when supplemented separately, these concentrations were found to have the optimal effects on the macrophage phenotype as well as on erythroblast expansion during co-culture.

**FIGURE 4.13. Surface marker expression after simultaneous addition of both dexamethasone and IL10 to human macrophages.** *Cell surface levels of the glycoproteins CD163, CD169, VCAM1, CD14, HLA-DR and CD80 were determined by direct staining and detection by flow cytometry. The mean and SEM of 3–5 independent biological repeats are shown. The different treatment conditions are compared to untreated macrophages, unless indicated otherwise. *P<0.05, **P<0.01 (one-way ANOVA with Tukey's post-test correction).*



Treatment of macrophages with both dexamethasone and IL10 (dexamethasone + IL10) induced similar VCAM1, CD14, HLA-DR and CD80 surface levels as macrophages that were treated with only dexamethasone (**Figure 4.13**). This suggests dexamethasone was more potent in its effects on macrophage surface marker expression than IL10. Although not statistically significant, there was a trend towards slightly further elevated surface expression of CD163 in dexamethasone + IL10 treated macrophages than that in dexamethasone or IL10 only stimulated cells. Therefore, this suggests that IL10-induced CD163 expression was mildly strengthened by glucocorticoid stimulation of macrophages and hence it is possible that the two molecules may activate different signal transduction pathways that augment CD163 surface levels.

The morphology of dexamethasone + IL10 supplemented macrophages displayed common features seen when macrophages were cultured with each modulator alone. For example, most macrophages under these conditions were rounded, reminiscent of dexamethasone treatment, but also showed extensive vacuolisation as observed when IL10 was used alone (**Appendix 4.4**). Furthermore, dexamethasone + IL10 treatment did not adversely affect macrophage viability (**Table 4.4**).

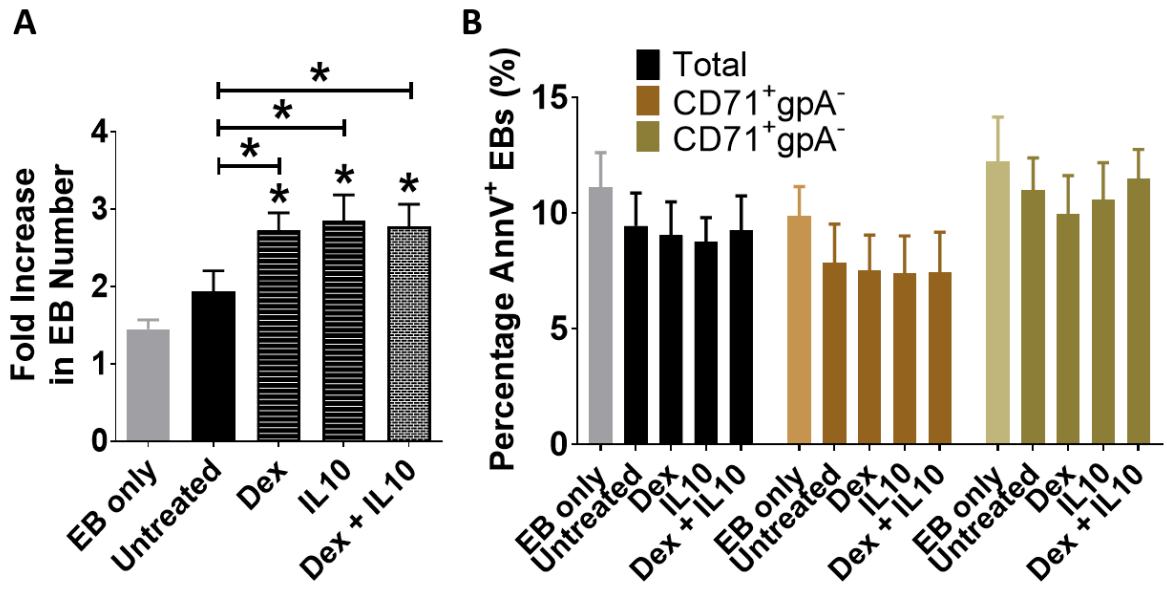
**TABLE 4.4. Effect of dexamethasone + IL10 treatment on macrophage viability.** *The percentage of viable (AnnV$^-$7AAD$^-$) macrophages following no treatment or treatment with dexamethasone ($10^{-6}$M), IL10 (10ng/ml) or dexamethasone + IL10 ($10^{-6}$M and 10ng/ml, respectively) was measured by dual staining for AnnexinV and 7AAD and subsequent detection on a flow cytometer. The mean and SEM of 3 biological repeats are shown. There is no significant difference in the viability of macrophages following their treatment with dexamethasone and IL10 individually or in combination; P > 0.05 in each case.*

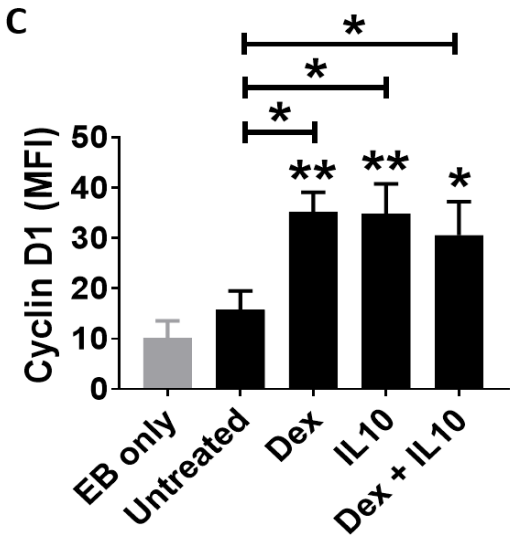
| Dexamethasone concentration (M) | AnnV$^-$7AAD$^-$ macrophages (%) |
|---|---|
| Untreated | 91.6 (± 5.9) |
| Dex | 88.8 (± 6.5) |
| IL10 | 90.3 (± 6.2) |
| Dex + IL10 | 87.9 (± 7.4) |

### 4.3.12 Erythroblast expansion during co-culture with macrophages simultaneously treated with dexamethasone and IL10

Having previously observed improvements in erythroblast expansion following co-culture with either dexamethasone or IL10 induced macrophages (**Figure 4.5**), it was of interest to explore whether simultaneously treated dexamethasone + IL10 cells could further elevate erythroid expansion in line with the trend for increased CD163 expression.

**FIGURE 4.14. Erythroid expansion, viability and cyclin D1 protein expression in erythroblasts cultured with dexamethasone and IL10 simultaneously supplemented macrophages.** *The fold increase in erythroblast number compared to the initial density (6 x $10^3$ cells/well) was determined after culture alone or with untreated, dexamethasone ($10^{-6}$M), IL10 (10ng/ml) or dexamethasone + IL10 ($10^{-6}$M and 10ng/ml) treated macrophages via haemocytometer counts of trypan blue excluded live cells (A). The percentage of apoptotic (AnnV$^+$) cells for the various erythroid populations (total, CD71$^+$gpA$^-$, CD71$^+$gpA$^+$) cultured under the different conditions was determined by staining for CD71, gpA and AnnexinV and detected on a flow cytometer (B). Cyclin D1 protein levels in the total erythroblast population was determined by intracellular staining and flow cytometric detection (C). The mean and SEM of 8 (for expansion) or 4 (for viability/cyclin D1) biological repeats are shown. *P<0.05, **P<0.01 compared to EB only unless indicated otherwise (one-way ANOVA with Tukey's post-test correction).*





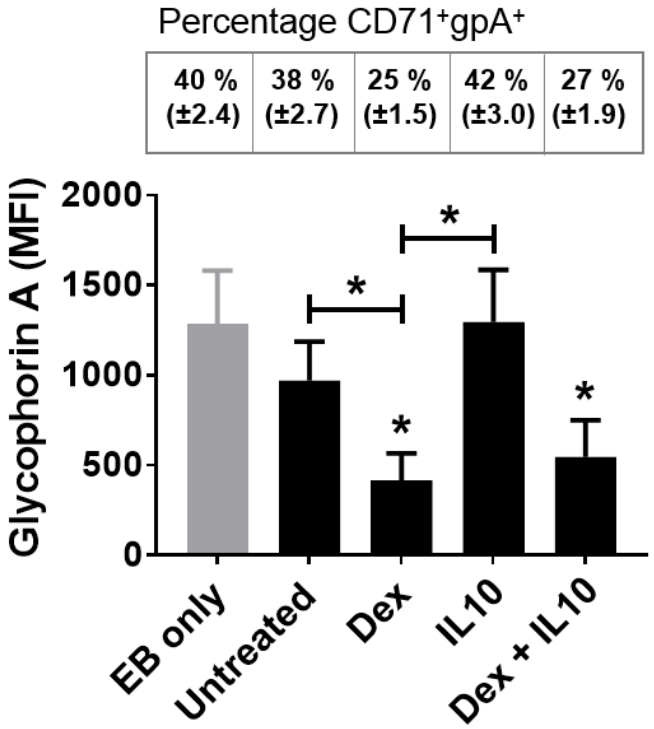

Dexamethasone + IL10 stimulated macrophages supported erythroblast expansion to a similar extent as macrophages treated with either molecule alone (**Figure 4.14A**). It was surprising that dexamethasone + IL10 simultaneously supplemented macrophages did not give a trend towards a further improvement in erythroblast expansion despite inducing slightly greater CD163 surface expression than macrophages modulated by dexamethasone or IL10 alone (**Figure 4.13**). This might indicate that the further increase in macrophage CD163 beyond the levels induced by dexamethasone or IL10 on their own was redundant in terms of further supporting erythroblast expansion. Moreover, no change in the percentage of $AnnV^+$ erythroid cells was found, for any erythroid population, during the co-culture with dexamethasone + IL10 modulated macrophages (**Figure 4.14B**) and cyclin D1 protein levels were also comparable to cultures involving either dexamethasone or IL10 only supplemented macrophages (**Figure 4.14C**). This suggests that the similar rates in erythroblast expansion for co-cultures involving the individual or dual treatment of macrophages with dexamethasone and IL10 were caused by

comparable increases in erythroid proliferation as opposed to any improvements in erythroblast survival.

### 4.3.13 Erythroid maturation following culture with macrophages simultaneously treated with dexamethasone and IL10

While dexamethasone + IL10 simultaneously treated macrophages did not further affect erythroblast expansion, it was of interest to examine if these macrophages differentially modified erythropoiesis by altering the maturation of co-cultured erythroid cells. For this reason, the maturation of erythroblasts under the various culture conditions was studied.

**FIGURE 4.15. Erythroid maturation following culture with dexamethasone and IL10 simultaneously modulated macrophages.** *Erythroblasts grown under the various culture conditions were collected after 48hours and stained for CD71, gpA and DAPI and subsequently detected on a flow cytometer. The surface expression of gpA on DAPI negative viable erythroblasts is shown to illustrate the gradual change in erythroid maturation. The percentages of mature (CD71⁺gpA⁺) erythroid cells are shown in the panel above the histogram. The mean and SEM from 6 different donors is shown. *P<0.05 compared to EB only or as indicated otherwise (one-way ANOVA with Tukey's post-test correction).*




Dexamethasone + IL10 treated macrophages blocked erythroid maturation to a similar extent as dexamethasone modulated macrophages, lowering the proportion of $CD71^{+}gpA^{+}$ erythroblasts by 11% compared to the co-culture with untreated

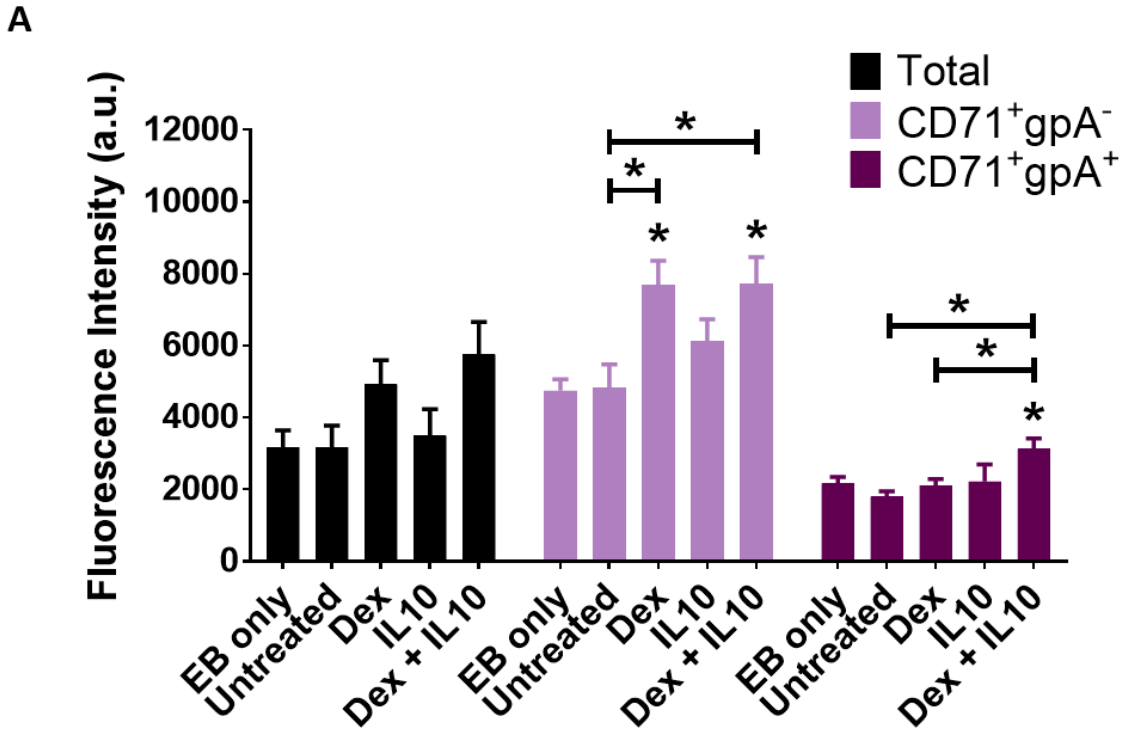
macrophages. Therefore, these findings suggest that the simultaneous addition of both dexamethasone and IL10 did not induce an altered macrophage phenotype that was any more advantageous to erythroblast expansion or maturation when compared to macrophages individually treated with only dexamethasone or IL10.

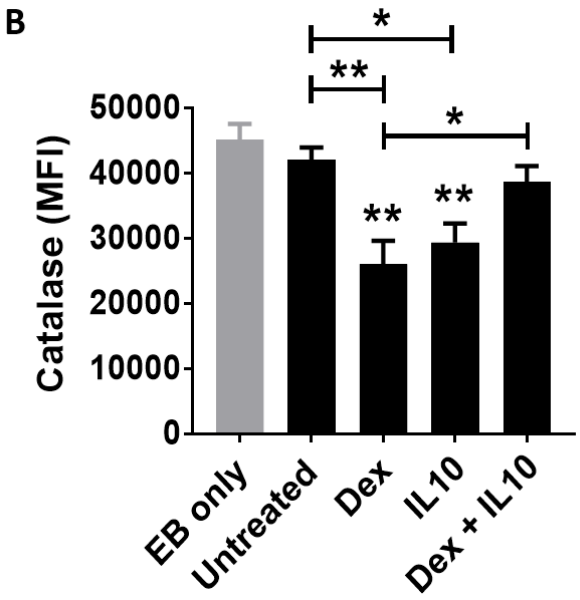
A defining feature of macrophages is their intrinsic phagocytic capabilities and hence it was speculated whether these cells might also have displayed altered erythrophagocytic capacities following their differential treatment with dexamethasone or IL10 that may have subsequently affected erythroid expansion and/or maturation. The trend towards slightly increased uptake of *E.coli* bioparticles by macrophages supplemented with dexamethasone or IL10, either individually or together, suggests the cells had somewhat increased phagocytic capabilities (**Appendix 4.5**). This may have been indicative of slightly heightened erythrophagocytic abilities of modulated macrophages which could potentially have played a part in reducing erythroid expansion or, if phagocytosing dead erythroid cells, in improving erythroblast viabilities. Nonetheless, the uptake of *E.coli* bioparticles may not be a reliable indication for the capacity of macrophages to phagocytose erythroid cells because *E.coli* is a pathogen that could potentially have activated Toll-like receptors or other ligands on the macrophage surface, thus modifying its functions.

### 4.3.14 ROS levels in erythroblasts following co-culture with the differentially stimulated macrophages

Having previously found differences in the intracellular ROS levels of erythroblasts cultured with dexamethasone or IL10 treated macrophages, it was of interest to determine whether dexamethasone + IL10 simultaneously modulated macrophages also affected free radical release by erythroblasts. Protein expression of the antioxidant catalase was also measured to see if this displayed the same pattern as erythroid cells grown with either dexamethasone or IL10 stimulated macrophages or whether it was associated to the intracellular ROS.

**FIGURE 4.16. Reactive oxygen species in erythroblasts cultured with the differentially treated macrophages.** *Intracellular ROS levels in the various erythroid populations (total, CD71$^+$gpA$^-$, CD71$^+$gpA$^+$) were measured via DCFDA staining and DCF excitation by flow cytometry of DAPI negative viable cells (A). Catalase protein levels in erythroblasts (total erythroid population) were measured by indirect intracellular staining of fixed cells and were detected on a flow cytometer (B). The mean and SEM for 4 independent biological repeats from different donors are shown. Data are the mean and SEM of 6 (for ROS) or 4 (for catalase) separate donors. *P<0.05, **P<0.01 compared to EB only or as indicated otherwise (one-way ANOVA with Tukey's post-test correction).*





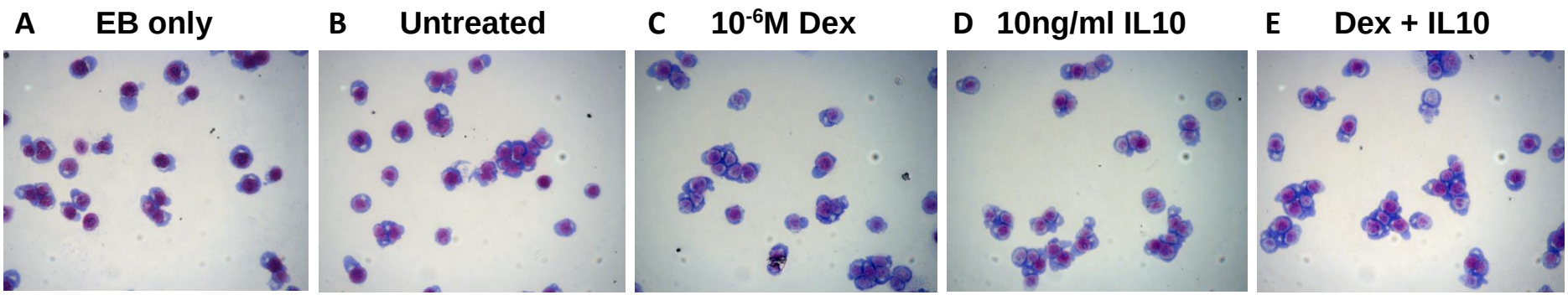

Again, dexamethasone + IL10 modulated macrophages had similar effects on erythroid cells as dexamethasone only treated macrophages in terms of the comparable elevation in intracellular ROS in immature ($CD71^{+}gpA^{-}$) erythroblasts (**Figure 4.16A**). However, simultaneous dexamethasone + IL10 supplemented macrophages also heightened free radical release in the more mature ($CD71^{+}gpA^{+}$) erythroblasts by 1.7-fold compared to the co-culture with untreated macrophages. This suggests that the dually treated macrophages triggered a more substantial oxidative stress that was present in both erythroid populations.

Catalase levels in erythroblasts co-cultured with dexamethasone + IL10 modulated macrophages were distinct from the catalase protein expression in erythroid cells grown with the individually targeted macrophages, with 1.5-fold greater levels compared to cultures involving dexamethasone only treated cells (**Figure 4.16B**). The changes in erythroid ROS and catalase levels following co-culture with dexamethasone + IL10 treated macrophages suggest altered intracellular signalling

cascades were induced and it is possible that the two may have been linked. More precisely, the heightened ROS levels may have activated antioxidant processes such as catalase enzyme expression in an attempt to overcome the increased oxidative burden.

Finally, morphological analyses of erythroblasts cultured under the various conditions revealed no significant differences and showed a majority of cells to be at the basophilic erythroblast stage (**Figure 4.17**).

**FIGURE 4.17. Erythroblast morphology following co-culture with differentially modulated macrophages.** *The morphology of erythroblasts is shown after culture for 48 hours alone without macrophages (A) or following co-culture with macrophages that were previously untreated (B) or treated with dexamethasone ($10^{-6}$M) (C), IL10 (10ng/ml) (D), or dexamethasone and IL10 ($10^{-6}$M and 10ng/ml, respectively) (E). Cells were cytospun and subsequently stained with Leishman's stain. Similar morphologies were observed in 3 separate donors, but images show erythroblasts derived from the same donor.*



## 4.4 Discussion

Although glucocorticoids and IL10 both induce a 'deactivated' M2c-polarised state of macrophage activation (Martinez and Gordon, 2014), with high CD163 but low MHC II expression, similarities and differences were observed in cell surface marker expression of macrophages treated with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) (**Figures 4.1** and **4.2**). Targeting macrophages with either of these molecules similarly increased the cell surface expression of CD163, CD169 and CD16, but reduced HLA-DR levels compared to untreated cells, with maximal effects seen with $10^{-6}$M dexamethasone or 10ng/ml IL10. Furthermore, while dexamethasone ($10^{-6}$M) treatment of macrophages diminished CD14, CD80 and VCAM1 receptor expressions, IL10 (10ng/ml) supplementation of macrophages elevated surface levels of the same glycoproteins. Hence, IL10 caused mostly upregulation of cell surface markers, while dexamethasone induced heightened expression of some receptors but decreased levels of others. These results illustrate the complexity of the macrophage phenotype even within the same M2c subclass, which possibly have even more intricate functions beyond the simple immunoregulation and tissue remodelling properties that have been attributed to this group (Martinez and Gordon, 2014). More precisely, downregulation of the antigen-presenting molecule HLA-DR and the costimulatory protein CD80 suggest long-term treatment with dexamethasone lowers adaptive immune mechanisms. This also differed from observations by van de Garde and colleagues who, in contrast, reported no effects on these molecules when dexamethasone was administered to maturing human macrophages (van de Garde et al., 2014). Although, similarly to our findings, the group found no difference in CD86 surface levels after adding dexamethasone for a range between 3 and 7 days

compared to untreated cells, they contrastingly saw no change in HLA-DR surface protein. This dissimilarity could be due to variations in the methods, for instance van de Garde and colleagues used a 10-fold lower concentration of dexamethasone and furthermore they added the steroid to monocytes from day 0 during maturation into macrophages, whereas the studies in this thesis added dexamethasone to day 6 monocyte-derived macrophages.

