The Role of Respiratory Viral Infection and Extracellular DNA in Allergic Sensitisation

by

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BSc Biomedical Science and Molecular Biology

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Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Danielle Suzanne Lawler
Abstract

The correlation between allergic disease and viral infections has been well established. While respiratory viral infections are strongly correlated with the development of allergic asthma, it is not known precisely how viral infection may produce, or alternatively protect, against allergic disease. Furthermore, little is known about the impact of viral infection on IgE sensitisation, due to conflicting and limited research. Recently, neutrophil extracellular traps (NETs) have been observed in viral infections and shown to induce a Th2 response. We aim to explore the link between virus infection and allergy and test if the link is mediated through NETs. We hypothesise that NETs are induced during viral respiratory infections, but that they do not adequately control the viral disease. Instead, they contribute to the development of allergic disease, and their removal will be beneficial to patients.

This hypothesis was addressed by sensitising rats to ovalbumin (OVA), during a respiratory viral infection and re-exposing the animals to OVA one week after sensitisation. To test the involvement of NETs, DNase-I was administered during viral infection to degrade NETs. OVA-specific IgE serum levels, along with cellular infiltrates into the airways, were compared between groups to assess allergic sensitisation before and after OVA re-exposure.

Although no effect on IgE sensitisation was observed with viral infection, DNase treatment reduced the risk of IgE sensitisation and increased T regulatory cell (Treg) proportions in the airways, which also displayed higher levels of FoxP3. We did not
observe a difference between any of the groups in respect to allergic recall response suggesting that the achieved sensitisation was not sufficient to induce clinical disease. Our findings nevertheless suggest that DNase treatment induces a regulatory response, which may protect against allergic disease. Future studies should explore this immunoregulatory response, as a novel strategy for allergy protection.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADLNs</td>
<td>Airway draining lymph nodes</td>
</tr>
<tr>
<td>Alum</td>
<td>Aluminium hydroxide adjuvant</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BN</td>
<td>Brown Norway rat strain</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribosenuclease- I, Pulmozyme</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>HRV</td>
<td>Human rhinovirus</td>
</tr>
<tr>
<td>GKN</td>
<td>Glucose phosphate buffer (2.1 buffers and solutions)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>mfi</td>
<td>Mean fluorescence intensity (a measure of cell marker expression)</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular trap</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>OVA-TR</td>
<td>Ovalbumin-Texas red (fluorescently tagged ovalbumin protein)</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated-danger molecule</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline <em>(2.1 buffers and solutions)</em></td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>Tfh</td>
<td>T follicular helper cell</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell/response</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>vMC0</td>
<td>Viral strain of Mengovirus, with Poly (C) length 0</td>
</tr>
</tbody>
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1. INTRODUCTION

1.1. Overview

There is a well-established link between respiratory viral infections and allergic disease. In particular, respiratory viral infections are strongly correlated with the development of allergic asthma, but it is not well known how infection alters the processes involved in IgE sensitisation. More recently, neutrophil extracellular traps (NETs) have been observed in viral infections and shown to induce Th2 immune cells, which are crucial to allergy development. These findings may explain the link between viral infection and allergic disease. The following review aims to collate the research surrounding virus and allergy development, with a focus on the role of neutrophil extracellular traps, or extracellular DNA, produced during respiratory viral infections.

1.2. Respiratory system

Every single minute a healthy human inhales 6 litres of air (1). This air contains the oxygen your body requires to carry out vital life processes, including the conversion of food into energy (respiration). The conducting portion of the respiratory system, including the nasal cavity, pharynx, larynx, trachea, bronchi and bronchioles, create a continuous passageway towards the respiratory zone. While in this conducting section, air is warmed, humidified and partially cleansed of particle matter, to protect the delicate respiratory tissues. The air then continues through a series of smaller and smaller bronchioles, until it reaches the respiratory bronchioles, and finally the alveoli. These alveoli have incredibly thin respiratory membranes, designed to facilitate gas exchange between the alveolus and surrounding pulmonary capillaries. (2).
Given that 6 litres of air pass through the lungs each minute, or approximately 9000 litres per day, the lungs are constantly exposed to a vast array of microbes, allergens and particulate matter. To combat these pathogens, both innate and adaptive immune cells are present in the respiratory system. The innate system provides rapid, first-line defence against pathogens and is present from birth. Natural barriers, such as epithelial and mucus layers, are components of this system. Immune cells, such as macrophages, granulocytes, dendritic cells (DCs) and natural killer (NK) cells are also part of the innate immune system and will be discussed in more detail later. Cells from this leg of the immune system initiate their defence roles through pattern recognition. They respond to microbial structures termed pathogen-associated molecular patterns (PAMPs), via pattern recognition receptors (PRRs). The innate response is incredibly rapid (minutes to hours) but is somewhat inefficient and unspecific when compared to the adaptive system. The adaptive system is triggered by cells of the innate system and takes days or weeks to develop. However, the benefit of the adaptive system is that protection is more efficient and long-lived, due to the production of clonally expanded, highly specific lymphocytes and soluble immunoglobulins (Ig), that reside in the bone marrow and affected tissues for up to several years (3).

The difficult job for the respiratory immune system is to distinguish the large amounts of highly immunogenic, but predominately harmless antigens, from the small amounts of pathogens requiring efficient clearance. If every single antigen presented evoked a strong, adaptive immune response, the detrimental effects from chronic airway
inflammation would be devastating (4). Hence, the immune system in the airways must be tightly controlled and regulated, to prevent and repair associated tissue damage.

This tightly controlled regulation occurs as a series of tiered, inter-related pathways, with multiple feedback and inhibition pathways. In general, signals are recognised by sensor cells, which can then exhibit direct pathogen clearance, directly recruit neighbouring effector cells, or trigger a tiered, cascade of intracellular events. Multiple innate immune cells can act as sensor cells, including epithelial cells, macrophages, DCs and mast cells. There is also a multitude of effector responses that may occur, involving both innate and adaptive responses (5). Due to the complexity of the potential immune responses possible in the respiratory system, the following sections will focus on how particular cell types change from steady state functions during a viral infection.

1.3. Innate immunity in the respiratory system

1.3.1. Epithelial cell barrier functions

The airway epithelial cells line the airways to provide a chemical, physical, and immunological barrier, as well as allowing for gas exchange in the respiratory regions. The epithelium acts as a barrier between the external and internal environment, by three general mechanisms. Firstly, the polarised epithelium is selectively permeable to ions and macromolecules, through receptors, transporters and tight junctions. In normal epithelial tissues, cell-cell junctions, namely intracellular tight junctions, prevent inhaled pathogens from entering and injuring the airways. Secondly, antimicrobial secretions, such as host defence proteins, help to destroy inhaled pathogens. Thirdly,
mucociliary escalator acts to trap and remove inhaled particle matter from the airways. (1, 5-9).

The upper portion of the conducting region is lined with pseudostratified epithelium; including ciliated, secretory and basal cells. Most cells in the conducting region are ciliated cells (over 50%), with approximately 200-300 cilia on its luminal surface. Immediately below these cilia are many mitochondria, which provide energy for ciliary beating. The other main cell type present in the conducting regions are secretory cells, which secrete mucus components into the airways. Mucus functions to trap inhaled particles and pathogens and prevent damage to the respiratory membranes. This mucus is then beaten away from the respiratory zones, up towards the pharynx, by cilia. Mucus production is highly regulated, as over-production may block the airways and impair mucociliary clearance, while under-production could allow foreign particles to damage the delicate respiratory tissues (1, 10, 11).

Along with this mucociliary escalator, the epithelium prevents pathogen invasion through antimicrobial secretions. Epithelial cells secrete an array of products into the airway lumen to destroy inhaled pathogens. Enzymes, protease inhibitors, oxidants, and antimicrobial peptides are a few examples of such substances (1, 9).

1.3.2. Epithelium during inflammation/ diseased epithelium

The early response to a respiratory infection can cascade down many different, interrelated pathways. Generally, these can be described as direct pathogen clearance,
direct effector cell response or a multi-tiered, multi-cellular response (5). The epithelium houses an array of resident immune cells, including DCs, macrophages, and some T and B cells (12) which act to sense and elicit responses to pathogen signals.

Along with barrier functions described above, epithelial cells also help to recognise pathogens by expressing pathogen recognition receptors (PRRs). They can then trigger an immune response through the release of an array of mediators, including; thymic stromal lymphopoitin (TSLP), interleukin (IL)-1b, IL-4, IL-3, IL-6, IL-25, IL-33, CXCL8, GM-CSF, and thus act to dictate the initial response to infectious stimuli by the respiratory, immune system (6, 9, 13, 14).

The subsequent response to infectious stimuli is responsible for efficiently clearing sensed pathogens. However, it is critical that proinflammatory signals are tightly controlled, as excessive inflammation can very quickly lead to detrimental effects. Hence, regulator components are a crucial part of the immune response (9). During the steady state, airway epithelial cells secrete regulatory cytokines, such as IL-10, to prevent the activation of resident immune cells. Disruption of this regulation, such as during a viral infection, indicates that the epithelial barrier has been damaged and acts as an activation signal for downstream anti-viral events (15).

During inflammation host factors, such as interferons (IFN) and transforming necrosis factor alpha (TNF-α), can disrupt tight junctions between epithelial cells, increasing permeability for immune cell infiltration. However, persistantly increased permeability
can allow unwanted allergens and pathogens passage through the epithelium. This can then further disrupt barrier functions and result in disrupted epithelial differentiation and repair processes. Downregulation of E-cadherin can lead to degradation of tight junctions, increasing allergen access to submucosal regions, stimulating a Th2 mediated response (6, 8, 14).

Furthermore, during airway inflammation, mucus production is often increased. Inflammatory mediators, such as TNF-α, IL-9, IL-13 and neutrophil elastase, as well as growth factors, allergens and microbe products, can stimulate mucus hypersecretion (1, 16-20). In addition to obstructing the airways, defective mucociliary clearance can result in recurrent and persistent respiratory infections, when pathogens fail to be cleared from the airways (1). This is evident in cystic fibrosis and common obstructive pulmonary disorder (COPD) patients (21, 22) Persistent infections then further drive the system away from homeostasis, by promoting an inflammatory response, which may further damage the epithelial layer (1).

1.3.3. Macrophages and dendritic cells

To allow for efficient clearing of potentially harmful microbes, the respiratory system houses many local immune cells capable of rapidly responding to the invading microorganism. Arguably one of the most important of these cells are alveolar macrophages and DCs. Alveolar macrophages travel round the airway spaces, while resident DCs are localised to the epithelial layer, with protrusions reaching into the airways to sample passing antigen. During inflammation, these cells can directly clear sensed pathogens via phagocytosis, produce cytokines to rapidly recruit effector cells,
or process and present antigens to the adaptive immune system. Importantly, this includes the ability to present antigen to antigen-specific memory CD4+ T cells in the lung and lymph nodes, allowing for more rapid adaptive responses. They also play an important role in the steady-state clearing of cellular debris and preventing aberrant inflammation (3, 15).

1.3.3.1. Macrophages

Resident tissue macrophages are believed to be derived from monocytes circulating in the blood and from GM-CSF induced local precursor proliferation. Alveolar macrophages are long-lived cells, with a turnover of approximately 40% in a year. They can display a high degree of heterogeneity, both between and within tissues (15, 23). Alveolar macrophages produce immunosuppressive prostaglandins and TGF-β, which suppress T cell activation. They may also enhance Treg development, by secreting TGF-β and retinoic acid (15).

Resident tissue macrophages can be broadly categorised into pro-inflammatory/classically activated (M1) and anti-inflammatory/alternatively activated (M2) macrophages. M1 macrophages appear to be IL-4 producing, while M2 appear to produce IL-13. There is a great deal of debate surrounding whether macrophages in healthy lung tissue are M1 or M2 in nature (15, 24).

In the steady state, epithelial cells produce cytokines and cell receptors to negatively regulate alveolar macrophages, such as IL-10, TGF-β and CD200. During the steady state,
these macrophages remain inactivated and mainly function to clear dead cells and debris by phagocytosis. They are quite poor at presenting antigen and have decreased phagocytotic activity, although they are capable of transporting antigen to airway draining lymph nodes (ADLN). They also produce regulatory cytokines themselves, such as TGF-β, which acts to suppress T cell activation (15).

A lack of these inhibitory signals, due to disrupted epithelial cell function, results in macrophage activation. Macrophages can also be directly activated by other activation signals. Examples of these include PAMPs binding to PRRs, or inflammatory cytokines (TNF, IL-1b) binding to their receptors. Activated macrophages have greater phagocytotic ability, become better at presenting antigen, and produce high amounts of inflammatory cytokines (15). Furthermore, pro-inflammatory conditions in the airways can result in recruitment of inflammatory monocytes, (or CCR2+Ly6C+ monocytes) which can then differentiate into macrophages (24). These recruited monocytes produce cytokines, such as type I IFNs, TNF and IL-6, which further enhance the recruitment of inflammatory cells (15).

1.3.3.2. Dendritic cells

DCs have the important role of continually “surveying” antigen-exposed sites and deciding on an immunogenic or tolerant response. Even in the steady state, DCs/monocyte precursors are constantly transporting antigen to ADLN and recruited back to the tissue (25). Failure to decide on the correct response could delay the inflammatory response to a pathogen or result in allergic disease (26).
Steady state, sentient DCs are unable to effectively present antigen to T cells, despite constantly migrating to ADLNs. However, co-exposure to antigens with PAMPs/danger-associated molecular patterns (DAMPs) results in DC activation. DCs then migrate to ADLNs to interact with T cells, where they play an important role in T cell activation (26) (*see adaptive immune section*).

Following activation, DCs display large amounts of processed antigen with MHC cell surface molecules, in conjunction with T cell co-stimulatory factors locally in the airways, where they activate memory lymphocytes. In this state, DCs are stronger T cell stimulators than other antigen presenting cells, including macrophages and B cells. Furthermore, inflammatory signals result in a rapid increase in pro-inflammatory DC recruitment, further driving the inflammatory process. (26).

Dendritic cells can be subdivided into plasmacytoid DCs (pDCs) and conventional DCs (cDCs). Broadly, pDCs are important for their early response to viral infections, due to their ability to produce large amounts of IFN-α/β. In rats, pDCs can be classified by their expression of MHC class II, CD172 expression and lack of CD11b and CD103 expression. Conventional DCs also express MHC class II, but at a lower level than pDCs. Conventional DCs further express CD11b with varied expression of CD103, CD172 and CD4. Both are found in steady-state airways, however, pDCs make up a very small proportion of the total airway mucosal DCs (27).
1.3.4. Granulocytes

1.3.4.1. Granulocyte recruitment

Granulocytes are not typically found within the alveolar spaces in the steady state; instead, they are rapidly recruited to the tissue in response to inflammatory signals, predominantly expressed by epithelial cells.

