The effects of atopy and asthma on *in vivo* human nasal responses to Toll-like receptor agonists

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Abstract

Acute respiratory viral infections cause significant morbidity and mortality, especially in vulnerable individuals, and it is important to study viral pathogenesis and the host immune response in humans. Toll-like receptors (TLRs) play a critical role in the detection of viral nucleic acids, and airway TLR receptors respond to nucleic acid patterns in the RNA viruses that cause respiratory infections. However, a reliable method of measuring mucosal innate immune responses to viral infections is lacking. TLR3 agonists (poly(I:C) and poly-ICLC) and the combined TLR7/8 agonist (resiquimod, R848) are synthetic analogues of double stranded RNA (dsRNA) and single stranded RNA (ssRNA) respectively. Nasal challenge with these TLR agonists was carried out, and serial sampling using nasosorption and nasal curettage was performed. Mucosal immune responses were measured and the effect of different host factors (e.g. asthma) on these responses was studied.

Poly(I:C) and poly-ICLC were well tolerated but failed to induce significant and reliable nasal mucosal innate immune responses. R848 at a higher dose (10 μg/100 μL per nostril) induced significant mucosal interferon and cytokine responses but caused mild to moderate flu-like symptoms in three out of nine volunteers. A lower dose of R848 (0.02 μg/kg/100 μL, mean dose 1.5 μg/100 μL) was subsequently utilised in three groups of volunteers: healthy non-atopic (n=12), allergic rhinitis (n=12) and allergic asthma (n=11). This was well tolerated and induced significant release of nasal mucosal IFNs (IFN-α, IFN-γ), proinflammatory cytokines (TNF-α, IL-2, IL-12p70) and chemokines (CXCL10, CCL2, CCL3, CCL4 and CCL13) when compared to saline. Participants with allergic rhinitis and allergic asthma had similar IFN-α, CCL3 and CCL13 levels that were increased compared to healthy volunteers. In volunteers with atopy, baseline nasal mucosal gene expression of the anti-inflammatory secretoglobin SCGB1A1 had a strong negative correlation with subsequent innate immune activation by R848. Eight hours after R848 challenge, several mucosal IFN stimulated genes (ISGs) were upregulated (IFIT3, OAS2, IRF7, MX1, MYD88, DDX58 and STAT1) as well as SOCS1, TLR3, TLR7, KRT5 and CLEC4C, whilst IFNAR1 and

ADGRG1 were downregulated. Volunteers with asthma had increased DDX58, MX1 and IFIT3 when compared to those with allergic rhinitis and healthy volunteers.

This research has led to the successful development of a non-invasive and well tolerated method to induce and precisely measure nasal mucosal innate immune responses to the ssRNA analogue and TLR7/8 agonist R848, and this methodology has been used to demonstrate heightened innate immune activation in volunteers with allergic rhinitis and asthma. This technique can be extended to examine responses in a range of host conditions and diseases as well as to assess the adjuvant potential of R848 in conjunction with mucosal vaccines targeted against infection and cancer.

Declaration

I hereby declare that this thesis is my own work and has been written by myself. Any work not done by me has been acknowledged in the text. Part of the work described in this thesis has been submitted for publication as a book chapter (Jha *et al*., Precision Mucosal Sampling and Biomarkers in Allergic Rhinitis and Asthma, *Handbook of Biomarkers and Personalized Medicine*, CRC Press).

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ALI	Air liquid interface
APC	Antigen presenting cell
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
cDC	Classical dendritic cells
CTL	Cytotoxic T lymphocyte
dsRNA	Double-stranded RNA
EBC	Exhaled breath condensate
ECP	Eosinophilic cationic protein
FeNO	Fractional exhaled nitric oxide
HRV	Human rhinovirus
IFN	Interferon
IFNAR	IFN-α/β receptor
IFNGR	IFN-γ receptor
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
LPS	Lipopolysaccharide
MABEL	Minimum Anticipated Biological Effect Level
MBP	Major basic protein
MDA-5	Melanoma differentiation-associated gene-5
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MSD	Mesoscale Discovery

MyD88	Myeloid differentiation primary response protein 88
NET	Neutrophil extracellular trap
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NOD	Nucleotide-binding oligomerization domain
NPA	Nasopharyngeal aspirate
PAMP	Pathogen associated molecular pattern
pDC	Plasmacytoid dendritic cell
Poly(I:C)	Polyinosinic:poly-cytidylic acid
Poly-ICLC	Polyinosinic:poly-cytidylic acid stabilized with poly-L-lysine
PRR	Pattern recognition receptor
R848	Resiquimod
RLR	RIG-1-like receptor
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
SAE	Serious adverse event
SAM	Synthetic absorptive matrix
ssRNA	Single-stranded RNA
TIR	Toll–interleukin 1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain containing adaptor inducing IFN-β
VOC	Volatile organic compound

1 Introduction

1.1 Airway mucosal immunity and infection

1.1.1 Immune response to infection

Our habitat is swarming with a multitude of infectious agents, some of which have the potential to cause disease and require host defences that are capable of combating the threat. The ability to recognize foreign “non-self” pathogens (distinguishing them from “self”) and eliminate them effectively necessitates an early response from both cellular and non-cellular components occurring within minutes and hours, predominantly effected by innate immunity. If this is inadequate and pathogen invasion is ongoing, vertebrates have evolved a second line of antigen-specific adaptive immunity that acts within hours and days to provide a targeted and powerful response against a foreign invader. It also has the capacity to possess immunological memory of previous infections and maintain a reserve of cells that can rapidly respond to a previously encountered pathogen (1). The immune response to infection is characterized by different phases: initiation, resolution and restoration, which in healthy individuals, results in successful elimination or containment of the pathogen without corresponding excessive inflammatory sequelae in the host. However, perturbations in any of these immune mechanisms can result in prolonged symptoms, enhanced severity of disease as well as secondary complications such as bacterial infection and even developmental lung disorders (2).

The early days of immunological research in the late 19th and early 20th century saw important progress being made in understanding mechanisms of infectious disease pathogenesis. Paul Ehrlich amongst others noted that specific antigens elicited the release of specific antibodies, developing the idea of humoral immunity. Elie Metchnikoff propounded the concept of a cellular mediated immune response with his work on phagocytosis by macrophages. In the second half of the 20th century, intense research in the field of immunology resulted in fundamental insights, such as the identification

of B cell and T cell cooperation in the immune response, clonal selection theory and T cell restriction to major histocompatibility complex (MHC). Whist the characterization of these key aspects of adaptive immune responses was of critical importance, the detailed mechanism by which invading pathogens were able to induce these effects remained elusive. The antigens used in experiments by researchers did not always lead to the production of cytokines as anticipated, with some having adjuvant activity, whilst others lacked it. This was highlighted by Charles Janeway in his seminal commentary (3):

"In order to obtain readily detectable responses to these antigens, they must be incorporated into a remarkable mixture termed complete Freund's adjuvant, heavily laced with killed Mycobacterium tuberculosis organisms or precipitated in alum and mixed with dead Bordetella pertussis organisms. I call this the immunologist's dirty little secret"

Charles Janeway, 1989

Janeway's hypothesis stipulated that pathogen associated molecular patterns (PAMPs) were detected by invariant innate immune receptors which led to the selection of antigens that would subsequently be recognized by T and B cells in conjunction with antigen presenting cells (APCs). He also proposed that this immune pathway was evolutionarily conserved across vertebrate and invertebrate species (with the latter lacking adaptive immunity). This radical concept laid the foundation for understanding how an immune response to foreign substances is initiated and provided a mechanism for understanding how adaptive immune responses are activated. This revolutionary framework brought research into innate immunity to the fore, resulting in intense efforts to investigate Its key role in the recognition of foreign microbes and culminating in the discovery of several pattern recognition receptors (PRRs) and innate immune mechanisms (4).

1.1.2 Airway mucosal innate immunity

1.1.2.1 Overview

The human airway mucosal surface forms the first line of the host defence against the constant barrage of bacteria, viruses, fungi and allergen that it encounters. The mucous membrane is made up of the ciliated epithelium, which extends from the nose to the respiratory bronchioles and in the healthy state, functions to form an effective physical barrier against pathogens. It can also produce mucus, act as a mucociliary escalator and secretes antimicrobial peptides. The innate immune system is especially focused on mucosal surfaces and co-ordinates an appropriate immune response to a diverse array of inhaled pathogens (figure 1.1). The respiratory epithelium forms a mechanical and immunological barrier with classical dendritic cells (cDCs) extending projections between epithelial cells to the mucosal surface, sampling the luminal fluid and sensing microbial flora. Plasmacytoid dendritic cells (pDCs) are interferon (IFN) factories that primarily produce IFNα and IFNβ in response to viral infections such as human rhinovirus (HRV) and respiratory syncytial virus (RSV) - the leading causes of asthma exacerbation and bronchiolitis respectively. Macrophages have a sentinel function in detecting inhaled microbes and act as professional antigen presenting cells for helper T cells. Natural killer (NK) cells recruited from the bloodstream have surface receptors that can be activated by components of microbes such as lipopolysaccharide (LPS) in bacterial cell walls. Neutrophils respond to local cytokines such as tumour necrosis factor (TNF) and are professional phagocytes. Eosinophils contain secretory granules that release eosinophilic cationic protein (ECP) and major basic protein (MBP) to effect the host defence against helminths as well as allergens, implicating it in allergic rhinitis and asthma. Mast cells release pre-formed histamine due to cross-linking of IgE with their FcεRI receptors in response to common aeroallergens such as grass pollen and house dust mite. Adaptive immunity and production of IgA antibodies are important at mucosal surfaces in combating respiratory pathogens but due to the time taken to generate this response, non-specific innate immunity becomes critical in the first line defence against microbes.

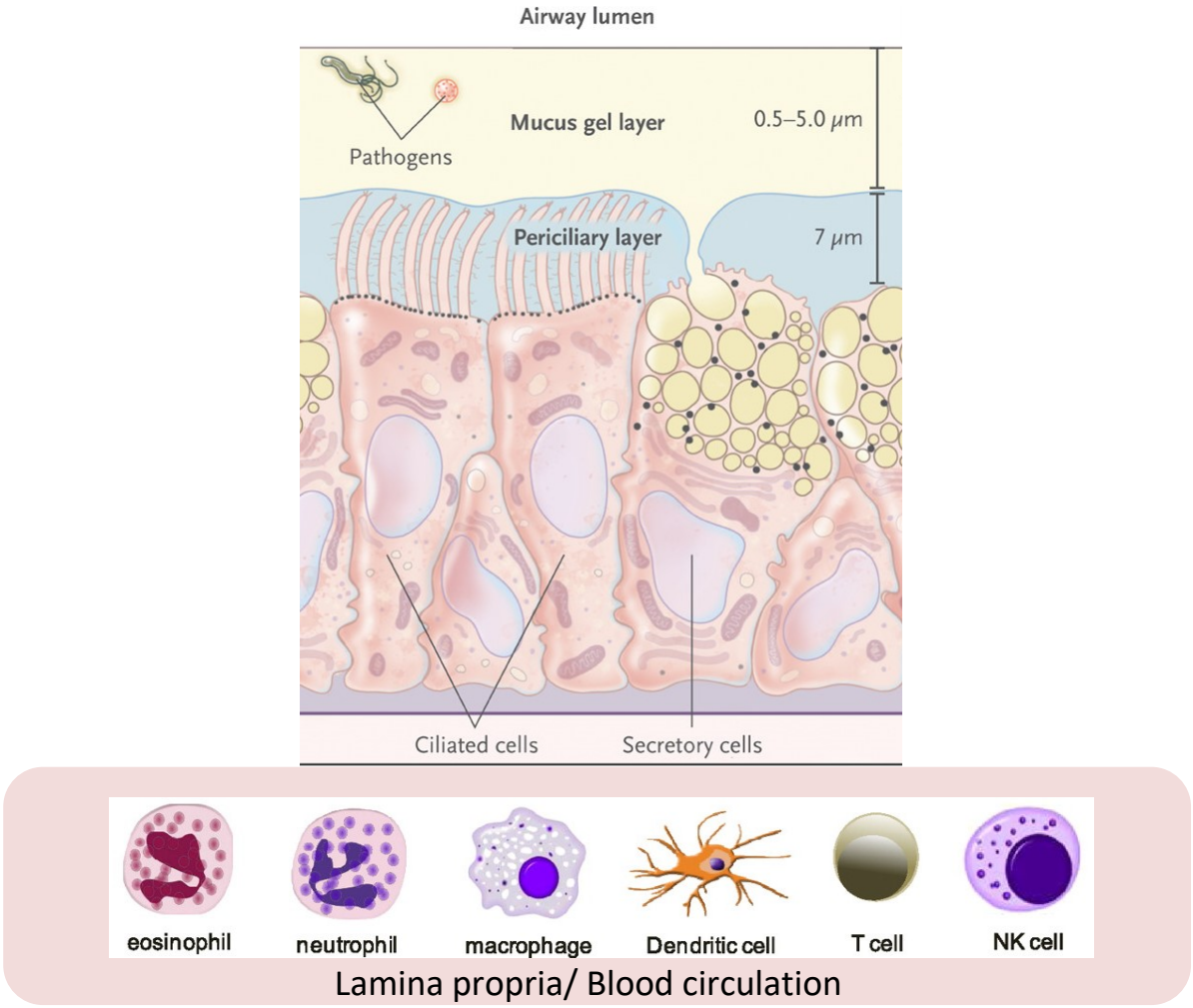


Figure 1.1 Airway mucosal immunity A single layer of pseudostratified columnar epithelial cells extends from the nose to the respiratory bronchioles and is interspersed with secretory cells containing granules and mucous. Whilst the periciliary layer is 7 µm deep throughout the conducting airways, the mucous gel layer extends in thickness from the distal to proximal airways. Innate and adaptive immune cells are either resident in the underlying lamina propria or can rapidly mobilise from the peripheral blood circulation to the mucosa in response to pathogenic or allergen stimuli. Reproduced with permission from (5, 6), Copyright Massachusetts Medical Society and American Society for Microbiology respectively.

Most respiratory pathogens enter through the upper airway to infect host cells. Respiratory RNA viruses typically cause infection in individuals due to inoculation of the nose and eyes via large-particle aerosols or direct contact with an infected person (7). Viral histochemistry studies demonstrate strong tropism of seasonal influenza virus and pandemic H1N1 towards upper airway epithelial and goblet cells. RSV infection typically infects and replicates in the upper airway epithelium with one third of cases progressing to the lower airways (8). Viral attachment and entry into target cells results in the release of double-stranded ribonucleic acid (dsRNA) or single-stranded RNA (ssRNA) intermediates that can bind to cytosolic and endosomal PRRs, initiating the production of proinflammatory cytokines and interferons. In a healthy airway with intact epithelium the innate immune system is able to identify and respond to PAMPs and facilitate the recruitment of macrophages and neutrophils, leading to a cascade of cytopathic cytokines, lipid mediators and reactive oxygen species release to fight infection. Although these have the potential to cause host tissue damage, immune regulatory mediators such as IL-10 and transforming growth factor-β (TGF-β) act to inhibit inflammation to provide a proportionate and controlled mucosal immune response. This balance can be disturbed in diseased states such as asthma and COPD, whilst other host factors, such as certain TLR genetic polymorphisms and the extremes of age can render individuals more susceptible to severe infection.

The airway mucosal innate immune response is reliant on its constituent parts: natural barriers, soluble mediators and cellular responses, with the latter group having overlapping roles in adaptive immunity.

1.1.2.2 Physical and chemical barriers

The mucosal epithelium consists of four major cells types: ciliated, secretory, and undifferentiated basal and intermediate cells, the latter having the capacity to differentiate in to ciliated and secretory cells in response to host damage. Epithelial cells are held together by tight junctions that form a

mechanical barrier, acting to effectively repel most infectious agents. Once activated, epithelial cells have the ability to produce proinflammatory cytokines, release antiviral mediators, recruit innate and adaptive immune cells, and stimulate the clearance of necrotic cells, all of which highlight their critical role in orchestrating the immune response (9). The rapid beating of cilia on ciliated epithelial cells in concert with mucous acts to form a mucociliary escalator in the airway tract, which in conjunction with the cough reflex aid in the clearance of foreign pathogens and particulate matter in a cephalad direction (10). Secretory goblet cells release viscous mucus containing heavily glycosylated proteins, assisting in hydrating the airway mucosa. The mucous blanket exists as two distinct layers with a basal aqueous layer that facilitates ciliary motility, and an overlying viscous mucous layer containing a multitude of glycoproteins, antimicrobial proteins, antimicrobial enzymes and secretory IgA. Whilst the traditional role of mucous has been ascribed to the entrapment of particulate matter including microbes, recent insights suggests a more complex relationship between host and pathogenic organisms. For example, respiratory mucous may play a role in viral transmission and also act as a host restriction factor for influenza virus, possibly via its sialic acid content (11). Microbes that can adhere, colonize or penetrate the epithelial barrier have the potential to cause more invasive infection. Therefore, host states that affect epithelial integrity or impact on effective mucociliary clearance, have impaired antimicrobial responses as seen in primary ciliary dyskinesia, cystic fibrosis, asthma and cigarette smoking (12).

1.1.2.3 Soluble mediators

Conducting airways and submucosal glands are involved in the secretion of a plethora of host defence molecules that serve to form the “humoral” component of innate immunity. Small antimicrobial peptides include cathelicidin (LL-37) and defensins (α and β), and are released from neutrophil granules or produced by epithelial cells, to cause disruption to the cell wall of bacteria, fungi and certain viral envelopes. Larger antimicrobial proteins include lysozomes that enzymatically degrade

bacteria and C-type lectins (Sp-A and Sp-D), secreted by epithelial cells that bind to conserved carbohydrate regions on microbes, facilitating their phagocytosis (13). This latter group also interacts with a major arm of innate immunity - the complement system, a network of more than 30 soluble proteins in the blood, which when activated leads to a cascade of reactions resulting in the direct lysis of pathogens or their opsonisation to facilitate killing by phagocytes (14).

1.1.2.4 Interferons

IFNs are key immune mediators with the ability to *interfere* with viral replication and was originally discovered by Alick Isaacs and Jean Lindermann in 1957 (15). Three IFN families have now been characterized. Type I IFNs include IFN-α (13 subtypes), IFN-β (1 subtype) and several less well defined single gene products; IFN-ε, IFN-τ, IFN-ζ, IFN-κ, IFN-ω, IFN-δ. They signal in a paracrine and autocrine manner via IFN-α/β receptors (IFNAR1 and IFNAR2), which are present on most cells in the body, and lead to a powerful anti-viral state in surrounding infected and non-infected bystander cells (16). Although inducing broadly conserved responses, type 1 IFNs and their subtypes can cause differential activation of downstream mediators and interferon stimulated genes (ISGs), which are likely mediated by differences in IFNAR binding affinity, duration of binding, the number of cell-surface receptors, activation of feedback mechanisms and cell-specific immunomodulation (17). The only type II IFN family member is IFN-γ, which signals via IFN-γ receptor (IFNGR) and is predominantly released by T cells and NK cells, to fight viral pathogens and parasites. The relatively recently discovered type III IFN family members comprise IFNλ1 (IL-29), IFNλ2 (IL28A), IFNλ3 (IL-28B) and IFNλ4 and its production is usually the preserve of epithelial cells. IFN production induces a cascade of ISGs that inhibit viral replication and help orchestrate the adaptive immune response. They are able to induce antigen presenting cells, stimulate DC maturation, help activate T and B cells and promote NK cell survival (18). The cellular sources of interferon production in the airway are varied (table 1.1).

Interferon	Interferon Subtypes	Target Receptors	Main Cellular Source
IFN-α (type I)	13	IFNAR1 IFNAR2	pDC
IFN-β (type I)	Single	IFNAR1 IFNAR2	pDCs, epithelial, fibroblast
IFN-γ (type II)	Single	IFNGR	NK, macrophages, Th1, CTLs, B cells
IFN-λ (type III)	4	IFNLR1 IL10RB	DCs, epithelial

Table 1.1 Main airway mucosal cellular sources of interferon production Summarised from references (16, 19, 20). Abbreviations pDC, plasmacytoid dendritic cells; NK, natural killer; CTLs, cytotoxic T lymphocytes; DCs, dendritic cells; IFNAR, IFN-α/β receptor; IFNGR, IFN-γ receptor; IFNLR, IFN-λ receptor; IL10R, IL-10 receptor.

1.1.2.5 Dendritic cells

Dendritic cells are key players in the immune response to pathogen infection and vaccination with their ability to act as antigen presenting cells and initiate adaptive immunity. In the resting state cDCs project long dendrites that interdigitate between mucosal epithelial cells to continuously sample the airway lumen (21). Once potential pathogenic antigens are encountered, DCs undergo functional maturation, coordinate innate effector cells in eliminating infection and travel to draining lymph nodes to rendezvous with and cause the differentiation of antigen-specific naive $CD8^+$ T cells (22). The importance of DCs in cross-presentation of antigens is highlighted by the lack of cytotoxic T lymphocytes (CTL) activation and susceptibility to infection seen in DC-depleted mice (23). The dynamic nature of DCs is evidenced by the transcriptional re-programming that takes place after stimulation with TLR agonists when compared to the homeostatic state (24). They are many different DC subsets, each with specialised and differing functions in response to varied stimuli, and characterized by a unique pattern of cell surface receptors (25). DC haematopoiesis occurs in the bone marrow and multiple different DC progenitors give rise to four major populations of DCs namely (i) monocyte derived DCs (CD14+, CD206+), (ii) CD141 (BDCA3+) cDCs, (iii) CD11c (BDCA1+) cDCs, and (iv) plasmacytoid DCs (BDCA2+) (26). CD141 cDCs express TLR3 and release type III IFNs in response to viral dsRNA and are especially efficient for $CD8^+$ T cell activation. CD11c cDCs are important in $CD4^+$ T-cell priming (with subsequent induction of Th2 and Th17 responses), whist pDCs expressing TLR7 (recognising ssRNA), readily produce large amounts of type 1 IFN (up to 1000 times more than other cells) in response to viral infection (27, 28).

1.1.2.6 Macrophages

Lung-resident macrophages include bronchial macrophages, alveolar macrophages (AMs) and interstitial macrophages (IMs) and have the ability to independently recognise, engulf and destroy foreign pathogens but also act as antigen presenting cells for helper T cells (29). Alveolar macrophages

have been the best studied and are yolk-sac derived long-lived resident cells that play an inherently suppressive role in lung homeostasis through its interaction with the epithelium via CD200, TGF-β and secreted IL-10 (30). During infection this negative regulation is released and AMs become critical in the innate immune response as seen in mice lacking in AMs that develop respiratory failure in response to influenza infection (31). Macrophages display plasticity due to their ability to polarize towards either a proinflammatory M1 phenotype (with TLR ligand or IFN-γ stimulation) or an M2 phenotype (with exposure to IL-4 or IL-13), that is primarily involved in remodelling and repair (32). The presence of macrophages in the nasal mucosa have been less well characterized but small-sized studies suggest that macrophages are present in the nasal mucosa in similar numbers to the bronchial mucosa of healthy volunteers (33).

1.1.2.7 Natural Killer Cells

NK cells are innate lymphocytes that circulate in the blood as well as being resident in peripheral tissues (10-20% of lymphocytes in the lungs being NK cells), to provide a rapid and powerful effector response to invading pathogens (34). Their importance in combating viral infections is highlighted by patients with NK cell deficiency who become susceptible to the early stages of herpes virus infection (35). They possess cytotoxic granules (containing granzyme and perforin) that are released on to the target cell membrane to induce programmed cell death and their ability to kill is potently enhanced in the presence of type I IFNs or IL-12 (36). NK cell activity occurs early on to contain viral replication before antigen-specific CD8 T cell killing occurs (14), and production of IFN-γ mediates $CD8^+$ T cell recruitment to the lungs and inhibits eosinophilia (37). In addition to these effects mediated through activating receptors, they possess inhibitory cell surface receptors that prevent host cell killing to ensure self-tolerance (38). If this finely tuned balancing act is disturbed, then excessive inflammation can ensue. Whilst NK cells of $CD56^{bright}$ phenotype have been isolated in the BAL of sarcoidosis patients

(39), there are no published studies that have characterized the distribution and function of lung tissue-resident NK cells in humans (34).

1.1.2.8 Neutrophils

Neutrophils are the most abundant human immune cells that circulate in the bloodstream, rapidly responding to signals of injury or infection, and extravasate towards sites of inflammation along a gradient of chemo-attractants (40). After recognising and engulfing pathogens, neutrophil killing occurs in their vacuoles due to the release of potent antimicrobial peptides and reactive oxygen species (ROS) - the "respiratory burst" (41). Neutrophils also have the ability to release neutrophil extracellular traps (NETs) to assist in trapping microbes and preventing overwhelming infection, particularly against bacteria. Whilst providing protective functions, excessive neutrophilic inflammation can lead to significant host tissue damage such as in acute respiratory distress syndrome (ARDS) (42). The role of neutrophils in anti-viral immunity is less well appreciated and may have both beneficial and harmful effects (43). For example, it has recently been shown that HRV infection is correlated with the release of host dsDNA and associated with NETosis, contributing to asthma exacerbations (44).

1.1.2.9 Eosinophils

The concept of eosinophils being primarily involved in defending against helminths has advanced substantially and they are now considered to be multifunctional granulocytes with important roles in innate and adaptive responses, as well as tissue repair (45). Eosinophils mature in the bone marrow under the influence of IL-3, IL-5 and granulocyte monocyte-colony stimulating factor (GM-CSF) and when released, constitute 1-3% of circulating lymphocytes (46). They migrate towards sites of tissue inflammation and the release of nitric oxide, ECP and MBP facilitates host defence. These toxic granules play a role in antiviral immunity by limiting viral replication and aiding the digestion of RNA

viruses such as RSV and parainfluenza (47, 48). However, they may also have detrimental effects in chronic inflammation and are regarded as a central player in the pathogenesis of allergic asthma (49).

1.1.2.10 Genetic determinants

Human immune system variability is a consequence of non-heritable factors such as age, gender, seasonal changes and circadian rhythm as well as heritable influences (50). Innate cytokine responses to PRR stimulation of blood in infants demonstrate significant variability depending on geographical location (51). Susceptibility to infectious diseases (as well as autoimmunity) has been associated with polymorphisms in genes encoding TLRs and downstream signalling pathways (52–54), and TLR7 and TLR8 polymorphisms are associated with respiratory disorders such as asthma (55).

1.1.2.11 Role of microbiome

The evolution of genetic sequencing techniques, which allow culture-independent methods of detecting bacteria has led to important insights in understanding the microbiome, moving away from a concept of "sterile" lungs in health to that of a resident steady state microbial flora (56). The upper airways and in particular the nose is colonized by bacteria early on in life (and heavily influenced by environmental factors such as breastfeeding), undergoing natural transition as individuals age (57, 58). The resident microbiome in healthy adult nasal cavities can be hugely variable but is dominated by actinobacteria (e.g. *corynebacterium*), and firmicutes (e.g. *streptococcus and staphylococcus*) (59). However, in conditions such as chronic rhinosinusitis, these same microbes (and in particular *staphylococcus aureus*) may develop a pathological niche and is associated with increased disease severity (60). In the nasal LPS challenge model, some volunteers with idiosyncratic immune responses in relation to LPS dose, had high levels of IL-6 and IL-1β at baseline, with a suggestion that this may be due to pre-activation with resident bacteria generating a mucosal immune 'tone' (61). The role of the resident microbiome may also be important in determining responses to viral infections such as

influenza and enhance susceptibility to secondary bacterial superinfection (62, 63). It may also interact with aeroallergens (64) and is disordered in the lower airways in asthma (65).

1.1.3 Respiratory viral infections and vaccination

Respiratory infections contributed to greater than 4 million deaths in 2013 with deaths due to pneumonia occurring in 41.7 per 100,000 population (66). Respiratory viruses are thought to play a role in 45% of children being hospitalized due to pneumonia (67) although some viruses such as rhinovirus and adenovirus are readily identified in both symptomatic and asymptomatic cases (68). RNA viruses have been identified as being key culprits in causing pathogenesis in susceptible groups of individuals. RSV has a global incidence of 33 million cases of acute lower respiratory tract infection per year causing nearly 60,000 deaths in children under 5 years of age (69). Influenza is estimated to cause up to ½ a million deaths per year globally with a greater toll on at-risk groups such as the elderly, resulting in the need for a global vaccination programme (70). Those with chronic respiratory diseases such as asthma are especially susceptible with more than 80% of exacerbations in children and more than half of exacerbations in adults considered to have viral aetiology, with two-thirds being due to HRV (71).

Vaccination is the most effective intervention to prevent infection and the introduction of vaccines against measles, mumps, rubella and meningitis amongst others have established itself in national childhood immunization schedules and transformed global public health. Vaccines against respiratory pathogens have been less forthcoming with notable exceptions being those directed against influenza and adenovirus, although other promising candidates are in the developmental pipeline (70). Despite great resources being devoted to combating respiratory infections, the appalling global death toll is testament to the amount of work that is still required to limit the burden of disease. The Battle against Respiratory Viruses (BRaVE) initiative launched by the World Health Organization (WHO) concludes:

"Our understanding of the mechanisms of transmission and disease pathogenesis in key patient groups is incomplete. Further basic and clinical research is needed".

The Battle against Respiratory Viruses (BRaVE) initiative, WHO, 2013

The key patient groups highlighted include children, the elderly and those with chronic respiratory diseases. In the latter group, asthma has been the focus of much research due to its prevalence in the general population, with infective exacerbations the cause of a substantial burden of disease and economic health impact.

1.1.4 Role of atopy and asthma in viral infections

Exacerbations of asthma can occur due to both infective (viral and bacterial) and non-infective (aero-allergens, occupational exposure) triggers. Whilst the underlying mechanisms may differ there is a common pathway of multicellular inflammation and increased airway hyperresponsiveness (72). The mucosal immune response to aeroallergens in atopic (those predisposed to developing allergic disease) and subjects with asthma have been well characterized (73). Allergic rhinitis subjects undergoing nasal allergen challenge with grass pollen experience type-2 innate lymphoid cells (ICL2) activation, release of type 2 inflammatory mediators (IL5 and IL-13) and inflammasome activation as well as release of complement (74, 75). Epithelial cells express PRRs that recognise allergens and produce chemokines (e.g. CCL17, prostaglandin D_2 (PGD_2) and eotaxins) that recruit DCs, basophils and ILC2s (76). The release of epithelial-derived cytokines such as thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 leads to the activation of DCs leading to a subsequent skew towards pro-Th2 inflammation (77). Although TLRs are abundantly expressed in airway epithelial and dendritic cells, they are also present on the surface of eosinophils and mast cells (49, 78).

Viruses and allergens are thought to have a synergistic role in triggering asthma exacerbations that can lead to increased hospitalization rates (79). Early life exposure to and interplay between

respiratory viral infections and allergen triggers have been linked with the inception of later wheeze and asthma in childhood as well as playing an important causative role in exacerbations (80). RSV inoculation of epithelial cells from children with asthma can induce TSLP, suggesting extensive crosstalk between anti-viral innate and type 2 immune pathways (81). Experimental proof of this is shown in adult subjects with allergic rhinitis undergoing segmental bronchus provocation with aeroallergen, who display enhanced histamine mediator release and eosinophil influx after live HRV challenge (82). The presence of atopy plays an important role in TLR-mediated eosinophil and mast cell activation, which leads to the production of IFN-α and IFN-β (78, 83). Since mast cells and eosinophils are upregulated in the nasal mucosa of allergic rhinitis subjects (84), they are likely to play a key role in anti-viral immune responses. The role of interferons in determining susceptibility to viral infections and in particular to HRV has been extensively studied but with conflicting results (85). Interferon deficiency has been described in cultured cells from volunteers with asthma following HRV infection (86–88), whereas robust IFN-γ and IFN-λ responses have been found in children with asthma (89, 90). A recent clinical trial of inhaled IFN-β therapy failed to meet its primary end-point of improvement in clinical symptoms, although it did boost blood and sputum innate immune markers (91).

