Pollutants and immune regulation in the human airway:

Modulation of dendritic cell function by environmental particulate matter

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Abstract

Ambient air pollution, including airborne particulate matter (PM) derived from combustion of fossil fuels (FF) or biomass (BM), has detrimental inflammatory effects on human health. Myeloid antigen presenting cells, including dendritic cells (DCs) regulate immune responses in the airway and sample inhaled PM. This study tests the hypothesis that PM interacts with multiple environmental sensing pathways in DCs with outcomes that depend on particle size and composition as determined by combustion source.

The effects of different sized PM (<10µm, PM10; <2.5µm, PM2.5), derived from the combustion of FF or BM, on human monocyte-derived or *ex vivo* sputum DCs, were examined. DC activation status, cytokine production and aryl hydrocarbon receptor (AhR) signalling were assessed by flow-cytometry, multiplex ELISA and qRT-PCR, following exposure to PM. Pathway-specific antagonists were used to explore underlying mechanisms.

Particle size and combustion source influenced the effects of PM on DCs. Irrespective of combustion source, PM10 but not PM2.5, induced MoDC maturation and stimulated production of inflammatory cytokines, including IL-1β and IL-18, indicative of inflammasome activation. These responses were dependent, at least in part, on TLR4 as was the induction of *IDO* by PM10. AhR signalling was induced by PM in both MoDC and *ex vivo* sputum DC. It was stimulated by both PM10 and PM2.5 and was induced more strongly by BM-derived PM. AhR activation was independent of DC maturation and TLR4 signalling. Additionally, BM- but not FF-derived PM increased NADH levels in DC suggestive of altered metabolism.

Thus, PM induces a complex programme of DC activation, influenced by size and combustion source, which includes classical maturation, inflammasome dependent cytokine release and AhR signalling as well as potential metabolic changes. In the airway, exposure to different PM and the changes in DCs induced by them may lead to altered responses to inhaled antigen.

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List of Abbreviations

7-AAD	7-Aminoactinomycin D
AhR	Aryl hydrocarbon Receptor
AIM2	Absent in melanoma 2
ALR	AIM2-like receptors
APC	Antigen presenting cell
ARNT	AhR nuclear translocator
ASC	Apoptosis-associated speck like protein containing a caspase recruitment domain
BM	Biomass
CARD	Caspase activation and recruitment domain
CB	Carbon black
CD	Cluster of differentiation
cDC	Classical dendritic cells
CFSE	6-Carboxyfluorescein succinimidyl ester
CLR	C-type lectin receptor
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DCIR1	Dendritic cell immunoreceptor 1
DEP	Diesel exhaust particles
DG	2-deoxyglucose
DRE	Dioxin responsive element
FF	Fossil Fuel
FICZ	6-formylindolo[3,2-b]carbazole
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Granulocyte and macrophage progenitor
HLA-DR	Human Leukocyte Antigen - antigen D Related
IDO	Indoleamine-2,3-dioxygenase
IFN-γ	Interferon- γ
IgE	Immunoglobulin E
IL	Interleukin

IRF	Interferon-regulatory factor
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
MAPK	Mitogen-activated protein kinases
MDP	Macrophage and DC precursor
MFI	Mean Fluorescence Intensity
MHC	Major histocompatibility complex
MLR	Mixed Leukocyte Reaction
MoDCs	Monocyte-derived dendritic cells
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NF- κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nucleotide-binding oligomerization domain-like receptors
NLRP3	NLR family pyrin domain contain 3
NOD	Nucleotide-binding oligomerization domain
PAH	Polycyclic aromatic hydrocarbons
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
pDC	plasmacytoid dendritic cell
PM10	Particulate matter with a size of less than 10μm
PM2.5	Particulate matter with a size of less than 2.5μm
PRR	Pattern recognition receptors
PS	Phosphatidylserine
RIG	Retinoic acid-inducible gene
RLR	RIG-I-like receptors
TCDD	2,3,7,8-tetrachlorodibenzop-dioxin
TCR	T-cell receptor
TGF-β1	Transforming growth factor beta 1
Th	T helper cell
TIR	Toll-interleukin (IL)-1 receptor
TLR	Toll-like receptors
TRIF	TIR-domain-containing adapter-inducing interferon-β

1. General Introduction

Overview

The main function of the respiratory tract is the supply of vital oxygen to the body by inhaling air. One of the main challenges of this major entry to the body with a large surface area is that, depending on the environment, pathogens and other potentially harmful substances suspended in the air, including a wide range of pollutants, can enter the body. Appropriate defence systems need to be in place to deal with these challenges. One of the key structures to provide this protection is the innate immune system, which acts in a non-specific way to ensure a quick immune response.

Antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs), play an important role in the innate immune response on a cellular level. They provide a first line of defence and are able to induce an adaptive immune response, thereby providing protection against incoming pathogens in the respiratory tract. In particular, DCs are important as they are the most potent type of APC and can activate naïve T cells, shape the differentiation of T cells to generate functionally distinct effector cells, and thereby establish antigen-specific immunological memory. Respiratory DCs encounter a wide range of environmental components that can impact their function, one major being ambient air pollution, which consists of both volatile and solid components. Gaseous substances include primarily carbon monoxide, nitrogen dioxide and ozone, whereas solid constituents include particulate matter (PM). Among solid air pollution components, PM with a size of less than 10µm (PM10) in diameter is of particular interest as it can induce a wide range of health effects (D'Amato, Cecchi, D'Amato, & Liccardi, 2010).

DCs in the respiratory tract confront PM both in developing countries, where easily available biomass is burned for energy generation, as well as in urban areas in industrialized countries, where air pollution mainly derives from the burning of fossil fuels in cars (Frank J. Kelly & Fussell, 2015b). Air pollution particles consist of a carbon core with additional components adsorbed to its surface. Hence, each particle type has a variable composition which will differ depending on the combustion source as well as the area where the particle was generated. Also, they not only vary in their composition but in their size. Taken together, all these factors may influence the impact different types of PM have on DCs.

The central question of this thesis is aimed to focus on two properties of the particles: their composition and their size in an effort to understand how both influence the effects particles

have on DCs. To this end, we have compared particles from urban UK environments, which are fossil fuel (FF)-derived, with particles from Africa, which are derived predominantly from biomass (BM) as a result of wood burning. We have also studied the influence of particle size by comparing FF-derived PM10 and PM2.5 from urban areas as well as biomass derived PM10 and PM2.5.

The introduction will provide a broad overview of the origin and function of DCs in general (Chapter 1.1.) and specifically in the respiratory tract (Chapter 1.2.). Subsequently, the generation and different types of air pollution and their effects on the human body will be discussed (Chapter 1.3.). Finally, this introduction will finish with the hypothesis and aims of this thesis (Chapter 1.4.).

1.1. Dendritic cells

DCs are bone marrow derived leukocytes. As part of the innate immune system, they act as APCs and thus their main function is to sample antigen and then initiate and shape adaptive immune responses (reviewed by Rossi, Young, & Alerts, 2005). They are part of the first line of defence against potential threats to the body. They are located throughout the body, including mucosal sites that are constantly exposed to the outside world such as the gastrointestinal tract and the lung. Hence, they face a variety of immunological challenges. After antigen uptake and processing, they migrate from non-lymphoid organs through the lymphatics to the lymph nodes where they activate T-cells. Thereby, they induce the adaptive immune response. This process is controlled by the upregulation of the chemokine receptor CCR7 and its ligands CCL19 and CCL20, which drive movement to the lymphatics (Förster, Davalos-Misslitz, & Rot, 2008). One distinct feature of DCs is their ability to induce primary immune responses by activating naïve T-cells as well as memory T-cells. Other APCs, like macrophages and B-cells, are only able to interact with memory T-cells. DCs are unique APCs because they are able to induce the establishment of immunological memory by inducing primary immune responses (Banchereau et al., 2000a; Banchereau & Steinman, 1998; Hart, 1997).

The role of DCs as APCs is vital since this is the key feature that brings together the innate antigen non-specific immunity and the adaptive antigen-specific immunity which then form the defence of the host organism (Medzhitov & Janeway, 1997). The distinctive characteristics of the adaptive immune system are: the ability to create a diverse set of antigen specific clones by rearranging immunoglobulin and T cell receptor genes; and expanding these clones to establish a state of antigen-specific immunological memory such that re-encounter with an antigen elicits

a more rapid and powerful response (Banchereau et al., 2000b; Banchereau & Steinman, 1998; Hart, 1997).

In 1868, Paul Langerhans discovered a population of skin cells which he mistakenly assumed were epidermal nerve cells due to their morphology, as their dendrites resemble the ones from neurons. Subsequently, these Langerhans cells in the epidermis were regarded as a population of bone marrow DCs in the skin. Recently, it has been shown that Langerhans cells are probably a macrophage like cell type and that the majority of these macrophages are derived from the yolk sac pre-birth (Ginhoux & Jung, 2014; Guilliams et al., 2014a; Paul Langerhans, 1868). Over 100 years later, in 1973, Ralph M. Steinman described a rare cell type obtained from mouse peripheral lymphoid organs with an irregular shape and dendritic protrusions. He proposed the term dendritic cell for this new cell type. At that time, their role in immunity was unclear (Steinmann & Cohn, 1973). Cells present in afferent lymph, called veiled cells because of their stellate morphology, were also recognised to be DCs (Hoefsmit, Duijvestijn, & Kamperdijk, 1982). In 2011, Ralph M. Steinman received the Nobel Prize in Physiology or Medicine for “his discovery of the dendritic cell and its role in adaptive immunity”.

1.1.1. Origin of dendritic cells

Our knowledge of the origin and development of DCs is mainly derived from mouse studies. All blood cells, including immune cells such as DCs, descend from one pluripotent hematopoietic stem cell of the bone marrow and are continuously replenished to ensure a constant supply of DCs in the periphery (Katz, Tamaki, & Sachs, 1979). From this progenitor cell develops the common myeloid progenitor (CMP), which is the precursor of macrophages, neutrophils and DCs, but no longer has the potential to give rise to lymphocyte populations, and has been identified in both mice and humans (Kondo et al., 2003). The CMP leads further to the granulocyte and macrophage progenitor (GMP), and further to the macrophage and DC precursor (MDP), which forms the basis of monocytes and DCs (A. Chow, Brown, & Merad, 2011; Fogg et al., 2006). Finally, the MDP gives rise to the common dendritic cell progenitor (CDP), which is then responsible for plasmacytoid DCs (pDCs) and pre-classical DCs (pre-cDCs) and therefore is the first progenitor that is limited to the DC lineage (Naik et al., 2007). During their development and their further commitment to the DC lineage, both CMP and the MDP lose their potential to give rise to granulocytes and monocytes respectively (A. Chow et al., 2011).

However, this model has been cast into doubt by new evidence and an alternative route has been suggested, which leaves out the MDP stage and suggests a direct lineage from early precursors (Sathe et al., 2014). Up to this stage, the formation of the cells takes place in the

bone marrow, where pDCs complete their differentiation, enter the blood circulation and migrate to non-lymphoid organs (Boris Reizis, 2010). Pre-cDCs leave the bone marrow and migrate through the blood stream to peripheral organs, where they terminate their differentiation and carry out their function as classical DCs (cDCs) (Liu, Victora, & Schwickert, 2009; Naik et al., 2006).

Less is known about the development of human DCs due to the lack of reliable markers, small numbers of DCs in the blood and limited access to human tissue. However, slow progress has been made on defining human dendritic cell progenitors. It has been shown that human cord blood and the bone marrow contain a progenitor that can give rise to granulocytes, monocytes and DCs (hGMPs), which then loses this potential and becomes a progenitor that can produce monocytes and DCs (hMDP) and finally leads to a specific DC progenitor, that can only give rise to cDCs and pDCs (hCDPs) (Lee et al., 2015). Also, a human pre-CDC progenitor cell has been identified, which leads to both subsets of cDCs (Breton et al., 2015).

Plasmacytoid DCs have been identified and extensively studied in the last 15 years (reviewed by Reizis, Bunin, Ghosh, Lewis, & Sisirak, 2011). Plasmacytoid DCs have a strong cytologic similarity to antibody producing plasma cells and hence share the name. They mainly mediate anti-viral innate immunity rather than present antigen (Merad & Manz, 2009), although they can acquire the ability to present antigen upon activation. Upon interaction by viruses, and the recognition of viral nucleic acids by pattern recognition receptors (PRR) including Toll-like receptors (TLR) 7 and TLR9, pDCs release high amounts of type I interferons, which leads to cellular expression of IFN-stimulated genes and apoptosis of infected cells (Blasius & Beutler, 2010; Gilliet, Cao, & Liu, 2008; Kawai & Akira, 2011; Y.-J. Liu, 2005; Swiecki & Colonna, 2015). Also known as interferon producing cells (IPC), pDC are the most potent producers of type I Interferons known.

Classical DCs are the dendritic cell type that performs the typical tasks DCs are known for: antigen presentation and T cell stimulation. They can be defined as all DCs other than pDCs. They have a high capacity to take up self and foreign antigens, process them, and then migrate loaded with these antigens to the T cell rich areas of the lymph nodes with the purpose of initiating T cell responses (reviewed by Merad, Sathe, Helft, Miller, & Mortha, 2013).

The developmental process from the pluripotent hematopoietic stem cell down to DCs and their subtypes is highly regulated by their cytokine environment as well as transcription factors (reviewed by Satpathy, Murphy, & Kc, 2011). One of the key cytokines regulating the process of pDC and cDC development is FMS-like tyrosine kinase 3 ligand (Flt3l) which is the ligand for the Flt3 receptor, also known as CD135 (K. Liu et al., 2009). CD135 can be used to distinguish

hematopoietic stem cells, which are CD135 negative, from other progenitor cells, which become CD135 positive. Mice lacking Flt3L or the Flt3 receptor exhibit a DC deficiency (McKenna et al., 2000).

As well as being controlled by cytokines, the development of DCs is influenced by transcription factors. PDCs and cDCs can be distinguished by the presence of E2-2, which is expressed by pDCs but not by cDCs. E2-2 is essential for pDC development in a cell intrinsic manner and is needed for the pDC-mediated IFN secretion (Cisse et al., 2008). Inhibitor of DNA binding 2 (ID2) is a repressor of E2-2 and absent in pDCs, but highly expressed in cDCs and therefore the balance between E2-2 and ID2 controls the differentiation fate of pDCs and cDCs. On the other hand, the transcription factor *Zbtb46* is expressed in cDCs and pre-DCs but not in pDCs and their precursors as shown in murine studies. However, it is not required for cDCs development and is also expressed in non-immune cells. Furthermore, it has been shown to be present in human DCs and is absent in monocytes and macrophages and therefore can be used to identify human cDCs (Meredith, Liu, Darrasse-Jeze, et al., 2012a; A. T. Satpathy et al., 2012). The different types of cDC subsets can be identified by their requirements for transcription factors. Basic Leucine Zipper ATF-Like Transcription Factor (BATF3) is required for the development of CD8αα^{+} cDCs and interferon-regulatory factor 4 (IRF4) contributes to the generation of CD4^{+} cDCs (Murphy, Tussiwand, & Murphy, 2013).

Resting state DCs are CDP-derived but, under inflammatory conditions monocytes, may be able to give rise to DCs and these DCs are therefore referred to as monocyte-derived DCs (MoDCs) or inflammatory DCs (Mildner, Yona, & Jung, 2013; Segura & Amigorena, 2013; Serbina, Salazar-mather, Biron, Kuziel, & Pamer, 2003). *In vitro*, granulocyte macrophage colony-stimulating factor (GM-CSF) in combination with interleukin-4 (IL-4) is used to generate DCs from monocytes (Inaba et al., 1992). However, GM-CSF is not essential for DC development in vivo as mice missing the GM-CSF receptor have a normal development of DCs (Vremec et al., 1997).

1.1.2. Classical dendritic cell subsets

It has been established over the years that DCs are not a homogenous population but rather consist of a wide range of subsets with specialized functions and locations with the purpose of providing specific immune responses to a variety of danger signals (Collin, McGovern, & Haniffa, 2013).

1.1.2.1. Murine dendritic cells

It is necessary to distinguish between the location, i.e. lymphoid and non-lymphoid tissues, as well as the state of the body, i.e. steady state or inflammatory state. Lymphoid tissue resident DCs consist of two major subpopulations which can be identified by the expression of the CD8αα homodimer or CD4. They spend their whole life span in the lymph nodes where they exhibit immune regulatory properties and promote tolerance to self-tissue (Sixt et al., 2005). Migratory DCs are found in the peripheral tissue and are also referred to as "non-lymphoid DCs" or "tissue DCs" and express CD103 or CD11b corresponding to CD8αα or CD4, respectively (Ansuman T Satpathy, Wu, Albring, & Murphy, 2012). Their function is to acquire antigen and migrate to the lymph nodes through the afferent lymphatics (Collin et al., 2013; Merad et al., 2013). Also, murine lymphoid organ-resident CD8αα DCs have been shown to be the most efficient at cross-presentation (O. P. Joffre, Segura, Savina, & Amigorena, 2012). In case of inflammation or infection where the composition of cells is radically altered, inflammatory DCs are generated, which are not present in a healthy organism. These DCs mainly derive from monocytes, which are recruited to the area of inflammation and cannot be found in the steady state and therefore are not present in a healthy organism (Shortman & Naik, 2007). The inflammatory DCs were initially identified as $CD11b^+$ $CD11c^+$ $F4/80^+$ $Ly6C^+$ DCs and share markers with tissue resident macrophages, such as CD64 (Segura & Amigorena, 2013). Their role is to help cDCs in case of a uncontrolled infection and hence act as a safeguard (Hespel & Moser, 2012).

1.1.2.2. Human dendritic cells

All human DCs express high levels of MHC Class II and lack lineage markers such as CD3 for T cells, CD19/20 for B cells and CD56 for natural killer cells (Collin et al., 2013). This definition of DCs has been used to study human respiratory tract DCs for cell sorting by flow-cytometry (see Chapter 4). Furthermore, cDCs can be distinguished from pDCs by the expression of CD11c and the expression of the transcription factor *Zbtb46*, which is specific for cDCs but not required for the development of DCs, at least in mice (Collin et al., 2013; Merad et al., 2013; A. T. Satpathy et al., 2012). Again, akin to murine DCs, human cDCs can be divided into subtypes by their location and function and can be found in tissue and lymphoid organs as well as blood (O'Keeffe, Mok, & Radford, 2015). The blood contains mainly precursors for tissue and lymphoid organs cDCs, such as $CD1c^+$ and $CD141^+$ immature cells. Non-lymphoid and lymphoid tissue DCs contain mainly $CD1c^+$ and $CD141^+$ cells, although $CD1c^+$ cells are the major population of DCs in all compartments and are more activated in peripheral tissue, as seen by expression of CD80, CD83, CD86 and CD40. $CD141^+$ cells have an enhanced ability to take up dead or necrotic cells (Collin et

al., 2013). A major obstacle to studying DCs was the lack of CD8αα expression of human DCs as it made comparing human and murine DCs difficult. It has been shown that $CD141^+$ cells are comparable to $CD8\alpha\alpha^+$ mouse DCs, while $CD1c^+$ are associated to $CD11b^+$murine DCs (Robbins et al., 2008). Corresponding to their murine counterpart human, $CD141^+$ DCs are also superior in cross-presentation compared to other DC subtypes (Cohn et al., 2013). To unify the nomenclature for human and murine DCs, it has been suggested that $CD141^+$ and $CD1c^+$ DCs are called cDC1 and cDC2 respectively and this new nomenclature would also apply to the corresponding murine DCs (Guilliams et al., 2014b).

During inflammation, another type of DCs can be found, which is derived from monocytes and is referred to as inflammatory DCs (O'Keeffe et al., 2015). The main characteristic of inflammatory DCs is their activated phenotype and a high production of pro-inflammatory cytokines showing a skewing towards a Th1 and Th17 T cell response and therefore suggesting that these cells play an important role in autoimmune diseases. GM-CSF, initially described as an hematopoietic growth factor and , however subsequent studies suggested its involvement in innate and adaptive immunity including the recruitment and differentiation of MoDCs (Wicks & Roberts, 2016). The presence of GM-CSF has been shown at inflammation sites, such as the synovial fluid of rheumatoid arthritis patients (Darrieutort-Laffite et al., 2014). The exact role of inflammatory DCs and difference to cDCs is still unclear, but it has been proposed that in addition to acting as safeguards they are mainly linked to innate immunity and T-cell activation as opposed to cDCs, which have been described to induce immunity and tolerance, hence , the two subsets may induce different responses (Hespel & Moser, 2012). As MoDCs have been used as a DCs model for *in vitro* studies for a long time and most of our knowledge about human DCs is based on this model, it needs to be pointed out that they are closely related to inflammatory DCs (O'Keeffe et al., 2015).

1.1.3. Dendritic cell function

1.1.3.1. Function of dendritic cells in the innate immune system

The innate immune system is the only mechanism of defence against infection for invertebrates, which make up 90% of all animals (Litman, Rast, & Fugmann, 2010). The main function of the innate immune system is to provide immediate defence against incoming threats and hence provide a first line of defence to the body. It prevents the spreading of infectious agents and forwards the information obtained to the adaptive immune system in organisms that have one. By doing so, it slows down the distribution of the pathogen in the organism and buys precious time for the adaptive immune response to activate in those species that have adaptive

immunity. Owing to the immediate nature of the response, the innate immune defence system acts in a non-specific, generic way since it does not rely on a clonal expansion of antigen-specific T lymphocytes like the adaptive immune response. Therefore, the main cells involved the innate immune function are phagocytic cells and APCs like granulocytes, macrophages and DCs.

DCs belong to the group of APCs, which also includes B cells and macrophages, that act at the interface of innate and adaptive immunity (Kambayashi & Laufer, 2014; Merad & Manz, 2009). To be able to fulfil their function as "gatekeepers" or "sentinels" of the immune system by providing a first line of defence against threats to the body, they are located throughout the body and have their main function at strategically important sites in the peripheral tissue, which are in constant contact with the environment. This process of patrolling is facilitated by their dendrites through which they are able to scan a bigger area and sample self and non-self-antigen. From the circulating blood, immature dendritic cell precursors enter the tissue. Immature DCs are present in the steady state and their phenotype is defined by a low surface expression of major histocompatibility complex (MHC) class II molecules and co-stimulatory markers such as CD80, CD86 and CD40 (D. Bell, Young, & Banchereau, 1999; Steinman RM, 2010). To be able to take up pathogens, they have a high endocytic activity, which is facilitated through receptors such as mannose receptors, which recognise patterns of carbohydrates on the surface of infectious agents (Stahl & Ezekowitz, 1998). Upon interaction with a danger signal such as microbial products, inflammatory cytokines (e.g., interferon-α, interleukin-1β as well as tumour necrosis factors), damaged host cells or infected host cells, DCs change from an immature to an activated or matured condition in order to induce an adaptive immune response (Matzinger, 1994; Reis e Sousa, 2006). As soon as they capture an antigen, they process it and undergo the process of maturation and lose the ability to take up more antigen (Banchereau & Steinman, 1998). The process of antigen uptake, maturation and the induction of the adaptive immune response will be fully discussed in the following sections.

1.1.3.2. Antigen uptake and processing

DCs are very versatile phagocytes and can detect a wide range of pathogens and other forms of danger signals. DC precursors constantly leave the bone marrow and develop in the peripheral and lymphoid tissue into immature DCs that are highly phagocytic (Merad et al., 2013). There, they encounter any pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Grace Chen & Nunez, 2010; Iwasaki & Medzhitov, 2010). PAMPs include molecules that are present on pathogens and are detected by PRRs. PRRs will be discussed in detail later in the section "Environmental sensing by DCs".

A number of different responses can be induced, depending on which PRR is engaged after interacting with a PAMP. DAMPS are molecules usually found inside cells that are released, as soon as the cell dies. Typical DAMPs include for example ATP and mitochondrial DNA (Grace Chen & Nunez, 2010). Some DAMPs are detected by receptors that detect PAMPs; therefore, the modulatory activity of these two activators may overlap (Zelenay & Reis e Sousa, 2013). Once the pathogen has been identified, it will be taken up through endocytosis and depending on the exact antigen, through receptor-mediated phagocytosis or macropinocytosis to be then presented on MHC Class II molecules (S. Gordon, 2016). For endogenous antigens such as viruses, antigens are delivered to MHC Class I molecules, to be then presented to CD8 T cells.

However, an exception to this rule is known as cross-presentation, allowing peptides derived from extracellular virus particles or infected cells to be presented on MHC class I to CD8 T cells, thereby circumventing the requirement of DCs to be infected in order to activate CD8 T cells. This phenomenon also uses receptor-mediated phagocytosis and macropinocytosis and is specifically carried out by CD8αα cDCs (Segura & Amigorena, 2014). Receptor-mediated phagocytosis is characterized by engulfing particles with a size of less than 0.5µm and forming a phagosome. Air pollution particles are known to be taken up through this pathway (Medzhitov et al., 2011). The phagosome then acidifies, which leads to the activation of proteolytic enzymes that degrade the antigen into small fragments (Wolf & Underhill, 2014).

On the other hand, macropinocytosis is a process, also called cell-drinking, that describes the uptake of large volumes of surrounding fluid by forming protrusions from the plasma membrane that fuse with themselves and thus take up the trapped fluid (Doherty & McMahon, 2009). This process is mediated through aquaporins, some of which are downregulated, once the DC has matured after uptake of antigen. Therefore, aquaporins represent a volume regulatory system in immature DCs to control the intake of fluid during constitutive macropinocytosis (de Baey & Lanzavecchia, 2000).

1.1.3.3. Stimulation of adaptive immune response

The adaptive immune response evolved under evolutionary pressure as a second, more specialized and long-lasting strategy of protection against infection for vertebrates in addition to the innate immune system (Litman et al., 2010). The induction of the adaptive immune response is the next step in fighting off threats to the body; it provides a very specific defence system. Initiation of an adaptive immune response is dependent upon an ongoing innate immune response; hence, without innate immunity, there would be no adaptive immunity (Iwasaki & Medzhitov, 2015). Whereas the main function of the innate immune system is to recognize

danger and induce an appropriate response, the adaptive immune system eliminates the pathogen and establishes immunological memory.

1.1.3.4. Maturation

A crucial step for DCs in order to fulfil their function as gatekeepers and activate the adaptive immune response is to undergo maturation as it enhances their ability to activate other immune cells (Banchereau et al., 2000a). Through their key function as APCs, DCs form the bridge between the innate and adaptive immunity and it has been shown that activated DCs are the most potent activators of T cell proliferation in a mixed leukocyte reaction *in vitro* (Steinman, Gutchinov, Witmer, & Nussenzweig, 1983). Immature DCs are usually found in non-lymphoid tissues and are able to take up antigen and process it, but do not have the ability to interact with T cells (Shortman & Liu, 2002). After encountering and taking up antigen, they go through the process of maturation, which involves phenotypic as well as functional changes (Reis e Sousa, 2006). Following the uptake of exogenous antigen through the processes mentioned above, peptides derived from the antigen are presented on the surface of the cell by loading them onto the MHC class II complex molecules, which are then transported from the intracellular endocytic compartments to the cell surface. After uptake, proteins from the pathogen are processed within the cell by lysosome enzymes to generate the short peptides which are presented on MHC class II molecules.

Endogenous antigens, such as those from viruses, are presented on MHC class I molecules, which involves the generation of peptides from cytoplasmic proteins by the proteasome. In the absence of infection, peptides derived from self-proteins are presented in a way that helps maintain self-tolerance. In order to fully initiate a naïve T cell response, three signals are necessary. The first signal is the presentation of antigen through MHC Class I and II molecules as described above. The second signal comes from co-stimulatory molecules such as CD40, CD80 and CD86. CD80 and CD86 signal through binding to CD28 on T cells, leading to IL-2 production, which stimulates expansion and survival of T cells (Linsley et al., 1991). When CD80 or CD86 interacts with cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) T cell responses are downregulated (Hubo et al., 2013). Both CD80 as well as CD86 are expressed soon in the maturation process, therefore are generally used to describe mature DCs, whereby CD86 is expressed at low levels constitutively on immature DCs, but not CD80 (Jonuleit et al., 2000). Consequently, CD80 is a more reliable marker to asses DC maturation in human DCs, as it is strongly expressed on mature DCs and not on immature DCs, whereas CD86 is expressed on immature DCs and gets further upregulated upon maturation (Hubo et al., 2013).

CD40, together with its ligand on T cells CD40L, plays an important role in T cell priming and differentiation by promoting the production of IL-12, which is essential for T cell stimulation (Lapteva et al., 2007; MacDonald et al., 2002). However, signalling through CD40 only is not sufficient to induce IL-12 and additional Interferon- γ (IFN-γ) is needed, therefore, mature DCs stimulated by CD40 alone can induce T cell anergy (Wiethe, Dittmar, Doan, Lindenmaier, & Tindle, 2003). As part of the maturation procedure, in addition to the expression of costimulatory molecules like CD40 and CD80 on their cell surface, they also express the chemokine receptor CCR7, which together with its ligands CCL19 and CCL21 in the lymph nodes is responsible for the homing of DCs to the lymph nodes (Förster et al., 2008). There, the interaction with T lymphocytes takes place leading to antigen presentation by DCs. T lymphocytes are defined by the presence of the T-cell receptor (TCR), which interacts with the antigen loaded MHC molecule.

MHC class I is loaded with intracellular antigens and interacts with $CD8^+$ cytotoxic T cells, whereas MHC class II presents extracellular antigens to $CD4^+$ helper T cells, although deviation from this rule are possible, known as cross-presentation (Bevan, 2006; Heath & Carbone, 2001). Cross-presentation describes the process by which exogenous antigens are presented on MHC class I molecules and presented to CD8 T cells. This pathway is required in order to present antigen from tumours and viruses, which did not infect DCs (Bevan, 2006). Once the DC has reached the lymph nodes, the activation processes is completed and the cell is fully mature.

Finally, the third signal is the secretion of cytokines including IL-12, IL-6, IL-10, IFN-α and IFN-γ, which leads to the differentiation of responding T cells towards alternative types of effector cells (Banchereau et al., 2000a). For example IL-12 is a typical cytokine released by DCs and leads to a type 1 immune response, which is characterized by the generation of T helper 1 cells (Th1) or cytotoxic T lymphocytes (CTL) through T-bet induction and stabilisation. IL-6 and IL-10 tend to promote Th2 responses; however, the exact signals that drive Th2 differentiation are poorly understood (O. Joffre, 2009; Wurtz, Bajénoff, & Guerder, 2004). In mice, it has been shown that type 1 interferon (IFN-1) secreted by DCs responding to Th2 antigen, such as egg antigen from the helminth Schistosoma mansoni, is essential for inducing Th2 responses (Webb et al., 2017). Also, the DC subset mainly involved in promoting Th2 and Th17 type immunity in mice are the $CD11b^+$ cDCs (Peter C. Cook & MacDonald, 2016). Vitamin A metabolite retinoic acid (RA) seems to also play an important role in Th2 type immunity. It has been reported that, RA exposure of DCs led to an induction of IL-10 by $CD4^+$ T cells (Jones et al., 2015). Furthermore, IL-4 seems to play an important role in DC induction of Th2 responses. Exposure of murine DCs to IL-4 leads to

the upregulation of RELMα and Ym1/2 in which RELMα plays an important role in instructing T cells to secrete IL-10 and IL-13 and therefore leading to a Th2 response (P. C. Cook et al., 2012).

Providing these three signals is the characteristic that defines a professional APCs and is necessary for a full effector T cell development (Banchereau & Steinman, 1998). Signal 1 in the absence of signal 2 and 3 generates inactive T cells through the induction of anergy, deletion of cells or the generation of inducible regulatory T cells.

1.1.3.5. Interaction with T cells

Once DCs have undergone functional maturation, they are no longer able to take up any more antigen and downregulate phagocytic activity. Also, the synthesis of MHC antigens is reduced, which helps to maintain sampled antigen at the cell surface. In the lymph nodes, DCs transfer the information gathered in the periphery onto T cells, by interaction of the MHC molecules and the TCR. Also, costimulatory molecules on the APC interact with their corresponding ligands on the T cells and cytokines secreted by the APC influence T cell differentiation. Presentation of antigens on MHC Class I molecules induces responses by naïve CD8 T cells, which differentiate into cytotoxic effector T cells. Naïve CD4 T cells have a more flexible repertoire of outcomes compared with CD8 T cells. They recirculate between lymph nodes scanning for the signals provided by MCH Class II molecules on DCs to then develop either effector T cells or in absence of signal 2 into regulatory T cells. Several different fates are known for CD4 T cells. These are dependent on signals which are determined by the nature of the infection or stimulus that is presented by the DC on the MHC Class II molecule. The subtypes, that evolve after interaction of DCs with a naïve CD4 T cell include Th1, Th2 as well as Th17 and regulatory T cells (Hooper & Macpherson, 2010). DCs provide key signals which determine the pathway of T helper cell differentiation. These fates can be influence by the subsets of DC involved but also DCs themselves are influenced by the PAMPs they sample and cytokines from other cells, which in turn may be influenced by pathogens. How exactly DCs are influenced and which pathways are involved will be discussed in detail in the next section.

Th1 effector cells are primarily characterised by the production of IFN-γ, IL-2 and GM-CSF (Mosmann, Cherwinski, Bond, Giedlin, & Coffman, 1986). They mainly assist with controlling intracellular bacteria and viruses, whereas Th2 cells are primarily involved in fighting off infections by parasites, such as helminths. In this process they mainly secret IL-4, IL-5 and IL-13, leading to the recruitment and activation of eosinophils, mast cells and plasma cells. Also, they are involved in the production of the Immunoglobulin E (IgE) antibody by B cells and are responsible for allergies such as asthma (Wills-Karp, 1998). Another potential outcome is the

differentiation of naïve T cells into Th17 cells, which secrete IL-17 that leads to the production of chemokines by mainly epithelial cells, resulting in the recruitment of neutrophils to the area of infection (Littman & Rudensky, 2010). Figure 1.1 shows a diagram of the different CD4 T cell fates after interaction with a DC.

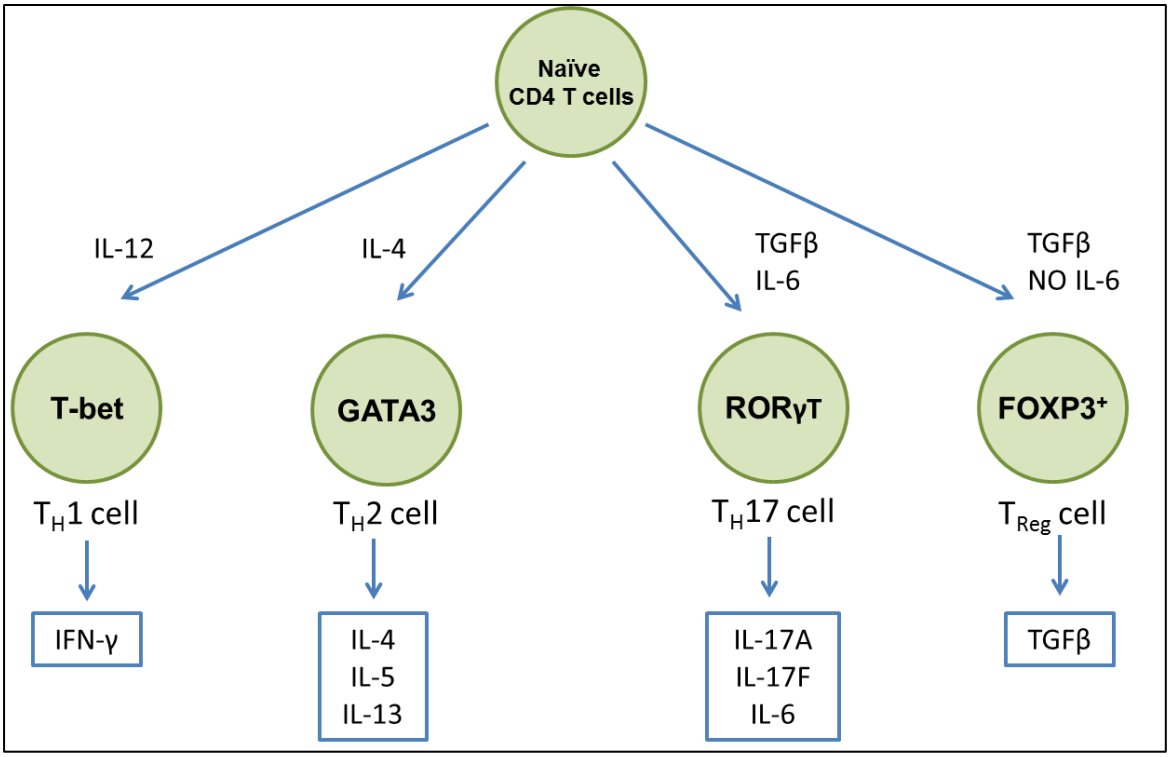

Figure 1.1: CD4+ T cell subset differentiation

CD4+ T cells differentiation fates depends on the cytokine-induced signals mediated by characteristic transcription factors. Each CD4 T cell subsets secretes their characteristic cytokine profile (Hooper & Macpherson, 2010).

In addition to orchestrating the adaptive immune response by inducing Th1, Th2 and Th17 responses, DCs can also have the opposite function, which is the prevention of immune responses and maintaining tolerance. It is necessary for an intact immune homeostasis to induce appropriate immune responses against harmful substances and at the same time prevent immune reactions against self-antigen and innocuous substances (Shortman & Naik, 2007). The inability to instigate an appropriate immune response to a specific antigen can lead to autoimmune and inflammatory diseases, like rheumatoid arthritis and ulcerative colitis, as well as allergies (Hopp, Rupp, & Lukacs-Kornek, 2014; John W. Upham & Stumbles, 2003). In this context, DCs are involved in two different types of tolerance: central and peripheral tolerance. Central tolerance takes place in the thymus where DCs eliminate newly generated T lymphocytes

that potentially could pose a risk to the body as they carry self-reactive receptors. Peripheral tolerance describes the process of inactivating harmful T lymphocytes after they have left the thymus. Immature DCs that lack the expression of co-stimulatory molecules contribute to peripheral tolerance by inducing T cell anergy, which refers to a state of non-responsiveness of T cells and the generation of regulatory T cells that preserves tolerance to self-antigens by suppressing immune reactions (Chung, Ysebaert, Berneman, & Cools, 2013).

1.1.4. Environmental sensing by dendritic cells

As the main role of DCs is to recognize threats to the body and induce appropriate T cell responses, they are plastic cells with the ability to respond to signals in their environment. These signals then in turn have downstream effects on adaptive immunity. To fulfil this role, DCs are equipped with various germ line-encoded receptors which sense the environment. The canonical environmental sensors are PRRs, which transduce danger signals to induce classical maturation, thereby leading to the expression of all three signals involved in T activation and the promotion of effector T cell responses rather than T cell anergy. However, other receptors and signalling pathways are likely to be involved too and these will modulate the functional maturation of DCs and the adaptive responses they induce. The major pathways will be discussed below.

1.1.4.1. Pattern Recognition Receptors

Their main role of PRRs is to identify evolutionarily conserved molecular patterns known as PAMPs, which are typical structures for invading pathogens and can be found on microbial products, as well as DAMPs that derive from damaged host cells like for example proteins (Janeway, 1989).

To be able to sense and respond to an immensely diverse group of danger signals in their local environment, PRRs can be found within the cytoplasm of cells, as well as associated with cell membranes on the cell surface or in endosomes. Currently, PRRs can be divided into five groups: Toll-like receptors (TLRs), C-type lectin receptor (CLR), Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and AIM2-like receptors (ALRs) (Brubaker, Bonham, Zanoni, & Kagan, 2014). All these receptors recognize PAMPs. An overview of all PRRs, their location as well as their ligands can be found in Table 1-1.

TLRs were the first to be identified and remain the most studied type of PRR; they are known to recognize microbial pathogens that include viruses, bacteria, protozoa and fungi. The human TLR family consists of 10 members all of which are membrane associated; some on the cell surface

and in the endosome. TLRs on the cell surface primarily recognize components of the microbial membrane such as lipids, lipoproteins and proteins, whereas intracellular TLRs recognize nucleic acids that derive from bacteria and viruses as well as from host cells during diseases such as autoimmunity (Blasius & Beutler, 2010). Upon activation of this pathway, the ectodomain with leucine-rich repeats (LRR) transfers the PAMP signal through to the cytoplasmic Toll-interleukin (IL)-1 receptor (TIR) domain, which then induces downstream signalling (van Duin, Medzhitov, & Shaw, 2006). This leads to the recruitment of TIR domain-containing adaptor proteins such as MyD88 and TIR-domain-containing adapter-inducing interferon-β (TRIF) and results in the activation of NF-κB, Interferon regulatory factors (IRFs) or mitogen-activated protein kinases (MAPK) that finally lead to the expression of inflammatory cytokines, chemokines and type 1 interferons. These then protect the host organism from the microbial infection (Kawasaki & Kawai, 2014). Lipopolysaccharide (LPS) is a typical TLR4 agonist used in *in vitro* studies that induces maturation of the DC. LPS is an element of the external membrane of gram-negative bacteria. Signalling through TLR4 induces the production of pro-inflammatory mediators, which then aim to eliminate the bacteria. This process is mediated through the dimerization of MyD88 and TRIF, whereby MyD88 leads to the production of inflammatory cytokines and TRIF drives the production of type I interferons (Płóciennikowska, Hromada-Judycka, Borzęcka, & Kwiatkowska, 2015).

CLRs are able to detect a wide range of microorganisms and compromise 17 different groups (Brubaker et al., 2014). It has been shown in mouse studies that deletion of CLR dendritic cell immunoreceptor (DCIR1), one of the major CLRs, leads to disease and disruption of immune homeostasis by inducing expansion of DCs, which results in autoimmune diseases, as shown in DCIR1 deficient mice (Fujikado et al., 2008). Dectin-1 is a member of CLR family of receptors and responds in DCs to fungal β-glucan carbohydrates, which are a common product from *Candida albicans* and *Aspergillus fumigatus* (Dennehy & Brown, 2007).

NLRs are able to detect PAMPs and endogenous molecules in the cytosol (reviewed by Kawai & Akira, 2009). In the cytosol, they are able to detect intracellular pathogen invasion as well as damage of cellular compartmentalization. For example, NOD2 recognizes bacterial muramyl-dipeptide (MDP) (Kofoed & Vance, 2012). Also, some NLRs are activated by the loss of cellular membrane integrity indicating that they are able to detect a different type of damage and may represent a back-up system if pathogen successfully avoided TLR detection (Krishnaswamy, Chu, & Eisenbarth, 2013). The NLRs NOD1 and NOD2 have been shown to enhance cytokine production in human and murine DCs together with TLR4, which in turn affects the fate of T cell differentiation (Fritz et al., 2007; Schwarz et al., 2013). It has been demonstrated that DC NOD

signalling was necessary in order to induce robust T cell responses and therefore NOD ligands are crucial for antigen-presentation (Magalhaes et al., 2011). Also, NLRs are involved in the formation of the inflammasome and the most fully characterized NLR is NLR family pyrin domain contain 3 (NLRP3), which is associated with particle toxicity on the lung. Upon activation, NLRP3 binds to the adaptor protein, apoptosis speck-like protein, containing a caspase activation and recruitment domain (CARD), (ASC), which then leads to activation of caspase-1 (Sayan & Mossman, 2016).

RLRs are mainly known to protect against RNA viruses, however, they also provide defence against DNA after generation of template RNA. RLRs have RNA helicase domains that recognise viral double-stranded RNA and contain a C-terminal regulatory domain that recognises single-stranded RNA. Also RLRs are able to distinguish foreign and self-RNA by the 5'-modification, which is present in self-RNA (Chiu, MacMillan, & Chen, 2009). In addition to RNA, RLRs are able to detect DNA from viruses and bacterial pathogens as well as self-DNA, which can induce autoimmune responses. It has been shown that in macrophages, which have lost the ability to digest self-DNA from apoptotic cells, RLRs induce production of IFN-β (Okabe, Kawane, Akira, Taniguchi, & Nagata, 2005; Yoshida, Okabe, Kawane, Fukuyama, & Nagata, 2005).

The ALR is part of the inflammasome, which will be discussed in detail in the next section. Briefly, absent in melanoma 2 (AIM2) induces ASC-dependent caspase-1 activation, which leads to the secretion of IL-1β and IL-18 (Fernandes-Alnemri, Yu, Datta, Wu, & Alnemri, 2009). Activation of AIM2 is triggered by microbial double-stranded-DNA or host DNA, which then leads to the assembly of the inflammasome. Typical viruses leading to AIM2 activation include Francisella, Listeria, Mycobacterium, mouse cytomegalovirus, vaccinia virus, Aspergillus and Plasmodium. Inappropriate reaction to self-DNA results in the development of autoimmune diseases, such as psoriasis, dermatitis and arthritis (Man, Karki, & Kanneganti, 2016).

Table 1-1: Pattern recognition receptors

The table shows the five families of pattern recognition receptors, their location and their ligands. (Abelson, Gamache, & McLaughlin, 2014; Brubaker et al., 2014)

PRR	Location	Ligand/PAMP	Ligand source
Toll-like receptors (TLRs)	Plasmamembrane (cell surface or endosomal)	Lipoproteins, DNA, RNA, Endotoxin, Endogenous danger signals	Bacteria, Viruses, Parasites, Self
C-type lectin receptor (CLR)	Plasmamembrane	Beta-glucans	Fungi
Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)	Cytoplasm	Endogenous danger signals, muramyl dipeptides	Self, Bacteria
Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs)	Cytoplasm	Double-stranded RNAs	RNA viruses
AIM2-like receptors (ALRs)	Cytoplasm	Intracellular DNA	Bacteria, Viruses

1.1.4.2. Inflammasomes

Inflammasomes were described recently, in 2002. They consist of a macromolecular complex that senses danger signals and induces inflammatory reactions and therefore plays an important role in innate immunity (Dagenais, Skeldon, & Saleh, 2012). The signals and pathways involved are illustrated in Figure 1.2. It is formed in the cytostolic compartment after the cell encounters PAMPs and DAMPs. This structural scaffold for the construction of the inflammasome consists of pyrin domains and CARD in the sensor, which enhances the activation of caspase-1 (A. Lu & Wu, 2015). The main outcome of inflammasome activation is the secretion of pro-inflammatory

cytokines IL-1β and IL-18 as well as the induction of pyroptosis, which is defined as a necrotic cell death characterized by swelling of the cells and rupture of the plasma membrane leading to IL-1β secretion and an inflammatory reaction (Rabolli, Lison, & Huaux, 2016). The interaction of several signals is necessary to induce these outcomes.

The first signal is provided by a TLR-agonist, which leads to the activation of the transcription NF-κB and induces transcription of pro-IL-1β pro-IL-18, as well as synthesis of structures needed for inflammasome activation, such as NLRs. In *in vitro* studies, cells need priming with, for example, LPS prior exposure to an inflammasome agonist (Rabolli et al., 2016). The second signal is required for activation of the inflammasomes complex leading to cleavage of the inactive pro-IL-1β and pro-IL-18 into its active form IL-1β and IL-18. The inflammasome consists of a sensor protein, an adaptor protein and the inactive form of the caspase-1 enzyme, pro-caspase 1. Sensor proteins include pattern recognition receptors NLR and ALR, which lead to the activation of the adaptor protein ASC which in turn cleaves pro-caspase 1 into its active form caspase-1 (Fernandes-Alnemri et al., 2009). Caspase-1 is then able to cleave pro-IL-1β as well as pro-IL-18 into their active forms IL-1β and IL-18 (Latz, Xiao, & Stutz, 2013). These are then secreted into the environment and lead to an inflammatory response (Gang Chen & Pedra, 2010). The caspase-1 dependent responses are the hallmark of inflammasome activation. This second signal can be induced by diverse stimuli, including cellular uptake of bacterial toxins as well as large particles like silica and asbestos (Martinon, Mayor, & Tschopp, 2009). It is thought that the action of these diverse stimuli may converge on a single effect such as efflux of potassium ions providing a common pathway for inflammasome activation.

NLRP3 is expressed in DCs and can be activated by endocytosis of silica particles (Rabolli et al., 2016). The involvement of inflammasome activation in DCs has been shown in several diseases. HIV-1 induces inflammasome activation in healthy volunteers, suggesting its contribution to the first steps of HIV-1 infection (Pontillo et al., 2012). Also, dying cancer cells resulting from chemotherapy have been reported to induce inflammasome responses in DCs by releasing ATP and ultimately leading to the secretion of IL-1β, which in turn primes IFN-γ producing $CD8^+$ T cells, which are important for the antitumor outcome of the therapy (Ghiringhelli et al., 2009). IL-1β contributes to the inflammatory processes in type 2 diabetes and it has been reported that islet amyloid polypeptide, from pancreatic islets, induces inflammasome activation in DCs (Masters et al., 2011). More importantly for DCs in the respiratory tract, activation of the inflammasome has been shown to be involved in the development and exacerbation of asthma. It has been demonstrated in asthmatics that IL-1β and IL-18 are increased as well as caspase-1 activity is enhanced (T.-H. Lee, Song, & Park, 2014). Also, administration of IL-1β induces airway

hyperreactivity, which is a typical feature of asthma (Zhang, Xu, & Cardell, 2009). As air pollution particles have been shown to contribute the pathology of asthma, it has also been reported that they may induce inflammasome activation (Hirota et al., 2012). However, whether PM is able to signal through the inflammasome in human DCs has yet to be determined.

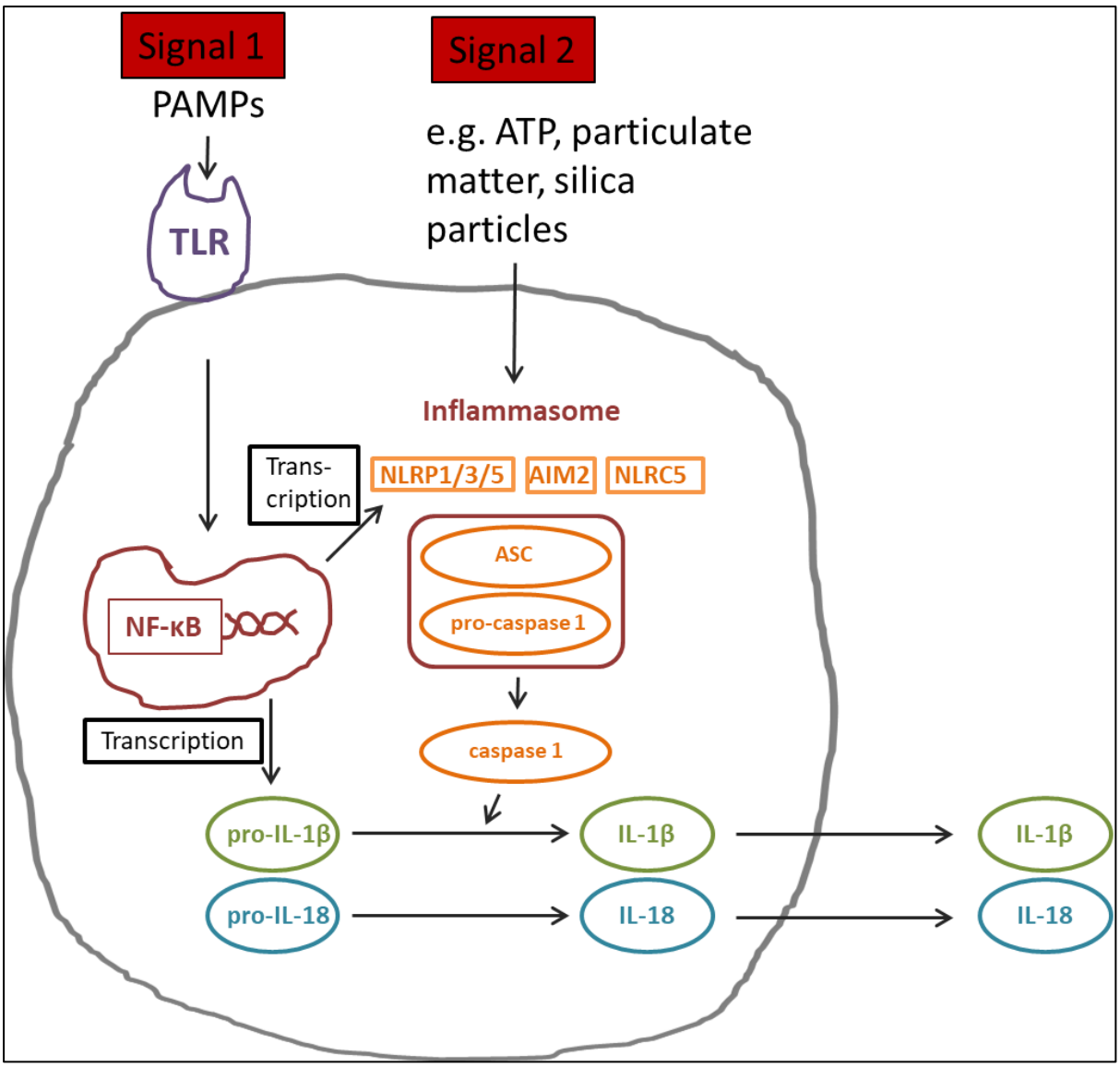

Figure 1.2: Inflammasome

Signal 1 induces the transcription of pro-IL-1β and pro-IL-18, whereas signal 2 activates the inflammasome and leads to the cleavage of pro-caspase-1 into its active form caspase-1. Caspase-1 then in turn cleaves pro-IL1-β and pro-IL-18 into their active forms IL-1β and IL-18 (InvivoGen, 2009).

1.1.4.3. Aryl-hydrocarbon Receptor

Another noteworthy receptor which potentially mediates immunological impacts on DCs in response to environmental signals is the aryl hydrocarbon receptor (AhR), which is a ligand activated transcription factor. This pathway was first investigated in order to understand the toxic effects of 2,3,7,8-tetrachlorodibenzop-dioxin (TCDD), which is a ligand for AhR. After interaction with its agonists, the AhR mediates the expression of several xenobiotic metabolizing enzymes, such as *CYP1A1*, *CYP1A2* and *CYP1B1*. Activation of this pathway is known to occur after interaction with polycyclic aromatic hydrocarbons (PAH), for example those derived from combustion of fossil fuels and adsorbed to the surface of environmental particulate matter (Andrysík et al., 2011). For an illustration of the canonical AhR pathway, please see Figure 1.3. Briefly, after passing the cell membrane, AhR ligands enter the cytosol and bind to the AhR, which leads to translocation of the complex into the nucleus. There, the ligand-AhR complex binds to the AhR nuclear translocator (ARNT), which leads to a high affinity of the complex to the DNA. This enables the ligand-AhR-ARNT complex to bind to the specific DNA recognition site, the dioxin - responsive element (DRE). Binding of the ligand-AhR-ARNT complex to the DRE leads to the transcription of genes such as *CYP1A1*, *CYP1A2* and *CYP1B1*, and many other AhR responsive genes. *CYP1A1* is present in most species and its expression is often used as a marker of AhR signalling. After transcription, the mRNA is transported to the cytosol, where the translation takes place and the enzyme is synthesized. The completed enzyme is then able to metabolize and inactivate AhR ligands and through this are able to prevent the cell from further damage (Chiaro, Patel, Marcus, & Perdew, 2007).

The presence of the AhR in DCs has been demonstrated and it has been shown that signalling through this pathway can lead to pro-inflammatory as well as anti-inflammatory effects (Frericks, Meissner, & Esser, 2007; S.-K. Huang, Zhang, Qiu, & Chung, 2015; Stockinger, Di Meglio, Gialitakis, & Duarte, 2014). Stockinger et al 2014 suggested that overall AhR activation seems to have anti-inflammatory effects on DCs because AhR appears to control the production of *indoleamine-2,3-dioxygenase* (*IDO)* and IL-10. Nonetheless, a systematic comparison of all DCs subsets has yet to be conducted. The detrimental effect of smoking on rheumatoid arthritis has been shown to be mediated through synovial DCs by signalling through AhR (Kazantseva, Highton, Stamp, & Hessian, 2012). In the lung, AhR has been investigated particularly in infection models, showing an immunomodulatory effect during influenza infection (Head & Lawrence, 2009). It has been shown in mice that exposure to dioxin, a synthetic AhR ligand, before an influenza infection decreases the expansion and activation of CD8 T cells in lung tissue leading to a reduction of IFN-γ secretion, which is in line with the immunosuppressive characteristic of AhR

(Warren, Mitchell, & Lawrence, 2000). It is unknown how exactly the T cell suppression is linked to AhR; however, it has been suggested that lung-resident DCs play an important role in immunosuppression (G. B. Jin, Moore, Head, Neumiller, & Lawrence, 2010). DCs in the respiratory tract are involved in antigen uptake which leads to T cell differentiation in the lymph nodes and also induces inflammation by secreting cytokines. In addition, it has been reported that IDO is controlled by AhR and may add to the reduction of immune responses observed, when AhR is activated (Nguyen et al., 2010; Vogel, Goth, Dong, Pessah, & Matsura, 2008). IDO as an immunosuppressive enzyme that acts through the depletion of tryptophan and therefore affects the growth of T cells (Mbongue et al., 2015).

It has been suggested that endogenous AhR ligands, which compromise compounds originating from within an organism, as opposed to exogenous, which originate from outside an organism, are involved in Th2 type immunity in the lung meditated through DCs. This was shown in an AhR-deficient mouse model; therefore, AhR in DCs may attenuate allergic airway diseases (Thatcher et al., 2016). It has been described that some effects of airborne particulate matter are partly mediated through AhR as PAH are one of the main components of PM; therefore, the AhR may link the exposure to air PM to the development of asthma and allergies (S.-K. Huang et al., 2015) In mice, exposure of mast cells to airborne PM components led to an enhanced mast cell signalling, degranulation, mediator and cytokine release and it has been shown that AhR ligands specifically regulate mast cell responses, suggesting that the AhR pathways links the exposure to environmental pollutants to the development of allergic diseases (Maaetoft-Udsen, Shimoda, Frøkiær, & Turner, 2012; Sibilano et al., 2012; Y. Zhou et al., 2015). However, how aromatic hydrocarbons that are present on air pollution particles affect human DCs in the respiratory tract has not been examined yet.

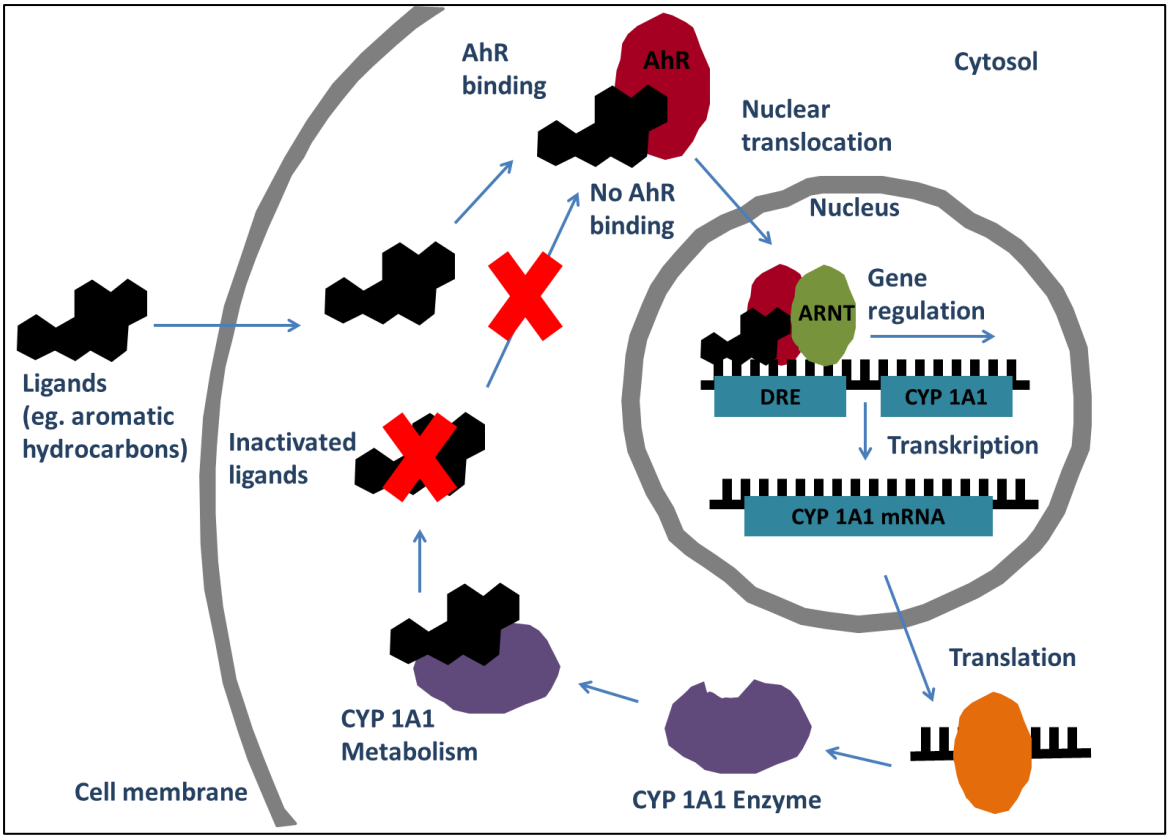

Figure 1.3: Aryl hydrocarbon receptor pathway

AhR ligands bind to the AhR and the complex translocates into the nucleus. There, after interaction with ARNT, the complex develops a high affinity to the DNA, leading to the binding of-AhR-ARNT to the specific DNA recognition site, the dioxin - responsive element (DRE). This is followed by the transcription of genes such as CYP1A1, CYP1A2 and CYP1B1, and several other AhR responsive genes. After this, the mRNA enters the cytosol and the corresponding proteins are translated. The enzymes then metabolize and inactivate AhR ligands and therefore are able to prevent further cell damage (Chiaro et al., 2007).

1.2. The respiratory tract and its immune system

The respiratory tract is the organ responsible for supplying essential oxygen to the blood circulation by breathing. It has several distinct properties that enable it to fulfil the task. The lung is easily accessible by air. The epithelial cell layer in the alveoli, where the gas exchange with the bloodstream takes place, has a large surface area. Through the pulmonary airway, air can be transported quickly to the alveoli after inhalation. This means that gases, liquids and solids dispersed in the inhaled air can easily reach different areas of the lung and can even enter the cardiovascular system, triggering local and systemic effects. This can be a beneficial characteristic, e.g., when delivering drugs to the lung, or it can be a detrimental characteristic, e.g., when harmful substances like bacteria and viruses or air pollution particles are present in the air, causing health problems after inhalation. Consequently, several mechanisms exist that protect the lung and the body from damage caused by inhaled antigen but still allow sufficient gas exchange (Strohl, Butler, & Malhotra, 2012; Weibel, 2009).

The respiratory tract is equipped with multiple layers of defence mechanisms (Nicod, 2005b). Immediately when entering the respiratory tract through the nose, small objects are retained by the nasal hair and objects like dust or sand are removed by sneezing. The epithelium in the airway is covered with mucus which entraps inhaled particles and transports them up through the movement of ciliated cells towards the oesophagus, where material is then swallowed and digested by the gastrointestinal tract or coughed up (Knowles & Boucher, 2002). In addition to that, there are immune cells below the mucus layer in the conducting airways, including DCs, which are responsible for taking up any antigen that managed to pass the thick mucus layer and potentially can initiate adaptive immune responses. If harmful materials still manage to get to the alveoli, alveolar macrophages are mainly responsible for their removal (Kopf, Schneider, & Nobs, 2014). The thick mucus layer and the ciliated epithelial cells that are present in the conducting airways are not present in the alveoli as they would interfere with the oxygen exchange through the thin blood-air-barrier in the alveoli. Also, another important aspect of the distal airways, where the gas exchange takes place, is that immune responses need to be carefully controlled to ensure that tissues involved in gas exchange do not get damaged and a constant supply of oxygen to the body is guaranteed. Therefore, a delicate balance between inflammatory and anti-inflammatory processes is needed for lung homeostasis (Moldoveanu et al., 2009).

DCs play a crucial role in immune responses towards inhaled pathogen to the lung and body. Firstly, they are the main immune cell type in the conducting airways where a large amount of

inhaled PM is retained. Secondly, as APCs and cells that are responsible for phagocytosis, they are in charge of uptake of inhaled antigen as well as self-antigen and therefore form the first line of defence against inhaled threats. Through this, they initiate immune responses and therefore determine the type of immune response that evolves in reaction to a particular threat. These functions are the reasons why DCs are involved in several lung associated illnesses such as asthma, which is also associated with the exposure to air pollution particles (Peter C. Cook & MacDonald, 2016).

1.2.1. Protective mechanisms in the respiratory tract

Inhaled air enters the respiratory system through the conducting airways, i.e. the nose and the mouth, and passes the pharynx and larynx, which form the upper respiratory tract. From there, the air flows into the lower respiratory tract by entering the trachea, which splits in two branches, the lung lobes, and enters the bronchi and bronchioles, which split several times until reaching the alveoli. The alveoli form the main respiratory region where the oxygen exchange takes place. All in all, the lung has a volume of 5l and processes around 8000-9000 litres of air per day. Due to its extensive branching of the conducting airways, the respiratory area of the lung exceeds a surface of 120m^2; it represents the body's second-largest mucosal surface area after the gastrointestinal tract (Burri, 2011; Kopf et al., 2014; Sato & Kiyono, 2012).

The key function of the conducting airways is not only to conduct inhaled air but also to filter unwanted substances like pathogens from the inhaled air. Consequently, the airways are equipped with facilities to enable this: the epithelium is covered by a thick layer of sticky and viscous mucus secreted by goblet cells located in the airway epithelium which entraps inhaled antigen, thereby acting as a physical trap (Langenback et al., 1990). Moreover, the mucus also has an antibiotic role since it contains anti-microbial peptides and oxidizing enzymes which counteract the microbial substances that are inhaled by themselves or can be present on the surface of air pollution particles (Ryu, Kim, & Yoon, 2010; Trompette et al., 2009). Furthermore, it also contains IgA, which contributes to the mechanism of mucocilliary clearance. Once inhaled, the pathogens are captured in the mucus layer. Ciliated epithelial cells are in charge of the movement of the mucous raft that floats on a watery fluid whereby the cilia propel the mucus layer towards the larynx, where it then either enters the oesophagus (where material is then swallowed) or is coughed up. This cleansing system is known as the mucociliary escalator and represents the primary innate defence mechanism in the human airways and is therefore part of the innate immune system (Hogg, 2004; Knowles & Boucher, 2002). Dysfunction of the mucociliary escalator leads to severe breathing problems and reoccurring infections since pathogens are not removed effectively, e.g., as observed in cystic fibrosis or chronic obstructive

pulmonary disease (COPD) (Cole PJ., 1986). Underneath the mucus, the epithelial cells are equipped with a protective barrier of tight junctions that prevent solutes passing through and therefore form another obstacle for inhaled pathogens like bacteria and viruses as well as inhaled air pollutants (Patton, 1996). In addition to that, immune cells are present on the mucosal surface acting as another protective barrier against inhaled threats.

After passing the conducting airways, inhaled air reaches the respiratory areas, which consist of the respiratory bronchioles, alveolar ducts and the pulmonary alveolus. The gas exchange mainly takes place in the alveoli. Before reaching the alveoli, the airways bifurcate approximately 16–17 ending in roughly 480 million alveoli, which are then surrounded by pulmonary capillaries (Lorenz, 1966; Ochs et al., 2004; Patton, 1996). Again, since the main function of the respiratory surfaces is to supply oxygen to the body and to release carbon dioxide from the blood through the lungs into the environment, the alveoli show differences in the morphology to the conducting airways, which enable them to do so. Firstly, the epithelium becomes thinner the closer it gets to the alveoli, reaching a thickness of 200nm at the thinnest point, which is essential to facilitate oxygen exchange between the air in the alveoli and the blood in the capillaries. In addition, the number of tight junctions decreases further down in the airways; it is five times lower in the alveoli compared to the conducting airways, which again facilitates the gas exchange (Patton, 1996). Secondly, the thick and viscous mucus layer is not present in the alveoli since it would block the very thin tissue barrier and impair the gas exchange. To still be able to protect itself from inhaled threats that managed to escape the clearance mechanisms in the conducting airways, the alveoli have their own protective barriers such as alveolar macrophages, small numbers of DCs and humoral (soluble) immune mechanisms in the surfactant that is lining the alveolar surface (Hasenberg, Stegemann-Koniszewski, & Gunzer, 2013).

1.2.2. Cellular immune regulation in the lung

In order to match their distinct functions, the conducting and respiratory areas have distinct populations of immune cells which reside in different locations to be able to address the specific threats that reach each area. It is crucial that immune responses in the lung a carefully controlled to avoid an overreaction which may endanger the constant air flow to the lung and therefore the oxygen supply to the body (Desch, Henson, & Jakubzick, 2013).

In the conducting airways, the first cell type interacting with inhaled threats are the epithelial cells, which form not only a barrier against environmental pollutants but also secrete a range of defence molecules such as mucins and surfactant proteins. (Holt, Strickland, Wikstroem, &

Jahnsen, 2008a). Furthermore, they are involved in the modulation of nearby cells, such as DCs, and contribute to their maturation after exposure to environmental pollutants (Bleck, Tse, Lafaille, & Zhang, 2008). In addition, APCs such as macrophages and DCs are present; the latter form the predominant immune cell type. Moreover, T cells can be found in the conducting airways: $CD8^+$ T cells are present within the epithelium and $CD4^+$ T cells are located in the lamina propria, just below the epithelial cell layer. Furthermore, the lamina propria also contains mast cells, plasma cells and B cells (Holt, Strickland, Wikstroem, & Jahnsen, 2008b).

As well as playing an important role in the conducting airways, epithelial cells are also present in the lung parenchyma, where two different types can be found: type I and II. Although Type I alveolar epithelial cells are present in lower numbers compared to the type II cells, they cover most of the surface of the alveoli, providing a thin lining as they have a flat shape. On the other hand, type II alveolar epithelial cells are round in shape and produce and store surfactant, which prevents the collapse of the alveoli upon expiration (Hasenberg et al., 2013). In addition to that, they replace the type I cells in case of damage (Weibel, 2009).

