Investigation of the immunomodulatory effects of MSC-derived extracellular vesicles on human monocyte-derived macrophages

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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To Elizabeth,

best of mothers, best of women and best of friends

“It is not the critic who counts; not the man who points out how the strong man stumbles, or where the doer of deeds could have done them better. The credit belongs to the man who is actually in the arena, whose face is marred by dust and sweat and blood; who strives valiantly; who errs, who comes short again and again, because there is no effort without error and shortcoming; but who does actually strive to do the deeds; who knows great enthusiasms, the great devotions; who spends himself in a worthy cause; who at the best knows in the end the triumph of high achievement, and who at the worst, if he fails, at least fails while daring greatly, so that his place shall never be with those cold and timid souls who neither know victory nor defeat.”

Excerpt from the speech “The man in the arena: Citizenship in a republic”, delivered by Theodore Roosevelt in 1910.

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Abstract

Acute kidney injury (AKI) is a common medical condition characterised by an abrupt decrease in kidney function. AKI can lead to chronic kidney disease (CKD), which has a global prevalence of approximately 10% and is linked to high morbidity and mortality. Despite the high prevalence of kidney injury, the pathophysiological mechanisms that elicit either tissue scarring or function recovery are poorly understood. Due to this lack of understanding, there is a shortage of options for effective treatments and depending on the severity of the disease the only available resources are dialysis and kidney transplantation. Hence, it is of utmost importance that new strategies are developed for the treatment of kidney injury.

Macrophage polarisation has a crucial role in the context of kidney injury, as there is evidence that a shift in macrophage phenotype aids in tissue repair and function recovery. The means by which this shift takes place is still not completely elucidated, but mesenchymal stromal cells (MSCs) appear to display immunomodulatory properties that would prime macrophages towards an anti-inflammatory phenotype. MSCs have been also investigated as a means for treatment of renal diseases and recently there is a reckoning that the mechanism of action is not through engraftment and differentiation, but instead through paracrine factors, including extracellular vesicles (EVs).

Therefore, the aim of this study was to assess the effects of MSC-derived EVs in activated human monocyte-derived macrophages (hMDMs), so as to verify whether these EVs would elicit a change in macrophage phenotype. For this purpose, MSCs were characterised and an isolation protocol was optimised. hMDMs were co-incubated with EVs and surface marker expression was evaluated through microscopy, cytometry and cytokine secretion.

MSCs displayed required characteristics, including expression of CD73, CD90 and CD105. The method used for EV isolation was serum starvation followed by ultracentrifugation of medium supernatant. EVs had an average diameter ranging from 150 to 200 nm and expressed CD9, CD63 and CD81.

MSC-derived EVs had an effect on hMDM morphology, but did not change surface marker expression or cytokine secretion. When hMDMs were incubated with human umbilical cord MSCs it was possible to notice changes in expression of CD14, CD80 and CD163, suggesting that soluble factors, including cytokines, are necessary to elicit a shift in phenotype. Angiogenic assays were put in place to confirm EV potency, but EVs did not promote vessel formation.

Taken together, even though there was no significant change in surface marker expression, these results contributed to a better understanding of the role of MSC-derived EV in hMDM polarisation. Among the reasons for the lack of effect of MSC-derived EVs could be the low number of samples analysed and the high inter-individual variability regarding hMDMs, indicating the need for further investigations.

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Abbreviations

AKI – Acute kidney injury

APC – Allophycocyanin

bFGF – Basic fibroblast growth factor

BSA – Bovine serum albumin

Casp-1 – Caspase 1

CFSE – Carboxyfluorescein diacetate succinimidyl ester

CKD – Chronic kidney disease

DAMP – Danger-associated molecular pattern

DAPI – Diamidino-2-phenylindole

DC – Dendritic cell

DMSO – Dimethyl sulfoxide

DPBS – Dulbecco's phosphate buffered saline

EDTA – Ethylenediaminetetraacetic acid

EGF – Epidermal growth factor

EV – Extracellular vesicle

EXO-FBS – Exosome-depleted foetal bovine serum

FBS – Foetal bovine serum

FITC – Fluorescein isothiocyanate

GFR – Glomerular filtration rate

GM-CSF – Granulocyte macrophage colony stimulating factor

hBMMSC – Human bone marrow mesenchymal stromal cells

HDMEC – Human dermal microvascular endothelial cell

HDNF – Human dermal normal fibroblast

hMDM – Human monocyte-derived macrophage

hUCMSC – Human umbilical cord mesenchymal stromal cell

HUVEC – Human umbilical vein endothelial cell

IFN-γ – Interferon gamma

IGF – Insulin growth factor

IGFR – Insulin growth factor receptor

IL-1β – Interleukin 1 beta

IL-1ra – Interleukin 1 receptor antagonist

IL-4 – Interleukin 4

IL-10 – Interleukin 10

IMDM – Iscove's modified Dulbecco's medium

IRI – Ischemia-reperfusion injury

LPS – Lipopolysaccharide

M-CSF – Macrophage colony stimulating factor

MEM-α – Minimum essential medium alpha

MHC – Major histocompatibility complex

MMP – Matrix metalloproteinase

mRNA – Messenger RNA

miRNA – Micro RNA

MSC – Mesenchymal stromal cell

MVB – Multivesicular body

NK – Natural killer cell

NTA – Nanoparticle Tracking Analysis

PAMP – Pathogen-associated molecular pattern

PBMC – Peripheral blood mononuclear cell

PE – Phycoerythrin

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RPC – Renal progenitor cell

sCR – Serum creatinine

SEM – Standard error mean

TBS – Tris-buffered saline

TBST – Tris-buffered saline 0.1% Tween 20

TGF-β – Transforming growth factor beta

Th1 – T helper 1

Th2 – T helper 2

TLR – Toll-like receptor

TNF-α – Tumour necrosis factor alpha

Treg – Regulatory T lymphocyte

VEGF – Vascular endothelial growth factor

Chapter 1 – Introduction

1.1. Acute kidney injury: a search for mechanisms and treatments

Acute kidney injury (AKI) is a major public health issue worldwide and is linked to high morbidity and mortality. Understanding the pathophysiology of the disease and how immune cells contribute to either repair or permanent loss of kidney function is paramount to the development of new strategies for effective treatment.

1.1.1. AKI: definition and potential outcomes

AKI is defined by the abrupt decrease in kidney function, which is resultant of both structural damage (injury) and impairment of function [1]. AKI is diagnosed based on a decrease on the glomerular filtration rate (GFR), which is assessed through the measurement of serum creatinine (sCr) and/or urine output over a given period of time [1-3]. It is a common medical condition that is estimated to occur in 1 out of 7 of all hospital admissions in England with reported increase in incidence over time [4], and can lead to chronic kidney disease (CKD) and permanent loss of function [5]. CKD has a global prevalence of almost 10% and might demand dialysis and/or kidney transplantation depending on the severity of the disease [6].

Different models to investigate AKI in pre-clinical studies have been developed. Chemical compounds can be used to induce AKI, such as gentamicin and cisplatin, an antibiotic and a chemotherapeutic agent, respectively [7, 8]. Glycerol can also be used to induce AKI decurrent from rhabdomyolysis [9], a breakdown of skeletal muscle that releases toxic

intracellular contents into the blood, that has AKI as a potential and serious complication [10]. Among non-pharmacological approaches, ischaemia-reperfusion (IR)-induced AKI, which was developed as a rodent model of acute tubular injury [11], has been extensively used and reported to display clinical relevance [12]. These models aid in the investigation of pathophysiological mechanisms, but no method can perfectly mirror human conditions.

Interestingly, AKI can be spontaneously resolved, and kidney function restored, through tubular epithelial cell proliferation and an important switch in macrophage phenotype that helps prevent chronic inflammation, as shown in Figure 1.1. Nevertheless, there is the possibility of maladaptive repair, particularly when the kidney has been previously injured; in those cases, there is an increased chance of a predominance of persistent inflammatory macrophages, due to the absence of a phenotype switch, that will cause chronic fibrosis and eventual loss of function [13].

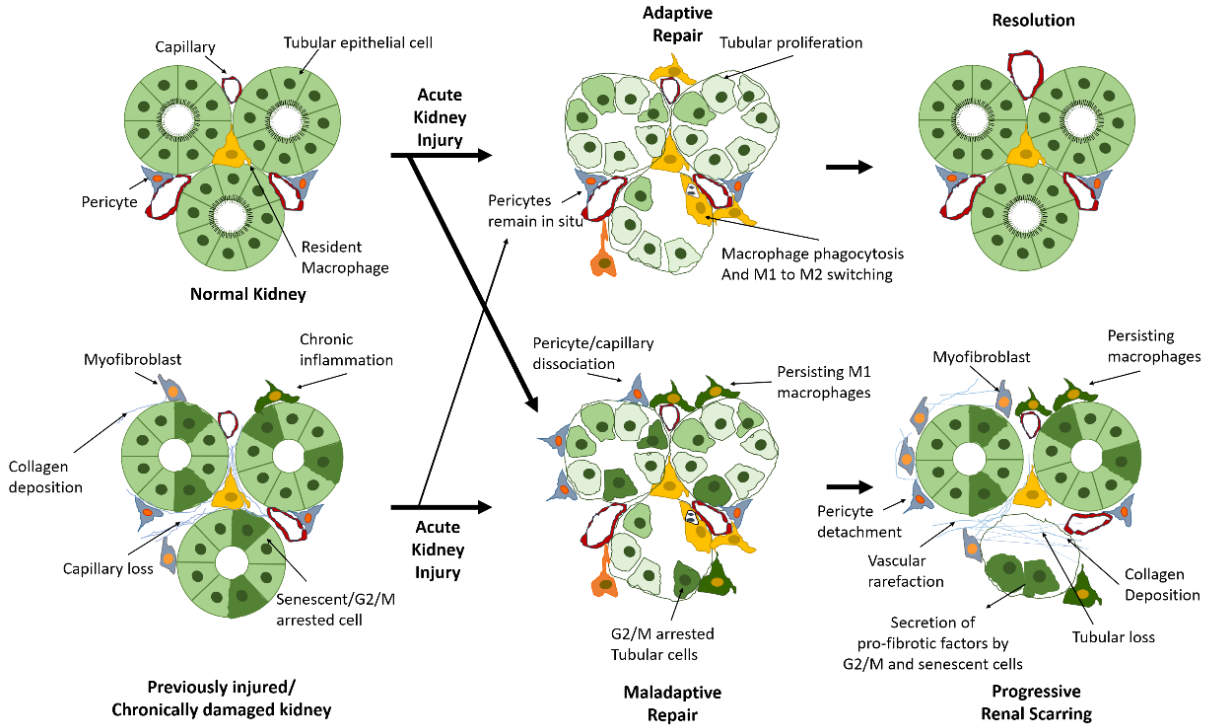

Figure 1. 1 Acute kidney injury and maladaptive repair.

Kidneys which are previously injured are less likely to undergo complete adaptive repair after a renal insult. In the context of pre-injury fibrosis, the kidney is more likely to repair maladaptively with increased tubular loss and scarring. While a normal kidney can respond to injury with adaptive repair it is also recognized that with greater levels of injury and increasing age, maladaptive repair to progressive loss of function is more likely. Adapted from [13].

The precise mechanisms that determine the outcome of an AKI episode and whether it will progress to chronic kidney disease are not entirely understood. Age, underlying conditions, such as hypertension and diabetes, senescence and microvascular loss are known contributors to progression to CKD [14-16]. Importantly, macrophage phenotype switching to an anti-inflammatory profile has been consistently reported as occurring during adaptive repair, and not in maladaptive repair, and thus is considered to be a determinant event to kidney function restoration [17].

Given that currently the only available treatments for kidney injury are haemodialysis and transplantation, which are replacement therapies and do not treat the causes of injury, the focus of regenerative medicine research has been to identify individual mechanisms that could be explored in order to permanently restore kidney function.

1.2. Macrophage polarisation and kidney injury

Macrophages are cells of the innate immune system that have defence functions. They perform these functions via many mechanisms, including pathogen phagocytosis, induction of tissue-specific metabolic responses and initiation of systemic inflammation [18]. In order to perform such a heterogeneous repertoire of roles, macrophages display different phenotypes according to the environment they are in and the functions they need to perform; this is what is referred to as macrophage polarisation. These phenotypes can change as a response of signalling provided by other cells, including other immune cells. This section will address how immune cells, particularly macrophages, can impact kidney injury prognosis.

1.2.1. The role of the immune cells in kidney injury

Kidneys have resident dendritic cells (DCs), macrophages and lymphocytes to maintain tissue homeostasis [19-22]. DCs are determinant in recruiting neutrophils and producing cytokines, aiding in the response against infections [23]. Mast cells are also present in the kidney, particularly in the tubule-interstitium, but their role remains unclear [24-26]. Moreover, it is known that kidneys have lymph nodes responsible for maturing T cells during renal inflammation [27], and these lymph nodes contribute greatly to build immune tolerance against innocuous antigens [28].

Immune cells play a crucial role in disease progression and the loss of kidney function. DCs and T cells can actively contribute to glomerular damage [29] and it is shown that infiltration of mononuclear cells is decisive for tissue scarring and maintenance of local chronic inflammation [30]. This unbalanced immune response that actively promotes chronic inflammation can lead to CKD, in which functional nephrons are replaced by fibrotic tissue [31]. Figure 1.2. is an example of the dynamics of immune cells after an ischemic event.

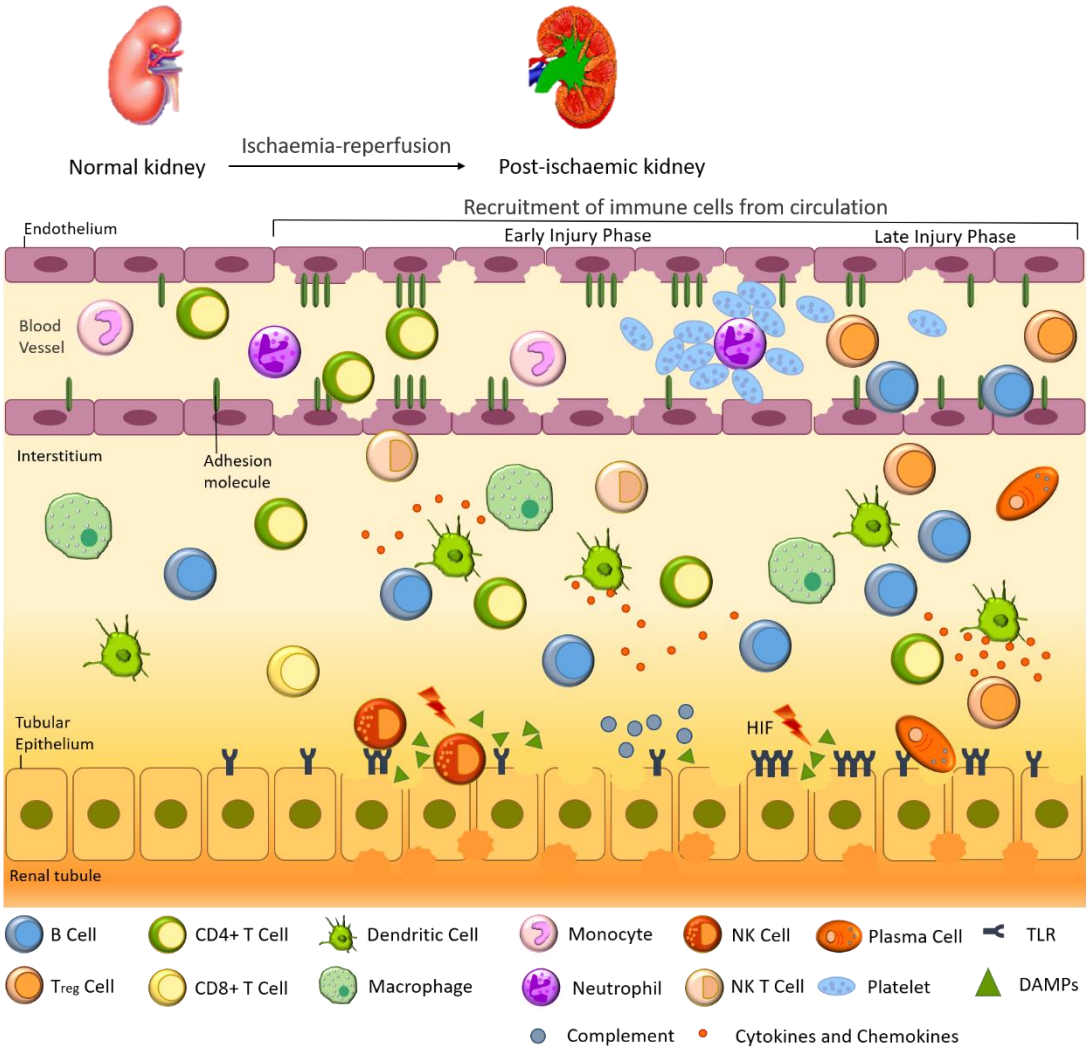

Figure 1. 2. Immune response in a normal kidney and after ischaemia-reperfusion.

Following ischemia, there is a massive influx of immune cells through the endothelium. TLRs, adhesion molecules and DAMPs released from dying cells act on the recruitment and activation of immune cells. The complement system and pro-inflammatory cytokines and chemokines contribute to leukocyte infiltration. Macrophages, dendritic cells, NK cells and T cells are major players in the pathophysiology of renal injury after ischaemia-reperfusion. Abbreviations: AKI = acute kidney injury; DAMPs = damage-associated molecular patterns; HIF = hypoxia-inducible factor; IRI = ischaemia-reperfusion injury; NK = natural killer; TLR = Toll-like receptor; Treg cell = regulatory T cell. Adapted from [32].

The question of why the immune response in some cases of kidney injury is not controlled and actually contributes to persistent inflammation is not completely answered. Nevertheless, the kidney displays some properties that

aid to exacerbate immune responses and can explain, at least partially, how the immune system can promote constant activation of certain cells.

One interesting property is that kidneys have a variety of parenchymal cell types that display pattern recognition receptors able to respond to Pathogen-Associated Molecular Patterns (PAMPs) and Danger-Associated Molecular Patterns (DAMPs) and trigger innate immune responses to cause renal inflammation [33]. As soon as inflammation is initiated, neutrophils, natural killer (NK) cells and T cells are stimulated to release inflammatory cytokines. Those cytokines induce a pro-inflammatory phenotype on macrophages, which enhance inflammation and sustain stimuli for inflammatory cytokine release, enabling a vicious cycle of cell death and inflammation [34]. These mechanisms create a positive feedback loop that would be broken once pathogens are eliminated. However, kidney injuries are usually sterile injuries, which do not present pathogens to be eliminated [35]; hence, this initial immune response can be detrimental to kidney function and should be counterbalanced to give rise to repair.

1.2.2. The role of macrophages in kidney injury pathogenesis

To date, the role of macrophages in kidney injury pathogenesis is still under debate, but it is known that they participate in both inflammation/injury and repair phases. Within hours of an insult to the kidney, there is a recruitment of innate immune cells to the site of injury, particularly neutrophils and NK cells. Monocytes are the next to migrate and differentiate into inflammatory macrophages, as the function of these cells is to eliminate pathogens [36]. All of these cells are part of the innate immune system, which provides a fast

response, but unspecific, failing to distinguish between sterile lesions and infections.

This is the first phase, when there is a predominance of inflammatory signals aimed at eliminating whatever caused the challenge. But as previously mentioned, from this point, either adaptive or maladaptive repair will occur, leading to tissue regeneration or chronic kidney disease, respectively. Macrophages are present in both outcomes, and their phenotype are crucial to determine which path will be taken [13].

Distinct subsets of macrophages can co-exist at the site of injury at different phases of disease, but it is paramount that during the reparative stage of disease, an anti-inflammatory phenotype takes predominance for adaptive repair to occur (Figure 1.3) [37]. Inflammatory macrophages can cause substantial damage to the tissue and in cases where there is not a clear shift in macrophage phenotype, the persistent inflammatory process causes fibrosis and permanent loss of function.

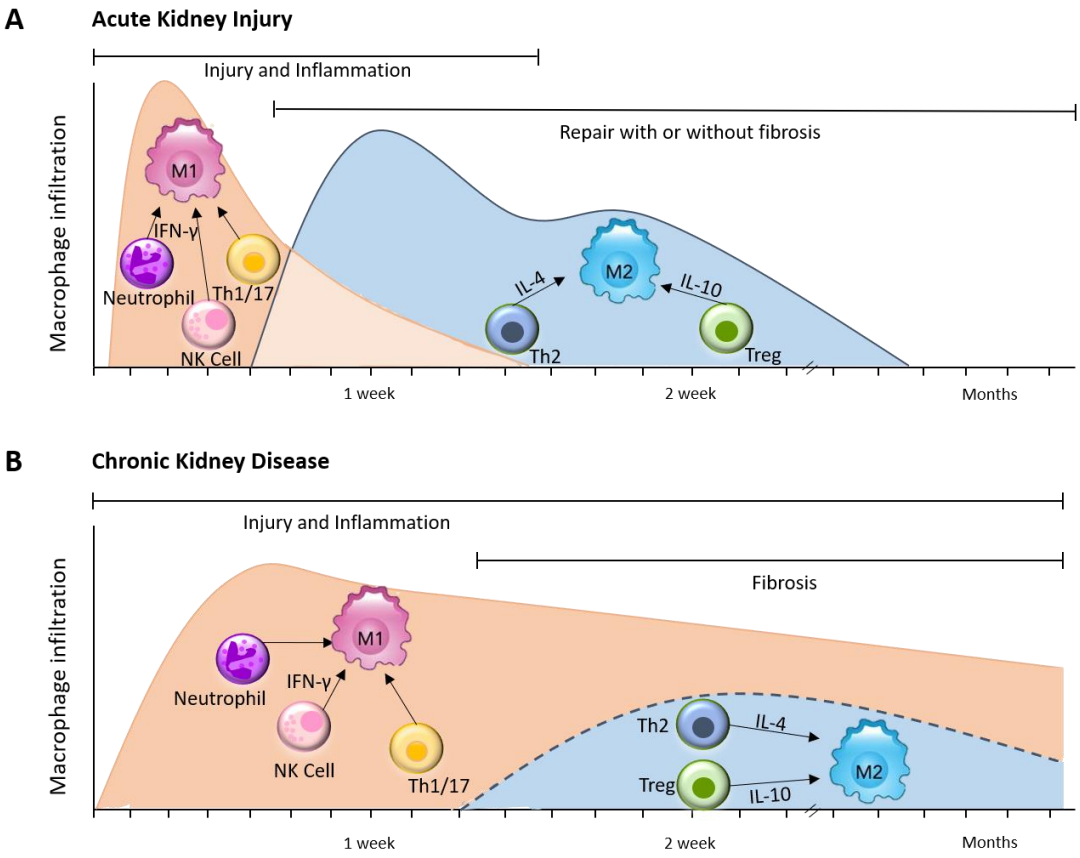

Figure 1. 3. Phase-dependent changes in macrophage phenotype during the progression of kidney disease.

Macrophage phenotypes varies throughout the stages of kidney injury. **A.** Acute kidney injury promotes recruitment of neutrophils and NK cells. Monocytes infiltrate to the site of the tissue injury after neutrophils, where they differentiate into macrophages and polarise into pro-inflammatory macrophages (M1) by inflammatory mediators, such as IFN-γ, released by neighbouring cells. Subsequently, T helper 2 (Th2) and regulatory T lymphocytes (Tregs) are recruited into the tissue and promote a switch in macrophage polarisation towards an anti-inflammatory phenotype (M2), which will induce phagocytosis of apoptotic cells. Fibrosis may occur depending on the severity of the injury. **B.** M1 macrophages are polarised due to the influence of neighbouring cells, but in contrast to what occurs in acute kidney injury, due to progressive injury and persistent inflammation, these cells persistently surround sites of damaged tissue. Small numbers of Th2 cells and Tregs are recruited into the kidney, but there is a predominance of an inflammatory response. Adapted from [37].

Considering Figure 1.3, it is clear that the so-called M2 macrophages play a crucial role in kidney repair, meaning that in the context of kidney injury it is advantageous to shift macrophage polarisation towards an anti-inflammatory

phenotype to prevent permanent tissue damage. Therefore, it is imperative to understand what macrophage polarisation means.

1.3. The macrophage polarisation paradigm

The topic of macrophage polarisation and how plastic this process can be is of great interest but also of great controversy in scientific circles. Macrophages are pleiotropic cells and as such can adapt to the environment surrounding them. In the previous subsection two distinct phenotypes were mentioned: M1 and M2. In this section there is a brief summary regarding the origin and rationale of this classification.

In the early 1990s, a classification was proposed which describes antagonistic phenotypes of macrophages: one pro-inflammatory, a product of IFN-γ and/or lipopolysaccharide (LPS) stimulation and promoter of an inflammatory response, and the other a result of IL-4 activation and involved in tissue regeneration and immune-regulatory functions [38]. The origin of this classification can be placed in the study of T helper (Th) lymphocytes in rodents by Mosmann and Coffman [39]; this investigation demonstrated the existence of differently activated lymphocytes, hereafter termed T helper 1 (Th1) and Th2 lymphocytes, which could release IFN-γ and IL-4, respectively [39, 40]. These cytokines are responsible for two opposite immune responses, indicated as type-I and type-II responses, which are characterised by mechanisms for tackling intracellular pathogens or humoral mechanisms, respectively, with IFN-γ and IL-4 being among the predominant products of these opposing phenotypes [40].

In studies with macrophages, it was previously shown that these cells are able to release either IL-12 or IL-10, cytokines able to elicit the production of IFN-γ and development of Th1 cells or secretion of IL-4 and Th2 cells proliferation, respectively [41, 42]. As a parallel to the Th1/Th2 nomenclature, these opposing macrophages were termed M1 and M2, or classically versus alternatively activated macrophages.

1.3.1. Macrophage classical activation

Classically activated or M1 macrophages are inflammatory cells responsible to produce cytokines that will aid in the destruction of microbial and intracellular organisms [43]. These cells are in the front line of infection responses and thus have Pattern Recognition Receptors, such as Toll-like receptors (TLR) that recognise PAMPs, among which LPS is a major example. They display MHC-II and costimulatory receptors CD80 and CD86 [44] and are generated by IFN-γ and/or LPS stimulation. Due to their role in microbial infection elimination, these cells have enhanced endocytic function and ability to kill intracellular pathogens.

M1 macrophages produce high levels of inflammatory cytokines, including TNF-α, IL-6, IL-12 and IL-23, reactive oxygen species (ROS), nitrogen intermediates and produce low levels of IL-10 [45].

Classically activated murine macrophages can release nitric oxide (NO) from L-arginine, as a result of inducible nitric oxide synthase (iNOS) activity, a specific isoform of NO synthase produced by IFN-γ stimulation. There is great debate, nevertheless, whether NO is released by its human counterparts, and our current understanding is that macrophages in humans can only release

NO in very specific circumstances [46], showcasing metabolic differences between species.

1.3.2. Macrophage alternative activation

Based on the simplistic classification of opposite phenotypes of macrophages, all macrophages which are not activated by IFN-γ or LPS would be considered alternatively activated macrophages. These cells were initially known to be activated by IL-4 [47] and to display a predominant anti-inflammatory phenotype. IL-13 has been also included as an activator of M2 macrophages due to fact that both IL-4 and IL-13 share a common receptor chain [48], leading to the understanding that IL-13 would elicit the same pathway activation as IL-4 and perform overlapping functions [49].

However, studies with macrophages have shown that IL-4/IL-13 are not the only activators of the alternative phenotype. Glucocorticoids, IL-10 and Fc receptors ligands have been investigated as activators of macrophages with pleiotropic actions, but mainly inducing responses towards a Th2 profile [50]. Therefore, because the stimuli are so diverse, there is great heterogeneity in these cells, leading to a further classification in M2a, M2b and M2c, according to the functions identified so far and the stimuli used to activate them. M2a are the cells generated from exposure to IL-4/IL-13, which are reported to be involved in wound healing processes [51]; M2b-like macrophages are the product of stimulation with immune complexes and LPS and are deemed to be regulatory cells [52]; finally, M2c-like macrophages are cells involved in resolution of inflammation or suppressors of immune response and would be generated by the use of glucocorticoids [53]. An additional phenotype would be tumour-associated macrophages, which promote angiogenesis [54]; these

macrophages have a M2 phenotype, but do not fit in the M2a/M2b/M2c description, so would probably be an extra phenotype M2d.

1.3.3. Macrophage phenotypes in kidney injury

Based on what has been mentioned regarding macrophage polarisation, it is clear that even though macrophages can assume a predominantly inflammatory phenotype or, on the contrary, a phenotype that resolves inflammation, there is not a defined delimitation of phenotypes, meaning macrophage polarisation is a spectrum (Figure 1.4).

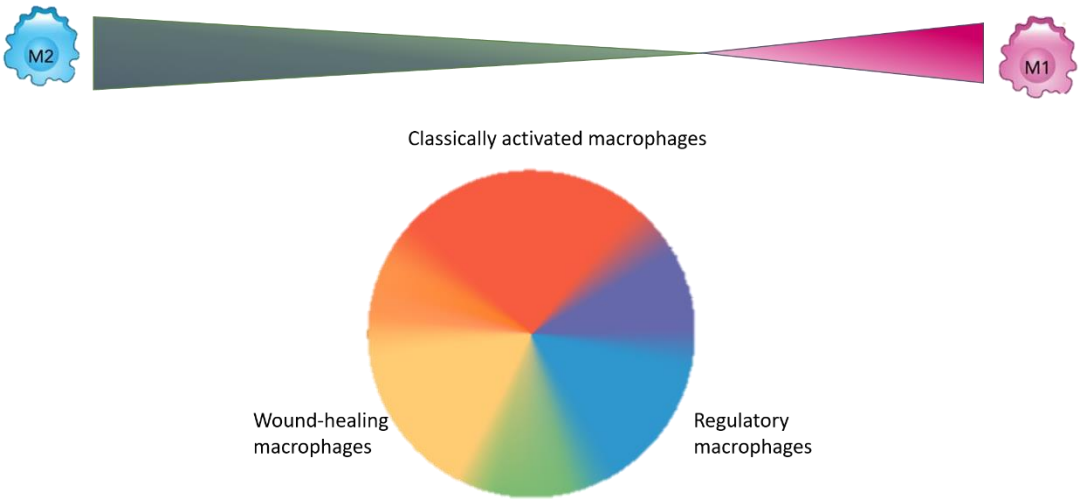

Figure 1. 4. Proposed spectrum of macrophage polarisation.

Macrophages can assume different phenotypes according to the environment they are in. As their phenotypes are a continuum it is challenging to assess their properties in vitro. Adapted from [43].

Therefore, if macrophage phenotypes can have many nuances and overlapping properties, if the aim of the investigation is to assess a switch in macrophage polarisation, it is paramount to determine parameters to distinguish phenotypes. Macrophage polarisation generates cells that are functionally and phenotypically diverse, developing not only according to the

cytokines used to activate them [44], but also depending on interactions with other immune cells and the microenvironment. These interactions are very challenging to be mimicked in *in vitro* studies, and even in animal models.

For the purposes of this study, more important than achieving the exact macrophage phenotypes found in the site of injury for both the inflammatory and reparative phases, was to consistently obtain macrophages with distinct pro- and anti-inflammatory phenotypes. The reasons for this approach are:

- Different types of injury might produce different macrophage phenotypes, and treatments can also impact phenotype. For instance, M2c-like macrophages were found to be the predominant phenotype on renal biopsies from patients with lupus nephritis [55], and it is not clear whether this predominance derives from the fact that glucocorticoids are the most used substances to treat lupus nephritis.
- Macrophage polarisation is a fluid process, and as a consequence, phenotypes change over time and assume intermediate nuances.
- The interest of this study is to determine phenotype switch, rendering the concern of specific alternative activation phenotype secondary.

Therefore, the first objective of an *in vitro* investigation concerning whether macrophage polarisation can be reversed would be the generation of inflammatory macrophages, representing the majority of macrophages during the inflammatory stage, and an opposing anti-inflammatory phenotype, to serve as a generic token of an anti-inflammatory phenotype.

Now the question rests on what would be an appropriate tool that could potentially cause a switch in macrophage polarisation. It should be a

compound or therapy which has shown positive results in the treatment of acute kidney injury, given that this is the condition this study is inserted into, and have immunomodulatory properties. Mesenchymal stromal cells (MSCs) and their derived extracellular vesicles fit well into these criteria, and are thus valid candidates for testing.

1.4. Mesenchymal Stromal Cells (MSCs)

MSCs have been investigated in a variety of pre-clinical models [56-60] and are currently been tested in clinical trials for different medical conditions. Despite their widespread use, the mechanisms by which they act and the reasons explaining variability in effects are still not entirely known. This is a brief overview of the history and use of MSCs and how they fit in as a potential therapy for the treatment of kidney injury.

1.4.1. Features and properties of MSCs

MSCs are a heterogeneous class of cells, classified as multipotent, that can be sourced from different tissues in the adult organism. Traditionally, these cells were isolated by centrifugation of bone marrow aspirates [61], but they can be sourced by less invasive procedures from adipose tissue [62], umbilical cord [63] and dental pulp [64], to mention but a few. Cells from different tissues do not necessarily share differentiation and lineage commitment properties [65], suggesting that they might have diverse effects for therapeutic use.

MSCs were first described by Friedenstein during the 1960s and 1970s as fibroblast-like cells from bone marrow which were able to form colony units and which supported the proliferation and differentiation of hematopoietic stem cells and osteogenesis [66, 67]. However, after these first descriptions these

cells were isolated from a variety of tissues and linked to diverse regenerative processes, leading the International Society for Cellular Therapy to issue a statement determining the minimal criteria for cells to be considered as MSC. In short, MSC need to present three characteristics: plastic-adherence, expression of CD105, CD90 and CD73, while lacking expression of CD45, CD34, CD14, CD19 and HLA-DR surface molecules, and they must differentiate into osteoblasts, chondrocytes and adipocytes [68].

CD105 or endoglin is a co-receptor for TGF-β involved in different physiological functions, such as proliferation, migration, adhesion and differentiation [69]. CD73 is an enzyme, more specifically an ecto-5'-nucleotidase, involved in the generation of extracellular adenosine via coordination with ATPases and hence with a very crucial metabolic role [70]. CD90 is a cell-adhesion molecule which plays a role in cell-cell adhesion between inflammatory mediators [71]. The functions of these markers are still under investigation, including how they might impact MSC physiology [72].

1.4.2. MSC applications and potential mechanisms of action

MSCs have been in the spotlight for some decades as a potential tool in the treatment of various pathologies, including steroid-refractory Graft versus Host Disease (GvHD), heart failure and enterocutaneous fistular disease [56]. The attention received by MSCs was initially due to their differentiation capacity, or "stemness", and it was hypothesised that MSCs would home to the site of injury, proliferate and differentiate into the injured cell types, repopulating the damaged tissue [73, 74]. There is evidence provided by multimodal imaging that this is not the case [75]. Indeed, MSC were able to confer amelioration of

kidney injuries without engraftment and trans differentiation in the tissue [57-60]. This ability raised questions about the repair mechanisms of MSC and the possibility that paracrine factors and released extracellular vesicles might be the effectors in tissue repair [76].

Immunomodulation is one of the most investigated features of stromal cells from different sources and a potential repair mechanism used by MSCs. There is growing evidence that MSCs have an immunosuppressive role regarding T cells and NK cells, while stimulating regulatory T cells [77], as shown in Figure 1.5.

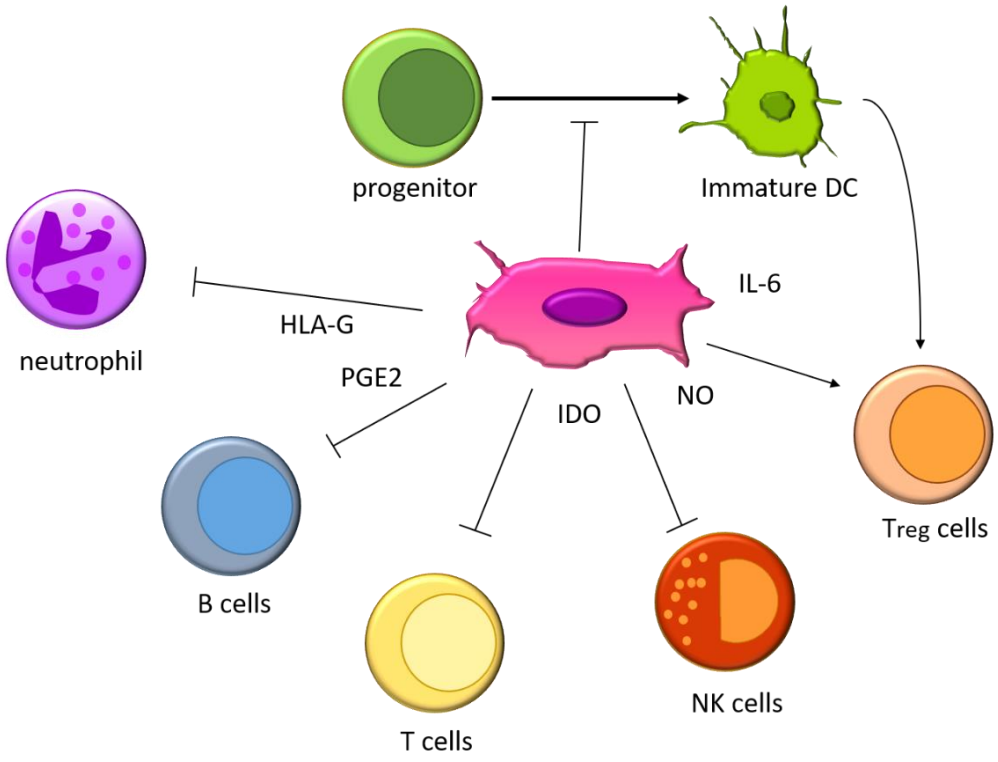

Figure 1. 5. Schematic representation of the interaction between mesenchymal stromal cells and immune cells.

MSCs secrete soluble mediators – such as nitric oxide (NO), prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO), IL-6, and human leukocyte antigen (HLA)-G. Production of these mediators regulates the proliferation and function of a variety of immune cells as well as the induction of regulatory T cells (Treg) either directly or indirectly through the generation of immature dendritic cells (DC). NK, natural killer. Adapted from [77].

1.4.3. Kidney injury as a target for stromal cell therapy

MSCs and their by-products have been tested in a variety of kidney injury animal models, including unilateral ureteral obstruction (UUO) [78], ischemia-reperfusion [79], adriamycin-induced kidney injury [80] and cisplatin-induced kidney injury [81], and have consistently ameliorated injuries. Again, the initial mechanism proposed for such improvements was direct engraftment and differentiation into tubular epithelial cells [82], but advances in the field have shown that MSCs do not home to injured kidneys [75] and some of the previously mentioned studies used conditioned medium or extracellular vesicles; therefore, paracrine effectors are probably the main players in regeneration conferred by MSCs (Figure 1.6).

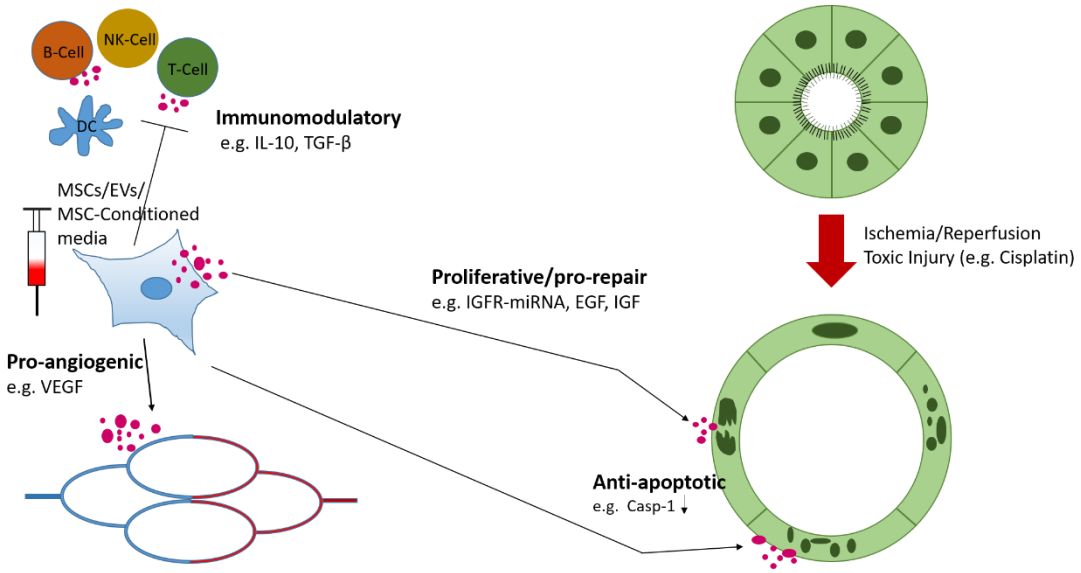

Figure 1. 6. Model of paracrine actions of MSCs on the injured tubule and surrounding tissue.