Conversely, the augmentation of the costimulatory molecule CD86 by IL10 also varied to the observations by van de Garde et al. who in comparison had no significant differences in CD86 protein levels compared to untreated cells. Again, this could be due to the fact that they added the cytokine to monocytes for five days as opposed to treatment of more mature cells for four days, suggesting IL10 might only promote T-cell activating capabilities in macrophages but not monocytes. Nevertheless, the group found the same elevation in CD14, indicating that both immature and more mature macrophages improve their capacity to detect bacterial LPS in response to IL10 stimulation. Therefore, while this thesis found that IL10 improved this component of the innate immune response, dexamethasone appeared to weaken it.

Apart from the clear phenotypic changes in terms of cell surface marker expressions, treatment of human macrophages for four days with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) also led to morphologic alterations. The addition of dexamethasone caused macrophages to diminish their microvilli formation and round up, as has been observed in several other studies (Falchi et al., 2015; Giles et al., 2001; Kraaij et al., 2011), whereas IL10 treated macrophages maintained their elongated shape (**Figure 4.3**). The changes in morphology, especially the rounding up of

macrophages following dexamethasone supplementation, were molecule specific induced effects that were not reflective of a decline in viability (**Tables 4.1** and **4.2**). Electron microscopic examination also revealed a substantial decrease in pseudopodia in macrophages treated with dexamethasone, which likely accounted for their rounder cell shape (**Figure 4.4**). Interestingly, dexamethasone ($10^{-6}$M) and especially IL10 (10ng/ml) stimulation were also found to trigger extensive cytoplasmic vacuolisation, which is reminiscent of degenerative changes and probably represented swelling of the rough endoplasmic reticulum. The vast majority (approximately 80-90%) of dexamethasone or IL10 modulated macrophages showed these changes in heightened vacuole formation, which is suggestive of cellular stress.

The co-culture of erythroblasts with either dexamethasone or IL10 treated macrophages significantly improved erythroid expansion, with maximal effects at each time point (days 8, 10 and 12) with the use of $10^{-6}$M dexamethasone or 10ng/ml IL10 (**Figure 4.5**). This elevated expansion supported by either of the differentially modulated macrophages was found to be as a result of a faster progression through the cell cycle rather than due to an increase in erythroblast survival (**Figures 4.6 – 4.8**). Thus, stimulation of macrophages with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) activated signalling pathways in erythroblasts for heightened proliferation.

The heightened expansion was associated with a blocked maturation of erythroid cells following co-culture with $10^{-6}$M dexamethasone treated macrophages, which reduced the proportion of $CD71^{+}gpA^{+}$ erythroblasts by 13.5% compared to cultures involving untreated macrophages (**Figure 4.9**). In contrast, IL10 (10ng/ml)

supplemented macrophages did not affect erythroid maturation. This indicates that although both types of differentially modulated macrophages promoted erythroid proliferation, only IL10 treated cells were able to simultaneously maintain their maturation. Several studies have already demonstrated the direct role of dexamethasone in augmenting erythroid progenitor proliferation whilst inhibiting ordered maturation (Leberbauer et al., 2005; von Lindern et al., 1999). However, the work carried out in this thesis differs from previous studies in that it involved targeting dexamethasone directly to macrophages prior to co-culture with erythroblasts, so that any changes in erythroid development were as a consequence of an altered macrophage phenotype resulting from glucocorticoid or IL10 treatment as opposed to effects of the molecules on erythroblasts themselves. It is unlikely that the observed effects on erythroid development were as a cause of any residual dexamethasone or IL10 remaining in the wells during the addition of erythroblasts to cultures because in each case the culture medium was completely aspirated and the wells briefly washed before supplementing with the co-culture medium. Thus, one can confidently conclude that the differences in erythroblast development are due to the influences of the modulated macrophages themselves.

Differences in erythroid oxidative stress were also found during co-culture with the differentially modulated macrophages (**Figure 4.10**). Dexamethasone ($10^{-6}$M) supplemented macrophages enhanced free radical generation specifically in immature ($CD71^{+}gpA^{-}$) but not in more mature ($CD71^{+}gpA^{+}$) erythroblasts whereas IL10 (10ng/ml) treated macrophages did not affect the intracellular ROS levels in either erythroid population. The augmented erythroid oxidative burden for co-cultures including dexamethasone modulated macrophages could be explained by

the diminished catalase enzyme expression but, surprisingly, erythroblasts cultured with IL10 stimulated cells also displayed significantly lower catalase levels (**Figure 4.11**). Therefore, IL10 treated macrophages may have activated other processes in erythroblasts downstream of the pathways implicated in the inhibited catalase expression that subsequently brought free radicals back to homeostatic levels. One possibility is that erythroid cells grown in the presence of dexamethasone versus IL10 stimulated macrophages displayed altered metabolic activities that led to the different ROS levels. For instance, IL10 treated macrophages may have induced a shift in erythroid cells towards the pentose phosphate pathway, generating intracellular NADPH that acts as an antioxidant (Birben et al., 2012). Another possibility is that the accumulation of such vast quantities of pro-inflammatory cytokines, such as CCL2 and IL8, in co-cultures containing dexamethasone modulated macrophages may have driven the heightened intracellular ROS in erythroid cells because chronic inflammation has been shown to induce oxidative stress and excess ROS (Bartsch and Nair, 2006).

The investigation of cytokines and chemokines in co-culture supernatants revealed a number of senescence-associated molecules, namely CCL2, IL8, IL1ra, Serpin E1 and GROα, most of which normally have important roles as chemoattractants during an inflammatory response. All of the soluble molecules detected by the human cytokine array (**Figure 4.12**) were macrophage-derived as opposed to being secreted by erythroblasts, as confirmed by macrophage only culture supernatants. Therefore, treatment of macrophages with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) not only altered their cell surface receptor expression but also affected their secretory profiles.

The ELISAs more reliably measured the levels of the senescence-associated factors in supernatants following co-culture with the differentially modulated macrophages (**Table 4.3**). The enhanced production of CCL2 and IL8 by dexamethasone treated compared to untreated macrophages (1.9- and 1.5-fold, respectively), along with the observed differences in surface glycoprotein expression (**Figure 4.1**), suggest there were highly strengthened innate immune elements in dexamethasone modulated cells that might provoke an inflammatory reaction. However, the levels of both of these cytokines were found to be lower and comparable in supernatants involving untreated or IL10 modulated macrophages. Furthermore, it was found that dexamethasone stimulated macrophages secreted a greater amount of Serpin E1, whereas IL10 treated macrophages produced more GROα. Interestingly, IL1ra, the only anti-inflammatory cytokine detected in co-culture supernatants, was released at similar levels by either untreated or IL10 modulated macrophages but at approximately 65% by dexamethasone treated cells. An early study found that injecting CBA/H mice with recombinant human IL1ra at both seventeen and two hours prior to sacrifice diminished the number of BFU-Es but did not affect reticulocytosis or erythrocytosis (Jovcic et al., 1996). Therefore, it would be noteworthy to determine whether the reduced levels of IL1ra in human *in vitro* co-cultures containing dexamethasone treated macrophages in any way contributed to the elevated erythroblast expansion via increased erythroid progenitor numbers. Another possibility is that IL1ra plays a role in dampening the senescence-associated inflammation and hence potentially explaining why dexamethasone treated macrophages had such an extreme senescence-associated secretory phenotype.

Nevertheless, future work to uncover the precise functions of each of the soluble molecules would be required.

The different cytokines detected in co-culture supernatants under the various treatment conditions provide further evidence for altered macrophage phenotypes during dexamethasone or IL10 stimulation. In line with the findings of reduced antigen presenting and costimulatory molecule expressions, stimulation of macrophages with dexamethasone appeared to negatively modulate the IFN pathway but positively regulate the MyD88 pathway. Thus, this would suggest that in the case of an infection, these dexamethasone modulated macrophages would spare anti-viral reactions while amplifying antibacterial responses. This is in accordance with previous studies that used the synthetic steroid fluticasone propionate (van de Garde et al., 2014). Contrastingly, IL10 treatment of macrophages seemed to induce more adaptive components, probably triggering similar, if not the same, signalling cascades initiated by TLR4/IFN receptor engagement for heightened anti-viral responses. This is suggested by the simultaneous increases in surface costimulatory molecules (CD80 and CD86) and also of Fc receptors (CD16 and CD64) that normally function in antibody-dependent cell mediated cytotoxicity, in addition to the unaltered secretion of the pro-inflammatory cytokines CCL2 and IL8 compared to untreated macrophages. Therefore, it is evident that although dexamethasone and IL10 both induced what is known as an M2c macrophage phenotype, which is characterised by elevated CD163 but reduced MHC II expression, the supplementation of macrophages with either of these molecules differentially affected other surface markers and secreted factors.

These altered macrophage phenotypes may in turn affect erythropoiesis in distinct ways.

The simultaneous administration of dexamethasone and IL10 (dexamethasone + IL10) to macrophages may have resulted in some cooperation between glucocorticoid nuclear receptor-dependent pathways and IL10R-mediated signalling cascades. Dexamethasone + IL10 treatment of macrophages elevated CD163 surface marker expression to a small extent when compared to dexamethasone or IL10 supplementation alone, but this was not statistically significant (**Figure 4.13**). Macrophages treated with both dexamethasone + IL10 had no significant change in CD16 or CD169 surface levels but expressed VCAM1, CD14, HLA-DR and CD80 to similar amounts as dexamethasone only stimulated cells. The viabilities of macrophages simultaneously treated with dexamethasone and IL10 were comparable to those of cells stimulated by either molecule alone (**Table 4.4**). These data illustrate the plasticity of macrophages and their ability to adopt various phenotypes in order to respond to different signals in their microenvironmental milieu.

The trend towards somewhat heightened CD163 expression by dexamethasone + IL10 treated macrophages, albeit not statistically significant, when compared to the dexamethasone or IL10 only modulated cells was not associated with a further improvement in erythroblast expansion (**Figure 4.14**). This was corroborated by the unchanged viability and cyclin D1 protein expression of erythroid cells, suggesting they had unaltered survival and proliferative rates. Thus, it would appear that the extra surface expression of macrophage CD163 had no further effect on erythroblast

expansion and so there may be a threshold level for CD163 beyond which it becomes redundant in its effects on erythropoiesis.

Although dexamethasone + IL10 treated macrophages did not further affect expansion, they inhibited erythroid maturation to a similar extent as dexamethasone only stimulated macrophages (**Figure 4.15**). The dually modulated macrophages lowered the glycophorin A surface expression on erythroblasts by 0.6-fold, which equated to an 11% reduction in the proportion of $CD71^{+}gpA^{+}$ cells compared to co-cultures including untreated macrophages. Furthermore, dexamethasone + IL10 modulated macrophages also augmented ROS levels in immature ($CD71^{+}gpA^{-}$) erythroblasts in a similar manner as dexamethasone only treated macrophages, while significantly elevating the oxidative stress in more mature ($CD71^{+}gpA^{+}$) erythroid cells as well (**Figure 4.16**). In contrast to macrophages individually supplemented with either dexamethasone or IL10, dexamethasone + IL10 treated macrophages did not block catalase protein expression in co-cultured erythroblasts and this may have been necessary as a protective mechanism to counterbalance any further increase in ROS, preventing death of these cells. Thus, the treatment of macrophages with both dexamethasone and IL10 overall induced a phenotype and altered erythropoiesis in a fashion that was more akin to the effects caused by dexamethasone only modulated macrophages and may indicate that the glucocorticoid was more potent in activating downstream erythroid signalling cascades than IL10. Nevertheless, the morphology of all erythroblasts cultured under the various conditions was normal, with a substantial proportion at the basophilic erythroblast stage (**Figure 4.17**).

## 4.5 Summary of Findings

- MCSF differentiated macrophages treated with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) for four days induced 'deactivated' M2c-polarised states but with differences in certain cell surface markers (**A**) and secreted cytokines (**B**) compared to untreated cells:

**A**

| | **Dexamethasone ($10^{-6}$M)** | **IL10 (10ng/ml)** |
|---|---|---|
| CD163 | ↑ | ↑ |
| CD169 | ↑ | ↑ |
| CD16 | ↑ | ↑ |
| HLA-DR | ↓ | ↓ |
| **CD14** | ↓ | ↑ |
| **CD86** | **=** | ↑ |
| **CD80** | ↓ | ↑ |
| **VCAM1** | ↓ | ↑ |
| **CD64** | **=** | ↑ |

**B**

| **Dexamethasone ($10^{-6}$M)** | **IL10 (10ng/ml)** |
|---|---|
| ↑ CCL2, ↑ IL8, ↑ Serpin E1, ↓ IL1ra | ↑ GROα |

- Supplementation with either $10^{-6}$M dexamethasone or 10ng/ml IL10 to M2 macrophages caused either a rounder or more elongated morphology, respectively.

- Dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) supplemented macrophages significantly elevated erythroid expansion, as well as increasing $G_0/G_1$ to S and $G_2$/M erythroid cell cycling on day 8.

- Dexamethasone ($10^{-6}$M) treated macrophages blocked erythroid maturation, whereas IL10 (10ng/ml) treated macrophages did not alter the percentage of $CD71^+gpA^+$ erythroblasts.

- Addition of both dexamethasone ($10^{-6}$M) and IL10 (10ng/ml) to macrophages gave a trend towards further increased surface CD163 levels, but this was not significant, when compared to cells treated with only dexamethasone or IL10.

- Dexamethasone + IL10 stimulated macrophages did not further enhance erythroid expansion compared to co-cultures with dexamethasone only or IL10 only treated macrophages.

- Dexamethasone + IL10 supplemented macrophages reduced erythroid maturation to a similar extent as co-cultures involving dexamethasone treated macrophages.

- Dexamethasone ($10^{-6}$M) as well as dexamethasone + IL10 treated macrophages increased erythroid intracellular ROS in the $CD71^+gpA^-$ erythroid population but not in $CD71^+gpA^+$ cells, whereas IL10 supplemented macrophages did not alter ROS levels.

- Erythroblasts co-cultured with either dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) treated macrophages had reduced catalase levels, whilst erythroid cells cultured with dexamethasone + IL10 modulated macrophages had comparable catalase levels to erythroblasts grown with untreated cells.

# Chapter 5: Macrophage mediated modification of erythroid FOXO3

## 5.1 Introduction

Tight regulation of erythroid cell proliferation, survival and differentiation is necessary during homeostasis. When this balance is compromised, it may lead to dyserythropoiesis and anaemia in acquired or congenital genetic disorders, and is central to pathology in malarial anaemia (McDevitt et al., 2004; Rivella, 2009). Specific nutrient deficiencies (particularly iron deficiency) also cause these imbalances. Growth factors and cytokines, for example EPO and SCF, act through their respective receptors to target transcription factors that control lineage commitment, growth and differentiation of erythroid cells (Bakker et al., 2004; Dolznig et al., 2002). The Forkhead box, Class O (FOXO) family of transcription factors control key cellular processes such as enhanced apoptosis, reduced cell cycle progression and redox balance (Brunet et al., 1999; Marinkovic et al., 2007). Mammals contain four related FOXO factors (FOXO1, FOXO3, FOXO4 and FOXO6) but erythropoiesis has been associated chiefly with FOXO3 (Bakker et al., 2007; Liang et al., 2015; Marinkovic et al., 2007; Zhang et al., 2014).

Numerous studies involving Foxo3$^{-/-}$ mice have demonstrated the critical role of FOXO3 in erythroid maturation (Zhang et al., 2014; Franco et al., 2014; Liang et al., 2015). The transcription factor has been found to promote terminal erythroid maturation via BTG1 induction and to facilitate both enucleation and mitochondrial clearance (Liang et al., 2015). FOXO3 activity has also been implicated in the control

of oxidative stress in erythroid cells through miR-451-mediated inhibition of 14-3-3ζ, which retains FOXO3 in a transcriptionally active form in the nucleus (Marinkovic et al., 2007; Yu et al., 2010). However, there are fewer studies that delve into the function of the transcription factor in repressing erythroid cell proliferation (Bakker et al., 2007) whereas copious investigations in the field of oncology have revealed the crucial role of inactivated FOXO3 in promoting cell proliferation (Cho et al., 2014; Lin et al., 2011; Lv et al., 2015; Wei et al., 2016).

The previous chapter identified an increase in cyclin D1 and decrease in catalase expression when erythroblasts were co-cultured with dexamethasone or IL10 treated macrophages. Cyclin D1 is negatively regulated whereas catalase is positively regulated by FOXO3. Falchi and co-workers suggested that FOXO3 activity may be altered in erythroid cultures stimulated with dexamethasone (Falchi et al., 2015) and it is also known that FOXO3 blocks cell cycle progression. Therefore, it was hypothesised that dexamethasone or IL10 modulated macrophages inactivated FOXO3 in erythroblasts to support erythroid expansion.

FOXO3 transcriptional activity can be affected in a number of ways, namely by its phosphorylation, subcellular localisation, DNA-binding capacity or altered transcriptional efficiency that results in varied FOXO3 protein levels (Boccitto and Kalb, 2011; Khatri et al., 2010). For this reason, several factors were measured in erythroblasts as indicators of altered FOXO3 activity: phospho-FOXO3 levels, total cellular FOXO3 abundance and 14-3-3ζ protein levels.

## 5.2 Methods

General methods are described in **chapter 2**. This section describes the methods specific to this chapter only.

### 5.2.1 Western blotting of phosphorylated and total FOXO3 in erythroblasts

1 x $10^6$ day 8 erythroblasts were harvested after 48 hours of growth alone or following co-culture with dexamethasone ($10^{-6}$M), IL10 (10ng/ml) or untreated macrophages. The cells were washed in 2ml ice-cold PBS and subsequently lysed in RIPA lysis and extraction buffer supplemented with Complete protease inhibitor cocktail tablet (Roche), 10mM EDTA, 1mM sodium orthovanadate (prepared in house) and 0.4mM Pefablock for 15 mins at 4ºC. The lysates were then centrifuged at 15,000 rpm at 4ºC for 10 mins. The total protein concentration per lysate was measured following the Bio-Rad DC Protein Assay protocol (Bio-Rad). The same concentration of total protein for each sample was diluted 1:4 in 4X Laemmli Sample Buffer (Bio-Rad) and heated at 95ºC for 1 min, followed by cooling on ice. The tank was set up for gel electrophoresis with a Criterion Tris-HCl Gel 4-20% (Bio-Rad) gel and filled with 1X running buffer (25mM Tris, 193mM glycine, 0.1% SDS, pH8.3, Bio-Rad). 30µl of each sample was loaded into a well of the gel along with 10µl of Precision Plus Protein Kaleidoscope Standards (Bio-Rad) for the molecular weight ladder and run for approximately 40 mins at 140V. The gel was then electrotransferred to a nitrocellulose membrane using the iBlot® system (Invitrogen) for 7 mins. The nitrocellulose membrane was then incubated in 25ml of blocking buffer (1X Tris Buffered Saline (TBS) + 0.1% Tween-20 + 3% BSA (Stem Cell Technologies)) for 1 hour at room temperature. The membrane was washed three

times for 5 mins each in 15ml of wash buffer (1X TBS + 0.1% Tween-20). One of the primary antibodies, either anti-FOXO3(phospho-T32) (Millipore) or anti-FOXO3(total cellular) (Upstate Biotechnology), was added at 1:1000 to the membrane, along with anti-Beta Actin (ab6276, Abcam) antibody at 1:12,000 as a control, in 10ml of blocking buffer and incubated with gentle agitation overnight at 4ºC. After three washes, as before, the membrane was incubated with the secondary goat anti-rabbit 680RB (LI-COR) and goat anti-mouse 800CW (LI-COR) antibodies (both at 1:5,000) in 10ml blocking buffer for 1 hour at room temperature. Four washes in 15ml wash buffer and two washes in 1X TBS were performed before detecting the proteins on the Odyssey® CLx Imaging System (LI-COR).

### 5.2.2 Immunofluorescence microscopy of FOXO3 subcellular localisation in erythroblasts

1 x $10^5$ erythroblasts, following 48 hours of co-culture with pre-treated macrophages, were collected and incubated on poly-L-lysine coated coverslips for 5 mins. The cells were gently washed in PBS and stained for surface glycophorin A by adding 5µl of CD235a antibody (Clone JC159, Dako) for 30 mins at 4ºC. The cells were then washed twice in PBS and fixed with 4% paraformaldehyde for 10 mins at room temperature. The fixed erythroblasts were washed twice with PBS and permeabilised by adding 0.05% Triton X-100 in PBS for 20 mins at room temperature. Blocking buffer (1% BSA in PBS) was then added and incubated for 1 hour at room temperature, followed by incubation with anti-FOXO3a (Upstate Biotechnology) at 0.5µg/ml overnight at 4ºC. The coverslips were again washed twice with PBS and subsequently incubated with goat anti-mouse IgG (H+L) Alexa Fluor 488 (Invitrogen) and goat anti-rabbit IgG (H+L) Alexa Fluor 555 (Invitrogen)

in blocking buffer for 1 hour at room temperature. After washing twice with PBS, one drop of VectorShield Hard Set mounting solution with DAPI (Vector Lab) was used to mount each coverslip onto slides and left to set for 24 hours in the dark. Standard fluorescence was observed under a wide-field microscope (DeltaVision Elite, MerckMillipore). Confocal images were obtained under a Zeiss 780 inverted microscope (Zeiss). At least 50 images per sample condition, with z-stacks of multiple planes to determine the middle of the cell, were analysed using a script created by Dominic Waithe (Weatherall Institute of Molecular Medicine) that assessed the fluorescence intensity of FOXO3 in the nucleus versus the cytoplasm of each cell.

### 5.2.3 Flow cytometry of intracellular proteins in erythroblasts

Erythroblasts from the different co-culture conditions were separately stained for the intracellular proteins cyclin D1 or 14-3-3ζ. After harvesting erythroblasts, they were washed in 1ml of FACS buffer and centrifuged at 300xg for 5 mins at 4ºC. For cyclin D1 intracellular staining, cells were fixed in 1ml of cold Fixation/Permeabilisation solution (Miltenyi Biotec) for 30 mins at 4ºC, whereas for 14-3-3ζ staining the cells were fixed in 80% ice-cold methanol for 5 mins at 4ºC. Erythroblasts were washed in 1ml FACS buffer and permeabilisation buffer was added (80µl 1X Permeabilisation buffer (Miltentyi Biotec) for cyclin D1; 80µl 0.1% PBS-Tween for 14-3-3ζ. 20µl FcR Blocking Reagent (Miltenyi Biotec) was added onto the cells already containing permeabilisation buffer and incubated for 5 mins at 4ºC. Then, FITC anti-cyclin D1 (BD Biosciences) or anti-14-3-3ζ (Abcam) was added and the cells incubated for 30 mins either at 4ºC (cyclin D1) or at 20ºC (14-3-3ζ). The cells were washed in 1ml cold 1X Permeabilisation Buffer (Miltenyi Biotec). For the directly conjugated

antibody (anti-cyclin D1) the cells were then run through the flow cytometer (FACScan LSR II, Becton Dickinson). However, for the detection of 14-3-3ζ a secondary antibody step was necessary before analysis by flow cytometry. This involved the addition of goat anti-rabbit Alexa Fluor 488 (Invitrogen) at 1:200 and incubating for 30 mins at room temperature.