Granulocyte recruitment begins with changes to the endothelium, due to inflammatory mediators. Endothelial cells increase expression of cellular adhesion molecules, such as P-selectin and E-selectin. These selectins then bind to their ligands and work to tether free-flowing neutrophils on the endothelial surface. Similarly, eosinophils are drawn to endothelial cells by multiple chemoattractant molecules (such as IL-5, IL-13, GM-CSF and RANTES), and bind to the endothelial surface via cell adhesion molecules. Granulocytes then roll along the endothelial surface via these adhesion molecules, in the direction of blood flow. While rolling, granulocytes are exposed to several chemokines needed to induce activation. CXCL8, CXCL1, CXCL2 and CXCL5 are important examples of these. The granulocyte then adheres to the endothelial surface and begins to crawl towards a chemical gradient. They then transmigrate out from the blood vessel, either travelling paracellularly or transcellularly. Paracellular transmigration occurs most frequently, at endothelial cell-cell junctions (28-30).

1.3.4.2. Neutrophils

Once neutrophils have reached their recruitment site, neutrophils have several effector functions to aid in eliminating the threat. They are most commonly known for their efficient phagocytosis, where infected cells are engulfed and destroyed by reactive
oxygen species (ROS). Neutrophils also degranulate to release toxic granule proteins, such as myeloperoxidase (MPO), elastase and defensins (31). Finally, they can release a net-like structure composed of chromatin and granule proteins to entrap and destroy pathogens (3, 32).

1.3.4.2.1. Neutrophil extracellular traps

Neutrophils are capable of releasing a net-like structure composed of chromatin and granule proteins, thought to assist in defence by entrapping and destroying pathogens (32). Since the discovery of these neutrophil extracellular traps (NETs) in 2004, they have been explored in bacterial, fungal and viral infection studies (33-36). There seems to be no single trigger that leads to their formation, but rather a wide variety of signals. TLR-ligands, complement, and activated platelets are examples of these (37). Viral particles can also trigger their formation via PRR, as can secondary signals from infected cells, such as GM-CSF (38). This ability to be induced via secondary signals may be particularly useful in viral infections, by allowing the immune system to recognise virus that may otherwise remain “hidden” inside infected host cells. It also aids in recognition of multiple, changing viral surface particles.

NET formation appears to be related to the production of reactive oxygen species (ROS) by NADPH oxidase, as disruption of this system prevents their formation (34, 39). The production of ROS by NADPH oxidase leads to the breakdown of granule membranes, and the granule contents are translocated to the nucleus. Peptidyl arginine deiminase (PAD4) and elastase trigger histone modifications, causing chromatin de-condensation. PAD4 appears to be another essential part of the NETosis pathway, as PAD4 deficient
mice are unable to produce NETs (40). The nuclear membrane then breaks down, and granule proteins associate with DNA. Finally, the plasma membrane bursts, leaking the contents into the extracellular space (41). This process is distinctly different from other forms of cell death (34) and may not always involve the death of the cell (42, 43).

1.3.4.3. Eosinophils

Eosinophils can be recruited by many interacting molecules and pathways, including C5a, platelet-activating factor, IL-2, IL-3, IL-5 and GM-CSF, many of which are released by epithelial cells. They are involved in defence against helminths and play a role in allergic disease. In response to mediators, such as histamine, they degranulate to release ROS, cytokines, enzymes, growth factors and granule proteins (3, 29). An increased number of eosinophils in the blood (eosinophilia) is a clinical marker for many diseases. In allergic pathologies, eosinophil titres often correlate with severity (44, 45). Excessive numbers of eosinophils can lead to tissue damage from cytotoxic granule proteins.

1.3.5. Other innate cells

Innate lymphoid cells (ILCs), are lymphoid- like cells that assist with regulating tissue homeostasis and inflammation. Group 1 ILC lineage includes cells that produce type one cytokines, most notably interferon gamma (IFN-γ) and tumour necrosis factor (TNF) NK cells are a component of this group (46, 47) NK cells recognise cells lacking MHC class I and cause cell death. They play a role in the early viral response, prior to CD8+ T cell activation and expansion (see adaptive section). Group 2 ILCs produce IL-5 and IL-13. They are thought to contribute to allergic pathologies, by producing these Th2 type
Furthermore, group 2 ILC populations may increase in allergic asthma models (47, 48). A third group of ILCs has also been defined, that are dependent on RORγ, and secrete IL-12 and IL-22 (46, 47).

1.4. Adaptive immunity in the respiratory system

In the steady state, to limit excessive inflammation, only a small number of adaptive immune cells are present in the lungs, namely tissue-resident memory T cells and regulatory T cells. Below, describes the processes of lymphocyte activation and the different subtypes involved in an inflammatory or regulatory response.

Lymphocytes are produced in the thymus and bone marrow. During development, selection and re-combination processes yield immature lymphocytes, capable of responding to antigens. During selection, the majority of cells which respond to self-antigen are destroyed or inactivated, yet, a small number escape this process, giving rise to autoimmune disorders (3).

1.4.1. T cell activation

T cells are unable to bind to antigen directly, instead, they respond to MHC bound antigen peptides, displayed by antigen presenting cells. To recognise these antigen peptides, T cells express antigen-specific T cell receptors (TCRs), in a complex with CD3. CD3 is a signal transduction molecule and plays an important role in the early phases of T cell activation. The interaction between the MHC bound antigen peptide on the
antigen presenting cell, and the TCR-CD3 complex (TCR complex) expressed on the T cell, is the first signal required for T cell activation (*Figure 1.1*) (3).

TCRs are also expressed in association with CD4 or CD8 co-receptors. The CD4 or CD8 co-receptor is involved in the first activation signal (*Figure 1.1*). CD4 binds with MHC class II bound on APCs and in general, used to present extracellular antigens, while CD8 binds with MHC I, expressed on all nucleated cells, including APCs and in general used to present intracellular antigens. Expression of CD4 or CD8 distinguishes T cells into two subset populations; CD4+ and CD8+ T cells (3).

T cell activation also requires co-stimulatory pairs, to enhance the signal delivered to the TCR complex (*Figure 1.1*). There is an extensive number of such co-stimulatory pairs, such as B7 with CD28, CD40 with CD40L, and ICAM-1 with LFA-1 (3, 49).

Adhesion molecules also assist in T cell activation, by slowing the movement of T cells near APCs, allowing more time for presentation in the ADLN. Examples of such molecules expressed on T cells include CD2 and ICAM-3 (3, 49).
Figure 1.1. *T cell activation by antigen presenting cells.* T cell activation requires two signals; one is provided by antigen bound to MHC, in conjunction with a co-receptor, and the second by co-stimulatory pairs. Adapted from Figure 11.1, Coico R, Sunshine G. Immunology: a short course: John Wiley & Sons; 2015 (3).

Following these initial activation signals, a series of intracellular signal cascades occur, transmitting the signal through the cytoplasm and into the nucleus. IL-2 and IL-2 receptor (CD25/IL-2Rα forms part of this receptor complex) synthesis increases, leading to rapid T cell expansion. Towards the end of this expansion phase, activated T cells differentiate into effector and memory T cells (3).

1.4.2. T cell effector functions

As previously mentioned, T cells can be broadly categorised by their expression of CD4 or CD8. The main function of CD4+ T cells is to produce cytokines. Hence, they are involved in influencing immune responses and are also often called “T helper” (Th) cells. CD8 T cells are predominately involved in killing infected cells and are often called “killer T cells” or “cytotoxic T cells”.

1.4.2.1. CD4+ T cells

CD4+ T cells can then be further subdivided according to the cytokines they synthesise once activated. The main subsets include T regulatory (Treg) and T effector cells, which can further be classed as Th1 or Th2 cells. However, there is some discussion regarding the simplicity this categorisation implies. Response to pathogens and the subsequent cytokines produced are likely to have considerable overlap.

1.4.2.1.1. Th1

An increase in Th1 type cells is often seen in response to viral infection. Th1 cells develop in the presence of IL-12, which is produced by DCs, NK cells, and other innate cells as an early response to intracellular infections. Th1 cells synthesise IFN-γ and IL-12, which promote activation of macrophages, CD8+ T cells and NK cells (3).

1.4.2.1.2. Th2

Th2 type cells develop in the presence of helminth infections, and in response to allergens. Furthermore, the airways are typically more skewed towards a Th2 response from birth (SO). Th2 cells typically synthesise IL-4, IL-5 and IL-13. IL-4 production further drives the production of more Th2 cells. It also induces the formation of T follicular helper (Tfh) which continue to produce IL-4 and IL-13. Both influence B cell class switching to IgE isotypes, which will be explained in more detail below. IL-5 is involved in activating eosinophils (3, 45).
1.4.2.1.3. T regulatory cells

Tregs play an important role in suppressing unwanted immune activation and limiting the expansion of activated lymphocytes. This is incredibly important for preventing excessive inflammation and autoimmune disorders. They can be categorised into natural Tregs (nTregs), which are involved in maintaining peripheral tolerance and limiting chronic inflammation, and induced Tregs (iTregs), which are recruited during adaptive responses to limit inflammation. Tregs predominantly exert their suppressive effects with TGF-β and IL-10 signalling molecules (51).

An important distinguishing marker for Tregs is the transcription factor forkhead box P3 (FoxP3), as FoxP3 has a critical role in Treg function (52). Foxp3 was first identified as a defective gene in Scurfy mice, who harbour a hyperactive CD4+ T cell disorder (53) prompting researchers to explore its role in Treg function. FoxP3 has since been shown to be a critical component for Treg differentiation from α/β TCR-positive T cells in the thymus. Transduction of Foxp3 into CD4+CD25− T cells can convert them into CD4+CD25+ Treg-like cells, which display immune suppressive properties (54). Transduction of Foxp3 into naïve T cells up-regulates some Treg cell-surface molecules, including CD25, and down-regulates production of IL-2, IFN-γ, and IL-4. Foxp3 deficient mice have fewer CD4+CD25+ Tregs. Furthermore, treating Scurfy mice with CD4+CD25+ Tregs from normal mice prevents CD4+ T cell-mediated inflammation (55). Foxp3 overexpression increases the amount of CD4+CD25+ T cells, and also results in CD4+CD25− and CD4−CD8+ T cells displaying suppressive activity (56).
IL-2 is another molecule critical to Treg function. CD25 forms part of this receptor (ILRα) and is used as a marker for cell activation. IL-2 has multiple effects on multiple target cells, including aiding the differentiation and expansion of CD4+ T cells, CD8+ T cells and NK cells. (57, 58). IL-2 maintains FoxP3+ Treg populations, by facilitating their differentiation (59) or acting as a Treg growth factor (57). Evidently, CD25 deficiencies are associated with autoimmune and allergic disorders (60-63).

Contrary to other thymic T cells, functionally mature Tregs cells can be found in the thymus, prior to encountering antigen in peripheral lymphatic tissues. These Tregs are involved in suppressing naïve T cells from recognising self-antigens (58, 64). Naïve T cells in the periphery are also capable of developing into Treg cells, under a number of stimuli, including IL-2 (59). However, the functional capabilities of these induced Tregs are still unclear (58).

FoxP3+ Tregs specific for tissue self-antigens are found in regional lymph nodes (65) Tregs also display a number of receptors for homing molecules, such as CCR7, to localise Treg functions to particular areas (58).

Following antigenic stimulation, Tregs downregulate an anti-apoptotic protein, Bcl-2 (66). This indicates that cell death helps to maintain Treg homeostasis, much like other activated T cell populations. However, it still remains to be seen whether some of these activated Treg cells differentiate into memory-type cells (58).
Tregs suppress both the proliferation of naïve T cells and their differentiation into effector cell subtypes. They also suppress the effector functions of CD4+ T cells, CD8+ T cells, NK cells, B cells, macrophages and DCs. Tregs suppressive functions are believed to occur via cell-cell contact with DCs downregulating DC functions, sequentially downregulating T cell activation. (58, 67-70).

1.4.2.2. CD8+ T cells

CD8+ T cells can be activated by APCs, or by APCs and CD4+ T cells, leading to rapid clonal expansion. In the case of virus infection, activated CD8+ T cells attach to infected cells and activate apoptotic pathways. These CD8+ T cells recognise infected cells when they express both the viral antigen and MHC class I molecule seen during their activation (3, 71).

1.4.3. Memory T cells

As previously explained, activation of T cells leads to clonal expansion and antigen-specific T cells. Following the elimination of the pathogen, most of these cells are eliminated. However, some of these cells survive, sometimes for years, as memory T cells. These memory cells allow for a more rapid and effective response if there is a re-exposure to the specific antigen (3, 71, 72).

1.4.4. B cells and antibody production

Naïve B cells circulate through the blood to secondary lymphoid tissues, such as the ADLNs. Here, naïve B cells are activated by interacting with antigen and TfH in germinal
centres. Once activated, B cells can differentiate into antibody-secreting plasma cells in the germinal centre of lymph nodes. These cells synthesise and secrete a specific antibody isotype, specific for a given antigen. These plasma cells also express CD27, which allows them to be distinguished from other B cell stages (3, 73).

The potential antibody (immunoglobulin, Ig) isotypes are IgM, IgG, IgA or IgE. IgG, IgA and IgM are involved in anti-viral immunity, with the most important of these being IgM, due to its role in complement-mediated cell lysis. IgG plays a large role in opsonisation, and therefore facilitating the phagocytotic destruction of invading pathogens. Secretory IgA plays a role in preventing virions from entering host cells. IgE is associated with allergic disease and defence against helminths. Class switching of B cells to IgE is promoted by IL-4 and IL-13 cytokines (3).

Plasma cell populations can survive for days to years. Long-lived plasma cells can differentiate into memory B cells. These cells do not proliferate but can survive for years in the bone marrow or affected tissue. Upon re-exposure to antigens, these memory cells can differentiate back into plasma cells (3, 73).

1.4.5. B and T cell interactions

Activated T- helper cells migrate to the B cell region of the ADLNs where they mediate B cell antigen-specific activation. This type of T cell display CXCR5 receptor on its surface and secretes IL-21 (3).
B cells also interact with T cells as antigen presenting cells. Unlike T cells, B cells can capture antigen using their BCR and internalise the antigen for processing. B cells are then able to present the peptide with MHC class II to a CD4+ T cell with the appropriate TCR epitope (3).

