DEVELOPMENT OF AN *IN VITRO* MIMIC OF THE *IN VIVO* DEPOSITION OF VIRUS-CONTAINING PARTICULATE MATTER ONTO THE BRONCHIAL EPITHELIUM

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Abstract

The association between exposure to particulate matter (PM) air pollutants and respiratory viral infection is well recognized, suggesting potential interaction between PM and viruses. We developed an *in vitro* mimic of the *in vivo* event of exposure to PM contaminated with the Respiratory Syncytial Virus (RSV). Concentration of an RSV stock solution without affecting its infectivity and a particle levitation apparatus were the foundations of the methodology developed to generate a specific number of distinct particles of engineered chemical composition prior to their dry deposition directly onto human lung epithelial cell cultures in a controlled fashion. The viruses carried by these PM mimics (PM_{Mimics}) remain capable of viral infection upon dry-delivery onto cell cultures; in fact, *ex vivo* PM_{Mimics} comprised of RSV and carbon (P_{C+RSV}) remain capable of infection months longer than *ex vivo* PM_{Mimics} comprised of RSV (A_{RSV}). Cells dosed with A_{RSV}, P_{C+RSV}, or PM_{Mimics} comprised of carbon (P_C) responded differentially as exemplified by the secretion patterns of IL-6 and IL-8. Regression analysis of the secretion of these two mediators 12 and 36 hours post infection with increasing concentrations of A_{RSV} or P_{C+RSV} confirmed higher secretion of both IL-6 and IL-8 upon infection with the latter and at earlier time points. In conclusion, our system provides an approach to study the airway epithelial-environmental interaction and the PM-viral interaction in the pathogenesis of respiratory diseases involving inhalation of environmental agents.

Dedication

Para mi abuelito, quien me infundo el amor al conocimiento; para mi abuelita y su encantadora disposicion siempre tan positiva; para mi mama, quien me ensena dia con dia lo que es ser una persona extraordinaria y completamente desprendida; para mis hermanos, quienes me inspiran a ser una mejor persona cada dia…

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As with any story worth telling, drama found its way through my research and I would like to thank Dr. Plettner and Dr. Walker for helping me gain perspective and for their supporting and encouraging words.

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"No man is an island, entire of itself. Everyman is a piece of the continent, a part of the main."

John Donne, Devotions upon Emergent Occasions, 1624

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Abbreviations

A-549	Adenocarcinomic human alveolar epithelial cells
AC	Alternating current
AD	Aerodynamic diameter
Ab	Antibody
AEC	Primary airway epithelial cell
AELF	Airway epithelial lining fluid
Ag	Antigen
ALI	Air liquid interface
AM	Alveolar macrophage
AMP	Antimicrobial peptides
ANOVA	Analysis of variance
APC	Antigen presenting cell
A_{RSV}	PM_{Mimics} consisting of RSV alone
ATCC	American type culture collection
ASL	Airway surface liquid
BCKDF	British Columbia Knowledge Development Fund
BEAS-2B	Human bronchial epithelial cells transformed by Adenovirus SV40
BSA	Bovine serum albumin
C	Celsius

CBP	Coarse biological particle
Cc	Slipflow
CC	Colloidal carbon
CCL	Chemokines that have to adjacent cysteines near their amino terminus
CFI	Canadian Foundation of Innovation
CLA	Canadian Lung Association
cm	Centimetre
CMI	Cell mediated immunity
COPD	Chronic obstructive pulmonary disease
Cox	Cyclo-oxygenase
CXCL	Chemokines in which two amino terminal cysteines are separated by another amino acid
d	Diameter
DAPI	4,6-diamidino-2-phenylindole
DC	Direct current
DCs	Dendritic cells
DEP	Diesel exhaust particles
DIC	Differential interference contrast
E*	Estimated viral dose
E_{AC}	Component of the AC field
ECP	Eosinophil cationic protein
E_{DC}	Component of the DC field
EDN	Eosinophil derived neurotoxin
EDTA	Ethylenediaminetetra-acetic acid

EHC-93	Environmental Health Centre 93
ELISA	Enzyme linked immunosorbent assay
ET	Endothelin
F	Fussion protein of RSV
FACS	Fluorescence-activated cell sorter
FBP	Fine biological particle
FBS	Foetal Bovine Serum
FESEM	Field emission scanning electron microscopy
FSC	Forward scatter
g	Gravitational acceleration
G	Attachment protein of RSV
GAG	Glycosaminoglycans
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRO	Growth regulated protein
GSK	GlaxoSmithKline
GTP	Gas to particle conversion
GTPase	Enzyme that hydrolyzes guanosine triphosphate
h	Hour
HBEC	Human bronchial epithelial cell
HeNe	Helium Neon
HEp-2	Human carcinomic laryngeal epithelial cells
HO	Hemoxygenase-1

Hz	Hertz
IFN	Interferons
Ig	Immunoglobulin
IL	Interleukins
IMPACT	Integrated and Mentored Pulmonary and Cardiovascular Training
IP	IFNγ inducible protein
IU	Infectious units
Kd	Stokesian drag parameter
km	Kilometre
kV	Kilovolt
L	RSV polymerase
LB	Lower bound
LDH	Lactose dehydrogenase
LRI	Lower respiratory infection
LRT	Long range transport
LT	Lymphotoxin
M	Mass
M	Matrix protein of RSV
M2-1	RSV transcription elongation factor
M2-2	RSV transcription regulation factor
MALDI	Matrix-assisted laser desorption/ionization
MCP	Monocyte chemotactic protein
M-CSF	Macrophage-colony stimulating factor

MEM	Minimal essential media
min	Minute
MIP	Macrophage inflammatory protein
mL	Millilitre
mM	Millimolar
mm	Millimetre
MOI	Multiplicity of infection
mRNA	Messenger RNA
MSFHR	Michael Smith Foundation for Health Research
m/z	Mass to charge
N	Nucleoprotein of RSV
NF	Nuclear Factor
NHBE	Normal human bronchial epithelial cell
NK	Natural killer cell
nm	Nanometre
NS	Non-structural protein
NSERC	National Sciences and Engineering Research Council of Canada
OSM	Oncostatin M
P	Nucleocapsid phosphoprotein of RSV
PAF	Platelet activating factor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
P_C	PM_{Mimics} consisting of carbon alone

$P_{C + MEM}$	PM_{Mimics} consisting of carbon and MEM
$P_{C + PBS}$	PM_{Mimics} consisting of carbon and PBS
$P_{C + RSV}$	PM_{Mimics} consisting of carbon and RSV
PCL	Periciliary liquid layer
pfu	Plaque forming units
pg	Picogram
pL	Picolitre
PM	Particulate matter
$PM_{0.1}$	PM having an AD smaller than 0.1 microns and larger
$PM_{0.1-2.5}$	PM having an AD smaller than 2.5 microns and larger than 0.1 microns
$PM_{2.5}$	PM having an AD smaller than 10 microns and larger than 2.5 microns
$PM_{2.5-10}$	PM having an AD smaller than 10 microns and larger than 2.5 microns
PM_{10}	PM having an AD smaller than 10 microns
PM_{Mimics}	Particulate matter mimics
PMN	Polymorphonuclear leukocyte
PRR	Pattern recognition receptors
Q	Quartile
r	Displacement from the centre
RANTES	Regulated on activation, normal T expressed and secreted
RNA	Ribonucleic acid
RSV	Respiratory Syncytial Virus
s	Second
S	Secretory cells activated by antibodies

SARS	Severe acute respiratory syndrome
SD	Standard deviation
SEM	Scanning electron microscopy
SH	Small hydrophobic protein
SFU	Simon Fraser University
SP	Surfactant proteins
SPP	Secreted phosphoprotein
SS*	Starting solution
SSC	Side scatter
SSD	Statistically significant difference
ssRNA	Single stranded RNA
TARC	Thymus and activation regulated chemokines
T_H	T helper cell
TNF	Tumour necrosis factor
TSP	Total suspended particles
UAP	Urban air particles
UB	Upper bound
UfCp	Ultrafine carbon particles
URI	Upper respiratory infection
v	Velocity
VEGF	Vascular endothelial growth factor
VPF	Vascular permeability factor
v/v	Volume by volume

μ	Viscosity of gas
μm	Micrometre
0-P	Zero to Peak
1HAEo-	Normal human airway epithelial cells transformed with Simian virus 40
9HTE	Human tracheal epithelial cell line

Glossary

Accumulation mode	Large PM (0.1 – 1.0 μm) that results from the coagulation of smaller particles and/or by condensation of gas vapour onto existing aerosols
Acquired Immunity	See adaptive immunity
Acute episode	Intense and short-lived occurrence
Acute-phase proteins	Proteins that participate in the initial stages of host defense against infection and thus are found in the blood shortly after infection
Adaptive Immunity	The development of immunologic memory and the response of antigen-specific lymphocytes to antigen. It increases with repeat exposure to a given pathogen
Aerodynamic diameter	Diameter of a perfect sphere of water that would have the same terminal velocity as a given particle in air
Aitken mode	Small PM(0.01 - 0.1 μm) formed by gas to particle conversion
Anthropogenic source	Resulting from human activities
Antibody	See immunoglobulin
Antioxidant	A molecule that inhibits the oxidation of other molecules
Apoptosis	Natural process in which cells activate an internal death program
Atopy	Also atopic allergy. It is the tendency to produce immediate hypersensitive reactions to innocuous agents
Baltimore classification	Virus classification based on type of genome and replication mode

Boundary layer	The air layer above the ground affected by transfer of heat and/or moisture from the surface
Bronchiolitis	Inflammation of the bronchioles
Bronchitis	Inflammation of the bronchi
Bronchospasm	Sudden contraction of the muscles surrounding the bronchioles
Cathelicidins	Amphipathic cationic peptides that disrupt plasma membrane integrity and as such exhibit broad-spectrum antimicrobial activity
Cell mediated immunity	An adaptive immune response in which antigen-specific T cells are the main players
$CD4^+$ T cells	A subset of T cells exhibiting the co-receptor $CD4^+$ as to recognize proteins from intravesicular sources bound to MHC class II molecules on cell membranes. They differentiate into Helper T cells
$CD8^+$ T cells	A subset of T cells exhibiting the co-receptor $CD8^+$ as to recognize antigens from intracellular pathogens bound to MHC class I molecules on cell membranes. They differentiate into Cytotoxic T cells
Chemokines	Chemoattractant chemokines which induce migration and activation of receptive cells
Co-culture	Growth of different cell types in a single culture
Collectins	Soluble pattern recognition receptors that upon binding opsonise pathogens
Complement	Set of proteins that upon activation binds to extracellular pathogens, killing some directly and opsonising others.
Congenic	Organisms that differ uniquely in a specific location of their DNA sequence
Continuous sampling	Sampling without interruption
Cytokines	Proteins produced by several cell types that modify the behaviour of cells that express receptors to specific

	cytokines
Cytopathicity	Causing pathological changes in cells
Cytotoxic T cells	T cells capable of killing other cells and thus are important in defense against intracellular pathogens
Defensins	Cationic peptides that upon binding of a pathogen disrupt plasma membrane stability
Efferocytosis	Phagocytosis of apoptotic cells
Endothelins	Proteins that raise blood pressure by constricting blood vessels
Endothelium	Tissue that lines the inner wall of the blood vessels
Environmental persistence	The amount of time that a particular substance continues to exist in the environment
Epithelium	Tissue that lines body cavities and surfaces
Ex vivo	Experiments done on cells recently extracted from an organism
Exogenous	A substance originated outside the system
Fomite	An inanimate material contaminated with a virus
Gas to particle conversion	Formation of aerosols through condensation of saturated gases
Granulocytes	White blood cells with cytoplasmic granules and mulitlobed nuclei also known as Polymorphonuclear leukocytes
Granulocytosis	Increased number of granulocytes in the blood
Granzyme	Protease that induces apoptosis of virus-infected cells
Heterogeneous nucleation	Nucleation events that occur at phase boundaries using impurities as nucleation sites
Homogeneous nucleation	Nucleation events that occur at random locations in a supersaturated environment and involve the creation of a phase boundary

Humoral immunity	A form of adaptive immunity which is antibody mediated
Immunoglobulins	A family of plasma proteins which exhibit at least one immunoglobin domain. Also known as antibodies, bind specifically to their antigen
In vitro	Experiments performed in cells outside of an organism, in a controlled environment
In vivo	Within a living organism
Innate immunity	The early stages of the immune response to pathogens; it is not pathogen specific
Interferons	Cytokines that interfere with viral replication
Interleukins	Cytokines produced by leukocytes
Interstitium	Connective tissue between the cellular elements of a tissue
Knock out	Organisms in which a specific gene has been rendered inoperative
Lactoferrin	Iron binding antimicrobial protein present in secretions
Leukocytes	White blood cells
Leukocytosis	Increased number of leukocytes in the blood
Lymphocytes	White blood cells that exhibit specific antigen receptors and thus mediate the adaptive immune response
Lymphoid organ	Tissue in which great numbers of lymphocytes interact. Primary lymphoid organs are those where lymphocytes are generated, such as the bone marrow and thymus. Secondary lymphoid organs are those in which the adaptive immune response is initiated, such as the mucosal associated lymphoid tissues, lymph nodes and spleen
Lysozyme	Enzyme that hydrolyses bacterial cell walls

Memory Lymphocytes	Cells that mediate immunological memory. They respond very rapidly to reexposure to the antigen that originally induced them.
Monocistronic	Containing the genetic information for a single gene
Morbidity	Rate of incidence of a disease
Morphometry	Quantification of the variation and change of the cellular/ structural components of the lung
Mortality	Measure of the number of deaths due to a specific condition
Naïve Lymphocytes	Cells that have never encountered their specific antigen.
Nucleocapsid	A viral structure consisting of the viral genome and the capsid
Nucleation	The localized initiation of a new phase
Nucleation mode	Smallest PM (< 0.01 μm) generated by gas to particle conversion
Number concentration	Number of entities of a constituent in a mixture divided by the volume of the mixture
Opsonins	Molecules that when bound to a pathogen, targets the pathogen for phagocytosis
Opsonisation	Modification of the surface of a pathogen so that it can be readily phagocytised, usually by the binding of molecules recognized by phagocytic cells
Oxidative stress	Imbalance between the production of reactive oxygen species and the ability to process the reactive intermediates and/or repair the resulting damage
Particle bound water	The content of water of a given particle; depends of the chemical composition of the particle and on the relative humidity
Particulate matter	Pollutant liquid or solid particles suspended in the troposphere

Parenchyma	Functional parts of an organ; the lung parenchyma is composed by alveolar walls, surfactant and interstitial cells
Pathogenesis	The chain of events that lead to a disease
Perforin	Protein that forms pores when inserted in target plasma membranes
Peripheral blood mononuclear cell	Lymphocytes, monocytes or macrophages found in the blood
Permissiveness	When a pathogen is able to circumvent the host defences and is able to replicate
Phagocytosis	Cellular process involving the vesicular internalization of solid particles
Pneumonia	Inflammation of the lung; including the parenchyma
Pollutant	A substance that in sufficient concentrations could cause environment damage and/or be toxic to humans
Polymerase	Enzyme in charge of the polymerization of nucleic acids
Polymorphonuclear leukocyte	See granulocytes
Polymerase processivity	Number of nucleotides added by a polymerase per association events with the template
Pseudostratified mucociliary columnar epithelium	An epithelium formed by one layer of cells, some of which are ciliated, in which the cell nuclei are not aligned so that it seems that there is more than one layer of cells and that is covered by mucus
Simple cuboidal epithelium	Epithelium formed by a monolayer of cells that have the same height, depth and width
Simple squamous epithelium	Epithelium formed by a monolayer of flat cells
Stratosphere	It is the layer of the atmosphere directly above the

	troposphere and still part of the biosphere
Systemic Inflammation	Inflammation involving the endothelium and several organs
Terminal velocity	The velocity at which the force of gravity and the drag force balance each other
T_H1 cells	Subset of $CD4^+$ T cells mainly involved in activating macrophages
T_H2 cells	Subset of $CD4^+$ T cells mainly involved in activating B cells for antibody production
Transcription	RNA synthesis from a complementary template
Transcription antiterminator	Protein that allows the RNA polymerase to ignore termination signals
Transcription attenuation	Regulatory mechanism that results in the premature termination of transcription
Transcytotic trafficking	Transport of molecules through the interior of a cell
Transgenic	Organism expressing genes transferred from a different species
Translation	Protein synthesis from a mRNA template
Troposphere	Lowest portion of the Earth's atmosphere
Vehicle-borne transmission	Infection caused by contact with a fomite
Virulence	Degree of pathogenicity measured by the ability of the pathogen to cause disease and its severity
Wheezing bronchitis	Bronchitis in which the inflammation of the bronchi causes so much narrowing that a whistling sound is heard when breathing
Xenobiotic	Compounds foreign to an organism's normal biochemistry

1: Introduction

Viruses are extraordinarily simple organisms with a remarkable adaptation rate, which has resulted in various capabilities that have enabled them to successfully colonize every other organism on the planet, making virology an area of active research. As such, great insight into their different modes of replication, diverse life cycles and evasion mechanisms of the host's immune system has been attained.

Traditional strategies employed in virology range from dosing a monolayer of cells with a solution containing the virus of interest to aerosolizing this solution[1-4] in order to explore numerous possible mechanisms of infection[5]. While a myriad of information has been gained using these approaches, solution chemistry based experimental strategies limit the understanding of important processes involving the microenvironment of those viruses that are either vehicle-borne or airborne[5-7]. In vehicle-borne transmission, the host is infected simply by coming in contact with a fomite (an inanimate material contaminated with the virus)[8], while in the airborne scenario the host is infected by inhaling ambient particles contaminated with the virus[9]. What these two modes of transmission have in common is the potential role of contaminated aerosols, both solid and liquid, under ten microns in diameter (PM_{10}) found either suspended in the troposphere, or simply settled onto surfaces, to be the means of infection[5,10]. The airborne mode of infection is of overwhelming importance in impoverished countries,

while in developed countries concern tends to be focussed toward certain occupational fields, such as health care and waste disposal[7,11,12].

PM_{10} acting as an infectious reservoir for viruses[13] is of interest as particulate pollution itself is known to ellicit numerous adverse health effects[14]. This thesis introduces of a new methodology that allows for the dry deposition of a controlled number of particulate matter mimics (PM_{Mimics}) containing viable virions capable of infection directly onto a cell culture, thus recreating the *in vivo* event of deposition of virus contaminated PM_{10} onto the airway epithelium.

2: Airway Epithelium

"Prana, [breath], is a subtle invisible force. It is the life-force that pervades the body. It is the factor that connects the body and the mind, because it is connected on one side with the body and on the other side with the mind. It is the connecting link between the body and the mind."

Swami Chidananda Saraswati,
The Philosophy, Psychology, and Practice of Yoga

The airway epithelium is the threshold between the internal milieu and the troposphere – and everything suspended in it. Consequently, it is entrusted with many different and important functions. In particular, it is the site of gas exchange, as well as the first line of defense against environmental pollutants and pathogens[15]. As such, the cell populations lining the airways are extremely diverse and complex.

Upon inhalation, the air makes its way through a branching labyrinth from the nose passage to the sites of gas exchange, the alveoli. As the proximal airways branch into the distal airways, there is an increase in surface area of approximately an order of magnitude[16]. In addition, both, the epithelium lining the airway[17] and the airway surface liquid[18] (ASL) change as the conducting

airways transform into the respiratory regions of the lung (Figure 2.1). The conducting airways are lined by a pseudostratified mucociliary columnar epithelium[19]; starting at the bronchioles, the respiratory portion of the lung is lined by a ciliated simple columnar/cuboidal epithelium, whereas the alveoli are lined by a simple squamous epithelium[20]. Similarly, the epithelium in the upper respiratory tract is covered by a thick continuous layer of ASL, which in the conducting region of the airways is no longer a continuous layer and progressively decreases in thickness towards the distal airways[18].

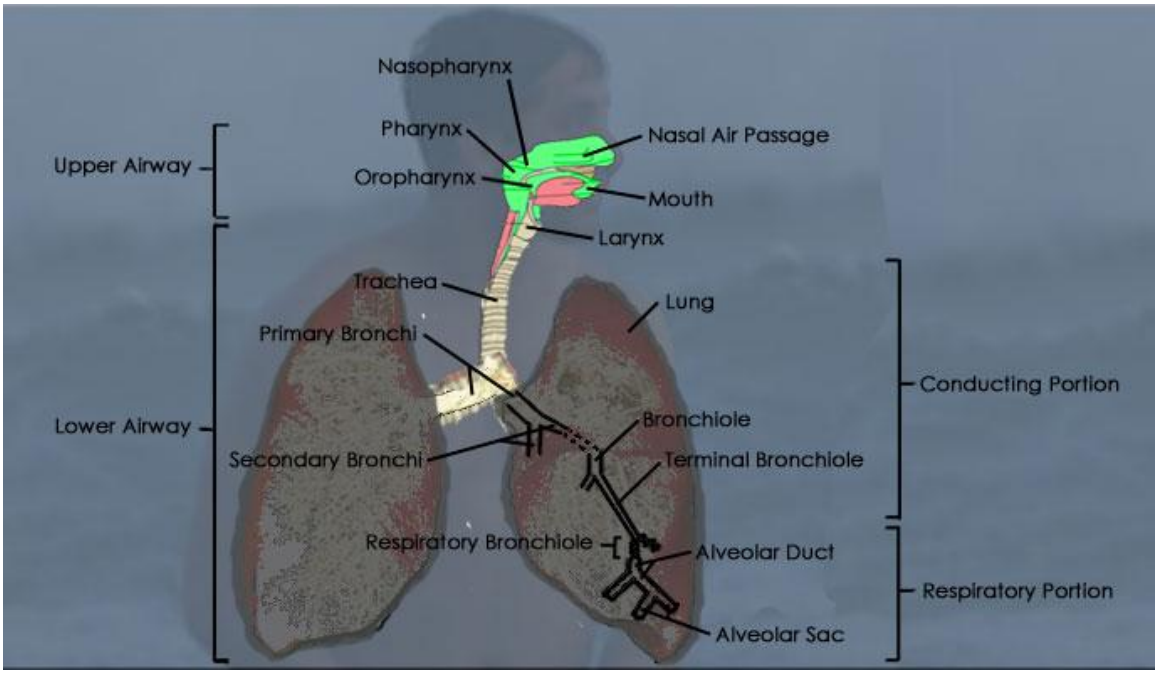

Figure 2.1 Airway Anatomy

2.1 The Cells of the Airway Epithelium

As the functionality of the airways change from air transport, filtration and humidification to gas exchange, the cellular population lining the airway changes as well, becoming more specialized towards the distal airways. At least five cell types line the conducting airways: ciliated cells, Clara cells, mucous goblet cells, neuroendocrine cells, and serous cells[17,21-24]. However, the diversity of cells lining the respiratory portion of the lung is much lower, with the bronchioles being lined mainly by ciliated cells, with some goblet and Clara cells interspersed. Finally, the alveoli are lined with Type I and Type II pneumocytes. For a brief description of cell function and location[17] see Table 2.1.

Table 2.1 The Cells of the Airway Epithelium

Cell Type	**Airway Location**	**Function**
Ciliated Cells[17]	Upper Respiratory Tract and Conducting Airways	Generate mucous flow
Clara Cells[17]	Respiratory Region	Surfactant secretion[15] Collectin secretion[25] Xenobiotic metabolism[26]
Mucous Goblet Cells[21]	Upper Respiratory Tract and Conducting Airways	Mucin secretion Airway lactoperoxidase secretion
Neuroendocrine Cells[22]	Upper Respiratory Tract and Conducting Airway	O_2 sensing Airway repair
Serous Cells[23]	Conducting Airway	Kallikrein secretion[27] Bactericidal secretion[23] IgA secretion[26] Lysozyme[28]
Type I pneumocytes	Alveoli	Gas exchange
Type II pneumocytes[24]	Alveoli	Surfactant secretion[15]

2.2 The Airway Surface Liquid

The airway surface liquid (ASL) is the solution that lies between the airway epithelium and the gases in the lumen. Starting from the epithelium towards the lumen, ASL consists of a hypophase, the periciliary liquid layer (PCL), and an epiphase, the mucus layer, separated by a surfactant layer[16,18,29].

PCL, also known as the sol layer, is a watery, low-viscosity layer whose thickness spans almost the entire length of the outstretched cilia (4-6 mm)[16,18] of the ciliated cells in the airway epithelium and is necessary for proper cilia function, clearance of noxious hydrophilic solutes[16], hydration of the upper ASL layers[29] and regulation of the immune response[30]. As such, optimal thickness, volume and composition of PCL are ensured by the airway epithelial cells, which in addition to replenishing PCL with fluid, electrolytes and antimicrobial components, secrete inflammatory mediators into PCL upon exogenous stimuli [30].

The next layer, which is the surfactant layer, consists of lipids (~92%), of which the most abundant are phospholipids (80%) and cholesterol, interspersed with surfactant proteins[29]. The surfactant layer preserves PCL integrity, prevents entanglement of cilia and facilitates mucus spreading[29] and thus enhances mucociliary clearance of contaminants[31]. In addition, surfactants are necessary for gas exchange homeostasis[32] and prevent alveolar collapse by regulating the surface tension at the air-liquid interface[29]; in particular, surfactant proteins (SP) – B and C, which are highly hydrophobic, are necessary for the integrity of the surfactant film at the air/liquid interface[33]. SP - A and D, on the other hand, play

an important role in the immune response against invading pathogens including bacteria, fungi and viruses[29].

The outermost layer, the mucus layer, consists mostly of an interwoven matrix of mucins, which being heavily glycosylated proteins efficiently bind and trap inhaled particles and pathogens so that they can be disposed of by expectoration or by mucociliary clearance[16,29], which itself could free the distal airways completely from exogenous particles in 6 hours[16]. As mentioned earlier, the characteristics of the ASL change from one location of the airway to the next. In fact, as the conducting airways narrow into the distal airways, so does the ASL. Whereas the upper airways are lined by a continuous three-layered liquid, in the smallest bronchioles there is no longer mucus. In addition, the thickness of the mucus layer varies immensely not only with the degree of branching of the airway, but within the same generation, i.e. degree of branching[18].

2.3 The immune response as it applies to the airway epithelium

The immune system can be thought of as a well-orchestrated army with many layers of defense, most obviously the innate and acquired/adaptive immunity[34]. The innate immunity is an immediate response based on the invariant recognition of general pathogen-associated motifs, which depends on granulocytes, also known as polymorphonuclear leukocytes (PMNs), and natural killer (NK) cells. The acquired immunity, on the other hand depends on lymphocytes, takes days to be established, is pathogen specific and, in some cases, confers protection to reinfection with the same pathogen. Table 2.2

provides a brief description of the activities of the cellular players of the immune system, also known as white blood cells or leukocytes[34].

Table 2.2 Leukocytes of the immune system

Immune branch	Lineage	Cell	Activated function
Innate	Granulocytes	Basophil	S
		Dendritic Cell (DC)	Main Ag presentation cell
		Eosinophil	S, Kills Ab covered parasites
		Mast Cell	S, histamine and vasodilator release
		Monocyte / Macrophage	Phagocytosis, activation of Inflammation, Ag presentation
		Neutrophil	Phagocytosis, Ag presentation
		Natural Killer Cells (NK)	S, Kills virus infected cells
Adaptive	Lymphocytes	Plasma B Cell	Ab production
		Memory B Cell	Immunologic memory
		T Helper Cells (T_H)	Differentiates into T_{H1} or T_{H2}
		Cytotoxic T Cells	Kills virus infected cells
		Memory T Cells	Immunologic memory
		Regulatory T Cells	Ends CMI

Ab = antibody, Ag = Antigen, CMI = Cell Mediated Immunity, and S = Secretory cells activated by Ab.