Intravenous administration of MSCs, MSC-derived EVs or MSC-conditioned media accelerate repair by supporting proliferation and inhibiting apoptosis of tubular cells, induce vascular growth and inhibit inflammation/modulate immune responses, e.g. in AKI patients after kidney transplantation. IL-10 = Interleukin 10; TGF-β = transforming growth factor β; IGFR = Insulin-like growth factor receptor; EGF = epidermal growth factor; IGF = Insulin-like growth factor; Casp-1 = caspase 1; VEGF = vascular endothelial growth factor. Adapted from [82].

Despite this lack of complete understanding regarding the mechanisms by which MSCs confer kidney function amelioration, MSCs are currently used in hundreds of clinical trials, as reported on www.clinicaltrials.gov. This is due to the great burden that kidney diseases represent, both for individuals and as a public health concern; new effective treatments are desperately needed. Current treatments are very demanding for patients, particularly in cases of CKD and end-stage renal disease, requiring hospitalisations and long-term use of immune-suppressants in cases of transplantation. Shortage of donors has prompted the use of older and higher risk organs, increasing the risk of delayed graft function and primary nonfunction [83]. In this scenario, MSCs are been used to prevent ischemic injuries in transplanted organs and increase graft function and survival [84].

1.4.4. Limitations of cell therapy

In the context of kidney injury, it is urgent to develop new treatments to tackle both acute and chronic kidney disease, in order to prevent patients from progressing to end-stage kidney disease and be dependent on dialysis and/or transplantation. Cell therapy has been tried as a very promising strategy to promote the recovery of kidney function, but as with any treatment, it has its downfalls and potential risks.

At the onset of stem cell research, there were great expectations regarding the possibilities for therapies, given that it was believed that these cells would reconstitute damaged organs [85]. Therefore, initially there was a search for cells able to differentiate into multiple cell types. Stem cells from different sources, including from amniotic fluid, can be used as a more plastic

alternative to cells from adult tissues and have the advantage of being considered, depending on the developmental stage, pluripotent [86], but this is debatable. Soon enough though, unexpected results, such as teratoma, demonstrated that stem cells needed to be administered with caution.

Due to ethical concerns in the use of embryonic material, there was increasing interest in investigating the properties of stem and progenitor cells from adult tissues and whether these tissues would contain progenitor or stem cells. Adult renal progenitor cells (RPCs) are cells found in the kidney parenchyma that are reported to be multipotent and hence could be potentially used for kidney function recovery [87-91]. Nevertheless, not all results were reproducible [92], suggesting the need of further elucidation.

The treatment of kidney injury with MSCs has generated promising pre-clinical data. MSCs can be isolated from different sources, and currently there is a predominance of studies using MSCs from adult tissues. When these cells are administered at high dosages and at multiple times, there is increased risk of uncontrolled expansion capacity, differentiation and, consequently, tumour formation [93]. There are studies also showing that MSCs might be involved with higher re-incidence of malignancies or can promote tumour growth, particularly in cases of blood malignancies, as a result of their immunosuppression effects [94, 95].

A recent trend in the cell therapy field is to use by-products of cells, such as conditioned medium or extracellular vesicles, and thus have cell-free therapies. By doing so, many, if not all, of the risks linked to cell therapy would be minimised or eliminated.

1.5. Extracellular vesicles

The field of extracellular vesicles is an emerging field that has gained attention mainly from the years 2000s onward. Because it is a recent modality, it has not reached consensus over important factors, such as classification and methods of isolation. It is beyond the scope of this study to attempt to review and standardise all methods described in the literature for EV characterisation and isolation, but the following sub-sections include a brief description of the most established concepts regarding EV physiology and their roles in health and disease, with a focus on kidney injury.

1.5.1. Origins and functions

Extracellular vesicles can be released by healthy cells and also stressed and senescent cells, suggesting that they play a role in both physiology and potentially, pathophysiology. Most data so far have classified EVs in roughly two different categories: microvesicles (MVs), which are vesicles shed directly from the plasma membrane of the cell and can size from 100 nm to 1 μm, and exosomes, which are smaller particles with a size ranging from 50 to 100 nm, are derived from the endosomal pathway and released upon multivesicular bodies fusion with the plasma membrane. Apart from these two classes of vesicles released from viable cells, there are apoptotic bodies, which are fragments of cells undergoing programmed cell death that may contain not only proteins and nucleic acids, but whole organelles. As these particles are fragments of cells they are usually bigger, with a size ranging from 1 to 5 μm [96-98]. Details regarding size usually vary among publications. Markers to distinguish vesicles from the different categories are currently under

investigation, including tetraspanins and proteins from the endosomal pathway [99]. Tetraspanins are a superfamily of proteins characterised by their four transmembrane domains; these proteins are highly enriched in EVs and are involved in motility, membrane fusion, cell adhesion, protein trafficking and signalling [100].

Figure 1.7 shows a schematic example of the populations of EVs released by non-apoptotic MSCs and their potential interactions with target cells.

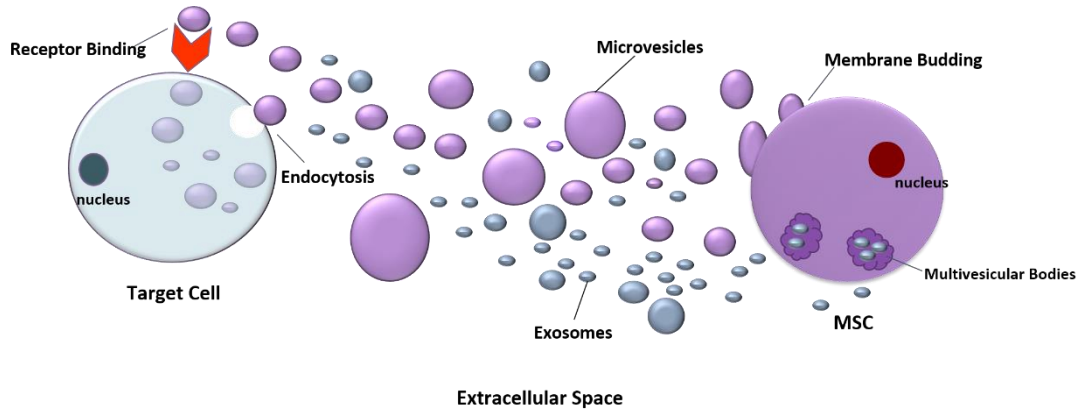

Figure 1. 7. Extracellular vesicles populations.

Extracellular vesicles are a heterogeneous population of microparticles, mainly composed by exosomes and microvesicles. In particular, exosomes (in blue) are stored within multivesicular bodies (MVBs) of the late endosome and are released in the microenvironment after fusion with the cell membrane, whereas microvesicles (in purple) originate by direct budding from the cell surface. After their secretion, EVs exert their effects on adjacent or distant recipient cells through receptor binding and membrane fusion. Adapted from [96].

EVs are known to contain DNA, lipids, messenger RNA (mRNA), microRNA (miRNA) and proteins. These particles are deemed to be vectors on intercellular communication, delivering goods to neighbouring and remote cells. There is growing evidence that EVs participate in physiological

processes through horizontal transfer of their contents, with particular emphasis on non-coding RNAs [101-103].

1.5.2. MSC-derived EVs in kidney injury

In light of the disadvantages of cell therapy, the scientific community is moving towards the development of cell-free therapies that would enable the same beneficial effects while reducing the risks associated with cellular therapy [104]. The advent of inorganic and organic nanoparticles which can be loaded and engineered according to the purpose of treatment has been extensively studied; currently nanoparticles are under investigation for a variety of clinical settings [105-107]. The complexity of clinical conditions, such as chronic kidney disease, hinders the advancement and use of engineered nanoparticles for complex diseases, as in some cases it remains unclear which pathways are involved in the pathophysiology of the condition and how tissue repair takes place.

Therapy using MSCs has showed promising results in rodents, but the exact mechanisms for tissue repair are not fully elucidated. Paracrine action is deemed to be one of the mechanisms used for repair, given that cells do not engraft in the injured tissue [108]. EVs derived from bone marrow and umbilical cord have already been used in *in vivo* models of ischemia-reperfusion with reported protective effects [109-112]. The precise mechanisms responsible for the amelioration conferred by EVs are still under investigation, but it is known that EVs promote tubular proliferation and angiogenesis and inhibit fibrosis and inflammation (Figure 1.8).

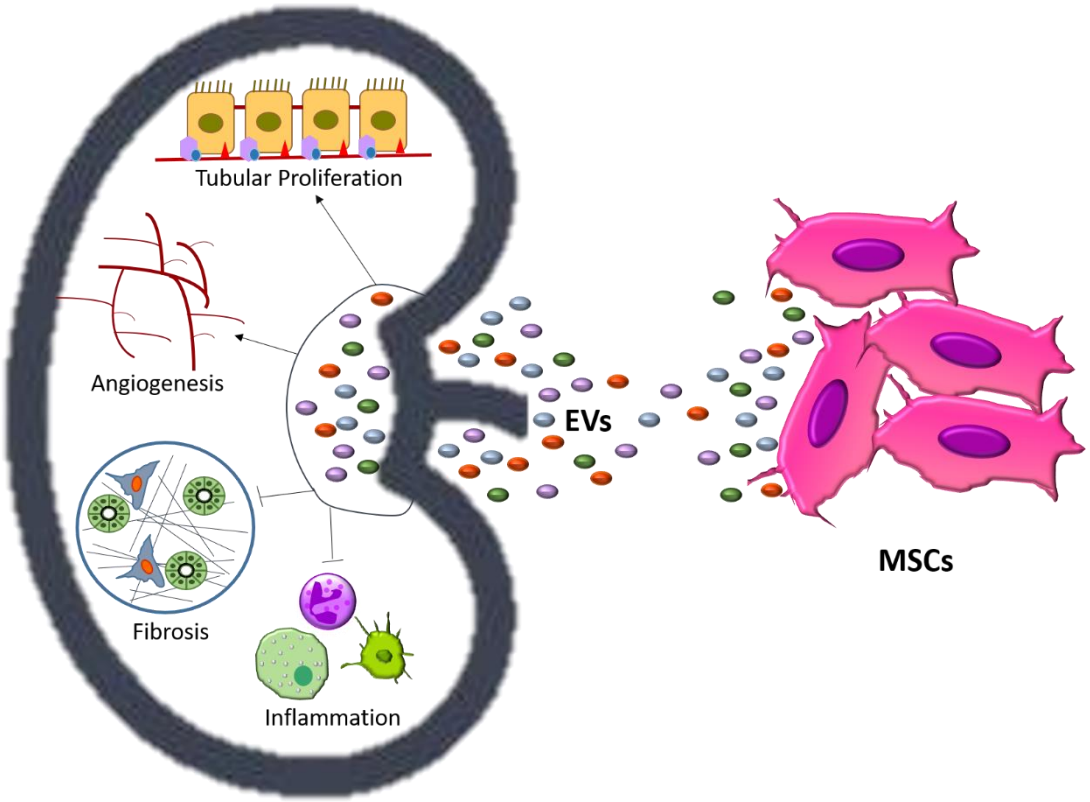

Figure 1. 8. Schematic representation of mechanisms of MSC-derived EVs in kidney injury.

Among the main mechanisms proposed for EV-induced repair, tubular proliferation, angiogenesis, inhibition of fibrosis and regulation of inflammation are the most investigated and reported. Adapted from [98].

EVs do not need to act by one exclusive mechanism, as they can perform poly-functionally by reducing inflammation and increasing tubular cell proliferation [113]. In ischemia-reperfusion models, angiogenesis and inhibition of fibrosis are apparently main players in repair [109]. Nevertheless, there is also evidence that EVs from adipose tissue stem cells can shift macrophage polarisation by transferring the transcription factor STAT3, enhancing the accumulation of M2 macrophages [114]. This would be of great importance in kidney injury.

Given all the evidence in the literature demonstrating that MSCs have elicited injury amelioration without engraftment, and considering that one of the

hallmarks of kidney repair is the shift in macrophage polarisation towards an anti-inflammatory phenotype, it is valid and reasonable to investigate whether MSC-derived EVs impact on macrophage polarisation. If so, this could aid in other conditions in which macrophages play a pivotal role. In the context of this thesis, the mechanism explored is macrophage polarisation and the possibility of switching polarisation *in vitro* via exposure to mesenchymal stromal cells (MSCs) and/or their derived extracellular vesicles (EVs).

1.6. Phases of the study

In order to investigate the effects of EVs on the expression of surface markers on macrophages the study requires several stages from proper characterisation of MSCs and their derived EVs to finally testing EVs on macrophages (Figure 1.9). Analysing EV cargo was originally planned, as to determine RNAs, and particularly miRNAs, which might be responsible for the immunomodulatory effects. Instead, angiogenic potential of EVs was performed for extra validation of how functional EVs were, given that there was a lack of effect on monocyte-derived macrophages.

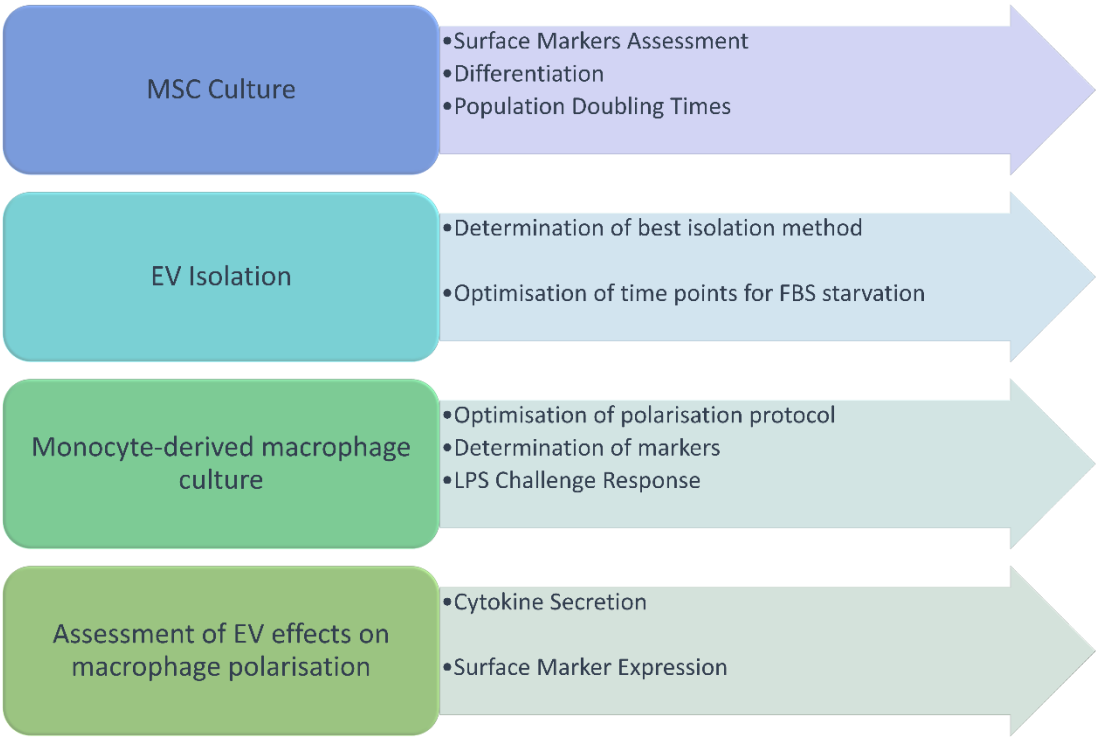

Figure 1. 9. Schematic diagram to illustrate phases of the study.

The scheme illustrates the phases of the project in terms of milestones in the investigation of the effects of extracellular vesicles on human monocyte-derived macrophage polarisation.

These phases are consecutive and aid in building robustness into the system that is tested. Despite how robust the system might become, it is important to emphasise that both macrophage polarisation and MSC-derived EVs mechanisms are currently not completely understood, so interpretation of results might change in light of new discoveries. The scope of this study is therefore limited by the markers which were used and the protocols employed, which unfortunately may not be necessarily representative of the exact macrophage phenotypes *in vivo* during kidney injury progress.

1.7. Hypothesis

Extracellular vesicles derived from MSCs have an effect on human monocyte-derived macrophages *in vitro*.

1.8. Aims and Objectives

The main aim of this study was to assess the effects of extracellular vesicles in activated human monocyte-derived macrophages. To achieve this, this thesis is broken down into the following main objectives

i. **To characterise umbilical cord MSCs and their derived EVs.** Characterisation followed the basic principles defining MSCs: morphology, adherence to plastic and expression of surface markers. Cell culture conditions were assessed in order to optimise proliferation and survival. EVs were characterised regarding size, concentration and expression of CD9, CD63 and CD81. Functionally, EVs were assessed regarding their immunomodulatory and angiogenic properties.

ii. **To develop a robust protocol for monocyte-derived macrophage culture and polarisation.** Culture conditions regarding medium and cytokines used for maturation and polarisation were optimised and tested mainly via flow cytometry to assess whether cells were expressing the expected surface markers.

iii. **To test the effects of lipopolysaccharide (LPS) in human monocyte-derived macrophages.** Macrophages were challenged with LPS, a potent pro-inflammatory stimulant, and assessed on expression of surface markers and secretion of cytokines in order to determine whether LPS was eliciting an appropriate response.

iv. **To evaluate the effects of EVs derived from umbilical cord and bone marrow mesenchymal stromal cells on the expression of**

surface markers and secreted cytokines in monocyte-derived macrophages. This was achieved by co-incubation of EVs with polarised macrophages for different periods of time and also assessing differences after LPS challenges. Flow cytometric analysis and cytokine secretion assays were performed for this assessment.

v. **To investigate EV cargo, particularly non-coding RNA content.** Assessment of EV cargo would be performed via RNA sequencing and miRNA profile to further investigate whether reported miRNAs linked to kidney injury repair were present in EV samples used for this study.

vi. **To investigate EV angiogenic potential.** Assess, via *in vitro* angiogenic assays, whether EVs were able to elicit vessel-like structures formation, as a means to confirm EV potency.

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Chapter 2 – Characterisation of hUCMSCs and their derived EVs

2.1. Introduction

Despite the fact that MSCs ameliorate kidney injury by inhibiting the degree of renal fibrosis and improving kidney function, there is increasing evidence, provided by multimodal imaging, that MSCs do not home to the damaged kidney tissue [1]. This suggests that, in contrast to some claims of earlier studies [2, 3], MSCs do not confer amelioration of kidney injury by homing to the site of injury and differentiating into renal cells, but rather stimulate and/or induce physiological changes through paracrine factors that are able to promote kidney amelioration via processes, such as immunomodulation and/or angiogenesis, etc. One mechanism in which MSCs might promote kidney function amelioration is through the release of extracellular vesicles (EVs).

2.1.1. MSC-derived EVs characterisation and their use in kidney injury repair

The term EVs encompasses vesicles budded from the plasma membrane and also endosomal-derived particles released by most, if not all, cells. Moreover, apoptotic bodies, which are fragments of cells undergoing apoptosis, are also classified as EVs. [4-6]. As this study was not aiming to investigate specific subpopulations of EVs, the general term was adopted. There is a regular attempt by the International Society for Extracellular Vesicles to standardise

procedures and classify vesicles according to determined characteristics, including size and membrane protein expression. However, these attempts have proved to be difficult to implement on an experimental basis, given that the amount of knowledge and evidence is until now both limited and ever-changing [7]. Moreover, until now the functional role of extracellular release, particularly the endo-lysosomal system is not completely understood [8]; differences between microvesicles, originated from plasma membrane budding, and exosomes released via exocytosis are not clear and it has been hypothesised that despite their diverse biogenesis and release, their functions might be analogous depending on the context [9].

Substantial differences in EV cargo and function are therefore to be expected, depending not only on the cell type from which EVs are derived and the protocol employed for isolation, but also the physiological state of the parent cells [10]; the reasons underlying these differences remain to be fully elucidated. It is shown that the repertoire of released EVs is variable from cell to cell, in terms of protein and nucleic acid content, and can also vary even when considering one single cell type [11, 12].

The knowledge that EV quantity and quality are highly impacted by the protocol of isolation highlights the importance of tailoring the protocol to the requirements of the research investigation. As this study is focused on the potential immunomodulatory and renal protective effects of EVs, the choice of cells, methods of isolation and quantification, as well as characterisation of these particles were in line with previous studies investigating the effects of EVs in renal injury [13-15]. Ultracentrifugation is the most used method of isolation and has been consistently reported within the context of kidney injury

treatment [16-18]. For characterisation, the methods were chosen based on available resources and relevance of the acquired data, following guidelines from the main extracellular vesicle societies [19]. Human umbilical cord MSCs (hUCMSCs) were chosen as they have reported benefits on kidney injury studies and can be easily obtained [20, 21].

2.1.2. Hypothesis

MSCs have been extensively used in pre-clinical models and clinical studies with varying degrees of effectiveness and associated risks. The hypothesis to be tested is that hUCMSC-derived EVs can display consistent parameters in regard to their size, concentration and surface receptor expression following a standardised protocol for hUCMSCs culture *in vitro*.

2.1.3. Aims

In order to provide clarity, the sequence of experiments was designed in a logical order to build a robust investigation and provide reliable results. The first part of the investigation, which is the subject of this chapter, is the characterisation of hUCMSCs and the establishment of a protocol to isolate and characterise their derived EVs, as shown in Figure 2.1. Therefore, the aims for this chapter are as follows:

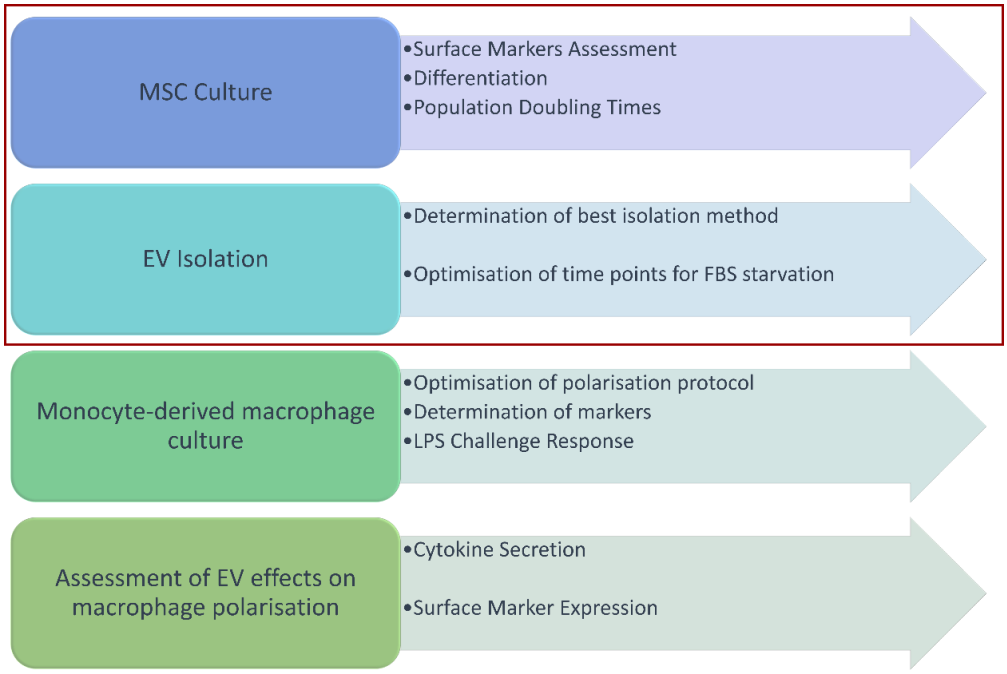

Figure 2. 1. Schematic diagram of stages in the study.

The results in this chapter deal with the initial stage of the study (red rectangle), which is the characterisation of hUCMSCs and their derived EVs. FBS – Foetal bovine serum.

i. To characterise human umbilical cord MSCs (hUCMSCs). Assessment of hUCMSCs properties, including surface marker expression, morphology and proliferation rate.

ii. To optimise a method of isolation of EVs derived from hUCMSCs. Determination of optimal conditions to isolate EVs using ultracentrifugation and evaluation of differences in EV characteristics resultant from changes in the protocol of isolation.

iii. To characterise hUCMSC-derived EVs. Investigation of EVs concerning size, concentration, expression of proteins and how consistent these characteristics are between batches.

2.2 Material and Methods

2.2.1. hUCMSC Culture and EV isolation

hUCMSCs were obtained from the National Health Service – Blood and Transplant (NHSBT) in Passage 2 and cultured in MEM-α medium (Gibco, cat. Number 32561-029) with four types of supplementation:

1. 10% Foetal Bovine Serum – FBS (Sigma, cat. Number F7524);
2. 10% FBS + 5 ng/mL of human recombinant basic Fibroblast Growth Factor (bFGF), (Life Technologies, cat. Number PHG0266);
3. 10% exosome-depleted FBS (Systems Biosciences, cat. Number EXO-FBS-250A-1);
4. 10% exosome-depleted FBS + 5 ng/mL of bFGF.

Cells were kept in humidified incubators at 37°C and 5% CO_2 and sub-cultured using 1x TrypLE Select Enzyme (Gibco, cat. Number 12563-011). All cells were cultured between 5 and 7 days following re-plating, when the cells were at least 80% confluent. For subculture, cells were incubated with 500 µl of TrypLE and 1 ml of PBS for 3 minutes at 37°C and gently detached from dish using a pipette. 2 ml of supplemented medium were used for inactivation of the enzyme. Cells were centrifuged at 400 x g for 3 minutes; supernatant was discarded and pellet re-suspended in fresh medium for seeding in new dishes or, when the purpose was freezing, re-suspended in 90 % FBS 10% dimethyl sulfoxide (DMSO, Sigma, cat. Number D2650) (v/v). Cells were seeded at a density of 5000 cells/cm^2. For freezing procedures, a maximum of 1 ml of liquid per cryovial was used and vials were placed at a Mr. Frosty cooling container for at least 2 hours at -80°C for gradual temperature decrease and then

transferred to a liquid nitrogen tank for permanent storage. Morphology was assessed using a Leica microscope system.

For EV isolation, hUCMSCs were cultured until approximately 80% confluent and then dishes were rinsed three times with sterile Dulbecco's Phosphate Buffered Saline (DPBS, Sigma, cat. Number D8537) and incubated with MEM-α medium for 24, 48 and 72 hours. Supernatant was collected and centrifuged at 500 x g for 5 minutes and at 2000 x g for 10 minutes. Ultracentrifugation was performed at 4°C at 100,000 x g for 2 hours and part of supernatant had an additional round of 2-hour ultracentrifugation to compare once and twice centrifuged samples. Pellets were re-suspended in sterile-filtered PBS and samples of EVs were kept at -80°C freezer until further analysis. Electron microscopy was performed to confirm EVs were intact after -80°C freezing.

2.2.2. Population Doubling Times

hUCMSCs were seeded at a density of 5000 cells/cm^2, cultured until approximately 80% confluent, and assessed regarding their population doubling times (PDT) in the different supplementation conditions. The formula below was used to calculate PDT:

$$T = t\,(\frac{\ln 2}{lnN1 - lnN0})$$

T: Population doubling time

t: Time that cells were cultured until passaging (days);

N0: Initial number of cells;

N1: Final number of cells.

Cells were counted both with a Neubauer haemocytometer (Baxter Scientific, Illinois, USA) and an automatic cell counter (BD, New Jersey, USA) to compare results obtained by these methodologies. At least three measurements were taken from each sample and the average was considered as the final number of cells.

2.2.3. Measurement of released ATP

In order to assess the metabolic state of the cells by monitoring ATP release throughout consecutive days, Cell Titer-Glo (Promega, cat. Number G7570) was used. hUCMSCs were seeded in 96-well plates at the amount of 500 cells per well and cultured with the already described 4 different supplementations. Wells containing only medium were used for blank measurements. The assay was performed twice for at least 7 consecutive days with cells on Passage 7.

2.2.4. EV characterisation by Nanoparticle Tracking Analysis (NTA)

Nanoparticle detector equipped with a 403-nm laser diode (NanoSight NS300; Malvern Instruments Inc, Massachusetts, USA) was used to characterize EVs. The equipment relies on the measurement of Brownian motion of the particles, providing information regarding size and concentration of vesicles found in the sample. In simple terms, a laser beam is directed to the sample and the software is able to quantify the scattered light and thus measure the number of particles and their sizes. That occurs because the Brownian motion of vesicles suspended in a fluid gives an indication of the size of particles and

can be used in the Stokes-Einstein equation to calculate size distribution and the number of tracks counted to give a precise concentration of particles within the sample [22]. The light scatter mode was used for quantification and scatter distribution according to the manufacturer's protocols. Before each measurement the solvent used for EV resuspension (PBS) was checked regarding the presence of any particles. Because PBS can crystalize in cold temperatures, all samples of PBS used for NTA were kept at room temperature, and sterile PBS was further filtered to remove large aggregates or crystals that could be counted as particles.

The charge-coupled device high sensitivity camera displays the detected vesicles in real time. Five 1-minute recordings were performed for each sample. Collected data were analysed with NTA software (NTA, version 3.4, Malvern Panalytical, USA), which provided high-resolution particle size distribution profiles and EV concentration measurements. EVs were expressed as number of particles per mL and the predominant size was displayed both as the mean and the number referent to the mode, as the presence of very small and very big particles could bias the calculated mean size. At least two dilutions were used and the average number of particles was considered as the final number as to minimise this bias.

2.2.5. Cytofluorimetric analysis - hUCMSCs

hUCMSCs were assessed regarding the expression of surface markers. Cells were harvested and incubated with antibody diluted in PBS 0.5% bovine serum albumin - BSA (w/v), (Sigma, cat. Number A4503) for ten minutes in the dark

in the fridge according to manufacturer's instructions. All antibodies were from Miltenyi Biotec (Bergisch Gladbach, Germany) and were REAffinity antibodies, meaning they do not require an FcR blocking step, and were engineered with a mutated Fc region that provides both reduction of background signal and also the possibility of one universal isotype control that would be suitable for all REAffinity antibodies conjugated with a specific fluorochrome. The antibodies used were human CD105-FITC (Clone REA794, cat. Number 130-112-169), human CD90-Vioblue (Clone REA897, cat. Number 130-114-866), human CD73-APC (Clone REA804, cat. Number 130-111-909) and human CD14-APC (Clone REA599, cat. Number 130-110-520). Isotype Controls were REA Control for surface staining for FITC (Clone REA293, cat. Number 130-113-437), for Vioblue (Clone REA293, cat. Number 130-104-609) and for APC (Clone REA293, cat. Number 130-113-434).

Table 2. 1. Antibodies used for cytofluorimetric analysis of hUCMSCs.

Antibody	Clone	Isotype	Fluorochrome	Dilution
CD105	REA794	IgG	FITC	1:100
CD73	REA804	IgG	APC	1:100
CD90	REA897	IgG	Vioblue	1:100
CD14	REA599	IgG	APC	1:100

Abbreviations used FITC: fluorescein isothiocyanate, APC: allophycocyanin. All antibodies used were anti-human.

2.2.6. Flow cytometry analysis of extracellular vesicles

Extracellular vesicles were incubated with 10µl of aldehyde/sulphate latex beads (Life Technologies, cat. number A37304, 4 µm, 4% w/v) for 15 minutes at room temperature. This is a reported method to detect EVs via conventional flow cytometry [23]. The final volume for this first incubation period was 50µl. The number of vesicles used was $1x10^9$. After the incubation, 950µl of PBS were added and the solution was incubated at 4°C overnight in a rotator. In order to saturate remaining free binding sites, a solution of glycine was added to a final concentration of 100mM and the solution was left to incubate for 30 minutes at room temperature. The solution was centrifuged at 4000 rpm for 3 minutes (this speed and time was used for all subsequent centrifugations). Supernatant was carefully discarded and the beads were washed twice with PBS 0.5% BSA (w/v). The pellet was re-suspended in 98µl of PBS 0.5% BSA and 2µl of primary antibody were added to incubate in the dark for 30 minutes. The antibodies used were human CD9 – FITC (Clone REA1071, cat. Number 130-118-806), human CD63 – PE (Clone REA1055, cat. Number 130-188-077), human CD81 – APC (Clone REA513, cat. Number 130-119-787), all purchased from Myltenyi Biotech and all REAffinity antibodies. Isotype Controls were REA Control for surface staining for FITC, for PE (Clone REA293, cat. Number 130-113-438) and for APC, also purchased from Miltenyi Biotec. After the incubation period, 900µl of PBS 0.5% BSA were added and the solution was centrifuged and washed twice. The pellet was then re-suspended in PBS 0.5% BSA and flow cytometry analysis was performed with 30,000 acquired events per sample. Data acquired using BD FACS Calibur were acquired using Cell Quest software and data acquired in MACS

were acquired with MACS Quant software. Histograms and percentages of positive events were obtained using FCSalyzer through fcs.files.

Table 2. 2. Antibodies used for cyfluorimetric analysis of hUCMSC-derived EVs

Antibody	Clone	Isotype	Fluorochrome	Dilution
CD9	REA1071	IgG	FITC	1:100
CD63	REA1055	IgG	PE	1:100
CD81	REA513	IgG	APC	1:100

Abbreviations used FITC: fluorescein isothiocyanate, PE: phycoerithrin, APC: allophycocyanin. All antibodies were anti-human.

2.2.7. Statistical Analysis

One-way ANOVA with Kruskal-Wallis correction test for multiple comparisons was used to compare one parameter for more than two groups and two-way ANOVA with Tukey's correction for multiple comparison was used to compare more than one parameter for more than two groups. GraphPad Prism 7 was used for statistical analysis, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Statistical analysis was performed for biological and technical replicates, and not for technical replicates only.

2.3. Results

2.3.1. Characterisation of hUCMSCs

In order to investigate potential differences in cell morphology and the time-point at which cells became senescent, hUCMSCs were cultured from Passage 5 until growth arrest with the four different supplementations described in the Methods section; i.e., standard and exosome-depleted FBS with or without bFGF. Medium was changed every two days and cells were

sub-cultured upon reaching 80% of confluence. Images were taken on the same day for each of the four groups, when cells had already adhered and were proliferating; therefore, cells were cultured for the same period of time. hUCMSC cultured with exosome-depleted FBS would initially take longer to proliferate, as it was possible to notice that cells were less confluent on the first days in comparison with conventional FBS, but morphology was not altered, as cells remained adherent and spindle-like shaped (Figure 2.2). By Passage 13 cells started to display signs of senescence, as many flat cells, with a non-fibroblastic aspect, could be noticed (white arrows); all four groups of cells were unable to reach confluency on Passage 14 due to growth arrest and more cells were enlarged, flattened and not following a morphological pattern.

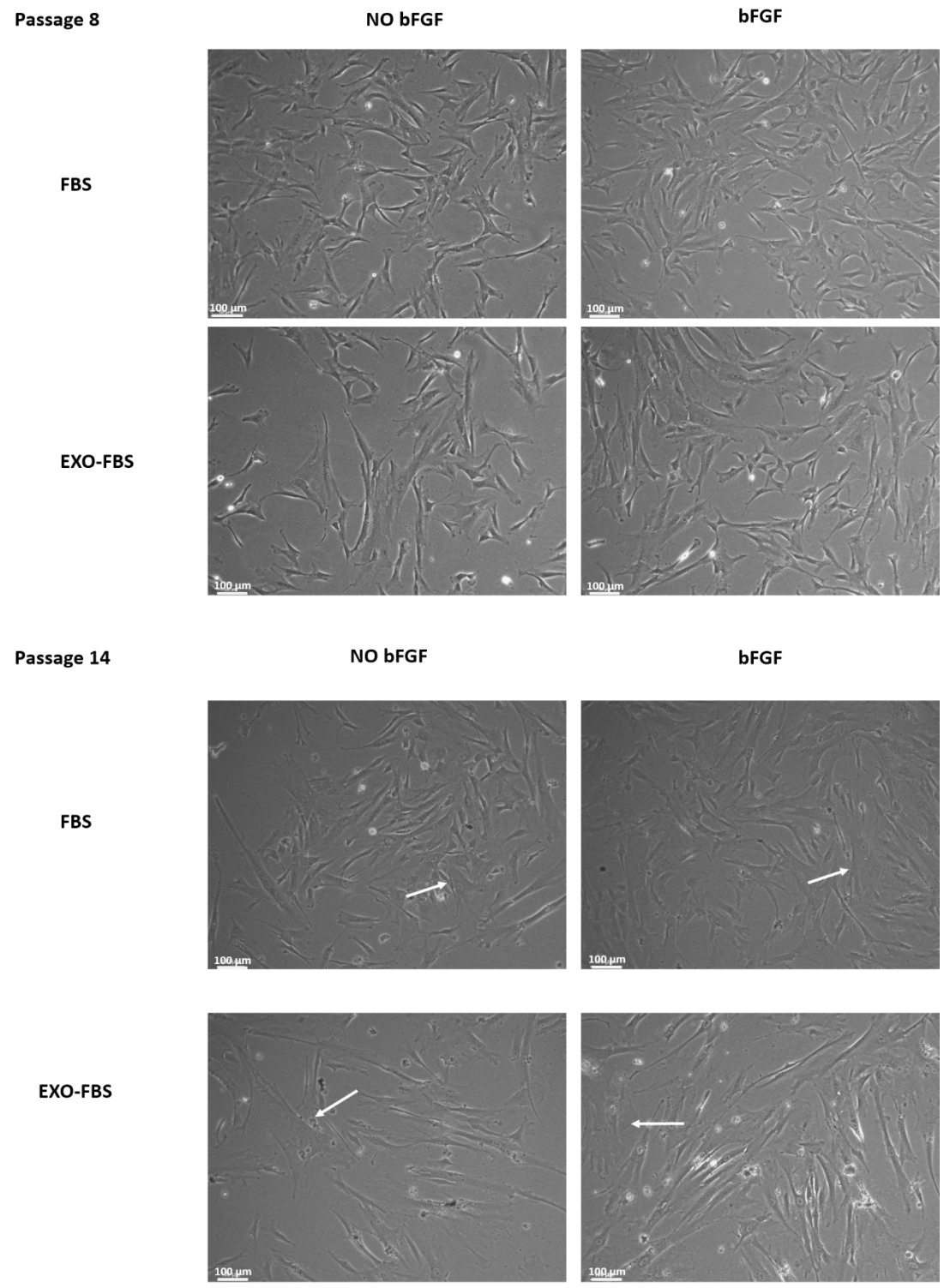

Figure 2. 2. Exosome-depleted foetal bovine serum and bFGF effects on hUCMSC morphology.

Morphology of cells appeared similar in early passages (e.g. Passage 8) regardless of the type of medium supplementation. By Passage 14 cells were no longer able to reach confluency for all groups analysed, even with regular media changing and prolonged days in culture. Arrows are pointing to flattened cells. Scale bar is 100 µm.

As Exosome-depleted FBS and bFGF do not seem to affect morphology, experiments were undertaken to investigate if they had any impact on cell proliferation and metabolism. Population doubling time was calculated over multiple passages, starting at Passage 5 and finishing at Passage 11, and using both a haemocytometer (Figure 2.3.A) and an automatic cell counter (Figure 2.3.B). There was significant difference between the FBS + bFGF and EXO-FBS groups in terms of doubling time. Although the absolute values were different for the methods used, the pattern was the same and the statistical analysis also provided similar results. bFGF did not change cell proliferation significantly, except in comparison to the exosome-depleted FBS without bFGF group.

In order to analyse the metabolic state of the cells, ATP release was measured using Cell Titer-Glo throughout 7 to 8 consecutive days. This method is based on evidence that the content of ATP in cell culture medium correlates to the number of cells in the medium [24]. All groups displayed the same pattern of ATP release, with a consistent increase ATP measurement reflecting the increasing number of cells analysed, and a decrease on the last day probably due to cell death as a result of overgrowth within the well (Figure 2.3.C and 2.3.D). The graphs contain information of one independent experiment, as luminescence absolute values varied between experiments, but the assay was repeated throughout several passages and a consistent pattern was obtained.