1.4.6. NKT cells

NKT cells are a type of immune cell that express TCR, as well as typical NK cell molecules. There are many subtypes of this cell type, but generally, they function as immune regulators, and to kill cells by triggering cell death pathways. Once activated, they secrete IL-4 and INF-γ (3, 74).

1.5. Inflammatory resolution

Once a viral infection is under control and infected cells have been removed, recruited and expanded cell populations must be removed to prevent aberrant inflammation, which can quickly lead to excessive tissue damage. Failure to correctly harbour the inflammatory response can lead to chronic inflammation and fibrosis, or scar tissue formation.

Anti-inflammatory (M2) macrophages play a critical role in this phase, by secreting anti-inflammatory cytokines and clearing up cellular debris. Activated and recruited cells undergo apoptosis, primarily induced by apoptotic signals expressed by M2 macrophages. Macrophages also release soluble mediators to stimulate fibroblasts, which are cells that synthesise the extracellular matrix. Stimulation of these cells is
important for healing tissue damage caused by inflammation, but can also lead to fibrosis, following chronic inflammation (75, 76).

1.6. Allergy/atopy

Although the respiratory, immune system is tightly regulated, hypersensitivity reactions may occur, where an exaggerated immune response is induced. Allergic reactions are an example of such a response, wherein the typical innocuous allergen is recognised as harmful, and an IgE-mediated reaction is induced (3, 77).

1.6.1. Mechanisms

Mechanistically, an allergic reaction occurs in two phases; the initial exposure or sensitisation phase, and the re-exposure or re-call phase, which results in disease symptoms. Allergies can be localised to several tissues, including the skin and gastrointestinal system. However, the following sections will focus on allergy induced in the respiratory tissue.

1.6.1.1. Sensitisation

Following inhalation of the typically innocuous allergen, often when co-exposed with other molecules, the immune system responds as it would to a harmful pathogen. The allergen interacts with receptors found on sensor cells, such as those on resident DCs and epithelial cells. DCs, and other antigen presenting cells recruited by secreted cytokines migrate to ADLNs, where they present allergen to adaptive immune cells. In the case of allergy development, T cells differentiate into Th2 cells and secrete IL-4, IL-
13, and IL-5 cytokines. IL-4 drives further Th2 differentiation and drives B cell class switching to IgE in conjunction with IL-13. Th2 cells migrate to airways, where they reside as memory T cells. Allergen-specific IgE circulates in the blood and binds to FCERI receptors found on mast cells and basophils in the airways. Once bound to FCERI, IgE can persist on the cell surface for weeks, leaving these cells primed to react to the allergen upon re-exposure (3).

1.6.1.2. Recall /re-exposure

Upon re-exposure, where the antigen is inhaled and recognised by IgE in the airways, cross-linking of FCERI occurs on mast cells. This triggers the mast cells to release pre-formed mediators and synthesise inflammatory molecules. (Figure 1.2.). The most notable of these pre-formed molecules is histamine. Once released histamine binds rapidly to a variety of cell types via H1 and H2 receptors. On smooth muscle cells the binding of histamine results in constriction, and on endothelial cells binding results in increased vascular permeability. Histamine is also involved in increased mucus secretion. In the airways, this results in a narrowed airway diameter and increased...
mucus secretion. Vascular permeability leads to increased cell and cytokine infiltration, increasing inflammation associated with the allergic response (3).

Figure 1.2. Cross-linking of IgE bound to FcεRI on mast cells, resulting in degranulation. Adapted from Figure 15.2, Coico R, Sunshine G. Immunology: a short course: John Wiley & Sons; 2015 (3).

1.6.2. Allergic airway diseases

Allergic immune responses are commonly induced due to airway antigens, such as pollen and house dust mites. Major allergic diseases in the airways include; allergic rhinitis and allergic asthma. Allergic rhinitis, commonly known as hay fever, involves nasal symptoms of sneezing, itching, congestion and rhinorrhea. Symptoms involving the eyes, ears and throat usually accompany nasal symptoms (78). Asthma defines a complex, heterogeneous, inflammatory disease of the airways, broadly categorised by airway hyperresponsiveness to a stimulus. Symptoms include cough, wheeze, shortness of breath, chest tightness and sputum production (79).
1.6.3. Epidemiology

Higher incidences of allergic disease are typically seen in developed countries. The highest prevalence for asthma was seen in the UK, Australia, New Zealand and Republic of Ireland, followed by North, Central and South America, in a 1998 international comparison study. The lowest rates were observed in Eastern European countries, Indonesia, Greece, China, Taiwan, Uzbekistan, India, and Ethiopia (80). Furthermore, as part of the international study of asthma and allergies in childhood (ISAAC), prevalence of wheeze in children surveyed ranged from 0.8% in Tibet, China to 32.6% in Wellington, New Zealand. Ecological, economic analyses showed a significant increase in wheeze for higher income countries, however, this trend was reversed for severe symptoms (81).

Allergy prevalence is also increasing worldwide and has been doing so for at least the last 50 years. Currently, 300 million people have asthma, and this is expected to rise to 400 million by 2025 (82).

In Australia, allergy is a leading cause of chronic illness, with almost 20% of the population having an allergic disease (83). Allergic diseases affect individuals of all ages and often result in persistent, gradual health deterioration over an individual's lifespan (83). Furthermore, in Australia alone, the total yearly cost for asthma and rhinitis is $9.4 million (84).
1.6.4. Development

Due to a global disparity in allergy incidence, multiple environmental factors have been suggested as risk factors for allergy development. A few examples of these are described below.

1.6.4.1 Early life infections

The integral role of the lung microbiome was first considered in 1989 as the “hygiene hypothesis”, as a mechanism to explain higher rates of asthma and allergic diseases in developed countries (85). This hypothesis proposes that exposure to bacterial and viral agents induces a Th1 response which skews the system away from the Th2 responses involved in allergy (86). Since then, the theory has evolved, and evidence to support the theory have been observed in several studies involving family size and daycare attendance (85, 87-89). However, lower respiratory infections have also been repeatedly associated with increased risk of asthma development (87, 90). More recent studies, therefore, propose that exposure to non-pathogenic microbes could potentially be more useful in guiding the immune response, mediating protection from allergy (91).

Most applicable to our study is the associated risk between early life infections and allergic disease development. For example, infant respiratory syncytial virus (RSV) infection has been identified as an important risk factor for asthma and allergy development in later life (92).
1.6.4.2. Rural vs urban

Another theory to explain the higher prevalence of allergy in developed countries is that rural environments may be more protective than urban. As with early infection, there is conflicting evidence regarding the proposed lowered risk of atopic diseases in rural populations (93-96). Children reared on farms typically display lower rates of atopy, but this is again controversial (97, 98). One proposed mechanism for lower atopy rates with a farming lifestyle have involved exposure to farm milk, and an associated increase in immune regulatory cells Evidence for this idea was provided by a study conducted in 2014, which found increased Treg numbers with farm milk exposure, and a correlated reduction in atopic sensitisation (99).

A particularly interesting study was conducted in 2016, comparing Amish and Hutterite populations. Both these populations display similar genetics and lifestyle factors, however, Amish follow traditional farming practices, while Hutterites use industrialised farming practices. Despite the similar genetic ancestries and lifestyles, allergic sensitisation was 6 times lower in the Amish compared to the Hutterites, while endotoxin levels were almost 7 times higher. Furthermore, intranasal instillation of dust extracts from Amish homes, but not Hutterite homes, was found to significantly inhibit airway eosinophilia (100).

1.6.4.3. Family history

Family history is another strong indicator for allergic disease (101). Hence, there is likely a genetic component to allergic disease development. Genome-wide association studies have observed variances in genes associated with activation and differentiation of Th2
responses, as well as uncovering disease heterogeneity (102-104). However, disease development is much more complex than simple genetics, due to numerous gene-environment interactions.

Another factor worthy of mention involves the progression of atopic dermatitis during infancy to allergic rhinitis and asthma in later years. This phenomenon is termed “atopic march”. A recent study observed that atopic dermatitis alone was not associated with increased asthma risk. However, atopic dermatitis and allergic sensitisation together displayed strong interactive effects towards asthma (105). Together, these findings evidently show the increasing complexity regarding the development of allergy.

1.7. Virus and allergy

As briefly described above, there is a well-known correlation between allergic disease incidence/severity and viral infections. However, whether viral infections are a cause, or consequence of allergic disease, is still of great debate. When rates of infection are similar, allergically sensitised children display higher rates of viral illness (106), indicating that atopy may influence viral disease. However, multiple studies have indicated that infections may enhance allergic disease symptoms and allergy development (107, 108).

Acute asthma exacerbations are often prevailed by respiratory viral infection (91, 92, 109-114). Since the advancements in sensitive molecular technologies, viruses have been found in 80% of wheezing episodes in children, and 50-75% of episodes in adults (115). Of these, the most commonly indicated are human rhinoviruses (HRVs) and
respiratory syncytial viruses (RSVs). RSV and HRV infections are also the main pathogens responsible for acute bronchiolitis in children (116, 117), leading to childhood wheeze, and potentially the induction or exacerbation of asthma. While many viral respiratory infections are self-limiting, wheezing episodes in early life have been indicated as a major risk factor for later asthma diagnosis (118).

This may be explained, by how infant RSV bronchiolitis has many similarities to acute asthma. Both exhibit symptoms of wheezing, rapid breathing and airway inflammation, and roughly one-third of children with initial wheezing will have recurrent wheezing episodes (119). Hence, severe RSV or HRV infection during infancy has been postulated as a cause of asthma development.

Evidence for this theory is shown by how RSV infection requiring hospitalisation, together with a family history of asthma, increase the likelihood of allergic disease development (120). Furthermore, infants born prior to the winter virus season were found to have an increased risk of developing asthma. Those born four months prior to the winter virus peak were at the highest risk, being 29% more likely to develop asthma compared to those born 12 months before the peak (121).

This risk of allergic disease with the viral disease has also been associated with non-asthmatic atopic diseases and explored in animal models (107, 122-125).
It has been pointed out that viral infections are unlikely to be the single cause allergy development, but rather a small part that adds to the likelihood of disease development. For example, a Perth birth cohort study found HRV wheezing to be a risk factor for asthma development, but only if there were signs of atopy (126). Some studies suggest that RSV infections are not the cause of asthma, but instead, a shared genetic disposition for both RSV bronchiolitis and asthma exists (127). Furthermore, it is unlikely viral infections are the single causative factor behind allergic disease development.

Regardless, it is clear virus-induced airway inflammation leads to an increased disease burden. Understanding the mechanisms of this inflammation may uncover novel new therapies to reduce this burden.

1.7.1. Proposed mechanisms of virus and allergen interactions

Multiple mechanisms have been proposed to explain the mechanisms surrounding virus and allergen interactions. As previously explained, allergic diseases are widely accepted to be the result of an exaggerated Th2 immune response, while virus infections are predominately overcome with a Th1 response.

Some studies have found an inverse relationship between FceR1 receptors on pDCs and virally induced IFN-α responses (128, 129). Furthermore, multiple studies have suggested that virally-induced IFN responses may be impaired in asthmatics and other atopic individuals.(128-133).
Both allergens and virus particles act on the epithelium to trigger innate immune responses. In particular, they both induce TSLP, IL-22 and IL-25, which promote innate lymphoid cells and suppress Tregs (113). Tregs play an important role in both virus and allergy responses, as they are involved in balancing immune effector responses and tolerogenic responses. There is some evidence that Tregs may be impaired in atopy (113). Treg populations expressed by atopic individuals have been shown to be less effective at suppressing CD4+ T cells (134). Asthmatic individuals may also have lower Treg proportions, as shown by a lower number of Tregs in BAL fluid from asthmatic children. However, reductions in Treg numbers appear to be resolved with inhaled-corticosteroid treatment (135). Furthermore, early RSV infection has been shown to suppress Treg development, and increase allergic susceptibility, in mice (136).

1.7.1.1. DNA/NET-associated mechanisms

Recently, chromatin released from apoptosis and necrosis of virally infected and responding immune cells were reported to skew the immune system towards an allergic Th2 response (137, 138). Additionally, with a similar mechanism, NET released from neutrophils during viral infections may be influencing the immune response.

NETs have been shown to enhance pathogenesis numerous times, bringing into question the effectiveness of their defence. Extracellular DNA, and the associated granule proteins found in NETs are both cytotoxic, allowing them to easily harm the surrounding tissue (138, 139). NETs directly contribute to epithelial and endothelial cell death in a concentration-dependent manner (140). Interestingly, this cytotoxic effect was unable to be resolved with DNA digestion using DNase. Similarly, treatment with
elastase inhibitors failed to reduce NET-associated cytotoxicity (140). The role of histamine in contributing to this cytotoxicity was examined by incubating epithelial and endothelial cells with histones. Histones were found to prevent cell growth and aggravate cytotoxicity in a concentration-dependent manner (140). Another study reported histones contribute to endothelial dysfunction, organ failure and death during sepsis (141). This alludes to the cytotoxic effects of extracellular DNA, regardless of whether this DNA was released via NETosis.

Furthermore, the possible use of NETs in assisting with pathogen clearance has been questioned. PAD4 deficient mice are incapable of forming NETs, and yet they still maintain immunity against influenza infection (142). It has been argued that while quite effective at ensnaring microbes, the killing ability of NETs is much less effective. Degradation of NETs with DNase was shown to release live bacteria, and NET formation seemed to interfere with phagocytotic killing, rather than aid (143). Together, these findings suggest NETs may not provide useful defence against virus infections and instead contribute to tissue damage by fueling Th2 inflammation.

Last year, Toussaint and colleagues indeed showed that rhinovirus infection induces NET release in the airways of mice and that this release is related to Th2 cytokine induction, and asthma severity. Furthermore, they found exogenous DNase treatment prevented asthma exacerbation (144). Toussaint proposed that DNA and associated peptides from NETs promotes macrophage/DC recruitment during allergen challenge, driving the Th2 response (144). Furthermore, antimicrobial peptides associated with NETs may also promote a Th2 response. Toussaint investigated this by inhibiting neutrophil elastase...
and found viral-induced airway hyperresponsiveness could be reduced, indicating neutrophil elastase may contribute to disease (144). However, little is known regarding if NETs are involved in the initial allergy sensitisation process.

