$P2X_7R$-driven IL-1 responses in differentiated murine Dendritic Cells; comparison with Macrophages.

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LIST OF ABBREVIATIONS:

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
APC	Antigen presenting cell
ASC	Apoptosis-associated, a speck-like protein containing a CARD
BBG	Brilliant blue G
BCL	B-cell lymphoma
BM	Bone marrow
BM-MΦ	Bone marrow derived macrophages
BSA	Bovine serum albumin
BSF	Biological safety facilities
BzATP	Benzoylbenzoyl adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
caMΦ	Classically activated macrophages
CCL	Chronic lymphocytic leukemia
CCR	Chemokine receptor
CD	Cluster of differentiation
cDC	conventional dendritic cells
CLA	Cutaneous lymphocyte associated protein
CLEC4A4	C-type lectin family 4, member A4
CSF	Colony stimulating factor
DAMP	Damage associated molecular patterns
DC	Dendritic cell
dDC	Dermal dendritic cell
DNA	Deoxyribonucleic acid
EBP	IgE binding protein
ECD	Extracellular domain
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EpCAM	Epithelial cell adhesion molecule
Flt-3L	Fms-like tyrosine kinase-3 ligand
FSDC	Folliculo-stellate dendritic cells

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony stimulating factor
hCAP-18	human cathelicidin protein-18
HBSS	Hank's balance salt solution
HEK	Human embryonic kidney
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HSC	Haematopoietic stem cell
HSP	Heat shock protein
HUVEC	Human umbilical vein endothelial cells
Id2	Inhibitor of DNA binding
IFN-	Interferon
IGIF	IFN-γ-inducing factor
IL	Interleukin
IL-1RA	Interleukin-1 receptor antagonist
IL-1RacP	IL-1 receptor accessory protein
IL-1RI	Type I Interleukin-1 receptor
IL-1RII	Type II Interleukin-1 receptor
ILV	Intraluminal vesicles
IP3	Inositol triphosphate
IRAK	Interleukin-1 receptor-asssociated kinases
JNK	c-Jun N-terminal kinases
KN-62	4-[(2S)-2-[(5-Isoquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl] phenylisoquinolinesulfonic acid ester
KO	Knockout
LAMP-1	Lysosomal-associated membrane protein 1
LDH	Lactate dehydrogenase
LC	Langerhans' cell
LPS	Lipopolysaccharide
aMΦ	Alternatively activated macrophages
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage-colony stimulating factor
MDP	Muramyl dipeptide
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MMP	Matrix metalloproteinase
MRC-1	mannose receptor 1
MVB	multivesicular bodies
MyD88	Myeloid differentiation primary response gene (88)
NFATc1	Nuclear factor of activated T-cells, cytoplasmic 1
NF-κB	Nuclear factor κB
NFIL-6	NF-κB like site 6
NK	Natural killer
NLR	Nucleotide-binding domain leucine-rich repeat
NLRC4	NLR-family CARD domain containing protein 4
NLS	Nuclear localization sequence
NOD	Nucleotide-binding oligomerization domain receptor
NPC	Nuclear pore complex
oATP	Oxidized adenosine triphosphate
PALS	Periarteriolar lymphatic sheaths
PAMP	Pathogen associated molecular patterns
pDC	Plasmacytoid dendritic cells
PLC	Phospholipase C inhibitor
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'- disulfuric acid
PPR	Pattern recognition receptors
RA	Rheumatoid arthritis
RalB-	Ras-like small G protein
RER	Rough endoplasmic reticulum
Siglec-H	Sialic acid binding Ig-like lectins
SNP	Single nucleotide polymorphisms
TCF	Transcription factor
TGF	Transforming growth factor-β
TH	Helper T-cell
TLR	Toll-like receptor
TM	Transmembrane
TNF	Tumour necrosis factor
TRAF	Tumour necrosis factor receptor-associated factor
UDP	Uridine diphosphate
UTP	Uridine triphosphate

YO-PRO	Quinolinium, 4-[3-methyl-2-(3H)-benzoxazolylidene)methyl]-1-[3-triethylammonio)propyl]di-iodide

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ABSTRACT:

The $P2X_7R$ is a functionally distinct member of the P2X non-selective cation channels and has been implicated in the initiation of immune responses. One of the most extensively characterised immune responses of the receptor is to signal the rapid aggregation of the inflammasome complex and signal the release of IL-1β. These investigations have focused in providing direct comparisons of $P2X_7R$-driven IL-1 responses between DC and mouse macrophages (peritoneal macrophages [PMΦ] and bone marrow derived macrophages [BM-MΦ]). Expression of the $P2X_7R$ has been identified in all three populations both at the transcriptional (P2X7A variant) and protein levels. Activation with lipopolysaccharide (LPS) (2h) induced a rapid dose dependent release of IL-6 but not of IL-1β in BM-DC. Rapid (2h) IL-1β release required both LPS priming and ATP activation. Both signals were also required for IL-1β release in mouse BM-MΦ and PMΦ, however, at comparatively markedly lower levels. Furthermore, like with IL-1β, LPS did not induce IL-1α release in BM-DC. Interestingly, subsequent challenge with ATP evoked IL-1α release in BM-DC alone, with little or no detectable levels observed in activated BM-MΦ. This rapid IL-1β release (but not IL-6) was potently inhibited in both macrophages and DC with a $P2X_7R$-specific inhibitor (A-740003) providing evidence that is predominantly a $P2X_7R$-driven process. Treatment with A-740003 also potently inhibited IL-1α release from BM-DC suggesting that the ATP-$P2X_7R$ and caspase-1 activation might have a role in the release of the cytokine. Expression of gain-of-function P2X7K and loss-of-function P2X7J splice variants has been identified in both BM-DC and BM-MΦ, at the level of transcription. The possibility that a differential baseline or LPS-induced expression (at the transcriptional level) of P2X7J and P2X7K variants accounts for the diverse cytokine responses observed in BM-DC and BM-MΦ was also explored. However, the levels of expression for the various splice variants of interest (P2X7K and P2X7J) were found to be similar between the two cell types. The results of these investigations identify some subtle but intriguing differences in the mechanism of $P2X_7R$ activation and IL-1 release between DCs and macrophages. Purinergic signalling is increasing being implicated in the regulation of immune responses both in potentiating or suppressing inflammation. However, further work is required to decipher how the dynamic interplay between different purines can influence the immune activation of different cell types and indeed different cell subsets.

DECLARATION:

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Chapter 1:
Introduction.

1.0 The Immune system.

«Των εναντίων η φύσις γλίχεται και εκ τούτων αποτελεί το σύμφωνον»
Nature strives for the opposite and from it generates consonance.
-Herakleitos

The Egyptians first recognized the immune system in action during the mid-17th century B.C, but it was not until first century A.D. that Cornelius Celsius defined the process of inflammation according to four distinct features: swelling, redness, heat and pain. Even today, the precise sequence of events that unfold within injured tissues remains a complex mystery. Under normal conditions, inflammation is the host's defence response mechanism to constrain, and eventually destroy, invading pathogens and to promote repair and regeneration [1]. In mammals, the primitive innate immune system evolved to identify invading pathogens at sites of tissue injury and to signal efficiently the presence of danger to the adaptive arm of the immune response. The system includes phagocytes, natural killer (NK) cells and interferons (IFN). These cells are capable of recognizing distinct patterns that are shared between pathogens, such as double stranded viral RNA and bacterial polysaccharide (LPS). These are usually referred to as pathogen associated molecular patterns (PAMP) and are recognized by a family of specialized pattern recognition receptors (PRR) (see section 1.1) [2]. In contrast, the adaptive immune system is only present in vertebrate species and has evolved from a primitive innate system found in metazoans [3] The adaptive arm is activated by the innate immune system and acts to enhance the immune response with T-cell and B-cell responses. In most cases, however, it induces tolerance by 'silencing' lymphocytes. The necessity for the adaptive arm of the immune response arose from evolutionary pressures by the increasing diversity of microbes that continuously challenge the mammalian immune system [4, 5]. Despite its unparalleled diversity and complexity, the different parts of the immune system have evolved in parallel to complement each other functions. At the heart of this sophisticated system, lies a complex, heterogeneous family of leukocytes, 'energizing' and directing multiplex processes, the dendritic cell (DC) family [6].

The various cellular components of the immune system are well adapted to respond to their requirements in the various tissue compartments. Such is also the case with the largest organ of the human body, the skin. The skin constitutes the largest interphase

between pathogen and host and is constantly challenged with a heavy load of invading pathogens. However, the immune system is also responsible for maintaining tissue homeostasis and silencing immune responses to self-antigens [7]. Thus, in addition to pathogenic entities, the skin microenvironment is also in contact with a substantial number of man-made chemicals. These are usually harmless molecules with low molecular weight and because of this they are effectively invisible to immune surveillance. Certain chemicals possess specific properties that allow them to form hapten-protein conjugates and become immunogenic resulting in the development of contact dermatitis, termed allergic contact dermatitis (ACD) [8]. ACD results in the development of a disease with visibly unpleasant clinical symptoms, such as that of papules and dry skin and poses a serious environmental and health hazard. There is a clear need to improve the current screening techniques for identifying potential chemical allergens that abide to the ethical and societal stresses to eliminate the need for use of experimental animals [9]. DC populations in the skin lie at the heart of antigen and hapten-protein recognition in the layers of the skin and there is no question that they are responsible immune responses in the skin and therefore the development of ACD [10]. DC are avid producers of pro-inflammatory cytokines such as IL-1β which are essential elements for inducing migration of DC from the layers of the skin to present antigen to T-cells in the draining lymph nodes [11]. Evidence suggests that ion channels like the $P2X_7R$ play an important role in activating the release of pro-inflammatory cytokines from cells of the haematopoietic lineage [12]. The functional properties of the mouse $P2X_7R$ on DC remain obscure and since DC present an attractive indicator cell for the development of in vitro screening assays, it is important to improve our understanding of this peculiar ion channel on the biology of mouse DC and its function during DC responses to xenobiotics.

1.1 Dendritic cells as sentinels of the immune system

The route to discovery of DC begun when Steinman decided to examine the cellular components of the spleen, moving away from the peritoneal cavity, which was the main focus of macrophage research [13]. Soon after Steinman and his colleagues begun dissecting the spleen, they identified novel cells with long dendritic processes that they named DC. Phenotypically they were found to be different to macrophages and it was soon realized that these cells also possessed distinct functional characteristics [14, 15]. Soon it was realized that DC are abundant at body surfaces and are ideally positioned in sites such as the skin, lung, gut, pharynx, vagina and ectocervix, ready to capture antigens and more often to 'silence' the immune system by inducing tolerance and occasionally to initiate inflammation against noxious invading pathogens [16]. They are essentially the connective bridge between the innate and adaptive immune responses. In comparison with macrophages and B-cells, DC are able to capture, internalize, process antigen into peptides and present it as part of the major histocompatibility complex (MHC) to receptive T-cells with great efficiency. The composition and regulation of this antigen presenting machinery soon became the hallmark feature of DC that distinguishes them from macrophages [17, 18]. As such, DC are unquestionably the key regulatory components of immune responses. The vast virtual library of lymphocyte clones bearing randomly arranged antigen receptors and thereby T-cell clonal expansion are essentially controlled by DC [6]. It is DC that lie at the heart of the decision making process of whether to engage the adaptive arm of an immune response [19].

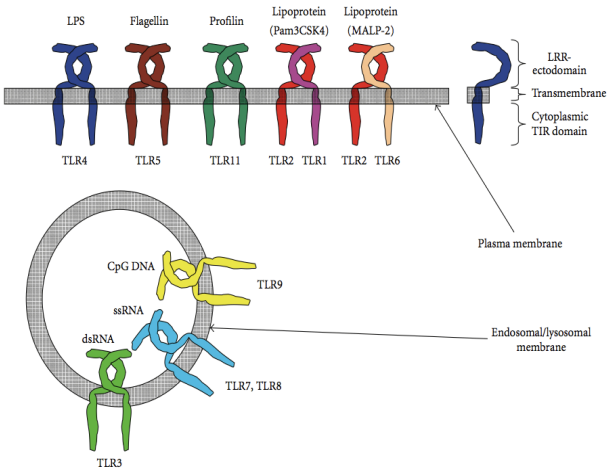
The DC family is a relatively small population of hematopoietic cells. Their name is derived from the Greek word for tree, 'dendron', which describes their unique 'stellate' morphology [14]. Newly formed DC progenitors emerge from the bone marrow, circulate in the bloodstream and home to non-lymphoid tissues in response to chemokine signals and other ligands, which guide their migration. Their differentiation progresses with the loss of developmental options as progenitors begin to commit to certain lineages [20]. What is not entirely clear is whether the commitment of haematopoietic cells (HSC) into the DC lineage for example is a process driven by intrinsic signals or whether the local microenvironment provides external cues along their differentiation route [20]. In the intrinsic model, lineage-specific genes are activated within HSC that dictate the expression of proteins and allow the cells to

become receptive to lineage-specific signals. Alternatively in the 'extrinsic model' successive instructive signals become highly concentrated in local microenvironments and depending on the level of expression of the receptor for a specific signal the HSC will commit to a particular lineage [21].

Dendritic cells are strategically positioned at the portals of antigen entry providing a robust shield against infection, regulating both innate and adaptive arms of the immune response [14]. They are characterized by their distinct stellate morphology and a high surface area that allows for simultaneous interaction with a large number of T-cells. Large delicate processes are extended in multiple directions away from the DC body to sample fluid from the extracellular compartment and to uptake antigens using several different mechanisms including micropinocytosis or receptor-mediated interactions (reviewed by [22]). They express a family of specialised receptors, the pattern-recognition receptors (PRR) that enables DC to invading pathogens. The complex system of detecting xenobiotics via PRR is illustrated in Figure 1.1. The most widely studied of the PRR is a family of receptors known as Toll-like receptors (TLR), that is able to recognise pathogen associated molecular patterns (PAMP) on viruses, bacteria, protozoa and fungi [23]. The discovery of these receptors is unquestionably a hallmark breakthrough for modern immunology. The TLR family consists of 12 highly homologous members in mouse tissues and 10 TLR in humans. Each TLR receptor has a structurally distinct extracellular domain suggesting that different TLR recognize different molecular patterns. For example, molecular patterns from Gram-positive bacteria are recognized by the TLR2/TLR1 or TLR2/TLR6 hybrid receptor whereas Gram-negative bacteria patterns, such as LPS, are recognized by TLR4. However, currently no known ligands have been shown to activate TLR11-13 in mouse tissues and the human TLR10 has no mouse orthologue. Aside from microorganism-derived moieties, TLR are also activated by certain endogenous ligands such as the heat shock protein (HSP)-60 -70, fibrinogen, necrotic cells, heparan sulphate which activate TLR4 [24]. Activation of TLR has a pleiotropic effect on DC morphology inducing cytoskeletal and morphological changes such as re-modelling of the endocytic network, and up-regulation and release of chemokines and cytokines. As it will be discussed later in greater detail, DC comprise of several distinct subsets, and the TLR repertoire differs in the different subsets and also between mammalian species. For example, no messenger RNA can be found for TLR2, 3, 4, 5, and 8 in human plasmacytoid DC

(pDC) and as a result these cells are not responsive to stimuli like LPS, lipoteichoic acid and peptidoglycan [25]. Differences in TLR expression and function in the various DC subsets are reviewed in detail by Sousa and colleagues [6].

a



b

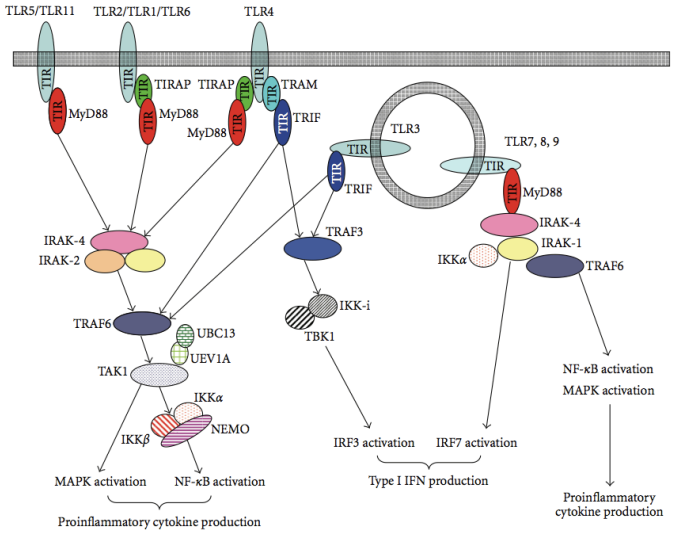


Figure 1.1: The family of Toll-like receptors.
A schematic diagram of the complex family of TLR **(a)**. The TLR family consists of 10 members in human cells and 13 members in murine tissues. TLR consist of 3 major domains: an extracellular domain that is rich in leucine repeats (LRR), a transmembrane domain and an intracellular TIR domain. The family is subdivided into two main groups according to their location. Whilst TLR1, 2, 4, 5, 6 and 11 are anchored on the cell surface membrane, TLR3, 7-9 are found expressed in intracellular compartments such as endosomes, lysosomes and the endoplasmic reticulum. The figure illustrates the various ligands associated with specific TLR receptors such as LPS for TLR4, and flagellin for TLR5. The intracellular signalling pathways associated with TLR activation **(b)**. Ligand binding of TLR results in the dimerization of the intracellular TIR domain that initiates of an array of signalling cascades culminating in the activation of NF-κB or MAPK and the expression of genes encoding pro-inflammatory mediators. The TIR domain of different TLR associates with adaptor proteins including MyD88, TRIF, TRAM, TIRAP which activate a specific intracellular pathway and generate specific cytokines production. For example, the MyD88-associated pathways mainly result in the production of pro-inflammatory cytokines or IRF-dependent type I IFN production. TLR3/4 signaling results in the activation of a MyD88-independent and TRIF-dependent pathway and lead to the expression of type I IFN. In a subset of DC known as plasmacytoid DC TLR7/9 activation also results in the expression of type I IFN in a MyD88-dependent mechanism. [26].

As will be discussed later, the precise phenotypic differences between DC subsets, particularly in humans, are not entirely clear. Therefore, the exact TLR repertoire for each subset cannot yet be defined. Nevertheless, information about the expression of a TLR receptor on a particular DC subset can provide insights regarding its function. What is clear is that TLR activation has a profound impact on the phenotype of DC [27]. However, DC are not only activated by TLR signalling; DC also express damage-associated molecular patterns (DAMP), cytosolic or nuclear proteins that detect stimuli derived from 'self' or necrotic cells and can also signal the maturation of DC. Finally inflammatory cytokines, like Interleukin (IL)-1 and tumour necrosis factor (TNF)-α released by cells in proximity can also cause DC maturation [28]. Helper T-lymphocytes (TH) can also induce DC maturation via CD40 interactions [29].

The cell surface membrane of DC is populated by a myriad of different receptors that allow the cells to recognize a wide variety of potential threats. The most abundant receptors expressed on DC are the C-type lectins, which are involved in the recognition of glycosylated antigens. These include the DEC205 and the mannose receptor [30]. Langerin is another C-type lectin that is expressed by certain skin DC subsets and is thought to be involved in the recognition of specific types of antigens. Additionally, Fc receptors that mediate the internalization of immune complexes are also found on the surface membrane of DC [31]. DC also express receptors for HSP that are involved in the internalization of HSP-antigenic complexes [32]. The cell surface membrane of DC is also very dynamic and their phenotype completely changes during the maturation process [33]. Apart from TLR, which coat the cell surface membrane of DC and macrophages, there is another family of receptors that play part in mediating host defence and are found in intracellular compartments. These are the NOD-like receptors (NLR) and consist of three distinct domains namely the C-terminal recognition domain, a caspase recruitment domain and an N-terminal effector domain. These proteins have the ability to activate caspases and therefore regulate the release of pro-inflammatory cytokines such as IL-1β and IL-18. Additionally, there is evidence to suggest that NLR, particularly NOD1 and NOD2 are also involved in sensing pathogens by recognizing different antigenic moieties and initiating inflammatory responses [34, 35].

1.2 Activation and maturation of Dendritic Cells.

DC are part of the mononuclear phagocyte system and like macrophages they are efficient in phagocytosis. They constitutively sample their microenvironment through micropinocytosis of extracellular fluid. They can engulf antigen by phagocytosis, receptor-mediated endocytosis or micropinocytosis [17]. Unlike other APC, they have the ability to quickly undergo complete genetic reprogramming upon the recognition of stimuli that signal the presence of a xenobiotic agent in their immediate microenvironment. Soon after the activation signal has been delivered, DC actively down-regulate their ability to induce phagocytosis or micropinocytosis of antigens [36, 37] but gain the ability to present antigen to T-cells. The cells initiate a complete transformation inducing the expression of a cohort of cell surface protein complexes that are involved in antigen presentation to T-cells. The process of antigen presentation involves the transport of the engulfed antigen through a complex network of endosomes. Newly synthesized MHC class II molecules, which are usually retained in lysosomal vesicles, are driven through a network of endosomes to be partnered with processed antigenic peptides. The whole process involves a specialized family of proteases, the cathepsins, which very efficiently manipulate the endosomal network of vesicles and fuse peptide-containing endosomes with the MHC class II compartments of the endosomal network. Once MHC class II molecules are loaded with a matching peptide, the complex is shuttled to the cell surface membrane for presentation to T-cells via their T-cell receptors. The maturation process of DC also involves the up-regulation of expression of co-stimulatory molecules like cluster of differentiation (CD)80/86 and CD40 and other molecules that are required to provide the co-stimulatory signal to T-cell associated CD28 during the formation of the ‘immunological synapse’ [38-42, 33].

Activated DC from the skin, lymphatics or blood need to migrate to the T-cell interfaces of the draining lymph node or the spleen. En route to the draining lymph node, DC receive ‘information’ that influence their state of activation. This information is processed by the recipient DC and will dictate the form of signal that will be ‘pushed’ to the recipient T-cell. In order to migrate through the various tissues activated DC manipulate the expression of a cohort of chemokine receptors, such as CCR7, that

allows them to travel towards the draining lymph node or the spleen. During the journey they begin to acquire a mature phenotype upregulating receptors required for antigen presentation and start to synthesize and release chemokines, proteases and pro-inflammatory cytokines like IL-1, IL-6 and TNF-α [43, 44]. For complete activation of T-cells, 3 independent signals are required. Signal 1 is antigen specific and the processed antigenic peptide is presented via the MHC class I complex for CD8+ cytotoxic T-cells or via the MHC class II complex for CD4+ T-cells. T-cells also simultaneously receive a second co-stimulatory signal from receptors such as CD80/86 that are upregulated by DC during their migratory pathway towards the lymph node. Immature DC in surveillance in various tissues lack substantial expression of such molecules and are therefore incapable of delivering an activating signal to T-cells. Finally, a third signal is required in the form of soluble cytokine and is delivered in a paracrine fashion [45].

Presentation of antigen is not restricted to MHC class II molecules. Intracellular peptides derived mainly from the cytosol like virus components are loaded onto MHC class I molecules and presented to cytotoxic T lymphocytes. Unlike most cells, DC have the ability to exchange material between the cytosol and endosomal compartments. This allows the cells to process endogenous antigens, derived from engulfed virally infected cells, in the cytosol and load them onto MHC class I complexes, which takes place in endosomes. DC are particularly efficient at presenting through this pathway [46]. Apart from T-cells, DC have the ability to present antigen to B-cells in the draining lymph nodes. They harbour antigenic pools in micropinosomes internally which they release to activate adjacent B-cells and induce humoral immunity. Due to their remarkable efficiency in antigen presentation, DC populations are the cells of choice for the development of antigen-specific immunotherapies both in cancer or other infectious diseases [2]. However, DC are a very heterogeneous population of cells and if therapeutic strategies are to yield success, our understanding of the phenotypic and functional differences of all the DC subsets needs to be improved [47, 33].

1.3 DC lineages and their role in immune responses.

It would be an impossible task for a single cell type to be responsible for the wide spectrum of effector functions that DC have been linked with. Several different subsets have been identified, often with opposing effector functions that are specialized to carry out specific tasks in specific tissue compartments. The identification of DC subsets occurred whilst in search for more molecular cell surface markers for DC. It was soon realised that specific cell surface receptors were not expressed by all DC populations at the steady state [48]. Additionally, the development of microarray databases and the identification of subset-specific transcription factors made it feasible to dissect the DC lineage route and present evidence for the existence of multiple DC subsets. However, the proposition that DC exist in multiple subsets was met once again with similar scepticism and strict criticism of complicating unnecessarily the field. It is now widely accepted that DC comprise multiple cell types with distinct phenotypes, anatomical locations and immunomodulatory functions. Whether the level of phenotypic and functional differences translates to a fundamentally different cell type, and how much plasticity exists in these different cell types is still disputed [49].

Two distinct pathways of DC development have been identified, the myeloid (derived from the blood, skin or other peripheral organs) and lymphoid (derived from the thymus, bone marrow, lymph node and other primary lymphoid organs). The myeloid-derived conventional DC commits to the DC lineage under the influence of granulocyte/macrophage colony-stimulating factor (GM-CSF), whereas another DC precursor has been identified in the thymus. The two DC lineages exhibit distinct phenotypic and functional properties. Both DC subsets express similar levels of MHC class II, CD86 and CD40, however, only lymphoid derived DC express the cell surface homodimer marker CD8α [50]. CD1d and CD205 are expressed at higher levels in lymphoid DC, but have been shown to be up-regulated in myeloid DC in vitro [51]. The two DC lineages localize in different areas of the lymphatic system, with lymphoid DC occupying the T-cell-rich areas of the periarteriolar lymphatic sheaths (PALS) in lymph nodes and in the spleen, whilst the myeloid DC are found in the marginal zone but are

induced to migrate [52]. Our knowledge for human DC subsets derives almost exclusively from blood derived DC since tissue is not readily available, particularly from primary lymphoid tissues, thus the functional and lineage origins of the various DC subsets are not well defined. [2]. From the limited data collected, DC with three different profiles have been identified in the human thymus, and lymph nodes and at least four different DC profiles have been identified in the spleen. The different subsets have been classified according to the expression of classic DC proteins such as the human leukocyte antigen (HLA)-DR (MHC class II), CD11c and CD13 (an N-aminopeptidase enzyme).

A similar diversity in DC subsets has also been identified in mouse tissue. In mouse however, tissues are more readily available and more markers have been identified for mouse DC. Therefore, more studies have been performed in mice regarding the commitment of cells to the DC lineage and their development in the various tissue compartments [53]. The different DC profiles/subsets identified in mouse and human lymphoid and non-lymphoid tissues is summarized in Table 1.1.

Table 1.1: *The profile of distinct DC populations.*
(Adapted from [54]).

Mouse DC subsets			
DC subset	**Phenotypic Markers**	**Location Identified**	**Class Description**
Plasmacytoid DC	$CD11c^{int}$ $B220^{+}$ BDCA-1^{+} (CD317) $CD11b^{-}$ Gr-1^{+} MHC-II^{+}	Spleen	Plasmacytoid DC
conventional DC $CD8\alpha^{+}$	$CD11c^{+}$ $CD8\alpha^{+}$ $CD4^{-}$ $CD205^{+}$ Langerin^{+} $CD11b^{-}$	Spleen, thymus	Lymphoid-resident DC
conventional DC $CD8\alpha^{-}$	$CD11c^{+}$ $CD8\alpha^{-/+}$ $CD4^{-}$ DCIR-2^{+} $CD11b^{-}$	spleen	Lymphoid-resident DC
Langerhans' cell	$CD11c^{int}$ MHC-II^{+} F4/80^{+} Langerin^{+} $CD11b^{+}$	Epidermis Cutaneous lymph nodes	Migratory DC
Dermal DC Langerin+	$CD11c^{+}$ $CD103^{+}$ Langerin^{+} $CD11b^{lo}$	Dermis Cutaneous lymph node	Migratory DC
Dermal DC Langerin-	$CD11c^{+}$ $CD103^{-}$ Langerin^{-} $CD11b^{+/-}$	Dermis Cutaneous lymph node	Migratory DC
Interstitial DC $CD11b^{hi}$	$CD11c^{+}$ $CD103^{-/+}$ $CD11b^{hi}$	Intestine, Lung/BAL, Liver, Kidney, Pancreas	Migratory DC
Interstitial DC $CD11b^{lo}$	$CD11c^{+}$ $CD103^{-/+}$ $CD11b^{lo}$	Intestine, Lung/BAL Liver, Kidney, Pancreas	Migratory DC
TIP-DC	$CD11c^{int}$ Mac-3^{hi} $CD11b^{int}$ MHC-II^{+}	Spleen	Migratory DC
Human DC subsets			
Plasmacytoid DC	$CD11c^{-}$ BDCA-4^{+} BDCA-2^{+} $CD123^{hi}$ HLA-DR^{+}	Bone marrow, Blood, Spleen, Tonsil, Lymph node	Plasmacytoid DC
Myeloid DC BDCA-1+	$CD11c^{+}$ BDCA-3^{+} DNGR-1^{+} XCR-1^{+} HLA-DR^{+}	Bone marrow, Blood Spleen, Lymph node	Migratory DC
Myeloid DC BDCA-3+	$CD11c^{+}$ BDCA-3^{+} DNGR-1^{+} XCR-1^{+} HLA-DR^{+}	Bone marrow, Blood Spleen, Tonsil	Lymphoid resident DC or Migratory DC
Langerhans' Cell	$CD11c^{int}$ BDCA-1^{+} Langerin^{+} E-cadherin^{+} $CD1a^{+}$ HLA-DR^{+}	Epidermis Cutaneous lymph node	Migratory DC
Dermal DC $CD14^{+}$	$CD11c^{hi}$BDCA-1^{+} Langerin^{-} $CD14^{+}$ $CD1a^{-}$ HLA-DR^{+}	Dermis Cutaneous lymph node	Migratory DC
Dermal DC $CD1a^{+}$	$CD11c^{hi}$ BDCA-1^{+} Langerin^{-} $CD14^{-}$ $CD1a^{+}$ HLA-DR^{+}	Dermis Cutaneous lymph node	Migratory DC
Interstitial DC	$CD11c^{hi}$ BDCA-1^{+} HLA-DR^{+}	Peripheral tissues Lymph node	Migratory DC

B220: often used as a lineage marker for B-cells; **BDCA-**integral membrane proteins found on endothelial cells and specific DC subtypes; **CD1α:** glycoprotein expressed by APC and is associated in antigen presentation of lipid molecules; **CD11c**: Integrin alpha X and DC marker; **CD11b:** also known as macrophage-1 antigen and is a heterodimeric integrin $\alpha_M\beta_2$; **CD14:** co-receptor for TLR4 for the recognition of PAMP (LPS mainly); **CD103**: also known as integrin αE and is associated with adhesion and homing of cells to tissue compartments; **CD123:** an important receptor that binds IL-3. **DCIR-2**: PRR for PAMP recognition; **DNGR-1:** a C-type lectin 1 involved in PAMP recognition; **E-cadherin:** glycoprotein involved in calcium-dependent cell-cell adhesion; **F4/80:** lineage marker for macrophage populations although found to be expressed by other cell types; **HLA-DR**: MHC-II receptor expressed by the human leukocyte antigen complex; **Langerin:** type II transmembrane protein that binds to HIV-1 and a marker for Langerhans' cells; **MHC-II**: expressed by APC and lymphocytes and is the immediate receptor for antigen presentation; **XCR-1**: a chemokine receptor also known as GPR5 and is involved in calcium influx-mediated signalling.

REFERENCES: [55-59, 4, 60-62].

1.3.1 Conventional DC.

The term conventional DC (cDC) usually describes the main population of DC, however, cDC are a heterogeneous population of haematopoietic cells with phenotypic differences according to their specific location in lymphoid or connective tissues. For example in the thymus of uninfected mice, a large percentage of the cells express CD8α and CD205 and lack CD4 and CD11b expression whereas a small population of DC express CD205 and lack expression of all the other aforementioned markers [63]. In the spleen, three different DC subsets have been identified according to the expression of the T-cell markers CD4 and CD8: CD4- CD8+, CD4+ CD8- and CD4- CD8-. The specific function of T-cell markers on DC, remains unknown [53, 63]. However, under steady-state conditions, CD8+ cDC have been identified in the T-cell areas of the spleen and lymph nodes, hence this DC subset is believed to play a part in mediating peripheral tolerance by presenting self-antigens to nearby T-cells. In contrast, CD8- cDC are found in abundance in the spleen, but only form a minor population in lymph nodes. They appear to localize in the marginal zones in the steady state and migrate to the T-cell areas following microbial challenge (e.g. LPS) [64-66]. To complicate things even further, CD8+ cDC were observed to mediate T-cell priming both in herpes simplex viral and bacterial infections. The question is whether the same DC subtype performs dual roles of immunity and tolerance, or whether this is the work of two different CD8+ cDC subtypes [4]. In humans, the majority of studies have been performed on freshly isolated blood. Human DC lack CD8 expression, this observation poses difficulties in comparing directly human DC subtypes with their murine counterparts. On the few occasions where human DC have been isolated from spleen or tonsils and analysed for their expression of cell surface markers, it has been observed that most splenic DC resemble CD8+ cells [67, 68]. Although the different cDC subtypes all have the capacity to present alloantigen and activate T-cells, they appear to have differential impacts on the subsequent fate of the signal-receiving T-cell [69, 70]. Trying to classify the various DC subsets proves extremely difficult simply because different markers are continuously being identified and employed to characterize DC and different authors employ different sets of markers to characterize the various populations of DC. To the present day cDC are recognized by their expression of MHC class II and the expression of the integrin CD11c. These markers, however, are also found on other immune cells, which highlights the necessity for the lineage specific markers for cDC and DC in

general [71]. For the purposes of this study it is important to emphasize the presence of multiple DC subsets found both in lymphoid and non-lymphoid tissue compartments. Additionally, table 1.1 provides an overview of the different functionally distinct DC subsets found in human and mouse tissues. Several reviews summarize in detailed the functional and phenotypic plasticity of DC in humans and mice [53, 72].

1.3.2 Plasmacytoid DC.

Plasmacytoid DC (pDC) were initially identified in the blood and due to their peculiar morphology which resembles that of plasma cells, they were erroneously considered to be plasma T-cells or monocytes [73]. pDC are found scattered throughout different tissues including the thymus, blood, liver and lymphoid organs [74-76]. Their name describes their immature state, cells with a large cytoplasm and a distinct gene expression profile that lack the characteristic protruding dendrites and have a more spherical, plasma cell-like structure. However, once activated pDC develop into fully functional DC that can efficiently activate T-cells [54]. They are usually distinguished by a set of expression markers, the CD45 receptor isoform of 220kDa (B220), sialic acid binding Ig-like lectins (Siglec)-H and CD303 in human pDC. This pDC precursor begins its journey along the common DC developmental pathway in the bone marrow, but diverges during haematopoiesis under the influence of cell-lineage distinct transcriptional factors such as transcription factor (TCF)-4 [77-79]. They maintain high surface levels of MHC class II expression and lose their phagocytic properties [80]. Upon activation, pDC reorganize, develop dendritic processes, up-regulate expression of co-stimulatory molecules and are therefore equipped for T-cell activation, a fact that raises questions as to whether this cell type is a distinct ontogeny of DC [81]. Nevertheless, pDC alone have the unique ability to induce a characteristic burst of IFN-α and -β in response to the presence of non-replicating viruses [82, 65]

1.3.3 Monocytes.

Both human and murine monocytes have the capacity to differentiate into either macrophages or CD11c+ DC in the presence of monocyte-colony stimulating factor (M-CSF) or GM-CSF [83, 84]. They are generally considered as the main reservoir for replenishing tissue-resident DC. Two subsets of monocytes have been identified with slightly different profiles, known as 'patrolling' and inflammatory monocytes. Patrolling monocytes (CD16+ in humans or Ly6Clo in mice) localize in proximity to blood vessels, allowing their rapid recruitment to inflammatory sites. Inflammatory monocytes (CD14+ in humans or Ly6Chi in mice) arrive with a somewhat delayed kinetics at the site of inflammation and are thought to constitute the main inflammatory cell at the site, replenishing the existing population of DC. They acquire a DC phenotype and up-regulate expression of CD11c [85-87]. In general, monocytes are considered as the early precursor of DC. However, a few in vivo transfer studies of monocytes in irradiated mice failed to give rise to substantial numbers of DC; thus, the precise role of monocytes in the DC lineage is not quite clear [88-90].

1.3.4 Langerhans' cells.

LC were identified much earlier than DC, by Paul Langerhans' in 1868, however, they were falsely described as intraepidermal nerve cells. For almost a century, these cells constituted an enigma until their true function as sentinels of the immune system and potent APC, was uncovered [91]. LC possess long dendrites and align to form a semi-continuous, net-like structure in the epidermis in order to capture invading antigens in the skin [92, 93]. LC are bone marrow-derived specialized DC located in the basal and suprabasal layers of the epidermis. Due to their specific location they act as the first immune barrier to external pathogens. Similar LC-like cells can also be found in the stratified epithelium of the oesophagus, vagina, oral cavity, cornea and uterine cervix [94]. These stellate shaped DC of the skin make up about 2-4% of the total cellular content of the epidermis. However, many fundamental questions regarding the exact immunological function of these cells remain unanswered [94].

Being leukocytes, human and mouse LC express the common leukocyte CD45 marker, the myeloid marker CD33 and even stain for CD8 at low levels. They are also

characterized by expression of CD11c and a variety of adhesion molecules (β1 integrins, CD44, CD54, cutaneous lymphocyte associated protein, [CLA] and E-cadherin) [95]. For example, LC constitutively express E-cadherin, which serves as an anchor to neighbouring keratinocytes and also to interact with epithelial T-cells [96, 95]. Their antigen uptake machinery comprises of a number of C-type lectins (e.g.: type II lectin Langerin [CD207] or dectin-1, employed during glucan and zymosan uptake) [97, 98]. Murine LC also express high levels of DEC205, a type-I lectin, whereas human LC up-regulate the expression of this receptor during the later stages of maturation only. They also express both high and low-affinity receptors for IgE and IgE binding protein (EBP), which are necessary for allergen uptake [99].

The skin's sophisticated DC surveillance system for identifying foreign entities is more complex than originally thought. LC are not the prime cellular player in detecting xenobiotics. Langerin$^+$ dermal DC were originally falsely identified as migrating LC. Recent evidence indicates that a minority population of dermal DC are Langerin$^+$, which according to the results of Kissenpfennig and colleagues, is a novel distinct population of LC. [100]. They can competently replace LC in detecting xenobiotics, and migrate, carrying antigen, to the draining lymph node [101, 102, 99]. "Langerhans' cells are sufficient but not required", Streilein elegantly concluded two decades ago [103]. In fact LC have relatively slow migration kinetics compared to Langerin$^+$ dDC. [104]. Thus, three DC populations are identifies in the layers of the skin and are distinguished by the cell surface expression of Langerin and CD103, a receptor thought to influence the activity of T-regulatory cells [105]. LC stain positive for Langerin expression but not for CD103 and they reside in the epidermal layers of the skin [106]. The underlying layers of the dermis house two cDC populations that are classified into the Langerin+ CD103+ dermal DC population or a more heterogeneous population of cDC that express neither Langerin nor CD103 [107].

Immature LC are characterized by: a relatively low level of expression of MHC class I and II; expression of invariant chain Ii/CD74 and co-stimulatory molecules, as well as the possession of high number of intracellular Birbeck granules; a hallmark cytoplasmic, tennis-racquet shaped organelle of LC that was identified in 1961 whose expression is regulated by Langerin [108, 109]. Controversy continues to shadow the life cycle of these specialized epidermal DC. LC are a long-lived tissue specific DC

subtype that constitutively migrate to the draining lymph nodes with an approximate 50% turnover every two weeks [110]. In resting conditions, LC exhibit a comparatively slow turnover and the traditional view is that the LC population is replenished from bone marrow precursors [111]. A different school of thought suggests that LC can replicate *in situ* to replenish the LC cell population. Merad and colleagues illustrate that both scenarios are valid and occur under different conditions. During inflammatory responses, LC are replenished and the DC pool is expanded by circulating precursors in a C-C motif chemokine receptor (CCR)2-dependent manner. Under normal conditions, the LC population can be replenished via two processes, *in situ* replication or recruitment of circulating precursors [112, 111].

Investigations using skin grafts provide evidence of a slow steady LC turnover over the course of 15 days [113] and can even persist in the skin for up to a period of 5-8 months. Thus, LC could either possess a long life span or could be replenished through transdifferentiation of a dermal or epidermal precursor present in the skin [114]. Further studies revealed that LC migrate towards the draining lymph node even if they have not been stimulated, although this flow is increased dramatically in the presence of a 'potential threat' [115]. Kissenpfennig and colleagues provide confocal images of motile LC in the epidermis during steady-state conditions, which in accordance with the results of Kamath and colleagues provide substantial evidence that LC-derived DC residing in cutaneous lymph nodes are replenished by skin emigrants [116, 117]. In a highly inflamed skin, however, LC in the skin employ a different pathway to replenish their population. Recent evidence provided by Hacker and colleagues reveals that during development, LC turnover is dependent on the inhibitor of deoxyribonucleic (DNA) binding (Id2) and is absent in Id2 deficient mice. This is not the case in an inflammatory scenario, where LC turnover is not dependent on Id2 [118, 119]. Table 1.2 gives a general description of the various DC subsets identified in various tissue compartments in the mouse.

Table 1.2: *A summary of a widely accepted classification system for DC subsets* [120].

Categories of Dendritic Cells

Pre-Dendritic Cells (pre-DC):

- Do not acquire the 'classic' DC form – may have a distinctive phenotype
- Have the capacity to fully develop into DC following stimulation by an inflammatory or microbial signal
- Examples: monocytes and plasmacytoid DC

Conventional Dendritic Cells (cDC):

- They exhibit the 'classic' DC phenotype but are subcategorized into several groups
    - *Migratory DC*
        - They are the sentinels of peripheral tissues
        - Migrate to lymph nodes carrying antigens for presentation to T-cells
        - Examples: Langerhans' cells and dermal DC
    - *Lymphoid tissue resident DC*
        - Usually restricted within one lymphoid organ
        - Engulf and present foreign and self-antigen within that lymphoid organ
        - Do not migrate through the lymph
        - Further categorized into: (i) $CD4^{+}CD8^{-}$ cDC, (ii) $CD4^{-}CD8^{-}$ cDC, (iii) $CD4^{-}CD8^{+}$ cDC
        - Examples: splenic cDC and cDC of the thymus

Inflammatory Dendritic Cells:

- Not usually present under the steady state
- They appear during inflammatory conditions
- Examples: TNF- and inducible nitric-oxide synthase-producing DC (Tip-DC), inflammatory monocytes can give rise to inflammatory DC

1.4 Heterogeneity between macrophages and DC.

The similarities in the functional properties between macrophages and the newly identified DC were originally assumed to be mutually exclusive. Macrophages are known to populate all tissues and their primary role is to maintain tissue homeostasis as well as phagocytosis and degradation of foreign entities [121]. In contrast, DC are more focused in presenting antigen and regulating the development of immune responses. For this reason perhaps phenotypically the largest distinction between DC and macrophages lies in their endocytic compartments for processing antigen. DC have evolved a more sophisticated system that delays the degradation of up taken proteins in lysosomal compartments, which allows the cells to present antigens if required. It is certain that the two cell types operate in collaboration *in vivo* but a better understanding of the other distinct cellular functions that characterize the two cell types is required. [122, 17, 123, 5].

The mononuclear phagocyte system was becoming increasingly complex as more markers were identified [124]. With CD11c (a classic DC marker) shown to be expressed on macrophage subtypes the classic view of what constitutes a DC is changing. The original description of DC provided by Steinman and Cohn suggested that DC did not express the macrophage marker F4/80 or Fc receptors and that they do not possess the capacity to induce phagocytosis, or the ability to adhere to fibronectin [125]. The confusion arose when cells isolated from the lamina propria were classed as DC, due to their CD11c expression, but were shown also to express F4/80, CD115 (the M-CSF receptor 1) and thus resemble macrophages [126]. This is a general problem faced by scientists as markers like F4/80, CD11c, CD11b that were once thought to be cell specific have been shown to be expressed by both DC and macrophage populations. Conversely, it is argued that in specific locations such as the spleen and lymph nodes, $CD11c^{hi}$ expressing mononuclear phagocytes should be classified as DC. There is sufficient evidence to suggest that macrophages and DC localized in spleen and lymph nodes exhibit functional differences [127-129]. It is the plasticity of DC and macrophages in non-lymphoid compartments, such as the intestine and the lungs, where there is a high and continuous load of new antigen that requires processing that the rules regarding lineage-specific markers do not apply. A good example is the epidermal DC (LC), which are found to express the macrophage-specific marker F4/80, have a

macrophage-like gene-expression profile and have low migratory capacity when in the steady-state [112, 130, 131]. However, their specific location which is under continuous challenge with foreign antigen demands that LC undertake specific macrophages functions [132].

Some extreme views even state that DC are nothing more than a glorified macrophage. Based on their observations on gene expression of both macrophages and DC, David Hume and colleagues suggest that DC are much like localized liver Kupffer cells, and thus are tissue specific macrophages that localize in lymphoid tissues [133]. A more logical approach by Professor Simon Gordon uses the analogy of the fox and the hedgehog foretold by the Greek philosopher Archilocus to describe the differences between macrophages and DC: "The fox knows many things, the hedgehog knows one big thing". In this allegory the fox is the macrophage with many different functions that possibly overlap DC functions (i.e.: antigen presentation) and the hedgehog is the DC specialized to present antigen to T-cells in an efficient manner [5]. It is without a doubt that DC are a specialized and functionally distinct population of bone marrow-derived leukocytes. They constitute the sentinels of peripheral tissue compartments and are extremely efficient in their ability to capture, process and present antigens to T-cells engaging the adaptive arm of the immune system to the inflammatory response [2, 30, 134]. Both DC and macrophages play important roles in immune responses, both in inducing inflammation and also playing roles during tissue recovery. What is clear is that we need to improve our understanding of the various functions of all the DC and macrophage subsets in vivo is required for the success of modern immune therapies and vaccine technology [132]. So far most studies focus on the expression of cell surface marker on the different subsets of DC and macrophages.

Instead of focusing on the similarities between the two cell types attention needs to be directed in elucidating the functional differences of macrophages and DC. For example, cross presentation of antigen to T-cells is a unique feature of DC. Although some argue that macrophages also have the ability to cross-present alloantigens, they do so with very low efficiency [135]. Thus, apart from investigating the capacity of each cell type to perform a function, it is also important to question the efficiency by which it performs and how this is important. For example, it is important to characterize the functional capacity as well as the efficiency by which the two cell types produce and

release pro-inflammatory cytokines such as IL-1 and IL-6 [71]. While no one can dispute the importance of *in situ* analysis of the different DC and macrophage subsets, isolating and quantifying these cells is a tedious and very inefficient process, and is not without undesirable side effects. The ability to generate DC and macrophages *in vitro* with their full panoply of markers and the full range of subsets is still a matter of science fiction. Most *in vitro* studies employ bone marrow (BM) cells and the standard M-CSF or GM-CSF cocktail cytokines to generate large numbers of macrophages or DC. This system of *in vitro* generation of large numbers of DC and macrophages is invaluable to investigators, simply because of its efficiency and consistency.

1.5 Macrophages and their role in inflammatory responses.

The discovery of macrophages preceded that of DC by more than a century; Ellie Metchnikoff first identified the cells during the 1800s [136]. They populate all tissue compartments including the brain and are able to respond very rapidly to changes in the microenvironment. They are considered as non-migratory resident cells and are generally inefficient at antigen presentation. They are, however, efficient scavengers of pathogens, dead cells and cell debris and they acquire a high proteolytic and catabolic capacity upon activation. In the absence of inflammation, macrophages help regulate tissue homeostasis and are a source of anti-inflammatory cytokines such as IL-10 [137, 138].

The importance of macrophages as a cellular mediator of immunity is best reflected by the fact that they occupy strategic locations in different tissues in the early stages of embryonic development. They are generally categorized according to their tissue/organ residency, for example liver Kupffer cells, splenic red-pulp cells, microglia in the brain and alveolar macrophages. Tissue specific macrophages do not, however, represent distinct lineages. In fact, there are only two distinct macrophage lineages, which are differentiated by the level of expression of two cell surface markers: CD11b and F4/80. Macrophages derived from HSC-derived monocytes express high levels of CD11b and intermediate levels of F4/80 ($CD11b^{hi}F4/80^{int}$), whereas a separate population of 'primitive' macrophages develops from the yolk sac, before the HSC cells commit to that lineage and express $CD11b^{int}F4/80^{hi}$. The two populations differ in the expression

of certain genes, but are both dependent on the M-CSF as a signal for the development [139].

1.6 The Immunobiology and functions of macrophages.

Macrophages appear early during embryonic development and scatter throughout all tissues, probing for pathogens and orchestrating innate inflammatory responses. Together with other effector cells, including basophils, eosinophils and neutrophils, they make up the first line of defence against invading pathogens. The differentiation of macrophages depends on a set of signals provided by cytokines, such as M-CSF, GM-CSF, as well as TNF family proteins and PU.1 transcription factors. Macrophages derive from a common monocyte progenitor with myeloid DC that circulate the bloodstream and constitutively drain to all tissue compartments, possibly either by replenishing or enhancing the resident tissue macrophage population during inflammatory responses (reviewed in [140]). Macrophages are arguably one of the first cell populations that arrive at the site of inflammation, if not already present. They are also a plastic population of cells with a wide variety of effector functions and they appear to acquire a specific phenotype according to the presence of specific microbial and cytokine signals. Stimuli such as IFN-γ and LPS activate macrophages to produce pro-inflammatory agents such as IL-1, IL-6 and IL-8. These are usually referred to as classically activated M1 macrophages. It is being realized that anti-inflammatory molecules such as IL-4 and IL-10 act as more than just suppressors of inflammation driving macrophages along the alternative pathway of activation. These polarized macrophages are usually referred to as M2 macrophages [141, 142].

1.7 Classical and alternative activation of macrophages.

The classical pathway of macrophage activation was observed for the first time in *Listeria monocytogenes*-infected mice, in which macrophages exhibited enhanced antimicrobial activity in a non-antigen-specific manner [143]. Since then, studies have shown that activation of these classically-activated macrophages (M1) depends on a cocktail of TH1- and NK cell-derived cytokines, such as IL-12, IFN-γ and IL-18 [144]. In the presence of this cytokine cocktail, macrophages augment complement-dependent phagocytosis of pathogens, up-regulate the synthesis and release of toxic molecules and

pro-inflammatory cytokines and acquire the capacity to present antigen to T-cells. In vitro M1 pro-inflammatory macrophages are polarized using IFN-γ and LPS treatments. They acquire a pro-inflammatory profile secreting cytokines such as TNF-α, IL-6, and IL-1β [140, 145].

The concept of an alternative pathway of macrophage activation (aMΦ) was proposed much later, around the 1990s, and is associated with cytokines such as IL-4 and IL-13. However, the source of these cytokines that drive the alternative activation of macrophages is less well defined [136]. Other cytokines have also been shown to play part in differentiation of aMΦ macrophages including IL-10, TGF-β and even glucocorticoids. IL-4/IL-13 treatment of peritoneal macrophages represses the induction of pro-inflammatory cytokines, induces MHC class II expression and enhances macrophage mannose receptor activity [146-148]. In fact, it is thought that alternative activation of macrophages results in the up-regulation of a distinct phagocytic receptor repertoire, importantly, mannose receptor 1 (MRC-1) [149]. Expression of MRC-1 has been studied extensively on aMΦ and is able to interact with a number of microorganisms, such as *Candida albicans* and *Leishmania* [150]. Activation of the receptor is also associated with the up-regulation of anti-inflammatory cytokines such as IL-10 and IL-1RA and expression of the IL-1 type II receptor IL-1RII (an IL-1β decoy receptor) [151]. aMΦ appear to localize in the lung and placenta of healthy individuals, possibly acting as a buffer against unnecessary inflammatory responses [152]. They have also been identified in chronic inflammatory diseases such as psoriasis, rheumatoid arthritis (RA) and during the processes of wound healing [153], failing to produce agents like IL-1, TNF-α and IL-6 [154].

The level complexity of immune responses is probably best reflected by the diversity of two of the key cellular components, DC and macrophages, that drive the development of immune responses, which were described in previous sections. It is therefore necessary to improve our understanding of the different roles of the cellular components and their various subsets, of the immune system, how they regulate the availability of their inflammatory products and how they mediate their specific effector functions.

1.8 Dissecting the processes of cutaneous inflammation.

The skin, being the largest organ of the body, is also the largest interphase between pathogens and the immune system. The skin is also a dynamic organ that constantly needs to adapt to tissue homeostasis, maintain a protective shield against invading pathogens and also induce tolerance [7]. DC lie at the heart of this system conveying environmental cues to the adaptive arm of the immune system. However, sometimes this fine-tuned balance is perturbed, for example, when chemicals are misread as potential threats and lead to the development of allergic sensitization. The mechanistic processes that form the basis for a chemical to become an allergen are well understood. However, the development of predictive testing of potential chemical sensitizers is very complex and has always involved animal testing. In order to develop powerful skin sensitizing tests that are able to provide in depth information about the skin sensitizing potential and of the relative potency of the chemicals in question but also attempt at devising successful therapeutic strategies for various 'ailments' of the skin, we first need to dissect the various molecular events that lead to the development of allergic responses in the skin [99]. The *stratum corneum* is situated in the outer aspect of the epidermis and is composed of dead, keratin-filled, enucleated cells that constantly migrate from the viable epidermis [155]. Allergens need to cross the *stratum corneum,* and reach the viable epidermis if they are to cause a cutaneous immune response [8, 156]. Upon antigen formation and subsequent uptake, a sequence of events is triggered which leads to the migration of LC or other DC subsets of the skin towards the regional draining lymph node. During this migration, the antigen-bearing DC is subjected to several phenotypic changes, described in an earlier section that enables the cell to successfully present antigen to receptive T-cells (sensitisation phase) [157, 158]. The crucial event for the acquisition of skin sensitization is the activation of allergen-specific T-lymphocytes by sensitizer-bearing DC, which results in their immediate clonal expansion. These T-cells drive aggravated, enhanced immune responses upon re-exposure to the same sensitizer (elicitation phase). Both memory $CD8^+$ and $CD4_+$ T-cells are thought to play important roles in the full development contact allergic responses, although other cells are most likely also involved in the regulation and development of both the immediate (mainly IgE responses) and later (cell-mediated) effector responses against allergens [159, 160].

Critical to triggering LC migration are 2 independent stimuli, the chief pro-inflammatory pleiotropic cytokines, IL-1 and TNF-α [157]. The importance of this pair of cytokines is illustrated by the results of Cumberbatch and colleagues, who demonstrated that intradermal injections of recombinant IL-1β or TNF-α induced the migration of LC away from the epidermis towards the regional lymph nodes [161]. Following the detection of an invading allergen or other activating stimulus, secreted IL-1β is believed to perform the following 2 main functions: first, to stimulate LC via IL-1R1 (autocrine stimulus) and second, to signal adjacent keratinocytes (paracrine stimulus) to initiate the production of TNF-α [162]. These 2 cytokines are believed to act in parallel in order to cause the mobilization and migration of the LC population [161]. If the activity of either cytokine is blocked with neutralizing antibodies, LC migration is inhibited [163-165].

An important function of IL-1β and TNF-α is to alter the expression of molecules that play key roles in the migration and maturation of LC. It was later realised that IL-1α can also exert similar functions in inducing migration and maturation of LC. Under normal conditions, LC employ E-cadherin, a homophilic adhesion molecule to form adherent junctions with neighbouring keratinocytes, which helps to retain LC within the skin [166, 167]. In the event of contact with allergen, LC are instructed to migrate; in turn, they must detach from neighbouring keratinocytes and move through the tissue matrix, cross the basement membrane that separates the dermis from the epidermis and move towards the draining LN [162]. For example, it is speculated that TNF-α release down-regulates the expression of a series of adhesion molecules, such as E-cadherin in a dose and time-dependent manner [168, 169].

IL-1β and TNF-α production also induces changes in integrin expression, particularly that of α6 and β1 [170], which are important for LC migration through the laminin-rich basement membranes of the epidermis for binding various extracellular matrix (ECM) proteins (including collagen, laminin and fibronectin) respectively [91, 171]. TNF-α and IL-1β expression are believed to influence the production of matrix metalloproteinases (MMP) expression (proteolytic enzymes), such as MMP-9, which are also thought to help LC emigrate from the epidermis and navigate through the ECM [91, 172]. IL-1 constitutes a necessary signal for the initiation of cutaneous immune responses and the development of contact sensitization. It has been shown to be a driving force of LC

migration from the skin [173]. Developing our understanding of the various mechanisms that regulate the mechanisms of IL-1 synthesis and release in the skin we will be able to regulate the dysfunctional responses of skin inflammation that result in the pathogenesis of skin diseases such as atopic dermatitis and psoriasis. If the level of complexity of how the mechanisms of synthesis for a cytokine are regulated can be used as an index for the biological significance of that cytokine, the IL-1 is undoubtedly a cytokine of exceptional importance. Dermatologists have first encountered IL-1 as an epidermal-derived factor that activates thymocytes [174]. Although our understanding of the biological activity and the mechanisms regulating its synthesis have greatly improved over the years, we still have a long way to go to learn how to manipulate the effector functions of the cytokine.

1.9 The superfamily of interleukin-1.

Cytokines are often described as the soluble regulators of inflammatory processes, the lack of which renders the immune system disabled. The family of IL-1 molecules are key pro-inflammatory mediators and are very attractive targets for therapeutic intervention. The IL-1 family currently numbers 11 members, and consists of both pro- and anti-inflammatory molecules. IL-1 was described over 50 years ago as an endogenous pyrogen due to its acute fever-inducing abilities. It was later discovered that IL-1 consists of two isoforms, IL-1α and IL-1β [175]. Despite being the product of two separate genes, the two isoforms share high sequence homology. Structurally, the two molecules acquire a similar 3D structure comprising all β-pleated sheets. Both isoforms are transcribed as large precursor proteins that require cleavage to adopt their mature, biologically active, form [176]. However, IL-1α and IL-1β are two functionally different molecules that are differentially transcribed, translated, processed and secreted from the cell [177]. Even though both molecules require processing IL-1α is also biologically active in its precursor form. Also, whereas IL-1α is perceived as a mediator of local inflammation with important intracellular functions, IL-1β is a hormone-like molecule that is actively released in bulk from cells, inducing a sequence of systemic events that can impact on a large number of different cell types [178].

A third member of the IL-1 family was identified as the IL-1RI. This is a cell surface membrane bound receptor (80kDa) that upon ligation recruits the IL-1R accessory

protein (IL-1RacP), forming a multi-protein signalling complex [179]. The receptor consists of a single transmembrane domain and a cytoplasmic domain. In brief, this signalling protein complex induces activation of mitogen-activated protein (MAP) kinases and nuclear factor κB (NF-κB), engaging in gene transcription pathways [180]. The receptor is expressed on a variety of different cell types, including endothelial cells, fibroblasts, keratinocytes, epidermal DC and T-cells [181].

A second IL-1 receptor was later identified as type IL-1RII and is believed to act as a decoy receptor. IL-1RII is also cell bound, but has not been shown to form heteromeric complexes with IL-1RI [182]. This decoy receptor has high affinity for IL-1β; however, ligand binding fails to recruit the IL-1RacP and initiate signal transduction [183, 184]. Up-regulation of surface expression of IL-1RII negatively impacts the biological responses of IL-1. Finally, a third IL-1 ligand was later identified that antagonizes the two aforementioned IL-1 isoforms. IL-1 receptor antagonist (IL-1RA) is a naturally occurring competitive inhibitor of IL-1α/β. The same cells that express the other IL-1 ligands can also express this molecule, however, its exact role *in vivo* has not been entirely defined [185]. What is known is that once IL-1β is secreted from the cell, it has to compete with IL-1RA and the soluble form of the IL-1RII to activate IL-1RI [178].

1.9.1 The mighty pyrogen IL-1β.

IL-1β is undoubtedly one of the key players in most inflammatory processes and incidentally one of the best-characterized molecules of the IL-1 family. Patients with viral, parasitic or fungal infections are found to have elevated levels of IL-1β; however, IL-1β is also implicated in autoimmune diseases, such as multiple sclerosis, in neurodegenerative disorders such as Alzheimer's and even in type 2 diabetes [1, 178]. Most of what is known about IL-1β regulation is derived from studies performed using either macrophage cell lines or human blood monocytes. The promoter region of the IL-1β gene contains a TATA box, a classical motif for gene activation. From what has been observed, endotoxin activation (LPS) induces a transient transcription of IL-1β mRNA, which can persist for approximately 4h and is subject to a transcriptional repressor [186]. Two independent promoter regions (a 50bp and an 80bp fragment) have been identified in the IL-1β gene and are thought to collaborate in regulating its expression. The 50bp fragment contains a cyclic adenosine monophosphate (cAMP)

response element, and is believed to initiate IL-1β transcription following histamine or prostaglandin E_2 activation; on the other hand, the 80bp fragment contains an additional NF-κB-like site (NFIL)-6, and is responsive to stimuli such as LPS [187]. Expression of the IL-1β gene is induced by a wide variety of pro-inflammatory stimuli including all microbial products, viruses and cytokines such as TNF [188]. These are generally classified into PAMP molecules and DAMP molecules. Production and release of IL-1β is reviewed in a later section.

IL-1β is often described as a leaderless cytokine because, like IL-1α, it lacks that leader peptide which guides proteins through the endoplasmic reticulum during translation and mediates their release. As the potent pro-inflammatory cytokine, IL-1 transcription requires a multifaceted, sophisticated regulatory mechanism. Another common feature of IL-1 isoforms is that they have been found to localize in cell nuclei of cultured microglia. Unlike IL-1α, IL-1β is thought to enter nuclei via passive diffusion [189]; however, its intranuclear function remains elusive. The multifaceted mechanisms that control its production and secretion from inflammatory cells are reviewed in detail below.

1.9.2 Interleukin-1α.

IL-1α has undoubtedly been overshadowed by IL-1β, as little has been done to investigate the distinct functions of both the precursor and bioactive IL-1α molecule. The lack of interest is due to the early observations that both IL-1 isoforms bind the same receptor and the two isoforms were thought to mediate similar effects *in vivo* [190]. The two IL-1 isoforms have distinct expression and processing profiles [153, 191]. IL-1α belongs to the family of alarmins, a group of molecules with dual functions; they mediate their effects intracellular in an autocrine/intracrine fashion or as they are released in the extracellular milieu they are internalized by neighbouring cells and mediate their effects in a paracrine fashion [192]. The biosynthesis and secretion of IL-1α is similarly unique to that of IL-1β. Unlike others, but like IL-1β, however, it lacks the leader peptide that guides proteins along the endoplasmic reticulum and along the classical pathway of secretion. Instead, IL-1α is synthesized along microtubule networks in the cytosol [193, 194]. The IL-1α precursor molecule (pro-IL-1α) (31kDa), unlike the IL-1β isoform, is fully active, and when released, both the precursor and

active form of IL-1α can activate the membrane bound IL-1 receptor I (IL-1RI) with similar potency to IL-1β. *In vitro* studies have shown that molecules such as HSP and adenosine triphosphate (ATP) can activate calcium-dependent cysteine proteases known as calpains, which mediate proteolytic cleavage of the precursor and actively release the mature form of IL-1α (17kDa) [195-197]. The general view is that *in vivo* IL-1α is retained intracellular by acting in an autocrine/intracrine fashion and is mainly released in cases of severe disease [178]. This is based on the fact that high concentrations of neutralizing autoantibodies were found present in healthy individuals (5-28%), probably because as the cytokine is retained intracellular, it evades the normal processes of immunological tolerance [198, 199].

Whereas IL-1β appears to be predominantly expressed by cells of the haematopoietic lineage, IL-1α predominates in primarily keratinocytes, fibroblasts and epithelial cells, [200, 201]. In fact the skin has been noted to house significant pools of IL-1α *in situ* suggesting a significant role of the cytokine in skin inflammation [202]. Aside from its soluble form, IL-1α can also be found bound on the cell surface membrane of monocytes, B-cells and other cells. This only accounts for 5% of the total IL-1α protein synthesized by the cell. Transport and expression to the cell surface membrane involves myristoylation of the protein during translation, after which the protein is anchored to the cell surface membrane via interaction with lectin [203]. Suppressed inflammatory conditions are observed in conditions where the secreted form of IL-1α is absent in IL-1α KO mice implicating the cell surface bound molecules in promoting inflammation [204].

One important property of IL-1α is its nuclear localization. There are sufficient experimental data to support the concept that IL-1α targets are intracellular. Firstly, binding of either the precursor or mature form of IL-1α to IL-1RI results in the internalization of the complex (or simply the ligand), and is found to localize in the nucleus [205, 206]. Both precursor isoforms of IL-1 (31kDa) are small enough to pass through the nuclear pore complex (NPC) (50kDa gap), but IL-1α has been shown to contain a nuclear localization sequence (NLS) motif on the pro-piece of the molecule [207]. The exact function of the pro-IL-1α appears to differ between cell types, and is generally reported to play a role in cell migration, proliferation and apoptosis.

Administration of exogenous IL-1α neutralizing antibodies or IL-1RA fails to inhibit these effects, demonstrating that it is the intracellular pro-IL-1α molecule that mediates these effects. For example, in a human osteosarcoma cell line (SaOS-2), enhancing pro-IL-1α expression appears to prevent cell proliferation [208], whereas in fibroblasts derived from skin lesions of patients with systemic sclerosis, it promotes proliferation [209]. Pollock and colleagues investigated the role of the IL-1α pro-piece and report that the presence of high levels of the pro-piece shifts the balance of B-cell lymphoma (BCL) molecules that in turn activate caspase-3 and initiate the process of apoptosis [210]. Using IL-1α-deficient mice it was possible to demonstrate that IL-1α is also involved in the development of atherosclerosis, diet-induced weight gain and contact allergen-induced T-cell activation [204, 211]. Together with TNF-α and IL-1β, IL-1α has been associated with the development of contact sensitization by inducing migration of LC. In a similar manner to IL-1β, it is thought to be involved in LC migration by regulating E-cadherin junctions and the upregulation of chemokines such as CCR7 which are involved in the guiding the transmigration of LC through the layers of the skin towards the draining LN [166]. It is suggested that IL-1α is also involved in the activation of allergen-specific T-cells during the sensitization phase of allergic contact dermatitis [211]. In a different setting, the role of IL-1α in cell migration is rather controversial. McMahon and colleagues reported that transfection of the IL-1α pro-piece in human umbilical vein endothelial cells (HUVEC) down-regulates the migratory capacity of the cells in comparison to mature IL-1α–transfected HUVEC cells [212]. Conversely, transfection of the IL-1α- pro-piece in ECV309 cells (a spontaneously transformed HUVEC cell line) increases cell motility, which is antagonized by the intracellular form of IL-1RA [213]. The method of choice to assess cell migration could perhaps explain the differences in results.

1.9.3 The extended family of IL-1.

The family of IL-1 consists of a long list of 11+ cytokines with potent inflammatory capacity, however, much of the focus has so far been directed to the four main ligands, IL-1α, IL-1β, IL-18 and their natural inhibitor the IL-1RA. IL-18 was originally identified in mice challenged with *Propionibacterium acnes,* which led to the development of endotoxaemia and the rise of circulating levels of IL-18 [214]. It was initially named IFN-γ-inducing factor (IGIF), due to its capacity to induce IFN-γ production [215]. Interestingly, the cytokine shares a number of similar characteristics with IL-1β. Like IL-1β, IL-18 is transcribed as a biologically inactive precursor (23kDa), which requires activation by cysteine-aspartic acid protease (caspase)-1 [216]. The position of the gene was identified on chromosome 11 in humans and on chromosome 9 in mice. However, IL-18 synthesis and release does not appear to be regulated as strictly as IL-1β. The collective evidence suggests that IL-18 precursor molecules are transcribed and accumulate in the cytosol waiting to be secreted. Therefore, unlike IL-1β, IL-18 is only regulated at the level of activation and release. Pro-IL-18 molecules have been identified in a number of cells, such as Kupffer cells, chondrocytes, fibroblasts as well as macrophages, LC and keratinocytes [217-220]. The IL-18 receptor complex is surprisingly similar to that of the IL-1RI. The complex even employs the same adaptor molecules after activation, such as the myeloid differentiation primary response gene (88) (MyD88), TNF-receptor associated factor (TRAF)-6 and interleukin-1 receptor-associated kinase (IRAK); it even initiates the same intracellular cascades (NF-κB, p38 MAPK and c-jun N-terminal kinases [JNK]) [221].

IL-18 is a pleiotropic cytokine that is involved during the early stages of innate immune responses [222]. The exact functions of IL-18 have not been identified, but it is clear that the cytokine is a crucial component of the innate immune responses against intracellular pathogens and viruses. It is speculated that, where IL-18 acts upstream of IL-1β, TNF-α amplifies the immune response by inducing the expression of the aforementioned pro-inflammatory cytokines, as well as that of IFN-γ, GM-CSF and IL-18 [223]. Intradermal injections of IL-18 have been shown to promote LC migration towards the draining lymph node, responses which have also been observed with TNF-α and IL-1β. IL-18 induced LC mobilization is dependent on the availability of IL-1β and TNF-α. The exact mechanisms involved in IL-18-mediated LC migration are not

entirely understood, however, it is recognized that the cytokine is required element for the initiation and development of cutaneous immune responses. It is speculated that IL-18 is produced by allergen-stimulated keratinocytes to signal the release of TNF-α and IL-1β, which then drive LC migration and the development of sensitization to the specific allergen [224]. IL-18 has also been associated with skin conditions such as psoriasis since high levels of the cytokine found present in the serum and psoriatic scales of the skin [225]. The source of IL-18 in these conditions is not entirely clear. On one hand keratinocytes are known to constitutively produce IL-18 but are unable to process it because they lack caspase-1. On the other hand LC and DC populations of the skin can also produce IL-18 and incidentally possess caspase-1 molecules to process the cytokine for release [226, 227].

Like with IL-1β the mechanisms responsible for IL-18 release, however, remain largely undefined. Following experiments by Grobmyer and colleagues on healthy, LPS-injected, human subjects, it was concluded that a secondary signal, other than LPS, is required to increase the circulating levels of IL-18. It is speculated that a Caspase-1 activator is required to stimulate IL-18 release [228]. In accordance with the observations of Grobmyer and colleagues, the results of Seki and colleagues demonstrated that substantial levels of IL-18 are released from LPS-primed murine Kupffer cells in a caspase-1 dependent pathway [229]. The current notion is that IL-18 release follows a similar pathway to that of IL-1β. Treatment with apyrase or $P2X_7R$ inhibitors appears to prevent IL-18 maturation in primary human monocytes. Additionally, a number of danger signals and activators of the inflammasome, such as uric acid promote the release of the cytokine from cells indicating that IL-18 follows a similar if not the same pathway of release to IL-1β [230].

1.10 Regulating IL-1 production and release.

Being such a potent pro-inflammatory cytokine, it should not be surprising that IL-1β is regulated at so many levels. Subnanomolar concentrations are sufficient to evoke a systemic inflammatory response manifesting in fever, thrombocytosis, hypotension, leukocytosis and the production of other cytokines such as IL-6 [231]. It is clear that the cytokine holds a significant role in inflammation, so its production is carefully regulated

at multiple levels [178]. Several pro-inflammatory stimuli can induce activation of the IL-1α/β genes such as cellular injury, complement components and LPS. The different mechanisms responsible for the production and release of the molecule have only recently begun to be elucidated [175].

IL-1β mRNA is not constitutively expressed in any cell type. Some form of TLR ligand activation, such as LPS or muramyl dipeptide, is required to activate the transcription of IL-1β. Activation of PAMP, DAMP or nucleotide-binding domain leucine-rich repeat containing receptor (NLR) leads to rapid aggregation of the inflammasome complex. The inflammasome is a molecular scaffold of multicomplex proteins whose main function is to activate caspases in response to pathogen or stress/damage-associated stimuli. They are considered to be important regulators of the immune response and have been identified in several tissues, mainly epithelial cells lining mucosal surfaces and also immune cells. There are several different subtypes of inflammasome platforms that take their name from the molecules that activate them. NLRs are part of a family of intracellular PAMP receptors that consists of 22 different sensors in humans and 34 NLR genes in mice, including NLRP1, NLRP3 and NLRP12. The receptors are able to sense PAMP and DAMP floating in the intracellular compartment, using a leucine-rich repeat motif embedded in the C-terminal region. Upon activation, they are signalled to cleave inactive caspase molecules; to do so, they require a caspase recruitment domain (CARD), which is provided by a different protein complex, known as apoptosis-associated, a speck-like protein containing a CARD (ASC). The amino-terminal interacts with NLR receptor, whilst its carboxy-terminus recruits inactive caspase molecules for proteolytic cleavage [232-234].