2.3.1 Innate Immunity

The first layer of defense against inhaled pathogens and pollutants is the anatomical barrier that separates the internal milieu and the external world: the airway epithelium. As mentioned earlier, the airway epithelium is coated by mucus, which minimizes adherence of pathogens and PM to the epithelium and facilitates their clearance. To complement mechanical clearance, epithelial

cells secrete antimicrobial agents, as well as antioxidants to prevent damage by noxious chemicals. Three types of antimicrobial agents are secreted by the airway epithelium: antimicrobial proteins[28], cationic antimicrobial peptides (AMP)[29] and antimicrobial soluble molecules. Two important antimicrobial proteins in the airway epithelium are lactoferrin and lysozyme, which actively kill bacteria by disrupting their plasma membranes among other mechanisms[28]. Cationic AMPs, such as cathelicidins and defensins[35], also lyse microbial cells, whereas soluble antimicrobial molecules target pathogens for phagocytosis, a process known as opsonisation. Opsonins[36] include collectins[25], which are pattern recognition receptors (PRR) that recognize microbial carbohydrates[37], and surfactant proteins[15] (SP) – A and D[29]. Finally, in order to protect the airways from oxidative damage, the airway epithelial lining fluid (AELF) contains antioxidants such as α-tocopherol, ascorbate, glutathione, LPO[21], mucins and uric acid[15].

If the injury or infection cannot be contained by the anatomical barriers, the resident macrophages, alveolar macrophages (present in the alveolar lumen[38] and in charge of phagocytizing PM_{10} when it reaches the lower respiratory tract[39]) and dust cells (fixed macrophages present in the interstitium), become activated and secrete the lipid mediators of inflammation, which are leukotrienes and prostaglandins. In addition, both, resident macrophages and affected epithelial cells, secrete pro-inflammatory mediators, known as cytokines (Table 2.3), such as interleukins (IL) and chemokines (chemotactic cytokines)[35]. Therefore, if initial innate immunity does not resolve the infection, inflammation is established.

Table 2.3 Cytokines and their Functions

Cytokine (alternative names)	**Producer Cells**	**Actions**	**T_H1/T_H2 Immunity**
CCL-1 (TCA-3)	T cells[40]	Neutrophil, T cell[34]	$T_H1 > T_H2$*[40]
CCL-2 (MCP-1)	Macrophage, Epithelial cells[41,42]	T cell, Monocyte, Basophil[34]	$T_H2 > T_H1$[41]
CCL-3 (MIP-1α)	Macrophage, Epithelial cells[41,43]	Monocyte/Macrophage, T cell, NK, Basophil, immature DC[34]	$T_H1 > T_H2$[34,44]
CCL-4 (MIP-1β)	Macrophage[41]	Monocyte/Macrophage, T cell, NK, Basophil, immature DC[34]	$T_H1 > T_H2$[34,44]
CCL-5 (RANTES)	Epithelial Cells[41,42]	Monocyte/Macrophage, T cell, NK, Basophil, Eosinophil, DC[34]	T_H2[45] /T_H1&[34,44]
CCL-7 (MCP-3)	Mononuclear cells[46]	T cell, Monocyte, Eosinophil, Basophil, DC[34] and NK[46]	$T_H1 < T_H2$[44]
CCL-8 (MCP-2)	Mononuclear cells, Fibroblasts[46]	T cell, Monocyte, Eosinophil, Basophil[34]	T_H1[47]
CCL-11 (Eotaxin)	Epithelial Cells[42]	Eosinophil, Macrophage, Lymphocyte Eotaxin[48]	T_H2[44,49]
CCL-13 (MCP-4)	Fibroblasts, Epithelial cells[50]	T cell, Monocyte, Eosinophil, Basophil, DC[34]	$T_H1 < T_H2$[44]
CCL-15 (MIP-5)	Lymphatics[51]	T cell, Monocyte, Neutrophil, DC[34];	T_H1*[51]
CCL-16	Macrophages[52]	Monocyte[34], Lymphocytes, Eosinophils, and DCs[52]	T_H1[52]
CCL-17 (TARC)	DC, Epithelial cells[53]	T cell, immature DC, NK, thymocyte[34]	$T_H1 < T_H2$[53]
CCL-18	Monocytes, Macrophages, DC[54]	Naïve T cell > T cell[34]	T_H2[54]
CCL-19 (MIP-3β)	Lymphatic tissues[55]	Naïve T cell, mature DC, B cell[34]	T_H1[55]
CCL-20 (MIP-3α)	T cell, Epithelial cells, Macrophages[56]	T cell, PBMC, DC[34]	T_H1[56]
CCL-21	Lymphatic tissues[55]	Naïve T cell, B cell, Mesangial cells[34], DC[57]	T_H1[55,57]
CCL-23 (MPIF-1)	DC, Monocytes,	Monocyte, T cell,	T_H2[58]

	Macrophages[58]	Neutrophil[34]	
CCL-24 (Eotaxin-2)	Epithelial cells[59]	T cell, Eosinophil, Basophil, Macrophage, Lymphocyte [34]	$T_H1 < T_H2$[50]
CCL-25 (TECK)	DC[60]	Macrophage, thymocytes, DC[34]	$T_H1 > T_H2$[61]
CCL-26 (Eotaxin-3)	Epithelial cells[62]	Eosinophil, Macrophage, Lymphocyte[48]	T_H2[62]
Cox-2	Epithelial cells[63], Macrophages, Neutrophils[64]	Limits mucosal T_H2 activity[64]	T_H1[64]
CXCL-1 (GROα)	Epithelial cells[65]	Neutrophil[34], Angiogenesis[66]	T_H1/ T_H2[50]
CXCL-2 (GROβ)	Epithelial cells[67]	Neutrophil[34]	$T_H2 > T_H1$*[67]
CXCL-3 (GROγ)	Epithelial cells[68]	Neutrophil[34]	$T_H2 > T_H1$*[68]
CXCL-5	Epithelial cells, Endothelial cells[66]	Neutrophil[34], Angiogenesis[66]	$T_H1 > T_H2$*[66]
CXCL-6	Epithelial cells[69]	Neutrophil, Monocyte, DC[70]	T_H2*[70]
CXCL-8 (IL-8)	Macrophages, Epithelial cells[41]	Neutrophil[34,43], Basophil, T cell[34], Angiogenesis[66]	$T_H1 > T_H2$[71]
CXCL-9 (Mig)	Epithelial cells, Endothelial cells and Fibroblasts[72]	Activated T cell[34,72] and NK[72]	T_H1[44]
CXCL-10 (IP-10)	Epithelial cells, Endothelial cells, Monocytes Neutrophils and Fibroblasts[72]	Activated T cell[34,72] and NK [72]	$T_H1 > T_H2$[34,44,49]
CXCL-11	Epithelial cells[66]	Activated T cell[34]	T_H1*[66]
CXCL-12	Epithelial cells, Endothelial cells[73]	T cell, DC, B cell, naïve B cell, activated CD4 T cell[34]	T_H2[74]
CXCL-14	Ciliated cells[75],	B cells and Monocytes[76]	T_H1 / T_H2[61]
GCSF	Fibroblasts and Monocytes[34]	Stimulates the bone marrow[77] Stimulates Neutrophil development and differentiation[34]	T_H1[61]
GM-CSF	Macrophages, T cells[34]	Stimulates turnover and release of granulocytes and Macrophages from bone marrow[34,77,78]	$T_H1 > T_H2$[45&61]

IFNα	DC[42]	Enhances T cell survival; antiviral replication[42]	$T_H1 > T_H2$*[42]
IFNγ	T cells[45]	Inhibits T_H2 activity[45]; activates Macrophages[42]	T_H1[45]
IL-1α	Macrophages Epithelial Cells[34]	Fever, T cell and Macrophage activation[34]	$T_H1 > T_H2$[61]
IL-1β	Macrophages Epithelial Cells[34]	Fever, T cell and macrophage activation[34]	$T_H1 > T_H2$[61]
IL-1F5	NK[79]	Anti-inflammatory cytokine; induces IL-4 expression[80]	T_H2[80]
IL-1F6	Leukocytes[79],Eoithelial cells[80]	Pro-inflammatory cytokine[80]	?
IL-1F7	Lymph node[79], PBMCs	Anti-inflammatory cytokine; inhibits IL-1β and TNFα[80]	?
IL-1F8	Lung tissue[79]	Pro-inflammatory cytokine[80]	?
IL-2	T cells	T cell proliferation[34]	T_H1[45]
IL-3 (M-CSF)	T cells, Epithelial cells[34]	Synergestic action in early hematopoiesis[34]	$T_H1 > T_H2$[61]
IL-4 (BCGF-1)	T cells[34,42], Mast cells[34]	B cell activation, IgE switch, suppresses T_H1 cells[34]	T_H2[42,45]
IL-5 (BCGF-2)	T cells[34,42], Mast cells[34]	Eosinophil growth and differentiation[34,42]	T_H2[42,45]
IL-6	T cells, Macrophages, Endothelial Cells[34] and Epithelial cells [45]	T and B cell growth/differentiation, acute phase protein production, fever[34]	T_H2[45]
IL-9	T cells	Mast-cell enhancing activity[34]	T_H2[34]
IL-10	T cells, DC[42]	Inhibits growth and function of T_H1[45]; suppression of allergic inflammation[42]	T_H2[45]
IL-11	Stromal fibroblasts[34]; Epithelial cells[45]	Synergistic action with IL-3 and IL-4 in hematopoiesis[34]; stimulates B cells in ab production[45]	$T_H1 > T_H2$[61]
IL-13	T cells[42]	B-cell growth and	T_H2[42,45]

		differentiation, inhibits macrophage inflammatory cytokine production and T_H1 cells[34]	
IL-17C	T cells[81]	Induce cytokine production by epithelia, endothelia and fibroblasts[34]	T_H1[81]
IL-22	T cells[82]	Regulates expression of β-defensins[82]	$T_H1 > T_H2$[82]
LIF	Bone marrow stroma and Fibroblasts[34]	Maintains embryonic stem cells[34]	T_H2*[83]
LT-α	Lymphocytes[84]	Lymphoid organogenesis[84]	$T_H1 > T_H2$[61]
LT-β	T and B cells[34]	Lymphoid organogenesis[34]	$T_H1 > T_H2$[61]
MIF	T cells, Macrophages[85]	Inhibits macrophage migration, stimulates Macrophage activation, induces steroid resistance[34]	T_H1/T_H2[85]
OSM	T cells, Macrophages[34]	DC maturation[86]	T_H1[86]
SPP1	Macrophages[87], DC, Neutrophils, T and B cells, Osteoblasts, Fibroblasts[88]	Chemoattractant for Neutrophils, Mast cells[89], Macrophages[90]	T_H1[88]
TGF-β	Monocytes, T cells[34]	Inhibits cell growth, anti-inflammatory induces IgA secretion[34]	T_H1/T_H2[61]
TNF-α	Macrophages, NK cells, T cells[34]	Local inflammation Endothelial activation[34]	T_H1[45]
VEGF/VPF	T cells[91]	Angiogenesis, leukocyte chemoattractant[91]	T_H2[91]

*Cytokines listed are those reportedly secreted by the airway in response to RSV infection or PM stimuli. CCLs and CXCLs are chemotactic factors for the cells listed under actions; *Inference from article; & contradictory results.*

2.3.1.1 Inflammation

The inflammatory response is important in combating infection, clearance of PM and injury repair in three ways. First, it increases the amount of effector

molecules (such as the complement components, which upon assembly form a complex capable of opsonising and even lysing pathogens, including enveloped viruses[92,93]) and effector cells at the site of infection. Second, it creates a physical barrier, through the activation of clotting mechanisms by the platelet activating factor (PAF), to prevent the spreading of infection. Finally, it promotes efferocytosis and tissue repair[94].

During inflammation, and near the site of injury, blood flow increases locally, the endothelium expresses adhesion molecules and there is an increase in vascular permeability. These changes in blood vessels contribute to extravasation of leukocytes from the blood into the tissues as well as to the accumulation of serum proteins at the site of infection or injury. Neutrophils are the main and the first phagocytic granulocyte recruited to the site of injury/infection, which are then followed by monocytes that once in the tissues differentiate into macrophages. In the case of viral infections, Interferons (IFN) - α and β are secreted by infected epithelial cells, thus activating NK cells, whose job is to kill virus infected cells[35].

Chemokines secreted by the aforementioned cells and chemotactic complement fractions can further expand and extend the immune response by attracting more neutrophils and other granulocytes such as eosinophils, basophils and mast cells, which upon recruitment and activation result in further vascular dilation and permeability[35]. The prevalence of these mediators is speculated to contribute to the pathogenesis of atopy, allergies and hyperreactivity[95-98]. As such, in order to prevent negative, long-term effects from inflammation, the inflammatory response needs to be actively terminated.

Just as lipid mediators are believed to initiate the inflammatory cascade, they are also believed to trigger the termination sequence of inflammation[99]. Leukocyte-platelet interactions result in the secretion of lipoxins, which stop PMNs extravasation into the tissues and also result in down-regulation of pro-inflammatory cytokines, which among other things causes neutrophil apoptosis to increase. Macrophage phagocytosis of apoptosed neutrophils induces the release of anti-inflammatory cytokines. In addition, it activates macrophages so that they can induce apoptosis of other leukocytes, whose phagocytosis reprograms the macrophage into tissue repair mode and finally emigration.

2.3.2 Acquired/Adaptive Immunity

The inflammatory response is important in the initiation of adaptive immunity as it is characterized by increased lymph and blood flow that result in more antigens and antigen presenting cells being delivered to the lymphoid tissues where they can activate lymphocytes.

Dendritic cells are the main antigen presenting cells. In fact, most adaptive responses start when naïve T cells recognize the antigen presented by dendritic cells. Thus primed $CD4^+$ T cells then differentiate into T helper (T_H) cells.; there are two types of T_H cells, T_H1 and T_H2. T_H1 cells effect cell mediated immunity, whereas T_H2 cells mediate humoral immunity. Whether a T_H cell becomes T_H1 or T_H2 cell depends on the cytokines produced during innate immunity (Table 2.3). After differentiation, T_H1 cells will leave the lymphoid organ to effect cell mediated immunity, whereas T_H2 cells will remain behind to activate antigen-binding B cells, thus mediating humoral immunity. Note that a

fragment of both activated/effector T cells and activated B cells will become memory lymphocytes, thus providing protective immunity against reinfection.

2.3.2.1 Cell Mediated Immunity

Cytotoxic T cells ($CD8^+$) are integral in immunity against intracellular pathogens. They recognize cells harbouring pathogens by the pathogenic fragments displayed in their plasma membranes. Binding to these surface-antigens causes $CD8^+$ cells to secrete perforin and granzyme to the site of contact. Perforin then punches holes in the plasma membrane of the infected cell through which granzyme can access its cytosol and induce apoptosis. In addition, $CD8^+$ cells secrete IFN-γ, which inhibits viral replication.

T_H1 cells are also important effectors of cell mediated immunity. They are tasked with activating infected macrophages so that they can kill both intracellular and digested pathogens. Additionally, T_H1 cells can kill senescent macrophages and stimulate the production of new ones.

2.3.2.2 Humoral Immunity

Antibodies are immunoglobulins (Ig) integral in the defense against extracellular pathogens. In their simplest action mode, neutralization, they prevent pathogens from entering the cells by binding to them. This binding also opsonises the pathogen and serves as one of the triggers of the complement cascade.

There are five main isotypes of antibodies, which are differentiable based in their structure and differ both in their distribution and in their effector

mechanisms. IgM is secreted only during the very early stages of antibody production. Later on, IgG and IgA antibodies are the most important isotypes, although IgG is more abundant. Finally, and only in some cases, IgE or IgD are produced. There is redundancy integrated into humoral immunity, so that many antibody isotypes effect the same outcomes, although in different degrees. For instance, IgM is very efficient activating complement. IgA, which is the main antibody found in secretions such as tears, saliva and breast milk, induces opsonisation by neutrophils and macrophages. IgG, activates complement and triggers pathogen phagocytosis by neutrophils, macrophages and eosinophils. In addition, it is the only antibody capable of sensitizing for killing by NK. IgD activates mast cells and basophils causing them to release antimicrobial and immune-stimulating factors[100]. Finally, IgE causes degranulation of eosinophils, basophils and mast cells and as such it is involved in allergic reactions and atopic states[96].

2.4 Respiratory Infections

Depending on whether the upper or lower airways are affected (Figure 2.1), respiratory infections are often categorized as upper and lower respiratory infections. Upper respiratory infections include the cold, the flu and pharyngitis, whereas lower respiratory infections include bronchitis, bronchiolitis and pneumonia. Usually, both upper and lower respiratory infections result from viral infections, among which the most common culprits are respiratory syncytial virus (RSV), influenza, parainfluenza and adenovirus[15].

In addition to affecting the levels of respiratory related morbidity and mortality directly through the aforementioned conditions, viral infections are also the main cause of exacerbation of respiratory chronic conditions, such as chronic obstructive pulmonary disease and asthma[15].

3: Particulate Matter

"Air is the creative force, the sprit moving over the face of the waters... It is the first of the elements that create and support our lives.

Air also embodies ideas in speech and language, in song and in the sweet airs of music...

Air is our element; we live inside the atmosphere, the envelope of mixed gases that forms the outer layer of the planet...

And just as air has shaped and sustained living beings, so living beings created and still sustain the air...

...air always remains within us and is as much a part of our bodies as any tissue or organ. We are a part of the air, which in turn is a part of all green plants and every other breathing creature."

David Suzuki, The Sacred Balance, 1997

3.1 Air Pollution and Particulate Matter

An air pollutant is a substance in the air that in sufficient concentration could be hazardous to the environment in general and/or to humans[101]. Various types of pollutants, gaseous to particulate are found in the planet's atmosphere, all with different chemical and physical properties and thus different environmental persistence, atmospheric transport and environmental impact[102]. Particulate matter is a category of air pollutant, which consists of an array of solid and/or liquid particles[12] of different composition and physical properties[103] suspended in the troposphere[102,104].

3.2 Sources of Particulate Matter

There are numerous natural and anthropogenic sources of PM, which can be classified into primary and secondary sources. Primary sources of PM are those processes that emit PM directly into the atmosphere, whereas secondary sources involve processing of gaseous emissions within the troposphere (and stratosphere) that lead to the production of more oxidized compounds. Increased oxidation increases the propensity of compounds to condense via homogeneous and heterogeneous nucleation giving rise to secondary PM in the atmosphere[105]. Regardless of the source, PM is removed from the atmosphere through precipitation or gravitational settling.

3.2.1 Primary Sources of Particulate Matter

Anthropogenic sources of primary PM include stationary and mobile sources of fossil fuel combustion[106], vehicular traffic related emissions from, and erosion of, unpaved roads, mechanical break up of materials involved in industrial manufacturing processes, agriculture and construction activities. Natural sources of primary PM include sea spray[107], volcanic eruptions[108], forest fires[109] and windblown dusts such as desert storms[110]. As such, primary sources of PM constitute the only route for biological materials such as viruses, moulds, pollens, bacteria and low volatility components thereof (eg. endotoxin) to be introduced into the troposphere.

Regardless of whether the source is anthropogenic or natural, primary sources tend to produce larger PM (1 – 100 μm)[105] than secondary sources. PM larger than 10 μm tends to be removed quickly from the atmosphere by

gravitational settling[111], so that most of the primary PM found suspended in the atmosphere is within the 0.1 - 10 μm size range[105]. Primary PM tends to not coagulate with one another; however, it can undergo mass exchange with the gas phase, thus contributing to the accumulation mode of secondary PM[105].

3.2.2 Secondary Sources of Particulate Matter

Secondary sources of PM utilize gaseous compounds emitted into the troposphere by both, anthropogenic and natural primary sources[112]. The simplest secondary process is gas to particle conversion[112]. In this process, liquid aerosols are formed through gaseous saturation that leads to condensation in a process known as nucleation, which can be either homogeneous or heterogeneous[105,112]. Homogeneous nucleation is possible due to favourable collisions of gas molecules, whereas heterogeneous nucleation requires the presence of a condensation nucleus[112]. Gas to particle conversion gives rise to the smallest modes of PM present in the atmosphere[112]: nucleation mode (< 0.01 μm) and Aitken mode (0.01 - 0.1 μm) [105].

3.2.3 Accumulation mode

Larger secondary and primary PM can result from the coagulation of smaller particles and by condensation of gas vapour onto existing aerosols. Heterogeneous condensation is possible due to the oxidative nature of the atmosphere, where the dominant oxidants include ozone (O_3), hydroxyl radical (OH), nitrate radical (NO_3), and chlorine atom (Cl) that act on compounds in the atmosphere through homogeneous, heterogeneous, and multiphase reactions. Homogeneous oxidation produces gaseous organic and inorganic compounds

that are more polar, have higher molecular weight, and lower boiling points than their respective precursor compounds. These oxidized compounds are involved, along with water depending on the local relative humidity, in the accumulation mode, which leads to size increases in all particles suspended in the atmosphere[113]. In fact, the size range of PM generated in this manner is 0.1-1μm[105].

3.3 Particulate Matter Characterization

The particulate matter suspended in the troposphere is a dynamic entity, whose physical and chemical characteristics are ever-changing[114] as a function of source, geography, temporal meteorological conditions and atmospheric processing[115-117]. PM loadings in the boundary layer, from the planet surface up to an altitude of ~1 km, are monitored in cities as an important indicator of air quality[118]. Traditionally, characterization of PM has been performed mainly by measuring daily averages of its total mass using active and passive filters[119]. Coarse temporal and spatial information has been gained using this approach; however, more detailed understanding of PM, in particular, its sources at high resolution (eg. within city variability, from street to street) and its chemical composition, is being pursued in an effort to better describe and understand observed health effects. As such, efforts have been launched to develop instrumentation that will allow for real time, continuous monitoring of physical and chemical characteristics of PM at high spatial and temporal resolution[115].

3.3.1 Physical Characteristics of Particulate Matter

Physical parameters of PM traditionally characterized include particle number concentration, size distribution, mass, volume, density and particle bound water[115,116]. Epidemiology studies have correlated increased pulmonary and cardiovascular morbidity and mortality with PM number concentration, density and size[114,120,121]. In fact, particle size and density are physical properties of a particle that determine its final deposition site within the respiratory tract, and that has given rise to the size-classifications used to describe PM, as defined in the following section[115,122,123].

3.3.1.1 Particulate Matter and its size

As mentioned earlier, PM is heterogeneous in nature; as such, each particle has a unique amorphous shape. To ease its characterization, the size of PM is traditionally described in terms of its aerodynamic diameter, which corresponds to the theoretical diameter of a perfect sphere of water that would have the same terminal velocity[111] as the aforementioned particle in air[124].

Three classifications of PM based on size are of medical interest: the coarse fraction that tends to settle in the upper airways[123], the fine fraction that tends to settle in the lower airways[123] and ultrafine fractions, which reportedly cross the alveolar epithelium[114,125] into the blood stream[126]. The coarse fraction consists of PM with aerodynamic diameter smaller than 10 μm, but bigger than 2.5 μm ($PM_{2.5-10}$); the fine fraction consists of PM with aerodynamic diameter smaller than 2.5 μm, but bigger than 0.1 μm ($PM_{0.1-2.5}$); and the ultrafine fraction includes PM with aerodynamic diameter smaller than 0.1μm ($PM_{0.1}$).

3.3.2 Particulate Matter Composition

The composition of PM is complex, varied and variable. It has even been shown to change within the same day[126,127]. Furthermore, once suspended in the troposphere, the chemical composition of a particle can be modified through chemical processing, either within, through multiphase reactions, or on its surface, through heterogeneous reactions[15,105,113]. Multiphase reactions occur once exogenous chemical species, such as gases or solids, are taken up by a liquid droplet, whereas heterogeneous reactions occur on the surface of a solid[113]. Regardless of whether the reactions that occur in the atmosphere are homogenous, heterogeneous or multiphase they lead to exchange of materials in PM and thus contribute to its compositional variability.

Generally speaking, the main components of primary PM are chemical inert elements such as automotive wear materials (eg. brake pads), biologics, crustal dusts (eg. silicates and carbonates), sea salts and soots (carbon); whereas the main components of secondary PM are sulphates, ammonium, organic compounds, nitrates and soots (carbon)[102,115,128]. Sulphuric acid is the most important nucleating substance in the troposphere, followed by oxidized iodine compounds essential for coastal nucleation[112]. Water, ammonia, organic acids, and ions are other species commonly associated with atmospheric nucleation events[112]. Note that the materials found on secondary PM can also condense onto primary PM.

The potential that different components and sizes of PM might have different environmental and health related implications has been recognized and results to date have not disproven this postulate[115,128]. As such an enormous

effort has been mounted to try to map the geographical and temporal variation of the size and composition of PM with improved resolution[115] as to enable differentiation between locally produced PM and atmospherically transported PM, as well as to better characterize health outcomes as a function of exposure to the aforementioned pollutants[115,129]. In spite of the development of instrumentation capable to undergo size characterization and, with limited success, chemical composition measurements of PM in real time[115,130-132], off line, filter-based methods continue to be the primary methodology used by regulatory bodies world wide[129]. Techniques for real-time chemical composition measurement of individual particles have not yet been proven to have the sensitivity and in particular, adequate chemical selectivity to generate knowledge[129].

With respect to filter-based sampling of PM, though the limitations are many, such as further chemical processing of the sample[133], loss of soluble compounds[134], loss of insoluble materials and the difficulty to characterize such, numerous analytical methods have been developed to circumvent, or control for, these limitations[134].

In spite of the limitations of off-line PM characterization, a wealth of information regarding the trends of chemical composition with respect to the source and size of PM has been gained. Due to its many sources, the spatial variation of the coarse fraction is the largest[135]; generally speaking, however, coarse PM tends to be composed of crustal and biological materials[136,137]. The fine fraction, on the other hand, contains higher variability in transition metals, hydrocarbons[138] and organic compounds, whereas the ultrafine fraction is rich in

sulphates and nitrates. In spite of these differences, one component common to all size fractions of PM is carbon; in fact, solid PM tends to have a carbon core[15,102,137,139].

3.3.2.1 Biological material in PM

It has been speculated that up to 28% of airborne PM is made up by biological material of various types such as animal and plant fragments, bacteria, fungal spores, pollen and virus[140,141]. Furthermore, it has been reported that desert dust clouds comprised of primary PM having crustal origins (eg. silicates, carbonates) carry microorganisms ranging from fungi to virus around the globe[13,110,142], many of which remain infectious[141]. For example, viable bacteria responsible for respiratory tract infections, meningitis, and urinary tract infections have been isolated from dust samples in North Africa, Kuwait and Mali[110]. In addition, many viruses, such as the Hantavirus are usually transmitted in dust[143]. Furthermore, long range airborne transmission across the Baltic Sea and the English Channel has been implicated in foot-and-mouth outbreaks in Denmark and the United Kingdom[144]; windborne dispersal of different fungi in the Pacific Northwest have been associated with human infections[145] and African dust storms have been implicated with gorgorian coral disease in the Caribbean[142].

The primary source of coarse biological particles are plants releasing pollen or spores into the environment, whereas the primary source of fine and ultrafine biological particles are anthropogenic activities such as industry, sewage plants[146], agriculture, sneezing and coughing[140]. As such, whereas

coarse biological particles might be found as independent particles, fine and ultrafine biological particles would most likely be found embedded or adsorbed onto a larger particle, or aggregated with smaller ones[141,147,148]. Bacteria, for example, are often found bound to other biological particles or to soil particles[141]. Similarly, airborne viruses could be found as re-suspensions of contaminated solid particles or as liquid aerosols[141,149].

3.3.2.1.1 Limitations of current sampling techniques to determine biologicals in ambient PM

A limiting factor in the characterization of the biological component of PM is the possibility of collection mechanisms rendering microorganisms no longer viable. A direct consequence derived from this dilemma, is the fact that most standard identification tests of biologicals involve viability assays in various media (the few exceptions involving direct counting of microscopic samples[150]), which would report false negative results if pathogens that would have tested positive when viable, had been rendered not viable by the sampling mechanisms. This is especially true in the case of viruses, for which it has been speculated that the number of viruses isolated from environmental samples may be up to two orders of magnitude smaller than the actual number[151]. As such, increased effort is being focused in developing sampling mechanisms and protocols that would preserve the viability of bioaerosols.

