Developing immunoresponsive tissue engineered oral mucosal equivalents to model oral inflammation and local drug metabolism

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Abstract

Innate immune cells such as macrophages and dendritic cells are recruited to tissue during inflammation, where they play a key role in the detection and elimination of invading organisms and foreign molecules and help orchestrate an immune response. There has been increasing interest in local drug delivery methods to treat inflammatory-mediated and other localised diseases, especially in the oral mucosa, although little research has been undertaken to identify drug metabolism in local tissue, and the potential for recruited inflammatory immune cells to participate in local metabolism. This study quantified expression of xenobiotic metabolising enzymes (XME) in monocyte-derived immune cells and developed a tissue-engineered model of buccal mucosa containing primary macrophages to better model the immune response and to assess drug metabolism in this tissue.

Primary monocytes were isolated from peripheral blood, differentiated into monocyte-derived macrophages (MDM) or dendritic cells (MoDC) and assessed for production of XME by gene array, qPCR, and western blot where they were found to have distinct expression profiles. As immune cells work in concert with other tissue resident cells to generate an immune response, investigations were undertaken to ensure MDM were suitable for inclusion into a tissue engineered oral mucosal model, including culture in a collagen matrix and optimisation of response to stimuli, then an MDM-oral mucosal equivalent (OME) generated by incorporating MDM into a collagen hydrogel with oral fibroblasts then seeded with immortalised oral keratinocytes and cultured at an air-to-liquid interface for 10 days. These models were challenged with *Escherichia coli* lipopolysaccharide (LPS) ± dexamethasone to examine changes in inflammatory markers.

MDM were suitable for inclusion in a tissue engineered model, as a measurable inflammatory response was conserved in response to *E. coli* LPS and dexamethasone in both monolayer and within collagen hydrogel. Addition of MDM into an OME had no effect on histology, and MDM-OME were immune-positive for CD68 and epithelial makers. MDM viability was confirmed using CD11c as an MDM-specific marker. MDM-OME responded to LPS with increased gene expression of inflammatory markers, and secretion of TNF-α was increased 10-fold in LPS-treated MDM-OME compared to all other conditions. Gene expression of relevant XME was detected in the MDM-OME, although most were unaltered by treatment conditions.

The data presented in this thesis suggests a potential novel role for inflammatory MDM in local drug metabolism, and further investigations may reveal additional insights which could impact on future drug design rationale. MDM-OME were generated that responded to inflammatory stimuli by shifting to a pro-inflammatory phenotype which was inhibited by a clinically used anti-inflammatory steroid. This immunocompetent oral mucosal model will aid studies that examine efficacy of novel pharmaceuticals and biomaterials and unravel the role of immune cells in local xenobiotic metabolism.

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Publications

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Presentations

Oral presentations

- **Generation of an immuno-responsive tissue engineered oral mucosal equivalent containing primary human macrophages.** Tissue and Cell engineering society (TCES) annual conference, Nottingham, 2019.
- **Academic writing.** Internal technical presentation, Sheffield, 2019.
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- **Enzyme-Linked ImmunoSorbent Assay (ELISA).** Internal technical presentation, Virtual, 2021.
- **Developing an immune model of the oral buccal mucosa to investigate local drug pharmacokinetics.** Lunchtime seminar, Virtual, 2021.
- **Incorporating Macrophages into Tissue-Engineered Oral Mucosal Equivalents Improves Inflammatory Response.** British Society of Dental Research (BSODR) annual conference, Birmingham, 2021. Finalist for Senior Colgate award.

Poster presentations

- **Generating an *in vitro* immuno-competent oral mucosal model.** Advanced Course in Mucosal Immunology (ACMI), Córdoba, Argentina, 2018.
- **Generating an *in vitro* immuno-competent oral mucosal model.** Biomaterials and tissue engineering group (BiTEG) annual meeting, Sheffield, 2018.
- **Generating an immuno-responsive tissue-engineered oral mucosal equivalent.** British Society of Dental Research (BSODR) annual conference, Leeds, 2019.
- **Generating Tissue-engineered Oral Mucosal Equivalents Containing an Immune Component.** 3D Bionet/IBIN joint meeting, London, 2020. Awarded first place prize.
- **Generating a Tissue-Engineered Oral Mucosal Equivalent Containing Primary Human Macrophages.** British Society of Dental Research (BSODR) annual conference, Virtual, 2020.

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ALI	Air-to-liquid interface
APC	Antigen presenting cell
APC	Allophycocyanin
ATP	Adenosine triphosphate
BHI	Brain heart infusion
BMDM	Bone marrow-derived macrophage
BDNF	Brain-derived neurotrophic factor
CD	Cluster of differentiation
CHI3L1	Chitinase-3-like protein 1
CNS	Central nervous system
COX	Cyclooxygenase
CPM	Carboxypeptidase M
CXCL	Chemokine (C–X–C motif) ligand
Cy3	Cyanine 3
CYP	Cytochrome P450-monooxygenase
DAPI	4′,6-diamidino-2-phenylindole
DC	Dendritic cells
DEAB	N,N-diethylaminobenzaldehyde
DED	De-epidermised dermis
DMEM	Dulbecco’s modified Eagle’s medium
DNCB	2,4-Dinitrochlorobenzene
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EV	Extracellular vesicle
FCS	Foetal calf serum

FITC	Fluorescein isothiocyanate
FMO	Flavin-containing monooxygenase
GCSF	Granulocyte colony-stimulating factor
GelMA	Gelatin methacryloyl
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GST	Glutathione s-transferase
H&E	Haematoxylin and eosin
HBSS	Hank’s balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid
HGF	Hepatocyte growth factor
hTERT	Human telomerase reverse transcriptase
IDA	Industrial denatured alcohol
IEL	Intraepithelial lymphocytes
IFN	Interferon
IHC	Immunohistochemistry
IKK	IκB kinase
IL	Interleukin
IMDM	Iscove’s modified Dulbecco’s medium
LDH	Lactate dehydrogenase
LC	Langerhans cells
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MDM	Monocyte-derived macrophages
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MM6	MonoMac-6
MoDC	Monocyte-derived dendritic cells
MoLC	Monocyte-derived Langerhans cells
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
NaHCO3	Sodium bicarbonate
NAM	N-acetyl muramic acid

NAT	N-acetyltransferase
NaOH	Sodium hydroxide
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEK	Normal human epidermal keratinocytes
NK	Natural killer
NOF	Normal oral fibroblast
OD	Optical density
OLP	Oral lichen planus
OOAC	Organ-on-a-chip
OPN	Osteopontin
OSCC	Oral squamous cell carcinoma
PAI	Plasminogen activator inhibitor
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principle component analysis
PCR	Polymerase chain reaction
PE	Phycoerythrin
PKC	Protein kinase C
PMA	Phorbol 12-myristate-13-acetate
PMN	Polymorphonuclear leukocytes
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
PTX	Pentraxin
RA	Retinoic acid
RAGE	Receptor for advanced glycation endproducts
RBP	Retinol binding protein
RLN	Relaxin
SDS	Sodium dodecyl sulphate
SHBG	Sex hormone-binding globulin
SULT	Sulfotransferase
TAK1	Transforming growth factor β-activated kinase 1

TAM	Tumour-associated macrophages
TCR	T cell receptor
TEMED	Tetramethylethylenediamine
TFF	Trefoil factor
TfR	Transferrin receptor
THBS	Thrombospondin
TLR	Toll-like receptor
TGF-β	Transforming growth factor β
TNF-α	Tumour necrosis factor α
UGT	UDP-glucuronosyltransferase
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
XME	Xenobiotic metabolising enzymes

Chapter 1 - Introduction

1.1 Immunity

Immunology is the study of innate and adaptive immunity that work in concert to protect our bodies from toxins, pathogens, and other factors which may cause harm (Hoebe et al., 2004). Innate immunity includes physical barriers that provide an unfavourable environment for excessive microbial growth, as well as specialised immune cells which rapidly secrete inflammatory cytokines, recruit additional immune cells to sites of infection, and directly act to neutralise threats (Turvey et al., 2010). Adaptive immunity requires cells to express antigen-specific receptors to identify and respond to a specific non-self-antigen, a process which includes cell-mediated and humoral immunity and often takes several days (Good et al., 1964; Chaplin, 2010). A summary of the key cell types of the innate and adaptive immune system is shown in Figure 1.1. Understanding how this process is initiated and regulated is key to developing new therapeutic agents that alter immune function to either enhance or (in the case of auto-immune diseases) diminish the capabilities of our immune system.

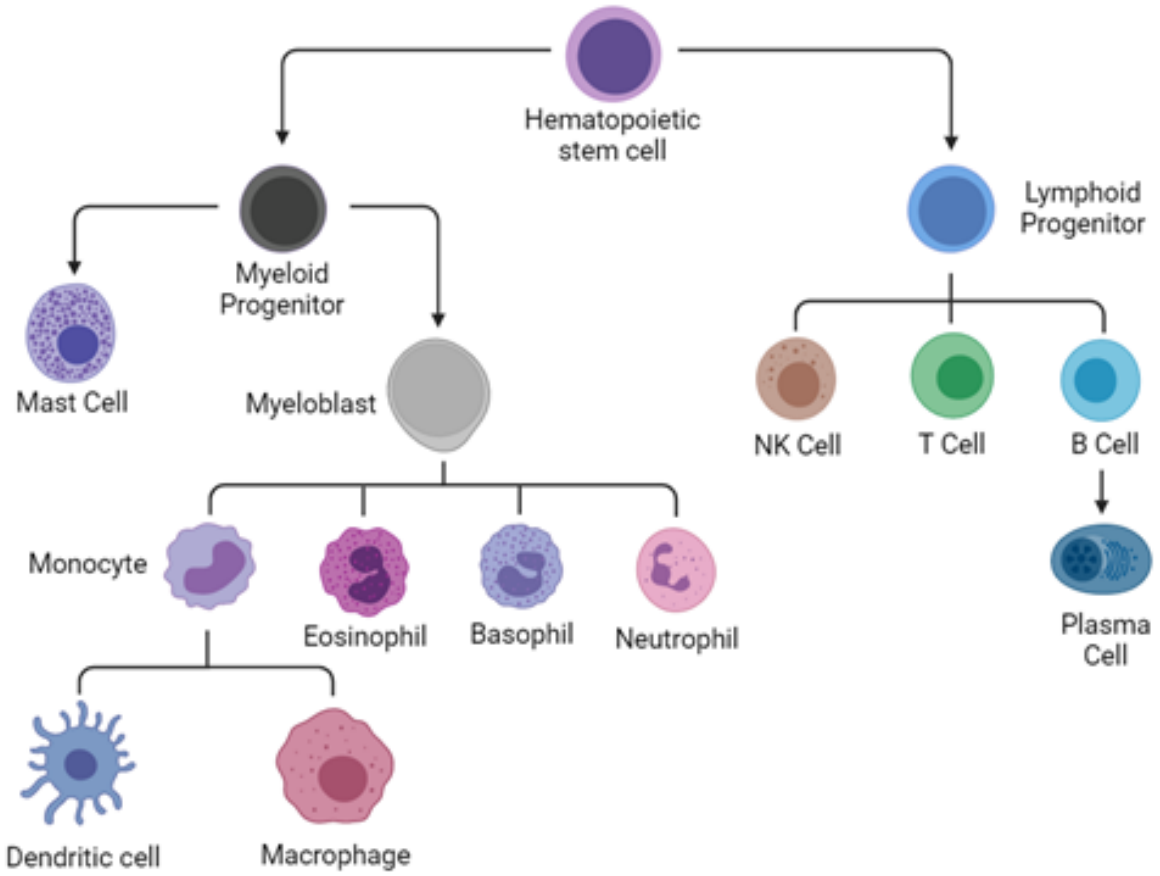


Figure 1.1. Innate and adaptive cell lineage.
Immune cells begin as stem cells which differentiate into myeloid and lymphoid precursors that produce distinct immune cell subtypes as indicated. Created with BioRender.com.

1.1.1 Innate immune cells

Specialised innate immune cells are found either circulating in peripheral blood, or resident in tissue. Monocyte-derived cells such as macrophages and dendritic cells are often tissue resident, and replenished by differentiation of peripheral blood monocytes when these cells are recruited from the circulation to tissues (Nichols et al., 1971; Coillard et al., 2019). Monocytes also act within the innate immune system by binding microbes, performing phagocytosis, and producing cytokines to alter immune responses (Passlick et al., 1989; Cormican et al., 2020). Lymphoid-derived natural killer (NK) cells clear senescent cells and eliminate cells lacking self-antigens, presented by MHC I, such as cancer cells (Wu et al., 2003), by secreting perforin and granzymes to induce cell apoptosis or lysis (Pardo et al., 2002; Paul et al., 2017).

Neutrophils, eosinophils, and basophils are together referred to as granulocytes due to the visible granules in the cytoplasm, or polymorphonuclear cells (PMN) due to their distinctive lobed nuclei (Manley et al., 2018). Neutrophils are the most abundant of these cells, making up over 60% of circulating leukocytes (Rosales, 2018). These cells are attracted to a site of infection, where they produce oxidising agents to directly attack pathogens (Ringel et al., 1984; Teng et al., 2017). Eosinophils release toxic proteins and free radicals, and act as antigen-presenting cells to regulate immune cell function (Gleich et al., 1986; Kita, 2011). Basophils have low abundance in blood, and typically bind to IgE to initiate release of histamine which contributes to the inflammatory response by causing local vasodilation and increasing vascular permeability (MacGlashan et al., 1980; Ashina et al., 2015), as well as prostaglandins which increase blood flow to the area (Chirumbolo, 2012). Mast cells are tissue resident cells which are activated by many stimuli, including pathogens, but most commonly known for IgE-mediated activation in hypersensitivity, and, like basophils, release histamine locally (Kulczycki et al., 1974; Krystel-Whittemore et al., 2016).

While T cells are discussed in more detail in Section 1.1.3, a subtype of T cells known as γδ T cells contain characteristics which place this cell type in between innate and adaptive immunity (Bergstresser et al., 1985; Born et al., 2006). Typically, these cells do not require antigen presentation to become activated, instead recognising lipid antigens (Luoma et al., 2013; Deseke et al., 2020), and can become activated by cell stress signals such as heat shock proteins (Hirsh et al., 2008), although they can

develop a memory phenotype characteristic of adaptive immune cells (Hu et al., 2012). A γδ T cell subtype is also resident in the epithelium of many tissue barriers such as the skin and oral mucosa where they are termed intraepithelial lymphocytes (IELs) (Ismail et al., 2011).

1.1.1.1 Macrophages

Macrophages commonly originate from peripheral blood monocytes which differentiate into macrophages as they cross the vasculature, but subpopulations of macrophages are also established *in utero* before birth from fetal monocyte progenitors (Epelman et al., 2014; Hoeffel et al., 2015). *In vitro* generation of macrophages can be achieved from primary monocytes by plastic adherence, with increased numbers produced on type 1 collagen coated plastic compared to tissue culture plastic alone (Wesley et al., 1998). As such differentiation *in vitro* is likely to occur through the same mechanisms used by migrating monocytes during differentiation to tissue-resident macrophages. In addition, treating monocytic leukaemia cell lines, such as THP-1 cells with phorbol 12-myristate-13-acetate (PMA) or vitamin D can also generate cells with a macrophage-like phenotype (Park et al., 2007; Arboleda Alzate et al., 2017), which occurs through activation of the protein kinase C (PKC) signalling pathway (Schwende et al., 1996; Richter et al., 2016).

Macrophages are distinguishable by physical location, and are able to alter phenotype according to the microenvironment in which they reside, with different sites producing macrophages capable of varied functions (Gordon et al., 2017). For example, Kupffer cells are specialised liver resident macrophages which play a role in host defence in the tissue, as well as participate in metabolism of various compounds, such as endogenous lipids, and apoptotic cells (Naito et al., 1998; Nguyen-Lefebvre et al., 2015). In the lung, there are two main macrophage populations, alveolar macrophages which reside on the surface of the alveolar epithelium, and interstitial macrophages that are found below the epithelium (Hu et al., 2019). Alveolar macrophages provide the first line of defence against pollutants and pathogens (Hussell et al., 2014), and have unique mechanisms to prevent inappropriate inflammatory responses (Holt et al., 1993; Allard et al., 2018), including induction of T cell inactivation (Blumenthal et al., 2001). Finally, macrophages residing with the

central nervous system (CNS) are termed microglial cells, and in addition to initiating an inflammatory response, are also involved in specialised functions such as synaptic organisation, myelin turnover, and control of neuronal excitability (Kreutzberg, 1995; Bachiller et al., 2018).

The main physiological role of macrophages is to recognise, phagocytose and eliminate material such as bacteria, virus, apoptotic cells and cell debris, which occurs following activation of a variety of receptors, including Fc receptors, complement receptors, Toll-like receptors, C-type lectin or scavenger receptors (Uribe-Querol et al., 2020). Activated macrophages also secrete a plethora of pro-inflammatory cytokines, such as IL-6 and TNF-α and chemokines such as CXCL8 which aid in tuning the local inflammatory response and guide immune cell migration respectively (Huber et al., 1981; Arango Duque et al., 2014). Furthermore, as well as dendritic cells, macrophages are known to act as antigen presenting cells (APCs) (Hume, 2008), with increasing evidence that various tissue-specific macrophages are capable of antigen presentation to T cells (Muntjewerff et al., 2020).

Upon pathogenic invasion, immune cells recognise pathogen-associated molecular patterns (PAMPs) through toll-like receptors (TLRs) or other recognition receptors (Mogensen, 2009). Of the many PAMPs identified, arguably the most widely studied are bacterial lipopolysaccharides (LPS); polysaccharide chains that are localised to the cell surface of Gram-negative bacteria. Most bacterial LPS is recognised specifically by TLR4, although there is evidence that TLR2 is required for signal transduction (Good et al., 2012). For example, LPS from *Porphyromonas gingivalis* (a keystone pathogen in periodontitis) has various structures which have been shown to interact with both TLR2 and TLR4 (Darveau et al., 2004; Maekawa et al., 2014). Other PAMPs such as lipoteichoic acid or peptidoglycan from Gram-positive organisms are also used by immune cells to recognise pathogens and can elicit distinct inflammatory cytokine release profiles (DeClue et al., 2012) due to differential TLR signalling (Paul-Clark et al., 2008).

Following macrophage activation by stimuli such as LPS (Sharif et al., 2007), the majority of inflammatory downstream effects are mediated by the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling pathway (Sen et al., 1986; Liu et al., 2017). The canonical pathway of NFκB is initiated by recognition of

inflammatory stimuli, including LPS, TNF-α, and IL-1 signalling. Upon initiation, the IκB kinase (IKKβ) is activated, which phosphorylates IκB proteins, leading to protein degradation by ubiquitination (Chen et al., 1999; Israel, 2010). The typical function of IκB proteins, such as IκBα, is to ensure the NFκB complex remains in the cytosol, and degradation allows NFκB to translocate into the cell nucleus to initiate transcription of inflammatory genes such as IL-1 and TNF-α (Lawrence, 2009). The NFκB complex in the canonical signalling pathway consists of p50 and p65, while the complex used in the non-canonical pathway (activated in response to CD40 and lymphotoxin signalling) often comprises p52/RelB, which allows for altered gene expression changes depending on activation mechanism (Sun, 2011). As well as inflammatory genes, NFκB activation also initiates production of IκBα, which inhibits further NFκB activation, forming a negative feedback loop to attenuate the overall inflammatory response (Verma et al., 1995).

Dysregulated activation of the NFκB pathways is linked to diseases including cancer where typically it acts in a tumorigenic manner (Xia et al., 2014), and inflammatory conditions such as arthritis and asthma (Tak et al., 2001), which has generated significant interest in this pathway as a drug target (Ramadass et al., 2020). Many anti-inflammatory drugs, such as dexamethasone, target the NFκB pathway (Auphan et al., 1995; Crinelli et al., 2000), mainly exerting their function through activation of the glucocorticoid receptor which prevents p65 translocation to the nucleus, a key step in NFκB activation (Nelson et al., 2003). In addition, small molecule inhibitors such as BAY 11-7082 and 11-7085 (Pierce et al., 1997) have been developed to specifically inhibit NFκB activation, which act by irreversibly inhibiting IκBα phosphorylation.

In recent years two main functional state macrophages have been identified and termed as M1 and M2 phenotypes (Figure 1.2). These were originally thought to be distinct and important in disease, as the M1 phenotype is pro-inflammatory which can exacerbate auto-immune conditions whereas the M2 phenotype is pro-wound healing and pro-angiogenic, and often found in the tumour microenvironment, where increased infiltration worsens patient outcome (Mori et al., 2011; Merry et al., 2012). To produce these distinct functions, M1 and M2 polarised macrophages have altered metabolic profiles (Abuawad et al., 2020), and utilise distinct metabolic pathways to produce energy. M1 macrophages preferentially utilise glycolysis for rapid energy production,

generating reactive oxygen species (ROS) as a by-product which is used to maintain inflammatory response elements. In contrast, M2 macrophages mainly rely on oxidative phosphorylation and fatty acid oxidation (Viola et al., 2019).

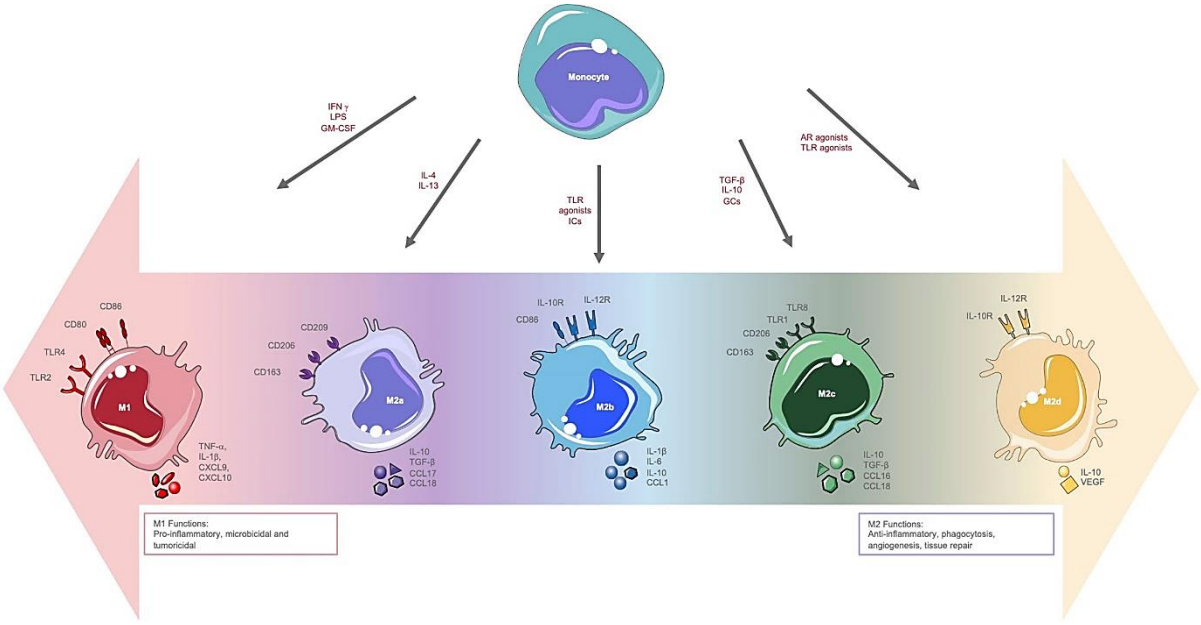


Figure 1.2. Human macrophage polarisation.
Overview of various polarisation states identified in human macrophages, with M1 inflammatory macrophages, and M2 alternatively activation macrophages split into four distinguishable subtypes. Image reproduced under a Creative Commons Attribution Licence, taken from (Chambers et al., 2021).

There are well used markers for each macrophage polarisation phenotype. M1 macrophages increase expression of co-stimulatory molecules CD80 and CD86, as well as genes associated with inflammatory cytokine production such as CXCL10 and 11, CCL5 and CCR7. In contrast, M2 macrophages increase expression of CD163, CD206, CD200R, TGF-β, and PPARγ, and production of cytokines such as IL-10 and CCL18 (Rostam et al., 2016). Macrophages in each polarisation state also respond differently to immune challenge with altered cytokine expression profiles (Jaguin et al., 2013). However, these phenotypes are generally created artificially in a laboratory under defined experimental conditions and the evidence pointing to their existence in such a clearly distinct polarised manner in a clinical setting is not so clear, with many reports noting that disease-associated macrophages adopt a mixed phenotype. For example, treatment of RAW264.7 murine macrophages with melanoma exosomes

stimulated secretion of both TNF-α (M1 associated), and IL-10 (M2 associated) as well as simultaneous upregulation of other M1 and M2 gene markers (Bardi et al., 2018). In agreement, macrophages isolated from ovarian cancer biopsies had increased gene expression of M2 markers such as CD163 and IL-10 as well as the M1 marker CD86 compared to MDM (Reinartz et al., 2014). Similarly, macrophages can shift between these functionally distinct phenotypes. For example, a M1-like phenotype is often adopted in response to acute bacterial challenge but macrophages can become M2 polarised after chronic exposure, or in response to specific bacterial by-products such as butyrate – a product of microbial fermentation (Benoit et al., 2008; Ji et al., 2016). Despite not mirroring the physiological environment, identification of these distinguishable phenotypes allows for research into the signalling that drives changes in macrophage behaviour and can also be used as an endpoint analysis when determining factors that can influence immunity.

1.1.1.2 Dendritic cells

Dendritic cells (DC) are the main immune cells that link innate and adaptive immune responses by taking up pathogenic material, forming antigens and through antigen presentation initiating the adaptive immune response. Conventional DC activate T cells through antigen presentation and cytokine signalling and comprise two subtypes: cDC1 and cDC2. cDC1 activate NK cells and produce T helper 1 cells through secretion of IL-12 (Sousa et al., 1997; Nizzoli et al., 2013) and can be identified by expression of XCR1, CD8α, CLEC9A and DEC205 (Shin et al., 2020). In contrast, cDC2 cells have a wider range of functionality and are often defined by CD11b, CD172a and CLEC10A expression (Shin et al., 2020). cDC2 cells can produce T helper 1, 2, and 17 cells, and T regulatory cells, depending on cytokine secretion patterns (Siegal et al., 1999; Sittig et al., 2016). Furthermore, cDC2 subtypes are defined as either DC-like or monocyte-like which preferentially prime T helper 2 and 17 cells, or T helper 1 cells respectively (Yin et al., 2017).

Monocyte-derived DC (MoDC) have been identified in inflammatory diseases (Segura et al., 2013), and mainly function to promote T cell polarisation instead of proliferation (Chow et al., 2016). MoDC can be generated *in vitro* from peripheral blood monocytes using a combination of interleukin 4 (IL-4) and granulocyte-macrophage

colony-stimulating factor (GM-CSF) at various concentrations (Posch et al., 2016; Lutz et al., 2017; Chometon et al., 2020). As monocytes are more plentiful in peripheral blood compared to dendritic cells, MoDC are a convenient source of DC-like cells and represent a valuable method to investigate *in situ* DC generation and inflammatory responses.

Langerhans cells (LC) are a subtype of dendritic cell found exclusively in the epidermal compartment of skin and mucosa. LC are conventionally activated by pathogens, which are processed into specific antigens. Activated LC migrate to the lymph nodes where they activate naïve T cells (Romani et al., 2012). LC reportedly maintain a near constant ratio of 2% of the epidermal cells (Bauer et al., 2001), and while the mechanism of *de novo* LC generation is still debated (Collin et al., 2016), the current theory is that LC are derived *in situ* from myeloid precursor cells, something that can be mimicked *ex vivo* by cytokine stimulation of peripheral blood monocytes (Geissmann et al., 1998; Guironnet et al., 2002) and *in vivo* by CD115 activation (Ginhoux et al., 2006). However, LC *in vivo* can be renewed following immunological challenge even when myeloid progenitor cells have been removed (Merad et al., 2002), suggesting multiple routes of *de novo* LC generation and renewal.

1.1.2 Adaptive immunity

The adaptive immune system is a highly specialised response to a specific non-self-molecule and, unlike innate immunity, includes immunological memory that improves immune response upon repeated exposure (Good et al., 1964; Ratajczak et al., 2018). The main cells of the adaptive immune system are T cells and B cells.

T cells are comprised of multiple subtypes with distinct roles which all express the cell surface T-cell receptor (TCR) complex (Allison et al., 1982; Yanagi et al., 1984). This complex is responsible for antigen recognition and initiating intracellular signalling pathways to enable T cells to respond to external stimuli (Gaud et al., 2018). The TCR is a heterodimer, with the majority of T cells utilising an α and β chain, while approximately 5% comprise a γ and δ chain (see section 1.1.1) (Morath et al., 2020). There are two main subtypes of T cells, $CD4^+$ helper T cells, and $CD8^+$ cytotoxic T cells, which are named according to primary functionality. Cytotoxic T cells are activated when an antigen bound to a class I MHC molecule on an APC is recognised

by the TCR, an association which is stabilised by CD8 (Zhang et al., 2011). Activated cytotoxic T cells can directly kill virus-infected and cancer cells by releasing cytotoxic agents such as perforin and granzyme, which induce cell apoptosis (Voskoboinik et al., 2015). In addition, cytotoxic T cells can secrete IL-2 and IFNγ to influence activity of local immune cells, notably macrophages and NK cells (Langston et al., 2003). Helper T cells are similarly activated by antigen recognition, but helper T cells recognise antigens bound to MHC class II. The main function of helper T cells is to secrete cytokines to activate and recruit immune cells locally and orchestrate the correct immune response to the nature of the threat, with helper T cell subtypes defined by the specific cytokines released (Murphy et al., 2002).

B cells can act as antigen presenting cells using cell surface monomeric immunoglobulin (Ig) specific to a particular antigen. Upon binding a reciprocal TCR and stimulation with Th2 secreted cytokines IL-4, -5, -10 and -13, B cells become activated, rapidly proliferate in a process termed clonal expansion (Martinez-A et al., 1981; Liu et al., 2020). Activated B cells differentiate into either plasma cells that produce large amounts of the specific antibody required (Fagraeus, 1948; Pioli, 2019), or memory B cells that retain the specific antibody involved in the initial exposure. When reactivated by subsequent exposure to the same presented antigen these cells proliferate and differentiate into plasma cells, allowing for a faster humoral response (Kurosaki et al., 2015).

1.2 Xenobiotic metabolism

Xenobiotic metabolism describes a set of metabolic processes which serve to chemically alter xenobiotic (foreign) compounds which can apply to drugs, environmental agents such as pollution and tobacco, and food (Murphy, 2001). Broadly this process detoxifies compounds and enables accelerated excretion from the body, although in some instances compounds can be metabolised to a more active metabolite (Patterson et al., 2010).

Xenobiotic metabolism is often split into either phase 1 (functionalisation) or phase 2 (conjugation) reactions. Functionalisation reactions generate or transform a functional group, while conjugation reactions transfer a chemical moiety onto the existing molecule. The most common functionalisation is an oxidation reaction to introduce an N-, O- or S- group as these are highly reactive. Conjugation reactions add glutathione, sulphates, or glucuronic acid groups to functionalised molecules, increasing their overall size and polarity and preventing passive membrane diffusion to increase the rate of elimination from the body (Penner et al., 2012). The functionalisation and conjugation reactions are performed by xenobiotic metabolising enzymes (XME) which are summarised in Table 1.1. These enzymes are generally concentrated in the liver, giving rise to the first pass effect, although they can be found in other tissues, such as the kidneys (Lock et al., 1998), lungs (Hukkanen et al., 2001), and skin (Oesch et al., 2018).

As described previously, the role of both macrophages and DC requires these cells to produce enzymes to degrade proteins and other biological molecules, to either present antigens or destroy the offending material. It is therefore likely that these cells also express enzymes involved in xenobiotic metabolism. A role for these resident immune cells in drug metabolism would be essential to investigate, due to their high density in diseases such as cancer where local treatment is potentially beneficial. Therefore, this section will summarise the function of clinically important xenobiotic metabolising enzymes (XME) families and discuss the evidence for expression of each of these XME by immune cells.

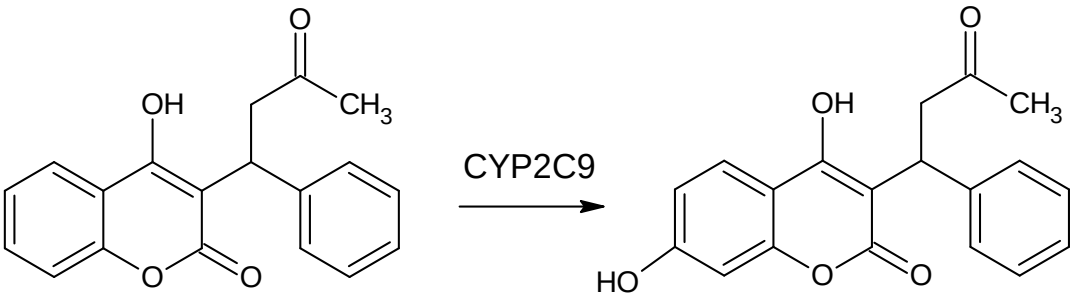
Table 1.1. Major phase 1 and phase 2 XME families

	Enzyme family	Specific isozymes	Function
Phase 1	Cytochrome P450 class I	1A1, 1A2, 1B1	Drug and steroid metabolism
	Cytochrome P450 class II	2A6, 2A7, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 2R1, 2S1, 2U1, 2W1	Drug and steroid metabolism
	Cytochrome P450 class III	3A4, 3A5, 3A7, 3A43	Drug and steroid metabolism
	Cytochrome P450 class IV	4A11, 4A22, 4B1, 4F2, 4F3, 4F8, 4F11, 4F12, 4F22, 4V2, 4X1, 4Z1	Fatty acid metabolism
	Other Cytochrome P450 enzymes	5A1, 7A1, 7B1, 8A1, 8B1, 11A1, 11B1, 11B2, 17A1, 19A1, 20A1, 21A2, 24A1, 261A, 26B1, 26C1, 27A1, 27B1, 27C1, 39A1, 46A1, 51A1	Varied
	Alcohol dehydrogenase	1A, 1B, 1C, 4, 5, 6, 7	Convert alcohol to aldehyde or ketones
	Aldehyde dehydrogenase	1A1, 1A2, 1A3, 1B1, 1L1, 1L2, 2, 3A1, 3A2, 3B1, 3B2, 4A1, 5A1, 6A1, 7A1, 8A1, 9A1, 16A1, 18A1	Convert aldehydes to carboxylic acids
	Flavin-containing monooxygenase	1, 2, 3, 4, 5, 6	Xenobiotic metabolism
Phase 2	Glutathione S-transferase	A1, A2, A3, A4, A5, K1, M1, M1L, M2, M3, M4, M5, O1, O2, P1, T1, T2, T4, Z1, MGST1, MGST2, MGST3	Glutathione conjugation
	UDP-glucuronosyltransferase class I	1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10	Glucuronidation
	UDP-glucuronosyltransferase class II	2A1, 2A2, 2A3, 2B4, 2B7, 2B10, 2B11, 2B15, 2B17, 2B28	Glucuronidation
	N-acetyltransferase	1, 2, 5, 6, 8, 8L, 9, 10, 11, 12, 13, 14, 15	Acetylation
	Sulfotransferase	1A1, 1A2, 1A3, 1A4, 1B1, 1C2, 1C3, 1C4, 1D1P, 1E1, 2A1, 2B1, 4A1, 6B1	Sulfation

1.2.1 Phase I metabolic enzymes

Cytochrome P450 (CYP) enzymes have been widely studied due their involvement in metabolism of pharmaceutical agents. While the CYP enzyme superfamily is made up of more than fifty isozymes (Table 1.1), only six metabolise the vast majority of clinically used drugs (Lynch et al., 2007), so these notable isozymes are discussed below.

CYP2C family contains four isozymes, CYP2C8, 9, 18 and 19 (Goldstein et al., 1994). These enzymes are involved in metabolism of long-chain polyunsaturated fatty acids to active epoxides, and notably metabolise clinically used drugs with a narrow therapeutic index such as warfarin (Herman et al., 2005)(Figure 1.3) and phenytoin (Silvado et al., 2018). In addition, expression can be inhibited by some therapeutics, such as gemfibrozil (Tornio et al., 2017). CYP2C9 was not detected in a panel of peripheral blood immune cells (Effner et al., 2017), but a separate study using a pan CYP2C primer which should identify all isozymes found it was expressed in bronchoalveolar macrophages, but not peripheral blood lymphocytes (Hukkanen et al., 1997).



Warfarin 7-hydroxywarfarin

Figure 1.3. Warfarin metabolism by CYP2C enzymes.

CYP2D6 is a highly polymorphic enzyme, which is involved in the metabolism of around a quarter of clinically used drugs. In general, CYP2D6 catalyses hydroxylation, demethylation and dealkylation reactions, with endogenous roles including catalysing the production of dopamine in brain tissue (Heit et al., 2013). Furthermore, clinically relevant substrates include drugs for neurological conditions such as antidepressants, antipsychotics, and opioids (Bertilsson et al., 2002). CYP2D6 is also involved in metabolising the chemotherapeutic prodrug tamoxifen to its active metabolites such

as 4-hydroxytamoxifen (Figure 1.4) (Goetz et al., 2008). CYP2D6 was expressed in most immune cell subtypes when examined by gene array, with $CD14^+$ monocytes containing the lowest expression (Effner et al., 2017). CYP2D6 gene expression has also been identified in U937 monocytic cells following differentiation to a macrophage phenotype (Jin et al., 2011).

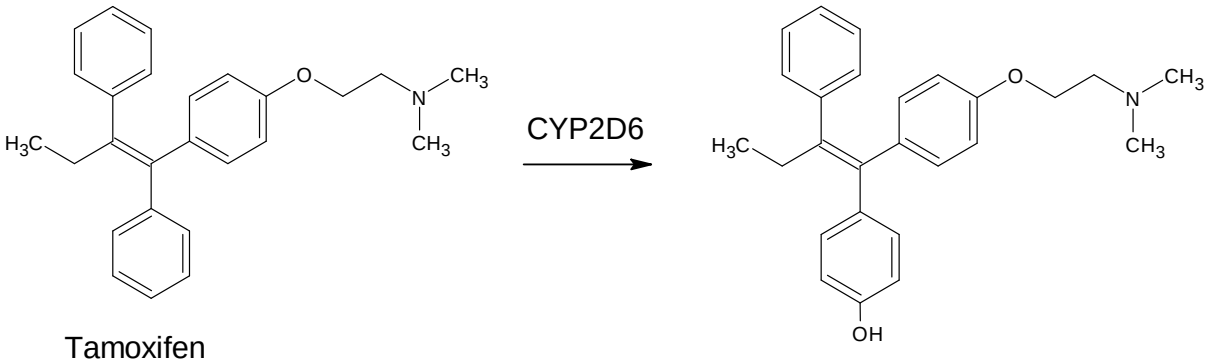


Figure 1.4. Tamoxifen metabolism by CYP2D6.

CYP3A enzymes are involved in around half of all CYP-mediated xenobiotic metabolism and are comprised of three main isozymes, CYP3A4, 5 and 7. Of these, CYP3A4 and 5 are more important for drug metabolism, with CYP3A4 predominantly expressed in the liver, and CYP3A5 mainly extrahepatic (Wright et al., 2019). These enzymes have a broad substrate specificity, and can utilise various mechanisms to metabolise xenobiotics, including hydroxylation, oxidation, dealkylation and dehydrogenation (Burk et al., 2004). By this mechanism, the CYP3A enzyme family can also bioactivate substrates including pro-drugs (Ortiz de Montellano, 2013) and pro-carcinogens (Yamazaki et al., 1995). Furthermore, expression of these enzymes is highly inducible by a range of clinical drugs, including glucocorticoids such as dexamethasone (Matsunaga et al., 2012), which can lead to drug-drug interactions and toxicity (Hakkola et al., 2020). CYP3A4 was not expressed in any human immune cells examined by gene array which included $CD14^+$ monocytes (Effner et al., 2017), although studies using alveolar macrophages have identified expression of CYP3A5 and inconsistent expression of CYP3A4 (Anttila et al., 1997), with further studies showing that the levels may be decreased in smokers compared to non-smokers (Piipari et al., 2000).

In addition to CYP enzymes, there are other enzyme types which contribute to xenobiotic metabolism, each with a specialised function and substrate specificity. The most clinically relevant enzymes families will be discussed here.

Alcohol dehydrogenases (ADH) catalyse the conversion between alcohols and aldehyde/ketones (Yin et al., 1999). There are five classes of ADH, but the naming conventions have changed over the years which has left some confusion in the literature. When discussed in this thesis, the following terms will be used: ADH1a-c (class I), ADH4 (class II), ADH5 (class III, sometimes termed ADH3), ADH6 (class V), and ADH7 (class IV). Clinically, ADH enzymes metabolise the pro-drug hydroxyzine (anti-histamine) to the active metabolite cetirizine (Di et al., 2021). In addition, polymorphisms and altered levels of expression of ADH enzymes have been linked to increased risk of alcoholism (Tolstrup et al., 2008). ADH enzymes are generally found throughout the body, and unlike other XME are not purely concentrated to the liver (Adinolfi et al., 1984). Murine Adh5 has been detected in various immune cell subtypes, including monocytes and immune progenitor cells (Dingler et al., 2020), but this has not been investigated in human cells, while human ADH6 is not expressed in bone marrow or peripheral leukocytes (Nishimura et al., 2006).

Aldehyde dehydrogenases (ALDH) catalyse the oxidation of exogenous and endogenous aldehydes to carboxylic acids, including the biotransformation of carbohydrates and lipids (Vasiliou et al., 2004), and metabolising the products of ADH metabolism (shown in Figure 1.5). ALDH enzymes also metabolise xenobiotic compounds such as ethanol and formaldehyde (Marchitti et al., 2008). It is a large family of enzymes, with nineteen isoenzymes identified to date (Table 1.1), although the majority of the metabolic activity is undertaken by ALDH1, which mainly functions in retinoic acid generation (Zhao et al., 1996; Marchitti et al., 2008), and ALDH2, which is known for oxidising acetaldehyde as part of ethanol metabolism (Shin et al., 2017), and activating nitroglycerin in blood (Lang et al., 2012). Increased ALDH1 has been linked to poor prognosis in breast cancer (Demir et al., 2018) suggesting a pro-tumorigenic function, and this enzyme has also been suggested as a marker for metastatic cancer (Rodriguez-Torres et al., 2016). ALDH1 enzymes are well known to be expressed by DCs, where they are involved in the production of retinoic acid from vitamin A (Yokota et al., 2009; Hall et al., 2011; Agace et al., 2012; Steimle et al., 2016), as well as haematopoietic cells (Chute et al., 2006). ALDH2 is also well known

to be expressed by immune cells including bone marrow-derived macrophages (BMDM) (Zhu et al., 2019) and peripheral blood leukocytes (Kimura et al., 2009). Finally, ALDH3B1 has been detected in in human monocyte-derived macrophages (MDM) (Ahmed et al., 2018), and Aldh3b1 in murine BMDM (Niu et al., 2016).

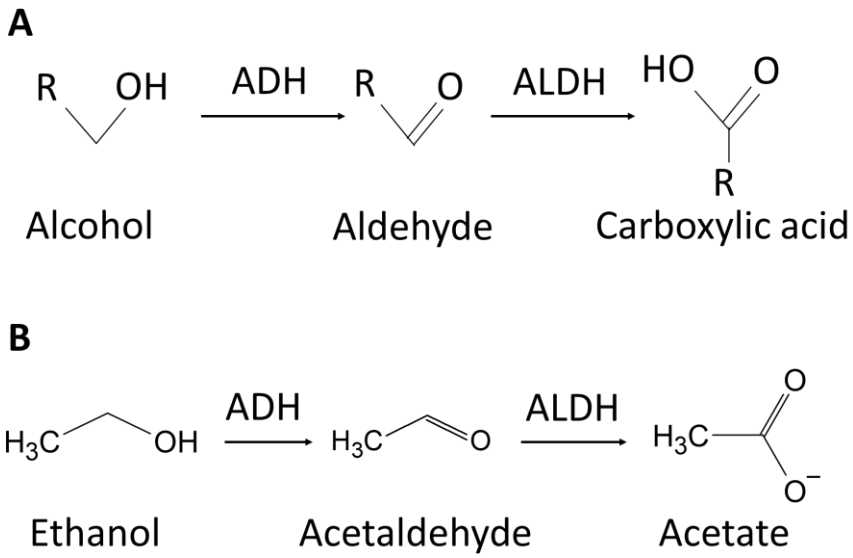


Figure 1.5. Summary of biotransformation by ADH and ALDH enzymes
A generalised reaction of ADH and ALDH enzyme activity (**A**), as well as a clinically relevant example of the metabolic pathway of dietary ethanol (**B**)

Flavin-containing monooxygenases (FMO) are involved in the oxidation of xenobiotics, in particular those containing amine (Figure 1.6A) and sulphide (Figure 1.6B) groups, using a flavin group as a cofactor (Eswaramoorthy et al., 2006), summarised in Figure 1.6. The FMO family of enzymes comprises five main subtypes (termed FMO1-5), which vary in tissue expression and substrate specificity (Cashman et al., 2006; Jones et al., 2017). Some clinically relevant substrates include benzylamine, ranitidine (Figure 1.6C), and chlorpheniramine (Krueger et al., 2005), and these enzymes also bioactivate the anti-inflammatory prodrug nabumetone to its pharmacologically active metabolite (Fiorentini et al., 2017). The expression of FMO enzymes is typically concentrated to the liver, lungs, and kidneys, but can also be found in the brain and small intestine (Yeung et al., 2000; Koukouritaki et al., 2002; Zhang et al., 2006). In addition, expression of FMO1 is upregulated in macrophages in response to haemoglobin (Schaer et al., 2006), but FMO1 and 2 are not expressed

by DC (Ogese et al., 2015). FMO4 and 5 gene expression has been detected in MoDC (Ogese et al., 2015) and peripheral blood mononuclear cells (Gagliardi et al., 2013).

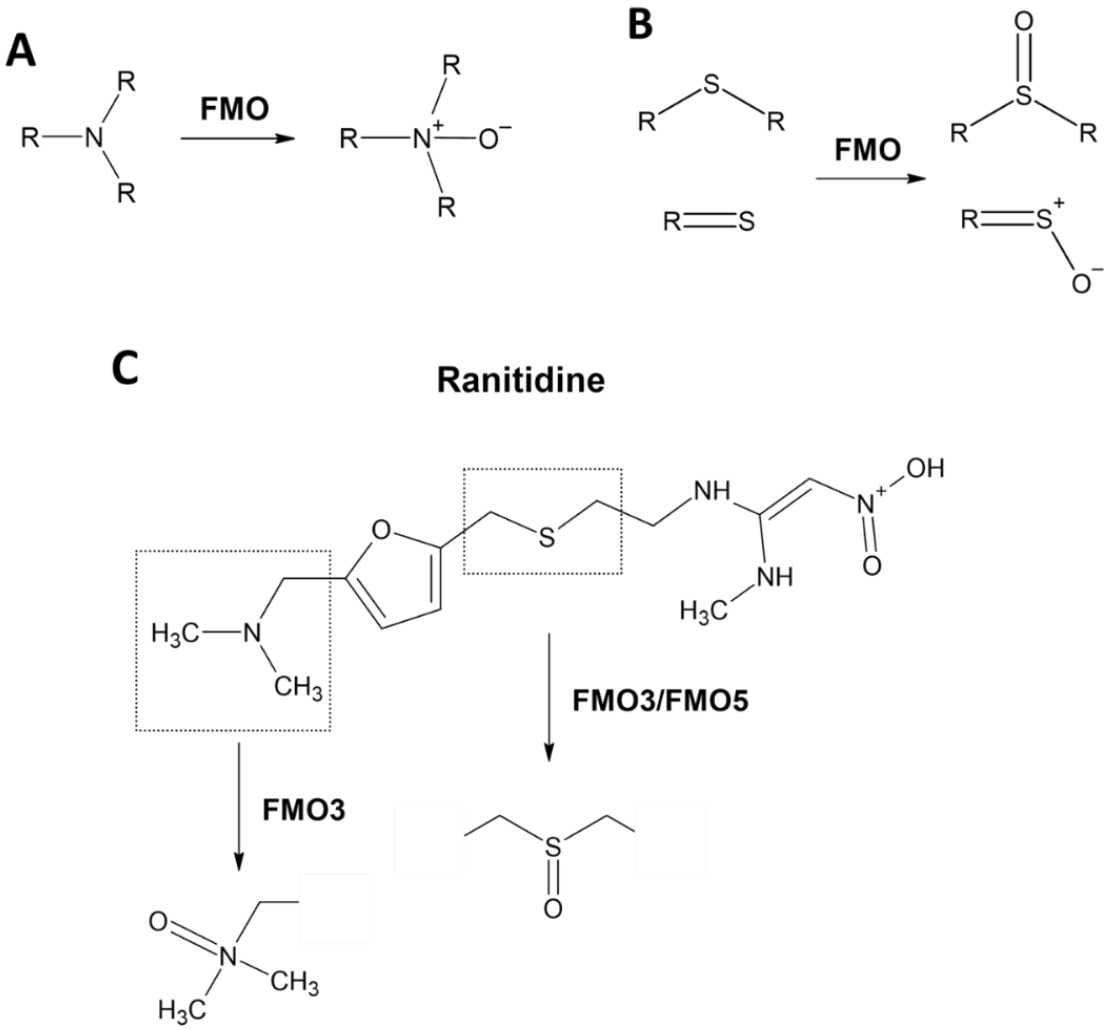


Figure 1.6. Summary of reactions catalysed by FMO enzymes
General mechanism of FMO enzyme activities (where R can be any group, including N, S, and H) of N-oxidation (**A**) and S-oxidation (**B**). A clinically relevant example is shown for metabolic pathways of ranitidine (**C**), adapted from Chung et al., 2000.

1.2.2 Phase II metabolic enzymes

Phase II enzymes generally catalyse reactions which transfer a chemical moiety onto the xenobiotic molecule, with the most common enzymes being glutathione S-transferases (GSTs), UDP-glucuronosyltransferases (UGTs), N-acetyltransferases (NATs) and sulfotransferases (SULTs) (Jancova et al., 2010). Each enzyme listed here functions to increase hydrophilicity of the substrate, which enables more rapid elimination from the body (Jancova et al., 2010). These main enzymes are discussed in further detail below.

GSTs are best known for catalysing the conjugation of glutathione (GSH) onto xenobiotic substrates (Sheehan et al., 2001), including allergen 2,4-Dinitrochlorobenzene (Harris et al., 2002)(Figure 1.7A), and also endogenously detoxify peroxidised lipids (Singhal et al., 2015). GSTs are often overexpressed in cancer tissue and have been linked to cancer development and chemotherapeutic resistance (Townsend et al., 2003). Most GST enzymes have been detected in a range of immune cell types (Effner et al., 2017). In alveolar macrophages, genes corresponding to most GST isoenzymes have been detected, where specific polymorphisms were linked to increased risk of smoking-associated lung disease (Butler et al., 2011). Furthermore, GSTP1 has been linked to dampening LPS-induced inflammation in murine RAW264.7 macrophages (Xue et al., 2005), and to chemotherapeutic resistance in a breast cancer cell line (Dong et al., 2020). In contrast, GSTO1 has been shown to be required for the pro-inflammatory response to LPS in murine J774.1A macrophages (Menon et al., 2015), where it is suggested to be involved in glycolysis (Hughes et al., 2017). These data suggest a complex role for GST enzymes in macrophages, where isozymes appear to work in opposition to regulate the inflammatory response.

UGTs catalyse the transfer of glucuronic acid onto small hydrophobic molecules, termed a glucuronidation reaction (Rowland et al., 2013). UGT enzymes notably play a key role in the blood-brain barrier to detoxify potentially harmful substances (Ouzzine et al., 2014), metabolise morphine to morphine-6-glucuronide which is responsible for most of the analgesic effect (Klimas et al., 2014), and also metabolise paracetamol (McGill et al., 2013)(Figure 1.7B). In addition, UGT dysregulation has been associated with the progression of several cancers, likely due to inactivation of endogenous

metabolites like steroids, that can affect the bioavailability of chemotherapeutic agents (Allain et al., 2020). Few studies have investigated the expression of UGT enzymes in immune cells, although isozyme specific expression has been found in rat peritoneal macrophages (Tochigi et al., 2005), and UGT1A9 expression is inducible in human MDM and THP-1 cells in response to Wy14643 and rosiglitazone, both agonists of peroxisome proliferator-activated receptors (PPARs) (Barbier et al., 2003).

NATs catalyse the transfer of acetyl groups from acetyl-CoA to aryl-amines, -hydroxylamines, and -hydrazines, in particular aromatic amines (Dupret et al., 1994), such as 4-aminobenzoic acid (Sim et al., 2008)(Figure 1.7C). There are two subtypes of NAT in humans termed NAT1 and NAT2 which have similar substrate specificities, and both are widely expressed in extrahepatic tissue (Windmill, 2000). NAT expression is increased in RAW264.7 macrophages following LPS stimulation, suggesting an involvement in inflammatory response (Muxel et al., 2012), and both NAT1 and NAT2 expression has been detected in human MoDC (Lichter et al., 2008).

Finally, SULTs catalyse the transfer of a sulfo group onto an alcohol or amine, which can affect many endogenous molecules such as lipids and steroids (Negishi et al., 2001). There are at least ten isozymes, and polymorphisms in these enzymes can impact susceptibility to some cancers, such as colorectal and breast cancer (Coughtrie, 2002). SULT1A1 has been found in MDM, but not $CD4^+$ T cells (Swann et al., 2016).

In conclusion, limited studies have been carried out to identify expression of phase 1 XME in immune cells, often specific to a single isozyme within an enzyme family, while phase 2 XME have been more broadly investigated. Of the studies that have been undertaken, many have established increased XME expression in inflammatory conditions, highlighting a potential role for inflammatory (M1) macrophages, although more research is required to confirm any functional implications of XME expression in local drug metabolism.

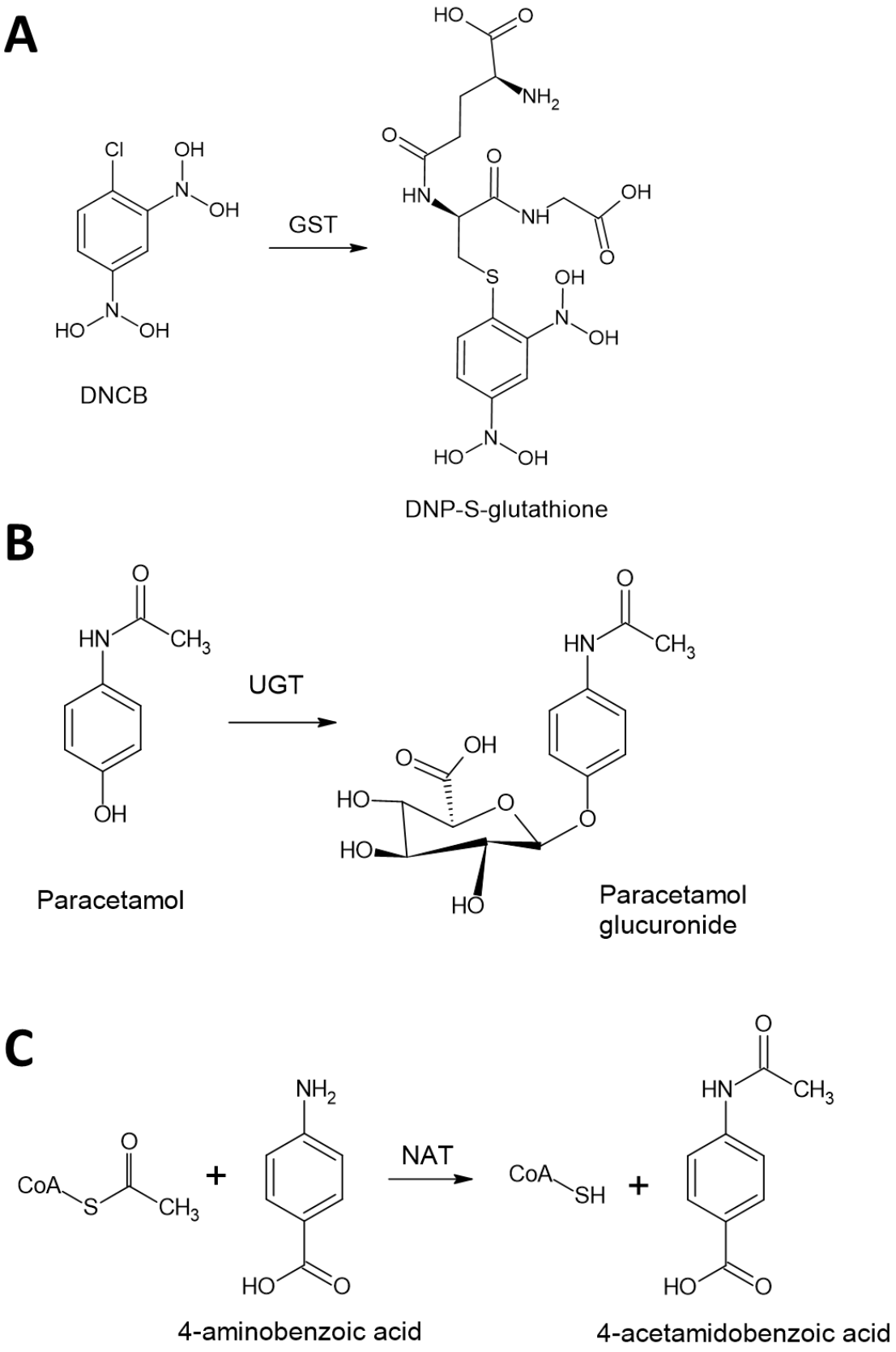


Figure 1.7. Examples of biotransformation by phase 2 metabolic enzymes
Example reactions for GST, which transforms allergen 2,4-Dinitrochlorobenzene (DNCB) into DNP-S-glutathione (**A**), UGT, which metabolises paracetamol to paracetamol glucuronide (**B**), and NAT, which acetylates 4-aminobenzoic acid (**C**).

1.3 Oral mucosa structure and function

The oral mucosa describes tissue barriers found within the oral cavity. Due to the variety of functions required within the mouth, there are functionally distinct regions of mucosa which have unique structures and morphological appearances. The structure of the oral mucosa will be discussed in more detail in section 1.3.1, but in general the apical side comprises an epithelium populated by resident keratinocytes and sits above the basement membrane composed of type IV collagen and laminin (Wilson et al., 1999). Underneath the basement membrane is the lamina propria, which contains extracellular matrix produced by resident fibroblasts, and hosts a diverse range of resident cells including myeloid cells such as macrophages and dendritic cells (Cutler et al., 2006; Merry et al., 2012; Hovav, 2014; Wu et al., 2014), and progenitor stem cells (Marynka-Kalmani et al., 2010; Davies et al., 2010).

1.3.1 Structure

The oral epithelium is the apical layer of all oral mucosal barriers, primarily comprising keratinocytes, water, and lipids, which act together as a barrier to prevent water loss and reduce access for xenobiotic compounds and pathogens. Oral keratinocytes found in the oral cavity typically display improved wound healing, proliferation, and migration compared to skin keratinocytes (Turabelidze et al., 2014).

The epithelium is further subcategorised into layers termed stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Figure 1.8). A single layer of oral keratinocytes is bound to the basement membrane by hemidesmosome contacts to form the most basal cell layer of the oral mucosa termed stratum basale. The subsequent oral mucosal epithelial layers are formed as the keratinocytes migrate away from the basement membrane and differentiate. The keratinocytes first form the stratum spinosum which is composed of polyhedral keratinocytes joined together by desmosomes and is where resident Langerhans cells are commonly found. Next, the stratum granulosum where cells accumulate dense basophilic lipid-containing granules which aid to prevent fluid loss and permeability through the epithelium. Finally, the stratum corneum is the outermost layer, often comprising multiple layers of flattened cells with no nuclei, which function to form a physical barrier. Within the

oral cavity, mucosa have specialised roles which affect epithelial structure. Of note, some are non-keratinised and lack a stratum corneum (such as the buccal mucosa), while others are highly keratinised (such as the hard palate) (Adams, 1976; Groeger et al., 2019).

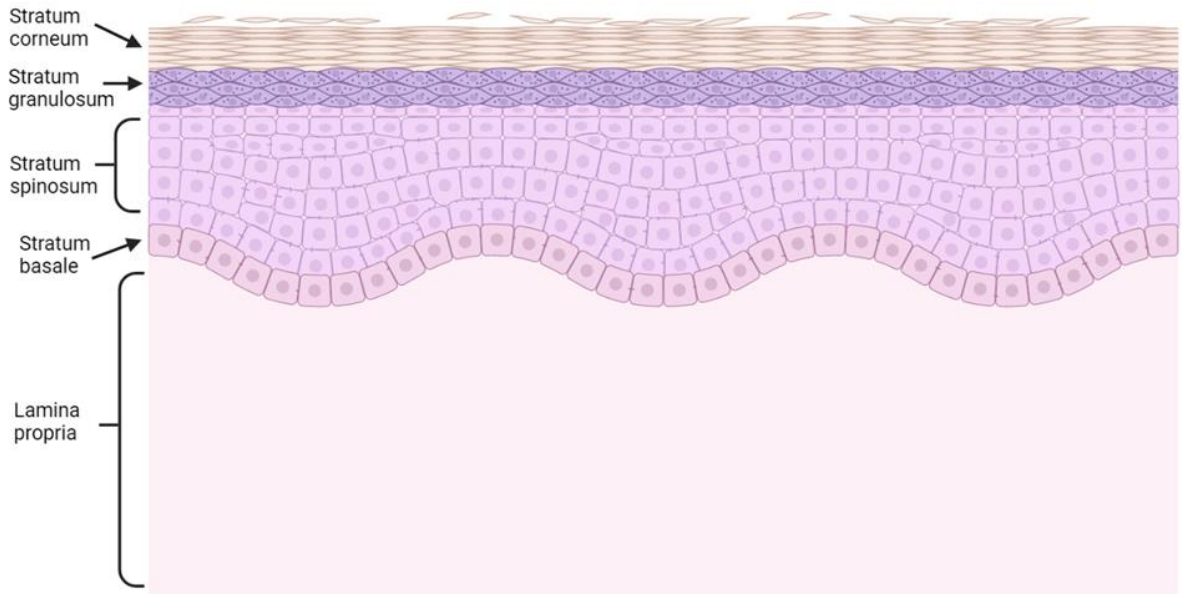


Figure 1.8. Generalised structure of the oral mucosa
Overview of the structure of the oral mucosa. The epithelium is composed of keratinocytes which differentiate to form the layers indicated, beneath which is the lamina propria containing fibroblasts and other resident immune cells. Created with BioRender.com.

In addition to keratinocytes, the basal cell layer of the epithelium also contains merkel cells which act as mechanoreceptors to sense pressure and vary in concentration between different mucosal tissues and disease states (Righi et al., 2006). There are also resident melanocytes, known to produce melanin for UV protection in skin, which can also neutralise harmful reactive oxygen species (ROS) and may have roles in mediating antimicrobial and immune responses, although their specific function in the oral mucosa is not well understood (Feller et al., 2014). Finally, epithelial-resident Langerhans cells are found in the basal and spinosum layers of the oral epithelium (Waterhouse et al., 1967). These cells represent the first immune challenge to pathogens, where they can act as antigen presenting cells, with a greater efficacy at activating T cells compared to skin LC (Hasséus et al., 2004). In addition, oral Langerhans cells can respond to allergens such as nickel (Kosten et al., 2016)

and have been linked to oral diseases such as oral lichen planus (OLP) and oral squamous cell carcinoma (Upadhyay et al., 2013). Finally, intraepithelial T cells can be found in the oral epithelium (Burkhardt, 1992; Wu et al., 2014), where they have been shown to act against opportunistic infections in an innate manner (Conti et al., 2014).

The connective tissue beneath the epithelium is mainly composed of type 1 collagen and elastin. The main roles are conserved across different tissue types: to support the epithelium, protect from mechanical damage and connect it to underlying structures such as a blood supply. As with the epithelium, the structure of the lamina propria varies within different tissue subtypes in the oral cavity with the masticatory mucosa tightly attached to underlying tissue while buccal mucosa is more elastic to allow for mobility (Squier et al., 2001). The main cell types found in this region are fibroblasts, macrophages, adipocytes, and endothelial cells. The most common cell type is the fibroblast which produce and regulate deposition of extracellular matrix proteins that mainly comprises the laminar propria. Fibroblasts also have a major role in wound repair (Bainbridge, 2013) and can initiate inflammation in response to pathogen invasion (Bautista-Hernández et al., 2017), although this is primarily the role of resident innate immune cells such as macrophages and DC. Furthermore, in comparison to dermal fibroblasts, oral resident fibroblasts express more glycoproteins and reduced HOX genes, indicating a tendency towards regeneration and away from senescence (Miyoshi et al., 2015). However, fibroblasts sampled from tumour sites find these cells adopt a cancer-associated fibroblast (CAF) phenotype, and can promote tumour growth by modulating immune responses and producing a favourable microenvironment (Tao et al., 2017).

1.3.2 Immunity in the oral mucosa

The oral mucosa is subjected to many external threats, from pathogens to environmental molecules. However, it also faces benign challenges, such as commensal pathogens/biofilms and food. Thus, it is imperative that the immune cells in the oral mucosa be specific and finely tuned to recognise and effectively respond to only harmful substances or increased levels of potentially pathogenic micro-organisms.

Tissue resident macrophages are constitutively present in the oral mucosa, with normal oral biopsies containing around 17 $CD68^+$ macrophages per mm^2 (Chiang et al., 2002). Resident oral macrophages are highly heterogeneous, which gives rise to a range of immune responses (Barbeau et al., 1989), including a subpopulation shown to contribute to the immune tolerance observed within the oral mucosa (Mascarell et al., 2011). In addition to tissue resident cells, macrophages are also highly recruitable to the oral cavity following infection and local inflammatory cytokine production. For example, *fusobacterium nucleatum* infection induced significant local recruitment of macrophages to dental pulp and gingival tissue *in vivo* (Johnson et al., 2018). Furthermore, oral bacteria are able to affect macrophage function, with *P. gingivalis* shown to influence local inflammation by inducing A20 (TNFAIP3) – a negative regulator of inflammation – in macrophages (Li et al., 2019). Macrophage-induced inflammation in the oral cavity can also be altered through crosstalk with other resident cells. For example, fibroblasts isolated from the oral periodontal ligament, but not gingiva, have been shown to dampen *P. gingivalis*-induced inflammatory cytokine secretion from THP-1 cells (Tzach-Nahman et al., 2017).

While constitutive presence of resident macrophages is essential for defence and tissue homeostasis, the presence of these cells have also been strongly linked to oral diseases. Patient biopsies of oral lesions, oral submucous fibrosis (OSMF), and oral squamous cell carcinoma (OSCC) have shown increased macrophage presence in these disease states, with elevated numbers associated with disease progression (Chiang et al., 2002; Pereira et al., 2015; Aghbali et al., 2018), and poor patient outcomes in oral cancer (Bingle et al., 2002). For example, there is a significantly higher density of macrophages in OSCC compared to normal tissue (Mostafazadeh et al., 2020). Similarly, in head and neck squamous cell carcinoma (HNSCC), there is a strong association between increased macrophage density and tumour progression (Marcus et al., 2004). Moreover, in OSCC, macrophages adopt a tumour-associated phenotype which has altered functionality to promote tumour growth and progression (Petruzzi et al., 2017).

Dendritic cells (DC), as well as the epithelial-specific subtype Langerhans cells (LC), are both constitutively present in the oral cavity, with LC found in the mucosal epithelium, and DC most often in the sub-epithelium (Jotwani et al., 2003). These cells typically act as the first immune defence against non-self or pathogenic material

(Romani et al., 2003) and can be recruited to the oral cavity from circulating DC precursors, such as monocytes, via the CCR6/CCL20 axis (Le Borgne et al., 2006). Within the oral cavity there are distinct DC phenotypes, notably Langerhans cells (LC), myeloid DC and plasmacytoid DCs, found at different densities in various oral tissues (Hovav, 2014). These cell types can implement distinct T cell priming mechanisms depending on cellular origin (Nudel et al., 2011). In comparison to DC from other tissue, oral resident cells have an improved ability to activate T cells (Reinartz et al., 2016). For example, gingival DC produced significantly higher basal concentrations of pro-inflammatory cytokines and cell surface markers than equivalent DC isolated from skin samples (Kosten et al., 2017) and LC isolated from oral tissue are able to active allogenic T cells more effectively compared to dermal LC (Allam et al., 2003; Hasséus et al., 2004).