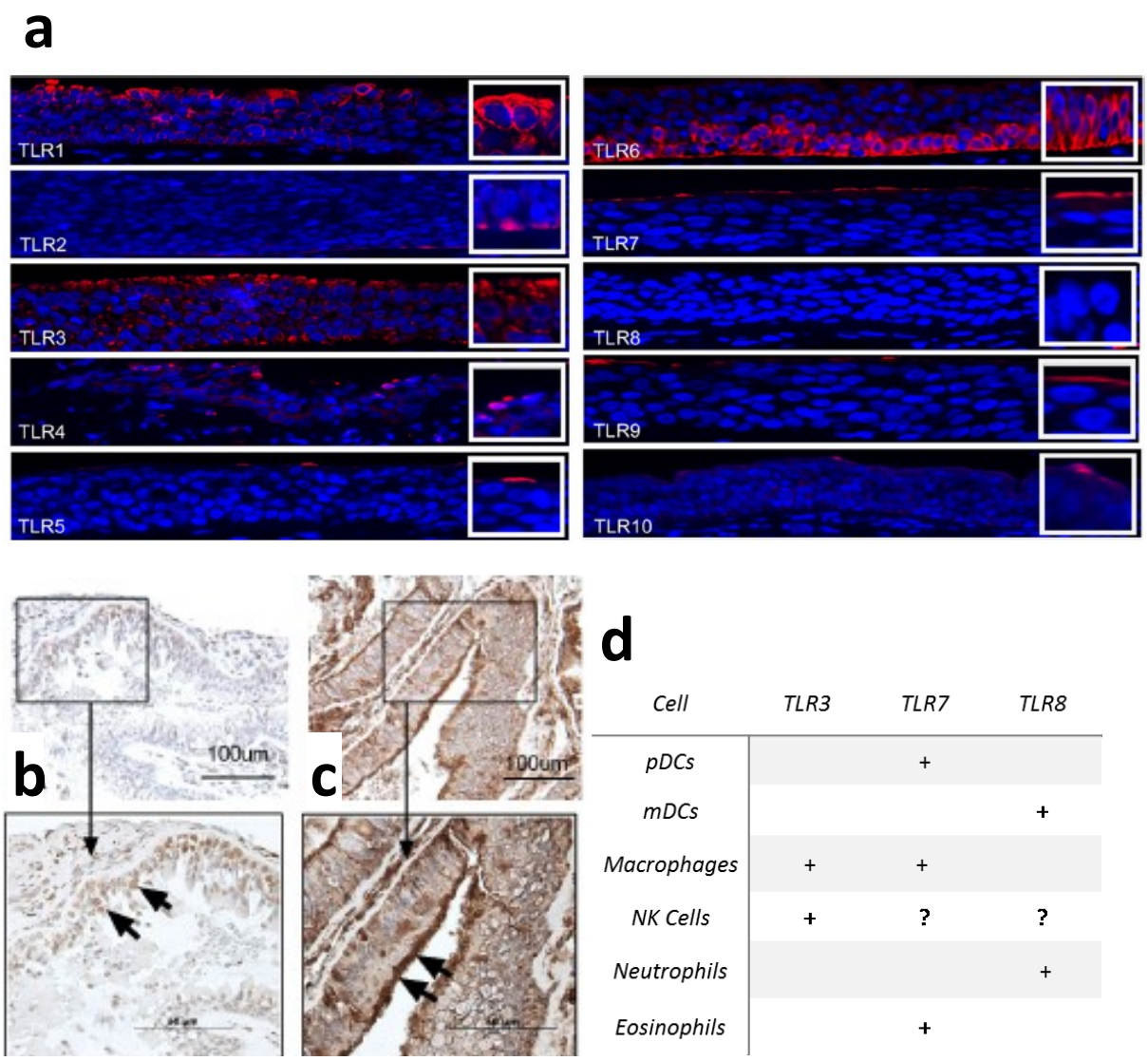
Further detailed understanding of the underlying immunopathogenesis of viral infections in allergy and asthma is therefore required for the development of new therapeutics (92).

1.2 Toll-like receptors

"Das war ja toll!"

Attributed to Christiane Nüsslein-Volhard, 1980s

The airway mucosa contains both epithelial and immune cells that are capable of responding to microbial invasion due to the presence of PRRs and in particular TLRs (figure 1.2).



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Cell	TLR3	TLR7	TLR8
pDCs		+	
mDCs			+
Macrophages	+	+	
NK Cells	+	?	?
Neutrophils			+
Eosinophils		+	

Figure 1.2 Airway mucosal cellular TLR receptor expression (a) TLR expression from human tracheal epithelial cells using immunofluorescence, from reference (93), reproduced with permission from the American Society for Microbiology; (b) Nasal biopsies expressing (b) TLR3 and (c) TLR7 as visualized by 3, 3'-diaminobenzidine (brown) and counterstained with haematoxylin (blue) with arrows indicating positive cells, from reference (94); (d) Relative expression of endosomal TLRs in innate immune cells, data summarized from references (95–97). Knowledge of NK cell expression and function of TLR7 and TLR8 is uncertain as responses may be dependent on activation status or presence of other cells such as pDCs and macrophages.

1.2.1 TLR signalling

Phagocytes, dendritic cells and epithelial cells all have PRRs that recognise conserved regions of microbes (PAMPs). These specialized PRRs consist of TLRs, RIG-1-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLR) and cytosolic DNA sensors, which are in constant communication with each other (98). The TLR family have been the most widely studied with ten known human TLRs that can form homodimers or heterodimers to recognise their cognate antigen. TLRs present on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6) typically recognise bacteria and fungi, whilst those situated in cytosolic structures such as endosomes (TLR3, TLR7, TLR8, TLR9) are focussed on identifying viral nucleic acids (figure 1.3). Intracellular Toll–interleukin 1 (IL-1) receptor (TIR) domains are common to all the TLRs and is required for downstream signalling. All TLRs except TLR3 signal through the adaptor molecule myeloid differentiation primary response protein 88 (MyD88). Activation through the MyD88-dependent pathway, with TLR2 and TLR4 additionally utilising TIR domain-containing adaptor protein (TIRAP), leads to the nuclear translocation of transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and/or activating protein 1 (AP1). There is a subsequent upregulation of genes encoding proinflammatory cytokines such as TNF and interleukin-6 (IL-6). Alternatively, TLR3 and also TLR4 (which uniquely has the ability to signal via both pathways) utilize both TIR-domain containing adaptor inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) to mediate production of type 1 interferons (99).

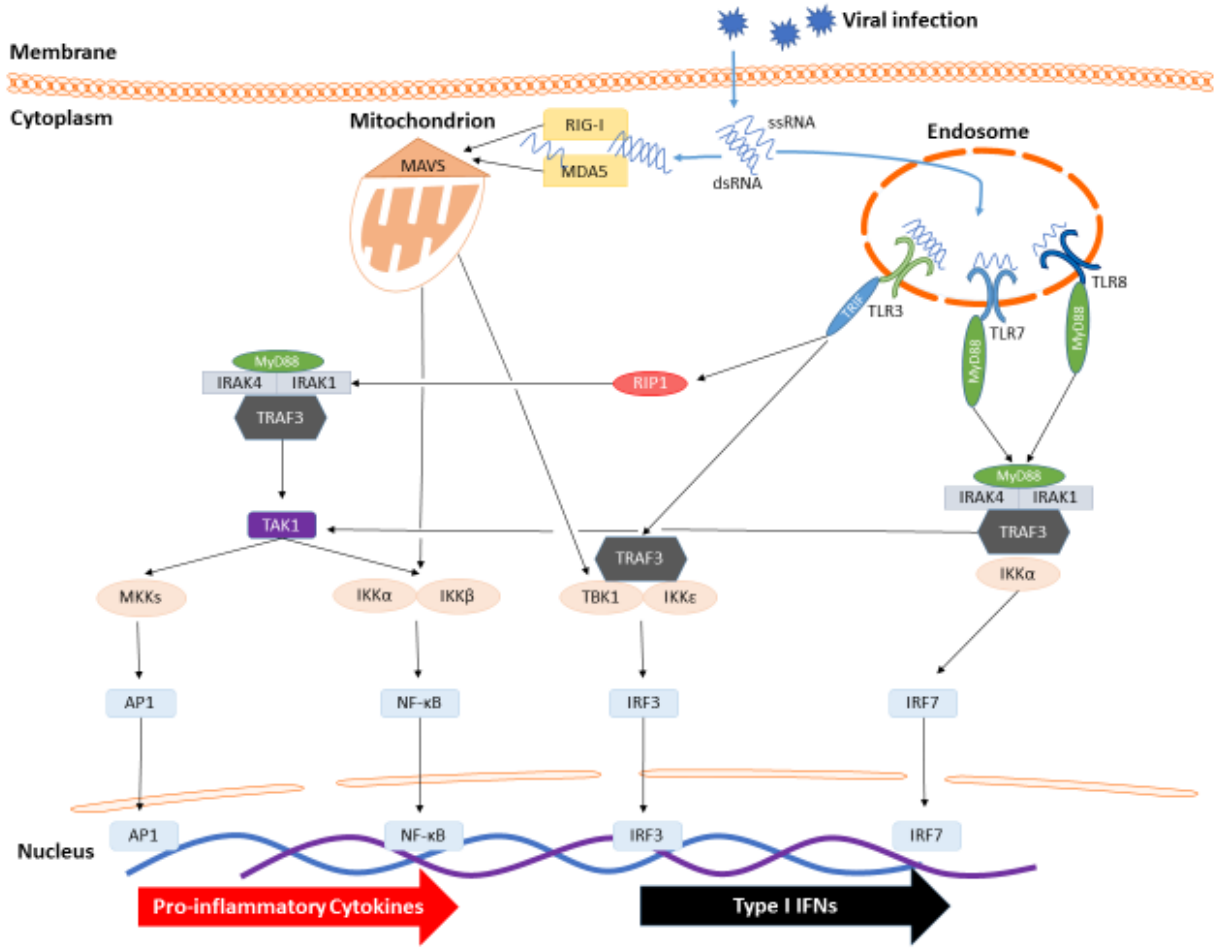
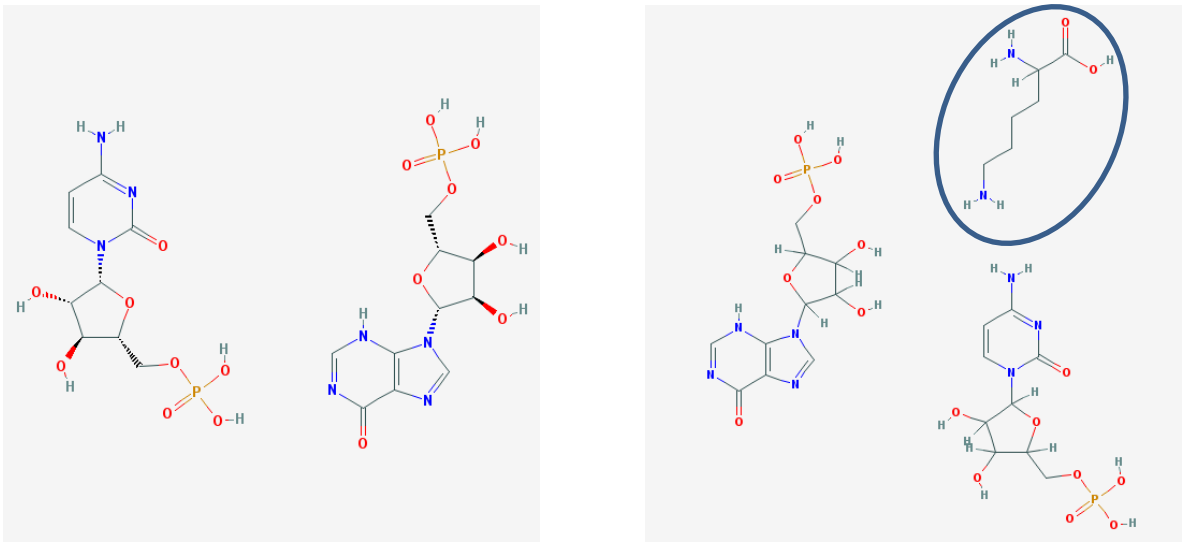


Figure 1.3 Viral nucleic acid recognition and cell signalling by TLRs and cytosolic receptors Viral infection results in the release of viral single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA) into endosomes or the cytoplasm. Endosomal TLR3 recognition of dsRNA results in the engagement of TIR domain-containing adaptor protein inducing IFNβ (TRIF), whilst endosomal TLR7 and TLR8 recognition of ssRNA results in the engagement of myeloid differentiation primary-response protein 88 (MYD88). Subsequent downstream signalling steps involve IL-1R-associated kinases (IRAKs), TNF receptor-associated factors (TRAFs), mitogen-activated protein kinases (MAPKs) and inhibitor of NF-κB kinases (IKKs). This results in the activation of either interferon regulatory factors (IRFs) or nuclear factor-κB (NF-κB)/activator protein 1 (AP1), which translocate to the nucleus and induce type I interferons (IFNs) or pro-inflammatory cytokine production respectively. Alternatively, viral nucleic acid recognition can occur in the cytoplasm by retinoic acid-inducible gene (RIG-I) or melanoma differentiation-associated gene 5 (MDA5). Subsequent recruitment of mitochondrial antiviral signalling protein (MAVS) results in a common pathway of IFN and pro-inflammatory cytokine induction. Abbreviations RIP1, receptor-interacting protein 1; TAK1, TGFβ-activated kinase; TBK1, TANK-binding kinase 1.

1.2.2 TLR3 modulators

TLR3 is important in recognising dsRNA, which are intermediate structures that are released during viral infections (100). The most widely used synthetic compounds that can stimulate TLR3 are polyinosinic:poly-cytidylic acid (poly(I:C)) and its more RNAse resistant analogue polyinosinic:poly-cytidylic acid stabilized with poly-L-lysine (poly-ICLC). It is important to note that these dsRNA analogues mediate diverse activation through its recognition by melanoma differentiation-associated gene-5 (MDA-5) and the cytoplasmic RNA helicase RIG-I to effect type 1 IFN signalling (101). Since RSV triggers IFN production via RIG-I, MDA-5 and TLR3, dsRNA analogues can therefore act as useful surrogates of live virus infection (102). Both poly(I:C) and poly-ICLC have been used directly to stimulate the immune system or used as adjuvants with vaccines in mice and rhesus macaques where they have been shown to protect against a variety of viral challenges (103). In humans, researchers have also exploited the anti-proliferative properties of poly-ICLC to perform trials in patients with brain tumours (104). Administration of poly-ICLC subcutaneously induces genes involved in IFN and NF-κB signalling, DC maturation and antigen presentation and has overlap with blood transcriptomic responses seen after administration of yellow fever vaccine (105). This ability to generate broad innate immune responses highlights the ability of dsRNA analogues to act as effective microbial mimics. One of the earliest studies of intranasal poly(I:C) demonstrated the potential for reducing symptoms when given just prior to live viral challenge but was not associated with any significant effect on intranasal interferon production or viral shedding (106). Nasal application of poly-ICLC in a phase I study has been performed in 57 healthy human volunteers by Dr Richard Davey's group at Bethesda, USA but the results have not yet been published (107). It is therefore not yet clear if humans exhibit a reliable mucosal innate response to poly-ICLC. Poly(I:C) has a molecular weight of 671.406 g/mol whilst poly-ICLC weighs 817.596 g/mol due to an additional L-lysine group (figure 1.4) (108, 109). This is thought to provide increased stability to the enzyme, making it more resistant to the effects of RNAse enzyme.



Poly(I:C) Poly-ICLC

Figure 1.4 Compound structures of poly(I:C) and poly-ICLC Blue oval represents extra L-lysine group in poly-ICLC compared to poly(I:C), acting to enhance its stability against RNAses. Downloaded from National Center for Biotechnology Information, PubChem Compound Database (108, 109).

1.2.3 TLR7 and TLR8 modulators

TLR7 and TLR8 recognize nucleic acid derived from ssRNA viruses and small molecule imidazoquinoline derivatives and its activation leads to production of IFN gamma, IL-12 and TNF alpha (110, 111). Resiquimod (R848), a combined TLR7 and TLR 8 agonist, is a member of the imidazoquinoline family and in contrast to the broad activation of PRRs elicited by poly(I:C) and poly-ICLC, R848 acts as a specific TLR7/8 agonist by binding to the uracil binding domain of TLR7 and TLR8. R848 has been trialled in humans in the form of a cream for the treatment for genital warts (112). Oral R848 has been used in a Phase IIa trial in patients with chronic HCV infection with doses of 0.01mg/kg being tolerated but 0.02mg/kg causing IFN-like side effects (113). Intranasal administration of R848 in an experimental murine asthma model resulted in suppression of allergic airway inflammation and hyperresponsiveness in response to inhaled allergen challenge (114). This was mediated by upregulation of Type I interferons and subsequent suppression of Th2 responses in an IFN-γ-dependent manner, with suppression of inflammation seen even after 1 month, making this route an attractive prospect in the treatment of allergic airway disease. TLR agonists including those acting on TLR7 have also been utilised in conjunction with immune checkpoint inhibitors and represent an important new therapeutic avenue for oncological therapies (115, 116). In terms of its structure, R848 represents a relatively small molecule combined TLR7/8 agonist with a weight of 314.389 g/mol (figure 1.5) (117).

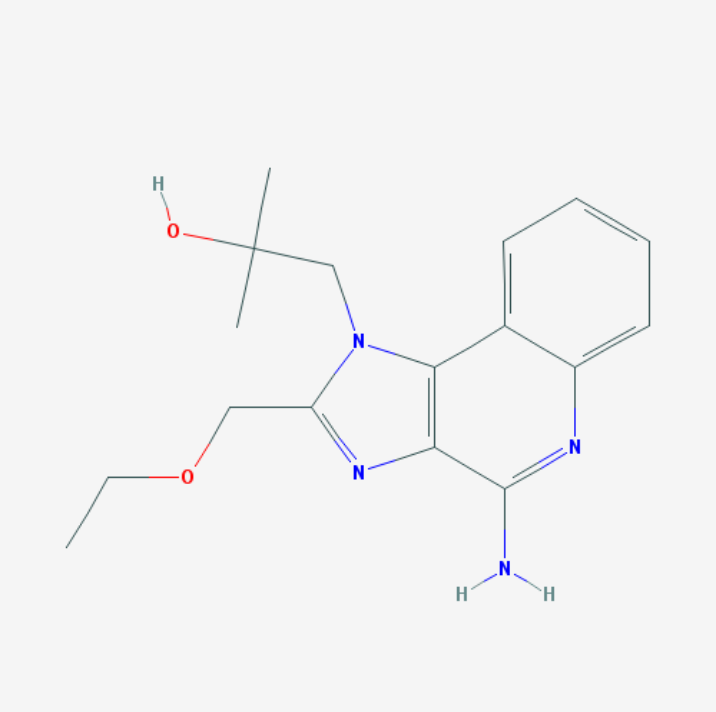


Figure 1.5 Compound structure of resiquimod (R848) Downloaded from National Center for Biotechnology Information, PubChem Compound Database (117).

1.3 Measuring airway inflammation

"When you can measure what you are speaking about, and express it in numbers, you know something about it"

Lord Kelvin, 1883

Research studies have focused on measuring T cells responses and systemic antibody titres to infection and are based on relatively straightforward methods of sampling (e.g. peripheral blood). In contrast, mucosal innate immunity remains difficult to quantify reliably, with a need for more accurate and precise methods to measure responses. Additionally, techniques that are non-invasive and easy to perform would facilitate its use in subjects with severe respiratory disease, who may be unable to tolerate more invasive procedures such as bronchoscopy. The latter does allow for direct measurements of lower airway responses to viral infections in bronchoalveolar lavage (BAL) but has the inherent demands for technical expertise, the use of sedative agents, cost implications and lack of repeatability. There have been a range of upper and lower airway sampling techniques to assess inflammatory responses, which have advantages and limitations that is context-specific and dependent on the rationale for sampling.

1.3.1 Airway structure and function

The nose is a highly specialized organ with multiple roles ranging from olfactory functions, humidification and filtering out of large particulate matter (8). Outside air enters the nose through the anterior nares and nasal vestibule, passing over shelf like protrusions called turbinates (inferior, middle and superior). The underlying vascular supply warms and humidifies the air before it continues through the internal nares and subsequently in to the lower airways consisting of the larynx, trachea, bronchi (primary, secondary and tertiary), bronchioles and alveoli. The airway mucosa consists of three layers, namely the epithelium, basement membrane and lamina propria. At the nasal vestibule

the inner lining of the nose transitions from a keratinized, stratified, squamous epithelium to a ciliated, pseudostratified, columnar epithelium. These cells are interspersed with goblet cells, which produce mucous to moisten the airway and trap particulate matter. Cilia present on epithelial cells transport debris-laden mucous towards the nasopharynx, beating at a rate of approximately 1000 times per minute (118). The epithelial cells are attached to the basement membrane with the lamina propria directly beneath this. The latter contains a rich plexus of leukocytes and fibroblasts, loose connective tissue, veins and serous glands with the latter being able to secrete bactericidal lysozymes. The mucosa has an intricate network of immune cells that regulate immune homeostasis as well as coordinating the response to infection (84).

1.3.2 One-airway hypothesis

Ciliated epithelial cells lining the nasal inferior turbinates represents the beginning of the respiratory epithelium, which extends to the bronchioles. The concept of a one-airway hypothesis is supported by both clinical and pathological research (119, 120). The shared characteristics between the nose and airways is based on similar responses to allergen, infection and other inflammatory stimulants (3), making the nose a potentially useful surrogate for pulmonary inflammation. Studies have demonstrated similarities between nasal and bronchial mucosa using histological techniques (121), transcriptomic analysis (122) and in clinical settings as evidenced in asthma patients by their improvement in lower airway symptoms with intranasal steroid treatment for allergic rhinitis (123). The presence of high IgE levels is a common factor between allergic rhinitis and asthma, which can lead to the triggering of immune cell activation and release of potent mediators after allergen stimulation (4). Whilst loss of barrier function is well recognised in the lower airway of individuals with asthma (77), comprised barrier integrity due to epithelial dysfunction has also been implicated in chronic rhinosinusitis (8). The similarities between the upper and lower airway may be especially pronounced in those with allergic rhinitis (with and without asthma). A study assessing gene

expression profiles in primary nasal and bronchial epithelial cells from allergic rhinitis and healthy control volunteers found 1988 genes differentially expressed between healthy upper and lower airways, whilst only 40 genes were differentially expressed in those with allergic rhinitis and asthma (124). This suggests that a common process of inflammation and cellular infiltration occurs in the upper and lower airway of those with allergy-mediated disease.

It is also important to note the differences between the structure and function of the two airway compartments. The nose does not have smooth muscle cells as compared to the bronchi, which may be lead to reduced production of fibrogenic growth factors and comparatively less remodelling. There may also be differences in the genetic predisposition to remodelling due to differences in the embryonic origin of upper and lower airways (6). Differences between the nose and bronchi also likely exist at the sub-mucosal level with highly developed vasculature in the former and a thick basement membrane in the latter (11). The cellular composition can differ, in particular when compared to the lower airways and alveoli, with the latter containing alveolar macrophages and surfactant-producing alveolar epithelial cells (9). The microenvironments of the anterior nose may have a unique ecological niche relative to the posterior nose and throat (9, 10). Functional differences to stimuli have also been demonstrated with a study in children, highlighting differential innate immune responses to RSV in cultured nasal and tracheal epithelial cells (5).

1.3.3 Upper airway sampling

The relatively easy accessibility of the upper airway has led to the development of several techniques that aim to measure immune responses by sampling the mucosal luminal contents either directly or indirectly. Sampling from the nose in particular can be performed serially and also has advantages of being able to performed in vulnerable populations such as children or hospitalized patients.

1.3.3.1 Nasal lavage

Due to its requirement for minimal equipment and training in procedural technique, the nasal lavage (or wash) method has been long been utilized as a method to assess nasal inflammation. It has been applied in a range of clinical settings to measure inflammatory mediators (125, 126), viral load (127, 128), neutrophil subsets (129) and mucosal IgA (130–132). Nasal lavage has also been used by clinicians to treat symptoms due to allergic rhinitis and upper respiratory tract infection (133, 134). Nasal lavage can be performed by asking the volunteer to extend their neck backwards and close their soft palate whilst saline is instilled. However, this has a greater risk of being performed incorrectly due to an inability by volunteers to retain saline in the nasal cavity (135). Therefore, alternative techniques have been developed involving forward flexion of the neck and instillation of a specified volume of saline (usually 5-10 mL) from a syringe either directly or via a nasal olive into the nasal mucosa through each nostril. Nasal lavage fluid is then aspirated back in to the syringe or a suitable receptacle with subsequent measurement of soluble mediators or immune cells. Overall however, there are limitations to the nasal lavage technique for monitoring nasal inflammatory responses. The amount of saline that is aspirated back can be variable with substantial dilution of mucosal secretions, leading to significantly lower levels of mediators being detected compared to direct sampling of the nasal mucosa (136–139). Although nasal lavage has been found to be reproducible (135), diagnostic nasal lavage can only be performed once a day to get results that are comparable (140).

1.3.3.2 Nasal curettage

Nasal curettage using the Rhinoprobe curette [Arlington Scientific] is a non-invasive technique directed at the inferior nasal turbinate to obtain a small quantity of mucosal tissue, and is an alternative to nasal biopsy (135). The sample that is obtained can be used for viral diagnosis, cytology, histology, transcriptomics, flow cytometry and has also recently been deployed for nasal epithelial cell culture at the air liquid interface (ALI) (141). Nasal curettage was found to be safe and easy to use in

studying 1257 infants and children with RSV infection (142), to look at gene expression after experimental HRV infection (143, 144), and to detect respiratory viruses in chronic rhinosinusitis (145). Nasal transcriptional signatures have also been assessed in serial samples during asthma exacerbations (146). Nasal cytology has been employed in allergic rhinitis (136, 147, 148), non-allergic rhinitis (137) (33) and cystic fibrosis patients (149, 150).

1.3.3.3 Nasal brush

Nasal epithelial sampling can also be performed using an interdental or cytology brush (150). This involves brushing the inferior surface of the inferior or middle turbinates and aspirating the contents in to a suitable medium. It has been used to assess epithelial cell markers and function in cystic fibrosis (67, 151) and childhood asthma (122) as well as to investigate changes in DNA methylation (119). Comparison of different nasal sampling procedures suggest that the nasal brushing method is optimal for obtaining epithelial cells for culture (152) whilst nasal curettage is preferable for quantifying leukocytes and eosinophils (148).

More invasive methods such as nasal biopsy can provide excellent architectural analysis of the mucosa for histology and immunohistochemistry but has the disadvantages of requiring anaesthesia and difficulty in repeatability (153).

1.3.3.4 Nasosorption

The concept of obtaining mucosal lining fluid from the nose using absorptive techniques with the aim of measuring inflammatory mediators has been established for more than 25 years (154). The use of filter paper relies on capillary suction to remove the serous sol phase of nasal mucous (155). However, the process of achieving a reliable, contaminant-free technique of measuring immune mediators in a reproducible manner has required extensive refinement of the methods and materials. Initially, the

filter paper used for nasal allergen challenge was based on natural cellulose derived from the cotton plant (156–161). The material has good absorptive capacity and represented an improvement in being able to reliably detect mediators such as eosinophilic cationic protein, compared to nasal lavage or other more basic techniques such as nose blowing or microsuction (155). Natural cellulose material has also been incorporated in to Weck-Cel sponges to sample salivary, cervical and vaginal mucosal secretions (162). However, all methods that utilised these natural sources had a major limitation in the variable binding to proteins that occurred, leading to variation between batches. Subsequently, filter papers consisting of a synthetic absorptive matrix (SAM) were designed to selectively bind cytokines and chemokines rather than larger proteins. Initially, manually cut strips were used but these had the potential for contamination with allergen and endotoxin, as well as electrostatic effects. The nasosorption device (Hunt Developments Ltd) was then developed and represented a synthetic, medical grade, allergen/LPS/microbe free and CE-marked device for use in mucosal sampling. An additional advantage compared to conventional sampling with cotton swabs was that it could be held in place in the nasal lumen with fingertip pressure, rather than the more vigorous brushing required with swabs, which may induce pro-inflammatory changes. Leukosorb (Pall Life Sciences) has been utilised in several studies including to detect IL-1β and IL-6 levels after LPS challenge (61), the epithelial cytokines IL-25 and IL-33 after HRV (163, 164) and to measure cytokines and viral load in RSV bronchiolitis (165). An extensive validation study of nasosorption comparing it to nasal lavage has been performed (138) and it has also been shown that recovery of cytokines from filter papers is unaffected in the first 24 hours whether stored at room or freezer temperature (166). A limitation to nasosorption is the small volume of elutant obtained, requiring the need for sensitive immunoassays to measure soluble mediators. There may also be differences in the cellular composition obtained using nasosorption compared to other sampling techniques. For example, nasosorption may absorb cells as well as mediators onto the matrix, which get lysed and release their intracellular contents. Therefore, subsequent assessment of inflammation may reflect the contribution of both intracellular

and extracellular mediators. This may be different to mediators that are measured in nasal lavage specimens, which likely contain a greater proportion of extracellular inflammatory mediators (139).

1.3.3.5 Nasal sponge

Synthetic sponges made from polyvinyl alcohol (PVA) and hydroxylated polyvinyl acetate (HOPVA) have been utilised to sample uterine cervical secretions (167). Seven different absorptive materials have been compared for sampling oral fluid prior to measuring antibodies (168), while polyurethane minisponges have been used to collect human tears (169). Sterile synthetic polyurethane sponges (or foam) ((RG 27 grau, Gummi-Welz GmbH & Co., Neu-Ulm, Germany) have been used for cat allergen challenge (170) and testing of allergen immunotherapy (171). It has shown good recovery of cytokines compared to filter papers made from cellulose and SAM (172).

1.3.3.6 Nasopharyngeal aspirate (NPA)

NPA is performed by inserting a catheter attached to a suction unit into a patient's nose whilst they are reclined at 45°. The catheter is advanced to the posterior nasopharynx and suction applied to collect secretions in to a suction trap. Saline can be can be suctioned through the tubing to improve the yield or alternatively saline can be first instilled in the nasal cavity and subsequently aspirated along with respiratory secretions (173). It has been considered the gold standard for the detection of respiratory viruses such as influenza and RSV (174, 175). However, it can be an unpleasant procedure, especially for children and nasal swab sampling have shown equivalent results for the detection of most respiratory viruses (7). More recently, NPA comparison with nasosorption has demonstrated that the latter is better at simultaneously recording viral load and inflammatory mediators in order to differentiate between RSV positive bronchiolitis and controls (165).

The frequency of nasal sampling may theoretically have an effect on the measurement of inflammatory parameters due to the potential for a mechanical and irritant effect on the nasal mucosa (172). Whilst the degree of effect may differ depending on the material used to perform sampling, it highlights the importance of including a saline control for all volunteers undergoing nasal challenge. This can help to detect changes in soluble mediator levels that may be due to the delivery of aerosolised spray on to the nasal mucosa as well as due to the effects of serial sampling.

1.3.4 Lower airway sampling

Samples from the lower airways can be obtained using patient-dependent methods such as spontaneous and induced sputum or the measurement of chemicals in exhaled breath. The more invasive technique of bronchoscopy can be used to obtain biopsies, epithelial brushings, mucosal lining fluid or luminal cells after bronchoalveolar lavage. These can be useful in obtaining tissue for transcriptomics and proteomics (176) as well as assessing cellular responses to viral challenge (177).

1.3.4.1 Sputum

Sputum has been used to study underlying disease states since before the time of Hippocrates (178). It can either be provided spontaneously by patients or induced – the latter relying on the use of hypertonic saline to aggravate patients into coughing. Standardised methodologies for sputum induction and processing have been established (179). Due to the potential for excessive bronchoconstriction, it is important that trained professionals who have access to resuscitation equipment perform the procedure, preferably using pre-treatment with salbutamol. The importance of the presence of eosinophils in the sputum of asthma patients to determine responsiveness to prednisolone was established by Morrow Brown (180), whose subsequent work heralded the transition to inhaled corticosteroids for the treatment of allergic asthma (181). The technique of inducing sputum using hypertonic saline to quantify eosinophils and assess response to steroids has

been pioneered by Freddy Hargreaves and colleagues (182, 183). More recently, omics platforms have been used on sputum samples to inform the search for biomarkers in inflammatory airway diseases (176). However, the analysis of fluid-phase mediators derived from sputum samples has a large number of technical problems (184): these range from degradation by proteases and bacteria, loss of protein secondary structure due to reduction by dithiothreitol [DTT], binding to mucus, contamination with saliva and oropharyngeal contents, variable leakage of mediators from apoptic cells and inability to produce sufficient volumes of sputum.

1.3.4.2 Bronchoalveolar lavage

Conventional flexible bronchoscopy is normally performed on patients with respiratory disease in order to carry out bronchial mucosal biopsy, bronchial brushings and BAL. The latter involves the instillation of a set amount of saline (typically 50-100mL) in a lobe of the lung (usually right middle), with aspiration of luminal contents. A European Society Task Force has issued guidelines for measurements of cellular components and standardization of BAL (185). Examples of the use of BAL in providing important mechanistic insights include the evaluation of inflammation in asthma (186), aiding the diagnosis of sarcoidosis (187), and assessment of T cell responses to RSV infection (177). Limitations of BAL include the unknown dilution and variability in obtaining lavage fluid, which makes accurate detection and quantification of soluble mediators difficult (188), whilst fever and malaise are acknowledged complications after the procedure (189).

1.3.4.3 Bronchial brush

Bronchial brushing is routinely performed in clinical and research settings. Bronchial brushing can yield positive tests for respiratory virus detection even when upper airway samples are negative (190). They have been used to characterize the epithelial cell basal transcriptome (191) and to assess antiviral responses in epithelial cells stimulated by HRV (192). In asthma, Prescott Woodruff, John Fahy and

colleagues have helped to define the molecular basis for heterogeneity seen in these patients by assessing IL-13 driven gene signatures in bronchial epithelial brushings and correlating these with cytokine expression in bronchial biopsies and responsiveness to inhaled corticosteroids (193).

1.3.4.4 Bronchosorption

Bronchosorption is a sampling method directed at the bronchial mucosa, which results in a much less dilute sample compared to BAL. A straw of SAM or synthetic sponge is attached to a leading plastic wire, and placed down a sheath within the operating portal of a bronchoscope. Under direct bronchoscopic vision the SAM is advanced against the mucosa of a main bronchus or segmental bronchus and then withdrawn back up the sheath. A limitation to the technique is the small volume of fluid that is obtained, requiring the need for a high sensitive assay for mediator assessment. A HRV infection model including volunteers with asthma and healthy controls, utilised both nasosorption and bronchosorption to measure IL-15 (194), IL-25 (163), IL-33 (164), IL-18 (195) and interferons (196).