Several instruments have been designed for the sampling of bioaerosols such as liquid impingers, impactors and filters. The efficiency of viable bioaerosol collection depends both on the microorganism and in the sampling mechanisms, with factors such as microorganism hardiness, sampling time, air

flow rate through the device, and the extraction process all affecting microbial viability[152]. For example, while excellent for fungal spore collection, filters have been shown to have much lower efficiency in the collection of viable virus, which are much more sensitive to environmental stress than bacteria and fungi[153], when compared to liquid impingers[152]. Liquid impingers are instruments capable of collection of viable bioaerosols for short periods of time, as collection for more than an hour results in reduction of microbial viability due to collection fluid evaporation, which in addition to the high air velocity employed, results in increased particle bounce and re-aerosolization[154]. Slit samplers overcome these limitations as the sampled organisms are collected directly onto a solid medium which also serves as a microbial nutrient during incubation[155]; as such sampling times can be much longer, with the limiting factor now being plate saturation[156]. In addition, when equipped with dividing plates so that different collection media can be used, a combination of bacteria and viruses can be collected[155].

Many advances have been made in the development of instrumentation for the characterization of bioaerosols, such as the implementation of surface enhanced Raman spectroscopy[157] and Matrix-assisted laser desorption / ionization (MALDI)[158] to characterize and differentiate microbes. However, since the collection instrumentation is still evolving, many unknowns remain, such as the actual size distribution of virus aerosols, which is thought to be affected by the suspension matrix[154].

3.3.2.2 Ambient Particulate Carbon

The importance of carbon as the core component of PM[78,102,128,137,159 160,161] and its impact on human health have been recognized[78,139,162,163]. In fact, it has been recommended to incorporate the use of instruments capable of continuously monitoring ambient particulate carbon into routine air quality monitoring techniques[115,162,164,165].

The main components of particulate carbon are elemental, black and organic carbon[115,129]. Elemental carbon and carbon black are better known as soot; this does not mean that the terms elemental carbon and carbon black are interchangeable, because this classification refers to parameters measured using different methods. elemental carbon refers to the quantification of the gas evolved from the thermal oxidation of carbon in a filter, whereas carbon black refers to the quantification of the absorption of light by particulate carbon in PM[115].

One of the main sources of elemental carbon and carbon black is the incomplete combustion of organic material[165,166] whereas the primary sources of organic carbon include natural, geological and industrial emissions of volatile organic compounds[164]. Secondary organic carbon is generated by the oxidation reactions of organic compounds, such as the condensation of products from photochemical oxidation[164,165]. In fact, studies across the USA[129] and in Chile[167] found that elemental carbon and carbon black correlate mainly with primary sources of PM emissions such as traffic patterns and forest fires, whereas the sources of organic carbon were found to be mostly secondary due to photochemical processing of PM[129]. In urban centres, one of the major

components of ambient PM are diesel particles, which are composed of many chemicals, among which both elemental carbon and organic carbon are important ones[168]; in fact the core is made of carbon[15]. In Kuwait, the main sources of elemental carbon and organic carbon were anthropological mobile and stationary sources[169], and after the Iraqi invasion in 1990, 50-60% of PM_{10} was composed of coarse PM, due to desert erosion. In London, elemental carbon was found both in local and long-range transported (LRT) aerosols, whereas organic carbon was found only in the core of LRT aerosols[170].

Around the globe, particulate carbon has been found to form an important fraction for ambient PM[171]. For example, during California winters, organic carbon and elemental carbon constitute 60-80% of PM[159]; furthermore, a study of locations across the USA found that the composition of ultrafine PM was entirely dominated by carbon compounds, whereas $PM_{2.5}$ was dominated in the morning by carbonaceous materials and in the afternoons by sulphates[129]. Samplings of PM in Mexico City, where carbon concentrations varied considerably from site to site, found that carbon concentrations were particularly abundant in the mornings and that two thirds of PM consisted of carbon, other crustal elements and ions; in fact, carbonaceous aerosols were the most abundant component in the fine fraction and constituted 30% of the coarse fraction[172]. Likewise, in Taiwan, carbonaceous species constitute up to 40% of ambient PM[173].

3.4 Epidemiology

The problem of PM air pollution is global[168]. Every location around the globe is a potential site for natural emission of PM, and nearly every location in the atmosphere, due to its photochemistry, is a potential site for homogeneous and heterogeneous oxidation reactions. Also, because the rate of mixing in the troposphere is efficient for natural and anthropogenic emissions of PM and gaseous compounds, there are pollutants in various forms present in virtually all locations around the globe.

At all locations where PM has been measured and characterized in epidemiology studies, there has been a consistent positive relationship between levels of ambient PM and the incidence of morbidity and mortality[120,121,137] via the pathogenesis of pulmonary and cardiovascular diseases[174,175]. In several studies of air pollution episodes across both the USA and Europe, an increase of 10 μg/mL of PM_{10} has been shown to be associated with an approximate increase of 1.0 - 1.5 % in respiratory related hospitalizations and an increase of 0.5 – 1.5% in mortality[128]. Likewise, long-term atmospheric PM exposure has been found associated with 6% increase in total mortality and an increase in both the incidence and exacerbation of respiratory conditions in both USA and Europe[128].

Apart from increasing mortality rates, particulate exposure also leads to numerous other low-grade responses in numerous organs of the body[176]. For instance, elevated levels of particulate pollution elicit acute events such as asthma exacerbations[177], and chronic obstruction pulmonary disease acute episodes[178]. Also of note is that diesel exhaust particles have been found to

induce apoptosis in throat cells[179]. Even skin diseases have been associated with exposure to ambient PM_{10}[180]; for example, vitamin D decreases as PM levels increase, scleroderma is related to prolonged exposure to silica particulates[181], and topical application of cigarette smoke-generated PM causes DNA damage in mice skin[182].

There has been considerable debate regarding the contribution to health effects of particles based on size classification according to where the particles are found to deposit in the human respiratory tract; as mentioned earlier, $PM_{2.5-10}$ generally deposits in the upper airways, whereas $PM_{2.5}$ tends to deposit in the lower airways or even cross into the blood stream[135,183]. While some studies have found $PM_{2.5}$ more relevant with respect to both cardiovascular and respiratory associated diseases and mortality[117,184], others conclude that $PM_{2.5-10}$ has stronger effects in morbidity[135] and in fact, there had been studies that had found the coarse fraction to be positively correlated with mortality[167], while many others have not found such correlation[135]. This inconsistency is not surprising, since the dynamic nature of PM lends itself naturally to high variability when sampled at high resolution (eg. time and location) which places stringent criteria on comparisons of one epidemiological study to the next[183]. Furthermore, there had been only several studies that have compared the respective effects of $PM_{2.5}$ and $PM_{2.5-10}$ as separate entities within the course of a single study[135]. In addition, the error associated with the measurements of $PM_{2.5-10}$ is larger than that for PM_{10} or $PM_{<2.5}$ as it is usually an indirect measurement[135]. Regardless of all this limitations, it is clear that it is the chemical composition, and therefore the

source, that determines the distinctive roles in health outcomes of the size fractions of PM[117].

In spite of the recognition that the composition of PM is most relevant for its negative health effects, debates still surrounds the specific contribution of the different constituents of PM[117,128]. A popular view is that one of the most toxic components are metals[163] because due to their redox chemistry can induce the formation of free radicals. However, it has been suggested that non-metal components of PM are necessary to enhance the cellular response to PM[117]. Moreover, it has been postulated that it is the combination of the materials that might be responsible for the effects of PM[117,185].

3.5 Particulate Matter and the host response

A myriad of research aiming to decipher the complex interactions between PM and the airway epithelium that lead to the biological mechanisms underlying the pulmonary and cardiovascular morbidity and mortality associated with PM pollution has been undertaken. Even though the physical characteristics and the chemical composition of PM is vast, there is a common theme to the mechanisms through which PM causes cellular damage. Free radicals and heavy metals interfere with the normal functioning of lipids, proteins and nucleic acids in different subcellular organelles[102], which can lead to apoptosis. Free radical accumulation, either directly from PM components or as a result of PM-dependent organelle dysfunction, can also lead to oxidative stress[15], which can then activate macrophages[186] and induce inflammation[187,188]. In addition to this "direct" effects, PM can also damage the lung epithelium by

reducing its ability to fight bacterial infections[189,190]. Moreover, human alveolar macrophages and monocytes have been shown to exhibit a lower phagocytosis rate[191] and secretion of oxidants upon exposure to PM_{10}[192].

Upon PM stimulation, alveolar macrophages and primary bronchial epithelial cells secrete mediators involved in various cellular responses, including inflammation and apoptosis (Table 3.1)[77]. Of particular interest are the bone marrow stimulation by IL-6 and IL-8[77] and the production of acute-phase proteins, which could lead to a systemic inflammatory response, initiated by IL-6[193]. Both *ex vivo* and animal studies show that both, inert carbon and ambient PM, trigger a monocytic response[194,195] as well as the bone marrow production of PMNs[196]. As such, both particle types are capable of generating an endemic response, although it is the chemical composition of the particle that is believed to determine its magnitude[194].

Table 3.1 Changes in Airway Cytokine Secretion in Response to PM_{10} Stimulus

Airway Region	Sample	Stimulus	Increased Secretion	Unchanged Secretion	Decreased Secretion
Trachea	Primary tracheal epithelium	TSP f 0-1500 μg	IL-8[197]		
Bronchial	BEAS-2B	TSP f 0-1500 μg	IL-8[197]		
			IL-6[197]		
		$PM_{2.5-10}$	CXCL-8[48]	CCL-5 (RANTES) [48]	
				CCL-11[48]	
				CCL-20[48]	
				CCL-26[48]	
				CXCL-1[48]	
				CXCL-3[48]	
				CXCL-5[48]	
				CXCL-10 (IP-10) [48]	
				CXCL-11[48]	
				CXCL-14[48]	
				TNFα[48]	
				LTβ[48]	
				IL-1α[48]	
				IL-1β[48]	
		$PM_{2.5}$		CCL-5 (RANTES) [48]	
				CCL-11[48]	
				CCL-20[48]	
				CCL-26[48]	
				CXCL-1[48]	
				CXCL-3[48]	
				CXCL-5[48]	
				CXCL-8[48]	
				CXCL-10 (IP-10) [48]	
				CXCL-11[48]	

				CXCL-14[48]	
				TNF-α[48]	
				LTβ[48]	
				IL-1β[48]	
				IL-1α[48]	
	HBEC	EHC-93: 10-500 μg/mL	IL-1β[78,198]		
			IL-8[78,198]		
			GM-CSF[78,198]		
			LIF[78,198]		
		soluble EHC-93: 100 μg/mL	GM-CSF[78]		
			IL-1β[78]		
			IL-8[78]		
			LIF[78]		
	NHBE	PM_{10}^{foam}	Cox-2[199]		
			IL-6[117]		
			IL-8[117,199]		
Alveoli Lumen	AM	EHC-93	GM-CSF [h194,198]	IL-6 [g200]	IL-8 [g200]
			IL-1β [h194,198]	M-CSF [h 194]	
			IL-6 [h194,198,201 r201]	MCP-1[198]	
			IL-8 [h 194,198]	MIP-1β[h 194]	
			MCP-1 [h 194]	OSM[198]	
			TNF-α[h 77,194,198,201 r201 b77 g200]		
		UAP	IL-1β[h202]		
			IL-6 [h 201,202]		
			TNF-α[h 201]		
		$PM_{2.5-10}$	Cox-2[h199]		
			IL-6[h117,199]		
			IL-8[h117]		
			TNF-α[h199]		
Co-cultures	AM/HBECs	EHC-93	GM-CSF[h198]	MCP-1[h41,198]	
			IL-1β[h198]	MIP-1β[h41]	

			IL-6[h198]	RANTES[h41]	
			IL-8[h41,198]		
			LIF[h198]		
			MIP-1α[h41]		
			OSM[h198]		
			TNF-α[h198]		

AM = primary alveolar macrophages; HBEC = primary human bronchial epithelial cells; BEAS-2B = human bronchial epithelial cells transformed by Adenovirus SV40; NHBE = primary normal human bronchial epithelial cells; [F] filter collected; EHC-93 = characterized ambient particles from Ottawa; TSP = uncharacterized total suspended particles; UAP = uncharacterized urban air particles; h= human samples; b=rabbit; r = rat; g = guinea pig

Acute episodes of air pollution have been shown to result in systemic inflammatory responses, which are associated with increased morbidity and mortality[77], following secretion of cellular mediators (Table 3.1). One adverse outcome of systemic inflammation is that the bone marrow is over stimulated so that immature PMNs and monocytes are released into the circulation[196]; in fact, leukocytosis, and in particular granulocytosis, is a marker of systemic inflammation[203]. Being that immature granulocytes are less chemotactic than mature ones, there is much opportunity for tissue damage away from the sites of inflammation[194].

When comparing the cellular response to stimuli with $PM_{2.5-10}$ versus $PM_{2.5}$, it was found that the coarse fraction was the main inducer of cytokine secretion and, as such, could induce more intense pulmonary infalmmation[117,163]. In fact, it has been found that the coarse fraction is the most potent stimuli in the induction of IL-6 and IL-8 secretion by both alveolar macrophages and normal human bronchial epithelial cells[117], whereas it is the chemical composition, on the other hand, that determines the ability of the particle to induce oxidative stress[117].

The importance of the chemical composition of PM in the host response has been shown *in vitro* by a study in which the soluble fraction of PM caused a smaller effect than PM as a whole, and colloidal carbon, from India Ink, alone elicited cytokine production[78].

3.5.1 Particulate Carbon and the host response

Animal studies indicate that two of the most important predictors of cytotoxic and inflammatory responses elicited by PM are elemental carbon and organic carbon[204]. In addition, *in vitro* studies have correlated IL-6 and IL-8 secretion by human airway epithelial cells to the levels of elemental carbon and organic carbon present in dust[163]. Also, organic carbon itself is known to induce oxidative stress in the lung[163] and has been shown to be sufficient to elicit cytokine secretion by both epithelial cells and alveolar macrophages (Table 3.2)[194].

Table 3.2 Changes in Airway Cytokine Secretion in Response to Carbon Stimulus

Airway Region	Sample	Stimulus	Increased Secretion	Unchanged Secretion	Decreased Secretion
Bronchial	BEAS-2B	UfCp	CCL-20[48]	CCL-1[48]	CCL-17 (TARC) [48]
			CXCL-1[48]	CCL-2[48]	CCL-19[48]
			CXCL-3[48]	CCL-3[48]	CCL-21[48]
			CXCL-10 (IP-10) [48]	CCL-4[48]	CCL-24[48]
			CXCL-11[48]	CCL-5 (RANTES) [48]	CXCL-6[48]
			IL-8[48]	CCL-7[48]	CXCL-13[48]
			TNFα[48]	CCL-8[48]	IL-1F5[48]
				CCL-11[48]	IL-1F7[48]
				CCL-13[48]	LTα[48]
				CCL-15[48]	SPP1[48]
				CCL-16[48]	
				CCL-18[48]	
				CCL-23[48]	
				CCL-25[48]	
				CCL-26[48]	
				CXCL-2[48]	
				CXCL-5[48]	
				CXCL-9[48]	
				CXCL-12[48]	
				CXCL-14[48]	
				IL-1α[48]	
				IL-1β[48]	
				IL-1F6[48]	
				IL-1F8[48]	
				IL-5[48]	
				IL-9[48]	
				IL-10[48]	
				IL-13[48]	
				IL-17C[48]	
				IL-22[48]	

				MIF[48]	
	HBEC	CC: 1% soln	IL-1β[78]	GM-CSF[78]	
			IL-8[78]		
			LIF[78]		
Alveoli	A-549	UfCp	IL-8[48]	CCL-5 (RANTES)[48]	
				CCL-17 (TARC)[48]	
				CXCL-10 (IP-10)[48]	
Alveoli Lumen	AM	CC: 1% soln	IL-6 h [194]	GM-CSF h [194]	
			TNF-α h [194]	IL-1β h [194]	
				IL-8 h [194]	
				MCP-1 h [194]	
				M-CSF h [194]	
				MIP-1β h [194]	

A-549 = adenocarcinomic human alveolar epithelial cells; AM = primary alveolar macrophages; HBEC = primary human bronchial epithelial cells; BEAS-2B = human bronchial epithelial cells transformed by Adenovirus SV40; TSP = uncharacterized total suspended particles; UAP = uncharacterized urban air particles; h= human samples; CC = colloidal carbon; UfCp = nanoparticles which in media formed aggregates of different sizes,

Alveolar macrophages are essential in the processing of PM by the airway epithelium and they have been shown to phagocytise at the same lower rate both inert carbon and ambient PM[205]; furthermore, this response was sufficient for systemic inflammation to take place[194]. In fact, animal studies have shown that systemic inflammation in response to carbon stimuli[195] includes stimulation of the bone marrow[196].

3.6 Shortcomings of current research in the effect of PM in human health

As mentioned earlier, the patterns of PM size and composition depend on local emissions and secondary processes, as well as meteorological patterns[117,206], which renders the synthetic generation of a "standard" PM composition virtually unattainable. In addition, different analytical laboratories

tend to use their own standards and methods, which results in poor transition of results from one analytical laboratory to another[116]. Even studies designed to circumvent the problems associated with PM collection by continuous sampling of air directly into a chamber without processing are subject to discrepancies/errors for which the sources remain undefined[116]. In addition to these analytical limitations, the treatment of the data generated tends to utilize temporal and even geographical averages[115,129], causing epidemiological studies to preclude information as to which components and sizes of PM elicit a specific effect[129,189]. Moreover, being that correlation does not reveal a causal connection, conclusions based on such observational studies, cannot draw more than associations between levels of PM and health outcomes[207]. Even when the study design allows for the establishment of a natural experiment, the casual observations drawn from it are always shadowed by the everlasting possibility of explanatory confounding factors that randomization could not account for[207].

In order to bridge the knowledge-gap regarding this issue, numerous laboratories have taken the approach of using ambient PM, or taking a subset of components known to form PM, for *in vitro* or *in vivo* dosing followed with readout of the host responses and/or health outcomes[163,189]. The causal associations drawn in such manner could then be developed into further hypothesis driven experimentation. In fact, advances in understanding the effect of PM on human health have been achieved following these approaches; however, shortcomings remain in the knowledge generated. In the *in vivo* methodological scenario, deemed as the more realistic one, the PM used in the

dosing studies has been modified simply by the process of collection (e.g. separation of soluble materials and even further chemical processing). Furthermore, the knowledge derived from these studies only indicates the nature of the host response to PM sampled from a specific area, with no information as to which components are responsible for the different aspects of the elicited response. The *in vitro* laboratory scenario addresses this problem in that the identity and the ratios and combinations, if any, of the chemicals used is known; so that any response elicited can be related to a specific chemical or combination thereof[189]. However, in the majority of the studies, solutions are used as the dosing strategies, which, taking into account that one does not breathe liquids, is a poor representation of the real events. In the more accurate approach of aerosolizing solutions, the solid component of ambient PM is overlooked and thus often excluded from the experimental dose. This is important, not only to stay true to the general characteristics of PM, but also, because compounds have been found to be retained more efficiently in the lung when associated to a particle than alone. The reason for this may be that solid PM could traverse the hypophase resulting in more direct contact between the cell and the particle[189].

3.7 Particulate Carbon as the starting point: a PM_{Mimic}

As mentioned earlier, the physical and chemical characteristics of PM are not a constant, but ever changing. As such, any experimental PM mimic would be just an approximation to the myriad of real particles that comprise environmental PM. In order to address the problem of causation of disease

states[129,163,189,207], and to facilitate the development of environmental legislation, PM mimics are understood as being an experimental tool that allows the exploration of the effects on cellular responses of individual components as particles of specific sizes, in either solid or liquids states, and in specific combinations.

Carbon, the principal element in soots, has been recognized as one of the main components of PM in all samplings at numerous locations around the globe[78,102,128,137,159 160,161]; in particular, ambient samplings have found it to be the principal component of solid PM[15,205]. Carbon black is known to have a long life in the atmosphere since it is chemically inert with respect to atmospheric oxidants and its only removal mechanism is wet deposition, yet unoxidized surface sites on carbon black are hydrophobic, as such, it is readily available for long-range atmospheric transport. In addition, carbon black is very porous[166] and therefore provides an excellent surface for other compounds, including biologicals, to get lodged onto and for chemical reactions to take place on. In addition to its suitable chemical and physical characteristics, a positive association between ambient carbon and negative human health outcomes has been recognized[139,162]. Moreover, carbon has been shown to elicit similar responses to ambient PM in *in vitro, in vivo* and *ex vivo* studies.

Due to the unique combination of its elegant simplicity and important role in long range transport and human health, carbon is an appropriate candidate as the material on which to build mimics of PM (PM_{Mimics}). A PM_{Mimics} comprised of carbon would allow the exploration of many important questions, while keeping things simple and controlled without confounding factors[208], so that

once a carbon based set of studies and characterizations has been performed, additional compounds could be added to further our understanding of PM effects. Therefore, the main component of the PM_{Mimics} used throughout my research was carbon.

4: Respiratory Syncytial Virus

" So, naturalists observe, a flea
Hath smaller fleas that on him prey
And these have smaller still to bite 'em
And so proceed ad infinitum. "

Jonathan Swift, On Poetry, A Rhapsody

4.1 Classification and Structure

The genome of RSV consists of a linear negative single stranded RNA ((-)ssRNA), which is strongly associated with the nucleoprotein (N) and therefore belongs to the Order of the *Mononegavirales*[209], within group V of the Baltimore Classification[210]. Being from the *Paramyxoviridae* family, RSV is an enveloped virus with a helical nucleocapsid composed by protein N and the viral polymerase (L)[211] and its cofactors, phosphoprotein P and transcription factor M2-1[212] (Figure 4.1). The envelope consists of three transmembrane glycoproteins[212,213], the attachment protein G, the fusion protein F[214] and a small hydrophobic protein SH, whose function has yet to be determined[215], embedded into a lipid bilayer derived from the host plasma membrane that surrounds the inner virion matrix protein M[212]. RSV is a vertebrate virus of the *Pneumovirinae*

subfamily[216]. RSV lacks neuraminidase activity, which is required for viral budding by many viruses, and thus belongs to *Pneumovirus* genus. Like the rest of the human viruses belonging to the *Pneumovirus* genus, RSV also lacks hemagglutinin activity, which is required for infection by many viruses[217].

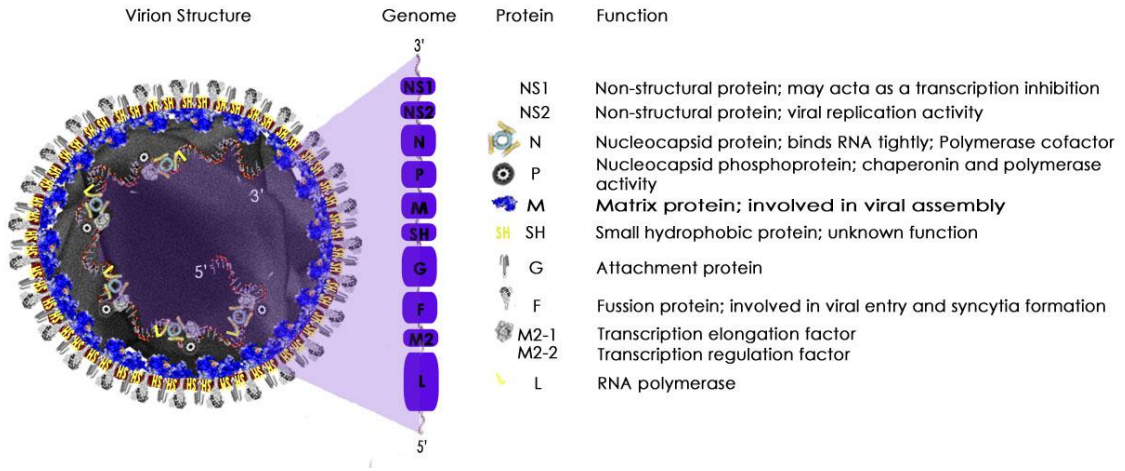

Figure 4.1 RSV Structure and Genome

Since its first isolation from ill children in 1957[218], two major antigenic strains have been determined, RSV A and RSV B. Within these subgroups, several subtypes have been identified, mostly based on their differences in protein G[219]. Several studies have been performed addressing the difference in virulence of the two subgroups. While some studies have reported no difference in virulence, others have reported higher disease severity in infants infected with RSV A[220]; although, even within a single subgroup, different subtypes have been reported to elicit different immune responses[221]. In addition, it has been found that RSV A replication results in higher viral titres *in vitro*[219]. For this reason, the RSV subtype selected for this study was "RSV A long".

4.2 Replicative Cycle

4.2.1 Viral Entry

Protein G mediates the first step in viral infection, which is the attachment of RSV to the cell surface[222]. The RSV receptor remains elusive; however, G has been shown to bind glycosaminoglycans on the plasma membrane[223] and anexin II has been postulated as a potential RSV receptor[224]. Following attachment, protein F facilitates nucleocapsid entry into the cytoplasm[225] by mediating fusion through the juxtaposition of the viral envelope and cellular plasma membrane[226].

4.2.2 RSV transcription and replication

A single polymerase complex, consisting of proteins N, P and L, is in charge of both genomic RNA replication and transcription of subgenomic messenger RNA (mRNA), both of which take place in the cytoplasm[225] without involvement of the host's nucleus[212]. There are two mechanisms in which the balance between RNA and genome synthesis is thought to occur (Figure 4.2). In the classic model, the polymerase has transcriptase activity, unless switched into the replicase mode, most likely by binding to N[227]. A more recent model postulates that the polymerase by itself has very low processivity and that binding to either a transcription or elongation factor is necessary to undergo either successful mRNA transcription or genomic replication[228].

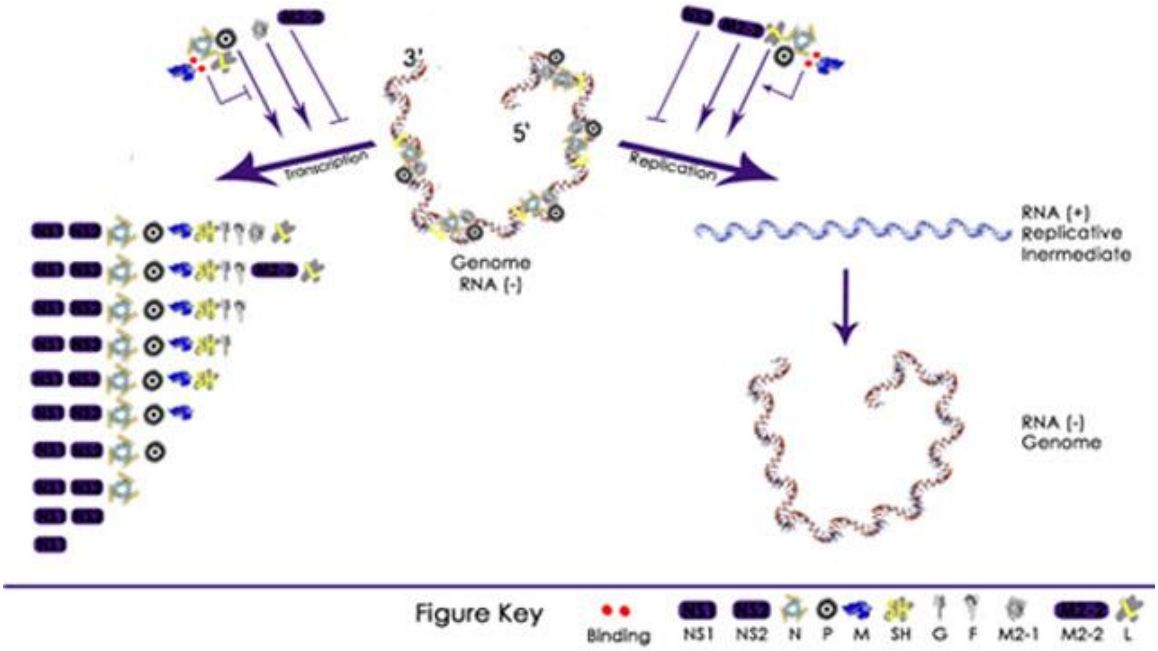

Figure 4.2 RSV transcription and replication

4.2.2.1 Transcription of RSV subgenomic mRNA

The first step in viral gene expression is to transcribe the (-)ssRNA genome into complementary mRNA[225] so that it can be translated into viral proteins by the host cell translation machinery, which is done quite efficiently as M renders the nucleus deficient for host transcription[212]. The RSV genome transcribes 10 monocistronic mRNAS coding for 2 non-structural (NS) and 10 structural proteins[229] (Figure 4.1). Transcription is guided by gene start and termination signals[230] and as such is modulated by transcription attenuation at gene junctions[231], which means that the amount of gene product is directly proportional to the distance of the gene from the 3' promoter[232]. The transcription elongation factor, M2-1, is necessary for transcription of promoter-distal genes as it increases transcriptase processivity[228,233] while acting as a transcription antiterminator[230]. As infection progresses and viral gene expression

increases, transcription is inhibited by the transcription regulation factor, M2-2[228], and NS-1[234].