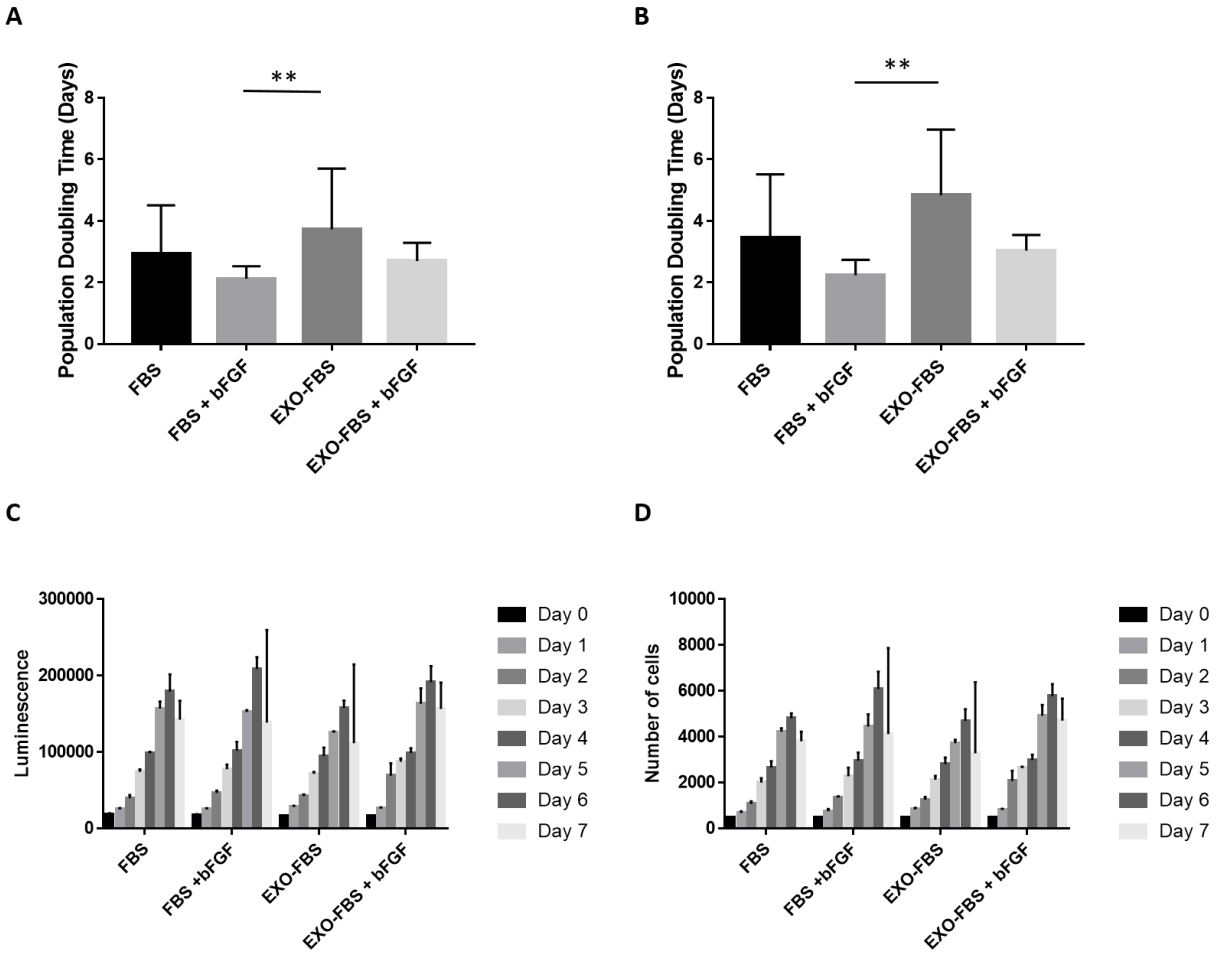

Figure 2. 3. Analysis of culture conditions on cell proliferation and ATP release.

hUCMSCs were counted using **A.** Automatic Cell Counter and **B.** Hemocytometer (Neubauer Chamber). **C.** Cell Titer assay to assess ATP release over a period of 8 consecutive days. There was no statistical significance in luminescence obtained from cells. **D.** Number of cells converted from luminescence did not differ between groups. ATP release graphs represent one independent experiment, which was repeated with similar results. Bars represent mean with standard deviation. No statistical significance was observed for ATP release assessment. Level of significance p set at 0.05. One-way ANOVA with Kruskal-Wallis correction test for multiple comparisons were used for statistical analysis of population doubling times analysis and Ordinary Two-way ANOVA with Tukey's multiple comparisons test was used for analysis of Cell-Titer Assay. ** is p value <0.01.

Following assessment of morphology, proliferation and metabolism, expression of surface receptors linked to mesenchymal cells was investigated. Expression of markers is displayed as percentage of positive events, rather than median fluorescence intensity to avoid any variations in absolute

numbers, and Figure 2.4 exemplifies how this percentage was determined for each sample.

Flow cytometry was used to assess whether the different culture conditions would affect expression of mesenchymal markers CD73, CD90 and CD105. hUCMSCs were assessed in terms of mesenchymal marker expression in Passages 9 (Figure 2.5.A) and 11 (Figure 2.5.B). These late passages were used to confirm whether cells were still displaying a mesenchymal phenotype at later stages. Dot plots comparing size and granularity of cells did not indicate existence of multiple populations and the need for detailed gating, but forward and side scatter were used as a simple gating strategy to prevent the inclusion of cell debris or duplets, just by excluding very small and large particles. In experiments where no isotype controls were included, unstained samples served as controls.

At both passages, the vast majority of cells in all groups were expressing CD90, CD105 and CD73, as Figure 2.5 shows. Monocyte marker CD14 was used in an additional analysis as a negative control (Figure 2.5 C), as well as isotype controls to determine whether there was significant unspecific binding. Isotype control staining displayed fluorescence similar to unstained samples and cells were negative for CD14, as expected.

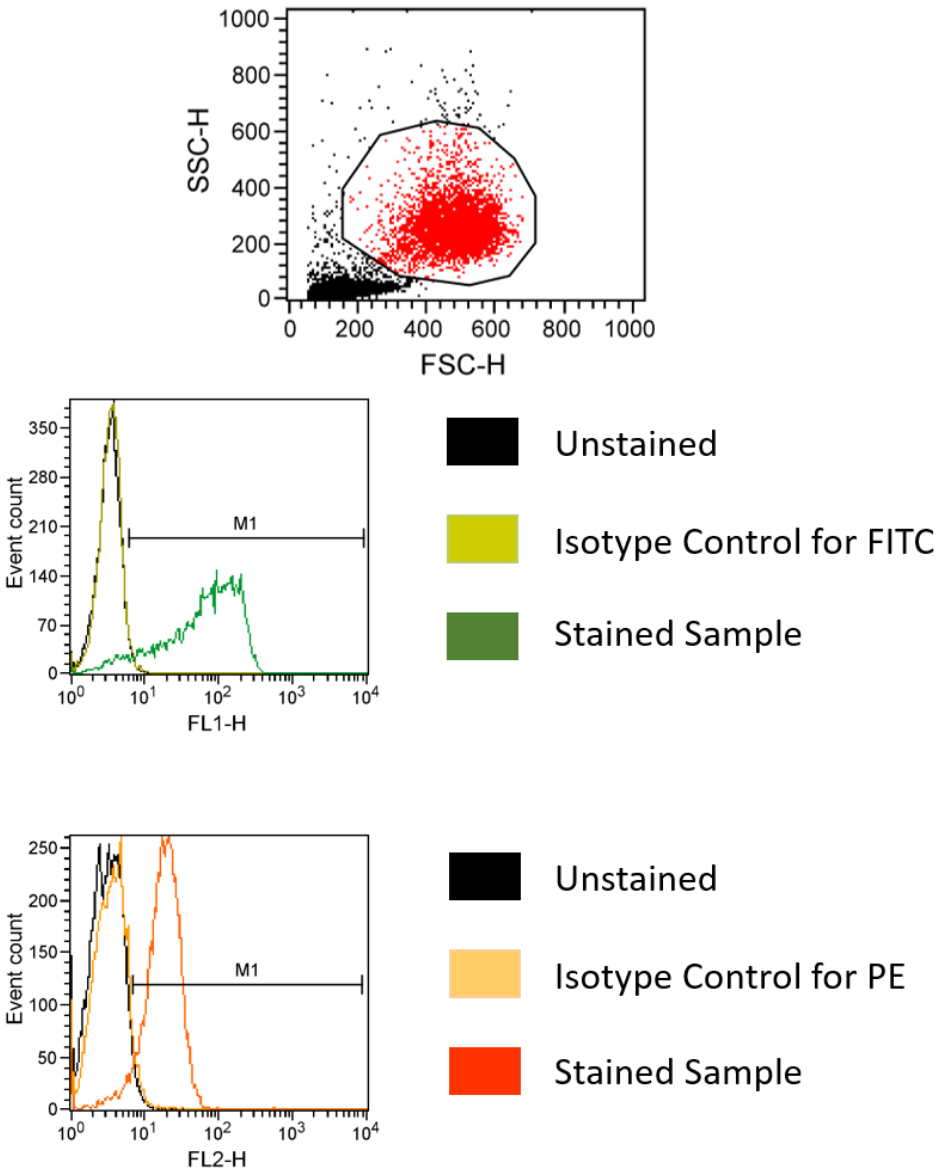

Figure 2. 4. Gating strategy example.

Gating was determined by side and forward scatter, as cells formed a distinct population. Cytometry data was analysed excluding the fluorescence presented by unstained samples and isotype controls. Isotype control histograms were used as the standard for gate drawing, as the fluorescence from surface markers isotype controls did not differ considerably from unstained samples. Gates to determine percentage of positive events, represented by the M1 intervals, needed to be manually set for each sample, as fluorescence varied greatly among MSCs, monocyte-derived macrophages phenotypes and blood donors. M1 here refers to the name of the interval. For samples analysed without isotype control, only the histograms in black, representing unstained samples, were used as standards for determining the percentage of positive events. This strategy was used for all cytometry data, acquired with BD FACS Canto II, BD Calibur, Miltenyi MACSQuant.

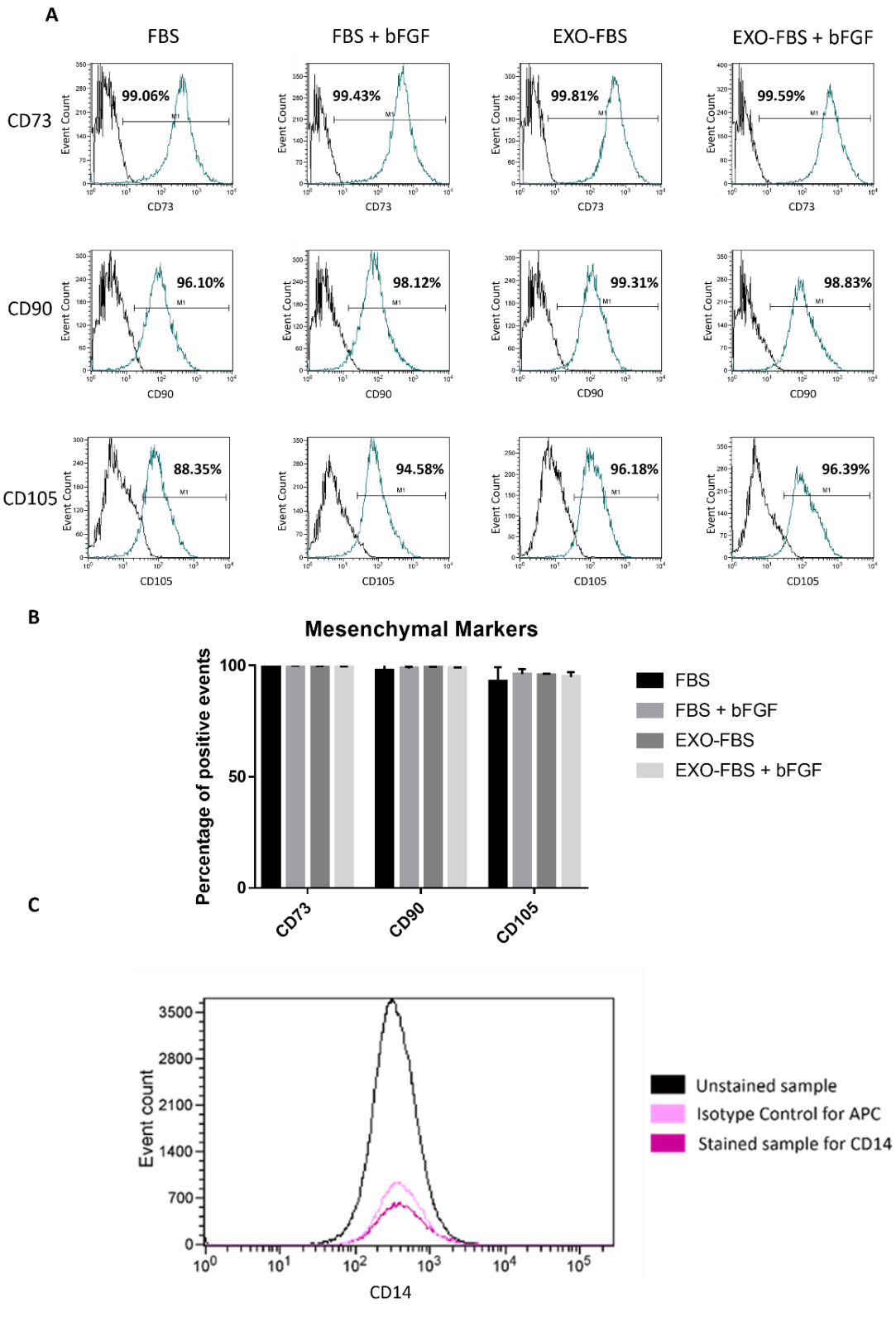

Figure 2. 5. hUCMSCs express mesenchymal markers in Passages 9 and 11.

Cells in Passage 11 (**A**); expression of CD73, CD90 and CD105 in hUCMSC in Passages 9 and 11 (**B)**. The vast majority of cells expressed all three markers analysed in both passages, showing that at least until Passage 11 cells did not lose their mesenchymal phenotype. **C.** Expression of CD14 in hUCMSC Passage 9 cultured with conventional FBS.

2.3.2. Establishment of EV isolation protocol and characterisation of EVs

Serum deprivation is a known mechanism to induce cell stress *in vitro* and thus stimulate EV release [25-27]. Deprivation was only performed when cells were already confluent, so as to induce as many cells as possible to release EVs. Figures 2.6 and 2.7 show hUCMSC cultured with conventional and exosome-depleted FBS (referred in Figure 2.7 as “EXO-FBS”). It is possible to notice that cells were adherent even after 72 hours of serum deprivation and cell detachment was not a major issue, especially for cells cultured with conventional FBS (Figures 2.6 and 2.7). Nevertheless, there was variability between dishes, and even areas within one dish, as exemplified in the picture of the EXO-FBS group after 48 hours of serum deprivation, where it seems to be an increased level of cell detachment. Overall, cells appeared to be stressed, as their morphology changed to more elongated; there was slight detachment and cells were not receiving enough nutrients to support their growth and maintenance, but even after the longest period of serum deprivation, more than 99% of cells were viable, as assessed by Trypan blue. Further evidence that the extent of detachment was negligible was the fact that when the supernatant was collected and centrifuged, no pellet could be visualised.

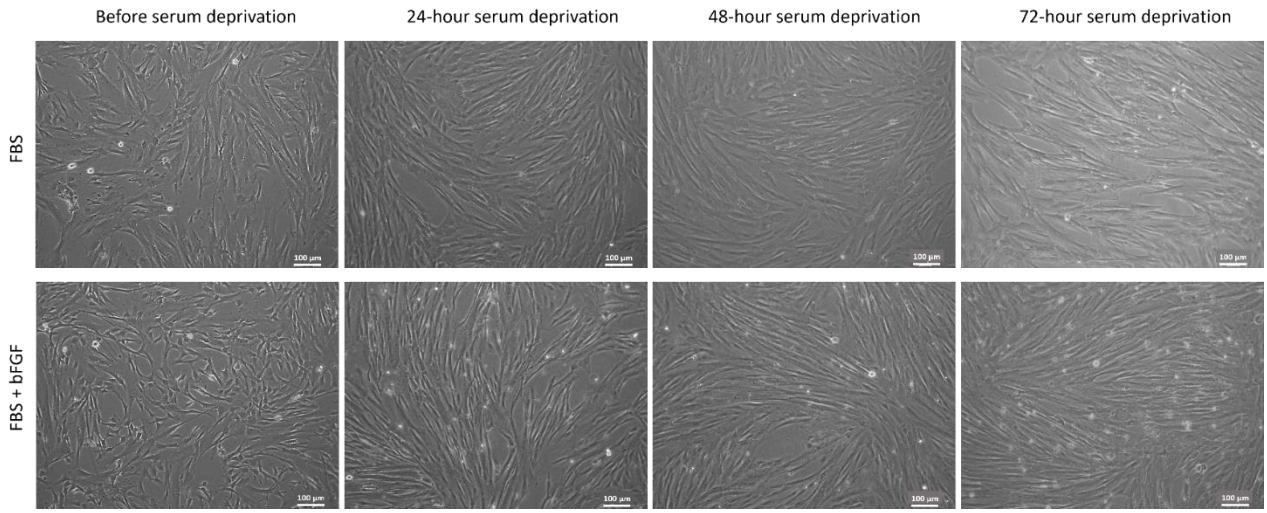

Figure 2. 6. hUCMSCs cultured with conventional FBS before and after serum deprivation.

Bright field microscopy of hUCMSC cultured with and without bFGF and deprived of FBS for up to 72 hours. Scale bar is 100 µm.

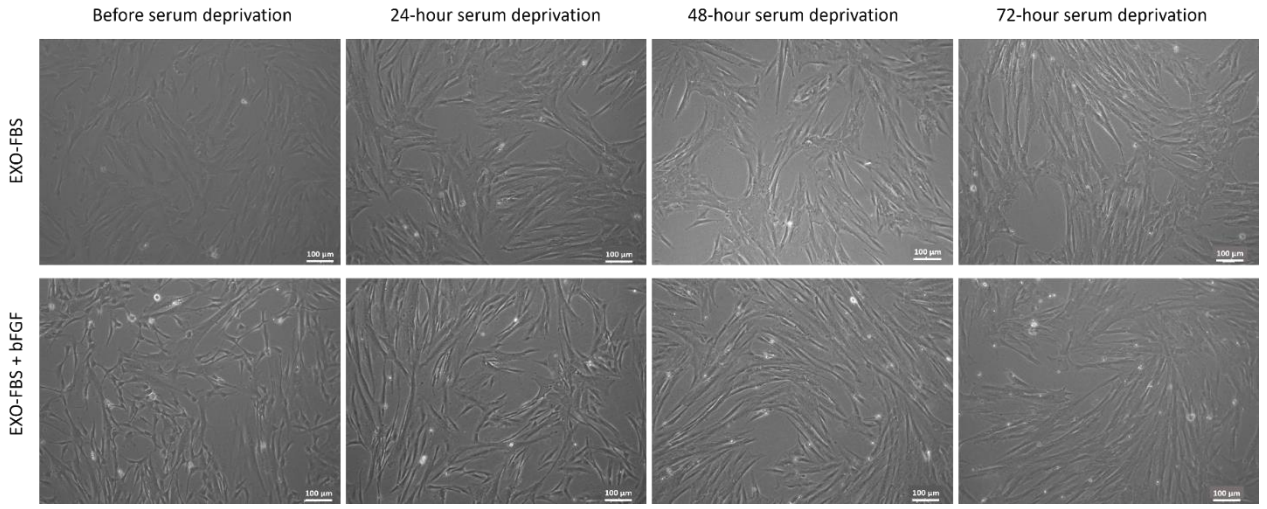

Figure 2. 7. hUCMSCs cultured with exosome-depleted FBS morphology before and after serum deprivation.

Bright field microscopy of hUCMSC cultured with and without bFGF and deprived of EXO-FBS for up to 72 hours. Scale bar is 100 µm.

Serum deprivation was used not only to increase the yield of EVs, but also to avoid contamination of samples with bovine EVs. This pilot test was designed to estimate differences in size and concentration of EVs caused by length of

serum deprivation. Size was assessed both as mean and mode, as depending on the size distribution of particles these values might vary considerably. EVs were isolated through ultracentrifugation of supernatant collected after serum deprivation. There was no significant difference in terms of size, considering both mean and mode, for the periods analysed of 24, 48 and 72 hours (Figures 2.8.A and 2.8.B). For particle concentration, there was an identifiable pattern of increase the longer the cells were deprived of serum (Figures 2.8.C and 2.8.D). Also, cells cultured with bFGF released the highest number of vesicles. Statistical analysis was not performed, given that only one sample per condition was analysed. Nevertheless, several dishes were used for this pilot test, as a great volume of supernatant was necessary to isolate a sufficient number of EVs in order for them to be within the optimal range for particle tracking analysis.

Two dilutions were used to acquire data in order to increase the reliability of the data, as it is known that NTA cannot distinguish between particle aggregates and single particles. Because this is an important issue to be considered when reporting concentration of particles, the graph on Figure 2.8.B shows the comparison between dilutions 1:10, 1:20 and 1:30 after correction. The closer the values obtained, the more accurate was the measurement. Dilution 1:30 was the least consistent, suggesting that the sample was too diluted for a precise measurement.

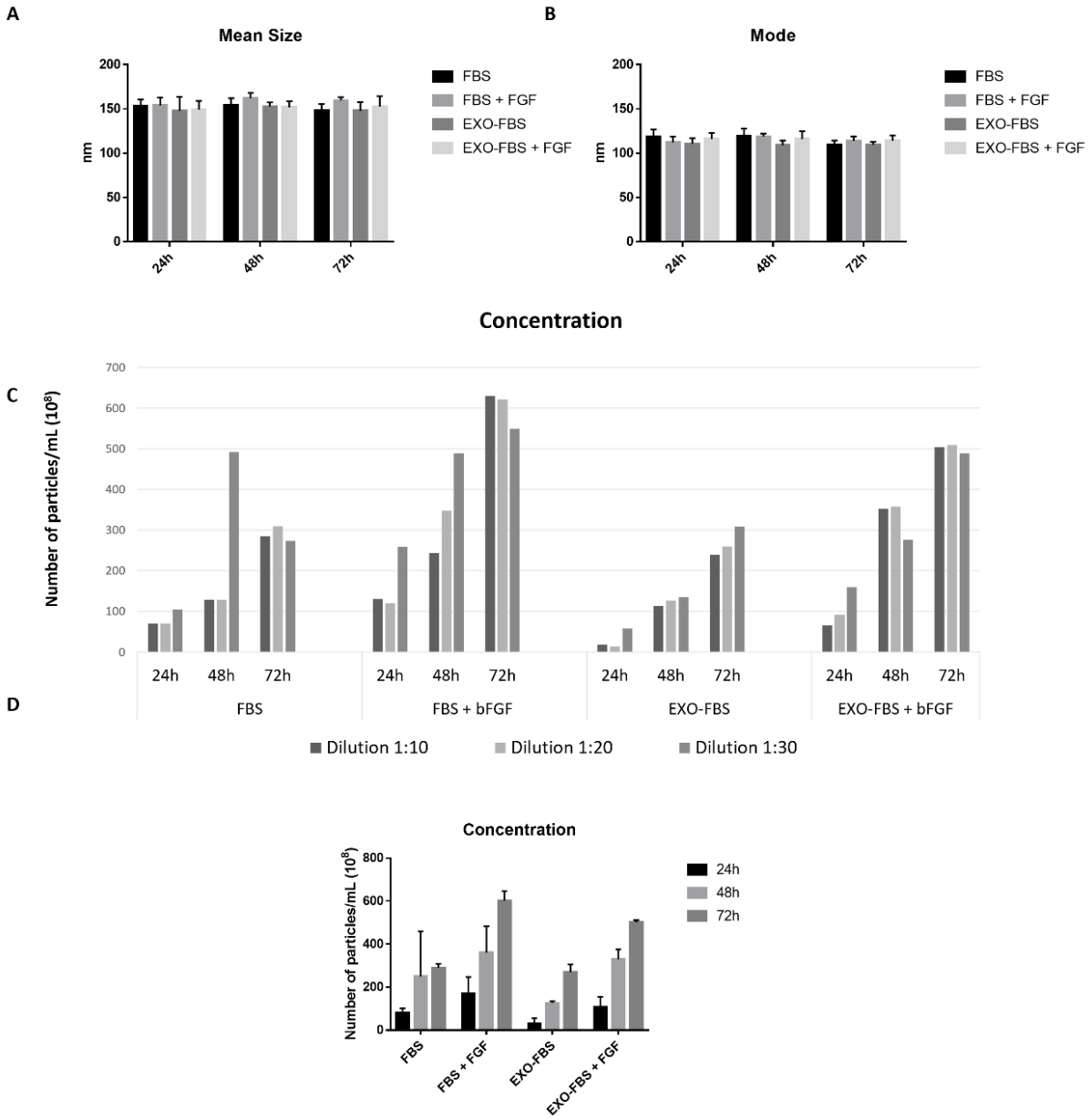

Figure 2. 8. Pilot test to determine optimal period of serum starvation in hUCMSC for EV isolation.

hUCMSC were deprived of FBS for 24, 48 and 72 hours in order to determine the most suitable protocol for EV isolation. EVs were isolated through ultracentrifugation of the collected supernatant and samples of EVs were analysed using Nanoparticle Tracking Analysis for characterisation of size and concentration. **A.** Mean size of particles with three technical replicates corresponding to the measurements of three dilutions; **B.** Size of particles reported as mode, or more frequent sizes, with three technical replicates per sample; **C.** Corrected values of number of particles per ml for the three dilutions used; **D.** Values from graph in **C**, pooled together to demonstrate that the longer the period of serum starvation the higher the concentration of particles. Bars represent mean plus standard deviation, except in graph displayed in **C.**, in which single mean values were plotted.

Because size is considered to be a determinant characteristic of EVs, used in the past to classify specific populations, it was important to assess the variability of size distribution between batches. Although there was a degree of variability, size distribution did not vary greatly. The mean diameter of 1 x 100,000 g and 2 x 100,000 g EVs was 183.3 ± 4.8 nm (mode: 146.7 ± 8.9 nm) and 182.3 ± 5.1 nm (mode: 132.5 ± 8.5 nm), respectively, as evaluated by NTA (Figure 2.9) in three different EV preparations (Batches A, B and C).

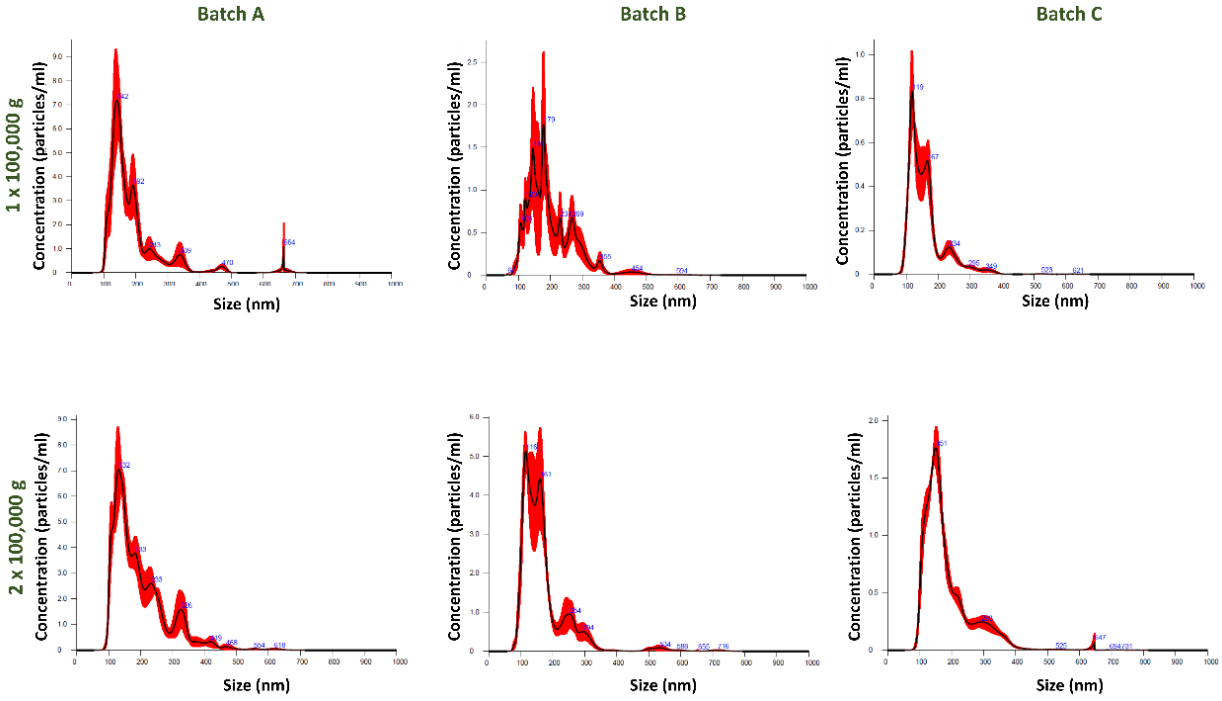

Figure 2. 9. Size distribution of EVs isolated by ultracentrifugation.

NanoSight representative images of EVs isolated by one or two rounds of ultracentrifugation at 100,000 g. Axis x and y represent size (nm) and concentration of vesicles (particles/ml), respectively.

As a further step in the characterisation of the vesicles, the expression of tetraspanin receptors was assessed using cytometry. Figure 2.10 is a summary of data from two batches of EVs, both acquired with one (1 x 100,000g) or two (2 x 100,000g) rounds of ultracentrifugation. EVs obtained

after two spins in general had a more intense expression of the surface proteins analysed, suggesting different subpopulations of vesicles.

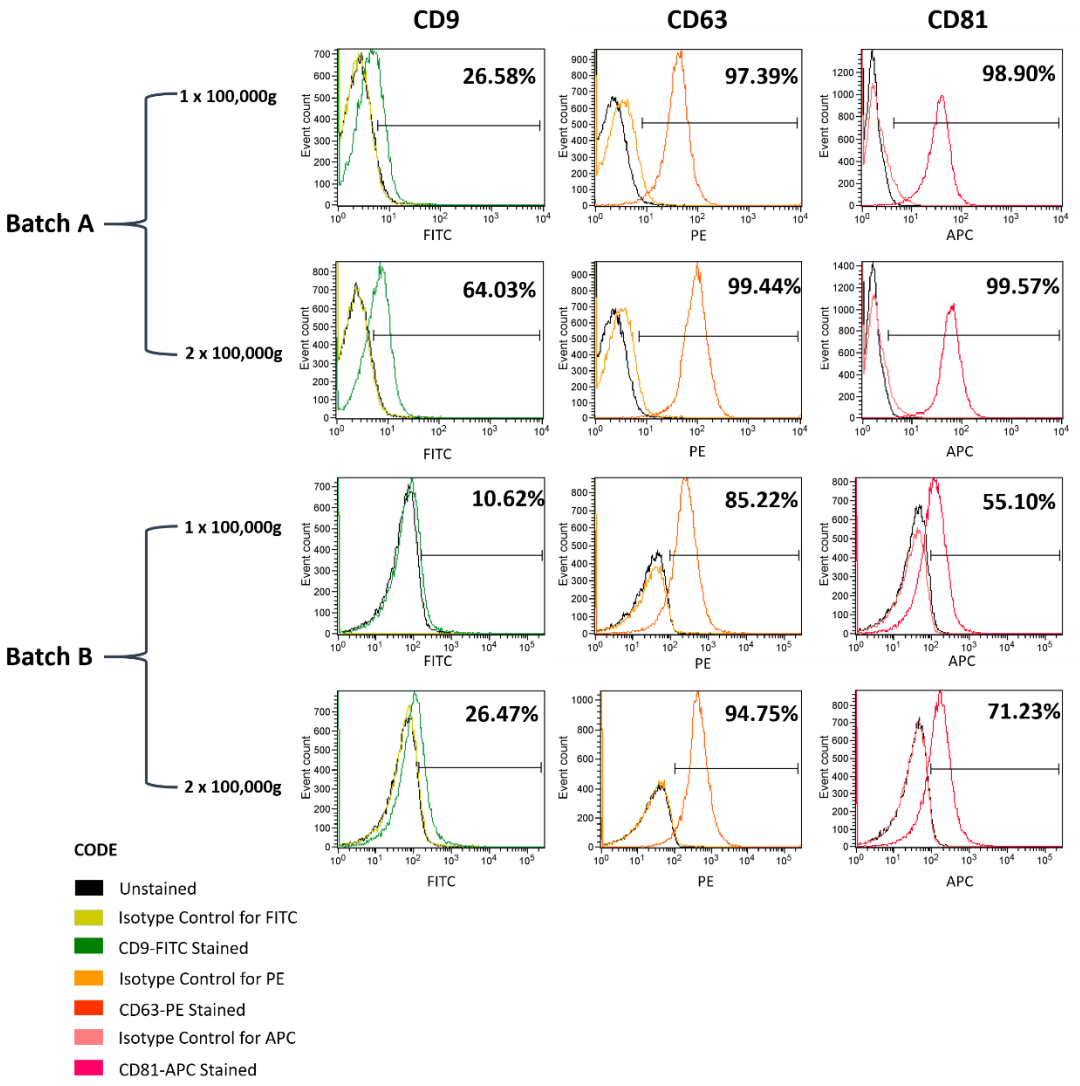

Figure 2. 10. Expression of tetraspanins in hUCMSC-derived EVs.

EVs isolated after one and two rounds of ultracentrifugation were incubated with aldehyde-sulphate beads and stained according to manufacturer's instructions. 30,000 events were acquired per sample and percentages express the number of positive events based on the strategy portrayed in Figure 1. One billion particles were used for each sample analysed. This procedure was repeated for 7 different EV preparations with similar results. FCSalyzer-0.9.18 alpha software was used for histograms and determination of regions.

EVs were assessed using transmission electron microscopy (TEM), to ensure they were still intact after long-term storage at -80°C. Figure 2.11 shows a micrograph of EVs which were frozen for 12 months.

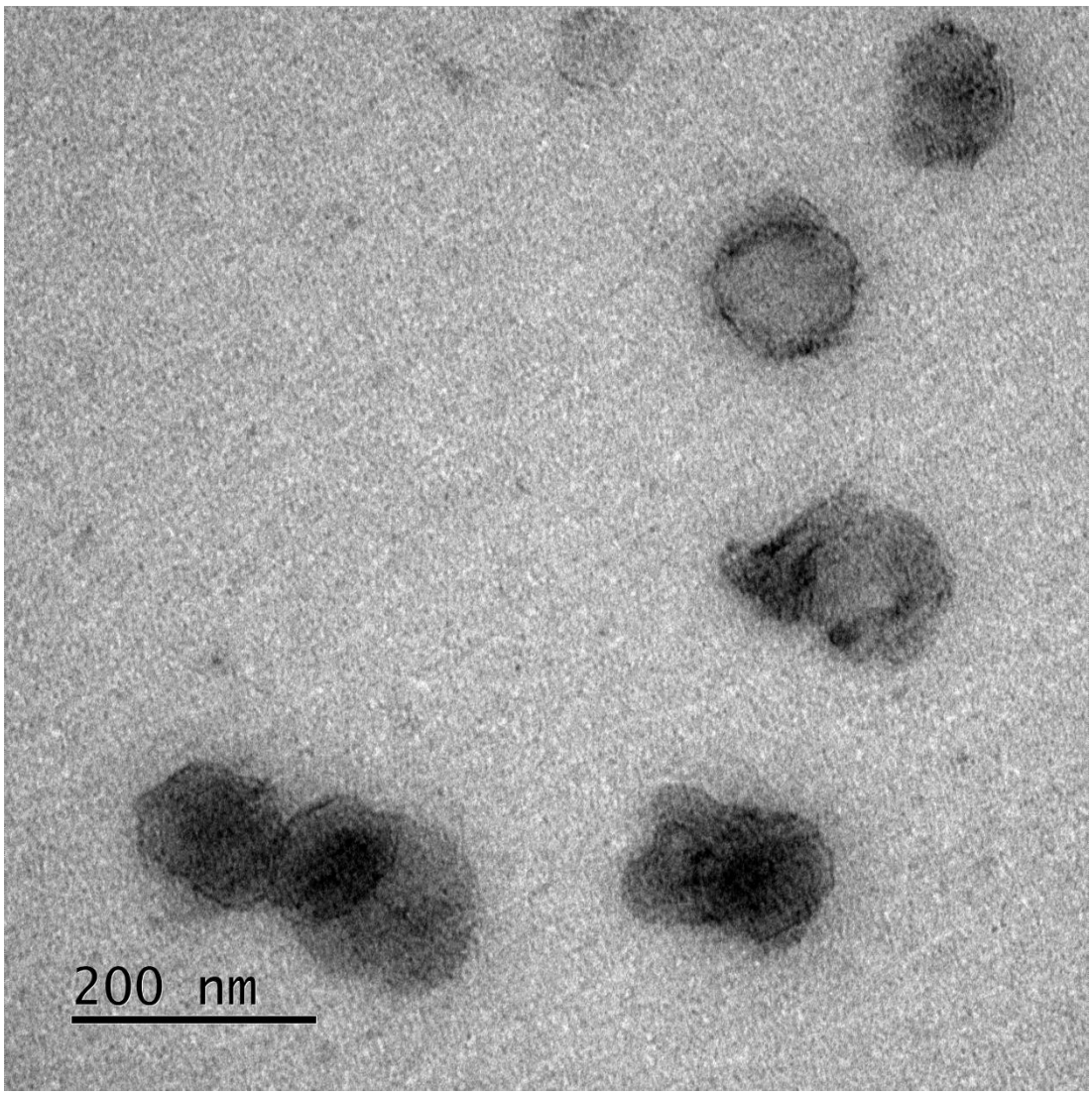

Figure 2. 11. Representative micrograph of transmission electron microscopy obtained from EVs isolated after two rounds of ultracentrifugation.

EVs were isolated from the supernatant of hUCMSCs deprived from FBS for 72 hours and after isolation via ultracentrifugation were immediately frozen at -80°C. The sample displayed at this micrograph was frozen for 12 months prior to electron microscopy assessment. FBS = Foetal bovine serum. Acknowledgement to Alison Beckett for performing electron microscopy.

2.4. Discussion

This initial characterisation was to determine the potential impact of bFGF on hUCMSC culture and to establish a reliable method of EV isolation. MSCs were characterised to comply with minimal criteria determined by the International Society for Cellular Therapy, and this included cell differentiation (Appendix, Figure A.1), plastic-adherence and surface marker expression. EVs were analysed regarding size, concentration and expression of the tetraspanins CD9, CD63 and CD81.

2.4.1. Exosome-depleted FBS impact on hUCMCS growth

Senescence is a process characterized by the loss of capacity to grow, with alteration in protein expression and morphology, although cells can remain viable and metabolically active [28]. Because the cells used for EV isolation were primary cells, they were expected to display a short lifespan [29]. Surprisingly, in Passage 11, hUCMSC were still expressing CD90, CD73 and CD105 in a very similar level to Passage 9, which is not completely in line with what was previously reported for human primary stromal cells, in which there is a noticeable decrease in mesenchymal marker expression in Passage 12 compared to Passage 3 [30], but is consistent with studies showing that MSCs can proliferate *in vitro* at different rates when passed at low or high cell density and cells would not be considered as senescent if they are still dividing [31, 32]. Nevertheless, there might still be robust expression of these markers in senescent cells, suggesting that senescence in MSCs cannot be only measured by mesenchymal marker expression.

Because cells would be incubated without FBS for at least 72 hours, β-galactosidase staining was used to assess whether these cells were senescent, and there was no detectable staining (Appendix, Figure A.2). Also, after the 72-hour serum starvation cells were harvested and re-seeded, to verify whether they would be able to attach and proliferate, and there was substantial attachment and growth, meaning that at least part of the population was not senescent (data not shown). Whether this would be of importance for the present study is mostly unknown, as there are no studies showing that EVs isolated from senescent cells would be more or less therapeutic within kidney injury or a macrophage polarisation context. But it is important to reiterate that all hUCMSCs used in this study were obtained from a single donor, and variability between donors should be expected. Therefore, additional studies comparing senescence in hUCMSCs from different donors should be performed, but cells used in these experiments were still proliferative after serum deprivation.