1.3.4.5 Exhaled breath

Exhaled breath has also been extensively studied as a non-invasive means to assess airway inflammation, by the measurement of mediators in exhaled breath condensate [EBC] (197). Richard Effros and colleagues have highlighted the issues of salivary contamination and dilution in condensed water vapour that occurs during collection of EBC (198, 199); and this is likely to be a serious obstacle to measuring EBC pH (200) and levels of inflammatory mediators that are in breath droplets. However, assessment of volatile organic compounds [VOCs] and metabolomics on EBC looks to be more promising (201–204).

Nitric oxide production in the bronchial mucosa is increased in asthma patients with eosinophilic inflammation and the measurement of fractional exhaled nitric oxide (FeNO) offers a quick, non-invasive and reproducible surrogate of airway inflammation (205–207). Whilst utilising FeNO may be

of benefit in monitoring inflammation, systematic reviews investigating the role of FeNO guided management on the use of inhaled corticosteroid therapy have been hampered by the heterogeneity of study design characteristics (208, 209). FeNO levels can also be influenced by age, medication use and smoking and disagreements have arisen in national and international guidelines for its use as a biomarker for diagnosis and monitoring of therapy in asthma patients (210, 211).

A comparison of upper and lower airway sampling methods is outlined in table 1.2.

Table 1.2 Comparison of upper and lower airway sampling techniques to measure inflammation

Sampling Method	Advantages	Limitations	Biomarkers	Key Refs
		Upper Airway		
Nasal lavage	Good tolerability Non-invasive	Variable dilution Limited repeatability (>24h)	Inflammatory mediators RSV and influenza viral load Secretory IgA	(125) (127) (128) (130)
Nasal curettage	Obtains epithelial and immune cells Ability to perform gene expression, epithelial cell culture and flow cytometry	Nasolacrimal reaction Limited repeatability (but can alternate nostrils) Fragile small sample	Detection of RSV PCR ILC2s on flow cytometry Cell-specific (e.g. eosinophil) gene signatures after HRV and allergen	(142) (144) (74) (75) (138)
Nasal biopsy	Full thickness mucosal and lamina propria Preserved architecture	Requires an expert clinician and local anaesthetic Bleeding	Immunohistology	(84)
Nasal brush	Non-invasive Ability to perform gene expression and epithelial cell culture	Obtains mainly epithelial cells	Epithelial cell markers, e.g. (EPCAM1, E-cadherin)	(152) (122) (67)
Nasosorption	Direct mucosal sampling Excellent tolerability Non-invasive Repeatable	Reduced sample volume Requires validation for viral load and mRNA detection	IFN response to HRV in asthma IL-5, IL-13 after allergen and HRV challenge	(196) (74) (164) (138) (148)
Nasal sponge	Direct mucosal sampling Non-invasive Repeatable	Can cause excessive nasolacrimal reaction	Type-2 mediators Assessing responses to allergen immunotherapy	(172) (170)
Nasopharyngeal aspirate (NPA)	Ability to collect large volume of respiratory secretions	Discomfort Limited repeatability	Influenza and RSV viral load Viral culture	(174) (175)
		Lower Airway		
Sputum	Non-invasive Easy to obtain Standardized techniques	Loss of protein structure with use of DTT Salivary contamination Limited repeatability (>24h)	Utilised for asthma phenotyping (eosinophilic, neutrophilic) Correlation between blood and sputum eosinophils has conflicting evidence	(180) (183) (212) (213) (184)
Bronchoalveolar lavage (BAL)	Good for ascertaining differential BAL cell counts Extensive literature base on range of respiratory conditions	Variability in dwell time and volume of aspirated fluid Variable dilution making measurement of soluble mediators difficult	Asthma phenotyping (Th1/Th2) Cellular and cytokine response to segmental and whole lung allergen challenge	(186) (185) (188)
Bronchial brush	Genome wide profiling Safe and well tolerated	Mainly obtains epithelial cells Occasional bleeding	IL-13 induced genes Role of POSTN, CLCA1, SERPINB2 genes in asthma and response to steroids	(186) (214) (193)
Bronchosorption	Direct mucosal sampling Detection of cytokines, interferons and DNA methylation analysis Well tolerated	Reduced sample volume	Increased IFN-γ and type 2 cytokines in asthma after HRV SerpinB3 and Uteroglobin increased in COPD after smoking	(196) (215) (216)
Exhaled breath	FENO is non-invasive, quick and can be used to monitor eosinophilic inflammation Measuring VOCs and metabolomics in EBC is promising	FeNO can be affected by smoking, age, medications and can be raised in other eosinophilic diseases Salivary contamination and dilution in condensed water vapour during collection of EBC	FeNO to guide ICS therapy is the subject of debate VOCs: Ability to differentiate asthma phenotypes and children with and without wheeze	(205) (211) (197) (201) (203)

Abbreviations RSV, Respiratory Syncytial Virus; IFN, Interferon; HRV, Human Rhinovirus; DTT, Dithiothreitol; VOC, Volatile Organic Compounds; EBC, Exhaled Breath Condensate; ICS, Inhaled Corticosteroids

1.4 Human challenge models

"I think your solution is just; but why think, why not trie the Expt"

John Hunter to Edward Jenner, 1775

The airway naturally experiences microbial and allergen challenge on a regular basis, most of which are contained by the highly efficient immune system. The ability to simulate these infectious insults by deliberately introducing a pathogen in to the airway mucosa has enabled the systematic study of host immune responses. This approach has important benefits over studying natural infection including the ability to control the exposure and dose of the microbial challenge, as well as knowledge of the time between exposure and onset of symptoms. Perhaps the most important feature of human challenge models is that it permits study of *in vivo* immune responses without reliance on cell lines, *ex vivo* tissue or the use of animal models, all of which may not accurately recapitulate the diverse host responses that occur in after an exposure to infection or allergen (217). There are clear ethical considerations that must be assessed early on in the design of human challenge studies including (a) careful volunteer selection, (b) safety considerations for volunteers, staff and the wider public and (c) ensuring full, informed consent from potential participants (218, 219). This needs to be matched with rigorous scrutiny from institutional review boards and the appropriate regulatory approvals (220) .

The classic early example of deliberate human microbial challenge is that of Edward Jenner who in 1796 inoculated James Phipps with cowpox virus, which later protected him from exposure to smallpox. This laid the foundation for the subsequent eradiation of the disease in 1979 and in helping to establish the concept of vaccines inducing long term protective immunity. In the 20th century, the Salisbury Common Cold Unit established that the main causative agent of the common cold was rhinovirus and explored its methods of transmission (221, 222). Research studies that utilise these infection challenge models have subsequently expanded exponentially with safety data, inoculation procedures and protocols established for a wide range of pathogens. Improved collaboration between

institutions to share knowledge and perform this research can help to accelerate understanding in mechanisms of disease pathogenesis and is the main aim of the recently established medical research council (MRC)-funded human infection challenge network for vaccine development (www.hic-vac.org).

Non-infectious challenge, in particular with allergens have also been important to study mechanisms of allergy mediated disease. The nasal allergen challenge model has been used to determine that the mucosal immune response consists of two phases: an early response characterized by mast cell degranulation and IgE cross-linking, followed by a late phase response involving release of multiple type-2 mediators and the efflux of inflammatory cells, in particular eosinophils (74, 223, 224).

Live viral challenges have been utilized to study mucosal immune responses in response to HRV, RSV and influenza, contributing important insights in to the underlying mechanisms and pathogenesis of these diseases (130, 196, 225). It is pertinent to note however that live viral challenges can be time consuming, resource intensive and require quarantine of volunteers due to the risk of transmissibility. An alternative approach can therefore be to utilize synthetic particles (such as TLR agonists) that mimic the main components of a viral or bacterial infection with respect to its ability to stimulate immune responses. This forms the rationale for performing the work described in this thesis.

1.5 Hypotheses, Aims and Objectives

The broad goals of the project were to develop a reliable and safe system of inducing and measuring mucosal innate immune responses to viral RNA analogues (TLR agonists) in a diverse group of subjects (healthy, atopic and those with asthma) with close monitoring of individual immune markers at multiple levels of profiling including soluble protein mediators and gene expression in correlation with clinical characteristics.

1.5.1 Hypotheses

- Nasally delivered TLR3 and TLR7/8 agonists are capable of inducing mucosal innate immune responses
- Nasosorption and nasal curettage are capable of measuring soluble mediator levels and mucosal gene expression in response to TLR agonists
- Nasal immune responses to innate stimulation are increased in volunteers with atopy and allergic asthma compared to healthy non-atopic volunteers

1.5.2 Aims

- To establish a safe and practical protocol for human nasal challenge with TLR agonists as a way to measure innate responses in the nose
- To characterise the molecular and cellular basis of the mucosal immune response to nasally delivered TLR agonists
- To investigate differences in mucosal immune responses to TLR agonists between healthy volunteers and those with atopy and asthma

1.5.3 Objectives

- To assess the tolerability and safety of subjects undergoing nasal challenge with TLR agonists
- To identify a suitable TLR agonist capable of inducing mucosal innate immune responses
- To collect nasal mucosal lining fluid to measure levels of interferons, cytokines and chemokines
- To obtain nasal mucosal cells for gene expression analysis to assess signalling pathways involved in the local innate immune response
- To identify quantitative and qualitative differences in mucosal innate immune responses to TLR agonists between healthy volunteers and those with atopy and asthma

2 Materials and Methods

2.1 Materials

2.1.1 Clinical equipment

Table 2.1 Clinical equipment used in research study

Name	Application	Source
ASI-Rhino-Pro Nasal Curettes	Collection of nasal mucosal tissue	Arlington Scientific, Utah, USA
Aspergillus fumigatus allergen	Skin Prick Tests	Allergopharma, GmbH & Co., Reinbeck, Germany
BD bidose device	Nasal spray	Aptar Pharma, Milton Keynes, UK
Cat allergen	Skin Prick Tests	Allergopharma, GmbH & Co., Reinbeck, Germany
Disposable micro-lancet	Skin Prick Tests	Allergopharma, GmbH & Co., Reinbeck, Germany
Disposable plastic olives	Nasal lavage	Hunt Development, Midhurst, UK
Dog allergen	Skin Prick Tests	Allergopharma, GmbH & Co., Reinbeck, Germany
Histamine 0.1%	Skin Prick Tests	Allergopharma, GmbH & Co., Reinbeck, Germany
Portable Headlight Green Series	Light source for nasal examination	Welch Allyn, New York, USA
House dust mite allergen	Skin Prick Tests	Allergopharma, GmbH & Co., Reinbeck, Germany
In-Check Nasal with Mask	PNIF measurement	Clement Clarke International, Harlow, UK
Methacholine	PC20	Unknown
Millipore assay buffer	Detergent buffer for protein elution	Millipore Ltd, Watford, UK
Nasosorption FX-i device	Nasosorption	Hunt Developments Limited, Midhurst, UK
Nasosorption SAM strips	Nasosorption	Hunt Developments Limited, Midhurst, UK
PAXgene Blood DNA Tubes	Peripheral blood DNA analysis	PreAnaltiX GmbH, Hombrechtikon, Switzerland
PAXgene Blood RNA Tubes	Peripheral blood RNA analysis	PreAnaltiX GmbH, Hombrechtikon, Switzerland
Portable spirometer	Spirometry	Micro Medical-Beckton Dickinson, New Jersey, USA
Six grass mix allergen	Skin Prick Tests	Allergopharma, GmbH & Co., Reinbeck, Germany
Thudicum nasal specula	Nasal examination	DP Medical Systems Ltd, Chessington, UK
Timothy grass pollen	Skin Prick Tests	ALK, Horsholm, Denmark
Tree mix allergen	Skin Prick Tests	Allergopharma, GmbH & Co., Reinbeck, Germany
Vacutainer blood tubes EDTA	Blood testing - full blood count	Becton Dickinson, New Jersey, USA
Vacutainer blood tubes SST	Blood testing - serum	Becton Dickinson, New Jersey, USA
Ventilated biological chamber	Administration of nasal challenge	Unknown

2.1.2 Laboratory instruments

Table 2.2 Laboratory instruments used in research study

Name	Supplier
Agilent 2100 Bioanalyzer	Agilent Technologies, Waldbronn, Germany
BioMark HD thermal cycler instrument	Fluidigm Corporation, San Francisco, USA
Conical Falcon tubes (15 mL and 50 mL, polypropylene, sterile)	Corning, New York, USA
Countess Automated Cell Counter	Invitrogen, California, USA
Cryogenic vials	VWR International, Pennsylvania, USA
Dynamic Array Integrated Fluid Chips (96.96)	Fluidigm Corporation, San Francisco, USA
ELISA Plate reader (SPECTRAMax PLUS S384)	Molecular Devices Ltd, Wokingham, UK
Hotplate and stirrer (Jenway 1000)	Grant Instruments Ltd, Cambridge, UK
IFC Controller HX	Fluidigm Corporation, San Francisco, USA
IKA vortex mixer MS3	IKA-Werke GmbH & Co., Staufen, Germany
Meso Scale Discovery SECTOR Imager 2400	Meso Scale Discovery, Rockville, MD, USA
Meso Scale Discovery SECTOR Imager Quickplex SQ120	Meso Scale Discovery, Rockville, MD, USA
Nuclease-free tubes	Invitrogen, California, USA
Refrigerated microfuge (Eppendorf 5415R)	Eppendorf, Hamburg, Germany
Rotary shaker	Jenway or equivalent
Spin filter centrifuge tubes without membrane, 0.22µm pore size, CLS9301	Sigma-Aldrich, Merck KGaA, Darmstadt, Germany
Temperature-controlled water bath	Clifton or equivalent
Vortexer	VWR International, Pennsylvania, USA

2.1.3 Buffers and Reagents

Table 2.3 Buffers and Reagents used in research study

Name	Application	Source
3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate	ELISA	Sigma-Aldrich, Merck KGaA, Darmstadt, Germany
4% paraformaldehyde	Fixation of nasal curettage samples	Dr Jie Zhu, Imperial College London
5% bovine serum albumin (BSA)	Block buffer, ELISA	Sigma-Aldrich, Merck KGaA, Darmstadt, Germany
Agilent RNA 6000 Nano Kit	RNA quality control and quantitation	Agilent Technologies, Waldbronn, Germany
Buffer RLT	Lysis buffer for RNA extraction	Qiagen, Hilden, Germany
DNA Suspension Buffer	RT-PCR	TEKnova, California, USA
Fetal calf serum (FCS, 10%)	PBMC cell culture	Sigma-Aldrich, Merck KGaA, Darmstadt, Germany
Fluidigm Delta Gene assays	RT-PCR	Fluidigm Corporation, San Francisco, USA
Fluidigm Reverse Transcription Master Mix	RT-PCR	Fluidigm Corporation, San Francisco, USA

Histopaque 1077	PBMC isolation	Sigma-Aldrich, Merck KGaA, Darmstadt, Germany
MSD Kit 30-Plex Panel	Soluble mediator analysis	Meso Scale Discovery, Rockville, MD, USA
MSD Kit Chemokine V-PLEX Panel	Soluble mediator analysis	Meso Scale Discovery, Rockville, MD, USA
MSD Kit Custom Tripleplex (IL-25, IL-29, IL-33)	Soluble mediator analysis	Meso Scale Discovery, Rockville, MD, USA
MSD Kit CXCL10/IP-10 Singleplex	Soluble mediator analysis	Meso Scale Discovery, Rockville, MD, USA
MSD Kit IFN-α2a Ultrasensitive Singleplex	Soluble mediator analysis	Meso Scale Discovery, Rockville, MD, USA
MSD Kit IFN-β Tissue Culture Singleplex	Soluble mediator analysis	Meso Scale Discovery, Rockville, MD, USA
MSD Kit IFN-γ Singleplex	Soluble mediator analysis	Meso Scale Discovery, Rockville, MD, USA
MSD Kit Proinflammatory V-PLEX Panel	Soluble mediator analysis	Meso Scale Discovery, Rockville, MD, USA
Nuclease-free water	Primer and probe dilution, RT-PCR	Promega, Madison, USA
Penicillin 100 U/mL and Streptomycin 100 μg/mL	PBMC cell culture	Sigma-Aldrich, Merck KGaA, Darmstadt, Germany
Phorbol 12-myristate 13-acetate (PMA)	Positive control for PBMC stimulation	Unknown
R&D Kit DuoSet IFN-γ	ELISA	R&D Systems, Minneapolis, USA
R&D Kit DuoSet IL-6	ELISA	R&D Systems, Minneapolis, USA
RNAprotect Cell Reagent	RNA lysis and storage	Qiagen, Hilden, Germany
RNeasy Mini kit	RNA extraction	Qiagen, Hilden, Germany
Roswell Park Memorial Institute (RPMI) medium	Cell suspension	Sigma-Aldrich, Merck KGaA, Darmstadt, Germany
SSOFast Evagreen with Low ROX	RT-PCR	Bio-Rad Laboratories, California, USA
Trypan blue solution, 0.4%	Cell viability	Invitrogen, California, USA
TWEEN-20 liquid	Detergent for washing	Sigma-Aldrich, Merck KGaA, Darmstadt, Germany
Wash Buffer	ELISA	Sigma-Aldrich, Merck KGaA, Darmstadt, Germany

2.1.4 TLR dose justification

2.1.4.1 Poly(I:C)

Poly(I:C) is a synthetic viral poly-inosine/cytosine molecule that stimulates TLR3. Unmodified poly(I:C) given systemically for cancer induced toxic side effects and this was probably caused by IL-12 production (226). High molecular weight poly(I:C) is employed for *in vitro* stimulation of human cells at doses of 5-100 μg/mL and has been used intranasally in mice at 100 μg in 20 μL (equivalent to 500 μg/100 μL) (227–231). The current study initially aimed to administer high molecular weight poly(I:C)

at incremental ascending single doses from 10 μg to 100 μg (in 100 μL) to 500 μg/500 μL, topically onto the nasal mucosa. A starting dose of 10 μg/100 μL poly-IC meant it was below the Minimum Anticipated Biological Effect Level (MABEL) (232).

During the course of the study, a pilot study utilising poly(I:C) nasal challenge was published by a group from Lund University, Sweden (233). Seven healthy atopic volunteers were challenged with a total of 800 μg poly(I:C), administered as eight separate doses of 100 μL delivered as a nasal installation into one nostril over two days. Four doses were given over an hour on day 1 and four doses 24h later on day 2. The challenge at this dose was tolerated well, causing no changes in total nasal symptom scores. Therefore, for the current study and after confirmation by the local ethics committee, the poly(I:C) dose escalation phase of the study was omitted and a decision made to directly utilise the top dose of 500 μg. As each actuation of the bidose spray device delivers a volume of 100 μL, a total of 500 μg/500 μL poly(I:C) into each nostril was delivered over one hour, in five equal doses (i.e. 15 minutes apart). It was not deemed practical to escalate beyond this dose as this would have meant the administration of 1000 μL volume of spray, which would require 10 actuations of the bidose device as well as the issue of administering an excess volume of challenge agent hat would not be retained in the nasal cavity.

2.1.4.2 Poly-ICLC

Poly-ICLC (Hiltonol, Oncovir, Washington DC, USA) is poly-IC stabilised with poly-L-lysine and carboxymethylcellulose, and has been designed to confer greater RNAse resistance, and has been administered in humans. Intramuscular poly-ICLC at 20 μg/kg has been used, equivalent to a 1.4 mg dose in a 70 kg person (234). Subcutaneous administration of 1.6 mg (1600 μg) of poly-ICLC caused some redness and induration at the site of injection, and some mild-to-moderate transient flu-like symptoms, with upregulation of blood innate transcriptional signals (105). Richard Davey (Baltimore,

MD, USA) has performed a human phase I dose escalation clinical study with poly-ICLC administered as nasal droplets installation (unpublished, https://clinicaltrials.gov/ct2/show/NCT00646152). Poly-ICLC was well tolerated at all doses between 0.25 mg and 2 mg (2000 μg) per nostril given 2 days apart (personal communication).

For the current study, poly-ICLC (maximum solubility of 2 mg/mL) was planned to be administered as a nasal spray (in contrast to droplets) at incremental ascending doses from 10 μg to 100 μg (in 100 μL) to 500 μg/300 μL topically onto the nasal mucosa. If the 500 μg dose was tolerated well, a top dose of 1000 μg in 500 μL would then be administered.

2.1.4.3 Resiquimod (R848)

Resiquimod is well tolerated in mice and rats in extensive tolerability studies (235, 236). Topical dermal resiquimod is safe and effective as a skin cream for the treatment of anogenital warts, actinic keratosis and skin cancer (237). Human studies have been carried out, giving topical resiquimod to inflamed skin with actinic keratosis at doses up to 0.25% (0.25 g in 100 mL= 250,000 μg/100 mL = 2500 μg/mL = 250 μg/100 μL) (237). Oral resiquimod has been given to patients with chronic hepatitis C virus (HCV) infection (113). Resiquimod was well tolerated orally at doses of 0.01 mg/kg (equating to 0.7 mg (700 μg) for a 100 kg man). In the current study, a dose-escalation phase involved the administration of R848 in incremental steps from 10 μg to 100 μg to 500 μg/100 μL as a nasal spray, to each nostril.

The pharmaceutical quality of the TLR ligands utilised for nasal challenge is outlined in table 2.4.

Table 2.4 Pharmaceutical quality of agents delivered by nasal spray

Challenge Agent	Manufacturer	Catalogue Dose	Production Purity
Poly-ICLC (poly inosine-cytosine stabilised with poly-L-lysine) Hiltonol Vaccine component (HTN code 3002200**)** Registered with FDA under IND 43984, held by Oncovir Inc. Lot PJ215-10-01	Dalton Chemical Laboratories for Oncovir Oncovir, Inc., 3203 Cleveland Ave, NW Washington, DC 200008-3450, USA asalazar@oncovir.com	2 mg/ mL 1 mL/vial	Chemical synthesis. Non-biological origin: does not contain any animal nor human components
Poly(I:C) (inosine-cytosine), high molecular weight, ultrapure, synthetic analogue of double stranded RNA	Invivogen, California 3950 Sorrento Valley Boulevard, Suite 100, San Diego, CA 92121, USA www.invivogen.com	Tlrl-pic 10 mg and 50 mg Lyophilised powder Reconstitute in endotoxin-free physiological water	Chemical synthesis. Non-biological origin: does not contain any animal nor human components, Endotoxin level <1.25 EU/mg
Resiquimod R848 VacciGrade TLR 7/8 agonist Synthetic vaccine grade	Invivogen, California 3950 Sorrento Valley Boulevard, Suite 100, San Diego, CA 92121, USA www.invivogen.com	5 mg lyophilized R848 Reconstitute in endotoxin-free physiological water CAS 144875-48-9	Non-biological origin: does not contain any animal nor human components. Endotoxin <1.25 EU/mg

2.2 Study conduct

2.2.1 Study design

For each TLR agonist administered, the study was divided in to two parts – a dose escalation phase (Part A, Figure 2.1) in healthy volunteers and those with allergic rhinitis, and then a top dose challenge in a larger number of volunteers (Part B, Figure 2.2). The aim of the first part of the study was to identify the optimal dose of TLR agonist to be given nasally, with the objective to be able to induce mucosal innate immune responses at a dose that was symptomatically tolerable for volunteers. The initial study design involved a cohort of eight volunteers (four healthy and four who had allergic rhinitis) for each nasal challenge agent (poly(I:C), poly-ICLC and R848). Each cohort would receive incremental ascending doses, initially with saline and then with single doses of between 10-1000 µg of challenge agent given in volumes ranging from 100-500 µL. Nasosorption was performed before administration of challenge agent and for up to eight hours after challenge, with assessment of mucosal cytokine and chemokine responses.

Once a suitable TLR agonist (either poly(I:C), poly-ICLC or R848) capable of inducing mucosal immune responses was identified, the second phase of the study utilised the top dose of this TLR agonist in a larger number of volunteers. The original study design envisaged this second phase to involve challenging 16 volunteers (8 healthy and 8 allergic rhinitis). As discussed in later results chapters, the identification of a single effective TLR agonist meant the study was subsequently adapted due to the increased capacity to be able to challenge a greater number of volunteers. The study design was also altered to include the addition of volunteers with asthma. Therefore, the final protocol planned to challenge 42 volunteers split in to four groups: healthy n=12; allergic rhinitis n=12; allergic asthma n=12; non-allergic asthma n=6 (in recognition of the fact that this group has traditionally been difficult to recruit). Comparison of immune responses was made with saline challenge and in between groups. In allergic rhinitis and allergic asthma subjects, all TLR agonist challenges were conducted outside of the UK grass pollen allergy season (mid-May to August) to reduce the confounding effect of a concomitant experimental TLR agonist and natural grass pollen allergen challenge.

As mentioned previously, due to the tolerability of poly(I:C) given at a dose of 800 μg in to one nostril (400 μg over one hour given daily for two days) in volunteers with allergic rhinitis (233), it was decided that for the purposes of the current research, a dose of 500 μg/500 μL per nostril of poly(I:C) could be used without the need for a dose escalation phase.

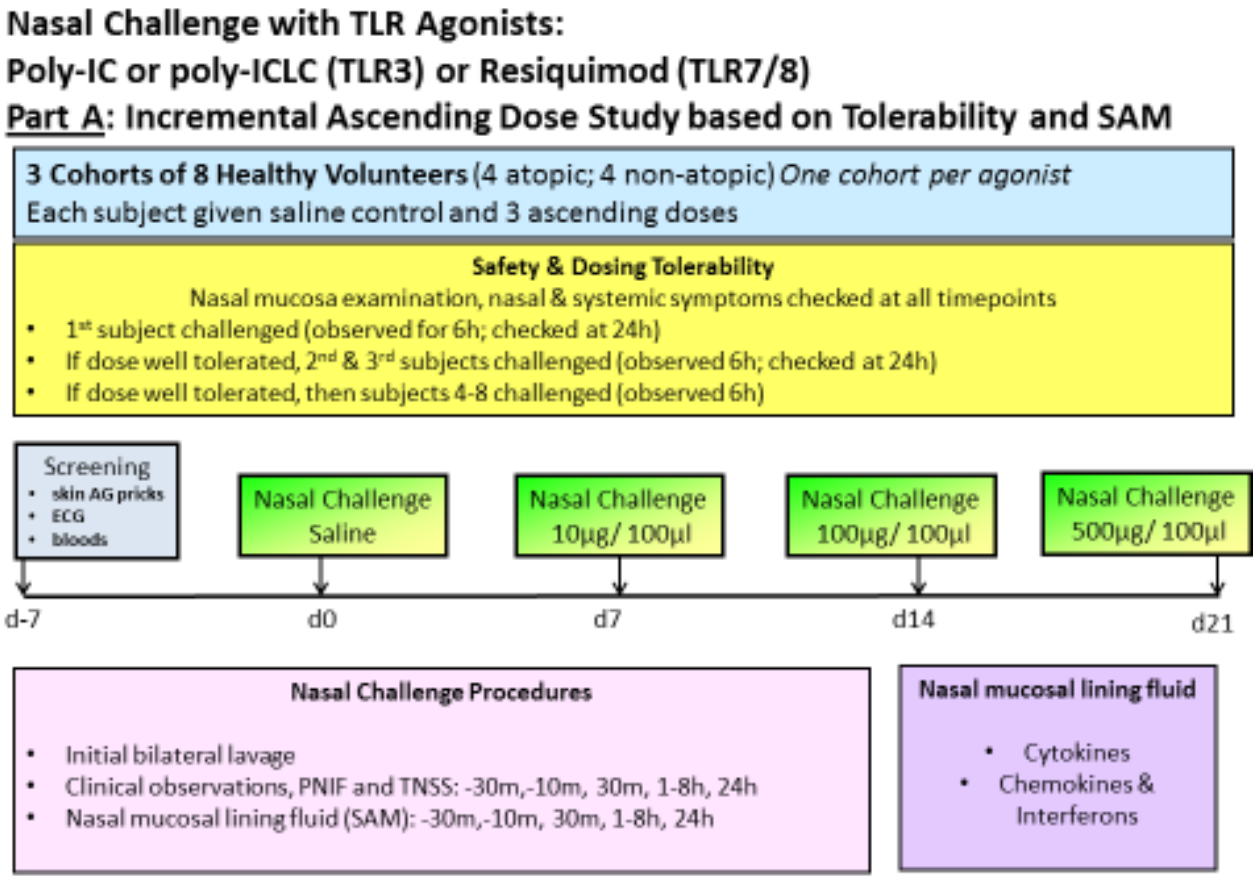


Figure 2.1 Original study design for incremental dose escalation nasal challenge Part A Abbreviations SAM, synthetic absorptive matrix; PNIF Peak nasal inspiratory flow; TNSS Total nasal symptom score

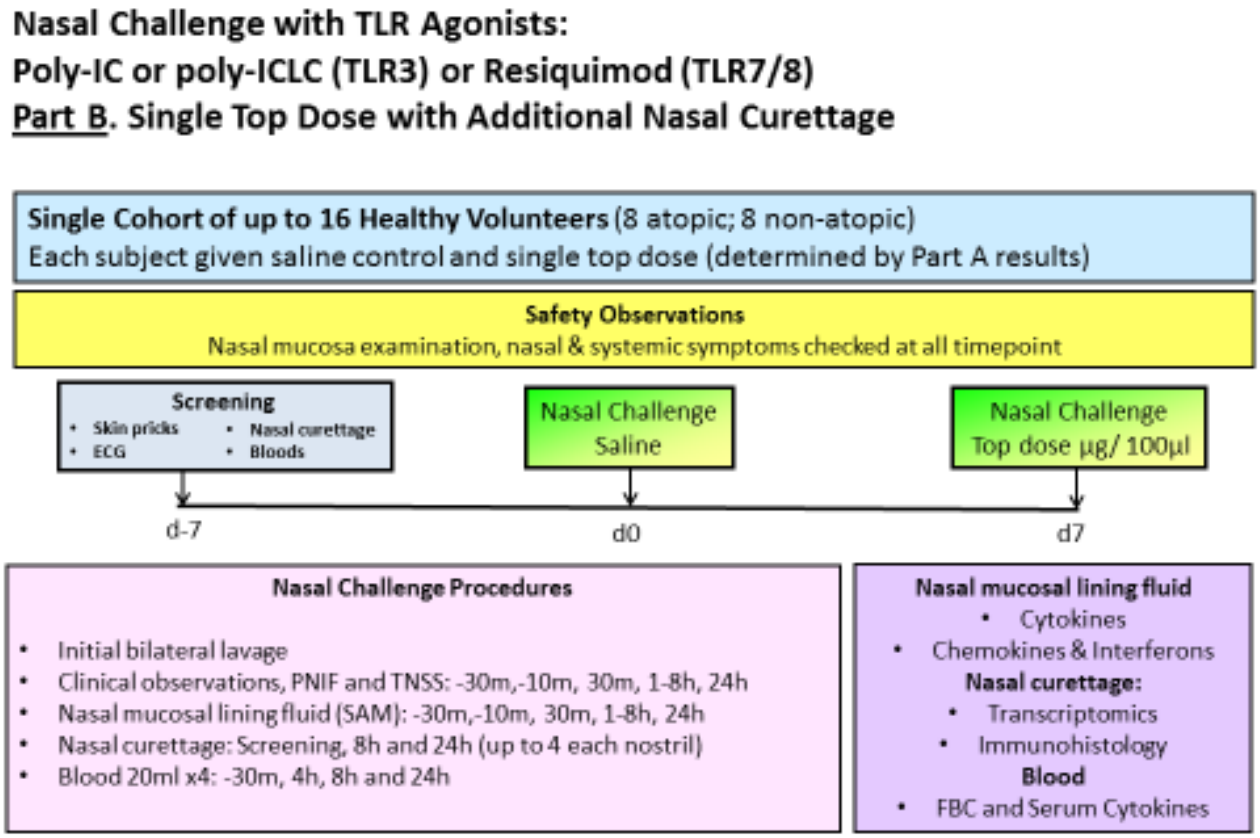


Figure 2.2 Original study design for top dose nasal challenge Part B Abbreviations SAM Synthetic absorptive matrix; PNIF Peak nasal inspiratory flow; TNSS Total nasal symptom score; FBC Full blood count

2.2.2 Safety measures

The importance of effective safety measures in first-in-human clinical studies is highlighted by the phase I trial of the anti-CD28 monoclonal antibody TGN1412 (238). This T-cell superagonist was given at 10-minute intervals to six healthy volunteers, but this lack of spacing in between administration of drug unfortunately led to a failure to recognise the severely deleterious side effects of the drug in a timely manner (239). Whilst the current research did not involve any first-in-human studies, there was no published data available for the administration of some challenge agents (e.g. R848) in to the respiratory tract in humans. With this in mind, the current study was carefully structured to provide adequate spacing in between the escalation of doses to ensure the safety of participants.

For each dosage cohort, a single subject was first assessed for safety over 24 hours. Subjects were monitored for local and systemic symptoms, with a record of vital signs, for at least six hours following each nasal challenge. Volunteers with asthma were additionally monitored using their forced expiratory volume (FEV_1) and if respiratory symptoms or spirometry deteriorated significantly, clinical staff were ready to promptly treat it, e.g. by administration of inhaled B_2 agonists via inhaler or nebulizer. For the dose escalation phase of the study (Part A), a telephone call was additionally made at 24 hours to ensure there were no persistent symptoms. If there were no adverse events, the study would continue to proceed to further subjects. Then the second and third subjects were observed over 24 hours, again only proceeding if there were no adverse events. Then the 4 to 8^{th} subjects were given nasal challenge simultaneously. There was at least four to seven days interval between each challenge agent in any one volunteer subject.