In contrast to the conducting airways, in the lung parenchyma, where the gas transfer takes place, alveolar macrophages are the main immune cell type present. They are located above the alveolar epithelium and in the subjacent parenchyma and typically 12-14 alveolar macrophages are patrolling in each alveolus (Stone KC, Mercer RR, Gehr P, Stockstill B, 1992). Alveolar macrophages account for 90% of the total leukocyte population, the rest being mainly DCs and T-cells (Holt et al., 2008a). Since the remaining immune cells are located under the surfactant that is lining the alveoli, they are forced to lie flat below the surfactant layer; hence, surfactant proteins may physically influence the cells (Hasenberg et al., 2013). Some of the proteins present in the surfactant also have immunological functions: they can bind bacterial lipopolysaccharide or they can cover the surface of pathogens, which then leads to pathogen aggregation and killing, or they can increase uptake and destruction activity of the surrounding immune cells (Hasenberg et al., 2013). In addition, surfactant proteins can also have immunregulatory functions, e.g., by affecting dendritic cell maturation or inhibiting T-cell proliferation (Wright, 2005). Finally, surfactant proteins, mainly SP-A and SP-D, have immunomodulatory functions since a lack of these proteins has been shown to result in a skewing of a protective T-helper 1 response into a non-protective T-helper 2 response, which is associated with hypersensitivity reactions (Madan, Reid, Singh, Sarma, & Kishore, 2005). It has been shown that SP-D plays a protective role in lung infection, allergy and inflammation by reducing IgE levels, eosinophilia as well as re-establishing Th1 and Th2 homeostasis. Also, SP-D gene deficient mice overexpress IL-

13, a cytokine commonly involved in Th2 immunity and allergic diseases (Mahajan, Gautam, Dodagatta-Marri, Madan, & Kishore, 2014; Qaseem et al., 2013).

1.2.3. Dendritic cells in the lung

DCs in the lung are mainly located in the conducting airways where they reside within and underneath the epithelial cell layer and reach in between the tight junctions of the epithelial cells into the airway with their dendrites and sample inhaled pathogens and other threats to the body without disrupting the protective epithelium (Jahnsen et al., 2006). Through their location and the protrusions into the airway lumen, they are one of the first immune cells to get in contact with inhaled particulates in the conducting airways, allowing them direct interaction with the threat (Jahnsen et al., 2006; Nelson, McMenamin, McWilliam, Brenan, & Holt, 1994). Due to their characteristics as phagocytes and APCs, they determine whether an immune response takes place and, if so, DCs influence the magnitude and the nature of the immune reaction. In the lung mucosa, DCs consist of classical or conventional and plasmacytoid DCs, although the cDC subset dominates, which again compromises different subsets. There is an increasing interest in pDCs since they play a role in tolerance induction to inhaled antigen (H. J. de Heer et al., 2004; Iwasaki, 2007). MoDCs can also be found in the lung although their function at steady state still continue to be unclear (Kopf et al., 2014).

1.2.4. Dendritic cell subsets and function in the lung

Our knowledge about respiratory tract dendritic cell subsets and their function in the lung is mainly based on animal models and their translation into humans can be a big challenge (C H GeurtsvanKessel & Lambrecht, 2008). In mice, cDCs are the predominant type of DCs in the lung; they carry out the main DC functions and can broadly be divided into cells that express integrin CD103 and integrin CD11b, which share the tasks among them. Both cDC subtypes express the lineage-specific transcription factor *Zbtb46* (Meredith, Liu, Darrasse-Jeze, et al., 2012b; Ansuman T Satpathy, Kc, et al., 2012). Lung $CD103^+$ require the transcription factor *BATF3* for their development, similar to lymphoid-resident $CD8\alpha\alpha^+$ cDCs (Edelson et al., 2010; Hildner et al., 2008). Also, $CD103^+$ cDCs require *IRF8* as well as *Id2* in order to fully develop (Ginhoux et al., 2009). For the development of $CD11b^+$ cDCs the transcription factor *IRF4* is needed (Schlitzer et al., 2013). It has been suggested that each of the DC subtype induces a distinct T helper cell outcome, whereby in a house dust mite model a Th2 response is initiated by $CD11b^+$ cDCs and in a fungal model a Th17 outcome (Plantinga et al., 2013; Schlitzer et al., 2013). $CD103^+$ cDCs have been shown to initiate Th1 and Th2 responses (Furuhashi et al., 2012; Nakano et al., 2012). Our knowledge of the function of lung DC subsets is mostly based on respiratory viral infections in

mice. Infection with the influenza virus leads to the CCR7-dependent migration of DCs to the lymph nodes and subsequent induction of an adaptive immune response by activating CD8 T cells (Corine H GeurtsvanKessel et al., 2008; A. K. Heer, Harris, Kopf, & Marsland, 2008). Generally, it has been shown that $CD103^+$ cDCs with regard to their cross-presentation abilities, uptake of apoptotic cells and their MHC Class I loading are superior compared to $CD11b^+$ cDCs, whereas $CD11b^+$ cDCs are the dominant subset to induce CD8 T cell responses on day 5 after infection. This suggests that $CD103^+$ cDCs are responsible for immune responses during acute infection and $CD11b^+$ cDCs are rather inducing long term immunity (Kopf et al., 2014).

Data from human and humanized mice showed that both human $CD1c^+$ and $CD141^+$ DCs were able to take up influenza virus *in vivo* and lead to cytotoxic $CD8^+$ T cell responses *in vitro*. But only $CD1c^+$ DCs led to expression of CD103 on $CD8^+$ T cells resulting in CD8+ T cell accumulation in the lung epithelium. This process was dependent on transforming growth factor beta 1 (TGF-β1), suggesting that DC subtypes induce $CD8^+$ T cell responses with different properties, in which $CD1c^+$ DCs regulate mucosal $CD8^+$ T cells (Yu et al., 2013).

1.3. Air pollution particles

Air pollution is the number one environmental cause of death in the world and accounts for approximately seven million deaths worldwide per year according to the World Health Organization (WHO) (S. B. Gordon, Bruce, Grigg, Hibberd, Kurmi, Lam, Mortimer, Havens, et al., 2014). The health effects associated with air pollution are wide-ranging, including effects local to the respiratory tract, such as development and exacerbation of asthma, COPD, the development of lung cancer and respiratory infections. Systemic health problems linked to polluted air comprise cardiovascular issues, such as high blood pressure, stroke, cardiac infarction and arrhythmia (Brook, 2008).

Air pollution is characterised by a broad mixture of substances containing liquids, gases (such as ozone and nitrogen oxides) and solids (such as carbonaceous PM) (Brook, 2008). These three different states of matter may interact with each other; for example, liquids may adsorb onto the surface of solid PM or may coexist in the air, forming a diverse air pollution mixture. However, of the different types of air pollution, PM has the largest adverse impact on human health (Brook et al., 2004a; J. C. Chow et al., 2006; EPA., 2004). Several events in history, such as the London fog of 1952, demonstrate the significant impact of PM on mortality rate; in this case, an estimated 1,200 people died because of the smog's effects on the respiratory tract (M. L. Bell, Davis, & Fletcher, 2003). Through this incident, awareness of the relationship between health and air pollution has increased, leading to an increase in environmental research and change in government policies.

Not only is it important to distinguish between different states of matter within air pollution but also between different sources of air pollution and the environment where humans are exposed to them, as well as the groups of the society that are exposed. Generally, polluted air is a bigger problem in low- and middle income countries; in more developed countries, more effective policies and technology exist which limit the emission of pollutants into the environment. However, in urban areas in developed countries, PM is widespread and underlines the adverse health effects of air pollution.

1.3.1. Development and origin of air pollution particles

The generation of air pollution particles is mainly based on the combustion of energy sources, such as wood or fossil fuels. Hence, air pollution particles all tend to be created through a similar burning process. However, even a broadly similar process can progress in different ways. For instance, an important part of the combustion process is the availability of oxygen, which is

essential for the burning itself. Insufficient oxygen will lead to an incomplete combustion and the formation of soot, which is elemental carbon.

$$CH_4 + O_2 \rightarrow H_2O + C$$

Methane + Oxygen → Water + Carbon

Figure 1.4: Combustion process of methane

The equation shows an incomplete combustion process with insufficient oxygen supply during the burning process leading to the formation of water and carbon ("http://environ.andrew.cmu.edu/m3/s3/09fossil.shtml," n.d.).

An incomplete combustion of organic substances such as methane due to a lack of oxygen leads to the formation of water and elemental carbon. Also, toxic carbon monoxide is produced through incomplete combustion. A complete combustion with sufficient oxygen present produces carbon dioxide (CO_2) and water.

Incomplete combustion will lead to the formation of various organic and inorganic compounds, including aromatic and aliphatic hydrocarbons. Smoke and vapour generated during the burning process will interact with the combustion product, adsorb it and form fragmented particles. Not only do carbon particles represent a health hazard themselves but they also provide a platform for other substances which are generated through incomplete combustion to be adsorbed to the carbon particles and form a mixture of pollutants.

PM is classified according to size. The size of particles is measured using the aerodynamic diameter, which facilitates the assessment of their dimension since particles usually have an irregular shape that makes an accurate measurement difficult. The aerodynamic diameter is defined as the diameter of the spherical particle with a density of 1,000kg/m^3 and the same settling velocity as the irregular particle (Hinds, 1999). Generally, several size fractions have been defined by the International Standards Organisations. The "inhalable" size fraction describes the particles that are able to enter through the nose and are referred to as PM10. Within PM10, two size fractions can be distinguished. First, the "thoracic" size fraction defines the particles penetrating beyond the larynx and contains particles with a size between PM10 and PM2.5. The second size fraction refers to "respirable" particles which are able to reach the deep lung and contain PM2.5. The most common definition of PM10 is that they contain air pollution particles with an aerodynamic diameter of less than 10µm, as described by the Environment Agency and the Health Protection Agency. However, the International Standards Organisation

defines them as “particles which pass through a size-selective inlet with a 50 % efficiency cut-off at 10µm aerodynamic diameter”. PM2.5 can be defined in a similar way. Widely used is the definition that they are PM with an aerodynamic diameter smaller than 2.5µm. Or, the International Standards Organisation defines them as “particles which pass through a size-selective inlet with a 50 % efficiency cut-off at 2,5 µm aerodynamic diameter” (USEPA, 2004). All of the different particle types have not only different sizes, but with the size come different physicochemical properties, which are linked to their exact composition (B. Wang et al., 2013). Since the definition suggested by the Environment Agency and the Health Protection Agency is more widely used, we will be referring to their definition of particle sizes in this thesis.

1.3.2. Composition of particulate matter

Air pollution particles are a complex mixture of different components, which vary depending on several different factors such as combustion process and source, environmental influences like pollen, dust and bacteria, and chemicals like metals and organic compounds which adsorb to the surface of the particle.

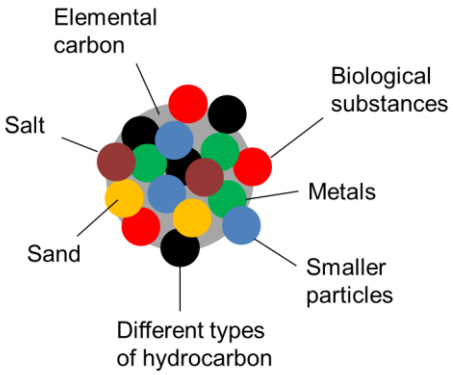

Figure 1.5: Composition of air pollution particles

Airborne particulate matter consists of an elemental carbon core with various substances adsorbed to its surface. Biological substances include bacteria, components derived from them and pollens and organic compounds contain different types of hydrocarbon derived from the combustion itself. Salt may be found on particles that were collected close to the coastline and sand is associated with the Sahara in Africa. Metals may derive from car abrasion such as brakes and include copper, iron and zinc (Brook et al., 2004b; B. Wang et al., 2013).

One component common to all particle types is the carbon core, which consists of elemental carbon and carries all additional substances on its surface. These substances then depend on the environment where the particles originate and can vary significantly (F J Kelly & Fussell, 2011).

The extra constituents and the size of the carbon core characterize the particle and are responsible for the specific health effects rather than the inert elemental carbon core, as the carbon core has been shown to be similar for all particle types, whereas additional components on the carbon core tend to be influenced by different factors and therefore vary considerably (Brook, 2008).

The area in which particles are collected will greatly affect their composition. In urban areas in the UK, the main source of air pollution is the combustion of fossil fuels from cars and industrial production, whereas in developing countries in Africa, the burning of biomass will add to the air pollution mixture. Biomass is mainly used as an energy source in African countries for cooking food and is therefore often found in indoor kitchens or outdoor cooking areas in courtyards. Burning of biomass fuels is associated with a high concentration of carbon black, potassium (K) and sulphur dioxide (SO_2) (Z. Zhou, Dionisio, Verissimo, Kerr, Coull, Arku, et al., 2013a). In developed countries, where air pollution in cities is mainly derived from cars, PM tends show high concentrations of zinc (Zn), lead (Pb), chromium (Cr), manganese (Mn) and cadmium (Cd) on top of the carbon content. Also, calcium (Ca), magnesium (Mg), iron (Fe) and aluminum (Al) are present as well as PAH, which are a strong indicator of motor vehicle emissions (Oliveira, Ignotti, & Hacon, 2011). Major components of PM in urban areas are road dust resuspension, break and tyre wear, whereas the combustion of fossil fuel from cars contributes 5-10% to the overall composition of urban PM. In general, the emissions in developed countries are tightly regulated, which tends to make it a smaller problem than in developing countries. However, legislation by authorities has led to a reduction of exhaust emissions, whereas non-exhaust emissions are hardly controlled (Wahid, 2017).

Other environmental factors also play a role in the composition of air pollution particles. For instance, PM from areas close to the sea have a higher concentration of salt, NaCl, derived from the seawater. PM from countries in the proximity of a desert, e.g., African states surrounding the Sahara, are likely to contain mineral oxides such as Al_2O_3 and SiO_2 during the dry and windy season called “Harmattan” (Z. Zhou et al., 2014). It has been shown that these variable components have biological effects on cells, such as SiO_2 as well as aluminium salts, which are involved in the activation of the inflammasome (Dostert et al., 2008; Hornung et al., 2008).

The size of particles will have an impact on their physical properties and this will then affect the composition. Ultrafine particles have a greater relative content of elemental carbon (J. A. Araujo et al., 2011). High molecular weight polycyclic hydrocarbons are found in higher concentrations the lower the size of the particle (B. Wang et al., 2013). Smaller particles are often associated with combustion metals like copper (Cu) and Zn, chromium (Cr) and Pb. Larger particles on the

other hand contain rock-forming elements, such as Fe, Mn, Nickel (Ni) and Cobalt (Co) (B. Wang et al., 2013).

Finally, airborne PM not only carries artificially generated pollutants but also naturally occurring ones, such as bacteria, fungal spores, viruses as well as allergens like pollen and house dust (Trompette et al., 2009). These than can then be spread through the air and ventilation systems and cause infections such as influenza and pneumonia as well as allergic reactions (La Duc, Stuecker, & Venkateswaran, 2007). It is likely that these additional components affect the overall effect the air pollution particle has on the immune system and on human health.

1.3.3. Distribution of particles in the lung after inhalation

In addition to being a complex mixture of substances, airborne PM consists of various sizes, which can be divided into three different size fractions. The different size fraction will settle in different areas in the lung after inhalation and have different effects.

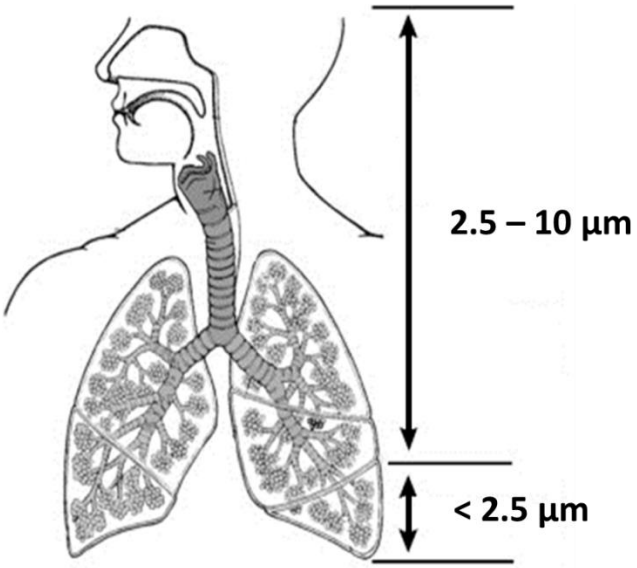

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Figure 1.6: Deposition of different particle size fractions in different areas in the lung

Particles with an aerodynamic diameter between 2.5 and 10µm will settle in the conducting airways and particles that are smaller than 2.5µm are able to advance further in the lung and reach the respiratory region of the lung. PM bigger than 10µm are unlikely to be inhaled since they are likely to fall to the ground due to their size and, if re-suspended, are likely to be retained early on.

The largest particle size fraction, PM10, consists of a wide range of sizes. After entering the airways through the nose or mouth some of these particles can already be trapped in the nose

by nasal hair (Ozturk A.B. Damadoglu E. Karakaya G. Kalyoncu A.F., 2011). The particles that manage to circumvent the first obstacle then face the thick layer of mucus with which the conducting airways are covered. This mucocilliary escalator removes some of the inhaled particles but some particles are able to pass this protective mechanism of the lung and reach the underlying epithelium and may therefore interact with immune cells like DCs (Langenback et al., 1990).

PM2.5 includes the particles that are able to migrate deeper in the lung down to the alveoli where the gas exchange with the blood takes place. The smallest fraction of PM0.1 is even able to pass the blood-air barrier and get into the bloodstream (Howard & Chb, 2009). There, it is able to cause systemic health effects such as cardiovascular problems (see Health effects of air pollution 1.3.5). These smaller particles also get trapped in the mucus of the conducting airways on their way down to the alveoli but they are more likely to escape that cleansing mechanism. The smaller the particles, the more negative is their surface charge and the repellent forces with the negatively charges mucins in the mucus are greater (Lai, Wang, & Hanes, 2009; B. Wang et al., 2013). In addition, larger particles are more easily trapped in the viscous layer of mucus; smaller particles, once embedded in the mucocilliary blanket, have less sterical resistance (Sanders et al., 2000). Once particles have reached the respiratory area, macrophages in the alveoli are another protective barrier. Therefore PM2.5 is more likely to be cleared away by macrophages, whereas PM10, which mainly settles in the conducting airways, encounter DCs (Nicod, 2005a).

Finally, since PM comes in a variety of sizes, smaller particles may stick to larger ones and separate again while moving through the air as well as after inhalation.

1.3.4. Indoor and outdoor air pollution

One aspect that needs to be taken into account are the circumstances under which individuals are exposed to air pollution particles as they may have a significant influence on the health effects. Indoor and outdoor air pollution may vary in a lot of aspects (Frank J. Kelly & Fussell, 2015a).

Indoor air pollution is a major problem in the developing world, where people often use wood, coal and dung as primary fuels for cooking and heating. The burning process is conducted using basic stoves that are not energy efficient leading to incomplete combustion and therefore higher levels of air pollution. This type of pollution mainly affects women, children and elderly people, who spend more time inside while either cooking themselves or around others who are cooking. Especially very young and very old people are prone to adverse health effects caused by air

pollution such as increased respiratory tract illnesses, exacerbation of asthma as well as decreased lung function; this can be particularly detrimental for very young children as their lung is still developing (American Academy of Pediatrics, 2004). Exposure of pregnant women to indoor pollution can have health effects on the unborn child and lead to preterm birth (Defranco et al., 2016). Also, lacking ventilation, especially in colder regions, where the energy from cooking is used to heat the house, leads to further accumulation of PM (S. B. Gordon, Bruce, Grigg, Hibberd, Kurmi, Lam, Mortimer, Havens, et al., 2014).

Outdoor air pollution is more related to car exhaust fumes, mainly from diesel combustion, factory emissions as well as natural causes, e.g., sand dust in Africa or pollen (F J Kelly & Fussell, 2011). The exposure to outdoor air pollution is temporarily, whereas indoor air pollution affects humans over a longer period of time. Also, outdoor air pollution is less concentrated as it gets diluted by the ambient air. Indoor air pollution on the other hand is concentrated within a confined room; hence, individuals are exposed to higher concentrations of PM. Also, behaviour and activities may influence exposure to air pollutants, such as cycling to work in urban areas (Nwokoro et al., 2012).

1.3.5. Health effects of air pollution

Exposure to outdoor and indoor pollution are both linked to detrimental health effects arising from exposure to fossil fuel and biomass derived combustion products. Health effects of air pollution are wide-ranging and depend on the type of air pollution as well as the manner of exposure, can be of local and systemic nature, and may also vary depending on short-term and long-term exposure.

It is well documented that exposure to air pollution increases morbidity and mortality (World Health Organization, 2012). General health effects of air pollution include cardiocerebral vascular disease and ischemic heart disease; they also include detrimental effects on the digestive and urinary system (Jiang, Mei, & Feng, 2016). As a mixture of components, air pollution includes solid and gaseous constituents. The main gases include ozone (O_3), sulfur dioxide (SO_2), nitrogen dioxide (NO_2) as well as carbon dioxide (CO) (American Thoracic Society, 2000). Of the solid components, PM is the most abundant and therefore well studied, and will be discussed in more detail later. O_3 has been shown to increase the risk of appendicitis and higher levels of ozone may even lead to a perforated appendicitis (Kaplan et al., 2013). Exposure to sulfur dioxide in urban areas has been linked to an increase in children's hospital admissions (Barnett et al., 2005). PM, NO_2, and SO_2 has been linked to increased mortality, whereas long-

term exposure to PM2.5 in particular can increase mortality from cardiovascular diseases (Carey et al., 2013; Dockery et al., 1993).

Exposure to outdoor particulate matter can lead to oxidative stress, airway hyper-responsiveness, and airway remodelling, which may present in combination with allergic sensitisation (Stanek, Brown, Stanek, Gift, & Costa, 2011). Short-term exposure to PM10 and PM2.5 has been associated with asthmatic symptoms in children and adults, and long-term exposure may lead to poorly controlled asthma and a decline in lung function (Bayer-Oglesby et al., 2006; Brunekreef & Holgate, 2002; Sunyer et al., 1997). Also, exposure during pregnancy and early life can lead to asthma at pre-school age (Clark et al., 2010). In general, it is well confirmed that ambient PM exacerbates existing asthma, and the majority of studies indicate that it may also lead to new cases of asthma (M. Guarnieri & Balmes, 2014). However, the strength of those studies is variable and does not allow us to draw a clear conclusion whether exposure of ambient PM leads to the development of asthma (M. Guarnieri & Balmes, 2014).

Even though a lot of adverse effects after exposure to air pollution are linked to the respiratory tract, significant health effects can also be found in the cardiovascular system (Brook et al., 2004a; J. C. Chow et al., 2006; EPA., 2004). The particle size fractions that have been linked to the systemic health effects are PM2.5 and UFP since, as a result of their small size, they can reach the respiratory area in the lung, enter the blood circulation and interact with organ systems (Geiser et al., 2005; Mills et al., 2006; Nemmar et al., 2002). UFP can pass into the blood stream where they can interact with organs and reach nearly every part of the body. Not only can they reach the cardiovascular system due to their small size, UFP can also cause more harm compared to the other particles sizes because they contain many of the combustion products that are known to have toxic potential, such as PAH and metals (B. Wang et al., 2013). UFP have a large specific surface area (i.e., their surface area is large in relation to their mass); hence, they can carry relatively large amounts of diverse toxic materials on their surface into the circulation (Brook, 2008). Ultrafine PM is known to induce a greater mitochondrial damage, induced by pro-apoptotic signals, which are triggered by mitochondrial perturbations, and have a higher redox activity compared to larger particle size fractions (J. a Araujo & Nel, 2009a). The systemic health effects of PM after inhalation are far-ranging and include systemic effects like cardiovascular inflammation and endothelial dysfunction, leading to atherosclerosis and hypertension, which, after plaque formation and ablation, can cause heart failure, stroke, arrhythmia and venous thromboembolism (J. a Araujo & Nel, 2009a; Brook, 2008; Martinelli, Olivieri, & Girelli, 2013). A study looking into the correlation of increased particulate air pollution concentrations and the triggering of myocardial infarction revealed an estimate odds ratio of 1.48 associated with an

increase of 25µg/m^3 PM2.5 during a two hour period before the onset. This study was based on data from 772 participants living in the greater Boston area (Peters, Dockery, Muller, & Mittleman, 2001).

Other systemic non-respiratory tract related health effects of ambient particulate matter include higher risk of type II diabetes, which has been reported as a 10-27% increase for a 10µg/m^3 increase of PM2.5 (Esposito, Petrizzo, Maiorino, Bellastella, & Giugliano, 2016). Also, effects on the brain have been reported, such as deposition of ultrafine particles in the animal brain as well as inflammatory responses, lesions and vascular diseases (Guxens & Sunyer, 2012). A study involving c. 400 older women who lived close to major roads, showed that long term exposure to PM leads to a mild decrease in cognitive function in older women, which in turn may be involved in the pathogenesis of Alzheimer's disease (Ranft, Schikowski, Sugiri, Krutmann, & Krämer, 2009). Also, another large population-based cohort study involving over 6 million adults in Canada showed that living less than 50m from a major road was associated with the development of dementia (H. Chen et al., 2017). All these detrimental health effects have an inflammatory component.

Indoor air pollution is a major health risk in low and middle income countries, where exposure to biomass derived PM is a significant issue, and in total 3 billion people are affected; outcomes of exposure to indoor air pollution include respiratory infections, chronic obstructive lung diseases and respiratory tract cancers (S. B. Gordon, Bruce, Grigg, Hibberd, Kurmi, Lam, Mortimer, Havens, et al., 2014). Although half of the world's population is exposed to household air pollution, only very little is known about their immunomodulatory effects and only a handful of studies investigated pulmonary inflammation induced by biomass smoke (A. Lee, Kinney, Chillrud, & Jack, 2015). These studies showed that exposure to biomass smoke leads to an oxidant imbalance and neutrophilic inflammation (Sussan et al., 2014). Similar to outdoor air pollution, endotoxins and microbial components play an important role in TLR activation (Bauer, Diaz-Sanchez, & Jaspers, 2012; Bovallius, Bucht, Roffey, & Anas, 1978). Similar to urban and ambient air pollution, PM derived from biomass lead to immunomodulation and an increased susceptibility to infection. This is mediated through inflammation induced by alveolar macrophages, disruption of barrier defences and recruitment of neutrophils. In addition to that, phagocytosis of alveolar macrophages is altered and receptors for pathogen invasion are upregulated (A. Lee et al., 2015). Biomass derived PM has been shown *in vitro* to lead to a dose-dependent increase in monocyte-derived macrophage carbon content as well as decrease in phagocytosis (Rylance et al., 2015). Induced sputum samples from people exposed to biomass

showed an inflammation with high levels of neutrophils, eosinophils, lymphocytes and alveolar macrophages as well as inflammatory markers (Dutta et al., 2007; M. J. Guarnieri et al., 2014).

However, most of these studies were based on macrophages; hence, the role of DCs in the respiratory tract in immune responses to biomass derived particulate matter needs to be explored further.

1.3.6. Effects of PM on dendritic cells

Given its complex composition and the presence of potentially biological active materials, it is likely that PM has wide-ranging effects on immune cells, including DCs, within the respiratory tract. By modulating DC activation and function, PM has the potential to impact the adaptive immune response to inhaled antigen. In order to study the *in vitro* effects of air pollution, different approaches have been used to address different questions. Ambient PM may be sourced from measuring stations, where air quality is monitored, in urban or rural areas. To study specific types of air pollution, such as diesel exhaust particles (DEP) or indoor air pollution from kitchens, PM can be collected directly from the exhaust pipe or close to the stove respectively. The most well studied air pollution particle type is ambient PM from urban areas, as it affects a large amount of people.

Exposure to ambient air pollution induces a wide array of effects on the innate immune system in the lung. After inhalation, they interact with the airway epithelium, if they manage to circumvent the mucus barriers. It has been shown that not only phagocytes such as macrophages and dendritic cell but also epithelial cells are able to take up and clear airway PM once exposed (Fujii, Hayashi, Hogg, Vincent, & Van Eeden, 2001; Mukae et al., 2001). These cells produce reactive oxygen species and inflammatory mediators therefore contribute to airway inflammatory responses as well as airway lesions in asthma and COPD when exposed to PM. Also, as these cell types reside in close proximity to each other, the interaction can amplify cytokine secretion (Bauer et al., 2012; Fujii et al., 2002). Post-mortem imaging of lung macrophages from children exposed to high concentrations of air pollution has shown that PM can be found within the cells *in vivo* (Mena, Woll, Cok, Ferrufino, & Accinelli, 2012).

More specifically for DCs, ambient PM has been shown to induce maturation of human (Williams et al., 2007) and mouse (Bezemer et al., 2011) DCs directly or indirectly via effects on epithelial cells (Bezemer et al., 2011; Bleck et al., 2006; Hirota et al., 2012; Williams et al., 2007) and can enhance the polarization of Th2 inflammatory responses (Bezemer et al., 2011). *In vitro* exposure of GM-CSF exposed human blood $CD1c^+$ DCs to urban particulate matter has been shown to lead to DC maturation and higher levels of naïve CD4 T cell proliferation as shown in an

allogeneic mixed leukocyte reaction (MLR). These proliferating T cells showed lower levels of IFN-γ, Il-13 and IL-5 secretion, all in all suggesting that PM exposure may impair Th1 responses in the lung (Matthews et al., 2013). It has also been shown that blood $CD1c^+$ DCs exposed to ambient PM drives proliferation of memory CD4 T cells, leading to Th1, Th2 and Th17 cells, confirming the role of urban PM in the both exacerbation of asthma and chronic inflammatory disease (Matthews et al., 2016).

The properties of PM that lead to DC activation and the mechanisms by which this occur are poorly understood. It is currently not known whether PM of different composition or size has similar effects on DC. Moreover, biologically active materials variably present in PM from different sources may impact upon other aspect of DC function. For instance, aromatic hydrocarbons can induce signalling through the AhR and PM have been shown to activate this pathway in epithelial cells (Ferecatu et al., 2010). AhR signalling can regulate expression of the enzyme IDO, which has been implicated in T cell anergy and the development of regulatory T cells (Jux, Kadow, & Esser, 2009), but induction of this pathway by PM has not been explored in DCs. In addition, inorganic matter such as silicates, which can be found in PM from some sources, can be potential activators of the inflammasomes, although the role of this pathway in the profile of cytokines released by DCs in response to PM exposure has also not been systematically addressed.

This project aims to explore the broad responses of DCs to PM of different size and composition. It has been suggested by Frank J. Kelly & Fussell, 2015b that one of the main aspects of fully understanding the adverse health effects of PM, is to understand its underlying biological toxicity and how this differs between PM from different sources.

1.4. Hypothesis and study aims

The overall hypothesis underlying the research project is that air pollution particles from different combustion sources and different sizes have distinct effects on DC activation and function. More specifically, we hypothesise that PM10 induce a higher level of DC activation compared to PM2.5. To address this hypothesis, the aims of the research project are:

- To determine the effects of PM on different pathways used by DCs to respond to their environment, including DC maturation, aryl hydrocarbon receptor signalling and activation of the inflammasome;
- To test the potential *in vivo* relevance of effects on DCs identified in *in vitro* models by analysing human respiratory tract DCs acquired from induced sputum;
- To determine the influence of particle size and composition on the effects of PM on DC by comparing PM10 and PM2.5 derived from the combustion of either biomass or fossil fuel.

2. Materials and Methods

2.1. Materials

2.1.1. Reagents and chemicals

- Agarose: polysaccharide polymer used for the separation of large molecules, such as DNA, by electrophoresis (Sigma, USA)
- CB: Carbon black particles, particles made of pure carbon with no additional substances on their surface, therefore represent the core of PM and serve as a particle control (Degussa, Germany)
- CFSE: 6-Carboxyfluorescein succinimidyl ester is widely used for cell tracking and proliferation studies. It can pass the membrane of cells easily and once inside the cell, proliferating cells can be identified by a decreasing level of fluorescence, which halves with each division of the cells. The stock solution was prepared in DMSO at a concentration of 1mM and aliquots were stored at -80˚C. For cell staining the stock solution was diluted with sterile PBS to a concentration of 5µM (Invitrogen, USA)
- CH223191: AhR antagonist (Choi, Lee, Dingle, Kim, & Swanson, 2012), the stock solution was prepared in DMSO at a concentration of 3mM and aliquots were stored at -80˚C (Sigma, USA)
- CLI-095: TLR4 signalling antagonist, the stock solution was prepared in DMSO at a concentration of 1mg/ml and further diluted to a concentration of 100µg/ml using complete cell culture medium
- EDTA: Ethylenediaminetetraacetic acid is a chelating agent used to bind ions and prevent clustering of the cells (Sigma, USA)
- FCS: Fetal calf serum is a supplement for *in vitro* cell culture and is also used in FACS buffer to inhibit nonspecific binding of the antibody during labelling (PAA, Austria)
- Ficoll-Paque Plus: High density polysaccharide solution used for separation of PBMCs from blood samples (GE Healthcare, UK)
- FICZ: 6-formylindolo[3,2-b]carbazole is an aryl hydrocarbon agonist, the stock solution was prepared in DMSO at a concentration of 350µM and aliquots were stored at -80˚C (Enzo Life Sciences, USA)
- Fixable Viability Dye eFluor 780: Viability dye that labels dead cells irreversibly prior fixation, pre-diluted aliquots were stored at -80˚C (eBioscience, Austria)
- GelRed: fluorescent nucleic acid dye in agarose gels (Biotium, USA))
- Hypperladder 50bp: Molecular weight marker for qualitative PCR (Bioline Reagents, UK)

- Ionomycin: Stimulates production of cytokines by T cells by increasing intracellular calcium levels, the stock solution was prepared in ethanol at a concentration of 1mg/ml and aliquots were stored at -80˚C (Merck Millipore, Germany)
- Leucoperm A and B: Combination of both reagents used for intracellular cytokine staining, reagent A is used for mild fixation of the cells, reagent B is used permeabilisation of the cells (AbD Serotec, UK)
- L-Glutamine: Amino acid used as a supplement for cell culture medium (Sigma, USA)
- Monensin: Widely used to disrupt the Golgi apparatus and retain newly synthesized cytokines within the Golgi apparatus by inhibiting the intracellular cytokine transport, the stock solution was prepared in ethanol at a concentration of 12mM and aliquots were stored at -80˚C (Sigma, USA)
- Pen/Strep (10000 units penicillin and 10mg streptomycin): Penicillin and streptomycin combination used in cell culture medium to control bacterial contamination (Sigma, USA)
- PMA: Phorbol 12-myristate 13-acetate activates the signal transduction enzyme protein kinase C and is commonly used to stimulate cytokine production by T cells, the stock solution was prepared in DMSO at a concentration of 1mg/ml and aliquots were stored at -80˚C (Merck Millipore, Germany).
- RPMI-1640 Medium (Dutch modification): Tissue culture medium containing both sodium bicarbonate and 20mM HEPES as buffering agents (Sigma, USA)
- Sodium azide: Used to prevent capping and shedding or internalization of the antibody-antigen complex after the antibodies bind to the antigens on the surface (Sigma, USA)
- Sputolysin: Concentrate of Dithiothreitol in PBS, reduces disulfide bonds and disrupts mucus in order to extract cells, pre-diluted with sterile PBS 1 in 10 before use (Merck Millipore, Germany)
- Trypan blue solution (0.4%): Vital stain that dyes dead cells blue (Sigma, USA)

2.1.2. Media and buffers

- 10 x TAE buffer: 1000ml distilled water containing 48.5g Tris base, 11.4ml acetic acid and 2.92g EDTA, 1 x TAE buffer was prepared by diluting 1:10 with distilled water
- Complete cell culture medium: RPMI-1640 Medium (Dutch modification) supplemented with 10% FCS, 100µg ml^{-1} penicillin, 100µg ml^{-1} streptomycin and 20mM L-glutamine
- FACS buffer: PBS containing 2% FCS, 1mM EDTA and 0.2% (w/v) sodium azide
- Minimacs buffer: PBS containing 0.5 % (w/v) BSA and 2 mM EDTA, sterile filtered through a 0.2µm cellulose acetate membrane syringe filter

- PBS: Phosphate-buffered saline solution, containing 137mM sodium chloride, 2.7 mM potassium chloride and 12mM phosphate in ultrapure water
- PFA: PBS containing 1% paraformaldehyde, used as a fixative, stored at 4˚C (Sigma, USA)

2.1.3. Antibodies

Table 2-1: Antibodies used for flow-cytometry

Antigen	Fluorochrome	Clone	Isotype	Supplier
CCR7	Pacific Blue	G043H7	Mouse IgG2a	Biolegend
	PerCP/Cy5.5	G043H7	Mouse IgG2a	Biolegend
CD3	PerCP/Cy5.5	HIT3a	Mouse IgG2a	Biolegend
CD4	FITC	RPA-T4	Mouse IgG1	Biolegend
CD40	FITC	LOB7/6	Mouse IgG2a	AbD Serotec
	PE/Cy7	5C3	Mouse IgG1	Biolegend
CD45RA	PE/Cy7	HI100	Mouse IgG2b	Biolegend
CD80	PE/Cy7	2D10	Mouse IgG1	Biolegend
CD83	APC	HB15e	Mouse IgG1	Biolegend
CD86	APC	IT2.2	Mouse IgG2b	Biolegend
	Brilliant Violet 510	IT2.2	Mouse IgG2b	Biolegend
	FITC	BU63	Mouse IgG1	AbD Serotec
HLA-DR	PerCP/Cy5.5	L243	Mouse IgG2a	Biolegend
	PE/Cy7	L243	Mouse IgG2a	Biolegend
Interferon-γ	PE/Cy7	B27	Mouse IgG1	BD Bioscience
Interleukin-10	PE	JES3-9D7	Rat IgG2a	Biolegend
Interleukin-17	PE	BL168	Mouse IgG1	Biolegend
Interleukin-4	PE	MP4-25D2	Rat IgG1	Biolegend
Interleukin-5	APC	TRFK5	Rat IgG1	Biolegend
Lineage Cocktail (CD3, CD14, CD16, CD19, CD20, CD56)	FITC	UCHT1, HCD14, 3G8, HIB19, 2H7, HCD56	Mouse IgG1, Mouse IgG2b	Biolegend

Table 2-2: Isotype controls used for flow-cytometry

Isotype	Fluorochrome	Clone	Supplier
Mouse IgG2a	Pacific Blue	MOPC-173	Biolegend
Mouse IgG2a	PerCP/Cy5.5	MOPC-173	Biolegend
Mouse IgG1	FITC	MOPC-21	Biolegend
Mouse IgG2a	FITC	LO-MG2a-7	AbD Serotec
Mouse IgG1	PE/Cy7	MOPC-21	Biolegend
Mouse IgG2b	PE/Cy7	MPC-11	Biolegend
Mouse IgG1	APC	MOPC-21	Biolegend
Mouse IgG2b	APC	MPC-11	Biolegend
Mouse IgG2b	Brilliant Violet 510	MPC-11	Biolegend
Mouse IgG1	FITC	N/A	AbD Serotec
Mouse IgG2a	PE/Cy7	MOPC-173	Biolegend
Mouse IgG1	PE/Cy7	N/A	BD Bioscience
Rat IgG2a	PE	RTK2758	Biolegend
Mouse IgG1	PE	MOPC-21	Biolegend
Rat IgG1	APC	RTK2071	Biolegend
Mouse IgG2b	FITC	MG2b-57	Biolegend

2.1.4. Cytokines

- GM-CSF: Granulocyte macrophage colony-stimulating factor used for the differentiation of $CD14^+$ monocytes into DCs together with IL-4, the stock solution was prepared in sterile and distilled water at a concentration of 100µg/ml and stored at -80˚C (Peprotech, UK)
- IL-4: Interleukin 4 is a cytokine used to differentiate $CD14^+$ monocytes into DCs together with GM-CSF, the stock solution was prepared in sterile and distilled water at a concentration of 100µg/ml and stored at -80˚C (Peprotech, UK)

2.1.5. Kits

- Control compensation particles set (BD Bioscience, USA)
    - Anti-Mouse Ig, κ
    - Negative control
- PCR (Qiagen, Germany)
    - RNA extraction: RNeasy Micro Kit
    - Reverse Transcription: QuantiTect Reverse Transcription Kit

  - SYBR Green real-time PCR: QuantiFast SYBR Green PCR Kit
  - Primer: QuantiTect primer assay for *AhR*, *CYP1A1*, *CYP1B1*, *IDO1, CD80, CD86, RPL30*

- Cell Separation (Miltenyi Biotec, UK)
  - CD14 MicroBeads, for separation of $CD14^+$ monocytes
  - Naive $CD4^+$ T Cell Isolation Kit II, for separation of naïve $CD4^+$ T cells
  - MS and LS columns: for isolation of magnetically labelled cells
- FlowCytomix™ Multiple Analyte Detection System (eBioscience, Austria)
  - Basic Kit
  - Simplex Kits: IL-1β, IL-6, IL-18, IL-10, IL-23, IL-12p70
- Cytometric Bead Array (BD Bioscience, USA)
  - Human Soluble Protein Master Kit
  - CBA Flex Set: IL-6, IL-10, IL-12p70, IL-1β
- NAD/NADH Quantification Kit (Sigma-Aldrich, USA)

2.2. Methods

2.2.1. Purification of different cell populations from blood

2.2.1.1. Peripheral blood mononuclear cell (PBMC) separation

50ml of blood was collected from healthy volunteers who gave informed written consent (REC: 05/Q0405/71) by venepuncture using sodium heparin blood collection tubes. The blood was then diluted 2:1 with RPMI-1640 medium Dutch modification and carefully layered on an equal amount of Ficoll-Paque PLUS in a 50ml Falcon tube. The tubes were then centrifuged at 650 x g for 20min with the lowest possible setting for the brake to avoid re-mixing of the cells after separation. After centrifugation, PBMCs were removed from the interface using a Pasteur pipette, pooled into a 15ml Falcon tube and centrifuged for another 10min at 650 x g with the brake fully switched on. After this, supernatants were aspirated completely and cell pellets were re-suspended in sterile filtered minimacs buffer if used for cell culture and pooled in a 5ml Falcon tube. In preparation for the magnetic labelling, the cell suspension was washed three times by adding 1-2ml of minimacs buffer and subsequent centrifuging for 5min at 400 x g.

2.2.1.2. $CD14^+$ monocyte separation

After three washes, the PBMC pellet was re-suspended in 100µl of minimacs buffer and incubated for 15min on ice with 10µl of CD14 MicroBeads per 10ml of initial blood volume. After incubation, the labelled cells were washed in minimacs buffer, re-suspended in 500µl of minimacs buffer and applied on a pre-cooled MACS column, which was placed in the magnet that was cooled to 4˚C and was rinsed with 500µl of minimacs buffer beforehand. Since the separation was conducted by positive selection, the MS columns was used as it has a capacity to hold back up to 10^7 cells, which is appropriate for an initial amount of 50ml of blood. Once the cell suspension had passed the column, the column was washed three times by adding 500µl of minimacs buffer onto the column to remove any unlabelled PBMCs. These cells were usually cultured for compensation purposes during FACS analysis (see section 2.2.6.5.) or processed further for naïve $CD4^+$ T cell separation. The $CD14^+$ monocytes were eluted by removing the column from the magnetic field, adding 1ml of minimacs buffer and applying the plunger to flush out the labelled cell fraction, which was held back by the magnetic field. The eluted cells were then washed into cell culture medium by centrifugation at 400 x g for 5min and counted.

2.2.1.3. Naïve $CD4^+$ T cell separation

The PBMC suspension was re-suspended in 40µl of minimacs buffer per 10^7 of cells, 10µl of the Naïve $CD4^+$ T Cell Biotin Antibody Cocktail II per 10^7 cells was added, mixed and incubated on ice for 10min. After incubation, the labelled cells were washed at 400 x g for 5min and re-suspended

in 80µl of minimacs buffer per 10^7 cells, 20µl of Naïve $CD4^+$ T Cell Microbead Cocktail II was added and cells were incubated for another 15min on ice. Next, cells were washed with minimacs buffer at 400 x g for 5min and re-suspended in 500µl of minimacs buffer and applied on a pre-cooled MACS column, which was placed in a pre-cooled magnet and was rinsed with 3ml of minimacs buffer before applying the cell suspension. Since the separation was performed by negative selection, a LS column was used, which can retain up to 10^8 cells. After the cell suspension has passed through the column, the column was rinsed 3 times with 3ml of minimacs buffer respectively to remove all unlabelled cells. As this is a negative selection method, the effluent contained the unlabelled naïve CD4 T cell fraction and was washed in sterile PBS at 400 x g for 5min in preparation for the CFSE labelling. Labelled cells were flushed out of the column by using the plunger after the column was removed from the separator. A proportion of these cells were usually cultured for compensation purposes during FACS analysis (see section 2.2.6.5.).

2.2.1.4. CFSE labelling

After cells were washed in sterile PBS, the supernatant was removed leaving 300µl of PBS and 300µl of a 10µM CFSE solution was added, resulting in a 5µM concentration of CFSE. The cell suspension was incubated at room temperature for 3 min and mixed half way through the incubation. The reaction was stopped by adding one volume of FCS, which causes quenching of the staining. The stained naïve CD4 T cell were washed in cell culture medium and counted for further experiments.

2.2.1.5. Cell counts

50µl of the cell suspension was diluted using 50µl of Trypan Blue and 150µl of RPMI-1640 medium dutch modification, mixed, applied on a Neubauer hemocytometer and covered with a cover slip. Cells were then counted three times in the 4 x 4 grid and the overall concentration of the cell suspension was calculated using the following equation:

$$\text{Average cell number of three counts} \times 5 \times 10^4 = \text{cell number/ml}$$

2.2.2. Particulate matter

2.2.2.1. Locations/Combustion source

To be able to compare different types of particulate air pollution, particles were collected from different areas in the UK and Africa, where different combustion sources are used for energy generation. In urban areas in the UK, the main source of particulate air pollution is the combustion of fossil fuels, whereas in Africa a large share is due to the burning of wood.

Therefore, the composition of particulate air pollution will vary and this variation may contribute to the different detrimental health effects of pollution exposure reported in these locations.

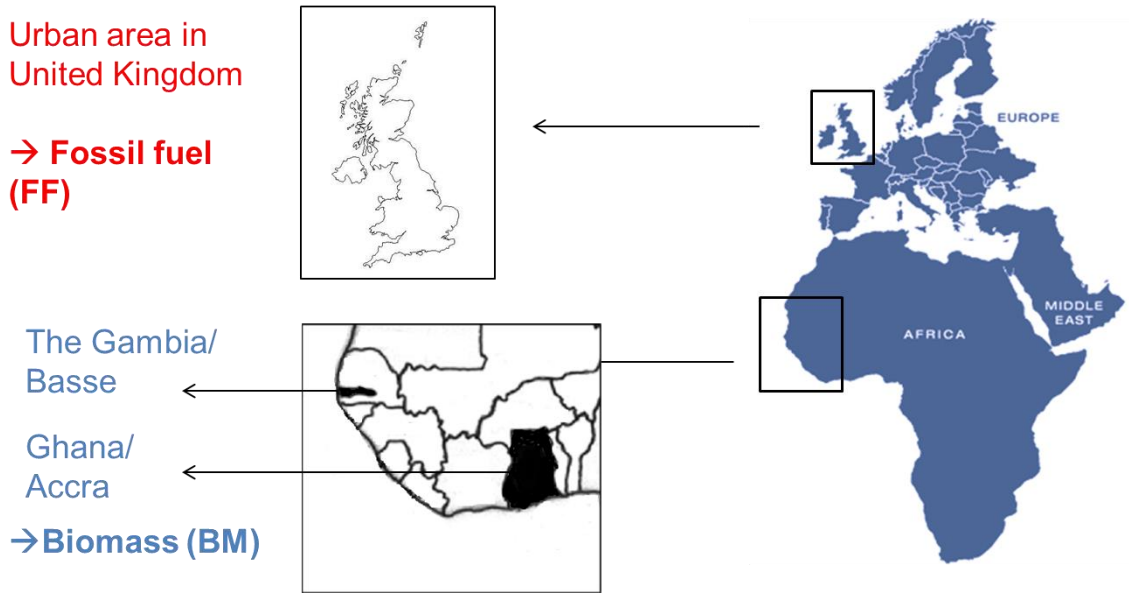


Figure 2.1: Locations of collected particulate matter

PM10 used was collected in an urban area in Leicester/UK, where the main source of ambient particulate air pollution is the combustion of fossil fuels by cars. London PM10 and PM2.5 were collected from exactly the same location to be used as a particle size control. The major energy source in western Africa is the combustion of biomass for cooking, which is mainly wood; therefore the composition of airborne particulate matter is derived from biomass burning. PM collected in The Gambia/Basse, originate from indoor kitchen areas, close to the fireplace, and therefore contain a high proportion of burned wood. These particles are PM2.5. PM10 from Accra/Ghana was collected in communal kitchen areas, where again mainly wood was used as the energy source.

The main particles used in this work are PM10 collected from the urban area in Leicester and, therefore, are mainly derived from the combustion of fossil fuel. As a comparison for particle size, PM10 and PM2.5 were collected in London in the United Kingdom, from the exact same location to avoid differences in composition. PM2.5 from Basse/The Gambia was collected indoor, in kitchens close to the cooking area where the primary energy source for food preparation is wood. Thereby, these particles were derived as purely as possible from biomass without outdoor influences like car exhausts from the combustion of fossil fuels (Dionisio et al., 2012). In order to have comparable particles smaller than 10µm in diameter derived from the combustion of biomass, PM10 was collected in communal kitchen areas in Accra/Ghana (Z. Zhou

et al., 2011). Also, ambient PM10 and PM2.5 were collected in an urban area in Accra/Ghana during the dry and windy season, called Harmattan, which is characterized by Saharan dust being blown into the city and therefore affecting the composition of particles. As a comparison, PM10 and PM2.5 from exactly the same location were collected during the rainy season (non-Harmattan). An overview of the particles used in this study can be seen in Table 2-3 and the location where these particles were collected can be seen on the map in Figure 2.1.

Table 2-3: Particles used for dendritic cell stimulation

Particle type	**Site of collection**	**Source**	**Supplier**	**Collection Date**
PM10	Urban area in Abbey Road, Leicester/UK	Fossil fuel	Jonathan Grigg (QMUL)	September 2012 and February 2014
PM2.5	Rural, indoor kitchen in Basse/The Gambia	Biomass	Majid Ezzati (Imperial)	November 2009 - May 2010
PM10	Urban, indoor communal kitchen, Accra/Gambia	Biomass	Majid Ezzati (Imperial)	November 2006
PM10	Urban area Shaftesbury Avenue, London/UK	Fossil Fuel	Ian Mudway (KCL)	May 2011 – January 2012
PM2.5	Urban area, Shaftesbury Avenue, London/UK	Fossil Fuel	Ian Mudway (KCL)	June 2011 – January 2012
PM10 (Harmattan)	Urban area in Accra/Ghana	Biomass, collected during dry season (Harmattan)	Majid Ezzati (Imperial)	N/A
PM2.5 (Harmattan)	Urban area in Accra/Ghana	Biomass, collected during dry season (Harmattan)	Majid Ezzati (Imperial)	N/A
PM10 (non-Harmattan)	Urban area in Accra/Ghana	Biomass, collected during rainy season (non-Harmattan)	Majid Ezzati (Imperial)	N/A
PM2.5 (non-Harmattan)	Urban area in Accra/Ghana	Biomass, collected during rainy season (non-Harmattan)	Majid Ezzati (Imperial)	N/A

2.2.2.2. Collection filters and sizes

Fossil fuel derived PM10 was collected on TX40 Teflon-coated glass fibre filter cartridges (Air Monitors Ltd, Tewkesbury, UK) by a fixed tapered element oscillating microbalance (Series 1400a TEOM Control Unit; Rupprecht and Patashnick Co, Inc, East Greenbush, NY) which measures the collected mass of PM continuously (Patashnick & Rupprecht, 1991). These monitors were set up in the central area of Leicester where most ambient PM10 is derived from the combustion of fossil fuels by cars. Air pollution monitors in The Gambia/Basse were located in household kitchens at about 1m above ground level and 1m away from cooking stoves. BM PM2.5 was collected on polytetrafluoroethylene (PTFE) filters with rings (Pall Life Sciences; Teflo, 0.2-µm pore size, 37mm diameter), back supported by a Whatman drain disc and placed inside a 37mm SureSeal Air Monitoring Cassette (SKC Inc., Eighty Four, PA). In The Gambia, PM2.5 samples were collected using a GK2.05SH (KTL) cyclone (BGI Inc., Waltham, MA) and PM mass was measured using a microbalance. In Accra/Ghana the same set up for particle collection was used as in The Gambia.

2.2.2.3. Extraction and quantitation of particle suspension

Particles on PTFE filters were extracted by vortexing for 4min and sonication in PBS after removal of the plastic ring. Several filters were pooled in 3ml of PBS in order to achieve a higher concentration. Sonication was conducted twice for 30s each using a probe sonicator (Imrich, Ning, & Kobzik, 2000). Two TEOM filters were vortexed in 3ml of PBS for 4min and then the particle suspension was transferred into a clean Bijou bottle and sonicated. The concentration of the particle suspension was measured by densitometry on a 96-well flat bottom plate at 340nm using a UV spectrophotometer. A calibration line was prepared using ultrafine carbon black particles (UfCB; Printex 90, Degussa) at a concentration ranging from 1mg/ml to 31.25µg/ml (Brown, Hutchison, Donaldson, & Stone, 2007).

2.2.3. *In vitro* cell stimulation with PM

2.2.3.1. DC and macrophage differentiation

To obtain MoDCs, the separated $CD14^+$ monocytes from PBMCS as described above were seeded at a density of 5 x 10^5 on a flat-bottom 24-well plate in a volume of 1ml of complete cell culture medium in the presence of GM-CSF and IL-4 at a concentration of 100ng/ml of each cytokine and incubated at 37˚C and 5 % CO_2 and 7 days. On day 3 or 4 of the incubation, 500µl of cell culture medium was carefully removed from the top of each well without disturbing the cell suspension layer at the bottom. 500µl of fresh cell culture medium was added, again supplemented with GM-CSF and IL-4, both at a concentration of 100ng/ml in 1ml of cell culture

medium in each well. The same procedure was conducted to obtain monocyte-derived macrophages with the difference that only GM-CSF was used during the differentiation process. After 7 days of incubation, the differentiation process was completed and the cells were ready for further experiments. The DCs in each well were re-suspended thoroughly, transferred into a 15ml falcon tube and the empty well was rinsed with 1ml of medium in order to capture as many DCs as possible. Cells were centrifuged at 400 x g for 5min, re-suspended in 1ml of cell culture medium and counted.

2.2.3.2. Stimulation of dendritic cells, macrophages and PBMCs

For the stimulation of cells with PM, cells were plated in a round-bottom 96-well plate at a density of 5 x 10^4 cells per well, except PBMCs, which were plated at a density of 10^5 cells per well. Particles were added at a concentration range of 10µg/ml, 25µg/ml and 50µg/ml. LPS was used as a TLR4 agonist for DC activation and added to the cells at a concentration of 0.01µg/ml. CB posed as a particulate control at a concentration range of 10µg/ml, 25µg/ml and 50µg/ml. FICZ is a widely used AhR agonist and was added to the culture at a concentration of 35nM; the AhR antagonist CH223191 was used at a concentration of 3µM, 30min prior stimulation and the TLR4 antagonist CLI-095 was added 1h before adding the stimulant at a concentration of 1µg/ml. Cells were incubated for 48h at 37˚C and 5 % CO2. After incubation, stimulated cells were thoroughly re-suspended in the well, transferred into 5ml FACS tubes and centrifuged at 400 x g for 5min. Supernatants were collected for further FlowCytomix™ Multiple Analyte Detection System measurements and stored at -80˚C. The cell pellet was washed in either PBS or FACS buffer for further experiments at 400 x g for 5min or re-suspended in cell culture medium and counted for the mixed leukocyte reaction. For PCR experiments the cell pellet was re-suspended in lysis buffer, vortexed for 1min and stored at -80˚C for further experiments.

2.2.4. Cytospins for Imaging with Light Microscopy

In order to examine cells by light microscopy, cytospins were prepared and photographs were taken. A small volume, usually around 10-20µl of the re-suspended cell suspension, was removed from the well. The absorbent paper on the cytofunnel was wetted and the cytofunnel was placed around a microscopy slide. The cell suspension was inserted in the funnel by leading the pipette tip to the bottom of the funnel and releasing the liquid carefully. The microscopy slides with the cytofunnel still attached were then placed in the cytospin centrifuge which was set for 3min at 1500rpm (254 x g). After centrifugation, the cytofunnels were removed from the microscopy slides and the slides were dried. After drying, the slides were dipped in methanol once for fixing. After drying, the slides were dipped briefly three times in red (eosin) staining

solution and after drying dipped in blue (azur) staining solution. As soon as the slide had dried, the residual staining solution was washed off by dipping the slide briefly into water and left to dry completely. Once dried, one small drop of mounting medium (Vectashield, Vector Laboratories, USA) was put on the cells and a cover slip was placed on top of the mounting medium and pressed down in order attach it firmly. As soon as the liquid had dried the slides were ready for imaging using light microscopy. Pictures were taken using a light microscope at a magnification of x100 in immersion oil to increase the resolution of the microscope. The images were taken using the ImageJ program.

2.2.5. Allogeneic Mixed Leukocyte Reaction (MLR)

In order to measure the stimulatory capacity of MoDCs, they were co-cultured with allogeneic naïve $CD4^+$ T cells separated from blood of healthy adult donors (see above). Stimulated MoDCs with PM, LPS or medium only were plated at a density of 300, 900, 2600 and 8000 cells per well in a 96-round-bottom well plate. 4 x 10^5 naïve $CD4^+$ T cells, that were labelled with CFSE beforehand, were added to each well and the volume was made up to 200µl per well in total with cell culture medium. The cell mixture was incubated for 5 days and subsequently analysed using flow-cytometry.

2.2.6. Flow-Cytometry

2.2.6.1. Surface staining

Cell surface phenotype was examined by antibody labelling and flow-cytometry. Cells were washed into sterile PBS and stained with a fixable viability dye in order to distinguish between dead and viable cells. Cells were incubated at a concentration of 1-10 x 10^6/ml with 1µl of fixable viability dye per ml for 30min on ice and protected from light. After that, cells were washed in FACS buffer, re-suspended in 100µl of FACS buffer and incubated with fluorescently labelled antibodies at pre-determined optimal concentrations for 15min on ice after mixing. The unbound antibody was removed from the cell suspension by washing in FACS buffer for 5min at 400 x g. After centrifugation cells were fixed in 300µl of 1% PFA and measured.

2.2.6.2. Intracellular staining

Intracellular staining was conducted on proliferating $CD4^+$ T cells to determine their cytokine profile after culture with allogeneic MoDCs for 5 days. Cells were transferred into 5ml FACS tubes and centrifuged for 5min at 400 x g, the supernatant was removed and fresh cell culture medium was added to re-suspend the cells. The cell suspension was centrifuged a second time for 5min at 400 x g and re-suspended in 300µl of a stimulation cocktail that contained 10ng/ml of phorbol-12-myristate-13-acetate (PMA), 500ng/ml ionomycin and 3µM of monensin and

incubated for 4h at 37˚C and 5 % CO_2 (PIM). Incubation in the PIM cocktail facilitates the staining and measurement of the cytokines, whereby PMA and ionomycin activate the cells, which will then produce the cytokines they have become committed to during the stimulation culture due to the "open" conformation of these loci. Monensin then causes retention of the cytokines.

2.2.6.3. FlowCytomix™ Multiple Analyte Detection System

Supernatants were collected and stored at -80˚C until analysed using FlowCytomix™ Multiple Analyte Detection System or the Cytometric Bead Array. For the FlowCytomix™ Multiple Analyte Detection System, 25µl of antibody coated beads specific for each cytokine of interest were added to 25µl of the supernatant derived from each stimulation condition. Subsequently, 50µl of a biotin-conjugated second antibody mixture was added, which interacted with the analytes captured by the first antibodies. Once mixed, the suspension was incubated for 2h at room temperature protected from light by an aluminium foil. After incubation, the labelled cytokines were washed with the Assay Buffer provided with the kit at 200 x g for 5min, supernatant were discarded carefully leaving 100µl of liquid in each tube. 50µl of the streptavidin-phycoerythrin solution, which binds to the biotin conjugate and emits a fluorescent signal, was added to each tube, mixed and incubated for 1h at room temperature in the dark. After incubation, the mixture was washed twice with assay buffer at 200 x g for 5min leaving 100µl of liquid in the tube; after discarding the supernatant, 500µl of assay buffer were added. Samples were measured subsequently using the BD FACSCanto II after mixing. Results were analysed by calculating the concentration based on the standard curve, which was prepared from the standards provided with the kit alongside the samples.

2.2.6.4. Cytometric Bead Array

As the FlowCytomix™ Multiple Analyte Detection System kit distributed by eBioscience was discontinued, the Cytometric Bead Array supplied by BD Bioscience was used in order to measure cytokine concentrations in cell culture supernatants. The method was similar to the one applied by the FlowCytomix™ Multiple Analyte Detection System kit. Standard dilutions were prepared and processed alongside the test tubes. Capture beads were added to the standards and the test samples and incubated for 1hour at room temperature. Subsequently, 50µl of PE Detection Reagent was added to each tube, gently mixed and incubated for 2 hours at room temperature. After a final wash with wash buffer, samples were measured using the BD FACSCanto and analysed using the Winlist software version 6.0 (Verity Software House, Maine) GraphPad Prism 5 (California, USA).

2.2.6.5. Controls for flow-cytometry and analytical approach

Single colour compensation controls were run with each experiment. These were prepared either with compensations for surface labelling beads or with cells for viability staining. Compensation was performed manually after acquisition of samples during the analysis using the Winlist software version 6.0 (Verity Software House, Maine). Also, isotype controls were used to label cells in order to control for non-specific background staining of the test antibodies. Data was analysed either as percentage positive cells in relation to the isotype control or as the overall MFI of the whole cell population.

2.2.6.6. Acquisition & data analysis

Flow-cytometry was performed on the BD FACSCanto II using the BD FACSDiva software. Once files were collected, data analysis was conducted using Winlist version 6.0 (Verity Software House, Maine).

2.2.7. Quantitative real-time PCR

All steps of the quantitative RT-PCR were performed using kits from Qiagen and were performed according to the manufacturer's instructions, summarised briefly below.

2.2.7.1. RNA extraction

Following stimulation, the cells were re-suspended in their culture medium and transferred from their cell-culture plate well into a 1.5ml Eppendorf tube. After centrifugation at 400 x g for 5min, the supernatant was removed carefully without disruption of the cell pellet. Cells were then re-suspended in 1ml of PBS and centrifuged again. Once the supernatant was aspirated completely, the cell pellet was re-suspended in 350µl of a lysis buffer, RLT buffer, and vortexed thoroughly for 1min in order to lyse the cells. The lysate was then stored at -80˚C until further processing. For the purification of the RNA, samples were defrosted and 350µl of 70% ethanol was added, to create conditions that help with the binding of the RNA to the column. Samples were then mixed properly until the ethanol and cell lysate looked homogenous and transferred to spin columns in 2ml collection tubes in order to extract the RNA from the lysate by binding the RNA to the silica-based column. The columns were spun at 8000 x g for 15s and the flow-through was discarded. 700µl of RW1 wash buffer was added to rinse the columns and the centrifugation was repeated and the flow-through discarded. 500µl of RPE buffer was added and the spin columns were centrifuged again to wash the membrane. 500µl of 80% ethanol was added to the spin column and the columns were spun for 2min at 8,000 x g and then again with open lids and fresh collection tubes for 5min at full speed to remove the remaining ethanol. To elute the RNA from the column, 14µl of RNase-free water was added to the middle of the column, which was placed

in 1.5ml collection tubes and columns were spun for 1min at full speed to get 12µl of RNA, as the dead volume of the column is 2µl. RNA samples were kept on ice to prevent degradation of the sample.

2.2.7.2. Reverse transcription

The reverse transcription step was conducted to translate the RNA into cDNA, which is also more stable. The first step was to eliminate genomic DNA by adding 2µl of gDNA Wipeout Buffer to each sample and incubate for 2min at 42˚C after transferring the samples into 200µl tubes. For the reverse transcription step itself, 1µl of the enzyme reverse transcription, 4µl of the reverse transcription buffer, which contains the dNTPs, and 1µl of the reverse transcription mix was added to the cDNA, mixed and incubated for 15min at 42˚C. The reaction was stopped by heating the samples to 95˚C for 3min, which denaturated and therefore inactivated the enzymes.

2.2.7.3. SYBR Green quantitative real-time PCR

12.5µl of SYBR Green PCR Mix and 2.5µl of the required primer (see Table 2-4 for a list of primers used) for each reaction were mixed beforehand and added to each well of a 96 – well PCR plate in duplicates. 1µl of cDNA sample was added to each well by placing the drop of liquid just below the edge of the well. The plate was centrifuged briefly in order to mix the liquids and covered with a self-adhesive polyolefin film. The reaction was performed using the 7,500 Real Time PCR Systems (Applied Biosystems, USA) and the 7,500 Software version 2.0.6. (Applied Biosystems, USA). The instrument was set to the manufacturers recommendations (see

Table ***2-5*** for the PCR cycling programme).

Table 2-4: Primers

Target	**Supplier**	**Catalogue number**
CYP1A1	Qiagen	QT00012341
CYP1B1	Qiagen	QT00209496
IDO1	Qiagen	QT00000504
AhR	Qiagen	QT00031437
Zbtb46	Qiagen	QT00053116
RPL30	Qiagen	QT00056651
CD80	Qiagen	QT00000497
CD86	Qiagen	QT00033915

Table 2-5: PCR cycling program

Reaction step	Temperature	Time	Repeats
1. Activation of DNA Polymerase	5min	95˚C	Once
2. Denaturation	10s	95˚C	Repeated 40 times
3. Annealing and extension	30s	60˚C	
4. Melt curve	30s	95˚C	Once

Data was analysed using the comparative Ct (ΔΔCt) method and therefore normalised to the medium control. The calculation was performed using the following equation:

$$\Delta\Delta\mathrm{Ct} = 2^{-(target\ gene\ ct - housekeeping\ gene\ CT) - (medium\ control\ ct - housekeeping\ gene\ CT)}$$

Either the normalised value or just the ΔCt value (not normalised to the medium control) was plotted. If no amplification took place for the medium control (Chapter 4), the value for the medium control was set to zero and the ΔCt value was illustrated.

2.2.8. Sputum

2.2.8.1. Sputum induction and processing

Sputum samples were obtained from healthy adult volunteers who gave informed written consent (REC 11-LO-1732) using the non-invasive sputum induction technique (Pizzichini E, Pizzichini MMM, Efthimiadis A.; Evans S, Morris MM, Squillace, D, Gleich, GJ, Dolovich, J, Hargreave, 1996). Volunteers were pre-treated with Salbutamol (Ventolin), which is a short acting β2-agonist, to prevent bronchoconstriction. FEV1 (Forced Expiratory Volume in 1 second) was monitored throughout the process in order to ensure that the lung function stays in acceptable limits. Inhalation of ultrasonic nebulised hypertonic (4.5%) saline solution induced mucosal secretion in the conducting airways which lead to coughing and subsequent discharge of sputum. The collected sputum was spread on a petri-dish and plugs were selected using forceps and transferred into a 15ml Falcon tube. The total amount of mucus plugs was weighed in grams and the 4-fold amount in ml of sputolysin was added, which was diluted 1:10 with sterile PBS beforehand. The suspension was vortexed thoroughly in order to break up the thick mucus plugs and incubated for 20min at 37˚C under continuous shaking. After that, the digested mixture was diluted with sterile PBS 1:1 and filtered through a 40µm cell strainer to exclude

undigested mucus clumps and cells. The filtered cell suspension was then centrifuged for 10min at 650g, the supernatant was aspirated completely and cells were re-suspended in 1ml cell culture medium. Cells were counted, divided equally in between two conditions and stimulated for 2-4h with either 50µg/ml fossil fuel derived PM10 or medium only.

2.2.8.2. Sputum antibody labelling and cell sorting

After incubation, cells were washed and centrifuged for 5min at 400 x g and re-suspended in FACS buffer for staining. Antibody labelling was conducted using PE/Cy7 anti-human HLA-DR antibody and the FITC anti-human lineage cocktail, which includes antibodies for CD3, CD14, CD16, CD19, CD20, CD56. Sputum cells were incubated with the antibodies for 15min on ice and washed with FACS buffer, centrifuged for 5min at 400 x g and re-suspended in 300µl of FACS buffer. For cell sorting, sputum DCs were identified as HLA-DR positive and lineage negative, whereas macrophages were cell sorted as lineage and HLA-DR positive. Cells were directly collected in lysis buffer, RLT buffer, that was supplied with the RNA extraction kit (RNeasy Micro Kit, Qiagen). After sorting, cells were lysed by vortexing for 1min and frozen at -80˚C for further analysis by quantitative or qualitative PCR.

2.2.8.3. Qualitative PCR

After quantitative real-time reverse transcription PCR (see section 2.2.7.), the finished PCR product was analysed by gel electrophoresis in order to confirm a single product for each primer. A 2% agarose gel was made with 1x TAE (Tris-acetate-EDTA) buffer containing GelRed dye at a dilution factor of 1:10,000, and then placed into the electrophoresis chamber. The chamber was then filled up with 1x TAE buffer to submerge the gel. 5µl of loading dye was added to 20µl of each PCR product and the samples were then loaded into individual wells on the gel. 6µl of 50bp hyperladder was added to a separate well. The gel was run for 45 minutes at 100V and then viewed with a UV transilluminator (BioRad ChemiDoc MP Imaging System, USA) to visualise the PCR products.

2.2.9. NAD(P)/NAD(P)H Quantification

2.2.9.1. Measurement of NAD(P)H

As a naturally fluorescent molecule, NAD(P)H can be measured without any antibody labelling. After UV excitation and detection in the Hoechst channel, NAD(P)H has an emission peak at 470nm, whereas NAD(P) is not fluorescent at all; therefore, this method can be used to specifically measure NAD(P)H. The measurement of NAD(P)H was performed on the BD LSR II and data analysis was conducted using Winlist version 6.0 (Verity Software House, Maine).

2.2.9.2. Measurement NAD/NADH using specific quantitation kit

As a second method of NAD/NADH quantification specifically a colorimetric assay from Sigma-Aldrich, USA was used. The assay was performed according to the manual provided with the kit. After incubation, cells were washed with cold PBS and counted, to make sure 2 x 10^5 cells were used for the assay. Cells were pelleted at 2000rpm (2440 x g) by centrifuging for 5min in a microcentrifuge tube. After removing the supernatants, cells were re-suspended in the NADH/NAD Extraction Buffer and lysed by two cycles of freeze-thawing on dry ice for 20min followed by 10min at room temperature. The lysis was followed by 10sec of vortexing and another centrifuge cycle at 13000 x g for 10min in order to remove the insoluble components. As cell lysates may contain enzymes that consume NADH, samples were spun through a 10kDa cut-off spin filter to deproteinize the sample. After this, samples were filled up to a final volume of 50µl with NADH/NAD Extraction Buffer.