2.4.2. bFGF effects on EV release

Calculations of population doubling times by both an automatic counter and haemocytometer, and also mesenchymal marker expression, did not point to a major role of bFGF in proliferation or surface protein expression for hUCMSCs. This might be due to the fact that FBS already contains FGF. Nevertheless, concentration of EVs obtained from cells cultured with bFGF was higher in comparison to cells cultured in the absence of this factor, and this pattern was seen for conventional and exosome-depleted serum.

bFGF has strong angiogenic action, and also promotes migration, differentiation and proliferation of diverse cell types [33, 34]. The role of bFGF in EV release is not completely understood, but there is evidence that in cultured hippocampal neurons bFGF facilitates the fusion of multi vesicular bodies (MVBs) with the plasma membrane, a crucial step in exosome release [35]. Exosome release, or more specifically fusion of MVBs with plasma membrane, can be regulated by calcium influx, similar to what occurs in neurotransmitter release, and thus involve calcium channels and soluble N-ethylmaleimide-sensitive factor (NSF) attachment receptor (SNARE) proteins [35-37], critical proteins for neuronal synapses and on neuronal vesicles [38]. One of the mechanisms by which bFGF can increase EV release would be by opening calcium channels [39, 40], one of the many possible effects of bFGF, but this body of evidence was built on studies using neuronal cells and not MSCs.

As a consequence of the obtained results showing that bFGF did not impact morphology and proliferation on hUCMSC, EV isolation for subsequent experiment using monocyte-derived macrophages was performed on cells cultured without bFGF. Further studies need to be performed in order to determine how FGF interferes with extracellular vesicle release and to what extent in terms of concentration and cargo.

2.4.3. Serum deprivation for EV isolation

Serum deprivation effectively elicited EV release, but this method does not rule out the presence of bovine EVs. FBS starvation is used for EV collection so as to minimise contaminants in the EV-containing medium and also to induce cell stress. These contaminants are bovine extracellular vesicles and all they might contain, such as protein and RNA contaminants. Many researchers have opted to ultracentrifuge FBS in an attempt to deplete it from vesicles and RNA, and this technique is used in most of the commercially available exosome-depleted FBS. Nevertheless, even after a 24-hour ultracentrifugation of FBS, there is still bovine RNA which was not pelleted and can be confounded by EV-RNA [41].

Some cells are particularly sensitive to serum starvation and cannot remain viable in the absence of serum [42], but it is essential to keep serum to a minimum while collecting EVs, as proteins and vesicles from FBS can contaminate the sample [43]. Serum deprivation is also a stress-inducing mechanism used to trigger EV release, which can potentially influence the cargo and amount of EVs [44].

In this study, cells were deprived of FBS for at least 24 hours for EV collection. Two groups of cells were cultured with commercial exosome-depleted FBS, which proved to support cell growth and mesenchymal marker expression, but had a lower yield of EVs in comparison to their conventional FBS counterparts. The undefined and variable composition between brands and batches of FBS limits the potential for investigation of which factors could be responsible for this effect.

It could be argued that cells cultured in the virtual absence of bovine exosomes would be less stressed when completely starved from FBS and thus would have less of the stimulus to release EVs. This argument could not only apply to exosomes but also to proteins and growth factors that could be potentially depleted or have a substantial lower concentration due to FBS ultracentrifugation, and this could be corroborated by the fact that cells cultured with exosome-depleted FBS and bFGF released a comparable amount of EVs to cells cultured with conventional FBS. Nevertheless, it cannot be ruled out that the difference in number of particles released is due to the presence of bovine EVs contaminating the sample, as it was previously mentioned that the exosome-depleted serum is not completely depleted.

When analysing the exosome-depleted serum from Systems Biosciences used for cell culture in this study in NTA, the concentration of particles was diminished by 10-fold in comparison to conventional FBS from Sigma. Although it is a considerable reduction, there were still some visible particles, or potentially protein aggregates. Exosome-depleted FBS from Gibco (cat. Number A2720803) was also analysed on Nanosight and had a lower concentration of particles in comparison to SBI FBS (Appendix, Figure A.3). However, depleted FBS from Gibco did not support cell growth and cells were easily detachable, suggesting the serum was also depleted of important growth factors. An in-house alternative was to deplete bovine extracellular vesicles from FBS using ultrafiltration, as described by Kornilov et al [45], but this method was also not suitable for *in vitro* expansion, as the pores of the filter would block and most of proteins would not be filtered, e.g. albumin.

Therefore, using FBS for cell culture, even exosome-depleted FBS, does not guarantee the absence of contaminants of bovine origin. Increasing the volume of serum-free medium used for EV collection, washing the dishes before isolation and complete starvation of serum are among the practices to minimise the proportion of contaminants, and those were practices used throughout this study.

2.4.4. Ultracentrifugation as an EV isolation method

The method of isolation determines the subpopulation of EVs that will be obtained [46], and that is of uttermost importance for the aim of the study. Ultracentrifugation is a widely used method for EV isolation, considered as a gold standard, and has been used for isolation of vesicles tested in kidney injury models [13, 47, 48]. Nevertheless, it has been associated with reduced RNA recovery and sedimentation of non-vesicular material [49]. Due to this sedimentation, some groups use two rounds of ultracentrifugation as a way of washing the sample from potential protein contaminants [50, 51].

Tetraspanins are membrane proteins that are also called exosomal-enriched proteins. They cannot be considered as exosomal markers given they are expressed on many cell types and can be expressed on microvesicles, and not only exosomes, but there is increasing evidence they are particularly abundant in EVs [52]. CD9, CD63 and CD81 are commonly used to assess EV populations, alongside other proteins, such as TSG101 [53].

Differences in expression intensity of tetraspanins may suggest that the extra round of ultracentrifugation is not only reducing protein contamination, but also

narrowing the final population towards a more exosomal predominance. Whether this is of importance for the purposes of this study remains to be elucidated.

2.5. Conclusion

MSCs from umbilical cord were successfully characterised regarding morphology, proliferation and surface marker expression. EV isolation using FBS deprivation and ultracentrifugation provided vesicles with a consistent size range and expressing tetraspanins reported to be present in EVs. Exosome-depleted FBS and bFGF did not disrupt neither MSC physiology nor EV release.

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Chapter 3 – Optimisation of monocyte-derived macrophage culture

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3.1 Introduction

Macrophages are cells from the innate immune system responsible for a variety of roles in development, inflammation and also tissue regeneration, as well as representing a bridge to components of the adaptive system. Although these cells are part of the innate immune system, which suggests they have a set of determined roles and characteristics and are not as flexible as their adaptive immunity counterparts, macrophages have a spectrum of phenotypes that determines their roles and the signals they send and respond to. Macrophages can be both residents in organs and originate from monocytes in the blood stream, thus explaining why they are usually among the first responders in injury. This chapter aims to specifically discuss macrophage physiology and the manner by which this study attempted to investigate phenotype nuances on these cells *in vitro*.

3.1.1. Monocytes and macrophages in kidney injury

As kidney injury imposes a major public health issue, many strategies to treat and improve outcomes have been tested and are currently under scrutiny. MSCs, as previously discussed, have emerged as a valuable tool for kidney disease treatment and are considered as immune suppressors. A major cause of kidney injury is ischemia/reperfusion [1], and as a consequence there is now a number of studies on acute kidney injury and chronic kidney disease using ischemia/reperfusion models in animals [2-4]. The first responders to a kidney

ischemic injury are neutrophils and natural killer T cells, which are recruited to the site of injury within hours after reperfusion. Monocytes are recruited after 24 hours of reperfusion and mature into mainly pro-inflammatory macrophages during the first 48 hours of injury [5]. However, macrophages are not only important in the early stages of injury, but are also present and functional in repair and late fibrosis [6].

There is an increasing body of evidence showing that there is a switch in macrophage phenotype within days of the initial challenge [7]. This shift is essential for tissue repair, as it limits inflammation and starts the process of tubular cell proliferation, but how it occurs remains to be fully elucidated. Figure 3.1 exemplifies some mechanisms involved in macrophage participation in kidney injury. Changes in macrophage phenotype are functionally and temporally dynamic, making it challenging to replicate it *in vitro*.

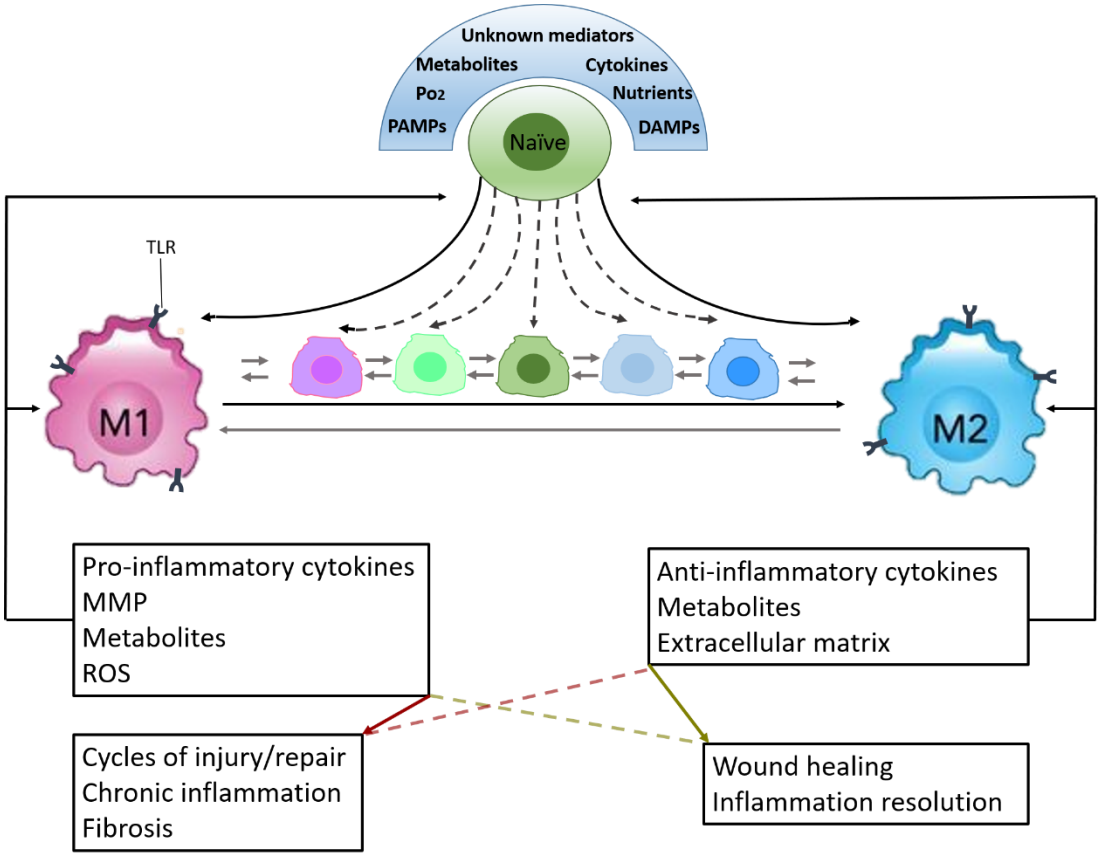

Figure 3. 1. Macrophage polarisation in pathogenesis of renal injury.

Macrophages are activated by DAMPs, PAMPs and other mediators. They can differentiate from monocytes and be polarised by activation of pattern recognition receptors, such as Toll-like receptors (TLR). Macrophages demonstrate dramatically diverse phenotypes in inflammation, injury-repair cycles and fibrosis over time, depending on the local environment, injury type, persistence, severity and reparative condition of the kidney. DAMP, Damage associated molecular pattern; PAMP, Pathogen associated molecular pattern; Po_2, Partial pressure of oxygen; MMP, Matrix metalloproteinase; ROS, Reactive oxygen species. Adapted from [8].

As previously mentioned in the introductory chapter, macrophage phenotypes can be characterised as a spectrum, rather than discreet entities. Indeed, the concept of only two opposing phenotypes in macrophages, M1 and M2, is deemed to be too simplistic, as reports show that different transcription factors, and consequently pathways, are activated depending on the cytokines that monocytes/macrophages are exposed to [9]. It is accepted that macrophages, particularly M2 macrophages, can assume diverse phenotypes depending on

their microenvironments [10]. Therefore, it would be ideal to choose markers sufficiently generic to allow detection of phenotype shifting.

3.1.2. Principles of *in vitro* macrophage culture

In vitro studies of macrophages have been performed mainly in murine *ex-vivo* models or cell lines, and differences between human and murine immune systems as well as differences between primary cells and cell lines are to be expected [11, 12]. It has been the aim of many researchers to standardise the culture and polarisation of macrophages *in vitro*, and there are certain culture conditions which became standard practice, such as use of the cytokines IFN-γ and IL-4 to polarise M1 and M2 macrophages, respectively [13].

However, there is a variety of differences in how monocytes are isolated and cultured and the manner by which macrophages can be activated, making it difficult to compare studies [14]. Optimisation of the protocols in this study aimed to obtain cells displaying the desired characteristics while simultaneously exposing them to widely used stimuli, so as not to substantially differ from established protocols.

Many in vitro studies are performed in rodents, particularly mice, and as a consequence there is abundant data of murine macrophages and their markers, but these markers do not necessarily have human equivalents. The markers chosen for this study are reported to be expressed or secreted by human macrophages. CD163 is one of the most widely used anti-inflammatory markers in the context of human macrophage polarisation [15-19] and CD80 is a well-known co-stimulatory molecule strongly associated with the M1-

phenotype [20-22]. Both IL-10 and IFN-γ are reported to induce macrophage M2 and M1 polarisation, respectively, and are also secreted by polarised macrophages [23-25]. This panel of markers would make it possible to verify whether cells were indeed polarised and, in future, if EVs are able to change polarisation.

3.1.3. Donor variability as a factor in immune cells studies

The immune system is known for its remarkable diversity in cell repertoire and functions. This diversity aids in an effective response to a wide array of pathogens and harmful organisms. However, the inter-individual variability coupled with differences in how immune cells respond in other species, mainly rodents, hinders the translation of pre-clinical studies into effective immunotherapies for humans [26-28]. These inter-individual differences can be heritable, but are mostly non-heritable, making it quite complex to extrapolate findings from a small number of individuals [29].

Because this study aims at the study of human monocyte-derived macrophages (hMDMs) obtained from the peripheral blood of healthy individuals, great variability in the expression of surface markers is expected [30]. Nevertheless, it is important to perform immunological studies using human samples, as opposed to only isogenic animal samples, in order to investigate how the human immune system behaves and find therapeutic strategies for as broad a population as possible.

3.1.4. Hypothesis

Macrophages are key players in kidney injury pathophysiology, and there is evidence that the phenotype of these cells greatly impacts prognosis. We

tested whether monocytes isolated from peripheral blood could be matured and polarised into anti- and pro-inflammatory phenotypes with distinct expression of surface markers and responsiveness to inflammatory stimuli. The hypothesis was that a standardised protocol for differentiation and polarisation could provide consistent expression of the selected markers.

3.1.5. Aims

This chapter establishes protocols that reliably lead to differentiation of macrophages from human monocytes and their further polarisation to M1 or M2 macrophage sub-types (Figure 3.2). This was an essential step before assessing the potential of hUCMSC-derived EVs to alter macrophage phenotype. Therefore, the aims were as follows:

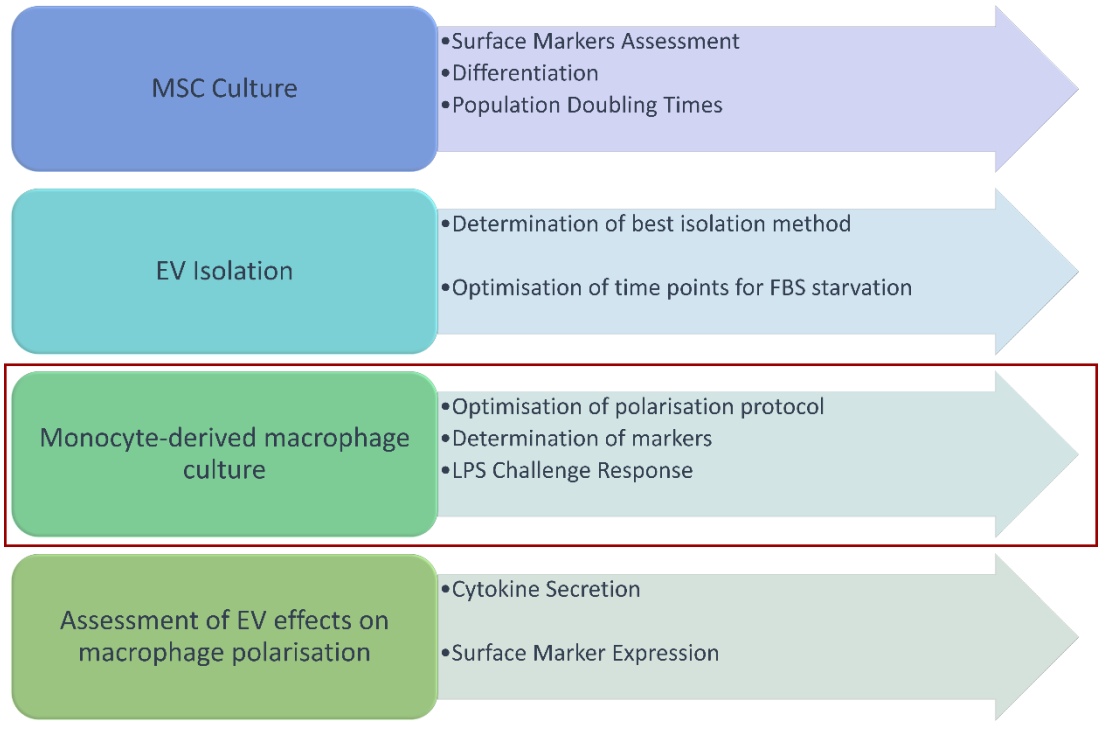

Figure 3. 2. Schematic diagram of study phases.

This chapter results refer to steps within the red rectangle, which is monocyte-derived macrophage culture and the optimisations required.

i. To develop a consistent hMDM culture system that provides the maximum yield and uniformity of cells. Optimisation of culture conditions to ensure monocytes were differentiating into macrophages. This included initial isolation of monocytes from buffy coats and culture protocol until polarisation.

ii. To successfully polarise macrophages towards a pro- and anti-inflammatory phenotype. This step refers to the optimisation of polarisation protocol, mainly according to results obtained through cytometric analysis.

iii. To determine the most suitable markers for assessment of polarisation and cell identity. Investigation of whether surface marker expression was coherent to expected phenotype.

iv. To assess cell responsiveness to LPS. Determination of cell responsiveness to a potent inflammatory stimulus, as is expected from mature macrophages, particularly M1.

3.2 Material and Methods

3.2.1. Monocyte isolation and maturation into macrophages

Monocytes were obtained through isolation of Peripheral Blood Mononuclear Cells (PBMCs) from Buffy Coats provided by National Health Service – Blood and Transplant (NHSBT). Buffy Coats were obtained as part of the Non-Clinical Issue system, aimed to provide surplus material for academic and clinical research. All experiments involving blood samples were approved by the Ethics Committee at the University of Liverpool and by the NHSBT. The

blood and the packing did not contain any personal or identifiable information of the donor. All samples were tested at NHSBT to be negative for Human Immunodeficiency Virus (HIV), Hepatitis C Virus (HCV) and Cytomegalovirus (CMV).

Upon arrival, blood was gently transferred to a tube containing Ficoll-Paque Plus (GE, cat. number GE17-1440-02). The ratio used was 15 mL of Ficoll-Paque to 30 mL of blood. Ideally both liquids do not mix with each other, but rather two clear and defined layers in the tube would be noticed. The sample was then centrifuged at 2000 rpm for 30 minutes without brake and after centrifugation, a cloudy, white layer of PBMCs was formed, along with other layers, such as red cells and plasma. Ficoll-Paque enables isolation of lymphocytes and monocytes based on the density of cells present on whole blood. Red blood cells are dense enough to penetrate the Ficoll-Paque layer and sediment on the bottom of the tube, as also occurs to granulocytes. Nevertheless, lymphocytes and monocytes, as well as platelets, are not as dense as red blood cells. As a result, after centrifugation, lymphocytes and monocytes are concentrated in a band formed between the original blood and Ficoll-Paque. This band is correspondent to the PBMC layer. The PBMC layer was aspirated using a Pasteur Pipette and transferred to another tube for an additional centrifugation with PBS. Cells were seeded in T-75 Nunc (Thermo Scientific, cat. number 156499) or T-25 Nunc (Thermo Scientific, cat. number 156367) flasks and cultured with IMDM (Gibco, cat. number 31980030) 10% FBS (Sigma, cat. Number F7524) overnight for cell attachment. Due to medium selection, only monocytes would firmly adhere to the flask. Red blood cells and platelets could compete for adherence with monocytes, so it was

very important to aspirate only the PBMC layer; medium selection would eventually enable only monocytes to remain in culture in case of aspiration of blood cells. On the following day, the supernatant was discarded and replaced by specific medium formulated to enable monocyte maturation and also polarisation towards either M1 or M2 phenotypes. The choice not to use CD14 magnetic beads to only culture $CD14^{positive}$ monocytes was aimed at increasing the yield, and not limiting the number of cultured cells, as the process of excluding all $CD14^{negative}$ cells would result in inevitable loss of monocyte populations that could potentially differentiate into macrophages.

For the first experiments with buffy coats, a defined xeno-free (DXF) Macrophage Generation Medium from PromoCell, which had different formulations for M1-like macrophage (cat. number C-28055) and M2-like macrophage (cat. number C-28056) polarisation and also came with precise instructions specifying days for adding and changing media, was used. For the naïve cells, Macrophage Base Medium DXF (PromoCell, cat. number C-28057) was used. Cells would be cultured for a total of 10 days from monocyte isolation until being considered as fully polarised macrophages. For these first experiments, macrophage detachment solution DXF (PromoCell, cat. number C-41330) was used to harvest mature macrophages just before flow cytometry analysis. Cells would be incubated with macrophage detachment solution for up to one hour at 4°C and then gently detached with a cell scraper.

This medium was replaced after testing three donors, not only to reduce costs, but also because surface marker expression proved to be highly variable. The replacement was for an in-house media using IMDM supplemented with either 20ng/mL GM-CSF (Miltenyi, cat. number 130-095-372) for M1 polarisation or

20ng/mL M-CSF (Miltenyi, cat. number 130-096-491) for M2 polarisation. The change enabled control of concentration and diversity of cytokines to which monocytes were exposed. Cells were activated/polarised with either IFN-g1b (Miltenyi, cat. number 130-096-873) during 24 hours for M1 polarisation or IL-4 (Miltenyi, cat. number 130-093-917) during 48 hours for M2 polarisation. Cell harvest was performed by incubating cells with a 2.5 mM solution of ethylenediaminetetraacetic acid (EDTA) for 15 minutes at 4°C, followed by gentle cell scraping.

As optimisation was necessary in terms of concentration of cytokines and periods of incubation; slight changes were performed according to cytometry results obtained from each buffy coat. These changes are described and explained in the Results and Discussion sections (pages 86-104).

3.2.2. Cytokine Secretion Assay

Cytokine secretion assay was performed using the supernatant of mature macrophages exposed to LPS for different periods. The kit used was Human Magnetic Luminex (R & D Systems, cat.number LXSAHM-04). The principle of the assay is the same used for ELISA. In summary, the supernatant was added to a 96-well plate pre-coated with analyte-specific antibody beads. Analytes tested were IL-1 beta/IL-1F2, IL-10 (BR22), IL-1ra/IL-1F3 (BR30) and IL-12 p70 (BR56), which standard values were in pg/mL 4,810, 1,190, 8,610 and 32,680, respectively. Biotinylated antibodies were used to form the antibody-antigen sandwich, followed by the addition of streptavidin conjugated with phycoerythrin (PE), to bind to the biotinylated antibodies and enable detection of the immune-complex. A Bio-Rad 200 Systems plate reader was used to detect luminescence from the 96-well plates and the Bio-Plex Manager

software was used to acquire and analyse data, including the standard curves for each analyte.

3.2.3. Flow Cytometry Analysis

Flow cytometry analysis was performed using BD FACS Calibur and Miltenyi MACSQuant equipment. The antibodies from Miltenyi used for intracellular markers were IL-10 (cat. number 130-112-729) and IFN-γ (cat. number 130-111-769), and cell blocking and permeabilization was initially performed using PBS 0.5% Bovine Serum Albumin (BSA, Sigma, cat. Number A4503) and PBS 0.2% Triton-X-100 (Sigma, T8787). Due to unspecific results, blocking and permeabilization were then performed with the kit InsideStain (Miltenyi, cat. number 130-090-477). Surface marker antibodies used were human anti-CD14 (cat. number 130-110-520), human anti-CD80 (cat. number 130-110-270) and human anti-CD163 (cat. number 130-112-132) – Table 3.1. As used in cytofluorimetric analysis of hUCMSC, Figure 3.2 illustrates the strategy to analyse samples and determine the percentage of positive events. For all analysis using isotype controls (IC), the IC histogram would be considered as the standard to start the interval from which the positive events were counted.

Table 3. 1. Antibodies used for cytofluorimetric analysis of monocyte-derived macrophages

Antibody	Clone	Isotype	Fluorochrome	Dilution
IL-10	REA842	IgG	APC	1:100
IFN-γ	REA600	IgG	Vio667	1:100
CD14	REA599	IgG	APC	1:100
CD80	REA661	IgG	PE	1:100
CD163	REA812	IgG	FITC	1:100

Abbreviations used FITC: fluorescein isothiocyanate, PE: phycoerithrin, APC: allophycocyanin.

Data acquired using BD FACS Calibur were acquired using Cell Quest software and data acquired in MACS were acquired with MACS Quant software. Histograms and percentages of positive events were obtained using FCSalyzer through fcs.files.

3.2.4. Statistical Analysis

One-way ANOVA with Kruskal-Wallis correction test for multiple comparisons was used to compare one parameter for more than two groups and two-way ANOVA with Dunn's correction for multiple comparison was used to compare more than one parameter for more than two groups. GraphPad Prism 7 was used for statistical analysis, *$p<0.05$, **$p<0.01$, ***$p<0.001$. Statistical analysis was performed for biological and technical replicates, and not for technical replicates only.

3.3 Results

3.3.1. Development of a consistent hMDM culture system

Cytofluorimetric analysis was the main technique performed in order to assess expressed markers and, due to the high variability of median fluorescence intensity absolute values among donors, the percentage of positive events was a more representative measurement of marker expression and enabled pooling of results from different donors. This measure was determined based on the fluorescence expressed by unstained or isotype control samples, exactly as performed with hUCMSCs and EVs in Chapter 2. The strategy to determine positive events is demonstrated on Figure 3.3.

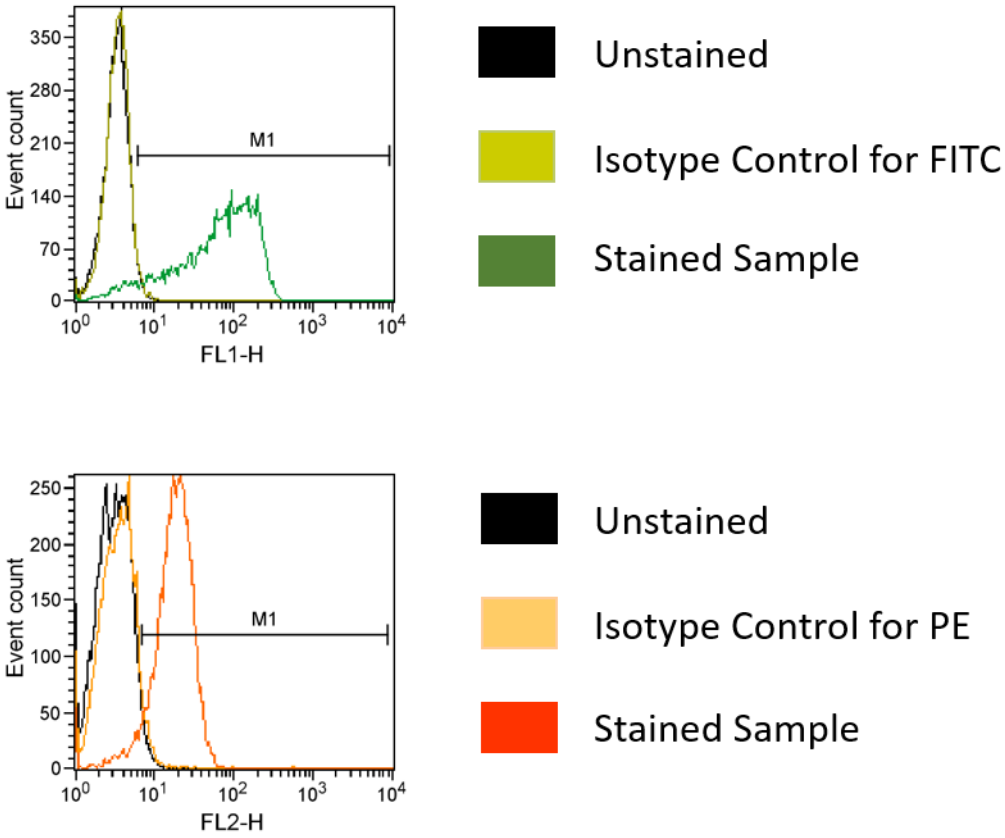

Figure 3. 3. Determination of percentage of positive events.

Cytometry data was analysed excluding the fluorescence presented by unstained samples and isotype controls. Isotype control histograms were used as the standard for gate drawing, as the fluorescence from surface markers isotype controls did not differ considerably from unstained samples. Gates, represented by the M1 intervals, needed to be manually set for each sample, as fluorescence varied greatly among MSCs, monocyte-derived macrophages phenotypes and blood donors. M1 here refers to the interval. For samples analysed without isotype control, only the histograms in black, representing unstained samples, were used as references for determining the percentage of positive events. This strategy was used for all cytometry data, acquired with BD FACS Canto II, BD Calibur, Miltenyi MACSQuant. Histograms are the same used in Figure 2.4.

Monocytes were initially cultured with ready-to-use PromoCell medium, following manufacturer's instructions, in order to obtain polarised cells. The markers CD80, CD163, IL-10 and IFN-γ were used to assess macrophage polarisation, as they are reported receptors and cytokines involved in macrophage polarisation. The objective of using a xeno-free and chemically defined medium was to avoid discrepancies potentially occasioned by in-

house preparations, such as differences in FBS batches and the complexity of fine-tuning cytokine concentrations and periods of incubation.

To investigate whether cells were expressing the markers as expected, hMDMs were cultured with PromoCell phenotype-specific medium and analysed using cytometry. Figure 3.4 shows cytometric analysis of hMDMs from three donors. IL-10 and IFN-γ had low expression in the first assessed donor (Figure 3.4.A), suggesting that maybe samples were not properly permeabilised. Because of this first result, cells from the two subsequent donors were permeabilised using the Miltenyi kit InsideStain, to test whether the first outcome resulted from actual lack of expression or that the cells were simply not successfully permeabilised with Triton-X-100. High fluorescence displayed by isotype control samples, for both IL-10 and IFN-γ antibodies, suggested that there was a high degree of unspecific binding (Figure 3.4 B); due to this result, IL-10 and IFN-γ were not used as markers for subsequent experiments.

Data obtained through cytometry shows that though CD80 was generally more highly expressed in M1-like hMDMs and CD163 was more highly expressed in M2-like hMDMs (as evidenced in two donors), there was a high degree of variability of expression between different donors, as evidenced by the fact that CD163 was robustly expressed by M1-like cells in one of the donors, but in other donor, this marker was barely expressed by M2-like cells, which are the cells expected to express this receptor. The results from these three first samples of hMDMs demonstrated that chemically defined medium was not enabling consistent outcomes regarding CD80 and CD163 expression. The percentages are summarised in Table 3.2.

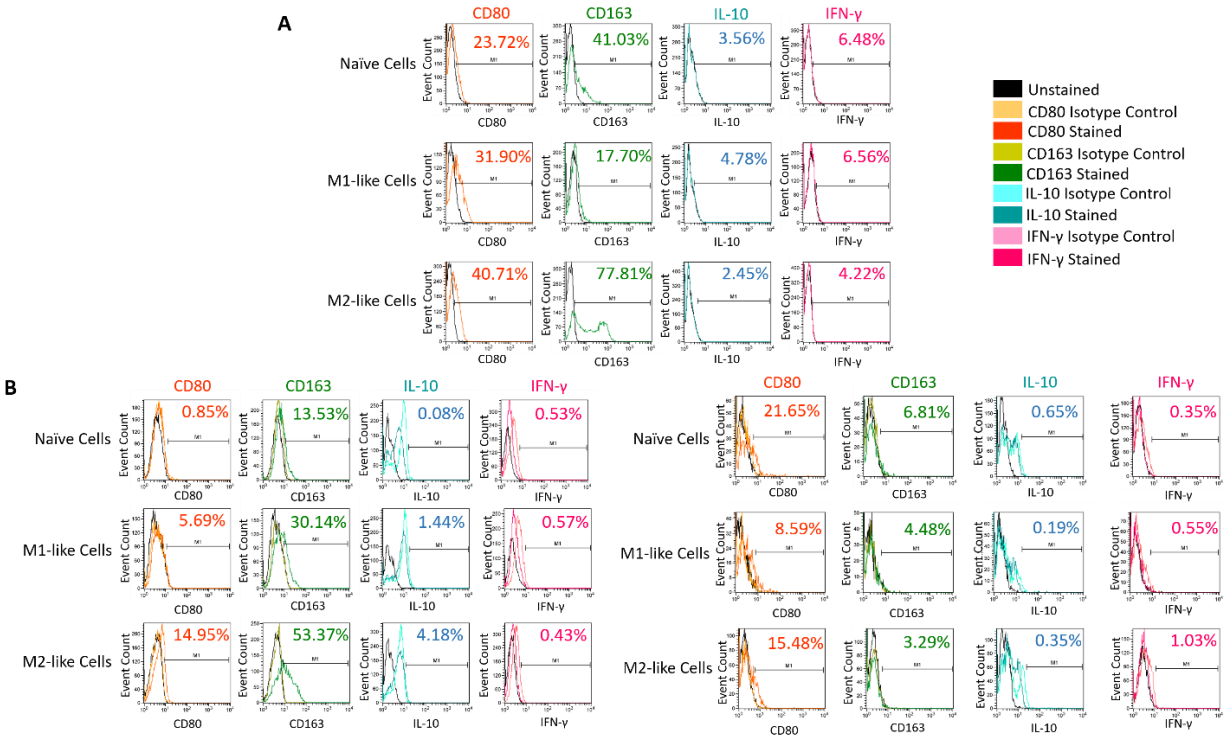

Figure 3. 4. Expression of CD80, CD163, IL-10 and IFN-γ in hMDMs cultured with ready-to-use medium.

A. PBMCs were isolated from a buffy coat and cultured with ready-to-use medium from PromoCell, according to the desired phenotype. Cells were stained according to manufacturer's instructions. For intracellular markers IL-10 and IFN-γ, cells were not permeabilized with the recommended kit from Miltenyi. Isotype Controls were not used. **B.** PBMCs were isolated and cultured with PromoCell medium. As the expression of IL-10 and IFN-γ was negligible in the sample shown in A, the two subsequent samples displayed in B were permeabilized using InsideStain kit from Miltenyi and isotype controls were added to the analysis. For surface markers CD80 and CD163, isotype control histograms fairly overlaid unstained sample histograms. Nevertheless, histograms for intracellular markers indicate high levels of unspecific binding, as isotype controls displayed considerable fluorescence. Surface markers CD80 and CD163 presented great variability between samples, even using chemically defined cell culture medium. Percentages indicate number of positive events according to how many samples fell within the determined M1 interval.

Table 3. 2. Expression of CD80, CD163, IL-10 and IFN-γ by hMDMs – Summary of percentages in Figure 3.4.

	CD80	CD163	IL-10	IFN-γ
Naïve	23.72%	41.03%	3.56%	6.48%
	0.85%	13.53%	0.08%	0.53%
	21.65%	6.81%	0.65%	0.35%
M1-like	31.90%	17.70%	4.78%	6.56%
	5.69%	30.14%	1.44%	0.57%
	8.59%	4.48%	0.19%	0.55%
M2-like	40.71%	77.81%	2.45%	4.22%
	14.75%	53.37%	4.18%	0.43%
	15.48%	3.29%	0.35%	1.03%

Due to the lack of consistency in terms of surface marker expression, it was necessary to test a different approach for hMDM culture and polarisation. Instead of ready-to-use medium, hMDMs were cultured with IMDM 10% FBS and medium was supplemented with either 20 ng/ml of GM-CSF for M1 polarisation or M-CSF for naïve cells and M2 polarisation. M-CSF was used in naïve cells so as to provide a stimulus for adherence and differentiation into macrophages. After 8 days in culture, cells were polarised with 20 ng/ml of IFN-g1b or IL-4 for M1 and M2 polarisation, respectively. During polarisation, cells were still exposed to either GM-CSF or M-CSF at the same concentration used for monocyte maturation. This was performed in one donor so as to assess CD80 and CD163 expression and if necessary change the protocol accordingly (Figure 3.5).

Results show the relatively high expression of CD163 in naïve cells and weak expression of CD80 in all phenotypes analysed, including M1-like cells that should be markedly expressing CD80. Due to the high expression of CD163, M-CSF was not added anymore into the culture of naïve cells, in an attempt to reduce CD163 expression in non-polarised cells. Therefore, naïve cells were subsequently cultured only with IMDM 10% FBS. Also, as a consequence of the weak expression of CD80, the period of culture before polarisation was extended from 8 to 10 days, so as to give cells additional time to differentiate, especially M1-like cells with GM-CSF.

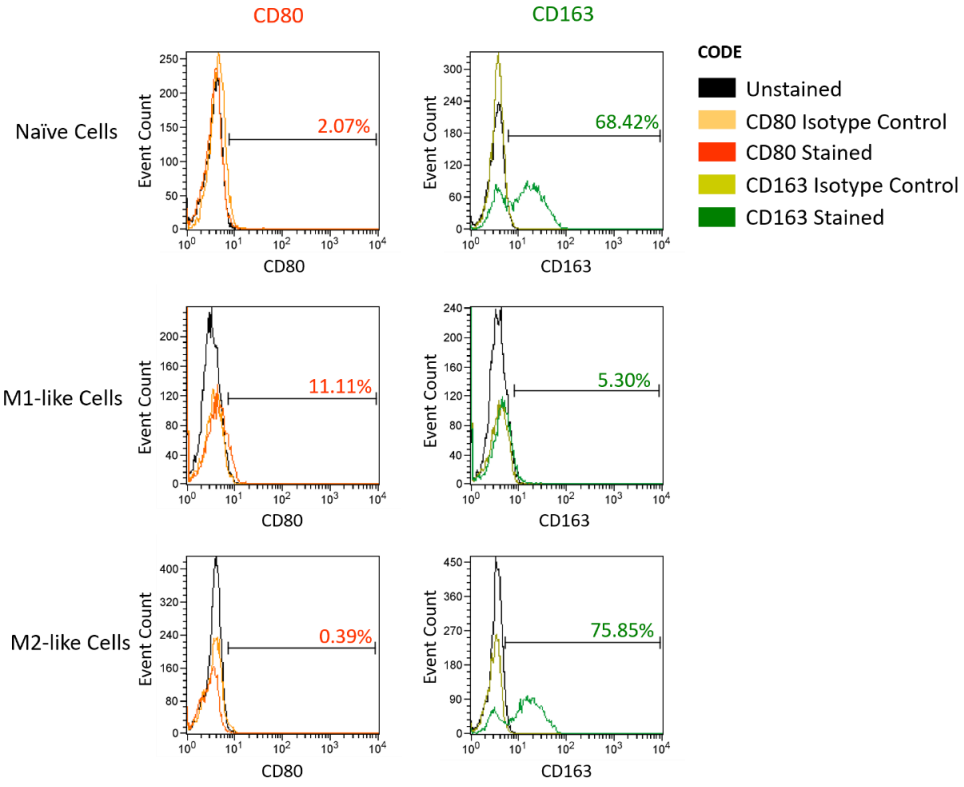

Figure 3. 5. Expression of CD80, CD163, IL-10 and IFN-γ in hMDMs cultured with IMDM medium and stimulated with M-CSF or GM-CSF.