To date, NLRP3 is one of the most-extensively studied inflammasome complexes. Unlike other inflammasome complexes, NLRP3 is not constitutively expressed in resting cells; instead, two signals are required to signal its transcription and translation [235]. Transcriptional induction of the inflammasome occurs downstream of TLR or nucleotide-binding oligomerization domain receptor (NOD) ligation mainly via the NF-κB pathway. Once primed, a plethora of secondary stimuli have been shown to activate inflammasome oligomerization including ATP, uric acid crystals, the potassium ionophore nigericin, the marine toxin maitotoxin, the antiviral imidazoquinolone

compounds R837 and R848 and aluminum adjuvant and even gut microbiota [236, 237]. However, the exact mechanism by which these compounds induce inflammasome activation remains a topic of controversy and it is important to decipher which stimuli induce transcription and which induce oligomerization of the protein. It is important to note that in most cells such murine macrophages, stimuli like muramyl dipeptide (MDP) and LPS can only prime the NLRP3 system, in human monocytes these stimuli are sufficient to induce oligomerization of the protein [236, 238]. One hypothesis postulates that ATP activates the $P2X_7$ receptor ($P2X_7R$), subsequently induces cell permeabilization and allows passage of microbial compounds that can directly activate the inflammasome [233]. In fact, it was thought that pannexin-1 hemichannels were involved in mediating passage of microbial compounds directly to the inflammasome since its activation is a necessary step for downstream caspase-1 activation [239]. Nevertheless, there is one problem with this hypothesis; $P2X_7R$ -induced pores can only allow passage of molecules up to 900 kDa, a pore perhaps too small for certain bacterial ligands [239]. A different school of thought proposes the K^+ efflux as a direct mediator of inflammasome activation, a common upstream signal for both NLRP1 and NLRP3 inflammasomes. Blocking K^+ efflux with a high extracellular K^+ concentration appears to inhibit activation of NLRP1 and NLRP3, but does not have the same effect with NLR-family CARD domain containing protein 4 (NLRC4) inflammasomes [240]. Alternatively, the pannexin-1 hemichannels and their recently identified splice isoforms have drawn considerable attention as possible candidates for direct inflammasome activators [241]. Inhibition of pannexin-1 with carbenoxolone inhibits caspase-1 activation and IL-1β release. The mechanisms mediated by pannexin-1 activation are currently not fully understood and therefore require further investigation [239].

Activation of the inflammasome complex results in proteolytic cleavage of pro-caspase-1 molecules, another level of regulation of IL-1β release. The activation of caspase-1 results in the proteolytic cleavage of pro-IL-1β molecules at position 116-117, between the aspartic acid-alanine positions and gives rise to the mature form 17kDa IL-1β molecule. Caspases are a family of cysteine proteases that possess the unique ability to cleave proteins at sites next to aspartic residues. Caspases are generally classified according to their main function, i.e.: caspase-1 is an inflammatory caspases whereas caspases-3 is an apoptotic caspase. Inflammatory caspases are involved in activating

cytokine precursors whereas apoptotic caspases are involved in regulating programmed cell death [35]. Apoptotic caspases are further subdivided into the initiator caspases, like caspase-1 and -11, and the executioner caspases, such as caspase-3 -6 and -7, depending at which point of the pathway of apoptosis they are involved. More importantly, evidence is continuously emerging suggesting that there is some form of 'cross-talk' between the two families of caspases. Caspase-1 deficient mice and macrophages are unable to secrete bioactive molecules of IL-1β and IL-18 [242, 216, 243]. However, in caspase-1 knockout (KO) mice, other proteases, such as granzyme A, are able to compensate for caspase-1 and induce IL-1β release. The levels of IL-1β release are comparatively lower and this compensatory mechanism has only been observed in neutrophils. What is not so clear is the level of contribution of non-caspase-dependent mechanisms of IL-1 processing and release [244, 245]. Alternatively, LC migration, which is dependent on IL-1 signalling (as well as TNF-α), is compromised in caspase-1 KO mice following challenge with contact allergens. These observations suggest that under these conditions caspase-1 appears to hold a key role in inducing IL-1 release and thereby regulating LC migration [246].

NLRP3 is not the only inflammasome platform that can mediate caspase-1 activation. For example, the NLRP1 inflammasome induces caspase-1 activation in response to the anthrax lethal toxin [247], whereas the NLRC4 inflammasome is activated upon intracellular infection with bacterial such as *Salmonella typhimurium* and *Legionella pneumophila* [248, 249]. In addition to IL-1β release, activation of NLRC4 induces programmed cell death known as pyroptosis. Pyroptosis is a process by which macrophages induce self-destruction to eliminate the invading pathogen, like *Salmonella typhimurium*, and at the same time mediate IL-1β release, raising a pro-inflammatory alarm. The released pathogens are then destroyed by the bactericidal mechanisms of neutrophils, which are recruited to the site [250, 251]. Thus, the mechanism of IL-1β release and thereby the levels of cytokine release may depend on the type of infection and the inflammasome subtype activated. What remains to be explored is whether cell specific inflammasome functions are in place *in vivo* and to identify the direct ligand that activates certain types of inflammasomes like the NLRP3.

1.11 The role of Autophagy in IL-1β production.

It is gradually becoming clear that autophagy is an important regulator of inflammasome function, and thereby IL-1β processing and release [252]. Autophagy was originally described as a catabolic process responsible for degrading cellular organelles during starvation. This is a fast-acting process that is able to sequester cytoplasmic organelles or proteins into a degradative 'autophagosome' within minutes. The autophagosome is very efficiently fused with a lysosome, in which the 'cargo' is hydrolysed before constituent monomeric units are recycled back to the cytoplasm for protein synthesis. Apart from being a degradative process, autophagy is considered an important regulatory mechanism of cellular homeostasis, a process whereby cells 'cleanse' their interiors [253].

Degradation of long-lived cytosolic proteins and organelles is a necessary process in order to maintain a continuous amino-acid turnover and other constituents for protein synthesis. There are 3 different types of autophagy classified according to how the substrate is delivered to the lysosome. Macroautophagy deals with the degradation and recycling of mitochondria, endoplasmic reticulum and generally other macromolecules. Chaperone-mediated autophagy is a 'selective' pathway, responsible for the degradation of cytosolic proteins [254]. Unlike macroautophagy, this type of autophagy does not require the formation of an intermediate vesicle. Instead, a cytosolic HSP (HSP70) recognises a motif (KFERQ) and tags proteins for lysosomal degradation. Finally, proteins can also be degraded by microautophagy, whereby a small portion of the cytoplasm is engulfed by a lysosome [255, 253].

The degradative processes of autophagy have been associated with immunity against intracellular pathogens such as *M. tuberculosis* [256] and *Listeria monocytogenes*, but also viruses such as *Herplex simplex virus* and protozoans like *Toxoplasma gondii* [257]. Microbes are ingested by autophagosomes and degraded by a fused autophagosome with a lysosome. Current investigations are focused in deciphering the signals that can potentiate autophagy, and how different molecular tags guide autophagosomes towards degradation in lysosomes. A connection between TLR signalling and autophagy has been uncovered by Delgado and colleagues in mouse

leukemic monocyte macrophage RAW 264.7 macrophages. It has also been observed that specific TLR receptors are more efficient activators of autophagy, something that may be attributed to the different downstream signalling cascades that they engage. For example, TLR7 is thought to be the strongest inducer of autophagy, compared to other TLR receptors investigated. TLR3 and 4 can also induce autophagy in murine macrophages [258, 259].

A different study has linked activation of the inflammasomes with the induction of autophagy, which thereafter regulates inflammasome activity by means of engulfing of inflammasome units. Blocking degradation of inflammasome units appears to enhance IL-1β production in macrophages. Also, known inflammasome recruiter signals appear to trigger Ras-like small G protein (RalB), which is a well-known activator of autophagosome formation. Down-regulating RalB expression also enhances IL-1β production. Although it is anticipated that the relationship between inflammasome activation and autophagy may be complex, it is important to unravel the various processes involved in the 'cross-talk' of these two mechanisms [252]

1.12 Mechanisms of IL-1β release.

Most proteins are synthesized as precursors containing a leader peptide. The leader peptide guides the proteins along the RER as they are synthesized during translation. A peptidase thereafter cleaves the leader signal, and the proteins are internalized in the lumen of the endoplasmic reticulum. Subsequently, the proteins acquire their tertiary structure, oligomerize and in some cases, even glycosylate in the endoplasmic reticulum [260] [261]. Following a strict quality control process, which removes incompletely folded or inappropriately glycosylated proteins, allowing the rest to be transported into the golgi apparatus; shuttle vesicles are slowly exported from the cell via a process known as exocytosis [262]. This method of protein secretion is known as the classical pathway and is responsible for the secretion of most proteins, such as TNF-α and IL-6.

Over the years, it has become apparent that for certain proteins, which lack a hydrophobic leader peptide like IL-1β, a different, less characterized, non-classical pathway is responsible for their secretion. In 1990, Rubartelli and colleagues employed two drugs, brefeldin A and monensin, to investigate whether IL-1β release follows the classical release pathway. Brefeldin A blocks the exit of proteins from the endoplasmic reticulum [263], and monensin inhibits transport of proteins along the golgi apparatus [264]. Whereas TNF-α and IL-6 release were inhibited in brefeldin and monensin-treated human monocytes, IL-1β release was unaffected, suggesting that this leaderless protein is release via non-classical pathway [193]. Figure 1.2 provides a schematic illustration of all the proposed mechanisms for IL-1β release.

To the present day, the mechanisms of IL-1β release remain elusive. IL-1β is a leaderless protein and several mechanisms have been proposed by which bioactive molecules of IL-1β could be exported from the cell. These include (a) via microvesicle release, (b) exocytosis of exosomes, (c) via cytolysis (leakage), (d) through specialized cell membrane transporters or (e) through exocytosis [265]. These mechanisms are considered in detail below.

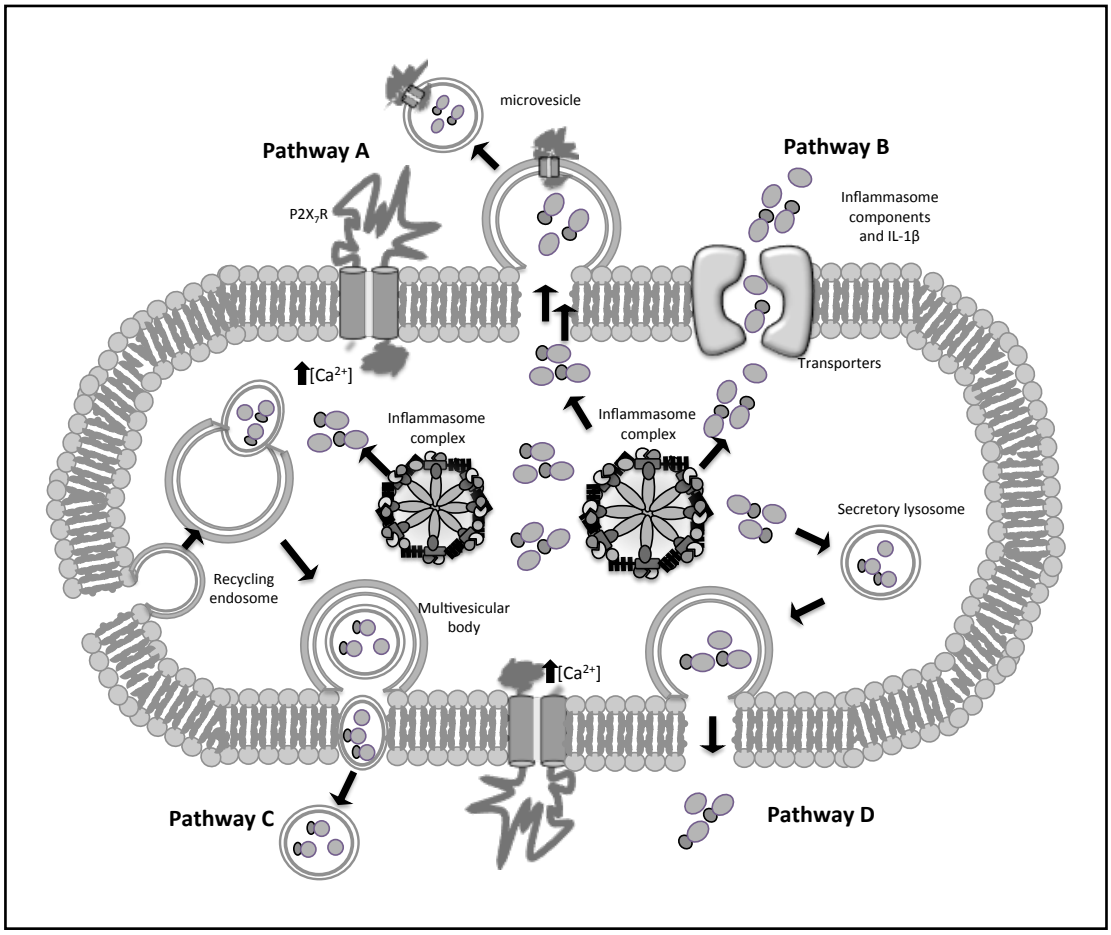


***Figure 1.2:* Mechanisms of IL-1β release.**
A schematic diagram of the various possible models for IL-1β secretion. **Pathway A**: Shedding of microvesicles. Both pro-IL-1β molecules and components of the inflammasome (Caspase-1 molecules) are driven into cell membrane formed and $P2X_7R$-expressing microvesicles. These are released into the extracellular milieu and lyse in the presence of high levels of ATP **Pathway B:** Secretion via cell membrane bound transporters. Specific cell surface membrane bound transporters recognise and transport active IL-1β molecules in the extracellular milieu. **Pathway C:** The secretion of IL-1β occurs via the formation of multivesicular bodies and the release of IL-1β-containing exosomes. **Pathway D:** IL-1β release via the secretion of lysosomes. The particular model proposes that IL-1β molecules are secreted from the cell via the lysosomal pathway [266].

1.12.1 Release of IL-1β through the microvesicles shedding.

It has been suggested that microvesicle shedding is the prevailing pathway of IL-1β release in murine microglia [267], human DC [268] and THP-1 monocytes [269]. Although this mechanism of IL-1β release was observed also in other cell types such as BM-MΦ [270, 271], it is believed that other pathways predominate to excrete IL-1β in murine BM-MΦ. Since membrane blebbing is an unequivocal downstream event of $P2X_7R$ activation, it was thought that it could contribute to IL-1β release in parallel with microvesicle shedding. However, the fine work of Verhoef and colleagues provide evidence of IL-1β release in murine macrophages treated with Rho-kinase membrane blebbing inhibitors, suggesting that IL-1β release and membrane blebbing are two distinct downstream pathways, at least in murine macrophages [272].

Microvesicles were isolated using either ultracentrifugation, or via positive selection using annexin-V-coated microbeads [268]. Whilst they were found to contain cytoplasmic content and plasma membrane components, they did not express any endosomal or lysosomal markers, thereby excluding the possibility that they were simply remnants of cell debris [269]. Their size can vary between 100nm-1μm, and they begin to form almost immediately following $P2X_7R$ activation. Their formation involves perturbation of the plasma membrane lipid bilayer, which begins to enfold and accumulate pro-IL-1β, bioactive IL-1β as well as caspase-1 molecules [268], forming microvesicles that are subsequently pinched off the plasma membrane [268, 273]. Since microvesicle formation was detected as soon as 5 minutes following $P2X_7R$ activation [269], and caspase-1 molecules were detected in isolated microvesicles, it is postulated that processing of IL-1β molecules into their active form must take place within the microvesicle during or after it has been released [267, 269, 268].

This theory of IL-1β release is somewhat incomplete, since there is no explanation of how IL-1β is released from the microvesicles into the extracellular compartment to exert its function. Based on their observations, Pizzirani and colleagues suggest that as microvesicles pinch off the cell membrane, they migrate towards sites of injury, which are rich in ATP. High levels of ATP activate the microvesicle-bound $P2X_7R$ inducing

releasing active IL-1β in a cytolytic manner [268]. Bianco and colleagues on the other hand propose an unidentified calcium-dependent mechanism is responsible for the release of bioactive IL-1β from the microvesicles [267].

1.12.2 IL-1β release via exocytosis of lysosomes.

The first evidence that exocytosis of lysosomes could be responsible for IL-1β release came from the work of Andrei and colleagues. Immunoelectron microscopy analyses colocalized pro-IL-1β and pro-caspase-1 molecules with lysosomal and endosomal membrane markers such as lysosomal-associated membrane protein 1 (LAMP-1) and cathepsin-D, suggesting that IL-1β is released enclosed in these organelles. Intriguingly, the kinetics of IL-1β release from human monocytes were very similar to that of cathepsin D; both molecules were identified in the extracellular medium 20 min after ATP challenge [274]. Additionally, the fine work of Carta and colleagues demonstrates how IL-1β secretion requires a functional microtubule network, which if disrupted or functionally impaired with nocodazole or taxol treatment, abrogates IL-1β release from human monocytes [275]. The same reduction in IL-1β secretion was observed when human monocytes were treated with histone deacetylase inhibitors responsible for hyperacetylation of tubulin. Tubulin is thought to be an integral part of vesicular transport and an essential component of mediating exocytosis of lysosomes in cells of haematopoietic origin [276]. Intriguingly, IL-1β release was not abrogated in N9 microglia cells treated with deacetylase inhibitors, since there is evidence to suggest that microvesicle shedding is the primary mediator of IL-1β release in these cells [275, 269]. Furthermore, Andrei and colleagues reveal that both lysosome exocytosis and IL-1β release are blocked from human monocytes following application of a calcium-dependent phospholipase A_2 inhibitor (AACOCF3) [277]. Rubartelli and colleagues go one step further and suggest that processing of IL-1β takes place within these lysosomes whilst these are trafficked towards the cell membrane, since no processed IL-1β was found in the cytosol of human monocytes [277]. On the other hand, Brough and colleagues report that IL-1β processing takes place entirely in the cytosol in mouse peritoneal macrophages, suggesting that different mechanisms of IL-1β release prevail in different cell types [270].

1.12.3 IL-β release via exocytosis of endosomes.

Multivesicular bodies (MVB) are usually responsible for the sequestration and degradation of proteins via the lysosomal pathway. Alternatively, MVB can fuse with the plasma membrane, inducing exocytosis of intraluminal vesicles (ILV) and are thereby referred to as exosomes [278]. Exosomes have been identified in a number of haematopoietic cells such as mast cells, DC, T-cells and platelets. Their formation is a strictly regulated process by which the 'cargo' is recognized before MVB are formed. However, the signalling pathway that stimulates the excretion of ILV into the extracellular milieu remains elusive. Immune-derived exosomes appear to carry all the necessary machinery required for antigen presentation such as MHC class II, adhesion molecules and co-stimulatory molecules [279, 280]. According to the maturation stage of the parent DC, these DC-derived exosomes have been shown to present MHC class II complexes to neighbouring DC and also to successfully present to and activate T-cells [281].

Exosome shedding was proposed as a possible mechanism of IL-1β release following the observations of Qu and colleagues in BM-MΦ. Both MHC class II molecules, and endosomal/lysosomal marker LAMP-1 molecules, were detected in ATP challenged BM-MΦ supernatants along with IL-1β. Nevertheless, these markers are not specific for exosomes and whether isolated BM-MΦ-derived exosomes contain IL-1β molecules remains to be seen. Interestingly, IL-1β release from BM-MΦ is evident both in the presence or absence of extracellular Ca^{2+}. Thus, since microvesicle shedding and lysosome exocytosis are both Ca^{2+}-dependent mechanisms, a separate mechanism must be responsible for IL-1β release from these cells [265, 282].

1.12.4 IL-1β release via cytolysis.

One of the most obvious pathways of IL-1β release would be simply the passive release of the molecule following cell lysis. High concentrations of ATP activate the $P2X_7R$ receptor and induce IL-1β release, but this also leads to the loss of membrane integrity, which could be the pathway of release of both pro- and mature IL-1β molecules. The fact that cytoplasmic lactate dehydrogenase (LDH) enzyme release follows a similar

pattern to that of IL-1β following ATP challenge in human monocytes is of interest; since LDH release is strongly associated with cell lysis, it is only natural to assume that IL-1β can simply passively diffuse out of cells as the cell membrane integrity is lost [283]. However, a number of studies have shown that, when using glycine to attenuate cell lysis, IL-1β release persists in human monocyte-derived DC and a murine macrophage cell line. Even though IL-1β release has been demonstrated via a cytolytic pathway, it is most likely a consequence rather than active 'pathway of choice' for the release of such a strictly regulated, potent pro-inflammatory cytokine [284, 272, 285].

1.12.5 Release of IL-1β via plasma membrane transporters.

Plasma membrane transporters are observed to translocate 'leaderless' proteins such as IL-1β, into the extracellular milieu in prokaryotes and eukaryotes. One such transporter is the ABC protein, whose knockdown ($ABCA1^{-/-}$) down-regulates the release of IL-1β from macrophages [286]. What is not clear is whether the ABC protein is the export machinery of IL-1β itself, or whether it regulates the intracellular concentration of ions, which in turn has an effect on IL-1β processing and release [265].

This mode of protein secretion is believed to be the prominent mechanism of IL-1β secretion in murine peritoneal macrophages, as demonstrated by the results of Brough and Rothwell. By the process of elimination, the group has employed different strategies to selectively inhibit or detect the processes of all other possible means of secretion, postulating that some form of protein transporter should be responsible for IL-1β in peritoneal macrophages. Thus, it is unlikely that IL-1β is released from peritoneal macrophages via lysosomal fusion with plasma membrane or the secretion of exosomes because: (a) neither the early endosome marker nor the late endosome/early lysosome marker, cathepsin D, were co-localized with IL-1β and (b) the signal for intracellular pro- and mature IL-1β was lost in digitonin-treated macrophages. Digitonin is a glycoside drug that binds to cholesterol and whilst permeabilizing the cell membrane, it allows for lysosomal cargo to be isolated intact [287]. When macrophages were treated with digitonin, it was not possible to detect intracellular pro- or active forms of IL-1β in isolated lysosomes, whereas cathepsin D (lysosomal marker) molecules were present, implying that IL-1β processing takes place in the cytosol, and

not in lysosomal bodies prior their release. Although there is lack of conclusive evidence, Brough and Rothwell postulate that since IL-1β processing takes place in the cytosol, then IL-1β cannot be released via microvesicle shedding [270]. The various aforementioned possible mechanisms of IL-1β release are depicted in figure 1.2.

Collectively, the data suggest that different cell types select different pathways to secrete pro-inflammatory cytokine such as IL-1β. Perhaps the capacity to engage a specific pathway of cytokine secretion is present in all cell types but different inflammatory conditions determine the route and therefore the volume of cytokine to be released from a cell. Although not much is known a with regards to DC and IL-1β release it appears that microvesicle shedding appears to be the method of choice for DC to release IL-1β [268] a method which arguably protects the cargo and also targets specific sites of release, sites rich in ATP required to lyse the vesicles. This, however, does not exclude the possibility that DC can engage other modes of cytokine release under different inflammatory conditions. What is particularly intriguing is the efficiency by which ATP signalling can induce externalisation of IL-1β from cells. ATP signals through the $P2X_7R$ and is one of the few potent physiological ligands that can activate the inflammasome through $P2X_7R$ activation [288]. Much of what we know with regards to ATP-mediated $P2X_7R$ inflammasome activation and cytokine release is derived from studies on monocytes and macrophages [283, 239]. Pro-inflammatory cytokines such as IL-1β hold an integral role in DC function and the role of $P2X_7R$ signalling in mediating pro-inflammatory cytokine release has only begun to be explored in these cells but from preliminary results $P2X_7R$ signalling appears to hold an important part in the IL-1β-driven development of ACD in mice [289].

1.13 Purinergic signalling.

ATP was first isolated in 1929 by Karl Lohman [290]. Shortly afterwards, Drury and Szent-Gyorgyi first proposed that adenine compounds act as extracellular transmitters in the heart [291]. These observations were neglected until the 1970s, when Khackh and Burnstock realised the importance of purinergic signalling and coined adenosine and pyrimidine as universal *extracellular* signalling molecules. Purinergic signalling is a primitive system found in green algae and has been conserved through evolution [292]. Although uridine di- (UDP) and triphosphate (UTP) have important roles in extracellular signalling, the unique properties of ATP have drawn considerable attention from the scientific community. ATP has a slightly negative charge under physiological conditions, ideal for binding positively charged moieties or proteins. ATP is typically found in packaged granules in nerve terminals of both adrenergic and cholinergic nerves; it is also found in cytosolic stores and is released following lysis of cells or other tissue injury. Additionally, platelets have been shown to contain platelet dense granules, which contain both ATP and serotonin. Finally, vascular endothelial cells can actively release ATP, via an as of yet unidentified mechanism. The concentrations of ATP released can attain millimolar levels between the intercellular spaces; however, the efficiency of ectonucleotidases in adjacent spaces can alter its biological effect [293]. The activity of these enzymes allows for a gradient of extracellular ATP that can increase by a 10^6-fold and activate purinoceptors. ATP is a by-product of inflammatory processes that plays an important part in the development of immune responses. However, various aspects of ATP signalling in the immune system are less well understood. ATP signalling is involved in a wide variety of cellular functions such as the release of histamines from mast cells, the production of prostaglandins and the release of cytokines from various different immune cell types [294]. The exact mechanisms involved remain obscure [295].

1.13.1 Purinergic receptors.

Purinergic receptors were first discovered in 1976; P1 purinoceptors respond to adenosine signalling but not ATP whereas P2 receptors that are activated predominantly by ATP, but ADP, UDP and UTP can also activate some P2 receptors [292, 296]. P1 purinergic receptors were characterized in 1989 [297] and are further classified into 4 subtypes, namely A_1, A_{2A}, A_{2B} and A_3. Like all G-protein coupled receptors, the purinoceptors contain 7 transmembrane domains with an amino-terminus protruding into the extracellular space and a carboxy-terminus protruding into the cytoplasmic side of the plasma membrane. P1 receptors couple to adenylate cyclase with A_1 and A_3 inhibiting adenylate cyclase and the two A_2 subtypes activating adenylate cyclase and inducing cAMP production [298]. The subfamily of P2 purinergic receptors was broadly categorized into 6 different phenotypes: P2X, P2Y, P2Z, P2U and P2T. In 1994, Abbracchio and Burnstock proposed the classification of P2 purinergic receptors into 2 broad families, according to their molecular structure and signal transduction mechanisms [299]. The ligand-gated ion channels were classified as P2X receptors, whereas the receptors that couple to heteromeric guanine nucleotide-binding proteins (G-protein) were named as P2Y. This nomenclature system is now widely accepted, with 7 subtypes identified as P2X receptors and 8 members making up the P2Y family of receptors [297]. The structure and downstream effects of purinergic receptor activation are illustrated in figure 1.3.

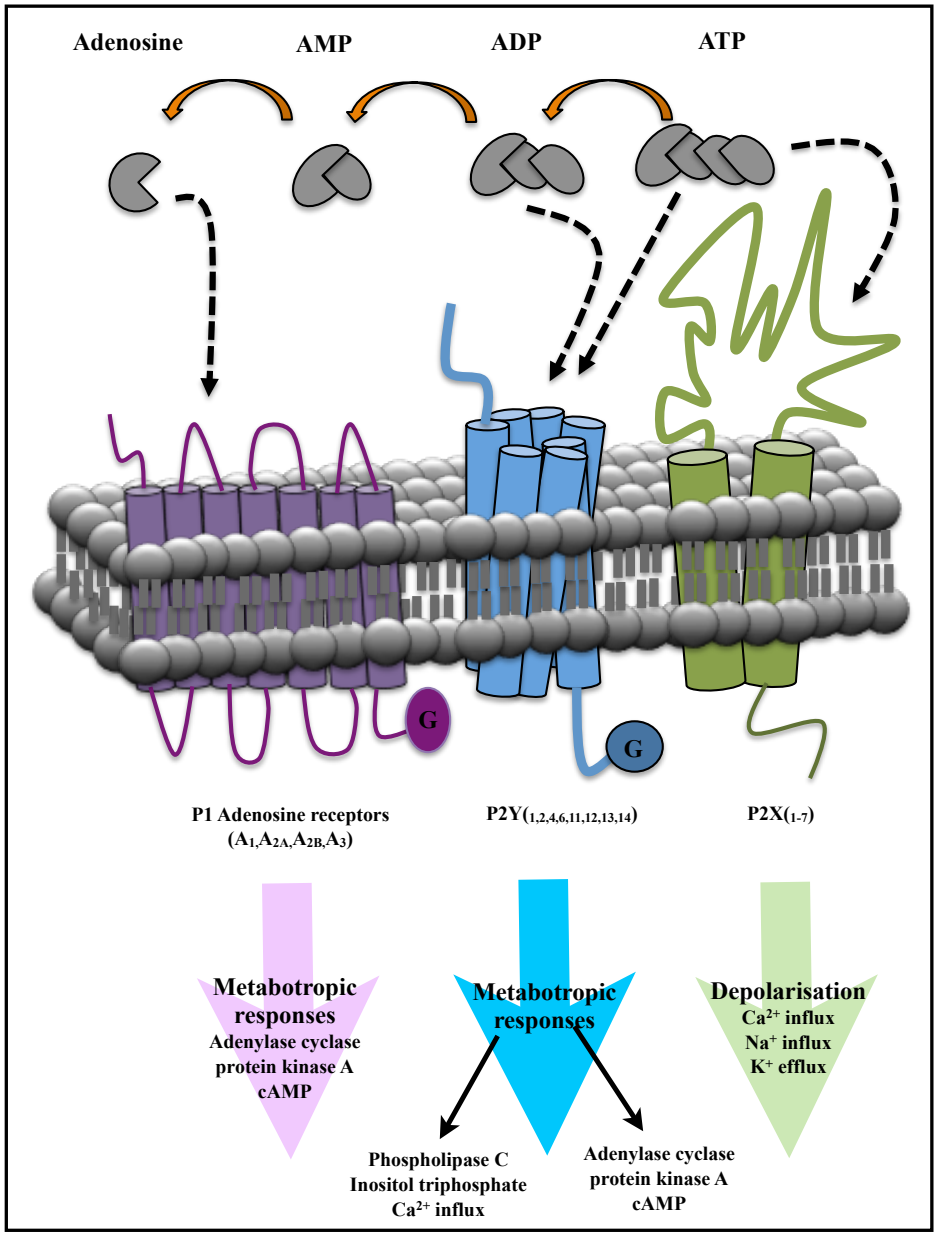


***Figure 1.3:* The superfamily of purinergic receptors.**
The P2X purinoceptors is a family of ionotropic channels whose activation results in the hyperpolasization (K+ efflux and Na+ and Ca2+) influx of the host cell. The P2Y family consists of G-protein coupled receptors whose activation results in the recruitment of phospholipase C and a rise of cytosolic calcium levels released from intracellular sources. Activation of P2Y receptors can also lead to recruitment of adenylase cyclase and its downstream signaling cascade. The P1 family of receptors consists of adenosine receptors that regulate the intracellular levels of cyclic AMP, cAMP. ATP is an agonist of both P2X and P2Y receptors but is also subject to ectonucleotidase enzymes that transiently degrade ATP to ADP, another agonist of P2Y receptors. Finally, Adenosine is the product of adenine nucleotide hydrolysis and is the activating ligand of P1 receptors. [300].

P2Y receptors were first cloned in 1993 and 8 subtypes have so far been isolated and cloned. Positively charged residue pockets in transmembrane (TM)3, 6 and 7 are believed to be important in agonist binding and activation of P2Y receptors [301]. In mammals 8 different P2Y receptors have so far been identified: P2Y1,2,4,6,11,12,13,14. The missing consecutive suffix numbers are either non-mammalian P2Y orthologues or new subtypes waiting to be identified in mammalian tissues. For example the P2Y8 receptor was cloned from embryonic amphibian tissue, which has been shown to be involved in the development of the neural tissue [302]. P2Y receptors are also classified into two less strict subtypes according to minor phylogenetic and structural differences. The protein sequence of P2Y1,2,4,6,11 receptors is quite distinct from P2Y12,13,14. Upon activation, P2Y receptors couple to either G-proteins activating cAMP production, or inositol triphosphate (IP3) second messenger systems. A striking feature of P2Y receptors is that different nucleotide agonists activate different P2Y receptor subtypes. For example, whereas ADP activates P2Y1, P2Y12 and P2Y13, the P2Y11 receptor has a high affinity for ATP. In the absence of specific inhibitors for each P2Y subtype it is proving quite challenging to investigate its exact role in various cellular process and intracellular signaling pathways or whether various P2Y and P2X receptor subtypes interact during a particular cell response. For example, the most commonly used P2Y blocker is a phospholipase C (PLC) inhibitor, U73122, which blocks intracellular calcium influx, a downstream effect of both P2Y and P2X signaling. Developing specific antagonists for P2Y subtypes is currently a hot topic amongst pharmaceutical companies [301, 303, 304]. In most cell types the library of P2 receptors extends to both P2X and P2Y receptors and what is important is that there is a bidirectional 'cross talk' between the purinoceptor subtypes.

The family of P2X receptors consists of 7 members ($P2X_1$- $P2X_7$) whose expression is widely distributed amongst all species of the vertebrate family [305]. Studying the expression of P2X receptors in the various classes of vertebrates reveals that expression of P2X receptors is not limited to the cell surface membrane, but can also be localized on intracellular sites as ion channels [306]. The 7 subunits of the P2X family share a substantial structural homology (30-50% sequence identity at the peptide level) [307]. P2X receptors consist of a large extracellular loop, 2 TM domains, an intracellular N and a C terminus. The size of the receptors ranges from 379 to 595 amino acids long

[308]. The extracellular loop contains 10 cysteine residues, which form disulphide bridges, and also houses the ligand-binding site situated at a hydrophobic H5 region, also known as the pore vestibule. The first TM (TM1) lines the intracellular N-terminus and is directly involved in channel gating whereas the second TM domain (TM2) is believed to form the ion pore and lines the C-terminus of the receptor. Receptor structure is depicted in a schematic diagram in figure 1.4. The receptors can be found in a stretched trimer form, or as a conjoined hexamer [309]. The pharmacology and functional aspects of the P2X receptors are thereby influenced by the stoichiometry of the trimer. Apart from the $P2X_7R$, all other P2X receptors can form functional heterotrimers in naturally occurring sites. To complicate things further, spliced variants of certain P2X subtypes can also influence the actions of ligand binding on the hetero/homotrimer [310, 311]. Interestingly, the loci of genes for P2X receptor subtypes, which share high amino acid sequence homology, are found clustered together on the same chromosome. For example, the genes for $P2X_4R$ and $P2X_7R$ receptors are located close together at the tip of the long arm of chromosome 12 [307] but vary in size and number of exons (11-13) [312].

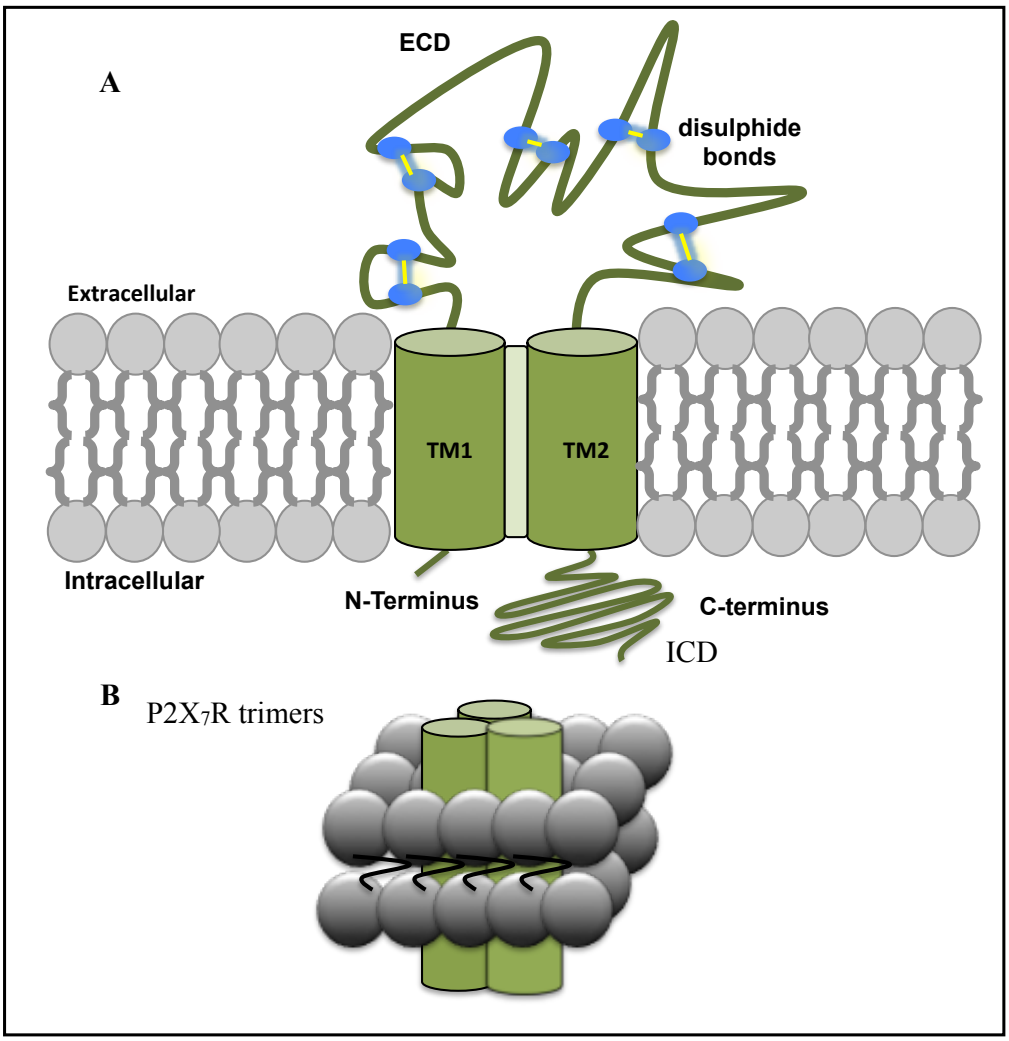


Figure 1.4: The structure of the P2X$_7$R.
A: The P2X$_7$ receptor comprises of two bulky transmembrane proteins, an extracellular domain (ECD) and a distinctive intracellular domain (ICD). The ECD is stabilized with 10 cysteine residues that form disulphide bonds between them. The ATP-binding sites are thought to be part of the 6-stranded β-pleated sheets that form part of the structure of the ECD. The distinctive feature of the receptor is its long (244 amino acid) cytoplasmic tail. The ICD is speculated to house a number of protein and lipid interaction motifs and plays an important role in the functional properties of the receptor. **B:** in its native form the receptor is found in a stretched trimer form. The P2X$_7$R is the only member of the P2X receptor that can only form homotrimers with other P2X$_7$R subunits, at least in its physiological form. The stoichiometry of the trimer impacts the function of the receptor [300].

P2X receptors have agonist binding sites (for ATP) widely termed as 'orthosteric sites' [313]. Early mutagenesis-based studies concluded that the ATP binding site is conserved within the members of the P2X family of receptors [314]. P2X receptors selectively bind ATP over other molecules such as the GTP or UTP. Binding of ATP on

these sites induces the appropriate conformational changes to allow opening of channels and ion flow. Application of the agonist (ATP) induces a rapid, strong inward current (activation phase) that slowly decays over time (desensitization phase); once the agonist is washed away, there is a rapid decay of inward current flow (deactivation phase). One of the striking differences between the various P2X receptors is their sensitivity to the agonist, and they exhibit different activation and desensitization profiles [315].

The kinetics of activation and rate desensitization is directly influenced by the concentration of ATP. The different P2XR have different supramaximal concentrations of ATP, which desensitize each receptor subtype. Whereas 10μM ATP is sufficient to desensitize $P2X_1R$ and $P2X_3R$, the $P2X_7R$ trimer requires at least a 10-fold higher (1 mM) concentration for desensitization. Additionally, the equivalent P2X receptors may have slightly different activation profiles in different mammalian species. For example, the rat $P2X_5R$ exhibits much lower current amplitudes in comparison with the human and chick $P2X_5R$ [316]. The same can be said about the $P2X_7R$, where human $P2X_7R$ homotrimers have a lower sensitivity to agonists than their rodent equivalents [317].

The $P2X_7R$ is increasingly implicated in various processes that unfold during inflammation. Unlike other P2X receptors, the $P2X_7R$ requires high levels of ATP to be activated. The level of cell surface expression of the $P2X_7R$ is increased by 5-fold as monocytes commit to the macrophage lineage and is generally found expressed at high levels in cells of the haematopoietic lineage such as microglia, macrophages and lymphocytes. Evidence suggests that the receptor regulates important cellular events during inflammation including the release of pro-inflammatory cytokines, paracrine signalling and recently with the differentiation of T-cells into a particular profile [318, 319]. The inflammatory properties of the receptor have attracted the attention of the scientific community, however, a more comprehensive understanding of the molecular events that are triggered by the $P2X_7R$ signalling during inflammation, is required [295]. Arguably, the receptor has distinct functional properties in different cell types and our knowledge of the physiological properties of the receptor derives largely from studies of macrophages or monocytes. More thorough characterization of the $P2X_7R$ properties in professional antigen cells (DC) is required.

1.14 The structurally and functionally distinct 7th member of the P2X family; the $P2X_7R$.

The various members of the P2X family of purinoceptors vary in their kinetics of activation, deactivation and inactivation. The $P2X_7R$ has a unique profile with regards to its pharmacokinetic properties. Perhaps the unique nature of the $P2X_7R$ in comparison with its sibling P2X receptors is best reflected by the fact that the very first observations of Khakh and Burnstock prompted the duo to classify the receptor in a completely different category, the P2Z [292]. The $P2X_7R$ was first isolated from the cDNA library of the rat and first cloned in human embryonic kidney (HEK-293) cells in 1996 by the Glaxo-Geneva group. The protein of the rat $P2X_7R$ structure has 35-40% homology with other P2X receptors, and is often described as a dolphin rising from the cell membrane [317]. Structurally, the $P2X_7R$ is composed of two hydrophobic transmembrane stretches (TM1 and TM2), an extracellular domain (ECD) and a long cytoplasmic tail. The extracellular domain forms a loop containing a sequence stretch of 170-330 amino acids, 10 cysteine residues that form disulphide bonds and 6-stranded β-pleated sheets. There is evidence to suggest that the ATP-binding site is situated within these β-pleated sheets of the ECD [320].

Finally, the long intracellular cytoplasmic C-terminal loop is the distinctive feature of the $P2X_7R$ and is arguably associated with its unique functional capacities. It contains an 18 amino acid cysteine-rich insertion that is distinct from other P2X receptors. The $P2X_7R$ interacts with numerous intracellular partners via the intracellular C-terminal loop. The loop contains multiple sites rich in protein and lipid interaction motifs, a structural platform ideal for activating several signalling cascades [316, 317]. For example, there is evidence for interaction with endothelial proteins [321], the cytoadherence accessory protein HMW3 of *Mycoplasma genitalium*, however, the most intriguing interaction is thought to be with the bacterial endotoxin LPS [322]. A conserved region situated at the 'tail' of the $P2X_7R$ is remarkably similar to the sequence motif of soluble LPS. Denlinger and colleagues designed a peptide identical to the sequence of the region in question and have shown that it successfully binds FITC-labelled LPS [323]. It is worth noting that destruction of the residues that allow LPS molecules to bind to this region of the receptor has a negative impact on receptor trafficking to the cell surface and also channel function, as assessed in $P2X_7R$-

transfected HEK-293 cells [323, 308]. A schematic diagram of the $P2X_7R$ structure is provided in Figure 1.4.

Although the exact gating properties of the $P2X_7R$ are not fully understood, it is clear that the receptor functions as a selective cation channel following brief agonist application. Sustained agonist application on the other hand results in a progressive pore opening, which allows passage of molecules up to ~800 Da (e.g.: the synthetic dye Quinolinium, 4-[3-methyl-2- (3H) -benzoxazolylidene) methyl] -1-[3-triethylammonio) propyl] di-iodide, YO-PRO). It was initially thought that the position of the pore was integral to the $P2X_7R$ channel [324, 325]; however, it was later revealed that this delayed permeabilization of cells to large molecular weight dyes is also evident to cells expressing $P2X_2$ and $P2X_4$ receptors [326, 327]. It is the unique profile of $P2X_7R$ sensitization that allows pore formation and activation of several signalling pathways. Sustained application of agonist at a sufficient concentration results in a progressive increase in current flow, with a comparatively slow deactivation rate. The $P2X_7R$ has the lowest sensitivity to ATP of all P2XRs, but is markedly more sensitive to the ATP analogue benzoylbenzoylATP (BzATP) [328, 329].

Apart from exogenous application of ATP, emerging evidence suggests that other endogenous ligands can activate the $P2X_7R$. A potent antimicrobial peptide, LL-37, released by neutrophils and epithelial cells, was reported to induce $P2X_7R$-dependent and ATP-independent IL-1β release [330, 331]. LL-37 is the product of proteolytic cleavage of the human cathelicidin protein (hCAP)-18, and is significantly upregulated during inflammation [332]. Tomasinsig and colleagues have shown that LL-37 can induce Ca^{2+} and ethidium bromide influx in $hP2X_7R$, and potentiate Bz-ATP-induced Ca^{2+} influx and pore formation. More importantly, LL-37 induces pore formation in a C-terminus truncated form of the $hP2X_7R$ contradicting the general view that the C-terminus regulates pore formation. The hypothesis is that LL-37 might act as a linker-protein between the receptor and the downstream signalling cascade activating pore formation [333]. Additionally, the fact that LL-37 seems to potentiate $P2X_7R$ current flow and pore formation suggests that *in vivo* the molecule might collaborate with ATP to fully activate $P2X_7R$ in the absence of the ATP at the concentrations that are required to activate the receptor. The exact mode of interaction between the LL-37 and the receptor remains elusive, and other unidentified endogenous molecules could activate

the receptor *in vivo* also [334]. Tenidap, a cytokine modulating anti-inflammatory drug, for example has been shown to block IL-1β release from macrophages, but further work is required to decipher whether the molecule is a direct ligand of the receptor [335].

1.14.1 Tissue distribution of the $P2X_7R$.

Initially, it was thought that $P2X_7R$ expression was restricted to cells of the haematopoietic lineages. However, more recent evidence suggests that $P2X_7R$ has a more ubiquitous distribution of expression, albeit with varying orders of magnitude in levels of expression [336]. It is this ubiquitous distribution of receptor expression that has intrigued scientists regarding the functions in which the receptor might be involved [337].

Even though there have been considerable advances in the field of purinergic signalling, the truth is that the field is limited by the lack of P2X-specific and species specific 'tools' available to investigate the seventh member of the P2X family [338, 339, 308]. From what is known, in descending order, the levels of $P2X_7R$ expression in human haematopoietic cells is as follows: macrophages > DC > monocytes > natural killer cells > B-cells > T-lymphocytes > and erythrocytes. These differences in the levels of surface $P2X_7R$ expression could simply be accounted for by the difference in size and cell surface area between the larger monocytes and much smaller lymphocyte populations [340]. It is worth noting that the same is not reflected in canine haematopoietic cells and most probably in other mammalian species [341]. Our knowledge regarding the density for the mouse $P2X_7R$ orthologues is limited. The work of Persechini who based the density of expression of the receptor on the level of TO-PRO-1 uptake concluded that monocytes have higher density of expression for the receptor in comparison with T-lymphocytes and NK cells [339]. Sluyter and colleagues have recently identified expression of functional $P2X_7R$ in mouse keratinocytes and LC by immunoblotting and immunofluorescence staining [342]. The same group have previously reported the presence of functional receptors in human keratinocytes and monocyte derived LC [343]. Mast cells and eosinophils have also been shown to express $P2X_7R$, but the receptor was absent in neutrophils. Like all cells of haematopoietic origin, high levels of $P2X_7R$ were detected in intracellular locations of neutrophils and it is postulated that

these reserves are recruited to the surface upon activation of the cells. What is not clear is whether cells with cell surface expression of the receptor internalize the receptor following agonist binding, and whether it is later are recycled or simply replaced [340].

Collo and colleagues first reported in 1997 the presence of $P2X_7R$ mRNA in rat and mouse microglia and ependymal cells but not in peripheral or central neurons [316]. This was in accordance with earlier observations of Surprenant and colleagues that microglia respond to ATP challenges with an inward cation-selective current [239]. Later studies by a number of groups caused controversy in the field claiming that functional $P2X_7R$ receptors are present in neurons of the hippocampus and could modulate transmission at synaptic junctions [344, 345] These results, however, were inconsistent, and hence the expression of the $P2X_7R$ on neuronal subpopulations became an ongoing topic of controversy until a more careful approach of Sim and colleagues employed a number of different antibodies (with different antigenic specificities) and attempted to decipher whether or not a functional $P2X_7R$ is expressed in neurons of a normal adult rodent. In accordance with early observations, the group could not provide any evidence of $P2X_7R$ expression in neurons of the rodent brain, and questioned the specificity of the antibodies employed by previous investigators claiming $P2X_7R$ receptor expression in neurons [346].

$P2X_7R$ receptor expression has also been confirmed in glomerular mesangial cells of the kidney [347], reproductive and urinary tracts, exocrine glands and cells of the skin. Little is known of the physiological function of the $P2X_7R$ on epithelial cells, but it is thought the receptor plays an important role in mediating host defence against intracellular pathogens, such as *Chlamydia sp*., since individuals with a loss-of-function polymorphism of the $P2X_7R$ are more susceptible to such infections [343]. Additionally, P2X receptors have the ability to form both hetero- and homotrimers, which varies in both *in vivo* and *in vitro* conditions and also between different cell types. To complicate things even further, the main agonist of $P2X_7R$, ATP, once released, is quickly and efficiently degraded into adenosine di- (ADP) and mono-phosphate (AMP), by a number of ectonucleotidases, molecules which potently activate P2Y receptors, triggering downstream effects that are often mistaken for direct P2X mediated effects [348].

1.14.2 Polymorphisms and splice variant expression of the $P2X_7R$ gene.

The human $P2X_7R$ gene is located in chromosome 12 at position 12q24. Initiation of transcription has been identified at 91 nucleotides upstream of exon 1, whereas the active promoter region has been located between 249 and 59 nucleotides upstream of exon 1 [349]. Importantly, several targets for microRNAs have been identified, including miR-150, miR-186, which are thought to have the capacity of regulating receptor expression [350]. Certainly, other mechanisms such as hypermethylation can influence receptor expression.

A number of splice isoforms of the $P2X_7R$ have been recently characterized, which result from alternative splicing of the human P2XR7 gene. The full-length $P2X_7R$ receptor has been termed as P2X7A, encoding 595 amino acids whilst the various splice variants were designated with a subsequent Latin character i.e. P2X7B [340]. P2X7B lacks the last 171 amino acids, instead containing a different 18 amino acid sequence to the full length protein, which is a result of a new stop codon found within the intron between exon 10 and 11. P2X7C lacks exon 4, P2X7D lacks exon 5, P2X7E lacks exons 7 and 8 and P2X7F lack exons 4 and 8. All these variants have parts of the extracellular domain of the receptor missing. A number of the splice variants including P2X7B, P2X7C, P2X7E and P2X7G have a shorter carboxy intracellular terminus, whereas 2 (P2X7G and P2X7H) have an incomplete first transmembrane domain [351]. A schematic diagram of the human splice isoforms of the $P2X_7R$ is provided in Figure 1.5.

Of all the splice isoforms, P2X7B and P2X7H have been studied most extensively in human cells. Although the different isoforms have yet to be compared at the protein level, P2X7B mRNA is expressed at comparatively higher levels than is P2X7A and it is believed that both isoforms have equal tissue distribution [352]. Functionally, P2X7B differs from P2X7A in its ability to activate pore formation but also caspase -3 and -7. Isoform B forms heteromers with P2X7A and P2X7A/B signalling has been shown to increase intracellular Ca^{2+} and ATP levels which activate the nuclear factor of activated

T-cells, cytoplasmic 1 (NFATc1) pathway leading to cell growth [353]. P2X7H on the other hand, appears to be non-functional, with low levels of mRNA expression [352].

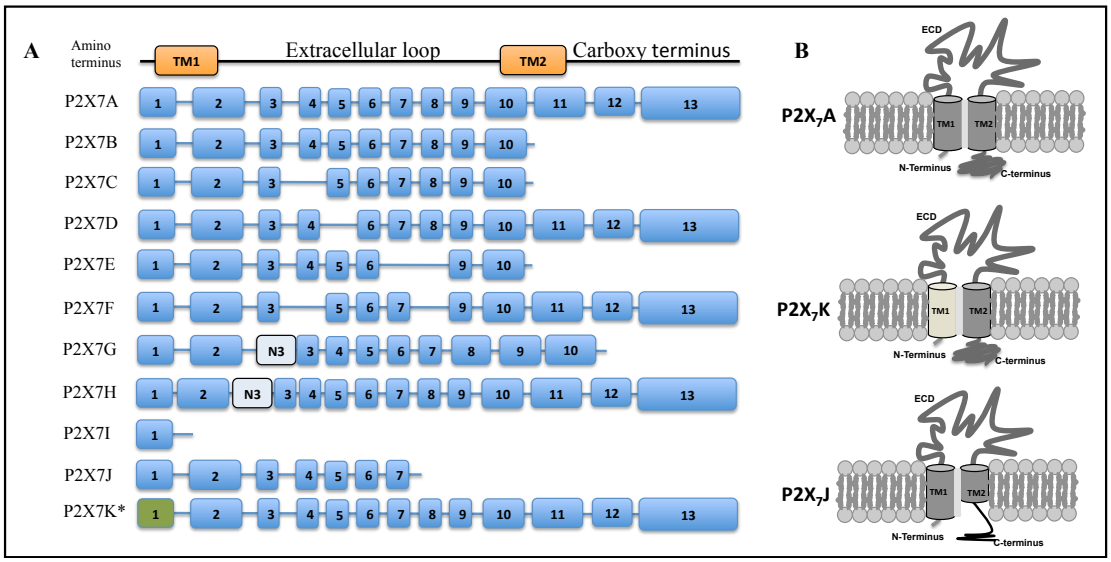


Figure 1.5: The splice variant isoforms of the P2X$_7$R.
A: The schematic diagram on the top illustrates the various segments of the P2X7R structure and the figure is aligned to the to illustrate which exon domains (represented by the numbered boxes) encode the particular segments of the receptor. The schematic diagrams below illustrate the various coding regions (exons) that make up the different P2X$_7$R splice variants (A-K). Additional exons present in the intron are represented with N3. This additional exon in P2X7G and H introduces a new start codon that results in the deletion of the first transmembrane protein. The P2X7K has an alternative exon 1 (illustrated in green) and currently there is no evidence for the presence of this splice exon variant in human tissues. **B**: A schematic diagram of the structure of three splice variants with distinct functional properties. P2X7A is the full functional splice variant. P2X7K has been shown to exhibit enhanced functional properties in comparison to the full variant whilst P2X7J is a truncated splice variant with limited, if any, functional properties [351].

Feng and colleagues have described a non-functional variant present in both normal and malignant cells of the cervix. P2X7J is co-expressed with P2X$_7$A forming non-functional heteromeric receptors. It is believed that in doing so, malignant cells are able to avoid ATP-induced apoptosis [354]. Our understanding of the polymorphisms of the mouse P2X$_7$R is limited since most studies have focused on the various polymorphisms of the human P2X$_7$R gene and their consequent effect on receptor function. However, one variant which exhibits enhanced functional properties has been identified in cells of the spleen. Its expression in human tissue remains to be verified. P2X7K carries an additional exon present at the intron between exons 1 and 2, which is thought to

enhance the sensitivity of the receptor to its agonists and potentiating pore formation [355]. Masin and colleagues have recently reported the expression of two distinct splice isoforms of the mouse $P2X_7R$. Both isoforms use different 13 exons have therefore been termed P2X713B and 13C. P2X713B has a shorter C-terminus whilst the 13C isoform has an additional 11 amino acids attached to the C-terminal domain. A neomycin cassette was introduced at exon 13 to disrupt the P2XR7 gene in mice and create the Pfizer $P2X_7R$ KO mice model. There is evidence that hints towards the presence of a functional $P2X_7R$ in Pfizer KO mice. Functional analysis of the P2X713B transfected in HEK-293 cells and co-expressed with P2X7A receptors reveals a dominant negative effect on the expression and function of the receptor with very small currents [356]. Previous investigations have also reported the presence of $P2X_7R$ in the brain of P2X7KO mice. Western blot analysis revealed the presence of a 65 and 77kDa $P2X_7R$ protein [357]. Further studies of the salivary glands of derived from Pfizer KO mice reveal residual ATP-induced responses associated with $P2X_7R$ activation (calcium influx and salivary secretions), functions that could be credited to P2X713B and/or 13C signalling [358, 359]. Splice variant expression is not a unique feature of the $P2X_7R$. Splice variants have been identified for $P2X_1$ $P2X_2$ and $P2X_4$ receptors. The $P2X_1$ splice variant was identified in cells of the bladder and lacks part of its second TM domain [360]. $P2X_2$ has several splice variants some of which alter the desensitisation properties of the channel [361]. The murine $P2X_4$ splice variant lacks part of the extracellular loop (27aa) that renders the receptor unable to form heteromers with the full variant [311].

According to the NCBI database, the human $P2RX_7$ gene hosts 686 single nucleotide polymorphisms (SNP) [362]. Only 28 SNP are non-synonymous, and 16 of these have been characterized for phenotype. Out of all SNP, the Glu496Ala (rs3751143) loss-of–function $P2X_7R$ polymorphism has attracted the most attention. Along with a few other loss-of-function SNP, like rs3533845, rs28360457 and rs2230911, they appear to alter ATP binding and impact on the ability of macrophages to kill mycobacteria and toxoplasma [363, 364]. On the other hand, a number of gain-of-function polymorphisms like rs208294, rs1718119 and rs7958311 appear to enhance ATP-driven IL-1β release [365]. The mechanisms by which the various SNP alter $P2X_7R$ function remain unclear [366]; however, amino acid substitutions found in the extracellular domain, [367] of the receptor, like rs28360457 are thought to impact on ligand binding. SNP found either on

the transmembrane domains or the intracellular C-terminus of the receptor modify current flow and or pore formation [368, 369, 365]. Finally, rs1653624 SNP has been reported to reduce trafficking of the $P2X_7R$ to the plasma membrane in human lymphocytes, which results in a loss-of-function polymorphism [370].

The association of $P2X_7R$ SNP and development of disease has been examined in a number of studies. The rs3751143 polymorphism has been associated with tuberculosis. It is most likely that the loss-of-function polymorphism impacts on IL-1β release from monocytes, affecting their ability to kill intracellular mycobacteria [371, 363, 372]. The specific polymorphism has also been associated with chronic lymphocytic leukemia (CLL) and this is possibly due to the impaired ability of lymphocytes to initiate apoptosis [373, 374]. Another SNP, rs2230912, has been associated with increased susceptibility to bipolar or unipolar disorders. Once again, this is thought to be due to increased circulating IL-1β, which could be responsible for alternating moods [365].

1.14.3 $P2X_7R$, pore formation and pannexin hemichannels.

Scientists are truly bewildered by the unique molecular complexity of this ion channel and how rapidly it converts from an ion channel to an enlarged pore. This is one of the striking features that separate the $P2X_7R$ receptor from its sibling P2X receptors. The receptor has the unique ability to induce cell permeabilization by activating the progressive opening of a non-selective large pore and allowing the rapid uptake of molecules with large molecular weight (>400Da in macrophages/DC and <400Da in lymphocytes) [375, 376, 307]. The relationship between $P2X_7R$ receptor activation and pore formation is not as straightforward as was initially thought. Originally, the hypothesis was that a sustained activation of the $P2X_7R$ led to the progressive dilatation of the channel itself [307]. Later on, however, a different hypothesis was postulated whereby the $P2X_7R$ activated a second non-selective pathway most probably mediated by a group of proteins known as pannexins [239].

The family of pannexin proteins consists of three members, namely Panx1, Panx2 and Panx3. The family is often associated with the vertebrate gap junction proteins, the connexins and innexins. Pannexins are structurally different from other gap junction proteins and are made of six subunits, which merge to form a pore complex. Panx1 has

the unique ability to form non-junctional channels, known as pannexons. These are voltage sensitive and are regulated by intracellular levels of calcium. The pannexin-1 gene is subject to alternative splicing, with three isoforms having being identified (Panx1a, b, bv). The expression and precise function of these isoforms remain unknown, but the fully functional Panx1a isoform is thought to be responsible for (a) ATP release from murine erythrocytes, T-cells, astrocytes and airway epithelia [377-381] and (b) engaging with inflammasome activation, to participate in programmed cell death and P2X and P2Y signalling [382].

The hypothesis that pannexin-1 is the $P2X_7R$ 'pore' was postulated by Pelegrin and Surprenant, who first observed that dye uptake was significantly reduced in ATP-challenged THP-1 monocytes, and when $P2X_7R$ -transfected HEK-293 cells were treated with a potent pannexin-1 channel inhibitor (carbenoxolone). A similar effect was observed when other pannexin-1 channel inhibitors were employed, including mefloquine and probenecid [239]. However, pannexin-1 blockers have inhibitory effects on other connexin channels, thereby making it difficult to designate pannexin-1 as the $P2X_7R$ 'pore'. Additionally, Schachter and colleagues report that no significant inhibition of dye uptake was detected in macrophages and $P2X_7R$-transfected HEK-293 cells treated with pannexin-1 blockers, in contrast with the earlier observations of Pelegrin and Surprenant [383]. Pelegrin and colleagues, however, argue that these observations could simply be due to the method employed. Pannexin-1 blockers inhibit the initial $P2X_7R$-induced dye uptake; given time, the inward diffusion of dye is inevitable, hence if dye uptake is quantified at a delayed time interval, the results could be misleading [300, 12, 383]. The findings of Schachter and colleagues are strengthened by the observations of Qu and colleagues who have employed pannexin-1 knockout mice to investigate the role of pannexin-1 in $P2X_7R$ -induced pore formation. In accordance with the results of Schachter and colleagues, dye uptake was neither inhibited, nor down-regulated in bone marrow derived macrophages (BM-MΦ) derived from pannexin-1 KO mice [384, 383].

The evidence supporting a role of pannexin-1 in $P2X_7R$ pore formation is, however, robust and hard to dispute. Firstly, immunoprecipitation studies place pannexin-1 hemichannels in the same interacting cell surface membrane complex, suggesting that there is likely to be 'cross-talk' between the two cell surface proteins [239]. Iglesias and

colleagues, 2008, transfected J774 macrophages with pannexin-1 siRNA and detected a significant reduction in dye uptake following ATP activation [378]. Finally, when pannexin-1 was over-expressed along with $P2X_7R$ receptors in HEK-293 cells, dye uptake and inward $P2X_7R$ channel currents were enhanced and subsequently blocked following application pannexin-1 inhibitors, evidence that strengthens the role of the hemichannel in $P2X_7R$ -induced pore formation [300, 239].

Going back to the findings of Schachter and colleagues, the group made an important observation, by noting the inability of ATP-challenged, $P2X_7R$-transfected HEK-293 cells to take up anionic dyes, an effect that is evident also in murine macrophages. Thus, the $P2X_7R$ appears to regulate two distinct selective dye uptake pathways in murine macrophages [383]. The general consensus that is beginning to emerge is that the primary $P2X_7R$ permeation pathway may simply differ in different cell types. Where in human cells pannexin-1 is evidently the primary permeation pathway activated by the $P2X_7R$, this might simply not be the case with murine macrophages. There is a number of other connexins, innexins or even pannexin-1 splice variants that make take the lead role of inducing this progressive cell permeabilization following $P2X_7R$ activation [300].

1.14.4 Pore formation and cytokine release.

The release of a potent pyrogen like IL-1β is unsurprisingly a tightly regulated, complex and multi-step process. It is clear that up-regulation of cytokine synthesis in its pre-cursor form is induced by PAMP activation, however, it is less clear how bioactive IL-1β molecules are expelled from the cell and whether this mechanism differs in different cell types. Following $P2X_7R$ activation with ATP, a secondary signal induces the assembly of the NLRP3 inflammasome complex, resulting in the proteolytic activation of pro-caspase-1 molecules into their active form. The bioactive form of caspase-1 can then cleave pro-IL-1β molecules into their active form in a regulated manner [385]. The various molecular events of $P2X_7R$-mediated IL-1β release are illustrated in Figure 1.6.

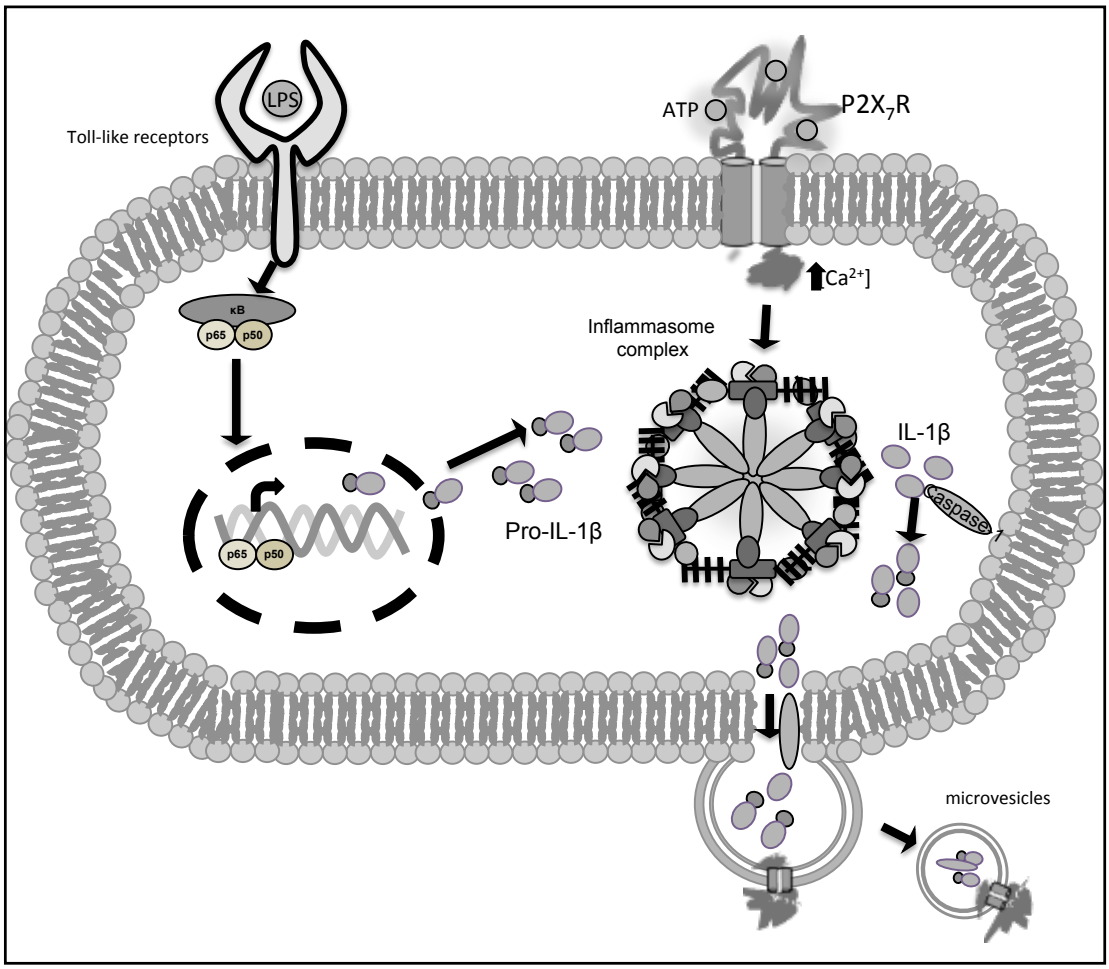


***Figure 1.6:* The role of the P2X$_7$R in IL-1β release.**
Toll-like receptors are able to recognize pathogen-associated molecular patterns, like LPS. This activates an intracellular signalling cascade, in most cases the NF-κB pathway that leads to the transcription pro-IL-1β molecules. A wave of pro-IL-1β transcription results in their accumulation below the inner leaflet of the cell surface membrane. The activation and release of pro-IL-1β molecules requires a secondary signal, such as ATP, which activates the P2X$_7$R. Activation of this ionotropic receptor leads to the hyperpolarisation of the cell (Na^+, $Ca2^+$ influx and K^+ efflux) and provides a signal that stimulates the assembly of the inflammasome (composed of, pro-Caspase-1, ASC, and NALP-3 molecules). Pro-Caspase-1 is then cleaved to Caspase-1 and is then able to activate pro-IL-1β molecules into active IL-1β molecules. Active IL-1β molecules are finally released into the extracellular compartment via different mechanisms, which are thought to be cell specific.

Naturally, pannexin-1 was one of the candidates to mediate IL-1β release. Interestingly, Pelegrin and Surprenant have shown that pannexin-1 was associated upstream at the level of inflammasome aggregation and caspase-1 activation in murine J774 macrophages [239]. However, the hypothesis that pannexin-1 is the sole exporter of IL-

1β molecules was swiftly withdrawn, since Pelegrin and Surprenant were able to demonstrate that nigericin and maitotoxin, two well-known activators of the inflammasome, were able to induce IL-1β release from LPS-primed J774 mouse macrophages. Nigericin cannot induce dye uptake from these cells, suggesting that IL-1β is not released via the $P2X_7R$-induced 'pore'. Additionally, maitotoxin is known to evoke dye uptake in macrophages but in a pannexin-1 independent manner. To complicate matters further, inhibiting pannexin-1 with either an inhibitor, gene silencing or using an inhibitory peptide, prevented IL-1β processing and release. Taken together, it appears that pannexin-1 is necessary for IL-1β processing and release in macrophages but is most likely not the primary pathway for dye uptake (pore formation) in these cells [386].

There are a number of hypotheses attempting to explain how pannexin-1 could play part in the processing and release of IL-1β; however, most of them face considerable contradictory evidence. Currently, the most plausible explanation is that pannexin-1 could act as a K^+ channel. K^+ efflux appears to be a necessary step for caspase-1 activation, since all three inducers ($P2X_7R$, nigericin and maitotoxin) of inflammasome activation mediate K^+ efflux. Furthermore, caspase-1 activation is inhibited in a high K^+-containing extracellular medium. The problem with this hypothesis, however, is that although pannexin-1 inhibitors block IL-1β release, they cannot prevent K^+ efflux. Although it could be argued that this is simply due to the mode of action of the inhibitors, it is most likely that K^+ efflux occurs via the $P2X_7R$ channel itself [271].

The role of pannexin-1 as a mediator of IL-1β release is reinforced by the work of Pelegrin and colleagues [239]. Based on their findings the group place pannexin-1 as an important mediator of not only IL-1β, but also IL-18 and importantly, IL-1α release from peritoneal macrophages. Firstly, using peritoneal macrophages from wild type and $P2X_7R$ knockout (KO) mice, the group demonstrate the importance of $P2X_7R$ activation in the release of all three IL-1-family member cytokines. Secondly, pannexin-1 mimetic inhibitory peptide (^{10}panx1), appears to inhibit the release of all three cytokines, emphasizing the role of the protein in IL-1 cytokine release. Furthermore, with the use of a potent caspase-1 inhibitor (YVAD-AOM), it was possible to show that caspase-1 activation is required for the release of all three cytokines from mouse peritoneal macrophages. What is interesting is that, unlike IL-1β and IL-18, IL-1α does not require

proteolytic cleavage by caspase-1; therefore, it is assumed that some sort of 'cross-talk' must take place between caspase-1 and capsaicin, which is the principal regulator of IL-1α release. Finally, the group demonstrated the release of pro-IL-1β from inflammasome-malfunctioning, ASC-depleted RAW264.7 and peritoneal macrophages, suggesting that $P2X_7R$ must be able to engage two distinct pathways of IL-1 cytokine release. Apart from the classic, $P2X_7R$-mediated, caspase-dependent pathway, which results in the release of active IL-1β, $P2X_7R$ can also activate a second, caspase-1 and pannexin-1-independent, non-cytolytic pathway which shuttles pro-IL-1β molecules outside the cell [12]. Taken collectively, it is unlikely that pannexin-1 channels offer a direct route for IL-1β egress from the cells. However, activation of pannexin-1 hemichannels is a necessary step for the inflammasome oligomerization and also as a mediator of transient ATP release, which in theory would potentiate P2X signalling and further cytokine release [387]. The contribution of pannexin hemichannels or other channels that might contribute to pore formation and inflammasome activation is less clear with regards cells other than macrophages. More data needs to be gathered to gain a better perspective of how the receptor is involved in inflammasome activation and cytokine release.

