The influence of stromal fibroblast on antigen-presenting cell function and alteration of their biology

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Author's declaration

I declare that the work in this dissertation was conducted in accordance with the requirement of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific references in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views mentioned in the thesis are those of the authors.

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Abstract

Dendritic cells (DC) and macrophages (Mϕ) are antigen presenting cells (APC) that orchestrate immune activation, immune and tissue homeostasis, and disease pathology. In the case of Mϕ that act in tissue repair and wound healing, it is important that local inflammation is tightly controlled. Disorder in inflammatory processes leads to impaired regeneration, excessive remodelling or immune suppression. In contrast, the major functions of DCs include priming and tolerization of immune responses, disruption of which has major impact on responses to infectious disease, malignancy, or can result in autoimmunity. Whilst a sizeable body of research has studied DCs and Mϕ, there has been relatively little attention paid to the role of stromal cells that share their tissue microenvironment.

In *vitro*, human laboratory models of Mϕ and DCs were differentiated from monocytes under the influence of cytokines; IL-4 and GM-CSF for DCs, or GM-CSF or M-CSF to differentiate GM-Mϕ or M-Mϕ, respectively. These two major polarized Mϕ populations exhibited unique cytokine profiles, ie GM-Mϕ expressed a pro-inflammatory cytokine profile including IL-12 and IL-23, whilst M-Mϕ exhibited elevated IL-10 with minimal IL-12.

Initial studies demonstrated the impact of fibroblasts (FB) on Mϕ in conventional planar co-culture and showed the presence of FB affected their cytokine secretion profile. However, further attempts to establish a more physiological model with 3D-co-culture of these cells in spheroids were promising to improve this system. In planar coculture, FB increased IL-23 secretion by GM-Mϕ but this was not observed in the spheroid model. Secretion of IL-10 by M-Mϕ was decreased in both planar and spheroid co-cultures in the presence of dermal fibroblasts. We explored the impact of ionising radiation (IR) delivered as a single dose. Irradiation (6Gy) altered the FB-mediated effect on Mϕ. In mono-culture, IR significantly increased IL-12 secretion by GM-Mϕ ($p<0.001$), suppressed IL-10 by GM-Mϕ and M-Mϕ ($p<0.001$ and $p<0.0001$), and decreased IL-23 secretion by Mϕ ($p<0.001$ and $p<0.01$). In contrast, irradiated co-cultures showed increased IL-12 expression by GM-Mϕ ($p<0.00001$), and suppressed IL-10 by M-Mϕ ($p<0.001$). This highlighted the potential for complex interactions between APC and stromal cells. Glucocorticoids (GCs) display both immunosuppressive and anti-inflammatory features, allowing them to be used for treatment

in various immune-mediated inflammatory disorders, we investigated the effect of Dex administration. Dex in acute and prolonged periods of culture suppressed cytokine secretion by the Mϕ. We explored that Dex at high concentration and treated with TGF-β significantly ($p<0.01$ and $p<0.001$) decreased IL-23 and IL-10 by GM-Mϕ. In contrast, Dex at low concentration and treated with TGF-β at low concentration significantly ($p<0.0001$) reduced IL-23 by M-Mϕ.

Recently, it has been shown that ECM can convey specific signals to cells. We therefore explored the hypothesis that FB-derived ECM regulates macrophage behaviour. We developed a model of Mϕ differentiation to investigate the influence of FB-derived ECM components on their differentiation and function.

Fibroblast cell lines and primary fibroblasts from breast cancer patients were cultured on plastic for an extended 10-day period to deposit ECM. Subsequently, monocytes were cultured on decellularized ECM in the presence of differentiating cytokines. Interestingly, the presence of ECM from BJ6 cell line fibroblast suppresses CD169 and CD86 on monocyte (CD14+)($p<0.05$ and $p<0.001$). BJ6-ECM down-regulates CD169 on GM-Mϕ ($p<0.05$), whereas BJ6-ECM down-regulates CD169 and CD86 on M-Mϕ ($p<0.05$ and $p<0.001$). Tumour-associated FB upregulates CD204 and PD-1 on M-Mϕ. Whilst there was also evidence of impact on cytokine secretion by Mϕ this was less clear. Taken together, this evidence suggests that phenotypic consequence of Mϕ regulate by FB-derived-ECM-and does represent the physiological situation and the potential promising to provide outlook on future experimental implications that may lead to the design of novel co-culture experiments. Further planned studies of this system (including sequencing the matrisome deposited by FB, and expression profiling of the resulting macrophages) have the potential to reveal key ECM proteins responsible for the development of the tumour-associated Mϕ phenotype.

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Table of Contents

List of Figures

List of Tables

Abbreviations

Ag	Antigen
APC	Antigen presenting cells
ArgI	Arginase I
ATM	Ataxia telangiectasia mutated
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
cDC	conventional DC
CDP	Common DC precursor
CREB	Ca2+/cAMP response element-binding protein
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CXCR	C-X-C motif chemokine receptor
CXCL	Chemokine (C-X-C motif) ligand
COX2	Cyclooxygenase 2
DC	Dendritic cells
Dex	Dexamethasone
DNA	Deoxyribonucleic acid
EC	Endothelial cells
ECM	Extra-cellular matrix
FBS	Foetal bovine serum
FcγR	Fc receptors for immunoglobulin G

GCS	Glucocorticosteroids
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GM-Mϕ	MoMϕ generated in the presence of GM-CSF
ICAM	Intracellular adhesion molecule
ICOSL	Inducible costimulator-ligand
IDO	Indoleamine 2,3 deoxygenase
IFN	Interferon, Ig Immunoglobulin
IL-1R	Interleukin 1 receptor
LFA-1	Lymphocyte function-associated antigen-1
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony-stimulating factor
Mϕ	Macrophages
MHC	Major Histocompatibility complex
M-Mϕ	MoMϕ generated in the presence of M-CSF
Mo	Monocytes
MoDC	Monocyte-derived DC
MoMϕ	Monocyte-derived macrophages
MR	Mannose receptor
PBS	Phosphate-buffered saline
PD-1	Programmed death-1
pDC	plasmacytoid DC
PGE2	Prostaglandin E2
PKA	Protein kinase A
PRR	Pattern recognition receptors

Rh	Recombinant human
ROS	Reactive oxygen species
SLE	Systemic lupus erythematodes
STAT	Signal transducer and activator of transcription
TAF	Tumour-associated fibroblast
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TIDC	Tumour-infiltrating DC
TIL	Tumour-infiltrating lymphocytes
TLR	Toll-like receptors
TNFα	Tumour necrosis factor α
T-reg	Regulatory T-cell
VEGF	Vascular endothelial growth factor
N ECM	Normal dermal extracellular matrix
S ECM	Surrounding extracellular matrix
T ECM	Tumour extracellular matrix
P/S	Penicillin Streptomycin
POSTN	Periostin
BGN	Biglycan
PD-L1	Programmed death ligand 1
mDC	Mature dendritic cell
FB	Fibroblasts
LPS	Lipopolysaccharide

PBMC	Peripheral Blood Mononuclear Cell
mAb	Monoclonal antibodies
MACS	Magnetic activated cell sorting
MDSC	Myeloid-derived suppressor cell
NK	Natural Killar
SSc	Side scatter
TcR	T cell receptor
TGF-β	Transforming growth factor β
TNF	Tumour necrosis factor
Vol./vol.	Volume/volume ratio
Wt./vol.	Weight/volume ratio
iDC	Immature dendritic cell
FITC	Fluorescein isothiocyanate
FCS	Foetal calf serum
FSc	Forward scatter
APC	Antigen presenting cell
TIL	Tumour infiltrating lymphocyte
BSA	Bovine serum albumin
CD	Cluster of differentiation
cpm	Counts per minute
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
dH2O	Distilled water
EDTA	Ethylene diamine tetra acetic acid

ER	Endoplasmic reticulum
FACS	Fluorescent-activated cell sorting
STAT	Signal transducer and activator of transcription
JAK	Janus Kinase
TAM	Tumour-associated macrophages
NAF	Normal-associated fibroblast
IR	Ionizing Radiation
HA	Hyaluronic Acid
LCs	Langerhans Cells
AKT	Serine/Threonine Protein Kinase
FG-7	Fibroblast Growth factor type 7
PDGF	Platelet Derived Growth Factor
bFGF	basic Fibroblast Growth Factor
CCL5	Chemokine, C-C motif Ligand 5, RANTES-regulated upon activation, normal T-cell expressed and secreted
DKK-1	Dickkopf-1 protein
ILC	Innate Lymphoid Cells
YS	Yolk Sac
WST	Water-Soluble Tetrazolium salts

NAD(P)H	Nicotinamide Adenine Dinucleotide Phosphate

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Chapter 1 : Introduction

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1.1 Immune system

Although innate immunity has provided rapid scanning and affected the elimination of a wide range of pathogenic organisms, the variety of common pathogenic molecular patterns that it can scan is limited. The innate immune system has developed in multicellular organisms to recognise and respond to conditions that compromise tissue homeostasis. It includes a variety of tissue-resident and peripheral leukocytes whose main job is to scan pathogens and tissue damage via hardwired receptors and eradicate harmful agents by mediating inflammatory processes. While indispensable to immunity, activated innate cells is that they can produce inflammatory mediators during microbial invasion or injury, while also contributing to an enhanced risk of cancer and tumourigenesis at the site.

In the context of the fight against foreign pathogens, innate immunity is the first line of defence when it comes to mechanisms which are qualified to specifically scan microbes and eliminate infection (Nowarski, Gagliani, Huber, & Flavell, 2013). Innate immunity comprises epithelial barriers which prevent microbes from entering, such as protective plasma proteins and tissue-resident or peripheral leukocytes, e.g. phagocytic macrophages and neutrophils, dendritic cells, natural killer cells, and innate lymphoid cells (ILCs) (Medzhitov, 2008; Nathan, 2002). Inflammation is induced by the recognition of pathogenic organisms or other danger signals, which causes innate immune cells to express receptors (Barton, 2008). During the initiation of an acute inflammatory response, various chemotactic molecules, cytokines, and inflammatory mediators are secreted by tissue-resident macrophages and dendritic and mast cells; these factors can recruit neutrophils and monocytes to the infected tissue. Through the production of antimicrobial elements, extensive phagocytosis, and extracellular matrix remodelling, followed by the activation of adaptive immune responses, innate immune cells play a key role in the elimination of noxious sources and restore tissue homeostasis (Medzhitov, 2007; Orkin & Zon, 2008).

The adaptive system has evolved to recognise the overwhelming variability of antigenic structures, and the pathogens are able to mutate to avoid host recognition (Bonilla & Oettgen, 2010; Cooper & Alder, 2006).

There exist a wide range of cells within adaptive immunity, such as the effectors of cellular immune responses, i.e. T lymphocytes, which develop in the thymus, and antibody-producing cells, i.e. B cells, which mature in bone marrow (Hedrick, 2008). In primary lymphoid organs, such as the thymus and bone marrow, lymphocytes are developed and characterised by high motility, migrating towards secondary lymphoid organs, including lymph nodes and the spleen, which derive to engulf circulating antigenic factors from lymph and blood, respectively. Adaptive immune responses exist in these sites, the process of which is often impacted by innate immunity signals driven either directly by peripheral pathogens or indirectly by pathogen-activated cutaneous or mucosal antigen-presenting cells (APCs) trafficking towards secondary lymphoid organs (Jenkinson, Jenkinson, Rossi, & Anderson, 2006; Takahama, 2006).

1.2 Antigen-presenting cells

Macrophages are one of the predominant immune cells that reside in different organs and have various phenotypic properities, such as Mϕ1 and Mϕ2, that are responsible for causing and activating inflammation. This process results in various inflammatory disease processes, including cancer elimination (Islam et al., 2018). Macrophages play a crucial role, not only in diseases like lung inflammation, acute lung injury or cancerous diseases, but also in the resolution of inflammation (H. Dong et al., 2013). DCs are identified as the most potent professional antigen-presenting cells (APCs), which can uptake, process and display different types of antigens (Ag), such as tumour antigens to tumour-specific T cells, leading to the regulation of the immune system and have the ability to stimulate adaptive immunity. Dendritic cells (DCs) and macrophages play an important role in the tumour microenvironment (Steinman & Witmer), resulting in a decrease in tumour progression in human malignant diseases. Tumour-infiltrating mature, activated DCs can promote immune responses and recruitment of effector cells and pathways. DCs can be the preferential target of infiltrating T cells (L. Zhang et al., 2003).

1.2.1 Key functions of APC

The adaptive immune system comprises of B cells, CD8+ and CD4+ T cells. Unlike B cells, T cells do not react to intact foreign proteins, rather they recognize polypeptide fragments of potential antigens. APCs display these fragments of polypeptides on their surfaces in association with MHC class I and MHC class II proteins. The priming of effective adaptive T cell responses is stimulated by recognition of processed antigens on DC (Roche & Furuta, 2015). Polypeptides are created by proteolysis of intact foreign proteins in endosomal/lysosomal antigen-processing compartments that bind MHC II. These compartments gain access to antigens by using several mechanisms including receptor-mediated endocytosis, macropinocytosis, phagocytosis and autophagy (M. S. Anderson & Miller, 1992; E. A. Elliott et al., 1994). In principle, antigenic peptides that access the endosomal/lysosomal compartments are capable of binding to MHC II; this in turn generates MHC II-peptide complexes throughout the endocytic pathway (Castellino & Germain, 1995). Whilst MHC-class I associating peptides are canonically obtained from the cytosolic compartment, the process of cross-presentation permits exchange of antigens between these systems.

1.2.2 Macrophages in health organs site

Leukocytes are comprised of many cell types, including monocytes and macrophages, which are involved in regulating tissue homeostasis and immunity. Monocytes are associated with modulating inflammation and pathogen challenge, whereas the tissue-resident macrophages regulate development, homeostasis and resolution of inflammation (Ginhoux & Jung, 2014). Tissue-resident macrophages are able to modulate some homeostatic functions including angiogenesis and clearance of cell apoptosis. Furthermore, the growth of blood vessels is induced by macrophages (Fantin et al., 2010; Stefater et al., 2011). In addition, the hematopoietic steady state is maintained when macrophages engulf neutrophils and eosinophils in the liver and spleen (Wynn, Chawla, & Pollard, 2013). Macrophages play a crucial role during inflammatory responses. They can involve mediators of inflammatory secretion, such as necrosis factor alpha (TNFα), IL-1β and nitric oxide. These mediators are associated with the killing of invading pathogens by the activation of antimicrobial defence mechanisms. The inflammatory macrophage response has the potential to cause tissue damage, which in turn causes macrophages to undergo apoptosis; they then change to an

anti-inflammatory phenotype in order to induce a balance resulting in a reduction in the pro-inflammatory response during wound healing (Duffield et al., 2005; Xue et al., 2014).

Evidence for monocyte subpopulations was provided using monoclonal antibodies and flow cytometry for clear identification based on the differential expression of CD14 and CD16 cell surface markers (Passlick, Flieger, & Ziegler-Heitbrock, 1989). In 2010, study propsed a nomenclature for the existence of different monocyte subpopulations in humans (Ziegler-Heitbrock et al., 2010). The proposed described the major population of $CD14^{high}$ cells circulated in human blood as classical monocytes and the population of cells with little CD14 and high CD16 as non-classical monocytes. There is another population in between these two subdivisions named intermediate monocytes (Ziegler-Heitbrock & Hofer, 2013). Studies have shown expansion of the intermediate monocyte population in several inflammatory situations are associated with diseases and these cells have been revealed to be of prognostic relevance in cardiovascular disease (Rogacev et al., 2012). It has been suggested that CCR2 or slan use as the additional markers for delineation of intermediate monocytes. Slan-positive cells populated in human blood have been described as DCs, but analysis of phenotype has described that cells are CD14-low and CD16-high. The same terminology as proposed for man can be utilized in other species (Ziegler-Heitbrock, 2014). The classical and non-classical monocyte populations are identified in species such as mouse as well, but various markers including CD115, Ly6C and CD43 are utilized (Ziegler-Heitbrock, 2015).

Peripheral blood mononuclear cells comprise monocytes in the circulation which are divided into two subsets following differentiation. These subsets can become distinct from one another based on expression on the cell surface. Expression of $LY6C^{high}$ $CX3CR1^{mid}$ $CCR2^{+}$ $CD62^{+}$ $CD43^{low}$ ($LY6C^{high}$) is found in inflammatory monocytes, whereas $LY6C^{low}$ $CX3CR1^{high}$ $CCR2^{-}$ $CD62^{-}$ $CD43^{high}$ ($LY6C^{low}$) are expressed in circulating monocytes. Chemokines, including macrophages-colony stimulating factor 1 (CSF-1), C-C motif chemokine ligand 2 (CCL2) and stromal cell-derived factor 1 alpha (SDF-1α), direct inflammatory monocytes towards sites of inflammation, such as cancer. They then extravasate from the blood vessels as a result of differentiation into monocyte-derived macrophages (Cortez-Retamozo et al., 2012; B. Z. Qian & Pollard, 2010).

It is becoming clear that macrophages are polarised into two types. M1 macrophages are activated by lipopolysaccharide (LPS) and interferon gamma (IFNγ), while M2 macrophages

are created in the presence of IL-4 and IL-13 (see Figure 1.1). Macrophages produce several types of pro-inflammmatory cytokines, IL-12, IFN-γ and TNFα, and generate reactive oxygen species, which result in the elimination of foreign organisms and cells. These cytokines are released by M1 macrophages, whereas M2 macrophages are associated with antiinflammatory cytokines (including IL-10), upregulation of scavenging receptors and the remodelling of tissue (Biswas & Mantovani, 2010; Murray et al., 2014).

1.2.3 Macrophages within the tumour microenvironment

The tumour microenvironment (Steinman & Witmer) is comprised of many molecules associated with regulating a M2-like polarization state, such as tumour-derived lactic acid or the secretion of cytokines, including IL-4, IL-10 and IL-13, that are released from different cells within the TME or by B cell-derived immunoglobulins (Colegio et al., 2014; Ruffell, Affara, & Coussens, 2012).

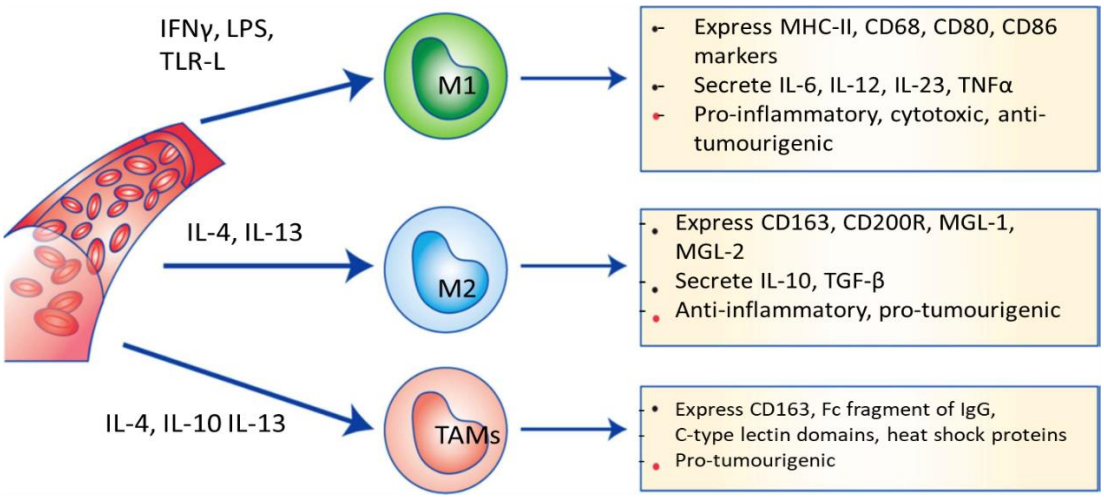

Figure 1.1 Differentiation of macrophages and their role within the tumour microenvironment. Classically activated M1 macrophages are induced by LPS/IFN-γ or TLR ligands, which release pro-inflammatory cytokines and play tumoricidal roles. IL-4 and IL-13 can stimulate alternatively activated M2 macrophages, which express anti-inflammatory cytokines IL-10 and TGF-β and act as tumorigenic roles. Cancer-associated macrophages (TAMs) express an M2-like phenotype and exert pro-tumorigenic features (Aras & Zaidi, 2017).

Hypoxia is a feature of the TME in a majority of malignant tumours. Also hypoxia can be influenced by TAMs in the TME, owing to their abnormal, pro-angiogenic factor secretion, leading to permeable blood vessels that lose ordinary structure and function. However, several studies demonstrate that TAM populations are associated with a level of hypoxia in distinct compartments within tumours (Casazza et al., 2013). Hypoxia plays a crucial role in driving macrophage recruitment and polarization within the TME (Boutilier & Elsawa, 2021; Huber et al., 2016).

1.2.4 Dendritic cells and their origin

In the 19th century, Paul Langerhans described cells, later named DCs by Ralph Steinman in 1973. These were bone marrow-derived cells that existed in blood, all tissues and lymphoid organs (Steinman, 2012).

Antigen (Ags) within peripheral tissue are captured by DCs using many complementary mechanisms. Initial immune responses are induced when DCs displaying an antigen complex in the form of peptide-MHC interact with naïve cells, i.e. T cells in lymph nodes. The fate of naïve CD4+ and CD8+ T cells following interaction with DCs is differentiation into antigen-specific memory T cells. CD4+ T cells can activate to T helper (Th1, Th2, or Th17) cells and T follicular helper cells (Tfh), which in turn help B cells differentiate into cells that produce antibodies or regulatory T cells (Tregs). These cells are involved in regulating other lymphocyte functions, whereas naïve CD8+ T cells can differentiate into cytotoxic T lymphocytes (CTLs).

The immune response results from a complex interaction between the innate and adaptive immune system which are antigen-non-specific and antigenspecific respectively, in which DCs play a crucial role. There are various molecules that are associated with activation of innate immunity, such as non-clonal recognition receptors including lectins, Toll-like receptors (TLRs), NOD-like receptors (NLRs) and helicases. In contrast, the adaptive immune system utilizes clonal receptors leading to an interaction with antigens or their bearing peptides in an extremely specific mode. Danger signals stimulate DCs and regulate the innate and adaptive immune response. They also provide signals within their environment via specific molecules

that act as sensors in order to receive danger signals that result in the transmission of information to lymphocytes (Banchereau & Steinman, 1998; Steinman & Banchereau, 2007). Pathogens or dying cells do not provide direct danger signals (molecules released during infections and/ or tissue damage and cellular stress) but are associated with disorders of tissue and cell homeostasis and may signal pathological stress. These signals, such as acidosis, hypoxia, and alterations in osmolarity, also play a role in inflammation and autoimmunity (Gallo & Gallucci, 2013; Land & Messmer, 2012).

1.2.4.1 DC sub-sets

DCs in human blood are divided into three main sub-sets based on the differential expression of surface molecules, including CD303 (BDCA-2), CD1c (BDCA-1), and CD141 (BDCA3) (Ueno et al., 2010). Cells expressing CD303 are known as plasmacytoid DCs (pDCs) and these cells play an essential role as a front-line defence against viral pathogens through their ability to express high levels of type I IFN in response to viral interaction of infection (Siegal et al., 1999). Major histocompatibility complex (MHC) class I and II molecules are comprised of peptide antigens. The surveillance function induces specific recognition between CD8+ T cells and antigen presenting cells (APCs). Migration of DCs to draining lymph nodes, viral peptides displayed on MHC I permit activation of anti-tumour CD8+ T cells in response to their interactions. Type I IFN expressed from pDCs can increase the immunogenic capacity of other DC populations and the activation of T cell clones. Tolerance is induced by the resting state of pDCs (Di Pucchio et al., 2008; Y. J. Liu, 2005). However, Toll-Like Receptor 3 (TLR3) is expressed on human CD141+CD1c-DCs that produce IL-12; these induce efficiently cross-prime CD8+ T cells once DCs are activated through the TLR3 ligand (Haniffa et al., 2012; Lauterbach et al., 2010). In addition, there are other human DC sub-sets, including Langerhans cells (Betiol et al.) and CD1c+ DCs, that also activate CD8+ T cells through interaction with cross-derived peptides (Jongbloed et al., 2010; Klechevsky et al., 2010; Mittag et al., 2011). Studies over several years have revealed that activation of antiviral effector CD8+ T cell is induced by both CD1c+ and CD141+DC sub-sets in humanized mice, human blood and lung tissue which acquire viral antigens, whereas differentiation of CD103+CD8+ mucosal T cells is induced by the ability of unique CD1c+DCs (C. I. Yu et al., 2013). Furthermore, the expression of CD103

on CTLs plays an important role in mediating adherent molecules to cadherin, resulting in reduction of tumour progression (Le Floc'h et al., 2007).

Epidermal Langerhans (Betiol et al.) and dermal interstitial DCs (dermal DCs) are present in skin. Dermal DCs are divided into further sub-classes such as CD1a+DCs and CD14+DCs. Previous studies of human skin DCs have found that initiation of activated B cells can be directly induced by interaction with human CD14+DCs, and can also help to activate naïve T cells into Tfh cells, thus possibly leading to the development of humoral immunity (Klechevsky et al., 2008; Palucka, Coussens, & O'Shaughnessy, 2013). The unique and shared activities of the different subsets of professional APC, together with cross-talk between different types of DC, are important for effective tolerance and immune activation when required.

1.3 Antigen processing, presentation and cross-presentation

Professional APCs include DCs, macrophages, B cells and thymic epithelial cells. MHC II expression is induced on exposure to gamma interferon (IFN-γ) resulting in cell activation. Phagocytic APCs (e.g. macrophages and dendritic cells) internalize bacteria and other foreign antigens and transfer this into phagosomes (Burgdorf & Kurts, 2008; Savina & Amigorena, 2007). Microbicidal and proteolytic degradation functions are balanced following fusion of the phagosome with the lysosomal compartment resulting in enzymatic generation of polypeptides for display on MHC class I and II molecules to CD4+ and CD8+ cells respectively (Kinchen & Ravichandran, 2008).

Cytosolic antigens within all nucleated cells are generated and displayed on MHC I, but the induction of internalizing and processing polypeptides can only be performed by phagocytic APCs for MHC I cross-presentation (Albert, Sauter, & Bhardwaj, 1998; Schaible et al., 2003). Many studies suggest that antigenic fragments within the cytoplasm may be exported by phagosomes for presentation to a conventional MHC I pathway in the endoplasmic reticulum (ER) or by phagosomes that can mediate the processing of peptides; MHC I complex themselves (Figure 1.2). There are various mechanisms of antigen cross-processing such as cross-processing in the phagosome to the cystol model involved in the export of antigens through the phagosomal membrane into the cytosol, resulting in degradation via the proteasome and presented on MHC I in the ER (Kovacsovics-Bankowski & Rock, 1995).

MHC II binds peptides produced by antigen proteolysis in endosomal/lysosomal antigen-presenting compartments. There are various mechanisms that allow antigens to have access to the compartments, such as receptor-mediated endocytosis, macropinocytosis, phagocytosis and autophagy. MHC II molecules are divided in a heterodimer of transmembrane α and β subunits and gain access to these compartments through correlation with an accessory protein, termed the invariant chain (I_i), rapidly after biosynthesis in the ER.

Alternatively, the vacuolar mechanism plays an important role in the processing of antigens cross through. This involves the movement of MHC I molecules to phagosomes. As a result of this, they bind to a polypeptide of whole proteins that are graded by vacuolar antigen proteolysis (Pfeifer et al., 1993; Roche & Cresswell, 1990; L. J. Shen, Sigal, Boes, & Rock, 2004). A third cross-processing mechanism revealed that the model of ER-phagocytosis can introduce molecules where the phagosome integrates with the ER, resulting in the transportation of these intact foreign proteins into the cytoplasm and transports a fraction of proteins back into the phagosome to present on MHC.

Macropinocytosis provides a route for delivery of antigens into the antigen-processing compartment that requires intact protein antigens, which must acquire access to the endocytic pathway of DCs. Extracellular material is captured by macropinocytosis; variants of extracellular protein size (from small molecules to whole bacteria and protozoa) can be part of the non-specific uptake by an endocytic process. Macropinocytosomes acquire extracellular material when they fuse with early endosomes, transporting gained proteins to the endolysosomal pathway for antigen processing (Lim & Gleeson, 2011; Norbury, Chambers, Prescott, Ljunggren, & Watts, 1997). The signalling pathway of Rho GTPase Cdc42 and Rac plays a role in controlling macropinocytosis by a reorganization of the cortical actin cytoskeleton (Garrett et al., 2000; M. A. West, Prescott, Eskelinen, Ridley, & Watts, 2000). Use of lipopolysaccharide to stimulate the activation of DCs *in vitro* reduces levels of Cdc42 and profoundly inhibits macropinocytosis. Conversely, some studies have revealed the maintenance of the activated DCs ability to acquire, process and present peptides to CD4 T cells *in vivo* (Drutman & Trombetta, 2010; Platt et al., 2010b; Ruedl, Koebel, & Karjalainen, 2001).

Additional mechanisms of antigen delivery includes receptor-mediated endocytosis. A range of cell-surface receptors expressed on APCs regulate antigen internalization. Macrophages

and DCs possess Fcy receptors, which in turn interact with immune complexes, where they are professionally transported to antigen-processing compartments (Platt et al., 2010a). Lectin receptors also express on DCs (including the mannose receptor (MR) and DE-205), carbohydrate residues on self-proteins, and some pathogens which can recognize these receptors and deliver them to receptor-mediated phagocytosis by a process of internalization. The efficacy of processing and presentation to anti-antigen T cells is dramatically promoted by the binding of antigens to their ligands on specific APC receptors (Bonifaz et al., 2004). MHC I cross-presentation is induced when antigens target the MR (CD206) leading to the transportation of molecules into early endosomes, whilst interaction between antigens and Fcy receptor or DEC-205 results in the transportation of late endosomes/lysosomes for effective processing of antigens and presentation by MHC II (Chatterjee et al., 2012).

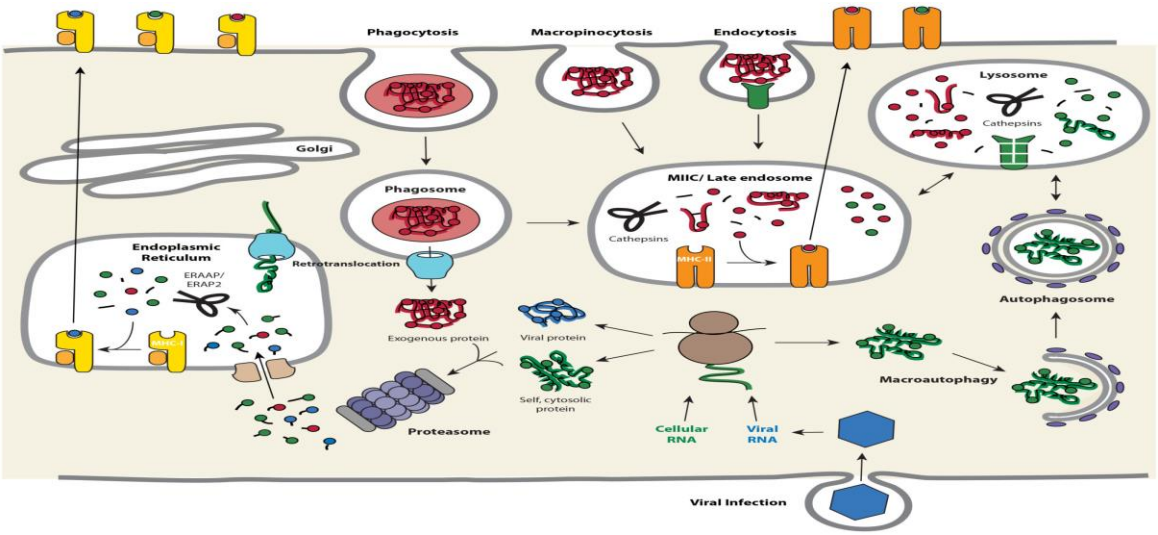

Figure 1.2 Antigen process and presentation with MHC. The proteasome can digest cytosolic proteins into small peptides that are then transferred to the lumen of the ER by TAP, where the peptides binds to nascent MHC-I molecules. ER peptides are trimmed to 8–10 residues by the action of ERAAP/ERAP1 and ERAP2. The MHC-I peptide complex then migrates to the ER before being transported to the plasma membrane. There are a variety of mechanisms involved in degrading proteins and late endosomes are rich in proteinases, classically named cathepsins. These compartments comprise of MHC-II molecules transported from the ER by association with a chaperone, named the invariant chain, shown as MIIC/late endosome in the figure above. MHC-II molecules that are ready for peptide binding are induced by proteinases degraded invariant chain in late endosomes. Following a set of peptide-editing processes, immunodominant MHC-II-peptide complexes are moved to the plasma membrane leading to recognition by CD4+ T cells. Induction of a cross-presentation process in professional APCs, namely DCs, is observed when proteins that enter cells through endocytosis/phagocytosis are retro-translocated into the cytosol, where, following proteasomal degradation, they may bind MHC-I (Roche & Cresswell, 2016).

1.4 T cell regulation and activation

Naïve T lymphocytes within peripheral lymphoid organs recognize tumour-derived peptides and other ligands on DCs and undergo proliferation and expansion of anti-tumour specific lymphocytes. These then differentiate into effector and memory lymphocytes (Acuto & Cantrell, 2000). This priming results from at least three signals, signal one, two and three.

1.4.1 Signal one

Naïve T cell priming is regulated by the interaction of the TcR with MHC class I/peptide complexes, which leads to the induction of signal one. The subsequent formation of an immunological synapse triggers TcR movement to lipid rafts on the cell membrane. At the centre (cSMAC) TcR and co-stimulatory molecules are generated. In contrast, on the side (pSMAC) adhesion molecules are presented (Montixi et al., 1998; Nel, 2002). Furthermore, central supramolecular activation clusters (cSMAC) contain high structural associations of several signalling clusters, which provide the effective activation of T cells; thereby it is recognized by the PDC protein kinase. The peripheral region surrounding the cosmic is where lymphocyte function-associated antigen-1 (LFA-1) and the cytoskeletal proteins are clustered (Potter, Grebe, Freiberg, & Kupfer, 2001). The TcR/CD3 complex consists of several molecules, such as α and β chains and the cytoplasmic signalling complex of molecules termed CD3, γ, δ, ε, and ζ.

1.4.2 Signal two

The two signal hypothesis states that, for the productive activation of naïve T cells not only must the cell receive MHC I/peptide complex signalling although their TcR, a second co-stimulatory signal is required. Numerous co-stimulatory molecules have been identified. T cells expressed CD28/CTLA-4 and APC expressed CD80/CD86 and are effective co-stimulatory molecules (Sharpe & Freeman, 2002b). The potent ones are CD28/CTLA-4 molecules which are expressed on T cells, binding B7.1 (CD80) and B7.2 (CD86) expressed on APC as their ligands. Up-regulation of B7.1 is induced upon activation, whereas expression of B7.2 reacts constitutively, and they are both shown to have interfering functions ((Sharpe & Freeman, 2002a). Interestingly, it has been shown that B7 expression is also essential for maintenance

of tolerance because of CD25+ Treg, which is absent in B7-deficient mice (Abbas, 2003). The recruitment of TcR is affected by lipid raft formation following the ligation of CD28 co-stimulatory molecules with its ligands (Viola, Schroeder, Sakakibara, & Lanzavecchia, 1999). Furthermore, this process has been found to inhibit the number of TcR required for activation. 8000 TcR are required without the process, while 1000 TcR are needed with it (Lanzavecchia, Iezzi, & Viola, 1999). The amplification of T cell expansion and survival can induce IL-2 as a result of the CD28-CD80/CD86 (Sharpe & Freeman, 2002a).

Another member of the CD28 co-stimulatory molecule family is ICOS, which is upregulated upon activation and interacts with its ligand (ICOSL) presented by the APC (Coyle et al., 2000; Wallin, 2001). Programmed death-1 (PD-1) is another member of the CD28 family. However, unlike ICOS, the interaction between PD-1 and its ligands PD-1L and PD-L2 act as inhibitory molecules; these ligands are expressed on a wide number of cell types such as APC, lymphocytes and epithelial cells (Freeman et al., 2000; Latchman et al., 2001). This interaction can suppress proliferation of CD8+T cell and IL-2 production, in turn inducing initiation of peripheral tolerance (Nishimura, Nose, Hiai, Minato, & Honjo, 1999).

CD40/CD40L are other important co-stimulatory molecules. ACP expresses CD40 on their surfaces, while CD4+ T cells and other cell types express CD40L. When CD40 interacts with CD40L, inducing APC maturation, this gives rise to the upregulation of their co-stimulatory molecules and cytokine secretions. In the absence of this binding, tolerance was induced (van Kooten & Banchereau, 2000).

ICAM-1 is an adhesion molecule which, upon binding to is ligand, LFA-1, on naïve CD8+T cells, has been shown to provide sufficient co-stimulatory signalling to prime naïve CD8+ cells to become CTLs (Jenkison, 2005).

1.4.3 Signal three

More recently, it has been shown that not only are signal one and two required for the priming of naive T cell activation, but a third signal in the form of cytokines and accessory molecules is also required for clonal expansion, differentiation and effector function. Such signals may be provided by secreted molecules such as IL-10, IL-12, IL-23, and type I IFN-α and

–β, as well as by accessory molecules such as Ly6C (Curtsinger et al., 1999; Schmidt & Mescher, 1999). Signal three polarising cytokines influence T cell responses.

Naïve CD4+ T cells differentiate into a range of effectors, including Th1, Th2, T-regulatory (Treg) or Th17 sub-types, by polarizing secretion of DC cytokines, like other APCs. Th1 cells play a crucial role in cell-mediated immunity resulting in compact intracellular pathogens, whilst humoral responses are regulated by Th2 cells, which then defend against extracellular pathogens. The regulation of autoimmunity is associated with the presence of Th17 cells, whereas the development of a toleraginic state can be mediated by Treg cells (J. Zhu & Paul, 2008). Secretion of IL-12 and IL-4 cytokines by DCs increases Th1 and Th2 polarization of adaptive immune responses respectively. IL-23 secretion plays an important role in the polarization of Th17 that is produced without simultaneous productions of the IL-12 transforming growth factor β (TGF-β). IL-2 and TGF-β are associated with the polarization of Treg cells (Constantinescu et al., 1998; Cua et al., 2003). Pro-inflammatory cytokines expressed by Th1 cells including IL-2 and IFN-γ, IL-4, IL-10, IL-13 and IL-5 are known anti-inflammatory cytokines produced by Th2 cells. IL-17 cytokine is expressed by Th17 cells and IL-10 is produced from Treg cells, including other cytokines (Blanco, Palucka, Pascual, & Banchereau, 2008; Langrish et al., 2005).

1.5 APC in peripheral tissues

Monocytes and DCs encounter antigens in peripheral tissues. In the bone marrow, a precusor of common monocytes and DCs is divided into many sub-sets, such as monocytes and other precursors known as DC precursors and pre-cDCs (K. Liu et al., 2009; Onai et al., 2007). Pre-cDCs transfer to both lymphoid and non-lymphoid tissues via circulating blood and give rise to $CD11^{high}$ and $MHC\text{-}II^{high}$ DCs. Hematopoietin and Flt3 ligand (flt3L) are required for DCs in the steady-state, whereas monocytes depend on the macrophage colony-stimulating factor (M-CSF) (D'Amico & Wu, 2003; Randolph, Ochando, & Partida-Sanchez, 2008).

The understanding of key components, such as TLRs, that interact with conserved proteins in pathogens was a significant discovery of how the host recognizes antigenic invasion; stimulates innate immune responses and leads to triggering of antigen-specific adaptive immunity (Kawai & Akira, 2011). TLRs have a high level of expression on monocytes; there are

approximately 20 times more than TLRs in DCs in blood and bone marrow (Leon & Ardavin, 2008). Cytokines play an important role in the migration and recruitment of monocytes towards inflammation sites considered critical for host defense (Charo & Ransohoff, 2006). Monocytes express CCR2 on their surface. This is associated with their activation through interaction with monocyte chemoattractant proteins (MCPs) (Gerard & Rollins, 2001). Studies have shown that immunity can be initiated as a result of the mobilization of monocytes and potent antigen-presenting DCs in peripheral organs may adopt upon the main service of monocytes as precursors, and also during inflammation (Boring et al., 1997; Leon, Lopez-Bravo, & Ardavin, 2007). Following on from the antigen processing and presentation on MHC to form MHC/peptide complexes, the migration is induced to reach downstream lymph nodes, which present the antigen to T cells (Cheong et al., 2010; Zhao et al., 2003).

A range of different TLR agonists are used to mimic certain types of antigenic pathogen that represent the roles of monocytes in antigen presentation *in vivo*. The naïve and memory T cells that interact with acquired peptides on the differentiated monocyte-derived DCs are activated (Qu, Moran, & Randolph, 2003). Within infection sites, formation of monocyte-derived DCs are important to induce efficient priming of T cells with APCs (Le Borgne et al., 2006; Qu, Nguyen, Merad, & Randolph, 2009).

1.5.1 Normal physiological function of APCs

DCs, macrophages (Mϕ) and B cells are a family of professional antigen presenting cells (APC) that chararacterize a highly hetrogeneous group of immune cells, which play a role in mediating the adaptive immune system to process and present antigens (Ag) to T cells and B cells, resulting in inducing an activating signal (Hamilos, 1989). Despite this, APCs can demonstrate several dynamic responses within the surrounding environment and other leukocytes. Induction of APCs may occur through secreted factors derived from stromal cells including cytokines and chemokines. In addition, lymphocyte function-associated antigen-1 (LFA-1),CD54, and CD40-CD40L can also influence APC by direct cell–cell contact (Hennen et al., 2011; Saalbach et al., 2015b; Sprent, 1995).

1.6 The influence of solid tumours on APC function

1.6.1 Tumour micro-environment

Genetically hetrogeneous cancer cells are a feature of the tumour microenvironment (TME). These cells comprise of endothelial cells, tumour-associated fibroblasts and different types of immune cells. Solid tumours show an abundance of immune cells with macrophages one of the most frequently observed cells types (Hanahan & Weinberg, 2011; B. Z. Qian & Pollard, 2010). High density of macrophages are associated with poor survival in pancreatic, breast, lung, cervix and bladder carcinomas and in Hodgkin's Lymphoma (Bingle, Brown, & Lewis, 2002; Scott & Steidl, 2014). Studies have used different breast cancer mouse models to demonstrate decreases in mammary tissue macrophages are linked with a reduction in timour size, whereas tumour-associated macrophages (TAMs) resulted in increased tumour growth. Expression of $CD11^{low}$ $MHCII^{high}$ $F4/80^{+}$ $CD64^{+}$ $MerTK^{+}$ on the surface of TAMs distinguishes TAMs from mammary-residing macrophages. In fact, TAM population results from direct differentiation from CCR2+ inflammatory monocytes in the TME (Franklin et al., 2014). Previous studies suggest that myeloid populations within a TME comprise of monocyte-derived LY6C+ inflammatory macrophages, which are induced from the circulating blood and the splenic reservoir and which subsequently differentiate into $LY6C^{low}$ TAMs. Moreover, these TAMs are a feature of heterogenous populations and they show an ability to differentiate into further separate populations according to the level of MHCII expression (Cortez-Retamozo et al., 2012; Movahedi et al., 2010).

Within the TME, cancer cells, stromal tissue, and the extracellular matrix influence both the priming and effector phases of anti-tumour immunity. Immunological deficiencies are associated with increased tumour growth in both rodent models and in humans (Chow, Moller, & Smyth, 2012; Smyth, Dunn, & Schreiber, 2006). Moreover, it has also been shown that in genetically immunocompromised mice, tumour progression is increased compared with progression in conventional mice (Girardi et al., 2001). It is now clear that the continual inflammation caused by certain chronic microbial infections can greatly increase the development of tumours (Aggarwal, 2009). Persistent inflammation creates a favourable microenvironment that promotes increased tumour development and growth caused by certain immunological-associated genes (F. Balkwill, Mantovani, A., 2001; Dunn, Bruce, Ikeda, Old, & Schreiber, 2002). Indeed, the immunoediting hypothesis shows that not only can the

immune system prevent tumour growth, but it can also help to promote tumour growth (Dunn, Old, & Schreiber, 2004). There are three distinct stages of immunoediting: 1) elimination, 2) equilibrium, and 3) escape (see Figure 1.3).

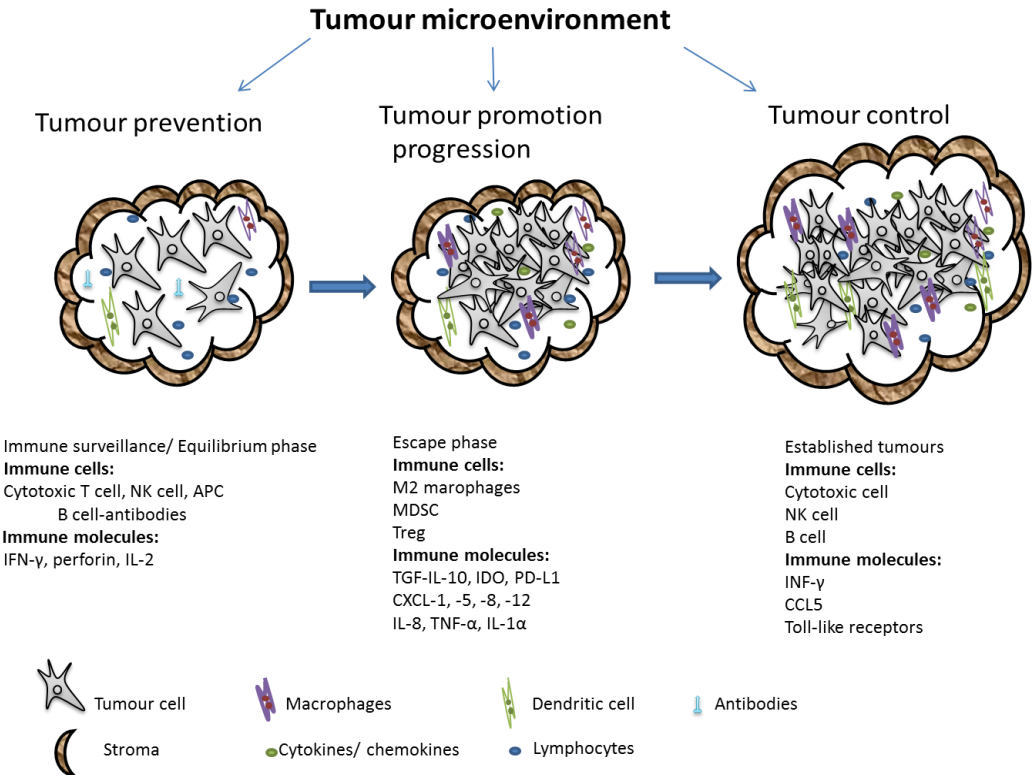

Figure 1.3 The mechanisms within the tumour microenvironment that play a role at each stage of tumour growth. CTL and Natural killer (NK) cells act at the initial immune surveillance stage helping to eliminate tumour cells. However, the next phase, known as the escape phase, is associated with the progressive accumulation of tumour cell mutations and alterations resulting in complete escape from immune surveillance. Several types of immune cells, such as myeloid–derived suppressor cells (MDSC), TAMs and Tregs, as well as multiple immune mediators within the TME favour tumour development.

1.6.2 Elimination and Equilibrium phases

It is becoming clear that the immune system is very important in combatting tumour progression. During the equilibrium phase, the tumour is being controlled by the immune response. The tumour can remain latent for long periods of time until the microenvironment becomes permissive for further tumour development. It is known that CD8+ CTL can partly control the dormant tumour; visceral metastases occur more rapidly if CD8+ T cells are depleted (Chew, Toh, & Abastado, 2012). During the process of immunoediting, tumour cells may mutate surface antigens, resulting in less immunogenic tumour cells. Thus, a highly invasive and less immunogenic tumour cells can escape the equilibrium phase of immune surveillance (Almog, 2010; Eyles et al., 2010).

The escape from immune surveillance is an important mechanism, as this selects out and then promotes the growth of poorly immunogenic cells (Browning & Bodmer, 1992). Tumour cell death is also prevented by the expression of anti-apoptotic molecules (Reed, 1999); growth factors that aid tumour formation, such as VEGF (X. Zhang, Nie, & Chakrabarty, 2010); immunosuppressive molecules, TGF-β, IL-10 and programmed cell death-ligand 1 (PD-L1) which eventually inhibits anti-tumour immune responses (Ben-Baruch, 2006; Gajewski, Meng, & Harlin, 2006). MDSC play a crucial role in suppressing naïve T cell expansion and differentiation; functions of T effector cells are blocked and Treg cells are generated by the release of IL-10 and TGF-β (Ostrand-Rosenberg & Sinha, 2009). However, it has been shown that the maturation of DCs is inhibited by the TME resulting in loss of function as effective antigen-presenting cells to activate anti-tumour immune response (L. Yang & Carbone, 2004). Certain pro-inflammatory cytokines, such as IL-6 and IL-8, and several chemokines are known to act as anti-apoptotic factors (Hodge, Hurt, & Farrar, 2005). Chemokines, including CXCL1 and CXCL8, are associated with an increase in tumour cell formation (Payne & Cornelius, 2002). CXCL5 and CXCL12 recruit neutrophils and MDSCs (L. Yang et al., 2008), and CXCL12 promotes the migration of tumour cells expressing the cognate receptor CXCR4 (F. Balkwill, 2004).

1.6.3 Established tumours

Although the immune system is often unable to inhibit tumour development, the immune response towards established TMEs plays an important role in controlling the outcome.

Studies in mice have shown that an established melanoma tumour could be reduced in size by activation of the anti-tumour specific CD8+ T cell responses using a peptide–based immunotherapy (Belnoue et al., 2004; S. B. Qian, Li, Qian, & Chen, 2001). Immunostimulatory cytokines IL-2 and IFN-α, as well as antibodies against tumour antigens may be utilized as adjuvants in combination with cancer vaccines or chemotherapy (Grillo-Lopez et al., 2000; Moschella, Proietti, Capone, & Belardelli, 2010). Indeed, there is an association between the presence of tumour-infiltrating lymphocytes (TIL) combined with a pro-inflammatory phenotype, and a favourable prognosis in various cancers including breast, colorectal, lung, ovarian cancer and melanoma (Budhu et al., 2006; Chew, Chen, et al., 2012; Chew V, 2012; Pages et al., 2010).

1.7 Mesenchymal stromal cells (MSCs) in the TME

Non-haematopoietic cells comprise of a subset of mesenchymal stromal cells (MSCs). These cells display homing and the potential to engraft to impaired tissue in several pathological conditions including inflammation, tissue repair and neoplasia (Bergfeld & DeClerck, 2010; Kidd et al., 2009). Uniquely, MSCs are not characterised or identified by a single cell surface marker. The haematopoietic cells markers CD34 and CD45 are typically absent on MSCs. However, MSCs are positive for the surface markers CD44, CD73, CD90, CD105, CD106 and STRO-1 (Salem & Thiemermann, 2010). Secretion of growth factors and cytokines by tumour cells induces the recruitment of MSCs into tumours from normal tissues. This leads to MSCs homing in on primary tumours. Mesenchymal and progenitor cells can migrate from the bone marrow to tumours (Hung et al., 2005). These cells are abundant in adipose tissue and these adipose tissue-derived MSCs can contribute to tumour stroma (Kidd et al., 2012). The cell surface marker that uniquely characterise adipose tissue-derived MSCs share a number of key properties with bone marrow-derived MSCs, such cell surface marker expression, plastic adherence and the potential to differentiate into cells mesenchymal lineage, including fat, bone, muscle and cartilage under suitable conditions (Dominici et al., 2006). These cells have been found to control the morphology and proliferation of cells by cell-to-cell interactions and the expression of chemoattractant cytokines and paracrine factors (Bergfeld & DeClerck, 2010). The role of MSCs in tumour growth are diverse and comprise of a modulatory function

on tumour cell proliferation, metastasis, angiogenesis and migration. MSCs affect tumour cells in four main ways.

- The immunomodulatory function of MSCs has an indirect role in tumour growth. The immunosuppressive function of MSCs can affect both innate and humoral immunity by suppression of Th1 lymphocytes, dendritic cells, B cells and NK cells (Le Blanc & Ringden, 2007).
- MCSs may have both a supportive and inhibitory function on tumour growth (Klopp, Gupta, Spaeth, Andreeff, & Marini, 2011).
- MSCs share the tumour vasculature by production of angiogenic factors. It has been found that proangiogenic factors, such as VEGF, angiopoietin, IL-6, IL-8, TGF-β, PDGF (platelet derived growth factor), bFGF (basic fibroblast growth factor) and FG-7 (fibroblast growth factor type 7), are secreted by MSCs. The stimulation of new blood vessel growth and the activation and recruitment of stromal fibroblasts by these cytokines leads to changed in normal tissue homeostasis (Grisendi et al., 2011).
- MSCs can increase tumour cell migration, cancer growth and metastasis to distant organs via expression of CCL5 (chemokine, C-C motif ligand 5, RANTES-regulated upon activation, normal T-cell expressed and secreted) (Karnoub et al., 2007).

The role of MSCs in tumour cells depends on their origin, their degree of differentiation, the dose and the administration route of the cell type they interact with (Klopp et al., 2011). Various mechanisms are involved in the initiation of mutual autocrine and paracrine signalling by secreted chemokines, apoptotic modulation and vascular support.

There are two mechanisms responsible for MSC-mediated tumour growth support. These are the direct impact on tumour cell proliferation and vasculogenesis (Prantl et al., 2010; Shinagawa et al., 2010; W. Zhu et al., 2006). Moreover, tumour incidence and size is increased in the presence of the coinjection of mesenchymal cells through SDF-1/CXCR4 signalling (Kucerova, Matuskova, Hlubinova, Altanerova, & Altaner, 2010; Muehlberg et al., 2009). Yan et al. (2012) reported compelling evidence that MSCs affect both proliferation and inhibition of apoptosis of breast cancer cells. Another study suggested that IL-10 secretion by MSCs can affect chemosensitivity in human breast cancer cell line SKBR3 *in vitro* (Greco et al., 2011). There are a number of biological roles mediated by MSCs that can affect tumour cells. These are attributed to their specific molecular profile and the unique characterisation of the

complex signalling pathways in response to soluble metabolites secreted into their microenvironment (Kucerova et al., 2011).

Several studies have shown that increased latency, decreased tumour size and metastases are associated with human MSCs-mediated tumour growth inhibition (Klopp et al., 2011). There are many mechanisms responsible for mediating this interaction, such as AKT (serine/threonine protein kinase) signalling, WNT signalling, DKK-1 (Dickkopf-1 protein) secretion or G1 cell cycle arrest (Cousin et al., 2009; Khakoo et al., 2006; L. Qiao, Xu, Zhao, Ye, & Zhang, 2008; Y. Zhu et al., 2009).

1.8 The role of fibroblasts in tumour micro-environments

Cancer-associated fibroblasts (Ngambenjawong, Gustafson, & Pun) play a role in the general host response to tissue destruction due to tumours. Fibroblasts remain alive, even if they undergo severe stress that is considered fatal to other cell. TAFs are found at all phases of disease development, such as metastasis. Furthermore, TAFs act as machines that create and deposit an extracellular matrix (ECM) structure; reprogramme metabolic and immune functions of the TME; and initiate synthesis of many different tumour elements resulting in resistance to chemotherapy (Hanahan & Weinberg, 2011; Kalluri, 2016b; Quail & Joyce, 2013).

The tumour stroma consists of cancer cells surrounded by components of normal organs including immune cells, capillaries, basement membranes, activated fibroblasts and ECM (Hanahan & Coussens, 2012; Pietras & Ostman, 2010). Fibroblasts are a major component of tumour stroma, and it has long been suggested that they play an important role in cancer progression and metastasis (Kalluri & Zeisberg, 2006; Ohlund, Elyada, & Tuveson, 2014). A wound healing response is usually comprised of activated fibroblasts that differentiate from quiescent cells, but the mechanism for converting quiescent and resting fibroblasts to activated cells is still unknown. Recent studies suggest the exciting new concept that activated fibroblasts can be divided into two types, reversible and irreversible (Tampe & Zeisberg, 2014; Zeisberg & Zeisberg, 2013). In addition, expression of α-smooth muscle actin (αSMA, also know as ACTA2), a cytoskeletal protein associated with smooth muscle cells, is a feature of activated fibroblasts (Micallef et al., 2012; Powell et al., 1999).

Such response to diverse types of acute injury can induce wound healing. Recruitment of inflammation processes, immune cells and fibroblasts induces the classic wound healing response, leading to an increase in angiogenesis and deposition of ECM (Darby, Laverdet, Bonte, & Desmouliere, 2014; Dvorak, 1986). The ECM and basement membranes comprise of many constituents, including type I, type III, type IV and type V collagens, several different laminins and fibronectin. All these are secreted by activated fibroblasts or myofibroblasts. Myofibroblasts were first identified in healing wounds in the skin (Tarin & Croft, 1969). ECM-degrading proteases, such as MMPs, are a major production of myofibroblasts, underscoring their key role in sustaining ECM homeostasis by modulation of ECM turnover (Simian et al., 2001). TGF-β-mediated signalling is associated with the induction of myofibroblasts, while vimentin and αSMA were proliferated and expressed by fibroblasts (Micallef et al., 2012).

Tumours can be characterised as wounds that do not heal (Dvorak, 1986). Due to the continuing development and accumulation of cancer cells in a given tissue, the process can be considered as a starting tissue injury with the induction of a chronic wound healing response into the cancer cells. Cancer fibrosis is recognised to be the result of chronic host repair response in the tumour. Myofibroblasts function in wound healing is becoming clear, whereas their role in cancer growth and metastasis seems to be complex and bimodal with both cancer-promoting and cancer-restraining actions (De Wever, Van Bockstal, Mareel, Hendrix, & Bracke, 2014; Dumont et al., 2013; Ohlund et al., 2014). In the 1970s, reports suggested that activated fibroblasts recruited by cancer cells are similar to those recruited in normal wound healing (Durning, Schor, & Sellwood, 1984; Ryan et al., 1973). This process is governed by release of growth factors that in turn regulate the recruitment of stromal fibroblasts. There are various phases of physiological diseases, including acute and chronic tissue damage, which are regulated by key cytokines, TGFβ, PDGF and fibroblast growth factor 2 (FGF2) (Aoyagi et al., 2004; Elenbaas & Weinberg, 2001). Restraining mechanisms of tumour progression and the potential elimination of the tumour is affected by activated fibroblasts in the presence of TGFβ stimulation within the TME (Dumont et al., 2013; Ishii, Ochiai, & Neri, 2016).

1.8.1 Cancer-associated fibroblasts support cancer development

Dermal fibroblasts not only create and regulate the ECM of skin, but they also communicate with each other and with other cell types, which in turn play an important role in modulating the physiology of skin (Ansel et al., 1996; Werner & Smola, 2001). Moreover, there are various cells of hematopietic origin that reside in skin, including a constitutive population of DCs and leukocytes such as monocytes/macrophages, neutrophils and lymphocytes (Lugovic, Lipozenocic, & Jakic-Razumovic, 2001; Nestle & Nickoloff, 1995). However, TAF differ substantially from normal fibroblasts.

The activated fibroblasts that are isolated from different human tumours have their own distinct properties when compared to those isolated from the culture of normal tissues (Tarin & Croft, 1969). The proliferative index of TAFs is higher than normal activated fibroblasts (NAFs). Nevertheless, the enhancement of migratory capacity and autocrine growth factor-induced signalling may be induced by TAFs, leading to in increase in the level of secretory molecules, such as growth factors and chemokines (Bechtel et al., 2010; Madar et al., 2009).

Several studies have used co-culture experiments to show that the tumorigenesis of cancer cells is enhanced in the presence of TAFs compared to that in the presence of NAFs (Orimo et al., 2005). Originally, it was found that the co-culture of Simian virus 40 (SV 40) transformed prostate epithelial cells of mice injected with this mixture. It was found that tumours similar to prostatic epithelial neoplasia formed with TAFs co-culture but not with NAFs. Invasive cancer cells from can be induced from non-invasive cells by TAFs (see Figure 1.4)(Dimanche-Boitrel et al., 1994; Olumi et al., 1999).

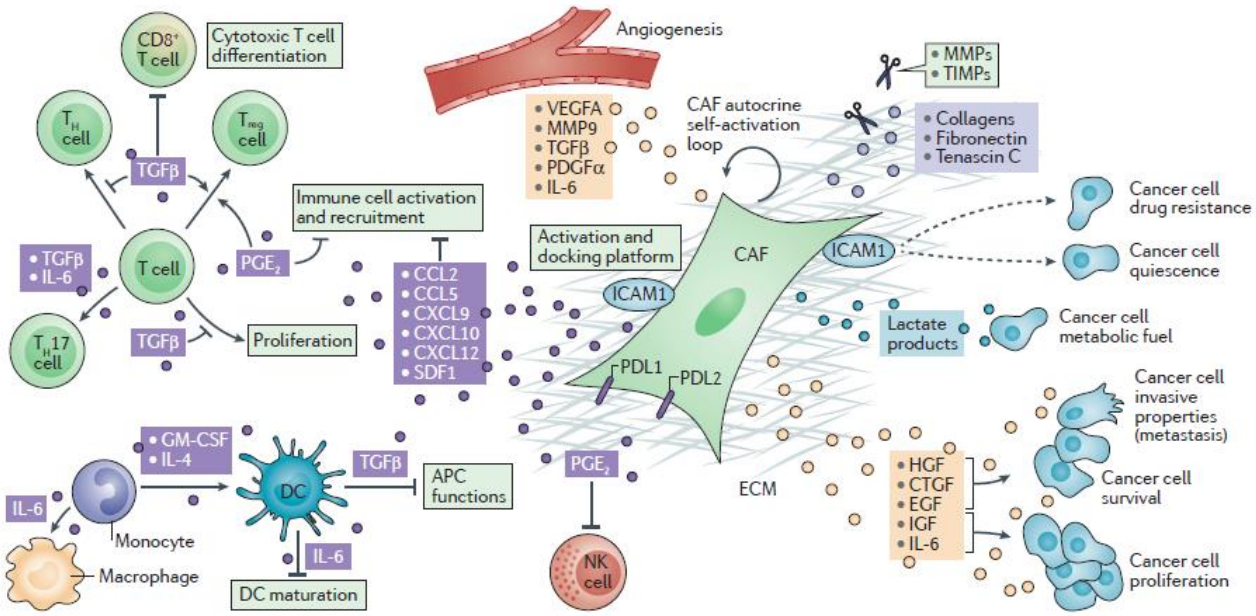

Figure 1.4 Role of TAFs in remodelling the stromal tumour. The TME is comprised of the cellular components together with ECM, which consists of actively regulated TAFs. Figure 1.4 represents regulation of immune-mediated secretory functions on the left;, self-maintained activation (centre); and interaction of cancer cells (right) resulting in increasing tumour progression, survival or resistance to therapy. Metabolic remodelling of TAFs are a crucial fuel for the TME and enhances the condition of tumour cells to growt. Tumour angiogenesis is associated with engagement with TAFs and by the action of indirect effects of immune cell recruitment and activation, tumour cell migration and invasion of features. TAFs express intercellular adhesion molecule 1 (ICAM1), which may act as a docking site for either the activation or suppression of immune cells. In addition, immunosuppressive roles may mediated by programmed cell death protein 1 ligands PDL1 and PDL2, which express on the TAFs. Cytokines secreted by TAFs may exert potent immunosuppressive actions on tumour immunity, innate immune cell recruitment and activation, and differentiation of the adaptive immune response (Kalluri 2016).

1.8.2 Immune regulation by TAFs

The pleiotropic immunodulatory functions of TAFs can be regarded as direct or indirect. TAFs are involved in a secretory phenotype, are able to synthesise ECM proteins, can release ECM-remodelling enzymes and can create high levels of cytokines and chemokines. Not only does can they maintain their activated state throughout tumour growth, but they can also dynamically develop during tumour progression (Costea et al., 2013; Lotti et al., 2013).

Generally, TAFs play a crucial role in the provision of immunosuppressive molecules within the TME (Harper & Sainson, 2014; Raffaghello & Dazzi, 2015). In the setting of tumour-induced hypoxia, a complexity of paracrine signalling responses are caused by the recruitment and interaction of TAFs, cancer cells, endothelial cells and immune cells. Established tumours comprised of TAFs express cytokines, chemokines and pro-angiogenic factors such as, IL-6, IL-4, IL-8, IL-10, TNF, TGFβ, C-C motif chemokine ligand 2 (CCL2), CCL5, C-X-C motif chemokine ligand 9 (CXCL9), CXCL10, SDF1, prostaglandin E2 (PGE2), nitric oxide (NO), HGF and human leukocyte antigen G (H. H. Chen et al.). This may have direct and or indirect impacts on tumour immunity (Patel, Filer, Barone, & Buckley, 2014; Soleymaninejadian, Pramanik, & Samadian, 2012).

Recent reports found that additional growth factors including TGFβ may adopt as a mediator for mainly immunosuppressive responses, and are also involved in the differentiation of T helper 17 cell (Th17) (Bailey et al., 2014; Wan & Flavell, 2007). In this regard, expression of the growth-promoting effect of TGFβ and HGF from TAFs may be affected by NAFs which leads to suppressed tumour growth (Goel, Wong, & Jain, 2012).

1.9 Experimental models of APC

1.9.1 Differention of DCs from monocytes

Since the discovery, 20 years ago, that blood monocytes are capable of differentiation into DCs or macrophages *in vitro*, many studies have utilised the human mononuclear phagocyte system to generate MoMϕ *in vitro* as primary cells cannot be sampled in adequate amounts (Haniffa, Bigley, & Collin, 2015; Kasinrerk, Baumruker, Majdic, Knapp, & Stockinger, 1993; Sallusto & Lanzavecchia, 1994). Discovery of the plasticity of macrophages provided a strategy which allowed the use of various stimuli to generate the most extreme classical forms of M1

(including toll-like receptor ligands and IFNγ) or an alternative activated M2 stimulated (Xue et al., 2014) by IL-4 (Murray et al., 2014). Several studies have shown that the human blood CD14+ monocytes have the ability to differentiate and polarize into sub-sets of different phenotypic macrophages and also into immature and mature MoDCs (see Figure 1.5) using flow cytometry to analyse their surface markers.

One study isolated $70x10^6$ CD14+ monocytes from human peripheral blood mononuclear cells (PBMCs) by anti-CD14 beads using magnetically-activated cell sorting. CD14 staining determined that the isolated monocytes had a purity of 99.8%. The differentiation of monocytes to macrophages were cultured using either a granulocyte-macrophage colony-stimulating factor (GM-CSF) or a macrophage colony-stimulating factor (M-CSF) for 7 days (Mantovani et al., 2004). Following this, M1 was activated using M1 Lipopolysaccharide (LPS + IFNγ) and M2a activation was induced using IL-4 stimuli. Monocytes were differentiated to macrophages in the presence of M-CSF, and then their activation was induced with IL-10 to give rise to the M2c subtype. GM-CSF and IL-4 cytokines were added to a culture of CD14+ cells for differentiation into DC for 7 days, whereas our lab utilized monocytes that were polarized for 6 days to M2 macrophages in the presence of M-CSF (Figure 1.5). Afterwards activation was induced with LPS for 24 hours. Activated DCs were induced with LPS for an additional 48 hours. Although a non-adherent state is a feature of MoDCs, all macrophages derived from monocytes were predominantly adherent and have a morphology reminiscent of a fried egg (GM-CSF) or are round to rod-like in shape (M-CSF) (Relloso et al., 2002; Verreck et al., 2004).

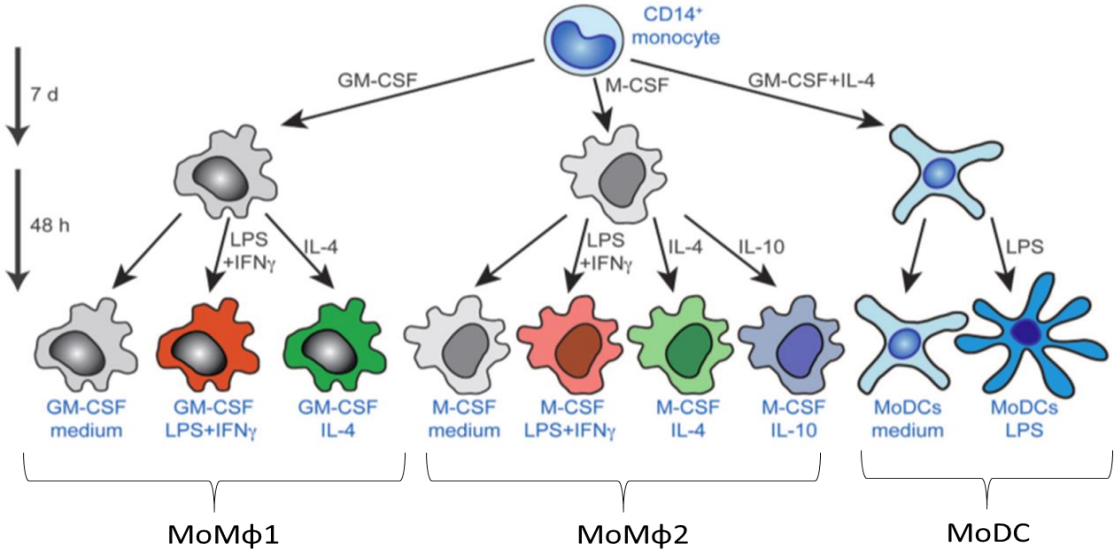

Figure 1.5 Schematic showing how human monocytes are differentiated into MoMϕ and MoDC cells. CD14+ human monocytes were isolated by magnetic-activated cell sorting (MACS) isolation kits and cells were cultured with either GM-CSF or M-CSF for differentiation into macrophages for 7 days. Afterwards, activation of macrophages were induced as indicated by either LPS/IFN-γ or LPS alone for 48 hours. Differentiation of CD14+ human cells into MoDC was induced by adding GM-CSF and IL-4 for 7 days. Their maturation was indicated by adding LPS/IFN-γ for an additional 48 hours (Ohradanova-Repic, Machacek, Fischer, & Stockinger, 2016).

Mantovani and colleagues (2002) revealed that macrophage stimuli are grouped on a continuum of two functionally distinct aspects, based on their selected immune-related ligands on a phenotype or effect on macrophages markers. IFN-γ combined with LPS or TNF are termed M1. M2 are divided into M2a, which are activated with IL-4 while IL-10 and CGs activate M2c (Figure 1.6). The subset termed M2b is activated with Fc receptors and immune complexes as described by Mosser (C. F. Anderson & Mosser, 2002; Mantovani et al., 2004; Martinez & Gordon, 2014).

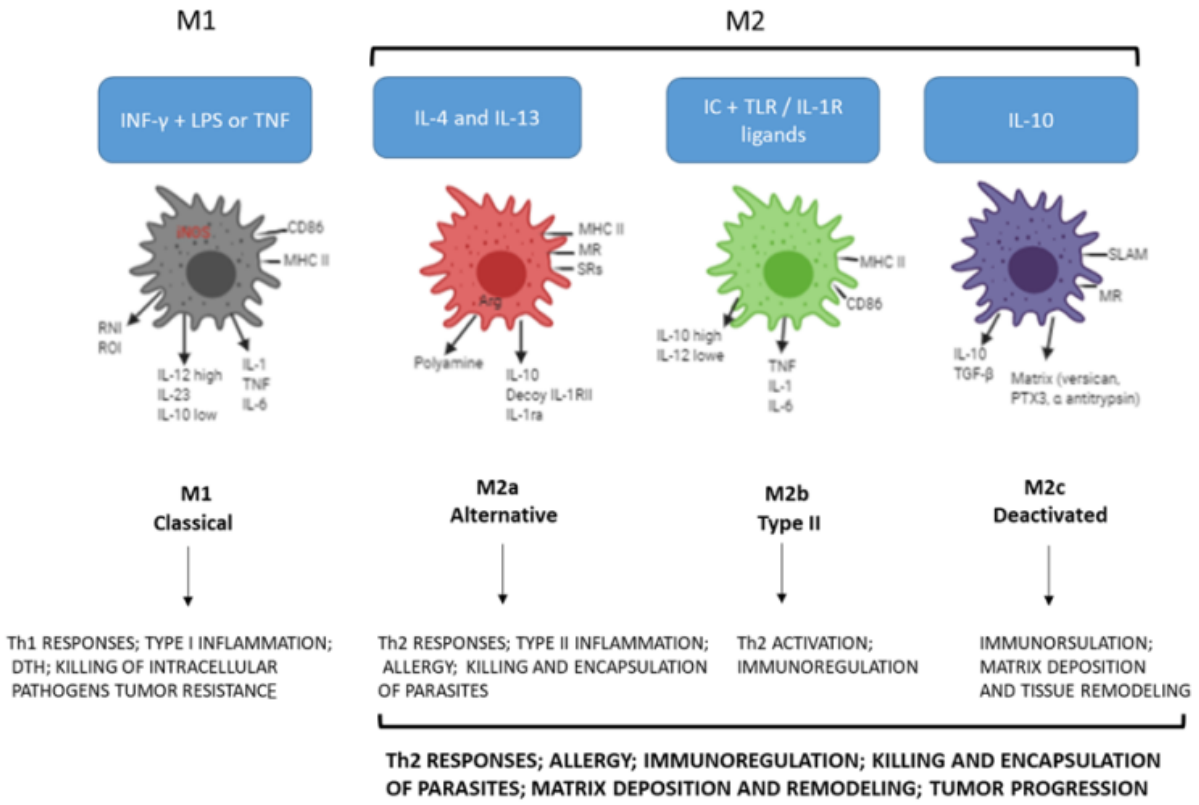

Figure 1.6 Diagram of M1 and M2 macrophage model. Activation of M1 induced by IFN-γ + LPS or TNF, and stimulation of IL-4 differentiates M2 into subdivisionsof M2a, and IL-10 and glucocorticoids binding M2c, whereas immune complex and Fc stimulate M2b.

1.9.2 Characterisation of tumour-associated macrophages (TAMs)

Cancer-associated inflammation involves monocytes and macrophages, which play crucial roles. Several microenvironmental stimuli can regulate the heterogeneity of macrophages. It is a widely accepted that there are two distinct subtypes of macrophages; the classical activation (Thomas B. Thornley, 2014) macrophage primed to microbial products and interferon-γ; and alternatively activated (M2) macrophages activated by IL-4, IL-13, and IL-10 (Jacques, Bleau, Turbide, Beauchemin, & Lamontagne, 2009; Sica et al., 2008). Many studies have revealed that tumour growth, angiogenesis, metastasis and immunosuppression are associated with tumour infiltrating M2 macrophages, also known as TAMs (S. Gordon, 2003; Varney, Johansson, & Singh, 2005). The histology gradient of malignancy in the human ovarian tumour is determined by unique features that are correlated with CD163- and CD204-positive macrophages. Consequently, these molecules are used as markers to distinguish activated TAMs in human samples. Furthermore, a high level of expression of CD163 on TAMs in malignant lymphoma, glioma, and kidney cancer indicates a poor clinical prognosis. However, there is no correlation between clinical prognosis and the level of CD204 expression on TAMs (Komohara, Jinushi, & Takeya, 2014; B. Z. Qian & Pollard, 2010). CD163 is a haemoglobin scavenger receptor and widely expressed by monocyte-macrophages system. Also, soluble CD163 may be useful as a reliable diagnostic parameter to demonstrate activated macrophages within inflammatory conditions (Komohara et al., 2013). Pollard et al. (Pollard, 2004) revealed that the M2 phenotype is polarized by tumour infiltrating macrophage tissues and is associated with the development of the TME due to angiogenesis and immune inhibition Previous studies have referred to specific markers, including CD163 and CD204, expressed by these M2-polarized macrophages as TAMs (Komohara, Niino, Ohnishi, Ohshima, & Takeya, 2015; B. Wang et al., 2015). Characterisation of CD163 function is fundamentally homeostatic and correlated with the binding of haemoglobin-haptoglobin complex. In addition, CD163-postive macrophages exist in high concentrations within inflamed tissue and are involved in the resolution of inflammation (Fabriek, Dijkstra, & van den Berg, 2005; Tentillier et al., 2016).

Expression of CD163 and CD204 and immunosuppression were examined in TAMs in oral squamous cell carcinoma (OSCC), showing that the localisation of CD163- and CD204-positive cells were clearly different. Interestingly, data showed that CD163- and CD204-positive cells

expressed by tumour infiltrating TAMs were located around tumours that produce IL10 and PD-L1 in higher levels compared to other TAM subsets when examined using a double immunofluorescent staining technique. IL-10 is positively correlated to PD-L1 expression, resulting in STAT3 being phosphorylated by IL-10 signalling. The interaction of IL-10:STAT3 signalling can produce a significant expression of PD-L1. Activation of T cell function and apoptosis are associated with existing CD163- and CD204-positive TAMs (Kubota et al., 2017; Wolfle et al., 2011).

