# The immunomodulatory impact of multipotential stromal cells on monocytes in healthy people and patients with rheumatoid arthritis
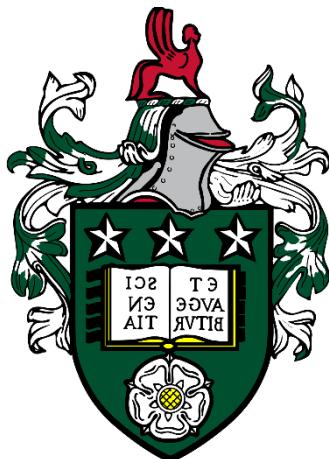
Priyanka Dutta

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Faculty of Medicine and Health
School of Medicine

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Primary Supervisor: Professor Michael McDermott
Secondary Supervisor: Dr Elena Jones
Secondary Supervisor: Professor Graham Cook

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Acknowledgements

Abstract

Multipotent stromal cells (MSCs) have the capacity for multilineage differentiation and are typically known for their applications for bone and cartilage regeneration. Additionally, MSCs have shown to have strong immunomodulatory properties, acting on both adaptive(Ma et al. 2014), and innate immune cells, including monocytes (J. Kim and Hematti 2009). Consequentially, MSC cell therapy have been proposed as an alternative new treatment for autoimmune diseases, such as rheumatoid arthritis (Luque-Campos et al. 2019). However, studies examining the interactions between MSCs and monocytes have commonly used magnetic cell separation methods or a monocytic cell line (Melief, Schrama, et al. 2013; Choi et al. 2011; Vallés et al. 2015). Therefore, this study investigated the immunomodulatory effects of MSCs or their conditioned media on healthy and RA monocytes, using a whole blood-based assay, which provided a more physiologically relevant setting. All MSCs used in co-culture experiments (IP006,Y201 and Y202 MSCs) conformed to the International Society of Cellular therapy phenotypic criteria for MSCs (Dominici et al. 2006). For the detection of intracellular tumour necrosis factor (TNF) and Interleukin 6 (IL-6) pro-inflammatory cytokine production via flow cytometry, healthy and RA monocytes were activated with lipopolysaccharide (LPS) and treated with Brefeldin A for 6 hours, using the whole blood assay. Results demonstrated that the addition of MSCs to healthy control monocytes, significantly reduced intracellular TNF and IL-6 levels. An even greater inhibition of TNF and IL-6 was observed when MSC CM was used instead of MSCs. Thus, for the treatment of early RA patients' response, bloods were treated with MSC CM only. This treatment showed statistically significant inhibition of TNF and IL-6 levels in activated monocytes, with the greatest potency being displayed in the immunosuppressive abilities of IP006 MSC CM treatment of established RA patients' bloods, from multi drug resistant patients.

In conclusion, MSCs and to a greater extent MSC CM showed potent immunosuppressive effects on monocytes in health and in both early and established RA. These results provide further evidence for a non-cell contact mechanism of MSC action and support the development of novel therapies for RA treatment.

Abbreviations

ACPA – Anti-citrullinated protein antibody

APCs – Antigen presenting cells

ASC – Adipose derived multipotential stromal cells

BD – Becton Dickinson

bDMARD – biological disease modifying antirheumatic drug

BM – Bone marrow

BM MSCs – Bone marrow derived multipotential stromal cells

CDAI – Clinical disease assessment index

CCP – Cyclic citrullinated peptide

CM – Conditioned media

csDMARD – conventional synthetic disease modifying antirheumatic drug

CTLA-4 – Cytotoxic T-lymphocyte associated protein 4

DAMPs – Damage-associated molecular patterns

DC – Dendritic cell

DMARDs – Disease modifying antirhuematic drugs

DPBS – Dulbecco's phosphate buffered saline

ELISA – Enzyme – linked immunosorbent assay

FBS – Fetal bovine serum

FSC – Forward scatter

GM-CSF – Granulocyte colony-stimulating factor

GvHD – Graft versus host disease

HGF – Hepatic growth factor

HLA – Human leukocyte antigen

HSP – Heat shock protein

IDO – Indoleamine 2,3-dioxygenase

IFN-β – Interferon- beta

IFN- γ – Interferon gamma

IL – Interleukin

ISCT – International society of cellular therapy

LPS – Lipopolysaccharide

M-CSF – Macrophage colony-stimulating factor

MCP-1 – Monocyte cehmo-attractant protein 1

MFI – mean/median fluorescence intensity

MHC – Major histocompatibility complex

miRNA – Micro RNA

mRNA – messenger RNA

MSC – Multipotential stromal cell

NF- κB – Nuclear factor kappa B

NSAIDs – Nonsteroidal anti-inflammatory drugs

OA – Osteoarthritis

P – Cell culture passage number

PADs – peptidyarginine

PAMPs – Pathogen-associated molecular patterns

PBMC – Peripheral blood mononuclear cells

PBS – Phosphate-buffered saline

PE – Phycoerythrin

PerCP – Peridinin chlorophyll protein-cyanine 5.5

PFA – Paraformaldeyde

PGE$_2$ – Prostaglandin E2

RA – Rheumatoid arthritis

S-MSCs – Synovial derived MSCs

SSC – Side scatter

STAT – signal transducer and activator of transcription

TCR – T cell receptor

TERT –Telomerase reverse transciptase

TGF-β – Transforming growth factor beta

TLR – Toll like receptor

TNF – Tumour necrosis factor

TSG-6 – Transforming necrosis factor-inducible gene 6

UCB – Umbilical cord blood

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Chapter 1 – Introduction

1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease affecting 0.5-1% of adults. In the UK, there are currently 430,000 patients living with this debilitating disease and over 21 million people worldwide. Women are affected with RA approximately three times more than men (Lin et al. 2020)

RA is a symmetrical arthritis, in that it typically it affects joints on both sides of the body in a similar pattern. RA usually manifests in the small joints of the hands and feet, first (proximal interphalangeal, metacarpophalangeal and metatarsophalangeal joints). And the disease is known as Polyarthritis as it can affect many joints in the body that become inflamed. The onset of RA is commonly between the ages of 40-60 and is characterised by inflammation and swelling of synovial joints, causing stiffness, pain and loss of mobility (Ulrich, Häupl, and Burmester 2020). While current biologic treatments have been revolutionary for the management of this painful debilitating disease, only approximately 65% of patients respond to these treatments. Moreover, even for those patients who are considered to have had a "successful" response to treatments, actually only have about a 50% reduction in symptoms, with the eventuality of most patients becoming non-responsive to treatment over time (Lin et al. 2020). Additionally, patients with RA have an increased cardiovascular risk and have a shortened life expectancy by 10 years and the disease can become very disabling (England et al. 2018). Furthermore, the involvement of the respiratory system in RA occurs in approximately 30-40% of patients and for 10-20% of them respiratory involvement is the first manifestation of the disease. Notably, 10-20% of mortalities in RA are due to these pulmonary manifestations of the disease and has been acknowledged as the second most frequent cause of death in RA patients behind cardiovascular disease (Alunno et al. 2017).

Typical clinical features of RA can vary, but includes morning stiffness in and around the joints, for a duration of at least 1 hour and typically early manifestation of RA involves the hands. Other features of the disease, includes, pain and swelling of the joints, fatigue, fever, weight loss and malaise. Along with extra-articular involvement, such as, vasculitis, rheumatoid nodules and hematologic abnormalities (Scott, Wolfe, and Huizinga 2010).

The synovial membrane surrounds the joints, which comprises a lining and sub-lining layer which, in health, is 1-3 cells thick. The synovium consists of two cell types: type A synoviocytes (fibroblast-like synoviocytes, FLS) and type B synoviocytes (macrophage-like cells) (Scott, Wolfe, and Huizinga 2010). In RA there is a heavy infiltration of immune cells in the synovium,

causing a pannus formation of the inflamed lining layer, which is an abnormal tissue growth caused by thickening of the synovium, containing activated FLS and monocytes/macrophages that facilitate the destruction of articular cartilage and bone. As discussed later in Section 1.1.3, normal synovium also hosts Synovial-Membrane Stem Cells (SM-MSCs), which, under physiological conditions, contribute to homeostasis and repair of joint tissues (C De Bari et al. 2001). In RA, as a consequence of dysregulated FLS proliferation, the relative frequency of MSCs is reduced (E. Jones, Churchman, et al. 2010). In preclinical murine models of RA, MSC intra-articular injections have been shown to exert beneficial therapeutic effects by reducing the proliferation of both innate and adaptive immune cells; however, other studies have reported that MSCs lose these immunomodulatory properties in the microenvironment of autoimmune arthritis (Djouad, Fritz, et al. 2005).

A possible explanation for the dysregulated control of the immune response by SM-MSC in RA is the effect of Toll-like receptor (TLR) activation, which is directly associated with RA pathogenesis (Huang and Pope 2009). In healthy synovium, TLR2 and 4 are scarcely detected, whereas they have greatly increased expression in the RA synovium (Radstake et al. 2004). In health, TLRs are a type of pattern recognition receptor (PRR) that play a vital role in the detection of pathogen associated molecular patterns (PAMPs) from various microbes, as part of the innate immune system. TLR2 and 4 ligands, such as Peptidoglycan and heat shock protein 22 (HSP 22) are abundantly expressed in the RA synovium and trigger TLR signalling and the secretion of pro-inflammatory cytokines by macrophages (Huang and Pope 2009). A summary of TLR activation and pathway is included (illustrated) in Figure 1.1. RA-induced cytokines, such as IL-12 and IL-18, along with IFN-γ, can also lead to an increase in TLR4 expression on MSCs which causes the induction of IL-6 and TNF expression (Radstake et al. 2004; B Bresnihan et al. 2002; Pope and Shahrara 2012). This illustrates how such licensing of SM-MSCs can reverse their immunosuppression ability, thus facilitating RA pathogenesis. Further investigation into the immunomodulatory abilities of SM-MSCs in RA is therefore required, including whether and how such functional changes occur throughout the progression of disease. This could enable exploitation of the therapeutic properties (abilities) of MSCs to modulate the immune response in RA. The main differences between a healthy and RA joint are briefly described in Figure 1.2.
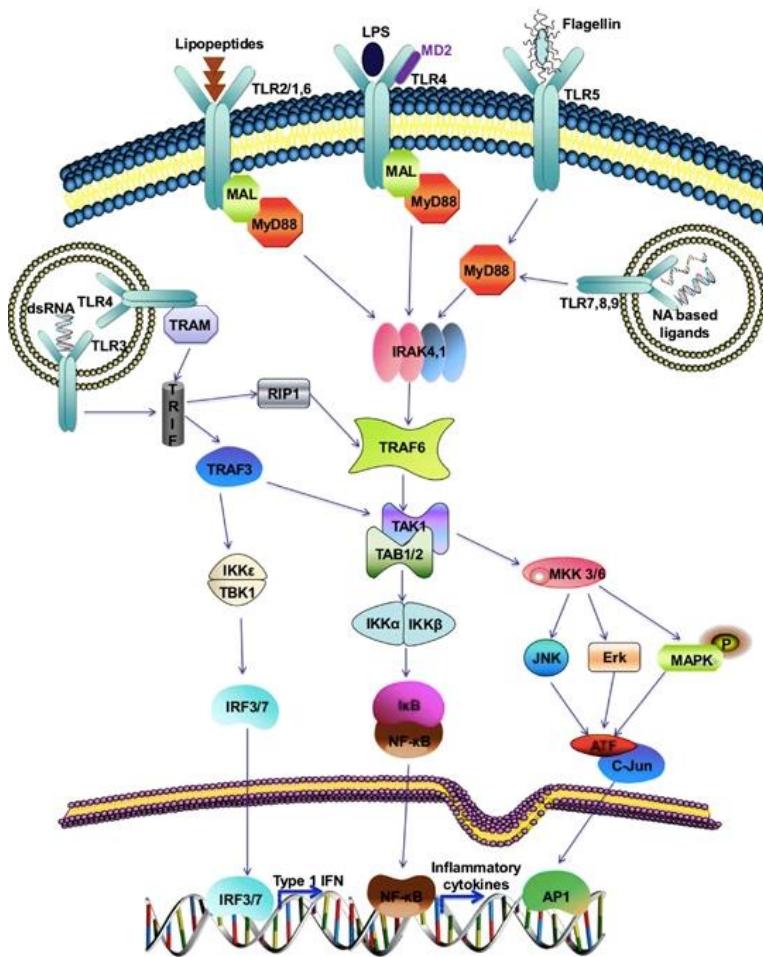
Figure 1.1 Outline of the Toll-like receptor (TLR) signalling pathway. After TLRs are stimulated with their corresponding ligands, they dimerise and recruit downstream adaptor molecules, such as myeloid differentiation primary response protein 88 (MyD88), MyD88-adaptator-like (MAL), Toll/interleukin (IL)-1 receptor (TIR)-domain containing adaptor-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM), that activate various downstream molecules causing activation of signalling cascades that come together at the nuclear factor-kB (NF-κB), interferon (IFN) response factors (IRFs) and mitogen-activated protein kinases (MAPKs). These pathways induce the transcription of various proinflammatory cytokines, like tumour necrosis factor (TNF), interleukin (IL)-6, IL-8, IL-12. Secretion of these molecules counteracts the threat posed by invading microbes and facilitates the activation of other immune components. (Ayaz et al 2013)
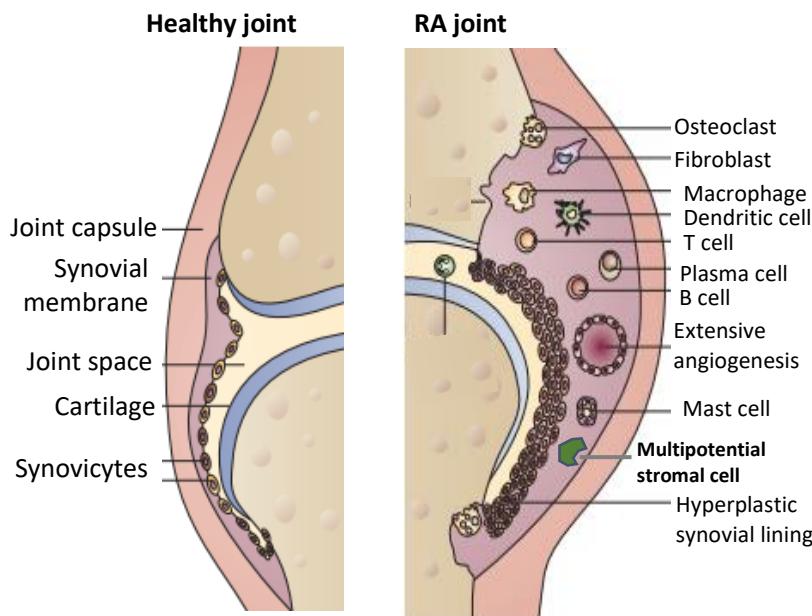
Figure 1.2 – Illustration of a normal versus RA joint (adpated from Strand, Kimberly, and Isaacs 2007)

1.1.1 Pathogenesis

RA has a worldwide prevalence of between 0.5-1% (Ulrich, Häupl, and Burmester 2020) with women being effected approximately three times more than men (Ulrich, Häupl, and Burmester 2020; Vollenhoven 2009). The etiology of RA is still largely unknown, however genetics, environmental factors, a dysregulated immune system and the microbiome all determine disease presentation and susceptibility.

RA can be classified into 2 main subtypes according to the presence or absence of anti-citrullinated protein antibodies (ACPAs) (Fang, Ou, and Nandakumar 2019; C. Y. Wu, Yang, and Lai 2020). ACPAs are autoantibodies that recognise self-peptides containing citrulline residues; citrulline is present as a consequence of post-translational modification (deamination/citrullination) of arginine, which is catalysed by peptidylarginine deiminases (PADs) (Alghamdi et al. 2019). Significantly, it is the process of citrullination that may modify the properties of self-peptides that results in their immunogenicity. Identification of ACPA in body fluids involves demonstrating their immunoreactivity with a number of cyclic citrullinated peptide (CCP) fragments of natural human proteins. This is assessed by an (the) enzyme-linked immunosorbent assay (ELISA) (Kurowska, Kuca-warnawin, and Radzikowska 2017). In this way, approximately, 67% of patients are ACPA positive and this is a helpful diagnostic measure for patients with early undifferentiated arthritis, and also gives an indication of the likelihood of disease progression to RA (Nishimura et al. 2007; Bizzaro et al. 2013). The ACPA positive cohort of patients with RA are more likely to develop a more aggressive disease

phenotype, compared with the ACPA negative subset (Malmström, Catrina, and Klareskog 2017). In the context of treatment, the ACPA negative subset have been shown to be less effective in terms of response to methotrexate (MTX), which is a conventional synthetic disease modifying antirheumatic drug (csDMARD) (Romão, Canhão, and Fonseca 2013), or rituximab, which is a biological DMARD (bDMARD) that targets CD20 on B cells, resulting in B cell depletion (Design 2014). This not only prompts the requirement to investigate alternative therapies for RA but also suggests that treatments should be stratified, dependent upon the RA subset to gain the biggest therapeutic benefit.

1.1.2 Genetics of RA

An elevated risk of RA has been correlated with genetic factors, with a high incidence of the disease amongst the native-American lineage (Hill et al. 2003). As with the case in many autoimmune diseases, the strongest genetic association in RA is with variants in the class II region of the major histocompatibility complex (MHC) on chromosome 6. Antigen presentation by MHC proteins is essential for adaptive immunity, as the MHC proteins carry out the responsibility of presenting peptides on the cell surface of antigen presenting cells (MHC II) or nucleated cells (MHC I) for T cell recognition (Wieczorek et al. 2017).

It has been shown that alleles of the HLA-DRB1 locus in the MHC of the majority of ethnic groups are associated with RA, with some alleles being associated with severe disease (e.g. HLA-DRB1 *0401,*0101, *0404,*0405 and *1402) (Seldin 2015; Geiler, Buch, and Mcdermott 2011). These HLA alleles share a conserved five amino amino sequence (QKRAA or QRRAA), known as the "shared epitope" (SE), which is very frequent among ACPA-positive patients (Gregersen, Silver, and Winchester 1987; Todd et al. 1988; Fontecchio et al. 2006). Along with the influential role of HLR-DRB1, there is growing evidence for a role of HLA-DP alleles; a single nucleotide polymorphism susceptibility (Ting et al. 2018; Hiwa et al. 2018; Z. Huang et al. 2018). Despite the strong association of RA with MHC class II alleles the shared epitope, the molecular basis for the association remains somewhat obscure. However more work is required to tease out the complex relationships between presenting SE molecules, such as HLA-DR4 and –DR1, and putative autoantigens, such as citrullinated self-peptides, in driving the pathogenesis of RA (Margulies 2018).

Apart from HLA loci, a number of non-HLA genes have also been associated with susceptibility to RA. Several studies have reported that other genetic loci are associated with RA; in particular there is are strong associations with the PTPN22 locus on chromosome 1p13 (Begovich et al. 2004) and the CTLA4 locus (Plenge et al. 2005).

Other susceptibility regions include promoter polymorphisms in cytokine genes that regulate their expression. For example, SNPs in the IL1B, IL37 and IL10 genes have been associated with increased disease susceptibility and severity in RA (Jahid, Rehan-Ul-Haq, Chawla, et al. 2018; El-Sayed et al. 2018; Jahid, Rehan-Ul-Haq, Avasthi, et al. 2018; Yuan et al. 2019).

Genome-wide association studies (GWAS) have also implicated several genetic variants in TNF superfamily genes in the development of RA, including SNPs in TNF-like ligand 1A (TL1A), TRAF1 and TRAF6 (Hassine et al. 2019) genes. Along with cytokines and growth factors, chemokines play a significant role in the inflammatory state of RA, and SNPs in their genes have also been examined (e.g. a polymorphism in the CCL4 gene; rs1719153). CCL4 is a chemokine involved in attracting monocytes, dendritic cells, natural killer cells and other immune effector cells to the site of damaged/inflamed tissue. The CCL4 gene polymorphism is linked to reduced cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) gene expression, subsequently leading to decreased risk of developing RA (Kuo et al. 2018). The CTLA-4 molecule competes with the co-stimulatory molecule CD28 for the ligands CD80 and CD86 expressed by antigen presenting cells. CTLA4 replaces the activation signals delivered by CD28 with inhibitory signalling, thereby decreasing T cell activation. Thus, the reduced CTLA-4 expression observed in the presence of certain CCL4 polymorphisms favours T cell activation and RA progression (Cutolo et al. 2016).

Other genes implicated in RA susceptibility include those involved in lymphocyte signalling, such as the CD226 gene (also known as DNAM-1), caspase genes (e.g. CASP5 rs9651713) and paraoxonase genes (PON1 L55m, rs854560) (Mosaad et al. 2018; Rui et al. 2018; Tanhapour et al. 2019). Finding out more details on how these genes are implicated is work in progress.

As well as protein coding genes, a number of microRNA molecules have also been implicated in RA progression. These are non-coding RNA molecules that are involved in the post-transcriptional regulation of gene expression. miRNAs interact via base-pairing with complementary sequences within the mRNA molecules. Consequently, they are able to "silence" these mRNA strands via mRNA cleavage and degradation, through shortening of the polyA tail of mRNA and by reducing the translation of the mRNA into protein (Brien et al. 2018). SNPs of microRNAs, such as miRNA-146a (rs2910164 and rs2710164) and miRNA-499 have been implicated in RA (rs3746444)(Shaker, El Boghdady, and El Sayed 2018; Ayeldeen et al. 2018; Fernandes et al. 2018).

There are now over 100 genetic loci associated with RA (Guo et al. 2018). However, in addition, epigenetic factors also contribute to disease susceptibility. In addition to miRNA expression, these include DNA methylation and histone modifications (Webster et al. 2018; Ai et al. 2018;

Guo et al. 2018; Scott, Wolfe, and Huizinga 2010). For example, the DNA methylation process can contribute to RA pathogenesis. Methylated genes are generally less expressed and hence there are differences in methylation regulated gene expression; Webster et al, showed a differentially variable DNA methylation signature (in the lack of differential methylation in RA) when studying discordant monozygotic twins. Therefore, this suggests that epigenetic variability plays a significant role in the development of RA (Webster et al. 2018; Ai et al. 2018).

1.1.3 The role of leukocyte populations in RA pathogenesis

As mentioned briefly in section 1.1, there is inflammation and infiltration of immune cells into the synovium of RA joints, causing a pannus formation, with destruction of cartilage and associated bone erosions. Both innate and adaptive immune cells are known to infiltrate the synovial lining layer and have been linked to RA pathogenesis (Fang, Zhou, and Nandakumar 2020; McInnes and Schett 2011).

Innate immune cells, such as monocytes, have been investigated in detail in numerous studies, in regards to their role in RA pathology. It has been shown that both RA-BM (bone marrow) and RA-PB (peripheral blood) monocytes are less mature and less differentiated BM and PB monocytes from non-RA patients (a control group with OA). These data suggest that there is a faster turnover of RA monocytes, that migrate from the bone marrow into the bloodstream then into the inflamed tissue (Smiljanovic et al. 2018). The same study also showed that the monocytes only became activated upon arrival at the joint tissue, confirming the role for local disease-specific stimuli (Smiljanovic et al. 2018). Furthermore, as will be mentioned in section 1.3.2, monocytes can differentiate into M1 pro-inflammatory macrophages and M2 anti-inflammatory macrophages. It has also been demonstrated that in RA patients that the ratio of M1/M2 macrophages positively correlates with RA disease severity and prognosis(Fukui et al. 2018), and that this greater pro-inflammatory activity is associated with disease severity. For instance, it has been observed that in ACPA-positive patients the M1/M2 ratio is increased, compared to ACPA negative patients (Fukui et al. 2018). Also, the increased M1/M2 ratio in these RA patients is positively correlated with elevated levels of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), which are inflammatory markers measured in the patients' blood. These data indicate that modulation of the monocyte/macrophage subsets represents a therapeutic target for treatment of RA disease.

Different monocyte populations impact disease activity in RA. As will be mentioned in section 1.3.1, monocytes can be divided into classical monocytes, with high CD14 but no CD16 expression (CD14++CD16- or CD14+CD16-), an intermediate subset with low expression of CD16 and high CD14 (CD14++ CD16+ or CD14+CD16+) and a non-classical subset

expressing high levels of CD16 but with much lower CD14 expression (CD14+CD16++ or CD14+ CD16+)(Fukui et al. 2018). It has been shown that the CD14++CD16+ subset is elevated in the PB of patients with RA, but decreased following anti-TNF or anti IL-6 treatment, alongside reduced disease activity (Tsukamoto et al. 2018).

A variety of soluble factors aid monocyte recruitment into the inflamed tissue of RA joints; these include the high mobility group protein (HMGB1, a damage associated molecular pattern or DAMP) and the chemokine CXCL12, which act in concert to escalate the migration of monocytes to the site of inflammation and to amplify the progression of inflammation in the diseased joint (Cecchinato et al. 2018). This suggests that down regulation of monocyte activation may be another therapeutic target for the treatment of RA, as monocytes are key players in the local inflammation and bone erosion.

Dysregulation of the adaptive immune response has also been implicated in the pathogenesis of RA; this includes both T and B lymphocytes. These cells are present in the synovium and circulate in the peripheral blood (PB). B lymphocytes secrete antibodies, including anti-citrullinated protein antibodies (ACPA) and rheumatoid factors (RFs), as well as pro-inflammatory cytokines, such as IL-1, TNF and IL-6, all of which contribute to RA progression and pathogenesis. B and T lymphocytes cooperate in that the B cells present antigen together with co-stimulatory signals to T cells and T helper cells are required for antibody production (Bugatti et al. 2014; Giltiay, Chappell, and Clark 2012; Browne 2012). The main function of T lymphocytes in RA is macrophage and fibroblast activation, thereby exacerbating their role in dysregulated tissue destruction and leading to increased production of pro-inflammatory, cytokines such as TNF, IL-1b, IL-6 and IL-15 (Cope, Schulze-Koops, and Aringer 2007). The immune cells implicated in RA and their pathogenic role are summarized in Table 1.1.

Immune Cell	Pathogenic Roles	References
Innate Immune Cell		
Macrophage	Antigen presenting cell (APC), T-Cell Activation, cytokine producer, promote angiogenesis and fibroblast proliferation	(Charles D Mills et al. 2012)
Dendritic Cell	APC and T cell Activation	(Wehr et al. 2019)
NK cells	Pro-Inflammatory cytokine producer	(Abel et al. 2018)
Mast Cells	Pro-inflammatory cytokine producer	(Mukai et al. 2018)
Adaptive Immune Cells		
B cells	Antibody producer, APC, T- Cell activation and cytokine-producer e.g. IL-4 and IL-10	(Browne et al. 2012)
T Cells	Th1- Macrophage activation, cytokine producer Th2- B- cell activation, cytokine producer, promotes Ig class switching to IgE Th17- MMP stimulation, cytokine producer, promote pannus growth, neoangiogenesis, osteoclastogenesis Treg- Suppress autoreactive lymphocytes	(Waldman, Fritz, and Lenardo 2020)

Table 1.1 – Innate and adaptive immune cells and their pathogenic roles (adapted from Yap et al. 2018)

1.1.4 Treatment of RA – biologics in RA, alternative therapies, new targets for therapy of RA

Although there is no cure for RA, at the moment, there are a number of treatments available to slow down disease progression and reduce disease activity/severity. A number of measurements are used to classify disease activity, such as the Disease Activity Score using 28 joints (DAS-28), the Clinical Disease Assessment Index (CDAI) and the Simplified Disease Activity Assessment (SDAI) (Ometto et al. 2010).

Treatments such as non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are provided to patients to relieve stiffness and pain; however, these treatments do not control disease progression (Moura et al. 2015).

Disease modifying anti-rheumatic drugs (DMARDs) are in the first line of treatment for RA and these have been shown to reduce disease activity and joint deformity. The most frequently administered DMARDs include methotrexate (MTX), hydroxychloroquine, leflunomide, sulfasalazine, azathioprine and cyclosporine (Grennan et al. 2001; Guo et al. 2018; Seegobin et al. 2014). These DMARDs each have a specific mechanism of action which basically interferes with the inflammatory cascade. For instance, Methotrexate stimulates fibroblasts to release adenosine, causes reduction in neutrophil adhesion, inhibits local IL-1 production, reduces IL-6 and IL-8 levels and inhibits cell-meditated immunity. Other DMARDs in this category aim to inhibit lymphocyte proliferation or dysfunction of them. Leflunomide works to inhibit dihydroorotate dehydrogenase which causes pyrimidine synthesis inhibition, thereby supressing lymphocyte proliferation. Hydroxychloroquine carries out its immunomodulatory capacity by inhibiting intracellular TLR-9. Whereas, Sulfasalazine exerts its anti-inflammatory effect by preventing nitrative, nitrosative and oxidative damage (Köhler et al. 2019).

Additionally, more targeted treatments are being prescribed to treat RA, such as variety of biological DMARDs based on antibodies or soluble receptors including anti-TNF (e.g. Infliximab) (Parameswaran and Patial 2012; Dulai et al. 2012; Schaible 2000) and anti-IL-6 (e.g. Tocilizumab) (Braun and Kay 2017; Navarro-Millán, Singh, and Curtis 2012; Burmester, Choy, et al. 2017; Tanaka and Kishimoto 2012). In addition, small molecule inhibitors, such as Tofacitinib, which is a Janus kinase (JAK) inhibitor (Döker, Dewenter, and El-Armouche 2014) are now being used in treatment to block signalling pathways associated with pro-inflammatory activity. Many reports have showed expression of various JAK isoforms, and the downstream signal transducer and activator of transcription (STAT) proteins in synovial tissue and synovial cells; several proinflammatory cytokines associated with RA pathogenesis bind to their cytokine receptors, which are dependent on the JAK-STAT pathway for signal

transduction (Walker, Ahern, Coleman, Weedon, Papangelis, Beroukas, Smith, et al. 2006; Walker, Ahern, Coleman, Weedon, Papangelis, Beroukas, and Smith 2006; Migita, Izumi, and Torigoshi 2013). This is an emerging field in the treatment for RA. However, it should be noted that these therapies can have some major side effects, referred to in Table 1.2.

Janus Kinase (JAK) inhibitors are the most recent class of disease-modifying medication to emerge for the treatment of RA. JAK inhibitors are small-molecule oral therapeutics that have become widely available. Three JAK inhibitors, baricitinib (Olumiant), tofacitinib (Xeljanz), and upadacitinib (Rinvoq), are approved by the FDA to treat rheumatoid arthritis. The significant discovery of the JAK and STAT components in cytokine signalling and the pathogenesis of RA has resulted in new targeted small molecules that represent low molecular mass drugs that are able to pass through the lipid bilayer of the cellular membrane. In this way, JAK inhibitors are considered as an improvement in targeted therapeutics due to their mechanism of action and oral route of delivery, which is much more appealing to the patients.

The first commercially available JAK inhibitor which was used for treatment of RA was Tofacitinib, which was developed in the mid-1990s and approved by the Food and Drug administration (FDA) in November 2012. Baricitinib and Upadacitinib were subsequently developed and received FDA approval in May 2018 and August 2019, respectively. Key differences in the JAK inhibitors include the greater selectivity of Tofacitinib for JAK1/JAK3, Baricitinib is a specific JAK1/JAK2 inhibitor and Upadacitinib only targets JAK1 pathway. (Harrington et al 2020).

Furthermore, even with these advancements, only approximately 65% of patients with RA respond to currently available treatments. Moreover, in these responders, only 50% of their symptoms are reduced and most patients cease responding to treatment over time (Horton et al. 2016). Consequently, this disease reduces life expectancy by approximately 5-10 years (Myasoedova et al. 2010). Therefore, there is a vital need to find alternative more effective therapies for over 50% of patients with RA. Other approaches, such as the use of anti-inflammatory MSCs, offer an exciting alternative route for the therapy for RA; this thesis will analyse this approach.

Classification	Name	Mechanism of action	Side effect	References
Conventional synthetic DMARDs	**Methotrexate**	**Analog of folic acid**	Increased liver enzymes, pulmonary damage	(Brown, Pratt, and Isaacs 2016)
	Leflunomide/ Teriflunomide	**Pyrimidine synthesis inhibitor**	Hypertension, diarrhea and nausea, hepatoxicity	(Bullock et al. 2019)
	Sulfasalazine	**Anti-inflammatory and immunosuppression**	Gastrointestinal, central nervous system and hematologic adverse effect	(Yee et al. 2020)
	Chloroquine/ Hydrochloroquine	**Immunomodulatory effects**	Gastrointestinal tract,skin, central nervous system adverse effect and retinal toxicity	(Rainsford et al. 2015)
Biological DMARDs（Antibody-based therapies）				
TNF targeted therapy	**Infliximab**	**TNF inhibitor**	Infection (pneumonia and atypical tuberculosis) injection-site reaction Hypertension Severe/anaphylactoid transfusion reaction	(Bullock et al. 2019)
	Adalimumab			
	Ethanercept			
	Golimumab			
B-Cell targeted therapy	**Rituximab**	**B cell depleting**	Infection, hypertension, hypogammaglobulinemia, viral reactivation, vaccination responses	(Bullock et al. 2019)
	Ofatumumab	**Inhibitors of B cell function**	Late-onset neutropenia Severe/anaphylactoid transfusion reaction	(Bullock et al. 2019)
	Belimumab			
	Atacicept			
	Tabalumab			

Classification	Name	Mechanism of action	Side effect	References
Biological DMARDs (Antibody-based therapies)				
T-cell targeted therapy	Abatacept	CD28/CTLA4 system	Infection, malignancy	(Mellado et al. 2015)
	Belatacept	CD80/CD86		
Interleukin targeted therapy	Tocilizumab	IL-6 inhibition	Infections (most notably skin and soft tissue), increases in serum cholesterol, transient decreases in neutrophil count and abnormal liver function	(Raimondo et al. 2017)
	Anakinra	IL-1 inhibition	Infection site reactions, infections, neutropenia, malignancy	(Cavalli and Dinarello 2015)
	Canakinumab			
	Rilonacept			
	Secukinumab	IL-17 inhibition	Infections, nasopharyngitis, candidiasis, neutropenia, safety data of mental health is limited	(E. K. Kim et al. 2017)
	Ixekizumab			

Classification	Name	Mechanism of action	Side effect	References
Biological DMARDs (Antibody-based therapies)				
Growth and differentiation factors	**Denosumab**	**RANKL inhibitor**	Low Ca2+ and phosphate in the blood, muscle cramps, cellulitis and numbness	(Takeuchi et al. 2019)
	Mavrilimumab	**GM-CSF inhibitor**	Safety profile needs further research	(Burmester, McInnes, et al. 2017)
Small molecules				
JAK pathway	**Tofacitinib**	**JAK1 and JAK3 inhibitor**	Zoster infection (advice is to vaccinate beforehand) and other potential side-effects should be monitored	(Winthrop et al. 2017)
	Baricitinib	**JAK1 and JAK2 inhibitor**		
	Filgotinib	**JAK1 inhibitor**		

Table 1.2 – Summary of current therapies used to treat RA, their mechanism of action and side effects (adapted from Guo et al 2018)

1.1.5 Key cytokines in RA: TNF and IL-6

Tumour Necrosis Factor (TNF) is a pro-inflammatory cytokine that was discovered by Lloyd J Old in 1975, being primarily identified for its tumour cytotoxicity (Carswell et al. 1975). However, it is now known to elicit inflammatory responses and is produced by T lymphocytes, NK cells, activated monocytes and macrophages. Also, TNF has involvement in many normal physiological functions in health and homeostasis and microbial immunity (Parameswaran and Patial 2012; Seymour and Henderson 2001; Sandler et al. 2007) TNF is a 26 kDa transmembrane protein expressed on the cell surface. This cell surface TNF can be cleaved (via the action of TNF alpha converting enzyme TACE, also known as ADAM17) and released into the extracellular environment as an active 17 kDa secreted factor (Solomon et al. 1999). TNF binds to TNF receptors 1 and 2 (TNFR1 and TNFR2) and activates key signalling pathways, such as the NF-κB pathway, RANK signalling, the ERK signalling pathway, the proapoptotic pathway and tumour progression locus 2 (TPL2) pathway. (Bazzoni and Beutler 2002). The TNFR1 molecule is expressed on all cell types, whereas TNFR2 is expressed mostly on immune cells and endothelial cells (Cope, Schulze-Koops, and Aringer 2007; Tetta et al. 1990) Notably, these TNFRs are also capable of being cleaved from the cell membrane to generate a soluble form. These soluble receptors (sTNFR), then behave as antagonists, via binding to soluble TNF, consequently preventing TNF from binding to the cell-surface TNFR and exerting their pro-inflammatory functions (Solomon et al. 1999).