1.7.2. Animal models

To investigate the underlying processes involved in allergic disease development, both human and animal models are used. Murine models are particularly useful, as several factors associated with allergic airway disease can be induced. These include airway inflammation, airway hyperresponsiveness, and mucus production.

Typical murine models of allergic sensitisation involve sensitising naïve animals to a protein, in conjunction with an adjuvant. Th2-high strains are preferred for this model, such as Brown Norway (BN) rats or Balb/C mice (27, 145, 146). Most models utilise the egg-white protein ovalbumin (OVA), often in combination with an aluminium hydroxide adjuvant (Alum), as this induces a strong Th2 dominated response. In an acute model of airway inflammation, animals are injected with an OVA/Alum solution intraperitoneally on days 0 and 14. They are then challenged with OVA on days 28-30 by nebulization with a 1% OVA solution. Inhaled exposure to OVA should occur for at least 20 minutes during these exposures. The allergic recall response is then analysed during the following 48 hours from the last challenge. This model depends on systemic sensitisation towards the OVA protein, and a response in the airways occurring following an inhaled challenge (123).
Strickland and colleagues have developed a murine model which mimics many features of chronic atopic asthma, including airway hyperresponsiveness. The BN rat strain is used, as they are hypersusceptible to allergic airway disease, due to their Th2-high inflammatory profile. OVA/Alum intraperitoneal injection is used to establish sensitisation. Challenge occurs via 1% OVA inhalation over 60 minutes (27). In the same model of allergic sensitisation, virus infection has been shown to induce an exaggerated airway response (147). Recently, intranasal sensitisation was also achieved in the same model following 4 consecutive intranasals of OVA. Challenge was achieved with another OVA intranasal one week later (148). This model is more suitable than traditional OVA/Alum methods, as removal of the Alum adjuvant allows for subtle differences in IgE sensitisation to be assessed.

1.8. Hypothesis, aims and significance of the study

Overall, genetics and environmental factors play essential roles in allergic disease development. Atopic individuals that are exposed to viral infections, often develop atopic diseases, such as allergic asthma. While there is an established link between virus infection and allergy development, it remains to be understood if/how viral infection alters the immune processes involved in allergen sensitisation. Viral infection appears to drive NET formation, and NETs appear to contribute to a Th2 response in sensitised individuals. However, it is not known if NETs contribute to the initial allergy sensitisation process.

We hypothesise that NETs are induced during viral respiratory infections and contribute to allergic sensitisation, and that their removal will reduce the rate of sensitisation.
To address this hypothesis, we aim to determine the role that virus infection, and subsequent induction of NETs/extracellular DNA released from dying cells, plays in the development of allergic disease, in an allergically sensitised model.

Our first aim will be to determine the role of virus infection in allergy sensitisation and the subsequent role of NETs/extracellular DNA. Our second aim will be to determine if a viral infection, and NET/extracellular DNA induction during sensitisation, alters the allergic recall response.

To address this, male adult Brown Norway (BN) rats will be infected with an attenuated mengovirus, which establishes a mild respiratory infection, similar to rhinovirus infection in humans (125). During the infection, rats will also be exposed to ovalbumin (OVA), as a non-pathologic model allergen, daily over four days to establish OVA-specific IgE sensitisation. Rats will then be re-exposed to OVA a week later, to induce an allergic recall response. To then test the involvement of NETs during the sensitisation process, DNase-I, which degrades extracellular DNA, will be administered and compared to untreated controls.

Our first aim will be addressed by comparing levels of OVA IgE as well as baseline immune profiles of allergically sensitised animals with and without the presence of a respiratory viral infection. Our second aim will be addressed, by analysing the allergic
recall response. The allergic recall response between treatment groups will be analysed by comparing any differences between pre- and post- OVA re-challenge.

We expect that virus infection will increase the level of allergic sensitisation, as determined by OVA IgE titres, and that NET degradation, with DNase treatment, will counteract this effect. We also expect that the increased sensitisation following viral infection, will increase the magnitude of the allergic recall response as determined by airway immune cell infiltration and that NET degradation will again reverse this effect. This project will determine if DNase treatment during viral infection could reduce the risk of allergy development, without affecting the outcome of viral infection. If this can be achieved, DNase could prove a promising new drug candidate for the reduction of allergic disease development, as a consequence of respiratory viral infections. This may then help to prevent the development of allergic diseases, such as atopic asthma.
2. MATERIALS AND METHODS.

2.1. Buffers and solutions

2.1.1. Glucose phosphate buffer (GKN)

Reagents (*Table 2.1.*) were dissolved and made up to 5 L in a volumetric flask with Baxter water. The solution was filter sterilised into ten 500 ml flasks and kept at room temperature.

*Table 2.1. GKN reagents.*

<table>
<thead>
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<th>Reagents</th>
<th>Concentrations</th>
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<tr>
<td>Sodium chloride (NaCl) (Sigma)</td>
<td>0.14 M</td>
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<tr>
<td>Potassium chloride (KCl) (Amresco)</td>
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<tr>
<td>Disodium hydrogen phosphate dodecahydrate Na$_2$HPO$_4$.12H$_2$O (Amresco)</td>
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</tr>
<tr>
<td>Sodium dihydrogen phosphate dihydrate NaH$_2$PO$_4$.2H$_2$O (Amresco)</td>
<td>5 mM</td>
</tr>
<tr>
<td>D-Glucose (Sigma)</td>
<td>11.1 mM</td>
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2.1.2. Phosphate buffered saline (PBS)

Reagents (*Table 2.2.*) were dissolved and made up to 5 L in a volumetric flask with Baxter water. The solution was filter sterilised into ten 500 ml flasks and kept at room temperature.
Table 2.2. PBS reagents.

<table>
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<th>Reagents</th>
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<tbody>
<tr>
<td>Sodium chloride (NaCl) (Sigma)</td>
<td>0.15 M</td>
</tr>
<tr>
<td>Potassium chloride (KCl) (Amresco)</td>
<td>2.68 mM</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate (Na$_2$HPO$_4$) (Amresco)</td>
<td>8.1 mM</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate (KH$_2$PO$_4$)</td>
<td>1.47 mM</td>
</tr>
<tr>
<td>HCL (Sigma)</td>
<td>0.4 mM</td>
</tr>
</tbody>
</table>

2.1.3. Heat inactivated fetal calf serum (FCS)

FCS (Serana, Bunbury, WA, Australia, 8050512FBS) was heat inactivated by incubating at 56 °C for 30 minutes. This was then stored in 10 ml aliquots at -20 °C. When required, FCS was left to thaw at room temperature. FCS was then added to GKN at 5-10% as required.

2.1.4. Bovine serum albumin (BSA)

Bovine serum albumin (Bovagen Biologicals, VIC, Australia) was dissolved in PBS at 5% (BSA) and stored in 10 ml aliquots at -20 °C. When required, BSA was left to thaw at room temperature prior to addition into appropriate buffers. Further reference to GKN BSA, indicates GKN with 0.1% BSA, while further reference to PBS BSA, indicates PBS with 0.1% BSA.
2.1.5. Red Blood Cell Lysis

Reagents (*Table 2.3.*) were dissolved in 2 L of Milli-Q water. The solution was filter sterilised into 500 ml flasks and kept at room temperature. The final pH of the resultant solution was 7.2. Both reagents were created in house.

*Table 2.3. Red blood cell lysis reagents.*

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium chloride (NH₄Cl)</td>
<td>0.14 M</td>
</tr>
<tr>
<td>Tris hydrogen chloride</td>
<td>17 mM</td>
</tr>
</tbody>
</table>

2.2. Animals, allergen sensitisation and virus infection

Male, 8-10-week-old, Brown Norway (BN) rats, bred at the Bioresources Centre of Telethon Kids Institute were used. All experimental protocols received prior approval by the Telethon Kids Institute Animal Ethics Committee (AEC #332). Animals were randomly assigned to experimental groups (*Table 2.4.*).

To resemble human rhinovirus (HRV), an attenuated viral strain of mengovirus, with Poly (C) length 0 (vMC0) was used (125, 149, 150). Rats were inoculated intranasally with 100 µl of 10⁷ plaque-forming units (PFU) of vMC0. Intranasals were performed under inhaled isoflurane anaesthesia. Animals were weighed immediately prior to vMC0 inoculation for weight change monitoring. vMC0 was prepared as previously described (125) and stored in 10 µl aliquots of 10¹⁰ PFU/ml at -80 °C.
vMC0 naturally infects rodents (151) and leads to a lower airway inflammatory response, consisting of neutrophils and lymphocytes. Due to these features, vMC0 has been previously described as a useful model for HRV infection (125).

The allergen sensitisation model involved sensitising all the rats with a 100 µl intranasal of 1 mg/ml ovalbumin (OVA, Sigma, 9006-59-1), in a solution of sterile PBS over four consecutive days. Some of the virus-infected animals were also given 200 µg/ml of endotoxin-free deoxyribonuclease-I (DNase-I, Sigma,100265251) in conjunction with the OVA intranasals. Since DNase is dependent on divalent cations, PBS was supplemented with 5 mM of MgCl₂, for all intranasals. Endotoxin-free DNase-I was initially dissolved to 5 mg/ml in PBS and aliquoted for storage. Aliquots were kept frozen at -20 °C until required. Intranasals were conducted under inhaled isoflurane anaesthesia, and weights were recorded immediately after each intranasal for animal monitoring.

**Figure 2.1. The allergy and virus model.** All animals received OVA on days 1, 2, 3 and 4. Viral groups were infected on day 0.5. DNase treatment groups received DNase in conjunction with OVA. Post-OVA re-challenge groups were challenged on day 7-11, and samples collected 24 hours later.
Exposure to OVA/DNase-I began 5 hours after virus infection so that initial exposure to the allergen (OVA) occurred during the peak of virus infection in those groups (125). To characterise the airway response during this regime, some samples were collected three days after viral infection, 24 hours after the second OVA/DNase intranasal. Some of the remaining animals were re-challenged 7-11 days later with 100 µl of 1 µg/ml ovalbumin-Texas Red (OVA-TR, ThermoFisher, 023021). So that re-challenge could be confirmed with flow cytometry analysis. Tissues were then collected 24 hours later. While groups were randomly assigned, attempts were made to include a pre and post sample for the chosen groups on each collection date (Figure 2.1).

### Table 2.4. Animal treatment groups used for DNase treatment experiments.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Number of pre-OVA re-challenge rats used</th>
<th>Number of post-OVA re-challenge rats used</th>
<th>Number of two-day exposure rats used</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA</td>
<td>6</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>OVA + Virus</td>
<td>6</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>OVA + Virus + DNase</td>
<td>5</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

2.3. Animal dissection and sample collection

For tissue collection, rats were anaesthetised with inhaled isoflurane. Once rats were unresponsive, approximately 1 ml of blood was collected via cardiac puncture. This blood was then placed into Eppendorf tubes and incubated at room temperature for 10 minutes before being transferred to ice. Animals were then culled with a 3 ml intraperitoneal injection of lethobarb (pentobarbitone sodium, Virbec, Milperra, NSW,
LETHA450). An initial incision was made into the abdominal region, and the diaphragm punctured to retract the lungs. The thoracic cavity was then exposed, and the ribs cut away adjacent to the spine. The heart was then removed. Airway draining lymph nodes were extracted *(Figure 2.2)* and placed into GKN-BSA on ice.

A bronchoalveolar lavage (BAL) was then performed with 8 ml of GKN to flush out airway cells from the bronchial and alveolar spaces. To do this, the trachea is firstly separated from the surrounding tissues using forceps. A piece of thread is then loosely tied around the trachea. To allow for both lung and BAL fluid to be collected, without compromising the lung tissue, the middle lobe was then also tied off using a piece of thread. A small incision was made into the trachea, below the loose knot. The lavage needle is then inserted into the incision, and the knot firmly tightened. The lavage needle was attached to tubing, connected to a syringe filled with GKN. The lungs are slowly filled with GKN and then aspirated back into the syringe. This was then repeated. In the case that the initial lavage failed (leaked), the knot was tightened, the syringe refilled with 8 ml of GKN, and a single lavage conducted. The recovered BAL fluid was placed into tubes and placed on ice. The middle lung lobe was then excised and placed in GKN-BSA on ice.
Figure 2.2. Schematic drawing of ADLNs collected (shown in bold). Figure taken from Lehmann C, et al. Lymphocytes in the bronchoalveolar space reenter the lung tissue by means of the alveolar epithelium, migrate to regional lymph nodes, and subsequently rejoin the systemic immune system. The Anatomical Record: An Official Publication of the American Association of Anatomists. 2001;264(3):229-36. (152).

2.4 Sample preparation

2.4.1. Lung tissue processing and cell counts

To extract cells from harvested tissues, lungs were cut into 0.5 mm sections using a McIlwain Tissue Chopper. Tissues were then incubated with digestion enzymes in 10 ml of GKN containing 10% FCS (GKN-10% FCS), shaking at 37 °C. Lung tissue was incubated with 1.5 mg/ml of collagenase IV (Worthington Biochemical Corporation, 43E14252), and 0.1 mg/ml of DNase-I (Sigma, 9003-98-9) for 90 minutes. During the last 30 minutes, a further 0.5 ml of 0.1 mg/ml of DNase-I was then added to each lung digest mixture.
Following enzymatic digestion, the samples were thoroughly mixed and filtered through a nylon filter, into a 15 ml tube coated with FCS. GKN containing 5% FCS (GKN-5% FCS) was used to wash the filter and flask for any residual cells. The samples were then centrifuged at 754 relative centrifugal force (RCF) for 5 minutes at 4 °C.

The supernatant was removed from the samples using suction. Lung samples were then incubated for four minutes at room temperature in 2 ml of red blood cell lysis buffer. Following the addition of GKN, samples were centrifuged for a further 5 minutes at 754 RCF at 4 °C.

To count the extracted cells, the cell pellets were resuspended in 1000 µl of PBS. 10 µl of the cell suspension solution was diluted 1:5 with trypan blue (Thermofisher, 15250061). 10 µl of the cell-trypan blue solution was then loaded onto a haemocytometer for cell counting. At least 100 live cells (defined by non-permeability to trypan blue) were counted under a light microscope.