The incidence of hepatocellular carcinoma (HCC) has increased, and it is now the second leading cause of cancer deaths globally. Postoperative recurrence is frequent and consequently, there is often a poor response to systemic chemotherapeutic treatments (X. Li et al., 2017; Maluccio & Covey, 2012). The most abundant infiltrating immune cell within TME is TAM, which is involved in distinct functions in HCC development. Furthermore, one of these cells including CD68+ TAMs indicate multiple differentiated immune cells mostly comprising CD86+ anti-tumour M1 macrophages and CD206+ pro-tumour M2 macrophages.

Evidence of CD68+ expression was determined in all macrophages, indicating CD68+ as a pan-macrophage biomarker (P. Dong et al., 2016; Falini et al., 1993). However, M1 and M2 subtype macrophages are not efficiently discriminated by CD68+. Previous data suggests that expression of CD86+ and tumour necrosis factor α (TNF-α) on M1 macrophages is observed in high levels, whereas CD206, CD163 and IL-10 were relatively highly expressed by M2 macrophages (Biswas & Mantovani, 2010; Olsson et al., 2015). Interestingly, Tan et al. (Tan et al., 2015) implied that in HCC, CD86 expression of M1 macrophages is significantly increased relative to TNF-α and IL-12, while increased CD206 expression of M2 macrophages increased relative to IL-10 and transforming growth factor β (TGF-β). Many studies have demonstrated the association of TAMs with HCC development, therefore may be a promising prognostic factor and therapeutic target. However, in HCC patients, CD68+ TAMs alone are not promising for prognostic value, indicating that whole macrophages have no effect on HCC prognosis. A correlation between invasive tumour phenotypes and TAMs has been found in the presence of a low level of CD86+ expression and a high level of CD206+ expression. Furthermore, the combination of CD86+ and CD206 analysis may have a better prognostic value for HCC patients than individual analysis of those molecules (T. Ding et al., 2009; P. Dong et al., 2016; Shirabe et al., 2012).

1.10 Role of TGF-β in modulating immune response

1.10.1 GF-β

TGF-β is a family of homodimeric polypeptide with a molecular weight of ~25 KDa. This family has five isoforms of TGF-β (Fawthrop, Frazer, Russell, & Bunning, 1997; Sporn, Roberts, Wakefield, & de Crombrugghe, 1987). TGF-βs play a crucial role in modulating many processes including connective tissue repair. In this context, the ECM components comprise of collagen I and fibronectin that are synthesized by TGFβ (Ignotz & Massague, 1986; Sporn & Roberts, 1989).

Originally, TGF-β1 was thought to be a protein expression that stimulated transformation and growth of normal fibroblasts (Todaro, Delarco, Fryling, Johnson, & Sporn, 1981). However, it is becoming clear that it is a multifunctional molecule and plays a role in regulating various fibrotic disorders including pulmonary and hepatic fibrosis (Inagaki & Okazaki, 2007; Leask & Abraham, 2004). Indeed, several physiological mechanisms such as cell growth, differentiation, and tumorigenesis can be regulated by TGF-β1, which acts as multifunctional cytokine. Many tumour cells and others within TME produce TGF-β1 that plays a role in promoting the tumour growth through the actions of inducing angiogenesis, immune-escape and metastasis (Go, Li, & Wang, 1999; Mumm & Oft, 2008; Siegel, Shu, Cardiff, Muller, & Massague, 2003). It is becoming increasingly apparent that lung fibrosis, including idiopathic pulmonary fibrosis (IPF), can be associated with promotion of lung cancer. On the other hand, it has also been shown that fibrosis within a lung tumour is not a precursor of cancer but acts as secondary phenomenon. It was identified that the existence of fibrosis in high levels led to tumour growth and poor prognosis. However, the mechanism of growth of fibrosis in lung tumour is not well understood (Noguchi et al., 1995; Shimosato et al., 1980). Epithelial mesenchymal transition (EMT) is a phenotype change induced by the action of TGF-β leading to differentiation of epithelial cells into fibroblast-like mesenchymal cells. EMT is correlated with wound healing and tumour growth in adult tissues (Sabe, 2011; Thiery, Acloque, Huang, & Nieto, 2009). Although the involvement of EMT-derived fibroblast-like cells in fibrosis is well established, TGF-β1 and the signalling mechanisms to induce biological events in tumour cells are less understood (Radisky, Kenny, & Bissell, 2007; Willis & Borok, 2007).

Therefore, autoimmune diseases, cardiovascular disease, fibrosis-related diseases and cancer are associated with abnormal modulation of TGF-β-dependent signalling pathways. Synergies

of TGF-β and connective tissue growth factor expression promoted by TGF-β can induce fibrosis (Bhowmick, Neilson, & Moses, 2004; Roberts & Wakefield, 2003). TGF-β further induces epithelial to mesenchymal transition further contributes to the fibrotic response. However, in addition to the well-established role of TGF-β as a profibrotic molecule, it has also recently been shown to play a crucial role as an antifibrotic regulator through the action of cyclooxygenase-2 (COX-2) signal pathways *(K. J. Gordon & Blobe, 2008; Rouzer & Marnett, 2008; Verrecchia, Mauviel, & Farge, 2006)*.

1.10.1.1 TGF-β regulation of Smad family

TGF-β signal transmission from the cell surface is a crucial intracellular pathway which is produced by components of the Smad family of signal transduction molecules. Studies have defined three distinct classes of the Smad family: the receptor-regulated Smads (R-Smads), such as Smad1, 2, 3 and 8; the common-mediator Smad (co-Smad), Smad4; and the antagonistic or inhibitory Smads (I-Smads) which include Smad6, and 7 (Attisano & Wrana, 1998; Heldin, Miyazono, & ten Dijke, 1997). Specific R-Smads can be phosphorylated through a conserved carboxy terminal SSXS motif when activated by type I receptors. However, dissociation of the phosphorylated R-Smad from the receptor generates a heteromeric component with the co-Smad (Smad4). These allow the complex to translocate into the nucleus. Once this complex transfers to nucleus, Smads are able to target several DNA binding proteins resulting in regulation of transcriptional responses (Attisano & Wrana, 2002; Moustakas, Souchelnytskyi, & Heldin, 2001; Wu et al., 2000).

Phosphorylation of Smad2 and 3 is induced by TGF-beta stimulation at their carboxyl termini via the receptor kinase 465 and 467 on smad2, and serine 423 and 425 on Smad3, through TbetaR-I. Phosphorylated Smad2 and 3 are combined with the co-Smad family member, Smad4, resulting in formation of a heteromeric complex (Figure 1.7). These complexes can be bound to DNA and regulate gene transcription (Abdollah et al., 1997; X. Liu et al., 1997; Souchelnytskyi et al., 1997).

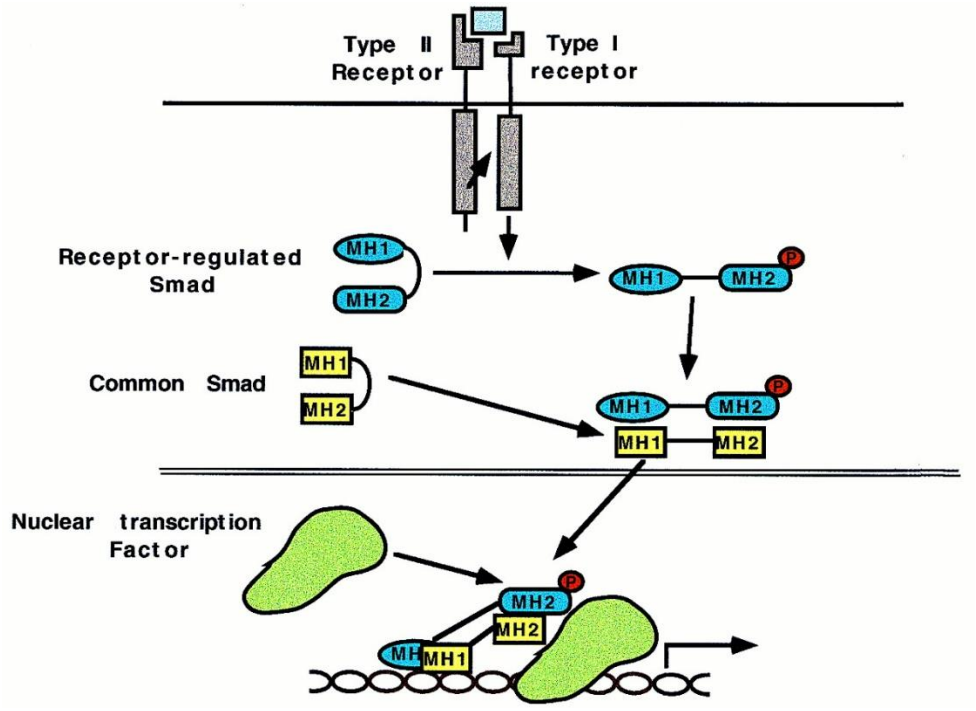

Figure 1.7 A general scheme of signal transduction molecules denoting members of the Smads family that regulate TGF-beta superfamily signals as transducers (Whitman, 1998).

1.10.2 Cyclooxygenase

Cyclooxygenase (COX) acts as the rate-limiting enzyme in prostanoid synthesis. COX is divided into 2 subclasses; COX-1 is constitutively expressed, whereas is COX-2 inducible. They pay a role in regulating inflammation. COX-2 expression is promoted in several tumour tissues such as lung cancer (F. Li et al., 2011; Soslow et al., 2000). It has also been shown that COX-2 is expressed in high levels in many lung cancers and leads to proliferation, resistance to apoptosis and induction of Treg cells (Baratelli et al., 2005; Stolina et al., 2000).

COX-1 is expressed constitutively in most tissues and plays a role in regulating homeostasis. However, induction of COX-2 is commonly absent under basal conditions and is induced by growth factors and cytokines (Gasparini, Longo, Sarmiento, & Morabito, 2003; Vancheri, Mastruzzo, Sortino, & Crimi, 2004). Evidence has revealed that several physiological and pathogenetic pathway can be regulated via the COX-2-prostanoid pathway, such as those involved in fibrosis, cancer, inflammation, angiogenesis, haemodynamics, and renal function (Araki et al., 2003; Koki & Masferrer, 2002).

Prostaglandin E2 (PGE2) is the major product of COX-2, and its role is largely considered to be as an effective proinflammatory mediator, it also acts to give antifibrotic effects (Wilborn et al., 1995). It has been documented that, in response to TGF-β, fibroblasts can increase secretion of PGE2 in high levels which are responsible for its antiproliferation effects. Expression of collagen and proliferation are suppressed in an autocrine fashion. The result of the inhibition of COX-2 up-regulation and the following PGE2 expression is the loss of the antiproliferative effects of TGF-β (Keerthisingam et al., 2001; X. H. Yang, Hou, Taylor, & Polgar, 1997). Furthermore, studies have revealed that COX-2-deficient mice have enhanced susceptibility to pulmonary fibrosis and cardiac fibrosis. Fibroblasts release the anti-fibroproliferative effects of TGF-beta by TGF-β-stimulation of COX-2 induction, which are thought to be essential biological processes (Bonner et al., 2002; Morteau et al., 2000).

Moreover, recent lines of study have confirmed that tumour growth and development is associated with abundance of tumour-associated stromal fibroblasts. Reports have shown colon adenomas have been found to have a high level of COX-2 expression from stromal fibroblasts that are considered to be the predominant source of COX-2 (Bierie & Moses, 2006; Sonoshita, Takaku, Oshima, Sugihara, & Taketo, 2002). However, an increase in COX-2 does not only exist in cancer cells, but also in fibroblasts surrounding invasive carcinomas. COX-2 expression is considered to be an up-regulator for the prostaglandin cascade that plays an important role in tumorigenesis, tumour invasion and metastasis. The induction of TGF-β-mediated COX-2 production in fibroblasts is envisioned as a crucial contributory factor within the tumour microenvironment (Adegboyega et al., 2004; Crawford et al., 2009).

Prostaglandin E2 (PGE2) exerts a potent biological effect through G protein-coupled receptors (including EP1—EP4) (Riedl et al., 2004; Sawaoka et al., 1998). It has been revealed that tumour progression, immunosuppression, or angiogenesis can be modulated by PGE2 (Greenhough et al., 2009; Sawaoka et al., 1998).

1.11 Role of dexamethasone in cancer patients

The most effective factor for manipulating Ag-specific immune responses is immunization. However, induction of an attenuated agent for established pathological responses responsible for autoimmune disease, allergy or graft rejection by using immunization can be

difficult, because the responses of pathogenic T cells can be triggered by immunogens and has the potential to worsen the condition.

Alteration of the host immune response to immunogens has long been established by using adjuvants for immunization (Coffman, Sher, & Seder, 2010). It has been found that adjuvants can comprise both of immunogenic means to promote immunity, and the potentiation of tolerance induced by tolerogenic agents. However, all adjuvants in use today encompass immunogenic means. Studies have detected immunosuppressive responses that are regulated by both suppression of pathogenic T cells responses and potentiation of tolerogenic antigen presenting cells via APCs (X. Chen, Oppenheim, Winkler-Pickett, Ortaldo, & Howard, 2006; G. Zheng et al., 2013).

Dexamethasone (Dex) is one of the glucocorticoid (GC) steroids that is used as a co-medication for patients with cancer with the aim of inducing immunomodulatory effects. The field of Cancer immunotherapy has rapidly developed methods to promote or direct the immune response of patients with tumours (Cook, McDonnell, Lake, & Nowak, 2016).

The synthesis of glucocorticoid immunosuppressive dexamethasone (Dex) is a critical factor as it has been shown that *in vivo* Dex favourably induces the apoptotic effector T cells while sparing Treg cells (X. Chen, Murakami, Oppenheim, & Howard, 2004). Moreover, *in vitro* trials performed with DCs, revealed that the function and phenotype of DCs were altered, were rendered tolerogenic (Hackstein & Thomson, 2004b). With these immunological events in mind, studies have used an adjuvant comprised of Dex, as tolerogenic agent, combined with antigens for immunization in a strategy named suppressed immunization (Kang, Xu, Wang, Chen, & Zheng, 2008).

Despite these interesting outcomes, the mechanism of Dex action as an adjuvant agent is still not fully understood. Surprising, all DCs within the spleen and peripheral lymph nodes (LNs) are actually depleted by Dex; the function of a subset of macrophages observed as enriched tolerogenic APCs, thus this study showed that mechanism of Dex's adjuvant is associated with selection of macrophages *in vivo*.

1.11.1 Dex promotes CD11c^{low} cells by depleting CD11c^{hi}

In the past few years, a study utilising the spleen and LNs derived from mouse models has revealed that Dex induces tolerogenic DCs *in vitro*. This study reasoned that Dex acts as an adjuvant agent by inducing tolerogenic DCs *in vivo* (Hackstein & Thomson, 2004a). However, induction, redistribution and apoptosis of the majority of DCs *in vivo* by Dex was observed, resulting in depletion of the cells was mediated the glucocorticoid receptor using a specific blocker of the glucocorticoid receptor as mifepristone (Abe, Colvin, & Thomson, 2005; Fauci, Dale, & Balow, 1976; G. Zheng et al., 2013).

In addition, in this investigation was conducted to detect whether DCs were reprogrammed to Dex-enriched CD11c^{low} cells, they tested their lineage markers. It was found that Dex differentiates monocytes into CD11c^{low}macrophages. In the LNs, these cells were monocytes (LY6c^{low}CD62L^{low}) and CCR7^{-}blood-borne, which are identical to the monocyte-derived DCs (Nakano et al., 2009). Unexpectedly, these cells also can express F4/80 and CD68 markers on the macrophages, indicating they were monocyte-derived macrophages. On the other hand, the Dex-depleted CD11b^{hi} cells comprised dermis-derived DCs, the Langerhans cells, and CD11b+ DCs (Nakano et al., 2009; Yona & Jung, 2010).

In the spleen, expression of the LY6c^{low} CD62L^{low} monocyte marker was also expressed by Dex-enriched CD11c^{lo}. However, the CD11c^{lo} cells in the spleen are not identical to those in LNs due to further differentiation into macrophages F4/80^{hi} and CD68^{hi} (Kohyama et al., 2009; Taylor et al., 2005; G. Zheng et al., 2013).

Dex has a range of effects on DCs, leading to inhibition of maturation, disruption of migration and recruitment to tolerogenic phenotypic DCs (Cumberbatch, Dearman, & Kimber, 1999; Kadmiel & Cidlowski, 2013). In response to activating stimuli, expression of MHC class II and the co-stimulatory molecules CD86 (B7.2), CD80 (B7.1), CD83 and CD40 can be down-regulated on cell surface of DCs by Dex treatment (Kitajima, Ariizumi, Bergstresser, & Takashima, 1996; Piemonti et al., 1999; Vanderheyde, Verhasselt, Goldman, & Willems, 1999). mRNA expression for pro-inflammatory cytokines IL-1, IL-6, and IL-12 is decreased. It is well established that the addition of Dex can act as a potent inducer of apoptosis in immature DCs (Bros et al., 2007; Moser et al., 1995). There is increasing evidence from the literature suggesting that Dex treatment is not the cause of increased maturation and migration in the frequency of myeloid (BDCA-1^{+}) and plasmacytoid (BDCA-2^{+}) DCs subsets.

Indeed, expression of MHC class II, CD80, CD86, and CD40 as maturational markers was not seen. However, after migration has induced, low levels of expression of these markers with terminal differentiation were observed in peripheral blood DCs.

Dex-treated DC cell lines of murine model have been utilized to promote generation of Tregs, and then to prevent autoimmunity or a graft-vs-host response (Rutella, Danese et al. 2006).

1.12 Transduction of the lipopolysaccharide receptor signal through Toll-like receptor-4

Understanding the complexity of lipopolysaccharide and TLR is crucial in the effort to provide a molecular basis for the potent effect of LPS during septic shock and to develop novel immunotherapies (S. J. Kim & Kim, 2017). Gram-negative bacteria comprise of lipopolysaccharides on their outer membrane that represents major components of membrane, which renders them major targets for recognition by the immune system (Schumann et al., 1990). There are several bacteria, such as *Staphylococcus aureus, that* produce LPS which are toxic to humans and accountable for the dangerously lowered blood pressure leading to septic shock. Expression of a broad range of pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α) and interferon-β (IFN-β), can be induced by the host defence in response to LPS. LPS-binding protein (LBP) plays an important role in aiding LPS to dock at the LPS receptors complex by recognizing LPS and then developing a ternary complex with CD14, consequently allowing LPS to be bound to the LPS receptors complex such as Toll-like receptor-4 (TLR4) (Hailman et al., 1994; Tobias, Soldau, Gegner, Mintz, & Ulevitch, 1995). CD14 is divided into two forms. Soluble CD14 (sCD14) exists in cells that lack membrane-bound CD14 e.g., endothelial and epithelial cells; sCD14 occurs in plasma where it helps to transmit LPS signalling, (Ferrero & Goyert, 1988; Haziot et al., 1988). The second form of CD14 has been more widely studied. Membrane bound (mCD14) are attached to the membrane surface of cells such as myeloid cells. They are attached via a glycosyl-phosphatidylinositol tail, allowing the CD14 to be membrane proximal regardless of lack of a transmembrane domain (Simmons, Tan, Tenen, Nicholson-Weller, & Seed, 1989; Wright, Ramos, Tobias, Ulevitch, & Mathison, 1990). Subsequent studies have since found that TLR-4 is the effective receptor for LPS. Since this finding, research has focused on CD14 and its involvement in TLR-4 signalling, which seems to interact with LPS and thus, consequently

presents it to MD-2 and TLR-4. In addition, studies have showed that TLR-2 signalling acts in different manner through a membrane receptor of CD14, and subsequent binding of bacterial products other than LPS, such as peptidoglycan and lipoarabinomannan, presenting these to TLR-2 (Muroi, Ohnishi, & Tanamoto, 2002; Poltorak et al., 1998). Muroi et al. (2002) have mapped the essential CD14 regions that are involved in the binding of LPS and transfer to MD-2/TLR-4 that leads to optimum signalling by TLR-4 and includes amino acids 35-44, 144-153, 235-243 and 270-275 (Muroi, Ohnishi et al. 2002, Summers, Broxton et al. 2004). However, Viriyakosol and Kirkland (Viriyakosol & Kirkland, 1996) found that the C-terminal portion of human CD14 beyond amino acid 152 was not required by CD14 to provide a signalling molecule for LPS (Viriyakosol and Kirkland 1996).

The activation of TLR-4 by priming of LPS is essential for LPS signalling. This induces involvement of the secreted glycoprotein, MD-2, which acts as an extracellular adaptor protein. In the past few years, studies have found that LPS responses were completely abolished using a mutant form (C95Y) in TRL-4 expressing cells which lack MD-2, whereas LPS responsiveness was restored in wild-type MD-2 (Schromm et al., 2001; Visintin, Mazzoni, Spitzer, & Segal, 2001). Visintin et al. (Visintin, Latz, Monks, Espevik, & Golenbock, 2003) reported that LPS does indeed attach to MD-2 before TLR-4 and MD-2 interaction. Association with the extracellular leucine-rich repeats of TLR-4 result in inducing signal transduction and TLR-4 aggregation and demonstrates that LPS does not bind to MD-2. Studies utilizing knockout mice have revealed that MD-2 is a crucial key in LPS responses, because LPS responsiveness was abolished within these MD-2-deficient mice (Hoshino et al., 1999; Nagai et al., 2002).

There is growing understanding of the mechanism of LPS transduction signalling through TLR-4. TLR-4 initially termed as human Toll (hToll) but was later renamed (Medzhitov, Preston-Hurlburt, & Janeway, 1997). Preliminary investigations revealed that a constitutive active form of TLR-4 gave rise to a fusion protein of TLR-4 with CD2, resulting in an increase the costimulatory ligand to CD28 (B7.1 expression) and production of inflammatory cytokines. In addition to this, several molecules can exhibit LPS- mimetic impacts on murine cells when they bind to TLR-4, including lipoteichoic acid (LTA); fibronectin; the fusion protein mediated to respiratory syncytial virus (RSV); and taxol, a plant diterpene that is structurally unrelated to LPS (Kawasaki et al., 2000; Kurt-Jones et al., 2000; Okamura et al., 2001).

There are many members of TLR family; 13 have been described in mice and 10 in humans. key features of type 1 receptors are the extracellular leucine-rich repeats, a single transmembrane region, and the intracellular TIR domain (which represents the TLRs domain that relate sequence similarity with Drosophila Toll) (Dunne & O'Neill, 2003). TRLs within the TIR domain can be shared by the family of the interleukin-1 receptor (IL-1R) and by the signalling adapter proteins in the IL-1R-related family. There is a small different between the extra- and intracellular regions of TLRs, leading to distinct responses to specific microbial products (Figure 1.8).

In addition to the LPS signalling complexity, different strains of bacteria produce different LPS that appears to interact with different receptor clusters; this recognition gives rise to different cellular responses (Hirschfeld et al., 2001; Werts et al., 2001). One study has shown that interaction of LPS derived from *Escherichia coli* as a ligand for TLR-4 is a critical for inducing the response of a cell to LPS, thus indicating an LPS with a conical shape A proteins (M. Triantafilou et al., 2004). Several non-enterobacteria, e.g., *Rhodobacter sphaeroides* and *R. capsulatus,* are comprised of a cylindrical-shaped lipid A proteins of LPS. On the other hand, precursors and analogues of toxic lipid A e.g., pentaacyl LPS (pLA) and Iva derived from *E.coli,* LPS give rise to, bind and activate TLR2 and can even act as antagonists to TLR-4 (Byrd et al., 1999). A good example for this in the lipid Iva of LPS analogues that appear to act as antagonists to LPS in human cells but can act as LPS agonists in mouse cells. Furthermore, it was found that suppression of NF-kB occurred when there was less recruitment of TLR-4/MD-2 within lipid rafts, and consequently increasing activation of the mitogen-activated protein kinase (MAPK) cascades. It is widely accepted that the structure of LPS plays a fundamental role in the formation of the specific TLR receptor clusters in response to a variety of bacterial products (Heine et al., 2003; K. Triantafilou, Triantafilou, & Dedrick, 2001).

To obtain a clearer picture of these signalling pathways, transduction signalling via activation of TLR-4 in response to LPS using adapter proteins to function has been extensively studied (Burns et al., 1998). These adapter proteins are divided into several forms, including myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal, also known as TIRAP), TIR-containing adapter molecule (TRIF, also named TICAM-1), and TRIF-related adapter molecule (TRAM, also named TICAM-2). From the evidence, activation of TLR-4 in priming of

LPS can be categorized into two classes: an early MyD88-dependent response and a delayed MyD88-independent response.

Studies have utilized a working model for the early MyD88-mediated signalling pathways once they have interacted with LPS (Figure 1.8). MD-2 and TLR-4 bind to LPS that appears to be assessed by CD14. Recruitment of MyD88 and Mal to the receptor complex occurs when homodimerization of TLR-4 is induced (Poltorak et al., 1998; Y. Xu et al., 2000).

Downstream signal pathways of MyD88-dependent pathway activation via LPS, give rise to active NF-kB and the MAPK pathways. An illustrated model shows NF-kB activation is induced when IRAK-1 and IRAK-4 are associated with the receptor complex. It is becoming clear that auto-phosphorylation of IRAK-1 (with TOLLIP acting as a negative mediator) follows in two sub-steps, leading to hyperphosphorylated IRAK-1, which induces dissociation from the receptor complex and binding of IRAK to TRAF6 (Cao, Xiong, Takeuchi, Kurama, & Goeddel, 1996; Ninomiya-Tsuji et al., 1999). TRAF6 then converts, activates, and links with TAB-2, which triggers the MAPK kinase transforming growth factor-*β*-activated kinase (TAK1), resulting in constitutive association with its adapter protein, TAB1. At this point, NF-kB, as well as of the p38 and c-jun N-terminal kinase (JNK) MAPK pathways, are regulated through TAK-1, which acts as a common activator. Regardless of NF-kB, P38 and JNK MAPK, LPS binds to the early MyD88-dependent signalling, inducing an early IRF3 activation and production of pro-inflammatory cytokines, such as TNF-α and IFN-β (Shirakabe et al., 1997; Yamaguchi et al., 1995).

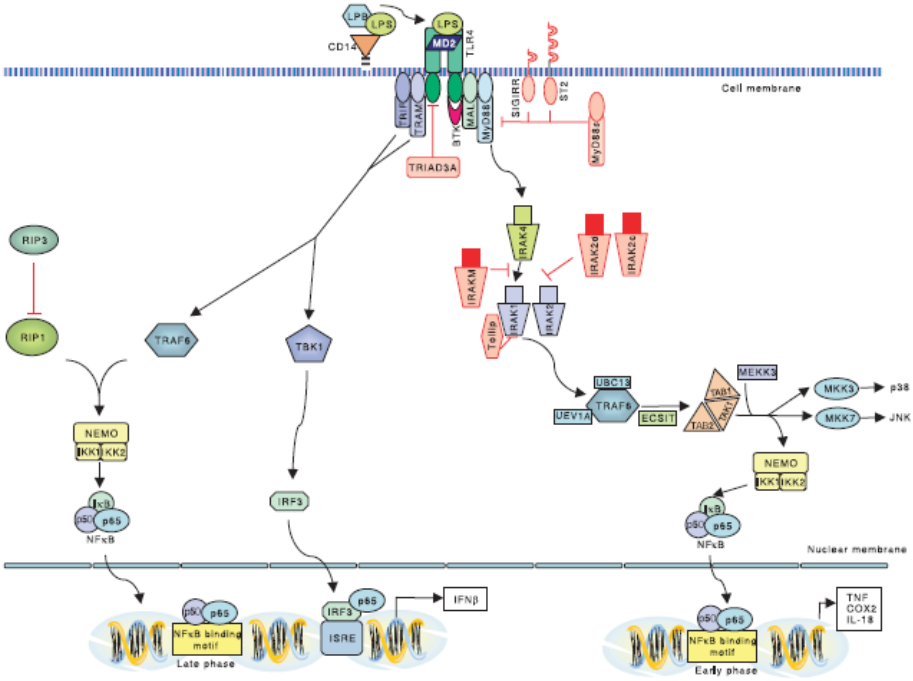

Figure 1.8 Induction of lipopolysaccharide (LPS) signalling transduction. LBP circulation can recognize LPS within the plasma and present it to CD14. This plays a role in the loading of LPS on to the receptor complex, comprising of dimerized TLR-4 receptors in addition to two other molecules, such as extracellular adapter MD-2. Consequent signals stimulated by TLR-4 can be subdivided into those dependent on MyD88 (and Mal), which induce early (referring to the procedures shown on the right side of the diagram), and those independent of myD88, which happen later and use the adapters TRIF and TRAM as mentioned on the left. The early activation of NF-kB, IRF3 and MAPK kinase pathways are induced by LPS signalling, therefore the adapters MyD88 and Mal are involved to mediate this transduction process. Furthermore, TRAF6 becomes activated after the subsequent activation and phosphorylation of IRAK, triggering the expression of several pro-inflammatory genes. As a later response to LPS, the activation of TRAF6 and TBK1 occurs through TLR-4-LPS interaction, a process mediated by the adapters TRIF and TRAM (C. Wang et al., 2001).

1.13 The role of the extracellular matrix (EMC) in the promotion of tumour growth

The immense diversity of EMC is evidenced by the variety of its biochemical and biophysical properties. ECM comprises of the non-cellular tissue component that provides essential structure to the cellular components. The ECM does not only serve as intracellular space, it instructs cell–cell communication, cell adhesion, and cell proliferation as a physically active constituent of living tissue (Frantz, Stewart, & Weaver, 2010).

Fundamentally, resident cells in the living tissue produce the ECM, which is an abundant interlocking network of water, minerals, proteoglycans, and fibrous proteins. These components play a key role as unique elements to serve a definite tissue-specific purpose for every organ (Bonnans, Chou, & Werb, 2014). This occurs by a feedback loop of dynamic biophysical and biochemical actions through cellular components and their microenvironment throughout tissue growth (Kai, Laklai, & Weaver, 2016; S. H. Kim, Turnbull, & Guimond, 2011). Resident cells secrete several elements of the ECM for any tissue depending on the demands of the tissue. The ECM produces essential fibrous proteins, including collagen, elastin, and laminin, and adapts to periods of embryonic development and disease evolution. In the induction of this dynamic arrangement, the ECM produces various biochemical agents, such as proteinases, that are consistently remodelling the ECM, which in turn degrade and modify their local environment (Jablonska-Trypuc, Matejczyk, & Rosochacki, 2016; P. Lu, Takai, Weaver, & Werb, 2011). Initiation of the balance is orchestrated by the ECM, which is largely responsible for the modification of cells, tensional homeostasis, and individual organ features, including plasticity and compressive/tensile strength. *In vitro*, the rate of viability of animal cells is only maintained when they adhere to a substrate, leading to the protrusion, adherence, and spatial interaction with the nearby ECM (K. D. Chen et al., 1999; Gumbiner, 1996; Katsumi, Orr, Tzima, & Schwartz, 2004). Indeed, studies have revealed that modulation of gene transcription occurs by transducing cues from the ECM, including spatial milieu and mechanical stiffness, to regulate signals and events of key morphological organisation (Engler, Sen, Sweeney, & Discher, 2006). Accordingly, excessive ECM deposition leading to increased stiffness can significantly contribute to cell fate through several fibrotic conditions as a consequence of down-regulated ECM remodelling (Cox & Erler, 2011).

1.13.1 The main ECM components

Collagen is the basis of ECM and exists as a "mesh". It is one of the most plentiful proteins within human tissue and has been classified into 28 unique subtypes (Mouw, Ou, & Weaver, 2014; Myllyharju & Kivirikko, 2004; Ricard-Blum, 2011). Homotrimers or heterotrimers exist in left-handed helical α chains, which in turn form a right-handed triple helix structure (Shoulders & Raines, 2009). In addition, tendons, cartilage, skin, and cornea consist of fibrillar collagens from fibrous components. Several subtypes of collagen make up each collagen fibre in response to the tissue site. The most abundant type of fibrillar collagen is type I collagen, which makes up the connective tissue within the skin, bones, corneas and tendons (Muiznieks & Keeley, 2013; Ricard-Blum & Ruggiero, 2005).

Laminins connect the ECM to the cell and are trimeric glycoproteins, including α, β, and γ chains, that are common in the basal lamina and in some mesenchymal compartments. There are 60 unique laminins that can theoretically be created by the 12 mammalian α, β, and γ chains (Domogatskaya, Rodin, & Tryggvason, 2012; Hohenester & Yurchenco, 2013; Iozzo & Schaefer, 2015). The laminins have a variety of different cell type-specific functions, such as adhesion, differentiation, migration, phenotype maintenance and apoptotic resistance. The interaction of laminins with integrins induces the dynamic between the cell and the ECM (Beck, Hunter, & Engel, 1990; Engel et al., 1981).

Fibronectin is an EMC component that is mechanosensitive and connects the cell to the ECM. Furthermore, the single fibronectin gene generates twenty isoforms in humans because of alternative mRNA splicing. Like collagen, the fibronectin in the ECM is in the form of a fibrillary mesh (Schwarzbauer & DeSimone, 2011; P. Singh, Carraher, & Schwarzbauer, 2010). Fibronectin exists in a dimer form outside the cell, mediated by two cysteine disulphide bonds, which in turn impacts on its ability to assemble in a fibrillar manner (Schwarzbauer, 1991). Alternative fibronectin formation is induced through the actin cytoskeleton pulling on fibronectin molecules once they bind to the cell surface via integrins (Galante & Schwarzbauer, 2007). This alternative fibronectin acts as binding sites for different ECM proteins and is involved in physiological functions such as associating with collagen type I. Previous studies have shown that the accumulation of collagen fibrils does not occur in the absence of fibronectin, suggesting a reciprocal relationship between collagen and its role in enhancing fibronectin assembly (Colombi et al., 2003; Dallas et al., 2005).

1.13.2 ECM function

The regulation of biochemical and biophysical contexts can be influenced by the plethora of unique ECM molecules that perform several functions in the cell simultaneously (N. H. Brown, 2011). Previously, the ECM was considered to be an inactive scaffold solely providing an architecture for the cells. Over the last two decades, its involvement in modulating the functions and phenotypes of cells has become well understood. The ECM plays a crucial role to serve as epitope sites, controlling both the adhesion and migration of cells. A barrier between epithelial cells and the interstitial stroma is created through the action of the complex structure and components of the basement membrane (P. Lu et al., 2011; Rozario & DeSimone, 2010). Growth factors are associated with several binding sites of the ECM components, controlling their secretion and presentation to target cells (Entchev & Gonzalez-Gaitan, 2002). Finally, the transduction of mechanical signals through the cells is transmitted by the ECM, in turn, activating various intracellular signalling pathways and cytoskeletal filaments (Uhler & Shivashankar, 2017).

Tissue homeostasis is regulated by the ECM, which acts as a highly dynamic structure. The constant deposition, degradation, and modification of the ECM maintains tissue homeostasis. This process is important as it is associated with maintaining the cell phenotype, as well as with physiological events including wound healing, angiogenesis, and bone remodelling (Gattazzo, Urciuolo, & Bonaldo, 2014; Rohani & Parks, 2015). The interaction between the cells and ECM occurs through receptors and focal adhesion complexes. In turn, the cells express ECM components and enzymes following signal pathways from the EMC (Starr & Fridolfsson, 2010). Weaver et al. (1997) identified the key role of the ECM in sustaining tissue homeostasis, showing how the malignant breast cancer cell phenotype converted and reverted to a normal phenotype using breast cancer cells cultured on basement membrane-based 3-D substrates coated with antagonists of integrin β1 (Weaver et al., 1997).

1.13.3 ECM in the tumour microenvironment

It is widely accepted that the traditional viewpoints of tumours have changed to consider the crucial role of the ECM in the modulation of proliferation, migration, and apoptosis (Friedl & Wolf, 2008; Gritsenko, Ilina, & Friedl, 2012). It is now understood that the proliferation of

cancer cells is significantly affected by small alterations in the microenvironment homeostasis. The primary functional features of the ECM are changeable and dictated by collagen, the most significant ECM constituent. Indeed, collagen can be altered via deposition or degradation leading to the loss of ECM homeostasis (Fang, Yuan, Peng, & Li, 2014; Provenzano et al., 2006).

During the proliferation of tumour cells, the (ECM) goes through substantial structural alterations as a result of the dynamic interplay between the surrounding space and resident cells. Secretion of fibronectin and collagens I, III, and IV are increased through the action of these changes. In turn, tumour development demands a continuous interaction of the ECM and tumour cells (Malik, Lelkes, & Cukierman, 2015). Several factors play a key role in promoting tumour progression, including amplification of the deposition of matrix proteins via correlating with cell polarity, cell–cell contact, and eventual magnification of growth factor signalling (Paszek et al., 2005). Previous studies have demonstrated the accumulation of collagen crosslinking and deposition, indicating that tumour growth occurs by promoting integrin signalling (Karagiannis et al., 2012; Levental et al., 2009). Interestingly, however, malignant behaviour can be promoted via deletion of fibrillar collagen I and III, suggesting that biomechanical forces induced by collagen deposition led to two aspects including beneficial and detrimental effects on tumour growth, as shown in Figure 1.9. (Arnold et al., 2010; Ozdemir et al., 2014). ECM signalling-derived cell proliferation is a key cellular event, which can induce differentiation and defer apoptosis (Huveneers & Danen, 2009). In brief, the cell needs to sense its mechanical environment so that it can survive.

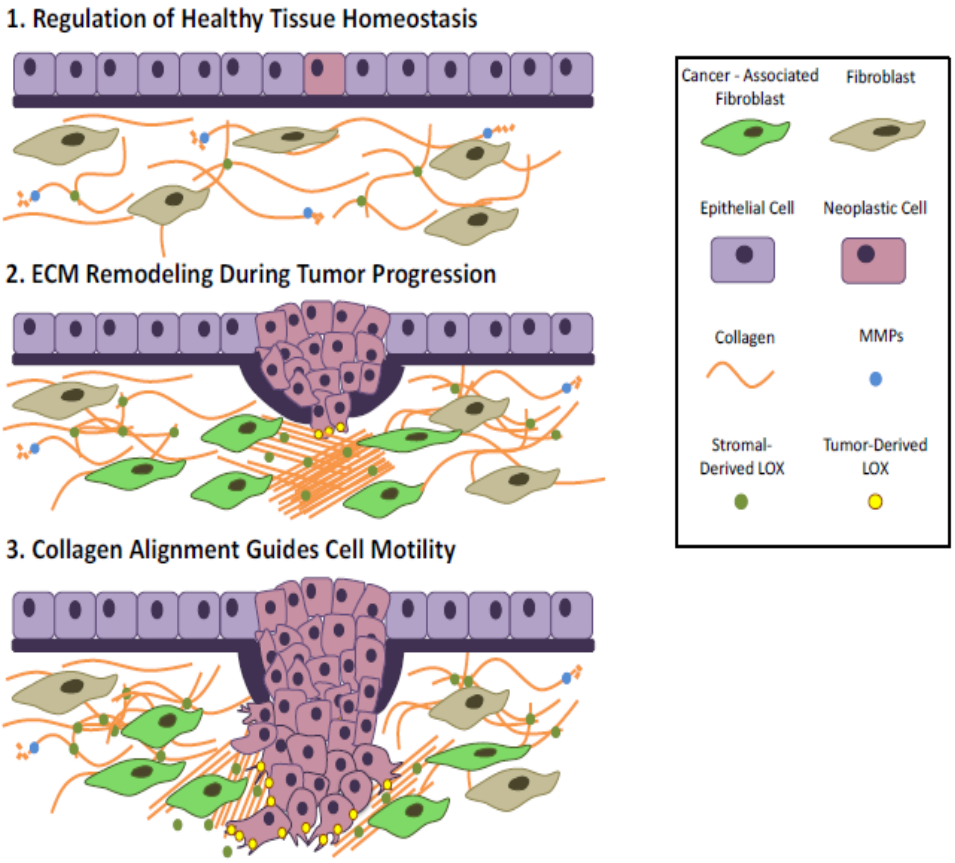

Figure 1.9 ECM remodelling in tumour growth and initiation.

1) Rapid proliferation of epithelial neoplastic cells induces clones on the basement membrane.
2) The basement membrane bulges due to the mechanical strain, with collagen deposition promoted by adjacent cancer-associated fibroblasts and collagen is aligned by stromal-derived lysyl oxidase (LOX).
3) Neoplastic cells breach the membrane and migrate along the aligned collagen (P. Lu et al., 2011).

1.13.4 ECM mediation for tumour initiation and migration

The ability of carcinoma and other cancer cells to breach the adjacent basement membrane to facilitate their migration through surrounding tissues is an essential hallmark of these cells (Morrissey, Hagedorn, & Sherwood, 2013). However, cells need to migrate around the body in healthy tissue homeostasis. Cancer cells have adapted several methods of penetrating the collagenous barrier, such as mechanical force. Studies have increasingly shown that mechanical force acts as a compelling factor for stimulation, and in turn, penetration of the basement membrane, as shown in Figure 1.9 (Kelley, Lohmer, Hagedorn, & Sherwood, 2014). Another method is the penetration of anchor cells through the basement membrane as a protrusion of cells by f-actin filaments named invadopodia (Hagedorn et al., 2013).

1.13.5 ECM regulates macrophages within the tumour microenvironment

Interestingly, several ECM constituents play an important role in TAM polarisation. At least in cell culture, macrophage polarization is driven by hyaluronan or hyaluronic acid (HA) promoting a protumorigenic and anti-inflammatory M2 phenotype (Tariq et al., 2017). Previously, it was reported that the effect of ECM on macrophage polarisation was driven by collagen I, which in turn differentiated macrophages into M2 (Wesley, Meng, Godin, & Galis, 1998). Conversely, the cytotoxic activity of macrophages was induced by fibronectin-rich ECM, maintaining M1 polarization and acting against tumour cells (Perri et al., 1982).

1.14 Role of the matrix component biglycan

ECM comprises of members of the family of small leucine-rich proteoglycans (SLRPs) named biglycan (BGN). The biological function of biglycan has not been fully elucidated, however, it has been investigated in macrophages as an endogenous ligand of TLR4 and TLR2, and it thought to play a role in regulating innate immunity, and has been indicated to be a rapidly activated factor for the stimulation of P38, ERK, and NF-kB to produce TNF-α and macrophage inflammatory protein-2 (MIP-2) (Iozzo, 1999; Schaefer et al., 2005). BGN organises the ECM into an irregular collagen fibril morphology and an osteoporosis-like phenotype correlated with the disorder of the targeted BGN gene. Furthermore, cell signalling pathways are

transduced by the direct involvement with BGN (Ameye et al., 2002; Moreno et al., 2005; T. Xu et al., 1998).

There is a growing body of evidence that inflammatory signal transduction may be conveyed by ECM molecules (He et al., 2004). In addition, previous studies have reported that expression of BGN in renal inflammation is increased, and infiltrating BGN-expressing macrophages have been observed (Schaefer et al., 2002). Previous data indicates that the regulation of cytokine activity is associated with BGN capacity, which may involve the binding of TGF-β and TNF-α (Tufvesson & Westergren-Thorsson, 2002). Growth and differentiation requirements for monocytic-lineage cells seem to be enhanced by BGN (Kikuchi et al., 2000).

1.14.1 The ECM composition from an immunological perspective

To date, little consideration has been given to the influence of host ECM on major cellular processes such as the immune response. Moreover, pathogens interact directly with substrates in the individual components of the ECM or degrade, facilitating adhesion, leading to pathogen infiltration of the host, as shown by a growing body of evidence starting over 40 years ago (Chagnot, Listrat, Astruc, & Desvaux, 2012; Kuusela, 1978). Recently, it has been accepted that specific signals can be conveyed to cells through the ECM, thereby playing a key role in regulation of essential immune responses, immune cell migration into and amongst infected tissues, activation of immune cells and proliferation and polarization. In the infected host, significant changes in the ECM composition are induced and associated with specific immune cell types.

Most immune responses to infections are tissue specific. There are around 300 different proteins in each tissue that contain distinct ECM signatures arising from the complex and dynamic combination of such proteins in varying concentrations and geometries within the three-dimensional extracellular shape. These proteins can be promoted in association with matrices (the matrisome) that comprise of approximately 43 collagen subunits, 36 proteoglycans and 200 glycoproteins (Hynes & Naba, 2012). Amplification of ECM molecular diversity and complexity is regulated by post-translational modifications, which in turn, unveils cryptic epitopes or regulates new ones to trigger immune responses and alternative splicing, which then plays a key role in generating various isoforms, each with different

functions (Boyd, Pierce, Schwarzbauer, Doege, & Sandell, 1993; Leeming et al., 2011). For example, during leucocyte recruitment, migration to inflammation spaces occurs as a result of the contribution of laminin α4 and α5 isoforms within the basement membrane with immune cell type selectivity (Kenne et al., 2010).

Basic forms of ECM are divided into interstitial ECMs and the loose fibril-like matrices that fill the tissue stroma, basement membranes, and laminar sheets, which support cell layers on underlying tissues. Additionally, studies have revealed that in secondary lymphoid organs, the combination of the properties of these two ECM structures occurs as specialised reticular fibre networks (Lokmic et al., 2008; Sixt et al., 2005).

The one feature of ECM is that it is highly dynamic yet acts as a strict regulator for tissue constitution. Normal function and components of ECM are determined and maintained through the action of a fine equilibrium between ECM synthesis controlled by cytokines, including transforming growth factor-β (TGF-β), and turnover accomplished by matrix metalloproteinases (MMPs); a disintegrin and metalloproteinases (MMPs); and a disintegrin and metalloproteinases with thrombospondin motifs (ADAMTSs), the function of which is modulated by tissue inhibitors of metalloproteinases (TIMPs) (P. Lu et al., 2011). Monocytes/macrophages in activated profiles as immune cells produce these enzymes during injury and infection to promote immune cell migration into sites of infection and tissue damage, suppressing the capability to boost inflammatory responses (Chou, Chan, & Werb, 2016).

The key dogma, described in matrix biology, was that structural support for the cell was provided by the ECM and associated with the unique tissue structure. There are several mechanisms involved in communication between the ECM and cells. One such mechanism is signalling via adhesion molecules on the cell surface and receptors, such as integrins and discoidin-domain receptors (Campbell & Humphries, 2011; Leitinger, 2011). Others consist of binding, storage, activation and release of secreted molecules with strong immunomodulatory function, such as cytokines, chemokines and growth factors (Doyle, Gerber, & Dietz, 2012). For example, the impact of TGF-β on activation, proliferation and differentiation of most immune cell types has been determined both *in vitro* and *in vivo* (Travis & Sheppard, 2014). Furthermore, tissue digestion can derive bioactive ECM fragments

such as hyaluronan and heparan sulphate oligosaccharides and ECM molecules, the expression of which is precisely produced upon injury e.g., fibronectin isoforms including extra domain A, versican and biglycan that stimulate inflammation leading to pro-inflammatory gene expression or display features of a chemoattractant (G. Y. Chen & Nunez, 2010; Piccinini & Midwood, 2010). A class of endogenous damage-associated molecules patterns are formed from these molecules, which in turn, activate pattern recognition receptors (PRRs), including TLRs, in immune cells (macrophages and dendritic cells) as well as in non-immune cells (fibroblasts and epithelial cells). This process occurs as the immune system responds to damage and infection and acts to eliminate pathogens and repair tissue (S. Gordon, 2002). Hence, there is synaptic intertwining of the ECM and the immune system underlying the induction of signals from the ECM to regulate immune responses. Consequently, immune cells trigger ECM repair and regeneration, producing cytokines, including tumour necrosis factor (TNF), IFN-γ and TGF-β. These modulate the release of various ECM molecules.

1.14.2 ECM-pathogens crosslinking and penetration of barriers to attack the host

The interaction between specific ECM and microbial pathogens has been reported (B. Singh, Fleury, Jalalvand, & Riesbeck, 2012). In 1978, Kuusela et al. published the first report of a pathogen binding to host ECM via a *Staphylococcus*-fibronectin interaction. Years later, the N-terminus and C-terminus were identified as two different binding regions on fibronectin (Kuusela, Vartio, Vuento, & Myhre, 1984). Characterization of the interaction between this ubiquitous and promiscuous ECM glycoprotein and *Staphylococcus aureus* and *Streptococcus pyogenes* was reviewed by other groups and was found to be associated with the binding of a remarkably large number of fibronectins to adhesins (Kreikemeyer, Klenk, & Podbielski, 2004). Furthermore, ECM is comprised of several proteins that play a crucial role in the specific binding of pathogenic bacteria, such as laminin, collagen, heparan and chondroitin sulphate, vitronectin, thrombospondin, elastin, bone sialoprotein and tenascin-C (Ljungh, Moran, & Wadstrom, 1996) (Table 1.1).

ECM molecule	Pathogen	Adhesin	Effect of interaction	Disease	References
Fibronectin	Enterohaemorrhagic *Escherichia coli* O157: H7	Lpf fimbriae (LpfA1 major subunit)	Colonization of GI tract	Acute diarrhoea; bloody diarrhoea; haemolytic uraemic syndrome	130
	Streptococcus pyogenes (group A streptococci, GAS)	Protein F1 (functional upstream domain)	Fibronectin links F1 to integrin receptors, helping bacterial uptake	Tonsillopharyngitis; necrotizing fasciitis; myositis; streptococcal toxic shock syndrome	131–133
		Protein F2 (C-terminal domains)	Host cell adhesion and internalization		33
		SfbII (C-terminal domain)	Host cell adhesion and internalization		134
		Fba	Host cell adhesion and internalization		31*
	Staphylococcus aureus	MntC	Mucosal colonization	Nosocomial infections, septicaemia, osteomyelitis, endocarditis, etc.	135
	Salmonella typhimurium	MisL (N-terminal non-conserved region)	Intestinal colonization	Gastroenteritis	136*
		ShdA			137
Plasma fibronectin	*Borrelia burgdorferi*	BBK32	Binding to fibronectin 13FnIII repeat module and intestinal colonization	Lyme disease	35
Fibronectin N-terminal proteolytic fragments (30 and 70 kDa)	*Streptococcus pyogenes*	Protein F2(C-terminal domains)	Fibronectin polymerization and colonization of vascular surfaces	Tonsillopharyngitis; necrotizing fasciitis; myositis; streptococcal toxic shock syndrome	33 130
Laminin	Enterohaemorrhagic*Escherichia coli* O157: H7	Lpf fimbriae (LpfA1 major subunit)	Eukaryotic cell adhesion and internalization	Acute diarrhoea; bloody diarrhoea; haemolytic uraemic syndrome	130
	Enterohaemorrhagic*Escherichia coli* O157: H7	Lpf fimbriae (LpfA1 major subunit)	Colonization of GI tract	Acute diarrhoea; bloody diarrhoea; haemolytic uraemic syndrome	135
	Staphylococcus aureus	MntC	Colonization of GI tract	Nosocomial infections, septicaemia, osteomyelitis, endocarditis, etc.	138
	Streptococcus gallolyticus (*gallolyticus* endocarditis isolates)	FimB, gtf and pilB	Mucosal colonization	Infective endocarditis	139
Collagen I	*Borrelia burgdorferi*	ErpX	Adhesion and invasion of endothelial cells	Lyme disease	138
	Streptococcus gallolyticus (*gallolyticus* endocarditis isolates)	FimB, gtf and pilB	Long-term host tissue colonization	Infective endocarditis	137
	Salmonella typhimurium	ShdA	Adhesion and invasion of endothelial cells	Gastroenteritis	140*

ECM molecule	Pathogen	Adhesin	Effect of interaction	Disease	References
Collagen II	*Enterococcus faecium*	Pilus subunits EmpA and EmpB	Intestinal colonization and persistence	UTIs, bacteraemia, and infective endocarditis	138
Collagen IV	*Streptococcus gallolyticus* (*gallolyticus* endocarditis isolates)	FimB, gtf and pilB	Adherence to host tissue and biofilm formation	Infective endocarditis	130
	Enterohaemorrhagic*Escherichia coli* O157: H7	Lpf fimbriae (LpfA1 major subunit)	Adhesion and invasion of endothelial cells	Acute diarrhoea; bloody diarrhoea; haemolytic uraemic syndrome	135
	Staphylococcus aureus	MntC			138
	Streptococcus gallolyticus (*gallolyticus* endocarditis isolates)	FimB, gtf and pilB	Colonization of GI tract	Nosocomial infections, septicaemia, osteomyelitis, endocarditis, etc.	136*
Collagen V	*Salmonella typhimurium*	MisL (N-terminal non-conserved region)	Mucosal colonization	Infective endocarditis	141
Collagen VI	*Enterococcus faecium*	EcbA	Adhesion and invasion of endothelial cells	Gastroenteritis	142*
Tenascin-C	*Legionella pneumophila*	Mip	Intestinal colonization	UTIs, bacteraemia, and infective endocarditis	138
Vitronectin	*Streptococcus gallolyticus* (*gallolyticus* endocarditis isolates)	FimB, gtf and pilB	Host tissue adhesion and biofilm formation	Legionellosis	138
	Streptococcus gallolyticus (*gallolyticus* endocarditis isolates)	FimB, gtf and pilB	Adhesion to lung tissue and bacterial dissemination	Infective endocarditis	143*
	Yersinia enterocolitica	YadA	Adhesion and invasion of endothelial cells	Infective endocarditis	46
Thrombospondin	*Trypanosoma cruzi*	TcCRT	Adhesion and invasion of endothelial cells	Enteric and systemic diseases	144*
Decorin	*Borrelia burgdorferi*	DbpA and DbpB	Adhesion to host cells and tissue; improved bacterial survival	Chagas disease	141
Nidogen 1 and 2	*Enterococcus faecium*	SgrA	Enhancement of cellular infection	Lyme disease	145,146
Soluble and immobilized fibrinogen (α- and β-chains)	*Staphylococcus aureus*	ClfA, ClfB	Specific localization to decorin-rich niches in the tunica adventitia and myocardial connective tissue; persistence of infection	UTIs, bacteraemia, and infective endocarditis	
			Host tissue adhesion and biofilm formation	Nosocomial infections, septicaemia, osteomyelitis, endocarditis, etc.	
			Colonization of biomaterial implants; bacterial spread		

Table 1.1 Examples of specific host ECM–pathogen interactions. Examples of specific host ECM–pathogen interactions that facilitate microbial adhesion to tissues and pathogenesis (animal models of infection were used in the study are indicated) (Henke, Nandigama, & Ergun, 2019).

The infected host, caused by the invasion of colonised pathogens to tissues, is a crucial mechanism to fight primary and/or secondary host infections, the crossing of interstitial matrices and basement membranes. Modification of the ECM occurs by the development of several distinct ways (Pizarro-Cerda & Cossart, 2006; Steukers, Glorieux, Vandekerckhove, Favoreel, & Nauwynck, 2012). Hyaluronidase and collagenases can directly degrade ECM components. This involves the action of invasins or bacterial tissue-degrading enzymes followed by damage to local tissue. The growing body of evidence reviewing these interactions is presented in table 1.2 (Steukers et al., 2012).

ECM molecule	Pathogen	Microbial enzyme	Effect of ECM cleavage	Disease	References
Laminin	*Psedomonas aeruginosa*	Elastase; alkaline protease	Tissue invasion and necrosis	Necrotizing pneumonia, septic shock, UTI, skin and soft-tissue infections	147#
	Clostridium difficile	Cwp84	Tissue integrity loss; facilitation of toxin diffusion	Pseudomembranous colitis and nosocomial diarrhoea	148
Collagen I	*Porphyromonas gingivalis*	Gingipains in *P. gingivalis* supernatant	Tissue degradation	Periodontal disease	149
	Vibrio parahaemolyticus	Metalloprotease VppC	Tissue damage	Acute gastroenteritis	150
Collagen I, II, III, IV, V and VI	*Clostridium histolyticum*	Class I and II collagenases (ColG, ColH)	Necrotic tissue degradation; promote keratinocyte migration	Gas gangrene, infective endocarditis	151,152
Collagen IV	*Streptococcus gordonii*	Serine protease	Basement membrane breakdown	Infective endocarditis	153
Fibronectin	*Clostridium difficile*	Cwp84	Tissue integrity loss; facilitation of toxin diffusion	Pseudomembranous colitis and nosocomial diarrhoea	148
	Porphyromonas gingivalis	Gingipains in *P. gingivalis* supernatant; HRgpA and RgpB gingipains	Cleavage and inactivation of cell-binding region of fibronectin; gingival fibroblast detachment and death; tissue destruction	Periodontal disease	149,154#
Vitronectin	*Clostridium difficile*	Cwp84	Tissue integrity loss; facilitation of toxin diffusion	Pseudomembranous colitis and nosocomial diarrhoea	148
Tenascin-C (large isoforms)	*Porphyromonas gingivalis*	HRgpA, RgpB and Kgp gingipains	Enhanced anti-adhesive activity of tenascin-C; gingival fibroblasts detachment, apoptosis and tissue destruction	Periodontal disease	154#

Cwp84, putative cell surface-associated cysteine protease; HRgpA and RgpB, arginine-gingipains; Kgp, lysine-gingipains; UTI, urinary tract infection.

Table 1.2 Examples of specific ECM–pathogen interactions. Examples of specific ECM–pathogen interactions that facilitate host invasion through direct degradation of ECM components (ex vivo mammalian tissue degradation models were used in the study) (Henke et al., 2019).

Induction of ECM degradation not only facilitates the spread of pathogens, but is also involved in tissue necrosis, diffusion of bacterial toxins, host cell adhesion, migration, and viability. Host proteolytic systems can be hijacked or misused by pathogens. For instance, pathogenic activity derived from common bacteria, *Staphylococcus aureus*, *Haemophilus influenza* and *Pseudomonas aeruginosa,* manipulates the plasminogen-plasmin system to degrade laminin and fibronectin and degradation of all types of (ECM) proteins can be induced by activating MMP zymogens. Additionally, this process can regulate many biologically active molecules, such as receptors on the cell surface and cytokines, which regulate behaviour and host defence (Tomlin & Piccinini, 2018). Furthermore, secretion of hyaluronidases by group B streptococci and other gram-positive bacteria is a key factor in evading immune responses in tissue invasion. Specifically, pro-inflammatory hyaluronan fragments degrade into disaccharides though a process of secretion of hyaluronidases, which inhibit TLR2/4 signalling activated by host-derived hyaluronan fragments and pathogenic ligands, such as lipopolysaccharide (LPS) and in turn leads to evasion of immune surveillance (Kolar et al., 2015).

1.14.3 ECM mediated recognition of macrophage activation

PRRs are an important recognition site in the innate immune system that employ highly conserved receptors that bind conserved motifs in pathogenic bacteria, namely pathogen-associated molecular patterns. Secreted ECM proteins, including the mindin-F-spondin family comprising various members such as mindin, are unique molecules who play a role in microbial pathogen pattern recognition within the ECM fand may also play a crucial function embedded in the innate immune response (He et al., 2004). He et al. (He et al., 2004) evaluated *in vivo* resistance to LPS-induced shock and systemic *Salmonella typhimurium* and *Streptococcus pneumonia* infections in the genetic depletion of mindin. Clearance of bacterial pathogens in the lungs with gram-positive group B Streptococcus or *Haemophilus influenza* was impaired and emerged in the mindin-null mice feature. Suppression of TNF-α and IL-6 secretion by macrophages and mast cells due to the deficiency of mindin led to defective phagocytosis *in vitro* (Jia, Li, & He, 2008; Tomlin & Piccinini, 2018). Tandem repeats of leucine-rich motifs are characteristic of the proteoglycan lumican, which is similar to PRRs. It plays a role in the interaction with CD14 expressed by macrophages and neutrophils, increasing

CD14-TLR4-mediated responses to LPS (Figure 1.10). Hence, septic shock was induced in lumican-null mice that were hyporesponsive to LPS (F. Wu et al., 2007). Failure to clear of bacteria from lung tissues of lumican-deficient mice infected with *Pseudomonas* aeruginosa was noted, along with increased mortality rate. This study reported that macrophages of CD14-mediated phagocytosis through the action of *Escherichia coli* and *Pseudomonas aeruginosa* bacteria are impaired in the absence of lumican and Tyr-20 is a crucial residue for CD14 interaction and phagocytosis (Shao et al., 2012). Galectin-3 is another member of ECM proteins, whose function is sensing several microbial pathogens, binding carbohydrate structures on glycoproteins and glycolipids including *N*-acetyl-D-lactosamine and LPS produced from mycobacteria, protozoan parasites, and yeast (Sano et al., 2003).

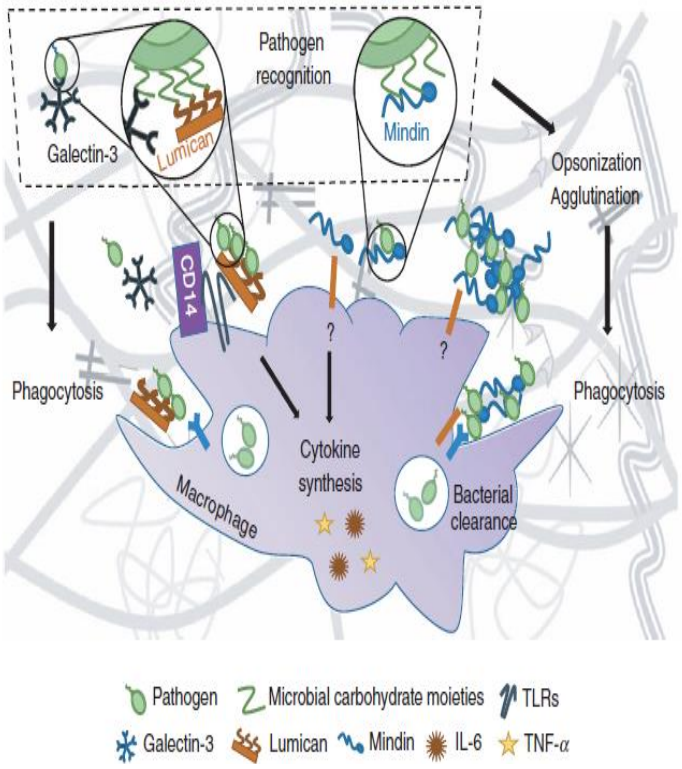

Figure 1.10 Schematic depiction of mindin, lumican and galectin-3 signals. These extracellular sentinels recognise and interact with moieties on the cell surface of various types of pathogens, promoting the phagocytosis of bacteria by macrophages. Opsonisation and agglutination are induced by binding of mindin to bacteria, facilitating their phagocytosis by macrophages. Induction of the synthesis of pro-inflammatory cytokine is promoted in the presence of mindin, whereas lumican binds to the surface of macrophages via CD14, promoting both CD14-TLR4-mediated responses to LPS and CD14-mediated phagocytosis (Tomlin & Piccinini, 2018).

1.14.4 The extracellular matrix in the tumour microenvironment and role of TAF

It is essential to closely investigate TAFs and their ECM as the main component in tumours (Bagordakis et al., 2016). All solid tumours include TAFs. Normal connective tissue comprises of quiescent, metabolically inactive fibroblasts (Puram et al., 2017). This type of tumour can often strongly drive the source of TAFs. The original TAFs were derived from stellate cells, bone-marrow-derived mesenchymal stem cells, and mesenchymal stem cells (Borriello et al., 2017; Ohlund et al., 2017). Not surprisingly, TAFs derive from various origins and their heterogeneous cell population can strongly differ in morphology, cell–cell interaction, and expression profile. However, they have characteristics in common, such as synthetic activation, migration, and invasion, as well as promotion of proliferation and the immune response (Kalluri, 2016b).

TAFs are associated with chemoresistance, as suppression of caspase-3 activation induces TAFs to produce the cytokine PA1-1, resulting in apoptotic tumour following chemotherapeutic stress. TAFs promote the production of IL-6 to stimulate tumour cells to express resistance-mediating CXCR7 (Che et al., 2018; Y. Qiao et al., 2018). Expression of TGF-β is induced by TAFs, especially under hypoxic conditions, which differentiates stem cells into tumour-like cells, promoting resistance to chemotherapy (Y. A. Tang et al., 2018). Finally, the ECM composition of TAF plays a key role in the therapeutic response, forming a barrier to shield and promote protective signalling via the interaction with integrins and cadherins (Jakubzig, Baltes, Henze, Schlesinger, & Bendas, 2018; Naik et al., 2018).

1.15 Periostin function in tumour growth

Periostin (POSTN), also known as OSF-2, is a member of the fasciclin family and is a disulphide-linked cell adhesion protein (S. Bao et al., 2004). Tumour development is associated with the dynamic interaction of cancer cells with stromal cells, as well as the ECM crosslinking the cells. A supportive microenvironment for the recruitment and progression of the primary tumour can be induced by ECM proteins that are produced by stromal cells and tumour cells, which then can initiate the metastatic dissemination of tumour to distant secondary locations. Periostin is a crucial constitutional factor and a secreted ECM protein and is not only

associated with inflammation, but it also acts as an active contributor to tumorigenesis and metastasis. Periostin protein is significantly increased in several types of solid tumours, such as colon, breast, head and neck, pancreatic, lung, papillary thyroid, ovarian, gastric and liver carcinoma, as well as in neuroblastoma (Conway et al., 2014; Gillan et al., 2002; Ruan, Bao, & Ouyang, 2009).

Periostin in the colon and ovary exerts pro-metastatic effects through the action of α vβ3 and/or α vβ5 receptors by promoting cell motility and survival *in vitro* and *in vivo* (S. Bao et al., 2004; Tai, Dai, & Chen, 2005). Periostin is an adhesion molecule which facilitates invasion in the tumour microenvironment and is a novel tumour-invasive molecule in oesophageal cancer. EGFR signalling and mutant p53 play a critical role in the induction of periostin (Michaylira et al., 2010), which can induce excessive expression of ECM proteins, such as collagen 1, and fibronectin, in turn, promotes tumour progression in the presence of nutrient deprivation, hypoxia and chemotherapeutic pressure (Erkan et al., 2007). The proliferation of melanoma cells is accelerated by the stromal periostin produced from normal human dermal fibroblasts *in vitro* and in vivo. Indeed, secreted periostin may have the potential to serve as a key target for future diagnostic and therapeutic strategies to treat cancer (Kotobuki et al., 2014; A. Y. Liu, Zheng, & Ouyang, 2014).

Many tumorigenic processes are associated with POSTN regulation via signalling transduction, including protein kinase B/phosphoinositide-3 kinase, integrin, and Wnt-1 (Baril et al., 2007; Malanchi et al., 2011). Zhou et al. (J. Zeng et al., 2018) used clinical specimens and an animal model of glioblastoma to investigate the role of POSTN) and showed that it acted as a chemoattractant to recruit M2 TAMs and was produced by stem cells. A study previously revealed that CD44+ intrahepatic cholangiocarcinoma (ICC) stem cells highly express POSTN and the presence of CD206+ TAMs has been observed with secreted POSTN in ICC.

1.16 Immune inhibitory receptors localised within macrophages are associated with tumour growth

The immune system is regulated by several molecules, including immune inhibitory receptors, which play a crucial role in maintaining immune homeostasis via monitoring of immune

responses (Sharpe, Wherry, Ahmed, & Freeman, 2007). In cancer, expression of immune inhibitory receptors is often on tumour cells, which in turn suppress the anti-tumour immune responses as an adaptive escape mechanism (Pardoll, 2012; Ribas, 2015). Checkpoints among immune cells are divided into many types, such as programmed death 1 receptor (PD-1) expression. TILs interact with its ligand, programmed death ligand 1 (PD-L1), on tumour cancer cells. This has been demonstrated as a key immunosuppressive molecule in cancer (H. Dong et al., 2002; Freeman et al., 2000; Zou, Wolchok, & Chen, 2016).

Several clinical studies have found that PD-1/PD-L1 axis of antagonists can block this pathway signalling, providing durable clinical responses resulting in an unprecedented survival rate (Gettinger et al., 2015; Herbst et al., 2014). Mechanistic studies have identified the PD-L1 function of macrophages using mouse models and have showed that the immunotherapeutic strategy of the blockade of PD-L1 expression influences PD-L1 pathway signalling in host cells (H. Tang et al., 2018). However, few studies have investigated the expression of PD-L1 in human tumours. Furthermore, a study that evaluated the colocalization of PD-L1 in three immune cell subtypes of 457 non-small cell lung cancer (NSCLC) patients' tissue samples using confocal microscopy showed CD68+ macrophages expressed the majority of PD-L1 (Y. Liu et al., 2020).

Macrophages are not the only immune cells to express PD-L1. PD-L1 has been identified to be expressed by cytotoxic T cells and NK cells, but at a lower level than haematopoietic and non-haematopoietic cells, such as B cells, dendritic cells, Treg cells, etc. (Curiel et al., 2003; Dorfman, Brown, Shahsafaei, & Freeman, 2006; Francisco, Sage, & Sharpe, 2010). In addition, in cases of increased recurrence, the probability of TAMs macrophages in the early-stage increases. T cells are not affected by TAMs, suggesting that PD-L1 expression in TAMs in early-stage lung cancer does not suppress the function of effector T cells (Mony & Schuchert, 2018; Singhal et al., 2019).

It has been widely accepted that the use of immunotherapies to promote host anti-tumour cells is a key cancer treatment strategy. However, despite recent clinical trials successfully using checkpoint antagonists and adaptive transfer of T cells, induction of a durable anti-tumour response in solid tumours has often failed when these immunotherapies were utilised in patients with cancer (Garon et al., 2015; Ribas & Hu-Lieskovan, 2016). This poor response to immunotherapies suggests that a deeper understanding of the interactions between

tumour-specific T cells and other immune cells within the TME is required to improve the effectiveness of cancer immunotherapy.

Accumulation of monocyte/macrophage lineage cells (MMLCs) in several types of human and murine model tumours is understood to play a key role in the mechanisms of tumour progression, such as anti-tumour T cells functions (Galdiero et al., 2013; Sica et al., 2012).

The successful therapy of anti-PD-L1 in a subset of patients with lung cancer is associated with boosting TAMs functions (Herbst et al., 2014). It appears that PD-L1 is significantly expressed by tumour cells, in turn inhibiting T cell function, whereas the role of PD-L1 expression in MMLCs is unclear and remains controversial in the regulation of effector T cells in both humans and mouse models (Juneja et al., 2017; Lau et al., 2017). Interestingly, a study also suggested that PD-L1 expressed by TAM may maintain the efficiency of effector T cell activation and protect against TAM apoptosis through effector T cells (S. N. Mueller et al., 2006). In addition, a study found that the significance of the high expression of PD-L1 on the surface leads to prevention of TAMs expressing an MHC I from being targeted by anti-tumour effector T cells. This data suggests that the scenario of PD-L1 pathway signalling blocking as a therapy might be contrary to the results in cancer patients. Blocking of PD-L1 tumour cells would prevent its inhibitory role, consequently increasing the function of cytotoxic effector T cells to combat these tumour cells. However, professional APCs blocked by anti-PD-L1 antibodies might be associated with the elimination of pre-existing anti-tumour cytotoxic T cells, thereby leading to restoration of the function of existing anti-tumour effector cells in tumours (Ngambenjawong et al., 2017; B. Z. Qian et al., 2011).

Although mechanism of the blockade of the PD-1/PD-L1 axis to restore T cells is well understood, the role of this pathway signalling in TAMs is not clear. A poor prognosis in human cancers was associated with the presence of TAMs (Pollard, 2004). However, an investigation previously revealed that macrophages express PD-1 in the environment of pathogenic infection (Huang et al., 2009; L. Shen et al., 2016). A study using a colon cancer mouse cell line CT26 to determine the expression of PD-1 on TAMs showed that macrophages within the TME produced around 50% of the surface PD-1, whereas levels of PD-1 expressed by circulating monocytes or splenic macrophages were undetectable. In addition, CD68 TAMs were clearly the most abundant population of cells that expressed PD-1 (Mantovani, Sica, Allavena, Garlanda, & Locati, 2009). Environmental stimuli can often polarize TAMs towards

an inflammatory M1 or pro-tumour M2 state. Analysis of TAMs populations by flow cytometry found that almost all TAMs with an M2-like surface profile expressed PD-1, whereas PD-1$^-$ TAMs expressed an M1-like profile. PD-1 expression of TAMs was highly variable in human colorectal cancer. Notably, M2 population was observed to expressing significantly more PD-1 than the M1 population. Furthermore, the frequency of PD-1 TAMs correlated with the increasing disease stage, but only within the M2 phenotype (Dahan et al., 2015; Maute et al., 2015).

1.17 *In vitro* models of immune function

1.17.1 Conventional 2-dimensional culture systems

Many cell lines (e.g. epithelial, endothelial, fibroblastoid) grow as a monolayer on a plastic substrate in a culture flask and form conventional, adherent 2D cultures by attaching to the planar surface (Breslin & O'Driscoll, 2013). Such 2D model systems have the advantage that they provide a highly-reproducible, technically simple,and relatively inexpensive cell culture system for basic research or drug screening. However, there are some limitations associated with 2D culture models, which at best over-simplify the physiological situation (Figure 1.11). In this system, paracrine interactions of the cell–cell extracellular environment do mimic biological sites in the tumour mass. Cell differentiation, proliferation, vitality, gene and protein expressions, responsiveness to stimulation, drug metabolism and other cellular functions are regulated through these types of cell–cell interactions (Bissell, Rizki, & Mian, 2003; F. Pampaloni, E. G. Reynaud, & E. H. K. Stelzer, 2007b). Cells that are isolated and cultured into a 2D condition been observed to have altered morphology when compared to the mode of cell division. This can affect the function of cells, cause the inability to organization the particular organelle inside the cell and distrupt cell signals and production (Debnath & Brugge, 2005; Nelson & Bissell, 2006). In addition, cell culture in 2D conditions can result in a decrease in the diverse phenotype (Kilian, Bugarija, Lahn, & Mrksich, 2010b; Mahmud et al., 2009; Petersen, Ronnovjessen, Howlett, & Bissell, 1992). The instability of the external environment within cell–cell interactions in 2D conditions and the effect of growing adherently results in a loss of polarity (Mseka, Bamburg, & Cramer, 2007), leading to various phenomena that change the responses of these cells leading to apoptotic cells (Weaver et al., 2002). Another stumbling block of working with 2D cultures is that the ingredients of the

medium, including oxygen, nutrients and the metabolites, means that the signalling pathway is not able to penetrate properly into the cells in the monolayer formation (Birgersdotter, Sandberg, & Ernberg, 2005; F. Pampaloni, E. G. Reynaud, & E. H. Stelzer, 2007a). Cancer cells *in vivo* have variable access to the ingredients such as nutrients, oxygen etc. due to the natural structure of the mass of the tumour. Furthermore, the gene expression and splicing, topology and biochemistry inside the cells are altered by using and growing cells in a 2D culture (Fuchs, Tumbar, & Guasch, 2004; C. Li et al., 2006). In 2D conditions, cells are growing adherently as monocultures that allow only one type of cells to be cultured, resulting in a lack of a TME that would be found *in vivo* for tumour-initiating cells (Fischbach et al., 2007; Gilbert et al., 2010).

Owing to the fact that many limitations have been observed in 2D cultures, it is important to find alternative ways to better represent the physiological architectures of a tumour mass for example, by using multi-cellular and /or 3D cultures.

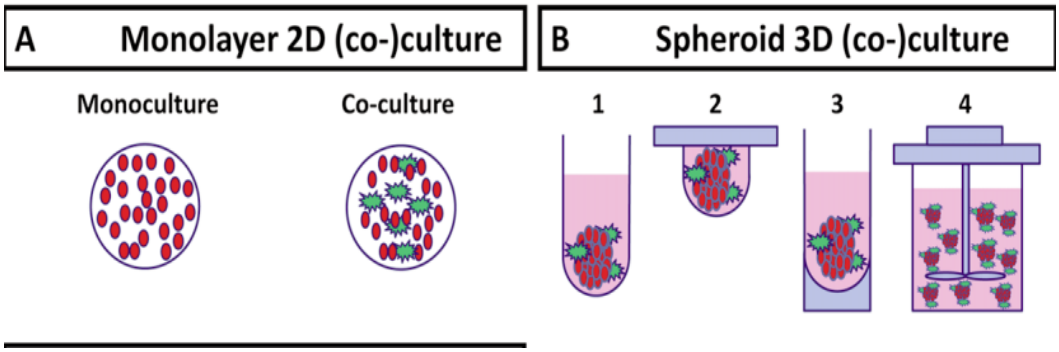

Figure 1.11 Schematic illustration of planar 2D model and 3D cell culture systems to illustrate key differences in the spatial relationship of two different cell types (red and green) . (A) Schematic representation of the monoculture and culture approach in 2D. (B) Schematic representation of the experimental approach. Tumour cells were inoculated as single cells in stirred-tank vessels to increase cell aggregation. After 3 days, tumour spheroids and single cells, such as TAF and cell line FBs or PBM, were mixed and encapsulated in alginate, in turn microcapsules circle the three cellular components.