1.14.5 The role of the $P2X_7R$ in cytokine release.

Apart from inducing IL-1β release, the $P2X_7R$ has been implicated in the synthesis and release of other important pro-inflammatory cytokines, such as IL-18 and the newly identified member of the IL-1 family, IL-33. Relatively little is known about the mechanisms driving the synthesis and release of IL-18. However, in individuals with the $Glu^{496}Ala$ loss-of-function $P2X_7R$ polymorphism, the production of IL-18 is remarkably low [388]. Additionally, when BzATP challenged monocytic THP-1 cells were treated with $P2X_7R$ antagonists, both IL-18 and IL-1β releases were significantly suppressed (Finlayson and colleagues, unpublished data). Regarding the newest member of the IL-1 super-family, there is currently no direct evidence to link IL-33 synthesis, or its release, with $P2X_7R$ signalling. In common with IL-18, IL-33 shares a similar phenotype with IL-1β, with respect to its transcription and recruitment of signalling pathways. Therefore, it is possible that both IL-18 and IL-33, like IL-1β, engage the $P2X_7R$ during their synthesis and release [389, 390].

The $P2X_7R$ appears to have a role also in TNF-α production in microglia cells of the brain. The cytokine exhibits both neurotoxic and neuroprotective effects during inflammation in the brain. Hide and colleagues have shown that ATP and BzATP induce TNF-α release from microglia cultures via a Ca^{2+}–dependent mechanism. The group has not, however, examined the effect of $P2X_7R$ specific blockers; therefore other P2X or P2Y receptors could also participate [391]. Conversely, using p55 TNF-α receptor 1-deficient mice Bruce and colleagues demonstrated how the lack of TNF-α signalling enhances brain injury under ischemic conditions [392].

$P2X_7R$ activation has also been implicated in the release of IL-6 in patients with RA and even atherosclerosis. In these inflammatory scenarios, fibroblasts are exposed to high concentrations of ATP and the $P2X_7R$ is activated to stimulate the release of IL-6 [393]. Furthermore, $P2X_7R$ activation induces up-regulation of IL-6 and TNF-α in murine mast cells, which has raised a lot of interest given the important role of mast cells in the development of allergy [394]. Finally, Gourine and colleagues have demonstrated the role of the $P2X_7R$ in IL-1β, TNF-α and IL-6 during LPS-induced systemic pyroxia. The concentrations of these pro-inflammatory cytokines are significantly reduced following treatment with suramin and more selective $P2X_7R$ antagonists like brilliant blue G (BBG) and pyridoxalphosphate-6-azophenyl-2',4'- disulfunic acid (PPADS) [395].

1.14.6 Other functions associated with $P2X_7R$ signalling.

Apart from cytokine release $P2X_7R$ signalling has been associated in wide variety of functions including the release of a complement component receptor 2 (CD21) from B-cells [396], MMP9 from peripheral blood mononuclear cells [397], the release of a low affinity IgE receptor, CD23 from lymphocytes [398] as well as cathepsins from macrophages [399]. The exact signalling pathways that lead to these events remain obscure [400].

Kim and colleagues have investigated the downstream signalling pathways associated with $P2X_7R$ activation in macrophages, cells in which the receptor is expressed constitutively. Their results demonstrate that the receptor can engage cytoskeletal

proteins directly. In particular, the rat $P2X_7R$ has been identified to engage with 11 proteins, including 8 intracellular proteins (e.g. α-actinin 4, β-actin, supervillin, HSP-90, -71 and -70), the integrin β2 intermembrane protein and the extracellular matrix protein laminin α3 protein [401]. These proteins, particularly the intracellular cytoskeletal proteins, are believed to be involved in the formation of multinucleated giant macrophage cells. The formation of polykarions is particularly important during granulomatous inflammation, an essential process for eliminating resistant pathogens, with Falzoni and colleagues demonstrating that $P2X_7R$ acquires an important physiological role in the process [402]. More precisely, $P2X_7R$-transfected macrophage cell lines appear to fuse spontaneously *in vitro*, a process which is blocked by oxidized ATP (oATP, a potent inhibitor of $P2X_7R$ signalling) [403].

The $P2X_7R$ has also been associated in the process of antigen presentation. The receptor is highly expressed in APC and its expression is upregulated by IFN-γ, a well-recognised potent inducer of antigen presentation. Mutini and colleagues show that treatment of mature DC with oATP induced a marked decrease in T-cell priming. This was also confirmed with experiments conducted with isolated low-expressing $P2X_7R$ clones from wild type folliculo-stellate DC (FSDC) providing strong evidence that the $P2X_7R$ could be involved in antigen presentation. However, during the priming of T-cells by APC, there is a bi-directional flow of signals through an array of co-stimulatory molecules including the release of IL-1β [404]. Thus, a contradictory school of thought suggests that it is the absence of IL-1β release that is responsible for the relative inefficiency of cells for antigen uptake and presentation antigen [404]. A similar scenario was observed by the work of Weber and colleagues where DC derived from $P2X_7R$ KO mice were unable to sensitize wild type mice to contact allergens. Additionally, treatment with $P2X_7R$ antagonists (KN-62) and apyrase appears to suppress sensitization to allergens. Cytokines such as IL-1β and IL-18 have been proven to be critical in mediating sensitization to allergens by driving LC migration from the skin and these results highlight the importance of $P2X_7R$ signalling in inducing IL-1 release under these conditions [289]. The results of this study also highlight the importance of $P2X_7R$ inhibitors as tools to investigate $P2X_7R$ signalling in detail and also the possibility of these being used as therapeutic agents.

1.14.7 $P2X_7R$ antagonism.

The landscape of $P2X_7R$ antagonism has changed dramatically over the last decade, with field of purinergic signalling gaining attention as a point for intervention. Both pharmaceutical companies and academia have presented a number of new classes of antagonists over the years as potential candidates for possible use in the clinic. The conventional $P2X_7R$ antagonists like suramin, PPADS, BBG, oATP and KN-62 (4-[(2S)-2- [(5- Isoquinolinylsulfonyl) methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl) propyl] phenylisoquinolinesulfonic acid ester) have gradually been replaced by a plethora of structurally distinct molecules, such as the cyclic amides [307]. Compounds such as suramin and oATP showed little promise because of their weak specificity for $P2X_7R$ receptors. Subsequently KN-62, a calmodulin-dependent protein kinase II inhibitor had received much attention. The impediment with this compound, as with most $P2X_7R$ antagonists, is its unequal potency between receptors from different species. KN-62 blocks human $P2X_7R$ currents, but has a weak affinity for its rat isoforms [405]. Weber and colleagues have employed the KN-62 in an attempt to elucidate the role of the $P2X_7R$ in the development of contact sensitization. Their results demonstrate that KN-62 prevents the development of sensitization to contact allergens in mice and also exhibiting efficacy of this obscure inhibitor on mouse $P2X_7R$ [289]. The observations of Donnelly-Roberts and colleagues reinforce the notion that KN-62 is a potent inhibitor for mouse as well as human $P2X_7R$, at least in blocking YO-PRO dye uptake, in a transfected cell line [328]. The bases for these discrepancies are yet to be identified, but similar patterns are observed with other compounds such as BBG and the polysulfonic dye, Coomassie [314] and newly designed compounds like the AZ116453743 [406].

Abbott Laboratories first reported the family cyanoguanidines in 2006 as potent and selective $P2X_7R$ inhibitors. Jarvis and colleagues presented the highly potent A-740003 (N-[1-[[(Cyanoamino) (5-quinolinylamino) methylene] amino] -2,2-dimethylpropyl]-3,4 dimethoxybenzeneacetamide) with high selective properties for the $P2X_7R$ receptor. At an advantage to other inhibitors, the compound exhibits minimal discrepancies in potency across the different mammalian $P2X_7R$ receptors. It has therefore become an attractive candidate for *in vivo* studies in rodents. *In vitro*, the inhibitor has been shown

to inhibit a number of $P2X_7R$ -related events, such as Ca^{2+}influx (IC_{50} 40nM for human $P2X_7R$ and 18nM for rat $P2X_7R$), pore formation (IC_{50} 92nM for human $P2X_7R$) and IL-1β cytokine release (IC_{50} 156nM for human $P2X_7R$) from human macrophages [407, 408] The structure of the A-740003 is very similar to a compound that is an ATP-sensitive potassium channel (K_{ATP}) ligand. Interestingly, this compound and other K_{ATP} inducers are extremely weak activators of the $P2X_7R$. Changing different parts of the molecule, such as introducing a piperazine unit enhanced the selectivity of the molecule for the $P2X_7R$ [409-411]. Carroll and colleagues summarized the development of different types of inhibitors including cyanoguinines in a review [407]. A recent addition to the family of cyanoguanidine molecules is the tritium-labelled A-804598. The compound shows remarkable selectivity for $P2X_7R$ receptors and an IC_{50} value of 10nM for mouse rat and human receptors [412].

Astra Zeneca presented the adamantyl benzamides in 2003. However, the first batch of compounds showed inhibition of only the human $P2X_7R$ [413]. With systematic modifications of the structure of the compounds, and more precisely, with substitution at the aromatic rings (position 5), it was possible to widen the selectivity of these compounds to rats and mouse $P2X_7R$ [414, 384]. One of latest and much improved adamantyl benzamides has been employed as a promising treatment for chronic pain and inflammation, particularly RA [415]. Substantial improvement has unquestionably been noted in the field of $P2X_7R$ antagonism. Novel, potent and selective inhibitors have been identified and there has been marked improvement in the drug-like properties of new compounds. This process, however, is almost exclusively based on high throughput screening methods, because no crystal structure of the receptor has been identified so far. Much of the field of $P2X_7R$ antagonism is still uncharted territory, and requires further exploration. New compounds need to be potent, specific and most importantly their potency be equal across all mammalian $P2X_7R$ orthologues. In addition, factors such as solubility of the compound and pharmacokinetic properties need to be taken into account [407, 416].

1.15 The role of the $P2X_7R$ in innate immune responses; a point of therapeutic intervention?

Much of the focus on $P2X_7R$ activation lies in the downstream induction of pro-inflammatory cytokine release. Learning how to manipulate the release of cytokines like IL-1β and TNF-α could be advantageous in certain immune disease states like for example suppressing the development of sensitization to allergens [289] In other instances, potentiating the release of IL-1β could be beneficial for limiting mycobacterial viability in macrophages. However, a systemic rise in IL-1β levels could result in undesirable widespread $P2X_7R$ -mediated cell death [417]. Conversely, administering a $P2X_7R$ antagonist systemically could dysregulate programmed cell death, potentially leading to carcinogenesis, or the development of autoimmune diseases. In this context it is of interest that the immune system in $P2X_7R$-deficient mice is reported to be generally quiescent but not immunocompromised [418].

Additionally, the absence of receptor expression by osteoblasts and osteoclasts (derived from $P2X_7R$ KO mice) results in an abnormal skeletal phenotype, suggesting that the receptor exhibits strong potential for future therapeutic strategies in osteoporosis [419]. Pharmaceutical companies have made significant investments in order to elucidate the pharmacokinetic properties of $P2X_7R$ blockers with the hope of these being used in the development of clinical treatments. Indeed, Astra Zeneca's AZD9056 is in the third phase of clinical trials as part of a treatment scheme against rheumatoid arthritis (RA) [420]. RA is a chronic inflammatory disorder whose pathogenesis is dependent on the synergistic effects of IL-1β and IL-18, two cytokines whose release is mediated by $P2X_7R$ signalling. AZD9056 has been shown to be a potent and selective inhibitor of the $P2X_7R$ and has been used to target $P2X_7R$ -mediated cytokine release in RA patients. The results from a phase IIa trial of clinical treatment with the inhibitor for 6 months have been extremely encouraging providing evidence of AZD9056 acting as a therapeutic agent. In fact 65% of recipients of the drug have shown significant reduction in the RA symptoms such as joint swelling. Unfortunately, a subsequent longer-phase IIb clinical trial suggests that the inhibitor had no efficacy in alleviating the symptoms of RA. A decade after it was first cloned, the $P2X_7R$ is an important focus of scientific attention. The $P2X_7R$ channel is a very attractive target for many pharmaceutical companies to manipulate biological responses. More careful design and analysis is

required for future approaches and for this to occur we need to improve our understanding of how the receptor functions in different cell types, characterize the various downstream effector functions of the receptor and investigate the effect of these inhibitors in blocking $P2X_7R$ -mediated events [421].

1.16 Conclusion and Hypothesis.

The brilliantly complicated immune system under the continuous pressure of an everchanging number of potential threats has evolved sophisticated processes to provide long-term immunity to its host. The largest interface between pathogen and host is the multilayered microenvironment of the skin, which houses an intricate system of surveillance. However, the immune system is far from perfect and can often mistake harmless agents for potential threats that initiate unwanted immune responses. One such group of agents are chemicals that have been incorporated to everyday life in our modern societies. Exposure to chemical sensitizers can lead to contact allergy or asthma in susceptible individuals [422]. There are considerable efforts by chemical companies and health institutions to improve the current assays for accurately, reliably and efficiently identifying chemical sensitizers to reduce the number of occupational and consumer health incidences. The ultimate goal is to design novel *in vitro/in silico* methods that will identify chemicals with the potential of initiating a cutaneous immune response [423]. To realise such a goal attention needs to focus on better understanding the intricate mechanisms that drive cutaneous immune responses at the molecular and cellular level. This study will therefore investigate the role of $P2X_7R$ signalling in mediating pro-inflammatory cytokine release from DC, particularly IL-1β.

1.17 Experimental Design.

DC are undoubtedly the chief initiators of primary immune responses [14], however, the small sample size of DC compartments poses a challenge in investigating DC biology during inflammatory responses. As described previously, the isolation procedures of the various DC populations are rigorous, technically demanding and often lead to undesired and non-physiological changes in the phenotype of the cells. Currently, the preferred method for expanding large numbers of mouse DC for subsequent experimentation on DC function was presented by Lutz and colleagues in 1999. It involves propagating several hundred millions of DC-like cells from progenitor cells in the BM, using specific cytokine cocktails. The procedure is easy, highly reproducible and results in the generation of high yields of relatively homogeneous DC-like cells. The phenotype of BM-DC is comparable to that of immature DC exhibiting marked expression of MHC class II, CD54, the integrin CD11c and other co-stimulatory molecules. Thus, these cells are considered suitable representatives of DC/LC *in vivo* and will therefore be employed to investigate the aims of this project [424, 425]. In a similar manner isolation of macrophages from mouse tissues involves complex procedures that also result in relatively low yields. Instead, cells with a macrophage-like phenotype can be expanded from BM precursors using M-CSF, an essential factor for the differentiation of committed precursors to the macrophage lineage [426]. Importantly, both DC and macrophage populations were derived simultaneously from precursors of the same BM pool culture in the presence of different cytokine profiles. The expanded homogeneous population of derived macrophages is an invaluable method to study macrophage biology and presented an appropriate isogenic control.

The overall scope of this thesis was first to characterize the expression of the $P2X_7R$ on BM-DC and subsequently to examine the role of ATP-induced $P2X_7R$ signalling on cytokine release, particularly with regards to IL-1β responses, in direct comparison with macrophages. This receptor has the unique functional capacity to permeabilize the cell membrane to molecules of large molecular weight, following prolonged activation of the receptor (10-60 seconds). This allows for large fluorescent molecules to accumulate in the cytosol thereby constituting a very useful tool to trace $P2X_7R$ activity since these

molecules, in theory, due to their large molecular size, can only enter a cell via the $P2X_7R$-induced pore. The large molecular weight YO-PRO dye was employed to assess the properties of the receptor on activated BM-DC and in comparison with that expressed by macrophages generated from precursors of the same BM tissue. YO-PRO dye uptake is a well-established method used to assess receptor activity in a number of different cell types [427]. Although this receptor-evoked cell membrane permeabilization is not clearly understood, it is generally accepted that permeation is non-selective to molecules smaller than 900Da and is related to the release of IL-1β molecules (at least in macrophages) [428]. There has been significant progress in identifying novel chemotypes that are potent and selective for $P2X_7R$. Many such antagonists like the A-740003 exhibit drug-like properties providing a powerful tool for investigating $P2X_7R$-specific functions both *in vitro* and *in vivo*. The A-740003 is a fast acting pharmacophore presented by Tocris that has previously been shown to potently block receptor activity and as such has been employed to investigate $P2X_7R$ responses in DC and macrophages [408]. The specific aims of this study are summarised below:

Aims and Objectives:

1. Characterise murine BM-DC as a model for DC and establish the optimum timeframe in culture to propagate a high-yield of naïve DC-like cells.
2. Assess the ability of murine BM-DC to respond to inflammatory TLR ligands by up-regulating their antigen-presenting machinery and inducing pro-inflammatory cytokine release.
3. Since much of the work on $P2X_7R$ has, thus far, been carried out in macrophage populations, identify an appropriate isogenic control macrophage model and provide a sufficient phenotypic analysis of this cell type.
4. To characterize $P2X_7R$ expression in murine BM-DC in direct comparison with macrophage populations.
5. To investigate the physicochemical properties of the murine $CD11c^+$-expressing BM-DC-bound $P2X_7R$.
6. Identify any distinct functional properties between DC and macrophage populations in mediating $P2X_7R$-driven pore formation.
7. Characterise $P2X_7R$-associated cytokine responses between murine DC and macrophage populations.
8. Finally, investigate whether the TLR4/2 ligand LPS has a distinct impact on the transcriptional expression of $P2X_7R$ splice isoforms in DC and macrophage populations and therefore whether splice variant expression constitutes a robust regulatory mechanism for $P2X_7R$ function.

Chapter 2: Materials and Methods.

2.1 Experimental animals.

All animals were housed in the *Biological Species Facility* (BSF) unit of the University of Manchester. The animals were allowed free access to food and water whilst environmental conditions comprised a 12h dark/light cycle at 21°C ±1°C and 55% ±10% humidity. Maintenance and termination of animals were carried out as dictated by the Animals (Scientific Procedures) Act 1986. Procedures were performed on female BALB/c mice.

2.2 Generating BM-DC from bone marrow progenitors.

Female BALB/c mice (6-8 weeks old) (Harlan Olac, Bicester) were used to obtain femurs and tibias and the bones were flushed with sterile phosphate buffered saline (PBS, Fisher Scientific, Loughborough, UK) to extract the bone marrow. The cell suspension was centrifuged at 168*g* for 5 min at room temperature (RT) and a viable cell count was performed by trypan blue (Sigma-Aldrich, Poole, Dorset, UK) exclusion. Cells were resuspended in pre-warmed, foetal calf serum (FCS; Invitrogen, California, USA) supplemented media (RPMI-1640 containing 25 mM HEPES, 400μg/ml penicillin/streptomycin (Invitrogen), 292μg/ml L-glutamine, 0.1% (v/v) 2-mercaptoethanol (Invitrogen), 10% (v/v) heat inactivated FCS (RPMI-10% FCS) and enriched with GM-CSF (Peprotech, New Jersey, USA) at 20ng/ml. BM cells were cultured at $2x10^6$ per 10ml in Petri dishes at 37°C in a 5% CO_2-rich environment.

Every 3 days, 10ml of exhausted medium was removed and replaced with 10ml of fresh medium (RPMI-10% FCS enriched with 20ng/ml of GM-CSF). On day 6, 8 or 12 of culture, the cells were rinsed gently from the Petri dish using a Pasteur pipette and were collected in a Falcon tube. The cell suspension was centrifuged at 168*g* for 5 min at RT. The viable cells were counted by trypan blue (Sigma-Aldrich) exclusion and seeded into a 24-well tissue culture plate at $1x10^6$ cells in 0.9ml per well.

2.3 Generating BM-MΦ from bone marrow progenitors.

BM-MΦ and BM-DC were cultured concurrently using BM progenitor cells from the same 6-8 weeks old female BALB/c mice. To expand BM-MΦ in culture, BM

progenitors were extracted from tibia and femur bones of female BALB/c mice as described in section 2.2. The cells were cultured with pre-warmed Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 400μg/ml penicillin/streptomycin, 292μg/ml L-glutamine, 0.1% (v/v) 2-mercaptoethanol, 10% (v/v) heat inactivated FCS and 30% L-929 conditioned medium (DMEM-10%FCS) (see section 2.5). BM cells were cultured at $2x10^6$ per 10ml in Petri dishes at 37°C in a 5% CO_2-rich environment.

Every 3 days following the 1st day of culture the culture medium along with non-adherent cells was removed and replaced with 10ml of fresh DMEM-10%FCS medium containing 30% L-929 conditioned medium. On day 8 or 12 of culture, the non-adherent cells were rinsed from the Petri dish using a Pasteur pipette before 10ml of ethylene diamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) was added to each Petri dish to detach the adherent population for 10-15min at 37°C in a 5% CO_2-rich environment. Subsequently, the cell suspension was centrifuged at 168*g* for 5 min at RT. The viable cells were counted by trypan blue (Sigma-Aldrich) exclusion and seeded into a 24-well tissue culture plate at $1x10^6$ cells in 0.9ml per well.

2.4 Isolation of mouse peritoneal exudate cells (PMΦ).

Adult (4-6 months old) female BALB/c mice were sacrificed and the skin was excised to expose the parietal peritoneum and approximately 5-8ml of pre-warmed RPMI-10%FCS medium was injected into the peritoneal cavity of each mouse. The medium was recovered 10min later and pooled with lavage from 2-3 other mice. The cells were washed with Hanks balanced salt solution (HBSS, with phenyl red, without Ca^{2+} and Mg^{2+}; Sigma-Aldrich), collected by centrifugation (168*g* for 5 min at RT) and cell viability was assessed by trypan blue (Sigma-Aldrich) exclusion. Subsequently, cells were cultured in a 24-well tissue culture plate at $2x10^6$ cells/ml with pre-warmed, RPMI-10% FCS medium. Finally, the cells were allowed to adhere overnight at 37°C in a 5% CO_2-rich environment before the non-adherent population was removed by thorough washing. PMΦ were allowed to rest for 2h at 37°C and subsequently used for experimentation.

2.5 Mouse L-929 fibroblast cells in vitro culture.

The mouse L-929 fibroblast cell line was kindly donated by the *Alan North Lab, Faculty of Life Sciences, The University of Manchester*. The cells were cultured at $1x10^6$ cells per 12ml of DMEM-10% FCS at 37°C in a 5% CO_2-rich environment until they reached confluency (80-100%) (3-4 days). Subsequently, the medium from several culture flasks (5-6) was collected, filtered (0.2μm filters) and stored at -70°C. This M-CSF-containing medium was used to prepare the conditioned medium for BM-MΦ cultures within 1 month after collection (see section 2.3). In the meantime, the cells were incubated with Trypsin/EDTA (0.2mg/ml of EDTA, 0.5mg/ml of porcine trypsin, 0.09% NaCl)(Sigma-Aldrich) solution for 2 min at 37°C in a 5% CO_2-rich environment to detach the adherent cell population. The cells were counted, their viability was assessed by trypan blue (Sigma-Aldrich) exclusion and re-plated at $1x10^6$ per 12ml of fresh pre-warmed medium. Cells were discarded following 4-5 passages and replaced with a fresh batch of cells.

2.6 Culturing Human Embryonic Kidney (HEK)-293 cells.

The Human Embryonic Kidney (HEK)-293 cell line was also kindly donated by the *Alan North Lab, Faculty of Life Sciences, The University of Manchester*. The cells were cultured at $1x10^6$ per 12ml in a T75 tissue culture flask with DMEM-10% FCS and incubated at 37°C in a 5% CO_2-rich environment until the cells were found to be confluent (80%) monolayer, usually after 3 days. At this point the medium was removed and the cells were washed thoroughly with PBS (w/o Ca^{2+} and Mg^{2+}, Fischer Scientific) and incubated with Trypsin/EDTA for 2 min at 37°C in a 5% CO_2-rich environment to detach the cells. The cells were collected by centrifugation (at 168*g* for 5 min at RT) counted, and their viability was assessed by trypan blue (Sigma-Aldrich) exclusion. Finally, the cells were re-plated in T75 flasks at $1x10^6$ per 12ml of fresh pre-warmed medium.

2.7 Preparation of undifferentiated splenocytes.

The spleens of adult female 6-8 weeks old BALB/c mice were excised and sliced into smaller fragments. Using the plunger end of a sterile syringe, the fragments were pressed through a sterile metal gauze strainer and the cells were washed through the

strainer with excess RPMI-10% FCS. The homogenized solution was centrifuged at 168*g* for 5 min at RT to collect the cells. Red blood cells were lysed by resuspending the pellet in pre-warmed RPMI-10% FCS supplemented with 0.85% ammonium chloride for 5min. Subsequently, the cells were washed thoroughly with RPMI-10% FCS, counted and cell viability was determined by trypan blue (Sigma-Aldrich) exclusion. Finally, splenocytes were plated at $2x10^6$/ml and allowed to adhere overnight at 37°C in a 5% CO_2-rich environment before used for experimentation.

2.8 Activation of BM-DC, BM-MΦ and PMΦ populations.

In all experiments, unless otherwise stated, BM-DC, BM-MΦ were plated in 24-well tissue culture plates at $1x10^6$/ml. PMΦ were freshly isolated and prepared as described in section 2.4. Ligands, with the exception of A-740003 (Tocris Bioscience, Bristol, UK), were dissolved either in RPMI-10% FCS for stimulation of BM-DC and PMΦ or in DMEM-10%FCS for stimulation of BM-MΦ. The $P2X_7R$ inhibitor, A-740003 (Tocris Bioscience), was dissolved in dimethyl sulfoxide (DMSO) and dilutions prepared such that the content of DMSO was equal at 0.5% for all concentrations employed.

Treatments with LPS (24h): cells were incubated with LPS (Sigma-Aldrich) at a final concentrations in 1ml volume (with 0.9ml cells): 1, 10, 100 or 1000ng/ml for 24h at 37°C in 5% CO_2.

Treatments with LPS and Apyrase (24h): cells were incubated with apyrase (Sigma-Aldrich) at a final concentration of 0.2, 2 20 or 200μg/ml together with 1000ng/ml LPS (Sigma-Aldrich) for 24h at 37°C in 5% CO_2.

Treatments with LPS (2h) and ATP: cells were cultured with LPS for 2h at a final concentration of 1, 10, 100 or 1000ng/ml at 37°C in 5% CO_2 and received treatment with medium alone or ATP (Sigma-Aldrich) at a final concentration of 0.5, 1, 5 or 10 mM, for the last 30 min of the incubation period.

Treatments with LPS (2h), ATP and A-740003: cells were cultured with LPS (Sigma-Aldrich) for 2h at a final concentration of 1000ng/ml at 37°C in 5% CO_2

and received treatment with medium alone or A-740003 (Tocris Bioscience) at a final concentration of 0.1, 1, 10 and 100μM, for the final 40min of the end of the incubation period. Finally, 10min after treatment with the $P2X_7R$ inhibitor, the cells were challenged with medium alone or ATP (Sigma-Aldrich) at a final concentration 5 mM for the last part of the incubation period.

Treatments with LPS, ATP and apyrase: cells were cultured with LPS (Sigma-Aldrich) for 2h at a final concentration of 1000ng/ml at 37°C in 5% CO_2 and received treatment with medium alone or Apyrase (Sigma-Aldrich) at a final concentration of 2 and 20μg/ml, for the final 30min of the incubation period. Additionally, 2-3min following treatment with apyrase, the cells were challenged with medium alone or ATP (Sigma-Aldrich) at 1 mM for the last part of the incubation period.

2.9 Collection of cell supernatants and lysates for analysis.

At the end of every series of treatments with the various ligands, supernatants were collected whilst cells were treated with 100μl/well of lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 20 mM ethylenediaminetetraacetic acid [EDTA], 10% glycerol, 0.5% Ipegal, phenylmethylsulfonyl [1 mM] and protease inhibitor cocktail [1:100] (Calbiochem, San Diego, USA) to collect lysates. The fact that cell lysates were collected in a total volume of 100μl was taken into consideration when the intracellular cytokine content was quantified by ELISA analysis. All aliquots were stored at -70°C for further analyses. Importantly, parallel treatments cells were carried out with the various ligands to determine cell viability using propidium iodide (Sigma-Aldrich) staining.

2.10 Assessing membrane marker expression using flow cytometry.

2.10.1 Staining for MHC class II, CD86 and CD54 marker expression.

Following the various treatments, BM-DC and BM-MΦ were assessed for the expression of the various membrane markers of interest using flow cytometry. At the end of the incubation period, the cells were treated with EDTA solution (0.5 mM) for 10-15 min, subsequently flushed and the cell suspension was centrifuged at 168g, for 5min, at 4°C to collect the cells in pellet form and was re-suspended in 0.6ml of fluorescence-activated cell sorter (FACS) buffer (10% FCS/PBS). The cells were then transferred into round-bottomed 96-well plates and were stained for MHC class II (purified rat anti-mouse MHC class II [clone 2G9]; diluted at 2.5µg/ml; BD Biosciences, Princeton, New Jersey, USA), CD86 (purified rat anti-mouse CD86 [clone GL1] and CD54 (purified rat anti-mouse CD54; diluted at 10µg/ml; BD Biosciences) using primary antibodies diluted in FACS buffer and incubated for 30 min on ice. The isotype control used for these primary antibodies was IgG2a (diluted at 10µg/ml; BD Biosciences). Subsequently, cells were washed using FACS buffer and then centrifuged at 242g for 5min at 4°C. Cells were then re-suspended in FACS buffer and the process was repeated three times. Secondary antibody (STAR69 goat anti-rat IgG fluorescein isothiocyanate [FITC]-labelled polyclonal antibody; AbD Serotec, Kidlington, Oxford, UK) was diluted at 7µg/ml in FACS buffer and added to the cells for 30 min on ice. Cells were washed for a further 3 times and at the end of the final wash cells were re-suspended in sodium azide buffer (0.05% sodium azide [BDH from VWR, Leicestershire, UK] 1% FCS/PBS). A FACScalibur machine and CellQuest Pro software (BD Biosciences) were used for analysis during which 10,000 cells were acquired. Propidium iodide (PI; diluted at 10µg/ml; Sigma-Aldrich) was used to gate out non-viable cells.

2.10.2 Staining for CD11c, CD11b and F4/80 marker expression.

For characterization of CD11c, CD11b (BD Biosciences) and F4/80 (E Biosciences, San Diego, California, USA) expression, BM-DC, BM-MΦ and PMΦ were collected and prepared as described in sections 2.2, 2.3 and 2.4. Cells were re-suspended in FACS buffer and transferred into 96-well round-bottomed plates. They were then blocked for 10 min on ice with purified rat anti-mouse CD16/32 (100µl/well at 5µg/ml; mouse Fc receptor block; BD Biosciences). Three 5-min washing cycles were performed as described in section 2.10.1 before the cells were stained with phycoerythrin (PE)-labelled hamster anti-mouse CD11c (diluted at 4µg/ml in FACS buffer; BD Biosciences), PE-labelled purified rat anti-mouse F4/80 (diluted at 4µg/ml in FACS buffer; E Biosciences) or PE-labelled purified rat anti-mouse CD11b (diluted at 10µg/ml in FACS buffer; BD Biosciences) for 30 min on ice. Purified PE-labelled hamster IgG1κ (for CD11c, diluted at 4µg/ml; BD Biosciences), IgG2a (for F4/80, diluted at 4µg/ml; E Biosciences) or IgG2b (for CD11b, diluted at 10µg/ml; BD Biosciences) isotype control diluted in FACS buffer and added to the appropriate wells for the same incubation period on ice. Cells received another set of washes, as described above, after which they were re-suspended in sodium azide buffer. A FACScalibur machine *CellQuest Pro* and *FlowJo 8.8.6* software were used to acquire and analyse 10,000 cells and 7-aminoactinomycin (AAD; BD Biosciences) was used to gate out non-viable cells. A full list of all antibodies and the concentration at which they were employed for flow cytometry can be found in table 2.4

2.11 Identifying $P2X_7R$ expression using flow cytometry.

For characterization of $P2X_7R$ expression using flow cytometry, cells were collected and prepared as described in section 2.2 and 2.6. BM-DC, splenocytes or HEK-cells were re-suspended in FACS buffer and transferred into 96-well round-bottomed plates. When the Alomone Labs (Jerusalem, Israel) and Epitomics (Burlingame, California, USA) anti-$P2X_7R$ antibodies, which are directed against an intracellular epitope, were employed to identify $P2X_7R$ expression, the following 2 steps preceded: (1) cells were fixed with 2% formaldehyde solution prepared in PBS for 10min at RT and subsequently washed with excess 5%FCS/PBS, (2) cells were treated with 0.01% saponin solution prepared in PBS for 10min on ice to permeabilize the cell surface

membrane (3) incubations with the primary and secondary antibodies as well as washing steps were performed with 0.01% saponin solution in PBS. In contrast, these two steps were excluded when the Santa-Cruz anti-$P2X_7R$ was employed.

To reduce the possibility of non-specific binding, the cells were treated with purified rat anti-mouse CD16/32 (diluted at 5µg/ml in FACS buffer) and subsequently 10% mouse serum for 10 min with each blocking buffer on ice. The cells were washed for three consecutive times in between each block step as described in section 2.10.1 before the cells were stained with either (1) rat anti-$P2X_7R$ antibody (diluted at 1, 5 or 10µg/ml in FACS buffer) (Santa-Cruz, Dallas, Texas, USA) or (2) rabbit polyclonal anti-$P2X_7R$ (diluted at 3µg/ml in 0.01% saponin in PBS; Alomone Labs) or (3) rat monoclonal anti-$P2X_7R$ (1:100) (Epitomics) for 30 min on ice. A rabbit polyclonal IgG (diluted at 3µg/ml in 0.01% saponin in PBS; R&D Systems) or rat IgG2b (1:100 prepared in 0.01% saponin in PBS; BD Biosciences) was employed as the isotype control and added to the appropriate wells for the same incubation period on ice. Following a second round of washes the secondary antibody goat anti-rabbit IgG Alexa Fluor 488-labelled (Invitrogen) or goat anti-rat IgG FITC-labelled was diluted in FACS buffer (diluted at 20µg/ml in FACS buffer) and added to the cells for 30 min on ice. Finally, cells received another set of washes, as described above, after which they were re-suspended in sodium azide buffer. A FACScalibur machine *CellQuest Pro* and *FlowJo 8.8.6* software were used to acquire and analyse 10,000 cells.

2.12 Assessing the expression of proteins of interest using immunocytochemistry.

In order to detect $P2X_7R$ in BM-DC, cells were collected and prepared as described in section 2.2, plated on 8 mm sterile glass coverslips at $250x10^3$ with pre-warmed, RPMI-10% FCS medium and incubated overnight at 37°C in a 5% CO_2-rich environment. The following day, the cells were fixed with 2% formaldehyde solution prepared in PBS for 10min at RT and subsequently washed with excess 5%FCS/PBS. The cells were treated with 0.01% saponin solution prepared in PBS for 10min on ice to permeabilize the cell surface membrane and received 3 5-min wash cycles with 5%FCS/PBS. To block non-specific antigen binding sites, the cells received treatment with purified rat anti-mouse CD16/32 (at 5µg/ml in 0.01% saponin in PBS) for 10min on ice after which the cells

were washed as described in section 2.10.1. The cells were also incubated with 10% FCS for 10 min on ice and received one cycle of 5-min wash as described in section 2.10.1. Subsequently, the cells were incubated with the rabbit polyclonal anti-$P2X_7R$ (diluted at 3μg/ml in 0.01% saponin in PBS, Alomone Labs) for 30 min on ice. A rabbit polyclonal IgG (BD Biosciences) was employed as the isotype control and added to the appropriate wells (at 3μg/ml) in FACS buffer for the same incubation period on ice. Following a second round of 5-min washes, the secondary antibody goat anti-rabbit IgG Alexa Fluor 488-labelled (Invitrogen) diluted in 0.01% saponin in PBS at 20μg/ml was added to the appropriate wells for 30 min on ice. Cell were also incubated with the secondary antibody alone to control for non-specific binding of the antibody. As a positive control, the cells were incubated with the PE-labelled hamster anti-mouse CD11c (diluted at 4μg/ml in FACS buffer; BD Biosciences) or its isotype control PE-labelled hamster IgG1κ (diluted at 4μg/ml in 0.01% saponin in PBS; BD Biosciences) for the same amount of time on ice. Finally, cells received another set of washes, as described above and coverslips were dabbed in distilled H_2O to remove residual buffer salts. Excess water was removed before the glass coverslips were mounted on glass microscope slides with Vectashield mounting medium (DAKO, Glostrup, Denmark). The slides were stored at 4°C and images were collected using an Olympus (BX51) upright fluorescent microscope with a 40x/0.74 PlanFLN objective, captured using a coolsnap ES camera through *MetaVue* software (Molecular Devices, San Francisco, USA) and analyzed using *ImageJ 1.46R*.

Additionally, a similar protocol was followed to stain BM-DC, BM-MΦ and PMΦ for the expression of CD11c, CD11b and F4/80. However, for these experiments the fixation with PFA and permeabilization with saponin steps were excluded. The cells were stained with PE-conjugated antibodies for all the markers of interest or the appropriate isotype controls for 30 min on ice.

2.13 Determining $P2X_7R$ expression by Western Blot analysis.

2.13.1 Lowry protein assay.

The BIO-RAD detergent-compatible assay was used to determine the protein concentration in lysates of BM-DC, BM-MΦ, PMΦ, undifferentiated splenocytes and HEK-293 cells. Lysates of the various cell types of interest were collected using the procedure described in section 2.9. BSA protein was used to create a 15-point standard curve. A 10mg/ml stock of BSA was diluted in PBS to yield the 15 different desired concentrations of BSA ranging from 0-4mg/ml. Lysates were added (neat or diluted 1:2 and 1:5 in PBS) in duplicates and standards (dilutions of BSA) in triplicates whilst PBS was added in blank rows (5µl/well). Solution A (alkaline copper tartrate) was mixed with Solution S (surfactant solution) at a 50:1 ratio and added to all wells at 25µl/well. Subsequently, wells received 200µl of Solution B (dilute Folin's Reagent) and incubated for 15min at RT on the orbital shaker [429]. A Titertek Multiscan Plus MKII plate reader and *Genesis software* were used to read the plates at 700nm and determine the protein concentration in each sample.

2.13.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

A 10% SDS polyacrylamide gel was prepared as follows: the two glass plates used to prepare the gel were firstly thoroughly cleaned with ultrapure water and ethanol to remove dust particles and also provide a clean air bubble-free adherent surface for the gel to form. A 0.75 mm glass plate with spacers and a shorter plate were clamped together forming a small but sufficient gap in between them to form the gel.

Table 2.1: *List of the components of the Resolving gel.*

10% Resolving gel	Final Concentration
1.5M Tris-HCL	0.45M
Sodium dodecyl sulphate	0.12 %
Aqueous acrylamide solution	40%
TEMED	0.18%
10% ammonium persulphate	0.432%

The various components of the resolving gel listed in Table 2.1 were added in their appropriate (listed) volumes with TEMED and 10% APS added last. The solution was thoroughly mixed in 30ml bijou tube and pippetted in the gap created by the two glass plates. Sufficient space (approximately 1cm) was left for the stacking gel. In order to prevent oxygen from penetrating the gel and interfering with the polymerization reaction and to ensure the formation of a levelled gel, propan-2-ol was added on top of the resolving gel. The gel was allowed to polymerise for approximately 30 minutes at RT, after which the propan-2-ol was carefully removed.

In a similar manner, the components of the stacking gel, listed in Table 2.2 were thoroughly mixed before the solution was pippetted on top of the polymerized resolving gel. A 10-well comb was inserted avoiding the formation of air bubbles below the teeth of the comb. The gel was allowed to polymerize for 30 minutes at RT and subsequently the comb was carefully removed.

Table 2.2: *List of the components of the Stacking gel.*

0.75 mm Stacking gel	Final Concentration
Deionised H_2O	
0.5M Tris-HCL	0.125M
Sodium dodecyl sulphate	0.4%
Aqueous acrylamide solution	4%
TEMED	0.14%
10% ammonium persulphate	0.3%

2.13.3 Sample preparation and gel electrophoresis.

Following the lowry protein assay described in section 2.13.1 the diluted samples containing equal amounts of protein received 2X Laemelli sample buffer (BIO-RAD, Berkeley, California, USA) at 1:1 ratio. The samples were then boiled at 80°C for 10 minutes at the end of which they were stored at -70°C for future use. The polymerised gels were inserted in a BIO-RAD Protean system tank and and the tank was filled with Tris/Glycine running buffer (BIO-RAD; 25 mM Tris, 250 mM glycine, pH 8.3 and 0.1% SDS). Samples were allowed sufficient time to thaw at RT before 10µg of each sample was carefully loaded in each well. Running buffer was used to completely fill

the centre of the tank and partially fill the outer part. Electrophoresis was performed at 110V and subsequently arrested once the bromophenol blue front reached the bottom end of the gel.

2.13.4 Chemiluminescence Western blotting.

A nitrocellulose membrane of the same size as the gel was soaked in Transfer buffer (10X stock: 0.25M Tris pH 8.3 and 1.92M glycine). A 'blotting sandwich' was formed consisting of fibre pad, filter paper, the gel, membrane, a second piece of filter paper and another piece of fibre (blotting apparatus). The blotting apparatus was firmly shut and inserted in a Transfer buffer-filled tank together with a block of ice (to prevent overheating). A current of 170V was run through it for 1h. At the end of the transfer, the membrane was removed from the blotting apparatus and transferred to 10ml of 5% blocking buffer (5% non-fat dry milk, Marvel) and incubated for 1h at RT shaking. The blocking buffer was freshly prepared in Tris-buffered saline Tween (TBST; 10X stock: 200 mM of Tris-HCl, 5M NaCl and 0,1% Tween), centrifuged at 168*g* for 10 minutes and filtered before use. Subsequently, the membrane was incubated with the primary anti-$P2X_7R$ Alomone antibody (polyclonal rabbit anti-mouse $P2X_7R$ prepared at 3µg/ml in 2% milk-TBST) overnight at 4°C.

The membrane was then washed 4 times in 10-minute cycles on the orbital shaker with 5ml of TBST. The secondary antibody (horseradish peroxidase [HRP]-conjugated goat anti-rabbit IgG; AbD Serotec) was diluted at 50ng/ml in 2% milk-TBST and incubated with the membrane for 2h at RT shaking. The membrane was washed again for another 4 times in 10-minute cycles during which the chemiluminescent reagents were prepared. A Super Signal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to prepare sufficient volume of the luminol-based solution by mixing Reagent A and B at 1:1 ratio. The membrane was washed in ultrapure water before being submerged in the solution for approximately 2 minutes.

2.13.5 Image acquisition.

Membranes were attached in light-exclusion film cassette and were developed on a hyperfilm (Amersham Biosciences/GE Healthcare, Little Chalfont, Buckinghamshire, UK) using a medical film processor (Xograph Imaging System, Compact). The exposure time was set at 30 sec, 1, 5min.

2.14 Electrophysiological recordings of BM-DC using whole-cell patch-clamp.

BM-DC were stained for the expression of CD11c (BD Biosciences) before the cells were clamped to measure electrophysiological currents in BM-DC following ATP (Sigma-Aldrich) activation. For these experiments, BM-DC were prepared on glass coverslips and immunocytochemistry was performed as described in section 2.5.1 with the exception of the fixation and permeabilization steps and all procedures were carried out at RT. Additionally, the cells were incubated with a lower concentration of CD11c (2μg/ml) (BD Biosciences) antibody or its isotype control (IgG1κ) at 37°C in 5% CO_2.

Recording pipettes were prepared from borosilicate glass (Harvard Apparatus, Kent UK) with resistances of 2-5 MΩ. The intracellular pipette solution contained: 147 mM NaCl, 10 mM HEPES and 10 mM ethylene glycol tetraacetic acid (EGTA). The extracellular recording solution contained 147 mM NaCl, 10 mM HEPES, 13 mM glucose, 2 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$. All solutions were maintained at 300-320 mOsm/L and pH 7.3 (adjusted with NaOH). Whole-cell patch clamp recordings were made at room temperature using a HEKA EPC9 patch clamp amplifier and Pulse acquisition software. Recordings were made at a holding potential of -60 mV. The data were low-pass filtered at 3kHz and sampled at 1kHz. ATP (5 mM) and A-740003 (100μM) (Tocris Bioscience) were applied using an RSC-160 rapid perfusion system (BioLogic, Claix, France), positioned approximately 100nm away from the cell under investigation.

2.15 YO-PRO dye uptake assay.

At day 8 of culture, BM-DC or BM-MΦ were transferred into black with clear bottom 96-well microtiter tissue culture plates (Corning Incorporated, Massachusetts, USA) at $3x10^5$ cells per 0.2ml of media and incubated overnight at 37°C and 5% CO_2. The following day cells were checked to have formed a confluent monolayer and were either left untreated or primed with 1000ng/ml of LPS (Sigma-Aldrich) for 2h at 37°C and 5% CO2. Subsequently, they were washed with PBS before 100µl/well of YO-PRO (10 µM, Tocris, Bioscience) solution, prepared in standard extracellular solution (ECS; 136 mM NaCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.8 mM KCl, 1.2 mM $CaCl_2$, 5 mM $NaHCO_3$, 20 mM Hepes and 5.5 mM of glucose) was added to the cells. Immediately after, cells were treated with either medium alone or with A-740003 (100µM; Tocris-Bioscience) for 10min at 37°C and 5% CO_2. At the end of the incubation period the plate was inserted in a fluorescent laser imaging plate reader (Flex Station 3; Molecular devices), which measures YO-PRO fluorescence (495nm/515nm excitation/emission). ATP (Sigma-Aldrich) at 1, 5 or 10 mM final concentration (50µl) was automatically added 30sec later, with or without A-740003 (100µM; Tocris Bioscience) in all wells, primed or not-primed with LPS (Sigma-Aldrich). YO-PRO (Tocris Bioscience) fluorescence was measured at 3-second intervals and a mean fluorescence was obtained for 3 identical wells. Fluorescence recordings of YO-PRO (Tocris Bioscience) dye uptake were assessed in BM-MΦ and BM-DC at the same time.

2.16 FITC/BSA dye uptake assay.

To assess the capacity of BM-DC and BM-MΦ to capture foreign antigen, cells were treated with FITC labelled bovine serum albumin (BSA). BM-DC and BM-MΦ were collected and prepared as described in sections 2.2 and 2.3. Parallel treatments were carried out at (a) 37°C and 5% CO_2 and (b) 4°C: cells in both groups received treatment with medium or sodium azide (NaN_3) (20 mM) for 1h. Subsequently, cells were treated with either medium alone or FITC-BSA (Sigma-Aldrich) (200µl/well), diluted in 10% FCS-medium at final concentration of 1mg/ml, and incubated for 30min at 37°C and 5% CO_2 or at 4°C. Cells received 5 cycles of 5-min washes, as described in section 2.10.1, after which they were re-suspended in 5% FCS/PBS buffer. A FACScalibur

machine, CellQuest Pro and FlowJo 8.8.6 software were used to acquire and analyse 10,000 cells.

2.17 Quantifying intracellular and released cytokine in BM-DC, BM-MΦ and PMΦ with ELISA.

2.17.1 Quantifying IL-1β and IL-1α.

Following the various treatments of the cells (BM-DC, BM-MΦ and PMΦ) described in sections 2.8 supernatants and lysates were analyzed for IL-1α and IL-1β content using the DuoSet ELISA Kits (R&D Systems). It is important to note that the recombinant IL-1β antibody used in the assay does not discriminate between the precursor and bioactive forms of IL-1β or IL-1α. Rat anti-mouse IL-1β or IL-1α monoclonal antibody (R&D Systems) was diluted in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na2HPO_4$ and 1.5 mM KH_2PO_4, pH 7.2-7.4) to coat 96 well plates immunoplates (50µl/well at 4µg/ml for IL-1β and 2µg/ml for IL-1α) and incubated overnight at 4°C. Plates received three 5min washing cycles with PBS containing 0.05% Tween-20 (Sigma-Aldrich) before wells were blocked with 1% BSA/PBS for 1h at 37°C. Plates received another round of three 5min washing cycles with PBS containing 0.05% Tween-20 at the end of the incubation period.

Samples and standard recombinant mouse IL-1β or IL-1α (R&D Systems) were diluted in RPMI-1640 media containing 10% FCS (samples at: 1:2, 1:5, 1:10, 1:20, 1:50 and standards at 10000pg/ml). A 9-step serial dilution of the top standard concentration (10,000pg/ml) recombinant IL-1β or IL-1α antibody was performed to create a standard curve. Samples (lysates and supernatants) were added in duplicates, standards in triplicates and RPMI/10% FCS, was added in blank rows at 100µl/well. Plates were incubated for 2h at RT on the orbital shaker. Plates received another set of 3 washing cycles before the biotinylated secondary antibody goat anti-mouse IL-1β or IL-1α (R&D Systems), diluted in reagent diluent (for IL-1β: 0.1% BSA, 0.05% Tween, 20 mM Trizma base, 150 mM NaCl PBS; for IL-1α: 1% BSA in PBS) was added to all wells (50µl/well at 1µg/ml for IL-1β and 0.1µg/ml for IL-1α) and incubated for 2h at RT. A third round of 5min washes was performed and a 100µl/well of streptavidin conjugated to HRP (R&D Systems), diluted 1:200 in reagent diluent was added to the

plates. Plates were incubated for 2h at RT on the orbital shaker at the end of which another set of three 5min washes was performed. Tetramethylbenzidine (TMB; Sigma-Aldrich) substrate was added to the plates at 100µl/well and incubated for 15min in the dark at the end of which the reaction was stopped by adding 50µl/well of stop solution (H_2SO_4, 2M) Finally, the plates were read using a Titertek Multiscan Plus MKII plate reader and Genesis software at 450nm.

2.17.2 Quantifying IL-6.

To analyse IL-6 intracellular content and release, rat anti-mouse IL-6 monoclonal antibody (R&D Systems) was diluted to 2.5µg/ml in coating buffer (15 mM Na2C03 [Fisher Scientific], 35 mM NaHC03 [Fisher Scientific], pH 9.6) to coat 96 well plates immunoplates (50µl/well at 2.5µg/ml) and incubated overnight at 4°C. The following day, plates were washed with PBS containing 0.05% Tween-20 (Sigma-Aldrich) for three 5min cycles. Wells received 150µl/well of 10% FCS/PBS for 30min at 37°C during which, samples (lysates or supernatants) were diluted in RPMI-1640 media containing 10% FCS (1:20, 1:50, 1:100). A 9-step serial dilution of the top standard recombinant mouse IL-6 (Genzyme) concentration (20,000pg/ml) was performed to create a standard curve. Subsequently, samples were added in duplicates and standards in triplicates whilst RPMI-1640/10% FCS was added in blank rows (100µl/well). Plates were incubated for 6h at RT on the orbital shaker. A further three 5-min washing cycles were performed after which the biotinylated secondary goat anti-mouse IL-6 antibody (R&D Systems), diluted in 10% FCS/PBS to 0.2µg/ml, was added to all wells (100µl/well) and incubated overnight at 4°C. Plates received another 3 cycles of 5min washes and then 100µl of ExtrAvidin Peroxidase (Sigma-Aldrich), diluted at 2µg/ml in 10% FCS/PBS, was added in each well. The plates were incubated with the ExtrAvidin for 2h at RT on the orbital shaker after which, the plates received a final set of 3 washing cycles. O-phenylenediamine dihydrochloride (OPD) (0.04g) was reconstituted in citrate-phosphate buffer (47 mM $Na_2HPO_412H_2O$ [Fisher Scientific], 26 mM Citric Acid [Riedel-de Haen, from Sigma-Aldrich] together with 250µl urea hydrogen peroxide adduct (UHP; 96g) dissolved in 25ml citrate phosphate buffer) and the solution was used as a substrate. Finally, the cells received 100µl/well of the substrate and incubated for 15min in the dark at the end of which, the reaction was stopped by

adding 50µl/well of citric acid (0.5M). A Titertek Multiscan Plus MKII plate reader and Genesis software were used to read the plates at 450nm.

2.18 Quantifying relative transcript expression of $P2X_7R$ splice isoform by RT-PCR analysis.

BM-DC and BM-MΦ were prepared as previously described. At day 8 of culture were either medium-treated or incubated with LPS. LPS was diluted in medium-10% FCS to yield a final concentration of 1000ng/ml of LPS in 1ml volume (with 0.9ml cells). Cells were treated for 2h at 37°C in 5% CO_2 before lysates were collected with 1mL of Trizol and frozen until use. Undifferentiated splenocytes and HEK-293 were also prepared as described in section 2.6 and 2.7 and used as positive and negative controls. Samples of each cell type were collected in three independent experiments. Total RNA was extracted from all the cell populations using an RNA isolation system (TRIzol RNA minikit, Invitrogen) according to the manufacturers instructions and subsequently treated with DNase (Ambion, Life Technologies, Carlsbad, California, USA). An equal amount of RNA (300ng) was reverse transcribed using a high capacity RNA-to-cDNA kit (Invitrogen). Reactions in the absence of the reverse transcriptase enzyme were performed in parallel as a control. The cDNA samples were kept at -20°C until used.

A dye based (SYBR Green) qPCR was employed to obtain relative quantification of mRNA levels of the various $P2X_7R$ isoforms of interest with that of the expression levels of hypoxantine phosphoribosyltranferase (HRPT; endogenous reference control gene). All primers were obtained from Sigma-Aldrich and were checked against GenBank for selectivity. The primer sequences are listed in table 2.3. A melt curve was also performed for all primers employed to ensure that these recognize and amplify a single product ensuring the specificity of the primers. qPCR reactions were performed with a SensiFAST no-ROX Kit (Life Technologies) according to the manufacturers instructions using (a) 400nM (final concentration) of forward and reverse primer (table 2.3) (b) 5µl of template cDNA or water (negative control) and (c) the SensiFAST SYBR no-ROX mix or water (negative control) in a total volume of 20µl. All samples were run in triplicates in an ABI StepOnePlus PCR machine (Applied Biosystems, Life Technologies) using the following conditions: 95°C for 2 min and then 40 cycles at: 95°C for 5sec (denaturation), 60-65°C for 10sec (annealing) and 72°C for 5-20 sec

(elongation). Samples were considered positive if the amplification curve crossed the set threshold (set automatically). Fold changes in gene expression for each $P2X_7R$ splice isoform were normalized to the endogenous reference gene (HPRT) and calculated using the 2-ΔΔCT method where:

$$\Delta\Delta C_T = 2^{(CT\ target - CT\ reference)\ of\ calibrator - (Ct\ target - Ct\ reference)of\ sample}$$

PCR samples along with a 100bp interval ladder (BIOLINE, London, UK) were separated in a 1% agarose gel with ethidium bromide dissolved in TBE buffer (Promega). The bands were visualized using a UV-transluminator (Syngene, Cambridge, UK) and a snapshot was taken using a Polaroid camera.

***Table 2.3**: List of PCR $P2X_7R$ and endogenous reference control primers and various reagents.*

PCR primers	Sequence 5' to 3'
HPRT	Forward: GGG CTT ACC TCA CTG CTT TC Reverse: TCT CCA CCA ATA ACT TTT ATG TCC
P2X7A	Forward: CAC ATG ATC GTC TTT TCC TAC Reverse: GGT CAG AAG AGC ACT GTG C
P2X7K	Forward: GCC CGT GAG CCA CTT ATG C Reverse: GGT CAG AAG AGC ACT GTG C
P2X7J	Forward: TTT CAG ATG TGG CAA TTC AGA TA Reverse: AAG TAG GAG AGG GTT GAG CC

2.19 Statistical Analyses.

Data were analyzed using Prism 6.0 and multiple comparisons were considered between groups using one-way ANOVA and Dunnett's post hoc tests. Tukey's was employed when comparisons were made between all treatment groups and two-way ANOVA and Sidak's multiple comparison post-hoc tests were employed for comparisons between different treatments of two cell types of interest. The results were expressed as the mean ± SEM and were considered statistical significant when the p value was less than 0.05.

***Table 2.4**: List of antibodies employed.*

Primary antibodies for phenotypic analysis of cells	
Product name:	**Manufacturer:**
Rat anti-mouse MHC class II (at 2.5µg/ml)	BD Biosciences
Rat anti-mouse CD86 (at 10µg/ml)	BD Biosciences
CD54 (at 10µg/ml)	BD Biosciences
Rat anti-mouse CD11b - PE-labelled (at 10µg/ml)	BD Biosciences
Hamster anti-mouse CD11c- PE-labelled, (at 4µg/ml)	BD Biosciences
Rat anti-mouse F4/80- PE-labelled, (at 4µg/ml)	E Biosciences
Rat anti-mouse CD16/32 (FcGIII/II Receptor), (at 5µg/ml)	BD Biosciences
Isotype controls and secondary antibodies:	
Rat anti-mouse IgG2a (at 10µg/ml)	BD Biosciences
Rat anti-mouse IgG2a -PE-labelled (at 4µg/ml)	BD Biosciences
Rat anti-mouse IgG2b - PE-labelled (at 10µg/ml)	BD Biosciences
IgG1κ- PE-labelled (at 4µg/ml)	BD Biosciences
Star 69: goat anti-rat IgG - FITC-labelled (at 7µg/ml)	ABD serotec

$P2X_7R$ antibodies and Isotype controls	
Product name:	**Manufacturer:**
Polyclonal anti-$P2X_7R$ (3µg/ml)	Alomone Labs, Reactivity: mouse, rat and human Intracellular epitope (576-595 a.a. in the C-terminus)
Monoclonal rat anti-$P2X_7R$ (Hano 43) (1, 5 or 10µg/ml)	Santa Cruz, Reactivity: mouse and rat, Extracellular epitope
Monoclonal rat anti-$P2X_7R$ (used at 1:100, concentration n/a)	Stratech Scientific, Epitomics, Reactivity: mouse, rat and human, Intracellular epitope
Rabbit polyclonal IgG isotype control (3µg/ml)	R&D system
Goat anti-rabbit IgG Alexa Fluor 488-labelled (20µg/ml)	Invitrogen

IL-1β ELISA -DY401 (R&D Systems)		
Product name:	**Manufacturer:**	**General info:**
Capture Antibody: Rat anti-mouse IL-1β	R&D Systems	Concentration employed: 4µg/ml
Secondary Antibody: Biotinylated goat anti-mouse IL-1β	R&D Systems	Concentration employed: 1µg/ml
Standard: recombinant IL-1β	R&D Systems	Top concentration used: 10ng/ml
Antibodies for IL-1α ELISA – DY400 (R&D Systems):		
Capture Antibody: Rat anti-mouse IL-1α	R&D Systems	Concentration employed: 2µg/ml
Detection Antibody: Biotinylated goat anti-mouse IL-1α	R&D Systems	Concentration employed: 100ng/ml
Standard: recombinant mouse IL-1α	R&D Systems	Top concentration employed: 10ng/ml
Antibodies for IL-6 ELISA:		
Product name:	**Manufacturer:**	**General info:**
Capture Antibody: Rat anti-mouse IL-6	R&D Systems	Concentration employed: 2.5µg/ml
Standard: recombinant mouse IL-6	Genzyme	Top concentration:20ng/ml
Detection Antibody: Biotinylated goat anti-mouse IL-6	R&D Systems	Concentration employed: 100ng/ml

Chapter 3:
Phenotypic and functional properties of murine bone marrow derived dendritic cells (BM-DC).

3.1 Introduction.

Ralph M. Steinman first described the distinct stellate population of classic DC in 1973, which he isolated from the mouse spleen. This was a minor population irregular in shape and phenotypically different to macrophages and lymphocytes. Since then DC have been identified in all tissues and it is thought that each tissue has several distinct local DC populations. They are part of the mononuclear phagocyte population and they are effective sensors for the presence of foreign antigen with which they potently activate naïve T-cells [15, 54]. As such, DC have become a major cell target for therapeutic applications and attract considerable attention in examining their biology and cellular functions.

Efforts to isolate DC from animal tissues are time-consuming and relatively ineffective, yielding low numbers of cells [430]. For example, only about ten thousand DC can be isolated from a single mouse spleen or thymus [431]. In 1986 Bowers described an alternative method for deriving myeloid DC in vitro by expanding rat BM-derived haematopoietic precursors in the presence of GM-CSF for 6-8 days. The protocol was later applied to murine [83] and human tissues [432]. Treating BM marrow cells with GM-CSF for about 6-8 days consistently generates approximately 5 million cells from one mouse. Therefore, generation of DCs with GM-CSF treatment has since become a popular tool for immunological investigations [433, 434]. Usually, DC populations have a relatively short half-life in their various DC compartments and are therefore continuously replenished by HSC or progenitor cells. GM-CSF was the first cytokine to be discovered that induces the differentiation of DC in vitro from progenitor cells [435]. GM-CSF is produced by fibroblasts, endothelial cells and activated lymphocytes in the mouse although it is thought that most cells have the capacity to synthesize the factor. The basal levels of the factor are undetectable in the circulation [436] whilst systematic administration of GM-CSF in mice results in a surge of inflammatory cells such as neutrophils and macrophages in the circulation [437]. Additionally, the absence of GM-CSF or the common β chain of its receptor has little impact on the DC population in vivo [438]. The targeted deletion of both GM-CSF and Flt3-L from mice results in the complete obliteration of a subset of dermal DC suggesting that there is some sort of compensatory mechanism between the two cytokines to regulate DC homeostasis [439, 129, 89].

In vitro, the non-adherent population of cultured BM-DC with GM-CSF was found to develop a DC-like phenotype expressing MHC class II, the classic DC-associated integrin CD11c and ICAM-1/CD54. The cells also exhibit high endocytic activity, are capable of up-regulating the production and release of pro-inflammatory cytokines as well as chemokines and express a pattern of TLRs similar to those found expressed by isolated epidermal LC [83, 440]. In general, the phenotype of the homogeneous population of GM-CSF-derived DCs resembles that of CD8- cDC found in the spleen and in lymphoid tissues based on their pattern of cell surface marker expression (MHC class II^+, $CD11c^+$, $CD11b^+CD8^-$) [4]. These cells constitute a popular tool for investigating DC biology particularly due to the capacity to consistently provide sufficient numbers for experimentation [83].

Different combinations of soluble factors have been used to generate myeloid DC precursors from BM cells in vitro. Whereas culture of BM progenitors with GM-CSF alone is particularly successful for the development of mouse BM-DC, human BM progenitors require a combination of GM-CSF and TNF-α. Additionally, IL-4 is usually required for culturing rat and cattle BM-DC or when GM-CSF becomes a limiting factor whilst IL-3 is used in BM cultures to support the development of DCs and mast cells [441]. Stem cell factor (SCF) and foetal liver tyrosine kinase 3 ligand (Flt-3L, CD135) are also often used to enhance the proliferative capacity of the DC progenitors. However, the use of Flt3-L has been shown to result in the development of a mixture of both pDC and conventional DCs (cDC) [442] but also a number of natural killer (NK) cells [443].

Generating DCs from murine BM precursors was quickly established as the method of choice for generating large numbers of a relatively homogeneous population of DC precursors in a reliable manner. Inevitably, despite this general homogeneity, there are aspects of the phenotype of DC generated *in vitro* that will exhibit inter-experimental variation. A major issue with the use of BM-DC cultures is contamination by small populations of neutrophils, mast cells and macrophages. A number of factors can influence the purity of the DC precursors such as the concentration of GM-CSF, seeding density and time in culture. In the studies conducted herein, the conditions for BM-DC culture were kept as closely as possible to the conditions outlined by Inaba and colleagues, particularly with respect to the aforementioned parameters such as the

concentration of GM-CSF [83]. Thus, the main objective of the investigations described in this chapter was to establish the optimum time frame in culture to propagate a high-purity yield of naïve BM-DC precursors with DC-like phenotypic characteristics and whether these acquire a mature inflammatory phenotype in response to inflammatory stimuli.

3.2 Results.

3.2.1 Characterizing untreated (control) BM-DC with regards to surface marker expression.

BM-DC were employed as the most suitable representative of primary cell expanded DC to investigate the various objectives of these experiments. The protocol for generating DCs from BM precursors was adapted from that published by Inaba and colleagues [83]. Fresh BM cells were cultured *in vitro* in the presence of GM-CSF as is described in Chapter 2 section 2.2. The non-adherent population of cells was isolated following 6, 8 or 12 days in culture and analysed for various phenotypic markers by flow cytometric analyses. The gating strategy for BM-DC populations is illustrated in figure 3.1. Analysis of side scatter (granularity) versus forward scatter (size) revealed that the cell population varied in size but had similar granularity (Figure 3.1a). The cells were stained with propidium iodide (PI) to assess the viability of the cells and the stained cells (gate R2) were excluded from subsequent analyses for the expression of the markers of interest (Figure 3.1b). For each marker of interest, cells were stained with an appropriate antibody and its specific isotype control. The results were plotted as histograms. Figure 3.1c illustrates how cells that stained more strongly with the antibody for the marker of interest than with the isotype control antibody than (point of intersection; M1) have been selected to determine the percentage of the population that was positive for the marker of interest (% positives) and the intensity of expression for that marker per cell (mean fluorescence intensity; MFI).

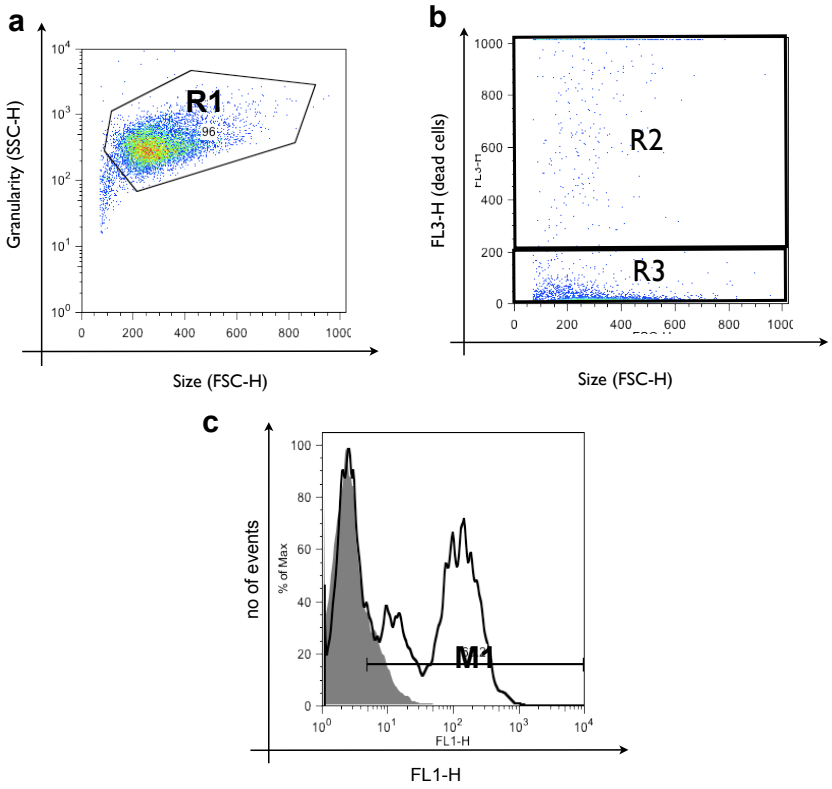


***Figure 3.1:* The gating strategy for day 6 BM-DC for analysis of cell surface marker expression by flow cytometry.**
Cells were first gated by forward scatter for their size (FSC-H) and by side scatter for their granularity (SSC-H) and the selected cells were denoted as gate R1 (a). Propidium iodide was used to stain for dead cells: denoted as gate R2. These were excluded from further analysis. Gate R3, which represents live cells, was included in further analysis (b). A representative histogram is included to illustrate how the intensity of expression of the marker of interest was determined. The histogram plotted for cells positive for the marker of interest (blue line) was overlaid on that plotted for the appropriate isotype control (red line). A line (M1) was drawn at the point of intersection of the two histograms thus selecting the cells whose MFI was higher than that of the isotype control (cells positive for the expression of the marker of interest). Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis.

Initially, the effect of time in culture on the viability of cells *in vitro* was examined. As described previously, cells were stained with PI and analysed using flow cytometry. The results are shown in Figure 3.2. The viability of cells was determined in 3 independent experiments following culture for 6, 8 and 12 days, the mean and standard errors of which are shown in Figure 3.1. The viability of BM-DC decreased from approximately 90% at day 6 to 80% at day 8 and 12 of culture. However, the effect was not statistically significant.

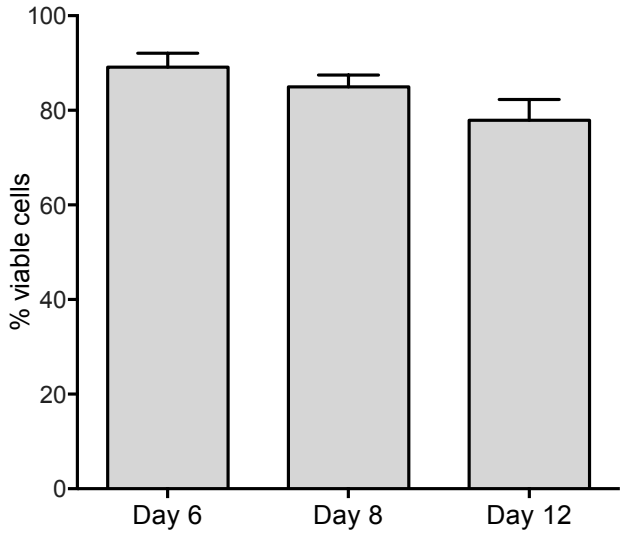


Figure 3.2: Changes in viability of BM-DC over time in culture.
Following 6, 8 or 12 days in culture in the presence of GM-CSF, BM-DC at $1x10^6$ cells/ml were incubated with medium alone for 24h. Cells were stained with propidium iodide and cell viability was determined using flow cytometry. Data shown are mean (n=3) ±SEM. Statistical differences between groups were considered by one way ANOVA followed by Tukey's multiple comparison post-hoc test. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. No group comparisons were statistically significant.

3.2.2 Analysis for phenotypic markers of untreated (control) BM-DC by flow cytometry.

Inaba and colleagues report that 7-8 days is the optimal period to propagate BM-DC *in vitro* in the presence of GM-CSF [83] [444]. Therefore the effect of time in culture on the phenotype of BM-DC was explored. The cells were analysed for the antigen presenting complex MHC class II, the co-stimulatory molecules CD86, CD54 and the integrin CD11c following 6, 8 or 12 days in culture with GM-CSF. Figure 3.3 shows a selection of representative histograms of flow cytometric analyses, which were used to calculate the percentage of expression and MFI for each of the markers of interest.