Cell concentrations were then calculated according to the following formula:

\[
\text{cell concentration (cells/ml)} = \frac{\text{cells counted}}{\text{rows counted}} \times \text{total rows} \times \text{dilution factor} \times 10^4
\]

Total cell numbers could then be calculated by accounting for the resuspension volume:

\[
\text{total cells extracted} = \text{cell concentration (cells/ml)} \times \text{resuspension volume}
\]
2.4.2. Airway draining lymph node (ADLN) processing and cell counts

Cells were extracted from airway draining lymph nodes (ADLNs) by mincing the samples by hand using a sterile surgical scalpel. This was then incubated with digestion enzymes in 10 ml of GKN-10% FCS, shaking at 37 °C for 30 minutes. Digestion enzymes included; 0.75 mg/ml of collagenase IV, and 0.1 mg/ml of DNase-I. Samples were then thoroughly mixed and filtered into a 15 ml tube coated with FCS, through a nylon filter. Samples were then centrifuged (Allegra X-12R centrifuge, Beckman Coulter, Indianapolis, United States) for 5 minutes at 754 RCF at 4 °C.

The supernatant was aspirated, and the cell pellets resuspended in 1000 µl of PBS. Lymph nodes required no red blood cell lysis due to low red blood cell numbers. 10 µl of the cell suspension solution was diluted 1:5 with trypan blue. 10 µl of the cell-trypan blue solution was then loaded onto a haemocytometer. Cells were counted, and concentrations calculated, as previously described.

2.4.3. Bronchoalveolar (BAL) fluid processing and cell counts

Bronchoalveolar lavage fluid was centrifuged at 754 RCF for 5 minutes at 4 °C. 1 ml of supernatant was collected and frozen at -20 °C for later analysis. The remaining supernatant was aspirated, and samples were incubated for 4 minutes in 2 ml of red blood cell lysis buffer. After incubation, GKN was added, and the samples were again centrifuged at 754 RCF for 5 minutes at 4 °C.
To count extracted cells, the supernatant was aspirated, and the cells resuspended in 100 µl of PBS. 10 µl of the cell suspension solution was diluted 1:2 with trypan blue. 10 µl of the cell-trypan blue solution was then loaded onto a haemocytometer for cell counting as described above.

20 000 cells from the BAL cell suspension were spun onto a glass microscope slide using a cytocentrifuge. Some of these slides were fixed with 4% formaldehyde and sent off to an external collaborator for NET detection staining (Figure 3.3.).

The rest of the slides were stained with a Diff-Quick staining kit (IHC, 64851) and counted using a light microscope. Macrophages, neutrophils, eosinophils and lymphocytes were distinguished by appearance (Figure 2.3.). Cells were only counted if they could be clearly classified as one of these four cell types. Areas of the slide that had stained poorly were avoided in favour of areas where cells could be more easily distinguished. At least 300 total cells were counted to allow different cell type proportions to be accurately obtained. In the case that 300 cells could not be counted, due to poor staining or low cell numbers, the highest number possible was counted.
2.4.4. Blood preparation

Blood samples were left to coagulate at room temperature for 10 minutes prior and then transferred to an icebox for approximately 7 hours. The blood clot was centrifuged for 5 minutes at 754 RCF at 4 °C and the remaining serum/supernatant was collected. The resulting serum was frozen at -20 °C for later IgE/IgG analysis.

2.5. Cell staining for flow cytometry analysis

For each BAL, lung and ADLN sample, one million cells were transferred to FACS tubes and suspended in 1 ml of PBS with 0.1% BSA (PBS-BSA). Samples were then centrifuged at 754 RCF for 5 minutes at 4 °C. The supernatant was aspirated, and the cell pellet
resuspended in 100 µl of primary antibody master mix (*Table 2.5.*) for 15 minutes at 4 °C away from light.

Following incubation, samples were washed with 2 ml of PBS-BSA and centrifuged at 754 RCF for 5 minutes at 4 °C. The supernatant was aspirated, and the cell pellet was then resuspended in 100 µl secondary antibody master mix (*Table 2.5.*) and incubated for 15 minutes 4 °C away from light.

Samples were again washed with 2 ml of PBS-BSA and centrifuged at 754 RCF for 5 minutes at 4 °C. The supernatant was aspirated, and the cell pellet resuspended in 500 µl of permeabilisation buffer (1:4 dilution of fixation concentrate [eBioscience, cat: 00-5123-43 with fixation diluent [eBioscience, cat: 00-5223-56) This was incubated for 30 minutes, or left overnight, at 4 °C.

Samples were then washed with permeabilisation wash buffer (1:10 dilution of permeabilisation) and centrifuged at 754 RCF for 5 minutes. For intracellular staining, the supernatant was removed by tipping, and the cells resuspended in 10 µl of FoxP3-PE antibody solution (1:100, E Bioscience, 12-5773-82) along with any residual buffer (approximately 80 µl) that was not removed by tipping. Samples were incubated at 4 °C for 30 minutes. Finally, samples were spun at 754 RCF for 5 minutes and resuspended in 150 µl of PBS-BSA.
Single stain controls and no stain controls were prepared as above, using 25 000 cells from lymph nodes, suspended in 1 ml of PBS-BSA.

Samples were acquired using the LSRII Fortessa (BD Biosciences) using FACSDiva Software (version 8.0.1, BD Bioscience). In general, 600 000 - 800 000 events were collected, while 50 000 events were collected for single stains. Excitation wavelengths for each antibody are shown in Figure 2.4. Samples were kept at 4 °C prior to analysis.
Figure 2.4. Laser excitation wavelengths for each antibody/fluorochrome. Adapted from a figure created by Yasmine Khandan.
Table 2.5. Antibody concentrations.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Fluorochrome /Host</th>
<th>Company</th>
<th>Cat #</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Fluorochrome</th>
<th>Company</th>
<th>Cat #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD127</td>
<td>Sheep</td>
<td>ThermoFisher</td>
<td>PA5-47758</td>
<td>1:25</td>
<td>Anti-sheep</td>
<td>APC</td>
<td>InVitro</td>
<td>F0127</td>
<td>1:10</td>
</tr>
<tr>
<td>CD161</td>
<td>APC-Cy7</td>
<td>Miltenyi</td>
<td>130-102-715</td>
<td>1:50</td>
<td></td>
<td></td>
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<tr>
<td>CCR7</td>
<td>PE-CF594</td>
<td>Jomar</td>
<td>130-102-715</td>
<td>1:50</td>
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<tr>
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<td>BD</td>
<td>562108</td>
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<tr>
<td>CD4</td>
<td>PE-Cy7</td>
<td>Bio legend</td>
<td>201516</td>
<td>1:100</td>
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<tr>
<td>CXCR5</td>
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<td>Abcam</td>
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<td>Anti-rabbit</td>
<td>BV650</td>
<td>Thermo</td>
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<td>743594</td>
<td>1:400</td>
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</tr>
</tbody>
</table>
2.6. Flow data compensation and gating schemes

Following acquisition using the LSR Fortessa, FlowJo LLC software (version 10.1r7) was used for data analysis. Single stain and no stain controls were used to compensate. Gating schemes are shown in Figures 3.7, 3.10 and 3.11.

2.7. IgE and IgG ELISA analysis

IgE and IgG enzyme-linked immunosorbent assay (ELISA) protocols were optimised prior to serum analysis (Appendix 2.).

Ovalbumin (5 µg/ml) or 1% BSA, in 50 µl of PBS was coated onto Maxisorb 96 well plates (ThermoFisher, 44-2404-21), and left to incubate overnight at 4°C. Following coating, plates were washed with PBS containing 0.05% Tween-20 (Sigma, P2287-500ML), using a BioTec plate washer (W760). To block unspecific binding, plates were incubated with 200 µl of PBS containing 1% BSA (PBS-1% BSA) for two hours at room temperature.

Serum samples, as well as positive (serum from an 8-week-old male BN rat with confirmed OVA-specific IgE) and negative (serum from naïve 8-week-old male BN rat) controls, were thawed and diluted in PBS-0.1% BSA to a final concentration of 12.5% serum. Positive and negative control serums were obtained from a previous study. Each sample was added to OVA-coated wells in duplicates, or BSA-coated control wells in singles, and incubated for one hour at room temperature.
Plates were washed and incubated with mouse anti-rat IgE antibody (1:2000 in PBS with 0.1% BSA, Bio-Rad, MCA193) for one hour at room temperature. After another wash, plates were incubated with sheep anti-mouse-HRP antibody (Amersham Biosciences, NA931) (1:2000 in PBS with 0.1% BSA) for 30 minutes at room temperature.

After another wash, 50 µl of tetramethylbenzidine (TMB) solution (Bio-Rad, BUF056A, 170811). This solution produces a deep colour during the enzymatic degradation of H₂O₂ by HRP. The reaction was then stopped after 30 minutes using 50 µl of orthophosphoric acid (1 M). Absorbance was then immediately read using at 450 nm using an EnSpire multi-plate reader (Perkin Elmer).

Absorbance values obtained were minused from a blank well value for each plate. BSA-coated well values were then subtracted from each matched duplicate sample, and the two resultant duplicate values were averaged. Average OVA-specific IgE titres, OVA-specific IgG titres and IgG:IgE ratios, were then calculated for each group.

2.8. DNA quantification

The DNA content of BAL samples was analysed using a Quant-iT Picogreen dsDNA assay kit (Invitrogen, P7581).

Quant-iT Picogreen dsDNA reagent (in a solution of dimethyl sulfoxide) was diluted 1:200 in Tris and EDTA (TE) buffer (x20, diluted with Baxter water). This was added to a
96 well Maxisorb plate (50 µl per well). BAL sample, or lambda DNA standard (eBioScience, 108038000) (50 µl) was then sequentially added to the plate in duplicates. Serial dilutions of lambda DNA were diluted in GKN. Fluorescence was read immediately at 480 nm using the EnSpire multi-plate reader.

2.9. Statistical analysis

Prism (version 7.0a, GraphPad Software Inc.) was used for statistical analysis and graphing of data. Statistical significance between the three baseline experimental groups was determined using two-way ANOVA tests with multiple comparisons (Tukey’s multiple comparisons test). Statistical significance between the two pre- and post- OVA re-challenge groups was determined with multiple t-tests.

Prior to statistical testing, daily weights were transformed \((weight/initial\ \weight)\) for each date.

For IgE/IgG analysis, outliers due to experimental error were removed. Values that >50% the next lowest sample in that group were removed. This resulted in one value being removed from the naïve group. Furthermore, pre- and post- re-challenge values were baseline corrected using the following formula (for each of the three treatment groups):

\[
(mean\ \value/\ pre-re-challenge\ \mean) \times 100
\]

Differences between corrected means were analysed using two-tailed paired t-tests.
Furthermore, naïve and OVA pre-challenge IgE titres were compared using a one-way ANOVA. To explore the proportion of sensitised animals, a Fishers Exact test was used to compare each sensitised group to the OVA- naïve group.

The DNA content in BAL was compared between the three treatment groups using a one-way ANOVA and Tukey’s multiple comparisons test.

Statistical significance was defined as p<0.05 for all statistical tests.
3. RESULTS

In our project, we aimed to determine the role of NETs, or extracellular DNA released from dying cells, in allergic disease development, following a viral infection. We theorised that NETs are induced during viral respiratory infections, but that they do not adequately control the viral disease. Instead, they contribute to the development of allergic disease, by contributing to the allergic inflammatory cycle, and their removal will be beneficial to patients.

To test this, male adult BN rats were infected with vMC0, as a model for human rhinovirus infection. The rats were then sensitised to OVA, as a non-pathogenic model allergen. To then test the involvement of NETs or extracellular DNA, half were treated with DNase during infection, as a means to efficiently degrade extracellular DNA. These groups were then compared to uninfected, sensitised controls.

Approximately one week after the initial OVA exposures, differences between OVA-specific IgE and airway inflammation was assessed with viral infection and DNase treatment. In addition, samples were collected before and after an OVA re-challenge, so that the degree of allergic recall response could be compared.
3.1. vMC0 disease model

Reduction in body weight, or a reduction in body weight gain, can be used as a measure of respiratory viral infections in rodents (125). Animal weights were recorded daily following viral infection. According to previous studies, vMC0 titres peak 1-3 days post-infection, and drop to low/undetectable levels by day five (125). Four days after viral infection, weight had not significantly changed between the groups (Figure 3.1.A). This suggests only a mild level of infection was established with vMC0.

Bronchoalveolar lavage (BAL) is a method of obtaining cells in the bronchial and alveolar spaces of the lungs. BALs were collected three days post virus infection, halfway through the OVA exposure scheme, to assess airway inflammation during peak viral infection.

The total number of cells in BAL fluid did not differ between any of the treatment groups (Figure 3.1.B.). The total number of macrophages in BAL did not differ between OVA and OVA+Virus exposed animals, however macrophage numbers were significantly lower in OVA+Virus+DNase animals compared to OVA exposed animals (p<0.05). There were no significant difference for any other any other cell types, between any treatment groups. However, an increased trend of eosinophils can be seen in the OVA group (p=0.17 and p=0.18, when compared to OVA+Virus and OVA+Virus+DNase, respectively) (Figure 3.1.C.).
Figure 3.1. vMCO disease model. Animal weight change during vMCO infection (days 1-4, with vMCO infection on day 0.5) (A.). Data is presented as means (n ≥ 9) with standard deviation indicated by dotted lines. As weights were collected prior to OVA re-challenge, pre- and post-OVA-rechallenge groups were combined. BAL cell numbers during peak vMCO infection (B and C.). BAL fluid samples were collected on day 3 post viral infection, 24 hrs after the second OVA intranasal. Statistical difference was calculated using a two-way ANOVA (with Tukey’s multiple comparisons test) and indicated as *p<0.05. Data is shown as means (n = 3) with standard deviations.
3.2. OVA sensitisation model

To investigate if virus infection influenced sensitisation to OVA, virus infected rats were exposed to OVA. Following OVA exposure, each experimental groups OVA-specific IgE levels were compared to those of non-sensitised, OVA-naive controls. This was done to confirm that OVA sensitisation was established in our animals and was achieved using an enzyme-linked immunosorbent assay (ELISA). OVA-specific IgE titres were significantly increased in the OVA exposed and OVA+Virus groups, compared to naïve controls (p<0.01 and p<0.05, respectively). OVA-specific IgE titres were not significantly different in the OVA+Virus+DNase group compared to naïve animals (p=0.22) (Figure 3.2.A.).