TNF and TNFRs are expressed at numerous locations within the synovial membrane, and are secreted locally by synovial lymphocytes and macrophages that infiltrate the joint synovium (Deleuran et al. 1991). Increased levels of TNF and sTNFR have been observed in the synovial fluid (Cope, Schulze-Koops, and Aringer 2007; Tetta et al. 1990). Furthermore, TNF plays a fundamental role in the induction and growth of fibroblast like synoviocytes (FLS), along with, metalloproteinase production, and angiogenesis and, thus, it orchestrates joint damage and destruction (Brennan and McInnes 2008).

In the 1980s and 90s, work by Feldmann et al, demonstrated that TNF blockade resulted in the inhibition of many other pro-inflammatory cytokines, such as IL-1, IL-6 and IL-8 (Feldmann and Maini 2008; Elliott et al. 1994; Brennan et al. 1989). This is because TNF production is a very early event in the proinflammatory cascade and, thus, blocking TNF action has considerable effects on downstream cytokines. This has highlighted TNF as a key target for treating RA, when considering new alternative treatments, such as MSC therapies (Simon and Yocum 2000; Augustsson et al. 2006; Emi Aikawa et al. 2010; Combe 2008). As discussed in Section 1.1.4, although key biologics, such as TNF inhibitors have revolutionised the management of RA, and their side effects are usually mild but they still may have some major

side effects, due to suppressing immune function and are only effective in treating a percentage of patients, who, over time, may also cease to respond to therapy(Ja et al. 2013).
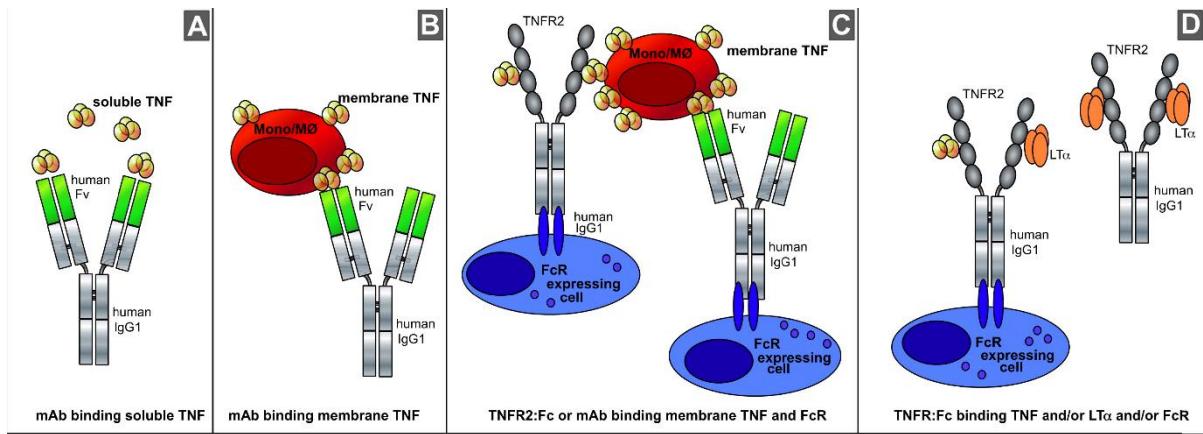
Figure 1.3 – Diagram showing mechanism of action of anti-TNF biologics. Anti-TNF biologics bind and neutralise soluble TNF **(A)** and membrane TNF **(B)**. Additionally, they co-engage with Fc-R expressing cells **(C)** (Sedger and McDermott 2014).

Interleukin 6 (IL-6) is a 26 kDa glycoprotein and has pleiotropic activity. Initially, it was identified as B cell stimulatory factor (BSF-2), a T cell-derived, soluble factor, which triggers the induction of differentiation of activated B cells into antibody producing cells (Kehrl et al. 1984; T Kishimoto 2003). Subsequent studies have revealed that IL-6 performs a number of essential functions in inflammation, immune regulation and oncogenesis. It has been highlighted as a key player for the progression of numerous chronic inflammatory and autoimmune diseases, including RA (Hirano et al. 1990; Akira, Taga, and Kishimoto 1993; Tadamitsu Kishimoto 2005; Ogata et al. 2013).

The IL-6 signalling system is activated through IL-6 binding to an 80 kDa transmembrane IL-6 receptor (IL-6R); this complex then associates with gp130 (signal transducing molecule)(Yamasaki et al. 1988; Tadamitsu Kishimoto, Akira, and Taga 1992). Consequently, this results in the activation of the JAK/STAT signalling pathway in cells (Lütticken et al. 1994; Rose-John 2012). This particular signalling pathway is known as the classical signalling pathway (Rose-John 2012).

Additionally, a soluble version of IL-6R exists in serum and has a comparable affinity to IL-6 as transmembrane IL-6R. The soluble IL-6R and IL-6 complex can also bind to gp130, causing activation of the signalling cascade - this is called the trans-signalling pathway (Rose-John 2012). Increasing evidence indicates that the IL-6 classic signalling pathway is required for regenerative or anti-inflammatory activities, while the trans-signalling pathway is pro-inflammatory (Rose-John 2012)

IL-6 is secreted by a range of cell types including T cells, B cells, fibroblasts and monocytes. In patients with RA, IL-6 levels are increased in the synovial fluid; monocytes are known to be the key producers of IL-6, as well as TNF (Hirano et al. 1988; HOLT, COOPER, and HOPKINS 1991; Dasgupta et al. 1992; Madhok et al. 1993). In RA pathogenesis, IL-6 promotes pannus formation, via elevated vascular endothelial growth factor (VEGF) expression and increased bone resorption, due to osteoclastogenesis and oxidative stress in leukocytes (Kotake et al. 2009; Suzuki et al. 2010; Nakahara et al. 2003). These activities highlight IL-6 as another crucial target for the treatment of RA that might provide longer term remission and fewer side effects compared to other current biologic drugs.
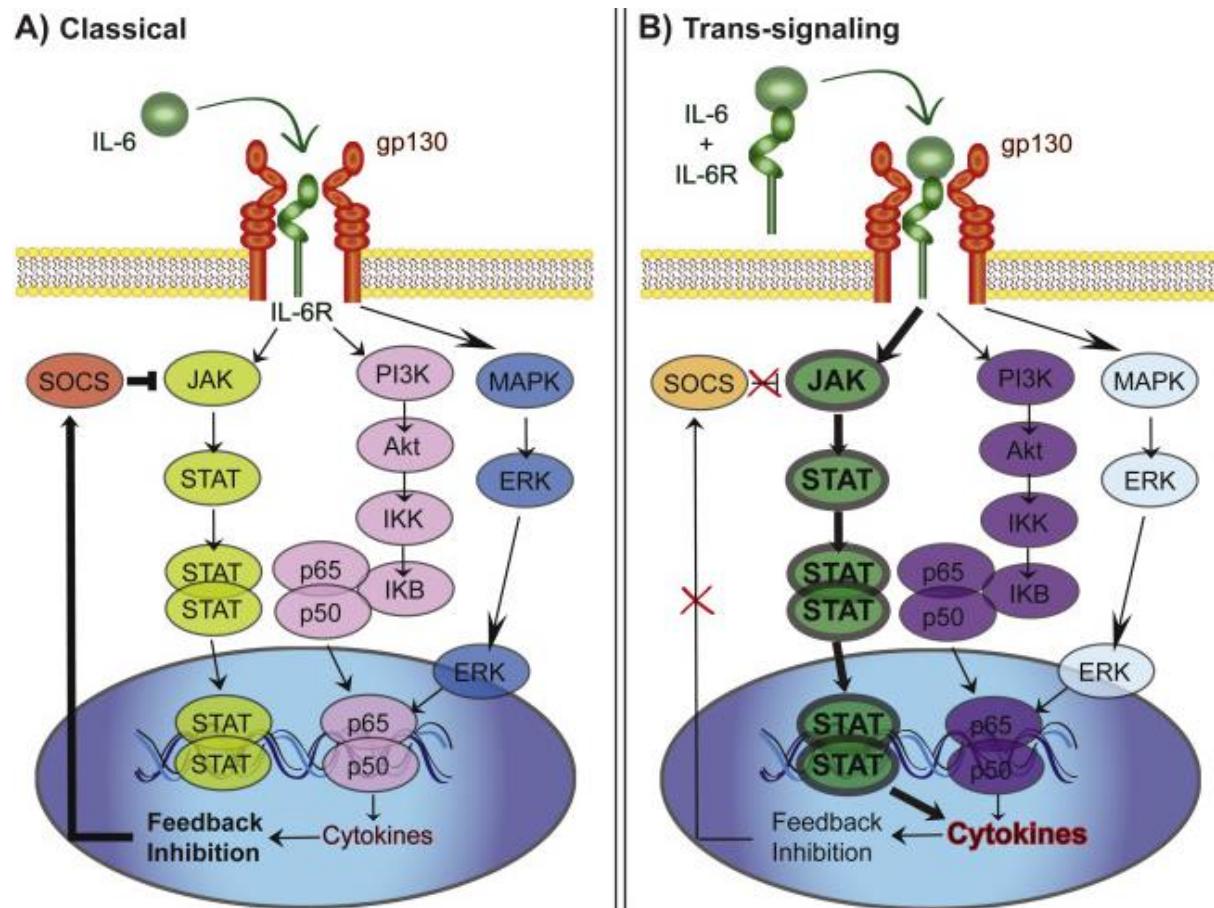
Figure 1.4 – Schematic diagram showing comparing molecular profiles of classical IL-6 signalling and trans-signalling pathways. **A)** The classical signalling pathway results in activation of the JAK/STAT, AKT and MAPK pathways to modify transcription. After the release of cytokines, SOCS1 provides a negative feedback loop. **B)** The trans-signalling pathway via suppression of SOCS1 and changes to NF-kB, causes further JAK/STAT signalling than MAPK. This increased activation of JAK/STAT and lack of feedback inhibition by SOCS1, results in increased transcription of pro-inflammatory cytokines. (Russo, Hodes, and Caroline 2016; Ja et al. 2013)

1.2 Multipotent stromal cells (MSCs)

Multipotent stromal cells are a heterogeneous population that are present throughout the body and have self-renewal, differentiation and regenerative capabilities, as well as immunoregulatory capacities (Ma et al. 2014). As a minimum, MSCs are characterised by their ability to differentiate into chondrocytes, adipocytes and osteocytes. These cells play an important role within the joint, including the regeneration/maintenance of cartilage, adipose storage, hormone production and bone formation and their dysregulation is thought to play a role in various inflammatory articular diseases, including RA).

As mentioned in section 1.1, RA is characterised by synovial joint inflammation and erosion of articular cartilage, leading to disabling joint deformities and loss of function in multiple joints (Klareskog, Catrina, and Paget 2009). Currently, there are no treatments that can restore or reverse cartilage/bone erosions. Therefore, there remains a pressing need to identify novel methods to prevent/reverse cartilage destruction in this diseases, and MSCs were originally proposed to be used for the treatment of RA, based on their cartilage and bone-regeneration potentials (Sutton and Bonfield 2014). This study will focus on the potential exploitation of the immunosuppressive abilities of MSCs for treatment of RA.

Friedenstein and colleagues were first to observe that a rare fibroblast-like cell population could be derived from the bone marrow (BM)(Friedenstein, Chailakhjan, and Lalykina 1970). In culture over a period of time, these single colony-forming cells (also termed units) divided prolifically and produced expanded populations of fibroblastic clones. These fibroblast-like cells were plastic-adherent and the adherent cultures were later denoted as MSCs, because they could be stimulated, both in vitro and in vivo, to differentiate into the mesenchymal-lineage cells, namely osteoblasts, adipocytes, chondrocytes, and connective stromal cells, such as ligamentous fibroblasts (Kopen, Prockop, and Phinney 1999).

The pleiotropic characteristics of MSCs have consequently led to some discrepancies in their identification. However, some common features, shared by cultured MSCs, are used to aid in their identification, as agreed by the International Society for Cellular Therapy (ISCT). The ISCT outlines the minimal criteria for defining MSCs as; adherence to plastic in standard culture conditions, in vitro differentiation into osteoblasts, adipocytes and chondroblasts, specific surface antigen expression, in which 95% or more of the cells express CD105, CD73 and CD90, whilst lacking the markers CD45, CD34, CD14/CD11b, CD79a/CD19 and HLA-DR (2% or less). These criteria are applicable to both human and mouse MSCs (Dominici et al. 2006). To date, there is no single surface marker that is unique to MSCs. Differences in expression of markers exist also between in vitro and in vivo settings, as well as between species and tissue source of MSCs (Boxall and Jones 2012). Tables 1.3 and 1.4 describe the ISCT MSC phenotypic markers and their functions.

Positive MSC marker	Function
CD73/5' nucleotidase (Pittenger 1999)	Involved in the catalysis of AMP to extracellular adenosine (Resta, Yamashita, and Thompson 1998)
CD90/Thy1 (Colter, Sekiya, and Prockop 2001)	Involved in cell matrix and cell-cell interactions Involved in wound repair (Rege and Hagood 2006)
CD105/ Endoglin (Pittenger 1999)	Involved in interactions with TGFb receptors to modulate MSC response to TGFb influence. Has a strongly pro-angiogenic role (Duff et al. 2003)

Table 1.3 – Positive MSC surface markers, as stipulated by ISCT and their proposed functions on the MSC cell surface.

Negative MSC markers	For the exclusion of:
CD34 (Colter, Sekiya, and Prockop 2001)	Endothelial cells and primitive hematopoietic cells (Fackler, Civin, and May 1996)
CD45 (Colter, Sekiya, and Prockop 2001; Pittenger 1999)	Leukocytes (Hermiston, Xu, and Weiss 2003)
CD14 (Colter, Sekiya, and Prockop 2001; Pittenger 1999)	Monocytes/macrophages (Mazzone and Ricevuti 1995)
CD79 and CD19 (Lubis et al. 2011)	B cells (Tabera et al. 2008)
HLA class II (Lubis et al. 2011)	Lymphocytes and antigen presenting cells (Thorsby 2009)

Table 1.4 – 'Negative' MSC surface markers, for exclusion of other cell lineages.

MSCs represent (are) a small population of cells in the bone marrow, known to represent between 0.01% and 0.03% of all nucleated bone marrow cells As this modest percentage of cells decreases further with age, there is a necessity to isolate MSCs from other tissues, besides the bone marrow (some different sources of MSCs are discussed below), (Laranjeira et al. 2012; Caplan 2007). MSCs can be sourced from dental pulp, adipose tissue, fetal tissues, skin, lung, peripheral blood, and synovial membrane (Caplan 2007).

The ideal source of MSCs used for each particular disease is an important matter; there is evidence that MSCs from different sources may have an influence on the clinical outcome. It

has previously been shown, that human MSCs isolated from different tissues, and tested under the same conditions, provided differential immunosuppressive capacities (Secco et al. 2009). Even with the common phenotypic features and main functional characteristics that differentiate between MSCs from different tissues, it is acknowledged they present differing characteristics, which are ultimately related to their function, e.g. in their immunosuppressive capabilities. Also, it is known that culture conditions during cell expansion can impact MSC phenotype and functional characteristics. This would be expected when considering their high sensitivity to micro-environmental cues and may be one of their key features, which may provide a clue to their success as an alternative therapy for RA. Another factor to consider is the inter-donor variability regarding the MSCs' immunosuppressive ability, which has been also observed (Secco et al. 2009).

Overall, these considerations cause complications in evaluating the results from different studies, and highlight some key issues regarding the optimal protocol in MSC isolation for clinical use. This is evident in studies that compared MSCs isolated from a variety of tissue sources of the same donor, such as UCM-MSC and UCB-MSC; and from dental pulp and periodontal MSC, derived from tooth ligament (Secco et al. 2009). These factors must be considered when comparing MSC functionalities between studies/experiments. For all these reasons above, it is imperative to compare qualitatively MSC sources, passages, batches and donors.

1.2.1 Bone Marrow derived MSCs (BM-MSCs)

MSCs can be derived from the bone marrow, alongside haematopoietic stem cells (HSCs). The first report describing BM-MSCs was published in 1966 by Friedenstein and colleagues, whereby the isolated cells were derived from murine bone marrow and, for the first time, cultured in vitro to form fibroblastic colonies (Friedenstein, Chailakhjan, and Lalykina 1970). Subsequent studies, for example, by Castro-Malaspina et al.(Castro-Malaspina et al. 1980), and Fei et al. (Fei, Penn, and Wolf 1990) provided an enhanced description of the biological characteristics of BM-MSCs in vitro, such as their fibroblast-like morphology, proliferative abilities and absence of the basic properties of endothelial cells and macrophages (other types of plastic-adherent cells from the BM). Following on from this, the multipotent differentiation abilities of BM– MSCs into osteogenic, adipogenic and chondrogenic lineages were established (Castro-Malaspina et al. 1980; Fei, Penn, and Wolf 1990; Ashton et al. 1980; Bennett et al. 1991). Furthermore, BM-MSCs are considered to form the micro-environmental niche for HSCs, via their secretion of cytokines and growth factors (Patt, Maloney, and Flannery 1982; Bennett et al. 1991; Wexler et al. 2003). In comparison to other sources of MSCs, BM-MSCs are present at a much lower frequency (Patt, Maloney, and Flannery 1982)

and have been demonstrated, in one particular study, to be the least potent in proliferation potential compared to MSCs derived from other sources (Yoshimura et al. 2007). The methodology for the isolation of BM-MSCs is different to that used for their isolation from other sources. This is primarily due to the minimal collagenous extracellular matrix present in the bone marrow. Thus, collagenase digestion is not carried out, but mechanical disruption (via pipetting) is used to produce a suspension of stromal and hematopoietic cells. Once the BM-MSCs are plated, they quickly adhere to plastic (Bianco et al. 2001). However, the adherent BM-MSC population remains a 4 heterogeneous population of cells (Bianco et al. 2001) and constitutes a variety of tri-, bi- and uni-potent cells at different frequencies (Muraglia, Cancedda, and Quarto 2000). This is as a result of individual MSCs being at different stages of maturity. Furthermore, the heterogeneous nature of MSC cultures is additionally affected by the methodology used for their isolation and passage number. Regardless of this known heterogeneity, numerous publications have now reported on the scope of the differentiation potential of MSCs to adipose, tendon, muscle, cartilage, and bone (Dennis et al. 1999)(Young et al. 1998; Galmiche et al. 1993; Kadiyala et al. 1997; Bruder, Jaiswal, and Haynesworth 1997).

1.2.2 Adipose derived MSCs (ASCs)

Zuk et al., in 2001, isolated adipose-derived MSCs (ASCs) and demonstrated them to be a desirable alternative source of MSCs compared to BM-MSCs (P. a Zuk et al. 2001). The ASCs are obtained via liposuction of fat, and carried out under local anaesthesia. The liposuction aspirates are processed to obtain the fibroblastic fraction (processed lipoaspirate). The processed lipoaspirate, when cultured in vitro, generates cultures similar to BM-MSCs (P. a Zuk et al. 2001). The surface markers expressed by ASCs are CD13, CD29, CD44, CD71, CD90, CD105, CD73, and STRO-1. They lack the expression of haematopoietic lineage markers CD14, CD16, CD31, CD34, CD45, CD56, CD61, CD62E, CD104, and CD106 (P. A. Zuk 2010). Although ASCs were only identified relatively recently, their ease of harvest, and abundance, place them in a unique position relative to other types of MSCs.

1.2.3 Synovial MSCs (S-MSCs)

MSCs have been identified in the synovial membrane (SM-MSCs), almost 20 years ago (C De Bari et al. 2001). The synovial membrane is also a source of relatively homogeneous, fibroblast-like, multipotent MSCs. Synovial-derived MSCs have very similar phenotypic characteristics to that of type B synoviocytes, as they contain characteristic lamellar bodies and express surfactant protein A (Vandenabeele et al. 2003). SM-MSCs are CD34−, CD45−, CD31−, CD14− and CD44+, CD73+, CD90+, CD105+, a phenotype similar to that of MSCs

derived from other tissues of origin (C De Bari et al. 2001; Vandenabeele et al. 2003). SM-MSCs differentiate into adipogenic, chondrogenic, osteogenic and myogenic lineages (C De Bari et al. 2001; Djouad, Bony, et al. 2005). SM-MSCs have been shown to be superior to bone- marrow and adipose tissue, with regard to colony number per nucleated cell, colony number per adherent cell, and cell number per colony (Yoshimura et al. 2007). Notably, SM-MSCs have shown the highest ability for differentiation to chondrocytes, making them the preferred candidate for cartilage regeneration, compared with MSCs from other sources (Yoshimura et al. 2007). Additionally, MSCs can also be found in the synovial fluid (E. A. Jones et al. 2004) and from joint ligaments, menisci and adipose tissue (Barry and Murphy 2013).

Synovium can be obtained arthroscopically and, due to the high regenerative capacity of the synovial membrane, donor site morbidity is low (Sakaguchi et al. 2005). It must be highlighted that, in comparison to BM-MSCs, the role of immunomodulation by synovial MSCs has been less clearly documented (not least because of the logistic and technical difficulties associated with their isolation). SM-MSC thus provide a novel and exciting area of study that this thesis aims to investigate.

1.2.4 MSCs as a therapy

There has been an increasing interest around the therapeutic potential of MSCs. Initially this was, from the perspective that MSCs have the ability to differentiate into chondrocytes, osteocytes and adipocytes and might thus be able to aid the regeneration of damaged tissue. This can be achieved with MSCs by replacement of the damaged cells or by secretion of trophic factors (Augello and De Bari 2010). Additionally, MSCs have been shown to demonstrate an immunomodulatory effect in treating many diseases. They have reported to have a potent immunomodulatory impact in the treatment of autoimmune and inflammatory diseases, where the immune response has become exacerbated and dysregulated (Ma et al. 2014; Griffin et al. 2013; P.-M. Chen et al. 2014; Luque-Campos et al. 2019).

MSCs have shown the ability to migrate to injured tissue and inflamed sites and to differentiate in a tissue-specific manner. This contributes to the modulation of immune response and also influences the behaviour of the neighbouring cells.

MSC, whether delivered locally or systemically, are able to retain their characteristics and provide an alternative therapy, as a cell-based treatment for autoimmune diseases, such as RA. They have been examined for the treatment of a broad spectrum of diseases that are associated with a variety of aetiologies and pathophysiologies, from myocardial infarction, spinal cord injury, wound healing, multiple sclerosis to type 1 insulin dependent diabetes,

GvHD and arthritis (Fernández Vallone et al. 2013; Ennis, Sui, and Bartholomew 2013; Sutton and Bonfield 2014; Ankrum, Ong, and Karp 2014)

The therapeutic potential MSCs has been demonstrated in the many clinical trials that are underway for the treatment of numerous diseases. At the time of writing, there are a total of 972 MSC-related trials registered on the NIH Clinical Trials Database (https://clinicaltrials.gov/). These include 251 clinical trials targeting autoimmune diseases, of which 52 are clinical trials targeting RA.

However, an ongoing concern is the long-term safety of MSC-based therapy, due to the risk of malignant transformation. Furthermore, some studies suggest that MSCs may favour the growth and metastatic potential of existing tumours (Luo et al. 2018). Another significant factor to consider in MSC therapy is the length of time of the treatment. As Lucchini et al. suggests, the response to MSC treatment can vary depending on different time points of MSC infusion in their clinical history, even within the same patient. (Lucchini et al. 2010). In other words, the clinical response is related to time of infusion.

Another issue to be considered for MSC therapy is whether allogenic or autologous MSC should be utilised. In theory, autologous MSCs will not trigger a donor specific immune response, unlike allo-transplants. However, even MHC-mismatched MSC are able to carry out immunosuppressive effect on T cells (Haddad and Saldanha-Araujo 2014). Thus, MSC are considered to be "immune privileged" cells and a promising alternative cellular therapy. On the contrary, more recent reports indicate that MSCs may not actually be immune privileged, with studies showing that allogenic MSCs triggers both a B and T cell response (Lohan et al. 2017). Whether rejection of donor MSC influences the efficacy of allogenic MSC is yet to be determined. It should be emphasised that, to date, no clinical advantage has been shown for use of autologous MSC over allogenic MSC. Nevertheless, it is necessary to remember that culture expanded MSC consist of a heterogeneous population expressing varying phenotypes and functional properties.

Furthermore, an autologous MSC transplant may not be not be optimal for an already unwell patient, for example with GvHD. Using, the patient's own BM-MSC could be detrimental, because a malignant cell may be existing. Furthermore, isolation of MSC from myelodysplasic patients had the same genetic alterations, whereas, MSCs isolated from patients with other diseases showed no genetic changes (Corradi et al. 2018). In addition, bone marrow MSCs are a rare population, and with acute illness, collection of autologous MSC would not be feasible. A readily available source of allogenic MSC, suitable for multiple recipients, would be a distinct advantage. The use of MSC as a cellular therapy has further potential, in that

MSCs can be manipulated in culture to modify their phenotype to target a particular disease type (e.g. by using transgenes, RNA interference or CRISPR/Cas9 technology).

Additional factors that require consideration for successful use of MSC as a cellular therapy are the route of administration, optimal time for administration, MSC dose, number of infusions and cell preparation techniques. Further research is required to establish the optimal protocol for specific diseases that can benefit from MSC-based therapies.

1.3 Monocytes/Macrophages

Monocytes are mononuclear phagocytic leukocytes that have common morphological features, including, for example, a spherical cell shape, a kidney/ovoid shaped nucleus, a high cytoplasmic to nuclear ratio and azurophilic granules. These monocytic cells originate from the BM, where they develop from a hematopoietic stem/progenitor cell and progress through the cell lineage of the common myeloid progenitor (CMP) (Akashi et al. 2000), the granulocyte-macrophage progenitor (GMP) (Fogg et al. 2006), the common macrophage and dendritic cell precursor (MDP) (Akashi et al. 2000) and, finally, the committed monocyte progenitor (cMoP).

Monocytes circulate in the blood, and are present in the BM and spleen; they constitute ~10% of the total leukocytes in humans. Monocytes circulate in the bloodstream, for up to 1-2 days, following which they are recruited into tissues throughout the body. When they're not recruited to tissues they die and are removed. Monocytes provide an essential reservoir of myeloid precursors for the renewal of tissue-resident macrophages, although, many macrophage subpopulations (for instance, brain microglia) develop directly from the BM, independently of monocytes (Ajami et al. 2007).

Recruited monocytes have been shown to be innate effectors of the inflammatory response to microbes, and orchestrate the killing of pathogens by phagocytosis, production of reactive oxygen species (ROS), nitric oxide (NO) and inflammatory cytokines (Serbina et al. 2008). In some instances, monocytes can also trigger and polarise T-cell responses (Serbina et al. 2008; Evans et al. 2009), which may further contribute to their role in RA pathogenesis.

1.3.1 Human Monocyte subsets

Heterogeneity in the monocytic population has led to the classification of three functional subsets of human monocytes. This nomenclature has grouped the monocytes into three subsets based on the expression of CD14 and CD16 surface markers and has been approved by the Nomenclature Committee of the International Union of Immunologic Societies (H. W. L. Ziegler-Heitbrock and Ulevitch 1993; Coulthard et al. 2012). The main population of human

monocytes (90%) are termed classical monocytes, with high CD14, but no CD16 expression (CD14++CD16- or CD14+CD16-). The remaining 10% is further divided into an intermediate subset with low expression of CD16 and high CD14. (CD14++ CD16+ or CD14+CD16+) and a non-classical subset expressing high levels of CD16 but with much lower CD14 expression (CD14+CD16++ or CD14+ CD16+) (L. Ziegler-Heitbrock et al. 2010). The main cytokines released by the classical monocytes, depending on stimulus received, are proinflammatory (e.g. TNF and IL-6), yet they can also produce anti-inflammatory cytokines such as IL-10 (Skrzeczyńska-Moncznik et al. 2008). The CD14dim monocytes with high CD16 expression, are the predominant TNF and IL-1b producing monocytic cells (Belge et al. 2002; Coulthard et al. 2012).

The CD14 marker present on the cell surface of monocytes is a co-receptor (along with Toll like receptor 4) for bacterial lipopolysaccharide (LPS), acting in conjunction with LPS- binding protein (LBP) (H. W. L. Ziegler-Heitbrock and Ulevitch 1993). CD16 is the low affinity Fc receptor for IgG, involved in orchestrating antibody cell–mediated cytotoxicity (ADCC) (Boot, Geerts, and Aarden 1989).

Tissue-resident macrophages are heterogeneous cells that show great plasticity and are found in all tissues throughout the body. The ability of macrophages to migrate into, and specialise their function in certain microenvironments, accounts for their heterogeneity. Macrophages resident in different tissues are thus given different names accordingly (e.g. osteoclasts in bone, alveolar macrophages in the lung, microglial cells in the CNS and Kupffer cells in the liver). Certain key functions are universal for all tissue-resident macrophages. These include being involved in the regulation of tissue homeostasis (via senescent or apoptotic cell clearance), tissue architecture and immune responses to pathogens, via initiating and resolving inflammation (Swirski et al. 2009; Nguyen et al. 2013; Epelman, Lavine, and Randolph 2014; Maus et al. 2002).

During the initial stages of an inflammatory response there is the recruitment of blood monocytes into the tissues, driven by resident macrophages alongside other tissue cells. Recruited blood monocytes are a source of inflammatory macrophages, also known as monocyte-derived inflammatory monocytes or BM-derived macrophages. Additionally, there is an increase in the proliferation of tissue-resident macrophages due to their ability to self-renew (Hashimoto et al. 2013).Once monocytes have undergone tissue recruitment they are polarised by the local microenvironment and differentiate into the distinct resident macrophages, under varying tissue-specific cues. Differentiation of these monocytes, according to various site-specific cues, results in substantial phenotypic and functional alterations, thereby creating a diverse range of macrophage subpopulations throughout the

body (L. Ziegler-Heitbrock et al. 2010). Under steady-state conditions, macrophages are involved in homeostatic processes for tissue development and healing, through their phagocytic activity, angiogenic, growth factor production and via their secretion of proteolytic enzymes to degrade the extracellular matrix (ECM). Contrastingly, in inflammatory responses, TLR and inflammatory cytokine signals skew the functional characteristics of macrophages towards pathogen defence and immunity, including phagocytosis, MHC class II-mediated antigen presentation, secretion of pro-inflammatory cytokines and generation of cytotoxic reactive oxygen and reactive nitrogen species (ROS/RNS) (Sica and Mantovani 2012). These varying functional capacities of macrophages, depending on the polarisation cues received in the local microenvironment, enable their categorisation into two distinct groups; classically activated M1 macrophages (pro-inflammatory) and alternatively activated M2 macrophages (anti-inflammatory) (Sica and Mantovani 2012). This classification is largely for convenience and is mostly based on data from mice. In humans, macrophages exist along a spectrum, with M1 and M2-like cells at the extremities (Bertani et al. 2017; Yang et al. 2014)

1.3.2 Macrophage polarisation

In vitro, M1 macrophages are generated by interferon gamma (IFNγ) stimulation, either alone or in combination with other cytokines, such as TNF and granulocyte-macrophage colony-stimulating factor (GM-CSF)(Lacey et al. 2012). Additionally, M1 macrophages can be activated via microbial stimuli, such as the TLR4 ligand, LPS. Contrastingly, M2 macrophages are activated according to their M2 subsets (Mosser and Edwards 2008); M2a macrophages are activated via Interleukin (IL)-4 and IL-13 whereas M2b macrophages are activated by immune complexes (IC), LPS or IL-1R ligands and M2c macrophages are induced by IL-10 and glucocorticoids (Martinez and Gordon 2014; L. xun Wang et al. 2019).

The main functions of M1 macrophages are in T-helper 1 (Th1) activation, via their enhanced ability to present antigens and their increased levels of inflammatory cytokine secretion, namely IL-1β, IL-6, IL-12, IL-23, and TNF (C D Mills et al. 2000). Thus, M1 macrophages have cytotoxic "killer" functions towards phagocytosed tumour cells and intracellular pathogens, with their cytotoxic effector functions being carried out by releasing toxic molecules, such as reactive oxygen intermediates, nitric oxide and TNF (Martinez and Gordon 2014; Le xun Wang et al. 2019).

By contrast, M2 macrophages have low antigen presentation capacity, and are characterised as having a low IL-12, but high IL-10 cytokine production, resulting in their minimal ability to elicit an inflammatory and Th1 adaptive immune response. Instead, M2a and M2b

macrophages elicit Th1 suppression and T-helper 2 (Th2) activation respectively and, therefore, M2 macrophages may play a role in defence against parasitic infection. Thus, M2b (and also M2c macrophage have key functions in immunosuppression, tissue remodelling and wound healing (Mosser and Edwards 2008). However, under pathological conditions, M1 macrophages act to promote chronic inflammation, tissue damage and autoimmunity; the mechanisms by which they do this are unclear. Additionally, M2 macrophages can play a significant role in cancer, fibrosis and epithelial hyperplasia (Wynn, Chawla, and Pollard 2013).

The polarisation of macrophage is achieved via different activation mechanisms through which macrophages can carry out their functions. Macrophages are able to regulate their metabolic functions, from wound healing/growth (M2 macrophages) to a killing/inhibitory function (M1 macrophages) (Charles D Mills 2012a; MacMicking, Xie, and Nathan 1997). The key discriminating factor between these subsets is that in M2 macrophages, there is metabolism of arginine produces ornithine and polyamines, yet in M1 cells it results in formation of nitric oxide (NO) and citrulline (Charles D Mills 2012b). NO produced by M1 macrophages is a key molecule for microbicidal and inhibition of cell proliferative functions (MacMicking, Xie, and Nathan 1997) whereas ornithine, produced by M2 macrophages, promotes cellular proliferation and repair. Understanding of the metabolic status of macrophages not only aids in characterising the distinct functions of the macrophages, but it also gives rise to potential therapeutic targets in disease. For instance in M1 macrophages, succinate (a Krebs cycle intermediate) regulates HIF-1α, which is involved in the production of the pro- inflammatory cytokines, like IL-1β (Tannahill GM, 2013) whereas, in M2 macrophages the metabolic programmes triggered are the electron transport chain and fatty acid β-oxidation, providing anti- parasitic and wound repair phenotypic functions (Epelman, Lavine, and Randolph 2014).

It should be noted, that the literature varies on the definition of macrophage polarised subsets, with in vitro characterisation not necessarily translating to the in vivo setting, reflecting the oversimplification of the M1-M2 classification. However, the macrophage subpopulations have been more clearly described in murine models (Hamilton 2008; Hamilton and Tak 2009).