### 5.2.4 siRNA targeted knockdown of surface CD163 on macrophages

Macrophages were cultured and differentiated with MCSF as described in section **2.1.3** (**chapter 2**). They were treated with IL10 to increase their surface CD163 expression as well as to induce their proliferation so that they would reach approximately 70% confluency on the day of transfection (day 9). 3µl of Lipofectamine-2000 (Life Technologies) were diluted in 150µl Opti-MEM I reduced serum medium (Life Technologies), vortexed briefly and left for 5 mins at room temperature. Meanwhile, siRNA (5, 50 or 100nM) was diluted in 150µl of Opti-MEM I medium, vortexed briefly and incubated for 5 mins at room temperature. The diluted siRNA was then added to the diluted Lipofectamine-2000 at a 1:1 ratio and incubated for at least 20 mins at room temperature. The 300µl of siRNA-lipid complex was then added straight to the macrophages in 1.2ml IMDM + 10% FBS (Sera Lab) without any penicillin/streptomycin or glutamine and placed in the 37ºC incubator. The media was changed to fresh IMDM + FBS without penicillin/streptomycin or glutamine every 24 hours. To determine whether macrophages were capable of taking up small interfering RNA (siRNA) in the first place, macrophages were transfected with the AllStars negative control siRNA (Qiagen), which is known to enter the RNA-induced silencing complex (RISC) and uptake by cells can be detected easily via fluorescence in the FITC channel. Once it

was established that macrophages could easily take up the AllStars siRNA, three different siRNAs for the targeted knockdown of CD163 were tested: siRNA1 (s17838), siRNA2 (s17839), siRNA3 (s17840) (Life Technologies). The transient knockdown of CD163, as well as potentially of any other surface markers, was measured by flow cytometry 2, 2.5, 3, 4.5 and 7 days post transfection. The most effective knockdown of macrophage CD163 with minimal toxicity was achieved with 5nM siRNA2 and the subsequent effects on erythroblasts were determined 48 hours after co-culture with these targeted macrophages.

### 5.2.5 Addition of cytokines to erythroblasts

The effects on erythroblasts of the main cytokines that were previously detected in co-culture supernatants were studied by adding recombinant human CCL2 (10,300, 31,000, 93,000 pg/ml), IL8 (900, 3,600, 14,400 pg/ml), IL1ra (22, 220, 2,200pg/ml), GROα (11, 110, 1,100 pg/ml) (PeproTech) and Serpin E1 (10, 100, 1,000 pg/ml) (BioLegend) directly into the culture medium of erythroblasts. The doses of each cytokine to be added to erythroblasts were chosen based on the highest concentration detected in the co-culture supernatants (the middle dose used) in addition to one higher and one lower dose. Functional studies into the effects of the various concentrations of these cytokines on erythroblasts were studied.

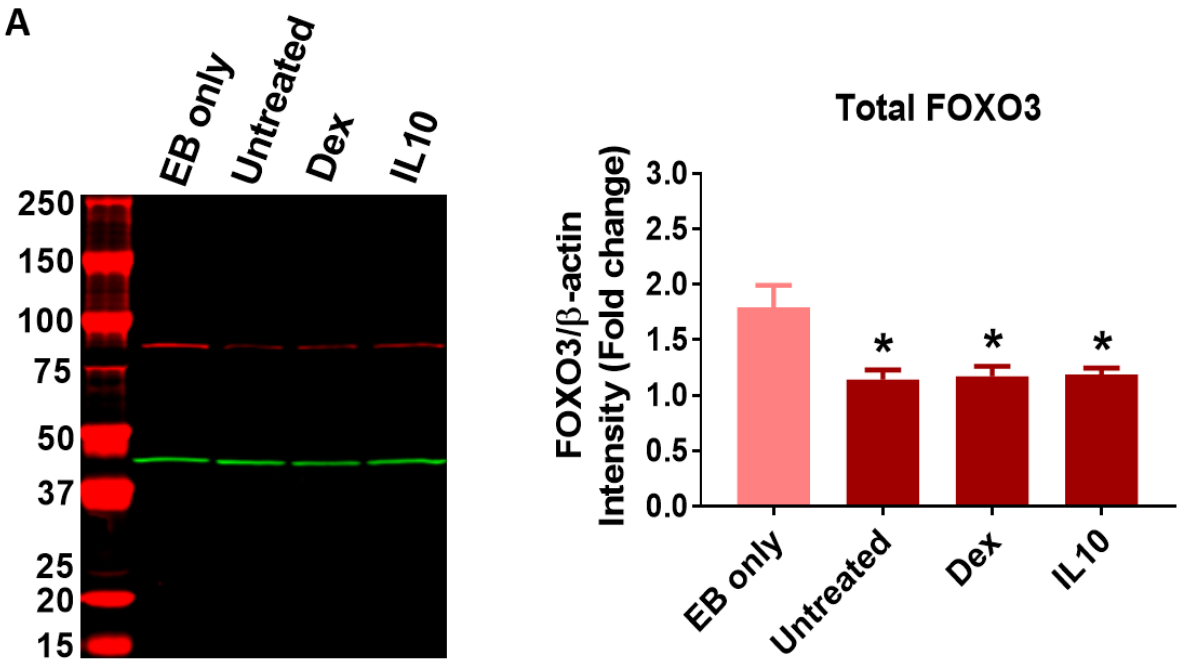
## 5.3 Results

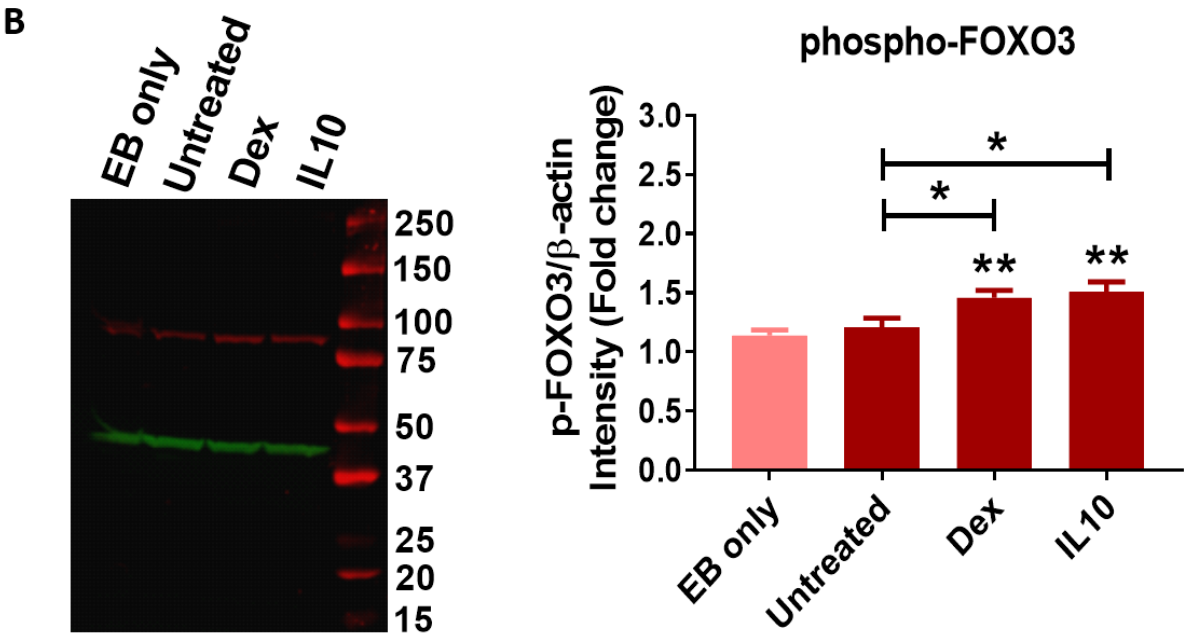
The decreased catalase and heightened cyclin D1 levels found in erythroblasts cultured with dexamethasone or IL10 modulated macrophages (**chapter 4**) suggested a potential role for reduced FOXO3 activity because FOXO3 directly regulates both catalase and cyclin D1 expression. In this chapter, various aspects related to FOXO3 activity are investigated in conditions where erythroblasts were grown with dexamethasone or IL10 treated macrophages, but not with both dexamethasone + IL10 stimulated cells. This is because the simultaneous treatment of macrophages with dexamethasone and IL10 provided no further benefit to erythroid expansion and inhibited erythroid maturation in a similar manner as macrophages treated with dexamethasone alone.

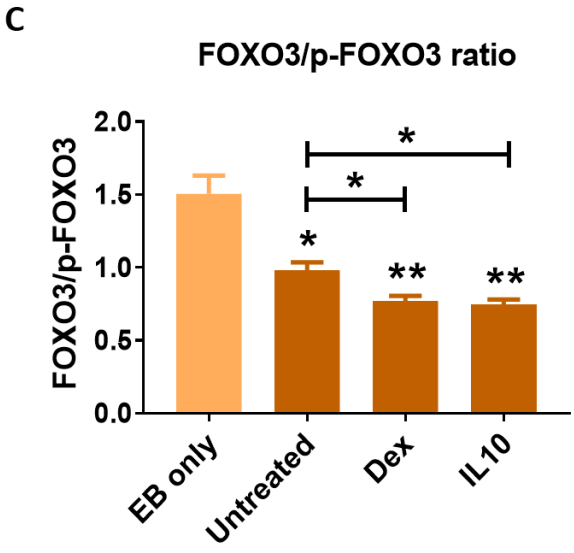
### 5.3.1 Total cellular levels and phosphorylation of FOXO3

Since the activity of the FOXO3 transcription factor is affected by its total protein levels and phosphorylation status (Biggs et al., 1999; Rinner et al., 2007), the abundance of FOXO3 as well as the amount of phosphorylated FOXO3 at residue T32 under each treatment condition was detected by western blot.

**FIGURE 5.1. Total cellular FOXO3 and phospho(T32)-FOXO3 protein levels in erythroblasts following culture alone or with untreated, dexamethasone or IL10 treated macrophages.** *'A' shows the total FOXO3 levels as red bands (~90kDa) with β-actin as the internal control shown by the green bands (42kDa) (left panel), and the fold change in total FOXO3/β-actin intensity is presented (right panel). 'B' shows the phospho-FOXO3(T32) levels as red bands (~90kDa) also with β-actin as the positive control as green bands (42kDa) (left panel), and the fold change in phospho-FOXO3/β-actin intensity is presented (right panel). 'C' illustrates the ratio of total FOXO3/phospho-FOXO3 as an indication of FOXO3 activity. The western blots in 'A' and 'B' (left panels) are representative blots of erythroblasts derived from one donor, while the right panels represent the mean and SEM for 4 independent biological repeats from separate donors. *P<0.05, **P<0.01, ***P<0.001 compared to EB only unless indicated otherwise (one-way ANOVA with Tukey's post-test correction).*






These data show that the presence of macrophages, regardless of whether they were untreated or treated with dexamethasone or IL10, had a similar effect of reducing the total cellular FOXO3 levels in co-cultured erythroblasts (**Figure 5.1A**). However, it was found that the culture of erythroblasts with dexamethasone or IL10 modulated macrophages also augmented the phosphorylation at residue T32 of

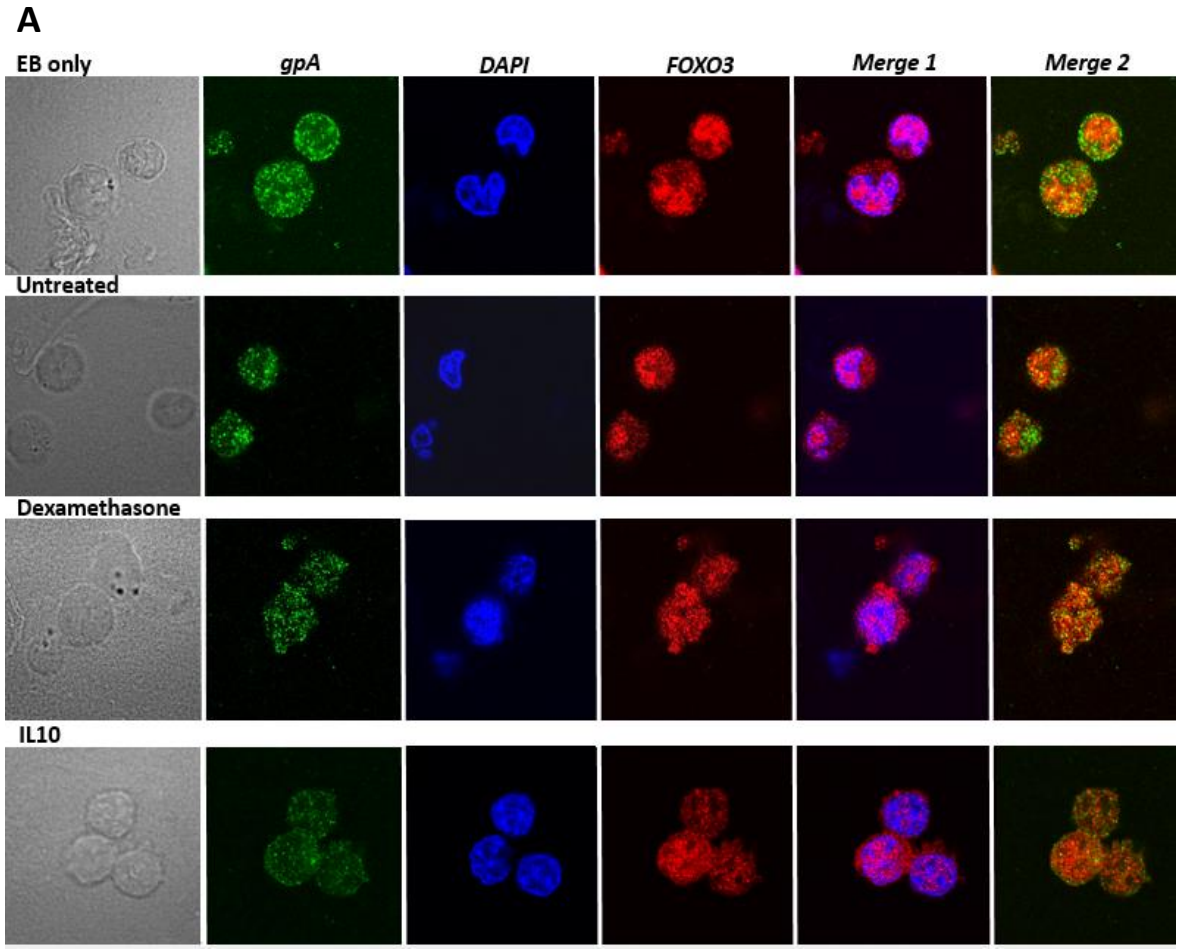
FOXO3 by 1.2-fold (p=0.0481) and 1.3-fold (p=0.0488), respectively, compared to the co-culture with untreated macrophages (**Figure 5.1B**).

Given that FOXO3 is inactivated by diminished total cellular levels as well as by its phosphorylation, presenting the ratio of FOXO3/phospho-FOXO3 is suggestive of the relative FOXO3 activity under each culture condition (**Figure 5.1C**). The reduced FOXO3/phospho-FOXO3 ratio for erythroblasts grown with dexamethasone or IL10 treated macrophages suggests these cells had the lowest FOXO3 activity, while the highest FOXO3/phospho-FOXO3 ratio for erythroblasts grown alone suggests they had the greatest FOXO3 activity.

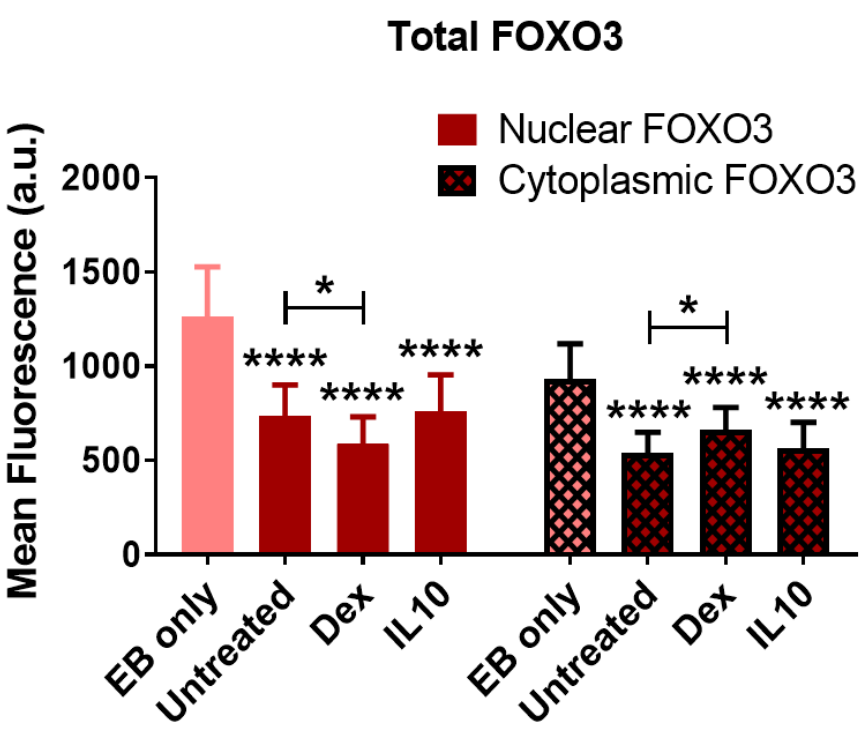
### 5.3.2 Subcellular localisation of FOXO3

It was important to determine the subcellular distribution of the FOXO3 transcription factor within erythroblasts because this contributes to its activity and ability to induce transcription of target genes involved in downstream signalling pathways that mediate erythroid development.

**FIGURE 5.2. Subcellular localisation of FOXO3 in erythroblasts cultured alone or with untreated, dexamethasone or IL10 modulated macrophages.** *'A' shows representative confocal images of erythroblasts from the same donor grown under the different conditions. Glycophorin A (gpA, green), cell surface stain; DAPI (blue), nuclear stain; FOXO3 (red), transcription factor subcellular localisation. Merge 1 are DAPI and FOXO3 combined; Merge 2 are gpA and FOXO3 combined. 'B' shows the fluorescence intensities of FOXO3 in the nucleus (pink/red) or cytoplasm (pink/red cross-hatched) of erythroblasts. Fluorescence intensities were measured in widefield (DeltaVision) microscopy images (N=60-65 per sample). The mean and SEM from 3 biological repeats are shown. *P<0.05, ****P<0.0001 compared to EB only or as indicated otherwise (one-way ANOVA with Tukey's post-test correction).*



**B**




All erythroblasts grown *in vitro* under the different culture conditions had detectable FOXO3 within the nucleus and cytoplasm of the cell. However, erythroblasts cultured alone had the greatest amount of FOXO3 both in the nucleus (on average 1.7-fold greater compared to erythroid cells co-cultured with macrophages) and also in the cytoplasm (approximately 1.6-fold greater abundance compared to erythroblasts cultured with macrophages). Interestingly, erythroblasts from co-cultures containing untreated or IL10 stimulated macrophages had a comparable FOXO3 abundance in the nucleus and cytoplasm. In contrast, dexamethasone treated macrophages significantly altered erythroid FOXO3 nuclear and cytoplasmic levels when compared to co-cultures involving untreated macrophages, inducing a 0.8-fold ($p=0.0497$) and 1.2-fold ($p=0.0499$) FOXO3 abundance in the nucleus and cytoplasm, respectively.

The subcellular distribution of FOXO3 determined by microscopy (**Figure 5.2**) also indicated altered total cellular levels between different culture conditions. The following table shows the mean total fluorescence intensity of the transcription factor per erythroid cell.

**Table 5.1. Mean total FOXO3 fluorescence intensity per erythroblast.** *Results are the mean and SEM per culture condition from 3 different donors. The total number of cells analysed per condition per donor was 70. ****P<0.0001 compared to EB only (one-way ANOVA with Tukey's post-test correction).*

| | Donor 1 | Donor 2 | Donor 3 |
|---|---|---|---|
| **EB only** | 2079 (± 511) | 2212 (± 582) | 2086 (± 526) |
| **Untreated** | 1276 (± 339) **** | 1456 (± 394) **** | 1301 (± 362) **** |
| **Dexamethasone** | 1226 (± 295) **** | 1393 (± 367) **** | 1394 (± 399) **** |
| **IL10** | 1275 (± 328) **** | 1480 (± 415) **** | 1360 (± 385) **** |

**Table 5.1** supports the findings in **Figure 5.1A** and illustrates that the total cellular FOXO3 levels in erythroblasts were substantially greater for erythroblasts cultured alone without the presence of any macrophages (approximately 1.6-fold higher compared to co-cultures involving untreated macrophages). However, total cellular FOXO3 levels were comparable for erythroblasts that were grown with untreated or with dexamethasone or IL10 modulated macrophages. This supports the conclusion that the culture of erythroblasts with macrophages, regardless of their treatment condition, inhibits total FOXO3 protein levels (both nuclear and cytoplasmic) in erythroid cells.

### 5.3.3 Chaperone 14-3-3ζ as an upstream regulator of FOXO3

The chaperone protein 14-3-3ζ binds to phosphorylated FOXO3 and exports it to the cytoplasm, maintaining FOXO3 in an inactive state. Thus, measuring the levels of 14-3-3ζ could potentially indicate altered FOXO3 transport and transcriptional activity within erythroblasts.