To explore the proportion of sensitised animals in each experimental group, Fisher’s exact tests were performed. Animals were classed as sensitised if their IgE level (absorbance value) was greater than the mean of the OVA-naïve groups, plus two standard deviations. OVA and OVA+Virus exposed groups had significantly higher proportions of sensitised animals (100%) when compared to OVA-naïve controls (p<0.01). The OVA+Virus+DNase group did not display a significantly higher proportion of sensitised animals (60%) when compared to OVA-naïve controls (Figure 3.2.B). This suggests that DNase treatment influenced OVA sensitisation.
**Figure 3.2. OVA sensitisation.** IgE titres for OVA sensitised (pre-OVA re-challenge) experimental groups, compared to OVA-naïve controls (A.). The dotted line represents the sensitisation cut off (naïve group mean plus two standard deviations) used for Chi-Square analysis. Statistical significance was calculated using a one-way ANOVA and shown as *p<0.05. Data is shown as means (n ≥ 4) with standard deviations. The proportion of OVA sensitised and non-sensitised animals based on OVA-naïve controls (B.). Statistical difference was calculated using a Fishers exact test, between naïve and each treatment group. Significance is shown as *p<0.05, **p<0.01 and ns = non-significant.
3.3. Quantification of DNA content in BAL samples

DNase is commonly used to cleave extracellular DNA released by necrotic neutrophils. In our model, we used DNase to break down NETs formed during viral infection. DNA and MPO content (two major NET components) in BAL samples were confirmed with fluorescent staining techniques by an external collaborator (Figure 3.3).

![Potential NETs on a virally infected BAL slide](image)

**Figure 3.3. Potential NETs on a virally infected BAL slide.** BAL slides were stained for DNA (blue) and myeloid peroxidase (MPO) (green) by an external collaborator.

To assess the effect of DNase treatment on DNA in the airways, DNA was quantified in BAL fluid three days after virus infection, after two daily DNase intranasals (Figure 2.1.). The DNA content in BAL did not significantly differ in OVA, OVA+Virus or OVA+Virus+DNase groups (Figure 3.4.). This suggests that DNase does not alter BAL DNA content.
Figure 3.4. DNA content in BAL samples (three days post-vMCO infection). Data is shown as means (n ≥ 4) with standard deviation. Statistical significance was calculated using a one-way ANOVA (non-significant).
3.4. OVA-specific IgE and IgG titres

To then assess how virus infections and extracellular DNA influenced the allergic recall response, OVA-specific IgE was measured 24 hours post-OVA re-challenge and compared to pre-re-challenge levels. OVA-specific IgG was also measured post-re-challenge and compared to pre-re-challenge values, as an important antibody in the anti-viral response.

As previously described (Figure 3.2.), baseline (pre-re-challenge) OVA-specific IgE titres did not significantly differ between treatment groups. Similarly, baseline OVA-specific IgG titres did not significantly differ between treatment groups (Figure 3.5.A.).

Allergic recall was then compared for each treatment group, by comparing IgE levels pre- and post-allergic re-challenge. No significant difference in the allergic recall was seen for any of the treatment groups, suggesting that a re-call response was not observed (Figure 3.5.B.). Additionally, no difference was observed between pre- and post-IgG levels (Figure 3.5.C.).

Finally, to account for competitive binding of IgE and IgG, the ratio of IgG:IgE was also analysed (Figure 3.5.D.). The ratio of OVA-specific IgG to OVA-specific IgE did not significantly differ between any groups.
Figure 3.5. Serum IgE and IgG titres pre- and post- OVA re-challenge. IgG pre-re-challenge titres (A.). Data is presented as means (n ≥ 4) with standard deviations. IgE pre-re-challenge titres are shown in Figure 3.2.

IgE (B.) and IgG (C.) titres from serum samples. Data has been corrected to pre-re-challenge means (n ≥ 4) and shown as corrected means with standard error means. Statistical difference between pre- and post-re-challenge groups was determined using a paired t-test. No significant differences were seen (ns).

As IgG may have competitively bound to OVA in the IgE assay, IgE titres were also analysed as a ratio with IgG (D.). Data is presented as means (n ≥ 4) with standard deviations.

Statistical significance between treatment groups at baseline (pre-re-challenge) was determined using a two-way ANOVA with Tukey’s multiple comparisons test. No significant differences were seen.
3.5. Cellular airway infiltration

We further assessed how viral infections and extracellular DNA influenced the allergic recall response, by assessing airway cell infiltration in response to OVA re-challenge.

3.5.1 Innate immune response

Assessing total cell numbers in BAL did not reveal any difference between any of our three treatment groups prior to OVA re-challenge (Figure 3.6.A.). In addition, none of the groups appeared to respond to OVA re-challenge with infiltration of cells into the airways, as pre- and post- OVA, re-challenge cell numbers, did not significantly differ for any cell type (Figure 3.6.).

3.5.2. Adaptive immune response

To further analyse the cellular differences in airways after virus infection and OVA sensitisation, we used multiparameter flow cytometry analysis. Three airway tissues were collected (lung, ADLNs and BAL fluid), however, due to time constraints, only lung data was statistically analysed for this project. As innate immune cell proportions were analysed in BAL differential counts, analysis focused on cell involved in adaptive immunity; namely T cells, B cells and DCs.

3.5.2.1 T cells

T cells were differentiated according to Figure 3.7. They were firstly subdivided into CD4+ T cells and CD4- T cells. Our CD8 marker was unable to be used due to poor staining. CD4+ T cells were then further divided by their expression of FoxP3, to split CD4+ T cells into Treg (FoxP3+) and non-Tregs (FoxP3-).
Firstly, baseline levels of T cell subtypes were compared for our three experimental groups. Baseline CD4+ or CD4- T cells did not differ between any groups. However, when CD4+ T cells were subdivided into Treg (FoxP3+) and non-Tregs (FoxP3-), Treg cells were markedly increased in OVA+Virus+DNase groups, when compared to OVA+Virus and OVA groups (p<0.01 and p<0.001) (Figure 3.8.C.).

Next, allergic re-call was assessed, by determining if there were any differences between pre- and post- OVA-re-challenge for any of the experimental groups. No differences were observed between pre-and post- OVA-re-challenge for any of the experimental groups (Figure 3.8.).

Furthermore, mean fluorescent intensities (mfi’s) were compared from different T cell subtypes. Baseline CD25 mfi’s did not differ between experimental groups for Treg, non-Tregs and CD4- T cells. Baseline FoxP3 mfi’s were significantly amplified in OVA+Virus+DNase when compared to OVA (Figure 3.9.B.). However, neither CD25 or FoxP3 mfi’s differed with OVA re-challenge for any treatment groups (Figure 3.9.). Together this suggests that DNase treatment may have had an immune regulatory effect.
Figure 3.6. BAL cell numbers. Total cell numbers recovered from BALs (A). Eosinophil (B.), macrophage (C.), neutrophil (D.) and lymphocyte (E.) total cell numbers, from BAL differential counts. No significant difference (ns) was seen between pre- and post- OVA re-challenge groups, or between any of the treatment groups (as shown by multiple t-tests and a two-way ANOVA, respectively). Data is shown as means (n ≥ 4 per group) with standard deviations. All groups were sensitised with four daily OVA intranasals and samples were collected one week after sensitisation. Post- groups received an OVA re-challenge 24 hours prior to sample collection.
Figure 3.7. T cell gating scheme. Firstly, forward and side scatter was used to create a cell gate (A.). T cells were defined as CD11b-, CD3+ (B.) and CD45+ (C). T cells were then subdivided into CD4+ and CD4- subtypes (D.). CD4+ T cells were further classified based on their expression of FoxP3 (E.).
Figure 3.8. T cell proportions. CD4+ T cell proportions (A.), CD4- T cell proportions (B.), FoxP3+ CD4+ T cell proportions (C.) and FoxP3- CD4+ T cell proportions (D.). Statistical significance between groups was determined using a two-way ANOVA with Tukey’s multiple comparisons, shown as **p<0.01, ***p<0.001. Statistical significance between pre- and post- samples were determined using multiple t-tests, and shown as ns = no significance. Data is shown as means (n ≥ 4 per group) with standard deviations.
Figure 3.9. CD25 and FoxP3 mfi's for T cells. Tregs CD25 (A.) and FoxP3 mfi's (B.). Non-Tregs CD25 mfi (C.). CD4+ T cell CD25 mfi (D.). Statistical significance between groups was determined using a two-way ANOVA with Tukey’s multiple comparisons, shown as **p<0.01. Statistical significance between pre- and post- samples were determined using multiple t-tests and shown as ns = no significance. Data is shown as means (n ≥ 4 per group) with standard deviations.
3.5.2.2 Dendritic cells and monocytes

DCs and monocyte proportions were assessed, due to their role in antigen presentation to lymphocytes. DCs can be subdivided into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs were gated according to Figure 3.10., while pDCs were gated according to Figure 3.11. Baseline cDC proportions did not differ between any of our experimental groups (Figure 3.12.). Similarly, pDC proportions at baseline did not differ between any treatment groups (Figure 3.12.). DCs were also assessed for any proportion changes with OVA re-challenge. DC proportions did not significantly change with OVA re-challenge for any treatment groups (Figure 3.12.).

Monocyte gating can be seen in Figure 3.10. Monocyte baseline proportions did not differ between any of our treatment groups. Monocyte proportions were also unchanged between pre- and post- OVA re-challenge for any of the treatment groups (Figure 3.13.B.).

3.5.2.3 B cells

B cells were gated according to Figure 3.11. Baseline B cell proportions did not differ between treatment groups. Furthermore, B cell proportions did not differ with OVA re-challenge for any treatment group (Figure 3.13.A).

8.5.2.4 Adaptive immunity findings

Together, these findings suggest the adaptive immune response did not differ between virally infected, and non-infected sensitised animals. However, DNase treatment did
induce a Treg response, as observed by increased Treg proportions, and increased Treg FoxP3 mfi’s, in the OVA+Virus+DNase experimental group, compared to OVA and OVA+ Virus groups.
Figure 3.10. Conventional dendritic cell (cDC) and monocyte gating scheme. Forward and side scatter was used to create a cell gate (A.). cDCs were defined as CD11b+, CD3- (B.), CD161- (C.), and MHCII+ (RT1B+) (D.). cDCs were then further subdivided by CD4 and CD172 expression (E.). Monocytes were defined as CD11b+, CD3- (B.), CD161- (C.), MHCII- (RT1B-) (D.), CD4+ and side scatter (F.).
**Figure 3.11. Plasmacytoid dendritic cell (pDC) and B cell gating scheme.** Forward and side scatter was used to create a cell gate (A.). Both were defined as CD3-, CD11b- (B.) and CD45R+ (C.). B cells and pDCs were then distinguished by their CD4 and MHCII (RTB1) expression (D.).
Figure 3.12. Plasmacytoid dendritic cell (pDC) and conventional dendritic cell (cDC) proportions (out of cell gate). cDCs were then analysed as DP (CD4+ and CD172+) cDCs (C.), DN (CD4- and CD172-) cDCs (D.) and CD4-, CD172+ cDCs (E.). ns = no significance between pre- and post-rechallenge. Data is shown as means (n ≥ 4 per group) with standard deviations.
Figure 3.13. **B cell (A.) and monocyte (B.) proportions** (out of cell gate). *ns* = no significance between pre- and post-rechallenge. Data is shown as means (*n* ≥ 4 per group) with standard deviations.
4. DISCUSSION

Although respiratory viral infections are strongly correlated with development of allergic asthma, little is known about its impact on IgE sensitisation, due to conflicting and limited research. Furthermore, it is not known precisely how viral infection may produce (or alternatively protect) against allergic disease. Our project aimed to explore this link between virus and allergy. In addition, we tested if this potential effect was mediated through NETs, given these were recently reported to be induced during viral infections (41, 153) and induce a Th2 response (144).

To do this, rats were infected with vMC0, as a model of HRV infection. Animals were then given four daily OVA exposures, as a non-pathogenic model allergen. The BN rat strain was used, as they are naturally susceptible to Th2 inflammation. This both removed the need for an adjuvant and provided a more clinically relevant model, as previous skewing towards a Th2 effector response often proceeds allergy development (102, 154). To then test the effect of extracellular DNA on allergic sensitisation, DNA was degraded using DNase, as this has been previously shown to degrade NETs (41). In addition to assessing virus and NET influences on IgE sensitisation, we determined if viral infection and NETs influenced the subsequent allergic recall response, by re-exposing the animals to OVA one week after their last sensitising exposure.

4.1. vMC0 infection model

Rhinovirus does not naturally infect rodents (149) and thus does not establish the clinical and immunological outcomes seen in HRV infection. vMC0 belongs to the same
*Picornaviridae* virus family as HRV (149, 155) and establishes a lower airway inflammatory response in BN rats, thus mimicking features of HRV inflammation (125).

Previous studies found infection with $10^7$ PFU of attenuated vMC0 resulted in significant reductions in weight gain percentage three days after viral infection, compared to those who received the vehicle control (supernatant from uninfected HeLa cultures). Furthermore, they saw no significant difference in weight gain percentage when UV-inactivated vMC0 was compared to vehicle controls (125). Interestingly, our model of vMC0 infection saw no significant differences in weights between our three experimental groups. Four days post-infection, both virally infected and non-infected groups displayed no differences in weight gain percentages (*Figure 3.1.A*). This suggests only a mild level of vMC0 infection was established in our model. This is in accordance with previous work from our research group, where the virus can be detected in BN rats 1-2 days post-infection, even in the absence of inflammatory symptoms (147).

To further explore the effects of vMC0 infection, cellular infiltrates were analysed during suspected peak of viral infection. Peak infection was expected to occur between days 1-3 post-inoculation, as significant viral titres have been observed in lung and BAL samples between these days, in a similar model (125, 147). BAL samples were collected on day 3 post-inoculation, and the number of airway-infiltrating cell types were compared between our treatment groups. This analysis also occurred mid-way through the sensitisation process, with and without virus infection/extracellular DNA, and thus the cellular response during sensitisation could be observed, as previously demonstrated in OVA sensitisation models (27, 123). The total number of BAL cells did not significantly differ between treatment groups (*Figure 3.1.B*). The number of macrophages in BAL
fluid did not differ between OVA and OVA+Virus groups. However, total macrophage numbers were reduced in OVA+Virus+DNase animals, compared to the OVA only group (Figure 3.1.C.). No other cell type displayed significant changes in numbers when our three groups were compared. Yet, there was a trend that eosinophils cell numbers were increased in the OVA group when compared to the OVA+Virus and the OVA+Virus+DNase groups. These results are different to what was previously observed during vMC0 infection in BN rats, where a significant infiltration of neutrophils and lymphocytes were observed three days post-infection (125).