Monocyte/macrophage subset	Surface markers	Activation Stimuli	Cytokines/Chemokines & receptors	Effector molecules	Key functions	Alterations in RA
Human Monocytes						
Classical	CD14++ CD16- CD14++ CD16-	LPS	CCR2+ CXC3CR1-	ROS	"Inflammatory" monocytes, Phagocytosis	Increased significantly in blood of patients in both early and late RA (Coulthard et al. 2012).
Intermediate	CD14++ CD16+	LPS	CCR2- CX3CR1+ TNF IL-1b		"Inflammatory" monocytes	Increased significantly in blood of patients in both early and late RA (Coulthard et al. 2012).
Non-classical Macrophages	CD14+CD16++	TLR7/8 dependent stimuli	CCR2- CXCR1+ IL-10		"Alternative/patrolling" monocytes- survey endothelium for pathogens & tissue damage	CD14dim monocyte subset significantly only in late RA (Coulthard et al. 2012).
Human Macrophages						
<u>M1</u>	CD68, CD86, TLR2, TLR4, CD16, CD32, CD64 (FcgI,II,III)	IFNgTNF, LPS	TNF, IL-1, IL-6 , IL-12, IFN, IL-1-R,CXCL9,CXCL10, CCR2, CCR7	iNOS, (ROS),ROI	Cytotoxic "killer" functions against tumours & intracellular pathogens, Th1 activation	Increased infiltration of synovial macrophages in the sub lining and lining layer in inflamed joint, CD68+ sub lining macrophages can be used as biomarker for disease severity. CD68+ sub lining macrophages can be used as biomarker for disease severity (Barry Bresnihan et al. 2009). CD68+ macrophage number correlates with disease activity (Tak et al. 1997). Importantly, synovial macrophage populations have not been characterised into their M1 or M2 phenotypes in RA.

Monocyte/ macrophage subset	Surface markers	Activation Stimuli	Cytokines/Chemokines & receptors	Effector molecules	Key functions	Alterations in RA
M2a	CD68, CD163, CD206, Scavenger receptor, Mannose receptor, FCeR	IL- 4,IL-10, IL-13	IL-10, IL-4R, Decoy IL-1R (type 2), CCL2, CCL17, 22, CXCR1-2	Arginase	Th1 suppression, Th2 activation, defence against parasites	It is well established that cytokines such as IL-1 & TNF are increased in RA (cytokines released from M1 macrophages); in addition, cytokines such as IL-10 is reduced in RA (associated with M2 phenotype) (Kennedy et al. 2011)
M2b	CD68, CD86, MHCII	Immune complexes (IC), LPS,IL-1r	IL-1, IL-6, IL-10, TNF, CCL1	iNOS, RNI	Th2 activation, Immunoregulation	
M2c	CD68,CD163, CD150, (SLAM)	IL-10, Glucocorticoids	IL-10, IL-4R, IL-10R, CCL16, CCL18	Arginase	Immunosuppression, tissue remodelling, wound healing	

Table 1.5 – Summary table of Monocyte/macrophages subsets and functions (Adapted from Italani & Rorgschi et al. 2014).

1.4 Immunomodulation by MSCs

In addition to the role of MSCs in tissue regeneration, MSCs also regulate T cell responses. More than a decade ago it was discovered that BM-MSCs inhibited T cell proliferation, and this initiated a series of studies into the immunomodulatory capacities of MSCs on both innate and adaptive immune cells (Bartholomew et al. 2002). This line of enquiry was further encouraged by the first successful clinical application of MSCs as immunosuppressive agents in the treatment of graft versus host disease (GvHD), in 2004 (Le Blanc et al. 2004) (Y. Wang et al. 2014; Le Blanc et al. 2004). Moreover, from more recent studies, the plasticity of MSCs has become more clearly understood, with studies showing their ability to not only exert immunosuppression but also promote an immune response, depending on the prevailing microenvironmental conditions (Y. Wang et al. 2014). The exciting prospect of modulating MSC immunomodulatory functions via their environmental cues, both in vitro and in vivo, has led to the development of MSC-based treatments for a range of inflammatory conditions.

However, their exact mechanisms of action remain to be elucidated. For example, MSCs exert their immunosuppressive abilities via the inhibition of DC maturation, by reducing IL-2 induced proliferation and secretion of IFN in NK cells, as well as suppressing B and T cells (Jiang et al. 2005; Aggarwal and Pittenger 2005). The main focus of my thesis is the analysis of the immunomodulatory functions of MSCs on monocytes and macrophages.

The first report showing that MSCs could polarise macrophages from their M1 proinflammatory phenotype to a M2 anti-inflammatory phenotype was published in 2009 (J. Kim and Hematti 2009). They discovered that human macrophages, when co-cultured with human BM-MSCs, displayed high expression levels of M2-type macrophage markers. Consequentially, these macrophages produced high levels of IL-10 and low levels of IL-12 and TNF. Additionally, on a functional level, macrophages co-cultured with MSCs showed an increased level of phagocytic activity, which fits with the M2 macrophage phenotype. Further studies have shown that MSC-mediated polarisation of M2 macrophages depends on the secretion of soluble factors, such as prostaglandin (PGE2), TNF–stimulated gene 6 (TSG-6), IL-6, indoleamine 2,3-dioxygenase (IDO), monocyte chemoattractant protein-1 (MCP-1) and TGF-β1, by the MSCs (Németh et al. 2009; Melief, Geutskens, et al. 2013; François et al. 2012; Choi et al. 2011). For example, Németh et al. showed that monocytes and macrophages, isolated from mice with sepsis, had a significant increase in their IL-10 anti-inflammatory cytokine production, when co-cultured with BM-MSCs (Németh et al. 2009). The MSCs skewed macrophage differentiation, by increasing cyclooxygenase-2 (Cox-2) activity. This led to an increased secretion of PGE2, which in turn acted on prostaglandin EP2 and EP4 receptors on

macrophages, reprogramming the macrophages to have an increased IL-10 cytokine production, and an anti-inflammatory phenotype.

It has also been shown that MSCs activated by inflammatory signals secrete the anti-inflammatory molecule, TSG-6. It is the interaction of TSG-6 with the CD44 receptor on resident macrophages that decreases TLR2 signalling in a zymosan-induced peritonitis murine model (Choi et al. 2011). This immunosuppressive ability of MSCs to skew monocytes towards an anti-inflammatory IL10 secretory profile is further supported by Melief et al. They reported that the addition of MSCs to monocyte cultures inhibited the differentiation of monocytes towards immunogenic dendritic cells and shifted monocyte differentiation towards an anti-inflammatory IL-10-secretory phenotype, by the constitutive secretion of IL-6 by MSCs (Melief, Geutskens, et al. 2013).

Furthermore, MSCs from healthy volunteers up-regulated IDO expression in the presence of TNF and IFN-γ (François et al. 2012). Increased IDO activity of MSCs results in the differentiation of monocytes into an IL-10-secreting M2 macrophage phenotype. Consequently, these monocyte-derived M2 macrophages act as bystanders in the suppression of T-cell proliferation, via an IL-10-independent pathway, thus amplifying the immunosuppressive effect generated by MSCs.

In addition, MSCs have demonstrated their widespread immunosuppressive properties via MSC-induced generation of T regulatory (Treg) cells, in which the presence of monocytes is essential. By prompting the survival of monocytes and their differentiation towards an M2 phenotype (with increased secretory levels of IL-10 and CCL-18), MSCs promote regulatory T cell induction, thereby amplifying the immunosuppressive effect of MSCs (Melief, Geutskens, et al. 2013).

Prockop (Prockop 2013) reviewed two negative feedback loops that facilitate the MSC-macrophage crosstalk. Under certain inflammatory states, macrophages release proinflammatory cytokines that activate MSCs, eliciting an increase in the secretion of PGE2 through up-regulated expression of COX2, a component of the arachidonic acid pathway, which then, causes the shift of macrophages to an M2 phenotype (Prockop 2013). The second feedback loop arises when MSCs, activated under an inflammatory environment (settings), secrete TSG-6, an anti-inflammatory molecule that binds to CD44 on macrophages, disrupting its interaction with TLR2, thus inhibiting TLR2-mediated NF-kB signalling and decreasing the inflammatory response.(Prockop 2013) In addition, it has been shown that MSCs exert their immunosuppressive abilities by inhibiting the NLRP3 inflammasome activation and IL-1b mediated inflammation, mediated by decreasing ROS production in macrophages, via the release of stanniocalcin-1 (Oh et al. 2014).

Furthermore, MSCs secrete HGF, which initiates IL-10 production in monocytes, via the ERK 1/2 pathway, and promote their immunosuppressive effects. HGF secreted by the MSCs bind to the c-met receptor on CD14+ monocytes, eliciting M2 macrophage polarisation (P.-M. Chen et al. 2014).

Interestingly, in vitro studies, incorporating 3D culturing of MSCs, have been utilised to provide a more reflective insight into their immunoregulatory functions on macrophages in vivo. Such reports using hMSCs, as 3D spheroids, show that they are self-activated, partially by intracellular stress responses, to produce PGE2, that directs stimulated macrophages into an anti-inflammatory phenotype, inhibiting activated macrophages from secreting pro-inflammatory molecules TNF, CXCL2,IL-6, IL-12p40 and IL-23, and, in contrast, to the increased levels of IL-10 secretion (Bartosh et al. 2010). Other studies, utilising 3D cultures of MSCs to monitor their immunoregulatory effects, have shown increased levels of pro-inflammatory cytokines, TNF, IL-1β and IFNγ in macrophages, cultured on hydrogels harbouring MSCs (King et al. 2014). Such differences, observed in various in vitro models, emphasise how MSCs immune functionality may be modulated by the microenvironment and surrounding cells.

MSCs undergo 'licensing', or "priming" that can regulate and enhance their immunoregulatory abilities by pro-inflammatory cytokines, such as TNF, IL-1 and IFNγ (Krampera 2011) This priming of MSCs has been shown to increase their production of soluble IDO, iNOS and PGE2 and is linked to the expression of Toll-like receptors (TLRs) on the MSC surface (Ren et al. 2008; Krampera et al. 2006). Waterman et al. showed how MSCs can be polarised to either a pro-inflammatory or anti-inflammatory state, by the manipulation of the type of TLR activated on MSCs. Basically, MSCs can be categorised into MSC1 (pro-inflammatory) and MSC2 (anti-inflammatory). The BM-MSCs primed with TLR3 ligands resulted in a MSC1 phenotype, whereas BM-MSCs primed with TLR4 ligands led to an MSC2 phenotype.(Waterman et al. 2010) In this way, the immunomodulation of MSCs can be skewed, depending on the local microenvironment, hence providing a possible explanation for the lack of success, to date, of their immunosuppressive therapeutic effects in autoimmune diseases such as RA. A summary of the effects of MSCs on monocytes/macrophages, depending on their pro-inflammatory/inflammatory state is illustrated in Figure 1.5.

Pro inflammatory   **Anti-inflammatory**

MSC 1   **MSC 2**

IL-6, IL-8,   IDO, PGE2, IL-4, IL-
TGF-β   1Ra, RANTES, IL-
                              10, TSG-6

Monocyte (CD14+)   **Monocyte (CD14+)**

Type I macrophage   **Type II macrophage**

Antigen Presentation   Phagocytosis
TNF/IL-6   CD206
NO   IL-10
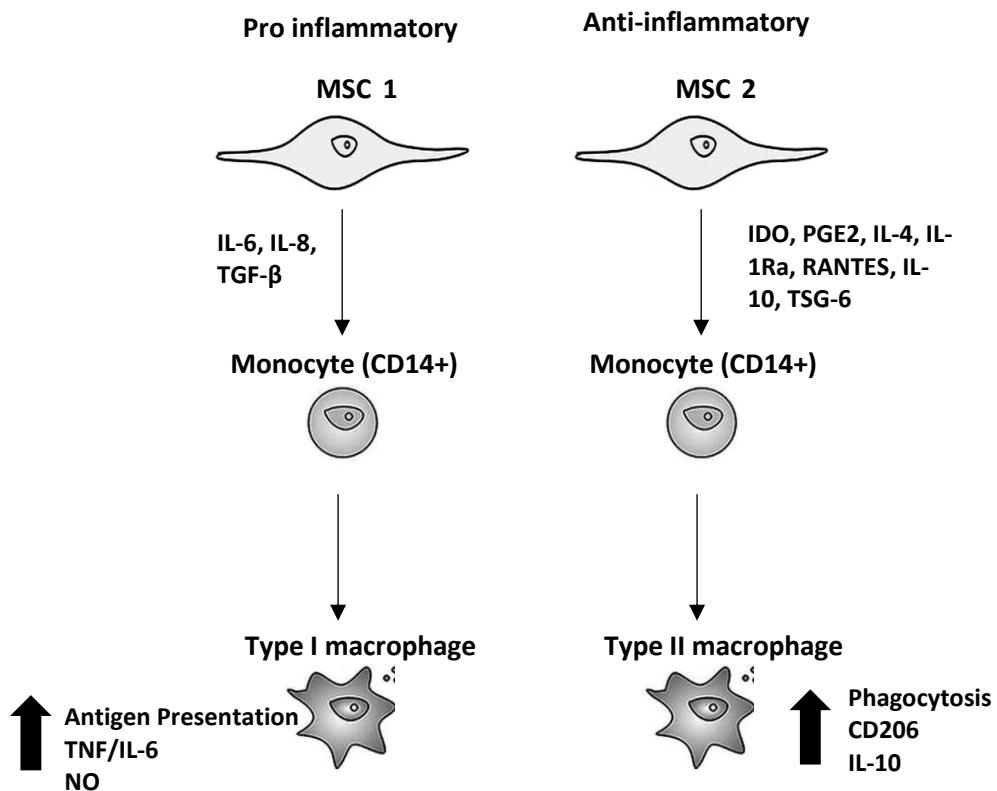
Figure 1.5 –The types of macrophages resulting from MSCs, and their pro-inflammatory or anti-inflammatory state. Type 1 MSC have a pro-inflammatory phenotype and secrete key cytokines such as IL-6, IL-8 and TGF-β, which then polarise the monocyte/macrophage to a M1 macrophage phenotype. Type 2 MSCs have an anti-inflammatory phenotype and secrete key immunomodulatory factors like IDO, IL-10 and TSG-6, that then act upon the monocyte/macrophage and skew their phenotype to a type 2 anti-inflammatory macrophage phenotype (Adapted from Melief et al. 2013).

MSC Immunoregulatory factor	Function	Effect on monocytes/macrophages
IDO (Indoeamine 2,3 dioxygenase)	Catalyses oxidative catabolism of L-tryptophan to N- formlkynurenine (KYN)	These factors act on macrophages to induce their Type II alternatively activated phenotype increase in CD206 expression, IL-10 production & phagocytic activity. And a decrease in IL-12 and TNF production).
PGE$_2$ (Prostagladin E$_2$)	MSCs produce PGE$_2$ via COX-2 enzyme. COX-2 converts Arachidonic acid to PGE$_2$	
IL-4	Cytokine prompt M2 macrophage activation	
IL-1Ra (IL-1 receptor antagonist)	Acts to prevent release of TNF by activated macrophages	
RANTES (CCL5)	Ability to block monocyte development. Produced by MSCs in an autocrine fashion to further recruit MSCs to sites of inflammation and exert their immunomodulatory properties	
IL-10	Pleiotropic cytokine involved in immune tolerance- inhibit M1 macrophage and induce M2 macrophages	
TSG-6 (TNF stimulated gene 6)	Upregulated in inflammation and in presence of pro-inflammatory mediators. Acts as part of a negative feedback loop anti-inflammatory response. Interferes with TLR2 NF-κB signalling in macrophages, thus macrophage activation is inhibited.	

Table 1.6 – Summary of MSC immunomodulatory factors, their functions and effects on monocytes/macrophages

1.5 Current MSC- based approaches for RA

As it has been previously outlined in Section 1.4 the use of MSCs as an immunosuppressive therapeutic has been largely accepted as these MSCs have the ability to inhibit a range of immune cells from B and T cells to monocytes and NK cells. This has been clearly demonstrated both in vitro and in vivo preclinical models, however there are far fewer studies evaluating the impact of MSC soluble factors on monocytes within the realm of RA, more specifically.

In murine models of RA, allogenic MSC administered intraperitoneally have demonstrated to successfully treat collagen induced arthritis. Even, with such advantageous use of allogenic MSCs, there is some contradictory evidence that has emerged in the context of RA treatment. Allogenic BM MSC and UC MSC were rejected in a murine model of graft versus host disease and lost their immunosuppressive capacity that was shown in vitro (Eliopoulos et al. 2005). Although studies conducted on patients with autoimmune diseases have shown the successful use of allogenic MSC, such as the intravenous infusion of allogenic MSCs into a group of anti-TNF resistant cases, resulting in a temporary short term clinical improvement but not on a long term follow-up (Liang et al. 2012). Furthermore, the safety and potential efficacy of allogenic MSCs as an RA therapy in a larger number of patients has been demonstrated (Wang et al. 2013). Additionally, conventional therapy combined with allogenic MSCs has been shown to improve RA cases both serologically and clinically, yet again this study does not factor in the direct impact ion RA monocytes. Thus, this would suggest that MSCs to be an effective treatment for refractory autoimmune diseases, such as RA, but further clinical trials are required to verify these findings. Also, the optimal tool for the delivery of MSCs is yet to be determined along with further clarification on exactly how MSC treatment impacts RA monocytes. MSCs have the ability to migrate to specific sites of inflammation, so MSCs administered systemically could provide anti- inflammatory effects to multiple sites within the body (Devine et al. 2003). Although, this systemic delivery of autologous could cause worsening of the disease, as demonstrated in a CIA model, but further investigation is required on the mechanism behind this (Sullivan et al. 2012)Therefore, the emerging delivery of MSCs is via intra-articular injections, which has shown to be a practical and safe route for treating rheumatic disorders (Toupet et al. 2013).

It is vital to further investigate the relationship between MSC and other immune cells, particularly monocytes/macrophages in patients with RA, in order, to consider MSCs and their soluble factors to be considered as a strong candidate for an alternative therapy for RA. As discussed, it is agreed that MSCs have an immunomodulatory/immunosuppressive effect,

however they can warrant side effects such as undesirable growth of tumours in RA. So, MSC depending on their microenvironment can have a beneficial or detrimental impact (Cosimo De Bari 2015)Thus, further examination is required of MSC CM as opposed to MSCs themselves as new therapy. Overall, the effects of MSCs, on RA monocytes are particularly poorly understood and those studies that have looked into this area have largely not considered the effects of MSC soluble mediators, as opposed to MSCs themselves.

1.6 Aims of the PhD project

Current literature suggests that MSCs are able to exert an immunomodulatory impact on both innate and adaptive immune cells, in both in vitro and in vivo studies prompting MSC therapy as an alternative therapy for the treatment of autoimmune diseases, such as RA. However, the majority of MSC immunomodulation studies have focussed on their interactions with B and T cells, with less known regarding their impact on monocytes in health and disease, particularly in RA. Therefore, the broad aim of the study was to increase our understanding of MSC immunomodulation in the context of monocytic activity, as there is some conflicting evidence in the literature concerning the immunomodulatory capacity of MSCs, because depending on their microenvironment MSCs could be skewed to be either immunostimulatory or immunoinhibitory in health and disease.

I have used an in vitro whole blood based assay, to study the response of activated monocytes to MSC treatment (and their conditioned media).

The main aims of this project are:

- Study the responsiveness of RA monocytes to the immunomodulatory effects of MSCs, in comparison to healthy control monocytes
- To investigate the immunomodulatory effects of MSC CM on healthy and RA monocytes

Chapter 2 – Materials and Methods

2.1 Materials

Flow cytometry is the main method implemented in the research conducted on the immunomodulatory abilities of MSCs on monocytes, in health and RA. This is outlined in detail in section 2.3. For experimental procedures, the following reagents and equipment were used:

2.1.1 List of antibodies used for flow cytometry

Flow cytometry antibodies			
MSC phenotyping			
Antibody	**Supplier**	**Clone**	**Catalogue Number**
Anti-Human CD105 FITC	Miltenyi Biotec	43A4E1	130-098-774
Anti-Human CD90 FITC	AbD Serotec	F15-42-1	609
Anti-Human CD73 BV421	BD Horizon	AD2	562430
Anti-Human CD14 PE	BD Pharmingen	M5E2	555398
Anti-Human CD19 PE	BD Pharmingen	HIB19	555413
Anti-Human CD34 PE	BD Pharmingen	563	550761
Anti-Human CD45 PeCy5	BD Pharmingen	HI30	555484
Monocyte/MSC Intracellular staining			
Antibody	**Supplier**	**Clone**	**Catalogue Number**
Anti-Human CD14 BV421	BD Horizon	MoP9	5625283
Anti-Human CD45 APC-Cy7	Biolegend	2D1	368515
Anti-Human TNF PE	BD FastImmune	6401.1111	340512
Anti-Human IL-6 PE	BD FastImmune	AS12	340527
Anti-Human IL-10 PE	Biolegend	JES3-19F1	506804
Anti-Human IL-12 APC	Biolegend	C11.5	501809
NK cell activation experiments			
Antibody	**Supplier**	**Clone**	**Catalogue Number**
Anti-Human CD3 FITC	BD Pharmingen	UCHT1	555332
Anti-Human CD56 APC	Miltenyi Biotec	REA 196	130-100-698

		Miltenyi	
Anti-Human NKp30 PE	BD Pharmigen	Biotec	558407
Anti-Human CD69 BV421	Biolegend	FN30	310930

Table 2.1 – List of flow cytometry antibodies

2.1.2 List of reagents used for experimental procedures

MSC culture reagents		
Reagent	**Supplier**	**Product Number**
Dulbecco's modified eagle's medium DMEM(1X)	Gibco Life Sciences	22320-022
Stem macs MSC expansion media	Miltenyi Biotec	130-091-680
Dimethyl sulphoxide (DMSO)	Sigma	D2650
Trypsin/EDTA solution (10X)	Sigma	59418C
Penicillin/Streptomycin solution	Gibco®, Invitrogen	14140
Lymphoprep	Axis-Shield	1114545
Trypan blue	Sigma	T8154
Foetal bovine serum (FBS)	Gilbco Life Sciences	10270
Roswell Park Memorial Institute RPMI) – 1640 medium	Sigma	RO883
Phosphate buffered saline (PBS)	Sigma	P4417- 100TAB

Table 2.2 – List of MSC culture reagents

Flow cytometry assay reagents		
Reagent	**Supplier**	**Product Number**
Lipopolysaccharide (LPS)	InvivoGen	Tlrl-eklps
Phosphate buffered saline (PBS)	Sigma	P4417- 100TAB
BD Vacutainer plastic sodium heparin blood collection tube	BD Vacutainer	367876
Brefeldin A	BD Golgi Plug	555029
BD Cytofix/Cytoperm plus fixation/permeabilisation kit	BD Biosciences	555028
Beckman Coulter Intraprep Permeabilisation reagent kit	Beckman Coulter	Ao7802
CountBright Absolute counting beads	Invitrogen	C36950

Table 2.3 – List of reagents used for the flow cytometry assay

ELISA kits		
Reagent	**Supplier**	**Product Number**
Human IL-10 ELISA ready-SET-Go!	Affymetrix Bioscience	88-7106-22
Human IL-6 Quantikine ELISA kit	R&D systems	D6050

Table 2.4 – List of ELISA kits used for examining secreted factors in MSC CM

2.1.3 List of plasticware and laboratory equipment used for experimental procedures

Plasticware equipment		
Reagent	Supplier	Product Number
Pasteur pipettes sterile 1ml	Gilson	1170915
Pipette tips (1000 μl)	Gilson	F167104
Pipette tips (200 μl)	Gilson	F167103
Pipette tips (10 μl)	Gilson	F167101
Stripette (25 ml)	Corning	4251
Stripette (10 ml)	Corning	4101
Stripette (5 ml)	Corning	4051
96 well plate (round bottom)	Corning	3799
Flask (25 cm2)	Corning	430639
Flask (75 cm2)	Corning	430641
Cryovials (2 ml)	Sarstedt	72.38
Centrifuge tubes (15 ml)	Corning	430790
Centrifuge tubes (50 ml)	Corning	430828
Freezing container, nalgene	Sigma	C1562-1EA
Polystyrene FACs tubes	BD	BD352002

Table 2.5 – List of plasticware equipment used in experiments

Equipment	Company	Product Number
Inverted microscope	Olympus	CKX41
Digital camera	Olympus	C-7070
Centrifuge	Eppendorf	5810R
Biological safety cabinet, class ll	ESCO	AC2-4E1
-80°C freezer	Sanyo	10020065
CO_2 incubator	Sanyo	50100783
BD LSRII 3 laser flow cytometer	BD Biosciences	-
Haemocytometer	Hawksley	BS.748
37°C water bath	Grant	-

Table 2.6 – List of laboratory equipment

2.2 Patient and healthy donor samples

2.2.1 Healthy control blood donors and MSCs

Healthy control blood donors were needed for the experiments carried out for the detection of intracellular TNF and IL-6 cytokines in activated monocytes, alone or with the addition of MSCs, using a whole blood assay. MSCs were added to the whole blood in order to assess their immunosuppressive effects on monocytes. Peripheral blood from 19 healthy adult volunteers was collected into heparinised blood collection tubes. Once collected, blood was kept at room temperature and was used the next day. These healthy blood samples were used for experiments examining the effects of MSCs and their conditioned media (CM) on healthy control monocytes, by measurement of TNF and IL-6 production, as described in detail in section 2.3.9.

2.2.2 Healthy control MSCs

Samples were obtained following patients' written consent. Sample collection was approved by the Yorkshire and Humberside ethics committee (06/Q1206/127 and 07/Q1205/27). Healthy control primary BM MSCs were obtained from 3 patients undergoing orthopaedic procedures for fracture fixation, but were otherwise healthy. The primary MSC donors were MSC 1 (87-year-old female donor), MSC 2 (22-year old, male donor) and MSC 2 (61-year old, female donor).

IP006 MSC clonal cell line were derived from the BM and obtained from another healthy individual (25-year old, male) from the Leeds General Infirmary (LGI) orthopaedic clinic. IP006 was a single-cell derived, highly proliferative clone. (P.-M. Chen et al. 2014).

Furthermore, MSC immortalized clonal cell lines, Y201 and Y202, were obtained from Professor Paul Genever, University of York. Use of these cell lines specifically for my project was covered by a MTA (material transfer agreement, registration number: RC000658). Y201 and Y202 MSCs were generated from primary human BM MSCS that were isolated from femoral heads during routine hip replacement procedures. These BM MSCs were then immortalised using human telomerase reverse transcriptase (hTERT), combined with human papillomavirus E6/7 (James et al. 2015).

2.2.3 RA patient blood samples and MSCs

Peripheral blood was collected from 2 main groups of RA patients, treatment naïve RA and refractory RA patients. This was to assess the potency of the immunosuppressive effects of healthy MSCs on RA monocytes across varying disease severity. Peripheral blood from a total of 18 treatment naïve RA patients and 15 refractory RA patients were collected into

heparinised blood collection tubes, at Chapel Allerton Hospital. Recruitment of volunteer patients and collection of their peripheral blood was covered by Rheumatoid Arthritis DiseAse Research (RADAR) study ethics, the Research Ethical committees approved (RADAR REC ref 09/H1307/98). Once collected, blood was transported over to St James' University Hospital (SJUH) and used the following day. Patient information was recorded, including disease activity scores (DAS 28), in order to assess RA disease activity of each patient, thereby enabling me to correlate the effectiveness of MSC immunomodulation on RA patient monocytes, with the patients' disease severity. DAS 28 is calculated by a complex mathematical formula with the following parameters: number of swollen and tender joints (out of 28), erythrocyte sedimentation rate (ESR) or C reactive protein (CRP) value and patients' global assessment score.

Treatment naïve patient blood samples				
RA patient	Gender	Age	DAS28 score (ESR)	DAS28 score (CRP)
RAM002	M	52	4.27	4.42
RAM004	F	71	6.65	6.39
RAM005	M	65	2.26	3.1
RAM006	F	68	4.5	4.95
RAM007	F	60	4.13	3.99
RAM008	M	64	No data	No data
RAM009	M	36	No data	2.87
RAM0010	F	86	2.8	2.79
RAM0011	M	67	6.12	6.46
RAM0012	F	52	7.45	6.78
RAM0013	M	32	5.09	4.52
RAM0014	F	56	4.06	3.83
RAM0015	F	52	4.97	6.15
RAM0016	F	46	5.96	5.73
RAM0017	F	83	4.12	3.77
RAM0018	F	55	5.34	5.01
RAM0019	F	72	No data	2.6
RAM0020	M	62	No data	No data

Table 2.7 – Key demographic and clinical data gathered from the treatment naïve RA patients

Refractory RA patient blood samples				
RA patient	Gender	Age	DAS28 score (ESR)	DAS28 score （CRP）
RAM0021	M	66	No data	5.17
RAM0022	F	54	No data	No data
RAM0023	F	69	6.01	5.55
RAM0024	F	46	No data	No data
RAM0025	F	31	2.36	2.29
RAM0026	F	54	No data	3.85
RAM0027	M	37	No data	6.28
RAM0028	F	56	No data	6.55
RAM0029	F	67	5	4.06
RAM0030	M	55	7.07	6.67
RAM0031	F	56	5.3	4.73
RAM0032	M	57	3.5	4.14
RAM0033	F	70	5.29	4.87
RAM0034	F	74	6.53	6.11
RAM0035	F	57	No data	6.28

Table 2.8 – List of key clinical data gathered from Refractory RA patients

Summary of RA patient charactertisics						
RA patient cohort	No. of Patients	Female:Male	Mean age	Mean DAS28 score (ESR)	Mean DAS28 score (CRP)	Mean CCP
Treatment naïve	18	11:7	60	4.8	4.5	73
Refractory	15	12:3	57	5.1	5.2	181

Table 2.9 – Summary of RA patient characteristics

Additionally, I tested synovial RA MSCs for their immunomodulatory effect on HC monocytes (E. Jones, Churchman, et al. 2010). The synovial RA MSCs obtained are listed below, in table 2.10. VAS stands for visual analog scale and is a unidimensional measure of pain that is completed by the patient for their assessment of what VAS score represents their pain intensity.

Synovial RA MSC donor	Gender	Age	VAS score
AR1214	Male	40	14
AR1388	Male	34	57
AR1390	Male	84	9
AR1400	Male	57	62
AR1404	Female	68	79

Table 2.10 – List of Synovial RA MSC donors

2.3 Methods

2.3.1 Isolation and culture of MSCs from bone marrow aspirates

MSCs from healthy donor bone marrow (BM) aspirates were isolated by transferring BM sample from a 5 ml EDTA blood collection tubes to a falcon tube, to which PBS was added at a 1:1 dilution. For each diluted aspirate, 20 ml of Lymphoprep was added to a separate falcon tube. Lymphoprep is a density gradient medium used for the isolation mononuclear cells (MNCs) from peripheral blood and bone marrow, by utilising differences in their cell density to separate MNCs from higher density cells such as Granulocytes and Erythrocytes, which would sediment through the lymphoprep layer during centrifugation. Each diluted aspirate was gently layered onto the lymphoprep and centrifuged at 650 g for 25 minutes at room temperature (RT), with brakes set at zero. After centrifugation, the buffy coat layer containing MNCs was carefully collected with a sterile Pasteur transfer pipette, into a new falcon tube, mixed with 10 ml of PBS and centrifuged at 400g for 5 minutes at RT. The supernatant was discarded and the pellet re-suspended in 5ml of PBS ready for cell counting.

2.3.2 Cell counting

Viable MNC count was carried out using a haemocytometer. Trypan blue was used to stain for any dead cells. It works on the principle that live cells have intact cell membranes that exclude certain dyes, such as Trypan Blue, but dead cells do not. Thus, a cell suspension is mixed with the dye and any viable cell will have a clear cytoplasm, however any non-viable cell will have a blue cytoplasm. Cells were diluted with Trypan Blue at a 1:1 dilution and mixed well, before being loaded onto the haemocytometer for cell counting under the microscope. Cells were counted in each "large" corner square. The formula used to calculate the total number of cells was:

Total cells/ml = (total cells counted per "large square"/ Number of "large squares" counted) x 2 x 10, 000 cells/ml

In the cell counting equation shown above, a multiplication of 10,000 is used because one "large" square has a volume of 1mm x 1mm x 0.1 mm= $0.1mm^3$ or 10^{-4} (10^{-4} ml).

2.3.3 Cell culture/passaging of MSCs

All cells were cultured in sterile conditions in a category two tissue culture hood, using aseptic technique.

To generate passage zero (p0) MSC cultures, 10^7 MNCs were seeded in T25 flasks in 10 ml of StemMACs MSC expansion media and placed in a 37°C incubator with 5% CO_2. Adherent cells were culture expanded and passaged upon reaching 80% confluency. For passaging, cells were washed twice with 5 ml of PBS and detached from the flask by incubation with 1xTrypsin – EDTA solution for approximately 2-3 minutes. Flasks were then gently tapped to dislodge any remained adherent cells. The trypsinised cells were then added to a centrifuge tube, containing equal volume of complete medium, to inactivate the trypsin. This was followed by a further 5ml of PBS used to collect any remaining cells from the flask and added to the centrifuge tube with the rest of the trypsinised cells collected from the flask previously. The cell suspension was then centrifuged at 500g for 5 minutes at RT. The supernatant was discarded and the pellet was re suspended in 1ml PBS, ready for cell counting using the haemocytometer. For further expansion, MSCs were seeded in further T75 flasks at an approximate density of 7.5 x 10^5 cells/flask. The 16ml medium was renewed every 2 days with half media change, until the cells reached 80-90% confluency.

IP006, Y201 and Y202 MSC cell lines and RA synovial MSCs were also culture expanded in T75 culture flasks, using the same methods mentioned above.

2.3.4 Freezing MSCs

Media was discarded and flasks containing MSCs washed with PBS, twice. Cells were treated with trypsin to detach cells from flask, as described in section 2.3.3. After centrifugation, the cell pellet was re-suspended in 1ml of freezing media, which was pipetted steadily to the cell pellet and mixed gently. The freezing media consisted of 10% Dimethyl sulphoxide (DMSO), 45% fetal bovine serum (FBS) and 45% DMEM. The cell suspension was then transferred to a cryovial, which was then placed into a "Mr Frosty" container (Nalgene), containing Isopropanol. This allowed the cells to freeze gradually, to preserve cellular integrity. The "Mr Frosty" was stored at -80°C for approximately one week; thereafter, the vials were transferred into liquid nitrogen for long-term storage. Approximately, 1.0x10^6 MSCs were frozen in each cryovial.

2.3.5 Thawing MSCs

Vials containing MSCs were thawed in a water bath and the contents transferred quickly into separate 15ml centrifuge tubes containing 5ml of DMEM supplemented with 10% FCS and 1% Penicllin/Streptomycin, mixed well and centrifuged at 652g for 5 minutes at RT. The supernatant was discarded and the cell pellet re suspended in StemMACs media. The cell suspension was then seeded into T75 flasks at an approximate cell density of 7.5 x10^5

cells/flask.

2.3.6 Preparation of MSC conditioned media (MSC CM)

MSCs were grown in T75 flasks and the conditioned media was harvested 4 days after MSCs had reached 80-90% confluence. Conditioned media from the flask was collected in a falcon tube and centrifuged at 2400g for 10 minutes, for the removal of cells. Thereafter, MSC CM was aliquoted in 4ml aliquots and frozen at -20°C, to be thawed at a later date for downstream experiments.