1.17.2 Alternative 3-dimensional culture systems

In response to the percieved limitations of conventional planar-culture systems, a number of different approaches have been explored. In the 1970s, a soft agar solution was used to grow cell cultures in one of the first 3D model systems (Hamburger & Salmon, 1977). Since then, cells grown in 3D culture have been found to have striking similarities in terms of morphology and behaviour compared with those found in a tumour mass (Mazzoleni, Di Lorenzo, & Steimberg, 2009; Pampaloni et al., 2007a). Advanced cell culture systems employ a number of major approaches including: i) using non-adherent plates to grow suspension cultures that often form clusters of cells; ii) using a concentrated medium or gel-like substance in cultures; and iii) growing cells on a scaffold.

The notion of 3D models is relied on the formation of spheroid structures in which cells form several layers. This culture condition has been developed a 3D model that mimics the dynamic interaction between tumour, immune and stromal compartments, combined with deposition of ECM and secreted factors. Several tumour cell lines have been cultured in 3D conditions and subjected to morphological analysis, including glioblastoma, astrocytoma, Wilms' tumour, neuroblastoma, head and neck squamous cell carcinoma, melanoma, lung, breast, colon, prostate, ovarian, hepatocellular and pancreatic cancer. The shapes and architecture of spheroid cultures can be divided into three distinct groups: i) tight spheroids, ii) compact aggregates, and iii) loose aggregates (Vinci, Box, & Eccles, 2015; Vinci et al., 2012). Importantly, multicellular, 3D cultures derived from the donor's tissues bear striking similarities to the architecture of the originating tissue and is a more accurate representation than is gained by using conventional cultures (Griffith & Swartz, 2006). This architecture of 3D conditions allows representation of cell–cell and cell–medium interactions that can imitate the natural structure of parental tissues, and can also provide the proper stimulation from the surronding environment that mimics the *in vivo* setting (Cawkill & Eaglestone, 2007; J. Lee, Cuddihy, & Kotov, 2008b).

The morphology and polarity of cells can be maintained in 3D culture conditions (Benya & Shaffer, 1982; Van Kirk, Cornell, & Jacoby, 1979; Yamada & Cukierman, 2007). Initiation of lumen formation in specific internal architecture occurred in some 3D conditions, such as acinar-like spheroids. As a result of induction, apoptotic cells in central parts of the spheroids was increased. Cell location plays a role in cell proliferation, with the periphery of spheroids

showing higher rates of proliferation (Pradhan-Bhatt et al., 2014; Pradhan-Bhatt et al., 2013; Pradhan et al., 2010). The natural structure of cell growth *in vivo,* such as metabolism, cellular topology, gene expression and signalling are similar to the attributes of 3D culture (Berthiaume, Moghe, Toner, & Yarmush, 1996; Ghosh et al., 2005; Marushima et al., 2011).

1.17.3 Comparison between 3D and 2D cell culture system

Type of culture	2D	3D
Time of culture formation	Intiation from minutes to a few hours	Within hours to a few days (S. F. Chen et al., 2012; Pampaloni et al., 2007a)
Culture quality	High conduct, repeatability, culture takes a while, easy to read, straightforwardness of culture	Worsening carry out and reproducibility, not easy to explain, culture more difficult to perform (Hickman et al., 2014)
In vivo mimic	Do not represent the biological stiuation of tumour mass	3D models mimic *in vivo* tissues and organs (Griffith & Swartz, 2006)
Cell interaction	There is no *in vivo*-like microenvironment and no niches due to deprivation of the cell–cell and cell–extracellular environment interactions	Provides appropriate conditions for cell–cell and cell–extracellular environment interactions, natural structure sof the tissue or tumour mass are created (J. Lee, Cuddihy, & Kotov, 2008a)
Charcteristics of cells	Morphology and way of divisions altered; diversity of phenotype and polarity is lost	Morphology and way of divisions are maintained; diversity of phenotype and polarity is observed (Kilian, Bugarija, Lahn, & Mrksich, 2010a)

Access to essential compounds	Oxygen, nutrients, metabolites and signalling molecules are availble with nolimitof access (contrary to *in vivo* conditions)	Access to oxygen, nutrients, metabolites and signalling molecules is variable (similar to *in vivo* conditions) (Pampaloni et al., 2007a)
Molecular mechanisms	Cells are changed with regards tro gene expression, mRNA splicing, topology and biochemistry	Cells are similar to those *in vivo* in terms of gene expression, splicing, topology and biochemistry (Birgersdotter et al., 2005)
Cost of maintaining a culture	Tests and the media are cheap; commercial test are readily available	Expensive, more time consuming, fewer commercially available tests (Sodunke et al., 2007)

Table 1.3 Comparison of 3D cultures and conventional 2D systems. This comparison illustrates cell culture formation time, ease of performance, representation of the biological situation *in vivo*, interaction between cells, loss of diversity phenotype, hypoxia, changes in gene expression and experimental expenditure.

The establishment of non-planar culture systems for solid tumours allows more complex systems to be devised and to include their population with immune components. In previous work, one study has established a 3D cell model (known as 3D-3-culture), where the compartment contains three enclosing cells; NSCLC cells form tumour spheroids, TAFs and monocytes. This study utilizes an alginate microencapsulation strategy model, which facilitates direct cell–cell and cell types contact, and allows monitoring and functional assessment, which is compatible for long-term culture using stirred systems (Estrada et al., 2016; Santo et al., 2016). Recently, this study has demonstrated that in the 3D model, human monocytes cultured with tumour tissue recruited their polarization into a M2-like phenotype in the absence of directed differentiation, such as exogenous cytokines. In turn reprograming the TAM phenotype *in vitro* (Rebelo et al., 2018).

In the 3D3-culture system, it was found that a proliferation of NCI-H157 tumour cells was a maintaining state and expressed phenotypic markers typical of aggressive stages of NSCLC,

including N-cadherin and vimentin and presented low E-cadherin expression. This indicates the pattern of typically late-stage epithelial-to-mesenchymal transition which is compatible with the previous immunohistochemical analysis of human tissue samples from primary tumours of lung squamous cell carcinoma and is relevant to late stage tumours (Prudkin et al., 2009). The proliferation and histological profiles of tumour cells showed no major differences upon interaction with TAF and myeloid cells, but growing evidence shows that remodelling of the different cell components can be observed within the alginate microcapsules through the culture (X. Q. Liu, Kiefl, Roskopf, Tian, & Huber, 2016).

Early reports suggest that tumour-immune interaction research can use spheroids as a suitable model and this is important for tumour architecture when developing the immunosuppressive TME in human tumours (Feder-Mengus et al., 2007). In the 3D-3-culture, detection of CD68 and CD163+ was expressed on macrophages polarized into M2-like phenotype (Lavin et al., 2017). Moreover, amongst tumour spheroids a high level of CD163+ cells were detected, indicating that the established model was favourable to cell migration, as is induced in infiltrating myeloid human lung cancers. Tumour-immune and TAF-immune co-culture controls expression of CD163+ cells, albeit at a lower level. Activation of monocytes within the TME was recruited in correlation with stromal compartments (Silzle et al., 2003a). An activated fibroblast phenotype is characterised as TAF. The secretory profile and ECM production were altered (Comito et al., 2014b). In fact, blood monocytes in a co-culture with breast cancer TAF spheroids found the level of monocyte migration was higher than in a tumour spheroid, demonstrating the association with overexpression of CCL2 phenotype (Ksiazkiewicz et al., 2010).

A cocktail of soluble factors and the cell contacts both play a crucial role in regulating the phenotype of macrophages in the 3D-3 culture system, since directed differentiation, in addition to cytokines, can promote the polarization of macrophages. In a previous work, it was found that the accumulation of soluble factors, including IL-6, IL-8, CXCL1 and Serpin, were induced in alginate capsules (Estrada et al., 2016). Therefore, the polarization of an M2-like phenotype was indicated through promotion of IL-4, IL-13, IL-10 and CXCL1 accumulation (Mantovani, Marchesi, Malesci, Laghi, & Allavena, 2017). Furthermore, during week three of the 3D-3-cultures, a distinct accumulation of CCL22 and CCL24 was detected. TAM is prominent for the production of both cytokines, whose functions are involved in the

recruitment and differentiation of regulatory T cells with TME; in turn they offer a poor prognosis and regulate suppression of a specific anti-tumour immune response (Noy & Pollard, 2014; Rebelo et al., 2018).

1.18 Hypothesis and aims

"Tumours govern adaptive immunity by modulating the behaviour of local fibroblasts that in turn impact on the function of antigen presenting cells." Much of the work has been done with cells in isolation and in a 2D setting. Consequently, this would lead into a section on the need for 3D models and co-culture models.

The project will address changes in the basic biology of the APC, and how these impact on their ability to communicate with key effector cells, including CD4+ and CD8+ T cells. We will establish co-cultures of tumour-conditioned fibroblasts and compare these with matched TAF/NAF to investigate their effect on the immunology of APCs, namely Mϕ and DCs. Initial studies will be limited to laboratory generated Mϕ and DCs (differentiated from CD14+ monocytes). There is reason to believe that tumour cells differentially condition FB in 2D vs 3D cultures in terms of their capacity to support cancer cells. However, to date there are no studies examining how this affects APCs. Therefore, the effect of these will be studied in 3D co-cultures and compared with conventional planar culture systems.

Chapter 2 : Materials and Methods

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2.1 Venous blood

Studies were undertaken with ethical approvals granted by The University of Nottingham Research Ethics Committee; reference: 10/H0405/6, BT20052010 and 09/H0408/75. Blood was collected from consenting healthy volunteers by venesection into sodium heparin solution. Donors with significant medical problems, currently on immunosuppressive medication or with anaemia were asked not to volunteer, although no specific screenings were performed or any data recorded.

2.2 Isolation of CD14+ monocytes

Peripheral blood mononuclear cells (PBMC) were separated from the whole blood using density centrifugation over Histopaque 1077 (Sigma, UK). Briefly, anti-coagulated blood (35mL) was layered onto Histopaque (15mL) in 50mL, contained in conical-based polypropylene tubes at room temperature. The gradient was subject to centrifugation (800g for 25 minutes). Heparinised whole blood was diluted with PBS as needed, layered without mixing over Histopaque-1077 (Sigma) and centrifuged at 800g for 25 minutes at room temperature with minimal deceleration. Peripheral blood mononuclear cells were collected from the plasma/Histopaque-1077 interface, washed three times with PBS to remove platelets, and washed once in cold MACS buffer (PBS + 1% FBS, 2mL 0.5M EDTA pH-8.0) before proceeding to magnetic separation. The purity of isolated cell subsets was routinely confirmed >95% by flow cytometry.

2.3 Generation of monocyte derived-macrophage (moMϕ)

CD14+ Mo were cultured in RPMI1640 medium containing 10% FBS and 1% sodium pyruvate in low adherence, 6-well plates (Corning Costar) at a concentration of $5x10^6$ cells per well per 5mL of medium for 5-7 days. For generation of GM-Mϕ, GM-CSF (Miltenyi Biotech) was added at a concentration 20U/mL while for generation of M-Mϕ, M-CSF (Miltenyi Biotech) was added at 10ng/mL. An additional complete medium with respective cytokines was added on

day 4. Immature Mϕ were collected by incubation on ice for 20 minutes in cold PBS and gentle pipetting, washed and plated in normal adherence cell culture plate (Nunclon) for experiments. A typical yield ranged between 25-40% of the initial cellular input. The cells were harvested on day 6 and plated in a tissue culture plate in Mϕ or M-CSF medium with GM-CSF for further experiments.

2.4 Generation of monocyte-derived dendritic cells (moDC)

CD14+ monocytes were isolated from peripheral blood mononuclear cells (PBMC) of healthy volunteers by positive selection with CD14+ microbeads (Miltenyi Biotech) and differentiated into MoDC with 1000U/mL IL-4 and 1000U/mL GM-CSF (Peprotech Ltd) at a concentration of 0.5-1x10^6 cells/mL in T75 flasks for 5-6 days. On day 4, the cells were fed with a half volume of the initial amount of the medium with cytokines. The cells were harvested on day 5 and plated in tissue culture plate in DCs medium with GM-CSF for further experiments.

2.5 Culture of primary tumour associated fibroblasts

The normal human dermal, together with surrounding and tumour FB cells were obtained from Iain Goulding and Jenny Gomm (Breast Cancer Now Tissue Bank, Queen Mary University). Frozen vials of FB were defrosted from liquid nitrogen stores by warming at 37^{o}C. As soon as the cell suspension was defrosted, it was transferred to a falcon tube and warmed FB medium (DMEM with 10% FBS with 1% P/S, 0.5µg/ml hydrocortisone, 10µg/ml apo-transferrin, 10ng/ml EGF, 5µg/ml insulin) was added dropwise whilst mixing the cell suspension. The cells were then centrifuged at 180xg for 5 minutes and resuspended in a fresh FB medium for culturing 0.15x10^6 cells in T25 or 0.5-1x10^6 in T75 flasks. Once the cell density reached the maximum as judged by microscopic examination, the cell suspension was aspirated and pelleted in a falcon tube. Then the cells were used up to 5^{th}-6^{th} passage.

2.5 Culture of BJ6, TIG and primary fibroblast cells

The human dermal FB cell lines BJ6 and TIG were obtained from Dr. Lloyd Hamilton (University of Nottingham). Primary dermal FB were kindly donated by Dr. Anja Saalbach (University of Leipzig). All cultures were tested free of mycoplasma infection. Frozen vials of FB were defrosted from liquid nitrogen stores by warming at 37^{o}C. As soon as the cell suspension was defrosted, it was transferred to a falcon tube and warmed FB medium (DMEM with 10% FBS with 1% L-glutamine or RPMI with10% FBS with 1% L-glutamine for TIG) was added dropwise whilst mixing the cell suspension. The cells were then centrifuged at 180xg for 5 minutes and resuspended in fresh FB medium for culturing in T25 or T75 flasks. Once the cell density reached the maximum as judged by microscopic examination, the cell suspension was aspirated and pelleted in a falcon tube. Then the cells were passaged at 1:3 or 1:5 for routine culture. BJ6 and TIG were used up to 12th passage, whereas primary fibroblasts were used up to 5th-6th passage.

2.6 Stimulation of Mϕ for phenotypic characterisation and secretory profile

Mϕ were counted, washed and seeded in 96-well normal adherence tissue culture plates in full Mϕ medium with GM-CSF or M-CSF at a concentration of 5x10^{4} cells/well in triplicates in 50µl Mϕ medium. Next Mϕ were rested for 1-2 hours in 37^{o}C and treated with respective drugs. Unless otherwise stated GM-Mϕ were activated with LPS (500 ng/mL and IFN-γ (1000 U/mL), while M-Mϕ with LPS (500ng/mL) only. 180µl of culture supernatants were collected after 24 hours and stored in -20 ^{o}C for analysis of cytokine secretion by ELISA.

2.7 Stimulation of MoDC for phenotypic characterisation and secretory profile

MoDC was plated in 96-well tissue culture plates in full Mϕ medium with GM-CSF at 8x10^{4} cells/well in triplicates in 80µl DCs medium. After resting for 1-2 hours (MoDC), cells were treated with LPS (500ng/mL) in a final volume of 200µl IFNγ (1000U/ml). After 24 hours, cells were microscopically confirmed to be free of microbial infection before 180µl of culture supernatant was collected and stored at -20^{o}C for subsequent cytokine secretion analysis by ELISA.

2.8 Production of spheroids

Cells grown as a monolayer were detached using trypsin/centrifuged, and the cell number was counted using a haemocytometer. Standard or Ultra Low Attachment (ULA) 96-well round-bottomed plates pre-coated with a hydrophilic polymer were used to allow for formation of one spheroid per well. Spheroids were seeded by diluting the single-cell suspensions in RPMI 1640 medium at a constant volume of 200ul per well. Replicates of 3–6 wells containing the same concentration were employed. The plates were centrifuged at 100g for 4 minutes in order to enhance the formation of a single spheroid and minimize cell death. Cells were incubated for 3 days. The growth of spheroids was monitored daily by taking pictures using a bright field microscope using 10x objective. The breast cancer cell line MCF7 was employed as a positive control for spheroid formation (personal communication Prof. A Grabowska).

The solid spheroids of the 3D system of culture were visualized by 10x fluorescence microscope.

2.9 Irradiation of cells

Co-cultures (APCs/FB) were irradiated in tissue culture plates immediately prior to Mϕ or DCs/Mϕ activation with LPS (500ng/ml, Sigma) and IFNγ (1000U/ml, R&D) or (500ng/ml, Sigma). Radiation (0-6Gy of 195kVp X-rays, 0.87Gy/min, 0.5mm Cu filter, 48.4 cm FSD) was delivered using a Gulmay Xstrahl cabinet irradiation facility within the department. Cell morphology was examined by phase-contrast microscopy (x10 or x40) after a further 24 hours of culture. 180µl of culture supernatants were collected and stored at -20^{o}C for subsequent cytokine secretion analysis by ELISA.

2.10 Cytokine ELISA

Supernatants were assayed by ELISA for human IL-12p70 (BD Bioscience, UK), IL-23 (Bioscience) and IL-10 (R&D Systems, UK) according to the manufacturer's instructions. Assays did not significantly cross-react with other proteins, and the sensitivities were 7.8 and 15pg/mL respectively. Samples were diluted between 1:5, 1:20 and 1:100 as needed and

assayed alongside duplicate serial dilutions of protein standard. Absorbance was measured at 450nm using a Tecan microplate reader.

2.11 Measurement of cell viability of live cells

Cells grown into 96-well round-bottomed plates pre-coated with a hydrophilic polymer were used to allow for formation of one spheroid per well. Spheroids were seeded by diluting the single-cell suspensions in RPMI 1640 medium at a constant volume of 200ul per well. The plates were centrifuged at 100g for 4 minutes in order to enhance the formation of a single spheroid and minimize cell death. Cells were incubated for 3, 5 and 7 days. The plates were incubated in a 5% vol/vol. CO2-humidified incubator at 37ºC. Cells were quantitated using a cell counting kit CCK-8 (Abcam, UK). Cell quantitation was performed according to the assay kit instructions. 100 µl of prepared CCK-8 is added to cell cultures in 96 well plates that have 200 µl of media. Cells are incubates for 4 hours. After incubation, the absorbance at 450 nm was determined using a Tecan microplate reader.

2.12 H3-thymidine-dependent proliferation

Cells grown into Ultra Low Attachment (ULA) 96-well round-bottomed plates pre-coated with a hydrophilic polymer were used to allow for formation of one spheroid per well. Spheroids were seeded by diluting the single-cell suspensions in RPMI 1640 medium at a constant volume of 200ul per well. Replicates of 3–6 wells containing the same concentration were employed. The plates were centrifuged at 100g for 4 minutes in order to enhance the formation of a single spheroid and minimize cell death. Cells were incubated for 3, 5 and 7 days. The plates were incubated in a 5% vol/vol. CO2-humidified incubator at 37ºC and pulsed with 1 µCi/well 3H-thymidine (Amersham Life Sciences), in 25µl serum-free media for the last 8hrs. Cells were harvested on fibre filter plates (Cox Science). Fibre filter plates were then dried over night. 3H-thymidine incorporation was read by a 1450 Microbeta liquid scintillation counter for Windows 2.7 using a TopCount NCT reader. Data was analysed using Microsoft Office Excel and Prism 7.0 software (Graphpad).

2.13 Preparation of a cell-derived matrix (CDM)

13mm glass coverslips were boiled three times in ddH2O, sterilised by autoclaving, and rinsed with 70% EtOH once and then placed in a 24-well plate. The coverslips were rinsed with PBS to remove EtOH and then coated for 60minutes using 0.2% sterile gelatin (autoclaved and sterilised by 0.2µm filtration before use) for 60 minutes at 37° C (gelatin type B). They were then rinsed with PBS to remove excess protein. Gelatin was crosslinked using 1% sterile glutaraldehyde for 30 minutes at RT and rinsed three times with PBS. Cross-linking was quenched using 1M sterile glycine (autoclaved) in PBS for 20 minutes at RT and rinsed three times with PBS. The coverslips were incubated with a normal growth medium (DMEM, 10% FBS, 1% P/S (penicillin/streptomycin)) for 30 minutes at 37°C and then rinsed three times with PBS. Following this, they were plated with fibroblasts from normal dermal, tumour or surrounding tissues at 60,000 cells per/well and cultured overnight until the cells were completely confluent. On the next day, the medium was supplemented with 50-µg/ml ascorbic acid and changed media (+50 µg/ml ascorbic acid) every two days. The cells were cultured for 3-10 days. The medium was removed to be decellularized. The coverslips were rinsed once with PBS. Pre-warmed (fresh) extraction buffer (10 mM NH_4OH, 0.25% Triton X-100 in PBS) was added and incubated at 37^oC for 4 minutes to allow cell lysis. Half of the buffer was removed and replaced with the same volume of PBS. This step was repeated until no visible intact cells remained. It was then rinsed twice with PBS, and finally PBS containing 1% P/S was added for storage at 4°C for up to four weeks. All ECMs were prepared by Dr. Tanya Shaw's lab in Kings College London. In this paper, N signifies normal ECM, S refers to ECM from surrounding tissue, and T indicates ECM from tumour.

2.14 Flow Cytometry

2.14.1 Staining cell surface

Isolated CD14+ cells were transferred into round-bottomed FACS tubes (BD Falcon^{TM}) in a total volume of 100 µl cold FACS buffer (PBS with 1% FBS and 0.1% sodium azide) with required antibodies and matched isotypes Anti (CD14). Flourochrome-conjugated mAb was directly added to 100µl cold FACS buffer to the $1x10^6$ cells and incubated at RT for 15-20 minutes. The cells were washed twice with 2 ml cold FACS buffer and centrifuged for 500 for

5 minutes to remove excess unbound mAbs. After each wash, supernatants were discarded. They were then resuspended in 200µl cold FACS buffer and analysed by flow cytometry. Samples were acquired on MACS Quant Flow Cytometer and analyzed using FlowJo software (Treestar Inc.). In some cases, samples were fixed in 2% formaldehyde (Sigma) and stored in the dark at 4^{o}C until acquisition.

2.14.2 Cell-surface marker expression by flow cytometry

An analysis of macrophage surface molecules was carried out with fluorescein isothiocyanate (FITC)-conjugated anti-CD204 mAb, anti-CD206 VioBlue, anti-CD86-PerCP-Vio700, anti-CD169 (Siglec-1) FITC, anti-CD204 APC and anti- CD279 (PD1)-PE by flow cytometry for surface immunostaining. Isotypes, Mouse IgG1-VioBlue, Mouse IgG1-PerCP-Vio700, REA Control-PE-Vio770, Mouse IgG1-APC, REA Control-PE-Vio615 were used. Briefly, cell suspension was centrifugated at 300xg for 10 minutes. Supernatant was aspirated completely, and the cells were resuspended. Then 2µl FITC-conjugated anti-CD204 mAb, anti-CD206 VioBlue, anti-CD86-PerCP-Vio700, anti- CD169 (Siglec-1) FITC, anti-CD204 APC and anti-CD279 (PD1)-PE were added. It was mixed well and incubated for 10 minutes in a dark refrigerator (2-8^{o}C). Cells were washed by adding 1-2 mL of buffer (PBS, pH= 7.2, 0.5% bovine serum albumin (BSA) and 2 mM EDTA by diluting a MACS BSA stock solution 1:20 with autoMACS rinsing solution) and then centrifugated at 300xg for 10 minutes. Supernatant was aspirated completely. The cell pellet was re-suspended in a suitable amount of buffer for analysis. CD206, CD86, CD169, CD204 and PD-1 surface molecules on CD14+ and Mϕs were determined based on forward- and side-scatter characteristics, following exclusion of debris. The results were analysed using FlowJo version 10.6.2 software (Treestar Inc., US).

2.15 Western blot (this experiment was carried out in Dr. Shaw's lab, King's College, London)

CDMs were harvested and lysed with RIPA buffer. The samples were stored in -80^{o}C until analysed. Nanodrop (ThermoFisher, US) was used to quantify the protein. Equal amounts were loaded into 8%-16% pre-cast gels (Invitrogen NuPAGE, UK). Gels were run for 90 minutes at 120V with Tris-Glycine running buffer. The gel was then transferred onto a nitrocellulose

membrane (Bio-Rad, UK) at 100V for 90 minutes. The transfer buffer was made up of 20% ethanol in 1X Tris-Glycine buffer.

After transfer, the membrane was blocked for 1 hour with 5% milk dissolved in Tris-Buffered Saline Tween 20 (TBS-T, Tween 20 at 1%), rinsed, and probed overnight in primary antibody (Table 1) diluted in 0.02% sodium azide, 1% BSA in TBS-T. The membrane was washed three times for 10 minutes in TBS-T and then a secondary antibody (Table 1) was added. After 1 hour, the membrane was washed again three times for 10 minutes in TBS-T and a quick rinse in TBS. Chemiluminescence was prepared by using 50% of component A and 50% of component B (ThermoFisher, US), and the signals were visualised by chemiluminescence. β-ACT was used as control.

Table 2.1 Characteristics of antibodies. Biglycan: BGN, POSTN: Periostin, β-ACT: Beta-actin

	BGN **(Proteintech, UK)**	POSTN **(Proteintech, UK)**	β-ACT **(Abcam, UK)**
Antibody species	Rabbit	Rabbit	Rabbit
Dilution of Primary	1:1000	1:1000	1:2000
Dilution of Secondary	1:5000	1:5000	1:5000
Molecular Weight (Ulubas Isik et al.)	42	93	42

2.16 Embedding spheroids in microarray mold

The Mold-maker was spray-coated with a silicone mold release spray and left to dry (30 sec). Hot (50–70 °C) Type IA agarose solution (2% wt/vol in deionized water, 2 mL) was dispensed in a pre-warmed (37–50 °C) stainless steel histology base mold (25 × 20 mm). The Mold-maker was placed on top of the warm agarose solution and the base-mold gently pressed and tapped to remove any potential air bubbles trapped underneath the Mold-maker. The agarose solution was left to gel at room temperature (2 min, 21 °C) and subsequently the mold was transferred to a laboratory freezer and placed on a level surface (1 min, −18 °C). The Mold-maker was removed leaving an agarose mold of 66 wells. The previously-fixed spheroids (4% wt/vol paraformaldehyde solution in PBS, 16–24 h at 2–8 °C) were taken up with fixation media (7–8 µL) from each well using a 20 µL pipette tip with the top section cut-off to facilitate spheroid collection. After each well was filled with aqueous media from the spheroid plate (4% wt/vol paraformaldehyde solution in PBS), the whole mold was centrifuged (1 min, 100 g). The agarose molds were centrifuged by taping the stainless steel molds to the top of a 50 ml swing rotor centrifuge adaptor. The mold was quickly warmed (5 s on a hotplate) to reach 37–40 °C and low-gelling agarose (0.5 mL, 2% wt/vol in deionized water) was slowly dispensed on the side of the mold to seal the spheroids. The agarose array was left to gel (5 min at room temperature, 1 min at −18 °C), placed in a histological cassette and processed in a tissue processor overnight. The samples were dehydrated in a series of alcohol solutions with increasing concentrations (one bath of 50%, 70%, 90%, and 4 baths of 100% methanol, 1 h each), cleared in xylene (3 baths, 1 h each) and infiltrated with molten paraffin (2 baths, 2 hours each). The molds were then embedded in paraffin on the next day.

2.16.1 Immunohistochemistry

Spheroid microarray molds were cut (4 µm sections) and placed on poly-lysine coated slides to dry (24 h, 25 °C). Paraffin section areas were lined with a solvent resistant marker (Securline). Slides were dewaxed in 3 xylene baths (5 min each) and rehydrated in 2 methanol baths (1 min). Hematoxylin and eosin staining (H&E) was performed with Mayer's Haemalum (3 min) and eosin (3 min).

2.17 Statistical analysis

Statistical analysis was undertaken. P values were calculated with the student paired t-test using the Prism 7, one-way AVOVA and two-way ANOVA software (GraphPad). P values of 0.05 0r less were considered significant.

Chapter 3 : Characterisation of *in vitro* APC model

Chapter 3 : Characterisation of *in vitro* APC model

3.1 Introduction

There are many variables involved in generating monocyte-derived APC that can be classified as follows:

- Donor-related (including genetic, sex, age, environmental)
- Venipuncture-related (e.g. time of day (diurnal variation), needle gauge, force, anti-coagulant)
- Experimenter variables (e.g. accuracy, efficiency, consistency)
- Reagent variables (e.g. those introduced when using undefined medium, batch-to-batch differences in reagents, endotoxin contamination) etc.

Such variables can reasonably be expected to impact on the quantity and quality of isolated leukocytes. Importantly, from an experimental point of view, this variability impacts on the function of sensitive cells such as APC and therefore it is important to validate the laboratory process to ensure reproducible data. Initial studies were therefore conducted to determine the range of cell yield, viability and function when monocytes were isolated from venous blood of healthy volunteers.

Leukocytes obtained from adult blood can be divided into two broad categories: lymphoid or myeloid. The lymphoid compartment is comprised of T, B, innate lymphoid and natural killer (NK) cells, whereas myeloid lineage includes distinct cell types in function and morphology, such as mononuclear phagocytes (monocytes and dendritic cells), granulocytes (neutrophils, basophils, and eosinophils) and platelets. The site of injury where these myeloid cells recruited rapidly via chemo-attractants are released from injured or infected tissue. these cells exist at the inflamed site and can regulate and play key effector functions (Akashi, Traver, Miyamoto, & Weissman, 2000) suggested that organization of hematopoiesis was a gradually process deriving from a common precursor cell. Indeed, it has been revealed that differentiation stages of hematopoietic stem cells that arise from bone marrow can be induced and become progressively more restricted into an eventually heterogeneous population of leukocytes (Akashi, Traver, Miyamoto, & Weissman, 2000). Nevertheless, there are several crucial factors that impacts on leukocyte generation. Several studies focused on

how age, ethnicity, diet, exercise, sleep and sex influence human monocyte numbers during homeostasis. In addition, circulating monocytes play an emergency role and their functions migrate at the injured site to induce pro-inflammatory and pro-resolving functions (Guilliams, Mildner, & Yona, 2018; Jakubzick, Randolph, & Henson, 2017; Yona & Gordon, 2015). Performance of flow cytometry was measured monocytes expressing high levels of LPS that binds CD14 receptor (Griffin, Ritz, Nadler, & Schlossman, 1981).

It is also important to understand lifestyle and genetic factors impacting monocytes and their development and function and monocyte-derived cells through pathology. However, healthy physiological conditions can play an important role for the behaviour of these cells. In addition, healthy homeostasis is not a lonely state, various agents often ignored, such as sex, diet, exercise and age impact the immune system. The impact of genetic traits which prevail over environmental signals related to the immune response is still poorly understood (Brodin et al., 2015; Mangino, Roederer, Beddall, Nestle, & Spector, 2017). The most apparent of these being their role in reproduction associated with various physical differences present between the sexes. Hormone concentration can be another obvious different. For example, a study by Fairweather, Frisancho-Kiss et al. (2008) found that Females were diagnosed with autoimmune disease over three quarters. Females afflict more than males in systemic lupus erythematosus (SLE), Sjogren syndrome, fibromyalgia, and rheumatoid arthritis (Regitz-Zagrosek, 2012), whereas ankylosing spondylitis, vasculitis and Good pasture syndrome predominantly diagnosed in males (Fish, 2008). Determine counts of monocytes was observed that at all stages of life have be reported that monocytes are consistently promoted in male (Y. Chen et al., 2016). Curiously, the previous study reported that monocytes are higher in Caucasian men than those in women over 400 individual cases were tested. This difference did not report in the Afro Caribbean population. Furthermore, ethnic diversity is a relatively association between disease susceptibility and different human populations, this can detect in patients infected with tuberculosis (TB) infection, autoimmune hepatitis and systemic lupus erythematosus (SLE) (Coussens et al., 2013). Keller et al. (2014) analysed that those population from European ancestry tend to have a higher monocyte count rather than African and Japanese individuals (Keller et al., 2014). Of note, populations from different ethnics can possibly reflect in monocyte function, for instance monocyte –derived cells from

Filipino, Chinese, and non-Hispanic whites challenged with Mycobacterium tuberculosis secreted different amounts of cytokines from 137 volunteers (Nahid et al., 2018).

Diet differs from person to person, the quantity and frequency of cells are associated to what they eat. Monocytes play a crucial role in various diet-related pathologies. In recent years, it is commonly accepted that a number of functional modifications was triggered in a high fat western diet. Elevation of circulating and splenic monocytes was determined in mice fed a western diet during weeks compared those fed on standard chow diet (Christ et al., 2018).

Sleep and wake cycle contributes to monocyte functions. However, immune cell gene expression can be modulated cortisol as known the archetypical neuroendocrine anti-inflammatory hormone (Cuesta, Boudreau, Dubeau-Laramee, Cermakian, & Boivin, 2016; J. Ehrchen et al., 2007). There is related between cortisol and a diurnal pattern, thus 30 minutes after waking in the morning this leads to its peak level and decreases during the day. Cortisol acts as an immunosuppressive and is used as an anti-inflammatory drug and is classified as the endogenous member of glucocorticoid family that can impact on many leukocytes, including monocytes, resulting in inducing a prompt reduction in circulating monocytes (Fingerle-Rowson, Angstwurm, Andreesen, & Ziegler-Heitbrock, 1998). Therefore, it would be interesting to a diurnal pattern is followed by several changes of function, such as cytokine secretion, level of expression of surface membrane protein, and phagocytic capacity (Shantsila et al., 2012). In addition, although during the day expression of TLR1, 2, 4 and 9 does not change, expression of costimulatory molecules are inhibited when activation of these receptors in the morning occurs (Lancaster et al., 2005), monocyte count was a higher in sleep-deprived individuals who remain awake throughout the night compared to individuals slept at this period (Born, Lange, Hansen, Molle, & Fehm, 1997).

The impact of circannual or seasonal rhythms on monocyte function was observed. Specifically, the amount of monocyte phagocytosis was higher in winter compared to spring and autumn. In response to LPS, production of both pro-inflammatory TNF-α and IL-6 and anti-inflammatory IL-10 cytokines was lower in isolated monocytes throughout the autumnal period (Linden, Larson, Prellner, Brattsand, & Laitinen, 1994; Myrianthefs et al., 2003).

Exercise is another factor affecting the human immune system and can cause leucocytosis (Shephard, 2010). Studies have shown that the period immediately following exercise sees a

rise in intermediate and non-classical monocytes (Steppich et al., 2000), whereas others have reported a change in classical and non-classical monocytes, and even all other populations (Slusher, Zuniga, & Acevedo, 2018). In one particular study the percentage of CD16+ monocytes was reduced by a 12-week exercise programme (Timmerman, Flynn, Coen, Markofski, & Pence, 2008). This reduction may be associated with loss of fat. Moreover, expression of TNF-α was significantly decreased subsequent to this 12-week exercise regime, and in contrast monocyte phagocytosis was found to be at a high level (Poitou et al., 2011).

Myeloid cells frequency changes with age. The number of monocytes is three times greater in newborn babies than in adults (Schefold et al., 2015). Some studies have reported a decrease in dendritic cells, whereas the number of monocyte subsets increased in the older aged group (mostly intermediate and non-classical monocytes) (Nyugen, Agrawal, Gollapudi, & Gupta, 2010). In older cohorts, plasma CCL2 was detected at a high level, which may lead to mobilisation of monocytes from the bone marrow. However, lower numbers of classical and intermediate monocytes were detected in advanced old age (81–100 years) (Seidler, Zimmermann, Bartneck, Trautwein, & Tacke, 2010; Verschoor et al., 2014). Coincidentally, CD16+ monocytes can be expanded in inflammatory conditions, and this is also promoted in older individuals. With these cells, in particular non-classical monocytes, secretion of TNF-α was higher in aged individuals (Ong et al., 2018). A consequence of this inflammatory environment may be dysregulation of innate immunity, such as impaired phagocytosis (Hearps et al., 2012).

It is well known that all the previously-mentioned physiological factors influence immune reactivity. There are also certain technical aspects of blood-drawing which can affect the viability, yield and function of immune cells that exist in peripheral blood. In a relevant study, blood was drawn with heparinised syringes or using recent vacuum-driven devices, such as BD Vacutainer^{R} tubes (BD, Franklin Lakes, NJ, USA) (Afonso et al., 2010). There was no significant difference either in terms of PBMC yield or T cell function following assessment which was performed using the human leukocyte antigen (HLA) class I multimer and INF-γ ELISPOT assays.

It is often assumed that T cell analysis is affected by ethylenediamine tetraacetic acid (EDTA), as anti-coagulated blood may suppress T cell activation through the action of its Ca^{2+}-chelating properties. Bull *et al.* compared EDTA, acid-citrate-dextrose (ACD) and sodium

heparin as anti-coagulants for drawing blood samples, and found that they did not differ in terms of cell recovery, viability and function (Bull et al., 2007).

An issue in blood storage until processing has been noted when it comes to thoroughly analysing the human immunodeficiency virus (HIV) area, where it was found that the most critical factor affecting subsequent cell recovery and function was associated with time from venepuncture to cryopreservation (Bull et al., 2007). In the above-mentioned study multi-centre study, other factors were considered, including type of anti-coagulant used, PBMC isolation method, and process of sample shipping. Increasing time from 8 h to 24 h before procedure and cryopreservation had a critical effect on sample quality. When PBMCs were collected from sodium heparin and layered by Ficoll gradient, reduction of cell recovery 24 h versus 8 h was determined by 30% (from 83% to 53%), viability by 4% (from ~96% to 92%), and by 36% to 56% in viral peptide-reactive T cells (INF-γ ELISPOT) (Kierstead et al., 2007).

It was found that in T cell assays for blood stored for prolonged periods, granulocyte contamination was increased in the PBMC preparation. This issue induces activation of granulocytes upon prolonged storage, resulting in separation which is insufficient by density gradient procedures (Schmielau & Finn, 2001). This point was addressed by a study which found that activate (CD11b+CD15+) granulocytes that are contaminated with PBMC within 8 h after venepuncture and stored at room temperature (2.3-fold increase), and contamination was promoted 11.3-fold by 24 h, compared to fresh blood PBMCs (< 3 h after venepuncture).

Dilution of blood in phosphate-buffered saline (PBS) or RPMI-1640 (1:1) prior to storage can reduce activation of granulocyte, resulting in limitation of both granulocyte contamination and suppression of T cell responses (Mannering et al., 2003). Macaque's study observed granulocyte contamination, in which the quality and quantity of spots in INF-γ ELISPOT assays were affected (De Rose, Taylor, Law, van der Meide, & Kent, 2005). It should be noted, however, that a comparison between samples kept under gentle agitation on a rocking platform and those in tubes stored horizontally without agitation revealed that recoveries were similar, but granulocytes were highly contaminated after prolonged (18 h) storage and non-agitation (Snow, Feldbush, & Oaks, 1980). This data suggests that 8-12 h after collection is the ideal time for processing blood (Thornthwaite, Rosenthal, Vazquez, & Seckinger, 1984).

Blood shipping and storage may impact on results, and it is important to identify conditions for shipping blood samples for the eventual PBMC isolation. Therefore, it is recommended that blood samples be held in insulated containers in order to avoid temperature variations (Martinuzzi et al., 2008). PBMC preparation may impact the product of PBMC separation upon blood-taking and storage. The effects on cell quality, quantity and/or function are likely associated with the addition of medium to whole blood before centrifugation, separation media from different sources, separation tubes, centrifugation speed and repetition and addition serum. PBMC preparation comprises several washing steps. The involvement of different washing media used, including RPM-1640, Hank's balanced salt solution (HBSS) or PBS, has not been thoroughly tested. However, improved final yields of PBMC were detected in the presence of human serum in the washing buffer (up to 40.5% median increase). Further promoting human serum concentrations did not increase PBMC counts further, but HLA tetramer staining was significantly decreased (40.0% median decrease), while there were no changes in the responses of INF-γ ELISPOT (Afonso et al., 2010; Mannering et al., 2003).

3.2 Hypothesis and aims

This initial results chapter aims to establish proficiency in techniques of cell isolation and differentiation, and to demonstrate the feasibility of generating sufficient different subsets of APC from circulating monocytes such that subsequent experiments may be conducted. Specifically, I aim to show that the number and purity of isolated cells is of a satisfactory level, and that when resting and stimulated the APC generated function as expected by secreting appropriate cytokine profile. Having stated that this is largely a technical chapter, it is nevertheless possible to test the hypothesis that the efficiency of generating different macrophage subsets, namely GM-Mϕ and M-Mϕ, differs.

3.3 Results

Our previous studies employed APC generated in the laboratory from human monocytes (Storr et al., 2017) and thesis of Malecka *(University of Nottingham, 2017)* In this system, isolated $CD14^+$ monocytes were differentiated into MoMϕ or MoDC cells under the influence of cytokines GM-CSF, M-CSF (for macrophage), or GM-CSF with IL-4 (for MoDC). APC

generated using this approach respond to activation signals, such as LPS and/ or IFNγ, giving rise to mature GM-Mϕ, M-Mϕ or MoDC that are expected to secrete a predicatable profiles of cytokines. These cytokines serve key functions involved in regulating adaptive immunity in T cells, therefore initial studies were conducted to demonstrate that the models of monocyte-derived APC were consistent with published data.

3.3.1 Donor characteristics

Several steps were taken to reduce variation in blood samples. Specifically these included

1. The exclusion of donors with chronic or acute healthy conditions (see our ethics for details), narcotic use etc
2. Phlebotomy undertaken between 8am and 12 to reduce diurnal variation.
3. A restricted panel of trained phlebotomists.
4. Standard equipment namely 60mL syringes with 19g butterfly set.
5. The sole use of sodium heparin from a single supplier as anti-coagulant.

3.3.2 Yield of PBMC from venous blood

The isolation of monocytes from PBMC is a key step for laboratory generated APC. Differences in the efficiency of this step may reduce the number of APC available for subsequent experimentation, and contamination of monocytes with other cells may impact on their function (Nair, Archer, & Tedder, 2012). Therefore, a series of standard preparations of PBMC were made from healthy-donor venous blood and the number of cells obtained determined. As shown (Figure 3.1), the number of PBMC obtained varied between individual isolations. The median number of PBMC obtained was $4.97x10^8$/100mL of venous blood with a wide variation between individual isolations was observed. These values were used when planning the volume of blood required to conduct subsequent experiments.

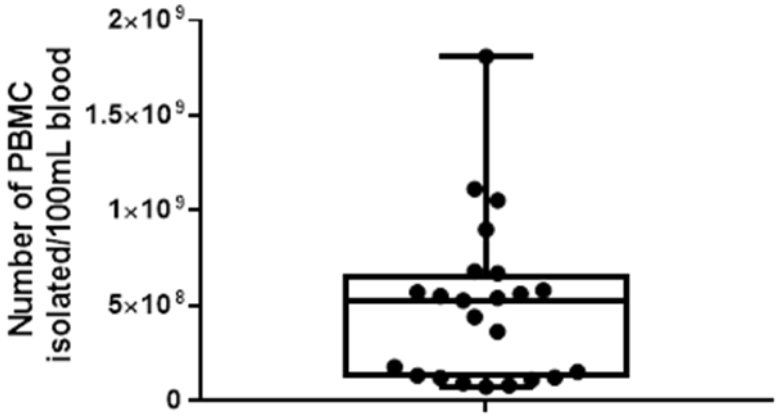

Figure 3. 1 The efficiency of isolation of PBMC from whole blood. PBMC were isolated from venous blood of healthy donors (n=23) by density centrifugation. After harvest the number of PBMC was determined with a haemocytometer. Dots indicate data from individual donors with box and whisker plot showing the median, 25% to 75% interquartile range, minimum and maximum values.

3.3.3 Yield of monocytes when isolated from PBMC

In order to understand the efficiency of monocyte isolation from PBMC we next conducted a series of standard magnetic positive selections and counted the cells obainted. As expected, the number of monocytes obtained varied between individual isolations (Figure 3.2). The number of monocytes obtained ranged from $0.78x10^7$ to $13x10^7$, with a median of ($4.945x10^7$ +/- $3.850x10^7$).

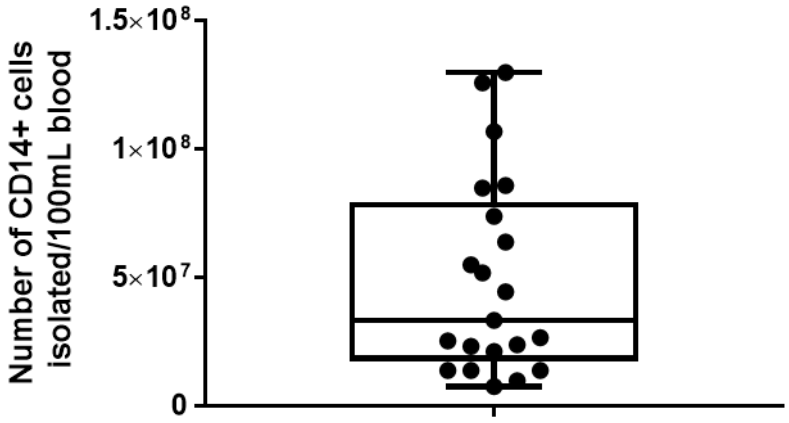

Figure 3.2 The efficiency of CD14+ monocytes isolation from whole blood. Isolated CD14+ monocytes from 21 healthy donors were obtained using positive magnetic cell selection. After harvest the number of selected cells was determined using a haemocytometer. Dots indicate data from individual donors with box and whisker plot showing the median, 25% to 75% interquartile range, minimum and maximum values.

We further analysed the efficiency of monocyte selection from PBMC by transforming the data to reflect the proportion of PBMC rather than whole blood as shown above. Whilst the efficiency was clearly still variable when data were expressed as a proportion of PBMC, the distribution of data appeared more normally distributed (Figure 3.3). On average approximately 15% of PBMC could be isolated as CD14+ monocytes and these data were important for designing later experiments in which APC were to be differentiated from isolated monocytes.

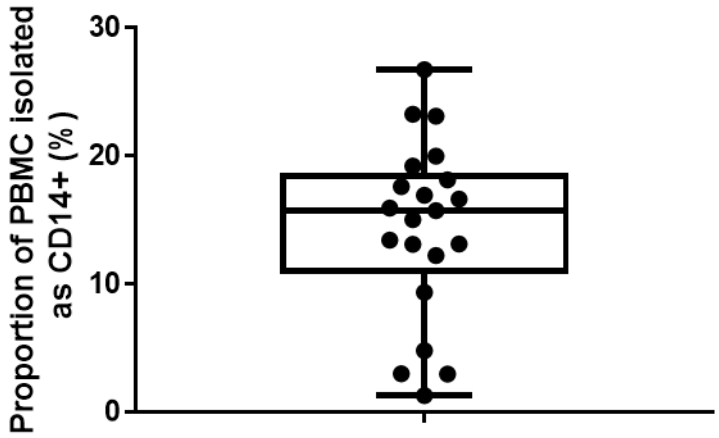

Figure 3.3 The efficiency of CD14+ monocytes isolation from PBMC. CD14+ monocytes were isolated from 21 healthy donors by magnetic cell selection. After harvest the number of selected cells was determined using a haemocytometer. Dots indicate data from individual donors with box and whisker plot showing the median, 25% to 75%percentile interquartile range, minimum and maximum values.

3.3.4 Purity of isolated CD14+ monocytes

The purity of isolated monocytes was next determined by flow cytometry with antibodies specific for CD14. The dot plots from representative donors show the presence of a clear population of cells that expressed the $CD14^{+}$ antigen (Figure 3.4). Subsequent experiments were therefore undertaken only when the purity of isolated monocytes was in excess of 90%. The distribution of monocyte purity was broadly in line with expected values from the laboratory (personal communication, A Jackson) (Figure 3.4).

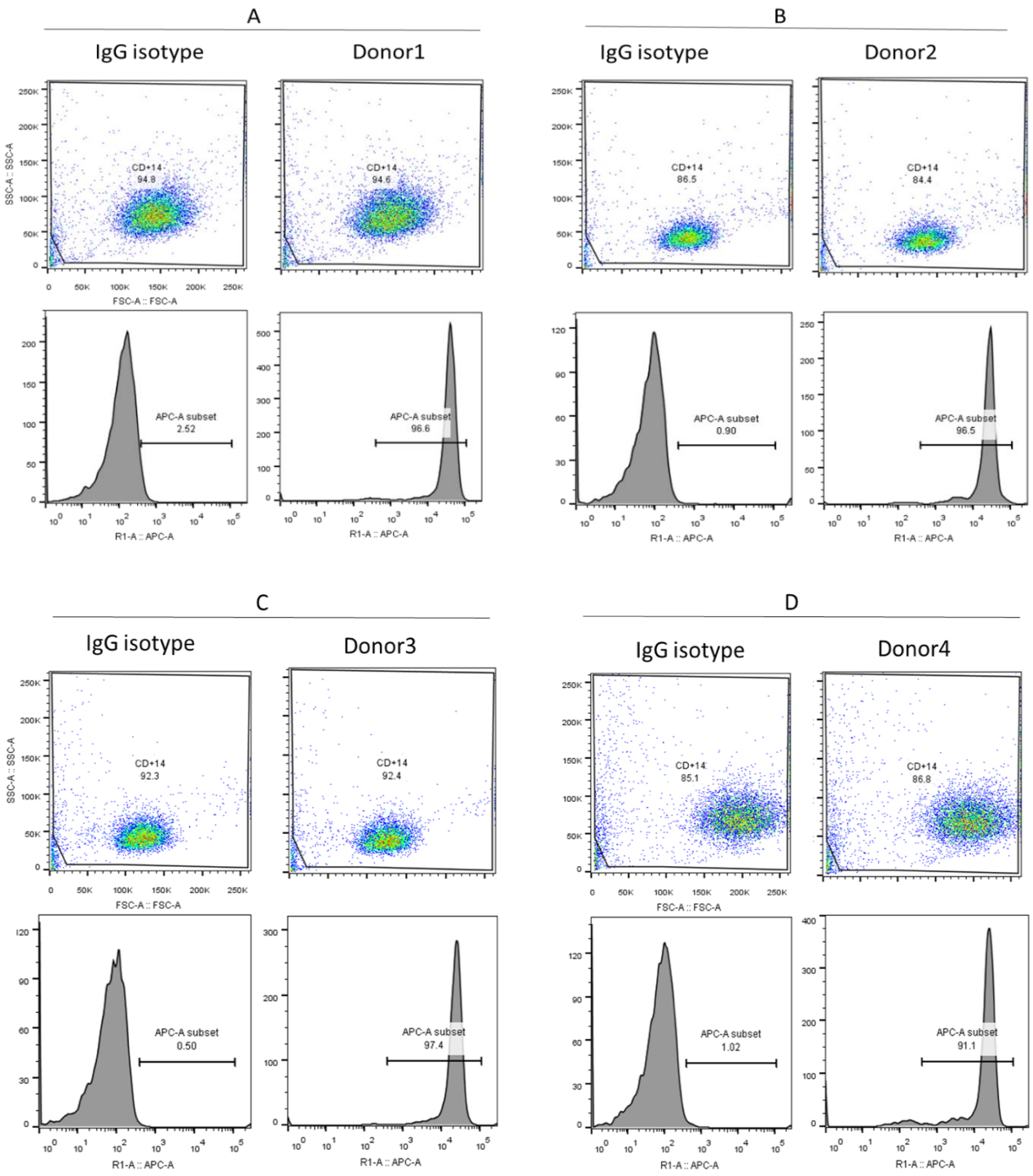

Figure 3.4 Representative purity of CD14^{+} cells isolated from PMBC.

Figure 3.4 representative purity of CD14+ cells isolated from PMBC. PBMC were obtained from blood from 4 donors (Klechevsky et al., 2010) by Histopaque gradient centrifugation and $CD14^+$ cells were isolated using magnetic beads. Isolated cells were stained with anti-IgG-APC isotype control mAb or anti-$CD14^+$ APC mAb and analysed by flow cytometry. Cells were gated on equal numbers of CD14+ (10^4). Dot plots show the forward scatter (FSC) and side-scatter properties of isolated cells, and histograms were drawn according to IgG staining and the numbers indicate the expression of CD14 with a marker showing the percentage of CD14+ cells obtained.

3.3.5 Generation of Mϕ

Recovery of GM-Mϕ and M-Mϕ After isolating and purifying CD14+ cells from circulating peripheral blood monocyte. It was important to differentiate CD14+ into different phenotypes are routinely classified into GM-Mϕ and M-Mϕ this allows us to undertake subsequent experiments. Purified CD14+ were differentiated in GM-Mϕ and M-Mϕ in the presence of GM-CSF or M-CSF respectively and the yield of cells determined after 6-7 days (Figure 3.5).

The yield of GM-Mϕ cells generated from CD14+ varied from $0.7x10^6$ to $4.5x10^6$, the median number of GM-Mϕ obtained was ($2.1x10^6$ +/- $1.2x10^6$). The number of M-Mϕ cells generated from CD14+ obtained varied from $0.3x10^6$ to $4.7x10^6$, the median number of cells obtained was ($2.2x10^6$ +/- $1.2x10^6$). The number of GM-Mϕ and M-Mϕ obtained at the end of differentiation was not significantly different and therefore both types of macrophage were generated with similar efficiency.

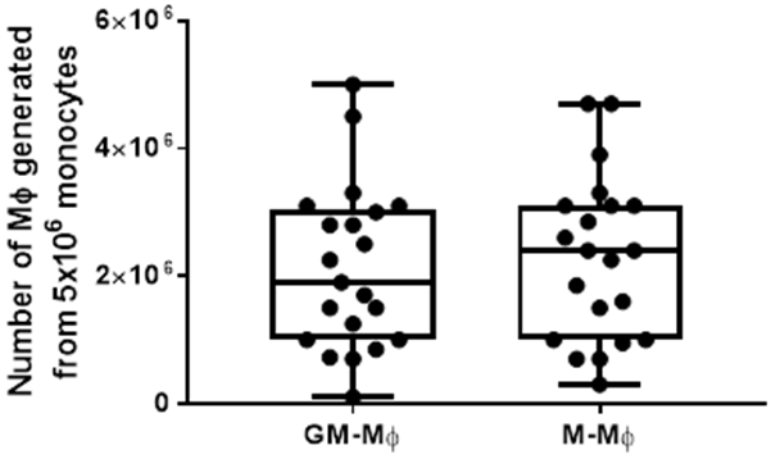

Figure 3.5 Box and whisker plot showing the efficiency of macrophage generation from monocytes. CD14+ cells were isolated and differentiated with the indicated cytokines (GM-CSF, M-CSF) for 7 days. At the conclusion of the culture cells were harvested and counted using a haemocytometer. Dots indicate data from individual donors with box and whisker plot showing the median, 25% to 75% interquartile range, minimum and maximum values.

We further analysed the efficiency of Mϕ generated from CD14+ cells by transforming the data to reflect the proportion of CD14+ rather than the total number of CD14+ as shown above. Whilst the efficiency was clearly still variable when data were expressed as a proportion of Mϕ, the distribution of data appeared more normally distributed (Figure 3.6). On average approximately 40% and 45% of CD14+ could be recovered Mϕ as polarizing GM-Mϕ and M-Mϕ, respectively. These data were important for designing later experiments in which APC were to be cultured in 2D and 3D system.

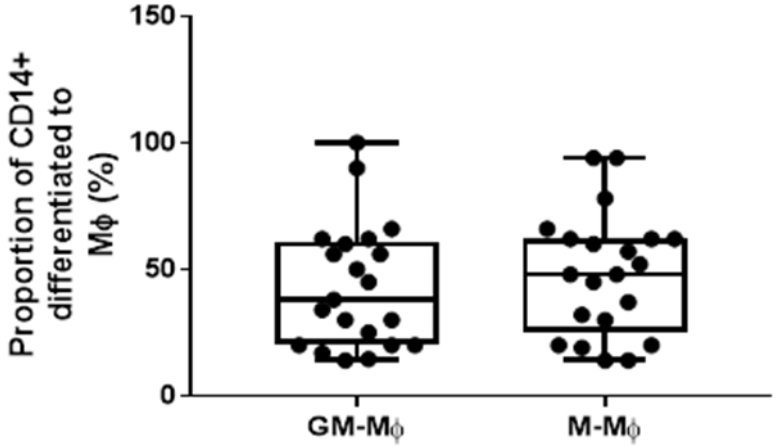

Figure 3.6 The efficiency of macrophage generation from CD14+ monocytes. Isolated CD14+ monocytes from 21 healthy donors were seeded in to 6-well plates and cultured for 6-7 days with the indicated cytokines. After harvest the number of recovered cells was determined using a haemocytometer. Dots indicate data from individual donors with box and whisker plot showing the median, 25% to 75% percentile interquartile range, minimum and maximum values.

3.3.6 Generation of DCs

Purified CD14+ cells were also differentiated to DCs using the combination of GM-CSF and IL-4 over 5-6 days. The yield of DC cells generated from purified CD14+ varied from $1.5x10^6$ to $10x10^6$ the median number of DCs obtained was ($5.2x10^6$ +/- $2.4x10^6$) (Figure 3.7).

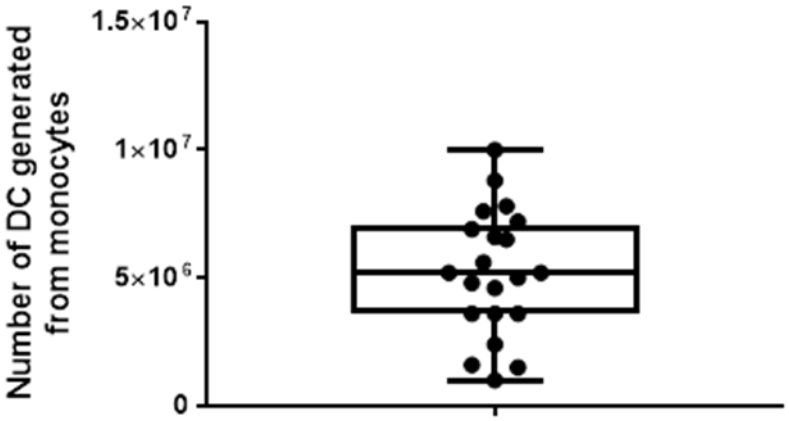

Figure 3.7 The box and whisker plot showing the efficiency of DC generation from monocytes. CD14+ cells were isolated and differentiated with the indicated cytokines (IL-4 and GM-CSF) for 6 days. At the conclusion of the culture cells were harvested and counted using a haemocytometer. Dots indicate data from individual donors with box and whisker plot showing the median, 25% to 75% interquartile range, minimum and maximum values.

We further analysed the efficiency of DC generated from CD14+ cells by transforming the data to reflect the proportion of CD14+ rather than the total number of CD14+ as shown above. Whilst the efficiency was clearly still variable when data were expressed as a proportion of DCs, the distribution of data appeared more normally distributed (Figure 3.8). On average approximately 65% of CD14+ were recovered as DC after *in vitro* differentiation. We observed a similar trend in efficiency of GM-Mϕ and M-Mϕ generated from CD14+ cells. The efficiency of DC generation was significantly higher ($p<0.01$) than for GM-Mϕ, and was also >1.4 fold greater than with M-Mϕ. These data were used to design later experiments in which APC were to be cultured in 2D and 3D systems.

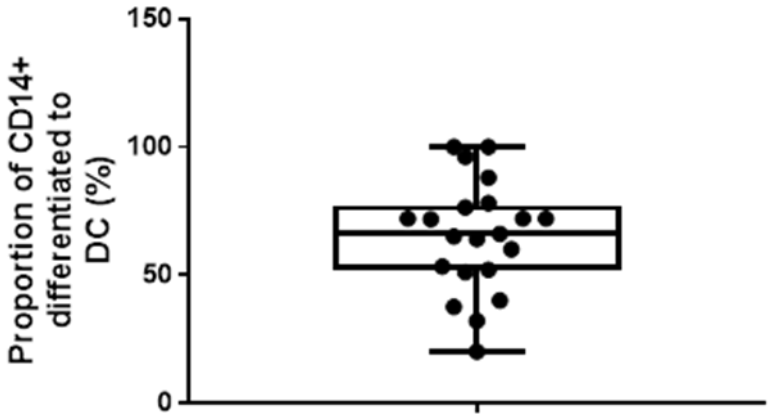

Figure 3.8 The efficiency of dendritic generation from CD14+ monocytes. Isolated CD14+ monocytes from 21 healthy donors were seeded in to 6-well plates and cultured for 5-6 days with the differentiating cytokines. After harvest the number of recovered cells was determined using a haemocytometer. Dots indicate data from individual donors with box and whisker plot showing the median, 25% to 75% percentile interquartile range, minimum and maximum values.

3.3.7 The cytokine profile of Mϕ and DCs upon exposure to TLR-4 agonist

Professional APC use a range of receptors to detect the presence of microbes (Nakano et al., 2009; A. P. West et al., 2011). The TLR-4 receptor is widely expressed and upon association with bacterial LPS activates signalling cascades that are important for the activation of APC, a process we refer to as maturation. LPS binding to TLR4 induces signalling pathways such as NFkB and MAPK that amplify the expression of pro-inflammatory cytokines (e.g., TNFα, IL-1β , IL-6, IL-12) and type-I interferons leads to regulate the inflammatory response and eventually pathogen destruction (Y. C. Lu, Yeh, & Ohashi, 2008; Swanson et al., 2020; Zettel et al., 2017).

Downstream of LPS binding to TLR4, MyD99-dependent signal pathways active NF-kB and the MAPK pathways. An illustrated model of NF-kB activation induces when IRAK-1 and IRAK-4 are associated with the receptor complex. Auto-phosphorylation of IRAK-1 (with Tollip acting as a negative mediator) induces dissociation from the receptor complex and binding of IRAK to TRAF6 (Cao et al., 1996; Ninomiya-Tsuji et al., 1999). TRAF6 in turn triggers the MAPK kinase TAK1 (transforming growth factor-*β*-activated kinase), results in association with its adapter protein, TAB1. At this point, NF-kB, the p38 and c-jun N-terminal kinase (JNK) MAPK pathways are regulated through TAK-1 which acts as a common activator. Regardless of NF-kB, p38 and JNK, LPS binds to the early MyD88 dependent signalling induces an early IRF3 activation and production of pro-inflammatory cytokines, such as TNF-α and IFN-β (Shirakabe et al., 1997; Yamaguchi et al., 1995).

Understanding how TLR signals give rise to the activation of different types of APC is fundamental to manipulating immune responses. Ultimately, when APC are fully activated by, for example TLR, this results in subsequent activation of leukocytes e.g. T cells. T-cell activation involves coordination of three signals (namely TCR, co-stimulation, and polarising cytokine) that are influenced by TLR-activation on APC. The differential expression of TLR by different subsets of APC (and other cells such as epithelia) conveys alternate responses to pathogens and other endogenous danger signals.

Previous studies showed the expression of type 1 IFN by pDC in response to TLR7 agonists, whilst IL-12p70 secretion by MyDC was activated through the same agonist (Ito et al., 2002). Oppman and colleagues demonstrated initial expression of IL-23 from activated MoDC. Initially described by Roses et al., IL-23 secretion from MyDC occurs upon activation of TLR agonists such as TLR2, 4 and 7/8. Remarkably, none of the individual TLR receptors stimulated

IL-12 showing differential recruitment of these two cytokines despite possessing a shared subunit with IL-12 (Kunze, Forster, Oehrl, Schmitz, & Schakel, 2017). In human MoMϕ activation upon LPS has been demonstrated to secrete IL-23p19 (Verreck et al., 2004). Additionally, it has been found that high level secretion of IL-23 but not IL-12, by Mϕ upon mycoplasma infection (Q. Wu et al., 2007). Furthermore, although IL-23 secretion by Mϕ increased in response to Sendai virus, influenza virus does not induce a similar response (Siren et al., 2004). IL-23 secretion by human Mϕ can be caused by infection by *Francisella* and in the microglia of patients with multiple sclerosis (MS) (Correa et al., 2011).

Hematopoietic stem and progenitor cells (HSPC) in circulating blood can be triggered through a range TLR-agonists which stimulate expansion and differentiation of specific myeloid subsets. For example, differentiation of CD34+ cells into CD11c+CD14-DCs is triggered by TLR7/8 ligands, whilst TLR2 ligands induce the differentiation into CD11c+CD14+Mϕ (Sioud & Floisand, 2007).

Many studies report that connective tissue cells regulate functional TLR receptors on their surfaces. TLR 1-6 and 9 express on epidermal keratinocytes (KC) (Lebre et al., 2007), in return, promoting distinct immune responses upon their activation (Miller & Modlin, 2007).

Not all bacterial LPS are identical, and there are several different sources for research-grade LPS that differ in the bacteria of origin (e.g *Salmonella sp., E. coli sp.*), and the method of purification. Additionally, the purity of LPS varies with some suppliers (Invivogen) assuring only the presence of TLR4 agonist with others (Sigma) contain additional contaminants. The effect of contaminants is unclear. We hypothesise that LPS supplied by Sigma activates MoDC and MoMϕ to produce higher levels of cytokine expression than Invivogen LPS. To test this hypothesis, we will carry out experiments to assess the relative impact of these on secretion of Signal 2 cytokines by antigen presenting cells. Cytokine secretion will be determined for LPS-treated DCs and Mϕ by Elisa for IL-23, IL-12 and IL-10 at a range of LPS concentrations.

3.3.8 Cytokine response of MoMϕ to the TLR4 agonist, LPS

Since previous studies revealed heterogeneity in the response of macrophage towards LPS (Meng & Lowell, 1997), we undertook experiments to determine the relative response of

macrophages generated under conditions in our laboratory by comparing two different sources of LPS with the secretion of IL-10, IL-12 and IL-23.

Most notably, secretion of interleukin-10 was significantly increased with mature GM-Mϕ with LPS from Sigma as compared to Invivogen (Figure 3.9). In contrast, IL-12 and IL-23 secretion were indifferent to the source of LPS. A pairwise comparison of both LPS stimulations using a t-test revealed significant differences in IL-10 secretion by GM-Mϕ with LPS provided by Sigma resulting in higher levels of IL-10 secretion (Table 3.1).

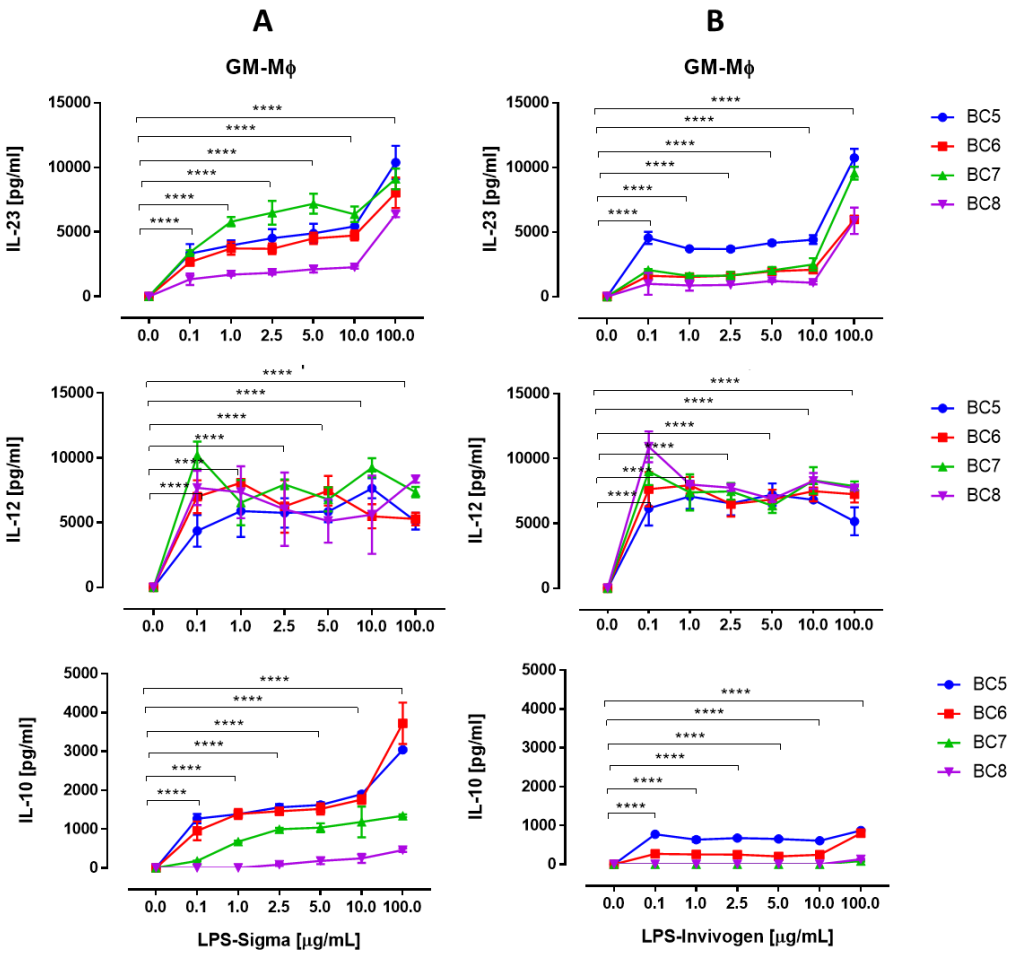

Figure 3.9 Effect of LPS at different concentrations in the presence on cytokine secretion by GM-Mϕ. $CD14^+$ cells (5×10^6) were cultured in low attachment 6 well plates in the presence 20U/ml of GM-CSF for 6 days. On day 7, cells were counted, washed and transferred into 96 well plates. A) Shows GM-Mϕ were activated with increasing concentrations of LPS) supplied by Sigma and 1000U/ml of IFNγ. B) Shows GM-Mϕ were activated with the same previous concentrations of LPS supplied by Invivogen and 1000U/ml of IFNγ. Data shown mean cytokine secretion amongst of triplicate well +/- SD and is representative 4 separated experiments. BC5, 6, 7 and 8 indicate donors. The secretion of IL-10, IL-12 and IL-23 were assayed at 24 hr by ELISA and statistically analysed by two-way ANOVA (*:0.05>p value≥0.01, **:0.01>p value≥0.001, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

Cytokine	LPS-Sigma Mean+/-SD (pg/mL)	LPS-Invivogen Mean+/-SD (pg/mL)	P Value
IL-12	6358.1+/-1443.2	7117.8+/-1174.7	Ns
IL-10	2144.3+/-1379.5	467.3+/-385.2	<0.001
IL-23	8476.2+/-1743.3	8234.5+/-2307.1	Ns

Table 3.1 Secretion of IL-10, IL-12 and IL-23 by GM-Mϕ in response to LPS supplied by Sigma or Invivogen. Cells were activated using different concentrations of LPS and 1000U/ml of IFNγ and cytokine secretion measured by ELISA after 24h. ns not significant.

When M-Mϕ were matured with Sigma LPS, their cytokine profile differed from those exposed to Invivogen LPS Release of IL-10 from M-Mϕ was significantly promoted from even low concentrations of LPS (Sigma), whereas release of IL-23 was unclear. We also observed a significantly lower IL-10 expression of M-Mϕ treated with LPS supplied by Invivogen compared to Sigma. Interestingly, LPS supplied by Sigma did stimulate IL-12 compared to Invivogen (Figure 3.10). This evidence demonstrates that these LPS obtained from two different resources (Sigma and Invivogen) can be induced equal levels of cytokine secretion and also low doses of LPS can prime hyper responsive macrophage leading to abundant cytokine expression (Table 3.2).

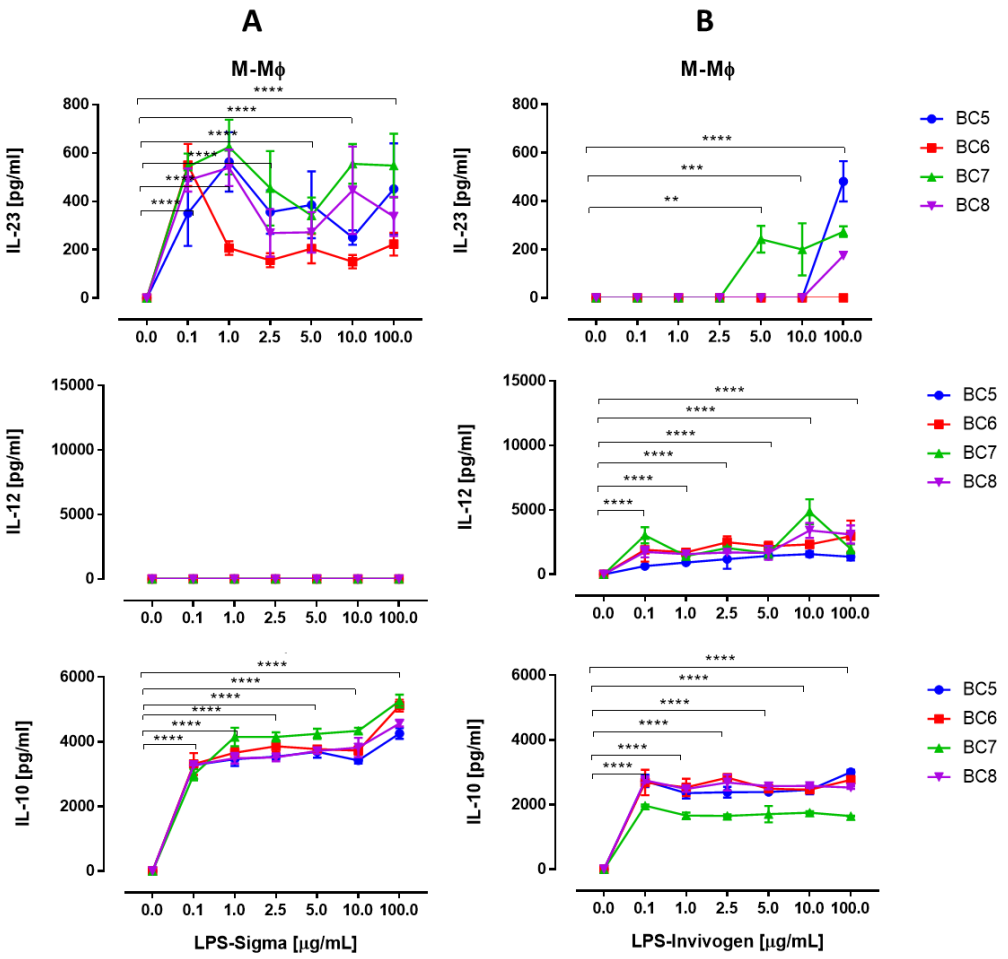

Figure 3.10 Effect of LPS at different concentrations on cytokine secretion by M-Mϕ. $CD14^+$ cells (5 x 10^6) were cultured in low attachment 6 well plates in the presence 10 ng/mL of M-CSF for 6 days. On day 7, cells were counted, washed and transferred into 96 well plates. A) Shows M-Mϕ were activated with increasing concentrations of LPS supplied by Sigma. B) Shows M-Mϕ were activated with the same previous concentrations of LPS supplied by Invivogen. Data shown mean cytokine secretion amongst of triplicate well +/- SD and is representative 4 separated experiments. BC5, 6, 7 and 8 indicate donors. The secretion of IL-10, IL-12 and IL-23 were assayed at 24 hr by ELISA and statistically analysed by two-way ANOVA (*:0.05>p value≥0.01, **:0.01>p value≥0.001, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

Cytokine	LPS-Sigma Mean+/-SD (pg/mL)	LPS-Invivogen Mean+/-SD (pg/mL)	P Value
IL-12	-	2374.4+/-963.3	<0.00001
IL-10	4801.4+/-447.9	2488.9+/-539.3	<0.0001
IL-23	390+/-165.3	232.8+/-185.6	Ns

Table 3.2 Secretion of IL-10, IL-12 and IL-23 by M-Mϕ in response to LPS supplied by Sigma or Invivogen. Cells were activated using different concentrations of LPS only and cytokine secretion measured by ELISA after 24h. ns not significant.

3.3.9 Response of MoDC to LPS

MoDC were activated with a range of doses of LPS (Sigma and Invivogen) in the presence of IFNγ and characterised by their cytokine profile (Figure 3.11). Secretion of IL-10 and IL-23 were similar regardless of increasing doses of LPS (Sigma) except at 100 μg/mL. IL-12 was similarly regulated except with 1μg/mL LPS. The cytokine responses were greater with DCs exposed to LPS supplied by Sigma than those activated with Invivogen's LPS (Table 3.3).