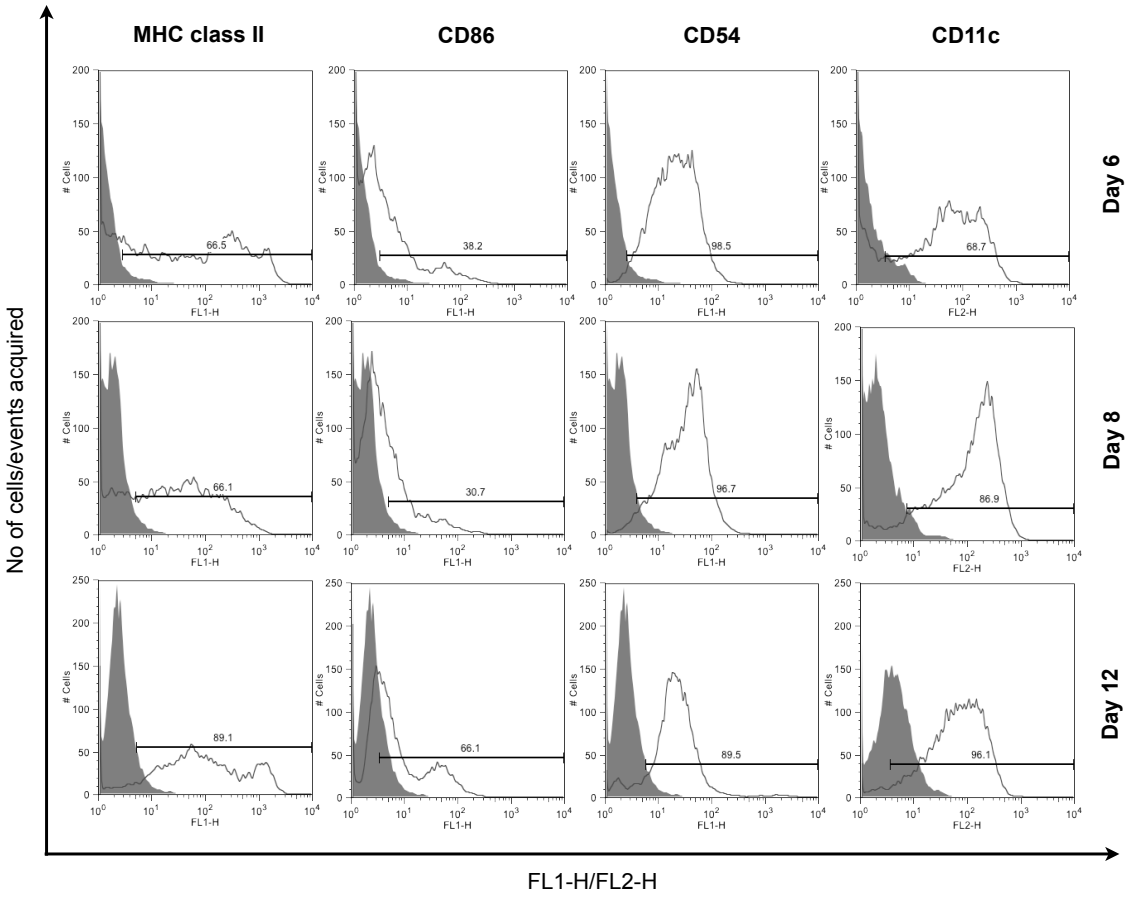


***Figure 3.3:* Expression of various cell markers was assessed by flow cytometry on resting day 6, 8 and 12 BM-DC: representative FACS plots.**
Day 6, 8 and 12 BM-DC were cultured for 24h at $1x10^6$ cells/ml in the presence of medium alone. The expression of the markers of interest is shown as plotted histograms (open line) overlaid with the appropriate isotype control (closed line). Expression of the various markers of interest was determined using the 3 step gating system as described in Figure 3.1. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. Representative plots are shown for each marker at each time point.

The flow cytometric data acquired were analysed to determine the percentage of the population that was positive for the marker/receptor of interest (Figure 3.4a) and for the density of expression of the marker/receptor per cell analysed (expressed as MFI in arbitrary units [au]) (Figure 3.4b). Three independent experiments were conducted with day 6, 8 and 12 cells. As shown in Figure 3.4, the percentage of cells expressing MHC class II increased significantly from approximately 40% at day 6 to 80% at day 12. The MFI levels for MHC class II expression, however, remained fairly constant over the same time period (ranging from 150 to 200au). There was no change in the frequencies of cells positive for the co-stimulatory molecule CD86 (35%) and the adhesion molecule CD54 (80%) between day 6 to day 12. The MFI for CD86 expression were comparatively low, remaining at approximately 15-20 au. An up-regulation of CD54 expression was observed from approximately 30au for day 6 cells to 65au for day 12 cells, however, such did not reach statistical significance. The percentage of cells expressing CD11c increased significantly from 60% at day 6 to 80% at day 8 and day 12. Levels for CD11c expression (measure as a function of MFI) also increased significantly from approximately 60au on day 6 cells to 200au on day 8 cells, followed by a decrease to 100au on day 12 cells.

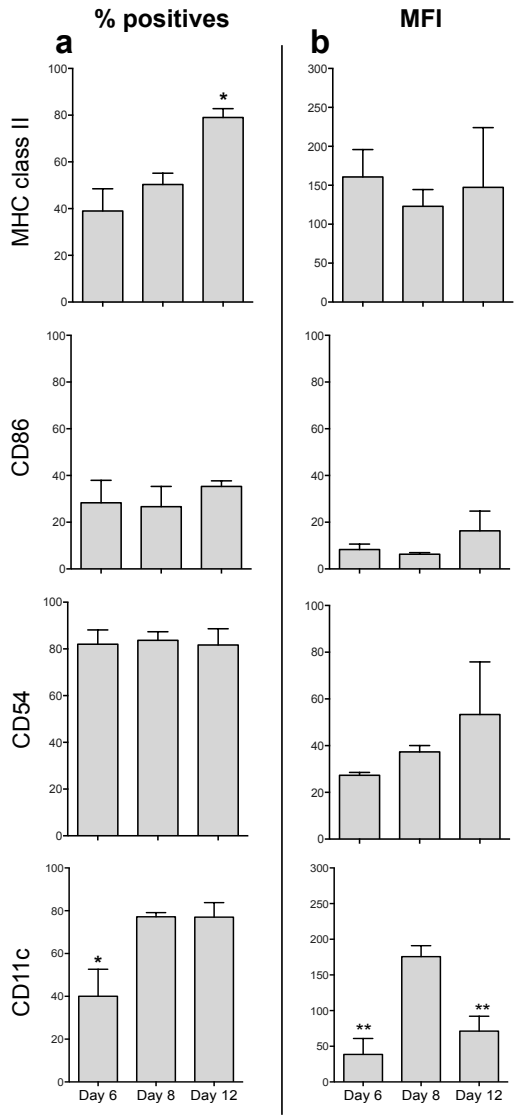


***Figure 3.4:* The kinetics of changes in expression and frequency of cells positive for the surface DC markers of interest on resting BM-DC.**
Day 6, 8 or 12 cultured BM-DC were incubated in medium alone for 24h at $1x10^6$ cells/ml. Cells were analysed by flow cytometry for expression of the cell surface markers of interest (MHC class II, CD86, CD54 and CD11c) and results are recorded as (a) the percentage positive cells and (b) mean fluorescence intensity (MFI), over time. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000); CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. Data shown are mean (n=3) ±SEM. Statistical significance of differences between groups was assessed by one way ANOVA and Tukey's multiple comparison post-hoc test (*= $p<0.05$ and **=$p>0.01$).

3.2.3 Phenotypic changes in BM-DC following activation with LPS.

The next objective was to analyse the effect of endotoxin (LPS) treatment on the phenotype of day 6, 8 and 12 BM-DC. At first, the effect of increasing concentrations of LPS on cell viability, assessed with PI staining, was investigated. Figure 3.5 shows the impact of LPS treatment on the percentage of viable cells at days 6, 8 and 12 in culture. The cell viability of day 8 and day 12 BM-DC was affected at the top concentration of LPS (10, 100 and 1000ng/ml) with a significant decrease from 90% to 80% viable cells for day 8 BM-DC and from 80% to 60% for day 12 BM-DC. Although there was small decline of cell viability with LPS treatment for day 6 BM-DC, this was not statistically significant. Day 12 BM-DC were found to be more susceptible to cell death in response to culture with LPS. Cell viability of day 12 BM-DC was significantly lower compared with those recorded for day 6 at all concentrations of LPS.

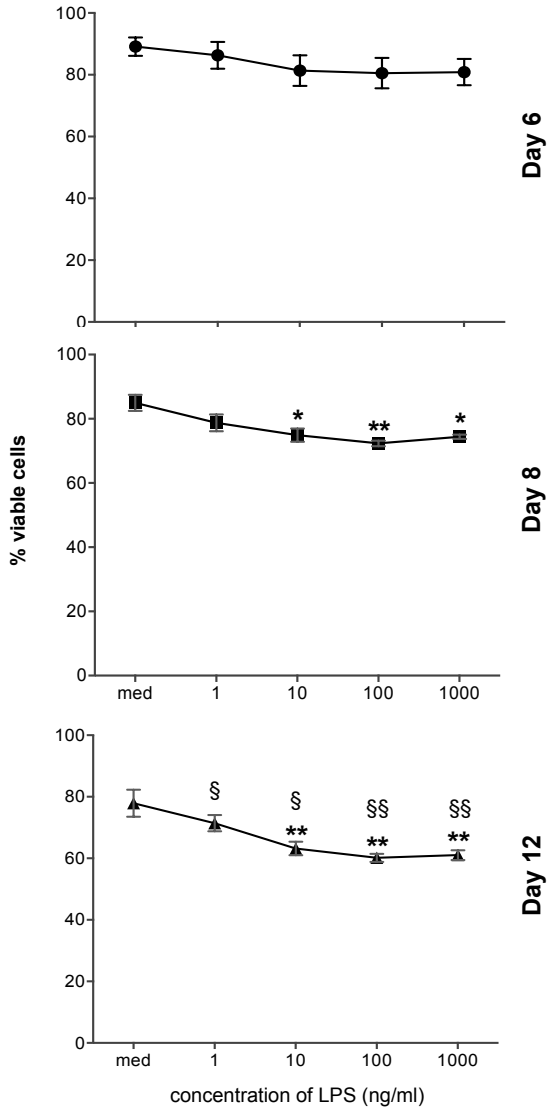


***Figure 3.5:* Changes in cell viability of BM-DC following activation with LPS, over time.**

Day 6, 8 or 12 cultured BM-DC at $1x10^6$ cells/ml were incubated with medium alone (med) or activated with LPS at 1, 10, 100 and 1000ng/ml for 24h. Cells were stained with propidium iodide and cell viability was determined at days 6, 8 and 12 using flow cytometry. Data shown are mean (n=3) ±SEM. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. Statistical significance of differences between groups (media-treated cells as a comparator) was assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (*= $p<0.05$ and **=$p>0.01$). Statistical significance of differences was also considered between cells that have received identical treatment but at different stage of culture in vitro (day 6, 8 and 12) (day 6 cells as a comparator) by one way ANOVA and Dunnett's multiple comparison post-hoc test (§= $p<0.05$ and §§=$p>0.01$).

Changes in the phenotype of BM-DC following endotoxin activation was also investigated by flow cytometric analyses of selected membrane markers. Figure 3.6 shows representative histograms for the expression of the markers of interest on day 6 BM-DC, which were either treated with medium alone or pulsed with LPS at different concentrations (1 to 1000ng/ml) for 24h. MHC class II expression in medium-treated cells was rather heterogeneous, however, a peak shifting to the right was observed with LPS treatment. Similarly, CD86 expression in medium-treated cells was largely negative which also had shifted to the right with LPS treatment. CD54 expression was found rather heterogeneous in medium-treated cells, however, 2 distinct peaks become apparent with LPS treatment characteristic of two subpopulations expressing different levels of CD54. CD11c was found to be somewhat heterogeneous with no marked change observed following treatment with LPS.

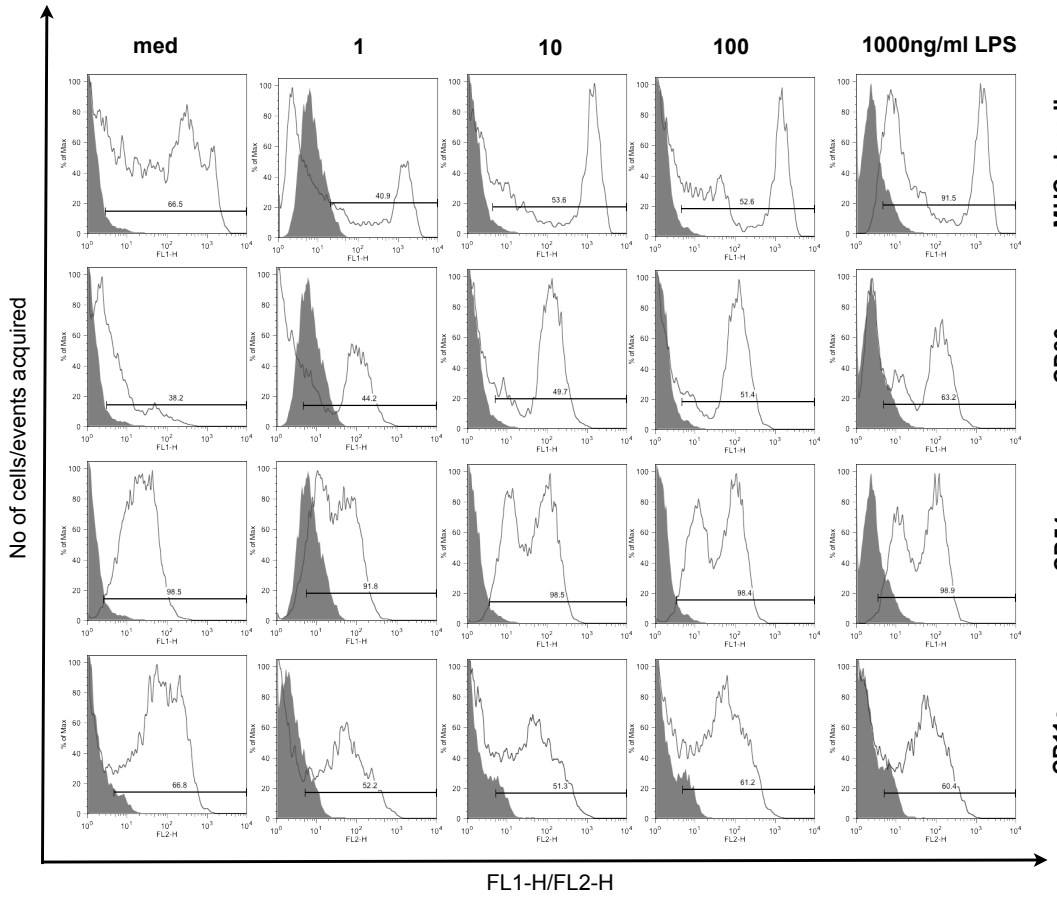


***Figure 3.6:* Flow cytometric analyses of DC cell surface markers on day 6 BM-DC following activation with LPS.**
Day 6 cultured BM-DC at $1x10^6$ cells/ml were incubated with medium alone (med), or stimulated with LPS at 1, 10, 100, or 1000ng/ml for 24h. Cells were then analysed by flow cytometry for the expression of the markers of interest (representative histograms shown). The histograms were used to derive the percentage of BM-DC expressing the various cell surface markers of interest (percentage positive) and the mean fluorescence intensity for each marker. The closed lines on the graphs represent the isotype control (IgG2a or IgG2b) whereas the open lines represent the expression of each cell surface marker. The point of interception of the two lines was determined and used to calculate the percentage of cells expressing the different markers of interest and their mean fluorescence intensity. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis.

Day 6 BM-DC were investigated for signs of maturation following activation with LPS as depicted in Figure 3.7a. The percentage of cells expressing MHC class II, the co-stimulatory molecules, CD86 and CD54 and CD11c did not change significantly with increasing concentrations of LPS. Approximately 45-50% of the population of BM-DC were found to express MHC class II and 30-40% of cells expressed CD86. A comparatively higher percentage of cells expressed CD54 receptors, ranging from 50-80%. Finally, approximately 40-50% of day 6-BM-DC expressed the classic DC marker, CD11c.

In contrast, as depicted by the figures in figure 3.7b, a significant LPS-induced increase in the expression of MHC class II molecules was noted on the cell surface of BM-DC (from approximately 200au to 500au). CD86 MFI expression was 10au in the media-treated group and this increased significantly to 75au at the highest concentration of LPS. Expression of CD54 was increased 2-fold (from approximately 30 to 70au) by culture with LPS, however, such did not reach statistical significance. CD11c expression was unaffected by LPS treatment, remaining at approximately 40au.

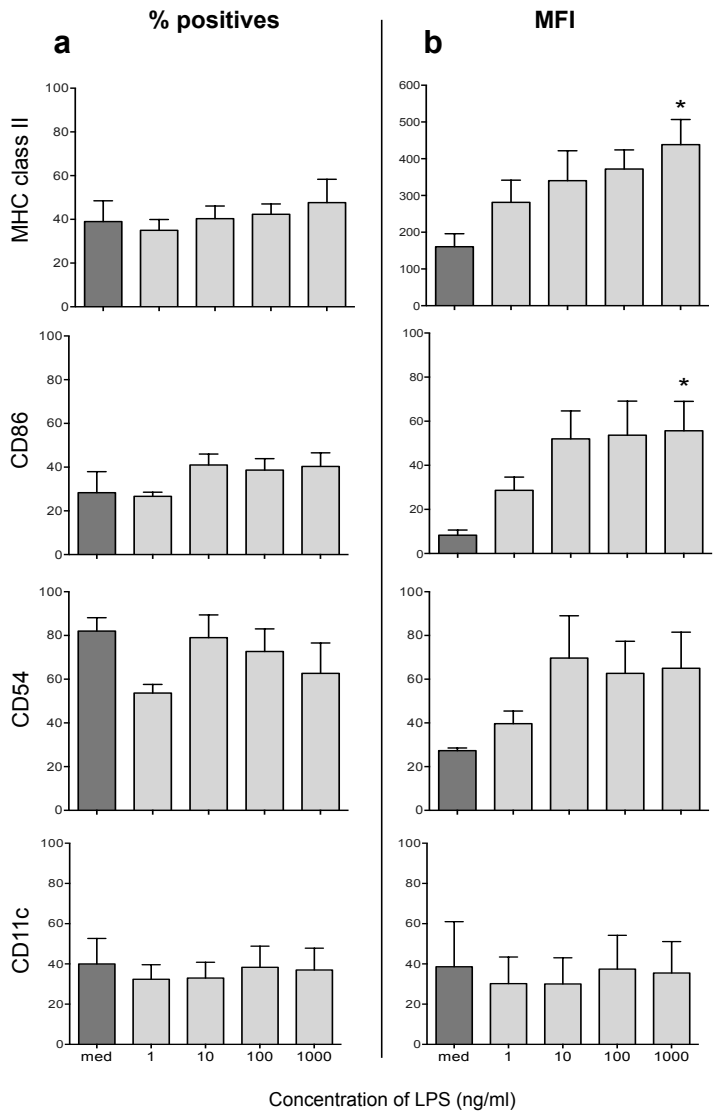


Figure 3.7: Frequency of cells positive and level of expression for DC markers on day 6 BM-DC: impact of LPS.

Day 6 cultured BM-DC were incubated with medium alone (med), or stimulated with LPS at 1, 10, 100, or 1000ng/ml for 24h at $1x10^6$ cell/ml. Cells were analysed by flow cytometry for surface expression of the markers of interest (MHC class II, CD86, CD54 and CD11c) and data are shown with respect to (a) the percentage positive cells and (b) the mean fluorescence intensity (MFI) with increasing concentrations of LPS. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. Data shown are mean (n=3) ±SEM. Statistical significance of differences between groups (media-treated cells as a comparator) was assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (*=$p<0.05$).

Representative histograms for the expression of the markers of interest on day 8 BM-DC, treated with medium alone or with LPS at different concentrations for 24h are shown in figure 3.8. The histograms depict the phenotypic changes of BM-DC induced with endotoxin treatment. Like with day 6 BM-DC MHC class II expression of medium-treated day 8 BM-DC was rather heterogeneous, however, F4/80 a distinct shift to right was observed with LPS treatment. CD86 expression was largely negative in medium-treated cells with a clear shift to the right detected with LPS treatment. CD54 expression was found homogeneous with no marked change detected following treatment with LPS. CD11c expression was largely homogeneous in medium treated cells. Expression of the DC integrin was slightly more heterogeneous in LPS treated BM-DC in comparison with untreated cells.

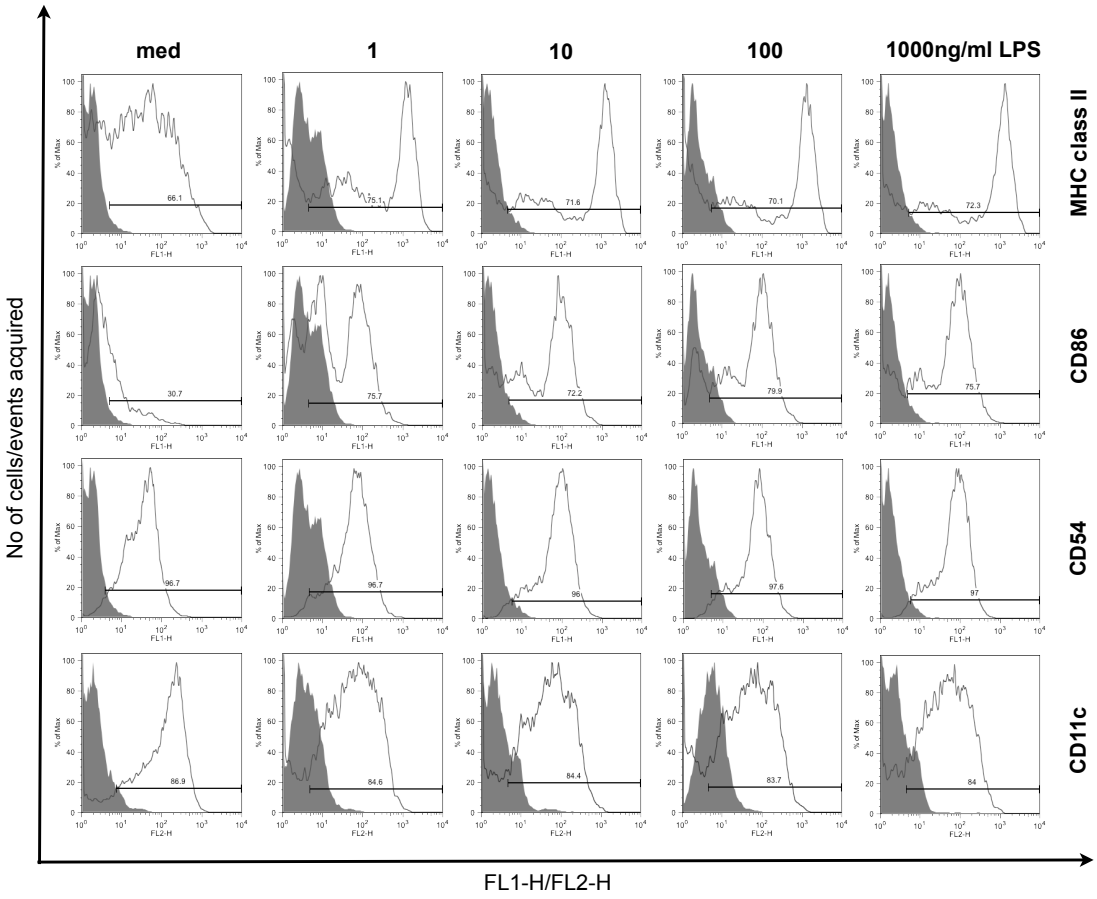


***Figure 3.8:* Flow cytometric analyses of DC cell surface markers on day 8 BM-DC following activation with LPS.**
Day 8 cultured BM-DC at $1x10^6$ cell/ml were incubated with medium alone (med), or stimulated with LPS at 1, 10, 100, or 1000ng/ml for 24h. Cells were then analysed by flow cytometry for the expression of the markers of interest (representative histograms shown). The histograms were used to derive the percentage of BM-DC expressing the various cell surface markers of interest (percentage positive) of interest and the mean fluorescence intensity for each marker. The closed lines on the graphs represent the isotype control (IgG2a or IgG2b) whereas the open lines represent the expression of each cell surface marker. The point of interception of the two lines was determined and used to calculate the percentage of cells expressing the different markers of interest and their mean fluorescence intensity. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis.

Changes in the profile of BM-DC with endotoxin activation was also analysed on day 8 of culture. As shown by the results in Figure 3.9 at day 8 approximately 55% of the BM-DC population expressed MHC class II molecules which was unaffected by LPS activation even at the top doses. Conversely, there was a significant dose-dependent increase in CD86 expressing cells with LPS activation, from 30% to 60%. This was not reflected in CD54 or CD11c expression where LPS treatment was without effect. Approximately 75-80% of both medium-treated and LPS-treated of day 8 BM-DC were found to express CD54 and 80% of the cells expressed the CD11c integrin, regardless of exposure to endotoxin.

The LPS-induced changes in the phenotype of day 8 BM-DC were more marked with respect to the MFI readings of the markers of interest shown in figure 3.9b. A significant 3-fold increase in the levels of MHC class II expression was observed with the top 3 concentrations of LPS (from 200au in the medium-treated population to 800au in the 1000ng/ml LPS-treated group). A similar pattern, albeit with more inter-experimental variation, was observed for the expression of CD86, with baseline expression of 10au peaking at 75au in the 100ng/ml LPS-treated group. A similar increase was observed for CD54 expression, however, such did not reach statistical significance. LPS treatment was without significant effect for CD54 or CD11c levels of expression

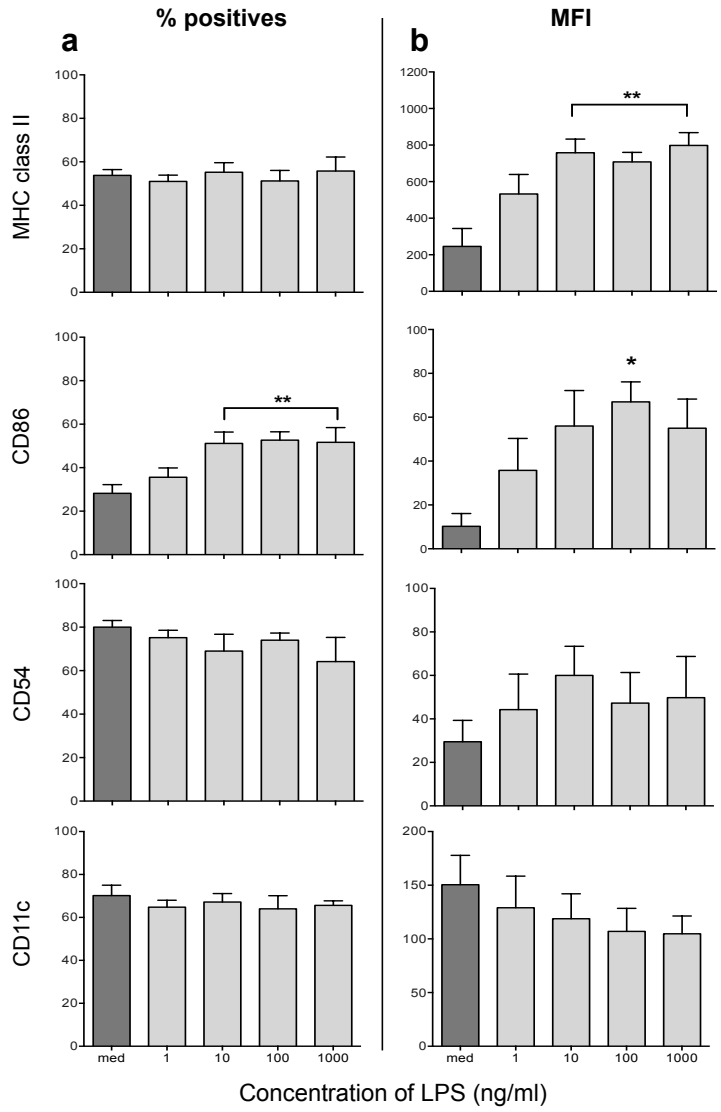


***Figure 3.9*: Frequency of cells positive and level of expression for DC markers on day 8 BM-DC: impact of LPS.**

Day 8 cultured BM-DC at $1x10^6$ cells/ml were incubated with medium alone (NT), or stimulated with LPS at 1, 10, 100, or 1000ng/ml for 24h. Cells were analysed by flow cytometry for surface of expression of the markers of interest (MHC class II, CD86, CD54 and CD11c) and data are shown with respect to (a) the percentage positive cells and (b) the mean fluorescence intensity (MFI) with increasing concentrations of LPS. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. Data shown are mean (n=3) ±SEM. Statistical significance of differences between groups (media-treated cells as a comparator) was assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (*= $p<0.05$ and **=$p<0.01$).

Figure 3.10 shows representative histograms for the expression of the markers of interest on day 12 BM-DC, treated with medium alone or with LPS at different concentrations for 24h. A clear shift to the right was observed with MHC class II expression in LPS-treated BM-DC in comparison to untreated cells. CD86 expression was largely negative in medium-treated BM-DC. LPS treatment cause a clear shit to the right with two distinct peaks detected in BM-DC treated with 1ng/ml LPS. The second peak became much less obvious following treatment with higher levels of LPS. Expression of both CD54 and CD11c was found homogeneous with no marked changes observed following LPS treatment.

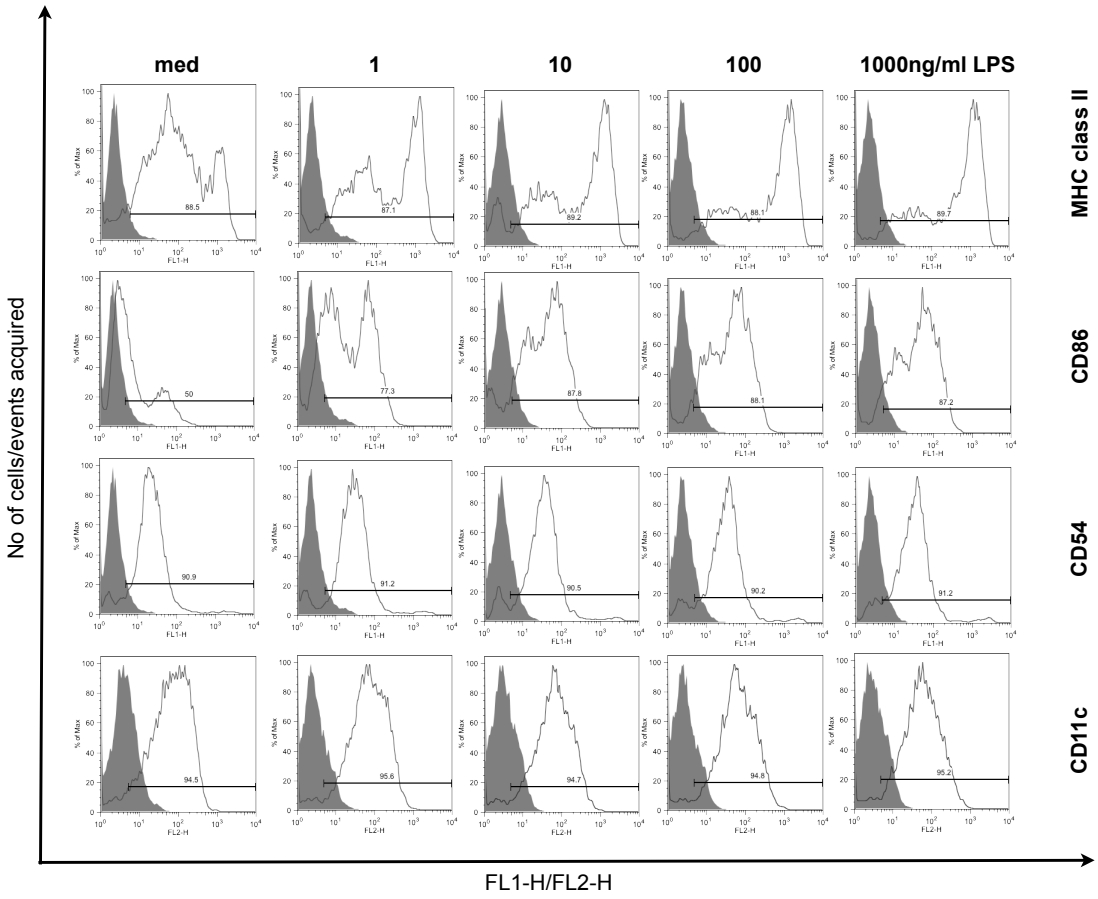


Figure 3.10: Flow cytometric analysis of DC cell surface markers on day 12 BM-DC following activation with LPS.

Flow cytometric analysis of DC cell surface markers on day 12 BM-DC following activation with LPS Day 12 cultured BM-DC at $1x10^6$ cells/ml were incubated with medium alone (med), or stimulated with LPS at 1, 10, 100, or 1000ng/ml for 24h. Cells were then analysed by flow cytometry for the expression of the markers of interest (representative histograms shown). The histograms were used to derive the percentage of BM-DC expressing the various cell surface markers of interest (percentage positive) of interest and the mean fluorescence intensity for each marker. The closed lines on the graphs represent the isotype control (IgG2a, IgG2b or IgG1λ) whereas the open lines represent the expression of each cell surface marker. The point of interception of the two lines was determined and used to calculate the percentage of cells expressing the different markers of interest and their mean fluorescence intensity. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis.

In subsequent experiments BM-DC were also isolated after 12 days in culture to analyse changes in the profile of DC marker expression induced by endotoxin activation. As shown in figure 3.11a, the constitutive expression of the various markers of interest is comparatively higher than day 6 and day 8 BM-DC, characteristic of a more mature DC phenotype. Approximately 80% of media-treated BM-DC expressed detectable MHC class II and this was not significantly affected by the introduction of LPS. Culture with LPS was without effect on the percentage of cells expressing CD86 (a minor increase was observed but did not reach statistical significance), CD54 or CD11c of the two markers.

Considerable inter-experimental variation characterised the expression levels of all the markers of interest on day 12 BM-DC, particularly with respect to MHC class II and CD86 expression as can be seen in figure 3.11b. The MFI levels for MHC class II expression CD54 and CD11c remained largely unchanged with LPS treatment. The MFI readings of CD86 expression saw a minor increase with LPS treatment but failed to reach statistically significant levels.

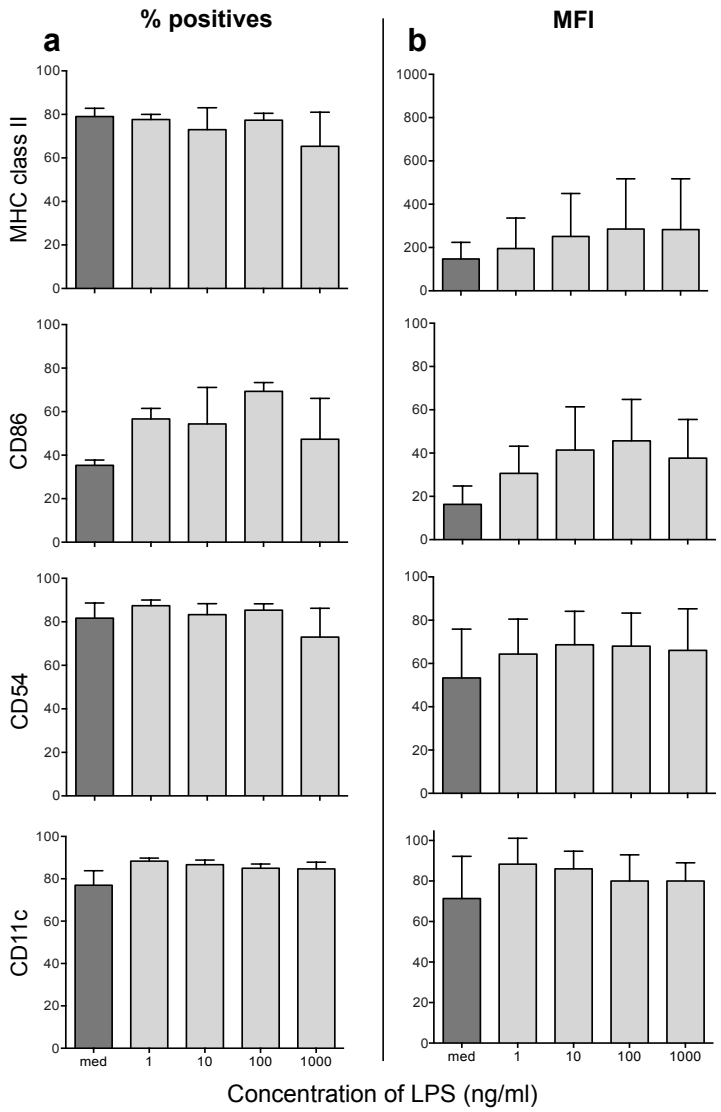


***Figure 3.11:* Frequency of cells positive and level of expression for DC markers on day 12 BM-DC: impact of LPS.**
Day 12 cultured BM-DC at $1x10^6$ cells/ml were incubated with medium alone (med), or stimulated with LPS at 1, 10, 100, or 1000ng/ml for 24h. Cells were analysed by flow cytometry for surface of expression of the markers of interest (MHC class II, CD86, CD54 and CD11c) and data are shown with respect to (a) the percentage of positive cells and (b) the mean fluorescence intensity (MFI) with increasing concentrations of LPS. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. Data shown are mean (n=3) ±SEM. Statistical significance of differences between groups (media-treated cells as a comparator) was assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test. There were no statistically significant differences between groups.

3.2.4 Endotoxin activation of BM-DC induces pro-inflammatory cytokine release.

Another important endpoint of BM-DC maturation is pro-inflammatory cytokine release, such as IL-6. Thus, BM-DC were harvested after 8 days in culture and treated with increasing concentrations of LPS or medium alone for 2 or 24h. Figure 3.12 shows the dose dependent secretion of IL-6 following LPS activation. In the absence of LPS, baseline cytokine expression was below the limit of detection at 2h and approximately 0.186 ng.ml after 24h. There was significant dose-dependent increase in IL-6 release that peaked at about 15ng/ml at the top concentration of LPS after 2h of culture. A similar profile of IL-6 secretion was observed in BM-DC cultured with LPS for 24h. The levels of IL-6 release were substantially higher and reached maximal levels at approximately 40ng/ml with 10ng/ml LPS treatment.

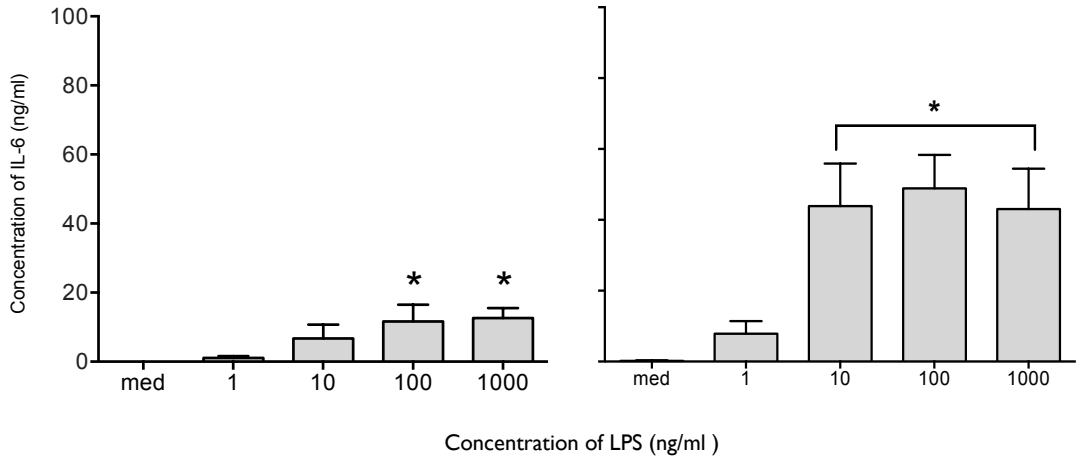


Figure 3.12: IL-6 release from BM-DC following LPS treatment.
Day 8, cultured BM-DC at $1x10^6$ cells/ml were incubated in medium alone (med), or stimulated with LPS at 1, 10, 100 or 1000ng/ml. Cells were treated with LPS for 2 or for 24h. The levels of IL-6 were measured using a cytokine-specific ELISA. Data shown are mean (n=3) ±SEM. Statistical significance of differences between groups (medium-treated BM-DC were used as a comparator) was assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (*= $p<0.05$).

3.3 Discussion.

3.3.1 Mapping the profile of murine in vitro cultured BM-DC.

The results herein demonstrate that 8 days *in vitro* culture with GM-CSF is the most appropriate time-frame for BM progenitors to differentiate into DC-like profile (based on the expression of the DC-associated CD11c integrin). When the cells were challenged with the potent endotoxin LPS, they up-regulated the necessary components required for the formation of the immunological synapse with T-cells for antigen presentation. The antigen presenting machinery includes molecules that provide co-stimulatory signals and also include soluble, inflammatory mediators (IL-6 secretion) that complete T-cell activation and clonal expansion. The results of these investigations are in accordance with the previous observations by Dearman and colleagues who performed a thorough analysis of the responsiveness of BM-DC to TLR ligands. Based on their observations the group conclude that the cells display similar characteristics to freshly isolated mouse epidermal LC following TLR activation [424].

DC populations in the mouse system are in fact small in size and involve complicated procedures to isolate the cells, which often impact on the phenotype of the cells. For example, Vremec and colleagues raised concerns that trying to isolate DC from the thymus using a low buoyant density fraction by centrifugation results in the formation of dense, non-adherent clusters of thymocyte cells and DC [431]. In contrast, expanding DC from BM progenitors in the aegis of GM-CSF results in a naïve DC-like population that constitutes a physiological relevant cell type and a better choice than immortalized cell lines to investigate DC function. Many cell lines harbour incomplete or malfunctioning signal transduction pathways that can lead to incorrect conclusions regulating the phenotype or functional activities of DC. For example even though the cells of the human acute monocytic leukemia THP-1 cell line harbour all the necessary inflammasome components, they do not respond to known activators of the inflammasome such as ATP [445]. Instead, one of the preferred methods for investigating DC is to expand progenitor cells from murine BM using specific cytokine cocktails [424].

Inaba and colleagues pioneered the system of generating DC from BM precursors under the aegis of GM-CSF [83]. The group reported the presence of three different types of cells in these cultures. The first type of cell exhibited a mononuclear profile, larger in size than regular phagocytes and assuming an irregular shape with protruding dendritic processes. The colonies of these cells grew in suspension, were loosely adherent (could be easily dislodged by swirling of the plate) and also stained strongly for MHC class II. A second population of cells was characterised by numerous cytoplasmic vacuoles, was relatively large in size, circular in shape and stained positive for macrosialin, a protein expressed on cytoplasmic granules of macrophages. Finally, the third population of cells was characterised by a distinct irregularly shaped nucleus much like granulocytes was also observed. An important difference was that the macrophage population was firmly adhered to the bottom of the culture dish whereas DC arose as single cells in suspension from marrow stroma aggregates. Finally, granulocytes were also found as a population in suspension [83]. Thus, using simple adherence criteria a DC-enriched population can be easily selected and used for further experimental analysis.

It is important that any *in vitro* DC cultured population should share a similar profile, express the same biomarkers that distinguish DC *in vivo* and share similar functional properties with these cells under different conditions. For example, upon activation by bacterial products, such as LPS, the representative DC cell line is expected to up-regulate the expression of MHC class II molecules, other components of their antigen presenting machinery and adhesion molecules. Thus, BM-DC were analysed with respect to MHC class II, CD86 CD54 and CD11c expression. Careful consideration was taken with regards to the cell culture protocol employed to derive BM-DC adhering to the specific instructions provided by Lutz and colleagues including using the appropriate concentration of GM-CSF, to generate DC-like cells from BM [446].

Evidence suggests that the dosage of GM-CSF is one of the defining factors for BM-DC generation. GM-CSF is required at concentrations around 200U/ml (=20ng/ml which was the concentration employed herein) for the generation of unstimulated cells with a DC-like profile that are prone to further maturation *in vitro*. The use of lower doses supports the generation of DC populations that are resistant to further maturation *in vitro* [446]. Arguably, the dose of GM-CSF should outweigh the presence of other factors released by the cells in the culture that might influence the differentiation of the

BM precursor cells or induce the spontaneous maturation of the cells. The sex of the mice is yet another factor that impacts the generation of BM-DC. Female mice were preferred as donors since males are typically more aggressive and therefore prone to injury, which can impact the yield of BM-DC [447]. The age of the BM donor mice is a third important factor. Lutz and colleagues investigated the levels of MHC class II expression in BM-DC derived from young donor mice aged 1-6 months old. Although an age-related increase in body weight and therefore the BM cellularity was evident, no significant differences were detected in the total numbers of cells expressing MHC class II or the intensity of expression of the marker in the different cell preparations [447]. Thus, to limit variation between the differentiation stage of DC employed for our investigations, mice aged only between 2-4 months old were used to generate BM-DC cultures. Nevertheless, some inter-experimental variation in the phenotype (expression of the different markers) of cells was evident between different animals and different cell preparations and this was observed for all, day 6, 8 and 12 BM-DC. Finally, the *in vitro* expansion of BM-DC was intentionally performed in bacterial quality Petri dishes, the specific adherence conditions of which hampers the development of macrophages and favours that of DC in suspension [446, 434]. Finally, to enhance the percentage purity of BM-DC the loosely adherent cells and those growing in suspension were transferred to fresh plates before being pulsed with LPS [446].

The commitment of precursors to the DC lineage requires signals from specific cytokines and transcription factors, that are also important for the development of other haematopoietic cells [120]. Interestingly, Vremec and colleagues observed that the DC population remained unaffected when the GM-CSF receptor was perturbed in mice. However, the group had only focused on lymphoid-resident DC and it is thought that GM-CSF is more important in the differentiation of monocytes into DC during inflammation [438]. Additionally, many groups report that the use of supplementary ligands/cytokines, such as IL-4 and Flt3-L, as cofactors is important for the expansion of BM-DC in culture. IL-4, for example, maybe used as a cofactor with a lower dose of GM-CSF. Flt3L has an important contribution for the development of DC in the steady state, the absence of which results in very low DC numbers in mice. However, the ligand favours the development of a mixed population of cDC and pDC [442]. Furthermore, based on *in vivo* observations, there are also concerns regarding the presence of NK cells in cultures that use Flt3-L as cofactor [443].

One of the most important factors that impact on the phenotype of expanded DC from BM progenitors is the concentration of GM-CSF. Evidence suggests that when BM precursors are grown in culture with GM-CSF, a distinct population of floating clusters was observed in the medium that differentiates towards the DC lineage. The longer BM precursors remain in culture with GM-CSF, the higher the proportion of differentiated ($CD11c^+$) cells that is obtained. However, when culture with GM-CSF is prolonged, the cells begin to drift towards the macrophage lineage or a mis-differentiated mature DC phenotype [448] [447], two undesirable phenotypes for studying DC biology. Additionally, with more time in culture, the BM cell density increases and the availability of GM-CSF becomes a limiting factor. As a result the cells begin to release various factors to sustain their viability, some of which can act as bystander antigens and induce spontaneous maturation of the DC population. [445, 447].

Thus, GM-CSF was used at the concentration dictated by Inaba and colleagues [83], and the profile of BM-DC following culture 6, 8 and 12 days has been characterized using flow cytometry. Initially, the baseline expression of the five markers of interest was assessed over time. Subsequently, MHC class II and CD86 expression were used as indicators for DC activation following culture with increasing concentration of LPS. LPS are cell wall component of Gram-negative bacteria and in their different classes they activate different TLR receptors. This study has employed the *Escherichia coli* LPS, which is a potent immunostimulatory ligand for TLR4 and TLR2 receptors [449] and has also been shown to evoke immune responses of the TH1 profile *in vivo* [42]. Other TLR ligands such as flagellin (TLR5) or poly:IC (TLR3), were considered, but have been previously classed as either weak inducers of inflammatory responses (poly:IC) or failed to induce complete maturation of murine BM-DC (flagellin) with regards to both up-regulation of pro-inflammatory cytokines and membrane marker expression [424].

Consistent with the data generated during this study, LPS has been previously shown to elevate activation markers such as MHC class II and CD86 in murine BM-DC [424]. Dearman and colleagues provide a thorough analysis of BM-DC responses, both cytokine and cell surface marker expression, to a range of TLR ligands. The TLR repertoire on DC populations is something that has intrigued scientists for a long time because it is inconsistent between DC subsets as well as mammalian species and strains.

The general consensus is that different TLR provoke specific cytokine and chemokine pattern expression that skews the immune response to the respective TH profile. Evidence suggests that TLR ligands such as TLR3 and TLR4 promote immune responses of a TH1 profile [450, 451] whereas ligands that bind to TLR2 lead to the development of the TH2 response [452]. Such observations raise questions as to whether division of labour is in place for DC subsets to better combat invading pathogens but they also invite further investigations into understanding DC biology. The panoply of TLR on murine BM-DC has been carefully screened by Dearman and colleagues and found to be representative of that expressed by epidermal LC [424, 453]. Human LC however were found to express a different repertoire of TLR and the results are conflicting between different authors. Some authors report the presence of TLR1, 2, 3, 5, 6 and 10 (TLR4, 7, 8, 9 were absent) [454] whereas others demonstrate the presence of TLR1, 3, 6 and 7 (TLR2, 4 5 and 8 were absent) in human LC at the level of transcription [455, 456]. Additionally, human LC were found to respond to TLR2 but not TLR4 ligands with regards to cytokine release and elevation of activation/maturation markers strengthening perhaps the results reported by Flacher and colleagues [454].

A difficult challenge faced by many in the scientific community is isolating native DC populations from lymphoid and non-lymphoid tissue for experimentation. Murine epidermal LC certainly do not constitute an exception, however, the fine work of Dearman and colleagues demonstrate that unstimulated BM-DC exhibit a profile that is representative of murine epidermal LC [424]. A significantly higher percentage of day 8 BM-DC (80%) was positive for CD11c. CD11c is an integrin and a receptor for complement component 3. It is a transmembrane protein whose high levels of expression have come to be associated with the DC lineage, even if the integrin has also been identified on other cell types, albeit at much lower levels [125]. Thus, in lack of a better DC lineage specific marker, CD11c will continue to be a reference cell surface marker for DC populations. Based on the observations of this study, a similar profile of day 6 and day 8 activated BM-DC was observed with the exception of CD11c expression. A more homogeneous population of cells, with regards to CD11c expression was characterised following 8 days in culture with GM-CSF. Additionally, cell viability was sustained at high levels in response to LPS challenge in day 8 BM-DC, whilst those recorded for day 12 BM-DC were significantly lower even at the lower

doses of endotoxin challenge. Untreated day 12 BM-DC exhibited a mature phenotype with significantly higher levels of MHC class II expression and CD11c. As a consequence, day 12 BM-DC were less responsive to increasing concentrations of LPS treatment, as they already were found to exhibit a mature (activated) phenotype, and were also more prone to LPS-induced cell death. Taking into consideration (i) the percentage levels of CD11c expression, (ii) MHC class II and CD86 expression in untreated BM-DC populations and how these change in the presence of LPS as well as (iii) the viability of the cells, it was concluded that day 8 BM-DC were more representative of immature DC and were employed for further experiments.

Finally, day 8 BM-DC were assessed for their ability to synthesise and release pro-inflammatory cytokines. Soluble inflammatory cytokines provide the third signal for T-cell activation. First identified as a T-cell derived factor, IL-6 is a pleiotropic cytokine that was observed to induce antibody secretion from B-cells and was therefore originally named B-cell differentiation factor. IL-6 is believed to be involved in cell growth, differentiation and migration of DC during the development of inflammatory responses [457]. Injection of a bacterial ligand such as LPS has been reported to induce a rise in IL-6 plasma levels and other pro-inflammatory cytokines *in vivo* [455]. A more comprehensive study reveals that IL-6 continuously acts as a differentiation signal for steady-state DC of the spleen and lymph nodes but is also thought to assist the transition of DC from their immature state to become activated after bacterial activation, *in vivo* [458]. IL-6 binds to its membrane-bound receptor, IL-6Rα inducing the gp130 signal transduction pathway that activates the STAT-3 signalling cascade, which subsequently initiates gene expression of important pro-inflammatory molecules [459]. It was therefore important to demonstrate that cultured BM-DC exhibit a fully activated profile and are able to synthesize and release pro-inflammatory cytokines such as IL-6, an unequivocal aspect of DC biology.

Consistent with previous observations of other authors on murine BM-DC and splenic DC [424, 453], day 8 BM-DC released substantial levels of IL-6 in response to LPS in a dose dependent but also time dependent manner. As to be expected, considerably higher levels of IL-6 were detected following culture with LPS for 24h in comparison with 2h. Previous observations of IL-6 production over time in murine BM-DC reveal that LPS initiates IL-6 transcription almost immediately with a peak of production recorded at

approximately 2-4 hours after challenge. A similar pattern was also observed in murine splenic DC [460]. Kinetic analyses of IL-6 release from murine BM-DC reveals that substantial levels of the cytokine are released as early as 8-10h after LPS activation [461]. Warger and colleagues demonstrate that a combination of TLR ligands (LPS+poly:IC) activating TLR4 and TLR3 receptors results in a synergistic effect on IL-6 release [462]. IL-6 is released via the classical pathway of exocytosis whereby proteins are transported through the golgi apparatus and is soon shuttled through vesicular release unlike cytokine such as IL-1 which require further processing by inflammasome components before release [193].

3.4 Conclusion.

The elegant system of growing progenitor cells under the aegis of GM-CSF to generate high yields of DC is the choice of preference for many to study various aspects of DC biology. In the absence of a better system that provides a high yield of DC-like cells in a consistent and reproducible manner, BM-DC will continue to be widely used to study the functional properties of DC. Care was taken to replicate the most appropriate conditions to nurture the majority of progenitor cells to differentiate towards a DC-like phenotype and with similar functional properties of DC subsets observed *in vivo*. Conclusively, our results demonstrate a high-yield, pure DC population can be derived from BM cells, according to the instructions provided by Lutz and colleagues and our findings are in general agreement with the findings of Inaba and colleagues that 8 days is the optimal time frame to generate BM-DC using GM-CSF [83]. Thus, based on the observations of this study and others [424] BM-DC were employed as a suitable representative for mouse epidermal LC to investigate the functional properties of the obscure $P2X_7R$ in DC function and more particularly in regulating pro-inflammatory cytokine release.

Chapter 4: Identifying $P2X_7R$ expression on murine BM-DC and BM-MΦ.

4.1 Introduction.

Found in abundance in sites of inflammation, ATP is the most potent physiological ligand of the $P2X_7R$. Functional $P2X_7R$ receptors have been previously identified in both epidermal LC (human and mouse) and keratinocytes (mouse) in the layers of the skin [343, 342]. Among P2 receptors, the $P2X_7R$ receives considerable attention due to its multiplex of cellular functions, particularly during the progression of inflammation [463]. The $P2X_7R$ belongs to the superfamily of purinoceptors first identified by Burnstock in the 1970s [464]. The receptor was later cloned in 1996 by Surprenant and colleagues from brain tissue of the rat and expressed in HEK-293 cells [325]. It is a ligand-operated ion channel with a widespread tissue distribution predominantly in endothelial and immune cells. Activation of the $P2X_7R$ leads to the exchange of mono- and divalent cations (Na^+, Ca^{2+} and K^+) that results in the depolarization of the cell surface membrane triggering a diversity of downstream responses (reviewed by [307]).

$P2X_7R$ signalling is most associated with the release of IL-1 molecules [385], the shedding of the low affinity IgE receptor CD23 [465], apoptosis [375], and degranulation of mast cells. Furthermore, transient activation of the receptor with low levels of agonist has been shown to promote cellular growth and survival [375]. Perhaps the more distinctive and more elusive function of the receptor is its pore forming capacity that has been associated with both cytolysis and cytokine release. Pore formation allows the flow of large organic molecules (<900 Da), like YO-PRO (MW 629) or ethidium bromide (MW 394), and is one of the most distinctive features of the $P2X_7R$. Although the prolonged uninterrupted opening of the pore results in inevitable cell death, the events that unfold during short-term reversible pore formation, remain obscure [466].

The mechanism(s) that facilitate the progression from receptor channel to a pore is also a topic of much controversy. Several hypotheses have been proposed as to how the pore is formed including the expansion of the receptor channel, which loses its cation selectivity to become a non-selective pore [467], the oligomerization of the receptor with other $P2X_7R$ subunits to form a central pore [468] and simply the recruitment of a separate channels, like the pannexins [12]. What is certain is that the signal initiating pore formation derives from the C-terminal region of the receptor. The C-terminus of the receptor is 120 amino acids longer in length than other P2X receptors and altering

the last 177 amino acids of the C-terminus has been shown to render the receptor unable to form a pore, whilst the properties of the channel remain intact [469, 325]. Additionally, the C-terminal region of the receptor is also thought to house an LPS binding domain [322], and has been associated with modulating its cell surface expression [470] as well as its sensitivity to agonist binding [469]. Interestingly, the ability of P2X$_7$R receptors to form pores differs between species [471]. For example, the rat P2X$_7$R exhibits 'enhanced' pore forming abilities in comparison to its mammalian orthologues [317]. Furthermore, the P2X$_7$R-induced permeation pathway exhibits distinct differences even between different cell types. In peripheral lymphocytes and thymocytes, the size of the 'pore' is slightly smaller allowing the passage of molecules of maximum size 400Da [472-474]. The lymphocyte-bound P2X$_7$R exhibits further functional differences whereby nicotinamide adenine dinucleotide (NAD) molecules constitute an additional indirect ligand for this receptor interfering with the sensitivity of the receptor to ATP, a feature that is not shared by the macrophage P2X$_7$R [475, 476].

Progress in deciphering the functional properties and the expression profile of the P2X$_7$R was mainly achieved with the use of potent and selective P2X$_7$R inhibitors. However, most of the early available antagonists did not exhibit equal potency across different mammalian receptors. KN-62, for example, is a potent antagonist for the human P2X$_7$R but exhibits weak antagonism against its rat orthologue [477]. In contrast, BBG is a selective potent antagonist for the rat P2X$_7$R but is rather weak against other mammalian orthologues [314]. Thus a new series of highly selective antagonists like the A-408598 and A-740003 molecules have been shown to competitively block P2X$_7$R activity *in vitro* and exhibit anti-nociceptive properties *in vivo* and have quickly replaced the old generation of inhibitors [408, 478]. With the availability of such tools, there has been a surge in interest in investigating the P2X$_7$R and its downstream cellular functions, particularly during inflammation.

Observations regarding the functional properties of the receptor in cells of the dendritic lineage are very limited. Even though the presence of P2X$_7$R mRNA has been identified in a number of different tissues, such as lung, liver, pancreas and spleen [317], functional studies have focused mainly on macrophages (and monocytes) [479] microglial cells [466], mast cells [468] and LC [480]. The presence of a functional

$P2X_7R$ has also been reported in both mouse and human DC [481], however, the functional properties of the receptor on these APC have not been explored in detail.

DC are found scattered among tissues sitting at the interface between host and the external environment in peripheral tissues, and routinely scan peripheral tissues for the presence of pathogens. They are the main APC of the immune system with the capacity to induce robust T-cell responses both *in vitro* and *in vivo* [482]. They are also capable of presenting alloantigen through MHC class I to cytotoxic CD8 T-cells [483] and constitute a vital element against viral infections. Macrophages also have a wide distribution within and among tissues and they are also an essential component of both innate and adaptive immune responses. They have the unique ability to recognize and restrain the majority of invading pathogens during their early phase of infection and at the same time provide danger signals for the recruitment of additional cell types to the 'battle' against pathogen invasion [294]. Macrophages, however, are mainly confined to their specific tissue compartments and they are more involved in innate defence against invading pathogens and the maintenance of the hosts' tissues [484]

Much of the work on $P2X_7R$ responses, thus far, has focused on macrophages [481, 317] and the role of the receptor in DC function has only recently begun to be explored. Murine BM-MΦ were employed as an isogenic reference cell type for BM-DC to examine the functional properties of the $P2X_7R$ and these were also compared with those observed in freshly isolated murine PMΦ. Subsequently, the aim was to provide robust evidence for the presence of a functional $P2X_7R$ on murine BM-DC that also expressed the DC-associated integrin CD11c. Finally, it was also important to identify any distinct functional properties between the DC and macrophage $P2X_7R$ in mediating pore formation in cells.

4.2 Results.

4.2.1 Phenotypic analysis of BM-DC, BM-MΦ and PMΦ.

The first objective of this chapter was to characterise the phenotype of the macrophage populations of choice. In brief, BM cells were isolated from the femur and tibia of female Balb/c strain mice and subsequently cultured in the presence of GM-CSF for BM-DC and L-cell medium (LCM) for BM-MΦ for 8 days. The BM-DC population was grown in suspension in the presence of GM-CSF, isolated following 8 days of *in vitro* culture and plated at $1x10^6$ for most experimental procedures. The BM-MΦ population represents the adherent population of BM precursors grown in the presence of L929 cell medium (LCM) for 8 days *in vitro* and also plated at $1x10^6$ for further experimentation. Finally, peritoneal exudate cells were cultured for 24h *in vitro*, non-adherent cells were removed and the adherent population used for further experiments representing the PMΦ population. The three cell populations were characterized for the expression of a number of cell surface markers by flow cytometry. BM-DC and BM-MΦ were similar in size and granularity (figure 4.1a) whereas BM-DC were slightly more heterogeneous in size. PMΦ were found to be less granular than BM-DC or BM-MΦ. Figure 4.1a also illustrates how the different cell populations were gated with regards to granularity and size for further analysis of cell surface marker expression. Photographic images of the three different cell populations cultured in medium are shown in figure 4.1b. Phenotypically, BM-DC were found to be circular in shape and slightly larger in comparison to BM-MΦ and PMΦ. They were a semi-adherent cell population that lacked the distinctive dendritic processes indicative of their immature state. BM-MΦ were phenotypically a more heterogeneous population; some of the cells were circular in shape whilst others assumed an elongated, spindle-like formation. Finally, PMΦ were smaller in size with a significant proportion of the population assuming a spindle-like phenotype, much like BM-MΦ (figure 4.1).

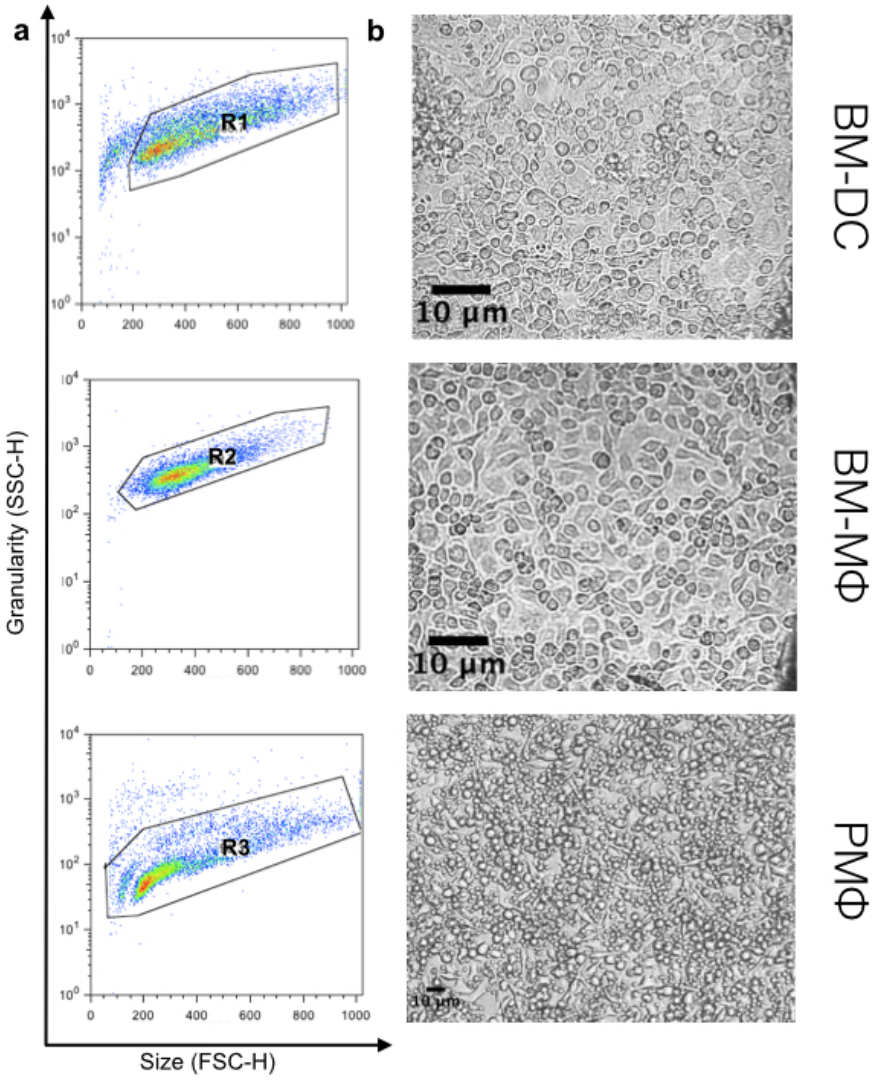


***Figure 4.1:* The gating strategy for flow cytometric analyses of BM-DC, BM-MΦ and PMΦ.**
Cells were first gated by forward scatter for their size (FSC-H) and by side scatter for their granularity (SSC-H) and the selected cells were denoted as gate R1 for BM-DC, R2 for BM-MΦ and R3 for PMΦ (a). Propidium iodide was used to stain for dead cells and these were excluded from further analysis as described in the legend to figure 3.1. BM-DC and BM-MΦ were cultured for 24h at $1x10^6$ cells/ml in the presence of medium alone. Cells from peritoneal exudates (PMΦ) at $1x10^6$ cells/ml were cultured for 24h before non-adherent cells were removed with thorough washing. Images were collected using an Olympus (BX51) upright fluorescent microscope with a 40x/0.74 PlanFLN objective, captured using a coolsnap ES camera through MetaVue software (Molecular Devices) and analysed using ImageJ 1.46R (b).

4.2.2 Phenotypic analysis of untreated (control) BM-MΦ by flow cytometry.

BM-MΦ precursors were grown in the presence of LCM. The LCM contains M-CSF, a macrophage lineage specific factor that drives differentiation of myeloid progenitors down the macrophage lineage *in vitro* [485]. The adherent population of BM-MΦ progenitor cells reached 80-100% confluency following 8 days in culture. The phenotype of the adherent population of cultured cells was analysed for the expression of MHC class II, the macrophage marker F4/80, the DC marker CD11c and also the integrin CD11b, 8 or 12 days after initiation of culture. Figure 4.2a shows representative flow cytometric histograms for the expression of the different markers of interest on day 8 BM-MΦ. There is a clear defined peak for both F4/80 and CD11b expression distinct from that of the isotype controls demonstrating that most of the BM-MΦ population expressed both markers of interest. This is not evident with MHC class II expression which in the absence of a clear defined peak on the histogram, was rather heterogeneous. Finally, there was no clear shift for CD11c expression in comparison to the isotype control.

The histograms were used to calculate the percentage expression and the MFI for each of the marker of interest on day 8 BM-MΦ (grey bars) shown in figures 4.2b and 4.2c respectively. Approximately 95% of the population expressed F4/80 and CD11b with the MFI levels of 150 and 75au, respectively. In contrast, only about 10% of the population was found to express MHC class II and 18% expressed CD11c with considerable inter-experimental variation. The MFI levels for MHC class II were approximately 25AU whereas for CD11c expression was negligible. BM-MΦ were also analysed at day 12 to investigate the effect of time in culture on the phenotype of the population. As revealed by the histograms in figure 4.8a, there was a similar distinct peak in the histograms for F4/80 and CD11b, evidence of relatively homogeneous expression of the two markers of interest. MHC class II and CD11c expression appeared rather heterogeneous. There was no statistically significant difference in the percentage of cells expressing F4/80, CD11b or MHC class II between day 8 and day 12 BM-MΦ. In contrast there was a significant increase in the expression of CD11c on day 12 BM-MΦ with approximately 50% of the population expressing the classic DC integrin. Only minor differences were identified in the baseline MFI levels for the expression of F4/80

and CD11b between the two cell types. The MFI levels for MHC class II expression were found unchanged (values of 50au recorded) with more inter-experimental variation. Importantly, the MFI levels for CD11c were found at markedly higher levels at 100au, albeit with high inter-experimental variation.

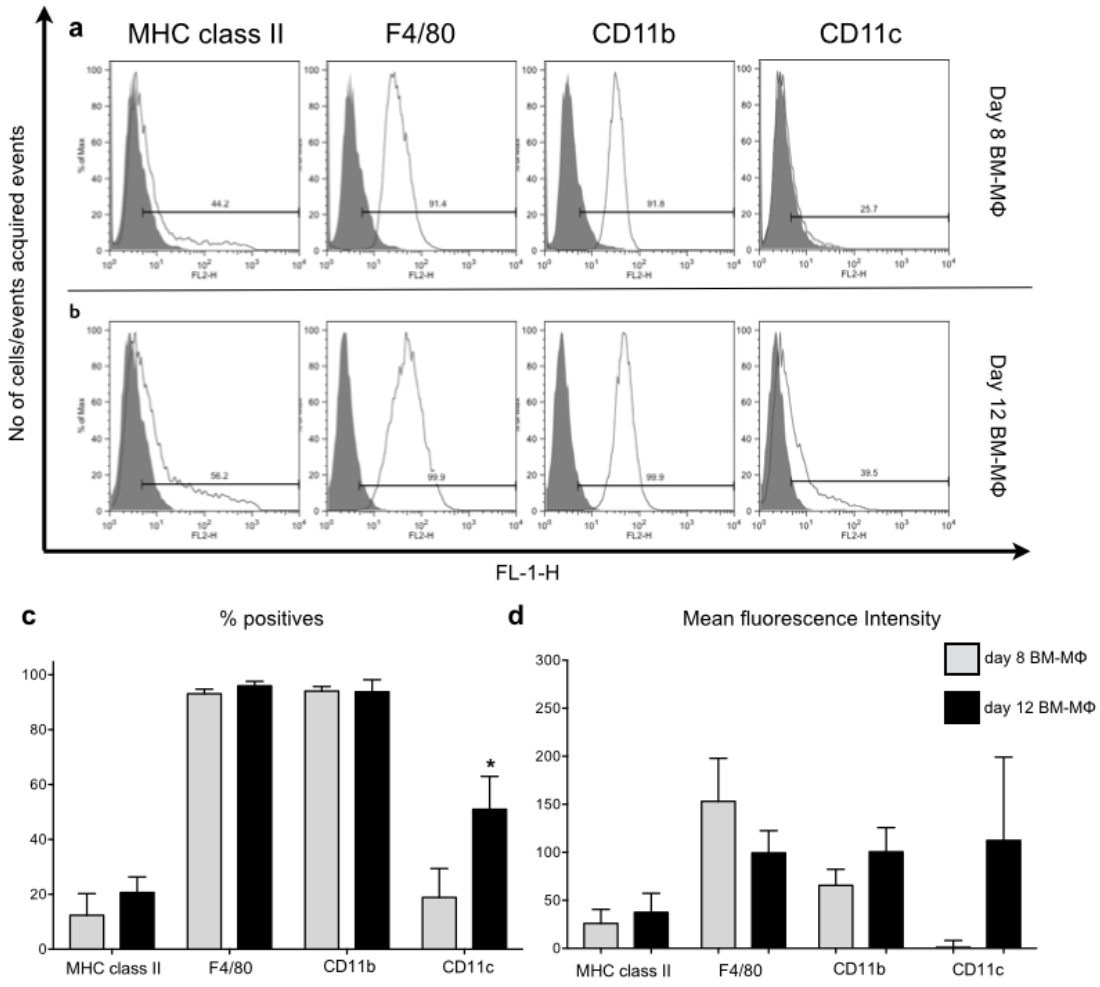


***Figure 4.2:* Differences in the profile of day 8 and day 12 BM-MΦ. Day 8 and 12 BM-MΦ were cultured for 24h at 1x10^6 cells/ml in the presence of medium alone.**
(a): The expression of the markers of interest is shown as plotted histograms (open line) overlaid with the appropriate isotype control (closed line). Expression of the various markers of interest was determined using the 3 step gating system described in chapter 3. Representative plots are shown for each marker of interest. Data are shown with respect to (b) the percentage positive cells and (c) the mean fluorescence intensity (MFI). Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. Data shown are mean (n=3) ±SEM. Statistical significance of differences between groups was assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (*=p<0.05).