2.2.3 Adverse event reporting

An adverse event (AE) is defined as any untoward medical occurrence in a patient or clinical study subject. A serious adverse event (SAE) refers to any untoward and unexpected medical occurrence or

effect that (i) results in death, (ii) is life-threatening, (iii) requires hospitalisation, or prolongation of existing inpatients' hospitalisation, (iv) results in persistent or significant disability or incapacity or (v) results in a congenital anomaly or birth defect. Important AEs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, was also considered serious. Non serious AEs, whether expected or not, were recorded and reported to the chief investigator of the study. A plan was established to report any SAEs to the Chief Investigator and the Sponsor within 24 hours as well as the NRES Committee London-Harrow if the event was deemed 'related' and 'unexpected'.

2.2.4 Regulatory compliance and ethical approval

All of the challenge agents used in this study were used to elicit an inflammatory response, and not employed for therapeutic benefit. Hence these agents were regarded by the UK regulatory authority (the Medicines and Healthcare Regulatory Authority, MHRA) as Non-Investigative Medicinal Products (non-IMP) (240). For this reason, the challenge agents were not required to be manufactured according to Good Manufacturing Practice (GMP). A proposal to employ these nasal challenges therefore did not require supervision by the MHRA (communication with the Clinical Trials Department and Dr Elaine Godfrey).

The study was conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions (241). The study and subsequent amendments received ethical approval from the National Research Ethics Service Committee London-Harrow (13/LO/1899) and R&D approval from Imperial College Healthcare NHS Trust. For the later stages of the study, genetic tests were collected and stored as per the Human Tissue Act, and data handled in a confidential manner, with no reporting back to the

subject. The study was registered on the clinicaltrials.gov database as trial number NCT02090374 (https://clinicaltrials.gov/ct2/show/NCT02090374).

2.3 Participants

2.3.1 Recruitment

Volunteers were recruited from St Mary's Hospital, Paddington, London, research volunteer databases, registration on clinicaltrials.gov website (indexed by specialist research study search engines) and with the use of adverts in the London Evening Standard and Metro newspapers. Participants that were interested were provided with participant information sheets (PIS) and remuneration for their time and travel expenses were provided (up to £250).

2.3.2 Screening

Interested subjects underwent screening at the ICRRU, St Mary's Hospital where consent was obtained and eligibility determined after interview, physical examination (including bedside observations) and review of screening investigations. For the dose-escalation studies, healthy volunteers and those with allergic rhinitis were sought, whilst the second phase of the study additionally recruited subjects with allergic asthma. Intra-epidermal skin allergen prick tests were performed using a panel of common aeroallergens (timothy grass pollen, six grass pollen mix, tree pollen mix, cat hair, dog hair, house dust mite, aspergillus fumigatus spores) and compared to positive histamine and negative saline controls. Screening blood tests comprised full blood count (including differential cell count), electrolytes, renal function, liver function, C-reactive protein (CRP) and total IgE. All women of childbearing potential also underwent a pregnancy test. A standardised screening case report form was utilised to record the above information (see appendix I).

2.3.2.1 General inclusion criteria

For all participants undergoing TLR agonist challenge, the general inclusion criteria were as follows:

- Males and females aged 18 to 60 years
- Body mass index in the range 18-39

2.3.2.2 General exclusion criteria

For all participants undergoing TLR agonist challenge, the general exclusion criteria were as follows:

- Current smokers (in the last year) or a smoking history of ≥5 pack years
- Recent infections in past 14 days before screening: especially upper respiratory tract illnesses (including colds and influenza), sore throats, sinusitis, infective conjunctivitis.
- Lower respiratory tract infection in past 28 days
- Signs or symptoms of significant nasal anatomical defects, hypertrophy of turbinates, major septum deviation, nasal polyposis or recurrent sinusitis
- Nasal mucosal defects, injury, ulceration
- Previous nasal or sinus surgery
- Tuberculosis at any stage in life
- ENT disease
- Active infectious disease including hepatitis
- Respiratory disease (other than asthma)
- Renal disease
- Autoimmune disease
- Rheumatological disease and vasculitis
- Dermatological disease
- Neoplastic conditions
- Metabolic diseases and extreme obesity
- Severe depression and psychiatric disorders

- Medical therapy other than that permitted for contraception or for chronic conditions unlikely to affect the results of the study as determined by the study physician.
- Treatment with local or systemic corticosteroids during the previous one month
- Use of anti-histamines within the previous 72 hours
- Anti-inflammatory therapy: including non-steroidal anti-inflammatory drugs (NSAIDs)
- Participation in a therapeutic drug trial in the prior 30 days.
- Inability or unwillingness to use contraception if the patient is female of child-bearing age.
- Pregnant or breast feeding women
- Inability to provide informed consent

2.3.2.3 Subject-specific criteria

For healthy non-atopic volunteers, mixed inclusion and criteria were as follows:

- No clinical history of allergic rhinitis, allergic asthma or eczema
- Negative skin prick tests to a range of common aeroallergens
- Normal blood eosinophil count
- Normal baseline forced expiratory volume (FEV1) i.e. ≥80% (for second phase of study)
- Negative methacholine challenge (PC20 >8mg/mL) (for second phase of study)

For volunteers with allergic rhinitis, mixed inclusion and exclusion criteria were as follows:

- A clinical history of seasonal grass pollen allergic rhinitis: sneezing, running and itching nose, nasal drip in the UK grass pollen summer season (May-July).
- Specific allergy confirmed by positive intra-epidermal skin prick test to Timothy grass pollen extract (Soluprick, Phleum pratense; ALK, Horsholm, Denmark), a positive reaction being a raised wheal of diameter >3mm larger than a negative saline control.
- Normal baseline forced expiratory volume (FEV1) i.e. ≥80% (for second phase of study)

- Either negative or positive methacholine challenge (for second phase of study)

For volunteers with allergic asthma (only included in the second phase of the study), mixed inclusion and exclusion criteria were as follows:

- Physician diagnosed asthma up to stage 2 BTS/GINA guidelines
- Seasonal grass pollen allergic rhinitis: sneezing, running and itching nose, nasal drip in the UK grass pollen summer season (May-July).
- Specific allergy confirmed by positive intra-epidermal skin prick test to Timothy grass pollen extract, a positive reaction being a raised wheal of diameter >3mm larger than a negative saline control.
- Baseline forced expiratory volume (FEV1) ≥75%
- Positive methacholine challenge (PC20 <8mg/mL)

For volunteers with non-allergic asthma (only included in the second phase of the study), mixed inclusion and exclusion criteria were as follows:

- Physician diagnosed asthma up to stage 2 BTS/GINA guidelines
- No clinical history of allergic rhinitis
- Negative skin prick tests to a range of common aeroallergens
- Baseline forced expiratory volume (FEV1) ≥75%
- Normal blood eosinophil count
- Positive methacholine challenge (PC20 <8mg/mL) (for second phase of study)

2.3.3 Consent

Consent to enter the study was sought from each participant only after a full explanation was given, an information leaflet offered and time allowed for consideration of the study material. Signed

participant consent forms were obtained. All participants were free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing further treatment.

2.3.4 Data handling

All study related documentation was stored in a secure, locked room on the premises of St Mary's Hospital, Paddington, London. All electronic data and communications were stored on a secure trust network that was restricted to researchers from the Imperial Clinical Respiratory Research Unit (ICRRU) based at St Mary's Hospital, Paddington, London.

2.4 Nasal challenge

All participants were invited to the ICRRU at St Mary's Hospital in the morning of their nasal challenge. The timing of administration of nasal challenge was kept consistent in between saline and TLR agonists as well as between volunteers due to the increasingly well recognised effects of the circadian rhythm on immune function (242). On the day of challenge, volunteers had baseline nasal sampling performed before and after nasal lavage. After nasal challenge, sampling was performed alongside clinical observations by the study physician or one of two trained study nurses at regular intervals. For the dose ascending Part A, participants were observed for up to eight hours on the unit and for part B, participants were observed for up to 10 hours on the unit and also returned at 24 hours. Tables 2.5 and 2.6 summarise the procedures in both parts of the nasal challenge study.

Table 2.5 Summary of procedures for ascending dose nasal challenge Part A

Time point	**Screening**	**Before nasal challenge** Total duration = 30 min			**Nasal Challenge (NC)**	**After nasal challenge** Total duration=8h	
	D-7	-30m	-20m	-10m		15, 30, 45m	1h – 6h (later extended to 8h)
Nasal challenge Saline/TLR agonist (variable doses)					X		
Nasal lavage (for washing only)	X		L/R				
Nasal mucosal examination	X	X				X	X
Total nasal symptom scores (TNSS) and Peak Nasal Inspiratory Flow (PNIF)		X				X	X
Nasosorption (Left and Right)		L/R		L/R		L/R	L/R

Table 2.6 Summary of procedures for top dose nasal challenge Part B

Time point	**Screening**	**Before nasal challenge** Total duration = 30 min			**Nasal Challenge** (NC)	**After nasal challenge** Total duration=24h		
	D -7	-30m	-20m	-10m	0m	30m	1-10h	24h
Nasal challenge Saline / Resiquimod					X	X		
Nasal lavage (for washing only)	X		L /R					
Nasal mucosal examination	X	X				X	X	X
Total nasal symptom scores (TNSS) and Peak Nasal Inspiratory Flow (PNIF)		X				X	X	X
Nasosorption (Left and Right)		L /R		L /R		L /R	L /R	X
Nasal curettage (Up to 4 samples)	X						Left (8h)	Right
Blood (up to 20mL)	X		X				4h, 8h	X
Spirometry (volunteers with asthma)	X	X					1,2,4,8h	X

2.4.1 Nasal Lavage

Prior to nasal challenge, nasal lavage was performed on all volunteers (figure 2.3). Subjects were seated with their necks held in a flexed position and chin tucked in to their chest. A sterile 10 mL syringe containing 5 mL 0.9% saline was instilled into each nasal cavity through a disposable plastic olive device (Hunt Development, Midhurst, UK). Saline was aspirated and reinstilled 10 times with disposal of the collected fluid.

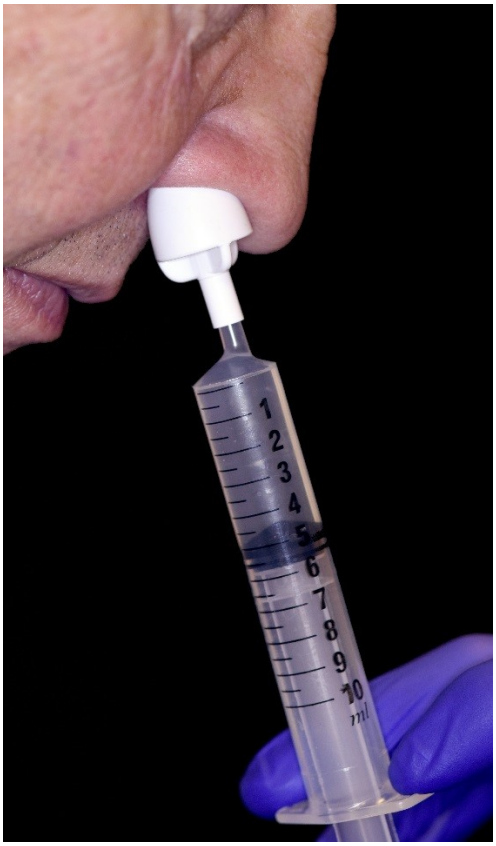


Figure 2.3 Technique of nasal lavage for washing nasal mucosa 5 mL of 0.9% saline was instilled in to each nasal cavity through a nasal olive, which formed a seal around the inner nostril ensuring saline was retained inside the nasal cavity. The saline was then aspirated and reinstilled using a 10 mL syringe up to 10 times.

2.4.2 Administration of TLR agonists

All challenge agents were prepared in advance of nasal challenge experiments with aliquots stored at -20°C (poly(I:C), R848), 4°C (poly-ICLC) or room temperature (0.9% saline). On the day of challenge, challenge agents were thawed to room temperature with the correct dose made up and recorded in the presence of the study physician and post-doctoral scientist. The final concentration was

subsequently loaded in to a BD bidose device (Aptar Pharma, Milton Keynes, UK) and labelled with the name and dose of the challenge agent. All subjects were then invited to be seated inside a dedicated ventilated biological chamber with a carbon filter attached to the ventilation unit in addition to a HEPA filter (figure 2.4). At the onset of challenge, the bidose was inserted along the floor of the nasal cavity towards the ipsilateral ear with each actuation delivering 100 µL challenge agent (figure 2.5). Unilateral allergen challenge has been previously shown to induce secretions of equivalent weight as well as prostaglandin D2 from unilateral and contralateral nostrils (243, 244). However, elevated histamine and nasal airway resistance is only seen on the ipsilateral side suggesting that neural mechanisms contribute to contralateral responses (243, 245). Therefore, to ensure reproducibility of mediators being measured between nostrils, the study was designed to deliver TLR agonists as a spray in to both nostrils.

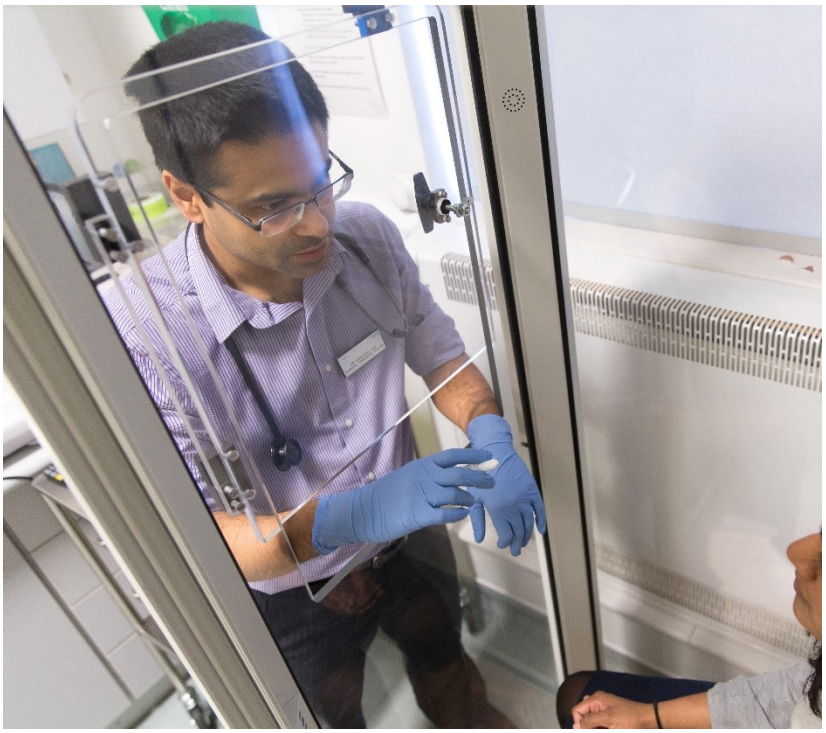


Figure 2.4 Delivery of nasal challenge agent in a biological safety chamber All challenge agents were delivered inside a ventilated chamber with a carbon filter attached to the ventilation unit in addition to a HEPA filter

Figure 2.5 Delivery of nasal challenge agent using bidose device The bidose device was inserted in to each nostril along the floor of the nasal cavity and pointed towards the ipsilateral ear. Each actuation delivered 100 µL volume of challenge agent.

2.4.3 Clinical correlates of nasal obstruction

Alongside nasal sampling, subjective and objective markers of nasal obstruction were recorded. The total nasal symptom score (TNSS) is recommended by the Food and Drug Administration (FDA) as a reliable and repeatable outcome measure to assess subjective allergic rhinitis symptoms in clinical trials (246). It is calculated by asking volunteers to assess the degree of nasal congestion, rhinorrhoea, sneezing, and nasal itch on a scale from 0 to 3 (0=no symptoms, 1=mild, 2=moderate, 3=severe symptoms) to obtain a total maximum score of 12. The objective measure of peak nasal inspiratory flow (PNIF) is the best non-invasive validated technique for assessment of airflow through the nose, including after nasal provocation (153, 247). It involves the placement of a face mask to form a seal around the nose and mouth with subjects asked to perform a maximal forced nasal inspiration with measurement (in L/min) of their peak nasal airflow (In-Check Nasal with Mask, Clement Clarke International, Harlow, UK, 3109751) (248). This was performed by volunteers, pre-challenge and post challenge at simultaneous times to nasosorption with the best of three scores recorded on the case

report form. For volunteers with asthma their forced expiratory volume during the first second (FEV1) was additionally monitored.

2.4.4 Systemic observations

As part of their screening all participants had basic clinical observations performed including temperature, pulse, blood pressure, respiratory rate and oxygen saturations. For the ascending dose Part A of the study, observations were taken before challenge and at 24 hours. Once the top dose was established in Part B, volunteers undergoing TLR agonist challenge had observations taken on a more frequent basis (hourly).

2.5 Sample collection

2.5.1 Nasal mucosal lining fluid and elution method

During earlier dose escalating part A nasal challenge experiments, nasal mucosal lining fluid was collected using sterile (endotoxin-free) strips of SAM (Nasosorption, Hunt Developments Limited, Midhurst, UK) inserted in to the nose using sterile forceps. During later top dose part B experiments, Hunt Developments produced an improved nasosorption device with the SAM strip mounted on to a handle, (Nasosorption FX-i), which could be inserted directly into the nasal cavity without the need for forceps. Nasosorption was performed pre-nasal lavage, post-nasal lavage, 30 minutes after challenge and at hourly intervals for up to eight hours with a final sample collection at 24 hours. The technique of performing nasosorption involved placing the nasosorption device alongside the inferior turbinate of a volunteer whose neck was held in a partially flexed position (figures 2.6a, b). During earlier part A experiments, the SAM strip was left within the nasal cavity for 30 seconds but as discussed in section 3.4.5 further experiments using a new nasosorption FX-R device, a duration of one minute was found to be optimal and therefore for all further experiments, nasosorption was placed in the nose for one minute. Finger pressure was applied after which the device was taken out

of the nose (figure 2.6c). The SAM strip was removed from the handle using sterile disposable forceps and placed inside a centrifuge tube containing 300 µL of pre-chilled assay buffer (Millipore Ltd, Watford, UK, AB-33K) to assist in elution of soluble mediators, where it resided on ice until transfer to the laboratory (between 5-60 minutes later). The tube was pulse vortexed for 10 seconds and the SAM strip and buffer was transferred to a filter cup within a centrifuge tube (Corning Costar Spin-X, 0.22 µm pore size, CLS9301) (figures 2.6d, e). The acetate membrane usually lining the bottom of the filter cups had previously been removed by the company to prevent the loss of proteins during the centrifugation stage. The tube was placed in a centrifuge pre-chilled to 4°C (Eppendorf, 5415R) and centrifuged at 16,000 x *g* for 20 minutes. The filter cup containing the SAM strip was discarded and the filtered eluate was gently pipetted to breakdown any residual mucous debris. The volume of detergent buffer (300 µL) was subtracted from the total volume of fluid recovered and recorded. Aliquots containing a minimum volume of 100 µL were stored at -80°C. Additional nasosorption samples were collected and directly stored at -80°C (without detergent buffer) for subsequent microbiome analysis by the genomic laboratory group led by Professors Miriam Moffatt and William Cookson (Imperial College London) and is not discussed in this thesis.

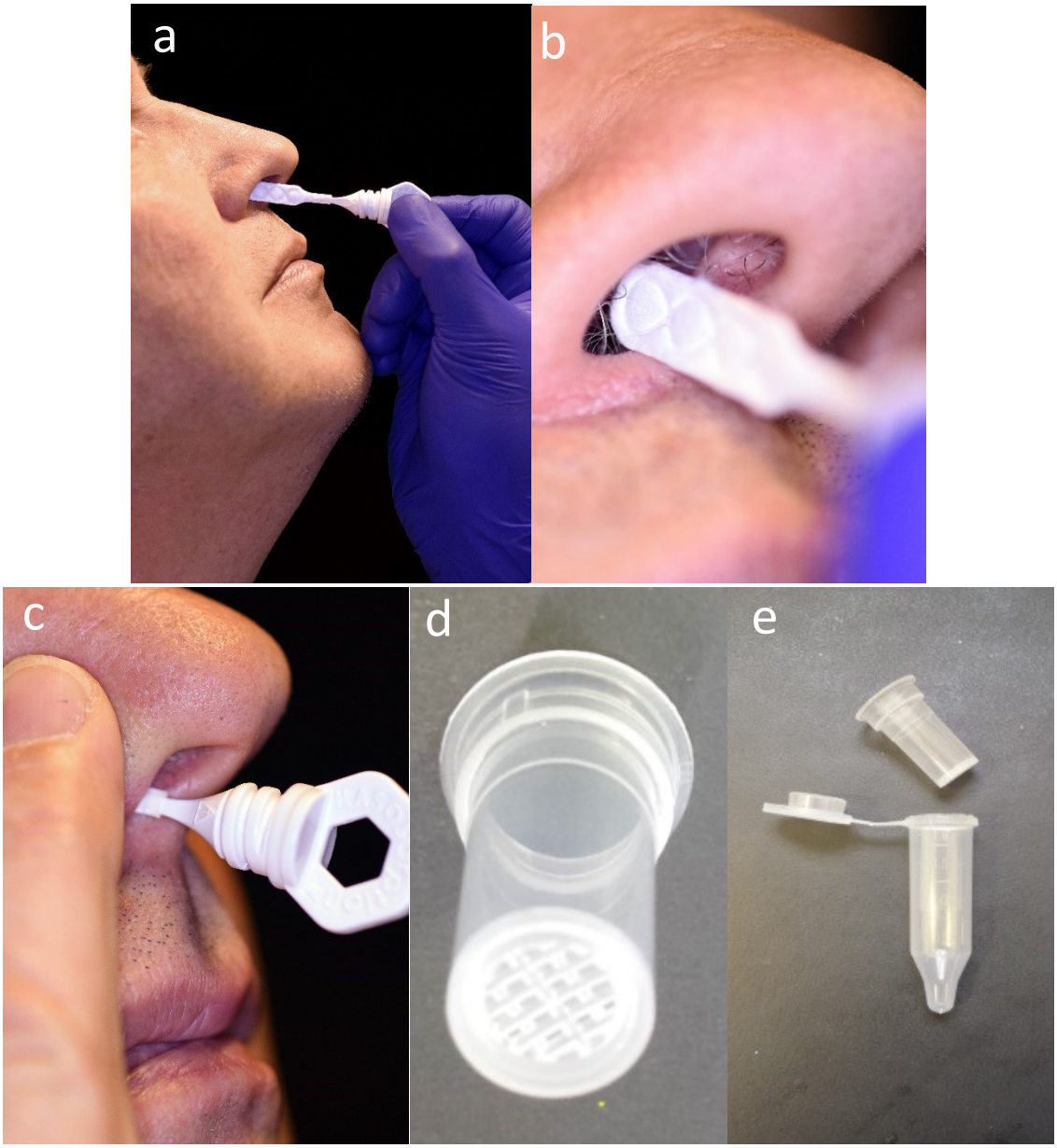


Figure 2.6 Technique of performing nasosorption and elution method (a) Nasosorption device was inserted with neck partially extended with (b) insertion alongside the inferior turbinate and (c) held in place for one minute under the pressure of a finger. The synthetic absorptive matrix strip was then removed and placed in a centrifuge tube with pre-chilled detergent buffer and subsequently transferred to (d) a filter cup without an acetate membrane contained within (e) a tube for subsequent centrifugation.

2.5.2 Nasal mucosal tissue

Nasal curettage was performed at the screening visit and at 8 and 24 hours after both saline and TLR agonist challenge. Throughout the study, nasal curettage was performed by a single study physician so as to maintain a consistent specimen collection technique with reduced variability during sampling.

A head mounted light source (Portable Headlight Green Series, Welch Allyn, NY, USA, 46074R) was utilised in conjunction with Thudicum nasal specula (DP Medical Systems Ltd, Chessington, UK, DPI2002) to clearly illuminate the nasal cavities (figure 2.7a). Two ASI-Rhino-Pro Nasal Curettes (Arlington Scientific, UT, USA) were used to gently scrape the mid-inferior region of the inferior turbinate (figure 2.7b). For transcriptomics analysis, the two curettes were placed in 500 µL RNAprotect Cell Reagent (Qiagen, Hilden, Germany, 76526) in 15 mL falcon tubes for RNA stabilization. This was subsequently pulse vortexed for 30 seconds to degrade the tissue and transferred to 2mL cryogenic vials (VWR International, 479-1262) for storage at -80°C. For immunohistology, the two curettes were carefully placed in 240 µL of 4% formaldehyde (provided by Dr Jie Zhu, Imperial College London) in cryogenic vials (with minimal disturbance to reduce degradation) and left at room temperature for at least four hours with subsequent storage overnight in a fridge at 4°C.

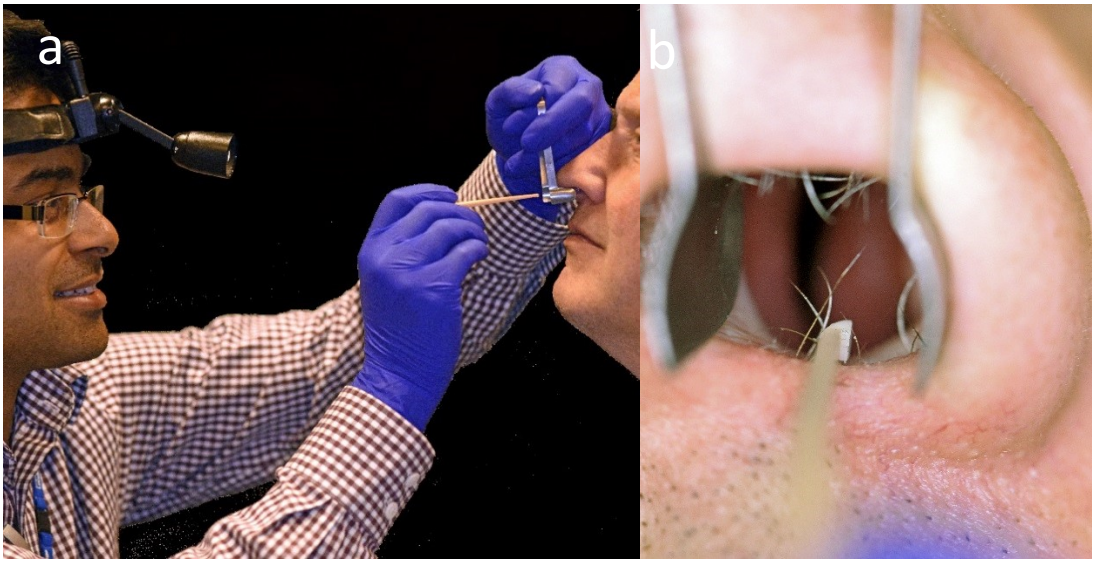


Figure 2.7 Technique of performing nasal curettage (a) A good light source is important to illuminate the interior nasal cavity with (b) curettage performed on the mid-inferior part of the nasal inferior turbinate – the beginning of the pseudostratified respiratory epithelium.

2.5.3 Blood

During the top dose Part B of the study, peripheral blood sampling was performed using standard phlebotomy techniques based on hospital trust guidance at baseline at 4, 8 and 24 hours after nasal challenge and sent to the hospital laboratory for assessment of differential white cell counts

(neutrophils, lymphocytes and eosinophils). Blood was also collected at baseline and 24 hours after challenge for peripheral blood RNA analysis (PAXgene Blood RNA Tube, PreAnaltiX GmbH, Hombrechtikon, Switzerland) and stored at room temperature for at least two hours prior to short-term storage at -20°C and subsequent long-term storage at -80°C. At the screening visit, participants were also offered the option to provide peripheral blood samples for genomic analysis (PAXgene Blood DNA tube, PreAnaltiX GmbH, Hombrechtikon, Switzerland). Peripheral blood RNA and DNA samples have been bio-banked for future use if required and are not discussed in this thesis.

2.5.4 Serum

Peripheral whole blood was also collected for serum analysis (BD SST II Vacutainer) and allowed to clot for at least 30 minutes. It was subsequently centrifuged at 1000 x *g* for 10 minutes at room temperature and 1000 µL aliquots were produced and stored at -80°C.

2.6 Measurement of soluble immune mediators

2.6.1 Electrochemiluminescence

2.6.1.1 Assay principles

Soluble mediators in nasal mucosal lining fluid and serum was measured using ultrasensitive immunoassay kits purchased from Meso Scale Discovery (MSD, Rockville, USA). These assays have several advantages over traditional enzyme-linked immunosorbent assay (ELISA) methods including the requirement for very small samples volumes (25 µL), high sensitivity at low concentrations (<1 pg/mL) and wide dynamic range (0.1-10,000 pg/mL). MSD plates consist of wells containing spots that are pre-coated with capture antibodies with the potential to include up to 10 spots within a single well permitting multiplex immunoassay profiling from a single small-volume sample. After incubation with sample and proprietary electrochemiluminescence compound (MSD SULFO-TAG) labelled detection antibodies, a sandwich immunoassay is formed. After addition of read buffer, plates are placed in an

MSD SECTOR Imager (2400 or Quickplex SQ 120) instrument whereby a voltage applied to electrodes present at the bottom of the plates induce the bound labels to emit light. The intensity of the emitted light is compared to known standard dilutions of mediator and therefore is a quantitative measure of the concentration of mediator present in the sample. Some of the MSD kits are V-PLEX assays, which have additional quality standards applied to improve lot-to-lot consistency.

2.6.1.2 Selection of immunoassays

Due to the generation of a large number of samples (numbering in the thousands) and the cost of MSD immunoassays, a practical decision was taken for the earlier stage experiments to restrict the number of mediators and/or the number of samples analysed. An iterative approach was taken to select the most suitable mediators and the specific detail of which assays and samples were utilised for each analysis is outlined in the relevant results chapters. Data from live viral challenge studies utilising RSV (unpublished, Max Habibi, Chris Chiu, Peter Openshaw) suggested that CXCL10/IP-10 and IFN-γ were immune mediators that were likely to be upregulated by TLR agonists, since they activate similar receptors to ssRNA and dsRNA viruses, and were therefore chosen to form part of the preliminary analysis. For later challenge experiments utilising the top dose of TLR agonist, a more comprehensive and pre-defined set of immunoassays were utilised on all samples collected. A list of immunoassays utilised in this study is outlined:

- CXCL10/IP-10 Singleplex
- IFN-γ Singleplex
- IFN-α2a Ultrasensitive Singleplex
- IFN-β Tissue Culture Singleplex
- Custom Tripleplex (IL-25, IL-29/28B [IFN-lambda 1/3], IL-33)
- Proinflammatory V-PLEX Panel (IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, TNF-α)

- Chemokine V-PLEX Panel (CXCL8/IL-8, CXCL10/IP-10, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL11/eotaxin-1, CCL13/MCP-4, CCL17/TARC, CCL22/MDC, CCL26/eotaxin-3)
- 30-Plex Panel - including the proinflammatory and chemokine panels listed above as well as a cytokine panel (GM-CSF, IL-12/IL-23p40, IL-15, IL-16, IL-17A, IL-1α, IL-5, IL-7, TNF-β, VEGF-A)

2.6.1.3 Assay method

Nasal mucosal lining fluid and serum samples were defrosted to room temperature on the day of analysis. Concentrated stock calibrators were diluted using serial 4-fold dilutions to generate seven standards of decreasing concentration with the inclusion of a zero standard (diluent alone). The assay protocol was followed as per the manufacturer's product insert. Briefly, the general assay method for proinflammatory, chemokine and cytokine multiplex panels as well as IFN-γ and CXCL10 singleplex assays was as follows: (a) the pre-coated (with capture antibody) 96-well plates were washed three times with 150 µL/well Wash Buffer (0.05% Tween-20 + PBS, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany); (b) singlet samples were diluted-in-plate two-fold by adding 25 µL of neat sample to 25 µL assay diluent to each well as well as the addition of 50 µL standard in duplicate to empty wells; (c) plates were sealed and incubated at room temperature on a rotary shaker at 600 *x* rpm for two hours; (d) plates were washed again as previously described and 25 µL of detection antibody solution was added to each well, with the plate sealed and incubated on a shaker for two hours; (e) after a final wash step, 2X strength of 150 µL Read Buffer was pipetted into each well and incubated for 5-10 minutes; (f) the plate was analysed on a MSD SECTOR Imager instrument.

For the IFN-α2a ultrasensitive singleplex plate, 25 µL Diluent 2 was initially dispensed into each well and sealed and incubated for 30 minutes on a shaker. After the addition of 25 µL sample or 25 µL standard (without an initial wash stage), the remaining steps were identical to those described above. For the IFN-β tissue culture plate, the MSD plate first required coating with capture antibody solution,

and was then sealed and incubated at room temperature on a rotary shaker for one hour with the remainder of steps as described previously.

For the custom tripleplex panel, standards, capture antibodies and detection antibodies were sourced from non-MSD suppliers (IL-25, Pebrotech; IL-29/IL-28B and IL-33, R&D Systems) and prepared as per the manufacturer's product inserts. After pre-coating with capture antibodies, 150 µL of 5% bovine serum albumin (BSA, Sigma-Aldrich) was added to each well as a block buffer, sealed and incubated for one hour with shaking. After washing the plate as described above and addition of standard and sample all steps were as outlined previously.