Human models assessing BAL cell numbers in HRV infected allergic individuals, typically see increased eosinophil infiltration (156-159). Hence, it interesting that we did not observe a similar increase in eosinophil numbers, in our model of virus infection and sensitisation. Such disparities between previous studies and our research project may indicate that a lower respiratory infection was not achieved in our model. As a previous study still observed a significant increase in neutrophils and lymphocytes when the vMC0 dosage was lowered ten-fold (125) it is unlikely the dosage we used was too low to achieve infection. However, it is important to note that we did not have a naïve control group, as non-infected animals were exposed to OVA, which would have likely affected immune cells in the respiratory tissues.

Another reason why we may not have observed cellular differences is that vMC0 had already been resolved by day three post-infection. While one previous study of vMC0 infection in BN rats still observed significant cellular infiltration and viral titres day three post-infection, the highest levels were observed one day post-infection (125). Furthermore, previous
studies by our group found that viral loads peaked at day one and had largely resolved by
day three (147). These results may explain why we failed to see any significant cellular
infiltration in response to virus three days after vMC0 infection. The same study found total
BAL cellularity was significantly increased at day one post-infection when compared to
uninfected controls. They further observed an influx of macrophages with virus infection,
and a notable lack of neutrophil recruitment in BAL fluid when sensitised BNs were
compared to a Th2-low strain. This may explain why we failed to see the typical neutrophilic
anti-viral response. They suggested that BN models likely exhibit a defective antiviral
immune response, as observed in some models of human allergic disease (147). For
example, this type of defective anti-viral response has been seen in peripheral blood
mononuclear cells from atopic asthma patients in response to HRV. Papadopoulos and
colleagues observed significantly lower levels of IFN-γ and IL-2 compared to non-atopic
patients, indicating that the Th1 response to viral infections may be defective in atopic
individuals (130).

Other murine models of Th2 high HRV infection have typically involved Balb/c mice (122,
160-162). These models typically display acute neutrophilic inflammation following viral
infection. Potentially, these models may be a more accurate model for analyzing the effect
of virally induced-NETs with DNase degradation. Future studies should look to confirm vMC0
infection by assessing viral titres in lung tissues three days post-inoculation, as well as
assessing cellularity and weight changes. Furthermore, cellular infiltrates and viral titres
should also be assessed one-day post-inoculation.
4.2. OVA sensitisation model

BN rats were used for OVA sensitisation, as their Th2 pre-disposition leads to a more pronounced allergic response, compared to less Th2-prone strains (147). Traditional methods of OVA sensitisation involves an intraperitoneal injection with an OVA/Alum solution, and re-challenging via nebulisation with 1% OVA solution (123). However, more recently, OVA sensitisation has been achieved with four consecutive daily OVA intranasals (148). This model is particularly suitable, as sensitisation is achieved without an adjuvant which may mask subtle differences in degree of sensitisation. We confirmed that OVA sensitisation had been achieved in our model by comparing OVA-specific IgE levels in serum (in pre-OVA re-challenge groups) to naïve controls, using an ELISA (Figure 3.2).

4.3. Effect of virus/DNase treatment on allergic sensitisation

OVA-specific IgE was significantly higher in OVA and OVA+Virus groups, compared to naïve controls (Figure 3.2.). In these groups, 100% of our animals (n=6) were sensitised (sensitisation was defined as titres above two standard deviations of the mean of naïve rats). There appeared to be no difference between OVA and OVA+Virus groups, suggesting virus infection did not alter sensitisation. Interestingly, OVA+Virus+DNase groups failed to establish significantly higher OVA-specific IgE titres, compared to naïve controls, indicating that DNase treatment may be reducing the risk of OVA sensitisation. Only 60% of animals in this group (n=5) established OVA sensitisation (Figure 3.2.).

In addition to the level of IgE, induction of additional Ig isoforms may influence allergic disease. Therefore, we also measured OVA-specific IgG titres in our three groups and
compared IgG:IgE ratios (*Figure 3.5.*). No difference was observed between any of the groups, suggesting the effect observed in the OVA+Virus+DNase group was specific to IgE induction, and not IgG. However, a low sample size (n ≥ 4), substantially reduces the power of this result. This was an over-arching limitation of our study design.

To assess why DNase might reduce the rate of OVA sensitisation, the DNA content in BAL fluid was measured three days post-vMC0-inoculation, following two OVA/DNase intranasals. DNA content in BAL fluid did not significantly differ between our three treatment groups (*Figure 3.4.*), indicating that virus infection and DNase treatment did not alter DNA content. This suggests differences to sensitisation occurred independently of DNA in BAL fluid and not due to reduced DNA content. During our optimisation of DNase dosages (*Appendix 6.1.*), increased DNase concentrations interestingly correlated with an increased trend in BAL DNA content, in female BN rats with elevated baseline airway inflammation (*Section 6.1., Figure 6.3.*). In a bovine model of viral respiratory infection (with a bovine respiratory syncytial virus), DNase treatment resulted in NET degradation, and an increase in BAL DNA content (41). While at first counter-intuitive, this increase in DNA suggests that DNase treatment is fragmenting and “freeing” DNA previously bound in NETs or inflammatory mucus plugs allowing detection in BAL (41). We may not have observed a similar change in our main treatment experiments, due to the low level of inflammation in BN males, compared to BN females (unpublished preliminary data). This reflects what is seen in another murine model of allergic sensitisation, where female Balb/C mice are typically more susceptible to Th2 inflammation than males (162). Additionally, the bovine model described above was characterised by neutrophilic inflammation, typical of severe RSV lower tract infection.
in children (41, 163, 164). Hence, this suggests that in our model of mild respiratory infection, substantial amounts of NETs/ extracellular DNA may not have been produced. Using confocal microscopy, our collaborators did detect signs of NETs in BAL fluid (Figure 3.3.). However, this is yet to be systematically analysed.

To further assess how viral infections and extracellular DNA influence the airway immune composition, cellular airway infiltrates were examined. Both innate and adaptive responses were considered, by analyzing both BAL fluid and lung flow cytometry data, respectively. Baseline cell composition in BAL did not significantly differ between the three groups, when samples were collected at one week after infection/sensitisation, prior to re-challenge. This is particularly interesting, as a decrease in macrophages was seen for our OVA+Virus+DNase group, compared to OVA only rodents, when samples were collected day three-post infection. This would suggest that DNase may alter the acute cellular response to infection, but that this difference is not noticeable one week after infection. This is not unusual, as innate responses are typically rapid, but short-lived (165). As no differences were observed for any cell types between our three experimental groups a week after infection, and no differences for any other cell types three-days post-infection, it would appear that virus infection is not altering the cellular response during the sensitisation process.

Since no difference was observed in major cell subtypes with virus infection and DNase treatment, the decreased rate of sensitisation in our OVA+Virus+DNase group may not translate to a difference in airway composition. In Cortjens and colleagues non-sensitised model, observing no difference between infected and non-infected groups suggested that NETs were not integral to the host-anti-viral response (41). However, observing the same in
our model, more likely suggests that NETs/ extracellular DNA may be involved in the sensitisation process, but not required for viral defence. However, due to the proposed defective anti-viral response in the rat strain used (147), it may be useful to confirm this finding in a non-Th2 high strain, while still allergically sensitising, to confirm if NETs play a greater role in viral defence. However, designing such a model would be complicated, as Th2 skewing is a typical pre-disposition observed in atopic individuals, and currently required for most atopic animal models (122, 123, 166, 167).

Furthermore, as our samples were collected one week after infection, we expected to observe variations between our three groups considering the expected activation of the adaptive immune response during the viral infection (3, 91, 168). To analyse this, proportions of cells involved in viral immunity was assessed with multiparameter flow cytometry. T cells, B cells, monocytes and DC proportions were considered, as the major cell types involved with the adaptive immune response. DC and monocyte proportions were considered, due to their role in antigen presentation to B and T cells (3). We expected possible changes reflecting a Th1 (viral) or Th2 (allergic) response. Virus infection during allergy development has been proposed to be both protective and inductive towards allergy sensitisation in previous models (169-172).

We uncovered no differences in B cell proportions between our three treatment groups (Figure 3.13.). Furthermore, no differences were seen between baseline proportions for monocyte and DCs (Figure 3.13.). Baseline CD4+ and CD4- T cell proportions further uncovered no significant differences between treatment groups. However, when CD4+ cells were divided into Treg and non-Treg cells, DNase treatment was found to markedly
heighten Treg populations, when compared to both OVA and OVA+Virus groups (Figure 3.8).

To explore this difference further, expression levels (as mfi’s) were compared for two main Treg activation markers, CD25 and FoxP3. CD25 forms part of the IL-2 receptor (IL-2 receptor alpha chain) together with CD122 and CD132 and is expressed on activated Tregs. FoxP3 is a master regulatory transcription factor crucial for Treg development and function (58). Considering the mfi’s of these markers, we could examine the level of expression within each treatment group, and thus determine Treg activation levels for each treatment group. Interestingly, Treg CD25 mfi’s did not significantly differ between our treatment groups, suggesting that while a higher proportion of Tregs were observed with DNase treatment, these cells were not significantly more activated. Yet, Treg FoxP3 mfi’s were higher for DNase treatment groups, but only when compared to OVA exposed animals. This suggests DNase treatment increases FoxP3 expression on Tregs, as well as increasing Treg proportions.

Tregs are a subset of T cells involved in immune suppression, by actively preventing the activation and expansion of activated lymphocytes. This is an important function, in particular for preventing excess-inflammatory damage following an infection and preventing autoimmune disorders. Depletion of Tregs has been shown to augment the immune response to both self and non-self antigens. Tregs have been shown to suppress allergy, thus, Treg deficiencies can evoke a T cell-mediated autoimmunity and immunopathology (171, 173).
The transcription factor FoxP3 expression is a critical component for Treg differentiation. Disruption of FoxP3 in both human and murine models result in proliferative lymphocyte diseases (56, 58, 174, 175). Foxp3 deficient mice lack Tregs, while mice that over-express Foxp3 possess elevated levels of Tregs (55, 58). Hence, increased expression of FoxP3 by Tregs, likely induced more Treg differentiation, increasing Treg proportions (56, 70, 176).

Expansion of Tregs has been used to induce tolerance following tissue transplants, by suppressing graft rejection, through inhibiting activation and expansion of Teffs. Additionally, recruited naïve T cells may differentiate into Tregs, due to FoxP3 expression, further boosting immuno-suppression. The same principle could be applied to autoimmune disease and allergy (58, 68, 70, 160, 171, 173, 176-178). Hence, the higher proportions of Tregs observed with DNase treatment, likely indicates a protective function towards atopy and possible association with lower levels of OVA-specific IgE, in this group. This in an exciting finding, as it alludes to an inducible protective response with DNase treatment. The debate regarding whether viral infections are inductive or protective against allergic disease indicates the complex nature of allergic disease development. Here, we show that DNase treatment following a viral infection may induce a regulatory response, capable of reducing allergy sensitisation.

4.4. Effect of virus/DNase treatment on allergic recall

Along with comparing differences between our treatment groups at baseline, each group was additionally re-challenged with OVA one week after sensitisation/infection,
to determine if virus infection/DNase treatment altered the response to allergen re-challenge.

OVA-specific IgE titres were expected to increase in response to allergen-re-challenge, indicative of an allergic response. However, this was not seen for any of our treatment groups (Figure 3.5). Similarly, analysis of innate and adaptive cells uncovered no differences between pre-and post-OVA re-challenge groups, for any treatment groups.

The lack of observable re-call response suggests that our animals failed to establish symptomatic allergic disease, despite establishing OVA sensitisation. This result mimics a common situation in the human population. In the Western world, 50% of people will react positively to a skin prick test, indicating that they are sensitised to a particular antigen. However, only a further 50% of these individuals will actually develop allergic symptoms if exposed to the allergen (179-181).

A previous model of OVA sensitisation and vMC0 infection similarly saw no significant differences in OVA-specific IgE titres, when OVA sensitised animals were compared to those co-exposed to virus and OVA. However, this model involved sensitising with an intraperitoneal OVA/Alum injection, two weeks prior to vMC0 infection (147). Including an adjuvant in this way may be required to generate high IgE titres that result in an allergic recall response. This finding highlights the complexity of the allergic disease, by emphasising the importance of sensitisation in conjunction with an adjuvant. Viral infection, in this case, does not appear to enhance the immune response in the same
way as the Alum adjuvant, indicating that something else may be required at the time of allergen exposure to trigger allergic disease. Future studies should consider using a stronger viral infection model, such as a higher dose and/or more virulent virus strain.

4.5. Future directions

Early life respiratory infections may be both protective or inductive of allergic disease. NETs, or extracellular DNA, induced by viral infection, were proposed to be inductive, by inducing a Th2 response. In the current model, we failed to observe any effect of viral infection on IgE sensitisation. However, a protective function of DNase treatment was observed. Rather than reducing the effect seen from viral infection, DNase treatment induced Treg differentiation or recruitment to the airways. Tregs have been shown to protect against infection, by suppressing the Th2 effector response. Hence, it would appear an immunomodulation approach, by increasing regulatory cells, is likely to promote protection against allergy development.

However, this effect has only been seen in a single rat strain. As shown by the abundant, conflicting evidence regarding virus infection and allergy, allergic disease development is a complex and developing field. This model includes one genetic strain, with high baseline Th2 inflammation, and a potentially defective viral response. Furthermore, this study only included a single, low virulence viral strain, where a low level of respiratory infection was produced. The ability of DNase treatment to induce a protective, regulatory response, will need to be applied to multiple genetic phenotypes, including testing in both genders, to establish its applicability to human disease models. Moreover, all experiments were conducted in adult rats, while human allergy
development typically occurs in younger individuals. Nevertheless, these findings add to the ever-increasing mass of knowledge surrounding allergic disease development, and will hopefully contribute to reducing the ever increasing burden of allergic disease on human health.
5. CONCLUSIONS

This project initially aimed to explore how virus infection may contribute to allergic disease development, and test the involvement of NETs or extracellular DNA, recently reported to be produced during viral respiratory infections. This was done by treating virally infected animals with DNase, a drug previously shown to efficiently degrade NETs. Although no effect on IgE sensitisation was observed due to virus infection, DNase treatment, reduced the risk of IgE sensitisation and increased Treg proportions in the lungs. Furthermore, Tregs in DNase treated animals displayed higher levels of FoxP3, compared to OVA exposed animals. This likely suggests that DNase treatment is encouraging a regulatory response, potentially protecting against allergic sensitisation.