NAD and NADH could be detected together, but to measure NADH only, NAD had to be removed from the solution. This was done by heating the samples to 60˚C for 30min in a heating block. After cooling on ice and a spin at 2000rpm (2440 x g) for 5 min to remove precipitates, NAD is decomposed and up to 50µl of the decomposed sample can be added in duplicates on a 96-well flat-bottom plate as well as the samples containing both NAD and NADH. In the meantime, the NADH standards were prepared by diluting 10µl of the 1mM NADH standard with 990µl of NAD/NADH Extraction Buffer to get a 10µM solution which was used to generate 0 (blank), 20, 40, 60, 80 and 100pmole/well standards. These were then topped up with NAD/NADH Extraction Buffer to a final volume of 50µl. 100µl of the Master Reaction Mix containing NAD Cycling Buffer and NAD Cycling Enzyme Mix was added to each reaction and incubated for 5min at room temperature by shaking. 10µl of NADH Developer was added to each well, and after an incubation time of 1-4h the absorbance was measured at 450nm. For the analysis, the background value for the blank was subtracted from the test readings and the values measured from the NADH standard were used to plot a standard curve.

2.2.10. Statistics

Statistical analysis was performed using GraphPad Prism 5 (California, USA). Comparisons between two groups were carried out using the nonparametric t-test, the Wilcoxon matched paired test. To assess the normality of a distribution, it was determined whether the values came from a Gaussian distribution using the D'Agostino-Pearson test. None of the data analysed passed the normality test. Comparisons between several groups were performed using a non-parametric one-way-analysis of variance (ANOVA), the Kruskal-Wallis test. To compare different stimulation conditions, the Dunn’s Multiple Comparison Test was performed. The results from

the MLR were analysed using a two-way-analysis of variance and a Bonferroni post-test to compare replicates. P-values <0.05 were regarded as statistically significant, p-values ≤ 0.05 were marked with one star, p-values ≤ 0.01 were marked with two stars and p-values ≤ 0.001 were marked with three stars.

2.2.11. Summary of Experimental Design

The diagram below (Figure 2.2) illustrates the overall experimental approach for the testing of different particle types on MoDCs. In brief, $CD14^+$ monocytes were separated from human PBMCs, cultured with GM-CSF and IL-4 for 7 days and stimulated for another 2 days. After activation, the expression of maturation markers, RNA and cytokine production was measured, or activated DCs were used to stimulate allogeneic naïve CD4 T cells, and proliferation and cytokine profile of T cells was measured via flow-cytometry. Human MoDCs were chosen as the preferred experimental model, as they were readily accessible in large numbers and provided a uniform and reproducible cell type. When stimulated with particles, a range of concentrations were used, making sure that inconsistencies in PM concentrations were covered, as accurate measurement of the particle suspension has proven difficult due to sedimentation of the particles.

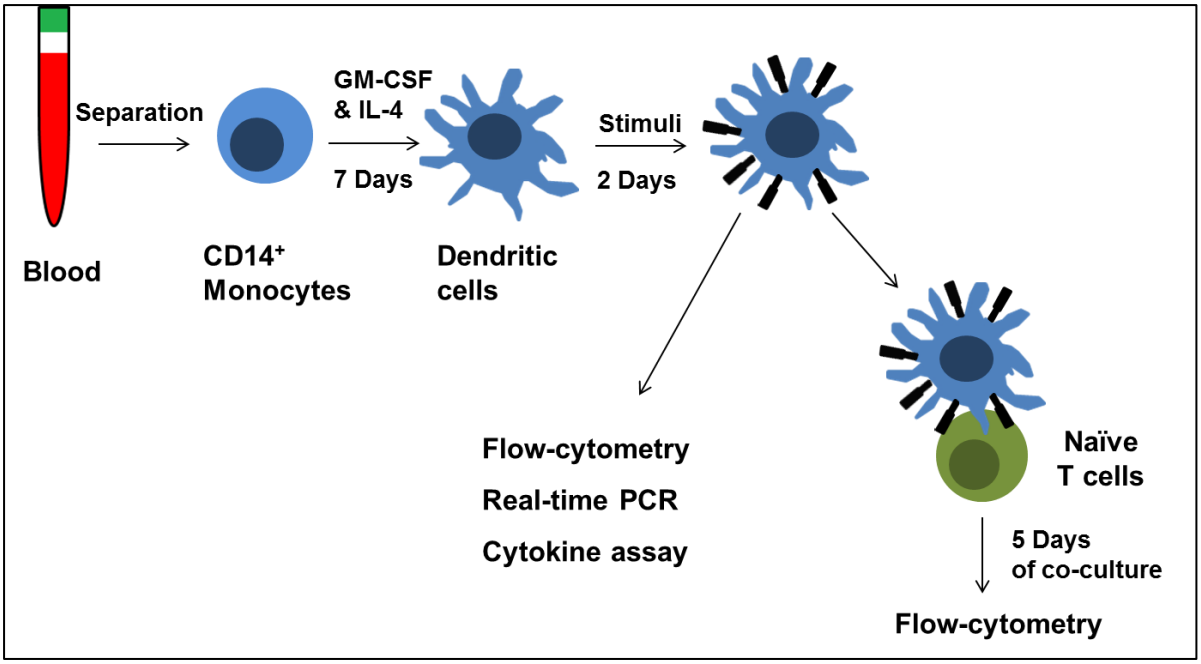


Figure 2.2: Overview of methods for PM testing

$CD14^+$ monocytes were separated from PBMCs obtained from blood from healthy volunteers through separation using Ficoll-Paque Plus and grown in the presence of GM-CSF and Interleukin-4 for 7 days. On day 7, the cells were exposed to PM and other stimuli like LPS, FICZ and carbon black for another 2 days. The expression of surface markers was measured using flow-cytometry, the expression of RNA using quantitative real-time PCR and the secretion of cytokines from DCs in the supernatants using FlowCytomix™ Multiple Analyte Detection System. Additionally, stimulated DCs were co-cultured with CFSE labelled naïve CD4+ T cells for another 5 days in order to measure the proliferation and cytokine profile of the T-cells.

3. Fossil fuel-derived ambient PM induces a distinct and complex programme of activation in MoDCs

3.1. Chapter summary

This section focuses on how human DC phenotype and function are influenced by exposure to ambient PM <10μm diameter (PM10) collected from an urban environment in the UK. Using human MoDCs as a model system, we observed that exposure to these predominantly fossil fuel (FF)-derived PM10 induced substantial expression of maturation markers including CD40 and CD86, the MHC class II molecule HLA-DR, and lymph node homing receptor CCR7. These effects were TLR4 dependent, since a specific antagonist of TLR4 signalling blocked the upregulation of CD80, CD83 and HLA-DR. It is therefore possible that the ability of FF PM10 to activate DCs is due in part to bacterial endotoxins which have previously been identified as a significant component of PM. Consistent with their mature phenotype, FF PM10-stimulated DCs had an enhanced ability to stimulate the proliferation of naïve allogeneic $CD4^+$ T cells and induced a Th1 cytokine profile that included substantial production of IFN-γ.

Moreover, DCs activated with FF PM10 themselves secreted multiple pro-inflammatory cytokines including IL-1β, IL-6, IL-12, IL-18 and IL-23. Secretion of IL-1β and IL-18 is associated with activation of the inflammasome, and these cytokines were produced by DCs after exposure to FF PM10 but not after exposure to the common microbial TLR4 agonist LPS. In addition, blocking the signalling of TLR4 led to a decrease in IL-6 and IL-1β secretion - but not IL-12 and IL-10 - suggesting that production of IL-6 and IL-1β is at least partly TLR4 dependent. FF PM10 exposure also increased expression of genes that are targets of the AhR signalling pathway: *CYP1A1*, *CYP1B1,* which encode enzymes that metabolize xenobiotics, and *IDO1,* which encodes indoleamine 2,3-dioxygenase, an enzyme involved in tryptophan metabolism. These effects were blocked by the AhR antagonist CH223191, confirming the involvement of this receptor. However, AhR signalling did not influence either the FF PM10 induced maturation of DCs or their production of cytokines. Conversely, inhibition of TLR4 prior to PM exposure did not inhibit MoDC of expression of AhR-dependent genes, such as *CYP1A1* and *CYP1B1*, but did inhibit *IDO1*, suggesting that these key immunomodulatory pathways are operating independently in our experimental system *in vitro*, and could potentially also exert distinct influences on DC function in the human lung *in vivo*.

3.2. Introduction

DCs play an important role in human immunity by detecting danger signals in body tissues and subsequently acting as potent APCs via migration to the draining lymph nodes and interaction with specific T cells. In the airways, they shape the immune response to inhaled antigen and are likely to sample inhaled pollutants as well. The rapid industrialisation of the modern world has radically altered the external environment in recent decades; hence DCs must now be able to detect bacteria and viruses against an increasingly complex backdrop of novel environmental pollutants. In the case of the respiratory tract, the pollutants that primarily affect the health of humans are airborne PM, which consists of solid particles that are suspended in the air. The particles that affect the health of humans are PM with a size of less than 10μm diameter (PM10) that are already known to exert major effects on human health. PM10 mainly settles within the conducting airways, whereas PM2.5, which is a part of PM10, is able to penetrate further in the respiratory tract, even reach the alveoli, pass the blood-air barrier and enter the cardiovascular system and cause systemic effects.

As well as consisting of different sizes, PM contains a wide range of substances. The core of these particles consists of elemental carbon and on its surface different substances are absorbed, such as metals, PAH, pollen, sand and smaller particles. Some of these substances will also be able to induce immunological effects when inhaled. These additional substances present on the particle core will vary greatly depending on the energy source, combustion conditions and environment and therefore make the type of air pollution highly variable and unique depending on the area where they have been collected. Not only can the additional substances on the surface of the particles determine some characteristics of the PM itself, for instance surface charge and endotoxin content, they can also affect the interaction of these particles with cells, e.g., immune cells like DCs, which are likely to interact with inhaled air pollution particles in the airways. It is therefore probable that a diverse particle surface induces a diverse set of effects on DCs.

For instance, endotoxins will presumably activate DCs in a TLR4 dependent manner. Aromatic hydrocarbons, which derive from the combustion itself, are known to induce activation of the AhR. Most likely, the different components on the particle surface will induce different pathways, which most likely will lead to activation of several pathways within the cell and result in combined outcomes for the cell. One example for a combination of several signals by different components is the secretion of inflammasome-dependent cytokines like IL-1β and IL-18, which need two signals to be released: one to start the transcription of pro-IL-1β and pro-IL-18 and

another one to initiate caspase 1-dependent cleavage of pro-IL-1β and pro-IL-18. Previous reports have shown that PM10 can activate human DCs and thereby elicit T cell responses (Porter et al., 2007; Williams et al., 2007). It has also been shown that they induce cytokine secretion. Because of the presence of aromatic hydrocarbons on their surface, it is likely they interact with the AhR.

However, since very little is known about how these pathways interact, we sought to establish the effects of ambient urban PM10 (mainly fossil fuel-derived material) on a wide range of environmental-sensing pathways in human MoDCs and assessed whether interplay between these different pathways could exert significant influence on key DC functions. MoDCs were chosen for the experiments, although being an *in vitro* model, they allowed the generation of large numbers of uniform cells in order to allow the comparison of air pollution particles at a wide range of concentrations.

3.3. Hypothesis and study aims

The hypothesis underlying this chapter is that urban fossil fuel derived PM10 influences multiple pathways of activation in MoDCs. More specifically, the aims of this research chapter are:

- Determine whether urban FF PM10 influences DC maturation and function
- Identify the pathways by which FF PM10 can affect MoDCs
- Determine how these different pathways might interact

3.4. Results

3.4.1. Uptake of FF PM10 by dendritic cells

In order to identify the effects and interactions of fossil fuel-derived PM10 with DCs, $CD14^+$ monocytes were first differentiated into DCs by 7 days culture in the presence of GM-CSF and IL-4. The resulting MoDCs were then exposed to FF PM10 or medium only for 2 days. After applying the cell suspension on a microscope slide by cytospin, the cells were fixed and stained using the Hemacolor kit. Pictures were taken at a magnification of x100 with oil immersion.

A **B**

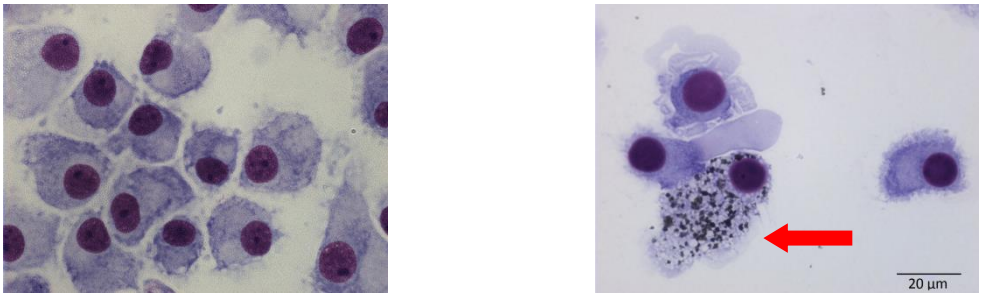


Figure 3.1: Uptake of PM10 by dendritic cells

Light microscopy pictures of MoDCs after 48h exposure to FF PM10 or medium only. After incubation cells were centrifuged onto glass slides by cytospin, fixed with methanol, and then differentially stained with azur dye (blue) and eosin (red). Pictures were taken using Picture Frame at a magnification of x100 with oil immersion and only one experiment was conducted. ***A****: Picture showing unstimulated DCs.* ***B:*** *Photo illustrating DCs from a cell suspension that was exposed to FF PM10. The red arrow indicates a cell that has taken up PM.*

MoDCs take up PM10 particles (Figure 3.1) B; see particle-laden cell at the bottom of the picture as indicated by the red arrow). Vesicles were observed to form around the black particles,

indicative of phagosome formation and putative cell stress. Activated DCs increased in size, as seen in comparison with unstimulated DCs (Figure 3.1 A).

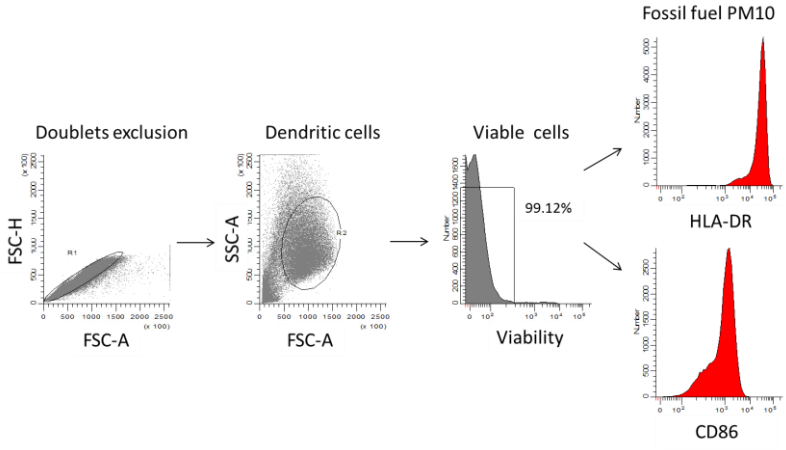
3.4.2. Fossil fuel PM10 induces TLR4-dependent dendritic cell maturation

Activation of DCs is the first and essential step in the development of an immune response since non-activated DCs would lead to T–cell anergy and tolerance. Typical markers of maturation are HLA-DR, CD86, CD83, CD80, CD40 and CCR7. As this initial step is significant for the outcome of the immune reaction, the modifications caused by the interactions of DCs with PM are important. Also, the presence of PM in the airways and the contact with DCs may have an influence on how DCs respond to inhaled antigen.

HLA-DR is a molecule of the MHC Class II Complex, CD40 and CD80 are both costimulatory proteins involved in the interaction of antigen presenting cells with T cells, and CCR7 is a lymph node homing receptor and leads to the migration of the cells to the lymph nodes. The expression of these markers was determined as the MFI measuring the level of expression.

In this set of experiments, MoDCs were exposed to PM10 that was collected in the urban area of Leicester and hence is mainly derived from the combustion of fossil fuels. In order to mature cells, LPS was used, which is a widely used activator of DCs and is an endotoxin that is present on the surface of gram-negative bacteria and constitutes a major component of the outer membrane of the bacteria. To test whether the core of the particles, without any additional components on its surface, can activate DCs, CB was included in the assay. CB is elemental and inert carbon and therefore represents the core of the particles.

A



B

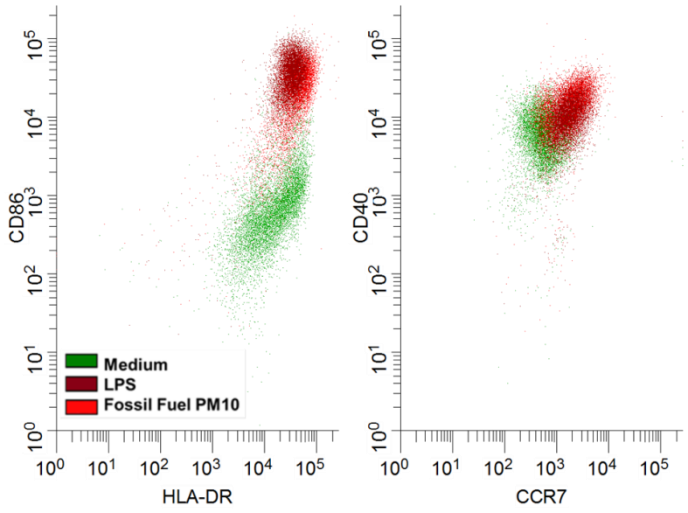


Figure 3.2: Gating strategy for the measurement of FF PM10 exposed MoDCs

Human MoDCs were stimulated for 48h with 50µg/ml of FF PM10, 0.01µg/ml LPS or medium only, subsequently antibody labelled for activation markers and measured using flow-cytometry. All in all, this experiment was conducted 36 times with no technical repeats and different blood donors each time. ***A****: The illustration shows one example of the gating sequence for the analysis of DC maturation after activation with FF PM10. Cells were labelled for HLA-DR and CD86 after incubation and measured using flow-cytometry.* ***B****: One representative scatter plot showing the expression of CD86, HLA-DR, CD40 and CCR7 on DCs that were activated with FF PM10, LPS or medium only for 2 days.*

The gating strategy to measure the expression of activation markers on MoDCs after activation with FF PM10 was to first eliminate any doublets from the analysis (Figure 3.2 A). Cell clumps can be generated during the process of the experiment and can falsify the data by generating a very high signal, as two cells stick together and double the signal but are measured as one cell. Through using the forward scatter height and area scatter, these can be eliminated, so only signals from truly single cells are taken into account. After gating on the singlets, the forward and side scatter shows a population of DCs which are taken forward to determine viable cells. This step separates cell debris from the rest of the cell suspension. During the antibody labelling process, cells were stained with a viability dye, which stains for dead cells. DCs that are negative, which usually was a very high proportion of cells, are taken into account for the analysis. Finally, once single and viable DCs were filtered from the rest of the cell suspension, the activation markers could be analysed. This was done using the MFI. MFI indicates the magnitude of the expression of a marker and can provide information on how positive cells are for a particular marker.

Figure 3.2 B shows a representative example of how CD86, HLA-DR, CD40 and CCR7 are expressed on DCs after they were stimulated with FF PM10, LPS or cultured in medium only. HLA-DR was highly expressed on all conditions, whereas CD86 was highly expressed only after stimulation with FF PM10. CD40 and CCR7 showed a similar pattern; by which medium only exposed DCs express the lowest level of the marker and stimulated cells show a high level of expression. Both FF PM10 and LPS-exposed cells show a similar expression of CD40 and CCR7.

In order to assess the kinetics of FF PM10-stimulated effects on DC at the level of gene and protein expression, different time points were examined. For this, $CD14^{+}$ monocytes were magnetically sorted from PBMCs extracted from blood donated by healthy volunteers. After 7 days of incubation with GM-CSF and IL-4, MoDCs were harvested and stimulated with fossil fuel PM10 for 2,4,6,24,and 48 hours. Subsequently, CD80, CD86 and HLA-DR were measured after extracellular antibody staining by flow-cytometry. In addition, cells were lysed, RNA was extracted, reverse transcripted and gene expression of CD80 and CD86 was measured by quantitative real-time PCR.

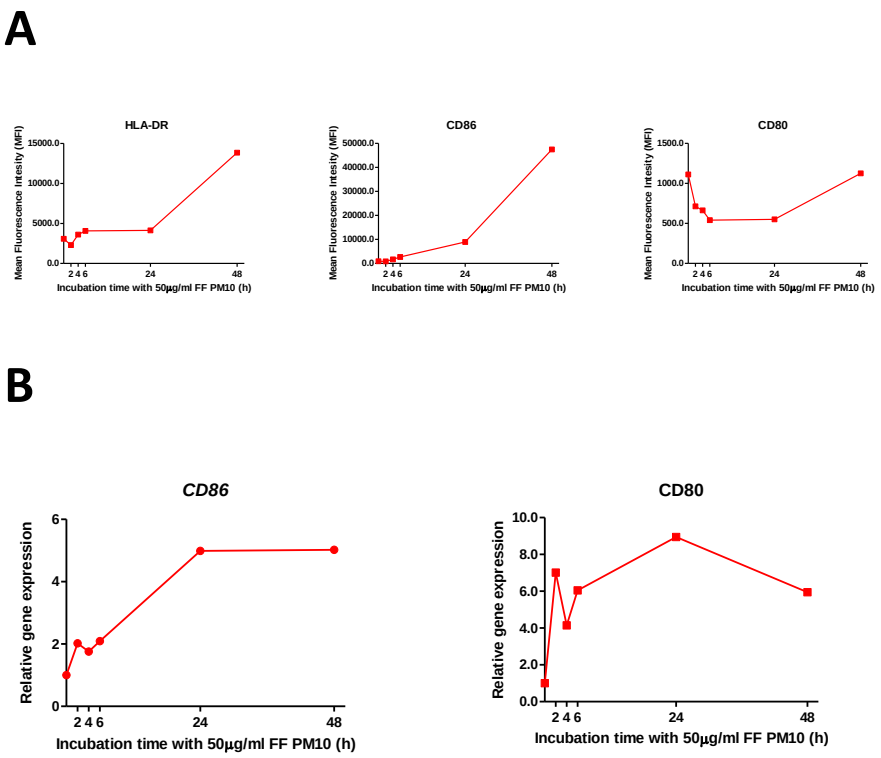


Figure 3.3: Fossil fuel PM10 induces expression of maturation markers on DCs

DCs derived from human blood monocytes were exposed to 50µg/ml of FF PM10 for variable incubation times. After stimulation, cells were either antibody stained for maturation markers and measured by FACS or lysed for qRT-PCR and the expression of activation markers was measured at RNA level. This experiment was conducted once, with one blood sample and no technical repeats. ***A****: The graphs show of the expression of DC maturation markers HLA-DR, CD80 and CD86 as measured by flow-cytometry after surface antibody labelling. Cells were incubated with FF PM10 for 2, 4, 6, 24 and 48 hours.* ***B****: The diagram illustrates the gene expression of CD80 and CD86 in DCs after an incubation time with FF PM10 for 2, 4, 6, 24 and 48 hours.*

All markers examined were expressed by MoDCs after incubation with 50µg/ml of FF PM10, either by surface marker measurement through flow-cytometry or qRT-PCR. The expression shows a small dip at 2-4 hours, but an increase after that, except for CD86, which decreases further and increases in expression after 6 hours. All markers increased in expression, reaching their highest level at 48h, except for the RNA expression of CD80, which has its highest expression at 24h. Therefore, it was decided to conduct all experiments after an incubation time of 48h.

In order to assess the effect of FF PM10 on the maturation of DCs, MoDCs were differentiated and exposed to FF PM10 and CB either at concentrations 10, 25 and 50μg/ml and medium only or at 50μg/ml for FF PM10 and CB and 0.01μg/ml of LPS. After antibody labelling for HLA-DR, CD86, CD40, CCR7, CD80 and CD86 cell suspensions were measured through flow-cytometry. Costimulatory markers were measured at a range of PM concentrations in order to cover any inconsistencies due to concentration differences, as the assessment of the concentration of the particle suspension was very difficult.

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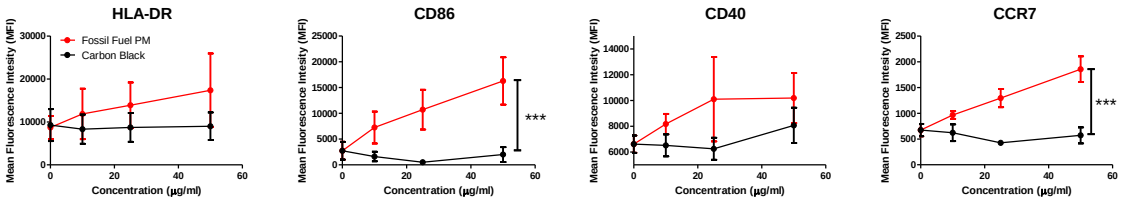

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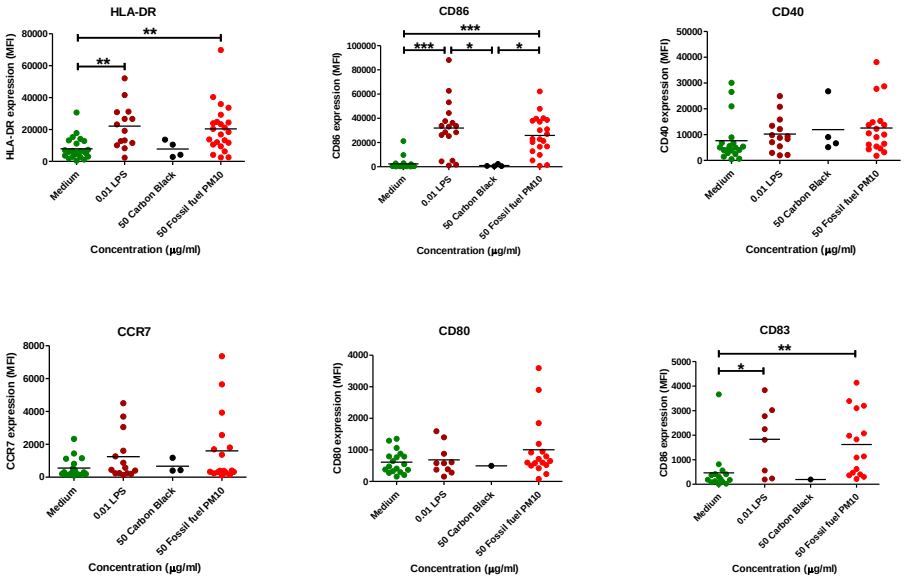

Figure 3.4: Fossil fuel PM10 induces DC maturation

Human MoDCs were stimulated for 48h in medium only and LPS as well as various concentrations of FF PM10 and CB. After incubation, expression of the activation markers was measured by antibody surface labelling and subsequent flow-cytometry. Each dot represents one independent experiment from a separate blood donor. ***A****: The graph shows the mean and standard deviation of six (for HLA-DR and CD86), five (for CD40) and four (for CCR7) independent experiments. The MFI was measured of maturation markers and chemokine receptor CCR7 after MoDC stimulation with FF PM10 and CB at 0, 10, 25 and 50µg/ml. The data was analysed using a two-way analysis of variance.* ***B****: Summary data of HLA-DR and co-stimulatory molecules CD40, CD86, CD80 and CD83 as well as CCR7 expression after incubation of DCs with 10ng/ml LPS, 50µg/ml CB and FF PM10 and medium only. Each dot represents one independent experiment. Statistical analysis was conducted using a one way analysis of variance based on the Kruskal-Wallis test.*

Stimulation of maturation markers HLA-DR, CD86, CD40 and lymph node homing receptor CCR7 with a range of concentrations of FF PM10 and CB resulted in a dose-dependent activation pattern after exposure to FF PM10, which was highly significant for CD86 and CCR7 (Figure 3.4 A). Stimulation with various concentrations of CB led to a flat line. CB is elemental inert carbon which constitutes the core of air pollution particles and is therefore used to test whether carbon particles without any additional components can cause activation of DCs. This data indicate that the inert elemental carbon core is not responsible for the activation of the cells and suggests that the additional substances adsorbed on the particle surface induce the expression of the maturation markers rather than the carbon core.

When repeating the experiment several times, we saw that the initial finding, i.e., that exposure of DCs to FF PM10 induces expression of the maturation markers HLA-DR, CD86, CD40, CD80, CD83 and CCR7 was a representative observation (Figure 3.4 B). The overall expression of the maturation markers and chemokine receptor was similar to the LPS control. LPS is one of the main elements of the outer membrane of gram-negative bacteria and acts by signalling through TLR4. It is widely used as an activator of DCs. HLA-DR expression was significantly higher after FF PM10 exposure as well as LPS exposure compared to the medium control. FF PM10 and LPS stimulation of DCs induces a highly significant expression of CD86; additionally the difference in stimulation between FF PM10 and LPS to CB is significant. Although the differences for CD40 and CCR7 between stimulated and non-stimulated DCs are not significant, a trend is noticeable, showing a slightly higher expression of CD40 and CCR7 in DCs after FF PM10 and LPS stimulation. Again, for CD80 the incubation of DCs with FF PM10 resulted in a small increase of the maturation marker compared to the medium control. A highly significant result was observed for the expression of CD83 after FF PM10 and LPS stimulation of MoDCs compared to the unstimulated control. The exposure of DCs to CB, which represents the particle core of air pollution particles without any additional components on the surface, did not lead to any significant result for all markers that were measured, indicating that the adsorbed substances on PM are responsible for the observed effects.

In addition, it was checked whether, as a true positive control, LPS in conjunction with a particle core, CB, induced a different type of activation pattern. This was done to affirm whether carbon particles had any inhibitory effect on DCs. But this was not the case. LPS, either bound on a particle or in solution, induced the same effects on MoDCs; therefore, the particle control did not influence the effects of LPS (data not shown).

Since all the particles were collected on filters, any filter components released during the extraction procedure to remove the particles from the filter surface could theoretically affect DCs during the *in vitro* experiments. Through this process, small filter parts may split off from the filter, enter the particle suspension and cause DC activation. Therefore, we tested whether filter components induce DC maturation, by performing the extraction procedure on an unused and empty filter and using the suspension from the extraction on DCs, exposing them for two days. At the same time, filters with collected particles on them were used as a control.

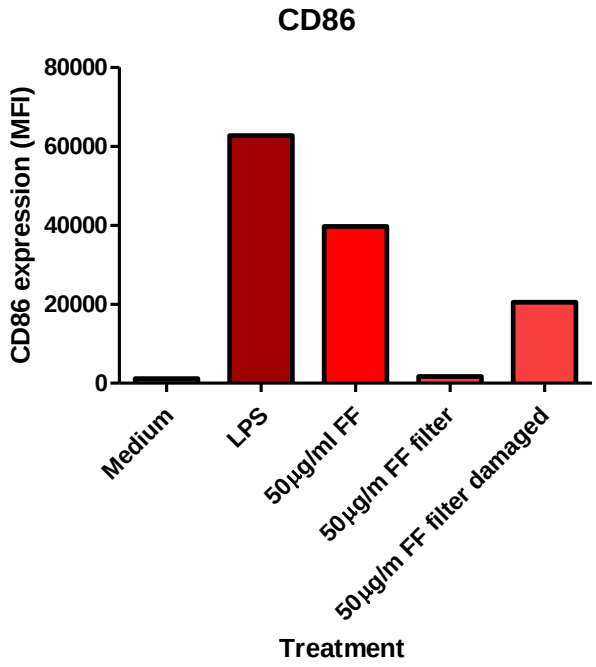

Figure 3.5: PM filter does not induce DC maturation

Human blood monocytes were cultured with GM-CSF and IL-4 for 7 days to obtain DCs, which were exposed for 2 days to 0.01µg/ml of LPS, 50µg/ml FF PM10 or medium only. Also, unused and empty filters which were used to collect the PM were put through the extraction procedure without carrying any particles, to test whether any substances from the filter itself induce activation of DCs. One set of filters was put through exactly the same procedure as for extracting the cells and another set of filters was sonicated beyond the standard treatment to the point at which the filter got visibly damaged and small fibres came loose in the suspension. Both suspensions were used at the corresponding volumes as the particles to incubate the cells with. The graph shows one experiment with no technical repeats using a blood sample from one donor.

Blank filters put through our standard extraction procedure did not activate DCs as measured by the expression of CD86 (Figure 3.5). This normal extraction process did not release any visible filter fibres into the suspension. To test whether fibres released by extended sonication beyond this point have the potential to activate DC sonication was prolonged until the filter was damaged. Extensive extraction damaged the filter releasing fibres in the suspension which were able to cause a low level of DC activation. This indicates that filter components have the potential to induce DC maturation if the filter is sufficiently damaged. However, this is unlikely to contribute in our standard extraction method where there is little evidence of damage to the filter.

The set of experiments above showed that the effects observed on the maturation of MoDCs after FF PM10 activation were similar to the effects induced by TLR4 dependent effects of LPS stimulation. Therefore, we wanted to determine if the effects of FF PM10 also involve TLR4 signalling.

For this, MoDCs were pre-treated with CLI-095, a specific TLR4 antagonist, prior stimulation with FF PM10 and LPS and the expression of maturation markers was measured by FACS.

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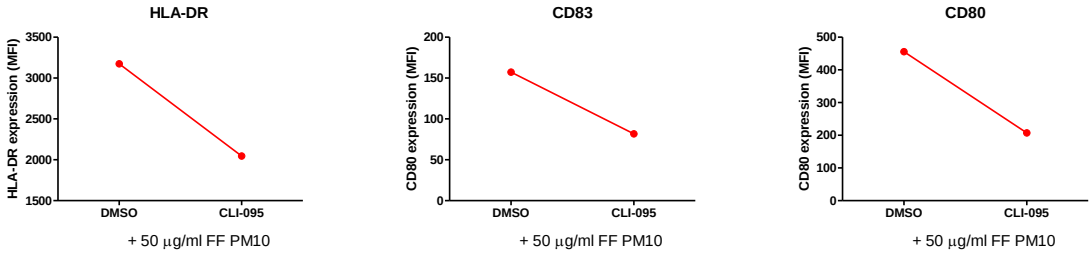

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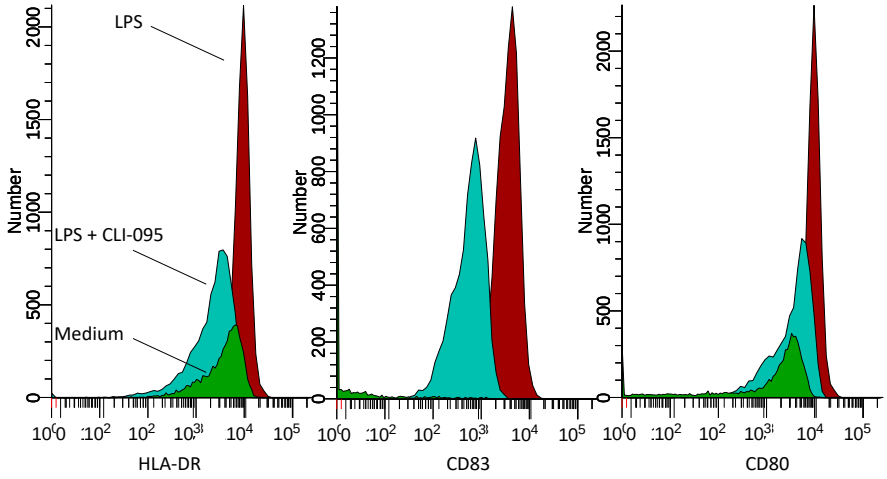

Figure 3.6: DC activation by FF PM10 is TLR4-dependent

DCs were derived from CD14^{+} monocytes by culturing with IL-4 and GM-CSF for 7 days. After differentiation, cells were cultured with 50µg/ml FF PM10, 0.01µg/ml LPS, medium only or pre-treated with TLR4 antagonist CLI-095 for 1h and subsequently cultured with 50µg/ml FF PM10 and 0.01µg/ml LPS. DCs were labelled for HLA-DR, CD83 and CD80 and measured by flow-cytometry. ***A****: Diagrams showing the expression of the activation markers of DCs. Cells were pre-treated with CLI-095 and subsequently stimulated with FF PM10 or the vehicle control DMSO.* ***B****: FACS plots showing the surface expression of HLA-DR, CD80 and CD83 on DCs after stimulation with LPS, CLI-095 and successive activation with LPS, or cells that were cultured in medium only. The magnitude of the peaks indicates the acquired cell numbers by FACS. The graph shows one experiment with no technical repeats using a blood sample from one donor.*

HLA-DR, CD83 and CD80 expression was reduced in MoDCs when cells were incubated with CLI-095 prior FF PM10 stimulation (Figure 3.6 A). When cells were stimulated with LPS, instead of FF

PM10, the difference in maturation marker expression for cells pre-treated with the TLR4 blocker compared cells that were not blocked prior to stimulation appeared similar to the pattern seen when cells were stimulated with FF PM10 (Figure 3.6 B).

In summary, this section shows that FF PM10 activates MoDCs in a TLR4 dependent manner.

3.4.3. Fossil fuel PM10 activated DCs show an increased ability to stimulate allogeneic naïve $CD4^+$ T cells

The above results showed an increased activation of DCs after exposure to FF PM10. To investigate the functional consequence of that matured phenotype induced by FF PM10, a MLR with allogeneic naïve $CD4^+$ T cell was performed. *In vivo*, the next step after the activation of DCs and migration to the lymph nodes is to induce proliferation of T cells in the lymph nodes; once the T cells are present in sufficient numbers, they migrate to the site of infection and fulfil their intended function.

For this, $CD14^+$ monocytes were incubated with GM-CSF and IL-4 for 7 days and subsequently incubated with FF PM10, LPS, CB or in medium only. After 2 days, pre-treated cells were co-cultured at different concentrations with allogeneic and CFSE labelled naïve CD4 T cells for another 5 days.

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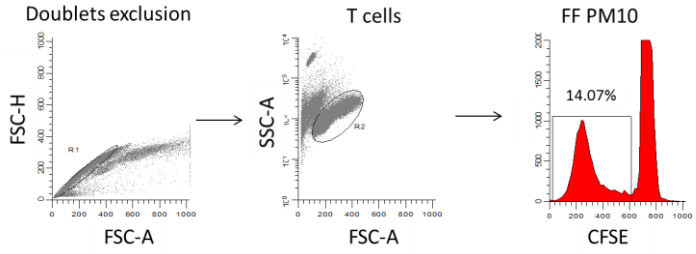

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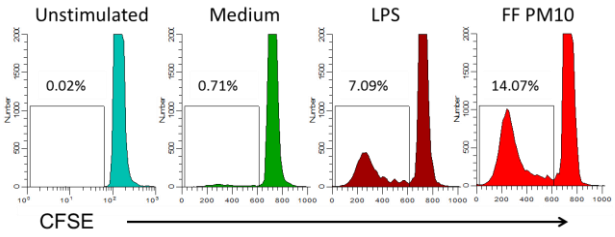

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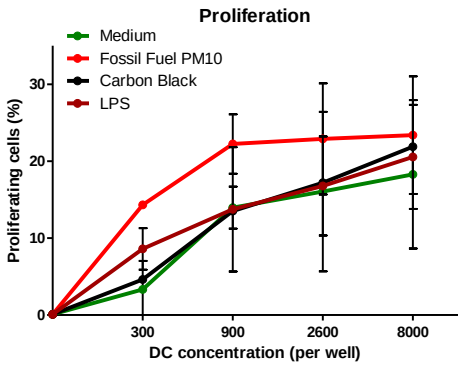


Figure 3.7: Proliferation of naïve CD4^{+} T cells cultured with allogeneic DC

DCs were derived from human blood monocytes and were exposed to 25µg/ml PM10 and CB and 0.01µg/ml LPS or the medium control. After 48h of exposure, cells were washed and plated at different concentration (300, 900, 2600 and 8000 DCs per well). 400,000 naïve T cells, which were labelled with CFSE beforehand to track their proliferation, were added to each concentration of DCs and incubated together for 5 days and then measured using flow-cytometry. ***A****: A representative example of the gating strategy showing the exclusion of doublets, T cell region and the proliferating T cells.* ***B****: Flow-cytometry plots of CFSE labelled T cells stimulated with either medium only exposed DCs or LPS and FF PM10 stimulated cells or unstimulated T cells.* ***C****: Percentage of proliferating T cells after 5 days of stimulation with various numbers of previously exposed DCs. The graph shows the mean proliferation data pooled from six independent experiments with standard deviation. A two-way analysis of variance was performed on three independent experiments (which provided a complete set of results) out of 6 independent experiments with six different blood donors. Bonferroni post-tests were conducted to compare the individual conditions.*

Part A of Figure 3.7 shows the gating strategy used for these experiments. Firstly, doublets were excluded to make sure that clumps of cells were not included in the analysis and only single cells were taken into account. Figure 3.7 B illustrates the flow-cytometry plots of one representative experiment showing proliferating and non-proliferating allogeneic naïve $CD4^+$ T cells, which were labelled with CFSE. These T cells were stimulated with various numbers of DCs, which were previously exposed to FF PM10, LPS, CB or the medium control. The far left plot shows T cells that were left unstimulated for the time of the incubation. The fluorescent staining dye CFSE is commonly used to track cell division. It is quickly taken up by cells after a short time of incubation and works by getting further diluted each time the cell divides. Therefore, non-dividing cells exhibit a higher fluorescence compared to proliferating cells that went through several divisions. This leads to a decreasing fluorescein signal, in which CFSE is detected and less CFSE is present in each cell; hence, the peak moves to the left in the FACS plots. The three different high peaks on the right hand side around 10^4 represent the non-proliferating T cells, as they demonstrate a high level of fluorescence. To the left of the high and narrow peak, and towards the lower fluorescence levels on the horizontal axis, the smaller and wider peak of the dividing cells can be seen. The proportion of proliferating T cells was the highest when DCs were previously exposed to FF PM10. A slightly smaller peak could be seen for T cells that were co-cultured with LPS-exposed DCs and the smallest peak was observed for those T cells that were activated by unstimulated DCs.

Part C of Figure 3.7 demonstrates the percentage of proliferation T cells plotted against the number of DCs that were added to the co-culture with T cells. DCs exposed to FF PM 10 stimulated more allogeneic naïve CD4 T cell proliferation compared with T cells that were stimulated with CB, LPS or medium only exposed DCs. The differences of proliferation due to activation of DCs were more prominent in the range of concentration of DCs between 300 ($p < 0.01$) and 900 ($p < 0.05$) cells compared to the medium control. Therefore, the difference between the treatments was most noticeable at lower concentrations of DCs and is more sensitive in that region. Even small numbers of DCs were able to stimulate naïve T cells. Higher concentrations of FF PM10 stimulated DCs did not induce a significantly higher proliferation of allogeneic naïve CD4 T cells compared to the medium only exposed DCs.

Also, it was checked whether the particles themselves could induce proliferation of naïve CD4 T cells in the absence of DC, as during the process PM10 was not washed off completely and so may interact with the T cells, but that was not the case (data not shown).

Overall, this section shows that, as expected, activated DCs induced proliferation of allogeneic naïve CD4 T cells in which FF PM10-exposed DCs caused the highest level of proliferation

followed by CB-stimulated DCs and medium only cultured DCs. In general, differences between the various stimuli were more prominent at lower cell concentrations.

3.4.4. Exposure of DCs to fossil fuel PM10 does not affect cytokine profile induced in CD4 T cells

Asthma is a disease that involves immune dysregulation in the airway and is known to be associated with a Th2-type cytokine response, which includes secretion of Il-4 and IL-5 (Hansen, 2001). Environmental PM, especially from urban areas where the main combustion source is fossil fuel, is known to be linked to the development and exacerbation of asthma. It was therefore hypothesised that exposure of DCs to FF PM10 would skew the cytokine profile of the naive $CD4^+$ T cells they activate towards a Th2 type immune response, by secreting IL-4, IL-5 and IL-10. IL-17 gives an indication whether the response is a Th17 related immune response and IFN-γ production is indicative of a Th1 immune reaction.

The production of IFN-γ, IL-17, 4, 5, 10 by T cells after activation with stimulated and unstimulated DCs was measured. DCs were cultured with CB, FF PM10 or in medium only. T cells were again stained with CFSE to be able to distinguish between proliferating cells and non-proliferating cells, as the proliferating proportion of cells is mainly responsible for producing and secreting cytokines. After 5 days, cells were re-stimulated with the PIM cocktail and intracellularly stained and measured by flow-cytometry.

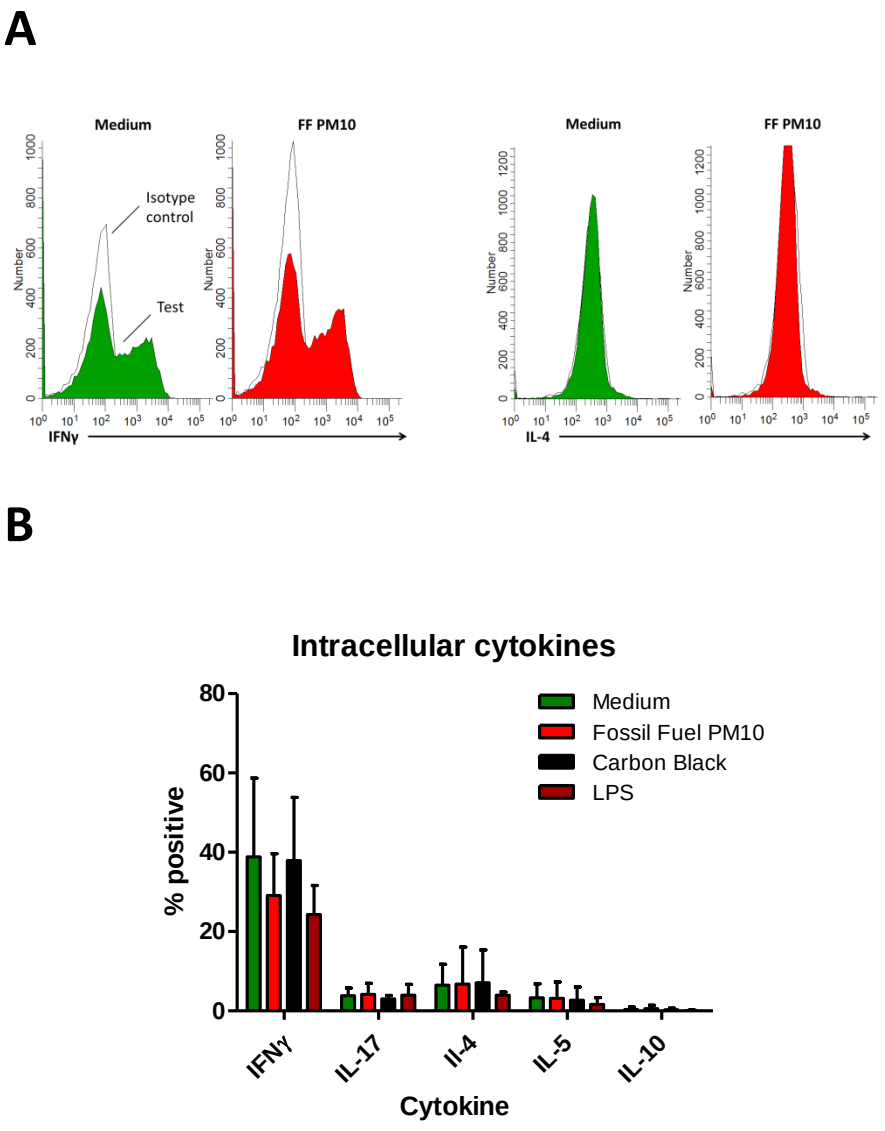


Figure 3.8: Cytokine profile of proliferating allogeneic naïve CD4$^+$ T cells after stimulation with PM10 exposed DCs

MoDCs were exposed to medium only, 25µg/ml of FF PM10 and CB and 0.01µg/ml LPS for 48h. After incubation and washing, 8,000 DCs were co-cultured in an allogeneic MLR with 400,000 naïve CD4$^+$T cells for 5 days. Subsequently, cells were fixed, permeabilized and labelled for intracellular cytokines, which were then measured by flow-cytometry. ***A:*** *Representative example of flow-cytometry histograms showing IFN-γ and IL-4 intracellular expression in naïve CD4 T cells after co-culture with FF PM10 stimulated DCs. The grey line in the background shows the matching isotype control.* ***B:*** *Summary data of intracellular measurement of IFN-γ, IL-17, IL-4, IL-5 and IL-10 in naïve CD4 T cells after stimulation with FF PM10 pre-incubated DCs. The graph shows the mean percentage of positive cells pooled from three independent experiments and the standard deviation.*

The expression of IFN-γ, IL-17, 4, 5, 10 is illustrated as the percentage of positive cells in relation to the isotype control (Figure 3.8). 25-40% of the proliferating T cells produced and secreted IFN-γ, whereas only around 5% of the cells produced IL-4, 5 and 17. IL-10 was only found in very low numbers, approximately 1-2%. The treatment of DCs, which was FF PM10, CB, LPS or untreated, seems to be irrelevant to the outcome, as all 4 conditions showed a similar frequency of production for the respective cytokines. Regardless of the type of exposure the DCs were subjected to, it did not influence the outcome. DCs that were cultured for 2 days in cell culture medium and therefore not exposed to any stimuli showed the same pattern of cytokine production, i.e. high production of IFN-γ and lower production of IL-17, 4, 5, and 10.

3.4.5. Secretion of proinflammatory cytokines by FF PM10 stimulated DCs

The secretion of cytokines during an immune response is one part of the function of DCs to coordinate the immunological process. IL-6 is an important cytokine in terms of activation of lymphocytes, whereas IL-12p70 is involved in the Th1 immune response by stimulating and maintaining cellular activity. IL-1β and IL-18 indicate that the inflammasome, which is involved in inflammatory processes, is activated. Specifically, both cytokines increase antimicrobial properties of phagocytes by inducing Th1 and Th17 immune responses. IL-23 stimulates T cells and can induce the production of IFN-γ.

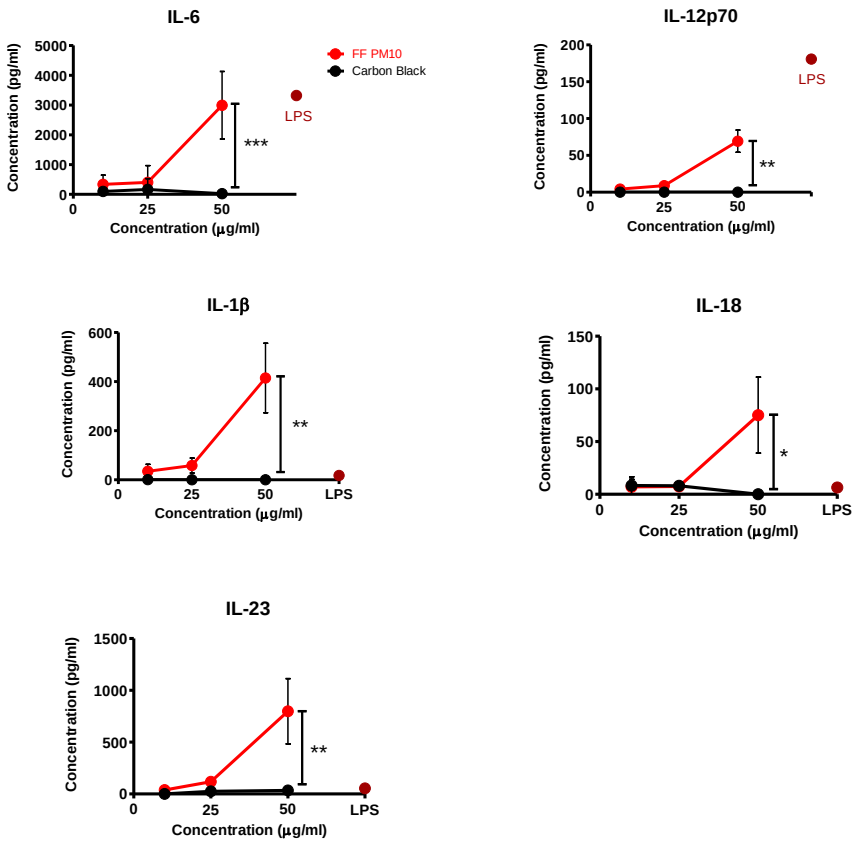


Figure 3.9: Secretion of proinflammatory cytokines by PM10 stimulated DCs

DCs derived from human blood monocytes were stimulated with FF PM10 and CB (10, 25, 50µg/ml) and LPS (0.01µg/ml) for 48h and the secretion of the cytokines IL-6, IL-12, IL-1ß, IL-18 and IL-23 was determined. The soluble cytokines in the supernatants of the cultures were assayed using a Multiplex ELISA kit and a subsequent measurement with flow-cytometry. The figures show the mean cytokine concentration in the supernatants of four separate experiments with no technical repeats. Statistical analysis was performed using a two-way analysis of variance.

The cytokine release in the supernatant of the DCs during 48h of incubation with various stimuli or without stimuli in medium only was analysed (Figure 3.9). Exposure of DCs to FF PM10 induced expression of IL-6, IL-12, IL-1β, IL-18 and IL-23 at overall variable levels, ranging up to 3000pg/ml for IL-6, up to 100pg/ml for IL-12p70, up to 400pg/ml for IL-1β, up to 80pg/ml for IL-18 and reaching up to 800pg/ml for IL-23. Stimulation of DCs with FF PM10 induced a significantly higher expression of the measured cytokines compared to the stimulation with CB. The largest difference can be observed at around 50µg/ml. The particulate control CB did not induce the expression of all the cytokines measured, regardless of the concentration used and

showed an almost flat line for all the cytokines. This indicates that the core of the particles was not responsible for the cytokine secretion, but rather the additional components on its surface lead to the effects observed. LPS, the microbial activator of DCs, induced a varied secretion pattern of the cytokines. Whereas both FF PM10 and LPS induced production of IL-6 and IL-12p70, only FF PM10 exposure led to the secretion of IL-1β, IL-18 and IL-23 by DCs. IL-1β and IL-18 are associated with activation of the inflammasome and production of inflammasome dependent cytokines like IL-1β and IL-18 requires two signals. The first signal starts the transcription of pro-IL-1β and pro-IL-18 and the second signal is required to activate the inflammasome leading to caspase-1 dependent cleavage of pro-IL-1β and pro-IL-18 into the active forms IL-1β and IL-18. FF PM10 seems to provide both signals, whereas CB and LPS lack one or the other or both signals and therefore IL-1β and IL-18 were not secreted.

In summary, FF PM10 induced secretion of the cytokines IL-6, IL-12p70, IL-1β, IL-18 and IL-23 in MoDCs, while LPS stimulation of DCs only lead to secretion of IL-6 and IL-12p70. CB did not initiate any cytokine production, which again indicates the relevance of the adsorbed substances on the particle surface for the activation of DCs.

In order to check whether the effects seen in the previous section were dependent on the TLR4 receptor, we blocked the TLR4 receptor in MoDCs with CLI-095, the specific TLR4 antagonist, as used before and stimulated DCs with FF PM10 afterwards. Cytokine concentrations in the supernatants were measured using a Cytometric Bead Array (CBA).

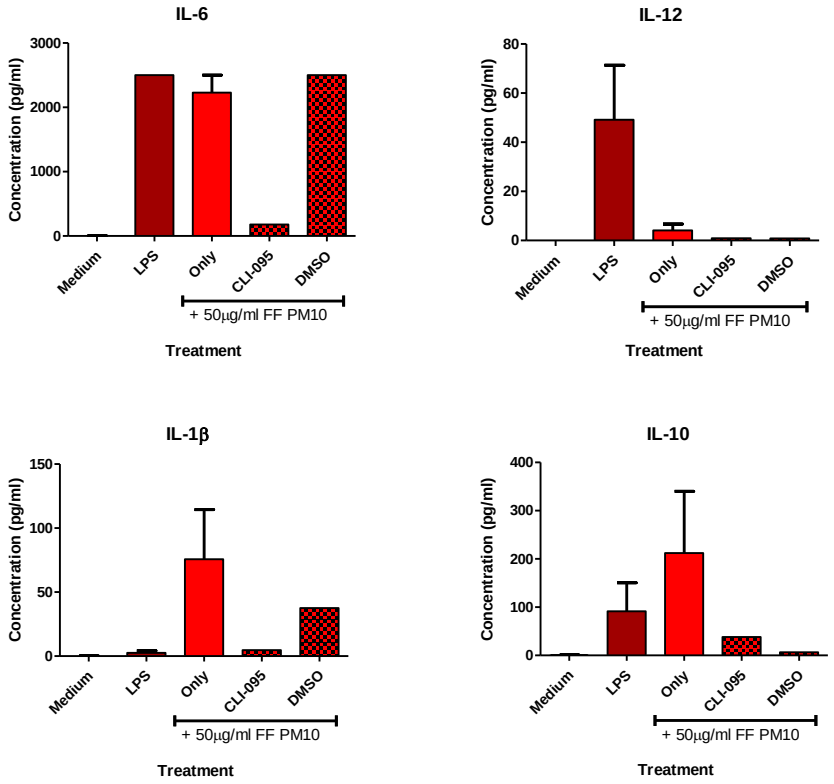


Figure 3.10: IL-6 and IL-1β production after stimulation with FF PM10 in DCs is TLR4-dependent

$CD14^+$ monocytes were separated from human PBMCs and cultured with IL-4 and GM-CSF for 7 days to obtain DCs. DCs were incubated with 0.01µg/ml LPS, 50µg/ml FF PM10 and medium only or cells were pre-treated with the specific TLR4 antagonist CLI-095 or the vehicle control for 1h and then stimulated with 50µg/ml FF PM10. Secretion of IL-6, IL-12, IL-1β and IL-10 in the supernatants was measured using a Cytometric Bead Array (CBA). The graphs show the mean and standard deviation of two independent experiment with blood samples from two separate donors.

IL-6, as seen in the previous section, was secreted by DCs after stimulation with LPS suggesting that its production is TLR4 dependent; hence, TLR4 blockade led to a decrease of this cytokine, as expected. Similar to IL-6, IL-12 was also produced after LPS activation by DCs. However, when cells were exposed to the TLR4 blocker and the vehicle control DMSO before activation with FF PM10, no substantial difference was detected between both conditions, which makes the interpretation of the results difficult. IL-1β is known to require a TLR signal to start the transcription of pro-IL-1β. Therefore, as expected, CLI-095 pre-treatment of DCs prior FF PM10 stimulation led to a lower secretion of the cytokine. IL-10 did not show any dependence on the TLR4 signalling pathway; thus, blocking the pathway did not influence the concentration of the

cytokine. However, similar to the observation with IL-12, interpretation of the result is difficult, as the vehicle control DMSO alone had a considerable effect on the secretion of IL-12 by DCs after stimulation with FF PM10.

All in all, as expected IL-6 and IL-1β concentration was decreased after pre-treatment with CLI-095 and IL-10 secretion was not affected by the antagonist. However, although expected, IL-12 production was not affected by the TLR4 antagonist.

3.4.6. Induction of the aryl hydrocarbon signalling pathway in DCs by FF PM10

The AhR is a ligand-activated transcription factor that can be activated by aromatic hydrocarbons. PM10 is a combustion product and therefore likely to contain combustion-derived aromatic hydrocarbons that may function as AhR ligands. Originally recognised for its ability to induce synthesis of xenobiotic-metabolizing enzymes such as *CYP1A1* and *CYP1B1,* AhR signalling is now known to affect immune cells through the induction of *IDO1* as well as other pathways. IDO1 is an enzyme involved in the kynurenine pathway and is responsible for the depletion of tryptophan, which can inhibit the growth of T cells, thereby limiting immune responses. *CYP1A1* and *CYP1B1* are members of the cytochrome P450 superfamily of enzymes and are involved in xenobiotic and drug metabolism (Smith, Stubbins, Harries, & Wolf C R, 1998). Currently, nothing is known about interactions between PM and the AhR pathway in DC.

Therefore, in order to assess the activation of the AhR pathway in DCs, we first measured whether the receptor was expressed on MoDCs. For this, MoDCs were cultured with FF PM10 or medium only and the expression of the *AhR* was measured by qRT-PCR.

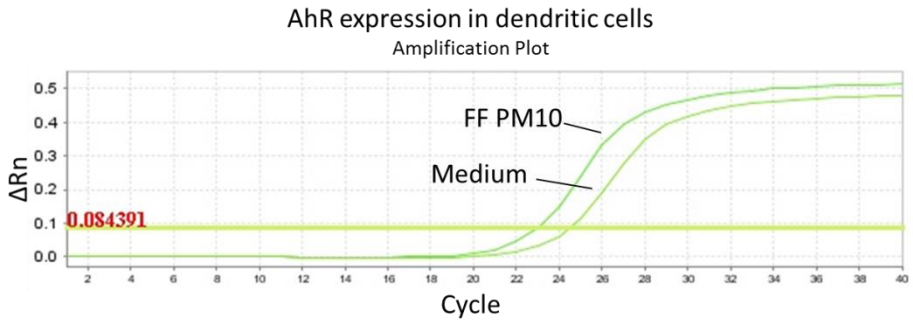


Figure 3.11: MoDCs express the AhR

Human MoDCs were exposed for 48h to 50µg/ml of FF PM10 or medium only. After the incubation, cells were lysed and quantitative real-time PCR was performed to measure the expression of the AhR. The diagram shows one representative example of the amplification plot out of 38.

Figure 3.11 shows that *AhR* is expressed in MoDCs and can be detected by qRT-PCR at a cycle threshold at around 24. When cells were stimulated with 50µg/ml of FF PM10, the expression of AhR increased and the threshold moved to 23. This indicates that the exposure of DCs to fossil fuel-derived PM10 induces a small increase in the expression of *AhR* on a transcriptional level. All in all mature and immature DCs express the AhR.

As a next step, an incubation time had to be determined in order to assess when the expression of *CYP1A1*, *CYP1B1* and *IDO1* is at its highest to be able to detect it. For this, MoDCs were grown and exposed to FF PM10 for variable incubation times. Subsequently, cells were lysed and qRT-PCR was performed to measure *CYP1A1*, *CYP1B1* and *IDO1*.

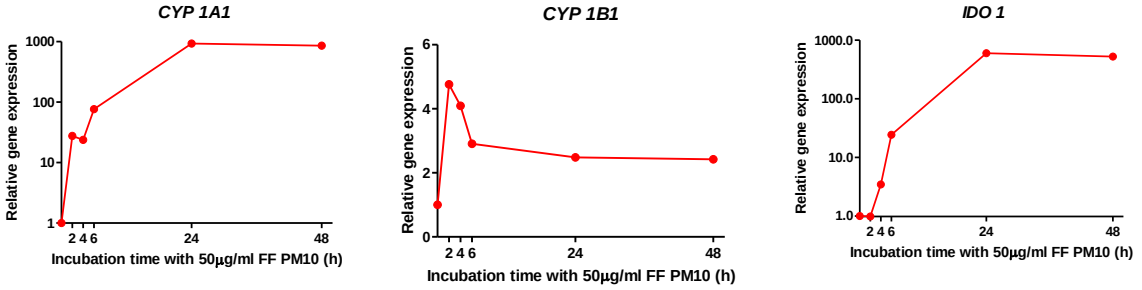


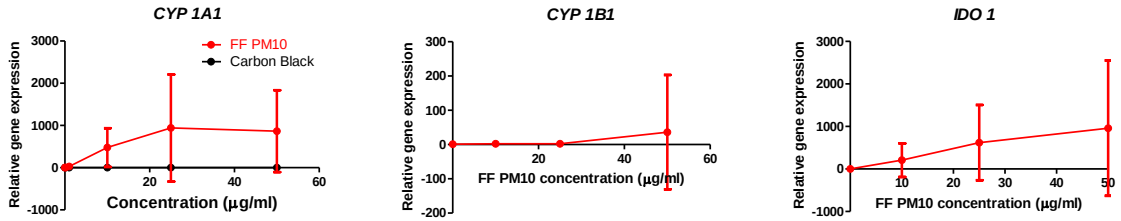
Figure 3.12: The AhR pathway is induced in DCs after exposure to FF PM10 in a time dependent manner

$CD14^+$ monocytes were separated from PBMCS and differentiated into DCs for 7 days. DCs were exposed to 50µg/ml of FF PM10 for 2, 4, 6, 24 and 48 or medium only. RNA expression of CYP1A1, CYP1B1 and IDO1 was measured after lysis of the cells by PCR. The graphs represent one experiment conducted aith a blood sample from one donor.

The expressions of *CYP1A1* and *IDO1* show their highest point after 48h, although expressions of both decrease at the beginning, *CYP1A1* at around 4 hours and *IDO1* at 2 hours. Interestingly, *CYP1B1* showed a different expression pattern. After reaching its peak at 2 hours, the RNA expression decreased, reaching its lowest level at 48h. However, at this time the expression was around 2-3 times larger than the medium control and still detectable.

Since we had confirmed that DCs express the *AhR*, and the incubation time needed, we could next look at how the receptor was influenced by looking at AhR-related genes known from the literature, such *CYP1A1*, *CYP1B*1 and *IDO1*. These were measured after two days of exposure of the cells to FF PM10, CB, LPS and FICZ or medium only.

A



B

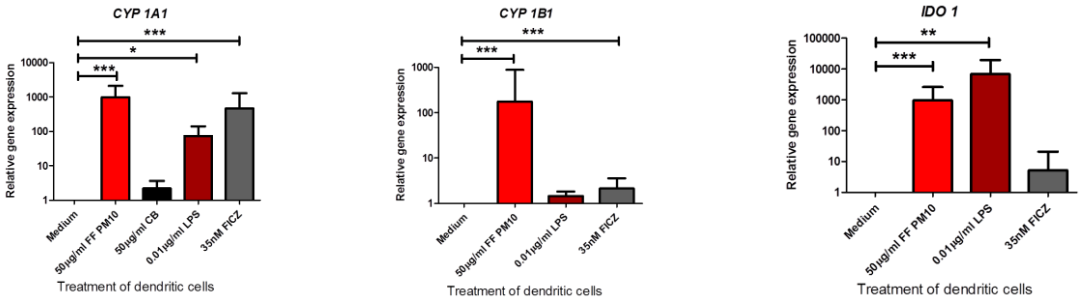


Figure 3.13: The AhR pathway is induced in DCs after exposure to FF PM10

Human monocytes were separated from PBMCs and cultured with IL-4 and GM-CSF to obtain MoDCs. These were then stimulated with several concentrations of fossil fuel derived PM10 and CB, as well as 0.01µg/ml LPS and 35nM of FICZ for 2 days. Subsequently, cells were analysed through PCR for the expression of CYP1A1, CYP1B1 and IDO1. ***A:*** *DCs were cultured with FF PM10 and CB at concentration of 0.1, 1, 10, 25, 50µg/ml or in medium only and the relative gene expression of CYP1A1 was measured. For the measurement of CYP1B1 and IDO1 DCs were exposed to FF PM10 at concentrations of 10, 25, 50µg/ml or medium. The graphs show the mean and standard deviation of 37 independent experiments using blood samples from different donors.* ***B:*** *Cells were cultured in the presence of 50µg/ml FF PM10, 50µg/ml CB (for CYP 1A1 only), 0.01µg/ml LPS and 35ng FICZ or in medium only. The graph shows the mean relative gene expression of CYP1A1, CYP1B1 and IDO1 of 38 separate experiments. The error bars represent the standard deviation. Statistical analysis was performed using a one-way analysis of variance, the Kruskal-Wallis test, after conducting a normality test. All results were analysed using the comparative C_T method and normalised relative to the medium control. The results are plotted on a log scale.*

CYP1A1, *CYP1B1* and *IDO1* were expressed in MoDCs after stimulation with FF PM10 in a dose-dependent manner (Figure 3.13 A). CB particles did not induce expression of *CYP1A1* at any of the concentrations used, again indicating that those additional components on the particle core, and not the core itself, were responsible for the observed effects. When looking at the summary data, after repeating the experiment several times, the results confirm that CB did not induce any significant expression of *CYP1A1* (Figure 3.13 B). When looking at the effect of FF PM10 on the genes measured in DCs, all of them showed a significant increase. LPS induced the expression of *CYP1A1* albeit to a lower level, as well as *IDO1*, but not *CYP1B1*. FICZ, the AhR antagonist, induced a highly significant increase in RNA levels of *CYP1A1* and *CYP1B1*, but interestingly the same effect was not observed with *IDO1*.

In summary, this section shows that the additional substances present on the carbon core of the particles induced activation of the AhR as measured by the expression of *CYP1A1*, *CYP1B1* and *IDO1*. The core itself, however, did not lead to the same effects. The specific AhR-agonist FICZ also induces expression of AhR-controlled genes, yet *IDO1* expression is not affected by FICZ. Interestingly, LPS induces expression of *CYP1A1* and *IDO1*.

The previous section showed that FF PM10 induced expression of *CYP1A1*, *CYP1B1* and *IDO1*, which we assumed was AhR dependent, as reported in the literature. To assess the dependence of the expressed genes on the AhR pathway, we pre-treated DCs with the AhR antagonist CH223191 prior stimulation with FF PM10 for 30 min.

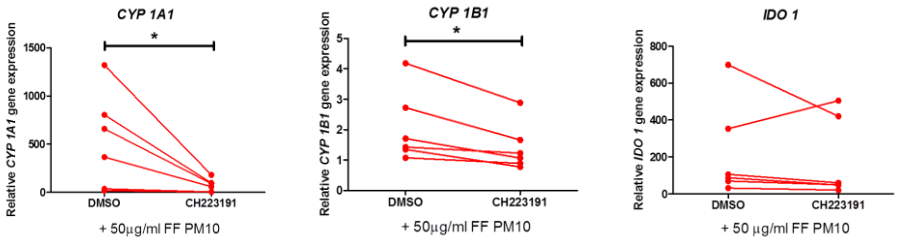


Figure 3.14: CYP1A1 and CYP1B1 expression in MoDCs is AhR-dependent

MoDCs were incubated with 3µM CH223191 or DMSO for 30 min and subsequently 50µg/ml of FF PM10 to both conditions was added and cultured for 48h. After incubation, cells were lysed and gene expression was measured using qRT-PCR. Each pair of dots represents a separate experiment with a different blood donor. All results were analysed using the comparative C_T method and normalised relative to the medium control. Statistical analysis was performed using the Wilcoxon matched pairs test.

The expression of *CYP1A1* and *CYP1B1* was significantly decreased after using the specific AhR-blocker CH223191 (Figure 3.14). The reduction of *IDO1* expression after exposure of DCs to CH223191 and subsequent FF PM10 stimulation was not significant. This section demonstrates that induction of *CYP1A1* and *CYP1B1* by FF PM10 was at least in part truly AhR-dependent. Although the literature indicates that *IDO1* expression can be induced by AhR signalling, its induction by FF PM10 was AhR independent as it was not decreased by the AhR-antagonist. This finding is in line with the data shown in the previous section, where we did not see a significant increase of *IDO1* levels after stimulation of DCs with the AhR-agonist FICZ. Also, *IDO1* expression was significantly increased after LPS stimulation, and for *CYP1A1* and *CYP1B1* less so, which indicates that other pathways may be involved in *IDO1* regulation.

In summary, this set of experiments demonstrates that PM10 activates AhR signalling as indicated by *CYP1A1* and *CYP1B1* expression. *IDO1* is also induced under similar conditions but, in contrast to observations in other systems, is likely to be induced independently of AhR.