DC have been linked to various immune-mediated diseases of the oral mucosa. For example, there is a significantly increased number of LC and DC in epithelium and stroma of biopsies from patients with oral lichen planus (Santoro et al., 2005) and chronic periodontitis (Jotwani et al., 2003; Anjana et al., 2012; Wilensky et al., 2014), compared to healthy controls. Similarly, patients with allergic rhinitis and/or asthma, both diseases linked to immune dysregulation (Kiboneka et al., 2019) have significantly higher DC and LC presence in the oral mucosa compared to healthy controls (Reinartz et al., 2016). In contrast, increased CD1a+ DC infiltration into tongue carcinoma tissue was associated with improved overall survival, while later stage carcinomas had reduced DC presence (Goldman et al., 1998). These examples highlight the complexity of DC function within the oral mucosa, with these cells typically functioning to protect native tissue during carcinoma, while increased infiltration of DC and LC into oral tissue is also linked to diseases with an immune-dysregulating component.

Innate lymphoid immune cells, including NK cells, are constitutively present in some oral tissues such as the gingiva, and can be recruited to the oral cavity during infection (Panda et al., 2019). In addition, NK cell count is lower in OSCC tissue compared to healthy tissue (Dutta et al., 2015) with increased cell numbers associated with an improved prognosis (Bisheshar et al., 2020). Furthermore, NK cells are often associated with periodontitis that occurs in gingival tissue, where they can act in both a pro- and anti-inflammatory manner, although there is much conflicting data as a

result of different model systems and patient-derived cells and tissue (Seidel et al., 2020).

Finally, adaptive immune cells such as T cells and B cells can be attracted to a specific mucosal site within the oral cavity as a result of cytokine signalling which can be exacerbated or modulated in diseases such as periodontitis and oral cancers (Sahingur et al., 2015). T cells populate the oral mucosa at steady state with the proportion of $CD8^+$ cytotoxic T cells significantly higher than $CD4^+$ helper T cells, in contrast to peripheral blood which has the opposite trend (George et al., 2015). There are also populations of γδ T cells which are constitutively resident in the oral mucosa, termed intra-epithelial lymphocytes (IEL), which can be considered part of the innate immune system (Wu et al., 2014), and can contribute to pathogenic inflammatory responses (Simpson et al., 1997). Furthermore, chronic infiltration of T cells is linked to immune diseases such as oral lichen planus, where these cells induce apoptosis in basal epithelial cells, leading to chronic inflammation and extensive tissue damage (Scully et al., 2008).

1.3.3 The oral mucosa as a site of drug delivery

There are many factors which make the oral mucosa an attractive site for local and systemic drug delivery. The site is easily accessible and this, along with the high vascularisation, allows for rapid systemic delivery of drugs administered to the oral mucosa (Hearnden et al., 2012). In addition, hepatic first pass metabolism can cause significant issues for drug bioavailability if a therapeutic is metabolised to an inactive form before substantial clinical effect is observed (Pond et al., 1984). By delivering therapeutic agents via the oral mucosa, the first pass effect can be bypassed to increase drug bioavailability (Zhang et al., 2002).

Some clinically used drugs are delivered through the oral mucosa, such as nitroglycerin given sublingually to treat angina pectoris (Zhang et al., 2002), and midazolam for treatment of seizures (Ülgey et al., 2012). Both of these examples are acute conditions which require treatment with drugs that are rapidly absorbed and efficacious, and in addition, the close proximity of the oral mucosa to the heart and CNS respectively allows the drugs to reach the site of action more quickly than other delivery sites.

The buccal mucosa has also been investigated as a site of drug delivery for systemic circulation using novel biomaterials. Specific drugs such as carvedilol (Rana et al., 2013), didanosine (Jones et al., 2014), selegiline (Al-Dhubiab et al., 2016), and prednisolone (Kumria et al., 2016) have been investigated for systemic drug delivery. In addition, this site has been suggested for delivery of drugs to treat oral diseases locally to avoid potential side effects from systemic delivery. For example, valdecoxib, a COX-2 inhibitor, was incorporated in a buccal film to treat oral sub mucous fibrosis, where most of the drug was released locally and only a small proportion was found to reach the systemic circulation (Averineni et al., 2009). Furthermore, lidocaine HCl, used to treat local oral pain, has been incorporated into an oral patch where it was detected within oral tissue, spatial localisation identified, and functionality confirmed, which suggest these patches could be useful to treat oral pain locally (Clitherow et al., 2019). However, the oral epithelium is often impermeable to hydrophilic compounds (Kulkarni et al., 2009) which can prevent absorption into the tissue. To enhance drug delivery through the buccal mucosa, permeation enhancers can be included in the drug formulation, which act to increase drug absorption through the mucosa. Enhancers generally function as detergents, affecting the hydrophobic plasma membrane which allows passage of molecules. Examples include sodium taurocholate (Averineni et al., 2009), oleic acid, and linoleic acid (Prasanth et al., 2014), and dodecyl-2-(N,N-dimethylamino) propionate hydrochloride (L. Hu et al., 2011), and comparisons between permeation enhancers show drug-specific efficacy (Wei et al., 2012).

Current use of the oral mucosa as a site of drug delivery, as well as recent studies to optimise novel delivery methods suggests it will remain an excellent site of drug delivery in the future, both to deliver local and systemic acting drugs. However, while many recent studies have focussed on the delivery of functional compounds through the oral epithelium, very few have considered the possibility of drug metabolism occurring locally in this tissue that could limit bioavailability. The available data on xenobiotic metabolism in the oral mucosa is discussed in the following section.

1.3.4 Phase 1 XME expression in the oral cavity

Extrahepatic metabolism can occur throughout the body and provides a mechanism for direct metabolism without requiring transportation to the liver. Skin is often examined as a site for such metabolism due the barrier function, and as a site of drug delivery through pastes, creams, and patches, as well as cosmetics. Several studies have investigated the presence of XME in the oral mucosa. As discussed above this site is of increasing importance as a novel mode of drug delivery (Colley et al., 2018), and has also been used to deliver drugs locally such as nicotine (Wadgave et al., 2016) and anaesthetics (Giovannitti et al., 2013). Historically these studies have additionally emphasised the importance of enzymes for transforming procarcinogens such as benzo[a]pyrene found in the environment and tobacco (Chi et al., 2009; Sacks et al., 2011). Therefore, fully characterising the expression of XME in the oral mucosa is vital to elucidate its role in extrahepatic metabolism and procarcinogen activation.

Gene expression of ADH5 (class III), responsible for metabolising alcohols, but no other ADH isozymes have been detected in normal and immortalised oral buccal epithelial cells, as well as buccal biopsies where expression was concentrated to the basal epithelial layers (Hedberg et al., 2000; Staab et al., 2008). ADH5 is therefore likely to be the isozyme responsible for local alcohol metabolism, but interestingly, is also involved in the metabolism of formaldehyde, found in some cigarettes (Baker, 2006), to the less reactive formate (Reingruber et al., 2018).

Gene expression of multiple ALDH isozymes (1A3, 2, 2A2, 4A1, 7A1 and 9A1) have been detected in different oral-derived cell lines (Hedberg et al., 2001; Dressler et al., 2002; Staab et al., 2008). In addition, ALDH1A1 and 3A1, although not found in untreated cells, were upregulated in normal human epidermal keratinocytes (NHEK), 101A, and premalignant oral leukoplakia cell lines (Leuk1 and Leuk2) following treatment with cigarette smoke extract (Nagaraj et al., 2006), and ALDH3A1 was found to be increased in oral biopsies from smokers compared to non-smokers (Boyle et al., 2010; Cao et al., 2015; Richter et al., 2019), suggesting inducibility and a role within the oral cavity.

COX-1 and COX-2 enzymes, involved in regulating inflammation, have also been investigated in the oral cavity. COX-1 was expressed in normal, dysplastic and OSCC oral biopsies, with higher expression in cancer tissue (Mauro et al., 2011). COX-2

expression was low in normal tissue but inducible by areca nut extract and smoking (Chang et al., 2014; Richter et al., 2019), and stage-dependent increases in oral dysplasia and OSCC have been observed (Mauro et al., 2011; Seyedmajidi et al., 2014; Thomas et al., 2019). Bioadhesive black raspberry gel applied orally was investigated as a chemopreventive by inhibition of COX-2, where it was found to be metabolised locally to produce active anthocyanidins, prolonging chemopreventive activity, and demonstrating enzyme functionality (Mallery et al., 2008, 2011; Mallery, Tong, Shumway, et al., 2014). These data highlight a role for COX enzymes in oral cancer development and progression and use of enzyme functionality to deliver locally activated chemotherapeutics.

CYP1A1 and 1B1 are key enzymes in metabolism of tobacco products and can activate environmental procarcinogens so expression in the oral cavity is of great interest. CYP1A1 was detected in primary and immortalised keratinocytes in early studies (Farin et al., 1995; Vondracek et al., 2001), but later investigations did not corroborate this (Dressler et al., 2002; Vondracek et al., 2002) which may be a result of interpatient variation. Similarly, CYP1B1 was detected in simian virus large T antigen immortalised (SVpgC2a) oral epithelial cells and some primary oral keratinocytes (Vondracek et al., 2002) but not others (Dressler et al., 2002). However, there is agreement in the literature that both of these enzymes are highly inducible by tobacco and smoking, with increases observed following tobacco extract treatment in cell lines (Nagaraj et al., 2006; Gümüş et al., 2008; Boyle et al., 2010; Sacks et al., 2011; Woo et al., 2017), 3D models (Schlage et al., 2014; Zanetti et al., 2016), and oral biopsies from smokers compared to non-smokers (Gümüş et al., 2008; Chi et al., 2009; Boyle et al., 2010; Cao et al., 2015). Betel quid, a known carcinogen, has also been shown to upregulate CYP1B1 expression (Chang et al., 2014).

Other CYP enzymes have also been investigated, with CYP2E1 found in all cell types investigated (Farin et al., 1995; Vondracek et al., 2001; Reichl et al., 2010), CYP2A6 and 2B6 not found in any cell type (Farin et al., 1995; Vondracek et al., 2001, 2002; Sarikaya et al., 2007), CYP2C and 2D6 only found in non-cancer cells, and CYP3A4 only found in primary cells (Farin et al., 1995; Vondracek et al., 2001; Sarikaya et al., 2007). In addition, CYP24A1 was induced following treatment with cigarette smoke concentrate (Woo et al., 2017). When examined in oral biopsies, CYP2E1 was found in normal and cancer tissue (Sarikaya et al., 2007; Mallery, Tong,

Michaels, et al., 2014), CYP2A6 was found in the basal epithelium of normal tissue (Mallery, Tong, Michaels, et al., 2014), and CYP2D6 expression was variable between patients (Vondracek et al., 2001; Sarikaya et al., 2007). Reports of CYP3A4/5 expression in oral biopsies are conflicting, with some showing expression of the enzyme in normal, dysplastic and OSCC tissue (Li et al., 2011; Mallery, Tong, Michaels, et al., 2014), and others only detecting CYP3A5 in normal biopsies (Martinez et al., 2007; Sarikaya et al., 2007). CYP3A4/5 expression may also be linked to disease, with a reduction seen in oral submucous fibrosis and HPV-negative OSCC biopsies compared to normal tissue (Martinez et al., 2007; Li et al., 2011). Detection of CYP3A4/5 is arguably the most important finding, as this subgroup of CYP450 enzymes is involved in metabolism of a large proportion of clinically available therapeutics, although it is important to note that experiments to quantify enzyme activity were unsuccessful, so functionality has not been shown in within the oral cavity.

In conclusion, many xenobiotic metabolising enzymes have been investigated in the oral cavity, in normal, tobacco-treated, and disease tissues. While functional studies have typically not revealed detectable levels of activity, the presence of these enzymes suggested a functional role within the oral cavity, which should be investigated further as a source of prodrug activation, carcinogen detoxification, and to ensure drugs delivered for systemic use are able to enter the bloodstream in their active form.

1.4 Tissue engineered models

In vivo oral mucosal models utilise whole organisms and are currently the gold standard for pharmaceutical research. The most commonly used model for oral research is the dog and pig given that their oral mucosal structure is most comparable to humans (Štembírek et al., 2012). Wider fields of research can also encompass the use of other animals such as zebrafish, small mammals (such as mice, rats, and rabbits) and higher primates (Hatton et al., 2015). Rodent models have been used extensively, but are not ideal due to inter-species variation, lack of non-keratinised mucosa, and especially with regard to the structure and function of the immune system (Haley, 2003). Simplified monolayer cells are often used as a cost-effective and efficient means to test hypotheses with primary cells or cell lines. It can also be beneficial to test theories in monolayer before utilising a more complex model. However, cells cultured in this environment lack 3D cell-cell and cell-matrix interactions which can cause changes in morphology and both gene and protein expression (Edmondson et al., 2014). Monolayer culture also tends to yield cells which are more susceptible to drug treatments compared to 3D equivalents, with 3D responses offering a better predictor of *in vivo* results (Hongisto et al., 2013). In cancer models this is especially true, given the essential role of the tumour microenvironment in chemotherapeutic resistance, cells grown in monolayer are unable to fully recapitulate *in vivo* resistance compared to 3D spheroids (Karlsson et al., 2012). Therefore, while monolayer studies use human cells but lack complexity, and *in vivo* studies are complex but use non-human species, tissue engineering provides a vital middle ground which adds increasing complexity (allowing for studying cell-cell interactions), while still using human-derived cells and tissue (Bédard et al., 2020).

The field of tissue engineering is varied, and comprises many model types which are broadly defined as a model tissue generated with multiple cell types in a 3D structure (Langer et al., 1993). One subtype is termed organoids, which are simplified models that can be used to mimic many organ tissues including cardiac (Drakhlis et al., 2021) and cerebral (Lancaster et al., 2013). Organoids are typically produced by fewer distinct cell types, often pluripotent stem cells, which self-organise to form an organ-like structure (Lancaster et al., 2014). Other emerging tissue engineering technologies include organ-on-a-chip (OOAC), which uses microfluidics and

connected chambers to enable distribution of nutrients throughout the model and mimic both organ systems and the interaction between different tissue types (Wu et al., 2020). OOAC is especially valuable for examining cell patterning such as hepatic zonation (Deng et al., 2019) and the alveolar-capillary interface in the lung (Huh et al., 2010). The subtype of model generated in this thesis is an epithelial model of the buccal mucosa, which is comparable to other epithelial models such as skin and produced by seeding a scaffold with tissue-specific fibroblasts and topped with keratinocytes grown at an air-to-liquid interface.

One of the key choices in epithelial tissue engineering is the scaffold in which cells are deposited and cultured as this can affect cellular activity. For example, oral fibroblasts cultured in different scaffolds had material-dependent viability and protein deposition (Mangera et al., 2013). A common choice of scaffold is type 1 collagen, as collagen is the most abundant protein in the extracellular matrix (ECM) in human tissue (Dong et al., 2016). Type I collagen can be isolated from almost every living animal, although typically is taken from sources such as bovine dermis, porcine dermis, and rat tail, due the availability of these tissues which allows production on a large scale (Parenteau-Bareil et al., 2010). However, collagen properties can vary between species (Lin et al., 2006) and some instances of immunogenicity have been reported (Lynn et al., 2004). Another often used scaffold is the human-derived de-epidermised dermis (DED), which can be manipulated to form altered phenotypes depending on the cells used for seeding (Lee et al., 2000; Colley et al., 2011). However, this scaffold is typically obtained from cadavers which requires additional ethical considerations and limits production scale and can also present a challenge for incorporating additional cells. Furthermore, a number of novel techniques have been used to produce native or manufactured scaffold alternatives, such as using fibroblast-derived extracellular matrices (Scherzer et al., 2015), electrospun (Edmans et al., 2020), 3D printed (Wang et al., 2021), and commercial scaffolds such as Alevtex (Knight et al., 2011). In addition, novel biomaterials are consistently in development for use in tissue engineered models. One example is gelatin methacryloyl (GelMA) (Kaemmerer et al., 2014) which has been shown to modulate the immune response when compared to tissue culture plastic (Donaldson et al., 2018), other biomaterial hydrogels (Cha et al., 2017) and micropatterned gels with the same chemical composition (Singh et al.,

2017). It is therefore essential to have a variety of scaffold options to enable selection of an appropriate scaffold for the model requirements.

1.4.1 Tissue engineered models of the oral mucosa

In the past 15 years, there has been a considerable increase in publications utilising 3D *in vitro* oral models (Moharamzadeh et al., 2012). Studies have investigated wound repair in the gingiva (Buskermolen et al., 2016), immune-mediated gingival inflammation (Bao et al., 2015), and inflammation in normal buccal mucosal models (Jennings et al., 2016) using primary oral keratinocytes or oral keratinocytes immortalised by over-expression of human telomerase reverse transcriptase (hTERT) or expression of oncogene HPV16-E6/E7 to utilise the non-disease cellular origin and inherent reproducibility. Efforts to complexify these models include incorporating immune cells (see section 1.4.3), generating a gingival-bone model by adhering a 3D oral mucosal model to engineered bone (Almela et al., 2016), and co-culture of fibroblasts, endothelial cells, and keratinocytes to generate a vascularised model (Heller et al., 2016). Each model type has the potential to answer more specific research questions and improves the capabilities of tissue-engineering technology to fully recapitulate *in situ* cellular behaviour.

While many of these studies have generated models aimed at replicating normal tissue, 3D oral models have also been utilised to investigate disease states and cell interactions within a relatively controllable environment. Of note, the progression of oral cancer has been modelled using cancer cell lines, and matched cells isolated from cancer patients to replicate normal, dysplastic, and cancerous tissue (Gaballah et al., 2008; Colley et al., 2011; Sawant et al., 2016). More recently, complex models have been utilised to investigate chemical inhibition of tumour migration (Väyrynen et al., 2019), and the role of extracellular vesicles (EVs) from cancer-associated fibroblasts in migration and invasion (Dourado et al., 2019). Similarly, models of oral mucositis, a side effect of radiation or chemotherapy, have been used to identify cell-specific roles (Colley et al., 2013), and methods of disease prevention (Lambros et al., 2015; Walladbegi et al., 2018). These studies were able to investigate cell function and motility in 3D space, and the impact of cellular crosstalk, by utilising 3D model systems, highlighting their importance for accurately modelling the oral cavity.

In addition to modelling normal and diseases tissues for study, tissue engineered oral models have the potential to be used as a tool to develop better *in vitro* diagnostic methods (Moharamzadeh et al., 2012), as well as providing a better testing platform for pre-clinical drug development. For example, commercially available SkinEthic™ Human Oral Epithelial models has been used to predict the irritation potential of ingredients in healthcare products (Hagi-Pavli et al., 2014), and a buccal mucosal model used to show release and permeability of clobetasol-17-propinoate into the oral mucosa (Colley et al., 2018).

1.4.2 Immune-competent tissue engineered models

To improve the complexity of *in vitro* modelling one route is to include immune cells which can be used to investigate cellular crosstalk, immune responses to stimuli (such as bacteria or drug compounds) and roles in human disease (Cianci et al., 2018). Primary immune cells are of particular relevance as they are closer to cells observed *in situ*, better recapitulate the variation observed in the human population, and are the cell type of interest in this thesis. Therefore, the incorporation of primary innate immune cells into models of various tissue types will be the focus of this section, and relevant publications are summarised in Table 1.2.

Firstly, immune-competent tissue engineered oral mucosal models made with primary monocytes typically use peripheral blood mononuclear cells isolated from whole blood. These primary monocytes have been a popular choice for inclusion into oral models of the tongue (Al-Samadi et al., 2017) and gingiva (Tschachojan et al., 2014; Björnfot Holmström et al., 2017; Lira-Junior et al., 2020). In addition, monocytes have been used in a 3D flowing model of the vasculature containing primary endothelial cells and smooth muscle cells within bioengineered vessels and was used to investigate monocyte migration into surrounding tissue in response to environmental cues and stimuli (Robert et al., 2017).

Monocyte-derived macrophages have been incorporated into models from various tissue types. For example, MDM were incorporated into a skin model made with fibroblasts isolated from patients with diabetic foot ulcers, and showed MDM adopted a proinflammatory phenotype in this environment (Smith et al., 2021). Another

group generated vaginal mucosal models, incorporated MDM, and used this immunocompetent model to study the response to HIV infection (Saba et al., 2021). Finally, a 3D model of the large intestine, comprising colonoids, was made immunocompetent by addition of MDM to the outer scaffold, and found that in inflammation, increased migration of MDM towards the epithelium was observed, alongside increased secretion of pro-inflammatory cytokines (Roh et al., 2019).

Monocyte-derived dendritic cells (MoDC) have also been used as an immune source in some tissue engineered models. For example, a skin model containing primary keratinocytes, fibroblasts and MoDC found the inclusion of MoDC had no effect on histological characteristics, and upon treatment with allergen DNCB, the immune cells migrated into the dermal compartment, although no cytokine-mediated inflammatory response was detected (Chau et al., 2013). Similarly, MoDC have been incorporated into a lung model comprising lung epithelial and fibroblast cell lines. In these models MoDC migrate into the basal region of the epithelium without any external signalling, while treatment with house dust mite extract and LPS cause MoDC migration into the apical region of the epithelium (Harrington et al., 2014).

Finally, monocyte-derived LC (MoLC) have been included in multiple models of the skin, but no other tissue types, likely due the specialised role in immune surveillance LCs carry out in barrier tissues (Collin et al., 2018). An early study used DED scaffold and seeded it with human keratinocytes and melanocytes to generate a pigmented model. To this, LC derived from $CD34^+$ monocyte progenitor cells were added to form an immune model. While no functional data was provided, both IHC and electron microscopy proved presence of these cells in the model (Régnier et al., 1997). Similarly, LC from $CD34^+$ progenitor cells, alongside primary keratinocytes, were seeded onto an EpiSkin dermal support to generate an immune model which was then challenged with allergens, irritants, and UV light to assess cell behaviour. Here, LC morphology and phenotype altered in response to allergens, but not irritants, and UV damage to LC was preventable by topical application of UV protection, highlighting two potential uses for this model system (Facy et al., 2005). A later study also incorporated LC into a skin model to assess the response to UV damage, finding the treatment increased secretion of pro-inflammatory cytokines and induced migration of LC into the dermal compartment (Bechetoille et al., 2007). A more recent study sought to compare function of MoLC and MUTZ-3 Langerhans-like cells within a skin model.

Both cell types permitted model formation, although some functional differences were observed, notably in the response to DNCB, where MoLC-containing models increased secretion of IL-6 and CXCL8, while immune cell migration into the dermal compartment was observed in MUTZ-3-containing models, suggesting utility for both cell types (Bock et al., 2018).

Table 1.2. Tissue engineered barrier models containing primary monocytes and monocyte-derived cells

Immune cell type	**Tissue type**	**Reference**
Monocytes	Tongue	(Al-Samadi et al., 2017)
	Gingival	(Tschachojan et al., 2014; Björnfot Holmström et al., 2017; Lira-Junior et al., 2020)
	Vasculature	(Robert et al., 2017)
Macrophages	Skin	(Smith et al., 2021)
	Vaginal mucosa	(Saba et al., 2021)
	Large intestine	(Roh et al., 2019)
Dendritic cells	Skin	(Chau et al., 2013)
	Lung	(Harrington et al., 2014)
Langerhans cells	Skin	(Régnier et al., 1997; Facy et al., 2005; Bechetoille et al., 2007; Bock et al., 2018)

1.4.3 Immune-competent tissue engineered models of the oral mucosa

While the oral cavity is generally more immune-tolerant than other tissue, there are resident immune cells which respond to pathogens, environmental triggers, and, although uncommon, can mediate diseases in the oral cavity (Ji et al., 2013; Wu et al., 2014; Moutsopoulos et al., 2018; Saccucci et al., 2018). There has been a recent drive to develop immune competent models, summarised in Table 1.3 and detailed below, which can more accurately represent an inflamed oral cavity, and could be used to test safety and activity of novel treatments. Most often, models have comprised gingival resident cells (fibroblasts ± keratinocytes), to model the gingiva and incorporated an immune component to more effectively model periodontitis and the local inflammatory response. Typically, the immune component is a cell line, often

preferred for ease of use and reproducibility. However, these cell lines are established from cancer cells – commonly lymphomas or myelomas – which raises potential issues of likeness to normal 'non-diseased' cells (Wilding et al., 2014).

Immune cell lines THP-1, U937, and MonoMac-6 (MM6) have all been used to model monocyte and macrophage function within a model of the gingiva. THP-1 cells have also been used to model macrophage polarisation into M1 and M2 phenotypes in a tongue carcinoma model. Interestingly, models containing M2 THP-1 cells secreted significantly higher pro-tumorigenic factors compared to M1 or immune-free models, displaying striking similarities to the tumour microenvironment, which makes these models potentially useful for investigating cancer progression and therapeutics (Pirilä et al., 2015). In addition, Xiao *et al* described a methodology for using PMA-differentiated THP-1 cells in conjunction with primary gingival fibroblasts and the HaCaT skin keratinocyte cell line to generate an immune-gingival model (Xiao et al., 2018). However, while presence was confirmed by CD14 staining, no functional data of the response to LPS challenge was provided, which could be an issue as THP-1 cells display a reduced response to inflammatory stimuli compared to primary cells (Tedesco et al., 2018) so a measurable response may not be achievable in these models. In contrast, U937 monocytic cells co-cultured with gingival fibroblasts in a type I collagen matrix to produce a gingival model responded to LPS with increased secretion of inflammatory MMP, which was reduced by treatment with green tea extract (Morin et al., 2017). Finally, Bao *et al* used HPV-E6/7 immortalised gingival fibroblast populated porcine collagen sponges, perfused with HPV-immortalised gingival keratinocytes then MM6 monocytic cells to produce an immune gingival model. These models were challenged with a multispecies bacterial biofilm grown in hydroxyapatite discs to mimic sub-gingival plaque, displaying significant increases in inflammatory cytokine and chemokine secretion in infected models compared to unstimulated controls (Bao et al., 2015). Taken together, these examples suggest that the choice of cell line may be an important consideration when generating immune models.

In addition, the MUTZ-3 cells have been used to represent resident dendritic cells in a gingival model containing primary gingival keratinocytes and fibroblasts, which was used to examine response to allergenic stimuli compared to skin models. Here,

comparable to the *in situ* behaviour, the immune cells in both model types migrated out of the tissue upon exposure to allergens (Kosten et al., 2016).

While many models have used cell lines to represent an immune component, there are also examples that use primary immune cells, typically isolated from peripheral blood. Peripheral blood mononuclear cells (PBMC) incorporated into a tongue carcinoma model, exhibited migration into the model which was dependent on the cancer cell line type, and activated immune cell status (Al-Samadi et al., 2017). PBMC have also been used to generate a gingival model including immortalised gingival keratinocytes and dermal fibroblasts, which was treated with X-ray irradiation (Tschachojan et al., 2014). These immune-competent models secreted higher levels of IL-6 and CXCL8, and increased expression occurred at a later time point compared to immune-free models, suggesting some immune regulation delayed an inflammatory response. Similarly, PBMC in a gingival model comprised of primary gingival fibroblasts and OKF6/TERT2 immortalised oral keratinocytes was used to model periodontitis. By incorporating undifferentiated mononuclear cells, they were able to examine cell differentiation *in situ*, observing increases in macrophage markers after 7 days. For example, following LPS ± IFN-γ stimulation, the modes displayed increased expression of inflammatory markers CD80 and TNF-α (Björnfot Holmström et al., 2017). Further studies found increased secretion of S100A12 in periodontitis which was recapitulated following inflammatory stimulation of the immune gingival model, and by using this model system it was possible to show direct cell contact was required to induce the effect (Lira-Junior et al., 2020), highlighting a benefit of using multicellular tissue engineered models.

Finally, Schaller *et al* used a commercial oral epithelial mode (SkinEthic RHE oral epithelial model) infected with *Candida albicans* and supplemented with granulocytes (polymorphonuclear leukocytes; PMN) to model the immune response to oral candidiasis. The addition of PMN had a protective effect against *C. albicans* infection, showing less fungal penetration into the model, and reduced epithelial damage. In addition, all models produced inflammatory cytokines such as IL-6 and CXCL8 in response to infection, but immune-containing models secreted more IFN-γ and TNF-α compared to immune-free models (Schaller et al., 2004).

These studies represent a recent drive to incorporate an immune component into oral models to study inflammation, disease, and therapeutic anti-inflammatory agents. However, there are some gaps in the current published literature. Firstly, specific immune cells derived from primary monocytes (macrophages, DC, LC) have not been used in oral models, despite these cells comprising a substantial proportion of cells recruited during inflammation (Coillard et al., 2019). Additionally, while most studies investigate the gingiva, only one modelled the buccal epithelium, and none have used a full thickness buccal model (including laminar propria), despite the importance of this site for local and systemic drug delivery (Shojaei, 1998; Zhang et al., 2002; Colley et al., 2018; Hua, 2019). Finally, although immunological processes typically rely on crosstalk between immune cell types, and also with the ECM (Bhattacharjee et al., 2019), there have been no studies containing different multiple immune cell types. Therefore, while these studies represent a step forward in modelling oral immunity *in vitro*, there are still significant advances required to generate complex models capable of fully recapitulating the immune response in these tissues.

Table 1.3. Immune-competent tissue engineered oral models

Immune cell type	Immune cell origin	Site modelled	Theme	Reference
Macrophage	THP-1 cell line	Gingiva	Methodology	(Xiao et al., 2018)
		Tongue	Cancer	(Pirilä et al., 2015)
	U937 cell line	Gingiva	Inflammation	(Morin et al., 2017)
	MM-6 cell line	Gingiva	Periodontitis	(Bao et al., 2015)
Langerhans cells	MUTZ-3 cell line	Gingiva	Allergenic response	(Kosten et al., 2016)
Mononuclear cells	Primary, from buffy coat	Tongue	Cancer	(Al-Samadi et al., 2017)
		Gingiva		(Tschachojan et al., 2014)
			Periodontitis	(Björnfot Holmström et al., 2017; Lira-Junior et al., 2020)
Polymorphonuclear leukocytes	Primary, from whole blood	Buccal epithelium	Oral candidiasis	(Schaller et al., 2004)

1.5 Hypothesis, aims and objectives

1.5.1 Hypothesis

Monocyte-derived cells express detectable levels of XME, and in macrophages this expression is dependent on inflammatory state. Monocyte-derived macrophages are a suitable cell type for inclusion into a tissue engineered model and will retain functionality within a model of the oral mucosa.

1.5.2 Aim

Identify a role for immune cells in local xenobiotic metabolism and optimise production of a novel immune oral model to further assess local inflammation and metabolism.

1.5.3 Objectives

To address the overall aim of this thesis, the following specific objectives were followed:

- Generate and characterise MDM and MoDC.
- Determine gene and protein expression of Phase I XME in MoDC and polarised MDM.
- Optimise culture of MDM to produce an inflammatory response in monolayer.
- Ensure the inflammatory response is conserved when MDM are embedded and cultured in a 3D collagen hydrogel.
- Examine the effects of an exogenously delivered drug (dexamethasone) to inhibit the inflammatory response.
- Generate a tissue engineered model of the oral mucosa containing MDM and confirm functionality in response to both inflammatory (LPS) and anti-inflammatory (dexamethasone) stimuli.
- Investigate XME expression in immune-free and immune-containing oral models to determine if expression is altered by inclusion and stimulation of macrophages.

Chapter 2 – Materials and Methods

2.1 Materials

Unless otherwise stated, all materials were purchased from Sigma-Aldrich (Dorset, UK).

2.2 Cell culture

2.2.1 Thawing cryopreserved cells

Cells were removed from long term storage in liquid nitrogen and warmed in a 37°C water bath until thawed. Immediately afterwards, cells were diluted with culture media to a total volume of 10 mL and centrifuged for 5 minutes at 200 g. The supernatant was aspirated, the pellet resuspended in 10 mL of culture medium, transferred to a T75 flask, and incubated at 37°C with 5% CO_2 in a humidified incubator.

2.2.2 Passaging cells

Cells were passaged when they approached 90% confluency. The media was aspirated, and each flask washed twice with sterile phosphate-buffered saline (PBS) before incubation with 2.5 mL 0.05% trypsin/0.02% EDTA solution (Gibco, Life technologies, Warrington, UK) for a T75 flask, (adjusted accordingly for flask size) for up to 10 minutes at 37°C and 5% CO_2. Once the cells had detached the enzymatic activity was inactivated by addition of FBS-containing media at a 3:1 ratio. The cell suspension was pelleted by centrifugation for 5 minutes at 200 g, supernatant removed, and the pellet resuspended in appropriate media before seeding in a fresh flask.

When required, cells were counted using a Neubauer haemocytometer (Weber Scientific International, Middlesex, UK) with the total cell count given by the following equation:

Cell count = average count per quadrant x 10,000 x dilution factor x volume (mL)

2.2.3 Cryopreserving cells

For long term storage, cells were kept in liquid nitrogen in freezing medium (90% FCS with 10% DMSO). When flasks were 80-90% confluent, cells were detached by trypsinisation and counted as described above. Cells were resuspended in freezing medium at 1 x 10^6 cells/mL and 1 mL aliquots transferred to cryopreservation vials (Greiner bio-one, Gloucestershire, UK). Vials were placed in a freezing container (Mr Frosty, Thermo Scientific, Leicestershire, UK) and cooled to -80˚C overnight before being placed in liquid nitrogen for long-term storage.

2.2.4 Imaging cells

When required as part of routine cell culture, cells were viewed using phase contrast light microscopy with a Nikon Eclipse TS100 light microscope and imaged using SPOT software (version 5.1).

2.2.5 Routine cell culture media

Media used was specific to each cell type and described in the tables below.

Table 2.1. Medium used to culture fibroblasts

Component	Final concentration
Dulbecco's Modified Eagle's Medium (DMEM)	90%
Foetal Calf Serum (FCS)	10%
Penicillin	100 IU/mL
Streptomycin	100 µg/mL

Table 2.2. Complete IMDM medium used to culture immune cells.

Component	Final concentration	Supplier
Iscove's modified Dulbecco's medium (IMDM)	97%	Thermo Fisher
Human AB serum	2% v/v	-
Penicillin	100 IU/mL	-
Streptomycin	100 µg/mL	-

Table 2.3. Complete Green's medium

Component	Final concentration
Dulbecco's Modified Eagle's Medium (DMEM)	66%
Ham's F12 medium	21.6%
Foetal Calf Serum (FCS)	10%
#Epidermal Growth Factor (EGF)	10 ng/mL
*Hydrocortisone	0.4 µg/mL
Adenine	0.18 mM
Insulin	5 µg/mL
Transferrin	5 µg/mL
L- Glutamine	2 mM
Triiodothyronine	0.2 µM
Amphotericin B	0.625 µg/mL
Penicillin	100 IU/mL
Streptomycin	100 µg/mL

Additives omitted when isolating primary cells from human biopsies.
* Additives omitted in media used with immune cells.

2.2.6 Culture of cell lines

FNB6 cells (a kind gift from Professor Keith Hunter) are hTERT immortalised oral keratinocytes originally isolated from normal female buccal mucosa (McGregor et al., 2002). The cells were cultured in Green's medium (Table 2.3) and media changed every 2-3 days.

2.2.7 Isolation and culture of primary oral fibroblasts

Biopsies were collected with written, informed consent and processed in line with NHS ethical review (approval number 09/H1308/66). Samples on arrival were removed from transport medium and submerged in trypsin/EDTA overnight at 4°C. Following trypsinisation, Green's medium (without EGF; Table 2.3) was added to neutralise enzymatic activity. The sample was finely minced with a scalpel blade and incubated in 10 mL 0.5% collagenase A (w/v) for 3-5 hours at 37°C with 5% CO_2. Following collagenase treatment cells were centrifuged at 200 g for 10 minutes and the cell pellet resuspended in DMEM media (Table 4.1). The isolated fibroblasts were plated in a T25 cell culture flask and incubated at 37°C with 5% CO_2.

Normal oral fibroblasts (NOFs) isolated from primary human oral biopsies using this protocol were cultured in supplemented DMEM (Table 4.1) with media changed weekly. Cells were passaged once 80% confluency was reached and used at passage 3 to 8.

2.2.8 Isolation and differentiation of human peripheral blood monocytes

Buffy coat was obtained from the NHS blood transfusion service and used after local ethical review (application number 12597). The blood was mixed at a 1:1 ratio with Hank's balanced salt solution (HBSS, without Ca^{2+} and Mg^{2+}) and, using a 50 mL centrifuge tube, 30 mL of this solution gently overlaid on 20 mL of Ficoll-Paque (GE Healthcare, Buckinghamshire, UK) before centrifuging at 400 g for 40 minutes at room temperature without deceleration.

Following separation, the mononuclear layer (predominantly monocytes and lymphocytes) was visible between the Ficoll and serum as a distinct white layer (buffy coat), which varied in size, colour, and viscosity between samples. The mononuclear cells were removed by aspiration with a sterile Pasteur pipette and washed three times in HBSS. The cells were resuspended in complete IMDM (Table 2.2) and seeded either in a 10 cm Petri dish (2×10^8 cells) or 6-well plate (5×10^7 cells). After a minimum of two hours incubation at 37°C and 5% CO_2, monocytes were purified by plastic adherence by washing at least three times with HBSS before fresh complete IMDM was added and the cells replaced in the incubator. Typically, 1×10^9 mononuclear cells were isolated per buffy coat, with a conversion rate of between 1-10% into viable monocytes. Monocytes were differentiated through 7-day treatment with specific cytokine stimulation as detailed in Table 2.4. Media was refreshed every 3-4 days and contained fresh cytokines relative to the cell type.

Table 2.4. Cytokines used for monocyte differentiation

Cell type	Cell subtype	Human recombinant cytokine	Concentration
Monocyte-derived macrophages (MDMs)	M0	-	-
	M1	GM-CSF	10 ng/mL
	M1 (day 6)	*E. coli* LPS	100 ng/mL
		Interferon γ (IFNγ)	20 ng/mL
	M2	M-CSF	25 ng/mL
	M2 (day 6)	IL-4	20 ng/mL
Monocyte-derived dendritic cells (MoDCs)	Dendritic cells (MoDC)	GM-CSF	50 ng/mL
		IL-4	40 ng/mL
	Langerhans cells (MoLC)	GM-CSF	50 ng/mL
		IL-4	40 ng/mL
		TGFβ	20 ng/mL

Cytokines were all purchased from Peprotech (London, UK) and diluted in complete IMDM medium (Table 2.2).

2.2.9 Stimulation of monocyte-derived cells in monolayer culture

Cells were removed from the culture dish by gently scraping and reseeded in 6-well plates at a density of 5 x 10^5 per well 24 hours prior to treatment. Bacterial lipopolysaccharides (LPS) at a final concentration of 500 ng per 1x10^6 MDM was used to treat MDM for 24 hours to stimulate an inflammatory response. NFκB inhibitors were used to block LPS-mediated MDM activation and study inhibition of the inflammatory response. The inhibitors used were BAY 11-7085 at 0.05-500 μg/mL and dexamethasone at 0.01-100 μg/mL (both Abcam, Cambridge, UK), both used to pre-treat MDM for 4 or 24 hours. Inhibition was compared to LPS-treated MDM as a positive control. Cytokine release was quantified by ELISA (section 2.3.5) and phenotypic changes quantified by qPCR (section 2.3.1).

2.2.10 Generating 3D tissue engineered models

Type 1 collagen was isolated from rat tails kindly donated by Dr Emma Bird (School of Clinical Dentistry, University of Sheffield) and stored at -20℃ until required. Tails were thawed overnight at 4℃, following which the skin was broken to expose the underlying tissue. Rat tail tendon collagen was identifiable by the white colouration, removed from the tail and washed in PBS. The collagen fibres were then dissolved in 0.1 M acetic acid for 7 days at 4 °C with stirring. This solution was freeze-dried (VirTis

Benchtop K Manifold freeze drier; SP Scientific, Suffolk, UK) for 3-4 days. The resultant collagen was weighed and aliquoted, then stored at -20 °C until required, at which point it was reconstituted in 0.1 M acetic acid to a stock concentration of 5 mg/mL and stored at 4 °C for use within 6 months.

To generate collagen hydrogels, the components described in Table 2.5 were added in descending order and mixed gently before the pH was adjusted to 7.4 by the addition of 2 M NaOH. Following this, NOF were added at a concentration of 2.5 x 10^5 per model. The subsequent hydrogel solution was gently pipetted into cell culture inserts (1 mL per insert; 0.4 µL Polyethylene Terephthalate (PET); MerckMillipore, Darmstadt, Germany) placed in 12-well plates. The gels were incubated at 37°C with 5% CO_2 until set. Once solidified, the gels were seeded with 5 x 10^5 FNB6 cells, and Green's medium (Table 2.3) added both underneath (1 mL), and on top (0.5 mL) of the gels. Media was replenished after 24 hours. Following 48 hours culture the models were raised to an air-to-liquid interface (ALI) by removal of the media and replacing the media (1 mL) underneath only. Models were cultured for 10 to 14 days, with the media changed every 1-2 days.

Table 2.5. Components of a collagen-based hydrogel

Component		Final concentration	Volume added per model (µL)	Supplier
DMEM (10X)		1X	100	-
Reconstitution buffer (10X):	260 mM $NaHCO_3$	1X	100	BD Biochemical, Poole, UK
	200 mM HEPES			
	59.5 mM NaOH			BDH AnalaR, Poole, UK
FBS		8%	80	-
L-Glutamine		2 mM	10	-
Type 1 rat tail collagen		3.35 mg/mL	670	Produced in house

To generate immune competent models, MDM were first differentiated for 6 days, then incorporated into a collagen matrix (1 x 10^6 cells per model) in the same step as

NOF as described above. Immune-cell containing collagen matrices were then cultured as detailed above for up to 14 days before analysis. Models were interrogated for functionality by the addition of stimuli as described in section 2.2.9. The conditioned media was collected, aliquoted, and analysed by ELISA. Models were fixed for histological analysis (section 2.5), and RNA was isolated as described in section 2.3.1. Modes were also digested with 2 mg/mL type 1 collagenase (Gibco, Life technologies, Warrington, UK), passed through a cell strainer (Corning, Flintshire, UK) and analysed for cell viability by flow cytometry (section 2.3.2.2).

2.3 Molecular biology

2.3.1 Gene expression analysis

RNA was isolated using a Monarch total RNA miniprep kit (New England Biolabs, Hitchin, UK) as per manufacturer guidelines. For adherent cells, lysis buffer was added directly to the culture plastic and cell lysate removed by cell scraping. For suspension or loosely adherent cells, prior to lysing, cells were scraped from the culture plastic, resuspended in appropriate media, and centrifuged for 5 minutes at 200 g. The supernatant was aspirated, and the pellet lysed using lysis buffer. To isolate RNA from 3D models, first the collagen was incubated in RNA protect until dissolved, vortexed briefly and debris pelleted, then supernatant combined with an equal volume of lysis buffer and mixed. RNA was then isolated from cell lysates using the same provided protocol. In brief, the RNA solution was passed through a gDNA column to remove contaminating genomic DNA, then combined 1:1 with 100% ethanol. This mixture was passed through an RNA purification column to capture RNA. The columns were then primed and washed before elution of RNA into nuclease-free water. Total RNA concentration was determined by NanoDrop 1000 (ThermoFisher Scientific, Leicestershire, UK).

cDNA was generated using high-capacity cDNA reverse transcription kit (ThermoFisher, Leicestershire, UK) as per manufacturer guidelines. Briefly, a 2 x RT master mix consisting of RT buffer, dNTP mix, RT random primers, and MultiScribe reverse transcriptase was combined on ice, then 10 µL diluted 1:1 with a set amount

of total RNA and made up to 20 µL total with nuclease free water. Final RNA concentration used was between 100 and 500 ng/mL and was consistent within each experiment. cDNA was transcribed by heating to 25°C for 10 minutes, 37˚C for 120 minutes, then 85°C for 5 minutes. After transcription, cDNA was stored at -20°C until required.

Quantitative PCR was carried out using a Rotor-Gene Q (Qiagen, Manchester, UK). In all cases β2-microglobulin (B2M control mix; Applied biosystems, Life Technologies, Warrington, UK) was used as an endogenous control to normalise gene expression. All TaqMan primers were purchased from ThermoFisher Scientific and detailed in Table 2.6 and Table 2.7.

For the phase I XME enzyme gene array, RNA was transcribed using the RT2 first strand kit, and cDNA analysed by gene array (both Qiagen, Manchester, UK; gene array PAHS-068Z), as per manufacturer instructions.

Table 2.6. TaqMan primers for immune cell characterisation and activation

Target	Assay ID
β2-microglobulin (B2M)	Hs00187842_m1
CD1a	Hs00381754_g1
CD11c (ITGAX)	Hs00174217_m1
CD14	Hs02621496_s1
CD36	Hs01567185_m1
CD80	Hs00175478_m1
CD86	Hs01567026_m1
CD115 (CSF1R)	Hs00911250_m1
CD163	Hs00174705_m1
CD204 (MSR1)	Hs00234007_m1
CD206	Hs00267207_m1
CD207	Hs00210453_m1
CPM	Hs01074151_m1
CDH1	Hs01023895_m1
CXCL8	Hs00174103_m1
HLA-DRA	Hs00219575_m1
IL-6	Hs00174131_m1
TLR2	Hs01872448_s1
TLR4	Hs00152939_m1
TNF-α	Hs01113624_g1
Vimentin	Hs05024057_m1

Table 2.7. TaqMan primers for assessing XME expression

Target	Assay ID
β2-microglobulin (B2M)	Hs00187842_m1
ALDH2	Hs01007998_m1
CYP1A1	Hs01054796_g1
CYP1B1	Hs00164383_m1
CYP2A6	Hs00868409_s1
CYP2C9	Hs00426397_m1
CYP2D6	Hs04931916_gH
CYP2E1	Hs00559367_m1
CYP3A4	Hs00604506_m1
CYP3A5	Hs01070905_m1
FMO1	Hs01032912_m1
FMO2	Hs01025544_m1
FMO4	Hs00157614_m1
FMO5	Hs00356233_m1
PTGS2	Hs00153133_m1
UCHL3	Hs04334565_m1

2.3.2 Flow cytometry

2.3.2.1 Analysis of cell surface proteins

Analysis was carried out on either a FACSCalibur or LSRII (BD Biosciences, UK). Cells grown as monolayers were prepared for analysis by scraping directly into culture media and centrifuging at 200 g for 5 minutes. The pellet was resuspended at a concentration of 5 x 10^5 cells/mL in FACS buffer (PBS, pH 7.4; 0.1% BSA; and 0.1% sodium azide). The cell suspension was separated into 1 mL aliquots and centrifuged for 2 minutes at 1800 g. Following this the pellet was resuspended in 30 µL FACS buffer and incubated with 5 µL Fc blocking reagent (MACS, Miltenyi Biotech, Surrey UK) on ice for 5 minutes. The samples were further incubated with antibodies as described in Table 2.8 for 20 minutes at 4˚C in the dark. To prevent further staining the samples were diluted with 1 mL FACS buffer and centrifuged at 1800 g for 2 minutes and resuspended in 300 µL FACS buffer. Samples were analysed directly after staining and 5 µL of the DNA binding dye, propidium iodide (PI; final concentration 5 µg/mL) was added to exclude dead cells immediately prior to analysis. Analysis of flow cytometry data were undertaken using FlowJo software (version 10)

and normalised median fluorescence intensity (nMFI) calculated by dividing the MFI of the target marker by the isotype control, allowing for comparison between independent experiments.

Table 2.8. Antibodies used for flow cytometry

Target	Clone	Supplier	Catalogue number	Dilution	Conjugate
IgG	P3.6.2.8.1	eBioscience (Leicestershire, UK)	17-4714-81	1:8	-
CD14	61D3		17-0149-42	1:8	APC
CD163	eBIOGHI/61		17-1639-41	1:8	APC
CD1a	REA736	Miltenyi Biotech (Surrey, UK)	130-112-023	1:20	APC
CD11c	REA618		130-114-110	1:20	APC
CD36	AC106		130-095-475	1:8	APC
CD207	REA770		130-112-369	1:20	APC
HLA-DR	REA805		130-111-943	1:20	APC

2.3.2.2 Assessing cell viability

To assess MDM viability, a fixable LIVE/DEAD flexible blue stain (ThermoFisher Scientific, Leicestershire, UK) was used. Cells were stained as per manufacturer guidelines and analysed using a LSRII flow cytometer (BD Biosciences, Oxford, UK). Briefly, cells from monolayer culture, or extracted from collagen hydrogels (section 2.2.10), were washed and resuspended in 1 mL PBS. 1 µL of dye was added to each cell suspension an incubated for 30 minutes at room temperature protected from light. After incubation cells were washed then fixed with 100 µL 10% neutral-buffered formalin (v/v) for 15 minutes. Cells were washed and resuspended with PBS with 1% BSA. Stained and fixed cells were stored at 4˚C protected from light until required and analysed within a week of staining. When additional staining was required, cells were either stained before fixation, or immediately prior to analysis, depending on individual antibody datasheets.

2.3.3 Western blotting

Total cell protein was isolated in RIPA buffer (ThermoFisher Scientific, Leicestershire, UK) containing EDTA-free protease inhibitor (Roche, Hertfordshire, UK), and the concentration quantified by BCA assay (Pierce BCA protein assay Kit; ThermoFisher Scientific, Leicestershire, UK) as per manufacturer instructions. Protein was diluted in RIPA to a standard concentration, mixed 1:1 with SDS buffer, and heated at 95˚C for 5 minutes to denature the proteins prior to loading onto a western blot gel.

To run the western blot, polyacrylamide gels (final concentration 10%) cast in house using standard recipes were used. Either 20 µg of isolated protein, or 1 µg of liver microsomes (positive control) were loaded per well, and DNA protein ladder used to determine protein sizes. Gels were run in SDS running buffer (0.025M tris base, 0.032M glycine, 1% SDS in ddH_2O), for 1 hour at 120V, or until the SDS band reached the bottom of the running gel. Proteins were transferred onto a nitrocellulose membrane using the Trans-Blot Turbo™ transfer system (BioRad, Hertfordshire, UK) in semi-dry transfer buffer (0.05M Tris base, 0.025M glycine, 0.4% SDS and 20% methanol in ddH_2O). Successful protein transfer was confirmed by Ponceau S stain, then the membrane washed in TBST solution (8g NaCl, 0.2g KCl, 3g Tris base in ddH_2O).

Next, membranes were blocked with 5% skim milk powder in TBST for 1 hour at room temperature, then incubated with a primary antibody (Table 2.9) overnight at 4˚C. The membrane was washed in TBST, then incubated with the corresponding secondary antibody (Table 2.9) for 1 hour at room temperature. After washing, the membrane was developed using chemiluminescence (Clarity™ Western ECL Substrate, BioRad, Hertfordshire, UK) and imaged using a C-DiGit blot scanner (Li-cor, Cambridge, UK) with associated software. Each membrane was stripped of antibodies using Restore western blot stripping buffer (ThermoFisher Scientific, Leicestershire, UK), re-blocked in blocking solution, and then re-probed with a primary antibody specific to β-actin (loading control).

Table 2.9. Antibodies used for western blotting

Target	Host species	Supplier	Catalogue number	Dilution
ALDH2	Rabbit	Abcam	Ab108303	1:1000
CYP2A6	Mouse	Abcam	Ab56069	1:1000
CYP2D6	Rabbit	Abcam	Ab185625	1:1000
CYP3A5	Rabbit	Abcam	Ab108624	1:400
FMO4	Rabbit	Abcam	Ab191141	1:1000
FMO5	Rabbit	Abcam	Ab189516	1:2500
PTGS2	Rabbit	Cell signalling	122825	1:1000
B-actin	Mouse	Sigma	A1978	1:10,000
HRP-linked anti-mouse IgG	Horse	Cell signalling	7076S	1:1000
HRP-linked anti-rabbit IgG	Goat	Cell signalling	7074S	1:1000

2.3.4 Quantifying enzyme activity

In order to preserve enzyme activity and prevent protein denaturation, an adapted protocol for protein isolation was used. Cells were scraped into PBS and pelleted before incubation with TNT buffer (0.05M Tris•HCl, 0.15M NaCl and 1% triton X-100 in ddH_2O) for 15 minutes on ice. The resulting solution was centrifuged at 4˚C for 15 minutes at 16k rpm to pellet insoluble protein and cell debris. The supernatant was transferred to a fresh tube and protein quantified by BCA assay (Pierce BCA protein assay Kit; ThermoFisher Scientific, Leicestershire, UK) as per manufacturer's instructions.

Enzyme activity was measured using an activity kit specific for COX (Abcam, Cambridge, UK; ab204699) or CYP2D6 (Abcam, Cambridge, UK; ab211078) as per manufacturer's instructions. Immune cell protein was loaded as 500 µg per well, and liver microsomal protein (50 µg per well), was used as a positive control. Data was graphed and analysed as per manufacturer's guidance.

2.3.5 Analysis of cytokine release

Media was conditioned by 24-hour incubation of samples in medium containing either LPS, or relevant controls, and was stored at -20°C until required for analysis. Conditioned media was assessed for individual cytokine concentrations by ELISA kits

specific to IL-6, CXCL8, TNF-α and IL-10 (R&D systems, Abingdon, UK) as per manufacturer instructions. In short, plates were coated overnight in capture antibody diluted in PBS, then blocked for 1 hour with 1% BSA in PBS. Samples were then incubated for 2 hours, either neat (TNF-α and IL-10) or diluted in assay diluent (IL-6 and CXCL8, between 1:20 and 1:100 as required). High binding 96 well plates (Greiner, Gloucestershire, UK) were incubated with detection antibody for 2 hours, washed, then coated in streptavidin-HRP solution for 20 minutes. After a final wash, TMB substrate solution (KPL SureBlue™, SeraCare, Massachusetts, US) was added to the plates for up to 30 minutes, and once the highest standard showed a dark blue colour, the reaction was stopped with 2N H_2SO_4. Plates were read at 450 nm with a 570 nm correction using a Tecan infinite M200 plate reader and Magellan software (version 7.2). Sample concentrations were calculated from the standard curve using GraphPad software to interpolate a standard curve (hyperbole standard curve).

In addition, conditioned media was assessed by cytokine array (Proteome Profiler Human XL cytokine array kit; ARY022B; R&D systems, Abingdon, UK). Array was carried out as per manufacturer instructions with no alterations. Membranes were imaged using a C-DiGit blot scanner (Li-cor, Cambridge, UK) with associated software. Images were semi-quantified using the protein array analyser plugin for ImageJ.

2.3.6 Determining cytotoxicity by lactate dehydrogenase (LDH) assay

Conditioned media was also assessed on the same day for LDH release by cells as an indirect measure of cell damage using the CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega, Southampton, UK) as per manufacturer instructions. Briefly, 50 µL conditioned media was pipetted into a 96-well plate and 50 µL substrate mix added to each well. After a 30-minute incubation, the reaction was stopped by addition of 50 µL stop solution (1 M acetic acid). Plates were read at 492 nm using a Tecan infinite M200 plate reader and Magellan software (version 7.2).

2.3.7 Quantifying endotoxins in collagen

Concentration of endotoxins (LPS) in collagen was measured using the Pierce™ Chromogenic Endotoxin Quant Kit (ThermoFisher Leicestershire, UK),

carried out as per manufacturer's instructions. In short, using endotoxin free plastics, collagen samples were diluted by at least 1:10 in endotoxin free water to neutralise pH while preventing collagen gel polymerisation. In a 96-well plate 50 µL of the diluted collagen was combined with 50 µL amoebocyte lysate reagent and incubated in a plate heater pre-heated to 37°C for 30 minutes. Following this, 100 µL of pre-warmed chromogenic substrate was added and the plate incubated at 37°C for a further 6 minutes. The reaction was then stopped by addition of 50 µL 25% acetic acid, and the plate read at 405 nm using a Tecan infinite M200 plate reader and Magellan software (version 7.2).

2.4 Microbiology

2.4.1 Growth of bacteria in culture

Tannerella forsythia (ATCC 43037) was cultured on fastidious anaerobe agar (Acumedia, Ayr, UK) supplemented with 5% horse blood (Thermo Scientific, Hampshire, UK) and 0.17 mM (w/v) N-acetyl muramic acid (NAM), and maintained in an anaerobic cabinet (CO_2, N_2, H_2) at 37°C. *Aggregatibacter actinomycetemcomitans* (strain isolated in-house denoted as SHEF30) was cultured on Brain Heart Infusion agar (BHI) and maintained in a 37°C incubator with 5% CO_2.

2.4.2 Isolating bacterial lipopolysaccharides

Bacterial LPS was isolated using the LPS Extraction Kit (iNtRON Biotechnology, South Korea) as per manufacturer guidelines with the following additions. Bacteria extract was incubated with DNase I (100 µg/mL; Bioline, London, UK) and RNase A (100 µg/mL; Bioline, London, UK) for an hour at 37°C, followed by incubation with proteinase K (250 µg/mL; Qiagen, Manchester, UK) for an hour at 50°C prior to LPS purification steps. Ultrapure LPS from *Porphyromonas gingivalis* and *Escherichia coli* was purchased from Invivogen (Toulouse, France).

2.4.3 Determining LPS purity by silver staining

Isolated LPS was combined 1:1 with 2x SDS (sodium dodecyl sulfate) buffer then 10 µL loaded onto an LPS gel (10% polyacrylamide gel with 2.4 g urea) and run at 120 V for 1 hour. The gel was soaked overnight (40% ethanol and 5% acetic acid solution in distilled water), then incubated in periodic acid solution (0.7% periodic acid, 5% acetic acid, 40% ethanol in distilled water) for 10 minutes before washing in distilled water for 2 hours with shaking. The gel was then incubated in silver staining reagent (0.4% concentrated ammonium hydroxide, 18.7mM NaOH, 0.67% silver nitrate in distilled water) for 10 minutes then washed in distilled water (3 x 15 minutes). The gel was developed with a silver-stain developing solution (BioRad, Hertfordshire, UK), used as per the manufacturer instructions until bands appeared (typically 10-15 minutes), and the reaction stopped by addition of 5% acetic acid. The gels were viewed under white light using an InGenius3 dark box, imaged with a Synoptics camera and images captured with Genesys software (version 1.6.1.0).

2.5 Histology

2.5.1 Preparing formalin-fixed paraffin-embedded tissue

After models reached culture endpoint, they were fixed in 10% neutral-buffered formalin (v/v) for 24 hours before overnight processing using a Shandon Citadel 2000 tissue processor (Table 2.10). Processed models were bisected and embedded in paraffin wax (Leica EG1160 embedding centre; Leica Microsystems, Germany) and stored at room temperature until use.

Table 2.10. Processing schedule for formalin-fixed models

Solution	Time	Repeats
10% neutral buffered formalin	1 hour	1
70% alcohol	1 hour	2
90% alcohol	1 hour	2
Absolute alcohol	1 hour	3
Xylene	1 hour 30 minutes	2
Paraffin wax	2 hours	2

2.5.2 Sectioning paraffin-embedded tissue

Blocks were cooled on ice for 30 minutes prior to sectioning at 5 µm (Leica RM2235 microtome, Leica Microsystems, Germany). Sections were floated in a mounting bath (Barnstead Electrothermal, Staffordshire, UK) and transferred to a Superfrost plus micro slide (VWR, West Sussex, UK) which was warmed in an oven at 60°C for 20 minutes before further staining.

2.5.3 Haematoxylin and eosin staining

Haematoxylin and eosin (H&E) staining was carried out with a Leica ST4040 linear stainer (Leica Microsystems, Germany) using a standard H&E staining protocol (Table 2.11). After staining, sections were mounted using DPX mounting media topped with a coverslip and left to dry overnight. Slides were imaged using an Olympus BX51 microscope and Cell^D software (version 2.8).

Table 2.11. Staining schedule for H&E staining tissue sections

Solution	**Repeats**
Xylene	3
99% IDA	3
Distilled water	2
Harris' haematoxylin	4
Running tap water	1
0.1% acid alcohol	1
Running tap water	1
Scott's tap water substitute	1
Running tap water	1
Eosin Y (aqueous)	3
Running tap water	1
99% IDA	3
Xylene	4

2.5.4 Immunohistochemistry of paraffin-embedded tissue

Routine immunohistochemistry (IHC) staining was carried out by the core histopathology unit within the Royal Hallamshire Hospital (Sheffield). Immunostaining for CD68 was carried out in house using the following protocol. 5 µm sections were dewaxed by 3 minutes each of xylene, 100% ethanol, 75% ethanol, 50% ethanol and ddH_2O. Endogenous peroxidases were then quenched for 20 minutes by 3% H_2O_2 in methanol, followed by two 5 minute PBS washes. Heat mediated antigen retrieval was carried out in a pressure cooker as per manufacturer's instructions in Tris/EDTA buffer (10 mM Tris Base, 1.2 mM EDTA, 0.05% v/v Tween20 in ddH_2O, pH 9). Slides were washed in PBS, sections bordered with a wax pen then blocked in normal goat serum for 20 mins at room temperature. Sections were incubated with CD68 primary antibody (Table 2.12) for 1 hour at room temperature, then washed. Next, sections were incubated with anti-mouse IgG secondary antibody (Vector laboratories, California, US) for 20 minutes at room temperature. After further washing, slides were incubated with Vectastain Elite ABC-HRP kit, then stained with DAB (both Vector laboratories, California, US), as per manufactures guidance. The reaction was halted in dH_2O, then counterstained with haematoxylin, dehydrated, and mounted in DPX as described in section 2.5.3.

2.5.5 Immunofluorescence of paraffin-embedded tissue

For immunofluorescence staining sections were prepared as described in section 2.5.4 and blocked in normal goat serum for 20 mins at room temperature. Each primary antibody was combined in PBS with its corresponding fluorescent conjugated antibody (Table 2.12) and incubated at room temperature for 20 minutes, then 200 µL added to blocked slides and incubated in the dark for 1 hour at room temperature. The slides were washed with excess PBS + 0.05% v/v Tween20, and then incubated with the other pre-incubated primary/secondary antibody for 1 hour. The slides were washed, counterstained with DAPI (5 µg/mL) for 5 minutes at room temperature and washed again. Slides were mounted in ProLong diamond anti-fade mountant (ThermoFisher Scientific, Leicestershire, UK), covered with a glass coverslip, and cured for 24 hours at room temperature in the dark then moved to 4˚C for storage.

Fluorescent images were taken with a Zeiss Axioplan 2 microscope with the Image ProPlus v7.0.1 software (Media Cybernetics Inc., MD, USA).

Table 2.12. Antibodies used for immunofluorescence

Target	Host species	Concentration	Supplier	Cat no
CD68	Mouse	0.4 µg/mL	Abcam	Ab955
TNF-α	Rabbit	8 µg/mL	Proteintech	17590-1-AP
Anti-mouse FITC	Goat	1 µg/mL	Abcam	Ab6669
Anti-rabbit CY3	Donkey	1 µg/mL	JacksonImmuno	711-165-152

2.6 Statistical and computational analysis

Principal Component Analysis (PCA) and heatmaps were generated using ClustVis software with vector scaling (Metsalu et al., 2015). Unless otherwise stated, data were analysed using GraphPad Prism 7 or later and presented as mean ± SD. When comparing two data sets, significance was determined using Student's t test (paired or unpaired as appropriate). When comparing multiple data sets, an ANOVA was used to determine significance. Data was considered significant when $p<0.05$.

Chapter 3 – Expression of XME in primary monocyte-derived immune cells

3.1 Introduction

Xenobiotic metabolism refers to the reactions which chemically alter foreign compounds, such as drug compounds and environmental carcinogens (Lang et al., 1999). These reactions are typically oxidative (addition of oxygen or removal of hydrogen molecule) or utilise water molecules to break chemical bonds through hydrolysis reactions (Omiecinski et al., 2011). Xenobiotic metabolising enzymes (XME) are mainly expressed in the liver, which is the site of first-pass metabolism. However, many XME are expressed in extrahepatic tissue (Krishna et al., 1994), predominately the kidney (Knights et al., 2013), but in many other surface tissues such as skin (Kazem et al., 2019) and lungs (Enlo-Scott et al., 2021). The enzymes involved in xenobiotic metabolism are often also involved in the metabolism of endogenous compounds such as lipids, fatty acids, and hormones (Rekka et al., 2019). Despite well-known roles for both macrophages and dendritic cells in the breakdown of substrates affected by XME, such as fatty acids (Kelly et al., 2015), very little work has been carried out to date to identify expression of XME in these cells. Better understanding the role of immune cells in drug and xenobiotic metabolism is vital to determine local drug bioavailability, and how local drug metabolism can be altered in different disease states. For example, tumour-associated macrophages are typically found at high density in the tumour microenvironment (J. Zhou et al., 2020). If these cells could metabolise chemotherapeutics, then this would limit local drug bioavailability. However, if this knowledge was incorporated into the drug development process, then prodrugs could also be developed which take advantage of these cells to activate drugs to the bioactive state locally.

This chapter sought to generate and characterise immune cells differentiated from peripheral blood monocytes and investigate a potential role for these cells in the metabolism of xenobiotics. *In vivo*, once across the vasculature, peripheral blood monocytes are able to differentiate into both tissue macrophages and dendritic cells under steady state and inflammatory conditions (Epelman et al., 2014; Collin et al., 2018). This process can be replicated *in vitro* to generate monocyte-derived macrophages (MDM) (Italiani et al., 2017) or monocyte-derived dendritic cells (MoDC) (Cechim et al., 2019). The low numbers of macrophages and dendritic cells available in the peripheral blood and the technical challenges in isolating tissue resident cells

(Allen et al., 2017) means that deriving these cells from circulating monocytes is currently the only viable method to achieve high numbers of these primary cells for experimentation.

Differentiation of monocytes into MDM (M0 phenotype) can occur without external cytokine signalling, mediated by adherence to tissue culture plastic (Nielsen et al., 2020), although addition of granulocyte-macrophage colony-stimulating factor (GM-CSF), or more often macrophage colony-stimulating factor (M-CSF) aids the differentiation process (Bender, 2004). Macrophages can be further polarised in an inflammatory (M1) phenotype by additional culture with GM-CSF, IFN-γ and LPS, while a wound healing or tumour-associated (M2) phenotype can be generated by culture with M-CSF and IL-4 (Mantovani et al., 2004). Furthermore, MoDC can be generated by culturing with GM-CSF and IL-4 to produce a dendritic cell-like phenotype (Guironnet et al., 2001; Colic et al., 2003) and Langerhans cells by additional culture with TGFβ (Geissmann et al., 1998).

Here, peripheral blood monocytes were differentiated into macrophage and dendritic cell phenotypes and characterised. Macrophages were further polarised into M0, M1 and M2 phenotypes and, together with dendritic cells, were assessed for phase 1 XME expression by unbiased gene array, and relevant hits investigated further. A particular focus was given to cytochrome P450 enzymes, as these metabolise the majority of drugs in clinical use, and thus would have the greatest clinical impact.

Chapter aim: To investigate the capacity of monocyte-derived innate immune cells to metabolise xenobiotics by quantifying expression and function of cytochrome P450 enzymes.

Objectives:

- Differentiate monocytes into M0, M1 and M2 polarised MDM, MoDC, and Langerhans cells and quantify phenotypic changes.
- Complete and validate a gene array to identify basal mRNA expression of phase 1 metabolic enzymes for M0, M1 or M2 MDM and MoDC.
- Examine protein abundance of a key phase I XME.
- Perform functional assays to confirm enzyme activity.

3.2 Methods

- Primary immune cell isolation and monocyte differentiation (Section 2.2.8)
- qPCR and gene array (Section 2.3.1)
- Flow cytometry (Section 2.3.2)
- Western blot (Section 2.3.3)
- Enzyme function assay (Section 2.3.4)

3.3 Results

3.3.1 Confirming change in cell phenotype following differentiation from peripheral blood monocytes

Initially, cell differentiation from monocyte to monocyte-derived cells was assessed by changes in gene expression. A panel of makers were chosen to confirm differentiation to the anticipated phenotype, quantifying pan immune cell markers, macrophage polarisation, dendritic cell markers, and other cell specific markers, detailed below. To further assess cell differentiation, changes in abundance of cell surface proteins corresponding to key immune and differentiation markers were examined by flow cytometry. The use of this method allows for population analysis and can show subpopulations that have different levels of protein abundance, so provides complementary analysis to gene expression, which quantifies average mRNA expression of a whole cell population.