4.2.2.2 RSV Genome Replication

Replication occurs later in infection, as the level of viral proteins involved in genome replication, encapsidation and inhibition of transcription increases[233]. It proceeds through a positive sense replicative intermediate, which is the template for the production of the (-)ssRNA, which constitutes the RSV genome[228] (Figure 4.2). Replication requires of cosynthetic encapsidation, as such, it has been found to positively correlate with increased levels of protein N[235]. It is negatively regulated by proteins NS-1 and NS-2[234] and positively regulated by protein M2-2 in deletion mutants[233], although it was found to inhibit replication in a minireplicon system[228].

4.2.3 RSV Assembly and Budding

As they are synthesized, viral envelope glycoproteins are targeted to the plasma membrane, where they concentrate in patches that exclude cellular proteins[212]. As infection progresses, protein M interacts with the cytosolic tails of the envelope proteins and with the preformed nucleocapsid. In addition to promoting virion assembly through its tendency to oligomerize, M works together with proteins NS-1 and M2-2 in rendering the nucleocapsid quiescent prior to packaging[212,228].

As with protein translation, where the virus occupies the host's machinery, virus assembly and budding necessitate cytoskeletal proteins[236] and take

advantage of the host's endosomal transcytotic trafficking through the apical recycling endosome[237].

4.2.3.1 Syncytia Formation

In addition to the creation of infectious virions, RSV propagates by infecting nearby cells through cell membrane fusion[213], which is mediated by protein F through its interaction with the cellular GTPase, RhoA[238,239]. The resulting multinucleated giant cells are called syncytia, hence the name of the virus[218]. The role of RhoA in syncytia formation has not been elucidated, but is probably related to its control of actin reorganization and cell morphology[240].

4.3 RSV Infection in Humans

RSV is responsible for a considerable number of respiratory-related hospitalizations in children[161,241], the elderly[242] and immunocompromised adults[243]. In fact, most infants would be infected with RSV by age two, although not all of them would develop severe disease[244]. However, infection continues to be the leading cause of infant hospitalization[245-247] as it is the main source of serious upper and lower respiratory infections in infants and children[247].

RSV transmission into the conjunctivae and the nasal mucosa is known to occur through large-particle aerosols or through self-inoculation from infective virus on inanimate objects known as fomites[248,249]. Virus survival depends on the medium in which it is found[248]. Replicative and infectious RSV have been recovered from aerosols for up to one minute[250], from skin for up to 20 min[249],

from clothing for up to 45 min[249], from countertops for up to six hours[249] and from solutions for up to six days[251].

The main target of infection is the airway epithelium. These airway epithelial cells constitute the first line of defense against infection and, as such, are the focus of the associated inflammation[213]. Generally, RSV infections present only cold-like symptoms as infection is contained within the upper respiratory tract. In some cases however, as with immunocompromised patients, the infection spreads to the lower airways where inflammation and cell debris can result in airway obstruction and even impaired gas exchange and thus it can lead to bronchiolitis and even pneumonia[39,247,252,253]. In fact, RSV is the main cause of wheezing bronchitis[254], bronchiolitis[246,255] and pneumonia[247] in infants. The repercussions of these illnesses can be long lived with wheezing and airway hyperreactivity reportedly persisting for up to 10 years after acute infection[247]. In fact, infantile bronchiolitis has been associated with the pathogenesis of asthma[256,257].

4.3.1 Epidemiology

The distribution of RSV around the globe is ubiquitous[246,258], and as such, annual RSV epidemics affect more than a third of the human population, afflicting people of all age groups[259]. RSV infectious outbreaks follow a seasonal pattern[260,261], which is different in distinct geographical locations[262]. For example, it is most common in the colder months in areas with temperate weather, but in tropical countries, it is most common during the rainy season[260]. Furthermore, in separate areas, different environmental factors correlate differently with RSV

infection; for example, in Mexico City, 41% of its activity depends on dew point, while in Miami, 26% of its activity depends on temperature and UVB radiation[261].

In spite of all these advances in the understanding of the epidemiology of RSV, no research has been done as to discover meteorological patterns that could account for the seasonality of RSV outbreaks and its ubiquitousness. This is especially surprising when taking into consideration that it has been suggested that RSV onsets are not necessarily spread from location to location as would be expected for community to community infection, but developed locally, thus signaling within community infections[263].

4.3.1.1 Host determinants

Ethnicity and race do not seem to influence the outcome of RSV infection[258]; however, some predisposing genetic factors have been reported[264]. Patient sex does not seem to affect incidence, but does seem to affect disease severity; reportedly, more males tend to require hospitalization[258]. Similarly, lower income populations require hospitalization much more often than middle income patients do[265]. Finally, age does not seem to affect incidence as much, probably due to the fact that no long-term immunity is established upon infection and so reinfections are a commonality[244]; however, disease severity is more common on infants and the elderly[241,242].

4.4 Human Response to infection

There has been a lot of research concerning the host response to RSV infection, as almost every aspect of the immune system has been implicated in

the development of the severe lower respiratory tract disease, which sometimes accompanies the infection[266-268]. Such cases are usually followed by recurrent wheezing or even asthma[256,257].

Regardless of the efforts to date, it is still not clear whether it is the RSV infection that is responsible for these serious consequences, or if it is the same host factors that predispose to bronchiolitis, which also facilitate the occurrence of such obstructive airway diseases[268].

4.4.1 Innate Immunity

As mentioned earlier, the main target of RSV infection is the airway epithelium[213]; although not as effectively as its main target, RSV will also infect monocytes[269], macrophages[270], neutrophils[200,271], eosinophils[272] and T cells[56]. The airway epithelium and the macrophages constitute the first line of defense against inhaled pathogens, the former acting as a physical barrier and the latter as an active soldier/cleanser[200,213], which inhibits viral spread to lower airways[39]. As such, both the airway epithelial cells and macrophages regulate the innate immune response to RSV.

Even prior to infection, epithelial cells secrete opsonins and collectins, which facilitate RSV opsonisation[273]. After infection, both epithelial cells and macrophages secrete cytokines chemotactic for neutrophils, eosinophils and T cells, as well as lymphocyte activator interleukins (Table 4.1)[39,213]. As such, neutrophils[274], followed by degranulated eosinophils[43,275], have been found to be the predominant cell type in upper and lower airways infected with RSV. Note that these innate leukocytes play an important role in inflammation. In fact, the

edema characteristic of RSV-bronchiolitis is believed to be due to viral associated necrosis and to increased concentration of mediators that cause blood vessel permeability alterations[276].

Interestingly, natural killer cells, the innate leukocytes classically entrusted with viral clearance, do not appear to have a strong role in recovery for RSV infection[258].

Table 4.1 Changes in Airway Cytokine Secretion in Response to RSV Infection

Airway Region	Samples	Increased Secretion	Unchanged Secretion
Nasal	Epithelial explants	IFN-γ[277]	Gro-α[278]
		IL-2[277]	IL-8[278 $ 279&]
		IL-4[277]	MCP-1[278 $]
		IL-5[277]	MIP-1α[278 $]
		IL-6[280]	TNF-α[281]
		IL-8[280,281 $]	
		MCP-1[279 $]	
		MIP-1α[279 $]	
		RANTES[278-280]	
	Lavage	VEGF[276]	
		IL-1β[282]	
		IL-6[282]	
		IL-8[279,282]	
		MCP-1[279]	
		MIP-1α[279,283]	
		TNFα[282]	
		RANTES[279,283]	
		VPF[276]	
Laryngeal	HEp-2	IL-8[43]	
		MIP-1α[43,283]	
		RANTES[43,283]	
Tracheal	Aspirations	IL-8[43]	
		MIP-1α[43]	
		RANTES[43]	
	9HTE	IL-11[284]	
Bronchial	BEAS-2B	GM-CSF[285]	IL-1[286]
		IL-6[285,286]	IFN-α[285]
		IL-8[39,285,286 #]	IFN-γ[285]
		RANTES[39,280]	MIP-1β[39,199]
		MIP-1α[39]	MCP-1[39,280]
			MCP-3[280]
	HBEC	IL-6[287]	IL-1β[287]

		IL-8[287]	
		RANTES[287]	
	NHBE	VEGF[276]	MCP-1[280]
		RANTES[280]	MCP-3[280]
		VPF[276]	MIP-1β[280]
Alveoli	A549	BEGF[276]	
		IL-1α[288,289]	
		IL-1β[288,289 @]	
		IL-6[290]	
		IL-8[289-292]	
		IL-11[284]	
		TNF-α[288,289 @]	
		VPF[276]	
Alveolar Lumen	AM	IL-1[293]	
		IL-6[286,293]	
		IL-8[286]	
		MCP-1[39]	
		MIP-1 beta[39]	
		RANTES[39]	
		TNF-α[286,293]	
Co-cultures	BEAS-2B / AM*	IL-8[39]	
		MIP-1α[39]	
		MIP-1 beta[39]	
		MCP-1[39]	
		RANTES[39]	
	A549/ PBMC^		IL-8[290]
	A549/PMN^		IL-8[290]
Peripheral Blood	PBMC/monocytes	IL-6[294]	
		IL-8[294]	
		TNF-α[294]	

*AM = primary alveolar macrophages; A-549 = adenocarcinomic human alveolar epithelial cells; HBEC = primary human bronchial epithelial cells; BEAS-2B = human bronchial epithelial cells transformed by Adenovirus SV40; NHBE = primary normal human bronchial epithelial cells; HEp-2 = human carcinomic laryngeal epithelial cells; 9HTE=immortalized human tracheal epithelial cell line; *AM co-cultured with infected BEAS-2B monolayers, ^Co-cultures infected with RSV at the same time. #No differential mRNA synthesis found[280] although the same authors have found IL-8 expression by ELISA upon RSV infection of BEAS-2B with an MOI of 1[39,285,286].@A study using ELISAs with higher limits of detection didn't find expression of this mediators in response to RSV infection[292]. $These contradictory results could be simply due to biological diversity of the samples, or to the fact that*

whereas Saito et al purified their cell cultures by filtration[278], Becker et al did not[279,280]. In fact, Becker et al comments that < 5% of his cultures consists of inflammatory cells[280], whereas Saito reports 98% of his cultures as being epithelial cells[278]. &This study assayed mRNA synthesis instead of protein expression.

4.4.2 Acquired/Adaptive Immunity

Studies have reported that a primary cell mediated immunity response against RSV is mounted within 10 days of infection[295]. As mentioned above, in classical immunology, it is believed that intracellular pathogens are dealt by cell mediated immunity, which is mediated by T_H1 cells. Being that RSV is a virus, an obligatory intracellular pathogen, it should elicit T_H1-mediated responses; however, evidence for both, T_H1 and T_H2 immune responses to RSV infection, has been found[213,266,296,297].

As mentioned earlier, dendritic cells regulate the differentiation of T cells into T_H1 or T_H2 cells. Conflicting studies have reported RSV to induce dendritic cells maturation, but also to cause them to acquire an immunosuppressive phenotype[42].

A possible clue to the factors determining the differentiation of T_H cells into either T_H1 or T_H2 cells is provided by vaccination trials with two different vaccines, one containing alum-precipitated formalyn-innactivated virus and the other one containing live attenuated virus[46]. It has been reported that vaccination with the former vaccine induced a T_H2 immune response, whereas the latter vaccine induced a T_H1 immune response[298]. These results seem to indicate the possibility of the requirement of the presence of an adjuvant, in addition to RSV, to drive the immune balance towards T_H2 mediated immunity.

Regardless of whether RSV infection drives T_H1 or T_H2 immunity, both $CD4^+$ and $CD8^+$ T cells have been shown to both clear RSV and to increase pulmonary damage; although it seems that T_H2 responses might be more immunopathogenic[296].

As with cell mediated immunity, there have been contradictory reports regarding the role of humoral immunity in RSV clearance or pathogenesis. It has been found that RSV-specific antibodies do not confer absolute protection against RSV infection[244]. In fact, RSV is unique in its ability to cause severe disease in infants carrying RSV-specific maternal antibodies[299]; however, high titres of IgA, IgG and IgM antibodies do correlate with decreased severity[265,300]. In addition, reinfections are found to decrease in severity as their occurrence boosts the RSV-specific antibodies[301]. On the other hand, if the humoral response resulted in a strong and lengthy production of IgE antibodies, which are important in allergic and hypersensitivity reactions, increased severity is to be expected[302,303]. In fact, elevated levels of mediators conducive to bronchospasm and inflammation have been found in children with RSV-bronchiolitis[43,275].

As mentioned earlier, the main effector cell of cell mediated immunity is the Cytotoxic T cell. Interestingly, $CD8^+$ cells do not recognize the envelope glycoprotein G, but recognize the inner virion protein M, the nucleocapsid protein N and the non-structural protein NS2[213]. In spite of this apparent deficiency, the presence of RSV-specific $CD8^+$ cells in blood of infected patients correlates with decrease clinical symptoms[213].

In spite of the wealth of knowledge gained regarding the role of adaptive immunity in the pathogenicity of RSV, much more remains to be discovered, specially as human studies continue to produce contradictory T_H1 versus T_H2 cytokine profiles in patients with RSV-bronchiolitis[213].

4.5 Current experimental designs concerning the investigation of RSV infectivity in humans

RSV has been recognized as a very important pathogen in humans. As such, a lot of research has been performed trying to understand the molecular mechanisms behind its pathology. Experimental approaches range from the simple pipetting of RSV stocks onto cell monolayers to animal exposure to inhalable aerosolized RSV stocks[304-306].

Traditional *in vitro* studies are excellent for mechanistic studies[189] as airway epithelial cell lines exist that allow for a very high level of control and a very high number of subjects to be used so that statistically significant conclusions can be easily derived. However, any information gained from this approach is limited to the cells used in the experiment; moreover, being in a confined environment, there is a lack of the stimuli otherwise generated by other cell types, tissues and even distal organs in the body. Some of this limitations can be compensated by co-culturing studies[39] in which different cell types can be brought together in order to explore their interactions resulting from RSV infection. Alternatively, primary airway epithelial cells can be cultured onto air-liquid interfaces (ALIs) as to allow them to differentiate into the diverse cell types present in the tissue the cells were sampled from[304,307]. This would approximate the *in vivo* environment so

that responses to RSV infection by that "airway tissue" as a whole can be explored. In spite of the advantages conferred by using differentiated primary cells in co-culturing studies, the only way to approximate the human response to RSV related pulmonary infection is through animal models.

Several animal models have been considered for their use in RSV dependent pulmonary infection. High cost and ethical concerns preclude viable research in animals genetically similar to humans. Terminal experiments are forbidden in chimpanzees, which are the most genetically similar to humans and there is a lack in availability of species-specific immunological reagents against both, chimpanzees and monkeys, as well no sufficient inbreeding to control for genetic variablility[308]. Research on small animals is preferred due to its much lower cost, availability of species-specific immunological reagents, as well as of congenic, knock out and transgenic strains. The permissiveness to RSV infection of mice and hamsters is very low; in fact, an RSV dose of five orders of magnitude larger than the dose for humans is required for bronchiolar inflammation to develop[308,309]. Moreover, pulmonary infection is only developed in older mice, whereas infant humans are the ones susceptible to it[309]. The permissiveness to RSV in cotton rats is improved[308,310]; however, pulmonary infection is reportedly developed at all ages[309,310] and there is a lack of species-specific reagents and of congenic, knock out and transgenic strains, which in contrast, are readily available for mice[309]. Ferrets develop pulmonary infection only during their infancy; in fact, they are reportedly the only animal model in which age-dependent pulmonary infection takes place; however, they face the same experimental limitations that cotton rats do[308]. Guinea pigs offer the advantage

of having a much higher RSV permissiveness[311], a similar gross lung anatomy to humans[312] and of being able to develop both acute and persistent RSV infection[311,313]; however, research in these animals is also faced with the same the limitations as the research on rats[308]. As such, it seems that a perfect animal model for RSV induced pulmonary infection does not exist.

The limitations possessed by each *in vitro* and animal experimental design seem to complement each other so that when combining the information obtained from both approaches, great insight has been obtained into the pathogenicity of RSV.

4.6 So why RSV?

As it can be deduced from the impressive amount of experimental models designed to investigate the pathogenicity of RSV infection, this pathogen is a major public health concern, causing millions of deaths worldwide and costing millions of dollars yearly[247]. As such enormous amounts of resources have been allocated to its characterization; in fact, RSV has become the prototype of the *Pneumovirus* genus[314].

In addition to its impact in human health and its being very well characterized, RSV elicits a very unique and interesting immune response in humans. Its alleged potential to sensitize the airway epithelium towards allergens makes it extremely interesting in the context of air pollution. It is for these reasons that I decided to use RSV in my research.

5: Particulate Matter and Respiratory Viral Infections

" See, through this air, this ocean, and this earth,
All matter quick, and bursting into birth.
Above, how high, progressive life may go!
Around, how wide! how deep extend below!
Vast chain of being, which from God began,
Natures ethereal, human, angel, man,
Beast, bird, fish, insect! what no eye can see,
No glass can reach! from infinite to thee,
From thee to nothing!–On superior pow'rs
Were we to press, inferior might on ours:
Or in the full creation leave a void,
Where, one step broken, the great scale's destroy'd:
From nature's chain whatever link you strike,
Tenth or ten thousandth, breaks the chain alike.

And, if each system in gradation roll
Alike essential to th' amazing whole,
The least confusion but in one, not all
That system only, but the whole must fall...."

Alexander Pope, An Essay on Man: Epistle 1

One of the main causes of morbidity and mortality in developing countries are lower respiratory infections[315]. Epidemiological studies have also found a positive correlation between ambient PM levels and respiratory related morbidity and mortality[15]. As such, the necessity to understand the effects of ambient PM on viral infections has been recognized. This work investigated lung cell responses due to infection by RSV-containing carbon particles.

5.1 Epidemiology

Due to technical challenges, there have been few direct epidemiological studies on the effect of PM levels and viral infections. Instead, numerous studies have looked at the association between levels of ambient PM and the incidence of respiratory conditions known to be often caused by viral infections[15,316,317]. Morbidity due to respiratory infections was found to be associated with increased $PM_{2.5-10}$ levels in Canada[318]; a Nordic study found a positive correlation between ambient PM and upper respiratory infections[319] and a study across USA found a correlation of increased morbidity due to respiratory infections and levels of $PM_{2.5}$[320]. More specific studies conducted in the USA, found a positive association between PM_{10} levels and the incidence of bronchitis[321] and pneumonia[322]. Similar associations were found in Europe, where in addition to pneumonia[323], laryngotracheitis[324] cases were found to increase with ambient PM_{10}.

In addition, infection with virus-contaminated PM has been postulated as an explanation of respiratory viral epidemics[143]. For example, infections with virus-laden aerosols reportedly account for the temporal and spatial transmission

of the severe acute respiratory syndrome virus in Hong Kong[325]. Moreover, virus-PM interactions could account for long-distance atmospheric transport of infectious virions. In fact, attachment of influenza virus to dust particles moving across the Pacific has been modelled as a likely means of transport of the virus from Asia to America[110,326].

5.1.1 Epidemiology: PM and RSV

Most epidemiological studies conducted regarding RSV morbidity and environmental factors, have found a positive correlation between RSV related respiratory infections and air pollution[161,246]. Many studies do not look directly at the incidence of RSV infection, but instead use the incidence of wheezy bronchitis and bronchiolitis as an estimate of RSV related morbidity. Concerning the effects of $PM_{2.5}$ on RSV related morbidity, conflicting results have been found. $PM_{2.5}$ was found to be a strong indicator of the incidence of wheezy bronchitis from April to October in infants younger than 1 year of age by a Chilean study[327]. On the other hand, a five year study in California, found only a weak positive correlation between $PM_{2.5}$ and the incidence of bronchiolitis in premature infants[328].

There have only been a few epidemiological studies looking at the short-term effects of PM_{10} in RSV associated morbidity and their results were contradictory. A first study followed RSV consultations and admissions at one hospital in Chile over a single RSV epidemic (March to September of 1998) over which there was no trend in the levels of ambient PM_{10}[329] and therefore found no association between the two parameters. In a second set of studies, direct

association between RSV and PM_{10} was not investigated; rather, the effect of each parameter in Chilean morbidity at a given hospital from Autumn to Spring (2001[330] and 2002[331]) was documented and it was found that only RSV infectivity was associated with lower respiratory related morbidity. The most recent study, did not focus on the incidence of RSV infection per se after the initial stages of the surveillance period, instead it followed Parisian bronchiolitis cases during three years (1997-2001) in at least 34 hospitals, and it found a positive correlation between the levels of PM_{10} and RSV associated morbidity[332].

Many factors could contribute to the conflicting results obtained from the aforementioned studies, among which, not having accounted for the composition of the PM used in each study, could be an important one.

5.2 Host Response to Exposure: PM and respiratory viruses

The cellular responses to viral infection and PM separately are, by themselves, extremely complicated. Therefore, exposure to both stimuli can only elicit much more intricate responses, assuming that the mechanisms of injury from each stimulus are additive or more. Independently of this assumption, one can infer that the cellular responses to both stimuli might result from a combination of the mechanisms of injury of each stimulus. For example, in addition to oxidative stress, PM can sensitize the airway epithelium to infection by disrupting the function of surfactant proteins[333] and by decreasing the phagocytic rates of macrophages[39,200]. The morbidity associated with viral infections could also be further increased by an exaggerated inflammatory

response due to PM[15]. In fact, it has been shown that antioxidant treatment prior to PM exposure neutralizes the usual enhancement of influenza[15].

5.2.1 Host Response to Exposure: PM and RSV

Ex vivo studies have looked at the effects of PM_{10} exposure in RSV infection of alveolar macrophages. These studies have looked at the effect of exposure to carbon before and after infection and it has been found that regardless of the order of events, alveolar macrophages show less phagocytic ability and less cytokine secretion[200] (Table 5.1). In an elegant co-culture study of PM_{10}-exposed human alveolar macrophages and RSV-infected human airway epithelial cells, a decrease in phagocytic activity and in some cytokine secretion[39] (Table 5.1) was observed.

An animal study found that upon exposure with diesel exhaust particles, RSV infection of mice increased and resulted in a higher inflammatory response[333]. There have been a number of animal studies looking at the effects of PM in RSV infection using a suspension of carbon black particles as a mimic of PM. It has been found that regardless of the order of exposure to carbon the morbidity associated with lower respiratory RSV infection is increased[208,334]. Pre-infection exposure to carbon results in a reduction in earlier responses, such as lymphocyte recruitment and cytokine secretion (Table 5.1), which results in a stronger infection and an increased inflammatory response later in infection[334]. Similarly, carbon exposure post-infection results in increased cytokine secretion (Table 5.1) and lymphocyte recruitment later in infection[208].

Table 5.1 Changes in Airway Cytokine Secretion in Response to PM stimuli and RSV Infection

Airway Region	Samples	Stimuli	Changed Secretion	Unchanged Secretion
Alveolar Lumen	AM	EHC-93 + RSV	IL-6 ↓[200]	TNF-α[200]
			IL-8 ↓[200]	
		RSV + EHC-93	IL-6 ↓[200]	TNF-α[200]
			IL-8 ↓[200]	
Co-cultures	BEAS-2B / AM*	EHC-93 + RSV	MCP-1 ↓[39]	IL-8[39]
				MIP - 1α[39]
				MIP – 1β[39]
				RANTES[39]
Mice	LH	DEP_{EC} + RSV_{LI}	IFN-γ ↑[333]	
			TNF-α ↑[333]	
	BAL, LH	CB_{ITI} + RSV_{ITI}	IL-13 ↑[334]	RANTES[334]
			TNF-α ↓[334]	MCP-1[334]
			IP-10 ↓[334]	MIP-1α[334]
			IFN-γ ↓[334]	MIP-1β[334]
	BAL, LH	RSV_{ITI} + CB_{ITI}	MCP-1 ↑[208]	
			MIP-1α ↑[208]	
			RANTES ↑[208]	

AM = primary alveolar macrophage; BEAS-2B = human bronchial epithelial cells transformed by Adenovirus SV40; BAL= bronchoalveolar fluid, LH = lung homogenate; CB = carbon black; EHC-93 = characterized ambient particles from Ottawa; DEP = diesel exhaust particle; ITI = intratracheal instillation; LI = lung instillation

5.3 PM and its possible effects on virus survival

Since at least the early1900s, the potential role for virus-PM interactions in microbial survival had been recognized[10,335]. Field studies have provided evidence for enhancement of survival of enteroviruses by their association/attachment to solid particless[336-338]. Influenza, an enveloped virus, has been found to persist for several weeks on dust[339] and *in vitro* studies have shown the ability of artificial test soil in solution to protect non-enveloped viruses from disinfectants[340].

The exact mechanisms of protection are not well understood; however, one could speculate that the PM onto which the viruses adsorb or aggregate with[147,148] act as a protective encasing[4] that could shield the virus from environmental factors that would normally inactivate it, such as tropospheric oxidants[338], UVB radiation[261] or enzymatic stress[148].

5.4 Shortcomings of current experimental designs concerning the investigation of potential PM and RSV interactions

The significance of the potential of PM_{10} acting as an infectious reservoir for viruses has been recognized[341,342] and, as such, much research concerning the interactions between PM_{10} and viruses has been undertaken[343]. Methodologies aiming to unravel the enigma of RSV-PM_{10} interactions range from the traditional pipetting of solutions onto cell monolayers to elegant animal models where the subject is exposed to inhaled diesel exhaust particles followed by RSV infection either through nasal or intratracheal instillation[15].

As mentioned above, both *in vitro* and animal studies have limitations of significance. In addition, regardless of the host model, experimental designs aimed to study RSV-PM_{10} interactions, usually involve the systematic sequential addition of each stimulus. This adds to the challenges intrinsic to both *in vitro* and animal experimental designs, in that the possibility of viable virions existing on airborne PM[147,148,344] is not accounted for in these scenarios. In addition, none of the dosing strategies employed, with the exception of the aerosolization of solutions (typically consisting either of PM or of virus), is a good representation of inhalable PM. As such, it was the goal of the research presented here to develop a methodology that would allow for both, the creation of RSV-containing PM_{Mimics} and their direct dry-deposition onto airway cellular cultures.

5.5 Moving on: an *in vitro* mimic of the *in vivo* event of RSV-infected PM deposition onto the airway epithelium

The ability to create virus-containing PM_{Mimics} and to deposit such PM_{Mimics} directly onto a differentiated epithelium grown on ALIs will prove beneficial in at least three ways. First, it will enable study of the mechanisms of respiratory infections[7]. Second, it will provide an opportunity to investigate the potential existence of synergism between virions and PM_{10}, as well as to study the effects that one has on the toxicity and cytopathicity of the other. Third, it will allow the study of the polarization in the responses of a differentiated epithelium that is in direct contact with air on its apical side and bathed with medium on its basal side, just as the lung epithelium is in contact with air on one side and blood on the other.

The next chapter introduces methodology that allows for the dry-deposition of a controlled number of RSV-containing PM_{Mimics} onto ALIs, thus creating an *in vitro* mimic of the *in vivo* event of RSV-infected PM deposition onto the airway epithelium.