2.6.1.4 Calculation of mediator levels from standard curves and limits of detection

Calibration curves were established using MSD DISCOVERY WORKBENCH analysis software by fitting the electrochemiluminescence (ECL) signals of known standards to a four-parameter logistic regression model. Furthermore, a $1/Y^2$ weighting was applied to provide a better fit of the data to the curve over a wide dynamic range. Unknown sample concentrations were subsequently calculated (as pg/mL) by back-fitting their ECL signals to the calibration curve and correcting for dilution factor. The upper limit of detection (ULOD) was defined by the concentration of the highest standard used. The lower limit of detection (LLOD) represented the calculated concentration that was 2.5 standard deviations above the background zero standard. The LLOD for each mediator on each plate was recorded and the geometric mean of all LLOD (GM-LLOD) values was calculated to permit subsequent comparison between different plates. Any concentrations falling below this GM-LLOD value were also assigned this value.

2.6.2 Enzyme-linked immunosorbent assay (ELISA)

Sandwich ELISAs were used to measure IL-6 and IFN-γ (DuoSet, R&D Systems, Minneapolis, USA) in culture supernatant derived from stimulation of peripheral blood mononuclear cells (PBMCs) as described in 2.8.4. The general ELISA assay method was as follows: (a) A 96-well microplate was coated with 100 µL per well of capture antibody, sealed and incubated overnight at room temperature; (b) the plates were washed using 400 µL/well of Wash Buffer three times; (c) plates were blocked using 300 µL/well Reagent Diluent and incubated at room temperature for one hour; (d) after washing, 100 µL of sample or standard (in duplicates) were added to wells and incubated for two hours at room temperature; (e) after washing, 100 µL of detection antibody was added to each well and the plate sealed and incubated for two hours at room temperature; (f) after washing, 100 µL of streptavidin-HRP was added to each well and incubated for 20 minutes in the dark; (g) after washing, 100 µL of Substrate Solution TMB was added to each well and incubated for 20 minutes in the dark; (h) after observing for change in colour for up to five minutes, 50 µL of Stop Solution was added to each well and placed on a microplate reader set to 450 nm to determine absorbance. The average zero standard optical density was then subtracted from each standard and sample reading. Calibration curves were established from standards using a linear regression model, from which sample concentrations were subsequently determined.

2.7 Nasal mucosal transcriptomics

The work done on nasal mucosal mRNA extraction and cDNA library preparation was greatly supported by Dr Ryan Thwaites (post-doctoral scientist, Imperial College London), and the real-time RT-PCR using the Fluidigm microfluidics platform (Fluidigm Corporation, San Francisco, USA) was assisted by Dr Nick Jordan (Field Application Specialist, Fluidigm).

2.7.1 mRNA extraction and quantitation

Nasal curettage mucosal tissue samples stored in RNAprotect were defrosted to room temperature and a RNeasy Mini kit (Qiagen, 74104) was used to purify mRNA. This process aimed to enrich mRNA by purifying molecules longer than 200 nucleotides in length (since most other RNAs are less than 200 nucleotides). The manufacturer's instructions were followed but the steps are outlined here: (a) cells were vortexed for 10 seconds and subsequently homogenized by passing the sample through a 20-gauge needle attached to a syringe 30 times. (b) cells were pelleted by brief centrifugation at 5,000 *x* g at 4°C for three minutes; (c) 600 µL of lysis buffer (Buffer RLT, Qiagen) was added to the sample and mixed; (d) the lysate was centrifuged at 16,000 x *g* at 4°C for three minutes; (e) the follow-through was transferred to a new tube and 600 µl ethanol (70%) added; (f) 700 µL of lysate was transferred to an RNeasy spin column and centrifuged (and repeated if excess volume present) to allow RNA to bind to the membrane and aide the removal of contaminants; (g) on-column DNase digestion was then performed: the lysate was washed with 350 µL buffer RW1, 10 µL DNase stock solution was added to 70 µL buffer RDD, mixed by inversion, 80 µL was added to each (dry) column membrane and incubated on the benchtop for 15 minutes, and finally 350 µL of buffer RW1 added and centrifuged at 8,000 *x* g for 15 seconds; (h) 700 µL of buffer RW1 was added to the column and centrifuged at 8000 *x* g for 15 seconds with the follow-through discarded; (i) 500 µL of buffer RPE was added to the column, centrifuged 8,000 *x* g for 15 seconds and repeated; (j) The spin column was placed in a new 1.5 mL collection tube and eluted with 30-50µl of RNase-free water.

RNA quality control and quantitation was then performed using an Agilent 2100 Bioanalyzer instrument (Agilent Technologies, Waldbronn, Germany) and accompanying reagents (Agilent RNA 6000 Nano Kit). The manufacturer's instructions were followed and are outlined here: (a) reagents were allowed to equilibrate to room temperature; (b) RNase decontamination of the bioanalyzer electrodes was performed using 350 µL of RNaseZAP loaded on to an electrode cleaner chip for one

minute and then repeated with a chip with 350 µL RNase-free water; (c) 550 µL RNA 6000 Nano gel matrix was placed into the top receptacle of the supplied spin filter and centrifuged at 1,500 *x* g for 10 minutes with 65 µL aliquots produced (and optionally stored at 4°C); (d) dye concentrate was vortexed and 1 µL added to 65 µL of filtered gel and vortexed again; (e) the mixture was centrifuged at 13,000 *x* g for 10 minutes; (f) a new Nano chip was placed on (a correctly assembled) priming station; (g) 9µl of gel-dye mix was dispensed in to a well, marked 'G'; (h) the syringe was clipped in to place, the syringe was depressed and after 30 seconds the clip was released and the syringe then pulled back to the 1 mL position; (i) 9µl of gel-dye mix was pipetted in to each remaining 'G' wells; (j) 5µl of Nano marker was pipetted in to each sample well as well as the well, marked with a ladder icon; (k) a ladder aliquot was thawed on ice and heat denatured for two minutes at 70°C; (l) 1 µL of ladder was pipetted in to the well, marked with a ladder icon; (m) 1µl of sample was added to wells 1-12; (n) The chip was vortexed (IKA vortex mixer MS3, IKA-Werke GmbH & Co., Staufen, Germany) at 2400 *x* rpm for 60 seconds and analysed within five minutes.

2.7.2 cDNA library creation

Extracted RNA from nasal curettage specimens was converted to cDNA for use in PCR using Fluidigm Reverse Transcription Master Mix (containing buffer, deoxyribonucleotide triphosphates (dNTPS), primers, ribonuclease inhibitor and an engineered RNaseH+ Moloney Murine Leukemia Virus (MMLV) reverse transcriptase). The manufacturer's instructions were followed using the following sample volumes: 1 µL of Master Mix, 3 µL RNase-free Water and 1 µL of sample RNA. The reactions were placed in a standard thermal cycler and incubated for 25°C for five minutes, at 42°C for 30 minutes and at 85°C for five minutes. To enhance the expression of genes with low abundance, preamplification for 12 cycles was performed using the manufacturer's instructions. Exonuclease I (E. coli, #M0293 New England Biolabs, Massachusetts, USA) was used to remove remaining primers prior to PCR.

2.7.3 Gene selection and primers for Real-Time PCR

Fluidigm Delta Gene assays containing specific primers to the gene of interest were utilised (see Appendix II for forward and reverse primers). They were designed to cross an intron wherever possible to avoid amplifying genomic DNA and aimed to detect all known isoforms when possible. A total of 43 genes were selected (table 2.7) to be run in duplicate and referenced to housekeeping genes GAPDH and HPRT1, along with non-template controls. Genes were selected based on knowledge of well-characterized intracellular signalling pathways after TLR agonist activation as well as known interferon stimulated genes (ISGs) (16, 144, 249). Nasal mucosal tissue after allergen challenge has been used to validate eosinophil-associated genes (74). To extend this concept of "cellular footprinting", genes associated with specific immune cells were selected based on literature search and review of the GeneCards database (http://www.genecards.org/) to identify tissue-specific expression. This included cells that were known to be involved in the immune response to viral infections including dendritic cells (250–254), NK cells (255, 256), macrophages (257, 258), neutrophils (74), eosinophils (74, 122), mast cells (74, 257) as well as epithelial cells and their derived cytokines (12, 193, 259, 260).

Table 2.7 Selected genes with cell-associated markers for gene expression array (n=43)

TLR Signalling	IFN-stimulated genes	Epithelial cells	Dendritic Cell	Macroph-age	Neutrophil	Natural Killer Cell	Eosinophil	Mast Cell
TLR3	IFIT3	IL25	CD1C (BDCA1)	CD163	MME	ADGRG1 (GPR56)	CLC	FCER1A
TLR7	OAS2	IL-33	CLEC4C (BDCA2)	CHIT1	MPO	KLRD1	SIGLEC8	KIT
TLR8	MX1	TSLP	THBD (BDCA3)	MMP12	CXCR2	NCAM1	LGALS12	TPSAB1
DDX58 (RIG-I)	STAT1	POSTN	NRP1 (BDCA4)					
IRF3	IFNAR1	FOXJ1						
IRF7	TNFAIP3	KRT5						
MYD88	IRAK3	SCGB1A1						
NFKB1	SOCS1	CLCA1						

TLR Toll-like receptor; DDX58 DExD/H-Box Helicase 58; IRF Interferon Regulatory Factor; MYD88 Myeloid Differentiation Primary Response 88; NFKB Nuclear Factor Kappa B Subunit 1; IFIT IFN Interferon Induced Protein With Tetratricopeptide Repeats; OAS2'-5'-Oligoadenylate Synthetase; MX MX Dynamin Like GTPase; STAT Signal Transducer And Activator Of Transcription; IFNAR Interferon Alpha And Beta Receptor Subunit; TNFAIP3 TNF Alpha Induced Protein 3; IRAK Interleukin 1 Receptor Associated Kinase; SOCS Suppressor Of Cytokine Signalling; TSLP Thymic Stromal Lymphopoietin; POSTN Periostin; FOXJ1 Forkhead Box J1; KRT Keratin; SCGB1A1 Secretoglobin Family 1A Member 1; CLCA Chloride Channel Accessory; CD1C CD1c Molecule; CLEC4C C-Type Lectin Domain Family 4 Member C; THBD Thrombomodulin; NRP Neuropilin 1; CD163 CD163 Molecule; CHIT Chitinase 1; MMP Matrix Metallopeptidase; MME Membrane Metalloendopeptidase; MPO Myeloperoxidase; CXCR C-X-C Motif Chemokine Receptor; ADGRG1 Adhesion G Protein-Coupled Receptor G1; KLRD1 Killer Cell Lectin Like Receptor D1; NCAM1 Neural Cell Adhesion Molecule 1; CLC Charcot-Leyden Crystal Galectin; SIGLEC Sialic Acid Binding Ig Like Lectin; LGALS Galectin; FCER1A Fc Fragment Of IgE Receptor Ia; KIT KIT Proto-Oncogene Receptor Tyrosine Kinase; TPSAB Tryptase Alpha/Beta 1.

2.7.4 Real-time RT-PCR using microfluidic platform

A microfluidics platform was used to perform RT-PCR (Fluidigm). The use of integrated fluidic chips (IFC, 96.96 Dynamic Array) that use nanolitre volumes to automate PCR permits high throughput sample processing by performing 9,216 parallel reactions (figure 2.7). The manufacturer's kit and instructions and are briefly outlined here: (a) Control line fluid (150 µL) was first injected in to accumulators on the chip and pressurized using an IFC controller (IFC Controller HX, Fluidigm); (b) Sample pre-mix was made using 3 µL SSOFast Evagreen with Low ROX (Bio-Rad Laboratories, California, USA) and 0.3 µL of DNA Binding Dye (Fluidigm) and added to 2.7 µL Preamplified and Exonuclease I-treated sample; (c) The assay mix was prepared using 3 µL Assay Loading Reagent (Fluidigm), 2.7 µL DNA Suspension Buffer (TEKnova, California, USA) and 0.25 µL 100 µM combined forward and reverse primers (the final concertation of each primer being 5 µM in the inlet and 500 nM in the final reaction); (d) once the sample and reagents were loaded, the components were pressurized using the IFC controller and combined; (e) the IFC was then loaded on to an internal thermal cycler instrument (BioMark HD, Fluidigm) and a pre-specified thermal cycling protocol utilised (GE 96x96 Fast PCR+Melt v2, table 2.8), with fluorescence detected using proprietary software.

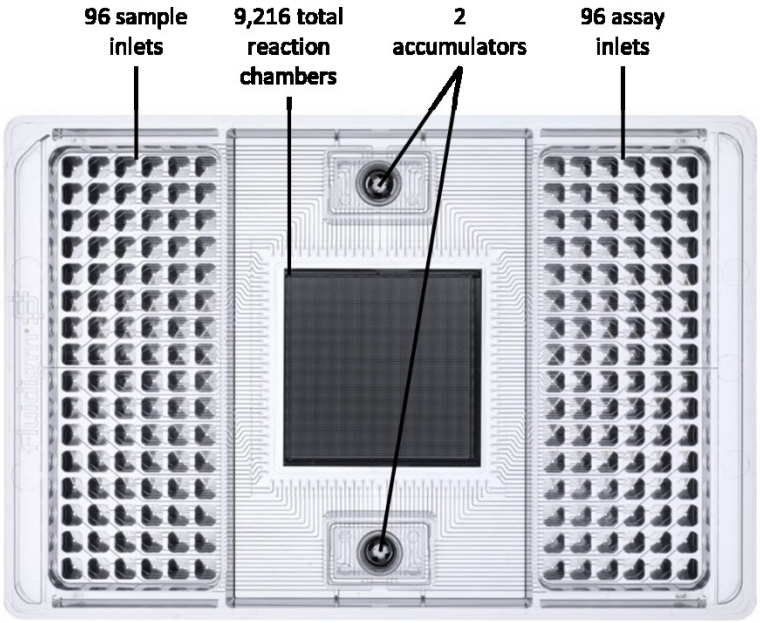


Figure 2.8 Fluidigm Dynamic Array Integrated Fluid Chip (96.96) for gene expression The left hand side of the chip consists of 96 sample inlets, the right side 96 assay inlets (or fewer if gene assays run in duplicate) and 2 accumulators for injection of control line fluid. The central portion of the chip contain reaction chambers connected by a network of microfluidics to permit 9,216 parallel reactions.

Table 2.8 Thermal cycling protocol used for Real-Time PCR

Segment	Type	Temperature (°C)	Duration (seconds)
1	Thermal Mix	70	2400
		60	30
2	Hot Start	95	60
3	PCR (30 Cycles)	96	5
		60	20
4	Melting Curve	60	3
		60-95	

2.8 Immunohistology of nasal curettage specimens

To help identify cellular sources of immune mediators and characterization of TLR expression, an experimental approach using immunohistology on nasal curettage samples was attempted. This was done in collaboration with colleagues at Imperial College London; Miss Marta Jamroziak (Professor Rob Goldin's group) performed sectioning of tissue samples, and Dr Jie Zhu performed processing, embedding and staining of tissue for hematoxylin and eosin (H&E) and immunohistochemistry, and generated images of nasal curettage specimens. Specimens fixed in 240 µL 4% paraformaldehyde were transferred to the Airway Disease laboratory for processing, embedding and staining using standard techniques based on trust guidelines. Immunohistochemistry was then performed to characterize IFN-γ expression and TLR receptor expression. IFN-γ positivity was detected using the EnVision Peroxidase staining method; (a) paraffin sections of nasal curettage were dewaxed, heated up in 0.01M Citrate Acid Buffer(PH 6.0) for antigen retrieval, incubated with peroxidase-blocking solution (Dako, Cambridge, UK) and then incubated overnight at 4°C with primary rabbit anti-IFN-γ monoclonal antibody (1:50 dilution, Abcam, Cambridge, UK, Ab133566); (b) the sections were incubated with EnVision System-HRP labelled polymer goat anti-rabbit secondary antibody solution for 30 minutes (Dako, K4003); (c) after washing, sections were incubated with chromogen diaminobenzidine (DAB) liquid and peroxide buffer (Dako) and stained antigen sites were detected as a brown product; (d) non-specific rabbit IgG, polyclonal-isotype control (Abcam, Ab171870) at the same concentration as those used above was used as a control primary antibody. The immunostaining intensity for IFN-γ on nasal epithelium was semi-quantitatively scored ranging from 0-3 (0-negative, 1-weak, 2-moderate and 3-strong staining) in a blinded manner. Several fields at 400x magnification were scored to cover all epithelial areas from each biopsy. An average of scores was taken to represent entire epithelial staining intensity of two to three biopsies from each subject. Anti-TLR7 monoclonal antibody (1:100 dilution, Abcam, Ab124928) and anti-TLR8 monoclonal antibody (1:150 dilution Abcam, Ab180610) were processed using the same technique described above.

2.9 PBMC isolation and stimulation

To study *in vitro* immune responses to TLR ligands, PBMCs were collected from volunteers and stimulated with TLR agonists. A preliminary experiment using poly(I:C) and poly-ICLC involved taking blood from a single donor, whilst subsequent experiments using R848 as an additional TLR agonist, utilised PBMCS donated by six donors. PBMCs were isolated using a standard protocol (261) but the steps are outlined here: (a) blood was collected using three EDTA bottles (BD) and processed at room temperature within four hours; (b) whole blood was diluted with Roswell Park Memorial Institute (RPMI)-1640 media (supplemented with L-glutamine and sodium bicarbonate, Sigma-Aldrich) in equal volumes (1:1 dilution) and gently mixed; (c) 25 mL of diluted cell suspension was layered over 17 mL of Histopaque (Sigma-Aldrich, 10771) in a 50 mL falcon tube; (d) this was centrifuged at 2,200 *x* rpm for 25 minutes at 21^0C in a swinging bucket rotor without brake; (e) the upper layer was aspirated, leaving the monolayer (containing lymphocytes, monocytes and thrombocytes) in the interphase; (f) the mononuclear cell layer was aspirated in to a new 50 mL tube and 50 mL RPMI-1640 added; (g) this was centrifuged at 1,200 *x* rpm for 10 minutes and the supernatant discarded; (h) after resuspension in 50 mL RPMI-1640, the cells were centrifuged at 1,200 *x* rpm for 10 minutes; (i) the supernatant was discarded completely and re-suspended in 20 mL RPMI-1640; (j) cells were counted using a haemocytometer; (k) For the preliminary experiment on sample from one volunteer, PBMCs were used immediately by transferring to two 48 well plates at a concentration of $2x10^6$/well (250 µl/well). PBMCs were stimulated with poly(I:C) or poly-ICLC at concentrations of 1 µg/mL, 10 µg/mL and 100 µg/mL, as well as with Phorbol 12-myristate 13-acetate (PMA, 25 ng/mL) as a positive control and blank media. All samples were run in duplicate, cultured at 37°C with 5% CO_2. The culture supernatant was collected at 24h, 48h and 72h and stored at -80°C for analysis later using ELISA. For the later experiments on samples from six volunteers, PBMCs were frozen at -80°C. When required, they were suspended in RPMI as well as 10% heat-inactivated foetal calf serum (FCS, Sigma-Aldrich), Penicillin (100 U/mL) and streptomycin (100 µg/mL). PBMCs were cultured at $1x10^5$ cells/well in 96-well plates

in 100 µL total well volume and were left in media alone or stimulated with R848 (1 µg/mL), poly(I:C) (10 µg/mL) or poly-ICLC (10 µg/mL). Stimulations were performed in triplicate at 37^{o}C with 5% CO_2. Culture supernatants were collected at 3, 8 and 24 hours and stored at -80^{o}C prior to later analysis using ELISA.

2.10 Evolution of nasosorption devices and optimal duration of nasosorption sampling

The optimal dwell time for SAM strips or nasosorption devices in the nose for the absorption of mucosal lining fluid is unknown. Most experiments that have previously utilised manually pre-cut strips of SAM, have been placed in the nose for two minutes in unchallenged adults (138) and children (262), as well as after allergen (74) and HRV challenge (164). Nasal sponges have been used for a similar duration after allergen challenge (171, 172). However, a more recent study has obtained good recovery of cytokines using nasosorption for 30 seconds in infants hospitalised with RSV (165).

In collaboration with Hunt Developments Ltd, an opportunity arose to improve the technique of absorbing nasal mucosal lining fluid by employing a nasosorption device with two SAM strips (bonded together ultrasonically) and mounted on to a handle (figure 2.8). This had advantages over using older iterations of SAM strips as there was no need to manually pre-cut strips and removed the need to use forceps to insert SAM strips in to the nasal cavity, which often led to buckling of the strips as they encountered the inferior turbinate. An intermediate device (Nasosorption FX-R) was initially developed with a spine running through the middle of the SAM strips to introduce a greater degree of rigidity and allow the strips to be inserted alongside the inferior turbinate without any buckling. However, this was found to cause increased nasolacrimal reaction in volunteers therefore, a final device (Nasosorption FX-i) was designed with a shorter handle, which was softer and had an appropriate balance between ease of insertion and tolerable symptoms.

To study the optimal duration for nasosorption in its ability to consistently absorb mucosal lining fluid, an experiment was designed with varying nasosorption dwell times. Six healthy unchallenged volunteers (three with skin prick positivity to grass pollen and three non-atopic) had a nasosorption FX-R device placed inside the right nostril for varying durations of 30 seconds, 60 seconds and 120 seconds. A Latin square design was employed to determine the sequence of sampling times for each volunteer to reduce sources of variation. The nasosorption device was weighed before and after insertion in the nose to calculate the weight of the mucosal lining fluid, and the different dwell times were compared.

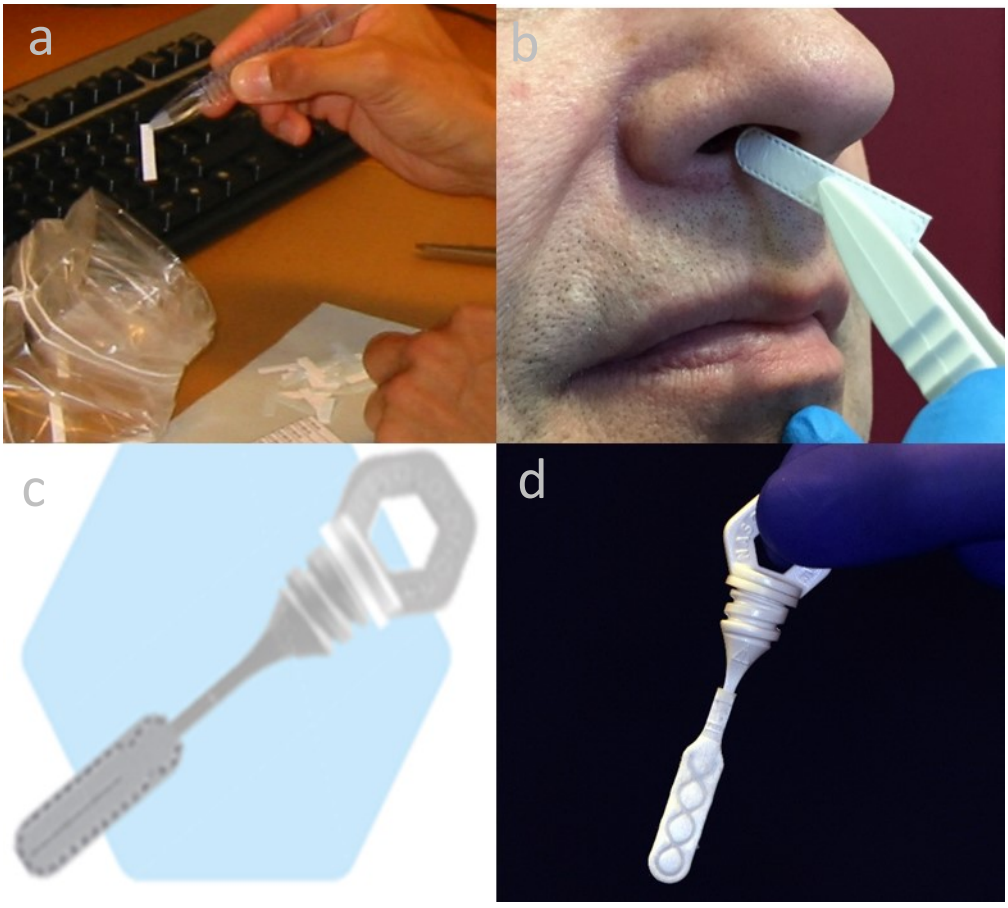


Figure 2.9 Evolution of nasosorption devices (a) The earliest iteration of nasosorption (prior to the current study, ref (263)) was performed using pre-cut pieces of SAM strips in a non-sterile manner; (b) endotoxin-free nasosorption strips were then manufactured by Hunt Developments Ltd and inserted using plastic forceps; (c) the Nasosorption FX-R device consisted of SAM strips mounted on a handle with a spine running through the entire length of the SAM strips for increased rigidity, removing the need for forceps but caused increased nasolacrimal reaction in volunteers, hence (d) the Nasosorption FX-i device was produced with a shorter spine, which permitted easy insertion into the nasal cavity and improved tolerability for volunteers.

2.11 Statistical analysis

A commercial software package (Graphpad Prism Version 7.00) was used to perform statistical analyses. Shapiro-Wilk testing confirmed a non-parametric distribution of data for analysis of soluble mediators and therefore Mann-Whitney rank test (with multiple correction were appropriate) was predominantly used. Area Under Curve (AUC) values were $\log_{10}$ transformed and compared using paired t-tests. For PNIF analysis, the difference from baseline was calculated for individual subjects with mean values compared to baseline by repeated measures Analysis of Variance (ANOVA) and Bonferroni correction for multiple comparison. For TNSS analysis, mean values were compared to baseline using Friedman test and Dunn's post-test. A very small number of missing clinical data points were imputed as the mean of all the values from that challenge. A P-value of < 0.05 was taken to indicate statistical significance. This was a pilot study and therefore formal power calculations were not performed. The specific statistical tests used are described in the relevant results chapters.

2.12 Software

Clinical and preliminary mediator data was processed using Microsoft Excel (Microsoft Corporation, Seattle, USA) including basic calculations, e.g. geometric mean, as well as calculating ΔΔCT values for gene expression analysis. Discovery Workbench Software (MSD) and SoftMax Pro 5 (Molecular Devices, California, USA) was utilised to determine all soluble mediator values. Graphpad Prism was used to generate graphs and dot plots for mediators and mRNA. Assessment of RNA purity, degradation and quantitation from nasal curettage samples was performed using Agilent 2100 Expert software. An algorithm analysis of the electropherogram was subsequently performed to calculate the RNA integrity number (RIN) and RNA concentration (expressed in ng/μL) for each sample. Fluidigm Real-Time PCR Software (v.4.1.3) and BioMark HD Software (v.3.0.2) was used to quantify changes in gene expression by providing amplification curves, heat maps and Ct values (set at threshold of 0.65) for each well. Heat maps were generated using R statistical analysis software (v.3.2.2).

3 TLR3 agonist nasal challenge: results of dose finding studies

3.1 Synopsis

The TLR3 agonists and dsRNA analogues poly(I:C) and poly-ICLC were administered to a panel of healthy volunteers and those with allergic rhinitis to assess their tolerability and ability to induce mucosal IFNs and cytokines. Both agents were safely tolerated but did not cause significant dose-dependent nasal innate immune responses, despite poly(I:C) inducing the release of IFN-γ and IL-6 in PBMCs.

3.2 Introduction

Analogues of dsRNA such as poly(I:C) and poly-ICLC have been utilised as a tool to study its therapeutic effect against viruses in animals (264, 265) as well as exploiting its capacity as an adjuvant for the delivery of vaccines against infections and cancer (266, 267). TLR3 agonists also have the potential to be used as a tool to investigate mucosal innate immune responses to understand differences in host immunity that is present at the extremes of age (268) as well as conditions that cause dysregulated epithelial cell function such as asthma (269). Early studies involving systemic administration of stand-alone poly-ICLC demonstrated its ability to induce serum IFN but also led to fever, hypotension, leukopenia, polyarthralgia and multi-organ toxicity (270–272). Poly-ICLC (1.6mg) has been well-tolerated when given subcutaneously with induction of systemic interferon production (105). More recently, poly-ICLC has been administered as a cancer vaccine adjuvant leading to enhanced immune activity (273, 274). However, there has been limited research performed to study its effects on *in vivo* respiratory tract immune responses and in particular to successfully detect mucosal interferons (106). Therefore, the purpose of the current study was to ascertain whether either poly(I:C) or poly-ICLC given intranasally was able to safely induce mucosal interferon and cytokine production.

3.3 Poly-ICLC dose escalation studies

3.3.1 Baseline clinical characteristics

Poly-ICLC was initially tested in eight volunteers (atopic n=4, non-atopic n=4) at escalating doses: 10 μg/100 μL, 100 μg/100 μL and 500 μg/300 μL per nostril. One subject developed an upper respiratory infection in between saline and poly-ICLC 10μg challenge and therefore the latter challenge was delayed by 8 weeks. Another volunteer developed an upper respiratory tract infection in between their poly-ICLC 100μg and poly-ICLC 500μg challenges and therefore the latter was delayed by six weeks. No adverse events were noted at any of the doses. The baseline characteristics of the eight volunteers undergoing poly-ICLC challenge are outlined in table 3.1.

Subject characteristic	Atopic (n=4)	Non-Atopic (n=4)
Age	30 (24 - 46)	25 (23 - 27)
Gender (F:M)	0:4	4:0
Skin Prick to Timothy Grass (mm)	5.25 (4-7)	1.25 (1 - 2)
Eosinophil Count ($x10^9$/L)	0.23 (0.2-0.3)	0.13 (0.1 - 0.3)
Total Serum IgE (IU/ml)	102.63 (50.3 – 175)	30.71 (8.54 – 73.7)

Table 3.1 Baseline characteristics of volunteers undergoing poly-ICLC dose escalation challenge Figures expressed as arithmetic mean (range).

3.3.2 Comprehensive nasal immune mediator profile in selected individuals

Since there were a very large number of samples generated from the poly-ICLC dose escalation studies, a decision was made at this preliminary stage to restrict the number of samples analysed for the MSD multiplex immunoassay. To assess the effects of varying doses of poly-ICLC (10 μg, 100 μg, 500 μg) on nasal immune responses, all samples from a single volunteer were analysed using a proinflammatory and chemokine panel of 20 mediators (appendix III). On visual inspection of the graphs, no mediators were noted to be reliably induced by poly-ICLC in a dose-dependent manner.

Subsequently, nasal samples from the most symptomatic volunteer (with the highest TNSS) after poly-ICLC 500 µg challenge was analysed using a comprehensive 30-plex immunoassay (proinflammatory, chemokine and cytokine panels). Visual inspection of the graphs again failed to show reliable differences in mediators between saline and poly-ICLC 500 µg challenge (appendix IV).

3.3.3 Nasal CXCL-10 and IFN-γ response in all volunteers at 500 µg dose

As mentioned previously, unpublished data from live RSV challenge studies suggested that CXCL10 and IFN-γ were likely to be upregulated by TLR agonists, since they activated similar receptors to ssRNA and dsRNA viruses. Therefore, nasal mucosal lining fluid samples from all volunteers undergoing poly-ICLC 500 µg / 300 µL challenge were analysed for CXCL10 and IFN-γ levels using the MSD platform (figure 3.1 and 3.2). When comparing AUC (0-6h) values after poly-ICLC challenge to saline control, CXCL10 was not found to be statistically significant ($P = 0.382$), whilst IFN-γ was significantly increased ($P = 0.0156$). The individual level data (figure 3.1) suggested that changes in IFN-γ occurred five hours after challenge, with minimal detection prior to this. One volunteer experienced an exaggerated CXCL10 after saline challenge. Since they were asymptomatic at the time of challenge, this may have been due to a sub-clinical infection.

Overall however, there were minimal changes in immune mediators noted at multiple doses of poly-ICLC up to six hours after challenge. Therefore, the next phase of the study involved escalating the dose of poly-ICLC further, increasing the duration of sampling to 24 hours and progressing to poly(I:C) challenge.