Monocytes were cultured using IMDM medium supplemented with 20ng/ml of GM-CSF or M-CSF for M1-like and M2-like polarisation, respectively. Naïve cells were initially cultured with M-CSF so as to promote maturation from monocytes into macrophages. M1-like cells were activated during 24 hours with IFN-g1b and M2-like cells were activated during 48 hours with IL-4, both at the concentration of 20 ng/ml. Percentages indicate number of positive events for the determined fluorochrome within the M1 interval.

3.3.2. Evaluation of cell responsiveness to LPS

As CD80 expression was not prominent, an additional step to ensure M1-like cells in particular were polarised was to use standard LPS as an inflammatory stimulus. Following differentiation and polarisation, cells were exposed to LPS at a concentration of 100 ng/ml for 24 hours. Again, this protocol was tested in only one donor so as to assess the response and change the protocol accordingly. Figure 3.6 displays the histograms indicating a shift in the expression of CD80 after 24 hours exposure to LPS, demonstrating an increase in expression in all phenotypes analysed. Even though CD80 expression by M1-like cells is not high, these cells are expressing this marker more than their naïve and M2-like counterparts. Not as robust, but still noticeable, is the increase in expression of CD163 after LPS exposure, suggesting that LPS has the effect of maximising the phenotype, even if it is an anti-inflammatory one.

This result was not conclusive as to determine whether cells were responsive to LPS as expected. Therefore, different time-points were tested to further investigate hMDM responsiveness to LPS.

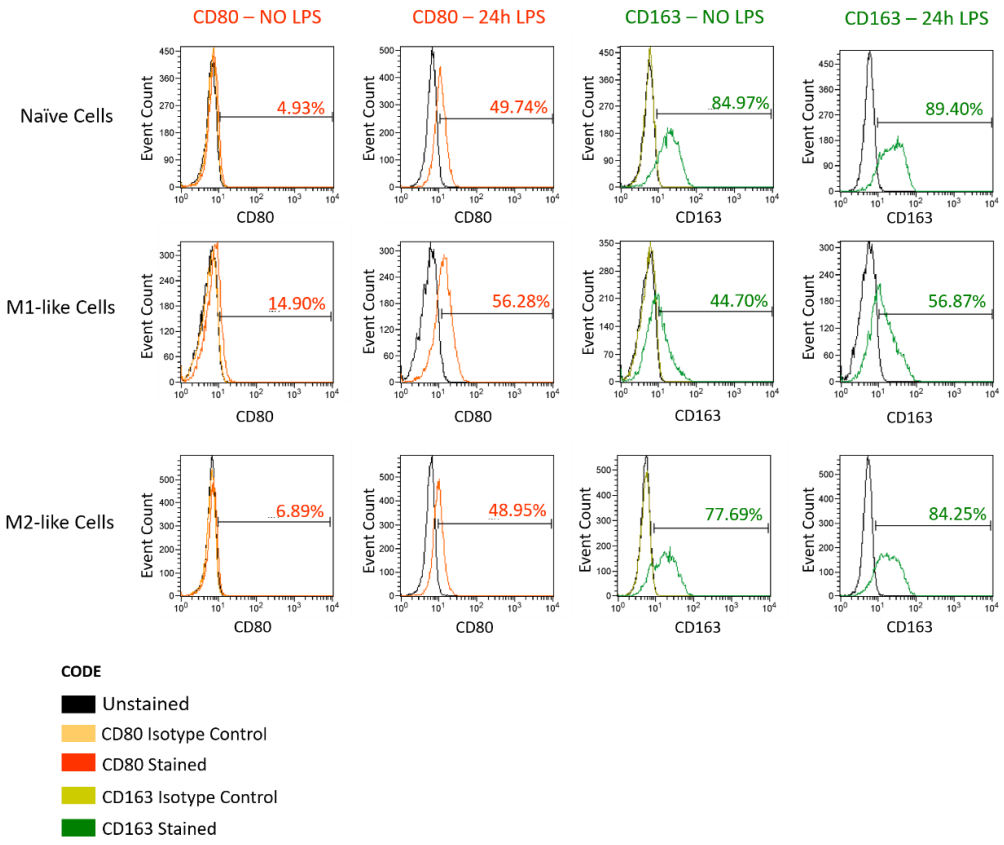

Figure 3. 6. Assessment of the effect of standard LPS on the expression of CD80 and CD163 in hMDMs.

Cells were incubated during 24 hours with Standard LPS from E. coli (Invivogen) at the concentration of 100 ng/ml and assessed by cytometry regarding the expression of CD80 and CD163. Cells exposed to LPS had increased expression of CD80 and CD163 for all phenotypes analysed. Percentages represent number of events within M1 interval.

hMDMs were exposed to 100 ng/ml of standard LPS for consecutive periods of 12 hours. This was to evaluate whether response to LPS was time-dependent, but also to determine which time points were the most suitable for experiments with EVs. Figure 3.7. shows the morphology of hMDMs after polarisation and exposure to LPS. The 12-hour time-point appears to be changing morphology in all phenotypes, leading to a predominance of cells with a “fried-egg” shape, which is not seen with longer exposures. LPS also appears to increase adherence of naïve cells, and this effect can be seen particularly on the 24- and 36-hour time-points.

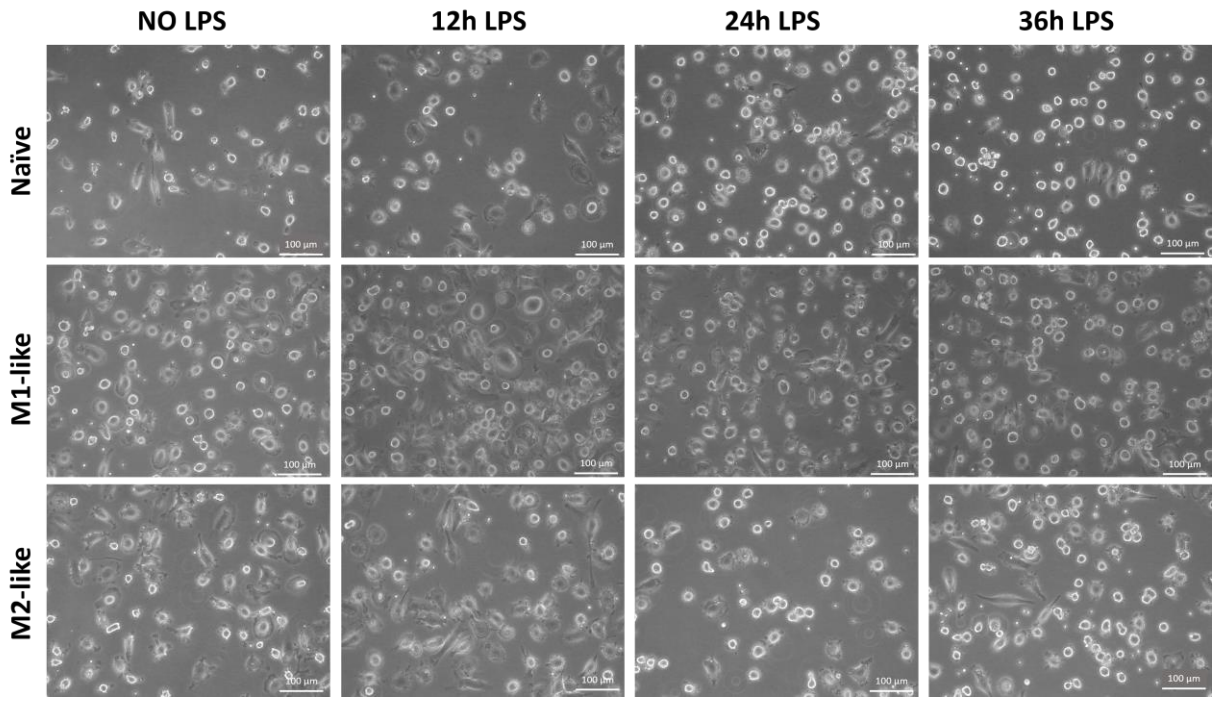

Figure 3. 7. Morphology of hMDMs upon exposure to standard LPS for consecutive periods of 12 hours.

hMDMs were polarised and incubated with 100 ng/ml of Standard LPS. Scale bar is 100 µm.

To investigate the effects of LPS on expression of CD80 and CD163, cells were harvested and analysed using cytometry. CD80 expression was not as high as expected in M1-like hMDMs, as shown in Figure 3.8. Stimulation with LPS substantially increased expression of CD80 in all phenotypes and indeed showed an element of time-dependence.

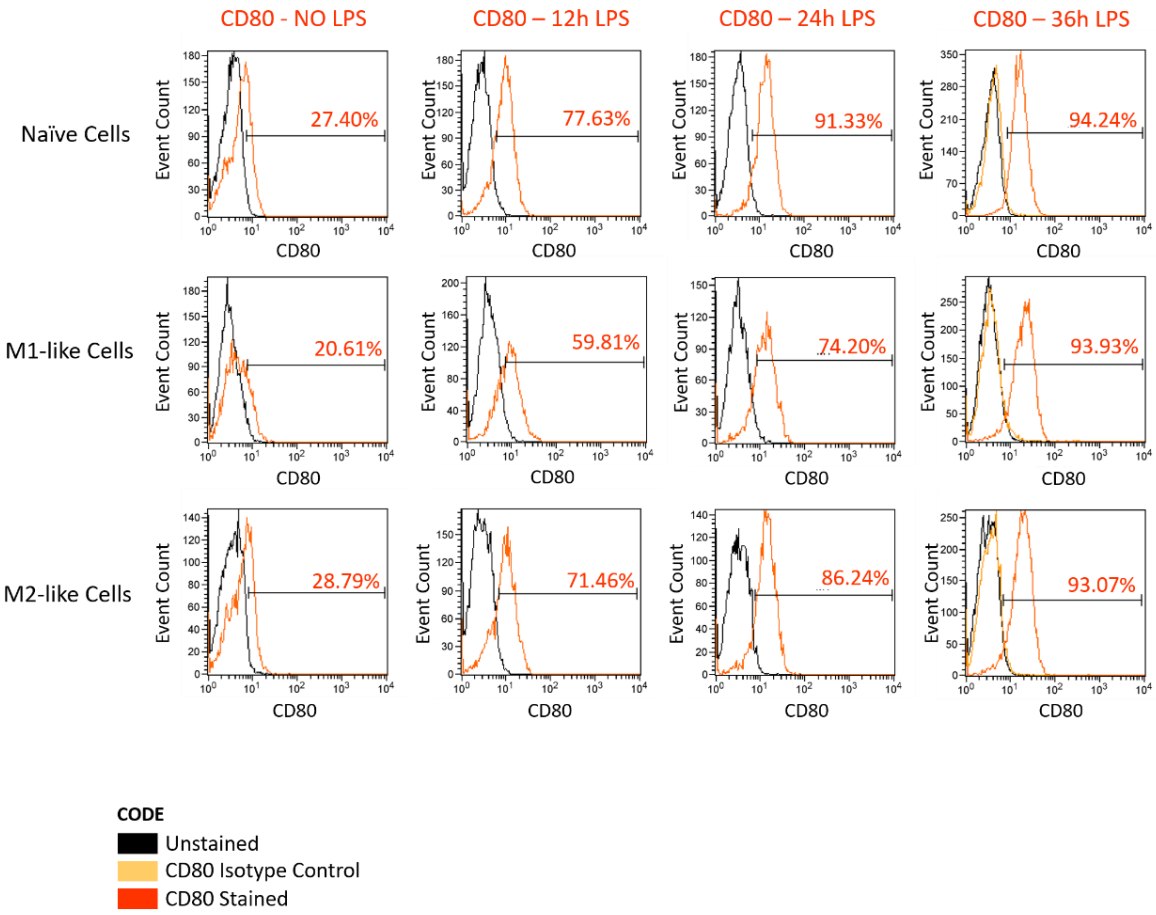

Figure 3. 8. Assessment of the effects of standard LPS on hMDM expression of CD80.

hMDMs were polarised with either IFN-g1b or IL-4 and exposed to standard LPS (Invivogen) at the concentration of 100 ng/ml for 12, 24 and 36 hours. Statistical tests were not performed as only one sample from a single donor was assessed. Isotype controls were only used at the 36-hour time point to verify unspecific staining. Percentages indicate proportion of positive events for CD80.

CD163 expression was impacted by LPS, but not following a clear pattern (Figure 3.9). 36 hours of LPS exposure appears to be the time point in which CD163 expression is uniformly increased across phenotypes, but shorter exposures generally did not have substantial impact.

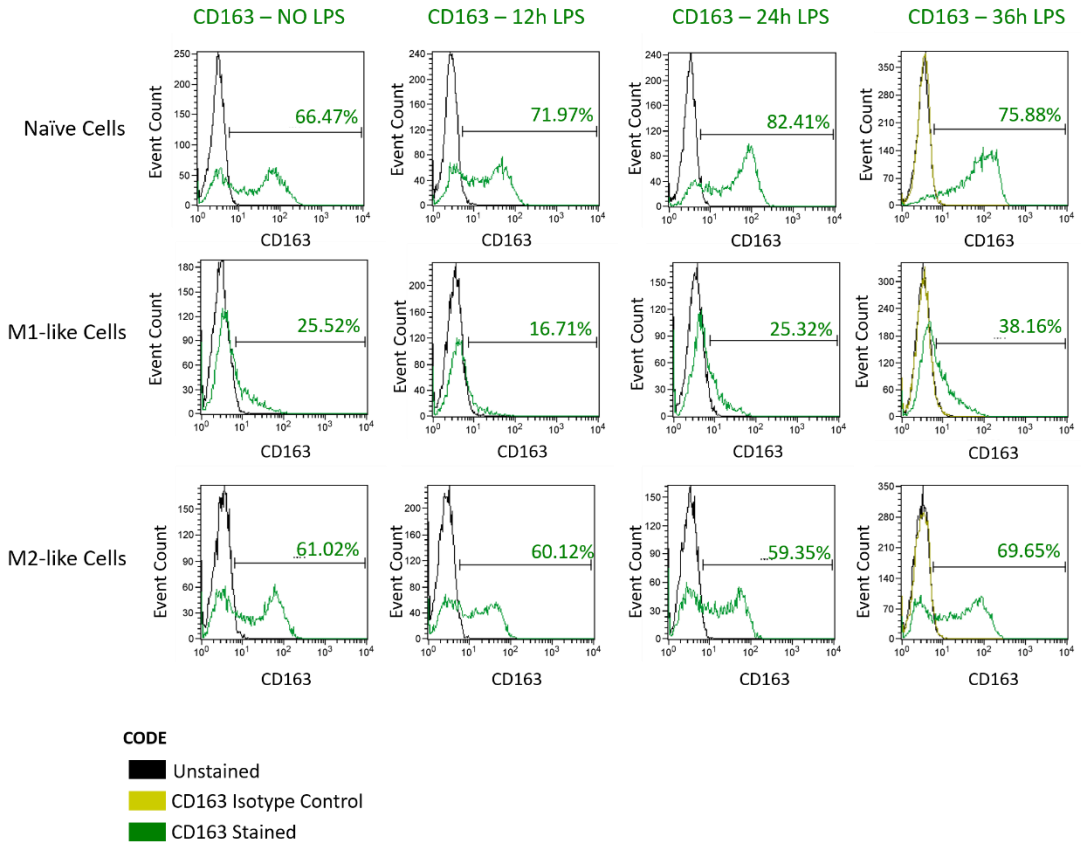

Figure 3. 9. Assessment of the effects of standard LPS on the expression of CD163 by hMDMs.

hMDMs were polarised with either IFN-g1b or IL-4 and exposed to standard LPS (Invivogen) at the concentration of 100 ng/ml for 12, 24 and 36 hours. Statistical tests were not performed as only one sample from a single donor was assessed. Isotype controls were only used at the 36-hour time point to verify unspecific staining. Percentages indicate proportion of positive events for CD163.

Results of experiments performed with LPS showed that CD80 expression is substantially increased upon stimulation, but effects on CD163 remained inconclusive. To test whether these results were linked to the fact that standard LPS can activate both Toll-like receptors 2 and 4, the same experiment was repeated using Ultrapure LPS, which can only activate Toll-like receptors 4 (TLR-4), in three different donors, as Figure 3.10 shows. For the first time, CD14 was used as a monocytic marker, mainly to ensure that naïve cells were still retaining monocytic characteristics.

The effect in CD80 expression was consistently time dependent, as in the experiment with standard LPS and the effect on CD163 expression, despite the variability, showed an opposite pattern of time dependence for M1- and M2-like cells, with expression decreasing with longer exposure times. Expression of CD14 was increased in naïve cells after 24 hours of LPS exposure. Isotype controls were used for all time-points.

Considering only the 0-hour time-point, it is possible to notice that M1-like cells are expressing more CD80 in comparison to other phenotypes, as expected. Also, CD163 expression without LPS is predominant in naïve and M2-like cells. CD14 is robustly expressed by all phenotypes.

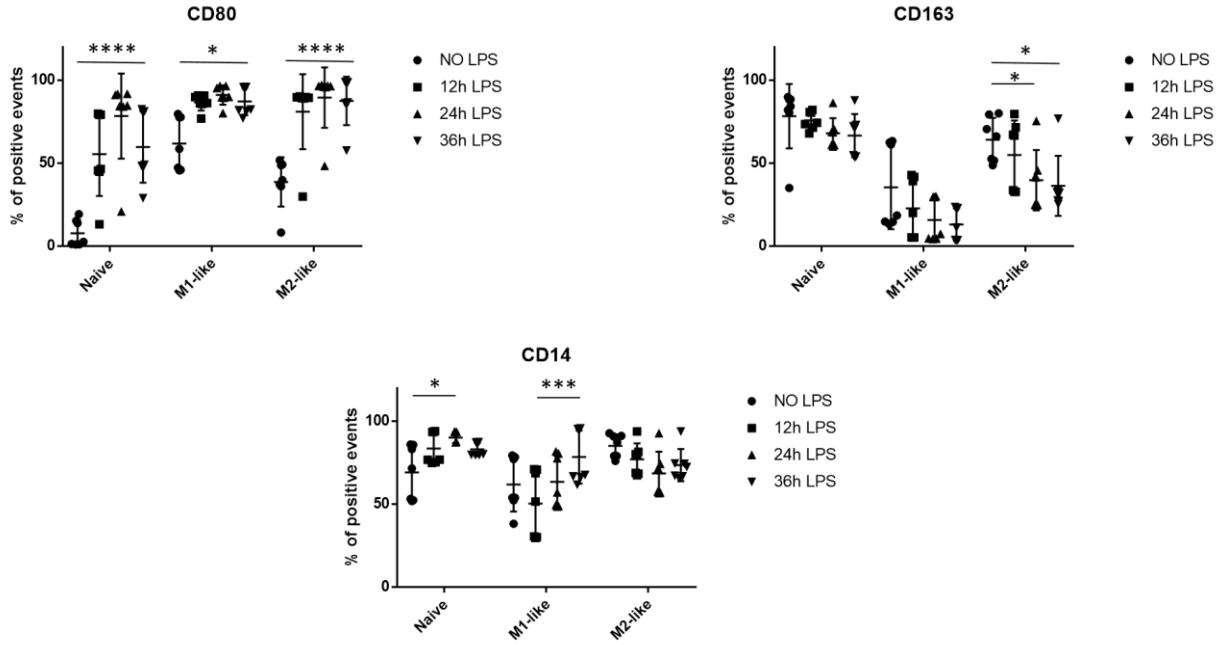

Figure 3. 10. Assessment of the effects of ultrapure LPS on the expression of CD14, CD80 and CD163 by hMDMs.

hMDMs from three donors were polarised and tested regarding the expression of CD14, CD80 and CD163. Measurements were taken in duplicates, except for one donor, therefore the five measurements for each condition as displayed. Kruskal-Wallis test followed by Dunn's were performed. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Lastly, to investigate whether ultrapure LPS would impact cytokine secretion, a bioluminex assay was performed using the supernatants of cells previously assessed for CD14, CD80 and CD163 expression. IL-1beta and IL-10 were the only cytokines that had detectable levels. Figure 3.10 shows that exposure to Ultrapure LPS increased the secretion of IL-10 in naïve and M2-like MDMs, not following a time dependant pattern, but again suggesting that exposure to LPS can maximise the phenotypes, even a non-inflammatory one. There were no significant changes in IL-1beta secretion.

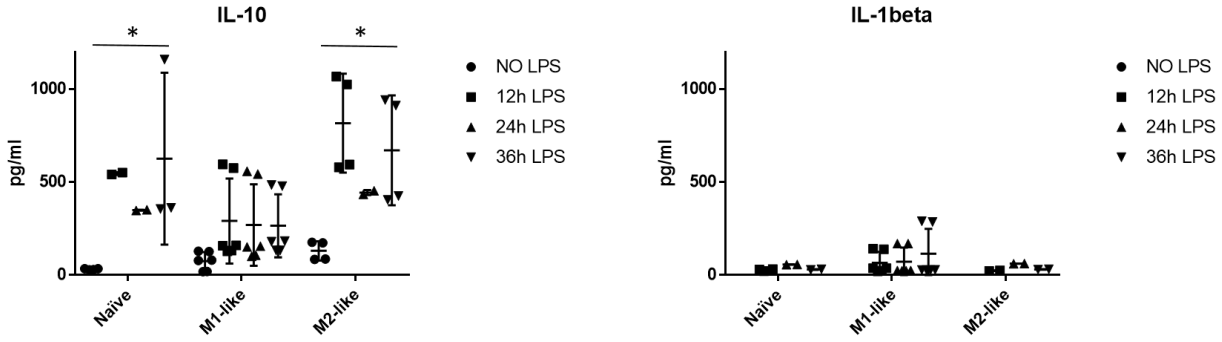

Figure 3. 11. Secretion of IL-10 and IL-1beta by hMDMs exposed to ultrapure LPS for 12, 24 and 36 hours.

Luminex assay performed with supernatant of hMDMs from 3 donors. The number of measurements per condition varies as some measurements were not within the range of the standard curve and needed to be excluded. Kruskal-Wallis test followed by Dunn's were performed. *$p<0.05$.

3.4. Discussion

3.4.1 Monocyte differentiation into macrophages

There is a variety of protocols in order to isolate peripheral blood mononuclear cells (PBMCs) from buffy coats or non-processed peripheral blood. Many groups use Ficoll-Paque [31-35] or alternative density gradient media, such as Ficoll-Hypaque or Histopaque [36, 37]. The method used in this study was based on plastic adherence and negative selection, and it is important to state that these methods can provide fundamentally different cells with different phenotypes in comparison to $CD14^{positive}$ selection [38]. A low level of contamination with platelets and lymphocytes is expected and buffy coats can vary in several aspects, including haemolysis, which impacts on how clear and delimited the PBMC layer will turn after centrifugation with Ficoll-Paque and consequently the purity of the cells seeded. However, even in the face of contamination with platelets, negative selection and plastic adherence should provide a final sample majorly comprised of monocyte-derived macrophages displaying different phenotypes on the spectrum. Furthermore, the use of cytokines also contributes to uniformity, as the cytokines used were specific for macrophage differentiation and activation.

Periods of differentiation from monocyte to macrophage also vary, ranging from 5 to more than 10 days, depending on the combination of cytokines used for macrophage differentiation and following polarisation, making it difficult to standardise monocyte culture [39-41]. Initially, monocytes were cultured for only 8 days prior to the start of the process of activation/polarisation with either IFN-g1b for M1 polarisation or IL-4 for M2 polarisation, so that the total of days in culture would coincide with the initial PromoCell protocol. Nevertheless, M1-

like hMDMs were not robustly expressing CD80 in flow cytometry analysis and naïve cells were very similar to M2-like macrophages in CD163 expression. As a strategy to increase CD80 expression and ensure that macrophages were fully mature, the protocol was extended so that activation would start on Day 10, instead of Day 8, and the days of media changing would be exactly the same as in the protocol with the ready-to-use media.

There is evidence that monocytes can in fact spontaneously differentiate into macrophages without the need for supplemented differentiation factors [42, 43]. Notwithstanding, if the purpose of the experiment is to generate polarised macrophages, it is necessary to stimulate cells with a specific set of cytokines. There are differences in concentrations of cytokines, cytokines used to obtain each phenotype, including polarisation with normal human serum instead of individual cytokines, and also the period of culture from monocytes to fully differentiated monocyte-derived macrophages [12, 31, 44-46]. Such diversity contributes to the difficulty in assessing hMDMs and also compromises the comparison of studies employing different culture procedures.

It is widely accepted that exposure to IL-4 and IFN-γ will prime hMDMs towards M2 and M1 phenotypes, respectively, alongside other cytokines and factors which are also used to maximise the potential of polarisation. For M2 polarisation it could be mentioned the use of IL-10, IL-23 and M-CSF; for M1 polarisation, besides IFN-γ, it is usual to perform stimulation with Lipopolysaccharide (LPS) and GM-CSF [47-49]. Variations in concentrations of cytokines and periods of exposure would be expected to influence the phenotype of the cells.

Regardless of the cytokines used, *in vitro* conditions do not provide a complete resemblance of the behaviour of immune cells *in vivo*. Even *in vivo* studies have demonstrated that macrophage polarisation is not as simplistic as proposed, as will be discussed in more detail in Chapter 4.

For the purposes of this study, it was imperative to demonstrate that monocytes were successfully differentiated into macrophages and were responsive to stimuli. Results obtained from cells and supernatant give strong evidence that these cells successfully matured into macrophages and were robustly responsive to LPS. Whether the phenotypes obtained resemble macrophage phenotypes in kidney injury pathophysiology *in vivo* remains to be investigated.

3.4.2 Macrophage polarisation

The classification proposed by Mantovani is the most widely used in basic research and states that macrophages can be either classically activated (M1) or alternatively activated (M2), representing two extremes of a spectrum of polarisation [47]. This classification was used in the wake of T_H1 – T_H2 dichotomy, proposed by Coffman and colleagues [50]. M1 macrophages are inflammatory and release mainly interleukin (IL) 12 and IL-23, which promotes a T_H1 response, as opposed to M2 macrophages, which are anti-inflammatory and have increased expression of pattern recognition receptors [6].

This classification is useful to explain contrasting functions of macrophages in injury and repair, but there is increasing evidence that macrophages are much more complex than two opposing phenotypes acting in reverse directions. As

the aim of this study was not to deeply investigate all phenotypes potentially displayed by macrophages, concentrations of cytokines and reagents were not extensively compared. It would be important that regardless of the phenotype, cells would have the plasticity of switching from an activated state to another, and there is evidence that macrophages *in vitro* have this ability [51, 52].

3.4.3 Markers used to assess macrophage differentiation and polarisation

As a highly heterogeneous type of immune cells, macrophage expression of surface receptors and cytosolic proteins is conditional to the state of activation and also the microenvironment in which they find themselves in. There is an ongoing discussion in the literature regarding whether some markers used in murine *in vitro* and *in vivo* models can be extrapolated to human macrophages, such as arginase and nitric oxide synthase (NOS) [53]. Therefore, markers used for this investigation should be reported to be expressed specifically by human monocyte-derived macrophages.

Macrophages can respond differently to LPS depending on their phenotype, but it is expected that such a strong pro-inflammatory stimulus will elicit a measurable response on phagocytes. For this study it was of uttermost importance to choose markers which would enable assessment of polarisation, and results from cytometry indicate that cells are expressing markers and responding to LPS as expected, even though sometimes without statistical difference.

The initial design for the panel of markers was to have both intracellular and surface markers, as to provide a broader range of investigated proteins. However, most “intracellular” markers for macrophages are actually secreted and, therefore, not suitable for cytometric analysis. Hence the need for cytokine secretion assays. Surface markers were chosen with the objective of providing evidence that cells had either pro- or anti-inflammatory properties; ideally naïve cells would not strongly express any polarisation marker, as they would resemble unstimulated monocytes.

CD163 is a haptoglobin-haemoglobin scavenger receptor with one single transmembrane domain expressed only on the monocytic-macrophage lineage [54]. Although it has been reported that IL-4 down-regulates the expression of CD163, this down-regulation is mostly reported in the context of dendritic cell maturation [16]. Moreover, the fact that naïve cells in this study robustly expressed CD163 to similar levels of M2-like cells confirms that IL-4, at the concentrations used here, does not reduce CD163 expression. This receptor has been widely recognised as an anti-inflammatory marker for macrophages.

CD80, on the other hand, has been identified in several studies using human samples as overexpressed on inflammatory macrophages and is considered to be a prototypical M1 marker [55]. As a co-stimulatory receptor on the surface of antigen presenting cells, CD80 has some overlapping roles with CD86 given that both receptors can bind to CD28 on the surface of T cells [56]. This receptor is expressed on the surface of dendritic cells, macrophages and B cells, but can also be found on non-immune cells, such as endothelial cells upon inflammatory stimuli [57], indicating its upregulation on

inflammatory settings. CD80 favours maintenance of a T_H1-driven environment and it is linked to development and exacerbation of inflammatory conditions [58, 59], including renal injury, by facilitating accumulation of T_H1 effectors and macrophages [60].

CD14 was used not only as a monocyte marker, but in the context of LPS stimulation, as it is an endotoxin cell surface receptor particularly expressed on human peripheral blood monocytes [61], that binds the complex LPS-LBP, inducing the production of TNF-α [62]. LPS is expressed on Gram-negative bacteria walls and is one of the most potent inflammatory stimuli, classically used for monocyte and macrophage activation [63], and used in this study to provide the inflammatory environment encountered at the site of early kidney injury.

These markers showed robust expression during optimisation phase and were efficient in displaying differences between M1- and M2-like phenotypes, suggesting that culture conditions were successful in polarising cells. The next phase of the project is the assessment of EV effects on polarised hMDMs.

3.5. Conclusion

Monocytes could be differentiated into macrophages and successfully polarised, as assessed by expression of CD14, CD80 and CD163. Cell morphology also changed according to the phenotypes, indicating that cells had the expected differences in activation. Responsiveness to LPS, particularly ultrapure LPS, showed that hMDMs were activated and surface

marker expression was a reliable parameter to investigate macrophage polarisation.

3.6. References

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Chapter 4 – Effects of EVs and hUCMSCs on monocyte-derived macrophages

4.1. Introduction

MSCs have been used in a panoply of renal injury models and clinical trials and have been reported as enablers of tissue amelioration [1-13]. The precise mechanisms remain to be elucidated, but given the prominent role of immune cells in kidney homeostasis it is reasonable to infer that they are involved in repair. As EVs are regarded as a means for cellular communication, and macrophages are among the first responders of immune cells in the renal injury context, this chapter will focus on the assessment of the potential effects of EVs derived from MSCs on human monocyte-derived macrophages (hMDMs) as a step to investigating the therapeutic mechanisms of MSCs in kidney injury amelioration.

4.1.1. Immune modulation by MSCs and EVs – potential mechanisms

As mentioned in previous chapters, inflammation plays a critical and detrimental role in kidney injury, promoting persistent fibrosis and consequent function loss [14-16]. The precise mechanisms by which MSCs might elicit immunomodulation are not yet fully understood. MSCs are able to supress T cell proliferation [17, 18] and induce the formation of regulatory T cells [19], mainly through release of soluble factors [20]. The mainstream evidence-based assumption is that MSC immunosuppression is major histocompatibility complex (MHC)-independent, non-antigen specific, and can generate cell

cycle arrest in distant immune cells [21-24]. Therefore, the mechanisms of immunomodulation could be theoretically assessed *in vitro*, using direct and indirect co-incubation of MSCs and immune cells. Whether this direct immune activation is different from the paracrine-mediated one remains to be elucidated.

Both umbilical cord and bone marrow are reported sources of MSCs for kidney injury treatment [25, 26], with evidence that umbilical cord cells might have a more potent anti-inflammatory effect in comparison to their bone-marrow counterparts, as they were more effective in reducing macrophage infiltration and inducing polarisation towards the M2 profile [27]. Hence the predominant use of umbilical cord MSCs in this study.

More recently it has been demonstrated that MSCs might undergo apoptosis and be phagocytosed by immune cells, and that would be the means by which the stromal cells would confer change in the phenotype of immune cells [28]. However, another potential mechanism is that extracellular vesicles, including apoptotic bodies, could be effectors by signalling through their cargo to distant cells and eliciting tissue regeneration. This is the mechanism predominantly evaluated in this study.

MSCs immunomodulatory potential has been assessed, including by co-culture with macrophages [29-31], which was attempted in this study. Moreover, there is endorsement that EVs impact shifts in macrophage polarisation [32-34], and promote elevated cytokine storms that modulate immune responses [35]; these reports corroborate that MSC-derived EVs are

good candidates to promote immunomodulatory effects, including in human cells [32].

The immunomodulatory potential of MSCs was evaluated on hMDMs and assessed mainly according to change in expression of CD14, CD80 and CD163. LPS was used as an inflammatory stimulus, following preliminary data showing responsiveness from 12 hours of exposure.

Beyond surface marker changes, another question regarding macrophage modulation by EVs to be addressed is whether these vesicles would need to be uptaken to elicit effects. Although EVs are reported to have immunomodulatory properties [32], the precise mechanisms are not entirely known. Attempts on labelling EVs for microscopy and/or cytometry investigation have been made, but the small size of the vesicles poses a challenge for individual labelling. The use of PKH has been reported as an effective method, but there is evidence that the dye can be incorporated by cell membranes and other lipidic entities [36]. DiD, another fluorescent lipidic dye, has also been reported as a reliable alternative for EV labelling [37, 38], usually added at one of the ultracentrifugation rounds to minimise the contamination of sample with free dye. Carboxyfluorescein succinimidyl ester (CFSE) is an advantageous option, as its use to label EVs has been shown to be specific [39] and more reliable than PKH-labelling [40]. In this study, CFSE was used for its ability to easily label cells and potentially indirectly label EVs. This approach was used to assess whether EVs were uptaken by hMDMs.

Finally, this study also investigated the angiogenic potential of EVs as part of their functional characterisation. Angiogenic effects of MSC-derived EVs have

been extensively reported [41-44], showing that they promote endothelial migration and proliferation. This front of investigation would confirm the potency of EVs.

4.1.2. Hypothesis

Preliminary studies have showed that MSCs do not act through engraftment and differentiation. We hypothesised that their derived EVs could be potential effectors of tissue regeneration, as for example in the context of kidney injury by shifting macrophage phenotype towards an anti-inflammatory profile that would benefit tubular epithelial cell proliferation and angiogenesis.

4.1.3. Aims

Following the sequence of experiments determined at the beginning of the study, the results displayed in this chapter refer to the assessment of EVs on hMDMs (Figure 4.1). The original plan was to investigate EV cargo but, due to the lack of effect on macrophage surface markers, the effects of EVs on endothelial cells were assessed instead, as a means to confirm that EVs were functional. Therefore, the aims were as follows:

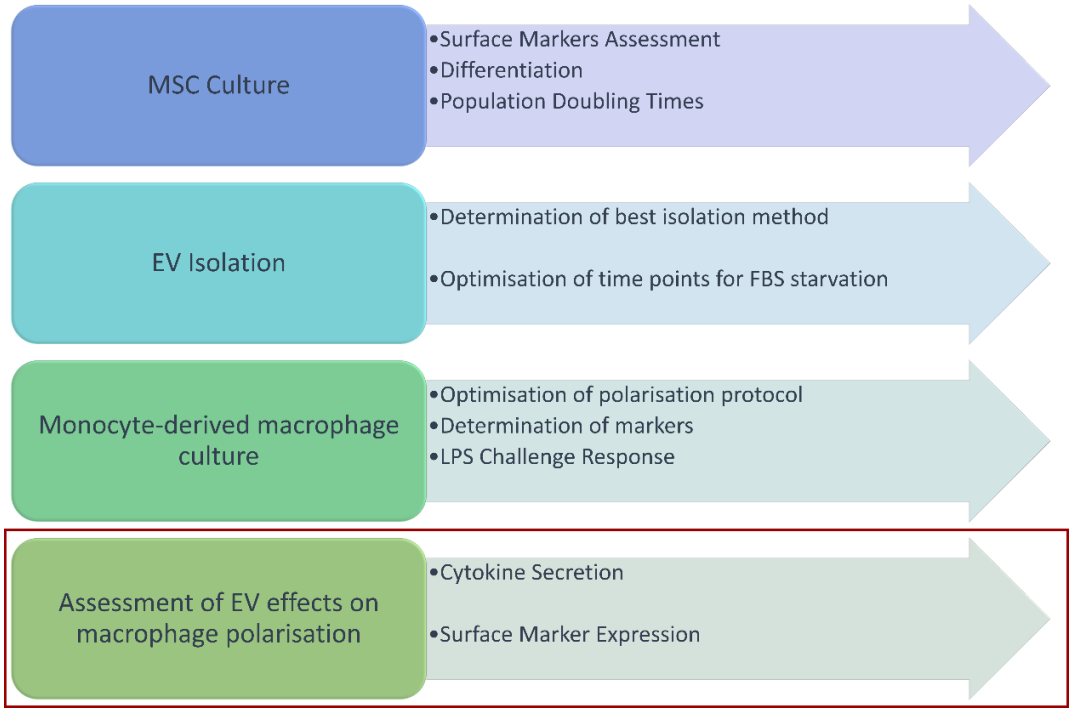


Figure 4. 1. Schematic diagram of stages in the study.

The results on this chapter deal with the final stage of the study, emphasised by the red rectangle which is the evaluation of extracellular vesicles effects on hMDMs.

i. To determine the effects of hUCMSC-derived EVs on the expression of CD14, CD80 and CD163 in hMDMs. As previous evaluation confirmed the suitability of these markers in the context of macrophage polarisation, the effects of EVs would be measured in light of changes in the expression of these markers.

ii. To determine the effects of hUCMSC-derived EVs on cytokine secretion in hMDMs. Secretion of cytokines reported to be secreted by macrophages was evaluated, as changes in secretion are linked to polarisation shift.

iii. To compare the effect of hUCMSC-derived EVs on macrophage polarisation with that of hBMMSCs. Investigation of whether EVs derived from a different source of MSCs would elicit the same effects.

iv. To compare the immunomodulatory effects of EVs obtained from hUCMSCs following different periods of serum starvation. This comparison was performed due to the possibility that the period of serum starvation could seriously impact MSC metabolism and consequently the populations of released EVs.

v. To compare the immunomodulatory effects of indirect incubation of hUCMSCs and hMDMs. Indirect incubation with cells was to investigate the effects of soluble factors on hMDM polarisation.

vi. To optimise a reliable EV labelling method for uptake assessment. In order to determine whether EVs were being phagocytosed by macrophages an indirect labelling method was tested and hMDMs incubated with labelled EVs were assessed by microscopy and cytometry.

vii. To determine the effects of EVs on endothelial cells. An additional testing system was set with endothelial cells to test EV potency, as EVs are reported to have angiogenic potential.

4.2. Material and Methods

4.2.1. Co-incubation of hMDMs and EVs

hMDMs were obtained through the maturation of peripheral blood monocytes following the optimised protocol for macrophage maturation and polarisation. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats

(NHSBT) using Ficol-Paque Plus (GE) and cultured with medium enriched with either GM-CSF or M-CSF for M1- and M2-like maturation, respectively. Polarisation was performed with IFN-g1b or IL-4. Naïve cells were cultured only with IMDM supplemented with 10% FBS.

EVs were isolated from both umbilical cord and bone marrow mesenchymal stromal cells after 72 hours of serum deprivation. Briefly, MSCs were cultured until 80% confluent, washed three times with PBS and deprived of FBS with non-supplemented MEM-α medium for 72h. Supernatant was collected and centrifuged at 500 x g for 5 minutes for debris removal, subsequently at 2000 x g for 10 minutes to remove dead cells and ultracentrifuged once or twice at 100,000 x g for 2 hours at 4°C. EVs were first assessed by NTA to determine size and concentration and were further characterised via the expression of CD9, CD63 and CD81 using flow cytometry with the aid of sulphate/aldehyde latex beads 4µm.

For the co-incubation assays, EVs were diluted in the culture medium and directly co-cultured with cells at concentration of 1 x 10^8 vesicles per ml. During co-incubation periods, culture medium was supplemented with exosome-depleted FBS (Systems Biosciences, cat. Number EXO-FBS-250A-1) in order to avoid potentially misleading results originated from bovine exosomes present on FBS. Colour-coded plots were obtained using Python (script provided in Appendix, Figure A.4).