6: Formation and Dry-Deposition of a Stable Mimic of Ambient Particulate Matter Containing Viable Infectious Respiratory Syncytial Virus

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6.1 Contributions

This work was possible thanks to a fortunate collaboration between the Agnes lab and the Mucosal Immunity group at the James Hogg Research Centre, Providence Heart + Lung Institute at St. Paul's Hospital, which had as principal investigators Tony R. Bai, Delbert R. Dorscheid, Richard G. Hegele, Darryl A. Knight and Stephen F. van Eeden. I benefited enormously from this collaboration as I received invaluable mentorship from these brilliant minds, the funding for my research and precious training in a variety of experimental techniques from their knowledgeable fellows and staff. Gurpreet K. Singhera did not only introduced me to most of the standard operating procedures at the hospital and trained me in cell culture and immunocytochemistry, but also took

upon herself to make sure I always had whatever I needed; David Marchant trained me in everything virus related; Tillie Hackett trained me in primary cell culture and the preparation of ELISAs, and also taught me a lot about confocal microscopy; finally, Anna Meredith trained me in flow cytometry. At SFU, Allen E. Haddrell trained me in the capricious art of levitation and was always ready to answer any questions regarding the inner working of the levitation apparatus and to assist me in the design and construction of my own apparatus; Ryan Lekivetz was always there to answer any statistical question I could have and to make sure that all my assumptions and chosen tests have been correct.

It seems redundant to say that this manuscript could not have happen without each and every one of my co-authors. In fact, the particle characterization by FACS and SEM were undertaken by Anna Meredith and Derrick Horne respectively. The rest of the experiments and all the method development, experimental design, and data collection and processing were performed by myself.

6.2 Abstract

Epidemiological associations of worse respiratory outcomes from combined exposure to ambient particulate matter (PM) and respiratory viral infection suggest possible interactions between PM and viruses. To characterize outcomes of such exposures, we developed an *in vitro* mimic of the *in vivo* event of exposure to PM contaminated with *Respiratory Syncytial Virus* (RSV). Concentration of infectious RSV stocks and a particle levitation apparatus were the foundations of the methodology developed to generate specific numbers of

PM mimics (PM_{Mimics}) of known composition for dry, direct deposition onto airway epithelial cell cultures. Three types of PM_{Mimics} were generated for this study: solid PM_{Mimics} consisting of carbon alone (P_C), solid PM_{Mimics} consisting of carbon and infectious RSV (P_{C+RSV}) and aerosols consisting of RSV (A_{RSV}). P_{C+RSV} were stable in solution and harboured infectious RSV for up to two months. Airway epithelial cells exposed to P_{C+RSV} secreted higher concentrations of cytokines (interleukin (IL)-6 and IL-8) than cells exposed to P_C or A_{RSV}. In conclusion, this experimental platform provides an approach to study the combined effects of PM-viral interactions and airway epithelial exposures in the pathogenesis of respiratory diseases involving inhalation of environmental agents.

6.3 Introduction

Ambient particulate matter, comprised of numerous different compounds each at different abundances, and measuring <10 μm in aerodynamic diameter (PM_{10}), is associated with increased pulmonary and cardiovascular morbidity and mortality[14,15]. Respiratory viral infections are also a major cause of morbidity and mortality worldwide[345,346] and the effects of upper and lower respiratory tract viral infections have been reported to increase with high ambient levels of PM_{10}[15]. Previous studies have reported that PM_{10} can act as a reservoir for viruses[110,336], but the potential interaction between PM_{10} and viruses and the resulting host inflammatory and antiviral responses in the airway are not currently well characterized. The ability to address such questions is evolving owing to emerging *in vitro* and *in vivo* methodologies[4,341,342]; however, the dimensional

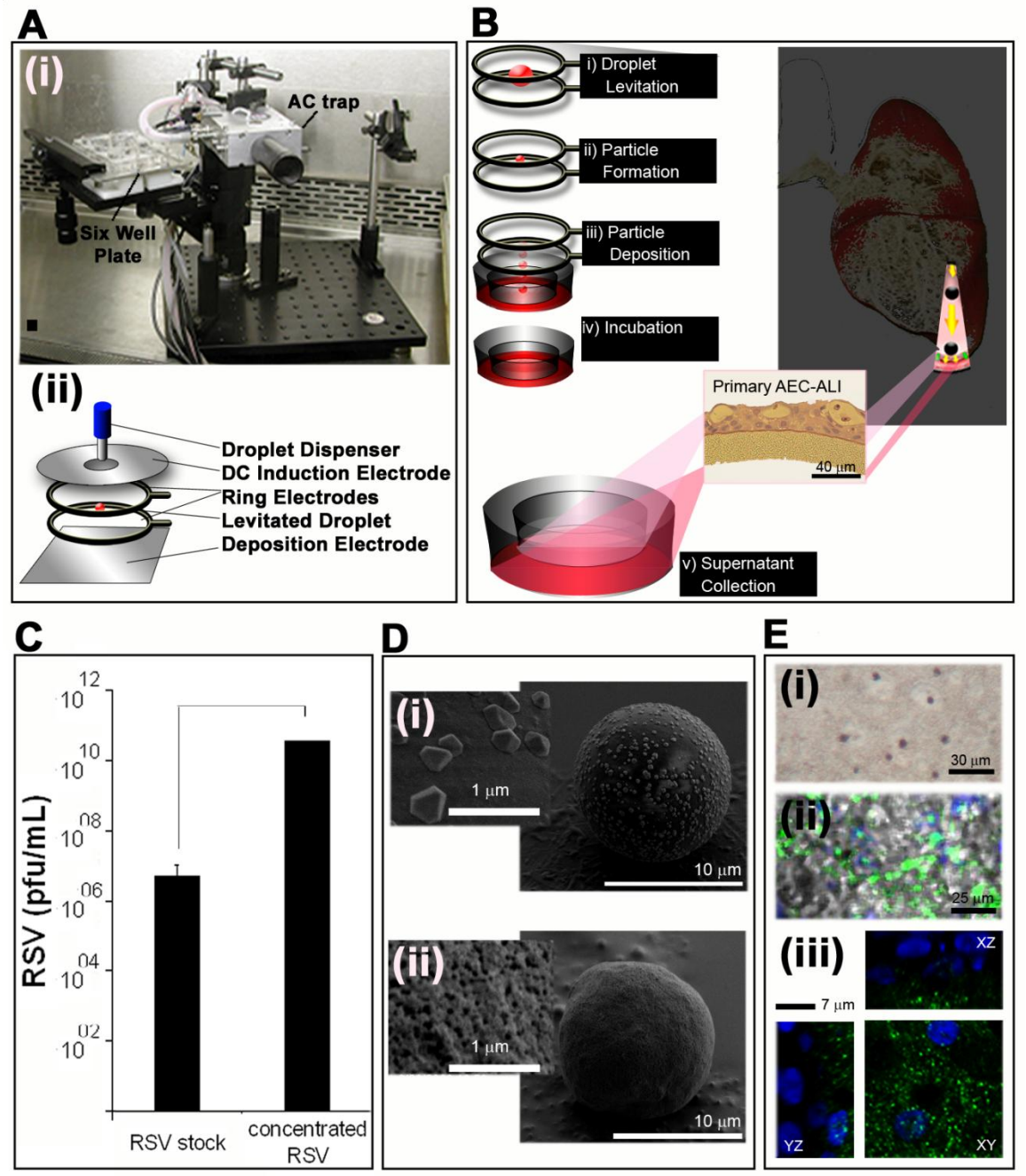
environment of an experimental system that spans from nanometers to micrometers as airborne virions are presented to a host, either alone or harbored in ambient particles, is a technical challenge[7,141].

We introduce an apparatus and associated methodology that enables creation of virus-containing PM mimics (PM_{Mimics}) and their dry deposition directly onto a differentiated primary airway epithelium grown on air-liquid interfaces (ALI) (Figure 6.1). Levitation trap technology has been applied to the study of cellular responses elicited by specific chemical and biological components in PM_{10} as it allows for the creation of particles of desired size and composition[347,348]. With respect to PM_{10}-virus interactions, our methodology will enable study of their potential to either enhance or reduce the toxicity of PM and/or the infectivity and cytopathic effects of the virus. Also, by more closely resembling airway microenvironments, our methodology affords opportunities to study mechanisms of respiratory infections by viruses associated with PM_{10}[7,349] and of the polarization in the responses of a differentiated epithelium.

The components of the ambient PM_{Mimics} used in this study were elemental carbon nanoparticles and Respiratory Syncytial Virus (RSV), selected because carbonaceous residues are a central component of PM[171,350], while RSV is associated with a considerable number of respiratory-related hospitalizations in children[241] and the elderly[242]. Studies have implicated exposure to ambient PM with increased susceptibility to RSV infections *in vitro*[200,39] and *in vivo*[333,334]. Therefore, we created solid PM_{Mimics} comprised of carbon particles (P_C) or RSV-containing carbon particles (P_{C+RSV}) and aerosols consisting of RSV (A_{RSV}), and measured their effects on cellular infection and cytokine response to

demonstrate the capabilities of this methodology in the investigation of the effects of virus-containing PM on the airway epithelium.

(A) Levitation apparatus: (i) Photograph of an AC levitation trap apparatus used for direct deposition of PMMimics onto cell monolayers and ALI inserts; (ii) cartoon of the main components of the levitation technology. (B) Snapshot representation of steps in the process of dispensing, trapping and levitating, and



depositing particles as used for infection of primary AEC-ALI and a comparison of the in vivo deposition of PM onto the airway epithelium and the in vitro deposition of PMMimics onto a differentiated primary AEC-ALI (insert). (C) The effect of concentrating the RSV stock solution on its infectivity (for $p < 0.001$). (D) Scanning electron micrographs of P_{C+RSV} (i) and PC (ii). (E) Bright field and confocal micrographs of Primary AEC-ALI infected with P_{C+RSV} and stained for RSV infection (green) and nuclei (blue) : (i) Bright field photograph illustrating the particles that are observed as opaque objects circular in appearance; (ii) Representative enface apical section (shown here overlapping an enface DIC image of the epithelium and a P_{C+RSV}); (iii) Representative three-dimensional reconstruction of confocal images; the XY inset corresponds to the enface apical surface of the AEC-ALI.

Figure 6.1 Photograph of AC levitation trap and representation of the methodology to create virus-containing PM_{Mimics} that are dry deposited directly onto monolayers and ALI of airway epithelial cell cultures.

6.4 Materials and Methods

6.4.1 Cell culture

Human epithelial-2 (HEp-2) cells, a human airway epithelial-like tumor cell line obtained from the American Type Culture Collection (ATCC) (Manassas, VA), were used for virus propagation, quantification and for dose-response studies. 1HAEo- cells, normal human airway epithelial cells transformed with Simian virus 40 (SV_{40}), were used for the characterization of viral survival *ex vivo*. Cells were grown in Minimal Essential Medium (MEM), supplemented with 10 % Fetal Bovine Serum (FBS), 2 mM L-glutamine, 1.0 mM sodium pyruvate, and 0.1 mM non-essential amino acids at 37 ° C and 5 % CO_2. Depending on the experiment, HEp-2 cells were cultured in either T75 flasks, 12 well plates, or in six well plates with or without coverslips. 1HAEo- cells were cultured in sterile 12 well plates.

Primary Airway Epithelial Cell - Air Liquid Interface (AEC-ALI) cultures grown on transwell plates were obtained from MatTek Inc. (Ashland, MA). These primary AEC-ALI were grown from bronchial epithelial cells obtained from brushings from a healthy 22 year old male airway donor as per standard protocol[351]. Twenty-four hours prior to an experiment, the medium of both the apical and basal surfaces was replaced with new media.

6.4.2 Histochemical staining of Primary AEC-ALI

Primary AEC-ALI sections were deparaffinized and rehydrated prior to Hematoxylin and Eosin staining[352] to assess epithelial differentiation and morphology. After dehydration, the sections were then mounted with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI).

6.4.3 Virus Culture and Isolation

The A strain of the Respiratory Syncytial Virus (RSV), originally obtained from ATCC and a recombinant Green Fluorescent Protein expressing RSV-A2 (GFP-RSV)[353], were propagated in HEp-2 cells as previously described[200]. It was determined that the concentration of the RSV stock was 6 X 10^6 plaque forming units of RSV per milliliter (pfu/mL) and of the GFP-RSV stock was 5 X 10^5 infectious units (IU) of GFP-RSV per milliliter (IU/mL). IU is defined as the product of the total number of cells per well and the fraction of fluorescent cells in the population as enumerated by flow cytometry 18 hr post-infection.

6.4.4 Virus Quantification

Virus quantification was performed using measurements of infectious virions, as opposed to quantifying the total amount of virus particles present in the stock solutions. RSV viral titer was determined by performing a methylcellulose plaque assay over dilution factors of the virus stock spanning 10^{-1} to 10^{-11} [354]. To quantify GFP-RSV, virus-exposed cells were harvested with EDTA Trypsin 0.05 % for 5 min and fixed with 2 % formalin (Sigma) and GFP-RSV infection was enumerated by flow cytometry using a Beckman Coulter Epics_XL flow cytometer (Beckman Coulter, Miami, FL). GFP fluorescence of samples gated

based on forward (FSC) and side (SSC) scatters using sham-infected cells as a control was done using Summit software (Dako Cytomation, Capritneria, CA). Titers were determined for each dilution in duplicate and only the solutions whose values were within the linear range were used to determine the titer.

6.4.5 Virus Concentration through Ultra-filtration

To concentrate RSV without losing its infectivity (Figure 6.1C), the RSV stock solution (~10 mL) underwent centrifugation at 3000 x *g* until 150 - 200 µL remained unfiltered (~20 min) through a 100,000 Molecular Weight Cutoff column (Millipore, Amicon Ultra, Bedford, MA). After discarding the filtered fraction, 15 mL of PBS were added to the top of the column and then centrifuged at 3000 x *g* until 150 - 200 µL remain unfiltered (~20 min). The virus stocks thus obtained were then each divided into 50 µL aliquots and frozen at – 80 °C. The concentrated RSV stock contained 3.3 $X10^{10}$ pfu/mL and GFP-RSV stock contained 7.2 $X10^{7}$ IU/mL.

6.4.6 Alternating Current (AC) Levitation Apparatus

The particle levitation apparatus built for this study was based on technology previously developed in our laboratory[348,355] and modified as to allow particle levitation and deposition onto various cell culture platforms, including six well plates and transwell inserts (Figure 6.1A). In the apparatus built for this study, the induction electrode was situated 17 mm above the top ring electrode. The ring electrodes were built by shaping 0.5 mm diameter copper wire into 12.8 mm diameter rings, and mounting two of these rings parallel to each other at a separation of 5 mm. These dimensions allowed for particle capture, levitation

and deposition onto targets situated ~40 mm below the bottom ring electrode. The waveforms used to functionalize the AC trap, and the methodologies developed to trap and levitate particles, are described in the next section.

6.4.7 Particle Generation, Levitation and Deposition

To generate simple mimics of soot particles, carbon particles (P_C) were created using India ink (Speedball, product #3338, Statesville, NC) as the carbon source[356] within the AC levitation trap described above (Figure 6.1A). The starting solutions (SS*) used to generate all the solid PM_{Mimics} consisted of 5 % India ink by volume in different solutions. To generate P_C, the solution used was sterile de-ionized water; to generate P_{C+RSV}, the solutions used were the RSV virus stocks with concentrations outlined in Table 6.1.

Table 6.1 Estimated (E*) RSV content of particulate matter mimics (PM_{Mimics})

[RSV] in starting solution (pfu/mL)	E* RSV virions per particle	No. of Particles Delivered	Total E* RSV dose (total number of virions)
$1.04 \pm 0.004 \times 10^9$	339 ± 14	700 ± 100	$2.37 \pm 0.007 \times 10^5$
$2.08 \pm 0.004 \times 10^9$	678 ± 20	700 ± 100	$4.75 \pm 0.008 \times 10^5$
$4.16 \pm 0.004 \times 10^9$	$136 \pm 3 \times 10^1$	700 ± 100	$9.49 \pm 0.01 \times 10^5$
$8.32 \pm 0.004 \times 10^9$	$271 \pm 4 \times 10^1$	700 ± 100	$1.90 \pm 0.02 \times 10^6$
$3.33 \pm 0.0004 \times 10^{10}$	$109 \pm 0.8 \times 10^2$	700 ± 100	$7.59 \pm 0.03 \times 10^6$
$3.33 \pm 0.0004 \times 10^{10}$	$109 \pm 0.8 \times 10^2$	490 ± 70	$5.32 \pm 0.03 \times 10^6$
$3.33 \pm 0.0004 \times 10^{10}$	$109 \pm 0.8 \times 10^2$	350 ± 50	$3.80 \pm 0.02 \times 10^6$
$3.33 \pm 0.0004 \times 10^{10}$	$109 \pm 0.8 \times 10^2$	210 ± 30	$2.28 \pm 0.02 \times 10^6$
$3.33 \pm 0.0004 \times 10^{10}$	$109 \pm 0.8 \times 10^2$	70 ± 10	$7.59 \pm 0.01 \times 10^5$

Values were calculated for 5 μm diameter PM_{Mimics} generated by dispensing droplets having an initial volume of 326 $\pm$ 194 pL. Values are reported as mean +/- SD.

To account for cellular response due to components present in the different RSV containing solutions, carbon particles were also generated using sterile phosphate buffered saline (P_{C+PBS}), or MEM treated as if harvesting the virus from an uninfected HEp-2 culture (P_{C+MEM}). Finally, the SS* for A_{RSV} consisted of the RSV stock solution alone.

The procedure to generate charged droplets involved transferring a 10 μL aliquot of SS* into the reservoir of a droplet on demand dispenser (MJ-AB-01-60, MicroFab Technologies Inc., Plano, TX) equipped with a 60 μm diameter nozzle. Monodisperse droplets having volumes of 330 $\pm$ 190 pL (mean $\pm$ standard deviation (SD))[357] were dispensed by applying a pulse waveform at 100 Hz to the piezoelectric ceramic of the droplet dispenser for 2 s, so that the concentric piezoceramic actuates causing an aliquot of the liquid within the solution reservoir to be expelled, with 70 ± 10 droplets captured per droplet dispensing event. A net charge was induced onto each droplet during its formation event by applying a direct current (DC) potential to an induction electrode positioned 2 mm away from the nozzle of the droplet dispenser (Figure 1A). The resultant image charge exhibited by each droplet was opposite in polarity to the DC potential applied and it allowed the droplet's trajectory to be manipulated by electric fields. The DC fields of the induction and bottom electrode assist in stabilizing the mean vertical position of the droplets by offsetting the force of gravity with an axial restoring force; whereas the AC field, created by applying a sinusoidal waveform to the ring electrodes (4.5 kV_{0-P}, 30-130 Hz), controls the horizontal position of the droplets by providing a radial restoring force towards the centre of the AC trap. Note that with imperfect electric fields, as those

present in this device, due to the non-quadrupolar electrode geometry used, the time dependent electric field also imparted a vertical force on the droplets.

To create P_C and P_{C+RSV}, the droplets created were captured in the AC trap and levitated for 1 min. Note that only charged droplets having a certain mass to charge (m/z) ratio within a certain range will have stable trajectories within the stability region of the AC trap. The mobility of these charged droplets can be characterized by the modified second order differential Mathieu equations, in which correction factors for Stokesian drag and collisions with background gasses have been included[358].

Equation 6.1

Where m is the mass of the droplet/particle, r is the displacement of the droplet/particle from the centre of the AC trap (both in the axial and radial directions), K_d is the drag parameter, v is the velocity of the surrounding gas molecules, g is the gravitational acceleration, q is the charge of the particles, E_{AC} and E_{DC} represent components of the AC and DC fields respectively and F_i accounts for any other forces on the droplet/particle. K_d accounts for the drag force of a droplet/particle of diameter d due to the viscosity of the surrounding gas (μ) and slipflow (C_C) as follows:

Equation 6.2

Following the evaporation of solvent, typically within 20 s, the low-volatile components present in the starting solution precipitated/agglomerated to form a single residue that was viewed to be spherical in appearance (Figure 6.1 E(i)) using a bright-field microscope. Specifically, for particles containing carbon, the ~20 nanometer dimension carbon black in India Ink aggregated and agglomerated into a single spherical in appearance particle. The droplets destined to become A_{RSV}, on the other hand, were not levitated; rather they were dispensed and allowed to pass through the AC trap directly onto a cell culture because the mass of low volatility solids in these droplets was too low for their residues to be readily levitated.

The particles and aerosols were deposited directly onto a cell culture or cell culture dish positioned below the ring electrodes (Figure 6.1B). The cell cultures were either grown on a coverslip from which the growth medium had been replaced with 15 μL of fresh 2 % FBS MEM, grown in a well of a six well plate from which all the media had been replaced with 200 μL of fresh 2 % FBS MEM, or grown onto ALI from which the mucus on the apical surface was removed with a PBS wash prior to particle deposition. This treatment of the cells is important in that it is meant to resemble the hypophase layer of bronchoalveolar fluid.

6.4.8 Flow Cytometry

P_C, P_{C+MEM} and P_{C+RSV} were generated from their respective starting solutions as indicated above and deposited into 50 μL of water. To minimize the loss of the particles through adhesion to the container surface, after the delivery of 350 particles, the solution was transferred to a fluorescence-activated cell sorting (FACS) tube. For each experimental condition, ~1050 particles were gathered into a single FACS tube and then incubated at 4 C for three days, prior to their characterization by FACS, to ensure that the virus-carbon interaction was lasting.

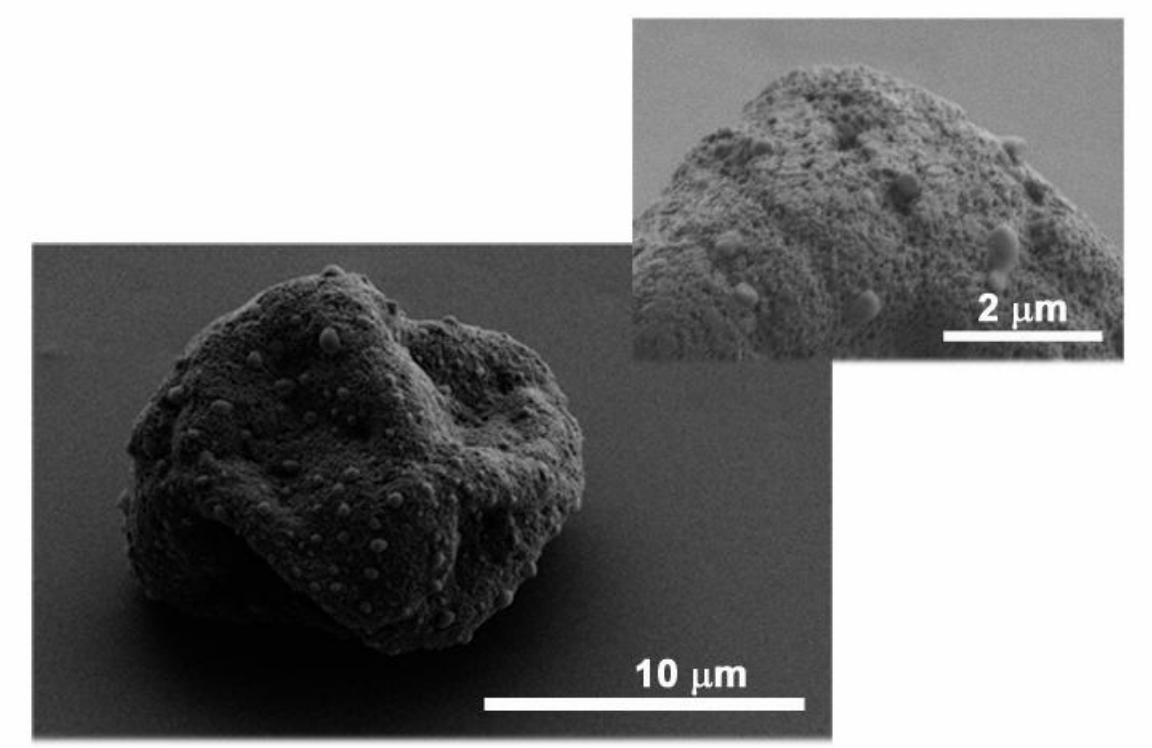
Samples were characterized using fluorescence in a MoFlo High Speed Cell Sorter (DakoCytomation, Fort Collins, CO) equipped with 305, 488 and 633 nm laser lines. FSC and SSC parameters were acquired and used to calibrate the instrument using 1 μm (Dako), 6 μm (APC) and 10 μm diameter (Beckman Coulter) latex beads. Only the population of P_{C+MEM}, P_C and P_{C+RSV} particles that fell within the area corresponding to the 5 μm bead population having high granularity were analyzed for fluorescence using Summit analysis software (Dako Cytomation, Capritneria, CA).

The fluorescence positive control consisted of 50 μL of the virus stock (6 X 10^6 pfu/mL) diluted into 200 μL of distilled water and incubated at 4 C for two days. The samples of P_C, P_{C+MEM} and P_{C+RSV}, collected above in water, and the fluorescence positive control were incubated at room temperature for 1 h, fixed with 10 % formalin and incubated for 10 min at with 0.4 μL of either 4,6-diamidino-2-phenylindole (DAPI) (InVitrogen, Eugene, OR), Hoechst 33342^{359} (InVitrogen,

Eugene, OR), Picogreen (InVitrogen, Eugene, OR) or Ribogreen (InVitrogen, Eugene, OR), prior to introducing the solutions to the flow cytometer.

6.4.9 Scanning Electron Microscopy (SEM)

Three different starting solutions containing 10 % India ink were used to generate particles for characterization using SEM. This higher concentration of India ink was chosen as to facilitate ready location of larger (~10 μm diameter) particles by SEM. P_C, P_{C+RSV}, P_{C+PBS} (Figure 6.2), were generated from their respective starting solutions and deposited onto separate circular 100 mesh nickel grids coated with Formvar (Fort Washington, PA). The grids were then immobilized onto an SEM stub and coated with a 2 nm thick layer of gold and palladium. The particles were visualized with a Hitachi S-4700 Field Emission Scanning Electron Microscope (FESEM) (Hitachi, Tokyo) using an accelerating voltage of 1.5 kV.



PM_{Mimics}, generated from a starting solution containing 10% India Ink and PBS, were deposited onto a nickel grid and then processed for scanning electron microscopy.

Figure 6.2 Representative scanning electron micrograph of P_{C+PBS}.

6.4.10 RSV infection of Primary AEC-ALI and HEp-2 monolayers

Primary AEC-ALI were infected with 700 P_{C+RSV}. 70-80 % confluent HEp-2 cells, grown on six well plates with or without coverslips, were dosed with 70, 210, 350, 490 or 700 P_{C+RSV} (Figure 6.9 carbon and E). For estimated (E*) viral doses for all experiments see Table 6.1 Estimated (E*) RSV content of particulate matter mimics (PM_{Mimics}). Alternatively, HEp-2 cells were dosed with 700 particles (Figure 6.9 D and F) prepared from five different starting solutions, all of which contained 5 % India ink with RSV titers of 1.0 X 10^9 pfu/mL, 2.1 X 10^9 pfu/mL, 4.2 X 10^9 pfu/mL, 8.3 X 10^9 pfu/mL, or 3.3 X 10^{10} pfu/mL. For cells grown on coverslips, 30 min after deposition, 50 μL of 2 % FBS MEM were added. For controls, cells were dosed

with 700 P_C, or with no particles, or with RSV alone in multiplicities of infection (MOI) of 1, 0.1 and 0.01 following the previously described methodology[200]. After rocking the cell cultures every 15 min for 2 h following particle deposition, 1.5 mL of 2 % FBS MEM were added. After 96 h, the primary AEC-ALI were assayed for RSV infection via immunocytochemistry, while the HEp-2 monolayers were assayed for the presence of syncytia on each slide (Figure 6.9 A and B).

6.4.11 Quantification of interleukin (IL)- 6 and IL- 8 secretion

HEp-2 monolayers were dosed with 700 P_C, RSV alone in multiplicities of infection (MOI) of 1, 0.1 and 0.01, or with various E* RSV doses in the form of P_{C+RSV}, which were achieved either by varying the number of RSV virions loaded onto each of the 700 P_{C+RSV} (Figure 6.10) or by varying the number of P_{C+RSV} (Figure 6.11) as outlined in Table 6.1. 100 μL supernatant aliquots were collected from each HEp-2 culture 12 h post-infection and the levels of human IL-6 and IL-8 present measured using commercially available ELISA Duosets (Biosource Europe S.A., limits of detection 0.5 pg/mL) and the concentration corrected for total protein using protein concentrations obtained with Pierce bicinchoninic acid (BCA) Protein Assay (Thermo Fisher Scientific, Rockford, IL). The manufacturer's protocol was followed for each protein determination assay and for each ELISA.