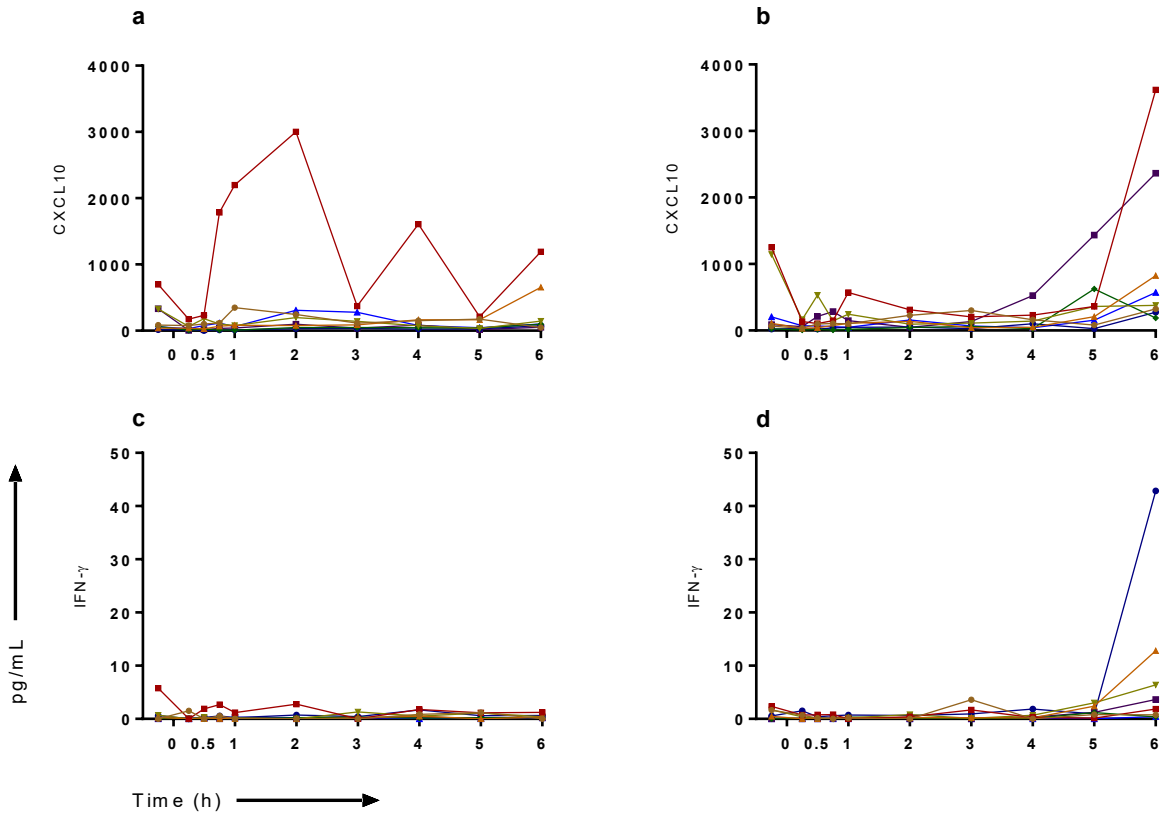


Figure 3.1 Individual nasal CXCL10 and IFN-γ responses after saline and poly-ICLC 500 µg Nasal mucosal lining fluid samples from eight volunteers (atopic n=4, non-atopic n=4) challenged with saline (a, c) and poly-ICLC (b, d).

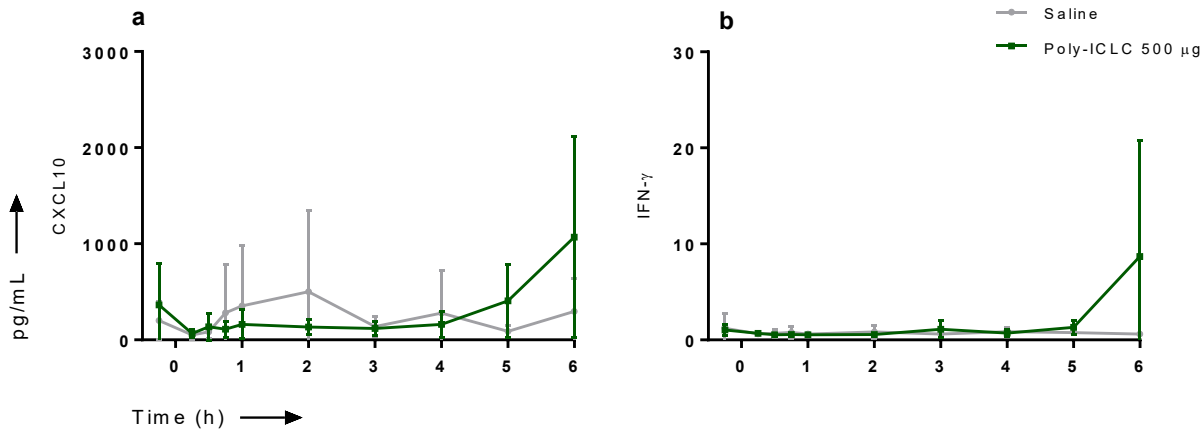


Figure 3.2 Group nasal (a) CXCL10 and (b) IFN-γ responses after saline and poly-ICLC 500 µg Nasal mucosal lining fluid samples from eight volunteers (atopic n=4, non-atopic n=4). Values expressed as mean with 95% CI.

3.4 Highest dose poly-ICLC and poly(I:C) challenge

After discussion with the ethics committee, a higher dose of poly-ICLC (1000 μg / 500 μL per nostril) was subsequently used for a final set of nasal challenges and performed alongside nasal challenge with poly(I:C). With regards to the latter, during the course of this research, a pilot study based in Sweden reported the use of intranasal delivered poly(I:C) at a dose of 800 μg in one nostril (400 μg over one hour given daily for two days) in volunteers with allergic rhinitis (233). As this dose was safely tolerated with no adverse reactions reported, it was decided for the purposes of the current research and after discussion with the ethics committee, that a dose of 500 μg / 500 μL per nostril of poly(I:C) could be used without the need for the originally planned dose escalation phase.

Since the bidose delivery device could only deliver 100 μL per actuation per nostril, in order to deliver 500 μL volume of challenge agent, five actuations were required to administer the challenge agent. A decision was made to deliver these five actuations over one hour in 15-minute intervals based on the dosing schedule of a previous study using intranasal challenge with recombinant IL-8, which successfully elicited neutrophilic inflammation in atopic and non-atopic volunteers (275).

Additionally, in response to the fact that poly-ICLC had not reliably induced consistent mucosal immune responses at lower doses, the study design was altered to perform a more limited study in a fewer number of volunteers than originally envisaged (n=4 rather than n=8 for each challenge agent). Therefore, four volunteers (two atopic and two non-atopic) were challenged with poly-ICLC (1000 μg / 500 μL) and four volunteers (two atopic and two non-atopic) were challenge with poly(I:C) (500 μg / 500 μL) with mucosal innate immune responses compared to saline challenge.

3.4.1 Baseline clinical characteristics

The subject characteristics of volunteers undergoing poly-ICLC (1000 μg/500 μL) and poly(I:C) (500 μg/500 μL) challenge are listed in table 3.2.

	Poly-ICLC (1000 μg, n=4)		**Poly(I:C) (500 μg, n=4)**	
Subject Characteristic	Atopic (n=2)	Non-Atopic (n=2)	Atopic (n=2)	Non-Atopic (n=2)
Age	32 - 51	24 - 30	23 - 23	25 - 33
Gender (F:M)	2:0	0:2	1:1	2:0
Skin Prick to Timothy Grass (mm)	6 - 13	0 - 1	6 - 9	0 - 1
Eosinophil Count ($x10^9$/L)	0.2	0.1	0.2	0.1 – 0.2
Total Serum IgE (IU/ml)	38.2 – 395	<2 – 90.8	<2 - 485	4.87 – 11.2

Table 3.2 Baseline characteristics of volunteers undergoing poly-ICLC (1000 μg) and poly(I:C) (500 μg) challenge Figures expressed as a range.

3.4.2 Clinical response

Subjective (TNSS) and objective (PNIF) correlates of nasal obstruction were measured serially after nasal challenge (figures 3.2 and 3.3). There was no difference noted between saline and poly-ICLC challenge and between saline and poly(I:C) challenge. There was also no difference seen in observations taken before and after challenge (data not shown).

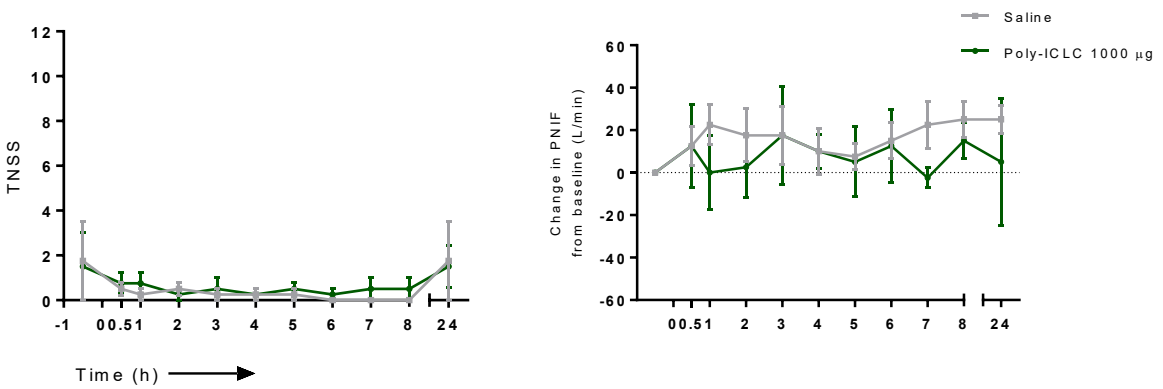


Figure 3.2 Clinical correlates of nasal obstruction after saline and poly-ICLC 1000 µg Data from four volunteers (atopic n=2, non-atopic n=2). Total nasal symptom score (TNSS) plotted as mean±SEM and comparison versus baseline by Friedman and Dunn's post hoc test. Change in peak nasal inspiratory flow (PNIF) from baseline plotted as mean±SEM and comparison versus baseline by repeated measures one-way ANOVA with Greenhouse-Geisser correction and Dunnett's multiple comparison test.

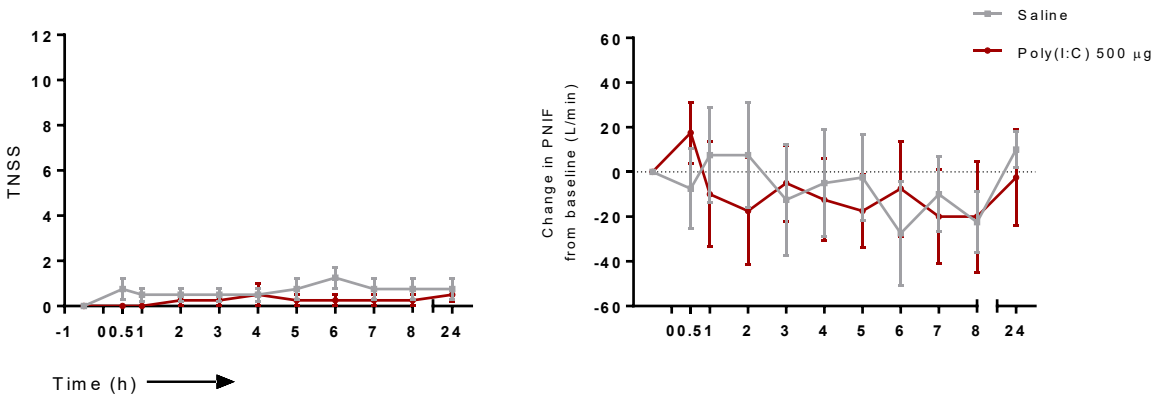


Figure 3.3 Clinical correlates of nasal obstruction after saline and poly(I:C) 500 µg Data from four volunteers (atopic n=2, non-atopic n=2). Total nasal symptom score (TNSS) plotted as mean±SEM and comparison versus baseline by Friedman and Dunn's post hoc test. Change in peak nasal inspiratory flow (PNIF) from baseline plotted as mean±SEM and comparison versus baseline by repeated measures one-way ANOVA with Greenhouse-Geisser correction and Dunnett's multiple comparison test.

3.4.3 Nasal immune mediator response

Nasal mucosal lining fluid samples from both poly-ICLC (1000 μg) and poly(I:C) (500 μg) challenge were analysed using a multiplex MSD panel to measure interferons and proinflammatory cytokines: IFN-α, IFN-β, IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13.

There were no differences noted in mean immune responses (selected mediators shown in figures 3.4 and 3.5) or individual immune responses (data not shown) between saline and poly-ICLC or between saline and poly(I:C) challenge as measured when comparing AUCs (tables 3.3 and 3.4).

3.4.4 Nasal curettage and serum collection

Nasal curettage tissue, as well as blood and serum samples were collected during challenge experiments. However, as there was a lack of consistent soluble mediator response seen, these were not processed for further analysis.

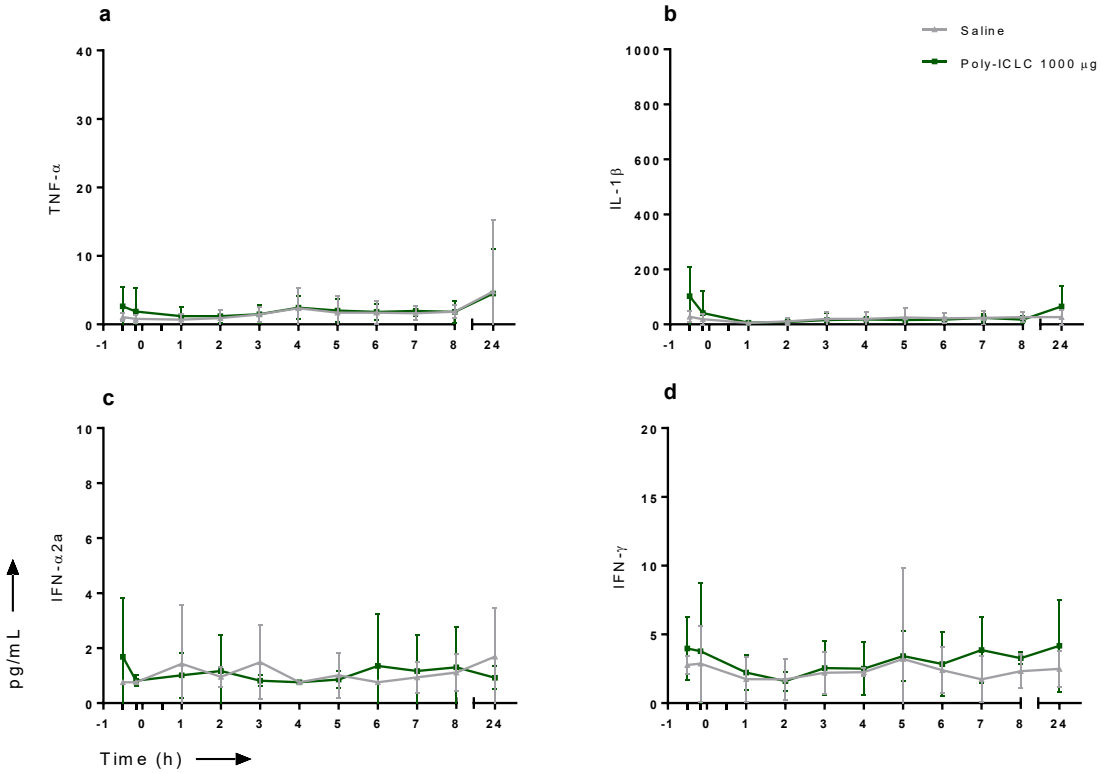


Figure 3.3 Nasal immune responses after saline and poly-ICLC 1000 µg Nasal mucosal lining fluid levels of a) TNF-α, (b) IL-1β, (c) IFN-α and (d) IFN-γ from four volunteers (atopic n=2, non-atopic n=2). Values expressed as mean with 95% CI.

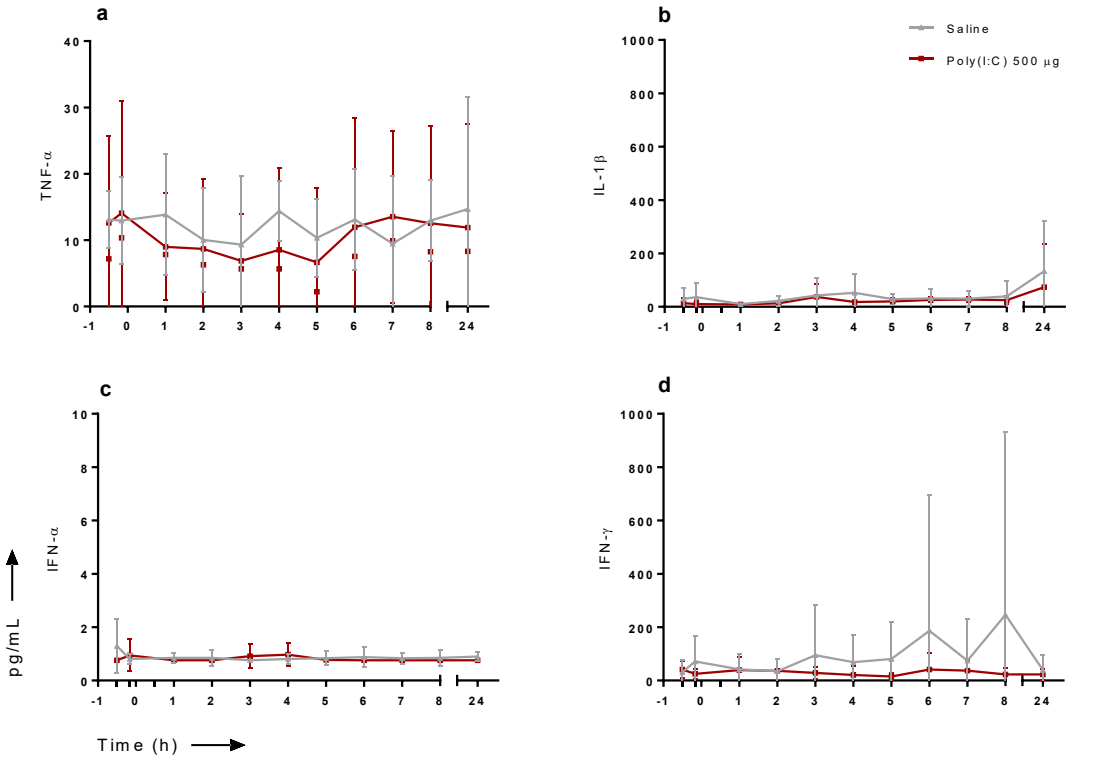


Figure 3.4 Nasal immune responses after saline and poly(I:C)-500 µg Nasal mucosal lining fluid levels of (a) TNF-α, (b) IL-1β, (c) IFN-α and (d) IFN-γ from four volunteers (atopic n=2, non-atopic n=2). Values expressed as mean with 95% CI

Mediator	Saline (n=4)	Poly-ICLC (n=4)	*P*-value
IFN-α	0.65 (0.13 – 1.17)	0.61 (-0.34 – 1.57)	0.9223
IFN-β^	0.16 (-0.33 – 0.65)	0.26 (-0.83 – 1.34)	0.7839
IFN-γ	2.36 (0.57 – 4.15)	3.29 (1.84 – 4.74)	0.2455
TNF-α	7.09 (4.22 – 9.97)	7.89 (5.94 – 9.85)	0.4908
IL-1β	15.55 (12.48 – 18.62)	15.66 (14.91 – 16.41)	0.9174
IL-2	4.58 (1.65 – 7.52)	5.24 (3.50 – 6.99)	0.5604
IL-4	3.73 (-1.26 – 8.72)	3.78 (-0.23 – 7.79)	0.9797
IL-6	9.70 (6.14 – 13.25)	9.45 (7.94 – 10.95)	0.8441
CXCL8/IL-8	25.28 (22.82 – 27.73)	26.78 (25.07 – 28.49)	0.1605
IL-10	8.2 (3.71 – 12.69)	10.31 (5.96 – 14.67)	0.3236
IL-12p70	1.06 (0.41 – 1.71)	1.56 (0.37 – 2.75)	0.2849
IL-13	4.49 (3.62 – 5.37)	5.48 (3.55 – 7.42)	0.1872

Table 3.3 Comparison of nasal immune responses after saline and poly-ICLC 1000 µg AUC of $\log_{10}$ transformed values between 0-8 hours was initially calculated for each mediator in individual subjects (n=4) after nasal challenge with saline and poly-ICLC. Subsequently, AUC values were compared between groups using a paired t-test. The baseline parameter for AUC was set at the lower limit of detection (LLOD) for each mediator. Samples collected prior to challenge were excluded, ^n=3. AUC values expressed as mean (95% CI).

Mediator	Saline (n=4)	Poly(I:C) (n=4)	*P*-value
IFN-α	0.25 (-0.23 – 0.72)	0.17 (-0.01 – 0.36)	0.6606
IFN-β	1.08 (-0.03 – 2.19)	1.12 (-0.11 – 2.34)	0.9629
IFN-γ	11.94 (6.37 – 17.51)	9.42 (4.76 – 14.08)	0.3122
TNF-α	12.98 (9.58 – 16.38)	11.94 (7.69 – 16.26)	0.5669
IL-1β	17.5 (15.22 – 19.78)	16.75 (15.52 – 17.98)	0.3927
IL-2	11.78 (10.89 – 12.67)	9.94 (4.82 – 15.07)	0.3051
IL-4	12.23 (10.18 – 14.27)	10.53 (2.85 – 18.22)	0.5235
IL-6	13.93 (10.9 – 16.95)	11.72 (9.82 – 13.62)	0.0966
CXCL8/IL-8	28.32 (25.87 – 30.77)	27.77 (25.95 – 29.6)	0.5908
IL-10	13.66 (10.33 – 16.98)	12.00 (9.37 – 14.64)	0.2621
IL-12p70	11.23 (7.94 – 14.52)	9.51 (0.69 – 18.33)	0.5817
IL-13	6.47 (5.72 – 7.22)	5.81 (3.59 – 8.04)	0.4084

Table 3.4 Comparison of nasal immune responses after saline and poly(I:C) 500 μg AUC of $\log_{10}$ transformed values between 0-8 hours was initially calculated for each mediator in individual subjects (n=4) after nasal challenge with saline and poly(I:C). Subsequently, AUC values were compared between groups using a paired t-test. The baseline parameter for AUC was set at the lower limit of detection (LLOD) for each mediator. Samples collected prior to challenge were excluded. AUC values expressed as mean (95% CI).

3.5 PBMC stimulation with poly(I:C) and poly-ICLC

Given the minimum nasal mucosal immune responses seen after poly-ICLC challenge and lack of response seen after poly(I:C) challenge, *in vitro* immune responses to these TLR ligands were assessed. PBMCs were isolated from a single atopic donor and cultured at $1x10^5$ cells/well in 96-well plates in 100 µL total well volume. They were either left in media alone or stimulated with a positive control (PMA) or with poly(I:C) or poly-ICLC at varying doses: 1 µg/mL, 10 µg/mL and 100 µg/mL. Culture supernatants were collected at 24 h, 48 h or 72 h and IFN-γ and IL-6 response analysed using ELISA (figure 3.5). The PBMC response to poly(I:C) was time and dose dependent with peak IFN-γ release occurring at 72 h, with peak IL-6 release occurring at 24 h. Poly-ICLC was able to induce IL-6 at higher doses but at significant lower levels when compared to poly(I:C) and caused minimal release of IFN-γ even at higher doses.

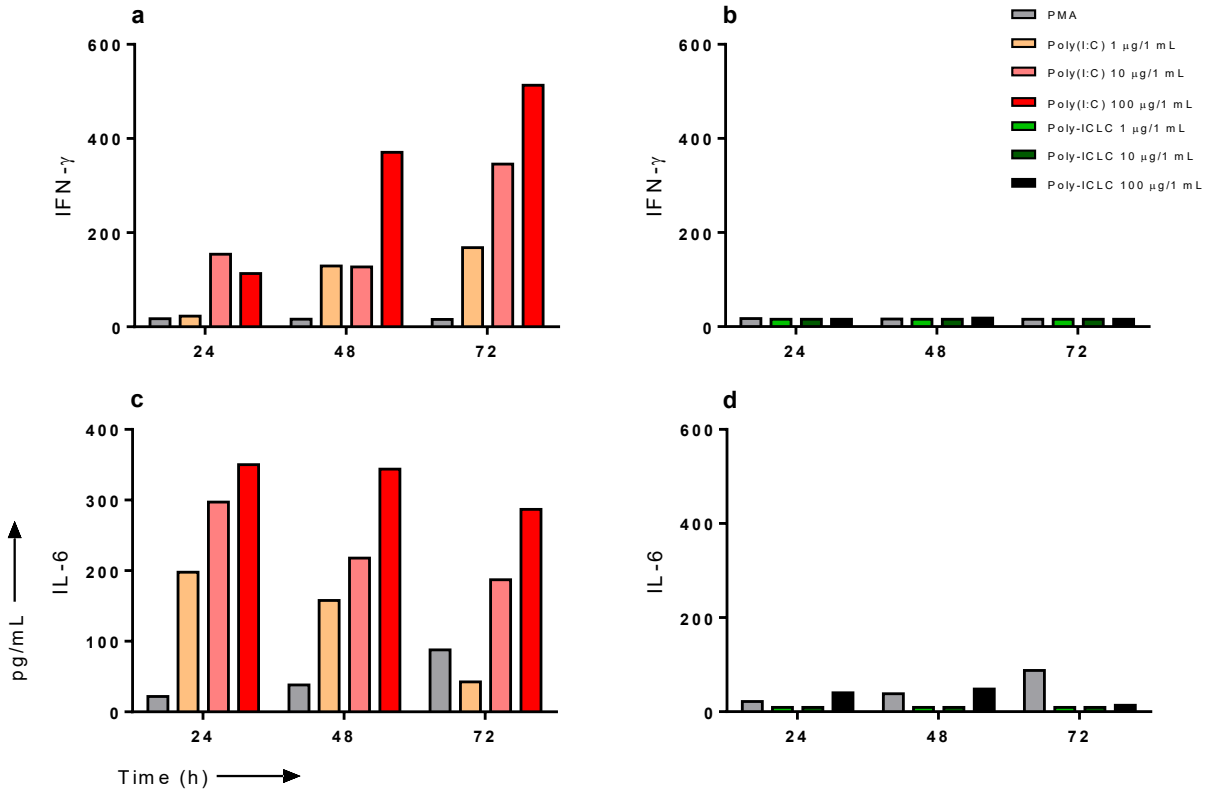


Figure 3.5 PBMC responses to poly(I:C) and poly-ICLC stimulation PBMCs from a single atopic volunteer were isolated and incubated at varying doses (1 µg/mL, 10 µg/mL, 100 µg/mL) of poly(I:C) (a, c) or poly-ICLC (b, d) as well as a positive control (PMA). Stimulations performed in triplicate at 37°C with 5% CO_2. Cell culture supernatant collected at 24 h, 48 h and 72 h and analysed using ELISA for IFN-γ and IL-6.

3.6 Optimal duration of nasosorption sampling

An additional experiment was performed to investigate the optimal duration of nasal sampling for a new nasosorption device (nasosorption FX-R), which was modified from previous versions by mounting the SAM fibre strip on a handle with a spine. Six unchallenged volunteers (three atopic to grass pollen and three non-atopic) had the nasosorption device placed inside the right nostril for varying durations of 30 s, 60 s and 120 s with a Latin square design employed to determine the sequence of sampling for each volunteer. The mucosal lining fluid weight was measured by weighing the nasosorption device before and after sampling and the mean weight from all volunteers for each dwell time compared using Friedman test with Dunn's multiple comparison. There was no significant difference in mean weight between 30 s and 60 s dwell time (P >0.9999) and between 60 s and 120 s dwell time (P 0.2498), whilst a significant difference was noted between 30 s and 120 s dwell time (P 0.0281). However, on inspection of individual mucosal lining fluid weights at different dwell times (figure 3.6), there was greater individual variability with 30 s and 120 s dwell times. The nasosorption device had relatively consistent absorption amongst all volunteers with 60 s dwell time, whilst a 120s dwell time led to increased absorption in certain individuals (volunteers 2, 4 and 6). One reason for this variability may have been due to the design of the nasosorption FX-R device, which had a stiff spine running through the middle of the SAM strips. This may have had a greater propensity to trigger a nasolacrimal reaction in volunteers (especially those with atopy who are likely to have a more sensitive mucosa) and lead to an increase in the volume of fluid absorbed. Using this feedback, Hunt Development Ltd produced another (final) iteration of the nasosorption device (nasosorption FX-i), which had a much shorter spine resulting in a softer and better tolerated sampling device, which was used for the remainder of the study. Based on the results of this experiment, for subsequent nasal challenge studies, a nasosorption dwell time of 60 s was used.

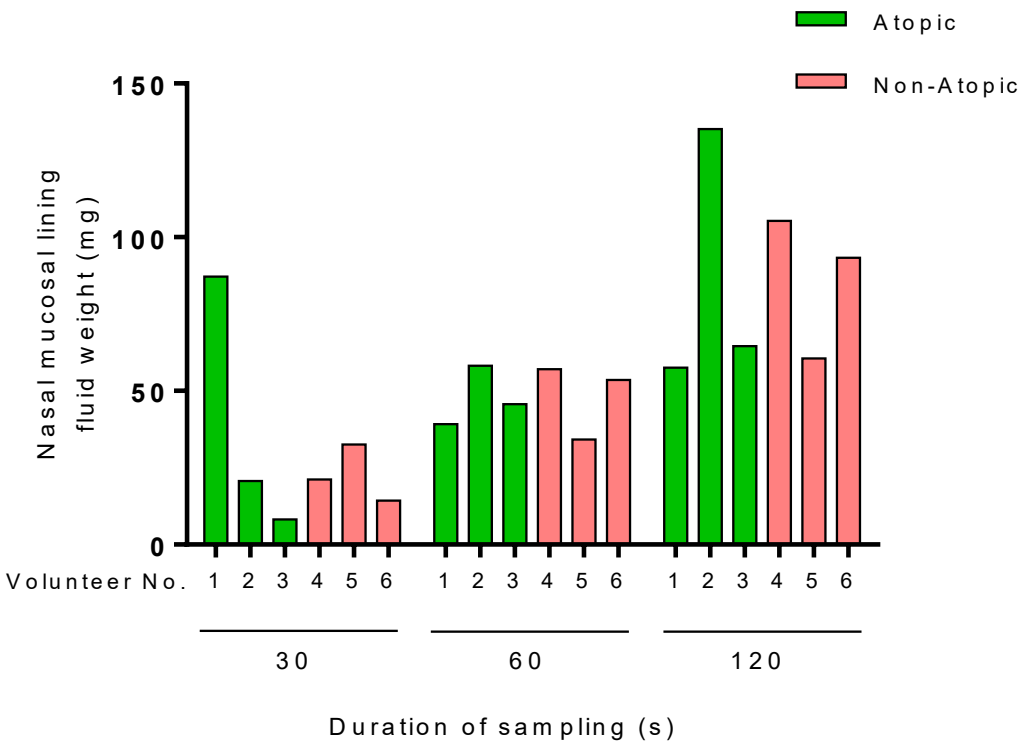


Figure 3.6 Optimal duration of nasosorption sampling A new nasosorption device (Nasosorption FX-R) was placed inside the right nostril of six unchallenged volunteers for varying durations (30 s, 60 s and 120 s) with measurement of nasal mucosal lining fluid (MLF) weight.

3.7 Discussion

Nasal delivery of poly-ICLC and poly(I:C) at all doses was safely tolerated by all volunteers with no adverse events noted. However, poly-ICLC did not induce dose-dependent changes in nasal immune mediator production. Although a significant increase in nasal IFN-γ was seen after poly-ICLC 500 μg challenge, particularly after five hours, this was not reproducible at a higher dose of 1000 μg. Similarly, poly(I:C) at a dose of 500 μg failed to induce nasal mucosal immune responses. This was in parallel with a lack of local clinical response seen in terms of TNSS or changes in PNIF. The PBMC stimulation studies demonstrated as expected that poly(I:C) induced IFN-γ and IL-6 release in a dose and time-dependent manner. However, poly-ICLC failed to cause any significant *in vitro* release of immune mediators.

The explanation for limited immune responses seen both mucosally and systemically using poly-ICLC and the absence of mucosal immune responses seen using poly(I:C) is likely to be multifactorial. This can be broadly divided in to factors relating to the challenge agent and those relating to the host. Prior to performing the current research, poly-ICLC (Hiltonol) had been sourced directly from the manufacturer (Oncovir) and stored at a consistent temperature of 4°C, with the stability of the lot confirmed by the manufacturer. However, further subsequent discussions with the manufacturer suggested that there was some lot to lot variability in the capacity for poly-ICLC to induce immune responses, which therefore may have played a role in the lack of immune response seen in the current study. A wide range of doses of poly-ICLC was used (up to 1000 μg) but might an even higher dose cause increased local immune activation? The delivery of poly-ICLC 1000 μg/500 μL required five actuations of the bidose device in to each nostril. Therefore, when given in aerosolised form, delivery of higher doses would require an even greater number of actuations, which may not be practical or desirable. Poly-ICLC was prepared neat without dilution at a concentration of 2 mg/mL, and since this was at the limit of solubility, preparation of a greater concentration (in a reduced volume) of challenge

agent was therefore not possible. Furthermore, other unpublished studies from the USA using poly-ICLC at doses between 0.25-2 mg given as droplets in to the nose confirmed that whilst poly-ICLC was safely tolerated at higher doses, it did not cause any clinical nasal inflammatory response, although specific nasal immune mediators were not measured (personal communication - Richard Davey, Bethesda and Marina Caskey, New York). Additionally, they reported that nasal droplet installation in volumes greater than 1 mg/0.5 mL caused significant leakage of challenge agent from the nasal mucosa, suggesting that the highest dose of poly-ICLC used in this study (also 1 mg/0.5 mL) was the maximum feasible dose.

An alternative method of administration might have been to deliver the challenge agent in split doses over two to three days and this approach has demonstrated that TLR3 agonists can successfully protect against lethal influenza challenge in mice (276). Recently, poly(I:C) given as droplets via a pipette in to the nose over a two-day period, was shown to increase IFN-β, IFN-λ1 and IL-32 gene expression in volunteers with allergic rhinitis during the allergy season (233). However, this study was limited by a lack of data on soluble mediators. Additionally, saline and poly(I:C) was administered simultaneously into separate nostrils and sampling performed from each nostril to study changes in gene expression. This relies on the assumption that nostrils are immunologically separate compartments despite evidence to suggest that contralateral changes can occur after ipsilateral challenge (243, 244).