2.3.7 Phenotyping of MSCs by Flow Cytometry

Immunophenotyping for MSCs was carried out as follows. MSCs were trypsinised, counted and $1x10^5$ cells were placed in cytometer tubes and stained with antibodies against CD14, CD19, CD34, CD45, CD73, CD90, CD105, DAPI (DAPI staining is for discriminating between live/dead cells) and appropriate isotype controls for 30 minutes in the dark at 4°C. To minimise spectral overlap, when later analysing flow cytometry data, MSCs were split into 2 main cytometer tubes. Tube 1 consisted of: CD105 FITC, CD73 BV421 and CD45 PeCY5. Tube 2 consisted of: CD90 FITC and all the antibodies that are examining the negative markers for MSCs according to the ISCT, as CD14 PE, CD19 PE, CD34 PE. Next, cells were washed with FACs buffer and centrifuged at 500g for 5 minutes at 4°C. Then, the supernatant was discarded and the cell pellet re-suspended in 200mL of FACs buffer ready for acquisition on the flow cytometer. The BD LSRII 3 Laser flow cytometer was used to acquire a minimum of $1x10^4$ events, per sample.

Flow cytometry is a common cell biology technique that uses laser based technology to sort, count and profile cells in a mixed cell suspension. Using a flow cytometer, the cell suspension is passed in a single file fashion through a laser beam and interaction with the light is measured by an electronic detector as light scatter and fluorescence intensity. If cellular components are fluorescently labelled the laser beam causes them to be excited and emit light at a longer wavelength than the light source, which is detected by the various detectors in the flow cytometer. Thus, the detector picks up a combination of scattered and florescent light emitted and the fluorescence intensity will represent the amount of that particular cell component. This data are then analysed by a computer, using special flow cytometry analysis software, that is attached to the flow cytometer. Flow cytometry is a powerful tool as it is a rapid and quantitative method for purification of cells in a mixed suspension, as well as determining the phenotype and function of cells being studied.

2.3.8 Intracellular staining for TNF in LPS activated monocytes using a whole

blood based assay, with BD biosciences fixation/permeabilisation kit

For the detection of intracellular TNF in activated monocytes a whole blood assay, modified from work carried out by Riberio *et a*l 2014, was carried out. Blood samples (9 ml) were collected in heparinised blood collection tubes from healthy blood donors. Blood samples for each donor were distributed into aliquots of 200µL per test, into polystyrene FACs tubes. Whole blood was stimulated with 200µL of LPS at a final concentration of 1ng/m in RPMI, and 2µL of BD biosciences Golgi Plug, Brefeldin A was added to the polystyrene FACs tubes for the correct controls (1µL per 1ml). BFA was added to allow the accumulation of cytokines in the Golgi apparatus, increasing the intracellular detection of TNF by immunofluorescent staining and flow cytometry analysis. LPS-stimulated blood was next diluted with RPMI to give a final dilution of 1 in 10 (for example, for 200µL of blood overall volume of FACs tube would be 2ml, including the addition of LPS and RPMI) and kept for 6 hours in a 37°C incubator with 5 % CO_2.

Negative controls included unstimulated (-LPS) blood, stimulated (+LPS) blood but without the Brefeldin A and another tube with stimulated blood with LPS, for staining with PE antibody isotype control instead of TNF-PE antibody.

After 6-hour stimulation, blood was treated with red blood cell (RBC) lysis buffer for 5 minutes at RT and cells pelleted at 500g for 5 minutes at RT. Supernatant was discarded and RBC lysis step was repeated. Pelleted cells were then re-suspended in 200µL staining buffer (1% FBS in PBS) and centrifuged at 500xg for 5 minutes at RT. Cell surface staining for monocytes was next carried out by re suspending cell pellet in 100µL of staining buffer containing 5µL of CD14-BV421 monoclonal antibody and incubated for 15 minutes at RT. Cells were then washed with staining buffer and centrifuged for 5 minutes at 500g at RT. Next, to fix and permeabilise cells, 500µL BD fix/perm solution was added to the cell pellet, vortexed well and left to incubate for 20 minutes at RT. After, the cell suspension was centrifuged at 500g for 5 minutes at RT. Then, 2 ml of BD perm wash solution was added to cells for 10 minutes at RT, and subsequently centrifuged at 500g for 5 minutes at RT. Next, intracellular staining for TNF was carried out, using 20mL of TNF-PE monoclonal antibody and 100µL of BD perm wash buffer to re-suspend the cells. Staining for intracellular TNF or isotype control was carried out for duration of 20 minutes at RT in the dark. Subsequently cells were washed with 2 ml of BD perm wash buffer by centrifugation at 500g for 5 minutes and fixed with 500µL of 2% of Paraformaldehyde (PFA). The data were acquired on BD LSRII 3 laser flow cytometer within 24 hours.

2.3.9 Intracellular staining for TNF and IL-6 in LPS activated monocytes using a

whole blood based assay, with the Beckman Coulter Intraprep Permeabilisation kit

The same experimental conditions and controls were used in experiments utilising the Beckman Coulter Intraprep Permeabilisation kit for detection of intracellular TNF in activated monocytes. After the 6-hour stimulation, surface staining for monocytes was carried out by adding 5µL of CD14 BV421 and CD45-APC Cy7 antibody per test, for 15 minutes at RT in the dark. Then, 250µL of Reagent 1 (fixation agent called formaldehyde) was added to samples, mixed thoroughly and left to incubate for 10 minutes at RT in the dark. Samples were then washed with 1mL of PBS and centrifuged at 1500 rpm for 5 minutes at RT. Subsequently, the supernatant was discarded and 220µL of reagent 2 (permeability agent consisting of saponin) was added, mixed vigorously and incubated for 5 minutes at RT in the dark. Subsequently, 20mL of TNF-PE antibody or IL-6-PE was added and incubated for 20 minutes at RT in the dark. A further 2 washing steps followed, with the addition of 1mL of PBS and centrifugation at 500g for 5 minutes at RT. The supernatant was discarded and cells re-suspended in 500µL FACs buffer ready for FACs analysis on BD LSRII 3 laser flow cytometer within 24 hours. In some experiments, 50mL of CountBright absolute counting beads were added to the 500mL cell suspension in FACs buffer and mixed thoroughly, prior to flow cytometer acquisition. Counting beads were added to determine absolute cell counts of the cells in the sample.

2.3.10 Co-culture of whole blood with MSCs to study their immunosuppressive effects on monocytes by measuring intracellular TNF and IL-6

For co-culture experiments P3 BM MSCs (n=3 donors) were thawed as described in section 3.6. Experiments were performed on three different occasions with blood from the same healthy blood donor. MSCs were split into aliquots of 1.5×10^5, 4×10^5 and 8×10^5 cells, re-suspended in RPMI in separate polystyrene FACs tubes and centrifuged at 500g for 5 mins at RT. The supernatant was discarded and the same protocol from whole blood stimulation with LPS was followed (section 2.3.9). The MSC cell pellet was broken by gentle tapping of the FACs tube followed by the MSCs being thoroughly mixed with the blood sample.

For co-culture experiments using the IP006, Y201 and Y202 MSC cell lines the same protocol for the whole blood stimulation with LPS was followed, as outlined in section 2.3.9. In these co-culture experiments 8×10^5 MSCs were added.

2.3.11 Co-culture of whole blood with MSC CM to study their

immunosuppressive effects on healthy and RA monocytes, by measuring intracellular TNF and IL-6

MSC CM was collected as outlined in section 2.2.6. For each experiment MSC CM aliquots (4ml) from IP006, Y201, Y202 and RA synovial MSCs were thawed and added to monocytes using the same whole blood assay. In sterile polystyrene FACs tubes heparinised blood was diluted 10-fold in MSC CM, with 1ng/ML LPS and 2mL Brefeldin A. The sealed tubes were placed in an incubator for 6 hours at 37°C and 5% CO_2. After the 6-hour incubation, the same procedure was carried out, as described in section 2.3.9. The above protocol was also carried out for RA synovial MSC CM cultured with HC monocytes, using the whole blood based system.

Control experiments were carried out whereby heparinised blood was stimulated with 1ng/ml LPS and treated with 2mL Brefeldin A (as mentioned above), in sterile polystyrene FACS tubes and diluted 10-fold in StemMACs media. The same procedure was carried out as described in section 2.3.9. The sealed tubes were placed in an incubator for 6 hours at 37°C and 5% CO_2. This was in order, to check that the different cell culture media that the MSCs were cultured in (StemMACs media) did not affect the detection levels of TNF and IL-6 in activated monocytes, in comparison to RPMI.

2.3.12 Flow cytometry: Acquisition and analysis

The BD LSRII 3 LASER flow cytometer was used to collect data acquiring a minimum of 1.0×10^4 events per sample. BD FACs DIVA version 7 offline analysis software was used to analyse the data.

2.3.13 Statistics

Statistical analysis and graphs were produced using Windows Excel for Mac 2011 and Graph Pad prism software version 7 for mac OS X. To determine the statistical significance of the differences observed between different conditions, paired t-tests were performed. Statistically significance differences were considered when $p < 0.05$ and were denoted $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$.

Chapter 3 – Optimisation of a whole-blood based assay for detection of pro-inflammatory cytokines in monocytes

3.1 Introduction

To test the hypothesis that MSCs modulate pro-inflammatory cytokine production by monocytes required the development of an assay in which monocytes were stimulated to produce pro-inflammatory cytokines, in the presence or absence of MSCs.

My aim was to develop a test that would be simple enough to repeat using small amounts of fresh clinical samples (i.e. the assay should not require multiple preparative steps in which these small samples would be processed over several hours, and during which small and valuable cell samples might lose or change functional capacity). Cytokines relevant to the pathology of RA include TNF and IL-6, as underlined (shown) by the therapeutic success of biologics targeting these cytokines (Kim et al. 2015, Radner and Aletaha, 2015). The production of both TNF and IL-6 can be induced in blood-derived monocytes via stimulation of TLR4 with bacterial endotoxin, also known as lipopolysaccharide (LPS) (Baqui et al 2000).

Cytokine production is frequently tested using an enzyme linked immunosorbent assay (ELISA), which quantitates soluble material secreted from cells. However, my aim was to quantitate TNF and IL-6 production by monocytes from within the complex mixture of cell types present in a whole blood sample. Thus, I used a flow cytometric assay in which cytokines were assayed as they accumulated in the producing cell type. This required the blocking of cytokine secretion (using Brefeldin A) and the permeabilisation of cells (to enable the (allow) entry of fluorescent conjugated antibodies). Despite these necessary manipulations, this intracellular staining assay has the distinct advantage that it can be combined with cell surface staining and gating of particular cell populations in order to assay cytokine production by the monocytes within the whole blood. Using whole blood also has the advantage that it provides a more physiologically relevant setting for the assessment of MSC and monocyte interactions in the presence of other cell types, which *in vivo* would play a role in modulating the monocyte-MSC crosstalk. This is in contrast to all other MSC-monocyte co-culture assays whereby the monocytes are purified from whole blood (J. Kim and Hematti 2009; Melief, Schrama, et al. 2013; Oh et al. 2014) or a monocytic cell line is used (such as the THP-1 monocytic cell line (Vallés et al. 2015)).

Initially, I selected TNF for measurement as it is a key pro-inflammatory mediator, known to be produced from monocytes during inflammation and it also contributes to the pathogenesis of many diseases, including RA (Belge et al. 2002; Vasanthi, Nalini, and Rajasekhar 2007;

Kalden and Schulze-koops 2017).

3.2 Optimisation of a whole-blood based assay for detection of pro-inflammatory cytokine TNF in monocytes

I first used the BD Biosciences FastImmune intracellular staining kit to detect the presence of TNF. Briefly, whole blood samples were treated with LPS (1ng/ml) for six hours and then treated with red blood cell (RBC) lysis buffer. After, which cell surface staining for monocytes was next carried out using CD14-BV421 monoclonal antibody, followed by fixation and permeabilisation steps. Next, intracellular staining for TNF was carried out. Subsequently cells were washed with wash buffer and fixed with paraformaldehyde (PFA). The data were acquired on BD LSRII 3 laser flow cytometer within 24 hours.

TNF production was assayed using the gating strategy, shown in Figure 3.1. Monocytes, granulocytes and lymphocytes were identified by their distinct light scatter characteristics, based on their size (in the forward light scatter channel; FSC) and granularity (in the side scatter channel; SSC). Monocytes were identified by their expression of cell surface CD14. Expression of TNF in these cell populations was then determined by analysing the staining with anti-TNF antibody in the monocyte, granulocyte and lymphocyte gates (Figure 3.1); intracellular TNF levels were assayed in blood samples in both the presence and absence of LPS stimulation.

The flow cytometry data for a single blood sample are shown in Figure 3.1. The data indicates that LPS induces intracellular TNF production in monocytes. There was also evidence of low-level production in granulocytes in response to LPS, but no detectable TNF in the lymphocytes.
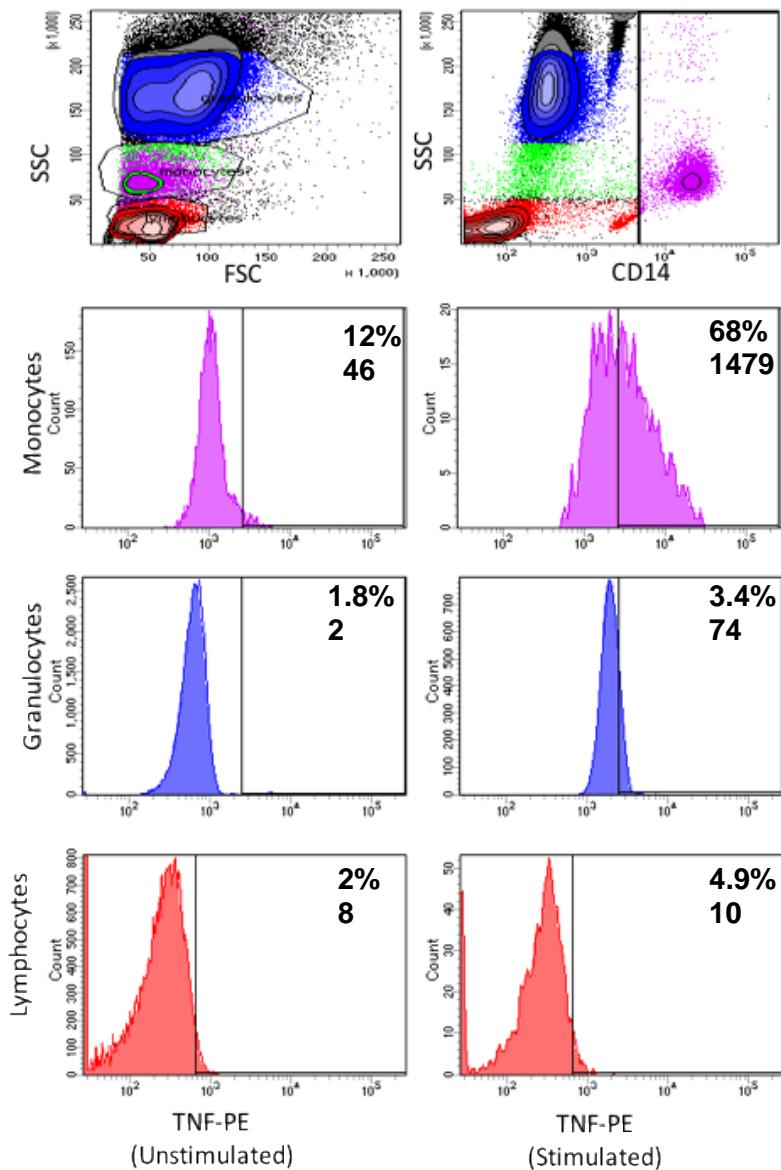
Figure 3.1 – Gating strategy used for the detection of intracellular TNF in LPS stimulated monocytes (identified by CD14 expression), using the BD Biosciences Fast Immune Cytokine Staining kit. Monocytes, granulocytes and lymphocytes (shown in purple, green and red respectively) were identified using FSC/SSC plots (top). Further gating was carried out to identify monocytes by their CD14 expression. Histograms are shown for the detection of intracellular TNF for both unstimulated and LPS stimulated CD14 positive monocytes, granulocytes and lymphocytes, based on isotype control staining.

This whole blood assay was then used to measure intracellular TNF levels from LPS stimulated monocytes, using five blood donors as healthy controls (Figure 3.2). This assay tested the effect of LPS stimulation and whether BFA treatment was essential to the detection. The expression of TNF in the different cell populations was determined in two ways; i) the percentage of gated cells expressing intracellular TNF and ii) the mean fluorescence intensity (MFI) of the TNF staining, as a measure of the level of TNF in these expressing cells. My

results showed that activation of monocytes with LPS is required for the induction of intracellular TNF, as there was a significant difference in the percentage of TNF expressing monocytes in untreated versus LPS-treated cells (P<0.01) and in the level of TNF expression (based on the MFI: P<0.05). Contrastingly, very low levels of intracellular TNF were detected in granulocytes and lymphocytes, regardless of LPS stimulation. Additionally, the results showed that the addition of BFA was essential for the successful detection of LPS-induced intracellular TNF in monocytes, as only minimal levels of TNF were detectable from LPS-stimulated monocytes without BFA treatment. These results were expected given that BFA blocks transport from the Golgi apparatus to the ER; in the absence of BFA, TNF is transported to the cell surface and (via the action of TNF cleaving enzymes) shed from the cell. By contrast, in the presence of BFA, TNF accumulates at the Golgi and post-Golgi secretory compartments, thereby facilitating its detection via intracellular staining.
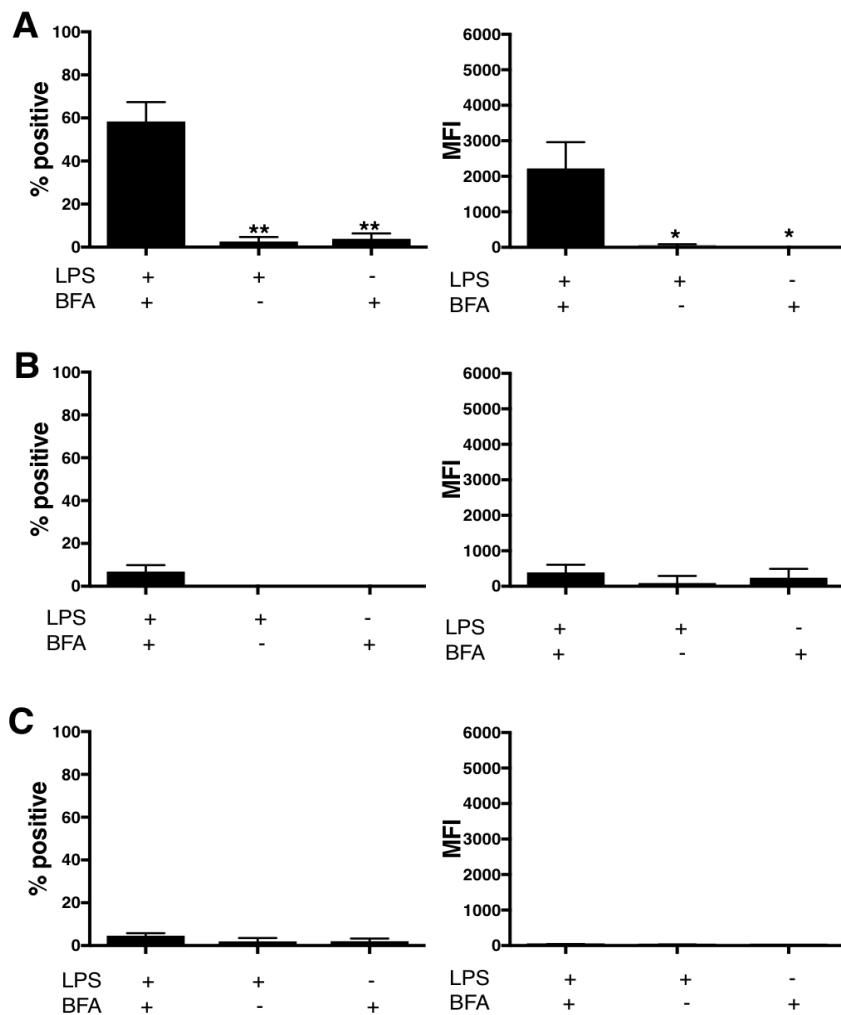
Figure 3.2 – Detection of intracellular staining for TNF using the BD Biosciences FastImmune intracellular staining kit. Intracellular staining was carried out using the whole blood assay, with cells being treated with 1ng/ml of LPS for 6 hours. Bar charts show intracellular TNF measurements using percentage positive **(left)** and MFI values **(right)** for: CD14 positive monocytes **(A)**, Granulocytes **(B)**, and Lymphocytes **(C)** (n=5). Error bars represent SEM. Statistical significance of intracellular TNF, detected on LPS activated monocytes in comparison to unstimulated monocytes and LPS stimulated monocytes with no BFA treatment, was carried out using paired T tests. $^{*}P<0.05$ and $^{**}P<0.01$.

To test if the intracellular staining for TNF in activated monocytes could be improved by using different fixation and permeabilisation conditions, an alternative intracellular staining kit was also used (the BC Intraprep Permeabilisation kit for intracellular staining of TNF). Using the same treatment conditions and gating strategy described above, this procedure generated similar results, with LPS-induced TNF production readily observed in CD14+ monocytes, but not in granulocyte or lymphocyte populations (Figure 3.3).
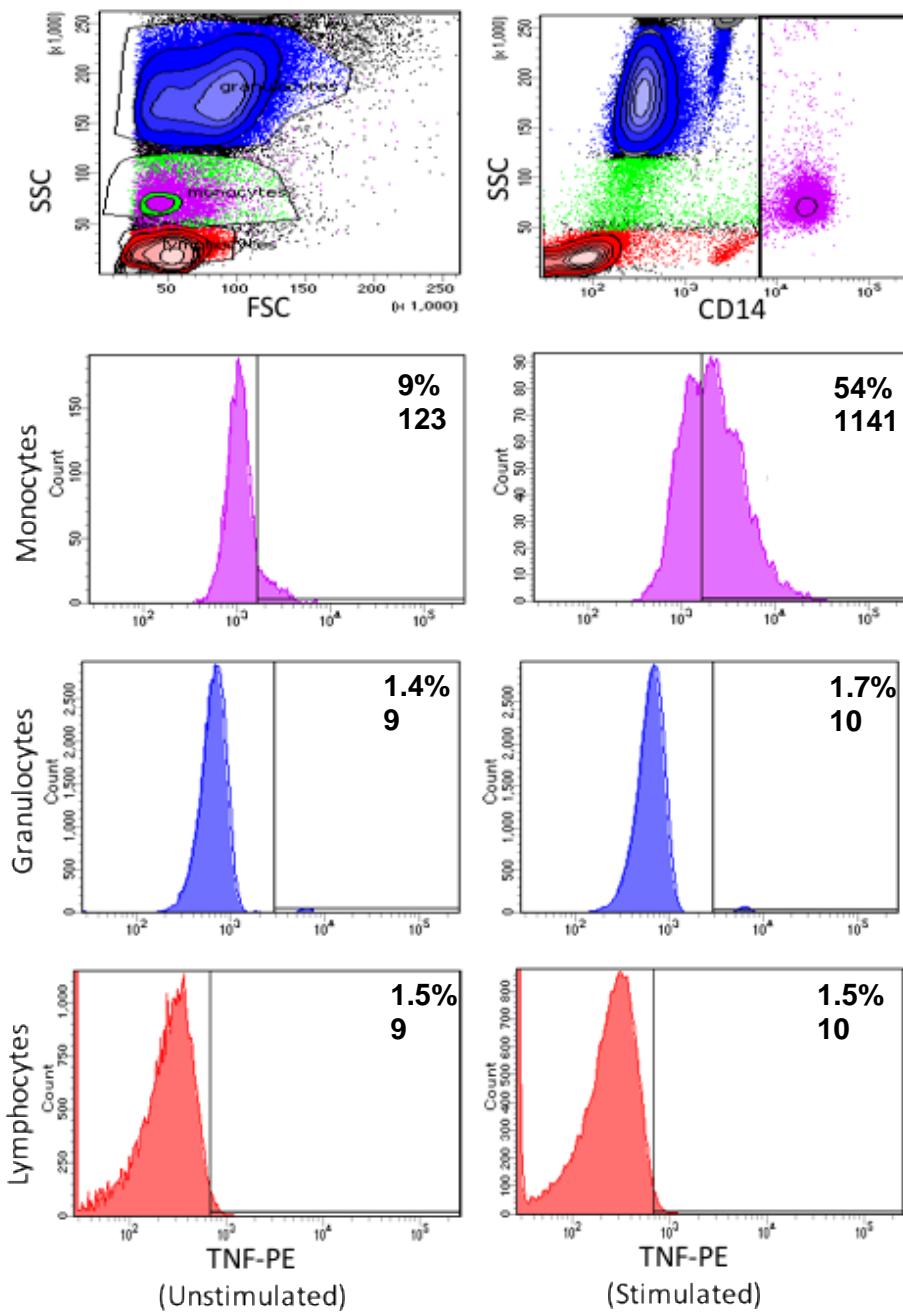
Figure 3.3 – Gating strategy used for the detection of intracellular TNF in LPS stimulated monocytes (identified by CD14 expression) using the Beckman Coulter Intraprep Permeabilisation Kit. Monocytes, Granulocytes and Lymphocytes were identified using FSC/SSC plots. Further gating was carried on monocytes for CD14 expression. Histograms are shown for the detection of intracellular TNF for both unstimulated and LPS stimulated CD14 positive monocytes, granulocytes and lymphocytes, based on isotype control staining.

Using this assay on blood samples from the five healthy donors showed that LPS treatment significantly induced the production of TNF in the monocyte population (Figure 3.4), based on both the percentage of expressing cells ($P<0.01$) and the TNF levels (MFI; $P<0.05$). As found

with the BD Biosciences Fastimmune intracellular staining protocol (Figures 3.1 and 3.2), LPS did not stimulate significant TNF production in the granulocyte or lymphocyte populations; a similar requirement for BFA was also evident.
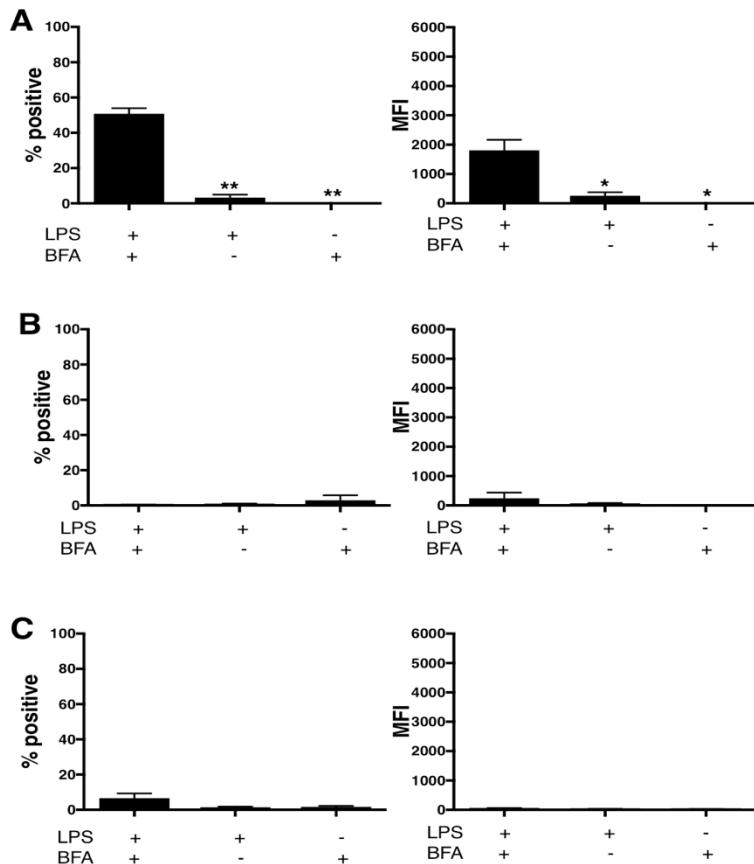
Figure 3.4 – Detection of intracellular staining for TNF. Intracellular staining was carried out using the Beckman Coulter Intraprep Permeabilisation Kit, with cells being treated with 1ng/ML of LPS for 6 hours. Bar charts show intracellular TNF measurements using percentage positive **(left)** and MFI values **(right)** for: CD14 positive monocytes **(A)**, Granulocytes **(B)**, and Lymphocytes **(C)** (n=5). Error bars represent SEM. Statistical significance of intracellular TNF detected on LPS activated monocytes in comparison to unstimulated monocytes and LPS stimulated monocytes with no BFA treatment, was carried out using paired T tests. $^{*}P<0.05$ and $^{**}P<0.01$.

I next determined whether the two intracellular staining kits generated statistically different results. I obtained three donor blood samples and split each into two; one aliquot for use with the BC kit and one for the BD kit. Comparing the results of LPS stimulation using the two kits, I found no significant difference in their assay of intracellular TNF in CD14+ monocytes, this was true for both the percentage of TNF producing monocytes and the MFI (Figure 3.5). Although the two kits generated the same data, the BC kit was faster and more user friendly

and was used for all subsequent intracellular staining experiments on LPS activated monocytes.
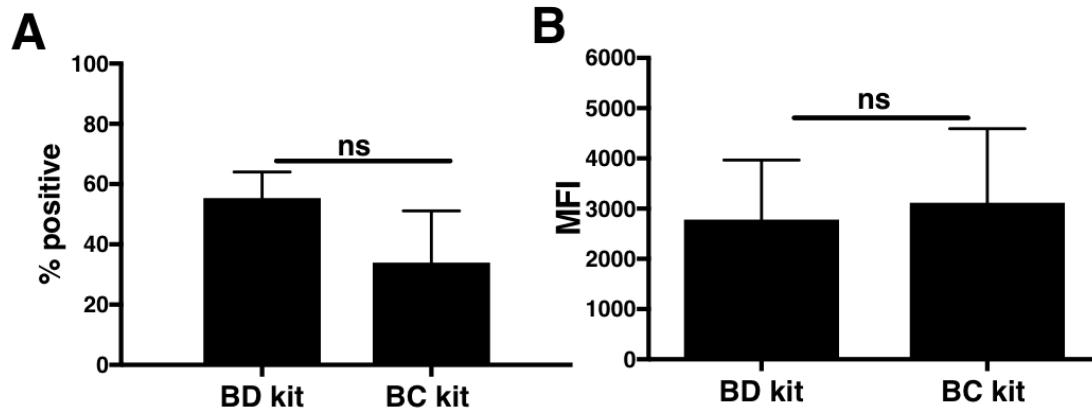
Figure 3.5 – Bar charts show comparison of intracellular TNF detected in LPS stimulated CD14 positive monocytes using the BD Biosciences FastImmune Intracellular staining kit (BD kit) and the Beckman Coulter Intraprep Permeabilisation kit (BC kit). Both percentage positive cells (**A**) and MFI values (**B**) were recorded, by flow cytometry analysis (n=3). Error bars represent SEM. Paired T tests (P>0.05 not significant; ns).

The assays described above demonstrate robust detection of TNF following treatment with 1ng/ml of LPS for six hours. Riberio *et al.* 2016, treated monocytes with up to 5ng/ml of LPS for the optimal detection of TNF production. Therefore, I titrated the LPS dose, using 1ng, 10ng, 100ng and 1000ng/ml of LPS and repeated the intracellular staining protocol for TNF. Figure 3.6 shows representative histograms of intracellular TNF detected in CD14+monocytes in three blood samples from healthy donors, with the collated data shown in Figure 3.7. Overall, results showed that increased concentrations of LPS treatment did not significantly increase the TNF expression in monocytes (Figures 3.6 and 3.7). However, the results did demonstrate a dose-dependent trend, with increased production of TNF in monocytes following stimulation with higher levels of LPS (the exception was the MFI of TNF at 10ng/ml of LPS). A 1000-fold increase in LPS generated an ~8-fold increase in TNF levels (based on the MFI) and a less than 2-fold increase in the percentage of TNF expressing monocytes (Figure 3.7). These modest increases in TNF production were outweighed by the highly non-physiological LPS doses used and, henceforth, further experiments were set up using 1ng/ml of LPS to activate the monocyte population.
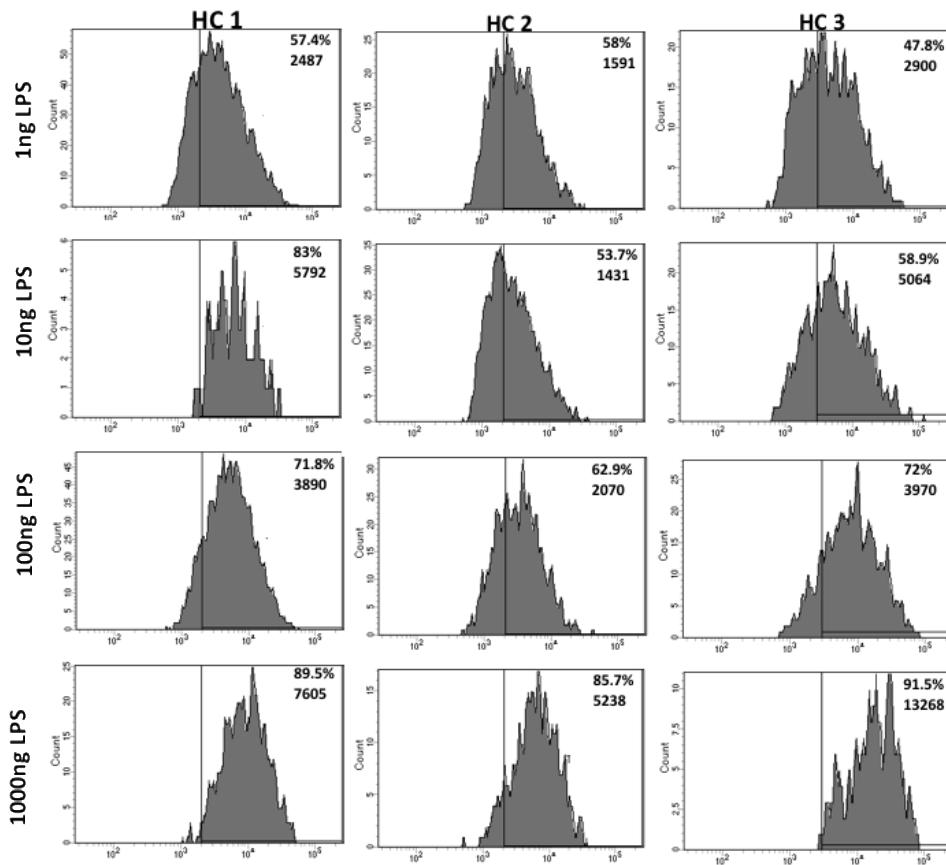
Figure 3.6 – Histograms showing LPS dose titration for the measurement of intracellular TNF on CD14 positive monocytes, for 3 representative HC blood donors. Both percentage positive and MFI values for intracellular TNF is noted, on the top right hand side of each histogram.
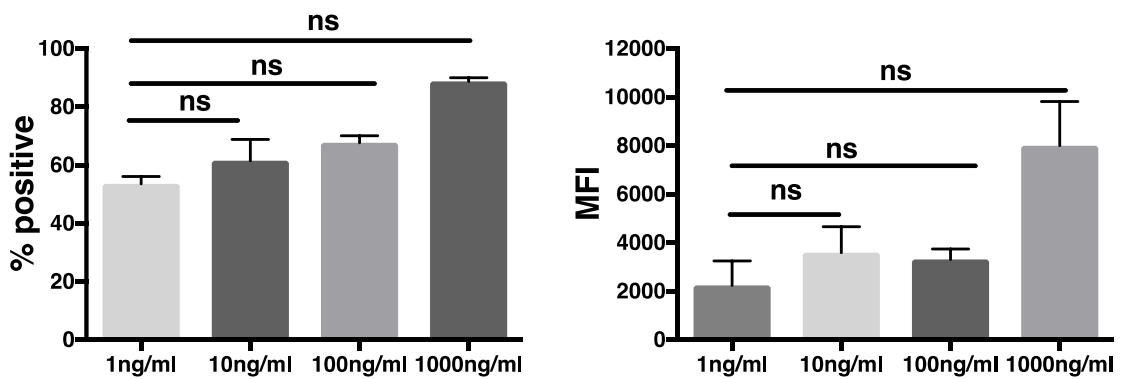
Figure 3.7 – LPS dose titration experiments. Bar charts presenting the detection of intracellular TNF in CD14 positive monocytes treated with different LPS dosages. Percentage positive (left) and MFI (right) values of intracellular TNF was recorded. (n=4, healthy control blood donors). Nonparametric paired T tests.