3.3.1.1 Monocyte-derived macrophages (MDM)

To first assess phenotypic changes from monocytes, morphology was examined. Adherent monocytes (24 hours after plating onto tissue culture plastic) were uniformly spherical, dark in appearance and approximately 10 μm in diameter (Figure 3.1A). Following 6 days in culture, monocytes had differentiated into a population of cells displaying a mixed phenotype comprising large spherical cells with dark nuclei and light cytoplasm (often referred to as a ‘fried egg’ morphology) which were typically 30-

40 μm in diameter, alongside more elongated cells which were up to 100 μm in length (Figure 3.1B), morphology typical for MDM.

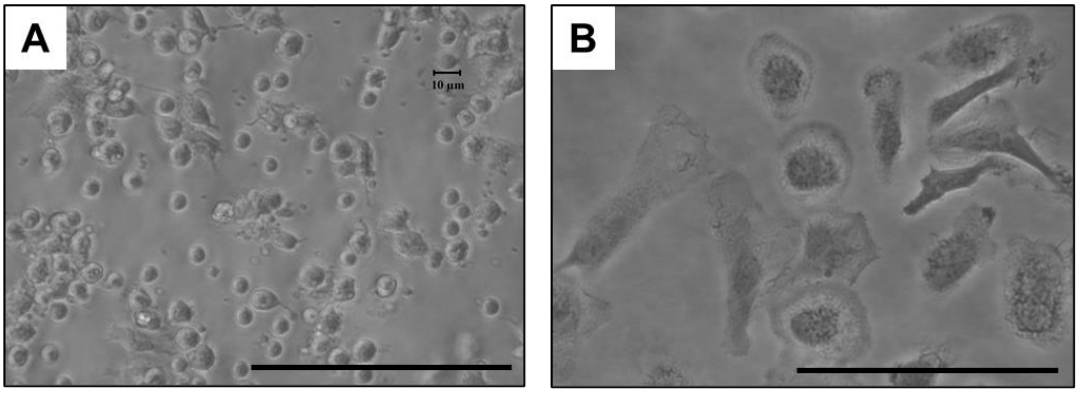


Figure 3.1. Monocyte and MDM morphology.
Adherent monocytes (**A**) and MDM following 6 days of differentiation (**B**). Images are representative of multiple isolations. Scale bar = 100 μm.

Next, gene and protein markers of MDM differentiation were assessed and compared to monocytes.

Firstly, CD11c, HLA-DR and CD115 (M-CSF receptor) were used as pan immune cell markers to ensure the overall immune cell profile was unchanged (Figure 3.2). Gene expression of CD11c ($p=0.47$) and CD115 ($p=0.58$) were unchanged, while HLADR was reduced 4-fold ($p=0.015$) in MDM compared to monocytes. When quantifying cell surface protein abundance (Figure 3.3) CD11c was similarly unchanged between cell types ($p=0.36$), while HLADR was increased (3-fold; $p=0.017$) in MDM compared to monocytes. These data suggest that both monocytes and MDM are of myeloid origin and express the expected markers.

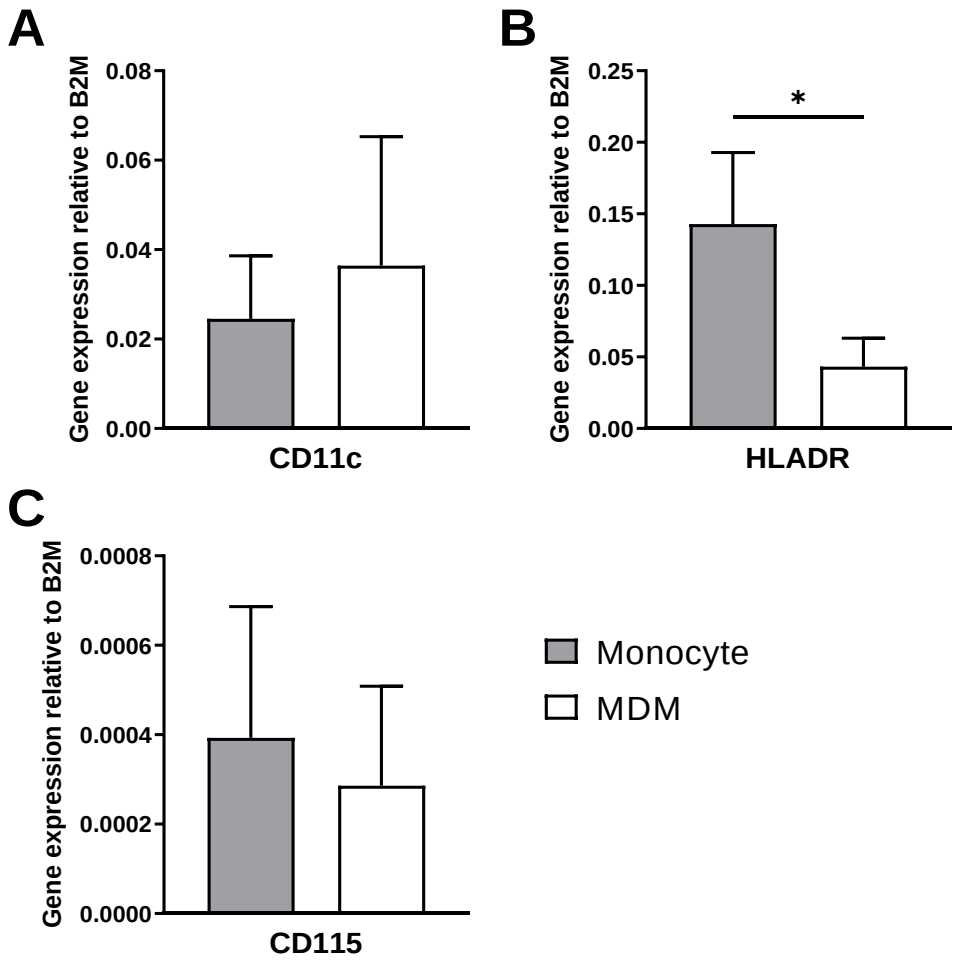


Figure 3.2. Gene expression of pan immune cell markers in monocytes and MDM.

Monocytes isolated from buffy coat on the same day (grey) compared to MDM following 7-day culture (white). Gene expression of pan immune cells markers *CD11c* (**A**), *HLADR* (**B**), and *CD115* (**C**) were analysed by qPCR, calculated relative to the reference control *β2-microglobulin*, Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by paired Student's t test; *p<0.05.

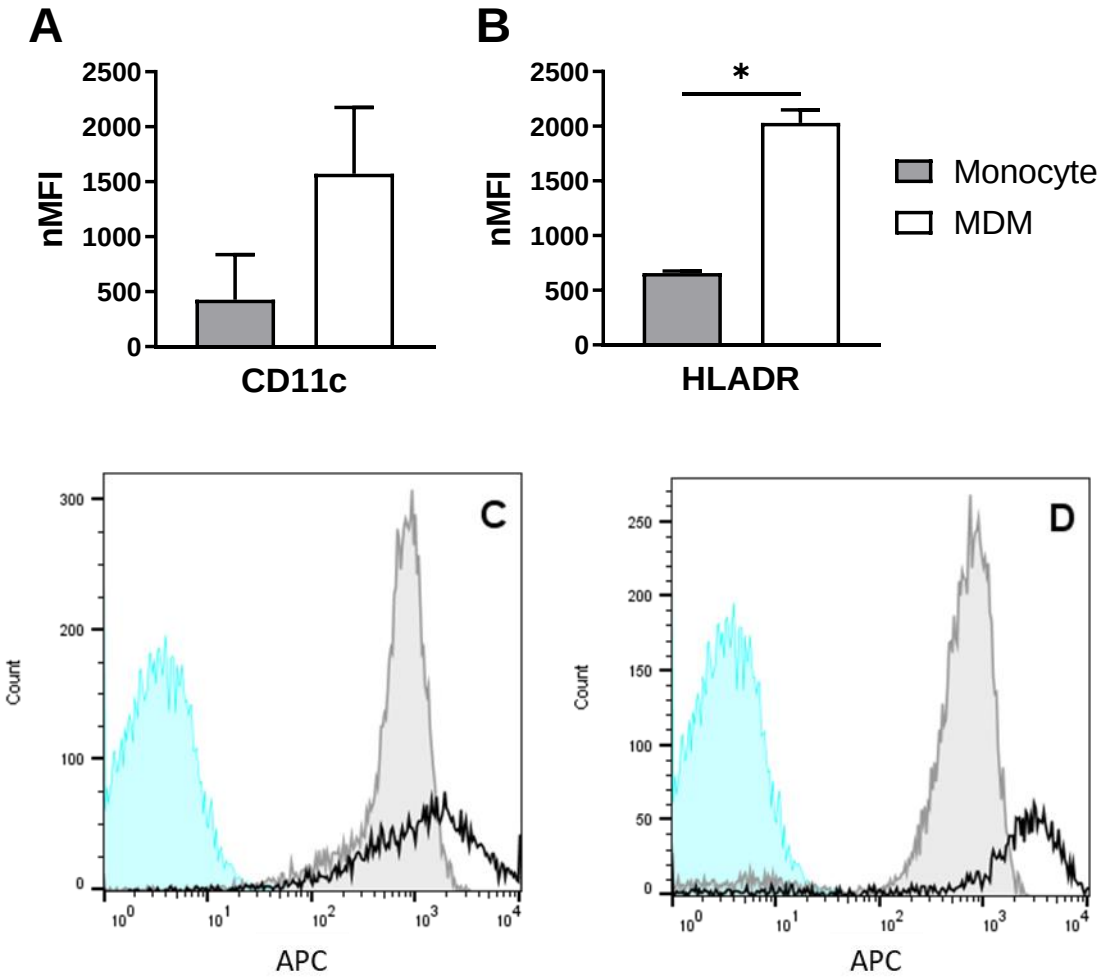


Figure 3.3. Cell surface protein abundance of pan immune markers in monocytes and MDM.

Monocytes isolated from buffy coat on the same day (grey) compared to MDM following 7-day culture (white). Abundance of pan immune markers CD11c (**A**) and HLA-DR (**B**) were measured by flow cytometry, shown as median fluorescence index (MFI) normalised to the IgG control, with representative histograms displayed for CD11c (**C**) and HLA-DR (**D**) comparing IgG control (blue, filled), monocytes (grey, filled) and MDM (black, unfilled). Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by paired Student's t test; *p<0.05.

Next, markers for monocytes, MDM, and multi-cell markers were examined, to confirm cell phenotype (Figure 3.4). CD14, a monocyte marker, was reduced in MDM (9-fold; p=0.029), while CPM, an MDM marker was increased (5-fold; p=0.015). CD36 was increased in MDM compared to monocytes (13-fold; p=0.0084), as was CD204 (2-fold; p=0.033). Protein abundance of some of these markers (Figure 3.5) revealed CD14 was decreased in MDM (5-fold; p=0.0045), although subpopulations of MDM appear to differentially express CD14. Additionally, no change was observed in CD36 (p=0.060) compared to monocytes. Unfortunately, it was not possible to obtain an antibody to assess protein abundance of CPM. Taken together these data confirm that monocyte have successfully differentiated into an MDM phenotype.

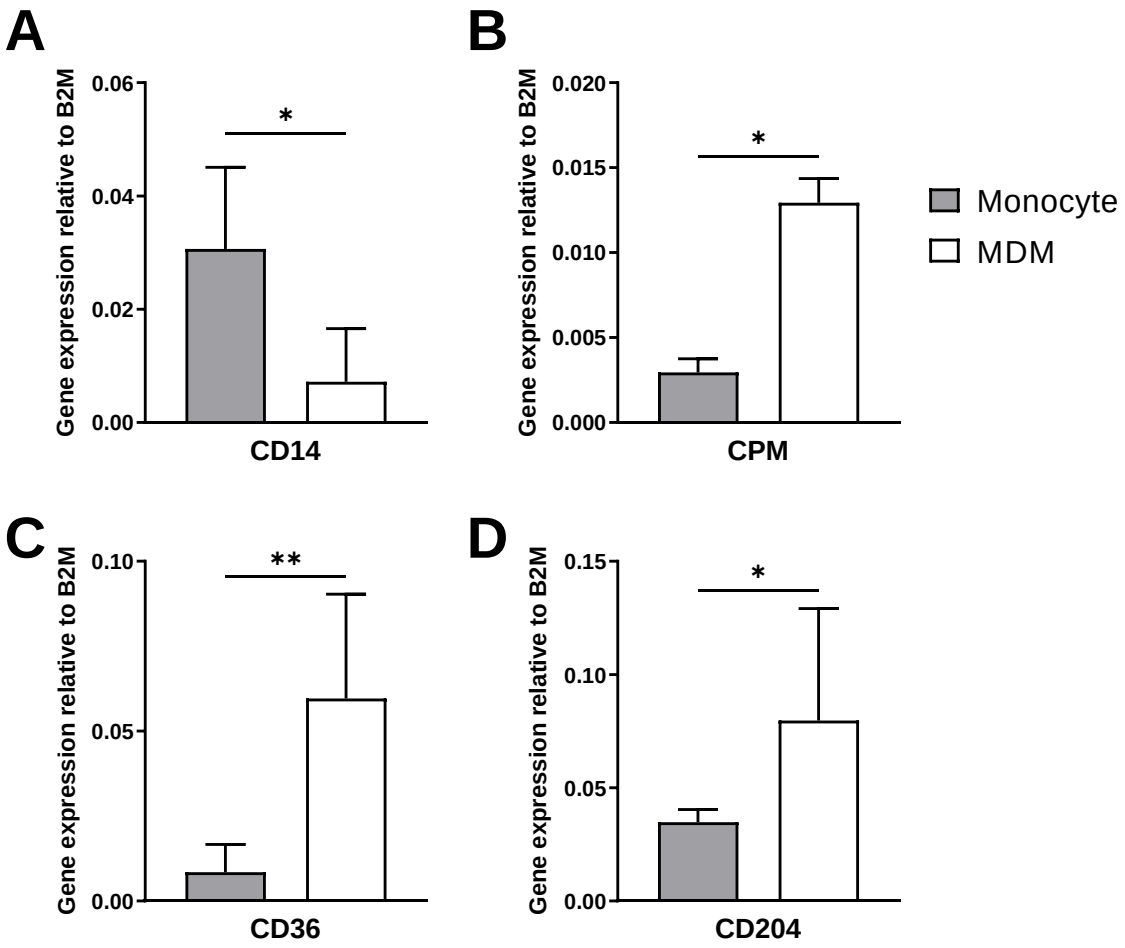


Figure 3.4. Gene expression of additional markers in monocytes and MDM.
Monocytes isolated from buffy coat on the same day (grey) compared to MDM following 7-day culture (white). Gene expression of additional markers for *CD14* (**A**), *CPM* (**B**), *CD36* (**C**), and *CD204* (**D**) were analysed by qPCR, calculated relative to the reference control *β2-microglobulin*, Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by paired Student's t test; *p<0.05, **p<0.01.

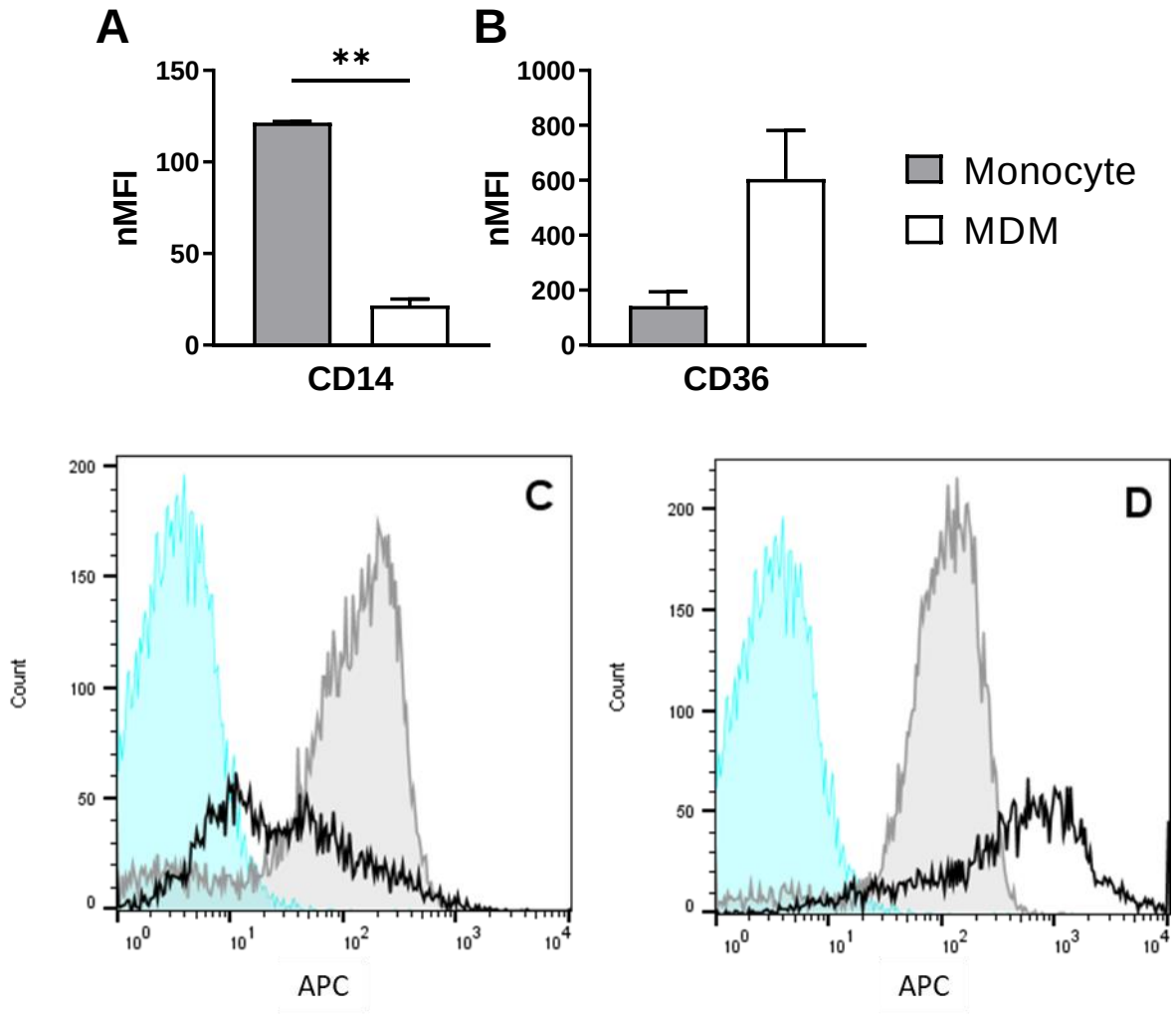


Figure 3.5. Cell surface protein abundance of additional immune markers in monocytes and MDM.

Monocytes isolated from buffy coat on the same day (grey) compared to MDM following 7 day culture (white). Abundance of additional immune markers CD14 (**A**) and CD36 (**B**) were measured by flow cytometry, shown as median fluorescence index (MFI) normalised to IgG control, with representative histograms displayed for CD14 (**C**) and CD36 (**D**) comparing IgG control (blue, filled), monocytes (grey, filled) and MDM (black, unfilled). Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by paired Student's t test; **p<0.01.

Next, markers of MDM polarisation were investigated to determine if MDM produced by monocyte differentiation were polarised towards an inflammatory (M1) or wound-healing (M2) phenotype (Figure 3.6). M1 marker CD80 was reduced 16-fold in MDM compared to monocytes (p=0.0025), while CD86 was unchanged (p=0.61). M2 markers CD163 (p=0.37) and CD206 (p=0.39) were unchanged between the two cell types. These data suggest that differentiating monocytes into MDM without additional cytokine supplements causes MDM to adopt a neutral M0 phenotype and not polarise to M1 or M2 phenotypes.

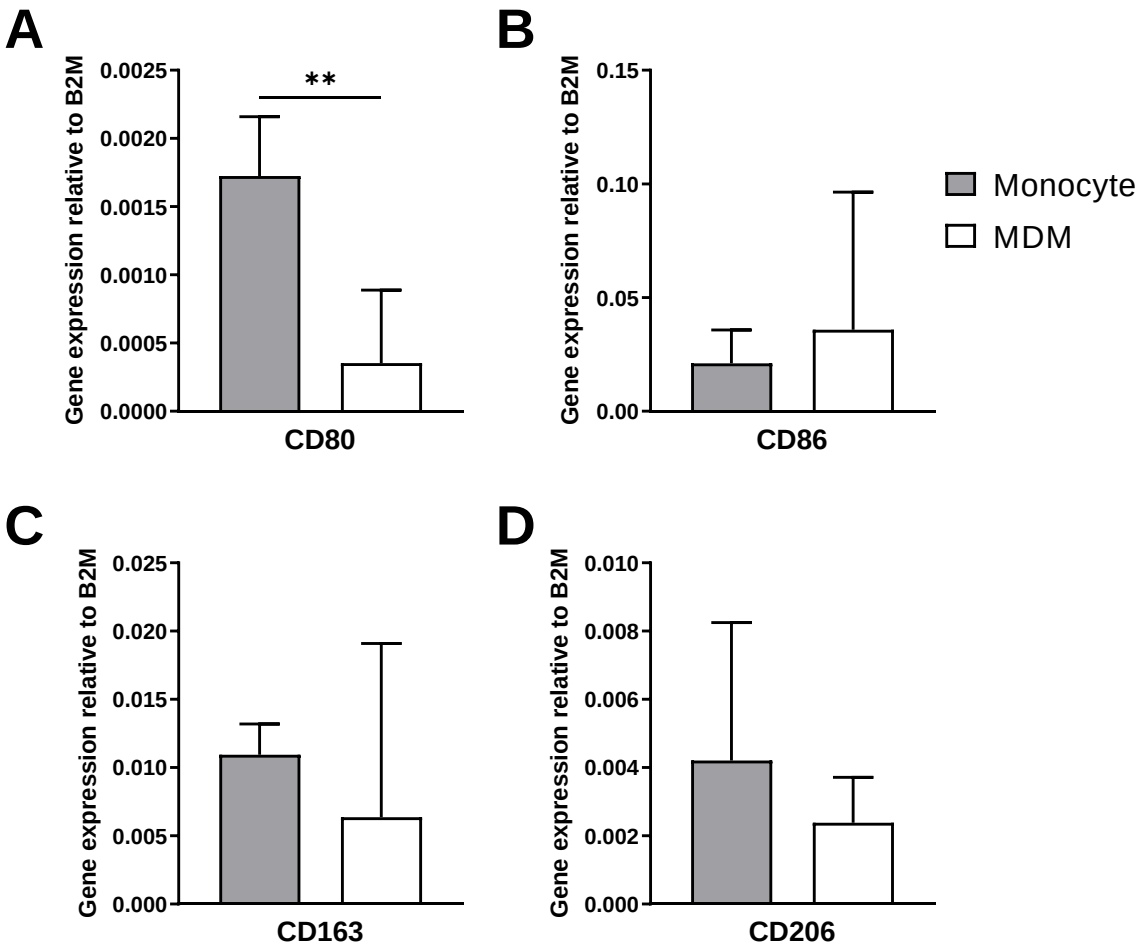


Figure 3.6. Gene expression of MDM polarisation markers in monocytes and MDM.

Monocytes isolated from buffy coat on the same day (grey) compared to MDM following 7 day culture (white). Gene expression of MDM polarisation markers *CD80* (**A**) and *CD86* (**B**) for M1, and *CD163* (**C**) and *CD206* (**D**) for M2 phenotypes were analysed by qPCR, calculated relative to the reference control *β2-microglobulin*, Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by paired Student's t test; **p<0.01.

Additionally, markers of dendritic cells were investigated to ensure MDM had not instead adopted a dendritic cell phenotype, as these cells can also be derived from monocytes (Figure 3.7). CD1a (p=0.44) and CDH1 (p=0.52) were unchanged between the two cell types, while CD207, a Langerhans cell specific marker was significantly reduced in MDM compared to monocytes (37-fold; p=0.045). When assessing protein abundance, CD207 was similarly unchanged (p=0.067), although CD1a was slightly increased (1.5-fold; p=0.013) in MDM compared to monocytes despite minimal difference compared to IgG control (Figure 3.7). Together these data suggest that MDM do not adopt a dendritic cell phenotype.

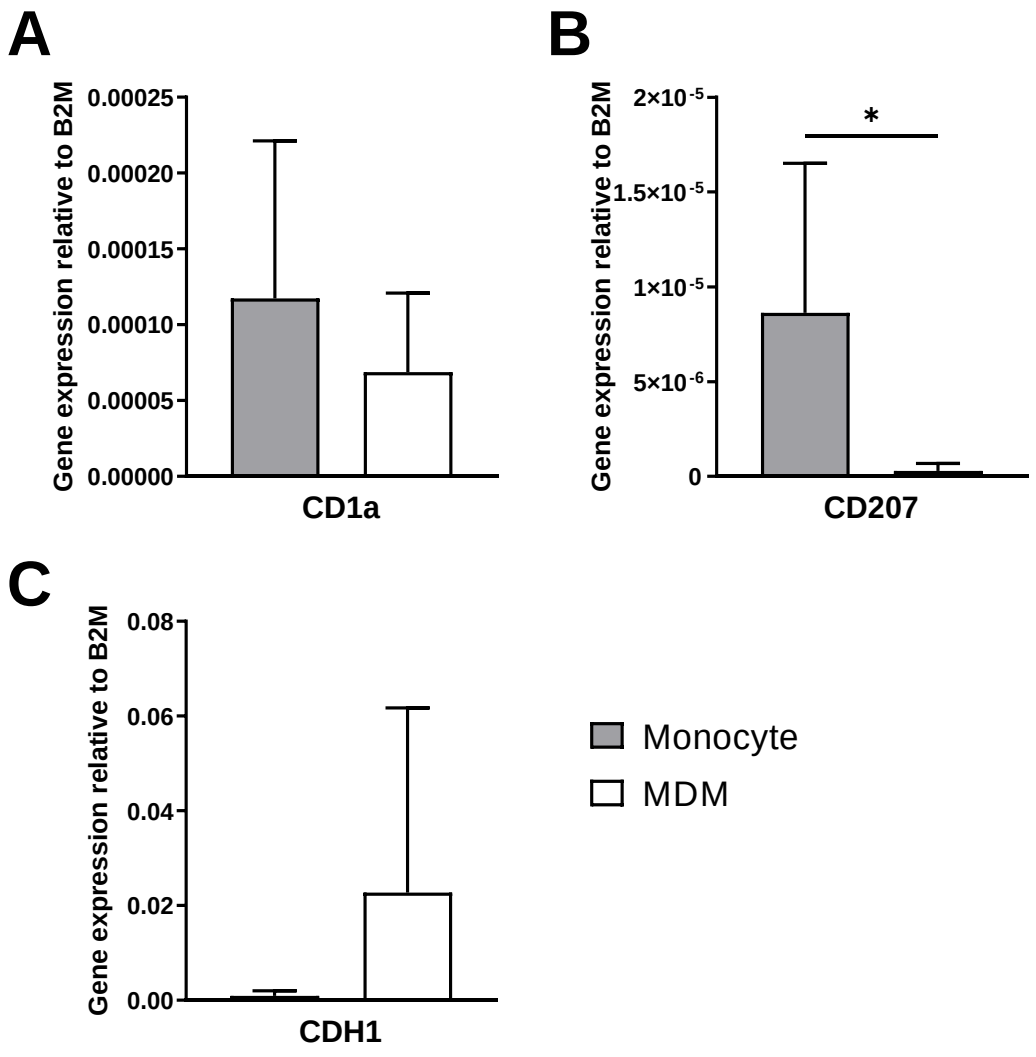


Figure 3.7. Gene expression of DC and LC markers in monocytes and MDM.
Monocytes isolated from buffy coat on the same day (grey) compared to MDM following 7-day culture (white). Gene expression of DC and LC markers *CD1a* (**A**), *CD207* (**B**), and *CDH1* (**C**) were analysed by qPCR, calculated relative to the reference control *β2-microglobulin*, Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by paired Student's t test; *p<0.05.

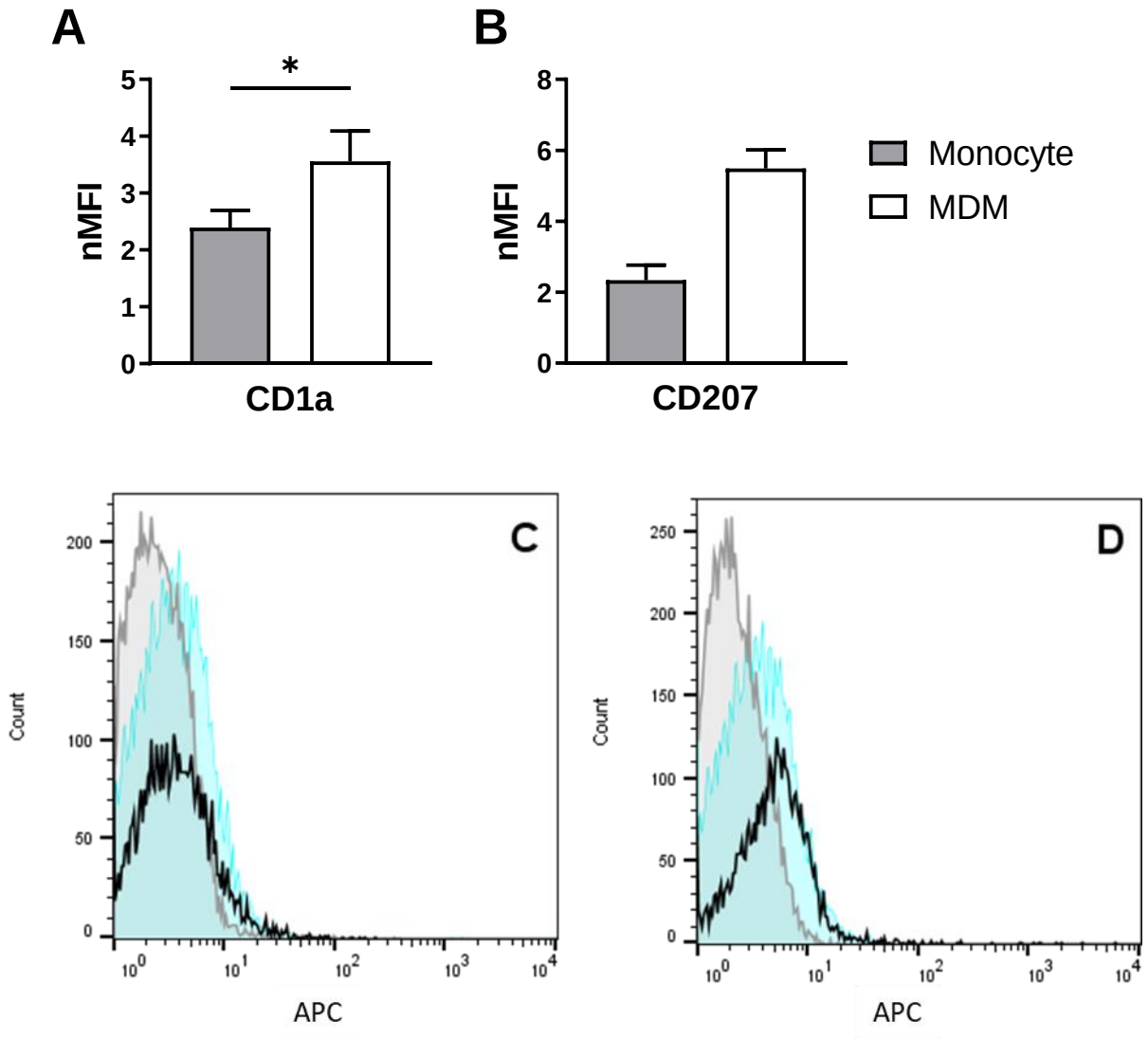


Figure 3.8. Cell surface protein abundance of DC markers in monocytes and MDM.

Monocytes isolated from buffy coat on the same day (grey) compared to MDM following 7-day culture (white). Abundance of DC markers CD1a (**A**) and CD207 (**B**) were measured by flow cytometry, shown as median fluorescence index (MFI) normalised to IgG control, with representative histograms displayed for CD1a (**C**) and CD207 (**D**) comparing IgG control (blue, filled), monocytes (grey, filled) and MDM (black, unfilled). Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by paired Student's t test; *p<0.05.

To further assess shift in marker expression between monocytes and MDM, principal component analysis (PCA) was used. PCA of the gene expression data showed completely separate clustering of monocytes and MDM (Figure 3.9A) while the protein abundance data showed some overlap, likely due to the reduced number

of markers used in this analysis, but remained largely distinct (Figure 3.9B), confirming that this gene and protein panel can determine the differentiation status of these cells.

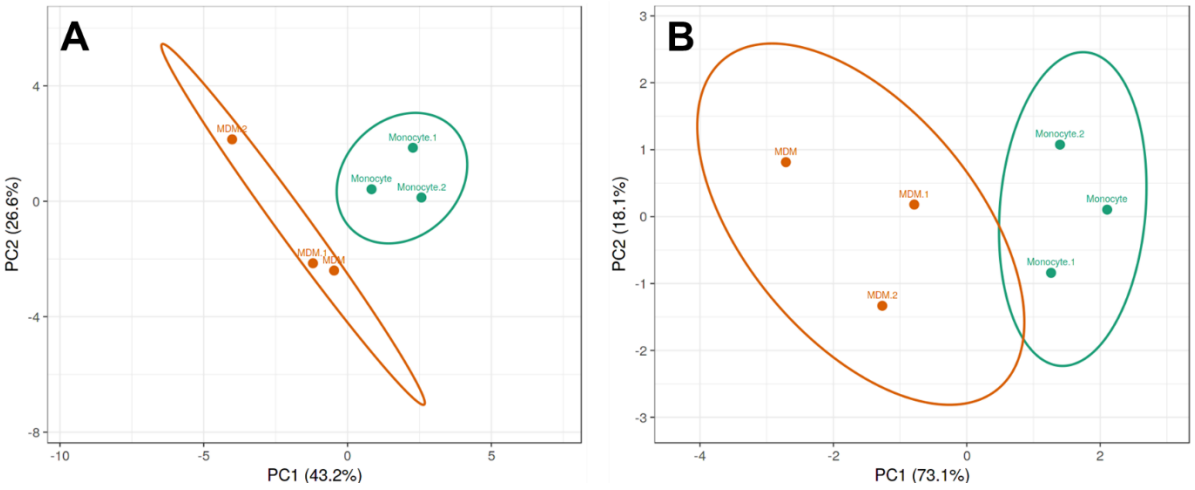


Figure 3.9. PCA of monocyte and MDM phenotypes
PCA analysis from gene expression data (**A**) and flow cytometry data (**B**), calculated with ClustVis software, comparing monocytes (blue) and MDM (orange); n=3.

3.3.1.2 Monocyte-derived dendritic cells (MoDC)

Initial observations of MoDC morphology showed dendrite shaped cells, typically 50-150 µm in length. These cells were dark throughout without any visible nuclei or other intracellular features observed under light microscopy. This contrasts with monocyte morphology described previously, of uniformly spherical cells that were 10 µm in diameter (Figure 3.10).

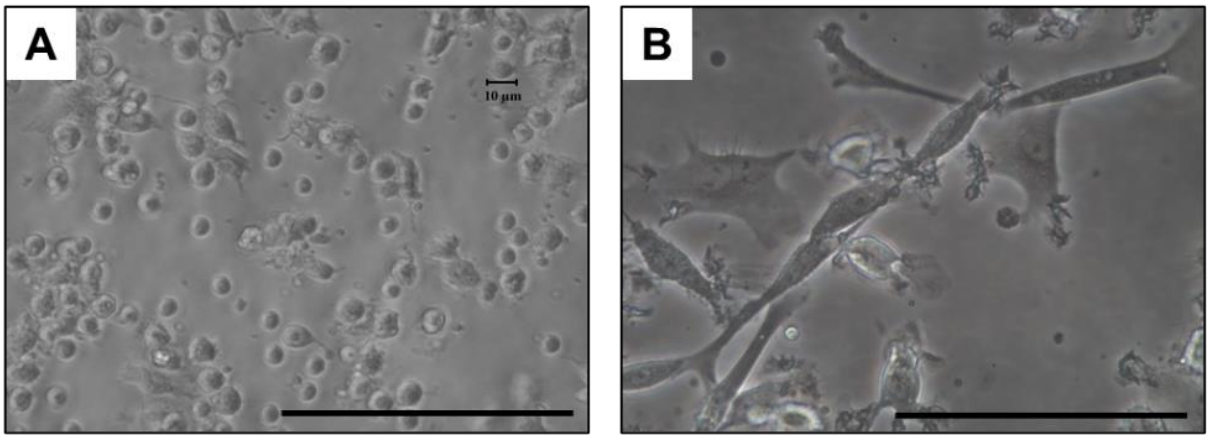


Figure 3.10. Monocyte and MoDC morphology.
Adherent monocytes 24 hours after plating on standard tissue culture plastic (**A**) and MoDC following 6 days of differentiation (**B**). Images are representative of multiple isolations. Scale bar = 100 µm.

Next, gene and protein markers of MoDC differentiation were assessed and compared to monocytes.

The gene expression of pan immune cell markers (Figure 3.11) was unchanged between monocytes and MoDC (CD11c p=0.95; HLA-DR p=0.061; CD115 p=0.79). Similarly, protein abundance for CD11c (p=0.99) and HLADR (p=0.34) were unchanged between the two cell types (Figure 3.12). These data show both monocytes and MoDC are of myeloid origin and similarly express these markers.

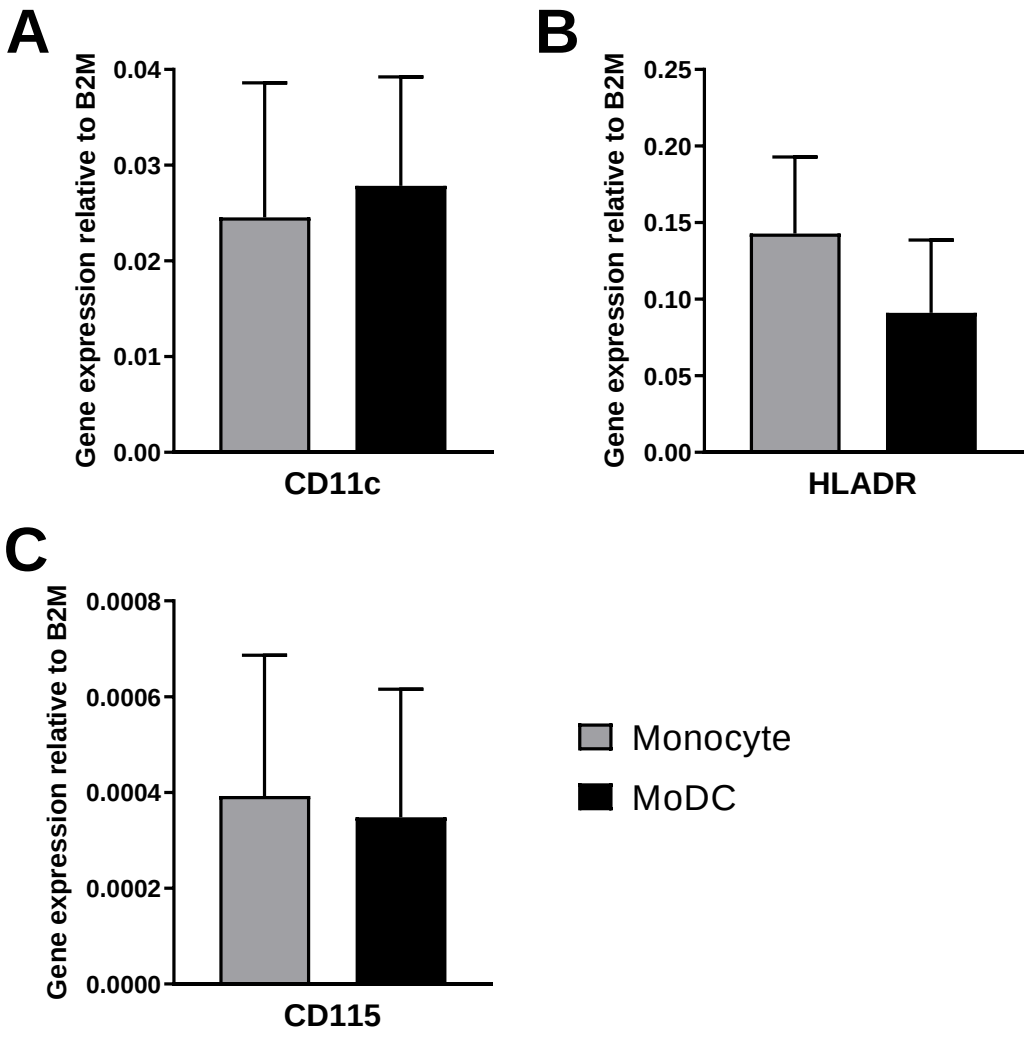


Figure 3.11. Gene expression of pan immune cell markers in monocytes and MoDC.

Monocytes isolated from buffy coat on the same day (grey) compared to MoDC following 7 day culture (black). Gene expression of pan immune cells markers *CD11c* (**A**), *HLADR* (**B**), and *CD115* (**C**) were analysed by qPCR, calculated relative to the reference control *β2-microglobulin*, Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by paired Student's t test.

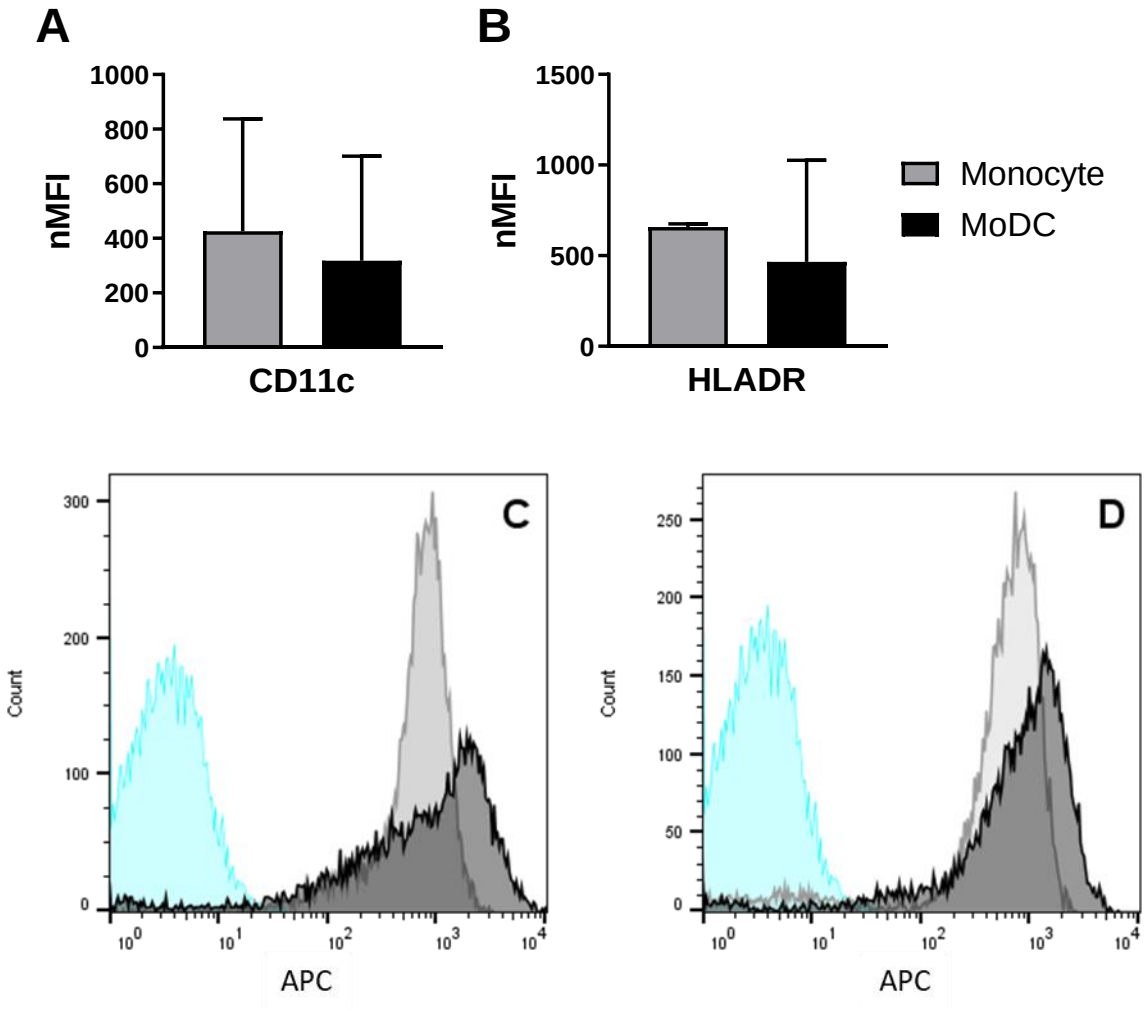


Figure 3.12. Cell surface protein abundance of pan immune cell markers in monocytes and MoDC.

Monocytes isolated from buffy coat on the same day (grey) compared to MoDC following 7 day culture (black). Abundance of pan immune markers CD11c (**A**) and HLADR (**B**) were measured by flow cytometry, shown as median fluorescence index (MFI) normalised to IgG control, with representative histograms displayed for CD11c (**C**) and HLADR (**D**) comparing IgG control (blue), monocytes (grey) and MoDC (black). Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by paired Student's t test.

Next, markers for monocytes, MDM, and multi-cell markers were examined, first at gene level (Figure 3.13). CD14, a monocyte marker, was reduced in MoDC (32-fold; p=0.040), while CPM, an MDM marker, was unchanged (p=0.22). CD36 was unchanged in MoDC compared to monocytes (p=0.22), while CD204 was increased in MoDC compared to monocytes (2-fold; p=0.046). At protein level, CD14 was similarly decreased in MoDC (55-fold; p=0.0070), but a reduction in CD36 was also seen (3-fold; p=0.0063) compared to monocytes (Figure 3.14). These data confirm that monocytes have differentiated into a non-MDM phenotype, as CPM was not significantly increased, but CD14 was strongly downregulated at both gene and protein level.

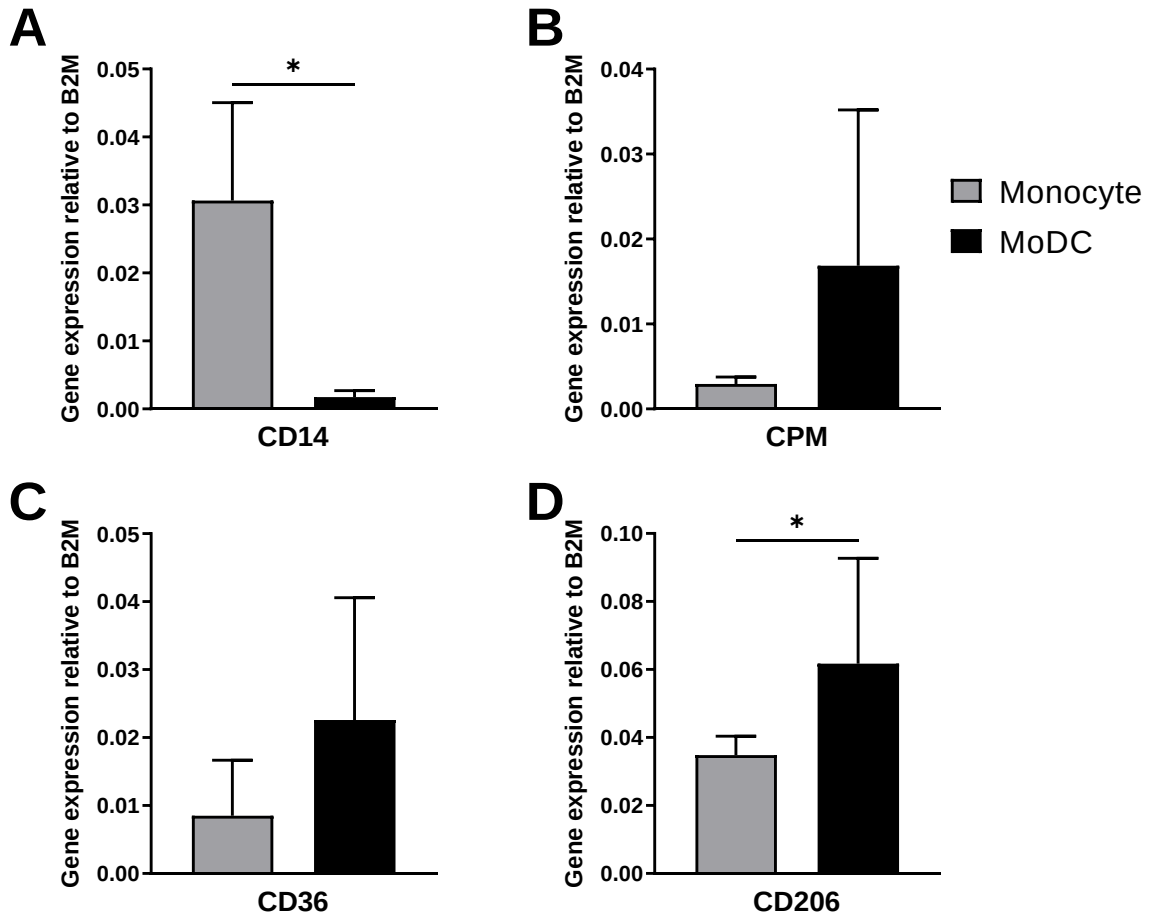


Figure 3.13. Gene expression of additional markers in monocytes and MoDC. Monocytes isolated from buffy coat on the same day (grey) compared to MoDC following 7-day culture (black). Gene expression of additional markers *CD14* (**A**), *CPM* (**B**), *CD36* (**C**), and *CD204* (**D**) were analysed by qPCR, calculated relative to the reference control *β2-microglobulin*, Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by paired Student's t test; *p<0.05.

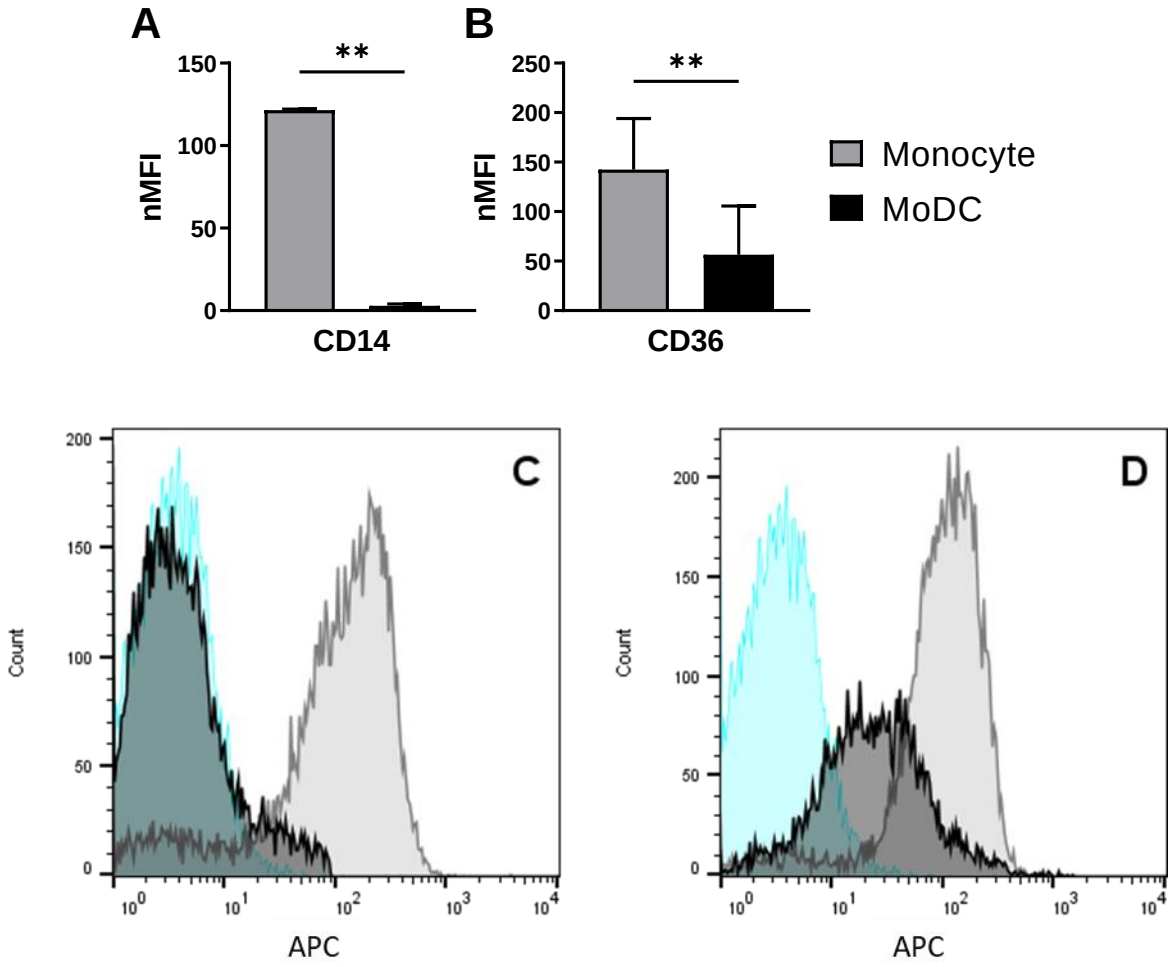


Figure 3.14. Cell surface protein abundance of additional immune markers in monocytes and MoDC.

Monocytes isolated from buffy coat on the same day (grey) compared to MoDC following 7-day culture (black). Abundance of additional immune markers CD14 (**A**) and CD36 (**B**) were measured by flow cytometry, shown as median fluorescence index (MFI) normalised to IgG control, with representative histograms displayed for CD14 (**C**) and CD36 (**D**) comparing IgG control (blue), monocytes (grey) and MDM (black). Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by paired Student's t test; **p<0.01.

In addition, markers of MDM polarisation were examined in MoDC (Figure 3.15). This revealed M1 MDM markers were unchanged (CD80 p=0.11; CD86 p=0.65). However, CD163 was reduced in MoDC compared to monocytes (60-fold; p=0.001), and CD206 was increased (23-fold; p=0.021).

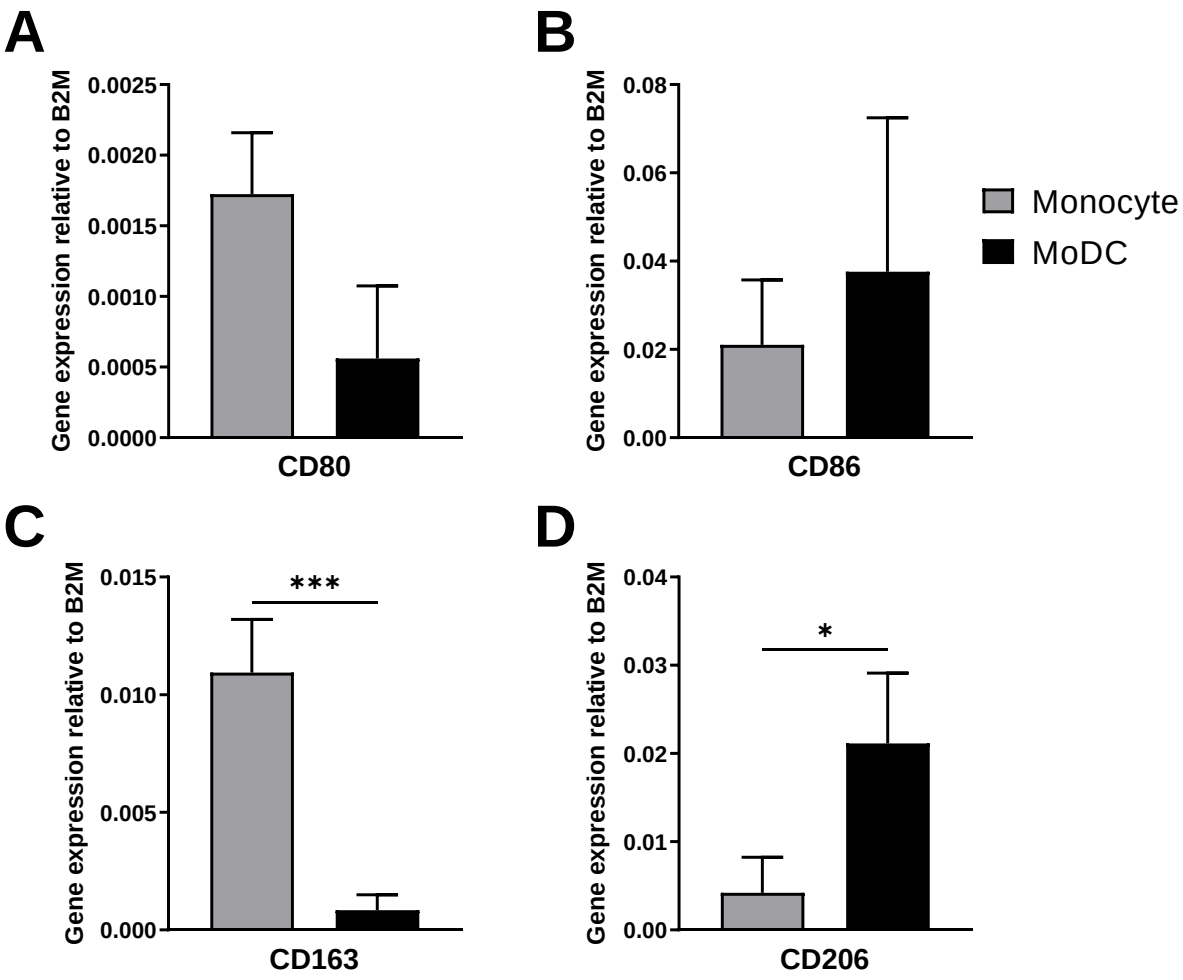


Figure 3.15. Gene expression of MDM polarisation markers in monocytes and MoDC.

Monocytes isolated from buffy coat on the same day (grey) compared to MoDC following 7-day culture (black). Gene expression of MDM polarisation markers *CD80* (**A**) and *CD86* (**B**) for M1, and *CD163* (**C**) and *CD206* (**D**) for M2 phenotypes were analysed by qPCR, calculated relative to the reference control *β2-microglobulin*, Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by paired Student's t test; *p<0.05, ***p<0.005.

Finally, markers of dendritic cells were investigated to confirm MoDC had adopted the correct phenotype at gene level (Figure 3.16). CD1a, a DC marker, was increased in MoDC compared to monocytes (6-fold; p=0.043). Similarly, increases in CD207, a marker for Langerhans cells, was seen in MoDC (7-fold; p=0.036) and CDH1 (23-fold; p=0.019), suggesting MoDC could potentially produce a Langerhans cell phenotype. Similar changes were observed at protein level, with increases seen for both CD1a (3-fold; p=0.019) and CD207 (2-fold; p=0.025) compared to monocytes (Figure 3.17), although typically these changes were similar to the IgG control antibody. These data confirm that MoDC adopt a DC-like phenotype following differentiation from monocytes.

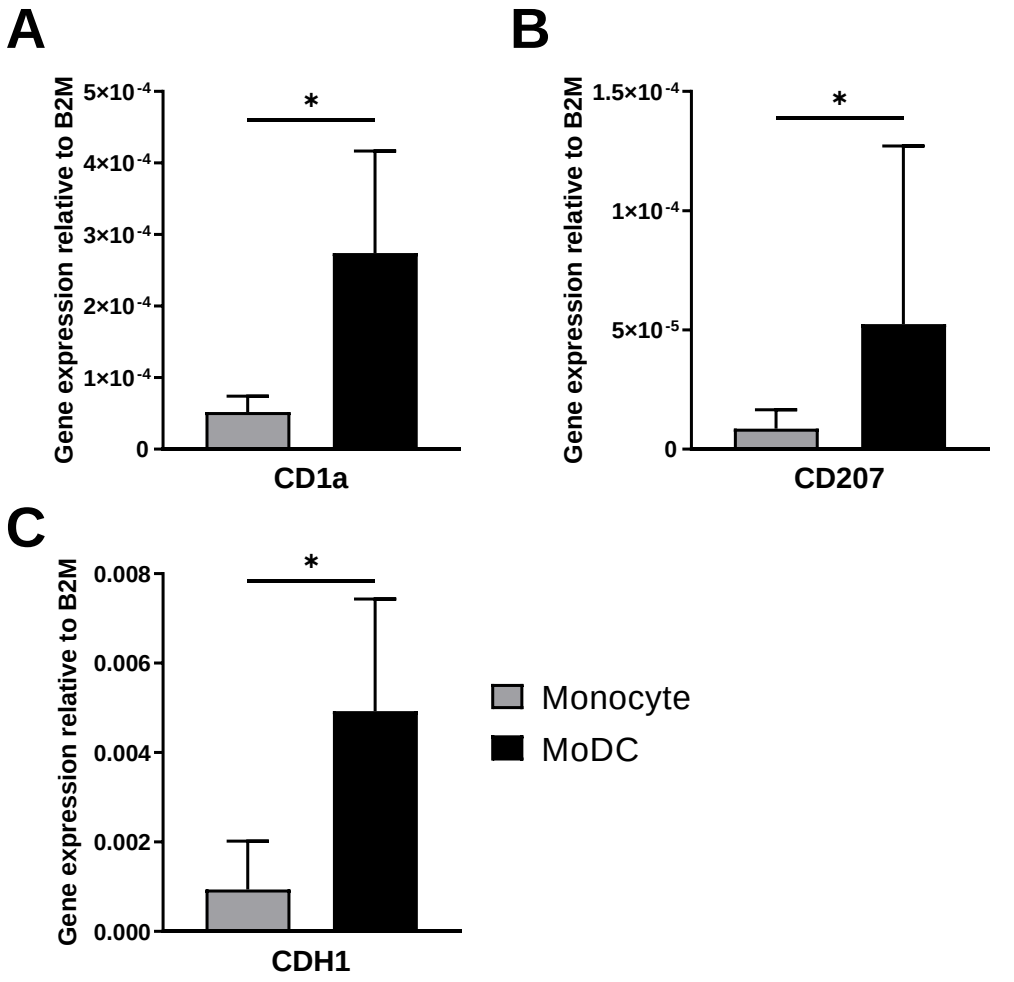


Figure 3.16. Gene expression of DC and LC markers in monocytes and MoDC.
Monocytes isolated from buffy coat on the same day (grey) compared to MoDC following 7-day culture (black). Gene expression of DC and LC markers *CD1a* (**A**), *CD207* (**B**), and *CDH1* (**C**) were analysed by qPCR, calculated relative to the reference control *β2-microglobulin*, Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by paired Student's t test; *p<0.05.

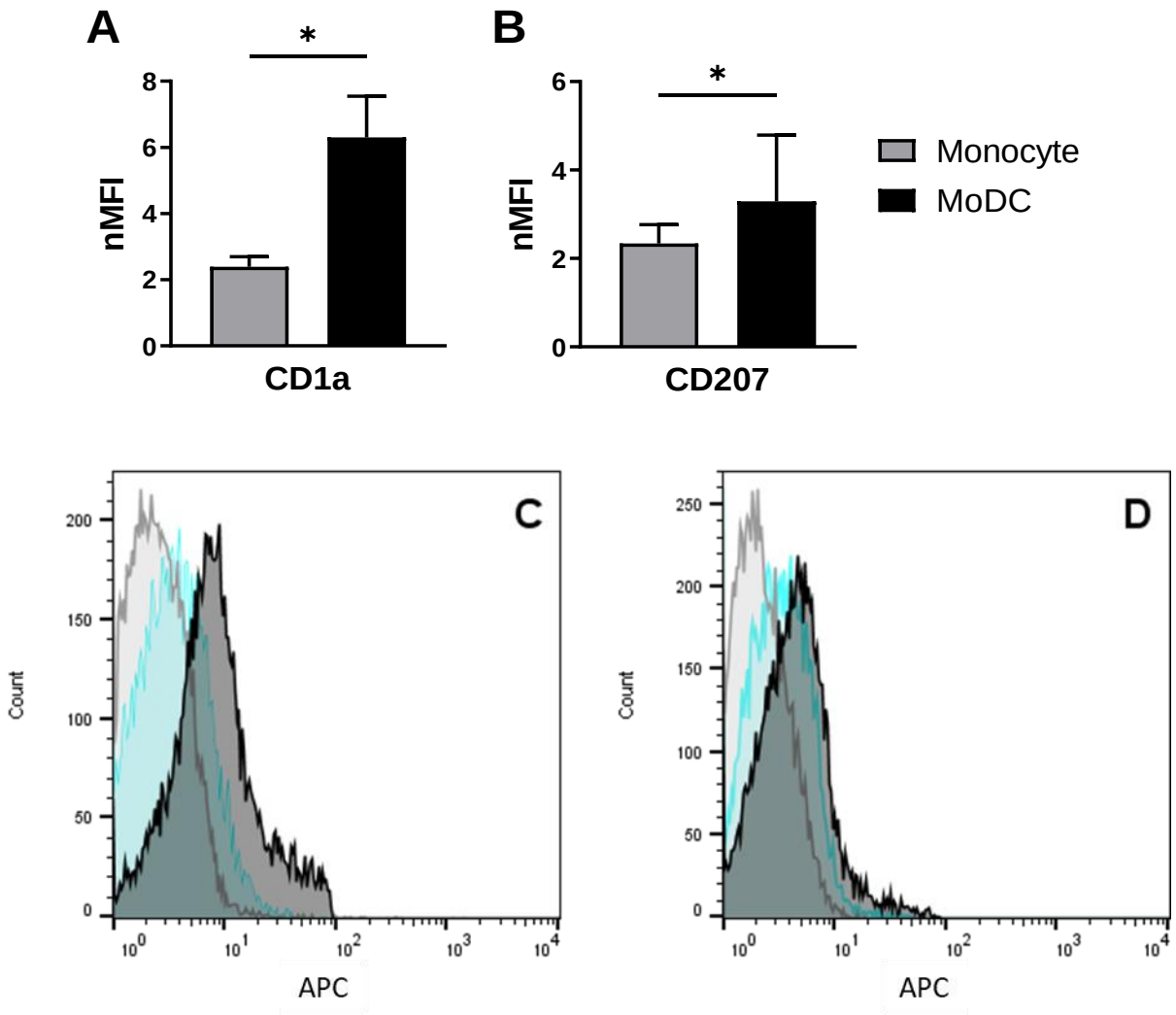


Figure 3.17. Cell surface protein abundance of DC markers in monocytes and MoDC.

Monocytes isolated from buffy coat on the same day (grey) compared to MoDC following 7-day culture (black). Abundance of DC markers CD1a (**A**) and CD207 (**B**) were measured by flow cytometry, shown as median fluorescence index (MFI) normalised to IgG control, with representative histograms displayed for CD1a (**C**) and CD207 (**D**) comparing IgG control (blue), monocytes (grey) and MDM (black). Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by paired Student's t test; *p<0.05.

A shift in gene expression profile was further confirmed by PCA, which showed monocytes and MoDC clustered separately and thus have distinct phenotypes (Figure 3.18A). The same was seen when assessing protein levels with PCA, with the two cell types clustering separately, confirming that this gene and protein panel could correctly identify the differentiation status of these monocyte-derived cells (Figure 3.18B).

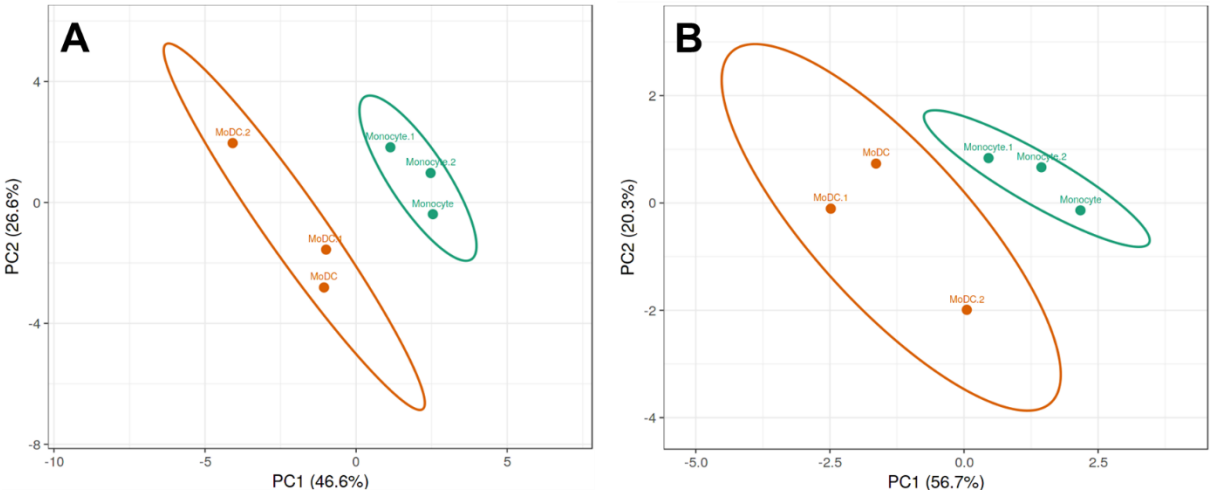


Figure 3.18. PCA of monocyte and MoDC phenotypes.
PCA analysis from gene expression data (**A**) and flow cytometry data (**B**), calculated with ClustVis software, comparing monocytes (blue) and MoDC (orange); n=3.