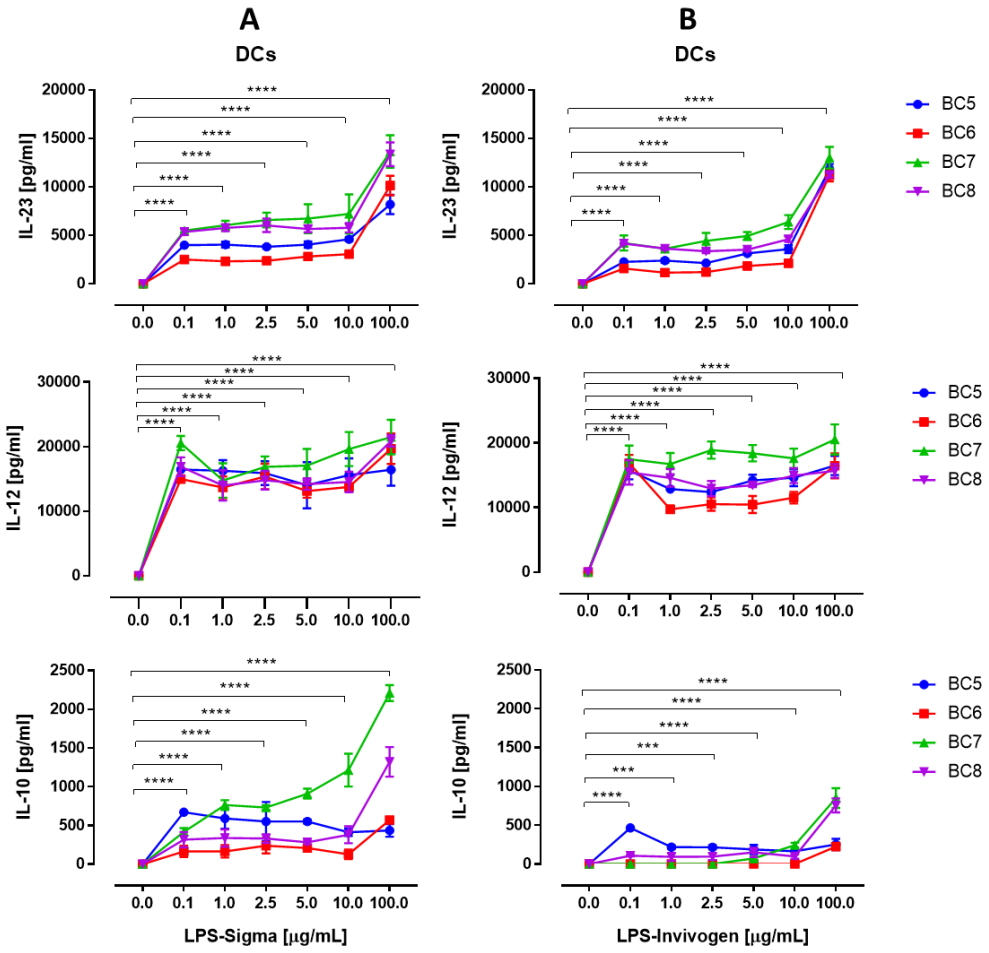

Figure 3.11 Effect of LPS on cytokine secretion by MoDC. A) DCs were activated with increasing concentrations of LPS supplied by Sigma and 1000U/ml of IFNγ. B) Shows DCs were activated with the same previous concentrations of LPS supplied by Invivogen and 1000U/ml of IFNγ. Data shown mean cytokine secretion amongst of triplicate well +/- SD and is representative 4 separated experiments. BC5, 6, 7 and 8 indicate donors. The secretion of IL-10, IL-12 and IL-23 were assayed at 24 hr by ELISA and statistically analysed by two-way ANOVA (*:0.05>p value≥0.01, **:0.01>p value≥0.001, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

Cytokine	LPS-Sigma Mean+/-SD (pg/mL)	LPS-Invivogen Mean+/-SD (pg/mL)	P Value
IL-12	19623.3+/2779.8	17299+/-2487.2	<0.01
IL-10	1117.9+/-776.5	521.4+/-306.2	<0.01
IL-23	11442+/-2691	11819.9+/-972.6	Ns

Table 3.3 Secretion of IL-10, IL-12 and IL-23 by DCs in response to LPS supplied by Sigma or Invivogen. Cells were activated using different concentrations of LPS and 1000U/ml of IFNγ and cytokine secretion measured by ELISA after 24h. ns not significant.

3.3.10 Characterising MoMϕ by cytokine secretion

MoMϕ can be characterised by their secretion of anti-inflammatory cytokines such as IL-10 (Kittan et al., 2013; Mills, Kincaid, Alt, Heilman, & Hill, 2000; Mills & Ley, 2014). Furthermore, MoMϕ also share a fried egg-like morphology as attached cells. Obtaining a large quantity of Mϕ from human tissue is difficult, and in this regard differentiation from monocytes has proven a useful research approach. Several protocols exist to generate Mϕ from monocytes (Brugger, Kreutz, & Andreesen, 1991; Plesner, 2003) but no standard protocol or definition of polarised Mϕ exists. Therefore, we elected to use a procedure which had previsouly given reliable Mϕ as shown by cytokine secretion (A Malecka, PhD 2017).

As expected, resting MoMϕ did not release cytokines (Figure 3.12). In contrast, MoMϕ activated with LPS and IFNγ or LPS alone released cytokines. Whislt GM-Mϕ secreted IL-12. activated M-Mϕ did not. Interlekuin-10 was produced by activated M-Mϕ and this cytokine was less-well produced by GM-Mϕ. Whilst IL-23 was secreted by both types of Mϕ, M-Mϕ released considerably lower levels. As indicated in Figure (3.12), there were significant differences between macrophages and their cytokine profiles.

IL-23 secretion by GM-Mϕ was significantly upregulated compared to immature cells, whereas IL-23 expression by M-Mϕ was significantly upregulated compared to immature cells, but at a lower level than with those in GM-Mϕ as shown in (Table 3.4). Figure 4.10 shows the typical morphology of Mϕ that were firmly attached to the substrate, despite using ULA plates. It was anticipated that macrophage morphology would agree with previous studies (Lacey et al., 2012; Rey-Giraud, Hafner, & Ries, 2012).

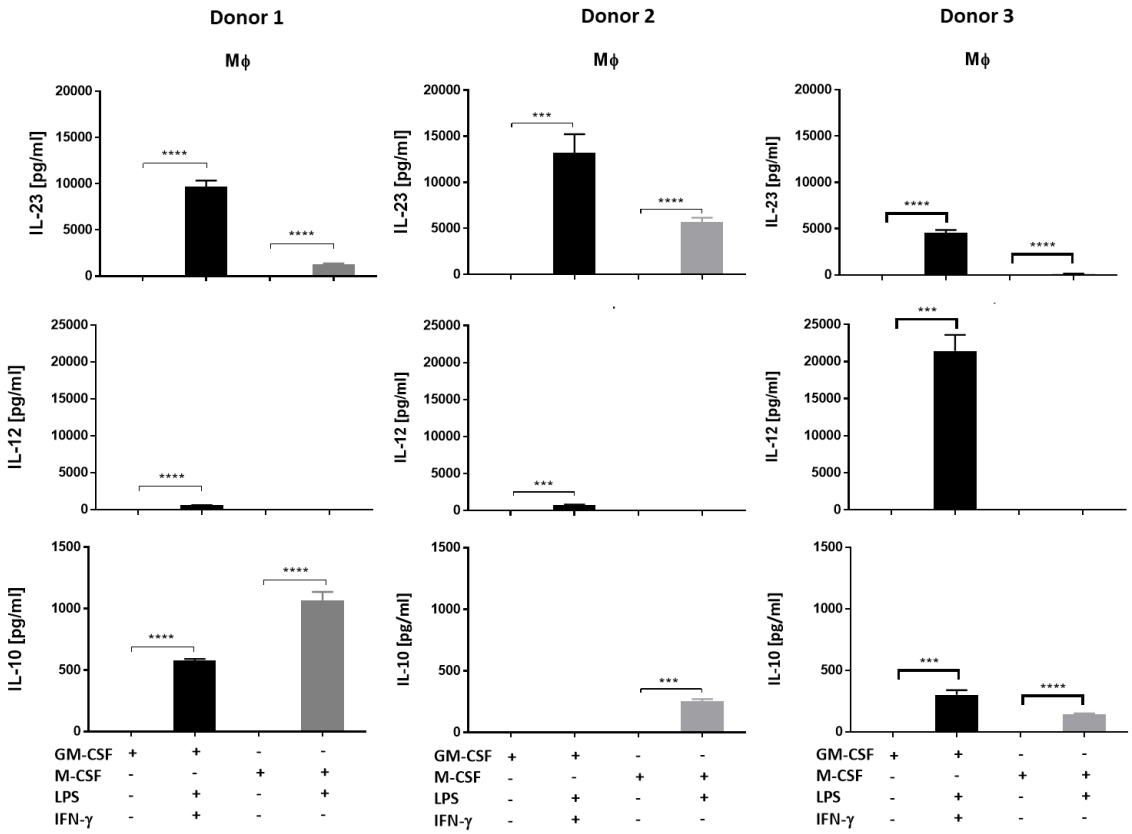

Figure 3.12 Cytokine profile of GM-Mϕ and M-Mϕ generated in ultra-low attachment 6-well plates. $CD14^{+}$ cells (5 x 10^{6}) were cultured in low attachment plates in the presence GM-CSF or M-CSF for 6 days. On day 7, cells were counted, washed and transferred into 96 well plates, rested for 1–2 hr before activation with LPS/IFNγ or LPS only for 24 hr. Data shown mean cytokine expressions amongst of triplicate well +/- SD and is representative 24 separated experiments. Cytokine secretion was quantified by ELISA and statistically analysed by t-tests.

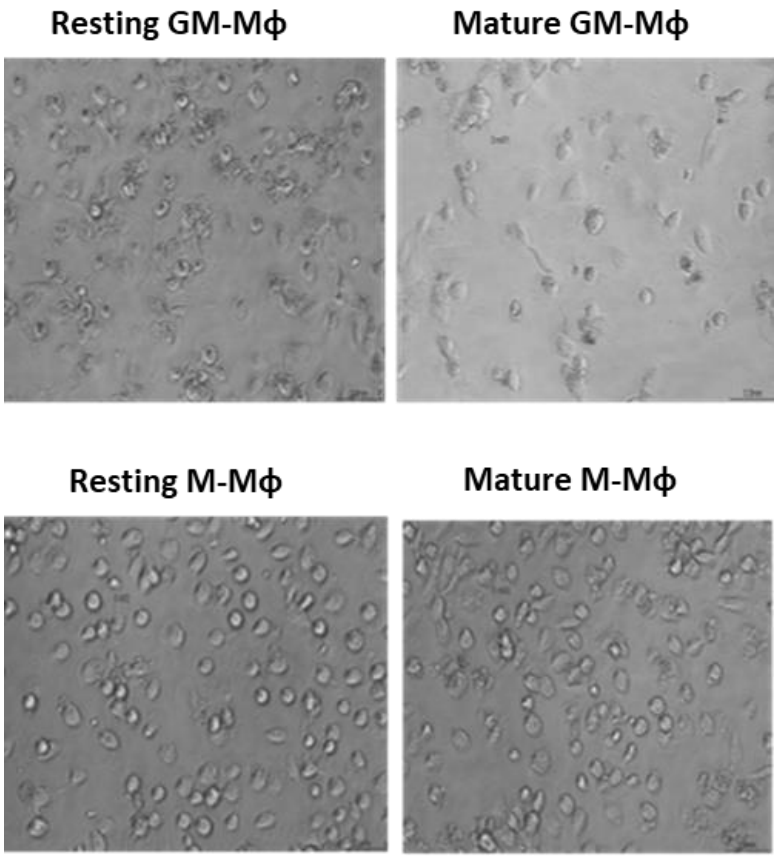

Figure 3.13 Morphology of GM-Mϕ and M-Mϕ. Phase contrast micrographs were taken under 10x magnifications on day 7 prior.

Cytokine	G-Mϕ Mean+/-SD (pg/mL)	M-Mϕ Mean+/-SD (pg/mL)	P Value
IL-12	4159.7+/-5277.6	1017.1+/-1674.9	<0.0002
IL-10	963.8+/-1684.4	1316.6+/-1816.4	Ns
IL-23	4939.8+/-3349.5	686.7+/-1349.6	<0.0001

Table 3.4 Characterising Mϕ by cytokine secretion of IL-10, IL-12 and IL-23 in response to LPS and IFNγ or LPS only. Mϕ were activated using a standard concentration of LPS/IFNγ (5 µg LPS/1000U/ml IFNγ) and cytokine secretion measured by ELISA after 24h. ns not significant.

3.3.11 Characterising MoDC by cytokine secretion

Previous studies in our lab examined the efficiency of MoDC generation, morphology, their phenotypic and secretory profile. MoDC assessed by microscope were found to be unattached. Activated DCs primarily secrete cytokines such as IL-12 and IL-23 (Collison & Vignali, 2008). The IL-12 and IL-23 cytokines are unique, even if there are similarities in their receptors, downstream signalling components and structure, but they often have different biological functions. Pro-inflammatory DCs comprise both IL-12 and IL-23, however Th1 responses upregulated by stimulation of IL-12 promote anti-microbial resistance, also chronic inflammation is stimulated through activation of Th17 by IL-23 (O'Shea & Paul, 2002; Stritesky, Yeh, & Kaplan, 2008).

This experiment was performed to demonstrate whether there is abundant cytokine secretion by activated DCs, including IL-12, IL-10 and IL-23. Purified $CD14^{+}$ cells were cultured in T75 flasks for a total of 5 days in the presence of GM-CSF and IL-4. On day 6, cells were harvested, washed, counted and seeded in 96 well plates and rested for 1 hr. Rested cells were activated with LPS/IFNγ for 24 hr before cytokine secretion was quantified by ELISA. Interestingly, the expression of IL-12 by DCs from all 3 donors was significantly increased compared to immature cells and DCs expressed IL-23 it was observed that cells from 3 of 3 donors secreted a high level of IL-12 compared to IL-10 and IL-23, whilst IL-23 secretion was significantly increased compared to IL-10. In contrast, donor 3 expressed high-level IL-12 compared to donor 1 and 2. However, donor 3 secreted a low level of IL-10 and IL-23 compared to others (Figure 3.14). It is possible that the differences between donors may be due to ethnic or biological variation, health condition or age of the donors. (Figure 3.14) shows that DCs from all 3 donors significantly expressed IL-12, IL-10 and IL-23 compared to inactivated DCs, with a higher level of IL-12 secretion compared to IL-10 (Table 3.5). These results are in agreement with previous findings (A Malecka, PhD 2017), that human monocytes were differentiated into immature DCs in the presence of GM-CSF combined with IL-4, resulting in the growth of an efficient number of DCs (Colic, Stojic-Vukanic, Pavlovic, Jandric, & Stefanoska, 2003).

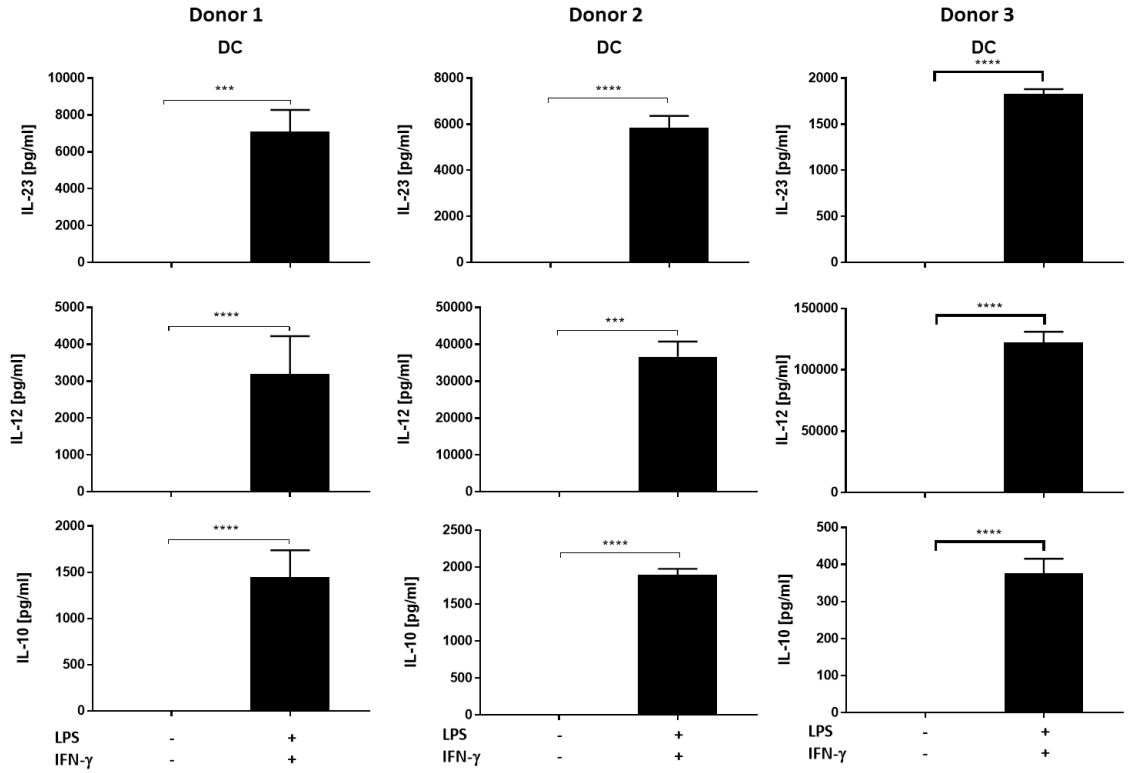

Figure 3.14 Cytokine profile of MoDC. CD14^{+} cells (15 x 10^{6}) were cultured in T75 flasks in the presence of 1000U/ml of GM-CSF and 1000U/ml of IL-4 for 5 days. On day 6, cells were counted, washed and transferred into 96 well plates and rested for 1–2 hr before activation with LPS/IFNγ for 24 hr. Data shown mean cytokine levels of triplicate wells +/- SD. Cytokine secretions were quantified by ELISA and statistically analysed using t-tests.

Cytokine	Mean+/-SD (pg/mL)	P Value
IL-12	25957+/-37050.5	<0.0001
IL-10	1184.8+/-1450.53	<0.0001
IL-23	4398+/-2507.9	<0.0001

Table 3.5 Characterisation of DCs by cytokine secretion in response to LPS and IFNγ. DCs were activated using a standard concentration of LPS/IFNγ (5 µg LPS/1000U/ml IFNγ) compared to DCs only. And cytokine secretion measured by ELISA after 24h.

3.4 Discussion

The differentiation of monocytes into Mϕ *in vitro* is characterised by the secretion of cytokines, namely IL-12, IL-10 and IL-23, which play a crucial role in regulating immune responses. Moreover, the *in vivo* and *in vitro* differentiation of monocytes into Mϕ is modulated by many exogenous stimulations, such as TLR ligands, cytokines, cell–cell contact, and surface attachment, leading to tissue and condition-specific Mϕ phenotype profiles (Rey-Giraud et al., 2012; M. Shen & Horbett, 2001; Y. Yang et al., 1996). There are many ways in which to generate MoMϕ, and these approaches vary from laboratory to laboratory, depending on whether low-attachment plastic surfaces or standard-attachment plates or flasks are used (Ambarus et al., 2012; Duluc et al., 2007). A previous study showed that the direct use of GM-CSF polarises the phenotype of MoMϕ into GM-Mϕ, while M-CSF differentiates MoMϕ into Mϕ2 (Fan et al., 2003). Additionally, the morphology of MoMϕ differentiated on low-attachment surface plates, regardless of the presence of GM-CSF or M-CSF, is characterised by a "fried egg" shape under microscopic examination (Rey-Giraud et al., 2012).

Following the successful generation of MoMϕ, we then investigated pro- and anti-inflammatory Mϕ, discovering that GM-Mϕ activated with LPS/IFNγ was characterised by IL-12 and high IL-23 secretion and lower IL-10 secretion, while additionally M-Mϕ was characterised by IL-10 and low IL-23 secretion without IL-12. These findings are in agreement with previous studies (A Malecka, PhD thesis, University of Nottingham, 2017, Xue et al. 2014) demonstrating the production of pro- and anti-inflammatory cytokines, such as IL-12, IL-10 and IL-23, by polarised GM-Mϕ and M-Mϕ when stimulated with LPS/IFNγ or LPS only, respectively.

The secretion of cytokines by MoDC was also investigated. Sallusto et al. showed that, *in vitro*, monocytes stimulated with GM-CSF/IL-4 differentiated into MoDC (J. Liu et al., 2016). IL-4 in combination with GM-CSF is typically used to polarise human monocytes into MoDC, as it suppresses the generation of Mϕ (Menges et al., 2005). With regard to infectious diseases, the priming of DCs to LPS derived from pathogens can be stimulated by TLR binding to their ligand on DCs, with the host cells at the infection site secreting pro-inflammatory cytokines such as IFNγ (Banchereau et al., 2000; Heath & Carbone, 2001; Steinman & Banchereau, 2007). In the present study, the activation of polarised MoDC with LPS/IFNγ *in vitro* induced

the production of high levels of IL-12, but lower levels of IL-23 and IL-10. This is in line with recent findings which showed that, following LPS/IFNγ activation, human monocytes underwent differentiation into MoDC, producing pro- and anti-inflammatory cytokines in the combined stimulation with GM-CSF and IL-4 (A Malecka, PhD 2017), (Gardner et al., 2018).

Dendritic cells are prominent and unique for mononuclear phagocytes existing in blood, lymphoid organs, and all tissues. They play a central role in digesting materials, including pathogens, and present processed epitopes to T cells, indicating the regulation of innate and adaptive immune responses (Haniffa et al., 2015). Over the past decades, the heterogeneity of DCs has been identified, comprising several subtypes with prominent functions in mice and humans. However, there are many factors related to DCs that are still unclear, such as how their subtype exists, how they relate to each other, and how they vary from other mononuclear phagocytes (Villani et al., 2017). However, DCs are still biasedly defined by the limited markers available for identifying, isolating and manipulating the cells. Such biases would affect the role of the function and ontogeny of the DCs subtype (Mildner & Jung, 2014; Schraml & Reis e Sousa, 2015). Single-cell RNA sequencing (scRNA-seq) was used to resolve the problem posed by certain limitations (as mentioned) in order to better assess the diversity of blood DCs and monocytes. This allowed us to confirm new subtypes of DCs and monocytes, refine their current classification, and also pinpoint cDC precursors in blood (Grun & van Oudenaarden, 2015; Trapnell, 2015; Villani et al., 2017).

Previous studies showed that the classification of human blood DCs is divided into pDC and cDC populations. Another study classified DCs into six populations: DC1 is characterised by the cross-presenting CD141/BDCA-3+ cDC1, of which CLEC9A is the best marker; DC2 and DC3 correspond to CD1C/BDCA-1+cDC2, which are new subdivisions; DC4 refers to CD1C-CD141-CD11C+DC, which is best marked by CD16 and shares characterisation with monocytes; DC5 corresponds to the DCs subtype, i.e. AS DCs; and DC6 is unique to the interferon-producing pDC, purer than the population of previously-identified pDC that was assessed by standard markers (e.g. CD123, CD303/BDCA-2+) and contaminated with AS DCs (Villani et al., 2017). Although macrophage development is well established by the roles of M-CSF and CSF-IR, intracellular signalling pathways constitute poorly understood information which is used to regulate macrophage generation. Stimulation through M-CSFR engagement leads to the

activation of multiple signalling cascades, such as Tyk2, STAT1, STATs, MAPK and PI3K (Novak et al., 1995; Yusoff, Hamilton, Nolan, & Phillips, 1994).

Since IFN-γ is a unique molecule in the polarisation of M1 macrophages, it is of little surprise that responses are induced by the mediation of JAK1, JAK2 and STAT1, as this cytokine stimulates these major signalling molecules. The activation of STAT1 homodimers can be triggered by IFN-γ; in turn, the transcriptional gene of classic M1 macrophages was induced (Darnell, Kerr, & Stark, 1994). IL-10, arginase 1, and the chemokines CCL17 and CCL22 are expressed at high levels by M2 macrophages (Bonecchi et al., 1998). The polarisation of the M2 phenotype corresponds to STAT6 – a signalling cascade stimulated by IL-4 and IL-13 (Takeda et al., 1996). Conversely, STAT1 phosphorylation inhibits IL-10, and inhibits signals predominantly through STAT3, which triggers ARG1 upregulation in mycobacteria-infected macrophages (H. S. Li & Watowich, 2014). The role of STAT3 has been well established: to suppress the secretion of pro-inflammatory cytokines in macrophages and neutrophils, resulting in lethal intestinal inflammation mediated by the cells, which was inhibited *in vivo* (O'Shea & Murray, 2008; Takeda et al., 1999).

Early studies reported that a primitive macrophage lineage can exist via self-renewal capacity and arises earlier in the development of definitive haematopoiesis, in the absence of a monocyte intermediate (Naito et al., 1996). The first identification of primitive macrophages was in the blood islands of the extra-embryonic yolk sac (YS) around embryonic day 8 (E8); from E8.5 to E10 they migrate to several tissues, giving rise to the proliferation of foetal macrophages independently of monocytes (Naito, Takahashi, & Nishikawa, 1990; K. Takahashi, Yamamura, & Naito, 1989). In mice, in YS blood islands on day E7.5, which comprise the first haematopoietic cells, primitive erythrocytes and macrophages were produced without lymphocytes (Bertrand et al., 2005; Palis, Robertson, Kennedy, Wall, & Keller, 1999). In humans, the third week of haematopoiesis development can be initiated in the extra-embryonic YS, and erythromyeloid cells are limited (Tavian & Peault, 2005).

There are several physiological agents that may affect immune sufficiency (including nutritional status, hormone levels, and circadian rhythms). There are also some technical characteristics of blood drawing that can influence how immune cells are derived from whole blood in terms of viability, yield and function (Mallone et al. 2011).

We have examined the effectiveness of the isolation of monocytes in PBMC from whole blood obtained from several donors, avoiding the contamination of monocytes with other cells that may influence their function. The yield of monocytes obtained from purified from PBMC and generated laboratory APC was assessed. Given the lack of literature on these technical aspects in the isolation and protection of CD14+ monocytes and the subsequent differentiation into Mϕ and DCs, we collected details from previous studies.

The population of the resident macrophages may contribute with phenotype of monocyte-derived macrophages, depending on the nature of the injury and the organ. Accumulating evidence has shown that Kupfer cell necrosis was induced through infection with *Listeria monocytogenes*; in this case, the contribution of monocyte-derived macrophages to repopulating the liver macrophage population was initiated (Bleriot et al., 2015; Lavin, Mortha, Rahman, & Merad, 2015). However, the proliferation and expansion of resident Kupfer cells are associated with paracetamol-induced injury, and the contribution of monocytes to the resident macrophage pool is not remarkable (Zigmond et al., 2014). Dendritic cells, monocytes, and macrophages are categorised by the mononuclear phagocyte system (MPS), which have several functions during immune responses. Historically, monocytes are considered to be the primitive progenitors of macrophages and DCs (Chomarat, Banchereau, Davoust, & Palucka, 2000). Macrophages are characterised by a large vacuolar shape that excels in the elimination of apoptotic cells, cellular debris and pathogens, as well as phenotypes that have been identified in mice as F4/80^{hi} cells (Austyn & Gordon, 1981). Conversely, DCs are distinguished as stellate-shaped cells that have been well established in terms of their ability to present antigens on MHC molecules and prime naive T cells (Steinman & Witmer, 1978). In mice, CD11c^{hi}MHC class II+ is a unique expression on DCs (Guilliams et al., 2014).

The monocyte-derived model of human DCs has been the mainstay of laboratory investigations for several decades. There is no direct correlate of moDC to be found in humans. In humans, DCs derive from pre-cDC and pre-pDC precursors, which, in turn, derive from CDP (Villani et al., 2017). Furthermore, the naturally occurring DCs subsets have substantially enhanced the properties of antigen-processing, specialised cytokine profiles and, importantly, cross-presentation.

In contrast with the DCs model, the monocyte-derived MO system has direct physiological relevance to our understanding of MO biology (Guilliams et al., 2014). While the organ-specific specialised MO (e.g. Kupfer cells, microglial cells, and Langerhans cells in the liver, brain, and skin, respectively) originate from embryonic stem cells under the control of cytokines CSF1, CSF2, IL-34 and TGFβ, in contrast, tumour-associated macrophages do not originate from stem cells, but rather from circulating CD14+ monocytes that enter tissues or are actively recruited. Under the control of the STAT1 transcription factor, CD14+ monocytes will differentiate into an M1-like cell capable of producing IL-12. In contrast, where the STAT3 transcription factor is activated, monocytes polarise to M2-like cells that secrete IL-10 amongst other cytokines. Thus, the monocyte-derived model of MO has biological relevance and can be used to dissect the role of TAM in cancer.

We establish the restriction in Mϕ plasticity and provide the microenvironmental conditions required in the early stages of differentiation of Mo into Mϕ for a terminal polarised Mϕ status. Furthermore, we demonstrate the importance of the initial activation on Mϕ in response to prolonged exposure to stimuli as a result of innate memory.

We refer to the previous protocols for Mϕ generation by using GM-CSF and M-CSF to provide GM-Mϕ and M-Mϕ, respectively. The significance of these initial phenotypes is frequently neglected because of the high plasticity of Mϕ (Duluc et al., 2009; Watkins, Egilmez, Suttles, & Stout, 2007). Smith et al. showed that MoMϕ consecutively or simultaneously treated with Mϕ1/Mϕ2 stimuli can induce different phenotypes where cells express Mϕ1 and Mϕ2 markers to some degree (Smith, Tse, Read, & Liu, 2016). Similarly, the phenotype of the resident tissue Mϕ often exhibits the profile of both pro- and anti-inflammatory Mϕ (Y. C. Liu, Zou, Chai, & Yao, 2014; Smith et al., 2016). We hypothesised that the differentiation of monocytes within environmental conditions can push it towards Mϕ, which may induce a specific signature on Mϕ and lead to the impact of a further response to pro- and anti-inflammatory stimulations that, in turn, leads to varied activation phenotypes. The activation of GM-Mϕ polarisation is often used with the combination of LPS and IFNγ for triggering. However, a comparison has never been made between activated Mϕ with LPS, and INFγ combined with GM-CSF or M-CSF.

Our findings regarding the importance of the monocyte differentiation into Mϕ phenotype indicate that the activation of Mϕ generated by using GM-CSF with the combination of LPS and INFγ expressed IL-10 in some donors.

In this investigation, we demonstrated that MoMϕ differentiated in the presence of GM-CSF or M-CSF exhibited a stable phenotype. *In vivo*, the exposure of Mϕ to constantly low levels of M-CSF is essential to sustaining the viability of Mϕ, and enhances an Mϕ2-like polarisation state (Hamilton, 2011; Lombardo, Alvarez-Barrientos, Maroto, Bosca, & Knaus, 2007). Mϕ viability is also maintained by GM-CSF, but its concentrations are promoted through inflammatory conditions and it can influence the phenotypic properties of Mϕ towards Mϕ1 (Lacey et al., 2012; J. Li, Hsu, & Mountz, 2012). It is essential to mention that initial studies are limited to laboratory-generated Mϕ and DCs using a single type of activation and polarisation stimulus, as well as short-term cell culture following activation for 24h. Further planned studies of this method will expand our findings on the different types of activation, in addition to offering a longer timeline of the culture, as Mϕ are long-living cells. The ultimate aim of this is to further the understanding of the impact of differentiation protocols on the response of Mϕ to activation.

We also examined the response of macrophages generated under conditions in our laboratory by comparing two different sources of LPS with the secretion of cytokines. Interestingly, LPS from both sources (Sigma and Invivogen) induced significantly strong secretions of IL-23, IL-12 and IL1-10 by Mϕ. Unexpectedly, LPS obtained from Invivogen induced IL-12 secretion by M-Mϕ. Furthermore, LPS from both sources (Sigma and Invivogen) induced significantly strong secretion by DCs. Our findings show that the purity of LPS obtained from Invivogen differs and assures only the presence of the TLR4 agonist, with others (Sigma) containing additional contaminants.

In summary, these findings demonstrate that the study of Mϕ and DCs polarisation can be used to assess the impact of FB on cytokine secretion by Mϕ and DCs, and then to further investigate 2D and 3D models.

Chapter 4 : Beyond 2-dimentional mono-culture of APC

Chapter 4 : Beyond 2-dimensional mono-culture of APC

4.1 Introduction

The tumour microenvironment can be highly heterogeneous and such differences are crucial factors that impact on tumour characteristics, including proliferation, invasion, metastasis, drug resistance and also immune escape (Y. Kim, Stolarska, & Othmer, 2011; Spano & Zollo, 2012). Solid tumours are not comprised of cancer cells alone but include a range of stromal cells, such as cancer-associated fibroblasts (CAF), tumour-associated macrophages (TAM), endothelial cells, mesenchymal stem cells, fat cells and immune components (Kalluri, 2016b; Shiga et al., 2015). Understanding the roles and importance of such a heterogeneous and dynamic mixture of cell-types in malignancy remains a considerable challenge. The majority of investigations sit at opposite poles of the cancer research spectrum which ranges from the patient (or animal model) to *in vitro* mono-layer cultures of single cell lines.

In recent years a growing number of studies have aimed to bridge the gap between single cell-line research and the patient by assembling increasingly complex experimental systems. These approaches include *in vitro* co-culture of different cell types (e.g. T-cells and tumour cells, DCs and T-cells, APC and fibroblasts) in conventional tissue-culture plastics, 3-dimensional tumour culture systems in contrast to mono-layer culture (e.g. tumour spheroids, tumours grown in matrix), and a combination of both these systems. As expected, important findings have resulted from such methodologies as they permit a more-physiological *in vitro* model in which cell-cell contact, hypoxia, and interactions between multiple cell types can be investigated. Nevertheless, these studies are in their infancy and further attention to such models is required.

Anticancer drug screening has traditionally been undertaken using the two-dimensional culture systems of cancer cell lines (Marino, Bishop, de Ridder, Delgado-Calle, & Reagan, 2019). However, there is poor correlation between efficacy in preclinical 2-D culture systems and efficacy in clinical trials (DiMasi, Grabowski, & Hansen, 2015). There are significant differences between the *in vitro* culture conditions of cancer cell lines to those of *in vivo* cancer environment with 2 important differences;

1) Most cancer tissues consist of cancer cells and various stromal cells (Shiga et al., 2015). Therefore, to mimic the cancer tissues, rather than using single cell line monolayers which do not represent the real physiological situation, co-culture or multi tri-culture of cancer cells with the stromal cells, including CAF and TAM should be considered;

2) The 2D monolayer cell culture does not represent and mimic the 3D structure of the *in vivo* cancer environment. Thus, it is important to establish 3 D cell culture techniques that better represent the in vivo environment.

In the body, it is well known that the biological functions, including proliferation, cell-cell interaction, and enzyme production are enhanced through cells interacting with other cells in a 3D manner (Fukuda, Sakai, & Nakazawa, 2006; Kurosawa, 2007; Nii, Makino, & Tabata, 2020). The range of technologies of cancer 3D tissue engineering have been reported (Burdett, Kasper, Mikos, & Ludwig, 2010). Cell aggregates have been tried, but poor oxygen and nutrient supply leads to loss of the function and cell death (Nii, Makino, & Tabata, 2019b). Gelatin hydrogel microspheres (GM) have been investigated as a distinct biomaterial for tissue regeneration that can be incorporated in cell aggregates to enhance cell survival (Kimura & Tabata, 2010; Ogawa, Akazawa, & Tabata, 2010). In addition, GM are able to release growth factors or to further promote more physiological cell functions. Of importance to this thesis, Haung et al studied TAM using GM (Huang, Li, Fu, & Xin, 2018). Cells present in TAM aggregates were incorporated with GM to allow survival of TAM and boost their functions. Moreover, the TAM functions were enhanced by adding adenosine (A), which exerts potent positive effects on TAM functions, the GM allows adenosine to impregnate for the enhanced release in TAM aggregates (3D TAM-GM-A). Then, co-culture of the 3D TAM-GM-A was performed with liver cancer cells (HepG2) across a model basement membrane on both sides to examine the invasive cells (Murray et al., 2014; Nii, Kuwahara, Makino, & Tabata, 2020). We demonstrate the potential system of the interaction between Mϕ and FBs in mixed cell 3D cultures, and discuss the evidence for the direct impact of FB on Mϕ function.

Another method of establishing 3D systems is to establish spheroids. It is becoming clear that understanding the creation of spheroid components of 3D spheres is based on the formation of numerous layers (Okochi et al., 2009). This culture condition mimics a more physiological structure seen in solid tumours and includes both physical and biochemical features such as cell-cell contact and juxtacrine signalling respectively. Many different tumour cell lines have been cultured in 3D conditions and subjected to morphological analysis including glioblastoma, astrocytoma, Wilms' tumour, neuroblastoma, head and neck squamous cell carcinoma, melanoma, lung, breast, colon, prostate, ovarian, hepatocellular and pancreatic cancer (Chitcholtan, Asselin, Parent, Sykes, & Evans, 2013). This analysis shows that according to the shapes of the architecture of the spheroid they can be divided into three distinct groups: i) tight spheroids, ii) compact aggregates and iii) loose aggregates (Vinci et al., 2015; Vinci et al., 2012).

Our collaborator (Professor A Grabowska, University of Nottingham) has established spheroid cultures with tumour cells e.g. MCF7 breast cancer cell line and we used this model to establish our 3D cultures.

The measurement of nicotinamide adenine dinucleotide phosphate (NAD(P)H) concentration has been demonstrated by a colourimetric approach using tetrazolium salts (Luo, Wang, Long, & Liu, 2006). Tetrazolium salts have been utilized generally in biological and clinical researches, although their use is limited by the low solubility of the formazan product (Hamasaki, Kogure, & Ohwada, 1996; Ngamwongsatit, Banada, Panbangred, & Bhunia, 2008). There are various water-soluble tetrazolium salts (WST) such as WST-1, WST-3, WST-4, WST-5, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], and WST-9, which have been synthesized (Ishiyama, Miyazono, Sasamoto, Ohkura, & Ueno, 1997). Several electron mediators, including 1-methoxy-5-methylphenazium methylasulfate (1-mPM, can affect WST, which is readily decreased by NAD(P)H to produce a formazan product that can be measured by monitoring absorbance in the range 430-550 nm. The absorption of formazan is proportional to the concentration of NAD(P)H, in turn the WST-based colourimetric assay shows potential for several qualitative and quantitative applications (Chamchoy, Pakotiprapha, Pumirat, Leartsakulpanich, & Boonyuen, 2019).

WST-8 is one of the most of commercially available tetrazolium salts and is supplied as WST-8/1-mPMS (Dojindo). WST-8 relies on the NAD(P)H detection approach in cell proliferation and enzymatic assays. NAD(P)H plays a crucial role in reducing WST-8 to produce a formazan product which is characterized by a strong orange dye with maximum absorption at 450 nm (Tsukatani et al., 2009; Tsukatani et al., 2012).

Solid malignancies contain large numbers of FBs, termed tumour-associated fibroblasts (TAF), which differ substantially from FB in normal tissues (NAF). Whilst their role in would healing and tissue remodelling cancer is well established, their involvement in the modulation of local and systemic anti-cancer immunity is less well understood. However, recent studies have shown that FBs interact with APC, namely dendritic cells (Barnes & Adcock) and macrophages (Mϕ) (Kalluri, 2016a; Malecka et al., 2016; Schirmer, Klein, von Bergen, Simon, & Saalbach, 2010). These APC are largely responsible for orchestrating the immune system.

Crosstalk between FB and Mϕ produces signals that substantially alter APC cell biology. As a result of this interaction, TNF-α/IL-1β are secreted from activated APCs, and the stromal fibroblasts promote PGE2 secretion. PGE2 can stimulate APC to release IL-23 which plays a crucial role in activation of Th17, resulting in an increase tumour growth, angiogenesis etc. (Schirmer et al., 2010).

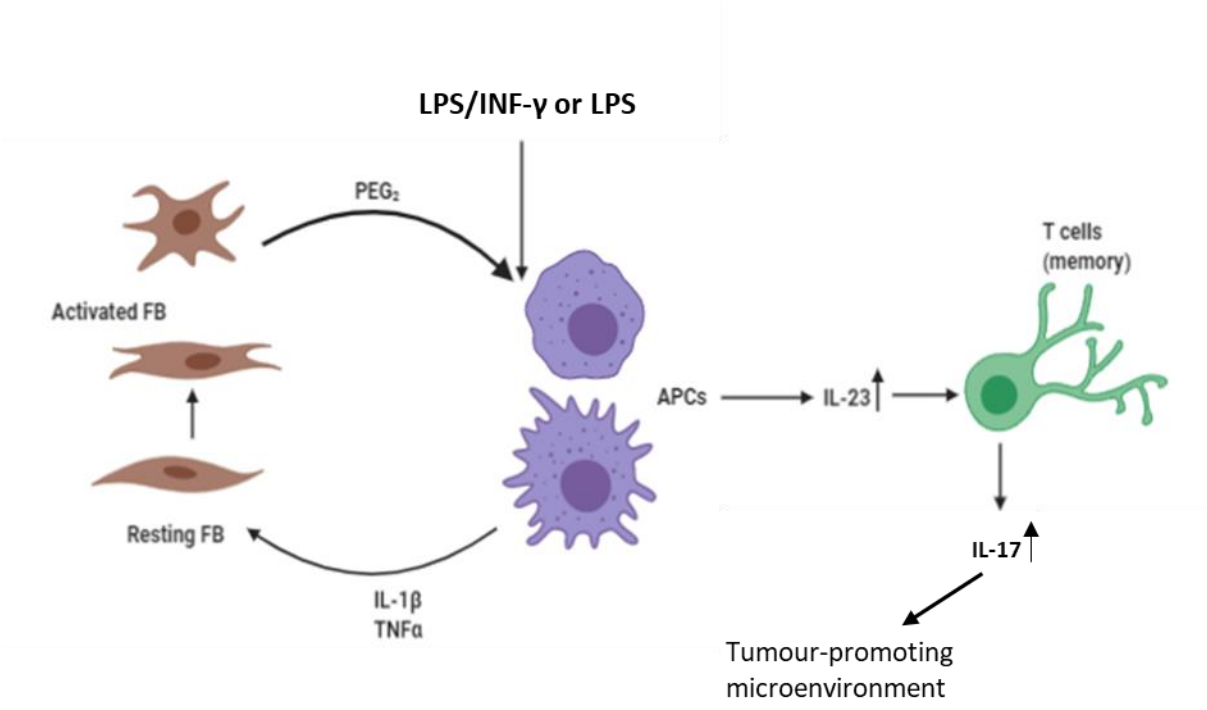

Figure 4.1 Model of interaction between fibroblasts and APCs and how they stimulate IL-23 secretion by pre-activated APCs resting leading to proliferation of Th17 cells.

4.2 Hypothesis and aims

The hypothesis that we aimed to test was| that "Tumours govern adaptive immunity by modulating the behaviour of local fibroblasts that in turn impacts of the function of antigen presenting cells."

The project will address changes in the basic biology of the APC, and how this impacts on their ability to communicate with key effector cells including CD4+ and CD8+ T-cells. We will establish co-cultures of tumour-conditioned fibroblasts and compare these with matched tumour-associated fibroblast/normal dermal fibroblast for their effect on the immunology of APC, namely Mϕ and DCs. In the first year Initial studies were limited with laboratory generated Mϕ and DCs (differentiated from CD14+ monocytes). Recently we have been established FBs/APCs co-culture experiments in 2D vs 3D models to identify how their interaction with FB affects functions of APCs. There is reason to believe that tumour cells differentially condition FB in 2D vs 3D cultures in terms of their capacity to support cancer cells. However, to date there are no studies examining how this impacts on APC. The effect of these will therefore be studied in 3D co-cultures and compared with conventional 2D culture systems.

4.3 Results

This study aimed to test the hypothesis that tumour cells differentially condition FB cells in 2D vs 3D cultures in terms of their capacity to support tumour progression through the influence of cytokine secretion by Mϕ, including IL-23.

The aim of this chapter was to investigate the impact of FB on cytokine production by Mϕ. Initially we did this by;

1. Optimising spheroid production of MCF7 and BJ6.
2. Measuring viability of MCF7 and BJ6 using WST8 solution-dependent metabolic activity of living cells.
3. Determining proliferation of MCF7 and BJ6 upon incorporation of ^{3}H-thymidine.
4. Determining the appropriate starting number of Mϕ in co-culture that allows FB to regulate cytokine secretion by APCs.

4.3.1 Growth and production of MCF7 breast cancer cell and fibroblast spheroids

Our collaborator (Professor A Grabowska, University of Nottingham) has established spheroid cultures with tumour cells e.g. MCF7 breast cancer cell line which we employed as a positive control prior to the generation of FB spheroid. To study the production of MCF7spheroids in 3D culture, FB cells were cultured in ULA 96-well (round bottomed) plates.

4.3.1.1 Optimization of BJ6 and MCF7 spheroids

The main ways for preparation of 3-D cultures include: i) using non-adherent plates to grow suspension cultures; ii) using a concentrated matrix or gel-like substance in cultures and iii) cultures use of a scaffold.

Based on previous studies by Professor A Grabowska (University of Nottingham) where spheroid cultures with breast tumour cell lines e.g. MCF7 were established the optimum number of MCF7 required for spheroid formation was (1000-2000). In contrast, the same number of FB results in minimal spheroids formation. It has been noticed that it is important to study their physiological behaviours, morphology and volume as a comparison and consequently, we can be established our spheroid model. To study the generation of spheroids in 3D culture, MCF7 cells or FB cells were cultured in ULA 96-well (round bottomed)

plated at a range of densities (1000-20000), the plate was centrifuged at 100 g for 4 min, then incubated for up to 7 days.

Spheroids were observed in both cultures Figures (4.2 and 4.3) confirming that spheroids could be generated with both MCF cells and FBs. Interestingly, as shown in in Figure 4.4, the volume of FB spheroids gradually decreased with time while the size of MCF7 increased.

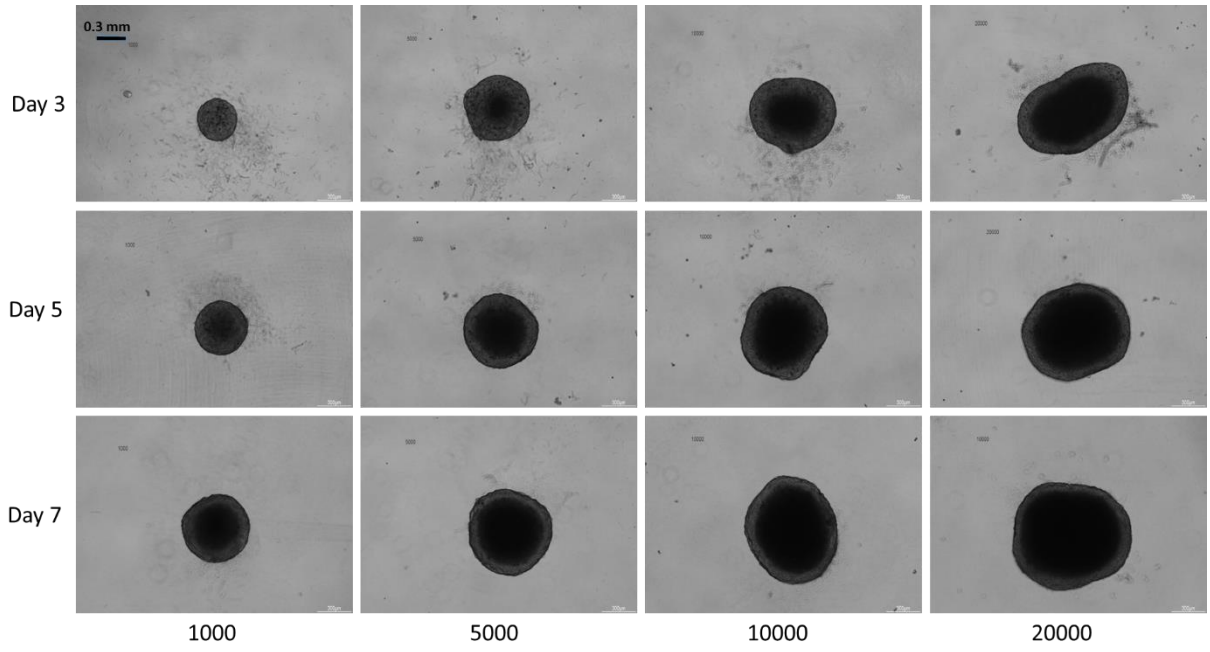

Figure 4.2 Macroscopic images of MCF7 tumour spheroids seeded at the indicated cell number in ULA 96-well (round bottomed) plates. Images of the same spheroid were taken at days 3, 5 and 7. MCF7 cells were cultured in ULA 96-well (round bottomed) plates at indicated cell numbers, the plate was centrifuged at 100 g for 4 min before culture at 37^{o}C and documentation by low-power microscopy at the indicated time points.

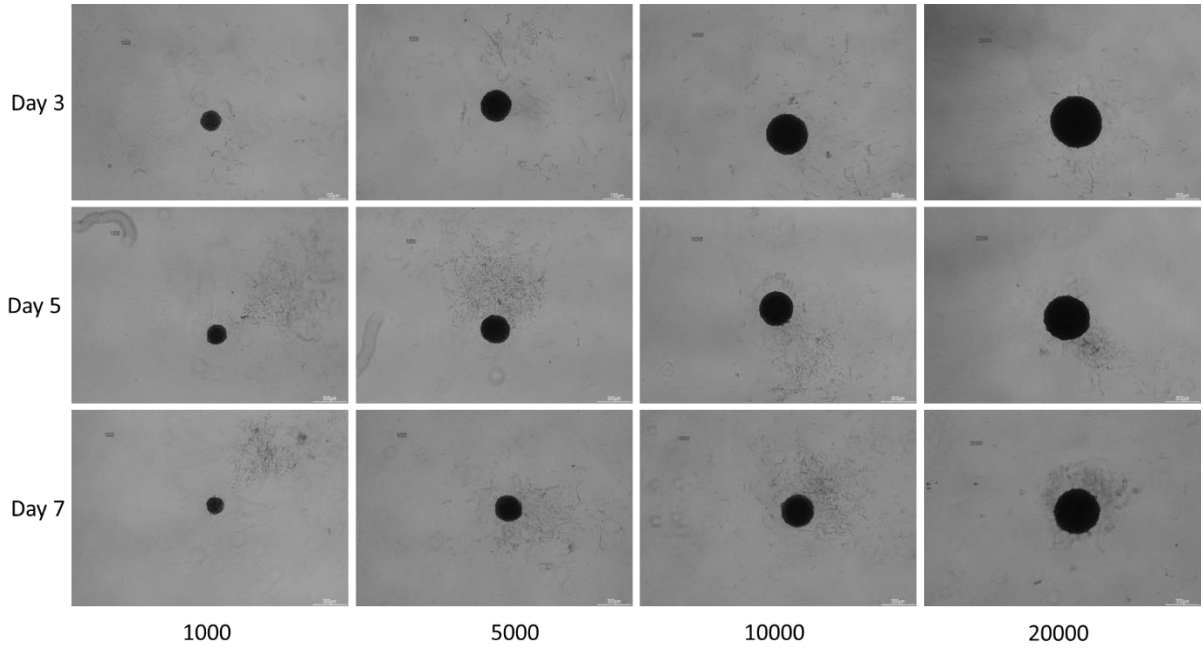

Figure 4.3 Macroscopic images of FB spheroids seeded at the indicated cell number in ULA 96-well (round bottomed) plates. Images of the same spheroid were taken at days 3, 5 and 7. FB cells were cultured in ULA 96-well (round bottomed) plates at indicated cell numbers, the plate was centrifuged at 100 g for 4 min at 3 different time points.

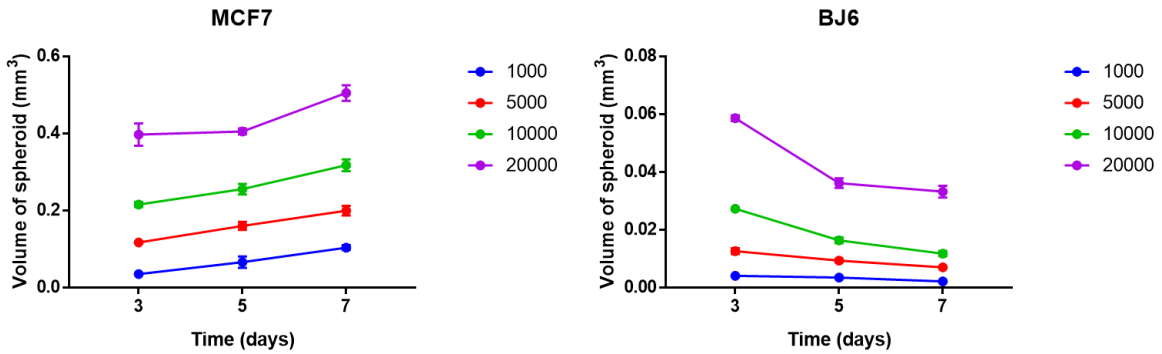

Figure 4.4 Growth of MCF7 and FB spheroids (mm^3) over the course of 1 week. Cells were cultured in a ULA 96-well (round bottomed) at the concentrations shown after 3, 5 and 7 days of culture in triplicate wells. Volumes were calculated based on the diameter as determined using Image J for the replicate wells +/-SD and is representative of three separate experiments.

4.3.1.1 Viability of MCF7 and BJ6 in spheroid production

On day 3, 5 and 7 WST8 solution was added to cells in parallel plates for overnight and the absorbance was measured at 460 nm using a plate reader. Increases in the absorbance (OD) of MCF7 and FB cells were detected with increased cell numbers and with increasing except the absorbance OD of 10000 FB on day 7 was slightly decreased (Figure 4.5).

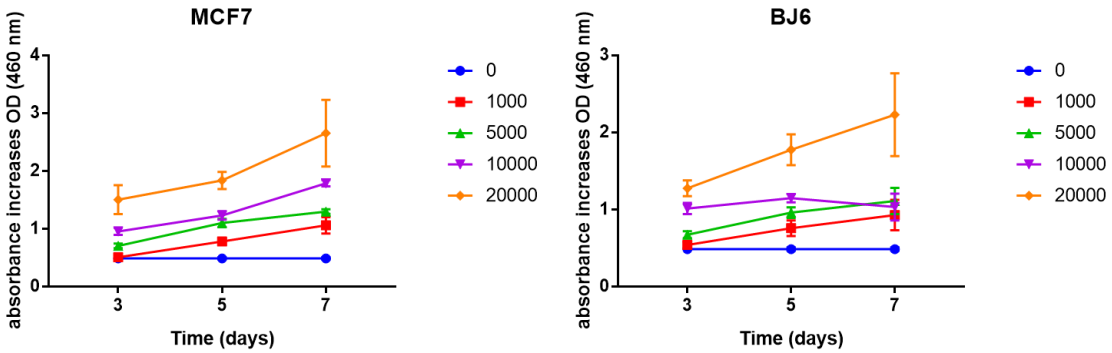

Figure 4.5 Metabolic activity of living FB and MCF7 cells. Cells were cultured in a ULA 96-well (round bottomed) at the cell numbers shown after 3, 5 and 7 days of culture in triplicate wells. WST8 solution was added to all wells for the last 8 hrs of culture in different time points as shown. The data shows the mean WST8 solution represented as absorbance increases OD (460 nm) for the replicate wells +/- SD and is representative of three separate experiments**.**

4.3.1.2 Determination of proliferation in BJ6 and MCF7 spheroids using ^{3}H-thymidine

This experiment was carried out in parallel the previous experiment using the same conditions including numbers of cells and time points of culture. Plates were pulsed with standard 3H-thymidine-incorporation for overnight on day 3, 5 and 7. Proliferation was assessed by measuring thymidine incorporation during the last 8 hrs (Figure 4.6). Figures show that the proliferation of FB spheroids was inhibited in each day and concentration compared to the actual starting number. In contrast, proliferation of MCF7 spheroids were increased dramatically on day 7 compared to day 3 and 5.

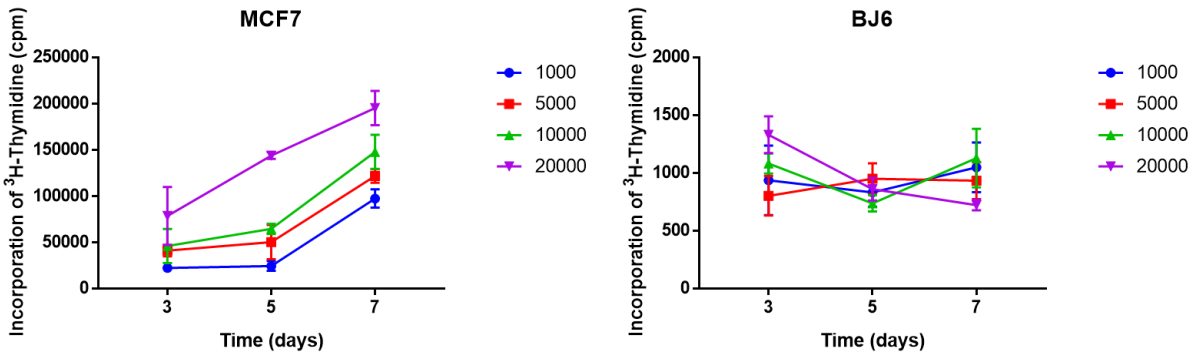

Figure 4.6 Proliferation of FB and MCF7 cells. Cells were cultured in a ULA 96-well (round bottomed) at the concentrations shown after 3, 5 and 7 days of culture in triplicate wells. ^{3}H-thymidine was added to all wells for the last 8 hrs of culture in different time points as shown. The data shows the mean incorporation of ^{3}H-thymidine represented as counts per minute (cpm) for the replicate wells +/-SD and is representative of three separate experiments.

4.3.2 Microscopic examination of spheroids

After production of FB spheroids we carried out microscopic examination of the spheroids to identify whether development of a necrotic core was observed. To this end, FB spheroids in different cell numbers (1000, 5000 and 10000) we prepared. Samples were fixed by 4% paraformaldehyde on day 3 and embedded in paraffin wax and then paraffin blocks were sectioned (3 µm) for Haematoxylin & Eosin staining. On microscopy at day 3 spheroids were formed properly without necrotic cores (Figure 4.7).

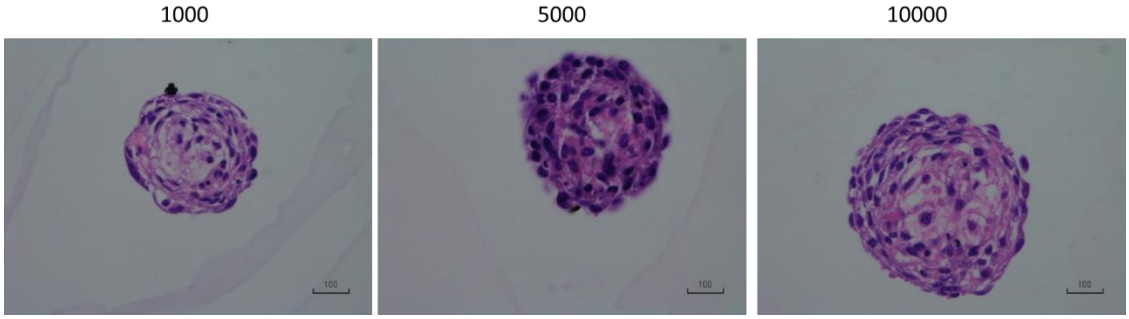

Figure 4.7 Characterisation of FB spheroids in 3D culture. Staining of representative FB spheroids (3 days old). Spheroid were carefully harvested, fixed and mounted in paraffin before 3µm thick sections were cut using a microtome. Mounted sections were stained with H+E dye for nuclei (blue) and cytoplasm (pink) respectively. Indicated are the number of cells with which spheroids were seeded. Scale bar show corresponds to 100mm.

4.3.3 Populating fibroblast spheroids with macrophages

We combined BJ6 fibroblasts with either GM-CSF treated monocyte derived macrophage (GM-Mϕ) or MCF treated monocyte derived macrophage (M-Mϕ) at different cell numbers:

16000 FB + 1600 Mϕ (10:1 ratio), 16000 FB + 3200 Mϕ (5:1 ratio), 12000 FB + 1200 Mϕ (10:1 ratio), 12000 FB + 2400 Mϕ (5:1 ratio) and examined them with light microscopy.

Figure (4.8) shows that the light microscope picture of 3D BJ6, 3D BJ6/resting GM-Mϕ and 3D BJ6/mature GM-Mϕ at different ratios as shown. Figure (4.9) shows that the light microscope picture of 3D BJ6, 3D BJ6/resting M-Mϕ and 3D BJ6/mature M-Mϕ at different ratios as shown. Moreover, on day 4 formation of spheroids was observed and solid, but disaggregation of some cells was attached at the bottom of wells irrespective of culture BJ6 only or co-culture of BJ6/Mϕ.

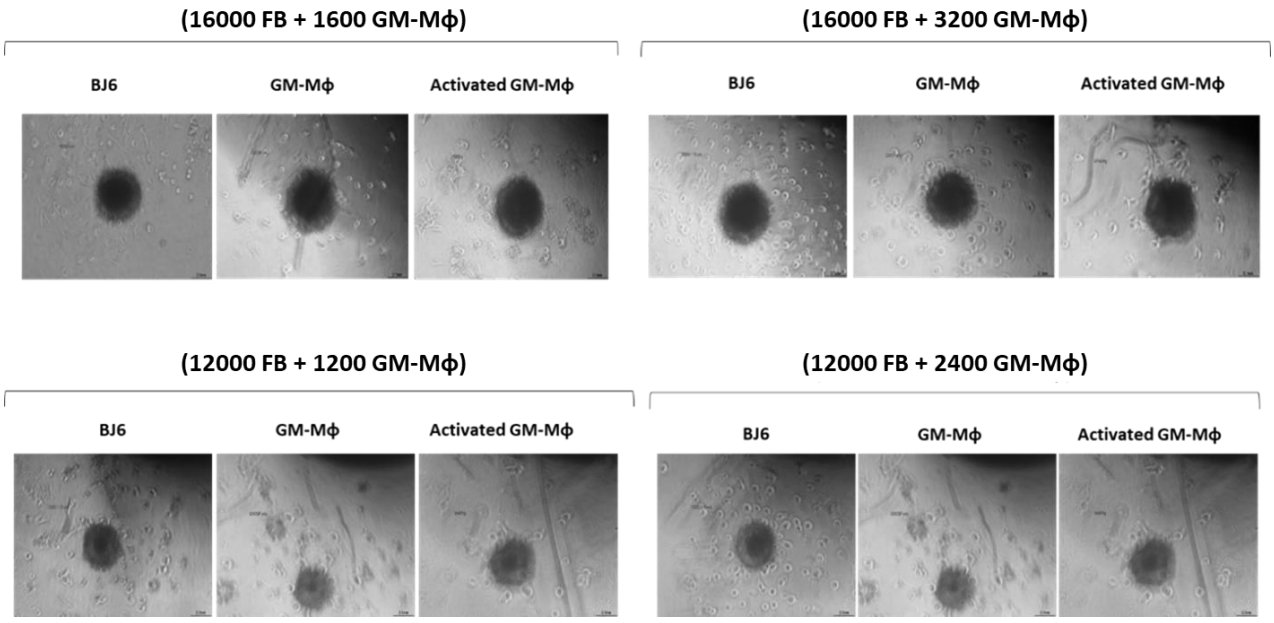

Figure 4.8 Optimizing Images of three-dimensional double culture of BJ6 FB/GM-Mϕ using phase contrast and light. Monocytes ($5x10^6$) were cultured in Ultra-low attachment 6 well plate and treated with 20U/ml of GM-CSF. After 6 days of the culture, cells were harvested and transferred into ULA- 96 well palates mixed with FB. Spheroid shapes at day 4 contains normal FB cultured with GM-Mϕ. From the top row and bottom rows the mixture of BJ6 FBs/GM-Mϕ and BJ6 FBs/GM-Mϕ (proportional FB/GM-Mϕ 1:10 and 1:5 respectively) for 4 days. Maturation was induced with LPS and IFN-γ for 24hr. Cells were spun at 100 g for 4 minutes and incubated for 3 days as shown.

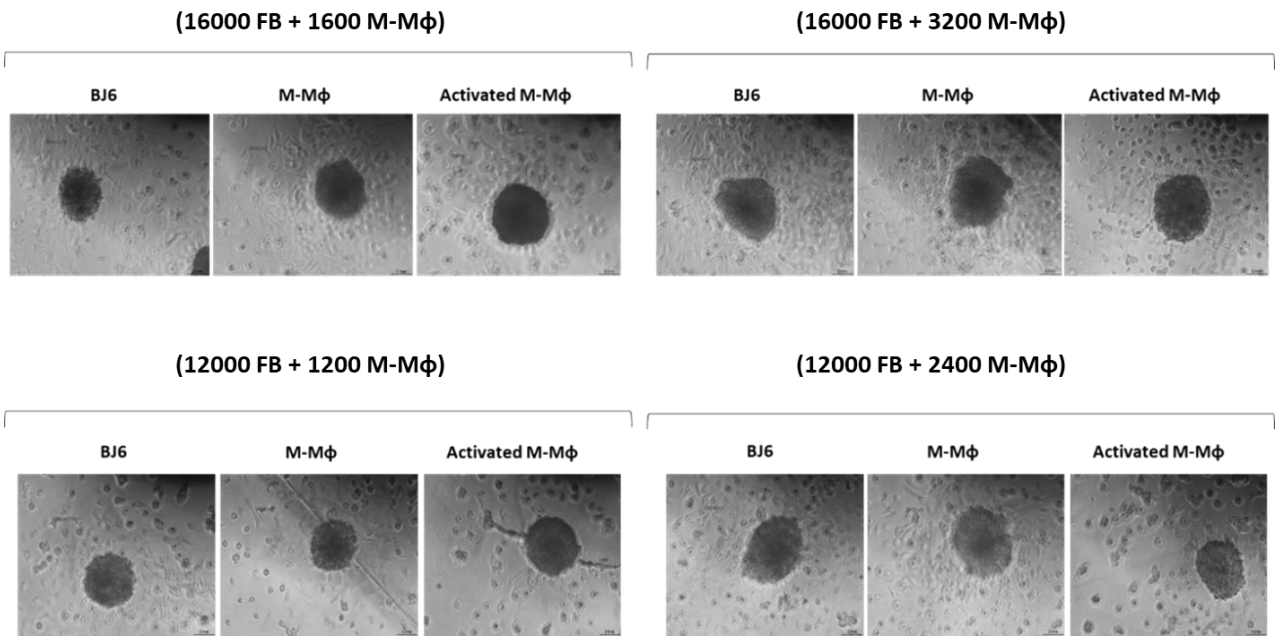

Figure 4.9 Optimizing Images of three-dimensional double culture of BJ6 FB/M-Mϕ using phase contrast and light. Monocytes ($5x10^6$) were cultured in Ultra-low attachment 6 well plate and treated with 10ng/ml of M-CSF. After 6 days of the culture, cells were harvested and transferred into ULA- 96 well palates mixed with FB. Spheroid shapes at day 4 contains normal FB cultured with M-Mϕ. From the top to the bottom rows the mixture of BJ6 FBs/M-Mϕ (proportional FB/M-Mϕ 1:10 and 1:5 respectively) for 4 days. Maturation was induced with LPS only for 24hr**.** Cells were spun at 100 g for 4 minutes and incubated for 3 days.

Previous studies have shown that the impact of FB on DCs and Mϕ (Comito et al., 2014a; Saalbach et al., 2007). Unfortunately, data revealed that cytokine secretion by Mϕ did not determine in 3D. Taken together, data suggest that this culture has two 2D and 3D in the same well, and concentration of soluble cytokines produce by Mϕ was low levels in a total volume of medium. On other hand, culture of Mϕ in these different numbers did not produce detectable levels of cytokines including IL-10, IL-12 and IL-23 (Data not shown) in 3D.

In this experiment and based on the previous experiment in (Figure 4.8, 4.9) it was decide to increase numbers of BJ6 and Mϕ cells in co-culture, we tried to demonstrate the ability of dermal FB to regulate MoMϕ cytokine expression in 2D model vs 3D system (Figure 4.10, 4.11). To better visualise FB and Mϕ in the spheroids we labelled BJ6 with red fluorescent protein and Mϕ with green fluorescent protein. A clearly well-defined spheroid border can be detected for all FBs as shown in both phase contrast and red protein labelled BJ6 FB fluorescent lights. BJ6 FB co-cultured with Mϕ spheroids that indicate in red protein labelled BJ6 FB and green CTV dye labelled Mϕ, increasing the number of cell did not production a consistent pattern of growth in spheroids size. However, non-uniform aggregates of spheroids were observed in an irrespective of co-culture of BJ6/mature or immature Mϕ (Figure 4.10 and 4.11).

We also prepared spheroids using NDF12 FB in Figure (4.12) shows spheroids made of NDF12 FB co-cultured with mature GM-Mϕ formed uniform and solid spheroids, where a distinct spheroid border was observed compared to those spheroids made of NDF12 co-cultured with immature GM-Mϕ. Spheroids made of ND12/M-Mϕ shows cell aggregates shaped as radial invasive patterns irrespective of the absence or the presence of LPS (Figure 4.13). These data would suggest that interaction of FB with Mϕ spheroids was promoting MMP productions, their functions to induce collagen and laminin degradation, resulting in radial invasive cells around and attached at the bottom of wells. Results are in line with the previous findings of data show that, interaction of CAF and TAM with cancer cells, leading to a higher production of matrix metalloproteinase (MMP), which promotes the invasive cancer cells, because MMP can degrade collagen and laminin, which are the major components of basement membrane (L. Liu, Ye, & Zhu, 2019).

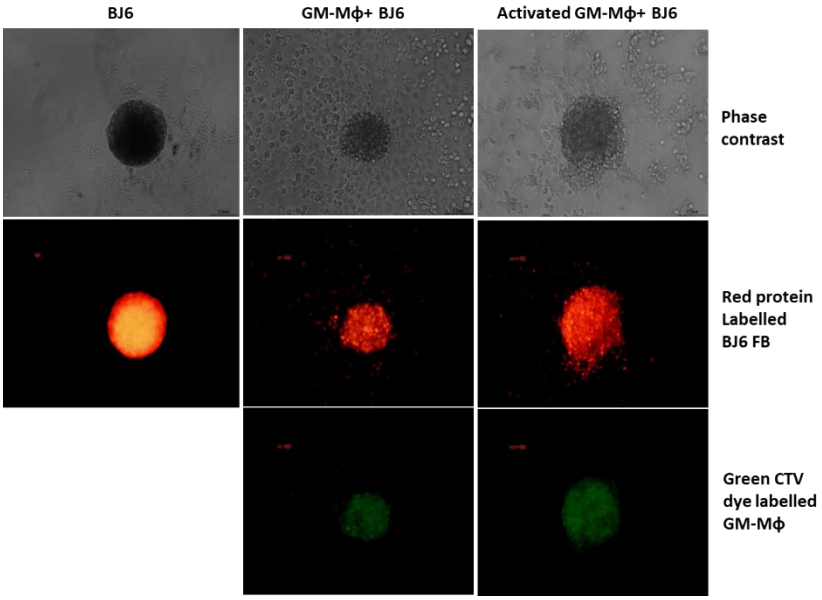

Figure 4.10 Images of three-dimensional double culture of BJ6 FB/GM-Mϕ using phase contrast, green and red fluorescent lights. Monocytes ($5x10^6$) were cultured in Ultra-low attachment 6 well plate and treated with 20U/ml of GM-CSF. After 6 days of the culture, cells were harvested and transferred into ULA- 96 well palates mixed with FB**.** Spheroid shapes made of BJ6 FB/GM-Mϕ at day 4 contains normal FB cultured with GM-Mϕ that are stained with cell trace violet (CTV) dye as green. Maturation was induced with LPS and IFN-γ for 24hr. BJ6 FB exposed red colour cultured with GM-Mϕ (green) were stained with cell trace violet (CTV) dye as shown.

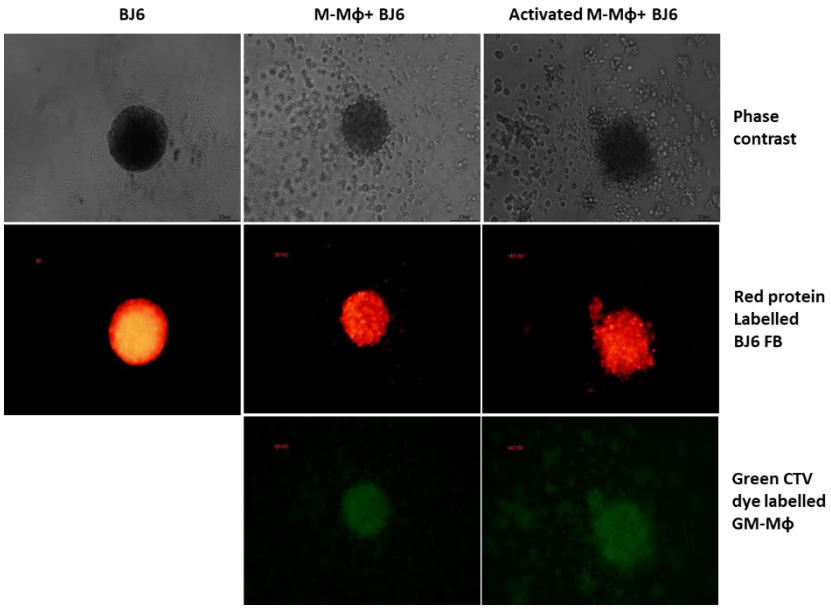

Figure 4.11 Images of three-dimensional double culture of BJ6 FB/M-Mϕ using phase contrast, green and red fluorescent lights. Monocytes ($5x10^6$) were cultured in Ultra-low attachment 6 well plate and treated with 10ng /ml of M-CSF. After 6 days of the culture, cells were harvested and transferred into ULA- 96 well palates mixed with FB. Spheroid shapes at day 4 contains normal FB cultured with M-Mϕ that are stained with cell trace violet (CTV) dye as green. Maturation was induced with LPS only for 24hr. BJ6 FB exposed red colour cultured with M-Mϕ (green) were stained with cell trace violet (CTV) dye as shown.

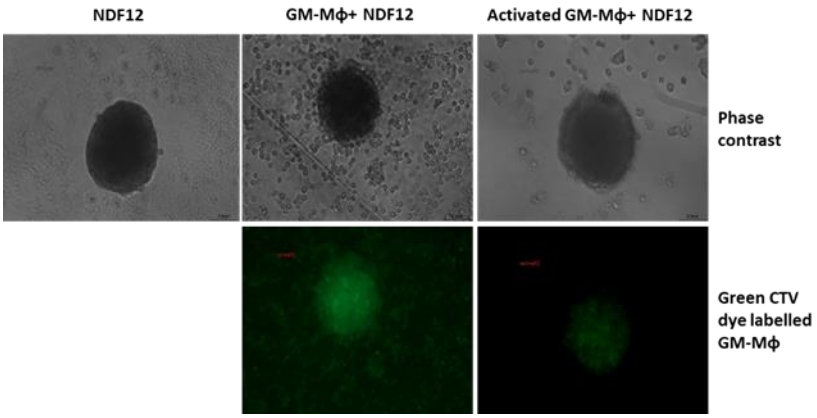

Figure 4.12 Images of three-dimensional double culture of NDF12 FB/GM-Mϕ using phase contrast and green fluorescent lights. Monocytes ($5x10^6$) were cultured in Ultra-low attachment 6 well plate and treated with 20U/ml of GM-CSF. After 6 days of the culture, cells were harvested and transferred into ULA- 96 well palates mixed with FB. Spheroid shapes at day 4 contains normal FB cultured with GM-Mϕ that are stained with cell trace violet (CTV) dye as green. Maturation was induced with LPS and IFN-γ for 24hr. NDF12 FB cultured with GM-Mϕ were stained with cell trace violet (CTV) dye as shown.

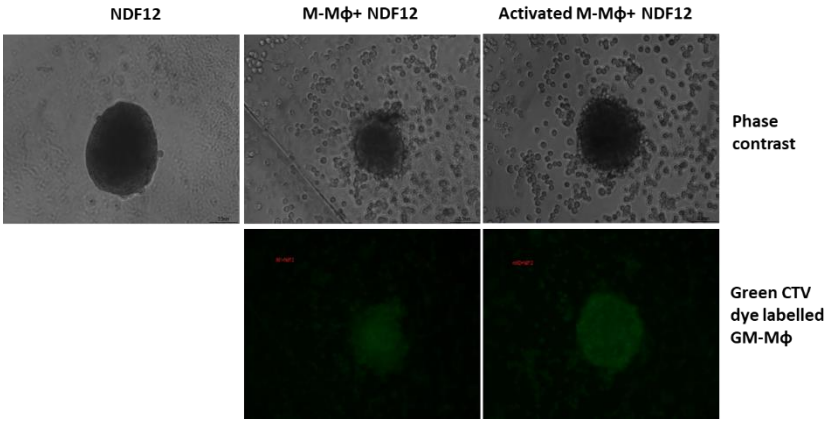

Figure 4.13 Images of three-dimensional double culture of NDF12 FB/M-Mϕ using phase contrast and green fluorescent lights. Monocytes ($5x10^6$) were cultured in Ultra-low attachment 6 well plate and treated with 10ng /ml of M-CSF. After 6 days of the culture, cells were harvested and transferred into ULA- 96 well palates mixed with FB. Spheroid shapes at day 4 contains normal FB cultured with M-Mϕ that are stained with cell trace violet (CTV) dye as green. Maturation was induced with LPS only for 24hr. NDF12 FB cultured with M-Mϕ were stained with cell trace violet (CTV) dye as shown.