4.2.2. Indirect Co-culture - Transwell Assays

Tissue culture treated 6 well plates containing 24mm Transwell inserts with a 0.4µm polyester membrane (Costar, cat. number 3450) were used for indirect

co-culture of hMDMs and hUCMSCs. Monocytes were isolated from buffy coats, seeded on the wells and differentiated into macrophages, as previously described. Polarisation was performed with IFN-g1b and IL-4 for M1- and M2-like activation, respectively. Throughout the maturation and polarisation of macrophages the inserts remained empty, only containing a fraction of the medium from the wells that would pass through the membrane. hUCMSCs were cultured separately until confluence using MEM-α 10% FBS.

Following hMDM polarisation, hUCMSCs were seeded at 250,000 cells per insert and medium of both chambers was changed to IMDM 10% exosome-depleted FBS. Cells were co-cultured for 72 hours, which was the same time point used for EV isolation, after which MDMs were harvested, stained and analysed through flow cytometry for investigation of CD14, CD80 and CD163 expression.

4.2.3. CFSE-labelling of mesenchymal stromal cells

CFSE was purchased from Abcam (cat. number ab113853) and used according to the manufacturer's instructions. Concentrations of 1, 2.5, 5 and 10 μM were tested so as to determine the optimal dose to enable EV fluorescence. The concentration of 5 μM was deemed the best to dye cells without causing toxicity or cell detachment.

Confluent hUCMSCs were washed with PBS and incubated with 5 μM of CFSE diluted in PBS for 10 minutes at 37°C. The dye was then aspirated and dishes were washed three times with PBS and deprived of FBS for 72 hours as standard for EV isolation in this study (Chapter 2). Supernatant was collected and centrifuged at 500 x g and 2000 x g and then ultracentrifuged twice at

100,000 x g. These vesicles were analysed through flow cytometry and compared to control vesicles derived from non-labelled cells. They were also co-stained with CD81-APC in order to confirm their vesicular nature. 50,000 events were acquired for the analysis.

Co-incubation of CFSE-EVs and hMDMs was performed after macrophage differentiation and polarisation and lasted for 24 hours. The time point of 24 hours was to provide more time for uptake and maximise the chances of cytometric detection of fluorescent hMDMs. hMDMs were stained with CD14, given that this marker was expressed at variable levels by all phenotypes in previous experiments.

4.2.4. Normal Human Dermal Fibroblast (NHDF) and Human Microvascular Endothelial Cell (HDMEC) Co-Culture *in vitro* Angiogenesis Assay

NHDF-cryopreserved (cat. number C-12300) and HDMEC-cryopreserved (cat. number C-12210), were cultured using Fibroblast Growth Medium 3 (cat. number C23130) and Endothelial Cell Full Growth Medium MV2 (cat. number C-39221), respectively, all purchased from Promocell. Fibroblasts were cultured for more than 5 passages before the start of the experiment, as to ensure they were under *in vitro* culture conditions for several passages and with stable characteristics.

Assays were performed on 24-well plates and every condition was analysed in duplicate. Sterile 30 mm coverslips were placed on each well and coated with 0.5% gelatin for at least 15 minutes. NHDFs were plated at a seeding density of 20,000 cells/well and cultured for 3 days with 1 ml of Fibroblast

Growth Medium (Figure 4.2). On Day 4, medium was aspirated and cells were washed with PBS. HDMECs were plated on top of fibroblasts at a seeding density of 30,000 cells/well and incubated with 0.5 ml of Endothelial Full Growth Medium. Medium was aspirated the following day and replaced with fresh Endothelial Growth Medium MV2 (Promocell, cat. number C-22221) without growth factors and containing only 1% of FBS plus Recombinant Human Vascular Endothelial Growth Factor-165 (VEGF-165 – Peprotech, cat. Number 100-20) and the EVs to be tested. On the third day after this initial incubation, medium was refreshed with a fresh dose of EVs for an additional 48 hours.

After 10 days in culture, counting from the day fibroblasts were initially plated, medium was aspirated, cells were washed twice with PBS and fixed for 15 minutes at room temperature with 2% paraformaldehyde. Cells were permeabilised with 0.2% Triton X-100 for 10 minutes, blocked with 0.1% Tween-20, 1% BSA and 5% donkey serum. Primary antibodies used were monoclonal Mouse anti-CD31 (Dako, cat. number M0823) and monoclonal Rabbit anti-VE-Cadherin (Cell Signaling, cat. number 2500S), both diluted 1:400 in Tris-Buffered Saline 0.1% Tween-20 (TBST) containing 1% BSA. Incubation with primary antibodies was 90 minutes at room temperature.

Secondary antibodies used were Alexa Fluor 488 Donkey anti-rabbit and Alexa Fluor 568 Donkey anti-mouse, diluted 1:1000 in TBST 1% BSA. Incubation was for 90 minutes at room temperature in the dark. After incubation ended, cells were washed twice with TBST. Coverslips were transferred to glass slides and mounted on ProLong Gold Antifade Mountant with DAPI (Invitrogen, cat. number P36935) and left to settle on the slides

overnight at room temperature in the dark. After nail varnish sealing, samples were analysed using fluorescence microscopy.

Images obtained from coverslips were analysed using AngioTool v 0.6a (02.18.14) and the main parameters considered were vessel percentage area and number of junctions. Analysis was performed with images obtained at 50x magnification.

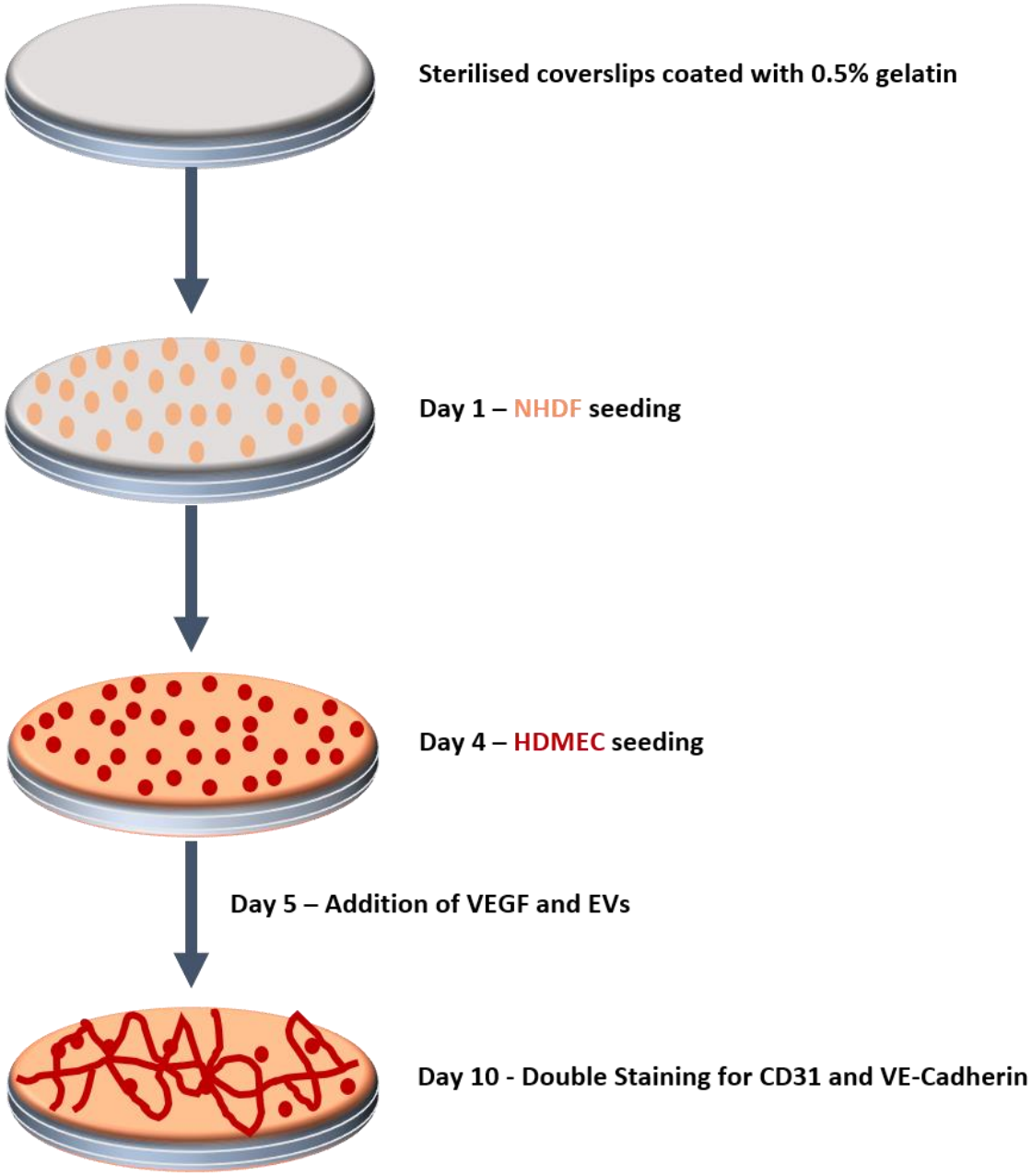


Figure 4. 2. Schematic representation of *in vitro* angiogenesis assay.

NHDFs were plated on gelatinised coverslips on Day 1 and cultured for 72 hours. By Day 4 there was a confluent monolayer and HDMECs were seeded on top of fibroblasts. VEGF and EVs were added on Day 5 and medium was changed on Day 8. The experiment ended on day 10 and cells were double stained with CD31 and VE-Cadherin for investigation of tube formation.

4.3. Results

4.3.1. Determination of the effects of hUCMSC-derived EVs on the expression of CD14, CD80 and CD163 on hMDMs

To investigate if the EVs had any obvious effect on the morphology of the monocytes or macrophages, naïve, M1- and M2-like macrophages were incubated in the presence of EVs that had been isolated from the medium of hUCMSCs following 72h of serum starvation. The EVs were isolated by using 1x or 2x ultracentrifugation steps. This was to assess whether the differences in tetraspanin expression between EVs centrifuged once or twice, as showed in Chapter 2, would impact macrophage polarisation.

Following 72 hours of culture, cells were imaged under the microscope (Figure 4.3). Differences in the number of centrifugations used for EV isolation do not appear to impact macrophage morphology. M1-like cells are usually more adherent to plastic, bigger and more circular in comparison to the other phenotypes. As a consequence, they are usually in greater number, particularly in comparison to naïve cells, which have less stimulus to adhere, as they are not cultured with M- or GM-CSF and are not activated with any cytokine.

Naïve cells and M2-like macrophages incubated with EVs seem to change their morphology, as both appear to acquire a more circular morphology, resembling a fried-egg, as described in the literature [45].

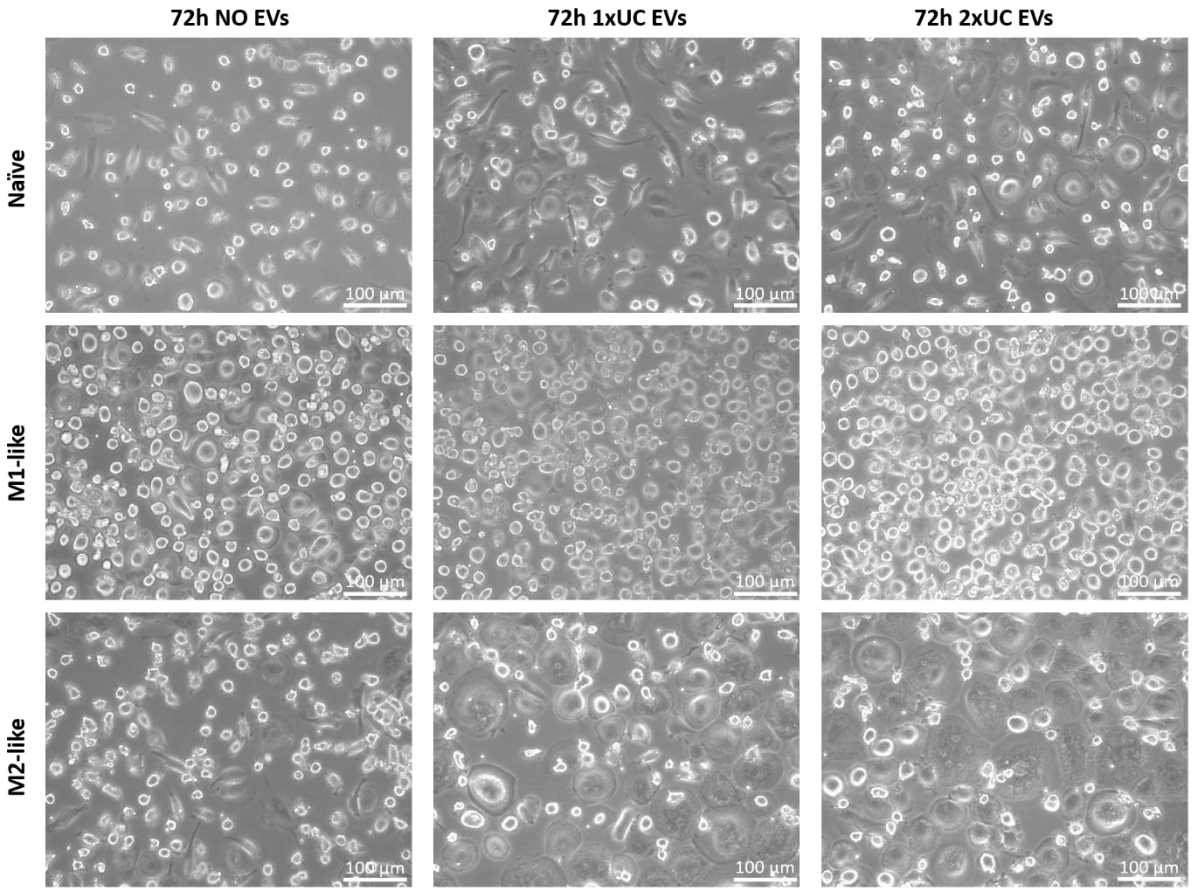


Figure 4. 3. Morphology of hMDMs on 72-hour experiments with hUCMSC-derived EVs.

hMDMs were polarised and incubated with 1 x 10^8 hUCMSC-derived EVs/ml for 72 hours. Scale bar is 100µm.

LPS was used in this study as an inflammatory stimulus. During the optimisation phase of macrophage culture, its use was to verify cell responsiveness, particularly of M1-like macrophages. In the context of EV assessment, LPS was used to mimic the inflammatory environment of the site of kidney injury and whether EVs would be able to impact polarisation even after such a potent stimulus. In Chapter 3, macrophages were exposed to LPS for consecutive periods of 12 hours to assess whether changes in surface marker expression was time-dependent. The first time-point tested, 12 hours, was sufficient to elicit a response, particularly in CD80 expression (Chapter 3,

Figure 3.10). Therefore, the 12-hour time-point was chosen for this stage of the study as a more prolonged exposure to LPS might significantly and irreversibly change cells towards an inflammatory phenotype.

Results showed that LPS occasioned a change in morphology in naïve cells, as they appear to be more elongated, and M2-like macrophages have an apparent reduction in the number of flat cells (Figure 4.4). Again, the number of centrifugations does not have a visible impact on cell morphology.

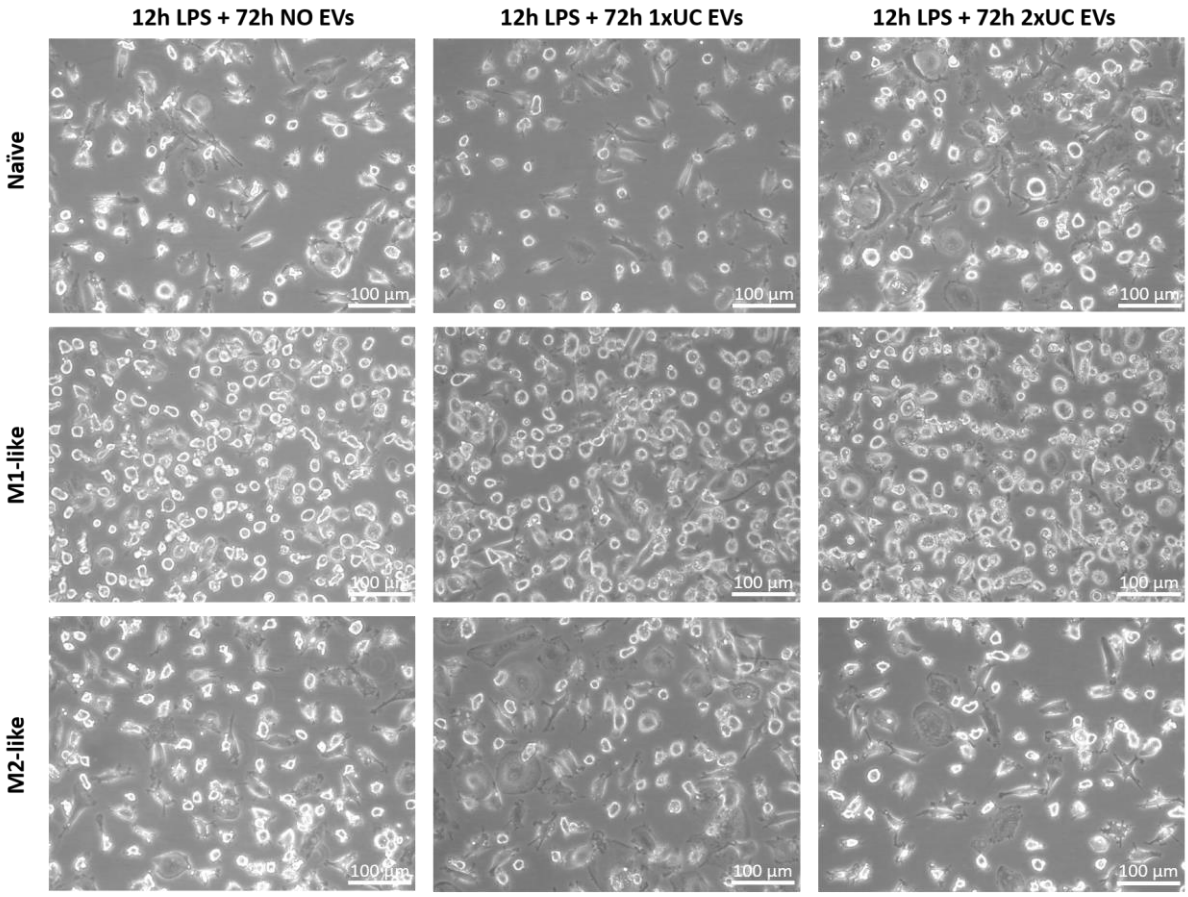
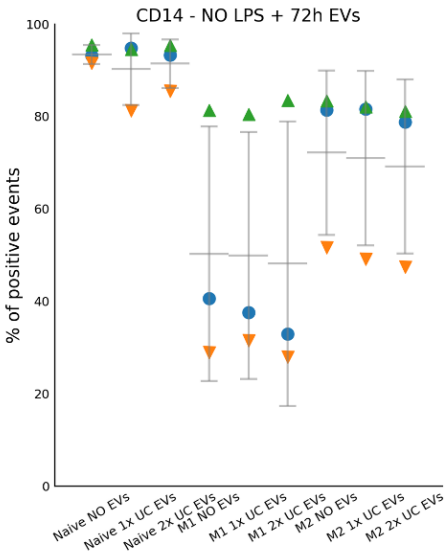


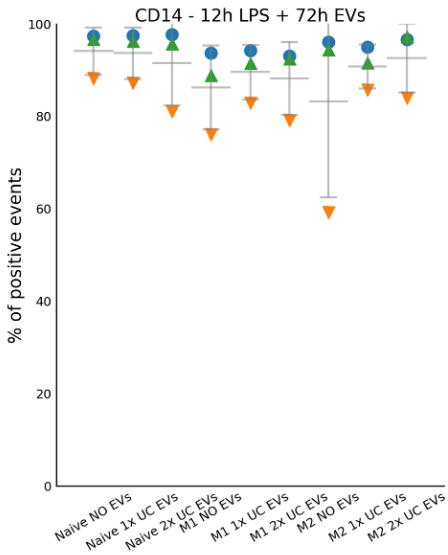
Figure 4. 4. Morphology of 12-hour stimulated LPS hMDMs on 72-hour experiments with hUCMSC-derived EVs.

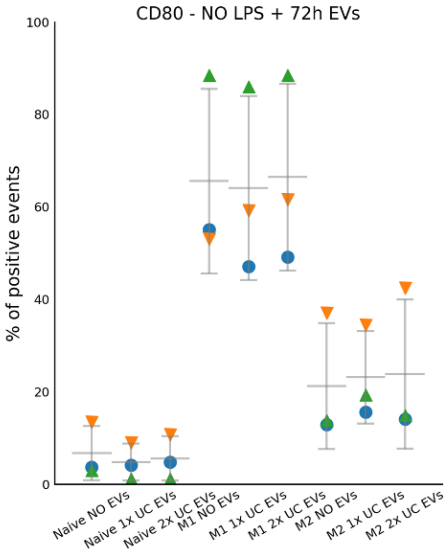
hMDMs were polarised, exposed to 100ng/ml of ultrapure LPS for 12 hours and incubated with 1 x 10^8 hUCMSC-derived EVs/ml for 72 hours. Scale bar is 100µm.

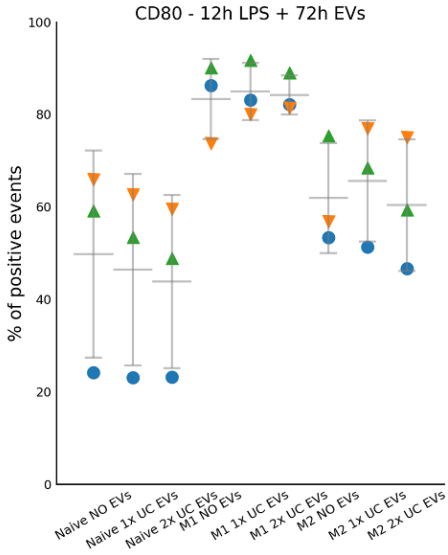
After microscopy, cells were harvested and analysed using cytometry. Exosome-depleted FBS was used during incubation with EVs and cells were gently detached with a cell scrapper after exposure to EDTA to avoid bias in the results.

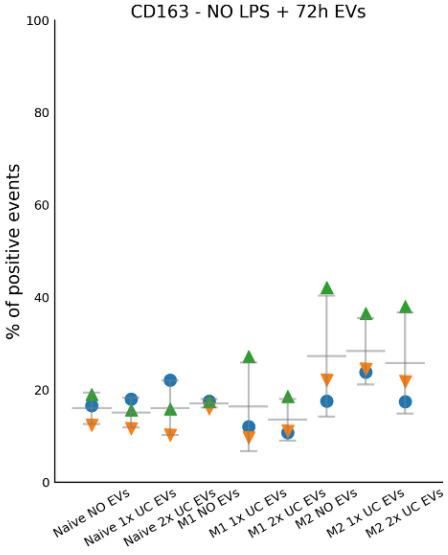
Ultrapure LPS was used in order to provide an inflammatory stimulus, given that in injured kidneys, the environment is predominantly inflammatory at the initial stages of the injury. Both once and twice ultracentrifuged vesicles, referred to as 1 x UC and 2 x UC, were used (Figure 4.4), so as to investigate a potential difference in the effects on hMDMs resultant from EVs expressing different levels of tetraspanins.


CD14 - NO LPS + 72h EVs
% of positive events
0
20
40
60
80
100
Naive NO EVs
Naive 1x UC EVs
Naive 2x UC EVs
M1 NO EVs
M1 1x UC EVs
M1 2x UC EVs
M2 NO EVs
M2 1x UC EVs
M2 2x UC EVs


CD14 - 12h LPS + 72h EVs
% of positive events
0
20
40
60
80
100
Naive NO EVs
Naive 1x UC EVs
Naive 2x UC EVs
M1 NO EVs
M1 1x UC EVs
M1 2x UC EVs
M2 NO EVs
M2 1x UC EVs
M2 2x UC EVs


CD80 - NO LPS + 72h EVs
% of positive events
0
20
40
60
80
100
Naive NO EVs
Naive 1x UC EVs
Naive 2x UC EVs
M1 NO EVs
M1 1x UC EVs
M1 2x UC EVs
M2 NO EVs
M2 1x UC EVs
M2 2x UC EVs


CD80 - 12h LPS + 72h EVs
% of positive events
0
20
40
60
80
100
Naive NO EVs
Naive 1x UC EVs
Naive 2x UC EVs
M1 NO EVs
M1 1x UC EVs
M1 2x UC EVs
M2 NO EVs
M2 1x UC EVs
M2 2x UC EVs


CD163 - NO LPS + 72h EVs
% of positive events
0
20
40
60
80
100
Naive NO EVs
Naive 1x UC EVs
Naive 2x UC EVs
M1 NO EVs
M1 1x UC EVs
M1 2x UC EVs
M2 NO EVs
M2 1x UC EVs
M2 2x UC EVs

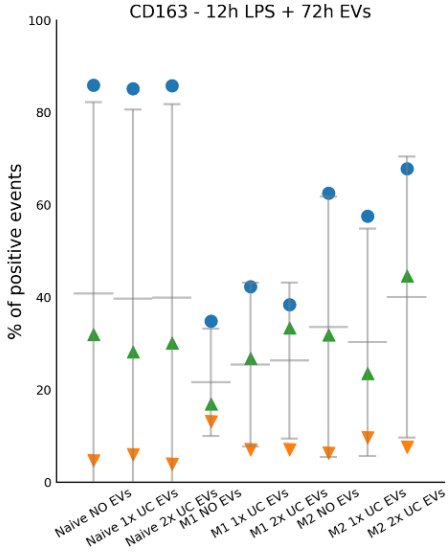

CD163 - 12h LPS + 72h EVs
% of positive events
0
20
40
60
80
100
Naive NO EVs
Naive 1x UC EVs
Naive 2x UC EVs
M1 NO EVs
M1 1x UC EVs
M1 2x UC EVs
M2 NO EVs
M2 1x UC EVs
M2 2x UC EVs

Figure 4. 5. Cytometric analysis of hMDMs incubated with hUCMSC-derived EVs for 72 hours.

Mature and polarised macrophages were incubated with once and twice ultracentrifuged (1x UC/2x UC) EVs for 72 hours. Cells on the LPS group were exposed to ultrapure LPS for 12 hours prior to incubation with vesicles. Each colour represents one donor. Statistical analysis was performed using Kruskal Wallis and Dunn's tests. There was no statistical difference between samples incubated with EVs and controls.

4.3.2. Determination of the effects of hUCMSC-derived EVs on cytokine secretion by hMDMs

A Luminex kit was used to assess cytokine levels on a bioplex assay in order to investigate a potential difference in cytokine secretion of hMDMs due to EV immunomodulation. The assay assessed secretion of IL-1beta, IL-1ra (receptor antagonist), IL-12p70 and IL-10. Only IL-10 concentrations were within the acceptable range determined by the standard curve and only cells exposed to ultrapure LPS for 12 hours had detectable levels. It is possible to notice differences depending on the phenotype of the cells, but there was no statistical change between samples exposed or not to EVs (Figure 4.6).

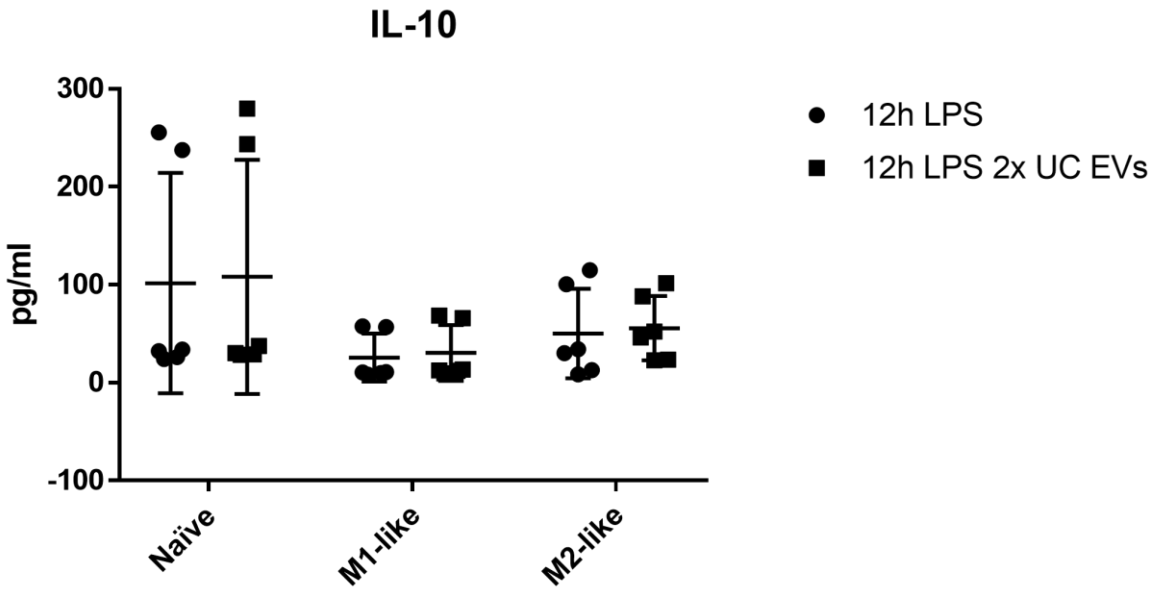


Figure 4. 6. IL-10 secretion on hMDMs exposed to LPS and EVs.

IL-10 secretion was measured through a Bioplex assay. The graph contains data from supernatant collected from 3 different donors analysed in duplicates. Supernatant corresponds to medium collected from cells which were analysed through flow cytometry. Statistical test used was Kruskal Wallis and Dunn's test. No statistical difference was found between samples incubated with vesicles and controls.

4.3.3. Comparison of the effect of hUCMSC-derived EVs on macrophage polarisation with that of hBMMSCs.

EVs were isolated from human bone marrow MSCs (hBMMSCs) following the same protocol used for umbilical cord derived EVs. Only twice ultracentrifuged EVs were used for co-incubation with hMDMs, as in previous experiments there was no significant difference with the once ultracentrifuged EVs and two rounds of ultracentrifugation should provide a higher level of purity in terms of protein contaminants, given that the second ultracentrifugation round is also considered to be a washing step.

Consistent with previous results showing great variability in expression of the chosen markers, it is possible to notice again very discrepant expression

depending on the donor (Figure 4.7, each colour represents one donor). Importantly, M1-like cells tend to express more CD80 and less CD163 in comparison to the other phenotypes. EVs did not elicit any effect on surface marker expression.

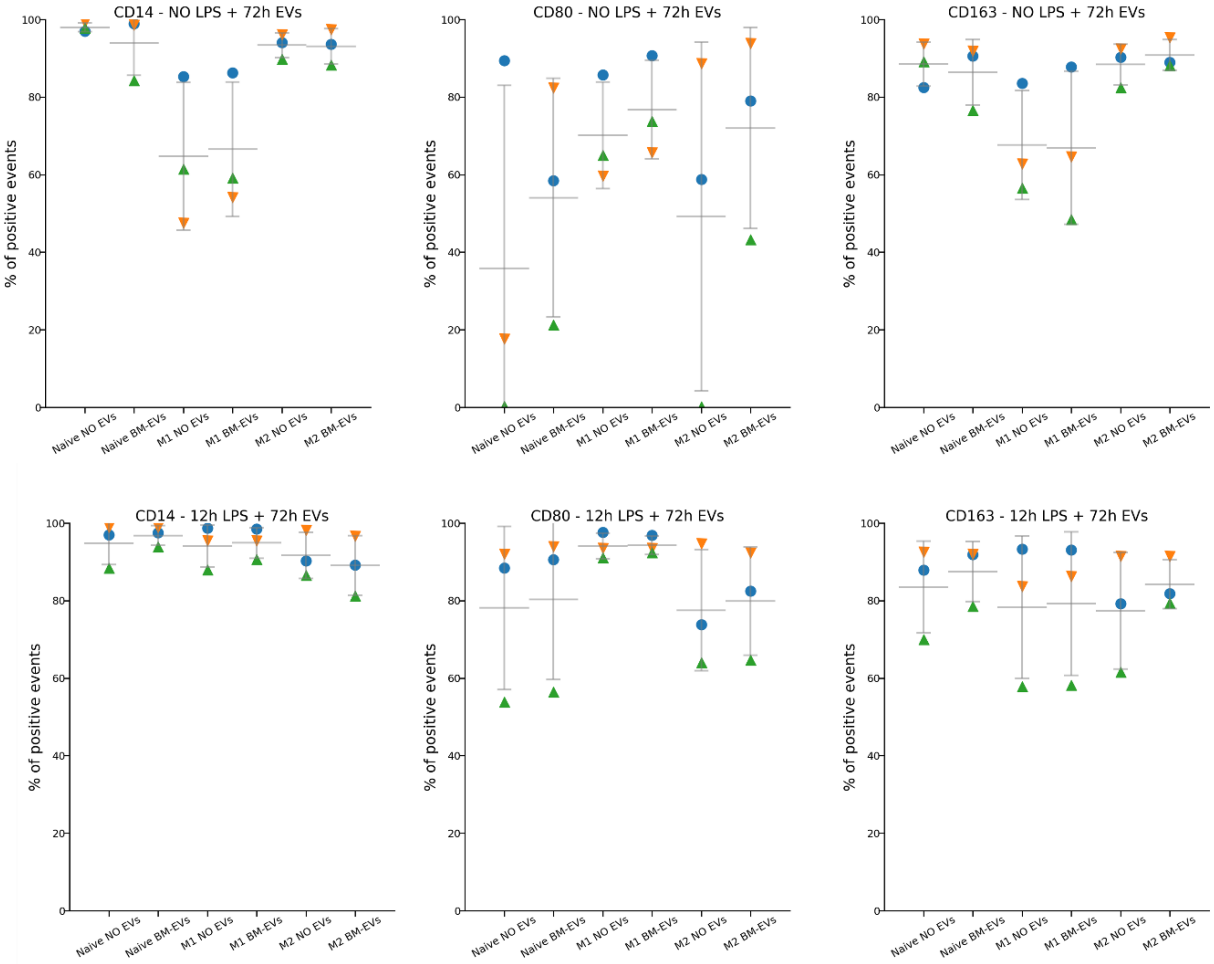


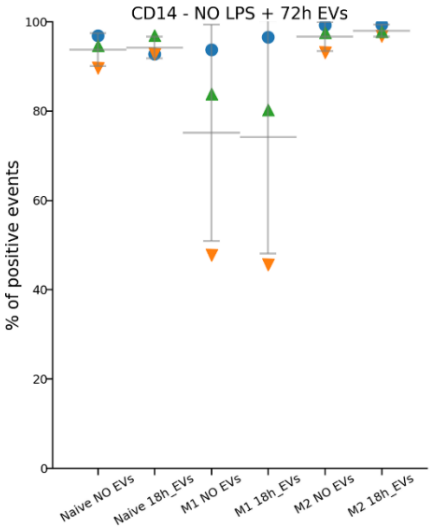
Figure 4. 7. Cytometric analysis of hMDMs incubated with hBMMSC-derived EVs for 72 hours.

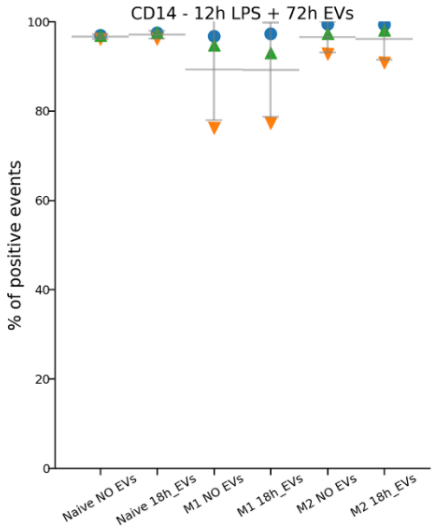
Mature and polarised macrophages were incubated with EVs derived from hBMMSCs for 72 hours. Cells were exposed to ultrapure LPS for 12 hours prior to incubation with vesicles. Each colour represents a different donor. Statistical analysis was performed using Kruskal Wallis and Dunn's tests. There was no statistical difference between samples incubated with EVs and controls.

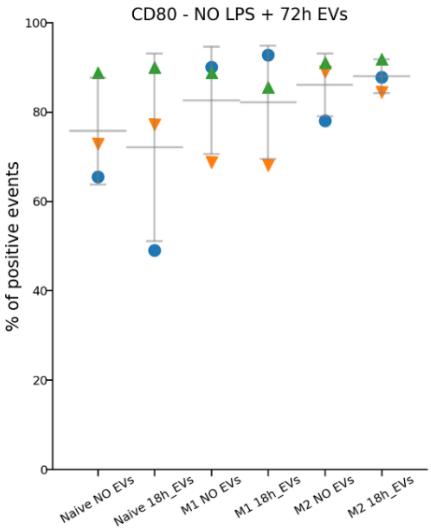
4.3.4. Comparison of the immunomodulatory effects of EVs obtained from hUCMSCs following different periods of serum starvation.

As 72 hours of serum deprivation for EV isolation might raise concerns regarding MSC viability and a potential change in the EV populations obtained, hUCMSCs were deprived from FBS for 18 hours and vesicles were isolated following the same protocol as the EVs obtained after 72 hours of FBS starvation. These EVs are referred to as “18h_EVs”.

hMDMs were exposed to 1 x 10^8 EVs per ml for 72 hours. In the LPS group, cells were stimulated with ultrapure LPS 12 hours prior to incubation with EVs. No difference was found between samples incubated with EVs and controls (Figure 4.8).


CD14 - NO LPS + 72h EVs
% of positive events
0
20
40
60
80
100
Naive NO EVs
Naive 18h_EVs
M1 NO EVs
M1 18h_EVs
M2 NO EVs
M2 18h_EVs


CD14 - 12h LPS + 72h EVs
% of positive events
0
20
40
60
80
100
Naive NO EVs
Naive 18h_EVs
M1 NO EVs
M1 18h_EVs
M2 NO EVs
M2 18h_EVs


CD80 - NO LPS + 72h EVs
% of positive events
0
20
40
60
80
100
Naive NO EVs
Naive 18h_EVs
M1 NO EVs
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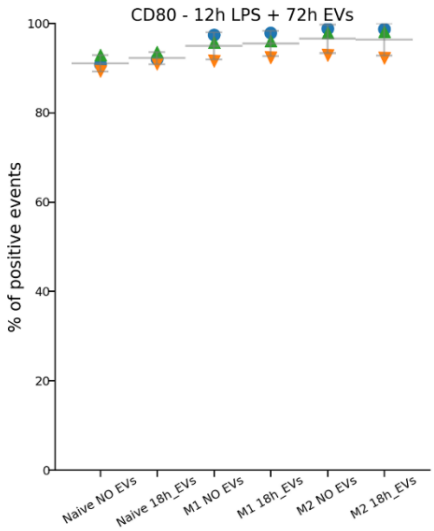
M2 NO EVs
M2 18h_EVs


CD80 - 12h LPS + 72h EVs
% of positive events
0
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80
100
Naive NO EVs
Naive 18h_EVs
M1 NO EVs
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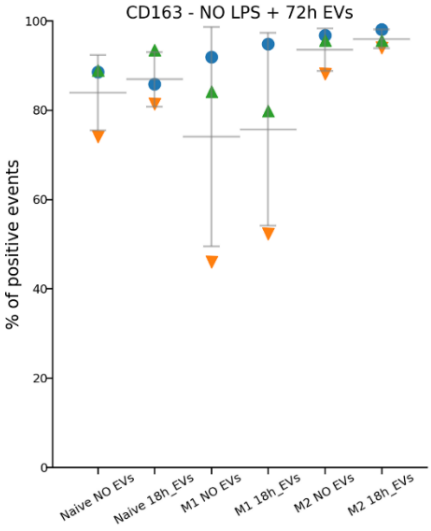
M2 NO EVs
M2 18h_EVs


CD163 - NO LPS + 72h EVs
% of positive events
0
20
40
60
80
100
Naive NO EVs
Naive 18h_EVs
M1 NO EVs
M1 18h_EVs
M2 NO EVs
M2 18h_EVs

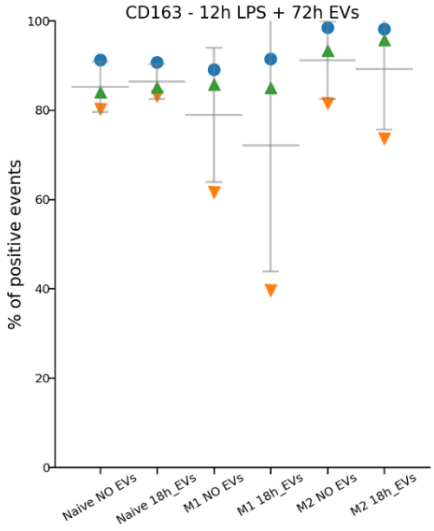

CD163 - 12h LPS + 72h EVs
% of positive events
0
20
40
60
80
100
Naive NO EVs
Naive 18h_EVs
M1 NO EVs
M1 18h_EVs
M2 NO EVs
M2 18h_EVs

Figure 4. 8. Cytometric analysis of hMDMs incubated with 18-hour FBS deprived hUCMSC-derived EVs for 72 hours.