3.3.1.3 Monocyte-derived Langerhans cells (MoLC)

Monocyte-derived Langerhans cells (MoLC) morphology was comparable to MoDC, both in irregular shaping and size, however these cells lacked visible dendrites (Figure 3.19).

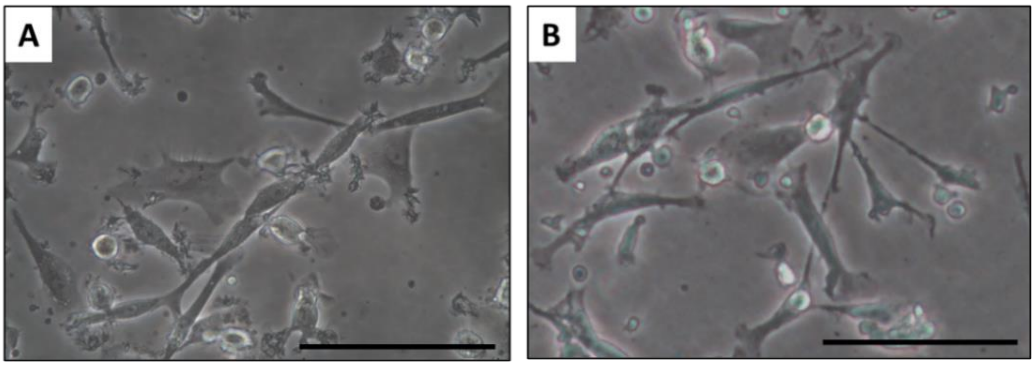


Figure 3.19. MoDC and MoLC morphology.
MoDC (**A**) and MoLC (**B**) following 6 days of differentiation. Images are representative of multiple isolations (MoDC) and n=3 isolations (MoLC). Scale bar = 100 µm.

Gene expression changes between monocytes and MoLC were also examined, although only key differentiation markers were tested for MoLC, notably CD1a (MoDC), CD14 (monocyte) and CD207 (MoLC). No significant changes were observed for any of these markers ($p>0.06$), contrary to the expected phenotype. CDH1 was

also examined, as it has previously been used as a marker for MoLC differentiation, but similarly this was unchanged compared to monocytes. These data suggest a lack of differentiation away from the monocyte phenotype, despite changes in cell morphology. Therefore, further analysis was not undertaken, and these cells were not implemented in future experiments.

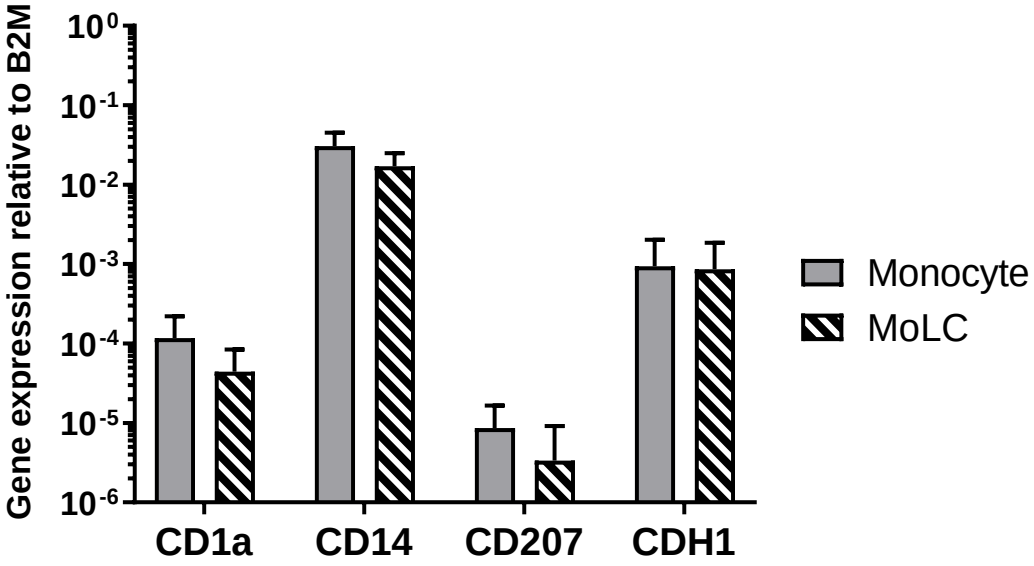


Figure 3.20. Gene expression of monocyte and MoLC markers.
Monocytes isolated from buffy coat on the same day (grey) compared to MoLC following 7-day culture (black striped). Gene expression of *CD1a*, *CD14*, *CD207,* and *CDH1* were analysed by qPCR, calculated relative to the reference control *β2-microglobulin*, Data are presented as mean ± SD of n=3 independent experiments.

3.3.1.4 Comparative expression of key markers in monocytes, MDM and MoDC

To further confirm differentiation of monocytes into MDM or MoDC, a direct comparison of key cell specific markers was undertaken (Figure 3.21). CD14, a marker of monocytes was significantly reduced in both MDM (9-fold; p=0.014) and MoDC (31-fold; p=0.010). CPM, a marker of MDM, was significantly increased in MDM compared to both monocytes (5-fold; p=0.0001) and MoDC (2-fold; p=0.014), although this marker was also increased in MoDC compared to monocytes (3-fold; p=0.023). Finally, CD1a, a marker for dendritic cells and MoDC was increased in MoDC compared to MDM (10-fold; p=0.025), although in contrast to previous data (Figure

3.16; compared by paired t test) this increase was not significant compared to monocytes (p=0.089).

As a result of these comparisons, it was determined that MDM and MoDC have distinct phenotypes, despite having the same monocyte origin, confirming the effect of differentiating these cells in media spiked with different cytokines.

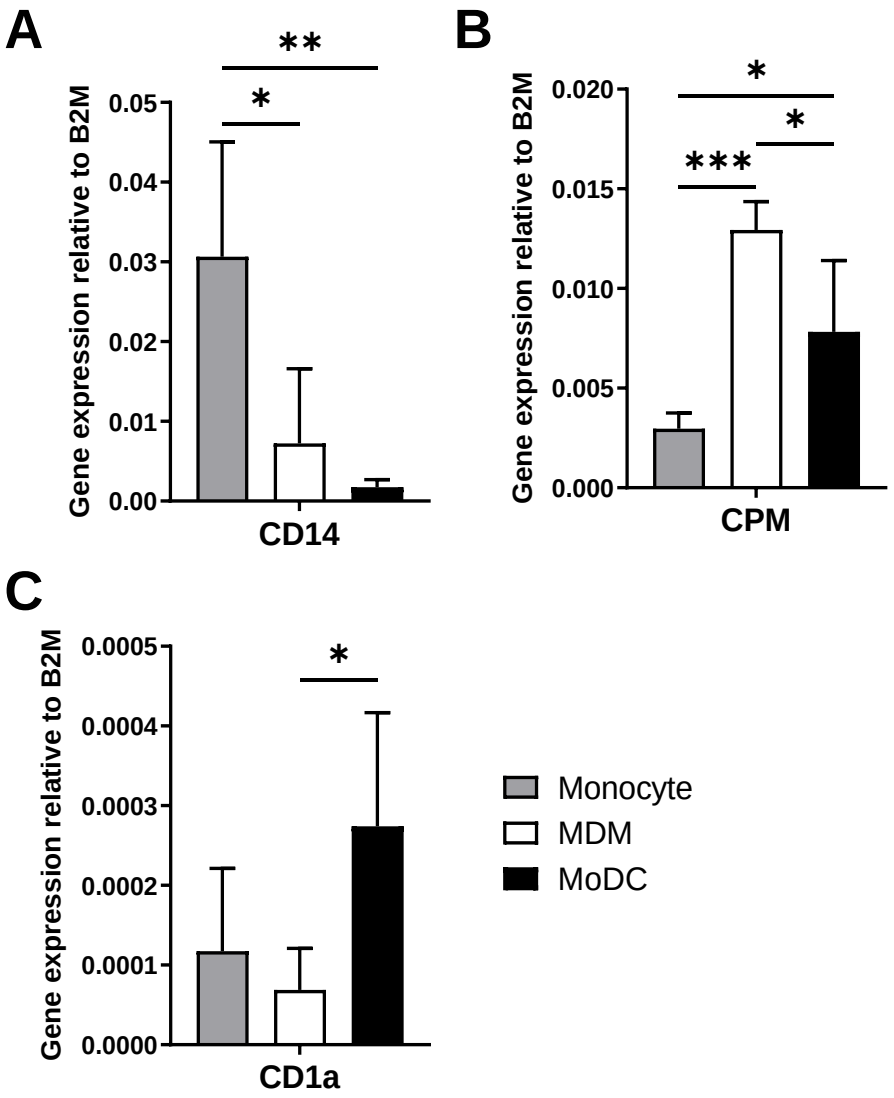


Figure 3.21. Gene expression of key monocyte, MDM, and MoDC markers.
Monocytes isolated from buffy coat on the same day (grey) compared to MDM (white) or MoDC (black) following 7-day culture. Gene expression of monocyte marker *CD14* (**A**), MDM marker *CPM* (**B**), and MoDC marker *CD1a* (**C**) were analysed by qPCR, calculated relative to the reference control *β2-microglobulin*. Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by one-way ANOVA; *p<0.05, **p<0.01, ***p<0.005.

3.3.1.5 MDM polarisation to M0, M1 and M2 phenotypes

To further assess the range of MDM activation states, polarisation into M1 (inflammatory) and M2 (wound healing) phenotypes was attempted. These cell phenotypes represent the extremes of macrophage functionality when exposed to certain microenvironmental conditions. In this study, MDM were polarised to M1 by addition of GM-CSF, and 24-hour treatment with LPS and IFN-γ, and to M2 by addition of M-CSF and 24-hour treatment with IL-4.

Firstly, MDM morphology was compared (Figure 3.22). In unstimulated M0 MDM (Figure 3.22A), populations typically comprised a mixed phenotype, with some cells retaining a 'fried egg' appearance, and others appearing more spindle shaped, with no defined nuclei. In contrast, M1 polarised MDM (Figure 3.22B) predominately appeared spindle shaped, while M2 polarised MDM (Figure 3.22C) tended towards a 'fried egg' appearance, with some visible dendrite-like structures.

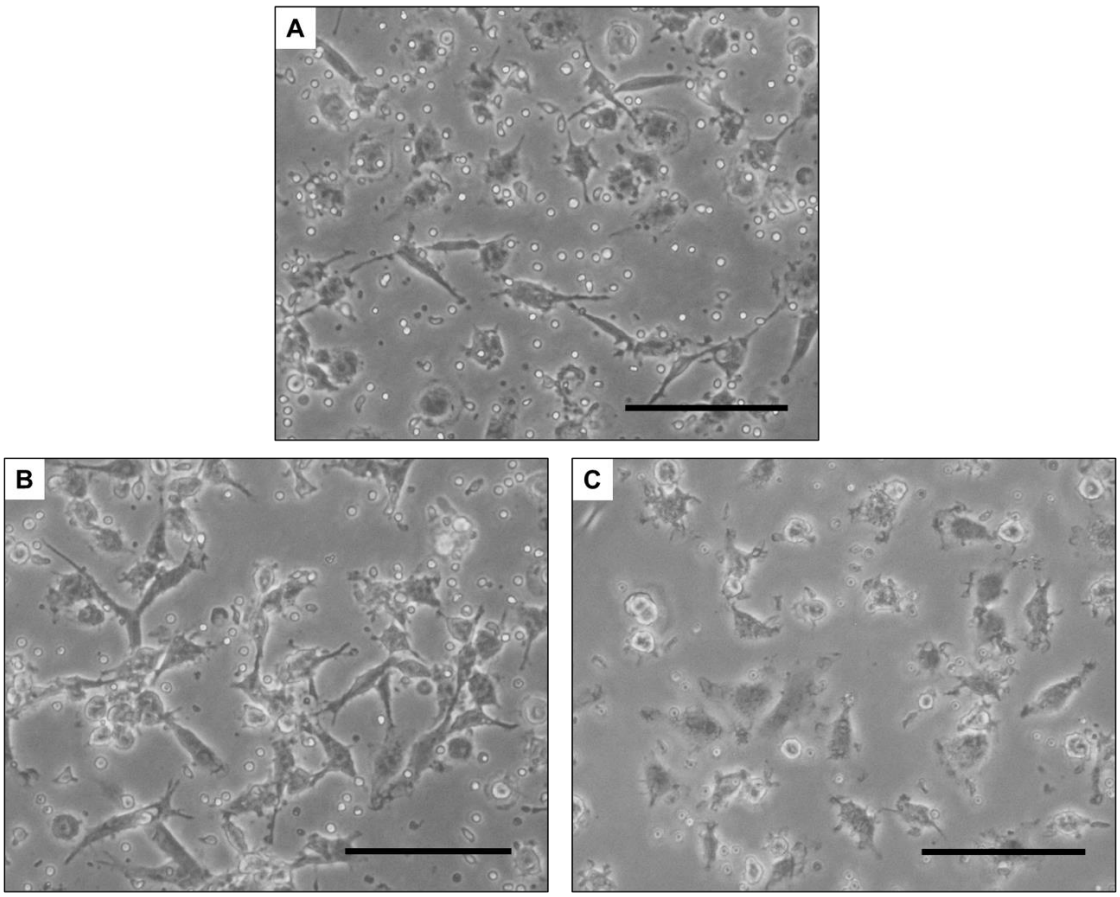


Figure 3.22. Morphology of M0, M1 and M2 MDM.
Unstimulated MDM (**A**), MDM stimulated to M1 phenotype by GM-CSF, IFN-γ, and LPS (**B**), or MDM stimulated to M2 phenotype by M-CSF and IL-4 (**C**) after 6 days of culture. Images are from a single matched isolation and representative of multiple isolations. Scale bar = 100 μm.

In addition, gene expression of polarisation markers was examined (Figure 3.23). CD80, a marker of M1 inflammatory macrophages, was increased in M1 polarised MDM compared to M0 (5-fold; p=0.046) while CD86 was increased in M1 MDM compared to both M0 (3-fold; p=0.039) and M2 (2-fold; p=0.049) MDM. Both CD163 and CD206, markers of M2 MDM, were increased in M2 MDM compared to M1 (2-fold; p=0.049 and 4-fold; p=0.045 respectively) but not M0 MDM (p>0.2), suggesting M0 are closer to an M2 phenotype than M1. Finally, CD1a, classically a DC marker, was used to ensure MDM had not differentiated to a DC phenotype as similar cytokine cocktails are used to polarise MDM. However, no differences were observed in CD1a expression between these cell types (p>0.1), indicating an MDM phenotype was preserved.

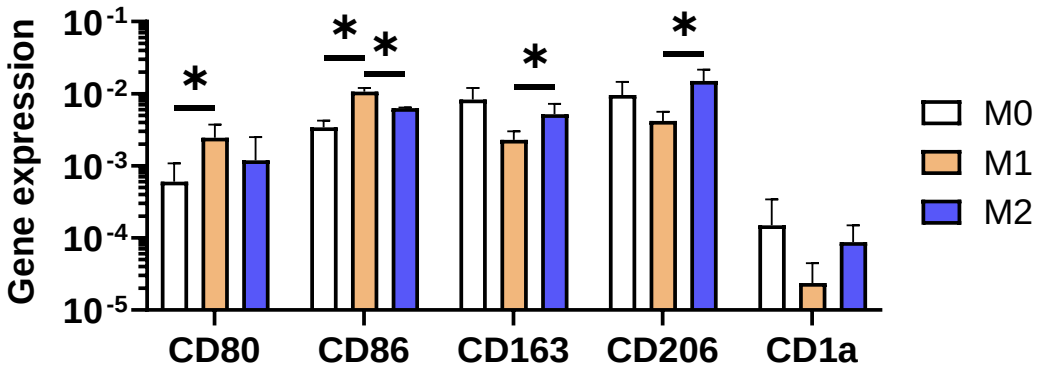


Figure 3.23. Gene expression of MDM polarisation markers in M0, M1, and M2 polarised MDM.
Unstimulated MDM (white) or MDM polarised to an M1 (tan) or M2 (purple) phenotype by 7-day culture. Gene expression of M1 markers *CD80* and *CD86*, M2 markers *CD163* and *CD206*, and DC marker *CD1a* were assessed by qPCR relative to the reference control *β2-microglobulin*. Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by one-way ANOVA; *p<0.05.

3.3.2 Quantifying gene expression of XME by gene array

Following characterisation of MDM and MoDC, and successful polarisation of MDM to M1 and M2 phenotypes, further investigations into the capacity of these cells to express phase 1 metabolic enzymes was assessed by gene array. When described here, expression is normalised to *β2-microglobulin* as a reference control, and CT categorised as low expression when $\leq 10^{-5}$, medium expression when $10^{-5} < - < 10^{-3}$, and high expression when $10^{-3} \leq$.

Firstly, flavin-containing monooxygenases (FMO) enzymes were measured (Figure 3.24). FMO1, 2 and 3 subtypes were either not detected or found at low levels across all cell types. In contrast, FMO4 and 5 had medium expression which was consistent between all cell types examined.

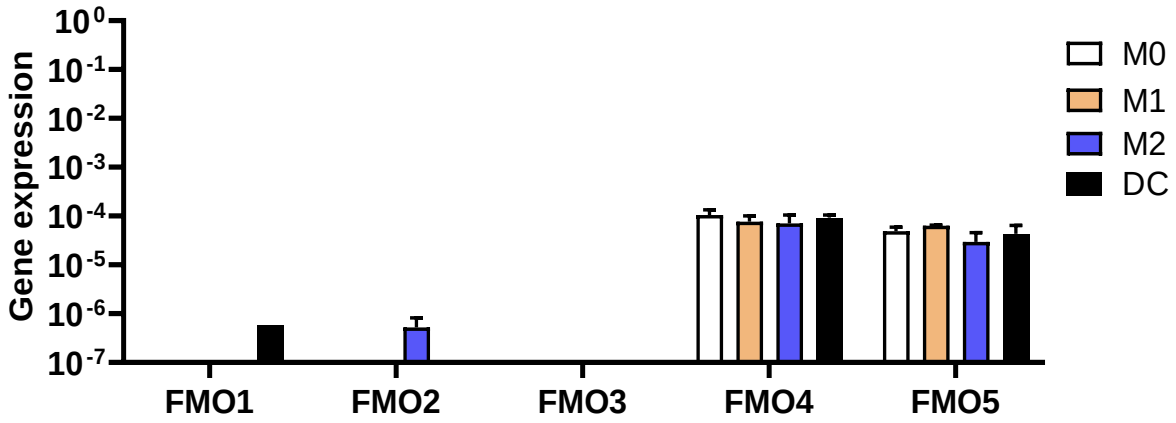


Figure 3.24. Expression of flavin-containing monooxygenase (FMO) by gene array.

MDM polarised to M0, M1 or M2 phenotypes, or MoDC following 7-day culture. Gene expression of flavin-containing monooxygenase enzymes was analysed by gene array, calculated relative to the reference control *β2-microglobulin*. Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by one-way ANOVA.

Next alcohol dehydrogenase (ADH) expression was analysed. ADH1 subtypes were not identified in any cell types. ADH4 was found at low levels in M0 only, and low levels of ADH6 were seen consistently between cell types, while high expression of ADH5 was found in all cell types. These data suggest that ADH5 is the main ADH isozyme in all cell types examined.

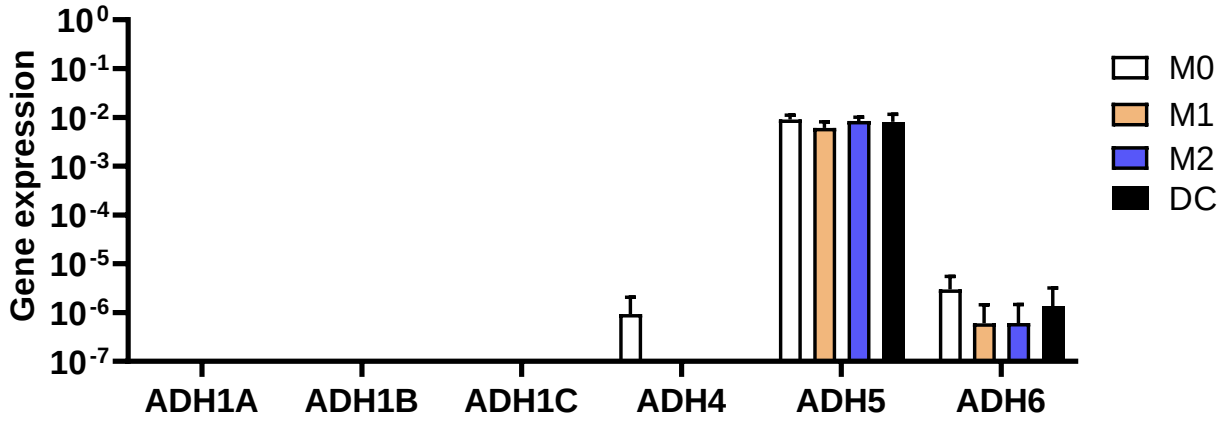


Figure 3.25. Expression of alcohol dehydrogenase (ADH) by gene array.
MDM polarised to M0, M1 or M2 phenotypes, or MoDC following 7-day culture. Gene expression of ADH enzymes was analysed by gene array, calculated relative to the reference control *β2-microglobulin*. Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by one-way ANOVA.

The expression of all isozymes of aldehyde dehydrogenase (ALDH) were detected by gene array to some extent (Figure 3.26). Low expression of ALDH1A3, 3A1, 3B2 and 8A1 and medium expression of ALDH 1B1, 4A1, 6A1, and 7A1 were detected in all cell types. High expression of ALDH2, 3A2, 3B1, and 9A1 was also observed which was unchanged between cell types. ALDH1A1 was expressed more highly in M0 MDM compared to all other cell types ($p<0.042$) while ALDH1A2 was expressed more highly in MoDC than all other cell types ($p<0.001$). Additionally, M1 MDM expressed significantly less ALDH5A1 compared to M0 (5-fold; $p=0.015$) and M2 MDM (3-fold; $p=0.05$).

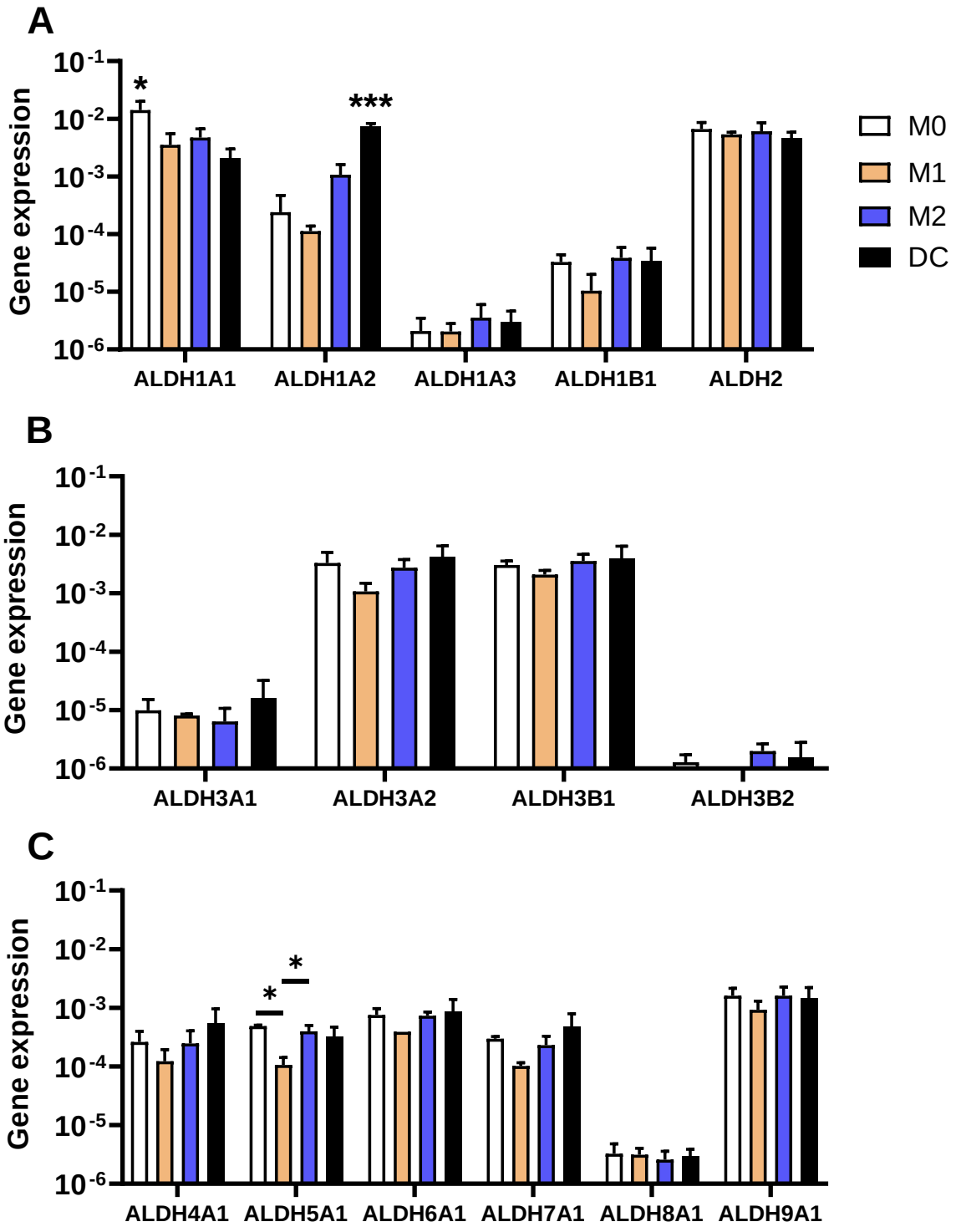


Figure 3.26. Expression of aldehyde dehydrogenase (ALDH) by gene array.

MDM polarised to M0, M1 or M2 phenotypes, or MoDC following 7-day culture. Gene expression of ALDH enzymes was analysed by gene array, calculated relative to the reference control *β2-microglobulin*. Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by one-way ANOVA; *p<0.05, ***p<0.005.

Next, cytochrome P450 superfamily (CYP) were assessed, however due to the large number of genes examined in this enzyme superfamily, data for isozymes which were undetected or had low relative expression ($\leq 10^{-5}$) will not be stated but can be seen in Figure 3.27 and Figure 3.28.

In subclass 1-3 (Figure 3.27), CYP1A1, 2D6, 2E1, 2R1 and 2W1 had medium expression, while 1B1, and 2S1 were highly expressed, all consistently between cell phenotypes. Medium expression of CYP3A5 and 3A7 was seen, with a significant increase in M1 MDM compared to MoDC for both 3A5 (6-fold; $p=0.030$) and 3A7 (9-fold; $p=0.030$). Additionally, when examining subclass 4-27 (Figure 3.28), CYP19A1, 21A2 and 24A1 had medium expression, while 27A1 and 27B1 were both highly expressed. CYP19A1 was significantly increased in MoDC compared to all other cell types (10-22-fold; $p<0.004$), while CYP21A2 was increased in MoDC compared to M0 (2-fold; $p=0.031$) and M1 MDM (7-fold; $p=0.0084$), but not M2 MDM ($p=0.072$).

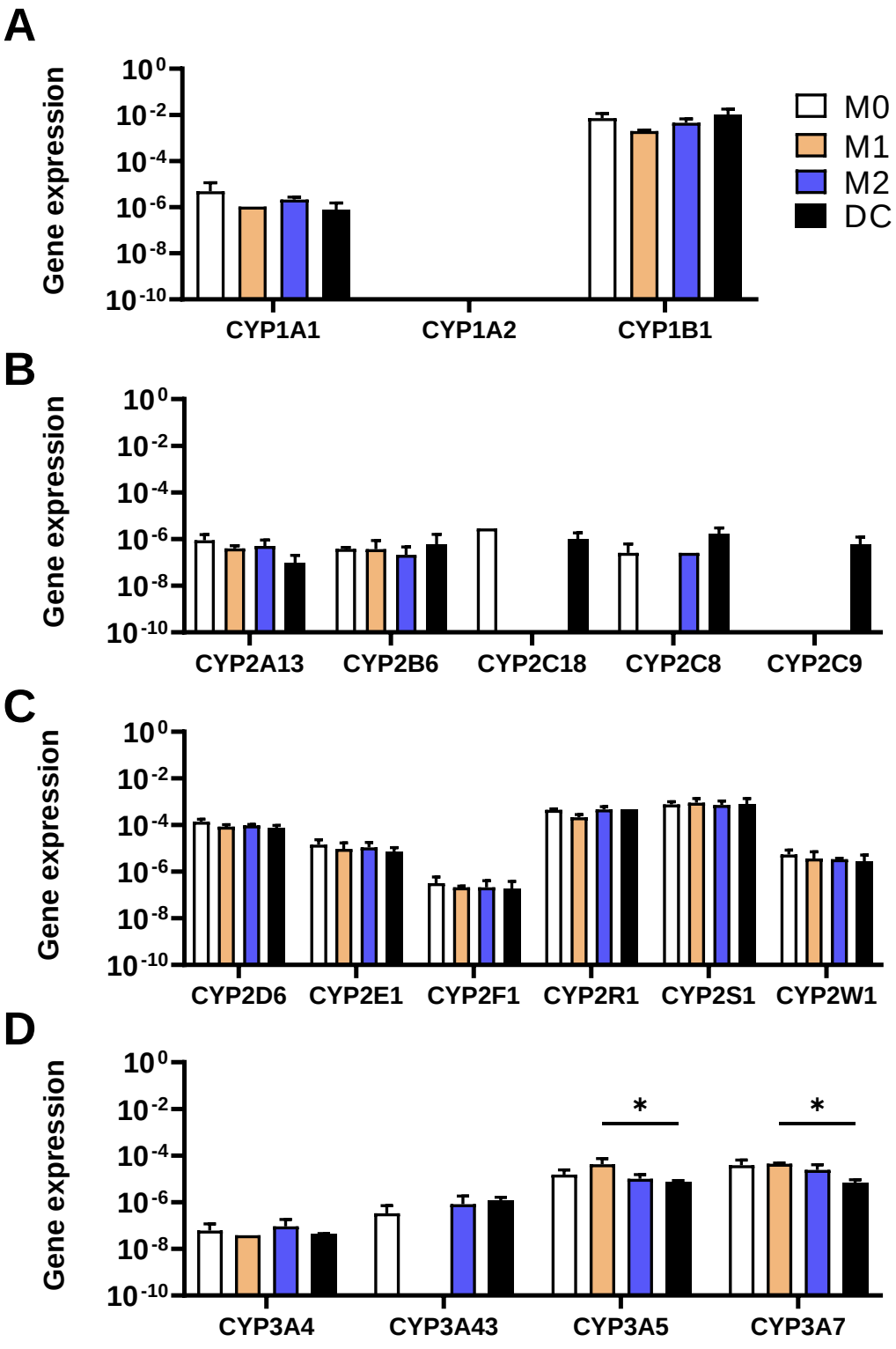


Figure 3.27. Expression of cytochrome P450 class 1-3 enzymes by gene array.

MDM polarised to M0, M1 or M2 phenotypes, or MoDC following 7-day culture. Gene expression of cytochrome P450 enzymes (subclass 1-3) was analysed by gene array, calculated relative to the reference control *β2-microglobulin*. Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by one-way ANOVA; *p<0.05.

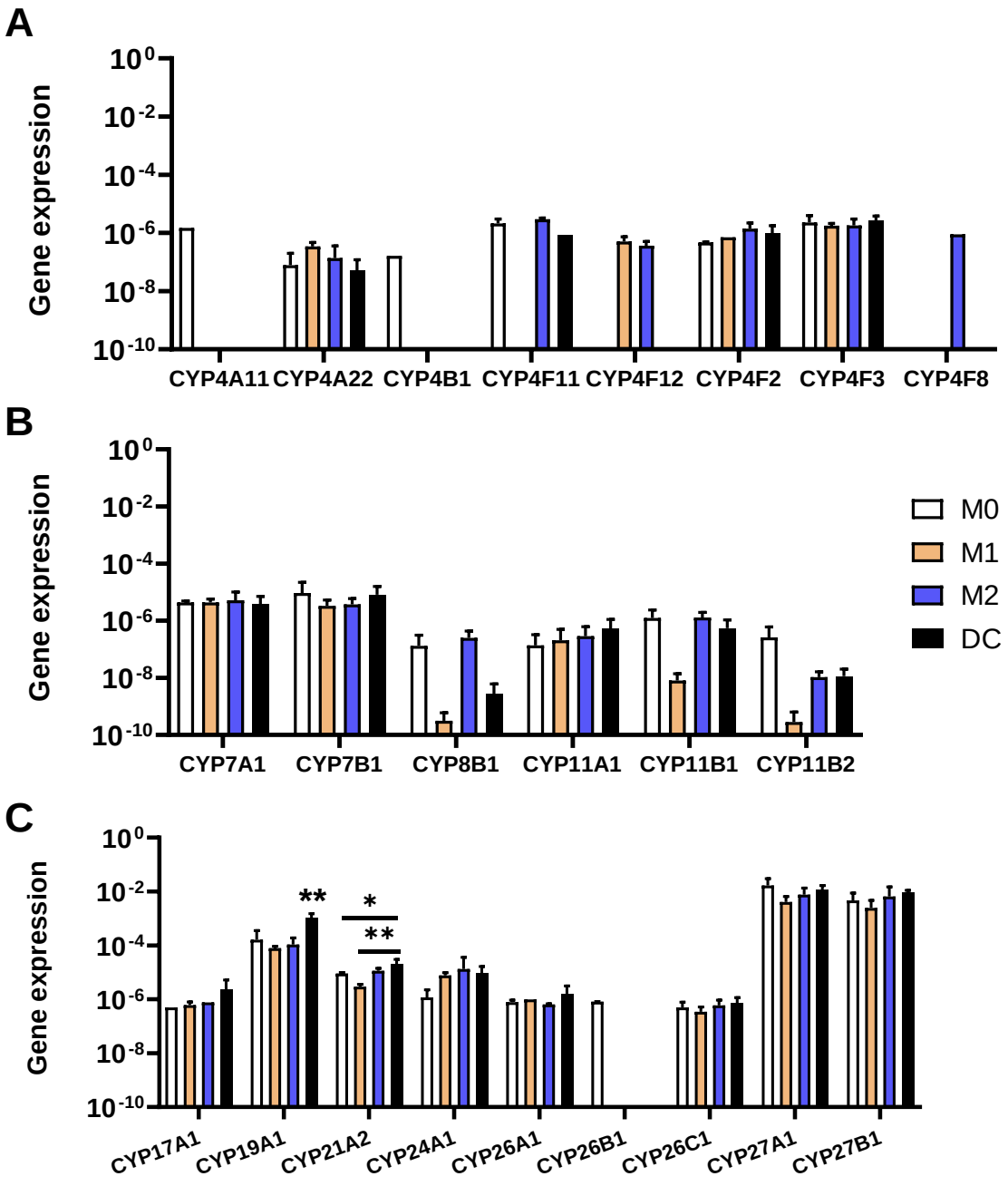


Figure 3.28. Expression of cytochrome P450 class 4-27 enzymes by gene array.

MDM polarised to M0, M1 or M2 phenotypes, or MoDC following 7-day culture. Gene expression of cytochrome P450 enzymes (subclass 4-27) was analysed by gene array, calculated relative to the reference control *β2-microglobulin*. Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by one-way ANOVA; *p<0.05, **p<0.01.

Finally, additional enzymes not part of an enzyme superfamily were examined separately (Figure 3.29). DHRS2, XDH and MAOB had low expression in all cell types, while CEL had medium expression. Most enzymes examined had high expression, notably DPYD, HSD17B10, GZMA, GZMB, MAOA, and PTGS1, which were unchanged between cell types.

ESD was increased in MoDC compared to M1 (2-fold; p=0.038), while PTGS2 was increased in M1 MDM compared to all other cell types (43- to 77-fold; p=0.0003). In addition, UCHL1 was increased in MoDC compared to M1 (11-fold; p=0.0093) and M2 (3-fold; p=0.026) MDM, while UCHL3 was increased in MoDC compared to all other cell types (2- to 7-fold; p<0.0033).

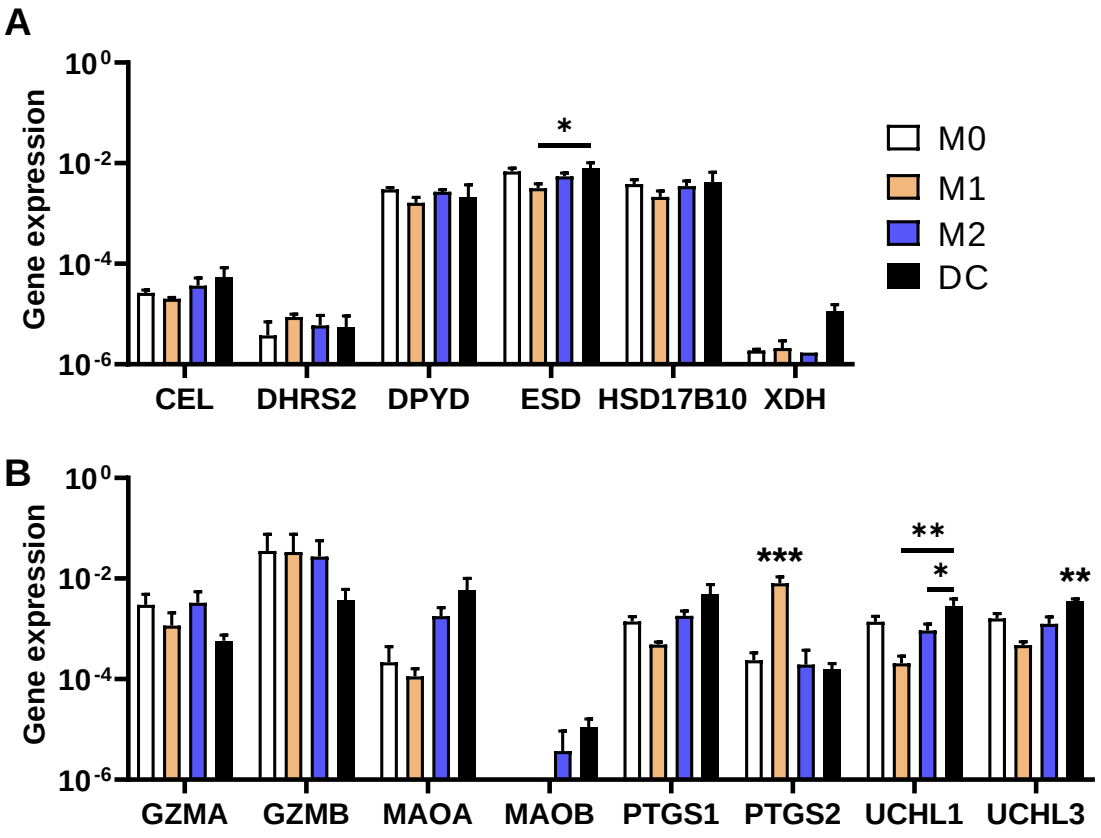


Figure 3.29. Expression of additional enzymes by gene array.

MDM polarised to M0, M1 or M2 phenotypes, or MoDC following 7-day culture. Gene expression of additional phase 1 XME was analysed by gene array, calculated relative to the reference control *β2-microglobulin*. Data are presented as mean ± SD of n=3 independent experiments with statistically significance differences determined by one-way ANOVA; *p<0.05, **p<0.01, ***p<0.005.

To further compare global XME expression between cell types, comparisons were made between M0 MDM and MoDC to examine the differences between the two cell types, as well as comparisons between M0, M1 and M2 MDM to examine differences between XME capacity in differentially polarised MDM.

Firstly, M0 and MoDC were compared by both heat map alignment, and PCA clustering, which both confirm that these cell types have distinct expression of the XME assessed by this gene array (Figure 3.30).

Similar comparisons between M0, M1, and M2 MDM found that M1 MDM were distinct from M0 and M2 MDM, clustering separately by heat map and PCA, but M0 and M2 MDM were indistinguishable by expression of XME genes (Figure 3.31).

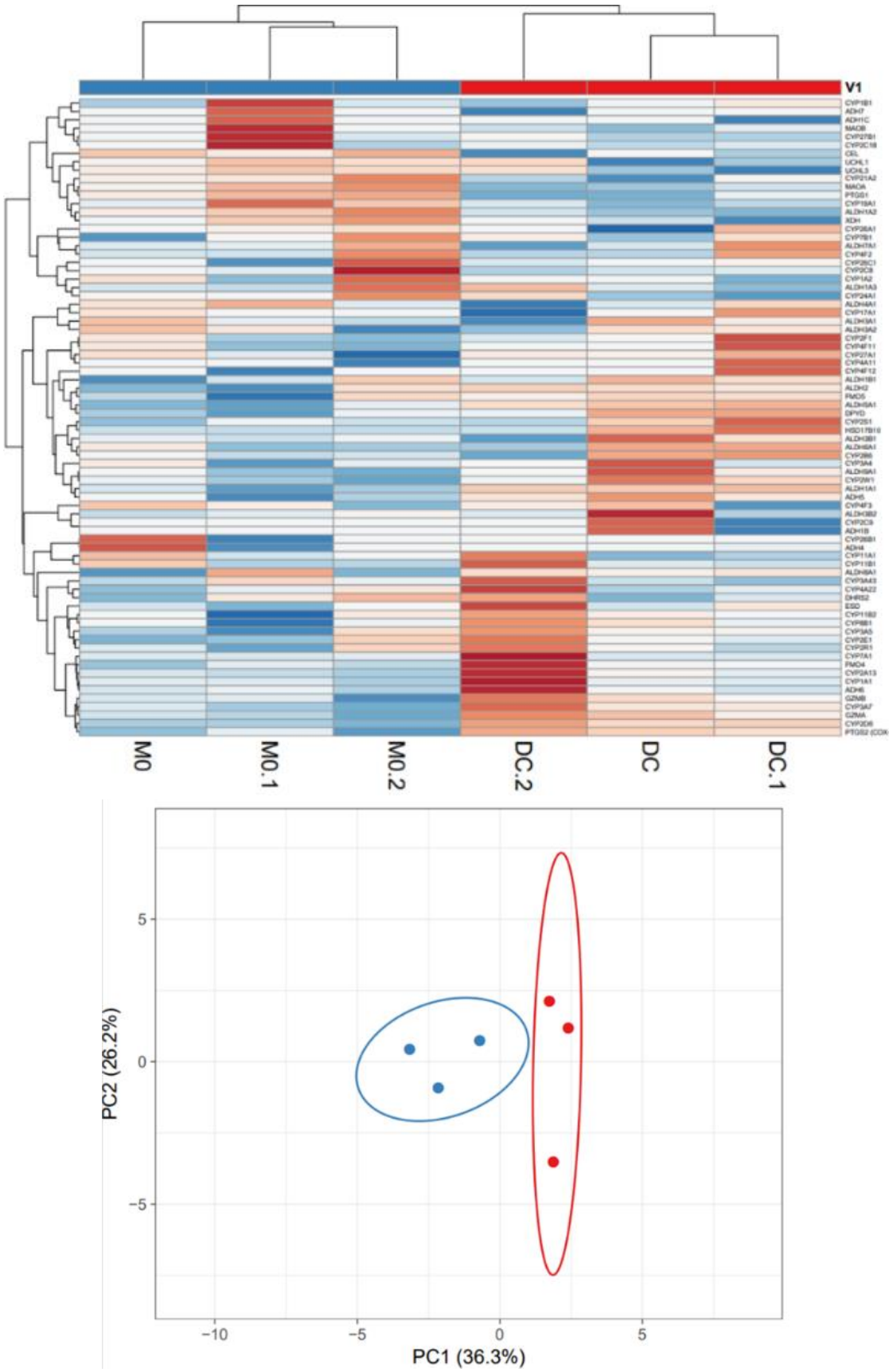


Figure 3.30. Cluster analysis of M0 MDM and MoDC XME gene expression.
M0 MDM (red) and MoDC (blue) following 7-day culture. Global gene expression of phase 1 XME was assessed by heatmap (top) and PCA (bottom). n=3.

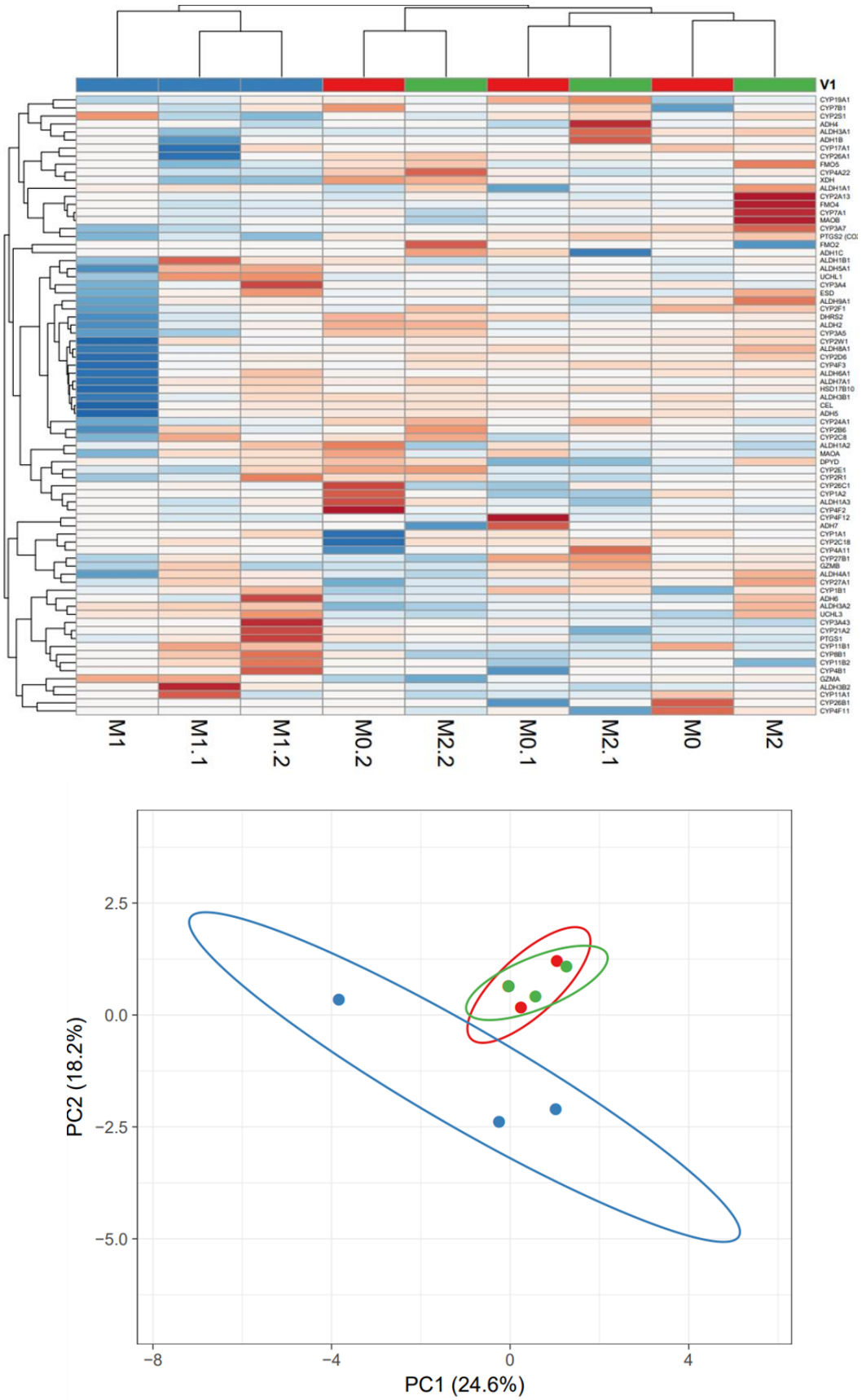


Figure 3.31. Cluster analysis of M0, M1 and M2 MDM XME gene expression.
MDM polarised to M0 (red), M1 (blue) or M2 (green) phenotype following 7-day culture. Global gene expression of phase 1 XME was assessed by heatmap (top) and PCA (bottom). n=3.

3.3.3 Validating gene array data by qPCR

In order to validate the results seen by gene array, a panel of XME were chosen to examine individually by qPCR. This method allows for technical repeats, and additional biological repeats, both of which add confidence in the results and improve statistical power.

Firstly, FMO enzyme subtypes 1, 2, 4 and 5 were examined (Figure 3.32). By gene array, only FMO4 and 5 were expressed above the limit of detection (Figure 3.24). In agreement with the gene array data, FMO1 was minimally expressed when measured by individual qPCR, while FMO2 was not detected (data not shown). Additionally, FMO4 was highly expressed by all cell types consistently ($p>0.15$) and FMO5 was highly expressed by all cell types but was significantly higher in M1 MDM compared to M2 MDM (3-fold; $p=0.039$).

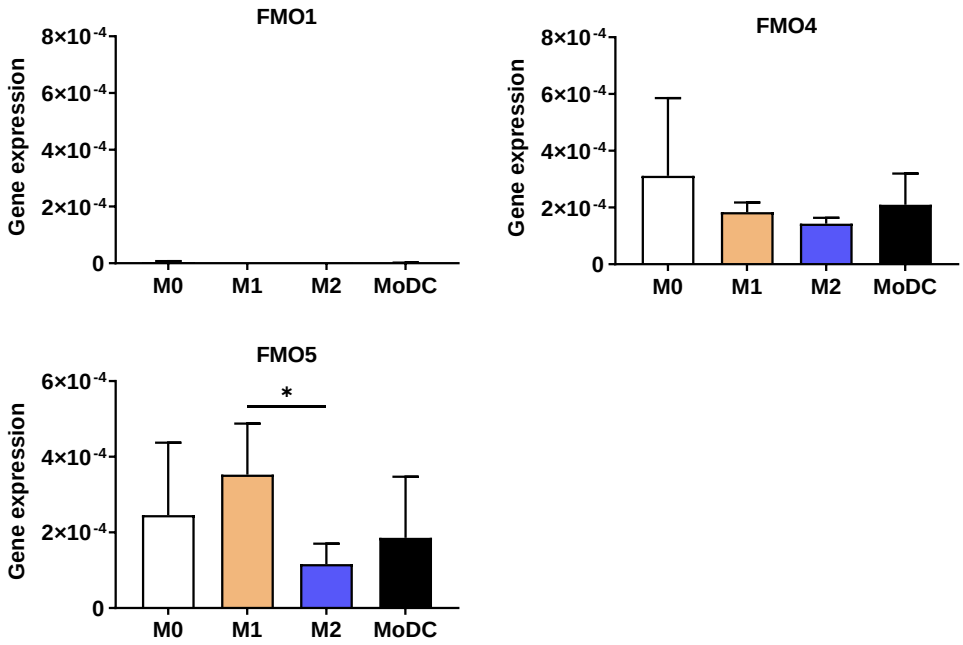


Figure 3.32. Gene expression of flavin-containing monooxygenases (FMO).
Unstimulated MDM (white), MDM polarised to an M1 (tan) or M2 (purple) phenotype and MoDC (black) following 7-day differentiation. Gene expression for *FMO1*, *FMO4* and *FMO5* was analysed by qPCR, calculated relative to the reference control *β2-microglobulin*, Data are presented as mean ± SD of n=4 independent experiments with statistically significance differences determined by one-way ANOVA; *p<0.05.

Next, expression of various cytochrome P450 enzymes was quantified (Figure 3.33). CYP1A1, 2C9, 2E1 and 3A4 had low basal expression, which was consistent between cell types, while CYP1B1 had higher basal expression which was also consistent. These results mostly agree with the gene array data, with the exception of CYP2C9 which was undetected by array, but measurable by qPCR, a more sensitive method. CYP2A6 was expressed more highly in MoDC compared to all MDM polarisation states, with significance reached compared to M2 MDM (18-fold; p=0.024). In contrast, CYP2D6 was more highly expressed in M0, and M1 MDM compared to M2 and MoDC, with significance reached between M0 and M2 (5-fold; p=0.047), M0 and MoDC (5-fold; p=0.026) and M1 and MoDC (4-fold; p=0.033). Finally, CYP3A5 expression was increased in M1 MDM compared to M0 (13-fold; p=0.033), M2 (6-fold; p=0.018) and MoDC (14-fold; p=0.016).

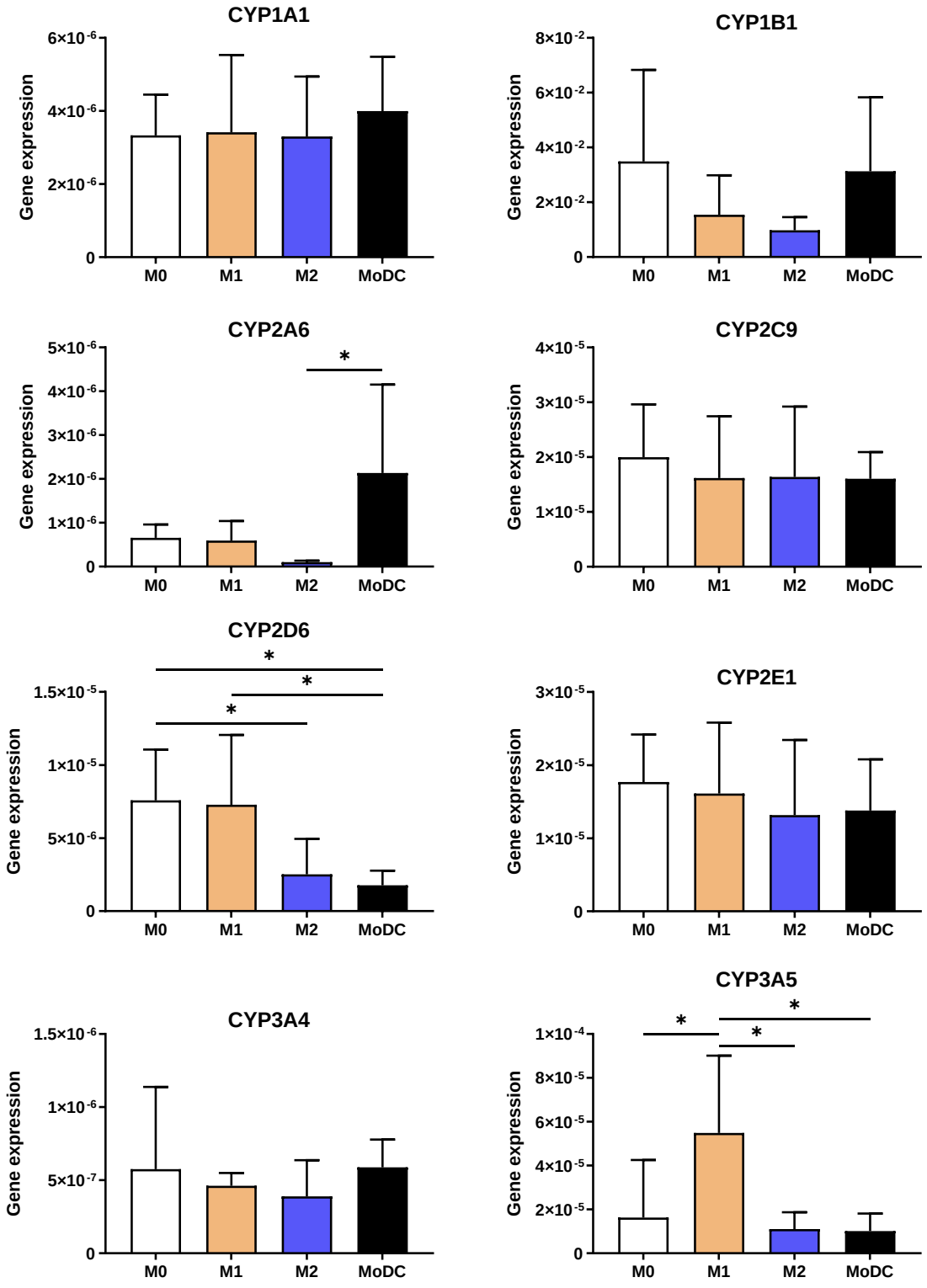


Figure 3.33. Gene expression of cytochrome P450 enzymes.

Unstimulated MDM (white), MDM polarised to an M1 (tan) or M2 (purple) phenotype and MoDC (black) following 7 day differentiation. Gene expression for the indicated enzymes was analysed by qPCR, calculated relative to the reference control *β2-microglobulin*, Data are presented as mean ± SD of n=4 independent experiments with statistically significance differences determined by one-way ANOVA; *p<0.05.

Finally, additionally enzymes were quantified by qPCR (Figure 3.34). ALDH2, in agreement with the gene array data, was highly expressed by all cell types. UCHL3 expression was significantly increased in MoDC compared to M0 (3-fold; p=0.008), M1 (5-fold; p=0.003) and M2 (4-fold; p=0.003), in agreement with the observed trend by gene array. Finally, PTGS2, also known as cyclooxygenase-2 (COX-2), can be used as a marker of inflammatory macrophages (Tarique et al., 2015). As expected, PTGS2 expression was significantly increased in M1 MDM compared to M0 (22-fold; p=0.014), M2 (20-fold; p=0.015) and MoDC (34-fold; p=0.013), similarly to the results generated by gene array, although in that instance statistical significance was not reached, while the higher statistical power of individual qPCR allowed for a significant increase.

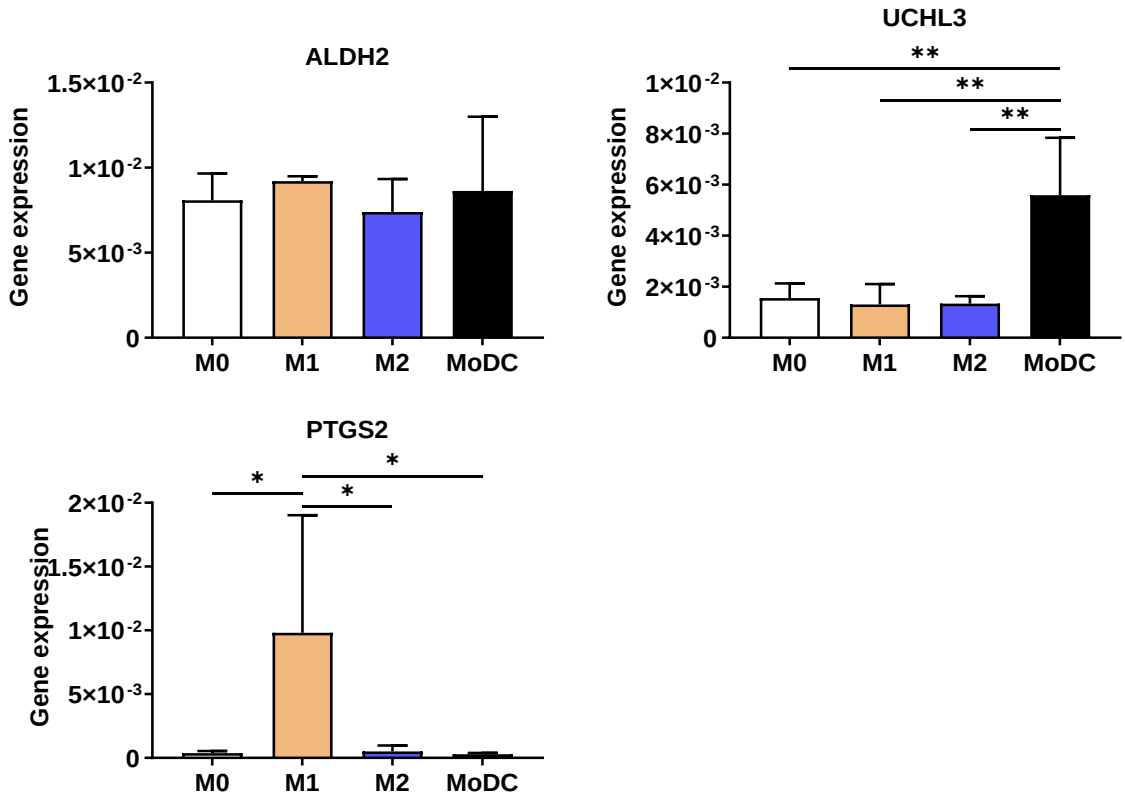


Figure 3.34. Gene expression of additional XME.
Unstimulated MDM (white), MDM polarised to an M1 (tan) or M2 (purple) phenotype and MoDC (black) following 7-day differentiation. Gene expression for *ALDH2* (**A**), *UCHL3* (**B**) and *PTGS2* (**C**) was analysed by qPCR, calculated relative to the reference control *β2-microglobulin*, Data are presented as mean ± SD of n=4 independent experiments with statistically significance differences determined by one-way ANOVA; *p<0.05, **p<0.01.

3.3.4 Protein abundance of XME produced at gene level

Following confirmation of XME expression at gene level, the production of associated proteins was examined by western blot. In these experiments, human liver microsomes were used as a positive control, as they contain high concentrations of most enzymes used in this study. Additionally, FMO4, FMO5, CYP3A5 and CYP2A6 were probed for but, despite a strong band in liver microsomes, nothing was observed for immune cell protein, so these blots were not included here.

Firstly, PTGS2 (COX-2) was examined (Figure 3.35). In line with the gene data, only M1 MDM protein contained a measurable concentration of PTGS2, including comparisons with the liver microsome positive control (data not shown). As no banding was visible for any condition except M1, densitometry for this protein was not undertaken.

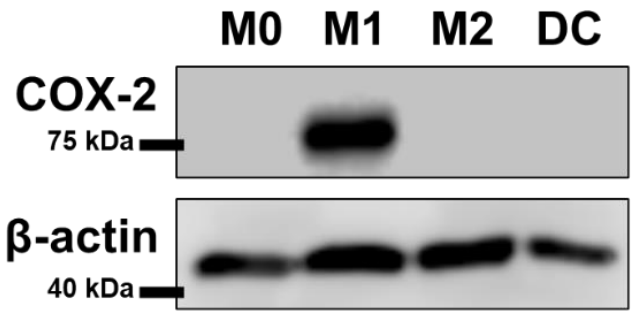


Figure 3.35. PTGS2 (COX-2) protein abundance.
Unstimulated MDM (M0), MDM polarised to an M1 or M2 phenotype and MoDC following 7-day differentiation. Protein abundance of PTGS2 and β-actin in immune cells (20 µg) was analysed by western blot. Blot is representative of n=4 independent experiments.

Next, ALDH2 was highly abundant in all cell types (Figure 3.36). This agrees with the gene array and qPCR validation data, where the ALDH2 gene was highly, and consistently, expressed. In fact, the protein abundance is comparable between immune cell and liver microsome protein relative to β-actin. While these protein types were isolated by different methods and thus cannot be directly compared, this nonetheless highlights the high abundance of ALDH2 in immune cells.

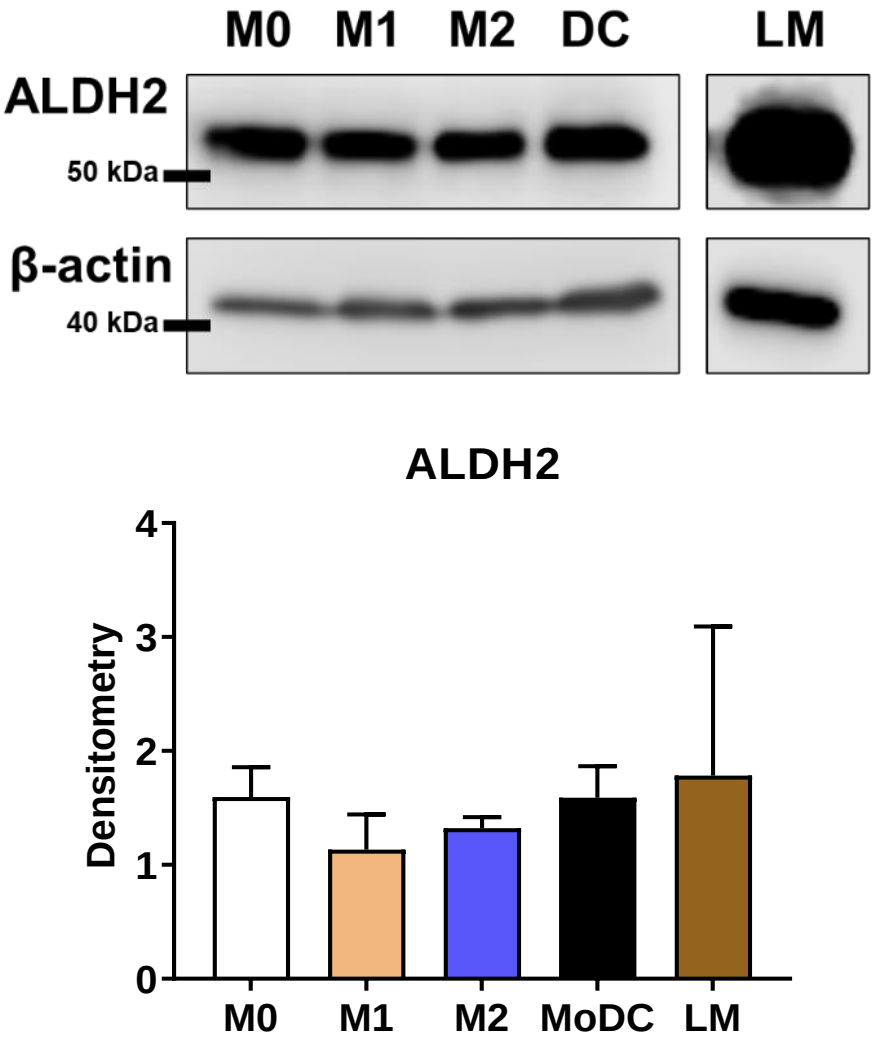


Figure 3.36. ALDH2 protein abundance.
Unstimulated MDM (M0; white), MDM polarised to an M1 (tan) or M2 (purple) phenotype and MoDC (black) following 7-day differentiation. Protein abundance of ALDH2 and β-actin in immune cells (20 µg) and liver microsomes (brown; 1 µg) as a positive control was analysed by western blot. Densitometry was calculated relative to the reference control β-actin; data are presented as mean ± SD of n=4 independent experiments with statistically significance differences determined by one-way ANOVA. Blot is representative of n=4 independent experiments.

Finally, CYP2D6 protein abundance was examined (Figure 3.37). At gene level, M0 and M1 MDM had higher gene expression compared to M2 and MoDC. However, at protein level, M1 MDM contained a significantly higher abundance of CYP2D6 compared to M0 (43-fold; p=0.023), M2 (23-fold; p=0.024) and MoDC (band not detected) when compared by densitometry, although this was still significantly less than the liver microsome positive control (37-fold; p<0.001).