4.3.4 Detecting the effects of FBs on cytokine secretion by Mϕ in 3D vs 2D

We measured cytokine levels in the supernatant of the experiments above.

When FB were cultured alone they did not express IL-12, IL-10 and IL-23 in either 2D or 3D systems. In 3D culture, IL-23 secretion by mature GM-Mϕ co-cultured with BJ6 and NDF12 was significantly ($P<0.001$ and $P<0.05$) decreased compared to mature GM-Mϕ alone in 1/3 donors, whereas IL-23 was significantly ($p<0.05$ and $p<0.01$) increased in another 1/3 donors. Expression of IL-23 in M-Mϕ did not change 1 out of 3 donors interacting with BJ6 (Figure 4.14).

Secretion of IL-23 by M-Mϕ co-cultured with BJ6 and NDF12 was inhibited in one donor. IL23 was significantly ($p<0.01$) decreased in the presence of NDF12 but IL-23 was inhibited when cultured with BJ6 in 1 donor. But in another donor expression of IL-23 by M-Mϕ was significantly ($p<0.0001$) decreased in the presence of BJ6.

In 2D system, we studied the effect of BJ6 and NDF12 on Mϕ (Figure 4.15). Expectedly, mature GM-Mϕ produced IL-23. In this setting interacting with BJ6 significantly ($p<0.001$) increased secretion of IL-23 but NDF12 did not affect IL-23. Interaction of BJ6 and NDF12 did not affect secretion of IL-23 by mature M-Mϕ.

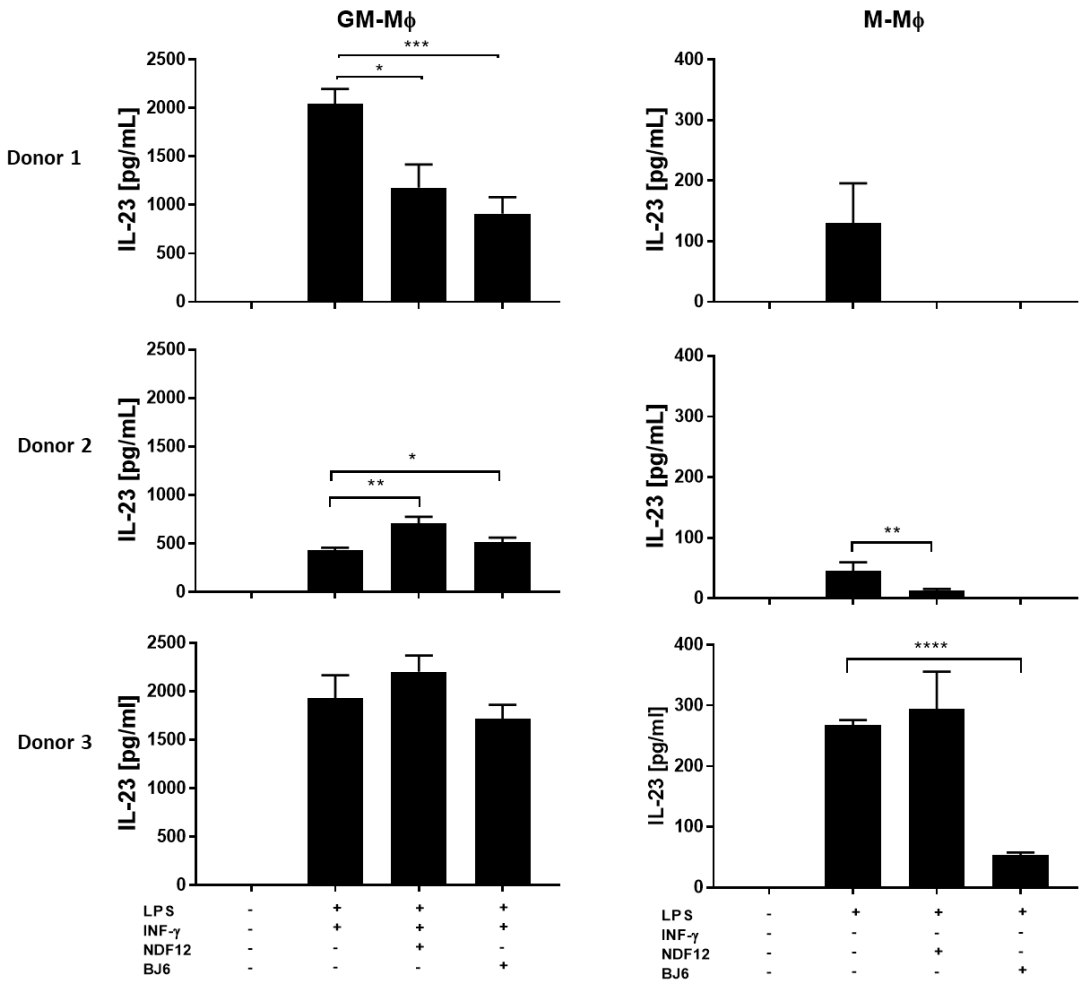

Figure 4.14 3D: Expression of IL-23 by activated Mϕ in the presence or absence of FBs. 5x10^6 monocytes were cultured in Ultra-low attachment 6 well plate with 20 U/ml of GM-CSF or 10 ng/ml of M-CSF. After 6 days, Mϕ were harvested. Next, the mixture of FBs/Mϕ (were cultured into ULA-96 well plates for 4 days. Cells were spun at 100 g for 4 minutes and incubate for 3 days. On day 3 spheroids were activated with 5µg/ml of LPS and 1000 U/ml of INFγ or 5µg/ml of LPS only for 24h. Next day supernatants were collected and cytokine secretion assayed by ELISA. Data showed mean cytokine secretion amongst of triplicate well +/- SD and is representative 3 experiments of 3 donors. Statistical differences determined using t-test.

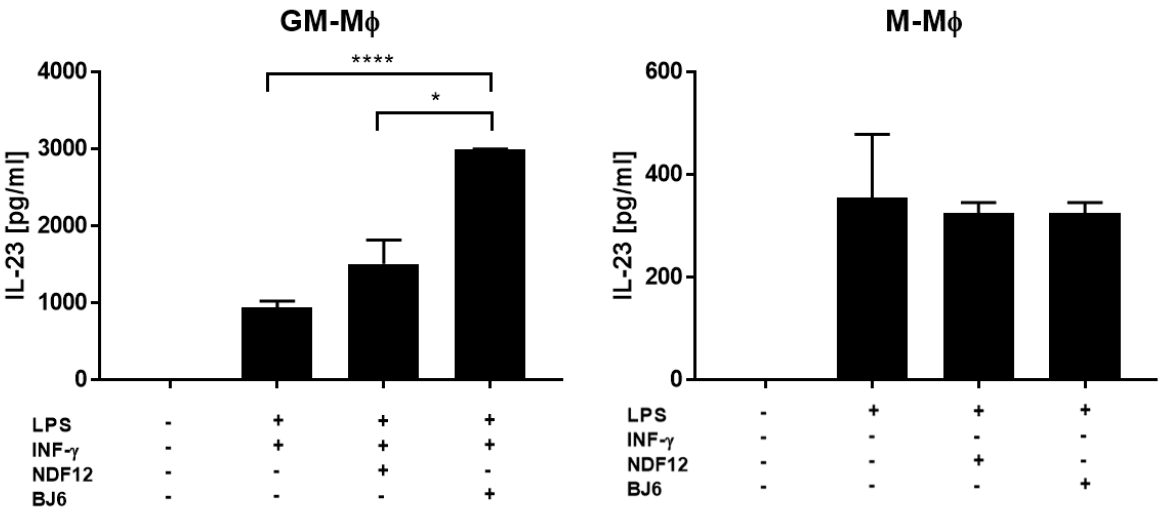

Figure 4.15 2D: Expression of cytokine by activated Mϕ in the presence or absence of FBs. 5x10^6 monocytes were cultured in Ultra-low attachment 6 well plate with 20 IU/ml of GM-CSF or 10 ng/ml of M-CSF. FB were cultured into 96 well plates for 24 hr. After 6 days, Mϕ cells were harvested and added and co-cultures were incubated for 1 hr to allow cells to rest. In triplicate well from each of these culture after 1hr resting following by stimulation with 5µg/ml of LPS and 1000 IU/ml of INFγ or 5µg/ml of LPS only for 24hr. Next day supernatants were collected and cytokine production assayed by ELISA. Data have shown mean cytokine production amongst of triplicate well +/- SD and is representative 3 production of 3 donors. Statistical significance assessed with t-test.

This finding allowed us to carry out the same experiment but in this time it was important to increase the cell ratio that aligns with the actual number of FB/Mϕ co-culture were cultured in 3D and 2D models. Production of cytokines in MoMϕ were observed in both 2D and 3D culture systems (Figure 4.16-4.18). There was detectable expression of IL-12, IL-10 and IL-23 in mature Mϕ monoculture. However, IL-23 secretion by mature GM-Mϕ cultured with FB was slightly increased in 2 donors but IL-23 was not affected compared to mature GM-Mϕ only in 1/3 donors. Unlike, IL-23 secretion by FB/mature GM-Mϕ co-culture, IL-12 secretion in the presence of FB was significantly ($p<0.05$) decreased in 1/3 donors and IL-12 was inhibited in other 2 donors. Mature GM-Mϕ in the presence of FB produced a less IL-10 production than mature GM-Mϕ and this was only in 1/3 donors. In this setting cross talking with FB decreased secretion of IL-10 by mature GM-Mϕ in 1/3 donors (Figure 4.16).

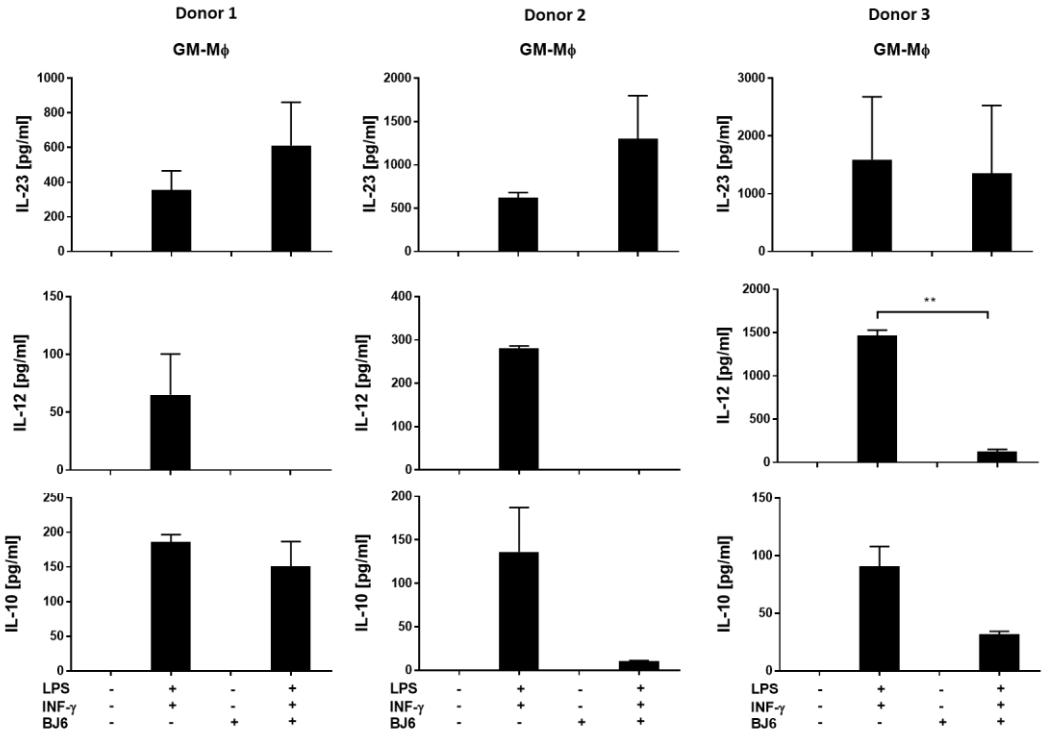

Figure 4.16 The effect of FB on cytokine secretion by activated GM-Mϕ in a 3D model. 5x10^6 CD14+ cells were cultured in Ultra-low attachment 6 well plate treated with 20 U/ml of GM-CSF. After 6 days, GM-Mϕ were harvested. Next, mixture of FBs/GM-Mϕ were cultured (proportional FB/Mϕ 1:1-20,000 FB, 20,000 Mϕ) into ULA-96 well plates for 4 days. Cells were spun at 100 g for 4 minutes and incubate for 3 days. On day 3 spheroids were activated with 5µg/ml of LPS and 1000 U/ml of INFγ for 24h. Next day supernatants were collected and cytokine production assayed by ELISA. Data have shown mean cytokine production amongst of triplicate well +/- SD and is representative 3 experiments of 3 donors. Statistical data were carried out using t-test.

In addition, in Figure (4.17) shows FB had a significant ($p<0.05$) impact on IL-10 secretion by mature M-Mϕ compared to mature M-Mϕ only. However, one donor of 3 donors did express IL-23 by mature M-Mϕ, the second donor expressed IL-23 by mature M-Mϕ but FB did inhibit expression of IL-23 while the rest remained unaffected.

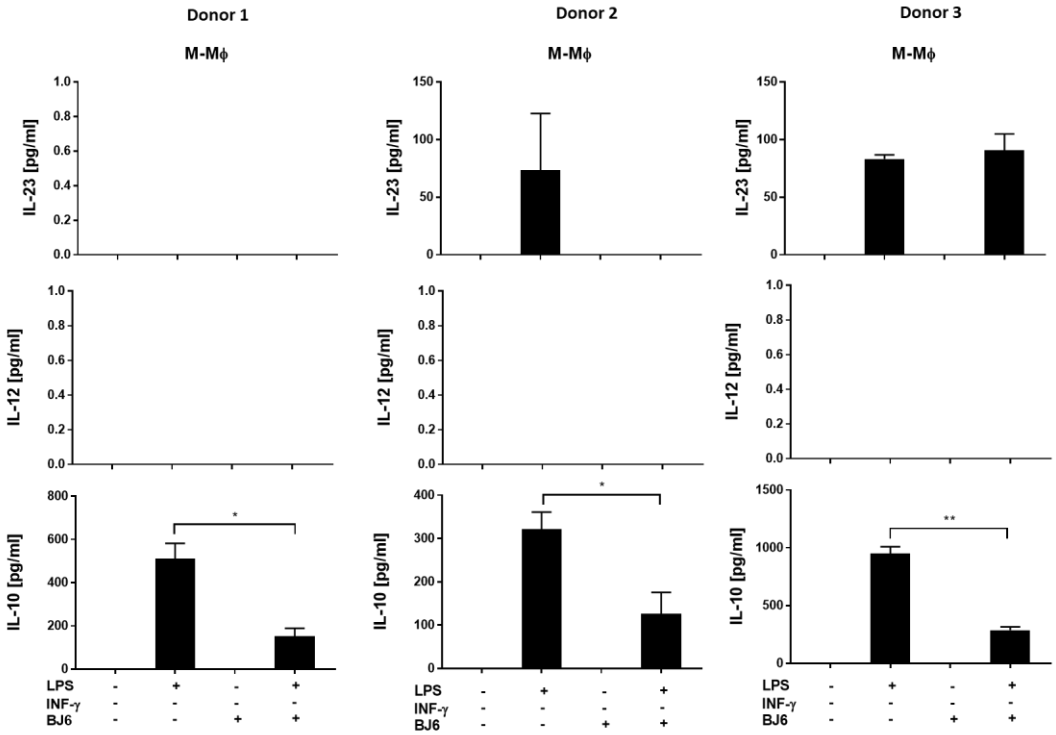

Figure 4.17 Cytokine secretion by activated M-Mϕ in the presence or absence of FBs in the 3D model. Monocytes ($5x10^6$) were cultured in Ultra-low attachment 6 well plate treated with 10 ng/ml of M-CSF. After 6 days of the culture, M-Mϕ cells were harvested. Next, mixture of FBs/M-Mϕ were cultured into ULA-96 well plates for 4 days. Cells were spun at 100 g for 4 minutes and incubate for 3 days. On day 3 spheroids were activated with 5µg/ml of LPS only for 24h. Next day supernatants were collected and cytokine secretion assayed by ELISA. Data show the mean cytokine secretion amongst of triplicate well +/- SD and is representative of 3 individual experiments Statistical data were carried out using t-test.

Interestingly, in Figure (4.18) secretion of cytokines (IL-12, IL-10 and IL-23) by FB/mature GM-Mϕ co-culture in 2D model was increased compared to mature GM-Mϕ only. Mature M-Mϕ did not secrete IL-12, whereas IL-10 was observed and FB induced slightly decreased IL-10 compared to mature M-Mϕ. FB slightly increased production of IL-23 by mature M-Mϕ.

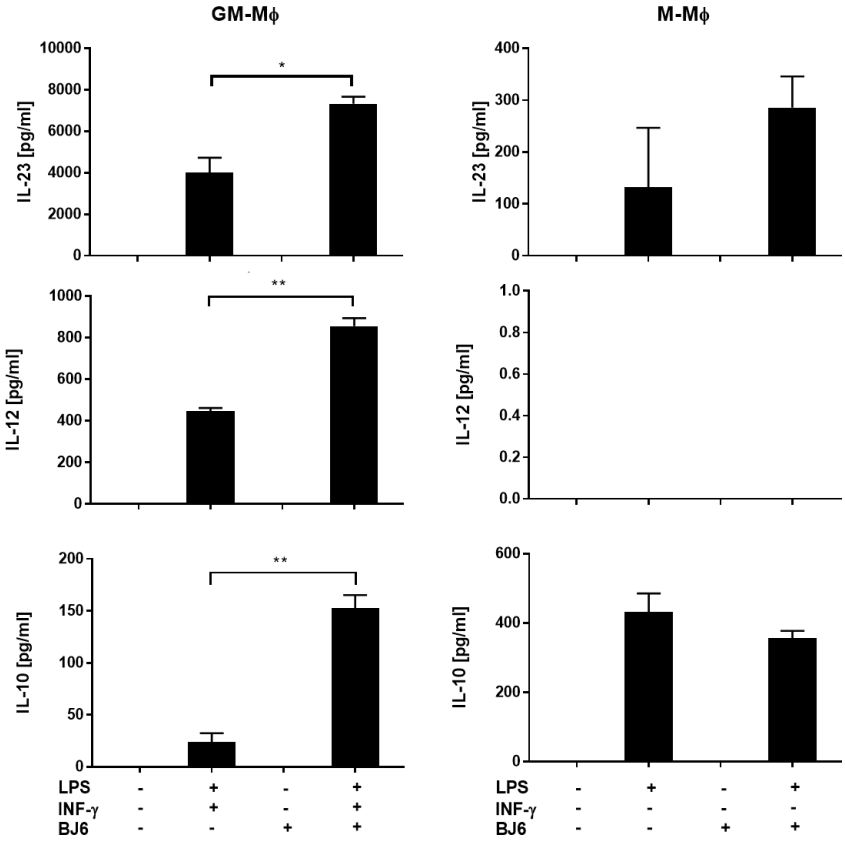

Figure 4.18 Cytokine secretion by activated Mϕ in the presence or absence of FBs in the 2D model. Monocytes ($5x10^6$) were cultured in Ultra-low attachment 6 well plate treated with 20 IU/ml of GM-CSF or 10 ng/ml of M-CSF. FB were cultured into 96 well plates for 24 hr. Next, after 6 days of the culture, Mϕ cells were harvested and added and co-cultures were incubated for 1 hr to allow cells to rest. In triplicate well from each of these culture after 1hr resting following by stimulation with 5µg/ml of LPS and 1000 IU/ml of INFγ or 5µg/ml of LPS only for 24hr. Next day supernatants were collected and cytokine secretion assayed by ELISA. Data show the mean cytokine levels from triplicate wells +/- SD and is representative 3 individual experiments. Statistical data were carried out using t-test.

4.4 Discussion

Extensive studies have revealed that the tumour microenvironment (Steinman & Witmer, 1978) plays an important role in disease progression and drug response (Rebelo et al., 2018; Shurin et al., 2016). For this reason, attention has been paid to establishing more complex culture systems to better represent complex cellular environments and replicate the physiological setting (Tang, Qiao, & Fu, 2016). Here we attempted to devise a two-cell 3D culture system combining alginate microencapsulation and stirred culture in order to form tumour spheroids consisting of cancer-associated fibroblasts and polarising macrophages, wherein the crosstalk interaction between each compartment can be analysed.

In the 3D culture, the expression of CD68 and CD163 macrophage markers was detected, thereby indicating macrophage differentiation into an M2-like phenotype (Almatroodi, McDonald, Darby, & Pouniotis, 2016; Lavin et al., 2017). However, at lower levels, stromal cells within the tumour microenvironment were also primarily involved in the regulation and activation of monocytes, as the activated fibroblast phenotype is a unique property of CAF; in turn, the secretory profile and ECM production were altered (Comito et al., 2014a; Silzle et al., 2003b). In this work, we show the development of FB/Mϕ co-culture in 3D vs 2D culture models. There are important biological molecules involved, including nicotinamide adenine dinucleotide (NAD^+/NADH) and nicotinamide adenine dinucleotide phosphate ($NADP^+$/NADPH), which play a role as cofactors in many enzyme reactions for cellular metabolism, protection against oxidative stress, signal transduction, mitochondrial functions, and cell death (Belenky, Bogan, & Brenner, 2007; Ying, 2008). One approach used to measure the NAD9 (P) H amount is the colourimetric method using tetrazolium salts (Dunigan, Waters, & Owen, 1995; Luo et al., 2006).

Tetrazolium salts are reduced to a formazan product by NAD(P)H, which is a technique widely used to detect the metabolic activity of cells, indicating cell viability. The study has described WST-8 solution as an assay for the quantitative measurement of the enzyme activity of dehydrogenase (Chamchoy et al., 2019). Enzyme activity was determined by WST-8 solution at different concentrations. Absorbance increases were dramatically boosted on the last day compared to previous days, suggesting that they demonstrated the metabolic activity of cells and as an indicator of cell viability.

Our data determined the proliferation of FB and MCF7 spheroids that were formed. However, the proliferation of FB spheroid culture was barely detectable. In contrast, the proliferation of the MCF7 spheroid was increased by half on day 7 of the culture compared to other days, suggesting that, over time in 3D culture, MCF7 cells exhibit more proliferation than do fibroblast cells.

As part of the optimisation of FB spheroids and to avoid hypoxia and necrosis in the core, the spheroids composed on average of 1,000, 5,000 and 10,000 cells were paraffin-embedded for sectioning and staining with H+E dye. We saw that, in each concentration, spheroids maintained their structures and displayed blue nuclei and pink cytoplasm. We were able to successfully generate MCF7 and fibroblast spheroids, and demonstrated viability and proliferation. Further ways of characterising the nature of the spheroids could include the evaluation of cell death and apoptosis markers as well as proliferation markers.

The establishment of developed ontogeny in the normal fibroblast cell system in *in vitro* and *in vivo* vertebrate and mammalian organisms is unknown. Human fibroblasts are categorised by a heterogeneous cell population, existing in adult bodies that can display embryonic and functional diversity (Sriram, Bigliardi, & Bigliardi-Qi, 2015). In fact, although most fibroblasts are derived from mesodermal cells, some research has revealed that neck skin fibroblasts are considered to be neural crest tissue (Buckley et al., 2001). The homoeostasis of the extracellular matrix can be preserved by fibroblasts, but can also gain an immunoregulatory phenotype. In fact, although activated fibroblasts are well placed to produce high levels of COX-2 and pro-inflammatory cytokines, the fibroblast activation period extends depending on the type of tissue (Vaheri, Enzerink, Rasanen, & Salmenpera, 2009). Apoptosis of activated fibroblasts was induced into apoptotic cell death at the end of the wound healing, whereas *in vitro* fibroblasts also undergo a programmed necrosis-like cell death, named nemosis (Bizik et al., 2004). In particular, human dermal fibroblasts can produce clusters *in vitro*, named spheroids, and the activation of fibroblasts increases the expression of COX-2, prostaglandins, pro-inflammatory cytokines, and growth factors, thus leading to nemosis associated with spheroid decomposition (Salmenpera, Karhemo, Rasanen, Laakkonen, & Vaheri, 2016).
In this study it was demonstrated that growing the human fibroblast cell line in suspension in 3D resulted in cell aggregates and the production of solid spheroids, while the volume of spheroids during their maturation decreased after 96 hours. This small decrease is owing to

a process of compaction, which is consistent with the results of previous reports concerning the determined generation and analysis of clusters such as spheroids derived from human primary cutaneous myofibroblasts that formed after 96 hours of seeding (Granato et al., 2017).

The development of better cancer therapy is a key priority of cancer research, and cancer invasion and metastasis are important issues to be solved, owing to metastasis being the most common cause of a cancer patient's death (Nii, Makino, et al., 2020). To enable the improvement of invasion investigations, it is essential to develop *in vitro* cell culture models, providing the opportunity for invasion assessment. In one trial, an evaluation of invasive cancer cells was conducted in a 3D manner using the conventional membrane chamber assay. Cancer cells were co-cultured with CAF, TAM, and endothelial cells to induce the cancer environment mimicking the pathological condition. However, during *in vitro* trials, the natural interaction of cancer cells with TAM is not always high enough in bioactivity (Kuen, Darowski, Kluge, & Majety, 2017; X. Q. Liu et al., 2016).

It has been shown that stromal cells are cultured at a concentration approximately more than twice the number of cancer cells in the body, although the cancer region is key to this process (Shiga et al., 2015). As one trial reported, a 1:2 ratio was fixed for a mixture of cancer and TAM. In this study, TAM culture was used for 10 days. Remarkably, there was no importance in the rate between the 2D TAM and the TAM-free adenosine groups. This can be clarified in terms of the distance between TAM and cancer cells. The interaction may be prevented by the distance between cancer cells and TAM. Furthermore, a previous study revealed that the interaction between cancer cells and CAF was difficult due to distance (Nii, Kuwahara, et al., 2020; Nii, Makino, & Tabata, 2019a).

Previous reports have suggested that the recruitment and activation of monocytes within TME are associated with the interaction of the stromal compartment (Silzle et al., 2003a), as CAF fibroblasts express activated phenotypes in addition to an altered secretory profile and ECM production (Turley, Cremasco, & Astarita, 2015). In fact, in 3D systems including blood monocytes with breast cancer or CAF spheroids, the migration of monocytes was increased towards CAF spheroids, resulting in a correlation between a phenotype and an overexpression of CCL2 (Ksiazkiewicz et al., 2010).

Our results demonstrated that 3D co-culture allowed the activation of Mϕ to secrete a considerable level of cytokines. These findings illustrated that primary FB and cell line FB significantly decreased IL-23 secretion of GM-Mϕ in one out of three donors. They also significantly increased IL-23 secretion by GM-Mϕ in one out of three donors, while FBs did not alter the expression of IL-23 in another donor. Furthermore, IL-23 secretion was inhibited in FBs co-cultured with M-Mϕ in two out of three donors. IL-23 secretion was significantly decreased in cell line FB co-cultured with M-Mϕ in one out of three donors. Furthermore, in a 2D culture system, FBs altered the GM-Mϕ function, pointing towards the potential expression of IL-23, whereas they did not alter the production of IL-23 from M-Mϕ, which is aligned with our previous findings, detailed in Chapter 5.

In addition, the same previous experiment was conducted using the same ratio of co-culture. Our data showed that a planar co-culture of GM-Mϕ and FB reduced Il-12 and increased IL-23 secretion. In contrast, 3D co-cultures did not increase IL-23 expression by GM-Mϕ. Neither resting Mϕ alone nor Mϕ/FB co-cultures secreted cytokines. When M-Mϕ were co-cultured with FB in 2D, cytokine secretion was largely unaffected. However, in 3D, IL-23 production was inhibited by FB.

Interestingly, Mϕ culture at different concentrations (either 5,000 or 20,000) produced detectable levels of cytokines including IL-12, IL-10 and IL-23, whereas, when co-cultured at lower concentrations (1,200, 1,600, 2,400 and 3,300), it did not secrete detectable levels of cytokines in 3D. Levels of IL-12, IL-10 and IL-23 cytokines by Mϕ in 3D were expressed more highly than was the case with those secretions in 2D culture. This finding is in line with previous reports suggesting that, in a 3D-3-culture model, M2-like phenotypes were observed to accumulate and release IL-10 and IL-12 (Rebelo et al., 2018). FB exerted potent suppression on cytokines secreted by Mϕ culture in 3D compared to those induced in the 2D model. An FB ratio of a propositional 1:1 induced a slight increase in the level of IL-23 expression, whereas, at a propositional 2:1, it induced a slight decrease in IL-23 secretion by GM-Mϕ.
Macrophages cannot produce collagen but they are able to release matrix metalloproteinases (MMPs) and TIMPs, which change EMC turnover and composition (Wynn & Barron, 2010). Macrophages produce MMP-2, -9 and -13, which enable digesting EMC and reverse scarring at the resolution stage of wound healing, and this is associated with reducing ECM degradation by depleting macrophages (Henderson & Iredale, 2007). This MMP is produced

by macrophages, giving rise to consequences beyond ECM consumption. The alteration of fibroblast behaviour was determined by MMPs and TIMPs when fibroblasts recognised their ligand with ECM, leading to changes in their composition and flexibility (Barron & Wynn, 2011; Henderson & Iredale, 2007).

Future work should build on this model to overcome some of its limitations. While a 3D system is clearly of interest, the spheroid model was challenging to build and further work should explore the alginate (Andrlova et al., 2017) microencapsulation of cells or stirred culture systems for spheroid assembly.

Chapter 5 : Co-culture of APC with stromal FBs and their ECM

Chapter 5 : Co-culture of APC with stromal FBs and their ECM

5.1 Introduction

Current therapeutic strategies focus principally on immunosuppression rather than on the mechanisms determining inflammation. Glucocorticosteroids (GCs) are the most commonly used anti-inflammatory drugs and target immune cells via cell specific mechanisms (Ma et al., 2004; Shapouri-Moghaddam et al., 2018). Resolution of inflammation can be induced by different subtypes of monocytes and macrophages, which are activated by microbial products or endogenous danger signals, to produce pro-inflammatory mediators that initiate the inflammatory response. These processes are associated with the downregulation of pro-inflammatory cytokines from macrophages and monocytes by an efficient inhibitor of GCs (Boehncke & Schon, 2015; Malissen, Tamoutounour, & Henri, 2014). The ability to migrate towards inflammatory stimuli was increased in GC-induced anti-inflammatory monocytes. In contrast, naïve monocytes and macrophages stimulate the action of GCs, induce anti-inflammatory mediators, and differentiate into anti- inflammatory phenotypes (Ehrchen, Roth, & Barczyk-Kahlert, 2019; Van den Bosch & Coates, 2018). GCs limit the amplification of inflammation, which is associated with repressed pro-inflammatory macrophage activation. Additionally, actively promoting the resolution of inflammation in anti-inflammatory monocytes and macrophages populations is relevant to the induction of GCs. Indeed, GCs play a pivotal role in inhibiting the transcription of several pro-inflammatory cytokines in human monocytes and macrophages, such as, interleukin (IL-1β), IL-6, IL-12, and tumour necrosis factor α (TNFα) (Cain & Cidlowski, 2017).

Transforming growth factor-β (TGF-β) is important for tumour growth, due to its function in differentiation and migration. Through the canonical and non-canonical signalling pathways, TGF-β acts as both a tumour suppressor and a tumour promoter (Massague, 2004; Siegel & Massague, 2003). Macrophages, dendritic cells (DCs), neutrophils and natural killer (NK) cells are also affected by TGF-β, which stimulates their recruitment, activation, and immune suppression (Flavell, Sanjabi, Wrzesinski, & Licona-Limon, 2010). Consequently, recognition of tumour cells is decreased as TGF-β antagonises immune function (Gigante, Gesualdo, & Ranieri, 2012). In this regard, TGF-β is associated with the induction of M2 macrophage polarisation and suppression of monocyte-mediated cytotoxicity via the expression of inducible nitric oxide synthase (iNOS) (Gong et al., 2012; Murray & Wynn, 2011). NK cells lose their functional maturation upon exposure to TGF-β, impairing the recognition and elimination of tumour cells (Marcoe et al., 2012). This impedes their downstream activity including activation of DCs and interferon γ (IFNγ) production (Laouar, Sutterwala, Gorelik, & Flavell, 2005; Leavy, 2012).

Initial research revealed that TGF-β function is crucial to controlling the adaptive immune system, for example the development of autoimmune-mediated inflammation in several tissues was observed as a result of systemic impairment of TGF-β1 expression. Furthermore, the loss of the TGF-β pathway particularly in T cells, is associated with an autoimmune response (Kulkarni et al., 1993; Marie, Liggitt, & Rudensky, 2006). Interestingly, initiation of TGF-β expression can drive suppression of the adaptive immune response through Treg cells (Chen et al., 2003; Tone et al., 2008). Naïve T cells treated with a combination of IL-6 and TGF-β can be differentiated into an entirely different phenotype such as immunosuppressive Treg cells. The addition of IL-6 and TGF-β initially supresses forkhead box P3 (FOXP3) in cells. In addition, the function of Th17 cells is primarily mediated through the secretion of IL-17, which associated with promoted tumorigenesis mediated by neutrophil recruitment (Mangan et al., 2006; von Boehmer & Daniel, 2013).

The investigation of the role of TGF-β in the regulation of myeloid cells revealed that the pro-inflammatory response of macrophages activated with TLR ligands or stimulation was largely suppressed by TGF-β (Li, Wan, Sanjabi, Robertson, & Flavell, 2006). However, initiation of various inflammatory cytokines was promoted in myeloid cells stimulated with TGF-β in the absence of TLR ligands or other cytokines (Turner, Chantry, & Feldmann, 1990). Monocytes

and macrophages isolated from human peripheral blood migrated in the presence of TGF-β, and this was associated with enhancing the adherence of monocytes (Bauvois, Rouillard, Sanceau, & Wietzerbin, 1992; Sanjabi, Oh, & Li, 2017). TGF-β controls the development and functions of innate cells, including macrophages, DCs and NK cells. Collectively, it maintains peripheral tolerance against self- and innocuous antigens, and modulates the immune response to pathogens. The study aimed to determine whether and how TGF-β controls the inflammatory reaction and induced M2-like polarisation in LPS-stimulated distinct macrophage cell populations *in vitro*. It was hypothesised that TGF-β produced within the tumour microenvironment stimulates inflammatory cytokines (IL-1β/TNFα) to recruit macrophages that elicit prostaglandin E_2 (PGE_2) secretion by fibroblasts, therby amplifying IL-23 expression, and anti-inflammatory cytokines by macrophages. As a consequence, this system alters the balance of Th cell responses, and preferentially supports expansion of Th17 cells, which are highly inflammatory, thus further potentiating the tumour-promoting microenvironment.

Tumour growth reflects malignant transformation, and also the impact of the tumour stroma (Kalluri, 2003). Several factors, such as angiogenesis and inflammatory cells, are associated with tumour progression. Moreover, it is becoming increasingly clear that cancer progression is prominently modified by structural cells such as fibroblasts (de Visser, Korets, & Coussens, 2005; Elenbaas & Weinberg, 2001). Our knowledge of the phenotypes of fibroblasts in cancer, including resting and activated cells, continues to evolve, and supports the notion of subpopulations of fibroblasts, the so-named cancer-associated fibroblasts, that act as important promoters of tumour growth and progression (M. M. Mueller & Fusenig, 2004).

Fibroblasts were first described in the late 19th century dependent on their location and microscopic appearance. They represent a principal cellular component of the connective tissue and are non-inflammatory, non-epithelial and non-vascular cells which exist embedded within the fibrillar matrix of connective tissue (Kalluri & Zeisberg, 2006). They play important roles in deposition of extracellular matrix (ECM), regulation of inflammation, epithelial differentiation, and wound healing (Parsonage et al., 2005). Several components of the fibrillary ECM such as type I, type III and type IV collagen, and fibronectin were up-regulated by fibroblasts (Rodemann & Muller, 1991). FB are therefore classified as key agents of ECM-degrading proteases, including metalloproteinases (MMPs), which highlights their importance in ECM-homeostasis by regulating ECM turnover (H. Y. Chang et al., 2002; Simian et al., 2001).

It is widely accepted that the tumour microenvironment of a given tissue comprises the basement membrane, immune cells, capillaries, fibroblasts and ECM surrounding the cancer cells constituting the tumour stroma (Ronnov-Jessen, Petersen, & Bissell, 1996). It was observed that the reactive stroma during wound healing is similar to the stroma is associated with *carcinoma in situ* (CIS). However, the impact of the stroma on cells and vice versa in the regulation of CIS is unknown but there is a growing body of evidence of crosslinking between components amongst the basement membrane barrier. There is much interest in the differences between normal and tumour stroma. In normal stroma, a minimal number of fibroblasts communicate with the physiological ECM (Dvorak, Form, Manseau, & Smith, 1984), whereas the number of fibroblasts is increased within the reactive stroma, leading to enhanced capillary density, as well as type I collagen and fibrin deposition. It has been demonstrated that increased oncogenic signals provided by reactive stroma facilitate tumorigenesis (Dolberg, Hollingsworth, Hertle, & Bissell, 1985; Sieweke, Thompson, Sporn, & Bissell, 1990).

Vascular endothelial growth factor (VEGF), which acts as vascular permeability factor (VPF), is involved in the emerging reactive stroma (L. F. Brown et al., 1999). The central source of host-derived VEGF are several types of cells can release VEGF, such as fibroblasts and inflammatory cells, in addition to cancer cells themselves (Fukumura et al., 1998). The induction of microvascular permeability can be triggered through the action of VEGF, in turn, increasing the extravasation of plasma proteins such as fibrin, as well as the influx of

fibroblasts, inflammatory cells and endothelial cells (Dvorak et al., 1991; Senger et al., 1983). These cells are a crucial resource of ECM production, providing an abundance of fibronectin and type I collagen, both of which play a role in tumour angiogenesis (Feng et al., 2000).

The tumour microenvironment includes TAFs that are present in high levels as stromal cells which produce cytokines, thereby impacting cancer cells and stroma (Deying et al., 2017; X. Zhang et al., 2018), causing tumour progression and migration, and are an alternative for extracellular matrix. Within the tumour milieu, TAMs are the abundant cell type, accounting for up to 50% of the cancer tissue bulk (Solinas, Germano, Mantovani, & Allavena, 2009). Polarised macrophages are divided into two types: classically activated (Thomas B. Thornley, 2014) macrophages which are involved in the immune response (Martinez, Helming, & Gordon, 2009), and alternatively activated (M2) macrophages which have a crucial role in promoting tumour growth (P. Zheng et al., 2018). Furthermore, TAMs and M2 polarised macrophages have similar properties.

Previously, colorectal cancer-derived TAFs (CC-TAFs) isolated from human colorectal cancer (CRC) tissue were shown to affect monocytes in terms of adhesion, recruitment, and polarisation of macrophages (C. Zhang et al., 2017). IL-8 secretion by TAFs can attract monocytes and the secretion of IL-6 increased adhesion between monocytes and CRC cells. Moreover, the suppressive effect of TAFs promoted the polarisation of M2 macrophages; this synergistic axis inhibits recruitment of NK cells (Parkhurst, Riley, Dudley, & Rosenberg, 2011; R. Zhang et al., 2019). TAMs are a phenotypic heterogeneous population that polarise from circulating monocytes or tissue-localised macrophages and are involved in several tumour-promoting processes, such as pro-inflammatory signalling, enhanced angiogenesis, invasion, metastasis and therapy resistance (Cassetta et al., 2019; B. Z. Qian & Pollard, 2010). Many factors within the tumour microenvironment can affect the functional differentiation of TAMs. Recently, TAFs were shown to act as novel effector cells through the polarisation of TAMs into an immunosuppressive phenotype in addition to their role in monocyte regulation. TAF-derived Chi3L1 is a genetically modified target of Chi3L1 expression in fibroblasts and was shown to attenuate macrophage recruitment and their reprogramming to polarised M2 as well as promote Th1 within the tumour microenvironment in bone morrow-derived macrophages isolated from a mouse model of breast cancer (Cohen et al., 2017; Comito et al., 2014b). Moreover, TAFs isolated from human tumours instigated M2 polarisation of the

phenotype differentiated from patient-derived CD14+ myeloid cells to induce ARG1, IL-10 and TGF-β, which act as potent suppressive molecules on the proliferation of autogenous T cells (H. Takahashi et al., 2017). However, the mediation of M2-like polarisation regulated by underlying TAF-derived factors was not identified.

A growing body of evidence that M2-polarised cells are derived from TAMs, and non-small cell lung cancer comprises approximately 70% of M2-like derived from TAMs, however the basis of this imbalance remains unknown (Sun, Sun, Xiao, Zhang, & Tian, 2015). In the TME, there are multiple agents which may control the phenotype of macrophages, including stromal cells (e.g. TAFs) that secrete immunosuppressive factors (Karakasheva et al., 2018; X. Zhang et al., 2018). Cytokines, growth factors, lipid mediators and prostaglandin E2 regulate a tumour-promoting phenotype (Hayakawa & Smyth, 2006; Takeda et al., 2001).

When TAM were cultured *in vitro* under standard conditions, they were able to restore their function to express IL-12 and TNF (Saccani et al., 2006). Initiation of cancer can be triggered through a chronic stage of the inflammatory environment comprising of macrophages. Recently, inflammatory cytokines IL-12, IL-10 and IL-23 produced from the presence of TAM and IL-17 were implicated in initiating cancer progression (Salmaninejad et al., 2019).

5.1.1 Characterisation of ECM fibroblast crosslinking CD14+ monocytes and macrophages

5.1.1.1 BJ6 cell line fibroblast-ECM interaction with CD14+ monocytes and macrophages

In vitro and in vivo, hypersensitivity and hyposensitivity are induced under experimental conditions, modulated by the biological activity of LPS (Salomao et al., 2012). Administration of minute amounts of LPS to animals or humans might induce a state of hyporesponsiveness to a second LPS challenge, a phenomenon recognised as “endotoxin-tolerance”. This process is a protective cellular mechanism to limit detrimental inflammation, the initial excessive activation of monocytes and macrophages by LPS is decreased, and the inhibition of the production of inflammatory cytokine is regulated, such as TNF-α (Cavaillon & Adib-Conquy, 2006; de Lima et al., 2014; Fernandes, Mendes, Brunialti, & Salomao, 2010). A study revealed that inflammatory cytokine production by tolerant monocytes activated by LPS was inhibited, these cells associated with the cell phenotype express CD206 and CD163. Furthermore, following LPS activation, CD206 expression was downregulated by tolerised cells (Alves-Januzzi, Brunialti, & Salomao, 2017).

The previous studies reviewed showed that the interaction of innate and adaptive immunity includes monocytes/macrophages and this process is increasingly recognised in HIV pathogenesis (Centlivre et al., 2011; Laforge et al., 2011). They initiate immune responses and act as the first cells to interact with pathogens, including HIV and SIV (simian immunodeficiency virus). CD169 (sialoadhesin, Sn, or Siglec1) is a distinct monocyte marker recognises early virus infection and is involved in the activation of the adaptive immunity (Klaas & Crocker, 2012). The sialylated bacteria and viruses can internalise by binding to this endocytic receptor. Also, CD8+ T lymphocytes and tumour cells bind to CD169 as a mediate interaction through its counter receptors CD43 to MUCI, respectively. Detection of CD169 expression has been observed in high levels on blood monocytes from patients with systemic sclerosis, systemic lupus erythematosus, and primary biliary cirrhosis (G. Bao et al., 2010; Biesen et al., 2008). Expression of CD169 also occurs on monocytes increased in patients infected with Epstein-Barr virus enteritis and HIV (Ashokkumar et al., 2012).

Monocytes express the co-stimulatory molecules CD80 and CD86 which are essential co-stimulatory ligands for adaptive immunity (Linsley et al., 1994). Monocytes have properties as heterogeneous phenotypes presenting unique subsets depending on the expression of the

surface molecules CD14 and CD16 (Mukherjee et al., 2015; Thaler et al., 2016). Furthermore, the immune system can regulate by monocytes as the cornerstones for critical drivers of inflammatory reactions (Zawada et al., 2011). Circulating intermediate CD14+CD16+ monocytes upregulated expression of CD86 on their surfaces and correlated with clinical severity in the epidermis and dermis of psoriatic plaques (Nguyen et al., 2018).

The study hypothesis raises important questions including:

i. Does the ECM play a role in regulating differentiation of CD14+ and macrophages rather than simply providing a scaffold for cell adhesion or being a barrier to breach?
ii. Which ECM-FB interactions significantly impact the capability of FB to modulate CD14+ and macrophages and influence how cells respond to the ECM?
iii. Can these interactions be targeted for new immunotherapeutic strategies or to improve current therapies?

Herein, we examine the interaction of myeloid cell function, and discuss the evidence for the direct impact of FB-derived ECM on the phenotype of monocytes and macrophages.

5.2 Results

5.2.1 The effects of fibroblast cell lines and primary fibroblasts on macrophages

5.2.1.1 Titration of FBs/APC co-culture in 2D model

Although distinct from cells of the classical immune system, a growing body of evidence shows that fibroblasts (FB) substantially modify immune responses and a growing body of evidence highlights that FB regulate functions of macrophages (Kalluri, 2016b). The purpose of this chapter was to explore the impact of FB on cytokine secretion by macrophages. Initially we aimed to determine the appropriate starting number of FB that would influence the biological physiology of Mϕ during interaction.

Initially we examined the effects of FB cell lines (BJ6 and TIG) and primary FBs (obtained from normal dermis) on cytokine production by resting Mϕ. Co-culture of GM-Mϕ with FB cell lines resulted in an increase in the secretion of IL-23 (Figure 5.1 A and B). The secretion of IL-23 correlated with the number of FB seeded. In contrast, no effect of FB on IL-12 or IL-10 secretion was observed. It is important to note that IL-12 was only observed from 1 donor with the positive control conditions. We next examined the effect of primary FB on macrophage function. As with long-term cell lines, co-culture with primary dermal FB markedly increased the secretion of IL-23 (Figure 5.2 C and D). Neither IL-12 nor IL-10 was detected in the presence of FB.

When M-Mϕ were co-cultured with normal dermal FB (Figure 5.3 G, H), but not cell lines (Figure 5.4 E, F), an increase in IL-23 secretion was observed. It is important to note that which IL-23 was secreted the levels obtained were considerably lower than achieved with M-Mϕ. FB did not affect IL-12 of IL-10 levels when co-cultured with M-Mϕ.

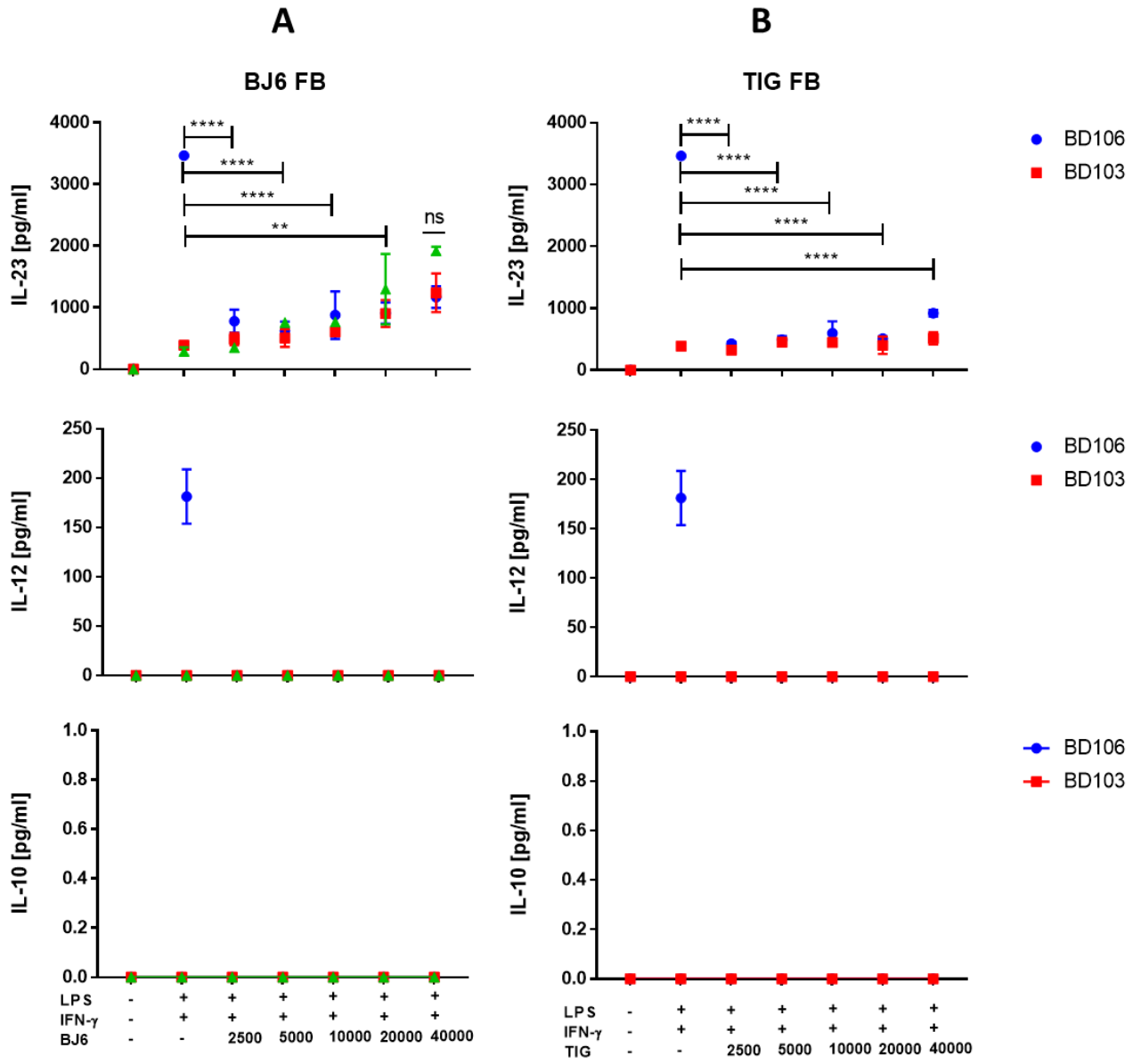

Figure 5.1 A and B Effect of BJ6 and TIG FB cell lines on cytokine production by GM-Mϕ. 5x10^6 CD14+ cells were cultured in Ultra-low attachment 6 well plate treated with 20U/ml of GM-CSF. FB were cultured at different concentrations into 96 well plates for overnight. Next, after 6 days of the culture, 1x10^4 GM-Mϕ cells were harvested and added and co-cultures were incubated for 1 hr to allow cells to rest. After 1hr resting following by stimulation with 5µg/ml of LPS and 1000U/ml of IFN-γ for 24hr. Supernatants were collected and cytokine expression assayed by ELISA. Data shown mean cytokine expressions amongst of triplicate well +/- SD and is representative 3-4 separated experiments. BD106 and BD103 represent donors. Statistical data were carried out using two-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

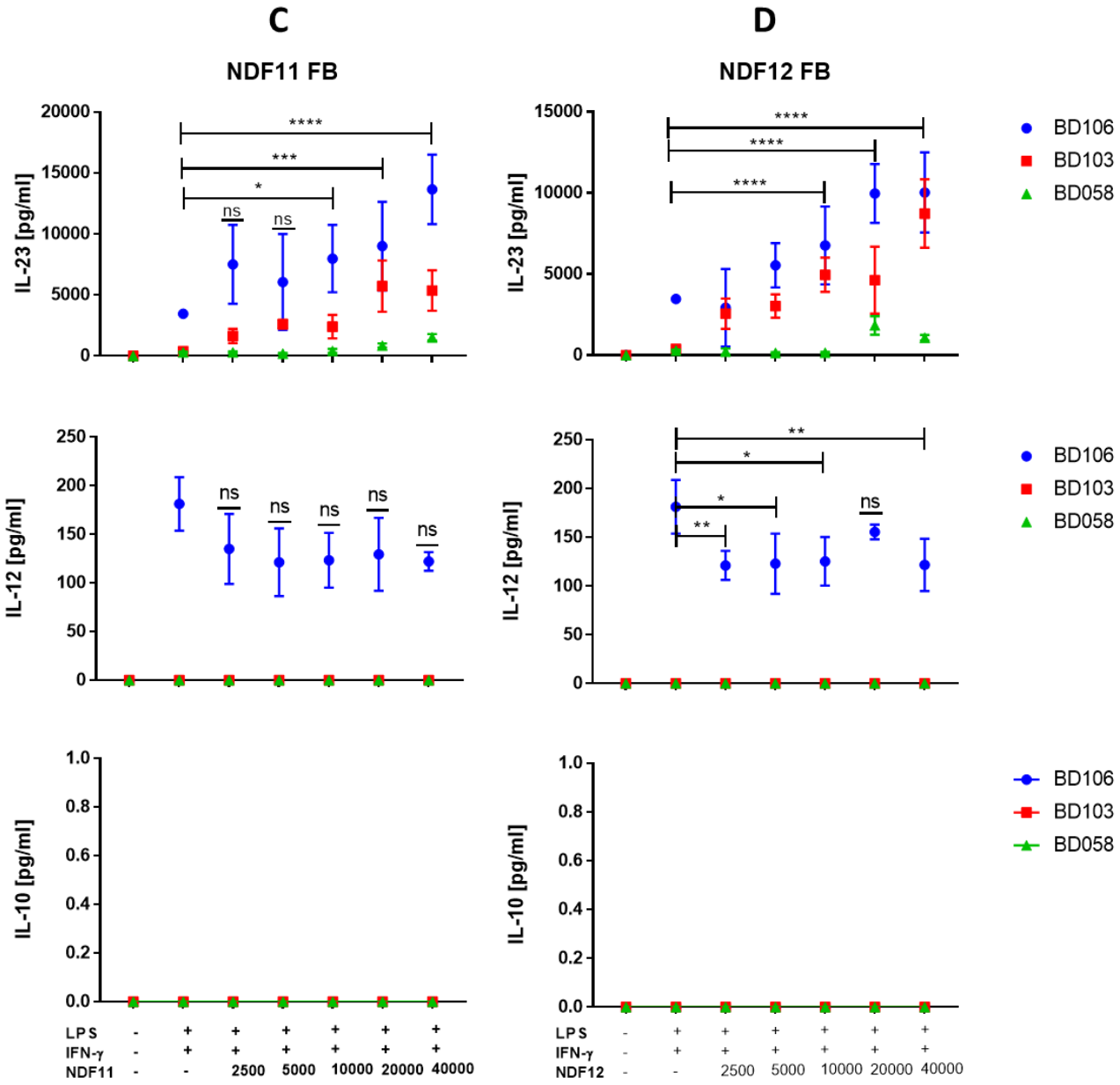

Figure 5.2 C and D Effect of ND11 and ND12 primary dermal FB on cytokine production by GM-Mϕ. 5x10^6 CD14+ cells were cultured in Ultra-low attachment 6 well plate treated with 20U/ml of GM-CSF. FB were cultured at different concentrations into 96 well plates for overnight. Next, after 6 days of the culture, 1x10^4 GM-Mϕ cells were harvested and added and co-cultures were incubated for 1 hr to allow cells to rest. After 1hr resting following by stimulation with 5µg/ml of LPS and 1000U/ml of IFN-γ for 24hr. Supernatants were collected and cytokine expression assayed by ELISA. Data shown mean cytokine expressions amongst of triplicate well +/- SD and is representative 3-4 separated experiments. BD106, BD058 and BD103 represent donors. Statistical data were carried out using two-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

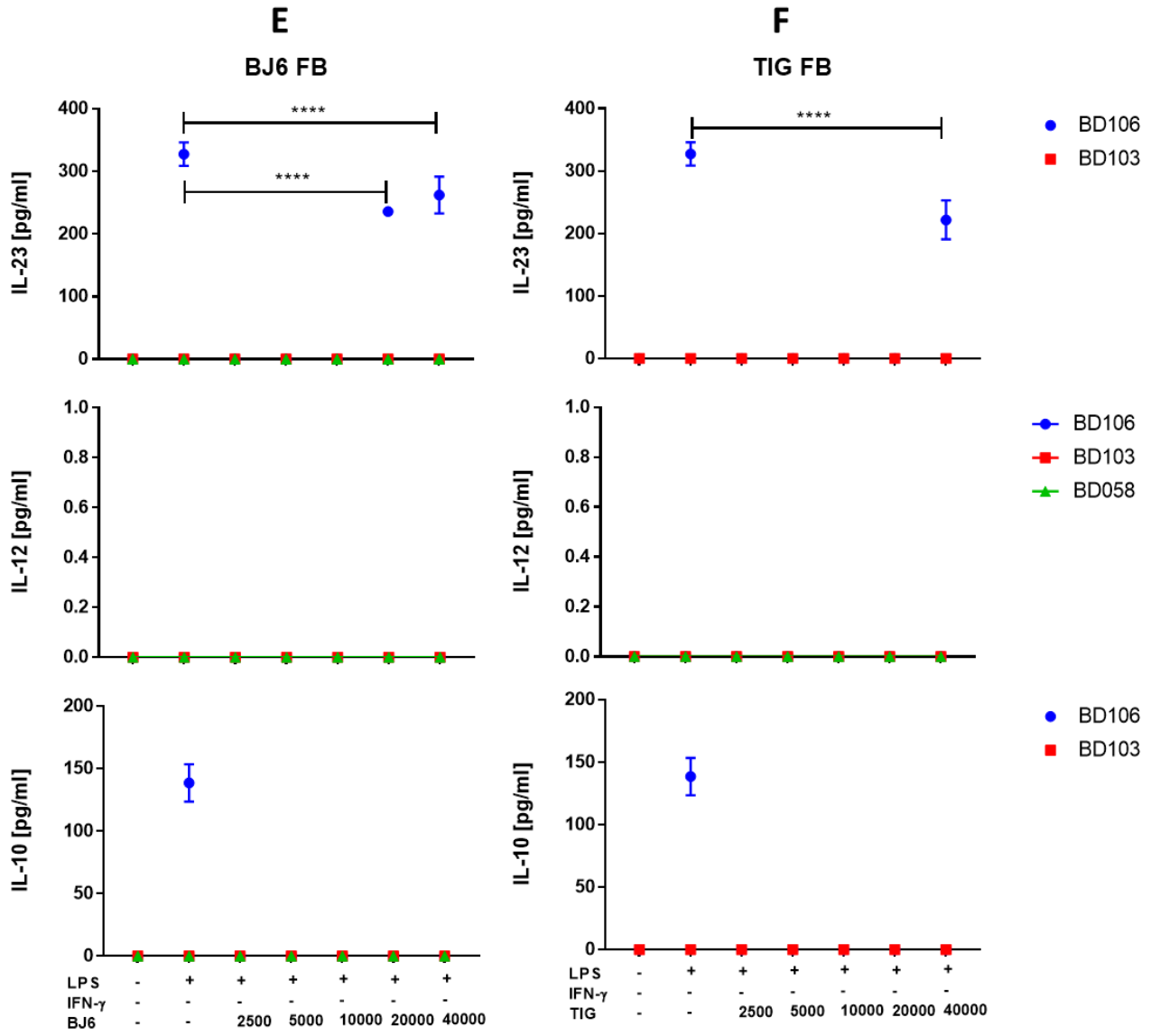

Figure 5.3 E and F Effect of BJ6 and TIG FB cell lines on cytokine production by M-Mϕ. $5x10^6$ CD14+ cells were cultured in Ultra-low attachment 6 well plate treated with 10 ng/ml of M-CSF. FB were cultured at different concentrations into 96 well plates for overnight. Next, after 6 days of the culture, $1x10^4$ M-Mϕ cells were harvested and added and co-cultures were incubated for 1 hr to allow cells to rest. After 1hr resting following by stimulation with 5µg/ml of LPS only for 24hr. Supernatants were collected and cytokine expression determined by ELISA. Data shown mean cytokine expressions amongst of triplicate well +/- SD and is representative 3-4 separated experiments. BD106 and BD103 represent donors Statistical significance was determined by two-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

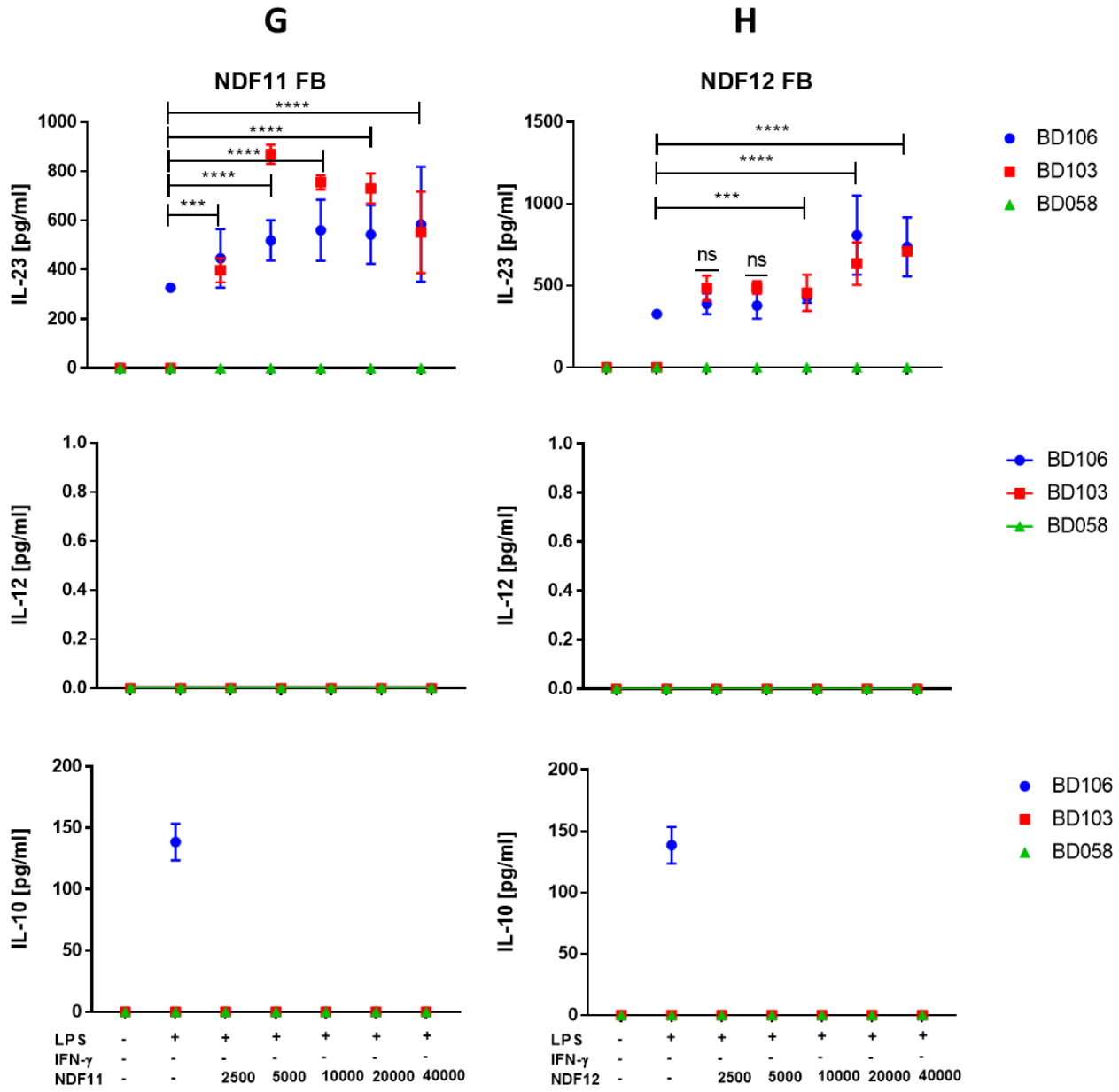

Figure 5.4 G and H Effect of NDF11 and NDF12 primary dermal FB on cytokine production by M-Mϕ. $5x10^6$ CD14+ cells were cultured in Ultra-low attachment 6 well plate treated with 10 ng/ml of M-CSF. FB were cultured at different concentrations into 96 well plates for overnight. Next, after 6 days of the culture, $1x10^4$ M-Mϕ cells were harvested and added and co-cultures were incubated for 1 hr to allow cells to rest. After 1hr resting following by stimulation with 5µg/ml of LPS only for 24hr. Supernatants were collected and cytokine expression determined by ELISA. Data shown mean cytokine expressions amongst of triplicate well +/- SD and is representative 3-4 separated experiments. BD106 and BD103 represent donors Statistical significance was determined by two-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

Having established the baseline conditions for the effect of FB on resting/immature Mϕ, we next explored the impact of FB on maturing Mϕ. This was examined using the respective maturation stimuli for GM- and M-Mϕ, and with increasing numbers of FB and Mϕ in the co-cultures. As shown in Figure 5.5A, GM-Mϕ secreted high levels of IL-23 and low levels of IL-10 upon exposure to LPS/IFNγ. Co-culture with TIG or BJ6 FB cell lines resulted in a marked and significant increase in IL-23 levels and this related to the number of FB in the culture system. Whilst there were some significant decreases in secreted IL-10, these were only observed with the TIG cell line.

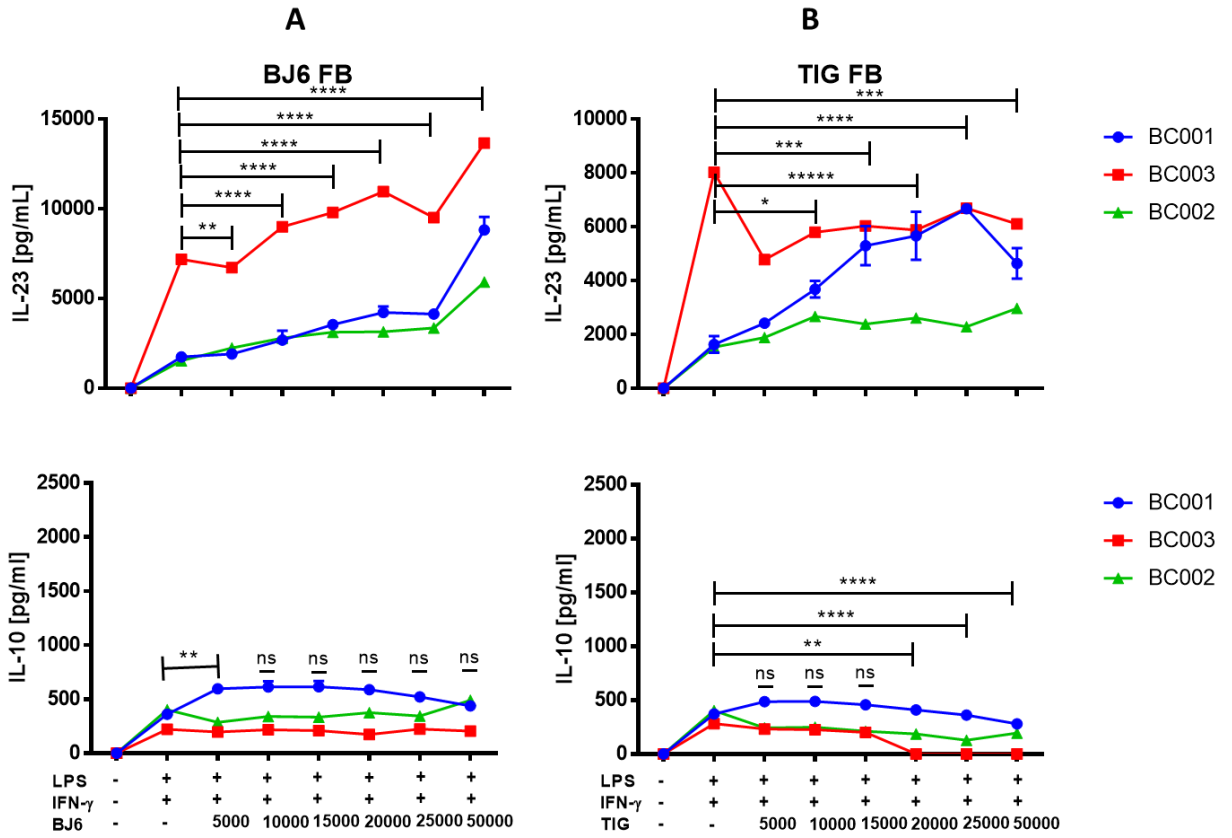

Figure 5.5 A and B Effect of BJ6 and TIG FB on mature GM-macrophage. 5x10^6 MACS-purified CD14+ cells were cultured in Ultra-low attachment 6 well plate treated with 20U/ml of GM-CSF. FB were cultured at different concentrations into 96 well plates for overnight. After 6 days of the culture, 2.5x10^4 Mϕ were added and co-cultures rested for 1 hr. Cells were stimulation with LPS and IFN-γ for 24hr. Next day supernatants were collected and cytokine levels determined by ELISA. Data shown mean cytokine expressions amongst of triplicate wells +/- SD and is representative 3-4 separated experiments. BC001, BC002 and BC003 represent donors. Statistical data were carried out using two-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

In addition, we determined IL-10 cytokine levels in culture supernatants of co-culture BJ6 or TIG/M-Mϕ (Figure 5.6 C, D). IL-10 cytokine levels significantly decreased in the supernatants of all BJ6/M-Mϕ co-culture compared to mature M-Mϕ only (Figure 5.6 C). TIG also reduced IL-10 secretion by mature M-Mϕ from 2/3 donors compared to mature M-Mϕ, although this decrease was not reach overall statistical significance (Figure 5.6 D). Interleukin-23 was not secreted by M-Mϕ (data not shown).

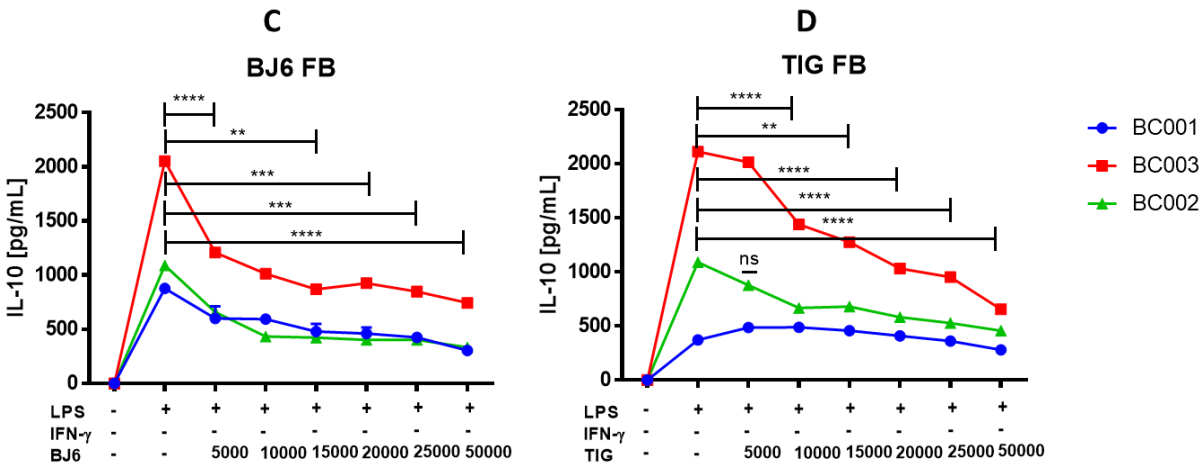

Figure 5.6 Effect of BJ6 and TIG FB on mature M-macrophage. $5x10^6$ MACS-purified CD14+ cells were cultured in Ultra-low attachment 6 well plate treated with 20U/ml of GM-CSF. FB were cultured at different concentrations into 96 well plates for overnight. After 6 days of the culture, $2.5x10^4$ Mϕ were added and co-cultures rested for 1 hr. Cells were stimulation with LPS for 24hr. Next day supernatants were collected and cytokine secretion assayed by ELISA. Data shown mean cytokine expressions amongst of triplicate wells +/- SD and is representative 3-4 separated experiments. BC001, BC002 and BC003 represent donors. Statistical data were carried out using two-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).).

It was decided to study the effect of further increasing the number of Mϕ in subsequent co-culture experiments. With a higher number of Mϕ in the co-cultures we observed FB cell lines to increase the IL-23 response to maturation (Figure 5.7 A, B). The relatively low levels of IL-10 produced by mature GM-Mϕ were suppressed by the addition of FB and this effect was particularly evident with the TIG FB cell line.

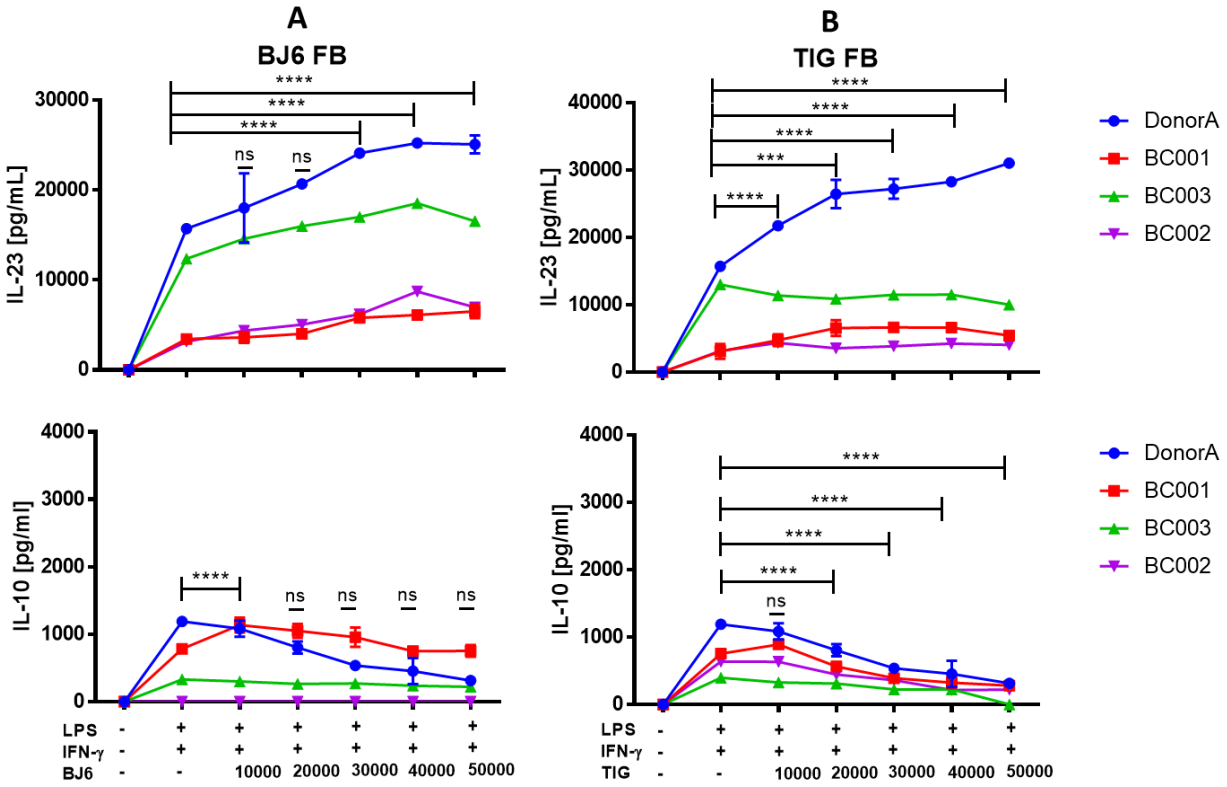

Figure 5.7 A and B Effect of BJ6 and TIG FB on mature macrophage. 5x10^6 MACS-purified CD14+ cells were cultured in Ultra-low attachment 6 well plate treated with 20U/ml of GM-CSF. FB were cultured at different concentrations into 96 well plates for overnight. After 6 days of the culture, 5x10^4 Mϕ were added and co-cultures rested for 1 hr. Cells were stimulation with LPS and IFN-γ for 24hr. Next day supernatants were collected and cytokines quantified by ELISA. Data shown mean cytokine expressions amongst of triplicate wells +/- SD and is representative 4 separated experiments. DonorA, BC001, BC002 and BC003 represent donors. Statistical data were carried out using two-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

When matured M-Mϕ were co-cultured with FB cell lines there was a prominent dose-dependent suppression of IL-10 secretion after 24hrs (Figure 5.8C & D). Once more, this was evident with both of the tested FB cell lines, but the effect was strongest with TIG cocultures (Figure 5.8D).