Next, the optimal time for 1ng/ml of LPS stimulation of monocytes was tested;a 6 hour, 10 hour and 24 hour incubation times were tested. Previously, it was shown that the optimal time for detection of TNF is 6 hours, as well as the cytokine being produced abundantly at 8 hours and 24 hours (Ribeiro et al. 2016a). However, results recorded in Figure 3.8 show that the optimal LPS stimulation time for TNF production, using this assay, was 6 hours, after which TNF expression dramatically decreased. At 24 hours, there was minimal levels of TNF being produced by the activated monocytes. Thus, further experiments continued to use the 6 hour incubation time to activate the monocytes with LPS.
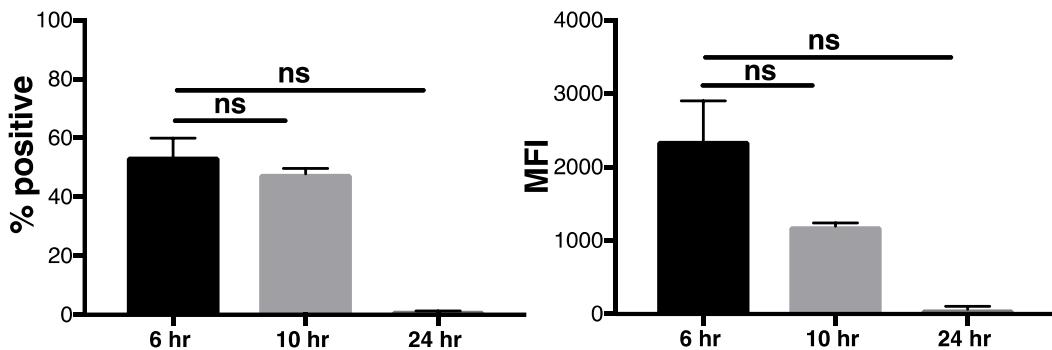
Figure 3.8 – Comparison between incubation times on TNF production in activated monocytes. Bar charts presenting the detection of intracellular TNF in CD14 positive monocytes treated with 1ng/ml of LPS. Percentage positive (left) and MFI (right) values of intracellular TNF was recorded. (n=3, healthy control blood donors). Non-parametric paired T tests.

3.3 Detection of IL-6 pro-inflammatory cytokine in LPS-stimulated monocytes, using a whole blood assay

Previously published work (Riberio et al. 2014) measured only the production of intracellular TNF in activated monocytes using a whole blood assay. However, as well as TNF, activated monocytes produce IL-6, another key pro-inflammatory cytokine implicated in the pathogenesis of RA (Duque and Descoteaux 2014) (Srirangan and Choy 2010). Like TNF, expression of the IL-6 gene is regulated by NF-KB and both IL-6 and TNF are induced by LPS stimulation of monocytes via TLR4 (Ploder et al. 2003). This suggests that LPS stimulated monocytes would also produce IL-6 within the context of the whole blood assay. I therefore repeated the LPS stimulation of whole blood and analysed intracellular IL-6 expression via flow cytometry. LPS induced expression of IL-6 in monocytes, but not in granulocytes or lymphocytes (Figure 3.9).
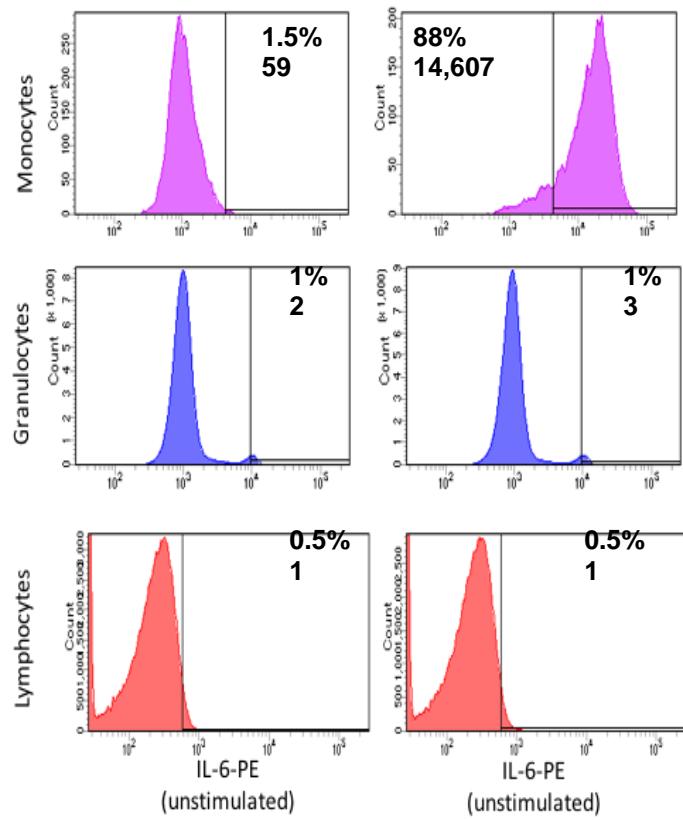
Figure 3.9 – Detection of intracellular IL-6 in LPS stimulated monocytes using the Beckman Coulter Intraprep Permeabilisation kit. Monocytes, granulocytes and lymphocytes were identified using FSC/SSC plots. Histograms are shown for the detection of intracellular IL-6 for both unstimulated and LPS stimulated Monocytes, Granulocytes and Lymphocytes.
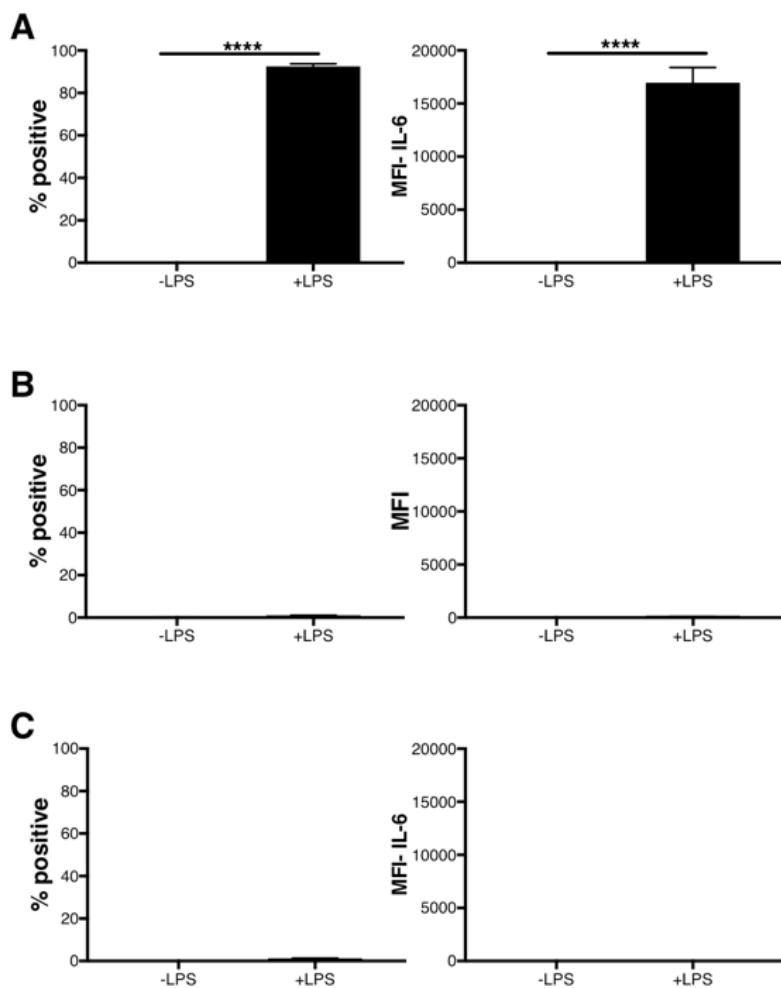
Figure 3.10 – Detection of intracellular staining for IL-6. Intracellular staining was carried out using the whole blood assay, with cells being treated with 1ng/ML of LPS for 6 hours. Bar charts show intracellular IL-6 measurements using percentage positive **(left)** and MFI values **(right)** for: CD14 positive monocytes **(A)**, Granulocytes **(B)**, and Lymphocytes **(C)** (n=6). Error bars represent SEM. Statistical significance of intracellular IL-6 detected on LPS activated monocytes in comparison to unstimulated monocytes, was carried out using paired T tests. ****P<0.0001.

Furthermore, analysis of data from six healthy donors showed this result was highly reproducible and statistically significant (Figure 3.10). These results indicate that the whole blood assay developed here is a simple and reliable assay for the LPS induction of both TNF and IL-6, key pro-inflammatory cytokines in RA and other inflammatory diseases. This assay can now be used as a means to assess whether MSCs can dampen these pro-inflammatory responses. This will be determined by culturing the MSC populations under test within the whole blood population, in the presence and absence of LPS. Performing these MSC-blood co-cultures required i) the culture and analysis of primary human MSCs and ii) an improved gating strategy to ensure that the monocytes could be easily distinguished from MSCs in these co-cultures.

3.4 Co-culture assay assessing the immunosuppressive effect of BM MSCs on LPS stimulated monocytes

Firstly, I characterised the cell surface marker expression of culture expanded bone marrow cells to prove their MSC identify, as outlined by the International Society for Cellular therapy (ISCT) (Dominici et al., 2006). The ISCT definition of MSCs is as follows: MSCs must be plastic adherent, *in vitro* they must express CD105, CD73, CD90 and lack expression for CD34, CD45 or CD11b, CD79 or CD19 and HLA-DR and must be able to differentiate into adipocytes, osteoblasts and chondroblasts (Dominici et al., 2006).

I cultured three MSC isolates MSC-1, -2 and -3. These were derived from healthy control bone marrow aspirates. Growth of MSCs originated from single colonies, grown to confluence, after which the adherent cells were passaged. Cultures were expanded and cryopreserved, as described in section 2.2.3; MSC isolates were thawed and used for ISCT MSC phenotyping experiments to investigate surface marker expression levels. Expression analysis by flow cytometry is shown in Figure 3.11. The primary BM MSCs displayed characteristic ISCT MSC phenotype, with expression of CD73, CD90 and CD105 and a very low/negative expression of the haematopoietic cell marker antigen CD45 and other markers expressed outside of the MSC lineage, such as CD34, CD14, CD19 and HLA-DR (the negative marker cocktail in Figure 3.11). These cells, therefore, met the ISCT criteria based on cell surface marker expression.
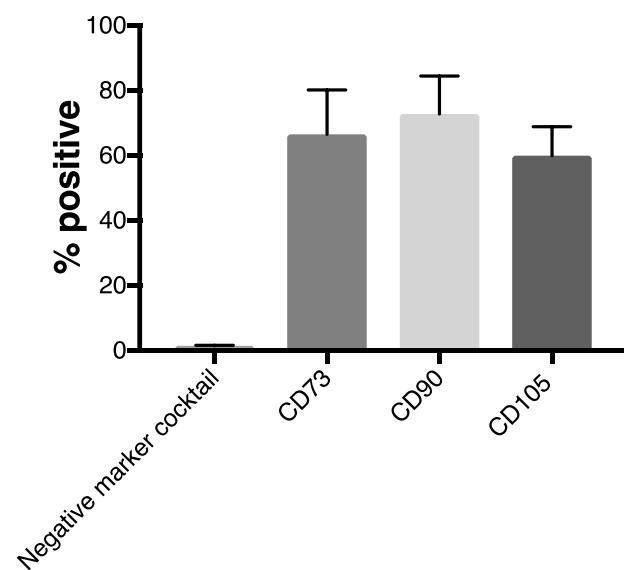
Figure 3.11- ISCT characterisation of MSCs. Bar chart showing % positive expression of MSC surface markers by flow cytometry analysis (n=3). Error bars represent SEM of mean values.

Co-culture experiments were then set up containing the primary BM MSCs and whole blood to determine the immunosuppressive abilities of MSCs on monocytes. A modified gating strategy was used; the assay contained counting beads and incorporated detection of CD45, as well as the CD14 detection, to distinguish the hematopoietic derived cells (CD45+) from the MSCs (CD45-). The same gating strategy was employed, as shown in Figure 3.3. Co-culture experiments (n=3) were carried out using one healthy control blood donor and three different BM MSC cultures, on different days. The results in Figure 3.12 show that MSC-1, -2 and -3 exert different levels of inhibition on the LPS-induced TNF production. MSC-1 demonstrated a clear dose-dependent suppression of TNF production by activated monocytes. Co-culture with MSC-2 and MSC-3 showed a reduction of TNF production by activated monocytes as the MSC:monocyte ratio increased. However, the increments of inhibition of TNF detected (for both % positive expression and MFI values) were less than those seen with MSC-1. Importantly, these co-culture experiments showed that LPS-induced TNF production was variable in the activated monocytes alone (the positive control) in three assays (48%, 62% and 41.5% respectively), when using the same healthy control blood donor. Thus, variation exists even for the same blood donor when assays are performed on 3 different days, presumably due to deterioration of sample quality.
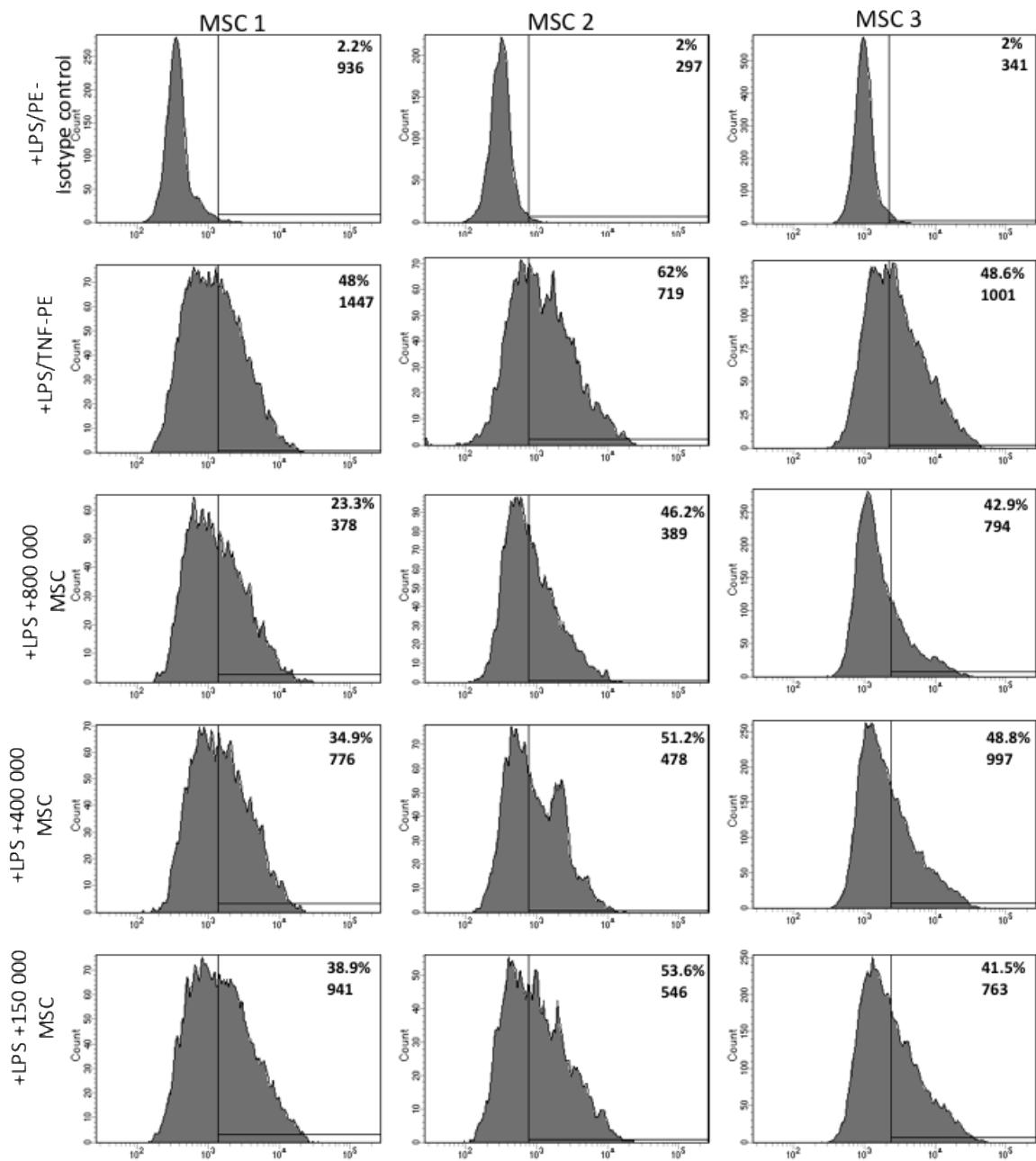
Figure 3.12 – Histograms for TNF staining, measuring intracellular TNF levels in activated monocytes co-cultured with BM MSCs, in the whole blood assay (n=3). Heparinised blood was diluted 10X and monocytes were stimulated for 6 hours with 1ng/ML of LPS in culture with different numbers of BM MSC. Both percentage positive and MFI values are noted on the top right hand side of each histogram.

Despite this donor variability, the collated results from all three co-culture experiments indicated a general trend for a dose-dependent suppression by the MSCs of LPS-induced TNF production. This same trend applied when using both percentage positive (Figure 3.13, **A**) and MFI measurements (Figure 3.13, **B**). Although statistical significance was not achieved, these results underline the immunosuppressive potential of BM MSCs on LPS-activated monocytes, using the whole blood assay.

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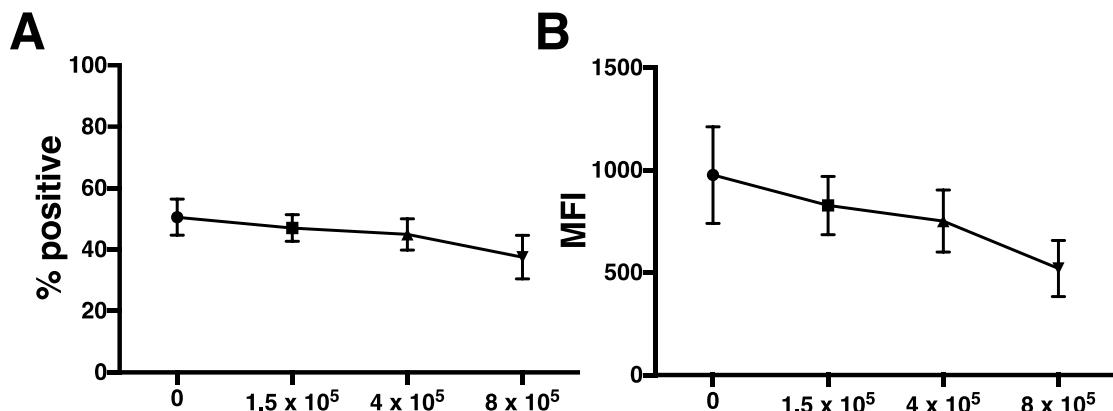
Figure 3.13 – TNF levels in LPS activated Monocytes in co-culture with different doses of BM MSCs. Assessing the immunosuppressive abilities of BM MSCs on LPS activated monocytes, by monitoring intracellular TNF levels (n=3). Both percentage positive (**A**) and MFI (**B)** values for intracellular TNF was recorded. Blood from the same healthy control donor was used for monocytes in co-culture with BM MSCs from different donors. Monocytes were stimulated with 1ng/mL of LPS in culture with BM MSCs (at different ratios) for 6 hours. Data represent mean values and error bars represent SEM. Paired T test (ns). P values for % positive results were; 1.5 x10^5 (p=0.58), 4x10^5 (p=0.49), 8 x10^5 (p= 0.23). P values for the MFI results were; 1.5 x10^5 (p=0.56),4x10^5 (p=0.48), 8 x10^5 (p= 0.29).

In summary, experimental conditions have been determined for the successful detection of intracellular TNF on activated monocytes, treated with 1ng/ml LPS for an incubation of 6 hours, using the whole blood assay. This assay was proven to be sufficiently robust across two different intracellular staining kits. In addition to previously published work (Ribeiro et al. 2016), my results in this chapter have shown that these conditions can also be used for the detection of IL-6 cytokine production in activated monocytes. Furthermore, the addition of primary BM-derived MSCs to the LPS-stimulated whole blood demonstrated a reduction in TNF production, consistent with an inhibitory role for MSCs in pro-inflammatory cytokine production. The greatest reduction in production of TNF in the activated monocytes was observed at the 8 x 10^5 MSCs per blood sample, confirming previous findings (Ribeiro et al. 2016). Therefore, all subsequent MSC co-culture experiments were with whole blood, used MSCs at this dose.

3.5 Discussion

Testing the ability of MSCs to modulate pro-inflammatory cytokine production from monocytes requires the development of suitable assays. The experiments presented in this chapter had two main aims. Firstly, to establish a simple physiologically relevant assay, applicable to fresh clinical samples, for optimal detection of the production of pro-inflammatory cytokines TNF and IL-6 from activated monocytes. Secondly, to examine the immunomodulatory impact of

BM MSCs on monocytic TNF and IL-6 production. The results demonstrate an optimal detection of intracellular TNF and IL-6 in LPS-activated monocytes, using a whole blood assay. Moreover, the addition of primary BM MSCs to the LPS-treated monocytes resulted in a reduction in TNF production.

The whole blood assay is a novel methodology developed to examine the immunomodulatory ability of MSCs on monocytes. This is an improved assay, as previous studies investigating the interactions between MSCs and monocytes have typically used a monocytic cell line or monocytes isolated using magnetic cell separation methods. Using magnetic cell separation kits, to isolate monocytes, not only requires more hours in the laboratory to process samples, but increases the chances of undesirable manipulation of the sample, which could affect the results. Also, using immortalised cell lines such as the THP-1 human monocytic cell line, provides a less physiologically relevant model compared to the whole blood assay. This is due to such cell lines as THP-1 being of malignant origin, thus they are more likely to alter their properties and be a less realistic representation in their natural environment, when compared with normal somatic cells. Using, the whole blood assay is preferable in that monocytes are able to interact with the MSCs, as well as being still have cross talk with the surrounding cells, as would be the case in normal physiological settings.

Monocytes were the chosen target cell population for investigation as they are known to respond swiftly to activation by pathogen-associated and damage associated molecular patterns (PAMPS and DAMPs). Additionally, it is known that monocyte levels are elevated in the peripheral blood of RA patients and are associated with increased disease severity and worse prognosis in patients with RA, as discussed in the introduction (Fukui et al. 2018). This is further supported by a study by Coulthard et al. that demonstrated that there were significant differences in absolute cell counts of monocytes between healthy control and patients with RA. Prior to any therapy (baseline) CD14bright (which are the main TNF producing monocyte subset) and granulocytes were in increased in both patients with early and late RA. Additionally, CD16+ granulocytes, NK cells and CD14dim monocytes all expressed higher levels of membrane associated TNF (mTNF) in patients with RA (Coulthard et al. 2012).

 TNF and IL-6 were the two cytokines chosen to be measured in this assay because the kinetics of production after activation is very rapid. These pro-inflammatory cytokines are induced via LPS stimulation via TLR4, which drives their transcription, within 2-6 hours of stimulation. Additionally, both TNF and IL-6 mRNA molecules have AU rich regions in the 3-untranslated regions, which results in the rapid degradation of the TNF and IL-6 genes that are typically active most of the time. However, with LPS stimulation the destabilisation machinery is switched off and therefore a pool of premade mRNA can make new protein very

rapidly (Stumpo DJ et al 2010). Additionally, TNF and IL-6 are key multifunctional cytokines involved in in the regulation of a range of biological processes, including the regulation of haematopoiesis, immune response and inflammation (Srirangan and Choy 2010; Bazzoni and Beutler 2002). Importantly, TNF and IL-6 are key players known to be produced from monocytes in RA pathology and, as such, they are key therapeutic targets for treatment of this disease (Ogata et al. 2013; Braun and Kay 2017)(Coulthard et al. 2012; Smiljanovic et al. 2018). However, other cytokines that might contribute to RA disease pathology could also be tested in this assay, such as IL-1 and IL-12 and the chemokine, IL-8. These are mainly produced by monocytes and macrophages and are involved in the pathogenesis of RA (Duque and Descoteaux 2014).

Several parameters in the whole blood assay were further considered and optimised. For example, the presence or absence of BFA, commonly referred to as the 'Golgi block' was examined. Protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus is inhibited by BFA (Schuerwegh et al. 2001; Bueno et al. 2002; Ritzenthaler and Robinson 2002), resulting in the accumulation of proteins in the cytoplasm and secretory pathway, which allows for optimal detection of cytokines via intracellular staining protocols. Without the addition of BFA, it wasn't possible to successfully detect intracellular TNF from LPS activated monocytes. BFA was chosen over the alternative protein transport inhibitor, monensin. Other researchers have shown that LPS-stimulated intracellular detection of cytokines such as TNF, IL-6 and IL-1β is more sensitive in BFA-inhibited monocytes compared to monensin-inhibited monocytes, with increased viability of monocytes when using BFA (Schuerwegh et al. 2001). In other words, for optimal detection of intracellular TNF and IL-6 produced from activated monocytes, BFA is a more potent and less toxic protein inhibitor of cytokine secretion than monensin.

Initially, using this whole blood assay, intracellular TNF from activated monocytes was successfully measured using the Becton Dickinson (BD) kit. However, to examine if the intracellular staining of TNF in activated monocytes could be further enhanced, an alternative intracellular staining kit was also used – the Beckman Coulter (BC) kit. The BC kit generated similar results to the BD kit, being able to detect intracellular TNF in activated monocytes, but not in granulocytes or lymphocytes. This demonstrated the reproducibility and robust nature of the whole blood assay. Although no significant difference was observed in intracellular TNF levels in monocytes, when comparing the two intracellular staining kits, all further experiments were carried out using the BC kit. The BC kit was faster and more user-friendly and had previously been successfully used in TNF intracellular staining experiments by Ribeiro et al. The BC kit allowed for fewer processing steps and combined the fixation, permeabilization and RBC cell lysis steps, by using two simple reagents, as part of the BC kit. This was in contrast to the BD kit which required additional steps, reagents and further washing steps.

As well as detecting intracellular TNF, experiments were also conducted to test for intracellular IL-6 production in monocytes. Detection of IL-6 was performed using the whole blood assay, employing an updated gating strategy. This included the addition of counting beads and a CD45 marker to be included and measured by flow cytometry. Counting beads were added to samples prior to acquisition on the flow cytometer for absolute cell counts, in particular to standardise the experiment from sample to sample. This was in contrast to previous intracellular cytokine profiling of monocytes which had been carried out using either THP-1 cells or purified monocytes from whole blood using magnetic beads, where the numbers of monocytes used in various experiments were known precisely (Eggenhofer and Hoogduijn 2012; Secco et al. 2009; Vallés et al. 2015; Melief et al. 2013). This would be useful in later MSC: monocyte co-culture experiments. Also, with the updated gating strategy, monocytes were further classified by their CD14/CD45 expression. CD45 is a protein-tyrosine phosphatase that is expressed on all haematopoietic cells. However, the monocytes in these experiments were not further classified by the 3 main monocyte subset groups, as described in the introduction (Section 1.3.1).

A limitation of the assay used here is that the separate monocyte subset populations cannot be evaluated, because following in vitro culture, and especially after LPS activation, CD16 expression is downregulated (Ong et al. 2019; Ribeiro et al. 2016a), thus, making it impossible to identify intermediate and non-classical monocytic subpopulations (Ribeiro et al. 2016; Belge et al. 2002). Moreover, in the future other substitute markers could be used, such as IREM-2, HLA-DR, CD33 or CD38 (Picozza et al. 2013), as well as a combination of the following five-marker alternative to the CD14-CD16 classification to identify the three monocyte subsets, which includes HLA-DR, CD33, CD86, CD64 and CCR2. The advantages of such markers are that, unlike CD16, they are stable markers for differentiating between monocyte subsets. As such, compared to CD14/CD16, they have been shown to be stable markers under culture conditions and are not downregulated under conditions where monocytes are being stimulated by LPS (Ong et al. 2019).

LPS was the chosen stimulant to activate the monocytes as it is the main component of the outer membrane of Gram-negative bacteria and one of the most potent PAMP that stimulates monocytes. It is well known that LPS binds to TLR-4 and its co-associated proteins, CD14 and LBP, on the surface of cells such as monocytes, macrophages and dendritic cells (Park and Lee 2013). LPS has previously been shown to be the optimal monocyte stimulus within the context of the whole blood assay, compared to other activation stimuli, such as: phorbol 12-Myristate 13-acetate (PMA), and other PAMPs such as polyinosinic-polycytidylic acid (PolyIC, a dsRNA mimic and a ligand of TLR3), pam3CysSerLys4 (a lipopeptide mimic and TLR1 and 2 ligand), and muramyl dipeptide (MDP, a component of bacterial peptidoglycan and

inflammasome ligand). These PAMPs induce TNF expression by monocytes within the 6-hour stimulation period; however they do so to a lesser extent than LPS (Ribeiro et al. 2016).

LPS titration experiments were carried out to assess the optimal detection of TNF from activated monocytes. Previous studies have used various concentrations of LPS (up to 1000ng/ml) for the successful detection of TNF from monocytes (Borz et al. 2013; Sinistro et al. 2007; Schildberger et al. 2013). Riberio et al. previously titrated LPS from 0.5-20ng/ml LPS, with peak TNF production being found at 1ng/ml, after which elevated levels of TNF were detected from the monocytes with up to 20ng/ml of LPS, but no significant increases in TNF was achieved. The LPS dose was titrated at 1ng, 10ng, 100ng and 1000ng/ml and the intracellular staining protocol was repeated for TNF production in monocytes. Although the results showed a dose-dependent trend with elevated production of TNF post LPS stimulation corresponding with increased levels of LPS, no significant difference were observed in TNF expression between the varying LPS treatments. Therefore, the LPS dose was fixed at 1ng/ml to activate the monocytes, as higher doses of the LPS are highly non-physiological, with only modest concomitant elevations in TNF production. In addition to determining the optimal LPS dose, the optimal time for LPS stimulation of monocytes was also examined. In concordance, with previous studies, optimal detection of TNF was at 6 hours post-stimulation, after which TNF production considerably decreased (Ribeiro et al. 2016). This is consistent with the known rapid kinetics of TNF production following activation (Pages 1991).

It is of note that TNF was not detected from granulocytes or lymphocytes post stimulation with 1ng/ml LPS for 6 hours. This is due the inability of granulocytes, such as neutrophils, to induce TLR4 activation by LPS, thereby failing to engage the "TIR domain-containing adaptor protein inducing interferon β (TRIF)/TRIF related adaptor molecule (TRAM)" dependent pathway (Tecchio, Micheletti, and Cassatella 2014; Tamassia et al. 2007; Van Bruggen et al. 2010). While mast cells also express TLR 4 and can produce many pro-inflammatory cytokines, such as TNF, IL-5, IL-10, and IL-13, the release of TNF from mast cells requires IL-4 priming and serum as a source for sCD14 (Varadaradjalou et al. 2003). Again, there is some debate regarding the absence or presence of certain TLRs, such as TLR4, on eosinophils; however, it has been shown that eosinophils are unresponsive to LPS and do not express CD14 proteins or TLR4 (Kvarnhammar and Cardell 2012). This is in agreement with results presented here, in that intracellular TNF was not detectable in the granulocyte population when carrying out the flow cytometry intracellular staining protocol. Lymphocytes, such as B cells, do not express TLR4 and are unresponsive to LPS (Bekeredjian-Ding and Jego 2009), whereas T cells express almost all TLRs including TLR4; however, their ability to express TLRs is modulated by TCR signaling (Hornung et al. 2002; Kvarnhammar and Cardell 2012; Zarember and Godowski 2002) .

Once the basic cytokine detection assay was assessed, I performed MSC growth and phenotyping and established conditions for MSC-blood co-cultures, using primary BM MSCs and whole blood to assess the immunosuppressive abilities of MSCs on monocytes. The primary BM MSCs were characterized for a number of cell surface marker expression, as proposed by the International Society for Cellular Therapy (ISCT)(Dominici et al. 2006). These primary BM MSCs displayed a characteristic ISCT phenotype. The ISCT MSC cocktail is a combination of positive and negative cell surface markers to characterize MSCs; however, these positive markers (CD105, CD90 and CD75) are co-expressed on a range of cells, so they do hold some limitations in exclusively identifying MSCs. Other MSC markers that have been successfully used in phenotyping MSCs include Stro-1. The Stro-1 antigen, first reported in 1991, has had a critical role in many studies where it was used for the isolation and/or identification of MSCs from a range of tissues (Shwun Lin et al. 2013). Additionally, as well as their cell surface phenotype the ISCT protocol indicates that accurate identification of MSCs includes that the cells can be differentiated into osteoblasts, adipocytes and chondroblasts, in vitro. The BM aspirates used in these experiments have been tested previously by others in our laboratory for their successful MSC differentiation potential, thus further verifying them as MSCs from the bone marrow and therefore such experiments weren't repeated here.

These primary BM MSCs were then added to the whole blood assay to determine their immunosuppressive abilities on monocytes. Interestingly, my experiments showed a general trend for dose-dependent suppression by the MSCs of TNF production from the LPS-activated monocytes. The greatest reduction in TNF production was observed at 8 x 10^5.

MSCs treatment per blood sample, which is in agreement with previous findings (Ribeiro et al. 2016). This indicates that assessment of the potential immunosuppressive activity of BM MSCs on monocytes maybe achieved by using the whole blood assay.

Reduction of TNF production from monocytes by the immunosuppressive abilities of MSCs has been demonstrated previously (J. Kim and Hematti 2009; Vallés et al. 2015; Oh et al. 2014; P.-M. Chen et al. 2014). However, others have preferentially used either the THP-1 monocytic cell line or purified CD14 monocytes from whole blood, using magnetic beads (Kim and Hematti 2009; Vallés et al. 2015; Melief et al. 2013). THP-1 is a human monocytic cell line derived from patient with acute monocytic leukaemia; this cell line is a commonly used model for the study of monocyte/macrophage activities. However, as previously mentioned a key shortcoming of the THP-1 cell line is its malignant origin, which is likely to alter its properties in comparison to normal somatic cells in their natural environment; this is a common disadvantage encountered in using tumour-derived cell lines. Additionally, potentially relevant interactions between target cells and surrounding cells, as in normal physiological settings

within tissues, cannot be easily mimicked when using this cell line (Chanput, Mes, and Wichers 2014; Schildberger et al. 2013). Such properties may underlie the observation that the THP-1 cell model differs from monocytes, PBMCs and whole blood, with regard to cytokine release after stimulation with LPS (Schildberger et al. 2013). Although the THP-1 model has been shown to produce similar levels of TNF after LPS stimulation, this cell line produces reduced levels of IL-8 chemokine and no IL-6 or IL-10 cytokines (Schildberger et al. 2013). Therefore, the whole blood assay provides a more physiologically relevant setting for the investigation of MSCs on activated monocytes, compared to previously used models (Kim and Hematti 2009; Vallés et al. 2015; Schildberger et al. 2013; Chanput, Mes, and Wichers 2014). One additional advantage is that minimal manipulation of monocytes is required prior to investigating the effects of MSC treatment on their activity.