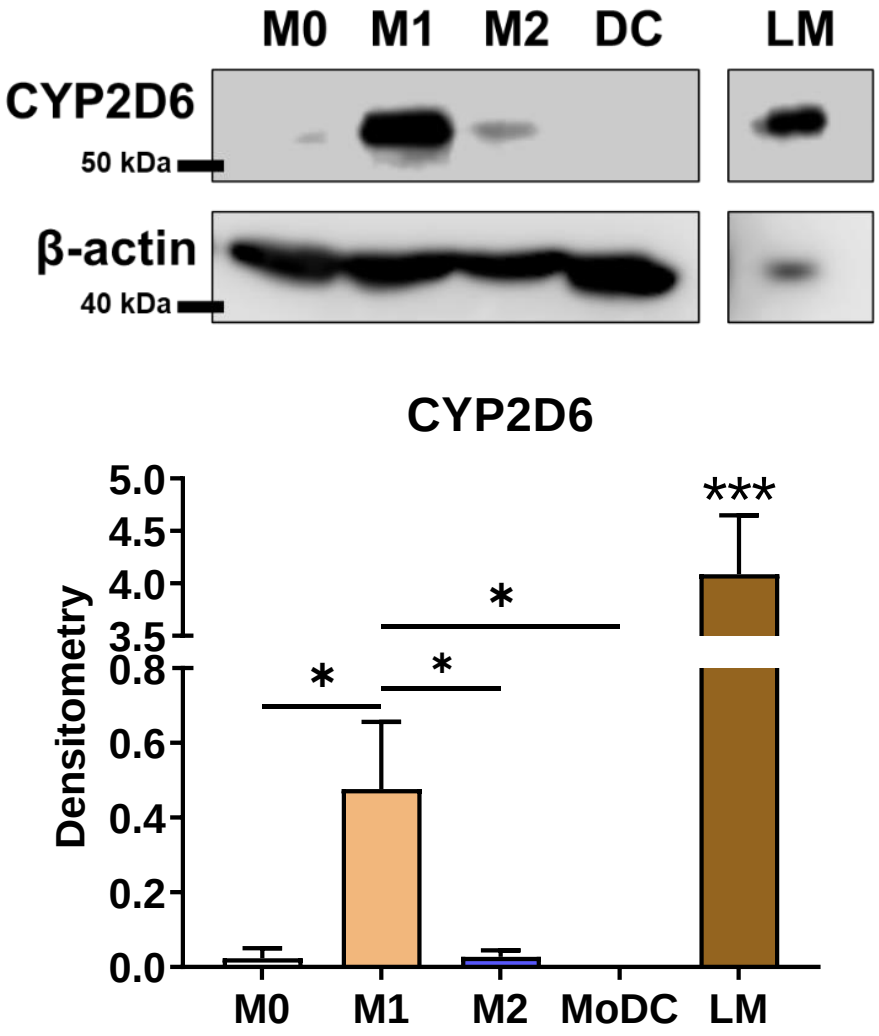


Figure 3.37. CYP2D6 protein abundance.
Unstimulated MDM (M0; white), MDM polarised to an M1 (tan) or M2 (purple) phenotype and MoDC (black) following 7-day differentiation. Protein abundance of CYP2D6 and β-actin in immune cells (20 µg) and liver microsomes (brown; 1 µg) as a positive control was analysed by western blot. Brightness and contrast were altered between immune cell and liver microsome images for better visualisation, blot is representative of n=4 independent experiments. Densitometry was calculated relative to the reference control β-actin on unaltered images and data presented as mean ± SD of n=4 independent experiments with statistically significance differences determined by one-way ANOVA; *p<0.05, ***p<0.005.

3.3.5 Functional study of XME in immune cells

Finally, to confirm enzyme function was similarly altered between cell types, functional assays were attempted on isolated protein for both PTGS2 and CYP2D6. First, PTGS2 activity was assessed by a non-specific PTGS assay. Total PTGS activity (Figure 3.38A) showed similar levels of activity between cell types. Treatment with a PTGS2-specific inhibitor allowed for quantification of PTGS2 activity as a proportion of total activity (Figure 3.38B). However, the inhibitor failed to significantly decrease the activity of any samples, except in M1 MDM and liver microsomes, where a small decrease was observed, showing slight PTGS2 activity in these samples. Furthermore, assays to quantify CYP2D6 activity were attempted multiple times, but no positive results were obtained (data not shown). This is likely due to technical limitations on amount of protein that could be isolated, and the relatively low abundance of this enzyme in total immune cell protein.

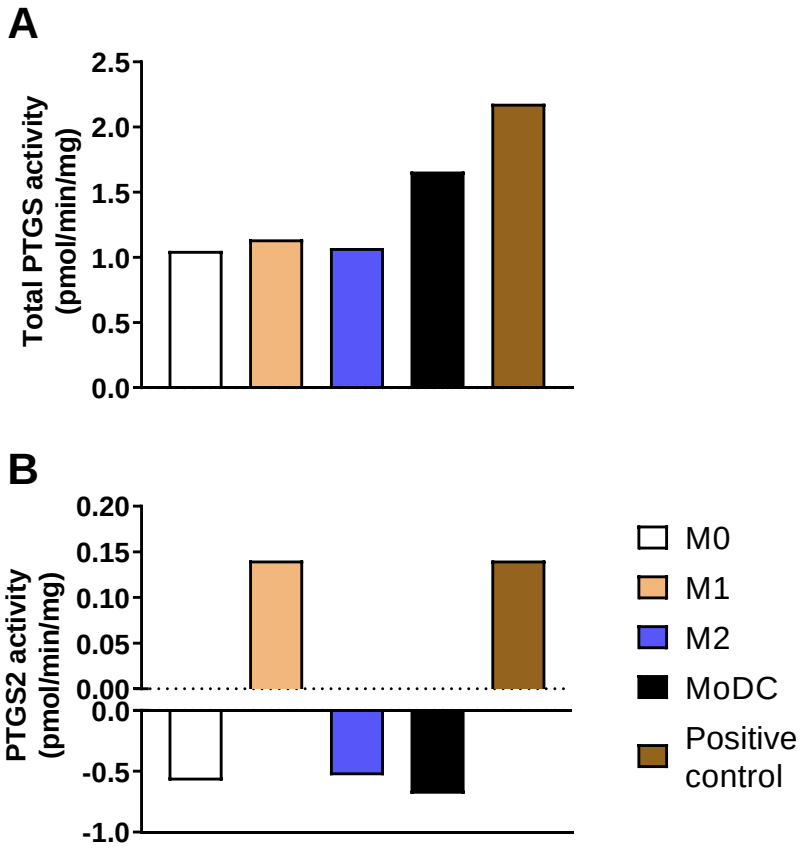


Figure 3.38. Pan PTGS, and PTGS2-specific enzyme activity.
Unstimulated MDM (M0), MDM polarised to an M1 or M2 phenotype and MoDC following 7-day differentiation. Total protein was isolated and quantified for enzyme activity for total PTGS (**A**) and PTGS2 specific activity (**B**) and compared to liver microsomes as a positive control; n=2.

3.4 Discussion

3.4.1 Generating monocyte-derived immune cells

The data presented here utilised monocytes isolated from peripheral blood to generate multiple immune cell phenotypes. These monocytes are often used as a source of macrophages and dendritic cells (Coillard et al., 2019), and can also be used to generate other distinct cell types such as osteoclasts (Sørensen et al., 2006) and hepatocyte-like cells (Ruhnke et al., 2005). Monocytes are desirable as an immune source as they are abundant in peripheral blood which makes them easy to obtain in high numbers. Equally, there are only three distinct subtypes, categorised by expression of CD14 and CD16 (Kapellos et al., 2019), and most monocytes isolated and purified by the method described here have a classical $CD14^{++}/CD16^{+}$ phenotype (Nielsen et al., 2020), which allows for relative consistency between samples. The data described here comprises the use of peripheral monocytes to generate MDM, MoDC and MoLC.

Firstly, unpolarised macrophages were differentiated by culture adhered to tissue culture plastic, a process which has been used in many previous studies (de Mulder et al., 1983; Rehli et al., 2000; Eligini et al., 2013). Furthermore, polarised macrophages were generated by culture in GM-CSF enriched media to produce M1 MDM, and M-CSF enriched media to produce M2 MDM and cells further simulated to opposing phenotypes by addition of IFN-γ and LPS, and IL-4, respectively (Tarique et al., 2015).

In addition to generating macrophage-like cells, this chapter also described the generation of dendritic cells from monocytes. There has been some controversy with their use as a dendritic cell type, with some arguing that as MoDC are only found *in situ* under inflammatory conditions, they are only representative of an inflammatory phenotype (Collin et al., 2018). However, it is challenging to isolate primary dendritic cells at the high numbers required for *in vitro* modelling while maintaining the heterogeneity observed in human populations. Therefore, it was decided to use MoDC with the caveat that these cells may not be fully representative of human immunology at steady state.

Previous reports have shown that culturing monocytes with the DC cytokine cocktail and adding TGFβ is sufficient to generate a Langerhans cell phenotype (Geissmann et al., 1998; Peiser et al., 2004; Bock et al., 2016). Langerhans cells play an essential role in epithelial immunity and as such feature in many fields of research including inflammation and allergenic responses (Seré et al., 2012; Picarda et al., 2016). However, as they typically only make up 2% of cells in the epithelium (Bauer et al., 2001) it is difficult to isolate these primary cells for *in vitro* use. A cancer cell line, MUTZ-3, has often been used in place of primary Langerhans cells (Laubach et al., 2011; Bock et al., 2018) although some differences have been observed in response to immune activators compared with primary cells, suggesting some limitations (Bock et al., 2018). Therefore, part of this work sought to generate primary Langerhans cells derived from monocytes.

3.4.2 Characterising cell differentiation away from a monocyte phenotype

Initial experiments differentiated primary monocytes into macrophages, dendritic cells, and attempted to also generate Langerhans cells, with morphology compared between cell types. Monocytes were spherical, dark, no defined nuclei, around 10 µm in diameter, which was comparable to previous reports (Pickl et al., 1996; Ruhnke et al., 2005). MDM displayed a distinct morphology typical of macrophages with a mixed phenotype comprising elongated and round cells (Eligini et al., 2013; Vogel et al., 2014; Tedesco et al., 2015). The observed morphology of MoDC agreed with previous reports, with visible dendrites (te Velde et al., 1988; Grassi et al., 1998) while MoLC had a similar appearance to MoDC but lacked visible dendrites (Geissmann et al., 1998).

Cell types were then compared using qPCR and flow cytometry, each of which have method-specific advantages. Measuring gene expression by qPCR highlights differences in total RNA, and additional genes can be reanalysed using different primers to improve breadth of results. In contrast, flow cytometry measure cell surface protein abundance which can only be carried out once on a sample but gives more detailed information at a single cell level and can identify cell subsets within a heterogeneous population. By combining these methods, a large range of markers could be assessed at the gene level, and population changes observed at the protein level, both providing distinct and complementary information.

3.4.2.1 Expression of pan immune cell markers

CD11c (ITGAX) is a transmembrane protein which, in combination with CD18, forms the complement receptor CR4 that can bind to complement fragments, matrix molecules, and ICAM-1 (Sadhu et al., 2007), and is also involved in the direct response to LPS (Ingalls et al., 1995). CD11c is found on many immune cell subtypes, including monocytes, tissue resident macrophages and dendritic cells and plays a role in phagocytosis and cell migration (Sadhu et al., 2007). In this study, CD11c was highly expressed at gene and protein level in monocytes, MDM and MoDC, in agreement with previous studies (Ancuta et al., 2000; Lukácsi et al., 2020).

Human leukocyte antigen (HLA) is an MHC class II surface receptor. It is upregulated in response to bacterial mediators (Heinzelmann et al., 1997) and is primarily involved in antigen presentation (Pinet et al., 1995). It can be found in a range of immune cells, primarily antigen presenting cells including monocytes (Basham et al., 1983) and dendritic cells (MacDonald et al., 2002), but also T cells (Arruvito et al., 2014), and non-immune cells such as keratinocytes when activated by IFN-g (Aubock et al., 1986). In this study MDM gene expression of HLA-DR was reduced, while protein abundance was increased compared to monocytes as seen previously (Bertho et al., 2000). In contrast, no changes were seen between monocytes and MoDC at either the gene or protein level. Previous work has shown increases in HLA-DR between monocytes and MoDC (Ancuta et al., 2000), and a study investigating the effect of IL-4 concentrations of HLA-DR protein abundance in MoDC found a dose-dependent increase, indicating that a higher IL-4 concentration may have yielded an increase in this marker (te Velde et al., 1988).

CD115 (CSF1R) is the transmembrane receptor for macrophage colony-stimulating factor 1 (M-CSF) which can influence macrophage differentiation and function. In addition, this receptor is overexpressed in many cancers and tumour-associated macrophages (Jeannin et al., 2018). In this study CD115 was only examined at gene level, where no changes were seen between monocytes, MDM or MoDC, in agreement with a recent study which described the use of CD115 as a pan-phagocyte lineage marker (Combes et al., 2021).

3.4.2.2 Expression of additional immune cell markers

CD14 encodes a transmembrane receptor which, in association with toll-like receptors, acts to recognise bacterial LPS and other pathogen-associated molecular patterns (Thomas et al., 2002; Zanoni et al., 2013). This marker is commonly used to identify monocytes but is also expressed by MDM at a reduced level (Landmann et al., 2000). Here, monocytes displayed high levels of CD14 at the gene and protein level compared to MDM and MoDC, but not MoLC. Reduction in CD14 has often been used as a marker of monocyte to MoDC differentiation (Grassi et al., 1998; Ancuta et al., 2000), and reduction of CD14 was more pronounced in MoDC, while some expression was retained in MDM, likely due to the role of CD14 in mediating MDM inflammatory responses (Thomas et al., 2002).

Carboxypeptidase M (CPM) is a membrane-bound enzyme which cleaves C-terminal amino acids from proteins and peptides, and while the endogenous function is still not fully understood, it appears to play a role in inflammation (Deiteren et al., 2009), and has been used as a marker of monocyte to MDM differentiation (Krause et al., 1998; Rehli et al., 2000). Here, CPM gene expression was upregulated in MDM, but not MoDC compared to monocytes, as expected. However, it was not possible to obtain a CPM antibody that was compatible with flow cytometry, so this change was not confirmed at the protein level.

CD36 is a scavenger receptor involved in cellular fatty acid uptake (Pepino et al., 2014) and has been shown to be the main receptor for LDL recognition and uptake in macrophages (Podrez et al., 2000). The data shown here saw an increase in CD36 gene expression in MDM compared to monocytes, and a non-significant increase at protein level. A similar study showed 8-fold increases in CD36 gene and protein levels in macrophages compared to monocytes, in partial agreement with the data here (Huh et al., 1996). In contrast, no difference was seen in CD36 gene expression in MoDC compared to monocytes, but there was a significant reduction in protein abundance. Interestingly, CD36 has been linked to MoDC maturation (Urban et al., 2001), but is typically expressed at lower levels compared to monocytes (Villani et al., 2017; Collin et al., 2018), in line with the data shown here.

CD204 is also a scavenger receptor which is expressed in myeloid cells, including macrophages and dendritic cells (Yi et al., 2009), which mediates

endocytosis of low density lipoproteins (Kelley et al., 2014). Here, both MDM and MoDC had increased expression of CD204 compared to monocytes which may be due to length of culture time as this receptor has been implicated in mediating cell adhesion to tissue culture plastic (Robbins et al., 1998).

3.4.2.3 Expression of MDM polarisation markers

To ensure macrophages derived from monocytes had not been polarised to an M1 or M2 phenotype, two markers of each polarisation state were examined.

CD80 and CD86 are membrane proteins which often work in tandem and can both interact with CD28 and CD152 found on the surface of T cells, resulting in T cell activation, proliferation, and differentiation. CD80 and CD86 can be found on the surface of various immune cells, including monocytes (Fleischer et al., 1996), macrophages (Foss et al., 1999), and dendritic cells (Delgado et al., 2004). Interactions with microbes and inflammatory cytokines can increase expression of CD80 and CD86 and as such both can be used as a marker of M1 inflammatory macrophages (Tarique et al., 2015). The data shown here found gene expression of CD80, but not CD86 was decreased in MDM compared to monocytes. Previously, monocyte populations were shown to be around 70% positive for CD80 when assessed by flow cytometry (Lahat et al., 2003), which would complement the gene data produced here. In addition, no changes were seen in the gene expression between monocytes and MoDC. The protein abundance of these markers has been shown to increase in immature MoDC compared to monocytes (Ancuta et al., 2000), so it may be that this change was not observable at gene level. A similar study assessed expression of immune markers by flow in MDM and MoDC, finding CD86 more so than CD80 was abundant in these cell populations, although no monocytes were examined for comparison (Santin et al., 1999).

CD163 is a scavenger receptor which can recognise both Gram-positive and Gram-negative bacteria (Van Gorp et al., 2010) and is specifically expressed by cells of a monocyte/macrophage lineage (Fabriek et al., 2005). CD163 is typically upregulated by anti-inflammatory signals (Sulahian et al., 2000), and downregulated by pro-inflammatory signals (Buechler et al., 2000) and as such can be used as a

marker of M2 MDM polarisation (Van Gorp et al., 2010; Rőszer, 2015), although it may not be an M2-specific marker (Barros et al., 2013) so should be used in conjunction with other markers. The data shown here found no differences in CD163 expression between monocytes and MDM at gene or protein level, while MoDC had significantly less gene expression compared to monocytes, as has been previously described (Sulahian et al., 2000), a trend which was also seen at protein level, but was not significant. A similar study compared CD163 gene expression in monocytes, MoDC and MDM, finding low expression in MoDC (in agreement with the data here), but upregulated expression in MDM, which was not repeated here (Buechler et al., 2000).

CD206 encodes the macrophage mannose receptor, which is primarily found on the surface of macrophages and immature dendritic cells (Azad et al., 2014). It functions to aid in homeostatic clearance of endogenous sugars, as well as pathogen recognition which can act to initiate the innate response to bacteria and fungi (Taylor et al., 2005; Azad et al., 2014). CD206 upregulation is often seen in tumour-associated macrophages (Jaynes et al., 2020) and is used as a marker of M2 polarisation (Rőszer, 2015) so was examined in conjunction with CD163. The data shown here saw no change in CD206 expression between monocytes and MDM, while CD206 was significantly increased in MoDC compared to monocytes.

3.4.2.4 Expression of MoDC and MoLC markers

To measure adoption of a MoDC or MoLC phenotype, and to confirm the absence of this phenotype in MDM, three markers were used.

CD1a has a key role in antigen presentation (Kaczmarek et al., 2017) and is found on antigen presenting cells (Dougan et al., 2007). In particular, MoDC are known to have CD1a high and CD1a negative subpopulations (Ancuta et al., 2000; Chang et al., 2000; Gogolak et al., 2007; Cernadas et al., 2009), which was also seen in this study when protein abundance was measured by flow cytometry and would explain the small significant increase observed at the gene level compared to monocytes. However, when MoLC were assessed for CD1a expression, no difference was seen compared to monocytes, suggesting a lack of differentiation to the expected LC phenotype (Georgiou et al., 2005). This was compounded by a lack of increase in LC

marker CD207 (Langerin), which mediates antigen presentation and can be found in subpopulations of DC (Idoyaga et al., 2008), but is primarily expressed by LC in the skin and oral mucosa (Romani et al., 2010). The first study that described the production of MoLC (Geissmann et al., 1998) was published prior to the discovery of CD207 in 2000 (Valladeau et al., 2000) and instead used E-cadherin (CDH1) as an LC marker. E-cadherin is typically used as a keratinocyte marker where it is involved in regulating epithelial differentiation (Charest et al., 2009) but was also examined as an immune cell differentiation marker. However, CDH1 was similarly unchanged in LC compared to monocytes, while both CD207 and CDH1 were increased in MoDC, implying these cells had a closer phenotype to LC than the MoLC generated in this study.

Finally, MDM had minimal increases in CD1a and CD207, as per previous studies (Van den Bossche et al., 2009, 2012; Ohradanova-Repic et al., 2016), confirming these cells did not adopt an unwanted phenotype. In addition, MDM did not increase expression of E-cadherin, which in macrophages is associated with an M2 MDM phenotype (Van den Bossche et al., 2015), further confirming that the MDM produced in this study did not polarise towards an M2 state.

3.4.3 Production of xenobiotic metabolising enzymes by immune cells

Having fully characterised the production of distinct myeloid cell phenotypes, these cells were assessed for gene and protein levels of various XME. M1 and M2 MDM were chosen as these represent opposing sides of macrophage polarisation (Tarique et al., 2015), so could best identify any potential regulatory mechanisms of macrophage XME function in response to different microenvironments. Furthermore, M1 and M2 MDM have distinct endogenous cellular metabolic mechanisms, as M1 MDM tend towards anaerobic glycolysis, and M2 MDM rely more on oxidative phosphorylation for energy generation (Viola et al., 2019). As many XME have endogenous roles in energy generation pathways such as fatty acid and carbohydrate metabolism, it is likely that polarised MDM would differ in expression of these enzymes. In addition, unpolarised MDM were used as an unstimulated phenotype, and MoDC were also examined to better understand how XME gene expression might

change between cell types derived from the same monocytic origin. In this section, results will be separated and discussed as enzyme families where possible for ease of description.

3.4.3.1 Flavin-containing monooxygenase (FMO)

FMO enzymes are important proteins involved in the metabolism of xenobiotics, which act by catalysing the oxygenation of lipophilic compounds (Eswaramoorthy et al., 2006). The FMO family of enzymes comprises five main subtypes (termed FMO1-5), which vary in tissue expression and substrate specificity (Cashman et al., 2006; Jones et al., 2017). When assessed by gene array in this study, only FMO4 and FMO5 were detectable, with consistent levels between cell types. In addition, when confirmed by individual gene qPCR FMO1, 4 and 5 were all detectable, but not FMO2, and while FMO1 and 4 were expressed at comparable levels between cell types, FMO5 was significantly higher in M1 MDM compared to M2 MDM. However, when protein abundance of FMO4 and 5 was examined, it was not detected.

FMO1 is typically found in foetal liver tissue, with a genetic switch to FMO3 in adult liver tissue (Koukouritaki et al., 2002), although in adults FMO1 appears to be an exclusively extrahepatic enzyme, with high concentrations found in the kidney (Yeung et al., 2000) that can be inhibited by bacteria in mice (Zhang et al., 2009). Furthermore, FMO1 has been detected in other extrahepatic tissue including dermal fibroblasts (Tabib et al., 2018), but not keratinocytes (Fabian et al., 2013). Immune cells have also been shown to express FMO1, with MDM upregulating expression in response to haemoglobin, suggesting inducibility (Schaer et al., 2006). However, MoDC have been shown to express low to no expression of FMO1 and 2 (Ogese et al., 2015), in partial agreement with the data presented here.

FMO4 function has historically been difficult to elucidate as the protein is unstable and multiple RNA splice variants with altered functions have been found (Itagaki et al., 1996; Lattard et al., 2004). Nonetheless, FMO4 expression in liver and extrahepatic tissue has been investigated, finding comparable expression levels in multiple tissues including liver, kidney and lung (Zhang et al., 2006). Furthermore, FMO4 gene expression has been detected in MoDC (Ogese et al., 2015) and peripheral blood mononuclear cells (Gagliardi et al., 2013), in agreement with the data here.

FMO5 differs in reactivity compared with other FMO subtypes as it acts as a Baeyer-Villiger monooxygenase by catalysing oxidation of ketones and cyclic ketones to esters and lactones, and has activity against a diverse range of carbonyl compounds (Fiorentini et al., 2016; Tolmie et al., 2019), including metabolising anti-inflammatory prodrug Nabumetone to its pharmacologically active metabolite (Fiorentini et al., 2017). Few studies have investigated expression of FMO5 in human immune cells, although it has been detected in MoDC (Ogese et al., 2015) and peripheral blood mononuclear cells (Gagliardi et al., 2013), in agreement with the data presented here. However, no studies investigating FMO5 expression in macrophages (in inflammatory or non-inflammatory conditions) could be found, so the upregulation in M1 MDM compared to M2 MDM observed in this study could not be confirmed or contested by other studies. In fact, the research into the endogenous role of FMO5 is still underway (Phillips et al., 2019), with recent studies linking this enzyme to glucose homeostasis, weight gain and insulin sensitivity (Scott et al., 2017). Furthermore, in $Fmo^{-/-}$ mice, proteins involved in glycolysis and carbohydrate metabolism were downregulated compared to wild type, implicating FMO5 in these pathways (Gonzalez Malagon et al., 2015). This has been further shown in cell lines overexpressing FMO5, in which ATP production was increased compared to control, and proteomics revealed increases in amino acid and energetic metabolic pathways (Huang et al., 2021). Therefore, M1 MDM, which rely on glycolysis for energy generation, may upregulate FMO5 to increase metabolic capacity, which is not required in M2 MDM, and could explain the data shown here.

3.4.3.2 Alcohol dehydrogenase (ADH)

The ADH family of enzymes can metabolise primary alcohols to aldehydes and secondary alcohols to ketones, with substrates including ethanol and retinol, and can also catalyse the reverse reaction, producing alcohols (Yin et al., 1999). When assessed by gene array in this study, only ADH5 (class III) and ADH6 (class V) were detectable, with consistent levels between cell types. ADH5 was notably higher than ADH6, which would suggest it is the primary ADH enzyme present in these immune cells.

ADH5 (also termed S-nitrosoglutathione reductase) metabolises S-nitrosoglutathione, S-hydroxymethylglutathione (a by-product of formaldehyde metabolism), and some alcohols (Barnett et al., 2017). ADH5 has been detected in most human tissue, including brain, liver, spleen and thymus (Adinolfi et al., 1984). ADH5 has also been found in the oral mucosa, and is suggested to be the primary formaldehyde oxidation enzyme in this tissue (Hedberg et al., 2000). The murine equivalent *Adh5* gene has been detected in various immune cell subtypes, including monocytes and immune progenitor cells (Dingler et al., 2020), although no studies examining ADH5 expression in human immune cells could be found for comparison.

ADH6 function is currently unknown, although it shares sequence homology with other ADH family members (Yasunami et al., 1991; Östberg et al., 2016). Early investigations found little ADH6 expression in liver and extrahepatic tissues (Engeland et al., 1993). More recently ADH6 has been detected at low concentrations in extrahepatic tissue, although no expression was detected in bone marrow or peripheral leukocytes (both sources of monocytes) (Nishimura et al., 2006), in contrast to the data presented here.

3.4.3.3. Aldehyde dehydrogenase (ALDH)

The ALDH family of enzymes comprise 19 genes which catalyse the oxidation of aldehydes to carboxylic acids. These enzymes are involved in biological processes such as biotransformation of carbohydrates and lipids (Vasiliou et al., 2004), as well as metabolism of xenobiotic compounds such as ethanol and formaldehyde (Marchitti et al., 2008). ALDH enzymes are further delineated into three enzymes classes based on expression pattern, subcellular location, and substrate binding affinity (Marchitti et al., 2008). In this study all ALDH subtypes examined were detected by gene array, with differences seen in ALDH1A1 (increased in M0), ALDH1A2 (increased in MoDC), and ALDH5A1 (decreased in M1).

The ALDH1 subgroup of enzymes comprising ALDH1A1, 1A2 and 1A3, have a highly conserved function to catalyse oxidation of retinal (metabolite of retinol) to retinoic acid (RA), and as such are sometimes referred to as retinaldehyde dehydrogenase (1-3) (Zhao et al., 1996; Marchitti et al., 2008). All three subtypes were

detected by gene array in the cell types examined, with ALDH1A1 increased in M0 MDM, and ALDH1A2 increased in MoDC. ALDH1 is expressed by haematopoietic cells, and can be inhibited by ALDH inhibitor N,N-diethylaminobenzaldehyde (DEAB) (Chute et al., 2006). Interestingly, dendritic cells are well known to express these enzymes, and to be involved in the production of retinoic acid from vitamin A, notably in the intestine as this tissue has the highest concentration of dietary vitamin A (Yokota et al., 2009; Hall et al., 2011; Agace et al., 2012; Steimle et al., 2016). Retinoic acid treatment has been shown to increase expression of CD80 and CD86, which would suggest an involvement with T cell activation by DC (Iwata, 2009; Ross et al., 2009). This was later shown in retinoic acid treated MoDCs, which preferentially induced IL-10 producing T cells compared to untreated MoDC (Bakdash et al., 2015). Furthermore, in macrophages, vitamin A is involved in differentiation to a tissue resident phenotype (Gundra et al., 2017), while retinoic acid is known to inhibit inflammatory responses in macrophages (Oliveira et al., 2018). Thus, vitamin A, and its metabolite retinoic acid, play a key role in regulating macrophage and dendritic cell activity, and the ability to catalyse this conversion is likely to be important for self-regulation in these cells.

ALDH2 is an important enzyme for oxidation of acetaldehyde as part of ethanol metabolism, with polymorphisms leading to decreased alcohol tolerance and associated with increased cancer risk of cancer (Shin et al., 2017). In addition, ALDH2 is also the principle enzyme to activate nitroglycerin in blood (Lang et al., 2012). Here, consistently high production was observed both at gene and protein level, in agreement with previous studies. For example, *Aldh2* expression was altered by oxLDL treatment in murine bone marrow-derived macrophages (BMDM) (Zhu et al., 2019). PBMC have also been transfected with human *Aldh2*, which increased expression compared to non-transfected control, and had a protective effect against hydrogen peroxide damage (X. Hu et al., 2011). Finally, peripheral blood leukocytes isolated from volunteers had increased ALDH2 gene expression following ingestion of alcohol, suggesting an inducible system in response to environmental cues (Kimura et al., 2009).

All examined ALDH3 enzymes were detected in these cells, although ALDH3A2 and 3B1 were highly expressed. ALDH3A2 (also known as fatty aldehyde dehydrogenase) is involved in fatty acid synthesis from fatty alcohol, amongst other

substrates (Ichihara et al., 1986; Marchitti et al., 2008). This enzyme has been found to be upregulated in cancer tissue, where it was suggested to play a role in regulating cellular metabolism (Yin et al., 2020), something which is essential for regulation and maturation of immune cells and overall immune response (Biswas et al., 2012; Ganeshan et al., 2014) which may explain the biological significance of high expression of this enzyme. ALDH3B1 has substrate specificity towards medium and long chain aliphatic aldehydes and is highly expressed in the liver, kidney and regions of the brain (Marchitti et al., 2007), and is upregulated in many cancer tissues compared to normal controls (Marchitti et al., 2010). It is thought that the main endogenous role of ALDH3B1 is detoxification of free radicals formed by lipid peroxidation, and thus providing a cellular defence against oxidative stress (Marchitti et al., 2008). ALDH3B1 has been measured in human MDM (Ahmed et al., 2018), and the equivalent murine gene in bone marrow-derived macrophages (Niu et al., 2016), in general agreement with the data shown here.

Finally, ALDH4-9 subtypes were also detected at various expression levels, consistently between cell types. These subtypes all contain distinct substrate specificity, but are typically expressed in the liver, kidney and brain regions (Marchitti et al., 2008).

3.4.3.4 Cytochrome P450

Cytochrome P450 enzymes are arguably the most important class of enzymes in terms of xenobiotic drug metabolism and are involved in the metabolism of the vast majority of drugs in clinical use. This superfamily of enzymes comprises almost 60 subtypes, which all function as monooxygenases to oxidise a range of substrates including endogenous hormones and fatty acids, as well as most xenobiotic compounds (Zanger et al., 2013). In this section, the cytochrome P450 enzymes will be described in groups of enzyme class, and only those isozymes which were moderately or highly expressed will be discussed.

Cytochrome P450 class I

CYP1A1 and 1B1 metabolise aromatic hydrocarbons by hydroxylation or conversion to an epoxide. 1A1 and 1B1 both have similar substrates, but form different

metabolites, and are commonly studied in the oral cavity due to their ability to activate environmental procarcinogens such as benzo[a]pyrene and polycyclic aromatic hydrocarbons found in tobacco products (Hanna et al., 2000; Walsh et al., 2013). In this study, expression of both enzymes was confirmed by individual qPCR, and were consistently expressed by all cell types with CYP1A1 moderately expressed and CYP1B1 highly expressed by these cells. In comparison, human lymphocytes also express CYP1A1 and 1B1, with a similar trend of higher CYP1B1 expression (van Duursen et al., 2005), which agrees with the data shown here. Furthermore, pulmonary alveolar macrophages have been shown to specifically metabolise benzo[a]pyrene to carcinogenic metabolites (Harris et al., 1978). It is therefore likely that macrophages and dendritic cells which reside in the oral cavity and respiratory tract, including alveolar macrophages, contribute to the local metabolism of environmental procarcinogens in tobacco smoke and pollution.

Cytochrome P450 class II

The CYP2 family includes many of the most important enzymes for hepatic drug metabolism, as well as enzymes expression in extrahepatic tissue. These enzymes can be further delineated into subgroups including CYP2A, CYP2C and CYP2D, which have distinct functionality (Zanger et al., 2013). When assessed by gene array, most CYP2 enzymes were detected consistently between cell types. Of note CYP2E1 which is involved in alcohol metabolism (Heit et al., 2013) was detected consistently between cell types, in agreement with a previous study quantifying expression in MDM (Hutson et al., 1999). Furthermore, CYP2A6 was not included in the gene array, but was examined by individual qPCR due to the role of this enzyme in xenobiotic metabolism of many substrates, including nicotine (Rahnasto et al., 2008). Notable differences were observed in CYP2A6, which was increased in MoDC compared to M2 MDM. Interestingly, nicotine is known to have an immunosuppressive role in MoDC (Nouri-Shirazi et al., 2003, 2012; Givi et al., 2015) but the extent to which dendritic cells are able to metabolise nicotine to its metabolite cotinine has not been investigated.

Finally, CYP2D6 is a key metabolic enzyme for a range of clinically relevant drugs (Bertilsson et al., 2002) and qPCR revealed an upregulation of this enzyme in M0, and M1 MDM compared to M2 and MoDC, while enzyme protein was significantly increased in M1 MDM compared to all subtypes, suggesting an upregulation in

inflammatory conditions. Interestingly, it has been shown previously that systemic treatment of patients with chronic hepatitis C with IFN-γ increased CYP2D6 activity (Becquemont et al., 2002), and increased CYP2D6 activity has been observed in multiple inflammatory conditions including HIV and cancer (Shah et al., 2015). This provides a link between enzyme expression and systemic inflammation, although no previous studies could be identified that investigated the effect of inflammation on local CYP2D6 expression. However, CYP2D6 expression has been identified in leukocytes (from whole blood, no purification) which was inhibited by treatment with morphine and a nitric oxide donor (Mantione et al., 2008), showing CYP2D6 enzyme expression can be altered in immune cells by local stimuli. Of note, nitric oxide often has an anti-inflammatory effect (Sharma et al., 2007), and is a known inhibitor of CYP2D6 (Hara et al., 2002). It would therefore stand to reason that if CYP2D6 expression is decreased by anti-inflammatory stimuli, that the reverse is also possible, which aligns with the key result here. To further understand CYP2D6 function in innate immune cells, functional studies were attempted in this study. Unfortunately, these experiments were unsuccessful due to technical limitations, as the maximum quantity of protein which could be isolated was insufficient to detect CYP2D6 enzyme activity. It is therefore likely that CYP2D6 has relatively low abundance, although there are other factors that should be considered. For example, CYP2D6 is well known to be highly polymorphic which can affect enzyme activity (Ingelman-Sundberg, 2005), and while this was not investigated in the present study, these polymorphisms could occur in immune cells as well. Additionally, the functional assay did not disclose the CYP2D6 substrate used, but the CYP2D6 identified in M1 MDM may have distinct substrate specificity which affected the assay result. Furthermore, the enzyme produced by M1 MDM may be similar enough in gene sequence and protein structure to be identified as CYP2D6 but with altered or no functionality.

Cytochrome P450 class III

This class of P450 enzymes comprises four genes, CYP3A4, 3A5, 3A7 and 3A43. These enzymes are arguably the most important XME as together they metabolise up to 60% of drugs in clinical use, as well as hormones, toxins and carcinogens (Burk et al., 2004). Within this enzyme family, CYP3A4 and 5 share similar substrate specificity, but differ in tissue expression with CYP3A4 predominantly expressed in the liver, while CYP3A5 is mostly extrahepatic (de Wildt et al., 1999).

Here, both CYP3A4 and 3A43 had low gene expression, and neither were altered between immune cell type. In contrast, both CYP3A5 and 3A7 were moderately expressed, and notably expression of CYP3A5 was significantly increased in M1 MDM compared to all other cell types, implying upregulation in inflammatory conditions. However, no protein was found when examined by western blot suggesting that the overall translation of CYP3A5 gene into protein is low. In agreement with these data, a previous study found expression of CYP3A5, but not CYP3A4, in peripheral blood cells (in particular PMN and mononuclear cells), although activity was not confirmed (Janardan et al., 1996). Due to the varied role of CYP3A4/5 it is difficult to determine any specific functions in MDM, although some speculations can be made. For example, testosterone is a CYP3A5 substrate (Kandel et al., 2017), which is known to dampen inflammatory responses (Rettew et al., 2008; Becerra-Diaz et al., 2020), so macrophages may upregulate CYP3A5 expression during inflammation to mitigate any anti-inflammatory effects. Similar arguments could be made for other hormones, such as estrogen, which can also modulate the inflammatory response (Straub, 2007). However, further investigations are required to elucidate any specific regulatory mechanisms and functional consequences of upregulating expression of CYP3A5 in inflammatory conditions.

Cytochrome P450 class IV

CYP4 enzymes can catalyse hydroxylation of terminal carbons of an alkyl chain, which most commonly is used in metabolism of fatty acids (Edson et al., 2013). Gene array of CYP4 enzymes showed most isozymes had low or undetected expression, with the exception of CYP4F2 and 4F3 which had consistent moderate expression. CYP4F2 (also known as leukotriene-B(4) omega-hydroxylase 1) and CYP4F3 (also known as leukotriene-B(4) omega-hydroxylase 2) catalyse the degradation of inflammatory mediator leukotriene B4, and have clinically relevant substrates such as warfarin (Alvarellos et al., 2015). Due to this anti-inflammatory function, it was anticipated that these enzymes may have been upregulated in M2 MDM compared to M1 inflammatory MDM, but this was not observed here. Notably CYP4F3 is known to be expressed in myeloid cells (Christmas et al., 2003), in general agreement with these data.

3.4.3.5 Additional enzymes

Finally, enzymes not belonging to a specific XME enzyme family, or limited to two isozymes, were examined. Overall, these enzymes were expressed more highly than other categories.

Carboxyl ester lipase (CEL), also known as bile salt-stimulated lipase, is a lipolytic enzyme which is primarily produced in the pancreas (Johansson et al., 2018), but can also be found in other tissues (Hui et al., 2002). In this study, CEL had medium expression, which was consistent across all cell types. Previous publications have identified consistent gene expression of CEL in peripheral blood monocytes, MDM, and THP-1 monocytic cells (Li et al., 1997; Bengtsson et al., 2002), in agreement with the data presented here.

Dehydrogenase/reductase (SDR family) member 2 (DHRS2), also known as HEP27, is a carbonyl reductase which catalyses the reduction of dicarbonyl compounds, and also has activity in lipid and endogenous hormone metabolism (Li et al., 2021). In this study, DHRS2 had medium expression, which was consistent across all cell types. A study comparing DHRS2 gene expression in monocytes, MoDC, MDM, and various immune cell lines found only MoDC expressed the enzyme (Heinz et al., 2002), in partial agreement with the data presented here.

Dihydropyrimidine dehydrogenase (DPYD) catalyses the initial step in pyrimidine catabolism, through the reduction of uracil and thymine (Offer et al., 2014). Of clinical note, it is also involved in degradation of chemotherapeutic fluoropyrimidines such as 5-fluorouracil (5-FU), and some DYPD polymorphisms have been linked to increased risk of adverse drug reactions to these therapies (Del Re et al., 2019). Here, DPYD was found to be highly expressed across all cell types. As M2 MDM are often found in the tumour microenvironment (Bingle et al., 2002; Petruzzi et al., 2017), which correlates with poor prognosis (Alves et al., 2018), expression of this enzyme in MDM may have implications for chemotherapeutic metabolism, and activation of prodrugs (such as 5-FU) locally by infiltrating immune cells. Indeed, a recent study found human MDM expressed DPYD, which was upregulated in hypoxic conditions (as seen in solid tumours), and conferred some chemoresistance in cancer cell lines in response to 5-FU treatment (Malier et al., 2020). These data suggest resident macrophage expression of DPYD in the tumour microenvironment could be a source of

chemoresistance to 5-FU, although further study would be required to better elucidate the mechanism.

Esterase D (ESD), also known as S-formylglutathione hydrolase, is a carboxylesterase, which primarily acts with the formaldehyde detoxification pathway by catalysing metabolism of formylglutathione to yield glutathione and formate (Uotila et al., 1974). In this study, ESD was highly expressed in all cell types, but was significantly decreased in M1 MDM. Glutathione is a well-known antioxidant, which is often downregulated in inflammation (Forman et al., 2009), and therefore it is expected that enzymes involved in yielding free glutathione would be downregulated in inflammatory macrophages, which would agree with the data presented here.

Hydroxysteroid (17-β) dehydrogenase 10 (HSD17B10) is a mitochondrial dehydrogenase which can metabolise a diverse range of substrates including steroids, fatty acids and xenobiotics (Yang et al., 2011). In this study, HSD17B10 was highly expressed across all cell types.

Xanthine dehydrogenase (XHD) oxidises hypoxanthine to xanthine to uric acid in successive reactions, can also catalyse hydroxylation of heterocyclic compounds, and has been studied extensively (Wang et al., 2016). Expression of this enzyme has been identified in both liver and extrahepatic tissues (Pritsos, 2000; Al-Shehri et al., 2020). Here, XDH had the lowest expression of all enzymes in this subcategory but was still detected in all cell types. Interestingly, XDH has implications as an immunomodulatory enzyme as it is involved in production of reactive oxygen species which can be used directly as a cytotoxic response to pathogens, and indirectly by increasing production of pro-inflammatory cytokines (Yang et al., 2013). Therefore, the expression of XDH could be inducible during inflammation as part of host defence, although this was not observed here.

Granzyme A and B (GZMA, GZMB) are serine proteases classically secreted by cytotoxic T cells as a mechanism of inducing cell death in a target cell (Voskoboinik et al., 2015). In this study, GZMA was highly expressed consistently across cell types, while GZMB was highly expressed, but significantly lower in MoDC compared to MDM. Previous work has identified the importance of secreted granzyme A as an inducer of pro-inflammatory cytokine production (van Eck et al., 2017). Granzyme B is upregulated in monocytes following TLR activation (Elavazhagan et al., 2015), and

expressed by resident macrophages in chronic inflamed tissue such as rheumatoid arthritis (Kim et al., 2007). In addition, granzyme B expression is upregulated in macrophages following treatment with extracellular matrix proteins, suggesting a greater role for this enzyme in tissue-resident macrophages (Kim et al., 2007). This would align with the data presented here, as the MDM used are commonly generated *in situ* to replenish tissue-resident macrophages.

Monoamine oxidase A and B (MAOA, MAOB) catalyse deamination of amines, including dopamine and serotonin (Yeung et al., 2019). In this study, MAOA was more highly expressed by these cells, although both subtypes were found throughout, with no differences between cell types. Due to the importance of these enzymes in metabolism of neurotransmitters, most studies have used microglial cells (CNS macrophage subtype) to investigate MAO expression. Interestingly, higher expression of MAO in microglial cells has been linked to increased inflammatory markers such as ROS production (Trudler et al., 2014), which could explain the anti-inflammatory effects MAO inhibitors display in the CNS (Ostadkarampour et al., 2021). These data would imply that MAO expression may be increased in M1 MDM, but this was not seen here, which suggests MAO-mediated inflammation may be specific to microglial cells, perhaps in response to local tissue-specific cues in the CNS.

Prostaglandin-endoperoxide synthase 1 and 2 (PTGS1, PTGS2), also known as cyclooxygenase (COX) 1 and 2, are cyclooxygenases which primarily catalyse formation of prostaglandin endoperoxide from arachidonic acid, the first step in prostaglandin synthesis (Fitzpatrick, 2004). Prostaglandins are bioactive lipids which elicit a range of biological responses dependent on lipid structure but are often involved in the generation of an inflammatory response (Ricciotti et al., 2011). PTGS1 is generally thought to be constitutively expressed in most tissues, providing homeostatic functions such as inflammation in the stomach lining. In comparison, PTGS2 expression is highly inducible in inflammatory conditions, including in response to LPS stimulation, and similarly detected in most tissue types (Rouzer et al., 2009). In this study, both enzymes were highly expressed by all cell types, with individual qPCR for PTGS2 revealing a significant increase in M1 MDM compared to all other cell types, which was also confirmed at the protein level. These results were expected as PTGS2 is a well-established marker of inflammatory macrophages (Barrios-Rodiles et al., 1998; Martinez et al., 2006; Viola et al., 2019). Further studies attempted to

confirm enzyme functionality in isolated cell protein, but due to time constraints it was not possible to optimise the protocol to accurately measure activity.

Finally, ubiquitin carboxyl-terminal hydrolase L1 and L3 (UCHL1, UCHL3) are deubiquitinating enzymes, which function to hydrolyse ubiquitin adducts in order to generate free ubiquitin (Larsen et al., 1998; Mtango et al., 2012). These enzymes are primarily investigated in the field of neuroscience, as they are highly abundant in neurons (Bishop et al., 2016) and deficiencies in enzyme expression correlates with neurodegeneration (Reinicke, Laban, et al., 2019). In addition, UCH expression is often increased in cancer, including head and neck cancer (Rong et al., 2021), and notably UCHL3 is considered a tumour promotor (Fang et al., 2017). In this study, both enzymes were highly expressed by all cell types, and expression was significantly increased in MoDC compared to MDM. While little research has investigated UCH expression in macrophages, in DC deubiquitinase enzymes have been linked to cell maturation and function (Zhu et al., 2020). Furthermore, UCHL1 was recently shown to promote antigen cross presentation in dendritic cells, thereby reducing ability to prime T cells (Reinicke, Raczkowski, et al., 2019), suggesting that UCH expression in DC is directly linked to immune function.

3.5 Conclusion

In this chapter, monocytes were successfully differentiated to MDM and MoDC phenotypes, but not MoLC, while MDM could be further polarised to M1 and M2 states, each with distinct gene expression profiles. MDM and MoDC were found to express many XME, with altered gene expression found between polarised macrophage states for important drug metabolising enzymes such as CYP2D6 and CYP3A5, suggesting implications for local drug metabolism in inflammatory diseases, although further work to confirm functionality is still required.

Chapter 4 – Optimising production of an inflammatory response in MDM

4.1 Introduction

In the previous chapter, a role for polarised macrophages in xenobiotic metabolism was established. To further investigate the involvement of these cells in oral biology, the overall goal of incorporating these cells into a tissue engineered 3D inflammatory model was established. This chapter sought to optimise culture conditions prior to inclusion in a 3D model system.

Recent studies have sought to generate immune-competent oral models by incorporating primary monocytes, peripheral blood mononuclear cells (PBMC), or myeloid cancer cell lines such as MonoMac 6 (MM6), U937 or THP-1 cells, and observing changes in inflammatory markers and proteases following bacterial LPS (Morin et al., 2017; Björnfot Holmström et al., 2017; Xiao et al., 2018; Lira-Junior et al., 2020), bacterial biofilms (Bao et al., 2015) or X-ray treatment (Tschachojan et al., 2014). While the use of myeloid cancer cell lines presents fewer technical limitations and reproducibility compared to primary immune cells, there is good evidence that their phenotype and function is markedly altered compared to primary cells, with THP-1 cells shown to express changed levels of key macrophage phenotypic markers, and thus respond differently to LPS stimulation (Bosshart et al., 2016). Moreover, peripheral blood monocytes very rapidly differentiate into macrophages upon crossing the vasculature as they migrate into tissues and so incorporation of macrophages rather than monocytes into OME is more desirable. Therefore, use of primary macrophages is preferential for use in human *in vitro* OME as these cells better represent the innate immune component of human tissue and thus the culture conditions were optimised for these cells specifically.

Within this chapter, bacterial LPS was used to stimulate MDM in 2D and in 3D hydrogels. LPS are long-chain lipopolysaccharides present on the outer membrane of Gram-negative bacteria. LPS induces a robust inflammatory response in macrophages by first binding to LPS binding protein in serum which binds to CD14 and Toll-like receptor 2 and 4 (TLR4 and TLR2) on the macrophage cell surface. This interaction induces a intracellular signalling cascade which culminates in activation of the NFκB pathway (Alexander et al., 2001; Liu et al., 2017). Downstream effects can be summarised as a phenotypic shift to a more pro-inflammatory state (sometimes referred to as M1 macrophages), identifiable by increased secretion of a multitude of

pro-inflammatory cytokines such as IL-6, CXCL8 and TNF-α, as well as increased gene expression of inflammatory markers like CD80 and CD86 (Rossol et al., 2011; Carmody et al., 2013). To further assess MDM functionality, two inhibitors of the NFκB pathway were tested. First BAY 11-7085 (Pierce et al., 1997), a small molecule inhibitor which irreversibly inhibits IκBα phosphorylation, a key step in the NFκB signalling cascade, and dexamethasone, a glucocorticoid which also inhibits the NFκB pathway (Newton, 2000). Together, the stimuli used in this chapter were chosen as they all have a relatively low molecular weight, and thus should be able to diffuse through a collagen matrix and potentially activate cells in a 3D environment.

Chapter aim: Optimise culture conditions to activate and inhibit MDM inflammatory response in monolayer and within a collagen hydrogel.

Objectives:

- Quantify MDM activation by LPS from different Gram-negative pathogens.
- Ensure MDM activation occurs following long-term culture.
- Assess inhibition of MDM activation by NFκB pathway inhibitors.
- Determine optimal collagen species type to prevent immune activation.
- Test MDM viability and functionality when embedded in a collagen hydrogel by treating with bacterial LPS or inhibiting with NFκB pathway inhibitors.

4.2 Methods

- Monocyte differentiation (Section 2.2.8)
- MDM activation and inhibition (Section 2.2.9)
- qPCR (Section 2.3.1)
- Flow cytometry (Section 2.3.2)
- ELISA (Section 2.3.5)
- LDH (Section 2.3.6)
- LAL assay (Section 2.3.7)
- LPS isolation (Section 2.4)

4.3 Results

4.3.1 Optimising culture conditions for MDM activation

Serum can be a source of endotoxins (LPS) in cell culture (Kirikae et al., 1997), and although most modern suppliers ensure a low endotoxin level, the serum used throughout this study was stated to contain ≤10 EU/mL (~1 ng/mL) which could cause unwanted immune activation. Moreover, serum is known to contain essential co-factors that aid LPS recognition so may be crucial for full MDM activation (Alexander et al., 2001). Therefore, stimulating MDM in the absence or presence of serum was investigated.

MDM were treated with 100 ng/mL LPS (commercial products derived from *P. gingivalis* or *E. coli*) diluted in media with or without human serum for 24 hours. The conditioned media was then quantified for presence of IL-6 (Figure 4.1A), CXCL8 (Figure 4.1B) and TNF-α (Figure 4.1C).

Addition of serum to culture media did not alter basal secretion of IL-6 ($p>0.99$) or response to *P. gingivalis* LPS ($p=0.98$) but did improve response to *E. coli* LPS (73-fold; $p=0.018$) compared to the same treatment in serum-free media. Furthermore, in serum-free media, LPS treatment did not alter IL-6 secretion ($p>0.99$), while in serum-containing media *E. coli* LPS significantly increased IL-6 secretion compared to untreated control (159-fold; $p=0.0075$) and *P. gingivalis* LPS (5-fold; $p=0.028$).

Similarly, addition of serum to culture media did not increase basal secretion of CXCL8 ($p=0.32$) but did improve response to both *P. gingivalis* (4-fold; $p=0.0083$) and *E. coli* LPS (56-fold; $p<0.001$) compared to serum-free media. In serum-free media, LPS treatment did not change CXCL8 secretion ($p>0.99$), while in serum-containing media only *E. coli* LPS significantly increased release of CXCL8 compared to untreated control (4-fold; $p=0.0013$).

Finally, addition of serum to culture media did not increase basal secretion of TNF-α ($p>0.99$) or response to *P. gingivalis* LPS ($p=0.26$) but did improve response to *E. coli* LPS (31-fold; $p<0.001$) compared to serum-free media. In serum-free media, LPS treatment did not change TNF-α secretion ($p>0.99$), while in serum-containing media only *E. coli* LPS significantly increased release of TNF-α compared to untreated control (79-fold; $p<0.001$) and *P. gingivalis* LPS (4-fold; $p<0.001$).

Taken together, these data show that MDM cultured in serum-containing medium alone do not initiate an immune response, suggesting that contaminating LPS levels within the serum are too low to affect MDM activity. However, these data also show that the presence of serum is a key mediator of an LPS-inducible MDM pro-inflammatory response, and therefore all future experiments were carried out in serum-containing medium.

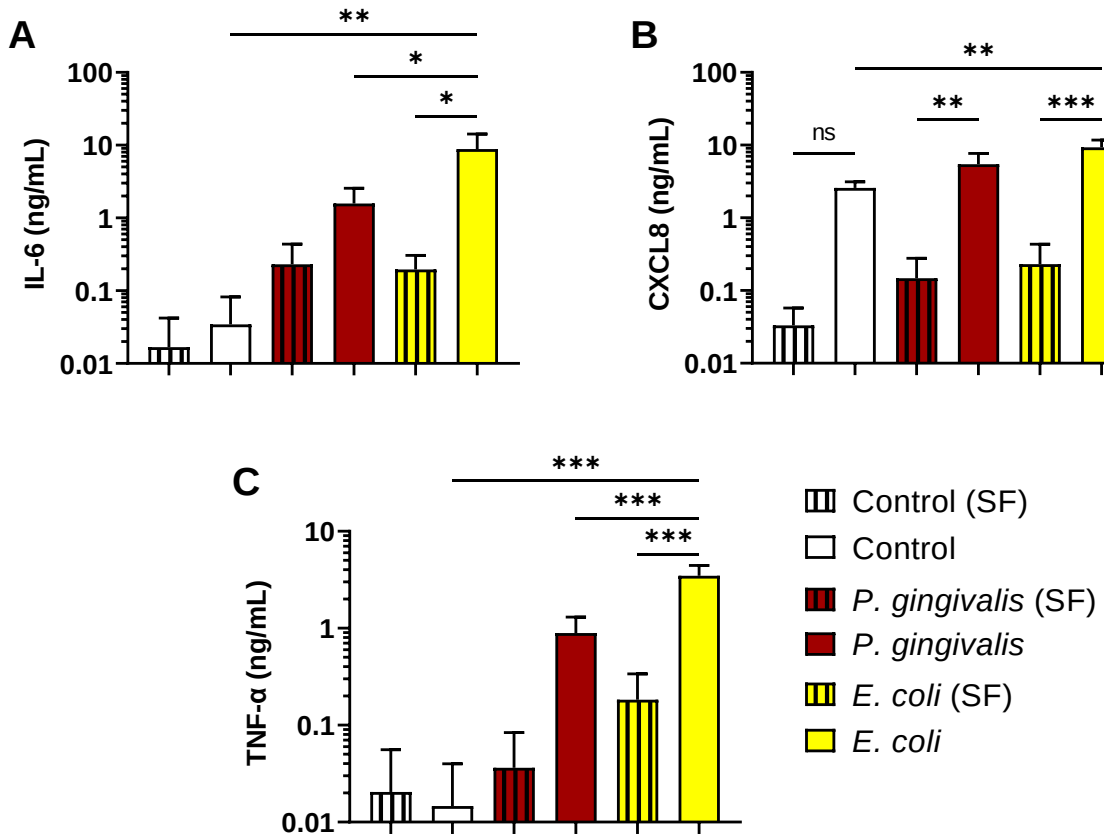


Figure 4.1. MDM response to LPS in serum-free or serum-containing media
MDM were stimulated with 100 ng/mL LPS from *P. gingivalis* or *E. coli* for 24 hours, in IMDM containing 2% serum, or serum free (SF) and compared to untreated controls. Secretion of IL-6 (**A**), CXCL8 (**B**) and TNF-α (**C**) into conditioned media was measured by ELISA. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA; *p<0.05, **p<0.01, ***p<0.005.

4.3.2 Characterising MDM response to LPS from oral bacteria

MDM response to LPS from bacteria involved in oral periodontitis was examined to identify an optimum stimulant to use in an oral mucosal model. The three bacteria selected, *P. gingivalis, T. forsythia* and *A. actinomycetemcomitans* were chosen as they are all Gram-negative species that have been linked to chronic periodontitis (Hajishengallis et al., 2012). *E. coli* was used as it is consistently found in the oral cavity, increases dramatically in patients with other oral lesions or systemic diseases, but is not directly linked to periodontitis (Zawadzki et al., 2016, 2017). Only *E. coli* and *P. gingivalis* LPS were commercially available, so LPS from other oral species was isolated in-house. After purification, LPS was examined by silver nitrate staining (Figure 4.2).

E. coli LPS displayed a well-characterised banding pattern which displaying decreasing concentrations of lower molecular weight LPS banding towards the bottom of the gel. *P. gingivalis* LPS displayed a similar banding pattern, but at a lower density and overall molecular weight compared to *E. coli*, with banding concentrated towards the bottom of the gel, indicating smaller LPS proteins produced by this species. However, for both *T. forsythia* and *A. actinomycetemcomitans*, a single light band was observed at the maximal weight of *E. coli* LPS, but other banding was not observed.

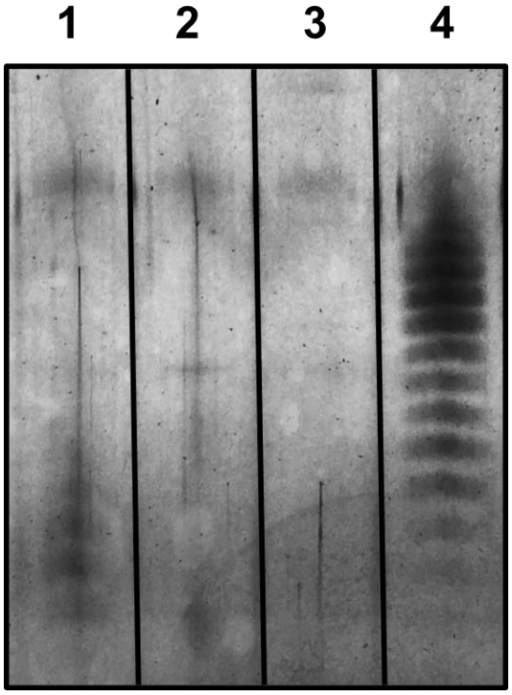


Figure 4.2. Structural analysis of bacterial LPS
LPS was separated on a 10% polyacrylamide urea gel and stained with silver nitrate. **1** *P. gingivalis* (1 µg/mL), **2** *A. actinomycetemcomitans* (5 µg/mL), **3** *T. forsythia* (5 µg/mL), **4** *E. coli* (1 µg/mL). Image is representative of multiple gels.

LPS from each bacterial species was tested for immunogenicity by treating 7-day MDM with LPS (500 ng/10^6 MDM for 24 hours) and the conditioned media examined for lactate dehydrogenase (LDH) release to assess cell viability (Figure 4.3A) and presence of pro-inflammatory cytokines by ELISA (Figure 4.3B-D). No significant differences were seen in LDH release following treatment with LPS from any species compared to controls, indicating LPS had no cytotoxic effects. Significant increases in cytokine secretion were observed following treatment with *E. coli* LPS for all inflammatory cytokines examined, compared to untreated control. IL-6 increased from 0.035 to 8.8 ng/mL ($p=0.0083$), CXCL8 from 2.57 to 9.33 ng/mL ($p=0.0041$) and TNF-α from 0.02 to 3.47 ng/mL ($p=0.0003$). However, while secretion was typically increased when MDM were treated with LPS from periodontal bacteria, it did not reach statistically significant levels (IL-6 $p>0.57$; CXCL8 $p>0.23$; TNF-α $p>0.35$), although notably LPS from *T. forsythia* did not induce any inflammatory response ($p>0.98$).

In addition to cytokine secretion, alterations in cellular gene expression were also measured after 24 hours treatment (Figure 4.4) and compared to untreated controls. The macrophage inflammation (M1) marker CD80 was increased by treatment with *E. coli* LPS (5-fold; $p=0.02$), but not periodontal bacteria, while expression of M1 marker CD86 was unchanged by LPS from all bacteria tested and M2 marker CD204 was decreased by *E. coli* LPS (2-fold; $p=0.005$), but not periodontal bacteria (Figure 4.4A). Gene expression corresponding to the inflammatory markers measured by ELISA was also examined (Figure 4.4B). IL-6 gene expression was significantly increased by *E. coli* (93-fold; $p=0.033$), but not periodontal bacterial ($p>0.57$). Similarly, CXCL8 gene expression was significantly increased by *E. coli* (13-fold; $p=0.037$), but not periodontal bacterial ($p>0.38$). Finally, TNF-α gene expression was unchanged by treatment with all LPS ($p>0.7$), despite a significant change in cytokine expression in *E. coli* treated cells. As a result of these data, only LPS from *E. coli* and *P. gingivalis* were used in future experiments.

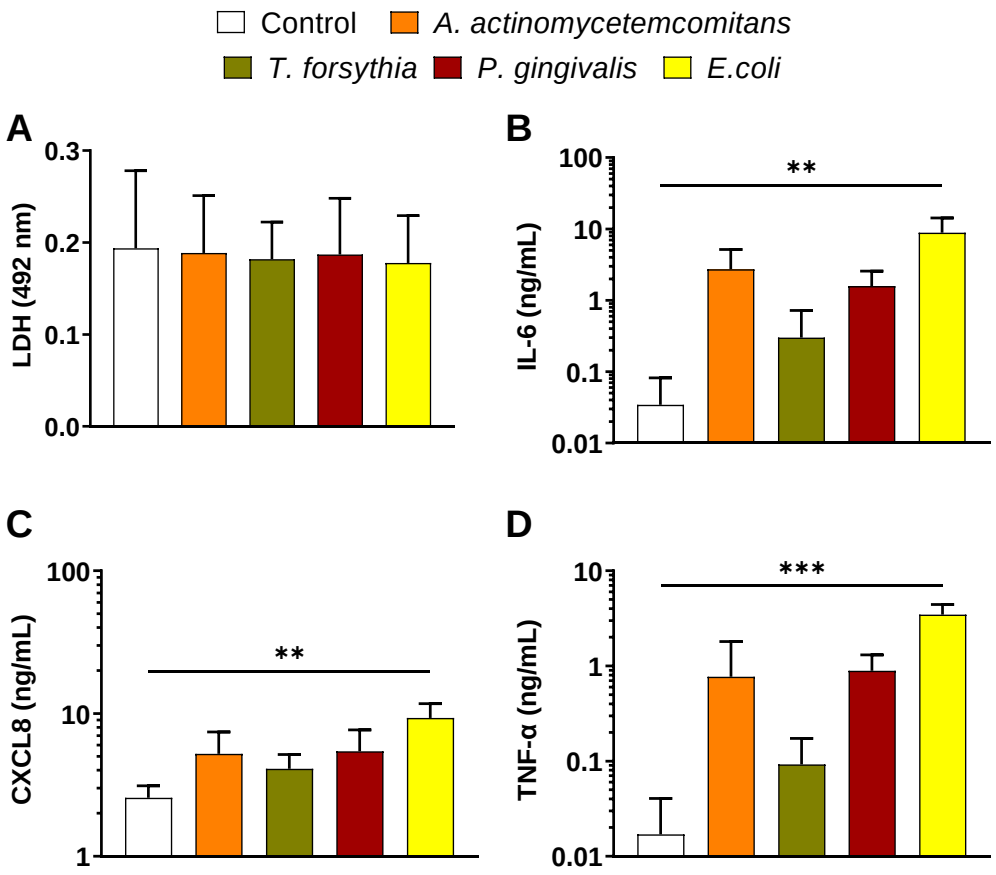


Figure 4.3. MDM inflammatory response to LPS from periodontal and non-periodontal bacteria

MDM were treated with 500 ng LPS per 10^6 cells for 24 hours and conditioned media analysed for LDH release (**A**) and for secretion of inflammatory cytokines IL-6 (**B**), CXCL8 (**C**) and TNF-α (**D**) by ELISA. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA; **p<0.01, ***p<0.005.

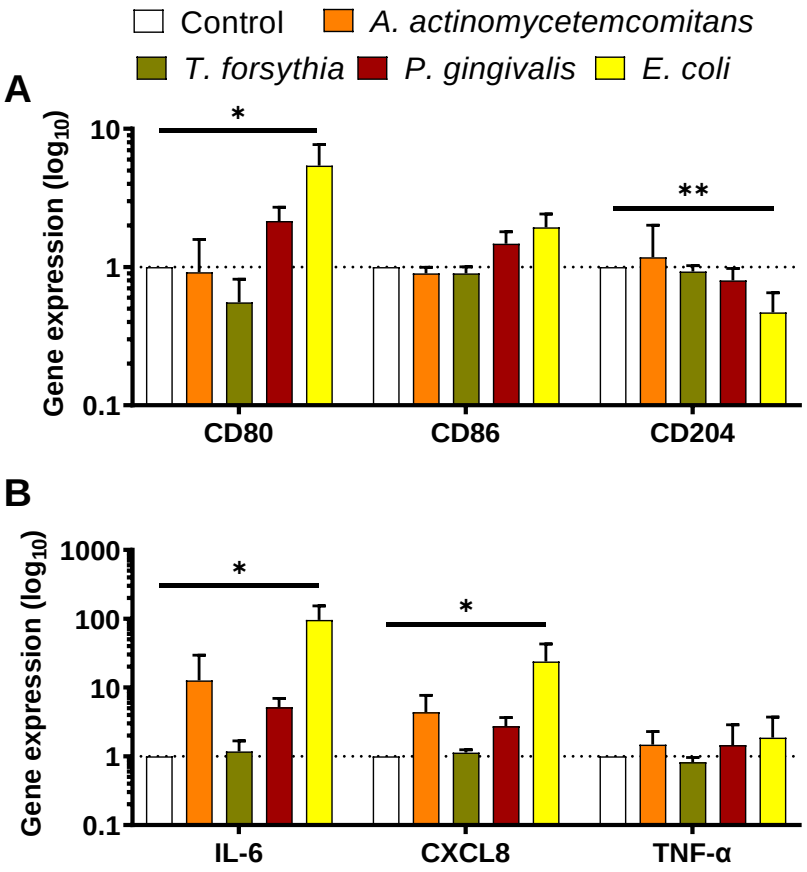


Figure 4.4. MDM gene expression response to LPS from periodontal and non-periodontal bacteria

MDM were treated with 500 ng LPS per 10^6 cells for 24 hours gene expression for a panel of inflammatory markers (**A**) and inflammatory cytokines (**B**) was analysed by qPCR, calculated relative to the reference control *β2-microglobulin,* and normalised to untreated control. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA; *p<0.05, **p<0.01.

4.3.3 MDM activation following long term cell culture

To incorporate functional MDM into a fully differentiated tissue engineered model, long term culture needs to be achieved. Firstly, monocytes need to be differentiated into MDM in 2D for 6 days before incorporation into a collagen hydrogel. Once MDM are incorporated into the connective tissue the OME requires a further 10 days in culture to allow formation of a stratified epithelium that is necessary for a mature tissue engineered model. Therefore, taking the longest potential culture period, MDM were grown for up to 21 days and tested to confirm these cells remain viable and functional at experimental end point.

MDM cell morphology remained stable over time with a dark nuclei and light cytoplasm (Figure 4.5). Over time, MDM size increased, and by day 14 the population phenotype had stabilised to the classical 'fried egg' appearance, compared to earlier time points where the phenotype was typically mixed.

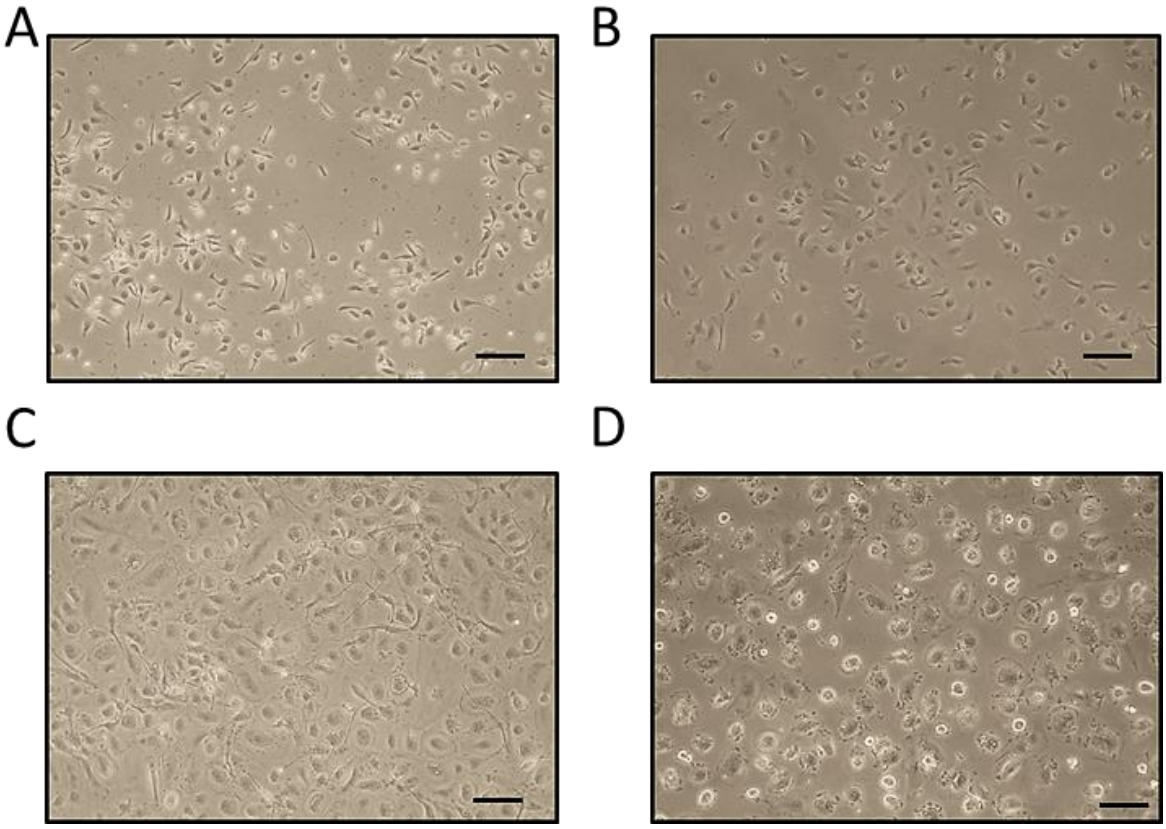


Figure 4.5. MDM phenotype during long-term culture
MDM were cultured for 3 (**A**), 6 (**B**), 14 (**C**), and 22 days (**D**). Images presented are representative from at least three independent experiments. Scale bar = 100 µm.