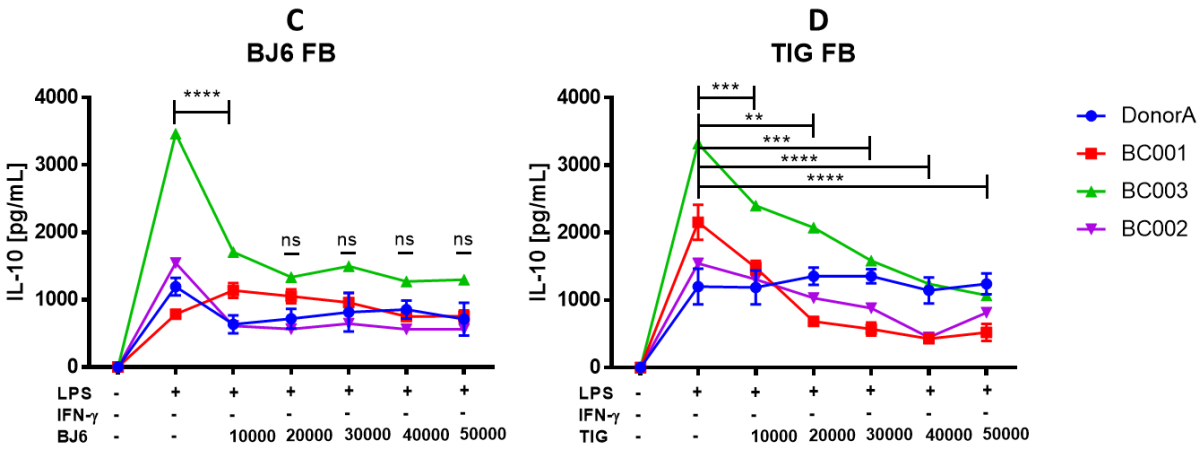

Figure 5.8 C and D Effect of BJ6 and TIG FB on mature M-macrophage. $5x10^6$ MACS-purified CD14+ cells were cultured in Ultra-low attachment 6 well plate treated with 20U/ml of GM-CSF. FB were cultured at different concentrations into 96 well plates for overnight. After 6 days of the culture, $5x10^4$ Mϕ were added and co-cultures rested for 1 hr. Cells were stimulation with LPS for 24hr. Next day supernatants were collected and cytokine levels assayed by ELISA. Data shown mean cytokine expressions amongst of triplicate wells +/- SD and is representative 4 separated experiments. DonorA, BC001, BC002 and BC003 represent donors. Statistical data were carried out two-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

5.2.1.2 The effect of Ionising Radiation (IR) on cytokine secretion by Mϕ in the presence of FB

Having established that FB may modulate the cytokine responses of Mϕ, we decided to use this model system to explore the possible consequences for a model of radiotherapy using external bean ionising radiation (IR). A number of studies have described that stromal Mϕ differentiate into Mϕ2 when exposed to IR (Crittenden et al., 2012; Gough, Young, & Crittenden, 2013; Seifert et al., 2016; Wunderlich et al., 2015). Furthermore, it is established that irradiation of Mϕ1 changes their differentiation pattern towards an M2-like phenotype (Crittenden et al., 2012). Our own group previously described that the presence of FB modulates the response of monocyte derived DCs to X rays (Malecka et al 2016). Therefore, we constructed an *in vitro* system to test the hypothesis that FB modulate the response of macrophages to ionising radiation. The model comprised the exposure of already-established FB-Mϕ cocultures to IR using a tissue culture cabinet to which an X-ray generator was attached (Gulmay). Co-cultures were exposed a single pulse of IR and the cytokine response to standard maturation stimuli assessed.

When macrophages were exposed to 6Gy ionising radiation their cytokine profile was altered. With GM-Mϕ, IR significantly reduced IL-10 and IL-23 secretion whist concomitantly increasing the release of IL-12 (Figure 5.9). A similar response was observed with following the irradiation of M-MΦ as, whilst they did not secrete IL-12, both IL-10 and IL-23 were inhibited. These observations are in agreement with previous findings that showed IR at 6Gy significantly inhibited IL-23 secretion by GM-Mϕ, IL-10 was downregulated in M-Mϕ, and there was no change in IL-12 in GM-Mϕ (A Malecka PhD 2017, University of Nottingham). Whilst IR increased IL-12 secretion by GM-Mϕ and co-culture with BJ6 FB resulted in a modest reduction in IL-12, the combination of FB with IR resulted in high levels of IL-12 secretion. Similarly, the effect of FB on potentiating IL-23 secreted from activated macrophages was preserved in irradiated co-cultures. The suppression of interleukin-10 secretion upon irradiation was not significantly affected by the presence of FB. Irradiation of Mϕ in the absence or presence of FB did not alter their morphology and cell retained their classical “fried egg” appearance.

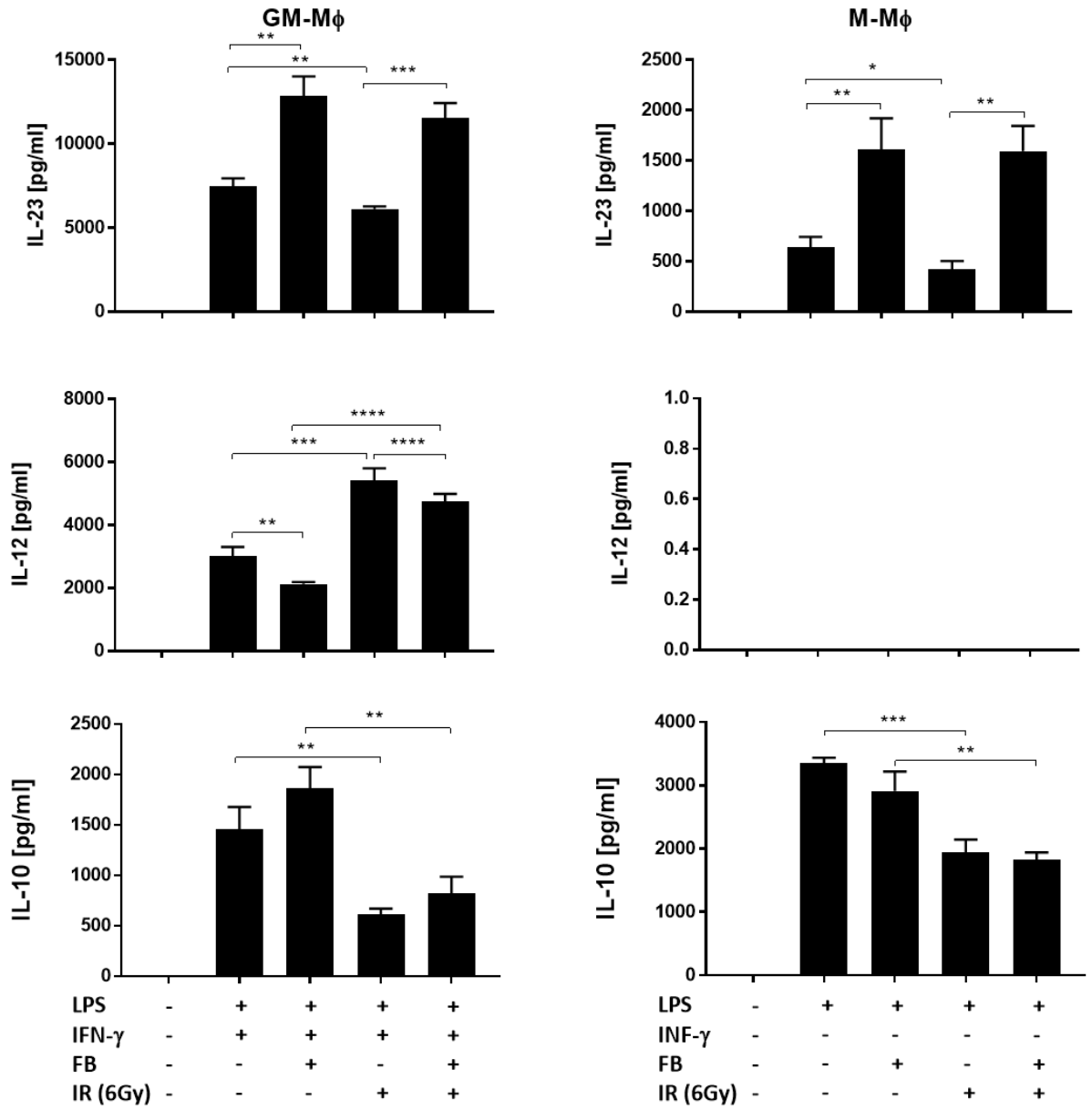

Figure 5.9 Effect of FB on the response of Mϕ to ionising radiation. 5x10^6 CD14+ cells were cultured in Ultra-low attachment 6 well plate treated with 20U/ml of GM-CSF or 10 ng/ml of M-CSF. BJ6 were cultured into 96 well plates for 24 hr. After 6 days, Mϕ were harvested and added to co-cultures before resting for 1hr. Cells were exposed to X-ray radiation at 0 or 6 Gy in triplicate before stimulation with LPS and IFN-γ or LPS only for 24hr. Next day supernatants were collected and cytokine secretion measured by ELISA. Data shown mean+/-SD cytokine levels from 5 discrete donors. Statistical significance was determined with a t-test.

5.2.1.3 Glucocorticoids (GCs) regulate macrophages

It is commonly accepted that macrophages can be viewed as a double-edged sword as the pro- and anti-inflammatory factors the release can both favour and suppress immune function. The cytokine profile of macrophages can be modulated by GCs and this generally tends to generate a less inflammatory environment. For example, decreased secretion of pro-inflammatory TNFα, IL-1, IL-6 by macrophages exposed to IFNγ in the presence of GCs was observed (Franchimont et al., 1999; Heasman et al., 2004). GCs also amplify IL-10 and TGF-β production by monocytes (Varga et al., 2008) and increase the display of membrane markers CD206, CD163 and CD169 (Heideveld et al., 2018). Exposure of macrophages to GCs leads to release of molecules that modify extracellular matrix and consequently play a role in matrix remodelling during the late phase of inflammatory responses. Glucocorticoids limit the production of collagenase, plasminogen and elastase activator by pro-inflammatory macrophages and these serve key roles for ECM remodelling (Desgeorges, Caratti, Mounier, Tuckermann, & Chazaud, 2019; Werb, Foley, & Munck, 1978). Lastly GC can impact on the number of Mϕ as illustrated by repeated dosing with Dexamethasone (Dex) which reduces the macrophage content of bronchial alveolar lavage fluid (BALF) (S. Zeng et al., 2017).

We therefore examined the response of our GM- and M-Mϕ models to the glucocorticoid, Dex. As shown in Figure 5.10, continues exposure to Dex significantly suppressed the production of IL-12 and IL-23 over the range of concentrations from 10^{-8}M to 10^{-6}M. Interestingly, there was a consistent observation of a Dex-dependent increase in both IL-12 and IL-23 by GM-Mϕ upon continuous exposure the lowest dose of Dex tested. The response of macrophages to GC occurred irrespective of the continuous presence of GM-CSF (Figure 5.10 blue vs red bars). We also examined the effect of transient exposure to Dex on cytokine production. To our surprise macrophages were rendered more sensitive to the suppression of cytokines by a brief (1hr) exposure to Dex (Figure 5.10). Dexamethasone did not induce the production of IL-10 under any of the conditions tested. We further analysed the data to obtain the IC_{50} (Figure 5.11). The IC50 of Dex does can be completely inhibited cytokine secretion is 10^{-7} M. This is in agreement with previous findings of an earlier study that showed high doses of Dex reduced IL-12 expression by alveolar macrophages (S. Zeng et al., 2017).

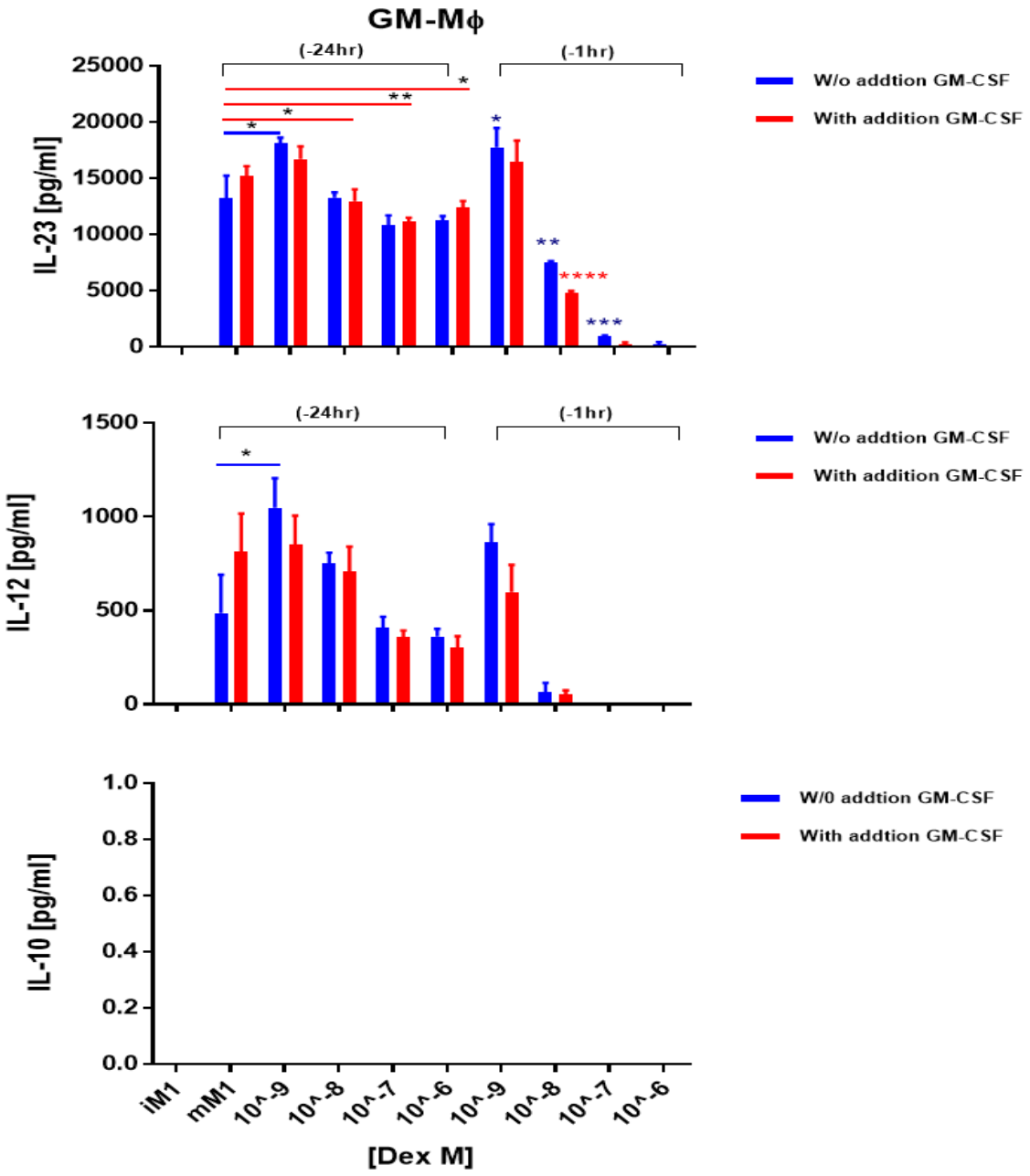

Figure 5.10 The effect of Glucocorticoids on the cytokine profile of GM-Mϕ. 5x10^6 CD14+ cells were cultured in Ultra-low attachment 6 well plate and treated with 20U/ml of GM-CSF. After 6 days of the culture, cells were harvested and transferred into 96 well plates at 5x10^4 cells per well. 0U/ml (Blue bars), 20U/l ml (Red bars) of GM-CSF was added for further culture. Dex was then added to triplicate wells at the indicated concentrations and maintained continuously in the culture (24hr) or was replaced with Dex-free medium after 1hr exposure. Maturation was induced with LPS and IFN-γ for 24hr. Supernatants were collected and cytokine secretion assayed by ELISA. Data shown mean cytokine levels amongst of triplicate well +/- SD and is representative 3 experiments of 3 donors. Statistical data were carried out using t-test.

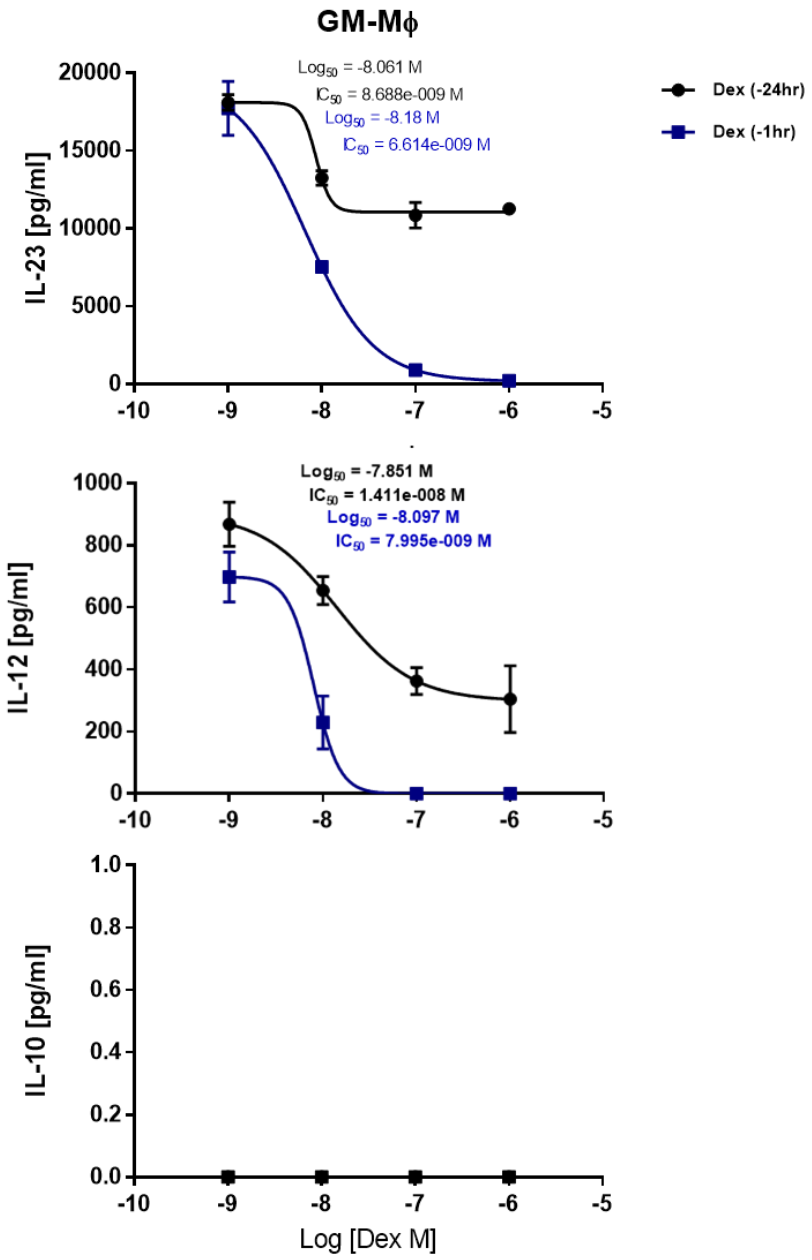

Figure 5.11 Determination of the Glucocorticoid dose (IC_{50}) that inhibits expression of cytokines by GM-Mϕ. $5x10^6$ CD14+ cells were cultured in Ultra-low attachment 6 well plate treated with 20U/ml of GM-CSF. After 6 days of the culture, cells were harvested and transferred into 96 well plates at $5x10^4$ cells per well. Dex was then added to triplicate wells at the different concentrations and maintained continuously in the culture (24hr) or was replaced with Dex-free medium after 1hr exposure. Maturation was induced with LPS and IFN-γ for 24hr. Supernatants were collected and cytokine secretion assayed by ELISA. Data shown log50 and IC50 of Dex doses are representative 3 experiments of 3 donors.

The prolonged addition of M-CSF did not change the pattern of cytokine responses from mature M-Mϕ (Figure 5.12 and 5.13). However, Dex did inhibit expression of IL-10 and IL-23. Low concentrations of Dex did not alter IL-10 secretion by GM-Mϕ however at higher does IL-10 was reduced in cells exposed to Dex for 24hr. Interestingly, when cells were treated with Dex 1hr before stimulation IL-10 production was inhibited 10^{-7}, 10^{-6} M, and induced a significant decrease at10^{-8} M. Interleukin-23 was markedly inhibited by Dex and this was again more prominent in cells briefly exposed to Dex than in those that were continuously treated. As expected, IL-12 was not observed by these cells.

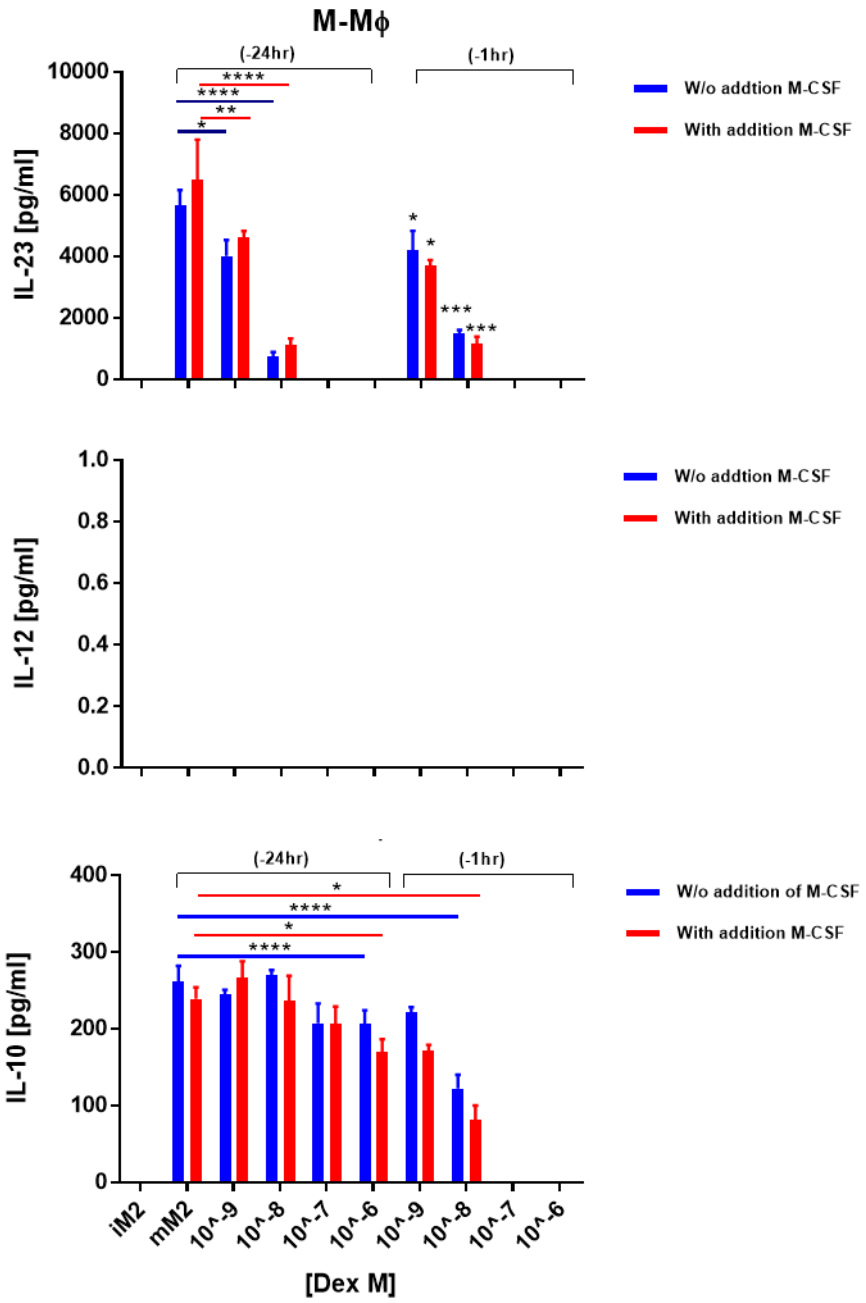

Figure 5.12 The effect of Glucocorticoids on the cytokine profile of M-Mϕ. 5x10^6 CD14+ cells were cultured in Ultra-low attachment 6 well plates and treated with 10 ng/ml of M-CSF. After 6 days of the culture, cells were harvested and transferred into 96 well plates at 5x10^4 cells per well. 0 ng/ml (Blue bars), 10 ng/ml (Red bars) of M-CSF was added for further culture. Dex was then added to triplicate wells at the indicated concentrations and maintained continuously in the culture (24hr) or was replaced with Dex-free medium after 1hr exposure. Maturation was induced with LPS for 24hr. Supernatants were collected and cytokine secretion assayed by ELISA. Data shown mean cytokine levels amongst of triplicate well +/- SD and is representative 3 experiments of 3 donors. Statistical data were carried out using t-test.

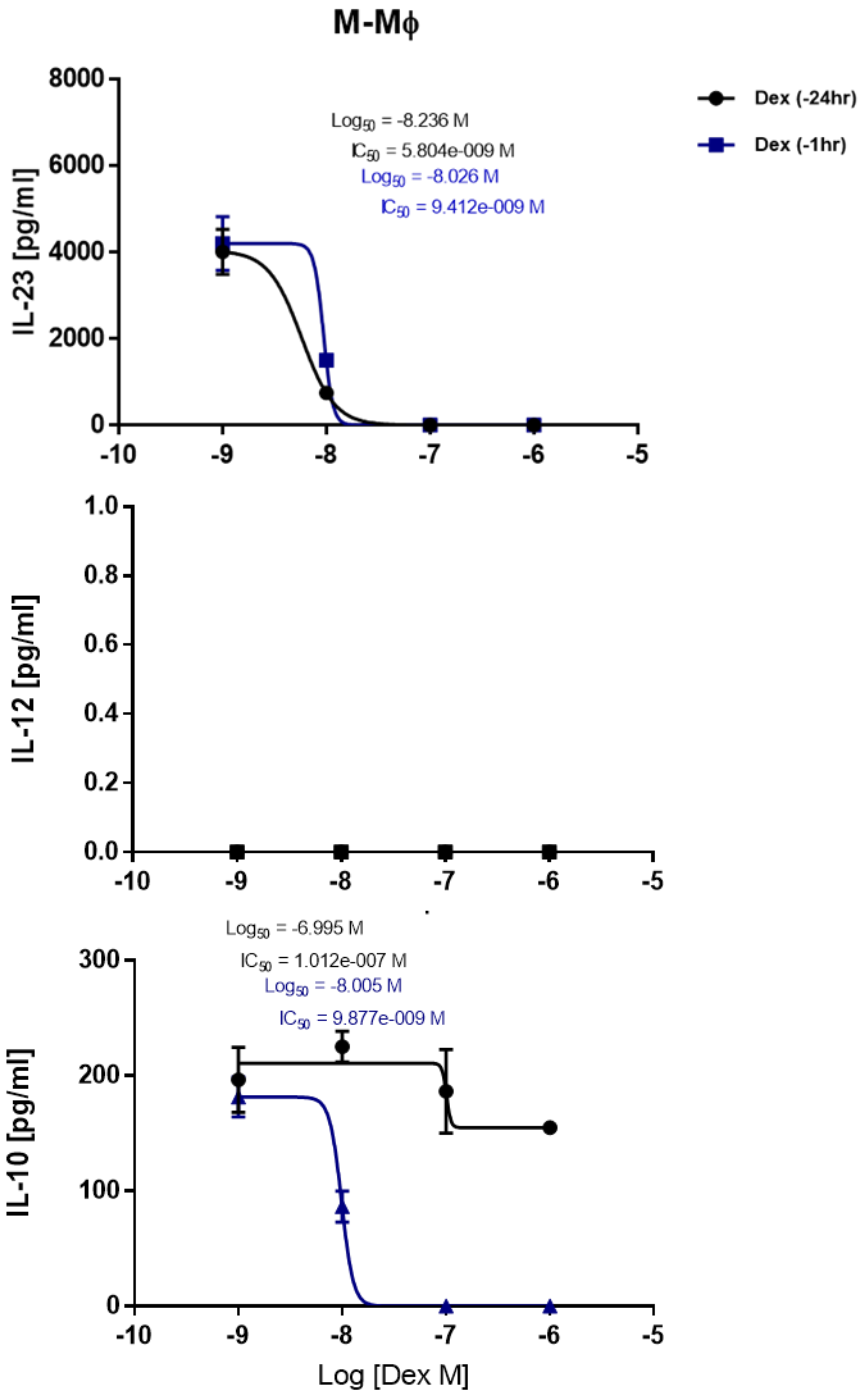

Figure 5.13 Determination of the Glucocorticoid dose (IC_{50}) that inhibits expression of cytokines by M-Mϕ. 5x10^6 CD14+ cells were cultured in Ultra-low attachment 6 well plate treated with 10 ng/ml of M-CSF. After 6 days of the culture, cells were harvested and transferred into 96 well plates at 5x10^4 cells per well. Dex was then added to triplicate wells at the different concentrations and maintained continuously in the culture (24hr) or was replaced with Dex-free medium after 1hr exposure. Maturation was induced with LPS for 24hr. Supernatants were collected and cytokine secretion assayed by ELISA. Data shown log50 and IC50 of Dex doses are representative 3 experiments of 3 donors.

5.2.1.4 The effects of TGF-β on FB-macrophage co-culture

To achieve the most robust effect of TGF-β, a low and a high concentrations of TGF-β were treated with MoMϕ. Secretion of IL-23 by LPS/IFN-γ-stimulated GM-Mϕ was a significant ($P<0.0001$) increase compared to GM-Mϕ stimulated with LPS only, whereas LPS/IFN-γ demonstrated production of IL-12 compared to GM-Mϕ stimulated with LPS only. Secretion of IL-10 by LPS/IFN-γ-stimulated GM-Mϕ was a significant ($P<0.05$) decrease compared to GM-Mϕ stimulated with LPS only. Unexpectedly, there was no effect on secretion of IL-23, IL-12 and IL-10 in the presence of TGF-β, Activation of M-Mϕ with LPS/IFN-γ further decreased ($P<0.0001$) secretion of IL-10 and IL-23 compared to M-Mϕ with LPS only. The secretion of IL-12 was induced while there was no significance irrespective of activation. There was a significant ($P<0.0001$) decrease IL-10 secretion by M-Mϕ in the presence of TGF-β at 5 ng/ml (Figure 5.14).

These changes, together with the literature reports, suggest that IFN-γ can prime cells for TLR4 responses and the production of pro-inflammatory cytokines (Duluc et al., 2009). Interestingly, IFN-γ was able to repolarize properties of M-Mϕ to a more pro-inflammatory phenotype by regulation of IL-12 secretion (Figure 5.14). Taken together, these results suggest that TGF-β at 5 ng/mL did regulate the macrophages phenotype.

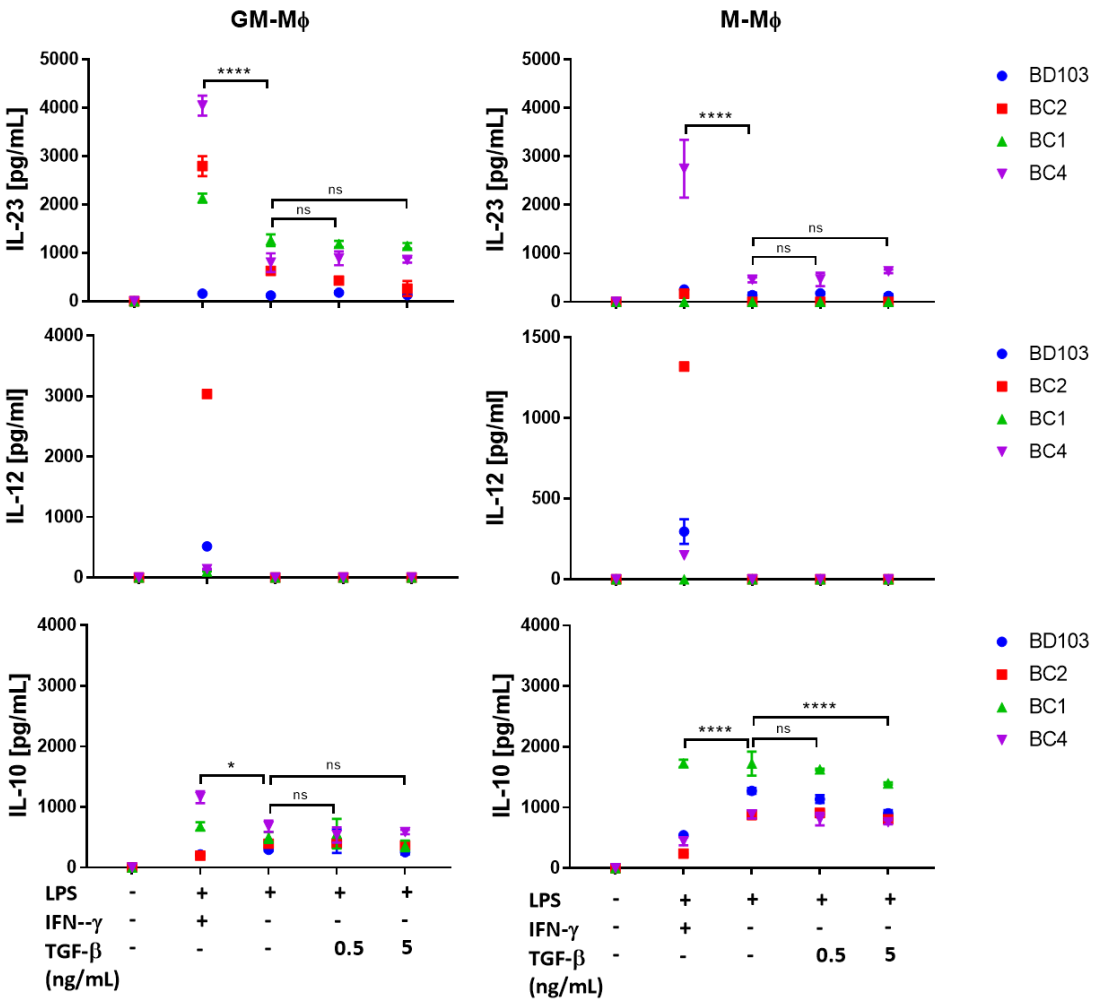

Figure 5.14 Effect of TGF-β regulate cytokine secretion by Mϕ through inhibit TLR ligands signalling pathway. 5x10^6 CD14+ cells were cultured in Ultra-low attachment 6 well plate and treated with 20U/ml of GM-CSF or 10 ng/ml of M-CSF. After 6 days of the culture, cells were harvested and transferred into 96 well plates at 5x10^4 cells per well. TGF-β was then added to triplicate wells at the indicated concentrations and maintained continuously in the culture (24hr). Maturation was induced with LPS and IFN-γ or LPS only for 24hr. Supernatants were collected and cytokine secretion assayed by ELISA. Data shown mean cytokine levels amongst of triplicate well +/- SD and is representative 4 experiments with 4 independent donors (BD103, BC1, BC2 and BC4). Statistical significance determined using two-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

5.2.1.5 Glucocorticoid (GCs) action on monocyte-derived macrophages in the presence of TGF-β

As described earlier the pattern of Mϕ were treated with TGF-β. However, after this experiment we investigated the combined impact of Dex plus TGF-β on cytokine secretion by macrophages. As shown in Figures 5.15 and 5.16, the effect of Dex was substantially altered by the presence of TGFβ irrespective of which macrophage population was studied. TGFβ at 0.5 and 5 ng/ml significantly ($P<0.05$ and $P<0.01$) suppressed secretion of IL-23 by GM-Mϕ in the presence of a high Dex concentration, and IL-10 was a significant ($P<0.001$) decreased in the presence of TGFβ at 5 ng/ml. TGFβ at 0.5 ng/ml significantly ($P<0.0001$) suppressed secretion of IL-23 by M-Mϕ in the presence of a low Dex concentration, Production of IL-23 by LPS/IFN-γ-stimulated GM-Mϕ was significantly ($P<0.0001$) increased in the presence of a high Dex concentration, whereas LPS/IFN-γ or with LPS only significantly ($P<0.0001$) suppressed IL-10 from GM-Mϕ and M-Mϕ. Interestingly, a high concentration of Dex inhibited IL-23 and IL-12, but increased IL-10 responses.

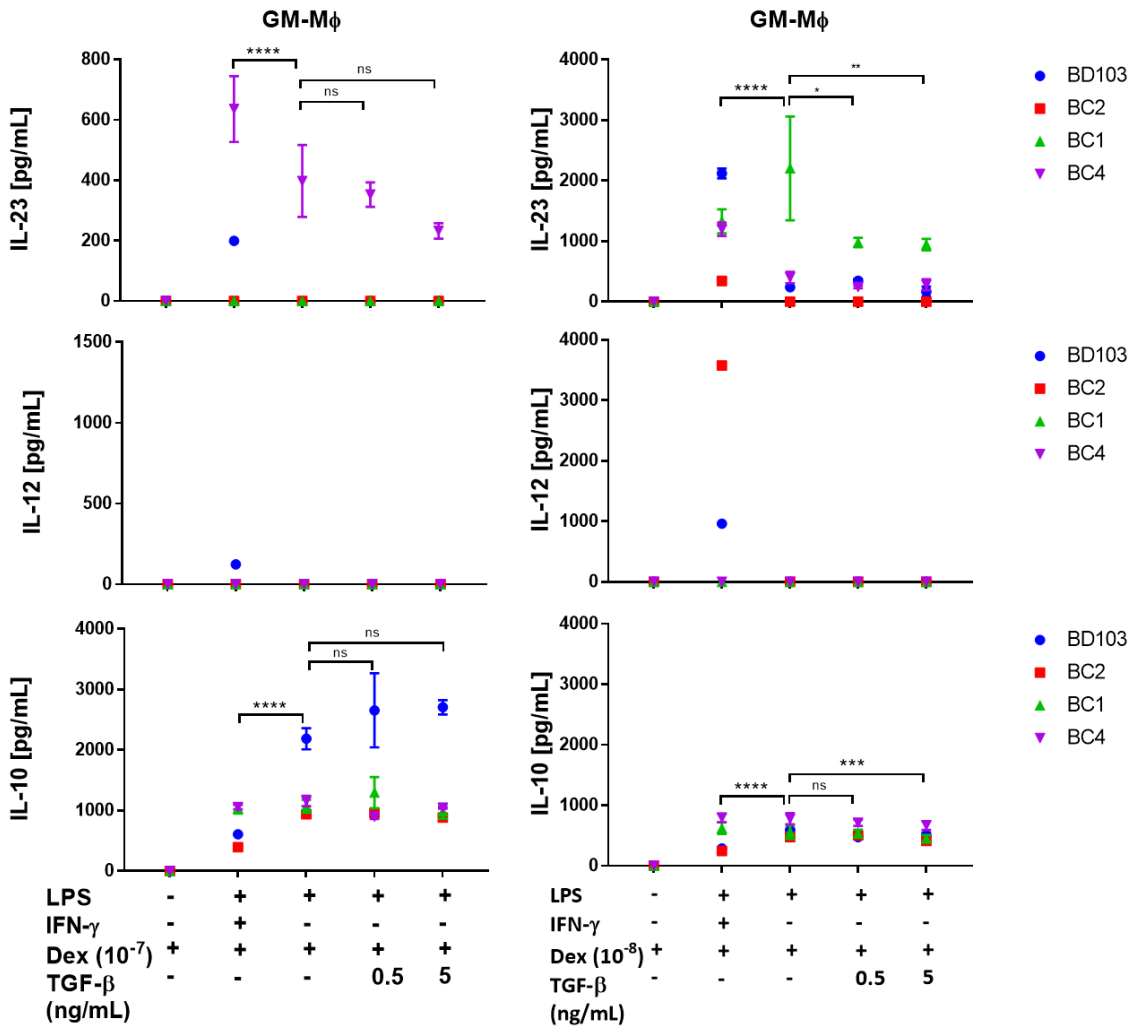

Figure 5.15 Anti-inflammatory effects of Dex exerts a potent anti-inflammation on cytokine secretion by GM-Mϕ in the presence of TGF-β. 5x10^6 CD14+ cells were cultured in Ultra-low attachment 6 well plate and treated with 20U/ml of GM-CSF. Dex was then added to ULA 6 well plates at the indicated concentrations. After 6 days of the culture, cells were harvested and transferred into 96 well palates at 5x10^4 cells per well. TGF-β was then added to triplicate wells at the indicated concentrations and maintained continuously in the culture (24hr). Maturation was induced with LPS and IFN-γ or LPS only for 24hr. Supernatants were collected and cytokine secretion assayed by ELISA. Data shown mean cytokine levels amongst of triplicate well +/- SD and is representative 4 experiments of 4 donors (BD103, BC1, BC2 and BC4). Statistical significance determined using two-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

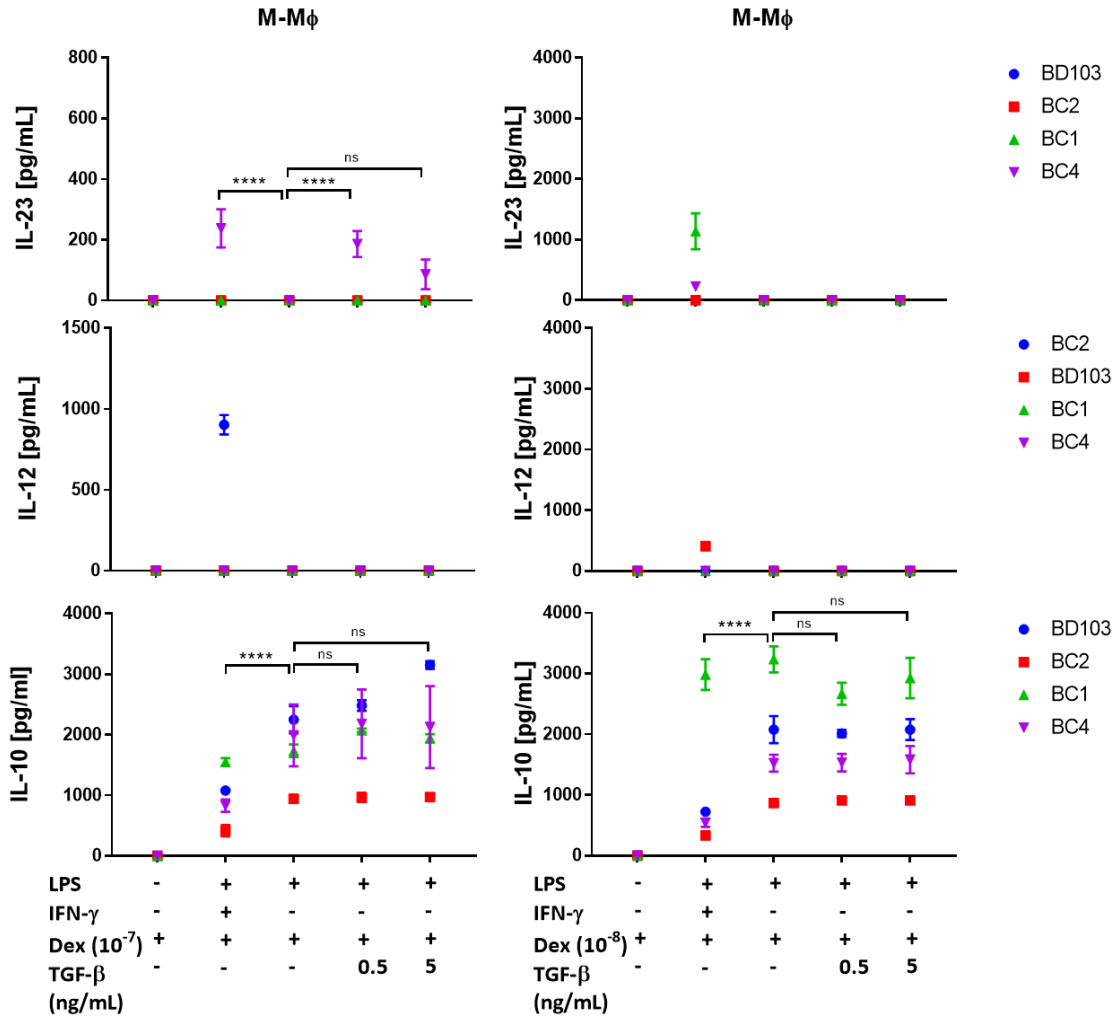

Figure 5.16 Anti-inflammatory effects of Dex exerts a potent anti-inflammation on cytokine secretion by M-Mϕ in the presence of TGF-β. 5x10^6 CD14+ cells were cultured in Ultra-low attachment 6 well plate and treated with 10 ng/ml of M-CSF. Dex was then added to ULA 6 well plates at the indicated concentrations. After 6 days of the culture, cells were harvested and transferred into 96 well palates at 5x10^4 cells per well. TGF-β was then added to triplicate wells at the indicated concentrations and maintained continuously in the culture (24hr). Maturation was induced with LPS and IFN-γ or LPS only for 24hr. Supernatants were collected and cytokine secretion assayed by ELISA. Data shown mean cytokine levels amongst of triplicate well +/- SD and is representative 4 experiments (BD103, BC1, BC2 and BC4). Statistical significance determined using two-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

5.2.2 The effects of FB cell lines and tumour-associated FB on macrophage differentiation

5.2.2.1 The impact of FB cell line-derived ECM on macrophage differentiation

Little is known of the effect of ECM on the differentiation of macrophages. Therefore, we utilised a system to coat tissue culture plastic in FB-derived matrix proteins (Barallobre-Barreiro et al., 2019) prior to the addition of monocytes and their differentiation in to macrophage. The methods requires extended culture of FB and therefore we document the microscopic appearance of 10 day old BJ6 cultures, and the resulting de-cellularised, ECM-coated wells (Figure 5.17). BJ6 cells achieved 100% confluence within 3 days when grown in FB medium and maintained a confluent layer throughout the culture period. This was not markedly affected by the addition of ascorbic acid. After cell lysis cells with extraction buffer cell bodies were no longer evident.

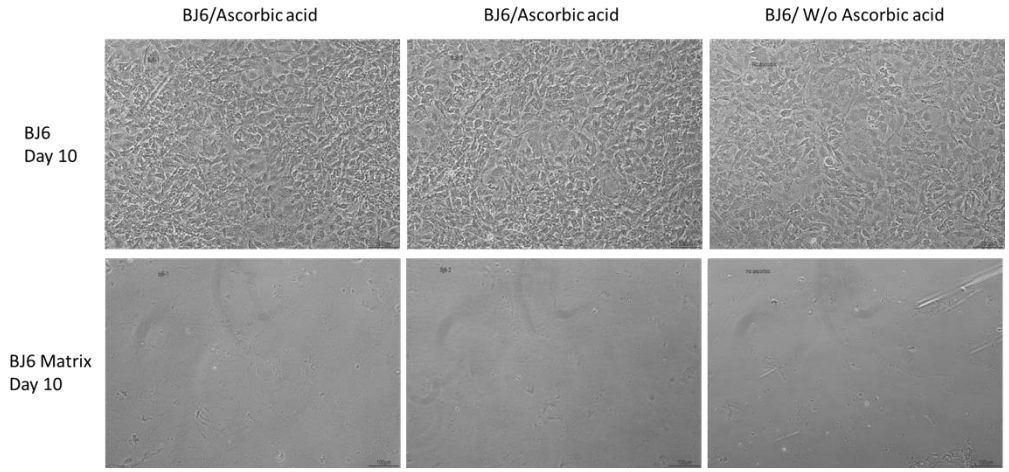

Figure 5.17 images of BJ6-ECM deposition on 13 mm glass coverslips placed on 24 well plates. Phase-contrast micrographs were taken under 10x magnification on day 10 of culture. Upper row indicates BJ6 cells confluence at 100% in the absence or the presence of ascorbic acid whilst the lower micrographs illustrate the resultant cell-free wells after decellularization by lysis of BJ6 cells with extraction buffer.

When freshly purified monocytes were plated on FB-derived ECM they displayed a uniformly circular morphology and were homogeneously distributed on the surface of the tissue culture plastic (Figure 5.18). In the absence of exogenous cytokine or maturation on ECM for seven days, however, a number of morphological changes were observed. Monocytes cultured on ECM deposited by the BJ6 FB cell line displayed a mixed morphology with a range of DC-like, spindle shaped, and macrophage-like 'fried egg' appearances. Upon the addition of maturation signals (LPS/IFNγ) (Figure 5.18), the morphology remained heterogeneous.

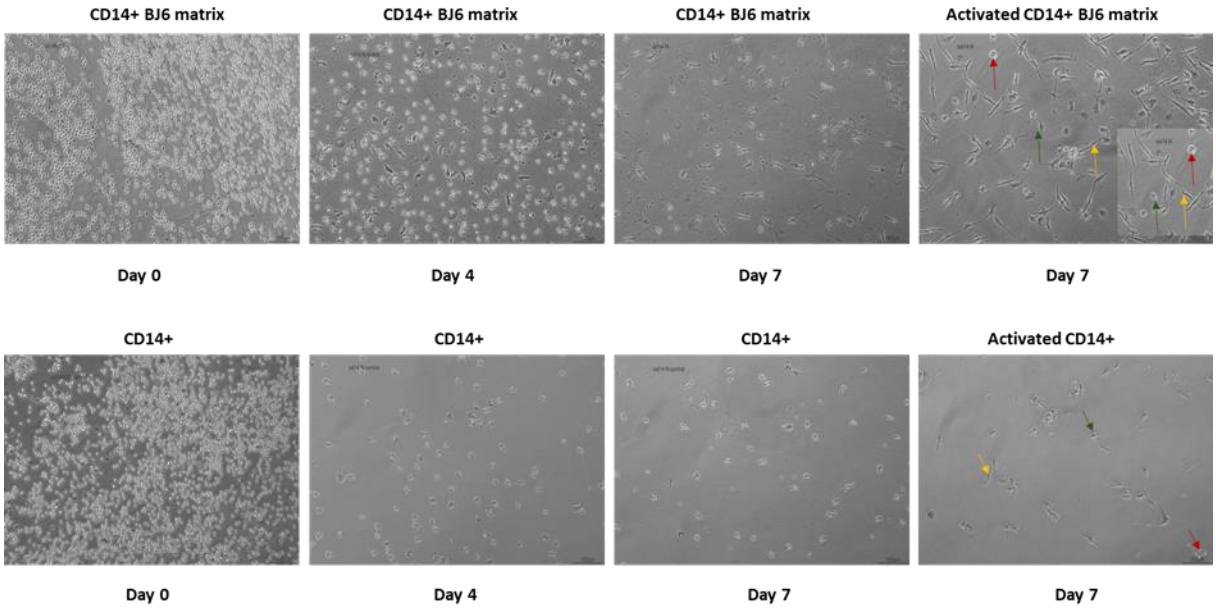

Figure 5.18 Morphology of CD14+ cells cultured on BJ6-ECM versus normal plastic. Cells were activated with LPS/IFN-γ for 24hr. Phase-contrast micrographs were taken under 10x magnifications at the indicate time points prior to harvest the supernatant and cells. Red arrow represents Mϕ-like cells, blue arrow represents DC-like cells and yellow indicates spindle shaped cells.

The morphological changes of macrophages when differentiated on ECM-coated plates were similar to monocytes. As expected, with immature GM-Mϕ in normal plates on day 7 the morphology was further altered with cell clusters or clumping, there were more adherent cells and little evidence of gross difference between cells cultured in either system (Figure 5.19). The addition of maturation resulted in cells with DC-like morphology, spindle shaped and classic macrophage-like appearances.

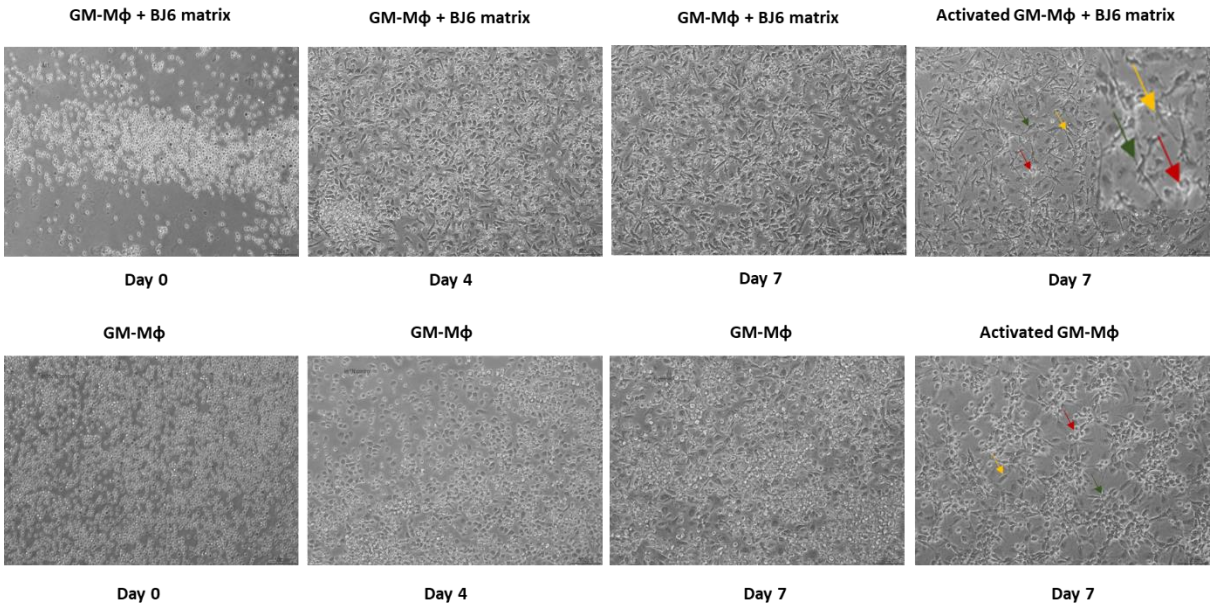

Figure 5.19 Morphology of GM-Mϕ differentiated on BJ6-ECM or on naked plastic. Cells were activated with LPS/IFN-Ɣ for 24hr. Phase-contrast micrographs were taken under 10x magnifications at the indicated time points prior to harvest the supernatant and cells. Red arrow represents Mϕ-like cells, green arrow represents DC-like cells and yellow indicates spindle shaped cells.

Immature M-Mϕs differentiated on ECM-coated plates also displayed a range of morphology (Figure 5.20). However, immature M-Mϕs on normal plastic showed a heterogeneous phenotype but little clustering whilst those plated on ECM displayed more clusters. Upon maturation, M-Mϕs became cell-like macrophages and cell-like DCs.

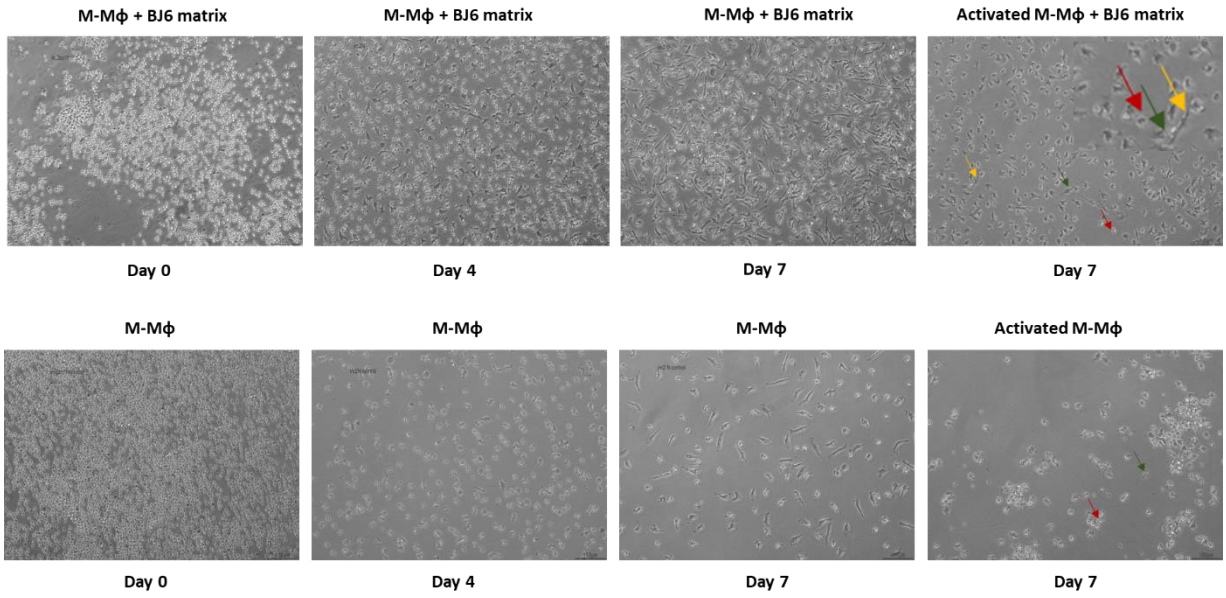

Figure 5.20 Morphology of M-Mϕ differentiated on BJ6-ECM or on normal plastics. Cells were activated with LPS only for 24hr. Phase-contrast micrographs were taken under 10x magnifications at different time points prior to harvest the supernatant and cells. Red arrow represents Mϕ-like cells, blue arrow represents DC-like cells and yellow indicates spindle shaped cells.

5.2.2.2 The impact of ECM on the phenotype of monocytes and macrophages

Having established that direct co-culture of Mϕ with FB on cytokine production, we next explored the possibility that FB-derived ECM modulates myeloid cell-function. Purified naïve CD14+ cells were cultured on FB-derived ECM in the absence/presence of LPS/IFN-γ and the phenotype and cytokine profile studied.

Culture of resting monocytes with FB-derived ECM resulted in some phenotypic changes (Figure 5.21). The presence of matrix significantly ($p<0.05$ and $p<0.001$) downregulated CD68, CD86 and CD169 respectively, with less change observed in CD206. These changes were different from those observed upon monocyte activation in the absence of ECM as these cells retained and increased CD169 and CD86 expression, whilst loosing CD68.

When monocytes were activated in the presence of matrix proteins their phenotype differed from activation alone. Activated monocytes in the presence of ECM had lower levels if CD206, the MRC1 mannose receptor but similar levels of CD68, CD86 and CD169. Taken together, these data confirm the activation of monocytes by exogenous maturation stimulus and suggest that their phenotype can be affected by the presence of FB-derived ECM.

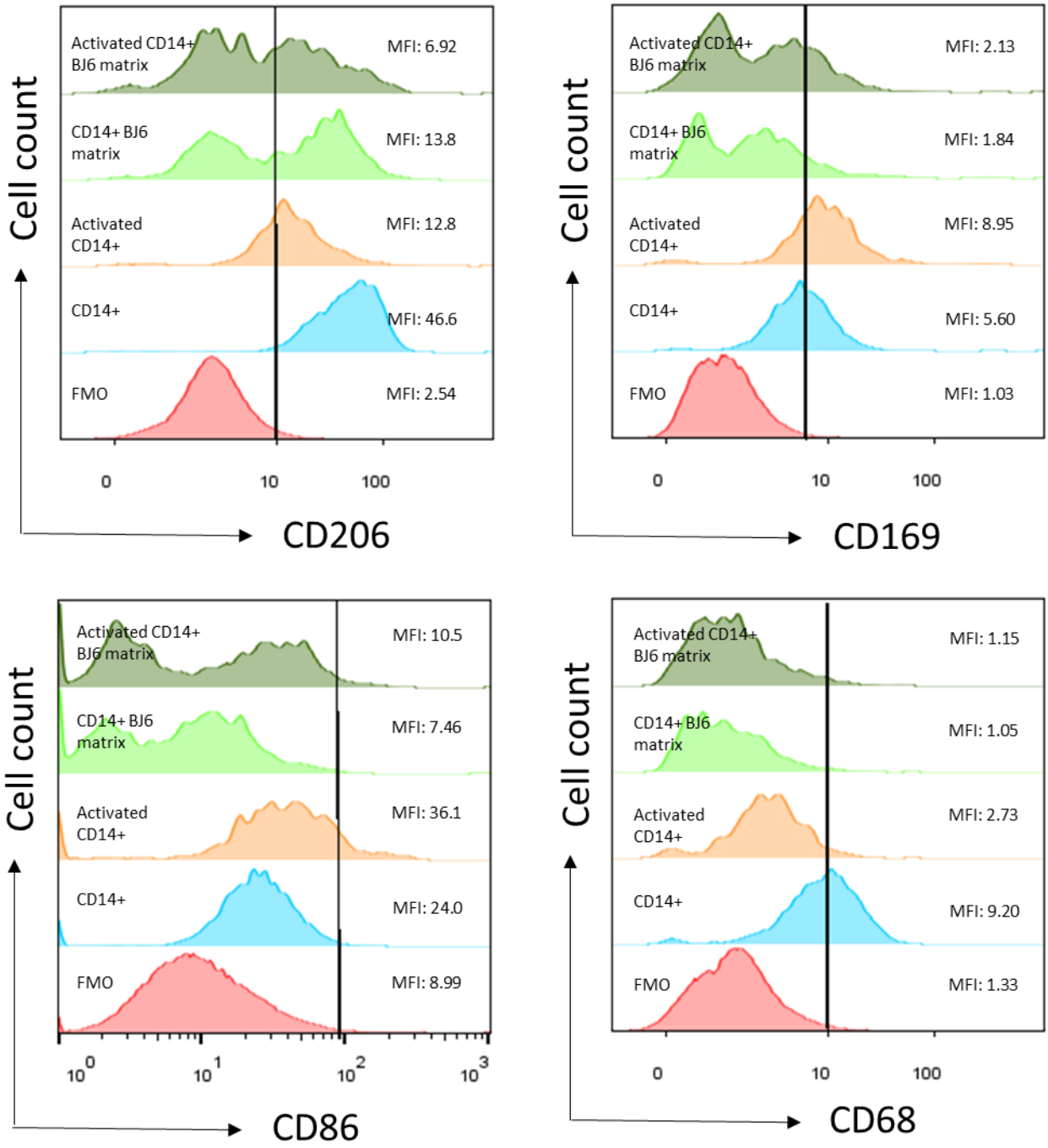

Figure 5.21 Analysis of expression of surface markers on monocytes cultured on ECM from BJ6 FB. Isolated CD14+ cells were cultured in BJ6-EMC coated 24 well plate in medium only. Next, after 6 days of the culture, cells were stimulated with 5µg/ml of LPS and 1000U/ml of IFN-γ for 24hr. Next day, cells were harvested and stained with anti-CD206 VioBlue, anti-CD86-PerCP-Vio700, and anti- CD169 (Siglec-1) FITC. Samples were analysed using flow cytometry. Cells were gated on equal number of cells ($2x10^4$) collected from CD14+ as shown. From left to the right histograms represent the median fluorescence intensity of CD206, CD169 and CD86. Histograms were drawn depending on fluorescent minus one (FMO). For the number indicates the MFI of CD206, CD169 and CD86 amongst CD14+ cells. Data shown is mean +/- SD of 4 separated experiments. Statistical data were carried out using *t-tests* (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

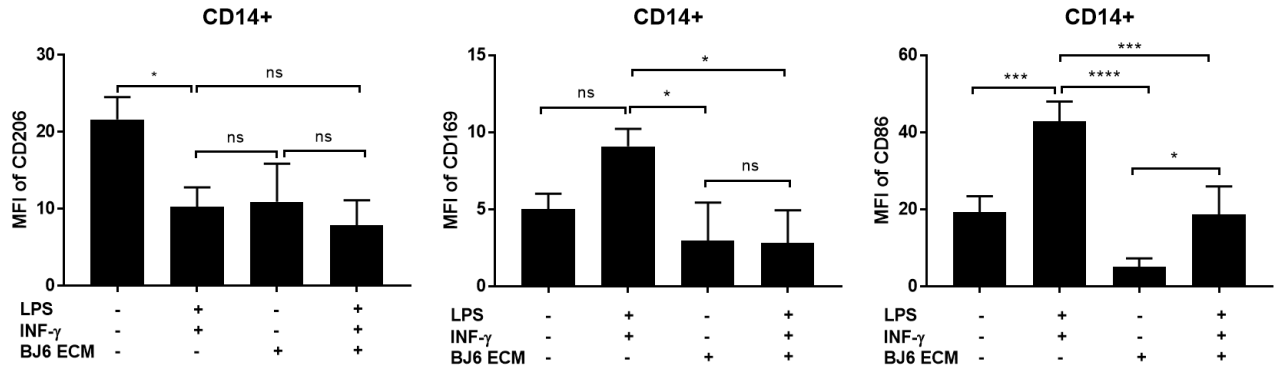

Figure 5.22 Median fluorescence intensity of CD206, CD169 and CD86 expression by monocytes differentiated on ECM. Resting CD14+ cells were cultured with ECM-FB in the absence/presence of LPS/IFN-γ. Expression of cell markers were determined in cultures of resting and activated CD14+, resting CD14+ crosslinking ECM-FB and activated CD14+crosslinking ECM-FB. Flow cytometry analysis and extracellular staining were performed according as per (Figure 5.21).

Markers	Mature CD14+ Mean+/-SD (MFI)	Mature CD14+/ECM Mean+/-SD (MFI)	P Value
CD206	10.3+/-2.5	7.9+/-3.3	NS
CD169	9+/-1.1	2.8+/-2.1	<0.05
CD86	42.9+/-5	18.7+/-7.3	<0.001

Table 5.1 The effect of BJ6-ECM on the expression of surface markers on CD14+ cells in response to LPS only. BJ6-ECM/CD14+ were activated using a standard concentration of LPS/IFNγ (5 µg LPS and 1000U/ml) compared to activated CD14+ only. And protein expression measured by flow cytometry after 24h.

To determine whether CD206, CD169 and CD86 expression by GM-Mϕ was modulated by BJ6-ECM, classical GM-Mϕ were generated in the presence of ECM. After activation their phenotype was determined (Figure 5.23), and as expected showed significantly ($p<0.01$) depressed levels of CD206. However, in the presence of ECM resting MO were more immature as demonstrated by significantly ($p<0.01$) increased CD206. CD169 was significantly ($p<0.0001$) upregulated upon activation. However, in mature GM-Mϕ, CD169 expression was more substantially increased with ECM than in immature cells on the same matrix. It was found that CD169 was displayed at a higher level by immature and mature GM-Mϕ when differentiated on ECM than on naked plastic. Whilst CD86 expression was elevated upon maturation, the presence of ECM further increased its display (Figure 5.24).

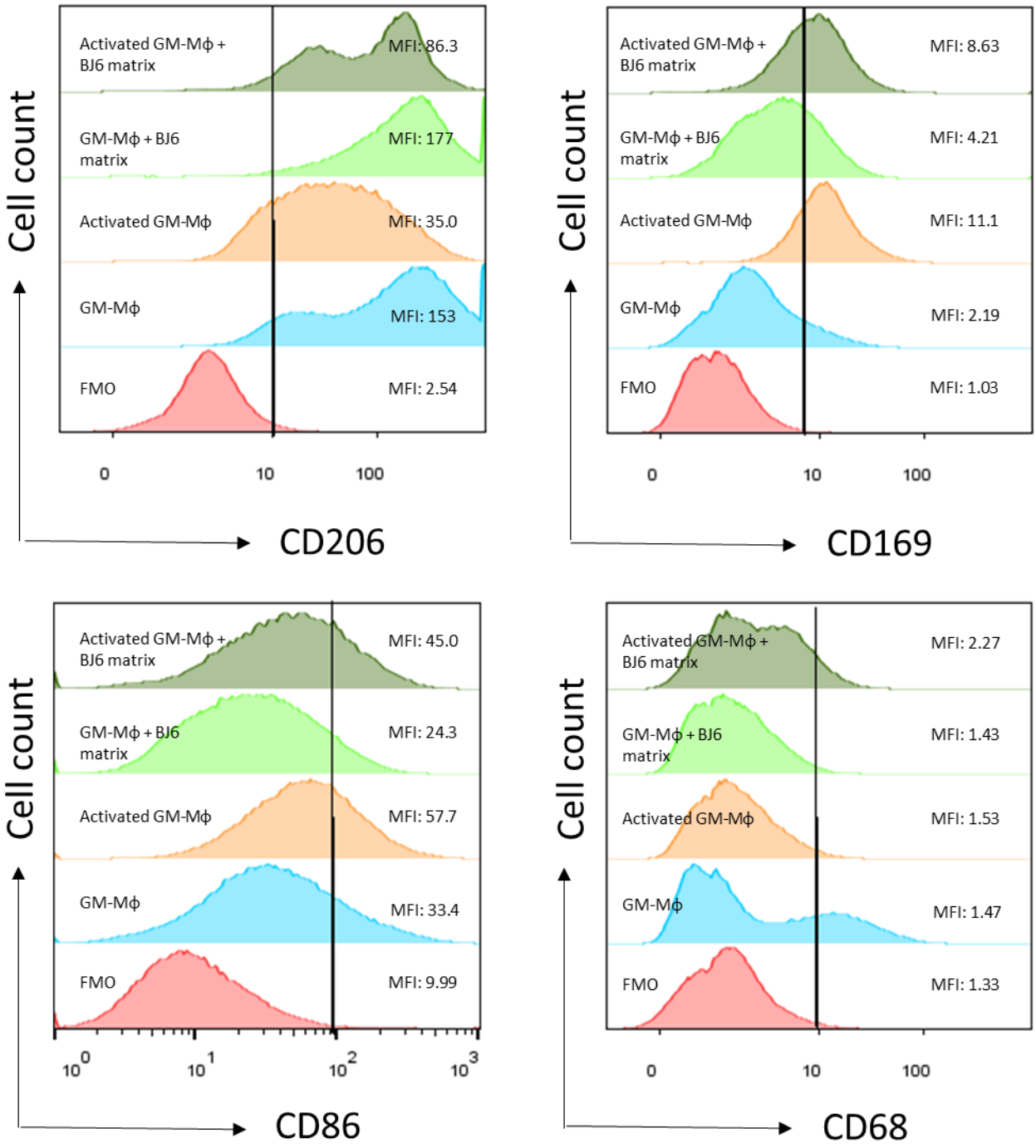

Figure 5.23 Analysis of expression of surface markers on GM-Mϕ differentiated on ECM. $0.7x10^6$ MACS-purified CD14+ cells were cultured in BJ6-EMC coated 24 well plate treated with 20U/ml of GM-CSF or medium only. Next, after 6 days of the culture, cells were stimulated with 5µg/ml of LPS and 1000U/ml of IFN-γ for 24hr. Next day, cells were harvested and phenotyped by flow cytometry. Cells were gated on equal number of cells ($2x10^4$). From left to the right histograms represent the median fluorescence intensity of CD206, CD169 and CD86. Histograms were drawn depending on fluorescent minus one (FMO). For the number indicates the MFI of CD206, CD169 and CD86 amongst GM-Mϕ cells. Data shown is mean +/- SD of 4 separated experiments. Statistical data were carried out using *t-tests* (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

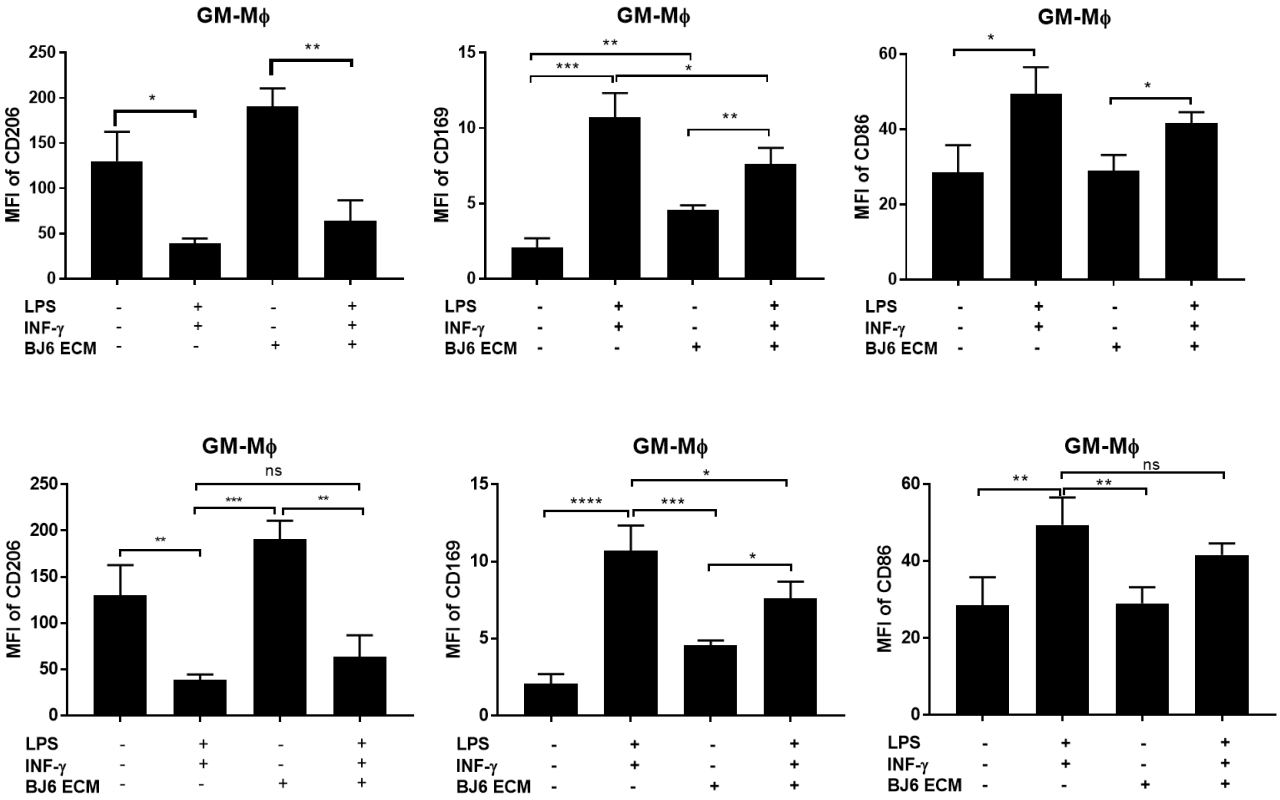

Figure 5.24 Median fluorescence intensity of CD206, CD169 and CD86 expression by GM-Mϕ in the presence of ECM. Resting GM-Mϕ cells were cultured with ECM-FB in the absence/presence LPS only. Expression of cell markers and cytokine production were determined in cultures of resting and activated GM-Mϕ, resting GM-Mϕ crosslinking ECM-FB and activated GM-Mϕ crosslinking ECM-FB. Flow cytometry analysis and extracellular staining were performed according to the Miltenyi protocol (Figure 5.23).

Markers	Mature GM-Mϕ Mean+/-SD (MFI)	Mature GM-Mϕ /ECM Mean+/-SD (MFI)	P Value
CD206	38.2+/-4.8	63.9+/-22.8	Ns
CD169	10.7+/-1.6	7.6+/-1	<0.05
CD86	49.4+/-7.2	41.6+/-3	Ns

Table 5.2 The effect of BJ6-ECM on the expression of surface markers on GM-Mϕ cells in response to LPS only. BJ6-ECM/GM-Mϕ were activated using a standard concentration of LPS/IFNγ (5 µg LPS and 1000U/ml) compared to activated GM-Mϕ only. And protein expression measured by flow cytometry after 24h.

Unexpectedly, CD206 expression by M-Mϕ was suppressed irrespective of the presence of ECM. The median fluorescent intensity of CD169 expression by mature M-Mϕ was significantly ($p<0.05$) lower than immature M-Mϕ (Figure 5.25), and CD169 expression of immature and mature M-Mϕ on ECM-coated plates was significantly ($p<0.05$) reduced compared to naked plastic. CD86 expression remained unaltered in immature M-Mϕ or mature M-Mϕ but its expression was significantly ($p<0.001$) decreased in the presence of ECM (Figure 5.26). This data suggests that FB-derived ECM BJ6 alter the phenotype of Mϕ. Further understanding of the impact of ECM myeloid cells was therefore undertaken using ECM deposited by primary fibroblasts from breast cancer patients.