Co-culture experiments, treating whole blood with primary BM MSCs from different donors, were carried out on different days to test the source of any observed variability. The results showed BM MSC donor variability in the differing levels of inhibition on the LPS-induced TNF production by monocytes. The assay was conducted on different days, as a limitation of using primary BM MSC cultures is that they do not always grow to confluency at the same rate, making it difficult to conduct multiple MSC-monocyte co-cultures at the same time (e.g. when testing multiple clinical samples). I have addressed this problem in the next chapter where I investigate the immunomodulatory impact of three immortalised MSC clonal cell lines (IP006, Y201 and Y202) to overcome any scarcity of primary BM MSCs for the treatment of activated monocytes in the whole blood assay.

In summary, data in this chapter reveal the optimal detection of intracellular TNF and IL-6 in LPS-activated monocytes, using a whole blood assay. Moreover, treatment of whole blood with primary BM MSCs showed a reduction of TNF production from monocytes, which suggests that MSCs have an immunosuppressive impact on activated monocytes. In the next chapter, the immunomodulatory abilities of three immortalised MSC lines and their conditioned media are investigated.

Chapter 4 – Immunosuppressive ability of immortalised MSC cell lines on monocytes from healthy donors

4.1 Introduction

In the previous chapter I demonstrated that donor-derived MSC (obtained from three healthy patients undergoing orthopaedic procedures for fracture fixation), were capable of suppressing the LPS-induced production of the pro-inflammatory cytokine, TNF, from healthy monocytes, within the context of a whole blood sample.

However, the primary BM-MSCs demonstrated donor variability, which was to be expected due to genetic/lifestyle factors, resulting in different levels of inhibition on LPS-induced TNF production from monocytes. Thus, the primary BM-MSCs showed donor variation in their immunosuppressive abilities. Additionally, the differing rate at which the primary BM-MSCs grew to confluency was another issue, making it difficult for MSCs to be harvested and ready at the same time for co-culture with patients' blood samples, as part of the whole blood assay examining the immunosuppressive ability of MSCs on monocytes.

I therefore switched from using donor-derived MSC to using the immortalised and clonal MSC cell lines, IP006 and Y201 and Y202. IP006 is a single-cell derived, highly proliferative MSC clonal cell line, derived from the BM of a healthy individual (E. Jones, English, et al. 2010). Y201 and Y202 MSCs are immortalised clonal cell lines that were generated from primary human BM-MSCs that were isolated from the femoral heads during routine hip replacement procedures. They were then immortalised using a lentiviral expression system to overexpress human telomerase reverse transcriptase (hTERT) (James et al. 2015). Previous work, confirms that the Y201 and Y202 MSCs still retain their MSC phenotype after immortalisation. The MSC profiles of Y201 and Y202 were assessed by flow cytometry and were positive for CD29, CD44, CD73, CD90, CD105 and CD166 expression; in addition they were negative for CD11b, CD14, CD169, CD1a, CD4, CD83, CD34 and CD45. This was a profile matching the typical profile of BM-MSCs and also matched their parental cells (James et al. 2015).

4.2 IP006 MSC immunophenotyping

In the first instance I performed an analysis of the cell surface phenotype of the IP006 cell line to confirm that it too had retained an MSC-like phenotype. The three primary MSC cultures used in Chapter 3 were CD73+, CD90+ and CD105+ and negative for a number of haematopoietic cell markers (Figure 3.11). I analysed the cell surface expression of characteristic markers on the IP006 cell line and found that this cell line expressed CD73,

CD90 and CD105, but lacked expression of CD34, CD45 and CD14, in agreement with their designation as an MSC cell line (Figure 4.1).
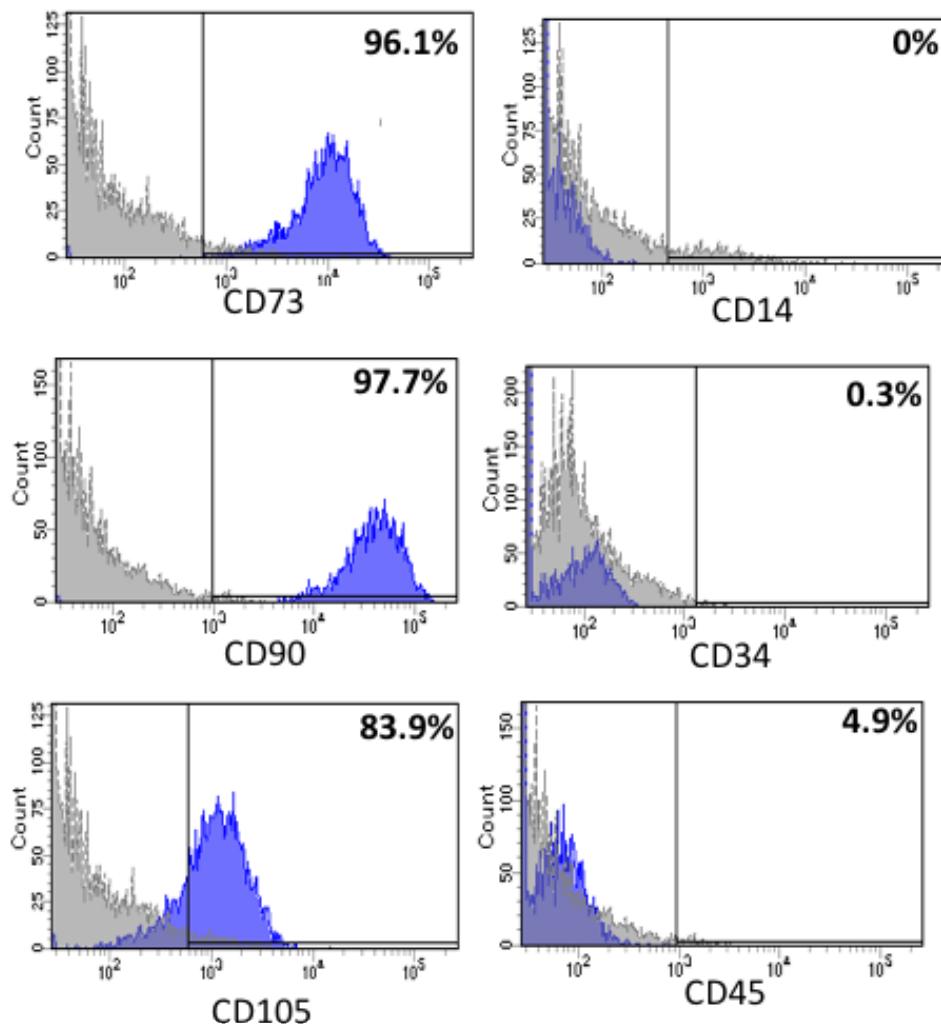
Figure 4.1 – Characterisation of IP006 MSC cell line by flow cytometry. Representative flow cytometry histogram plots for each ISCT surface marker (positive markers on left and negative markers on right). Data were obtained by gating on viable cells, (using DAPI) and cell surface marker expression (in blue) determined in comparison to isotype controls (in grey)

4.3 The immunomodulatory impact of IP006 MSCs on healthy control monocytes

Next, I determined whether the IP006 cell line would behave similarly to the primary MSC cultures and inhibit LPS-induced TNF production from monocytes in the whole blood assays described in the previous chapter. I treated whole blood from three healthy donors with LPS and analysed TNF production in the presence or absence of the IP006 clonal cell line. These three donors revealed that IP006 inhibited the production of TNF from healthy monocytes,

albeit at differing degrees (Figure 4.2A). Furthermore, extending this analysis to seven donors revealed that the IP006-mediated reduction in TNF was statistically significant when analysed in terms of both the percentage of TNF positive cells (P= 0.0154) and the MFI of fluorescence (P= 0.0071: Figure 4.2B).
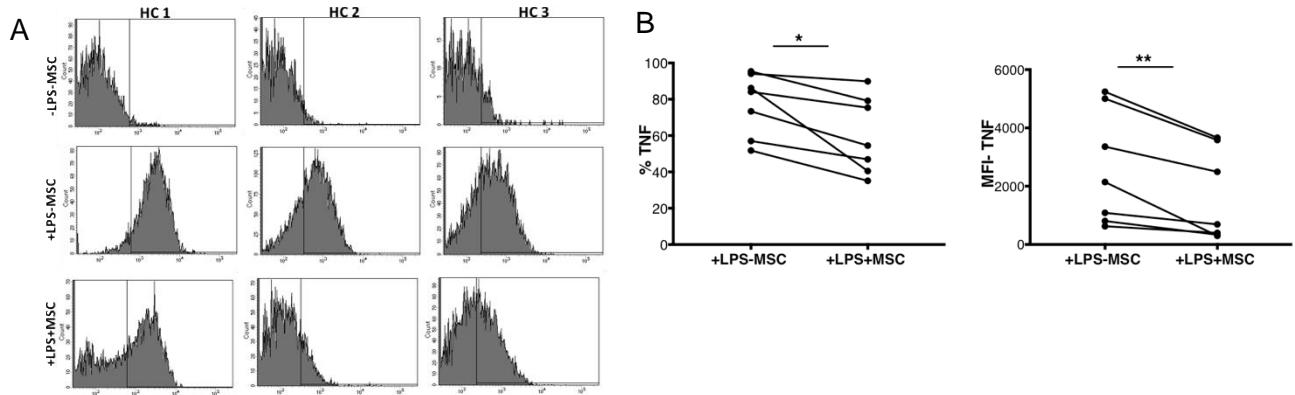
Figure 4.2 – **(A)** Histograms showing inhibition of intracellular TNF in activated healthy monocytes co-cultured with IP006 MSCs, in the whole blood assay (n=3). Heparinised blood was diluted 10X and monocytes were stimulated for 6 hours with 1ng/ml of LPS in culture with 8 x 10^5 IP006 MSCs.
B) TNF levels in LPS activated healthy monocytes with and without IP006 MSC treatment (n=7). Both percentage positive (left) and MFI (right) values for intracellular TNF was determined. LPS activated healthy monocytes were treated with 8 x10^5 IP006 MSCs. Monocytes were stimulated with 1ng/ml of LPS in culture with IP006 MSCs, for 6 hours. Data was analysed using a paired Student's T test.

To further confirm the immunosuppressive abilities of IP006 MSCs, I tested their ability to inhibit IL-6 expression by LPS activated monocytes. Blood from three healthy donors, with or without IP006 MSCs, was treated with LPS and intracellular IL-6 expression in the monocytes (CD14+, CD45+) was determined using flow cytometry. Like the situation with TNF (Figure 4.2), Figure 4.3 demonstrates that the IP006 MSC cell line reduces IL-6 expression in LPS activated monocytes, further supporting the immunosuppressive capacity of IP006 MSCs on healthy, LPS-activated monocytes.
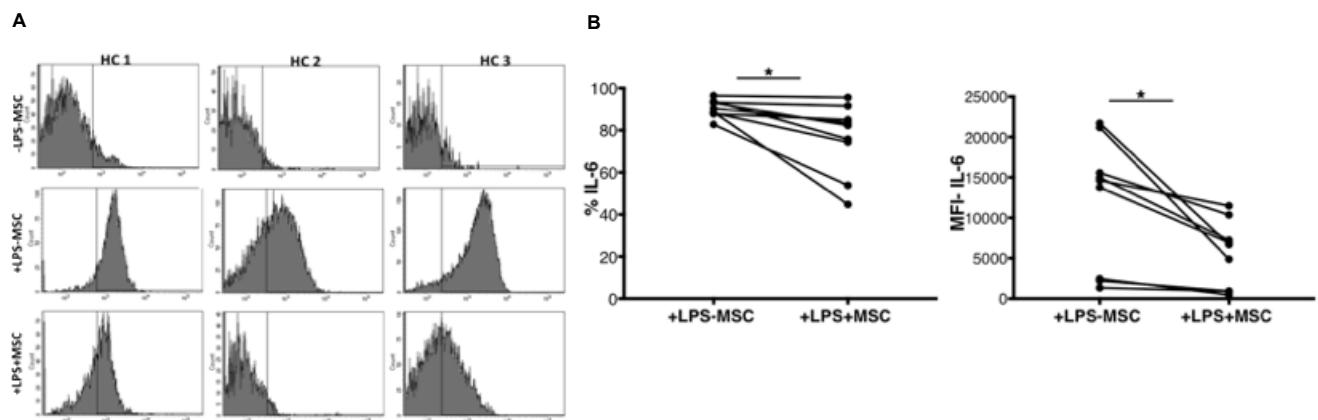
Figure 4.3 – (A) Histograms showing the inhibition of intracellular IL-6 in activated healthy monocytes co-cultured with IP006 MSCs, in the whole blood assay (n=3). Heparinised blood was diluted 10X and Monocytes were stimulated for 6 hours with 1ng/ML of LPS in culture in presence or absence of 8 x 10^{5} IP006 MSCs. **(B)** Graphs showing IL-6 levels in LPS activated healthy Monocytes with and without IP006 MSC treatment (n=10). Both percentage positive (left) and MFI (right) values for intracellular IL-6 was recorded. Activated healthy Monocytes were treated with 8 x10^{5} IP006 MSCs. Monocytes were stimulated with 1ng/ml of LPS in culture with IP006 MSCs, for 6 hours. Paired T tests.

The monocyte population was gated on CD45/CD14 positive cells and IL-6 expression was measured in comparison to isotype controls. This data was then expanded upon using ten donors (Figure 4.3B). The results show (Figure 4.3B) that IP006 reduced the LPS-induced IL-6 production from monocytes, in terms of both the percentage of monocytes expressing IL-6 (P= 0.0179) and the level of IL-6 expression (as determined by the mean fluorescence intensity of expression; P= 0.0104). These results demonstrate that the immortalised IP006 MSC line has immunosuppressive activity similar to the donor-derived MSC used in the previous chapter, specifically IP006 inhibits the LPS-induced TNF production and IL-6 production from blood monocytes from healthy donors.

4.4 The immunomodulatory impact of IP006 MSC conditioned media on healthy control monocytes

To examine whether the immunosuppressive effects of IP006 MSCs were dependent upon cell-cell contact or regulated by soluble factors I used IP006 MSC conditioned media (CM). I repeated the LPS stimulation of healthy blood samples but replaced the IP006 cell line with CM from the IP006 cells. Whole blood was treated with 1ng/ml of LPS and IP006 MSC CM for 6 hours before carrying out intracellular cytokine staining, gating on the on the monocyte population. Figure 4.4A shows representative flow cytometry histogram plots from 3 healthy control blood donors treated with or without IP006 MSC CM. The data shows that that the IP006 CM reduces LPS-induced TNF expression in monocytes, demonstrating that soluble

factors in the IP006 CM was responsible for this immunosuppressive activity of IP006 MSCs on monocytes. This assay was repeated using thirteen healthy donor blood samples and statistical analysis showed a significant reduction in both the proportion of monocytes expressing TNF (P= 0.0001) and the expression level of TNF (P= 0.0053) in the presence of the IP006 CM (Figure 4.4B). IP006 MSC CM reduced monocyte TNF production to a greater extent than IP006 MSCs, by 1.1-fold (%positive) and 2.1- fold (MFI) respectively. In fact, the level of inhibition observed with the IP006 CM (Figure 4.4) was greater than that observed using the IP006 cells themselves (Figure 4.2).
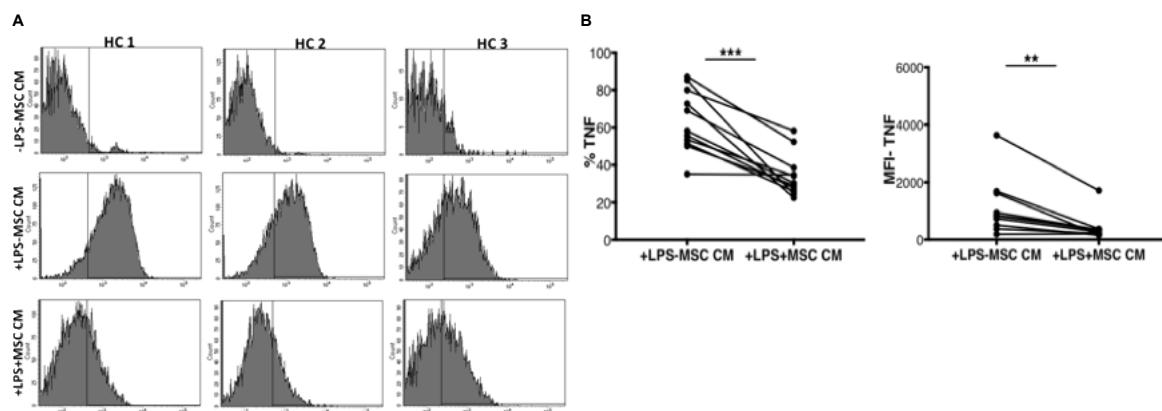

Figure 4.4 – (A) Histograms showing inhibition of intracellular TNF in activated healthy monocytes treated with IP006 MSC CM, using the whole blood assay (n=3). Heparinised blood was diluted 10X and Monocytes were stimulated for 6 hours with 1ng/ML of LPS in culture with 1.6ml of IP006 MSC CM. **(B)** Graphs showing TNF levels in LPS activated healthy monocytes pre and post IP006 MSC CM treatment (n=13). Both percentage positive (left) and MFI (right) values for intracellular TNF was recorded. Monocytes were stimulated with 1ng/ml of LPS in culture with IP006 MSCs, for 6 hours. Paired T tests.

These experiments using IP006 CM were repeated and the LPS-induced IL-6 production was analysed (Figure 4.5). My results, using nineteen healthy blood donors, show that the IP006 CM significantly reduced the proportion of IL-6 expressing monocytes (P= <0.0001) and IL-6 expression levels (P= 0.0008). Furthermore, like the situation with TNF above, the CM exhibited more potent immunosuppressive activity than the IP006 cells themselves (Figure 4.5).
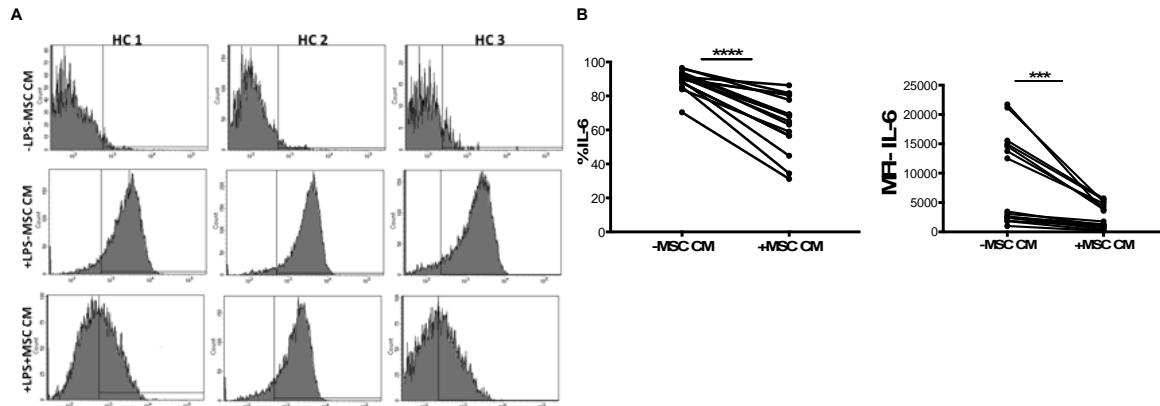
Figure 4.5 – (A) Histograms showing inhibition of intracellular IL-6 in activated healthy monocytes treated with IP006 MSC CM, using the whole blood assay (n=3). Heparinised blood was diluted 10X and Monocytes were stimulated for 6 hours with 1ng/ml of LPS in culture with 1.6ml of IP006 MSC CM.**(B)** Graphs showing IL-6 levels in LPS activated healthy Monocytes pre and post IP006 MSC CM treatment (n=19). Both percentage positive (left) and MFI (right) values for intracellular TNF was recorded. Monocytes were stimulated with 1ng/ml of LPS in culture with IP006 MSCs, for 6 hours. Paired T tests.

4.5 Control experiments – RPMI Vs StemMACs Vs MSC CM

One potential limitation of the experiments described above, using IP006 CM, is the different culture media used in control and test procedures. Controls used RPMI media whereas the IP006 cells were cultured in StemMACs and the CM was derived in this media.

Therefore, experiments were carried out to confirm that optimal detection of TNF and IL-6 was not affected by the 2 different cell culture medias used for the 1/10 dilution of LPS-stimulated whole blood in the intracellular cytokine staining assay. Monocytes in the whole blood assay were either treated with RPMI (positive control) or IP006 MSC CM, whereas the MSCs were grown to confluency in StemMACs media, prior to harvesting the CM (conditioned media) for future downstream experiments.

Figure 4.6 demonstrates that there was no significant difference in the detection of TNF (top) and IL-6 (bottom) in monocytes, when comparing the RPMI and StemMACs media treatments in which the LPS-stimulated whole blood was diluted. Again, the suppression of monocytic TNF and IL-6 production was evident when monocytes were treated with IP006 MSC CM, in comparison to the cell culture media that were not conditioned with IP006 MSCs.
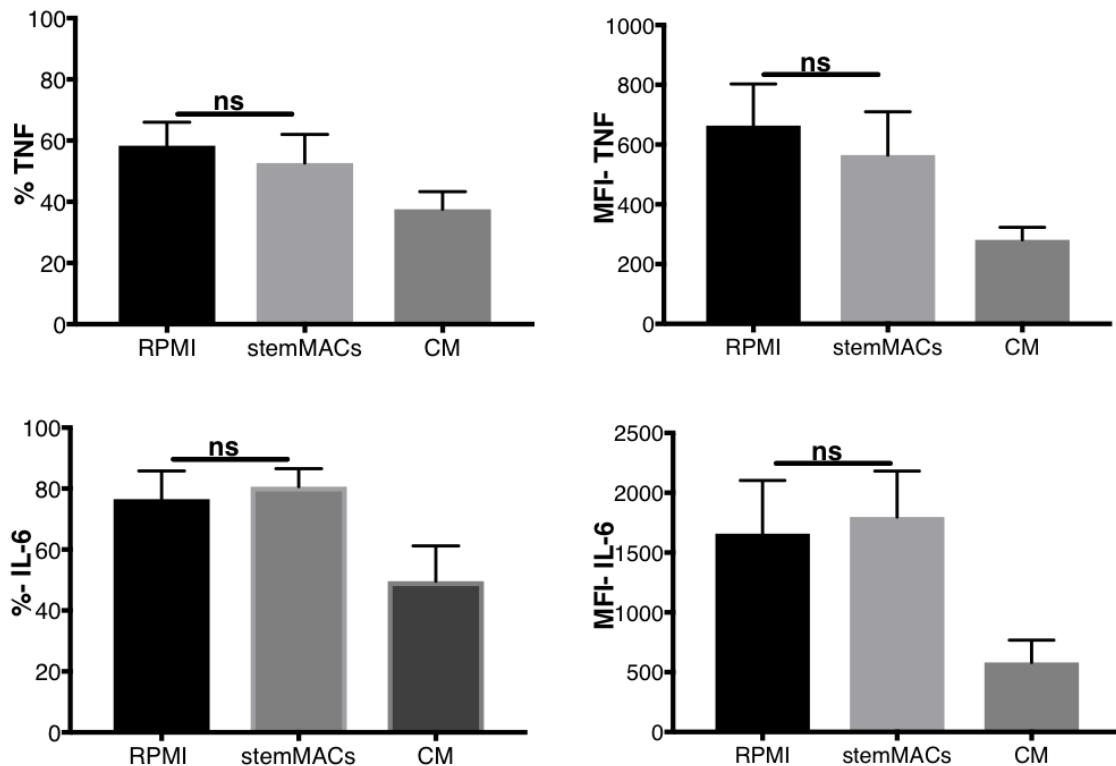
Figure 4.6 – Comparison between cell culture medias on TNF (top) and IL-6 (bottom) production in activated monocytes. Bar charts presenting the detection of TNF and IL-6 in CD14/CD45 positive monocytes treated with 1ng/ml LPS. Percentage positive (left) and MFI (right) values of TNF and IL-6 was recorded. (n=5, healthy control blood donors). Paired T tests.

4.6 Suppression of IL-6 and TNF production by multiple immortalised MSC lines

The data above show that the clonal MSC line IP006 has immunosuppressive activity similar to that observed with primary MSCs isolated from donors (chapter 3). However, it was unclear if this immunosuppressive activity was a common feature of immortalised MSC lines. I therefore obtained two additional MSC lines, Y201 and Y202 (which were generated by lentiviral-mediated transduction of primary MSCs with the telomerase component hTERT) (James et al. 2015). I performed a side-by-side comparison of the ability of IP006, Y201 and Y202 to inhibit LPS-induced TNF and IL-6 production. These experiments were performed using blood samples taken from three healthy donors. The results in Figure 4.7 show that, for both TNF and IL-6 production, IP006 produced the greatest inhibition, followed by Y201 and then Y202. However, whilst inhibition of cytokine production was observed, none of the results obtained demonstrated statistical significance. At first sight, this appears to disagree with the results observed using IP006 in Figures 4.2 and 4.3, above. However, the lack of statistical

significance more likely reflects the lower number of donors used (n=3), in contrast to the experiments using IP006 above, where a minimum of seven donors were used.
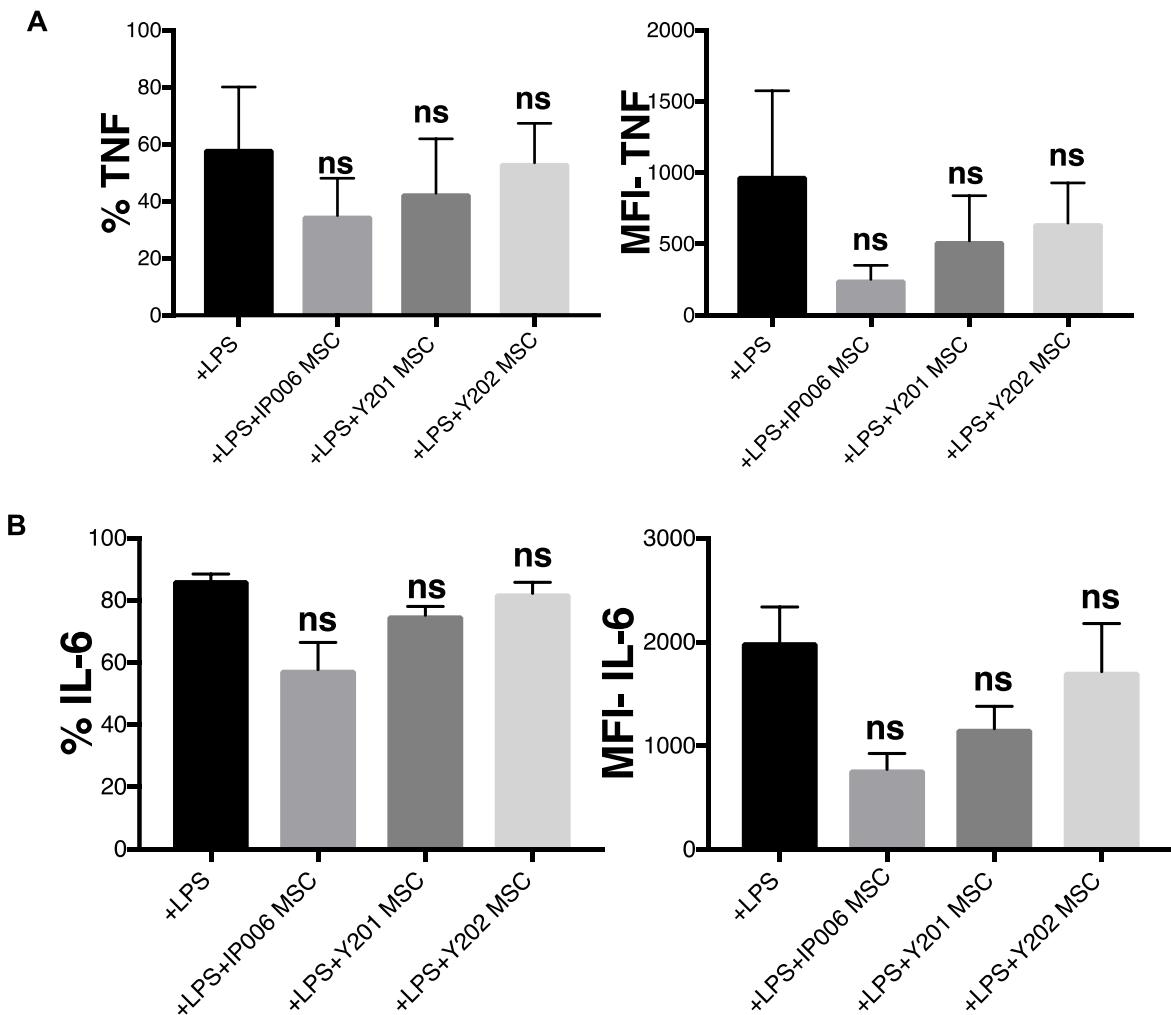
Figure 4.7 – **(A)** Graphs showing TNF levels in LPS activated healthy monocytes pre and post IP006, Y201 and Y202 MSC treatment (n=3). Both percentage positive (left) and MFI (right) values for intracellular TNF was recorded. Monocytes were stimulated with 1ng/ml of LPS in culture for 6 hours. Paired T tests. **(B)** Graphs showing IL-6 levels in LPS activated healthy monocytes pre and post IP006, Y201 and Y202 MSC treatment (n=3). Both percentage positive (left) and MFI (right) values for intracellular TNF was recorded. Monocytes were stimulated with 1ng/ml of LPS in culture for 6 hours. Paired T tests (Individual paired T tests were run against the +LPS as a control).

The IP006 cell line suppressed pro-inflammatory cytokine production in a contact-independent manner (Figure 4.4 and 4.5). To investigate whether this was also true of Y201 and Y202 MSCs, I generated conditioned media from these two MSC lines and the effect of this CM on TNF and IL-6 production from LPS stimulated monocytes was compared to that observed with IP006 CM. The results with these three CM show a similar pattern of inhibition to that seen when using the MSC lines themselves (Figure 4.8, below); the CM from IP006 had the greatest

suppressive activity (for both TNF and IL-6 production) followed by Y201 and then Y202. Statistically significant inhibition of both TNF and IL-6 production was achieved when using CM derived from IP006 and Y201, and blood from six healthy donors, with no significant results being obtained with the CM from Y202 (Figure 4.8).

These results show that the immunosuppressive activity observed with primary MSCs is retained following *in vitro* immortalisation of MSCs, but that there are variations in the potency of inhibition observed from cell line to cell line.
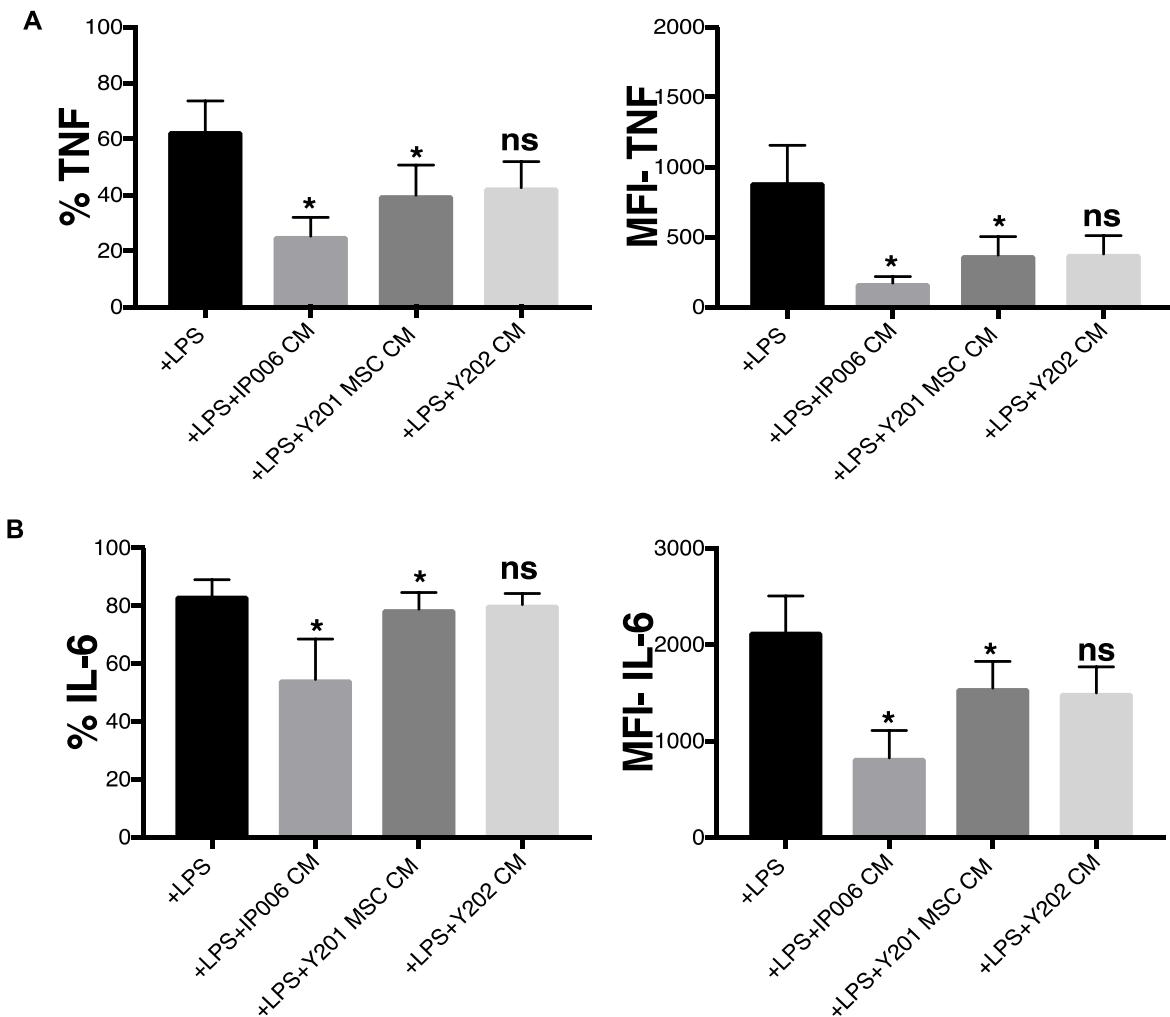
Figure 4.8 – **(A)** Graphs showing TNF levels in LPS-activated healthy monocytes, pre and post IP006, Y201 and Y202 MSC CM treatment (n=6). Both percentage positive (left) and MFI (right) values for intracellular TNF were recorded. Monocytes were stimulated with 1ng/ml of LPS in culture for 6 hours. Paired T tests. **(B)** Graphs showing IL-6 levels in LPS activated healthy monocytes pre- and post-IP006, Y201 and Y202 MSC CM treatment (n=6). Both percentage positive (left) and MFI (right) values for intracellular TNF was recorded. Monocytes were stimulated with 1ng/ml of LPS in culture for 6 hours. Paired T tests (individual paired T tests were run against the +LPS as a control). P value for % positive results were 0.042 (+LPS+IP006 CM) and 0.043, respectively.

4.7 Correlation between TNF and IL-6 cytokine inhibition

LPS is sensed by the toll-like receptor 4 (TLR4) and TLR4 engagement results in a cascade of signalling that activates NF-κB and induces pro-inflammatory gene expression, including genes encoding TNF and IL-6. This coordinated regulation suggests that the IL-6 and TNF levels could share a common mechanism by which MSCs can modulate their activity.