MDM were challenged with LPS at day 7, 14, or 21 to assess viability and function at these time points. Viability of these cells was measured indirectly by release of LDH, and functionality determined by release of pro-inflammatory cytokines IL-6, CXCL8 and TNF-α into conditioned media.

There was no significant increase in LDH release following LPS treatment (Figure 4.6A) at any time point examined, suggesting no toxic effect upon LPS treatment, or following long term culture.

In general, cytokine release profiles remained consistent and inducible throughout the culture period with *E. coli* LPS significantly increasing release of all cytokines at all time points with the exception of IL-6 at day 14.

Secretion of IL-6 was negligible from unstimulated MDM and only found above the ELISA limit of detection on day 14 at 0.6 ± 0.4 ng/mL (Figure 4.6B). When treated with *P. gingivalis* LPS, IL-6 secretion increased at all time points, although this was only significant at day 14 (15-fold; $p=0.02$). In comparison, *E. coli* LPS induced greater increase in IL-6 secretion, which was significant at day 7 (9-fold; $p=0.014$) and day 21 (9-fold; $p=0.017$), but not day 14 ($p=0.13$).

CXCL8 (Figure 4.6C) had the highest basal expression of all cytokines measured, although significant increases were observed when treated with *E. coli* LPS across all time points ($p<0.006$; fold change 9.5 day 7, 8.8 day 14, 36.9 day 21). In contrast, despite *P. gingivalis* LPS treated MDM consistently secreting higher levels of CXCL8 than control, this was not statistically significant at any time point examined ($p>0.06$).

Finally, TNF-α (Figure 4.6D) also had a very low basal secretion, which was typically undetectable. Significant increases when treated with *E. coli* LPS was observed ($p<0.048$; fold change 282.2 day 7, 349.8 day 14, 601.7 day 21), but not upon treatment with *P. gingivalis* LPS.

Taken together, these data show MDM are viable for at least three weeks in monolayer culture and retain functionality in response to bacterial LPS. Therefore, the time required to differentiate monocytes and grow a tissue engineered model is not functionally prohibitive, and these cells could be taken forward to experiments in a 3D environment.

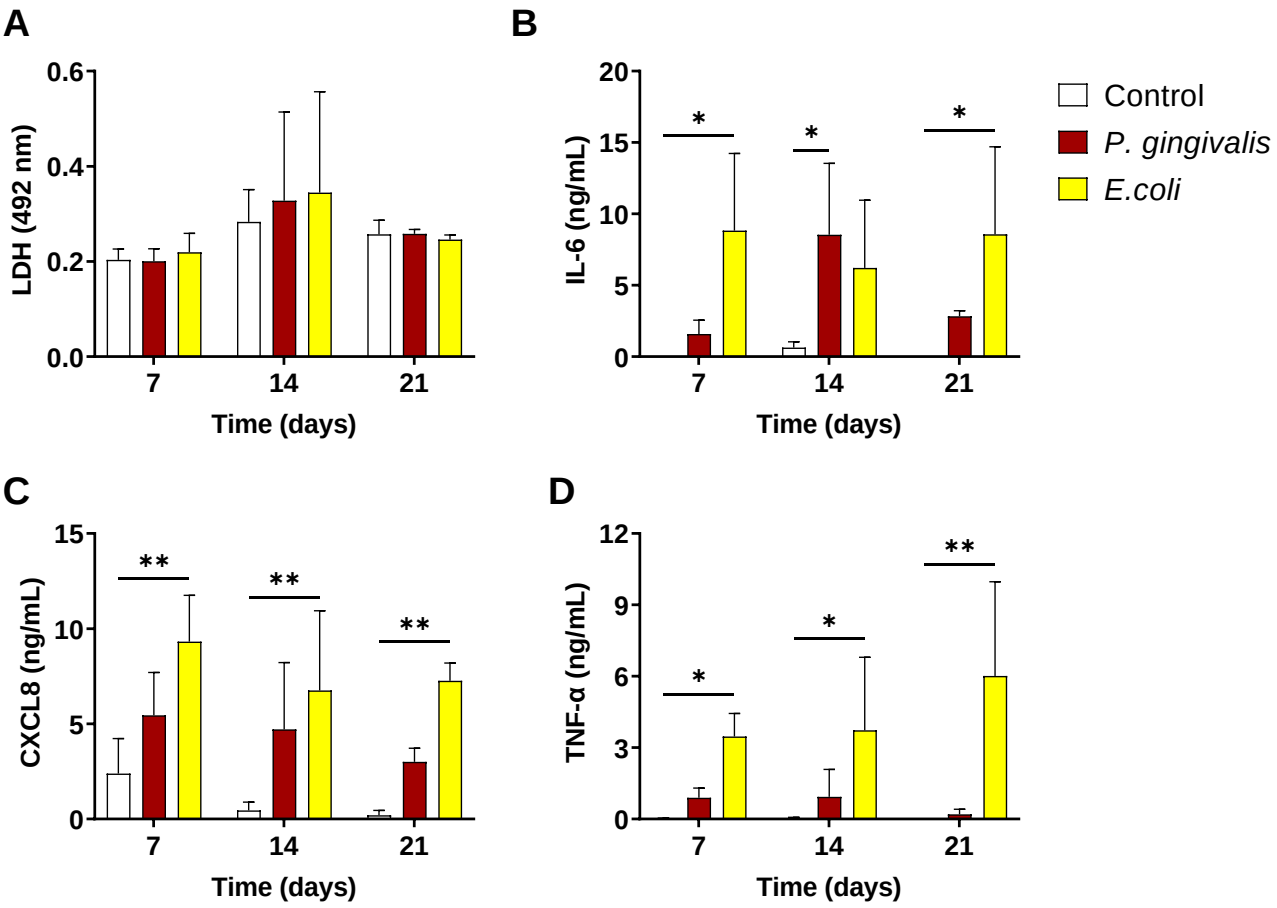


Figure 4.6. MDM viability and function during long term culture
MDM were cultured for up to 21 days and treated with LPS from *P. gingivalis* or *E. coli* (500 ng per 10^6 cells) for 24 hours at each endpoint of 7, 14 and 21 days. Conditioned media was analysed for LDH release (**A**) and for secretion of inflammatory cytokines IL-6 (**B**), CXCL8 (**C**) and TNF-α (**D**) by ELISA. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using two-way ANOVA; *p<0.05, **p<0.01.

4.3.4 Determining model optimal collagen type to model OME connective tissue

To identify any inflammatory properties of collagen hydrogels, collagen from three different animal species was tested to quantify potential MDM activating effects. MDM cultured in monolayer were overlaid with a collagen solution from either jellyfish (Jellagen, UK), bovine dermis (Dr Christopher Wright, Swansea University) or rat tail collagen (produced in-house), and collagen-free MDM were used as a control. After the solutions polymerised to form a hydrogel, media was added above the gels and the cells incubated for 24 hours, then *E. coli* LPS was added to the media for a further 24 hours to induce an inflammatory response. When used in this study, both rat tail and bovine collagen gels polymerised within 30 minutes, but even following 2 hours incubation jellyfish collagen did not. The experiment therefore proceeded with jellyfish collagen diluted in tissue culture media as opposed to providing a barrier between MDM and media. While this may have affected the end results, it was assumed that any collagen immuno-reactivity would induce an MDM inflammatory response regardless of gel consistency.

As shown in Figure 4.7A, no hydrogels induced a significant change in LDH release compared to monolayer control ($p>0.84$), suggesting no effect on cell viability. As shown previously, MDM in monolayer culture respond to LPS treatment with increases in IL-6 (66-fold; $p=0.041$) CXCL8 (5-fold; $p=0.045$) and TNF-α (9-fold; $p=0.0075$) (Figure 4.7B-D). In contrast, both jellyfish ($p>0.07$) and bovine ($p>0.14$) collagen prevented a significant increase in LPS-induced inflammatory cytokine production, typically due to increased cytokine secretion in the LPS-free control. Finally, in contrast to other collagens tested, rat tail collagen displayed no immune-reactivity and MDM cultured under these conditions displayed LPS-induced increased secretion of IL-6 (17-fold; $p=0.041$), CXCL8 (6-fold; $p=0.039$) and TNF-α (34-fold; $p=0.048$).

To further understand the source of collagen-induced inflammation, endotoxin levels were quantified by LAL assay in cell-free collagen. As shown in Table 4.1, the rat tail collagen contained endotoxin levels below the limit of detection of the assay, while bovine and jellyfish contained 7.3 and 30.94 EU/mL, respectively. These values equate to 0.7 and 3 ng/mL LPS, which is much lower than the concentration used to

treat MDM, but still may have induced a moderate inflammatory response. As a result of both this and the functional data collected in Figure 4.7, rat tail collagen was used exclusively in future experiments. Furthermore, only one batch of rat tail collagen was used throughout, to ensure consistency, and low endotoxin levels.

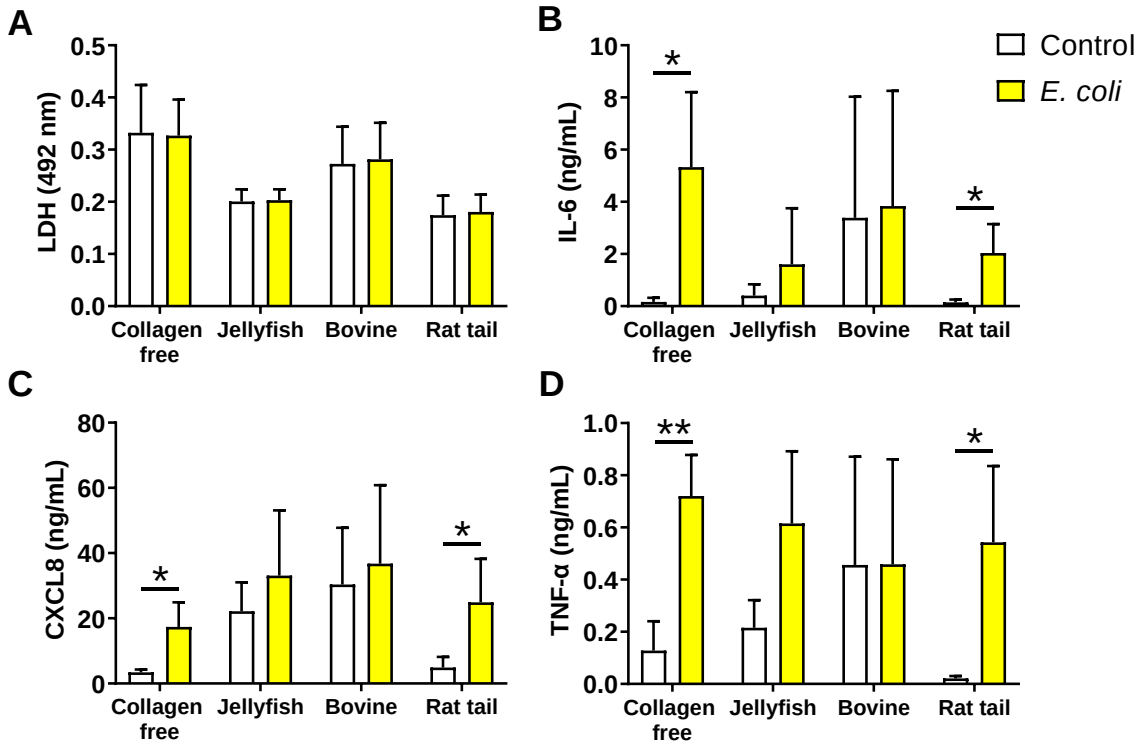


Figure 4.7. Effect of collagen from different species on MDM activation
MDM in monolayer were overlaid with collagen solution and incubated to form a polymerised gel. After 24 hours MDM were treated with *E. coli* LPS (500 ng per 10^6 cells) for a further 24 hours and conditioned media analysed for LDH release (**A**) and for secretion of inflammatory cytokines IL-6 (**B**), CXCL8 (**C**) and TNF-α (**D**) by ELISA. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using paired t test; *p<0.05, **p<0.01.

Table 4.1. Quantification of endotoxin concentrations in cell-free collagen

Collagen origin	Endotoxin concentration
Rat tail (produced in-house)	Below limit of detection (≤0.2 EU/mL)
Bovine dermis (commercial)	7.3 EU/mL
Jellyfish (commercial)	30.94 EU/mL

4.3.5 MDM activation in a 3D rat tail collagen hydrogel

After determining the optimal species-derived collagen to use, MDM function and response to stimuli was further investigated using MDM embedded within a collagen hydrogel to mimic a 3D tissue-like environment.

First, histology of MDM-containing hydrogels was examined. Minimal H&E staining was noted within cell-free hydrogels, confirming no cellular contamination (Figure 4.8A-B), while in MDM-containing hydrogel cells were evenly distributed within the matrix with no localised cell clustering observed (Figure 4.8C-D). Collagen staining appeared to be more intense compared to cell-free models, suggesting MDM were depositing protein into the extracellular space.

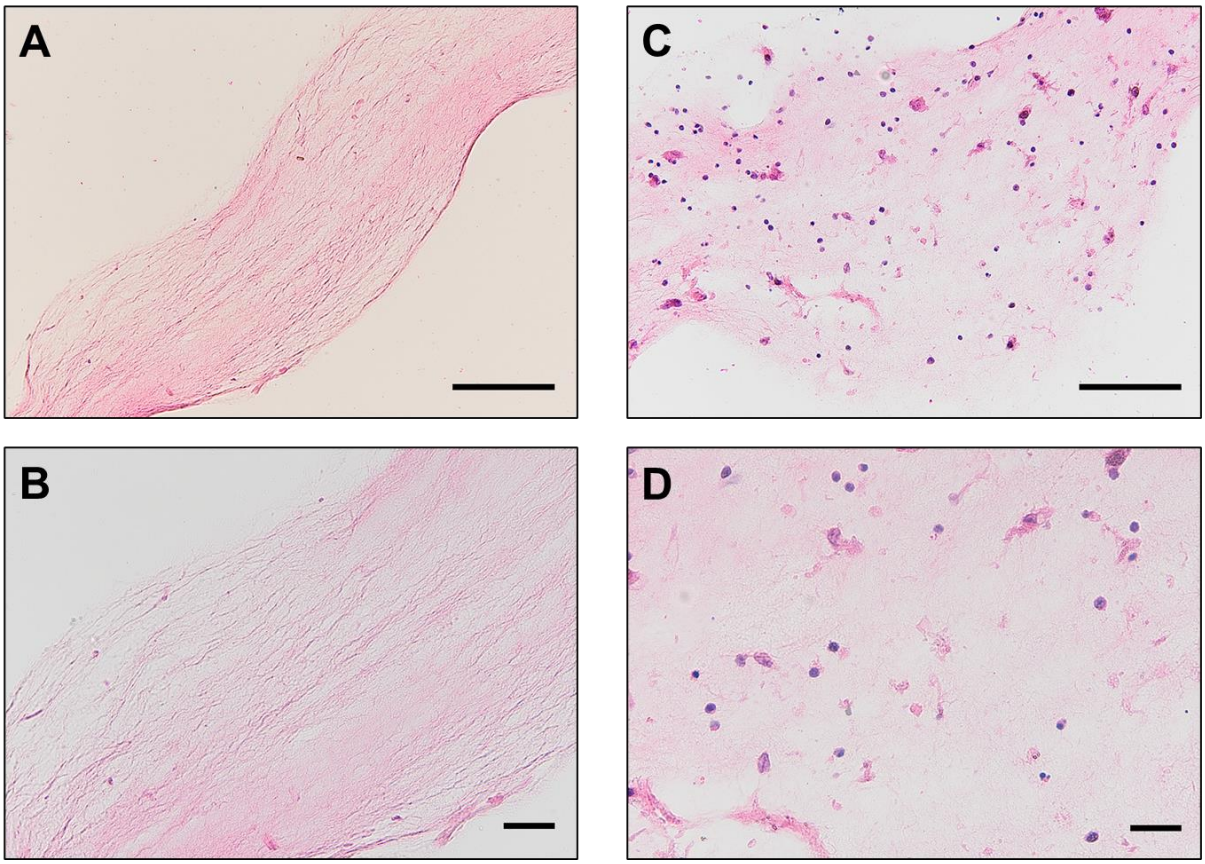


Figure 4.8. Histological analysis of MDM-containing collagen hydrogels
Cell-free (**A**, **B**) and MDM-containing gels (**C**, **D**) were cultured for 12 days, then formalin fixed, wax embedded, and H&E stained. Images are representative of n=4 independent experiments. Scale bar represents 100 µm.

Next, to determine MDM viability following 3D culture, MDM were collected from collagenase-treated hydrogels and compared to control cells grown in monolayer. Common stains for directly determining cell death such as trypan blue are ineffective as the dye is readily taken up by live MDM, so a fixable live dead stain was used to analyse cells by flow cytometry (Figure 4.9A). There was a significant difference in MDM viability between 2D and 3D culture ($p<0.0006$) with the mean live cells measuring 82 ± 8% and 24 ± 16% respectively, but no difference observed between 24 hours and 12 days culture ($p>0.8$) (Figure 4.9A). LDH release was also employed as an indirect measure of cell viability (Figure 4.9B), which showed a significantly higher LDH release at 12 days compared to 24 hours ($p>0.001$), and, surprisingly, in 2D compared to 3D at 12 days ($p=0.002$), but not 24 hours ($p=0.31$).

Taken together, these data suggest that the collagenase treatment used to isolate MDM from collagen is contributing to loss of viability, as opposed to length of culture period. Additionally, LDH could not be normalised to cell count, or maximal cell death and release may have been restricted by 3D culture so these data may not accurately reflect cell viability. Therefore, although viability may be underestimated when assessed by flow cytometry, it is a direct measure of cell viability and thus likely to give a truer value, so was used in all future 3D studies.

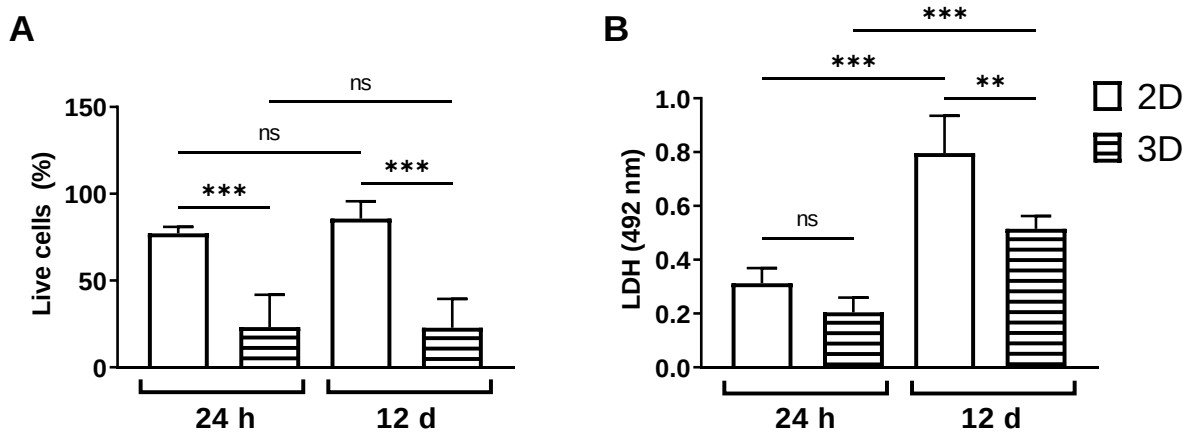


Figure 4.9. MDM viability in a collagen hydrogel
MDM were isolated from collagen after 24 hour or 12 day and assessed for viability using a live dead viability stain (**A**) and LDH release into conditioned media (**B**). Data shown are mean ± SD of live cells as a percentage of total cell count (**A**) or OD (**B**) from n=4 independent experiments with statistical significance determined using one-way ANOVA; **p<0.01, ***p<0.005.

MDM function within a collagen hydrogel was assessed by quantifying inflammatory cytokine release following stimulation with either *E. coli* or *P. gingivalis* LPS (Figure 4.10).

After 24 hours in a collagen hydrogel, *E. coli* LPS significantly increased expression of IL-6 (29-fold; $p=0.003$), CXCL8 (8-fold; $p<0.001$) but not TNF-α ($p=0.20$) compared to unstimulated controls, while no changes were observed after *P. gingivalis* treatment. Similarly, following 12-day culture in 3D, *E. coli* LPS significantly increased expression of IL-6 (29-fold; $p<0.001$), and TNF-α (10-fold; $p<0.001$), but not CXCL8 ($p=0.29$) compared to controls, while no changes were observed after *P. gingivalis* treatment ($p>0.98$). In addition, when comparing the effect of the two LPS treatments, *E. coli* had a significantly higher effect than *P. gingivalis* for IL-6 at both time points ($p<0.003$), CXCL8 at 24 hours ($p<0.001$) but not 12 days ($p=0.39$) and TNF- α at 12 days ($p=0.0013$) but not 24 hours ($p=0.20$). Finally, *E. coli* LPS treatment saw some time dependent variation in cytokine response where, compared to 24 hours, 12 days MDM produced significantly more IL-6 ($p=0.014$) and TNF-α ($p=0.013$), but significantly less CXCL8 ($p=0.0024$).

Therefore, at the LPS concentration used here, *P. gingivalis* was unable to induce a measurable inflammatory response, while *E. coli* LPS was shown to induce a robust inflammatory response in 3D MDM both immediately and following longer term culture. Thus, in future experiments *E. coli* LPS was employed as a suitable stimulant to study MDM inflammation in a tissue engineered model.

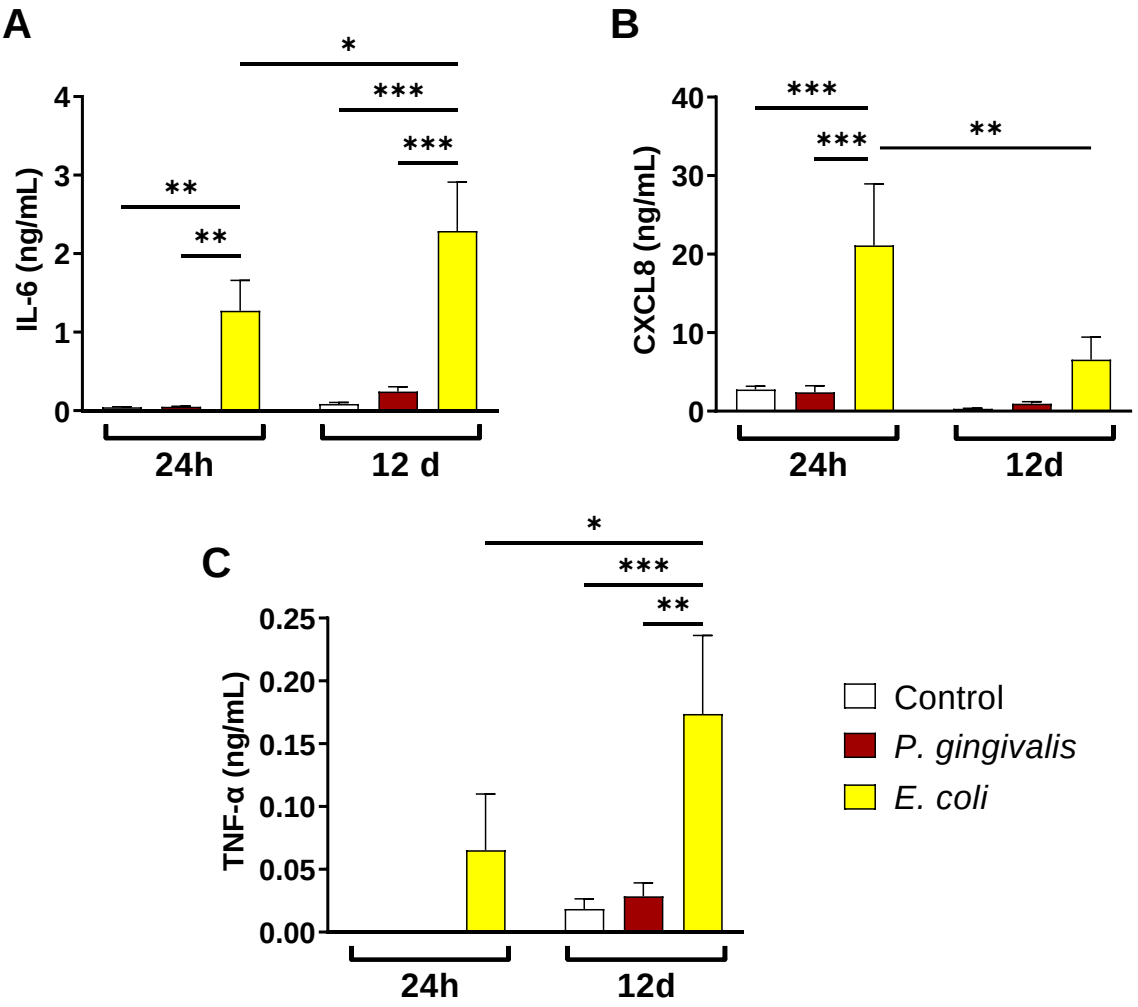


Figure 4.10. MDM function in a collagen hydrogel
MDM were cultured in a collagen hydrogel for 24 hours or 12 days, then stimulated for 24 hours with LPS from *P. gingivalis* or *E. coli* and cytokine release compared to untreated controls. Secretion of IL-6 (**A**), CXCL8 (**B**) and TNF-α (**C**) was measured by ELISA. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using two-way ANOVA; *p<0.05, **p<0.01, ***p<0.005.

4.3.6 Optimising MDM activation in the presence of NFκB inhibitors

In addition to testing the activation of MDM by LPS, the inhibition of this response was also investigated for the future testing of both inflammatory and anti-inflammatory effects. Two compounds were tested, a small molecule inhibitor, BAY 11-7085 (BAY) which irreversibly inhibits activity of IκB kinase (IKK), leading to NFκB inactivation (Pierce et al., 1997), and dexamethasone, a clinical drug used as an anti-inflammatory agent which acts as a glucocorticoid receptor agonist and disrupts inflammatory mechanisms, including the NFκB pathway (Scheinman et al., 1997). By inhibiting the NFκB pathway which mediates LPS activity, LPS-induced inflammatory effects should be reduced. Previous studies have used both BAY 11-7085 (Kahlenberg et al., 2005; Hoppstädter, Diesel, et al., 2019) and dexamethasone (van der Goes et al., 2000; Tedesco et al., 2015; Kim et al., 2017) to inhibit macrophage activation, but with a wide range of incubation times (30 minutes to 48 hours), and with doses ranging from 10 nM to 10 μM depending on cell type and study context. Therefore, a dose response study was performed for both inhibitors using two pre-incubation times (4 and 24 hours), prior to stimulation with LPS for a further 24 hours, to identify optimal inhibitory activity.

Cell viability in the presence of each compound was indirectly measured by release of LDH after incubation with the inhibitor alone for 4 or 24 hours. Higher concentrations of BAY 11-7085 (≥50 μg/mL) resulted in substantial loss of viability, which was significant after 4 hours, but not 24 hours, incubation (Figure 4.11A). In comparison, dexamethasone did not cause a significant increase in LDH release after either incubation time at any dose compared to the untreated control (Figure 4.12A).

The inhibitory activity of each compound was tested by stimulating with *E. coli* LPS following inhibition for 4 or 24 hours. BAY 11-7085 mediated inhibition was dose dependent, with significant reductions only observed at 50 and 500 μg/mL for all cytokines tested, where levels were reduced back to unstimulated controls (Figure 4.11). However, at these concentrations, significant increases of LDH were also observed, suggesting the reduction is due to loss of cell viability rather than chemical inhibition. In contrast, dexamethasone induced dose-dependent inhibition at doses of 0.1 μg/mL and above for all cytokines tested (Figure 4.12), while no loss of viability was observed. Finally, for all doses of both compounds, there was no significant difference observed between 4 and 24 hours incubation times.

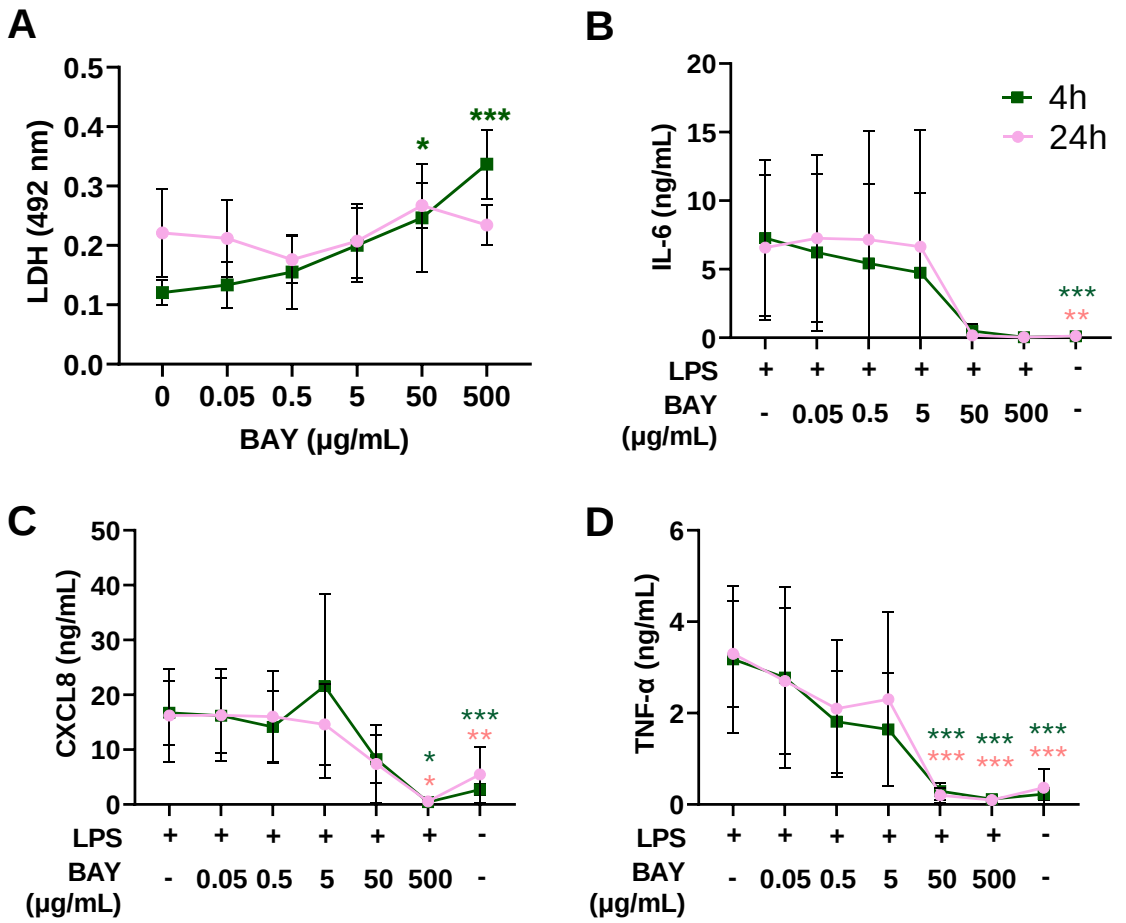


Figure 4.11. Dose response of inflammation inhibition by BAY 11-7085
MDM were treated with BAY 11-7085 (0.05 - 500 µg/mL) for or 4 or 24 hours and conditioned media tested for LDH release (**A**). Cells were further treated with *E. coli* LPS (500 ng per 10^6 cells) for 24 hours and secretion of IL-6 (**B**), CXCL8 (**C**), and TNF-α (**D**) measured by ELISA. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA compared to untreated control (**A**) or LPS positive control (**B**-**D**); *p<0.05, **p<0.01, ***p<0.005.

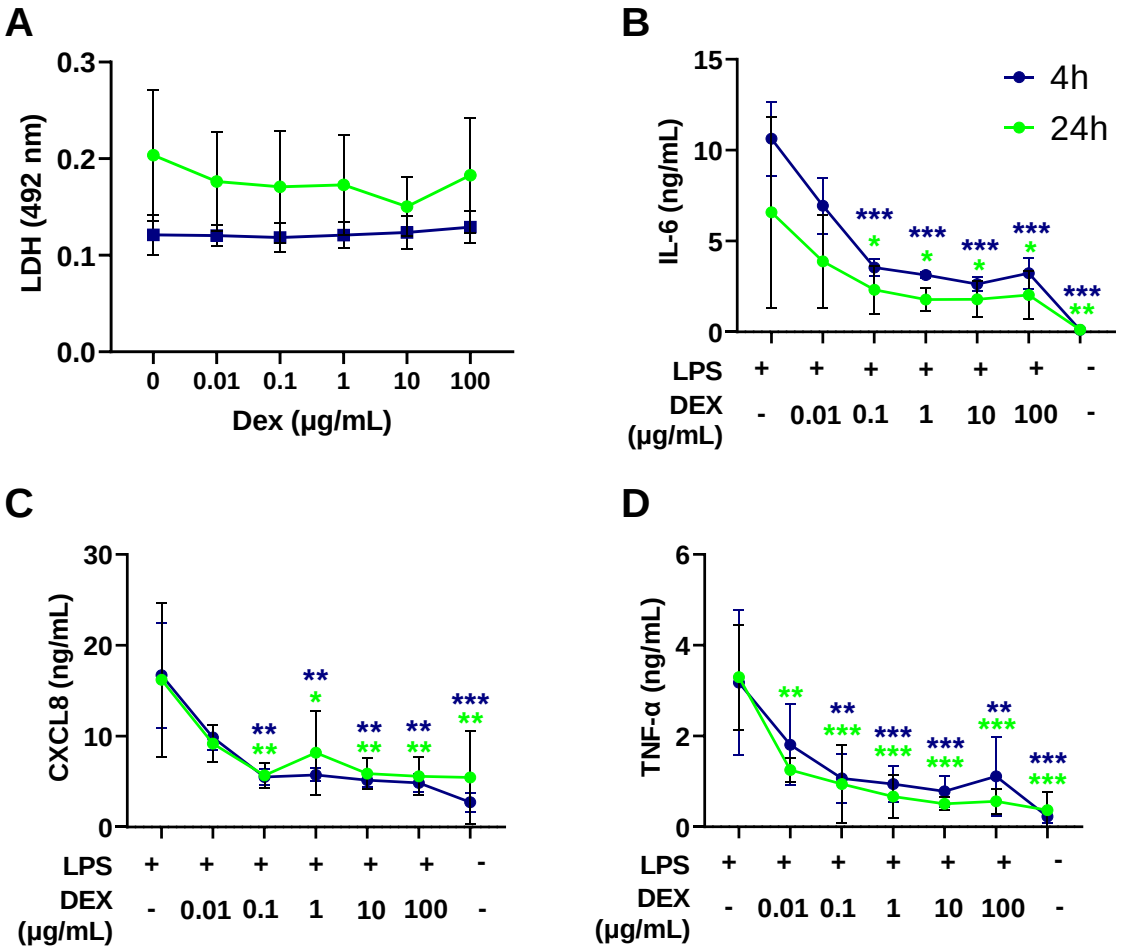


Figure 4.12. Dose response of inflammation inhibition by dexamethasone
MDM were treated with dexamethasone (0.01 - 100 µg/mL) for 4 or 24 hours and conditioned media tested for LDH release (**A**). Cells were further treated with *E. coli* LPS (500 ng per 10^6 cells) for 24 hours and secretion of IL-6 (**B**), CXCL8 (**C**), and TNF-α (**D**) measured by ELISA. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA compared to untreated control (**A**) or LPS positive control (**B**-**D**); *p<0.05, **p<0.01, ***p<0.005.

As a result of these preliminary investigations, dexamethasone was carried forward using a 4-hour pre-incubation regime, as this gave the most consistent results. In addition, while 1 µg/mL was a sufficient dose to induce maximal inhibition in 2D monolayers, both 1 and 10 µg/mL were carried forward to 3D investigations to allow for potential issues with permeation into a collagen hydrogel which may require the use of a higher concentration of inhibitor to achieve the desired effect.

To further assess the effect of treating 2D MDM with dexamethasone (1 µg/mL and 10 µg/mL), changes in gene expression were investigated (Figure 4.13), quantifying CD80 as an inflammatory (M1) marker, and both CD206 and CD163 as wound healing (M2) phenotype markers (Figure 4.13A), in addition to inflammatory cytokines IL-6, CXCL8 and TNF-α, and anti-inflammatory cytokine IL-10 (Figure 4.13B).

MDM treated with *E. coli* LPS increased gene expression of CD80 (6-fold; $p=0.0084$), and decreased expression of CD206 (4-fold; $p=0.037$), while CD163 was unchanged ($p=0.81$) compared to control. Dexamethasone pre-treatment prevented LPS-induced changes in CD80 and CD206, and increased CD163 expression compared to both unstimulated cells (40-fold; $p<0.01$) and cells treated with LPS alone (50-fold; $p<0.005$).

Furthermore, *E. coli* LPS increased gene expression of IL-6 (64-fold; $p=0.0005$) and CXCL8 (18-fold; $p=0.031$) but not TNF-α ($p=0.99$) or IL-10 ($p=0.84$), in agreement with data shown in Figure 4.3. Dexamethasone pre-treatment reduced LPS-induced expression of IL-6 (24-fold; $p<0.001$) and CXCL8 (13-fold; $p<0.041$). In contrast, TNF-α expression was reduced by dexamethasone pre-treatment (7-fold; $p<0.047$) compared to cells treated with LPS alone. Finally, no change was seen in IL-10 expression between any treatment conditions ($p>0.36$).

Taken together this confirms that treatment with *E. coli* LPS is sufficient to induce a pro-inflammatory phenotype and that both concentrations of dexamethasone can markedly prevent this phenotypic shift.

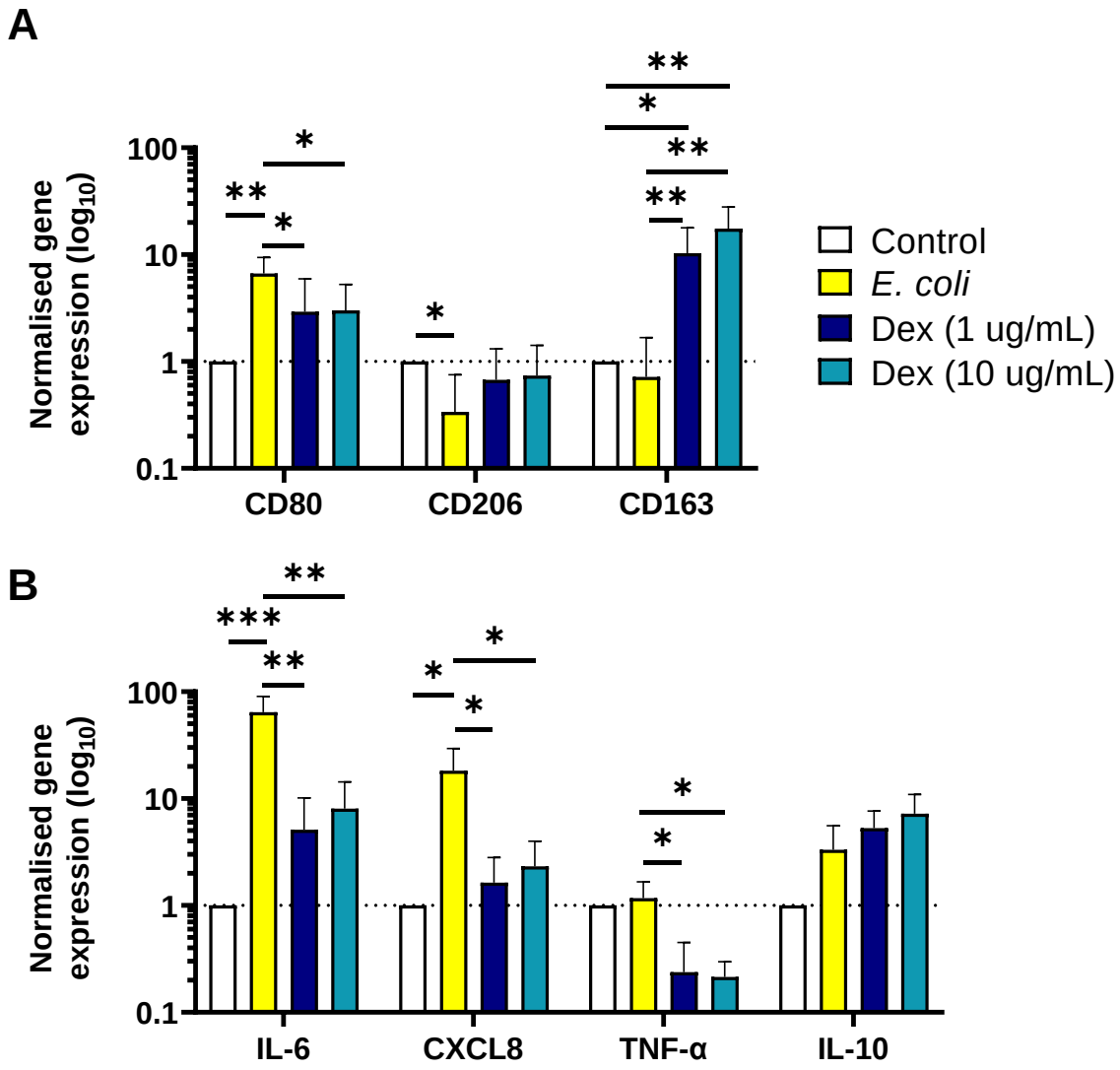


Figure 4.13. MDM gene expression following inhibition of inflammation by dexamethasone

MDM were treated with dexamethasone (1 and 10 µg/mL) for 4 hours then *E. coli* LPS for 24 hours. Gene expression for a panel of inflammatory markers (**A**) and cytokines (**B**) was analysed by qPCR, calculated relative to the reference control *β2-microglobulin* (B2M), and normalised to untreated control. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA; *p<0.05, **p<0.01, ***p<0.005.

4.3.7 MDM inflammation is inhibited by dexamethasone in a 3D collagen hydrogel

Following successful inhibition of LPS-induced inflammation in monolayer MDM, the efficacy to inhibit MDM activation in a 3D collagen hydrogel was examined. As highlighted earlier, the concentration of dexamethasone required to inhibit the LPS-induced activity of MDM in 3D may be different to those in 2D as the drug is required to penetrate through the matrix to reach the target cells, so both 1 and 10 µg/mL dexamethasone concentrations were tested. As with previous experiments, inflammation was measured by inflammatory cytokine release (Figure 4.14) and alterations in gene expression profiles (Figure 4.15).

In agreement with previous results, treatment of 3D MDM with *E. coli* LPS for 24 hours significantly increased expression of inflammatory cytokines IL-6 (9-fold; $p=0.0003$), CXCL8 (3-fold; $p<0.0001$) and TNF-α (2-fold; $p=0.0004$). When MDM were pre-incubated with dexamethasone for 4 hours, IL-6 secretion was reduced 3-fold compared to LPS treatment alone ($p<0.005$) (Figure 4.14A). Similarly, secretion of CXCL8 and TNF-α was decreased (2-fold; $p<0.0001$ and $p<0.02$ respectively) by dexamethasone pre-treatment compared to LPS treatment alone, although this was still significantly greater than unstimulated controls (Figure 4.15B&C). Release of IL-10, an anti-inflammatory cytokine, displayed no significant changes between any treatment ($p>0.07$) (Figure 4.14D). In all instances, no significant differences were observed between the two concentrations of dexamethasone tested.

Changes in gene expression were also measured in treated 3D-cultured MDM for markers of inflammation (Figure 4.15A) and cytokines (Figure 4.15B). In contrast to MDM in monolayer, 3D MDM gene expression of CD80 and CD206 were unchanged following LPS stimulation both in the absence or presence of dexamethasone compared to untreated control (Figure 4.15A). The expression of the M2 marker CD163 was decreased by LPS stimulation compared to control and dramatically increased upon pre-treatment with dexamethasone compared to both unstimulated control (33-fold; $p=0.049$) and LPS treatment alone (226-fold; $p=0.044$).

Furthermore, LPS increased IL-6 gene expression compared to control (2-fold; $p=0.007$) which was prevented by dexamethasone pre-treatment ($p<0.011$). CXCL8 gene expression was increased by LPS (7-fold; $p=0.005$) compared to control, although this was not significantly inhibited by dexamethasone pre-treatment ($p>0.07$).

Finally, expression of TNF-α ($p>0.99$) and IL-10 ($p>0.88$) was unchanged in all treatment conditions. For all genes examined, no differences were observed between the two doses of dexamethasone ($p>0.89$).

Taken together, these gene expression data suggest that some basal MDM inflammation may be occurring within the 3D environment, preventing a significant shift to an inflammatory phenotype following LPS treatment alone or that the gene expression response is in decline at this 24-hour time point, a time where transcription has likely passed and translation and protein production is prominent, as observed in the cytokine secretion data in Figure 4.14. Overall, treatment with dexamethasone appears to sufficiently inhibit the LPS-induced inflammatory phenotype, and thus allow for an inflammatory response to be measured and compared. In addition, as no differences were seen between the doses of dexamethasone, it was determined that the lower dose was sufficient to induce a maximal effect, and therefore 1 µg/mL was used going forward to model a more physiologically relevant response.

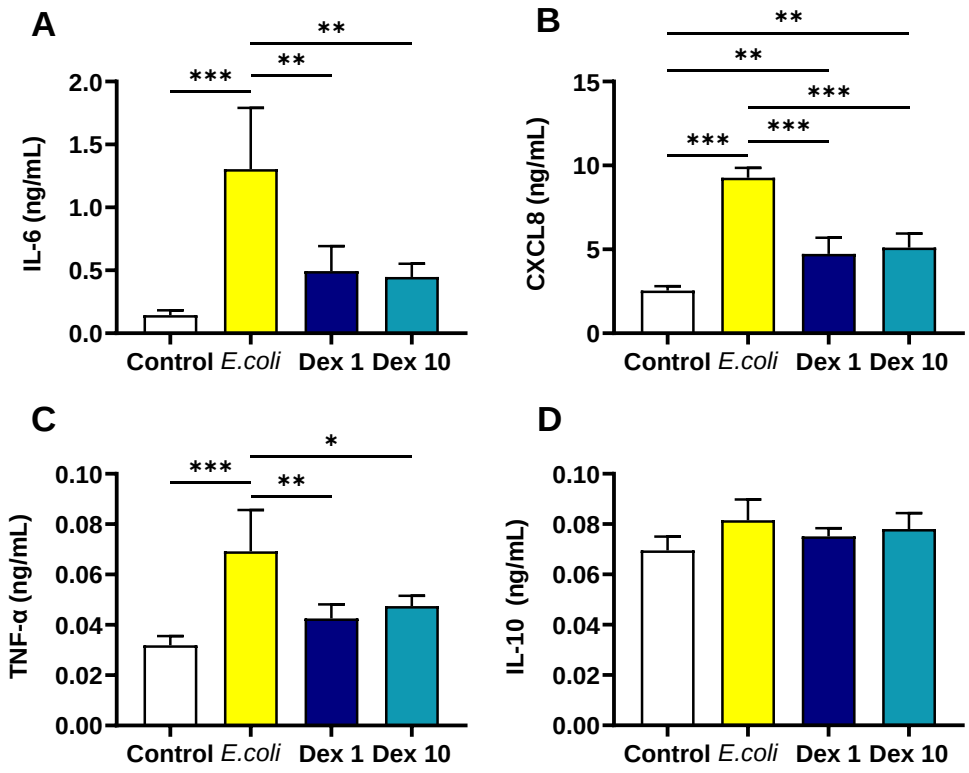


Figure 4.14. MDM function following short term inhibition of inflammation in a collagen hydrogel

MDM were cultured in a collagen gel for 24 hours, treated with dexamethasone (1 or 10 µg/mL) for 4 hours, then simulated with *E. coli* LPS (500 ng per 10^6 cells) for 24 hours with untreated and LPS only controls used. Secretion of IL-6 (**A**), CXCL8 (**B**), TNF-α (**C**) and IL-10 (**D**) was measured by ELISA. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA; *p<0.05, **p<0.01, ***p<0.005.

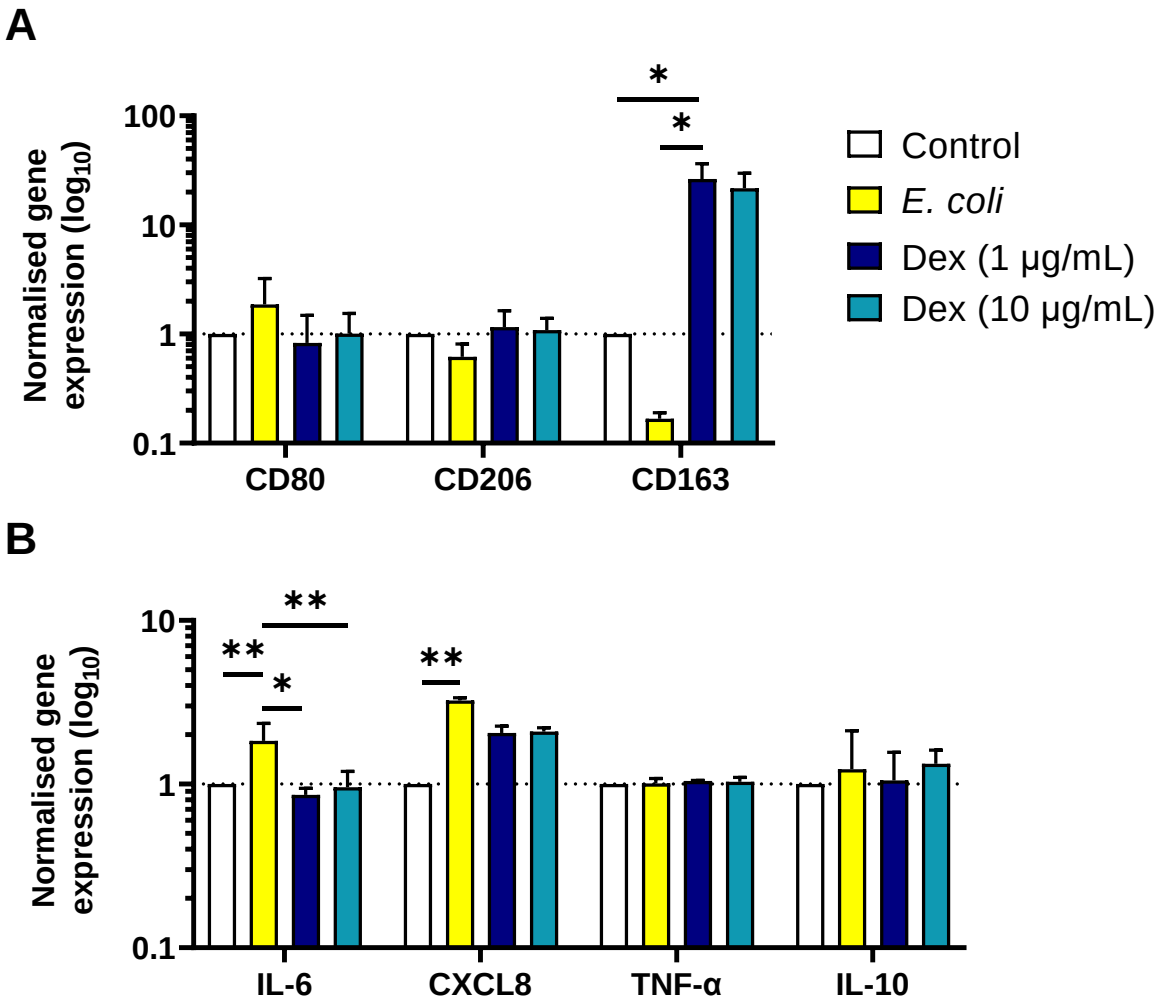


Figure 4.15. MDM gene expression following short term inhibition of inflammation in a collagen hydrogel

MDM were cultured in a collagen gel for 24 hours, treated with dexamethasone for 4 hours, then simulated with *E. coli* LPS (500 ng per 10^6 cells) for 24 hours with untreated and LPS only controls used. Gene expression for a panel of inflammatory markers (**A**) and cytokines (**B**) was analysed by qPCR, calculated relative to the reference control *β2-microglobulin* (B2M), and normalised to untreated control. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA; *p<0.05, **p<0.01.

4.3.8 MDM inflammation can be measured in different culture medium

Epithelial tissue engineered models require a complex media (termed Green's medium) with additional additives to allow the epithelium to stratify and differentiate. Prior to incorporating MDM into a tissue-engineered OME, it was important to ensure a measurable inflammatory response could be achieved when MDM are cultured in Green's medium. To examine this, MDM cultured as monolayer in IMDM for 6 days were switched to Green's medium for 24 hours before treatment with LPS ± pre-treatment with 1 µg/mL dexamethasone and cytokine responses compared to MDM cultured in IMDM medium alone.

In response to LPS treatment, IL-6 secretion (Figure 4.16A) was increased in IMDM (22-fold; p=0.0004) and Green's medium (20-fold; p=0.03) compared to control, although the response was lower in Green's medium compared to IMDM (3-fold; p=0.008). Additionally, MDM pre-treated with dexamethasone in IMDM displayed significantly reduced levels of IL-6 compared to LPS alone (p=0.007), although this was still higher than untreated control (p=0.037). In contrast, there was no significant reduction of IL-6 secretion by dexamethasone in Green's medium compared to LPS (p=0.09), but also no increase compared to untreated control (p=0.67). Following LPS stimulation, CXCL8 secretion (Figure 4.16B) was increased in IMDM (10-fold; p=0.028) and Green's medium (10-fold; p=0.047), while dexamethasone did not significantly inhibit secretion compared to control or LPS in both media (p>0.11). TNF-α secretion (Figure 4.16C) was increased in IMDM (24-fold; p=0.0002) and Green's medium (13-fold; p=0.001) following LPS treatment. Dexamethasone significantly reduced secretion in IMDM (3-fold; p=0.0029) and Green's medium (4-fold; p=0.0025). Finally, dexamethasone pre-treatment prevented a significant increase in TNF-α secretion in Green's medium (p=0.31) but not in IMDM (p=0.025). Finally, no differences were observed for either IMDM or Green's medium for IL-10 secretion (Figure 4.16D).

Taken together, these data suggest that MDM are still responsive to LPS and dexamethasone treatment when cultured in both IMDM and Green's medium and therefore should retain activity when cultured within OME.

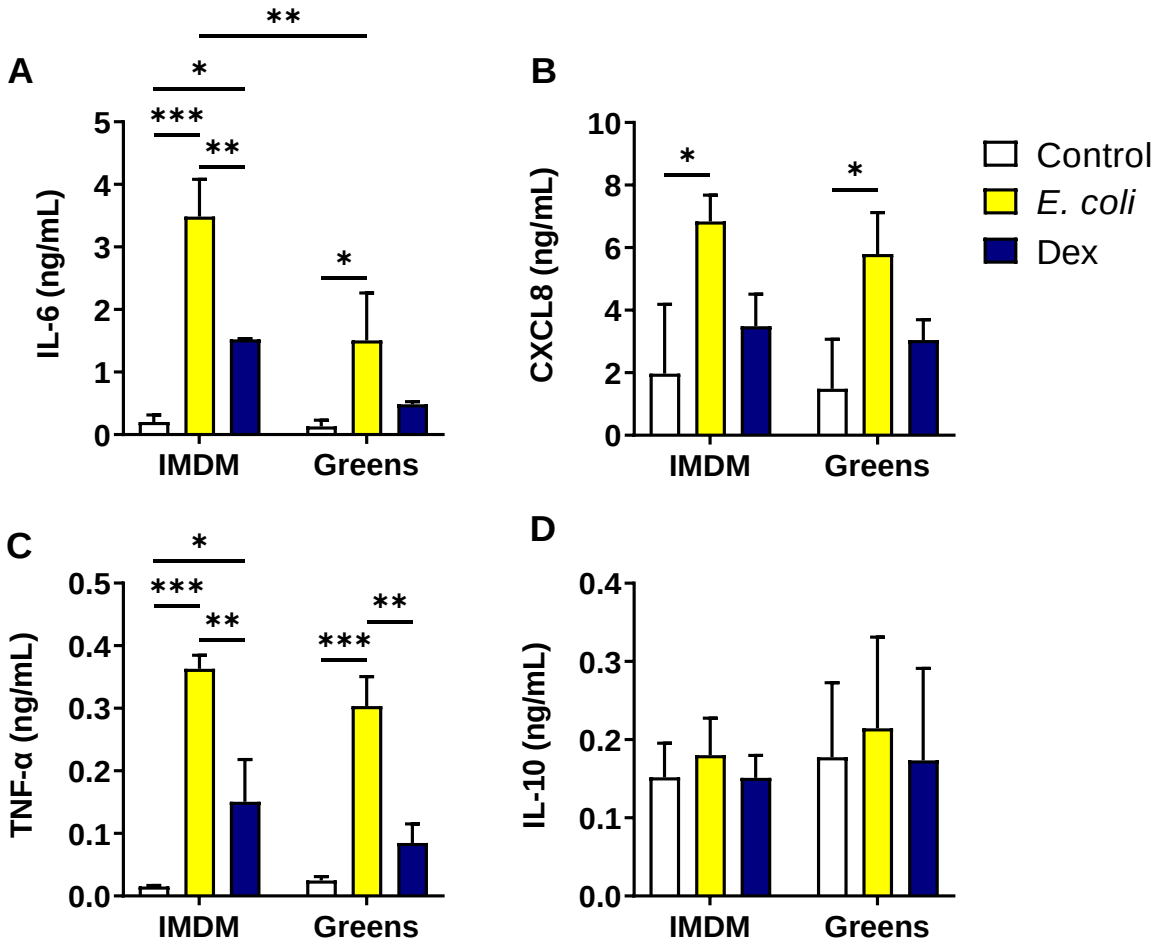


Figure 4.16. MDM activation and inhibition in IMDM and Green's medium
MDM in either IMDM or Green's medium were treated with dexamethasone for 4 hours, then simulated with *E. coli* LPS (500 ng per 10^6 cells) for 24 hours, compared to untreated and LPS only controls. Secretion of IL-6 (**A**), CXCL8 (**B**), TNF-α (**C**) and IL-10 (**D**) was measured by ELISA. Data are presented as mean ± SD of n=2 independent experiments with statistical significance determined using two-way ANOVA; *p<0.05, **p<0.01, ***p<0.005.

4.4 Discussion

The importance of macrophages in initiating an immune response in the oral mucosa as well as their significance in multiple oral diseases is well recognised (Merry et al., 2012; Aghbali et al., 2018; Alves et al., 2018). However, macrophage responses to external stimuli are often examined in isolation as monolayer cultures where cell-cell interactions and cell surface adhesion-matrix contacts are limited. *In vivo* macrophages experience life in a 3D context, therefore developing 3D tissue engineered *in vitro* models with increasing complexity is of particular importance to fully understand how cells function in native tissue. Although the use of tissue engineered OME is now relatively common in oral mucosal studies, the incorporation of additional cells into these systems, especially immune cells, is lacking. The data presented in this chapter describes measuring MDM function in response to microbial stimuli and their inhibition using anti-inflammatory agents, and the subsequent incorporation of these cells into collagen hydrogels to investigate function in a 3D environment.

4.4.1 Optimising culture conditions for MDM to produce an inflammatory response

Initial optimisation sought to examine the need for human serum in MDM culture medium. Concerns were raised that it could be a source of immune-activating endotoxins (Kirikae et al., 1997), and while suppliers ensure low endotoxin levels (typically ≤10 EU/mL) this may still activate MDM. However, it was observed that the addition of serum within the culture medium was not pro-inflammatory on its own, indeed, the presence of serum was necessary to induce an inflammatory response, in line with previous reports (Posch et al., 2013). The importance of serum is to provide a source of LPS binding protein which is essential for binding to LPS and presenting it to MDM cell surface receptors, such as CD14 (Yu et al., 1995; Thomas et al., 2002) and Toll-like receptors (Park et al., 2013). Purified LPS binding protein could be used but this approach would not account for other factors present in human serum which may aid in cell homeostasis and function; therefore, serum was used in future experiments.

Next, LPS was isolated from oral bacteria to investigate the inflammatory response induced by each species. Three species of oral bacteria linked to

periodontitis – *P. gingivalis, T. forsythia* and *A. actinomycetemcomitans* – and *E. coli,* an organism not often associated as an oral bacterium, although it has been identified in the oral cavity as a commensal organism and in specific oral diseases (Zawadzki et al., 2016, 2017). Both *P. gingivalis* and *T. forsythia* (alongside *T. denticola*) are termed the red complex, and associated with severe chronic periodontitis (Suzuki et al., 2013). The presence of *A. actinomycetemcomitans* is often found at significantly higher rates in periodontitis patients, a finding that was first described in the 1980s (Slots et al., 1980; Zambon, 1985), although a specific role in periodontitis is still subject to investigation (Fine et al., 2019). However, it is clear that the presence of both red complex bacteria and *A. actinomycetemcomitans* is associated with chronic and aggressive forms of the disease (da Silva-Boghossian et al., 2011). Thus, the ability of LPS from these bacteria to activate inflammatory responses was investigated.

Initially, the structure of LPS isolated from *T. forsythia* and *A. actinomycetemcomitans*, and commercially purified LPS from *P. gingivalis* and *E. coli* was investigated using silver-staining. LPS from *E. coli* displayed the expected well-defined laddering which decreased in density as protein size decreased (Han et al., 2014). Similarly, *P. gingivalis* LPS had a ladder appearance, with decreasing band density with both higher and lower protein sizes, in line with previous reports (Holden et al., 2014). In contrast, both *T. forsythia* and *A. actinomycetemcomitans* only displayed a single band, as per previous studies following silver staining (Darveau et al., 2004; Saito et al., 2012; Posch et al., 2013; Park et al., 2015), so it is likely that these species produce LPS that is either a homogenous size, or a molecular weight that is above or below the range of the gel used. Altogether the gel identified stark differences in LPS structure between species, which may cause altered identification and activation of local immune cells.

Next, MDM culture in monolayer were stimulated with the LPS isolated from each of these bacteria species to determine inflammatory effects. As expected, *E. coli* LPS significantly increased release of inflammatory cytokines IL-6, CXCL8 and TNF-α and the gene expression of inflammatory markers, as this is well-established (Rossol et al., 2011). In contrast, each LPS from oral bacterial was unable to induce a consistent significant inflammatory response at either gene or protein level. This is in contrast to data by Bodet *et al* who used U937 macrophage-like cells to investigate inflammation in response to 1 μg/mL *P. gingivalis* and *T. forsythia* LPS. They found that 1 μg/mL LPS from either species increased secretion of CXCL8, although no secretion of IL-

1β, IL-6 or TNF-α was detected (Bodet et al., 2006). In partial agreement, Posch *et al* used *T. forsythia* LPS to stimulate U937 cells, and found TNF-α and IL-6 secretion was increased by 10 and 100 ng/mL LPS respectively (Posch et al., 2013). The cause of the difference in results is unclear, as the same strain of *T. forsythia*, and similar LPS extractions methods were used, although Bodet *et al* used a higher concentration which suggests a dose-dependent effect. In addition, primary MDM have been shown to be more responsive to LPS stimulation than U937 cells (Sharif et al., 2007), so a greater response was expected, although greater interpatient variation is often observed with primary cells, which can reduce statistical power in smaller studies. Similarly, *A. actinomycetemcomitans* LPS (100 ng/mL) has been shown to elicit secretion of inflammatory markers in primary bone marrow-derived macrophages (Park et al., 2015) and primary human MDM (Kelk et al., 2011). Although these studies did not examine the same pro-inflammatory markers shown here, both measured an inflammatory response in primary macrophages in response to *A. actinomycetemcomitans* LPS, in contrast to the data generated here.

The observed differences between responses to LPS simulation may be a result of altered signalling pathways. For example, *E. coli* LPS is known to induce intracellular signalling cascades through interacting with TLR4 complexed with MD-2 (Park et al., 2009), an interaction which is well accepted (Park et al., 2013; Mazgaeen et al., 2020). In contrast, *P. gingivalis* LPS is often described as interacting with both TLR2 (Burns et al., 2006) and TLR4 (Jia et al., 2019), with signalling bias appearing to be dependent on factors such as LPS structure (Herath et al., 2013) and host cell expression (Kocgozlu et al., 2009). Additionally, outer membrane vesicles isolated from *P. gingivalis* and *T. forsythia*, which are enriched for virulence factors including LPS, can activate both TLR2 and TLR4, as well as other TLR subtypes (Cecil et al., 2016), while *T. forsythia* induces inflammation through TLR2-dependent signalling in mice (Myneni et al., 2011). Finally, *A. actinomycetemcomitans* upregulates TLR2 expression in THP-1 cells, inducing an increased inflammatory response (Kato et al., 2013), although many TLR subtypes have been implicated in *A. actinomycetemcomitans*-mediated inflammation in macrophages (Park et al., 2014). These differences in TLR-mediated signalling by the bacteria and associated LPS used in this study are likely the cause of the differences in response observed here, and may also be a mechanism by which commensal oral bacteria evade host immunity (Shang et al., 2019).

4.4.2 MDM inflammatory response is conserved during long term culture

To ensure that MDM could remain viable and functional for the time required to differentiate the cells and grow the 3D model, MDM were grown for 21 days and response to stimuli examined every 7 days. MDM have been shown to be viable in culture for up to 4 months (Zuckerman et al., 1979), although most studies do not require this cell type to be grown long term so do not carry out additional investigations to confirm ongoing cell viability and function.

During the study, MDM expanded in size over time as expected (Wang et al., 1985) and the morphology appeared to stabilise towards a spherical appearance, which is sometimes attributed to M2 polarisation. These same morphological changes have been observed in murine bone marrow-derived macrophages, where it was also shown that basal secretion of inflammatory factors (IL-6, CCL2 and CCL5) decreased over time, suggesting a trend away from an M1 inflammatory phenotype, although this was not accompanied by any notable changes in gene expression (Chamberlain et al., 2015). Next, assessment of MDM function shown here found that MDM secrete comparable concentrations of inflammatory cytokines IL-6, CXCL8 and TNF-α, in response to *E. coli* LPS, at each time point measured. Similarly, Jumeau *et al* examined human MDM response to LPS (species and concentration not given) at day 7, 14, and 21 and saw IL-1β and IL-6 gene expression increase at each time point, in agreement with the data presented here (Jumeau et al., 2019).

As a result of these data, it was determined that MDM retained sufficient functionality at the time points measured. Thus, these cells were carried forward for use in a 3D model system.

4.4.3 Assessing immune activating properties of collagen from different species

Collagen isolated from different species was investigated to ensure MDM were not activated by collagen interactions, and that inflammation could still be induced. Three different collagens were tested: rat tail collagen isolated in-house, bovine dermis collagen, and jellyfish collagen (both commercially available). Each collagen source is commonly used in the field of biomaterials and regenerative medicine, typically for implantation as a scaffold to aid in wound healing (Davison-Kotler et al., 2019).

Historic case studies of bovine collagen implants found that it could induce local inflammation in a small subset of patients (Cooperman et al., 1984a, 1984b), but most studies conclude little to no immune activation occurs, with some batch to batch variation (Lynn et al., 2004; Rahmanian-Schwarz et al., 2014; Davison-Kotler et al., 2019). The data presented here however, showed bovine collagen had high levels of endotoxin contamination, which likely caused the high basal inflammation seen, although there may have been other contaminants that induced immune activation. However, only a single batch of bovine collagen was used, and results from use of a different batch may vary.