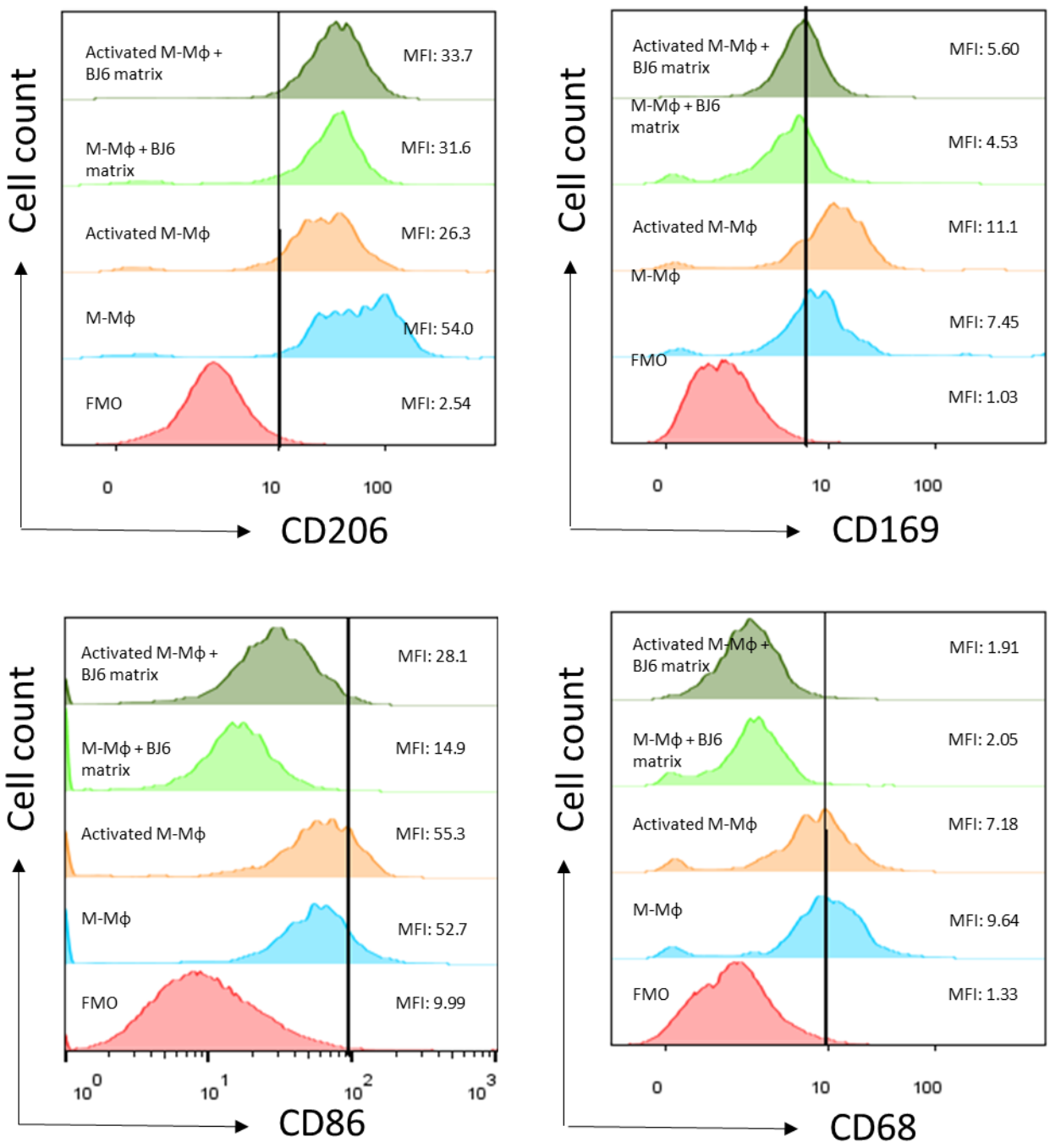

Figure 5.25 Analysis of phenotype of M-Mϕ cultured on FB cell line-derived ECM. 0.7x10^6 CD14+ cells were cultured in BJ6-ECM coated plates treated with 10 ng/ml of M-CSF or medium only. After 6 days, cells were stimulated with 5µg/ml of LPS for 24hr. Next day, cells were harvested and stained for flow cytometry with anti-CD206 VioBlue, anti-CD86-PerCP-Vio700, and anti- CD169 (Siglec-1) FITC. From left to the right histograms represent the median fluorescence intensity of CD206, CD169 and CD86. Histograms were drawn depending on fluorescent minus one (FMO). For the number indicates the MFI of CD206, CD169 and CD86 amongst M-Mϕ. Data shown is mean +/- SD of 4 separated experiments. Statistical data were carried out using *t-tests* (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

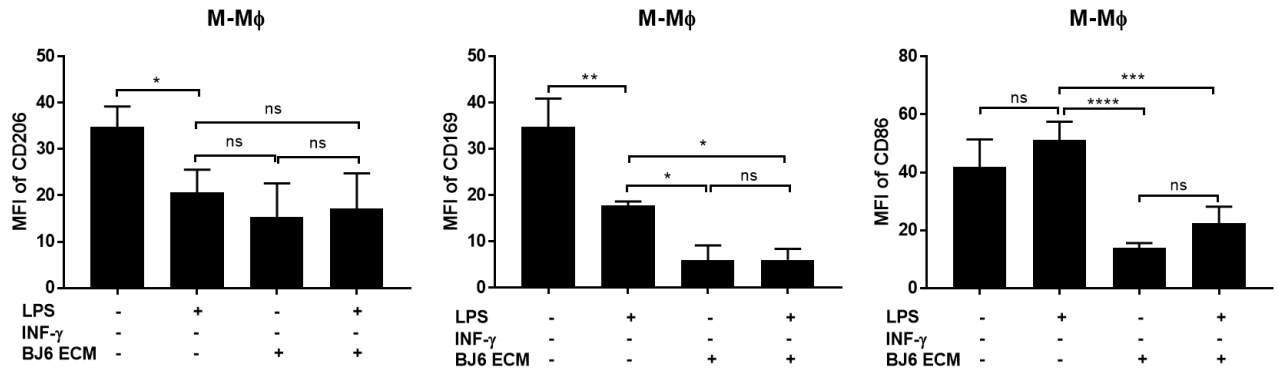

Figure 5.26 Median fluorescence intensity of CD206, CD169 and CD86 expression by M-Mϕ cultured with FB-derived ECM. Resting M-Mϕ cells were cultured with ECM-FB in the absence/presence LPS only. Expression of cell markers and cytokine production were determined in cultures of resting and activated M-Mϕ, resting M-Mϕ crosslinking ECM-FB and activated M-Mϕ crosslinking ECM-FB. Flow cytometry analysis and extracellular staining were performed (Figure 5.25).

Marker	Mature Mϕ-M Mean+/-SD (MFI)	Mature Mϕ-M /ECM Mean+/-SD (MFI)	P Value
CD206	20.6+/-4.9	17.1+/-7.6	NS
CD169	17.7+/-1	6+/-2.4	<0.05
CD86	51+/-6.4	22.4+/-5.7	<0.001

Table 5.3 The effect of BJ6-ECM on the expression of surface markers on M-Mϕ cells in response to LPS only. BJ6-ECM/M-Mϕ were activated using a standard concentration of LPS/IFNγ (5 µg LPS) compared to activated M-Mϕ only. And protein expression measured by flow cytometry after 24h.

5.2.2.3 The effect of ECM on cytokine production by monocytes and macrophages

The pathophysiology of rheumatoid arthritis (RA) includes cells from the myelomonocytic lineage (Firestein, 2003). The current concept reported that T cell polarisation may be involved in the polarisation of monocytes (Edwards, Zhang, Frauwirth, & Mosser, 2006). Monocytes express Toll-like receptors (TLR) which play a key role in the early, powerful and non-specific immune response through the action of danger signals. They are responsible for the immune reaction of the initial adaptive immune response, as well as modulating cytokine, chemokine secretion and co-stimulation (Iwasaki & Medzhitov, 2004).

Interleukin-12 is an important cytokine for macrophage and lymphocyte polarisation and differentiation. A study reported that tumour-bearing mice treated intravenously with recombinant IL-12 induced tumour regression and conversion of the anti-tumoural phenotype from TAM (Gabrilovich, Ostrand-Rosenberg, & Bronte, 2012; Watkins et al., 2007). There is growing evidence of the effects of colorectal cancer-derived EVs (extracellular vesicles) are taken up by monocytes and macrophages and regulate their phenotype and cytokine profile within the tumour microenvironment. Alteration of cytokine secretion in THP-1 monocytes and macrophages following culture with SW480 and SW620 EVs was observed; IL-23 secretion by M1 and M2 was induced, and IL-10 secretion by M2 was upregulated (Murawski et al., 2009; Sallusto & Lanzavecchia, 1994).

Gok et al. reported that TAFs not only regulate monocytes by stromal cell-derived factor-1 (SDF-1) and monocyte chemotactic protein-1 (MCP-1) but they were involved in the differentiation of monocytes into M2 macrophages expressing a high level of IL-10, therefore exerting a potent immunosuppressive molecule in breast cancer (Comito et al., 2014b; Yeh et al., 2016). IL-12 secretion by both TAF and breast cell-educated M1 macrophages was decreased, whereas IL-10 was slightly increased (Gok Yavuz et al., 2018). Within the tumour microenvironment and during tumour progression, tumour-infiltrating M1 expressed abundant IL-12 and low IL-10. Moreover, identification of this M2-like phenotype is characterised by a low IL-12 and a high IL-10 secretion (F. R. Balkwill & Mantovani, 2012; Sica & Mantovani, 2012).

We determine the effect of FB-derived ECM on monocytes (Figure 5.27). Monocytes produced little IL-10 production and this was only in ¼ donors. In this setting crosslinking by ECM decreased secretion of IL-10 by mature monocytes. IL-23 secretion by mature CD14+

cells was upregulated in three out of four donors. Interestingly, IL-23 secretion was observed in 2 donors when immature monocytes were cultured on ECM. Secretion of IL-12 by CD14+ cells was not observed.

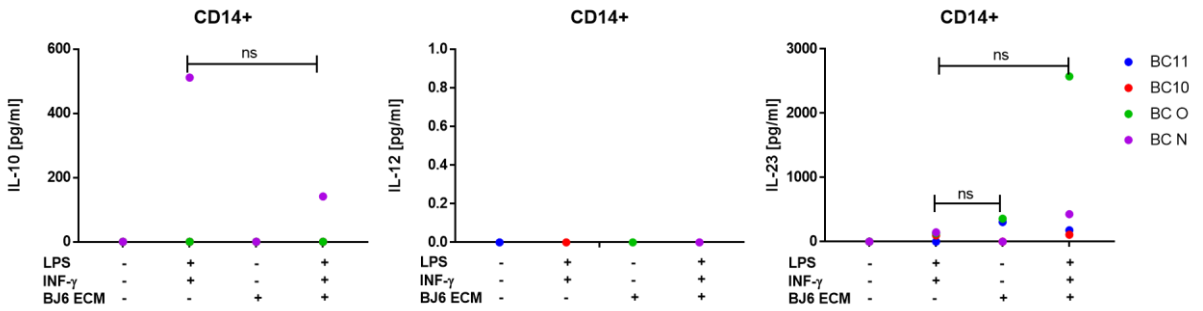

Figure 5.27 Expression of cytokines by monocytes cultured on BJ6-ECM. Monocytes ($0.7x10^6$) were cultured in BJ6-EMC coated 24 well plate. Next, after 6 days of the culture, cells were stimulated with 5µg/ml of LPS and 1000U/ml of IFN-γ for 24hr. Next day supernatants were collected and cytokine expressions assayed by ELISA. Data shown is mean +/- SD of 4 separated experiments (BC11, BC10, BCO and BCN). Statistical data were carried out using one-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

We studied the effect of FB-derived ECM on GM-Mϕ (Figure 5.28). As expected, activated GM-Mϕ produced IL-10, IL-12 and IL-23. In this setting crosslinking by ECM significantly ($P<0.5$) decreased secretion of IL-10 and IL-23 but did not affect IL-12.

When M-Mϕ were activated they expressed IL-10, with some IL-23 and minimal IL-12 (Figure 5.29). Crosslinking by ECM significantly ($P<0.5$) decreased secretion of IL-10 by mature M-Mϕ. Taken together, these data suggest that ECM may modulate the function of myeloid cells.

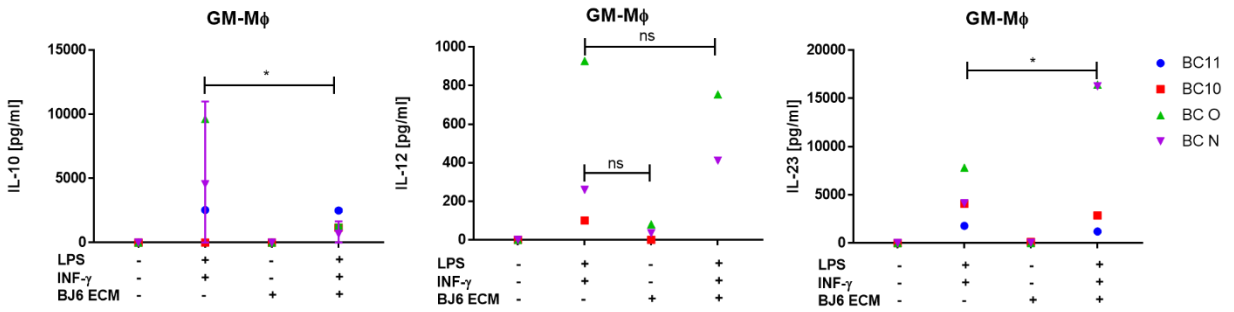

Figure 5.28 Expression of cytokines by GM-Mϕ cultured on BJ6-ECM. 0.7x10^6 MACS-purified CD14+ cells were cultured in BJ6-EMC coated 24 well plate treated with 20 IU/ml of GM-CSF. Next, after 6 days of the culture, cells were stimulated with 5µg/ml of LPS and 1000U/ml of IFN-γ for 24hr. Next day supernatants were collected and cytokine expressions assayed by ELISA. Data shown is mean +/- SD of 4 separated experiments. Statistical data were carried out using one-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

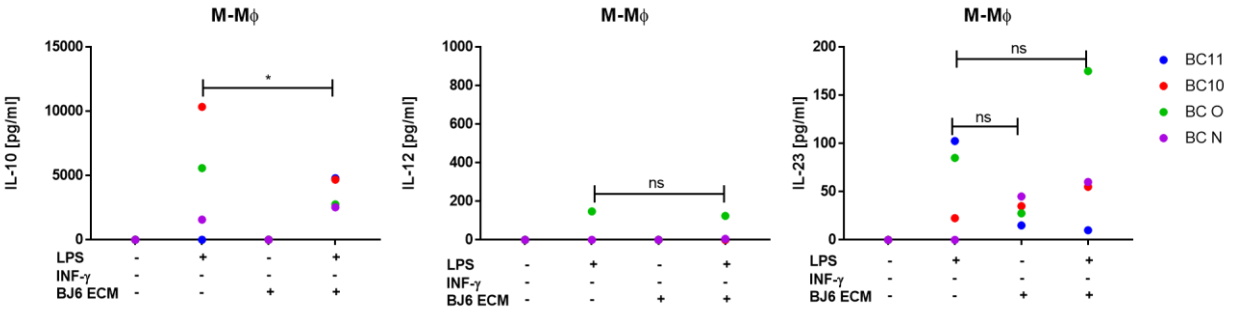

Figure 5.29 Expression of cytokines by M-Mϕ cultured on BJ6-ECM. 0.7x10^6 MACS-purified CD14+ cells were cultured in BJ6-EMC coated 24 well plate treated with 10 ng/ml of M-CSF. Next, after 6 days of the culture, cells were stimulated with 5µg/ml of LPS and 1000U/ml of IFN-γ for 24hr. Next day supernatants were collected and cytokine expressions assayed by ELISA. Data shown is mean +/- SD of 4 separated experiments. Statistical data were carried out using one-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

5.2.3 The role of ECM deposited by primary FB in the regulation of macrophage phenotype

5.2.3.1 Morphological changes of monocytes/macrophages in ECM ND, S and T

We utilised a system to coat tissue culture plastic in FB-derived matrix proteins as mentioned, prior to the addition of monocytes and their differentiation in to macrophage. The methods requires extended culture of FB and therefore we document the microscopic appearance of 10 day old ND, S and T FB cultures, and the resulting de-cellularised, ECM-coated wells (Figure 5.30). Cells achieved 100% confluence within 3 days when grown in FB medium and maintained a confluent layer throughout the culture period.

(Figure 5.31, 32, 33 and 34) displays examples of morphological changes of monocytes and macrophages the same as in section (5.2.2.1.1) as shown in (Figure 5.18, 19 and 20).

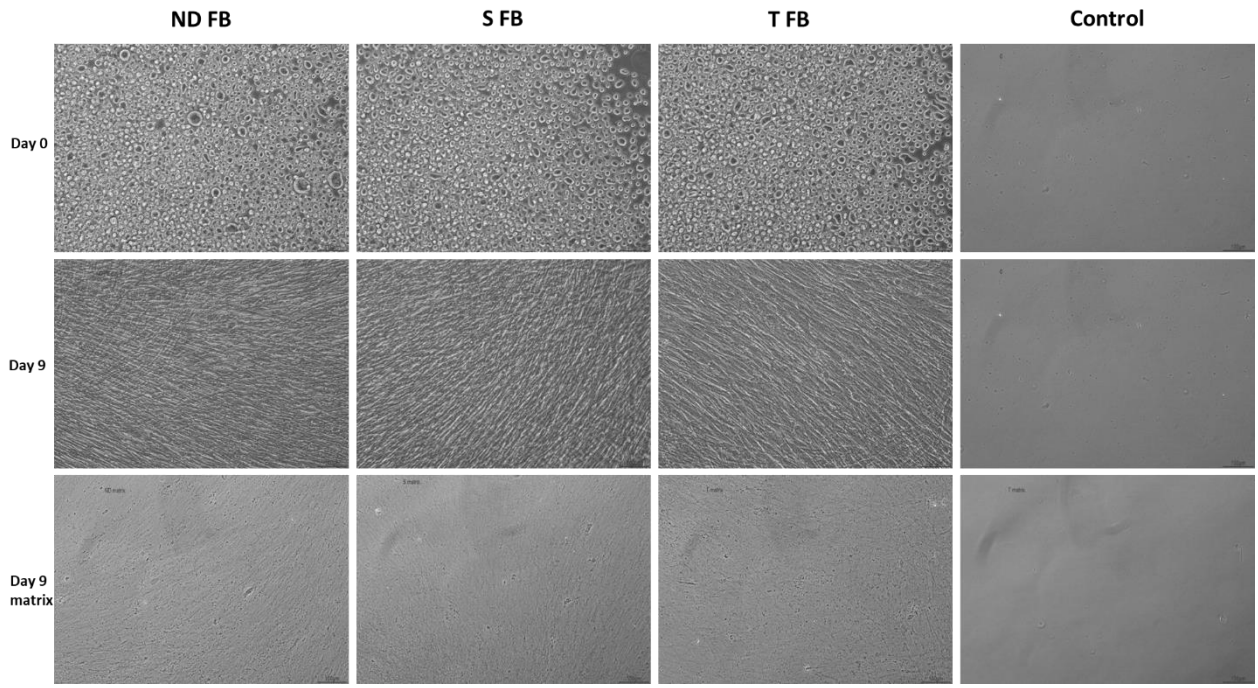

Figure 5.30 Micrographs of ND, S and T-ECM deposition on glass coverslips. Phase-contrast micrographs were taken under 10x magnifications as shown on day 0 and 9 of culture, from top row indicates actual starting numbers of cells on day 0, from middle row indicates cells confluence at 100% on day 9 and whilst the lower micrographs illustrate the resultant cell-free wells after decellularization lysis of ND, S and T cells with extraction buffer.

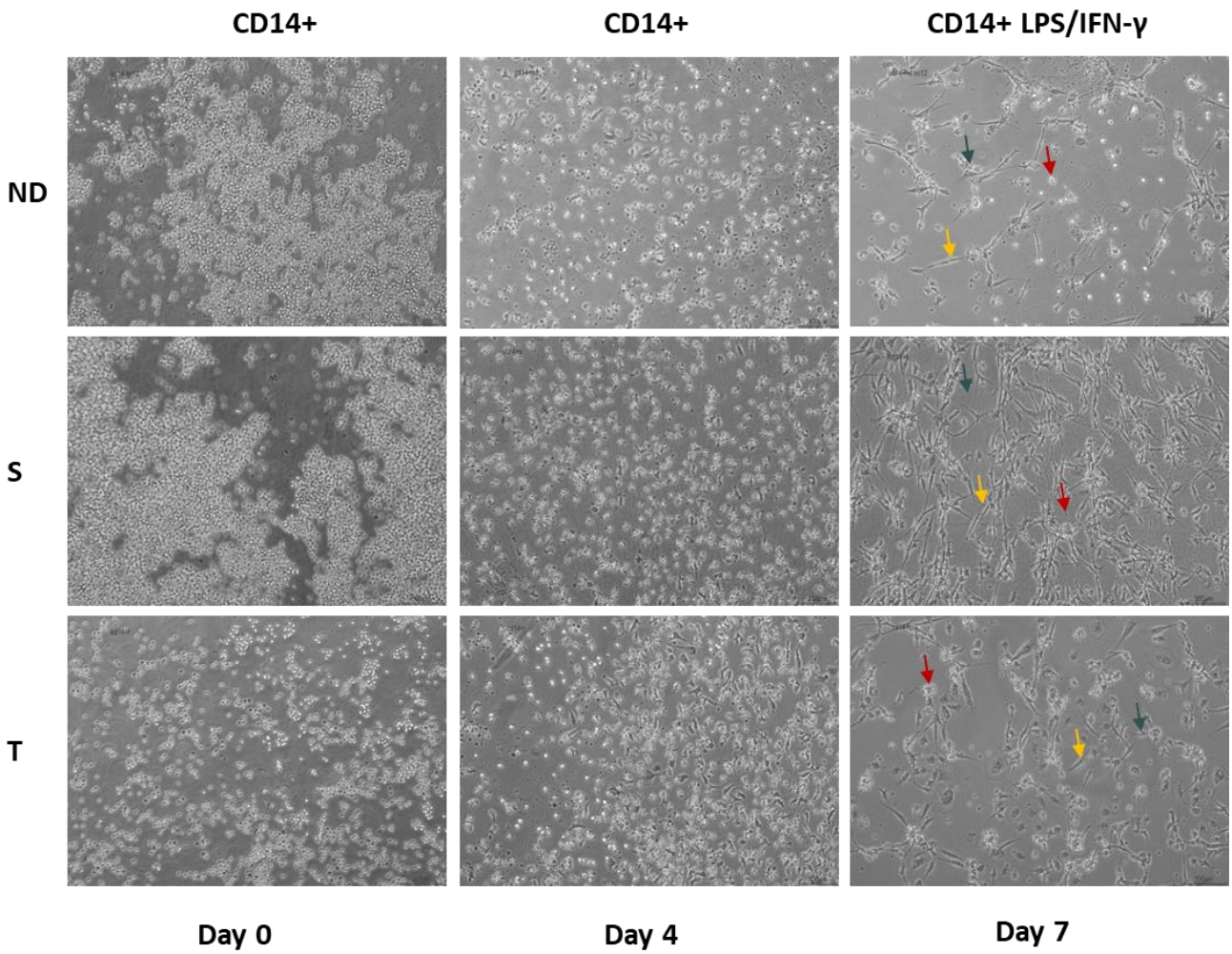

Figure 5.31 Morphology of monocytes were cultured on ND, S and T-ECM. Cells were activated with LPS/IFN-γ for 24hr. Phase-contrast micrographs were taken under 10x magnifications at the indicate time points prior to harvest the supernatant and cells. Red arrow represents Mϕ-like cells, blue arrow represents DC-like cells and yellow indicates spindle shaped cells.

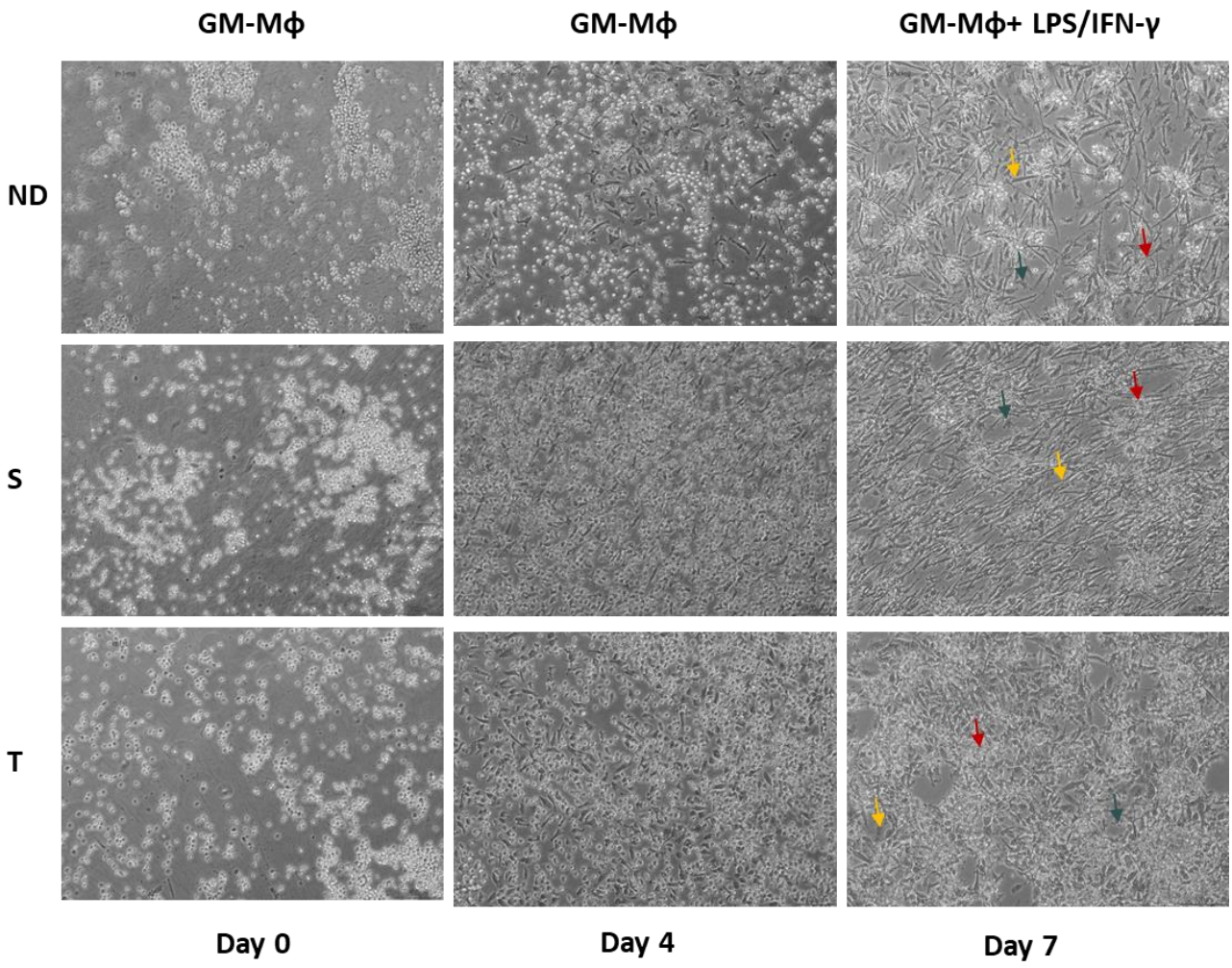

Figure 5.32 Morphology of GM-Mϕ cultured on ND, S and T-ECM. Cells were activated with LPS/IFN-γ for 24hr. Phase-contrast micrographs were taken under 10x magnifications at the indicated time points prior to harvest the supernatant and cells. Red arrow represents Mϕ-like cells, blue arrow represents DC-like cells and yellow indicates spindle shaped cells.

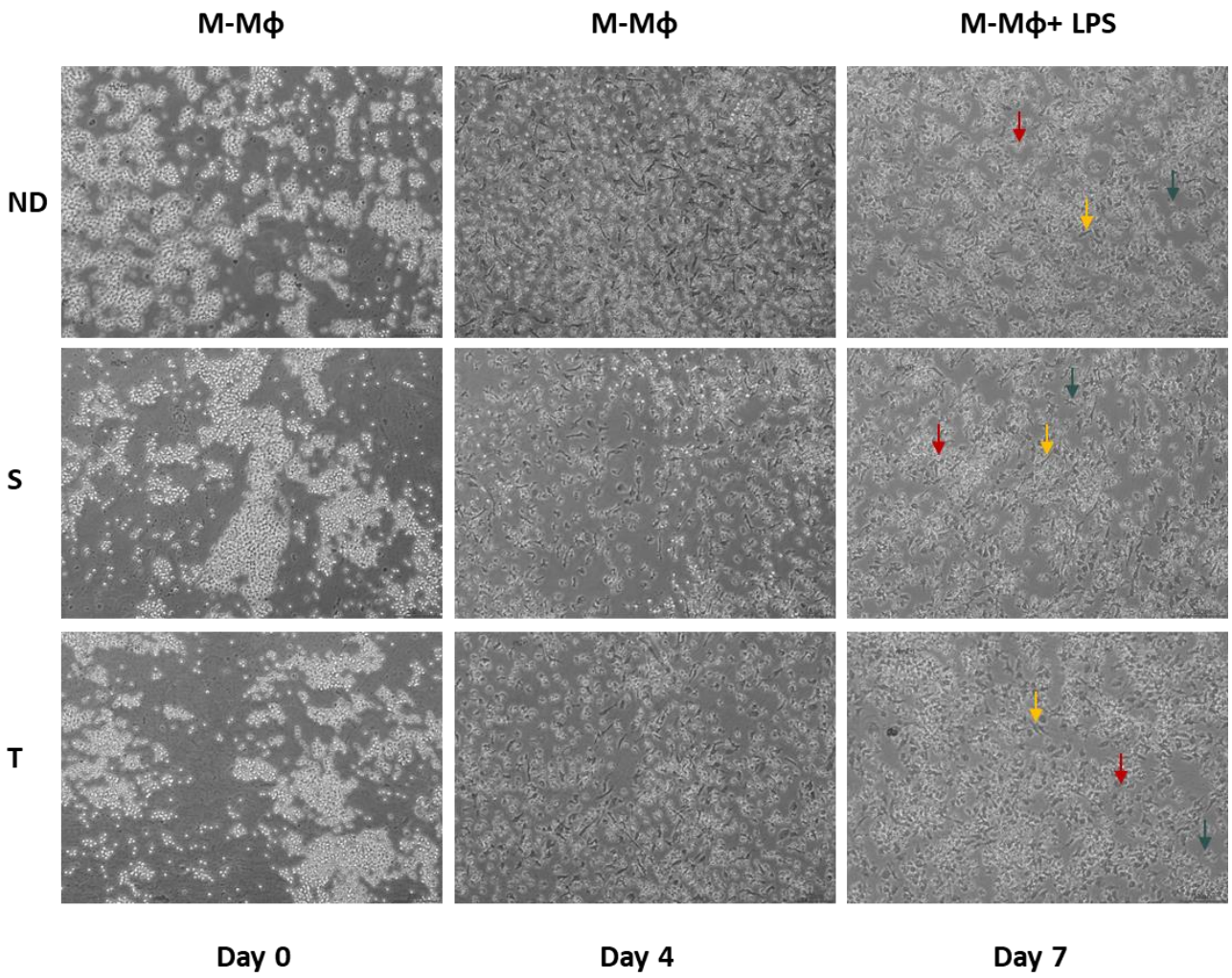

Figure 5.33 Morphology of M-Mϕ cells cultured on ND, S and T-ECM. Cells were activated with LPS only for 24hr. Phase-contrast micrographs were taken under 10x magnifications at the indicated time points prior to harvest the supernatant and cells. Red arrow represents Mϕ-like cells, blue arrow represents DC-like cells and yellow indicates spindle shaped cells.

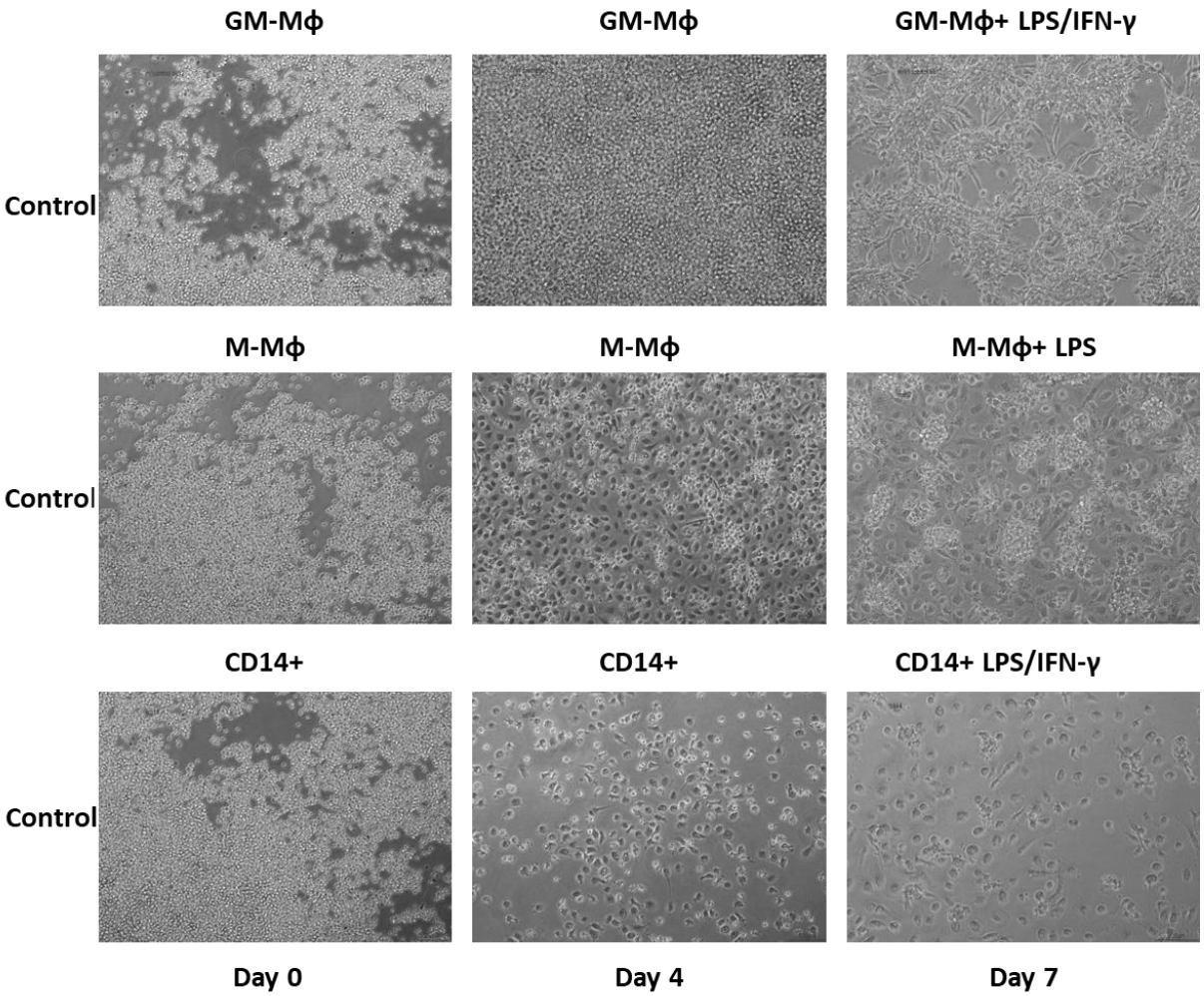

Figure 5.34 Morphology of monocytes, GM-Mϕ and M-Mϕ cultured on plastic surface. Cells were activated with LPS/IFN-γ or LPS only for 24hr. Phase-contrast micrographs were taken under 10x magnifications at the indicated time points prior to harvest the supernatant and cells. Mature GM-Mϕ are circular and clumpy, mature M-Mϕ display clumpy Mϕ-like cells, and mature CD14+ represents DC-like cells and Mϕ-like cells.

5.2.3.2 The effects of FB ECM on monocytes and macrophages phenotype

It was essential to perform experiments of ECM from normal dermal, surrounding and tumour fibroblasts based on the previous results in section (5.2.3.1) to investigate phenotypic consequence of differentiating Mϕ on matrix protein. This observation led to the hypothesis that ECM from primary fibroblasts influences the macrophage phenotype (Figure 5.35, 36 and 37).

Induction of CD80, CD163 and CD86 was observed on M1 macrophages, and CD206 for M2 macrophages (Bertani et al., 2017). Both resting and mature monocytes cultured on naked plastic displayed elevated CD206, CD86, CD169, CD204 and PD-1. CD206, CD86, CD169, CD204 and PD-1 expression by mature CD14+ cultured on ND, S and T ECM was lower but did not reach significance due to the variation in results from the different donors (Figure 5.36 A-E). Also, the expression of CD206, CD86, CD169, CD204 and PD-1 by mature monocytes on plated coated with T ECM was no effect, whereas expression of CD206, CD86, CD169, CD204 and PD-1 was lower in those cultured on S ECM.

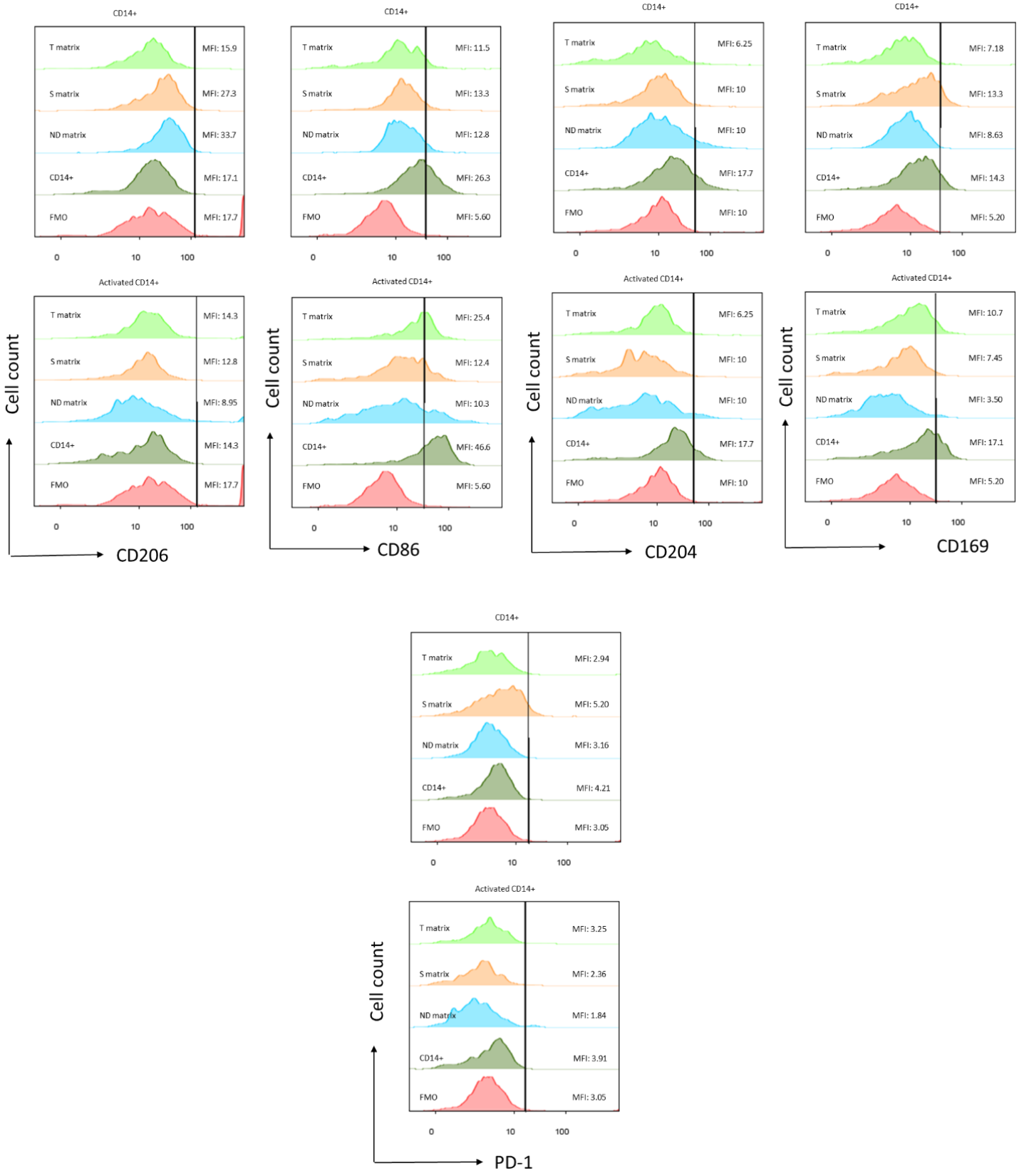

Figure 5.35 Effect of (Normal dermal) ND, (Surrounding) S and (Tumour) T-ECM on monocytes polarization.

Figure 5.35 Effect of (Normal dermal) **ND,** (Surrounding) **S and** (Tumour) **T-ECM on monocytes polarization.** 0.7x10^6 MACS-purified CD14+ cells were cultured in ND, S and T-ECM coated 24 well plate. Next, after 6 days of the culture, activated cells were stimulated with LPS and IFN-γ or LPS only for 24hr. Next day, cells were harvested and stained with anti-CD206 VioBlue, anti-CD86-PerCP-Vio700, anti- CD169 (Siglec-1) FITC, anti-CD204 APC and anti- CD279 (PD1)-PE. Samples were analysed using flow cytometry. Cells were gated on equal number of cells (1x10^4) collected from CD14+ as shown. From left to the right histograms represent the median fluorescence intensity of CD206, CD86, CD169, CD204 and PD-1. Histograms were drawn depending on fluorescent minus one (FMO). For the number indicates the MFI of CD206, CD86, CD169, CD204 and PD-1 amongst CD14+ cells. Data shown is mean +/- SD of 4 separated experiments. Statistical data were carried out using *t-test* (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

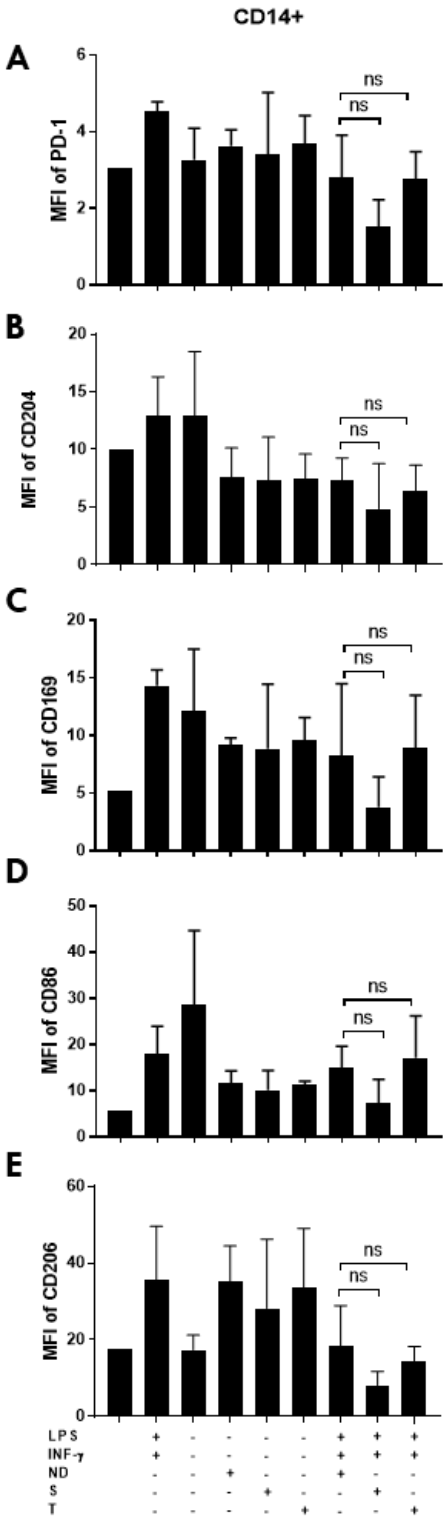

Figure 5.36 Median fluorescence intensity of CD206, CD86, CD169, CD204 and PD-1 on CD14+ cells cultured on primary FB ND, S and T-ECM. Resting CD14+ cells were cultured with ND, S and T-ECM in the absence/presence of LPS/IFN-γ. Expression of cell markers were determined in cultures of resting and activated CD14+, resting CD14+ crosslinking ECM-FB and activated CD14+ crosslinking ECM-FB. Flow cytometry analysis and extracellular staining were performed according as per (Figure 5.35).

Marker	CD14+ Type	ND-ECM Mean+/-SD	ESM Mean+/-SD	Value
CD206	S-ESM/CD14+	18.4+/-10.4	8.1+/-3.6	Ns
	T-ECM/CD14+	18.4+/-10.4	14.5+/-3.8	Ns
CD86	S-ESM/CD14+	14.8+/-4.8	7.4+/-5.1	Ns
	T-ECM/CD14+	14.8+/-4.8	17.1+/-9	Ns
CD169	S-ESM/CD14+	8.3+/-6.2	3.8+/-2.6	Ns
	T-ECM/CD14+	8.3+/-6.2	8.9+/-4.6	Ns
CD204	S-ESM/CD14+	7.4+/-1.9	4.8+/-3.9	Ns
	T-ECM/CD14+	7.4+/-1.9	6.4+/-2.3	Ns
PD-1	S-ESM/CD14+	2.8+/-1.1	1.5+/- 0.7	Ns
	T-ECM/CD14+	2.8+/-1.1	2.7+/-0.7	Ns

Table 5.4 The effect of ND, S and T-ECM on the expression of surface markers on CD14+ cells in response to LPS and IFNγ. S or T-ECM/CD14+ were activated using a standard concentration of LPS/IFNγ (5 µg LPS/1000U/ml IFNγ) compared to ND-ECM/CD14+. And protein expression measured by flow cytometry after 24h.

The expression of surface markers on macrophages was analysed (Figures 5.37 and 5.39). Both immature and mature GM-Mϕ cultured on naked plastic displayed elevated CD206, CD86, CD169 and CD204, whilst PD-1 was not observed. Expression of CD206 and CD86 was not regulated in mature GM-Mϕ cultured on ECM from tumour-derived FB (Figure 5.38 E and D). However, the expression of CD204 and CD169 in stimulated GM-Mϕ was upregulated by T-ECM in mature GM-Mϕ in (Figure 5.38 B and C). There was no effect of T-ECM on PD-1 display (Figure 5.38 A).

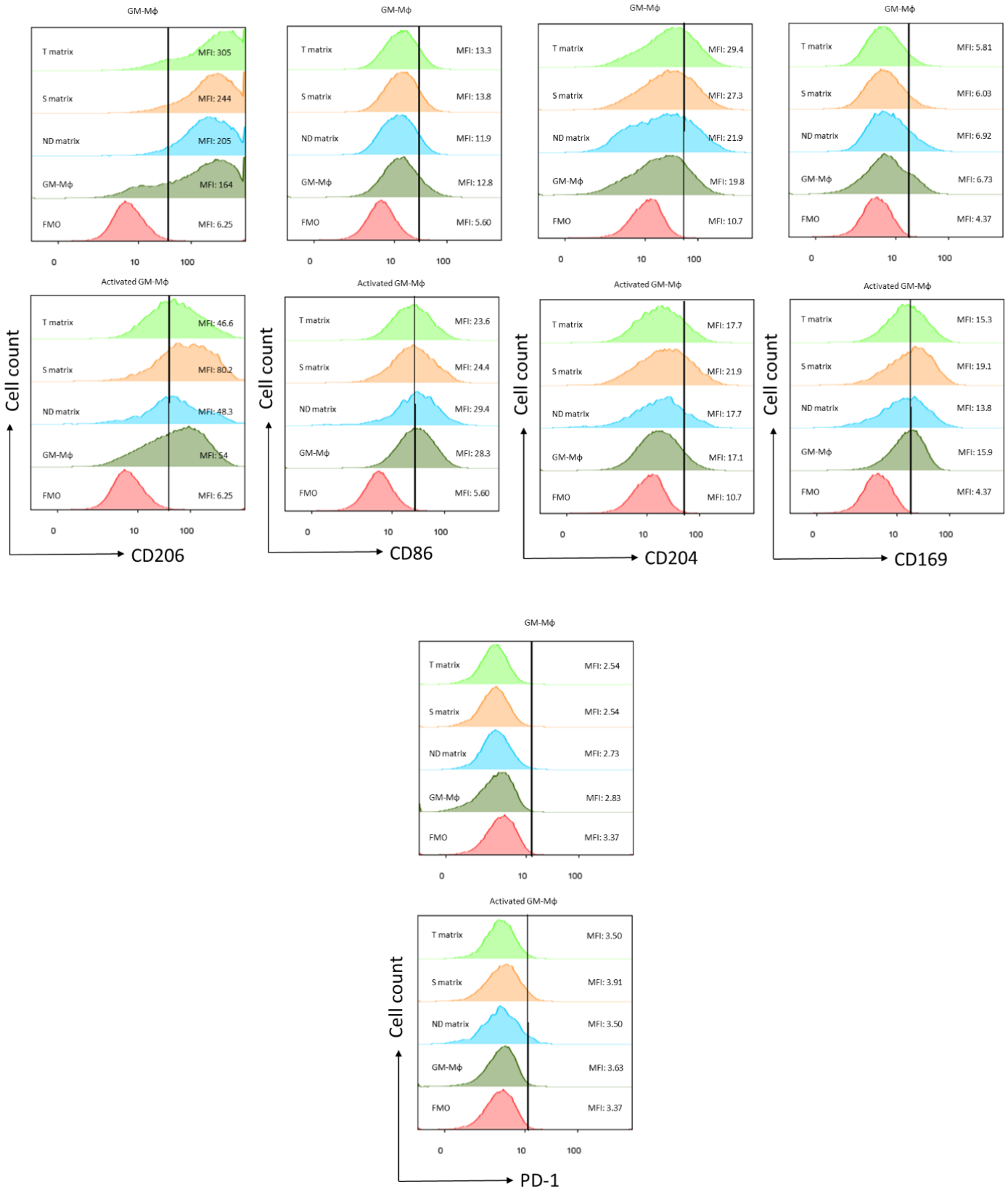

Figure 5.37 Effect of (Normal dermal) ND, (Surrounding) S and (Tumour) T-ECM on GM-Mϕ.

Figure 5.37 Effect of (Normal dermal) **ND,** (Surrounding) **S and** (Tumour) **T-ECM on GM-Mϕ.** $0.7x10^6$ MACS-purified CD14+ cells were cultured in ND, S and T-ECM coated 24 well plate treated with 20U/ml of GM-CSF. Next, after 6 days of the culture, cells were stimulated with LPS and IFN-γ for 24hr. Next day, cells were harvested and stained with anti-CD206 VioBlue, anti-CD86-PerCP-Vio700, anti- CD169 (Siglec-1) FITC, anti-CD204 APC and anti- CD279 (PD1)-PE. Samples were analysed using flow cytometry. Cells were gated on equal number of cells ($1x10^4$) collected from GM-Mϕ as shown. From left to the right histograms represent the median fluorescence intensity of CD206, CD86, CD169, CD204 and PD-1. Histograms were drawn depending on fluorescent minus one (FMO). For the number indicates the MFI of CD206, CD86, CD169, CD204 and PD-1 amongst GM-Mϕ cells. Data shown is mean +/- SD of 4 separated experiments. Statistical data were carried out using *t-test* (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

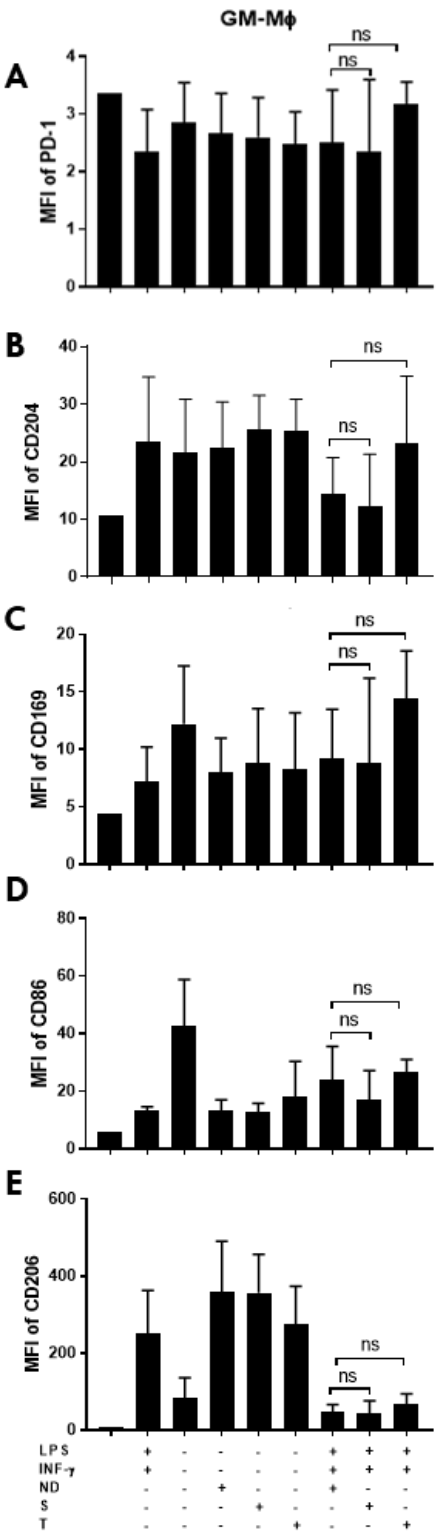

Figure 5.38 Median fluorescence intensity of CD206, CD86, CD169, CD204 and PD-1 expression by GM-Mϕ cells cultured on primary ND, S and T-ECM. Resting GM-Mϕ cells were cultured with ND, S and T-ECM in the absence/presence of LPS/IFN-γ. Expression of cell markers were determined in cultures of resting and activated GM-Mϕ, resting GM-Mϕ and activated GM-Mϕ cultured on ECM-FB. Flow cytometry analysis and extracellular staining were performed according as per (Figure 5.37).

Marker	GM-Mϕ Type	ND-ECM Mean+/-SD	ESM Mean+/-SD	Value
CD206	S-ESM/GM-Mϕ	47.1+/-9.5	8.1+/-3.6	Ns
	T-ECM/GM-Mϕ	47.1+/-9.5	67.7+/-27.1	Ns
CD86	S-ESM/GM-Mϕ	23.9+/-11.7	17.1+/-10	Ns
	T-ECM/GM-Mϕ	23.9+/-11.7	26.7+/-4.2	Ns
CD169	S-ESM/GM-Mϕ	9.2+/-4.3	8.8+/-7.4	Ns
	T-ECM/GM-Mϕ	9.2+/-4.3	14.4+/-4.1	Ns
CD204	S-ESM/GM-Mϕ	14.4+/-6.4	12.4+/-8.9	Ns
	T-ECM/GM-Mϕ	14.4+/-6.4	23.3+/-11.6	Ns
PD-1	T-ECM/GM-Mϕ	2.9+/-0.9	2.3+/-1.3	Ns
	T-ECM/GM-Mϕ	2.9+/-0.9	3.1+/-0.4	Ns

Table 5.5 The effect of ND, S and T-ECM on the expression of surface markers on GM-Mϕ cells in response to LPS and IFNγ. S or T-ECM/GM-Mϕ were activated using a standard concentration of LPS/IFNγ (5 µg LPS/1000U/ml IFNγ) compared to ND-ECM/GM-Mϕ. And protein expression measured by flow cytometry after 24h.

CD206, CD86, CD169, CD204 and PD-1 in M-Mϕ was regulated in any of the experimental processes cultured on naked plastic (Figure 5.39). In (Figure 5.40 A, B, C, D and E) shows the relative surface expression of CD206, CD86, CD169, CD204 and PD-1 on activated M-Mϕ cells grown of TECM was similar and was not clearly altered by T ECM.

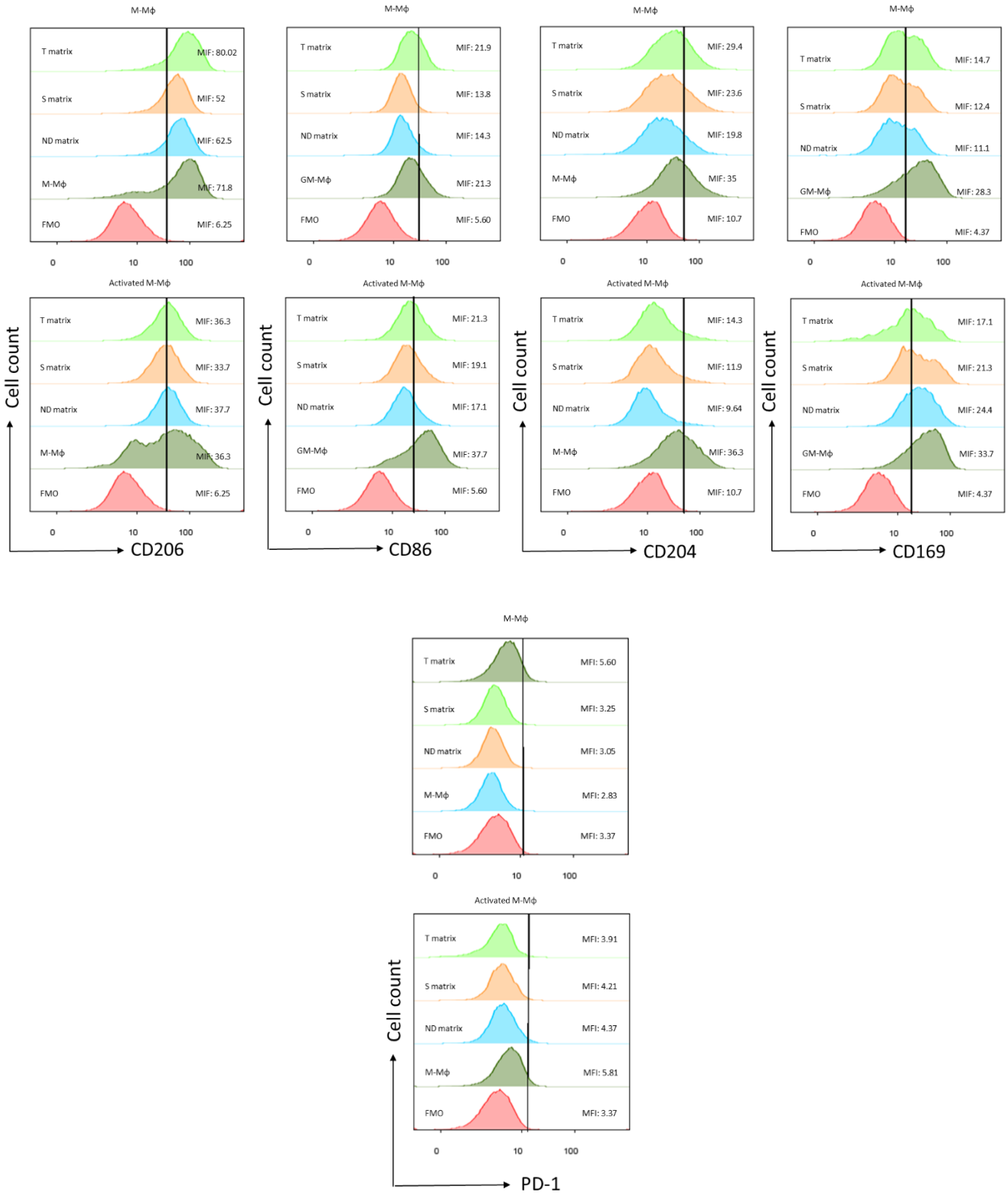

Figure 5.39 The effect of ECM deposited by (Normal dermal) ND, (Surrounding) S and (Tumour) T FB on the differentiation of monocytes to M-Mϕ.

Figure 5.39 The effect of ECM deposited by (Normal dermal) **ND,** (Surrounding) **S and** (Tumour) **T FB on the differentiation of monocytes to M-Mϕ.** $0.7x10^6$ MACS-purified CD14+ cells were cultured in ND, S and T-ECM coated 24 well plate treated with 10ng/ml of M-CSF. Next, after 6 days of the culture, cells were stimulated with LPS for 24hr. Next day, cells were harvested and stained with anti-CD206 VioBlue, anti-CD86-PerCP-Vio700, anti- CD169 (Siglec-1) FITC, anti-CD204 APC and anti- CD279 (PD1)-PE. Samples were analysed using flow cytometry. Cells were gated on equal number of cells ($1x10^4$) collected from M-Mϕ as shown. From left to the right histograms represent the median fluorescence intensity of CD206, CD86, CD169, CD204 and PD-1. Histograms were drawn depending on fluorescent minus one (FMO). For the number indicates the MFI of CD206, CD86, CD169, CD204 and PD-1 amongst M-Mϕ cells. Data shown is mean +/- SD of 4 separated experiments. Statistical data were carried out using *t-test* **(*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).**

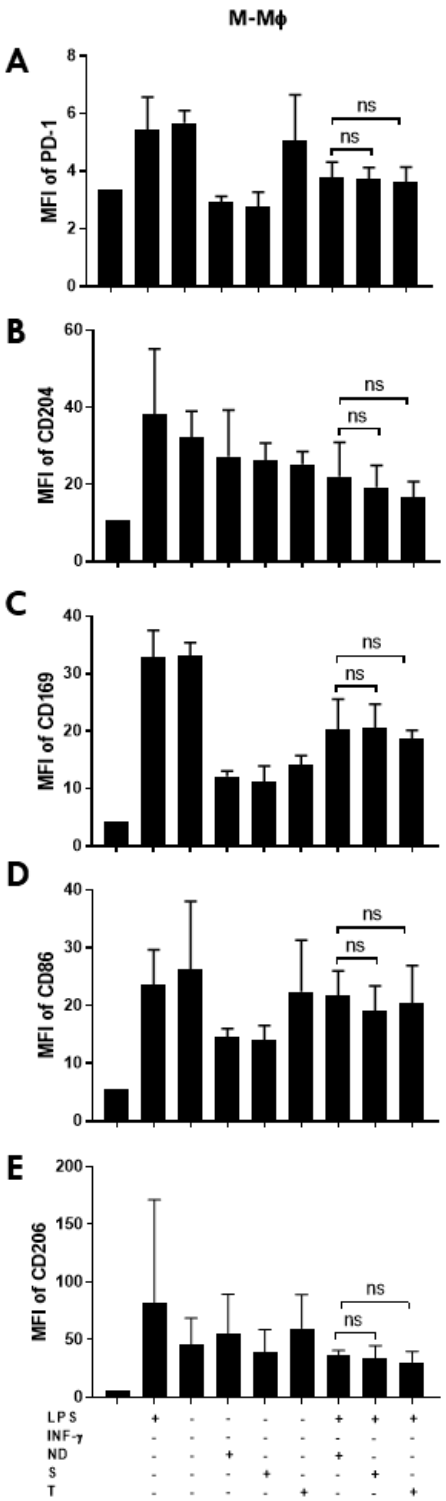

Figure 5.40 Median fluorescence intensity of CD206, CD86, CD169, CD204 and PD-1 expression by M-Mϕ differentiated on ND, S and T-ECM. Resting M-Mϕ cells were cultured with ND, S and T-ECM in the absence/presence of LPS. Expression of cell markers were determined in cultures of resting and activated M-Mϕ, resting M-Mϕ and activated M-Mϕ in the presence of ECM-FB. Flow cytometry analysis and extracellular staining were performed according as per (Figure 5.39).

Marker	M-Mϕ Type	ND-ECM Mean+/-SD	ESM Mean+/-SD	value
CD206	S-ESM/M-Mϕ	35.7+/-5.4	33.4+/-13.2	Ns
	T-ECM/M-Mϕ	35.7+/-5.4	29.1+/-10.2	Ns
CD86	S-ESM/M-Mϕ	21.7+/-4.3	19.1+/-4.3	Ns
	T-ECM/M-Mϕ	21.7+/-4.3	26.7+/-4.2	Ns
CD169	S-ESM/M-Mϕ	20.3+/-5.3	20.6+/-4.1	Ns
	T-ECM/M-Mϕ	20.3+/-5.3	18.8+/-1.4	Ns
CD204	S-ESM/M-Mϕ	22+/-8.9	19.3+/-5.7	Ns
	T-ECM/M-Mϕ	22+/-8.9	16.7+/-3.9	Ns
PD-1	T-ECM/M-Mϕ	3.8+/-0.5	3.7+/-0.4	Ns
	T-ECM/M-Mϕ	3.8+/-0.5	3.6+/-0.5	Ns

Table 5.6 The effect of ND, S and T-ECM on the expression of surface markers on M-Mϕ cells in response to LPS only. S or T-ECM/M-Mϕ were activated using a standard concentration of LPS/IFNγ (5 µg LPS) compared to ND-ECM/M-Mϕ. And protein expression measured by flow cytometry after 24h.

5.2.3.3 Effects of ECM from primary FB on cytokine secretion

To further investigate impact of TAF-derived ECM on monocyte and macrophage phenotypes, we detected the ability of ECM to regulate cytokine production by monocytes and macrophages. Figures 5.41, 42 and 43 show the main observations of analyses of IL-10, IL-12 and IL-23 secretions by CD14+ and Mϕ cultured on ND, S and T ECM.

We determine differentiation of CD14+ on ECM from surrounding and tumour FB (S and T) did alter CD14+ phenotype (Figure 5.41). The responses were donor dependent and it was difficult to observe reduction in cytokine levels as only 1-2 of 4 donors secreted detectable cytokine in the positive control conditions (Figure 5.41). Nevertheless, there was evidence of suppressed IL-10 and IL-23 secretion when monocytes were cultured on ECM deposited by primary FB, whereas IL-12 was slightly increased. For one donor secretion of IL-12 and IL-23 (but not IL-10 were enhanced by the presence of ECM.

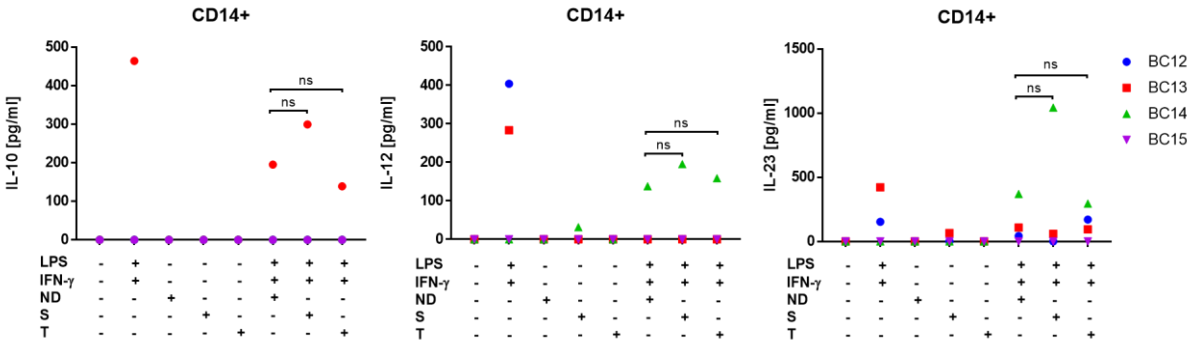

Figure 5.41 Expression of cytokines by by monocytes cultured on ND, S and T-ECM. 0.7x10^6 MACS-purified CD14+ were cultured in ND, S and T-ECM coated 24 well plate. After 6 days of the culture, cells were stimulated with LPS and IFN-γ for 24hr. Next day supernatants were collected and cytokine expressions assayed by ELISA. Data shown is mean +/- SD of 4 separated experiments (BC12, BC13, BC14 and BC15). Statistical data were carried out using one-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

We detected the responses of cytokine levels in all donors secreted detectable cytokine in the positive control conditions (Figure 5.42). Interleukin-12 production by GM-Mϕ was significantly (P<0.05) decreased in cross-linked ND-ECM compared to GM-Mϕ cultured in naked plastic However, there was evidence of increased IL-12 and IL-23 secretion when GM-Mϕ were cultured on ECM deposited by primary FB. For one donor secretion of IL-23 was supressed by the presence of ECM and two donors secretion of IL-10 and IL-12 were suppressed in the presence of ECM.

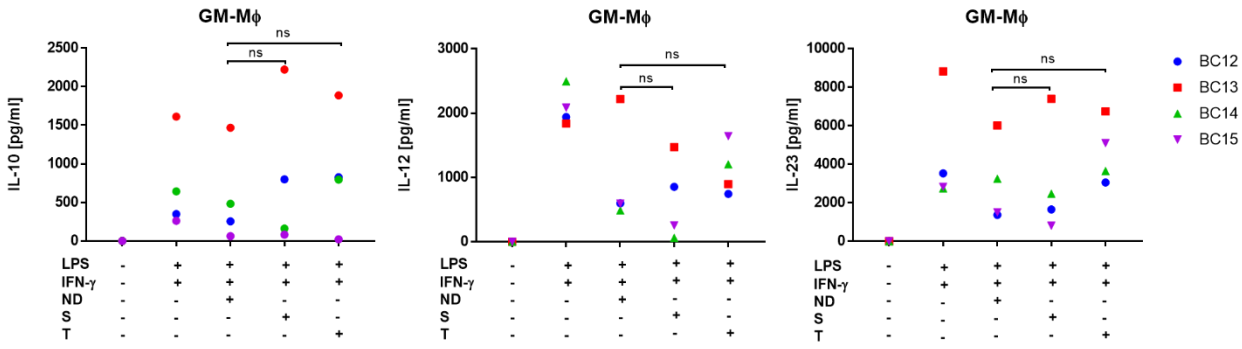

Figure 5.42 Expression of cytokines by GM-Mϕ cultured on ND, S and T-ECM. Monocytes ($0.7x10^6$) were cultured on ND, S and T-ECM coated 24 well plate treated with 20 IU/ml of GM-CSF. Next, after 6 days of the culture, cells were stimulated with LPS and IFN-γ for 24hr. Next day supernatants were collected and cytokine expressions assayed by ELISA. Data shown is mean +/- SD of 4 separated experiments (BC12, BC13, BC14 and BC15). Statistical data were carried out using one-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

Consistent with above results, we found that cytokine levels in response to stimulation were secreted detectable of IL-10 in all donors and IL-23 was donor dependent in secreted detectable as only two donors in the positive control conditions (Figure 5.43). Interleukin-10 production by M-Mϕ was significantly (P<0.05) decreased in cross-linked ND-ECM compared to M-Mϕ cultured in plastic plate. Nevertheless, there was evidence of increased IL-10 secretion when M-Mϕ were cultured on ECM. For two donor secretion of IL-23 was supressed by the presence of ECM.

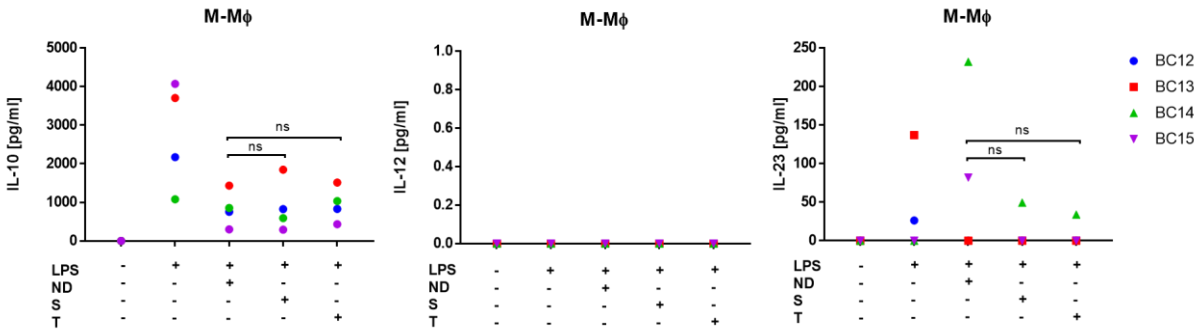

Figure 5.43 Expression of cytokines by M-Mϕ in the presence of ND, S and T-ECM. Monocytes ($0.7x10^6$) were cultured in ND, S and T-ECM coated 24 well plate treated with 10ng/ml of M-CSF or medium only. Next, after 6 days of the culture, cells were stimulated with LPS only for 24hr. Supernatants were assayed by ELISA. Data shown is mean +/- SD of 4 separated experiments (BC12, BC13, BC14 and BC15). Statistical data were carried out using one-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

This experiment was carried out using N, S and T-ECM coated 24 well plates (kindly provided by Dr Tanya Shaw's laboratory, King's College London), which were incubated with immature CD14+, primed GM-Mϕ and primed M-Mϕ for 6 days. In (Figure 5.44-46) displays morphological changes of monocytes and macrophages the same as in section (5.2.2.3.1) as shown in (Figure 5.31- 33).

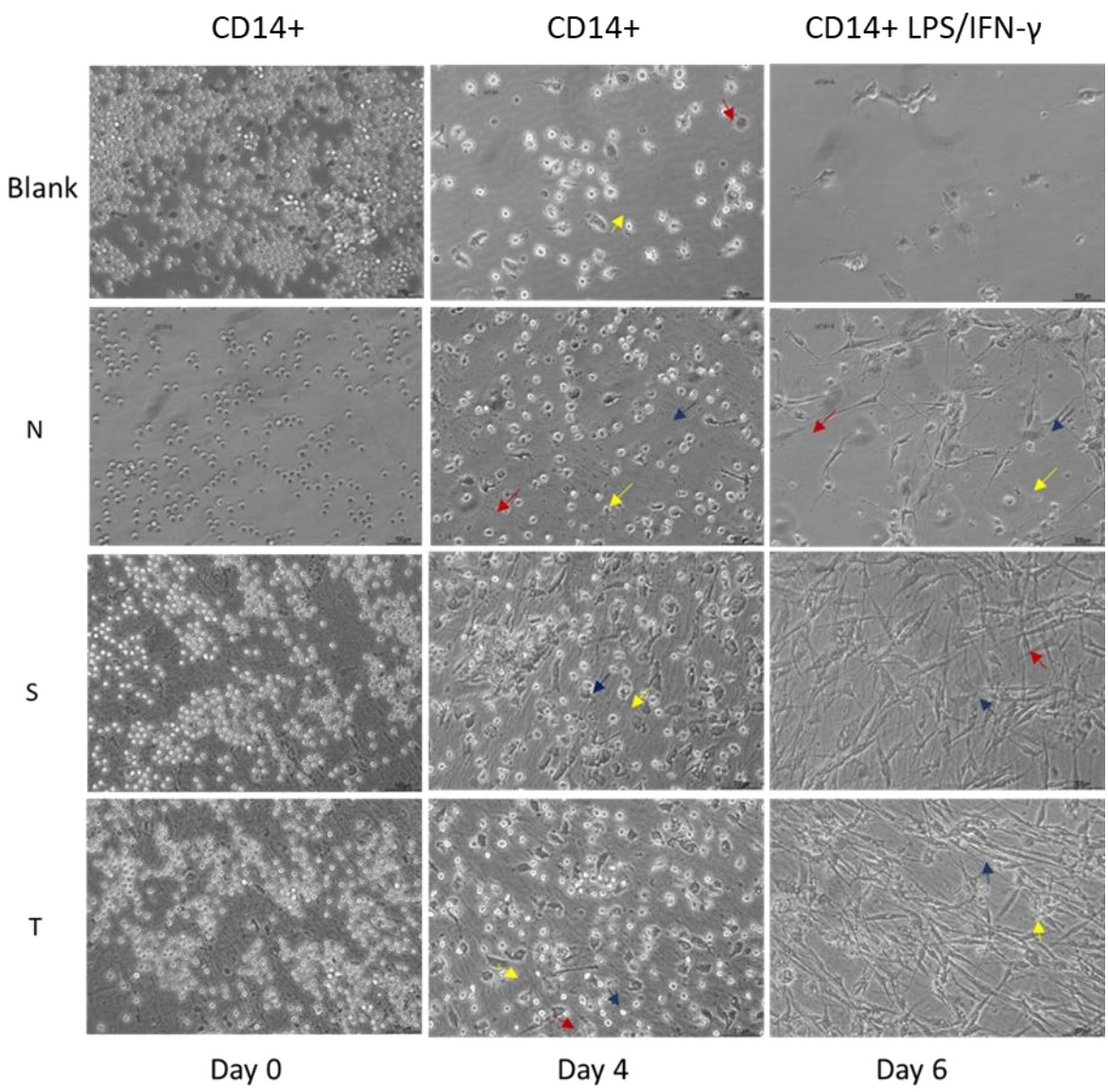

Figure 5.44 Morphology of monocytes cultured on ND, S and T-ECM. Cells were activated with LPS/IFN-γ for 24hr. Phase-contrast micrographs were taken under 10x magnification at the indicated time points prior to harvest the supernatant and cells. Red arrow represents Mϕ-like cells, blue arrow represents DC-like cells and yellow indicates spindle shaped cells.