Mature and polarised macrophages were incubated with 1 x 10^8 EVs per ml for 72 hours. Cells on the LPS group were exposed to ultrapure LPS for 12 hours prior to incubation with vesicles. Each colour represents one donor. Statistical analysis was performed using Kruskal Wallis and Dunn's tests. There was no statistical difference between samples incubated with EVs and controls.

Figure 4.9 shows a compilation of all 72-hour experiments performed with MDMs and both hUCMSC- and hBMMSC-derived EVs. Besides the great variability between donors, particularly regarding CD163 expression, it is possible to notice that EVs did not affect expression of CD14, CD80 and CD163 regardless of the protocol used for their isolation.

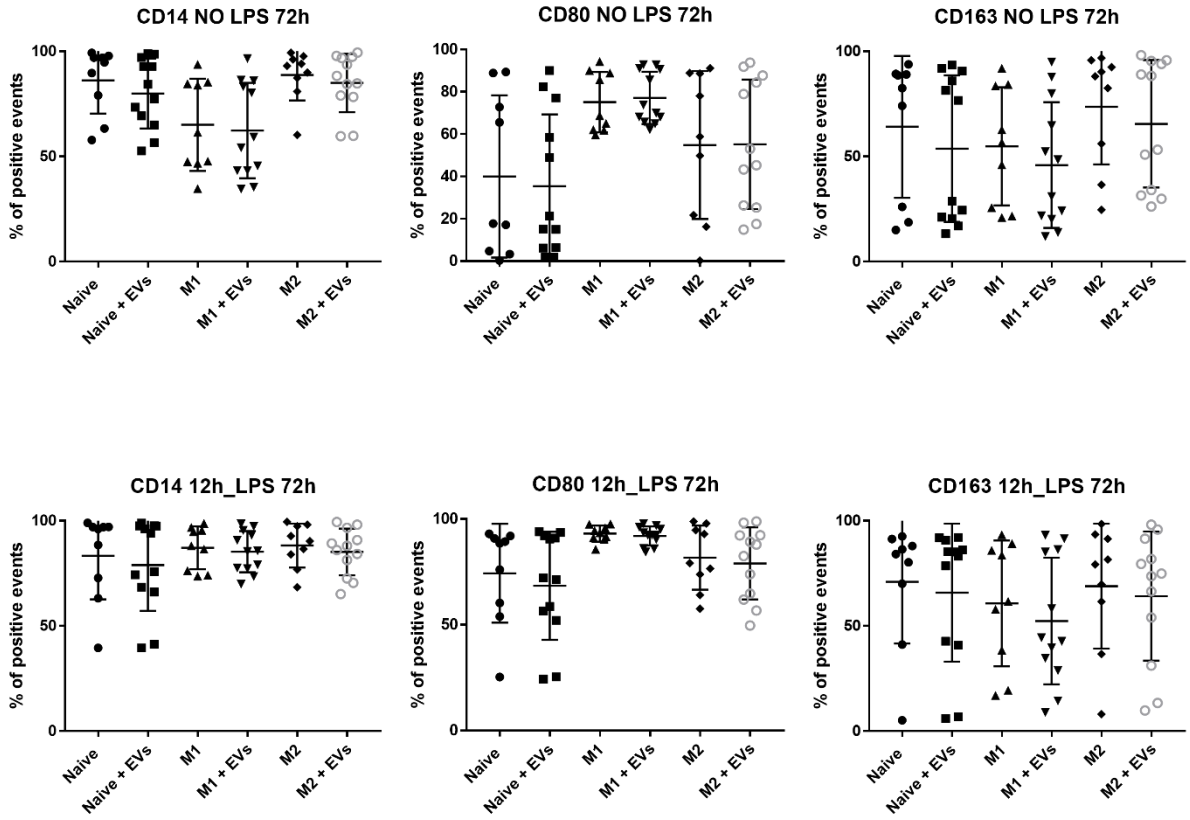


Figure 4. 9. Compilation of cytometric analysis of hMDMs incubated with MSC-derived EVs for 72 hours.

Mature and polarised macrophages were incubated with 1 x 10^8 EVs per ml for 72 hours. Cells on the LPS group were exposed to ultrapure LPS for 12 hours prior to incubation with vesicles. Each point corresponds to one donor. Statistical analysis was performed using Kruskal Wallis and Dunn's tests. There was no statistical difference between samples incubated with EVs and controls.

4.3.5. Comparison of the immunomodulatory effects of indirect incubation of hUCMSCs and hMDMs

Due to the lack of response after exposure to EVs, hMDMs were co-cultured with hUCMSCs during 72 hours using a 6-well plate with Transwell inserts to assess whether EV parent cells would elicit a response. hMDMs were matured and polarised following the same protocol of previous experiments. Once the polarisation was completed, 250,000 hUCMSC in Passages 7-8 were seeded

in the inserts and medium was changed to IMDM 10% exosome-depleted FBS.

Cytofluorimetric analysis showed that hUCMSCs seemed to change surface marker expression on hMDMs (Figure 4.10). M1-like cells had their expression of CD14, CD80 and CD163 increased after incubation with hUCMSCs without LPS, even though it was not statistically significant. In the group exposed to 12 hours of LPS before incubation with hUCMSCs, there was not a clear change, though it appears that LPS had the effect of slightly decreasing expression of the markers.

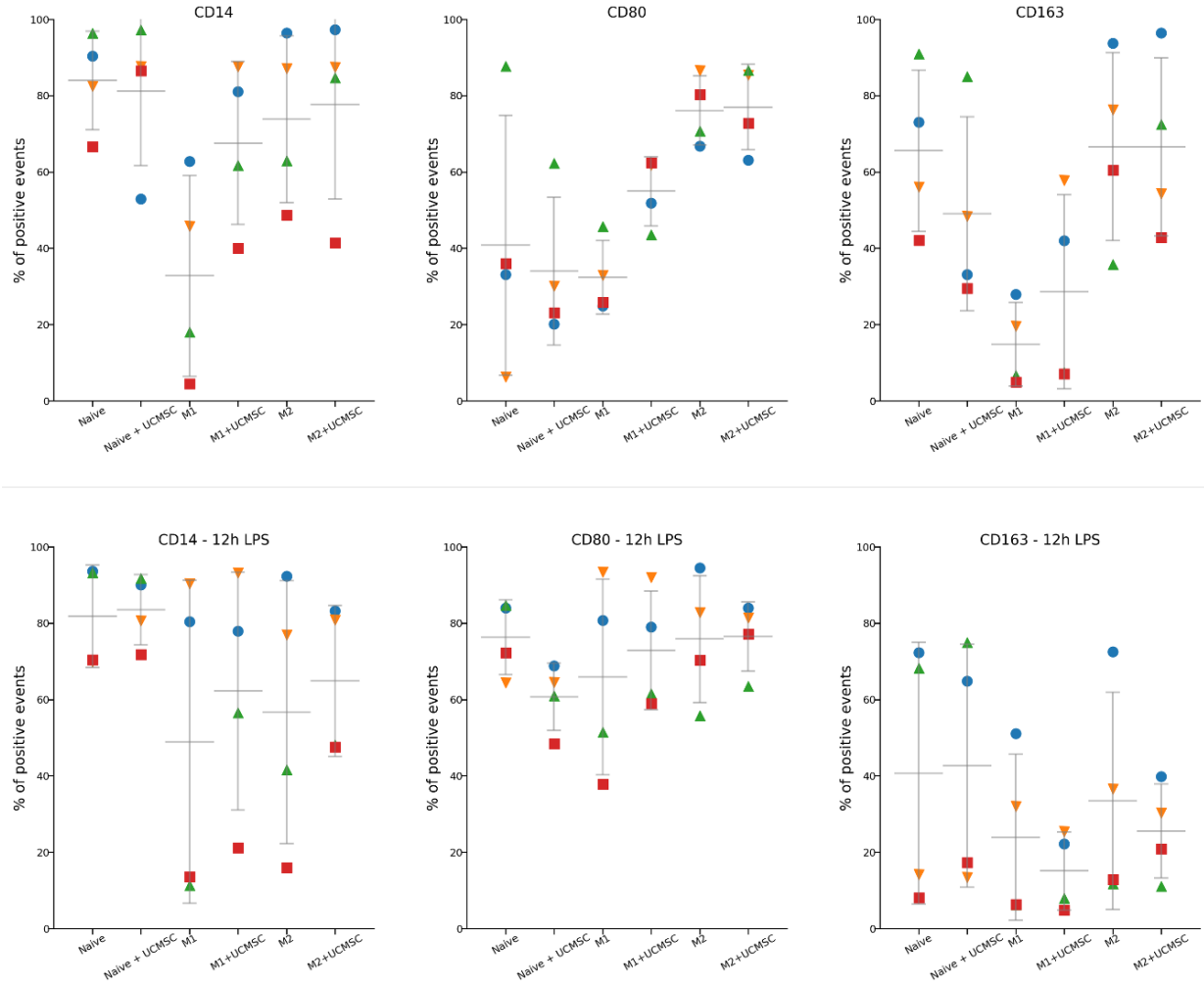


Figure 4. 10. Indirect co-culture of hMDMs and hUCMSCs using Transwell inserts.

hMDMs were co-culture for 72 hours with hUCMSCs. Each colour corresponds to a different donor. Statistical analysis was performed with Kruskall Wallis and Dunn's post-test and no statistical difference was found between control samples and co-cultured cells.

4.3.6. Optimisation of a reliable EV labelling method for uptake assessment.

In order to investigate whether the reason vesicles were not eliciting an effect was based on a lack of uptake by hMDMs, hUCMSCs were labelled with CFSE and EVs were isolated from labelled cells. This was to investigate whether the vesicles would also be stained and, if so, try to analyse EV uptake by hMDMs using fluorescence microscopy and cytometry.

In comparison with unstained EVs, vesicles from CFSE-labelled hUCMSCs displayed more fluorescence in the green channel, indicating that even after dye dilution due to the EV release process and potential hUCMSC proliferation during FBS deprivation, these EVs had enough fluorescence to be detected by the cytometer and potentially these EVs could be detected inside hMDMs (Figure 4.11). CFSE-labelled EVs expressed CD81, showing that cell staining did not alter the enrichment of this protein.

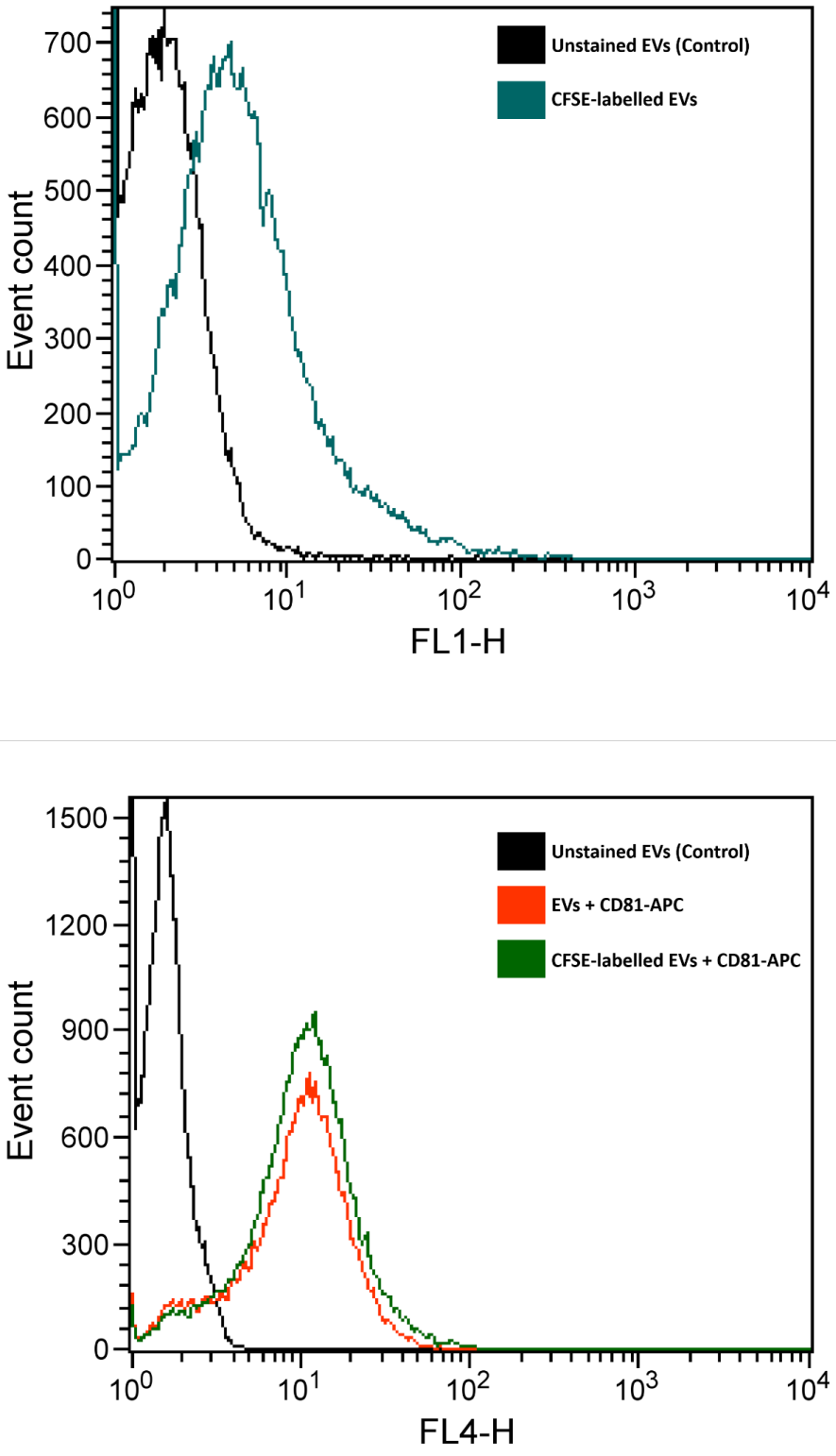


Figure 4. 11. Cytometric analysis of EVs derived from CFSE-labelled hUCMSCs.

Cytometric analysis was performed on EVs derived from CFSE-labelled hUCMSCs and EVs derived from non-labelled hUCMSCs as controls. CD81 was used to confirm that particles were extracellular vesicles. FL1 is the channel to detect green fluorescence and FL4 is the channel for red fluorescence.

To determine whether uptake could be evaluated, hMDMs were matured and polarised as in previous experiments and incubated for 24 hours with EVs derived from CFSE-labelled hUCMSCs at a concentration of 1 x 10^8 vesicles per ml. Fluorescence microscopy did not detect any visible fluorescent vesicle after 3 hours of incubation, even using the 63x objective, the highest magnification available. Therefore, cells were harvested and analysed through flow cytometry, but no fluorescence was found in the green channel suggesting that either cells did not uptake EVs or they did not uptake at a sufficient amount to be detectable (Figure 4.12). CD14 was used as a control, but was mainly expressed by naïve cells, not following the pattern of previous experiments when it was widely expressed by all phenotypes.

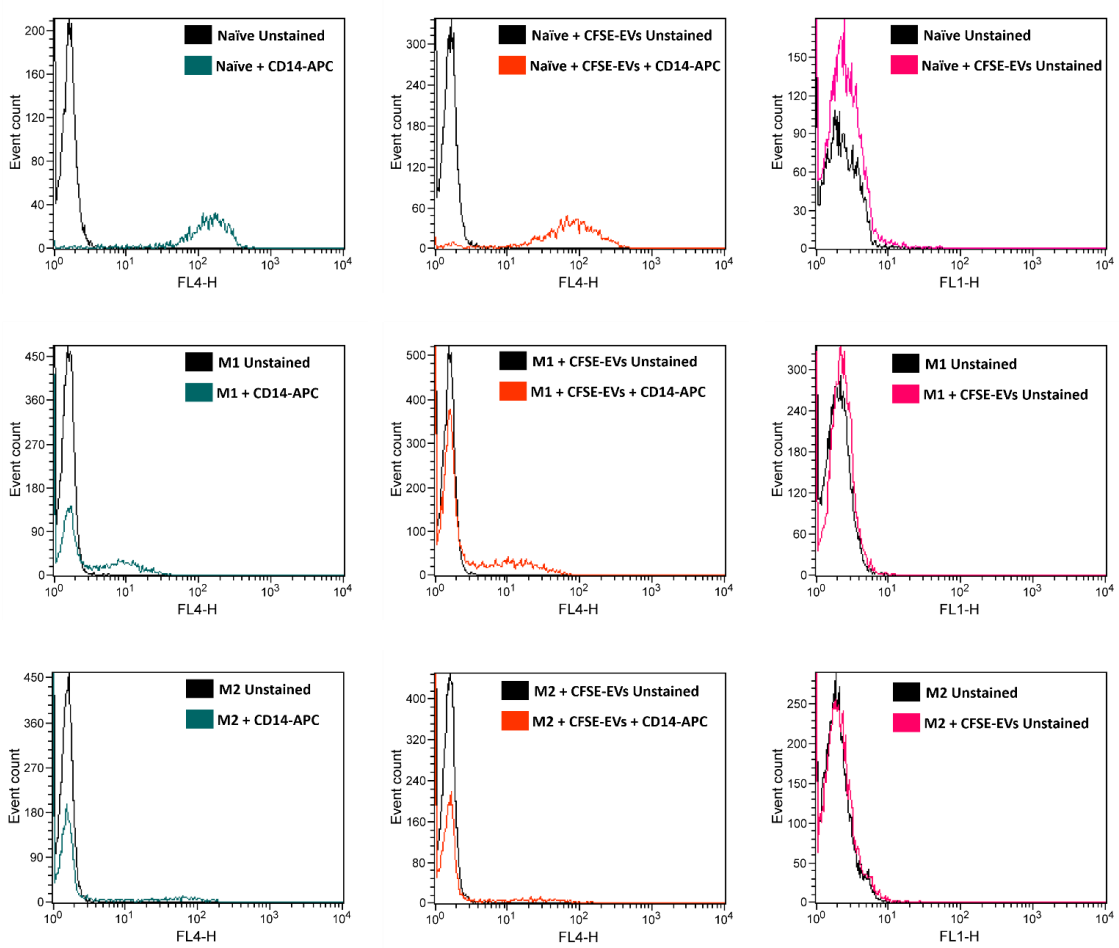


Figure 4. 12. Cytometric analysis of hMDMs incubated with CFSE-labelled EVs.

hMDMs were matured and polarised into M1-like and M2-like macrophages. Naïve cells were not activated with any cytokine. Cells were stained with CD14 conjugated with APC as a monocytic marker control. FL1 is the appropriate channel for CFSE detection and FL4 for APC.

4.3.7. Determination of the effects of EVs on endothelial cells

The initial plan for this study was to assess the immunomodulatory effects of hUCMSC-derived EVs on hMDMs and proceed to investigate EV cargo, so as to attempt to identify molecules responsible for those immunomodulatory properties. As throughout this investigation, EVs did not elicit any clear response on hMDMs, endothelial cells were used to assess whether these EVs are functional in promoting angiogenesis.

With this purpose, a co-incubation assay to analyse vessel formation was designed, using normal human dermal fibroblasts (NHDFs) and human dermal microvascular endothelial cells (HDMECs). NHDFs and HDMECs were co-cultured for 7 days to test the response to the agonist VEGF-165 and also different batches of EVs. Concentration of EVs was $1x10^8$ vesicles per well or 2×10^8 per ml, and medium was changed once during co-incubation, resulting in double exposure to EVs. Cells were stained with both VE-Cadherin and CD31; tubular formation was assessed using AngioTool.

This experiment was performed three times and Figure 4.13 shows representative images of one independent experiment. The agonist VEGF-165 clearly enhanced CD31 staining in comparison to samples exposed only to basal medium. All the EVs used for assessment of immunomodulatory changes on hMDMs were tested, including EVs isolated from hUCMSCs after 18 and 72 hours of FBS deprivation (referred as 18h_EVs and 72h_EVs).

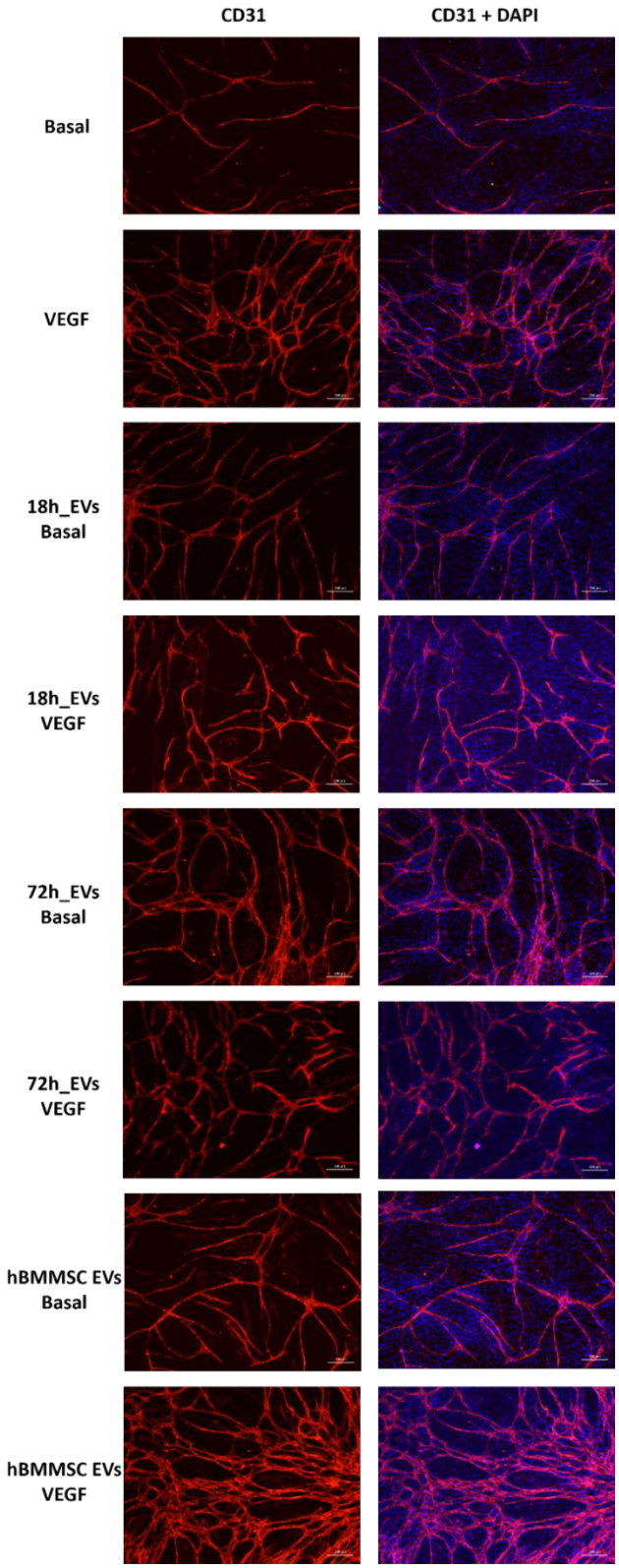


Figure 4. 13. Angiogenic assay using NHDFs and HDMECs to investigate effects of EVs on angiogenesis promotion.

Cells were co-cultured for 7 days for investigation of vessel formation and effects of EVs previously used on incubation with hMDMs. Representative images of CD31 staining on samples exposed or not to EVs using 50 x magnification. Scale bar is 200µm.

Figure 4.14 shows a compilation of data from independent experiments concerning the proportion of area occupied by vessels and number junctions. The size of error bars demonstrates great variability among samples. No statistical difference was found between samples. Analysis was performed with images obtained using 50 x magnification (5x objective) and coverslips showed great variability in vessel coverage for all conditions.

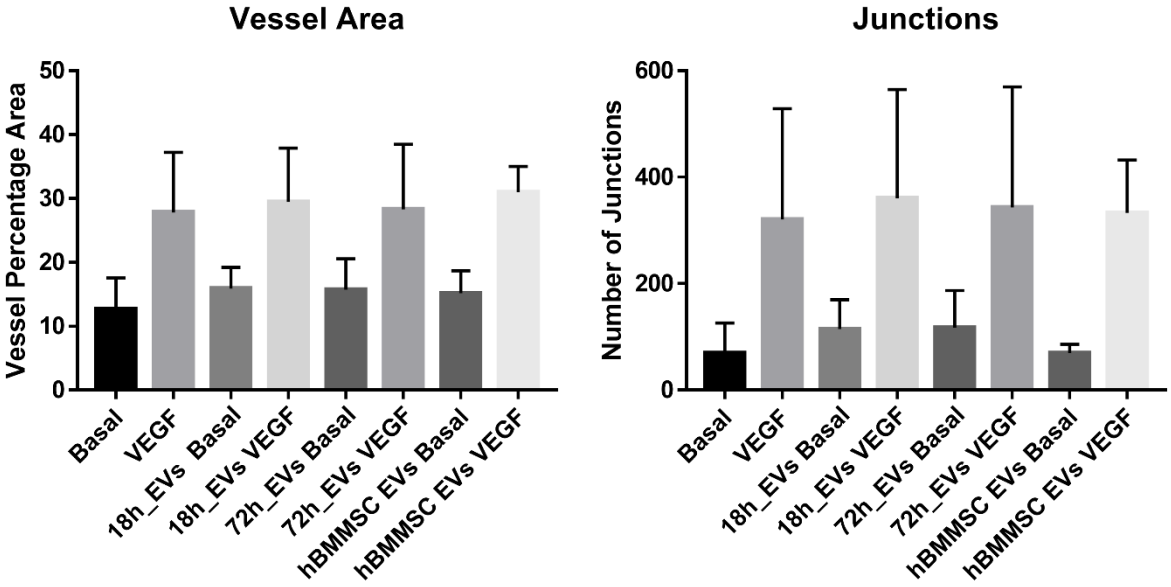


Figure 4. 14. Analysis of proportion of area occupied by vessels and number of junctions.

Vessel percentage area and number of junctions obtained via analysis using AngioTool. Data was obtained from images taken at 50 x magnification. Statistical analysis was performed with Kruskall Wallis and Dunn's post-test and no statistical difference was found between samples.

4.4. Discussion

The experiments performed in this chapter aimed to investigate the potential effects of MSC-derived EVs on important surface marker expression on hMDMs. Because there was no major shift in the expression of the chosen markers, Transwell assays were employed to further assess whether MSCs would change the expression of CD14, CD80 and CD163. The time points of 24 and 72 hours used for EV co-incubation with hMDMs were chosen for two main reasons: first, it would enable the comparison of results from the *in vivo* animal model of ischemia/reperfusion which used the same EVs as a therapeutic tool 24 hours after injury infliction; and second, a longer time-point would provide time for the cells to recover, especially from the potent LPS stimulus. This would provide an additional testing system to the study at the 24-hour time-point while maximising the possibility of detecting EV effects by also analysing cells at a later time point. Only results from 72-hour incubation experiments are shown in this chapter and results obtained from the shorter time point are listed in the Appendix (Figures A.5, A.6 and A.7). To further investigate EV potential, angiogenesis assays were performed in order to determine whether EVs would elicit tubular formation.

4.4.1. Immunomodulatory effects

EVs did not display immunomodulatory effects in the settings of this study. In order to determine whether this lack of response was related to the source of MSCs, both umbilical cord and bone marrow MSCs were used to isolate EVs and these EVs were tested on hMDMs. Besides, experiments to test the immunomodulatory effects of EVs isolated from MSCs exposed to

inflammatory cytokines and EV deprived MSC secretome were performed (data not shown). Those experiments were additional steps to validate the system being used for this study, particularly in face of the lack of difference between groups.

The absence of a shift in surface marker expression may not entirely rule out an immunomodulatory capacity, as it is reported that EVs, and not only their parent cells, have immunomodulatory effects on B cells, T cells and macrophages, among others [46].

Increased IL-10 secretion is one of the reported effects of MSC-derived EVs on immune cells [17, 47, 48]. There was no detectable secretion on cells not exposed to LPS, and no difference in secretion among cells exposed to LPS for 12 hours. Also, the other analysed cytokines could not be detected, implying that cytokine secretion was not as strong as initially expected.

Although IL-10 is an anti-inflammatory cytokine, it has been reported that LPS induces IL-10 secretion on hMDMs, a mechanism regulated by glycolysis [49]. This finding is in line with the enhanced secretion found on cells exposed to LPS. IL-12, a proinflammatory cytokine, is majorly secreted by macrophages under inflammatory conditions, and its secretion is stimulated by pathogens and their products, including LPS [50]. Nevertheless, IL-12 secretion is very complex given that IL-10 production can inhibit IL-12 secretion in monocytes and macrophages, but this production can be disrupted by some specific Fc receptors ligation [51, 52].

Surface marker expression did not change after incubation with EVs, suggesting that vesicles were either not functional or that the conditions of the

assays, including concentration of EVs and length of incubation, were not optimal. Given that data on EV immunomodulatory properties is not currently abundant, it was challenging to precisely determine the most suitable protocols for this investigation. Nevertheless, this study attempted to test as many conditions as possible within limitations imposed by time and resources.

Important to mention that there were changes in morphology of hMDMs after exposure to EVs and noticeable changes in surface marker expression on the hUCMSC-hMDM co-incubation experiments, suggesting that EVs are not completely innocuous. The effects elicited by hUCMSC-hMDM co-incubation also indicates that there is a possibility that cytokines and chemokines secreted by MSCs play a fundamental role in macrophage immunomodulation.

4.4.2. Angiogenic effects

Due to the lack of hMDM response in terms of phenotype shifting or differences in surface marker expression, the question was raised whether the EVs used in this study were not functional at all, or indeed they just did not elicit any effects on the expression of CD14, CD80 and CD163. For this purpose, an angiogenesis assay was performed as there is building evidence in the literature that EVs promote angiogenesis [53-57].

Although those reports suggest that EVs consistently favour vessel formation, there is a number of factors that need to be considered, such as EV dose or concentration, cell type of the parent cells tested on the angiogenic assay and methods of EV isolation in order to reach a conclusion regarding EV effects on angiogenesis. It is important to consider that MSCs exposed to an

inflammatory environment inhibit angiogenesis, and it is demonstrated that their derived EVs, in this case, also have anti-angiogenesis effects [58].

Examples from literature do not always provide a consistent picture of EVs effects on angiogenesis. Low doses (2 x 10^3 vesicles per well) of EVs derived from endothelial cells promoted tube formation, as opposed to an inhibitory effect provided by high concentrations (2 x 10^5 particles per well) [59]. This finding agrees with other studies, showing that concentrations higher than 10^5 particles were detrimental to angiogenesis promotion [60], and high concentrations of platelet-derived microparticles were necessary to stimulate revascularization of the myocardium [61]. However, it is not in line with a study which demonstrated that low concentrations of MSC exosomes were more effective for angiogenesis [62]; nevertheless, the absolute numbers were conflicting with another report of the optimal concentration for angiogenic effect *in vivo* and *in vitro* [63]. As methodologies and sources of EVs vary greatly, it is challenging to determine whether all EVs indeed have an angiogenic effect [64], and if so what are the optimal conditions to elicit this effect. Moreover, publication bias is an obstacle to objective evaluation of EVs effects, as the majority of articles tend to report positive effects and statistically significant data only.

The scope of this study did not encompass an in-depth evaluation of angiogenesis *in vitro*, as this is a complex and dynamic process which would require a panoply of techniques to be correctly assessed. Accordingly, the experiments performed here were means to obtain an additional system to evaluate EV functionality, given the absence of response on the immunomodulatory front.

The first attempts of assessing angiogenesis were with Human Umbilical Vein Endothelial Cells (HUVEC) on scratch assays, in order to determine cell migration. Those experiments did not work as planned, as cells were not responsive to their main agonist (VEGF) and scratches were not closing even when cells were incubated for 24 hours in the presence of VEGF (Appendix, Figure A.8), potentially due to differences in the width of the scratch.

Due to those inconsistencies, the co-culture of fibroblasts and endothelial cells was employed as an alternative manner of assessing the potential effects of EVs on endothelial cells by analysing vascular formation over several days and without the subjectivity brought by handmade scratches. Reiterating that this experiment was intended on representing an indication of effects on endothelial cells, and not a proper study on angiogenesis.

As this experiment was designed to provide a clear assessment of vascular formation, the markers chosen needed to be 1. Ubiquitous in tube formation, even if not present in all structures of the tube it should be necessarily present at a vascular formation, and 2. Abundant enough to be easily detected by fluorescence microscopy. The two chosen markers were CD31 and VE-Cadherin.

CD31 can be expressed by diverse cell types besides endothelial cells, such as monocytes, platelets and granulocytes. It is a 130 kDa molecule that has roles in inflammation, signal transduction and platelet function. This molecule plays a crucial role on endothelial cell adhesion, being present on endothelial cell junctions, and is also called Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) [65]. VE-Cadherin is also present at junctions and provides the

basic organisation of adherens junctions along with p120-catenin and β-catenin [66]. Both CD31 and VE-Cadherin expression was assessed in this study (data not shown for VE-Cadherin expression).

The concentration of EVs per well was 1 x 10^8 particles, as to follow experiments performed on hMDMs. A 10-fold lower concentration was also tested in a pilot experiment. Nevertheless, even the lowest concentration would fall under the high concentration criteria of previous published reports, but there were limitations regarding the number of conditions that could be tested. Analysis was performed using the 5x objective, as to provide a bigger coverage of each sample and thus minimise bias.

Due to variability and low number of samples, angiogenic potential of EVs was not substantially investigated. Nevertheless, given that different EVs were tested in independent experiments, it is reasonable to state that EVs did not promote angiogenesis under the settings used for the co-incubation assay. Whether this means that these EVs were not functional altogether or that different settings would elicit a different response is unclear.

4.5. Conclusion

Under the parameters of this study, EVs derived from MSCs, both from bone marrow and umbilical cord, were not effective in shifting expression of CD14, CD80 and CD163. This lack of response could be due to specifics of the study, such as low number of analysed donors and how macrophages were cultured. This outcome cannot be generalised and does not exclude the possibility of immunomodulatory effects under different settings. EVs were successfully

indirectly labelled using CFSE and fluorescence could be determined through flow cytometry, providing a potential useful tool for EV fluorescent analysis. The angiogenic potential of EVs used for this investigation was not clearly determined, and further analysis is needed to conclusively investigate how EVs can promote angiogenesis.

4.6. References

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Chapter 5 – Discussion and Future Work

The major aim of this study was to investigate the effects of MSC-derived EVs on macrophage phenotype. For this purpose, a reproducible protocol for polarising macrophages was established based mainly on the expression of the surface markers CD80 and CD163. However, EVs derived from hUCMSCs and hBMMSCs did not elicit a detectable shift in the expression of any of the markers chosen to assess macrophage polarisation. In contrast, when hMDMs were co-cultured with hUCMSCs, a noticeable, though non-significant shift in macrophage phenotype was observed, suggesting that MSCs and their derived EVs might have antagonistic effects on macrophages. This outcome has prompted questions regarding how robust were the testing systems used for this study and the reasons that could explain this lack of response.

We have shown how macrophages were assessed regarding maturation and polarisation and how an additional system with endothelial cells to evaluate angiogenesis was put in place in order to investigate EV effects. This emphasis on the optimisation of cell culture conditions was necessary as a consequence of the variety of protocols described in the literature, and also the need to prove that the absence of effects on EV treated samples was not caused by an inherent unresponsiveness of the cells. All this would aid in safeguarding that the results would be as accurate and consistent as possible within the chosen parameters. But due to the relatively low number of blood donors and constraints on how many different settings could be tested throughout the project, it is difficult to reach a definite conclusion on the effects of these EVs on macrophage surface marker expression. Even with a higher

number of samples there is substantial inter-individual variability that can account for differences in how cells respond.

This chapter will focus on the discussion of potential mechanisms behind MSC and their derived EVs effects on pre-clinical studies. It also gives insights into potential future work to investigate the immunomodulatory properties of MSC-derived EVs.

5.1. Understanding variability as a source of discrepancy

There is a focus, mainly on pre-clinical studies, to reduce biological variability in order to avoid confounders. Standardisation of methods to reduce variability has been long deemed as good practice [1], and the concept has been expanded to include the use of isogenic and immunodeficient animals to provide as homogeneous samples as possible and ensure that no genetic difference or immune abnormality could potentially impact outcomes. This approach has many advantages, particularly when the aim is to investigate pathophysiological mechanisms, but often result in fundamental differences when attempted in human samples [2]. There is evidence that EVs derived from MSCs are able to alter macrophage polarisation *in vivo* [3], but this study did not confirm this evidence. This section discusses the potential reasons for this apparent discrepancy, including intrinsic variabilities concerning MSCs, EVs and macrophages, and the manner by which the impact of this diversity could be addressed.

5.1.1. MSCs and the variability of their derived EVs

The diversity of MSCs are initially a result of the different tissues they can be obtained from. A certain degree of variability in the properties displayed by MSCs originating from different tissues is expected, though they should share some surface markers (CD73, CD90 and CD105) and adherence to plastic, among other characteristics [4]. Nevertheless, MSCs can substantially differ depending on the environment in which they are cultured and the procedures they are subjected to. For instance, freezing and thawing MSCs can impact their immunomodulatory properties [5], demonstrating that these properties are dynamic and susceptible to change depending on the environment, and not constitutive as once thought.

MSCs from the same tissue can also differ in how they respond to inflammatory stimuli, an example being exposure to IFN-γ that induces expression of indoleamine 2,3-dioxygenase (IDO) [6]. This might explain why some patients are responsive to MSCs in clinical trials, while others do not show improvement. IDO is consistently linked to MSC immunosuppressive abilities, essential in limiting inflammation and inducing the formation of regulatory T lymphocytes [7-9].

IDO is one of the most reported proteins associated with MSC immunosuppression, but cell surface factors like VCAM-1, ICAM-1, PDL1, PDL2 and CD95L also play a role on the effect MSCs have on T cells [10-13]. The level of these factors impacts the immunomodulatory ability of MSCs. It has been reported that kynurenic acid (KYNA), a metabolite of IDO, is an important regulator of TNF-stimulated gene 6 (TSG-6), another key immunosuppressive factor in MSCs [14]. Pre-treatment of hUCMSCs with

KYNA enhanced their therapeutic effect in a lung injury model [14], suggesting that cells can be artificially inclined to perform determined functions.

Nevertheless, not only IDO, but many pathways are involved in the metabolic shift necessary for macrophage phenotype switching [15], as exemplified by Figure 5.1.

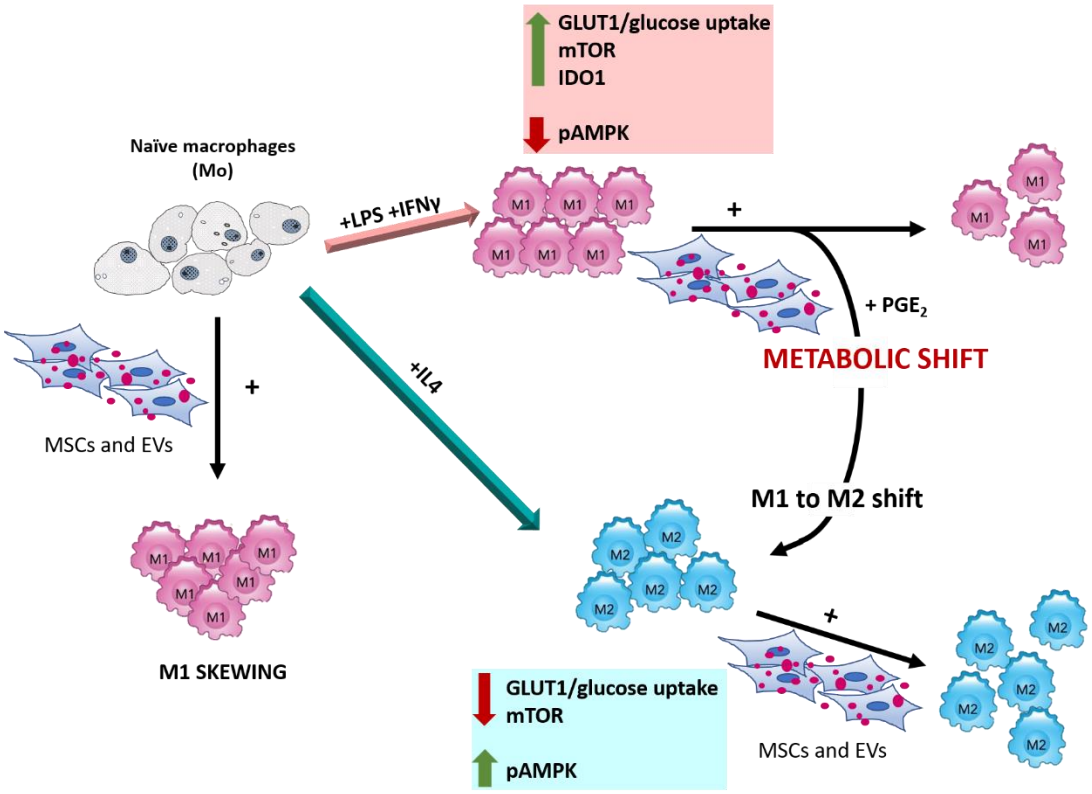

Figure 5. 1. Mechanistic insights into how macrophage plasticity mediated by MSCs occurs.