While rat tail and bovine dermis collagen have been in use for decades, the use of jellyfish collagen has only recently been developed, and thus feature in relatively few comparative studies. Paradiso *et al* compared amino acid composition of rat tail, bovine dermis, and jellyfish collagen, and also compared impact of culturing ovarian cancer cells on jellyfish and rat tail collagen, finding conserved protein expression between all collagen types, and concluding suitability as a 3D scaffold (Paradiso et al., 2019). In addition, cultured iPSC-derived microglial-like cells (iMGL) have been cultured on jellyfish and rat tail collagen and compared to tissue culture plastic controls to measure induction of inflammatory response. Microglial cells are the primary innate immune cell of the central nervous system, and share some inflammatory response mechanisms with peripheral macrophages (Bachiller et al., 2018), although many of the response elements are distinct (DePaula-Silva et al., 2019) and thus these cells may not respond in the same manner as MDM. In this publication, it was shown that following LPS stimulation (100 ng/mL; species not stated), production of both IL-6 and TNF-α were significantly increased by iMGL, to a comparable level between control and both collagen types (Mearns-Spragg et al., 2020). This contrasts with the data presented here, where culture with jellyfish collagen prevented significant increase of IL-6 and TNF-α production in MDM, although the response to rat tail collagen was comparable to monolayer. It is of note that both publications which used jellyfish collagen were completed by the company producing jellyfish collagen, and thus far no independent studies have been published.

As a result of these data, rat tail collagen was used as a scaffold for MDM-containing hydrogels, as it conserved a measurable inflammatory response, and a single batch was used throughout to ensure consistency between experiments.

4.4.4 MDM inflammatory response can be reduced by NFκB pathway inhibitors

Following optimisation of MDM activation, the potential to reduce this response using NFκB pathway inhibitors was also investigated. Two compounds were tested at a range of concentrations to examine inhibition and cytotoxicity.

BAY 11-7085 is a small molecule irreversible IκBα phosphorylation inhibitor, first described in 1997 (Pierce et al., 1997) and chosen as a small molecule that would likely permeate a collagen matrix efficiently. BAY 11-7085 was only able to significantly inhibit MDM activation at high concentrations (≥50 µg/mL), where MDM cytotoxicity was also observed, leading to the conclusion that reduced cytokine levels were likely due to cell death. These data are in contrast to published studies where BAY used at 1-10 µM was shown not to be cytotoxic and was used to inhibit LPS-induced effects in Bac1 (Kahlenberg et al., 2005) and RAW 264.7 (Ryu et al., 2015; McKenna et al., 2015) murine macrophage lines and the human THP-1 cell line (Zhang et al., 2019). However, primary human-derived cells, may be more sensitive to the cytotoxic action of BAY 11-7085 which may explain the differences observed compared to other studies. Although, 1 hour incubation of 10 µM BAY 11-7085 was sufficient to reduce LPS-induced release of TNF-α in primary MDM (Landes et al., 2015), suggesting a shorter incubation time may have reduced the cytotoxicity observed.

Dexamethasone, a clinically used anti-inflammatory glucocorticoid, was also investigated for efficacy against LPS stimulated MDM as it can inhibit macrophage activation and promote a pro-wound healing/M2 MDM phenotype (Desgeorges et al., 2019), although the mechanism of action is not yet fully understood (Chuang et al., 2017). In this study dexamethasone had a low cytotoxicity profile at all concentrations tested. This is in agreement with most published studies which conclude dexamethasone has an LD50 of 35 – 222 µg/mL, or found no loss of viability at the concentrations used in their studies (Sakai et al., 1999; Nestler et al., 2002; Santos et al., 2016; Kim et al., 2017). However, two studies have concluded that dexamethasone causes loss of cell viability in MDM at 1-100 µg/mL (Bartneck et al., 2014) or BMDM at 0.1-1 µM (Haim et al., 2015). It is of note that both publications which found cytotoxicity measured cell viability by MTT assay, compared to the other studies who used trypan blue, alamarBlue, or LDH release to assess viability. The MTT cell viability assay is known to be altered by macrophage activation state, for example LPS treatment increases macrophage metabolic rate and typically yields cell viability of

over 100% (Pozzolini et al., 2003). It is therefore likely that dexamethasone, whose mode of action inhibits macrophage activation, may inhibit overall cell metabolism, which would cause a result by MTT that would imply cytotoxicity. Thus, as most publications agree with the data presented here, it is concluded that dexamethasone is not cytotoxic at the concentrations used in this study.

Dexamethasone displayed moderate inhibitory effects at the lowest concentration tested (0.01 µg/mL) in this study, with a more pronounced and consistent effect seen at higher concentrations (≥.1 µg/mL). Many other studies have employed dexamethasone as an inhibitor of macrophage activation, although concentration is either expressed in µg/mL (as used in this study) or as µM. For comparison purposes, approximately 1 µg/mL is equal to 2.5 µM. MDM treated with 10 µg/mL for 24 hours (Bartneck et al., 2014) or J774 cells at a higher concentration of 20 µg/mL for 4-24 hours (Santos et al., 2016) reduced LPS-induced increases in TNF-α and IL-6 secretion. In THP-1 cells 0.01-1 µM dexamethasone was sufficient to prevent activation of the NFκB pathway and reduce secretion of CCL2 and MMP-9 (Kim et al., 2017). Similarly, 0.1-1 µM for 24 hours was sufficient to reduce LPS-induced increase in MIP-1α in BMDM (Haim et al., 2015). Higher concentrations of 1-10 µM have been used in MDM (van der Goes et al., 2000; Jumeau et al., 2019) and primary murine macrophages (Sakai et al., 1999) where similar inhibitory effects were observed. Overall, most studies use comparable concentrations of dexamethasone to treat macrophages and macrophage cell lines, finding consistent reductions in induced inflammatory responses, in line with the data presented here.

While collagen hydrogels are not restrictive to diffusion of larger molecules ≥25 kDa (Hettiaratchi et al., 2018), to account for any potential issues with diffusion or a higher dose being required to treat cells in a 3D environment (Sun et al., 2006), both 1 and 10 µg/mL doses of dexamethasone were brought forward to stimulate MDM in a 3D hydrogel as they significantly inhibited cytokine release with minimal variation. Further analysis of gene expression changes for these doses was undertaken which revealed both could equally prevent LPS-induced changes in CD80, CD206 and IL-6, while also reducing expression of TNF-α compared to control. Similar studies have found that 1 µM dexamethasone was a sufficient concentration to significantly reduce IL-6 gene expression 4-fold compared to MDM treated with LPS (species not given) alone (Jumeau et al., 2019) and 100 nM could shift MDM towards an M2 phenotype

with decreased CD80 and increased CD206 expression compared to M1 polarised MDM (Tedesco et al., 2015).

4.4.5 Culture within a collagen hydrogel does not affect MDM activation and inhibition, but may reduce cell viability

While it was shown that rat tail collagen did not contain any factors that could activate an unwanted inflammatory response, this was further confirmed by culturing MDM embedded in a rat tail collagen hydrogel. Initial H&E staining of the hydrogels showed well dispersed MDM, and interestingly the collagen staining appeared to be more intense compared to cell-free models, suggesting MDM were depositing protein into the extracellular space. Indeed, while macrophages typically secrete matrix metalloproteinases that degrade ECM (Newby, 2008), both THP-1 cells and primary human MDM can produce type VI collagen (Schnoor et al., 2008).

LDH is typically released when a cell membrane loses structural integrity and has been shown to correlate with viability in both THP-1 cells and primary human macrophages (Kelk et al., 2011; Ayesh et al., 2014). When assessing viability by LDH, the data suggested long term culture leads to loss of cell viability, with significantly increased release after 12 days compared to 24 hours in both monolayer and hydrogel cultured cells. It is of note that the LDH method employed here is colorimetric, reading at 492 nm, while phenol red in culture media can be read at 480-580 nm, more so when the media has a more acidic pH (Amran et al., 2019). At later time points, increased cellular production of acidic metabolites may have shifted the phenol red colour which could influence readings and lead to an overestimation at later time points. Additionally, while LDH readings were taken within 2 hours of collection, LDH has a short half-life of approximately 9 hours in culture media (Riss et al., 2004). While culture media was changed 24 hours prior to analysis, this does not account for cell death which may have occurred during the long-term culture period so may not accurately measure acute cytotoxicity.

In contrast, assessing direct viability by flow cytometry suggested that viability is unchanged by culture time, but culture in 3D induces loss of viable cells. Measuring viability directly by flow cytometry required isolating single cell suspensions from collagen by collagenase treatment (2 mg/mL for 2 hours) and multiple PBS washes. It is likely that the treatment is contributing to loss of cell viability (Hefley et al., 1981)

and that wash steps remove some dead cells and debris from analysis, both of which would affect the result measured. However, taken together with the LDH data, it suggests that both culture time and environment may have an effect of cell viability, but in either case remaining viable MDM are still able to produce a measurable inflammatory response.

Next, MDM response to both *P. gingivalis* and *E. coli* LPS in a hydrogel was assessed. As with the results in monolayer, *P. gingivalis* LPS failed to produce a significant inflammatory response. It is likely that a higher concentration would be required, such as 1 µg/mL which has been used successfully in other studies to induce inflammation in human and murine macrophages (Zhou et al., 2006; Holden et al., 2014). From a biological perspective *P. gingivalis* is a commensal organism in the oral cavity and produces LPS which elicits a relatively weak immune reaction (Jain et al., 2010), especially in comparison to *E. coli* LPS, which is known to be more potent, and capable of inducing a measurable response in THP-1 cells with as low as 1 ng/mL LPS (Martin et al., 2001). As a result, it was deemed that the concentration required to induce a measurable response was unlikely to be a physiologically relevant dose, and while it has use in examining bacterial function, it is not suitable for confirming functionality of a novel model system. Therefore, *P. gingivalis* LPS was not carried forward into further studies.

In contrast to *P. gingivalis*, *E. coli* LPS could effectively induce expression of all inflammatory cytokines examined, both after 24 hours and 12 days of culture in a hydrogel, further confirming the non-immunogenic properties of the rat tail hydrogel used and suitability of these cells for long term culture. In fact, while CXCL8 was reduced at the later time point, induced IL-6 and TNF-α secretion was significantly higher after a 12-day culture period. It is of note, however, that the concentration of TNF-α measured from hydrogels was much lower than the monolayer equivalent, in agreement with previous findings which identified that a GelMA hydrogel can deplete soluble TNF-α (Donaldson et al., 2018), a process that may be occurring here.

Moreover, 3D cultured MDM displayed increased expression of the classical LPS-induced M1 macrophage phenotypic markers CD80, CXCL8, and IL-6, with simultaneously decreased expression of the M2 marker CD206, similar to 2D polarised MDM (Martinez et al., 2006; Alasoo et al., 2015), indicating that these cells can alter phenotype within a tissue-like environment.

Finally, MDM inhibition in a 3D hydrogel was also examined. Two concentrations of dexamethasone were used to ensure any issue affecting potency was discovered, as seen in other 3D culture systems (Sun et al., 2006). Both concentrations significantly dampened LPS-induced secretion of IL-6, CXCL8 and TNF-α, and only CXCL8 was increased relative to control, indicating MDM are amenable to drug treatments in a 3D environment. Additionally, the anti-inflammatory cytokine IL-10, which has been found to be upregulated in primary murine macrophages following LPS (species not given) treatment (Boonstra et al., 2006) as well as human monocytes after 24 hours treatment with *E. coli* LPS (Planès et al., 2016), was not observed, suggesting it was not a mechanism by which dexamethasone-mediated inhibition was occurring. Gene expression changes mostly mimicked the results seen in monolayer, although CD163 was significantly increased by dexamethasone treatment. CD163 encodes a monocyte lineage-specific scavenger receptor which is upregulated by IL-6, IL-10 (Tsianakas et al., 2012) and glucocorticoids such as dexamethasone (Roth et al., 1994; Högger et al., 1998; Ehrchen et al., 2019). Finally, a comparison between the two doses of dexamethasone found no differences, implying the lower concentration can maximally inhibit LPS activation, which was therefore used in future experiments.

4.4.6 Cell culture medium had minimal impact on MDM function

In this study, monocytes were differentiated into MDM in IMDM, a commonly used media for this purpose (Murdoch et al., 2007; Dekkers et al., 2019). However, this differentiation can occur in a range of media, including RPMI (Tedesco et al., 2015; Björnfot Holmström et al., 2017; Jumeau et al., 2019; Lira-Junior et al., 2020), which would imply some flexibility for culture conditions. Another notable distinction is the origin of serum used in the media. Here, human AB serum was used in IMDM (Murdoch et al., 2007), but many studies opt to use bovine fetal calf serum (Tedesco et al., 2015; Dekkers et al., 2019) which is the serum used in Green's medium.

Green's medium is complex, containing many supplements required for epithelial differentiation and stratification. Often it can include the glucocorticoid hydrocortisone, however this drug was omitted in this study due to its well-known anti-inflammatory activity (Coutinho et al., 2011; Ehrchen et al., 2019). Of the other additions in Green's medium, some can induce inflammation, such as insulin (Manowsky et al., 2016) and

triiodothyronine (T_3) (Perrotta et al., 2014; Montesinos et al., 2019), while others inhibit inflammation, such as adenine (Kohno et al., 2015; Silwal et al., 2018) and epithelial growth factor (EGF) (D'Angelo et al., 2013; Zeng et al., 2019). Transferrin is also important for binding free iron which is taken up by macrophages *in situ* (Sukhbaatar et al., 2018), more readily in M2 compared to M1 macrophages (Corna et al., 2010), but has not been shown to directly influence inflammation. It was therefore important to ensure that changing media did not significantly alter MDM function, and that culture in Green's medium could facilitate appropriate MDM response to stimuli. Here, it was shown that while IL-6 release was significantly lower in Green's media, there were no other reductions seen between the two media types. Additionally, the use of Green's medium still allowed for a significant increase in LPS-induced inflammatory cytokine release, so would be suitable to stimulate MDM-OME.

4.5 Conclusion

In summary, MDM can be activated by LPS from *E. coli*, but LPS from periodontitis-associated bacteria did not achieve the same effect. The inflammatory response is inhibited by 1 µg/mL dexamethasone, but not BAY 11-7085. Further optimisation showed MDM retain an inducible response after 21 days, the culture time required for epithelial differentiation, and could produce a measurable response in culture media used for tissue engineered models, suggesting these cells are suitable for inclusion in this model system. In addition, rat tail collagen was non-immune activating compared to other collagen types, and when embedded within a rat tail collagen hydrogel, MDM remain viable and retain an inducible and inhibitable inflammatory response. Taken together these data show that MDM are suitable for inclusion into a tissue engineered model using rat tail collagen and remain responsive to pro- and anti-inflammatory stimuli. The following chapter will build on this optimisation to incorporate MDM into multicellular tissue engineered models with oral fibroblasts and keratinocytes to produce immune competent oral mucosal equivalents.

Chapter 5 – Generating an immunoresponsive tissue engineered model of the oral mucosa containing MDM

5.1 Introduction

In the previous chapters, a role for polarised macrophages in xenobiotic metabolism was established and functionality in response to stimuli optimised. To further investigate the involvement of MDM in oral biology, response to inflammatory stimuli, and functional implications of the contribution to local xenobiotic metabolism, this chapter sought to generate a tissue engineered 3D inflammatory model of the oral buccal mucosa containing MDM which could better model this tissue.

Tissue engineered oral mucosal equivalents (OME) have been used extensively to study the oral mucosa as improved model systems compared to *in vitro* cultured oral keratinocytes grown as 2D monolayers (Moharamzadeh et al., 2012). OME can be in the form of a reconstituted human epithelium (RHE) where keratinocytes alone are cultured on a porous membrane or as full-thickness cultures that are composed of a fibroblast-populated connective tissue topped with a stratified squamous oral epithelium. Collectively, these OME have been used in numerous studies to study oral mucosal microbial infection (Yadev et al., 2011; Tabatabaei et al., 2020), wound healing (Buskermolen et al., 2016; Schmitt et al., 2019), cancer progression (Colley et al., 2011; Sawant et al., 2016) and oral mucositis (Colley et al., 2013; Walladbegi et al., 2018), as well as to examine the response of the oral mucosa to biomaterials and to monitor toxicity, drug delivery and efficacy (Thakur et al., 2007; Zanetti et al., 2016; Colley et al., 2018).

Previous studies have sought to generate increasingly complex OME to better model the oral cavity. In particular, there has been recent efforts to produce immune oral models by incorporating primary monocytes, peripheral blood mononuclear cells (PBMC), or myeloid cancer cell lines such as MonoMac 6 (MM6), U937 or THP-1 cells, and observing changes in inflammatory markers and proteases following bacterial LPS (Morin et al., 2017; Björnfot Holmström et al., 2017; Xiao et al., 2018; Lira-Junior et al., 2020), bacterial biofilms (Bao et al., 2015) or X-ray treatment (Tschachojan et al., 2014). However, no previous studies have used differentiated primary macrophages in these models, despite the importance of macrophages in oral disease (Merry et al., 2012). In addition, the buccal mucosa is an important site of drug delivery (Shojaei, 1998; Zhang et al., 2002), and data presented here previously highlighted a potential role for immune cells in local drug metabolism, but no immune-competent buccal models have been produced. Thus, the data presented here fills this knowledge gap

in tissue engineered oral models, and the models produced could have wide ranging impacts for study in many fields, including oral biology and immunology, as well as investigating the xenobiotic metabolising potential of this site of drug delivery.

Chapter aim: To produce tissue-engineered OME that contain an immune component and carry out proof-of-concept tests to prove functionality.

Objectives:

- Generate co-culture models without an epithelium to examine MDM and NOF interactions.
- Generate OMEs both with and without immune cells.
- Optimise analytical techniques to allow for analysis of immune cells within a heterogeneous population, and changes within a whole model system.
- Test immune cell functionality in a 3D culture system by treating with bacterial lipopolysaccharide (LPS) or inhibiting with NFκB pathway inhibitors and measuring by ELISA for pro-inflammatory cytokine secretion.
- Quantify expression of XME in models with and without immune cells.

5.2 Methods

- Primary cell isolation (Section 2.2.7)
- Monocyte differentiation (Section 2.2.8)
- MDM activation and inhibition (Section 2.2.9)
- Generating tissue engineered models (Section 2.2.10)
- qPCR (Section 2.3.1)
- Flow cytometry (Section 2.3.2)
- ELISA (Section 2.3.5)
- Histology (Section 2.5)

5.3 Results

5.3.1 MDM and NOF co-culture in a 3D collagen hydrogel

Prior to generating a full tissue engineered immunocompetent OME, a co-culture of normal oral fibroblasts (NOF) and MDM in a collagen hydrogel was examined. Fibroblasts are essential for producing and remodelling collagen to maintain tissue homeostasis, and are able to alter local inflammation in the oral cavity (Tzach-Nahman et al., 2017). It was therefore important to examine if NOF influence the inflammatory response produced by MDM prior to inclusion in a more complex model system.

Histology for these models is shown in Figure 5.1. H&E staining revealed a dense collagen matrix which was stained strongly by eosin, with well dispersed cells identified by haematoxylin staining throughout the examined area.

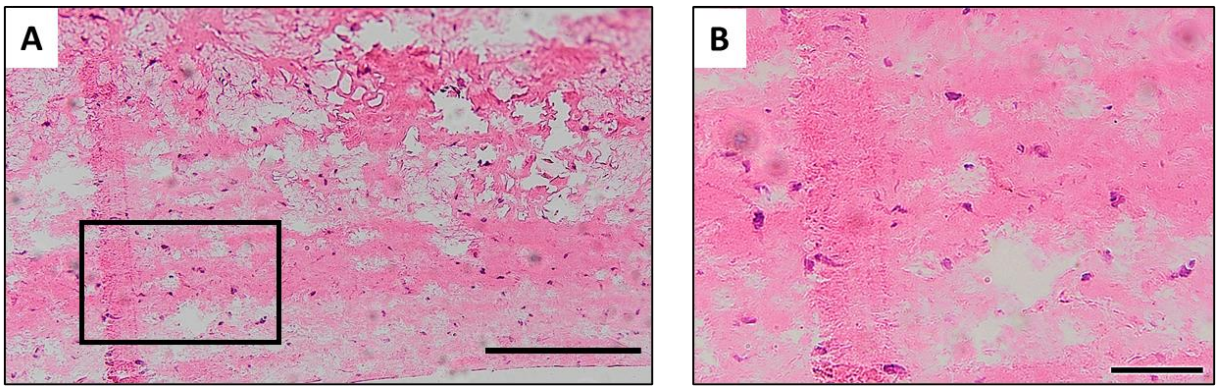


Figure 5.1. Histology of MDM-NOF co-culture
MDM and NOF were cultured for 10 days in a rat tail collagen hydrogel and analysed by histology with haematoxylin and eosin staining. Images are representative of a single technical repeat. Scale bar = 200 µm (**A**) and 20 µm (**B**).

Next, alterations in gene expression of MDM markers were examined (Figure 5.2A). CD80, a marker of MDM activation was increased by LPS treatment compared to both untreated (12-fold; p=0.019) and dexamethasone treatment (23-fold; p=0.021). CD163, a marker of M2 MDM was unchanged by LPS treatment (p=0.42), or dexamethasone pre-treatment (p=0.098), but was significantly increased by dexamethasone compared to LPS treatment alone (2-fold; p=0.019). Finally, CD206, also a marker of M2 MDM was significantly decreased by LPS compared to untreated control (5-fold; p=0.0087) as well as by dexamethasone pre-treatment (4-fold; p=0.020). Next, gene expression of inflammatory cytokines was quantified (Figure 5.2B). IL-6 was unchanged between treatments (p>0.29). In contrast CXCL8 was increased by LPS treatment compared to both untreated (13-fold; p=0.034) and dexamethasone pre-treated (24-fold; p=0.030) samples. Finally, TNF-α (p>0.36) and IL-10 (p>0.65) were unchanged between treatments.

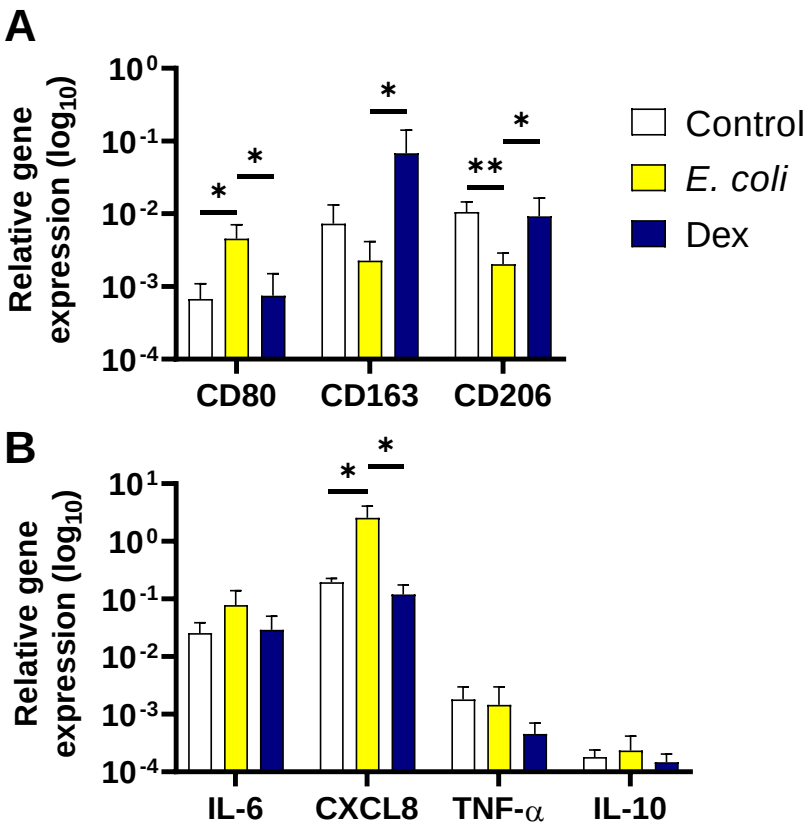


Figure 5.2. Altered gene expression in stimulated MDM-NOF 3D co-culture
MDM ($1x10^6$) and NOF ($5x10^5$) were cultured in a collagen hydrogel for 10 days, treated with dexamethasone for 4 h, then simulated with *E. coli* LPS for 24 h and compared to untreated and LPS only controls. Gene expression for a panel of inflammatory markers (**A**) and cytokines (**B**) was analysed by qPCR, calculated relative to the reference control β2-microglobulin. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA; *p<0.05, **p<0.01.

Following LPS treatment, IL-6 secretion was increased 1.4-fold (p=0.038), which was prevented by pre-treatment with 1 µg/mL dexamethasone (p=0.47 compared to control). Similarly, CXCL8 secretion was increased 2-fold by LPS (p=0.041), but not following dexamethasone pre-treatment (p=0.97 compared to control). TNF-α was increased 49-fold by LPS (p=0.021) and this response was inhibited 18-fold by dexamethasone pre-treatment (p=0.0028). Finally, IL-10 was not significantly altered by either treatment (p>0.11).

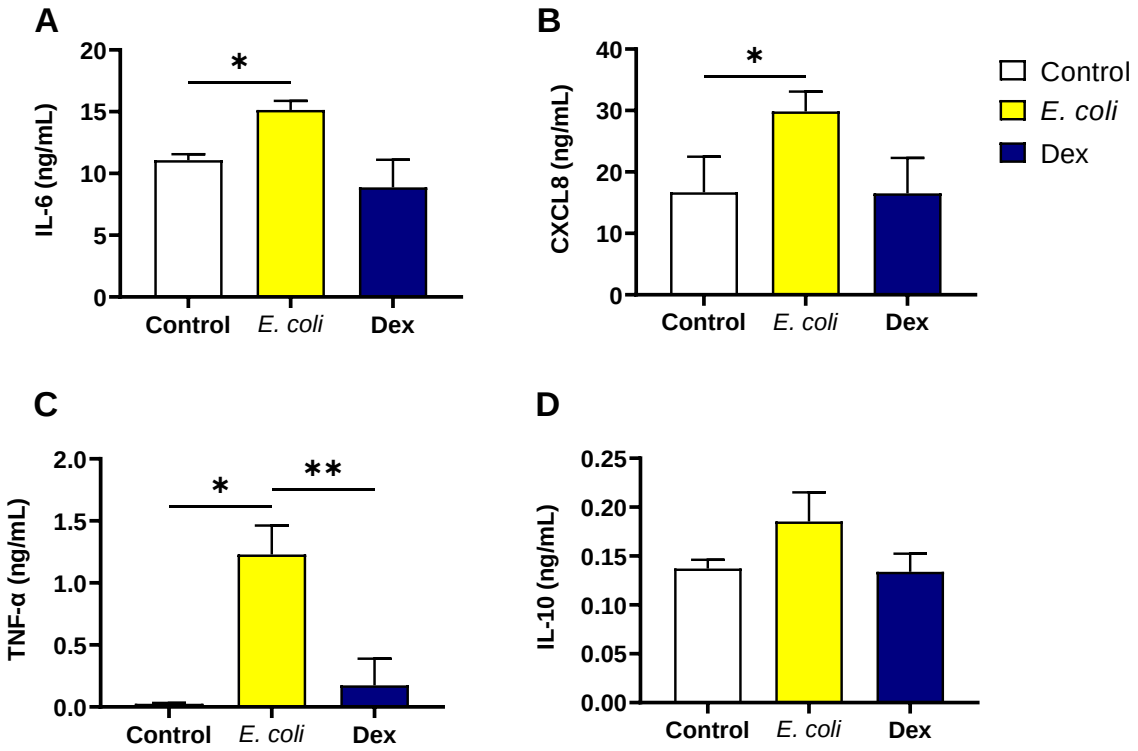


Figure 5.3. Cytokine secretion from stimulated MDM-NOF 3D co-culture
MDM ($1x10^6$) and NOF ($5x10^5$) were cultured in a collagen hydrogel for 10 days, treated with dexamethasone for 4 h, then simulated with *E. coli* LPS for 24 h and compared to untreated and LPS only controls. Secretion of IL-6 (**A**), CXCL8 (**B**), TNF-α (**C**) and IL-10 (**D**) was measured by ELISA. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA; *p<0.05, **p<0.01.

5.3.2 Incorporation of MDM into a 3D model of the oral buccal mucosa

To model an immune-mediated inflammatory response in the buccal mucosa, MDM were next incorporated into full thickness OME. During culture, the addition of MDM had no noticeable impact on gel contraction, colouration, or the rate at which media was exhausted compared to MDM-free models.

First, immuno-staining for macrophage marker CD68 was used to confirm presence of MDM within the model, revealing MDM throughout the connective tissue component of MDM-OME, but not OME (Figure 5.4). Therefore, MDM were retained within the model throughout the culture period and did not migrate out of the tissue. Furthermore, these cells were well dispersed, with no $CD68^+$ cell clusters observed.

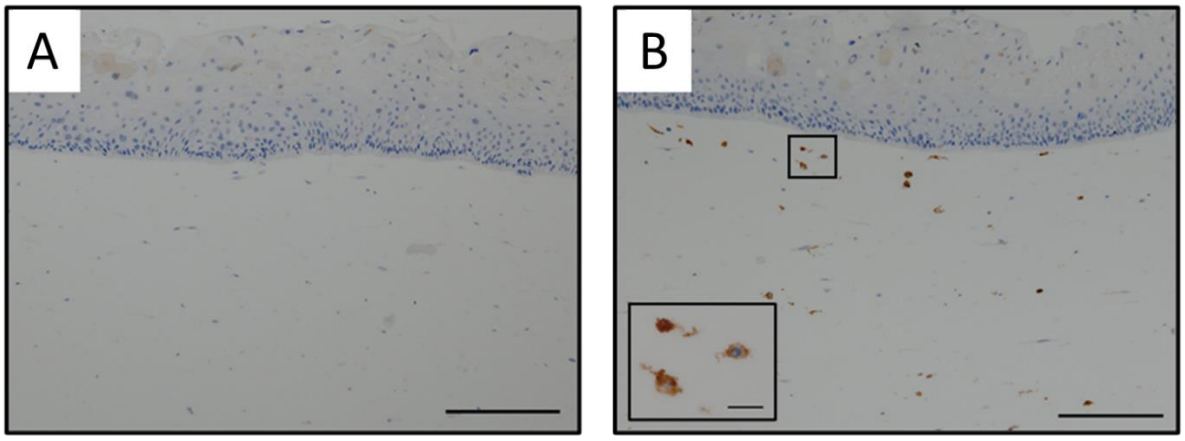


Figure 5.4. $CD68^+$ staining is observed in MDM-OME but not OME
OME (**A**) and MDM-OME (**B**) cultured for 10 days at air-to-liquid interface, were stained for macrophage marker CD68. Images are representative of a single technical repeat. Scale bar = 200 µm and 20 µm in inlay.

Furthermore, H&E stained sections were examined to compare tissue structure between native oral mucosa, OME and MDM-OME (Figure 5.5). Each example was comprised of a non-keratinised, stratified squamous epithelium consisting of oral keratinocytes that progressively differentiate toward the apical surface, along with a cell-populated connective tissue. However, while native mucosa contains the well characterised rete ridges, these are not recapitulated within this model system.

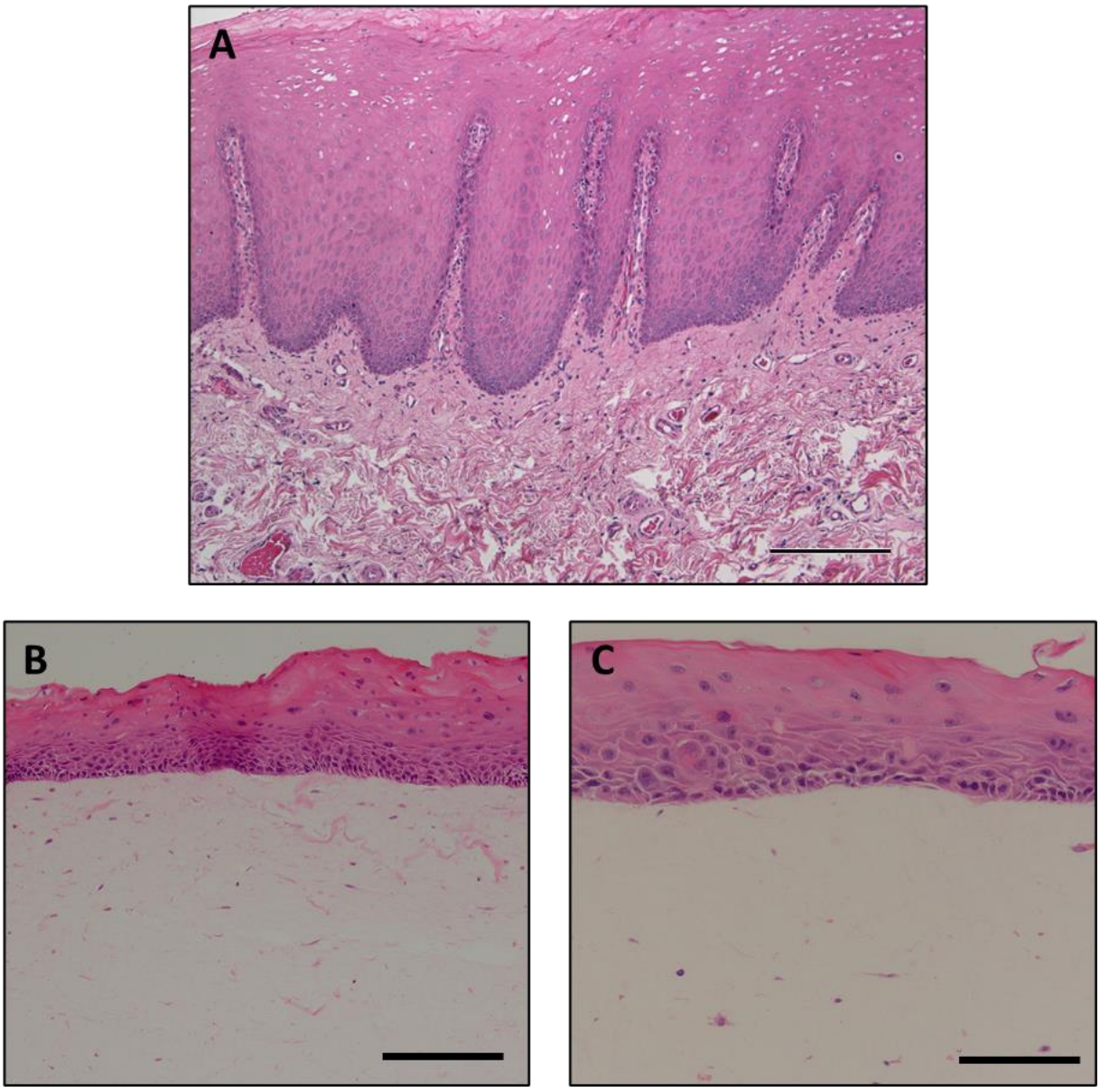


Figure 5.5. Histology of MDM-OME compared to OME and native oral mucosa
Native oral mucosa (**A**), and OME (**B**) or MDM-OME (**C**) cultured for 10 days at air-to-liquid interface, were analysed by histology with haematoxylin and eosin staining. Images are representative of a single technical repeat. Scale bar = 200 µm.

Next, immuno-staining for ki-67 was used to measure proliferating cells within the model (Figure 5.6). Ki-67 is a marker of proliferation, which is often used to determine unregulated proliferation in cancer cells, but is also expressed in the basal epithelium of normal tissue (Birajdar et al., 2014). In models, only the basal keratinocytes should be positive for ki-67 as other keratinocytes are terminally differentiated so should not express this marker, while both fibroblasts and MDM are minimally proliferative so are unlikely to express this marker. As expected, native tissue expressed ki-67 throughout the basal epithelium, and within the lamina propria. In contrast, OME and MDM-OME both expressed some ki-67 in the basal epithelium, but no expression was seen in the collagen, likely due to the lack of additional cell types found here in native tissue.

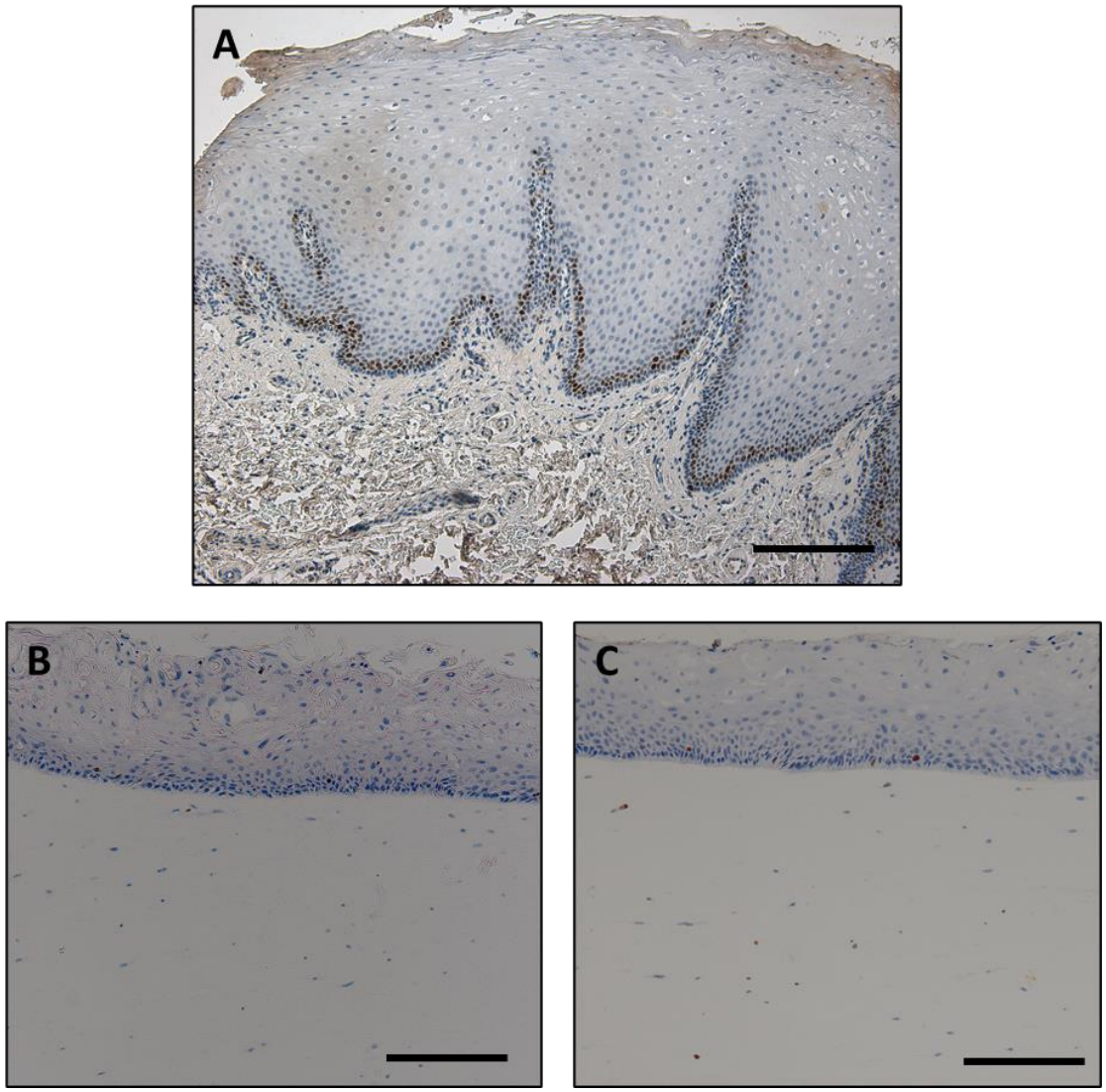


Figure 5.6. Ki-67 in MDM-OME compared to OME and native oral mucosa
Native oral mucosa (**A**), and OME (**B**) or MDM-OME (**C**) cultured for 10 days at air-to-liquid interface, stained for Ki-67. Images are representative of a single technical repeat. Scale bar = 200 µm.

Pan-cytokeratin antibody AE1/3 was also used to examine abundance of multiple cytokeratins which are typically found in surface epithelia (Figure 5.7). In all sections, positive staining was observed throughout the epithelium, and not in the lamina propria. However, in native tissue expression was more concentrated to the lower epithelium, while in OME and MDM-OME staining was seen consistently throughout the epithelium.

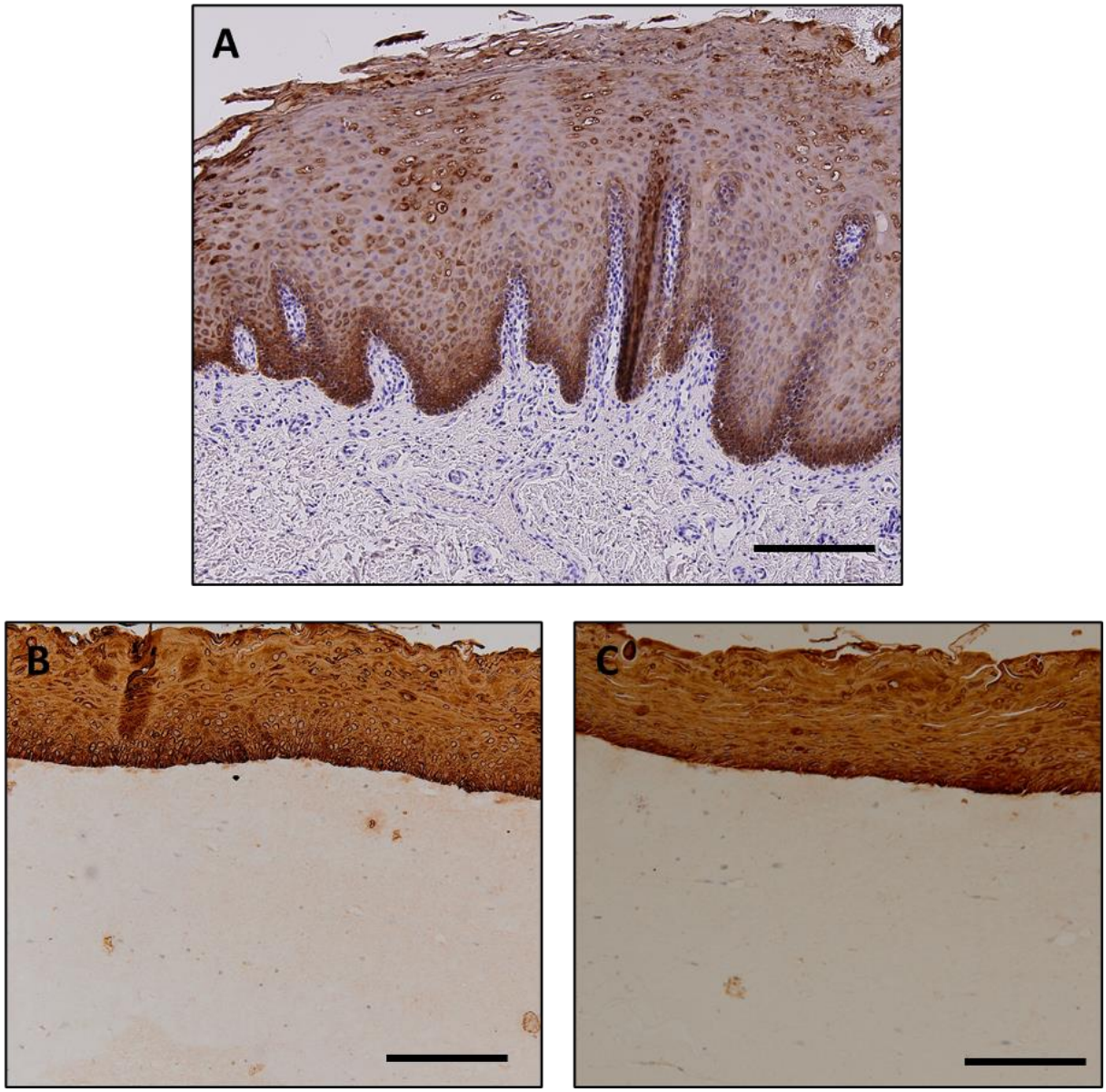


Figure 5.7. AE 1/3 in MDM-OME compared to OME and native oral mucosa
Native oral mucosa (**A**), and OME (**B**) or MDM-OME (**C**) cultured for 10 days at air-to-liquid interface, stained for AE 1/3. Images are representative of a single technical repeat. Scale bar = 200 µm.

Next, staining for e-cadherin was used to examine cell-cell junctions (Figure 5.8). In each of the sections, strong staining was observed in the epithelium, specifically in the extracellular space, indicating a well-formed epithelial network which was consistent between native tissue and both model types.

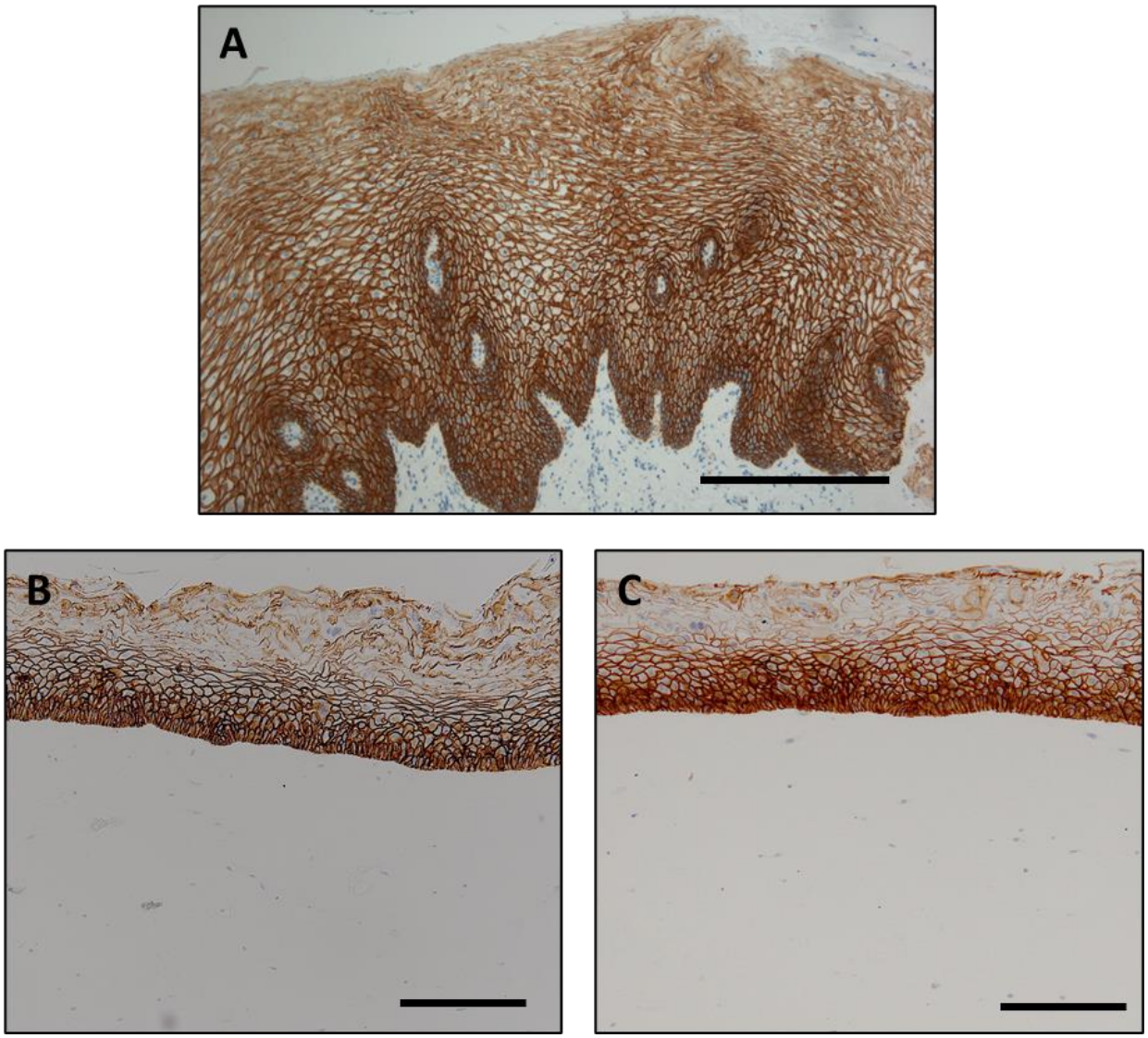


Figure 5.8. E-cadherin in MDM-OME compared to OME and native oral mucosa
Native oral mucosa (**A**), and OME (**B**) or MDM-OME (**C**) cultured for 10 days at air-to-liquid interface, stained for E-cadherin. Images are representative of a single technical repeat. Scale bar = 200 µm.

Finally, vimentin staining was used to identify fibroblasts and MDM in OME and MDM-OME (Figure 5.9). Positive staining was observed for all cells found in the connective tissue and was also seen in some basal keratinocytes.

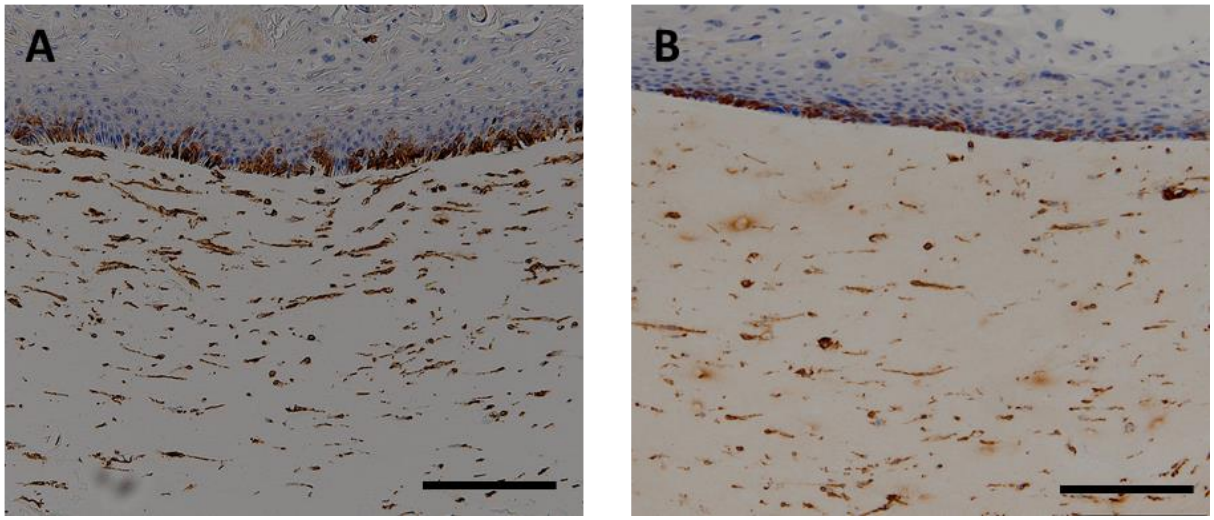


Figure 5.9. Vimentin in MDM-OME compared to OME
OME (**A**) or MDM-OME (**B**) cultured for 10 days at air-to-liquid interface, stained for vimentin. Images are representative of a single technical repeat. Scale bar = 200 µm.

5.3.3 Isolation and viability assessment of MDM from MDM-OME

To assess cell-specific viability, models were disaggregated by collagenase, passed through a cell strainer to obtain a single cell suspension for analysis by flow cytometry to quantify cell viability as well as cell surface marker expression (Figure 5.10). Cells were dual stained with a fluorescent viability dye and an APC-conjugated monoclonal antibody for the pan-monocyte-derived cell marker, CD11c, to specifically identify MDM within the heterogeneous cell population. Here, no $CD11c^+$ MDM were observed in OME (Figure 5.10A), but MDM within MDM-OME could be completely separated from the $CD11c^-$ cell population by fluorescence (Figure 5.10B). When segregated into $CD11c^-$ and $CD11c^+$ cells, MDM-OME contained 32 ± 10% $CD11c^+$ MDM, whereas OME contained <1% $CD11c^+$ due to non-specific antibody staining (Figure 5.10A+B). The viability of the $CD11c^-$ keratinocyte and fibroblast population was comparable between MDM-OME (68 ± 4%; Figure 5.10C), and OME (73 ± 4%), whilst the MDM $CD11c^+$ population viability in MDM-OME was 67 ± 8% (Figure 5.10D).

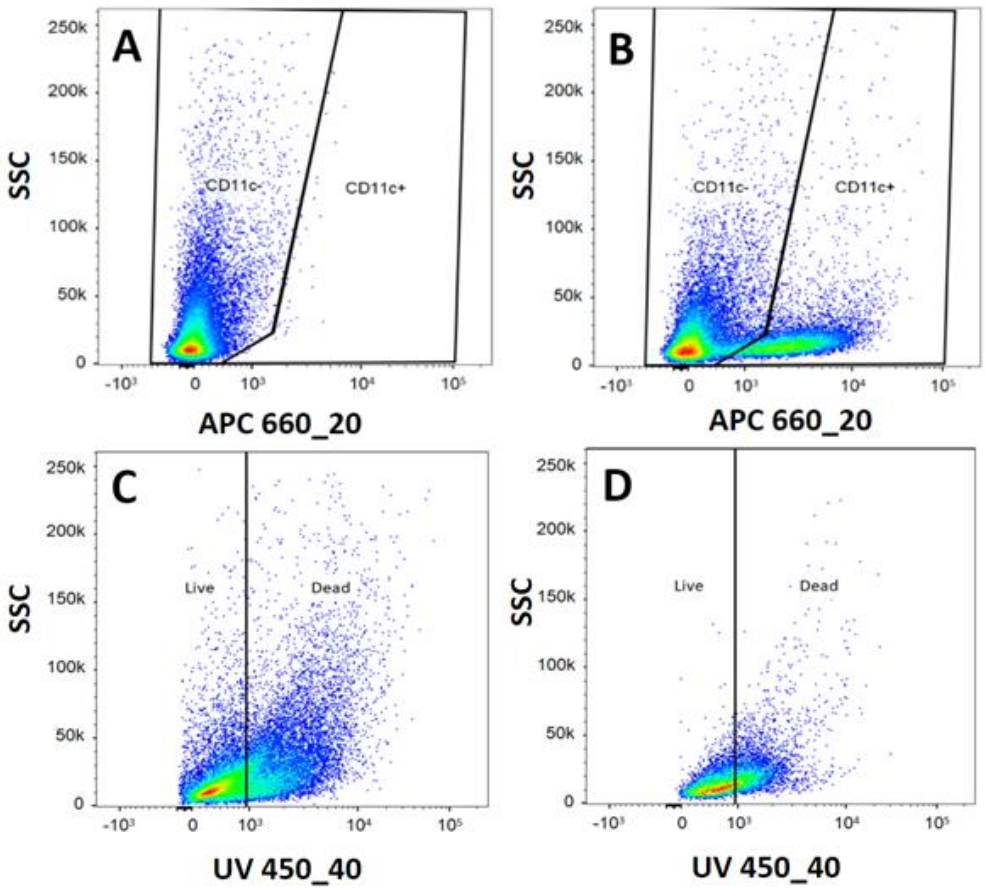


Figure 5.10. Viability of MDM within MDM-OME
Collagenase treated OME (**A**) and MDM-OME (**B-D**) stained with anti-CD11c-APC to identify MDM. Cells identified as CD11c- (**C**) or CD11c+ (**D**) were gated and assessed for viability. Graphs are representative; n=3.

5.3.4 Assessment of inflammatory response in MDM-OME

To confirm functionality of MDM within the OME, both total RNA in the models and cytokine secretion into conditioned media were measured.

5.3.4.1 Altered gene expression in MDM-OME

First, changes in expression of MDM polarisation markers were quantified in MDM-OME and compared to OME (Figure 5.11). Expression of CD80 and CD206 were not detected in OME, although expression of CD163 was increased 16-fold by dexamethasone compared to untreated OME (p=0.042; Figure 5.11A). In MDM-OME (Figure 5.11B), mRNA of the macrophage markers CD80 and CD206 were detected but there were no differences between control and treated samples. As with OME, CD163 was increased 6-fold by dexamethasone compared to control (p=0.025) and 13-fold compared to LPS treatment alone (p=0.018).

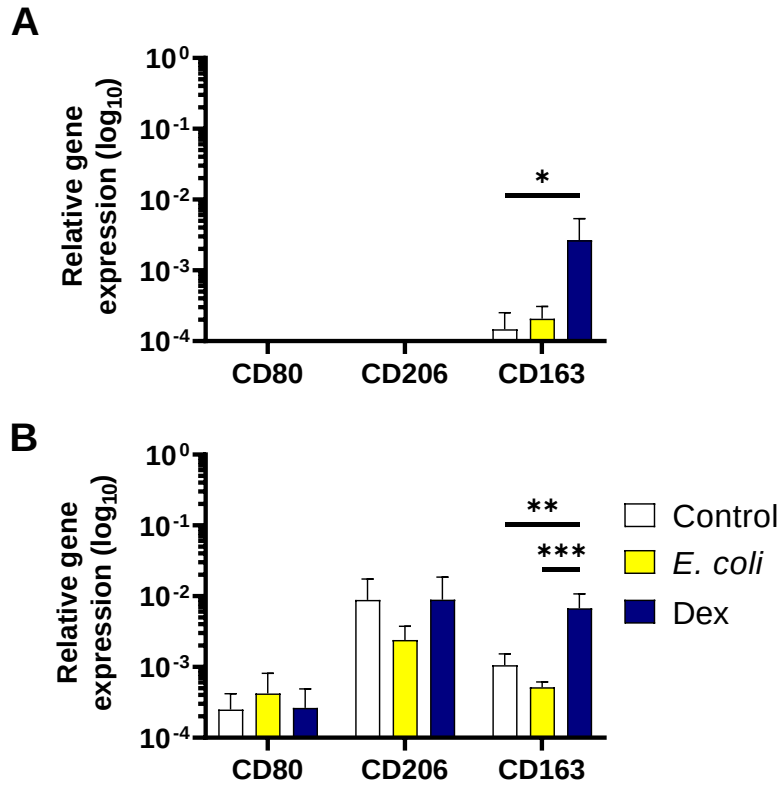


Figure 5.11. MDM-OME and OME expression of inflammatory markers
OME (**A**) and MDM-OME (**B**) cultured at ALI for 10 days were treated with dexamethasone for 4 h, then simulated with *E. coli* LPS for 24 h and compared to untreated and LPS only controls. Gene expression for inflammatory markers *CD80*, *CD206*, and *CD163* was analysed by qPCR, calculated relative to the reference control *β2-microglobulin* (B2M). Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA; *p<0.05, **p<0.01, ***p<0.005.

Next, expression of inflammatory cytokines IL-6, CXCL8 and TNF-α, as well as anti-inflammatory cytokine IL-10 was quantified. No changes in gene expression were observed in OME (p>0.07; Figure 5.12A). In contrast, MDM-OME (Figure 5.12B) expression of IL-6 was significantly inhibited by dexamethasone compared to LPS (8-fold; p=0.045) and control (15-fold; p=0.0064). A similar trend was observed for CXCL8, where expression was significantly inhibited by dexamethasone compared to LPS (14-fold; p=0.0005) but also increased in LPS compared to control (3-fold; p=0.035). Gene expression of both TNF-α and IL-10 were unchanged between all conditions (p>0.22).

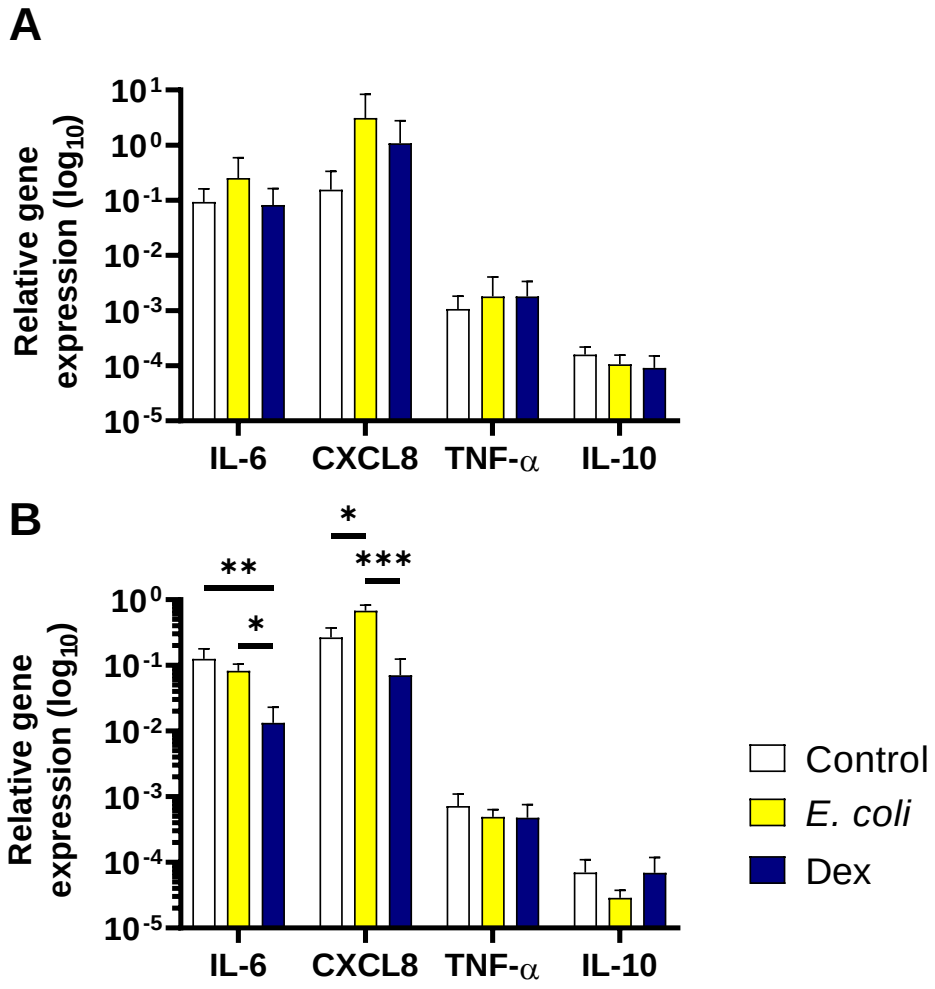


Figure 5.12. MDM-OME and OME expression of inflammatory cytokine genes
OME (**A**) and MDM-OME (**B**) cultured at ALI for 10 days were treated with dexamethasone for 4 h, then simulated with *E. coli* LPS for 24 h and compared to untreated and LPS only controls. Gene expression for inflammatory cytokines *IL*-6, *CXCL8, TNF-α,* and *IL-10* was analysed by qPCR, calculated relative to the reference control *β2-microglobulin* (B2M). Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA; *p<0.05, **p<0.01, ***p<0.005.

To further understand the effect of macrophage activation, expression of Toll-like receptors (TLR) 2 and 4 was also measured (Figure 5.13). In OME (Figure 5.13A), TLR2 expression was increased 7-fold by dexamethasone treatment (p=0.025). Similarly, MDM-OME (Figure 5.13B), TLR2 expression was increased 3-fold by dexamethasone treatment compared to control (p=0.012), and 3-fold compared to LPS treatment alone (p=0.030). In both models, TLR4 expression was unchanged across all treatments.

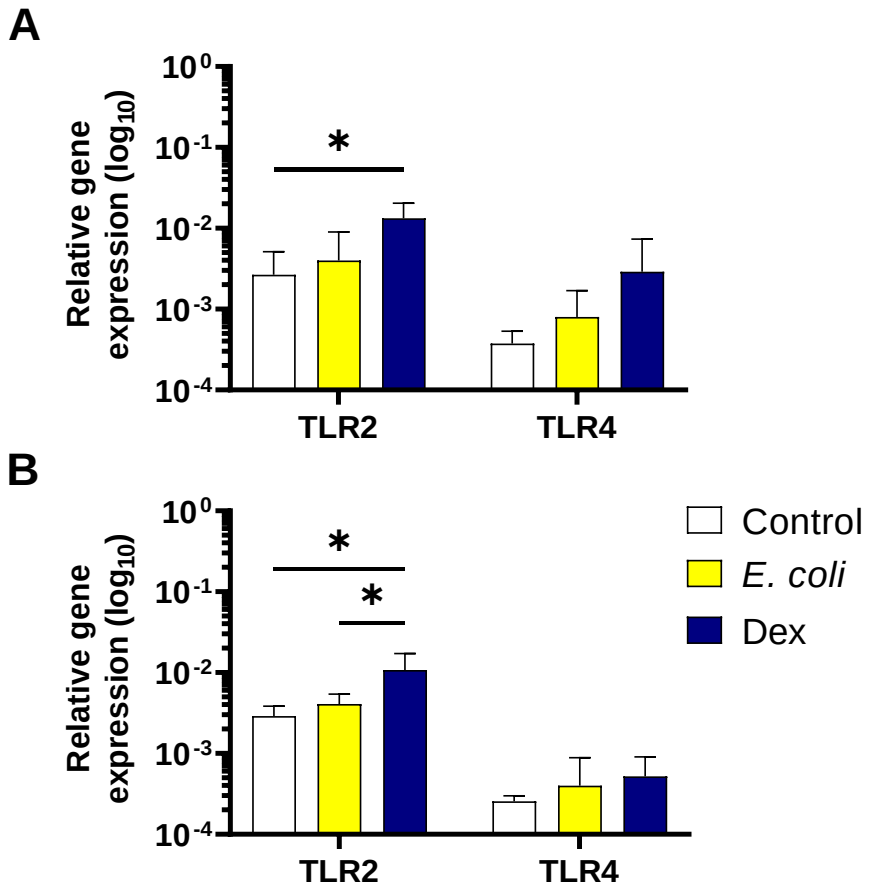


Figure 5.13. MDM-OME and OME expression of Toll-like receptors
OME (**A**) and MDM-OME (**B**) cultured at ALI for 10 days were treated with dexamethasone for 4h, then simulated with *E. coli* LPS for 24h and compared to untreated and LPS only controls. Gene expression for *TLR2* and *TLR4* was analysed by qPCR, calculated relative to the reference control *β2-microglobulin*. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using two-way ANOVA; *p<0.05.

5.3.4.2 Secretion of inflammatory cytokines

Next, the secretion of inflammatory cytokines in response to LPS stimuli was measured in MDM-OME conditioned media compared to OME (Figure 5.14). In contrast to MDM-NOF co-culture (Figure 5.3A), IL-6 secretion (Figure 5.14A) was unchanged by stimuli ($p>0.51$) in both OME and MDM-OME. CXCL8 secretion (Figure 5.14B) increased between 3- and 4-fold in LPS treated MDM-OME compared to immune-free controls ($p<0.05$). Dexamethasone treatment decreased LPS-induced secretion of both IL-6 and CXCL8 in MDM-OME, but not significantly. Basal secretion of TNF-α (Figure 5.14C) was consistently low in OME and both untreated and dexamethasone-treated MDM-OME which increased 10-fold in MDM-OME following LPS treatment ($p<0.005$). Dexamethasone significantly decreased the levels of TNF-α, inhibiting the actions of LPS. Finally, as with previous experiments, no change in IL-10 secretion was observed between models and treatments ($p>0.79$) (Figure 5.14D).