6.4.12 Quantification of Lactate dehydrogenase (LDH) activity in the supernatant

HEp-2 monolayers were untreated or dosed either with 700 P_C or with various E* viral doses in the form of P_{C+RSV}, which were achieved either by varying the number of P_{C+RSV} (Figure 6.9 carbon and E) or by varying the number of RSV virions loaded onto each of the 700 P_{C+RSV} (Figure 6.9 D and F) as outlined in

Table 6.1. 72 h post stimuli, 200 μL supernatant aliquots were collected from each HEp-2 culture. The level of LDH activity secreted into the supernatant was measured using a commercially available Cytotoxicity Detection Kit (Roche Applied Science, Laval, QC, Canada). The manufacturer's protocol was followed and the LDH activity was normalized to the background control.

6.4.13 Immunofluorescent staining of RSV antigens in Primary AEC-ALI

Primary AEC-ALI were fixed with 4 % v/v paraformaldehyde (Fisher, Ottawa, ON, Canada) for 2 hrs. After three washes in 0.1% v/v saponin in PBS, each 15 min in duration, the cells were blocked with 0.1 % v/v saponin and 5 % v/v BSA for 30 min. Subsequently, the primary AEC-ALI were incubated overnight at 4 °C with a mouse anti-RSV monoclonal antibody (NCL-RSV3, Novocastra Laboratories Ltd., Newcastle on Tyne, UK) (1:100 in PBS with 0.1 % v/v saponin and 5 % v/v BSA). After three washes, the cells were incubated overnight at 4 °C with a goat anti-mouse IgG Alexa Fluor 488 (InVitrogen, Eugene, OR) (1:200 in PBS with 0.1 % v/v saponin and 0.1 % v/v BSA). Prior to a 20 min incubation in 5 ng/mL of DAPI, the primary AEC-ALI were washed as before. After a final wash, the AEC-ALI was removed from the inserts and fixed on a slide with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI). For each solution defined and used in this section, 500 μL was aliquoted onto the apical side of the AEC-ALI and 1 mL onto the basal side.

6.4.14 Confocal microscopy of RSV infected AEC-ALI

Differential Interference Contrast (DIC) and confocal fluorescent images were captured with a laser scanning confocal microscope (Leica, model AOBS

SP2, Heidelberg, Germany equipped with version 3.2 Zeiss LSM S10 software, using a high resolution Leica 63 x / 1.4 Plan-Apochromat oil immersion objective). Argon (Ar) and Helium Neon (HeNe) gas lasers were used to generate 405 and 488 nm laser lines used to excite DAPI and Alexa-488 during confocal imaging. A UV diode was used to generate a 514 nm laser line for the DIC imaging. The voxel and the z-axis step dimensions were set as to satisfy the *Nyquist* criteria[360]. Volocity software (Improvision, Waltham, MA) was used to merge enface DIC and confocal fluorescent images, as well as to create three-dimensional reconstructions of confocal fluorescent images.

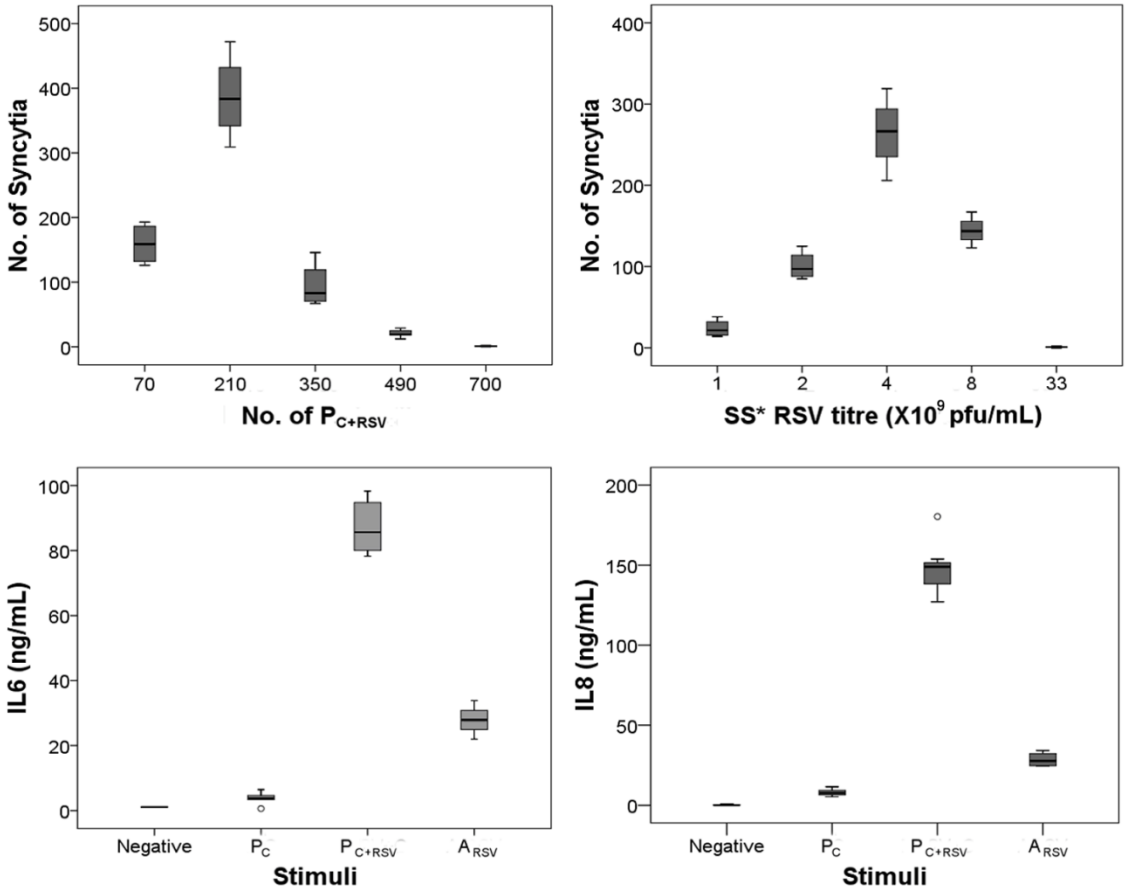
6.4.15 Assessment of viral survival ex vivo.

350 $P_{C+GFP-RSV}$ or $A_{GFP-RSV}$ were generated from a virus stock containing 7.2 $X10^7$ IU/mL and deposited onto separate wells on a sterile 12 well tissue culture plate. The plates were then stored at room temperature under ambient laboratory conditions. After incubation periods of 48 h, 2 weeks and 2 months, ~500,000 1HAEo- cells were seeded into each well. Infection was assessed using a fluorescent microscope after a 96 h incubation period at 37 C and 5 % CO_2.

6.4.16 Statistical analysis

Results were reported as mean +/- SD, unless otherwise stated. Statistical tests were done using Statistical Product and Services Solutions software v.16 (Polar engineering and Consulting, Chicago, IL), unless otherwise stated. Deviations from normality were assessed graphically with quantile plots (Figure 6.3) and confirmed with the Shapiro-Wilk test with α set to 0.05 [361] and homogeneity of variances tested using Levene statistic[362]. For each treatment

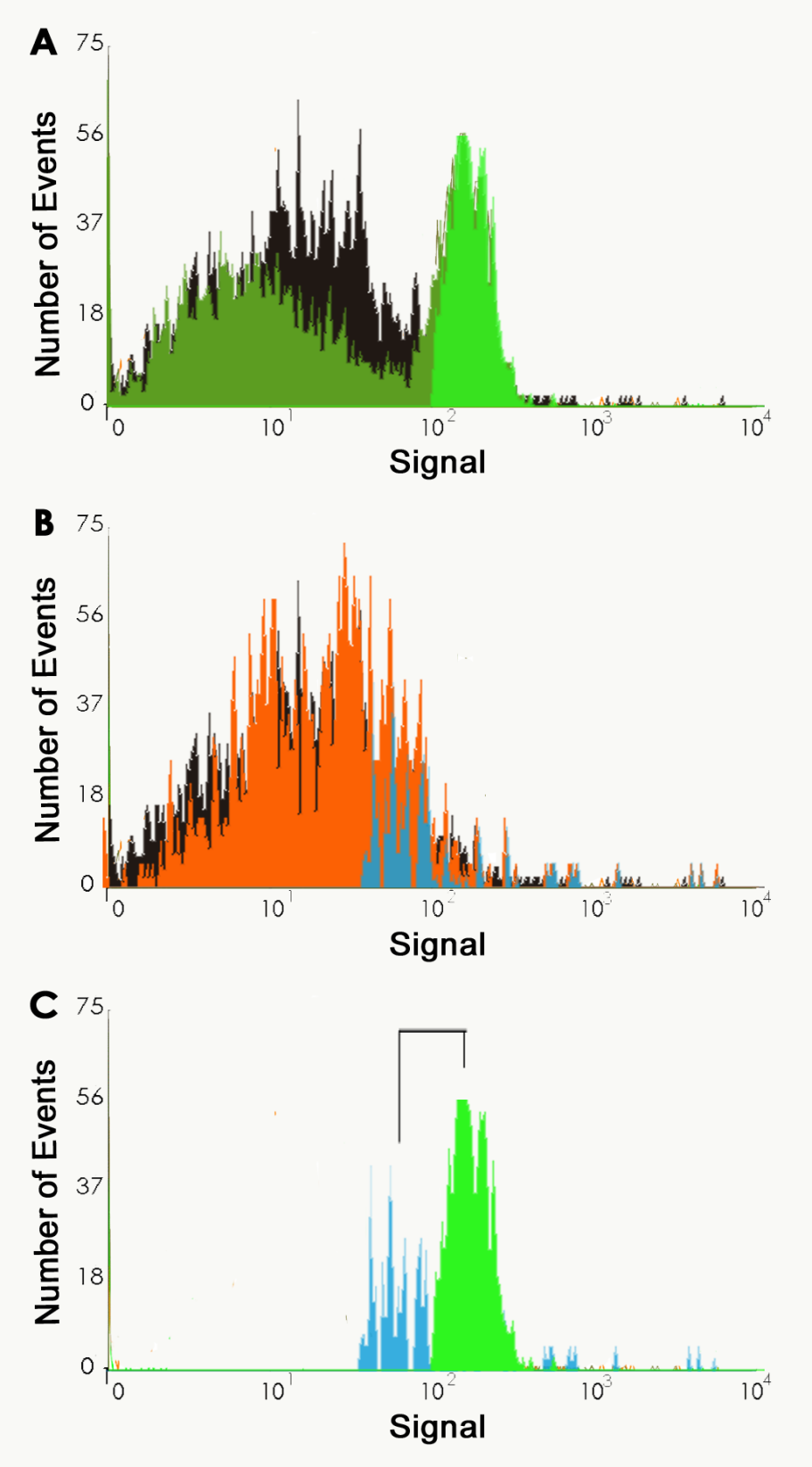
and for each experimental condition, the outliers were confirmed by applying the Grubb's test with α set to 0.05[363]. For data sets with heterogeneous sample sizes and variances, Welch's 2 sided t-tests were performed using the Welch-Satterthwaite equation to determine the degrees of freedom (DF)[361]. Unless otherwise stated, the data were processed with α set to 0.05 for all multiple simultaneous comparisons. Similarly, all error bars in graphical depictions of the data correspond to one SD, unless otherwise stated. Statistically significant differences (SSD) were taken as $p < 0.05$[361].



Box plots illustrating the median (thick line), first and third quartiles (box) and the range of the data (whiskers). ° indicates an outlier.

Figure 6.3 Graphical depiction of the five number summary of the distribution of infectivity and cell response data.

Syncytia quantification: Infections of HEp-2 monolayers with different doses of RSV-containing PM_{Mimics} were performed in triplicate and each dose tested three times over three different dates. For each experimental condition, an ANOVA was performed. After an ANOVA test confirmed SSD between the samples, *post-hoc* tests were applied as to determine the nature of such differences[361]. A Tukey's test[361] was performed for the cells subjected to particles with various amounts of RSV, whereas a Tamhane's T2 test was performed for the cells dosed with different numbers of PM_{Mimics} as their variances were heterogeneous[364]. For comparison of the number of syncytia obtained from similar E* RSV doses obtained by either modifying the number of P_{C+RSV} or the amount of virus per PM_{Mimics}, t-tests were performed using the Bonferroni correction for simultaneous multiple comparisons[365]. *Quantification of LDH activity:* For comparison of the level of LDH activity in the supernatant observed from similar E* RSV doses obtained by either modifying the number of P_{C+RSV} or the amount of virus per PM_{Mimics}, t-tests were performed using the Bonferroni correction for simultaneous multiple comparisons[365]. *Fluorescent data:* To minimize false positives originating from the overlap of the subpopulations present in a bimodal distribution, the Overton algorithm from the Summit analysis software was used to subtract the signal intensity distribution generated by the P_C from the signal intensity distributions generated by the media and the P_{C+RSV} (Figure 6.4)[366].



Panels A and B are a graphical depiction of the background subtracted data for P_{C+RSV} and P_{C+MEM} respectively. In panel C, Indicates $p < 0.05$ for the means of the signal intensity due to MEM and RSV. The black, dark green and orange curves represent the raw data for P_C, P_{C+RSV} and P_{C+MEM} respectively, whereas the light green and blue curves represent the background-subtracted data for RSV and MEM respectively.

Figure 6.4 True fluorescence of Hoescht-33342 stained PM_{Mimics}.

To assess the presence of a SSD in fluorescence emission, a student t-test was applied to the resultant fluorescent distributions of RSV and the media (Table 6.2).

Table 6.2 Statistics for Fluorescence data presented in Figure 6.4 and Figure 6.7.

PM_{Mimics}	**Mean**	**SD**	**Skew**	**LB**	**Q1**	**Median**	**Q2**	**UB**	**Major Mode**	**Minor Mode**
P_{C+RSV}	74.4	93.6	2.3	1	3.8	22.3	94.7	991.0	151.5	4.9
P_{C+RSV}*	141.5†	7.6	0.4	126		141		176	136	-
P_C	29.5	54.9	6.6	1	4.7	14.5	30.9	716.0	14.5	-
P_{C+MEM}	40.1	70.6	6.5	1	9.1	20.1	44.4	797.9	29.8	-
P_{C+MEM}*	126†	27	2.2	1		118		255	104	-
A_{RSV}	89.4	85.4	2.3	1	11.3	63.7	117.7	995.5	109.5	-

P_{C+RSV}, P_{C+MEM}, or P_C were deposited directly onto water and incubated for two days before staining them with Hoechst-43332. The particles were then analyzed in a FACS instrument and their fluorescence intensities recorded. The above are the statistics generated by the distribution of the fluorescence intensities of each population, where SD=standard deviation, LB and UB = lower and upper bound respectively, Q1 and Q2 indicate first and second quartile. Note that the statistics listed for P_{C+RSV} and P_{C+MEM}* correspond to the distribution obtained by subtracting the fluorescence intensities emitted by the negative control, P_C, from the fluorescence intensities emitted by P_{C+RSV} or by P_{C+MEM} respectively. † $p < 0.05$ for P_{C+RSV}* versus P_{C+MEM}**

Interleukin secretion: Experiments were performed in triplicate where the HEp-2 cells were dosed with A_{RSV}. Experiments where the cells were dosed with P_{C+RSV} were performed in triplicate and each experiment repeated thrice on three different days. In addition, the ELISAs for each sample were performed in duplicate. For any sample assayed for secretion of both, IL-6 and IL-8, if it was found that a given sample was an outlier for the secretion of either of the interleukins, that sample's data was removed from the data space of both cytokines. In addition, in order to decrease the possibility of a type 2 error and because the variances of the samples were different, a Tamhane's T2 post hoc

test was applied once the statistical significant difference among the levels of secretion of either interleukin had been established ($p > 0.05$). This statistical significant difference was established using either a one way or a two way ANOVA. Finally, when possible, the secretions of interleukins as a function of viral dose were modelled using linear regression analysis with an inclusion criteria of pF=0.05 and an exclusion criteria of pF=0.10. Finally, a focused analysis was done on the most important interleukin secretion data 12 hr post-stimuli; for each interleukin, an Independent Sample Kruskal-Wallis test was performed as to determine the presence of SSD among the samples[361]. T-tests were then performed to determine the SSD. To this end, the significance level was corrected for simultaneous multiple comparisons using the Bonferroni correction by setting α to 0.05/m, where m is the number of comparisons made[365]. *Ex vivo RSV survival:* Experiments were performed three times for each experimental condition and time point. A one tailed t-test was used to compare each time point.

6.5 Results

6.5.1 Concentration of RSV.

The ultracentrifugation protocol described concentrated the virus by a factor of ~5 X and resulted in an increase of RSV infectivity of ~6000 X (Figure 6.1C). This result was reproducible with 3 different stock preparations.

6.5.2 SEM of PM_{Mimics}.

Differences in appearance of P_{C+RSV} and P_C are observable in the representative scanning electron micrographs of these PM_{Mimics} (Figure 6.1D). The surface of P_{C+RSV} was observed as smooth yet laden with regularly shaped protrusions measured to be 240 $\pm$ 90 nm in size (Figure 6.1D (i)), whereas the surface of P_C was observed as rough and possibly porous (Figure 6.1D (ii)). Interestingly, the range of the dimensions of the structures observed across the surface of P_{C+RSV} are within the documented sizes of the pleomorphic capsid of RSV (80 – 500 nm) [258].

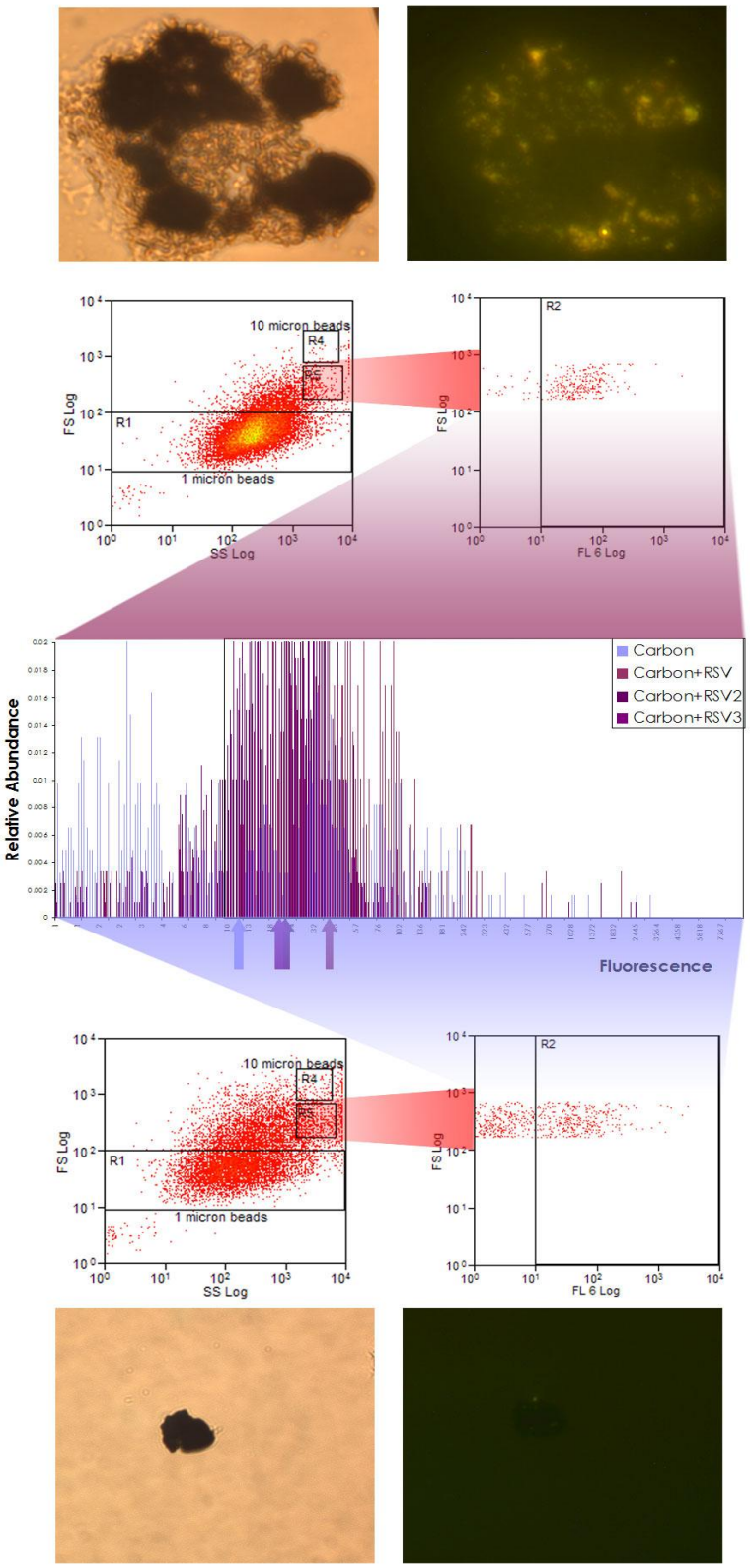
6.5.3 P_{C+RSV} infection of Primary AEC-ALI.

In Figure 6.1E, selected images illustrate that P_{C+RSV} dosed onto differentiated primary airway epithelium are capable of infection, which was observed to be localized to the apical side of primary AEC-ALI (Figure 6.1E (iii)).

6.5.4 Stability of RSV on P_{C+RSV}: RSV remains tightly associated with PM_{Mimics} days after deposition.

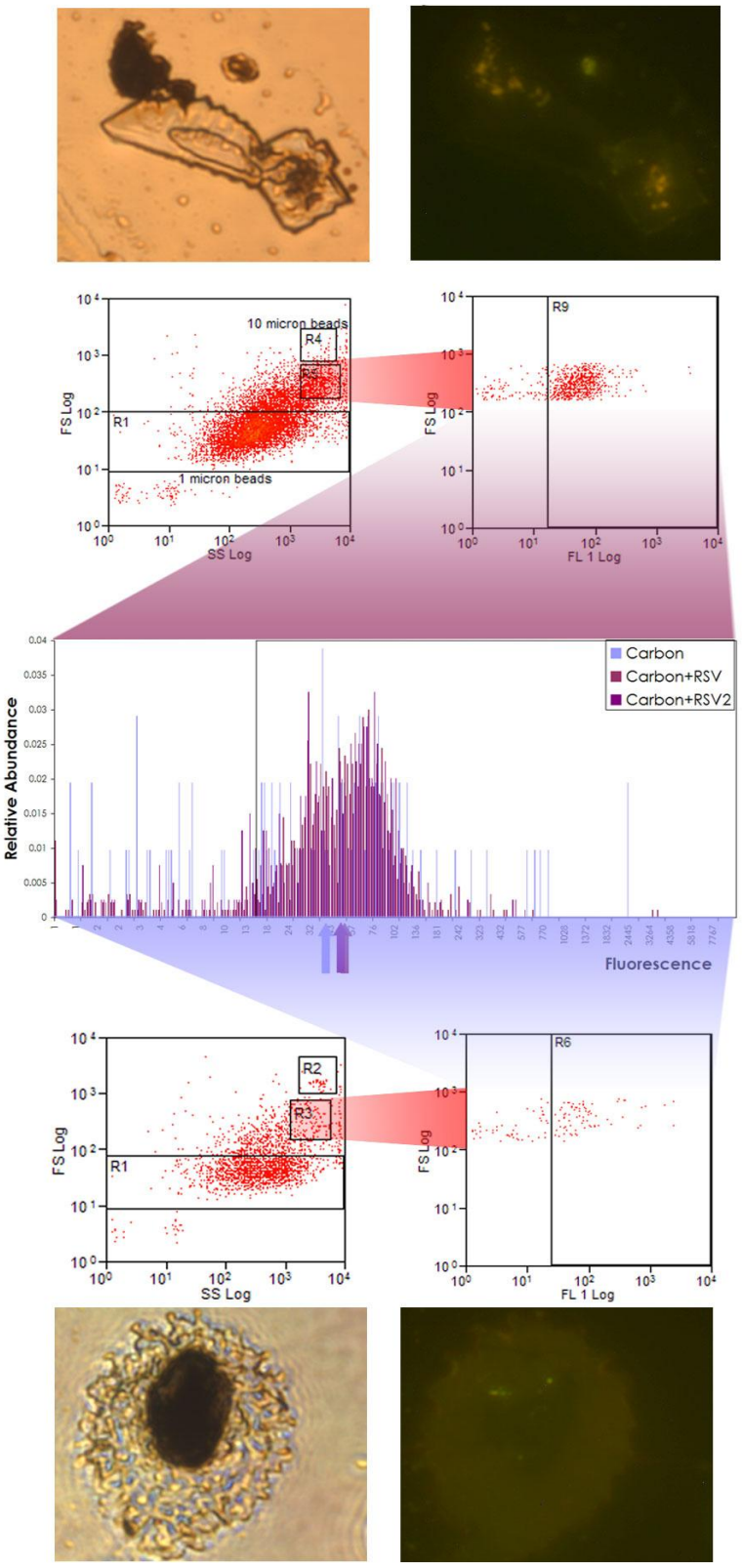
P_{C+RSV}, P_C and P_{C+MEM} were generated and incubated in water for three days over which time there was no observable change in the microscopic appearance of the particles. Bright-field microscopy of PM_{Mimics} (Figure 6.7A-C) indicated that they were granular in appearance and had an average size of 5.3 ± 0.5 μm. Four different nucleic acid dyes (RiboGreen, PicoGreen, DAPI and Hoechst-33342) were screened for selective binding to RSV and not to carbon. Using a fluorescence microscope, it was found that staining with DAPI and

Picogreen resulted in no signal for P_C and P_{C+MEM} and a positive signal from P_{RSV+C} (Figure 6.5 and Figure 6.6 respectively).



P_C (bottom panel and blue curve) or P_{C+RSV} (top panel and purple curves), were stained with DAPI after being incubated in water for two days. In each panel, the dot plots on the right represent the whole population of PM_{Mimics}. The mimics generated in this study are 5.3 + 0.5 μm; therefore, only the fluorescence emitted by the events within the 6 μm rectangle were studied. The raw data corresponding to the intensity of fluorescence emitted by the PM_{Mimics} gated by size is shown in the dot plots to the left in each panel.

Figure 6.5 FACS data for DAPI stained PM_{Mimics}.



P_C (bottom panel and blue curve) or P_{C+RSV} (top panel and purple curves), were stained with DAPI after being incubated in water for two days. In each panel, the dot plots on the right represent the whole population of PM_{Mimics}. The mimics generated in this study are 5.3 + 0.5 μm; therefore, only the fluorescence emitted by the events within the 6 μm rectangle were studied. The raw data corresponding to the intensity of fluorescence emitted by the PM_{Mimics} gated by size is shown in the dot plots to the left in each panel.

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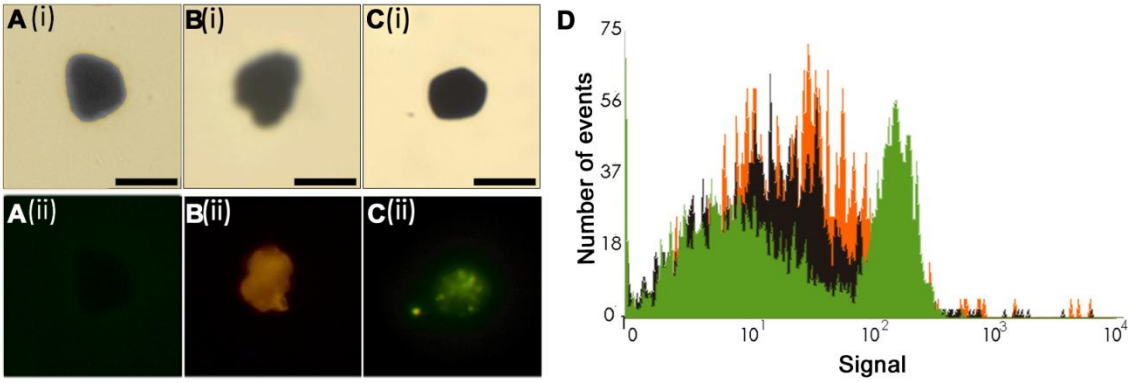
Figure 6.6 FACS data for Picogreen stained PM_{Mimics}.