**FIGURE 5.3. 14-3-3ζ protein levels in erythroblasts following culture alone or with modulated macrophages.** *14-3-3ζ was measured in erythroblasts after 48 hours of culture alone, with untreated macrophages, or with macrophages treated with dexamethasone or IL10. The protein levels of 14-3-3ζ were determined by indirect antibody staining and subsequent detection by flow cytometry (A). The mean and SEM for 6 biological repeats are shown (B). *P<0.05, **P<0.01 compared to EB only or as indicated (one-way ANOVA with Tukey's post-test correction).*

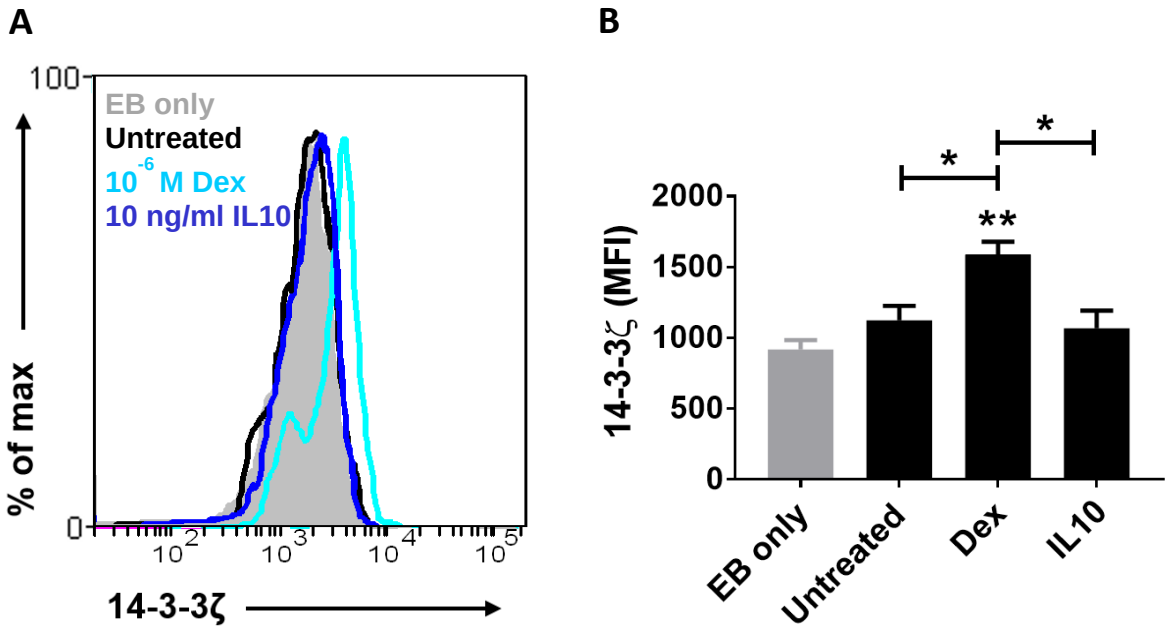


**Figure 5.3** shows that dexamethasone modulated macrophages augmented 14-3-3ζ levels in erythroblasts by 1.41-fold compared to untreated macrophages, while IL10 stimulated macrophages had no effect on erythroid 14-3-3ζ abundance. This suggests elevated binding and transport of phosphorylated FOXO3 to the cytoplasm when erythroblasts are co-cultured with dexamethasone but not with IL10 treated macrophages and is supported by the data in **Figure 5.2** showing reduced nuclear and increased cytoplasmic FOXO3 levels in cultures involving dexamethasone but not IL10 modulated macrophages. Taken together, the findings provide strong indications that implicate the FOXO3 signalling pathway during altered macrophage mediated erythropoiesis.

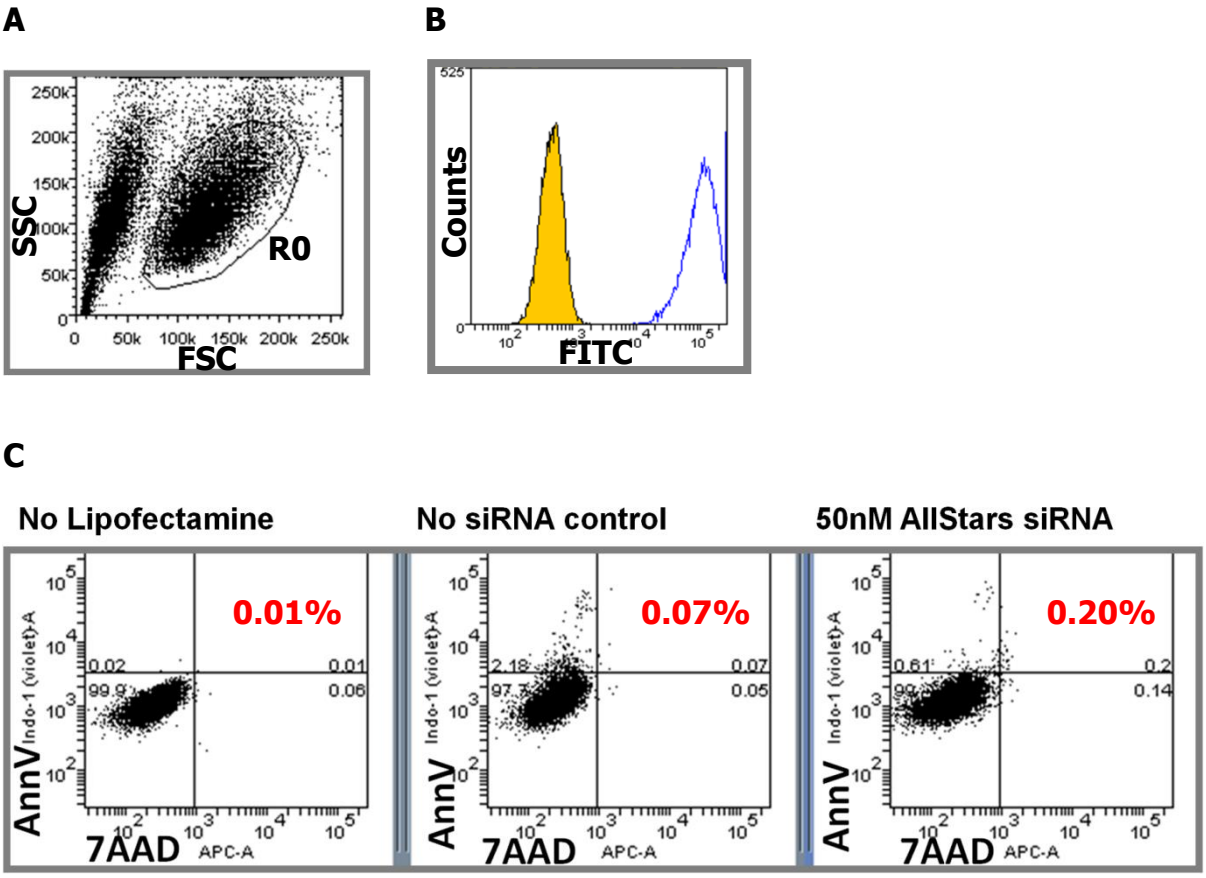
### 5.3.4 Transient knockdown of surface CD163 on macrophages

To determine whether the inactivation of FOXO3 in erythroblasts was mediated through binding to the macrophage CD163 receptor, CD163 on macrophages was targeted for knockdown using small interfering RNAs (siRNAs). The macrophage CD163 glycoprotein, which is involved in scavenging haemoglobin-haptoglobin complexes, plays crucial roles in binding erythroid cells via its second domain to promote their expansion (Fabriek et al., 2007). Therefore, it was hypothesised that CD163 was involved in the FOXO3 inactivation that was associated with elevated erythroblast expansion. In line with this, it was hypothesised that knocking down CD163 on human macrophages would increase FOXO3 activity in erythroblasts and as a result reduce erythroid expansion.

The first step was to establish whether primary human monocyte-derived macrophages were capable of taking up siRNAs. The AllStars negative control siRNA

(Qiagen), which has no homology to any gene in mammals, was tested because it is known to enter the RISC complex and its uptake by cells can be easily detected via fluorescence in the FITC channel.

**FIGURE 5.4. Uptake of AllStars siRNA by macrophages.** *50nM AllStars siRNA was added to macrophages and uptake after 48 hours was determined by fluorescence in the FITC channel. 'A' illustrates the gating strategy (R0) applied to DAPI$^-$ live cells. 'B' shows the uptake of 50nM AllStars siRNA (blue line) measured by fluorescence in the FITC channel compared to the negative control without siRNA (yellow filled line) and untreated cells without any lipofectamine added (black line). 'C' shows the percentages of cell death (red figures) under the different conditions as measured by Annexin V and 7AAD staining (AnnV$^+$7AAD$^+$) of macrophages in the R0 gate. Representative plots from one donor of 3 biological repeats are shown.*



These data demonstrate that macrophages could efficiently take up the AllStars siRNA, causing only minimal (less than 1%) cell death.

Therefore, target gene siRNAs to specific receptors on macrophages could subsequently be tested and compared to appropriate negative controls to investigate the effects of macrophage surface marker knockdown on erythroblast development.

**FIGURE 5.5. Effective knockdown of surface CD163 on human macrophages by siRNA2.** *Three different target siRNAs (siRNA1, siRNA2 and siRNA3) were tested and potential knockdown of CD163 determined via flow cytometry by measuring plasma membrane levels of CD163. siRNAs were added to day 8 cells and transfection medium changed every 24 hours for 3.5 days. 5nM (light blue line), 50nM (dark blue line) and 100nM (red line) of each target siRNA were tested. A negative control siRNA (yellow filled line), positive control siRNA for GAPDH (green line) and unstained cells (grey line) were also used in each case. Representative histograms are shown for one donor of 3 biological repeats.*

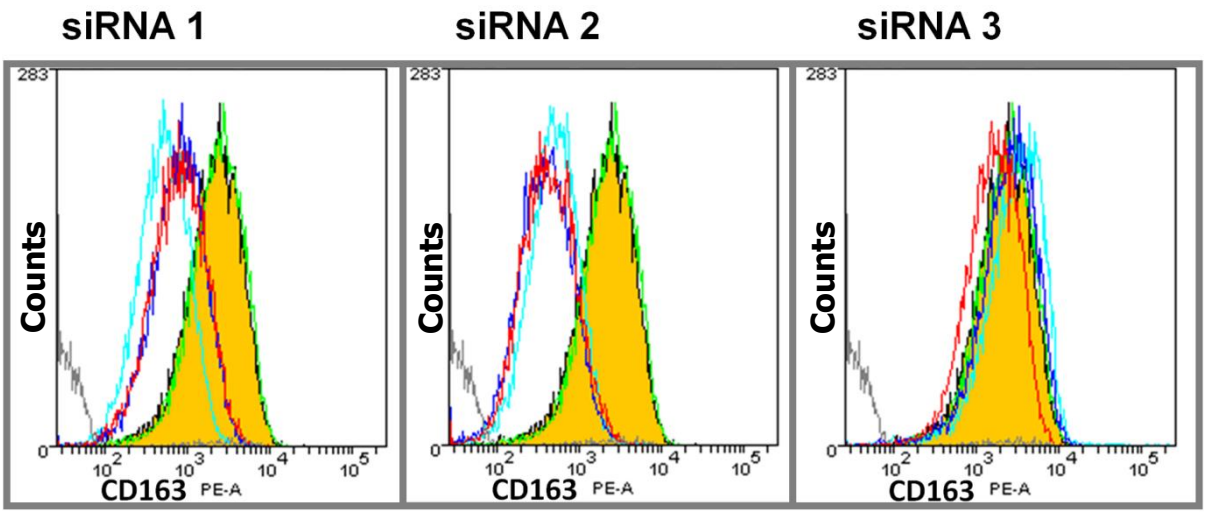


**TABLE 5.2. Mean CD163 protein expression on macrophages as a percentage of control after targeted knockdown by the various siRNAs.** *The mean relative surface CD163 protein expression as a percentage of the control was calculated based on the CD163 MFI of the target siRNA as a percentage of the CD163 MFI of the control siRNA. Three different concentrations of siRNA were tested (5, 50 and 100nM). Data are the mean and SEM for 3 different donors per siRNA tested at the various doses. *P<0.05, ***P<0.001 compared to control siRNA (100%) (one-way ANOVA with Tukey's post-test correction).*

| | **Relative CD163 Surface Expression (%)** | | |
|---|---|---|---|
| | **siRNA1** | **siRNA2** | **siRNA3** |
| **5nM** | 30.6 (± 9.6)*** | 20.2 (± 8.4)*** | 138.2 (± 8.5) |
| **50nM** | 36.4 (± 8.8)*** | 16.0 (± 8.2)*** | 113.5 (± 10.6) |
| **100nM** | 42.4 (± 14.1)*** | 26.8 (± 9.8)*** | 69.8 (± 15.6)* |

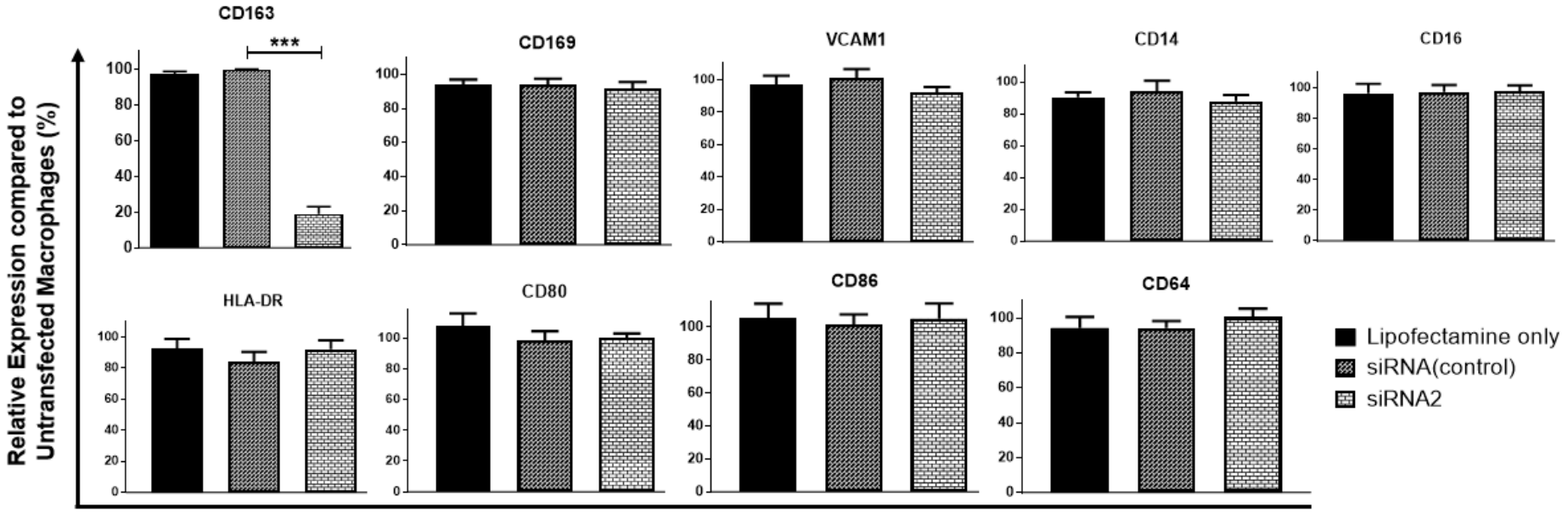
**TABLE 5.3. Macrophage cell death after targeted knockdown with several doses of siRNA2 and following 48 hours of culture with erythroblasts.** *The percentage of dead macrophages was determined by Annexin V and 7AAD staining ($AnnV^+7AAD^+$) after 48 hours in culture with erythroblasts once macrophages had been targeted for knockdown with either 5, 50 or 100nM of siRNA2. Data are the mean and SEM of 3 donors per concentration of siRNA2 used. ***P<0.001 compared to 5nM siRNA2 (one-way ANOVA with Tukey's post-test correction).*

| **siRNA2** | **Cell Death (%)** |
|---|---|
| **5nM** | 3.5 (± 0.5) |
| **50nM** | 56.8 (± 8.4)*** |
| **100nM** | 90.1 (± 9.2)*** |

The knockdown of CD163 on the macrophage surface was very successful, with up to 84% reduced protein expression on average with the use of 50nM siRNA2 (**Figure 5.5** and **Table 5.2**). However, the use of 50nM and 100nM siRNA2 to target macrophage CD163 subsequently caused excessive cell death during 48 hours of co-culture with erythroblasts (56.8 ±SEM 8.4%, p=0.0008 and 90.1 ±SEM 9.2%, p=0.0005, respectively) whereas the use of 5nM siRNA2 only resulted in 3.5 ±SEM 0.5% macrophage apoptosis (**Table 5.3**). Hence, the lower dose (5nM), which still significantly reduced surface CD163, was chosen for all future experiments and the effects on co-cultured erythroblasts were investigated.

Although the knockdown of CD163 was successful, it was crucial to establish whether this was specific or if it also altered the expression of other functionally important surface markers. This is particularly important since macrophages are immune cells with a tendency to alter their phenotype when presented with substances they perceive as foreign and potentially dangerous. Thus, the levels of other macrophage surface markers were measured to check that siRNA2 administration was specific to the knockdown of CD163 and that it did not modify the macrophage phenotype.

**FIGURE 5.6. The effect of transient CD163 knockdown by siRNA2 on macrophage surface markers.** *Surface levels of CD163, CD169, VCAM1, CD14, CD16, HLA-DR, CD80, CD86 and CD64 were measured by flow cytometry for macrophages transfected with lipofectamine only, control siRNA or siRNA2 and the expression shown as a percentage relative to untransfected cells. This was measured 3 days after transient transfections, just prior to the addition of erythroblasts in co-culture. Data are the mean and SEM of 3 biological repeats. ***P<0.001 compared to control (one-way ANOVA with Tukey's post-test correction).*

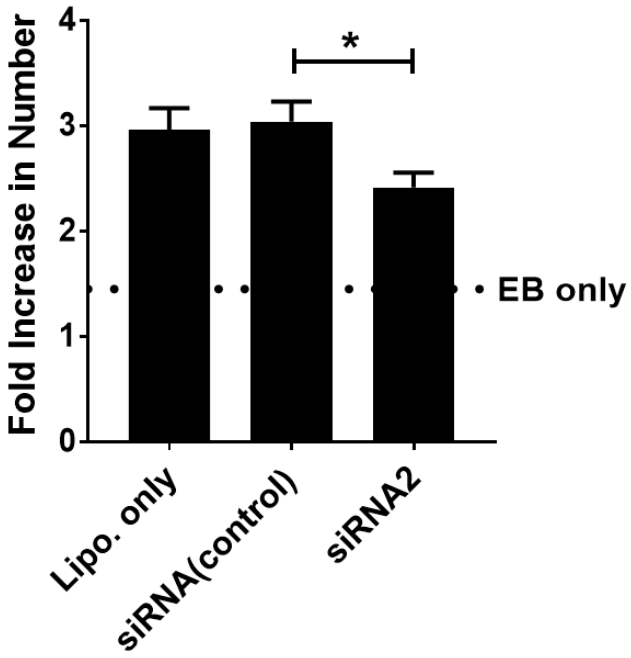


The results in **Figure 5.6** illustrate that siRNA2 did not affect the surface levels of any of the markers except CD163 when compared to siRNA(control) treated cells. This indicates that cultures using siRNA2 to knockdown CD163 and siRNA(control) as a negative control were appropriate for studying the subsequent functional effects on erythroblasts of downregulated CD163 on the surface of M2-polarised macrophages. Furthermore, there was no difference in the secretory profile of macrophages transfected with either siRNA2 or siRNA(control) (**Appendix 5.1**).

### 5.3.5 Functional effects of macrophage surface CD163 knockdown on erythroblast development

The effects of co-culturing macrophages deficient in plasma membrane bound CD163 with erythroblasts in direct physical contact were investigated. The aim of this was to identify the precise function and relative importance of erythroblast binding to CD163 as opposed to any soluble molecules secreted by macrophages and to try to determine any key signalling pathways implicated during macrophage mediated erythroid development.

**FIGURE 5.7. The effect of macrophage CD163 knockdown on erythroblast expansion.** *The fold increase in erythroblast number following culture with lipofectamine only treated, siRNA(control) or siRNA2 targeted macrophages was determined by haemocytometer cell counts of trypan blue excluded live cells and shown in relation to the fold increase in number of erythroblasts cultured alone (dotted line). Erythroid expansion was calculated as the fold increase in erythroid number compared to the density of erythroblasts on the day of co-culture (9 x $10^3$ cells/well). Data are representative of the mean and SEM from 13 different donors. *P<0.05 as indicated (one-way ANOVA with Tukey's post-test correction).*