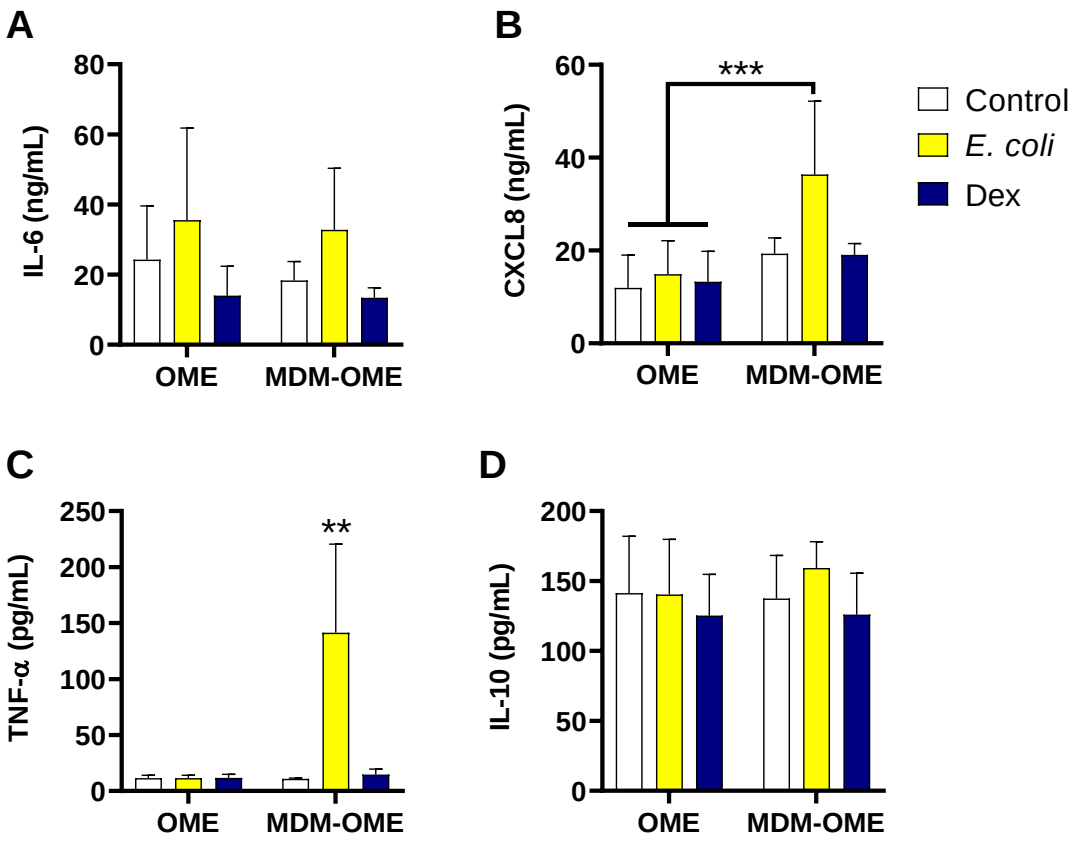


Figure 5.14. MDM enhance inflammatory cytokine secretion in OME
OME and MDM-OME cultured at ALI for 10 days were treated with dexamethasone for 4 h, then simulated with *E. coli* LPS for 24 h and compared to untreated and LPS only controls. Secretion of IL-6 (**A**), CXCL8 (**B**), TNF-α (**C**) and IL-10 (**D**) was measured by ELISA. Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using two-way ANOVA; **p<0.01, ***p<0.005.

To ensure that the secretion of TNF-α was MDM specific, wax tissue sections were dual stained with CD68 (MDM) and TNF-α. As shown in Figure 5.15A, TNF-α positive staining was increased in LPS-treated MDM-OME and decreased by dexamethasone. Moreover, staining of TNF-α co-localised with that of $CD68^+$ positive MDM and not $CD68^-$ NOF in LPS-treated MDM-OME (Figure 5.15B), indicating that LPS-mediated TNF-α release within the MDM-OME 3D models is MDM-specific.

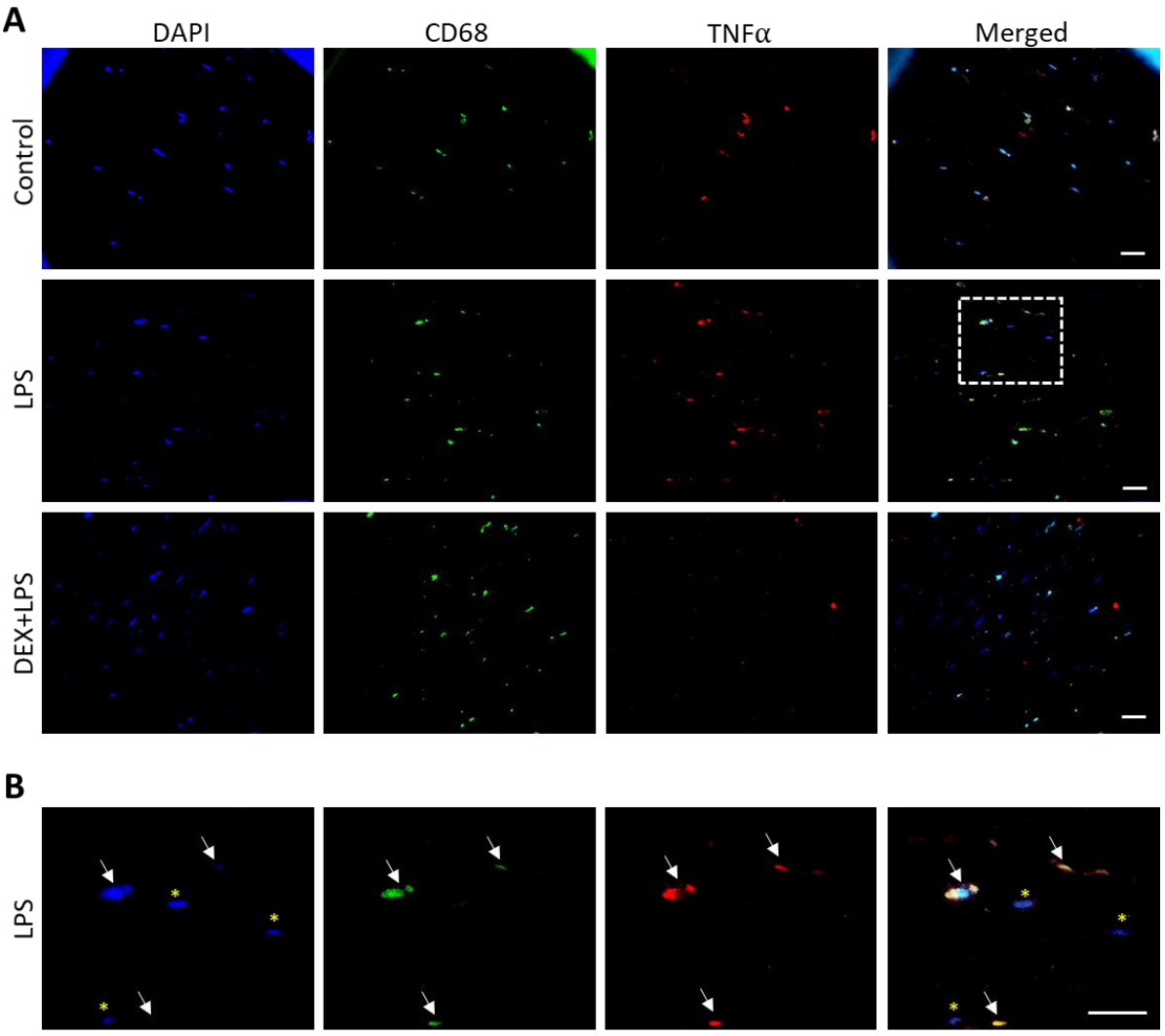
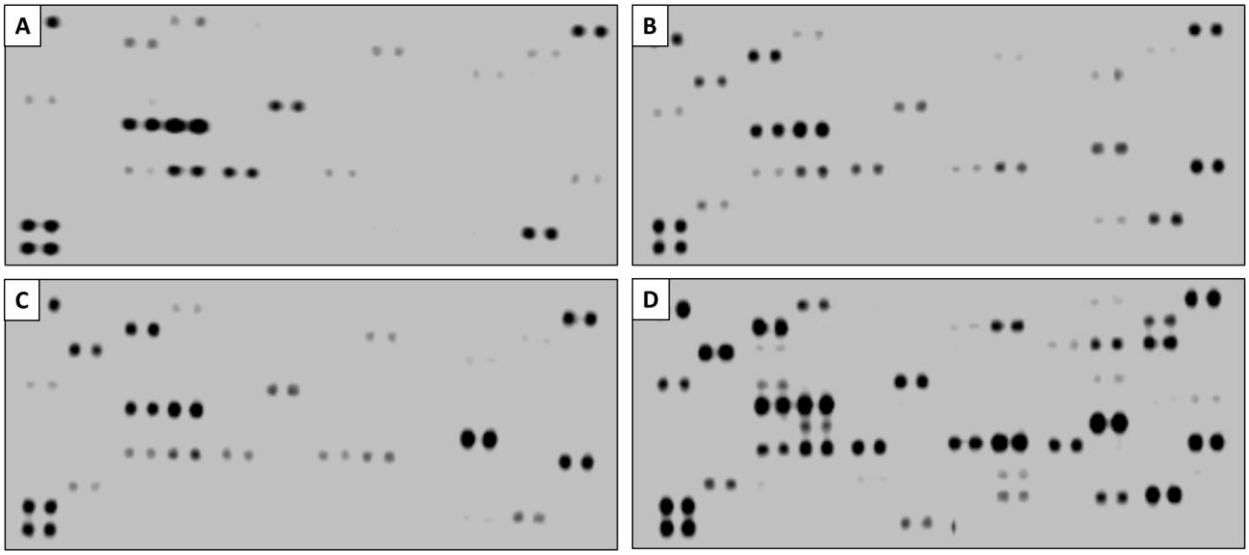


Figure 5.15. Only $CD68^+$ MDM produce TNF-α in MDM-OME
MDM-OME were stimulated with dexamethasone for 4 h, then *E. coli* LPS for 24 h and compared to untreated and LPS only controls. Immunofluorescence staining of tissue sections was performed for CD68 (green), TNF-α (red), counterstained for nuclei with DAPI (blue), and images merged (**A**). A magnified image is shown of the white square displayed in LPS-treated MDM-OME (**B**). Images presented are representative of a single technical repeat. Scale bar = 20 µm.

5.3.4.3 Analysis of conditioned media by cytokine array

To further assess the ability of MDM-OME to secrete inflammatory factors in response to stimuli, a broad cytokine array of human inflammatory cytokines and chemokines was undertaken (Figure 5.16). Due to the large number of analytes assessed by the array, only detected analytes will be reported here. Full results and list of analytes measured can be found in the appendix (Table 8.1).



	1+2	3+4	5+6	7+8	9+10	11+12	13+14	15+16	17+18	19+20	21+22	23+24
A	POS	Adiponectin	Apo A-1	Angiogenin	ANG1	ANG2	BAFF	BDNF	C5/C5a	CD14	CD30	POS
B		CD40L	CHI3L1	CFD	CRP	Cripto-1	CST3	Dkk-1	CD26	EGF	CD147	
C		CXCL5	CD105	FasL	FGF2	KGF	FGF19	FLT3LG	GCSF	GDF15	GMCSF	
D	CXCL1	HGH	HGF	CD54	IFN-γ	IGFBP2	IGFBP3	IL-1α	IL-1β	IL1ra	IL-2	IL-3
E	IL-4	IL-5	IL-6	IL-8	IL-10	IL-11	IL-12 p70	IL-13	IL-15	IL-16	IL-17A	IL-18 BP
F	IL-19	IL-22	IL-23	IL-24	IL-27	IL-31	IL-32	IL-33	IL-34	CXCL10	CXCL11	PSA
G	Leptin	LIF	LCN2	CCL2	CCL7	MSCF	MIF	CXCL9	CCL3/4	CCL20	CCL19	MMP-9
H	MPO	OPN	PDGF-AA	PDGF- AB/BB	PTX3	CXCL4	RAGE	CCL5	RBP4	RLN2	Resistin	CXCL12
I	PAI1	SHBG	IL1RL1	CCL17	TFF3	TfR	TGFα	THBS1	TNFα	CD87	VEGF	
J	POS		DBP	CD31	CD366	CD106						NEG

Figure 5.16. Cytokine array of representative OME and stimulated MDM-OME
OME (**A**), untreated MDM-OME (**B**), MDM-OME treated with dexamethasone for 4 h then simulated with *E. coli* LPS for 24 h (**C**), and MDM-OME treated with *E. coli* LPS for 24 h only (**D**) were assessed by cytokine array; n=1. Table describes the analyte in each position for reference, POS: positive control, NEG: negative control.

A number of inflammatory factors were exclusively secreted from LPS-treated MDM-OME (Figure 5.17A). Of note, GM-CSF, IL-24, and CCL3/4 were strongly detected by the array, with many of the other analytes weakly detected, suggesting low abundance in conditioned media. Furthermore, secretion of all analytes was comparable or increased in LPS treated MDM-OME compared to all other samples (Figure 5.17B). In addition, only CXCL10 was increased in dexamethasone treated MDM-OME compared to untreated, while these two conditions were indistinguishable for all other analytes, indicating that dexamethasone was able to inhibit secretion of a plethora of proinflammatory mediators in response to LPS.

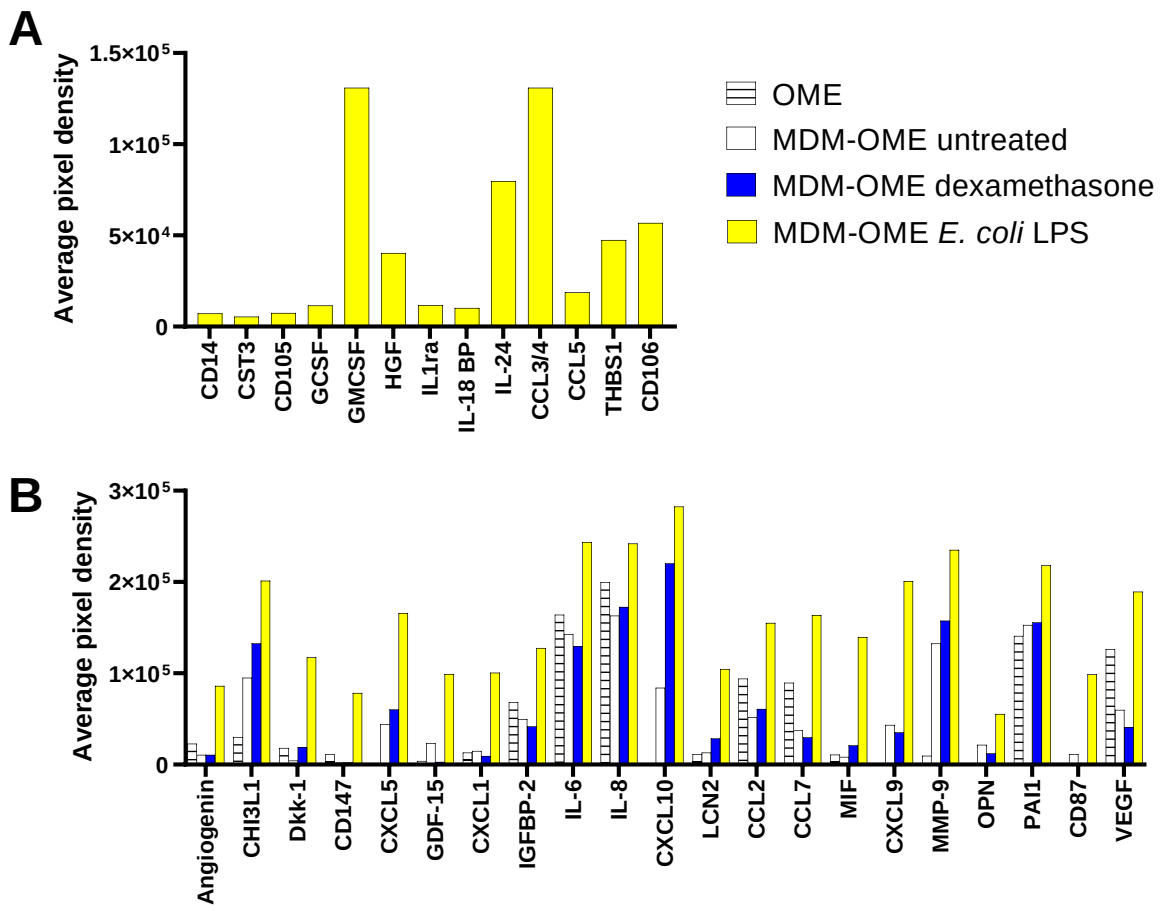


Figure 5.17. Quantification of inflammatory cytokine array
Cytokine array images were semi-quantified for pixel density. Analytes only found in *E. coli* LPS treated MDM-OME (**A**) and found in multiple samples (**B**); n=1.

5.3.5 Expression of xenobiotic metabolising enzymes in MDM-OME

Finally, preliminary work was undertaken to quantify any changes in gene expression of XME identified in chapter 3 to be altered in inflammatory (M1) MDM, and those which are especially relevant to the oral mucosa.

First, CYP1A1 and 1B1 are very important in the oral cavity, as they are the primary metabolisers of environmental procarcinogens, and those found in tobacco. Expression of both enzymes was identified in OME and MDM-OME, with no changes between treatment conditions (Figure 5.18). While direct comparisons could not be made between the two model types, expression of these enzymes appeared to be reduced in MDM-containing models, likely due to either lower relative expression in MDM compared to other cell types, or a negative regulatory effect of MDM which reduced expression.

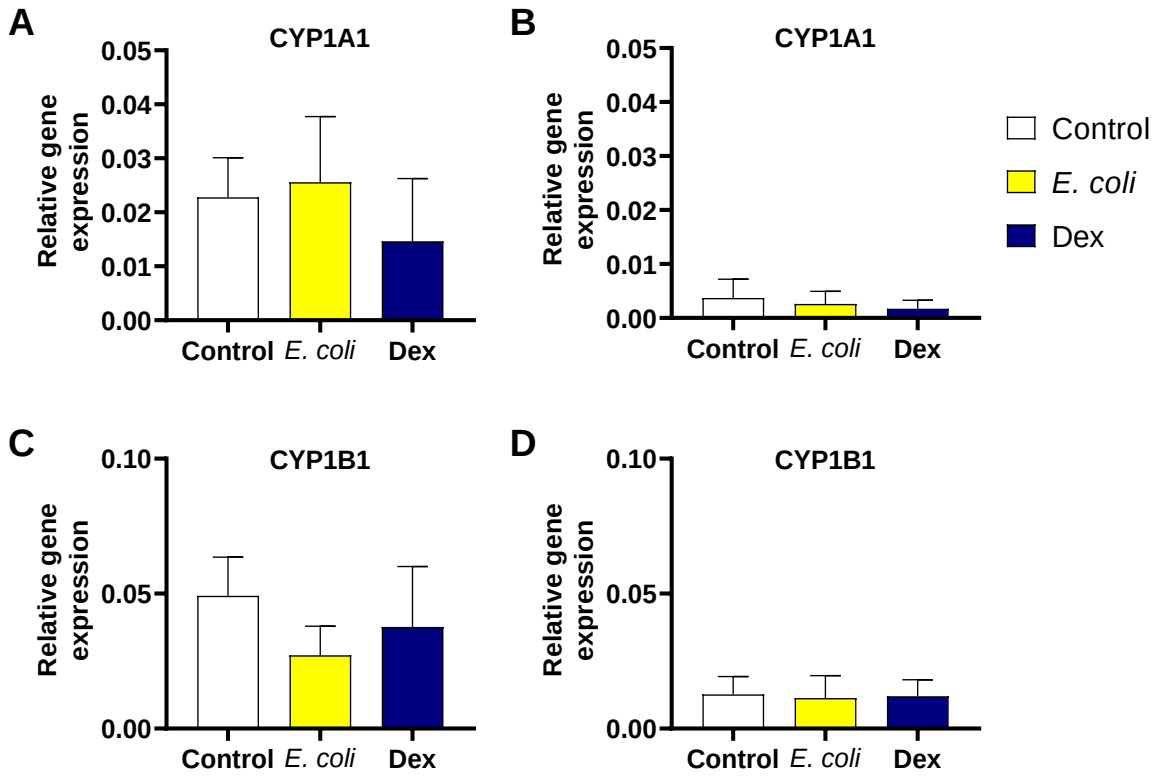


Figure 5.18. MDM-OME expression of CYP1A1 and CYP1B1
OME (**A**, **C**) and MDM-OME (**B**, **D**) cultured at ALI for 10 days were treated with dexamethasone for 4 h, then simulated with *E. coli* LPS for 24 h and compared to untreated and LPS only controls. Gene expression for *CYP1A1* (**A**, **B**), and *CYP1B1* (**C**, **D**) were calculated relative to the reference control *β2-microglobulin* (B2M). Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA.

Next, CYP2A6, CYP2D6 and FMO5 were examined (Figure 5.19), as these were all significantly increased in inflammatory MDM. CYP2A6 and 2D6 had comparable levels of expression in OME and MDM-OME which were not significantly altered between treatments ($p>0.17$). Expression of FMO5 was higher, although again was comparable between model types and treatment conditions ($p>0.28$).

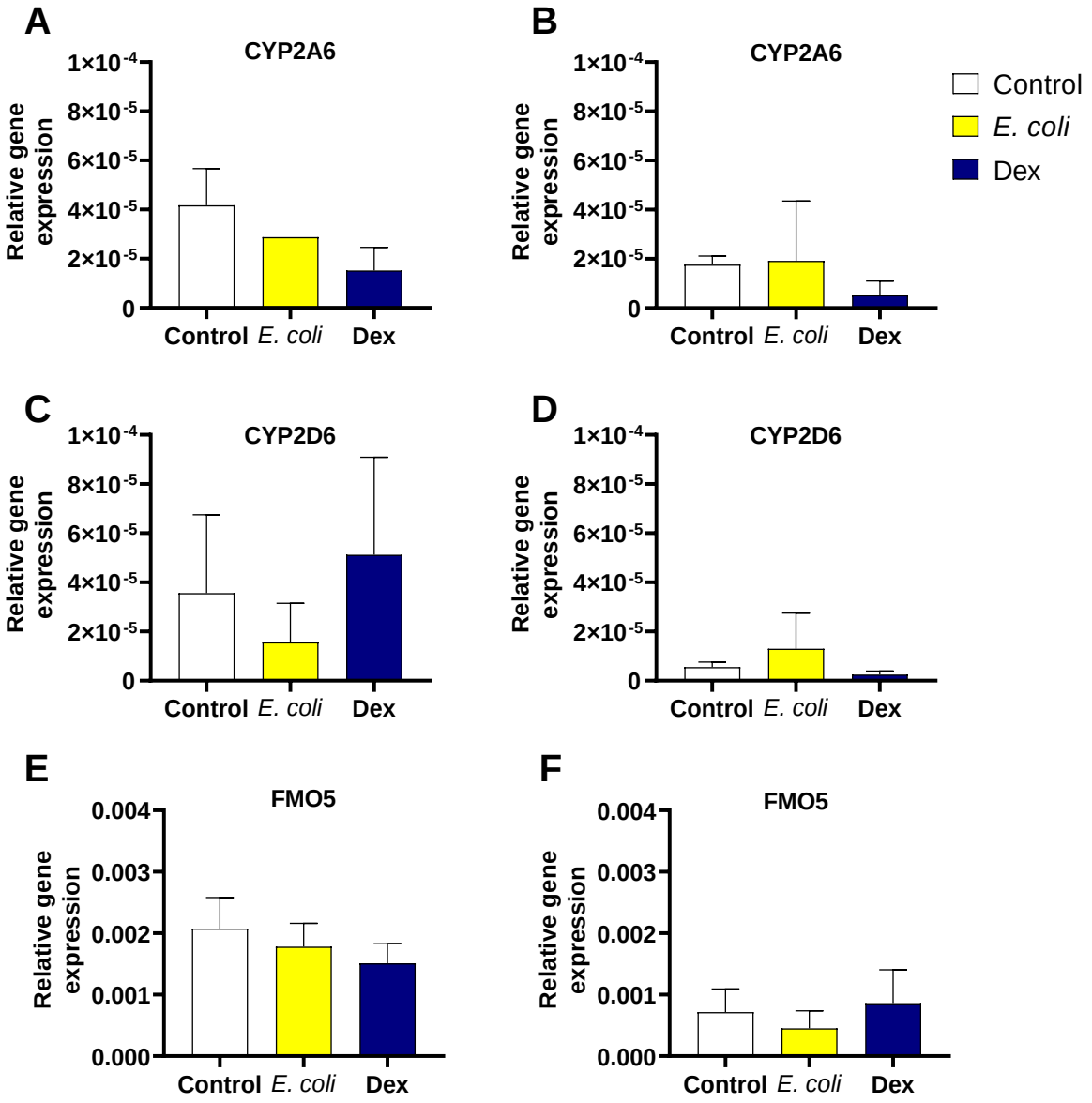


Figure 5.19. MDM-OME expression of CYP2A6, 2D6, and FMO5
OME (**A**, **C**, **E**) and MDM-OME (**B**, **D**, **F**) cultured at ALI for 10 days were treated with dexamethasone for 4 h, then simulated with *E. coli* LPS for 24 h and compared to untreated and LPS only controls. Gene expression for *CYP2A6* (**A**, **B**), *CYP2D6* (**C**, **D**), and *FMO5* (**E**, **F**) were calculated relative to the reference control *β2-microglobulin* (B2M). Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA.

Furthermore, CYP3A4 is a key metabolic enzyme, and can be induced by dexamethasone (Pascussi et al., 2001), so was examined to establish if the enzyme is present and inducible in these models (Figure 5.20). CYP3A4 was detected in both model types, with non-significant increases in dexamethasone treated samples in OME (p>0.19), and MDM-OME (p>0.06) compared to other treatments.

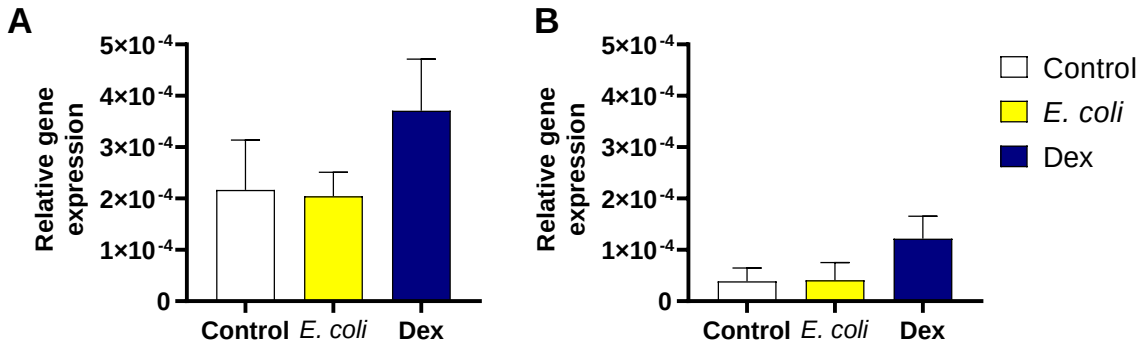


Figure 5.20. MDM-OME expression of CYP3A4
OME (**A**) and MDM-OME (**B**) cultured at ALI for 10 days were treated with dexamethasone for 4 h, then simulated with *E. coli* LPS for 24 h and compared to untreated and LPS only controls. Gene expression for *CYP3A4* was calculated relative to the reference control *β2-microglobulin* (B2M). Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA.

Finally, PTGS2 (COX-2) was detected in OME (Figure 5.21A), and unchanged between treatments (p>0.62). In contrast, expression in MDM-OME (Figure 5.21B), was significantly increased by LPS treatment compared to dexamethasone pre-treatment (12-fold; p=0.018), while neither were significantly altered compared to control (p>0.19).

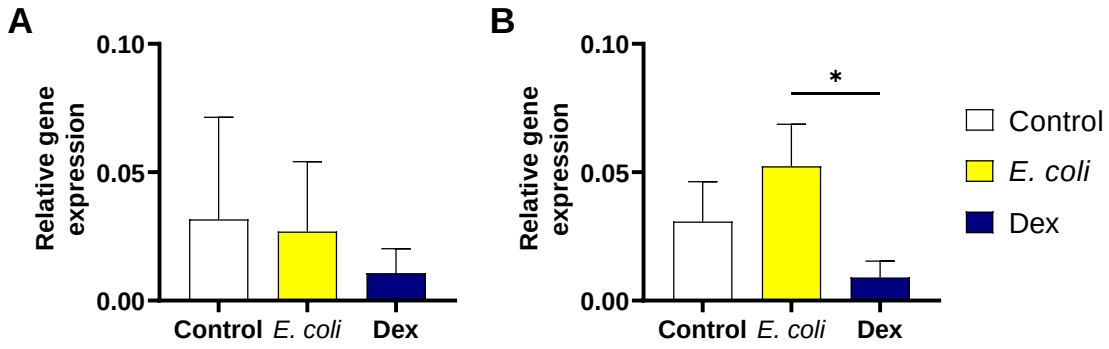


Figure 5.21. MDM-OME expression of PTGS2
OME (**A**) and MDM-OME (**B**) cultured at ALI for 10 days were treated with dexamethasone for 4 h, then simulated with *E. coli* LPS for 24 h and compared to untreated and LPS only controls. Gene expression for *PTGS2* was calculated relative to the reference control *β2-microglobulin* (B2M). Data are presented as mean ± SD of n=3 independent experiments with statistical significance determined using one-way ANOVA; *p<0.05.

5.4 Discussion

5.4.1 MDM-NOF co-culture

Macrophages in the oral mucosa maintain a symbiotic relationship with local microbiota (Belkaid et al., 2017) which requires a finely tuned tolerance to commensal bacteria while maintaining an effective response to pathogenic bacteria (Moutsopoulos et al., 2018). As a result, it is expected that resident macrophages of the oral mucosa have a reduced response to inflammatory stimuli compared to those localised to other tissues. To determine if this process can be controlled by environmental cues and crosstalk between resident macrophages and fibroblasts, a 3D co-culture in a collagen hydrogel was established.

Gene expression changes were examined in response to stimuli, finding that M1-associated CD80 was increased by LPS, and decreased by dexamethasone, and that the reverse was true for M2-associated CD163 and CD206. These markers are thought to be specifically produced by macrophages, with no evidence to our knowledge that these genes can be expressed by NOF. This demonstrated that macrophage polarisation markers could be altered in a co-culture with NOF.

Release of inflammatory cytokines in response to stimuli found LPS-induced increase in IL-6, CXCL8 and TNF-α was retained and partially prevented by dexamethasone, while altered gene expression was only observed for CXCL8. This trend is similar to the results seen in MDM cultured alone in a hydrogel, and thus culturing with NOF did not prevent an inflammatory response. Oral fibroblasts from the buccal mucosa typically secrete low basal levels of IL-6, but expression is higher in fibrosis-associated fibroblasts (Tsai et al., 2004). Interestingly, a fibroblast MDM co-culture comparing fibroblasts isolated from the periodontal ligament and gingiva found that periodontal ligament fibroblasts, but not gingival fibroblasts increased IL-6 secretion in response to *P. gingivalis* LPS (Tzach-Nahman et al., 2017), which was also shown in this study, implying some tissue-specific mechanisms. Moreover, gingival fibroblasts display interpatient variation in IL-6 production. Kent *et al* described two primary fibroblast lines, one non-responsive to LPS stimulation and the other producing ~3 ng/mL IL-6 in response to *E. coli* LPS (Kent et al., 1998), suggesting fibroblast heterogeneity. Imatani *et al* treated primary gingival fibroblasts in monolayer

and found basal secretion of IL-6 was less than 50 pg/mL, which was unchanged by 100 µg/mL *E. coli* LPS, although higher concentrations (1-10 µg/mL) significantly increased secretion to 250-500 pg/mL (Imatani et al., 2001), while another report found basal IL-6 expression was about 500 pg/mL (Kosten et al., 2015), further highlighting the variability of primary gingival fibroblasts. Taken together, these accounts place fibroblast basal and stimulated secretion of IL-6 significantly lower than the values observed here (10-15 ng/mL), so it can be concluded that in the co-culture MDM are the primary producer of IL-6. CXCL8 secretion from primary gingival fibroblasts is similarly varied, with reports claiming 1 ng/ml basal secretion (Kosten et al., 2015), while another stated 100 pg/mL which increased to 250 pg/mL when treated with 100ng/mL *E. coli* LPS (Imatani et al., 2001). In comparison, primary tonsillar fibroblasts have been reported to secrete negligible amounts of CXCL8, although this expression can be induced by the secretome of HPV- cancer cells (Al-Sahaf et al., 2019). Finally, TNF-α is minimally expressed (<20 pg/mL) by fibroblasts from the periodontal ligament and gingiva (Imatani et al., 2001; Tzach-Nahman et al., 2017), which is expected from a cytokine primarily produced by monocytes and macrophages (Parameswaran et al., 2010).

5.4.2 Comparable histology of MDM-OME, OME and native oral tissue

Incorporation of MDM into OME did not adversely affect model development, with similar differentiated epithelia observed compared to MDM-free OME (Jennings et al., 2016).

CD68 is often considered a macrophage-specific marker, although CD68 protein has been detected in skin-derived fibroblasts (Gottfried et al., 2008). However, in the immune-free models, no immunopositive staining was observed for CD68, so it is likely that the cells used in that model do not express CD68 to an appreciable amount, and that the staining observed in the immune model is specific to the macrophages, similar to previous studies (Björnfot Holmström et al., 2017).

Ki-67, a marker of cell proliferation, was also investigated, as it can be used as a marker of proliferating basal keratinocytes in tissue engineered models (Yadev et al., 2011; Buskermolen et al., 2016; Jennings et al., 2016) and is expressed in the basal epithelium of healthy tissue (Birajdar et al., 2014). The staining pattern here

agreed with these studies, with positive staining confined to the basal epithelium in both native tissue, OME, and MDM-OME.

Next, AE 1/3 was used as a pan marker for cytokeratins. These proteins are predominately expressed by epithelial cells and play an important role in intracellular signalling (Awasthi et al., 2016). Specifically in the oral mucosa, expression of specific cytokeratins varies in regions of the epithelium, as well as between keratinized and non-keratinized tissues (Belaldavar et al., 2016). Thus, a pan cytokeratin antibody should uniformly stain the oral epithelium, which was observed in the OME, but not native tissue, where staining was concentrated to the basal epithelium.

In addition, e-cadherin was examined as it is a cell adhesion protein which forms tight junctions and is important for structural integrity of the epithelium (van Roy et al., 2008). Here, native tissue and both models strongly express e-cadherin throughout the epithelium, indicated a well-formed cell network, in agreement with other published studies (Zhou et al., 2015; Buskermolen et al., 2016; Jennings et al., 2016; Björnfot Holmström et al., 2017).

Finally, vimentin was examined as a marker of fibroblasts and macrophages (Goodpaster et al., 2008). In native tissue positive staining was observed throughout the connective tissue, in agreement with previous reports (Zhou et al., 2015), and this was recapitulated in the models, with all cells in the collagen hydrogel expressing vimentin, as seen in similar tissue engineered models (Björnfot Holmström et al., 2017; Xiao et al., 2018).

5.4.3 MDM viability can be assessed by flow cytometry using CD11c as an immune marker

As described previously, CD11c is a transmembrane protein involved in phagocytosis and cell migration (Sadhu et al., 2007), which is found in myeloid immune cells. Although macrophage-specific markers like CD68 could have been used in this study, CD11c was instead chosen as can be used as a pan myeloid immune cell marker. This would allow for more flexibility in future model types which may incorporate different immune cells (such as monocytes and dendritic cells) to also use the same validated marker to identify these cells from within a heterogenous

population. Indeed, here CD11c was found to be a reliable marker to separate MDM from oral keratinocytes and fibroblasts by flow cytometry, in line with previous studies confirming by histology that CD11c is only expressed by immune cells in the oral mucosa (Santoro et al., 2005). The use of an MDM-specific marker allowed for viability of MDM and non-MDM populations to be calculated, which showed little difference between OME and MDM-OME, and comparable MDM viability to 3D single cell culture.

5.4.4 MDM-OME have an inducible inflammatory response which is enhanced compared to immune-free OME

To examine if a broad spectrum of inducible functionality was recapitulated in these MDM-OME, these models were treated with pro- and anti-inflammatory stimuli, and changes in inflammation associated gene expression, and expression of inflammatory cytokines were measured.

First, an inflammatory response was induced by treatment with *E. coli* LPS (500 ng/10^6 MDM) for 24 hours. Both LPS concentration and treatment timing has been used in other immune models (Bao et al., 2015; Björnfot Holmström et al., 2017), while others have used 48 hours (Xiao et al., 2018), and one study found inflammation could be measured after 2 hours (Morin et al., 2017). Short incubation times are advantageous for measuring gene expression as this can alter very rapidly, while longer incubation times can increase quantity of inflammatory cytokines secreted. Thus 24 hours was chosen in the present study, as it aligned with the majority of published regimes, and allowed for a compromise between gene expression and cytokine secretion.

Furthermore, some MDM-OME were pre-treated with dexamethasone for 4 hours to inhibit the inflammatory response. Another study used green tea extract as an anti-inflammatory agent, which had a detectable effect on LPS-induced inflammation following 2 hours pre-treatment (Morin et al., 2017), which is a comparable schedule to the timings used here. However, this study found a dose-dependent effect on production of inflammatory cytokines in their models (Morin et al., 2017), which was not investigated here in MDM-OME, as optimisation was carried out

in monolayer. Additional investigations would have been useful to determine if a dose dependent effect could be observed in these models as well.

5.4.4.1 Changes in gene expression

First gene expression of macrophage polarisation markers was investigated to determine if these are present in other immune cells and whether the changes in MDM expression could be measured in RNA isolated from a mixed cell population.

CD80, a marker of inflammatory macrophages was not detectable in OME, suggesting this protein is not expressed by oral keratinocytes and fibroblasts, in agreement with previous data which did not detect CD80 expression in oral keratinocytes and OSCC derived cells (Villarroel-Dorrego et al., 2005). In contrast, MDM-OME contained detectable expression of CD80, but no significant differences were observable, likely due to the dilution of MDM RNA in the total RNA.

Similar results were obtained for M2 marker CD206, with no expression seen in oral keratinocytes and fibroblasts, and non-significant changes in MDM-OME expression. While no previous studies could be found which examined CD206 expression in oral cells for comparison, it is known to be expressed in dermal cells (Sheikh et al., 2000; Szolnoky et al., 2001), which suggests tissue specific expression.

Finally, CD163 was investigated as a marker of M2 macrophages. Interestingly it was increased in both OME and MDM-OME following dexamethasone treatment, despite many considering it to be a monocyte and macrophage-specific marker (Etzerodt et al., 2013). Increased CD163 by dexamethasone has been observed in MDM (Buechler et al., 2000), as well as lung biopsies (Abdullah et al., 2012), and adipose tissue (Fjeldborg et al., 2014), implicating other resident epithelial cells in this mechanism, in agreement with the data found here.

In addition, analysis of gene expression corresponding to inflammatory cytokines was measured, and while all the genes measured were detected in OME, there were no changes between treatments, suggesting these models were non-responsive to the stimuli used. Similar analysis of gingival fibroblasts following treatment with *P. gingivalis* LPS found increased expression of IL-6 and CXCL8 which peaked at 4-8 hours, then sharply declined (Xie et al., 2018). By 24 hours most gene expression increases had resolved, which was the timepoint used in this study. Therefore, it is

possible that alterations in gene expression may have been detected if an earlier timepoint was examined. In contrast, in LPS treated MDM-OME increases in IL-6 and CXCL8, but not TNF-α and IL-10 gene expression were detected. Expression of TNF-α has also been measured in a gingival immune model, where *E. coli* LPS was similarly not sufficient to induce a measurable increase, while co-stimulating with IFNγ significantly increased TNF-α expression (Björnfot Holmström et al., 2017). It is likely that additionally treating MDM-OME with IFNγ would enhance the inflammatory response measured, although this was not investigated in the present study.

Further analysis was undertaken to TLR2 expression was increased by dexamethasone in OME, which has been shown in cutaneous keratinocytes stimulated with bacteria, but not fibroblasts (Shibata et al., 2009; Su et al., 2017). Alveolar macrophages have also been shown to upregulate TLR2, but not TLR4 in response to dexamethasone, in line with the data shown here (Hoppstädter, Dembek, et al., 2019). While this seems contradictory for an anti-inflammatory agent, Hoppstädter *et al* further investigated the consequences of increased TLR2 expression. They found that it did not correlate with enhanced signalling but did increase secretion of soluble TLR2 and TLR2 within extracellular vesicles, both of which have anti-inflammatory activity. Soluble TLR2 is able to bind to PAMPs and sequester them to prevent further inflammation (Henrick et al., 2016), while TLR2-EVs were found to act as decoy TLR2 receptors (Hoppstädter, Dembek, et al., 2019). This mechanism is likely conserved in the OME and MDM-OME developed here, although further investigation would be required for confirmation. However, this could be an interesting area of future research, as oral bacteria such as *P. gingivalis* preferentially activate TLR2 (Burns et al., 2006), so this could be a mechanism for immune tolerance in the oral mucosa and a potential druggable target to disrupt periodontitis.

5.4.4.2 Changes in inflammatory cytokine production

In this study, secretion of four key cytokines in inflammation were quantified by ELISA: IL-6, CXCL8, TNF-α, and IL-10. In general, OME secretion of these cytokines was unchanged by treatments, as observed previously (Jennings et al., 2016). In contrast, LPS induced a significant increase in TNF-α secretion in MDM-OME, compared to OME, which was prevented by dexamethasone treatment. Similar

immune models have also detected increases in TNF-α secretion following treatment with inflammatory stimuli. Bao *et al* reported an increase from around 0.1 pg/mL to 2 pg/mL following treatment of a gingival model with a multispecies biofilm (Bao et al., 2015). These values are lower than observed in the current study, likely due to the increased volume of receiving media, the use of MM6 cells to produce the inflammatory response, and using a biofilm instead of concentrated LPS to induce inflammation. In comparison, Holmström *et al* repeatedly stimulated their gingival model with LPS ± IFN-γ, finding after a week of dosing that TNF-α secretion was increased to around 100 pg/mL with LPS alone, which was further increased to around 250 pg/mL with the addition of IFN-γ (Björnfot Holmström et al., 2017). The values reported in that study are comparable to those found in this thesis, suggesting that the repeated dosing did not affect overall capacity of the model to produce TNF-α, although the co-dosing with IFN-γ raised the response above that observed here, so it is likely that it could improve the response observed in the MDM-OME. Fluorescence imaging showed TNF-α was specifically secreted by MDM within the collagen connective tissue, underscoring the importance of these innate immune cells in driving the inflammatory response. TNF-α is a key mediator in initiating the immune response, often considered the master regulator of pro-inflammatory cytokine production, and can affect neighbouring cells, such as fibroblasts and keratinocytes, to further orchestrate inflammation (Parameswaran et al., 2010). The ability of MDM-OME to produce TNF-α in response to stimuli compared to immune-free models highlights the importance of inducing MDM in models which are used to study the inflammatory process.

The implication of TNF-α secretion can be observe in MDM-OME as secretion of CXCL8, but not IL-6, was increased in LPS-stimulated MDM-OME compared to OME alone, likely as a consequence of increased TNF-α secretion. Furthermore, cytokine release may be induced further if measured at a later timepoint as gene transcription progresses to translation and protein secretion, as observed by Björnfot Holmström *et al* in their OME of gingival inflammation that was stimulated repeatedly with LPS for several days (Björnfot Holmström et al., 2017).

5.4.4.3 Assessment of inflammatory cytokine production by cytokine array

Other studies have used inflammatory markers not individually analysed in the present study, including MMP-3, -8 and -9 (Morin et al., 2017), lL1β, lL-2, and IL-4 (Bao et al., 2015), and M-CSF and MMP-12 (Björnfot Holmström et al., 2017). Therefore, to better understand the effect of inflammatory and anti-inflammatory stimuli on OME and MDM-OME, an inflammatory cytokine array was undertaken, comparing untreated OME to MDM-OME. In comparison with the results obtained by individual ELISA, both CXCL8 and IL-6 were similarly increased in LPS treated MDM-OME compared to other samples, while TNF-α and lL-10 were not detected, suggesting that assay was less sensitive than the ELISA used. As these cytokines have been discussed previously, further discussion will not be undertaken here. However, many inflammatory mediators were exclusively detected in LPS treated MDM-OME, illustrating the conserved pro-inflammatory response in this model system.

CD14 is a cell surface receptor which is used to stabilise TLRs to facilitation recognition of LPS by macrophages (Zanoni et al., 2013) and can also be secreted in a soluble form. Secreted CD14 is increased in MDM following LPS treatment and correlates with an acute decrease (after 1 hour of treatment) in cell surface abundance of the protein (Marcos et al., 2010). Secreted CD14 has also been shown to enable an LPS response in cells which do not produce CD14, such as gingival fibroblasts (Hayashi et al., 1996). Therefore, the low level of secretion observed in LPS treated MDM-OME is likely the maximal secretion of CD14 and could have improved the overall model inflammatory response by enabling the other cell types to detect and respond to LPS directly.

CD105 (Endoglin) is typically a cell surface protein which promotes angiogenesis through interactions with the VEGF receptor (Tian et al., 2018) and is secreted by MDM in inflammatory conditions, in an MMP-12 dependent manner (Aristorena et al., 2019). Here, low expression of CD105 was detected in LPS-treated MDM-OME only, while MMP-12 was not present in this array, so the mechanistic interaction could not be confirmed, although it is likely that MMP-12 production is increased in LPS treated MDM-OME, as it is associated with inflammatory disease phenotypes (Nénan et al., 2005).

Granulocyte colony-stimulating factor (GCSF) is a secreted glycoprotein with a primary function to produce and stimulate neutrophils (Roberts, 2005). Production can be induced by treatment with LPS and other inflammatory mediators, where it can act to suppress the production of proinflammatory cytokines (Martins et al., 2010). It is therefore likely that the low level of secretion seen here was the initiation of a reduction to the inflammatory response, and that if media was sampled at a later time point a higher concentration may have been observed. Granulocyte-macrophage colony-stimulating factor (GM-CSF) functions to promote immune cell differentiation with macrophages being a major source of this cytokine (Egea et al., 2010), notably in response to proinflammatory stimuli (Fleetwood et al., 2005). GM-CSF has a low basal level of secretion but can be rapidly produced during inflammation (Ushach et al., 2016), in agreement with the data here. Oral epithelial cells have also been shown to secrete GM-CSF in response to inflammatory stimuli such as *Candida albicans* (Dongari-Bagtzoglou et al., 2003), so this response may also be observed in the LPS treated OME.

IL-1 receptor antagonist (IL1ra) is secreted by many immune cell types and can modulate immune response via interactions with IL-1 receptor and inhibiting IL1α and IL1β to act in a broadly anti-inflammatory manner (Kaneko et al., 2019). Similarly, IL-18bpa inhibits pro-inflammatory IL-18 and IFN-γ production to also produce anti-inflammatory effects (T. Zhou et al., 2020). IL1ra secretion had been noted in macrophages following both resolving and persistent inflammation, with secretion peaking at around 24 hours (Italiani et al., 2020). In addition, IL-18bpa is produced in macrophages during inflammation in a transforming growth factor β-activated kinase 1 (TAK1)-mediated manner (Scarneo et al., 2018). TAK1 can be inhibited by dexamethasone (Bhattacharyya et al., 2010), which would explain the lack of this cytokine in the dexamethasone pre-treated models.

CCL3/4 (MIP-1-α and -β) and CCL5 (RANTES) are chemoattractants which recruit NK cells, PMN cells and T cells (Vilgelm et al., 2019). CCL3/4 are well known to be secreted by MDM in inflammation (Menten et al., 2002; Bhavsar et al., 2015), in agreement with the data presented here.

Many of the cytokines measured by this array were detected in multiple samples, indicating some basal expression by oral cells and MDM. For discussion,

these cytokines have been separated into three categories. Firstly, those secreted more highly in all MDM-OME compared to OME, suggesting higher basal secretion by MDM. Five cytokines in total were increased in all MDM-OME compared to OME, which were CXCL5, 9, 10, MMP-9 and chitinase-3-like protein 1 (CHI3L1). Notably, CXCL10, unlike other cytokines, was similarly expressed in LPS and dexamethasone samples, which has been observed clinically (Wark et al., 2007; Gauthier et al., 2017) and in MDM (Hu et al., 2003), and suggests regulation of CXCL10 expression can occur in an NFκB-independent manner. Next those where secretion by OME was higher than MDM-OME, namely CCL2, CCL7 and VEGF. This suggests MDM may be negatively regulating the basal inflammatory response in the tissue. In addition, these cytokines were more highly secreted in LPS-treated MDM-OME, suggesting inducible expression is nonetheless maintained. Finally, most cytokines were increased in LPS treated MDM-OME compared to other samples, demonstrating an MDM-mediated inducible inflammatory response was retained and measurable in MDM-OME.

5.4.5 Quantification of notable XME expression in OME and MDM-OME

As determined in chapter 3 in this thesis, MDM upregulate expression of many XME in inflammatory conditions. Therefore, the expression of these enzymes in OME and MDM-OME treated with inflammatory stimuli was investigated to determine if this effect was measurable in a tissue engineered model.

First, CYP1A1 and 1B1 were examined, as these enzymes are important for detoxification of environmental toxins. They have also been identified in the oral mucosa (Vondracek et al., 2001, 2002), macrophages (Hodges et al., 2000; Eder et al., 2009), and tissue engineered models of the oral mucosa (Schlage et al., 2014; Zanetti et al., 2016). Both enzymes were unaltered by treatments, and detectable in OME and MDM-OME, although expression appeared to be lower in MDM-OME. This would suggest that either MDM expression of these enzymes is lower, or that these cells are having an inhibitory effect on enzyme expression.

Next, CYP2A6 was examined as it was significantly decreased in M2 MDM in monolayer and is the primary enzyme responsible for nicotine metabolism (Raunio et al., 2012), so is a highly relevant enzyme in the oral cavity. Enzyme expression was

detected and similar in both OME and MDM-OME and was unchanged by treatments. This is in contrast to many studies using oral cells in monolayer, where no expression was observed (Farin et al., 1995; Vondracek et al., 2001, 2002; Sarikaya et al., 2007), but in agreement with abundance in oral biopsies (Mallery, Tong, Shumway, et al., 2014), as well as observations in skin where fibroblasts and melanocytes, but not keratinocytes express CYP2A6 (Saeki et al., 2002), highlighting the value of multi-cell tissue engineered models. In addition, while no significant difference was observed in MDM-OME between treatments, this may be a result of keratinocyte and fibroblast RNA masking any difference in MDM expression of CYP2A6, which may have been quantifiable by single cell RNA analysis.

In addition, CYP2D6 expression was examined as it is a key metabolic enzyme and was found to be highly upregulated in M1 MDM compared to other polarisation states. While CYP2D6 was detected in all samples, no changes were observed, although OME had a non-significant decrease following LPS treatment, and the reverse occurred in MDM-OME. As the RNA examined was from the heterogenous cell population, it is possible that decreased expression of CYPD6 in oral keratinocytes and fibroblasts masked any increase in MDM, preventing significance being reached, although further experiments would be required to confirm this. Expression of CYP2D6 is inconsistent in literature, with some buccal tissue positively staining for the enzyme (Vondracek et al., 2001), but none in another study (Sarikaya et al., 2007), and thus further investigations are warranted to determine expression in this tissue.

Similarly, FMO5 was notably increased in M1 MDM compared to M2 MDM so was investigated here. FMO5 was comparably expressed in both models and all treatments, suggesting a lack of inducibility in this model system. As mentioned previously, research into FMO5 functionality is still ongoing (Phillips et al., 2019), and thus no other studies examining enzyme expression in macrophages, oral cells or oral tissue could be identified for comparison.

CYP3A4 was also examined, despite a relatively low abundance in MDM, as it is a key metabolic enzyme, and induced by dexamethasone (McCune et al., 2000). Expression was measurable in both model systems, with a higher expression seen in OME, suggesting higher expression by oral keratinocytes and fibroblasts compared to MDM. In addition, non-significant increases were observed following dexamethasone treatment compared to control. It is possible that induction could be dose dependent,

which has been shown in hepG2 liver cells (Pascussi et al., 2001; Seah et al., 2015) and thus a higher dose may have achieved a significant induction in these models.

Finally, PTGS2 was examined as it is often used as a marker of M1 MDM (Viola et al., 2019) and plays a key role in local inflammation (Simon, 1999). Here, as expected, PTGS2 expression was increased by LPS treatment compared to dexamethasone in MDM-OME, but not in immune-free OME. Previous studies have identified PTGS2 expression in gingival keratinocytes (Chang et al., 2014), although in oral biopsies PTGS2 expression appears to increase as the tissue progresses towards cancer, with little expression observed in healthy tissue (Mauro et al., 2011). In addition, a skin co-culture with RAW264.7 cells found LPS similarly increased production of PTGS2 (Chung et al., 2014). In contrast, dexamethasone is known to inhibit production of PTGS2 by inhibiting p38 activity, which in turn destabilises PTGS2 mRNA (Lasa et al., 2001; Shah et al., 2014), in agreement with the data found here.

5.5 Conclusion

In this chapter, the generation of MDM-OME was described, and these models displayed an improved functional response to inflammatory stimuli compared to MDM-free models, notably with secretion of MDM-derived TNF-α. Preliminary investigations identified expression of multiple key metabolic enzymes in these models, highlighting their relevance to study local drug metabolism. The improved model system presented here has potential applications in several areas of oral bioscience including oral mucosal responses to microorganisms and analysis of host-pathogen interactions, chronic inflammatory conditions, drug delivery or adverse reaction to biomaterials.

Chapter 6 – Final conclusions and future work

6.1 Final conclusions

The innate immune system is a vital first line of defence against pathogens and toxins, but it can also become dysregulated in disease. For example oral lichen planus has a higher density of MDM (Ferrisse et al., 2021), and increased macrophage infiltration in oral squamous cell carcinoma correlates with a poor clinical outcome (Petruzzi et al., 2017). Often therapeutic agents delivered systemically can cause off target effects (Homayun et al., 2019), and thus local delivery of drugs to treat local diseases is preferable. The oral mucosa is one such site due to easy access and non-keratinised epithelium allowing for rapid absorption into the tissue (Hearnden et al., 2012). Despite this, little work has been done to measure the potential for local drug metabolism, both as a potential activator of prodrugs, and inactivator of local therapeutics. As interest increases in developing local drug delivery methods, more work is required to identify potential metabolic pathways which may affect local bioavailability.

The first chapter described the generation of MDM and MoDC from peripheral blood monocytes. These cells in particular were chosen because as part of the innate immune system, they are rapidly recruited to tissue during acute infection, and persist in chronic immune-mediated diseases (Ma et al., 2019), so data could be widely applicable to normal and diseased tissues. Here, it was shown for the first time, that inflammatory MDM have a distinct gene expression profile of XME compared to unpolarised, and M2 polarised MDM, and that MoDC and MDM had distinguishable XME expression. While no previous studies could be identified which examined the effect of macrophage polarisation state on XME expression, a recent study measured expression in a panel of peripheral blood immune cells by gene array and detected CYP2D6, but not CYP2C9 or 3A4 in most cell types (Effner et al., 2017), in general agreement with the data found here. Furthermore, polarised macrophages are increasingly found to have distinct metabolic profiles (Viola et al., 2019; Abuawad et al., 2020), which would provide a mechanistic rational for the changes observed here, as many XME have well established endogenous roles in energy generation and metabolism (Nebert, 1991). Further work would be required to confirm enzyme functionality, which could open up opportunities for improved rational drug design to target therapies to a tissue depending on local inflammation state. However, it is important to note that while alterations in XME expression occur in immune cell

subpopulations, there is still a contribution of other local cells, such as the epithelium or skin or lungs, to consider, which may surpass any differences noted here. For example, CYP2D6 mRNA expression has been detected in multiple oral cell types (Farin et al., 1995; Vondracek et al., 2001; Sarikaya et al., 2007), and protein in half of buccal tissue samples examined, while functional activity was below the limit of detection (Vondracek et al., 2001), as was the case here. It is therefore possible that the levels exceed that detected in M1 MDM, and as such, while differences were noted in these immune cells, further work is required to confirm the extent to which this is relevant *in situ*.

The subsequent work presented in this thesis sought to develop an MDM-OME model and test immune-responsivity to examine the role of MDM in the oral mucosa, and if altered XME expression in macrophages could be observed in a complex immune tissue engineered model. First, the use of MDM as the immune component was tested and optimised. Recent work has used MDM to provide an immune component in tissue engineered models of various tissues (Roh et al., 2019; Smith et al., 2021; Saba et al., 2021), although minimal preliminary work has been shown to justify inclusion of this cell type. Here, a detailed investigation proved that MDM remain viable and functional within a rat tail collagen hydrogel, and thus would be suitable to mimic the immune component of a tissue engineered model. A recent comparable study investigated human MDM response to LPS and dexamethasone over time and found conserved LPS-induced increases in inflammatory cytokine gene expression which was inhibited by dexamethasone (Jumeau et al., 2019), in agreement with the findings here. Furthermore, a decrease in inflammatory cytokine secretion was observed from MDM cultured in a hydrogel compared to monolayer, which has been reported previously in a biomaterial GelMA hydrogel where it was shown to deplete soluble cytokines (Donaldson et al., 2018) which may also be occurring here.

The final chapter describes the development of a tissue engineered model of the oral mucosa containing MDM, to better assess immune function and XME expression *in vitro*. As mentioned above, recent studies have utilised MDM to provide an immune component, but none of these have been in oral models. In addition, studies have produced oral models containing macrophage-like cell lines (Pirilä et al., 2015; Bao et al., 2015; Morin et al., 2017; Xiao et al., 2018), while others have used primary monocytes which were not differentiated into macrophages (Tschachojan et al., 2014; Al-Samadi et al., 2017; Björnfot Holmström et al., 2017; Lira-Junior et al.,

2020). Therefore, the model described in this thesis provides a novel platform to better understand the role of primary MDM in the oral mucosa using an immunoresponsive model system. This is important for two main reasons, firstly that it provides a model option for investigating MDM inflammation in the buccal mucosa, which was not previously possible, and secondly to further confirm that MDM are suitable for inclusion in oral epithelial models, which has previously been shown in other epithelial tissues (Roh et al., 2019; Smith et al., 2021; Saba et al., 2021).

However, there are some key limitations of this model which must be discussed. Firstly, primary cells are typically closer to cells found *in situ*, but this comes at the cost of reproducibility due to substantial interpatient variability and the lack of cell proliferation following differentiation. Furthermore, repeatedly isolating primary monocytes is time-consuming which limits scalability for high throughput applications. In comparison, the use of cell lines such as THP-1 provide better reproducibility and can be scaled which is preferable for use in industry and other applications, but all human monocyte/macrophage cell lines currently available are derived from cancers and thus may not fully recapitulate healthy cells. An improved option would be to generate and use an immortalised human immune cell line from primary cells, as these cells would have greater reproducibility while likely retaining improved responses to inflammatory stimuli. Another limitation of this model is the relevance to healthy tissue, as the model described here has a relatively large density of MDM. Typically, there is a low density of macrophages in the oral cavity in steady state, but a rapid increase during disease and acute inflammation (Parisi et al., 2018; Moutsopoulos et al., 2018), which would suggest the MDM-OME produced here are functionally closer to an inflamed model. While this model is still valuable for testing anti-inflammatory agents and investigating local inflammation in this tissue, it should be considered in conjunction with other available model systems and used only when appropriate for the specific scientific question being investigated.

Overall, the work presented in this thesis has firstly identified a potential role for innate immune cells in the metabolism of xenobiotics, especially for inflammatory (M1) macrophages. To better understand the local implications of XME expression, a tissue engineered model of the oral mucosa was developed which contained MDM. Upon treatment with inflammatory stimuli these models responded with increased inflammatory markers, so would be a suitable model system to assess implications of M1 polarised MDM.

6.2 Future work

This thesis has identified a potential role for immune cells in local drug metabolism and established a novel 3D model of the oral buccal mucosa containing macrophages which can be used to further understand macrophage biology. There are multiple avenues of research which could lead on from the foundation established here, which are detailed below.

6.2.1 Functionality of XME in immune cells

Firstly, in this thesis, differential expression of XME, notably CYP2D6, in polarised MDM was identified, although not confirmed by functional analysis using conventional kits. To take this further, initial work would need to confirm functional expression of this enzyme. One way this could be achieved is by treating polarised MDM in monolayer with a CYP2D6 substrate such as codeine (Kirchheiner et al., 2007), and quantifying metabolite production by tandem mass spectrometry using previously published protocols (Coles et al., 2007). Another option is to mass isolate MDM by pooling multiple samples, which may provide enough functional enzyme to detect by conventional assays. If a differential role is identified for polarised MDM in metabolism of clinically relevant substrates, this could be further examined in a 3D model, which has been shown to better represent drug pharmacology (Sun et al., 2006). The stimuli optimised in this thesis to polarise MDM *in situ* towards M1 (LPS) or M2 (dexamethasone) phenotypes could be used to quantify metabolite production by mass spectrometry. By examining xenobiotic metabolism in a multicellular model, it will allow for understanding the contribution of MDM compared to local cells (keratinocytes and fibroblasts) which have relatively well established expression of XME (section 1.3.4). If MDM are shown to substantially contribute to XME, this would be vital for our understanding of the implications of macrophage activation on local drug metabolism, especially during periods of inflammation when cell numbers dramatically increase. This information could be used to aid in better drug design to deliver local drugs to treat inflammatory conditions, as well as chemotherapeutics, and allow the design of prodrugs which would be metabolised to an active metabolite locally. Furthermore, if XME are substantially increased in inflammation by macrophages, then treating local inflammation with anti-inflammatory agents (such as

dexamethasone) could significantly prolong the half-life of some therapeutics and provide an opportunity to increase drug efficacy without increasing overall dose.

6.2.2 Using single cell RNA to better assess changes in MDM phenotype

Next, the data provided here showed CD11c was a reliable marker to distinguish MDM from other resident cells. Recent studies have used single-cell RNA seq to assess macrophage heterogeneity with great success (Arlauckas et al., 2021; Specht et al., 2021), however the number of macrophages found in tissue at steady state is low which limits the scale to which these cells can be investigated. By incorporating macrophages into a tissue engineered model, this allows for a greater quantity of these cells to be assessed, increasing the power of experiments.

In addition to the data presented here, monocyte-derived macrophages have also been incorporated into other tissue engineered models, such as skin (Smith et al., 2021) and the large intestine (Roh et al., 2019). However, the consequence of culture in different tissue types has not been directly compared. This could be an interesting avenue to better understand the local cues which drive macrophage differentiation into distinct phenotypes by analysing single cell RNA through RNA seq and comparing gene expression profiles of MDM isolated from different tissue engineered models.

Finally, this methodology would allow for the use of genetically modified human MDM (Moyes et al., 2017) to be examined in a 3D environment and quantify changes in gene expression profile in response to stimuli such as biomaterials and carcinogens. This would enable examination of the effect of specific genes in an *in vitro*, without the use of knockout *in vivo* models, in line with the 3Rs of animal research.

6.2.3 Using MDM-OME to generate tissue engineered models of oral disease

The MDM-OME described here is a suitable model to study immune activation in healthy tissue and would therefore be useful for testing efficacy of anti-inflammatory pharmaceutical agents, as well as novel delivery methods of existing drugs. For example, these models could be used to examine immune activation in the oral mucosa in response to novel biomaterials, and also investigate efficacy of drug-loaded biomaterials to reduce implantation-induced local inflammation. Furthermore, with

small alterations this model system could also be used to mimic oral diseases which would allow for better study of how these diseases arise and provide a robust testing platform to optimise treatments.

Recurrent aphthous ulcers are a common painful condition which presents with superficial tissue necrosis (Preeti et al., 2011). These sites often have high infiltration of $CD68^+$ macrophages, and can be categorised by increased TNF-α expression compared to healthy tissue (Natah et al., 2000). Methods have previously been developed in tissue engineered models to achieve a compromised epithelium, such as burning (Shepherd et al., 2009), or use of a micropipette tip to inflict a wound (Riabov et al., 2017). These methods could be applied to the MDM-OME generated here to model ulcerated tissue to provide a valuable platform to test potential therapeutics.

Oral lichen planus (OLP) is a chronic T cell mediated oral condition but also has a high infiltration of $CD68^+$ macrophages (Ferrisse et al., 2021). Adaptations to the model could be made by using oral cells isolated from OLP tissue which have been shown to differentially respond to inflammatory stimuli compared to cells isolated from healthy tissue (Wang et al., 2018). This model would allow for insight into the specific role of macrophages within this disease, and a better understanding of how cells in OLP interact with local immunity to produce chronic inflammation.

Tumour-associated macrophages (TAM) are present in the tumour microenvironment where they can create an immunosuppressive environment and promote tumour metastasis (Lin et al., 2019). Similar alterations to the MDM-OME could be made by using widely available cancer-derived oral cells, which have been used in spheroids and other 3D model systems (Chitturi Suryaprakash et al., 2020). Furthermore MDM could be polarised towards an M2 phenotype before inclusion as these are associated with more aggressive tumour characteristics (Jayasingam et al., 2020). This could aid in developing therapies which either target TAM by depleting cell numbers or shifting to M1 polarised TAM, or utilise TAM as drug delivery vectors, all of which have been underutilised as potential interventions in oral cancers thus far (Bruna et al., 2021).

Chapter 7 - References

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Chapter 8 - Appendix

Analyte	OME Control	MDM-OME Control	MDM-OME Dex	MDM-OME LPS
Adiponectin	-	-	-	-
Apo A-1	-	-	-	-
Angiogenin	+	+	+	+
Angiopoietin-1	-	-	-	-
Angiopoietin-2	-	-	-	-
BAFF	-	-	-	-
BDNF	-	-	-	-
Complement component C5/C5a	-	-	-	-
CD14	-	-	-	+
CD30	-	-	-	-
CD40 ligand	-	-	-	-
Chitinase 3-like 1	+	+	+	+
Complement factor D	-	-	-	-
C-reactive protein	-	-	-	-
Cripto-1	-	-	-	-
Cystatin C	-	-	-	+
Dkk-1	+	+	+	+
DPPIV	-	-	-	-
EGF	-	-	-	-
Emmprin	+	+	+	+
ENA-78	-	+	+	+
Endoglin	-	-	-	+
Fas Ligand	-	-	-	-
FGF basic	-	-	-	-
FGF-7	-	-	-	-
FGF-19	-	-	-	-
Flt-3 ligand	-	-	-	-
GCSF	-	-	-	+
GDF-15	+	+	+	+
GM-CSF	-	-	-	+
GROα	+	+	+	+
Growth hormone	-	-	-	-
HGF	-	-	-	+
ICAM-1	-	-	-	-
IFN-γ	-	-	-	-
IGFBP-2	+	+	+	+
IGFBP-3	-	-	-	-
IL-1α	-	-	-	-
IL-1β	-	-	-	-
IL1ra	-	-	-	+
IL-2	-	-	-	-

IL-3	-	-	-	-
IL-4	-	-	-	-
IL-5	-	-	-	-
IL-6	+	+	+	+
IL-8	+	+	+	+
IL-10	-	-	-	-
IL-11	-	-	-	-
IL-12 p70	-	-	-	-
IL-13	-	-	-	-
IL-15	-	-	-	-
IL-16	-	-	-	-
IL-17A	-	-	-	-
IL-18 Bpa	-	-	-	+
IL-19	-	-	-	-
IL-22	-	-	-	-
IL-23	-	-	-	-
IL-24	-	-	-	+
IL-27	-	-	-	-
IL-31	-	-	-	-
IL-32	-	-	-	-
IL-33	-	-	-	-
IL-34	-	-	-	-
IP-10	-	+	+	+
I-TAC	-	-	-	-
Kallikrein 3	-	-	-	-
Leptin	-	-	-	-
LIF	-	-	-	-
Lipocalin-2	+	+	+	+
MCP-1	+	+	+	+
MCP-3	+	+	+	+
M-CSF	-	-	-	-
MIF	+	+	+	+
MIG	-	+	+	+
MIP-1a/MIP-1b	-	-	-	+
MIP-3a	-	-	-	-
MIP-3b	-	-	-	-
MMP-9	+	+	+	+
MPO	-	-	-	-
OPN	-	+	+	+
PDGF-AA	-	-	-	-
PDGF-AB/BB	-	-	-	-
PTX3	-	-	-	-
PF4	-	-	-	-
RAGE	-	-	-	-
RANTES	-	-	-	+
RBP-4	-	-	-	-

RLN2	-	-	-	-
Resistin	-	-	-	-
SDF-1a	-	-	-	-
Serpin E1	+	+	+	+
SHBG	-	-	-	-
ST2	-	-	-	-
TARC	-	-	-	-
TFF3	-	-	-	-
TfR	-	-	-	-
TGFα	-	-	-	-
Thrombospondin-1	-	-	-	+
TNFα	-	-	-	-
uPAR	-	+	+	+
VEGF	+	+	+	+
Vitamin D BP	-	-	-	-
CD31	-	-	-	-
TIM-3	-	-	-	-
VCAM-1	-	-	-	+

Table 8.1. MDM-OME and OME cytokine array summary

OME, untreated MDM-OME, MDM-OME treated with dexamethasone for 4 h then simulated with *E. coli* LPS for 24 h (dex), and MDM-OME treated with *E. coli* LPS for 24 h (LPS) were assessed by cytokine array. Analytes observed to be secreted (+) or below the limit of detection of the assay (-); n=1.