Staining with Hoechst-33342 also resulted in a positive signal from P_{RSV+C} and a negative signal from P_C; however, P_{C+MEM} displayed a uniform low intensity and spatially differentiable fluorescence emission (Figure 6.7B (ii)) relative to that observed from P_{RSV+C} (Figure 6.7C (II)). The RiboGreen stain was the only dye observed to not enable differentiation of the different particle types.



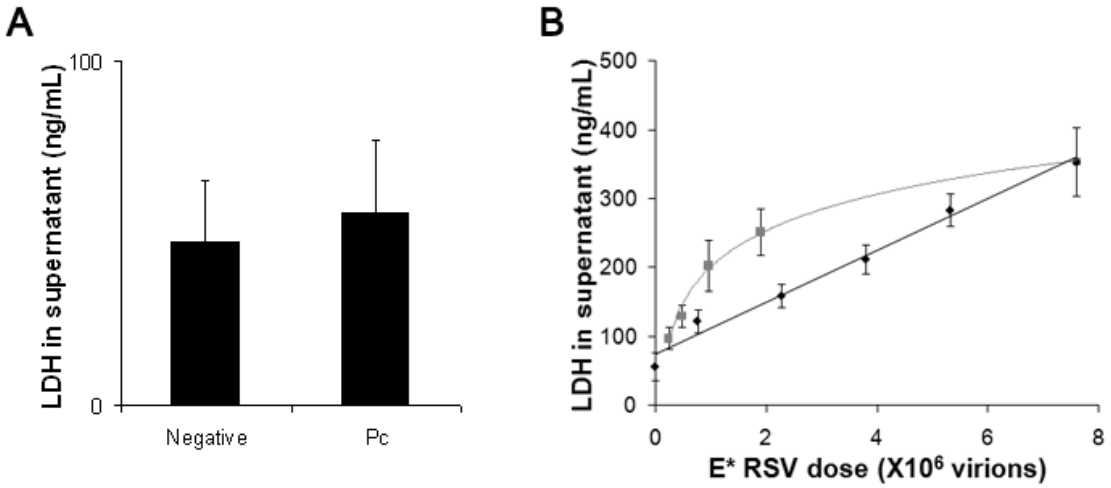
Representative photographs of P_C (A), P_{C+MEM} (B) and P_{C+RSV} (C). Scale bars represent 5 μm. Light microscopy photographs of PM_{Mimics} are shown in the upper panels (i), while the respective images obtained using fluorescence microscopy, using Hoechst-33342 as the fluorophore, are in the bottom panels (ii). Panel D shows the raw data corresponding to the intensity of the FACS signal detected for the PM_{Mimics} gated by size: the black curve represents P_C, the orange curve P_{C+MEM} and the green curve P_{C+RSV}.

Figure 6.7 RSV remains associated with the PMMimics days after deposition.

Flow cytometry analysis indicated that the median signal intensity detected from the particles dyed with either DAPI (Figure 6.5), Picogreen (Figure 6.6) or Hoechst-33342 exhibited a shift toward higher values for P_{RSV+C} relative to the median signal intensities measured for P_C. In addition, the distribution of Hoescht-33342 fluorescence intensity exhibited by the P_{C+RSV} was bimodal (Figure 6.7D) with the major mode exhibiting a fluorescence intensity much higher than the signal for P_C and P_{C+MEM}. Moreover, regardless of which of the three discernable fluorescent dyes was used, only P_{C+RSV} could be sorted based on a positive fluorescent signal.

6.5.5 P_{C+RSV} infection of HEp-2 monolayers.

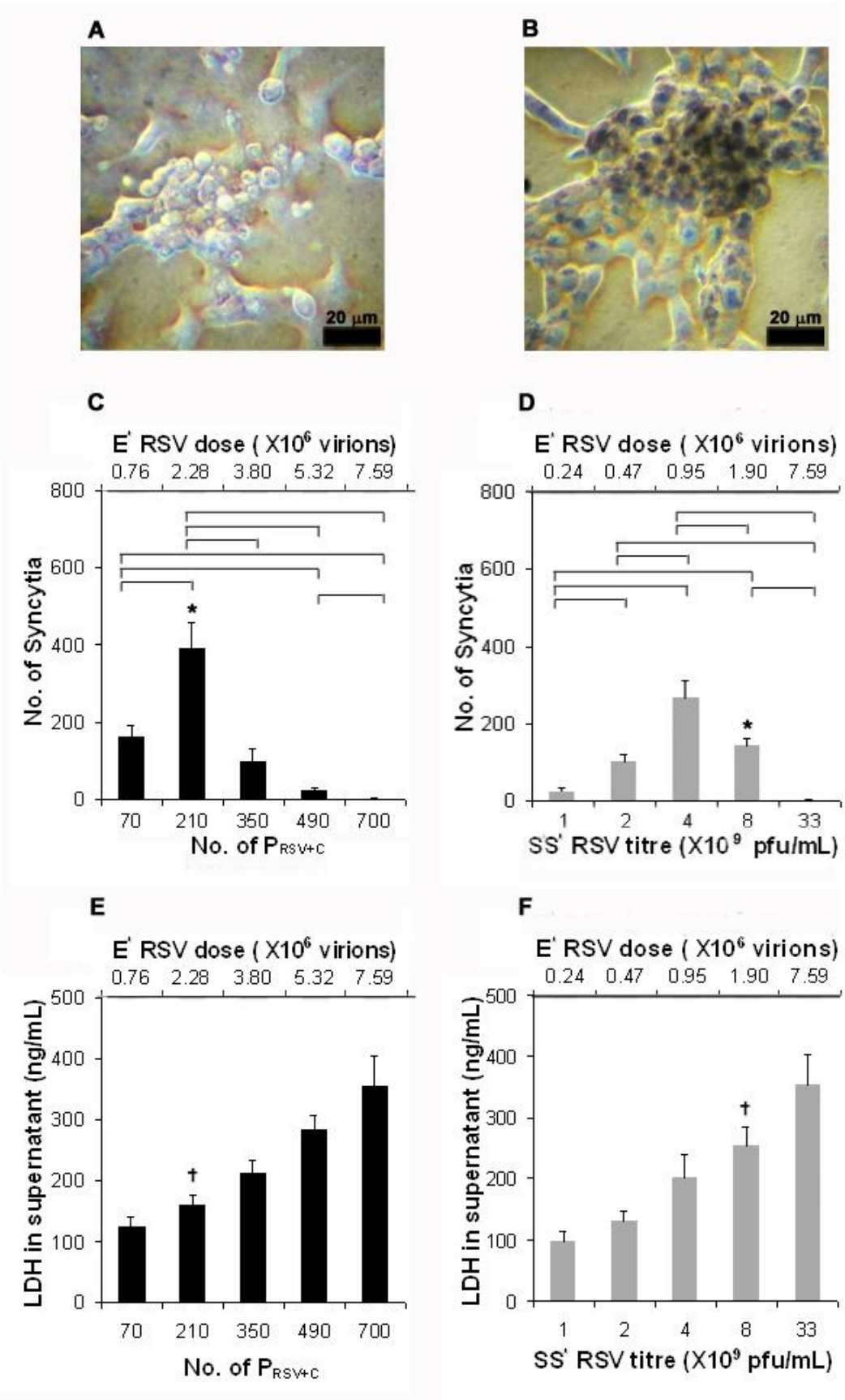
Figure 6.9 demonstrates the control that the methodology offers regarding systematic variation of the composition of the PM_{Mimics}, while preserving the infectivity of the virus. Syncytia generated by A_{RSV} were observed as being pale in color (Figure 6.9A), whereas syncytia generated by P_{C+RSV} were darker (Figure 6.9B), the latter attributable to the presence of carbon within the PM_{Mimics}. HEp-2 monolayers were dosed with either a different number of P_{C+RSV} prepared from a single virus stock (Figure 6.9 carbon and E) or 700 P_{C+RSV} prepared from four different virus stocks having various viral titres (Figure 6.9 D and F). E* RSV doses of 7.6 X 10^5 RSV virions, in the form of 70 P_{C+RSV} harboring each 109 X 10^2 virions, (Figure 6.9C) or 4.7 X 10^5 RSV virions, in the form of 700 P_{C+RSV} harboring each 1.36 X 10^3 virions, (Figure 6.9D) were sufficient for approximately 100 syncytia to have formed 96 h post-infection. The data indicated that increasing the RSV dose up to ~2.3 X 10^6 virions by increasing the number of P_{C+RSV} (Figure 6.9C) or up to ~9.5 X 10^5 virions by increasing the number of virions per PM_{Mimic} (Figure 6.9D) increases the number of syncytia observed at 96 h post-infection. The decrease in the number of syncytia observed at E* RSV doses higher than 2.3 X 10^6 virions was accompanied by an increase in cytotoxicity, as assayed by LDH activity released into the supernatant[367] (Figure 6.9 E and F), which followed a dose-response pattern and thus likely results from an aggressive cytopathic effect due to an overwhelming infection[368,369] (see Figure 6.8 to confirm that stimulus with P_C does not increase LDH activity with respect to untreated cells, and that the time dependences of LDH expression as a function of RSV dose are different depending on the delivery mode).



HEp-2 monolayers were dosed with 700 P_C (A) or various doses of RSV delivered in the form of P_{C+RSV} (B). The various E* RSV doses were achieved either by varying the number of P_{RSV+C} (solid diamonds) or by varying the amount of RSV loaded in each of 700 P_{RSV+C} (solid squares). 72 h after stimuli, the amount of LDH present in the supernatant was measured as an indicator of cytotoxicity.

Figure 6.8 Cytotoxicity resulting from various stimuli.

Of interest, is the disparity between the numbers of syncytia resulting from similar E* viral doses resulting from delivery of different PM_{Mimic}. Compare the ~400 syncytia obtained with an RSV dose of ~ 2 X 10^6 virions, in the form of 210 P_{C+RSV} where each particle harbored 1.1 X 10^4 RSV virions (Figure 6.9C), versus the ~125 syncytia obtained upon delivery of similar doses in the form of 700 P_{C+RSV} where each of these particles harbored 2.7 X10^3 RSV virions (* in Figure 6.9D)). This result demonstrates the capacity of PM to conjugate virions and augment infection of epithelial cells.


A
20 μm
B
20 μm
C
E' RSV dose (X10⁶ virions)
0.76 2.28 3.80 5.32 7.59
No. of Syncytia
800
600
400
200
0
*
70 210 350 490 700
No. of PRSV+C
D
E' RSV dose (X10⁶ virions)
0.24 0.47 0.95 1.90 7.59
No. of Syncytia
*
1 2 4 8 33
SS' RSV titre (X10⁹ pfu/mL)
E
E' RSV dose (X10⁶ virions)
0.76 2.28 3.80 5.32 7.59
LDH in supernatant (ng/mL)
500
400
300
200
100
0
†
70 210 350 490 700
No. of PRSV+C
F
E' RSV dose (X10⁶ virions)
0.24 0.47 0.95 1.90 7.59
LDH in supernatant (ng/mL)
†
1 2 4 8 33
SS' RSV titre (X10⁹ pfu/mL)

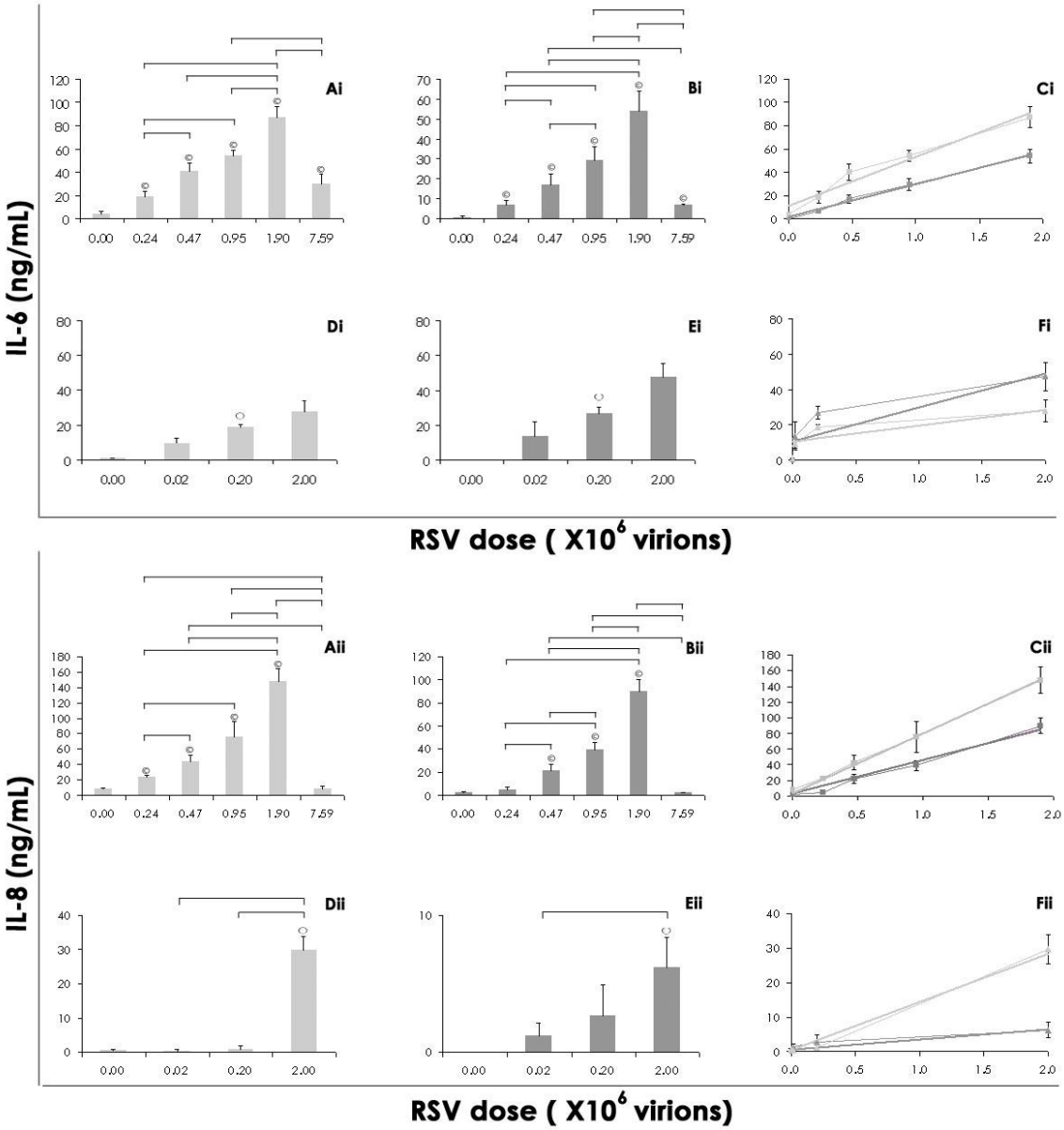
Bright field images of a syncytium formed by cells infected with A_{RSV} (A) or P_{C+RSV} (B). Panels carbon and D represent the number of syncytia observed 96 h after dosing HEp-2 monolayers with different quantities of RSV (E RSV doses in secondary X-axis, top) obtained by either varying the number of P_{C+RSV} while keeping RSV/particle constant (Panel C) or by varying RSV/particle (by utilizing different Starting Solutions (SS*) having different RSV concentrations) and keeping the number of P_{C+RSV} constant (Panel D). * Indicates a SSD between the syncytia observed upon infection with similar E* RSV doses obtained with either of the two aforementioned delivery systems of PM_{Mimics}, $p < 0.005$. Also, for each delivery system, [ ] indicates a SSD between the numbers of syncytia generated upon infection with the different RSV doses, $p < 0.05$. Panels E and F illustrate the relationship of LDH, as a measure of cytotoxicity, to infection with different E* RSV doses obtained with the two aforementioned doses. † Indicates a SSD between the level of LDH activity observed in the supernatant upon infection with similar E* RSV doses, $p < 0.05$.*

Figure 6.9 Infection of HEp-2 monolayers following exposure to P_{C+RSV}.

6.5.6 Differential Interleukin Secretion by HEp-2 cells in response to different stimuli.

Measurement of IL-6 and IL-8 concentrations in the supernatants of cell cultures incubated with P_C, P_{C+RSV} or A_{RSV} showed differential interleukin secretion upon dosage with the different stimuli. Overall, the degree of IL-8 secretion was higher than that of IL-6 in response to the same stimuli and with respect to the negative control. Cells dosed with P_{C+RSV} consistently secreted higher levels of these mediators than those secreted by cells stimulated with P_C, both at 12 and 36 hours post deposition (Panels A and B in Figure 6.10). Within both time points, a dose dependent pattern of cytokine secretion was observed when dosing the cells with 700 $\pm$ 70 P_{RSV+C} for total RSV doses ranging from $2.4X10^5 \pm 7X10^2$ to $1.9X10^6 \pm 1X10^3$ ($p < 0.05$: P_C vs P_{C+RSV} Panels A-C I in Figure 6.10). The decrease in the secretion of both cytokines upon infection with a total RSV dose of $7.6X10^6 \pm 3X10^4$ virions in the form of 700 $\pm$ 100 P_{C+RSV} ($p < 0.5$, panels A and B in Figure 6.10) is associated with an increase in cell death and thus a very low number of syncytia persisting 96 hr post infection (Figure 6.9, panels B and D). This increase in cell death is characteristic of the normal progression of RSV infection; as such,

a decrease in the secretion of IL-6 and IL-8 was observed over time, independently of the mode of infection (Panel C in Figure 6.10).



Hep-2 monolayers were dosed with 700 ± 100 PM_{Mimics}: P_C or P_{RSV+C} (panels A-C) or A_{RSV} (panels D-F). The levels of IL-6 and IL-8 secreted, 12 (A, and light gray in C) and 36 (B, and dark gray in C) hours post infection, were assayed by ELISA. ⊓ indicates a statistical significant difference in the amount of IL-6 or IL-8 secreted by the different doses of RSV ($p < 0.05$), ◎ indicates $p < 0.05$ with respect to cells dosed with 700 ± 70 P_C, and ○ $p < 0.05$ with respect to the negative control. Panels C and F show the lines modelled by a linear regression analysis on the data plotted in panels A and D as light gray lines, and in panels D and E as dark gray lines.

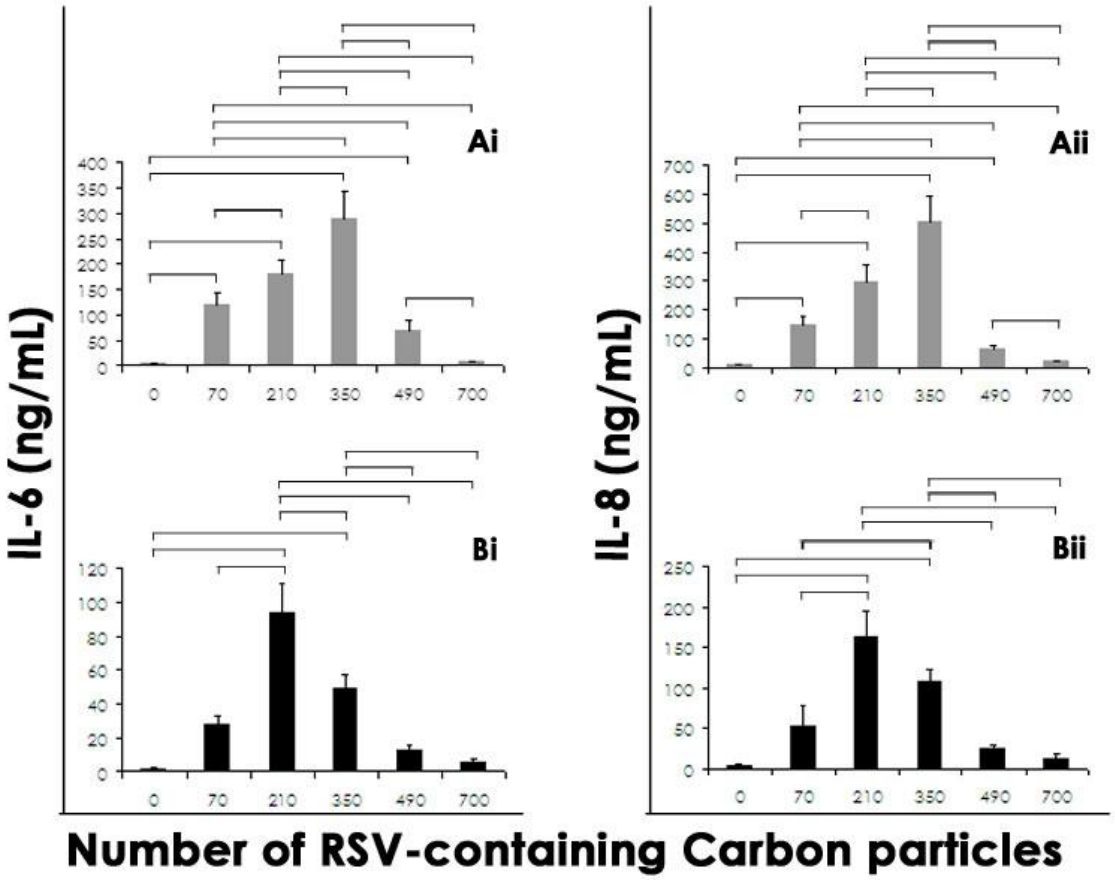
Figure 6.10 Differential secretion of IL-6 and IL-8 by Hep-2 cells upon different stimuli.

In contrast to infection with P_{C+RSV}, infection with only one A_{RSV} dose resulted in an increase in cytokine secretion with respect to the negative control: an A_{RSV} dose of 2 x 10^5 virions in the case of IL-6 and a dose of 2 x 10^6 virions in the case of IL-8 ($p < 0.05$, Panels A and B in Figure 6.10).

Infection with A_{RSV} resulted in an increase of cytokine secretion of ~ 26 X for IL-6 and ~ 63 X for IL-8 ($p < 0.001$). This cytokine response was further amplified upon exposure to P_{C+RSV}, which elicited the highest concentration of cytokines, with a synergistic increase of ~ 81 X for IL-6 and ~ 315 X for IL-8 ($p < 0.001$).

6.5.7 P_{C+RSV} Infection results in dose and time dependent secretion of ILs.

To determine whether there is a correlation with the number of syncytium observed 96 hrs post infection, viral doses between 2.0X10^6 (slightly higher than the viral dose used to obtain peak secretion of IL-6 and IL-8 when dosing the cells with P_{C+RSV}, each containing different concentrations of RSV) and 8.0X10^6 virions in the form of P_{C+RSV} were tested (Figure 6.11). Such doses were achieved by varying the number of similarly created PM_{Mimics} dosed onto Hep-2 cells, which also allows for the exploration of the possibility of synergism between RSV and carbon by increasing both carbon and viral doses simultaneously.



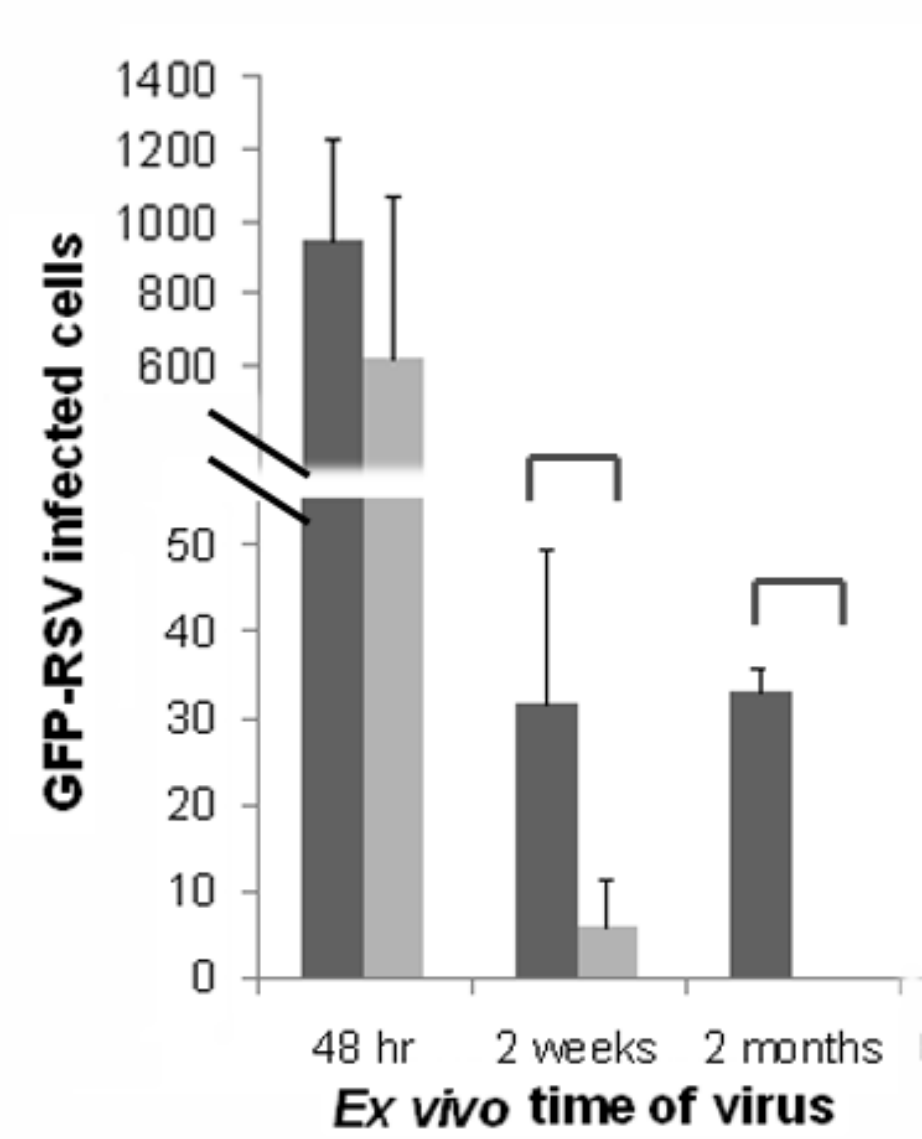
Hep-2 monolayers were dosed with different populations of P_{C+RSV}. The levels of IL-6 and IL-8 secreted by each culture, 12 (Panels A) and 36 (Panels B) hours post infection, were assayed by ELISA. ⌐¬ indicates a statistical significant difference in the amount of IL-6 or IL-8 secreted upon infection with different doses of P_{C+RSV} ($p < 0.05$).

Figure 6.11 Secretion of IL-6 and IL-8 by Hep-2 cells upon stimuli with P_{C+RSV} is time sensitive.

The highest secretion of IL-6 and IL-8 12 hrs post-deposition was observed in cells dosed with 350 ± 50 P_{C+RSV} ($p < 0.05$; panels A in Figure 6.11). At 36 hrs post-deposition, as infection progressed (and the number of cell deaths), there was a decrease in interleukin concentration in the supernatant ($p < 0.05$; Panels B in figure 5). In this trial, the maximum interleukin secretion was triggered by a smaller particle dose: 210 ± 30 P_{C+RSV}, which correlates with the maximum amount of syncytia observed 96 hrs post infection (Panels B and C in Figure 6.9).

6.5.8 Ex vivo P_{C+RSV} remain capable of infection longer than ex vivo A_{RSV}.

Figure 6.12 illustrates the infectivity of P_{C+RSV} and A_{RSV} after remaining stored dry under laboratory ambient conditions for extended periods. Results showed that while A_{RSV} was no longer infectious after 2 weeks, P_{C+RSV} remained capable of cellular infection up to at least 2 months after storage.



$A_{GFP-RSV}$ (light gray) or $P_{C+GFP-RSV}$ (dark gray) were stored dry at ambient laboratory conditions for up to 2 months and then assayed for infectivity of 1HAEo- cells. Note that the Y axis is truncated. ⌐¬ *indicates SSDs.*

Figure 6.12 *Ex vivo* RSV survival.