The size and molecular mass of TLR ligands could also impact on their ability to engage TLR receptors and generate subsequent immune responses. High molecular weight poly(I:C) has been shown to activate TLR3 with much greater efficiency than low molecular weight poly(I:C) (277). Whist high molecular weight poly(I:C) was used in this study, along with poly-ICLC, the uptake pattern by mucosal cells is relatively less well characterized compared to that of PBMCs. This may explain why there were

major differences noted in immune responses between *in vivo* nasal mucosal and PBMC stimulation by poly(I:C). In contrast, whilst poly-ICLC was capable of inducing mucosal IFN-γ at a dose of 500 μg, it largely failed to stimulate immune responses in PBMCs, which may have been affected by poor cellular uptake (e.g. due to ligand complex formation). Finally, TLR ligands given as alternative formulations may help to boost immune activation. Particulate delivery systems such as liposomal envelopes containing poly(I:C) or poly-ICLC have the potential to induce enhanced immune responses compared to using them alone (278, 279). This would require extensive toxicology testing prior to use in humans.

The absence of an immune response in the nose may also be due to host-related factors. Poly-ICLC generates potent systemic innate immune IFN responses in humans when given subcutaneously (105) but the current research failed to detect mucosal immune responses when given nasally. Whilst TLR3 is expressed on the basal and luminal surface and cytoplasm of human airway epithelial cells (93), there are important differences to consider between mucosal and systemic routes of administration, which may affect how TLR ligands access their intended site of action. In the mucosa, these include the presence of: a thick mucus layer; a ciliary escalator; bacterial biofilm; AMPs as well as the possibility of different concentration of RNAses, which might affect the extent of TLR ligand degradation. Finally, whilst poly(I:C) has been administered mucosally in animal models successfully to induce anti-viral immunity and protect against lethal influenza challenge, humans may have a different composition of airway cells and PRRs that make it difficult to compare immune responses between different species (280).

In summary, whilst both TLR3 agonists poly-ICLC and poly(I:C) were well-tolerated by volunteers and had *in vitro* immunostimulatory activity, they failed to reliably induce *in vivo* nasal mucosal immune responses. Therefore, the next phase of the study involved the administration of the TLR7/8 agonist resiquimod.

4 TLR7/8 agonist nasal challenge: results of dose finding study

4.1 Synopsis

The TLR7/8 agonist R848 was administered via the nose to a panel of non-atopic and atopic volunteers to assess tolerability and immune responses. All nine volunteers receiving R848 at a dose of 10 μg / 100 μL per nostril developed significant IFN and cytokine release in the nose compared to saline challenge. One volunteer receiving a dose of 100 μg / 100 μL per nostril developed extremely potent mucosal IFN and pro-inflammatory cytokine responses. Whilst there were no serious adverse events, three out of nine volunteers experienced flu-like symptoms with one volunteer demonstrating a high grade fever, tachycardia, systemic IFN production, neutrophilia and lymphopenia, prompting a revision of the study design to reduce the dose of R848 for future nasal challenge experiments.

4.2 Introduction

The discovery that ssRNA is the natural ligand for TLR7 and TLR8 (281, 282) accelerated research into synthetic compounds that could bind to TLR7/8. Small molecule imidazoquinoline derivative TLR7/8 agonists have been shown to regulate dendritic cell function (283), prime neutrophils for leukotriene B4 and prostaglandin biosynthesis (284), reverse $CD4^+$ regulatory T-cell function (285), and help activate NK cells (286). Imiquimod (a TLR7 agonist) has been licensed as a cream (Aldara, Meda Pharmaceuticals) against genital warts. Imiquimod cream has also been administered to the nares of non-human primates with induction of IFN-α and TNF-α mRNA (287). Oral R848 has been used in a Phase IIa trial in patients with chronic HCV infection with doses of 0.01mg/kg being tolerated but 0.02mg/kg causing IFN-like side effects (113). Whilst the immunostimulatory potential of TLR7/8 agonists such as R848 has been established, its effects on *in vivo* human respiratory tract immune responses are not known. The aim of the subsequent phase of the study was to establish whether R848 had the potential to induce mucosal interferon and proinflammatory cytokine responses in a manner that would be safe and tolerable to volunteers.

4.3 Baseline clinical characteristics

As per the original study design, eight volunteers (three atopic, five non-atopic) were successfully challenged with R848 10 µg / 100 µL per nostril. One volunteer (subject 13) proceeded to the next dose of 100 µg / 100 µL per nostril and tolerated this well. However, as two volunteers receiving R848 10 µg subsequently experienced systemic symptoms, it was decided to pause the study and analyse nasal immune mediators from all eight volunteers at the 10 µg dose and one volunteer at the 100 µg dose. Since immune responses were readily detectable at the 10 µg dose, the study progressed to the second part at this dose without the need for dose escalation. However, the next volunteer to receive R848 10 µg had reduced tolerability (described later in this chapter) and therefore the study did not continue further at the 10 µg dose. This volunteer from the second part of the study was combined with those from the first part of the study and the baseline characteristics of these nine volunteers (three atopic, six non-atopic) undergoing R848 10 µg challenge are shown in table 4.1.

Subject ID	Age	Gender	Ethnicity	BMI	Skin Prick to Timothy Grass (mm)	Eosinophil Count ($x10^9$/L)	Serum Total IgE (IU/mL)
13	45	Male	White British	25.4	0	0	23.5
15	29	Female	Other White	22.6	3	0.1	67.5
16	49	Female	Other Mixed	21.2	13	0.2	1085
25	35	Female	White British	22.4	12	0.2	142
28	34	Female	White British	24.3	1	0.3	54.1
30	27	Female	White British	21.5	0	0	32.8
31	47	Female	Other White	21.7	0	0	4.69
35	45	Female	Other White	23.8	0	0.1	16.4
53	32	Female	Other White	21.9	0	^	2.12

Table 4.1 Baseline characteristics of volunteers undergoing R848 10 µg challenge ^Data unavailable.

4.4 Nasal clinical response

After R848 10 µg administration, volunteers stayed on the ICRRU for eight hours and then had a telephone consultation at 24 h. It was therefore possible to assess TNSS (a subjective score that could be provided over the phone) over a 24 h period. PNIF (an objective score that was clinician-dependent) was assessed over 8 h. Overall, R848 caused a drop in PNIF between three to five hours after challenge but this was not statistically significant when compared to baseline (P = 0.0831 at five hours) (figure 4.1).

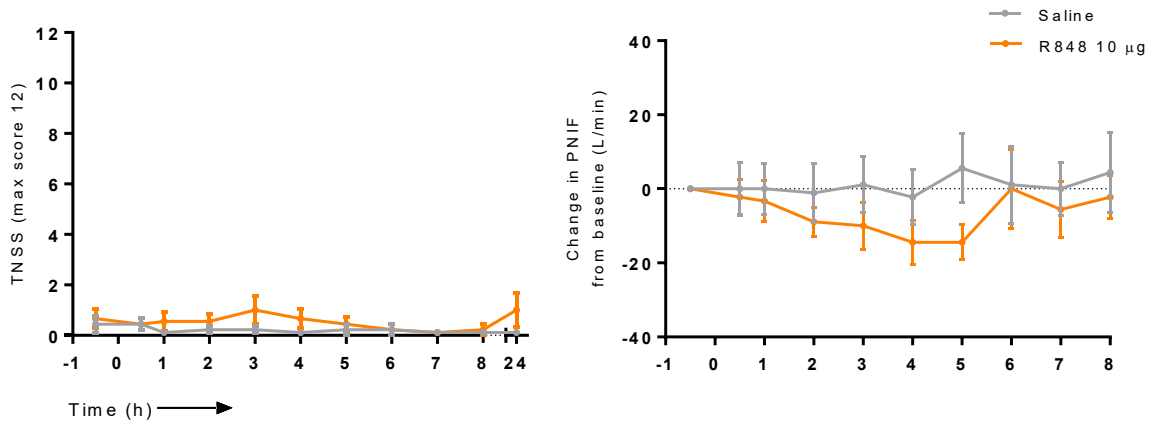


Figure 4.1 Clinical correlates of nasal obstruction after saline and R848 10 µg Data from nine volunteers (atopic n=3, non-atopic n=6). Total nasal symptom score (TNSS) plotted as mean±SEM and comparison versus baseline by Friedman and Dunn's post hoc test. Change in peak nasal inspiratory flow (PNIF) from baseline plotted as mean±SEM and comparison versus baseline by repeated measures one-way ANOVA (with the Greenhouse-Geisser correction) and Dunnett's multiple comparison test.

4.5 Systemic clinical and immune response

R848 10 µg nasal challenge was well-tolerated by most volunteers with no serious adverse events noted. All volunteers had observations taken before and at eight hours after challenge. Those reporting symptoms and those challenged at a later date (after the initial reports of systemic symptoms were noted), had additional observations taken on the day of challenge and were also invited back to return the following day. Six out of nine volunteers did not experience any systemic symptoms. Three volunteers did experience symptoms, which included myalgia, fatigue and headache (table 4.2). Out of the three volunteers with systemic symptoms (atopic n=2, non-atopic n=1), subjects 25 and 53 were able to return for additional sampling and observations at 24 h but subject 15 could only return at 48 h (figure 4.2). There was no change in blood pressure or oxygen saturations (data not shown).

Subject 15 experienced fatigue for up to 36 h after challenge, a temperature of up to 37.4°C, transient tachycardia of >20 beats per minute above baseline as well as myalgia and headache on the day of challenge. Subject 25 experienced shivering soon after administration of challenge agent, as well as flu-like symptoms overnight and a blocked, itchy nose 24 h after challenge. Subject 53 experienced flu-like symptoms for 24 h, which included a transient temperature rise up to 39.2°C for one hour that reduced to 37.8°C by the end of the first day as well as a transient tachycardia up to 30 beats per minute (bpm) higher than baseline. The subject also experienced a moderate headache, which required paracetamol ten hours after challenge. Full blood count samples were collected before and at 8 and 24 hours after challenge from subject 53 to ascertain the white cell count differential, which showed a transiently neutrophilia and lymphopenia (figure 4.3). These factors therefore temporarily met the moderate to severe criteria for clinical parameters in the FDA's grading scale for trials of preventative vaccines (288).

Subject ID	Nasal symptoms	Fever >37.2°C	Myalgia	Fatigue	Headache	Comments
13	N	N	N	N	N	Asymptomatic
15	N	N	Y	Y	Y	Fatigue up to 36 h
16	Blocked nose	Y	N	N	N	Blocked nose at 3 h Temperature rise to 37.4°C No systemic symptoms
25	Blacked + Itchy nose	N	Y	Y	Y	Shivering soon after administration for 1.5 h Flu-like symptoms overnight
28	N	N	N	N	N	Asymptomatic
30	N	N	N	N	N	Asymptomatic
31	N	N	N	N	N	Asymptomatic
35	N	N	N	N	N	Asymptomatic
53	N	Y	Y	Y	Y	Flu-like symptoms for 24 h Temperature rise to 39.2°C Moderate headache Tachycardia Lymphopenia & Neutrophilia
Total n (%)	2 (22%)	2 (22%)	3 (33%)	3 (33%)	3 (33%)	-

Table 4.2 Nasal and systemic symptoms in volunteers after R848 10 µg

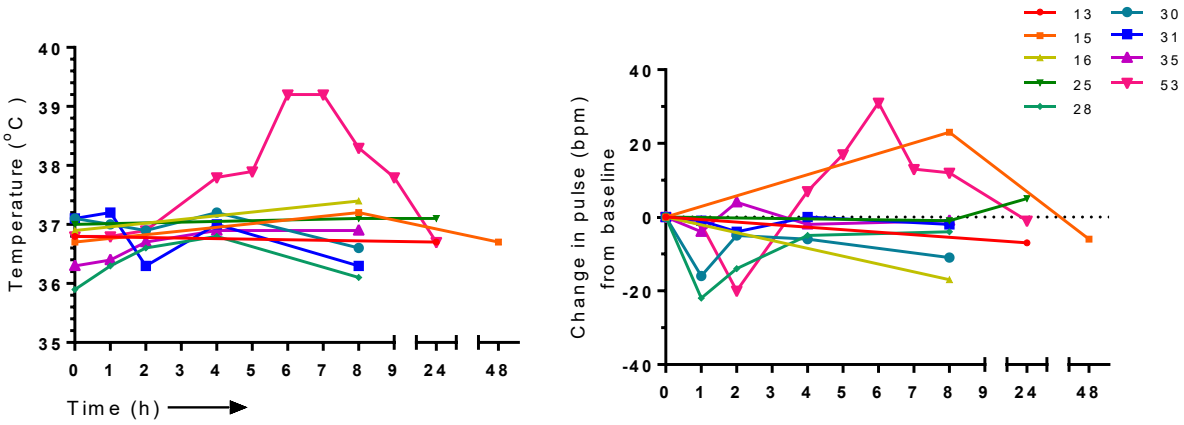


Figure 4.2 Systemic physiological response after R848 10 µg All volunteers (n=9) had observations measured at 0 h and 8 h with some volunteers observed more frequently based on symptoms.

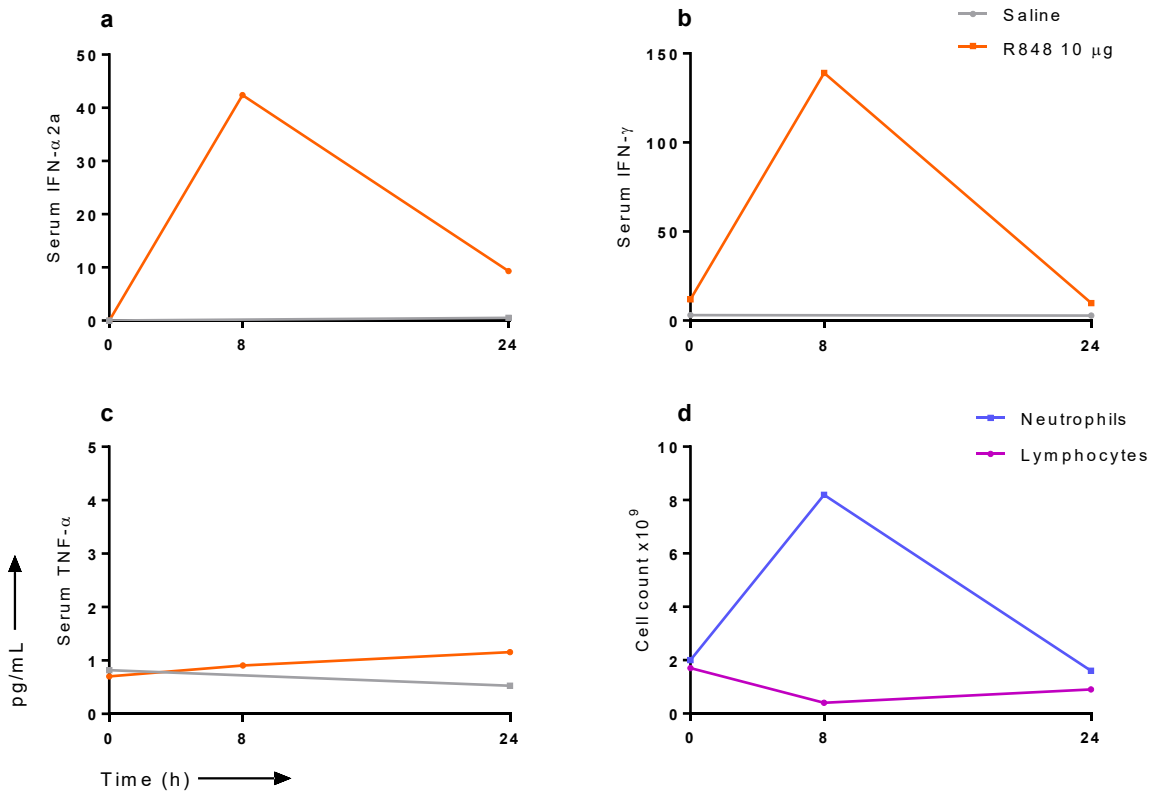


Figure 4.3 Serum immune response after saline and R848 in a symptomatic volunteer Serum (a) IFN-α, (b) IFN-γ and (c) TNF-α after saline and R848 10 µg challenge and (d) white cell differential count after R848 10 µg challenge. Data from a selected volunteer with systemic symptoms (subject 53).

4.6 Nasal immune mediator response

Nasosorption was performed before and at serial intervals for up to eight hours after both saline and R848 10 μg challenge. Three volunteers with systemic symptoms had additional sampling performed at 24 h or 48 h. A multiplex MSD panel was used to measure the following interferons and proinflammatory cytokines: IFN-α, IFN-β, IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13. When compared to saline, R848 caused significant induction of several mucosal innate immune mediators: IFN-α, IFN-γ, TNF-α, IL-1β, IL-10, IL-12p70, IL-13 (table 4.3). Amongst these mediators IFN-α, IFN-γ, TNF-α and IL-1β were very strongly induced in all volunteers and had characteristic kinetic profiles: TNF-α levels increased almost immediately and peaked at two hours; IFN-α peaked at three hours; IL-1β peaked at four hours and IFN-γ induction occurred much later on after four hours, with a peak at eight hours. All of these mediators subsequently returned to baseline after 24 h (figure 4.4). One volunteer (subject 16) experienced an inflammatory and interferon response after saline challenge despite being asymptomatic at the onset of challenge, which may have represented a subclinical occult infection. This volunteer went on to have a much greater IFN-α, IL-1β and IFN-γ response after R848 challenge. The three volunteers with systemic symptoms (subjects 15, 25 and 53) had variable mucosal immune responses. Subject 15 had high levels of nasal TNF-α, IFN-α and IFN-γ but relatively reduced IL-β response. Subject 25 had high levels of TNF-α, IFN-α and IL-1β but had relatively reduced levels of IFN-γ. Subject 53, who had experienced fever, increased serum IFN responses and neutrophilia, only had a moderate rise in nasal TNF-α, IFN-α, IL-1β and IFN-γ that was less pronounced than other volunteers. This suggested that mucosal immune responses may not necessarily predict systemic immune responses or the degree of systemic symptoms. Of interest, the three volunteers with the greatest IFN-α responses (subjects 15, 16, 25) were all atopic, having peak IFN- α levels between 800-1500 pg/mL, compared to <350 pg/mL for other volunteers. Although the number of volunteers at this stage was not sufficient for statistical comparison, this suggested that individuals with atopy are capable of mounting strong mucosal anti-viral responses.

Mediator	Saline (n=9)	R848 (n=9)	*P*-value
IFN-γ	2.21 (0.93 – 3.49)	8.20 (6.30 – 10.11)	<0.0001
IFN-α	2.03 (-0.54 – 4.60)	15.65 (11.73 – 19.56)	0.0001
IL-1β	16.00 (13.82 – 18.18)	20.22 (18.55 – 21.88)	0.0009
TNF-α	6.79 (4.58 – 9.00)	12.63 (11.33 – 13.92)	0.0015
IL-10	7.90 (6.42 – 9.38)	9.67 (8.22 – 11.11)	0.0116
IL-12p70	1.43 (0.48 – 2.38)	2.15 (1.47 – 2.84)	0.0164
IL-13	4.03 (2.26 – 5.81)	4.88 (3.37 – 6.39)	0.0202
IFN-β^	1.72 (-1.20 – 4.63)	4.65 (-0.84 – 10.14)	0.0617
IL-4	3.13 (1.99 – 4.26)	3.78 (2.64 – 4.93)	0.1127
IL-2	2.88 (1.80 – 3.95)	3.39 (2.20 – 4.58)	0.1648
IL-6	11.77 (9.37 – 14.16)	12.94 (11.40 – 14.48)	0.3458
CXCL8/IL-8	28.36 (26.67 – 30.05)	28.99 (27.40 – 30.58)	0.4064

Table 4.3 Comparison of nasal immune mediator responses after saline and R848 10 µg AUC of $\log_{10}$ transformed values between 0-8 hours after nasal challenge with saline and R848 10 µg was initially calculated for each mediator in individual subjects (n=9). Subsequently, AUC values were compared between groups using a paired t-test. The baseline parameter for AUC was set at the lower limit of detection (LLOD) for each mediator. Samples collected prior to challenge were excluded, ^n=7. AUC values expressed as mean (95% CI) and displayed in order of significance.

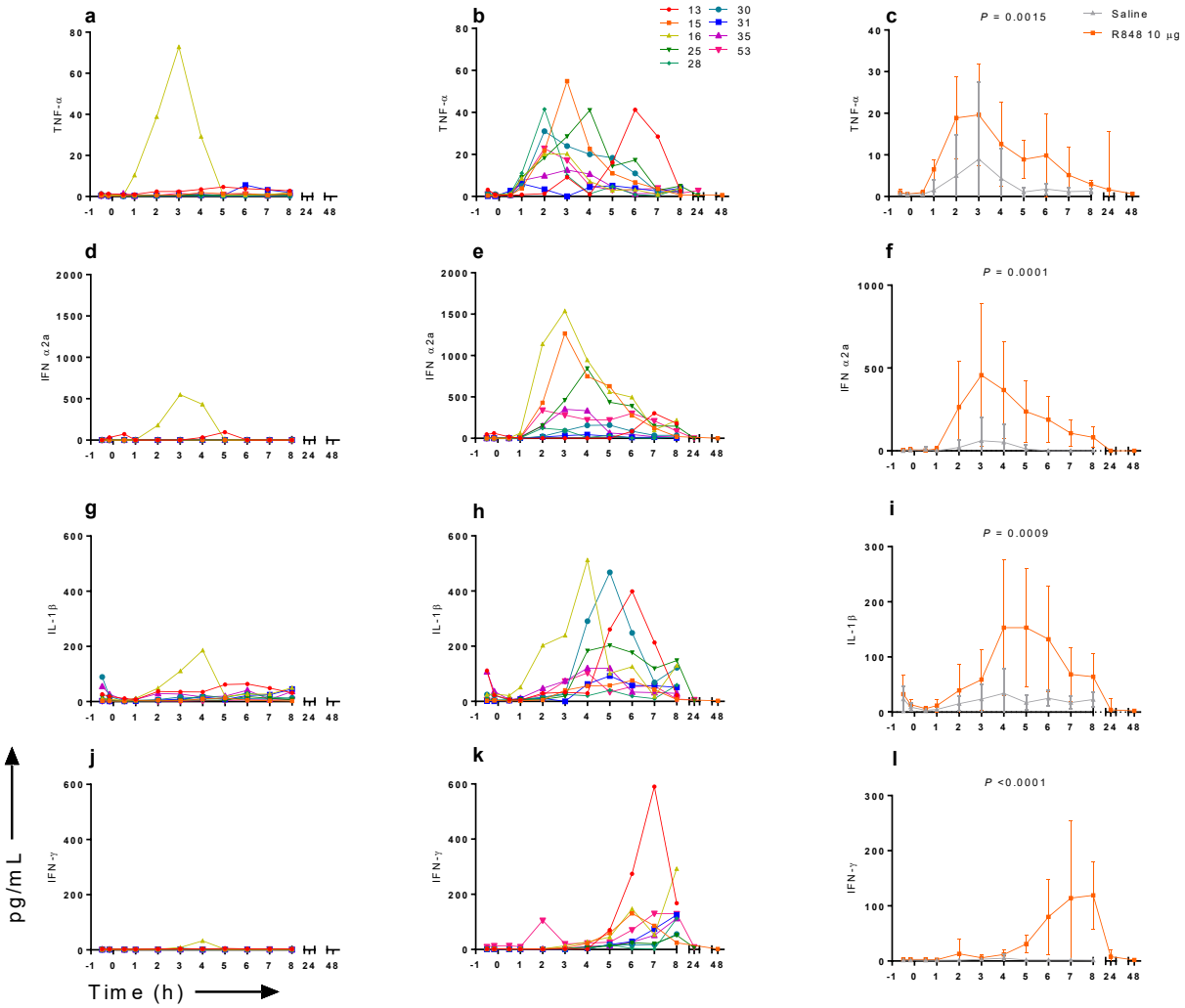


Figure 4.4 Nasal immune mediator responses after saline and R848 10 µg Individual responses after saline (a, d, g, j) and R848 (b, e, h, k), with grouped responses (c, f, i, l) expressed as mean and 95% CI from nine volunteers (atopic n=3, non-atopic n=6). AUC values compared between groups using a paired t-test.

4.7 Highest dose R848 nasal challenge

One male, non-atopic volunteer (subject 13) went on to receive nasal R848 challenge at a higher dose of 100 µg / 100 µL. He reported flu-like symptoms with myalgia between 5 and 24 hours after challenge. Observations recorded before and eight hours after challenge showed no difference in temperature (36.4°C and 36.8°C respectively), no difference in heart rate (63 bpm, 65 bpm) and a mild increase in blood pressure (108/62 mmHg, 129/72 mmHg). His total nasal symptom score was zero at all time-points on the day of challenge but increased to four at 24 h (figure 4.5). There were no differences in his peak nasal inspiratory flow when comparing saline, R848 10 µg and R848 100 µg (figure 4.5). R848 100 µg challenge led to a potent nasal mucosal interferon and pro-inflammatory response when compared to both saline and R848 10 µg, and in particular induced extremely high levels of IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12 (figure 4.6).

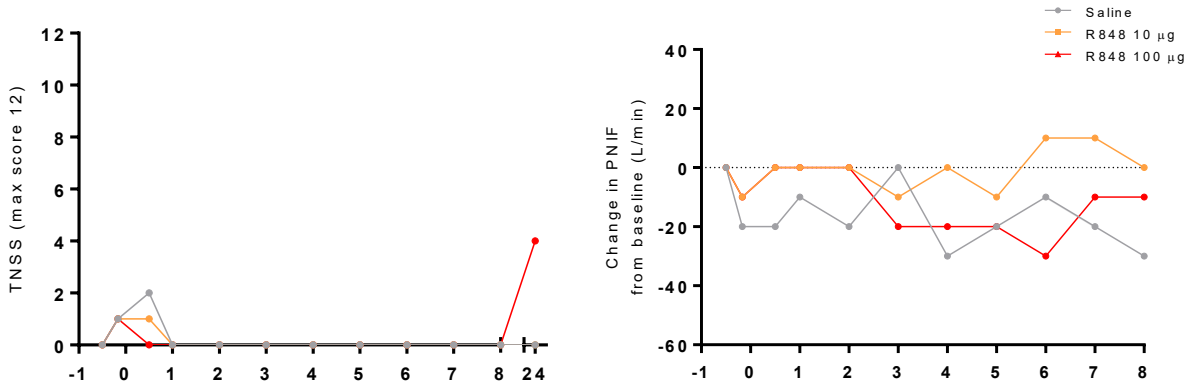


Figure 4.5 Clinical correlates of nasal obstruction after saline and R848 100 µg Data from a single volunteer (subject 13).

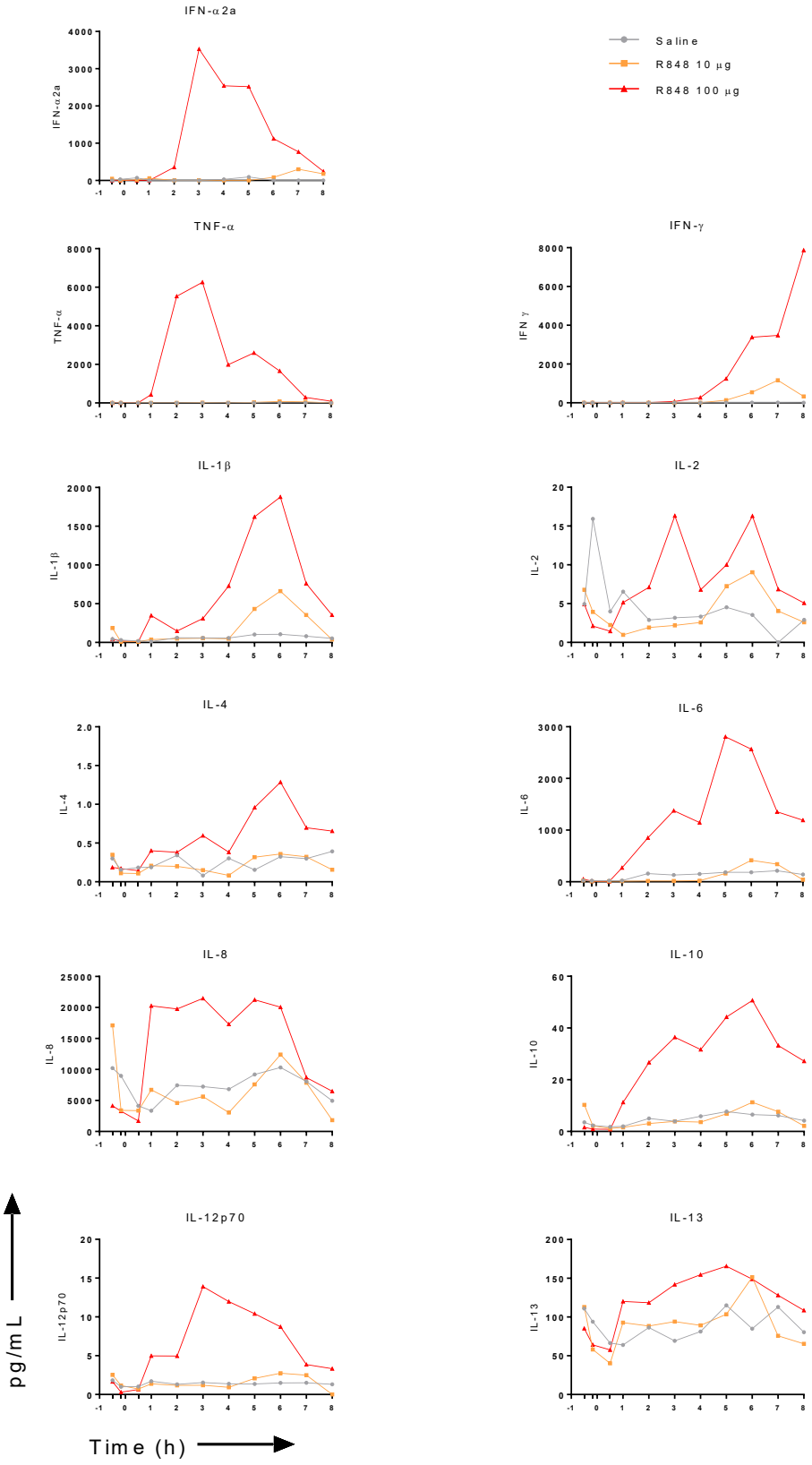


Figure 4.6 Nasal immune mediator responses after saline, R848 10 µg and R848 100 µg Data from a single volunteer (subject 13).

4.8 PBMC experiments

To assess and compare peripheral responses to TLR agonists, PBMCs were isolated from six volunteers and stimulated with media, R848 (1 µg/ml), poly(I:C) (10 µg/mL) or poly-ICLC (10 µg/mL). Culture supernatants were collected at earlier time-points than previous experiments at 3 h, 8 h and 24 h and immune responses assessed using the MSD platform (figure 4.7). This demonstrated that R848 induced high levels of TNF-α, IL-1β, IFN-α and IFN-γ at all time-points. Poly(I:C) was also capable of inducing these mediators especially at 24 h, but only IFN-α reached statistical significance at 24 h. In keeping with previous experiments, poly-ICLC failed to stimulate any meaningful PBMC immune responses. This confirmed that R848 was a reliable TLR agonist for inducing both *in vivo* mucosal immune responses and *in vitro* PBMC responses.

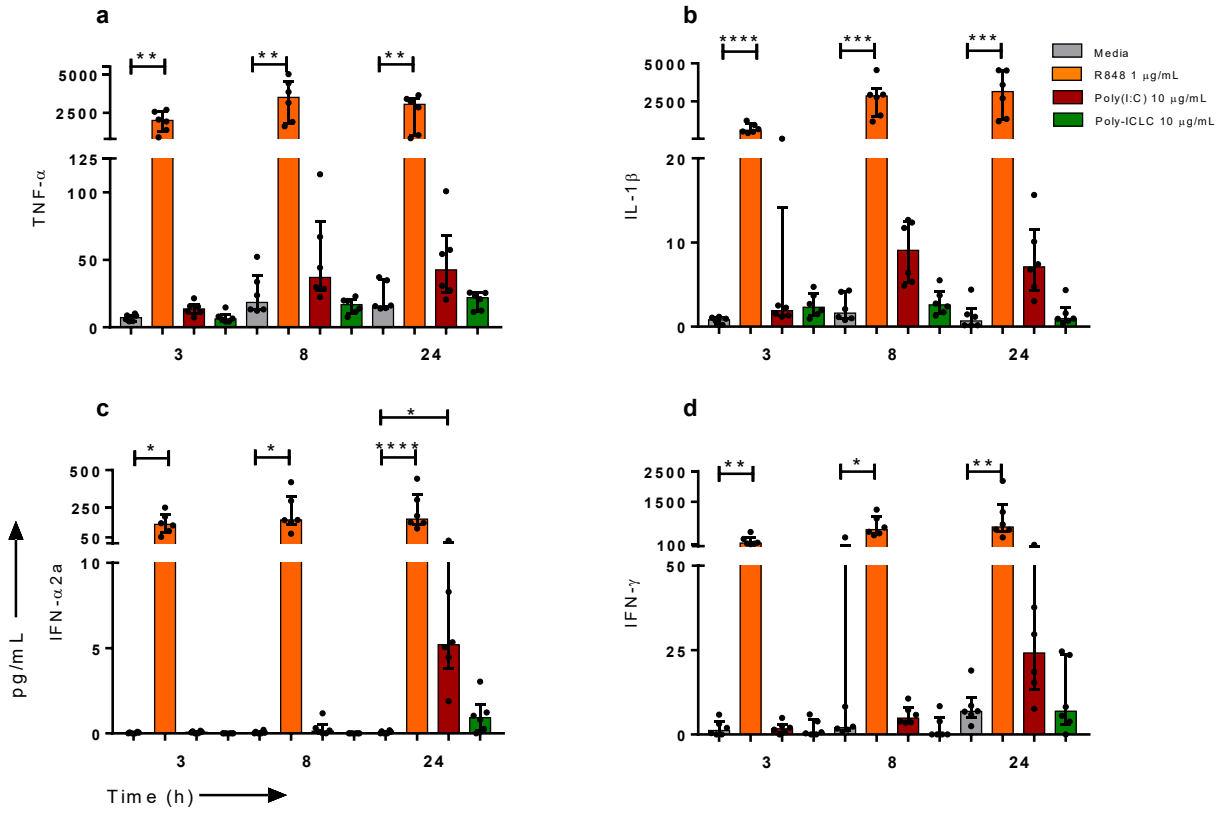


Figure 4.7 PBMC responses to R848, poly(I:C) and poly-ICLC stimulation The MSD immunoassay platform was used to measure levels of (a) TNF-α, (b) IL-1β, (c) IFN-α and (d) IFN-γ in culture supernatant harvested at 3 h and 8 h from PBMCs stimulated with media alone, R848 (1 µg/ml), poly(I:C) (10 µg/mL) or poly-ICLC (10 µg/mL). Represents data from six volunteers with supernatant samples in triplicate. Median ± interquartile range shown. Kruskal Wallis test performed, followed by Dunn's multiple comparison test, *P<0.05, **P<0.01, ***P<0.001.