MSCs facilitate polarisation of naïve macrophages to an inflammatory M1 activation module and enhance M2 activation. MSCs further attenuate inflammatory M1 macrophages, shifting them to an anti-inflammatory M2 activation state by instructing metabolic shifts in a PGE_2 dependent manner. EVs and soluble factors might be one of the mechanisms by which MSCs confer immunomodulation on macrophages. Adapted from [15].

The concept of MSC pre-treatment has been investigated in recent reports and it has been demonstrated that exposing MSCs to particular environments might enhance their therapeutic properties [16-20]. Of particular interest for

the scope of this study is the reported enhanced ability of MSCs to shift macrophage polarisation towards an M2 phenotype after being exposed to the pro-inflammatory cytokines IFN-γ and TNFα [21]. This report actually investigated the abilities of MSC-derived exosomes, giving evidence that the physiological state of EV parent cells play a role in released populations of EVs.

Indeed, EVs resemble the cells they have originated from, meaning that these sources of variability in MSCs can also impact the cargo and role of their derived EVs. EV diversity can originate from the conditions of their parent cells immediately before their isolation; for instance, EVs derived from cells subjected to hypoxia have a greater angiogenic potential in comparison to EVs derived from normoxic cells [22, 23]. Diversity also stems from the fact that EVs are heterogeneous and as such can be differentially enriched according to specific protocols of isolation (Figure 5.2). Different subpopulations of EVs might display different properties and elicit different effects [24].

EVs can also present protein signatures that reflect the environment they are inserted into [25]. Not only protein contents can vary, but also there is some evidence that in the case of MSC-derived EVs, there is intrinsic donor variability that can determine whether the released EVs are or not therapeutic (Madel RJ, Börger V, Dittrich R, Bremer M, Tertel T, Phuong NNT, Baba HA, Kordelas L, Buer J, Horn PA, Westendorf AM, Brandau S, Kirschning CJ, Giebel B. Independent human mesenchymal stromal cell-derived extracellular vesicle preparations differentially affect symptoms in an advanced murine Graft-versus-Host-Disease model. BioRxvi [**Preprint**]. December 22, 2020. Available from https://doi.org/10.1101/2020.12.21.423658). This is in line with

the assumption that EV potential is influenced by the characteristics of the parent cell, including donor, the conditions in which the cells release the vesicles and the method of isolation employed. Moreover, these variabilities reinforce the importance of testing different conditions, such as exposing cells to varied conditions and using supernatant depleted from EVs as a control, as was the case for this study.

Ultracentrifugation is a method of EV isolation that has been reliably reported to be appropriate in the context of kidney injury investigations [26-29]. Nevertheless, it is important to acknowledge that a different method of isolation could have yielded a distinct population of EVs that could elicit different effects on hMDMs.

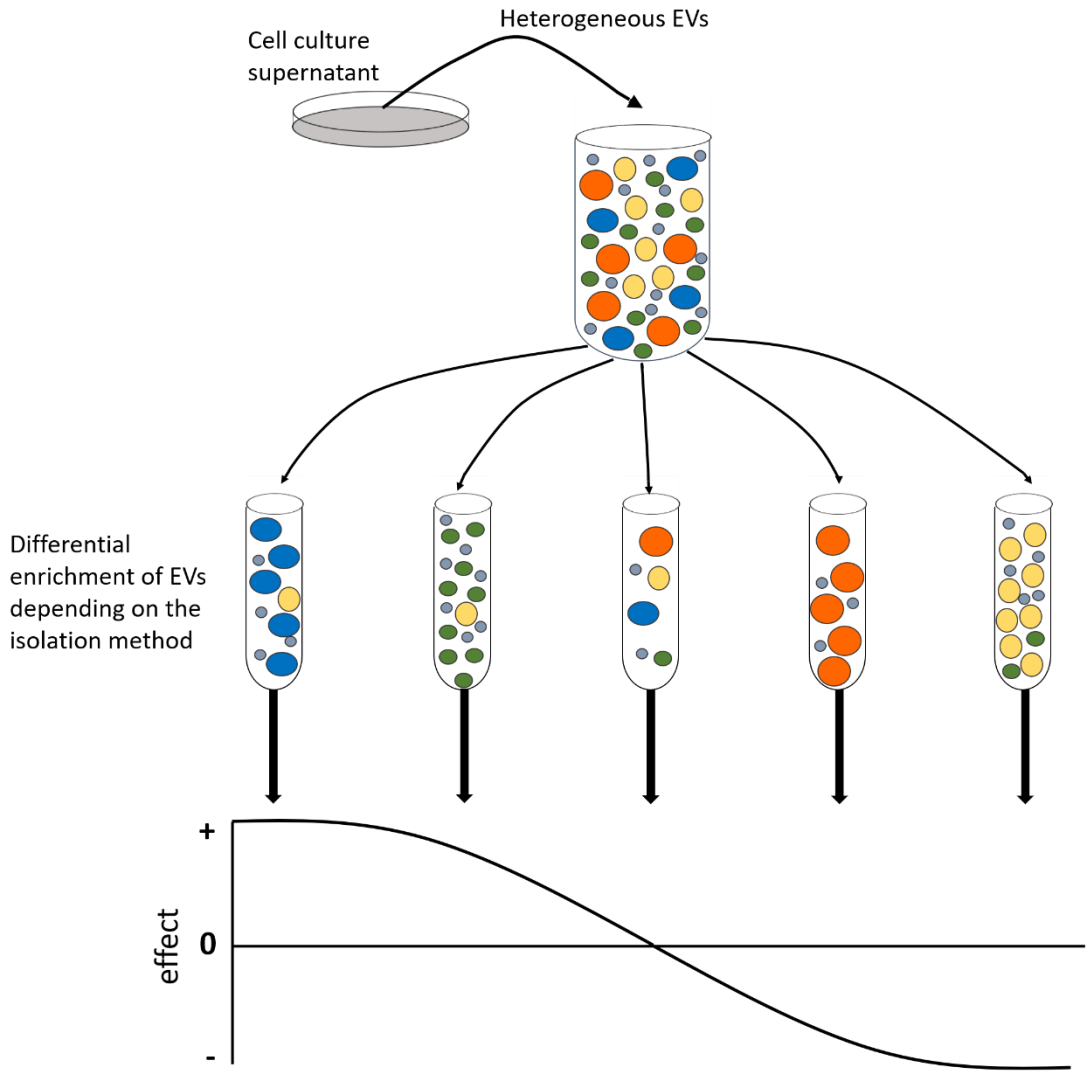

Figure 5. 2. Functional analysis of heterogeneous EVs populations.

When analysing the functionality of EVs released by a determined cell type, the results can be extremely biased depending on the isolation technique used. In the scheme, it is illustrated a case where both large blue EVs and small green EVs have a positive effect on the analysed target cells, while the large orange EVs and small yellow EVs have a negative effect. Depending of the ratio of these EVs in a given preparation, we can either observe a positive or a negative effect, or even no effect if the mixture of all EV subtypes compensates the respective effects. Adapted from [24].

5.1.2. Monocyte and Macrophage Variability

Inter-individual variability in immune cells has been extensively investigated and linked to failure in translating pre-clinical research to clinical settings, differences in vaccination responses and diversity in pathophysiology of

diseases [30-35]. Further to inter-individual variability, there is intra-individual variability; macrophages are known for their plasticity, as they are broadly distributed in the body and need to change according to the context and molecules they are exposed to [36, 37]. The spectrum of polarisation states reflects the variety of nuances that these cells can assume and many states are not fully understood regarding function, as these are intermediate states [36, 38-41].

Additional to these intrinsic variabilities, there is undeniable variability in how immune cells develop and function in humans and rodents. Great part of immunological research is based either on rodent animal research or immortalised cell lines, including research regarding macrophage physiology. Most of the animal research is performed on isogenic animals under standardised conditions, which not necessarily mirror physiological and pathological conditions in humans [42]. Immortalised cell lines lack many characteristics of their *ex vivo* and *in vivo* counterparts [43]. As a result, there is an abundance of data regarding immune response in murine models that in many cases cannot be replicated in pre-clinical human studies, due to differences in cellular mechanisms and physiological responses [44-46]. Moreover, macrophage metabolism and the response to inflammatory stimuli differ markedly in humans and rodents, affecting cytokine secretion, ATP production and mitochondrial function [47].

It can also be the case that the lack of response from hMDMs was due to the fact that macrophage function and phenotype depend, to a high degree, on signals from other cells, particularly other immune cells [48]. The *in vitro* environment provided in this study failed to recreate this *in vivo* dynamic.

Even though studies with human samples pose an extra layer of variability due to restrictions in controlling age, gender, diet and other factors, particularly when samples are provided in an anonymised manner, it is relevant to attempt and report immunological investigations with human material, as to elucidate physiological and pathological mechanisms.

That EVs can confer immunomodulation in the context of renal injury and malignancy is known [49, 50]; therefore, the conclusion of this study is not pointing to an inability of EVs in modulating the immune system. The degree of this modulation, however, besides its consistency and reproducibility, could be potentially enhanced by tailoring the conditions in which EVs are isolated as to better mimic the pathophysiological conditions of interest.

5.2. Future Work

The work conducted in this investigation did not exhaust by any means the possibilities of macrophage polarisation assessment. Limitations on the number of donors and conditions that could be tested restricted the scope of the study; moreover, the few conditions tested did not display differences between groups, providing additional questions. This section deals with potential research that can be pursued to answer some of the questions opened by this investigation.

5.2.1. Additional analysis of macrophages

Given the difficulties faced in assessing macrophage phenotype based on surface marker expression, an alternative approach would be to assess macrophage function. The plasticity of macrophage phenotype does impact

their function, and one of the primary roles of macrophages as innate immune cells is phagocytosis [48].

One molecule that affects phagocytosis is Kidney Injury Molecule-1 (KIM-1). KIM-1 is expressed by tubular cells and upregulated in the proximal tubule and is linked to the phagocytosis of apoptotic bodies and inflammatory mediators [51, 52]. Therefore, it is known that phagocytosis performed by tubular cells takes place during kidney injury at earlier stages of the disease. Analysing macrophage responsiveness to KIM-1 might offer valuable insight, including evaluation of whether KIM-1 impacts macrophage phagocytic ability. Phagocytosis can be quite crucial in the context of EV uptake, as the size of EVs does allow for this mechanism of uptake. Hence, evaluating phagocytosis in macrophages might provide relevant information regarding phenotype shifting and help to elucidate some of the potential mechanisms by which EVs can interact with phagocytes.

Although there are a number of possibilities for studying different facets of macrophage physiology, there is remarkable variability linked to human macrophages; thus, focusing on refining the study on the MSCs and extracellular vesicles front might be an easier approach, particularly in light of the advances in the field of extracellular vesicles bioengineering.

5.2.2. MSC and tailoring of extracellular vesicles

One of the reasons that could explain the lack of immunomodulatory effects in this study is the method of isolation, as exemplified in Figure 5.2. Nevertheless, the method of isolation here refers not only to the technique

used to isolate EVs, in this case ultracentrifugation, but also the condition of the parent cells before EV isolation.

As previously mentioned in section 5.1.1, there is evidence that the conditions in which EV parent cells are cultured can impact the obtained populations of vesicles. Bioengineering of EVs is an elegant approach that can be performed pre- or post-isolation and can aid in improving bioactivity, kinetics, targeting, biodistribution and cargo [53]. There is a plethora of mechanisms to enhance the immunomodulatory ability of both MSCs and EVs; Figure 5.3 provides some examples.

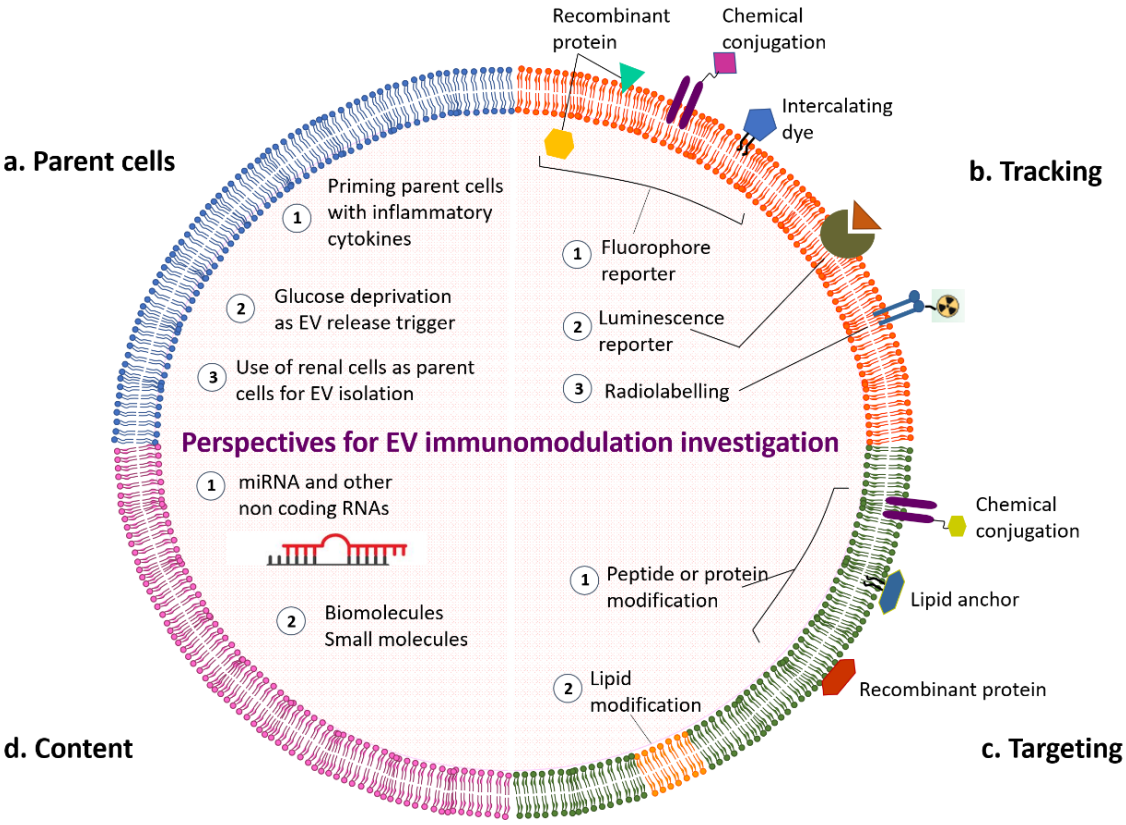

Figure 5. 3. Perspectives for future investigations of immunomodulatory effects of EVs in the context of renal injury.

a | Parent Cells. To increase the therapeutic potential of EVs, EV parent cells can be primed with inflammatory cytokines (1) or deprived from specific nutrients, such as glucose (2). Specific for the kidney injury realm, renal cells could be used as parent cells (3). **b** | Tracking. EVs can be labelled with fluorophores (1), luminescence reporters (2) or radiotracers (3). **c** | Targeting. To increase the targeting efficacy, EVs could be modified with exogenous peptides or proteins (1) or lipids (2). **d** | Content. EVs can be enriched with specific miRNAs (1) or bioactive molecules, such as lipids or proteins (2). Adapted from [53].

This thesis has attempted to investigate the immunomodulatory properties of MSC-derived EVs, more specifically their capacity to shift macrophage polarisation. The results did not show any impact of EVs on hMDMs, but this does not close the investigation. Some of the reasons behind the absence of effect have been discussed both in this chapter and in Chapter 4; the possibility that EVs indeed do not affect macrophage polarisation is real, but it might be that EVs have an effect, but at different settings.

The unanswered questions at the conclusion of this study exceed the number of the original questions at the beginning, mainly due to the lack of differences in the outcomes; this does not constitute an issue *per se*, as the data obtained from this investigation can aid in the development of more refined and focused approaches. This study offered insights, rather than solutions; but always insights have to precede solutions. There is a great deal of work to be performed for the elucidation of potential immunomodulatory effects of EVs on macrophage polarisation and hopefully this study could provide some understanding and relevant information for this future work.

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Appendix

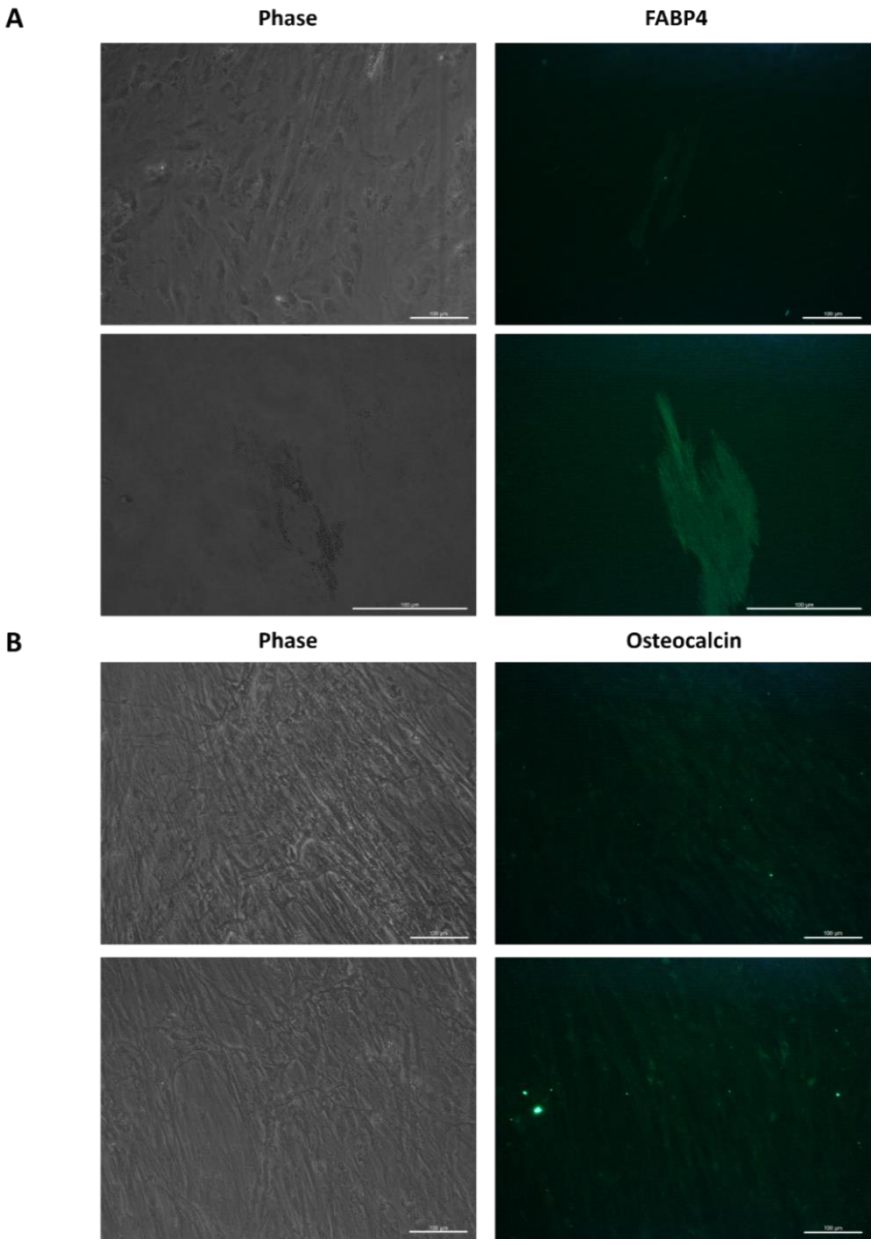


Figure A. 1. Adipogenic and osteogenic differentiation of hUCMSCs.

hUCMSCs were cultured with MEM-α medium and differentiation was induced using the media supplements included in the Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, cat. number SC006). **A.** Adipogenic differentiation using Goat Anti-Mouse FABP-4 Antigen Affinity-purified Polyclonal Antibody. **B.** Osteogenic differentiation using Mouse Anti-Human Osteocalcin Monoclonal Antibody for the confirmation of differentiation status. The cells were stained using Alexa-Fluor 488-conjugated Donkey Anti-Goat (Abcam, cat. number ab150129) or Anti-Mouse (Abcam, cat. number 150105) IgG Secondary Antibodies. Staining was dim for all conditions, suggesting most of cells could not differentiate. Chondrogenic differentiation did not occur. Scale bar is 100 µm.

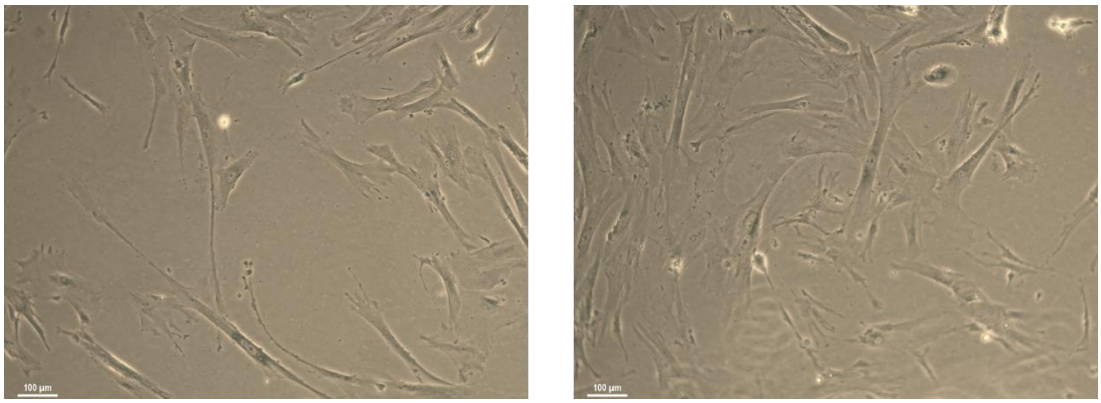
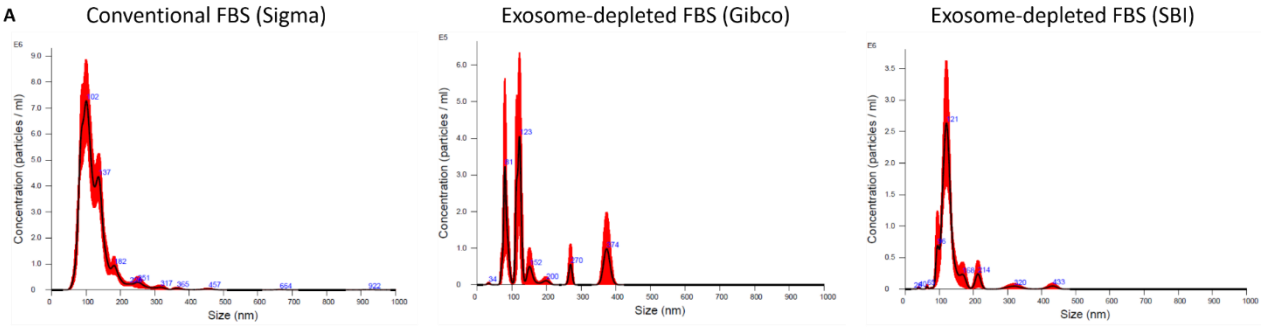


Figure A. 2. β-Galactosidase staining in FBS-deprived hUCMSCs.

hUCMSCs deprived from FBS for 72 hours were subjected to β-galactosidase staining using Senescence β-Galactosidase Staining kit (Cell Signalling, cat. Number 9860) according to manufacturer instructions to determine their senescent status. Cells did not display visible blue staining, indicating lack of β-Galactosidase expression. FBS = Foetal bovine serum. Scale bar is 100 µm.

A



B

	Mean (Size)	Mode (Size)	Concentration
Conventional FBS (Sigma)	124.4 +/- 4.3 nm	112.4 +/- 9.2 nm	4.69e+008 +/- 1.79e+007 particles/ml
EXO-FBS (Gibco)	140.5 +/- 26.9 nm	103.8 +/- 10.0 nm	1.47e+007 +/- 2.46e+006 particles/ml
EXO-FBS (SBI)	132.9 +/- 6.5 nm	116.1 +/- 7.1 nm	9.54e+007 +/- 2.03e+007 particles/ml

Figure A. 3. Assessment of exosome-depleted FBS using NTA.

A. Size distribution of conventional FBS (Sigma) and exosome-depleted FBS from Gibco and Systems Biosciences (SBI). **B.** Size and concentration of particles ± standard error. FBS = Foetal bovine serum; NTA = Nanoparticle tracking analysis.

```
import sys
import csv

from numpy import loadtxt
import numpy as np
import matplotlib.pyplot as plt

title   = "insert file title"
inline = number

infile = 'infile.csv'
my_x_ticks = ['categories']
v_lines = [inline, inline+1,inline+2]

outfile = title+'.png'

result=None
with open(infile,'r') as fin:
      reader=csv.reader(fin)
      result = [[float(s) for s in row] for i,row in enumerate(reader) if i in v_lines]

dataset = np.array(result)

dataT = np.transpose(dataset)

v_mean = [ np.mean(j) for j in dataT  ]
v_std = [ np.std(j,ddof=1) for j in dataT  ]
v_xerr= [ 0.5 for j in dataT  ]
#print result
#print np.transpose(dataset)
print dataset

plt.clf()
fig = plt.figure(figsize=(10,12), dpi=100)
ax = fig.add_subplot(111)

#plt.rcParams['figure.figsize'] = (5,10)
#plt.rcParams['figure.dpi'] = 100
plt.title(title, fontsize=25)

xlen = np.array(range(len(my_x_ticks)))

plt.xticks(xlen, my_x_ticks, rotation=30, fontsize=18)
my_y_ticks = [0,20,40,60,80,100]
plt.yticks(my_y_ticks, my_y_ticks, fontsize=18)
plt.plot( xlen,dataset[0], 'o',ms=18.)
plt.plot( xlen,dataset[1], 'v',ms=18.)
plt.plot( xlen,dataset[2], '^',ms=18.)
#plt.plot( xlen,dataset[3], 's',ms=18.) ###

plt.ylabel('% of positive events',fontsize=25)
plt.ylim([0.,100.])

ax.spines['top'].set_linewidth(0.)
ax.spines['right'].set_linewidth(0.)
ax.spines['left'].set_linewidth(2.)
ax.spines['bottom'].set_linewidth(2.)

ax.errorbar(xlen, v_mean,  yerr=v_std,  fmt='.k', ms=0, capthick=3,capsize=12, ecolor='grey', alpha=0.5, elinewidth=3,zorder=9)

ax.errorbar(xlen, v_mean, xerr=v_xerr,  fmt='.k', ms=0, capsize=0, ecolor='grey', alpha=0.5, elinewidth=3,zorder=9)

ax.tick_params(axis = 'both', which = 'major', length = 10, width=2)
#ax.axvline(linewidth=4)
#ax.axhline(linewidth=4,color='r')
#ax.yaxis.label.set_size(18)
#plt.show()
plt.savefig(outfile)
```

Figure A. 4. Python script for colour-coded plots.

Codes were written in Python 2.7 using NumPy 1.11.1 and MatPlotLib 2.0.0b3 for Python 2.7 64bit. Acknowledgement to Dr. Nikolaos Rompotis for coding.

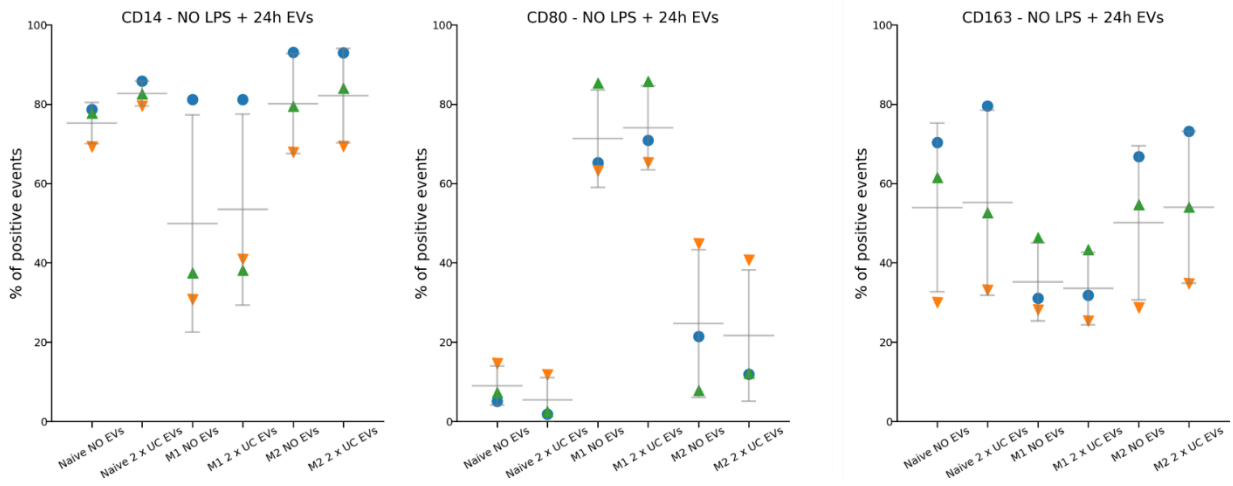


Figure A. 5. Cytometric analysis of hMDMs incubated with hUCMSC-derived EVs for 24 hours.

Mature and polarised macrophages were incubated with twice ultracentrifuged (2x UC) EVs for 24 hours. Each colour represents one donor. Statistical analysis was performed using Kruskal Wallis and Dunn's tests. There was no statistical difference between samples incubated with EVs and controls.

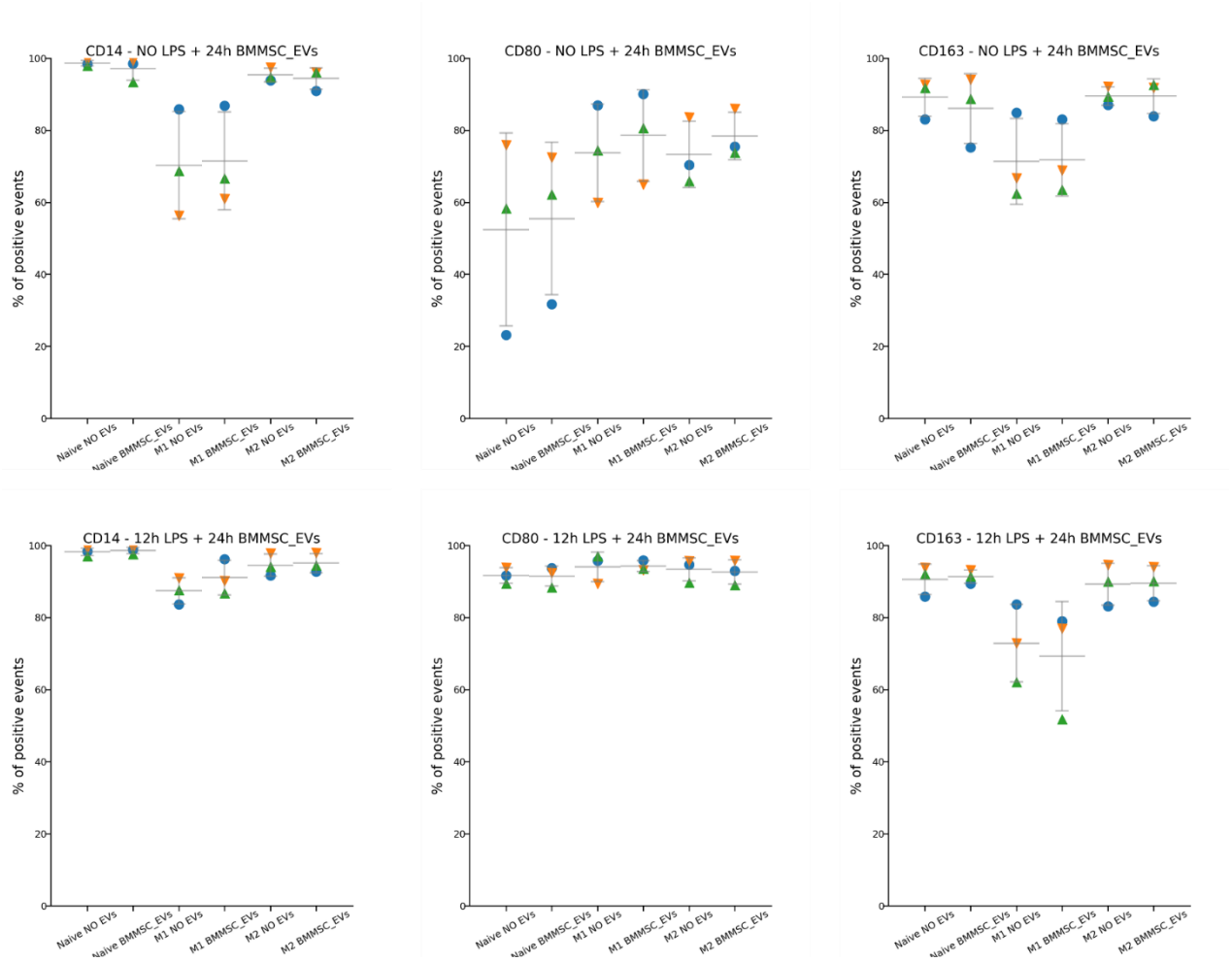


Figure A. 6. Cytometric analysis of hMDMs incubated with hBMMSC-derived EVs for 24 hours.

Mature and polarised macrophages were incubated with EVs derived from hBMMSCs for 24 hours. Cells were exposed to ultrapure LPS for 12 hours prior to incubation with vesicles. Each colour represents a different donor. Statistical analysis was performed using Kruskal Wallis and Dunn's tests. There was no statistical difference between samples incubated with EVs and controls.

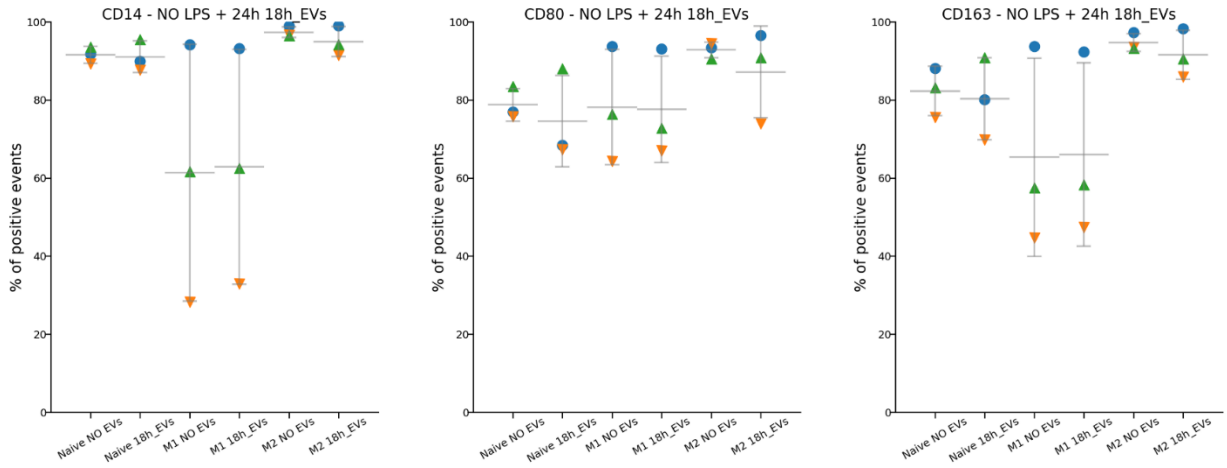


Figure A. 7. Cytometric analysis of hMDMs incubated with 18-hour FBS deprived hUCMSC-derived EVs for 24 hours.

Mature and polarised macrophages were incubated with 1 x 10^8 EVs per ml for 24 hours. Cells on the LPS group were exposed to ultrapure LPS for 12 hours prior to incubation with vesicles. Each colour represents one donor. Statistical analysis was performed using Kruskal Wallis and Dunn's tests. There was no statistical difference between samples incubated with EVs and controls.

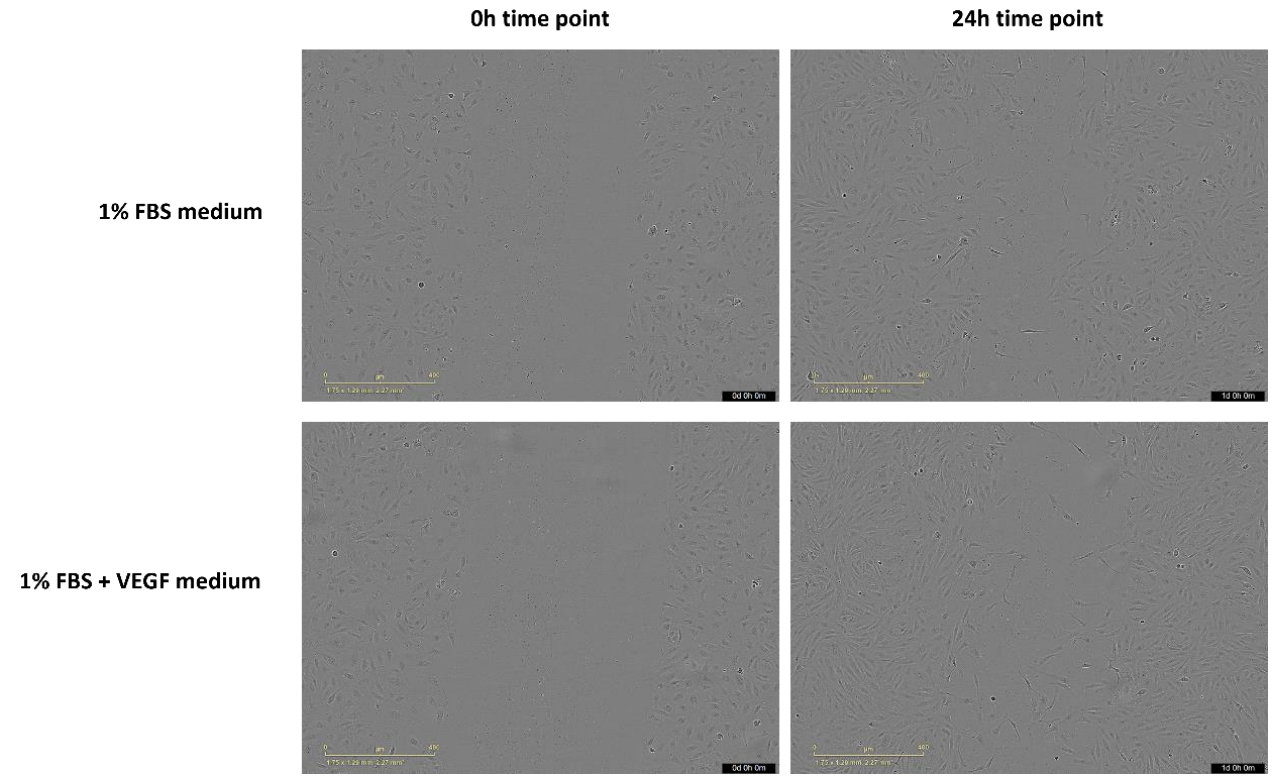


Figure A. 8. Cell migration assay with HUVECs at 0 hour and after 24 hours.

HUVECs were cultured with Endothelial Growth Medium (PromoCell) and exposed to 50 ng/ml of VEGF. Scratches did not close after 24 hours and cells were not responsive to VEGF, suggesting the assay lacked reliability.