3.4.7. Maturation, inflammasome activation and AhR signalling in DC are independent effects of FF PM10

As seen in the above results sections, exposure of DCs to fossil fuel PM10 lead to TLR4 dependent maturation of DCs as measured by expression of HLA-DR, CD80 and CD86 amongst others. Furthermore, several proinflammatory cytokines were secreted by MoDCs after incubation with FF M10, including inflammasome dependent IL-1β and IL-18. Finally, the AhR was triggered after activation of the cells with PM10 derived from fossil fuel. It has been reported that these pathways may be linked. For example, Abu-rezq & Millar, 2013 have reported that AhR-ligands induce secretion of proinflammatory cytokines such as IL-1β and IL-6. Exposure of mouse DCs to the AhR agonist TCDD affects differentiation and maturation of DCs, as shown by the increased level of MHC class II and the co-stimulatory molecule CD86, while the expression of CD11c and the secretion of IL-10 was reduced after DC exposure to TCDD (J.-A. Lee et al., 2007). Also, the interaction of AhR and RelB, a member of the NF-κB family, can lead to activation of both pathways, AhR and NF-κB, as the AhR/RelB dimer is able to target binding sites supporting the activation of AhR as well as NF-κB. This suggests that AhR with RelB coordinate inflammatory responses together (Vogel & Matsumura, 2009).

Hence, the interaction of all those pathways was investigated to determine whether the measured effects were dependent on each other. For this, MoDCs were pre-treated with specific antagonists, either blocking TLR4 signalling or the AhR pathway. Afterwards, cells were incubated with fossil fuel derived PM10 for 48h. Subsequently, the outcomes for maturation,

AhR signalling and the secretion of proinflammatory cytokines including inflammasome dependent IL-1β and IL-18 were measured.

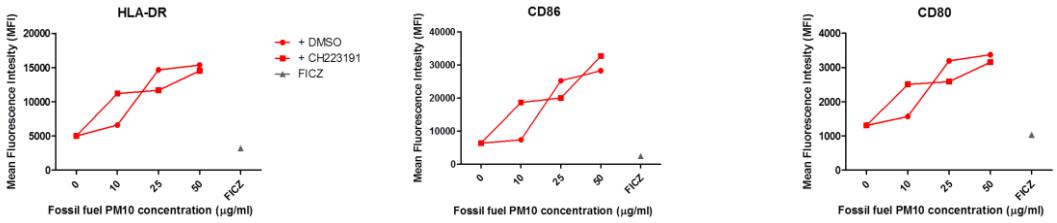


Figure 3.15: DC maturation is not dependent on AhR signalling

Human MoDCs were pre-incubated with 3µM of the AhR antagonist CH223191 or the vehicle control DMSO for 30 min and subsequently incubated for 48h with 10, 25 and 50µg/ml of FF PM10 and medium only. Cells were treated with the AhR agonist FICZ for 2 days after which HLA-DR, CD86 and CD80 were measured in order to determine whether an AhR agonist alone is sufficient to induce DC maturation. After surface antibody labelling and analysis using flow-cytometry, results were plotted showing the MFI. The experiment was conducted once with one blood donor.

FF PM10 stimulated MoDCs, which were pre-treated with either the AhR antagonist CH223191 or the DMSO control, expressed HLA-DR, CD86 as well as CD80, as measured after surface staining by flow-cytometry. CH223191 was used at a concentration which has been shown to block the effects of AhR-ligands (see section 3.4.6.). All markers were expressed at a similar level over concentrations of 10, 25 and 50µg/ml of FF PM10 or medium only, irrespective of whether cells were pre-incubated with the blocker or the vehicle control. This indicates that blocking the AhR signalling pathway does not influence the expression of activation markers on DCs after FF PM10 stimulation. Therefore, induction of maturation by FF PM10 was TLR4 dependent but does not require AhR signalling.

To see whether AhR signalling is necessary for the secretion of proinflammatory cytokines such as IL-1β, IL-6, IL-10, IL-12p70 and IL-23, AhR was again blocked by treating DCs with CH223191 or the DMSO control and subsequently stimulating cells with FF PM10. The concentrations of the cytokines were measured by multiplex ELISA in the supernatants after 2 days of culture.

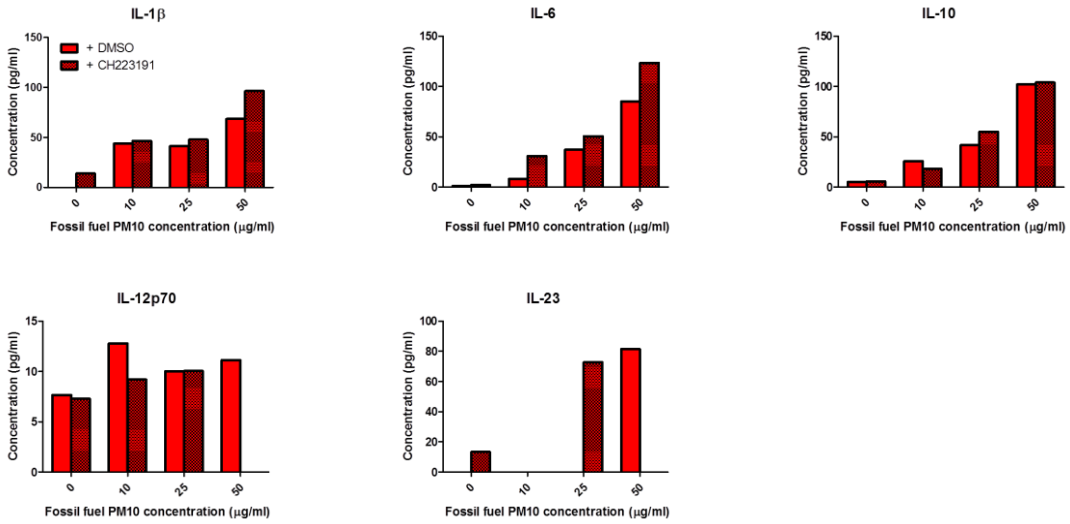


Figure 3.16: Proinflammatory cytokine secretion by MoDCs is AhR-independent

$CD14^+$ monocyte were separated from PBMCs and cultured with GM-CSF and IL-4 to obtain DCs. DCs were pre-incubated with 3µM of the specific AhR blocker CH223191 or DMSO for 30min. After that, cells were stimulated with 10, 25 and 50µg/ml of FF PM10 and medium only for 48h. IL-1ϐ, IL-6, IL-10, IL-12p70 and IL-23 in the supernatants were labelled using a Multiplex ELISA kit and measured by flow-cytometry. The graphs above show one experiment conducted with one blood donor.

Again, the use of CH223191 or the vehicle control DMSO prior the exposure of DCs to FF PM10 did not influence the concentrations of proinflammatory cytokines in the supernatants. IL-1β, IL-6 and IL-10 were secreted in a dose-dependent manner showing the same concentrations ranges between 0 and about 130pg/ml. IL-12p70 and IL-23 were lower expressed, ranging between 0 and 80pg/ml and not showing a dose-dependent pattern. These findings indicate that AhR signalling was not required for the production of IL-1β, IL-6, IL-10, IL-12p70 and IL-23 and blocking of AhR before FF PM10 stimulation did not alter cytokine concentration.

Finally, to evaluate whether TLR4-signalling may also be required for activation of the AhR signalling pathway, MoDCs were incubated with the TLR4-antagonist CLI-095 or the DMSO vehicle control for 1h before FF PM10 stimulation for 48h. Subsequently, the AhR dependent genes for *CYP1A1*, *CYP1B1* and *IDO1* were measured using qRT-PCR.

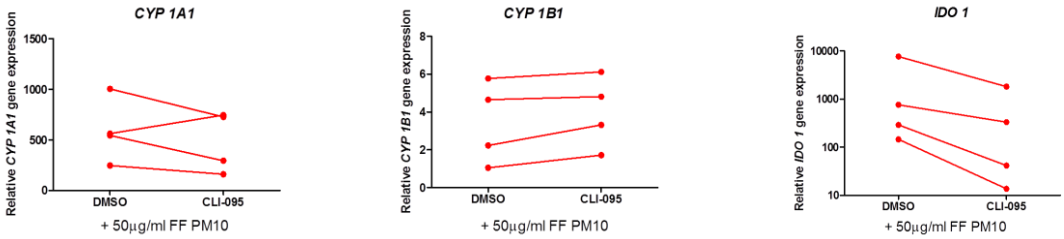


Figure 3.17: Induction of the AhR pathway is independent of TLR4-signalling

DCs cultured from human blood CD14^{+} monocytes were pre-treated with 1µg/ml of the TLR4 antagonist CLI-095 or the vehicle control DMSO for 1h and subsequently both conditions were incubated for 48h with 50µg/ml of FF PM10. Gene expression of CYP1A1, CYP1B1 and IDO1 was measured using qRT-PCR and the data was analysed using the comparative C_T method and normalised relative to the medium control. Each pair of dots represents one independent experiment with a separate blood donor.

Blocking of the TLR4 signalling pathways with CLI-095 prior to stimulation with the agonist FF PM10 did not affect the expression of *CYP1A1* and *CYP1B1* in DCs.

Although non-significant there was a trend towards partial inhibition of *IDO1*. In 4 independent experiments, *IDO1* was consistently lower expressed after cells were pre-treated with CLI-095 compared to the DMSO control. Taken together with the data above, this implies that regulation of *IDO1* is distinct from *CYP1A1* and *CYP1B1* and may be partially dependent on TLR4 activation.

In conclusion, this section of the chapter shows that maturation of DCs by FF PM10 was not dependent on the AhR signalling pathway, and that expression of the AhR target genes *CYP1A1* and *CYP1B1* was not dependent on the TLR4 signalling induced by FF PM10. However, several experiments have suggested that, in contrast with *CYP1A1* and *CYP1B1*, *IDO1* expression was not solely controlled by AhR, but may also be partially dependent on TLR4 activation. Furthermore, AhR signalling was not involved in the production of IL-1β, IL-6, IL-10, IL-12p70 and IL-23 by DCs in this system.

3.5. Discussion

The first aim of this chapter was to determine whether urban FF PM10 influences DCs maturation and function.

The fossil fuel derived PM10 was tested on MoDCs. One major benefit of MoDCs is that it is possible to generate large numbers of uniform cells under controlled conditions which allowed us to compare particles at a range of concentrations within one experiment, without running out

of cells. This permitted us to do a simple, convenient and specific for a cell type analysis. Although DCs are present in the blood, numbers are very low; hence, it is difficult to obtain large enough amounts to perform an extensive comparison of particles and testing a dose range. However, there are also limitations to the use of MoDCs as a model system. It should be kept in mind that MoDCs are an *in vitro* entity that may not reflect actual cells *in situ*. Also, as these cells are derived from monocytes, they may represent inflammatory DC type rather than steady-state DCs (Mildner et al., 2013; Segura & Amigorena, 2013; Serbina et al., 2003). Additionally, one disadvantage of *in vitro* MoDCs is that human organisms are very complex and this complexity is taken away in an *in vitro* study; therefore, results should be extrapolated carefully from *in vitro* studies back to whole organisms.

It has been shown *in vivo* that during pulmonary inflammation, such as viral or fungal infection, MoDCs or inflammatory DCs are recruited (Hohl et al., 2009; Lin, Suzuki, Nakano, Ramsburg, & Gunn, 2008). After exposure to DEP an increase in inflammatory DCs has been found in the lung interstitium (Hohl et al., 2009; Lin et al., 2008). Also, after exposure to an inflammatory stimuli, monocytes were recruited to the lung, upregulated CD11c and therefore became inflammatory DCs (Provoost, Maes, Joos, & Tournoy, 2012). Therefore, the use of MoDCs in our assay may mimic the cell environment in the lung after exposure to PM.The first observation was that DCs take up FF PM10 as they were found in the phagosome, which is typical of an APC. The interaction with the particles induced TLR4 dependent maturation of the cells. This could be seen through the expression of HLA-DR, CD86, CD40 and CCR7, which was dose-dependent and clear after 48 hours. This is partially consistent with previous findings (Williams et al., 2007), but an important aspect in these experiments is the origin of the particles, as not all particle types induced DC activation. Williams *et al.*, reported that exposure of human DCs to ambient PM induces non-classical activation of DCs that failed to upregulate HLA-DR, which was due to high baseline levels of HLA-DR. On the other hand, Porter *et al.,* demonstrated that diesel-enriched particles collected in a traffic tunnel induce human DC activation together with enhanced expression of HLA-DR, which is consistent with our findings. Diesel exhaust particles (DEP) collected directly from a running diesel engine do not seem to induce DC maturation at various concentrations, just as the CB does in this study (Bleck et al., 2006; Verstraelen et al., 2005).

This has likely to do with the area where the particles were collected and hence their composition, rather than with the combustion itself. The FF PM10 used in this study was collected in an urban area and therefore represent the ambient mixture of air pollution. In contrast, the DEP were collected directly from the exhaust pipe of a diesel car and represent a “cleaner” particle type. DEP only form a portion of our FF PM10, as diesel vehicles are found in

urban areas too, but DEP miss all the additional components found in the atmosphere. These observations suggest that the additional components, such as endotoxins found in the atmosphere, which cannot be found on particles that were collected directly from diesel engines, but on ambient PM, may be inducing those effects. This is supported by the fact that expression of some of the maturation markers was reversible when MoDCs were pre-treated with the TLR4 antagonist CLI-095, showing that those effects are TLR4 dependent.

Another observation in this experiment is that CB particles induced no maturation of DCs, as none of the maturation markers showed a significant increase after CB exposure. Since they represent the core of the particles and additional substances that are present on the surface of the FF PM10 are not found on CB particles, this suggests that these additional substances are responsible for the effects of FF PM10 seen in DCs. In general, this data suggests that the additional components on the surface of FF PM10 activate DCs.

All particles used in this study were collected on different types of filters from the ambient air and needed to be extracted and suspended in PBS in order to perform *in vitro* experiments. During this extraction process, small fibres and particles originating from the filter itself may go into the particle suspension as well and interact with the cells, too. Therefore, we checked that no fibres coming from the filter on which the particles were collected were in the suspension and induced the effects we have observed. For this, blank filters were put through the extraction process and it was tested whether the effects shown above are due to any substances that are released by the filters during the extraction. During the standard procedure and level of sonication that was used to release the particles, no visible fibres were in the suspension and no activation of DCs could be detected. Therefore, it can be concluded that the effects shown above are due to the particles themselves and not any substances deriving from the filter. However, when sonication was prolonged and visible filter disruption occurred, filter components were found in the suspension, which then led to activation of DCs. Hence, the method of particle extraction needs to be taken into account when designing *in vitro* experiments for PM testing.

The expression of MHC Class II indicates that cells are capable of presenting antigen on their surface and can interact with T cells. Increased expression of HLA-DR may be indicative of an enhanced ability to activate CD4 T cells by interacting with the T cell co-receptor CD4 and hence induce adaptive immune responses. Also, the upregulation of CD40, CD80 and CD86 on DCs suggests that cells can deliver enhanced co-stimulatory signals to T cells. Increased co-stimulation (signal 2) is linked to the development of effector responses in preference to the generation of regulatory cells and in the airway could increase responsiveness to inhaled antigens. Costimulatory molecules CD80 and CD86 interact with CD28 and CTLA-4 on T cells,

whereas signalling through CD28 leads to T cell proliferation and cytokine secretion and CTLA-4 signalling attenuates T cells responses.

How exactly these two opposing outcomes are initiated and controlled is unclear. It has been proposed that CD86 binds CD28 more effectively and therefore lead to an activating rather than inhibiting response (Collins et al., 2002). Consequently, in our case, a significantly higher expression of CD86, but not CD80, on PM-stimulated DCs would lead to a strong adaptive immune response. In addition to CD86, CD83 was also significantly increased in PM-stimulated DCs. Although the exact role of CD83 is unknown, some studies suggested that CD83 plays an important role in transplant rejection and autoimmune diseases; however, CD83 deficiency leads to a normal T cell response (Prazma & Tedder, 2008). CCR7 is the chemokine receptor, which ensures that DCs migrate to the lymph nodes under the influence of CCL19 and CCL21. This suggests that DCs may retain the capacity to migrate to lymph nodes where T cell activation can occur, although there was no evidence that PM10 exposure increased CCR7 expression.

Exposure to air pollution has been linked to the development and exacerbation of asthma and respiratory tract DCs have been shown to play an important role in the origin of asthma as well as the symptoms which accompany asthma (Miller & Peden, 2014; van Helden & Lambrecht, 2013). We have shown that upregulation of some of the maturation markers is TLR4 dependent and therefore environmentally acquired endotoxins on the particle surface may be primarily responsible for maturation of DCs after FF PM10 stimulation.

The previous result showed that FF PM10 exposed DCs expressed a higher level of HLA-DR and co-stimulatory molecules and therefore might be predicted to induce a higher level of T cell proliferation. Indeed, increased stimulation by PM10-exposed DCs was observed in allogeneic MLRs with $CD4^+$ T cells. Previous reports have also shown that PM10, both ambient and diesel-enriched, can activate human DCs and thereby elicit T cell responses (Porter et al., 2007; Williams et al., 2007). DCs that were stimulated with FF PM10 showed a higher ability to induce T cell proliferation of allogeneic naïve CD4 T cells in a mixed allogeneic leukocyte reaction compared with DCs that were not exposed to FF PM10, but cultured with CB or medium only. This indicates that the downstream consequence of DC activation by PM10 is an increased capacity to activate CD4 T cells. Therefore, when applied to an *in vivo* situation, it could be concluded that exposure to air pollution particles stimulated an innate and adaptive immune response, which may lead to lung inflammation and detrimental health effects.

The cytokine profile of the T cells activated in the MLR cultures was characterised by a high frequency of cells producing IFN-γ irrespective of DC pre-treatment. Few cells produced IL-4 or

IL-5. This indicates that without being exposed to any kind of treatment, human MoDCs induce a Th1 type cytokine response, which is characterized by an elevated IFN-γ production by T cells after activation with DCs. There was no evidence for a shift towards a Th2 response when DCs were treated with PM10, at least as determined by production of IL-4 and IL-5 by T cells. Williams *et al.*, reported that after co-culture with PM-exposed DCs, the stimulated T-cells produced a complex panel of cytokines including IL-5 and IL-13 together with IFN-γ, and thus did not display classical features of a Th2 response. However, a different type of DCs was used, which can lead different outcomes. As opposed to the MoDCs that were used in our study, Williams *et al* used DCs derived from $CD34^+$ progenitors and particles were collected from an outdoor urban area. All in all, there was no evidence for a Th2 directed immune response when dendritic cells were exposed to PM10. Therefore, we did not prove that PM10 can cause new cases of asthma as a TH2 type immune response would indicate, but rather an exacerbation of an existing asthmatic condition. Individuals with allergic asthma are already sensitized against certain environmental allergens. In situ, the activation level of already sensitized DCs after exposure to PM10 may reach a critical threshold and lead to T cell mediated exacerbation.

In a mouse model, Bezemer *et al.,* observed that *in vivo* exposure to ambient PM, induces activation of DCs while inhibiting their production of IL-10, and that *ex vivo* co-culture with naïve $CD4^+$ T cells induced a Th2 type immune response (Bezemer et al., 2011). Strikingly, in these experiments, the PM-stimulated DCs did not upregulate expression of CD40, CD80 or HLA-DR, suggesting that PM exposure can exert complex atypical effects on DC function. However, contrary to our study, this was conducted in a mouse model after ambient PM from urban areas was instilled in the oropharynx. This may explain the different outcomes. Special consideration must be given to the types of DC and PM as well as the overall experimental design used when investigating the cytokine profile of proliferating T cells that were activated with PM stimulated DCs.

Asthma is an allergic disease, which is characterized by a skewing of T cell responses towards a Th2 type T cell response. As ambient air pollution has been associated with allergic lung diseases and is a major factor in asthma, it could be hypothesized that exposure to urban air pollution leads to Th2 type immune responses in the lung (Barnes, 2001; Eggleston, 2000). Also, nasal challenge by DEP's of individuals with allergy induced a type 2 immune response as seen by IL-4, -5, -6 and -13 secretion of DCs (Fujieda, Diaz-Sanchez, & Saxon, 1998). However, in our study we were not able to show this correlation. Matthews et al., 2013 have reported that when GM-CSF was added to human blood $CD1c^+$, which were activated with urban PM to imitate the signals from surrounding epithelial cells in the lung, suppression of the Th1 response took place, but a

skewing towards a Th2 type response was not observed. In comparison, GM-CSF only with no addition of urban PM did show a lower level of Th1 cells, as measured by IFN-γ concentration. This highlights the importance of the model used and results should be interpreted in the light of the model system; hence, drawing conclusions with respect to an *in vivo* human system is difficult. However, in our case, as we did not show a skewing of T cell responses towards a Th2 type T cell immune reaction, using DCs from human induced sputum could help us show the correlation between air pollution and Th2 immune responses and provide results that are closer to a human *in vivo* model. Another possibility would be to use DCs separated directly from human blood without an *in vitro* differentiation step.

Overall, our results could be a characteristic of a particular model used and it may be important to use a different one. Moreover, other cells could be involved in the Th2-polarization of DCs, such as bronchial epithelial cells (Bleck et al., 2006; Bleck, Tse, Gordon, Mohammad, & Reibman, 2010). Therefore, one option could be that PM does not influence Th2 polarization directly, but rather through indirect effects, such as via epithelial cells.

In parallel with the effects on DC maturation, FF PM10 but not CB induced the secretion of inflammatory cytokines by DCs; FF PM10 exposure stimulated a significant increase in IL-6, IL-12, IL-1β, IL-18 and IL-23. The effect was particularly evident at a PM10 concentration of 50µg/ml (the highest tested). IL-6 and IL-12p70 were both also secreted after LPS stimulation; IL-6 production was similar following FF PM10 or LPS stimulation whereas more IL-12p70 was produced in response to LPS. These results are partly in line with the findings of Williams et al., 2007, who showed greater production of both IL-12p70 and IL-6 in response to LPS than in response to PM10, in which LPS was used at a 10-times higher concentration compared to our study and PM at 100µg/ml. Since IL-12p70 is associated with a Th1 cellular response, a higher expression of this cytokine is consistent with the previous result which showed a high production of IFN-γ by proliferating allogeneic naïve $CD4^+$ T cells. As PM10 induced a much higher secretion of IL-12p70 from DCs compared to CB, a higher concentration of IFN-γ could have been expected, however, this was not the case.

While some of the cytokines were produced by PM10 as well as LPS, this suggests that both stimuli induce similar pathways, which are all TLR4 dependent and indicates that endotoxins on the particle surface may be responsible for this effect. Increased secretion of IL-1β, IL-18 and IL-23 was seen after FF PM10 exposure of DCs but not with the LPS control, which suggests a distinct effect of the particles on the cells. Secretion of IL-1β and IL-18 indicates involvement of inflammasomes. The inflammasomes are a group of protein complexes which contain a sensor molecule that is triggered by a variety of substances, which includes triggers derived from

metabolic changes as well as from infections (Latz et al., 2013). Production of biologically active IL-1β and IL-18 by the inflammasome involves the following events: expression of pro-IL-1β and pro-IL-18 genes, inflammasome complex engagement, activation of caspase-1, caspase-mediated cleavage of pro-IL-1β and pro-IL-18 and finally secretion of the active cytokine forms (Rabolli et al., 2016). A form of inflammatory cell death, pyroptosis, can also be induced as a result of inflamamsome activation (Vince & Silke, 2016). For this whole process, two signals are required. The first signal, which can be provided by TLR ligands including LPS, is to prime the expression of the inactive cytokine forms as well as to increase expression of inflammasome components. The second step, called signal 2, activates the inflammasome itself and the secretion of the cytokines (Rabolli et al., 2016). The second signal is poorly understood but can be induced by diverse stimuli including ATP, nigericin, silica particles as well as PM10.

As FF PM10 induced production of biologically active IL-1β and IL-18, the implication is that it provides both signals required for inflammasomes activation. CB induced neither IL-18 nor IL-1β, suggesting that the particulate core fails to provide either or both of the two and the inflammasome was not activated. FF PM10-stimulated IL-1β production was inhibited with a TLR4 antagonist suggesting that the first signal for inflamamsome activation is provided via this PRR. It may be that small amounts of LPS present as environmentally acquired endotoxins on the PM surface signal via TLR4 to induce the transcription of genes and another as yet unidentified signal from the particles then activates caspase-1.

A common feature of signal 2 is that it leads to permeability of the cell membrane for K^+ and Na^+ which results in a reduction of intracellular K^+ concentration and then ultimately leads to caspase-1 activation. Involvement of inflammasome activation in the responses to fossil fuel derived PM10 has been shown before in epithelial cells and macrophages, but not specifically in DCs (Hirota et al., 2012; Rabolli et al., 2016). As secretion of IL-1β and IL-18 indicates activation of the inflammasome and an undergoing innate immune response, the primary aim is the death of infected cells. In mice, IL-1β secretion has been shown to be involved in pulmonary inflammation induced by exposure to DEP (Provoost et al., 2011). Both IL-1β and IL-18 has been reported to be involved in airway inflammation and the pathogenesis of Chronic Obstructive Pulmonary Disease (COPD) in mice after exposure to cigarette smoke (Eltom et al., 2014).

The secretion of IL-12 is needed to stimulate growth and function of T cells; we have seen above that T cells were stimulated after being activated with PM-exposed DCs. IL-12 is involved in type 1 immune responses and together with IL-23 is secreted upon pathogen detection by lung DCs before they migrate to the lymph nodes (Iwasaki, Foxman, & Molony, 2016). Secretion of IL-6, which is an important cytokine in the development of fever and is known to be initiating the

synthesis of PGE_2, leads to changes in body temperature. It also supports the growth of B cells and inhibits the development of a regulatory immune response. IL-23 is also secreted as a reaction to inflammation.

With all these measured cytokines it should be kept in mind that the simultaneous presence of PM may affect the overall levels of these cytokines, as secreted cytokines may adsorb to the surface of particles and therefore cannot be detected during measurement (Kocbach, Totlandsdal, Låg, Refsnes, & Schwarze, 2008).

The fossil fuel derived PM induced activation of the environmental sensor AhR in DCs. Given the increasing evidence that AhR signalling has immune effects, this pathway represents an additional way by which FF PM10 may modulate these cells. Highly expressed by DCs, the AhR has been described as the primary immune receptor for PAH. The AhR is a transcription factor involved in the regulation of responses to aromatic hydrocarbons which are found on the surfaces of PM. Activation of the AhR pathway can be assessed by measuring the expression of downstream target genes such as *CYP1A1* and *CYP1B1,* which has allowed other investigators to identify critical functions for this receptor in host protection against autoimmunity and inflammation (Cella & Colonna, 2015).

Exposure of MoDCs led to a significant and dose dependent expression of AhR-dependent genes *CYP1A1* and *CYP1B1.* Also, *IDO1* expression was increased dose-dependently. Blocking AhR with the specific antagonist CH223191 confirmed that PM10-induced *CYP1A1* and *CYP1B1* induction was dependent on AhR signalling. Expression of *IDO1,* a third gene known to be regulated by AhR, also increased following exposure FF PM10. However, *IDO* induction was not significantly reduced after AhR blockade but was reduced following antagonism via TLR4. Taken together with the observation that *IDO1* was not induced after stimulation of DCs with the AhR ligand FICZ, these finding suggest the *IDO1* is induced in DC by FF PM10 but it is via a TLR4-dependent, rather than an AhR-dependent mechanism. IDO is the first and rate limiting catabolic enzyme in the degradation pathway of the essential amino acid tryptophan. By cleaving the aromatic indole ring of tryptophan, IDO initiates the production of a variety of tryptophan degradation products called “kynurenines” that are known to exert important immuno-regulatory functions (Mbongue et al., 2015). However, to our knowledge, there have been no reports in the literature describing *IDO1* expression to be AhR-independent.

The exact impact of activation of the AhR-pathway in DC is unclear. Contradictory findings have been reported in the literature (Stockinger et al., 2014). Suggested outcomes of AhR activation include pro-inflammatory effects, such as the promotion of Th17 development (Veldhoen et al.,

2008) as well as potentially anti-inflammatory responses, including production of IL-10 and IDO expression (Stockinger et al., 2014) in DCs. It has been shown that the detrimental effect of smoking on rheumatoid arthritis may be mediated through synovial DCs by signalling through AhR (Kazantseva et al., 2012). Also, by signalling through AhR exposure to air pollution has been linked to the development and exacerbation of atopic dermatitis, which has been shown in mice (Hidaka et al., 2016).

However, in the work presented in this thesis, no evidence was found for both pro– and anti-inflammatory outcomes. Therefore, the exact effects of the AhR on DCs after exposure to FF PM10 need to be investigated further, which could be done with a microarray analysis. This type of analysis would permit measuring the expression of a large number of genes simultaneously, as opposed to only a small number of genes that can be analysed by PCR. Through this, the complex effects of PM could be measured leading to a better understanding of the impact of air pollution on DCs.

Theoretically, the identified effects of FF PM10 on DC, namely maturation, cytokine production and AhR signalling, could all be different manifestation of the same activating event stimulated by single PM component, or they may represent multiple independent effects stimulated by different components. To address these possibilities and explore potential interaction between pathways, the effects of blocking individual recognition components were tested. As a first step, it was determined whether AhR stimulation influences maturation and cytokine production or whether TLR4 inhibition impacts upon components of the Ahr pathway.

Blocking the AhR pathway with a specific antagonist did not influence FF PM10–induced expression of the maturation markers studied and did not cause any changes in the secretion of pro-inflammatory cytokines. This contrasts in part with the findings of Abu-rezq & Millar, 2013.They showed on bone marrow-derived DCs after stimulation with the AhR ligand Indole-3-carbinol (I3C), which was added during LPS stimulation, that aryl hydrocarbon ligands suppressed the expression of CD40, while not affecting the expression of other maturation markers. Furthermore, they showed that AhR-ligands, such as FICZ, I3C, curcumin and the ligand precursor tryptophan enhanced the expression of IL-6, 12 and 1β, which is not consistent with our findings. C. Wang, Ye, Kijlstra, Zhou, & Yang, 2014 have shown that the FICZ induced down-regulation of maturation markers, except CD83 in MoDCs and inhibited production of IL-1β, IL-6 and IL-23, which is partly in contrast with our findings. However, our study used FF PM10 to stimulate DCs, which contain AhR ligands, but also other components, which can provide a range of signals as opposed to using a specific AhR ligand such as FICZ. In terms of blocking the AhR pathway with the specific antagonist CH223191 and looking into the AhR regulated genes, the

expression of *IDO1* was not as much decreased as for example in the case of *CYP1A1*. In addition to that, blocking TLR4 did reduce expression of *IDO1*, indicating that *IDO1* may be controlled by TLR4 signalling, which has recently been reported in an independent study, showing that the mannose receptor is involved in the allergen induced downregulation of *IDO* activity in LPS-exposed MoDCs (Salazar et al., 2015). A lot of reports claim that *IDO1* is controlled by AhR, but to our knowledge, no study so far has shown that it is not controlled by AhR.

All in all, it can be seen that the effects of FF PM10 on DCs are complex and involve different mechanisms reflecting their complex composition. How exactly these different pathways interact and how these pathways then influence DCs has to be investigated further.

3.6. Conclusion

In conclusion, fossil fuel derived PM10 from an urban area have complex effects on MoDCs, including induction of classical maturation, inflammasome activation, inflammatory cytokine expression, and AhR activation. How these outcomes differ between particles derived from different sources and of different compositions and sizes will be the focus of the next chapters.

4. *Ex vivo* respiratory tract DCs and macrophages express AhR dependent genes

4.1. Chapter summary

The previous chapter explored the different pathways that are induced in MoDCs when exposed to fossil fuel-derived air pollution, including classical maturation as well as activation of the AhR. However, it remains unknown whether these pathways are also induced in DCs and macrophages in the human airway obtained from sputum samples. Therefore, this section focuses on the cell sorting followed by qRT-PCR analysis of respiratory tract DCs and macrophages from induced sputum samples given by healthy volunteers. The aim was to detect AhR-related genes among others and study the effects of fossil fuel derived PM10 on this pathway. As a first step after cell sorting, DCs and macrophages were analysed for the transcription factor *Zbtb46*, which is specific for cDCs and therefore confirms that sorted cells that were defined as DCs were truly DCs. Sorted DCs and macrophages both expressed the *AhR* at similar levels. Sputum cells were stimulated with 50µg/ml FF PM10 for 2-4 hours and subsequently sorted and lysed for reverse transcription real-time PCR. Both respiratory tract DCs and macrophages expressed the AhR-related genes *CYP1A1* and *CYP1B1* as well as *IDO1* in the absence of stimulation *in vitro*. After stimulation with FF PM10, expression of *CYP1B1* was significantly increased in both cell types, indicating that the AhR pathway was activated by FF PM10. For *CYP1A1* and *IDO1,* no significant difference was detected, although a trend towards higher expression was observed. As well as expressing AhR related genes and *IDO1*, sputum DCs expressed genes for maturation markers such as CD80 and CD86 *ex vivo* but no significant change in expression was observed following stimulation with FF PM10 *in vitro.*

4.2. Introduction

The AhR is expressed throughout the body, particularly in immune cells (Stockinger et al., 2014). Since the key function of the immune system is to provide protection against incoming pathogens, immune cells are also present at barrier sites such as the lung. It has been shown that AhR is highly expressed in lung cells, although the exact immunological processes in which AhR is involved are still unknown (Hayashi et al., 1994; Stockinger et al., 2014). As the respiratory tract is constantly exposed to airborne PM, which is known to consist of aromatic hydrocarbons amongst others, the AhR is likely to be involved in immune responses to inhaled PM (Ohura, Sawada, Amagai, & Shinomiya, 2009; Sun, Zeng, & Ni, 2013). Not only is AhR expressed in the airway, but AhR dependent genes like *CYP1A1* and *CYP1B1* are also expressed, suggesting that

the AhR pathway is activated in the lung tissue (Choudhary, Jansson, Schenkman, Sarfarazi, & Stoilov, 2003). A lot is known about the expression and function of AhR in epithelial cells: activation of AhR in lung epithelial cells leads to the productions of mucins, thereby supporting the protective barrier function against inhaled toxins (Wong, Vogel, Kokosinski, & Matsumura, 2010). In terms of lung resident DCs, it has been shown that AhR activation prior to influenza virus infection may lead to an immunosuppressive effect, whereby $CD8^+$ T cells are reduced, resulting in poorer response by cytotoxic T lymphocytes (G.-B. Jin, Winans, Martin & Lawrence, 2014). This is due to a reduced number of $CD103^+$ DCs migrating from the respiratory tract to the lymph nodes activating those cells and means that AhR signalling decreases the immune reaction to viral lung infection by altering the response of DC subsets. In addition to that, it has been claimed that AhR triggering in DCs may lead to the induction of IDO, which contributes to the immunosuppressive effect of AhR activation in DCs (Nguyen et al., 2010; Vogel et al., 2008). All these findings on AhR in the lung were obtained from mouse models and cell lines, but very little is known about AhR expression in actual human lung DCs and about how triggering AhR affects these cells. We have shown in the previous chapter that FF PM10 induces AhR signalling in MoDCs, but the effects of these particles on AhR in airway DCs and macrophages obtained from sputum remain unknown. We have also seen induction of *IDO1* in MoDCs by a mechanism that is at least in part independent of AhR, but very little is known about *IDO1* in sputum DCs and macrophages.

Therefore, we aimed to investigate the AhR pathway in lung DCs and macrophages from sputum samples given by healthy volunteers. Induced sputum is an easy, practical, safe and relatively non-invasive technique to study lung immune cells originating from the conducting airways and thus can be applied to investigate lung DCs and macrophages. It has been widely used to study airway inflammation in asthmatic subjects, but expression of AhR dependent genes and *IDO1* has not been examined (Bleck et al., 2015; Brugha, Mushtaq, Mccarthy, Stagg, & Grigg, 2015; Fahy et al., 2001). In order to trigger AhR, we used ambient PM from the urban area of Leicester/UK, which is mainly derived from the combustion of fossil fuels (FF PM10). Additionally, in order to assess the state of maturation in sputum DCs, and also to see whether it is possible to replicate the findings observed in MoDCs in respiratory tract DCs, we measured costimulatory molecules CD80 and CD86 in sputum DCs as we have seen before that they are expressed in MoDCs after stimulation with FF PM10.

4.3. Hypothesis and study aims

The underlying hypothesis of this chapter is that FF PM10 induce maturation and activation of the AhR pathway in respiratory tract DCs. Specifically, to address this hypothesis, the aims of this chapter are:

- Use cell sorting to purify DCs and macrophages from induced sputum in sufficient numbers and quality for PCR analysis
- Determine whether AhR and AhR-regulated genes are expressed in sputum DCs and macrophages *ex vivo*
- Determine whether *in vitro* exposure of sputum DCs and macrophages to FF PM10 affects expression of genes involved in AhR signalling and maturation as observed with MoDCs (Chapter 3)

4.4. Results

4.4.1. DCs and macrophages from the human respiratory tract differ in their expression of *Zbtb46*

In order to analyse the expression of different genes in respiratory tract DCs and macrophages, those cells had to be separated from the whole sputum sample by cell sorting first. Macrophages are also APCs and play an important role in the immune responses to inhaled antigen, but not much is known about sputum macrophages and how they respond to inhaled air pollution. Therefore, we decided to sort sputum macrophages, in addition to sputum DCs; the macrophages were defined as lineage and HLA-DR positive (McCarthy et al., 2007). For this, the samples were processed and the cell suspension was either incubated with FF PM10 or in medium only for 2-4 hours. As the number of sorted cells was expected to be very low and viability may have been reduced after sorting, we decided to stimulate the whole sputum sample with FF PM10 or medium only. A disadvantage of this is that it cannot be ruled out that other cells present in the sputum sample may have an effect on the stimulation of macrophages and DCs.

After stimulation, cells were labelled with antibodies for CD3, CD14, CD16, CD19, CD20, CD56 and HLA-DR and FACS-sorted directly into lysis buffer for analysis with real-time PCR. To confirm that sorted cells were DCs, the expression of the zinc finger transcription factor *Zbtb46* was measured, which is specific for classical DCs and their precursors and is not expressed in other immune cells (Meredith, Liu, Darrasse-Jeze, et al., 2012a; B. Reizis, 2012; A. T. Satpathy et al.,

2012). As can be seen in the table below, sputum samples varied in cell numbers, which led to some of the samples with low cell numbers not being analysed for gene expression.

Table 4-1: Cell yields from sputum samples used in this study

Overview of sputum samples collected and processed for this study. The table shows the number of the experiment, the weight of plugs collected from each sample and the number of cells before stimulation as counted using a haemocytometer through a light microscope. It also shows the number of cells for each cell type and condition, i.e. DCs and macrophages, medium and fossil fuel stimulated, as counted during the flow-cytometry cell sorting. The last column indicates whether the data was used in the final analysis or whether the experiment failed due to insufficient cells recovered or instrument failure.

Sample number (Experiment number)	**Amount of Plugs (g)**	**Cell number of whole sputum sample**	**Cell number after sorting**				**Used /fail**
			DCs		**Macrophages**		
			Medium	**FF PM10**	**Medium**	**FF PM10**	
59	1.35g	12.72×10^6	8372	8882	220850	310500	used
60	0.77g	2.33×10^6	1684	2504	15990	17708	used
61	8.77g	1.744×10^6	N/A	N/A	N/A	N/A	used
64	6.217g	2.32×10^6	5093	4890	85000	91600	used
71	6.2g	1.29×10^6	2034	1238	20308	19558	used
77	1.246g	2×10^6	5600	5168	68800	59829	used
82	8.5g	1.79 x 106	169	195	823	1072	Exp fail
95	1.149g	3.9×10^6	1487	1075	124332	114010	used
101	0.77g	0.2×10^6	73	44	1305	940	Exp fail
102	1.18g	0.78×10^6	9619	9048	88508	91.403	used
106	4.884g	0.85×10^6	2193	2290	10228	10983	used

108	0.326g	1.11×10^6	1796	1480	47798	35979	used
109	0.719g	1.34×10^6	9799	10244	84324	71310	Exp fail
111	0.597g	0.3×10^6	348	447	10100	12020	used
112	1.339g	0.64×10^6	1677	1854	22140	27465	used
113	2.346g	0.9×10^6	1652	2188	24554	27057	used

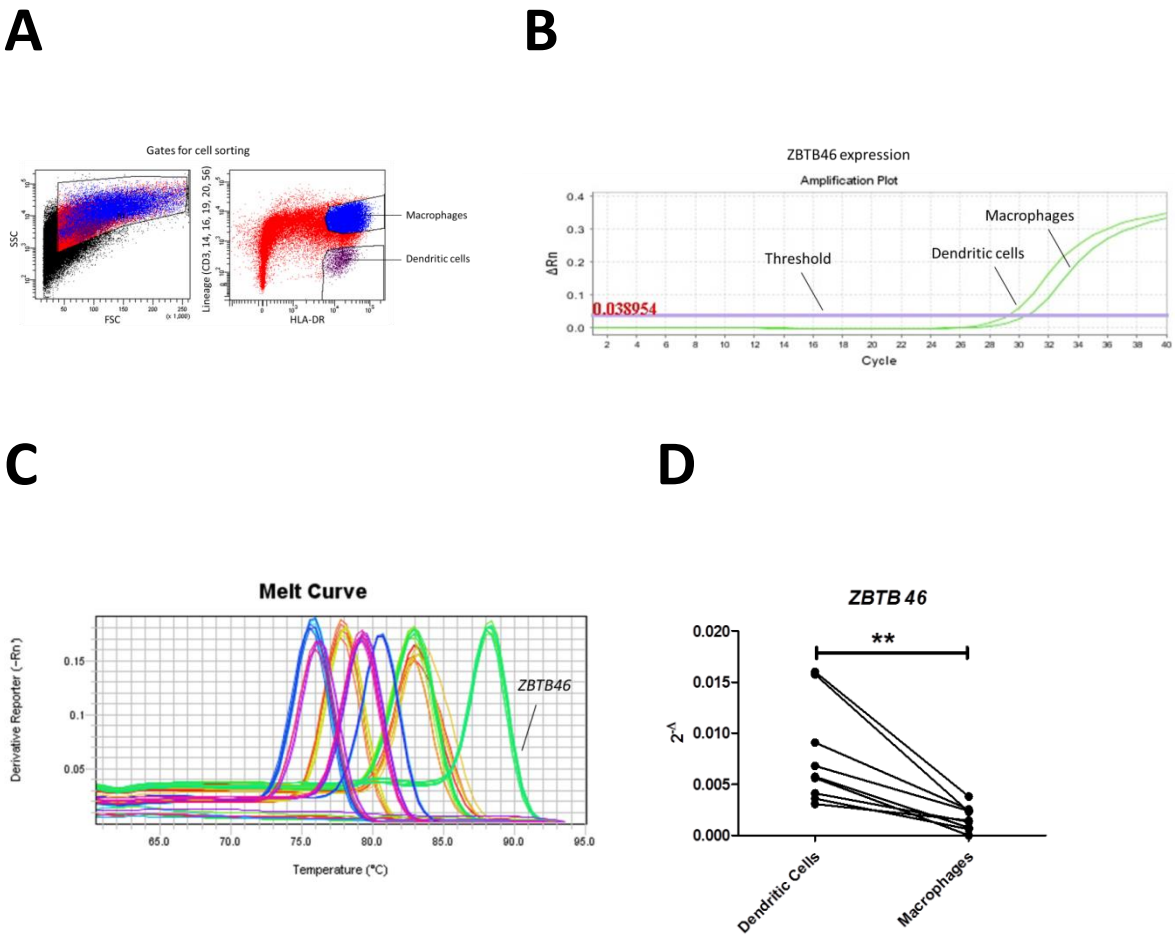


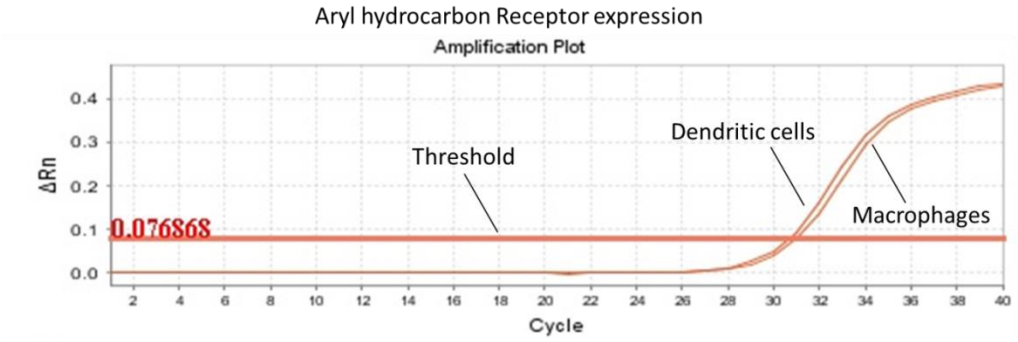
Figure 4.1: DCs and macrophages from the human respiratory tract differ by the expression of Zbtb46*Ex vivo lung DCs and macrophages were sorted from sputum donated by healthy volunteers and stimulated with 50µg/ml of FF PM10 or medium only for 2-4 hours. After incubation, cells were antibody labelled for CD3, CD14, CD16, CD19, CD20 and CD56 contained in the lineage cocktail as well as HLA-DR. Stimulated and unstimulated DCs and macrophages were directly sorted into a lysis buffer and lysed through vortexing. After disruption of the cells, gene expression of cells was measured using quantitative real-time PCR, followed by qualitative PCR. These experiments were conducted 16 times in total with separate sputum donors.* ***A****: One representative example of a scatter plot showing the gating for cell sorting. Respiratory macrophages were gated as HLA-DR and lineage positive, whereas lung DCs were identified as lineage negative and HLA-DR positive.* ***B****: Amplification plot showing one representative example of the amplification of Zbtb46 in respiratory DCs and macrophages.* ***C****: Representative example showing the melt curve after the PCR for Zbtb46.* ***D****: Summary data showing the expression of Zbtb46 in DCs and macrophages. Data was plotted as* $2^{-\Delta Ct}$ *and statistical analysis was performed using the Wilcoxon matched pairs test. Each pair of dots represents one independent experiment with a different sputum donor.*

DCs from induced sputum were identified as negative for CD3, CD14, CD16, CD19, CD20, and CD56 and positive for HLA-DR; whereas macrophages were sorted as positive for CD3, CD14, CD16, CD19, CD20, CD56 and HLA-DR. The cycle threshold value for *Zbtb46* in unstimulated DCs ranged from 29 to 35 and was highly dependent on obtained cell numbers from sputum samples, which varied between donors. Cycle threshold values were higher for sorted sputum macrophages and ranged between 29 and 36. In summary, the difference in *Zbtb46* expression between DCs and macrophages was significant with a p-value of 0.0023. From this we can conclude that with certainty the sorted DCs from sputum samples were genuine DCs, as defined by the expression of *Zbtb46*.

4.4.2. Respiratory tract DCs and macrophages express the AhR

The above results show that obtaining respiratory tract DCs and macrophages in sufficient numbers for qRT-PCR is feasible; hence, additional genes could be investigated. In order to analyse whether exposure of sputum cells to fossil fuel derived PM10 induces AhR activation, it was tested whether the AhR is expressed in respiratory tract DCs and macrophages *ex vivo* and can be detected. For this, sputum samples were processed, DCs and macrophages were sorted and the expression of the AhR transcript was measured using qRT-PCR.

A



B

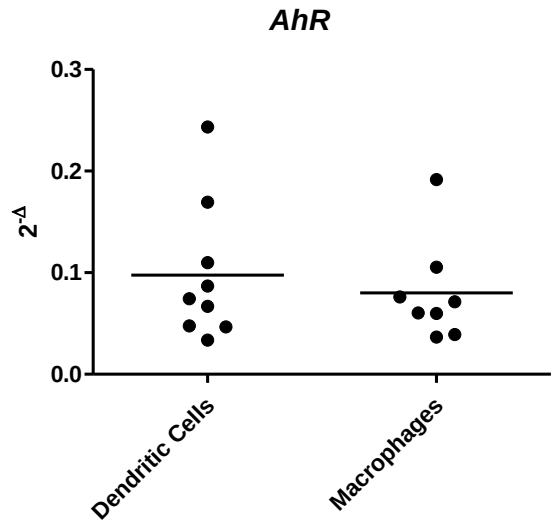


Figure 4.2: Respiratory tract DCs and macrophages express the AhR

After processing induced sputum samples from healthy donors, the cell suspension was split equally and incubated medium only for 2-4 hours. Subsequently, cells were labelled with antibodies for HLA-DR, CD3, CD14, CD16, CD19, CD20 and CD56, cell sorted and lysed followed by real-time PCR analysis. These experiments were conducted 16 times in total with separate sputum donors. ***A****: One representative example illustrating the amplification of the AhR gene in respiratory tract DCs and macrophages.* ***B****: Summary data demonstrating the* $2^{-\Delta Ct}$ *values for the expression of the AhR in respiratory tract DCs and macrophages. Each dot represents one independent experiment with a different sputum donor.*

Both unstimulated respiratory tract DCs and macrophages expressed the *AhR* transcript, which was detected at a cycle threshold between 27 and 34 for DCs and between 24 and 34 for macrophages (Figure 4.2 A). The gel electrophoresis showed a clear band, confirming the amplification of the PCR product in DCs (See Appendix Figure 4). The summary of all $2^{-\Delta}$ values shows no significant difference in the expression of the *AhR* transcript between the two cell types (Figure 4.2 B). Therefore, as we were confident that *AhR* is expressed in respiratory tract DCs and macrophages, we could then look at the expression and upregulation of AhR-related genes such as *CYP1A1* and *CYP1B1* when samples were stimulated with FF PM10.

4.4.3. AhR dependent genes are expressed by ex vivo respiratory tract DCs and macrophages and can be further upregulated after FF PM10 exposure *in vitro*

The previous results showed that respiratory tract DCs and macrophages are present in induced sputum samples from healthy volunteers and can be cell sorted in sufficient numbers to conduct quantitative PCR analysis. To be able to look at the downstream effects of AhR activation thus whether AhR-controlled genes are expressed in sputum DCs and macrophages, and at how they are then affected by the exposure of sputum cells to FF PM10, sputum cells were exposed to 50µg/ml of fossil fuel PM10 for 2 to 4 hours or medium only. Subsequently, cells were antibody labelled, cell sorted, lysed and qRT-PCR was performed. As cell numbers after sorting were expected to be low, we stimulated all sputum cells and performed the sorting after the incubation. Thereby, it cannot be excluded that other cells had an effect on the cells of interest, either before or after interaction with FF PM10 themselves and maybe being activated by PM.

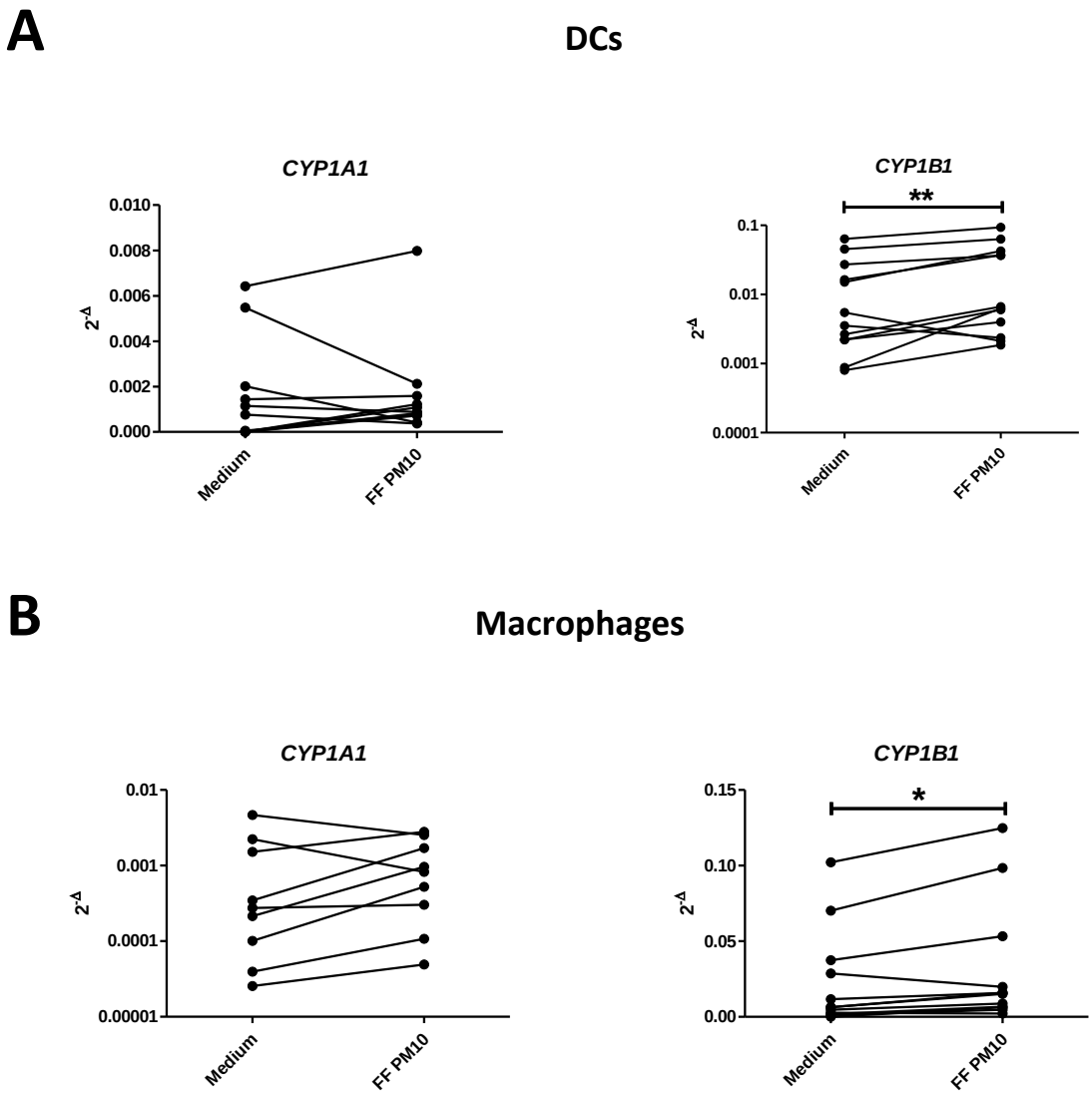


Figure 4.3: FF PM10 exposed respiratory tract DCs and macrophages express AhR regulated genes

Respiratory tract DCs and macrophages were identified by labelling for HLA-DR, CD3, CD14, CD16, CD19, CD20 and CD56 in processed sputum samples given by healthy volunteers after 2-4 hours of incubation with 50µg/ml FF PM10 or medium only. After antibody staining cells were sorted into lysis buffer and analysed for CYP1A1 and CYP1B1 using real-time PCR. These experiments were conducted 16 times in total with separate sputum donors. ***A****: Summary data illustrating the expression of CYP1A1 and CYP1B1 in cell sorted DCs from induced sputum as* $2^{-\Delta Ct}$*. Data was analysed using a paired t-test.* ***B****: Summary data showing the expression of AhR related genes as* $2^{-\Delta Ct}$ *in respiratory tract macrophages. Statistical analysis was performed using the Wilcoxon matched pairs test. Each pair of dots represents one independent experiment with a different sputum donor.*

The electrophoresis gel shows that *CYP1A1* and *CYP1B1* could be detected in unstimulated DCs (Appendix Figure 4). Since the expression of *CYP1A1* was very low without stimulation, and no Ct value was detected for some of the samples, the $2^{-\Delta ct}$ was set to zero. It can be seen that in unstimulated samples the level of expression of *CYP1A1* and *CYP1B1* was highly variable. After sputum cells were exposed to FF PM10, expression of *CYP1B1* increased significantly in DCs ($p = 0.0128$) (Figure 4.3 A). Although *CYP1A1* did not show a significant increase, a trend towards higher expression was detected as, out of 11 samples, 7 showed an increase in expression for *CYP1A1* of the target gene after stimulation of the cells with FF PM10.

A similar pattern was observed in respiratory tract macrophages. Again, *CYP1A1* and *CYP1B1* could be detected in macrophages at variable levels (Figure 4.3 B). Upon stimulation with FF PM10, *CYP1B1* showed a significant increase of gene expression in macrophages ($p = 0.0161$). For the expression of *CYP1A1,* no significant difference could be observed between FF PM10 stimulated or unstimulated cells, although again a trend towards an increase in stimulated cells could be observed. Overall, out of 10 samples, 8 showed an increase expression of *CYP1A1* in respiratory tract macrophages.

In summary, we saw that unstimulated respiratory tract DCs and macrophages express the AhR-controlled genes *CYP1A1* and *CYP1B1*, which could be due to natural exposure of AhR ligands or AhR polymorphisms. Upon incubation of cells with FF P10, a significant increase in *CYP1B1* expression can be observed for DCs as well as macrophages, whereas for *CYP1A1* a trend towards higher expression can be observed when samples are exposed to FF PM10.

4.4.4. *IDO1* is expressed by sputum DCs and macrophages

The previous chapter has shown that *IDO1* is expressed in MoDCs and is upregulated after exposure of DCs to FF PM10. However, we found that in contrast to some findings reported in the literature (Stockinger et al., 2014), we could not find any evidence that *IDO1* was controlled by AhR. Nonetheless, to look at whether *IDO1* is expressed in respiratory tract DCs or macrophages, and whether its expression is affected by the exposure of the cells to FF PM10, we stimulated sputum cells with 50µg/ml of FF PM10 for 2-4h or medium only, cell sorted DCs and macrophages and performed PCR analysis for *IDO1*.

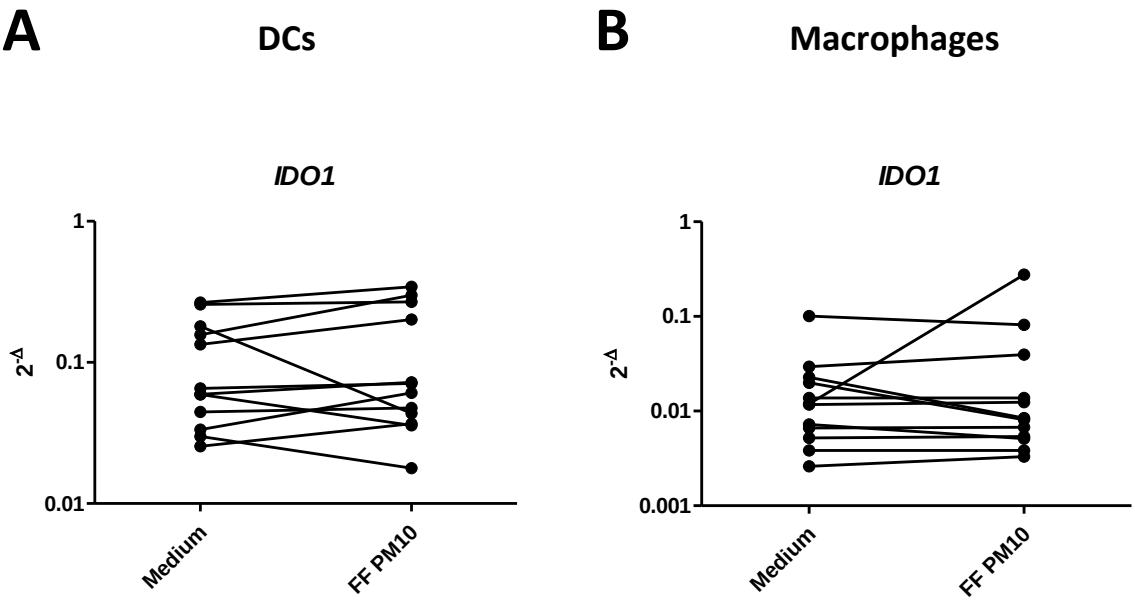


Figure 4.4: IDO1 is expressed in sputum DCs and macrophages

Sputum DCs and macrophages were detected by labelling for HLA-DR, CD3, CD14, CD16, CD19, CD20 and CD56 in processed sputum samples from healthy volunteers after 2-4 hour stimulation with 50µg/ml FF PM10 or medium only. After antibody staining cells were sorted into lysis buffer and analysed for IDO1 using real-time PCR. These experiments were conducted 16 times in total with separate sputum donors. ***A****: Summary data illustrating the expression of IDO1 in cell sorted DCs from induced sputum as* $2^{-\Delta Ct}$. ***B****: Summary data showing the expression of IDO1 genes as* $2^{-\Delta Ct}$ *in respiratory tract macrophages. Each pair of dots represents one independent experiment with a different sputum donor.*

IDO1 was expressed in unstimulated sputum DCs, as seen on the electrophoresis gel (Appendix Figure 4). Again, similar to the previous genes analysed, the summary data shows that *IDO1* was expressed at variable levels (Figure 4.4 A). When DCs were exposed to FF PM10 no significant increase was measured, although, when looking at the individual experiments 9 out of 12 samples showed a small increased expression of the target gene after stimulation of the cells with FF PM10.

For the expression of *IDO1* in sputum macrophages, no significant difference could be observed between FF PM10 stimulated or unstimulated cells, although again a trend was detectable (Figure 4.4 B). Overall, out of 10 samples, 7 showed an increased expression of *IDO1* in respiratory tract macrophages after stimulation with FF PM10. Again, as seen before, no

particular correlation was found between upregulation and sample quality for sputum DCs and macrophages.

4.4.5. Respiratory tract DCs express maturation markers

The previous chapter showed that FF PM10 induced a wide range of effects in MoDCs including AhR activation and classical maturation. MoDCs showed a highly significant upregulation of CD86 after stimulation with FF PM10, whereas CD80 showed a lower increase in expression compared to CD86. However, CD80, is not expressed in immature DCs and is strongly induced upon stimulation and is therefore a reliable marker of maturation (Jonuleit et al., 2000). The preceding sections showed AhR expression and induction in respiratory tract DCs and macrophages after FF PM10 exposure. It remains unknown whether incubation of the cell suspension with FF PM10 leads to classical maturation of DCs, including upregulation of costimulatory molecules CD80 and CD86. To test this, and also to determine whether the same pattern of expression of CD80 and CD86 as seen in MoDCs can be observed in respiratory tract DCs, human sputum samples were collected and equally split to incubate with FF PM10 or medium only for 2-4 hours. After incubation, cells were antibody stained and cell sorted. Respiratory tract DCs were lysed and PCR analysis was performed for *CD80* and *CD86* to assess the maturation status of the cells. As cell numbers of samples after sorting were low, and hence the number of analyses that could be done was limited, we decided to perform a PCR for *CD80* and *CD86* while analysing for *AhR*-related genes.

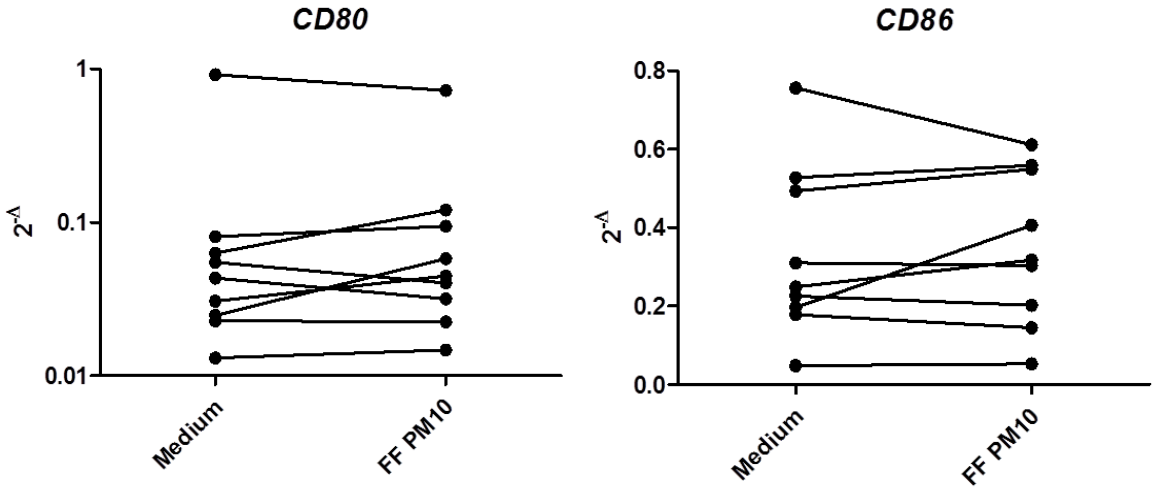


Figure 4.5: Respiratory tract DCs express maturation markers

Human induced sputum samples were processed and the cell suspension was incubated for 2-4 hours either with or without 50µg/ml FF PM10. Cells were stained for HLA-DR, CD3, CD14, CD16, CD19, CD20 and CD56 to detect DCs, sorted and lysed. The expression of CD80 and CD86 was measured by reverse-transcription real-time PCR. These experiments were conducted 16 times in total with separate sputum donors. The graphs illustrate the summary data of the gene expression for CD80 and CD86 as $2^{-\Delta Ct}$ in lung DCs. Analysis of statistical significance was performed with the Wilcoxon matched pairs test. Each pair of dots represents one independent experiment with a different sputum donor.

Expression of *CD80* and *CD86* was detected in stimulated and unstimulated DCs. The gel of PCR products from a single DC preparation shows that *CD86* and *CD80* are expressed in unstimulated cells (Appendix Figure 4). In general, *CD86* seems to be slightly higher expressed than *CD80* as indicated by the $2^{-\Delta}$ values. Overall, stimulation of DCs did not show a statistically significant further upregulation of *CD80* and *CD86.* However, 5 out of 9 samples showed further upregulation after stimulation of DCs with fossil fuel PM10 for both *CD80* and *CD86*. The remaining 4 samples did not show any anomalies in terms of cell numbers, overall gene expression or volunteers. All in all, sputum DCs showed expression of maturation markers *CD80* and *CD86* on a transcriptional level in unstimulated samples and partly a further upregulation when samples were exposed to FF PM10, although this is not consistent and not significant.

4.5. Discussion

In this chapter, respiratory tract DCs and macrophages were sorted from induced sputum samples from healthy volunteers for PCR analysis. The main focus of this section was to show that it is possible to analyse DCs and macrophages from the airway. It was originally claimed that DCs are not detectable in induced sputum (J. W. Upham, Denburg, & O'Byrne, 2002) but since then DCs have been identified by flow cytometric analysis in sputum collected from adults and even from children (Brugha et al., 2015; McCarthy et al., 2007). The induced sputum method is a non-invasive and safe technique, compared to bronchoscopy, which is more invasive and requires a more complex setup; it has enabled us to have easy access to respiratory tract cells for further analysis (Fahy et al., 2001). Sputum induction leads to phlegm from the lower airways to be coughed up, especially from the trachea and bronchi, i.e., originating predominately from the conducting airways. Bronchoscopy is able to provide cells from the bronchi and bronchioles. Depending on the donor and therefore on the quality of the sample, we were able to FACS sort respiratory tract DCs and macrophages in sufficient numbers to perform PCR analysis. However, not all the samples that have were collected were in sufficient quality and provided enough cells for further PCR analysis. Nonetheless, out of 16 samples collected, only three did not yield any results; in 2 out of the 3 samples, this failure could be attributed to low cell numbers leading to no amplification during the PCR, and in one sample a technical failure occurred.

The use of induced sputum samples for identification of biomarkers using flow-cytometry has been widely used by other research groups (Bleck et al., 2015; Brugha et al., 2015; McCarthy et al., 2007). However, not much PCR analysis has been performed on cell sorted DCs and macrophages, presumably due to low numbers of cells in these samples. To our knowledge, this is the first time that the *AhR* gene and AhR related genes and as well as the transcription factor *Zbtb46* have been detected in airway DCs and macrophages. In addition, genes for the costimulatory markers *CD80* and *CD86* were also detected in respiratory tract DCs.

Antibody labelling was limited to two fluorochromes to avoid extensive gating and compensation through which we would lose sample and therefore diminish numbers of cells for PCR analysis. DCs were defined as HLA-DR^{+}Lin^{-} cells, where Lin represents a mixture of markers expressed by non-DCs and macrophages were defined by co-expression of HLA-DR and Lin. It was important to confirm that the populations we defined did indeed correspond to DCs and macrophages. To this end, expression of the transcription factor *Zbtb46* was assessed. *Zbtb46* is a transcription factor only expressed in cDCs and its progenitors but not in pDCs, monocytes or macrophages or any other immune cell populations (Meredith, Liu, Darrasse-Jeze, et al., 2012b;

Ansuman T Satpathy, Kc, et al., 2012). Although *Zbtb46* expression in DCs was significantly higher, *Zbtb46* expression was also detected at low levels in the sorted respiratory tract macrophage population. Specific *Zbtb46* expression in DCs has been studied widely in mice and to a limited extent in humans, showing its expression in cDCs, but not in pDCs, monocytes, granulocytes, or T, B, or NK cells (Ansuman T Satpathy, Kc, et al., 2012). However, to our knowledge *Zbtb46* expression has not been studied in sputum DCs.

Moreover, our study has shown that sputum macrophages also express *Zbtb46*, although at a significantly lower level than sputum DCs. Again, we believe this has not been shown before. A low expression of *Zbtb46* in macrophages may suggest that there could have been some overlap of populations, although this is highly unlikely because it would have meant that DCs were sorted in the macrophage gate. Gating was performed very carefully and as cell numbers for macrophages were very high, the gate for macrophages was put far away from the gate for DCs to leave a large gap between gates. It is more likely that, since DC numbers were very low, the gate for DCs was set too generously and cut into the macrophage population, although the data does not immediately suggest that. It has been proposed that *Zbtb46* acts as a negative regulator for the gene expression of murine cDCs, showing that TLR stimulation of cDCs leads to downregulation of *Zbtb46* and therefore prevents DCs maturation in steady-state (Meredith, Liu, Kamphorst, et al., 2012). However, we did not see evidence for this in our experiments (data not shown).

First, before we were able to analyse the effects of fossil fuel PM10 on airway DCs and macrophages and how they affect the induction of the AhR, we had to make sure that these cells express the AhR. As AhR acts as an environmental sensor, it is expressed in innate immune cells at barrier sites, such as lung, gut and skin. It has been widely shown that both cell types generally express the AhR in mice and humans but it remains unknown whether different subsets in different parts of the body have express variable levels of AhR (Frericks et al., 2007). It has been shown that murine DCs from the mediastinal lymph nodes express the AhR as well as lung macrophages from mice (Esser & Rannug, 2015; G. B. Jin et al., 2010). However, specifically respiratory tract DCs and macrophages from induced sputum samples have not been investigated. Here, we have shown that sputum DCs and macrophages both expressed the AhR, and that DCs showed a slightly higher expression compared to macrophages, although this difference was not statistically significant. This data would indicate that AhR plays a role in environmental sensing in both cell types in the respiratory tract, although the exact immunological consequences of these sensing pathways in DC and macrophages are still unclear.