Finally, I therefore examined the correlation between the inhibition of TNF and IL-6 production in monocytes by IP006 MSC CM treatment (Figure 4.9). Percentage inhibition values of TNF and IL-6 levels of healthy control monocytes were calculated for both % positive (left) and MFI (right) values. The correlation between TNF and IL- 6 percentage inhibition was examined to determine whether the inhibition of both these pro-inflammatory cytokines, shared a common mechanism by which the IP006 MSC was able to reduce their production levels. However, it is clearly evident, in Figure 4.9, that there is no correlation between the levels of inhibition of TNF and IL-6. When considering the percentage positive values (Figure 4.9, left) there appears to be no linear relationship between the 2 cytokines, with the r value being 0.13 (Pearson's, r statistical test applied). Also, with regard to the MFI values (figure 4.9, right), in fact there is a weak (negative) linear relationship between the percentage inhibition of TNF and IL-6 cytokine levels, post IP006 MSC CM treatment, but this was not statistically significant.
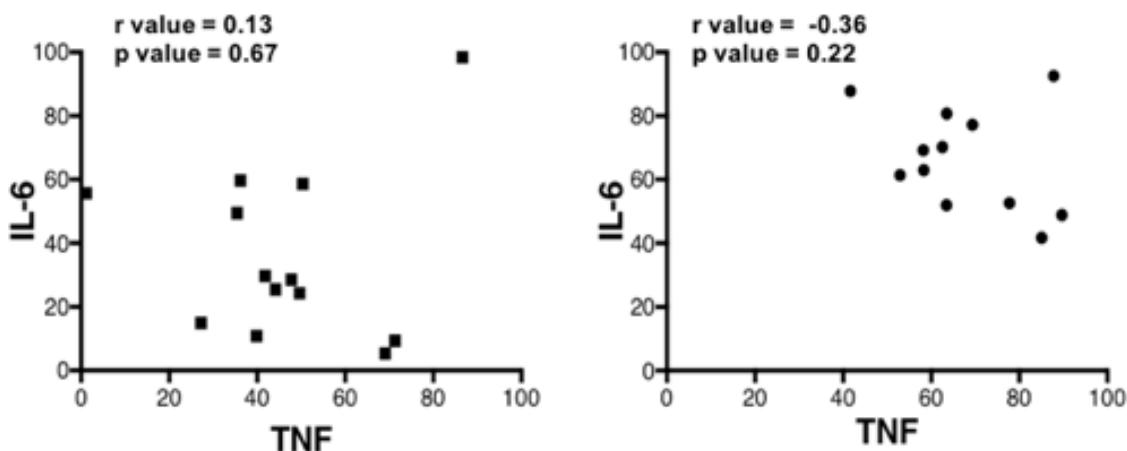
Figure 4.9 – Correlation between percentage inhibition of TNF and IL-6 from healthy control monocytes treated with IP006 MSC CM (left- % positive values and right- MFI values). N=12. Pearson's, r statistical test applied.

Overall, these results show the immunosuppressive abilities of MSCs on monocytes, with IP006 MSC having the most potent inhibitory effect on both intracellular TNF and IL-6 production levels. Furthermore, MSC CM displayed an even greater immunosuppressive

effect on monocytes in health, significantly reducing both the TNF and IL-6 levels. These results strongly suggest further evidence for a non-cell contact mechanism of MSC action. However, there was no detectable correlation between the inhibition of TNF and IL-6 cytokine levels, indicating a different molecular mechanism by which the IP006 MSC CM is able to act upon reducing the TNF and IL-6 cytokine levels.

4.8 Discussion

Upon establishing the immunosuppressive abilities of primary BM-MSCs on LPS-induced TNF production from monocytes (shown in Chapter 3), it was necessary to further establish the MSC immunosuppressive abilities by using immortalised MSC cell lines, to minimise the influence of donor variability. The experiments carried out in this chapter had the following aims: to determine the immunomodulatory impact of immortalised and clonal MSC cell lines on LPS-induced monocytic TNF and IL-6 pro-inflammatory cytokine levels, and to test whether the immunosuppressive ability of MSCs was as a result of cell-cell contact mechanisms or production of soluble factors. The results show that the strong immunosuppressive abilities of IP006 MSC clonal cell line, were further enhanced by MSC CM treatment (compared to MSCs themselves) of the LPS-induced monocytes, resulting in a further reduction of TNF and IL-6 levels.

In the previous chapter, it was shown that donor-derived BM-MSCs had an immunosuppressive capacity in suppressing TNF from healthy monocytes activated by LPS. However, the primary BM-MSCs showed donor variation in their immunosuppressive abilities. Also, there was the issue of the varying rates at which the primary BM-MSCs grew to confluency, making it difficult for them to be harvested and prepared at the same time for co-culture with patients' blood samples, as part of the whole blood assay assessing the immunosuppressive capacity of MSCs on monocytes. Therefore, in the experiments presented in this chapter I transitioned to using immortalised and clonal MSC cell lines: IP006, Y201 and Y202. As previously mentioned, IP006 is a highly proliferative, single-cell MSC clonal cell line derived from the BM of a healthy individual (E. Jones, Churchman, et al. 2010). Y201 and Y202 MSCs are both immortalised clonal cell lines from primary human BM-MSCs, which were isolated from the femoral heads during routine hip replacement procedures (James et al. 2015).

All MSC cell lines were previously assessed for their differentiation capacities into adipocytes, osteocytes and chondrocytes (James et al. 2015). All the MSC cell lines displayed the typical MSC cell surface phenotype, as outlined by ISCT, and shown in Figure 4.1 (James et al. 2015).

Results obtained from the immortalised clonal MSC cell lines demonstrated that the immunosuppressive activity of MSCs is retained upon immortalisation, although there was some variation in their potency. Healthy monocytes co-cultured with these MSC cell lines showed a reduction in the TNF and IL-6 pro-inflammatory cytokines levels compared to levels without MSC treatment. Furthermore, the addition of MSC CM produced an even greater inhibition of monocytic TNF and IL-6 compared to MSC treatment alone. This may be due to a number of reasons, including the fact that soluble mediators have time to accumulate in the MSC CM, whereas the cells themselves will produce much less soluble mediator in the 6 hours of the assay (it doesn't have time to accumulate). It is also could be possible that the MSC CM and MSCs actually mediate by separate pathways e.g. MSC CM might contain soluble factors such as IL-10, PGE_2, kynurenine, whereas the MSCs might use cell surface immunomodulatory molecules, such as CD73 to produce immunosuppressive adenosine from ATP/ADP/AMP (Tan et al. 2019). This often works alongside CD39 which can be present on other cells (e.g monocytes). This is important to consider, as CD73 was checked and it was demonstrated to be expressed on the MSC cell lines. Interestingly, CD73 might be present on exosomes of the MSC CM, which deserves further investigation (Tan et al. 2019).

Previous work conducted by James et al, investigated the immunomodulatory capacity of these Y201 and Y202 immortalised MSC cell lines, however, this was only in relation to their effect on T cells. This highlights the novelty of my experiments carried out on the immunomodulatory impact of MSCs on monocytes, using a whole blood assay.

Y201 and Y202 have also both been shown to retain/preserve other characteristics after immortalisation. Their BM MSC marker profile was revealed to be positive for CD166, CD105, CD90, CD73, CD44, CD29 and negative for CD34 and CD45; this profile matches their parental cells and is typical of primary BM MSCs (Dominici et al. 2006). However, there were distinct differences/characteristics observed in the Y201 and Y202 MSC lines. Y201 had a typical elongated fibroblastic morphology, whereas Y202 MSCs were flattened and spread out. Y201 MSCs possessed a migratory phenotype, having formed dispersed colonies with minimal cell contact, as opposed to Y202 MSCs which formed high density compact and regular colonies. In terms of their differentiation capacity, Y201 MSCs a greater adipogenic and chondrogenic differentiation and increased expression of genes associated with vascular growth. By contrast, Y202 MSCs showed an increased expression of genes regulating immunogenicity for T cell signalling, response to interferons, antigen processing and MHC class II protein expression. The Y202 MSCs also had elevated levels of endogenous expression of inflammation-induced genes compared to Y201 MSCs and parental cells; this observation implies the existence of a non-differentiating resident MSC subset with "unlicensed" immunomodulatory capacity associated with pro-inflammatory responses

(James et al. 2015).However, in immunosuppressive function assays conducted by James et al., it was discovered that all the immortalised BM MSCs were similarly able to supress anti-CD3/anti-CD28 antibody stimulated peripheral blood mononuclear cell (PBMC) proliferation, thereby demonstrating their inhibitory capacity. This conclusion is supported by results shown in Section 4, whereby both Y201 and Y202 cells and CM showed an immunosuppressive effect on healthy monocytes, with Y201 MSCs and their CM (conditioned media) having the greater effect. Additionally, Y202 MSCs have been shown to be more responsive to inflammatory cytokine-induced expression of factors involved in lymphocyte homing and development, such as CXCL10 and IL-7 (James et al. 2015).

This observable variation in phenotypic and immunoregulatory potency of the MSC cell lines is likely to be a reflection of their in vivo functional diversity and their biological requirements for distinct stromal subsets with specific functions in bone marrow maintenance. James et al. demonstrated, from their single-cell derived BM MSC clone analysis, that there is substantial variation in differentiation capacity (from nullipotency to tripotency) and also in characteristics such as immunomodulatory abilities in vitro (James et al. 2015). Again, this may suggest the presence of BM MSC subtypes with varied potencies and/or a hierarchical developmental progression.

Upon testing the inhibitory effect of IP006, Y201 and Y202 MSCs on the TNF and IL-6 levels from activated healthy monocytes, the CM from these MSC lines (MSC CM) were then added to activated healthy monocytes to determine whether the immunomodulatory capacity of the MSCs was cell-cell contact dependent or due to soluble factors. As already mentioned, the MSC CM showed an even greater immunosuppressive effect on the monocytic TNF and IL-6 levels than the MSCs alone, suggesting soluble factors to be a key mechanism for their immunomodulatory impact. Previously, it has been shown that MSCs, to some extent, can exert an immunomodulatory effect on monocytes via cell-cell contact mechanisms (Weiss and Dahlke 2019; Y. Wu et al. 2017; de Witte et al. 2018). However, the overarching evidence for the mechanism of action for MSC immunomodulation involves soluble factors. Yet, the immunomodulatory impact of MSCs, on monocytes/macrophages specifically, still is still largely unknown and needs further investigation. The fact that MSC express TLRs indicates that they play a role in regulating early immune responses (Le Blanc and Davies 2015). Often, the initiation of the innate immune response is carried out by the recognition of microbial PAMPs, such as LPS. In this whole blood assay, the LPS and MSC were cultured simultaneously; therefore there was a possibility that, in the presence of MSC, monocytes were not expressing pro-inflammatory cytokines, as the LPS was being bound by the added MSC, thereby not being available to bind to the monocytes. However, Riberio et al. conducted experiments whereby LPS was biotinylated, the results of which showed that at 1 µg/ml LPS

(an increased concentration to the one used in the whole blood assay, which was necessary for the staining to be revealed via flow cytometry), the LPS bound to monocytes and not to the MSC (Ribeiro et al. 2016).

The release of soluble immunomodulatory factors by MSCs, such as IDO, PGE2, TSG-6, IL-6, MCP-1, TGF-β and HGF has been implicated in their ability to inhibit the pro-inflammatory cytokine release from monocytes and to skew M1 macrophages to an M2 anti-inflammatory phenotype (François et al. 2012; Prockop 2013; Chiossone et al. 2016; Bartosh et al. 2010; Miranda et al. 2019; W. Chen et al. 2016; P.-M. Chen et al. 2014; Glenn and Whartenby 2014).

Nevertheless, the majority of studies pertaining to the mechanism of MSC immunomodulation have been conducted in relation to B cell and T cell inhibition, and the mechanism(s) whereby MSCs inhibit monocyte activation remain largely unknown. However, results obtained in section 4, strongly suggest that the IP006, Y201 and Y202 MSCs carry out their immunosuppressive effect on monocytes predominately via the release of soluble factors, as the MSC CM across all cell lines demonstrated the greatest inhibition of TNF and IL-6 release from activated monocytes compared to the MSC treatment.

As already stated, a limited amount of work has been carried out to reveal the relationship between MSCs and monocyte/macrophages, although it is known that soluble factors, released by MSCs, play a pivotal role in their interactions. Thus, more recent investigations underpinning the immunomodulatory impact of monocytes/macrophages have looked into the role of extracellular vesicles/exosomes released by MSCs, which have been shown to be immunologically active (Okoye et al. 2014; Matsuno et al. 2019). Exosomes are involved in transport of various molecules, and enable the transfer of proteins, mRNA and microRNAs (Phinney et al. 2015; Matsuno et al. 2019). It has been shown how exosomes are able to exert an immunomodulatory impact on monocytes/macrophages through this method of MSC-exosome secretion.

Zhang et al. have demonstrated that MSC-derived exosomes are able to activate monocytes via TLR signalling (MYD88-dependent pathway), which results in a M2 macrophage phenotype, with the release of anti-inflammatory cytokines and consequentially inducing T regulatory cell expansion (Zhang et al. 2014). Although, the study conducted by Zhang et al concluded that the immunosuppressive effects of MSCs play an important role in monocyte activity, they did not address the mechanism of interaction between monocytes and exosomes. It was just highlighted that LPS activation of monocytes leads to their pro-inflammatory phenotype, and conversely that MSC-exosomes attenuated monocytic pro-inflammatory cytokine release and increased their anti-inflammatory response (Zhang et al. 2014). Therefore, these data highlight a limitation in the work carried out using MSC CM treatment

on monocytes, as the nature of the key mediators in the CM are currently unknown. Later MSC-exosome studies, by Phinney et al. consolidated these findings of the MSC-exosomes skewing monocytes to an anti-inflammatory phenotype (Phinney et al. 2015). It has been shown that under oxidative stress, MSCs, during ex vivo culture transfer, depolarised mitochondria to macrophages to promote MSC survival and these mitochondria were then used via a process involving fusion to enhance macrophage bioenergetics. Furthermore, Phinney et al, showed that MSCs simultaneously secrete exosomes to desensitise macrophages to mitochondrial transfer by supressing TLR-signalling. Thus, the mitochondrial transfer from the MSCs to the macrophages can induce inflammation by the activation of PRRs, so the extracellular vesicles, such as the exosomes carry miRNAs that stimulate changes in TLR expression on the macrophages. The uptake of these MSC exosomes by macrophages induces translocation of NF-kB resulting in a significant shift in TLR transcript expression and the downregulation of TLR signalling involved in the MyD88-dependent, TRIF-dependent and TLR-signalling pathways.

MicroRNAs are highly conserved, small non-coding RNA molecules that play a key role in the regulation of gene expression, often negatively by repression via binding to the 3' UTR of their target mRNAs (Dexheimer et al. 2020). And, a key microRNA expressed highly in MSC-exosomes is miR-451. It is known to negatively regulate cytokine production in dendritic cells infected with influenza virus and macrophages transfected with miR-451 have shown a decreased TNF mRNA expression. These findings, would support a potential mechanism for how MSC CM (IP006, Y201, Y202) is able to regulate pro-inflammatory cytokine expression in monocytes presented in section 4. Although it is yet to be determined whether miR-451 reduces TNF directly or by regulating a different molecule, with the effect of that molecule being able to indirectly regulate TNF.

However, there are relatively few studies outlining MSC-exosome and monocyte/macrophage interactions, and thus further investigation are necessary to better understand the mechanisms of action between MSC-exosome secretion and monocyte uptake. The field of extracellular vesicles and exosomes is a rapidly emerging and exciting area of research that will allow us to better understand cellular interactions. Therefore, further studies need to be conducted to understand how MSCs are able to communicate with monocytes in the course of exosome release, in order to carry out their immunosuppressive abilities. Biochemical approaches, e.g the purification of the active components from the CM and their characterisation, or candidate approaches using neutralising antibodies or genetically modified MSC (by shRNA or CRISPR/Cas9 targeting) is an essential step to develop knowledge in this area.

In conclusion, data in this chapter show how immortalised and clonal MSC cell lines are able to exert an immunosuppressive effect on healthy monocytes in the whole blood assay, with a reduction in pro-inflammatory cytokine TNF and IL-6 expression levels, with IP006 MSC having the greatest inhibitory effect followed by Y201 and Y202 MSC. Furthermore, I have demonstrated that the CM from these MSCs were able to exert an enhanced reduction of TNF and IL-6 from monocytes. This suggests that MSC CM has a greater immunosuppressive ability on monocytes compared with MSCs, pointing to paracrine mechanisms being responsible for the immunomodulatory capacity of MSCs. In the next chapter, the immunomodulatory capacities of IP006, Y201 and Y202 MSC CM on monocytes from patients with RA are investigated.

Chapter 5 – The therapeutic potential of mesenchymal stem cells in rheumatoid arthritis

5.1 Introduction

Current therapeutic approaches in RA are focussed on blocking the action of pro-inflammatory cytokines, such as TNF and IL-6, thereby reducing downstream inflammation, joint damage and pain. However, these approaches (using anti-cytokine or anti-cytokine receptor antibodies or soluble receptors) are only effective in ~50% of patients and new therapeutic approaches are required. My data presented in chapters 3 and 4 shows that MSC (either primary or immortalised) secrete a factor(s) that reduces the production of TNF and IL-6, albeit in response to LPS. I hypothesise that this MSC-derived factor might inhibit the production of TNF and IL-6 in patients with RA and provide the basis of future therapeutic approaches in which MSC or MSC-derived factors are used therapeutically. Here, I test the ability of CM from IP006 cells to inhibit pro-inflammatory cytokine production from monocytes derived from patients with RA. I have included newly diagnosed RA patients who have not yet received treatment (treatment naive patients), as well as those with established disease that have failed multiple drug treatments.

5.2 The immunomodulatory potential of MSC CM in early rheumatoid arthritis

Blood samples were obtained from seventeen, treatment-naive, early RA patients. These patients had a disease duration of less than 6 months and received no prior DMARD/MTX treatment for their symptoms. Patients self-report on the length of their symptoms regarding any joint that is clinically involved at the time of assessment. Symptoms include swelling of small joint regions, tenderness, pain and stiffness. As well, as joint redness and joint warmth and patients often experience fatigue. At this stage, there is inflammation within the joint and swelling of the tissue with the synovium inflamed, however, there is no damage to the bone.

Experiments were performed as in chapter 4. Blood samples from these RA patients were stimulated with LPS in the presence or absence of CM from IP006 and monocyte production of TNF and IL-6 assayed using intracellular staining and flow cytometry. The results in Figure 5.1 show that the MSC-derived CM significantly reduced pro-inflammatory cytokine production in these early RA patients in response to LPS stimulation, as previously seen using the healthy donor blood samples in chapter 4. These effects were seen when analysed in terms of the

percentage of cytokine-producing cells, or the level of cytokine production per cell (using the MFI of fluorescence).
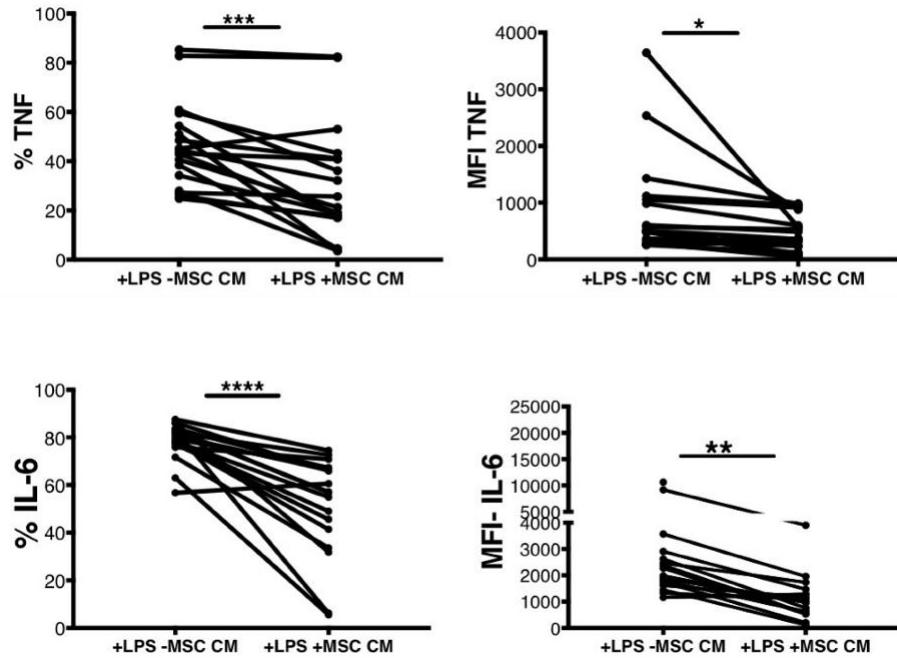
Figure 5.1 – Graphs showing TNF (top) and IL-6 (bottom) levels in LPS activated early RA (treatment naïve) monocytes pre and post IP006 MSC CM treatment (n=17). Both percentage positive (left) and MFI (right) values for intracellular TNF and IL-6 was recorded. Monocytes were stimulated with 1ng/ml of LPS in culture with IP006 MSC CM, for 6 hours. Paired T tests

Then, I investigated whether the ability of IP006 CM to inhibit TNF and IL-6 production in early RA was related to disease severity. An internationally agreed measure of disease severity in RA is the Disease Activity Score or DAS28 (CRP) score (Wells et al. 2009). This score combines joint involvement, patient wellbeing and a measure of C-reactive protein (CRP). The score can be used to classify patients into low (less then 3.2), moderate (>3.2 but < 5.1) or high (>5.1) disease activity, with remission being classified with a score of less then 2.6. In addition, a score of less than X is defined as remission; patients in this group have shown improvement in their disease activity, and for early RA, this remission is spontaneous, that is it occurs in the absence of treatment.

I classified the 17 early RA patients into remission, low, moderate and high disease activity and analysed the effect of the IP009 CM on LPS-induced TNF and IL-6 production, using the data from figure 5.1, above. This patient stratification revealed no obvious trend in the ability of the IP006 CM to inhibit pro-inflammatory cytokine production, when analysed either by fold change in cytokine or the MFI of expression (Figure 5.2).
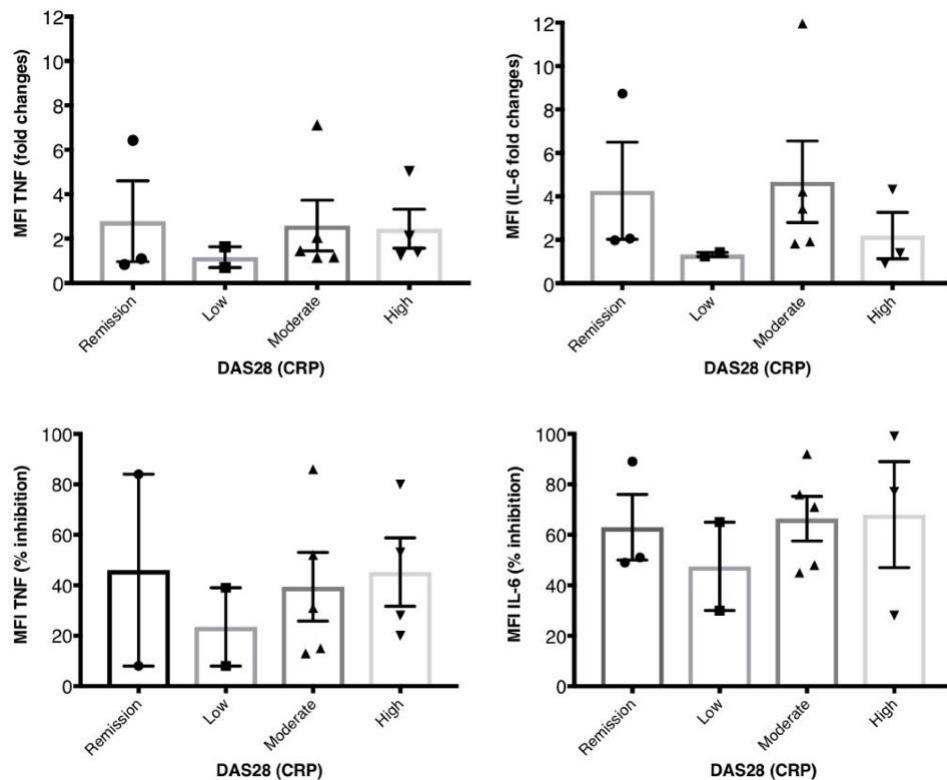
Figure 5.2 – Graphs comparing fold changes (top) and percentage inhibition(bottom) values of TNF (left) and IL-6 levels (right) post IP006 MSC CM treatment, across disease severity in LPS activated early RA (treatment naïve) monocytes. Monocytes were stimulated with 1ng/ml of LPS in culture with IP006 MSC CM, for 6 hours. RA disease severity of these treatment naïve patients with RA, was classified according to DAS28 (CRP) scores.

I refined this stratification by testing for any correlation between CM-mediated inhibition of cytokine production and RA disease severity (DAS28 CRP scores), removing the classification into the four disease activity groups. Similar to the analysis in (Figure 5.2), no correlation was found between the inhibitory effects of IP006 CM treatment and DAS28 CRP scores, when analysing either the fold change in expression (using MFI) or the percentage of expressing cells.
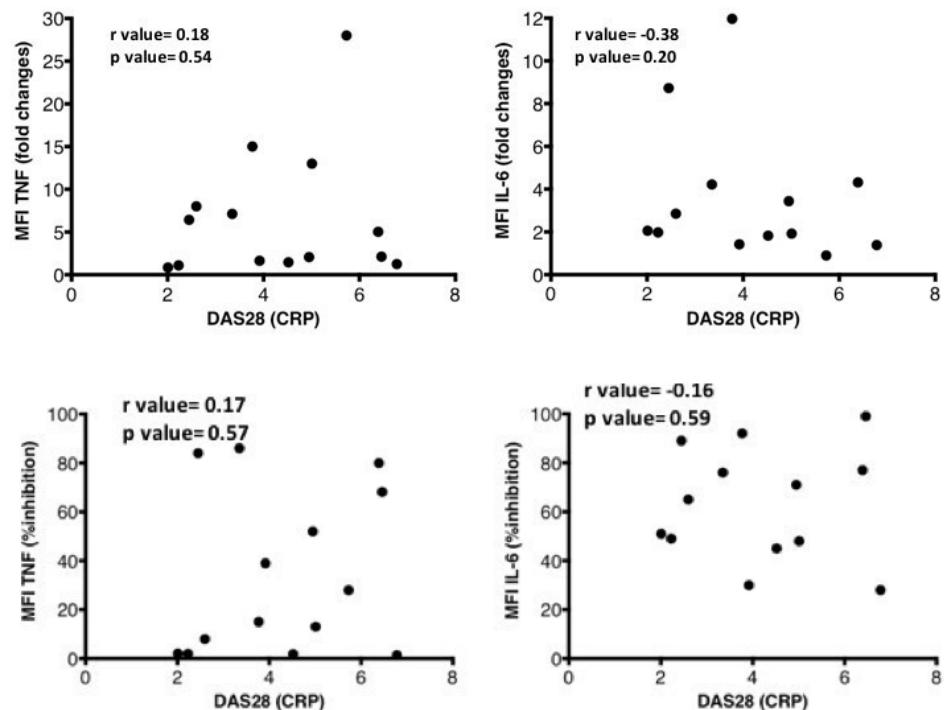
Figure 5.3 – Correlation between fold changes (top) and percentage inhibition (bottom) in TNF and IL-6 (MFI values) and RA disease severity (DAS28 CRP score), from early RA monocytes-after IP006 MSC CM treatment. (left- TNF, right- IL-6 values). Spearman r test. N=14.

Collectively, these results underline the strong immunosuppressive abilities of MSC CM on monocytes from patients with early RA, who have not received any treatment. The results demonstrate a potential alternative treatment for early RA, capitalising on the immunosuppressive effects of MSC CM. However, in terms of clinical need, it is likely that the most important application for new therapies will be in patients who do not respond to current therapies such as TNF and/or IL-6 blockade. It was therefore necessary to evaluate the effectiveness of IP006 CM in inhibiting pro-inflammatory cytokine production from monocytes derived from patients with established RA.

5.3 The immunomodulatory impact of MSC CM in established RA

Blood samples were obtained from fourteen, established RA patients. These patients had previously been treated with multiple conventional synthetic DMARDs biologic treatments and failed to respond. With more established disease compared with the early stages damage caused by inflammation extends beyond the synovium and destructs the cartilage and causes bone erosions. This causes heightened pain and swelling to patients, with a loss of mobility and increased muscle weakness.

The data in Figure 5.4 show that, for established RA patients, IP006 CM potently inhibits LPS-induced TNF and IL-6 production (in terms of the proportion of expressing cells and the level of expression).
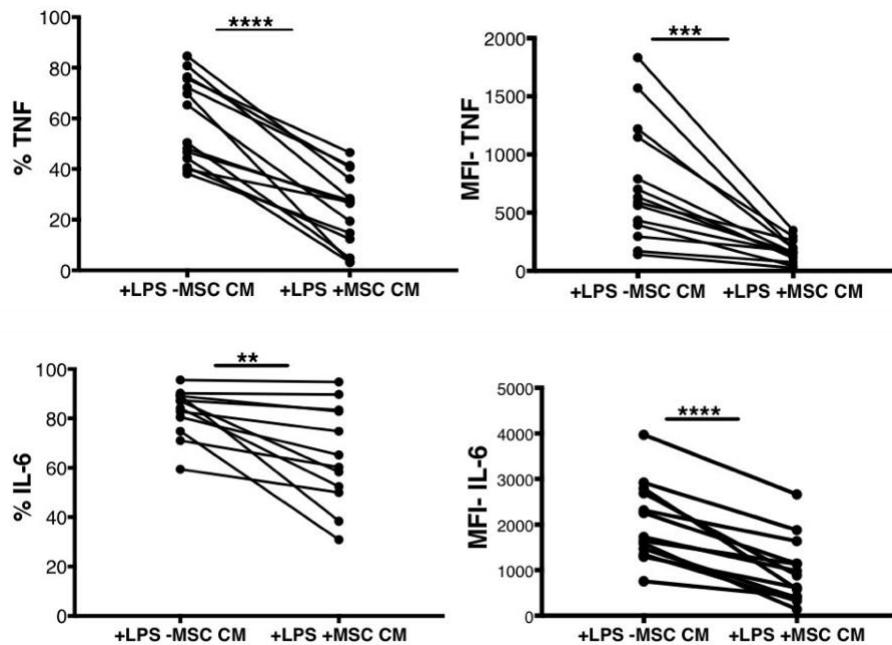
**Figure 5.4** – Graphs showing TNF (top) and IL-6 (bottom) levels in LPS activated established RA monocytes pre and post IP006 MSC CM treatment (TNF, n=14 and IL-6, n=12). Both percentage positive (left) and MFI (right) values for intracellular TNF and IL-6 was recorded. Monocytes were stimulated with 1ng/ml of LPS in culture with IP006 MSC CM, for 6 hours. Paired T tests

These results indicate that IP006 MSC CM is able to exert its immunosuppressive effects on monocytes from patients suffering from more advanced rheumatoid disease who have failed multiple therapies. Comparing results from early RA (Figure 5.1) or established RA patients (Figure 5.4) shows that the greatest inhibitory effects of IP006 CM (as indicated by the level of statistical significance) were observed for the inhibition of TNF from monocytes derived from established RA patients. The effectiveness of inhibition in this patient group is encouraging from the perspective of therapeutic need and potential.

Analysing the relationship between inhibition of TNF production and RA disease severity (DAS28 CRP scores), in established RA revealed a striking negative correlation within this cohort. There was a statistically significant, inverse relationship between inhibition of TNF production and disease severity score when analysing inhibition either as the fold change in TNF expression or the percentage of inhibition (Spearman r rank test, r= -0.7 and P=0.01 in both cases; Figure 5.5, left). However, this relationship was not observed for IL-6 production (Figure 5.5,right).
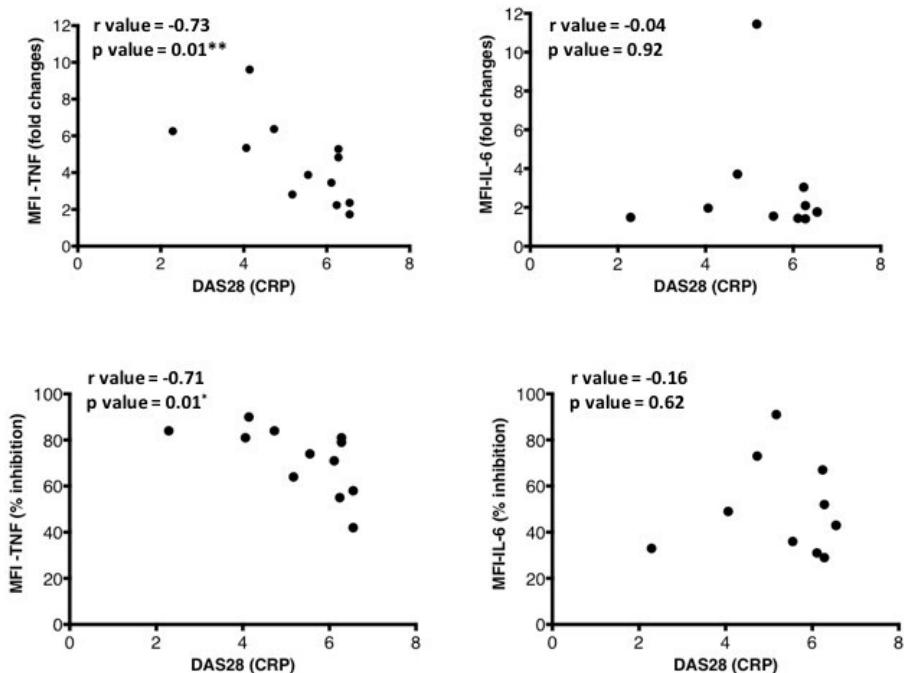
Figure 5.5 – Correlation between fold changes (top) and percentage inhibition (bottom) in TNF and IL-6 (MFI values) and RA disease severity (DAS28 CRP score), from established RA monocytes-after IP006 MSC CM treatment. (left- TNF, right- IL-6 values). Spearman r test. N=12.

5.4 The immunomodulatory impact of RA synovium-derived MSC CM on pro-inflammatory cytokine production from healthy control monocytes

The data presented to this point show that primary and immortalised MSC (and MSC-derived CM) from non-RA patients have the ability to inhibit LPS-induced pro-inflammatory cytokine production from monocytes derived from healthy donors and both early and established RA patients. The inflammation in RA occurs within the synovium, the lining of the joint also known as the synovial membrane, which is located between the joint space and whereby the synovial fluid is secreted for lubrication that ensures reduced friction between cartilage. The synovium contains resident MSC populations which presumably serve to replenish tissue, especially bone and cartilage, at these sites (C De Bari et al. 2001). I hypothesised that MSC derived from RA synovium might lack the ability to inhibit TNF and IL-6 production from monocytes, thereby contributing to the disease progression.

Synovial MSCs were obtained from tissue biopsies from the suprapatella pouch, and experiments were conducted to demonstrate they possessed a classic MSC phenotype and exhibited the typical MSC differentiation capacity of tripotential nature (E. Jones, Churchman,

et al. 2010) . Five separate MSC lines were established from RA synovium, AR1388, AR1214, AR1390, AR1400 and AR1404 and CM was derived from each. This CM was then used in the LPS stimulation assay (using blood from a single healthy donor) and the levels of TNF and IL-6 inhibition compared to that obtained with IP006 CM.

The results in Figure 5.6 show that CM from all five RA synovium-derived MSC inhibit LPS induced TNF production to some extent. However, CM from the RA synovium-derived MSC was not as effective as CM from the IP006 cell line; IP006 MSC CM reduced TNF production from activated healthy monocytes by 9.6-fold (MFI value) compared to AR1388 and AR1214 synovial RA MSC CM that showed TNF inhibition by 2.4-fold and 1.9-fold respectively (Figure 5.6,left).