4.2.3 Investigating the effect of endotoxin activation on the phenotype of BM-MΦ.

Macrophages have been previously shown to respond to endotoxin (LPS) activation, which modulates transcription factors [486]. Hence, the effect of endotoxin activation on the BM-MΦ profile was investigated next. The adherent population of BM-MΦ was isolated following 8 days in culture and grown in the presence of LPS for 24h at $1x10^6$ cells/ml. Figure 4.3 shows phenotypic changes in BM-MΦ following activation with LPS for 24h as depicted by flow cytometry histograms. Baseline expression of all the markers of interest was either rather homogeneous or largely negative on the BM-MΦ cell population. There was no change in the baseline expression of most of the markers of interest induced by culture with LPS. Both MHC class II and CD11c expression on medium-treated cells was largely negative and no shift was evident following treatment with either LPS concentration. The most striking effect of LPS treatment was observed on CD54 expression, which is illustrated by a clear shift to the right of the population indicative of up-regulation of CD54 expression. Baseline expression of the other markers of interest, F4/80, CD86 and CD11b, remained unaffected by LPS treatment.

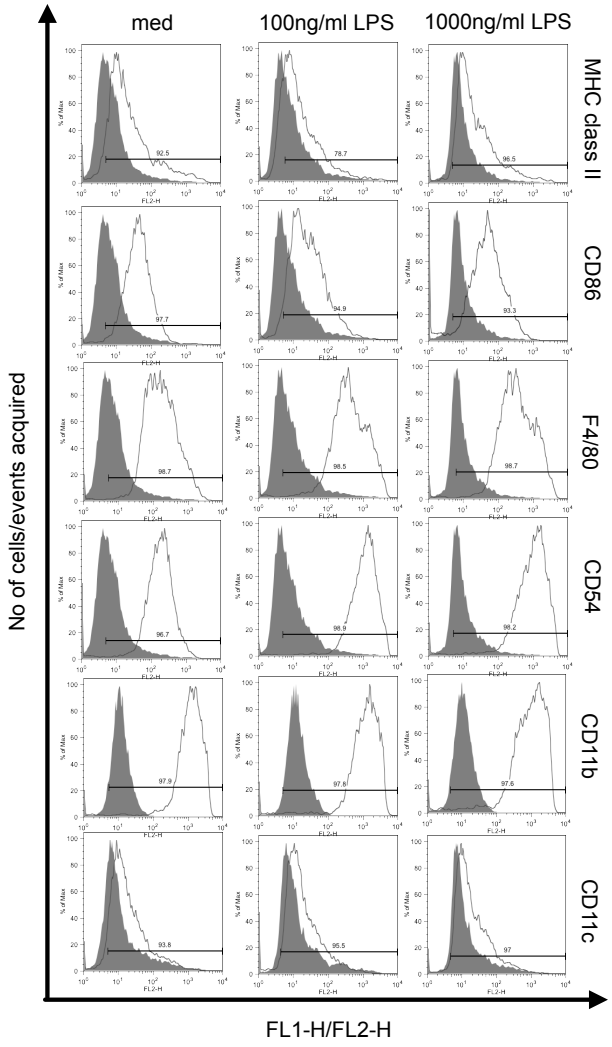


***Figure 4.3:* LPS-induced changes in the profile of day 8 BM-MΦ as detected by flow cytometry.**

Day 8 cultured BM-MΦ at 1x10^6 cell/ml were incubated with medium alone (med), or stimulated with LPS at 100, or 1000ng/ml for 24h. Subsequently, cells were analysed by flow cytometry for the expression of the markers of interest (representative histograms shown). The histograms were used to derive the percentage of BM-MΦ expressing the various cell surface markers of interest (percentage positive) of interest and the mean fluorescence intensity for each marker. The closed lines on the graphs represent the isotype control (IgG2a, IgG2b or IgG1λ) whereas the open lines represent the expression of each cell surface marker. The point of interception of the two lines was determined and used to calculate the percentage of cells expressing the different markers of interest and their mean fluorescence intensity. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis.

Analysis of the flow cytometry histograms gives a more accurate measure of the frequency of cells positive for the expression of the markers of interest (figure 4.4a) and their respective MFI levels (figure 4.4b). The percentage of cells positive for the various markers of interest was unaffected by culture with LPS. Similarly, LPS treatment of BM-MΦ was without marked effects on MFI levels of most of the markers of interest. Whereas a significant 2-fold reduction in the MFI levels of MHC class II was noted with both concentrations of LPS, the MFI levels of CD86, CD11b and CD11c remained largely unaffected. The MFI levels for CD54 expression saw a significant 2fold increase from 150au to 300au following activation with 100ng/ml LPS. No significant changes were observed with regards to the MFI levels of F4/80 expression.

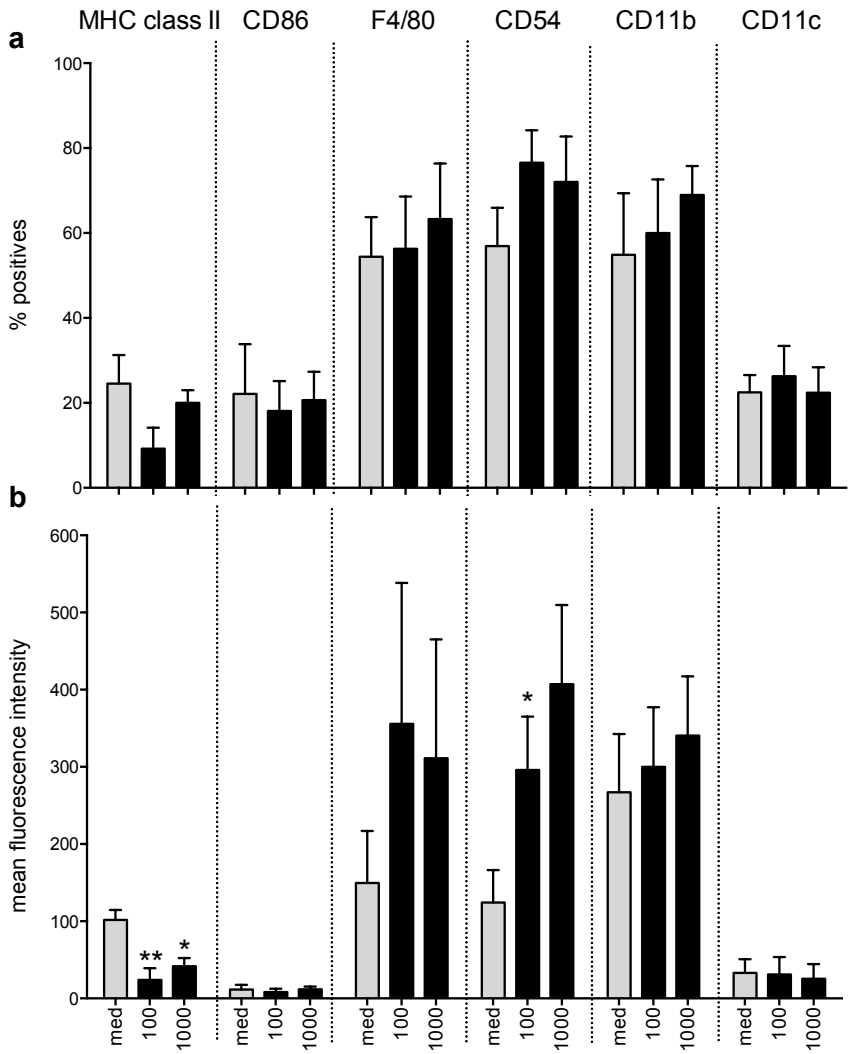


***Figure 4.4:* Frequency of cells positive and level of expression for DC and MΦ markers on day 8 BM-MΦ: impact of LPS.**
Day 8-cultured BM-MΦ at 1x10^6 cells/ml were incubated with medium alone (med), or stimulated with LPS at 100, or 1000ng/ml for 24h. Cells were analysed by flow cytometry for surface of expression of the markers of interest (MHC class II, CD86, F4/80, CD54, CD11b and CD11c) and data are shown with respect to (a) the percentage of positive cells and (b) the mean fluorescence intensity (MFI) with increasing concentrations of LPS. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. Data shown are mean (n=3) ±SEM. Statistical significance of differences between groups (media-treated cells as a comparator) was assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (*= $p<0.05$ and **=$p<0.01$).

4.2.4 Phenotypic differences between BM-DC, BM-MΦ and PMΦ.

For the purposes of these experiments, it was important to explore the phenotypic differences between naïve DC and macrophages differentiated from the same BM precursors before these were used for further experimentation. The phenotypes of day 8 BM-DC and BM-MΦ were also compared with the adherent population of freshly isolated peritoneal exudate cells, PMΦ. Day 8 BM-DC were characterized by a heterogeneous expression of MHC class II and CD11c and a more homogenous expression of F4/80. CD11b expression was also found to be homogeneous but at low levels since the positive signal was largely masked by that of the isotype control (figure 4.5a). Day 8 BM-MΦ were found to be predominantly negative for the expression of MHC class II and CD11c as expression of the two markers was almost entirely overlaid with the isotype control. In contrast, a well-defined distinct peak was observed for F4/80 and CD11b expression (figure 4.5b). A similar distinct peak was observed for CD11b expression on PMΦ. MHC class II expression was found to be rather homogeneous on PMΦ cells, unlike F4/80 expression, which was found to be rather heterogeneous. Two small peaks were observed at either end of the log scale indicating that some of the population expresses low levels of the macrophage receptor whilst high levels of expression characterized the rest. Similar to BM-MΦ expression of CD11c was negligible (figure 4.5c).

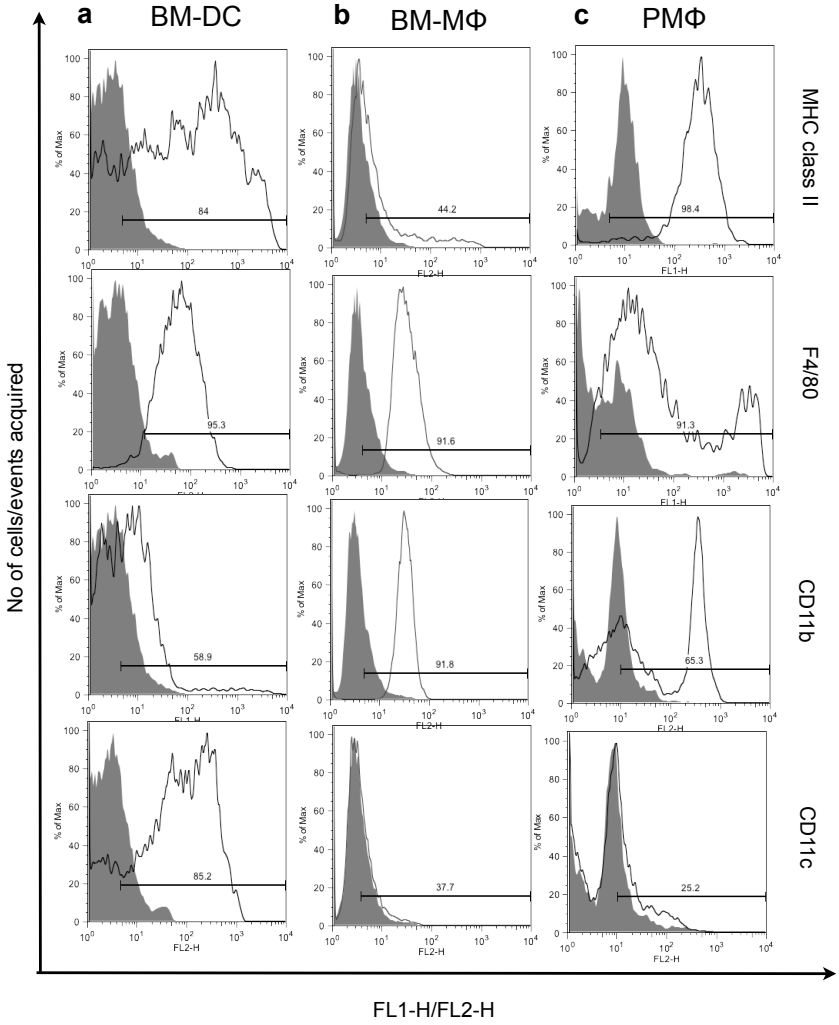


Figure 4.5: Flow cytometric analysis of surface marker expression on resting BM-DC, BM-MΦ and isolated PMΦ.

Day 8 BM-DC, BM-MΦ and the adherent population of cells from peritoneal exudates (PMΦ) 1x10^6 cells/ml were cultured for 24h in the presence of medium alone. The adherent cell populations were removed from plates by rigorous scraping of the plates before cells were stained for the expression of the markers of interest. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. The expression of the markers of interest (MHC class II, CD86, F4/80, CD11b and CD11c) is shown as plotted histograms (open line) overlaid with the appropriate isotype control (closed line). Expression of the various markers of interest was determined using the 3 step gating system as previously described. Representative plots are shown for each of the markers on (a) BM-DC, (b) BM-MΦ and (c) PMΦ.

The histograms in figure 4.5 were used to calculate the percentage expression and MFI levels for the markers of interest on BM-DC, BM-MΦ and PMΦ. With regards to percentage levels of expression there were significant differences between the populations for all markers of interest (4.5a). Importantly, the percentage of day 8 BM-DC expressing CD11c (60%) was significantly higher than that recorded for day 8 BM-MΦ (20%) and freshly isolated PMΦ (5%). Furthermore, BM-MΦ expressed significantly higher levels of F4/80 (70%) in comparison with PMΦ (28%). The low percentage for F4/80 expression (28%) in peritoneal exudate cells was a concern since it contradicted the findings published by others [487-489]. Thus, the possibility that the low levels for F4/80 expression in PMΦ was an artefact due to experimental procedures was further investigated in section 4.5 below.

The percentage of cells positive for MHC class II expression was found to be similar between BM-DC and PMΦ with levels at 55% and 45% respectively. However, only about 10% of BM-MΦ were found to express MHC class II. The percentage of cells positive for CD11b expression was very similar between BM-DC and BM-MΦ (90% positive) and this was significantly higher than that recorded for PMΦ, which was found to be approximately 35%. The phenotypic differences between the three cell types were also extended to the MFI levels for the expression of the markers of interest. The MFI levels for MHC class II were significantly higher in BM-DC (250au) in comparison to BM-MΦ (20au), whereas similar high expression was recorded for PMΦ (230au). In contrast, the MFI levels for F4/80 were more than 3-fold higher in both macrophage cell types (150au), however, due to substantial inter-experimental variation the difference in MFI values between BM-DC and PMΦ failed to reach statistical significance. The MFI for CD11b was found at approximately similar levels for all cell types of interest ranging from 50-75au. Finally, the MFI levels for the CD11c integrin were recorded at significantly higher levels in BM-DC (100au) in comparison with both macrophage cell types.

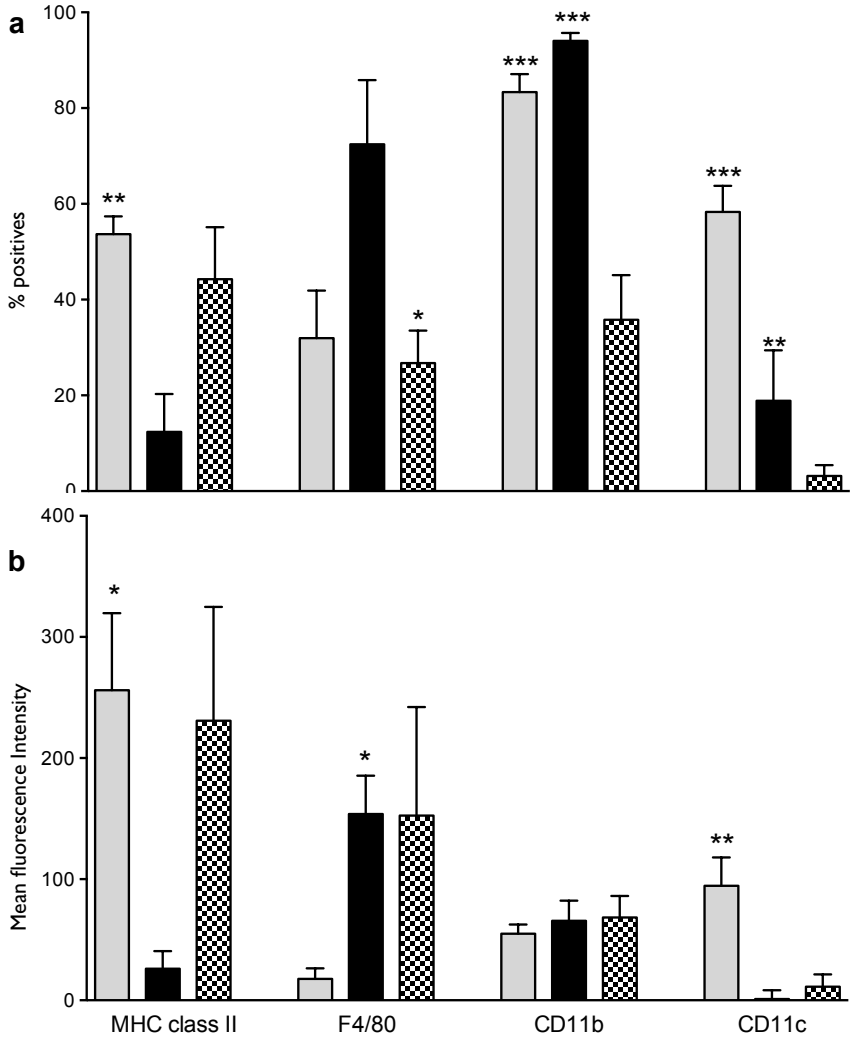


***Figure 4.6:* Frequency of cells positive and level of expression for DC and MΦ markers on day 8 BM-DC and BM-MΦ and isolated PMΦ.**
Day 8 BM-DC (grey bars), BM-MΦ (black bars) and the adherent population of cells from peritoneal exudates (PMΦ) (hatched bars) at 1x10^6 cells/ml were cultured for 24h in the presence of medium alone. The adherent cell populations were removed from plates by rigorous scraping of the plates before cells were stained for the expression of the markers of interest. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. Cells were analysed by flow cytometry for surface of expression of the markers of interest (MHC class II, F4/80, CD11b and CD11c). Data are shown with respect to (a) the percentage positive cells and (b) the mean fluorescence intensity (MFI) and are mean (n=6) ±SEM. Statistical significance of differences between groups was assessed by one way ANOVA and Tukey's multiple comparison post-hoc test (*= $p<0.05$, **=$p<0.01$, ***=$p<0.005$).

4.2.5 Immunohistochemical analysis of the phenotype of BM-DC, BM-MΦ and PMΦ.

As mentioned previously, the low expression of F4/80 freshly isolated PMΦ raised concerns, as well as the somewhat unusual pattern of CD11c expression recorded. Along with many others, Hu and colleagues 2012 [490] report 70% of peritoneal exudate cells express F4/80. Expression of the F4/80 antigen is subject to environmental stimuli and is downregulated in the presence of IFN-γ [491]. The phenotypes of the three cell types were therefore also analysed by immunohistochemistry to investigate whether processing of peritoneal exudate cells during flow cytometric staining and analysis had an impact on the expression of the F4/80 molecule. Figure 4.7 shows representative images for the expression of F4/80, CD11b and CD11c in the cell types. There was a high frequency of F4/80 expression in both BM-MΦ and PMΦ, however, the expression pattern of the macrophage marker was heterogeneous. F4/80 expression was absent in BM-DC as was CD11c expression in PMΦ. A very small number of BM-MΦ and PMΦ were found to express CD11c whereas almost the entire population of BM-DC expressed the classic DC integrin albeit with some variation in the intensity of expression. Finally, all three cell types were positive for CD11b expression.

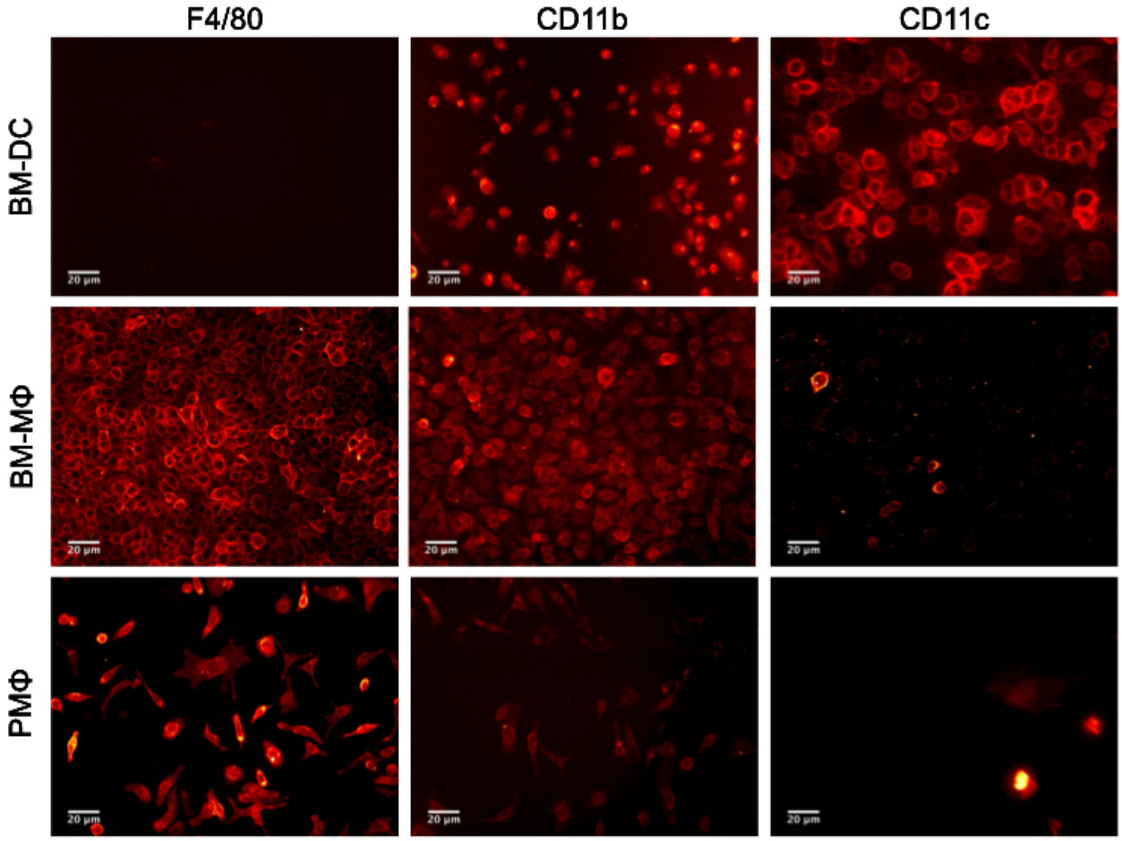


***Figure 4.7:* Immunohistochemical analysis of DC and MΦ markers on day 8 BM-DC and BM-MΦ and isolated PMΦ.**
Day 8 BM-DC, BM-MΦ and the adherent population of cells from peritoneal exudates (PMΦ) at $1x10^6$ cells/ml were cultured for 24h in the presence of medium alone. Cells were stained in situ in the tissue culture plate for the expression of the markers of interest (F4/80, CD11b and CD11c)(representative images shown) or the appropriate isotype control (images not shown). Cells stained for isotype controls were all negative. Images were collected using an Olympus (BX51) upright fluorescent microscope with a 40x/0.74 PlanFLN objective, captured using a coolsnap ES camera through MetaVue software (Molecular Devices) and analysed using ImageJ 1.46R.

4.2.6 Endocytic activity in BM-DC and BM-MΦ.

A well characterized function of naive DC and MΦ is their ability to 'sense' the microenvironment by capturing and processing exogenous antigen for presentation at T-cell areas [492]. FITC-labelled BSA was employed to investigate the ability of BM-DC and BM-MΦ to capture foreign particles. Day 8 BM-DC and BM-MΦ were cultured in medium or in the presence of sodium azide for 1h at 37°C or 4°C before the cells were loaded with FITC-labelled BSA for 30min. Endocytosis of FITC-BSA particles was assessed by flow cytometry. At 37°C, FITC-BSA uptake in both BM-DC and BM-MΦ was homogeneous among the populations as depicted by a narrow high peak on the histograms (open black line) in figure 4.8a shifted to the right of the medium control-treated cells. A clear shift to the left was noted for FITC-BSA uptake in the presence of sodium azide (light grey closed line) in both BM-DC and BM-MΦ and uptake was found to be more heterogeneous, particularly in BM-DC, with two distinct peaks evident in the histogram. Cells were also treated at 4°C, a temperature at which the cells are not metabolically active, these treatment groups were included as controls to determine the difference between FITC-BSA cell surface membrane binding and particle uptake by the cells. Both medium and sodium azide treated cells loaded with FITC-BSA particles were found to bind similar levels of the particles as the peaks in the histograms for the two treatment groups were superimposed on each other (open black line and light grey line). The histograms were used to quantify the levels of FITC-BSA uptake in BM-DC and BM-MΦ and the results are shown on figure 4.8c expressed as levels of mean fluorescence intensity. Similar MFI levels were observed in both FITC-BSA-loaded BM-DC and BM-MΦ (400au) at 37°C. These were markedly reduced in cells treated at 4°C with MFI levels recorded at 150au for both cell types.

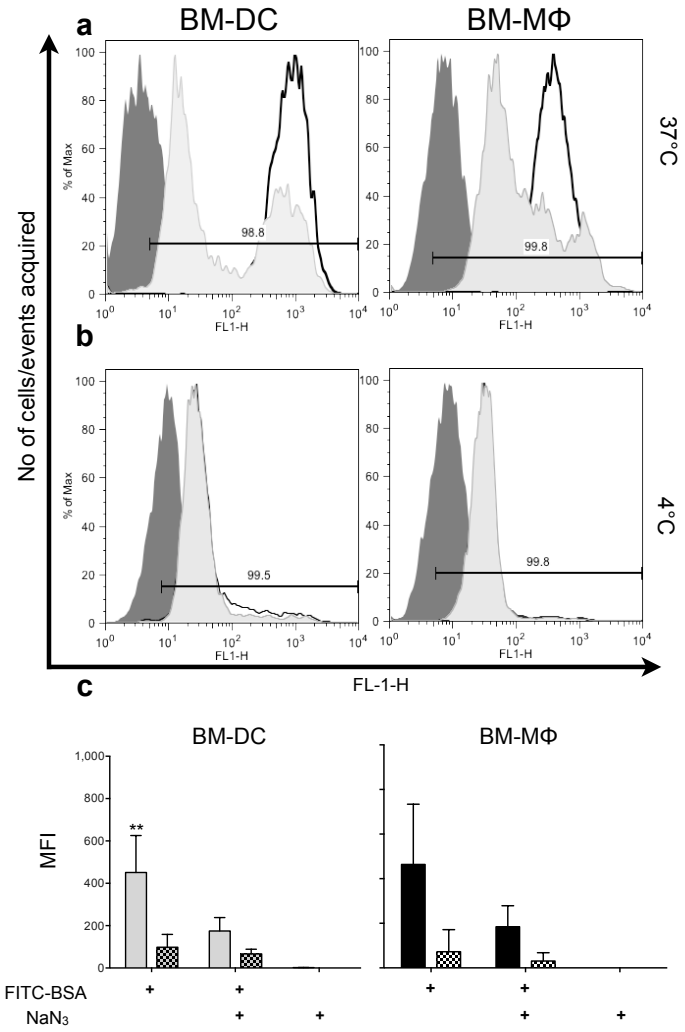


Figure 4.8: FITC-BSA uptake in Day 8 BM-DC and BM-MΦ.
Day 8 BM-DC, BM-MΦ at 1x10^6 cells/ml were incubated with sodium azide (NaNa3, 20 mM) or medium alone for 1h at 37°C or at 4°C (control). Subsequently, cells were loaded with FITC-BSA (1mg/ml) for 30 min and cells maintained at 37°C or at 4°C. FITC-BSA uptake was monitored by flow cytometry. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. (a): FITC-BSA uptake in BM-DC and BM-MΦ is shown as plotted histograms overlaid with the controls: medium-treated cells (dark grey line), FITC-BSA treated cells (open line) and FITC-BSA treated/NaNa3-treated cells (light grey line). Representative plots are shown for each cell type at 37°C and 4°C. (b): data are shown with respect to the mean fluorescence intensity (MFI) of FITC-BSA expression/uptake both at 37°C (closed bars) and 4°C (hatched bars), and are mean (n=3) ±SEM. Statistical significance of differences between groups was assessed by one way ANOVA and Tukey's multiple comparison post-hoc test (**=p<0.01).

4.2.7 Endotoxin-induced TLR activation stimulates cytokine release in BM-DC.

The ability of these three cell types to synthesize and release the pro-inflammatory cytokine, IL-1β, in response to different concentrations of LPS and in the absence of ATP stimulation was examined next. Cell viability was assessed with PI staining and the results are shown in figure 4.9a. Increasing concentrations of LPS was without marked cytotoxic effects on any of the cell populations with cell viability recorded at approximately 85% in media treated BM-DC, 80% in BM-MΦ and 75% in PMΦ independent of LPS addition. Expression of IL-1β by BM-DC was also compared with BM-MΦ and PMΦ under the same conditions (figure 4.9). Whilst LPS activation for 24h induced IL-1β synthesis in both DC and both types of MΦ in a dose dependent manner, IL-1β release was only detected in BM-DC (figure 4.9b). LPS activation induced dose-dependent IL-1β release in BM-DC that reached statistically significant levels at the top concentration of LPS; with the release of 3ng/ml of IL-1β following activation of BM-DC with 1000ng/ml of LPS. In contrast, levels of IL-1β released from endotoxin-matured BM-MΦ and PMΦ in the absence of exogenous ATP were found below the level of accurate detection (39pg/ml), whilst cytosolic IL-1β was detected in all 3 cell types. In fact, the levels of cytosolic IL-1β reached statistical significance in BM-MΦ (4ng/ml) at the top concentration of LPS.

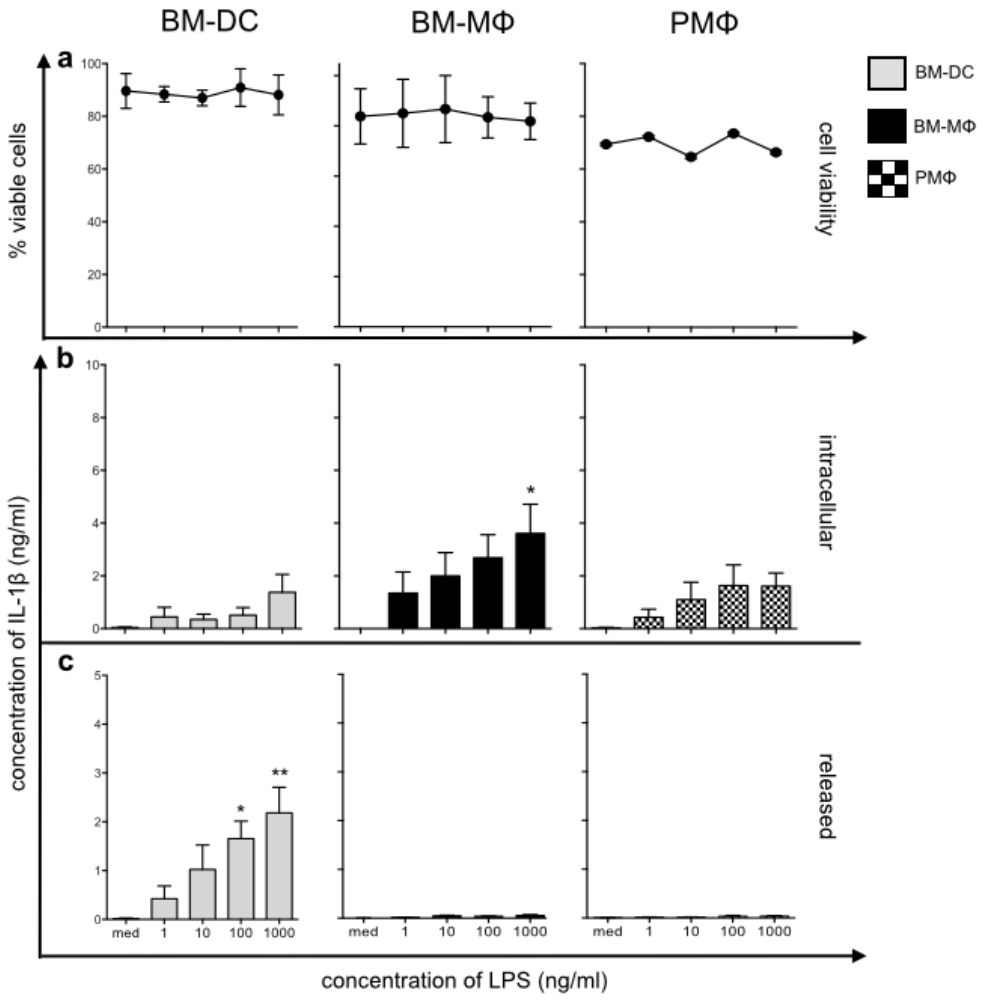


Figure 4.9: Endotoxin-induced IL-1β synthesis and release in day 8 BM-DC, BM-MΦ and isolated PMΦ.

Day 8 cultured BM-DC, BM-MΦ at 1x10^6/ml and the adherent population of cells from peritoneal exudates (PMΦ) were cultured for 24h in the presence of medium alone or stimulated with LPS at 1, 10, 100 or 1000ng/ml. LPS-induced changes in cell viability of all 3 cell types are shown in (a). Cells were stained with propidium iodide and cell viability was determined using flow cytometry. Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. Both intracellular (b) and released (c) levels of IL-1β were measured using a cytokine-specific ELISA. Data shown are mean (n=3-5) ±SEM. Statistical significance of differences between groups (medium-treated cells were used as a comparator) was assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (*= $p<0.05$, **=$p<0.01$).

4.2.8 BM-DC release comparatively higher levels of the non-inflammasome related IL-6 cytokine.

It was also important to investigate the ability of day 8 BM-DC to initiate the synthesis and release of other non-inflammasome-related cytokines such as IL-6 following endotoxin activation. The effect of long-term LPS activation (24h) on IL-6 synthesis and release was assessed on day 8 BM-DC, BM-MΦ and freshly isolated PMΦ (figure 4.10). Cells were activated with LPS concentrations ranging from 1-1000ng/ml, which activated IL-6 release in all 3 cell types in a dose-dependent manner. LPS induced similar amounts of IL-6 release in BM-MΦ and PMΦ that reached statistically significant levels (approximately 2ng/ml IL-6) at the top concentrations of LPS (100 and 1000ng/ml LPS). Importantly, LPS induced comparatively higher levels of IL-6 release in BM-DC even with lower concentrations of LPS, that peaked at 50ng/ml IL-6. No significant IL-6 production was detected in the cell lysates.

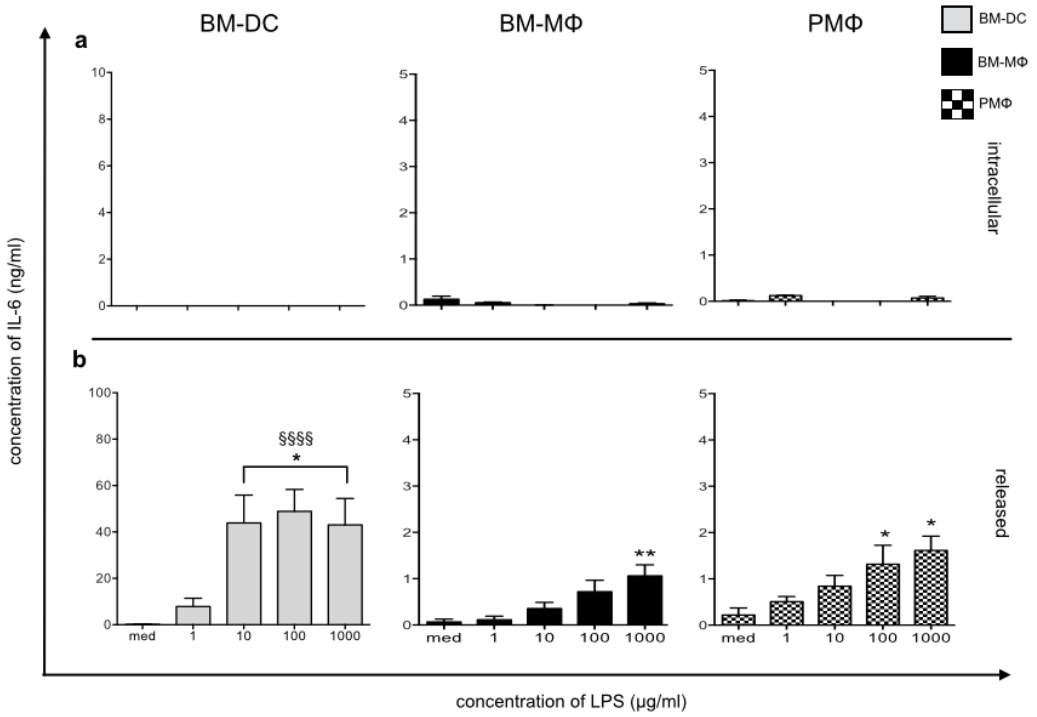


***Figure 4.10:* Endotoxin-induced IL-6 synthesis and release in day 8 BM-DC, BM-MΦ and isolated PMΦ.**
Day 8 cultured BM-DC, BM-MΦ at 1x10^6/ml and the adherent population of cells from peritoneal exudates (PMΦ) were cultured for 24h in the presence of medium alone or stimulated with LPS at 1, 10, 100 or 1000ng/ml. Both intracellular (a) and released (b) levels of IL-6 synthesis were measured using a cytokine-specific ELISA. Data shown are mean (n=3-5) ±SEM. Statistical significance of differences between groups (medium-treated cells were used as a comparator) was assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (*= $p<0.05$, **=$p<0.01$). Statistical significance of differences for each treatment group between different cell types were considered by two way ANOVA and Tukey's multiple comparison post-hoc test (§§§§= $p<0.01$).

The profile of BM-DC and the two macrophage populations (BM-MΦ and PMΦ) was assessed with regards to (a) their cell surface marker expression (b) their ability to produce and release pro-inflammatory mediators and (c) their ability to undergo macropinocytosis (assessed with the ability of the cells to take up FITC-BSA molecules), a necessary process for taking up antigen. On the basis of these analyses comparing these parameters the decision was made to use day 8 cells for further studies. Day 8 BM-DC and BM-MΦ would be employed to directly compare the functional properties of the $P2X_7R$. PMΦ were used as reference macrophage cells where possible to compare macrophage function with BM-MΦ.

4.2.9 Flow cytometric analysis of BM-DC for cell surface expression of P2X$_7$R.

Currently, our understanding regarding the expression profile and functional properties of the receptor in cells of the haematopoietic lineage other than macrophages, is lacking. One of the first priorities of this study was to provide substantial evidence of the expression of a functional P2X$_7$R in murine DC and BM-MΦ were employed as an appropriate comparator to assess the expression and functional profile of the P2X$_7$R. Thus, having established that the BM-MΦ profile presented exhibits a satisfactory macrophage-like phenotype, day 8 BM-MΦ were employed to assess the functional properties of the mouse P2X$_7$R in parallel with DC. P2X$_7$R expression was previously detected in human monocytes by flow cytometry [340] and the cell surface expression of the receptor is enhanced as monocytes commit to the macrophage cell lineage [493].

Day 8 murine BM-DC were therefore incubated with medium alone for 24h and the cells were stained with various concentrations (1, 5 and 10μg/ml) of a specific antibody directed against the P2X$_7$R (a monoclonal rat anti-mouse antibody with specificity for a P2X$_7$R extracellular loop epitope). Unfractionated mouse (Balb/c) splenocytes were previously reported to express detectable levels of the P2X$_7$R as demonstrated by Tsukimoto and colleagues by immunoblotting and the tissue was therefore as a positive control [494]. Flow cytometric analysis of cultured BM-DC and unfractionated splenocytes failed to reveal detectable levels of receptor expression with the specific antibody binding at equivalent levels to those observed for the isotype antibody (shown in figure 4.11a). Although the antibody employed was not directly conjugated, binding of the secondary antibody control for both MHC class II and P2X$_7$R expression was found at lower levels compared to those of the isotype control antibodies (data not shown). The analysis was repeated on three different DC and spleen preparations (data not shown), which in each case were negative for P2X$_7$R expression, whereas in each case positive staining was observed for MHC class II (figure 4.11b), a marker present on both cell types.

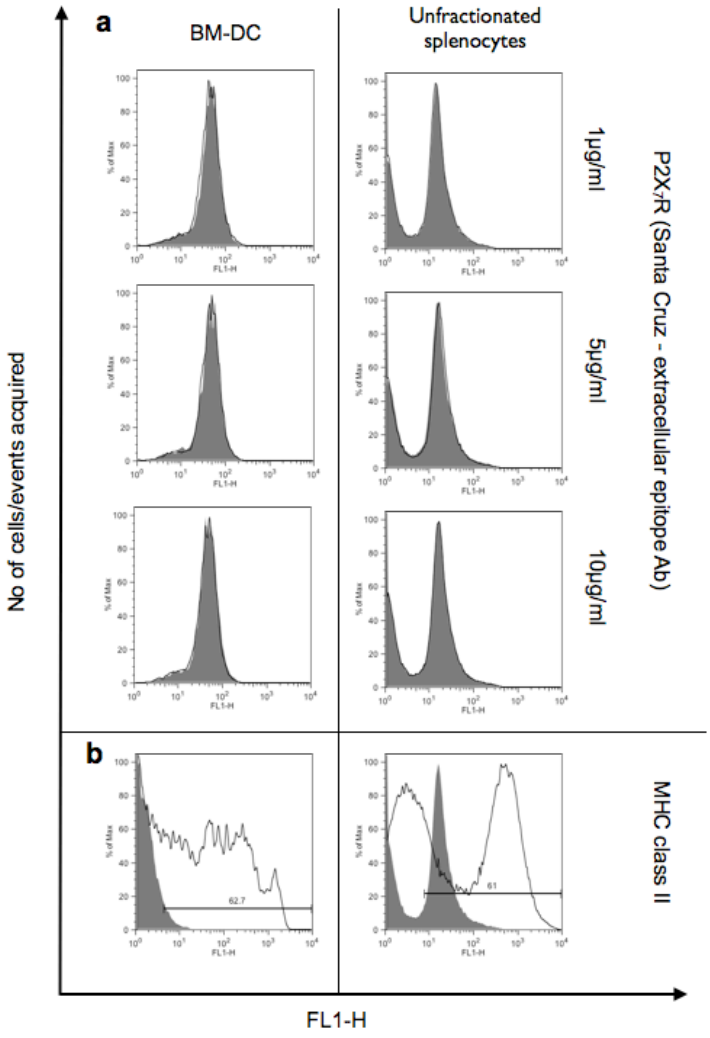


***Figure 4.11:* Flow cytometric analysis of the cell surface P2X$_7$R expression on day 8 BM-DC and unfractionated cultured splenocytes.**
Day 8 BM-DC and mechanically disaggregated, unfractionated splenocytes (positive control) at 1x10^6/ml were incubated in medium alone for 24h. Cells were stained for the cell surface expression of P2X$_7$R with (a) a P2X$_7$R-specific antibody designed to bind the extracellular P2X$_7$R -loop (Santa-Cruz antibody) at 1, 5 or 10µg/ml and also for the expression of MHC class II molecules (b). Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. The expression of MHC class II and P2X$_7$R is shown as plotted histograms (open line) overlaid with the appropriate isotype control (closed line).

4.2.10 Further efforts to detect $P2X_7R$ expression on BM-DC using flow cytometry.

In a further attempt to provide direct evidence of cell surface expression of the $P2X_7R$ on day 8 BM-DC but also gain a perspective of the levels of cell surface expression, two different antibodies directed against the same intracellular epitope on the C-terminus of the $P2X_7R$ were employed. BM-DC and non-transfected HEK-293 cells, a tissue used routinely as a negative control for $P2X_7R$, were stained for $P2X_7R$ and for MHC class II (used as positive control for membrane marker expression) and analysed by flow cytometry (figure 4.12). A polyclonal antibody known as the *Alomone Labs* antibody as well as a monoclonal antibody from *Epitomics,* were employed in an effort to detect $P2X_7R$ expression. Analyses revealed a clear peak positive for $P2X_7R$ expression present in BM-DC preparations stained with the two different types of antibodies and in each case positive staining was also observed for MHC class II. However, detectable levels of $P2X_7R$ were also found on non-transfected HEK-293 cell, suggesting that both antibodies bind non-specifically to the target tissue.

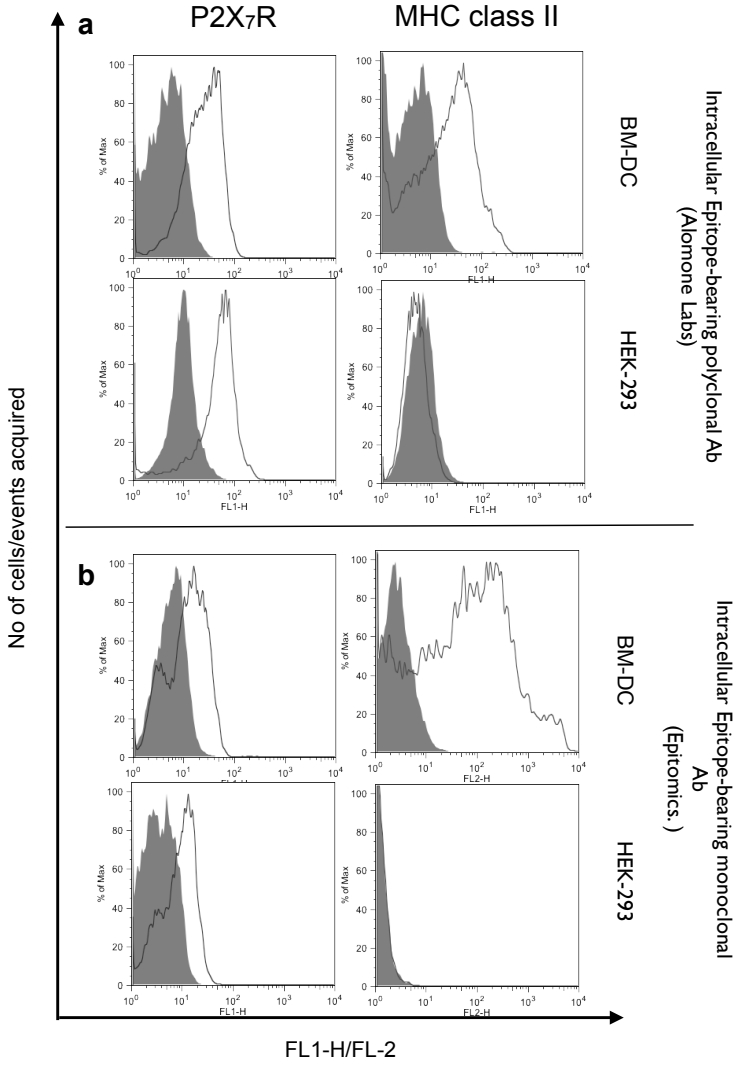


***Figure 4.12:* Flow cytometric analysis of the cell surface P2X$_7$R expression on BM-DC and human embryonic kidney (HEK)-293.**
Day 8 cultured BM-DC and non-transfected HEK-293 cells (negative control) at 1x10^6 cells/ml were stained with (a) a polyclonal (Alomone Labs) and (b) a monoclonal (Epitomics) P2X$_7$R-specific antibody designed to bind the P2X$_7$R C-terminus and also for the expression of MHC class II molecules (positive control). Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. The expression of MHC class II and P2X$_7$R is shown as plotted histograms (open line) overlaid with the appropriate isotype control (closed line).

4.2.11 Immunohistochemical analysis of BM-DC for $P2X_7R$.

Having failed to detect expression of the $P2X_7R$ with flow cytometry, BM-DC were next analysed for $P2X_7R$ expression using immunohistochemical analysis with the *Alomone Labs* antibody (intracellular epitope). BM-DC were grown in the presence of GM-CSF for 8 days before the cells in suspension were isolated and cultured in medium for 24h. The cultured cells were subsequently stained *in situ* with the specific antibody directed against the $P2X_7R$, or with anti-CD11c antibody (positive control) and their respective isotype controls (). BM-DC were found positive for CD11c, however, the isotype control for $P2X_7R$ expression was found to stain more strongly than the antibody itself, suggesting that the anti-$P2X_7R$ antibody binds non-specifically to the target tissue.

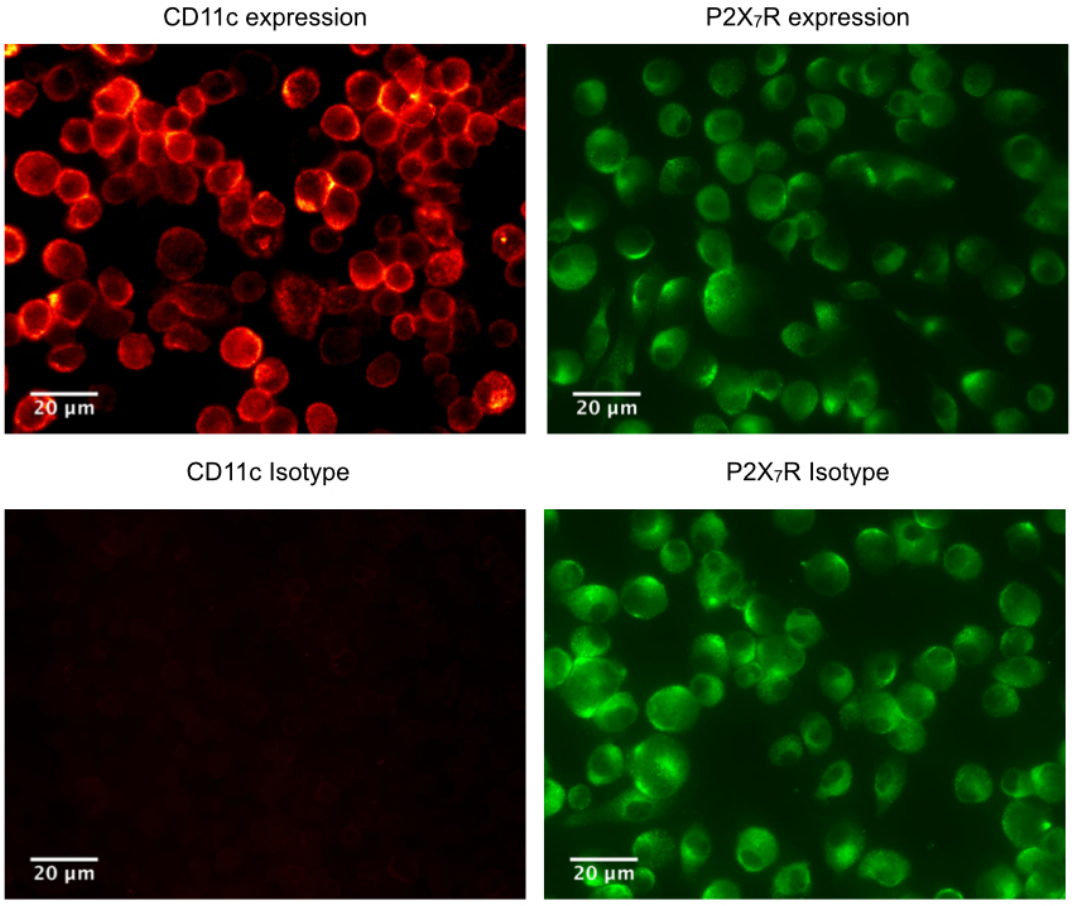


***Figure 4.13:* Immunohistochemical analysis of day 8 BM-DC for P2X$_7$R expression.**
Day 8 BMDC at 0.25x10^6/ml were incubated in medium alone for 24h. Cells were stained in situ in the tissue culture plate for the expression of P2X$_7$R (Alomone Labs antibody, green) and for CD11c expression (red), as well as with the appropriate isotype controls. Images were collected using an Olympus (BX51) upright fluorescent microscope with a 40x/0.74 PlanFLN objective, captured using a coolsnap ES camera through MetaVue software (Molecular Devices) and analysed using ImageJ 1.46R.

4.2.12 Western blot analysis of BM-DC reveals P2X$_7$R expression.

Given that the two methods (flow cytometry and immunocytochemistry) for directly detecting P2X$_7$R expression failed to provide convincing evidence of expression of this receptor by murine BM-DC, the next experimental approach involved analysing BM-DC for P2X$_7$R expression using Western blot. This technique has used successfully in this respect for other murine cell types such as macrophages [495]. Lysates from three different preparations of each of naive day 8 BM-DC, day 8 BM-MΦ, the adherent population of peritoneal exudates, non-transfected HEK-293 and unfractionated splenocytes were analysed for receptor expression using the polyclonal rabbit anti-mouse Alomone antibody with specificity for a P2X$_7$R intracellular loop epitope developed for Western blotting (Figure 4.14). A band representing protein expression at the appropriate molecular weight (75kDa) was detected in all cell types apart from HEK-293 lysates (negative control). Protein concentration was equilibrated for all samples using a colorometric Lowry assay. Of note is that a stronger signal was detected in lysates of day 8 BM-MΦ in comparison to PMΦ and day 8 BM-DC and a much weaker signal was detected in unfractionated splenocytes.

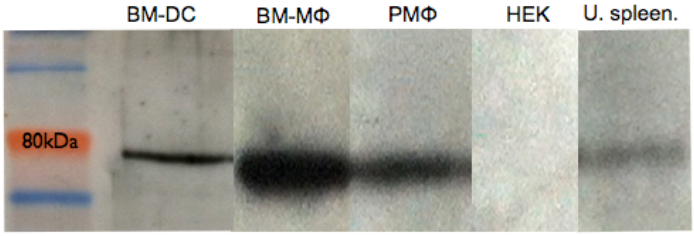


***Figure 4.14:* Western blot analysis confirms P2X$_7$R expression on day 8 BM-DC, BM-MΦ and isolated PMΦ.**
Day 8 cultured BM-DC, BM-MΦ, HEK-293, disaggregated splenocytes at 1x10^6 cells/ml and peritoneal exudates (PMΦ) were cultured for 24h in the presence of medium alone. Cell lysates were collected from BM-DC, BM-MΦ, the adherent population of cells from peritoneal exudates, HEK-293 cells (negative control) and undifferentiated splenocytes (positive control) and a colorometric Lowry assay to quantify the protein concentration in each sample. Equal amounts of protein (20µg) from each cell type was analysed for the expression of P2X$_7$R and representative images are shown from analyses of three independent preparations.

4.2.13 Facilitating of P2X$_7$R currents on CD11c$^+$ BM-DC.

Having established P2X$_7$R expression in day 8 cultured BM-DC using Western blot analysis the next step was to (a) use patch clamping to assess the electrophysiology of the P2X$_7$R on BM-DC and (b) confirm at the single cell level that a cell is both positive for CD11c and P2X$_7$R expression. This is important as approximately 30% of BM-DC were found to be negative of CD11c expression. Day 8 BM-DC were stained for the expression of CD11c or with the appropriate isotype control antibody (Figure 4.15a) and CD11c$^+$ cells were clamped *in situ* for further patch recordings. First, current flow from a whole-cell clamp was recorded following sustained (10s) applications of increasing concentrations of ATP (100μM to 5 mM). A dose-dependent growth in current flow was observed and the representative traces are shown in the graph in figure 4.15b. Clamped cells received repetitive applications of ATP (5 mM) to facilitate current flow. A rapid onset of inward flowing current was evoked following the fast-flow and sustained application of ATP, a characteristic response of a ligand gated ion channel. Figure 4.15c displays representative traces for current flow from a whole-cell clamp during sustained (10s) ATP (5 mM) applications. An approximately 2-fold increase in current amplitude was observed with repetitive applications of ATP that had reached maximal levels (recorded at 250pA) by the third application. Patch recordings of fully facilitated currents in CD11c$^+$ BM-DC revealed a range of amplitude up to 100pA at the maximal dose of ATP (figure 4.15c).

To investigate the role of the P2X$_7$R channel in the recorded ATP-evoked current, the impact of the A-740003 treatment was investigated next. A-740003 is a selective antagonist of the P2X$_7$R with strong potency for all P2X$_7$R orthologues [408]. Due to its pharmacokinetic properties the drug-like agent has become a popular tool in investigating the properties of the P2X$_7$R. Thus, CD11c-expressing BM-DC received repetitive applications of ATP (5 mM) to fully facilitate current flow before A-740003 treatment was applied at the same time as further repeat applications of ATP. Patch recordings of fully facilitated currents before A-740003 treatment revealed amplitudes of 50pA. Treatment with the P2X$_7$R specific inhibitor A-740003 was found to completely block ATP-evoked sustained currents (figure 4.15d).

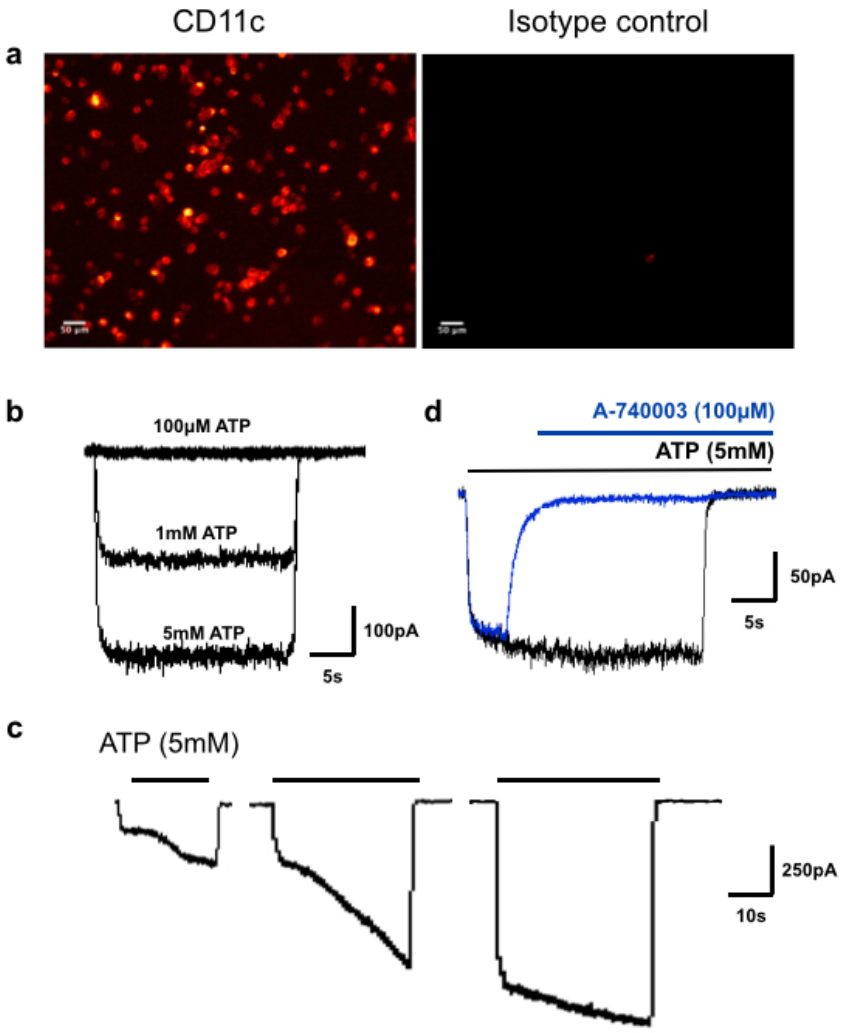


***Figure 4.15*: ATP-induced currents in CD11c$^+$ BM-DC.**
A: Day 8 BM-DC at 0.25x10^6/ml were cultured on glass coverslips and incubated in medium alone for 24h. Cells were immune-stained for CD11c expression (red) or with the appropriate isotype control antibody. Images were collected using an Axiovision with an upright microscope body with a 10x/0.13 PlanFLN objective, captured using a coolsnap ES camera through MetaVue software (Molecular Devices) and analysed using ImageJ 1.46R B: Concentration dependence of ATP-evoked currents. ATP was applied at three different concentrations examined (100μM, 1 mM and 5 mM) to fully facilitated CD11c$^+$ BM-DC channels (mean ± SEM; n = 6) C: Representative traces showing current facilitation in clamped CD11c$^+$ BM-DC following repetitive applications of ATP at 5 mM (for 30 s at 30 s intervals) (n = 6) D: Pharmacological profile of P2X$_7$R mediated currents. Responses to 5 mM ATP, were made in the presence (blue trace) and absence (black trace) of the specific P2X$_7$R inhibitor, A-740003 (100μM). A-740003 was applied for 20 s during a sustained application of ATP (5 mM, 30 s), as shown. An ATP control recording (30s) was made 30 s before the experimental sweep with A-740003.

4.2.14 Comparing the rate of endotoxin and ATP-induced YO-PRO dye uptake in BM-DC and BM-MΦ.

Currently, two of the most distinct cellular responses of the P2X$_7$R are the rapid release of pro-inflammatory cytokine and the recruitment of a dye (up to 900Da) permeable pathway [496, 239]. The exact subcellular constituents of this permeation pathway that allows the uptake of YO-PRO molecules are not entirely clear, however, dye uptake assays have become a popular tool in investigating P2X$_7$R physiology and function. The next experimental objective involved investigating the pore-forming ability of the P2X$_7$R activation using the YO-PRO fluorescent dye uptake assay in BM-DC and BM-MΦ. Dye uptake was quantified using the FlexStation, a widely used tool employed to investigate the functional characteristics of receptors such as the P2X$_7$R. It allows the automatic addition of fluids into a fluorescent plate reader and is a high throughput method for analysing functional properties of ion channels such as the YO-PRO dye uptake assay employed herein [497].

No dye uptake was detected in untreated or in LPS-primed but not ATP-activated BM-DC and BM-MΦ (figure 4.17a,b,c). Conversely, challenge of unprimed (LPS) BM-DC with ATP at 1 mM induced substantial dye uptake (>1000 RFU) (figure 4.16 a, c). Interestingly, activation of LPS-primed BM-DC with ATP (1 mM) appeared to have greatly enhanced the rates of dye uptake by more than a 3 –fold with the final levels of fluorescence recorded at approximately ~3500 RFU. With regards to BM-MΦ, the kinetics of dye uptake were similar between non-primed and LPS-primed cells following challenge with ATP with the cumulative levels of dye uptake recorded at around 1000 RFU. Furthermore, the final levels of fluorescence in LPS-primed BM-DC (~3500 RFU) challenged with ATP at 1 mM were significantly higher in comparison to those recorded in LPS-primed BM-MΦ (~1800) (figure 4.16c).

Both cell populations were also challenged with higher levels of ATP (5 mM), to investigate the kinetics of pore formation and dye uptake (figure 4.16d, e). No change in fluorescence was observed in the untreated cell populations or LPS primed only cells. Challenge with ATP at 5 mM of non-LPS-primed BM-DC or BM-MΦ evoked a substantial increase in the levels of fluorescence. Importantly, the accumulated levels of fluorescence were found at significantly higher levels in BM-MΦ (~3000) in

comparison to BM-DC (~1000) (figure 4.16f). Similar, levels and kinetics of dye uptake were were observed in LPS-primed cells challenged with ATP. Furthermore, the kinetics of pore formation and dye uptake were notably faster in BM-MΦ (~1000RFU 30 min post ATP application) in comparison with BM-DC (~1000RFU 90 min post ATP application) (figure 4.16d, e).

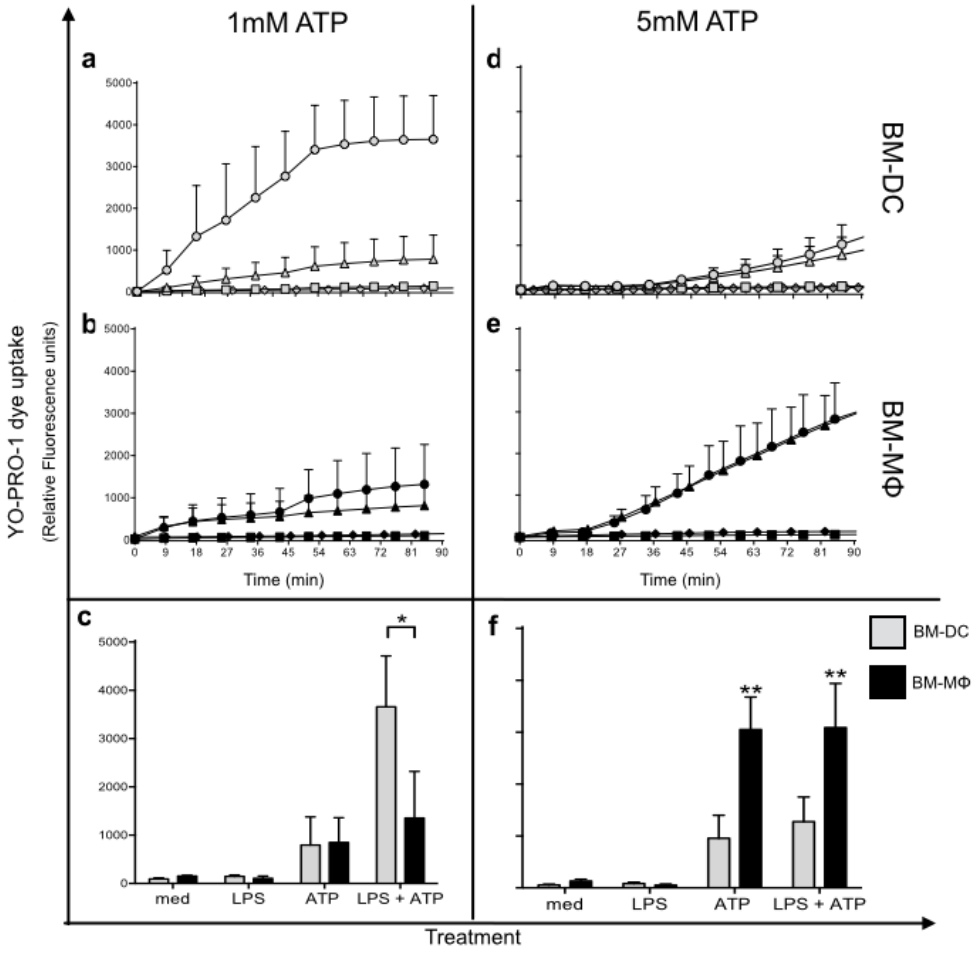


***Figure 4.16:* The rate of YO-PRO dye uptake on day 8 BM-DC and BM-MΦ.**
Day 8 BM-DC and BM-MΦ at 0.3x10^6/ml were incubated in medium alone or in the presence of 1000ng/ml LPS for 2h and subsequently treated with medium alone or with ATP at 1 (a, b, c) or 5 mM (d, e, f). Time-depended YO-PRO dye uptake was recorded as changes in fluorescence measured at 3-sec intervals for 90min, using a Flex Station 3. Recordings were made in the presence of a low divalent extracellular bathing medium. Traces shown are of medium- (squares), LPS- (diamonds), ATP- (triangles) and LPS with ATP-treated (circles) BM-DC (a, d), and BM-MΦ (b, e) and presented as real fluorescence units over time. The cumulative levels of dye uptake (relative fluorescence units) are compared between the different treatments of BM-DC (grey bars) and BM-MΦ (black bars) 90 min post ATP application at 1 mM (c) and 5 mM (f). Data shown are mean (n=3) ±SEM. Statistical significance of differences between groups was assessed by two way ANOVA and Sidak's multiple comparison post-hoc test (*= $p<0.05$).

The rate of dye uptake was also investigated following challenge of ATP at 10 mM in both BM-DC and BM-MΦ shown in figure 4.17. The traces of dye uptake induced by ATP challenge, at 10 mM, in both untreated and endotoxin-primed BM-DC and BM-MΦ were almost identical. Although some levels of dye uptake were observed following challenge with a high concentration of ATP a large majority of the cells observed to detach off the plate suggesting that this concentration was most likely toxic for the cells. As such, these experimental conditions were not repeated.

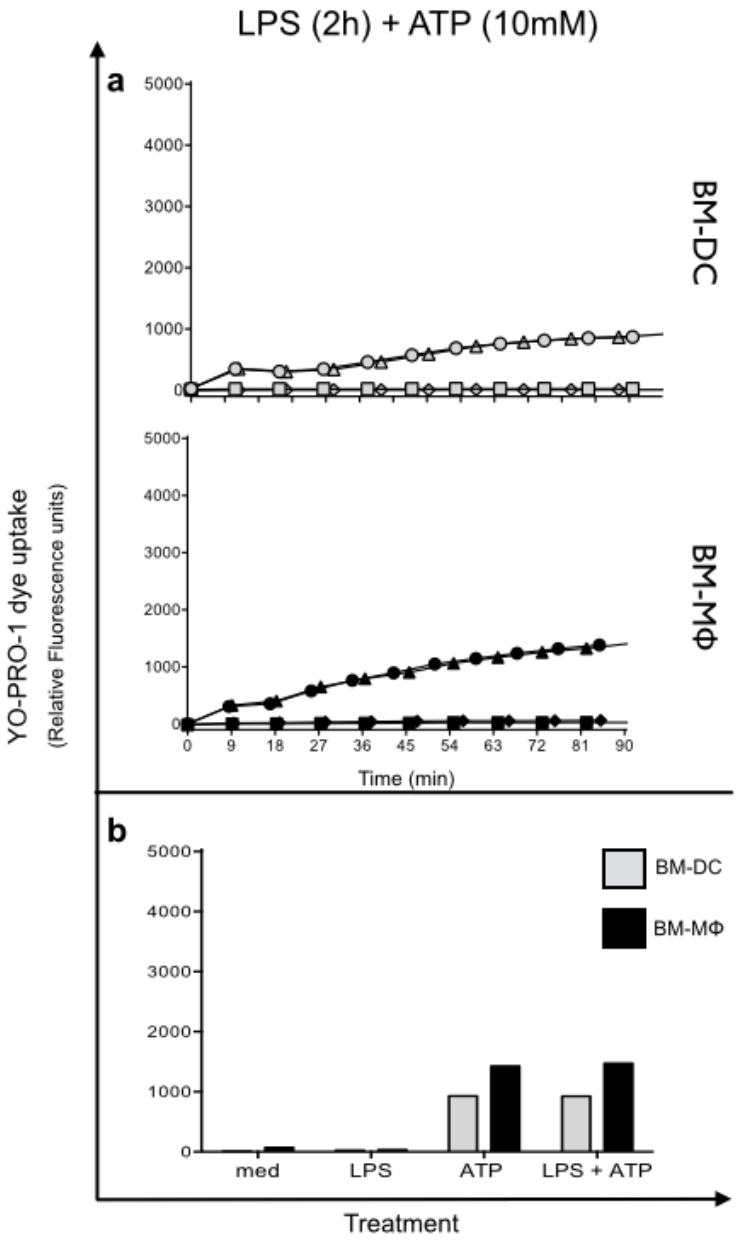


***Figure 4.17:* Day 8 BM-DC and BM-MΦ exhibit similar rates of YO-PRO dye uptake in the presence of high levels of exogenous ATP.**
Day 8 BM-DC and BM-MΦ at 0.3x106/ml were incubated in medium alone or in the presence of 1000ng/ml LPS for 2h and subsequently activated with medium or 10 mM of ATP. Time-depended YO-PRO uptake was observed as changes in fluorescence recorded at 3-sec intervals using a Flex Station 3, for 90min. Recordings were made in the presence of a low divalent extracellular bathing medium. Traces shown are of medium- (squares), LPS- (diamonds), ATP- (triangles) and LPS with ATP-treated (circles) BM-DC and BM-MΦ, presented as real fluorescence units over time (a). The cumulative levels of dye uptake (real fluorescence units) are compared between the different treatment groups of BM-DC (grey bars) and BM-MΦ (black bars) 90 min post ATP application (b).

4.2.15 A-740003 treatment blocks ATP-induced YO-PRO dye uptake in LPS-primed BM-DC and BM-MΦ.

The effect of A-740003 treatment on ATP-induced dye uptake in BM-DC and BM-MΦ was assessed by immunohistochemical analysis to provide visual evidence of both dye uptake by BM-DC and BM-MΦ and the effect of the A-740003 antagonist on this $P2X_7R$ -associated function. As shown in figure 4.18a no visible fluorescence was detected in control vehicle and A-740003 treated BM-DC and BM-MΦ (figure 4.18a and d). Activation of non-LPS primed BM-DC with ATP (5 mM) induced a small increase in the levels of fluorescence as assessed with microscopy. In contrast, visibly higher levels of fluorescence were observed in ATP-treated naïve BM-MΦ (figure 4.18b). The complete absence of fluorescence in medium-treated, A-740003 treated, ATP-activated BM-DC and BM-MΦ suggests that dye uptake was blocked by the $P2X_7R$-specific pharmacophore (figure 4.18c) providing strong evidence that YO-PRO dye uptake is $P2X_7R$-driven mechanism in both cell types.