The incubation time for the sputum experiments was chosen as 2-4 hours. One concern was that viability would be significantly reduced if culture time was prolonged. On the other hand, incubation time had to be long enough to ensure sufficient expression of the genes to be measured. Therefore, we tried to perform the experiment within one day, as others have done (Bleck et al., 2015). In MoDCs, we observed that after two hours of incubation with FF PM10 most of the genes of interest were upregulated, except *IDO1*, which showed a slight decrease compared to the medium control, but a dose-dependent increase after that. Therefore, based on the results from MoDCs presented in the previous chapter, sputum samples were stimulated for 2-4 hours to measure the genes of interest by PCR.

The AhR related genes in respiratory tract DCs and macrophages from different donors were expressed at variable levels. This may be due to genetically determined variability between donors as well as a variation of natural exposure of people to air pollution, which can lead to variable baseline levels of expression. Donors came from the urban area of London, hence were exposed to various amounts of urban air pollution, depending on exact location and activity; consequently, the level of AhR-controlled genes may have been induced at various levels in unstimulated sputum samples (Nwokoro et al., 2012). Also, the concentration of endogenous ligands may vary between donors and lead to variable levels of natural induction. Moreover, it has been shown that polymorphism of the AhR can have an effect on the activation of the AhR pathway as well as on the sensitivity and the protein-ligand binding strength of the coding proteins (Beischlag, Luis Morales, Hollingshead, & Perdew, 2008; Frericks, Burgoon, Zacharewski, & Esser, 2008). In addition to that, from the point of collection of the sample until the cell sorting and lysis for PCR, the sputum cells have been through a long procedure of processing, including a harsh treatment with Dithiothreitol (DTT) to break up the mucus, and the cell sorting itself. All these steps may influence and introduce variability in the level of expression of the genes of interest.

One possible option for the future which might help normalise baseline levels would be to allow the cells to rest, possibly overnight, and stimulate them the next day. However, there may be a cost to this approach in terms of cell viability and the potential for cells to change significantly during the resting period.

Nonetheless, both cell types expressed *CYP1A1* and *CYP1B1* at a similar level indicating similar levels of activity in the AhR pathway. In contrast, *IDO1* was significantly more highly expressed in sputum DCs than in macrophages ($p = 0.0068$, data not shown). This could have potentially interesting effects, as it could indicate that DCs in the lung play an important role in immunosuppression (Yeung, Terentis, King, & Thomas, 2015). Indoleamine 2,3-dioxygenase is an

enzyme that is involved in the catabolism of the essential amino acid tryptophan into the stable metabolite kynurenine. The depletion of tryptophan and the subsequent production of kynurenine are the main effects by which IDO affects the immune response. The lack of tryptophan leads to starvation of effector cells. The increased amount of kynurenine favours the expansion of regulatory T cells; hence, the suppression of effector cells and the increase in regulatory responses are the two main effects of IDO (Harden & Egilmez, 2012).

One could speculate that out of the two main antigen-presenting cells in the lung, DCs play an increased role in the active immune regulation that may limit responses to inhaled antigen. As DCs are the most potent APCs, it is beneficial that they play a greater role in immunosuppression. However, in some cases a high level of expression of *IDO1* in respiratory tract DCs may not be beneficial, for example in cases of lung cancer, in which case immunosuppression would be disadvantageous (Opitz et al., 2011).

After sputum cells were exposed to FF PM10, *CYP1B1* showed a significant increase in expression for both DCs and macrophages. Although *CYP1A1* and *IDO1* did not show a significant increase, a trend towards higher expression was detected, which may lead to statistical significance with more repeats. While *CYP1A1* did not show a significant upregulation, a trend towards higher expression was discovered as, out of 11 samples, 7 showed an increase in expression for *CYP1A1* after stimulation of the cells with FF PM10. After looking into the remaining 4 samples very closely, there was no particular differences regarding cell numbers or gene expression of unstimulated samples, and the volunteers were not exposed to a higher level of air pollution, cigarette smoke or other AhR-agonists compared to the other volunteers.

There was no correlation between cell numbers and expression of genes in unstimulated samples, which indicates that low cell numbers did not affect the quality of the experiment and did not limit the study. The prominent increase in expression of *CYP1B1* but not *CYP1A1* may be explained by the kinetics of gene expression, as observed in the previous chapter. *CYP1B1* mRNA showed a steep increase in MoDCs after 2 hours of incubation with FF PM10 and it is therefore likely that a similar response occurs in sputum cells in a similar time-frame. In contrast, for *CYP1A1* in MoDC, the PM10-induced increase in expression was relatively delayed and an analysis of sputum cells at 2-4 hours may not have been optimal to detect this. Similarly, *IDO1* expression was slightly decreased after 2 hours in MoDC but showed an upregulation after that.

We were also able to detect expression of genes for *CD80* and *CD86* in sputum DCs. Expression of both markers was conducted at a transcriptional level rather than through flow-cytometry. This is advantageous since a separate FACS analysis would not have been possible with same

sample due to low cell numbers and hence would have required a separate sputum induction with a different donor. *CD80* and *CD86* were also measured by Bleck et al., 2015 at a transcriptional level, as a comparison between healthy and asthmatic DCs from induced sputum samples, showing no differences in *CD80* and *CD86* expression in DCs between asthmatic and healthy individual. We were not able to detect a significant difference in the gene expression of either *CD80* or *CD86* after exposure of sputum DCs to FF PM10. The DCs may have become activated during the sputum isolation process. Alternatively, the expression of these markers in unstimulated cells may suggest that cells were already activated *in situ* as the result of exposure to air pollution or other activators. It should be borne in mind that this study was conducted in the urban area of London and sputum donors are likely to have been exposed to varying degrees of pollution. An unpublished study from our institute (Whitehouse, personal communication), showed that sputum DCs from children going to a school in an area of London highly affected by air pollution from traffic, expressed higher levels of maturation markers than sputum DC from children in low pollution areas. Therefore, the presence of *CD80* and *CD86* in cells that were not exposed to FF PM10 *in vitro* may have been caused by natural exposure *in vivo*.

As the sputum sample was stimulated as a whole before the cell sorting of DCs and macrophages, other sputum cells were present too, such as epithelial cells. It has been shown that these can be activated by PM10 and also interact with DCs and other immune cells (Bleck et al., 2010). Therefore, it cannot be ruled out that the expression of *CD80* and *CD86* are partly due to the influence of other activated cells. This also applies to the other genes measured in this chapter. We cannot exclude that *CYP1A1*, *CYP1B1* and *IDO1* expression was influenced by other cells present in the culture. However, to our knowledge, no findings on this topic have been published.

4.6. Conclusion

In conclusion, this chapter has shown that it is possible to purify respiratory tract DCs and macrophages by FACS sorting in sufficient numbers from induced sputum samples for quantitative PCR analysis of immunologically relevant genes. Evidence was obtained for expression of genes associated with AhR signalling and DC maturation in *ex vivo* cell populations that potentially is the result of exposure to ambient pollution, although further studies are needed. Furthermore, expression of *CYP1B1,* and in some donors *CYP1A1*, *IDO1* or co-stimulatory molecules, increased upon exposure to FF PM 10, further illustrating responsiveness to this component of ambient pollution.

5. Particle size and composition influence the effects of PM on DCs

5.1. Chapter summary

This chapter describes the effects of different types of airborne PM on the activation of DCs. We have shown in the previous chapters that FF PM10 from an urban environment activates MoDCs through several pathways, such as TLR4 and AhR signalling pathways. We have also shown that the AhR is activated in respiratory tract DCs. But how the activation of these pathways varies if DCs are exposed to alternative particle types is not known and is the subject of this chapter.

Ambient particles used in this chapter vary in terms of size and composition, as a result of derivation from different combustion sources. Specifically, the composition of PM derived from combustion of fossil fuels (FF) in urban environments is different from the composition of PM derived from the combustion of biomass (BM) in areas of the world where wood burning is common (Oliveira et al., 2011). In this chapter, the effects of particle size and composition on the modulation of DC by PM was examined by comparing FF-derived ambient particles from two urban locations (PM10 from Leicester as well as both FF PM10 and FF PM2.5 sampled from the same location in London) with PM10 and PM2.5 obtained from kitchen areas in Accra/Ghana and Basse/The Gambia, respectively, and derived mainly from BM.

Irrespective of their size or combustion source, particles were taken up by DCs to a similar extent. When DCs were stimulated with PM10 – regardless of their origin – they upregulated maturation markers such as HLA-DR, CD86, CD40, CD80 and CD83. PM2.5 – again independent of the area where they were collected – induced a lower level of expression of these markers or no expression at all. However, CCR7 was an exception, as it was upregulated to a higher extend after DCs were incubated with particles derived from biomass combustion compared to fossil fuel. PM10 from both UK urban environments, Leicester and London, had almost identical effects on DCs. Activated DCs induced proliferation of allogeneic naïve CD4 T cells in proportion to their maturation status. Thus, DCs derived from both fossil fuel and biomass combustion led to the highest level of proliferation, whereas PM2.5 exposed DCs induced an intermediate level of proliferation. This proliferation was greater than that stimulated by medium only exposed DCs but less that stimulated by PM10-treated DC. As reported in chapter 3, MoDCs generated a Th1-directed T cell response characterised by IFN-γ production and this was not altered by exposure to BM PM2.5. Cytokine release by DCs themselves was also influenced by particle size: PM10 induced production of more IL-6, IL-12p70, IL-1β, IL-10 and IL-23 by DCs compared to PM2.5, irrespective of combustion source. FF PM10 from two different locations, Leicester and London, had almost identical effects on cytokine production of DCs. With the exception of IL-6, FF PM10

induced a slightly higher expression of the cytokines by DCs compared with BM PM10 at a similar concentration. IL-6 was secreted at similar levels by DC exposed to both BM- and FF-derived PM10.

Although FF-derived and BM-derived PM10 were similar at stimulating many aspects of classical DC maturation and cytokine production, evidence was found that activation of the AhR signalling pathway was greater by BM-derived particles. In three independent experiments, induction of *CYP1A1* and *CYP1B1* expression was higher when the DCs were exposed to BM PM10 than when they were exposed to FF PM10. Furthermore, the smaller BM-derived PM2.5 also induced more *CYP1A1* and *CYP1B1* expression than FF PM10, even though FF PM2.5 were no better than matched PM10 at activating AhR signalling. In contrast to *CYP1A1* and *CYP1B1,* the expression of *IDO1* most closely reflected DC maturation and cytokine production and was similiar in DCs exposed to FF and BM PM10. Taken together with data presented in Chapter 3, these findings support the conclusion that induction of *IDO1* is largely AhR-independent in this system.

In summary, in terms of activating DCs, PM10 plays a critical role; especially particles from an urban environment have an enhanced ability to stimulate DCs compared to particles derived from biomass combustion. In particular, AhR activations seem to be increased after BM-derived PM exposure, indicating that BM PM is more likely to induce AhR-dependent outcomes in the immune system, be they pro-inflammatory or anti-inflammatory.

5.2. Introduction

Airborne PM is a major health problem worldwide, causing premature death and adverse health outcomes. It has been suggested that the toxicity of air pollution particles is dependent on their size; different size fractions have different local and systemic effects in the body because they can reach different areas in the respiratory tract. PM10 are known to settle in the upper airway whereas smaller particles, PM2.5, tend to be able to access the lower respiratory tract, pass the blood-air barrier and cause systemic health effects (Kim, Kabir, & Kabir, 2015). On a cellular level, it has been shown in cell culture studies with epithelial and macrophage cell lines that, generally, the bigger the particles are, the higher is the release of inflammatory cytokines such as TNF-α and IL-8; particles smaller than 0.4µm in diameter are the exception since they induce the highest level of cytokine release. Lastly, it has been shown that ultrafine particles are involved in the development of atherosclerosis (J. a Araujo & Nel, 2009b).

It has been reported that people in different parts of the world, where a particular energy sources is used and therefore the air pollution has a specific composition, health effects of the population in this area can vary compared to other areas with other types of air pollution. Air

pollution that derives from biomass is associated with respiratory infections, obstructive lung disease and lung cancer (S. B. Gordon, Bruce, Grigg, Hibberd, Kurmi, Lam, Mortimer, Asante, et al., 2014). Air pollution in urban areas that is primarily derived from the combustion of fossil fuel is linked to development and exacerbation of asthma (D'Amato et al., 2010; Sunyer et al., 1997). It is unclear whether the immunological effects of different kinds of pollution contribute to these different health effects.

Specifically, little is known about how these different types of particles affect immune cells and in particular DCs. The previous chapters have shown that PM10 derived from fossil fuel combustion induces a complex activation pattern in DCs. Therefore, we looked into how particles with different sizes and from different sources affect the function of MoDCs by looking at several pathways of activation.

To achieve this, we sought to compare particles, differing in their size and in their composition; the latter because they were collected from locations where different combustion sources predominate. For the purpose of this chapter, we compared PM mainly derived from the combustion of biomass to fossil fuel derived particles and both particles types came in two different sizes, PM10 and PM2.5. The primarily biomass derived PM2.5 was collected in household kitchens of rural Basse in The Gambia/Africa and the burning of biomass accounted for 74-87% of its mass (Z. Zhou, Dionisio, Verissimo, Kerr, Coull, Howie, et al., 2013) BM PM10 was collected from communal kitchen areas shared by several households in Accra/Ghana (Z. Zhou et al., 2011, 2014). Both kitchens used mainly biomass as their primary energy source; however, as the communal kitchen areas in Accra were open areas, the overall contribution of biomass to PM was lower compared to the Gambia PM2.5. Also, a small percentage of traffic related components was found in the PM from Accra. For the comparison with fossil fuel derived PM we used PM10 from the urban area of Leicester as well as FF PM10 and FF PM2.5 from exactly the same location in central London. All these different particle types were compared in terms of their effects on DCs activation.

5.3. Hypothesis and study aim

The hypothesis underlying this chapter is that PM10 induce a higher level of activation in DCs compared to PM2.5. The aim of this chapter is:

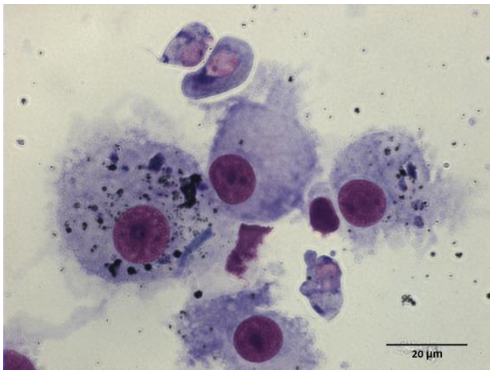
- Determine the effects of particles from different combustion sources and different sizes on MoDCs

5.4. Results

5.4.1. PM is taken up by DCs irrespective of their size

Since the interaction between particles and MoDCs can vary greatly, the first step was to see whether the particles are taken up by the cells. For this, human monocytes were separated from blood and cultured with GM-CSF and IL-4 for 7 days and then incubated with different types of air pollution particles for another 2 days. After this, cells were put on microscopy slides by cytospin. Subsequently, cells were fixed and stained and analysed using a light microscope.

A



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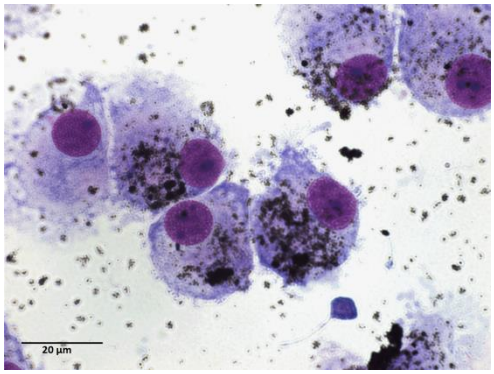

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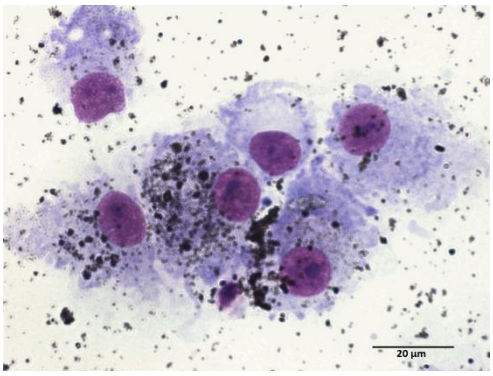

Figure 5.1: PM are taken up by DCs

*DCs derived from human monocytes were incubated with 50µg/ml of (**A**) BM PM2.5 and (**B**) London FF PM2.5 and (**C**) FF PM10. After two days of incubation, cells were centrifuged on glass slides by cytospin, fixed with methanol and stained with a blue solution (azur) and a red solution (eosin). Pictures were taken with a light microscope using Picture Frame at a magnification of x100 with oil. This experiment was conducted once.*

All three different particle types seem to show a similar interaction with MoDCs (Figure 5.1). In terms of their size, PM10 and PM2.5 refer to the size of the particles at the point of collection on the filter by the cyclone monitor, which separated PM2.5 and PM10 from the particle mixture (see Chapter 2 for exact specification). When particles were then extracted from the filter by vortexing and sonication, the size was not measured. Therefore, when adding particles onto the culture with DCs, it is not certain that these particles are exactly PM10 and PM2.5, since the agglomerates, which have built during the collection, may not have been broken up. Hence, the

size classification of PM10 and PM2.5 refers to the size when collected and not when added to the culture and thus the different particles look very similar in the pictures in terms of their size. After the incubation time, PM was located within the cells, as seen in vesicles, as well as on the cell surface, as seen by black dots without a vesicle around them. In terms of their uptake by MoDCs, they did not seem to differ.

5.4.2. PM10 induces a higher level of DC maturation than PM2.5

Activation of DCs is the first and crucial step of the immune response. As seen in chapter 1, FF PM10 leads to activation of MoDCs and expression of HLA-DR, CD86, CD40, CCR7, CD80 as well as CD83. But how the expression of these markers is affected once cells have been exposed to particles from different combustion sources and different sizes is unknown. Understanding this is the aim of this section. For this, we exposed MoDCs to various concentrations of Leicester FF PM10, London FF PM10 and London FF PM2.5, BM PM10 as well as BM PM2.5 for 2 days. A range of concentrations was chosen for testing to make sure results were comparable, which would not have been the case with a single concentration. Also, any inconsistencies in determining the concentration of the particle suspension can be compensated for by comparing a range of concentrations. Therefore, particles were used at concentrations of 10, 25 and 50µg/ml for DC stimulation. After incubation, cells were antibody stained for activation markers and analysed using flow-cytometry.

Initially, biomass derived PM2.5 was compared to FF PM10. BM PM2.5 was the first type of particle available to us, so we based our first comparison on these. Hence, these particles differed in their size as well as combustion source.

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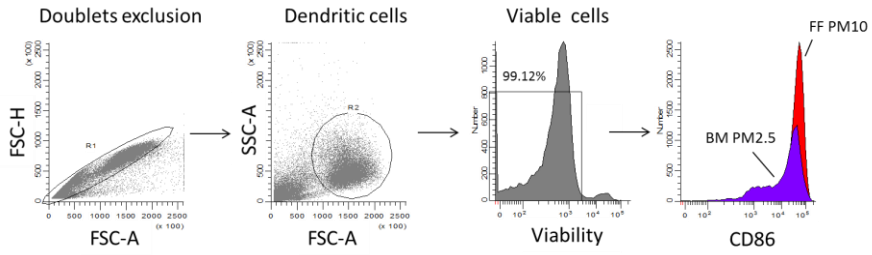

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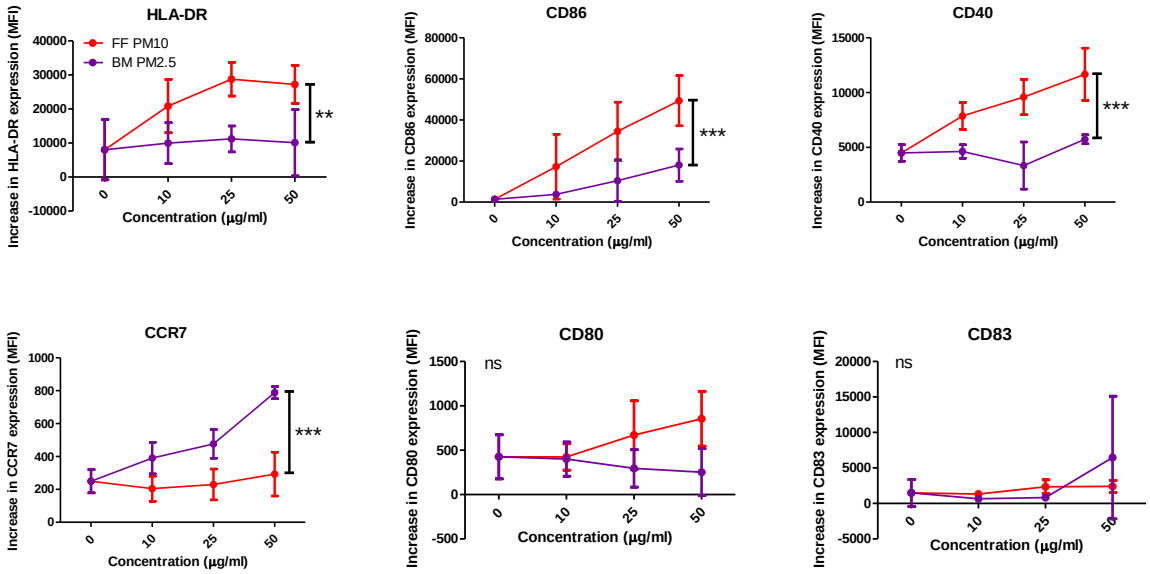

Figure 5.2: FF PM10 induces a higher level of maturation in DCs than BM PM2.5

Human MoDCs were stimulated for 48h in medium only or 10, 25 and 50µg/ml of FF PM10 from Leicester and BM PM2.5. After incubation, cells were washed with FACS buffer and antibody stained for HLA-DR, CD83, CD80, CCR7, CD86 and CD40. Subsequently, cells were labelled and measured using a flow-cytometer and the MFI was plotted. ***A****: One representative example showing the gating strategy, including the doublets exclusion, gating on DCs based on their size and granularity and further excluding dead cells. The last histogram illustrates the expression of CD86 on DCs after exposure to 50µg/ml of FF PM10 and BM PM2.5.* ***B:*** *Diagrams illustrating the expression of maturation markers on DCs after exposure to several concentrations of the different types of PM. The mean and standard deviation of three independent experiments was plotted and statistical analysis was performed using a two way analysis of variance.*

As seen in the previous chapter, exposure to fossil fuel derived PM10 leads to an increase in the expression of all DC activation markers that were measured, except for CD83 (Figure 5.2). In relation to this, biomass derived PM2.5 induced a non-significant increase in expression of maturation markers compared to FF PM10, except for CCR7 and CD40, which were increased significantly after BM PM2.5 stimulation ($p=0.0208$ and $p=0.0285$ respectively). For HLA-DR, CD86, CD40 and CD80, a dose-dependent increase of expression was observed after exposure of DCs to FF PM10, but not for CCR7 and CD83. The difference between the two particle types was statistically significant for the markers HLA-DR, CD86, CD40 as well as CCR7; a non-significant difference was measured for CD80 and CD83. CCR7 surface expression was an exception compared to the other maturation markers: BM PM2.5 induced a significantly higher expression of the marker compared with FF PM10 stimulation. However, the overall expression of CCR7 was low compared to the other markers.

As a next step, we were able to source BM PM10 as a comparison to FF PM10. This allowed us to compare particle of the same size which were different in only one respect, viz. the combustion source. As a result, we were able to draw conclusions about the impact of particle combustion source on the activation of DCs.

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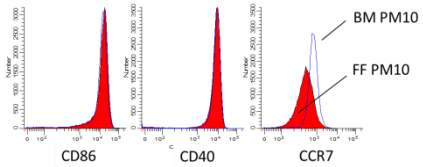

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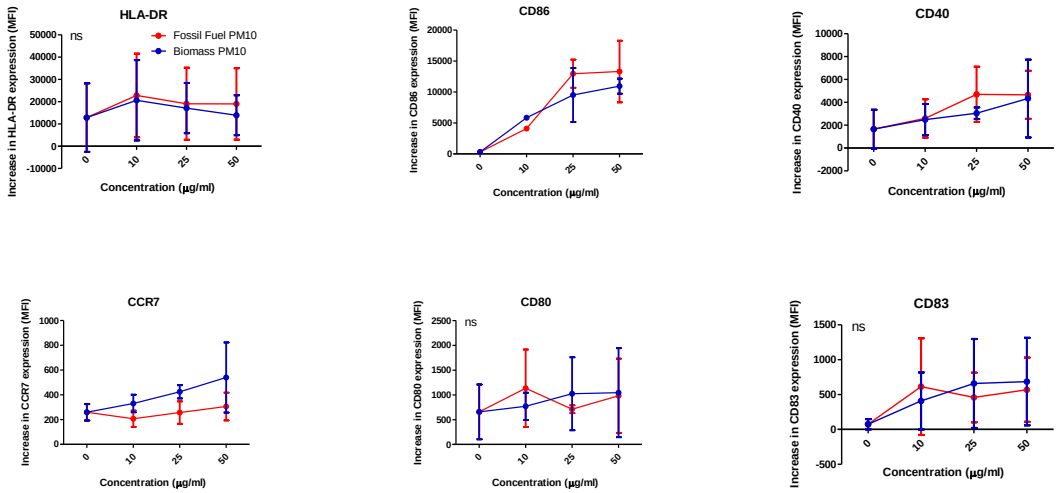

Figure 5.3: PM10, regardless of its combustion source, induces a similar level of maturation in DCs

DCs were derived from human monocytes in the presence of GM-CSF and IL-4 and after 7 days of differentiation DCs were cultured for another 2 days in medium only and 10, 25 and 50µg/ml of FF PM10 from Leicester and BM PM10. Subsequently, cells were labelled with antibodies for HLA-DR, CD83, CD80, CCR7, CD86 and CD40 and measured by flow-cytometry. ***A****: Flow-cytometry plots showing one representative example of the expression of CD80, CD40 and CCR7 on DCs after stimulation with 50µg/ml of FF PM10 and BM PM10. The red peak shows the magnitude of expression after FF PM10 stimulation, the blue lines indicate the incubation with BM PM10. This experiment was conducted three times in total.* ***B****: Diagrams showing the expression as MFI of various activation markers after the exposure of DCs to several concentrations of FF PM10 and BM PM10. HLA-DR, CD83 and CD80 expression was plotted showing the mean and standard deviation of 3 repeats. These experiments were analysed for statistical significance using a two-way analysis of variance. The expression of CD86, CD40 and CCR7 was plotted as the mean and standard deviation of the two repeats and no statistical analysis was performed.*

From the results shown in Figure 5.2., it was unclear whether the effects on DC were related to differences in particle size or differences in composition (due to differences in combustion source). BM PM2.5 was the first biomass particle type available to us for this project; BM PM10 was sourced subsequently to address these additional questions.

The next step was to compare particles with the same size but different source; to that end, FF PM10 and BM PM10 were compared. In general, both BM PM10 and FF PM10 had similar effects on DCs, characterised by dose-dependent induction of the maturation markers HLA-DR, CD86, CD40, CD80 and CD83 to similar overall levels of expression (Figure 5.3). With regard to CCR7, two independent experiments demonstrated that BM PM10, like the BM PM2.5 analysed previously, induced higher expression than was observed with FF PM10 (Figure 5.3 A). Statistical analysis using a two-way analysis of variance did not reveal a statistically significant difference for the expression of HLA-DR, CD80 and CD83 on DCs after incubation with different particles types. No statistical analysis was performed on the results for the expression of CD86, CCR7 and CD40 as only two repeats of the experiment were conducted. BM PM10 was only available at a late stage in the project; hence, only a limited number of experimental repeats could be conducted.

While stimulating DCs with biomass derived particles, a high level of autofluorescence was detected in some cytometer channels, which limited the use of some of the fluorochromes. The induction of autofluorescence by BM-derived PM is addressed specifically in the next chapter.

Finally, we were also able to source FF PM2.5 and FF PM10 from London. This way, we could examine the effects on size, as both particles types were collected in the exact same location, thereby eliminating any differences due to the combustion source. This allowed us the draw conclusions about the effect of particle size on the maturation on DCs.

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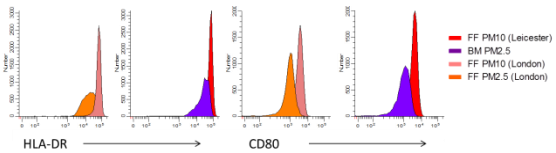

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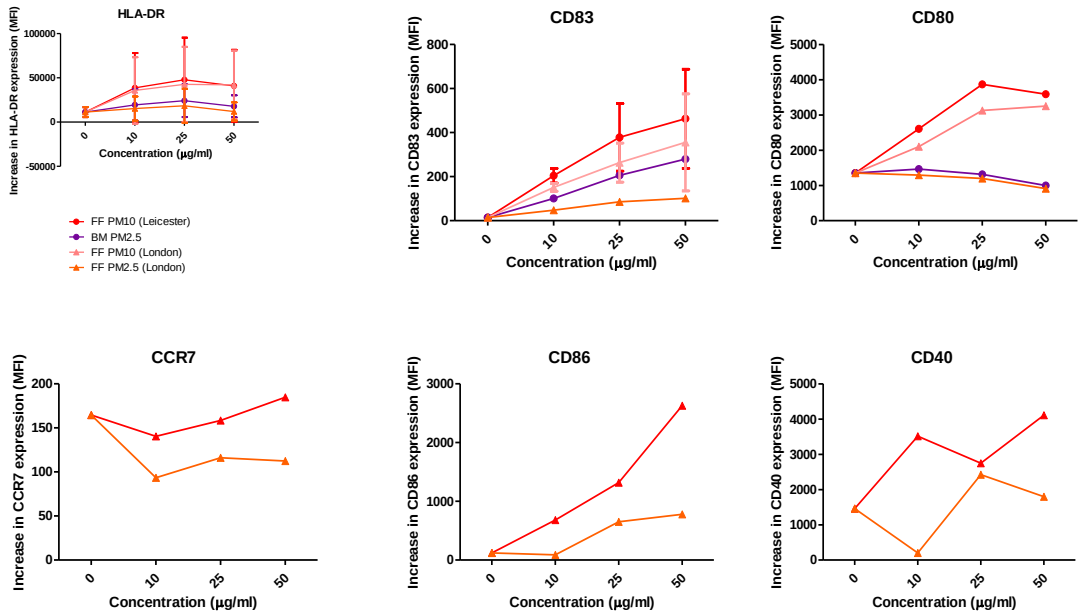

Figure 5.4: PM10 induces a higher level of maturation in DCs compared to PM2.5

MoDCs were incubated for 48h in medium only or 10, 25 and 50µg/ml of Leicester FF PM10, BM PM2.5, London FF PM10 and London FF PM2.5. After stimulation, cells were stained for HLA-DR, CD83, CD80, CCR7, CD86 and CD40 and measured by flow-cytometry. ***A****: Representative flow-cytometry plots showing the expression of HLA-DR and CD80 comparing the effects of the different particle types on DCs at a concentration of 50µg/ml.* ***B:*** *The diagrams show the expression of maturation markers on DCs after the exposure to the various particle types. The expression of HLA-DR, CD83 and CD80 were measured after DC stimulation with Leicester FF PM10, London FF PM10 and FF PM2.5 as well as BM PM2.5 at several concentrations. CCR7, CD86 and CD40 expression on DCs was measured after stimulation with London FF PM10 and FF PM2.5 at concentrations ranging from 0 to 50µg/ml. The experiments for HLA-DR and CD83 were conducted twice with two separate blood donors; the remaining markers were only measured once.*

FF PM2.5 was compared to the other particles types and sizes for its effects on DCs. For HLA-DR, CD83 and CD80, London FF PM2.5 and London FF PM10, BM PM2.5, Leicester FF PM10 were compared (Figure 5.4). Again, a similar pattern as seen before was observed. Both types of FF PM10 showed a dose-dependent induction of those maturation markers in DCs. Consistently, both types of PM2.5 induced either no expression at all or a very low level. For CCR7, CD86 and CD40 only one experiment was performed comparing London FF PM10 and FF PM2.5. Except for CCR7, a dose-dependent increase in expression was observed for both particle types; again, FF PM2.5 induced a lower overall expression of DCs compared to FF PM10. In the single experiment performed, there was little or no induction of CCR7 after exposure of DC to either FF PM10 or FF PM2.5 from London.

In summary, PM10, whether FF-derived from two independent UK locations or BM-derived from Africa, was a potent stimulator of classical DC maturation. Preliminary data suggests increased induction of CCR7 by BM PM10. In contrast, PM2.5, regardless of its combustion source, induced little or no DC maturation. Thus, PM10 may be the key ambient pollution particulate that acts via DCs in the airway to enhance local immune activity.

5.4.3. PM10-exposed DCs induce a higher level of T cell proliferation than PM2.5-stimulated DCs

The previous section had indicated that different sizes of PM, derived from biomass or from fossil fuel combustion, induce distinct levels of DC activation. To look into the functional significance of this, a MLR was performed to measure the stimulatory capacity of the activated DCs. Also, the cytokine profile of the proliferating T cells was analysed.

MoDCs were stimulated for 2 days with FF PM10 (from London or Leicester), FF PM2.5, BM PM2.5, and BM PM10 or cultured in medium only. Subsequently, different concentrations of these DCs were co-cultured with naïve allogeneic CD4 T cells for 5 days. T cells were previously labelled with CFSE to track their proliferation. For cytokine measurement, cells were stimulated, permeabilized and intracellularly labelled.

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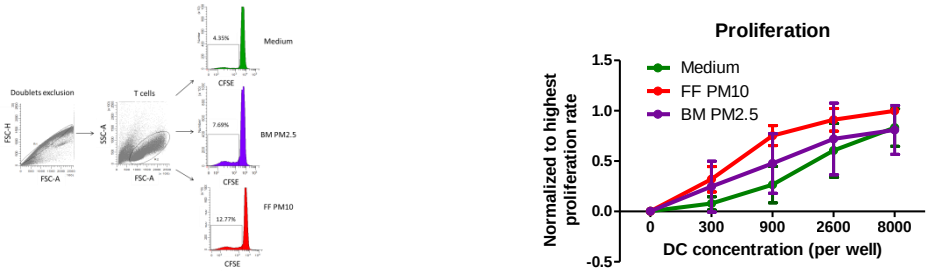

Figure 5.5: FF PM10-exposed DCs induce an increased proliferation of allogeneic CD4 T cells compared to BM PM2.5-stimulated DCs

CD14+monocytes were magnetically separated from PBMCs, differentiated into DCs and exposed to 25µg/ml of Leicester FF PM10 and BM PM2.5 from Gambia for 2 days. After incubation, 0, 300, 900, 2600, 8000 and 2400 DCs were co-cultured with 400,000 magnetically separated allogeneic naïve CD4 T cells for another 5 days. T cells were labelled with CFSE before stimulation in order to monitor their proliferation. ***A****: One representative flow-cytometry plot out of 3 experiments showing the gating strategy and the proliferation of CD4 T cells when exposed to pre-stimulated DCs at concentration of 900 cells. DCs were stimulated with FF PM10 from Leicester, BM PM2.5 or medium only.* ***B****: Summary data illustrating the proliferation of naïve CD4 T cells after stimulation with Leicester FF PM10 – and BM PM2.5 - exposed DCs. The diagram shows the mean and standard deviation of 3 independent experiments using 3 different blood donors for DCs as well as naïve T cells. Two technical replicates were performed for each experiment. The percentage of proliferating cells was normalized to the highest proliferation in each experiment and statistical analysis was conducted using a two-way-analysis of variance and Bonferroni post-tests.*

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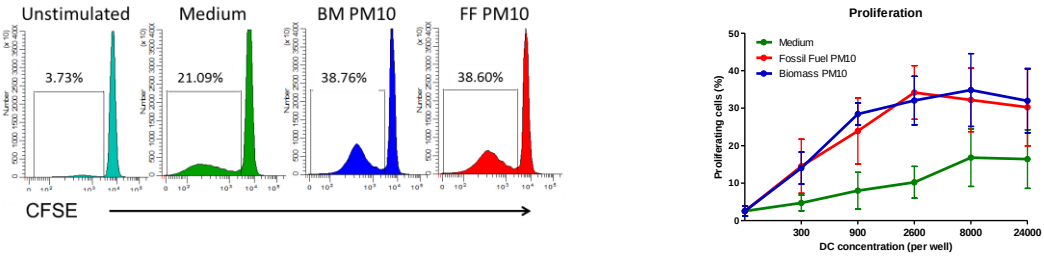

Figure 5.6: FF PM 10- and BM PM10-stimulated DCs induce a similar level of proliferation of allogeneic CD4 T cells

MoDCs were stimulated Leicester FF PM10 and BM PM10 for 48h at a concentration of 25µg/ml. After incubation, 0, 300, 900, 2600, 8000 and 2400 DCs were co-cultured with 400,000 naïve CD4 T cells for another 5 day, which were magnetically separated from PBMCs from a different blood donors and were labelled with CFSE prior co-culture. ***A****: Flow-cytometry histograms illustrating one representative example of the percentage of proliferating allogeneic naïve CD4 T cells, which were stimulated with 24,000 activated DCs for 5 days or cultured without DCs. DCs were previously exposed to FF PM10 from Leicester, BM PM10 or medium only.* ***B:*** *The diagram shows the mean and standard deviation of naïve CD4 T cell proliferation of 3 independent experiments conducted using three different blood donors and 2 technical repeats. T cells were activated with DCs, which were previously exposed to FF PM10, BM PM10 or medium only. Statistical analysis was performed using a two-way-analysis of variance and Bonferroni post-tests.*

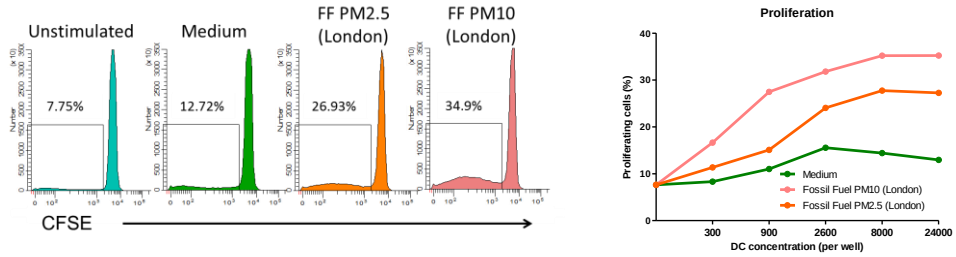

Figure 5.7: DCs stimulated with FF PM10 induce a higher proliferation of naïve CD4 T cells compared to DCs exposed to FF M2.5 sourced from exactly the same location

Human MoDCs were exposed to 25µg/ml of London FF PM10 and London FF PM2.5 for 2 days. After incubation, 0, 300, 900, 2600, 8000 and 2400 DCs were co-cultured with 400,000 magnetically separated and CFSE labelled allogeneic naïve CD4 T cells for another 5 days. ***A:*** *Flow-cytometry histograms showing the percentage of proliferated allogeneic CD4 T cells unstimulated or stimulated with London FF PM10 and PM2.5 exposed DCs or DCs that were cultured in medium only.* ***B:*** *Percentage of proliferating naïve CD4 T cells after stimulation with London FF PM10 and FF PM2.5 exposed DCs. The graph shows one experiment.*

Consistent with the findings of the previous chapter, FF PM10-activated DCs were more stimulatory for naïve CD4 T cells than DCs that were cultured in medium only (Figure 5.5 A and B). This was also consistent with their more activated phenotype. In line with their maturation level, BM PM2.5-exposed DCs were less stimulatory for allogeneic naïve CD4 T cells compared with FF PM10-exposed DCs, but induced higher T cells proliferation compared to medium only cultured DCs. A two-way analysis of variance and the Bonferroni post-tests indicated no significant difference in proliferation of T cells exposed to BM PM2.5-stimulated DCs compared with T cells that were co-cultured with unstimulated DCs. In this particular set of experiments, the difference between the T cell proliferation of FF PM10-stimulated DCs and unstimulated DCs was significant ($p < 0.05$) at a concentration of 900 DCs.

BM PM10 stimulated DCs and FF PM10 activated DCs induced a comparable level of proliferation, which is consistent with their matured phenotype (Figure 5.7 A and B). A two-way analysis of variance showed a significant difference between the proliferation of DC-exposed T cells when DCs were incubated with BM PM10 compared to medium only ($p < 0.01$ for DC concentrations of 900 and 2600 and $p < 0.05$ for 800 DCs). FF PM10-exposed DCs led to a

significant increase of T cell proliferation ($p < 0.01$) compared to T cells that were co-cultured with unstimulated DCs.

When comparing London FF PM10 and FF PM2.5 incubated DCs for their effect on naïve CD4 T cells, a difference in proliferation levels was observed. This indicates that FF PM10 activated DCs exhibit the highest stimulatory activity; T cells that were co-cultured with FF PM2.5 exposed DCs show a level in between T cells that were exposed to DCs incubated in medium only and FF PM10 (Figure 5.7 A and B). However, no statistical analysis was performed, as only one experiment using the London FF PM10 and FF PM2.5 was conducted.

Overall, the data tentatively confirms the relationship between PM-induced DC maturation and the capacity to stimulate CD4 T cells.

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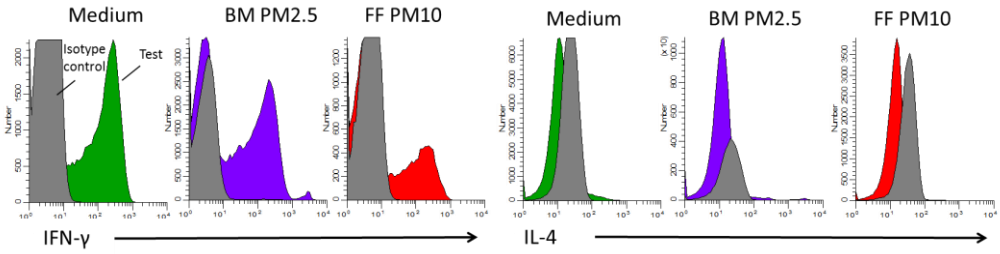

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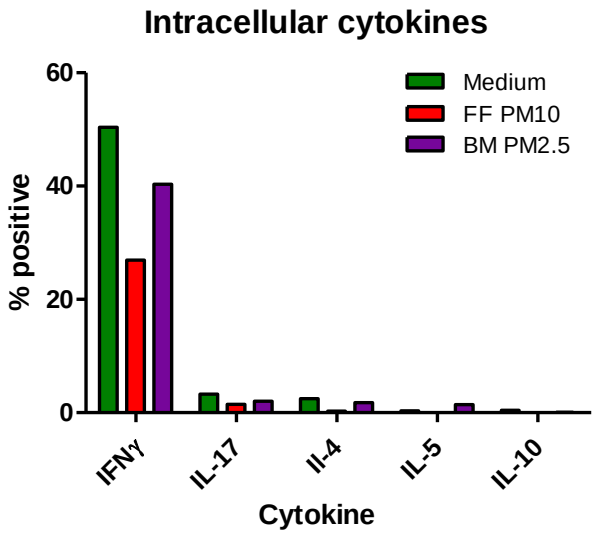

Figure 5.8: Unaltered cytokine profile of allogeneic CD4 T cells after stimulation with FF PM10- and BM PM2.5-exposed DCs

MoDCs were stimulated with 25µg/ml of Leicester FF PM10 and BM PM2.5 from Basse/The Gambia for 2 days. Subsequently, 0, 300, 900, 2600, 8000 and 2400 of stimulated and unstimulated DCs were co-cultured with 400,000 CFSE labelled allogeneic naïve CD4 T cells for another 5 days. For intracellular cytokine measurement, cells were stimulated and fixed after incubation, permeabilized and labelled with antibodies for IFN-γ, IL-17, IL-4, IL-5 and IL-10. ***A:*** *Representative example of flow-cytometry histograms showing IFN-γ and IL-4 intracellular expression in naïve CD4 T cells after co-culture with BM PM2.5-, FF PM10- or medium only stimulated DCs. The grey peak shows the matching isotype control.* ***B:*** *The diagram shows the cytokine profile of proliferating naïve CD4 t cells after co-culture with DCs that were previously stimulated with FF PM10 from Leicester and BM PM2.5. The chart shows one experiment.*

As reported in Chapter 3, the main cytokine produced in our system by proliferating naïve CD4 T cells after stimulation with MoDCs was IFN-γ (Figure 5.8). Pre-treatment with either FF PM10 of BM PM2.5 did not alter the predominance of IFN-γ. In the light of this result, the effects of the remaining PM were not examined.

All in all, the proliferation of allogeneic naïve CD4 T cells in an *in vitro* MLR stimulated with PM-exposed DCs matched their activation status. T cells stimulated with DCs exposed to PM10 derived from biomass or fossil fuel induced a similar level of proliferation, whereas T cells activated by PM2.5 stimulated DCs induced a lower level of proliferation, but still higher than the proliferation of T cells which were stimulated with medium only cultured DCs. In our system, we found no evidence for an effect of either FF or BM derived PM on the differentiation of CD4 T cells to different effector fates, as assed by cytokine production.

5.4.4. PM10 stimulates higher levels of cytokine secretion by DC than PM2.5

DCs influence immune responses by producing a variety of pro-inflammatory and regulatory cytokines. To assess the ability of different PM to induce cytokine production by DC, supernatants were collected from DCs cultured with PM or medium alone and subjected to a Multiplex ELISA.

For this, $CD14^+$ monocytes were cultured for 7 days with GM-CSF and IL-4 to differentiate DCs. These were then exposed to Leicester FF PM10, BM PM2.5, BM PM10 and London FF PM10 and FF PM2.5 for 2 days. After this, supernatants were collected and frozen for storage. Samples were measured for IL-6, IL-12p70, IL-1β, IL-10 and IL-23 using a Multiplex ELISA kit.

As a first step, FF PM10 and BM PM2.5 were compared in their ability to induce cytokine secretion in MoDCs, as these were the particles that were available to us at the beginning of this project.

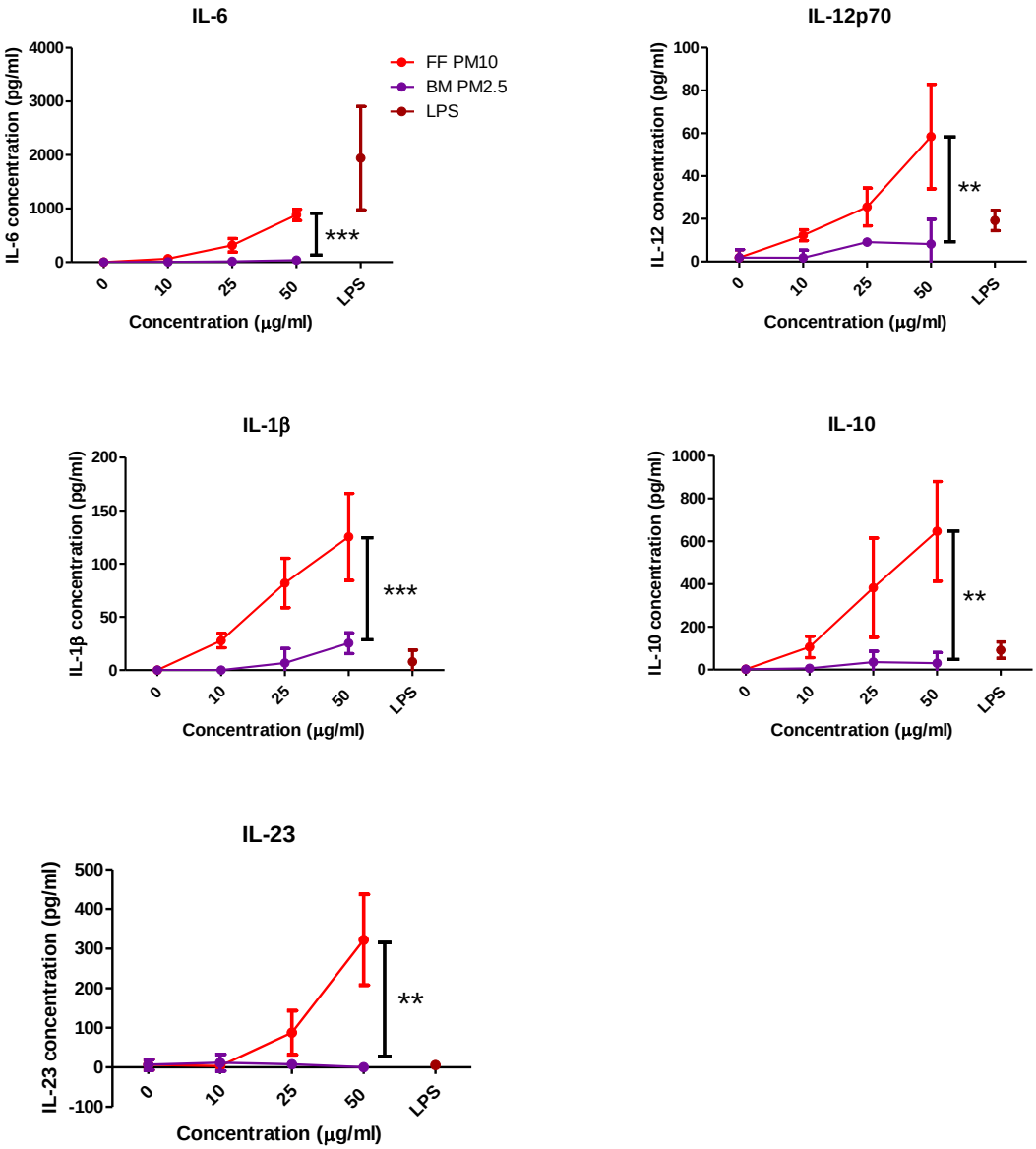

Figure 5.9: FF PM10 activated DCs induce higher levels of cytokine secretion than BM PM2.5 stimulated DCs

MoDCs were exposed to medium only or to 10, 25 and 50µg/ml of Leicester FF PM10, BM PM2.5 and 0.01µg.ml LPS for 48h. Subsequently, supernatants were aspirated carefully and stored at -80˚C for further analysis. A Multiplex ELISA (eBioscience) was performed after a sufficient number of samples were accumulated. Briefly, each sample was mixed with specific beads for the cytokines to be analysed and, after incubation, detector antibodies were added. Samples were then analysed using a flow-cytometer. The plots show the summary data illustrating the concentration of IL-6, IL-12, IL-1β, IL-10 and IL-23 in supernatants from cultures of PM-exposed DCs. The mean and standard deviation of 4 independent experiments were plotted and statistical analysis was performed using a two-way analysis of variance.

The secretion of IL-6, IL-12p70, IL-1β, IL-10 and IL-23 revealed a statistically significant difference between the cytokine concentrations released by DCs after stimulation with FF PM10 and BM PM2.5 (Figure 5.9). FF PM10-exposed DCs produced significantly higher concentrations of IL-6, IL-12p70, IL-10 and IL-23 as well as the inflammsome-dependent cytokine IL-1β, in a dose-dependent manner, whereas BM PM2.5 exposed DCs either did not produce the cytokines at all or at very low levels, not significantly different from the medium control. As previously seen, IL-6 and IL-12p70 were also secreted by DCs after stimulation with LPS to levels comparable to those induced by PM10, whereas production of IL-1β, IL-10 and IL-23 were not detected in response to LPS.

The previous section compared FF PM10 and BM PM2.5, i.e., comparing particles in terms of their size as well as their composition. As a next step, particles differing only in one aspect, their composition were assessed. For this MoDCs were stimulated with various concentrations of FF PM10 and BM PM10 and the cytokine secretion was measured.

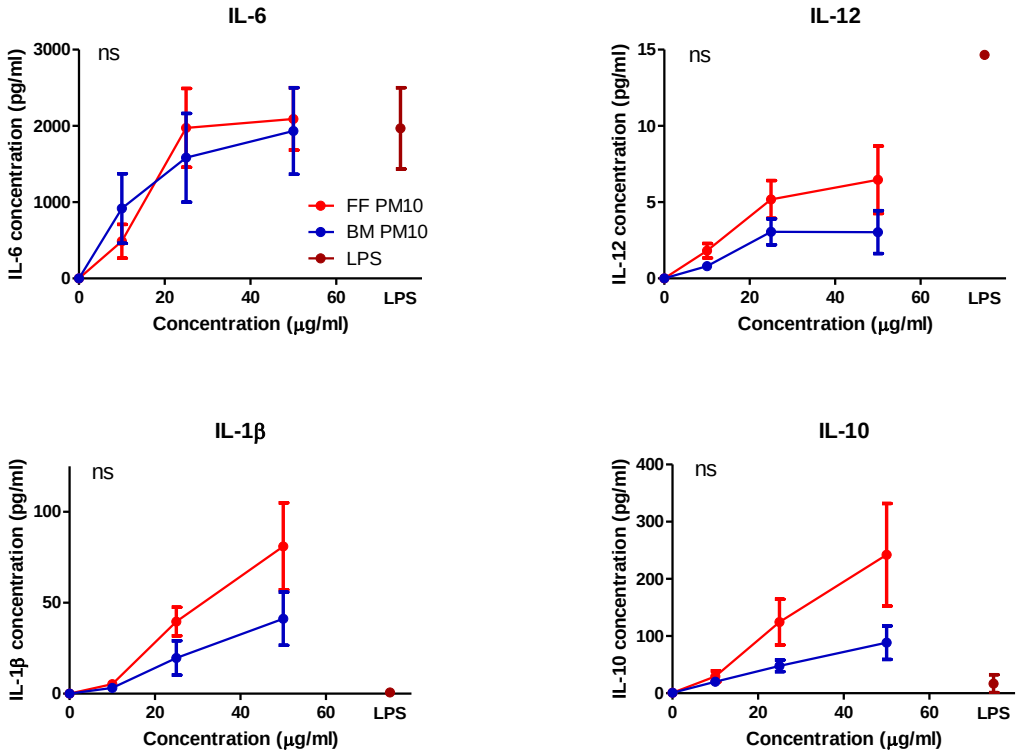

Figure 5.10: PM10 regardless of its combustion source induces a similar level of cytokine secretion in DCs

Human monocytes were cultured with GM-CSF and IL-4 for 7 days and subsequently exposed to 0, 10, 25 and 50µg/ml of Leicester FF PM10, BM PM10 and 0.01µg/ml of LPS for 2 days. Subsequently supernatants were removed and a Cytomteric Bead Array (BD Bioscience) was performed. For this, each supernatant was mixed with the cytokine specific beads and after incubation antibodies for detection were added. After this, samples were analysed using a flow-cytometer. The diagrams show the summary data illustrating the concentration of IL-6, IL-12, IL-1β and IL-10 in supernatants. The mean and standard deviation of 3 independent experiments was plotted and statistical analysis was performed using a two-way analysis of variance.

When particles with matching sizes were compared, FF PM10 and BM PM10, no significant difference between particle types was detected in terms of their ability to induce secretion of IL-6, IL-12, IL-1β and IL-10 by MoDCs (Figure 5.10). However, a trend towards higher expression was detected for IL-12, IL-1β and IL-10 consistently in 3 independent experiments after exposure of DCs to FF PM10. Also, although overall not significant, the Bonferroni post-test showed that

for IL-10 at a concentration of 50μg/ml FF PM10 induced a significant higher ($p < 0.05$) secretion of the cytokine compared with BM PM10.

Finally, we were able to source FF PM10 and FF PM2.5 from the exact same location in London; crucially, these PMs have the same composition and only differ in terms of their size.

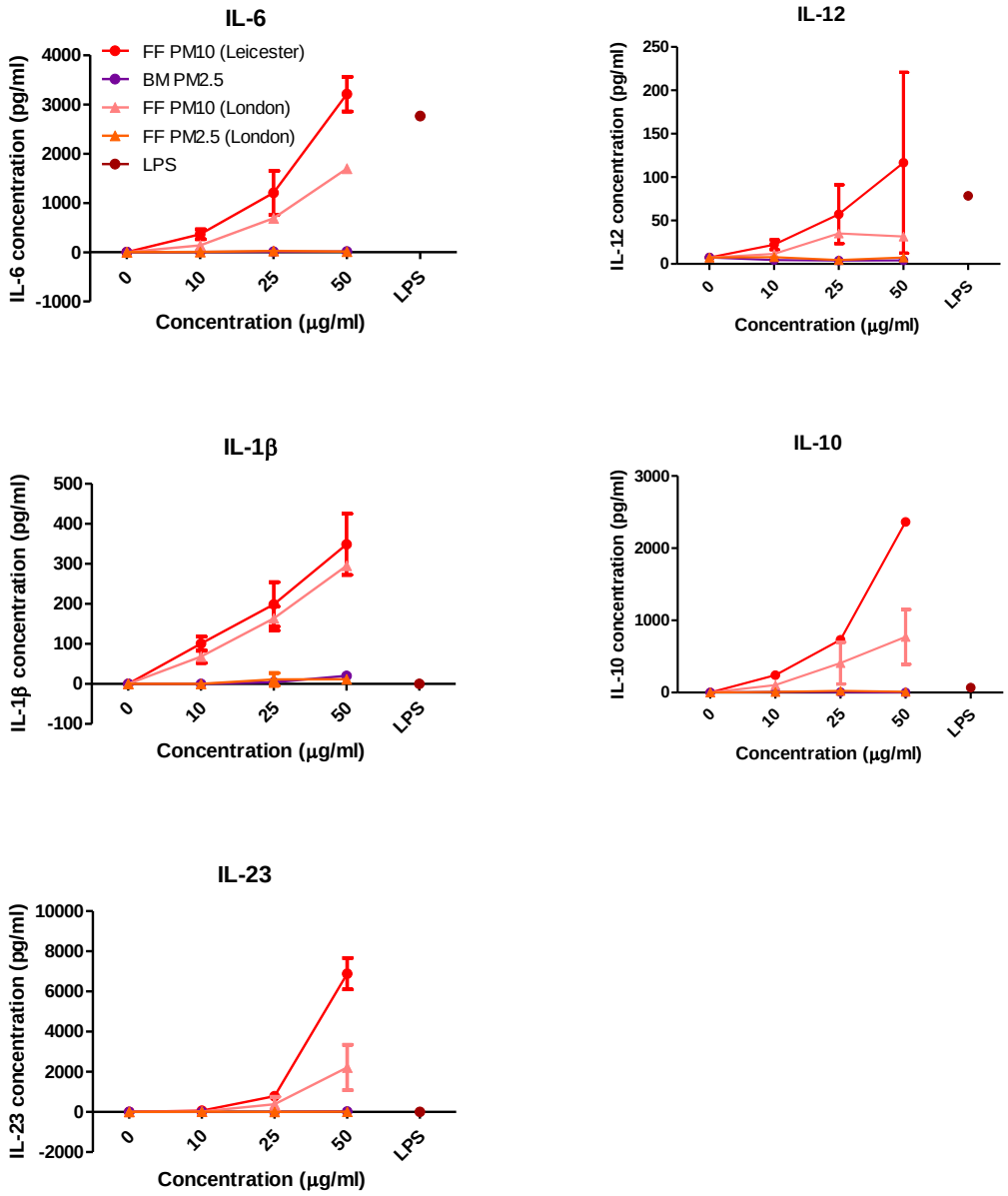

Figure 5.11: Exposure to PM10 induces a higher level of cytokine secretion in DCs than DC stimulation with PM2.5

DCs derived from human monocytes were stimulated with 10, 25 and 50µg/ml of Leicester FF PM10, BM PM2.5, London FF PM10 and FF PM2.5 as well as 0.01µg/ml of LPS for 48h. After collecting the supernatants, a Multiplex ELISA (eBioscience) was performed. For this each sample was incubated with specific beads for the cytokines to be analysed and detector antibodies. After incubation, samples were measured using a flow-cytometer. The plots illustrate the mean and standard deviation of two independent experiments showing the amount of IL-6, IL-12, IL-1β, IL-10 and IL-23 secreted by DCs after incubation with the different PM-types.

Comparing matched location and combustion sources revealed that, in general, FF PM10 induced secretion of the measured cytokines, whereas FF PM2.5 led to either no secretion at all or very low concentrations (Figure 5.11). Leicester FF PM10, BM PM2.5 and London FF PM10 and FF PM2.5 were used to stimulate DCs showing that both FF PM10 from Leicester and FF PM10 from London have an almost identical effect, secreting high concentrations of IL-6, IL-12, IL-1β, IL-10 and IL-23. Again, PM2.5, regardless of its origin, induced very low levels or no secretion at all of the cytokines measured. This set of experiments was conducted only twice, as certain air pollution particles were not available until late in the project; hence, there was insufficient time for further replicates.

In conclusion, as with activation measured by expression of maturation markers, particle size was a major determinant of cytokine production in response to PM: PM10, but not PM2.5, induced cytokine production, again suggesting the potential effects of particles of this size on immune activity in the airway. There was some evidence of a modifying effect of combustion source on the induction of cytokines by PM as FF PM10 induced a slightly higher secretion of some cytokines in 3 independent experiments, although this difference did not reach statistical significance overall in this small number of experiments.

5.4.5. AhR signalling in PM-stimulated DCs is influenced by size and combustion source of PM

In the preceding chapter, FF PM10 was shown to activate AhR in DCs as assessed by expression of *CYP1A1* and *CYP1B1*. If and how the expressions of these genes vary when cells are exposed to different types of particles is unknown.

To determine how the expression of AhR related genes is affected by PM from different locations and different sizes, MoDCs were stimulated with Leicester FF PM10, BM PM2.5, BM PM10 and London FF PM10 and FF PM 2.5 for 2 days, after which cells were lysed and gene expression of *CYP1A1* and *CYP1B1* was measured using qRT PCR.

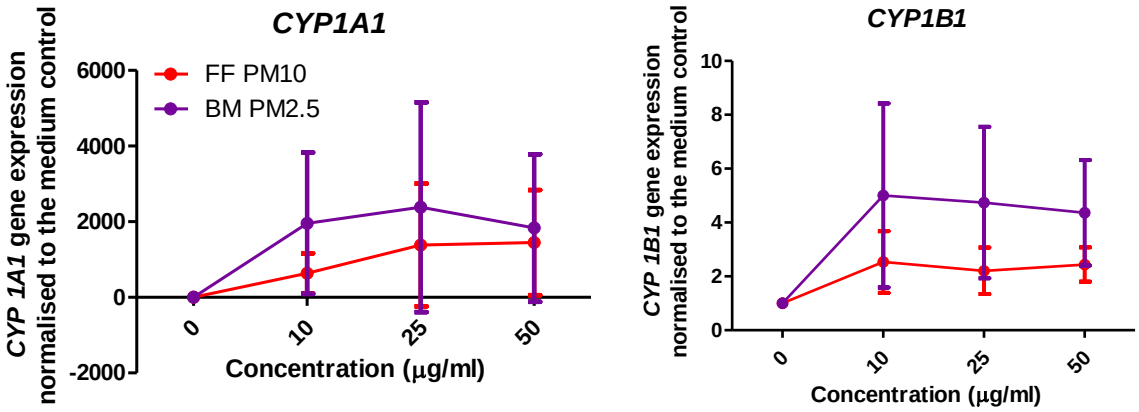

Figure 5.12: CYP1A1 and CYP1B1 are higher expressed in DCs after stimulation with BM PM2.5 over a dose-range compared to FF PM10 exposure

DCs derived from human blood monocytes were stimulated with Leicester FF PM10 and BM PM2.5 at 10, 25 and 50µg/ml and medium only for 2 days. After incubation, cells were lysed, RNA was extracted, reverse transcription was performed and finally a RT-PCR was conducted using SYBR Green reagents to measure the expression of the AhR-related genes CYP1A1 and CYP1B1. Data was analysed using the comparative C_T method and plotted relative to the medium control. The graphs show the mean and standard deviation of 6 repeats for CYP1A1 and 4 repeats for CYP1B1 in total.

Both FF PM10 and BM PM2.5 induced AhR signalling as indicated by an upregulation of *CYP1A1* and CYP1B1 (Figure 5.12). In 4 out of 6 experiments, BM PM2.5 induced a higher expression of *CYP1A1* at concentrations of 10 and 25µg/ml compared to the gene expression induced in DCs after stimulation with FF PM10. *CYP1B1* was induced in a dose-dependent manner by FF PM10 and BM PM2.5. In 3 out of 4 experiments, BM PM2.5 induced higher expression of *CYP1B1* compared with to the induction by FF PM10.

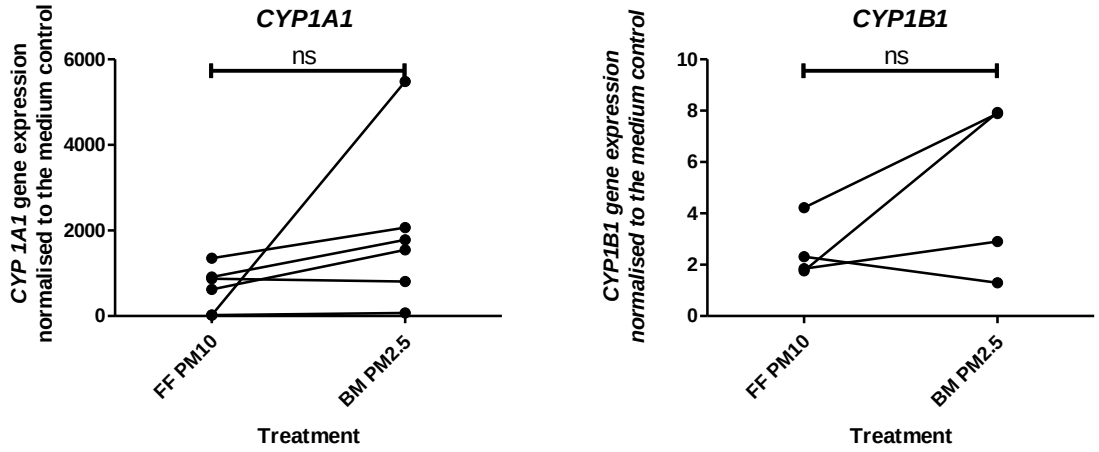

Figure 5.13: Difference between in the expression of AhR-dependent genes after FF PM10 and BM PM2.5 exposure is non-significant

Human MoDCs were stimulated with 10µg/ml of Leicester FF PM10 and BM PM2.5 for 48h. After incubation, quantitative real-time RT-PCR was performed using SYBR Green reagents and the expression of the AhR-related genes CYP1A1 and CYP1B1 was measured. The results were illustrated using the comparative C_T method and plotted relative to the medium control. The graphs show the summary data of the expression of CYP1A1 and CYP1B1 in DCs, where each pair of dots represents one independent experiment. Statistical analysis was performed using a paired T test, the Mann-Whitney test specifically.

The summary data, comparing the effects of FF PM10 and BM PM2.5 at a concentration of 10µg/ml on the expression of *CYP1A1* and *CYP1B1* in DCs showed a non-significant difference between both particle types (Figure 5.13). However, only 4 repeats were compared at a single concentration for *CYP1B1;* all except one experiment showed a higher expression of *CYP1B1* after DC stimulation with BM PM2.5 compared to FF PM10. For *CYP1A1,* 6 repeats were performed and in 5 experiments BM PM2.5 stimulation induced a higher expression of *CYP1A1*.

Again, as a next step, particles only differing in one aspect, their combustion source, were compared in their ability to induce AhR signalling in MoDCs.

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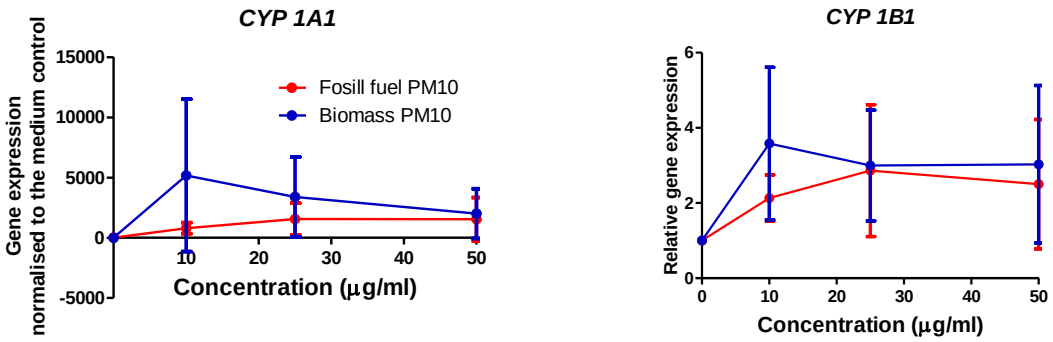

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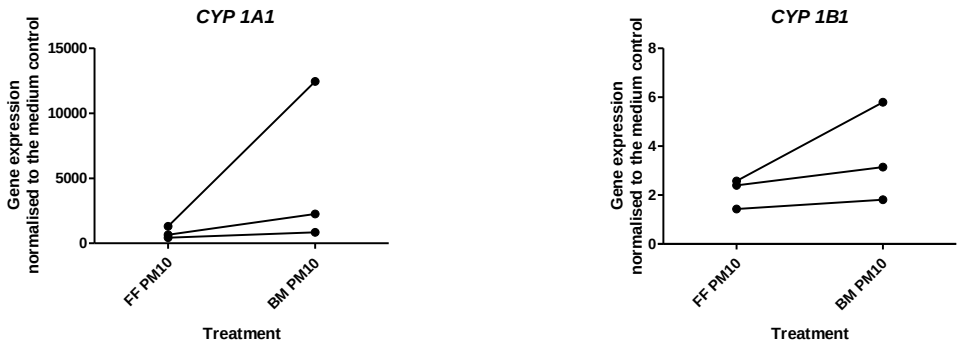


Figure 5.14: AhR-dependent genes are higher expressed in DCs after exposure to BM PM10 compared to FF PM10

Human blood monocytes were separated from PBMCs and differentiated into DCs by incubation with GM-CSF and IL-4 for 7 days. DCs were stimulated with Leicester FF PM10 and BM PM10 from Accra/Ghana at concentrations of 10, 25 and 50µg/ml or medium only for 2 days. Subsequently, cells were lysed, RNA was extracted, reverse transcription was performed and finally a RT-PCR was conducted to measure CYP1A1 and CYP1B1. Data was analysed using the comparative C_T method and illustrated relative to the medium control. ***A****: Graphs showing the expression of CYP1A1 and CYP1B1 after DC stimulation with FF PM10 and BM PM2.5 at a concentration ranging from 0 to 50µg/ml for 2 days. The graphs show the mean and standard deviation of three independent experiments.* ***B****: Diagrams illustrating the summary data of the expression of CYP1A1 and CYP1B1 in DCs after activation with 10µg/ml of FF PM10 and BM PM10. Each pair of dots represent one independent experiment with a separate blood donor.*

The comparison of FF PM10 and BM PM10 showed a clear difference between the two particle types in terms of their ability to induce the AhR pathway. In 3 independent experiments, both *CYP1A1* and *CYP1B1* were expressed at higher levels in DCs after BM PM10 than after FF PM10 exposure (Figure 5.14). Overall, although consistently observed, the difference between BM and FF PM10 did not reach statistical significance, probably due to the small number of experiments possible.