Next, the same healthy control monocyte donor was treated with IP006 MSC CM or synovial RA MSC CM AR1390 and AR1400 on a different day (Figure 5.6, middle). Again, IP006 MSC CM inhibited TNF production from activated healthy control monocytes the most (4.5-fold) compared to the AR1390 and AR14000 MSC CM, that demonstrated TNF inhibition by 1.3-fold and 1.7-fold respectively.

Finally, on a third occasion the immunosuppressive effects of healthy control MSC CM (IP006) and synovial RA MSC CM (AR1404) were examined on the same healthy control monocytes (Figure 5.6, right). Furthermore, it was confirmed that the healthy MSC CM (IP006) exerted the greatest TNF inhibition in healthy activated monocytes, compared to synovial RA MSC CM (AR1404). In Figure 5.6 (right), you can see the greatest reduction in TNF production from activated monocytes was after the treatment with IP006 MSC CM, by 7-fold, whereas synovial RA MSC CM AR1404 inhibited TNF production by 1.4-fold.
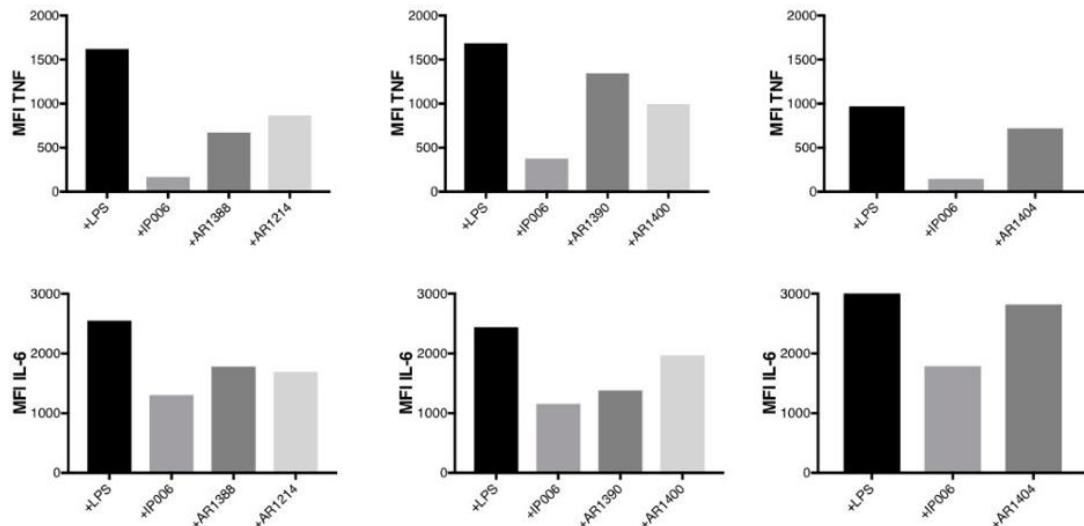
Figure 5.6 – Graphs showing TNF (top) and IL-6 (bottom) levels in LPS-activated healthy control monocytes pre-and post-synovial RA MSC CM treatment (AR1388,AR1214,AR1390,AR1400,AR1404). MFI values for intracellular TNF and IL-6 was recorded. Monocytes from the same healthy control donor were stimulated with 1ng/ml of LPS in culture with synovial RA MSC CM from 5 different donors or IP006 MSC CM, for 6 hours.

Next experiments were carried out to confirm if synovial RA MSC CM were capable of inhibiting healthy monocyte activation, by measuring changes in intracellular IL-6 expression. In order to, assess the immunosuppressive abilities of synovial RA MSC CM on healthy control monocytes.

Figure 5.6 (bottom) shows the differences in the intracellular IL-6 expression levels in activated healthy monocytes, pre-and post (6 hours) IP006 MSC CM or synovial RA MSC CM treatment. Figure 5.6 (bottom) illustrates how there is the greatest reduction of intracellular IL-6 expression in activated healthy control monocytes, post IP006 MSC CM treatment, when measuring MFI values, as compared to synovial RA MSC CM treatment (AR1388 MSC CM and AR1214, left. AR1390 MSC CM and AR1400 MSC middle. AR1404 MSC CM, right). It is clear that all synovial RA MSC CM are worse than IP006 MSC CM in their ability to exert an immunosuppressive effect on healthy monocytes from the same donor, when assessing the monocytic IL-6 production levels.

IP006 MSC CM reduced IL-6 production from activated healthy monocytes by 2.0-fold (MFI value) compared to AR1388 and AR1214 synovial RA MSC CM that inhibited IL-6 by 1.4-fold and 1.5-fold respectively (Figure 5.6, left).

Subsequently, the same healthy control monocyte donor was treated with IP006 MSC CM or synovial RA MSC CM AR1390 and AR1400 on a different day (Figure 5.6, middle). IP006 MSC CM inhibited IL-6 production from activated healthy control monocytes the most (2.1-

fold) compared to the AR1390 and AR14000 MSC CM, that demonstrated IL-6 inhibition by 1.7-fold and 1.2-fold respectively.

Thirdly, the immunosuppressive effects of healthy control MSC CM (IP006) and synovial RA MSC CM (AR1404) on the same healthy control monocytes was compared (Figure 5.6, right). Moreover, it was confirmed that the healthy MSC CM (IP006) exerted the greatest IL-6 inhibition in healthy activated monocytes, compared to synovial RA MSC CM (AR1404). In Figure 5.6 (right), you can see the IP006 MSC CM inhibited IL-6 production the most by 1.7-fold, while the synovial RA MSC CM AR1404 inhibited IL-6 production by 1.1-fold.

RA patients self-declare the pain they are suffering using the Visual analogue scale (VAS) score, a questionnaire-based assessment (Nikiphorou et al. 2016). I assessed whether the inhibition of TNF and IL-6 production by RA synovium-derived MSC was correlated with the VAS score of the RA patient donating the MSC. My hypothesis was that synovium-derived MSC from patients with a high VAS score (with higher disease activity) might be less effective at inhibiting pro-inflammatory cytokine production, contributing to the disease severity.

I plotted the fold change in pro-inflammatory cytokine production (measured by the MFI) in the presence of the RA synovium-derived MSC (as a measure of MSC effectiveness) against the VAS score for the donating patient. These values were then used to calculate correlation (using Spearman's r value; Figure 5.7). The results show that, for both TNF and IL-6, there was a negative correlation between VAS score and inhibitory activity (with negative r values obtained), revealing that higher disease activity (higher VAS score) was associated with decreased inhibitory activity (Figure 5.7). For TNF, this effect was weak (r=-0.2, P=0.78) but for IL-6 the effect was more pronounced, although it still did not reach statistical significance (r=-0.8, P=0.08). These results suggest that those patients with RA suffering with the most severe pain may have synovial-derived MSC CM with the weakest immunosuppressive activity against monocytic IL-6 production.
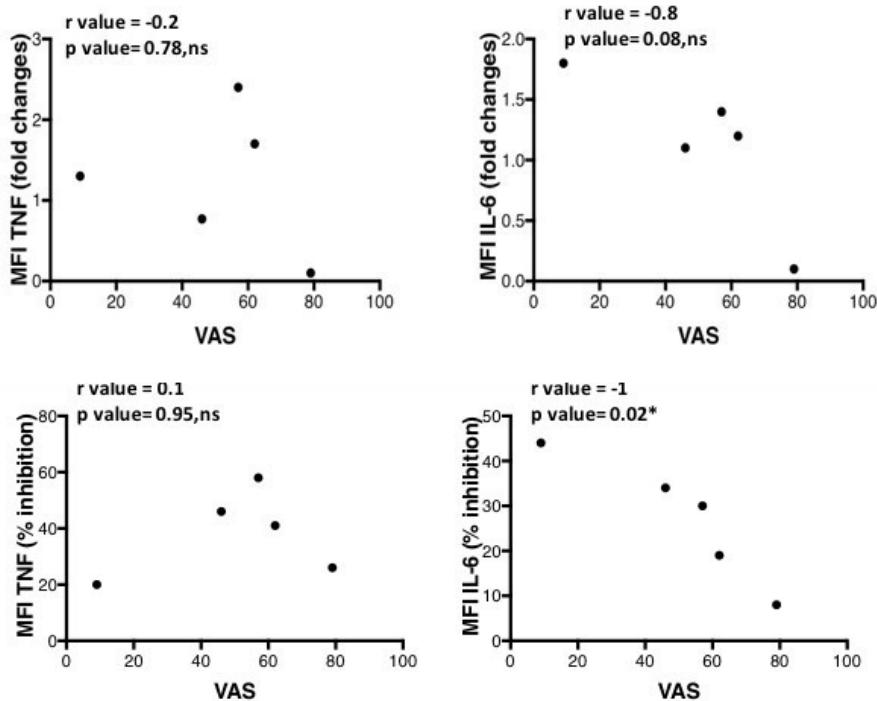
Figure 5.7 – Correlation between fold changes (top) and percentage inhibition (bottom) values in TNF and IL-6 (MFI) and the Visual Analog Scale (VAS score), of synovial MSCs from RA patients. (left- TNF, right- IL-6 values). Spearman r test. N=5.

I also assessed these correlations using the percentage inhibition of TNF and IL-6 in healthy monocytes by synovial RA MSC CM treatment. Inhibition of TNF production showed no apparent link to VAS score. However, there was a perfect negative relationship between the treatment with synovial RA MSC CM to reduce IL-6 and VAS score of patients with RA, which reached statistical significance (r=-1, P=0.02). This indicates that the strongest immunosuppressive activity (with respect to IL-6 production) resided in the CM from RA synovial-derived MSC from patients with the lowest VAS score. Thus, patients with RA suffering with the most severe pain may have synovial MSC with the weakest immunosuppressive ability to inhibit monocytic IL-6 production.

5.5 Discussion

Results presented in this chapter demonstrate that the CM from IP006 MSC was able to exert immunosuppressive effects on monocyte pro-inflammatory cytokine production from early RA (treatment naïve) patients as well as monocytes from patients suffering from more advanced RA, who have failed multiple drugs (Established RA). Conversely, CM from RA synovial MSCs had a reduced immunomodulatory capacity in the inhibition of pro-inflammatory cytokines TNF and IL-6 from healthy monocytes.

Firstly, results interestingly showed that IP006 CM potently inhibits TNF and IL-6 in patients with early RA who have had no prior treatment (treatment naïve cohort). This is of great interest as so far the study of MSCs in clinical trials has been limited to patients with severe RA resistant to standard therapies. Such MSC treatment could be more effective if administered at early stages of disease, to be able to "reset" the immune system by creating regulatory networks. And from my novel work using monocytes from patients with early RA, it would indicate such MSC treatments could be successful in treating the devasting disease and halting it's progression at a much earlier stage. However, when patient stratification was carried out on the early RA (treatment naïve) cohort, into remission, low, moderate and high disease activity, there was no apparent trend in their ability for IP006 CM to inhibit the pro-inflammatory cytokine production across these divisions. This could be due to the low number of samples in each sub-group and could be further explored with additional patient samples examined in each stratified group. However, to further test possible trends within the early RA, treatment naïve cohort, I refined stratification of this group by assessing for a possible correlation between IP006 CM-mediated inhibition of TNF and IL-6 cytokine production and RA disease severity (DAS28 CRP scores). This removed the classification into the four smaller disease activity groups. Similarly, there was no correlation between the DAS28 CRP scores and the inhibitory effects of IP006 CM treatment.

Nevertheless, monocytes from patients with established RA who have failed multiple conventional synthetic DMARDs and biologic treatments actually showed an exciting response to IP006 CM treatment, whereby the CM potently inhibited LPS induced TNF and IL-6 production. These results are of particular significance, as when comparing results from early RA and established RA cohorts, the greatest inhibitory effects of the CM were observed for the inhibition of TNF from monocytes derived from patients with established RA. Strikingly, there was a strong and statistically significant negative correlation between immunosuppressive activity of IP006 CM and TNF production from monocytes derived from patients with established RA (Figure 5.5, r= -0.73, p= 0.01). This indicates that as RA disease severity increases, the immunosuppressive ability of IP006 MSC CM lessens. The potent immunosuppressive impact of the CM on monocytes from patients with advanced aggressive disease, that have failed multiple conventional treatments, provides a promising potential alternative therapeutic for RA. Conventional RA therapeutics, such as anti-IL1, anti- IL-6, anti-TNF, anti CD20 and T cell co-stimulation blockers, as 30-58% of patients do not respond to such biologics (McInnes and Schett 2011; Smolen et al. 2012)and 30%-40% lose responsiveness over time (Braun and Kay 2017), with between 50-58% discontinue therapy within 2 years (Smolen et al. 2012; Rubbert-Roth and Finckh 2009). Moreover, as previously mentioned current RA biologic treatments can cause severe side effects, including

hypertension, lymphoma and increased risk of infection. Current treatments need continuous subcutaneous injection and are expensive. Therefore, results presented from my work suggest an exciting novel substitute for conventional RA therapeutics as there is a demand for treatments with higher efficacy, that are safer and more affordable.

Other studies, investigating the immunomodulatory impact of MSCs and their conditioned media in RA in preclinical models, have shown beneficial effects in reducing inflammation and joint destruction. However, such studies have predominately focussed more specifically on the effect of MSC treatment on B and T cells within RA compared to monocytes/macrophages and even fewer studies examining the impact of MSC-CM on monocytes within the context of RA. However, it has been previously been shown how in vivo studies using an adjuvant-induced model for arthritis (AIA) MSC CM had a significantly higher therapeutic impact over MSCs(Miranda et al. 2019). This study showed that the use of MSC-CM is an enhanced and viable strategy than using MSCs directly for reducing AIA related signs (Miranda et al. 2019). Riberio et al. examined the mechanism of action of MSCs immunomodulation on monocytes, in vitro using transwell cultures, whereby the MSCs and monocytes were cultured in flat bottom wells of plates with porous filter inserts. Results showed no reduction in monocytic TNF when treated with MSCs, suggesting that cell contact was more likely needed for the immunosuppressive impact on monocytes (Ribeiro et al. 2016a). Conversely, more recently it has been demonstrated that UCB MSCs are able to direct macrophage polarisation and block inflammasome activation to alleviate rheumatoid arthritis. A limitation of this study directly looking into the effect of MSCs on macrophages within RA, is that the CM from the MSCs was not tested on the macrophages to determine if this could further enhance the immunosuppressive effects to treat RA (Shin et al. 2016). Thus, my findings are novel in that it is further indicating the paracrine mode of action of MSCs specifically on monocytes obtained from patients with RA, using the whole blood assay.

Although, for the treatment of RA, MSC based therapy is one of the key autoimmune disease models used to examine the mechanism underpinning the therapeutic effect of MSCs, presently, MSC based clinical trials for RA has been the least studied within the autoimmune diseases.

Regarding this, exist 14 MSC-based therapy clinical trials for RA, of which it has been stated that intravenous infusion of allogenic BM and UC derived MSCs in a small group of refractory patients resistant to the anti-TNF monoclonal antibody therapy, resulted in an improvement on DAS28 clinical score, reduced erythrocyte sedimentation rate as well as the serum anti-cyclic citrullinated peptide (anti-CCP) antibody level, showing the efficacy of MSC treatment. Although, the documented clinical improvement was only temporary and partial due to the

short term follow up (Liang et al. 2012). Additionally, in another study, using MSCs for the treatment of RA, the safety and efficacy was shown in a greater number of patients (Liming Wang et al. 2013). As part of this study, MSCs and DMARDs were intravenously co-administered in 172 patients with active RA, leading to a great increase in the number of regulatory CD4+ T cells (Treg) in the blood, along with clinical improvement for up to 6 months. Furthermore, it was demonstrated that even after this period, repeated infusion of MSCs resulted in an increased therapeutic efficacy of the cells (Liming Wang et al. 2013). In another study, a phase Ib/IIa clinical trial, the intravenous administration of MSCs to 56 patients with a placebo group was shown to be a well tolerated and safe in refractory RA patients (Álvaro-gracia et al.2007).

Also, I showed that CM derived from RA synovial MSC was less effective than the IP006 CM in terms of immunosuppressive activity. This suggests that MSC from patients with RA might lose their immunosuppressive abilities. However, these differences might be due to technical effects, such as the concentration of the active components in the CM. Loss of these components might be due to their disease origin, but also might be due to culture conditions, such as growth rates of the IP006 cells compared to the RA derived MSC. Importantly, the immunosuppressive activity of MSC from RA patients with high VAS scores was reduced; as the VAS score increased, the ability of the synovial RA MSC CM to inhibit these pro-inflammatory cytokines from healthy monocytes, decreased. This is in line with the understanding that under physiological settings, MSCs resident in the joint are known to contribute to the maintenance and repair of joint tissues. Furthermore, since MSC have the ability to suppress effector cells and inflammatory responses, they can interact with immune cells and play a key role in the reduction of inflammation. Conversely, in the RA joint, the repair and immunoregulatory function of MSCs seems to be blocked by the inflammatory millieu, and importantly it should be noted that MSC and macrophages are part of synovial membrane stroma. As macrophages have an essential role in RA, it is essential to consider MSC-macrophage interactions. In this way, MSCs could perpetuate joint damage within the diseased joint in RA and enhance disease progression (Bari et al. 2015).

It is essential to further understand the relationship between MSC and other cells, especially macrophages, in patients with RA, especially if MSC and their conditioned media will be considered as an alternative therapeutic. The general consensus is that MSC are able to exert immunoregulatory/immunosuppressive effects, but it is also known that they can facilitate unwanted growth in tumours and FLSs in RA. Thus, MSC can have beneficial or detrimental effects, depending on their environment. Prompting further investigation of MSC CM as opposed to MSCs themselves as a new therapeutic (Bari et al. 2015).

Overall, the results provide further evidence for a non-cell contact mechanism of MSC action and supports the development of novel alternative therapies for RA treatment, across RA disease severity.

Chapter 6 – Overall discussion

The goal of this study was to explore the therapeutic potential of MSCs (or MSC-derived products) in the treatment of RA. In particular, whilst anti-TNF, anti-IL6 and B cell depletion based therapies (anti-CD20) have revolutionised RA treatment and had very positive outcomes for patients worldwide, there remains a pressing need for new treatments for the high proportion of patients with RA, who show no response to standard therapies, or who become refractory to their action during treatment.

In this study, I investigated the immunomodulatory effects of human MSCs on monocytes in health and in RA. Chapter 3 outlined a method for the assessment of the immunosuppressive potency of BM MSCs on monocytes in a whole blood assay. This relied on being able to record the reduction of intracellular expression of the pro-inflammatory cytokines in TNF and IL-6 in LPS activated healthy monocytes, with or without the addition of primary BM MSCs. A whole blood assay was developed where monocytes could be identified by their cell surface marker expression and expression of cytokines via simultaneous intra-cytoplasmic staining for the relevant protein. Results showed an optimal detection of intracellular TNF and IL-6 in monocytes activated with LPS and treatment of whole blood with primary BM MSCs demonstrated reduction in TNF production from monocytes. This assay indicates that MSCs have an immunosuppressive effect on activated monocytes, in agreement with the work of using others using different approaches (Vallés et al. 2015; Shin et al. 2016; François et al. 2012). Other studies, have focused more on the immunomodulatory interactions between B and T cells (Haddad and Saldanha-Araujo 2014; Fan et al. 2016) and have implemented co-culture systems whereby for the study of MSC-monocyte interactions THP-1 monocytic cell line has been used or purified monocytes from PBMCs using magnetic bead cell separation techniques. This results in further manipulation of samples and potential skewing of results and doesn't take into consideration the impact of crosstalk between other cells compared with examining how MSCs modulate monocytic activity in the presence of other cells as part of this whole blood assay. However, a limitation to my whole blood assay is the LPS stimulation required for the activation of monocytes to detect TNF and IL-6 expression, as this is unlikely to be relevant to cytokine production in RA.

Chapter 4 investigated the immunomodulatory capacity of three immortalised and clonal MSC lines and their conditioned media. It was shown how BM MSCs, even once immortalised retain their immunomodulatory characteristics, as Y201 and Y202 MSCs were able to reduce the pro-inflammatory cytokines TNF and IL-6 expression levels, with IP006 MSCs having the greatest immunosuppressive effect. Moreover, it was clearly evident that the conditioned media of these MSCs were able to have an enhanced immunosuppressive effect with further

reductions in TNF and IL-6 in healthy LPS activated monocytes. These results are of significance not only in highlighting that MSCs are immunosuppressive but indicating also their main mode of action being a paracrine mechanism.

Therefore, in chapter 5, CM was used to treat activated monocytes from patients with RA. Results shown here demonstrated the potent immunomodulatory impact of CM even on RA monocytes. These results were exciting and novel in suggesting CM as an alternative treatment to RA, in both early and established RA. Presently, MSC treatment of RA in clinical trials is only conducted on patients with refractory RA and not early RA. Furthermore, for MSCs, only cell-based treatment is currently being tested in patients and not the CM or soluble factor products. Use of CM or soluble products (especially if the latter can be produced by recombinant DNA technology) would be cheaper and easier in logistical terms than cell based treatments. Thus, my work presented in these chapters, shows promising results for the development of an alternative therapeutic for the treatment of RA at both early and established stages of disease.
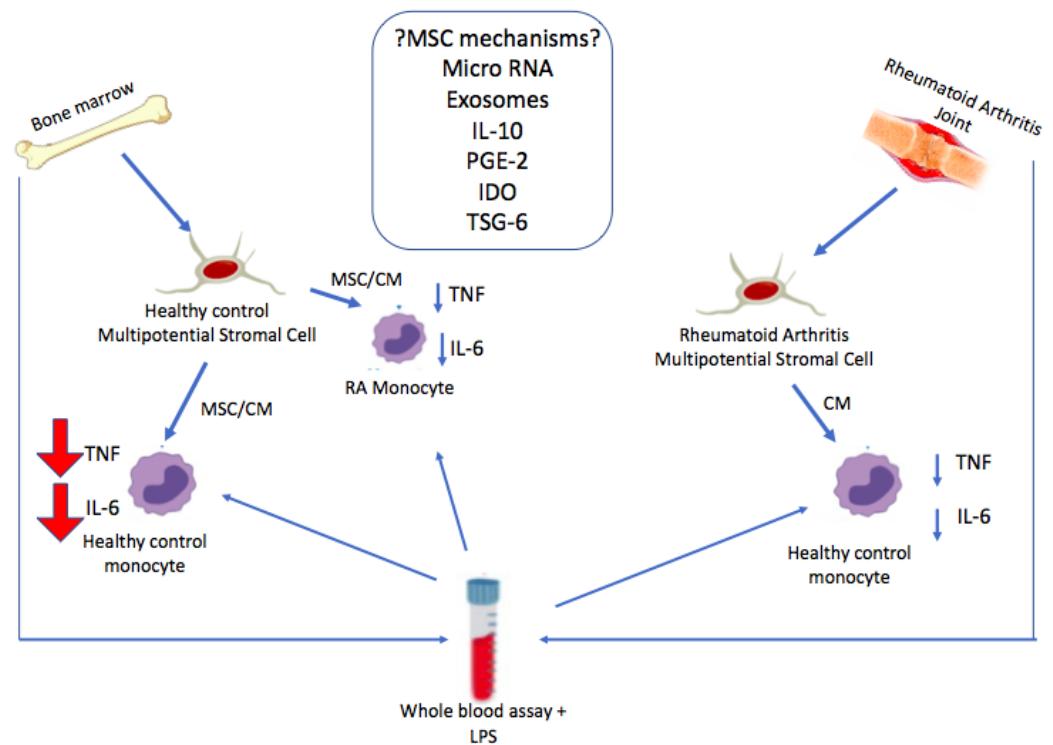
Figure 6.1. Schematic illustration summarising MSC immunomodulatory experiments carried out in both healthy controls and patients with RA, using the whole blood-based assay

Usually to determine the mode of action of MSCs on target immune cells, such as monocytes, co-culture assays are conducted to assess if the MSC has a direct cell contact or paracrine effect on effector cells. Another way to assess this, could be the methodology of neutralisation

of specific molecules, in order to better understand the role key molecules play between the interactions of MSC and immune cell. However, a key limitation of such assays is they cannot capture all of the complexity that unfolds *in vivo*, particularly as MSCs are sensitive to their microenvironment; MSCs are affected by cytokines, presence of other cells, and by PAMPs (Le Blanc and Davies 2015). Also, the source from where the MSC was isolated from is of importance. Typically, the majority of clinical studies have so far used autologous MSC infusion and in the context of where MSCs come from diseased patients, such as RA, they might already be primed towards a pro-inflammatory MSC sub-type. The whole blood assay used here will be useful to examine the differences in immunomodulatory activity of MSCs from different anatomical sites and indeed the assay could be used to test the immunomodulatory activity of particular MSC preparations on TNF and IL-6 production by the proposed patients' blood, as a preliminary test before therapy. However, with the whole blood assay it is not possible to determine how long the immunomodulatory effects of the MSCs/CM would last. To investigate the length of time the MSC treatment was able to exert an immunosuppressive effect on monocytes, further experiments would need to be incorporated using this whole blood-based assay, by including additional timepoints at which TNF and IL-6 production levels by the monocytes were measured after treatment with MSCs.

In relation to treating patients with cell-based therapies such as MSC treatment or their conditioned media, it is true that different patient's response is varied based on different cell numbers/CM treatment or according to MSC source (Brinke et al. 2014). Vaes et al. concluded that the dose-response is not consistent, as each individual has a varying optimal dose for such treatments (Vaes et al. 2012). As has been already discussed, many studies have shown MSC-CM to be effective in treating a variety of autoimmune diseases at the pre-clinical stage (Pawitan et al. 2014). However, this valuable property has yet to be transitioned into the clinical setting. An added benefit is that MSC-CM could be used as an off-the-shelf tool to treat patients promptly, such as those with RA, without the extra processing steps of MSC isolation and culturing. However, even with such benefits of using MSC-CM for treatment of autoimmune diseases, various issues remain to be tackled before it is successfully applied in the clinic. Presently, there is lack of recommendations for bioprocessing and quality control of MSC secretome-based therapeutics, which requires further clarification. For MSC conditioning, StemMACs expansion media was used, as this was previously defined by the lab to be the optimal media for culture of MSCs. However, it is yet to be determined which media is optimal for both the viability of MSC and long-term conditioning in treatment of autoimmune diseases, when delivering the CM to a patient. As the MSC-CM is a complex composition, the way forward in treating patients would require a focus on specific bioactive components (e.g. exosomes) from the CM as the therapeutic tool in clinical application. Interestingly, it has been

shown that the type of growth medium used has a marked effect on MSC secretory potential, so it would be necessary to standardise this factor before taking CM treatment to a clinical setting. Again, the route of administration and dose of MSC CM/bioactive component remains to be determined for the treatment of patients with RA. However, this work will help define future strategies to translate cell-targeted CM into clinical applications with the goal of suppressing immune cell activation, using naturally evolved stromal cell-based immune control mechanisms. The path to translation will involve further characterisation and IP protection of any MSC CM effector molecules that are specifically effective at the inhibition of immune activation in refractory RA patients, followed by collaborative pre-clinical work to test compounds' safety following local and systemic delivery.

The key aspect of developing the whole blood assay was to assess the mechanism of how MSC inhibited LPS activated monocytes, however the MSC interactions between MSC and immune cells is very complicated and is still not fully understood, making it difficult to pinpoint the exact mode of action for immunosuppression. My results indicate that the mechanism is likely to be a range of MSC-immune cell interactions and MSC soluble factors with the latter having a more potent immunosuppressive effect (Griffin et al. 2013).

There have been several studies that have attempted to develop assays to examine the immunomodulatory capacities of MSC. However, most of the studies use PBMC cultures rather than peripheral whole blood. This is important to highlight, as whole blood provides a more physiologically relevant setting, as opposed to isolating PBMC and using these in co-culture assays. As previously mentioned, using the whole blood assay is more realistic as it allows interaction between a variety of blood cell types and the molecules being secreted by them are able to be measured. Contrastingly, with assays involving the culturing of PBMCs only lymphocytes and monocytes are present, with granulocytes, red blood cells and the soluble components of the circulation absent.

Future work would be to further investigate the mechanism of action for the immunomodulatory ability of MSCs. As CM showed a greater immunosuppressive effect then the MSCs themselves, it would be paramount to unravel the key soluble components of the CM secreted by the MSCs that are involved in the potent immunoinhibitory effect. It would be interesting to see if key cytokines/soluble mediators were responsible, such as IL-10, PGE-2, TSG-6 and IDO and or secretion of extracellular vesicles (microvesicles and exosomes) and miRNAs. This can be tested using antibodies or by genetic manipulation of the MSC, e.g. using RNA interference or CRISPR/Cas9 technology. To examine key candidate cytokines that could be responsible for the immunosuppressive effect of the MSC CM a multiplex assay could be carried out, whereby multiple cytokines in the CM could be analysed simultaneously

via ELISA techniques using MSC supernatants. However, these experiments do not identify which cells these are directly being secreted by. Therefore, testing for cytokines/soluble mediators, such as IL-10, PGE-2, TSG-6 and IDO could be further verified by carrying out intracellular staining directly within the whole blood assay, by gating on MSCs and measuring the intracellular cytokine levels via flow cytometry. Using peripheral blood could also mean that the immunosuppressive ability of MSCs on monocytes could be due to another cell type, indirectly, for instance it has been reported that MSCs can exert an indirect immunosuppressive impact by inducing T regulatory cells (Azevedo et al. 2020). Thus, it would be ideal to include phenotyping and gating on MSCs and other cell populations in the whole blood assay to investigate their activity in relation to monocytes. The next step would be using a cell sorter to sort these cell types and check for gene expression and key pathways that may be implicated in the immunomodulatory ability of MSCs.

Additionally, as mentioned, it would be interesting to see if extracellular vesicles secreted from the MSCs were implicated in their immunomodulatory capacity. Exosomes and extracellular vesicles whose synthesis by endocytosis of multivesicular endosomes were discovered in 1983 (Doyle et al. 2019). Extracellular vesicles are secreted by cells into the extracellular space and are a type of lipid bound vesicle. The three main subgroups of extracellular vesicles are: microvesicles, exosomes and apoptotic bodies. These subgroups are determined based on their size, content, function and release pathways. The cargo of extracellular vesicles is made up of proteins, nucleic acids and lipids. It should be noted, that the field of extracellular vesicles has resulted in greater understanding in the area of cell-cell communication, as well as their use in the clinical setting as carriers of biomarkers for diagnostic purposes; however, the standardisation of methods for extracellular vesicles and their analysis remain to be further developed, if they are to become reliable tools in the clinical setting (Doyle et al. 2019). It has also been shown that MSCs can achieve their therapeutic effects by secreting micro RNA-containing exosomes that supress Toll-like receptor (TLR) signalling, thereby inhibiting macrophage activation and providing an exciting area of focus for future investigation of the mechanism of action involved in the immunomodulatory effects of MSCs (Phinney et al. 2014).

Extracellular vesicles could be extracted from MSC-CM by ultracentrifugation techniques. This method would extract the extracellular vesicle as a pellet, which could then be resuspended in the same volume of media as the CM and cultured with monocytes, in order to examine the immunomodulatory ability of the extracellular vesicles on monocytes. Their immunomodulatory capacity could be compared to the potency of the other soluble factors

remaining in the MSC CM, to evaluate the strongest candidate secreted by MSCs that causes the immunoinhibitory effect on monocytes.

Furthermore, extracellular vesicles from MSCs have already shown their potential as an immunomodulatory therapy, for instance with the treatment of the coronavirus (COVID-19). A first clinical trial has been recently conducted whereby 24 patients with severe COVID-19 pneumonia were treated with MSC extracellular vesicles. Patients were administered a single IV dose of MSC extracellular vesicles for a duration of 60 minutes. However, neither the dose nor the origin of MSC were stated in the published report. Results of this trial included a 83% survival rate and the study met its primary safety endpoints, furthermore, it should be highlighted that there was a significant decrease inflammatory markers and absolute neutrophil count (Lim et al. 2020). So, from current reports extracellular vesicles derived from MSCs could also be considered alongside MSCs, themselves as a potential therapeutic tool in a range of diseases, including inflammatory and autoimmune diseases. Additionally, it should be noted that MSC extracellular vesicles are cheaper and easier to produce, isolate, store and administer. They are also safer than their MSC of origin, too (Muraca et al. 2017).

Interestingly, biomarkers for use in novel cell-free therapeutic strategies for RA should be considered. It should be noted, that as well as their treatment potential, many studies have reported that miRNAs secreted by MSC-EVs (MSC-EV-miRNAs) regulate diverse signalling pathways by their targeting of specific proteins, thus influencing the development of RA (Liu et al. 2020). So, MSC-EV-miRNAs are possible biomarkers for use in novel cell-free therapeutic strategies for RA, alongside circulating cell free DNA (cfDNA) in patients with RA. It has been reported that patients with RA develop anti-DNA antibodies (Duvvuri et al. 2019). This is significant as DNA immune complexes have the ability to engage antigen receptor and TL9, simultaneously, resulting in B cell proliferation and antibody secretion. In addition to this evidence of DNA in RA, the potential release of cfDNA during inflammation has resulted in several studies investigating the potential of cfDNA as a biomarker of diagnosis, disease severity and/or treatment response in RA. Most studies investigating this have reported elevated levels of circulating cfDNA in the blood of patients with RA compared to controls, prompting cfDNA to be a potential biomarker for cell-free therapeutic strategies in RA (Duvvuri et al. 2019).

To date, utilising MSCs as an immunosuppressive therapeutic for the treatment of RA has largely focussed on their ability to inhibit B and T cells, with fewer studies investigating the impact of MSC soluble factors on monocytes/macrophages in RA. However, it is clearly evident in both in vitro and in vivo preclinical models that MSCs are able to exert an immunosuppressive effect on a range of immune cells and their biological properties.

For instance, in murine RA models, allogenic MSCs administered intraperitoneally have demonstrated to successfully treat collagen induced arthritis. However, other studies have produced contradictory evidence with the use of allogenic MSCs for treatment of autoimmune diseases. Allogenic UC MSC and BM MSC were rejected in a murine model of graft versus host disease (Eliopoulos et al. 2005). Nevertheless, the safety and efficacy of allogenic MSCs as a therapeutic tool to treat RA in a large cohort of patients has been shown. Furthermore, conventional therapy combined with allogenic MSCs has demonstrated to improve RA at serological and clinical level. Yet it should be emphasised this study did not factor in the direct impact of treatment on RA monocytes. Such studies indicate that MSCs are a promising treatment for refractory autoimmune diseases, such as RA (Devine et al. 2003). However, additional clinical trials are necessary to verify these findings, particularly when considering MSCs' mode of action and trying to unravel the way in which MSCs are able to exert their immunosuppressive therapeutic effect in patients with RA. In addition, to the mode of action the most efficient tool for the delivery of MSCs and its conditioned media are yet to be defined, along with to further unravel specifically how MSC treatment effects RA monocytes.

If indeed a new therapy for rheumatoid arthritis was to be found in the MSC supernatants used in my whole blood assay experiments the therapy could be delivered to patients via direct delivery to the disease-affected joint, similar to how current therapies such as anti-TNF are administered.

6.1 Conclusion

In conclusion a physiologically relevant whole blood assay was developed for the assessment of the immunomodulatory impact MSCs on monocytes, by measuring the intracellular levels of pro-inflammatory cytokines TNF and IL-6 from activated monocytes. Results showed how MSCs and to an even greater extent their conditioned media is able to exert a strong immunosuppressive effect on monocytes, in health and in patients with RA, both at early and established stages of disease. Thus, providing an exciting, novel alternative treatment for the devastating disease that is RA.

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