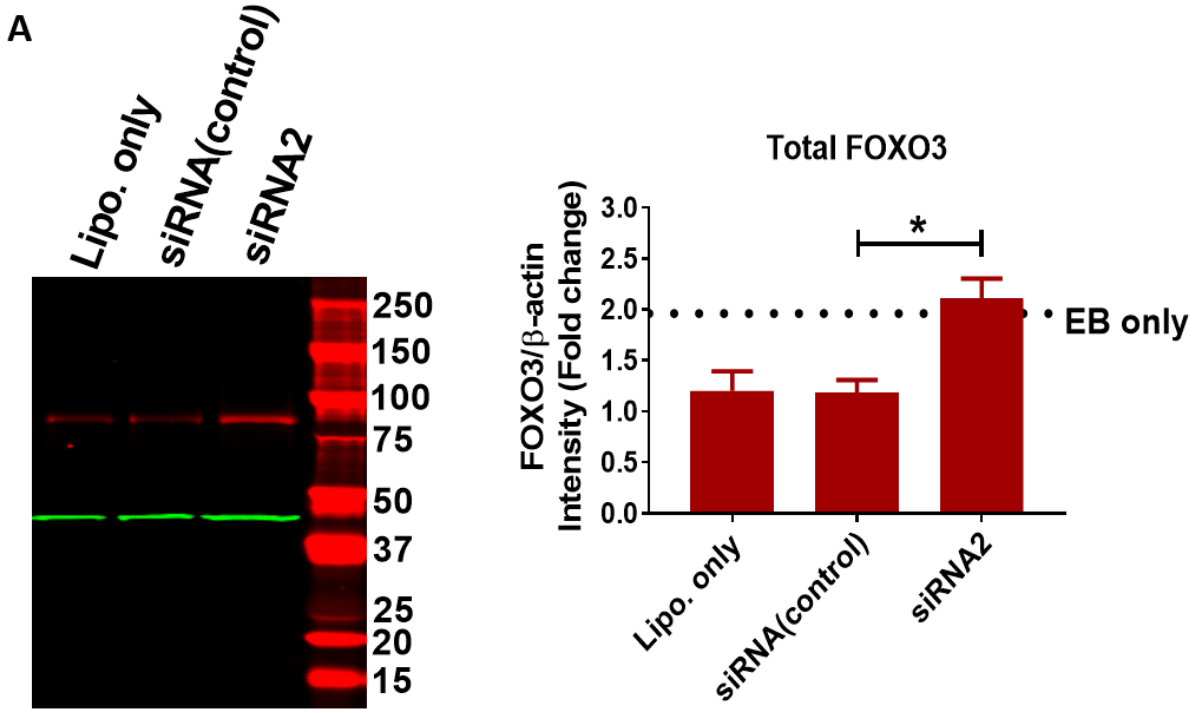


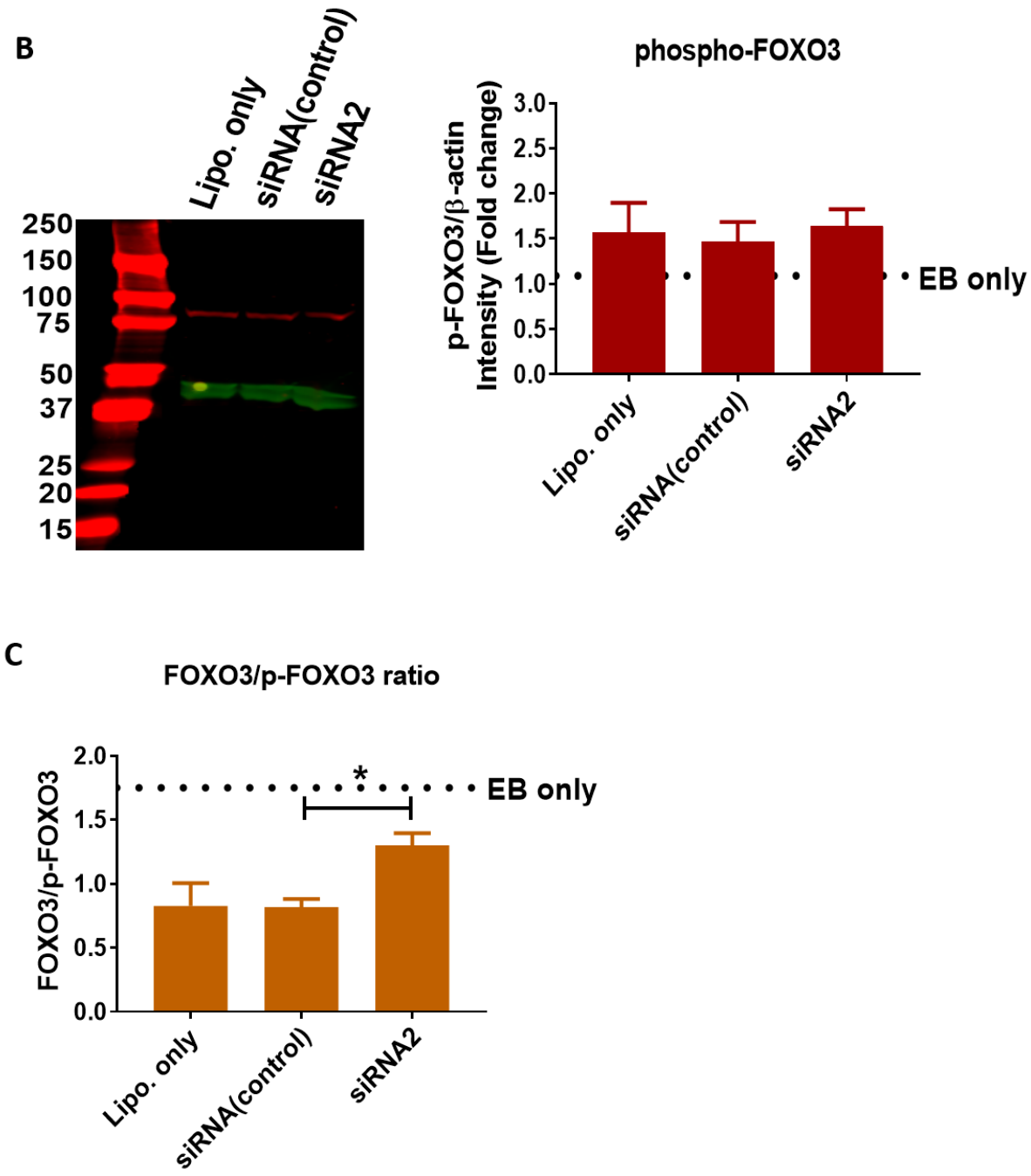

CD163 deficient macrophages (siRNA2) significantly inhibited erythroblast expansion compared to the culture of erythroid cells with control macrophages (siRNA(control)), supporting already established findings for a role of CD163 in erythroblast expansion (Fabriek et al., 2007). However, Fabriek and colleagues did

not identify the downstream signalling pathways that were activated in erythroblasts following attachment to the CD163 receptor and which promoted the augmented expansion.

Given that there is an inverse association between erythroblast expansion and FOXO3 activity (**Figures 4.6** and **5.1**), it was hypothesised that CD163 may exert its effects on improved erythroid expansion via reduced FOXO3 activity. For this reason, total cellular FOXO3 and phospho-FOXO3 levels, as well as the subcellular distribution of this transcription factor, were measured in erythroblasts cultured with normal (siRNA(control)) or CD163 deficient (siRNA2) macrophages.

**FIGURE 5.8. Total cellular FOXO3 (A) and phospho(T32)-FOXO3 (B) protein levels in erythroblasts following co-culture with lipofectamine only, siRNA(control) or siRNA2 targeted macrophages.** *'A' shows the total FOXO3 levels as red bands (~90kDa) with β-actin as the positive control shown by the green bands (42kDa) (left panel), and the fold change in total FOXO3/β-actin intensity is presented (right panel). 'B' shows the phospho-FOXO3(T32) levels as red bands (~90kDa) also with β-actin as the positive control (left panel), and the fold change in phospho-FOXO3/β-actin intensity is presented (right panel). The dotted line illustrates the band intensity relative to β-actin for erythroblasts cultured alone. 'C' illustrates the ratio of total FOXO3/phospho-FOXO3 as an indication of FOXO3 activity. The western blots in 'A' and 'B' (left panels) are representative blots of erythroblasts derived from one donor, while the right panels represent the mean and SEM for 3 independent biological repeats. *P<0.05 compared to siRNA(control) as indicated (one-way ANOVA with Tukey's post-test correction).*



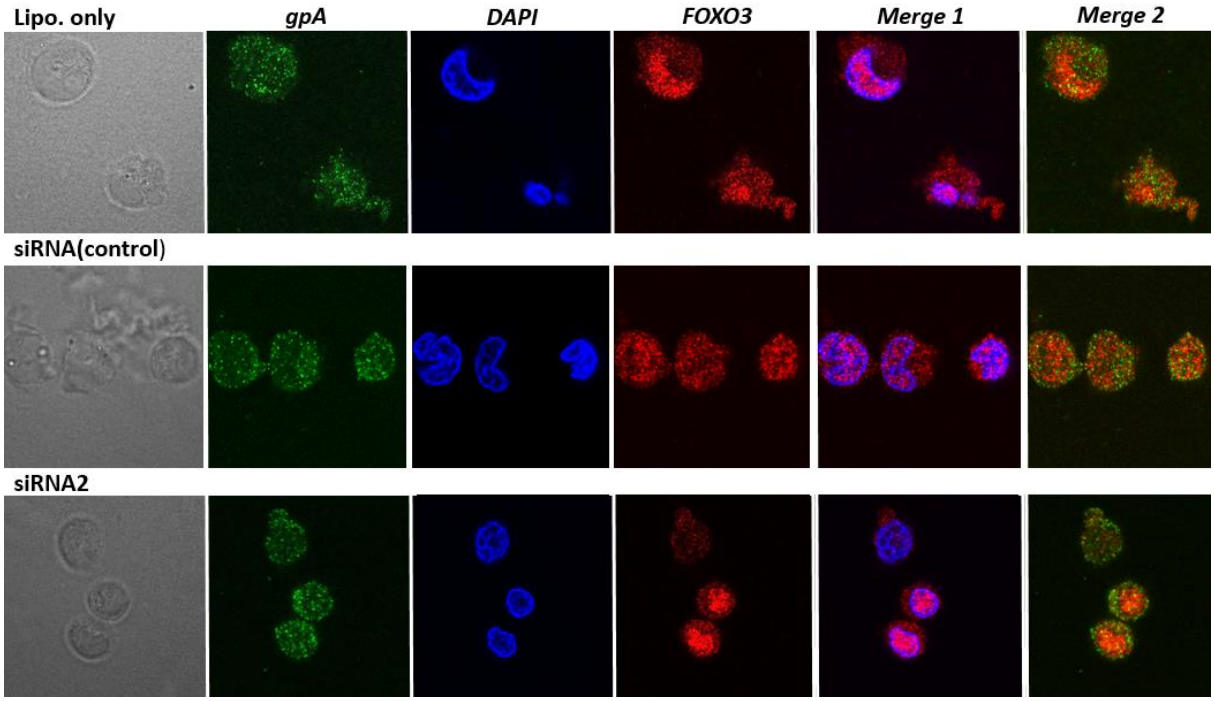



These data show that the knockdown of surface CD163 on macrophages heightened total cellular FOXO3 levels in co-cultured erythroblasts by 1.78-fold but had no effect on the phosphorylation of the same transcription factor (**Figure 5.8A** and **B**). The increased ratio of FOXO3/phospho-FOXO3 in erythroblasts illustrates that the lack of CD163 on macrophages augmented FOXO3 activity (**Figure 5.8C**), and the western

blots indicated this to be due to the increase in total FOXO3 levels as opposed to reduced phosphorylation.

**FIGURE 5.9. Effects of macrophage CD163 knockdown on the subcellular localisation of FOXO3 in erythroblasts.** *'A' shows representative confocal images of erythroblasts from the same donor grown with the differentially targeted macrophages. Glycophorin A (gpA, green), cell surface stain; DAPI (blue), nuclear stain; FOXO3 (red), transcription factor subcellular localisation. Merge 1 are DAPI and FOXO3 combined; Merge 2 are gpA and FOXO3 combined. 'B' shows the fluorescence intensities of FOXO3 in the nucleus (red) or cytoplasm (red cross-hatched) of erythroblasts cultured under the different conditions relative also to erythroblasts only (dotted lines). Fluorescence intensities were measured in widefield (DeltaVision) microscopy images (N=60–65 per sample). The mean and SEM from three biological repeats are shown. *P<0.05, ****P<0.0001 compared to siRNA(control) (one-way ANOVA with Tukey's post-test correction).*

**A**



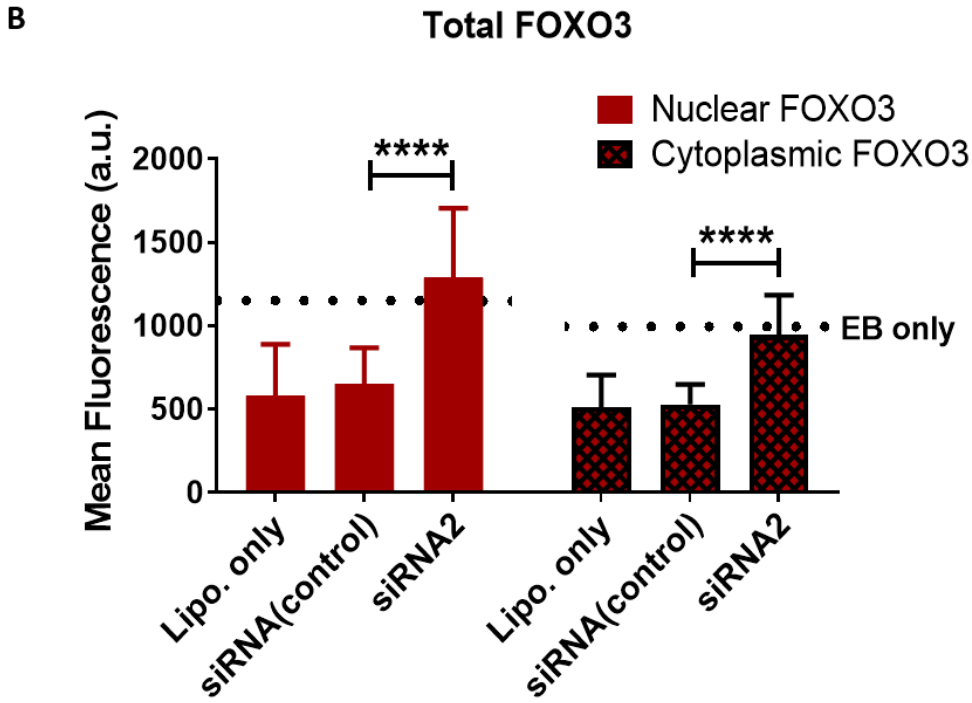


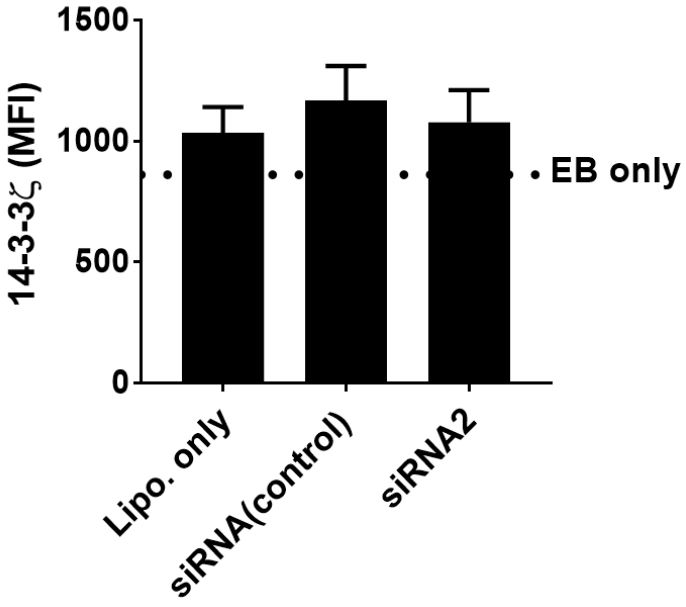

**Figure 5.9** shows a 1.97- and 1.79-fold increase in nuclear and cytoplasmic FOXO3 abundance, respectively, when erythroblasts are cultured with siRNA2 compared to siRNA(control) targeted macrophages. **Table 5.4** shows how this corresponds to, on average, a 1.80-fold elevation in the total cellular FOXO3 levels, which consistently is comparable to the total FOXO3 abundance in erythroblasts only. Furthermore, these data support the findings from **Figure 5.9A**. This suggests that CD163 deficient macrophages induce FOXO3 accumulation in erythroid cells. Moreover, the similar nuclear, cytoplasmic and total cellular FOXO3 levels in erythroblasts cultured with siRNA2 targeted macrophages as in erythroid cells grown alone suggest that CD163 is the main macrophage derived factor responsible for blocking transcription and/or translation of FOXO3.

**Table 5.4. Mean total FOXO3 fluorescence intensity per erythroblast.** *Results are the mean and SD per culture condition for each of 3 different donors. The total number of cells analysed per condition per donor was 70. ****P<0.0001 compared to EB only, ####P<0.0001 compared to siRNA(control) (one-way ANOVA with Tukey's post-test correction).*

| | Donor 1 | Donor 2 | Donor 3 |
|---|---|---|---|
| **EB only** | 2099 (± 520) | 1990 (± 497) | 2278 (± 538) |
| **Lipo. only** | 1117 (± 282) **** | 1046 (± 355) **** | 1451 (± 409) **** |
| **siRNA(control)** | 1246 (± 319) **** | 1483 (± 430) **** | 1122 (± 287) **** |
| **siRNA2** | 2252 (± 607) #### | 2603 (± 9113) #### | 2014 (± 512) #### |

The final part to these studies involved investigating whether siRNA2 targeted macrophages altered the levels of the adaptor protein 14-3-3ζ in erythroblasts, as a potential indication for altered transport of phosphorylated FOXO3.

**FIGURE 5.10. The effect of macrophage CD163 knockdown on erythroid 14-3-3ζ levels.** *The total levels of 14-3-3ζ in erythroblasts following co-culture with CD163 deficient (siRNA2) macrophages was measured by direct antibody staining and flow cytometry. Results are the mean and SEM of 3 biological repeats.*




The co-culture of erythroblasts with CD163 deficient macrophages did not affect erythroid 14-3-3ζ abundance compared to the culture with control macrophages. This suggests that the knockdown of surface CD163 on macrophages did not alter the export of phosphorylated nuclear FOXO3 via 14-3-3ζ in co-cultured erythroblasts. These results are also in line with the unchanged phospho-FOXO3 levels in erythroid cells cultured with the differentially targeted macrophages (**Figure 5.8B**).

Taken together, the findings from the CD163 knockdown experiments support the hypothesis for a role of CD163 in heightened erythroid expansion via reduced FOXO3

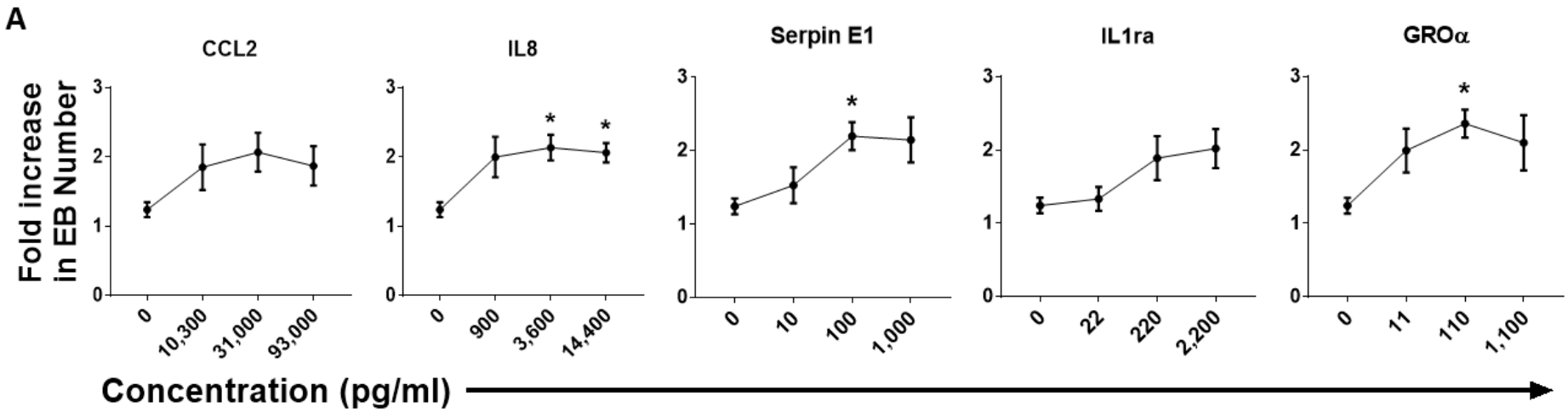
activity. More precisely, the data suggest that the CD163 glycoprotein inactivates erythroid FOXO3 by inhibiting total cellular levels rather than by promoting phosphorylation of the transcription factor. Hence, these studies put forward a mechanism for macrophage-dependent erythropoiesis which was previously unidentified.

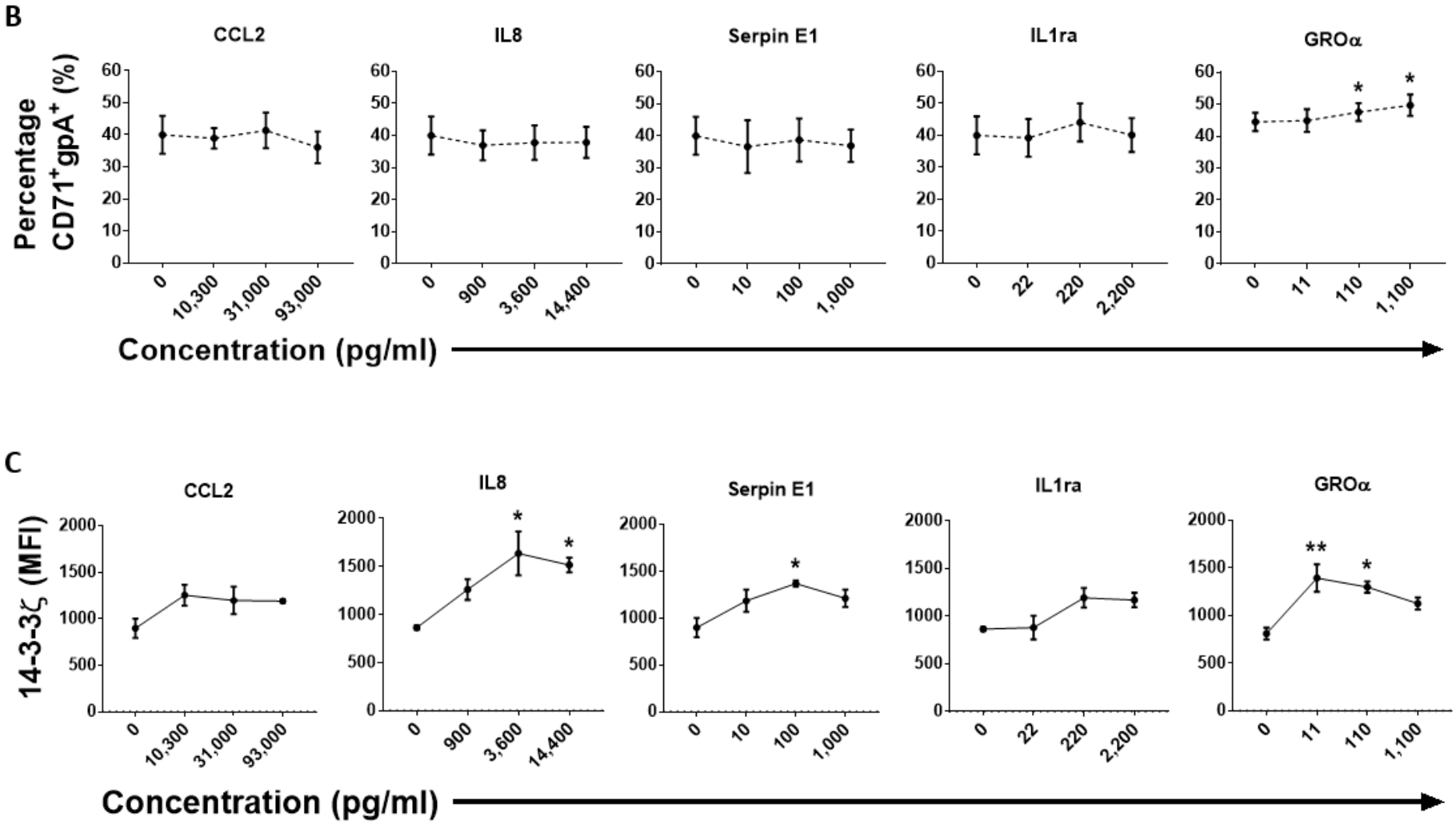
### 5.3.6 The effects of macrophage derived senescence-associated soluble molecules on erythropoiesis

Although knockdown of the macrophage receptor CD163 reduced erythroid expansion and augmented total cellular FOXO3 levels, neither expansion (**Figure 5.7**) nor the FOXO3/phospho-FOXO3 ratio (**Figure 5.8C**) were altered to the same extent as erythroblasts cultured alone without macrophages. This suggests there were other macrophage derived factors that were contributing to the improved erythroblast expansion via diminished FOXO3 transcriptional activity.

The macrophage phenotype can be affected through an altered secretory profile as well as through changes in cell surface markers. Therefore, given that several senescence-associated secretory molecules were identified in the supernatants of erythroblasts co-cultured with the differentially modulated macrophages (see **Figure 4.12** and **Table 4.3**), it was speculated whether these soluble molecules could also be contributing to the supported erythroid expansion. To explore this, CCL2, IL8, Serpin E1, IL1ra and GROα were added either individually at a range of doses (with the middle dose as the highest measured by ELISAs in supernatants) or at the combinations detected in the different co-culture supernatants to erythroblasts only to investigate whether they affected erythroid development.