Finally, London FF PM10 and FF PM2.5 were assessed in relation to their ability to signal through AhR in MoDCs. These particles differed only in terms of their size, not their composition.

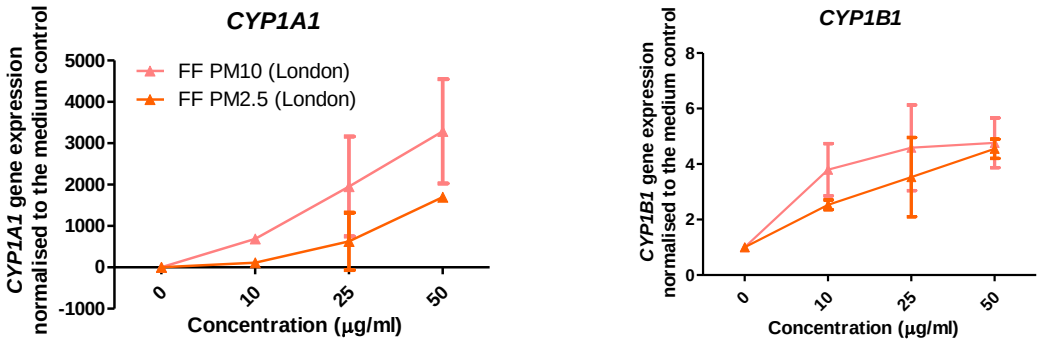


Figure 5.15: FF PM10 induces a higher level of CYP1A1 and CYP1B1 expression in DCs than stimulation with FF PM2.5

DCs derived from human blood monocytes were stimulated with 0, 10, 25 and 50µg/ml of London FF PM10 and FF PM2.5 for 2 days. After incubation, cells were measured for the expression of AhR-related genes CYP1A1 and CYP1B1 by qRT-PCR. Data was analysed using the comparative C_T method and plotted relative to the medium control. The plots show the mean and standard deviation of three independent experiments.

When comparing the effect of FF PM10 and FF PM2.5 from the same location in London, *CYP1A1* shows a distinct expression for each particle type, whereby FF PM10 induces higher RNA levels after incubation in DCs compared to FF PM2.5 (Figure 5.15). This was consistently observed in 3 independent experiments. RNA levels of *CYP1B1* were much lower than the levels of *CYP1A1* overall, but the pattern of induction was similar to that of *CYP1A1* after exposure to London FF PM10 and FF PM2.5.

The pattern of AhR activation in MoDCs by different types of BM follows a pattern distinct from that observed when assessing maturation, T cell proliferation and cytokine production. Specifically, BM PM2.5 was similar to FF PM10 in its ability to induce *CYP1A1* and *CYP1B1,* despite being a poor activator of maturation and cytokine production, and overall BM derived PM was superior to FF derived PM at inducing AhR dependent genes: BM PM10 induced a consistently higher level of *CYP1A1* and *CYP1B1* expression compared with FF PM10. In conclusion, this section shows – for the first time – that different combustion sources can make a strong differences with regard to the activation of the AhR pathway; in particular, BM PM2.5 induced the AhR pathway in the absence of classical activation or cytokine production.

5.4.6. FF PM10 induces a higher level of *IDO1* expression in DCs compared with BM PM10, FF PM2.5 and BM PM2.5 induce the lowest expression

The previous chapters have shown that *IDO1* is clearly induced in MoDCs as well as respiratory tract DCs and macrophages when exposed to PM. Also, in contrast to what has been suggested in the literature, no evidence has been found in the previous chapters for *IDO1* being controlled by the AhR pathway. As indoleamine 2,3-dioxygenase is an enzyme, which is involved in tryptophan metabolism and therefore plays an important role in immunological processes, we investigated the effect of different particle types on the expression of *IDO1*, the gene that encodes indoleamine 2,3-dioxygenase. For this, we stimulated MoDCs for 48 hours in medium only or with various concentrations of FF PM10 from Leicester, BM PM10 and BM PM2.5 as well as FF PM10 and FF PM2.5 from London. Subsequently, we measured *IDO1* expression by PCR.

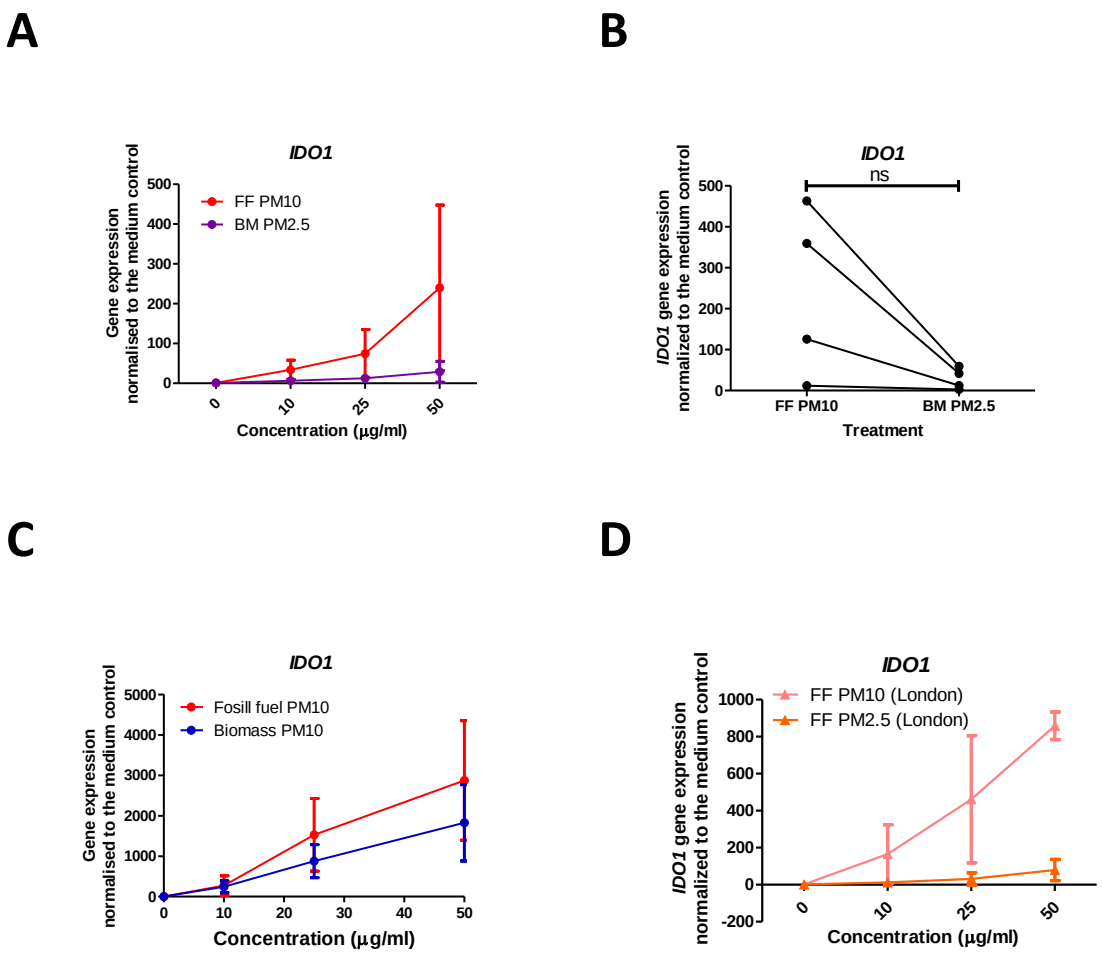


Figure 5.16: IDO1 expression in DCs depends on particle size and combustion source of PM

MoDCs were differentiated from CD14$^+$ cells separated from human PBMCs. DCs were stimulated with various concentrations of Leicester FF PM10, BM PM10, BM PM2.5, and London FF PM10 and FF PM2.5 for 48h. Subsequently, cells were lysed, RNA was extracted, reverse transcription was performed and finally a RT-PCR was conducted using SYBR Green reagents to assess the expression of IDO1. Data analysis was performed using the comparative C_T method and plotted relative to the medium control. ***A****: The graph shows the mean and standard deviation of 4 independent experiments illustrating the dose-dependent induction of IDO1 in DCs after exposure to Leicester FF PM10 and BM PM2.5 at 10, 25 and 50µg/ml and medium only.* ***B****: Summary data showing the expression of IDO1 in DCs after stimulation with 50µg/ml of Leicester FF PM10 and BM PM2.5. Each pair of dots represents an independent experiment.* ***C****: Figure showing the expression of IDO1 in DCs as mean and standard deviation of three independent experiments after stimulation with Leicester FF PM10 and BM PM10 from Accra/Ghana. Concentrations range from 0 to 50µg/ml.* ***D****: Dose dependent expression of IDO1 in DCs after stimulation with 0, 10, 25 and 50µg/ml of London FF PM10 and FF PM2.5. The graph shows the mean and standard deviation of three experiments.*

When considering the pattern of response observed with the various PMs studied, induction of *IDO1* was similar to DC activation and cytokine production and distinct from AhR activation. In most experiments, *IDO1* was induced following exposure to 50µg/ml of FF PM10 with little or no induction by BM PM2.5 (Figure 5.16 B). The same trend was observed when several concentrations were tested: over a concentration range of 10-50µg/ml, FF PM10 induces a higher gene expression than BM PM2.5 (Figure 5.16 A). Therefore, it is highly unlikely that a shift in the dose response curve can explain the difference between these two types of PMs. When FF PM10 and BM PM10 were compared, *IDO1* was induced by both with no significant difference between the two types of PMs (Figure 5.16 C). Finally, the comparison of FF PM10 and FF PM2.5 from London showed that larger particles induce a higher level of *IDO1* expression in DCs (Figure 5.16 D), suggesting again, that *IDO1* induction follows a different pathway unrelated to AhR signalling.

In summary, *IDO1* expression, like classical dendritic cell activation and cytokine production, was mainly influenced by particle size rather than combustion source; combustion source had a significant impact on the strength of AhR signalling induced.

5.4.7. Air pollution during Harmattan and non-Harmattan season has distinct effects on cytokine production of MoDCs

We happened to have had access to ambient PM10 and PM2.5 from Accra/Ghana that was collected during the dry as well as the rainy season. The dry and sandy season in Accra/Ghana between December and February, called Harmattan, is characterized by a dry wind blowing from the northeast of the Sahara carrying large amounts of Saharan dust. Therefore, the composition of the air pollution particles differs from the rest of the year as it contains more crustal particles. Outside the Harmattan season, ambient air pollution particles contained more sea salt, car emissions, tire and break particles as well as road dust (Z. Zhou, Dionisio, Verissimo, Kerr, Coull, Arku, et al., 2013b). Hence, we compared the effects of PM10 and PM2.5 collected during and outside the Harmattan with FF PM10 from Leicester on the cytokine secretion of MoDCs.

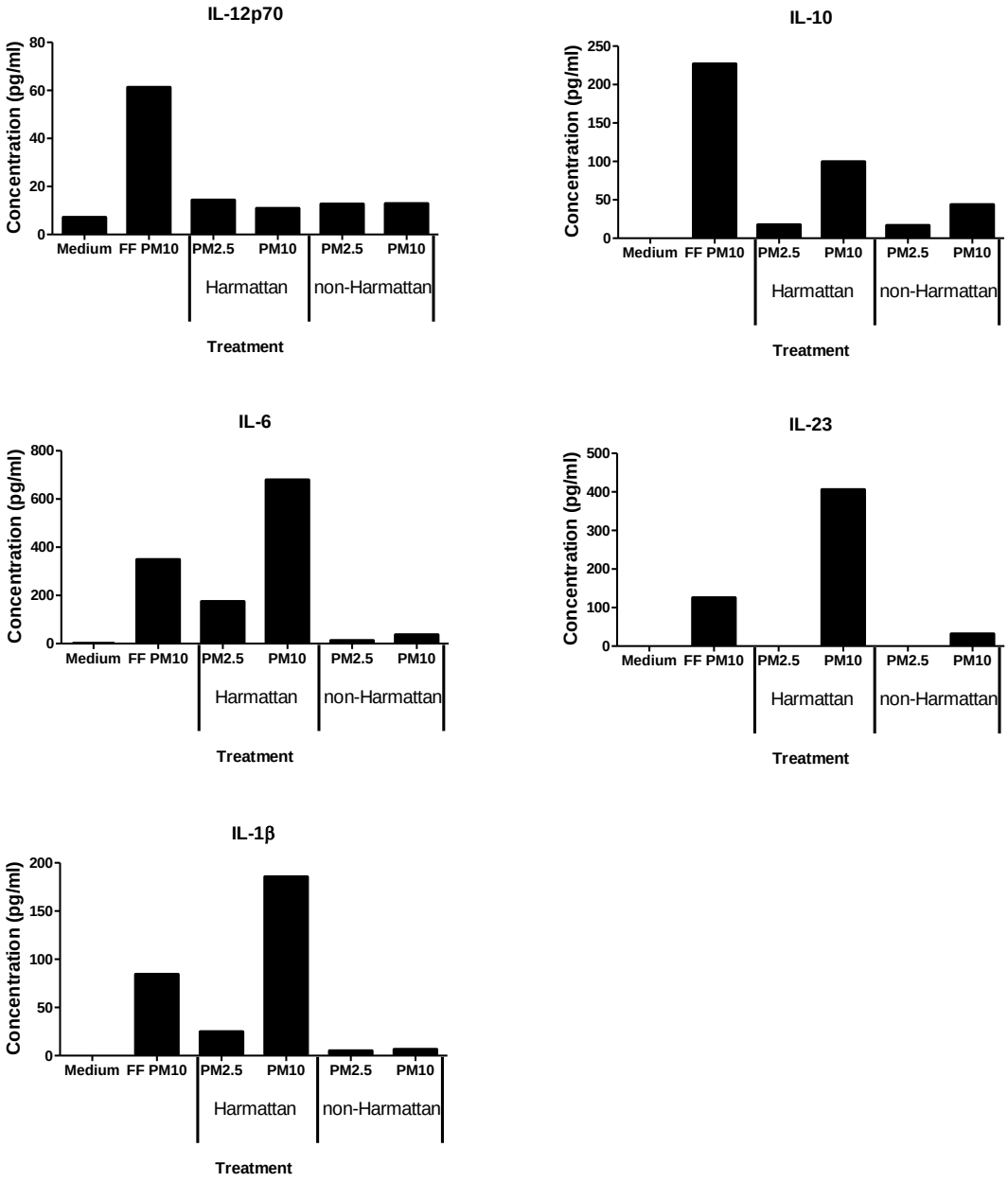


Figure 5.17: Air pollution during Harmattan and non-Harmattan season has distinct effects on cytokine production of MoDCs

DCs derived from human monocytes were cultured with 50µg/ml of Leicester FF PM10, PM10 and PM2.5 collected during Harmattan and non-Harmattan season in Accra/Ghana for 48h. After collecting the supernatants, a Cytomteric Bead Array (BD Bioscience) was performed. For this, each sample was incubated with specific beads for the cytokines to be analysed and the detector antibodies. After incubation, samples were analysed using a flow-cytometer. The diagrams illustrate one experiment showing the amount of IL-6, IL-12, IL-1β, IL-10 and IL-23 secreted by DCs after incubation with the different PM-types.

In general, particles collected during the Harmattan season induced a higher secretion of IL-6, IL-12, IL-1β, IL-10 and IL-23 for both PM10 and PM2.5 compared to non-Harmattan particles, except for IL-12p70, which showed an equal secretion by MoDCs after stimulation with both Harmattan PM10 and PM2.5 (Figure 5.17). Again, similar to the observations above, PM2.5, regardless of its composition, induced a lower expression of the measured cytokines compared to its PM10 counterpart. Harmattan PM10 induced a higher secretion of IL-6, IL-23 and IL-1β in MoDCs after stimulation compared to FF PM10.

Overall, Harmattan air pollution particles induced a higher secretion of pro-inflammatory cytokines compared to non-Harmattan particles. As seen before, Harmattan and non-Harmattan PM10 also had a higher effect on cytokine secretion compared to PM2.5.

5.5. Discussion

This chapter has shown how different types of particles varying in their origin and size influence different pathways of activation in DCs. Although at first glance the uptake of the particles did not differ between particle types since they all seem to interact in the same way after incubation when looking at them through the microscope, the extent to which pathways were activated was different. An important aspect that needs to be considered when conducting experiments with PM which were collected on filters and then extracted into suspension for *in vitro* studies is that the particle size that was collected may not be exactly the same particle size that is added to the cell culture. During the collection of particles, they stick firmly to the filter and to each other and thereby may form bigger particles. Once they are extracted through vortexing and sonication, these bigger particles may not be broken up completely. Hence, instead of adding a smaller particle, which was believed to be collected on the filter, a larger particle is added formed by several smaller particles firmly stuck together. Therefore, when comparing smaller and larger particle sizes originally, during the experiment the sizes may not represent the actual size that was collected, but rather a similar size of particles. Hence, where differences between PMs of different notional size are noted, these are likely to reflect properties of the particles as collected rather than size-dependent effects in the interaction with DC.

After uptake, the first pathway that was analysed was the expression of maturation markers. Particles were measured over a range of concentrations from 0 to 50µg/ml. This was done for several reasons. Firstly, it was difficult to assess the concentration of particles accurately, as this was done by UV spectrophotometry measuring the absorption of the particle suspension and then calculating the concentration based on a calibration line which was generated using carbon black particles. This method has been used by other groups (Brown et al., 2007; Mushtaq et al.,

2011). For us, doing these measurements accurately was difficult because the particles in suspension sedimented quickly, making it challenging to measure evenly distributed particles in a suspension. Also, carbon black particles were highly charged and of much larger size, which made the measurement even harder; due to their size, they sediment quicker while their charge leads them to stick to the walls of the cuvette or plate. Testing multiple concentrations helped to mitigate systematic inaccuracies in particle quantitation. Therefore, it is highly unlikely that any shift in the dose response curve can explain the difference between two types of PMs, but rather show the true different effects of the PM on DCs. Secondly, particles from different parts of the world with varying exposures to air pollution were measured, i.e. a comparison of indoor and outdoor air pollution was conducted in this work. Exposure to indoor and outdoor air pollution varies and hence the concentration of the particles inhaled varies too. For this reason, a range of concentrations was tested, making sure that inaccuracies due to the difficult quantification as well as the variations in exposure were covered in our model system.

The effect of fossil fuel derived PM10, as seen in the first results chapter, remains unchanged; we still observed expression of the activation markers HLA-DR, CD83, CD80, CCR7, CD86 and CD40 in a dose-dependent manner for both locations, Leicester and London FF PM10. The exception was CCR7, which was not expressed in a dose-dependent manner as was seen in the original series of experiments (Chapter 3) where CCR7 induction was observed in some but not all experiments. Both FF PM10 and BM PM10 induced a similar dose-dependent expression of maturation markers, suggesting that activation of DCs is a size-dependent effect rather than dependent on the combustion source. FF PM2.5 from London generally showed the same impact on MoDCs as on BM PM2.5, either inducing very low expression of maturation markers or none at all, which could be due to the amount of endotoxins present on the surface of the particles. As the activation of DCs is a process that is partly TLR4 controlled and can be activated with LPS, as seen in Chapter 3, endotoxins present on the surface of the particles may influence this pathway. It has been shown that smaller particles have less endotoxins on their surface compared to larger particles (Monn & Becker, 1999; B. Wang et al., 2013). Wang et al. has shown that the lowest amount of endotoxins can be found on particles with a size of 0.4-1.1µm in diameter. Taken together, these findings indicate that in our model system measuring the effects of maturation markers on DCs, PM10 seems to have a significantly greater impact than PM2.5, although PM2.5 is known to induce detrimental health effects, especially systemic outcomes, which is mainly due to its size as it is able to reach the whole body (Cohen et al., 2006). However, as for immunological effects specifically on DCs PM2.5, regardless of its combustion source, may have less impact. Therefore, when considering the immunological

effects of air pollution that may result from modulation of DCs, PM10 should be considered, rather than PM2.5. It could be speculated that the size of the particle influences their composition and this in turn affects the outcome on MoDCs (Akhtar et al., 2014). Smaller particles have less endotoxin, as endotoxins originate from gram-negative bacteria, which usually are associated with lager particles (Monn & Becker, 1999).

However, interestingly, it has been reported that larger particles show augmented effects on cell in *in vitro* studies, due to their increased size and higher gravity compared to smaller particles, which leads to an increased sedimentation rate and hence more opportunities to interact with the cell surface (Hetland et al., 2004; Osornio-Vargas et al., 2003). The concept of the *In vitro* Sedimentation, Diffusion and Dosimetry model (ISDD), which has been applied in *in vitro* studies using nanoparticles, takes into account the size of the particle and calculates how much of the actual dose interacts with the cell layer (Hinderliter et al., 2010). Applying this concept to *in vitro* PM studies could help evaluate size dependent effects more accurately, but in our case the exact size of the particles that were added to the culture would need to be determined first, making sure the size differences are relevant.

In terms of the ability of PM-exposed DCs to activate allogeneic naïve CD4 T cells the result reflected the maturation status of the cells. DCs stimulated with PM10 induced a higher level of T cell proliferation whereas PM2.5-exposed DCs induced a lower proliferation of T cells compared to PM10-stimulated cells, but still higher compared with T cells that were stimulated with DCs cultured in medium only. This indicates that both PM10 types were able to induce an adaptive immune response at a similar level *in vitro* and shows that individuals exposed to both types of air pollution responded similarly. Therefore, people with pre-existing lung conditions, such as asthma or COPD, are more likely to respond to inhaled antigen, which in turn may lead to exacerbations (Alexis & Carlsten, 2014; Schikowski et al., 2014). As both FF and BM PM10 induced similar effects in terms of T cell activation after DC-exposure it is likely that health outcomes are similar too. However, it needs to be kept in mind that exposure to BM-derived air pollution usually takes place in confined spaces and people are exposed for longer periods of time to a higher concentration of pollutants. This contrasts with ambient FF-derived PM exposure which occurs outdoors, for a limited amount of time; hence, the concentration of PM is lower due to the surrounding air. Also, different groups of people are exposed to air pollution; indoor BM PM is affecting primarily woman and children, whereas outdoor FF PM is inhaled by a wide range of people (S. B. Gordon, Bruce, Grigg, Hibberd, Kurmi, Lam, Mortimer, Havens, et al., 2014; Frank J. Kelly & Fussell, 2015b). However, as this *in vitro* assay only used naive CD4 T cells,

it did not give a complete picture of the immune response that is induced and of other cells that are involved.

The cytokine production of DC-stimulated T cells was similar to what we observed in Chapter 3: regardless of the type of particle the DCs were stimulated with or even whether they were cultured in medium only, the main cytokine secreted was IFN-γ.

When looking at the cytokines produced by DCs when exposed to PM, a similar pattern was observed, where PM10 generally induced a higher secretion of the measured cytokines compared to PM2.5. However, FF PM10 and BM PM10 show a small difference, as FF PM10 induced a slightly higher secretion of cytokines compared to BM PM10. Interestingly, unlike the expression of maturation markers in the previous paragraph, the observations in this section cannot be solely explained by the potential presence of endotoxins on the particle surface. Some pro-inflammatory cytokines, such as IL-6 and IL-12p70, were also expressed at a higher level when cells were stimulated with LPS, consistent with a potential role for PM-associated endotoxins in the activation of cytokine production by DC. Other cytokines, such as IL-1β, IL-10 and IL-23 were not induced by LPS; hence, a different characteristic on the particle surface irrespective of the endotoxin contents was likely responsible for this effect. It has been shown in several studies that PM induces the expression of inflammatory cytokines in immune cells, such as epithelial cells, macrophages and monocytes, and that larger particles lead to a higher concentration of released cytokines compared to smaller particles, although very small particles (below 0.4µm in diameter) have been shown to induce the highest inflammatory effects (Akhtar et al., 2014; Monn & Becker, 1998; B. Wang et al., 2013). These effects can be attributed to the characteristics of the particles, such as endotoxin concentration, zeta potential, metal content and redox ability, as they vary with particle size (Wang et al., 2013). It has been shown that with increasing size of the particles, zeta potential (which describes the surface charge of the particles) increases; if the specific surface area decreases, metal content decreases (B. Wang et al., 2013). Hence all these characteristics may contribute to the inflammatory potential of the particles. This would indicate that the FF PM2.5 used in our study has a lower zeta potential, a higher specific surface area and a higher metal content compared to FF PM10 and therefore lead to a lower production of inflammatory cytokines compared to FF PM10. However, many of the size-related differences in the activity of particles may be due to their physical characteristics, rather than size *per se*. Several studies have been published on how different characteristics on PM affect biological outcomes and ultimately their specific effects. The main characteristics are endotoxin content, zeta potential and concentration of hydrocarbons and metals on the particle surface as well as surface area. For instance, a study performed in Beijing investigating the effect

of different ambient PM-size fractions on an epithelial and macrophage cell line showed that the inflammatory potential, as measured by the secretion of IL-8 and TNF-α, increases with increasing content of endotoxins and polycyclic hydrocarbons, as well as size (Y. Lu et al., 2014). Although it is unlikely that the particles added to the culture in our experiments were of the nominal size of those collected due to clumping during processing, any differences in composition related to the physicochemical properties in the original native particles are likely to be retained into the cell cultures. Although a large literature review has been published about the composition of biomass and fossil fuel derived particles, there was not a clear and consistent difference between both particle types related to their size, which most likely had to do with the fact that particles were collected from different regions and environmental factors contributed to the variance together with seasonal differences. The main difference between biomass and fossil fuel derived air pollution particles was that air pollution from urban areas had a higher metal content (Oliveira et al., 2011). To our knowledge, nothing has been reported on how their physicochemical properties and particle size compare to each other and how this then relates to the effects on DCs.

Although both FF and BM derived PM10 induced similar degrees of DC activation, other effects did vary in relation to the combustion sources from which the particles derived. First of all, FF PM10 induced a higher secretion of inflammatory cytokines compared to BM PM10. This may indicate that the third signal required for T cell activation varies for both particle types; this may affect the adaptive immune response. In contrast, BM-derived PM consistently induced higher levels of AhR signalling as measured by *CYP1A1* and *CYP1B1* induction, compared with FF derived PM. Both BM PM2.5 and BM PM10 activated AhR, indicating that the smaller particles are not inert and are able to interact with DC and stimulate some aspects of environmental sensing. However, when individuals are exposed to air pollution particles during everyday life, they inhale the particle mixture and not the individual size fractions. Therefore, when looking at the effects of the inhalation of air pollution particles, the overall PM mixture and its effects need to be taken into account; hence, the effects measured for both particles sizes are likely to take place, and a combination of effects may be happening. This would mean that in terms of outcomes, for example DC maturation, PM10 would activate the cells and lead to an immune response, whereas PM2.5 would not induce stimulation at the cells at the same time, resulting in a decreased maturation status of the cells overall. In contrast to that, comparison of FF PM10 and FF PM2.5 from London showed an increased induction of AhR-dependent genes by larger particles. Clearly, the effects of AhR seemed to be size-dependent for fossil fuel derived particles, whereas biomass derived particles induced a higher level of activation regardless of

their size. This indicates that size is less critical for BM PM as it is more activating overall. This superior ability of BM PM to induce the AhR signalling may be due to a higher content of AhR-activating aromatic hydrocarbons in BM-derived PM.

A large literature review, which looked at air pollution studies in Brazil, examined the effects of air pollution particles from different areas in the country and therefore deriving from different combustion sources; it showed that biomass derived particles are less often tested for the content of polycyclic hydrocarbons than fossil fuel derived particles and therefore relatively little is known about their hydrocarbon content (Oliveira et al., 2011). Nonetheless, it has been shown that biomass particles have a higher content of benzene, a type of aryl hydrocarbon, compared with fossil fuel particles (Khalequzzaman, Kamijima, Sakai, Hoque, & Nakajima, 2010).

Furthermore, a study in Tianjin, China investigated the contribution of different combustion sources to the different types of polycyclic hydrocarbons; it revealed that biomass combustion was the major source of low molecular weight hydrocarbons during the non-heating seasons, whereas fossil fuel combustion accounted for most of the aryl hydrocarbons measured during the heating season (Tao et al., 2006). It has also been shown that with increasing size, the amount of polycyclic aromatic hydrocarbons decreases in particles; this holds only for the hydrocarbons with a high molecular weight though since low molecular weight hydrocarbons show no significant difference (B. Wang et al., 2013).

It has been shown that the molecular weight of hydrocarbons plays a crucial role when assessing the ability of a compound to activate the AhR (Wincent et al., 2012). Specifically, high molecular weight hydrocarbons are known to be more potent activators of AhR (Larsson, Hagberg, Giesy, & Engwall, 2014). However, when considering the potency of polycyclic hydrocarbons as AhR-ligands, other chemical and physical characteristics should be taken into account such as polarity and lipophilicity (Wincent, Le Bihanic, & Dreij, 2016). All in all, it is not clear why exactly BM PM, regardless of its size, induce a higher level of AhR activation. It seems that BM PM is a better activator of AhR overall compared to FF PM and that size is less critical for BM PM.

Although size is an important factor for some of the observed effects, such as maturation of DCs, for the activation of AhR, size seemed to be insignificant. Although it has been shown that size can be an important determinant of composition, showing in particular a higher concentration of low molecular weight hydrocarbons, which are less potent in activating AhR, the presence of stronger AhR ligands must outweigh this. For example, the high concentration of benzene on BM PM may compensate the effect of low molecular weight hydrocarbons. However, there could

also be other components on the particles that activate AhR, which have not been taken into account.

It could be argued that BM PM2.5 is able to induce AhR activation without inducing classical maturation at the same time; it seems like in this case, the role of AhR is limited to a xenobiotic response, without the involvement of an adaptive immune response including the activation of T cells. Therefore, its only job would be to clean away and render harmless the incoming air pollution to prevent it from causing further damage in the respiratory tract, instead of inducing a proper immune response. It would also leave out both immunological aspects of the AhR: anti-inflammatory and pro-inflammatory. Nonetheless, we have measured a limited number of pathways and the information these pathways provide does not allow us to draw conclusions about all the processes happening within the cell. As PM is a complex mixture of components, it may have a wide range of effects on additional signalling pathways, the investigation of which was beyond the scope of this work.

As we have seen *IDO* induction, an overall pro-regulatory response of AhR is likely as it has been described in the literature that *IDO* is controlled by AhR. However, we did not find any evidence that *IDO* is AhR dependent. It needs to be kept in mind that we only used MoDCs as an *in-vitro* model; other cell types as well as other methods may play an important role, which will need to be explored in the future. In addition to looking into different pathways, it is important to understand the interplay of different pathways, as for example co-stimulation may affect other signalling outcomes within the cell as well.

When looking at the expression of *IDO1* when exposed to the different types of particles, a similar pattern was observed as seen previously with, for example, maturation. In general, PM10 induced a higher level compared to PM2.5, regardless of its origin. This is most likely due to its endotoxin content and indicates that *IDO1*, as seen in previous chapters, is not controlled by the AhR pathway. Interestingly, when taking into account other pathways, such as TLR4-dependent activation of DCs together with the pro-regulatory effect of *IDO,* it could be argued that *IDO* signalling attenuates the effects of DC activation on T cell stimulation. By blocking of *IDO* with a specific antagonist the effects of *IDO* on DC maturation and T cell activation could be examined.

Finally, the comparison between Harmattan and non-Harmattan particles revealed that Harmattan particles induced a higher secretion of pro-inflammatory cytokines compared to non-Harmattan PM, except for IL-12. This may indicate that health outcomes may vary depending on the time of the year, leading to more pro-inflammatory effects during the dry and sandy season. Also, as seen before, PM2.5, both Harmattan and non-Harmattan, led to a lower secretion of the

measured cytokines, except for IL-12. Interestingly, as PM collected during the Harmattan season has a higher content of sand, this is also reflected in their increased ability to induce inflammasome activation, as seen by an increased level of IL-1β. Generally, the overall pattern of Harmattan PM compared to FF PM10 indicates that the effects of these particles are very distinct, which is most likely due to their composition.

5.6. Conclusion

In conclusion, many of the variable effects of PM on DC, including maturation, ability to induce T cell proliferation, cytokine secretion and *IDO1* expression can be attributed to particles size and/or the physicochemical characteristics of the particles that are influenced by size. Generally, smaller particles are known for their higher toxicity; however our observations have shown that larger particles have a higher inflammatory potential than smaller PM in DCs. Therefore, PM10 particles may be of particular importance for immunomodulatory effects via DCs and should be considered in terms of air pollution control and health. However, the combustion source of the particles, and therefore their composition, can also exert an influence independently of size, notably with regard to activation of AhR: biomass derived particles are more potent activators of AhR. The downstream effects of PM-induced AhR activation of DCs remain to be fully defined.

6. Biomass-derived PM induces metabolic changes in DCs

6.1. Chapter summary

This chapter focusses on the further exploration of the unexpected finding, reported in Chapter 5, that DC became highly autofluorescent upon exposure to biomass-derived PM but not FF derived PM. Particles referred to as "biomass-derived" in this study were obtained from Africa and were available as both PM10 and PM2.5. The biomass PM2.5 was collected from kitchens in rural areas in The Gambia, whereas the BM PM10 came from communal kitchen areas in Accra/Ghana. In both places residents mainly use wood as an energy source; therefore, the air pollution is mainly derived from the combustion of biomass. Both particle types induced a high level of autofluorescence in MoDCs after 2 days of incubation. This autofluorescence was not due to the particles themselves. Although dying cells tend to be autofluorescent, induction of autofluorescence by BM PM could also not be attributed to toxicity. NADH and NADPH, which are the main constituents in a cell that can give rise to autofluorescence, were increased in DCs when cells were exposed to biomass PM2.5. This was shown by flow-cytometry; more specifically, NADH was identified as the main reason for the autofluorescence as measured directly using a colorimetric assay.

NADH plays an important role as an electron carrier in redox reactions and hence is involved in metabolic processes such as glycolysis. During glycolysis, the energy from nutrients is released and transferred onto NAD^+ to form NADH. Glycolysis increases in immune cells, such as DCs and macrophages, upon activation due to danger signals and cytokines (O'Neill & Pearce, 2015). To test whether the increase in NADH observed following exposure of DCs to BM PM could be attributed to glycolysis, 2-deoxyglucose (DG) was used to block glycolysis and NADH measured. Also, as signalling through TLR4 is known to increase glycolysis in order to provide energy for the cells, we blocked TLR4 signalling with a specific inhibitor and assessed whether this had an effect on NADH concentrations. However, neither blocking TLR4-signalling nor blocking glycolysis influenced NADH levels. BM PM also induced autofluorescence and increased NADH in other cell types such as monocytes, monocyte-derived macrophages and lymphocytes. However, the effect was greatest on DCs and macrophages and less marked on monocytes and even inhibited in lymphocytes. Both PM10 and PM2.5 derived from biomass induced autofluorescence and higher NADH levels in DCs, although these effects were significantly greater with BM PM2.5 compared with FF PM10 at higher concentrations. All in all, this data suggests, that BM-derived particles, but not FF PM, induce metabolic changes in DCs, which may impact the function of DCs.

6.2. Introduction

The hypothesis tested in the previous chapter was that particles derived from different combustion sources will vary in their composition and hence their effects on DCs will vary, too. One of the main differences found between different particle types was that PM derived from the combustion from biomass, irrespective of their size, induced high levels of autofluorescence. This was discovered by chance, when MoDCs were incubated with BM PM2.5 and the expression of surface markers was measured using flow-cytometry. As pacific blue is a channel that picks up autofluorescence very well and is often used to assess viability of cells if left empty, the first indicator was the high MFI in that channel. DCs showed a very bright fluorescence in the pacific blue channel after BM PM2.5 exposure, but not with FF PM10 or medium only.

Autofluorescence is a natural emission of light from cells and is usually caused by substances present in the mitochondria or lysosomes such as NADH, NADPH and flavins and can affect flow-cytometry measurements (Georgakoudi et al., 2002; Monici, 2005). High autofluorescence levels can also be caused by a reduced viability of the cells, although the exact mechanisms are unknown (Hennings et al., 2009). One possible explanation is that the increased level of autofluorescence in dying cells is due to an increased number of denatured proteins (Majno & Joris, 1995; Majno G, La Gatutta M, 1960). In addition, the release of fluorescent molecules from the cells, such as NADH and NADPH, may be causing increased autofluorescence of dying cells (Liang et al., 2007). NADH and its oxidised form NAD^+ are known to be involved in energy metabolism, reductive biosynthesis and antioxidation. They are generated either through a *de novo* pathways as well as a salvage pathway, the latter being essential to humans. The salvage pathway recycles NAD^+ back into its active form by using precursors of NAD^+. Not only is NAD/NADH involved in energy metabolism but it also mediates cell death, calcium homeostasis and gene expression (Ying, 2008). Changes in metabolism can affect concentrations in NADH and this approach has been used, for example, to track the metabolic state at a cellular level (Vergen, Hecht, Zholudeva, Marquardt, & Nichols, 2012). NADPH plays an important role in cellular antioxidation systems and is involved in the production of reactive oxygen species (ROS). These are generated through the transfer of electrons from NADPH, oxidizing it to $NADP^+$, a process which is catalysed by the NADPH oxidase (Ying, 2008).

In this chapter, we try to establish the causes of the measured autofluorescence in BM PM2.5 exposed MoDCs and explore how it may affect the cells.

6.3. Hypothesis and study aims

The overall hypothesis of this chapter is that the observed autofluorescence in MoDCs after exposure to BM PM is due to metabolic changes within the cells. Specifically, to address this hypothesis, the aims of this chapter are:

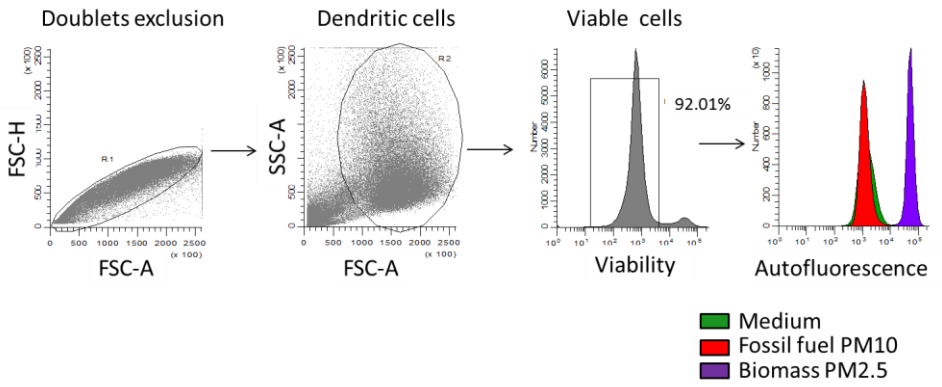
- Investigate the reason why biomass PM induces autofluorescence in MoDCs
- Determine whether high levels of autofluorescence reflect altered metabolism and assess the effect of this on DC function

6.4. Results

6.4.1. Biomass PM induces autofluorescence in MoDCs

In order to measure the level of fluorescence coming from the stimulated cells themselves, in the absence of antibody labelling, we cultured MoDCs with 50µg/ml of biomass PM for 48h or with medium alone. Subsequently, we measured using flow-cytometry, without previous antibody staining, to assess the level of autofluorescence. Also, we measured the fluorescence of the particle suspension without any cells in order to determine whether the particles were themselves fluorescent. The fluorescence was measured using the pacific blue channel, as it is the channel that picks up autofluorescence best.

A



B

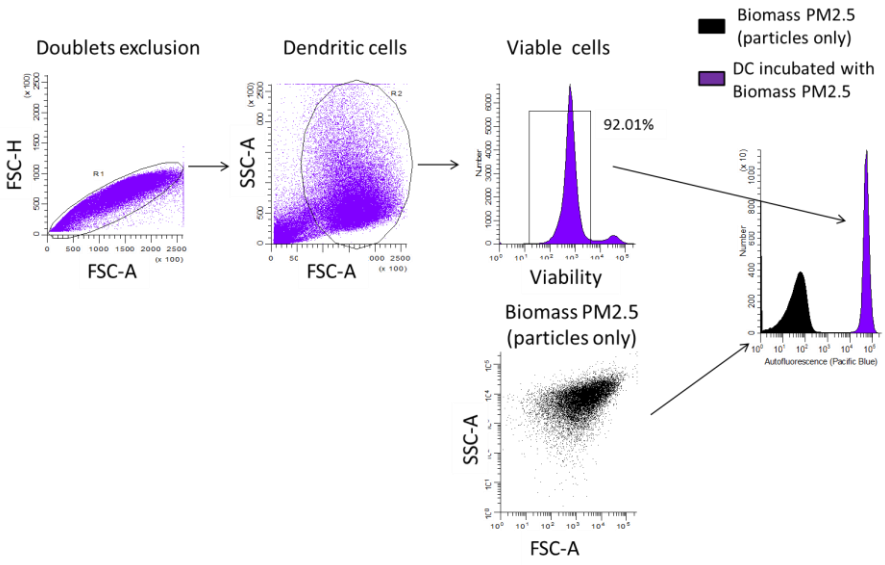


Figure 6.1: Biomass PM induces autofluorescence in MoDCs

DCs were derived from human blood monocytes and were exposed to 50µg/ml biomass PM2.5, fossil fuel PM10 or medium only. After 48h of exposure, cells were measured without any antibody labelling by flow-cytometry using the pacific blue channel. This experiment was conducted once. ***A****: Representative flow-cytometry plots showing the gating sequence for the analysis of autofluorescence after exposure of DCs to PM or medium only. The final histogram shows the measured autofluorescence of BM, FF or medium only exposed DCs.* ***B****: Flow-cytometry gating strategy illustrating biomass particles by themselves and DCs that were exposed to biomass particles and measured for autofluorescence in the pacific blue channel.*

MoDCs that were exposed to biomass derived PM2.5 for 2 days showed the highest level of autofluorescence as measured by flow-cytometry using the pacific blue channel (Figure 6.1 A). In comparison, DCs cultured in medium only or FF PM10 for two days showed the lowest level of autofluorescence. In order to check whether the particles themselves were autofluorescent, we assessed them in the absence of DCs (Figure 6.1 B). Biomass particles by themselves were not autofluorescent, whereas DCs exposed to BM PM2.5 showed a high level of autofluorescence. This indicated that autofluorescence resulted from the interaction of DCs with BM PM2.5. Therefore, the origin of fluorescence lies in stimulated DCs and changes in these cells needed to be investigated further.

6.4.2. BM PM-exposure of DCs does not reduce viability of cells

A factor that is a common cause of autofluorescence in cells is reduced viability of the cells. Therefore, we assessed the viability of DCs that were stimulated for 2 days with 50µg/ml of FF PM10 and BM PM2.5 or incubated with medium only. This was done using 7-Aminoactinomycin D (7-AAD) and Annexin-V. 7-AAD is a nucleic acid stain that, as soon as it intercalates with DNA, undergoes a spectral shift. Annexin-V, on the other hand, identifies apoptotic cells by binding to phosphatidylserine (PS); these usually are on the inner side of the plasmamembrane but move to the outside during early apoptosis and hence become accessible to Annexin-V. For this, gating was set on the unstained cells and dead cells, and apoptotic cells were analysed relative to the unstained cells. Also, as the forward and side scatter can give an indication about the apoptotic state of cells, this was recorded too.

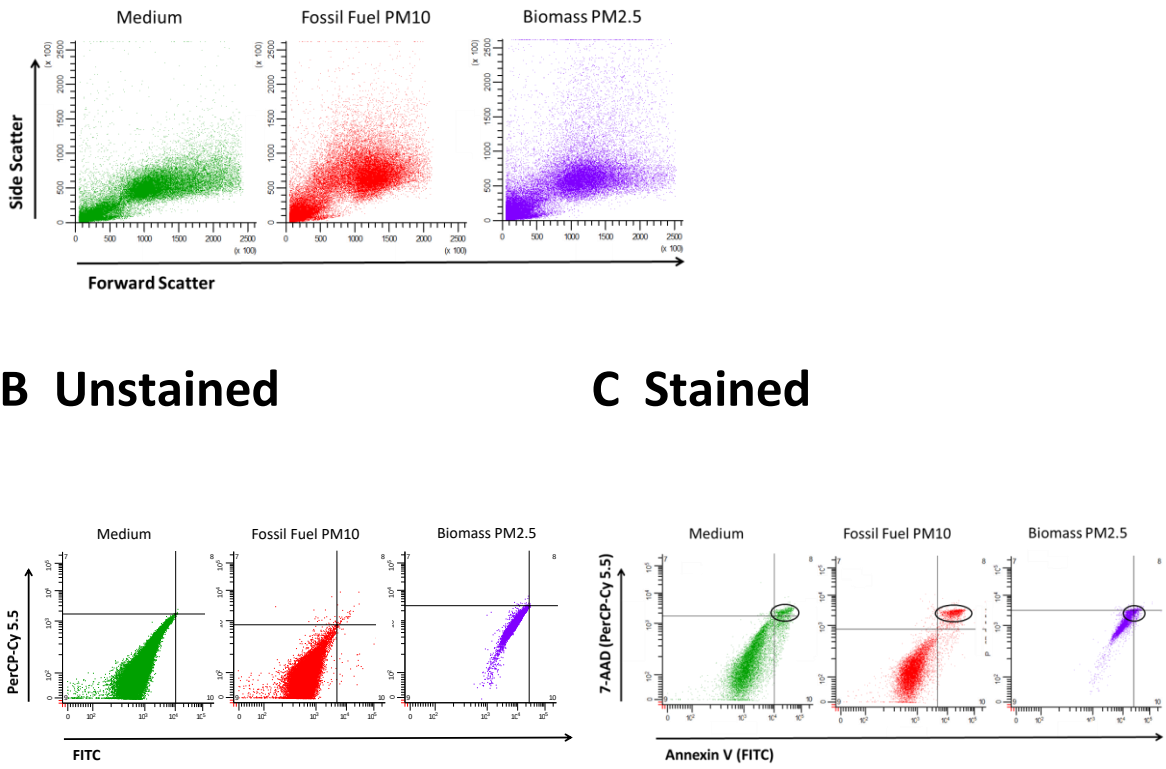


Figure 6.2: BM PM-exposure of DCs does not reduce viability

Human MoDCs were stimulated for 48h in medium only, 50µg/ml FF PM10 or BM PM2.5. After incubation, cells were washed with FACS buffer and fluorescence was measured by flow-cytometry, either unstained or stained to measure viability with 7-AAD and Annexin-V, which identify dead and apoptotic cells, respectively. This experiment was conducted once. ***A****: Forward and side scatter showing size and granularity of MoDCs incubated in medium only, FF PM10 and BM PM2.5.* ***B****: Scatter plot showing unstained, stimulated (with FF PM10 and BM PM2.5) or unstimulated (medium only) DCs in the PerCP-Cy 5.5 and FITC fluorescence channel.* ***C****: MoDCs were incubated with FF PM10, BM PM10 or medium only. After incubation, cells were stained for 7-AAD and Annexin V and measured with flow-cytometry. Scatter plot shows the gating based on the unstained control and the black circle highlights the population of dead cells.*

DCs that were exposed to FF PM10 and BM PM2.5 showed no changes in forward or side scatter, meaning that size and granularity remained the same (Figure 6.2 A). Measuring unstained, stimulated cells shows, again, that BM PM10 exposed cells were highly autofluorescent after stimulation, which could be detected in the PerCP-Cy5.5 and FITC channels (Figure 6.2 B).

When looking at the viability measured through staining cells with Annexin-V and 7-AAD, Annexin-V gives an indication of the state of the cell membrane. It binds to PS, which are present on the inner side of the cell membrane and translocate to the outer membrane during early apoptosis and can therefore be detected by a specific reagent (Figure 6.2 C). 7-AAD has a strong affinity to double-stranded DNA and can therefore be used as a fluorescent marker for DNA. It is efficiently excluded from viable cells. The medium only exposed DCs as well as the FF PM10 stimulated DCs showed a small, but clear and distinctive population of dead cells, as indicated by a small circle. This small population was also evident in BM PM2.5 exposed DCs, but it was harder to discriminate from the bulk of the cells due to increased level of autofluorescence in the major population. However, because of the small numbers and modest shift of cells, it is unlikely that the observed autofluorescence in BM PM2.5 is due to a toxic effect on the cells leading to reduced viability. In summary, there was no evidence that viability is reduced after cells are exposed to BM PM2.5. Hence, there must be a different explanation for the autofluorescence in DCs.

6.4.3. DCs stimulated with biomass PM have increased levels of NAD(P)H

The above result had shown that the autofluorescence in DCs after stimulation with BM PM2.5 was not due to their reduced viability. Thus, we investigated the expression of nicotinamide adenine dinucleotide (NADH) and Nicotinamide adenine dinucleotide phosphate (NADPH) in MoDCs after incubation with BM PM2.5. NADH and NADPH are the main components that can cause autofluorescence in cells detected during flow-cytometric measurements (Blacker & Duchen, 2016). NADH is a coenzyme involved in redox reactions, acting as an electron carrier; it also plays important roles in metabolism; NADPH on the other hand is a key determinant of biosynthetic pathways and antioxidant effects (Blacker et al., 2014). Both compounds are fluorescent and can be measured more specifically after excitation with a UV laser and assessing fluorescence emission in the Hoechst channel, with an emission peak at 470nm (Mayevsky & Rogatsky, 2007; Vergen et al., 2012). We stimulated human MoDCs with a range of concentrations of FF PM10 and BM PM2.5 or medium only for 2 days and measured the expression of NADH and NADPH using the Hoechst channel.

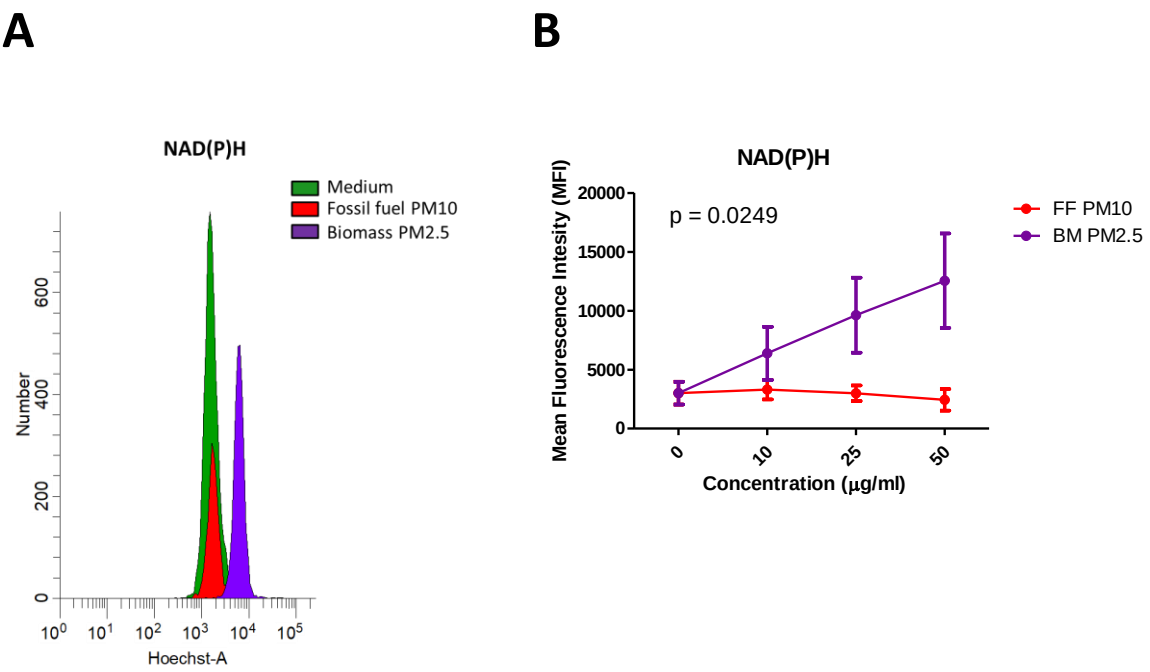


Figure 6.3: DCs stimulated with biomass PM show increased levels of NAD(P)H

MoDCs cultured from human PBMCs were exposed to various concentrations of fossil fuel PM10 and biomass PM2.5 for 2 days. After incubation, samples were measured for NAD(P)H contents. ***A****: Histogram illustrating the autofluorescence caused by NAD(P)H measured using the NAD(P)H-specific Hoechst channel with a UV laser. DCs were exposed to 50µg/ml of FF PM10, BM PM2.5 or medium only and analysed using flow-cytometry without any previous antibody labelling.* ***B****: MoDCs were stimulated with 10, 25 and 50µg/ml of FF PM10 and BM PM2.5 or medium only. The diagram shows the summary data of 3 independent experiments using blood samples from three independent donors and the p-value was calculated using a two way analysis of variance.*

Measurement of FF PM10 and BM PM2.5 exposed DCs or DCs incubated with medium only using the Hoechst channel showed an increased fluorescent signal for BM PM2.5 exposed DCs (Figure 6.3 A), suggesting an increased level of NAD(P)H. Fluorescence of FF PM10 stimulated DCs under UV excitation was similar to that of DC cultured in medium only. After several repeats of the experiment using a range of 10, 25 and 50µg/ml of FF PM10 and BM PM2.5 or medium only to stimulate MoDCs for 2 days, it was evident that only exposure of DCs to BM PM2.5 lead to a significant increase of NAD(P)H in those cells (Figure 6.3 B).

NADH plays an important role in metabolism, which may be affected by air pollution. Therefore, we used a second method to measure NADH, specifically in PM stimulated DCs, using a

commercially available colorimetric kit to measure NAD and NADH ratios as well as their concentrations in cells.

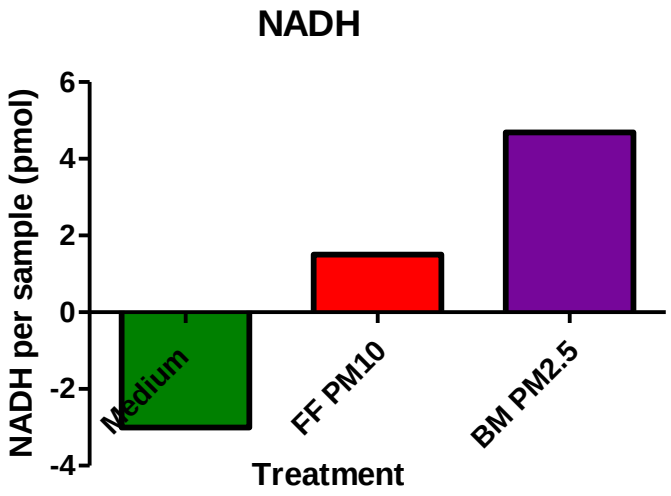


Figure 6.4: DCs stimulated with biomass PM show increased levels of NADH

Human monocytes were cultured in GM-CSF and IL-4 for 7 days and exposed to 50µg/ml of fossil fuel PM10, biomass PM2.5 or medium only for 48 hours. Subsequently samples were measured for NADH using a specific colorimetric assay. The bar chart demonstrates the amount of NADH per sample, measured by a NAD/NADH quantification kit. This experiment was conducted once.

In order to confirm that NADH is responsible for the autofluorescence in BM PM2.5-exposed DCs, we used a specific colorimetric assay to assess NADH levels (Figure 6.4). The assay worked via an enzyme reaction that specifically measured NADH and NAD, but not NADPH and NADP. It only detected the NADH total concentration; therefore, NAD had to be converted to NADH for the assay or decomposed to measure NADH only. In order to then calculate the NADH/NAD ratio, NAD concentrations had to be determined. This was done by subtracting the NADH content after NAD decomposition from the total NADH concentration after conversion of NAD to NADH. The NADH concentration in DCs exposed to 50µg/ml of BM PM2.5 was 4.69pg/sample; in contrast to that, DCs stimulated with 50µg/ml of FF PM10 led to 1.5pg/sample of NADH. The medium control had a very low concentration of NADH, even lower than the blank control, resulting in a negative result. All conditions had an equal amount of cells in them to make them comparable.

All in all, the results from the NAD/NADH colorimetric assay confirmed the findings from the flow-cytometric measurement and give further support to the notion that BM exposure of DCs

leads to increased levels of NADH. This also means that the ratio was shifted towards NADH, indicating the concentration of NAD^+ was decreased. However, this result represents only one experiment, as very large numbers of cells were required for one test therefore more repeats are needed. Still, this section tentatively confirms our assumption that the reason for autofluorescence in MoDCs after BM PM2.5 exposure is a higher level of NADH within the cells.

6.4.4. Increased NADH expression in DCs after BM PM-exposure is not dependent on glycolysis or TLR4 signalling

In the previous sections we have shown that the autofluorescence is due to NADH and NADPH, and more specifically NADH. As an electron carrier, NADH plays important roles in metabolic processes, like glycolysis, or the citric acid cycle. It has been reported previously that innate and adaptive immune responses require high energy levels which are supplied by glycolysis (Krawczyk et al., 2010). TLR4 signalling increases glycolysis in order to support the activation of DCs (Krawczyk et al., 2010) and we have seen in previous chapters that exposure to PM leads to TLR4 dependent effects on DCs. It is known that TLR4 activation leads to changes in NAD^+ and NADH (T. F. Liu, Vachharajani, Yoza, & McCall, 2012); therefore, we tested whether blocking this pathway affects NADH levels. Firstly, we blocked the TLR4 receptor using CLI-095. Secondly, we used DG, which blocks glycolysis, as it has a similar structure to a glucose molecule but has a slightly altered chemical composition; therefore, it cannot undergo further glycolysis and ultimately blocks the metabolic pathway.

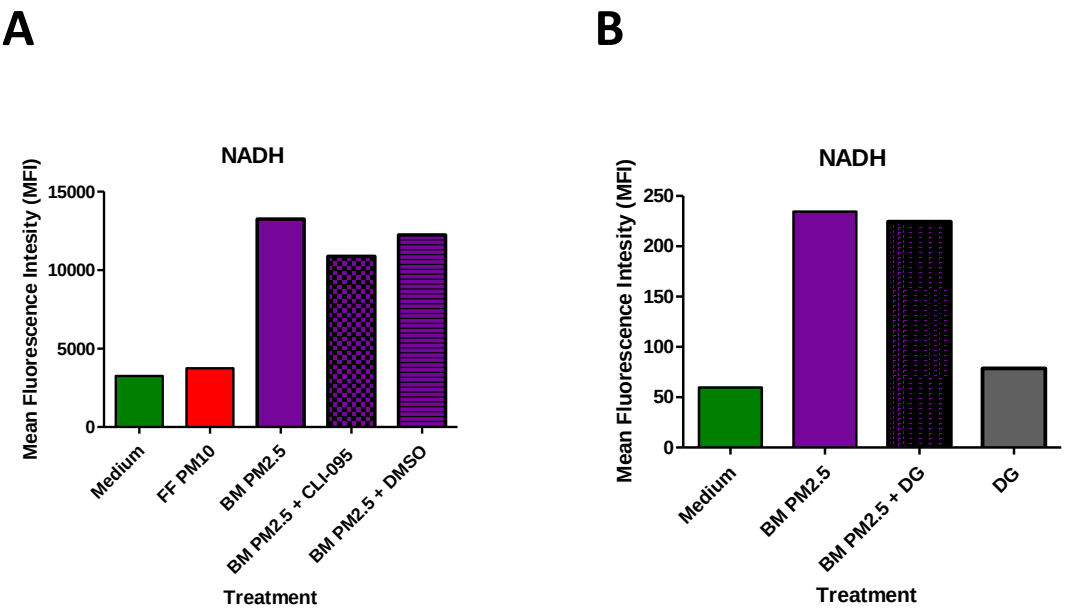


Figure 6.5: Increased NADH expression by DCs after BM PM-exposure is not dependent on glycolysis or TLR4 signalling

$CD14^+$ monocytes were separated from human PBMCs and cultured with IL-4 and GM-CSF for 7 days to obtain DCs. DCs were stimulated with 50µg/ml of FF PM10 and BM PM10 or medium only for 48h. In addition to that, cells were pre-treated with the glycolysis inhibitor 2- DG for 1h or the TLR4-antagonist CLI-095 for 1h. ***A****: MoDCs were stimulated with medium only, FF PM10, BM PM2.5 and BM PM2.5 with CLI-095 and BM PM2.5 with the vehicle control DMSO.* ***B****: DCs were incubated with BM PM2.5, BM PM2.5 and DG or DG and medium only. This experiment was conducted once.*

The inhibition of the pattern recognition receptor TLR4 did not change the level of NADH in BM-exposed DCs in comparison to the vehicle control DMSO (Figure 6.5 A). Also, inhibiting glycolysis itself prior to stimulation of the cells with BM PM2.5 did not lead to a significant decrease of NADH expression in DCs (Figure 6.5 B).Therefore, as the NADH level in the DCs does not seem to be affected by blocking glycolysis, the involvement of metabolism and glycolysis specifically needed to be investigated further in DCs that were stimulated with BM PM2.5.

6.4.5. Biomass PM exposure induces NADH expression in monocytes and macrophages

The occurrence of autofluorescence in DCs when exposed to BM PM2.5 in association with a higher concentration of NADH in those cells was highly consistent in all experiments conducted with the use of BM PM2.5. Since, so far, this effect was only seen in DCs, we wanted to

investigate whether this effect was observed specifically with DCs or whether it occured with other types of immune cells. For this, we exposed PBMCs and monocyte-derived macrophages (as an alternative monocyte-derived *in vitro* generated population) to FF PM10 and BM PM2.5 for 48h and measured the NADH concentration using the UV laser on the flow-cytometer. Within the PBMCs, we could measure NADH in monocytes as well as lymphocytes. We grew monocyte-derived macrophages by exposing $CD14^+$ blood monocytes to GM-CSF for 7 days, which were then stimulated for 2 days with BM PM2.5 and FF PM10. All different cell types were stimulated with a concentration range of 10, 25 and 50µg/ml of PM or medium only.

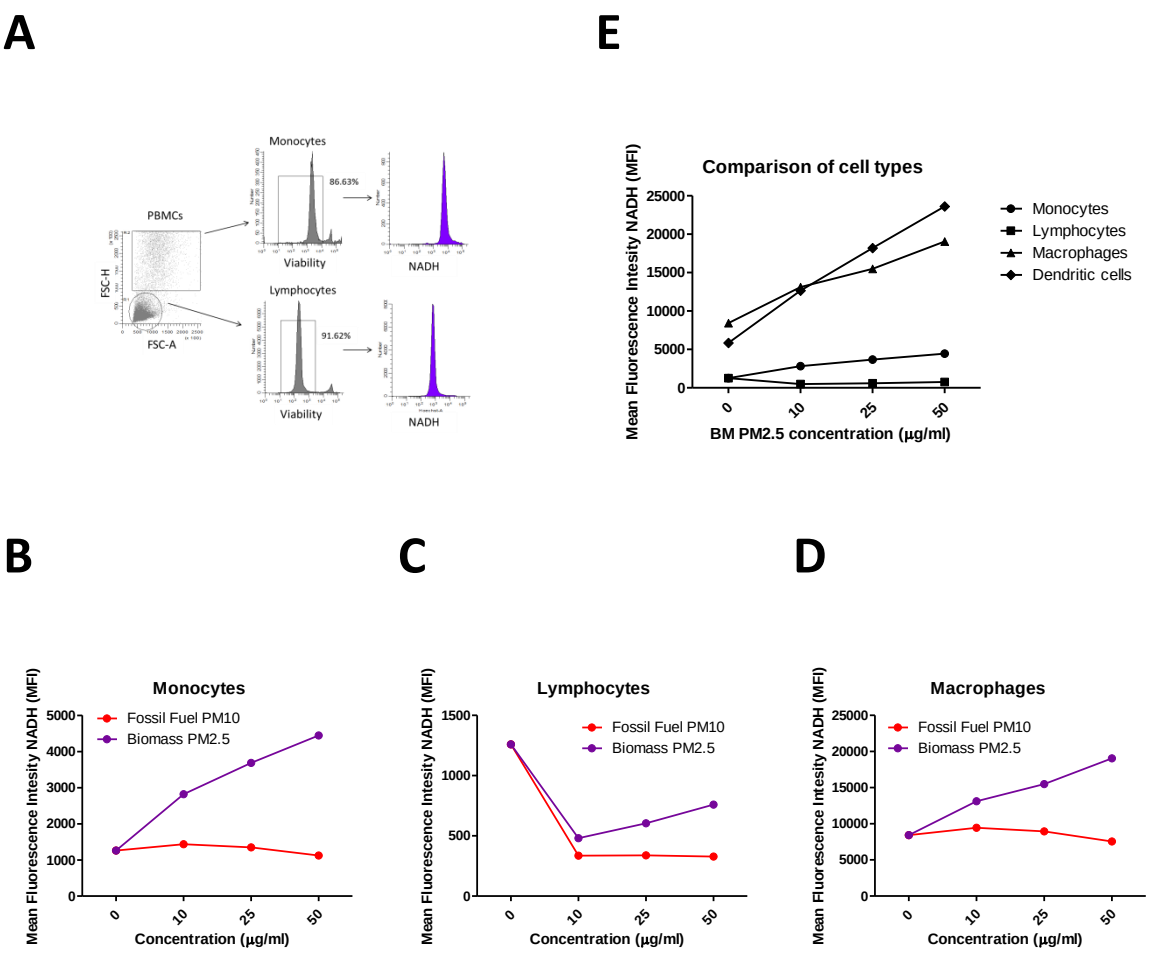


Figure 6.6: Biomass PM exposure induces NADH expression in monocytes and macrophages

Human PBMCs were separated from blood and stimulated with 10, 25 and 50µg/ml of FF PM10 and BM PM2.5 or medium only. Macrophages and DCs were derived from blood monocytes by culturing with GM-CSF only for macrophages or GM-CSF and IL-4 for DCs for 7 days. Both cells types were exposed to 10, 25 and 50µg/ml of FF PM10 and BM PM2.5 for 2 days and measured by flow-cytometry using the Hoechst channel to determine NADH levels. ***A****: Flow-cytometry plots showing the gating strategy for monocytes and lymphocytes. After identifying the cell populations in PBMCs through forward and side scatter, viable cells were measured for NADH contents at 470nm after exposure to 50µg/ml BM PM2.5.* ***B****: The diagram shows the expression of NADH in monocytes after stimulation with various concentrations of FF PM10 and BM PM2.5 or medium only.* ***C****: The plot illustrates the amount of NADH in blood lymphocytes after incubation with a range of concentration of FF PM10 and BM PM2.5 or medium only.* ***D****: Expression of NADH in monocytes-derived macrophages after incubation with several concentrations of FF PM10 and BM PM2.5 or medium only.* ***E****: Comparison of NADH expression of monocytes, lymphocytes, MoDCs and macrophages after stimulation with various concentrations of FF PM10 and BM PM2.5 or medium only. This experiment was conducted once.*

Blood monocytes and lymphocytes were identified in human PBMCs through forward and side scatter, dead cells were excluded from the analysis and autofluorescence, which gives an indication of NADH contents, was measured using the Hoechst channel (Figure 6.6 A). Levels of NADH in monocytes analysed *ex vivo* increased in a dose-dependent manner when exposed to BM PM2.5 but not when exposed to FF PM10 (Figure 6.6 B). In contrast, NADH levels in *ex vivo* lymphocytes did not increase when exposed to either BM PM2.5 FF PM10, and indeed may even have decreased slightly, most notably in cells exposed to FF derived PM (Figure 6.6 C). The cell populations differentiated *in vitro* had higher NADH levels at baseline compared with the monocytes from which they were derived. NADH levels increased dramatically and to a similar degree in macrophages as well as DCs following exposure to BM PM2.5 but not the FF derived particles (Figure 6.6 D and E).

In summary, this set of experiments shows that the effect of BM PM2.5 on NADH levels was cell-type specific and that MoDCs and macrophages, but not lymphocytes, were the main cell types affected by BM PM2.5. *Ex vivo* monocytes showed an intermediate response. Therefore, it could be concluded that the differentiation process, which the macrophages and DCs go through, impacts their ability to respond to BM PM2.5 exposure with higher NADH levels.

6.4.6. NADH increase in MoDCs is dependent on combustion source and not on particle size

The previous chapter has shown that particle size and the combustion source from which the air pollution particle is derived both play an important role in terms of the effects on DCs. Therefore, we determined whether particle size or derivation was the key determinant in the differential effect of BM PM2.5 and FF PM10 on NADH levels in DCs. For this, we cultured MoDCs with fossil fuel PM10, biomass PM10 and PM2.5 or medium only for 2 days at concentrations of 10, 25 and 50µg/ml.

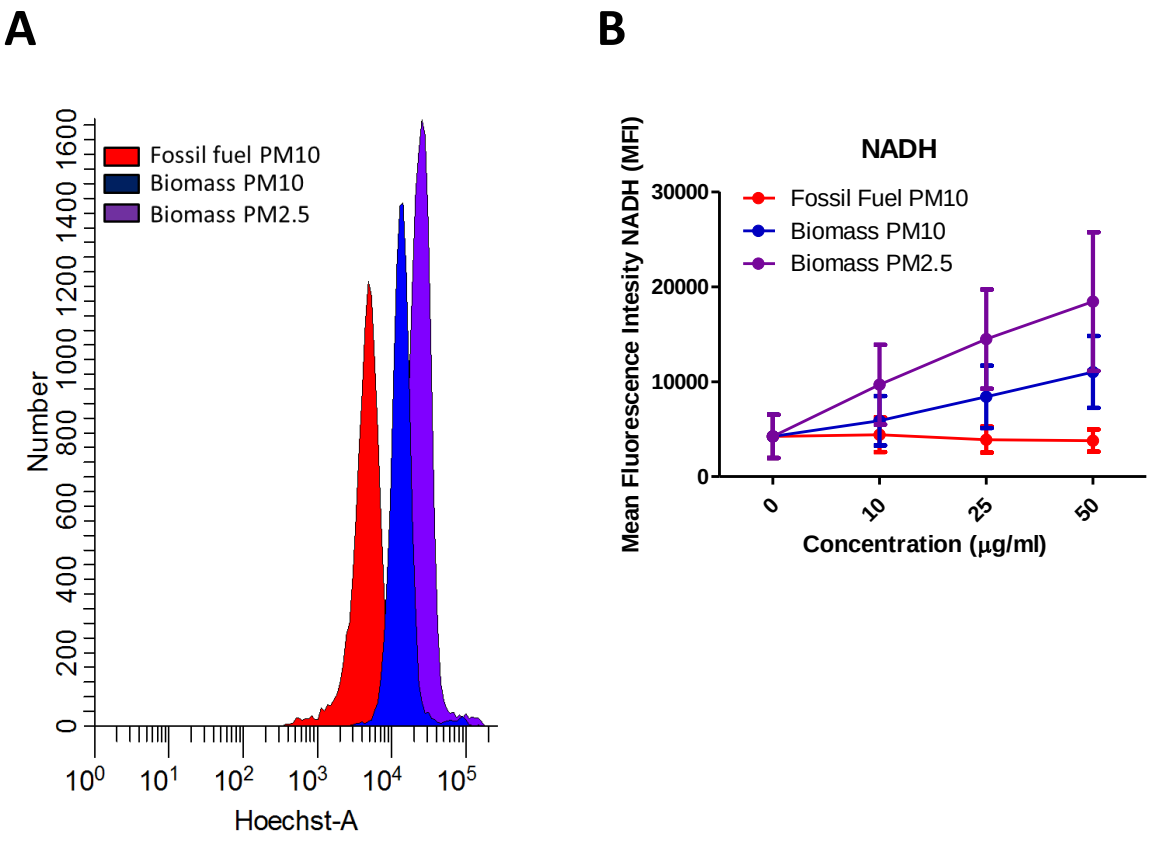


Figure 6.7: NADH increase in MoDCs is dependent on combustion source and not on particle size

Human MoDCs were stimulated for 48h with FF PM10, BM PM10, BM PM2.5 or medium only and measured using the UV laser by flow-cytometry. ***A****: Flow-cytometry histograms illustrating the MFI of NADH in MoDCs after stimulation with 50µg/ml of FF PM10, BM PM10 and BM PM2.5.* ***B****: DCs were stimulated with 0, 10, 25 and 50µg/ml of FF PM10, BM PM10 and BM PM2.5 for 2 days and the NADH contents was measured using the Hoechst channel. The mean and standard deviation of two independent measurements was plotted.*

BM PM10 as well as BM PM2.5 led to a dose-dependent increase of NADH in DCs after 2 days of exposure compared with DCs that were exposed to FF PM10 (Figure 6.7 A and B). When comparing the level of NADH in DCs after cells were exposed to different sizes of biomass, BM PM2.5 led to a higher level of NADH in DCs compared with BM PM10. When FF PM10, BM PM10 and BM PM2.5 were compared over a range of concentrations, both biomass types of particles stimulated a dose-dependent increase of NADH in DCs, whereas FF PM10 did not increase of NADH at any of the concentrations tested, as seen before (Figure 6.7 B). A two-way analysis of variance did not reveal a significant difference between the particle types overall; however, a

Bonferroni post-test showed a significant difference between FF PM10 and BM PM2.5 treated DCs at 25µg/ml and 50µg/ml (at 25µg/ml $p < 0.05$, at 50µg/ml $p < 0.01$). From this we can conclude that the induction of high NADH levels in MoDCs is related to the combustion source of the particles.