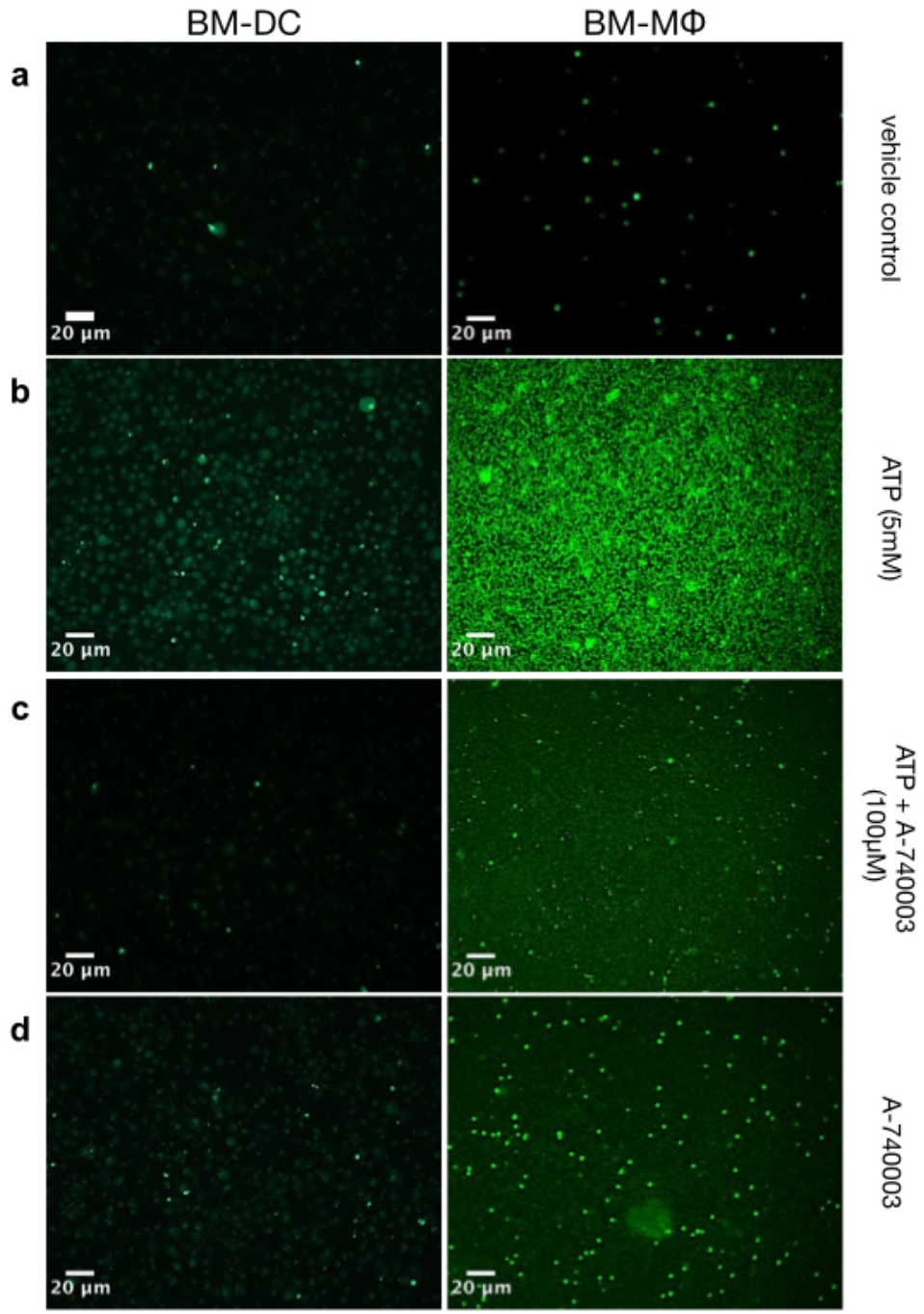


***Figure 4.18:* A-740003 blocks ATP induced YO-PRO dye uptake in naive day 8 BM-DC and BM-MΦ.**
Day 8 BM-DC or BM-MΦ at 0.25x10^6/ml were treated with A740003 (100μM) or DMSO at 0.5% (vehicle control) for 10 min before activated with ATP (5 mM). Treatments were conducted in the presence of a low divalent extracellular bathing medium. Changes in the levels of fluorescence and YO-PRO dye uptake were recorded 90 min following ATP activation with image acquisition. Images were collected using an Axiovision with an upright microscope body with a 10x/0.13 PlanFLN objective, captured using a coolsnap ES camera through MetaVue software (Molecular Devices) and analysed using ImageJ 1.46R.

As shown in figure 4.19, in the absence of exogenous ATP application, LPS priming and DMSO treatment (A-740003 vehicle control) of BM-DC and BM-MΦ did not activate the permeation pathway for dye uptake in either cell type. Dye uptake was also absent in control treatment groups (LPS with A-740003, figure 4.19b). Activation of both endotoxin primed BM-DC and BM-MΦ with ATP (5 mM) induced a visible increase in fluorescence as a result of YO-PRO dye uptake with comparatively higher levels of fluorescence evident in BM-MΦ and consistent with the previous flexstation data (figure 4.19c). The increase in fluorescence caused by dye uptake in LPS-primed and ATP-activated BM-DC and BM-MΦ was successfully blocked with A-740003 treatment suggesting that ATP-induced dye uptake is $P2X_7R$ mediated (figure 4.19d).

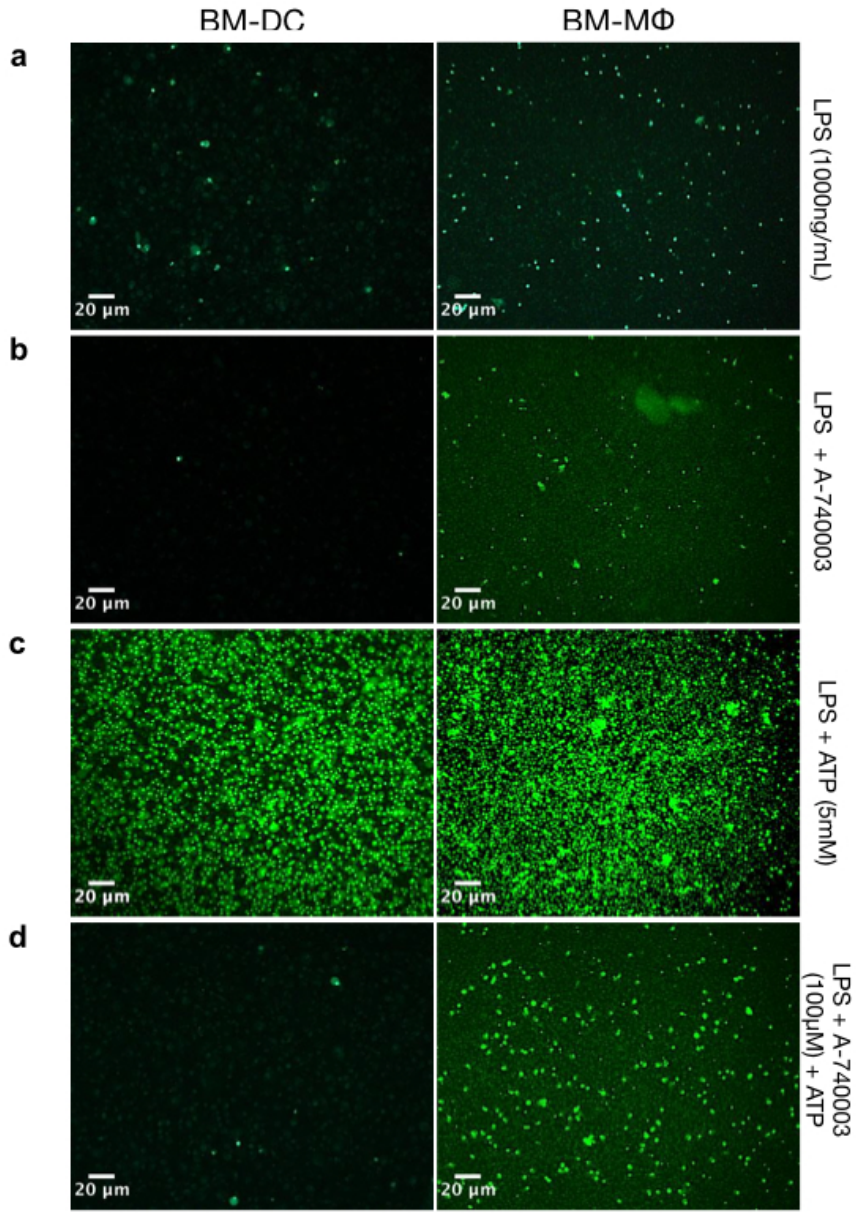


***Figure 4.19:* A-740003 blocks ATP induced YO-PRO dye uptake in LPS-primed day 8 BM-DC and BM-MΦ.**
Day 8 BM-DC or BM-MΦ at 0.25x10^6/ml were incubated in the presence of LPS (1000ng/ml) for 2h. Subsequently, cells were treated with A-740003 (100μM) or DMSO at 0.5% (vehicle control) for 10 min before activation with ATP (5 mM) or medium. Treatments were conducted in the presence of a low divalent extracellular bathing medium. Changes in the levels of fluorescence and YO-PRO dye uptake were recorded 90 min following ATP activation with image acquisition in the following treatment groups: (a) 1000ng/ml LPS, (b) LPS + A-740003 (c) LPS + 5 mM ATP, and (d) LPS + ATP + 100μM A-740003. Images were collected using an Axiovision with an upright microscope body with a 10x/0.13 PlanFLN objective, captured using a coolsnap ES camera through MetaVue software (Molecular Devices) and analysed using ImageJ 1.46R.

4.3 Discussion.

Since it was first cloned in the mid-1990s the $P2X_7R$ has received considerable attention due to its 'peculiar' nature and its important role in immune responses. The receptor displays high levels of expression in cells of the haematopoietic lineage, particularly in macrophages and microglia [267, 316, 498, 12] which has justifiably intrigued the scientific community. Therefore, much of the focus in investigating $P2X_7R$ physiology was directed to these two cell types or $P2X_7R$-transfected HEK-293 cells that provided an easy gateway in investigating the pharmacokinetic properties of the human $P2X_7R$ channel and all its mammalian orthologues [325]. Instead, these investigations have focused in examining $P2X_7R$ responses in primary DC and macrophage populations and preliminary evidence revealed some interesting differences between different cell types that are discussed below and more importantly, merit further work.

4.3.1 Phenotypic analysis of BM-MΦ colonies.

When assessing the functional properties of the $P2X_7R$ on DC it was important to identify an appropriate isogenic control for direct comparison with BM-DC and it was decided that macrophages were the most appropriate reference cell type, since a lot of previous work has focused on this cell type. Macrophages are a specialized heterogeneous population of phagocytes, found in both lymphoid and non-lymphoid tissue, that play an important part during both innate and adaptive immune responses. They are also a very 'plastic' population of cells that adapt their phenotypic characteristics in response to a wide variety of environmental stimuli [499, 138]. They are well known for their immune effector functions against pathogens, however, they are also an important component of tissue homeostasis under steady-state conditions [500]. One common source of macrophages used experimentally are BM precursors cultured in the presence of M-CSF to generate BM-MΦ. This is a preferred method for generating naïve macrophages *in vitro* as it yields a high number of homogeneous cells and is easily reproducible [501]. The cells have a high proliferative capacity and a long lifespan that can last up to 3 weeks in primary culture without any major phenotypic differences [502]. Macrophage cell lines, such as the J774A.1, RAW264.7 and U937, were another option, however, one major caveat when using *in vitro* cell line is the

uncertainty of whether the signalling pathway of interest is either functional or present [503, 504]. Instead BM-MΦ are a source of a well-defined homogeneous population and are usually preferred to resident or elicited populations [505].

Since cultured BM-MΦ were employed as a suitable isogenic control for BM-DC responses, both cell types were derived from the same BM progenitors. Protocols for generating macrophage from BM precursors are numerous and vary according to the strain of mice used, culture dishes, and the source of M-CSF. Previous studies have shown that M-CSF is a critical component for the differentiation of precursors to the monocyte/macrophage lineage. In the absence of functional M-CSF, mice are deficient in the macrophage and osteoclast populations and are susceptible to the development of osteoporosis [506, 502]. Most studies employ conditioned medium from L-929 fibroblast cell line supernatants in which to culture BM precursors, a rich source of M-CSF [507]. An obvious concern regarding the use of L-929 conditioned medium is the presence of unwanted cell products in the medium that can influence the level of maturation of the cells. The most notable of these factors is IFN-β which is constitutively produced by L-929 cells at low levels [505]. To minimize this effect, only 30% of the crude conditioned medium was used to culture the cells. To monitor any unwanted effects, untreated cells were analysed for differentiation markers such as F4/80 expression and activation markers like MHC class II. PMΦ were also employed as a reference cell type in addition to BM-MΦ, however, to a lesser extent. The peritoneal cavity provides an easily accessible site for harvesting considerable numbers of cells and they are a well-characterized population. The cells were isolated from the peritoneal cavity following the protocol of Davies and Gordon [508]. Thioglycollate activation was avoided because of concerns of the effect on the macrophage profile. For instance, Leijh and colleagues reported that thioglycollate-elicited PMΦ are unable to destroy ingested pathogens [509, 510].

To identify the optimum culture conditions for BM-MΦ, the phenotype of untreated cells was assessed following 8 and 12 days *in vitro* culture with L-929 conditioned medium to identify the optimum time frame for use of BM-MΦ for further experiments. Prolonged *in vitro* culture with L-929 conditioned medium appeared to impact on the expression of F4/80 and enhance the levels of CD11c expression, probably due to the prolonged exposure of unknown factors released by BM-MΦ themselves or derived

from the conditioned medium. Day 8 BM-MΦ were found to exhibit a more macrophage-like phenotype with lower levels of the activation marker, MHC class II and the DC-associated integrin CD11c, whilst the levels of a macrophage-restricted plasma membrane antigen marker, for murine macrophages, F4/80, were very high. BM-MΦ are known to grow in clusters and adhere very strongly to plastic culture dishes [511]. The cells needed to be detached, quantified and re-plated before any experimental analysis. Specialized bacterial petri dishes were utilized which allowed the detachment of the cells with EDTA treatment without any effect on cell surface marker expression. However, the strong adherence of re-plated cells presented a technical problem for analyses of cell surface marker expression by methods that required a single cell suspension, such as analysis of cell surface marker expression by flow cytometry. One potential approach to circumvent this problem would be to culture the cells in Teflon non-stick membranes to avoid attachment on the cells, although it is not clear what effect this may have on the phenotype of the population [511]. Vigorous shaking and sometimes scraping was therefore required to detach BM-MΦ for analysis.

A similar problem was observed with the PMΦ population, which were found to be strongly adherent and posed a real challenge for manipulation for experimentation. Peritoneal exudate cells were first cultured and allowed to adhere overnight. Non-adherent populations were discarded and the adherent cells needed to be quantified and re-plated for further experiments. Detaching the cells for quantification and further analysis, however, posed a difficult challenge. Several methods were employed in an attempt to detach the cells including treatment with EDTA, lidocaine and even pre-coating the plates with FCS, however, the cells would not detach without vigorous scraping [512]. None of the aforementioned approaches employed appeared to work efficiently and employing vigorous scrapping to remove the cells raised a few concerns. One of the main issues was the effect on cell surface membrane marker expression, as measured by flow cytometry, but also on the viability of the cells and their ability to produce pro-inflammatory cytokines. For example, levels of F4/80 expression were widely variable (see figure 4.6), cytokine production and release (IL-1β) was minimal (data not shown) and these were observations that contradict published observations [513]. Thus, peritoneal exudate cells were also employed. The cells were plated at $2x10^6$ per well and allowed to adhere overnight. Subsequently, the non-adherent

population was discarded the following day and the cells were used for further experimentation with 70-80% confluence of the adherent population

Both macrophages populations were analysed along with BM-DC for the expression of the markers of interest *in situ* to obtain a more realistic phenotypic analysis of the cells. The phenotypic profile of PMΦ was found to resemble that of BM-MΦ with high levels of F4/80 and moderate levels of CD11b expression. Although similar levels of CD11b expression were detected in BM-DC cells, the levels of F4/80 expression were undetected. Interestingly, flow cytometric analysis of BM-DC for the expression of F4/80 revealed 40% expression highlighting differences in the sensitivity between the two techniques. The opposite was observed with both macrophage populations where lower F4/80 levels were detected with flow cytometric analysis in comparison with immunohistochemical analysis *in situ,* which was probably the effect of rigorous scraping to detach the cells for analysis. Additionally, high expression levels of CD11c were detected in BM-DC but were very low in both BM-MΦ and PMΦ and these results reflected those obtained with flow cytometric analysis. Collectively, the phenotypic and functional analysis of BM-MΦ suggested that the *in vitro* culture conditions employed successfully nurtured the differentiation of BM precursors towards the macrophage lineage exhibiting a similar phenotypic profile to that detected in PMΦ [514, 515].

All three cell populations were also analysed for the expression of the antigen presenting machinery, MHC class II. The low levels of MHC class II expression in BM-MΦ but not PMΦ, came as a surprise. MHC class II expression is an important functional molecule for antigen presentation and its expression is enhanced challenge with endotoxin, in BM-DC (see Chapter 3 section 3.2.3). In contrast, MHC class II expression was relatively low and was downregulated with LPS treatment in BM-MΦ. In accordance with the observations of this study, Warren and Vogel and others, also report similar observations and show that the low levels of MHC class II expression in macrophages are due to a suppressive effect of prostaglandins. The upregulation of prostaglandins in BM-MΦ is the result of M-CSF signalling present in the cultured medium during their differentiation process from BM precursors [516, 505]. Consistent with this, it has been reported that MHC class II expression is upregulated in the absence of M-CSF and under IFN-γ signalling in BM-MΦ [517].

Thus far, the phenotype of BM-DC and BM-MΦ with regards to cell surface specific markers, their ability to up-regulate important components for migration and antigen presentation and their ability to induce the release of soluble inflammatory factors was examined in detail. An equally important functional property of APC is to engulf antigen. They do so via three main pathways: macropinocytosis, endocytosis and receptor-mediated endocytosis [518]. Previous reports show that both DC and macrophages take up a number of different macromolecules (such as FITC-Dextran, ovalbumin) from the extracellular milleu by a combination of receptor-mediated endocytosis and macropinocytosis. Therefore, in an attempt to assess the ability of BM-DC and BM-MΦ to engulf antigen the cells were treated with BSA protein that was coupled to a fluorescent tracer (FITC-BSA). It was important to discriminate between particles that have been internalized by the cells and those that simply bind with high affinity to the bilayer, therefore the uptake assay was concurrently carried out at 37°C and on ice. Although this process might not directly compare with the physiological conditions *in vivo*, in accordance with previous studies the results suggest that both cell types were able to take up copious amounts of the FITC-BSA pseudo antigen [519, 492, 518].

The current view dictates that the phagocytic capacity of macrophages is superior to that of DC, *in vitro* and *in vivo*, as well as LC [433, 520, 521]. Studies comparing the ability of macrophages and DC to uptake pseudoantigens are scarce and so these investigations attempted to compare the capacity of the two cell types to interact and engulf antigen. In discordance with previous observations the results of this study suggest that the phagocytic capacity for FITC-BSA molecules is comparable between DC and macrophages. The observations of Thiele and collaborators on blood derived DC and macrophages suggest that small particles like FITC-BSA are more efficiently engulfed by macrophages in comparison to DC. Conversely, the 'appetite' of DC and macrophages for larger particles (coated polystyrene particles) is equivalent between the two cell types [522].

The current view also dictates that properties such as the size, charge and structural features of the antigenic particle will affect the rate of uptake by DC and macrophages. Improving our understanding of how antigenic particles are taken up by APC will aid in the development of efficient antigen delivery system to alert DC for therapeutic

applications. The purposes of this study were limited in establishing the functional capacity of BM-DC and BM-MΦ in engulfing antigen in the form of FITC-BSA and satisfactory results were obtained.

4.3.2 Identifying $P2X_7R$ expression in murine BM-DC

$P2X_7R$ is expressed ubiquitously in mammalian tissues with surprisingly high expression levels in cells of the immune system. It is also expressed in glial, microglia, Schwann cells and oligodendrocytes [316, 523, 317]. Northern blot analysis of both rat and human tissues revealed the presence of $P2X_7R$ mRNA pancreas, liver, placenta testis and many other tissues [317]. Data for functional $P2X_7R$ responses are limited to mainly cells of the haematopoietic lineage, macrophages, microglia and LC [480, 524]. The development of a monoclonal antibody raised against an extracellular loop epitope of the human $P2X_7R$ by Buell and colleagues allowed for the investigation of receptor expression at least on human tissues [525]. Flow cytometric analysis by Gu and colleagues in various cell types of human blood revealed 5-fold higher expression levels of the receptor in monocytes compared with circulating B- T- and NK-cells. The functional relevance of this finding, however, remains unclear since monocytes are comparatively larger in size and would be expected to express higher levels of the receptor. In contrast, blood platelets or polymorphonuclear neutrophils do not express detectable levels of the receptor [319]. A weak baseline expression of the receptor was identified in human $CD14^+$ monocytes, which remained unaffected by endotoxin challenge [526]. Georgiou and colleagues have also reported expression of the receptor on human epidermal LC and monocyte-derived DC using flow cytometric analysis [343]. In mouse tissues direct expression of $P2X_7R$ and function has mainly focused on macrophages and microglia cells [316].

With regard to DC subpopulations, high levels of $P2X_7R$ expression have been detected in human DC, isolated from peripheral blood, using Western blot analysis and flow cytometry as reported by Ferrari and colleagues. In fact, due to its high levels of expression, the P2X purinoceptor could in theory constitute a phenotypic marker for these cells [495]. With regards to identifying $P2X_7R$ expression in murine tissues a popular approach is to first confirm expression at the transcriptional level and subsequently assess protein expression using various $P2X_7R$-associated functional

assays in conjunction with specific inhibitors. For example, Coutinho-Silva and colleagues report P2X$_7$R expression in a spleen derived murine DC cell line at the transcriptional level. Subsequently, the group recorded large inward fluxes of calcium ions following ATP activation, detected by changes in fluorescence emissions of cells, and uptake of large macromolecular fluorescent molecules (Lucifer yellow), an event associated with P2X$_7$R activation [527]. A similar study by Nihei and colleagues, reported a functional P2X$_7$R on mouse splenic DC using dye uptake assays in conjunction with oATP treatment (a P2X$_7$R -specific inhibitor) [338]. Immunofluorescence and flow cytometry are not as commonly used, however, a few studies report P2X$_7$R expression in epidermal cells including mouse LC by using these techniques. The same group have previously assessed the expression of P2X$_7$R on human LC using flow cytometry and were found to be substantially lower to those expressed by human keratinocytes. In contrast, mouse keratinocytes and LC were found to express comparable levels of the receptor, which begs the question as to what the functional relevance behind such discrepancies is [343, 342].

Direct evidence for P2X$_7$R expression on DC populations is scarce. To complicate things even further it is unclear whether the receptor is stored within intracellular pools or whether it is recycled to the cell surface following ligand binding. Large intracellular pools of the P2X$_7$R are found in platelets and neutrophils, which is rather unusual since cell surface expression is low. It is quite possible that these 'reserves' are recruited following activation of the cells. In general, little is known regarding the mechanisms regulating cell surface expression of the P2X$_7$R [340]. Characterizing the receptor using classical pharmacological studies (calcium ion and dye uptake technique) alone is increasingly regarded as inadequate, as the receptor could display unknown forms of shared activity with its 7 sibling receptors. Techniques like flow cytometry (and other molecular biology approaches) are now considered a prerequisite for studying the functionality of the receptor [338]. Flow cytometry provides for the rapid and quantitative analysis of a large number of cells, has a highly sensitive fluorescence detection system and can also distinguish between different cell populations based on the expression of specific cell markers, which are identified using multiple fluorescent tags. Thus, flow cytometry was first employed to detect receptor expression in murine BM-DC to provide a comparative expression profile between murine macrophages and

DC and possibly further explore the mechanisms of cell surface expression of the receptor on DC.

A *Santa-Cruz antibody* that bears an extracellular epitope was first employed to detect receptor expression in BM-DC using flow cytometry. No detectable levels of $P2X_7R$ were recorded in BM-DC or unfractionated splenocytes. One possible explanation could simply be that DC and splenocyte populations express low levels of cell surface receptor expression. The levels $P2X_7R$ expression reported by Tran and colleagues on mouse LC as detected by flow cytometry were particularly low [342]. In human monocytes and macrophages, the highest levels of $P2X_7R$ are located in intracellular stores, particularly in the membranes of the endoplasmic reticulum and are recruited to the cell surface membrane accordingly, for example, during the activation of phagocytosis in macrophages [528].

Certainly, based on the results of the Western blot analyses of DC and macrophages provided by this study, there is a noticeable difference on the baseline levels of expression between BM-DC and BM-MΦ. In contrast, using an *Alomone Labs* antibody targeted against an extracellular epitope, $P2X_7R$ expression was previously reported in murine peritoneal macrophages [357] suggesting possible differences in the cell surface expression of the receptor between murine macrophages and DC. Following a series of structure-function analyses on HEK-293 cells, Smart and colleagues concluded that receptor expression is controlled by a region between the 551-581 residues in the long cytoplasmic tail of the receptor, which incidentally resembles an LPS-binding motif. Induced mutation of the particular region abolishes cell surface receptor expression and consequently function [529]. $P2X_7R$ is a 'powerful' cytolytic receptor and it should not be surprising if different mechanisms are in place to regulate its cell surface expression and activation.

Subsequently an *Alomone labs* antibody, which bears an intracellular epitope, was employed to detect receptor expression using flow cytometry. Although a positive signal for receptor expression was detected in BM-DC populations, a similar signal was also observed in HEK-293 cells, which have been previously reported to be negative for receptor expression suggesting that the antibody was largely binding in a non-specific manner [525, 340]. The epitope target was situated in the C-terminal domain of the

receptor, and so the cells underwent fixation and permeabilization before staining and analysis. For the fixation process paraformaldehyde was used as a cross linking reagent to prevent loss of cohesiveness of the receptor as a unit. Subsequently, saponin was used as a detergent to permeabilize the cells. Saponin is made up by terpenoid and glycoside molecules that bind cholesterol molecules of cell membranes forming pores for fluorophore-conjugated antibodies to pass through [530]. It is widely known that fixation and permeabilization of cells can increase non-specific binding of primary and secondary antibodies, damage the light scatter properties of the cells compared to fresh preparations and also increase cell autofluorescence [531, 532]. Additionally, the *Alomone Labs* antibody employed was also a polyclonal antibody and can therefore bind to multiple aspects of the target antigen. Although polyclonal antibodies could be advantageous in certain occasions by emitting a more robust signal, they could also increase the levels of non-specific binding giving false positive signals, such as that observed in HEK-293 cells [533]. To reduce the possibility of non-specific binding non-specific binding, treatment with mouse or human serum was employed to block possible non-specific binding sites that could attract antibody binding. As a final attempt to detect P2X$_7$R expression using flow cytometry, a monoclonal antibody (*Epitomics*) targeted against an intracellular epitope was employed. Although the positive signal for P2X$_7$R expression in HEK-293 cells was reduced, this was still higher than that observed with the isotype control. It was therefore not possible to demonstrate P2X$_7$R expression on murine BM-DC using flow cytometry.

Given that several attempts to try and detect receptor expression using flow cytometry proved unsuccessful, Western blot analysis was next employed. The *Alomone antibody* targeted against an intracellular epitope was used since it was previously successfully employed to detect expression of P2X$_7$R in murine PMΦ [513]. In a similar manner, direct evidence of P2X$_7$R expression on murine BM-DC but also BM-MΦ and PMΦ is provided herein. Western blot analysis is possibly the most popular technique employed to detect direct expression of the receptor in macrophages and macrophage-like cell lines (specifically in RAW264.7) [383]. For example, Bianco and colleagues report P2X$_7$R expression by Western blot analysis in murine N9 cells [267] whilst Schachter and colleagues detect P2X$_7$R expression in mouse macrophages [383]. Although the exact reasons why using the same antibody (*Alomone Labs*) it was possible to detect receptor expression with immunoblotting but not with flow cytometry is not entirely

clear, it is argued that the sensitivity and accuracy of Western blot analysis is far greater to both flow cytometry and ELISA. This is mainly because Western blot analysis involves the separation of all the cell proteins according to size gel electrophoresis. As such the antigenic epitope is far more accessible for antibody binding and non-specific binding sites at the specific 'size level pocket' are reduced. In addition, the use of a polyclonal antibody in Western blot analysis amplifies the signal as several antibodies can bind to the same target making it possible to detect proteins expressed at very low levels.

4.3.3 Assessing the electrophysiological properties of the P2X$_7$R on BM-DC.

Having established P2X$_7$R expression in BM-DC using Western blot analysis it was important to assess the pharmacological profile of the receptor and patch clamping is one of the most common techniques adopted for profiling ion channels such as the P2X$_7$R. Since BM-DC preparations are not entirely pure with respect to CD11c-expressing cells it was particularly important to specifically target CD11c$^+$ BM-DC for patch clamping. P2X$_7$R expression has been previously reported in DC populations, however, the results of this study provide functional evidence of P2X$_7$R expression in CD11$^+$ murine BM-DC for the first time. Patch clamp was developed in 1976 by Neher and Sakmann to provide the possibility of investigating the electrophysiology of receptors individually or collectively and this technique is extensively used to study the physiology of all P2X receptors [534]. It has to be made clear that the recognized pharmacology of the P2X$_7$R is derived from studies conducted almost exclusively on ectopically expressed P2X$_7$R in HEK-293 cells [408]. P2X$_7$R activation in HEK-293 cells is generally characterized by two main features; high levels of ATP activation (>100μM) are required to activate the receptor and the current amplitude is sustained over time under repeated challenges by ATP [535].

Of all P2X receptors, P2X$_7$R has a characteristic slow current decay and is sensitive to the extracellular ionic environment, particularly divalent cations [338]. The experiments were therefore conducted in the absence of Mg^{2+} and Ca^{2+} ions, which have been shown to be potent negative regulators of the P2X$_7$R activation. Herein, BM-DC were challenged with repeated applications of ATP to facilitate currents through the receptor

and a sustained current flow with slow desensitization was observed. Desensitization describes the decline of current in the presence of the activating agonist, in this case ATP. Based on the observations of this study, the size of the current following ATP applications at 5 mM was of lower magnitude for fully facilitated channels on BM-DC (50-250pA) compared to that observed in native microglia channels (1nA) by other authors [324].

The mouse $P2X_7R$ in BM-DC was found to be insensitive to ATP applications. No currents were observed following ATP applications at 100μM, concentrations and a dose-dependent growth in current flow was observed with increasing concentrations of ATP. A similar study was performed by Chessel and colleagues, who found that the native channel in mouse microglia cells is more sensitive to ATP application with an EC_{50} value of 298μM. The operational characteristics of the native mouse $P2X_7R$ in BM-DC were also found to be different to that observed in microglia cells by Chessel and colleagues [324]. Repeated applications of ATP induced a distinctive current plateau in BM-DC, which was also observed by other authors in HEK-293 cells transfected with the rat and human $P2X_7R$. Interestingly, this characteristic plateau is absent in both mouse microglia and HEK-293 cells transfected with the mouse $P2X_7R$, which could be attributed to either a difference in the density of receptor expression or differences in the $P2X_7R$ complex between cell types and species. Additionally, the operational characteristics of the mouse $P2X_7R$ in BM-DC investigated herein were marked by a systematic growth in current flow, which is in accordance to the observations of Chessel and colleagues in mouse microglia cells and mouse $P2X_7R$ - transfected HEK-293 cells but is a characteristic that is absent in HEK-293 cells transfected with the human and rat $P2X_7R$ orthologues [324].

To confirm that the ATP-evoked current flow observed in murine BM-DC was the result of $P2X_7R$, one of the new series of specific inhibitors was employed, the A-740003. The new series of inhibitors have only recently become available [536, 405] and have been shown to block a number of $P2X_7R$ functions including calcium influx, pore formation and IL-1β release from differentiated THP-1 cells and an astrocytoma cell line transfected with rat and human $P2X_7R$ [408]. A-740003 treatment of ATP-challenged BM-DC was found to completely block current flow excluding the contribution of other ion channels. Taken collectively, the results of these investigations

confirm the presence of a functional P2X$_7$R on CD11c$^+$ BM-DC but also provide important insights into the operational characteristics of the ion channel in murine DC.

4.3.4 Assessing the pore forming ability of the P2X$_7$R in BM-DC and BM-MΦ.

These investigations also provide evidence that demonstrate significant differences in the cumulative fluorescence signal and the rate of dye uptake in BM-DC and BM-MΦ. Differences in the physiological properties and pore-forming ability of the receptor have been previously noted between different P2X$_7$R mammalian orthologues. Based on studies conducted on HEK-293 cells transfected with various mammalian orthologues, activation of the human receptor requires higher levels of ATP [317], yet the levels of YO-PRO dye uptake are lower, in comparison with its rat orthologue [477]. Although there is a wealth of studies investigating the functional properties of the P2X$_7$R ectopically expressed in HEK-293 cells, these are limited in primary cells and a direct comparison examining the pore forming ability of the P2X$_7$R in two different primary cell types is novel.

YO-PRO (MW 629Da) is the most widely used fluorescent dye for assessing the pore-forming ability of the P2X$_7$R as it presents two major advantages over other dyes. The dye remains largely unaffected by the processes of diffusion whilst the organic transporters that reside in the cytoplasm do not perturb the emitting fluorescence signal [338]. This dye becomes fluorescent only when it intercalates with nucleic acids within the nuclear envelope. It can therefore be used as a direct measure for the entry into the cells [307]. A major problem in assessing the findings of different groups studying the pore forming ability of the P2X$_7$R is that different groups employ different tools to investigate this functional aspect of the receptor that are not always comparable. For example Murgia and colleagues investigated the uptake of Lucifer yellow dye molecules by macrophages [537] whilst others use ethidium bromide, TO-PRO-3 or YO-PRO molecules, which might not share the same pathway of entry into the cell. Furthermore, the tool employed to identify the intracellular accumulation of the dye (such as flow cytometry, plate reader and immunohistochemical analysis), and finally the ligand employed (ATP or BzATP) to activate the receptor also varies between studies [332, 387, 538, 539]. For example, dye uptake was assessed in microglial

cultures following challenge with ATP at mM concentrations, which results in induced uptake of both Lucifer yellow dye and ethidium bromide [540, 498]. Although dye uptake is comparable to a certain extent between different studies the size and charge of the molecules will affect the rate and levels of uptake. It therefore becomes challenging to provide an accurate comparative analysis of the pore forming ability of the $P2X_7R$ based on the results of studies that have employed different tools to examine this functional property of the receptor.

The $P2X_7R$ carries ubiquitous expression, however, its functionality in specific cell types is often questioned [541]. The results herein provide a concurrent analysis of the pore forming ability of the $P2X_7R$ between murine DC and macrophages. The results of these investigations regarding YO-PRO dye uptake from murine BM-DC are in accordance to previous observations by Nihei and colleagues in the sense that ATP induces uptake of the dye in murine splenic DC [338]. The group, however, has employed flow cytometry to detect dye uptake in splenic DC and could not therefore characterize the kinetics of pore formation. The data presented herein provide for the first time, to our knowledge, a direct comparison of the rate of pore formation and dye uptake in murine BM-DC and BM-MΦ. Although dye uptake assays do not allow analysis for the density of receptor expression, they provide clues for the kinetics of pore formation and the level of permeabilization. These in turn provide clues for the functional properties of the receptor in the two cell types [307].

To confirm that pore formation and dye uptake was the result of receptor ligation and not diffusion, cells were treated with LPS alone. LPS is not the physiological ligand of the $P2X_7R$, therefore cannot directly activate the receptor, which is confirmed in the results reported herein. In contrast, application of high levels of ATP (the physiological ligand of the $P2X_7R$), successfully induced pore formation and dye uptake in both unprimed and LPS-primed BM-DC and BM-MΦ suggesting that dye uptake is an ATP-mediated effect and the result of $P2X_7R$ activation. The rate and levels of dye uptake in BM-MΦ were similar to those observed by Bianco and colleagues in a murine microglial cell line, exhibiting latency in dye uptake of 20 min. In contrast to their results, however, LPS treatment had no effect on the levels or kinetics of dye uptake. Unexpectedly, the levels of dye uptake were significantly lower exhibiting latency in uptake of approximately 45 min in BM-DC during exposure to 5 mM of ATP, an

optimum concentration of ATP for $P2X_7R$ activation [267]. In contrast, in the presence of lower concentrations of ATP (1 mM) the rate of dye uptake in LPS-primed BM-DC was faster and the signal of fluorescence was comparatively stronger to that observed in LPS-primed BM-MΦ. Confirmation that permeabilization of BM-DC and BM-MΦ to YO-PRO molecules was the result of $P2X_7R$ and not other P2 receptor signalling came from the following observations: (a) dye uptake was observed in BM-DC treated with high concentrations of ATP (5 mM), which desensitize other P2X receptors [542] more importantly, (b) pore formation and dye uptake were blocked when BM-DC and BM-MΦ were treated with the $P2X_7R$-specific A-740003 inhibitor. Although this effect was qualitatively assessed with immunocytochemistry, it appeared that the antagonist blocked pore formation and dye uptake with equal potency.

One possible explanation for the differences in the rate and levels of dye uptake between DC and macrophages comes from the observations of a recent study by Browne and colleagues. Using HEK-293 cells transfected with the rat $P2X_7R$ and site mutagenesis directed against the second and channel-forming transmembrane domain of the receptor, the group demonstrates that the receptor itself can restructure to form the pore [543]. Nevertheless, the possibility that other hemichannels, such as the pannexins, are recruited to contribute to the formation of the pore is still open [379]. Published reports have long favoured pannexin-1 as the mediator of the pore and evidence suggests that pannexin channels mediate the pore in macrophages allowing passage of molecules at a high rate in a non-selective manner [544, 381]. There are also reasons to suggest that dye uptake is dependent on the density of receptor expression [545]. Therefore, if cell surface expression of the $P2X_7R$ is higher on macrophages than DC and pannexin hemichannels contribute to pore formation and dye uptake then that could explain the higher levels of dye uptake observed in macrophages challenged with 5 mM ATP.

Differences in density of receptor expression, however, cannot explain (a) the prolonged latency in dye uptake observed in BM-DC and (b) the higher levels and faster rate of pore formation in BM-DC challenged with a lower concentration of ATP (1 mM). Lower concentrations of ATP are generally thought to activate other members of the P2X family. Other P2X receptors including the $P2X_4R$ and $P2X_2R$ have been shown to contribute to the uptake of large organic dyes. Although the capacity of the $P2X_4R$ to

form heterotrimers with other members of the P2X family has not been demonstrated in primary cells, this has proved possible in HEK-293 cells by Dubyak and colleagues [546]. In fact Casas-Pruneda and colleagues demonstrate that $P2X_4R$ and $P2X_7R$ receptors can form functional heterotrimers (in a 2:1, $P2X_4$:$P2X_7$ ratio) in transfected HEK-293 cells and permeabilize the cells to ethidium bromide [547]. Other authors also provide evidence that the $P2X_4R$ contributes in permeabilizing cells to large molecules. Khakh and colleagues show that $P2X_4R$ signalling contributes in permeabilizing rat neuronal cells to N-methyl-D-glucamine (NMDG) [326, 548]. However, it was later realized that $NMDG^+$ and $YO\text{-}PRO^+$ molecules are taken up by cells via two separate pathways providing further evidence that it is wrong to search for a single permeation pathway that is common between species and cell types [549]. Additionally, whether the $P2X_4R$ engages in some form of 'cross-talk' or is recruited to form heterotrimers with the $P2X_7R$ in primary cells of the haematopoietic lineage remains unclear. The former seems most likely [550] and the latter has only ever been observed in transfected cells. Cankurtaran-Sayar and colleagues have also challenged the idea that the $P2X_7R$ recruits separate permeation pathways for different molecules of different size or charge. The group examined the permeation pathway induced by the rat $P2X_7R$-transfected-HEK-293 and RAW264.7 macrophages and observed that two separate permeation pathways are responsible for dye uptake. Whereas the rat $P2X_7R$ activates a cation-selective and Ca^{2+}-dependent permeation pathway in HEK-293 cells, the $P2X_7R$-induced pore in RAW264.7 cells is both Ca^{2+}-independent and non-selective [428]. What remains unclear is whether such and other functional differences are species specific and/or cell specific. [239]. It could be argued that the permeation pathway responsible for uptake of YO-PRO molecules could be more prominent in macrophages than DC, which could provide an explanation for the observations of these investigations.

$P2X_2R$ has also been shown to contribute to $P2X_7R$-mediated pore formation, at least in Xenopus ocytes by Marques-da-Silva. The $P2X_2R$ also bears a slightly long C-terminus and appears to possess the ability to induce permeabilization of cells via a microtubule-mediated pathway, which is also recruited by the $P2X_7R$. A microtubule-mediated pathway of pore formation and dye uptake was also identified in mouse peritoneal macrophages suggesting that is both shared by the $P2X_7R$ and $P2X_2R$ receptor and has also been associated with pro-inflammatory cytokine release (IL-1β) [551]. It is

important to note that the $P2X_2R$ can form functional heterotrimers with other P2X receptors such as the $P2X_3R$ and in doing so can alter its physicochemical properties. Additionally, although the presence of functional $P2X_2R$ has not been examined in murine BM-DC, monocyte-derived DC have been shown to lack expression of this P2X receptor member [552, 523]. Taken collectively, it is very likely that DC and macrophages share distinct mechanisms in regulating pore formation, which could attribute to the discrepancies observed by the results of the uptake assays enclosed herein. Further work is required to elucidate the contribution of other P2X receptors and hemichannels in mediating pore formation in murine DC populations, particularly if such pathways are involved in mediating the egress of pro-inflammatory cytokines such as the IL-1β.

An important aspect in trying to provide possible explanations for the discrepancies detected in dye uptake between the two cell populations is keeping in mind that the $P2X_7R$ might be associated with the functional properties that are specific to DC and macrophages. Recent evidence suggests that in some cell types other P2Y receptors serve to prevent $P2X_7R$-mediated cell death by cytolysis. More specifically the results of Sugiyama and colleauges suggest that UTP, a potent $P2Y_4R$ ligand, prevents $P2X_7R$-induced dye uptake in retinal microvasculature cells, in a $Ca2^+$ influx- and PLA_2-dependent manner [553]. In a similar manner, $P2Y_4R$ receptors could also suppress the $P2X_7R$-mediated pore formation in DC exposed, which are often exposed to high levels of ATP in sites of inflammation. This could provide an explanation for the prolonged latency in dye uptake observed in BM-DC following challenge with ATP at 5 mM. Conversely, $P2X_7R$ -mediated cytolysis has been shown to be a key effector process for the destruction of mycobacteria [417]. Intracellular bacteria such as *Helicobacter pylori* and *Salmonella enterica* have been shown to prolong their viability by preventing $P2X_7R$-mediated host-cell death [294, 554]. Extracellular ATP released from damaged or infected cells activates the $P2X_7R$ on macrophages mediating necrosis and in this way alerting the innate immune system for the presence of infection [555].

It would make sense that DC, being the primary APC present at the site of inflammation, would be required to actively suppress excessive pore opening in order to maintain their viability in such ATP-rich microenvironments. Evidence suggests that naïve DC use ATP gradients to migrate towards sites of inflammation, a process that

involves P2Y signalling. This ability is lost following antigen capture and phenotypic maturation [556]. It would therefore seem illogical if DC use ATP gradients to navigate to sites of damage and inflammation just to undergo apoptosis. As professional APC they should, in theory, be able to regulate this pore-mediated cytolysis long enough to begin their migration and maturation to draining lymph nodes.

Interestingly, a recent study by Zanin and colleagues suggests that peritoneal macrophages challenged with LPS exhibit a reduced capacity to hydrolyse ATP due to a reduced expression of ATPase proteins such as NTPDase1 [557] It is postulated that LPS activation induces phenotypic changes which affect the cell surface expression of ATPases that regulate the availability of ATP in proximity and therefore P2X$_7$R activation. LPS signals the activation of the NF-κB pathway, which has been shown to suppress the transcription of the ATPase ecto-5′-NT [558]. In doing so inflammatory macrophages (M1) that have encountered LPS would suppress ATPase activity promoting ATP-signalling mediated clearance of invading pathogens. In contrast, M2 macrophages (often referred to as alternatively activated anti-inflammatory macrophages, see Chapter 1, section 1.7) were found to exhibit enhanced ATPase activity leading to the accumulation of adenosine. Adenosine is a physiological ligand of P1 receptors and has also been associated with suppressing the production of pro-inflammatory cytokines such as TNF-α from macrophages and promoting regeneration of tissue [559]. Interestingly, no difference was found in the levels of P2X$_7$R transcripts levels between M1 and M2 macrophages [560, 557].

Although the observations by other authors could provide a plausible explanation for the enhanced levels of dye uptake observed with BM-MΦ, they cannot explain the observations found in BM-DC. LPS would activate the NF-κB pathway also in DC, which has been shown to suppress ATPase activity. This would result in the accumulation of extracellular ATP, activation of the P2X$_7$R, the release of pro-inflammatory cytokines such as IL-1β and more importantly pore formation and cytolysis. Although ATP-induced cytolysis proves beneficial for macrophage populations in mediating microbial killing, this would be disastrous for DC populations, which are responsible for presenting antigen to receptive T-cells. Thus, it is very likely that in order to avoid ATP-mediated cell death and still maintain their pro-inflammatory

profile, DC have probably evolved a different mechanism to regulate their sensitivity to danger signals such as ATP.

4.3.5 Discrepancies in endotoxin (LPS)-induced cytokine release between DC and macrophages.

Finally, the ability of BM-DC, BM-MΦ and PMΦ to stimulate pro-inflammatory cytokine release following challenge with endotoxin (LPS) was also examined. Pro-inflammatory cytokines such as IL-1β provide the third signal for successful antigen presentation and T-cell activation [16]. The cells were therefore analysed for their ability to release IL-1β and IL-6. The level of IL-6 release was comparable between DC and the macrophage populations. In contrast, IL-1β was only detected in supernatants of BM-DC indicating a differential regulation of the inflammasome and IL-1β release between DC and macrophage populations. Surprisingly, the intracellular levels of IL-1β were comparable in all cell populations. Evidence for such discrepancies in IL-1β release between mouse DC and macrophage populations were recently reported by He and colleagues [561]. Similar differences were also observed between human macrophages and peripheral blood monocytes [238]. Briefly, the cytokine is synthesized as an inactive precursor and requires activation by caspase-1 activation and is reviewed in detail by Dinarello and colleagues [562]. IL-1β release from *in vitro* macrophage populations requires two signals, TLR ligation and P2X$_7$R-mediated inflammasome activation which results in the subsequent release of IL-1β-containing microvesicles [563, 239]. The fact that all three cell types are capable of synthesizing and releasing IL-6, a process which is not regulated by the inflammasome points to interesting differences between the two cell types with regards to IL-1 and this has been recently suggested by other authors. Based on their observations He and colleagues deduced that the differences observed in IL-1β release between DC and macrophages are partly due to the higher constitutive levels of NLRP3, at the protein level, and pro-IL-1β at the level of transcription and partly due to activation of the IL-1 receptors which acts as a feedback loop mechanism [561]. Furthermore, He and colleagues postulate that TLR signalling may be able to directly activate the inflammasome in BM-DC but not in macrophages [561]. Alternatively, the P2X$_7$R might share structural and physiological

(i.e. sensitivity to agonist) differences in BM-DC and macrophage populations. This will be explored in detail in chapter 5.

4.4 Conclusion.

How one single protein such as the $P2X_7R$ can mediate such a wide variety of functions is perplexing and it is postulated that the majority of these functions are regulated at the C-terminal domain of the receptor. Proteomic analysis by Kim and colleagues demonstrate that the C-terminal region harbours several interaction motifs with a multitude of intracellular proteins suggesting that the receptor might regulate a number of intracellular cascade [367, 321]. What is almost certain is that the signal initiating the recruitment of hemichannels and pore formation as well as cytokine release derives from particular motifs within this region [564]. The mechanism behind this remarkable functional diversity is currently, unclear. Interestingly, several splice variants have now been identified for the human $P2X_7R$ with different-sized C-terminal regions that can alter the function of the $P2X_7R$ oligomer [352, 355, 351]. A possible hypothesis suggests that $P2X_7R$ splice variant expression could provide a plausible model for modulating receptor function, impacting on current flow, sensitivity to agonist and the pore forming ability of the receptor [355, 476]. It was therefore of particular interest to examine the profile of macrophages and DC with regards to their profile of splice variant expression that could provide an explanation for the discrepancies observed in their pore forming ability. This will be addressed in detail in the next chapter.

Chapter 5: $P2X_7R$-driven responses in murine BM-DC; comparisons with macrophages.

5.1 Introduction.

Undoubtedly, IL-1 is one of the primary and most potent instigators of inflammation involved in a wide array of host immune responses against viral, fungal, parasitic and bacterial infections. The family of IL-1 consists of a number of different pro- and anti-inflammatory molecules that has progressively grown over the years and includes the 3 main ligands IL-1β and IL-1α, their natural inhibitor IL-1RA (also marketed as *Anakinra*), a membrane-associated as well as a decoy receptor [1, 178, 565]. IL-1α/β molecules bind to their specific membrane-associated IL-1RI (signalling receptor) and activate downstream signalling cascades such as the NF-κB to initiate the expression of other pro-inflammatory mediators such as IL-6 and prostaglandins [566, 567]. Although most cellular responses are common between the two isoforms of IL-1, there are reports of isoform-specific functions, for example, T-cell mediated antibody production [211]. Furthermore, IL-1β plays an important part in the initiation of inflammatory processes including sensitization to contact allergens [568, 11] and improving our understanding of the mechanisms that regulate its synthesis and release is paramount for the success of future therapeutic applications.

The primary source for IL-1β is cells of the monocytic lineage such as macrophages and DC. Due to its high inflammatory potency, the immune system has developed sophisticated mechanisms that strictly regulate its synthesis at several different levels including that of transcription, translation, maturation and secretion [569]. The P2X$_7$R has long been implicated in IL-1β synthesis at the level of processing and release. P2X$_7$R is an efficient mediator of K^+ depletion, a prerequisite for inflammasome activation and IL-1β release, at least in macrophages [239]. In truth, however, the molecular processes involved in regulating the transcription of IL-1 molecules have only begun to be unravelled.

Usually, IL-1 expression is induced by TLR signals derived from bacterial or viral components that result in the synthesis and accumulation of IL-1β precursor molecules (31kDa) (reviewed in [570]) beneath the inner leaflet of the cell surface membrane in macrophages and monocytes. A second stimulus, such as a DAMP signal, is required for the efficient and rapid release of the cytokine into the extracellular space [231]. In the absence of this secondary signal, the release of IL-1β molecules in the extracellular milieu is a relatively inefficient process. ATP constitutes one of the few physiological

DAMP signals that can induce inflammasome oligomerization and caspase-1 processing [288]. Caspase-1 mediates rapid processing of IL-1β molecules into their bioactive form and are subsequently externalized from the cell [231]. ATP fulfils several criteria that make it an ideal danger signal to potentiate immune responses. It has ubiquitous expression, is a hydrophilic molecule, readily available within the cytoplasm and is tightly regulated by ecto-ATPases [571]. ATP is also the physiological ligand of the P2X$_7$R driving inflammasome-mediated IL-1β responses in different cell types [572, 573].

However, the P2X$_7$R has been associated with a large multitude of functions including the activation of caspases [394, 574], the secretion of cytokines [575, 271], membrane blebbing [576, 329], production of ROS [577], T-cell maturation [578], induction of cell proliferation [267] and pore formation [579]. How a single transmembrane protein is involved in so many different functions remains elusive. Recently, several functional isoforms of the receptor have been identified [356, 355] that impact its functional profile and therefore its ability to regulate downstream cell effector functions thereby contributing to the functional diversity of the receptor.

A larger proportion of studies investigating the mechanisms of P2X$_7$R-mediated inflammasome activation and IL-1β release have focused in macrophage populations [283, 269, 239]. Thus, the main objective herein was to improve our understanding of the ATP-P2X$_7$R axis in mediating inflammasome activation and IL-1 release in murine BM-DC, in response to LPS priming and ATP challenges and in comparison to macrophages for use in future therapeutic applications. Additionally, it was also important to investigate whether LPS signalling has a distinct impact in regulating the transcriptional expression of P2X$_7$R splice isoforms in DC and macrophage populations.

5.2 Results.

5.2.1 LPS-mediated up-regulation and ATP-driven release of IL-1β in BM- DC and BM-MΦ.

An *in vitro* model has been employed to investigate the role $P2X_7R$-associated processes of IL-1β production and release in macrophages and DC populations. Cells were treated with LPS at different concentrations for 2h and challenged with ATP 20 min before the end of the incubation with LPS, the results of which are depicted in figure 5.1. Cells were primed with increasing concentrations of LPS for 2h and subsequently challenged with different doses of ATP. The levels of IL-1β were quantified in both lysates (intracellular content) and supernatants (release) of day 8 cultured BM-DC and BM-MΦ. A dose-dependent accumulation of IL-1β was observed in lysates of BM-DC in response to LPS; negligible levels (<1ng/ml) of the cytokine were detected in BM-DC treated with 1ng/ml LPS whereas approximately 22ng/ml were found in BM-DC treated with 1000ng/ml of this TLR ligand. In comparison, for BM-MΦ, substantial amounts of IL-1β (4ng/ml) were detected even at the lowest LPS concentrations (1ng/ml) and a 3-fold increase was observed following treatment with 10ng/ml of LPS. The intracellular levels of IL-1β in murine BM-MΦ peaked at just below 20ng/ml following treatment with 100 or 1000ng/ml of LPS, reaching similar levels to those observed in lysates of murine BM-DC cultured with high dose LPS (≈20ng/ml). No detectable IL-1β secretion was recorded in cells treated with medium or LPS at all experimental concentrations examined, in either BM-DC or BM-MΦ (<0.038ng/ml).

Following application of exogenous ATP, treatment with LPS induced a dose-dependent secretion of IL-1β. Low dose of ATP (0.1 mM) was without effect on cytokine secretion for all concentrations of LPS-priming. Incubation with LPS at 10ng/ml or higher primed BM-DC for responses to ATP. Maximal IL-1β secretion was recorded generally for 5 and 10 mM ATP with approximately 15-20ng/ml of the cytokine secreted from cells treated with 100 or 1000ng/ml LPS. At these concentrations of ATP that provoked marked secretion of cytokine, there was a concomitant decrease in intracellular IL-1β.

In contrast, the pattern of IL-1β secretion was distinct for BM-MΦ that received treatment with LPS at 10, 100 and 1000ng/ml. Incubation with LPS at these concentrations provoked a vigorous up-regulation of intracellular cytokine and secretion was detected at ATP challenge doses of 1 and 5 mM ATP, peaking at 1-2ng/ml. At the highest doses of ATP (10 mM), there was little cytokine production. Furthermore, peak cytokine secretion by BM-DC was some 10-fold higher than that recorded for BM-MΦ. As observed for BM-DC, a concomitant drop in intracellular expression of IL-1β was recorded for BM-MΦ in experimental conditions where cytokine secretion was observed.

The impact of LPS treatment and ATP activation on the viability of cells was also examined to exclude the possibility that IL-1β release was simply the consequence of cell death (figure 5.1c). BM-MΦ are a strongly adherent population of cells as opposed to BM-DC which are loosely adherent and easily detach following gentle re-suspension. For consistency, both cell types were treated with a low EDTA/Trypsin-containing solution to detach the cells avoiding scraping before viability analysis. LPS treatment (1-1000ng/ml) had no significant impact on the viability of either BM-DC or BM-MΦ. Activation of cells with ATP had a significant impact on the viability of BM-DC but not on BM-MΦ, particularly at the highest concentrations of LPS (100, 1000ng/ml). For example, the viability of medium-treated or LPS-primed (100ng/ml) BM-DC was recorded at approximately 95% and that dropped to approximately 50% following challenge with ATP at 5 mM. A similar pattern of reduction in cell viability following ATP challenge was observed with BM-DC treated with 1000ng/ml of LPS. Under these experimental conditions, where cell viability was dropped a concomitant cytokine secretion was observed. Furthermore, the effects on viability were reversed following activation with 10 mM ATP in BM-DC (90-95%) treated with the higher concentrations of LPS (100 and 1000ng/ml), however, a concomitant cytokine secretion was sustained.

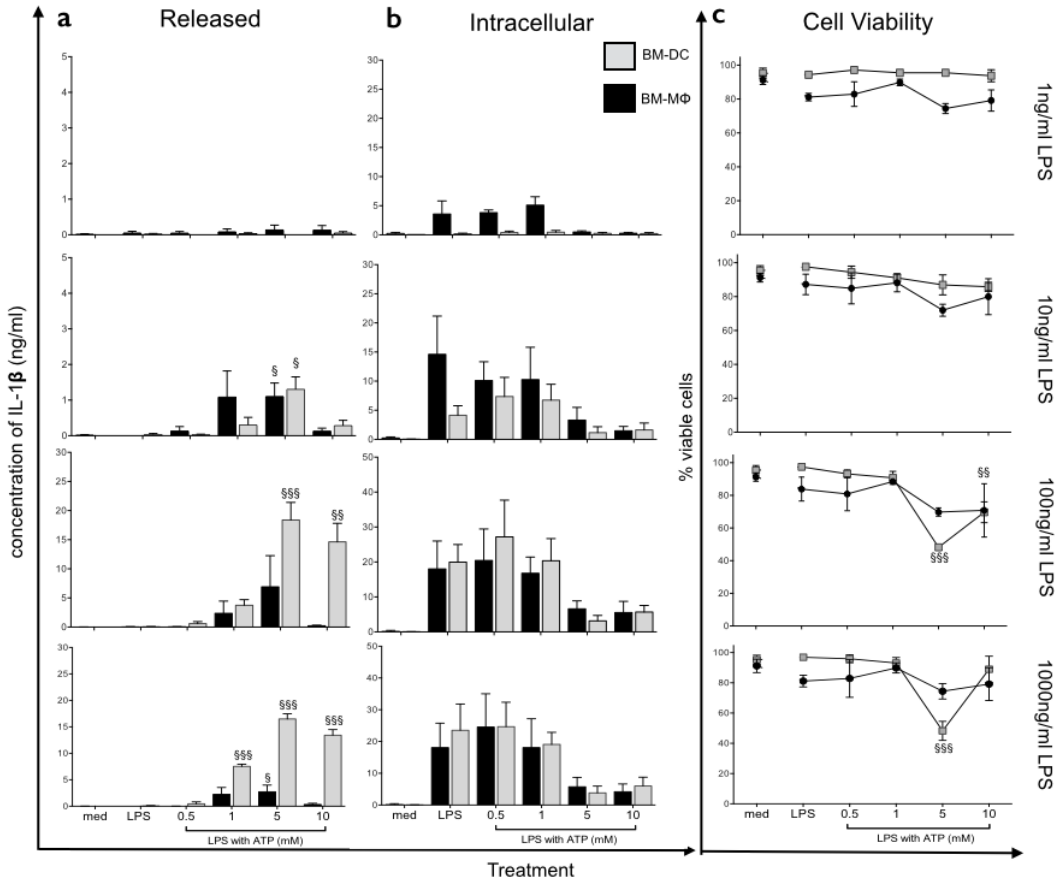


***Figure 5.1:* Comparison of intracellular and released levels of IL-1β between day 8 BM-DC and BM-MΦ primed with LPS and challenged with ATP.**
Day 8 BM-DC (grey bars) and BM-MΦ (black bars) at 1x10^6 cells/ml were cultured for 2h in the presence of medium alone or stimulated with LPS at 1, 10, 100 or 1000ng/ml and subsequently challenged with medium alone or ATP at 0.5, 1, 5 and 10 mM. Both released (a) and intracellular (b) levels of IL-1β were quantified with a cytokine-specific ELISA. Cells were stained with propidium iodide and cell viability of treated BM-DC (grey squares) and BM-MΦ (black circles) was assessed using flow cytometry (c) Data shown are mean (n=3) ±SEM. Statistical significance of differences was first considered between medium- and LPS-treated cells with a Mann-Whitney, two-tailed test. No statistically significant differences between groups were found. Statistical significance of differences was also considered between the LPS-treated groups (LPS alone-treated cells were used as a comparator) for each cell population and assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (§= $p<0.05$, §§=$p< 0.01$, §§§=$p< 0.001$).

5.2.2 LPS-mediated up-regulation and ATP-driven IL-1α responses in BM-DC and BM-MΦ.

In the same experiments, both intracellular content and released levels of IL-1α were measured (figure 5.2). The pattern of IL-1β and IL-1α production was strikingly similar as well as the nature of the dose responses to LPS and ATP. LPS treatment induced IL-1α production (intracellular content) in BM-DC but no detectable levels (< 0.039ng/ml) of the cytokine were recorded in the absence of exogenous ATP challenge. Maximal IL-1α production (intracellular content) was observed following treatment with LPS at 100 and 1000ng/ml (approximately 10ng/ml), and doses of 5 and 10 mM resulted in vigorous secretion (approximately 5ng/ml). As observed for IL-1β expression there was a concomitant down-regulation of intracellular cytokine in experimental conditions where vigorous secretion was induced. In contrast, IL-1α production followed a distinct pattern in BM-MΦ. Treatment with LPS induced IL-1α intracellular expression (approximately 2ng/ml), particularly at the high concentrations of LPS (100 and 1000ng/ml). Interestingly, the cytokine content of the lysates decreased to approximately 0.5ng/ml following challenge with exogenous ATP at 5 and 10 mM. Furthermore, under no conditions was there vigorous secretion of IL-1α by BM-MΦ.

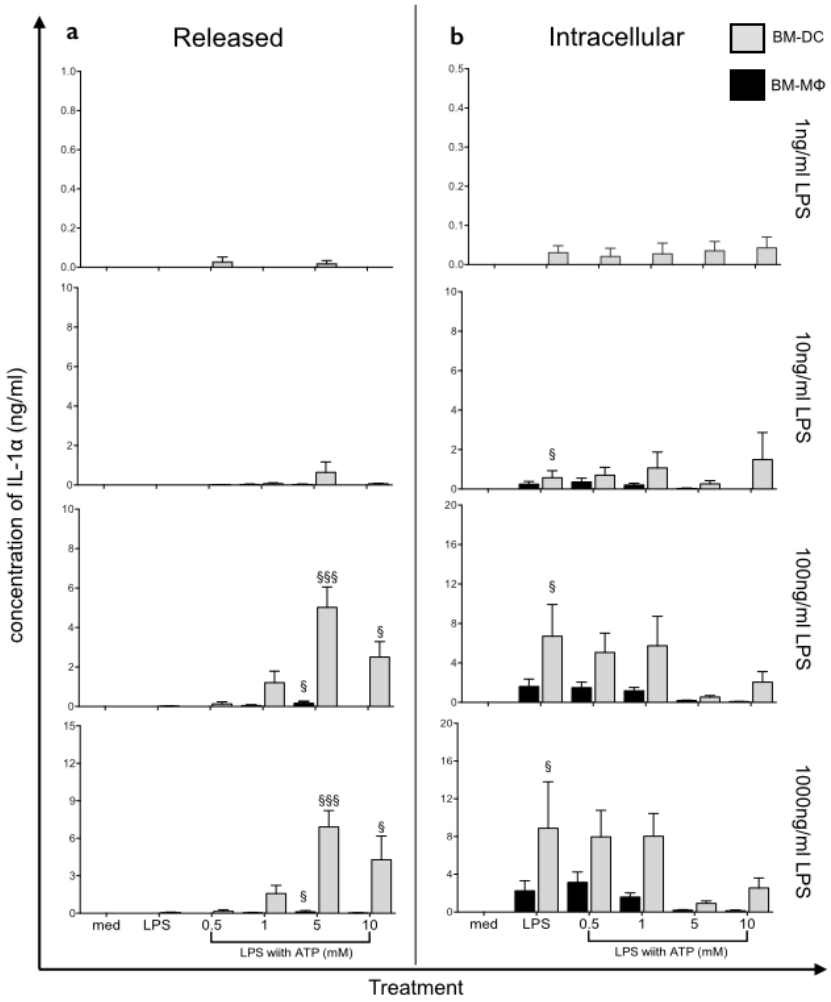


Figure 5.2: Comparisons of intracellular and released levels of IL-1α between day 8 BM-DC and BM-MΦ primed with LPS and challenged with ATP.
Day 8 BM-DC (grey bars) and BM-MΦ (black bars) at 1x10^6 cells/ml were cultured for 2h in the presence of medium alone or stimulated with LPS at 1, 10, 100 or 1000ng/ml and subsequently challenged with medium alone or ATP at 0.5, 1, 5 and 10 mM. Both released (a) and intracellular (b) levels of IL-1α were quantified with a cytokine-specific ELISA. Data shown are mean (n=3) ±SEM. Statistical significance of differences was first considered between medium- and LPS-treated cells with a Mann-Whitney, two-tailed test (§= $p<0.05$). Statistical significance of differences was considered between the LPS-treated groups (LPS alone-treated cells were used as a comparator) for each cell population and assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (§= $p<0.05$, §§§=$p< 0.001$).

5.2.3 Investigating the impact of ATP activation on IL-6 expression in LPS-primed BM-DC and BM-MΦ.

IL-6 is another important pro-inflammatory cytokine released early on during inflammation and importantly, does not share the same inflammasome-associated pathway of secretion with IL-1β. Therefore the impact of ATP signalling on the production and release of IL-6 was investigated in the same experiments (figure 5.3). Although the pattern of IL-6 expression showed marked inter-experimental variation such that most of the differences failed to reach statistical significance, an interesting pattern of cytokine release was observed. With regards to BM-DC, treatment with low concentrations of LPS (1 and 10ng/ml) had little effect on IL-6 production (intracellular content) or secretion (<1ng/ml). However, both 100 and 10000ng/ml LPS primed BM-DC for IL-6 expression. At the lower dose (100ng/ml), the intracellular content was recorded at approximately 2ng/ml, whilst 5ng/ml were secreted by the cells whereas at the higher dose (1000ng/ml), approximately 0.078ng/ml was recorded in the lysates (intracellular content) and a maximum of 9ng/ml was secreted. Importantly, there was no requirement for stimulation with ATP for IL-6, in contrast to the IL-1 cytokines.

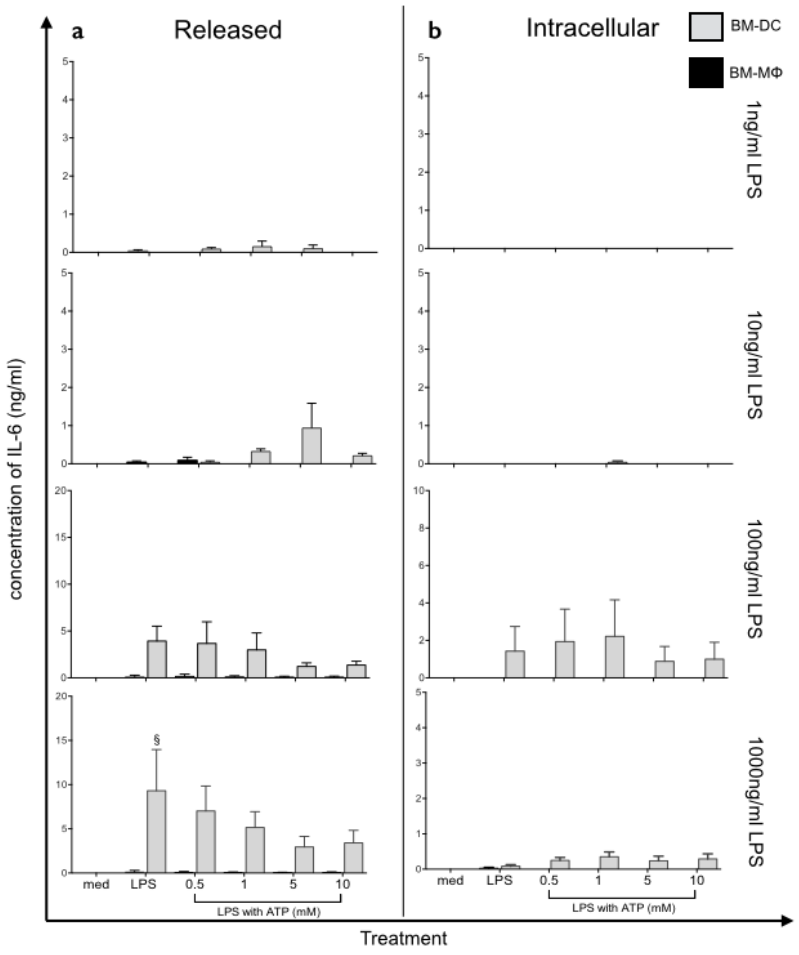


***Figure 5.3*: Comparisons of intracellular and released levels of IL-6 between day 8 BM-DC and BM-MΦ primed with LPS and challenged with ATP.**
Day 8 BM-DC (grey bars) and BM-MΦ (black bars) at 1x10^6 cells/ml were cultured for 2h in the presence of medium alone or stimulated with LPS at 1, 10, 100 or 1000ng/ml and subsequently challenged with medium alone or ATP at 0.5, 1, 5 and 10 mM. Both released (a) and intracellular (b) levels of IL-6 were quantified with a cytokine-specific ELISA. Data shown are mean (n=3) ±SEM. Statistical significance of differences was first considered between medium- and LPS-treated cells with a Mann-Whitney, two-tailed test (§= $p<0.05$). Statistical significance of differences was also considered between the LPS-treated groups (LPS alone-treated cells were used as a comparator) for each cell population and assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test. No statistically significant differences between groups were found

5.2.4 Comparison of total IL-1 and IL-6 cytokine levels in BM-DC and BM-MΦ following treatment with LPS and ATP.

In order to illustrate more clearly the impact of LPS and ATP signalling on the levels of cytokine synthesis *per se,* the total amount of each cytokine was calculated for both BM-DC and BM-MΦ under all the experimental conditions. Therefore, in the same experiments, total cytokine was determined by calculating the cumulative levels for each cytokine of interest in both lysates and supernatants (figure 5.4). Interestingly, although the pattern of IL-1β and IL-1α synthesis was broadly similar in BM-DC, distinct differences emerged in the synthesis of the two IL-1 cytokines in BM-MΦ (figure 5.4a, b). Macrophages were found to be seemingly much less sensitive with regards to IL-1α production. Very little IL-1α production was recorded at the lower doses (<0.1ng/ml) of LPS whilst the top concentration of LPS induced much lower levels of IL-1α in comparison to IL-β in BM-MΦ (<5ng/ml). The impact of ATP activation is also different in BM-MΦ.

Unlike with IL-1β, there was no marked enhancement in total IL-1α levels just a marked inhibition with the high doses of ATP. For example, LPS at 1000ng/ml provoked the synthesis of approximately 4ng/ml which was reduced to <0.5ng/ml following challenge with ATP at 10 mM. In contrast, in BM-DC LPS treatment induced a dose dependent increase in IL-1α synthesis (peaked at 5ng/ml at the top concentration of LPS) increased and remained largely unaffected following ATP activation. The levels of IL-1α synthesis were generally lower in comparison to IL-1β. For example, at 1000ng/ml LPS induced approximately 22ng/ml of IL-1β but only 5ng/ml of IL-1α. Finally, total IL-6 synthesis has also been examined and revealed some interesting patterns of IL-6 release in BM-DC. BM-DC did not produce large amounts of IL-6 (<1ng/ml in general) following treatment with low concentrations of LPS (1, 10ng/ml) (figure 5.4c). At high concentrations of LPS treatment (100, 1000ng/ml), BM-DC expressed substantial levels of IL-6 (up to 10ng/ml) and in contrast to the IL-1 cytokines some inhibitory effect was noted with high doses of ATP challenge but did not reach statistical significance.