**FIGURE 5.11. The effect of CCL2, IL8, Serpin E1, IL1ra or GROα at a range of concentrations on erythroblast expansion, maturation and 14-3-3ζ levels.** *Live erythroblasts were counted via haemocytometer cell counts with trypan blue exclusion and the fold increase in erythroblast number determined as the number compared to the initial seeding density (6 x $10^3$ cells/well) (A). Erythroid maturation was determined by staining for surface CD71 and glycophorin A and detected on a flow cytometer (B). Levels of 14-3-3ζ in erythroblasts were determined by indirect staining and detection on a flow cytometer (C). Expansion, maturation and 14-3-3ζ abundance were all measured 48 hours after the addition of each cytokine to erythroblasts only at the range of concentrations shown. Data are the mean and SEM from 6 (A), 3-7 (B) or 3 (C) separate donors. *P<0.05, **P<0.01 compared to 0 pg/ml (one-way ANOVA with Tukey's post-test correction).*



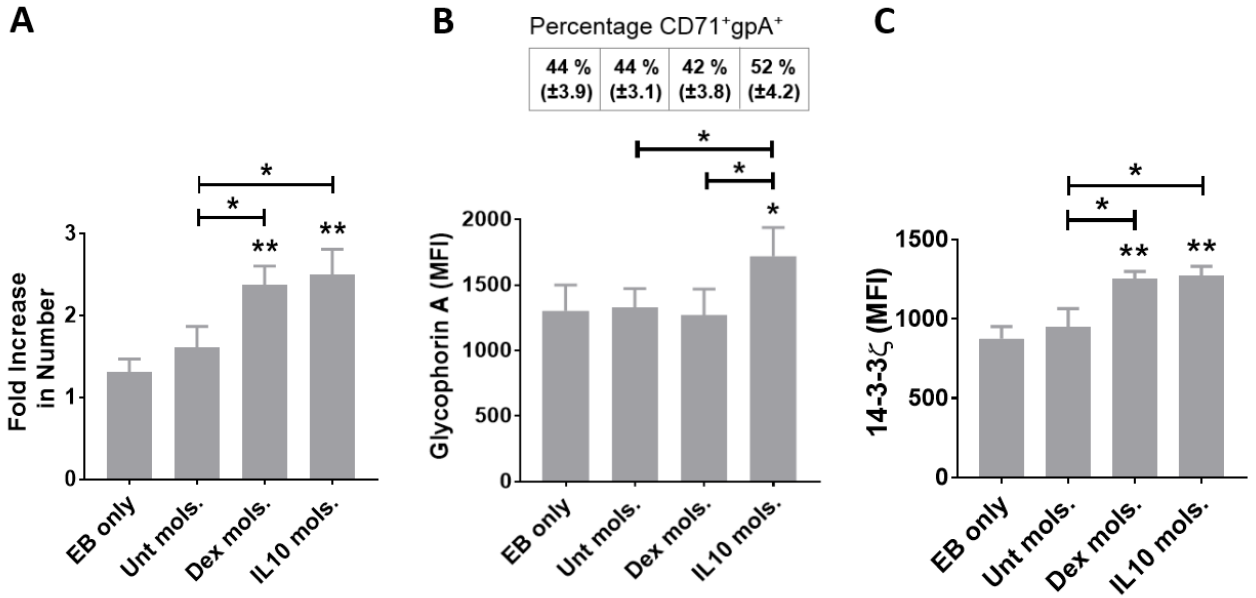

B
CCL2
IL8
Serpin E1
IL1ra
GROα
Percentage CD71+gpA+ (%)
0
10
20
30
40
50
60
0
10,300
31,000
93,000
0
900
3,600
14,400
0
10
100
1,000
0
22
220
2,200
0
11
110
1,100
*
*
Concentration (pg/ml)
C
CCL2
IL8
Serpin E1
IL1ra
GROα
14-3-3ζ (MFI)
0
500
1000
1500
2000
0
10,300
31,000
93,000
0
900
3,600
14,400
0
10
100
1,000
0
22
220
2,200
0
11
110
1,100
*
*
*
*
**
*
Concentration (pg/ml)

IL8 (3,600 pg/ml), Serpin E1 (100 pg/ml) and GROα (110 pg/ml) improved erythroblast expansion at the doses that were the highest detected in co-culture supernatants (involving dexamethasone or IL10 treated macrophages) (**Figure 5.11A**). However, IL8 (14,400 pg/ml) was also found to elevate erythroid expansion by 1.7-fold at a concentration 4 times that identified in the dexamethasone modulated macrophage culture supernatants.

Erythroblast maturation was not affected by the addition of any of the soluble molecules at any of the doses except for GROα, which elevated the percentage of $CD71^{+}gpA^{+}$ erythroid cells by 3.1 and 5.2% at 110 and 1,100 pg/ml, respectively (**Figure 5.11B**). Thus, GROα supported the simultaneous expansion and maturation of erythroblasts, with the maturation augmented in a seemingly dose-dependent manner. Although not tested, it would have been worthwhile investigating whether concentrations of GROα above 1,100 pg/ml could further improve erythroblast maturation and if this simultaneously affected erythroid expansion.

A substantial enhancement in the abundance of the adaptor protein 14-3-3ζ was found in erythroblasts following the addition of IL8 (3,600 and 14,400 pg/ml), Serpin E1 (100 pg/ml) or GROα (11 and 110 pg/ml) (**Figure 5.11C**). Interestingly, the increases in 14-3-3ζ levels often corresponded with the significant enhancement in expansion, suggesting a possible regulation of erythroblast expansion via reduced FOXO3 activity controlled by its export from the nucleus.

**FIGURE 5.12. The effect of adding the senescence-associated molecules together at combinations found in the various co-culture supernatants on erythroblast expansion, maturation and 14-3-3ζ levels.** *CCL2, IL8, Serpin E1, IL1ra and GROα were added simultaneously to erythroblasts at the concentrations detected in co-culture supernatants containing untreated (16,200, 2,500, 0, 200, 50 pg/ml), dexamethasone (31,000, 3,600, 100, 140, 60 pg/ml) or IL10 (12,300, 2,700, 50, 230, 110 pg/ml) treated macrophages. Erythroid expansion was determined by haemocytometer cell counts of live trypan blue excluded cells (A), while maturation was measured by staining for surface CD71 and glycophorin A (B) and 14-3-3ζ abundance by indirect staining and detection on a flow cytometer (C). Data are the mean and SEM for 7 (A and B) or 4 (C) different donors. *P<0.05, **P<0.01 compared to EB only or as indicated (one-way ANOVA with Tukey's post-test correction).*



The addition of the senescence-associated molecules at the combinations found in dexamethasone or IL10 treated macrophage co-culture supernatants enhanced erythroid expansion by 1.5- and 1.6-fold, respectively, compared to their addition at the concentrations present in supernatants involving untreated macrophages (**Figure 5.12A**).

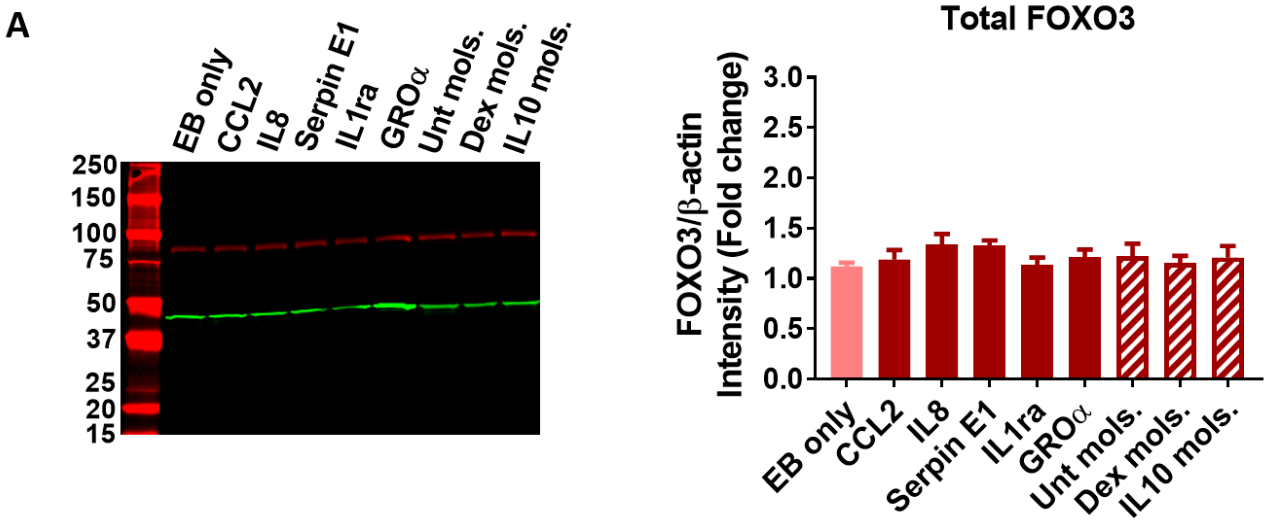
While this increase in expansion did not correspond with any change in maturation for erythroblasts supplemented with the dexamethasone supernatant molecule combinations, erythroid cells cultured with the IL10 treated molecule combinations had a 1.4-fold improvement in glycophorin A surface expression (**Figure 5.12B**). This was equivalent to an 8% increase in $CD71^{+}gpA^{+}$ erythroid cells compared to cells grown with the untreated supernatant molecule combinations and for one donor was especially significant, enhancing this proportion of mature erythroid cells by 20%. Thus, the cytokines in combination appear to support maturation only when their respective receptors on erythroblasts are engaged to an exact extent that induce downstream signalling cascades for erythroid differentiation.

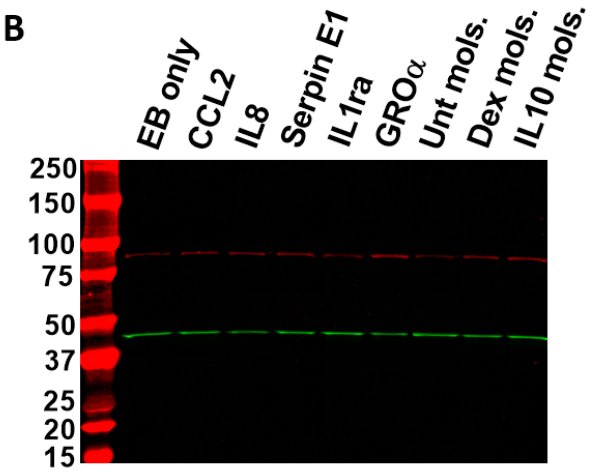
It was found that the addition of the dexamethasone or IL10 modulated molecule combinations augmented 14-3-3ζ abundance in erythroblasts by 1.3-fold in each case (**Figure 5.12C**). This suggests the senescence-associated molecules at either of these combinations may diminish FOXO3 activity by the action of the chaperone protein 14-3-3ζ transporting phosphorylated FOXO3 to the cytoplasm. However, 14-3-3ζ has also been described to combine with phosphatidylinositol 3 kinase (PI3K) and Shc to activate the transcription factor PKB, which is well known to phosphorylate and inhibit FOXO3 (Barry et al., 2009; Ursini-Siegel et al., 2012). Therefore, regardless of whether 14-3-3ζ functions mostly upstream (as a cause) or

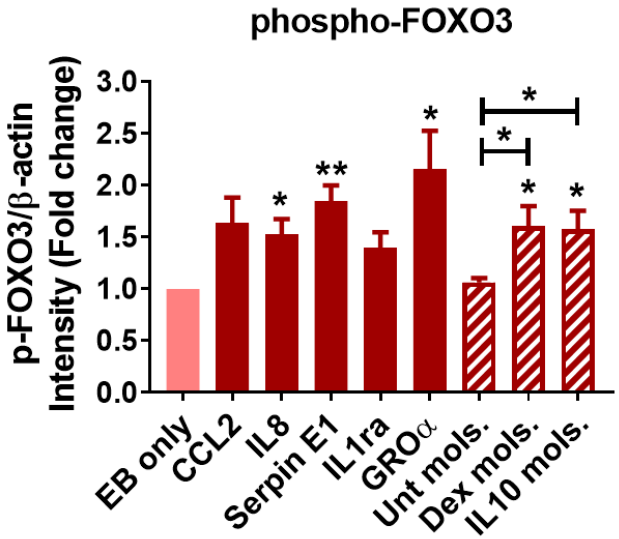
downstream (as a consequence) of FOXO3 regulation, its heightened levels implicate a potential role for phosphorylated FOXO3 under these conditions.

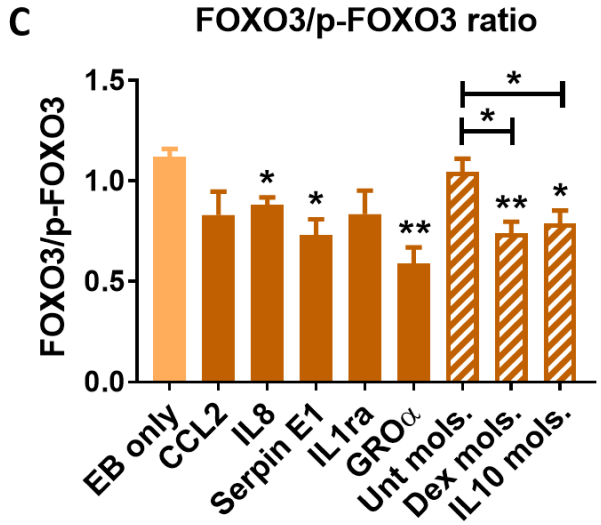
Given that the senescence-associated molecules elevated both erythroblast expansion and 14-3-3ζ levels when added at certain concentrations and in specific combinations (**Figures 5.11** and **5.12**), the possibility that FOXO3 inactivation via phosphorylation could contribute to this expansion was investigated next.

**FIGURE 5.13. The effect of adding the senescence-associated molecules either individually or together at combinations found in the co-culture supernatants on the total cellular FOXO3 (A) and phospho-FOXO3 (B) levels in erythroblasts.** *CCL2, IL8, Serpin E1, IL1ra and GROα were added to erythroblasts either individually at the highest dose detected in co-culture supernatants (31,000, 3,600, 100, 220, 110 pg/ml, respectively) or in combinations with the respective concentrations detected in the various culture supernatants (untreated mols: 16,200, 2,500, 0, 200, 50; Dex mols: 31,000, 3,600, 100, 140, 60; IL10 mols: 12,300, 2,700, 50, 230, 110 pg/ml, respectively). Total FOXO3 (A) and phospho-FOXO3 (B) levels in erythroblasts after culture were measured and the ratio of total FOXO3/phospho-FOXO3 determined (C). Data are the mean and SEM of 3-4 separate donors. *P<0.05, **P<0.01 compared to EB only or as indicated otherwise (one-way ANOVA with Tukey's post-test correction).*




B
EB only
CCL2
IL8
Serpin E1
IL1ra
GROα
Unt mols.
Dex mols.
IL10 mols.
250
150
100
75
50
37
25
20
15


phospho-FOXO3
p-FOXO3/β-actin
Intensity (Fold change)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
EB only
CCL2
IL8
Serpin E1
IL1ra
GROα
Unt mols.
Dex mols.
IL10 mols.


C
FOXO3/p-FOXO3 ratio
FOXO3/p-FOXO3
1.5
1.0
0.5
0.0
EB only
CCL2
IL8
Serpin E1
IL1ra
GROα
Unt mols.
Dex mols.
IL10 mols.

As hypothesised, the addition of some of the senescence-associated molecules and their precise combinations increased phospho-FOXO3 levels without affecting total cellular FOXO3 abundance in erythroblasts (**Figure 5.13A** and **B**). IL8, Serpin E1 and GROα induced FOXO3 phosphorylation when added individually to erythroblasts at 3,600, 100 and 110 pg/ml, respectively (**Figure 5.13B**). The addition of all the soluble molecules together (CCL2, IL8, Serpin E1, IL1ra and GROα) also augmented phospho-FOXO3 levels, but only when combined at the specific concentrations found previously in either the dexamethasone (31,000, 3,600, 100, 140, 60 pg/ml, respectively) or IL10 (12,300, 2,700, 50, 230, 110 pg/ml, respectively) treated macrophage co-culture supernatants. The elevated phosphorylation of FOXO3 under these conditions led to a reduction in the FOXO3/phospho-FOXO3 ratio accordingly, indicating a potentially inhibited FOXO3 activity that could explain the increase in erythroid expansion (**Figure 5.13C**).

## 5.4 Discussion

The observation in the previous chapter (**chapter 4**) that erythroblasts cultured with dexamethasone or IL10 treated macrophages had diminished catalase and augmented cyclin D1 protein levels, which are both directly controlled by the FOXO3 transcription factor, hinted to a possible inhibition of FOXO3 activity under these conditions. Hence, this chapter aimed to determine whether reduced FOXO3 activity in erythroblasts could be responsible for the observed improvement in expansion.

Culturing erythroblasts with dexamethasone or IL10 modulated macrophages reduced the total cellular FOXO3 abundance in erythroid cells while simultaneously enhancing the phosphorylation of FOXO3 on residue T32 (**Figure 5.1A** and **B**). Since FOXO3 is phosphorylated on T32 by the serine/threonine kinase PKB (Jang et al., 2007; Weidinger et al., 2011), this suggests there was a greater PKB inhibition of FOXO3 in erythroblasts during co-culture with dexamethasone or IL10 stimulated macrophages. The combined phosphorylation and blocked total FOXO3 protein levels resulted in the lowest FOXO3/phospho-FOXO3 ratio that is suggestive of substantially decreased FOXO3 activity (**Figure 5.1C**).

Interestingly, the co-culture of erythroblasts with any type of macrophage, whether previously treated with dexamethasone or IL10 or left untreated, similarly diminished total erythroid FOXO3 levels by almost half compared to erythroblasts grown on their own (**Figure 5.1A**). This was supported by the immunofluorescence microscopy data for FOXO3 subcellular distribution, which showed that FOXO3 levels were reduced in both the nucleus and cytoplasm of all cultures involving macrophages (**Figure 5.2** and **Table 5.1**). These findings propose an intrinsic ability of

macrophages to block FOXO3 production in erythroblasts. This inhibited production likely entails decreased FOXO3 transcription and/or translation, and perhaps also a lowered protein stability that results in greater proteasomal degradation. However, extensive and detailed investigations would be required to determine which of these cellular processes are implicated in the reduced erythroid total cellular FOXO3 levels.

Moreover, the immunofluorescence microscopy studies also elucidated that dexamethasone supplemented macrophages altered the subcellular localisation of FOXO3 compared to unmodulated macrophages, lowering nuclear levels by 0.80-fold and increasing cytoplasmic levels by 1.23-fold, while IL10 treated macrophages had no effect on the distribution of FOXO3 within erythroblasts (**Figure 5.2**). The augmented 14-3-3ζ in erythroid cells co-cultured with dexamethasone but not IL10 treated macrophages provides a possible explanation for this differential subcellular FOXO3 localisation (**Figure 5.3**). It suggests that while dexamethasone modulated macrophages would have promoted nuclear export of phosphorylated FOXO3 in erythroblasts via the binding of the 14-3-3ζ chaperone protein, IL10 stimulated cells on the other hand could not have altered transport of phospho-FOXO3 due to the unchanged 14-3-3ζ abundance. Therefore, given the elevated phosphorylation of FOXO3 but unaffected nuclear and cytoplasmic FOXO3 levels in erythroid cells grown with IL10 treated compared to untreated macrophages, one would hypothesise that the phospho(T32)-FOXO3 is retained within the nucleus where it is initially phosphorylated. However, further studies would be necessary to corroborate this.

The targeting of macrophage CD163 using 50nM siRNA2 reduced cell surface expression by an average of 84% (**Figure 5.5** and **Table 5.2**). However, the lowest dose of 5nM siRNA2 was chosen for all subsequent experiments because it caused

only very minimal macrophage death (**Table 5.3**), while still effectively knocking down surface CD163 by 79.8%. On the other hand, the use of 50nM or 100nM of siRNA2 induced much greater apoptotic death on the fifth day after the initial addition of the siRNA (approximately 56.8% and 90.1% macrophage apoptosis, respectively). Comparing the surface marker expression in siRNA2 versus siRNA(control) targeted macrophages revealed a significant knockdown of CD163 only and not of any other receptors (**Figure 5.6**). There was also no change in the secretory profile of the siRNA2-targeted compared to control macrophages (**Appendix 5.1**). This indicates siRNA2 did not affect the phenotype of macrophages and was specific in targeting only CD163 for knockdown.

The knockdown of the macrophage CD163 retarded erythroblast expansion but not to the extent of erythroid cells cultured alone (**Figure 5.7**). One would expect to find reduced erythroid expansion with a lack of CD163 because the glycoprotein has a motif within the second domain that has been established to bind erythroblasts and enhance their expansion (Fabriek et al., 2007). However, the fact that the absence of the CD163 receptor on siRNA2 targeted macrophages did not lower expansion to levels comparable for erythroblasts grown on their own suggests there were other macrophage derived factors also contributing to the erythroid expansion. Other than the lack of surface CD163 expression, siRNA2 targeted macrophages had a similar phenotype to IL10 treated cells, which were previously found to have a senescence-associated secretory profile (see **Figure 4.12** and **Table 4.3**). Thus, it was hypothesised that these senescence-associated molecules and/or potentially other soluble factors such as secreted miRNAs played a part in the erythroblast expansion (investigated later on in this chapter).

Given the mounting evidence throughout this thesis supporting a possible role for FOXO3 inactivation in heightened erythroid expansion, it seemed logical to explore whether CD163 exerted its effects on expansion through the FOXO3 transcription factor. Knockdown of CD163 on macrophages almost doubled total cellular FOXO3 levels in erythroblasts but did not affect phosphorylation of the transcription factor (**Figures 5.8** and **5.9**, **Table 5.4**). siRNA2 targeted macrophages increased the abundance of FOXO3 by a similar proportion in both the erythroid nucleus and cytoplasm, indicating no significant change in the cellular distribution of FOXO3 (**Figure 5.9B**). Further evidence for this is provided by the unaltered 14-3-3ζ levels, which suggest the transport of phospho-FOXO3 was unaffected (**Figure 5.10**). Hence, the elevated total FOXO3 abundance but unchanged phospho-FOXO3 levels induced by macrophage CD163 knockdown led to an increase in the FOXO3/phospho-FOXO3 ratio compared to co-cultures involving control macrophages (**Figure 5.8C**). Nonetheless, as with erythroid expansion, this did not reach the levels for erythroblasts cultured alone, suggesting CD163 is not the only factor contributing to enhanced expansion via FOXO3 inactivation.