However, as BM PM10 also induced an increase in NADH in DCs it may be speculated that another property of the BM PM2.5 must be responsible for the induction of NADH in DCs, such as the size, which in turn can affect the composition, as discussed in the previous chapter. However, one aspect that should be kept in mind is that both biomass derived particles, PM10 and PM2.5, do not come from the exact same location; therefore, differences in the composition of the particles may be affecting NADH levels.

6.5. Discussion

The experiments presented in this chapter aimed to explore further the chance observation that MoDCs became highly autofluorescent when exposed to BM PM2.5. As a first step, we checked whether the stimulated cells were the source of the autofluorescence or whether the cellular autofluorescence could instead be explained by uptake of air pollution particles that were themselves highly fluorescent. Analysis of the particles themselves by flow-cytometry revealed only low level fluorescence and therefore the signal from stimulated DCs is likely to be genuinely of cellular origin. Also, we checked whether any substances from the filter on which the particles were collected were responsible for the strong fluorescent signal. For this, we put empty unused filters through the same extraction procedure as filters that carried particles on them and used the PBS from the extraction to stimulate the MoDCs. Material from empty filters had no effect on the florescence of DCs (data not shown). Taken together with the failure of FF PM preparations from identical filters to induce autofluorescence, it is highly unlikely that filter components are a contributing factor. This confirmed our assumption that the fluorescence was coming truly from the stimulated cells themselves. Therefore, we had to further investigate what the reasons were for the strong autofluorescence when DCs were exposed to BM PM2.5.

The next step was to look into the viability of the cells, as cells with a reduced viability tend to fluoresce more than healthy cells. A first indicator of the state of stimulated cells is the forward and side scatter on the flow-cytometer. The forward scatter measured the size of the cells that pass through the flow-cytometer. Dying cells tend to swell initially and, after releasing their cell content, they deflate, which can be seen as a first increase followed by a decrease on the forward scatter; this gives an initial indication how healthy the cells are. BM PM2.5-exposed MoDCs did not show a strong shift in forward scatter compared with cells that were cultured in

medium only of with FF PM10. A small increase was observed in the granularity of the cells when stimulated with PM; therefore, on the side scatter, the cells shifted up a little bit. This was an expected finding, as we have seen before that DCs take up PM and therefore their granularity must change too.

The viability measurement of stimulated and unstimulated cells with Annexin V in the FITC channel and 7-AAD in PerCP-Cy 5.5 showed that stimulating cells with BM PM2.5 leads to a small population of dead cells. This was seen as a shift within the cell population and was not clearly distinguishable. DCs that were cultured in medium only or with FF PM10 showed a clearly defined and small proportion of dead cells. Again, BM PM2.5-exposed DCs showed a strong shift towards higher fluorescence in both fluorescent channels that were measured, even when cells were unstained, which was due to their own level of fluorescence. This strong autofluoresence covers the population of dead cells and makes them harder to distinguish from the rest of the cells.

Since by now we had confirmed that the reason for high fluorescence levels was the BM PM2.5-stimulated cells themselves, and that their viability was not the reason for the autofluorescence, we had to look further into the cause of this phenomenon. NADH and NADPH are the main components that can cause autofluorescence in cells; therefore, we utilised a method to measure NAD(P)H levels in cells (Heikal, 2010). Using UV light to excite the cells and the Hoechst channel to measure emissions at a wavelength of 470nm enabled fluorescence from NAD(P)H to be selectively measured. Using this method, we could confirm our first assumption, namely that elevated levels of NAD(P)H were responsible for the high autofluorescence levels that we saw in earlier experiments. More specifically, we measured NADH, which is the component involved in glycolysis, using a colorimetric assay which determines the specific concentration of NADH. This assay kit was very expensive and required a large number of cells. For those reasons, only limited experiments were possible. In this assay, NADH levels in unstimulated DCs were very low and indeed fell below the 'blank' value that was subtracted from all readings, leading to negative values for the medium control. Nonetheless, NADH levels were increased following stimulation, notably with BM2.5.

In order to further understand what the elevated levels of NADH in cells imply and what is causing the elevation, we looked into the various roles NADH plays within the cells (Ying, 2008). NADH is a coenzyme that acts as an electron carrier transporting electrons from one reaction to another; therefore, it can be found in two different states: the oxidized form NAD^+ and the reduced form NADH; only NADH is fluorescent after UV excitation. One of the reactions in which NAD^+/NADH plays an important role is glycolysis, where the energy gained from nutrients is

transferred to NAD^+, which results in the reduced form NADH. TLR4 signalling plays an important role in metabolic changes within the cells, as TLR agonists are known to increase glycolysis (Claire E. McCoy, 2016). Signalling through TLR4 may therefore increase the rate of glycolysis, which can then lead to an increased concentration of NADH. However, in our case, BM PM2.5 did not induce TLR4 signalling; it could still contribute to the overall change in metabolism that ultimately leads to increased NADH. We blocked the TLR4 receptor using the specific antagonist CLI-095.

However, the BM PM2.5 induced increase in NADH was not altered by the presence of CLI-095 in DCs. Therefore, we blocked glycolysis directly using DG, which is a glucose molecule, but has hydrogen instead of the 2-hydroxyl group; consequently it cannot undergo further glycolysis. Yet, we saw no evidence that blocking glycolysis decreases the levels of NADH in DCs after BM PM2.5 exposure. This could be due to the fact that NADH may be resulting from other processes within the cell, such as the citric acid cycle in the mitochondria. Also, we did not confirm whether glycolysis was truly blocked throughout the process. DG may have become degraded during the stimulation; therefore, glycolysis may have not been blocked throughout the whole incubation time. There is no data available on the stability of DG once it is in solution.

At the moment it is unclear what exactly causes the high levels of NADH; this needs to be investigated further. This could be done via a more in-depth analysis of cell metabolism using an approach called Seahorse analysis. Seahorse analysis measures the oxygen consumption rate, which is an indicator of mitochondrial respiration, and the extracellular acidification rate, which is a result of glycolysis, in intervals of 5-8 minutes while different substances and inhibitors are added. Furthermore, glucose oxidation, fatty acid oxidation, and aerobic glycolysis rates can easily be measured under a wide range of conditions. It has been used to investigate how environmental effects impact dermal fibroblasts, showing that glycolysis and NAD^+ synthesis are involved in the protective mechanism against oxidative stress (Rovito & Oblong, 2013). This approach would allow us to understand which pathways are involved after cells were exposed to BM PM2.5 and the role of metabolism in DC function. A recent study using the Seahorse analysis has described a protocol on how to use this method on DCs (Claire E. McCoy, 2016).

There is increasing evidence that metabolism is linked to the ability of an organism to initiate an immune response (Hotamisligil & Erbay, 2008). It is known that this link works in both directions: changes in metabolism are needed for appropriate immune responses and immune processes induce changes in metabolism. Triggering TLR in murine DCs leads to an increase in glycolysis and a decrease in oxidative phosphorylation. More specifically, it leads to TLR dependent production of nitric oxide which leads to a decrease of oxidative function in the mitochondria

and results in obstruction of the mitochondrial respiratory chain. ATP is then provided through glycolytic flux. In addition to that, the increase in glycolysis through TLR signalling mainly serves the anabolic requirement of the DCs to undergo maturation and express cytokines, which then leads in turn to T cell priming (Claire E. McCoy, 2016). It has also been shown that TLR agonists lead to an increase in glucose consumption and lactic acid production (O'Neill & Pearce, 2015). Hence, metabolic conditions are known to affect inflammatory diseases and vice versa (Schertzer & Steinberg, 2014).

There are number of ways in which an increase in cellular NADH may impact DC function. For instance, NADH is known to be a substrate for sirtuins and DC sirtuin 1 is involved in T-cell differentiation. It plays a critical role in directing the cytokine production by DCs and through this influences T cell fate. More specifically, SIRT1 is required for the secretion of IL-12, TGF-β1 in DCs as well as in the production of the corresponding receptors on T cells. (Liu et al., 2015). Also, it has been reported that the NADH oxidase NOX2, which catalyses the redox reaction of $NADH/NAD^+$, plays an important role in cross-presentation of murine DCs. NOX2, through production of low levels of ROS, preserves alkalinisation of the phagosome, making sure that antigen degradation is controlled to prevent destruction of peptides for T cell recognition; hence, NOX2 deficiency in DCs leads to compromised cross-presentation (Savina et al., 2006). Additionally, it has been shown that NOX2 induces lipid peroxidation, which leads to membrane disruption of the endosome, resulting in antigen leakage. In chronic granulomatous disease (CGD), patients have a dysfunctional NOX2; antigen release from the endosome is impaired in DCs (Dingjan et al., 2016). It can be speculated that people exposed to BM PM, who have a higher concentration of NADH and therefore an increased activity of NOX2, show a higher efficiency of antigen processing in DCs which would then affect immune responses to pathogens.

As all our observations regarding autofluorescence and NADH concentrations within cells were based on MoDCs, we wanted to look into whether this effect is specific for DCs or whether other cells experience the same when exposed to BM PM2.5. For this, we generated monocyte-derived macrophages by culturing $CD14^+$ human blood monocytes with GM-CSF for 7 days and incubated them with several concentrations of BM PM2.5. These cells were easy to generate under controlled conditions and therefore could be compared with the MoDCs in our study. Also, we used PBMCs as a whole and exposed it to BM PM2.5 to measure the impact of BM PM2.5 on lymphocytes and monocytes. Monocytes as well as macrophages and DC differentiated from them showed a dose-dependent increase in NADH expression after exposure to BM PM2.5, whereas lymphocytes did not. When all cell types are compared, DCs and macrophages show the highest levels of NADH across different PM concentrations. Monocytes

are less affected and lymphocytes do not seem to be affected by BM PM2.5 in terms of their NADH levels.

This suggests that the cell types most affected by BM PM2.5 are phagocytic cells in general, which are fully differentiated and MoDCs and macrophages are particularly affected by BM PM2.5. This indicates that the differentiation process and the derivation from monocytes in vitro affect their ability to increase NADH levels after encountering BM PM. As both MoDCs and monocyte-derived macrophages require GM-CSF for their differentiation, there could be a potential link between GM-CSF and NADH. It has been shown that supplying human podocytes with NADH as a substrate for the NAD(P)H oxidase leads to an increase in GM-CSF release by the cells through the production of ROS (Greiber, Müller, Daemisch, & Pavenstädt, 2002). Also, innate cells such as DCs, macrophages and monocytes are much more likely to act as environmental sensors and were more responsive to environmental triggers such as BM PM, which ultimately leads to increased susceptibility to higher NADH levels than lymphocytes.

Finally, we have observed that both combustion source and particle size influence the effects of PM on MoDCs. At first, BM PM2.5 was investigated and those particles led to the initial discovery of autofluorescence in DCs after exposure to those particles and the related increased NADH expression. FF PM10 and FF PM2.5 did not induce increased autofluorescence levels in DCs. Therefore, we sourced BM PM10 in order to confirm that the observed effects were due to the source of combustion and not the particle size. Although BM PM10 was obtained from a different location than the BM PM2.5, they were also collected in a kitchen area where wood was the main energy source. These experiments confirmed that combustion source is a primary influence on NADH upregulation since the upregulation was induced by both sizes of BM-derived PM but neither size of FF-derived PM. However, they also provided evidence for an effect linked to the size of collected BM PM: BM PM 2.5 induced more NADH that BM PM10. The previous chapter has shown that particle size is an important aspect when considering the effects on DCs and it also has been shown before by others that different particle sizes have different chemical and physical properties and therefore vary in their effects on APCs (B. Wang et al., 2013). However, it needs to be pointed out that BM PM10 and BM PM2.5 do not come from exactly the same location. Although both come from kitchens, where biomass was the predominant source of energy, kitchens were located in different countries, The Gambia and Ghana, whereas PM2.5 from The Gambia were collected in a rural area and PM10 in Ghana came from the capital Accra (Z. Zhou et al., 2011, 2014). It needs to be kept in mind that the environment affects the composition of the particles. For example, the time of the year when particles were collected plays an important role since during the dry and windy season in Accra/Ghana particles contain

more sand than during the rainy season. Also, proximity to the sea affects salt concentration on particles and proximity to industry will have an effect on composition, too (Oliveira et al., 2011; Z. Zhou, Dionisio, Verissimo, Kerr, Coull, Arku, et al., 2013a).

Interestingly, as seen in the previous chapter, BM particles induced a higher AhR activation compared to FF PM; BM PM2.5 induced a higher level of stimulation than BM PM10. Therefore, determining how AhR may be linked to an increase of NADH in DCs would be an interesting next step towards understanding how NADH concentration is increased in BM-exposed MoDCs. For this, the influence of AhR activation on NADH levels should be tested by pre-treating MoDC with the specific AhR antagonist CH223191 prior to BM PM stimulation and subsequent NADH measurement by flow-cytometry.

6.6. Conclusion

In conclusion, work in this chapter demonstrates that PM derived from the burning of wood, but not fossil fuel, induce autofluorescence in MoDCs and macrophages that can be attributed to increases in cellular NADH. This effect of BM PM is independent of TLR4 signalling and glycolysis, each of which can be linked to NADH, and may reflect an as yet undefined effect on the metabolic activity of certain immune cell populations. The impact of these perturbations on the function of these immune cell populations remains to be determined.

7. Final discussion

Air pollution is an increasing health risk in both developed and developing countries. PM, one of the major components of air pollution, is responsible for a wide range of detrimental health effects such as the development and exacerbation of lung diseases like asthma and COPD as well as systemic effects like cardiovascular events. DCs are the most potent APCs, provide a link between the innate and adaptive immune system and are the only cell type that can initiate primary immune responses by activating naïve T cells. As the first line of defence, they are located throughout the body at mucosal sites and are most likely to interact with incoming threats to the body as well as inhaled air pollution in the lung.

The main aims of this thesis were, firstly, to determine the effects of ambient PM on different pathways used by DCs to sense and respond to their environment and, secondly, to evaluate the influence of particle size and composition on these effects. These aims were addressed by sourcing ambient PM from two independent UK urban environments and from The Gambia and Ghana in Africa in order to compare PM10 and PM2.5 derived from the combustion of either fossil fuel or biomass. The use of MoDCs as an *in vitro* model provided sufficient cells for an in-depth analysis in which issues such as dosimetry could be properly considered. An analysis of *ex vivo* sputum DCs made it possible to explore the relevance of findings from this *in vitro* model to *in vivo* cell population.

The first key finding was that ambient PM is able to interact with multiple environmental sensing mechanisms used by DC including pattern recognition via TLRs, inflammasome activation and AhR signalling. The carbon core of the PM was largely inert in these cases and unlikely to be responsible for the effects on DC. Indeed, the complex nature of the interaction with DCs is likely to reflect the complex nature of the particles themselves and the overall effect of PM on DC and downstream immunological events are likely to reflect the net effect of multiple pathways activated in the cells. Although identification of individual PM responsible for the activation of DC sensing pathways was beyond the scope of this study, it is likely that multiple components will contribute. Ideally, those individual components of PM should be identified and the corresponding effect on DCs should be matched, however, as mentioned above, many components may interact and one single outcome may not be matched. Also, the aim of the project was to measure the effects of PM as a whole, not broken down into individual components.

The second major finding was that particle size is a major determinant of the effects of PM on DCs, irrespective of the combustion source from which the particles were derived or the location

from which they were sampled. Specifically PM10 but not PM2.5 was a potent inducer of DC maturation, cytokine production, inflammasome activation and AhR signalling. It is crucial to emphasise again that these size values represent what was sampled not the size of the material added into DC cultures. Therefore, these size-related differences are likely to reflect the physicochemical properties of the particles themselves rather than distinct handling of particles of difference sizes by DCs, although it is not possible to entirely exclude a contribution of this effect as no formal analysis of the material added to the cultures was conducted. In hindsight, a formal analysis of size after extraction of the particles should have been conducted, in order to match the effects of the particles to the actual size that was added to the culture.

Many of the effects of PM10 on DCs may be regarded as pro-inflammatory. DC maturation is associated with increased expression of MHC and co-stimulatory molecules, a phenotype associated with enhanced generation of effector T cell responses rather than anergy or the generation of regulatory T cells. In addition, PM10-treated DCs produced a number of inflammatory cytokines that would be predicted to enhance tissue inflammation and promote various effector T cell responses. Following inhalation of PM10, these pro-inflammatory effects may contribute to immune dysregulation in the airway, promoting disease exacerbation in asthmatic individuals, for instance. Currently, from a public health perspective, greater emphasis is placed on the effects of particles smaller that PM10 and the European Union has stricter yearly average limits for PM2.5 than for PM10.

Our data suggests, however, that the potential for PM10 to have detrimental effects should be reconsidered. Many of the pro-inflammatory effects of PM10 on DCs were dependent, at least in part, on TLR4 signalling. It is likely that these effects are due to environmentally acquired bacterial endotoxins, which are potent TLR4 agonists adsorbed to the particles. However, not all TLR4 ligands are microbial in origin (Peri & Calabrese, 2015) and it remains possible that PM10 contain an alternative, as yet unidentified, activator of TLR4. PM10 induced production of inflammasome dependent cytokines such as IL-1β and IL-18. They may also deliver the first signal required for inflammasome activation via TLR4, although a role for other TLRs or similar molecules has not been excluded. TLR signalling alone is insufficient to activate the inflammasome and the nature of the second essential signal provided by PM10 is unknown. One possibility is that the particulate nature of the material provides this second signal as other particulates such as silica crystals (Sayan & Mossman, 2016) have been shown to fulfil this role. In addition, it remains a theoretical possibility that PM10 induces IL-1β production by a means

that does not require classical caspase-1 dependent inflammasome activity; this possibility requires further investigation.

PM10 induced AhR signalling in DC as indicated by increased expression of specific AhR-dependent genes. The PM constituents responsible for this effect have not been established although likely candidates are aromatic hydrocarbons, known AhR ligands, found within PM (B. Wang et al., 2013). Interestingly, expression of AhR-dependent genes was detected in sputum DCs and macrophages analysed *ex vivo*, suggesting activation of this pathway *in vivo*. Levels of expression were highly variable and could reflect variations in natural exposure to pollution. This would merit further investigation with subjects rigorously screened for their pollution exposure. In general, the functional significance of AhR signalling in DC is poorly understood. AhR is not ubiquitously expressed and therefore its presence in DCs presumably fulfils a specific function. Although AhR has been reported to play a role in maturation of some DCs (Chaokui Wang et al., 2014), no evidence was found in this study of a role for AhR in PM-induced DC maturation. AhR signalling can have a pro-regulatory role by inducing the typtophan-depleting enzyme IDO (Stockinger et al., 2014). However, although IDO expression was induced by PM10 on our system, several lines of evidence suggested it was predominately induced via an AhR-independent mechanism, likely related to TLR4. Irrespective of the precise pathway by which it is induced, IDO may act to attenuate some of the other more inflammatory effects of PM10 and this possibility merits further investigation.

The third key finding of this study is that the combustion source from which ambient PM is derived influences their effects on DCs. Although PM derived predominately from the burning of fossil fuel or from biomass had similar effects on several aspects of DC activation, including maturation and cytokine production, there was also evidence of distinct effects associated with each type of PM. Firstly, although both types of PM induced AhR signalling, the magnitude of induction of AhR-dependent genes was consistently higher following stimulation with BM-derived PM, irrespective of the size of the particle. Since combustion source is likely to be the major determinant of PM composition, it is likely that this difference reflects a difference in composition, possibly with regard to the aromatic hydrocarbons that can act as AhR ligands. Most strikingly, BM-derived PM, but not FF-derived PM, induced dramatic changes in the DCs that were highly suggestive of altered cellular metabolism. This was evident as increased cell-associated autofluorescence in viable DCs following exposure to BM PM10 or BM PM2.5 that could be attributed, at least in part, to increased NAD(P)H. This interpretation was confirmed by preliminary biochemical analysis showing increased NADH in DCs treated with BM-derived PM.

NADH plays an important role in cell metabolism and in recent years it has become clear that metabolism and immune function are closely linked (O'Neill & Pearce, 2015). However, the full characterisation of the altered metabolism in DCs treated with BM-derived PM and the significance of these changes for the function of DCs remain to be determined in future work. In addition, it will be interesting to determine whether there is any link between altered metabolism and high levels of AhR signalling since both of the effects were determined to be specifically associated with BM-derived PM. It has been shown in mice that AhR deficiency leads to an increased insulin sensitivity and a reduced hepatic PPAR-α pathway activity, indicating a link between glucose metabolism and AhR signalling (Chun Wang et al., 2011). Addressing the significance of this finding would be an important next step of this project. Also, in order to truly understand the different effects induced by fossil fuel and biomass particles having more information on the contribution of each combustion source on the particle composition would be ideal.

All in all, the work in this thesis has shown that the effects of air pollution particles on DCs are complex and multiple pathways contribute to the overall activation of cells. How exactly different types of particles influence these pathways and what the exact outcomes are of the combination of different pathways on DCs will be the subject of further study.

The next steps of this project would include continuing the measurement of NADH with the colorimetric assay in order to confirm the metabolic changes, as we have only shown one experiment. Also, the characterization of the particles would need to be addressed. Further next steps can be seen under the 'Future Work' section, which also addresses the remaining questions raised in this thesis.

8. Future work

The findings resulting from this thesis can be taken further by addressing the following aspects and conducting the corresponding experiments.

During the course of the work of this thesis we were not able to find a definite answer to the question what the impact of AhR signalling on DCs in response to PM exposure is. Using transcriptomics technologies, such as RNA sequencing would allow us to understand the implications PM exposure has on the DCs by measuring which genes are going to be upregulated. Especially in comparison to PCR, which averages the gene expression over the cell population, high-throughput sequencing it would allow us to perform an analysis on a single cell level, which would take into account the heterogeneity of each cell. This method will take into

account all the possible genes that are upregulated due to PM stimulation and will therefore give us information not only about the effects of AhR signalling, but also about other pathways that are stimulated upon PM exposure. This would take into account the complex nature of particles, which may lead to a wide range of pathways being induced in DCs.

As the complex mixture of components of PM is an important determinant when assessing the impact of air pollution on DCs, the exact composition of the different particle types should be assessed. Since composition, size and physicochemical properties are closely linked, these should be all measured, making sure a complete picture of particle properties and composition is gained, especially since toxicity is size-dependent. Surface charge and particle size could be assessed with a Zetasizer, which performs the measurement using dynamic light scattering in suspension. This ensures that the actual size of the particle is measured while they are in the cell culture medium so it provides information about size and charge of the particle when it interacts with the cell. The carbon content of PM can vary between particle sizes, fine PM tend to have a higher carbon proportion as larger PM and we saw that the carbon core is inert and does not influence the activation of MoDCs. However, a larger carbon core and a larger specific surface area means that fine particles can adsorb more than coarse PM. The total carbon content can be measured with a carbon analyser, a widely used method. This method works by combusting a known amount of material in the presence of excess oxygen which leads to the production of water vapour and carbon dioxide and the carbon dioxide can then be further analysed, for example using infrared detection. PAH, known to induce AhR signalling, can be measured using gas chromatograph-mass spectrometer, which combines gas-chromatography and mass spectrometry, allowing one to determine the concentration of a range hydrocarbons. It needs to be distinguished between high and low molecular weight PAHs, as high molecular weight PAHs tend to be associated with smaller PM. Also the concentration of metals on the surface of PM can differ between particle sizes. Similar to PAH content, smaller particles tend to have a higher proportion of metals compared to coarse PM. Metal content can be measured by an inductively coupled plasma−atomic emission spectrometry, which is a type of emission spectrometry that permits the measurement of trace metals. Finally, endotoxin content can be assessed by a Limulus Amebocyte Lysate assay and a DTT assay would provide information about the oxidative potential of PM. All these components of PM influence the toxicity of the particle and would therefore allow us to understand how size and therefore composition of the particles affect the activation of DCs (B. Wang et al., 2013).

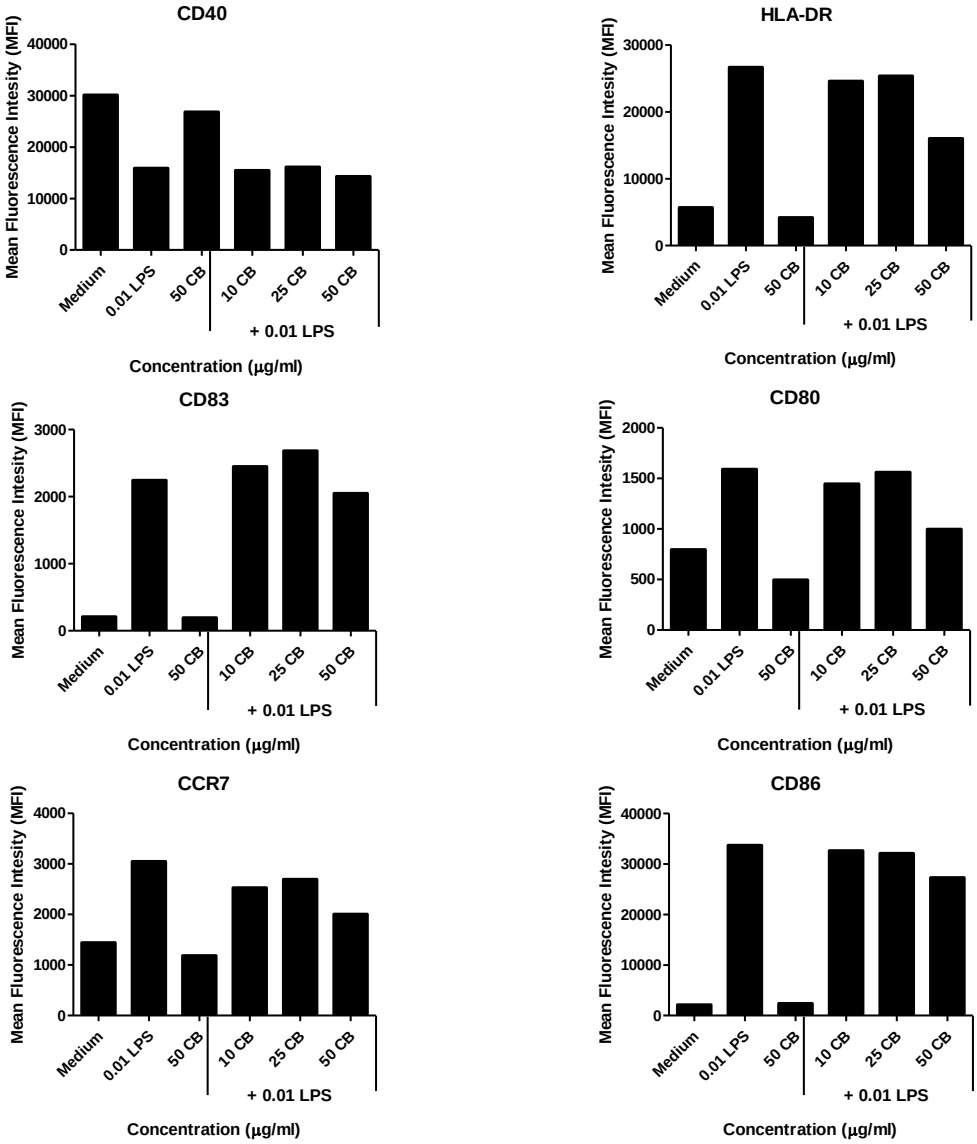
Also, an important consideration when analysing the chemical composition and physical properties of PM is to take onto account these measurements on single particles as well as in the bulk. Most measurements focus on the bulk of the particles, as these techniques focus on ensemble measurements rather than a single particle. Therefore, using a technique that can distinguish between the chemical composition of the bulk as a mixture and single particles will help understand the effects the composition has on cells. Secondary ion mass spectrometry can distinguish between the single particle surface and the surface of the bulk material (D. Huang et al., 2017). Furthermore, taking into account that the effects of these particles were measured *in vitro* it also needs to be considered how the cell culture medium affects the surface of the particles as it is known that proteins from the medium adsorb to the surface of the particles forming a protein corona. The protein corona can then affect the surface charge of the particle and composition as well as the interaction with the cell surface during *in vitro* studies (Gräfe et al., 2016).

All these characteristics, when measured for all particle types used in our study, would give us a good overall picture of their properties, which would allow us to do a more detailed analysis of our results.

Characterising particles would not only allow us to understand how exactly the composition is altered between particles from different combustion sources but also how these differences affect the outcomes in DCs. One clear difference between combustion sources was the induction of NADH, which was significantly increased after stimulation of DCs with BM PM, but not after stimulation with FF PM. NADH concentration is indicative of metabolic changes in cells; therefore, performing a Seahorse analysis of the cells would measure a wide range of metabolic processes connected to glycolysis and respiration. Analysing different aspects of metabolic alterations in cells in response to FF PM as well as BM PM would let us understand which signalling pathways may be affected. This would ultimately allow us to draw conclusions about the immunological changes within the cell as well as the health outcomes for individuals exposed to this type of air pollution.

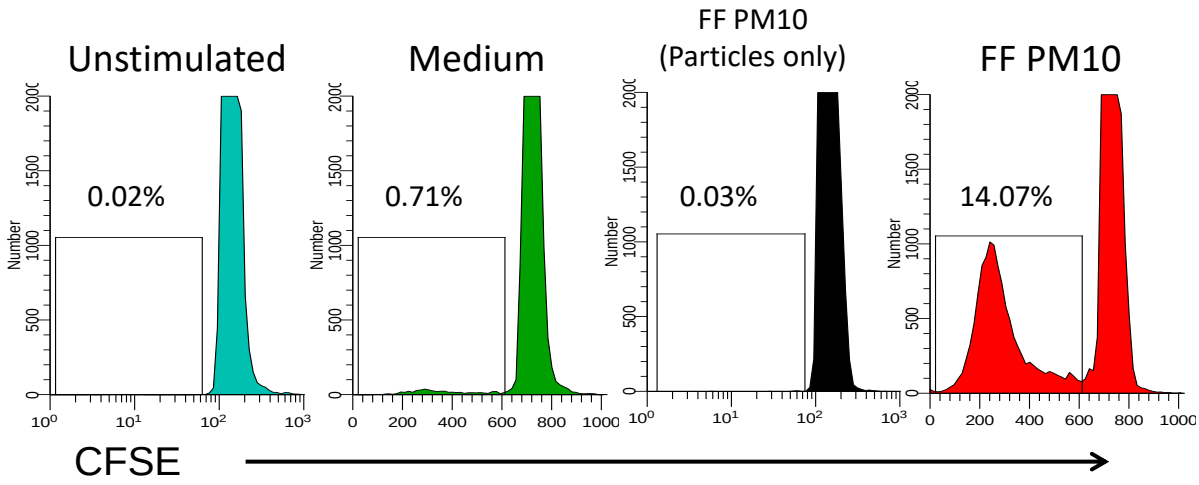
As a next step, one could look into how other cell types in addition to DCs such as macrophages and epithelial cells contribute to the immune responses arising from PM exposure. This would allow one to see how these different cell types interact and influence each other, which would shed further light on the complex health effects of air pollution. Also, moving towards an *in vivo* model to examine effects of PM would give us an approach that is closer to human exposure and therefore would provide a more realistic picture of the health effects of air pollution.

9. Appendix



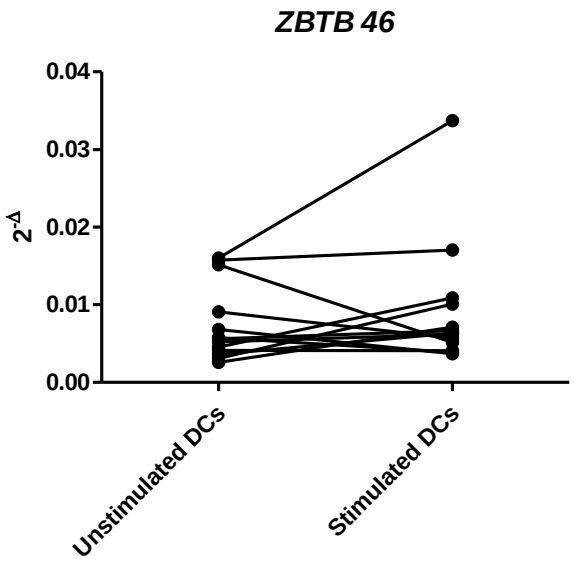
Appendix Figure 9.1: LPS bound on a particle has similar effects on dendritic cell maturation as in solution

Human MoDCs were stimulated for 48h with 50µg/ml CB or medium only as well as 0.01µg/ml soluble LPS and the same concentration of LPS bound on CB particles as a particle control. The particle control was added at concentrations of 10, 25 and 50µg/ml. After stimulation, cells were fluorescently labelled and the surface expression of CD40, HLA-DR, CD83, CD80, CCR7 and CD86 was measured. The graphs show one experiment representing one blood donor with no technical replicates.



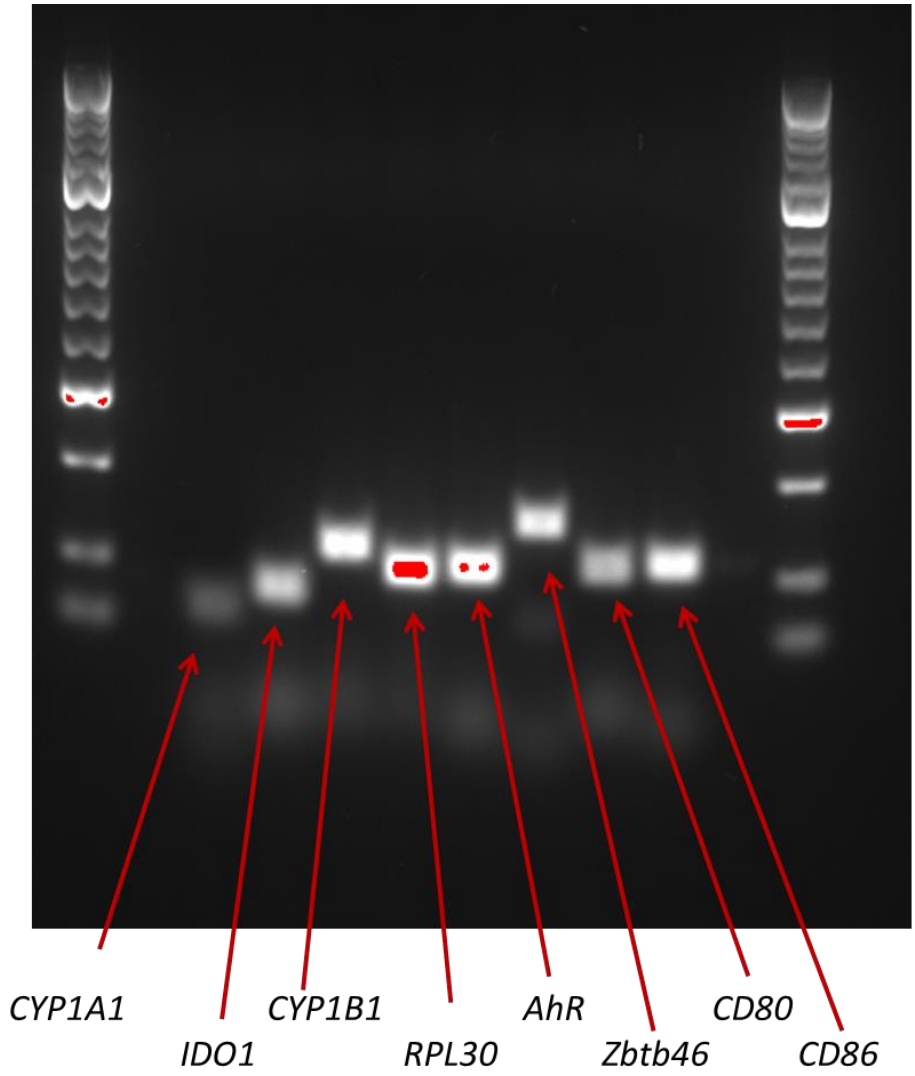
Appendix Figure 9.2: FF PM10 is suspension do not induce T cell proliferation in a MLR

DCs were derived from human blood monocytes and were exposed to 25µg/ml FF PM10 and medium only. After 48h of exposure, cells were washed and 300 DCs were plated with 400,000 naïve T cells, which were labelled with CFSE beforehand to track their proliferation. T cells were also incubated with 25µg/ml FF PM10 directly or in medium only without the addition of DCs. The MLR was cultured for 5 days and measured using flow-cytometry afterwards. The graph shows the flow-cytometry plots of CFSE labelled unstimulated T cells as well as T cells stimulated with medium only exposed DCs, FF PM10 stimulated DCs and T cells cultured with the particle suspension. The plots show one representative example out of six independent experiments using six independent blood donors.



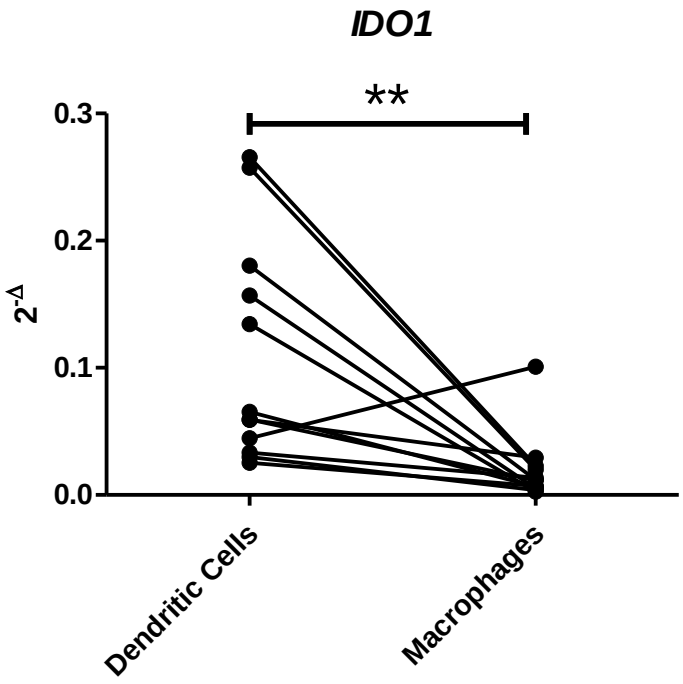
Appendix Figure 9.3: Stimulation of DCs does not influence their expression of Zbtb46

Human DCs were derived from CD14^{+} monocytes and stimulated with 50µg/ml FF PM10 or medium only for 2 days. After incubation, the expression of the transcription factor Zbtb46 was measured using PCR. The graph shows 13 independent PCR experiments conducted by analysing two technical replicates, each dot representing one independent blood donor. Statistical analysis conducted by using a paired two-tailed t test did not reveal a significant difference between the two conditions.



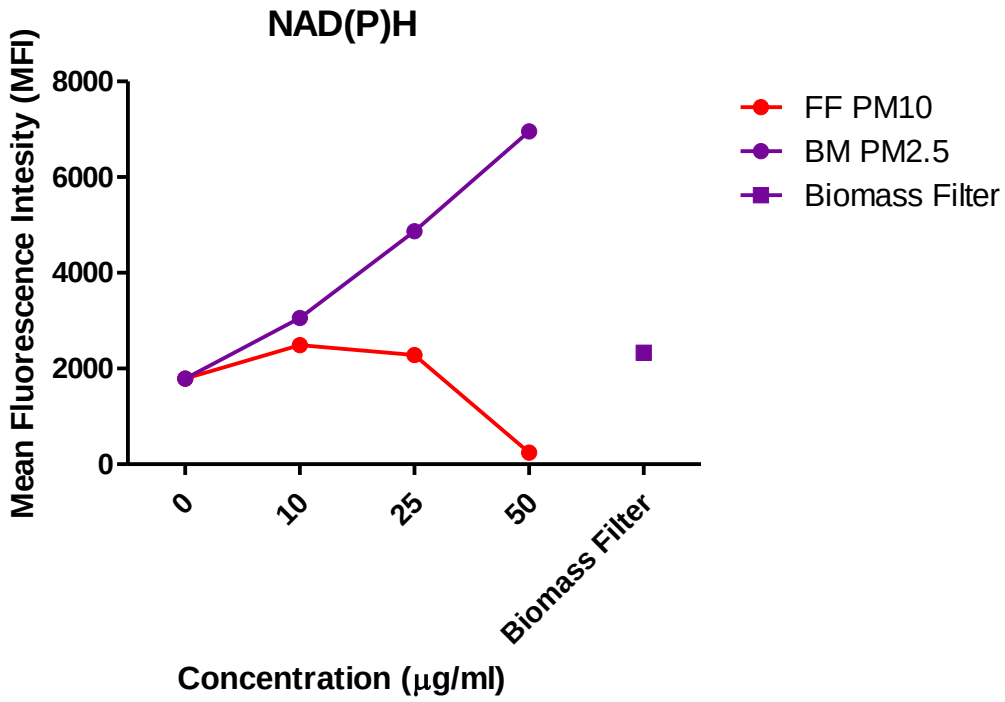
Appendix Figure 9.4: Unstimulated sputum DCs express AhR, AhR-related genes, maturation markers as well as ZBTB46 and IDO1

Sputum donated by healthy volunteers was stimulated with 50µg/ml of FF PM10 or medium only for 2-4 hours. After incubation, cells were antibody labelled for CD3, CD14, CD16, CD19, CD20 and CD56 contained in the lineage cocktail as well as HLA-DR. Stimulated and unstimulated DCs were directly sorted into a lysis buffer and lysed through vortexing. After disruption of the cells, gene expression of cells was measured using quantitative real-time PCR, followed by qualitative PCR. The agarose gel shows the electrophoretic separation of the PCR product CYP1A1, IDO1, CYP1B1, RPL30, AhR, Zbtb46, CD80 and CD86 in unstimulated DCs. The gel illustrates one experiment from one blood donor, without technical replicates.



Appendix Figure 9.5: Sputum DCs express significantly higher levels of IDO1 than sputum macrophages

After processing induced sputum samples from healthy donors, the cell suspension was incubated in medium only for 2-4hours. Subsequently, cells were labelled with antibodies for HLA-DR, CD3, CD14, CD16, CD19, CD20 and CD56, cell sorted and lysed, followed by real-time PCR analysis. The graph illustrates the expression of IDO1 in unstimulated ex vivo DCs and macrophages, showing 11 independent experiments from 11 different sputum donors. For the PCR, two technical repeats were conducted.



Appendix Figure 9.6: Material from the biomass filter does not have any effect on the autofluorescence of DCs.

Human MoDCs were stimulated for 2 days with 10, 25 and 50µg/ml FF PM10 and BM PM2.5 or medium only. Also, empty filters that were used for collecting biomass particles were put through the same extraction procedure and the suspension was used to stimulate DCs. After 48h cells were measured using the UV laser by flow-cytometry. The graph shows one experiment conducted without technical replicates using one blood donor.

10. References

Abelson, M. B., Gamache, D., & McLaughlin, J. (2014). Innate Immunity : A Question of Balance. *Review of Ophthalmology*, 70–90.

Abu-rezq, H. A., & Millar, D. G. (2013). Effects of aryl-hydrocarbon ligands on dendritic cell maturation, *1*(3), 24–33. http://doi.org/10.11648/j.iji.20130103.11

Akhtar, U. S., Rastogi, N., McWhinney, R. D., Urch, B., Chow, C. W., Evans, G. J., & Scott, J. A. (2014). The combined effects of physicochemical properties of size-fractionated ambient particulate matter on in vitro toxicity in human A549 lung epithelial cells. *Toxicology Reports*, *1*, 145–156. http://doi.org/10.1016/j.toxrep.2014.05.002

Alexis, N. E., & Carlsten, C. (2014). Interplay of air pollution and asthma immunopathogenesis: A focused review of diesel exhaust and ozone. *International Immunopharmacology*, *23*(1), 347–355. http://doi.org/10.1016/j.intimp.2014.08.009

American Academy of Pediatrics. (2004). Ambient Air Pollution: Health Hazards to Children. *Pediatrics*, *114*(6), 1699–1707. http://doi.org/10.1542/peds.2004-2166

American Thoracic Society. (2000). *What Constitutes an Adverse Health Effect of Air Pollution? Am J Respir Crit Care Med* (Vol. 161). Retrieved from http://www.atsjournals.org/doi/abs/10.1164/ajrccm.161.2.ats4-00

Andrysík, Z., Vondráček, J., Marvanová, S., Ciganek, M., Neča, J., Pěnčíková, K., … Machala, M. (2011). Activation of the aryl hydrocarbon receptor is the major toxic mode of action of an organic extract of a reference urban dust particulate matter mixture: the role of polycyclic aromatic hydrocarbons. *Mutation Research*, *714*(1–2), 53–62. http://doi.org/10.1016/j.mrfmmm.2011.06.011

Araujo, J. A., Barajas, B., Kleinman, M., Wang, X., Bennett, B. J., Gong, W., … Geffen, D. (2011). Ambient Particulate Pollutants in the Ultrafine Range Promote Early Atherosclerosis and Systemic Oxidative Stress, *102*(5), 589–596. http://doi.org/10.1161/CIRCRESAHA.107.164970.Ambient

Araujo, J. a, & Nel, A. E. (2009a). Particulate matter and atherosclerosis: role of particle size, composition and oxidative stress. *Particle and Fibre Toxicology*, *6*, 24. http://doi.org/10.1186/1743-8977-6-24

Araujo, J. a, & Nel, A. E. (2009b). Particulate matter and atherosclerosis: role of particle size, composition and oxidative stress. *Particle and Fibre Toxicology*, *6*, 24.

http://doi.org/10.1186/1743-8977-6-24

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y., … Palucka, K. (2000a). Immunobiology of dendritic cells, *18*, 767–811.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y., … Palucka, K. (2000b). Imunobiology of Dendritic Cells, (Figure 1), 767–811.

Banchereau, J., & Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature*, *392*(6673), 245–52. http://doi.org/10.1038/32588

Barnes, P. J. (2001). Th2 cytokines and asthma: an introduction. *Respiratory Research*, *2*(2), 64–5. http://doi.org/10.1186/rr39

Barnett, A. G., Williams, G. M., Schwartz, J., Neller, A. H., Best, T. L., Petroeschevsky, A. L., & Simpson, R. W. (2005). Air pollution and child respiratory health: A case-crossover study in Australia and New Zealand. *American Journal of Respiratory and Critical Care Medicine*, *171*(11), 1272–1278. http://doi.org/10.1164/rccm.200411-1586OC

Bauer, R. N., Diaz-Sanchez, D., & Jaspers, I. (2012). Effects of air pollutants on innate immunity: The role of Toll-like receptors and nucleotide-binding oligomerization domain-like receptors. *Journal of Allergy and Clinical Immunology*, *129*(1), 14–24. http://doi.org/10.1016/j.jaci.2011.11.004

Bayer-Oglesby, L., Schindler, C., Hazenkamp-Von Arx, M. E., Braun-Fahrländer, C., Keidel, D., Rapp, R., … Ackermann-Liebrich, U. (2006). Living near main streets and respiratory symptoms in adults: The Swiss Cohort Study on Air Pollution and Lung Diseases in Adults. *American Journal of Epidemiology*, *164*(12), 1190–1198. http://doi.org/10.1093/aje/kwj338

Beischlag, T. V, Luis Morales, J., Hollingshead, B. D., & Perdew, G. H. (2008). The aryl hydrocarbon receptor complex and the control of gene expression. *Critical Reviews in Eukaryotic Gene Expression*, *18*(3), 207–250. http://doi.org/10.2964/jsik.kuni0223

Bell, D., Young, J. W., & Banchereau, J. (1999). Dendritic Cells. *Advances in Immunology*, *72*, 255–324.

Bell, M. L., Davis, D. L., & Fletcher, T. (2003). A Retrospective Assessment of Mortality from the London Smog Episode of 1952: The Role of Influenza and Pollution. *Environmental Health Perspectives*, *112*(1), 6–8. http://doi.org/10.1289/ehp.6539

Bevan, M. J. (2006). Cross-priming. *Nature Immunology*, *7*(4), 363–365. http://doi.org/10.1038/ni0406-363

Bezemer, G. F. G., Bauer, S. M., Oberdörster, G., Breysse, P. N., Pieters, R. H. H., Georas, S. N., & Williams, M. a. (2011). Activation of pulmonary dendritic cells and Th2-type inflammatory responses on instillation of engineered, environmental diesel emission source or ambient air pollutant particles in vivo. *Journal of Innate Immunity*, *3*(2), 150–66. http://doi.org/10.1159/000321725

Blacker, T. S., & Duchen, M. R. (2016). Investigating mitochondrial redox state using NADH and NADPH autofluorescence. *Free Radical Biology and Medicine*, *100*, 53–65. http://doi.org/10.1016/j.freeradbiomed.2016.08.010

Blacker, T. S., Mann, Z. F., Gale, J. E., Ziegler, M., Bain, A. J., Szabadkai, G., & Duchen, M. R. (2014). Separating NADH and NADPH fluorescence in live cells and tissues using FLIM. *Nature Communications*, *5*(May), 3936. http://doi.org/10.1038/ncomms4936

Blasius, A. L., & Beutler, B. (2010). Intracellular Toll-like Receptors. *Immunity*, *32*(3), 305–315. http://doi.org/10.1016/j.immuni.2010.03.012

Bleck, B., Kazeros, A., Bakal, K., Garcia-Medina, L., Adams, A., Liu, M., … Reibman, J. (2015). Coexpression of type 2 immune targets in sputum-derived epithelial and dendritic cells from asthmatic subjects. *Journal of Allergy and Clinical Immunology*, *136*(3), 619–627.e5. http://doi.org/10.1016/j.jaci.2014.12.1950

Bleck, B., Tse, D. B., Gordon, T., Mohammad, R., & Reibman, J. (2010). Diesel Exhaust Particle-Treated Human Bronchial Epithelial Cells Upregulate Jagged-1 and OX40 Ligand in Myeloid Dendritic Cells via Thymic Stromal Lymphopoietin. *Journal of Immunology*, *185*, 6636–6645. http://doi.org/10.4049/jimmunol.1000719

Bleck, B., Tse, D. B., Jaspers, I., Curotto, M. A., Lafaille, D., Reibman, J., … Curotto de Lafaille, M. a. (2006). Diesel exhaust particle-exposed human bronchial epithelial cells induce dendritic cell maturation. *Journal of Immunology (Baltimore, Md. : 1950)*, *176*(12), 7431–7. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2757761&tool=pmcentrez&rendertype=abstract

Bleck, B., Tse, D. B., Lafaille, M. A. C. De, & Zhang, F. (2008). Diesel Exhaust Perticle-Exposed Human Bronchial Epithelial Cells Induce Dendritic Cell Maturation and Polarization via Thymic Stromal Lymphopoietin, *28*(2), 147–156. http://doi.org/10.1007/s10875-007-9149-0.Diesel

Bovallius, A., Bucht, B., Roffey, R., & Anas, P. (1978). Three year investigation of the natural

airborne bacterial flora at four localities in Sweden. *Applied and Environmental Microbiology*, *35*(5), 847–852. http://doi.org/10.5194/acp-9-9263-2009

Breton, G., Lee, J., Zhou, Y. J., Schreiber, J. J., Keler, T., Puhr, S., … Nussenzweig, M. C. (2015). Circulating precursors of human CD1c+ and CD141+ dendritic cells. *Journal of Experimental Medicine*, *212*(3), 401–413. http://doi.org/10.1084/jem.20141441

Brook, R. D. (2008). Cardiovascular effects of air pollution. *Clinical Science (London, England : 1979)*, *115*(6), 175–87. http://doi.org/10.1042/CS20070444

Brook, R. D., Franklin, B., Cascio, W., Hong, Y., Howard, G., Lipsett, M., … Tager, I. (2004a). Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation*, *109*(21), 2655–71. http://doi.org/10.1161/01.CIR.0000128587.30041.C8

Brook, R. D., Franklin, B., Cascio, W., Hong, Y., Howard, G., Lipsett, M., … Tager, I. (2004b). Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation*, *109*(21), 2655–71. http://doi.org/10.1161/01.CIR.0000128587.30041.C8

Brown, D. M., Hutchison, L., Donaldson, K., & Stone, V. (2007). The effects of PM10 particles and oxidative stress on macrophages and lung epithelial cells: modulating effects of calcium-signaling antagonists. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *292*(6), L1444-51. http://doi.org/10.1152/ajplung.00162.2006

Brubaker, S. W., Bonham, K. S., Zanoni, I., & Kagan, J. C. (2014). *Innate Immune Pattern Recognition: A Cell Biological Perspective*. *Annual Review of Immunology* (Vol. 33). http://doi.org/10.1146/annurev-immunol-032414-112240

Brugha, R., Mushtaq, N., Mccarthy, N. E., Stagg, A. J., & Grigg, J. (2015). Respiratory tract dendritic cells in paediatric asthma. *Clinical and Experimental Allergy*, *45*(3), 624–631. http://doi.org/10.1111/cea.12457

Brunekreef, B., & Holgate, S. T. (2002). Air pollution and health. *Lance*, *360*(9341), 1233–42. http://doi.org/10.1016/S0140-6736(02)11274-8

Burri, P. H. (2011). Development and Growth of the Human Lung. In *Comprehensive Physiology*. John Wiley & Sons, Inc. http://doi.org/10.1002/cphy.cp030101

Carey, I. M., Atkinson, R. W., Kent, A. J., Van Staa, T., Cook, D. G., & Anderson, H. R. (2013). Mortality associations with long-term exposure to outdoor air pollution in a national English cohort. *American Journal of Respiratory and Critical Care Medicine*, *187*(11), 1226–

1233. http://doi.org/10.1164/rccm.201210-1758OC

Cella, M., & Colonna, M. (2015). Aryl hydrocarbon receptor: Linking environment to immunity. *Seminars in Immunology*, *27*(5), 310–314. http://doi.org/10.1016/j.smim.2015.10.002

Chen, G., & Nunez, G. (2010). Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol*, *10*, 826–837.

Chen, G., & Pedra, J. H. F. (2010). The inflammasome in host defense. *Sensors (Basel, Switzerland)*, *10*(1), 97–111. http://doi.org/10.3390/s100100097

Chen, H., Kwong, J., Copes, R., Tu, K., Villeneuve, P. J., van Donkelaar, A., … Burnett, R. (2017). Living near major roads and the incidence of dementia, Parkinson's disease, and multiple sclerosis: a population-based cohort study. *Lancet*.

Chiaro, C. R., Patel, R. D., Marcus, C. B., & Perdew, G. H. (2007). Evidence for an Aryl Hydrocarbon Receptor-Mediated Cytochrome P450 Autoregulatory Pathway, *72*(5), 1369–1379. http://doi.org/10.1124/mol.107.038968.receptor

Chiu, Y.-H., MacMillan, J. B., & Chen, Z. J. (2009). RNA Polymerase III Detects Cytosolic DNA and Induces Type-I Interferons Through the RIG-I Pathway. *Cell*, *138*(3), 576–591. http://doi.org/10.1016/j.cell.2009.06.015.RNA

Choi, E.-Y., Lee, H., Dingle, R. W. C., Kim, K. B., & Swanson, H. I. (2012). Development of novel CH223191-based antagonists of the aryl hydrocarbon receptor. *Molecular Pharmacology*, *81*(1), 3–11. http://doi.org/10.1124/mol.111.073643

Choudhary, D., Jansson, I., Schenkman, J. B., Sarfarazi, M., & Stoilov, I. (2003). Comparative expression profiling of 40 mouse cytochrome P450 genes in embryonic and adult tissues. *Archives of Biochemistry and Biophysics*, *414*(1), 91–100. http://doi.org/10.1016/S0003-9861(03)00174-7

Chow, A., Brown, B. D., & Merad, M. (2011). Studying the mononuclear phagocyte system in the molecular age. *Nature Reviews. Immunology*, *11*(11), 788–98. http://doi.org/10.1038/nri3087

Chow, J. C., Watson, J. G., Mauderly, J. L., Costa, D. L., Wyzga, R. E., Vedal, S., … Dockery, D. W. (2006). Health effects of fine particulate air pollution: lines that connect. *Journal of the Air & Waste Management Association (1995)*, *56*(10), 1368–80. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17063860

Chung, C. Y. J., Ysebaert, D., Berneman, Z. N., & Cools, N. (2013). Dendritic cells: cellular

mediators for immunological tolerance. *Clinical & Developmental Immunology*, *2013*, 972865. http://doi.org/10.1155/2013/972865

Cisse, B., Caton, M. L., Lehner, M., Maeda, T., Scheu, S., Locksley, R., … Reizis, B. (2008). Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development, *135*(1), 37–48. http://doi.org/10.1016/j.cell.2008.09.016.Transcription

Claire E. McCoy. (2016). *Toll-Like Receptors*.

Clark, N. A., Demers, P. A., Karr, C. J., Koehoorn, M., Lencar, C., Tamburic, L., & Brauer, M. (2010). Effect of early life exposure to air pollution on development of childhood asthma. *Environmental Health Perspectives*, *118*(2), 284–290. http://doi.org/10.1289/ehp.0900916

Cohen, A. J., Anderson, H. R., Ostro, B., Pandey, D., Krzyzanowski, M., Küünzli, N., … Smith, K. (2006). The Global Burden of Disease Due to Outdoor Air Pollution. *Journal of Toxicology and Environmental Health, Part A*, *1528739059*(September 2011), 1301–1307. http://doi.org/10.1080/15287390590936166

Cohn, L., Chatterjee, B., Esselborn, F., Smed-Sörensen, A., Nakamura, N., Chalouni, C., … Delamarre, L. (2013). Antigen delivery to early endosomes eliminates the superiority of human blood BDCA3+ dendritic cells at cross presentation. *The Journal of Experimental Medicine*, *210*(5), 1049–63. http://doi.org/10.1084/jem.20121251

Cole PJ. (1986). Inflammation: a two-edged sword--the model of bronchiectasis. *Eur J Respir Dis Suppl.*, (147), 6–15.

Collin, M., McGovern, N., & Haniffa, M. (2013). Human dendritic cell subsets. *Immunology*, *140*(1), 22–30. http://doi.org/10.1111/imm.12117

Collins, A. V., Brodie, D. W., Gilbert, R. J. C., Iaboni, A., Manso-Sancho, R., Walse, B., … Davis, S. J. (2002). The interaction properties of costimulatory molecules revisited. *Immunity*, *17*(2), 201–210. http://doi.org/10.1016/S1074-7613(02)00362-X

Cook, P. C., Jones, L. H., Jenkins, S. J., Wynn, T. A., Allen, J. E., & MacDonald, A. S. (2012). Alternatively activated dendritic cells regulate CD4+ T-cell polarization in vitro and in vivo. *Proceedings of the National Academy of Sciences*, *109*(25), 9977–9982. http://doi.org/10.1073/pnas.1121231109

Cook, P. C., & MacDonald, A. S. (2016). Dendritic cells in lung immunopathology. *Seminars in Immunopathology*, 449–460. http://doi.org/10.1007/s00281-016-0571-3

D'Amato, G., Cecchi, L., D'Amato, M., & Liccardi, G. (2010). Urban air pollution and climate

change as environmental risk factors of respiratory allergy: an update. *Journal of Investigational Allergology & Clinical Immunology*, *20*(2), 95–102; quiz following 102. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20461963

Dagenais, M., Skeldon, A., & Saleh, M. (2012). The inflammasome: in memory of Dr. Jurg Tschopp. *Cell Death and Differentiation*, *19*(1), 5–12. http://doi.org/10.1038/cdd.2011.159

Darrieutort-Laffite, C., Boutet, M. A., Chatelais, M., Brion, R., Blanchard, F., Heymann, D., & Le Goff, B. (2014). IL-1 β and TNF α promote monocyte viability through the induction of GM-CSF expression by rheumatoid arthritis synovial fibroblasts. *Mediators of Inflammation*, *2014*. http://doi.org/10.1155/2014/241840

de Baey, a, & Lanzavecchia, a. (2000). The role of aquaporins in dendritic cell macropinocytosis. *The Journal of Experimental Medicine*, *191*(4), 743–8. http://doi.org/10.1084/jem.191.4.743

de Heer, H. J., Hammad, H., Soullié, T., Hijdra, D., Vos, N., Willart, M. a M., … Lambrecht, B. N. (2004). Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *The Journal of Experimental Medicine*, *200*(1), 89–98. http://doi.org/10.1084/jem.20040035

Defranco, E., Moravec, W., Xu, F., Hall, E., Hossain, M., Haynes, E. N., … Chen, A. (2016). Exposure to airborne particulate matter during pregnancy is associated with preterm birth: a population-based cohort study. *Environmental Health*, 1–8. http://doi.org/10.1186/s12940-016-0094-3

Dennehy, K. M., & Brown, G. D. (2007). The role of the beta-glucan receptor Dectin-1 in control of fungal infection. *Journal of Leukocyte Biology*, *82*(2), 253–258. http://doi.org/10.1189/jlb.1206753

Desch, N. A., Henson, P. M., & Jakubzick, C. (2013). Pulmonary dendritic cell development and antigen acquisition. *Immunologic Research*, *55*(0), 178–186. http://doi.org/10.1016/j.biotechadv.2011.08.021.Secreted

Dingjan, I., Verboogen, D. R., Paardekooper, L. M., Revelo, N. H., Sittig, S. P., Visser, L. J., … van den Bogaart, G. (2016). Lipid peroxidation causes endosomal antigen release for cross-presentation. *Scientific Reports*, *6*(February), 22064. http://doi.org/10.1038/srep22064

Dionisio, K. L., Howie, S. R. C., Dominici, F., Fornace, K. M., Spengler, J. D., Adegbola, R. a, & Ezzati, M. (2012). Household concentrations and exposure of children to particulate matter from biomass fuels in The Gambia. *Environmental Science & Technology*, *46*(6), 3519–27.

http://doi.org/10.1021/es203047e

Dockery, D. W., Pope, C. A., Xu, X., Spengler, J. D., Ware, J., Fay, M., … Speizer, F. (1993). An association between air pollution and mortality in six U.S. cities, (329), 1753–9.

Doherty, G. J., & McMahon, H. T. (2009). Mechanisms of endocytosis. *Annual Review of Biochemistry*, *78*, 857–902. http://doi.org/10.1146/annurev.biochem.78.081307.110540

Dostert, C., Pétrilli, V., Bruggen, R. Van, Steele, C., Mossman, B. T., & Tschopp, J. (2008). Innate Immune Activation Through Nalp3 Inflammasome Sensing of Asbestos and Silica, *320*(5876), 674–677.

Dutta, D., Sundaram, S. K., Teeguarden, J. G., Riley, B. J., Fifield, L. S., Jacobs, J. M., … Weber, T. J. (2007). Adsorbed proteins influence the biological activity and molecular targeting of nanomaterials. *Toxicological Sciences*, *100*(1), 303–315. http://doi.org/10.1093/toxsci/kfm217

Edelson, B. T., KC, W., Juang, R., Kohyama, M., Benoit, L. A., Klekotka, P. A., … Murphy, K. M. (2010). Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. *The Journal of Experimental Medicine*, *207*(4), 823–36. http://doi.org/10.1084/jem.20091627

Eggleston, P. A. (2000). Environmental causes of asthma in children. *Clinical Reviews in Allergy and Immunology*, *18*(5 S), 57–60. http://doi.org/10.1111/j.1399-3038.1994.tb00350.x

Eltom, S., Belvisi, M. G., Stevenson, C. S., Maher, S. A., Dubuis, E., Fitzgerald, K. A., & Birrell, M. A. (2014). Role of the inflammasome-caspase1/11-IL-1/18 axis in cigarette smoke driven airway inflammation: An insight into the pathogenesis of COPD. *PLoS ONE*, *9*(11). http://doi.org/10.1371/journal.pone.0112829

EPA., U. S. (2004). Air Quality Criteria for Particulate Matter (Final Report, Oct 2004). *U.S. Environmental Protection Agency, Washington, DC, EPA 600/P-99/002aF-bF*.

Esposito, K., Petrizzo, M., Maiorino, M. I., Bellastella, G., & Giugliano, D. (2016). Particulate matter pollutants and risk of type 2 diabetes: a time for concern? *Endocrine*, *51*(1), 32–37. http://doi.org/10.1007/s12020-015-0638-2

Esser, C., & Rannug, A. (2015). The Aryl Hydrocarbon Receptor in Barrier Organ Physiology, Immunology, and Toxicology. *Pharmacological Reviews*, *67*(2), 259–279. http://doi.org/10.1124/pr.114.009001

Fahy, J. V., Boushey, H. A., Lazarus, S. C., Mauger, E. A., Cherniack, R. M., Chinchilli, V. M., …

Szefler, S. J. (2001). Safety and reproducibility of sputum induction in asthmatic subjects in a multicenter study. *American Journal of Respiratory and Critical Care Medicine*, *163*(6), 1470–1475. http://doi.org/10.1164/ajrccm.163.6.9901105

Ferecatu, I., Borot, M.-C., Bossard, C., Leroux, M., Boggetto, N., Marano, F., … Andreau, K. (2010). Polycyclic aromatic hydrocarbon components contribute to the mitochondria-antiapoptotic effect of fine particulate matter on human bronchial epithelial cells via the aryl hydrocarbon receptor. *Particle and Fibre Toxicology*, *7*, 18. http://doi.org/10.1186/1743-8977-7-18

Fernandes-Alnemri, T., Yu, J.-W., Datta, P., Wu, J., & Alnemri, E. S. (2009). AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature*, *458*(7237), 509–13. http://doi.org/10.1038/nature07710

Fogg, D. K., Sibon, C., Miled, C., Jung, S., Aucouturier, P., Littman, D. R., … Geissmann, F. (2006). A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science (New York, N.Y.)*, *311*(November), 83–87. http://doi.org/10.1126/science.1117729

Förster, R., Davalos-Misslitz, A. C., & Rot, A. (2008). CCR7 and its ligands: balancing immunity and tolerance. *Nature Reviews. Immunology*, *8*(5), 362–71. http://doi.org/10.1038/nri2297

Frericks, M., Burgoon, L. D., Zacharewski, T. R., & Esser, C. (2008). Promoter analysis of TCDD-inducible genes in a thymic epithelial cell line indicates the potential for cell-specific transcription factor crosstalk in the AhR response. *Toxicology and Applied Pharmacology*, *232*(2), 268–279.

Frericks, M., Meissner, M., & Esser, C. (2007). Microarray analysis of the AHR system: Tissue-specific flexibility in signal and target genes. *Toxicology and Applied Pharmacology*, *220*(3), 320–332. http://doi.org/10.1016/j.taap.2007.01.014

Fritz, J. H., Le Bourhis, L., Sellge, G., Magalhaes, J. G., Fsihi, H., Kufer, T. A., … Philpott, D. J. (2007). Nod1-Mediated Innate Immune Recognition of Peptidoglycan Contributes to the Onset of Adaptive Immunity. *Immunity*, *26*(4), 445–459. http://doi.org/10.1016/j.immuni.2007.03.009

Fujieda, S., Diaz-Sanchez, D., & Saxon, A. (1998). Combined nasal challenge with diesel exhaust particles and allergen induces in vivo IgE isotype switching. *American Journal of Respiratory Cell and Molecular Biology*, *19*(3), 507–512. http://doi.org/10.1165/ajrcmb.19.3.3143

Fujii, T., Hayashi, S., Hogg, J. C., Vincent, R., & Van Eeden, S. F. (2001). Particulate matter induces cytokine expression in human bronchial epithelial cells. *American Journal of Respiratory*

Cell and Molecular Biology, *25*(3), 265–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11588002

Fujii, T., Hayashi, S., Hogg, J., Mukae, H., Suwa, T., Goto, Y., … van Eeden, S. (2002). Interaction of Alveolar Macrophages and Airway Epithelial Cells Following Exposure to Particulate Matter Produces Mediators that Stimulate the Bone Marrow. *Am. J. Respir. Cell Mol. Biol.*, *27*, 34–41. http://doi.org/10.1165/ajrcmb.27.1.4787

Fujikado, N., Saijo, S., Yonezawa, T., Shimamori, K., Ishii, A., Sugai, S., … Iwakura, Y. (2008). Dcir deficiency causes development of autoimmune diseases in mice due to excess expansion of dendritic cells. *Nature Medicine*, *14*(2), 176–180. http://doi.org/10.1038/nm1697

Furuhashi, K., Suda, T., Hasegawa, H., Suzuki, Y., Hashimoto, D., Enomoto, N., … Chida, K. (2012). Mouse lung CD103+ and CD11bhigh dendritic cells preferentially induce distinct CD4+ T-cell responses. *American Journal of Respiratory Cell and Molecular Biology*, *46*(2), 165–72. http://doi.org/10.1165/rcmb.2011-0070OC

Geiser, M., Rothen-Rutishauser, B., Kapp, N., Schürch, S., Kreyling, W., Schulz, H., … Gehr, P. (2005). Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. *Environmental Health Perspectives*, *113*(11), 1555–1560. http://doi.org/10.1289/ehp.8006

Georgakoudi, I., Jacobson, B. C., Müller, M. G., Sheets, E. E., Badizadegan, K., Carr-Locke, D. L., … Feld, M. S. (2002). NAD(P)H and collagen as in vivo quantitative fluorescent biomarkers of epithelial precancerous changes. *Cancer Research*, *62*(3), 682–687.