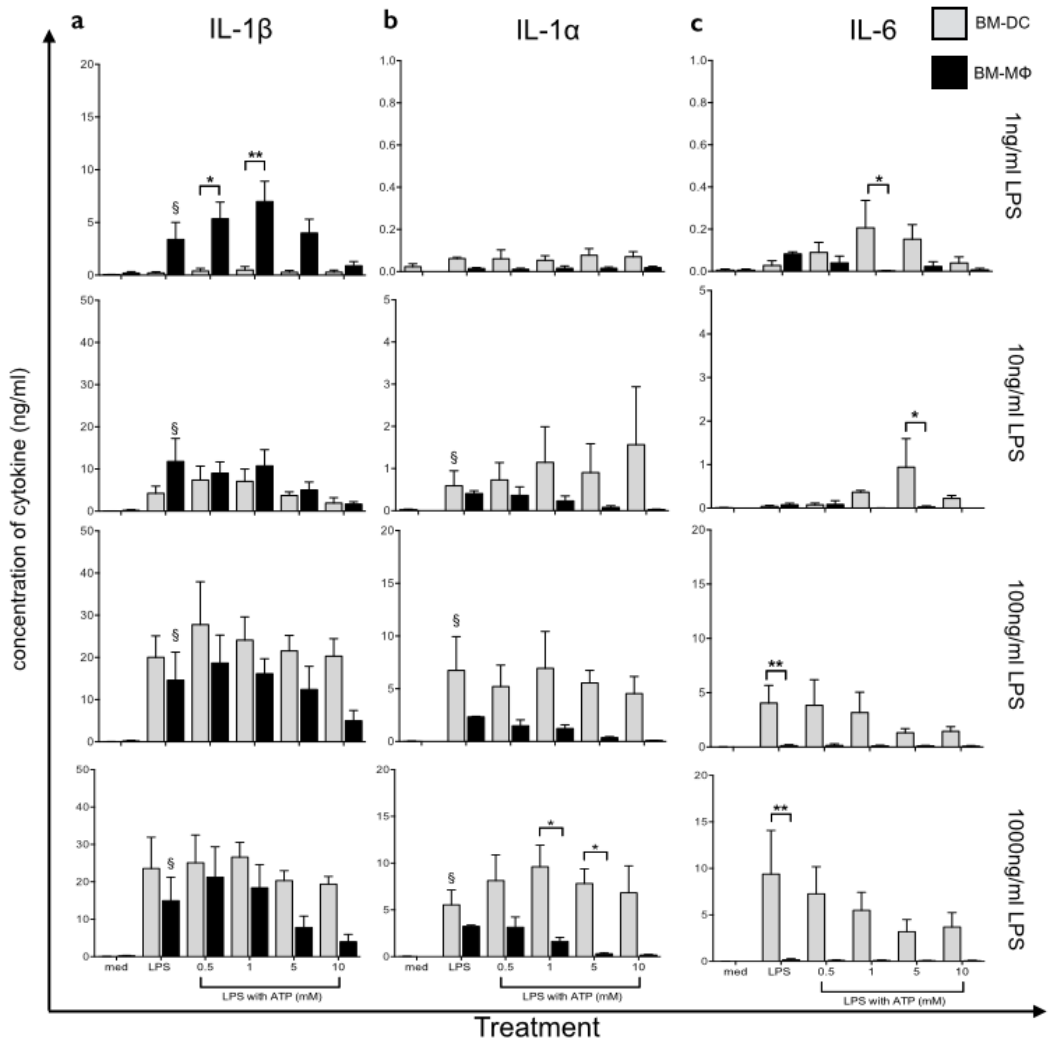


***Figure 5.4*: Comparison of IL-1 and IL-6 cytokine synthesis in endotoxin-induced day 8 BM-DC and BM-MΦ.**

Day 8 BM-DC (grey bars) and BM-MΦ (black bars) at 1x10^6 cells/ml were cultured for 2h in the presence of medium alone or stimulated with LPS at 1, 10, 100 or 1000ng/ml and subsequently challenged with medium alone or ATP at 0.5, 1, 5 and 10 mM. Both intracellular and released levels of (a) IL-1β, (b) IL-1α and (c) IL-6 were quantified with a cytokine-specific ELISA. Data shown are mean (n=3-4) ±SEM. Statistical significance of differences was first considered between medium- and LPS-treated cells with a Mann-Whitney, two-tailed test (§= $p<0.05$). Statistical significance of differences between BM-DC and BM-MΦ for each experimental condition were considered by two way ANOVA and Tukey's multiple comparison post-hoc test (*= $p<0.05$, **=$p<0.01$).

5.2.5 Comparison of IL-1 cytokine responses in BM-MΦ and PMΦ following LPS-priming and ATP activation.

Macrophages are well known for their phenotypic heterogeneity and the diversity of activities they engage *in vivo*. They are perfectly adapted to perform their function, as illustrated by the release of pro- or anti-inflammatory mediators among many others in their specific microenvironments [580]. Two distinct macrophage populations, BM-MΦ and PMΦ have therefore been employed for the purposes of these investigations. The functional profile of BM-MΦ with regards to their ability to produce and release pro-inflammatory cytokines was compared with that of PMΦ. However, it is important to note, that cultured PMΦ were not found to be a homogeneous population and were not cultured at exactly the same density with BM-MΦ see chapter 4 section 4.3.1 They were, however, employed as a reference cell type for comparisons with BM-MΦ (figure 5.5). Focus was first directed towards comparing IL-1β responses to LPS priming and ATP challenge between the macrophage populations of interest and the results are depicted in figure 5.5. LPS priming (at 100 and 1000ng/ml) induced substantial levels of IL-1β expression in lysates derived from both BM-MΦ (approximately 20ng/ml) and PMΦ (approximately 5-8ng/ml). The TLR ligand did not induce any detectable (<0.038ng/ml) levels of IL-1β release by either macrophage cell type.

Activation with ATP induced IL-1β release of similar magnitude in both macrophage cell populations even if the intracellular content of the cytokine was much higher in BM-MΦ. Additionally, a concomitant inverse dose–dependent decrease in the intracellular levels of the cytokine were noted in both macrophage populations with ATP activation. For example, the intracellular levels of IL-1β in BM-MΦ primed with 1000ng/ml of LPS and challenged with ATP at 0.5 mM peaked at approximately 25ng/ml and reduced to 5ng/ml at the top doses of ATP (figure 5.5b). Changes in cell viability were determined with PI staining and are depicted in figure 5.5a. The viability of medium-treated BM-MΦ was recorded at 90% whereas that of PMΦ at approximately 80% (figure 5.5c). For BM-MΦ a small decrease in cell viability (75-80%) was observed following treatment with the top doses of ATP (5 and 10 mM) and a concomitant release of the cytokine was observed under these experimental conditions. Similar patterns with regards to cell viability and cytokine release were observed in PMΦ.

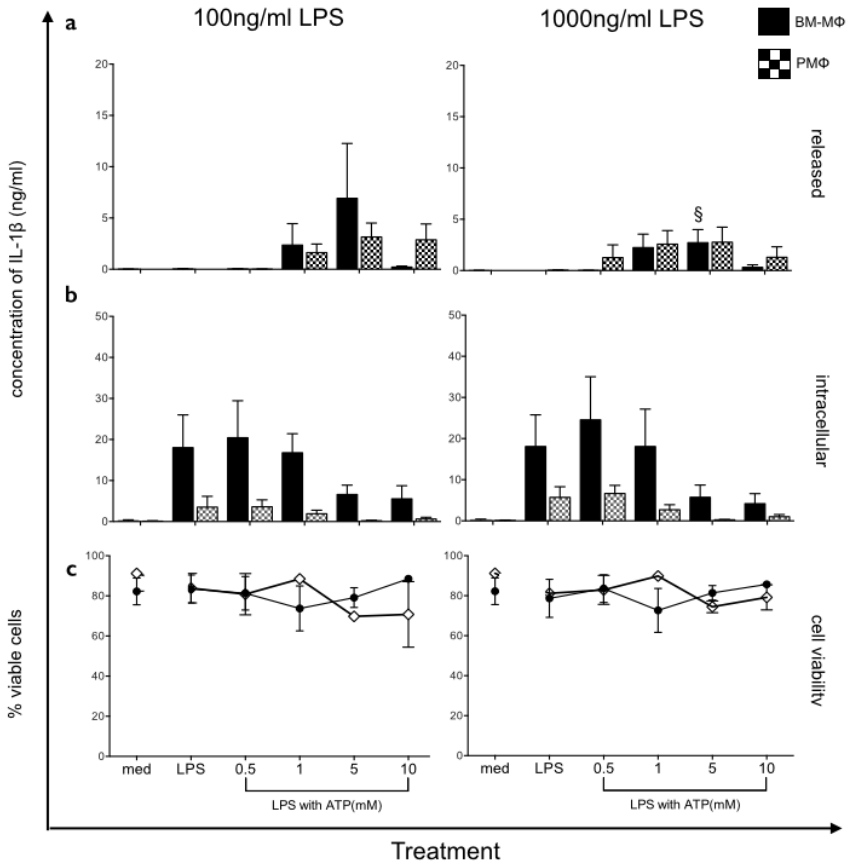


Figure 5.5: Comparisons of intracellular and released levels of IL-1β between day 8 BM-MΦ and isolated PMΦ primed with LPS and challenged with ATP.
Day 8 BM-MΦ (black bars) at 1x10^6 cells/ml and the adherent population of peritoneal exudates (PMΦ, hatched bars) were cultured for 2h in the presence of medium alone or stimulated with LPS at 100 or 1000ng/ml and subsequently challenged with medium alone or ATP at 0.5, 1, 5 and 10 mM. Both released (a) and intracellular (b) levels of IL-1β were quantified using a cytokine-specific ELISA. Cells were stained with propidium iodide and cell viability of treated BM-MΦ (black circles) and PMΦ (open diamonds) was assessed using flow cytometry (c). Data shown are mean (n=3) ±SEM. Statistical significance of differences was first considered between medium- and LPS-treated cells with a Mann-Whitney, two-tailed test. Statistical significance of differences was considered between the LPS-treated groups (LPS alone-treated cells were used as a comparator) for each cell population and assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (§=$p< 0.05$).

In the same experiments, the ability of LPS and ATP to signal IL-1α expression and release in PMΦ was also examined and compared to previous observations from BM-MΦ (figure 5.6). Both concentrations of LPS induced an up-regulation of IL-1α production (intracellular content) of similar magnitude (~2ng/ml) but the TLR ligand was insufficient to provoke cytokine secretion. In accordance with previous observations herein, ATP challenge failed to stimulate IL-1α secretion by LPS-primed BM-MΦ. In contrast, significant ATP-induced secretion was recorded in PMΦ. Maximal levels of 2ng/ml were observed following challenge with 5 mM ATP whilst these were reduced at the highest dose of ATP (10 mM) (figure 5.6a). Despite the differential pattern of secretion, a similar inverse dose dependent effect was noted in both cell types with ATP stimulation. In PMΦ for example, whereas 3ng/ml of IL-1α were detected in cells primed with 1000ng/ml of LPS and challenged with 0.5 mM ATP, these were reduced to <0.5ng/ml at the top doses of ATP.

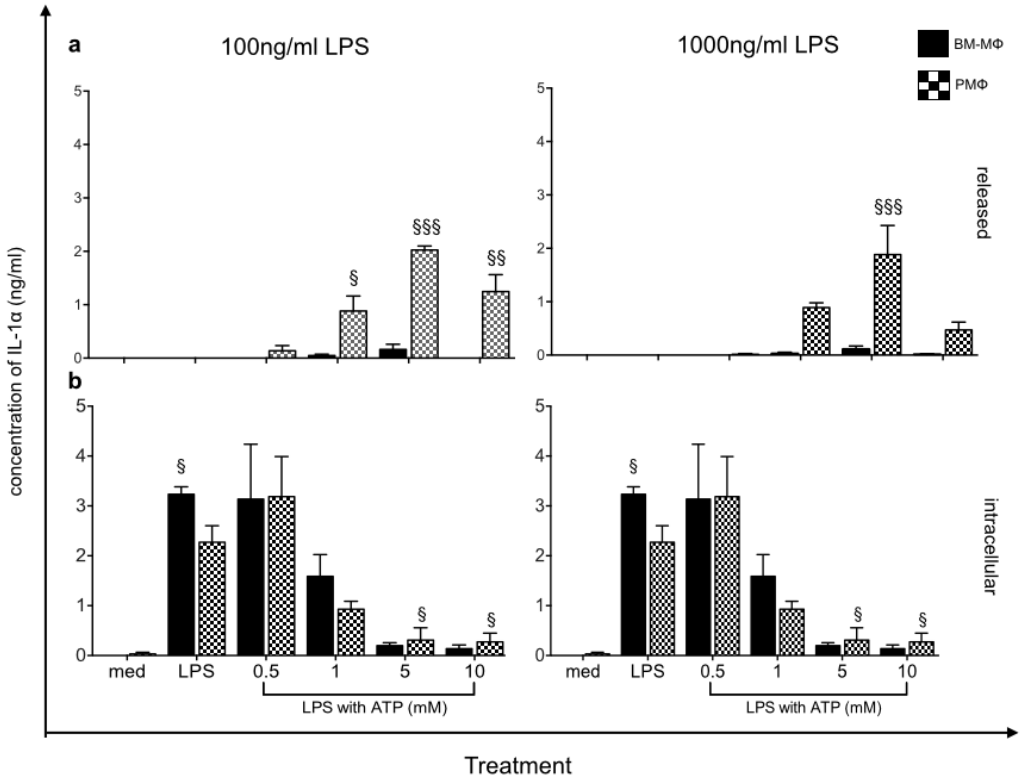


***Figure 5.6:* Comparisons of intracellular and released levels of IL-1α between day 8 BM-MΦ and isolated PMΦ primed with LPS and challenged with ATP.**
Day 8 BM-MΦ (black bars) at 1x10^6 cells/ml and the adherent population of peritoneal exudates (PMΦ, hatched bars) were cultured for 2h in the presence of medium alone or stimulated with LPS at 100 or 1000ng/ml and subsequently challenged with medium alone or ATP at 0.5, 1, 5 and 10 mM. Both released (a) and intracellular (b) levels of IL-1α were quantified using a cytokine-specific ELISA. Data shown are mean (n=3) ±SEM. Statistical significance of differences was first considered between medium- and LPS-treated cells with a Mann-Whitney, two-tailed test (§= $p<0.05$). Statistical significance of differences was considered between the LPS-treated groups (LPS alone-treated cells were used as a comparator) for each individual cell type and assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (§=$p< 0.05$, §§=$p<0.01$, §§§=$p< 0.001$).

5.2.6 Comparison of IL-6 responses in BM-MΦ and PMΦ following treatment with LPS and ATP.

In the same experiments, the ability of LPS and ATP to signal the synthesis and release of IL-6 was next examined in PMΦ and compared to those observed in BM-MΦ (Figure 5.7). Overall, little induction of IL-6 was observed in either macrophage cell types with intracellular levels recorded at approximately <0.1ng/ml and maximal levels of secreted product at 0.3ng/ml, in PMΦ. Comparatively, somewhat higher levels were recorded in PMΦ, however, these did not reach statistical significance.

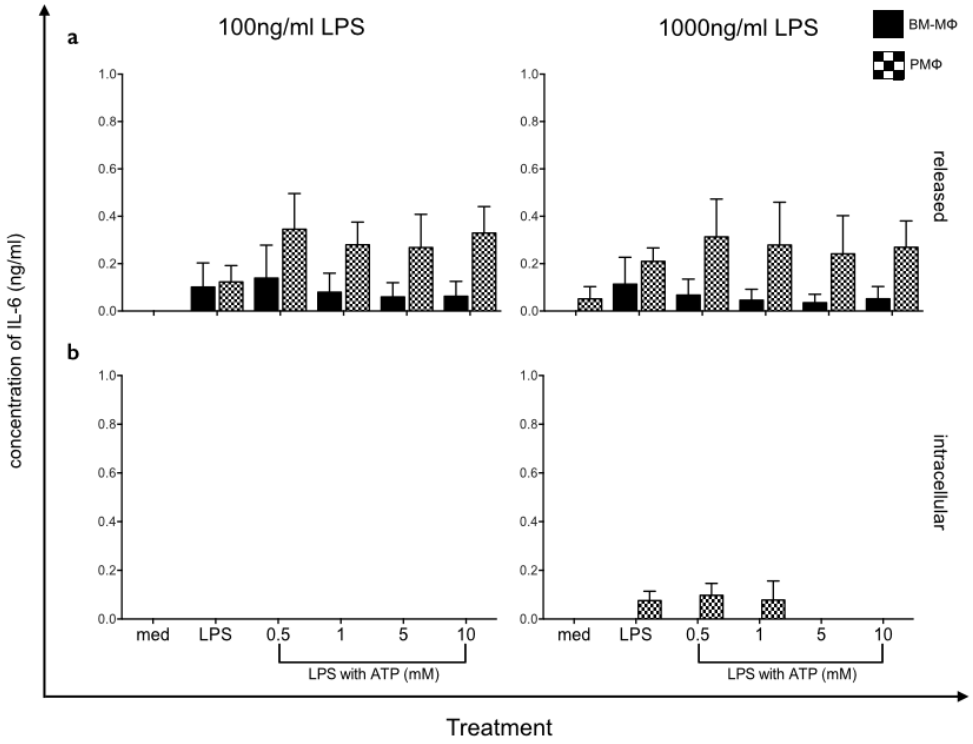


***Figure 5.7:* Comparisons of intracellular and released levels of IL-6 between day 8 BM-MΦ and isolated PMΦ primed with LPS and challenged with ATP.**
Day 8 BM-MΦ (black bars) at 1x10^6 cells/ml and the adherent population of peritoneal exudates (PMΦ) were cultured for 2h in the presence of medium alone or stimulated with LPS at 100 or 1000ng/ml and subsequently challenged with medium alone or ATP at 0.5, 1, 5 and 10 mM. Both released **(a)** and intracellular **(b)** levels of IL-6 were quantified using a cytokine-specific ELISA. Data shown are mean (n=3) ±SEM. Statistical significance of differences was first considered between medium- and LPS-treated cells with a Mann-Whitney, two-tailed test. Statistical significance of differences was considered between the all LPS-treatment groups (LPS alone-treated cells were used as a comparator) for each cell population and assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test. No statistically significant differences between groups were found.

5.2.7 Investigating the efficacy of A-70003 treatment in blocking ATP-induced IL-1β release from LPS-primed in BM-DC.

Important advances on P2X$_7$R pharmacology have been reported with the discovery of a new series of potent and competitive antagonists for the receptor such as the A-740003 [405], which allows more accurate analysis of the role the receptor in various signalling pathways. Thus, the efficacy of A-740003 in blocking IL-1β release from murine BM-DC was next explored. Generally, in the next series of investigations BM-DC were treated with LPS at 1000ng/ml for 2h and challenged with ATP at 5 mM, unless otherwise stated. Incubation with LPS alone induced substantial intracellular IL-1β production (14ng/ml) (figure 5.8b) but failed to provoke cytokine release (figure 5.8a), consistent with previous experiments. Further challenge of LPS-primed BM-DC with ATP (5 mM) or ATP in the presence of DMSO (vehicle control, 0.5%) stimulated a decrease in the intracellular levels of the cytokine (2.5ng/ml) followed by an equivalent rise in the levels of IL-1β detected in supernatants (approximately 15ng/ml). There was no impact of the presence of DMSO vehicle on intracellular or secreted cytokine levels. However, treatment with the A-740003 inhibitor (100μM) successfully blocked the ATP-induced IL-1β release (from 15ng/ml to <0.6ng/ml), such that the intracellular levels of the cytokine in A-740003-treated BM-DC were comparable with those detected in LPS-primed BM-DC without ATP challenge (14ng/ml). Treatment with lower concentrations of the inhibitor A-740003 (1 or 10μM) had no impact on cytokine release. The viability of medium-treated BM-DC was recorded at 98% and remained unaffected following treatment with LPS (figure 5.8c). Activation with ATP (or ATP with DMSO, 0.5%), however, resulted in a substantial drop in the viability of the cells to approximately 40%. A concomitant cytokine secretion of significant magnitude was observed under these experimental conditions. Importantly, treatment with A-740003 at 100μM, but not at lower concentrations, rescued the viability of the cells (~100%) and concomitantly inhibited IL-1β secretion.

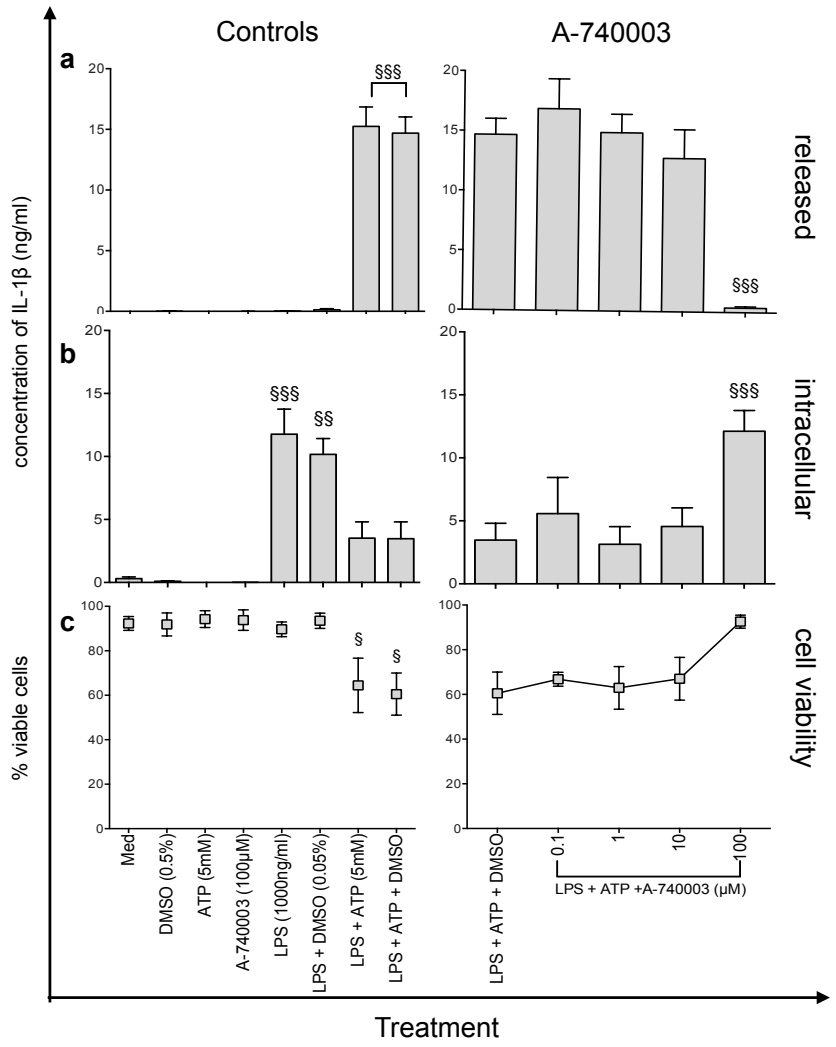


***Figure 5.8:* Investigating the effect of treatment with a P2X$_7$R-specific antagonist on IL-1β release from LPS-primed and ATP challenged day 8 BM-DC.**
Day 8 BM-DC at 1x10^6 cells/ml were cultured for 2h in the presence of medium alone or stimulated with LPS at a 1000ng/ml. Subsequently, cells received treatment with DMSO (vehicle control) or A-740003 at 0.1, 1, 10 or 100μM 10-15min before they were challenged with ATP at 5 mM for the final 20 minutes of treatment with LPS. Both released (a) and intracellular (b) levels of IL-1β were quantified using a cytokine-specific ELISA. Cells were stained with propidium iodide and cell viability of treated BM-DC was assessed using flow cytometry (c). Data shown are mean (n=3) ±SEM. Statistical significance of differences was considered between groups (medium treated-cell were used as a comparator in control groups and LPS+ATP+DMSO-treated cells were used as a comparator elsewhere) and assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (§§=p<0.01, §§§= p<0.0001).

5.2.8 The effect of A-740003 treatment on IL-1β responses in LPS-primed BM-DC and BM-MΦ.

In independent experiments, the P2X$_7$R specific inhibitor A-740003 was employed next to investigate the role of P2X$_7$R signalling in ATP (5 mM)-induced IL-1β synthesis concurrently, from LPS (1000ng/ml)-primed BM-DC and BM-MΦ (figure 5.9). Consistent with previous experiments, treatment with LPS induced comparable levels of intracellular IL-1β synthesis in BM-DC and BM-MΦ (10ng/ml) but was unable to provoke IL-1β release in either cell type (<0.039ng/ml). Activation with the P2X$_7$R ligand ATP induced vigorous secretion of IL-1β in BM-DC (15ng/ml) and consistent with previous observations, much lower levels of cytokine release in BM-MΦ (~1ng/ml) (figure 5.9a). A concomitant substantial reduction in the intracellular content of the cytokine was observed in both BM-DC (~5ng/ml) and BM-MΦ (~5ng/ml) (figure 5.9b).

Importantly, treatment with A-740003 (100μM) successfully blocked the ATP-induced IL-1β release from both LPS-primed BM-DC (97% inhibition) and BM-MΦ (91% inhibition). Treatment with the inhibitor also resulted in a concomitant increase in intracellular cytokine levels in BM-DC. Finally, cell viability was assessed using PI staining with medium-treated cells were found to be 95-99% viable. Treatment with LPS had no impact on the viability of either cell population. Activation of primed BM-DC with exogenous ATP significantly reduced the viability of the cells to approximately 60% (figure 5.9c) whilst that of BM-MΦ was reduced to 80% with a concomitant release of the cytokine noted in both cell types (figure 5.9b). In contrast, treatment with A-740003 rescued the viability of the cells, which was recorded at approximately 95% and as well as preventing cytokine release.

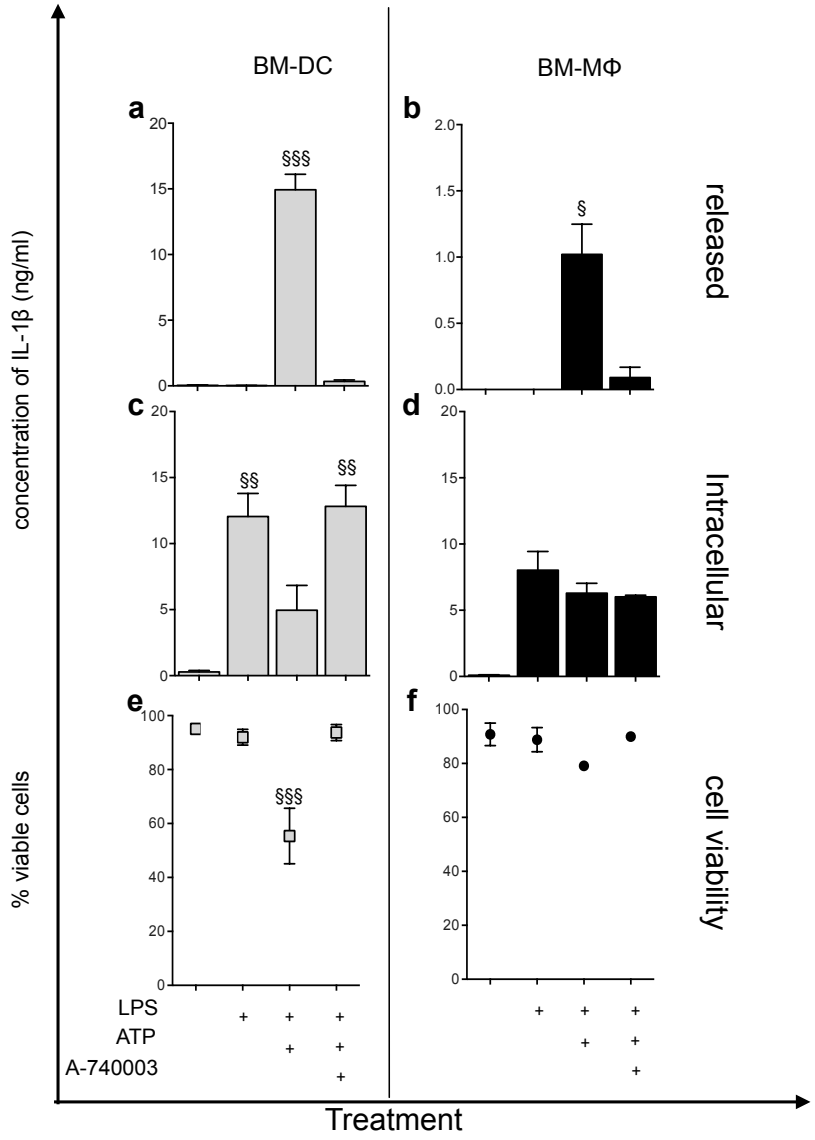


***Figure 5.9:* The impact of treatment with A-740003 on IL-1β release from LPS-primed and ATP challenged day 8 BM-DC and BM-MΦ.**
Day 8 BM-DC and BM-MΦ at 1x10^6 cells/ml were cultured for 2h in the presence of medium alone or stimulated with LPS at a 1000ng/ml. Subsequently, cells received treatment with DMSO (vehicle control) or with A-740003 at 100μM 10-15min before they were challenged with ATP at 5 mM for the final 20min of treatment with LPS. Both released (a, b) and intracellular (c, d) levels of IL-1β were quantified using a cytokine-specific ELISA. Cells were stained with propidium iodide and cell viability of treated BM-DC was assessed using flow cytometry (e, f). Data shown are mean (n=3) ±SEM. Statistical significance of differences was considered between groups (medium treated-cell were used as a comparator) and assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (§=p<0.05, §§=p<0.01, §§§= p<0.001).

5.2.9 The effect of A-740003 treatment on IL-1α and IL-6 responses from LPS-primed BM-DC.

In the same experiments, both (a) IL-1α release from murine BM-DC and BM-MΦ and (b) IL-6 release from BM-DC (since treatment with LPS for 2h in the presence or absence of ATP failed to induce detectable levels of IL-6 in BM-M), were determined following treatment with the A-740003 P2X$_7$R inhibitor (figure 5.10). LPS treatment (1000ng/ml) induced an up-regulation of IL-1α production (intracellular content) in both BM-DC (15ng/ml) and BM-MΦ (just below 5ng/ml) and consistent with previous observations failed to induce IL-1α secretion in either cell type. Vigorous IL-1α secretion was recorded in BM-DC (15ng/ml) following challenge with ATP (5 mM) and none was detected in BM-MΦ. Additionally, a concomitant substantial reduction in the intracellular content of the cytokine in both cell types even if IL- 1α was not secreted in BM-MΦ. In BM-DC, A-740003 treatment successfully blocked ATP-induced IL-1α secretion (<1ng/ml) (98% inhibition) with a concomitant rise noted in the intracellular content of the cytokine (13ng/ml). A similar rise in the intracellular cytokine content was also observed in BM-MΦ and since ATP could not signal IL-1α secretion in BM-MΦ, the inhibitor had no effect in blocking its release, but also did not induce it.

With regards to IL-6, although maximal levels of IL-6 release from BM-DC were considerably lower to those depicted in figure 5.3 the pattern of release, with regards to ATP dependence, was the same. LPS at 1000ng/ml in the absence of ATP activation induced IL-6 release of approximately 1.5ng/ml. Activation with exogenous ATP (5 mM) had no significant effect on the levels of IL-6 release nor did treatment with A-740003. Overall, the intracellular content of the cytokine was minimal (<0.4ng/ml) in BM-DC incubated with LPS and remained unaffected following treatment with ATP and the inhibitor.

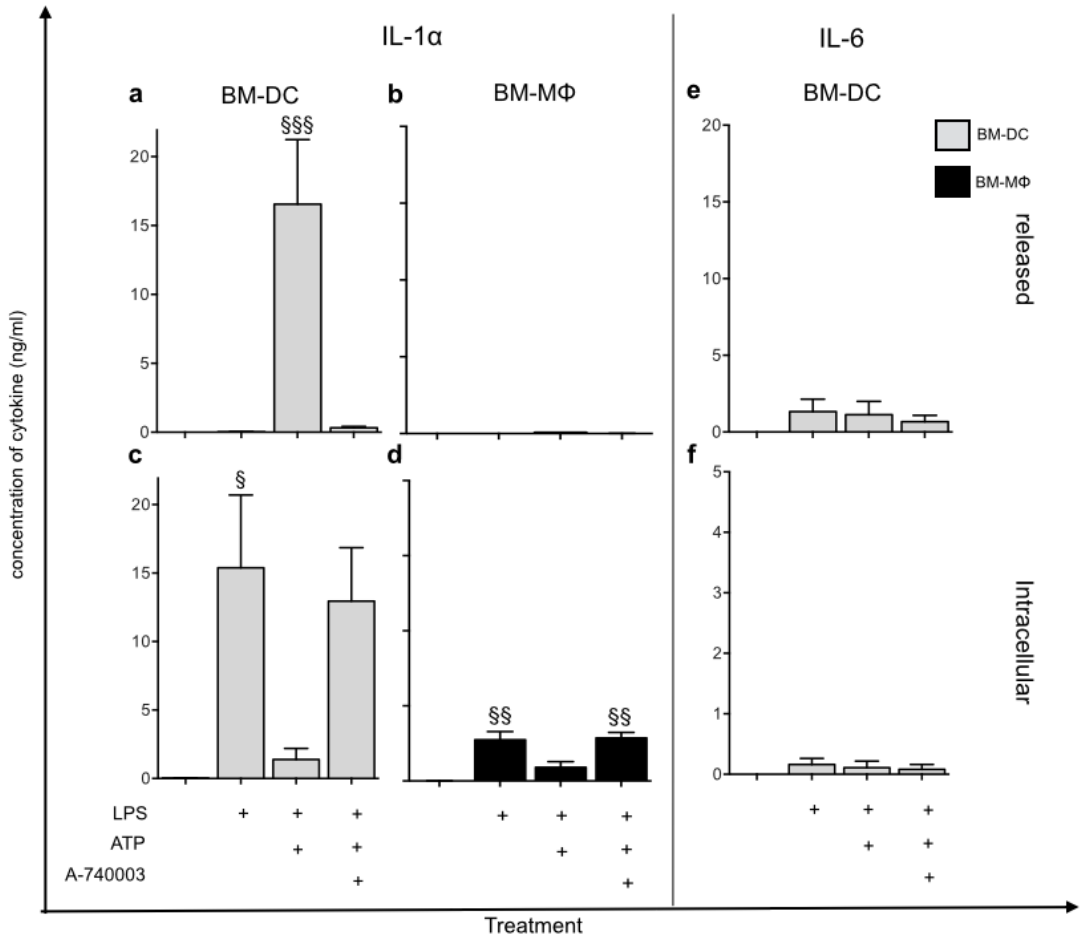


***Figure 5.10:* The impact of treatment with A-740003 on IL-1α and IL-6 release from LPS-primed and ATP challenged day 8 BM-DC and BM-MΦ.**
Day 8 BM-DC and BM-MΦ at 1x10^6 cells/ml were cultured for 2h in the presence of medium alone or stimulated with LPS at a 1000ng/ml. Subsequently, cells received treatment with DMSO (vehicle control) or with A-740003 at 100μM 10-15min before they were challenged with ATP at 5 mM for the final 20 minutes of treatment with LPS. Both released (a, b, c) and intracellular (d, e, f) levels of the cytokines were quantified using a cytokine-specific ELISA. Data shown are mean (n=3) ±SEM. Statistical significance of differences was considered between groups (medium treated-cell were used as a comparator) and assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (*=p<0.05, **=p<0.01, ***= p<0.001).

5.2.10 The impact of apyrase treatment on IL-1 release from LPS-primed and ATP-challenged BM-DC.

A potent ATPase enzyme, apyrase, was next employed to further implicate the role of ATP and exclude that of ADP and AMP signalling in mediating IL-1β as well as IL-1α secretion from LPS-primed BM-DC [513, 581]. Additionally, it was important to illustrate first the capacity of the enzyme to reverse cytokine responses in response to exogenous ATP before employing the ATPase to investigate the role of ATP signalling in the 24h model (see section 5.2.13). BM-DC were primed with LPS for 2h at 1000ng/ml and subsequently challenged with ATP for the last 20 minutes of the incubation period. BM-DC received treatment with apyrase at different concentrations (2, 20μg/ml) immediately before being challenged with ATP. BM-DC were initially challenged with ATP at 5 mM with/without apyrase treatment (0.2-20μg/ml) but the enzyme failed to prevent cytokine release under these experimental conditions. Additionally, higher concentrations of apyrase (>20μg/ml) were not employed since a 20% drop in viability was observed at this top concentration (data not shown). In contrast, apyrase treatment successfully blocked IL-1 cytokine release from BM-DC challenged with ATP at 1 mM and there was no effect on the viability of the cell at the top doses of apyrase (figure 5.11).

Consistent with previous observations, treatment with LPS induced both IL-1β (~12ng/ml) and IL-1α (~5ng/ml) production (intracellular content, figure 5.11b) and failed to induce secretion of either IL-1 cytokine (figure 5.11a). Additionally, no detectable levels (<0.039ng/ml) of IL-1β/IL-1α were detected in cells that received treatment with ATP (1 mM) or apyrase alone. Activation with ATP at 1 mM signalled both IL-1α (~5ng/ml) and IL-1β (10ng/ml) secretion from LPS-primed BM-DC whilst the intracellular content remained largely unaffected, in accordance with previous observations (see figures 5.1 an 5.2). Importantly, both IL-1β and IL-1α release were substantially reduced following pre-treatment with apyrase at 2μg/ml (54% inhibition was recorded for IL-1β and 74% inhibition for IL-1α) and almost completely abrogated at 20μg/ml (95% inhibition for IL-1β and 93% inhibition for IL-1α). No impact on the intracellular content of either IL-1β or IL-1α was noted. Finally, the viability of BM-DC was recorded at approximately 90% in medium-treated cells and remained largely unaffected with the various treatments (figure 5.11c).

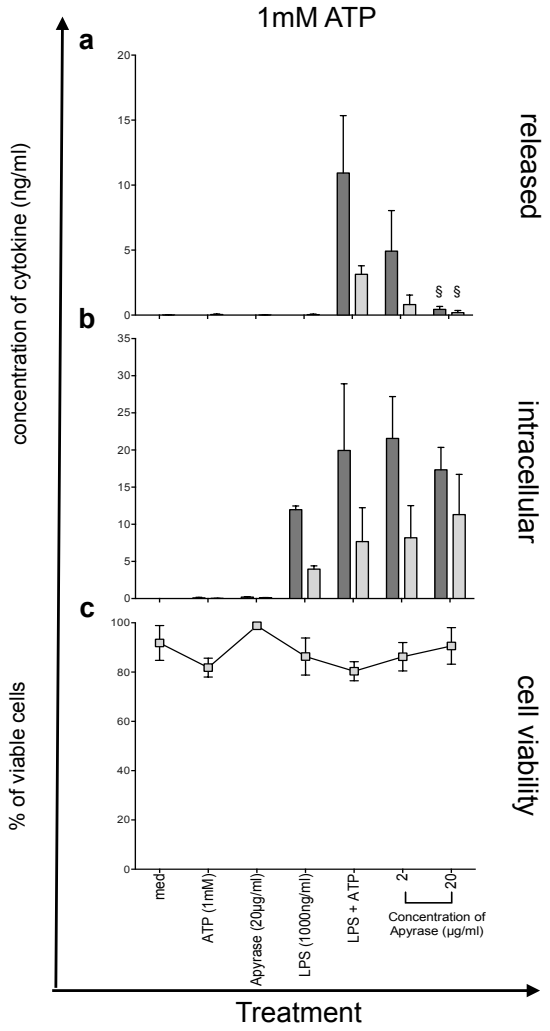


***Figure 5.11*: The effect of apyrase treatment on IL-1 release from LPS-primed and ATP challenged day 8 BM-DC.**
Day 8 BM-DC at 1x10^6 cells/ml were cultured for 2h in the presence of medium alone or stimulated with LPS at a 1000ng/ml. Subsequently, cells received treatment with medium or apyrase (2, 20µg/ml) shortly (5min) before they were challenged with ATP at 1 mM for the final 20 minutes of treatment with LPS. Both released (a) and intracellular (b) levels of IL-1β (dark grey bars) and IL-1α (light grey bars) were quantified using a cytokine-specific ELISA. Cells were stained with propidium iodide and cell viability of treated BM-DC was assessed using flow cytometry (c). Data shown are mean (n=3) ±SEM. Statistical significance of differences was first considered between medium- and LPS-treated cells with a Mann-Whitney, two-tailed test. No statistically significant differences between groups were found. Statistical significance of differences was also considered between all groups that received LPS treatment (LPS alone-treated cells were used as a comparator) and assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test (§=$p<0.05$).

5.2.11 P2X$_7$R splice variant expression in BM-DC and BM-MΦ.

Based on earlier observations provided herein, distinct differences in P2X$_7$R-associated cytokine responses were noted between DC and macrophage populations and BM-MΦ. In light of recent findings of different functionally distinct splice isoforms of the P2X$_7$R both in mice [355], it was postulated that either LPS impacted on the expression levels of the various splice variant isoforms of interest or the constitutive expression levels for each isoform varied in the two different cell types. Most commercially available anti-P2X$_7$R antibodies are directed against the carboxy terminus of the P2X$_7$R making them inappropriate for analysis of protein expression of the various splice variants in different tissues. For this reason, the expression of different P2X$_7$R splice variants in macrophages and DC at the messenger RNA (mRNA) levels was investigated next.

Primers were designed for each splice variant as described in the material and methods section in chapter 2, section 2.18. The specificity of each primer set was initially tested on splenocyte tissue using the equivalent cloned cDNA. A single band of PCR product was obtained with each set of primers (data not shown). Figure 5.12 shows expression of messenger RNA of the full-length variant P2X7A, the full-length variant with an alternative TM1 domain, P2X7K and the truncated form of the receptor P2X7J. The expression of mRNA was also investigated for isoforms P2X7B and 13b, however, the quantity of mRNA present in BM-DC, BM-MΦ and undifferentiated splenocytes was bordering the levels of accurate detection and identification of the PCR products with gel analysis was negative (data not shown).

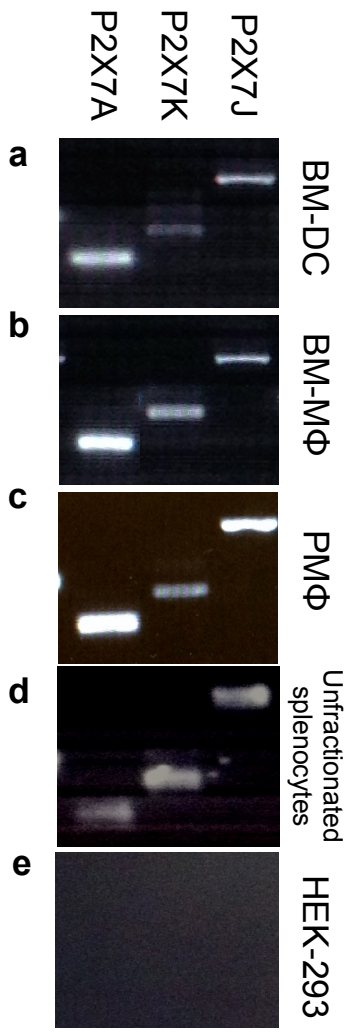


Figure 5.12: Detection of P2X$_7$R splice isoform transcript expression in DC and MΦ.
RNA was prepared from the different cell populations of interest and RT-PCR was performed as described in Chapter 2, section 2.18. Analysis of RT-PCR products for the expression of P2X$_7$R splice variants A, J and K in lysates of mouse day 8 BM-DC, day 8 BM-MΦ, the adherent population of freshly isolated peritoneal exudate cells (PMΦ), unfractionated splenocytes and HEK-293 cells. Images shown are representative of gel analysis of PCR products.

5.2.12 Endotoxin activation induces fold changes in mRNA levels of $P2X_7$ splice variants.

The possibility that LPS treatment impacts P2X7R expression at the transcriptional levels of distinct splice variants was investigated next using quantitative PCR. Both BM-DC and BM-MΦ were cultured in the presence or absence of LPS (1000ng/ml) for 2h, and changes in the mRNA levels for the various splice variant forms was assessed with quantitative PCR (figure 5.13). Transcript levels for the different splice variants were normalised to those recorded in unfractionated splenocytes. The levels of P2X7A transcript expression in medium-treated BM-DC and BM-MΦ were found to be 1000-fold higher in comparison to unfractionated medium-treated splenocytes and at the highest levels in comparison to all other splice isoforms of interest. Transcripts for the P2X7J truncated isoform were found to be approximately 100-fold higher in both cell types, than in splenocytes, however, these were more variable in BM-DC. Finally, expression of the P2X7K isoform at the mRNA level was found at similar levels to those recorded in unfractionated splenocytes. A 10-fold decrease in the mRNA levels of P2X7A was detected in both cell types as a result of LPS treatment. A similar LPS-induced decrease was observed for the mRNA levels of the truncated P2X7J isoform, albeit the extent of downregulation was less profound. This effect was found to be statistically significant in BM-MΦ. Finally, no effect on the relatively low mRNA levels of the $P2X_7K$ isoform was detected with LPS activation in either BM-DC or BM-MΦ. There were no detectable differences in the mRNA levels of all the splice isoforms investigated, between BM-DC and BM-MΦ in the presence or absence of LPS.

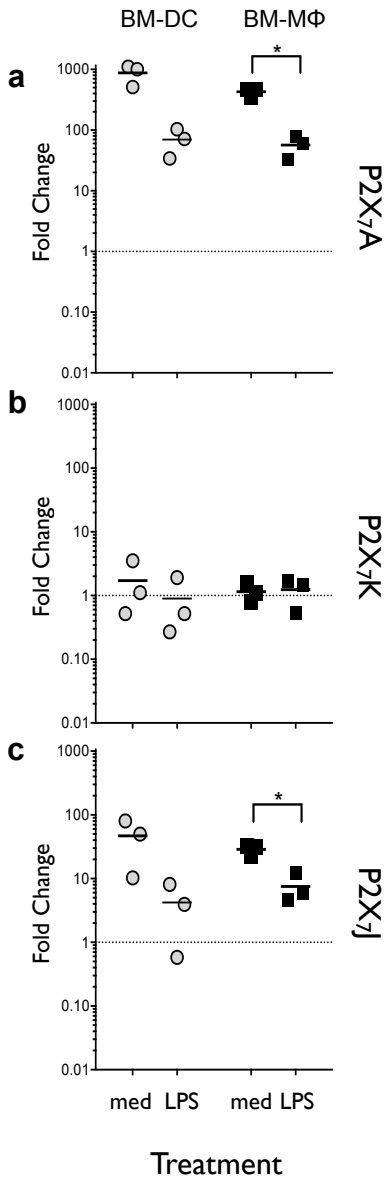


***Figure 5.13:* LPS-induced changes in mRNA levels of P2X$_7$R splice variant in BM-DC and BM-MΦ.**
Cells were incubated in medium alone or in the presence of LPS (1000ng/ml) for 2h at 1x10^6 cells/ml. Total RNA was extracted from each cell population and the mRNA expression for each splice variant was evaluated on the converted cDNA (150ng/ml) using quantitative PCR and specific primers. Levels of mRNA for (a) P2X7A, (b) P2X7K and (c) P2X7K were normalized to the expression levels of the housekeeping gene, HPRT and are expressed as fold changes relative to expression levels detected in unfractionated splenocytes. Data shown are mean (n=3) ±SEM of three independent BM-DC and BM-MΦ preparations. Statistical significance of differences between groups was assessed by one way ANOVA and Tukey's multiple comparison post-hoc test (*= $p<0.05$).

5.2.13 The impact of apyrase treatment on IL-1β released from day 8 BM-DC pulsed with LPS.

Finally, based on preliminary observations, it was possible to demonstrate a possible role of ATP signalling in LPS (24h)-induced IL-1β release using apyrase treatment (figure 5.14). No detectable levels of IL-1β were found in lysates (intracellular) or supernatants of medium-treated or apyrase-treated 200μg/ml BM-DC (figure 5.14a,b). LPS treatment induced the up-regulation of approximately 4ng/ml of intracellular IL-1β, which was further enhanced with apyrase treatment in a dose dependent manner; 8ng/ml of IL-1β was found following treatment with the top doses of apyrase, 200μg/ml. In this case, LPS treatment (24h) stimulated the release of approximately 1ng/ml of IL-1β, which was partially blocked with apyrase treatment at all concentrations of apyrase employed (0.2-200μg/ml of apyrase). The viability of medium-treated BM-DC was recorded at approximately 95% and remained unaffected following treatment with LPS and/or apyrase at all the different concentrations of apyrase employed (figure 5.14c).

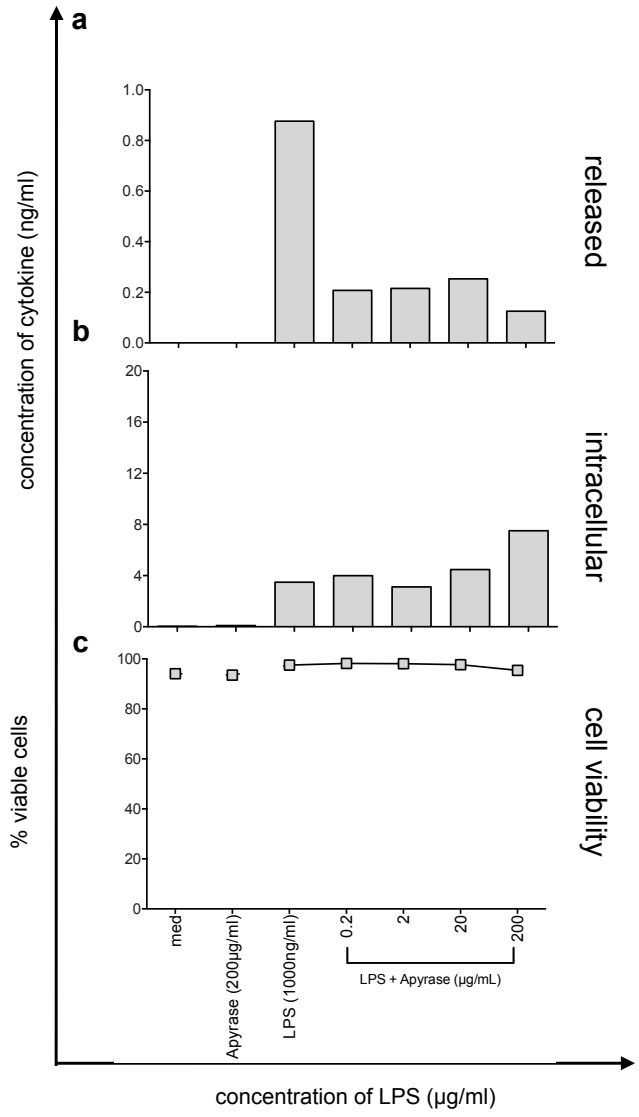


***Figure 5.14:* The effect of apyrase treatment on IL-1β release from LPS-pulsed day 8 BM-DC.**
Day 8 BM-DC at 1x10^6 cells/ml were cultured for 24h in the presence of medium alone or stimulated with LPS at a 1000ng/ml. Cells also received treatment with medium or apyrase (0.2, 2, 20, 200µg/ml). Both released (a) and intracellular (b) levels of IL-1β were quantified using a cytokine-specific ELISA. Cells were stained with propidium iodide and cell viability of treated BM-DC was assessed using flow cytometry (c).

5.2.14 LPS (24h)-induced IL-1β release in BM-DC and BM-MΦ in the absence of exogenous ATP.

Based on the preliminary results of these studies presented in chapter 4 (section 4.2.7) unlike with BM-MΦ and PMΦ, treatment with LPS (24h) alone prompted the release of substantial levels of IL-1β from BM-DC in a dose-dependent manner in the absence of exogenous ATP. These results, however, were not reproducible. It was of particular interest to further assess the role of both ATP and $P2X_7R$ signalling in these LPS-driven cytokine responses by murine BM-DC. Therefore, both BM-DC and BM-MΦ were cultured for 24h in the presence of LPS at 1, 10, 100 or 1000ng/ml and the results are shown in figure 5.15. However, in these repeat experiments, the marked differences between BM-DC and BM-MΦ were no longer observed (figure 5.15a) with the levels of IL-1β release barely rising above the lowest level of accurate detection (<0.039ng/ml). Additionally, the intracellular content of IL-1β was found to be comparable between the two cell types with approximately 7ng/ml detected in BM-DC and 6ng/ml in BM-MΦ following treatment with the top concentrations of LPS (1000ng/ml) (figure 5.15b). Finally, the viability of medium-treated BM-DC was recorded at approximately 90% whereas that of BM-MΦ was found just below 80%. Treatment with LPS had no impact on the viability of the cells (figure 5.15c).

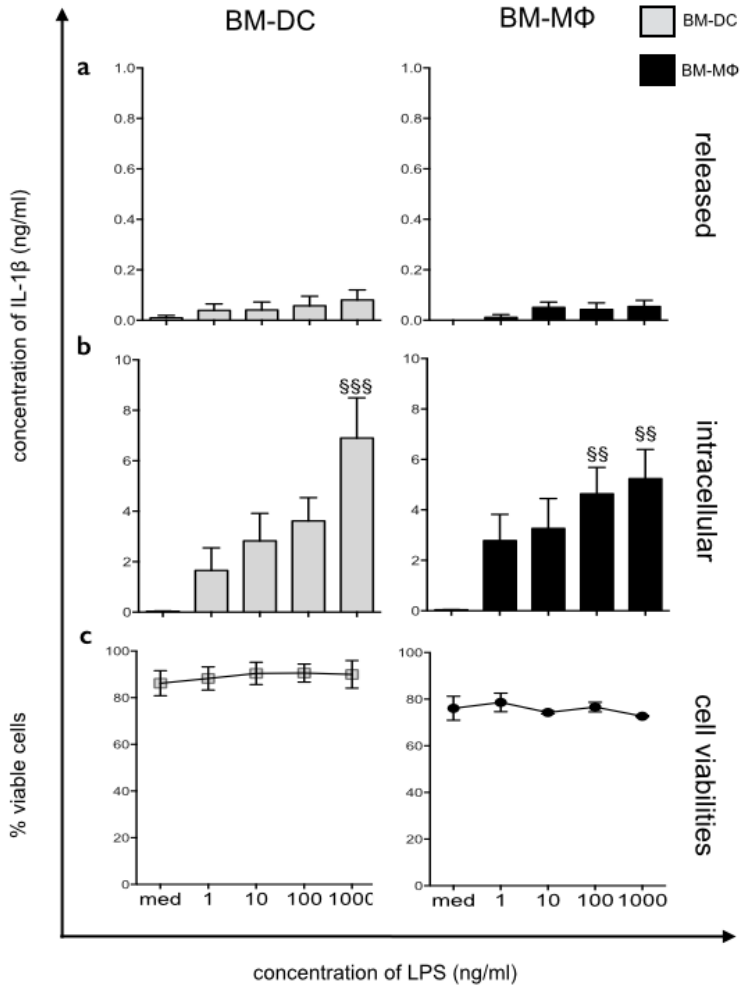


***Figure 5.15:* Endotoxin activation (24h) fails to induce significant levels of IL-1β release in day 8 BM-DC; comparisons with 8 BM-MΦ.**
Day 8 BM-DC (grey bars) and BM-MΦ (black bars) at 1x10^6 cells/ml were cultured for 24h in the presence of medium alone or stimulated with LPS at 1, 10, 100 or 1000ng/ml. Both released (a) and intracellular (b) levels of IL-1β were quantified using a cytokine-specific ELISA. Cells were stained with propidium iodide and cell viability of treated BM-DC (grey squares) and BM-MΦ (black circles) was assessed with flow cytometry (c). Data shown are mean (n=5-6) ±SEM. Statistical significance of differences between treatment groups for each cell type (medium-treated cells were used as a comparator) was assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test. (§§= $p<0.01$, §§§= $p<0.001$).

5.2.15 Assessing the responsiveness of BM-DC and BM-MΦ to LPS activation

As a first strategy to investigate possible reasons for the changes in cytokine responses observed in BM-DC, the ability of the cells to respond to higher concentrations of LPS was investigated. These were compared directly with cytokine responses observed from murine BM-MΦ challenged with equal concentrations of LPS (figure 5.16). Treatment with higher levels of LPS induced substantial up-regulation of IL-1β but failed to signal the release of IL-1β in either cell type (figure 5.16a). More precisely, intracellular IL-1β levels peaked at 8ng/ml in BM-DC and at 6ng/ml in BM-MΦ following treatment with LPS at 100 μg/ml and generally, these were comparable between the two cell types for both concentrations of LPS (figure 5.16b). Nevertheless, only minimal levels of the cytokine were detected in supernatants of both cell types. Finally, treatment with LPS at these high concentrations did not impact on the viability of the cells. Approximately 90% of medium-treated BM-DC and 80% medium-treated BM-MΦ were viable and LPS treatment had no further significant impact (figure 5.16c).

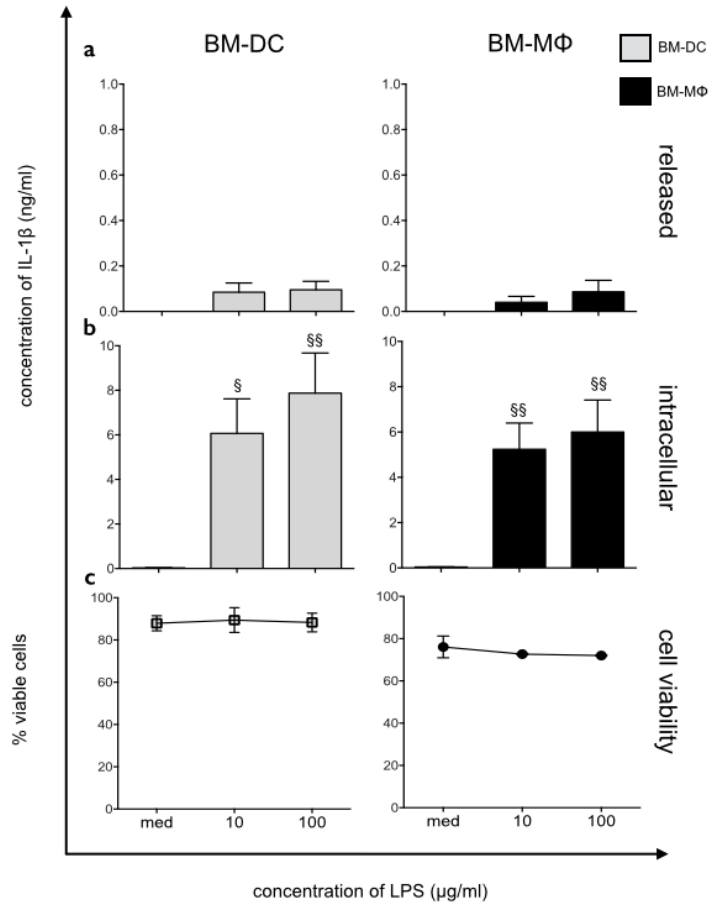


***Figure 5.16:* Higher concentrations of LPS (24h) fail to induce IL-1β release in day 8 BM-DC; comparisons with day 8 BM-MΦ.**
Day 8 BM-DC (grey bars) and BM-MΦ (black bars) at 1x10^6 cells/ml were cultured for 24h in the presence of medium alone or stimulated with LPS at 10,000 (10μg/ml), 100,000ng/ml (100μg/ml). Both released (a) and intracellular (b) levels of IL-1β were quantified using a cytokine-specific ELISA. Cells were stained with propidium iodide and cell viability of treated BM-DC (grey squares) and BM-MΦ (black circles) was assessed with flow cytometry (c) Data shown are mean (n=6) ±SEM. Statistical significance of differences between treatment groups for each cell type (medium-treated cells were used as a comparator) was assessed by one way ANOVA and Dunnett's multiple comparison post-hoc test. (§= $p<0.05$, §§= $p<0.01$).

5.2.16 The kinetics of IL-1β production and release from LPS primed BM-DC.

The next objective involved tracing IL-1β production (intracellular content) and release in BM-DC following treatment with LPS over a time course of 24h (figure 5.17). No detectable levels (<0.039ng/ml) of IL-1β were detected in supernatants or lysates of medium-treated cells at any time point investigated (data not shown). More importantly, no detectable levels of IL-1β were recorded (<0.039ng/ml), in cultured supernatants of LPS challenged BM-DC for all time-points analyzed (figure 5.17a). The intracellular levels of IL-1β in LPS-cultured BM-DC peaked at 15ng/ml within the first 2h of culture. A time dependent decline in the intracellular levels of the cytokine was evident with approximately 5ng/ml of the cytokine detected in lysates of BM-DC at the end of the treatment (24h) (figure 5.17b). Finally, the viability of BM-DC was recorded at approximately 99% at time 0 and remained largely unaffected over time in culture with LPS (figure 5.17c).

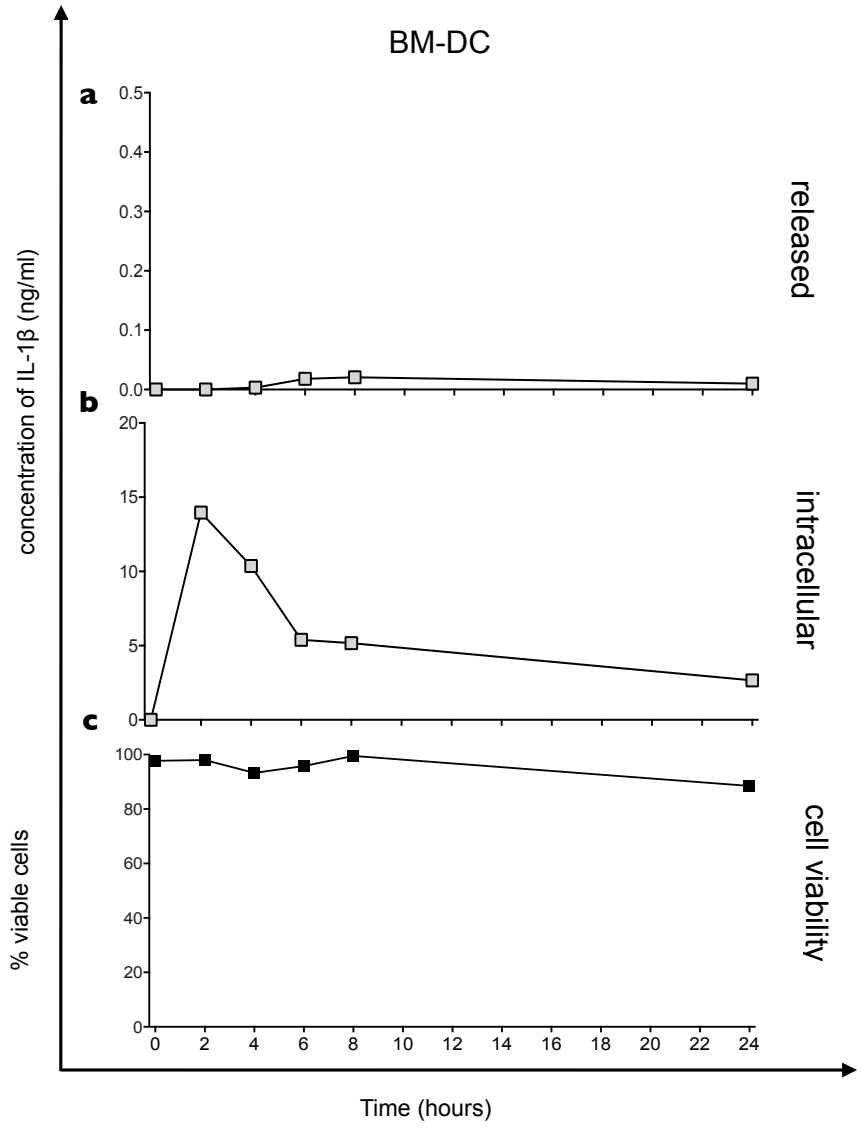


Figure 5.17: The kinetics of LPS-induced IL-1β production and release in day 8 BM-DC.
Day 8 BM-DC at 1x10^6 cells/ml were cultured for 0, 2, 4, 6, 8 or 24h in the presence of LPS at 1000ng/ml. Both intracellular (a) and released (b) levels of IL-1β were quantified using a cytokine-specific ELISA. Cells were stained with propidium iodide and cell viability was assessed with flow cytometry (c).

5.2.17 Investigating possible phenotypic changes in the profile of cultured day 8 BM-DC.

The development of DC-like cells (BM-DC) from BM precursors can be influenced by a number of variables that affect their differentiation towards a DC phenotype as well as their activation state. For example, the presence of bystander antigens in culture conditions can activate BM-DC and alter their responsiveness to further TLR stimulation [447]. Therefore, the phenotype of BM-DC was assessed particularly with regards to the expression of activation markers (MHC class II and CD86) and expression of the DC-specific integrin CD11c the results of which are depicted in figure 5.18. The phenotypic characteristics of the "unresponsive" BM-DC, the population that failed to release IL-1β in response to 24h incubation with LPS in the absence of exogenous ATP, was compared to that of "responsive" BM-DC, which secrete IL-1β following LPS activation alone. No differences were observed between the two populations of BM-DC with regards to the percentage of cell surface expression for the markers of interest. However, the MFI levels for both markers of activation, MHC class II (approximately 280AU) and CD86 (approximately 40AU), were found to be somewhat higher whilst those for the DC-associated integrin CD11c were found to be lower at approximately 120AU in the "unresponsive" population.

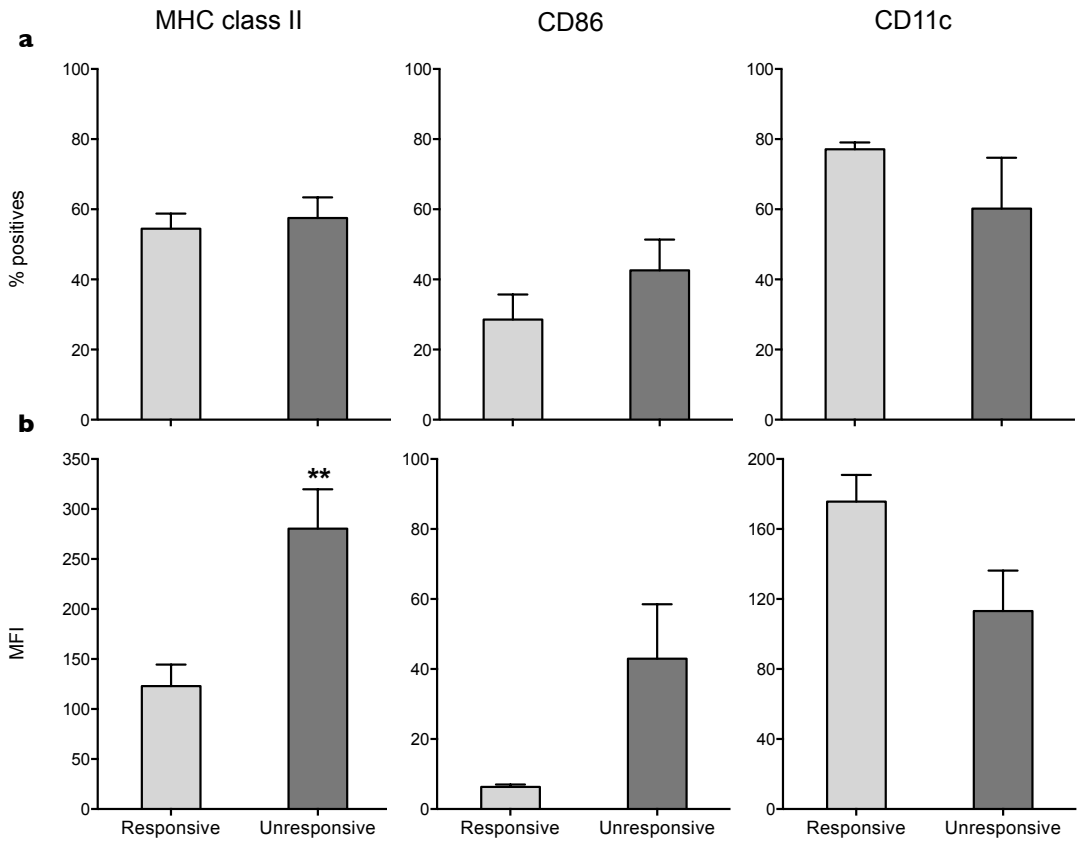


Figure 5.18: Phenotypic changes in cultured day 8 BM-DC.
Day 8 cultured BM-DC were analysed by flow cytometry for surface expression of the markers of interest (MHC class II, CD86 and CD11c) and data are shown with respect to (a) the percentage positive cells and (b) the mean fluorescence intensity (MFI). Cells (10,000) were acquired using a BD Biosciences FACSCalibur flow cytometer (2000), CellQuest Pro was used for data acquisition and FlowJo 9.5.2 for data analysis. Data shown are mean (n=3) ±SEM. Statistical significance of differences between groups was assessed by a two way ANOVA and Sidak's multiple comparison post-hoc test (**=$p<0.01$).

5.3 Discussion.

5.3.1 Cytokine responses in BM-DC and BM-MΦ.

The results provided herein reveal interesting distinct patterns of LPS and ATP-induced cytokine responses between murine BM-DC and BM-MΦ, particularly with regards to IL-1. Although the ratio of precursor to bioactive form of IL-1β was not distinguished in either intracellular content or in supernatants, some interesting patterns in production and secretion emerged between the two cell types. LPS priming induced similar levels of intracellular IL-1β expression in BM-DC and BM-MΦ, however, activation with ATP prompted comparatively higher levels of IL-1β secretion in BM-DC. Additionally, macrophages exhibit more sensitivity to LPS treatment with regards to IL-1β production (intracellular content), nevertheless, the levels of cytokine secretion were always found to be higher in BM-DC. Interestingly, macrophages were also more sensitive to the effects of high dose ATP, down regulating IL-1β expression. The exact mechanism by which IL-1β molecules are externalised from cells is currently a topic of controversy. IL-1β does not carry a leader peptide motif required to chaperone proteins through the classical, ER and Golgi apparatus-mediated pathway of exocytosis [261, 582]. A number of possible different mechanisms have been described to mediate IL-1β release and it is very likely that individual routes are of preference for different cell types. For example, evidence suggests that whilst in murine microglia cells IL-1β release follows microvesicle shedding [268], in mouse peritoneal macrophages, it is assisted through plasma-membrane transporters [270]. Such differences are thought to be the result of distinct functional requirements of different cell types. For example, murine DC are required to externalize larger volumes of IL-1 and are therefore required to employ a more efficient route of secretion than their phagocyte siblings.

Published reports suggest that the primary pathway of IL-1β release from murine BM-MΦ is via the release of exosomes. These are vesicular structures, small in size, that are formed following the invagination of the cells surface membrane and are characterized by their MHC class II and LAMP expression [271]. Conversely, Pizzirani and colleagues suggest that IL-1β release in human monocyte-derived DC occurs via the release of microvesicles. The mechanism driving microvesicle-shedding remains unclear, however, MacKenzie and colleagues demonstrate an important role of the P2X$_7$R activating their release, at least in HEK-293 cells [269]. These vesicles are

usually heterogeneous in size but comparatively larger in comparison to exosomes and have also been found to express MHC class II molecules. Additionally, microvesicles were found to express $P2X_7R$, which is believed to mediate their lysis to release their cargo in the presence of ATP [268]. Microvesicles therefore constitute a more efficient system for the delivery of large quantities of pro-inflammatory molecules to specific targets.

It is widely accepted that IL-1β is regulated at multiple levels before bioactive IL-1β molecules are shed from the cell surface membrane. Thus, a number of reasons could contribute to the discrepancies observed in cytokine responses between BM-DC and BM-MΦ. In accordance with previous observations, the results of these investigations suggest that activation of TLR with LPS (2h) induces comparable levels of IL-1β expression (intracellular) in murine BM-DC and BM-MΦ [561]. An interesting pattern emerged when LPS-primed cells were challenged with increasing concentrations of ATP. Intracellular levels of IL-1β were reduced in an ATP dose-depended manner in both LPS-primed BM-DC and BM-MΦ. In BM-DC, a concomitant dose-dependent increase in cytokine release was recorded whilst in BM-MΦ, the magnitude of cytokine release was significantly lower even though the intracellular content was comparable to that observed in BM-DC. Based on the observations of total cytokine levels for each cytokine (figure 5.4), it was postulated that IL-1β must be partially degraded in BM-MΦ following activation with the high doses of ATP (5 and 10 mM).