While we initially sought to utilise DNase to degrade NETs and reduce a virally induced Th2 response, we instead saw little difference between the immune profiles of virally infected and non-infected sensitised animals. Future studies should, therefore, use a more virulent virus strain to ensure detectable disease. Ideally, they should also utilize increased sample numbers to increase statistical significance. In summary, the protective, immunoregulatory response following DNase treatment, has uncovered a possible novel strategy of allergy protection and should be explored further.
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6. APPENDIX

This chapter contains the optimisation protocols and findings used to determine protocols used in the main experimental methods. DNase dosage was decided from a series of dose safety experiments. Final IgE and IgG ELISA protocols, were also based off a series of optimisation protocols. Both the methods and findings for both these optimisations are outlined below.

6.1. DNase safety testing

6.1.1. Experimental design

The OVA sensitisation and vMC0 infection protocols were established in the lab, however, before we could start using the model, DNase-I exposure needed to be optimised and deemed safe in our model. For this purpose, 8-week-old female BN rats received DNase-I intranasals, ranging from 0-200 µg/ml, according to Table 8.1. DNase was given intranasally in a solution of sterile PBS (100 µl), under isoflurane aesthetic. This treatment commenced for four consecutive days in accordance with further experimental plans (Figure 2.1.). To assess cell viability, cells from bronchoalveolar lavage (BAL) and lung tissue were collected 24 hours after the last DNase intranasal, as per Section 2.3.
Table 6.1. DNase concentrations and number of rats used for DNase safety testing

<table>
<thead>
<tr>
<th>DNase concentration (µg/ml)</th>
<th>Number of female BN rats used</th>
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<tbody>
<tr>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>50</td>
<td>3</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
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<td>200</td>
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Figure 6.1. DNA safety testing. Animals received four daily DNase intranasals. Lung and BAL samples were collected 24 hours after the last intranasal.
BAL fluid and lung tissue samples were processed as per method section 2.4. For lung and BAL samples, cells were stained with trypan blue to discriminate dead (positive) and live (negative) cells. Cells were then counted so that dead cell proportions could be obtained. Proportion of cell types in the BAL samples were also counted as previously described (Section 2.4.3.). To assess if DNase treatment had an impact on DNA released into the BAL, the DNA content of BAL samples was analysed as described in the DNA quantification section (Section 2.11.).

In addition to trypan blue staining, BAL and lung samples were stained using Annexin-V and Viability dye and assessed using flow cytometry. The staining protocol was as previously described (Section 2.5), however, included a different set of antibodies and viability stains to assess if different type of immune cells responded differently to DNase exposure (Table 8.2.). Single stains were measured for each antibody, as well as annexin-V and viability dye fluorescence minus one’s (FMOs).

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Fluorochrome /Host</th>
<th>Dilution</th>
<th>Company</th>
<th>Cat</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>FITC</td>
<td>1:100</td>
<td>BD</td>
<td>557354</td>
</tr>
<tr>
<td>MHCII</td>
<td>PerCP-Cy5</td>
<td>1: 100</td>
<td>BD</td>
<td>744131</td>
</tr>
<tr>
<td>Annexin-V</td>
<td>BV510</td>
<td>1:80</td>
<td>Biolegend</td>
<td>640937</td>
</tr>
<tr>
<td>Viability dye</td>
<td>BV450</td>
<td>0.1 ul per 100 ul</td>
<td>BD</td>
<td>562247</td>
</tr>
</tbody>
</table>
6.1.2. Dead cell proportions

The proportion of dead cells for each DNase dosage was determined using flow cytometry (*Figure 6.2.*) and trypan blue exclusion. Using either of the methods, no significant difference in dead cells with increasing DNase doses was observed for BAL or lung samples (*Figure 6.2.A.*) Furthermore, these two methods were compared, and no significant differences were observed at any DNase dose (*Figure 6.2.B.*).

Interestingly, both BAL and lung baseline dead cell proportions were high. This was likely due to the use of female BN rats. The BN strain display lungs with high baseline inflammation (145). In particular, females have a higher level of inflammation compared to males (27, 146). Although male rats were used for the main project, due to the limited availability of animals, females were the only available option for this optimisation in the limited timeframe. Despite this limitation, these findings show DNase did not influence cell viability even in an inflammatory environment.

6.1.3. BAL differential counts

BAL differential counts were also analysed, to determine if airway inflammation was affected by the different dosages. Interestingly, a significant increase in the number of eosinophils was seen with the highest DNase dose (200 µg/ml) (*Figure 6.2.D.*). Given the elevated baseline and variability observed within BN females (unpublished data), this dose was further tested in male rats. However, due to the limited time frame and available animals, a direct comparison of male and females was not achievable. Instead, samples from another aspect of the study were used. A small number of male rats received 200 µg/ml DNase and 1 mg/ml OVA intranasals over two days, and BAL samples were collected 24 hours after the last exposure (*Figure 2.1*). Although this is not directly comparable to the females, only limited eosinophil infiltration was observed at this timepoint in male rats (*Figure 6.2.E.*). This suggests the increased eosinophil infiltration
observed at 200 ug/ml DNase was a female only effect, associated with their high baseline inflammation.

While unable to conclude that this difference was caused by gender, due to the numerous variables introduced by this comparison, this outcome may explain why a high eosinophil proportion was observed with the highest DNase dose. We propose that the increase in eosinophils was due to the use of female test subjects, rather than the 200 µg/ml DNase dose, since it remained within the normal range of female variation. Future safety tests should utilise animals of the same gender, as well as strain and age, to test this idea.

6.1.4. DNA content of BAL samples

The DNA content in BAL fluid was quantified to determine the effect of increasing DNase concentrations (Section 2.11.). We hypothesised that increasing the DNase concentration would proportionally reduce the DNA content in BAL fluid. Interestingly, the opposite appears to be true. A significant increase in DNA was observed between 0 and 200 µg/ml, and 50 and 200 µg/ml of DNase (p<0.05, p<0.01, respectively) (Figure 6.3.). An explanation for this may be that the increased amount of DNase results in an increased amount of released DNA, airway inflammation and eosinophil infiltration.
**Figure 6.2. DNase safety testing.** (A.) Gating scheme used to assess cell viability. Cells were gated according to forward and side scatter. Dead cell proportions were then obtained using Annexin V and Viability dye. (B.) Dead cell proportions for BAL and lung samples. (C.) Comparison of trypan blue and flow cytometry-based methods. (D.) BAL cell type numbers for each DNase dose. Dosages were compared for each cell type using a two-way ANOVA with Tukey's multiple comparisons, and significance shown as **** p<.0001. (E.) Male and female BAL cell type proportions. Statistical significance between male and females was determined using multiple t tests, shown as **** p<.0001. All bar graphs are displayed as means (n=3) with standard deviations.
Increased eosinophil infiltration was seen with a 200 µg/ml DNase dose, indicating a trend between DNase-induced DNA release and inflammation. However, increased eosinophils were not seen in the next lowest dose, despite an increased DNA trend. This suggests that DNA was released into BAL, independent of eosinophil inflammation.

6.1.5. Reasoning for dosage decision

Since no significant difference in dead cell proportions was seen across the different dosage groups, the highest dose was used for further experiments to maximise the potential to degrade extracellular DNA.

However, it is important to note that the BAL differential count and DNA quantification data indicate this may not have been the best choice. Differential counts indicted the 200 µg/ml DNase dose alters eosinophil numbers and proportions, and thus the 100 µg/ml dose may have been a safer option. However, this finding was observed in female animals, which may explain this result. Preliminary data (unpublished) suggested this was not applicable to males. The 200 µg/ml dose also showed an increase in DNA content in BAL, compared to both the 0 and 50 µg/ml doses. This DNA release did not appear to correlate with eosinophil infiltration, hence, it appears that DNase does not induce inflammation, but unexpectedly lead to more DNA in BAL supernatant.

Future studies with DNase-I should involve further safety testing, preferably in animals of the same strain, age and gender. It may also be useful to test the source of increased DNA content in BAL supernantant. Potentially, known amounts of free DNA could be treated with DNase, to determine if increased DNA concentrations are primarily due to degradation.
Figure 6.3. DNA content in BAL supernatant for different DNase dosage groups. An increased trend was seen for both the 100 and 200 µg/ml DNase dosages. *p<0.05, **p<0.01. Data is displayed as means (n=3) with standard deviations.
6.2. IgE/IgG ELISA optimisation

The ELISA protocol to quantify OVA-specific IgE and IgG antibodies was developed from a series of optimisation experiments. Two different methods were considered. These methods were based on two strategies, as outlined below.

6.2.1. Capture IgE

The preferred method involved coating the plate with antibodies specific for IgE, removing other OVA specific antibodies that may interfere with detection. Since serum concentration of IgE is very low, this method would be preferred. IgE from serum was bound to these antibodies, and OVA-specific IgE was then detected using fluorescently tagged OVA. This was then detected using an EnSpire multi-plate reader (Perkin Elmer).

To test this method, 50 µl of mouse anti-rat IgE antibody (Bio-rad, MCA193) at 5 µg/ml in PBS, was coated onto Maxisorb 96 well (ThermoFisher, 44-2404-21), and left to incubate overnight at 4 °C. Following coating, plates were washed with PBS with 0.05% Tween-20 (Sigma, P2287-500ML), using a BioTec plate washer (W760). To block unspecific binding, plates were incubated with 200 µl of PBS- 1% BSA for one hour at room temperature.

Following washing, serial dilutions (same 50 µl volume) of positive and negative control samples (serum from a BN rat with confirmed OVA-specific IgE, and serum from a naïve BN rat, respectively) were added to the plates, and incubated for 90 minutes at room temperature. Both positive and negative samples were provided from a previous study.
After incubating and washing, 5 µg/ml or 50 µg/ml of OVA-AF488 (50 µl, Invitogen, 034781), was added in duplicates.

After a one-hour incubation at room temperature and washing, fluorescently tagged OVA was detected at 490 nm using the EnSpire multi-plate reader, before and after washing.

Assays were classed as successful if positive controls displayed a higher fluorescent signal when compared to negative controls. Ideally, negative controls values would display no fluorescence once minused from the blank control.

A successful result was only observed for the highest serum concentration (12.5%) and highest OVA-AF488 concentration (50 µg/ml) (Figure 8.4.B.). Due to these findings, the method was repeated, with a serum concentration of 20%, and serial dilutions of OVA-AF488. A differently labelled OVA (OVA-TexasRed, ThermoFisher, 023021) was also analysed as a series of dilutions when the method was repeated (Figure 8.4.C and 8.4.D.). We also included BSA coated plates to detect unspecific binding of OVA. Once unspecific binding was subtracted from the detected signal, no consistent difference in fluorescent signal was detected between positive and negative serum samples from any of the two OVA versions.
6.2.2. OVA coated plates

Since low levels of OVA specific IgE were expected to be present in serum samples which may not be detectable using a fluorescence based assay, a second method of detection was trialled. In this method, the plates were initially coated with OVA, which will bind OVA-specific antibodies from serum. Bound IgE was specifically detected by adding anti-rat IgE antibody. A secondary antibody, coupled to horseradish peroxidase (HRP) was then added, so that bound IgE could be quantified using colour-based absorbance.

To test this method, 5 µg/ml of OVA in PBS, was added to Maxisorb 96 well plates, and left to incubate overnight at 4 °C. Following coating, plates were washed with PBS with 0.05% Tween-20, using a plate washer. To block unspecific binding, plates were incubated with 200 µl of PBS- 1% BSA for one hour at room temperature.

Following washing, serial dilutions of positive and negative serum samples were incubated for 90 minutes at room temperature. Serum samples were the same as those used for the capture IgE method.

Two different dilutions (1:500 or 1:2000 in PBS with 0.1% BSA) of mouse anti-rat IgE antibody were then incubated for one hour at room temperature. After washing, plates were incubated with sheep anti-mouse-HRP antibody (Amersham Biosciences, NA931) (1:2000 in PBS with 0.1% BSA) for 30 minutes at room temperature.
After another wash, 50 µl of tetramethylbenzidine (TMB) solution (BUF056A, 170811, Bio-Rad) was added to each well. TMB produces a blue colour by donating a hydrogen during reduction of H₂O₂ that is facilitated by HRP. The reaction can be stopped using orthophosphoric acid. Absorbance was firstly read prior to stopping the reaction, at 650 nm using the Enspire plate reader. The reaction was then stopped after 30 minutes using 50 µl of orthophosphoric acid (1 M). Absorbance was then immediately read at 450 nm using the Enspire multi-plate reader. For both antibody dilutions, a dose-dependent increase in signal was observed for positive samples (Figure 6.5).

To confirm that the signal was specific to OVA specific IgE, this method was repeated to include BSA coated control wells. This allowed for background absorbance values to be observed. Using this method, a consistent difference, that increased with increasing serum concentrations, in positive and negative serum samples were obtained (Figure 6.5).

6.2.3. Method choice

No difference in fluorescence signal was observed between the positive and negative samples using the capture IgE method (Figure 6.4.). This likely occurred due to low detection of fluorescently tagged OVA. Due to these results, this method was not used as the final protocol.

A difference was observed between the positive and negative serum samples when the OVA coating method was used (Figure 6.5.). To calculate this difference, replicate values
were averaged, and BSA coated blanks used to remove background absorbance. The greatest difference was observed with larger serum concentrations (Figure 6.5.C.). Due to these findings, the OVA capture method, with a serum dilution of 12.5%, was used for IgE quantification.

A likely limitation of using the OVA capture method, involves the low levels of OVA-specific IgE in comparison to other OVA-specific antibodies. High levels of other OVA-specific antibodies are likely to interfere with IgE OVA binding sites, due to the low proportion of IgE typically present in serum (0.0001-0.001 mg/ml, compared to 0.3-5 mg/ml for IgG1 (182)). To account for this competition, we also detected OVA specific IgG bound to OVA using an anti-rat IgG1 antibody (eBioScience, 108039000). Using this method IgG:IgE ratios could be obtained for each sample. IgG is present in serum at high levels, and a difference in IgG levels may inaccurately indicate a significant change in IgE levels. By presenting IgE levels as a ratio of IgG, this inaccuracy will be reduced.
Figure 6.4. capture IgE method. Differences between positive and negative serum samples for differing capture-IgE optimisation methods. Data is shown as means (n=2).
Figure 6.5. OVA coated plates. Differences between positive and negative serum samples for differing OVA coated optimisation methods. Data is shown as means (n=2).