Surface markers, including CD163, comprise only one part of the macrophage phenotype, which can also be defined by changes in secreted molecules. **Chapter 4** identified that the treatment of macrophages with either dexamethasone or IL10 induced a senescence-associated secretory phenotype. Therefore, having determined that the CD163 glycoprotein is not the sole factor responsible for heightened erythroblast expansion, the possibility that these senescence-associated molecules could also play an important role was investigated. IL8, Serpin E1 and GROα were all found to improve expansion when added individually to erythroblasts

at the highest concentrations detected in co-culture supernatants (3,600, 100, 110 pg/ml, respectively) (**Figure 5.11A**). Each of these doses was present in culture supernatants that involved either dexamethasone or IL10 modulated macrophages, but not untreated macrophages, and thus provides a possible explanation as to why erythroid cells grown with either of the differentially stimulated macrophages experienced a further enhancement in expansion that was not observed when they were grown with untreated macrophages. Moreover, erythroblasts supplemented with a 4-fold greater dose of IL8 (14,400 pg/ml) also experienced a significant improvement in expansion compared to cells without any cytokine addition. Surprisingly, GROα also promoted erythroid maturation at 110 and 1,100 pg/ml, elevating the proportion of $CD71^{+}gpA^{+}$ cells by 3.09% and 5.21%, respectively (**Figure 5.11B**). In contrast, none of the other soluble molecules affected erythroblast maturation. The doses of cytokines that led to substantial elevations in expansion were also associated with simultaneous increases in 14-3-3ζ abundance, except for the lowest concentration of GROα (11 pg/ml) (**Figure 5.11C**). The 14-3-3ζ protein is known for its crucial function in translocating phosphorylated FOXO3 (Biggs et al., 1999; Rinner et al., 2007) but can also form a complex with PI3K and Shc to activate PKB that in turn inactivates FOXO3 through its phosphorylation (Barry et al., 2009; Ursini-Siegel et al., 2012). Therefore, this suggests a potential role for phosphorylated FOXO3 during the increased expansion observed with certain chemokines.

Nevertheless, the senescence-associated molecules were secreted into culture by the differentially modulated macrophages in specific combinations, so it was necessary to examine if their combined effects altered erythropoiesis. The addition of CCL2,

IL8, Serpin E1, IL1ra and GROα together at the doses found in dexamethasone (31,000, 3,600, 100, 140, 60 pg/ml, respectively) or IL10 (12,300, 2,700, 50, 230, 110 pg/ml, respectively) treated macrophage co-culture supernatants elevated both expansion and 14-3-3ζ levels compared to erythroid cells supplemented with the concentrations representative of the untreated macrophage secretory phenotype (16,200, 2,500, 0, 200, 50 pg/ml, respectively) (**Figure 5.12A** and **C**). However, the differential combinations of senescence-associated molecules did not have additive or synergistic effects on erythroid expansion or 14-3-3ζ abundance in either case. These findings provide further support for a possible involvement of phosphorylated FOXO3 in the heightened expansion. The fact that the IL10 modulated molecule combinations added to erythroblasts alone heightened 14-3-3ζ levels, which was contrary to the co-culture of erythroid cells with IL10 treated macrophages that had an unchanged abundance of the chaperone protein (**Figure 5.3**), suggests there were other factors from these IL10 treated macrophages that suppressed 14-3-3ζ expression. This could possibly be due to augmented miR-451 levels, which act to directly inhibit 14-3-3ζ mRNA (Yu et al., 2010) but this hypothesis would need to be tested.

While GROα alone at 110 pg/ml augmented erythroid maturation by 3.09%, its combination with the other four molecules at the doses present in IL10 treated macrophage supernatants increased maturation by 8% (**Figure 5.12B**). Therefore, the combination of senescence-associated molecules accentuates the effects of GROα, strongly inducing signalling pathways in erythroblasts for differentiation. GROα and IL8 both elicit their effects through the CXCR2 receptor and thus it may be the joint action of these two chemokines that activates the downstream signalling

cascades for maturation in erythroblasts. Experiments involving blocking antibodies to the receptors for each soluble molecule would help identify which are the major players in the augmented erythroid maturation.

Supplementing erythroblasts with IL8 (3,600 pg/ml), Serpin E1 (100 pg/ml) or GROα (110 pg/ml) individually or adding all the cytokines together at the dexamethasone or IL10 supernatant combinations induced FOXO3 phosphorylation without altering total cellular FOXO3 levels, resulting in significant reductions in the FOXO3/phospho-FOXO3 ratio (**Figure 5.13A**, **B** and **C**). Given their association to the elevated expansion, this provides evidence that these senescence-associated molecules support erythroid expansion via the phosphorylation and inactivation of FOXO3 when added to erythroblasts in culture alone. Interestingly, increased levels of IL8 have been detected in patients with polycythaemia vera (Hermouet et al., 2002) and hence it would valuable to investigate whether this molecule plays a part in the uncontrolled erythropoiesis through FOXO3 phosphorylation. GROα, on the other hand, has been described in erythropoiesis in the context of causing vaso-occlusive stress in murine models of sickle cell anaemia (Jang et al., 2011) or in regulating haematopoietic stem cell proliferation and survival via its receptor CXCR2 (Sinclair et al., 2016). Therefore, the effect of GROα on the expansion and maturation of erythroblasts is an interesting finding that suggests several avenues for further work.

Previous work in the field had already identified that macrophages support erythroid proliferation and aid in the recovery from induced anaemia via a mechanism independent of EPO and iron (Rhodes et al., 2008; Ramos et al., 2013). However, there had been no suggestions for the potential mechanism involved. The findings in

this chapter together propose a novel and critical role for the inactivation of the FOXO3 transcription factor in erythroblasts during macrophage mediated expansion. It finds that macrophages provide a multi-layered regulation of erythroid expansion by controlling the overall production of FOXO3 as well as its post-translational modification. More precisely, it identifies that the macrophage CD163 receptor inhibits total cellular FOXO3 levels in erythroid cells while senescence-associated molecules secreted by dexamethasone or IL10 modulated macrophages induce phosphorylation of the transcription factor. Furthermore, GROα on its own or the combination of senescence-associated molecules representative of the IL10 treated macrophage secretory profile substantially augment erythroid maturation in a FOXO3-independent manner.

Remarkably, there are many similarities between the macrophage dependent pathways that support erythroblast expansion and those activated in a number of tumours. For instance, senescent cells have been reported to release pro-inflammatory cytokines that promote tumorigenesis and the important function of tumour associated macrophages in various types of cancer are now being elucidated (Coppe et al., 2010; Davalos et al., 2010; Liu and Cao, 2015; Mantovani et al., 2017; Tchkonia et al., 2013; Yang and Zhang, 2017). Additionally, the FOXO transcription factors, including FOXO3, are commonly referred to in the context of oncology as tumour suppressors (Cho et al., 2014; Dansen and Burgering, 2008; Karube et al., 2011; Qi et al., 2011).

In conclusion, this thesis suggests how macrophages may alter the microenvironment to support the pathological erythropoiesis in conditions such as polycythaemia vera and how FOXO3 could hypothetically be targeted in erythroblasts

to ameliorate the outcome in patients suffering from dyserythropoietic anaemias or for those that fail EPO treatment. GROα may prove an especially valuable molecule in ineffective erythropoiesis given its ability to enhance erythroid maturation during increased expansion and thus might promote the adequate production of differentiated erythroid cells.

## 5.5 Summary of Findings

- Dexamethasone or IL10 treated macrophages enhanced erythroid expansion by reducing FOXO3 activity.

- Dexamethasone or IL10 modulated macrophages inactivated erythroid FOXO3 in two ways:

  1. by diminishing total cellular FOXO3 abundance (nuclear and cellular levels)

  2. by increasing the phosphorylation of FOXO3 on residue T32.

- The macrophage CD163 receptor is associated with blocking FOXO3 production in erythroblasts, while the senescence-associated molecules secreted by dexamethasone or IL10 treated macrophages are associated with the elevated FOXO3 phosphorylation.

- IL8 (3,600 pg/ml), Serpin E1 (100 pg/ml) and GROα (110 pg/ml) each improved the expansion of erythroblasts cultured alone via FOXO3 phosphorylation, while GROα simultaneously augmented erythroid maturation through a FOXO3-independent pathway.

- Combination of the senescence-associated molecules (CCL2, IL8, Serpin E1, IL1ra, GROα) at concentrations representative of the dexamethasone (31,000, 3,600, 100, 140, 60 pg/ml, respectively) or IL10 (12,300, 2,700, 60, 230, 110 pg/ml, respectively) treated macrophage secretory phenotypes significantly elevated erythroblast expansion. The IL10 modulated macrophage molecule combinations also augmented erythroid maturation, increasing the proportion of $CD71^{+}gpA^{+}$ cells by 8%.

# Chapter 6: Discussion, conclusions and future work

## 6.1 General Discussion

The overall objective of this thesis was to investigate the role that human macrophages play in enhancing erythroid development that may inform us of the key functions of central macrophages found in erythroblastic islands within the bone marrow. The thesis finds that the co-culture of erythroblasts with macrophages in direct physical contact had beneficial effects on erythroid expansion over their co-culture in separation (**chapter 3**). Moreover, it provides several insights that are novel to the field of erythropoiesis. Firstly, it identifies that human macrophages can be modulated by IL10 (10ng/ml) to induce a phenotype supportive of elevated erythroblast expansion (**chapter 4**). Secondly, the treatment of macrophages for four days with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) induced a senescence-associated secretory phenotype that promotes erythroblast expansion and, in certain combinations, also erythroid maturation (**chapter 4** and **5**). Finally, inactivation of the FOXO3 transcription factor in erythroblasts is found to contribute to their enhanced expansion, with the macrophage CD163 receptor diminishing total cellular FOXO3 levels and the senescence-associated molecules heightening its phosphorylation (**chapter 5**).

Previous work in the field had already established some of the crucial functions of macrophages in erythropoiesis. For instance, it is well known that central macrophages are important suppliers of iron for the synthesis of heme by maturing erythroid cells and that these macrophages phagocytose extruded nuclei (Alam et al., 2017; An and Mohandas, 2011; de Back et al., 2014; Korolnek and Hamza,

2015; Manwani and Bieker, 2008). A $CD15^{-}CD163^{+}CD169^{+}VCAM1^{+}$ population of human macrophages was also identified from healthy bone marrow aspirates, while peripheral blood derived macrophages from healthy, β-thalassaemia or polycythaemia vera patients have been shown to support erythroblast proliferation, albeit to differing extents (Chow et al., 2013; Ramos et al., 2013). Moreover, M2-like macrophages can form 'loose' interactions with erythroblasts to promote their expansion and the macrophage derived CD163 receptor is known to promote erythroid proliferation (Fabriek et al., 2007; Falchi et al., 2015). However, despite all this, there are still a number of crucial gaps in our knowledge in this field. For example, the corresponding ligand on erythroid cells for the CD163 receptor has not yet been identified. Furthermore, the macrophage-dependent signalling cascades activated in erythroblasts remain elusive. For this reason, the discovery in this thesis of how macrophages provide a multi-layered regulation of the FOXO3 pathway in erythroid cells is an important novel finding. Additionally, the observed role of GROα in augmenting erythroid maturation provides scope for future work to determine the FOXO3-independent pathway(s) that function to support erythroblast maturation during enhanced expansion.

There are various potential applications to understanding the molecular and cellular mechanisms involved during elevated erythropoiesis. These mechanisms may provide insights into not only normal but also pathological erythropoiesis *in vivo*, as a result of inherited or acquired disease, for example β-thalassaemia or polycythaemia vera. This is of significance because even though blood transfusions and phlebotomy may be used in cases of chronic anaemia or polycythaemia vera, respectively, neither is a panacea without the need for routine visits to the clinic or

without potential development of secondary complications, such as iron overload. Therefore, dissecting the signalling pathways implicated during macrophage-mediated erythropoiesis not only furthers our knowledge of this complex process but could also hypothetically lead to the development of new treatments. Moreover, this research might elucidate, at least some of the requirements for the therapeutic production of erythrocytes *in vitro*, which may be used for niche applications where it is difficult to find donors.

The following sections describe the objectives and findings of each chapter. The first aim was to establish macrophage and erythroblast culture conditions to study macrophage enhancement of erythropoiesis (**chapter 3**). Once the optimal macrophage density had been determined, the effects of dexamethasone or IL10 treatment of macrophages on erythroid development could be investigated (**chapter 4**). The final chapter involved examining the influence of these dexamethasone or IL10 modulated macrophages on FOXO3 inactivation in erythroblasts as a cause of the elevated expansion (**chapter 5**).

### 6.1.1 Examination of macrophage and erythroblast culture conditions

It was necessary as a first step to define the culture conditions for all future experiments and for initial studies that investigated how different seeding densities affected macrophage surface marker expression, viability and subsequently erythroid development (**chapter 3**). It was found that macrophages in co-culture could only enhance erythroid expansion at a certain seeding concentration (1 x $10^5$ cells/well) that significantly augmented surface CD163 and CD14 but simultaneously reduced CD16, CD86 and CD83 glycoprotein expression. However, macrophages plated at

1 x $10^5$ cells/well also had the greatest viability, which may also have contributed towards the heightened erythroblast expansion. Densities above or below this had no significant effect compared to erythroblasts grown alone, except for the lowest macrophage seeding density of 0.25 x $10^5$ cells/well that actually lowered erythroblast expansion by day 14 and was potentially due to the increased macrophage apoptosis. Nevertheless, it must be taken into consideration that macrophages at the lowest seeding density might also have had stronger erythrophagocytic properties.

Co-cultures of macrophages and erythroblasts either in direct contact or physical separation provided insight into the contributions of adhesive factors on macrophages versus secreted soluble molecules. There was a simultaneous increase in expansion and blocked maturation on day 14 but no change in maturation or enucleation on day 20 of erythroblasts co-cultured with macrophages in contact compared to culture in separation. Ramos and colleagues similarly found the greatest improvement in human erythroid expansion in direct contact with macrophages and observed that this simultaneously diminished the percentage of enucleated day 8 cells (Ramos et al., 2013). Therefore, this seems to suggest that while macrophages promote erythroblast expansion and inhibit maturation during erythroid development, they might not have such substantial effects on maturation or enucleation as erythroid cells approach the reticulocyte stage.

### 6.1.2 Modulation of macrophages to support enhanced erythropoiesis

While the work in **chapter 3** defined the appropriate macrophage seeding densities and highlighted the importance of having defined culture conditions to investigate

any effects on erythroblast development, **chapter 4** explored how a modulated M2-type macrophage could affect erythropoiesis. Dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) treatment of MCSF differentiated macrophages induced what is known as a 'deactivated' M2c phenotype but distinct differences in morphology, surface marker expression and cytokine secretion were found with the two molecules that could suggest further subdivisions of the macrophage phenotype might be applicable. The glucocorticoid tended to downregulate cell surface markers such as CD14, CD80 and VCAM1, whereas IL10 upregulated these markers as well as most other receptors, except for HLA-DR. Despite the well-established notion that glucocorticoids suppress antigen-presenting pathways in both mice and humans (Schwiebert et al., 1995; Snyder and Unanue, 1982), the link between repressed adaptive activation but intensified innate immune signatures following long-term exposure to glucocorticoids was only more recently established (van de Garde et al., 2014). Analogous to these findings by van de Garde and colleagues, this thesis found a hugely amplified (over double) secretion of MyD88-regulated CCL2 as well as elevated IL8 in co-cultures involving dexamethasone stimulated macrophages compared to untreated cells, suggesting there were strengthened innate immune components. Furthermore, Kratochvill and coworkers observed that the adherence of tumour associated macrophages (TAMs) reduced inflammatory mRNA expression (Kratochvill et al., 2015), which could provide another explanation for the overall lower levels of secreted pro-inflammatory cytokines by IL10 stimulated macrophages that were observed to be more adherent to plastic plates than dexamethasone treated cells.

Other molecules were also detected at different concentrations in co-culture supernatants, such as Serpin E1 and GROα from dexamethasone or IL10

supplemented macrophages. Interestingly, several studies have described the role of IL8, Serpin E1 and GROα during the induction of the senescence-associated secretory phenotype (SASP) in senescent cells (Acosta et al., 2008; Kortlever et al., 2006; Kuilman et al., 2008; Wajapeyee et al., 2008). This suggests that stimulation of human M2-polarised macrophages with dexamethasone or IL10 provoked inflammasome-dependent IL1 signalling and triggered a pro-inflammatory response that normally would be involved in paracrine senescence (Acosta et al., 2013; Herranz et al., 2015). The SASP has been found to induce opposing effects depending on the environmental setting, with both pro-tumorigenic (Coppe et al., 2006; Coppe et al., 2010; Coppe et al., 2008) and tumour suppressive (Hoenicke and Zender, 2012; Kuilman and Peeper, 2009) potential. In the setting studied in this thesis, it seems that the SASP may trigger signalling cascades in erythroblasts that are parallel to tumourigenic pathways, with reduced FOXO3 transcriptional activities (discussed in more detail with relevance to **chapter 5**).

A recent study using dexamethasone discovered that human macrophages can be divided into two distinct populations, namely $CD169^-$ macrophages that form 'tight' interactions with mature erythroblasts or $CD169^+$ cells that form 'loose' interactions with proerythroblasts and support their proliferation (Falchi et al., 2015). While these studies provide important contributions to our understanding of macrophage-erythroblast cell interactions during co-culture in the presence of the glucocorticoid, this thesis is novel in that it targets dexamethasone or IL10 (or both simultaneously) directly to macrophages prior to co-culture with erythroblasts. Hence, the work herein investigates the precise influence of these modulated macrophages on erythroid development.

It was found that dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) treated macrophages induced similar improvements in erythroblast expansion, with elevated cell cycle transition from $G_0/G_1$ to S + $G_2$/M compared to erythroblasts cultured alone. This was associated with increased cyclin D1 protein levels in erythroblasts, providing an explanation for the faster cell cycle transition during their culture with the differentially treated macrophages. A clear difference was that dexamethasone treated macrophages inhibited erythroid maturation and heightened intracellular ROS levels in immature ($CD71^+gpA^-$) erythroblasts whereas IL10 stimulated cells caused no change. Nevertheless, the consistencies in augmented erythroid expansion, increased cyclin D1 and reduced catalase expression in erythroblasts co-cultured with either dexamethasone or IL10 treated macrophages suggested there might have been common signalling pathways activated in erythroid cells.

### 6.1.3 The FOXO3 signalling pathway in erythroblasts

Cyclin D1 is negatively regulated by the FOXO3 transcription factor while catalase is positively regulated (Zhang et al., 2014; Yu et al., 2010; Marinkovic et al., 2007). This thesis found an upregulated cyclin D1 protein expression but downregulated catalase expression in erythroblasts cultured with either of the differentially supplemented macrophages. Furthermore, FOXO3 inactivation has been implicated in the elevated proliferation of numerous types of cancer cells (Liang et al., 2017; Lin et al., 2011; Lin et al., 2015; Lv et al., 2015; Wei et al., 2016). Thus, it was hypothesised that dexamethasone or IL10 modulated macrophages enhanced erythroid proliferation via reduced FOXO3 activity (**chapter 5**).

There appeared to be a clear link between FOXO3 inactivation and improved erythroid expansion, as suggested by both the increased phosphorylation (on residue T32) and reduced total cellular abundance of FOXO3 during the augmented expansion of erythroblasts co-cultured with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) treated macrophages. However, while higher levels of the adaptor protein 14-3-3ζ were positively associated with increased cytoplasmic FOXO3 in erythroblasts grown with dexamethasone modulated macrophages, this was not the case for erythroid cells cultured with IL10 stimulated cells.

Studies involving siRNA mediated knockdown of the macrophage CD163 receptor demonstrated that a lack of erythroblast binding to this glycoprotein significantly blocked their expansion and approximately doubled total cellular FOXO3 levels to an abundance comparable to that in erythroblasts grown without any macrophages. This highlights the dependence of erythroblasts on the macrophage adhesion molecule CD163 and on repressed FOXO3 signalling during augmented erythroid expansion.

In contrast, the addition to erythroblasts only of IL8, Serpin E1 or GROα at the highest concentrations detected in co-culture supernatants significantly improved expansion via FOXO3 phosphorylation. Similarly, supplementing erythroblasts with all the senescence-associated molecules (CCL2, IL8, Serpin E1, IL1ra and GROα) at the concentrations found in co-cultures involving either dexamethasone or IL10 stimulated macrophages also elevated erythroid expansion. Remarkably, the IL10 treated macrophage molecule combinations simultaneously increased the percentage of mature ($CD71^{+}gpA^{+}$) erythroblasts by 8% and, given that GROα on its own also augmented erythroid maturation, although to a lesser extent, suggests GROα was an

important soluble molecule involved in the enhanced maturation. This function of GROα as an erythropoiesis stimulating agent is a novel finding and could potentially be beneficial during the mass generation of erythrocytes *ex vivo*, either as a diagnostic or therapeutic tool.

Therefore, dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) modulated macrophages provided multiple levels of regulation for erythroblast expansion via CD163 and senescence-associated secreted molecules that blocked total cellular FOXO3 abundance and increased phospho-FOXO3 levels, respectively. These layers of regulation highlight the importance of the FOXO3 pathway in erythroid proliferation.

A summary of macrophage effects on erythroblasts is shown in **Table 6.1**.

**TABLE 6.1. Functional effects of modulated macrophages on erythroblasts following co-culture.** *The effects on erythroblasts of macrophages treated with $10^{-6}$M dexamethasone (Dex), 10ng/ml IL10 (IL10), or simultaneous $10^{-6}$M dexamethasone and 10ng/ml IL10 (Dex + IL10) are shown. ↑ or ↓ indicate an increase or decrease, respectively, compared to erythroblasts only (black) or untreated macrophages (red). More arrows mean there is a greater effect; ( ) means there is only a slight effect in the indicated direction; = means no change; N.D. means not done.*

| | Dex | | IL10 | | Dex + IL10 | |
|---|---|---|---|---|---|---|
| Expansion | ↑↑ | ↑↑ | ↑↑ | ↑↑ | ↑↑ | ↑↑ |
| Maturation | ↓↓ | ↓ | = | (↑) | ↓↓ | ↓ |
| Viability | = | = | = | = | = | = |
| ROS | ↑ | ↑ | = | = | ↑↑ | ↑↑↑ |
| Catalase | ↓↓ | ↓↓ | ↓↓ | ↓↓ | (↓) | = |
| Cyclin D1 | ↑↑ | ↑ | ↑↑ | ↑ | ↑ | ↑ |
| 14-3-3ζ | ↑↑ | ↑ | = | = | ↑ | (↑) |
| Total cellular FOXO3 | ↓↓ | = | ↓↓ | = | N.D. | N.D. |
| Phospho-FOXO3 | ↑ | ↑ | ↑ | ↑ | N.D. | N.D. |
| Nuclear FOXO3 | ↓↓ | ↓ | ↓ | = | N.D. | N.D. |

## 6.2 Conclusions

1. Macrophage cell surface marker levels and viabilities changed with varying seeding densities (**chapter 3**).
2. Treatment for four days of human peripheral blood monocyte-derived macrophages with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) altered morphology, cell surface marker expression and induced secretion of senescence-associated molecules (**chapter 4**).
3. Co-culture of erythroblasts with dexamethasone, IL10 or dexamethasone + IL10 treated macrophages similarly enhanced expansion by increasing proliferation while differentially affecting erythroid maturation (**chapter 4**).
4. Intracellular ROS was substantially increased in immature ($CD71^+gpA^-$) erythroblasts co-cultured with dexamethasone modulated macrophages. Dexamethasone + IL10 treated macrophages elevated ROS levels in both immature ($CD71^+gpA^-$) and mature ($CD71^+gpA^+$) erythroblasts (**chapter 4**).
5. FOXO3 was inactivated via reduced total cellular levels and heightened phosphorylation during enhanced erythroblast expansion. siRNA targeted knockdown of CD163 on macrophages identified this receptor as the major factor responsible for blocking FOXO3 abundance in erythroid cells. In contrast, IL8 (3,600 pg/ml), Serpin E1 (100 pg/ml) or GROα (110 pg/ml) added individually to erythroblasts only, or the addition of all the senescence-associated molecules (CCL2, IL8, Serpin E1, IL1ra, GROα) together at concentrations representative of dexamethasone or IL10 treated macrophage co-culture supernatants, induced phosphorylation of FOXO3 (**chapter 5**).