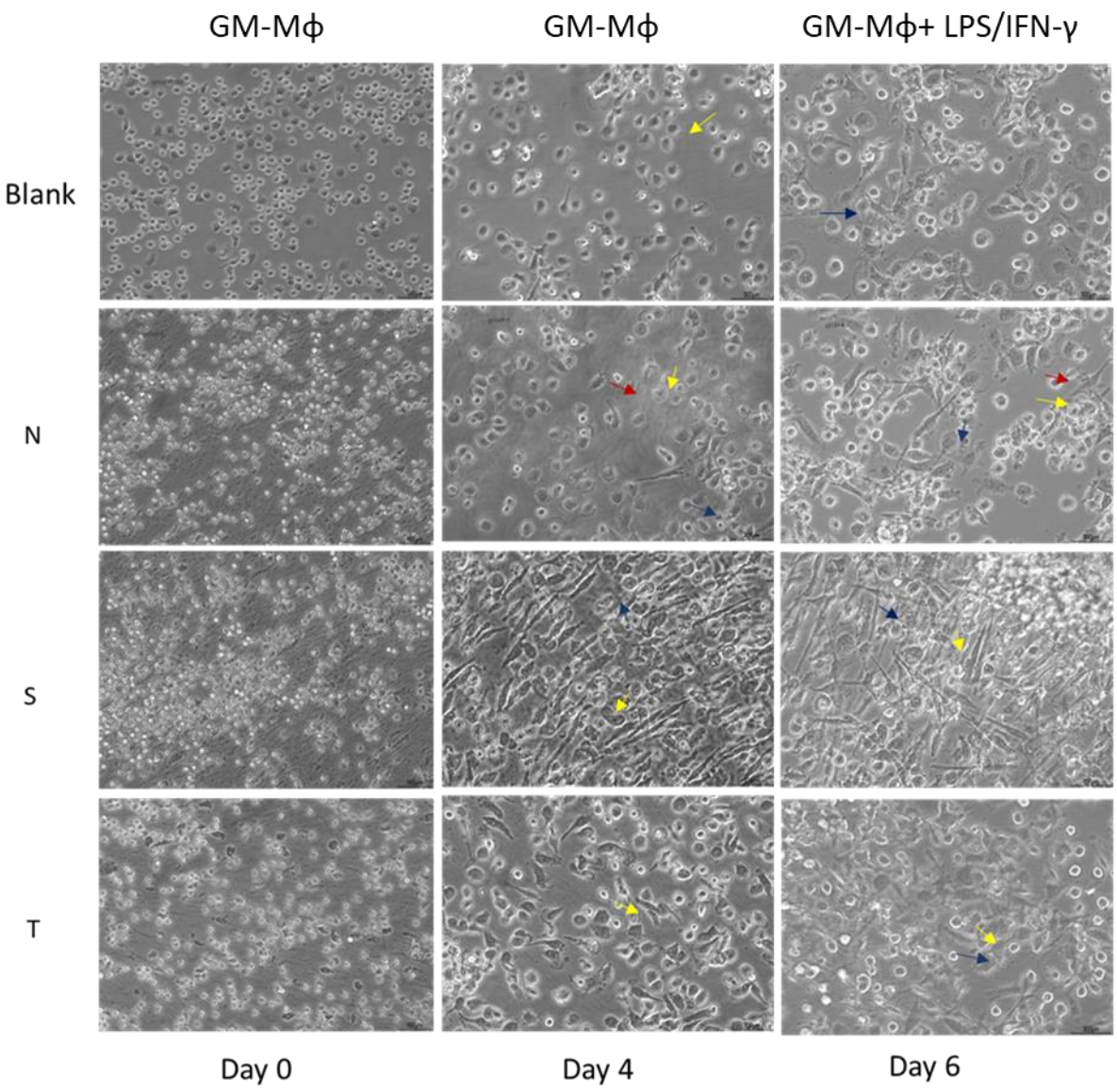

Figure 5.45 Morphology of GM-Mϕ cells were crosslinked on ND, S and T-ECM. Cells were activated with LPS/IFN-γ for 24hr. Phase-contrast micrographs were taken under 10x magnifications at the indicated time points prior to harvest the supernatant and cells. Red arrow represents Mϕ-like cells, blue arrow represents DC-like cells and yellow indicates spindle shaped cells.

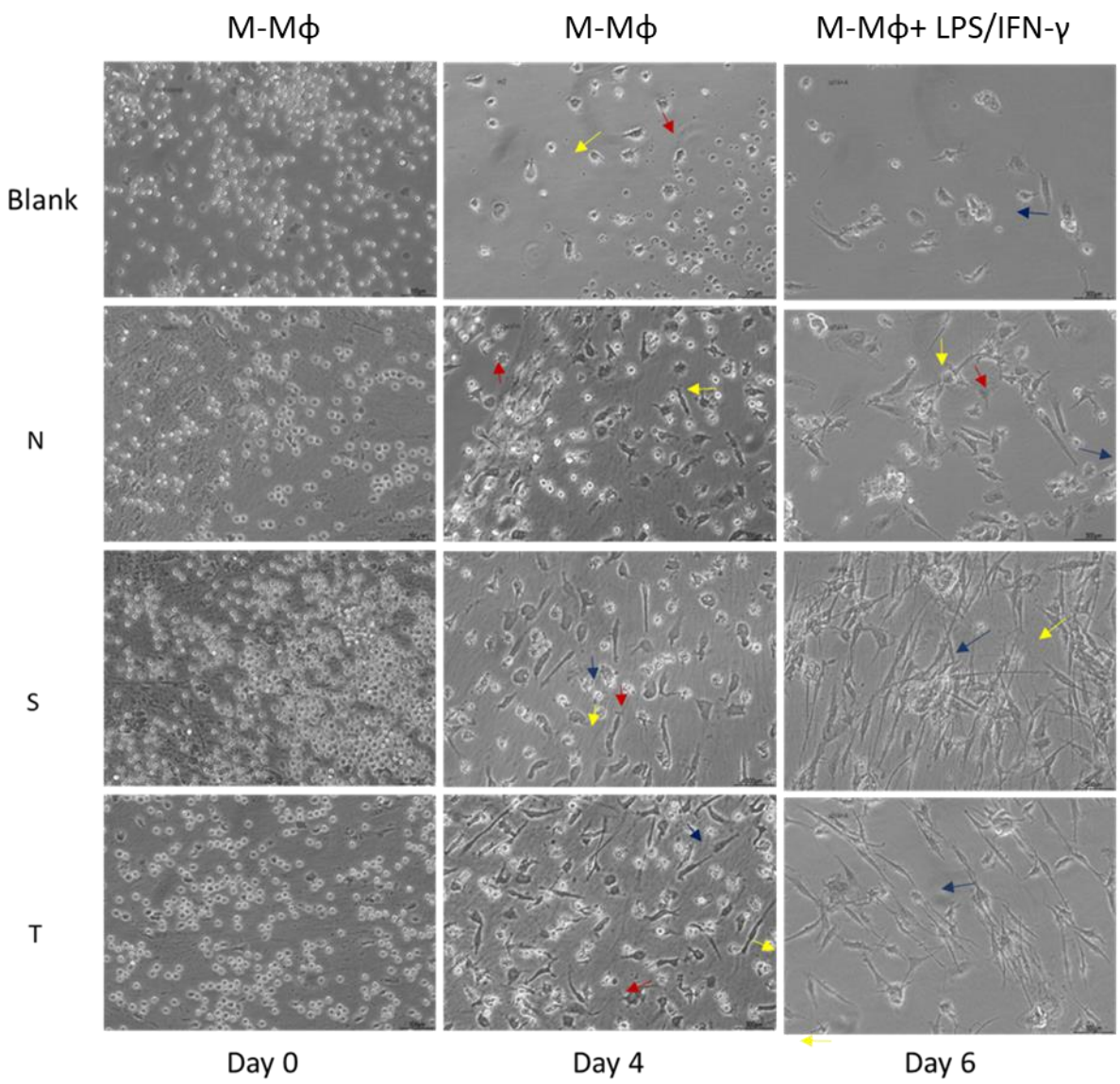

Figure 5.46 Morphology of M-Mϕ cultured in the presence of ND, S and T-ECM. Cells were activated with LPS/IFN-γ for 24hr. Phase-contrast micrographs were taken under 10x magnifications at the indicated time points prior to harvest the supernatant and cells. Red arrow represents Mϕ-like cells, blue arrow represents DC-like cells and yellow indicates spindle shaped cells.

We next investigated how the phenotype of monocytes was affected by the presence of primary FB-derived ECM. As shown In Figure 5.47, the presence of ECM did not affect CD204 expression.

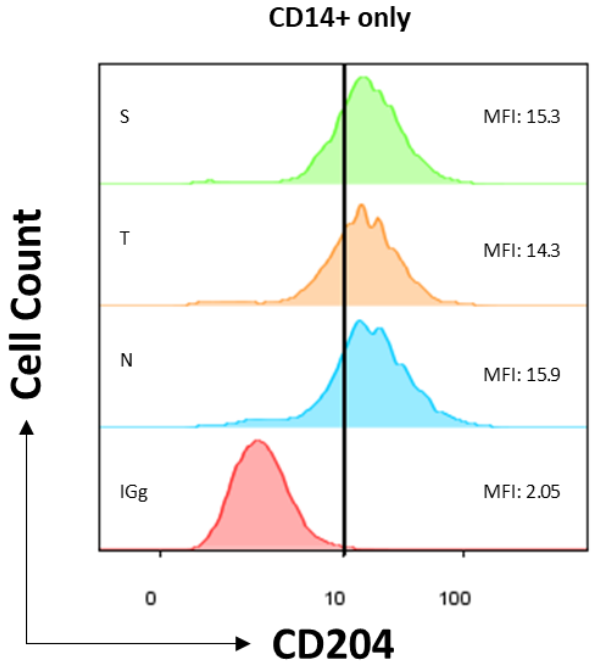

Figure 5.47 Analysis of CD204 expression by monocytes cultured on ECM from ND, S and T FB. Isolated monocytes were cultured in N, S and T-ECM -coated plates for six days the culture, cells were stimulated with LPS and IFN-γ for 24hr. Next day, cells were harvested and stained with anti-CD204 FITC and analysed by flow cytometry. Cells were gated on equal number of cells ($2x10^4$). Histograms were drawn depending on fluorescent minus one (FMO). For the number indicates the MFI of CD204. Data shown is mean +/- SD of 1 experiment.

In mature GM-Mϕ, CD204 expression was not substantially affect by the presence of any form of ECM (Figure 5.48).

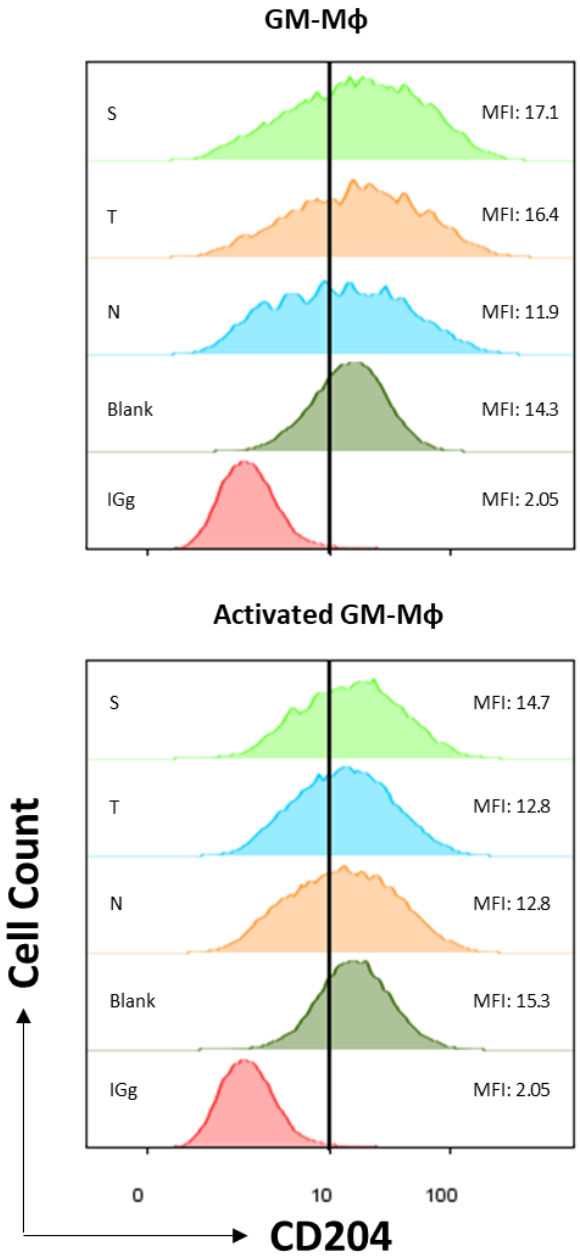

Figure 5.48 Analysis of CD204 expression by GM-Mϕ cultured on ECM from ND, S and T FB. 0.7x10^6 MACS-purified CD14+ cells were cultured in N, S and T ECM coated plates with 20U/ml of GM-CSF or medium only for six days the culture, cells were stimulated with LPS and IFN-γ for 24hr. Next day, cells were harvested and stained with anti-CD204 FITC and analysed by flow cytometry. Cells were gated on equal number of cells (2x10^4) collected from GM-Mϕ as shown. Histograms were drawn depending on fluorescent minus one (FMO). For the number indicates the MFI of CD204. Data shown is mean +/- SD of 1 experiment.

Interestingly, resting M-Mϕ expressed CD204 at a high-level when differentiated on ECM S and T compared to those on ECM derived from normal control FB (Figure 5.49). Upon activation CD204 expression was slightly elevated in in the presence of tumour FB-derived ECM. Similar to the mature M-Mϕ, immature M-Mϕ were cultured with the plate coated with S ECM expressed a greater level of CD204 than those cell on the plate coated with N ECM.

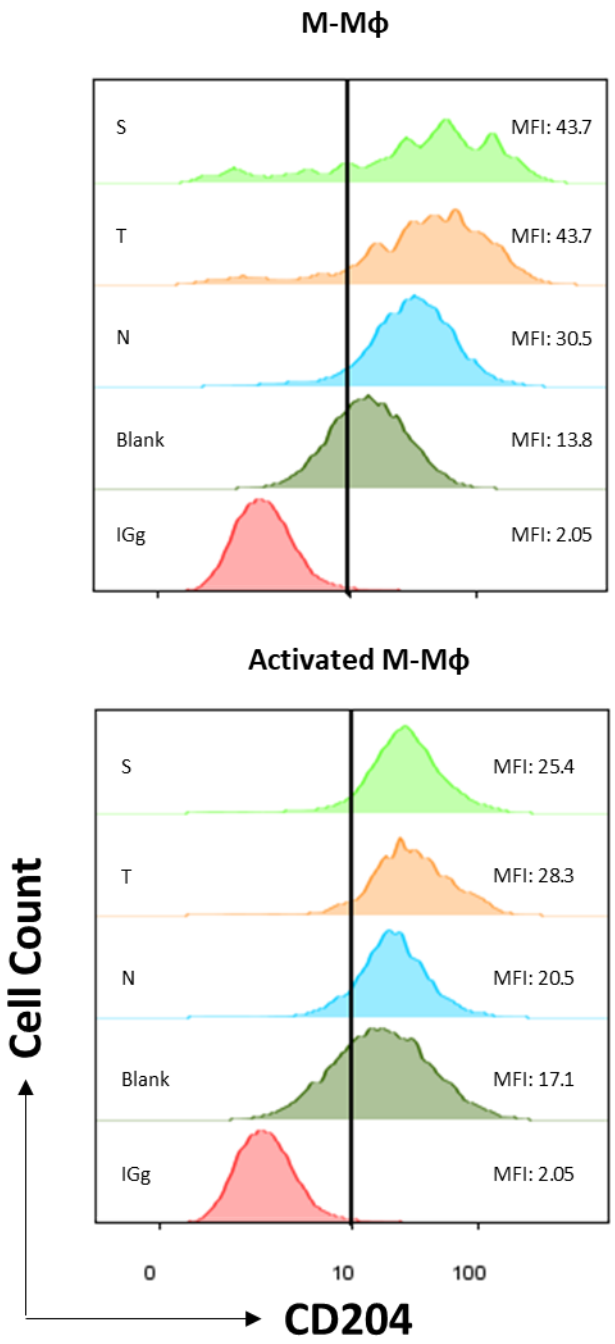

Figure 5.49 Analysis of CD204 display on M-Mϕ cultured on ECM from ND, S and T FB. 0.7x10^6 MACS-purified CD14+ cells were cultured in N, S and T ECM coated plates with 10ng/ml of M-CSF or medium only for six days the culture, cells were stimulated with LPS and IFN-γ for 24hr. Next day, cells were harvested and stained with anti-CD204 FITC and analysed by flow cytometry. Cells were gated on equal number of cells (2x10^4) collected from GM-Mϕ as shown. Histograms were drawn depending on fluorescent minus one (FMO). For the number indicates the MFI of CD204. Data shown is mean +/- SD of 1 experiment.

The responses were donor dependent and it was difficult to see production in cytokine levels in donors in the positive control conditions (Figure 5.50). Nevertheless, there was evidence of increased IL-10 and IL-12 secretions when monocytes were cultured on ECM deposited by primary FB as only 1 of 2 donors. Minimal IL-23 was produced.

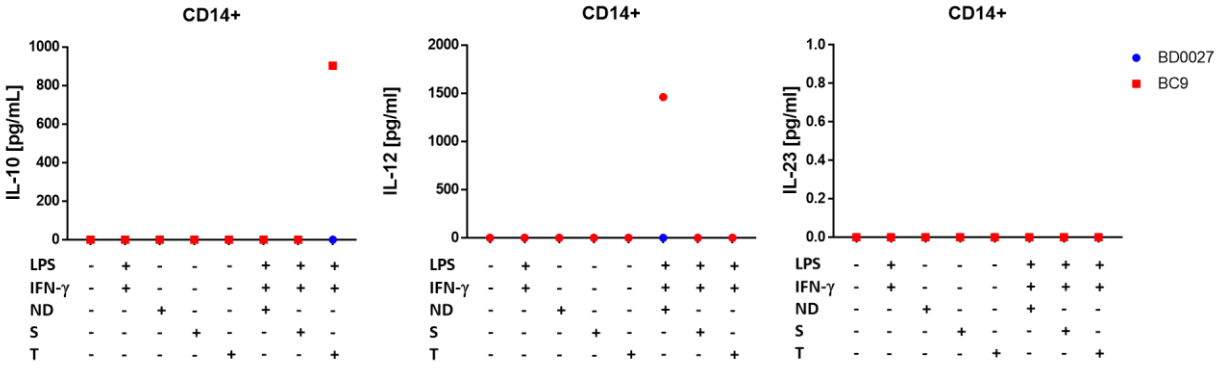

Figure 5.50 Expression of cytokines by monocytes cultured on ND, S and T-ECM. 0.7x10^6 MACS-purified CD14+ cells were cultured in ND, S and T-ECM coated 24 well plate. Next, after 6 days of the culture, cells were stimulated with LPS and IFN-γ for 24hr. Next day supernatants were collected and cytokine expressions assayed by ELISA. Data shown is mean +/- SD of 2 separated experiments BD0027 and BC9. Statistical data were carried out using one-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

When GM-Mϕ cultured in ECM, the responses of cytokine levels in all donors secreted detectable cytokine in the positive control conditions (Figure 5.51). However, there was evidence of increased IL-12 and IL-23 secretion when GM-Mϕ were cultured on ECM deposited by primary FB in all donors. For one donor secretion of IL-10 was increased by the presence of ECM.

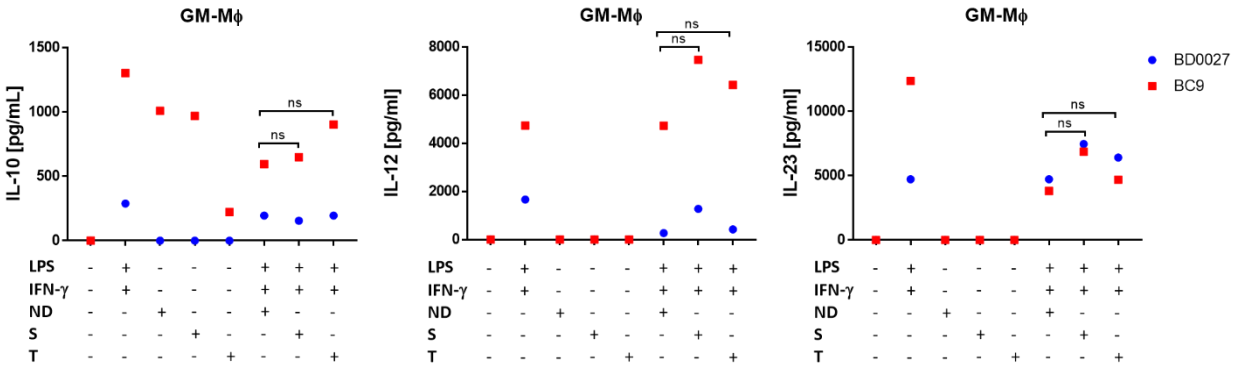

Figure 5.51 Expression of cytokines by GM-Mϕ in the presence ofND, S and T-ECM. 0.7x10^6 MACS-purified CD14+ cells were cultured in ND, S and T-ECM coated 24 well plate treated with 20U/ml of GM-CSF. Next, after 6 days of the culture, cells were stimulated with 5µg/ml of LPS and 1000U/ml of IFN-γ for 24hr. Next day supernatants were collected and cytokine expressions assayed by ELISA. Data shown is mean +/- SD of 2 separated experiments (BD0027 and BC9). Statistical data were carried out using one-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

similarity, the responses were donor dependent and it was difficult to see production in cytokine levels as only one of 2 donors secreted detectable cytokine in the positive control conditions (Figure 5.52). Nevertheless, there was evidence of increased IL-10 secretion when M-Mϕ were cultured on ECM as only one. For one donor secretion of IL-12 was increased by the presence of ECM. Minimal IL-23 was produced.

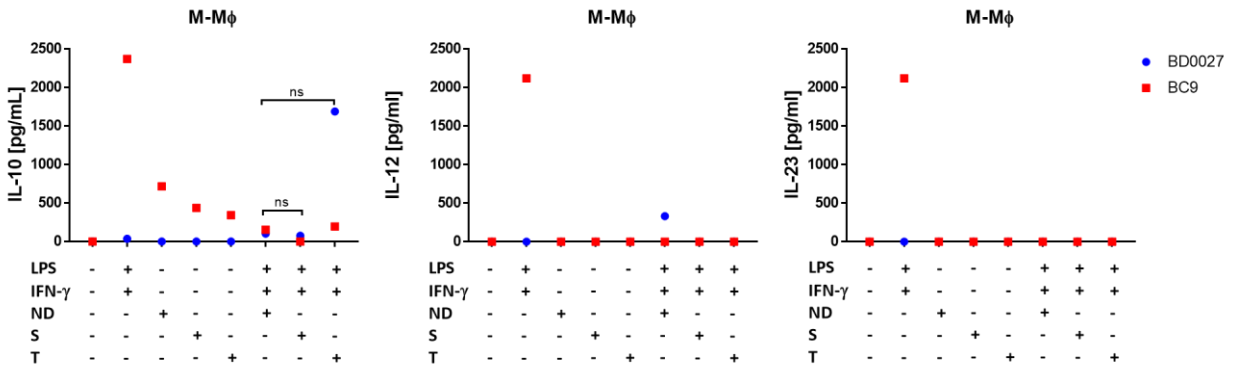

Figure 5.52 Expression of cytokines by M-Mϕ cultured on ND, S and T-ECM. 0.7x10^6 MACS-purified CD14+ cells were cultured in ND, S and T-ECM coated 24 well plate treated with 10ng/ml of M-CSF or medium only. Next, after 6 days of the culture, cells were stimulated with LPS and IFN-γ for 24hr. Next day supernatants were collected and cytokine expressions assayed by ELISA. Data shown is mean +/- SD of 2 separated experiments (BD0027 and BC9. Statistical data were carried out using one-way ANOVA (*: p value<0.05, **: p value<0.01, ***: p value<0.001, ****: p value<0.0001, ns: not significant).

5.2.3 Characterisation of fibroblast-derived ECM

Deposition of the different ECM proteins by tumour- or stromal-associated fibroblasts were initially characterised, using a western blot. The data showed expression of Biglycan (BGN) (Figure 5.53A) and Periostin (POSTN) (Figure 5.53B) was observed in the matrix deposited by TAFs (T) only from both patients studied. These agreed with the expected molecular weight as follows: BGN (42 kDa) and POSTN (93 kDa). Contrary, the matched stromal fibroblasts-derived matrix (S) was not detected BGM or POSTN expression. Importantly, all samples were probed for β-actin (42 kDa), which acts as a house keeping gene as it is expressed at relatively consistent levels.

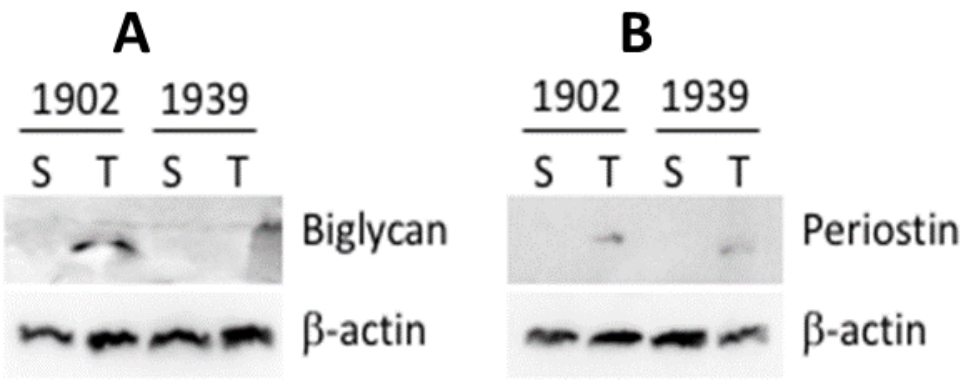

Figure 5.53 Characterisation of ECM deposited by primary S and T FBs. Western blot analyses of Biglycan and Periostin in cell-derived matrix (CDM) lysate taken from 1902 and 1939 indicate identifiers for two patients with breast cancer (Dr Tanya Shaw- King's College London).

The extracellular matrix (ECM) culture model enables the investigation of cell behaviours by providing cell-ECM interactions in monoculture, but also provides an opportunity for the co-culture of numerous cell types, which more closely mimics the *in vivo* situation. In this project, we investigated the hypothesis that the fibroblast-containing portion of the ECM may play an important role in modulating a pro-tumourigenic phenotype both in monocytes and macrophages. In addition to polarisation, the ECM affects other aspects of macrophage biology and their cell marker expression. However, to date there have been no studies establishing a model of ECM deposited by the fibroblast (FB) BJ6 cell line and the move to use primary patient derived FBs, examining and visualising how this impacts CD14+ and macrophage. Taken together, initial studies even were limited in terms of laboratory control, but we successfully established a complex and novel system of ECM culture that works and has the potential to deliver more robust data in further planned studies. In this model, the matrix is deposited, as is the signature of tumour tissues. Monocytes and macrophages cultured on ECM survive, function, and differentiate into three morphologically different cell types by upregulating surface markers such as CD204 and PD-1. Upon activation of on M-Mϕ with LPS and IFN-γ, there was evidence of cytokine secretion that was donor dependent. It was difficult to see production in cytokine levels, which may reflect different donors, as we did not use monocyte cell lines, instead utilising primary monocytes. The fact that the results vary as mentioned, and a lot of findings is not important. We anticipated analysis of sequencing matrices deposited by FBs by sending some samples to Dr Tanya Shaw's laboratory in King's College London. However, the COVID-19 lock down led to postponing this investigation.

5.3 Discussion

It is important to appreciate that cells outside of the classical immune system impact the function of major leukocyte subsets. This is perhaps not surprising, considering that many of these cells form the barriers on and inside our bodies, and are thus those that first suffer damage or encounter pathogens. Furthermore, the wide range of stromal FBs and endothelial cells inside tissues makes immune environments complex. Dermal fibroblasts (dFb) are a clear example of such cells, as they produce immunomodulators such as prostaglain-E2 (PGE2) and the tumour necrosis factor-inducible gene 6 protein (TSG6) upon inflammatory activation (Saalbach et al., 2015a).

Changes in Mϕ phenotype were observed in the presence of a range of different FBs, including cell-lines and primary FB. The inclusion of primary FB adds confidence to these findings and support the notion that this is not restricted just to immortalised cells. Nonetheless, further investigations were conducted to establish any differences between FB types in for example reducing inflammatory polarization. Whilst inconclusive in this regard, this model could certainly be employed to study tumour-associated FBs and the impact of external factors such as hypoxic conditions.

After the successful generation of M-Mϕ and establishment of a co-culture system of FBs/Mϕ, we then investigated pro- and anti-inflammatory Mϕ. Our findings examine the effects of FBs on Mϕ function during intimate planar co-culture. Dendritic cells function was influenced by the stromal microenvironment exerted under both inflammatory and steady-state conditions (Svensson & Kaye, 2006). Interestingly, IL-23 secretion by Mϕ was significantly augmented by FB, and IL-12 secretion was significantly decreased. In contrast, there were minimal changes in IL-10. Previous studies revealed that FBs do not release any IL-23 (Langrish et al., 2004). According to this data, FBs can modify pro-inflammatory macrophages towards a tumour-favouring phenotype. IL-23 is mainly expressed by activated macrophages and DCs that reside in peripheral tissues (McKenzie, Kastelein, & Cua, 2006). The secretion was implicated in autoimmune inflammatory disorders including arthritis, psoriasis, colitis and gastritis (Lankford & Frucht, 2003) to generate a novel pro-inflammatory profile (Oppmann et al., 2000). IL-23 stimulates IL-17, producing T-cells that contribute to the development and maintenance of autoimmunity (Hunter, 2005). PGE2 plays a crucial role in controlling the balance of IL-12 and IL-23 secretion by DCs, which

increases inflammatory responses (Hayashi, Yanagawa, Onoe, & Iwabuchi, 2010). Activation of NF-kB is required for IL-23 secretion prior to subsequent immune responses, leading to Th17 responses (J. Chang, Voorhees, Liu, Zhao, & Chang, 2010). In the murine setting, differentiation of naïve T cells to Th17 cells is regulated by TGF-β and IL-6, whilst IL-23 serves to amplify and expand such responses (Lankford & Frucht, 2003). IL-17 is stimulated by IL-1β and IL-23, while synergistic effects of IL-17 on TNF-α are modulated by IL-23 (Stojanovic, Cvjeticanin, Lazaroski, Stosic-Grujicic, & Miljkovic, 2009). There is a growing body of evidence supporting a role for Th17 responses to bacterial infections, and, in the setting of malignancy, IL-23 and IL-17 are thought to contribute to angiogenesis and subsequent tumour growth (Happel et al., 2005; Numasaki et al., 2003).

The effect of FBs on cytokine secretion by Mϕ is related to the number of FBs in the co-culture. Our experiments do not delineate the roles of soluble or cell-associated FB factors for enhanced IL-23 secretion. However, it is reasonable to propose that these mechanisms are similar to those previously observed with dendritic cells (Malecka et al., 2016; Walch-Ruckheim et al., 2019). Further experiments would be required to fully dissect FB-Mϕ co-cultures. The role of soluble factors could be determined with a Transwell system or with a supernatant transfer. Furthermore, a role for PGE2 could be demonstrated using a COX2 inhibitor, such as indomethacin (A Malecka, PhD 2017) (Malecka et al., 2016).

Functions of DCs, including maturation, homing to lymph nodes and following activation of T-cell responses, have been associated with stromal cells (Saalbach et al., 2010; Sugita et al., 2007). Our previous work established that stroma and its ability are involved in the outcome of therapeutic developments directed towards the immune system, also via establishing cytokine response inhibition by IR, in particular IL-23 of DCs and Mϕ (the thesis of Malecka (2017)). This work is based on a previous study describing increased IL-23 secretion by activated DCs as a result of interaction with stromal FB (Saalbach et al., 2010). Therefore, we investigated the previously well-understood concept of FB cells being relatively resistant in response to radiation, the function of APC support continuously in the presence of IR (Papadopoulou & Kletsas, 2011). Additionally, TAFs exposed to high radiation doses can be influenced by their secretory profile (Hellevik et al., 2013). One study revealed that when TAF cultures are treated with different radiation regimens, their mediated

immunoregulatory effects on macrophages are altered (Berzaghi et al., 2019). Radiation plays an important role in directly affecting macrophage phenotypes and their recruitment.

Next, we explored which external factor is involved in down-regulating cytokine production by GM-Mϕ in culture. Interestingly, irradiation of GM-Mϕ downregulated IL-23 and IL-10 and promoted IL-12. Whilst suppressing IL-23 and IL-10 by M-Mϕ. FBs exposed to repeated doses of IR show that viability is maintained and drives cells towards premature senescence, alteration of phenotype upregulating tumour progression and invasion, as well as allowing for adjacent tumours that are more resistant to radiation. FB exposed to a single dose of 4 Gy was observed to induce a prolonged growth arrest (Di Leonardo, Linke, Clarkin, & Wahl, 1994; Papadopoulou & Kletsas, 2011; Suzuki et al., 2012). Our findings are in line with the previous report and (A Malecka, PhD 2017), where a high single dose (10 Gy) caused no developing senescence in primary dFB.

In our study, we found a radiation dose at 6 Gy can restore function in macrophages which were cultured with FB. *Schrimmer et al.* determined upregulation of IL-23 secretion by DCs can be induced through action of COX2-dependent PGE2 production of activated FB (Schirmer et al., 2010). Our present study focused on this theory, supporting these results in the additional setting of IR. The IR downregulated suppression of COX2 lead to enhancing the effect of co-culture. In keeping with this observation, previous findings of studies have reported that *in vitro* and *in vivo* x-ray exposure between 2 and 5 Gy can induce reprograming macrophages from M2- to the pro-immunogenic M1-phenotype (Hildebrandt et al., 2003; Pinto et al., 2016). Interestingly, although IR significantly promoted IL-12 secretion by GM-Mϕ in the presence of FB (IR significantly downregulated IL-10 secretions by GM-Mϕ/FB), IL-23 was slightly altered. Likewise, IL-10 secretion by M-M-ϕ cultured with FB in the presence of IR was significantly suppressed. Furthermore, IR did not affect IL-23 secretion by M-Mϕ cultured with FB.

The expression of many cytokines and other pro-inflammatory mediators are increased and present in inflammation. These factors are important for modulating immune cells, but enabling them also induces severe impairments in the case of uncontrolled inflammation. Monocytes and macrophages are characterised by the prominent production of pro-inflammatory profiles, and the ability to limit overwhelming and continued inflammation can be mediated by GCs. In fact, GCs act as the most potent agents and inhibitors for the

transcription of several pro-inflammatory cytokines secreted by human monocytes and macrophages, such as IL-1β, IL-12, and TNF-α (Cain & Cidlowski, 2017; Ma et al., 2004).

The addition of the respective cytokines (GM-CSF or M-CSF) to culture did not change levels of cytokine secretion by Mϕ. Importantly, the effect of Dex using a high to low dose on cytokine secretion during the acute period was greater than in the chronic one. In these experiments, prolonged Dex inhibited IL-23 and IL-12 release by M-Mϕ. These effects are likely mediated by transcription factors, including NF-kB and downregulate pathways of synthesis of inflammatory mediators by the modulation of glucocorticoid receptors (Meduri, Muthiah, Carratu, Eltorky, & Chrousos, 2005). Consistent with previous findings, Dex was shown to suppress IL-12 and IL-10 production of LPS-stimulated alveolar macrophages (AMs) (S. Zeng et al., 2017). In addition, high-dose Dex combined with adjuvant treatment is an efficient/effective way to eliminate the side effects of immunotherapy (Sharma et al., 2015). This result might help to explain how to address the effect of prolonged and excessive doses of Dex used to treat anti-inflammations. Meanwhile, apoptosis of macrophages may be induced by Dex, so programmed cell death measurements (apoptosis) should be assessed.

Inhibition of macrophage and monocyte activation through the action of microbial agents or endogenous danger signals was initiated by inflammation GCs (Dalli & Serhan, 2017; Perretti & D'Acquisto, 2009). Pro-inflammatory activation of monocytes and macrophages can be simultaneously suppressed, while GCs play a key role in the differentiation of naïve monocytes into a long-lived pro-resolution phenotype (J. M. Ehrchen et al., 2019; Gilmour et al., 2006). Further investigations should aim to understand these mechanisms in order to improve therapeutic options targeting undesirable inflammation in autoimmunity.

The transforming growth factor β (TGF-β) is a pleiotropic cytokine that contributed to both suppressive and inflammatory immune responses. Previous work demonstrated that the regulation of TGF-β secretion is achieved by myeloid cells, indicating that TGF-β is involved with a great inhibition of pro-inflammatory macrophages, which had been stimulated by TLR ligands or cytokines (M. O. Li et al., 2006). Notwithstanding, the initiation of migration of monocytes and macrophages can also be induced by TGF-β (Wahl et al., 1987).

Trafficking of monocytes was observed in the intestine, and in turn induced differentiation of non-inflammatory macrophages residing in the tissue. Indeed, prolonged-dose TGF-β may

lead human blood monocytes of a less activated phenotype, indicated by innate response receptor expression through downregulated and decreased cytokine secretion (Smythies et al., 2005).

It is worth highlighting that not all experiments yielded the same induction of IL-23 in Mϕ in the presence of FB. Such inconsistencies may be due to donor variability, such as prostaglandin EP receptor polymorphisms and the consequent requirement for a stronger trigger to regulate cytokine secretion (Schroder & Schumann, 2005). It has been suggested that several signalling pathways, such as prostaglandins (PGs), TGF-β and IL-10, are linked to the development of tumour-associated macrophages (Ruffell et al., 2012).

Modulation of IL-10 transcription in macrophages is complex. Macrophages have a number of stimuli that trigger increased intracellular levels of cAMP, which then elicits PKA. Several studies have shown that enhancement of IL-10 transcription is associated with agents through the action of increasing cAMP (Avni, Philosoph, Meijler, & Zor, 2010; S. H. Kim, C. H. Serezani, et al., 2011), such as with GE2. PGs are lipid-derived molecules that can regulate the immune system. Of note, IL-10 secretion was promoted in the presence of PGE2 synergistically with LPS (Strassmann, Patil-Koota, Finkelman, Fong, & Kambayashi, 1994), co-culturing Mϕ with FB depressed levels of IL-10 seen in M-Mϕ. This result was not consistent with previous results of studies, which show that the PKA-salt-inducible kinase (SIK)-CREB-regulated transcription coactivator (CRTC)3-dependent pathway can be activated by PEG2, in a synergistic effect with TLP agonists on IL-10 production, by macrophages that become polarised toward a M2-like macrophage phenotype (MacKenzie et al., 2013).

Our data demonstrates the involvement of a critical cytokine signalling pathway, TGF-β, on Mϕ functions. Interestingly, TGF-β significantly decreased cytokine secretion by Mϕ, such as IL-10, but did not affect IL-12 and IL-23. Therefore, it is possible that TGF-β could have lost its activity and, therefore, its ability to induce cytokine secretion due to incorrect storage. These findings were not in line with the previous results of studies, which show that the anti-inflammatory cytokine IL-10 increased upon TGF-β stimulation, while IL-12 and TNF-α were suppressed in THP-1 macrophages in response to TGF-β. Likewise, activation of the M2-like phenotype isolated from murine BMDMs was characterized by high levels of IL-10 and low levels of IL-12 (F. Zhang et al., 2016). It seems that TGF-β can be influenced by the regulation of myeloid cells, which are associated with the identity of the cell. A good example of this is

that some subsets of macrophages appear more susceptible to TGF-β effects than others, depending on their anatomic origin (Sanjabi et al., 2017).

Importantly, upregulation of pro-inflammatory and anti-inflammatory profiles was observed. IL-10 and IL-23 in LPS-stimulated GM-Significantly downregulated, while IL-12 was suppressed in combination with Dex and TGF-β. Dex could inhibit IL-23 levels in LPS-stimulated M-Mϕ in the presence of TGF-β, whereas IL-23 in combination with a low dose of Dex and TGF-β restored it. In our model, addition of TGF-β to M-Mϕ treated with Dex did not affect IL-10. These results are in agreement with our previous findings, which show that the chronic period of Dex doses was able to restore cytokine production by Mϕ. Based on these results, we conclude that prolonged exposure to Dex impacts pro-inflammatory and anti-inflammatory cytokines in a way that would be beneficial to autoimmune diseases. In contrast, chronic exposure to Dex in the setting of cancer treatment may surprisingly enhance the immune response by restoring pro-inflammatory cytokine production. Further studies are required to investigate the effect of TGF-β on macrophages and the relationship between Dex and TGF-β.

The interstitial matrix organisation within healthy tissue penetrates as deep as the basement membrane, which is comprised of fibroblasts, resident immune cells, vasculature and lymphatics distributed within a loose ECM comprised of many proteins, including collagens I and III, elastin fibres and glycoproteins (Frantz et al., 2010). The organisation of an ECM includes more than 300 different proteins (Hynes & Naba, 2012) together with plenty of diversity, through the actions of splice variations, post-translational modifications (Yuzhalin et al., 2018) and protein-protein interactions (Rodriguez-Pascual & Slatter, 2016). Importantly, the structure, physics and composition of the ECM are heavily involved to regulate immune function, that the cancer ECM provides mechanistic immunity (Gordon-Weeks & Yuzhalin, 2020).

The ECM acts as a regulator for immune cell function. Furthermore, immune cell movement can be influenced by the ECM. The ECM impacts the polarisation of myeloid cell types, including macrophages (Sica & Mantovani, 2012). Here, we investigated the impact of different types of ECM derived from different cell lines, or primary fibroblasts originating from normal dermis, tumour-surrounding areas and tumour-cores, when encountered by monocytes and macrophages *in vitro*. Specifically, we describe in detail the underlying

morphology of monocytes and macrophages cultured on ECM driving and their differentiation into the same shapes, such as DC-like cells, Mϕ-like cells and spindle-shaped cells. Indeed, *in vitro* the organisation and deposition of ECM may be different, as well as its composition, structure and physical properties, when compared with properties for ECM progressed *in vivo* with regard to various stromal cells, immune cells, cell-cell contact and tumour cells. Therefore, it is of great necessity for us to find the unknown mechanisms that underpin the question: *Why do cells undergo the same fate of morphology regardless of types of ECM*? Some disparity can be observed in collagen fibres which are thicker, more organised and more densely packed in cancers than in healthy tissue (Drifka et al., 2016). Importantly, these experiments were performed in the absence of immunostaining of ECM depositions, which allowed us to distinguish structures and components within each ECM.

The results of our study revealed that the effect of BJ6 on CD206 expression levels in monocytes is that of a slight decrease, but of significant decreases in CD169 and CD86 expression. Moreover, BJ6 ECM slightly downregulated CD86, significantly decreased CD169, and slightly increased CD206 in mature GM-Mϕ. CD206 M-Mϕ saw no change in the presence of BJ6 ECM, while CD169 and CD86 were significantly suppressed. Consistent with results from the previous study, this showed that CD86 M1 TAMs were at a low level, while a high expression of CD206 M2 TAMs was associated with aggressive tumour phenotypes and a poor prognosis for HCC patients (Chavez-Galan, Olleros, Vesin, & Garcia, 2015; P. Dong et al., 2016).

In many studies, the expression levels of CD68 and CD86 (on M1), or CD163 and CD206 (on M2), are used to quantify and classify TAMs (Hu et al., 2016). When considering cell surface marker proteins, such as CD206, CD204, CD169 and CD86 expression by human monocytes and Mϕ cells, it is worth noting that these cells were cultured in plates coated with N, S, and T ECM, and the effect of these final two in modulating CD206 monocytes depends on signals received from the interaction, which decreased slightly. It also highlights the fact that the expression of CD86, CD169, CD204 and PD-1 were slightly affected by S ECM, whereas T ECM did not change their expression levels. Gordon *et al*. have found that mouse and human TAMs can display PD-1 upon activation of T cells. They also defined PD-1+TAMs and showed that M2-like surface profile expresses PD-1 within TAMs (S. R. Gordon et al., 2017).

Our results showed T ECM slightly upregulates CD206, CD86, CD169, CD204 and PD-1 expression by mature GM-Mϕ, thus S ECM induced a slight decrease in CD206, CD86, CD169, CD204 and PD-1. Given that multiple biomarkers of M-Mϕ were used to determine the effects of S and T ECM on their expression, these biomarkers were slightly decreased in the presence of S and T ECM, whereas the PD-1 immune inhibitory receptor was not affected. This finding is in agreement with previous study results showing the association between the prognosis of patients with hepatocellular carcinoma and the density of TAM biomarkers: a high density of CD86 M1 TAMs, while a high density of intra-tumoural M2 TAMs expressing CD206, CD204, or CD169 was associated with worse predicted survival rates (W. Ding et al., 2019; L. A. Elliott, Doherty, Sheahan, & Ryan, 2017; M. Yu et al., 2019) .

Angiogenesis, lymphangiogenesis, stroma remodelling, immune suppression and metastasis are regulated and promoted by TAMs (R. Wang et al., 2011). The high production of IL-10 is an important TAM-phenotype that resembles M2 polarisation. The activation of the TAM pathways results in enhanced IL-10 suppressing other important cytokines, such as IL-12 (DeNardo et al., 2009). In the light of this investigation, we focused on understanding whether a continual crosstalk exists, not only between tumour cells and immune cells, but also between extracellular matrix-derived from the cell line and tumour cells. In addition, N, S, and T fibroblasts might have a role in regulating pro- and anti-inflammatory profiles for monocytes/macrophages. The role of ECM in modulating cytokine secretion by macrophages has not been investigated before in our laboratory. We found that the BJ6 ECM crosslink to monocytes secrete low IL-10, high IL-23 and IL-12 was not observed.

Our findings demonstrate for the first time that matrix deposited by the BJ6 FB cell line significantly inhibited IL-10 and significantly increased IL-23 secretion by mature GM-Mϕ. This agrees with our previous findings, which show regulation of IL-10 and IL-23 in 2D and 3D models where IL-12 was slightly decreased: this is in line with our previous findings in 2D and 3D systems. In addition, our findings demonstrate that BJ6 ECM acts as a potent mediator to induce a significant suppression of IL-10 secretion by M-Mϕ, which is similar to the findings in 2D and 3D co-culture, and low IL-23 secretion by mature M-Mϕ. This is in line with 3D model results in Figure 4.17, but conflicts with our earlier findings.

Activation events for M1 and M2 macrophages within the tumour environment or in tumour tissues are transient and regulated through functional plasticity (X. Zheng et al., 2017). Our

in vitro ELISA assay findings and cytokine secretion results show that S ECM increased IL-10, IL-12 and IL-23 secretion by mature monocytes. IL-10, IL-12, and IL-23 secretion also decreased in T ECM cultured with mature monocytes. The polarisation of macrophages into pro-tumoural M2-like macrophages from anti-tumoural M1-like macrophages is a fundamental event in the development of the tumour microenvironment. Thus, studying the factors that play a crucial role in trans-differentiating pro-inflammatory M1 to anti-inflammatory M2 profiles in the tumour region is promising in terms of immunotherapeutic treatment (F. R. Balkwill & Mantovani, 2012). We observed that IL-10 and IL-23, which are secreted from crosslinking mature GM-Mϕ/S ECM, were slightly decreased and IL-12 was increased. The secretion of IL-10, IL-12 and IL-23 were slightly increased in mature GM-Mϕ cultured on T ECM, but this did not reach statistical significance due to variations between donors. This finding is in line with the previous data from the study, which shows that TAF cultured with M1 macrophages promoted IL-10 and IL-12 secretion.

In fact, our studies with ECM normal and breast cancer FBs (isolated from human samples) demonstrate a higher significant level of IL-10 by M-Mϕ cultured on N ECM compared to mature M-Mϕ cultured on naked plates. It has been shown that S and T ECM did not affect IL-10 secretion, while S and T ECM decreased secretion of IL-23 by M-Mϕ.

Over a period of six days, macrophages were cultured on stiff matrices, which induce a wound-healing (M2) phenotype, characterised as a high expression of IL-10 and inhibition of TNF-α (Friedemann et al., 2017).

We also assessed the effect of N, S, and T ECM coated plates on CD204 expression on monocytes and macrophages, discovering that they were unaffected. It was also determined that the expression of CD204 by immature GM-Mϕ was affected by S and T ECM, whereas S and T ECM did not change its level of expression through mature GM-Mϕ. Additionally, It should be noted that resting and mature M-Mϕ differentiated on S, while T ECM showed an increased CD204 expression.

To evaluate cytokine secretion, monocytes and macrophages were cultured on N, S, and T ECM, which resulted in increased IL-23 in the monocyte cross linked with T ECM. IL-10, IL-12, and IL-23 secretion by mature GM-Mϕ were increased in S and T ECM, which is in line

with the previous findings we used with the N, S, and T ECM deposited in our laboratory. Therefore, only T ECM was able to promote IL-10 secretion by stimulated M-Mϕ.

Functionally, we linked examining both biglycan and periostin expressed in cell-derived matrix (CDM) lysate derived from two patient samples with breast cancer by Dr Shaw (King's College London). Periostin and biglycan are ECM molecules expressed in the melanoma microenvironment, which promote invasiveness through increased tissue stiffness (Andrlova et al., 2017). Here POSTN has a role in regulating the integrin-dependent mechanisms through promoting extravasation of TAMs into the tumour stroma, resulting in changes to ECM structure (Zhou et al., 2015).

In summary, our data clarifies the correlation between high biglycan and periostin expression levels and a denser collagen architecture, leading to the impact on monocyte and macrophage differentiation, polarisation and function. Previous studies have extensively revealed that specific ECM proteins can bind to their ligands on microbial pathogens (B. Singh et al., 2012).

This study was mostly limited by the use flow cytometric analysis to measure and characterise cell protein markers on monocytes and macrophages, observe the morphological changes in these cells by fluorescent microscopy, and to characterise cytokine secretion by these cells using ELISA assays. Taken together, this chapter describes several key advances:

a) The development of a new system to examine the effect of FB-derived matrix on immune cells. This is challenging and novel, and the results from this chapter demonstrate that such a system is feasible for further experimentation.

b) The inclusion of primary cells from cancer patients renders this model increasingly physiological and sets the stage for further work with a broader panel of primary cells and extensive experimental repetition.

We suggest that this investigation should be extended and that the initial characterisation of previous ECM deposition phenotypes via immunofluorescence staining of ECM fibroblasts should be demonstrated. Here, we detailed the implications of an active effect for the T ECM in upregulation of both CD204 and PD-1, and the polarisation of monocytes and

macrophages, comparable with others, suggesting that these ECM compositions require further review of fibroblast-derived matrisome proteins, such as collagens, proteoglycans, glycoproteins can be using mass spectrometry to identify which have a profound impact on the specific signals and binding sites that the ECMs convey to monocytes and macrophages for remodelling, such as glycoproteins, proteoglycans and collagen molecules, along with transcriptionally profiling macrophages.

Chapter 6 : Final discussion

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6.1 Establishing novel culture systems of the effects of FB on Mϕ and their differentiation and function

Macrophages (Mϕ) and dendritic cells are crucial constituents of the mononuclear phagocyte system and are important players in mediating the immune response in tissues. These cells are unique in their noteworthy functions, such as priming naïve leukocyte responses and phagocytosis of pathogens through action of Fc receptors; use of pattern recognition receptors to identify pathogens; secretion of cytokines and other factors that shape inflammatory processes (Shi and Pamer 2011). These cells represent a key role in adaptive immunity by antigen presentation by T cells (Banchereau & Steinman, 1998).

Whilst they play a role in protection, Mϕ and DCs are also actively recruited in chronic inflammatory conditions. A growing body of evidence shows that macrophage and dendritic cells have phenotypic features of complex regulatory functions mediated by chemokines, cytokines, growth factors, angiogenesis promoting factors and proteolytic enzymes, which promotes disease progression (S. Gordon & Martinez, 2010; B. Z. Qian & Pollard, 2010).

Stromal cells, including fibroblasts, have traditionally been known as quiescent cells. They play a key role in depositing extracellular matrices, contributing to excessive remodelling of cancer tissue and in repairing tissue. These dedicated cells are associated with regulating immune responses through the effect on the differentiation, migration, and activation of immune cells. Fibroblasts are well known as ordinary structural components of organs but are also considered as dynamic contributors in orchestrating immune responses. Previous studies have discussed that the interaction between fibroblasts and immune cells occur by mechanisms such as the action of cytokine and chemokine signalling by paracrine, interactions by direct priming of juxtacrine, and by modulation behaviour of extracellular matrix remodelling. Finally, more recent studies describe the migration of the extracellular matrix microenvironment (Correa-Gallegos, Jiang, & Rinkevich, 2021).

In this thesis, I address the critical question of whether it is possible to study and understand how stromal fibroblasts and their ECM alter the biological behaviour of APC responses in terms of differentiation into phenotypic features and functions including cytokine secretion

and the impact of external factors such as ionizing radiation (IR) on FB-mediated function. To answer this question, we need to know the mechanisms that controls the function of Mϕ and DC activity *in vitro*. To do so, extensive experiments are underway to identify how to differentiate cytokines to polarize CD14+ cells into Mϕ or DC and to execute their functions; how they develop, activate and respond to their milieus; and more fundamentally, where fibroblasts and their ECM interact with APC and identification of differentiation and functions.

Experiments in this investigation address the consequences of generation of APC derived from CD14+ monocyte isolated from PBMC from healthy blood donors. These monocytes are a resource to further characterise cellular subpopulations such as macrophages and dendritic cells. We set out to demonstrate optimal, standardized conditions for the key steps leading up to APC culture, from blood taking to CD14+ monocyte isolation derived from PBMC.

When differentiating APC from CD14+ cells the yield of APC was different between DC and Mϕ. Likewise, the different efficiency in the same phenotype emerged between GM-Mϕ and M-Mϕ. The reason for this is not clear but may relate to the relative contribution of different monocyte subsets or to the challenges in recovering Mϕ, also which were more firmly attached to the plastic substrate.

It is becoming clear that there are several physiological agents that may affect immune sufficiency including nutritional status, hormone levels and circadian rhythms. There are also some technical characteristics of blood drawing that can influence how immune cells are derived from whole blood in terms of viability, yield, and function (Mallone et al., 2011).

We have investigated the efficiency of isolation of monocytes in PBMC from whole blood from several donors, avoiding contamination of monocytes with other cells that may influence their function. Yield of monocytes when purified from PBMC and generated laboratory APC is performed. Given the lack of literature on these technical aspects in the isolation and protection of CD14+ monocyte and subsequent differentiation into Mϕ and DC, we collected details from previous studies. In so doing, we determined that what does not work effectively for analysing characterisation of APC in 2D culture systems is more likely to work in the more challenging field of 3D systems.

We discuss the biology of monocytes, their relationship with DCs, and the potential to use monocyte-derived dendritic cells (moDC) in the design of vaccines against certain chronic infectious diseases.

In vitro experiments and studies generating human DCs from monocytes cultured under influence of GM-CSF and IL-4 can cross-present and have long been utilized as a model to address the biology, however, this culture system does not closely represent naturally inducing Mo-DCs that exist *in vivo* in inflammatory fluids (Goudot et al., 2017).

Studies have shown that *in vivo* in peritoneal ascites, human MoDC and MoMϕ are naturally induced and can be generated *in vitro* from culture of monocytes treated with M-CSF, IL-4 and TNFα (Segura et al., 2013), with some studies showing that monocytes can be differentiated into DCs the presence of GM-CSF and IL-4 *in vitro* as the cross-presentation of soluble antigens a vacuolar pathway and using a cytosolic pathway for others (Baleeiro & Walden, 2017).

Exposure of monocytes to cytokines leads to differentiation into a variety of macrophages and DC-like cells. Addition of GM-CSF and IL-4 to monocytes *in vitro* induces polarization of human and mouse monocytes into DCs, regardless of their subsets (Geissmann et al., 2010) and differentiation of monocytes into macrophages under the influence of M-CSF. Exposure to LPS and IFN-γ to M-CSF induces the polarization of M1-like macrophages, while M2-like macrophages are polarized by adding IL-4. M1 macrophages are activated with LPS and IFN-γ, display potent microbial feathers and express a high level of IL-12. In contrast, M2 macrophages play a key role in resolution of inflammation.

Macrophages, in particular, exert distinct fates owing to their phenotype of plasticity and their capacity to adjust receptor expression to the tissue microenvironment (Okabe & Medzhitov, 2014). Conventionally, classification of macrophages has been considered as a wide spectrum from the pro-inflammatory M1-like to anti-inflammatory M2-like. These tissue macrophages exhibit a massive phenotype complexity *in vivo* (Martinez & Gordon, 2014; Murray, 2017).

Here we demonstrated that our laboratory models enable studies of characterization of GM-Mϕ and M-Mϕ or DC using differentiating cytokines to polarize monocyte into these different phenotypes of APC in *vitro*. Therefore, it would be of great interest to detect

whether some of the environmental factors influence DCs and Mϕ behaviour. Another immunotherapeutic approach would be to attempt to manipulate Mϕ for their extreme plasticity, such as repolarization, determinations, depletion, blocking, inhibition or increasing the up-regulation of pro- and anti-inflammatory cytokines and down regulation of immune inhibitory receptors. However, our study found that there were important initial factors to direct Mϕ phenotype and restriction in their capacity to fully repolarize following challenge. Activated M-Mϕ with LPS were only sufficient in secretion of IL-10 and IL-23, and deficient in their ability to secrete IL-12, whereas Mϕ differentiated under influence of GM-CSF and activated with ILPS and IFN-γ were deficient in their ability to express IL-10.

Our study revealed major findings, showing expression of pro-inflammatory cytokine is associated with GM-Mϕ phenotype and maybe useful for distinguishing GM-Mϕ from an anti-inflammatory M-Mϕ. LPS-stimulated DCs in the presence of IFN-γ express increased levels of IL-12, IL-23 and lower IL-10. The consequences of differentiation conditions were associated markedly with high levels of IL-23 expression.

There were certain limitations conducting the study. Not all APC generated from monocytes possess the same arsenal of receptors, and the same phenotypic features may express different receptors depending on milieus. Further studies are planned to understand phenotypic diversity of polarizing macrophages and DCs and to identify the phenotypic signatures in different types.

Tumour-infiltrating immune cells are correlated with tumour progression (Kunk, Bauer, Slingluff, & Rahma, 2016). Interaction of different cells within the tumour microenvironment acts a crucial role in the progression of the tumour and tumour-mediated immune suppression. The situation *in vivo* reflects a greater response compared to in a 2D monolayer. 3D co-culture models can deliver superior insight into the inflammation action of the tumour microenvironment and its crosstalk between stromal fibroblasts and immune cells in tumour growth and resistance to therapy (Imamura et al., 2015; J. M. Lee et al., 2013). To establish 3D, several studies have focused on providing a more realistic *in vivo* condition, by using a co-culture of one or two cell lines to achieve cell-cell contact. However, co-culture of immune cells has to be taken into account with tumour cells and fibroblasts (Koeck et al., 2016). Using this model proliferation and viability of both FBs and MCF7 spheroids were investigated. Both cell lines in the monoculture strongly enhanced the ability to form

spheroids. All MCF7 spheroids showed increased growth and reached the largest size on day 7 compared to FB spheroids. In contrast, all FB spheroids increased cell survival until day 7 compared to MCF7 spheroids. These data indicate that fibroblasts support spheroid formation and cell survival.

The establishment of IHC was performed to identify the formation of fibroblast spheroids at different concentrations, the inner spheroid core and detection of viability of cells was performed using H&E staining.

Most solid tumours remain a challenge to treat with a high unmet medical need resulting from poor responses to standard patient therapies. Crosstalk of TAFs and the immune cells in the tumour microenvironment play a significant role in tumour immune escape and progression (Kuen et al., 2017). GM-CSF and M-CSF in the presence of other cytokines, such as IL-16 and IL-8, have been reported to be associated with recruitment and differentiation of myeloid cells towards M2 and MDSCs in the tumour irrespective of promoting tumour angiogenesis (Noy & Pollard, 2014). We aimed to establish 3D co-culture model with fibroblasts and macrophages to demonstrate the novel cellular mechanisms produced in the immunosuppressive tumour microenvironment. Establishing this model, we identify the functional effect of FBs on cytokine secretion by Mϕ. Phenotypic characterisation of Mϕ after 3D co-culture with FBs spheroids was conducted via analyse of the cytokine secretion. 3D co-culture of GM-Mϕ with fibroblasts induced the lowest level of IL-10, increased IL-23 and suppressed IL-12. IL-10 secretion by M-Mϕ is well understood for its function in polarization of M2 and inhibition of several types of myeloid cells and T cells. These cytokines were observed in the supernatants of our 3D co-culture model.

The data suggest that there is a limiting finding of 3D co-culture Mϕ and FBs spheroids that were not sustained by the strong enhanced spheroids within 5 days and that cells formed a radial invasive form towards at bottom of well, leading to delivery of 2D and 3D model at the same wells. Further studies will be useful to develop these 3D co-culture models, such as alginate microcapsulation model or gelatin hydrogel microspheres.

Understanding the mechanisms of a complete alteration of Mϕ polarization into a pro-inflammatory from an anti-inflammatory phenotype within the tumour microenvironment and vice versa could lead to the development of novel immunotherapeutic approaches.

However, an alteration of expression of various genes could induce a complete alteration of polarizing Mϕ as stated by one study (Martinez, Gordon, Locati, & Mantovani, 2006). Our data suggest that the function of Mϕ may be influenced by conditions instead of a complete repolarization. For example, low or inhibited levels of IL-23 secretion by Mϕ are essential to alter the balance of helper T-cell responses and preferentially supports Th17 cell expansion resulting in further potentiating the tumour-promoting microenvironment. Tumour-associated macrophages are prominent components in solid tumours. These are divided into a mixed phenotype and their phenotypic features include pro- and anti-inflammatory cytokines that can simultaneously promote tumour growth, invade, and metastasise. Tumours with infiltrating Mϕ1 are often correlated to a more favourable prognosis than Mϕ2, which continue to increase tumour progression by immunosuppressive factors such as IL-23 and prolonged inflammation (Langowski, Kastelein, & Oft, 2007). Consequently, the importance of the current research focuses on programmes that give rise to increasing desired features of Mϕ biology may provide a rational approach through complete repolarization. To date, our study concerning Mϕ differentiation has used two different procedures and activation with two different stimuli. Further planned studies highlight and expand on differentiation methods of diversity of Mϕ that will represent immune-inhibitory conditions within tumour microenvironments. Furthermore, we demonstrated that the impacts of environmental stimuli on Mo differentiation into Mϕ and the recruitment of innate memory was associated with the ability of Mϕ in response to LPS and IFN-γ. Our results are in line with previous findings showing that IFN-γ secreted by T-cells are required to gain full activation of Mϕ (Mackaness, 1969).

The biology of APC can be inadvertently modulated by chemotherapy or RT, in turn reshaping the tumour microenvironment. It showed that preservation of pro-angiogenic efforts were induced when pro-inflammatory Mϕ phenotype were promoted by RT (Merrick, Errington et al. 2005). Past studies reported that exposure of DC to RT inhibited the ability of DC to prime T cells against the process of endogenous antigen, whilst priming was enhanced against exogenous antigen indicating the complex interplay between radiation and DC function (Liao, Wang et al. 2004). Our results are in agreement with the findings of (A Malecka, PhD 2017), which shows that genes were not all targets of the immune-regulatory effect of IR. IR selectively suppressed IL-10 andIL-23 secretion by GM-

Mϕ. Interestingly, IL-12 secretion by GM-Mϕ was increased and IR caused altered FB-mediated effects. Furthermore, IL-10 and IL-23 secretions by M-Mϕ in the presence of IR were decreased.

It has been found that effect of FB on DC is up-regulation of IL-23 secretion (Presky et al., 1996). These interactions have implications for the response of APC to external factors such as IR that, in the absence of NAF, suppresses the IL-23 response of DC. However, co-culture of DC with NAF renders them resistant to the suppressive effect of IR on IL-23, as shown by (A Malecka, PhD 2017). Whilst of interest, studies to date have been restricted to the impact of FB on normal immune responses, the specific roles played by TAF in the tumour immuno-biology of APC remain to be addressed. Whilst this is important in DC biology, it may also be pertinent to other cancer-associated myeloid cells including Mϕ and myeloid derived suppresser cells (MDSC). Tumours contain large numbers of Mϕ and MDSC scattered throughout. In contrast, DC are relatively scarce and typically located at the margin of the tumour. Therefore, it is reasonable to propose that these two key APC cell types may be exposed to different types of FB (Schirmer et al., 2010). This will allow us to raise the question whether FB exposed to IR can maintain their effect on regulation of IL-23, IL-12 and IL-10 secretions by Mϕ. Indeed, our work determines that the mitigating effect of IR on IL-23 and IL-10 secretions by GM-Mϕ in the presence of FB were largely suppressed. In contrast, secretion of IL-12 was highly increased. IL-10 secretion by M-Mϕ cultured with FB in the presence of IR was decreased. IR did not affect IL-23 by M-Mϕ cultured with FB. These data suggest that establishment of Mϕ and DC function in co-culture of FB in 2D models, i.e. grown on plastic substrate, does not represent the physiological situation, showing the need for further comprehensive studies to develop co-culture 3D models.

Glucocorticoids (GCs) have pleiotropic effects on the immune system. GCs have important actions on immune cells, and macrophages are prominent targeted. GCs display both immunosuppressive and anti-inflammatory features, allowing them to be used for treatment in various immune-mediated inflammatory disorders. When cytoplasmic glucocorticoid receptors (GR) are activated by GCs, formation of signalling pathways that can translocate to the nucleus occurs. This allows binding to the glucocorticoid response elements (GRE) of a wide range of genes that can encode inflammatory mediators, and regulate their transcription (Barnes and Adcock 1993, Bamberger, Schulte et al. 1996,

Aaltonen, Adelman et al. 2010). Conversely, a variety of transcription factors are suppressed by another action of the GC mechanism, such as nuclear factor kappa B and AP-1 (Scheinman, Cogswell, Lofquist, & Baldwin, 1995). GCs are involved in the transcription of various cytokines that play a role in recruitment of inflammation, including IL-1, TNF-α, GM-CSF, IL-3, IL-4, IL-5, IL-6 and IL-8, and in down-regulation of the expression of the lipid mediators, prostaglandins (Auphan, DiDonato et al. 1995).

Here, we investigated the effect of Dex administration on the Mϕ function in acute and prolonged periods of culture in order to gain a better understanding of GCs actions in different clinical states, characterized by macrophages and their responsiveness to Dex added before or after activation. To our surprise, we found that culture of Mϕ administrated with Dex for 24 hours after activation with LPS and IFN-γ or LPS only potently inhibited the cytokine secretion by Mϕ compared to Dex added from day 0 of culture.

Combination of LPS and Dex elevate GR, thus LPS and GCs stimulate different, sometimes opposite, signalling pathways. Activation of GR is associated with the activation of transcriptional function of NFKB, while the consequence of LPS binding is to induce the phosphorylation and translocation of NFKB towards the nucleus. GCs trigger a phosphatase, which in turn de-activates the MAPK pathway (Bonin et al., 2013; Oakley & Cidlowski, 2013), whereas the MAPK pathway is activated by LPS (J. Brown, Wang, Hajishengallis, & Martin, 2011). In addition, JNK is involved in the MAPK pathway and its activation is responsible for phosphorylation of GR at a specific site, leading to rapid inhibition of GR-dependent gene expression (Rogatsky, Logan, & Garabedian, 1998). These molecular pathways were not detected in activation of Mϕ with LPS in the presence of Dex in this study, and this warrants further investigation.

TAMs are present in tumour stroma, developing under effects of tumour microenvironment where immunosuppressive molecules, such as TGF-β, are frequently present. Polarization of TAM modulated by TGF-β is responsible for stimulating the proliferation of tumour cells, indicating tumour immune escape. A model of polarizing GM-Mϕ and M-Mϕ that resembles prominent properties of TAM has previously been established (Gratchev, 2017). We showed that Mϕ incubated with Dex and treated with TGF-β at high and low concentrations responded in certain way. This allows the hypothesis that TGF-β signalling may regulate Mϕ function in the presence of Dex in different ways, depending on the functional condition of

the cell, indicating that IL-10 and IL-23 cytokine secretion by Mϕ is altered as a consequence of TGF-β signalling.

TGF-β is a key pleiotropic functional cytokine that is currently used to treat a number of human diseases such as cancer and inflammatory disorders and plays an important function in orchestrating immunity. Although a sizable body of studies has showed an understanding of TGF-β how regulates T cell responses, much less is understood about how it regulates innate immunity, such as regulation of macrophages and monocytes, and how the mechanisms of signalling pathways are changed in disease (Akhurst, 2017; Kelly et al., 2018).

Over the last 25 year, there has been a large shift in our approach to the study of the biology of solid tumours. Starting from the concept of the tumour microenvironment which comprises of the vasculature of tumour, connective tissue, infiltrating immune cells, and ECM. Over the last decade, many articles have focused on understanding the tumour ECM and its role in solid tumours and the response to therapy.

In this study, we considered the importance of the FB-derived-ECM in its role in regulating differentiation and function of monocytes and macrophages *in vitro*. We established a base for co-culture models of monocytes or macrophages cultured with ECM derived from BJ6 cell line, ECM derived from normal, surrounding and tumour fibroblasts derived from different breast cancer patients. We first recapitulated the particularities of monocytes and macrophages by detecting expression of cell markers and immune inhibitory receptors, in addition to characterising these cells by cytokine secretion.

TAMs are most common components that are found in the tumour microenvironment. These frequently occurring immune cells play a crucial role in regulating the adaptive immune response in cancer (Tariq et al., 2017). Several ECM depositions are correlated with polarization of TAM. In cell cultures, it was found that Hyaluronic acid (HA) is one protein of the ECM components that can strongly differentiate macrophage into a pro-tumorigenic and anti-inflammatory M2 phenotype (H. Kim, Cha, Jang, & Kim, 2019). In past studies, it was reported that the effect of ECM components on differentiation of macrophages involved Col 1 that modulated M2 polarization. Conversely, enhancement of the cytotoxic function of macrophages to tumour cells was strongly induced by fibronectin-rich ECM, following M1 polarization (Perri et al., 1982; Wesley et al., 1998).

Identification of deposition of various ECM tumours from the same tissue of origin can be categorized into subtypes by their molecular expression profile (Henke, Nandigama et al. 2019). These molecular subtypes can provide a range of information including the metabolism of tumour, misregulation of survival and apoptotic pathways, the occurrence of oncogenic drivers, and thus the sensitivity and resistance to diverse treatment regimes (Perou et al., 2000; Takai, Le, Weaver, & Werb, 2016).

In the previous study, M2-polarized macrophage receptor expression of CD204 was measured by immunohistochemistry within the tumour microenvironment (including TAMs) in 101 samples from patients with canine mammary tumour (CMT) (Seung et al., 2018). In this study, we confirmed that BJ6-ECM down-regulated CD169 on GM-Mϕ, whereas BJ6-ECM supressed CD169 and CD86 on M-Mϕ. S and T-ECM up-regulated CD204 expression on M-Mϕ. This result is in an agreement with previous findings of studies showing that the focusing of the expression of infiltrating-CD204-positive TAMs (M2-polarized TAMs) in benign and malignant CMT, rather than the total TAMs, results in increased CD204 in malignant CMTs compared to benign CMTs. This indicates that TAMs are correlated with the development of breast cancer in humans (Aaltonen et al., 2010; Rasotto, Berlato, Goldschmidt, & Zappulli, 2017). Poor prognosis is related to the presence of a high density of TAMs in a variety of tumours (Shabo, Stal et al. 2008). Studies concentrating on M2-polarized TAMs are essential to investigate the functions that play a protumoral role (Sica & Mantovani, 2012). Ma-polarized TAMs have prominent features that can be correlated with aggressive behaviour of human cancer (Shabo, Stal, Olsson, Dore, & Svanvik, 2008), which suggests that the protumoral properties of CD204-positive TAMs play a role in enhancing tumour growth and metastasis. Since induction of an aggressive tumour behaviour index in human is associated with highly infiltrating CD204-positive macrophages, our results suggest that TAMs that express a high level of CD204 may drive the development and behaviour of cancers. Furthermore, the findings suggest the use of CD204-positive macrophages as a novel use of biomarkers in to determine prognosis in cancer cases, as well as a providing a target for therapy to suppress tumour metastasis.

TAMs modulate tumour immunity. Previous studies found that M2 macrophages phenotype express PD-1. It has been shown that patients with muscle-invasive bladder cancer (MIBC)

display a negative survival indicator in the presence of PD-1-positive TAMs (Jiang et al., 2021).

PD-1 is one family of immune inhibitory receptors, which is expressed on activated T cells and associated with induction of immune tolerance (N. Wang et al., 2018). Their ligand, PD-L1, plays an important role in promoting the escape of tumour cells from immune system. However, tumour cells can gain the feature of immune escape by expression of PD-L1 (Mocan, Sparchez, Craciun, Bora, & Leucuta, 2019).

Recent studies have shown that PD-1 express in a high level on TAMs. These M2-polarized M2 expressed PD-1 have phenotypic characteristics and can enhance tumour proliferation by inhibiting tumour immunity (S. R. Gordon et al., 2017).

In this investigation, we aimed to identify the polarization of Mϕ cultured with normal, surrounding and tumour ECM derived from fibroblasts in patients with breast cancer and how these impact on up-regulation of immune inhibitory receptors such as PD-1. Consistent with this, we confirmed that T-ECM up-regulated PD-1 on M-Mϕ. PD-1-postitive TAMs were also associated with increasing undifferentiated histological patterns, which indicate poor diagnostic survival and worse clinical outcomes (Boegemann, Aydin, Bagrodia, & Krabbe, 2017; Jiang et al., 2021). Further planned studies should explore the mechanisms underlying the signalling pathway of PD-1/PD-L1 axis in TAMs, as it can directly affect tumour cell proliferation. We suggest that it is possible that macrophage function can be restored by using an immunotherapeutic strategy of anti-PD-1 and this has potential promise for future therapy for cancer treatment.

6.2 Conclusion and further experiments

Data from this thesis suggest that macrophages have their biology altered markedly in the tumour microenvironment due to interaction with stromal fibroblasts and their extracellular matrix. Like the other components of the TME, stromal fibroblasts and their ECM in solid tumours vary significantly from those in normal tissue. Further experiments are required to examine how the mechanisms of tumour suppressive matrisome deposited by FB contribute to the development of tumour-associated macrophages phenotype, following understanding of phenotypic diversity of macrophages. The ECM play a key role in

controlling intratumoral signalling, metabolism, transport mechanisms, oxygenation and immunogenicity.

6.2.1 Extracellular matrix in the TME and its impact on biomarkers in cancer therapy

Our work addressed the hypothesis that ECM derived from fibroblasts can influence of activation, proliferation and polarization of monocytes and macrophages. These findings demonstrate restrictions in differentiation of monocytes and macrophages by ECM in the presence of initial conditioning and suggest up-regulation of CD204 and PD-1 expression on M-Mϕ. We observed morphological changes of cells into cells-like Mϕ, cells-like DCs and cells-spindle shape. Our current work examined the key-representative traits of pro- and anti-inflammatory Mϕ, such as IL23, IL-12 and IL-10. However, results were unclear. Further studies should be planned to fully identify the different proteins in each matrisome derived from distinct FBs. This will aim to understand how polarization of phenotypic signatures of macrophages including tumour-associated macrophages at different stages of cancer development occurs by using developments in mass cytometry. This method can combine flow cytometry with mass spectrometry and has the capacity to detect up to 40 protein readouts in single cells. These strategies have the potential to identify promising proteins that are responsible for differentiation of macrophages and could be utilized to improve biomarkers in cancer immunotherapy.

6.2.2 Designing a promising 3D culture model to evaluate the effects of FB on Mϕ functions

We have shown that the 3D co-culture of FB/Mϕ drives activation of Mϕ to express a high level of cytokines. Spheroid shapes were observed that were made of FBs/Mϕ and showed cell aggregates shaped as radial invasive cells around and attached to the bottom of the well, suggesting a higher production of MMP can degrade collagen and laminin. Moreover, the spheroid model was challenging to establish and further studies should aim to improve the alginate microencapsulation of cells or the stirred culture model for spheroid assembly. To further improve TME research, it is necessary to develop *in vitro* cell culture systems capable of investigating the impacts of FB on Mϕ and their differentiation and function based on the combination 3D cell aggregates and alginate microencapsulation in the presence of LPS and

IFN-γ, to assay cytokine secretion and use IHC to identify protein markers expressed on Mϕ and FBs.

6.2.3 Blocking PD-1 and PD-L1 axis

We observed that M–Mϕ cultured with T-ECM upregulated the expression of PD-1 molecules when compared with N-ECM. It is possible that theses inhibitory receptors contribute to the effect of Mϕ function within tumour microenvironment. Moreover, understanding the blockade of PD-1/PD-L1 by inhibitors has set a new model for treatment of metastatic bladder cancer in patients who are unable to take or are resistant to cisplatin-based therapy (Tan, Tan et al. 2019). Therefore, adding anti-PD-1 and anti-PD-L1 mAbs (antagonists) into Mϕ that are cultured with ECM could help to repolarize Mϕ function *in vitro.*

6.2.4 Determining the role of CD39 and CD73 expressed by fibroblasts

Data from this thesis have shown that when APCs interact with FBs their biology is markedly altered. A prime example of this is the potentiation of IL-23 responses from human DC and Mϕ derived from monocytes (moDC and moMϕ) upon co-culture with NAF. This occurs by feedback loops of inflammatory cytokine (IL-1β/TNFα) production by DCs that elicit prostaglandin E_2 (PGE_2) secretion by NAF that then amplifies IL-23 expression by DCs (Schirmer et al., 2010). Another feedback loop of the effects of FBs on APC is the potent immunosuppressive activities of adenosine produced by up-regulation of ectonucleotidases CD39 and CD73 on FBs. High CD39 and CD73 may be directly linked with PD-1 and PD-L1 expression by Mϕ. It has been revealed that cancer cells, CAF and tumour-infiltrating lymphocytes overexpress ectonucleotidases in lung tumours (Giatromanolaki et al., 2020). Adding antagonists to adenosine receptors A1, A2A, A2B and A3 in a co-culture of FB/APC may alleviate adenosine-mediated suppression and consequently lead to down-regulation of immune inhibitory receptors on Mϕ. This may help to alter the FB-mediated effect.

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