Previous observations suggest that TLR activation of murine macrophages with LPS potentiates processes of autophagy [258, 583]. Harris and colleagues demonstrate that autophagy specifically targets the sequestration of pro-IL-1β molecules within autophagosomes and show that treatment with rapamycin, a potent enhancer of autophagal processes, successfully blocks IL-1β release from LPS-primed and ATP-challenged macrophages as well as suppressing the levels of plasma circulating IL-1β *in vivo* [584]. Autophagy is a process renowned for keeping the cellular interior 'clean' by recycling intracellular structures and organelles such as mitochondria (mitophagy). Mitochondria constitute a rich source of ROS and mitochondrial DNA, entities that have previously been shown to activate inflammasome oligomerization in human macrophages [585, 586]. In addition, autophagosomes have also been shown to target individual inflammasome components [587]. The role of autophagy in regulating IL-1β

release is two-fold; on the one hand it controls the availability of pro-IL-1β molecules in the cytosol and on the other hand it regulates the availability of individual inflammasome components and signals (such as ROS derived from mitochondria) that activate its aggregation. Furthermore, $P2X_7R$ activation is often linked with IL-1β release from macrophages and as previously mentioned activation of this receptor results in substantial efflux of K^+, a necessary step for IL-1β release [239] but not for its expression [585]. Harris and colleagues observed that blocking K^+ efflux whilst also suppressing autophagy (using the chemical inhibitor 3-methyladenine, 3-MA), unexpectedly prevents IL-1β secretion [584]. Together with previous observations, their results suggest that K^+ efflux (most possibly mediated by $P2X_7R$ activation) is required for IL-1β release in macrophages.

Whether autophagy follows distinct regulatory mechanisms in BM-DC and BM-MΦ has not been previously explored, however, Harris and colleagues demonstrate that autophagy is more strictly controlled in DC [584]. Studies using MyD88 and TIR-domain-containing adapter-inducing interferon-β (TRIF) KO mice (two intracellular pathways that are associated with TLR-mediated autophagy activation) suggest that whilst murine BM-MΦ require a MyD88-derived signal alone to initiate autophagy, in murine BM-DC both TRIF- and MyD88-derived signals are necessary [584]. In theory, if autophagy is more strictly regulated in BM-DC then this would result in (a) comparatively higher levels of inflammasome components present in BM-DC (reported in [561]) (b) higher levels of inflammasome-activating signals such as ROS and (c) higher levels of released cytokine. Certainly autophagy is a crucial element of the defence system of the cells against a number of bacterial, viral and parasitic pathogens delivering them into internal lysosomes for degradation [588, 589]. Nevertheless, autophagy is also an integral process of antigen presentation and it is an efficient pathway for accessing antigenic peptides for loading onto MHC class II molecules. The contribution of autophagy to MHC class II presentation [590], inflammasome activation and IL-1β processing in DC requires further attention but it should not be surprising if DC, as the primary APC, employs distinct mechanisms to regulate such processes [591].

He and colleagues provide similar observations, with regards to cytokine responses in murine BM-DC and BM-MΦ, to those reported herein. The group reports that LPS

(24h) signalling is sufficient to induce IL-1β release in BM-DC but not BM-MΦ [561]. A similar phenomenon was observed in our preliminary experiments and illustrated in chapter 4, figure 4.9. When investigating the constitutive levels of NLRP3 in macrophage (BM-MΦ) and DC (splenic and BM-DC) populations the group reported that BM-DC express higher levels of the protein both under the steady-state conditions and following LPS activation [561], in accordance with previous observations by Bauernfeind and colleagues [592]. The group therefore postulates that the presence of higher levels of NLRP-3 protein in BM-DC provides a 'head-start' for inflammasome activation and IL-1β processing [561]. Additionally, the group employed apyrase (a well-known ATPase) and $P2X_7R$ KO mice to investigate the role of $P2X_7R$ signalling in LPS (24h)-induced IL-1β release from murine BM-DC [561]. Their findings suggest that neither ATP nor $P2X_7R$ signalling are required for LPS-induced cytokine release. However, the group failed to investigate the effect of apyrase treatment in a range of concentrations, or at least do not provide any relevant evidence [561]. Perhaps, both higher concentrations of apyrase and repeated treatments were required to effectively degrade transiently released ATP and block downstream signalling. In contrast, our preliminary observations suggest that apyrase, at 20μg/ml, successfully prevented LPS-induced IL-1β release from murine BM-DC thereby implicating ATP signalling in LPS-induced cytokine responses from these cells.

Furthermore, following the identification of functional $P2X_7R$ splice variants present in $P2X_7R$ KO mice with gain-of-function properties (P2X7K) [355], the relevance of the $P2X_7R$ KO mouse model in examining $P2X_7R$ responses is often questioned. Further work is required to exclude the possibility that gain-of-function $P2X_7R$ splice isoforms in conjunction with other P2X receptors ($P2X_4R$) can compensate for the function of the full variant (P2X7A) in KO mice [593]. Arguably, the use of specific $P2X_7R$ antagonists such as the A-740003 is currently considered the most appropriate tool to investigate $P2X_7R$ function. The evidence provided by He and colleagues challenge the relevance of the ATP-$P2X_7R$ signalling axis for IL-1β release in DC populations, but at present, do not rule out. Undoubtedly, the ATP-gated $P2X_7R$ provides a potent stimulus for inflammasome activation and an efficient route for IL-1β processing and release [594]. In fact, LPS has been previously shown to induce transient release of ATP from murine microglia and macrophages [498, 595]. Evidence suggests that endogenous ATP

molecules are released via pannexin/connexin hemichannels to activate the P2X$_7$R in an autocrine loop, at least in macrophages [596, 384, 597].

The bioavailability of ATP in the extracellular milieu is strictly regulated by a number of ATPases. Interestingly, murine macrophages have been previously shown to degrade exogenous ATP more efficiently than human macrophages [597]. Perhaps, ATPases are less efficient in murine DC in comparison to macrophages resulting in more enhanced ATP-P2X$_7$R-driven responses. The result of ATP degradation is an accumulation of adenosine molecules (an ATP derivative) that signal through P1 receptors to counter-act pro-inflammatory processes [598]. Additionally, ATP does not only activate P2X$_7$R receptors but it is also a potent activator of other P2X receptors, whose functional properties often overlap with those of the P2X$_7$R. As previously discussed, Sakaki and colleagues in a recent study demonstrate an important role of P2X$_4$R signalling in P2X$_7$R-mediated IL-1β and IL-18 release from LPS-primed and ATP-activated murine BM-DC [599]. P2X$_4$R is highly permeable to Ca^{2+} [600] and evidence suggests that it provides a necessary initial peak of Ca^{2+} influx to initiate P2X$_7$R-driven IL-1β and IL-18 release [599]. A similar contribution of P2X$_4$R signalling in P2X$_7$R-associated functions including Ca^{2+} influx and pore formation was previously reported in murine macrophages. The role of P2X$_4$R signalling in P2X$_7$R-mediated cytokine release was not directly examined [601]. Importantly, the murine BM-DC population employed by Sakaki and colleagues is different to that BM-DC population employed by these investigations. Sakaki and colleagues also generated BM-DC using GM-CSF for 7 days, however, they employed the adherent population of cultured cells [599], with no assessment of cell surface expression of DC- and/or macrophage–associated markers (e.g. F4/80 or CD11c expression) for their investigations. The initial observations of Inaba and colleagues who pioneered the protocol for the generation of GM-CSF-derived BM-DC suggest that the adherent population of cultured BM-DC skews towards the macrophage lineage [83]. In contrast, these studies have employed the non- or loosely-adherent population of cells, having phenotypically analyzed the cells with regards to a number of cell surface markers (see Chapter 3). Based on the observations of this study with the A-740003 inhibitor (97% inhibition of IL-1β was recorded), it is postulated that ATP (5 mM)-induced IL-1β responses in BM-DC are almost exclusively the result of P2X$_7$R signalling. However, the possibility that some form of cross-talk exists between the P2X$_4$R and the P2X$_7$R cannot be excluded with absolute certainty at this stage.

It is important to keep in mind that macrophages are predominantly involved in the removal of erythrocytes and apoptotic tissue, wound healing and tissue remodeling. They provide the host essential 'cleaning' services and remain most of the time in a 'quiescent state' with little or no production of pro-inflammatory agents (usually described as M2 macrophages). However, they are sometimes required to assist in presenting antigens to T-cells to exacerbate the adaptive immune responses and are therefore required to maintain the capacity to produce and release potent pro-inflammatory cytokines such as IL-1β. As such, in the absence of inflammatory stimuli they need to more strictly regulatory the mechanisms for the production and release of such potent inflammatory molecules [602, 138].

Having observed quite distinct cytokine responses in the two cell types with much interest it was subsequently important to examine the underlying molecular mechanisms responsible for these observations. Thus, the role of ATP as well as P2X$_7$R signalling in IL-1β release from LPS-primed BM-DC and BM-MΦ was investigated next. This was achieved by employing (a) apyrase treatment and (b) P2X$_7$R-specific inhibitors. As previously mentioned, apyrase is an enzyme that targets ATP molecules for degradation to yield AMP or other inorganic molecules. The enzyme has previously been shown to counteract ATP-driven IL-1β responses in murine BM-MΦ [597, 289]. In agreement with these observations, the results provided herein demonstrate the capacity of apyrase to block ATP-driven IL-1β responses in LPS-primed BM-DC thereby implicating the role of ATP and P2X signalling in driving the release of inflammatory cytokines. Although this effect was largely anticipated, it was also important to demonstrate the capacity of the enzyme to degrade ATP molecules and block its associated cytokine responses in BM-DC. Apyrase was also employed to investigate the role of the ATP-P2X$_7$R-axis in the distinct LPS (24h)-induced cytokine responses reported in Chapter 4 section 4.2.7 the results of which are discussed in section 5.3.5. Treatment with apyrase results in the degradation of ATP molecules (therefore lowers the concentration of ATP) and enhances the accumulation of ADP and AMP molecules in the cultured medium. As a result, this prevents activation of P2X receptors, activated with high concentrations of ATP and at the same time provides ligands for P2Y and P1 as well as certain P2X activation (activated with lower concentrations of ATP) [603]. In a way the results obtained herein from BM-DC treated with apyrase exclude the contribution of P2Y and P1 signalling in IL-1β responses.

This study has also employed a P2X$_7$R-specific inhibitor to challenge the significance of the P2X$_7$R-axis in IL-1β release from LPS and ATP-challenged BM-DC [407, 408]. The interest in designing P2X$_7$R antagonists arose from early phenotypic observations of P2X$_7$R$^{-/-}$ mice that exhibited a suppressed response to chronic inflammatory and neuropathic pain. This has triggered a global quest for potent and selective receptor antagonists for use in pharmacological intervention [572, 418] among the major pharmaceutical companies. In agreement to previous observations of Honore and colleagues in human macrophages, the results provided herein demonstrate that the A-740003 inhibitor effectively blocks the release of IL-1β from both murine BM-DC and murine BM-MΦ [408]. Considering that one of the major characteristics of A-740003 is its specificity for the P2X$_7$R then inhibition of IL-1β release from BM-DCs and BM-MΦ using A-740003 provides indirect evidence that (a) a functional P2X$_7$R is expressed on both cell types and (b) demonstrates the requirement of P2X$_7$R signalling in IL-1β release from *in vitro* cultured DC and macrophages. Importantly, Riteau and colleagues provide evidence that in P2X$_7$R KO mice other mechanisms can compensate for the loss of the receptor in mediating inflammasome activation and IL-1β release [597]. Although evidence such as these provide a valid point, the fact is, that the ATP-gated P2X$_7$R provides an effective and efficient route for the release of a high magnitude of IL-1β molecules [604].

Indeed, the physiological relevance of the ATP-P2X$_7$R axis in mediating IL-1β release *in vivo* is often questioned particularly due to the high 'non-physiological' levels of ATP required for receptor activation. However, ATP concentrations have previously been shown to reach the mM range following trauma therefore the concentrations used in the *in vitro* experiments herein reflect those recorded *in vivo* at sited of injury [571, 605, 606]. The findings of Ferrari and colleagues suggest that activation of the P2X$_7$R is followed by a transient release of endogenous ATP molecules establishing an amplification loop for receptor activation [607]. Further, ATP is not the only physiological ligand of the P2X$_7$R; the microbiocidal molecule LL-37 has also been shown to induce P2X$_7$R activation and IL-1β release from endotoxin-primed blood derived human monocytes [332]. Non-nucleotide molecules can also act indirectly to activate the receptor by changing its sensitivity to agonist; for example P2X$_7$R in murine macrophages are activated in the presence of NAD molecules with low concentrations of ATP [475]. Conversely, NAD was previously reported to activate

P2X$_7$R of murine T-cells in an ATP-independent manner [476] highlighting the functional diversity of the receptor on different cell types.

The functional relevance of the P2X$_7$R axis might not constitute the primary platform for IL-1β release under all inflammatory circumstances *in vivo*, however, convincing evidence provides proof that this peculiar receptor is an important functional tool for inflammatory cells. Studies in monocytes derived from subjects with the loss-of-function Glu496Ala P2X$_7$R polymorphism are unable to release substantial levels of either IL-1β or IL-18, emphasizing the importance of the P2X$_7$R axis in IL-1 release, at least in human tissues [608, 609]. More importantly, the P2X$_7$R has been shown to play a part in mediating IL-1β release that drives the development of contact hypersensitivity in mice, *in vivo* [289]. In contrast, previous published observations suggest that non-caspase-1 processing of IL-1β is also an important axis for its release particularly during the development of sterile inflammatory responses in mice. Sterile inflammation is characterized by the presence of IL-1β-producing neutrophils. These cells have a relatively short-life span and a short amount of time to process IL-1β, the cytokine is released in its precursor form along with a proteinase-3 inhibitor, which cleaves IL-1β to its active form in the extracellular milieu [610].

Another important aspect of this study was to provide further evidence of the role of the P2X$_7$R in mediating IL-1α release from BM-DC and BM-MΦ. The data provided herein reveal some interesting distinct patterns of IL-1α release between the two cell types. Whereas incubation with LPS and activation with ATP provoked IL-1α secretion in BM-DC, little secretion of the cytokine was observed under all experimental conditions explored in BM-MΦ. IL-1α has been clearly marginalized over the years as most interest has focused in deciphering the pro-inflammatory functions of IL-1β. It was therefore important to improve our understanding of how IL-1α expression and release is regulated in DC populations. IL-1α shares similar functional properties with IL-1β, however, its post-translational processing differs from that of its sibling [195]. IL-1α is subject to enzymatic cleavage by a class of proteolytic enzymes known as calpains, however, the effect of this cleavage remains largely unknown since the cytokine is biologically active in both its precursor (31kDa) and cleaved forms (17kDa), although processing may increase its likelihood for secretion [197]. Recent evidence suggests that proteolytic cleavage of IL-1α by proteases including calpains, elastase and

granzyme B induces a necessary conformational change of the cytokine that enhances its affinity of the IL-1RI and potentiate its pro-inflammatory potency [611].

The route of IL-1α secretion is thought to be via passive diffusion from necrotic DC, following severe injury [612, 613] and this is an important process that alerts the immune system to initiate healing. Conversely, apoptotic cells have been shown to sequester IL-1α intracellularly [614] whereas, more recent evidence suggests that in intact cells IL-1α hijacks the classic inflammasome-dependent secretory pathway of IL-1β [615]. In accordance with the observations of Fettelschoss and colleagues the results of this study reveal that IL-1α is released by murine BM-DC in a P2X$_7$R-mediated inflammasome-dependent manner. Using BM-DC derived from NLRP3 and P2X$_7$R KO mice Fettelschoss and colleagues demonstrate the BM-DC require both components to release IL-1α as well as IL-1β in response to LPS (24h) priming and ATP activation (for the last 30min of the incubation). Although the group provides evidence for the LPS-induced intracellular up-regulation of both IL-1 cytokines with Western blot analysis, they fail to quantify these which would exclude the possibility that lack of cytokine release is due to low levels of intracellular cytokine content [616]. Additionally, in accordance with our observations of IL-1α release from peritoneal exudate cells, the findings of Perregaux and Gabel suggest that in PMΦ, IL-1β and IL-1α share a similar route for release following LPS priming and ATP activation [617].

Apart from being released as a bioactive molecule, IL-1α is also expressed as a cell surface lectin-anchored protein in murine BM-DC and macrophages [618, 616]. Previous published observations suggest that LPS induces the trafficking of IL-1α to the cell surface membrane in murine BM-DC, a process believed to occur via a distinct inflammasome-independent pathway [616]. The secreted and cell surface membrane-bound form of IL-1α are believed to share distinct biological functions and, most possibly patterns of expression [584]. Therefore, it is possible that the two cell types could regulate their cell-surface IL-1α expression differently due to distinct functional requirements. For example, IL-1α has been associated with the destruction of *Mycobacterium tuberculosis* organisms, a process more involved with macrophages than DC [619]. Perhaps cell surface expression of IL-1α is given priority in BM-MΦ. Alternatively, since IL-1α is thought to be able to hijack the IL-1β pathway for secretion, it may also be specifically targeted by autophagosomes for destruction, which

could also provide an explanation for the ATP dose-dependent reduction of intracellular IL-1α observed in BM-MΦ. Another interesting scenario emerges from the studies of Luheshi and colleagues in murine microglia suggesting that IL-1α is actively trafficked and retained within the nuclear envelope to suppress its release and consequent inflammatory responses from necrotic cells. In fact, the data presented in chapter 4 section 4.2.14 illustrating YO-PRO dye uptake in BM-MΦ suggest that ATP induces enhanced pore formation providing an accessible route for IL-1α release. LPS-primed BM-MΦ may adopt similar mechanisms to suppress the release of IL-1α following ATP activation [189].

The release of IL-1α constitutes a potent DAMP signal with important functions, particularly in sterile inflammation. A recent study by Kono and colleagues suggests that murine BM-DC and not macrophages provide a large proportion if not all of the IL-1α required to drive acute inflammatory responses. The group postulates that BM-DC have a higher content of pro-inflammatory cytokines in comparison to other cell types, a hypothesis which is strengthened by the observations of this study. Their *in vivo* observations also suggest the DC are the predominant source of IL-1 during inflammatory responses [620] and it is believed that whilst macrophages secrete IL-1α to alert the immune system for the presence of necrotic cells in a caspase-independent manner [621], DC-derived IL-1α among other functions, is more involved with sterile inflammation and the recruitment of neutrophils [621].

A third important inflammatory cytokine is IL-6 and so the release of IL-6 from BM-DC was also investigated and the results suggest that IL-6 expression and release is influenced more by LPS- than the ATP-P2X$_7$R signalling axis. Solini and colleagues are amongst the few who report an association between P2X$_7$R activation and IL-6 release in human fibroblasts derived from diabetic patients that exhibit an overactive P2X$_7$R compared to healthy controls. When fibroblast monolayers were primed with LPS (1μg/ml) and phorbol myristate acetate (PMA) (100nM) and subsequently challenged with ATP (0.1-1 mM), comparatively enhanced levels of IL-6 release were recorded. Treatment with KN-62 only partially inhibited IL-6 release and it was therefore postulated that its release might also be regulated by other P2 receptors or even other cytokines [622]. Based on the results of this study, IL-6 release from BM-DC remained largely unaffected by ATP. The results of further experiments with A-740003 treatment

collectively suggest that P2X$_7$R signalling is not required in IL-6 expression or release. Additionally, in accordance with previous observations by Eigenbrod and colleagues, murine BM-MΦ failed to synthesize detectable levels of IL-6 in response to LPS treatment [612] highlighting further the fact that DC and macrophages respond differently with regards to cytokine production to the same bacterial ligands and danger signals.

5.3.2 The effect of LPS signalling in P2X$_7$R splice variant expression.

In an attempt to decipher the underlying mechanisms responsible for the distinct cytokine responses observed in DC and macrophage populations, these investigations examined the presence of functional P2X$_7$R splice variants. Relatively recent reports for the presence of functional P2X$_7$R splice isoforms in mice that exhibit gain-of-function properties, the expression of the three different splice variants was examined in untreated and LPS-activated BM-DC and BM-MΦ [355]. This study provides evidence for the expression of P2X7A, P2X7J and P2X7K transcripts in murine BM-DC, BM-MΦ and PMΦ. The expression of P2X7J and P2X7K transcripts has not been previously described in murine BM-DC and BM-MΦ. The full P2X$_7$R variant (P2X7A) bears an excessively long intracellular C-terminus that is unique among the members of the P2X family and is thought to house multiple interaction motifs with intracellular signalling cascades. Importantly, the C-terminal region has been shown to regulate pore formation and K^+ efflux. Not all splice variant isoforms bear the full length of the C-terminal region and therefore their ability to mediate pore formation is compromised [352, 354]. Furthermore, Denlinger and colleagues demonstrate that the LPS binding motifs situated in the C-terminal region of the receptor are functionally associated with signalling the recruitment of the P2X$_7$R channels to the cell surface membrane in HEK-293 cells [322, 323]. It was therefore postulated that LPS signalling, has the ability to manipulate transcriptional expression of splice variant isoforms in order to regulate P2X$_7$R function including cytokine release.

The P2X$_7$R oligomerizes into functional stable trimeric channels at the cell surface membrane [307]. It is suggested, at least in primary tissue and possibly due to its structural diversity, that the P2X$_7$R cannot form functional trimers channels with other

P2X receptors [623]. The composition of these trimers has a consequent effect on the pharmacokinetic properties of the receptor. For example, in mouse endothelial cancer cells, the co-assembly of P2X7J isoforms (a truncated non-functional variant) with P2X7A (the full functional variant) has a dominant negative impact on the ability of the receptor to mediate cytolysis [356]. This has prompted the following questions: (a) whether specific intracellular signals, such as LPS-derived signals, can affect P2X$_7$R gene transcript expression and (b) whether these can enhance for example, the transcription of loss-of-function variants (such as P2X7H or P2X7J) to prevent caspase-mediated apoptosis, or gain-of-function variants (P2X7K) to promote cell death of, for instance, over-reactive T-cells cells [355, 578]. As previously mentioned, the functional P2X7K is also known as the 'death receptor isoform' because it exhibits enhanced functional properties with slower desensitization rates and enhanced levels of dye uptake in transfected HEK-293 cells. [355]. The splice variant bears an alternative exon 1 and therefore escapes inactivation in P2X$_7$R KO mice (Glaxo strain) [624]. Additionally, splice variants were also identified in the second strain of P2X$_7$R KO mice, known as the Pfizer strain, which received a Neomycin cassette insertion at exon 13 to disrupt expression of the receptor. Masin and colleagues identified functional splice variant isoforms with an alternative exon 13 that escape inactivation in this strain of KO mice (P2X713a-13c) [356]. Finally, another important splice variant is the P2X7J, which is a non-functional isoform truncated at exon 7 onwards and has been shown to form heterotrimers with the full functional variant (P2X7A) and down-regulate its function [354]. High levels of P2X7J are found expressed in cervical cancer cells, a strategy employed by these cells to escape P2X$_7$R-induced apoptosis [354].

Having detected differential P2X$_7$R-associated cytokine responses in murine BM-DC and BM-MΦ, it was next hypothesized that TLR ligands such as LPS can impact on the expression of P2X$_7$R splice isoform transcripts thereby regulating receptor function including cytokine release. This study has therefore examined LPS-induced changes in the expression of the full-functional variant (P2X7A), a truncated, loss-of-function variant (P2X7J) and a gain-of-function isoform (P2X7K) in murine BM-DC and BM-MΦ populations. The expression of P2X713b and P2X7B isoforms was also examined (data not shown) although neither BM-DC not BM-MΦ were found to express detectable levels of isoform transcripts.

To date, the process of $P2X_7R$-induced pore formation and whether this facilitates cytokine release remains obscure. With regards to how the pore itself is formed, one hypothesis suggests that existing $P2X_7R$ trimers undergo conformational changes to allow further dilatation of the existing channel pore to increase its diameter forming a larger pore. This was recently demonstrated using site mutagenesis directed at the channel structure showing that the $P2X_7R$ has the capacity to enlarge the channel pore allowing the passage of nanometer-sized particles, such as YO-PRO molecules, in transfected HEK-293 cells [543]. An alternative hypothesis suggests that the receptor quite simply employs existing cell surface hemichannels to facilitate pore functions [307, 548].

More importantly, it is widely acknowledged amongst researchers in the field that the C-terminal region of the $P2X_7R$ has a key role in pore formation [378]. Based on their observations, Pelegrin and Surprenant suggest that in murine macrophages pore formation is mediated by pannexin-1, which is also thought to facilitate IL-1β release [379, 239]. Their studies concluded that the $P2X_7R$ mediates rapid K^+ efflux, which is necessary for pannexin-1 activation and subsequent formation of the pore in murine macrophages. Pannexin-1 is also thought to be involved in inflammasome activation but currently there are no substantial evidence to demonstrate a direct link between the two components [625, 239]. The inflammasome is known to be rigorously activated by DAMP signals, such as ATP, which induce rapid IL-1β synthesis and release from cells [604]. Based on the results of these investigations, substantial levels of cytokine were released following treatment with ATP for 20 minutes alone. These were found to be significantly higher in BM-DC.

Interestingly, on one hand data from previous research indicate that IL-1β processing and release in macrophages derived from individuals carrying the *Glu496Ala* loss-of-function $P2X_7R$ short nucleotide polymorphism, is suppressed (78% lower to healthy individuals) in response to ATP [609]. On the other hand, a more recent study performed on an *ex vivo* whole-blood model reports enhanced IL-1β responses in LPS-activated plasma derived from individuals with the *Glu496Ala* polymorphism in comparison to the plasma derived from healthy donors. The ATP levels were comparable between the two groups and the group postulates that leukocyte cells carrying the *Glu496Ala* $P2X_7R$ polymorphism are more resistant to ATP-induced cell

death [626]. Although the second study did not examine specific leukocyte populations, one possible explanation would be that the particular polymorphism has a different impact on specific white blood cell subpopulations, which results in distinct cytokine responses.

In a similar manner it was postulated that LPS signalling could enhance the transcription of loss-of-function P2X$_7$R transcripts in murine BM-DC, protecting the cells from ATP-mediated cell death and potentiating IL-1β release. Conversely, gain-of-function P2X$_7$R isoforms transcripts (P2X7K) would be expected to increase in expression in murine BM-MΦ potentiating pore formation and ATP-mediated cytolysis, thereby suppressing IL-1β responses. However, the observations of this study revealed that expression levels for the different splice variant isoforms of interest followed similar fluctuating patterns in both cell types with LPS treatment. In brief, the expression of the full-functional variant P2X7A and loss-of-function variant P2X7J transcripts was suppressed whilst those of the gain-of-function P2X7K variant remained stationary. At this stage it was not possible to demonstrate whether LPS impacts on the protein expression of P2X$_7$R splice isoform and it is possible that LPS may have an impact on the formation of the functional P2X$_7$R trimer, or that the constitutive levels of expression the various isoforms differ between the two cell types. Future advancements of P2X$_7$R specific tools that can target specific splice variants of the P2X$_7$R will help decipher their physiological contribution to P2X$_7$R function. The idea that the different functions of this peculiar P2X receptor can be regulated in this manner is still an attractive hypothesis with potential future therapeutic applications.

Taken collectively, the results of these investigations with previous observations from studies using cells derived from individuals carrying the *Glu496Ala* P2X$_7$R polymorphism hint towards the fact that IL-1β release may be regulated differently in macrophages and DC. To elaborate, the observations of these studies reveal that treatment of murine BM-MΦ with LPS (a) results in comparatively lower levels of IL-1β and no IL-1α release following challenge with ATP and (b) in the suppression of P2X7A transcript expression. In addition, IL-1β release has been previously associated with pore formation and pannexin-1 activation in murine macrophages [239]. Similarly, human macrophages derived from individuals with the *Glu496Ala* P2X$_7$R polymorphism which is associated with a poor pore forming capacity, exhibit impaired

IL-1β responses [609]. Conversely, murine BM-DC employed herein reveal enhanced IL-1β responses to treatment with LPS whilst our data suggest that the expression of the various receptor isoforms follow the same pattern to BM-MΦ at the transcriptional level. It may simply be that unlike with macrophages, IL-1β release in DC is P2X$_7$R and K^+ efflux-dependent but IL-1β molecules do not egress through the pore.

Being the most efficient APC of the immune system [17] DC are undoubtedly a skilful army of sentinels, evolved to regulate the adaptive arm of the immune response. They are strategically positioned at sites of invading pathogens, such as the epidermal layers of the skin. Such microenvironments are sites of regular tissue damage, prone to invasion by pathogenic entities and are therefore often characterized by high levels of ATP [22]. DC are thought to have evolved from the primitive macrophage during the development of the adaptive arm of the mammalian immune system. Thus, DC might therefore have unwillingly inherited the P2X$_7$R signalling system as an efficient mechanism to activate the inflammasome, and not only, but at the same time were required to evolve sophisticated mechanisms to counteract ATP-induced, pore-mediated, cytolysis and develop novel mechanisms to mediate rapid pro-inflammatory cytokine release. In contrast, macrophages perhaps functionally require ATP-induced cytolysis to release pro-inflammatory cytokines along with pathogens to alert the immune system and potentiate immune responses during persisting infections. Previous published reports suggest that TLR ligands (such as LPS) can enhance the up-regulation of Fas receptors in macrophages rendering them more susceptible to apoptosis via Fas-mediated pathways [627]. Additionally, macrophages derived from human subjects homozygous for a loss-of-function P2X$_7$R polymorphism (1513C-allele polymorphism) reveal that cells show more resistance to ATP-induced cytolysis but fail to mediate efficient destruction of intracellular mycobacteria [371]. Thus, whilst in murine macrophages IL-1β release may rely on ATP-induced pore formation and cell death, in DC a more sophisticated approach is required for cytokine release that is still dependent on P2X$_7$R activation but independent of enhanced pore formation.

5.3.3 LPS constitutes a sufficient signal for IL-1β release in BM-DC

The ability of LPS to signal both IL-1β production and release in murine BM-DC but not in murine macrophage populations (BM-MΦ and PMΦ) as shown in chapter 4, section 4.2.7 and also previously observed by He and colleagues is beginning to attract more attention [561]. LPS is traditionally considered as a relatively inefficient signal to induce cytokine release but along with other fungal stimuli, the TLR ligand has been shown to mediate both IL-1β production and inflammasome activation in BM-DC or thioglycollate-elicited PMΦ, in agreement with the observations of these studies [628]. In contrast, BM-MΦ require a two-step signal for inducing pro-inflammatory cytokine release in response to bacterial or fungal ligands [629]. The findings reported above together with the preliminary observations of these studies prompted us to further question the role of P2X$_7$R activation as well as ATP signalling in LPS-mediated cytokine responses in murine BM-DC. In a later attempt to try and replicate this phenomenon in order to investigate the role of P2X$_7$R signalling LPS-induced cytokine responses in BM-DC, these earlier observations were difficult to replicate. LPS signalling no longer induced IL-1β release from BM-DC in the absence of exogenous ATP. BM-DC were challenged with higher LPS load and the pattern of IL-1β expression was assessed over the 24h period to provide possible explanations for the changes in the cytokine profile observed, however, with no real success. Phenotypic analysis of the cells showed that responsive and nonresponsive cells differed with regards to the levels of MHC class II, and CD11c expression, with cells that exhibited a more mature phenotype being nonresponsive. Lutz and colleagues provide a thorough analysis of the different possible variables that can affect the *in vitro* generation of BM-DC. The authors concluded that (a) the 'quality' of FCS (based on the levels of LDH and GOT content) and (b) GM-CSF are the most important factors that affect the differentiation of BM precursors to the DC lineage. High levels of LDH and GOT in FCS can act as bystander antigens and trigger spontaneous maturation of BM-DC whilst in culture. BM-DC can then become insensitive to further maturation by other ligands. Careful consideration was taken with regards to the 'quality' of the FCS employed, and several different variables that might have affected the quality of BM-DC culture, including the age of donor mice, the concentration of LPS challenge, and the seeding concentration of BM cells, have all been examined, but with no real success in identifying a responder phenotype (data not shown) [447]. The preliminary results

invite further work to elucidate the role of ATP signalling and P2X$_7$R activation in the LPS-mediated cytokine responses observed in murine BM-DC.

5.4 Conclusion.

With their multitude of functions, and their efficient antigen presenting capacity DC and their various subsets are an integral and unmistakably invaluable tool for the immune system. They are a primary source of pro-inflammatory cytokines such as IL-1 along with other signals that allow the host to establish immunity against pathogenic microorganisms. Certainly DC will continue to offer an attractive target for clinical therapy. The fact is, however, that we are only beginning to appreciate the extraordinary life of these stellate cells, how they can switch the immune system on/off, or how they promote inflammation but at the same time mediate tolerance. The results of these studies demonstrate that DC display different magnitudes of cytokine release, particularly IL-1, than other cell types. The mechanisms by which IL-1 molecules are externalized from cells remains a matter of controversy with several models proposed explaining how IL-1β release from different cell types. It would be foolish to think that there is only one route for the secretion of such a potent cytokine whose upstream expression and processing are so tightly-regulated and that this is common between different cell types. The ATP-P2X$_7$R offers an efficient platform for the processing and release of IL-1β and perhaps its role in IL-1β release is not central for all types of immune responses. Compensatory mechanisms for IL-1β release are present, functional and as expected numerous. However, evidence suggests that in certain inflammatory conditions such as the sensitization phase of contact hypersensitivity the P2X$_7$R holds 'centre stage' in mediating IL-1β responses [289] and since IL-1β is essential for development of contact allergy [11] the significance of the efforts in trying improve our understanding of P2X$_7$R-mediated IL-1β responses in murine DC, is further emphasized. Learning to manipulate important functions such as the release of potent pro-inflammatory cytokines will be an invaluable tool in redirecting immune responses for effective therapy, for example, to alleviate the symptoms of allergy.

Chapter 6: General Discussion.

6. General Discussion.

Continuously emerging evidence highlights the important role of ATP signalling and $P2X_7R$ function in health and disease. The $P2X_7R$ has been shown to have an important role in a number of inflammatory conditions including Alzheimer's disease, arthritis, granuloma formation and even cancer [630-632] therefore constituting an attractive target for therapy. The receptor is associated with a long list of diverse functions whose underlying molecular mechanisms are not well understood [633]. This study set out to improve our understanding of $P2X_7R$ function in DC, which acquire 'centre stage' during the development of many of the aforementioned clinical conditions. It was also particularly important to concurrently compare $P2X_7R$-driven responses with those observed in macrophages. One of the most extensively studied functions of the receptor is its contribution to inflammasome activation, IL-1β processing and release [239]. Therefore these investigations have primarily focused in characterizing $P2X_7R$-associated IL-1β responses in murine BM-DC using an *in vitro* culture system.

Identified first among cytokines, IL-1β [1] has since attracted considerable interest in deciphering the molecular mechanisms that govern its expression, processing and release from cells. Although the role of the $P2X_7R$ in IL-1β release has been well documented in mouse macrophages [601, 12, 266], it has only just begun to be explored in mouse DC. IL-1β production and release is necessarily a complex and tightly regulated [634]. *In vitro* observations in macrophages, reveal that IL-1β synthesis and release is a two-step process. TLR signalling induces expression of pro-IL-1β molecules and a second stimulus, such as ATP, is required to mediate efficient processing and release of the bioactive IL-1β molecule in the extracellular milieu [248, 231, 265, 404]. The requirement for a second stimulus is generally considered as an additional safeguard mechanism to ensure that IL-1β molecules are externalised only under conditions of pathogen infection or injury. In the absence of a secondary signal IL-1β cannot be processed and fails to be released from macrophages [635]. In contrast, as shown by the results of these investigations LPS activation alone is a sufficient to signal substantial amounts of IL-6 release in the extracellular milieu and this is largely due to differences in the mechanisms of release of the two cytokines. Although a rapid release of IL-1β at large magnitudes requires 2 signals, there is evidence to suggest (in accordance to preliminary observations of these investigations) [628, 561, 636] that in DC populations, TLR signalling alone has the capacity to signal IL-1β release in the

absence of the second stimulus. Members of the IL-1 family including IL-1α, IL-1β and IL-18, unlike most proteins including IL-6, do not follow the classical pathway of secretion. The evolution of this 'leaderless', non-classical secretion pathway is not entirely understood but it is postulated that it has evolved from a safeguard mechanism whose function was to quickly rid of toxic or missfolded proteins to prevent collapse of cellular homeostasis [637, 638].

Several mechanisms have been proposed for the secretion of IL-1β [639] including microvesicles, secretory vesicles, exosomes and even a non-vesicular pathway. It is likely though that the route of choice will depend on the type of cell, its maturation status, as well as the type and duration of stimulus [640, 265, 641]. Evidence indicates that the release of both IL-1β and IL-18 from mature DC is triggered by T-cell or NK-derived signals (including ATP) within the immunological synapse in an autocrine fashion. Morphological analysis demonstrates the interaction of mature DC with antigen-specific T-cells, which triggers the cytosolic migration of IL-1β and IL-18-containing lysosomes in DC, towards the point of contact (immunological synapse) [642, 643]. Evidence such as this illustrates that the mode of IL-1β release is cell type-specific suited to their specific function. Whereas monocytes adopt a more rigorous approach (exocytosis) to release inflammatory cytokines in bulk and these spread within the microenvironment, for particular functions, DC require a more localized approach (exosome-driven release) to specifically target the cell of contact [644].

Nevertheless, DC are a widely heterogeneous population with numerous subsets that differ in localization, functional properties and phenotype (reviewed in [54]). Perhaps all DC subsets may have the various non-classical mechanisms to secrete members of the IL-1 family at their disposal, however, the route of choice for cytokine secretion will most likely depend on factors such as the maturation stage of the cells but also the strength, type and duration of the stimulus, which will determine the volume, rate and direction of cytokine release. It is in fact foolish to think that all the possible routes for IL-1β secretion are mutually exclusive in a particular cell type. It is more likely that different mechanisms can be employed by the same cell type and quite even possibly that more than one mechanism are activated at the same time. For instance, release of IL-1β via microvesicles may be the route of choice following an extensive, but brief, traumatic tissue injury characterised by high levels of ATP [645]. Alternatively, the

apoptosis of neighbouring cells will provide a lower in strength but sustained ATP signal that will evoke cytokine release via the externalization of smaller exosomes [646]. Additionally, a DC subset in the presence of accumulating levels of ATP will irreversibly activate $P2X_7R$-induced pores and even pyroptosis-induced pores, which will eventually act as the gateway for cytokine release overtaking any other form of vesicular secretion.

Many questions remain unanswered with regards to how IL-1β is processed and released. For example, the molecular mechanism(s) that triggers IL-1β release following cytokine processing by the inflammasome remain elusive, as does the relative contribution of different inflammasome platforms in processing IL-1β. It is postulated that caspase-1 itself has an important role in signalling the export of processed IL-1β molecules from the cell by interacting with key proteins that are implicated in the non-classical pathway of protein secretion, however, there is considerable work to be done to decipher how such interactions determine how the cytokine will be released from the cell [647]. Additionally, the mechanism by which IL-1α is released from the cells is also unclear. An important difference between IL-1β and IL-1α is that the latter does not require proteolytic cleavage for it to be biologically active [648, 612] and the precursor protein is actively retained intracellular to mediate its additional nuclear functions [649, 650].

The general consensus is that IL-1α is passively released following cell lysis, most probably through pyroptosis-induced pores. Interestingly, a number of recent reports suggest that IL-1α is actively released from stressed but not necrotic cells and it is therefore postulated that since both cytokines bind the same receptor, IL-1α could 'hijack' the IL-1β route of exocytosis to potentiate the effect of IL-1β signalling, when required [445, 196]. Based on the observations of these investigations, the characteristics of the IL-1α responses were strikingly different between the two cell types of interest. The absence of substantial levels of IL-1α release in BM-MΦ suggests that the route of passive diffusion as a mode of IL-1α is secretion cannot be the only one. Although a significant drop in cell viability is observed in both cell types following challenges with ATP, IL-1α was only detected in supernatans of BM-DC. If IL-1α is simply passively released following cell lysis or through pyroptotic-induced pores, a lot more IL-1α would have been detected in supernatants of BM-MΦ. Additionally, ATP

induced a dose dependent reduction of intracellular levels in BM-MΦ, suggesting that the cytokine is perhaps actively directed to degrative lysosomes. Observations such as these are reminiscent of an M2 macrophage profile which will be discussed more extensively below. Thus, in lack of the right co-stimulatory signals (IFN-γ), which are known to shift macrophages towards a more pro-inflammatory phenotype, a well-orchestrated suppressive mechanism overrides other signals (such as LPS and ATP) that dictate the cell to express and release pro-inflammatory cytokines.

One important route for IL-1β and IL-1α secretion is through pyroptotic-induced pores. Pyroptosis is often confused with apoptosis as several of their features often overlap. Currently, pyroptosis has only been identified in DC and macrophages [284, 651]. The word pyroptosis derives from the Greek word *pyro (πυρά),* meaning fire and *ptosis (πτώσις),* meaning 'falling off', thereby describing the release of 'fire' (pyrogenic IL-1β molecules) from 'falling' (dying) cells. Pyroptosis is dependent on caspase-1 activation alone but apoptosis can still proceed in the absence of caspase-1 activation, for example in caspase-1 KO mice [243]. Additionally, certain proteins that are traditionally cleaved during apoptosis remain intact during pyroptosis and cytochrome *c* release from mitochondria a hallmark event of apoptosis does not occur during pyroptosis [652, 653]. What remains unclear is whether the $P2X_7R$-induced pore contributes to pyroptosis and IL-1β release. The $P2X_7R$-induced pore is known to facilitate rapid K^+ efflux that is required for caspase-1 activation [241, 574, 654] and caspase-1 activation is essential for pyroptosis-driven membrane pore formation.

The distinction between apoptosis and pyroptosis is not entirely clear and there is evidence to suggest that caspase-1 activation induces downstream activation of apoptotic caspases such as capsase-7 [655]. These investigations have not specifically examined the role of pyroptosis in IL-1β release but clues in the results provided herein suggest that pyroptosis might also be responsible for IL-1β release. Both DC and macrophage populations were stained with cell viability dyes that are thought to enter pyroptosis-induced pores interact with nuclear DNA and stain the cells positive [656, 284]. Although further morphological analysis is required to determine with certainty to which extent does pyroptosis contribute to cytokine release and whether this is the same between the two cell types, it was clear that cytokine release was closely associated with loss in the viability of the cells.

The relevance of such a mechanism *in vivo* was at first difficult to grasp. It seemed illogical that a cell would commit suicide simply to release inflammatory molecules, such as IL-1, particularly since there are numerous other mechanisms at its disposal. It later became apparent that this mechanism evolved to restrict intracellular pathogen growth; the cells facilitate rapid secretion of live bacteria through pyroptotic-induced pores thereby enhancing pathogen recognition and perpetuating innate and adaptive immune responses. In doing so, they limit pathogen replication and improve the chances for host survival. For instance, the *S. typhimurium* intracellular bacteria evade detection by the inflammasome by suppressing flagellin expression. Using an engineered *S. typhimurium* flagelling-expressing strain, Miao and colleagues demonstrate that mouse macrophages excrete live bacteria through pyroptosis to perpetuate their recognition and their killing by neutrophils [250]. Pyroptosis is mostly associated with macrophages, which are longer-lived cells and with less microbicidal activity (in comparison to neutrophils) and are therefore perhaps selectively targeted by bacteria. Pyroptosis has also been demonstrated in salmonella-infected DC [657], however, whether intracellular bacteria actively seek to infect macrophages or actively avoid DC subsets is not yet known. This will most likely depend on the site of infection and the available subsets of DC and macrophages. The same applies to the mechanism of IL-1β release selected by a particular cell type. For example, in the presence of a persistent intracellular bacterial infection DC would initiate pyroptosis to promote cytokine release and clearance of the pathogen. Alternatively, when DC target T-cells for clonal expansion a more directed approach would be appropriate; for example exosome-driven cytokine release.

An integral component of the IL-1β processing and secretion machinery is the inflammasome and it is anticipated that distinct regulatory mechanisms are at play between the two cell types regulating its activation. Inflammasomes are an expanding family of diverse and complex structures situated in the cytosol, able to sense a variety of structurally diverse PAMP and DAMP signals. [635]. They consist of various components situated in the cytosol whose oligomerization is triggered by signals that have been loosely grouped into three main pathways: the ATP-P2X$_7$R-, ROS- or lysosomal-derived signals. Certainly, the ATP-P2X$_7$R axis constitutes a robust and efficient route for inflammasome activation, IL-1β processing [230]. Evidence suggests that inflammasome-driven cytokine release persists in the absence of the ATP signalling

axis. For example, the pore forming, bacterial toxin and potassium ionophore nigericin is also able to disturb the intracellular ion balance and effectively activate the inflammasome resulting in IL-1β release from mouse or human macrophages [241, 658-660]. The physiological relevance of the ATP-$P2X_7R$ axis in mediating IL-1β release, *in vivo,* is therefore often questioned, mainly due to the particularly high levels of ATP that are required to activate the $P2X_7R$ in *in vitro* studies. However, taking into consideration the total amount of ATP used by the human body on a daily basis (50-75kg/per day) and the fact that at maximum conditions, a single mitochondrium is said to produce 600 ATP molecules per second, the possibility that ATP can reach the mM range within inflamed tissues is very plausible [661].

Many studies investigating the physiological relevance of the $P2X_7R$ *in vivo* often employ the various strains of $P2X_7R$ KO mice that are available and their results often exclude a role of the $P2X_7R$ axis in cytokine release [561]. However, in light of recent evidence that demonstrate the presence of functional splice variants in various cell types of $P2X_7R$ KO mice, particular caution is required when interpreting the results obtained from such studies, particularly [355]. Furthermore, both the human and mouse $P2RX_7$ genes house several non-synonymous-single nucleotide polymorphisms which give rise to several newly identified variants that are structurally and thereby functionally diverse (reviewed by [351]. Whether P2X7K, or indeed other $P2X_7R$ splice variants ($P2X_7R13b$), are able to compensate for the absence of the P2X7A in KO mice, is being investigated currently [356]. In fact, careful analysis of the phenotypic characteristics of the various $P2X_7R$ KO models suggests that the P2X7K is indeed able to compensate for the lack of the full P2X7A variant. For example, whereas the Pfizer KO mice develop skeletal abnormalities due to the absence of the $P2X_7R$ [662], the same is not observed in the Glaxo KO mice, which harbour the P2X7K variant. The Glaxo mice exhibit a normal bone phenotype and it is therefore suggested that the P2X7K is able to fulfil the requirements of $P2X_7R$ signalling in bone development [663].

Evidence of new splice variants of the receptor in mice are continuing to emerge [356, 664] together with the results of these investigations, which demonstrate the presence of the truncated P2X7J and gain-of-function P2X7K in murine BM-DC and BM-MΦ at the level of transcription. Despite these limitations, the $P2X_7R$ KO mice have constituted a useful tool in improving our understanding of the physiological function of the receptor

over the years and were critical in identifying functional splice variant isoforms of the receptor. Perhaps at present these KO mice models are not the most appropriate tool for investigating $P2X_7R$-mediated responses and should at least be used in conjunction with other available tools, such as $P2X_7R$-specific inhibitors [665]. It is also important to note that the $P2X_7R$ is only one member of a large family of purinergic P2X receptors which are thought to engage in cross-talk in order to mediate their functions and can arguably compensate for the loss of other members of the P2X family. For example, in mouse lung epithelial cells Weinhold and colleagues showed that the depletion of $P2X_7R$ results in the upregulation of $P2X_4R$ complexes as a compensatory mechanism [666].

What is clear is that activation of the $P2X_7R$ results in the opening of an intrinsic channel as well as a mediated pore leading to a rapid distortion of the cytosolic ion balance ($Ca2^+$ influx and K^+ efflux). The depletion of K^+ ions is believed to act as a surrogate danger signal that activates inflammasome complexes [386]. The molecular mechanisms by which the $P2X_7R$ communicates with the inflammasome platform are currently unknown. Interestingly, a link between inflammasome activation, autophagy and IL-1β release is beginning to emerge. Autophagy essentially provides a cleaning service for the eukaryotic cell effectively directing damaged organelles, misfolded proteins and DNA, which are potent activators of the inflammasome, towards degradation [667, 668]. It is therefore implicated in innate immune responses usually with an anti-inflammatory role limiting the presence of inflammasome activators. An alternative hypothesis suggests that autophagy could enhance IL-1β release; TLR ligation drives autophagosomes to engulf pro-IL-1β and pro-caspase-1 molecules that are subsequently either signalled to fuse with lysosomes or with the cell surface membrane, depending on the strength and duration of the signal, thereby regulating the IL-1β release [637, 669].

Whether upstream signalling cascades can intentionally regulate autophagal processes to skew immune responses towards a desired outcome is still hypothetical rather than proven. A recent study by Misawa and colleagues demonstrated how microtubules regulate mitochondrial transportation within the cytosol to facilitate spatial proximity of the different components of the inflammasome in murine BM-MΦ. Their results suggest that following treatment of BM-MΦ with various inducers of the inflammasome (e.g.

nigericin, ATP and silica crystals) the ASC components of the inflammasome, associated with the mitochondria, are shuffled along with the mitochondria towards the endoplasmic reticulum at the perinuclear region of the cytosol, where NLRP3 components of the inflammasome can be found [670]. Interestingly, the $P2X_7R$ intracellular complex has previously been associated with modulating cytoskeletal rearrangements [321], however, whether this mechanism forms the basis by which the $P2X_7R$ induces its activation is a question that needs to be addressed.

One of the core findings of these investigations was that macrophages and DC evoke distinct cytokine responses to the same TLR ligand and DAMP signals. Both the magnitude of cytokine release and the dose-response characteristics, varied between murine BM-DC in comparison with BM-MΦ. These results suggest that the machinery responsible for inflammasome activation and IL-1β release, but also IL-1α, is perhaps more 'at the ready' in BM-DC than in macrophages In contrast to macrophages, it appears that DC are highly responsive with regards to cytokine release under high levels of ATP stimulation. Brief exposure to high levels of ATP, conditions that mimic a traumatic tissue injury, should drive a massive influx of Ca^{2+} through $P2X_7R$ activation in both cell types. Perturbations in the intracellular ion balance have a number of downstream effects including inflammasome activation and pro-inflammatory cytokine release [645]. These comparatively 'suppressed' cytokine responses observed in macrophages, in some way, emphasize that DC are perhaps better to sense changes in the microenvironment and more prepared to alert the immune system with the release of pro-inflammatory cytokines and promote the infiltration of immune cells specialized in pathogen clearance (e.g. NK cells) and tissue repair. Even though macrophages are in theory capable of alerting the adaptive arm of the immune response and also secrete pro-inflammatory cytokines, DC are faster and more efficient, they are after all the driving force of the immune response both at activating and suppressing responses. It was remarkable to find that even at particularly high levels of ATP, DC remained responsive conditions, which for other cells would be toxic. Although this merits further investigation it is most likely that the DC cell surface is decorated with enzymes that can effectively shape the 'purinergic halo' (ATP:ADP:adenosine ratio) to favour pro-inflammatory responses. It is also likely that different patterns of ATPases and other enzymes decorate the cell surface of macrophages and that will differ between different macrophage subsets.

The importance of purinergic signalling in inflammation has been gaining momentum over the years. ATP is one of the oldest transmitters and constitutes a potent and more importantly a 'convenient' signalling molecule due to its abundant availability in cytosolic stores. The availability of ATP in the extracellular milieu is a tightly regulated process as it is subjected to ecto-nucleoside triphosphate diphosphorylases (NTPDases including CD39) and 5'-ectonucleotidase (CD73), which hydrolyse the phosphate groups of ATP molecules to generate ADP and subsequently adenosine molecules [671]. Adenosine molecules subsequently signal through P1 cell surface G-coupled receptors to modulate cell function [672]. Whereas ATP signals to enhance inflammatory responses, adenosine has been shown to counteract inflammatory responses, suppressing the production of TNF-α IL-6 and IL-12, and at the same time enhancing that of anti-inflammatory agents, such as IL-10, in LPS-induced macrophages [673-675]. Although the exact intracellular pathway by which adenosine mediates its effects is not entirely clear, it is postulated that the molecule acts on a central transcription factor such as NF-κB to down-regulate cytokine production in macrophages in response to a variety of TLR signals (TLR2, 3, 4, 7 and 9) [676]. Additionally, the bioavailability of adenosine at receptor sites does not simply rely on the hydrolysis of ATP molecules by NTDPases but also on the activity of (a) adenosine kinase, that ensures that ADP/AMP does not re-phosphorylate to ATP [298], and (b) that of adenosine deaminase that concomitantly facilitates further degradation of adenosine to a more stable product, uric acid [677]. Thus, the expression or availability of all these enzymes in a particular cell type will determine the fine balance between ATP and ADP and adenosine molecules which will impact the cytokine profile expressed by that cell type. For example, high ATPase and adenosine kinase and suppressed adenosine deaminase activity in DC or macrophages at sites of high ATP concentrations will actively suppress the maturation of that cell type towards a pro-inflammatory profile.

Macrophages are a heterogeneous population of cells associated with a wide variety of functions and are broadly classified into two main profiles [678]; the inflammatory M1 macrophages that promote Th1 responses and express a particular profile of inflammatory cytokines including TNF-α and IL-1β and the immunosuppressive M2 macrophages that are involved with wound healing and tissue remodelling. M2 macrophages are activated by IL-4 and secrete high levels of IL-10 [679, 142].

Nevertheless, it is expected that macrophages exhibit plasticity and can transdifferentiate between different phenotypes with the progression of inflammatory responses. Each macrophage profile is associated with a particular set of genes whose upregulation direct macrophages towards a particular profile. For example, TLR2, LY64 (lymphocyte antigen 64, a TLR4 associated transmembrane glycoprotein) and CD86 are associated with the M1 macrophage profile whilst genes such as arginase 1, IL-1RA and vascular endothelial factor (VEGF) direct macrophages towards the M2 profile [680]. The gene expression profile of the BM-MΦ employed by these investigations was not examined and it is unknown whether the BM-MΦ in culture were polarized towards one or the other profile particularly since the cells did not receive any cytokines that are involved in macrophage polarization. It would be interesting to examine whether differentiating BM precursors in the presence of L-cell supernatant nurtures the development of M1 or M2 macrophages. This would perhaps offer explanations for the 'dampened' cytokine profile observed by LPS-primed BM-MΦ employed herein [678]. Additionally, it would also be interesting to examine the effect macrophage polarization on the ATP-$P2X_7R$ driven IL-1 cytokine responses, particularly how do M2 macrophages dampen IL-1 responses. Adenosine is a broad inhibitor of the classical activation of M1 macrophages actively suppressing the expression of pro-inflammatory cytokines. At the same time, adenosine promotes the expression of anti-inflammatory agents in macrophages to regulate excessive inflammation and protect host tissues during immune responses [681, 675]. In fact, the nucleoside has recently been associated with promoting alternative activation of M2 macrophages *in vitro* by up-regulating the expression of key transcription factors that are associated with this macrophage profile (e.g. arginase-1) [682].

Although certain functional aspects of DC overlap with those of macrophages, they express an unparalleled ability to engage the adaptive arm in the presence of harmful pathogenic entities. DC have the unique ability to generate a variety of antigenic ligands for presentation to T-cells and thereafter manipulate T-cell responses. Apart from stimulating T-cell clonal expansion in the presence of pathogenic stimuli, DC have been observed to interact with T-cells in the absence of pathogen. It was soon realised that DC are key players in maintaining 'quiescence' (deletion or differentiation to T-regulatory cells) or 'silencing' to non-harmful 'self-antigens' during the steady-state (peripheral tolerance), albeit a specific DC subset specialised to mediate tolerance has

not yet been dedicated [683, 684]. In addition, they dictate the quality of the humoral response and are able to cross-present antigen to give rise to skilled cytotoxic $CD8^+$ killers. It is clear that DC hold the 'steering wheel' that drives immune responses. With their wide variety of functions and their abundance at different body surfaces, DC exhibit, a remarkable, but much required heterogeneity. As previously discussed, DC are largely classified into the cDC, the pDC that are more involved with viral infections and possibly tolerance as well as the LC, the specialised sentinels of the epidermis [5]. A particular tissue microenvironment is home to multiple DC subsets that exhibit distinct specialised functions. For example, in the mouse spleen DEC-205^+ DC are particularly efficient in cross-presenting antigen via MHC class I whilst C-type lectin domain family 4, member A4 $(CLEC4A4)^+$ DC are more efficient MHC class II presenters. A new hypothesis, however, comes to challenge the traditional dogma that DC universally detect pathogens in peripheral tissues and subsequently undergo maturation and migrate to draining lymph nodes to activate T-cells. It is now proposed that whilst a particular subset of DC specializes in detecting antigens (detector DC), a separate DC subset ('presentor' DC) specialises in migrating to present antigen to T-cells [685].

There is evidence to suggest that the activation and maturation of DC can be affected by the immediate bioavailability of molecules, such as ATP, that surround the APC forming a particular 'purinergic halo'. Under conditions of stress, the concentration of molecules purinergic molecules changes within the microenvironment and their bioavailability is tightly regulated by the activity of the various different enzymes that decorate the DC cell surface [686, 687]. Thus, the shape of the 'purinergic halo' will direct the immune response accordingly. The effects of adenosine signalling in DC are currently understudied but evidence suggests that BM-DC possess strong adenosine deaminase activity [688]. Discrepancies in the mechanism that regulate the bioavailability of ATP and adenosine offer an attractive model of how macrophages can remain 'quiescent' during tissue remodelling and how DC can quickly overcome the suppressive effects of adenosine. For example, in the absence of potent co-stimulatory signals, such as IFN-γ, that facilitate maturation of LPS-challenged macrophages towards an M1 inflammatory profile, ATP molecules are quickly dephosphorylated to adenosine which actively suppress P2X pro-inflammatory signalling and promote immunosuppressive P1 signalling. This could perhaps explain the 'dampened' immune

responses (lower levels of IL-1β and absence of IL-1α release) observed in BM-MΦ employed herein. In contrast, in the presence of the appropriate TLR ligands, DC expressing and even releasing high levels of adenosine deaminase, are perhaps more efficient in preventing ATP degradation. The overall balance of all these enzymes might shift when DC acquire a more regulatory role at the later stages of inflammation to dampen T-cell mediated responses. Mechanisms such as these will have a direct impact on P2X signalling, inflammasome activation, and therefore pro-inflammatory cytokine release. Extracellular nucleotide signalling merits further investigation in understanding how the different enzymes that regulate purinergic signalling, such as adenosine deaminase, NTPDase and ectonucleotidase (CD79), can alter immune responses in different cell types (see figure 6.1) [689].

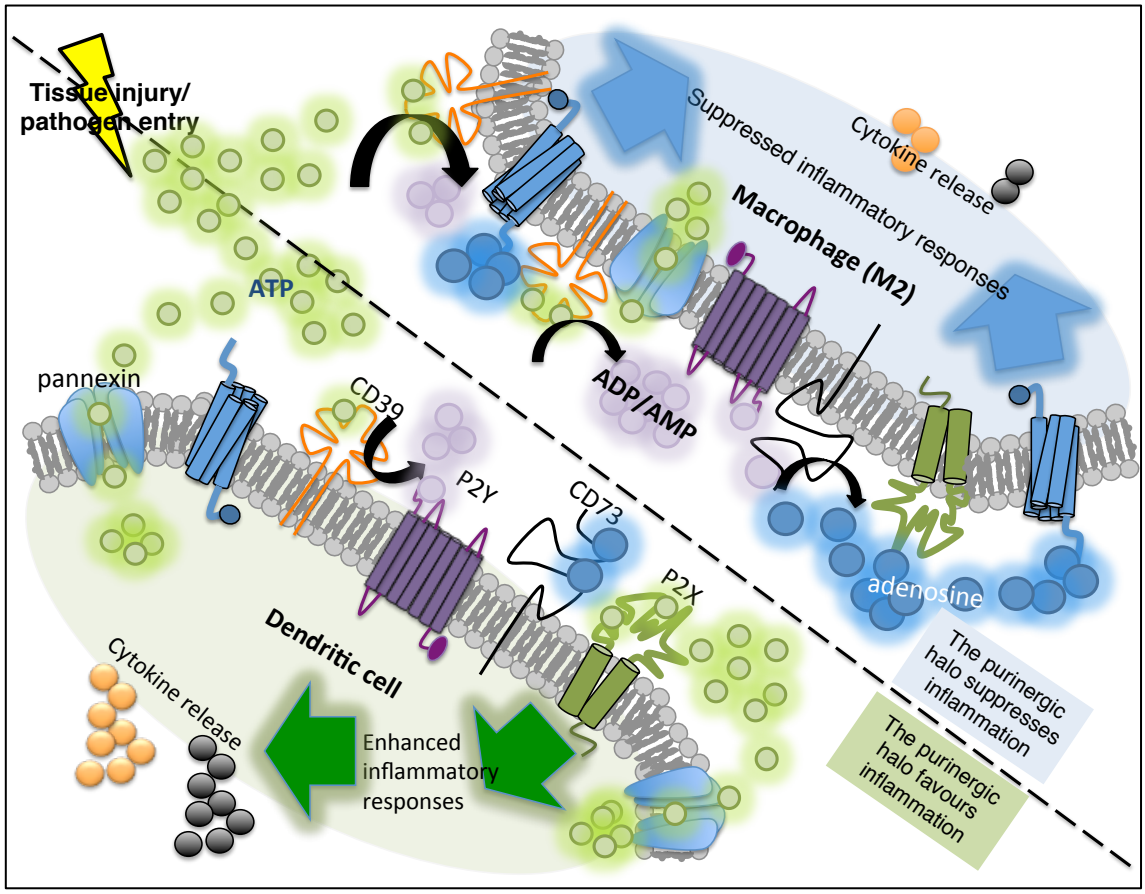


***Figure 6.1:* The nature of the 'purinergic halo' contributes to the outcome of the immune responses.**
Traumatic injury or pathogen invasion causing tissue injury can result in the accumulation of ATP. ATP molecules are sensed by immune cells as danger signals triggering an array of downstream pro-inflammatory responses. ATP released by infected or injured cells is readily subjected to dephosphorylation by ATPases such as CD39. ATP is phosphorylated to ADP/AMP and further broken down to adenosine molecules by CD73. The relative expression of these enzymes is determined by cytokines and other transcription factors. In turn they determine the ATP:adenosine ratio and therefore the nature of the 'purinergic halo' surrounding immune cells, which can either potentiate or suppress inflammatory responses such as cytokine release.

Other mechanisms are also important in regulating P2X receptor function that could also contribute in the cell-type diversity in $P2X_7R$ function. It is clear that different structural domains are responsible for different properties of the receptor. For example, the C-terminus is a quintessential feature of the $P2X_7R$ with important functions such as receptor trafficking and stability of expression on the cell surface membrane, pore formation and channel activity [323, 690, 529, 370]. This rather elongated C-terminal

domain extends the diversity of receptor functions including the formation of the infamous $P2X_7R$ pore [691]. Although much work has focused on the properties of the pore [692, 693], the molecular platform that forms the $P2X_7R$ pore remains elusive and it most likely varies between species and cell type. The evidence gathered thus far is controversial. A number of reports implicate pannexin-1 hemichannels as mediators of the pore in mouse peritoneal macrophages [694]. Others provide evidence that excludes a role for pannexin-1 channels as the $P2X_7R$ pore in the same cells as well as murine BM-MΦ [695, 696]. Furthermore, in HEK-293 cells transfected with the rat $P2X_7R$, using site directed mutagenesis, Browne and colleagues demonstrate that formation of the pore results from the structural rearrangement of the $P2X_7R$ ion conductive pathway, i.e. the channel itself [543]. Overall, the current consensus is that there is not a single $P2X_7R$ pore but rather a number of different channels including the pannexins that can be recruited by the $P2X_7R$ to form the pore and this most likely depends on the cell type and/or the type of inflammatory signal.

Part of the problem in trying to characterize the different properties of the pore lies with the fact that studies employ diverse approaches and different tools to investigate pore formation which are not directly comparable. Flow cytometry, fluorescent microscopy as well as plate readers are a few examples of tools employed to detect dye uptake. Additionally, the fluorescent large molecular weight dyes used to investigate pore formation differ in size and characteristic between different studies further complicating a conclusive analysis of the published literature [387]. Additionally, marked differences between the receptor exist between different species and even between different mouse strains, which introduces further challenges when trying to compare responses between different studies. For instance, evidence suggests that C57BL6/J mice carry a polymorphic allele that suppresses the function of the channel (low-activity) whereas Balb/c mice carry the high-activity allele [564, 697]. The other major problem that has hindered progress in deciphering the molecular basis of receptor function is the lack of selective and potent tools that target P2X receptors. For example, BBG has long been employed as a selective and potent inhibitor of the human $P2X_7R$, however, it is now realized that the inhibitor also blocks sodium channels as wells as $P2X_4R$ [698]. As such, certain aspects of studies that have employed the specific inhibitor to investigate $P2X_7R$ pharmacology need to be revisited.

Pharmaceutical companies have since made exhaustive efforts to screen for potent and selective $P2X_7R$ antagonists and a new series of potent and selective antagonists have recently been introduced to the market. [699-701] Unfortunately, the first clinical trials using the new series of $P2X_7R$ antagonists (AZD9056 and CE-224, 535) for the treatment of rheumatoid arthritis did not exhibit significant efficacy (in comparison with the placebo controls) emphasizing the need for an improved understanding of the specific processes associated with the $P2X_7R$ in various disease states [421, 702]. Perhaps pharmaceutical companies should focus on devising drugs that specifically block channel opening and others that prevent pore formation alone or even drugs that block specific splice variants. First, however, we need to understand the various cell-specific receptor functions and how these relate to different domains of the receptor.

The diversity of $P2X_7R$-associated cellular functions continues to puzzle researchers in the field with regards to how can a single protein regulate such diverse functions. Surprisingly, the $P2X_7R$ is the only receptor that cannot form heteromers with other P2X receptors in native cells, arguably due to its peculiar structure, which could offer some some degree of functional diversity [307]. Recently, splice variant expression has been proposed as an endogenous regulatory mechanism for $P2X_7R$ function bearing in mind that variations in $P2X_7R$ structure alter the physicochemical properties of the receptor including its sensitivity to agonist and that particular domains of the receptor, for example the C-terminus, are either shorter or absent. For example, P2X7K-expressing T-cells respond to both ATP and μM levels of NAD whereas murine BM-MΦ derived from $P2X_7R$ KO mice lack P2X7K expression and can respond only to ATP [550, 475, 355, 476]. Unfortunately, the currently available antibodies do not discriminate between various splice variants in wild type tissue, particularly those of equal size, and therefore hinder progress in the field.

These investigations have therefore examined whether splice variant isoforms contribute to cell-type-dependent diversity of $P2X_7R$ function, which could explain the different cytokine responses observed in BM-DC and BM-MΦ. Additionally, on the basis that localization and stabilization of the $P2X_7R$ on the cell surface membrane, at least in microglia and macrophages, is tightly regulated by a specific motif that sits within an LPS-binding motif [322] it was interesting to examine the effect of LPS priming on the expression of various splice variant isoforms of the receptor. Although,

the patterns of splice variant expression by resting and LPS-primed DC and macrophages were characterised, the functional differences observed between the two cell types could not be reconciled on the basis of differential splice variant expression. It is, however, possible that splice variant expression modulates $P2X_7R$ function at the level of translation rather than transcription and would therefore remain undetected. At present the topic of splice variants and their relative contribution to $P2X_7R$ function in primary tissues is still in its infancy, but once the appropriate tools become available it will be interesting to investigate which of the variants can form stable $P2X_7R$ trimers on the cell surface membrane and how these alter interactions of the receptors with the various adaptor, anchor or scaffolding proteins. To complicate things even further, the human $P2RX_7$ gene houses over 600 SNPs, which result in both gain- and loss-of-function $P2X_7R$ profiles and are increasingly associated with susceptibility to various diseases including diabetes, toxoplasmosis and tuberculosis [703-706].

Although there is no evidence of the $P2X_7R$ forming functional heteromers with other P2X receptors in primary tissues, it is thought that other P2X receptors, particularly $P2X_4R$, have important contributions in various functions of the receptor. Recently, $P2X_4R$ signalling has been associated with $P2X_7R$-directed cytokine responses in both macrophages and DC [599], however, not much is known about any distinct functional aspects of the receptor in DC versus macrophages. Interestingly, an extensive expression analysis by Xiang and colleagues [707] of rat Kupffer cells (liver macrophages) and DC of the liver reveals there is a lack of expression of $P2X_7R$ subunits in Kupffer cells but not in liver DC. Previous reports, however, provide substantial evidence of $P2X_7R$ expression in various macrophage subsets including microglia [316], osteoclasts [708], mouse BM-MΦ and PMΦ (based on the results of these investigations) and even various macrophage cell lines [709, 710], rat Kupffer cells express $P2X_4R$ subunits alone, whereas rat liver DC express both P2X receptors highlighting a tissue differential expression of P2X receptors of liver immune cells. Conversely, mouse Kuppfer cells appear to express functional $P2X_7R$ as elegantly shown by Hoque and colleagues [711]. Data such these raise important questions as to whether such differences are restricted to one particular species or whether particular subtypes of macrophages have the ability to modulate their expression of the $P2X_7R$. Indeed, if the $P2X_4R$ is the sole representative of the P2X family of receptors in rat

Kupffer cells, it would be interesting to demonstrate whether it is able to compensate for the various functions associated with $P2X_7R$ signalling.

The fact that similar signalling cascades might have different functional consequences, such as cytokine release, in different cell types, is not surprising. For example, the effect of cAMP signalling, which is often associated with innate immune responses, has distinct consequences in DC and macrophages. Whereas cAMP signalling enhances IL-6 expression in LPS-primed macrophages, it has the exact opposite effect in LPS primed murine BM-DC cells [712, 713]. Interestingly, the $P2X_7R$ signalling complex has been associated with regulating cAMP activity in monocytes [714, 715], however, further work is required to decipher how the $P2X_7R$ trimer interacts with individual intracellular proteins and how these are linked with specific receptor functions in different cell types. In doing so, it will be possible to devise therapeutic strategies that selectively block receptor functions in targeted cell types.

6.1 Conclusion.

The $P2X_7R$ is an important mediator of inflammation implicated in several disease states including asthma, tuberculosis, arthritis, mood disorders and even cancer [716, 630, 717] and so the receptor constitutes an attractive therapeutic target. Since the receptor was first cloned by Surprenant and colleagues in 1996 [325] it has received considerable attention; almost 20 years later, we now have a much better understanding of the basic principles that regulate its function. A new era is now emerging in purinergic signalling research with the field of purinergic signalling rapidly growing with the receptor being implicated in new immunomodulatory functions. It is becoming increasingly evident that the $P2X_7R$ has distinct physicochemical properties that are species but also cell-specific.

Despite the many reports focusing in $P2X_7R$ activation and IL-1β release, few have considered possible differences in $P2X_7R$-driven responses between different cell types. So far, focus has disproportionately been directed towards macrophages and even though DC and macrophages share the same myeloid lineage and perhaps overlapping functions, they also have quite distinct and specialised functions and should therefore be expected to distinctly regulate important functions such as the release of pro-

inflammatory cytokines [2]. The present investigations have identified important differences in $P2X_7R$-driven cytokine responses between macrophages and DC populations and have examined whether splice variant expression plays part in regulating receptor function. It appears that the certain aspects of $P2X_7R$ function are cell type specific and further work is required to decipher the works of this magnificent receptor in different immune cell types.

DC constitute a large family of heterogeneous cells with a leading role in shaping unfolding adaptive immune responses. Perhaps certain DC subsets such as LC situated within an intricate network in the epidermal layers of the skin might adopt a less constraint approach to release IL-1β in the microenvironment in order to trigger their migration towards the draining lymph node. In contrast mature DC, once within in T-cell compartments of the lymph node are required to switch to a more targeted approach to release pro-inflammatory cytokine to antigen specific T-cells within the immunological synapse. What is certain is that we need to further explore the mechanistic molecular cascades that lead to IL-1β release in different physiological and pathological inflammatory conditions and in different cell types. Further questions will arise but more importantly valuable insight for the design of sophisticated therapeutic strategies will be gained.

Chapter 7:
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