6. GROα augmented erythroid maturation through FOXO3-independent signalling when added to erythroblasts either individually (at 110 or 1,100 pg/ml) or in combination with the other senescence-associated molecules (at the doses found in IL10 treated macrophage co-culture supernatants) (**chapter 5**).

In conclusion, the data from this thesis together provide valuable insights into different ways in which modulated human monocyte-derived macrophages can affect erythroid development *in vitro* (**Figure 6.1**). It is evident that CD163 and senescence-associated factors (IL8, Serpin E1 and GROα) derived from macrophages contribute to the augmented erythroid expansion (**Figure 6.2**). Hence, the multifaceted effects of macrophages can suggest why the autonomous differentiation program of erythroblasts is often not sufficient to recapitulate the effects observed with erythroblastic islands. While the modulation of macrophages with dexamethasone ($10^{-6}$M) or IL10 (10ng/ml) is useful for clarifying the roles of macrophages in erythropoiesis, one would hypothesise that IL10 treated macrophages would be more valuable in future studies. This is because these macrophages were $CD163^{+}CD169^{+}VCAM1^{+}$, thus representing bone marrow macrophages more closely.

**FIGURE 6.1. Dexamethasone or IL10 modulated macrophages affect erythroid development in different ways.** *Dexamethasone ($10^{-6}$M) treated macrophages increased surface CD163 expression and secreted higher amounts of CCL2 (10kDa), IL8 (8.4kDa) and Serpin E1 (45kDa). Although IL10 (10ng/ml) supplemented macrophages also augmented CD163 receptor expression, they instead released IL1ra (17kDa) and GROα (11kDa) at greater levels compared to dexamethasone modulated macrophages. Dexamethasone treated macrophages improved erythroblast expansion while blocking maturation, whereas IL10 stimulated cells supported both enhanced expansion and maturation of erythroblasts.*

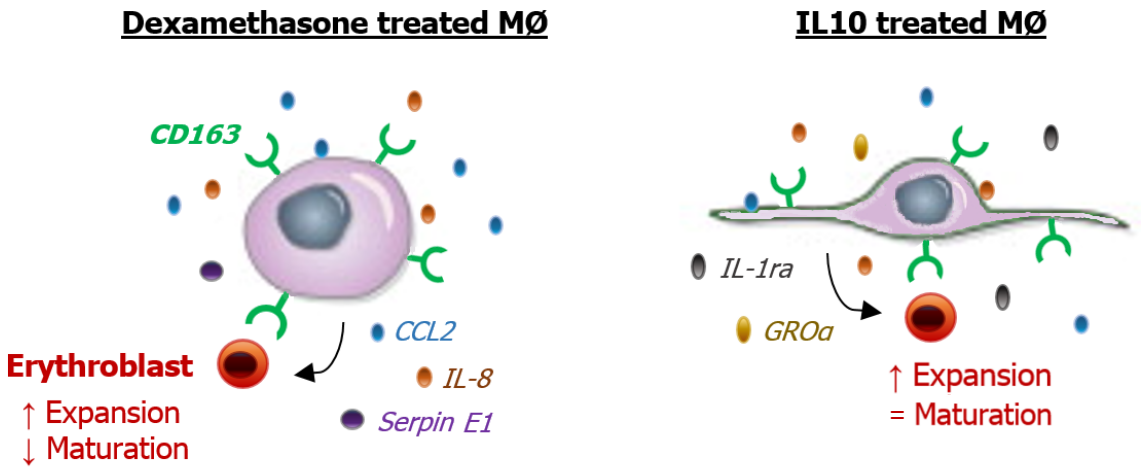
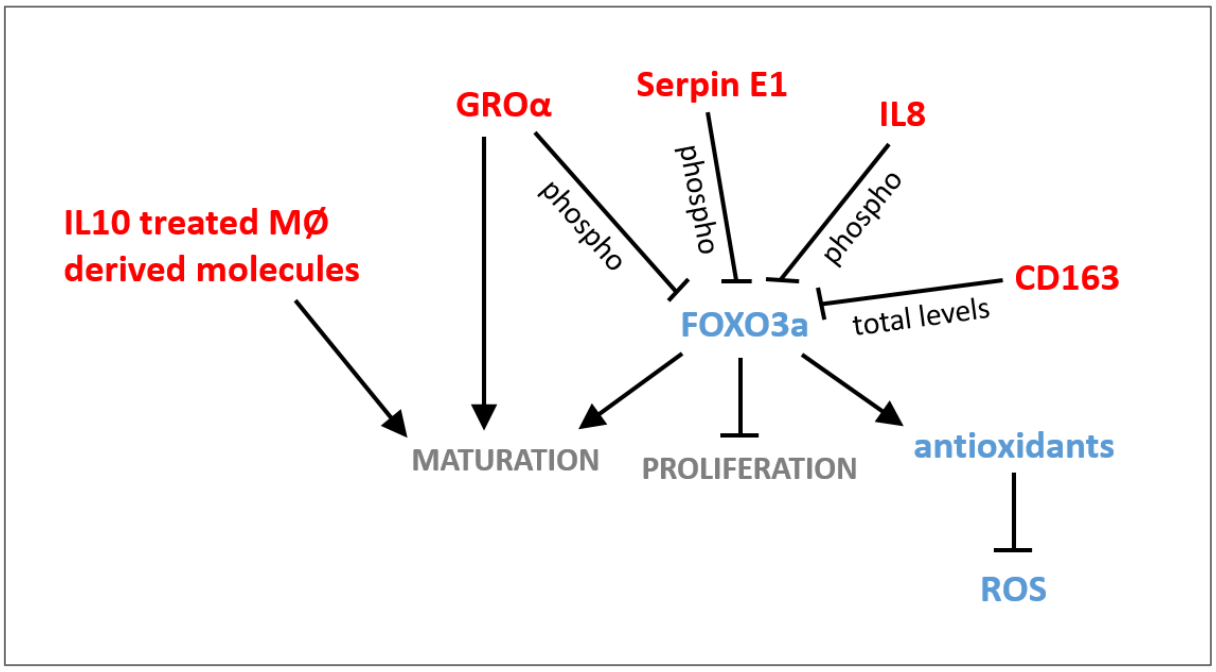


**FIGURE 6.2. The role of the FOXO3 signalling pathway in human erythroblasts following co-culture with autologous dexamethasone or IL10 treated macrophages.** *The role of the macrophage receptor CD163 as well as GROα, Serpin E1 and IL8 in the inactivation of erythroid FOXO3 and subsequent effects on erythroblast development are shown. CD163 inhibits FOXO3 by reducing total cellular levels whereas the soluble molecules inactivate FOXO3 through its phosphorylation. Question marks (?) illustrate that molecular mechanisms leading to those processes in erythroid cells are yet to be identified.*



## 6.3 Limitations

The major limitation to the work in this thesis is the fact that all investigations were performed using peripheral blood monocyte-derived macrophages as a representation of erythroblastic island macrophages in the human bone marrow. Although human macrophages can be obtained from bone marrow aspirates, the number of cells collected can be low and variable, making some experimental work difficult. Moreover, differentiating macrophages from peripheral blood monocytes for several days *in vitro* produces homogenous populations, which makes investigations into the roles of specific molecules easier.

Moreover, phagocytosis studies of the modulated macrophages involved measuring the uptake of *E.coli* bioparticles, meaning TLRs on macrophages would most probably have been engaged and hence the phenotype may have been affected. Therefore, these results are only interpreted as the maximal potential of the differentially treated macrophages to phagocytose substances.

## 6.4 Future Work

A number of studies could be performed to further elucidate the precise functions of human macrophages in erythroid development. Firstly, since CD163 and the senescence-associated molecules were on their own found to inactivate FOXO3, it would be of relevance to examine if the combined addition of CD163 together with the senescence-associated molecules to erythroblasts only recapitulates the extent of erythroid expansion and maturation seen in co-cultures with macrophages. If this were the case, it would suggest that the CD163 receptor and these soluble molecules are the main macrophage factors that drive erythroblast expansion and/or maturation.

In line with the finding that GROα enhanced both erythroid expansion and maturation when added to erythroblasts alone, it would be useful to add blocking antibodies against GROα in co-cultures involving IL10 stimulated macrophages to determine if this is the main molecule driving the improved erythroid maturation. Also adding blocking antibodies against IL8, which shares the same receptor on erythroid cells as GROα (CXCR2), would be beneficial to examine if the combined effect of these two soluble molecules is behind the substantially augmented erythroblast maturation that occurs when all the senescence-associated molecules are added at doses present in IL10 treated macrophage supernatants. Identifying any other factors that can promote erythroblast maturation would also be highly beneficial. It would be worthwhile determining whether other factors produced by macrophages, such as soluble microRNAs, also play a part in erythropoiesis.

The erythroid integrin $\alpha_4\beta_1$ is expressed at all stages of differentiation (Spring et al., 2013) and binds to the macrophage VCAM1, so it could be hypothesised that there are some fundamental roles associated with this interaction. To investigate this, one would ideally knockdown surface VCAM1 on macrophages and study the subsequent effects on erythropoiesis at various time points and erythroid differentiation stages. Blocking antibodies against VCAM1 could also be used.

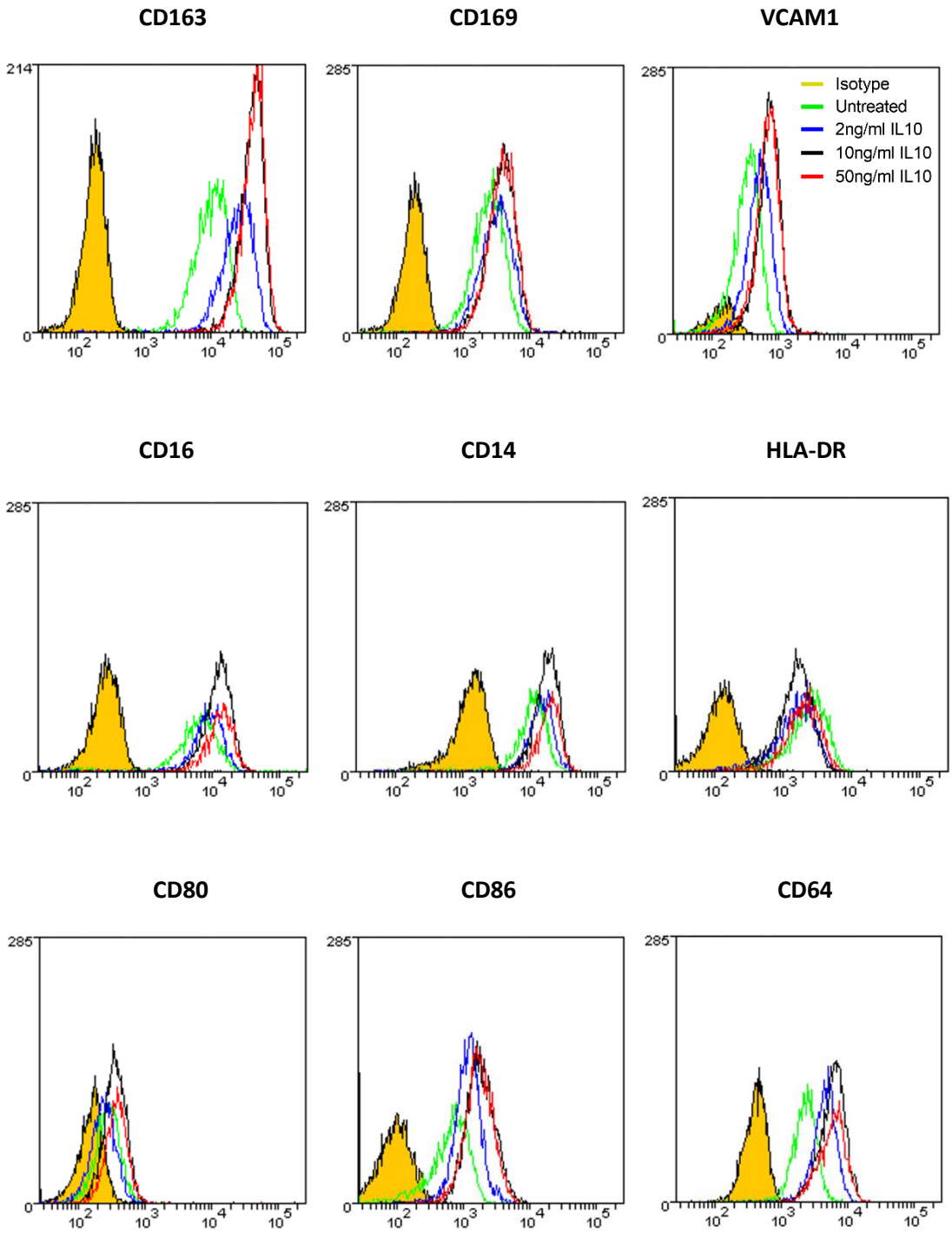
The erythroblast receptor for the macrophage CD163 glycoprotein is yet to be discovered. Despite the relevance of CD163 in heightened erythroid expansion (Fabriek et al., 2007), any attempts to identify the erythroid ligand may have been unfruitful due to the loose interactions that are involved (Falchi et al., 2015).

An extension of the work described in this thesis, with more extensive proteomic, metabolic and transcriptomic studies, would therefore confirm how dexamethasone or IL10 treated macrophages alter erythroblast expansion and maturation. Moreover, it would be beneficial to determine if GROα supports erythropoiesis *in vivo* and if, along with other current strategies, the molecule could aid patients with abnormal erythropoiesis. Other potential applications are in the development of methods that expand erythroid cells *ex vivo.* Finally, additional studies to examine the effector pathways induced by human bone marrow macrophages as opposed to peripheral blood derived macrophages would significantly advance our understanding of human erythroid development.

# Appendices

## Appendix 4.1

**Raw histogram plots showing the change in various surface marker levels for macrophages treated with the different doses of IL10.**



# Appendix 4.2

## Table showing the relative signal of other cytokines in the different co-culture supernatants as detected by the human cytokine array.

| | EB only | | Untreated | | Dex treated | | IL10 treated | |
|---|---|---|---|---|---|---|---|---|
| MIF | 963 | 927 | 987 | 962 | 1968 | 1968 | 862 | 864 |
| IL-13 | 396 | 424 | 392 | 384 | 396 | 461 | 397 | 383 |
| CXCL11/I-TAC | 287 | 231 | 219 | 163 | 300 | 337 | 220 | 279 |
| CCL1/I-309 | 349 | 220 | 301 | 295 | 451 | 508 | 284 | 245 |
| IL-16 | 354 | 296 | 331 | 352 | 465 | 399 | 282 | 261 |
| IL-2 | 299 | 307 | 192 | 324 | 282 | 570 | 232 | 236 |
| CXCL12/SDF-1 | 705 | 748 | 233 | 180 | 855 | 935 | 861 | 862 |
| TNF-a | 448 | 526 | 260 | 283 | 327 | 349 | 379 | 330 |
| IL-17A | 316 | 368 | 322 | 402 | 247 | 264 | 208 | 176 |
| IL-4 | 159 | 210 | 679 | 107 | 200 | 254 | 171 | 172 |
| G-CSF | 321 | 442 | 188 | 169 | 382 | 486 | 270 | 318 |
| TREM-1 | 586 | 514 | 306 | 422 | 418 | 503 | 190 | 140 |
| IL-17E | 764 | 742 | 320 | 242 | 760 | 531 | 353 | 176 |
| IL-5 | 303 | 264 | 80 | 108 | 152 | 164 | 82 | 60 |
| GM-CSF | 720 | 612 | 210 | 223 | 455 | 521 | 254 | 165 |
| CCL5/RANTES | 416 | 422 | 294 | 517 | 406 | 334 | 332 | 166 |
| IL-18/IL-1F4 | 377 | 401 | 200 | 220 | 745 | 863 | 225 | 188 |
| IL-6 | 330 | 305 | 242 | 337 | 1143 | 1380 | 282 | 279 |
| ICAM-1/CD54 | 378 | 344 | 289 | 250 | 1151 | 1322 | 414 | 332 |
| CD40 ligand/TNFSF5 | 964 | 1040 | 541 | 626 | 1250 | 1117 | 639 | 544 |
| IFN-g | 1020 | 1040 | 437 | 716 | 1546 | 1546 | 441 | 340 |
| Complement Component C5/C5a | 156 | 187 | 171 | 258 | 204 | 146 | 51.9 | 33.1 |
| IL-1β | 808 | 561 | 1256 | 681 | 373 | 511 | 565 | 729 |

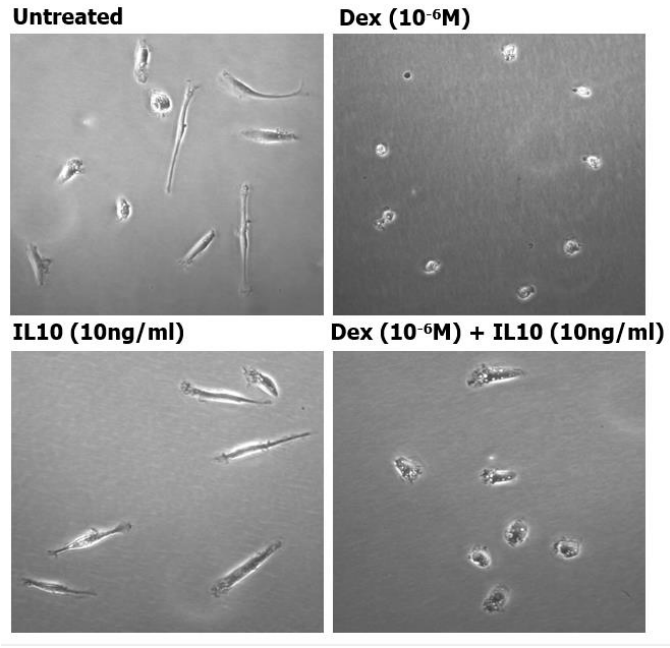
## Appendix 4.3

**Table describing the functions of some of the senescence-associated secreted molecules detected in co-culture supernatants.**

| Cytokine | Function |
|---|---|
| CCL2 | Monocyte chemoattractant; regulates migration and infiltration of monocytes and macrophages |
| GROα | Neutrophil chemoattractant |
| IL8 | Induces chemotaxis and phagocytosis |
| IL1ra | An inhibitor of IL1α and IL1β, thus regulating pro-inflammatory responses |
| Serpin E1 | A serine protease inhibitor that blocks the activities of tissue plasminogen activator (tPA) and urokinase (uPA) |

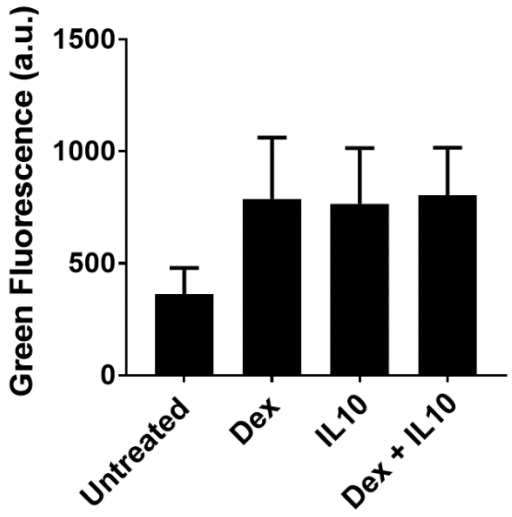
## Appendix 4.4

**Brightfield images of macrophages following their treatment with only MCSF (untreated), or with dexamethasone, IL10 or both dexamethasone and IL10 simultaneously.**



## Appendix 4.5

**The green fluorescence intensity of macrophages after 1hour as an indication of *E.coli* bioparticle uptake. Data are representative of cells from one donor in duplicate.**



# Appendix 5.1

**Table showing the relative signal of cytokines secreted by siRNA2-targeted compared to control macrophages as detected by the human cytokine array.**

| | siRNA(control) | | siRNA2 | |
|---|---|---|---|---|
| CCL2 | 12008 | 11985 | 12209 | 12031 |
| IL-8 | 2742 | 2513 | 2599 | 2684 |
| Serpin E1 | 50 | 41 | 58 | 50 |
| IL-1ra | 223 | 247 | 207 | 235 |
| GROα | 121 | 139 | 128 | 134 |
| MIF | 970 | 931 | 982 | 974 |
| IL-13 | 405 | 437 | 381 | 425 |
| CXCL11/I-TAC | 289 | 250 | 276 | 249 |
| CCL1/I-309 | 348 | 215 | 312 | 291 |
| IL-16 | 346 | 299 | 330 | 352 |
| IL-2 | 281 | 311 | 208 | 333 |
| CXCL12/SDF-1 | 300 | 334 | 259 | 287 |
| TNF-a | 306 | 274 | 291 | 254 |
| IL-17A | 338 | 386 | 355 | 394 |
| IL-4 | 305 | 210 | 277 | 219 |
| G-CSF | 200 | 165 | 198 | 156 |
| TREM-1 | 487 | 446 | 389 | 452 |
| IL-17E | 400 | 352 | 337 | 378 |
| IL-5 | 97 | 143 | 91 | 115 |
| GM-CSF | 256 | 210 | 298 | 245 |
| CCL5/RANTES | 447 | 424 | 359 | 433 |
| IL-18/IL-1F4 | 210 | 164 | 212 | 228 |
| IL-6 | 334 | 322 | 298 | 321 |
| ICAM-1/CD54 | 255 | 209 | 276 | 185 |
| CD40 ligand/TNFSF5 | 713 | 802 | 642 | 756 |
| IFN-g | 639 | 590 | 614 | 703 |
| Complement Component C5/C5a | 145 | 175 | 169 | 180 |
| IL-1β | 664 | 672 | 708 | 654 |

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