6.6 Discussion

Respiratory viruses are known to attach/conjugate to ambient PM [110,141,337,342]. Interaction of these viruses with ambient PM could affect their ability to infect airway epithelial cells. We have developed a method to generate mimics of virus-containing ambient PM_{10} using an AC trap. Levitation trap technology permits dry deposition of PM_{Mimics} directly onto cell cultures without compromising cell viability[355]. As such, it has been used to study cellular responses elicited by specific chemical and biological components in PM_{10}[347] and to measure differentiation in the response of the cells in direct contact with such PM_{Mimics} from the rest of the cells in the culture[348]. We have extended this methodology by generating stable conjugates of RSV and carbon particles, and dosed these P_{C+RSV} onto airway epithelial cells, while retaining RSV infectivity. With this methodology we were able to successfully infect primary AEC-ALI cultures, which had been shown to differentiate into an epithelium closely representing the epithelium of the human bronchus[307]. We further showed that P_{C+RSV} remained infectious up to two months in contrast to A_{RSV}, which lost infectivity after 2 weeks. This methodology can be used in systematic studies designed to determine particle effects in the infectivity and cytopathic effects of the virus and vice versa. The data presented in this manuscript supports this perspective in that cells responded differentially to A_{RSV} versus P_{C+RSV} versus P_C. Overall, our technology provides an *in vitro* mimic of the *in vivo* deposition of PM_{10} contaminated with respiratory viruses onto the airway epithelium.

Central to this methodology is a simple and efficient protocol to concentrate RSV without losing its infectivity[370,371], as well as a levitation

apparatus modified as to allow PM_{Mimic} deposition onto AEC-ALI. The concentration method described for the virus, based on our published protocol for purification of RSV from soluble macromolecules[200], involves the use of disposable purification columns and ultracentrifugation. The apparatus constructed for this study is a modification of the levitation apparatus previously developed in our laboratory[355], which allows for particle deposition onto a series of diverse cell-culture platforms, such as six well plates and AEC-ALI. As such, our methodology provides a unique approach to study the association of viruses with PM and how this may contribute to infection.

The PM_{Mimics} created in this study were stable entities capable of causing cellular infection. Fluorescence results indicated that RSV remained associated to the PM_{Mimics}, even after being immersed in water for three days. The final localization of the virions within the particle was not determined due to technical limitations. However, the bimodal distribution observed in the fluorescence intensity signal associated with P_{RSV+C} (Figure 6.7, panel F) indicates the possibility of the existence of two different types of P_{RSV+C}: one population consisting of particles having RSV virions available for staining with Hoechst-33342 and thus most likely on the surface, and another population depleted of RSV virions or having no virions accessible for staining due to their being isolated from the solution by being entrapped within a carbon shell. In spite of the likelihood of this possibility, the fraction of virions per particle within the core versus those on the particle surface and able to interact immediately and directly with the cell membrane is not known. It also remains unclear if it is necessary for the particles to be endocytosed first for the virions to be bio-available for infection (we

speculate that particle endocytosis does take place as suggested by the dark shade characteristic of syncytia derived from direct contact with P_{C+RSV} (see Figure 6.9, panel A); however, whether this is necessary for infection or is a byproduct remains unanswered).

Regardless of the exact localization of virus in the PM_{Mimics}, it has been found that the RSV contained by the PM_{Mimics} remains capable of infection even after remaining *ex vivo* for two months. Several studies have explored the survival of RSV *ex vivo* either as an aerosol[250], in solution[251] or on fomites[372], but none have explored the survival of RSV coupled to PM. RSV has been found to remain capable of infection up to six days at low temperatures[251]. In this study, we speculate that the longer survival period we observed for A_{RSV}, and the even longer for $P_{C+RSV,}$ was due to the formation of a protective encasing[4], salt and carbon respectively, that shielded the virus from environmental factors that would otherwise have effected its inactivation[261,337]. The finding of P_{C+RSV} being stable for a much longer period than A_{RSV} is in agreement with previous observations that particle association/attachment enhances viral survival[336,337] and may contribute to viral transmission in the community.

In addition to providing a methodology that improves the mimicry of *in vivo* events of PM inhalation and viral infection, our levitation technology provides control over the number, size[355], chemical composition and physical state of the PM_{Mimics}. This customizability of the composition and size of PM_{Mimics} to closely resemble ambient PM sampled at specific sites and in different scenarios allows for the exploration of relevant queries including the contribution of the infectious dose and aspects related to the presentation of the infectious agent

to the success of establishing infection, its extent and its impact in the cellular response. The data presented here suggest there is an interplay between the number of RSV-containing particles and the amount of the virus in PM, which determines the degree of infection as exemplified by the disparate amount of syncytia and cytotoxicity observed upon infection with similar E* RSV doses in different presentations. Specifically, our data showed that infection with an equivalent E* RSV dose in the presence of excessive carbon caused a ~50% increase in the cytotoxicity, but a ~65% decrease in the number of syncytia observed 96 h post-infection, suggesting there may be an optimal ratio of RSV to carbon for maximal infectivity with respect to P_{C+RSV}. Moreover, epithelial cell cultures exposed to different PM_{Mimics} using AC levitation responded selectively and differentially with regard to secretion of inflammatory mediators (Table 6.3). IL-6 and IL-8 concentrations were greatest from cells infected by RSV-containing PM_{Mimics} and, even though P_C dosed onto the cells elicited cytokine secretion, the levels measured were modest when compared to the response to RSV alone.

Table 6.3 Differential HEp-2 cytokine secretion 12 hr post-stimuli: Highlights from Figure 6.10.

Stimuli	**IL-6 (pg/mL)**	**IL-8 (pg/mL)**
Negative	1.08 ± 0.10	0.47 ± 0.4
P_C	3.76 ± 2.12 *†	8.04 ± 2.31 *†
P_{C+RSV}	87.4 ± 8.87 *	148 ± 17.1 *
A_{RSV}	27.9 ± 5.93 *†	29.7 ± 4.20 *†

HEp-2 monolayers were dosed with 700 P_C, or an E RSV dose of ~2 X 10^6 virions in the form of A_{RSV} or 700 P_{C+RSV}. SSDs ($p < 0.001$) with respect to the negative control and to P_{C+RSV} are indicated by * and † respectively.*

These responses were time dependent and sensitive to the chemical composition of each particle. Using regression analysis, these dose responses were modelled. The secretion of IL-6 in response to infection with P_{C+RSV} over time is described as y = 1.517 + 2.811 E-5 x + 9.373 z + 1.419 E-5 x z (Figure 6.10 panel C). Where y = IL-6 secretion in ng/mL, x = RSV dose in the form of total number of virions deposited in the form of P_{C+RSV} and z is a binary variable indicating the time point, with values of 0 for 12 hr and 1 for 36 hr. A stepwise collinearity diagnosis confirmed the validity and importance of each term in the model, which describes 94% of the variation observed in the secretion of IL-6. Similarly, the expression w = 3.348 + 4.343 E-5 x + 3.3298 E-5 xz was found to model 95% of the pattern of the secretion of IL-8 in response to deposition of P_{RSV+C} overtime (Figure 6.10 panel C). Note that in this model the z term was omitted in order to decrease multicollinearity and increase the tolerance of each term. Similarly, regression analysis for the secretion over time of IL-6 in response to infection with RSV alone returned a model that explained only 66% of the variation observed: y = 10.435 + 1.947 E-5 v –1.042 E-5 vz, where v = RSV dose in the form of total number of virions deposited in the form of droplets. The model for the secretion of IL-8, on the other hand, explained 95% of the variation detected over time and is defined as w = 0.495 + 2.890 E-6 v + 1.110 E –5 vz. Further analysis of the cellular responses to different doses of identical P_{C+RSV} indicates that, in addition to the nature of the stimulus, the dose in which the stimulus is presented also has an effect on the degree of cytokine secretion; thus confirming that there is a delicate balance between RSV infection, carbon challenge, time elapsed after exposure, and cellular response. A statistical analysis of this data indicates that

the particle dose accounts for 49% of the variation observed in IL-6 secretion ($p < 0.01$) and 59% of that observed in IL-8 secretion ($p < 0.01$), whilst the time elapsed after infection accounts for 20% and 15% of the variation observed in the secretion of IL-6 and IL-8 respectively ($p < 0.01$). The additional variation is accounted by the interaction between viral dose and time, which could be translated into syncytia formation, which as time progresses results in cell death and thus less cytokine secretion.

Further studies are needed to decipher the nature of this apparent synergistic response between PM and the virus harboured within. However, these mathematical models, in addition to confirming the effect that the different PM_{Mimics}, their chemical compositions, their numbers, and the time elapsed after infection have in the cellular response to stimuli, allow us, by quantification, to better understand the role that each of these variables has in eliciting the very fist steps of what, *in vivo*, could unfold into the immune response.

Traditionally, viral doses are expressed in terms of multiplicity of infection (MOI), which represents the number of virions that in average will infect each cell during the first round of infection. In our methodology, a small number of P_{C+RSV} is dosed onto a comparatively large cell culture, as such, expressing viral doses in terms of MOI is not appropriate for P_{C+RSV}; which necessarily presents challenges when comparing this methodology with traditional *in vitro* viral infection techniques. However, being that our model more closely resembles *in vivo* events of PM_{10} deposition onto the airway epithelium and its subsequent viral infection, we believe it is a reasonable compromise.

Epidemiological studies have reported that RSV outbreaks follow a seasonal pattern[260,261] and that various environmental factors correlate differently with RSV infection[261]. However, little is known about the occurrence of meteorological patterns that could account for regional to intercontinental atmospheric movement of the virus before, during, and after an outbreak. It has been reported that desert dust clouds can harbour microorganisms (including viruses) as they are dispersed around the globe[110]. Intercontinental transport of atmospherically dispersed airborne viruses have been implicated in the seasonal epidemiology of influenza[326] and a model of aerosol transmission of fecal fine $PM_{2.5}$ contaminated with H10N7 has been described[342]. We speculate that long-distance atmospheric transport of airborne RSV-infected PM_{10} could be relevant to the ubiquitous nature of RSV and its seasonal, region-wide infections. Further research is necessary to test this intriguing possibility; to understand the association of the virus with PM and how this may contribute to infection is but the start.

6.7 Conclusion

We present a method for generating mimics of virus-containing ambient PM_{10}. The PM_{Mimics} generated were stable and capable of infection. The results indicate that RSV survival was extended when the virus was harboured by PM_{10} and that the RSV-containing PM_{Mimics} increased the secretion of pro-inflammatory mediators (IL-6 and IL-8) by respiratory epithelial cell cultures as compared to the same dose of virus alone. By coupling enabling features of levitation traps with *in vitro* differentiated human bronchial epithelia, we have developed an *in vitro*

mimic of the *in vivo* interactions between an inhaled virus-containing ambient particle and the airway epithelium. Moreover, the methodology is amenable for use in hypothesis-testing experiments, whose scope can encompass the chemistry and biology of not only particle-viral interactions, but of ambient particle types and mixtures thereof present in distinct geographical regions with readout of the elicited responses of airway tissues incubated with those particles.

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Stephan F van Eeden is a MSFHR Senior Scholar and the GSK/CIHR Professor in Chronic Obstructive Pulmonary Disease.

7: Placing this *in vitro* mimic in the realm of research

"... the modern fertilizing process... excised ovary alive ... transferred to a porous receptacle ... immerse in a warm bullion containing free-swimming spermatozoa ...

A bokanovskified egg will bud ... From eight to ninety six buds, and every bud will grow into a perfectly formed embryo, and every embryo into a full-sized adult. Making ninety-six human beings grow where only one grew before. Progress...

Ninety-six identical twins working in ninety six identical machines! "

Aldous Huxley, A Brave World

If I could remove myself from the human condition and somehow retain the scientific mind, I would be fighting legislations and ethic boards so that the "ideal" mimic of the *in vivo* events of virus-containing PM inhalation, the subsequent infection and the host response could be in our future. This "ideal" mimic would consist of the ideal emission source, the ideal host and the ideal readouts. The ideal emission source would confer complete control to the researcher of the size, composition, presentation and sequential or parallel dosage of a variety of PM mimics. As a pre-requisite for these PM mimics to be relevant and truly representative of their real life counterparts, one would need thorough and complete understanding of ambient PM (ie, its true physical and

chemical characteristics and secondary chemical modifications once in the atmosphere) and its geographical distribution. The ideal host would require thorough knowledge of the human genome, epigenetics and frequencies of gene alleles and polymorphisms, as well as their association with diseases, so that a "racially neutral", healthy human clone population could be breadth – as well as any other population exhibiting a specific disease, or disease subtype, customized. Some of the aspects of the ideal readout have been portrayed in movies such as "Inner space". Imagine an army of nanobots, biologically inert and removed just enough as a biologist studying panthers, able to capture amazing photographic/video evidence of the cellular mechanisms that together would unfold into the host response involving the organism as a whole. Yet, capable of following the immediate molecular mechanisms, obtain biopsies and simply follow the biology of the cells in direct contact with the stimuli; then follow the "smoke signals" (i.e. hormones, lipids and proteins) produced by these cells to their receivers, both neighboring and distant cells, and the changes that, as a consequence of these signals, will then unfold on the receiver tissues, organs and even organ systems. Nanobots would also confirm how many PM mimics were inhaled by the experimental subjects and where along the airway they would deposit or follow them if they had crossed the epithelial barrier into the blood stream.

Currently, the three stages forming the *in vitro* mimics of the *in vivo* event of inhalation of virus-containing PM face various constraints. As such, one must be content with studying the events of infection before or after PM dosage onto *in vitro* systems, their infection and associated immediate cellular responses, or

the infection of experimental subjects before or after exposure to ambient PM and the elicited host response.

The aim of the following sections is to try to critically assess the *in vitro* mimic described herein as an emission source/dosing mechanism and as a host model in the context of current experimental methodologies and to compare this mimic to the "ideal" emission source and the "ideal" experimental host.

7.1 Regarding particulate matter and viral dosing mechanisms

The enigma surrounding the joint effects of ambient PM and viral infection on human health has elicited enormous interest. As such numerous protocols for its study have been developed. Most commonly, suspensions of ambient PM samples are either pipetted onto cell cultures or instilled into animal subjects, either before or after virus aliquots are similarly delivered to the experimental hosts. There are five major problems associated with these exposure methods. First, one does not breathe in liquids. Second, the physical and chemical characteristics of the PM dosed onto the experimental subjects is rendered different from those of the ambient PM sampled by the process of collection and delivery. Third, as particles tend to agglomerate in solution, the true size of the particles encountering the host remains unknown. Fourth, the chemical composition of each particle remains unknown so that it is impossible to relate an effect to any specific compound, instead any effect observed is assumed to result from the combination of compounds present in the sample. Fifth, the sequential design for the exposure to the two stimuli, does not account for the possibility of inhalation of virus-containing PM.

An improvement regarding the first limitation of the pipette/instillation technique consists in aerosolizing the solutions containing the stimuli[4], thus rendering the mimic of the stimuli more realistic as both PM and viruses are commonly found as aerosols. In spite of this improvement, this dosing approach is faced with the remaining four limitations that constrain the pipette/instillation technique.

A much more realistic dosing approach has been possible due to the development of technologies that allow for the direct exposure of experimental subjects to ambient PM sampled directly from the atmosphere or from diesel exhaust or cigarette smoke sources. To date, continuous ambient sampling and exposure has been applied only to cell cultures; however, diesel exhaust or cigarette smoke exposure chambers have been often used to dose animal subjects or human volunteers. These exposure protocols circumvent the first, second and third problems that limit the pipette/instillation technique, as the subjects are dosed with unmodified PM. However, any effects observed can still only be associated to the average chemical and physical characteristics of the PM dosed. In spite of the ability to dose with direct sources of ambient PM, these experimental protocols do require a separate step for infection with a virus stock, either before or after PM exposure, which in human volunteers is usually in the form of nasal spray, nasal drops or inhalation[373], and in animal subjects usually involves nasal, tracheal or bronchial instillation. The need to add viral infection with a stock to PM stimulus is because there is no technology capable to determine the percentage of ambient particles harboring pathogens, nor their identity or the combinations in which they might be present, as such, it is not

possible yet to design experiments to explore the effects of infection with virus-containing PM. These sequential dosing procedures still fail to account for the possibility of inhalation of virus-containing PM.

There have been several studies designed to explore the effects of particulates on virus survival in the context of soils and/or water, as such, the experimental design calls for infection with a suspension of particulates and viruses[337,338,374]. In the case of studies assessing virus survival in water, this approach seems quite valid, although it does not account for scenarios where viruses could have adhered to particles prior to entering the water[374]. However, in the studies addressing virus survival in soils, the dosage protocols might not necessarily reflect real life scenarios[337,338]. To the best of my knowledge, there has only been one group, in addition to ours, trying to address the possibility of infection with virus-containing ambient PM; in this study, Avian Influenza Virus (AIV) - coated chicken feces dust particles smaller than 2.5 μm were generated[342]. However, the investigators dosed the experimental subjects with a solution of these particles, thus modifying their physical characteristics.

The AC levitation trap technology that we use to generate virus-containing PM_{Mimics} allows the operator to control the physical state (solid or liquid), composition, size and number of PM_{Mimics} to be dry-deposited directly onto a cell culture, thus mimicking the inhalation of air in which suspended PM is encountered. This customizability of PM_{Mimics} allows us to circumvent the five limitations that faced the pipette/instilled techniques, providing the researcher with knowledge/control of the chemical composition and physical state of each particle and more importantly, providing the unique ability to dose with a

specific number of dry virus-containing PM_{Mimic} a differentiated epithelium, thus recreating the *in vivo* event of deposition onto the airway epithelium of PM harboring virus. However, being that our technology does not use continuous sampling of ambient PM, our PM_{Mimics} are limited by our understanding of ambient PM. In addition, our technology requires the operator to be more physically involved with potentially hazardous aerosols than the other methodologies. In addition to these safety concerns, our methodology requires much more practice and skill to be able to operate it with control and reproducibility.

7.2 Regarding host models / experimental subjects

Being that we have no access to the "ideal" experimental subjects, nor to the "ideal" readout / nanobots to follow the effects of each stimulus conferred onto it, the selection of host model depends on the questions needed to be answered. If the researcher wished to study the systemic effects of infection with virus-containing PM and the clinical pathology associated to it, then a whole organism would be necessary. However, if the aim of the research was to understand the virus biology, the responses of specific cells to stimuli and/or the resultant interactions among different cell types, then an *in vitro* or *ex vivo* (with or without co-culturing) system would be preferred.

As mentioned earlier, the ideal human experimental subjects would be an army of human clones. However, ethics forbid us from exploring that possibility. As such, research conducted on human experimental subjects requires a large number of human volunteers, for significant results to be attainable, as in small

sample sizes any responses to stimuli could be lost in the immense genetic and biological variability within the experimental subjects. In addition to the enormous expense this entails, it is unethical to terminate human lives in the name of science; as such, terminal experiments regarding RSV infectivity and/or the effects of PM are conducted in animal models, where the option exist of using species, which have been inbreed sufficiently as to minimize genetic variability and for which congenic, knock out and transgenic strains exist. The problems with this approach are the anatomical and physiological differences between animals and humans. In addition, to their respective limitations, the utilization of animal or human experimental subjects adds a level of uncertainty to dosage, as it is not possible to know where the stimuli deposits and whether all of it does it along the same area.

In spite of its limitations, *in vitro* platforms are excellent tools for mechanistic studies and to learn information regarding the immediate responses to stimuli. The problems faced by *in vitro* platforms are similar to those faced by whole organism studies; the researcher either chooses genetic uniformity and compromises authenticity, or chooses authenticity but is faced with the problems associated with genetic variability. Working with cell lines confers several advantages: there exists a wide variety of them so that one would be able to find a cell line originated from most body parts, many of them have been thoroughly characterized, there is low cost involved in their maintenance, they are easy to handle and, most importantly, they are clones from one another and as such are ideal for mechanistic studies. However, it is not uncommon to find that cell lines differ in several biochemical pathways and/or behaviours from

primary cells. On the other hand, primary cells are difficult to maintain, can only be passaged a very small number of times and could potentially harbour hazardous organisms; in addition, due to the immense genetic variability among humans, large numbers of primary cell sources are required for statistically significant results to be obtained. However, primary cells have not been modified in any way, so that one could expect that they would respond to stimuli much like they would in the intact organism. This is especially true when growing the cells onto ALIs, which allows their differentiation and allows the direct contact of the apical side with air in the troposphere, while providing the basal side with a nourishing liquid. To further improve on this mimic, co-culturing protocols have been designed, in which a differentiated epithelium is placed in contact with the same cells that it would be in contact within the whole organism, such as the endothelium[375] or with cells of the immune system[39].

There are several methods to measure the effects of different stimuli on experimental subjects, such as changes in lung capacity, morphometry, protein concentration in the bronchoalveolar lavage fluid and cellular counts and, once terminated, immunohistochemistry, protein and/or nucleic acid quantification at different anatomical sites. The readouts available for *in vitro* studies are similar to those available for terminal studies; however, these are usually easier to conduct in the *in vitro* scenario. Moreover, due to the localized nature of the stimuli dosed by the *in vitro* mimic presented in this thesis, it is possible to differentiate by visual means between the cellular responses of those at the site of PM_{Mimics} deposition, versus those further removed. Note that laser micro-dissection allows for isolation of tissue of interest (i.e. an airway section in direct contact with PM), so that

potentially, similar information could be obtained from terminal studies with experimental subjects; however, this requires processing enormous amounts of tissue.

7.3 Summary

The problems of air pollution and viral infections are ubiquitous having negative effects in human health that range from minor discomfort or infection, to respiratory and cardiovascular related mortality. As such, numerous research efforts have been established to understand the mechanisms of damage effected by these stimuli and how to prevent it. To realize this, various ingenious methodologies that allow for the study of the host responses to infection with PM contaminated with virus have been designed. However, the perfect mimic of these events does not exist, though several attempts have been made to develop experimental designs that allow access to answers of specific questions. The methodology presented herein provides an *in vitro* mimic that closer mirrors the *in vivo* events of infection with PM contaminated with virus, as it provides the unique ability to dry deposit virus-containing PM_{Mimics} of controlled size, chemical composition and in a controlled dose. This plasticity, unique to our methodology, allows for the exploration of scenarios which otherwise would remain untargeted.

8: Future directions

"And new Philosophy calls all in doubt, the element of fire is quite put out; the Sun is lost, and the earth, and no mans wit can well direct him where to look for it."
John Donne

The most exciting part about the development of new methodology is the number of possibilities that unfold in front of you upon its completion. At such junctures, the hardest part being deciding which mystery to explore with your new tool first! I suppose the most natural evolution of my work would be twofold, RSV virologists could suggest to explore the differences in infectivity between A_{RSV} and P_{C+RSV}, whereas physicians could opt to move from exploring the cellular response to infection with P_{C+RSV} in permissive cells to its study in primary cells. However, the control that this methodology allows us in the composition, size and number of particles created enables the exploration of many more questions of relevance to a broad audience, which could include toxicologists, virologists, health professionals and policy makers. In short, I believe that this methodology provides something for everyone…

8.1 For the adventurous microbiologist

As mentioned earlier, RSV is the prototype of the *Pneumovirus* genus[314], so one could expect that the effects of particulate carbon on RSV survival and infectivity would be representative of the genus, as viral classification is primarily based in physical, chemical and biological characteristics. Therefore, it would be desirable to move the target of investigation towards other airborne or vehicle-borne viruses possessing fundamentally different characteristics, such as naked RSV viruses, enveloped icosahedral RNA viruses (e.g. Rubella) and DNA viruses (e.g. Adenovirus) in general.

In addition to prolonging virus survival, other microbes have reportedly longer survival periods upon their association with solids; for example, laboratory studies have shown that entrapment of *lactobacillus* in calcium alginate beads enhances their survival during freezing processes[376]. In fact, viable bacteria and fungi have been recovered from ambient PM[377]. As such, it would be very interesting to see the effects of PM composition in bacterial and fungal survival and infectivity. The viability of different microbial types in a single particle, under different environmental conditions could also be assessed.

8.2 For the toxicologist and the virologist

It has been suggested that the most toxic components in PM are metals; in particular, chromium, copper, iron, vanadium and zinc as they are known to generate reactive oxygen species in tissue[163]. Studies with pure metals done at atmospherically relevant concentrations, have shown no effect; however, when the metals are dosed as part of a complex mix, strong cellular responses have

been observed[163]. As such, it would be interesting to systematically explore the effect of each metal and their combinations on the infectivity of RSV on primary cell cultures by creating P_{X+RSV} PM_{Mimics} (where X could or could not include carbon).

Such a study on the interaction of metals and RSV, its effects in RSV infection and in the cellular response, could be expanded into a mechanistic study. It is well known that an important mechanism by which metals induce lung injury is the catalysis of oxygen derived free radicals[163]. As such, it would be desirable to look at the differences in the cellular response when dosing the cells prior to exposure with a hydroxyl radical scavenger, such as mannitol, or an antioxidant, such as glutathione or superoxide dismutase[163]. Note that the hypoxic environment of cultured airway epithelial cells could underestimate the importance of oxygen-derived free radical cellular damage, as the partial pressure of Oxygen in cultured cells will be much lower than its *in vivo* counterpart[189]. As such, it would be beneficial to further develop the methodology described in this thesis to perform experiments in an oxygen rich environment.

Interestingly, the oxidative stress attributed to diesel exhaust particles exposure has been found to increase influenza infectivity[378]. It seems reasonable to revisit this finding in the context of infection with PM_{Mimics} consisting of diesel exhaust particles and influenza virus (P_{DE+Flu}) and P_{DE+RSV}. Specially, taking into consideration that it has been found that ICAM-1 expression increases in response to diesel exhaust exposure[379] and that certain PM_{Mimics} compositions

induce ICAM-1 expression in a dose response manner[347,348], as ICAM-1 is reportedly an invasion site for rhinovirus[379] and RSV[380].

Reportedly, diesel exhaust particles not only enhances influenza infectivity, but also the cellular response against it. Pre-infection exposure to diesel exhaust particles results in an enhanced inflammatory response to influenza. This is thought to occur by inducing an increase in expression of the toll-like receptor 3 (TLR3), which is a pattern recognition receptor involved in innate immunity against viral replication[160]. Unpublished results from our laboratory, suggests that exposure to PM_{Mimics} containing endotoxin results in the recruitment of intracellular TLR4 to the site of particle deposition. As such, it would be of interest to explore the effects of infection with P_{C+RSV} in TLR3 recruitment.

8.3 For the respirologist

During the development of this methodology, I touched only on a very small portion of the airway epithelium, that lining the bronchi and that lining the alveoli. While the cellular responses of these two areas to P_{C+RSV} infection remains uncharacterised –as the focus of the study was to develop a new tool that could later be used towards hypothesis-driven research-- the rest of the airways remain untouched. Being that viral respiratory infections constitute a huge expense both in economic terms and loss of productivity, it seems essential to explore the cellular responses to infection with virus-containing PM by all the areas of the respiratory tract. This could be facilitated by the use of co-culturing experiments to investigate the interactions among the different airway epithelial cells in

response to P_{C+V} (where V can be any respiratory virus or microbe in general) stimuli; this could be further extended to study epithelium-endothelium or epithelium-macrophage interactions in response to P_{C+V} infection.

Once the epithelial response to P_{C+V} infection was fully characterized, it could be compared to responses to the same stimuli from atopic, asthmatic or chronic obstructive pulmonary diseased epithelium. An understanding of the health effects of P_{C+V} infection in such diseases would prove extremely beneficial as it is known that both, PM and viral infections independently, are major contributors to their morbidity and mortality.

In addition to respirology, the ability to create virus containing-PM_{Mimics} will prove beneficial to other medical professionals in that it will enable study of the mechanisms of dermal and open wound infections[7], as well as surgery related infections due to laser-plume generated carbonized virus[341].

8.4 For …

It seems a recurrent theme in human history… the most amazing discoveries happening when someone with imagination takes something old, looks at it with a new eye and transforms it into something amazing!

I invite you to take the methodology presented, make it yours and let your imagination do the rest ... just remember …

"in the field of observation, chance favours only the prepared mind"

Louis Pasteur.

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