GeurtsvanKessel, C. H., & Lambrecht, B. N. (2008). Division of labor between dendritic cell subsets of the lung. *Mucosal Immunology*, 1–9.

GeurtsvanKessel, C. H., Willart, M. a M., van Rijt, L. S., Muskens, F., Kool, M., Baas, C., … Lambrecht, B. N. (2008). Clearance of influenza virus from the lung depends on migratory langerin+CD11b- but not plasmacytoid dendritic cells. *The Journal of Experimental Medicine*, *205*(7), 1621–34. http://doi.org/10.1084/jem.20071365

Ghiringhelli, F., Apetoh, L., Tesniere, A., Aymeric, L., Ma, Y., Ortiz, C., … Zitvogel, L. (2009). Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat Med*, *15*(10), 1170–1178. http://doi.org/10.1038/nm.2028

Gilliet, M., Cao, W., & Liu, Y.-J. (2008). Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nature Reviews. Immunology*, *8*(8), 594–606.

http://doi.org/10.1038/nri2358

Ginhoux, F., & Jung, S. (2014). Monocytes and macrophages: developmental pathways and tissue homeostasis TL - 14. *Nature Reviews Immunology*, *14 VN-r*(6), 392–404. http://doi.org/10.1038/nri3671

Ginhoux, F., Liu, K., Helft, J., Bogunovic, M., Greter, M., Hashimoto, D., … Merad, M. (2009). The origin and development of nonlymphoid tissue CD103+ DCs. *The Journal of Experimental Medicine*, *206*(13), 3115–30. http://doi.org/10.1084/jem.20091756

Gordon, S. (2016). Phagocytosis: An Immunobiologic Process. *Immunity*, *44*(3), 463–475. http://doi.org/10.1016/j.immuni.2016.02.026

Gordon, S. B., Bruce, N. G., Grigg, J., Hibberd, P. L., Kurmi, O. P., Lam, K. bong H., … Martin, W. J. (2014). Respiratory risks from household air pollution in low and middle income countries. *The Lancet Respiratory Medicine*, *2*(10), 823–860. http://doi.org/10.1016/S2213-2600(14)70168-7

Gordon, S. B., Bruce, N. G., Grigg, J., Hibberd, P. L., Kurmi, O. P., Lam, K. H., … Perez-padilla, R. (2014). The Lancet Respiratory Medicine Commission Respiratory risks from household air pollution in low and middle income countries, *2600*(14), 1–38.

Gräfe, C., Weidner, A., Lühe, M. V.D., Bergemann, C., Schacher, F. H., Clement, J. H., & Dutz, S. (2016). Intentional formation of a protein corona on nanoparticles: Serum concentration affects protein corona mass, surface charge, and nanoparticle-cell interaction. *International Journal of Biochemistry and Cell Biology*, *75*, 196–202. http://doi.org/10.1016/j.biocel.2015.11.005

Greiber, S., Müller, B., Daemisch, P., & Pavenstädt, H. (2002). Reactive oxygen species alter gene expression in podocytes: induction of granulocyte macrophage-colony-stimulating factor. *Journal of the American Society of Nephrology : JASN*, *13*(1), 86–95. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11752025

Guarnieri, M., & Balmes, J. R. (2014). Outdoor air pollution and asthma. *The Lancet*, *383*(9928), 1581–1592. http://doi.org/10.1016/S0140-6736(14)60617-6

Guarnieri, M. J., Diaz, J. V., Basu, C., Diaz, A., Pope, D., Smith, K. R., … Balmes, J. R. (2014). Effects of woodsmoke exposure on airway inflammation in rural Guatemalan women. *PLoS ONE*, *9*(3), 1–9. http://doi.org/10.1371/journal.pone.0088455

Guilliams, M., Ginhoux, F., Jakubzick, C., Naik, S. H., Onai, N., Schraml, B. U., … Yona, S. (2014a). Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny.

Nature Reviews. Immunology, *14*(July), 571–578. http://doi.org/10.1038/nri3712

Guilliams, M., Ginhoux, F., Jakubzick, C., Naik, S. H., Onai, N., Schraml, B. U., … Yona, S. (2014b). Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nature Reviews. Immunology*, *14*(8), 571–578. http://doi.org/10.1038/nri3712

Guxens, M., & Sunyer, J. (2012). A review of epidemiological studies on neuropsychological effects of air pollution. *Swiss Medical Weekly*, (January), 1–7. http://doi.org/10.4414/smw.2012.13322

Hansen, G. (2001). Das Th1 / Th2-Paradigma beim allergischen Asthma bronchiale. *Monatsschr Kinderheilkd*, 112–119.

Harden, J. L., & Egilmez, N. K. (2012). Indoleamine 2,3-Dioxygenase and Dendritic Cell Tolerogenicity. *Immunol Invest.*, *41*(0), 738–764. http://doi.org/10.3109/08820139.2012.676122.Indoleamine

Hart, D. N. (1997). Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood*, *90*(9), 3245–87. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9345009

Hasenberg, M., Stegemann-Koniszewski, S., & Gunzer, M. (2013). Cellular immune reactions in the lung. *Immunological Reviews*, *251*(1), 189–214. http://doi.org/10.1111/imr.12020

Hayashi, S., Watanabe, J., Nakachi, K., Eguchi, H., Gotoh, O., & Kawajiri, K. (1994). Interindividual difference in expression of human Ah receptor and related P450 genes. *Carcinogenesis*, *15*(5), 801–806. http://doi.org/10.1093/carcin/15.5.801

Head, J. L., & Lawrence, B. P. (2009). The aryl hydrocarbon receptor is a modulator of anti-viral immunity. *Biochemical Pharmacology*, *77*(4), 642–653. http://doi.org/10.1016/j.bcp.2008.10.031.The

Heath, W. R., & Carbone, F. R. (2001). Cross-presentation in viral immunity and self-tolerance. *Nature Reviews. Immunology*, *1*(2), 126–134. http://doi.org/10.1038/35100512

Heer, A. K., Harris, N. L., Kopf, M., & Marsland, B. J. (2008). CD4+ and CD8+ T cells exhibit differential requirements for CCR7-mediated antigen transport during influenza infection. *Journal of Immunology*, *181*(10), 6984–6994. http://doi.org/181/10/6984 [pii]

Heikal, A. A. (2010). Intracellular coenzymes as natural biomarkers for metabolic activities and mitochondrial anomalies. *Biomark Med*, *4*(2), 241–263. http://doi.org/10.2217/bmm.10.1.Intracellular

Hennings, L., Kaufmann, Y., Griffin, R., Siegel, E., Corry, P., Moros, E. G., & Shafirstein, G. A. L. (2009). Dead or alive? Autofluorescence distinguishes heat-fixed from viable cells, *25*(5), 355–363. http://doi.org/10.1080/02656730902964357.Dead

Hespel, C., & Moser, M. (2012). Role of inflammatory dendritic cells in innate and adaptive immunity. *European Journal of Immunology*, *42*(10), 2535–2543. http://doi.org/10.1002/eji.201242480

Hetland, R. B., Cassee, F. R., Refsnes, M., Schwarze, P. E., Låg, M., Boere, A. J. F., & Dybing, E. (2004). Release of inflammatory cytokines, cell toxicity and apoptosis in epithelial lung cells after exposure to ambient air particles of different size fractions. *Toxicology in Vitro*, *18*(2), 203–212. http://doi.org/10.1016/S0887-2333(03)00142-5

Hidaka, T., Ogawa, E., Suzuki, T., Funayama, R., Nagashima, T., Fujimura, T., … Yamamoto, M. (2016). The aryl hydrocarbon receptor AhR links atopic dermatitis and air pollution via induction of the neurotrophic factor artemin. *Nature Immunology*, *18*(1). http://doi.org/10.1038/ni.3614

Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., … Murphy, K. M. (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science (New York, N.Y.)*, *322*(5904), 1097–100. http://doi.org/10.1126/science.1164206

Hinderliter, P. M., Minard, K. R., Orr, G., Chrisler, W. B., Thrall, B. D., Pounds, J. G., & Teeguarden, J. G. (2010). ISDD: A computational model of particle sedimentation, diffusion and target cell dosimetry for in vitro toxicity studies. *Particle and Fibre Toxicology*, *7*(36), 1–20. http://doi.org/10.1186/1743-8977-7-36

Hinds, W. C. (1999). Aerosol Technology. *Wiley - Interscience*.

Hirota, J. a, Hirota, S. a, Warner, S. M., Stefanowicz, D., Shaheen, F., Beck, P. L., … Van Eeden, S. (2012). The airway epithelium nucleotide-binding domain and leucine-rich repeat protein 3 inflammasome is activated by urban particulate matter. *The Journal of Allergy and Clinical Immunology*, *129*(4), 1116–25.e6. http://doi.org/10.1016/j.jaci.2011.11.033

Hoefsmit, E. C. M., Duijvestijn, A. M., & Kamperdijk, E. W. A. (1982). Relation between Langerhans Cells, Veiled Cells, and Interdigitating Cells. *Immunobiology*, *161*(3–4), 255–265.

Hogg, J. C. (2004). Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. *Lancet*, *364*(9435), 709–21. http://doi.org/10.1016/S0140-6736(04)16900-6

Hohl, T. M., Rivera, A., Lipuma, L., Gallegos, A., Shi, C., Mack, M., & Pamer, E. G. (2009). Inflammatory Monocytes Facilitate Adaptive CD4 T Cell Responses during Respiratory Fungal Infection, *6*(5), 470–481. http://doi.org/10.1016/j.chom.2009.10.007.Inflammatory

Holt, P. G., Strickland, D. H., Wikstroem, M. H., & Jahnsen, F. L. (2008a). Regulation of immunological homeostasis in the respiratory tract. *Nature Reviews Immunology*, *8*, 142–152.

Holt, P. G., Strickland, D. H., Wikstroem, M. H., & Jahnsen, F. L. (2008b). Regulation of immunological homeostasis in the respiratory tract. *Nature Reviews Immunology*, *8*, 142–152.

Hooper, L., & Macpherson, A. (2010). Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nature Reviews Immunology*, *10*, 159–169.

Hopp, A.-K., Rupp, A., & Lukacs-Kornek, V. (2014). Self-Antigen Presentation by Dendritic Cells in Autoimmunity. *Frontiers in Immunology*, *5*(February), 55. http://doi.org/10.3389/fimmu.2014.00055

Hornung, V., Bauernfeind, F., Halle, A., Samstad, E. O., Rock, K. L., Fitzgerald, K. A., & Latz, E. (2008). Silica crystals and aluminum salts mediate NALP-3 inflammasome activation via phagosomal destabilization Veit, *9*(8), 847–856. http://doi.org/10.1038/ni.1631.Silica

Hotamisligil, G., & Erbay, E. (2008). Nutrient sensing and inflammation in metabolic diseases Gökhan. *Nat Rev Immunol*, *8*(12), 1–26. http://doi.org/10.1038/nri2449.Nutrient

Howard, C. V., & Chb, M. B. (2009). Particulate Emissions and Health, (June).

http://environ.andrew.cmu.edu/m3/s3/09fossil.shtml. (n.d.).

Huang, D., Hua, X., Xiu, G.-L., Zheng, Y.-J., Yu, X.-Y., & Long, Y.-T. (2017). Secondary ion mass spectrometry: The application in the analysis of atmospheric particulate matter. *Analytica Chimica Acta*, *989*, 1–14. http://doi.org/10.1016/j.aca.2017.07.042

Huang, S.-K., Zhang, Q., Qiu, Z., & Chung, K. F. (2015). Mechanistic impact of outdoor air pollution on asthma and allergic diseases. *Journal of Thoracic Disease*, *7*(1), 23–33. http://doi.org/10.3978/j.issn.2072-1439.2014.12.13

Hubo, M., Trinschek, B., Kryczanowsky, F., Tuettenberg, A., Steinbrink, K., & Jonuleit, H. (2013). Costimulatory molecules on immunogenic versus tolerogenic human dendritic cells. *Frontiers in Immunology*, *4*(APR), 1–14. http://doi.org/10.3389/fimmu.2013.00082

Imrich, a, Ning, Y., & Kobzik, L. (2000). Insoluble components of concentrated air particles

mediate alveolar macrophage responses in vitro. *Toxicology and Applied Pharmacology*, *167*(2), 140–50. http://doi.org/10.1006/taap.2000.9002

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., … Steinman, R. M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *The Journal of Experimental Medicine*, *176*(December), 1693–1702. http://doi.org/10.1084/jem.176.6.1693

InvivoGen. (2009). Activation of the NLRP3 Inflammasome. *InvivoGen Insight*, *9*(8), 8–11.

Iwasaki, A. (2007). Mucosal dendritic cells. *Annual Review of Immunology*, *25*, 381–418. http://doi.org/10.1146/annurev.immunol.25.022106.141634

Iwasaki, A., Foxman, E. F., & Molony, R. D. (2016). Early local immune defences in the. *Nature Publishing Group*. http://doi.org/10.1038/nri.2016.117

Iwasaki, A., & Medzhitov, R. (2010). Regulation of adaptative immunity bye the innate immune system. *Science*, *327*(5963), 291–295. http://doi.org/10.1126/science.1183021.Regulation

Iwasaki, A., & Medzhitov, R. (2015). Control of adaptive immunity by the innate immune system. *Nature Immunol.*, *16*(4). http://doi.org/10.1126/science.1183021

Jahnsen, F. L., Strickland, D. H., Thomas, J. a, Tobagus, I. T., Napoli, S., Zosky, G. R., … Holt, P. G. (2006). Accelerated antigen sampling and transport by airway mucosal dendritic cells following inhalation of a bacterial stimulus. *Journal of Immunology (Baltimore, Md. : 1950)*, *177*(9), 5861–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17056510

Janeway, C. a. (1989). Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor Protocols*, *54*(1), 1–13.

Jiang, X.-Q., Mei, X.-D., & Feng, D. (2016). Air pollution and chronic airway diseases: what should people know and do? *Journal of Thoracic Disease*, *8*(1), E31-40. http://doi.org/10.3978/j.issn.2072-1439.2015.11.50

Jin, G.-B., Winans, B., Martin, K., & Lawrence, B. P. (2014). New insights into the role of the aryl hydrocarbon receptor in the function of CD11c+ cells during respiratory viral infection. *June*, *44*(6), 1685–1685. http://doi.org/10.1016/j.surg.2006.10.010.Use

Jin, G. B., Moore, A. J., Head, J. L., Neumiller, J. J., & Lawrence, B. P. (2010). Aryl hydrocarbon receptor activation reduces dendritic cell function during influenza virus infection. *Toxicol Sci*, *116*(2), 514–522. http://doi.org/10.1093/toxsci/kfq153

Joffre, O. (2009). Inflammatory signals in dendritic cell activation and the induction of adaptive immunity - Joffre - 2008 - Immunological Reviews - Wiley Online Library, 234–247.

Joffre, O. P., Segura, E., Savina, A., & Amigorena, S. (2012). Cross-presentation by dendritic cells. *Nature Reviews Immunology*, *12*(8), 557–569. http://doi.org/10.1038/nri3254

Jones, L. H., Cook, P. C., Ivens, A. C., Thomas, G. D., Phythian-Adams, A. T., Allen, J. E., & MacDonald, A. S. (2015). Modulation of dendritic cell alternative activation and function by the vitamin A metabolite retinoic acid. *International Immunology*, *27*(11), 589–596. http://doi.org/10.1093/intimm/dxv020

Jonuleit, H., Giesecke, A., Kandemir, A., Paragnik, L., Knop, J., & Enk, A. H. (2000). Induction of tumor peptide-specific cytotoxic T cells under serum-free conditions by mature human dendritic cells. *Archives of Dermatological Research*, *292*(7), 325–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10966056

Jux, B., Kadow, S., & Esser, C. (2009). Langerhans cell maturation and contact hypersensitivity are impaired in aryl hydrocarbon receptor-null mice. *Journal of Immunology (Baltimore, Md. : 1950)*, *182*(11), 6709–17. http://doi.org/10.4049/jimmunol.0713344

Kambayashi, T., & Laufer, T. M. (2014). Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell? *Nature Reviews Immunology*, *14*(11), 719–730. http://doi.org/10.1038/nri3754

Kaplan, G. G., Tanyingoh, D., Dixon, E., Johnson, M., Wheeler, A. J., Myers, R. P., … Villeneuve, P. J. (2013). Ambient ozone concentrations and the risk of perforated and nonperforated appendicitis: A multicity case-crossover study. *Environmental Health Perspectives*, *121*(8), 939–943. http://doi.org/10.1289/ehp.1206085

Katz, S., Tamaki, K., & Sachs, D. (1979). Epidermal Langerhans cells are derived from cells originating in bone marrow. *Nature*, *15;282*(5736), 324–6.

Kawai, T., & Akira, S. (2009). The roles of TLRs, RLRs and NLRs in pathogen recognition. *International Immunology*, *21*(4), 317–337. http://doi.org/10.1093/intimm/dxp017

Kawai, T., & Akira, S. (2011). Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. *Immunity*, *34*(5), 637–650. http://doi.org/10.1016/j.immuni.2011.05.006

Kawasaki, T., & Kawai, T. (2014). Toll-Like Receptor Signaling Pathways. *Frontiers in Immunology*, *5*(September), 1–8. http://doi.org/10.3389/fimmu.2014.00461

Kazantseva, M. G., Highton, J., Stamp, L. K., & Hessian, P. a. (2012). Dendritic cells provide a potential link between smoking and inflammation in rheumatoid arthritis. *Arthritis Research & Therapy*, *14*(5), R208. http://doi.org/10.1186/ar4046

Kelly, F. J., & Fussell, J. C. (2011). Air pollution and airway disease. *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology*, *41*(8), 1059–71. http://doi.org/10.1111/j.1365-2222.2011.03776.x

Kelly, F. J., & Fussell, J. C. (2015a). Air pollution and public health: emerging hazards and improved understanding of risk. *Environmental Geochemistry and Health*, *37*(4), 631–649. http://doi.org/10.1007/s10653-015-9720-1

Kelly, F. J., & Fussell, J. C. (2015b). Linking ambient particulate matter pollution effects with oxidative biology and immune responses. *Annals of the New York Academy of Sciences*, *1340*, n/a-n/a. http://doi.org/10.1111/nyas.12720

Khalequzzaman, M., Kamijima, M., Sakai, K., Hoque, B. A., & Nakajima, T. (2010). Indoor air pollution and the health of children in biomass- and fossil-fuel users of Bangladesh: situation in two different seasons. *Environmental Health and Preventive Medicine*, *15*(4), 236–43. http://doi.org/10.1007/s12199-009-0133-6

Kim, K.-H., Kabir, E., & Kabir, S. (2015). A review on the human health impact of airborne particulate matter. *Environment International*, *74*, 136–143. http://doi.org/http://dx.doi.org/10.1016/j.envint.2014.10.005

Knowles, M. R., & Boucher, R. C. (2002). Mucus clearance as a primary innate defense mechanism for mammalian airways, *109*(5), 571–577. http://doi.org/10.1172/JCI200215217.The

Kocbach, a, Totlandsdal, a I., Låg, M., Refsnes, M., & Schwarze, P. E. (2008). Differential binding of cytokines to environmentally relevant particles: a possible source for misinterpretation of in vitro results? *Toxicology Letters*, *176*(2), 131–7. http://doi.org/10.1016/j.toxlet.2007.10.014

Kofoed, E. M., & Vance, R. E. (2012). Innate immune recognition of bacterial ligands by NAIPs dictates inflammasome specificity. *Nature*, *477*(7366), 592–595. http://doi.org/10.1038/nature10394.Innate

Kondo, M., Wagers, A., Manz, M. G., Prohaska, S., Scherer, D., Beilhack, G., … Weissman, I. (2003). Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu. Rev. Immunol.*, *21*, 759–806.

Kopf, M., Schneider, C., & Nobs, S. P. (2014). The development and function of lung-resident macrophages and dendritic cells. *Nature Immunology*, *16*(1). http://doi.org/10.1038/ni.3052

Krawczyk, C. M., Holowka, T., Sun, J., Blagih, J., Amiel, E., Deberardinis, R. J., … Pearce, E. J. (2010). Toll-like receptor – induced changes in glycolytic metabolism regulate dendritic cell activation, *115*(23), 6–8. http://doi.org/10.1182/blood-2009-10-249540.The

Krishnaswamy, J. K., Chu, T., & Eisenbarth, S. C. (2013). Beyond pattern recognition: NOD-like receptors in dendritic cells. *Trends in Immunology*, *34*(5), 224–233. http://doi.org/10.1016/j.it.2012.12.003

La Duc, M. T., Stuecker, T., & Venkateswaran, K. (2007). Molecular bacterial diversity and bioburden of commercial airliner cabin air. *Canadian Journal of Microbiology*, *53*(11), 1259–1271. http://doi.org/10.1139/W07-093

Lai, S. K., Wang, Y.-Y., & Hanes, J. (2009). Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. *Adv Drug Deliv Rev*, *61*(2), 158–171. http://doi.org/10.1016/j.addr.2008.11.002.Mucus-penetrating

Langenback et al., E. G. (1990). Supramicron-sized clearance from alveoli: route and kinetics. *Analysis*, 1302–1308.

Lapteva, N., Seethammagari, M. R., Hanks, B. A., Jiang, H., Levitt, J. M., Slawin, K. M., & Spencer, D. M. (2007). Enhanced activation of human dendritic cells by inducible CD40 and toll-like receptor-4 ligation. *Cancer Research*, *67*(21), 10528–10537. http://doi.org/10.1158/0008-5472.CAN-07-0833

Larsson, M., Hagberg, J., Giesy, J. P., & Engwall, M. (2014). Time-dependent relative potency factors for polycyclic aromatic hydrocarbons and their derivatives in the H4IIE-luc bioassay. *Environmental Toxicology and Chemistry*, *33*(4), 943–953. http://doi.org/10.1002/etc.2517

Latz, E., Xiao, T. S., & Stutz, A. (2013). Activation and regulation of the inflammasomes. *Nature Reviews. Immunology*, *13*(6), 397–411. http://doi.org/10.1038/nri3452

Lee, A., Kinney, P., Chillrud, S., & Jack, D. (2015). A Systematic Review of Innate Immunomodulatory Effects of Household Air Pollution Secondary to the Burning of Biomass Fuels. *Ann Glob Health*, *81*(3), 368–374. http://doi.org/10.1530/ERC-14-0411.Persistent

Lee, J.-A., Hwang, J.-A., Sung, H.-N., Jeon, C.-H., Gill, B.-C., Youn, H.-J., & Park, J.-H. (2007). 2,3,7,8-Tetrachlorodibenzo-p-dioxin modulates functional differentiation of mouse bone

marrow-derived dendritic cells Downregulation of RelB by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicology Letters*, *173*(1), 31–40. http://doi.org/10.1016/j.toxlet.2007.06.012

Lee, J., Breton, G., Oliveira, T. Y. K., Zhou, Y. J., Aljoufi, A., Puhr, S., … Liu, K. (2015). Restricted dendritic cell and monocyte progenitors in human cord blood and bone marrow. *The Journal of Experimental Medicine*, *212*(3), 385–99. http://doi.org/10.1084/jem.20141442

Lee, T.-H., Song, H. J., & Park, C.-S. (2014). Role of inflammasome activation in development and exacerbation of asthma. *Asia Pacific Allergy*, *4*(4), 187–96. http://doi.org/10.5415/apallergy.2014.4.4.187

Liang, J., Wu, W. L., Liu, Z. H., Mei, Y. J., Cai, R. X., & Shen, P. (2007). Study the oxidative injury of yeast cells by NADH autofluorescence. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, *67*(2), 355–359. http://doi.org/10.1016/j.saa.2006.07.035

Lin, K. L., Suzuki, Y., Nakano, H., Ramsburg, E., & Gunn, M. D. (2008). CCR2+ Monocyte-Derived Dendritic Cells and Exudate Macrophages Produce Influenza-Induced Pulmonary Immune Pathology and Mortality. *The Journal of Immunology*, *180*(4), 2562–2572. http://doi.org/10.4049/jimmunol.180.4.2562

Linsley, P. S., Brady, W., Grosmaire, L., Aruffo, A., Damle, N. K., & Ledbetter, J. A. (1991). Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *Journal of Experimental Medicine*, *173*(3), 721–730. http://doi.org/10.1084/jem.173.3.721

Litman, G. W., Rast, J. P., & Fugmann, S. D. (2010). The origins of vertebrate adaptive immunity. *Nature Reviews. Immunology*, *10*(8), 543–553. http://doi.org/10.1038/nri2807

Littman, D. R., & Rudensky, A. Y. (2010). Th17 and Regulatory T Cells in Mediating and Restraining Inflammation. *Cell*, *140*(6), 845–858. http://doi.org/10.1016/j.cell.2010.02.021

Liu, G., Bi, Y., Xue, L., Zhang, Y., Yang, H., Chen, X., … Yang, R. (2015). Dendritic cell SIRT1-HIF1α axis programs the differentiation of CD4+ T cells through IL-12 and TGF-β1. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(9), E957-65. http://doi.org/10.1073/pnas.1420419112

Liu, K., Victora, G., & Schwickert, T. (2009). In vivo analysis of dendritic cell development and homeostasis. *Science*, *324*(5925), 392–397. http://doi.org/10.1126/science.1170540.In

Liu, T. F., Vachharajani, V. T., Yoza, B. K., & McCall, C. E. (2012). NAD+ -dependent sirtuin 1 and 6 proteins coordinate a switch from glucose to fatty acid oxidation during the acute inflammatory response. *Journal of Biological Chemistry*, *287*(31), 25758–25769.

http://doi.org/10.1074/jbc.M112.362343

Liu, Y.-J. (2005). IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annual Review of Immunology*, *23*(3), 275–306. http://doi.org/10.1146/annurev.immunol.23.021704.115633

Lorenz, R. J. (1966). Weibel, E. R.: Morphometry of the Human Lung. Springer Verlag, Berlin-Göttingen-Heidelberg 1963; 151 S., 109 Abb., DM 36,-. *Biometrische Zeitschrift*, *8*(1–2), 143–144. http://doi.org/10.1002/bimj.19660080155

Lu, A., & Wu, H. (2015). Structural mechanisms of inflammasome assembly. *FEBS Journal*, *282*(3), 435–444. http://doi.org/10.1111/febs.13133

Lu, Y., Su, S., Jin, W., Wang, B., Li, N., Shen, H., … Tao, S. (2014). Characteristics and cellular effects of ambient particulate matter from Beijing. *Environmental Pollution*, *191*, 63–69. http://doi.org/10.1016/j.envpol.2014.04.008

Maaetoft-Udsen, K., Shimoda, L. M. N., Frøkiær, H., & Turner, H. (2012). Aryl Hydrocarbon Receptor Ligand Effects in RBL2H3 Cells. *J Immunotoxicol*, *9*(3), 327–337. http://doi.org/10.3109/1547691X.2012.661802.Aryl

MacDonald, A. S., Patton, E. A., La-Flamme, A. C., Araujo, M. I., Huxtable, C. R., Bauman, B., & Pearce, E. J. (2002). Impaired Th2 development and increased mortality during Schistosoma mansoni infection in the absence of CD40/CD154 interaction. *Journal of Immunology*, *168*(9), 4643–4649. http://doi.org/10.4049/jimmunol.168.9.4643

Madan, T., Reid, K. B. M., Singh, M., Sarma, U., & Kishore, U. (2005). Susceptibility of Mice Genetically Deficient in the Surfactant Protein (SP)-A or SP-D Gene to Pulmonary Hypersensitivity Induced by Antigens and Allergens of Aspergillus fumigatus1. *J Immunol*, *174*, 6943–6954.

Magalhaes, J. G., Rubino, S. J., Travassos, L. H., Le Bourhis, L., Duan, W., Sellge, G., … Philpott, D. J. (2011). Nucleotide oligomerization domain-containing proteins instruct T cell helper type 2 immunity through stromal activation. *Proceedings of the National Academy of Sciences*, *108*(36), 14896–14901. http://doi.org/10.1073/pnas.1015063108

Mahajan, L., Gautam, P., Dodagatta-Marri, E., Madan, T., & Kishore, U. (2014). Surfactant protein SP-D modulates activity of immune cells: proteomic profiling of its interaction with eosinophilic cells. *Expert Review of Proteomics*, *11*(3), 355–369. http://doi.org/10.1586/14789450.2014.897612

Majno, G., & Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *The*

American Journal of Pathology, *146*(1), 3–15. http://doi.org/7856735

Majno G, La Gatutta M, T. T. (1960). Cellular death and necrosis: chemical, physical and morphologic changes in rat liver. *Virchows Arch Pathol Anat Physiol Klin Med.*, *333*, 421–65.

Man, S. M., Karki, R., & Kanneganti, T. D. (2016). AIM2 inflammasome in infection, cancer, and autoimmunity: Role in DNA sensing, inflammation, and innate immunity. *European Journal of Immunology*, *46*(2), 269–280. http://doi.org/10.1002/eji.201545839

Martinelli, N., Olivieri, O., & Girelli, D. (2013). Air particulate matter and cardiovascular disease: a narrative review. *European Journal of Internal Medicine*, *24*(4), 295–302. http://doi.org/10.1016/j.ejim.2013.04.001

Martinon, F., Mayor, A., & Tschopp, J. (2009). The inflammasomes: guardians of the body. *Annual Review of Immunology*, *27*, 229–65. http://doi.org/10.1146/annurev.immunol.021908.132715

Masters, S. L., Dunne, A., Subramanian, S. L., Hull, R. L., Gillian, M., Sharp, F. a, … Kahn, S. E. (2011). Activation of the Nlrp3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1b in type 2 diabetes. *Nature Immunology*, *11*(10), 897–904. http://doi.org/10.1038/ni.1935.Activation

Matthews, N. C., Faith, A., Pfeffer, P., LU, H., Kelly, F. J., Hawrylowicz, C. M., & Lee, T. H. (2013). Urban particulate matter suppresses priming of Th1 cells by GM-CSF-activated human dendritic cells. *AmericanThoracic Society*, 1–37.

Matthews, N. C., Pfeffer, P. E., Mann, E. H., Kelly, F. J., Corrigan, C. J., Hawrylowicz, C. M., & Lee, T. H. (2016). Urban particulate matter-activated human dendritic cells induce the expansion of potent inflammatory Th1, Th2, and Th17 effector cells. *American Journal of Respiratory Cell and Molecular Biology*, *54*(2), 250–262. http://doi.org/10.1165/rcmb.2015-0084OC

Matzinger, P. (1994). TOLERANCE , DANGER , AND THE EXTENDED F AMIL y *, 991–1045.

Mayevsky, A., & Rogatsky, G. G. (2007). Mitochondrial function in vivo evaluated by NADH fluorescence : from animal models to human studies. http://doi.org/10.1152/ajpcell.00249.2006.

Mbongue, J., Nicholas, D., Torrez, T., Kim, N.-S., Firek, A., & Langridge, W. (2015). The Role of Indoleamine 2, 3-Dioxygenase in Immune Suppression and Autoimmunity. *Vaccines*, *3*(3), 703–729. http://doi.org/10.3390/vaccines3030703

McCarthy, N. E., Jones, H. A., Marks, N. A., Shiner, R. J., Ind, P. W., Al-Hassi, H. O., … Stagg, A. J. (2007). Inhaled allergen-driven CD1c up-regulation and enhanced antigen uptake by activated human respiratory-tract dendritic cells in atopic asthma. *Clinical and Experimental Allergy*, *37*(1), 72–82. http://doi.org/10.1111/j.1365-2222.2006.02631.x

McKenna, H. J., Stocking, K. L., Miller, R. E., Brasel, K., De Smedt, T., Maraskovsky, E., … Peschon, J. J. (2000). Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood*, *95*(11), 3489–3497.

Medzhitov, R., & Janeway, C. A. (1997). Innate immunity: the virtues of a nonclonal system of recognition. *Cell*, *91*(3), 295–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9363937

Medzhitov, R., Shevach, E. M., Trinchieri, G., Mellor, A. L., Munn, D. H., Gordon, S., … Garra, A. O. (2011). Highlights of 10 years of immunology in Nature Reviews Immunology. *Nature Reviews. Immunology*, *11*(10), 693–702. http://doi.org/10.1038/nri3063

Mena, M. A., Woll, F., Cok, J., Ferrufino, J. C., & Accinelli, R. A. (2012). Histopathological Lung Changes in Children due to Biomass Fuel, *1*(3).

Merad, M., & Manz, M. G. (2009). Dendritic cell homeostasis. *Blood*, *113*(15), 3418–27. http://doi.org/10.1182/blood-2008-12-180646

Merad, M., Sathe, P., Helft, J., Miller, J., & Mortha, A. (2013). The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting. *Annual Review of Immunology*, *29*(9), 997–1003. http://doi.org/10.1016/j.biotechadv.2011.08.021.Secreted

Meredith, M. M., Liu, K., Darrasse-Jeze, G., Kamphorst, a. O., Schreiber, H. a., Guermonprez, P., … Nussenzweig, M. C. (2012a). Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *Journal of Experimental Medicine*, *209*(6), 1153–1165. http://doi.org/10.1084/jem.20112675

Meredith, M. M., Liu, K., Darrasse-Jeze, G., Kamphorst, a. O., Schreiber, H. a., Guermonprez, P., … Nussenzweig, M. C. (2012b). Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *Journal of Experimental Medicine*, *209*(6), 1153–1165. http://doi.org/10.1084/jem.20112675

Meredith, M. M., Liu, K., Kamphorst, a. O., Idoyaga, J., Yamane, a., Guermonprez, P., … Nussenzweig, M. C. (2012). Zinc finger transcription factor zDC is a negative regulator required to prevent activation of classical dendritic cells in the steady state. *Journal of*

Experimental Medicine, *209*(9), 1583–1593. http://doi.org/10.1084/jem.20121003

Mildner, A., Yona, S., & Jung, S. (2013). A close encounter of the third kind: monocyte-derived cells. *Advances in Immunology*, *120*, 69–103.

Miller, R. L., & Peden, D. B. (2014). Environmental impacts on immune responses in atopy and asthma. *The Journal of Allergy and Clinical Immunology*, *134*(5), 1001–8. http://doi.org/10.1016/j.jaci.2014.07.064

Mills, N. L., Amin, N., Robinson, S. D., Anand, A., Davies, J., Patel, D., … Newby, D. E. (2006). Do inhaled carbon nanoparticles translocate directly into the circulation in humans? *American Journal of Respiratory and Critical Care Medicine*, *173*(4), 426–431. http://doi.org/10.1164/rccm.200506-865OC

Moldoveanu, B., Otmishi, P., Jani, P., Walker, J., Sarmiento, X., Guardiola, J., … Yu, J. (2009). Inflammatory mechanisms in the lung. *Journal of Inflammation Research*, *2*, 1–11. http://doi.org/10.2147/JIR.S4385

Monici, M. (2005). Cell and tissue autofluorescence research and diagnostic applications. *Biotechnol. Annu. Rev.*, *11*, 227–256. http://doi.org/http://dx.doi.org/10.1016/S1387-2656(05)11007-2

Monn, C., & Becker, S. (1998). Cytotoxicity and induction of proinflammatory cytokines from human monocytes exposed to fine (PM2.5) and coarse particles (PM10-2.5) in outdoor and indoor air. *Toxicology and Applied Pharmacology*, *155*(3), 245–52. http://doi.org/10.1006/taap.1998.8591

Monn, C., & Becker, S. (1999). Cytotoxicity and induction of proinflammatory cytokines from human monocytes exposed to fine (PM2.5) and coarse particles (PM10-2.5) in outdoor and indoor air. *Toxicology and Applied Pharmacology*, *155*(3), 245–52. http://doi.org/10.1006/taap.1998.8591

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., & Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of Immunology (Baltimore, Md. : 1950)*, *136*(7), 2348–57. http://doi.org/10.1111/j.1442-9071.2011.02672.x

Mukae, H., Vincent, R., Quinlan, K., English, D., Hards, J., Hogg, J. C., & Eeden, S. F. Van. (2001). The Effect of Repeated Exposure to Particulate Air Pollution (PM 10) on the Bone Marrow. *American Journal of Respiratory and Critical Care Medicine*, *163*, 201–209.

Murphy, T. L., Tussiwand, R., & Murphy, K. M. (2013). Specificity through cooperation: BATF–IRF

interactions control immune-regulatory networks. *Nature Reviews Immunology*, *13*(7), 499–509. http://doi.org/10.1038/nri3470

Mushtaq, N., Ezzati, M., Hall, L., Dickson, I., Kirwan, M., Png, K. M. Y., … Grigg, J. (2011). Adhesion of Streptococcus pneumoniae to human airway epithelial cells exposed to urban particulate matter. *The Journal of Allergy and Clinical Immunology*, *127*(5), 1236–42.e2. http://doi.org/10.1016/j.jaci.2010.11.039

Naik, S. H., Metcalf, D., van Nieuwenhuijze, A., Wicks, I., Wu, L., O'Keeffe, M., & Shortman, K. (2006). Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nature Immunology*, *7*(May), 663–671. http://doi.org/10.1038/ni1340

Naik, S. H., Sathe, P., Park, H.-Y., Metcalf, D., Proietto, A. I., Dakic, A., … Shortman, K. (2007). Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nature Immunology*, *8*(11), 1217–26. http://doi.org/10.1038/ni1522

Nakano, H., Free, M., Whitehead, G., Maruoka, S., Wilson, R., Nakano, K., & Cook, D. (2012). Pulmonary CD103+ dendritic cells prime Th2 responses to inhaled allergend. *Mucosal Immunology*, *5*(1), 54–56. http://doi.org/10.1038/nmeth.2250.Digestion

Nelson, B. D. J., McMenamin, C., McWilliam, A. S., Brenan, M., & Holt, P. G. (1994). Development of the Airway Intraepithelial Dendritic Cell Network in the Rat from Class II Major Histocompatibility (Ia)-negative Precursors: Differential Regulation of Ia Expression at Different Levels of the Respiratory Tract. *J. Exp. Med*, *179*(January), 203–212. http://doi.org/10.1084/jem.179.1.203

Nemmar, A., Hoet, P., Vanquickenborne, B., Dinsdale, D., Thomeer, M., Hoylaerts, M., … Nemery, B. (2002). Passage of inhaledpParticles into the blood circulation in humans. *Circulation*, *105*(4), 411–414. http://doi.org/10.1161/hc0402.104118

Nguyen, N. T., Kimura, A., Nakahama, T., Chinen, I., Masuda, K., Nohara, K., … Kishimoto, T. (2010). Aryl hydrocarbon receptor negatively regulates dendritic cell immunogenicity via a kynurenine-dependent mechanism. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(46), 19961–6. http://doi.org/10.1073/pnas.1014465107

Nicod, L. P. (2005a). Lung defences: an overview. *European Respiratory Review*, *14*(95), 45–50. http://doi.org/10.1183/09059180.05.00009501

Nicod, L. P. (2005b). Lung defences: An overview. *European Respiratory Review*, *14*(95), 45–50. http://doi.org/10.1183/09059180.05.00009501

Nwokoro, C., Ewin, C., Harrison, C., Ibrahim, M., Dundas, I., Dickson, I., … Grigg, J. (2012). Cycling to work in London and inhaled dose of black carbon. *The European Respiratory Journal : Official Journal of the European Society for Clinical Respiratory Physiology*. http://doi.org/10.1183/09031936.00195711

O'Keeffe, M., Mok, W. H., & Radford, K. J. (2015). Human dendritic cell subsets and function in health and disease. *Cellular and Molecular Life Sciences*, *72*(22), 4309–4325. http://doi.org/10.1007/s00018-015-2005-0

O'Neill, L. A. J., & Pearce, E. J. (2015). Immunometabolism governs dendritic cell and macrophage function. *The Journal of Experimental Medicine*, jem.20151570. http://doi.org/10.1084/jem.20151570

Ochs, M., Nyengaard, J. R., Jung, A., Knudsen, L., Voigt, M., Wahlers, T., … Gundersen, H. J. G. (2004). The number of alveoli in the human lung. *American Journal of Respiratory and Critical Care Medicine*, *169*(1), 120–4. http://doi.org/10.1164/rccm.200308-1107OC

Ohura, T., Sawada, K. I., Amagai, T., & Shinomiya, M. (2009). Discovery of novel halogenated polycyclic aromatic hydrocarbons in urban particulate matters: Occurrence, photostability, and AhR activity. *Environmental Science and Technology*, *43*(7), 2269–2275. http://doi.org/10.1021/es803633d

Okabe, Y., Kawane, K., Akira, S., Taniguchi, T., & Nagata, S. (2005). Toll-like receptor-independent gene induction program activated by mammalian DNA escaped from apoptotic DNA degradation. *The Journal of Experimental Medicine*, *202*(10), 1333–9. http://doi.org/10.1084/jem.20051654

Oliveira, B. F. A. de, Ignotti, E., & Hacon, S. S. (2011). A systematic review of the physical and chemical characteristics of pollutants from biomass burning and combustion of fossil fuels and health effects in Brazil Revisão sistemática das características físico- químicas dos poluentes atmosféri. *Cad Saude Publica*, *27*(9), 1678–1698.

Opitz, C. a, Litzenburger, U. M., Opitz, U., Sahm, F., Ochs, K., Lutz, C., … Platten, M. (2011). The indoleamine-2,3-dioxygenase (IDO) inhibitor 1-methyl-D-tryptophan upregulates IDO1 in human cancer cells. *PloS One*, *6*(5), e19823. http://doi.org/10.1371/journal.pone.0019823

Osornio-Vargas, A. R., Bonner, J. C., Alfaro-Moreno, E., Martínez, L., García-Cuellar, C., Ponce-de-León Rosales, S., … Rosas, I. (2003). Proinflammatory and cytotoxic effects of Mexico City air pollution particulate matter in vitro are dependent on particle size and composition. *Environmental Health Perspectives*, *111*(10), 1289–93. http://doi.org/10.1289/ehp.5913

Ozturk A.B. Damadoglu E. Karakaya G. Kalyoncu A.F. (2011). Does Nasal Hair (Vibrissae) Density Affect the Risk of Developing Asthma in Patients with Seasonal Rhinitis? *Int Arch Allergy Immunol*, *156*(1), 75–80.

Patashnick, H., & Rupprecht, E. (1991). Continuous PM10 measurements using the tapered element oscillating microbalance. *J Air Waste Manage Assoc*, *41*, 1079–1081.

Patton, S. (1996). Mechanisms of macromolecule absorption by the lungs, (95).

Paul Langerhans. (1868). Ueber die Nerven der menschlichen Haut. *Archiv Für Pathologische Anatomie Und Physiologie Und Für Klinische Medicin*, *44*(2–3), 325–337.

Peri, F., & Calabrese, V. (2015). Toll-like Receptor 4 (TLR4) modulation by synthetic and natural compounds: an update. *J Med Chem*, *57*(9), 3612–3622. http://doi.org/10.1021/jm401006s.Toll-like

Peters, A., Dockery, D. W., Muller, J. E., & Mittleman, M. A. (2001). Increased particulate air pollution and the triggering of myocardial infarction. *Circulation*, *103*(23), 2810–5. http://doi.org/10.1161/01.cir.103.23.2810

Pizzichini E, Pizzichini MMM, Efthimiadis A.; Evans S, Morris MM, Squillace, D, Gleich, GJ, Dolovich, J, Hargreave, F. (1996). Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid phase measurements. *Am. J. Respir Crit Care Med*, *154*, 308–17.

Plantinga, M., Guilliams, M., Vanheerswynghels, M., Deswarte, K., Branco-Madeira, F., Toussaint, W., … Lambrecht, B. N. (2013). Conventional and Monocyte-Derived CD11b+ Dendritic Cells Initiate and Maintain T Helper 2 Cell-Mediated Immunity to House Dust Mite Allergen. *Immunity*, *38*(2), 322–335. http://doi.org/10.1016/j.immuni.2012.10.016

Płóciennikowska, A., Hromada-Judycka, A., Borzęcka, K., & Kwiatkowska, K. (2015). Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling. *Cellular and Molecular Life Sciences*, *72*(3), 557–581. http://doi.org/10.1007/s00018-014-1762-5

Pontillo, A., Silva, L. T., Oshiro, T. M., Finazzo, C., Crovella, S., & Duarte, A. J. (2012). HIV-1 induces NALP3-inflammasome expression and interleukin-1beta secretion in dendritic cells from healthy individuals but not from HIV-positive patients. *Aids*, *26*(1), 11–18. http://doi.org/10.1097/QAD.0b013e32834d697f [doi]

Porter, M., Karp, M., Killedar, S., Bauer, S. M., Guo, J., Williams, D., … Williams, M. a. (2007). Diesel-enriched particulate matter functionally activates human dendritic cells. *American Journal of Respiratory Cell and Molecular Biology*, *37*(6), 706–19.

http://doi.org/10.1165/rcmb.2007-0199OC

Prazma, C. M., & Tedder, T. F. (2008). Dendritic cell CD83: A therapeutic target or innocent bystander? *Immunology Letters*, *115*(1), 1–8. http://doi.org/10.1016/j.imlet.2007.10.001

Provoost, S., Maes, T., Joos, G. F., & Tournoy, K. G. (2012). Monocyte-derived dendritic cell recruitment and allergic T(H)2 responses after exposure to diesel particles are CCR2 dependent. *The Journal of Allergy and Clinical Immunology*, *129*(2), 483–91. http://doi.org/10.1016/j.jaci.2011.07.051

Provoost, S., Maes, T., Pauwels, N. S., Vanden Berghe, T., Vandenabeele, P., Lambrecht, B. N., … Tournoy, K. G. (2011). NLRP3/Caspase-1-Independent IL-1{beta} Production Mediates Diesel Exhaust Particle-Induced Pulmonary Inflammation. *Journal of Immunology (Baltimore, Md. : 1950)*, *187*(6), 3331–3337. http://doi.org/10.4049/jimmunol.1004062

Qaseem, A. S., Sonar, S., Mahajan, L., Madan, T., Sorensen, G. L., Shamji, M. H., & Kishore, U. (2013). Linking surfactant protein SP-D and IL-13: Implications in asthma and allergy. *Molecular Immunology*, *54*(1), 98–107. http://doi.org/10.1016/j.molimm.2012.10.039

Rabolli, V., Lison, D., & Huaux, F. (2016). The complex cascade of cellular events governing inflammasome activation and IL-1β processing in response to inhaled particles. *Particle and Fibre Toxicology*, *13*(1), 40. http://doi.org/10.1186/s12989-016-0150-8

Ranft, U., Schikowski, T., Sugiri, D., Krutmann, J., & Krämer, U. (2009). Long-term exposure to traffic-related particulate matter impairs cognitive function in the elderly. *Environmental Research*, *109*(8), 1004–1011. http://doi.org/10.1016/j.envres.2009.08.003

Reis e Sousa, C. (2006). Dendritic cells in a mature age. *Nature Reviews Immunology*, *6*(June), 476–483.

Reizis, B. (2010). Regulation of playmacytoid dendritic cell development. *Current Opinion in Immunology*, *22*(2), 206–211. http://doi.org/10.1016/j.coi.2010.01.005.Regulation

Reizis, B. (2012). Classical dendritic cells as a unique immune cell lineage. *Journal of Experimental Medicine*, *209*(6), 1053–1056. http://doi.org/10.1084/jem.20121038

Reizis, B., Bunin, A., Ghosh, H. S., Lewis, K. L., & Sisirak, V. (2011). Plasmacytoid Dendritic Cells: Recent Progress and Open Questions. *Annu. Rev. Immunol.*, *29*, 997–1003. http://doi.org/10.1016/j.biotechadv.2011.08.021.Secreted

Robbins, S. H., Walzer, T., Dembélé, D., Thibault, C., Defays, A., Bessou, G., … Dalod, M. (2008). Novel insights into the relationships between dendritic cell subsets in human and mouse

revealed by genome-wide expression profiling. *Genome Biology*, *9*(1), R17. http://doi.org/10.1186/gb-2008-9-1-r17

Rossi, M., Young, J. W., & Alerts, E. (2005). Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. *Journal of Immunology (Baltimore, Md. : 1950)*, *175*(3), 1373–81. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16034072

Rovito, H. A., & Oblong, J. E. (2013). Nicotinamide preferentially protects glycolysis in dermal fibroblasts under oxidative stress conditions. *British Journal of Dermatology*, *169*(SUPPL.2), 15–24. http://doi.org/10.1111/bjd.12365

Rylance, J., Fullerton, D. G., Scriven, J., Aljurayyan, A. N., Mzinza, D., Barrett, S., … Gordon, S. B. (2015). Household air pollution causes dose-dependent inflammation and altered phagocytosis in human macrophages. *American Journal of Respiratory Cell and Molecular Biology*, *52*(5), 584–593. http://doi.org/10.1165/rcmb.2014-0188OC

Ryu, J.-H., Kim, C.-H., & Yoon, J.-H. (2010). Innate immune responses of the airway epithelium. *Molecules and Cells*, *30*(3), 173–83. http://doi.org/10.1007/s10059-010-0146-4

Salazar, F., Hall, L., Negm, O. H., Awuah, D., Tighe, P. J., Shakib, F., & Ghaemmaghami, A. M. (2015). The mannose receptor negatively modulates the Toll-like receptor 4–aryl hydrocarbon receptor–indoleamine 2,3-dioxygenase axis in dendritic cells affecting T helper cell polarization. *Journal of Allergy and Clinical Immunology*. http://doi.org/10.1016/j.jaci.2015.10.033

Sanders, N. N., De Smedt, S. C., Van Rompaey, E., Simoens, P., De Baets, F., & Demeester, J. (2000). Cystic fibrosis sputum: a barrier to the transport of nanospheres. *American Journal of Respiratory and Critical Care Medicine*, *162*(5), 1905–11. http://doi.org/10.1164/ajrccm.162.5.9909009

Sathe, P., Metcalf, D., Vremec, D., Naik, S. H., Langdon, W. Y., Huntington, N. D., … Shortman, K. (2014). Lymphoid Tissue and Plasmacytoid Dendritic Cells and Macrophages Do Not Share a Common Macrophage-Dendritic Cell-Restricted Progenitor. *Immunity*, *41*, 104–115. http://doi.org/10.1016/j.immuni.2014.05.020

Sato, S., & Kiyono, H. (2012). The mucosal immune system of the respiratory tract. *Current Opinion in Virology*, *2*(3), 225–32. http://doi.org/10.1016/j.coviro.2012.03.009

Satpathy, A. T., Kc, W., Albring, J. C., Edelson, B. T., Kretzer, N. M., Bhattacharya, D., … Murphy, K. M. (2012). Zbtb46 expression distinguishes classical dendritic cells and their committed

progenitors from other immune lineages. *Journal of Experimental Medicine*, *209*(6), 1135–1152. http://doi.org/10.1084/jem.20120030

Satpathy, A. T., Kc, W., Albring, J. C., Edelson, B. T., Kretzer, N. M., Bhattacharya, D., … Murphy, K. M. (2012). Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. *The Journal of Experimental Medicine*, *209*(6), 1135–52. http://doi.org/10.1084/jem.20120030

Satpathy, A. T., Murphy, K. M., & Kc, W. (2011). Transcription factor networks in dendritic cell development. *Seminars in Immunology*, *23*(5), 388–97. http://doi.org/10.1016/j.smim.2011.08.009

Satpathy, A. T., Wu, X., Albring, J. C., & Murphy, K. M. (2012). Re(de)fining the dendritic cell lineage. *Changes*, *13*(12), 1145–1154. http://doi.org/10.1016/j.biotechadv.2011.08.021.Secreted

Savina, A., Jancic, C., Hugues, S., Guermonprez, P., Vargas, P., Moura, I. C., … Amigorena, S. (2006). NOX2 Controls Phagosomal pH to Regulate Antigen Processing during Crosspresentation by Dendritic Cells. *Cell*, *126*(1), 205–218. http://doi.org/10.1016/j.cell.2006.05.035

Sayan, M., & Mossman, B. T. (2016). The NLRP3 inflammasome in pathogenic particle and fibre-associated lung inflammation and diseases. *Particle and Fibre Toxicology*, *13*(1), 51. http://doi.org/10.1186/s12989-016-0162-4

Schertzer, J. D., & Steinberg, G. R. (2014). Immunometabolism: the interface of immune and metabolic responses in disease. *Immunology and Cell Biology*, *92*(4), 303. http://doi.org/10.1038/icb.2014.12

Schikowski, T., Mills, I. C., Anderson, H. R., Cohen, A., Hansell, A., Kauffmann, F., … K??nzli, N. (2014). Ambient air pollution: A cause of COPD. *European Respiratory Journal*, *43*(1), 250–263. http://doi.org/10.1183/09031936.00100112

Schlitzer, A., McGovern, N., Teo, P., Zelante, T., Atarashi, K., Low, D., … Ginhoux, F. (2013). IRF4 Transcription Factor-Dependent CD11b+ Dendritic Cells in Human and Mouse Control Mucosal IL-17 Cytokine Responses. *Immunity*, *38*(5), 970–983. http://doi.org/10.1016/j.immuni.2013.04.011

Schwarz, H., Posselt, G., Wurm, P., Ulbing, M., Duschl, A., & Horejs-Hoeck, J. (2013). TLR8 and NOD signaling synergistically induce the production of IL-1?? and IL-23 in monocyte-derived DCs and enhance the expression of the feedback inhibitor SOCS2. *Immunobiology*,

218(4), 533–542. http://doi.org/10.1016/j.imbio.2012.06.007

Segura, E., & Amigorena, S. (2013). Inflammatory dendritic cells in mice and humans. *Trends in Immunology*, *34*(9), 440–445.

Segura, E., & Amigorena, S. (2014). Cross-presentation by human dendritic cell subsets. *Immunology Letters*, *158*(1–2), 73–78. http://doi.org/10.1016/j.imlet.2013.12.001

Serbina, N. V, Salazar-mather, T. P., Biron, C. a, Kuziel, W. a, & Pamer, E. G. (2003). TNF / iNOS-Producing Dendritic Cells Mediate Innate Immune Defense against Bacterial Infection University of Texas at Austin, *19*, 59–70.

Shortman, K., & Liu, Y.-J. (2002). Mouse and human dendritic cell subtypes. *Nature Reviews. Immunology*, *2*(3), 151–61. http://doi.org/10.1038/nri746

Shortman, K., & Naik, S. H. (2007). Steady-state and inflammatory dendritic-cell development. *Nature Reviews. Immunology*, *7*(1), 19–30. http://doi.org/10.1038/nri1996

Sibilano, R., Frossi, B., Calvaruso, M., Danelli, L., Betto, E., Dall'agnese, A., … Gri, G. (2012). The aryl hydrocarbon receptor modulates acute and late mast cell responses. *The Journal of Immunology*, *189*, 120–127. http://doi.org/10.4049/jimmunol.1200009

Sixt, M., Kanazawa, N., Selg, M., Samson, T., Roos, G., Reinhardt, D. P., … Sorokin, L. (2005). The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node. *Immunity*, *22*(1), 19–29. http://doi.org/10.1016/j.immuni.2004.11.013

Smith, G., Stubbins, M. J., Harries, L. W., & Wolf C R. (1998). Molecular genetics of the human cytochrome P450 monooxygenase superfamily. *Xenobiotica*, *28*(12).

Stahl, P. D., & Ezekowitz, R. a. (1998). The mannose receptor is a pattern recognition receptor involved in host defense. *Current Opinion in Immunology*, *10*(1), 50–55. http://doi.org/10.1016/S0952-7915(98)80031-9

Stanek, L. W., Brown, J. S., Stanek, J., Gift, J., & Costa, D. L. (2011). Air pollution toxicology-a brief review of the role of the science in shaping the current understanding of air pollution health risks. *Toxicological Sciences*, *120*(SUPPL.1), 8–27. http://doi.org/10.1093/toxsci/kfq367

Steinman, R. M., Gutchinov, B., Witmer, M. D., & Nussenzweig, M. C. (1983). Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *The Journal of Experimental Medicine*, *157*(2), 613–627. http://doi.org/10.1084/jem.157.2.613

Steinman RM, I. J. (2010). Features of the dendritic cell lineage. *Immunol Rev*, *234*(1), 5–17. http://doi.org/10.1111/j.0105-2896.2009.00888.x.

Steinmann, R. M., & Cohn, Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. *The Journal of Experimental Medicine*, *137*, 1142–1162.

Stockinger, B., Di Meglio, P., Gialitakis, M., & Duarte, J. H. (2014). The aryl hydrocarbon receptor: multitasking in the immune system. *Annual Review of Immunology*, *32*, 403–32. http://doi.org/10.1146/annurev-immunol-032713-120245

Stone KC, Mercer RR, Gehr P, Stockstill B, C. J. (1992). Allometric relationships of cell numbers and size in the mammalian lung. *Am J Respir Cell Mol Biol*, *6*(2), 235–243.

Strohl, K. P., Butler, J. P., & Malhotra, A. (2012). Mechanical properties of the upper airway. *Comprehensive Physiology*, *2*(3), 1853–72. http://doi.org/10.1002/cphy.c110053

Sun, J. L., Zeng, H., & Ni, H. G. (2013). Halogenated polycyclic aromatic hydrocarbons in the environment. *Chemosphere*, *90*(6), 1751–1759. http://doi.org/10.1016/j.chemosphere.2012.10.094

Sunyer, J., Spix, C., Quénel, P., Ponce-de-León, a, Pönka, a, Barumandzadeh, T., … Katsouyanni, K. (1997). Urban air pollution and emergency admissions for asthma in four European cities: the APHEA Project. *Thorax*, *52*(9), 760–765. http://doi.org/10.1136/thx.52.9.760

Sussan, T. E., Ingole, V., Kim, J. H., McCormick, S., Negherbon, J., Fallica, J., … Biswal, S. (2014). Source of biomass cooking fuel determines pulmonary response to household air pollution. *American Journal of Respiratory Cell and Molecular Biology*, *50*(3), 538–548. http://doi.org/10.1165/rcmb.2013-0201OC

Swiecki, M., & Colonna, M. (2015). The multifaceted biology of plasmacytoid dendritic cells. *Nature Reviews Immunology*, *15*(8), 471–485. http://doi.org/10.1038/nri3865

Tao, S., Li, X., Yang, Y., Coveney, R. M., Lu, X., Chen, H., & Shen, W. (2006). Dispersion modeling of polycyclic aromatic hydrocarbons from combustion of biomass and fossil fuels and production of coke in Tianjin, China. *Environmental Science and Technology*, *40*(15), 4586–4591. http://doi.org/10.1021/es060220y

Thatcher, T. H., Williams, M. A., Pollock, S. J., Mccarthy, C. E., Lacy, S. H., Phipps, R. P., & Sime, P. J. (2016). Endogenous ligands of the aryl hydrocarbon receptor regulate lung dendritic cell function. *Immunology*, 41–54. http://doi.org/10.1111/imm.12540

Trompette, A., Divanovic, S., Visintin, A., Blanchard, C., Hegde, R. S., Madan, R., … Karp, C. L.

(2009). Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature*, *457*(7229), 585–8. http://doi.org/10.1038/nature07548

Upham, J. W., Denburg, J. A., & O'Byrne, P. M. (2002). Rapid response of circulating myeloid dendritic cells to inhaled allergen in asthmatic subjects. *Clinical and Experimental Allergy*, *32*(6), 818–823. http://doi.org/10.1046/j.1365-2222.2002.01375.x

Upham, J. W., & Stumbles, P. a. (2003). Why are dendritic cells important in allergic diseases of the respiratory tract? *Pharmacology & Therapeutics*, *100*(1), 75–87. http://doi.org/10.1016/S0163-7258(03)00094-9

USEPA. (2004). Air Quality Criteria for Particulate Matter. *Air Quality Criteria for Particulate Matter*, *I*(October), 900. http://doi.org/EPA 600/P-99/002aF-bF

van Duin, D., Medzhitov, R., & Shaw, A. (2006). Triggering TLR signaling in vaccination. *Trends in Immunology*, *1*(27), 49–55.

van Helden, M. J., & Lambrecht, B. N. (2013). Dendritic cells in asthma. *Current Opinion in Immunology*, *25*(6), 745–754. http://doi.org/10.1016/j.coi.2013.10.002

Veldhoen, M., Hirota, K., Westendorf, A. M., Buer, J., Dumoutier, L., Renauld, J.-C., & Stockinger, B. (2008). The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature*, *453*(7191), 106–9. http://doi.org/10.1038/nature06881

Vergen, J., Hecht, C., Zholudeva, L. V, Marquardt, M. M., & Nichols, M. G. (2012). Metabolic imaging using two-photon excited NADH intensity and fluorescence lifetime imaging. *Microsc Microanal.*, *18*(4). http://doi.org/10.1017/S1431927612000529.Metabolic

Verstraelen, S., Van Den Heuvel, R., Nelissen, I., Witters, H., Verheyen, G., & Schoeters, G. (2005). Flow cytometric characterisation of antigen presenting dendritic cells after in vitro exposure to diesel exhaust particles. *Toxicology in Vitro : An International Journal Published in Association with BIBRA*, *19*(7), 903–7. http://doi.org/10.1016/j.tiv.2005.06.017

Vince, J. E., & Silke, J. (2016). The intersection of cell death and inflammasome activation. *Cellular and Molecular Life Sciences*, *73*(11–12), 2349–2367. http://doi.org/10.1007/s00018-016-2205-2

Vogel, C. F. A., Goth, S. R., Dong, B., Pessah, I. N., & Matsura, F. (2008). Aryl hydrocarbon receptor signaling mediates expression of indoleamine 2,3-dioxygenase. *Biochemical and Biophysical Research Communications*, *375*(3), 331–335. http://doi.org/10.1016/j.bbrc.2008.07.156.Aryl

Vogel, C. F. A., & Matsumura, F. (2009). A new cross-talk between the Aryl hydrocarbon receptor and RelB, a member of the NF-κB family. *Biochemical Pharmacology*, *77*(4), 734–745. http://doi.org/10.1126/scisignal.2001449.Engineering

Vremec, D., Lieschke, G. J., Dunn, a R., Robb, L., Metcalf, D., & Shortman, K. (1997). The influence of granulocyte/macrophage colony-stimulating factor on dendritic cell levels in mouse lymphoid organs. *European Journal of Immunology*, *27*, 40–44. http://doi.org/10.1002/eji.1830270107

Wahid, S. M. S. (2017). Automotive brake wear: a review. *Environmental Science and Pollution Research*, 1–7. http://doi.org/10.1007/s11356-017-0463-7

Wang, B., Li, K., Jin, W., Lu, Y., Zhang, Y., Shen, G., … Tao, S. (2013). Properties and Inflammatory Effects of Various Size Fractions of Ambient Particulate Matter from Beijing on A549 and J774A.1 Cells.

Wang, C., Xu, C.-X., Krager, S. L., Bottum, K. M., Liao, D.-F., & Tischkau, S. a. (2011). Aryl Hydrocarbon Receptor Deficiency Enhances Insulin Sensitivity and Reduces PPAR-α Pathway Activity in Mice. *Environmental Health Perspectives*, *119*(12), 1739–1744. http://doi.org/10.1289/ehp.1103593

Wang, C., Ye, Z., Kijlstra, A., Zhou, Y., & Yang, P. (2014). Activation of the aryl hydrocarbon receptor affects activation and function of human monocyte-derived dendritic cells. *Clinical and Experimental Immunology*, 1–24. http://doi.org/10.1111/cei.12352

Warren, T. K., Mitchell, K. a, & Lawrence, B. P. (2000). Exposure to 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) Suppresses the Humoral and Cell-Mediated Immune Responses to Influenza A Virus without Affecting Cytolytic Activity in the Lung. *Toxicological Sciences*. http://doi.org/10.1093/toxsci/56.1.114

Webb, L. M., Lundie, R. J., Borger, J. G., Brown, S. L., Connor, L. M., Cartwright, A. N., … MacDonald, A. S. (2017). Type I interferon is required for T helper (Th) 2 induction by dendritic cells. *The EMBO Journal*, *36*(16), 2404–2418. http://doi.org/10.15252/embj.201695345

Weibel, E. R. (2009). What makes a good lung? *Swiss Medical Weekly*, *139*(27–28), 375–86. http://doi.org/smw-12270

Wicks, I. P., & Roberts, A. W. (2016). Targeting GM-CSF in inflammatory diseases. *Nature Reviews Rheumatology*, *12*(1), 37–48. http://doi.org/10.1038/nrrheum.2015.161

Wiethe, C., Dittmar, K., Doan, T., Lindenmaier, W., & Tindle, R. (2003). Enhanced effector and

memory CTL responses generated by incorporation of receptor activator of NF-kappa B (RANK)/RANK ligand costimulatory molecules into dendritic cell immunogens expressing a human tumor-specific antigen. *J Immunol*, *171*(8), 4121–4130. http://doi.org/10.4049/jimmunol.171.8.4121

Williams, M. A., Porter, M., Horton, M., Guo, J., Roman, J., Williams, D. A., … Georas, S. N. (2007). Ambient particulate matter directs nonclassic dendritic cell activation and a mixed TH1/TH2-like cytokine response by naive CD4+ T cells. *The Journal of Allergy and Clinical Immunology*, *119*(2), 488–97. http://doi.org/10.1016/j.jaci.2006.10.022

Wills-Karp, M. (1998). Interleukin-13: central mediator of allergic asthma. *Science*, *282*, 2258–2261. Retrieved from http://dx.doi.org/10.1126/science.282.5397.2258

Wincent, E., Bengtsson, J., Mohammadi Bardbori, A., Alsberg, T., Luecke, S., Rannug, U., & Rannug, A. (2012). Inhibition of cytochrome P4501-dependent clearance of the endogenous agonist FICZ as a mechanism for activation of the aryl hydrocarbon receptor. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(12), 4479–84. http://doi.org/10.1073/pnas.1118467109

Wincent, E., Le Bihanic, F., & Dreij, K. (2016). Induction and inhibition of human cytochrome P4501 by oxygenated polycyclic aromatic hydrocarbons. *Toxicol. Res.*, 788–799. http://doi.org/10.1039/C6TX00004E

Wolf, A. J., & Underhill, D. M. (2014). Phagocytosis. *Macrophages: Biology and Role in the Pathology of Diseases*, 91–109.

Wong, P. S., Vogel, C. F., Kokosinski, K., & Matsumura, F. (2010). Arylhydrocarbon receptor activation in NCI-H441 cells and C57BL/6 mice possible mechanisms for lung dysfunction. *American Journal of Respiratory Cell and Molecular Biology*, *42*(2), 210–217. http://doi.org/10.1165/rcmb.2008-0228OC

World Health Organization. (2012). Burden of disease from joint effects of household and ambient air pollution for 2012.

Wright, J. R. (2005). Immunoregulatory functions of surfactant proteins. *Nature Reviews. Immunology*, *5*(1), 58–68. http://doi.org/10.1038/nri1528

Wurtz, O., Bajénoff, M., & Guerder, S. (2004). IL-4 mediated inhibition of IFN-γ production by CD4+ T cells proceeds by several developmentally regulated mechanisms. *International Immunology*, *16*(3), 501–508. http://doi.org/10.1093/intimm/dxh050

Yeung, A. W. S., Terentis, A. C., King, N. J. C., & Thomas, S. R. (2015). Role of indoleamine 2,3-

dioxygenase in health and disease. *Clinical Science*, *129*(7), 601–672. http://doi.org/10.1042/CS20140392

Ying, W. (2008). NAD+/NADH and NADP+/NADPH in cellular functions and cell death: regulation and biological consequences. *Antioxidants & Redox Signaling*, *10*(2), 179–206. http://doi.org/10.1089/ars.2007.1672

Yoshida, H., Okabe, Y., Kawane, K., Fukuyama, H., & Nagata, S. (2005). Lethal anemia caused by interferon-β produced in mouse embryos carrying undigested DNA. *Nature Immunology*, *6*(1), 49–56. http://doi.org/10.1038/ni1146

Yu, C. I., Becker, C., Wang, Y., Marches, F., Helft, J., Leboeuf, M., … Palucka, K. (2013). Human cd1c+ dendritic cells drive the differentiation of cd103+ cd8+ mucosal effector t cells via the cytokine tgf-β. *Immunity*, *38*(4), 818–830. http://doi.org/10.1016/j.immuni.2013.03.004

Zelenay, S., & Reis e Sousa, C. (2013). Adaptive immunity after cell death. *Trends in Immunology*, *34*(7), 329–335.

Zhang, Y., Xu, C., & Cardell, L. (2009). Long-term exposure to IL-1beta enhances Toll-IL-1 receptor-mediated inflammatory signaling in murine airway hyperresponsiveness. *Eur Cytokine Netw*, *20*, 148–56.

Zhou, Y., Tung, H., Tsai, Y., Hsu, S., Chang, H., Kawasaki, H., … Huang, S. (2015). Aryl hydrocarbon receptor controls murine mast cell homeostasis, *121*(16), 3195–3205. http://doi.org/10.1182/blood-2012-08-453597.The

Zhou, Z., Dionisio, K. L., Arku, R. E., Quaye, A., Hughes, A. F., Vallarino, J., … Ezzati, M. (2011). Household and community povety, biomass use, and air pollution in Accra, Ghana. *Pnas*, *108*(27), 11028–11033. http://doi.org/10.1073/pnas.1019183108/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1019183108

Zhou, Z., Dionisio, K. L., Verissimo, T. G., Kerr, A. S., Coull, B., Arku, R. E., … Ezzati, M. (2013a). Chemical composition and sources of particle pollution in affluent and poor neighborhoods of Accra, Ghana. *Environmental Research Letters*, *8*(4), 44025. http://doi.org/10.1088/1748-9326/8/4/044025

Zhou, Z., Dionisio, K. L., Verissimo, T. G., Kerr, A. S., Coull, B., Arku, R. E., … Ezzati, M. (2013b). Chemical composition and sources of particle pollution in affluent and poor neighborhoods of Accra, Ghana. *Environmental Research Letters*, *8*(4), 44025. http://doi.org/10.1088/1748-9326/8/4/044025

Zhou, Z., Dionisio, K. L., Verissimo, T. G., Kerr, A. S., Coull, B., Howie, S., … Ezzati, M. (2013). Chemical characterization and source apportionment of household fine particulate matter in rural, peri-urban, and urban West Africa. *Environmental Science & Technology*, *48*(2), 1343–51. http://doi.org/10.1021/es404185m

Zhou, Z., Dionisio, K. L., Verissimo, T. G., Kerr, A. S., Coull, B., Howie, S., … Ezzati, M. (2014). Chemical Characterization and Source Apportionment of Household Fine Particulate Matter in Rural, Peri-urban, and Urban West Africa. *Environmental Science & Technology*, *48*(2), 1343–51. http://doi.org/10.1021/es404185m