4.9 Discussion

Nasal delivery of the TLR7/8 agonist R848 was capable of generating significant mucosal innate immune responses for a duration of 24 hours after administration, with no serious adverse events noted. The mucosal response was characterized by an early peak in pro-inflammatory cytokines (TNF-α and IL-1β) as well as IFN-α and a later induction of IFN-γ, with a reduction of these mediators back to baseline by 24 hours. Additionally, a single volunteer who received two different doses of R848 (10 μg and 100 μg) showed that mucosal immune responses were dose-dependent. The robust mucosal immune responses seen after R848 administration is in stark contrast to the lack of response seen after nasal challenge with TLR3 agonists poly(I:C) and poly-ICLC.

Whilst R848 did not cause any significant increase in clinical correlates of nasal obstruction, one in three volunteers did experience significant systemic symptoms involving myalgia, headache and lethargy. One volunteer (subject 53) was noted to have a transient temperature rise in excess of 39°C and had elevated systemic IFN-α, IFN-γ levels, neutrophilia and lymphopenia at eight hours, with all of these measures returning to baseline by 24 hours. R848 delivery in mice suggests a narrow therapeutic index and the potential to cause hypotension and lymphopenia when given at higher doses (289). Type 1 IFNs have been shown to play a key role in stimulating B cells and T cells and regulating lymphocyte recirculation (290). Whilst serum R848 levels were not measured in this study, it is possible that R848 was absorbed systemically from the nasal mucosa. However, the lack of systemic proinflammatory cytokine production suggests that the systemic clinical response is likely due to mucosal-derived IFNs that "spill-over" in to the systemic circulation. An ideal challenge agent would have characteristics that would cause induction of local mucosal immune mediators with minimal systemic symptoms. The TLR7 agonists GSK2245035 and AZD8848 have been developed with the aim of generating mainly local type 1 IFN responses (rather than pro-inflammatory cytokines typically induced by activation of TLR8), which have therapeutic potential for the treatment of allergic

rhinitis (291, 292). However, as a tool to measure mucosal innate immune responses to viral RNA analogues in different host groups (e.g. asthma, allergic rhinitis, COPD and the elderly), R848 is an ideal challenge agent due to its ability to stimulate multiple cell types such as pDCs (TLR7) and myeloid dendritic cells (mDCs) (TLR8). This has to be balanced however by limiting the possibility of inducing undesirable systemic clinical symptoms and risk of cytokine release syndrome (293). Hence, for the second part of the study, it was decided to use a lower dose of R848. To limit the possibility of body weight playing a confounding role in determining immune responses (in the event of systemic absorption of R848), the dosage would be based on the volunteer's body weight. A dosage of 0.02 μg/kg/100 μL was chosen, which would correlate to a dose of between 1-2 μg/100 μL per nostril in a volunteer weighing 50-100kg. This would be an approximately five to ten-fold lower dose than the doses used thus far in the study.

It was of interest to note that the three volunteers with the greatest IFN-α responses were all atopic. However, a greater number of participants (as planned for the second part of the study) would be required to support the hypothesis that atopic subjects have exaggerated anti-viral responses compared to non-atopic volunteers. It was noted that one female atopic volunteer (subject 16) had enhanced mucosal responses to both R848 and saline control challenge. The volunteer had a simultaneous increase in TNF-α, IFN-α, and IL-1β levels after saline challenge suggesting the presence of nasal mucosal inflammation rather than representing spurious findings. This may have been due to a sub-occult upper respiratory tract infection at the time of saline challenge that did not present with symptoms and were not apparent on clinical examination prior to challenge.

5 TLR7/8 agonist nasal challenge in allergy and asthma

5.1 Synopsis

A nasal spray containing a lower dose of R848 (0.02 μg/kg/100 μL, mean dose 1.5 μg) was administered to healthy volunteers and those with atopy or allergic asthma. This dose of R848 was well-tolerated by all volunteers (with negligible effects on nasal obstruction and symptoms) and the kinetics of mucosal cytokine and chemokine production was delineated: an early rise in IFN-α; a later release of IFN-γ and CXCL10; and a sustained production of CCL2, CCL3, CCL4 and CCL13 (all $p<0.05$, AUC compared to placebo challenge). The results also demonstrate remarkably robust innate immune responses in volunteers with allergic rhinitis (n=12) and allergic asthma (n=11) compared to healthy volunteers (n=12), with an associated increase in ISGs seen in volunteers with asthma. Nasal R848 administration did not cause systemic IFN or proinflammatory cytokine release but was associated with a significant early drop in blood eosinophils and a late drop in lymphocytes amongst volunteers with allergic rhinitis and allergic asthma. These results confirm the suitability of nasal delivery of R848 as a non-invasive tool to assess mucosal innate immune immunity and highlights an important role for atopy in determining the immune response to viral RNA analogues.

5.2 Introduction

As the tolerability of nasal R848 administration in healthy volunteers and those with allergic rhinitis had been established, the final part of the study involved its use in volunteers with asthma. Viral infections in asthma are important triggers for exacerbations in both children and adults, with TLR7 and TLR8 known to play a key role in viral nucleic acid recognition (294). Studying the *in vivo* effects of IFN production in response to live viral challenges in humans poses some difficulties. Whilst the initial viral inoculation dose can be standardized between volunteers, *in vivo* viral titres may be higher in individuals with asthma (164), which may affect interferon production. Therefore, a non-replicating

viral RNA-like immunomodulatory agent such as R848 has the important advantage of being able to induce immune responses without the confounding factor of viral load.

Approximately half of patients with mild to moderate asthma have persistently non-eosinophilic disease that is poorly responsive to inhaled corticosteroid treatment and is associated with lower total IgE levels (295). Neutrophils have been implicated in asthma and viral infection (296) and have been associated with increased severity of asthma (297). Neutrophils express both TLR7 and TLR8, especially the latter (95, 298), and enhanced TLR7/8 mediated CXCL8 release has been reported in peripheral neutrophils from subjects with asthma (299). This heterogeneity of asthma endophenotypes has been demonstrated in large cluster-based studies and has advanced the field away from basing assessment of asthma simply on clinical phenotypes such as the presence of allergic symptoms (300, 301). However, for the purposes of studying the effects of atopy on *in vivo* responses to TLR agonist challenge, a non-atopic asthma group (defined by a lack of allergic rhinitis symptoms and negative skin-prick response to aeroallergens) was also included in the initial study design. Additionally, most clinical studies that investigate mucosal immunity after viral challenge involve the comparison of immune responses between non-atopic healthy volunteers and those with allergic asthma, with differences in results being ascribed to the presence of asthma. However, few studies include volunteers with allergic rhinitis and without asthma, and therefore this group serves as a useful control population for comparison of immune responses.

The study therefore aimed to recruit four separate groups of volunteers: healthy non-atopic (n=12), atopic (n=12), allergic asthma (n=12) and non-allergic asthma (n=6) (figure 5.1). A reduced number of volunteers with non-allergic asthma was planned due to the known difficulty in recruiting this group of volunteers. The dosage regimen to be used for this study would be 0.02µg/kg/100µL, approximating to a dose of between 1-2µg in a 50-100kg individual. However, as volunteers with asthma had not

been included thus far in the study, half this dose (0.01 µg/kg/100 µL) was initially trialled in four volunteers with allergic asthma. As it was safely tolerated with no adverse events (data not shown), the remainder of the study proceeded at the 0.02µg/kg/100µL dose.

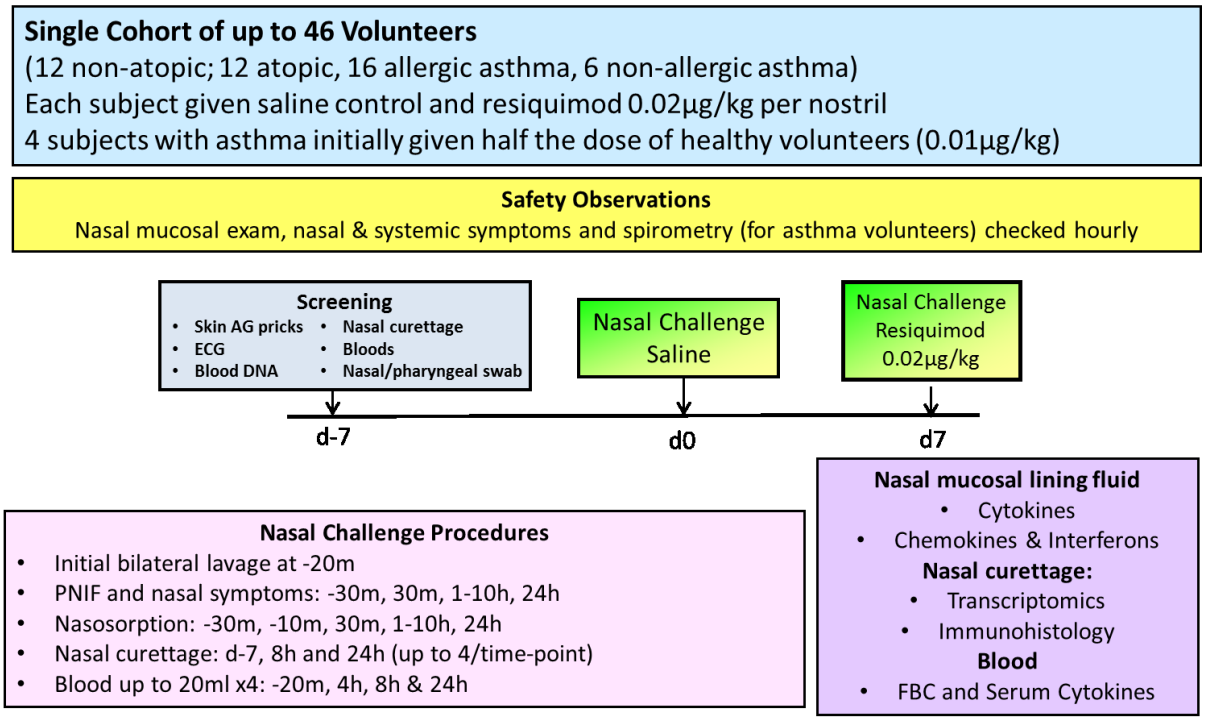


Figure 5.1 Final study design for R848 challenge in healthy, atopic and asthma volunteers Abbreviations SAM, synthetic absorptive matrix; PNIF Peak nasal inspiratory flow; TNSS Total nasal symptom score

5.3 Baseline clinical characteristics

Recruitment for most volunteer groups was successful (healthy non-atopic n=12, atopic n=12, allergic asthma n=11). However, despite manual curation of volunteer databases as well as online and newspaper advertising, only two volunteers with non-allergic asthma were recruited. Due to the small number of subjects, this group was therefore removed from further analysis. The baseline characteristics of participants included in the study are outlined in table 5.1. The mean total dose administered of R848 for all participants, which was based on body weight (0.02 µg/kg/100 µL) was 1.5 µg and was not significantly different between groups (table 5.1).

Clinical Characteristic	**Healthy n=12**	**Atopic n=12**	**Allergic Asthma n=11**	***P*-value**
Gender M:F	5:7	5:7	5:6	0.9781
Age	37.3 ±3.1	43.3 ±4.3	31.6 ±2.8	0.0781
Weight (kg)	70.7 ±4.3	75.0 ±3.8	74.4 ±3.5	0.7068
Skin Prick Grass (mm)	0	6.9 ±0.8	6.9 ± 0.5	<0.0001
Serum Total IgE (IU/mL)	29.09 ±11.16	160.8 ±47.71	379.4 ±151.5	0.0276
Baseline Blood Eosinophil Count ($x10^9$/L)	0.09 ± 0.02	0.11 ± 0.03	0.30 ± 0.04	<0.0001
FEV1% predicted*	95.5 (85 - 102.5)	87.5 (83.25 – 92.5)	87 (77 – 116)	0.4149
Histamine PC20 (mg/mL)*	16 (16 - 16)	6 (1.25 – 16)	0.48 (0.24 – 2)	<0.0001
Asthma Control Questionnaire (ACQ) Mean ±SEM	0	0	0.7649 ±0.1393	<0.0001
Inhaled Corticosteroid Dose (Beclometasone Equivalent)*	0	0	50 (0-200)	<0.0001
Dose of R848 administered (µg)^	1.42 ±0.09	1.50 ±0.08	1.49 ±0.07	0.8335

Table 5.1 Baseline characteristics of volunteers undergoing R848 (0.02 µg/kg/100 µL) challenge Values expressed are mean ±SEM and comparison performed using one-way ANOVA. *Values expressed as median and interquartile range and compared using Kruskal-Wallis test. P-values are uncorrected for multiple comparison.

5.4 Nasal and airway clinical response

Volunteers were observed and sampled for eight hours after saline challenge and for ten hours after R848 challenge, with all volunteers returning for a 24 h visit. There was no difference in TNSS scores and change in FEV1 after saline or R848 challenge (figure 5.2). R848 caused a small reduction in PNIF at three time points compared to baseline: 0.5 h (mean difference -13.7 L/min, 95% CI -23.6 to -3.9, *P* = 0.0028), 2 h (-13.4 L/min, 95% CI -26.2 to -0.7, *P* = 0.0343) and 8 h (-15.4 L/min, 95% CI -29.9 to -1.0, *P* = 0.0311). The group analysis showed no difference in change of PNIF values compared to baseline (figure 5.2).

Taken together, this data demonstrates that R848 at a dose of 0.02 µg/kg/100 µL did not induce any consistent change in nasal or airway correlates of obstruction.

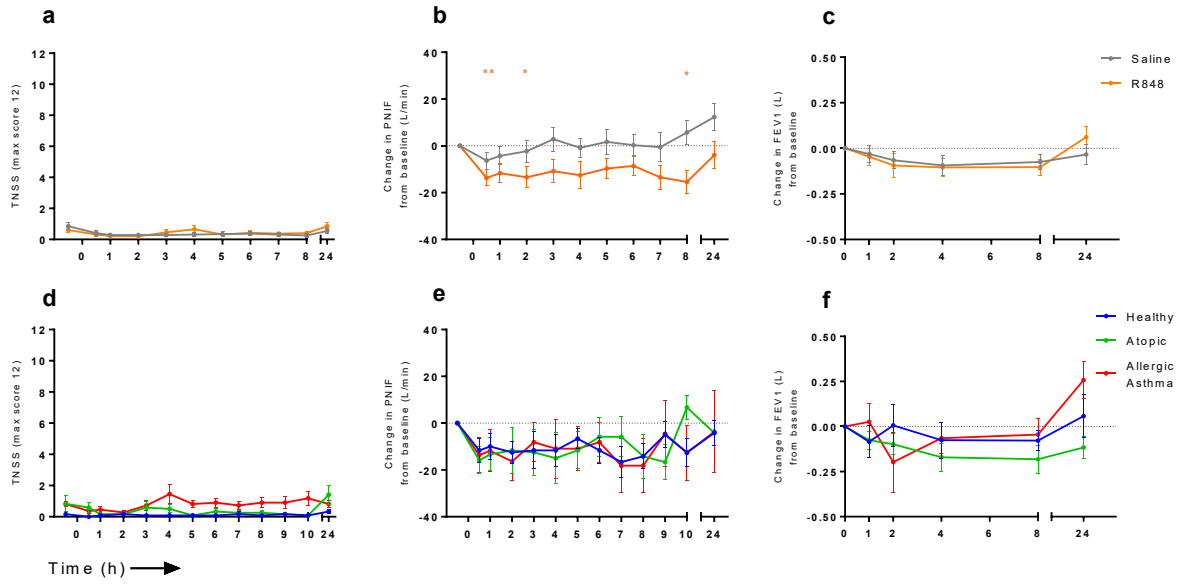


Figure 5.2 Clinical correlates of airway obstruction after saline and R848 (0.02 µg/kg/100 µL) Data from 35 volunteers (healthy n=12, atopic n=12, allergic asthma n=11). Total nasal symptom score (a, d) plotted as mean±SEM and comparison versus baseline by Friedman and Dunn's post hoc test. Change in peak nasal inspiratory flow (b, e) and FEV1 (c, f) plotted as mean±SEM and comparison versus baseline by repeated measures one-way ANOVA with Greenhouse-Geisser correction and Dunnett's multiple comparison test.

5.5 Systemic clinical response

Systemic observations of vital physiological signs were performed at 0 h, 8 h and 24 h after saline challenge and on an hourly basis after R848 challenge (figure 5.3). Eight hours after saline challenge, there was a small reduction in pulse rate compared to baseline (mean difference -5.9 bpm, 95% CI -10.4 to -1.3, *P* = 0.0097). Eight hours after R848 challenge, there was a small reduction in the diastolic blood pressure (-3.7 mmHg, 95% CI -6.7 to -0.6, *P* = 0.0154). After R848 challenge, healthy volunteers had an increase in pulse at 24 h compared to baseline (mean difference 15.3 bpm, 95% CI 2.3 to 28.2, *P* = 0.0127) whilst atopic volunteers had a reduction in pulse rate at three time points: 8 h (-7.3 bpm, 95% CI -14.01 to -0.655, *P* = 0.0236), 9 h (-7.8 bpm, 95% CI -14.5 to -1.2, *P* = 0.0127) and 10 h (-7.1 bpm, 95% CI -13.8 to -0.41, *P* = 0.0317). Eight hours after R848 challenge, atopic volunteers had a small reduction in diastolic BP (-6.9 mmHg, 95% CI -13.3 to -0.53, *P* = 0.0267). Four hours after R848, volunteers with asthma had a small reduction in their diastolic BP (-7 mmHg, 95% CI -13.4 to -0.6, *P* = 0.0258). Although, there was no difference in temperature between saline and R848 challenge, group-specific analysis showed that healthy volunteers had a small increase in their temperature at three time points compared to baseline: 4 h (mean difference 0.3°C, 95% CI 0.02 to 0.61, *P* = 0.0285), 8 h (0.3°C, 95% CI 0.03 to 0.62, *P* = 0.0228) 10 h (0.3°C, 95% CI 0.04 to 0.63, *P* = 0.0181).

Taken together, this data demonstrates that R848 at a dose of 0.02 µg/kg/100 µL did not induce any consistent change in systemic clinical responses.

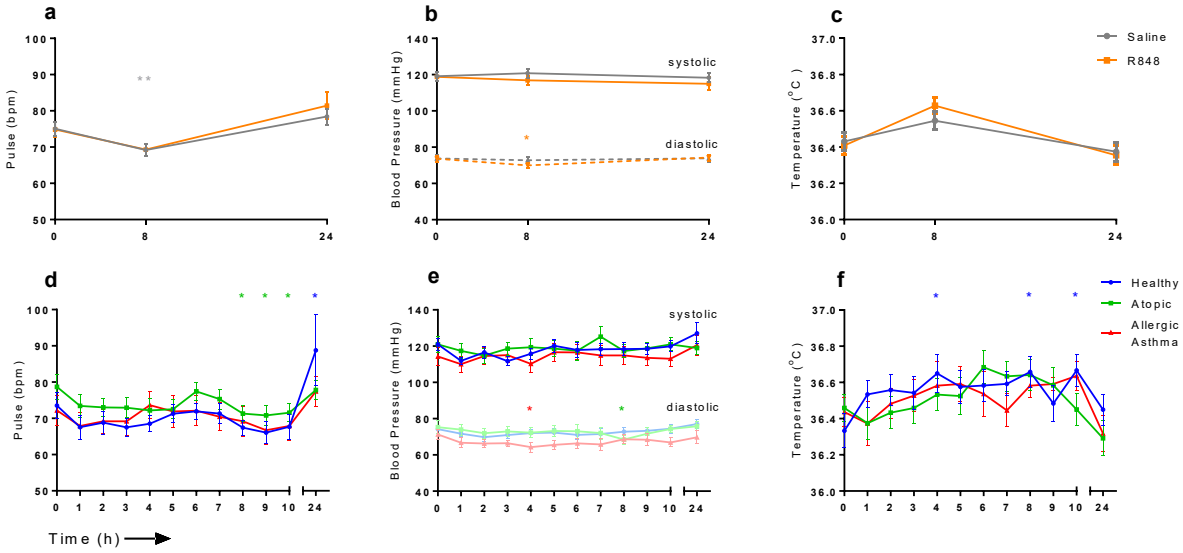


Figure 5.3 Systemic physiological observations after saline and R848 (0.02 µg/kg/100 µL) Data from 35 volunteers (healthy n=12, atopic n=12, allergic asthma n=11). Pulse (a, d), blood pressure (b, e) and temperature (c, f) plotted as mean±SEM and comparison versus baseline by repeated measures one-way ANOVA and Dunnett's multiple comparison test, *P<0.05, **P<0.01.

5.6 Nasal immune mediator response

5.6.1 Saline versus R848

Nasosorption was performed before and at serial intervals for eight hours after saline and ten hours after R848 challenge, as well as an additional 24 h time-point after both challenges. Multiplex MSD panels were used to measure interferons (IFN-α, IFN-β, IFN-γ), proinflammatory cytokines (TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13) and chemokines (CXCL8/IL-8 high assay, CXCL10/IP-10, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL11/eotaxin-1, CCL13/MCP-4, CCL17/TARC, CCL22/MDC, CCL26/eotaxin-3). A custom tripleplex was also used to measure the epithelial derived cytokines IL-25 and IL-33 along with IL-29/28B (IFN-λ 1/3). Unfortunately, it was not possible to detect IL-29/28B in the vast majority of samples and was therefore excluded from any further analysis. When compared to saline, R848 (mean dose 1.5 μg) challenge significantly induced several mediators: IFN-α, IFN-γ, CXCL10, CCL3, CCL4, CCL13, IL-2, TNF-α, CCL2 and IL-12p70 (table 5.2 and figure 5.4).

An additional analysis comparing AUC values between saline and R848 within each subject group identified that amongst volunteers with asthma (but not healthy or atopic volunteers), R848 also induced IL-1β, IL-6, IL-8, IL-10 and CCL11 (figure 5.5). In keeping with higher dose R848 10 μg challenge, the pattern of response was characterized by an early rise in TNF-α (peak 2 h) and IFN-α (peak 3 h), followed by a later rise in IFN-γ (peak 8 h) and CXCL10, with the latter having high levels even at 24 h. CCL2, CCL3, CCL4 and CCL13 all started to increase approximately 1-2 hours after R848 challenge and continuously increased until about 8 h. At 24 h, all chemokines with the exception of CCL13, showed no difference in levels when compared to saline challenge. Although IL-2 and IL-12p70 were statistically significantly increased after R848 compared to saline, the response curves over time failed to show meaningful differences for most of the challenge period (figure 5.4). These results therefore suggest that at a lower dose R848 adequately induces IFN and chemokine responses, whilst a higher dose tends to cause a significant release of both IFNs and proinflammatory cytokines.

Mediator	Saline	R848	*P*-value
IFN-α	0.05 (-0.03 - 0.13)	5.13 (3.708 - 6.557)	<0.0001
IFN-γ	1.98 (1.31 - 2.64)	5.10 (3.88 - 6.32)	<0.0001
CXCL10/IP-10	25.63 (24.5 - 26.76)	28.44 (27.5 - 29.39)	<0.0001
CCL3/MIP-1α	5.54 (4.59 - 6.49)	7.35 (6.32 - 8.38)	<0.0001
CCL4/MIP-1β	11.56 (9.97 - 13.13)	13.73 (12.23 - 15.24)	<0.0001
CCL13/MCP-4	2.03 (1.52 - 2.54)	2.99 (2.32 - 3.66)	<0.0001
IL-2	0.92 (0.53 - 1.30)	2.48 (1.59 - 3.37)	0.0013
TNF-α	9.60 (8.73 - 10.47)	11.40 (10.45 - 12.35)	0.0042
CCL2/MCP-1	22.26 (21.58 - 22.94)	23.14 (22.39 - 23.9)	0.0065
IL-12p70	1.89 (1.40 - 2.38)	2.61 (1.99 - 3.23)	0.0322
CCL22/MDC	5.86 (4.87 - 6.84)	6.59 (5.65 - 7.52)	0.0584
IL-10	28.98 (25.93 - 32.03)	30.2 (27.03 - 33.37)	0.0601
IL-1β	20.05 (19.11 - 20.99)	20.91 (19.96 - 21.86)	0.0658
CCL11/eotaxin-1	6.73 (5.78 - 7.68)	7.20 (6.27 - 8.13)	0.1442
CCL17/TARC	11.6 (10.11 - 13.08)	12.15 (10.6 - 13.7)	0.1349
IL-33^	6.281 (4.231 - 8.33)	7.202 (4.297 - 10.11)	0.1355
IL-4	2.74 (1.84 - 3.63)	3.36 (2.28 - 4.43)	0.1356
CXCL8/IL-8 High	6.08 (5.28 - 6.89)	6.47 (5.88 - 7.06)	0.2406
CXCL8/IL-8 Low	30.39 (29.69 - 31.08)	30.67 (30.18 - 31.16)	0.4637
IL-13	6.99 (5.97 - 8.02)	6.70 (5.61 - 7.80)	0.5108
IFN-β	3.08 (2.19 - 3.97)	3.309 (2.47 - 4.15)	0.5630
IL-6	14.60 (13.46 - 15.74)	14.39 (13.34 - 15.43)	0.7456
CCL26/eotaxin-3*	5.59 (4.14 - 7.03)	5.63 (4.06 - 7.21)	0.9093

Table 5.2 Comparison of nasal immune responses after saline and R848 (0.02 μg/kg/100 μL) AUC of $\log_{10}$ transformed values between 0-8 hours was initially calculated for each mediator in individual subjects after nasal challenge with saline and R848 (n=35). Subsequently, AUC values were compared between groups using a paired t-test. The baseline parameter for AUC was set at the lower limit of detection (LLOD) for each mediator. Samples prior to challenge were excluded, ^n=10, *n=33. AUC values expressed as mean (95% CI) and displayed in order of significance.

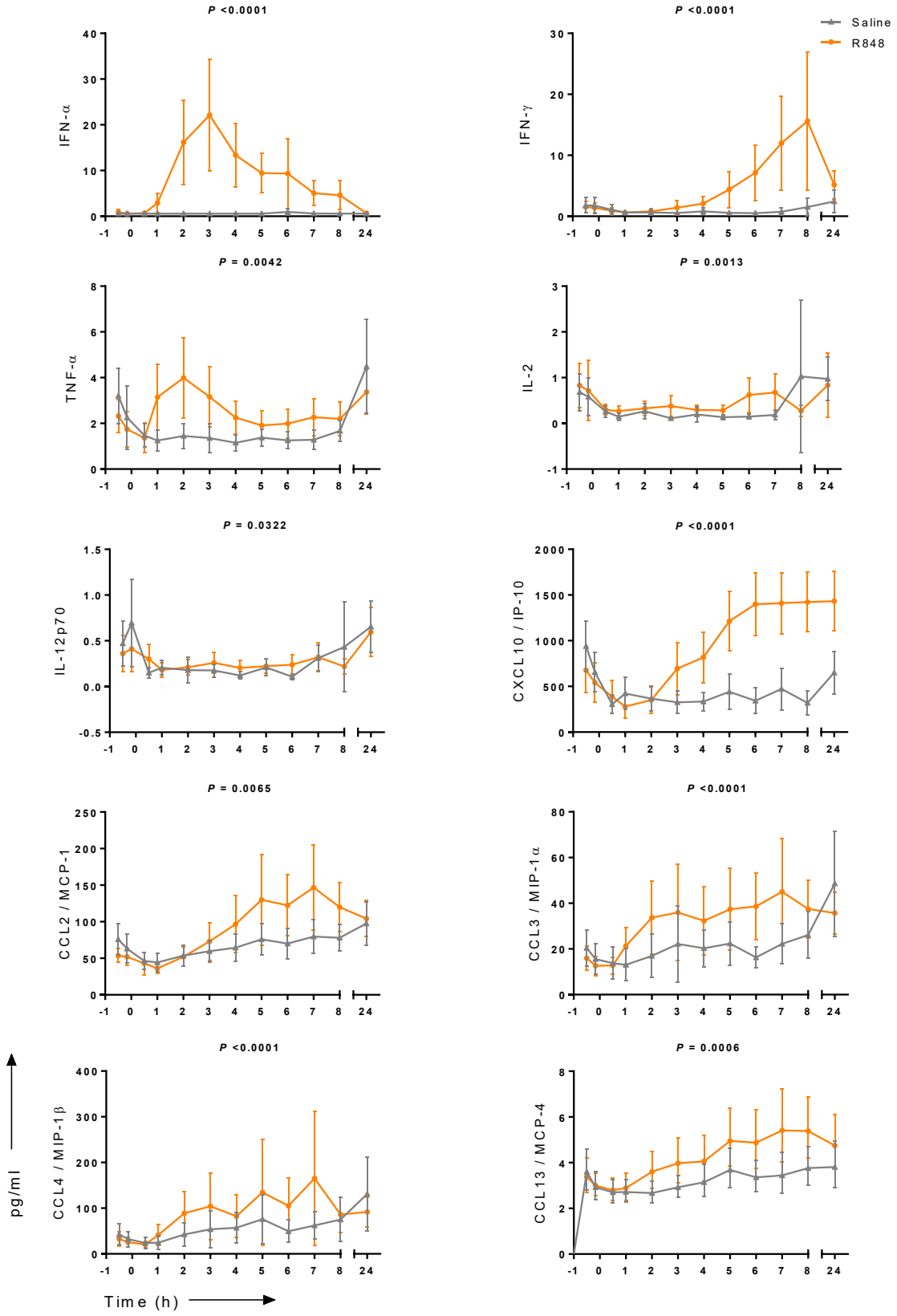


Figure 5.4 Nasal immune responses after saline and R848 (0.02 µg/kg/100 µL) Values expressed as mean with 95% CI, n=35 (healthy n=12, atopic n=12, allergic asthma n=11). AUC of log_{10} transformed values between 0-8h compared between groups using a paired t-test.

5.6.2 Group-specific responses

Nasal immune mediator responses were compared between groups (figures 5.5). This demonstrated that volunteers with atopy and asthma had enhanced IFN and chemokine responses after R848 administration throughout the period of sampling. With regards to IFN responses, volunteers with atopy and asthma had significantly greater IFN-α production at three hours, and earlier but non-statistically significant increases in IFN-γ and CXCL10 compared to healthy volunteers. Volunteers with atopy and asthma also had a remarkably similar and enhanced pattern of chemokine responses with early and sustained increases in CCL2, CCL3, CCL4, CCL11 and CCL13 for up to ten hours after challenge, with CCL13 being significantly higher than healthy volunteers at almost all time-points. The response in healthy volunteers was characterized by a relatively reduced magnitude of IFN and chemokine production but which also peaked at a later stage, with statistically higher levels at 24 h of IFN-γ (compared to volunteers with asthma) and IL-2 (compared to both volunteers with atopy and asthma). Whilst TNF-α and IL-6 levels were higher amongst volunteers with atopy and asthma, in general the proinflammatory response after R848 was less pronounced compared to the IFN and chemokine response. Analysis of IFN-α and IFN-γ levels after R848 at the level of individual volunteers confirms the distinct kinetics of IFN production (figure 5.6). IFN-α is produced in some volunteers within one hour of challenge, peaking at three hours with levels back to baseline in all volunteers by 24 h. In contrast, IFN-γ production begins at five hours in atopic and allergic asthma volunteers and from six hours in healthy volunteers, with a peak between nine to ten hours and reducing (but not returning to baseline) by 24 h. A heat map to compare the ten most significant mediators induced by R848 suggested individual variability in the pattern of the immune response (figure 5.7). Interestingly, eight volunteers (five with atopy and three with asthma) clustered together with strong IFN-α, CCL2, CCL3, CCL4, CCL13, IL-12p70 responses and whose nasal mucosa were therefore the most sensitive to the stimulant effects of R848. In contrast, the six volunteers who clustered together with the weakest responses to R848 consisted of four healthy volunteers, one with atopy and one with allergic asthma.

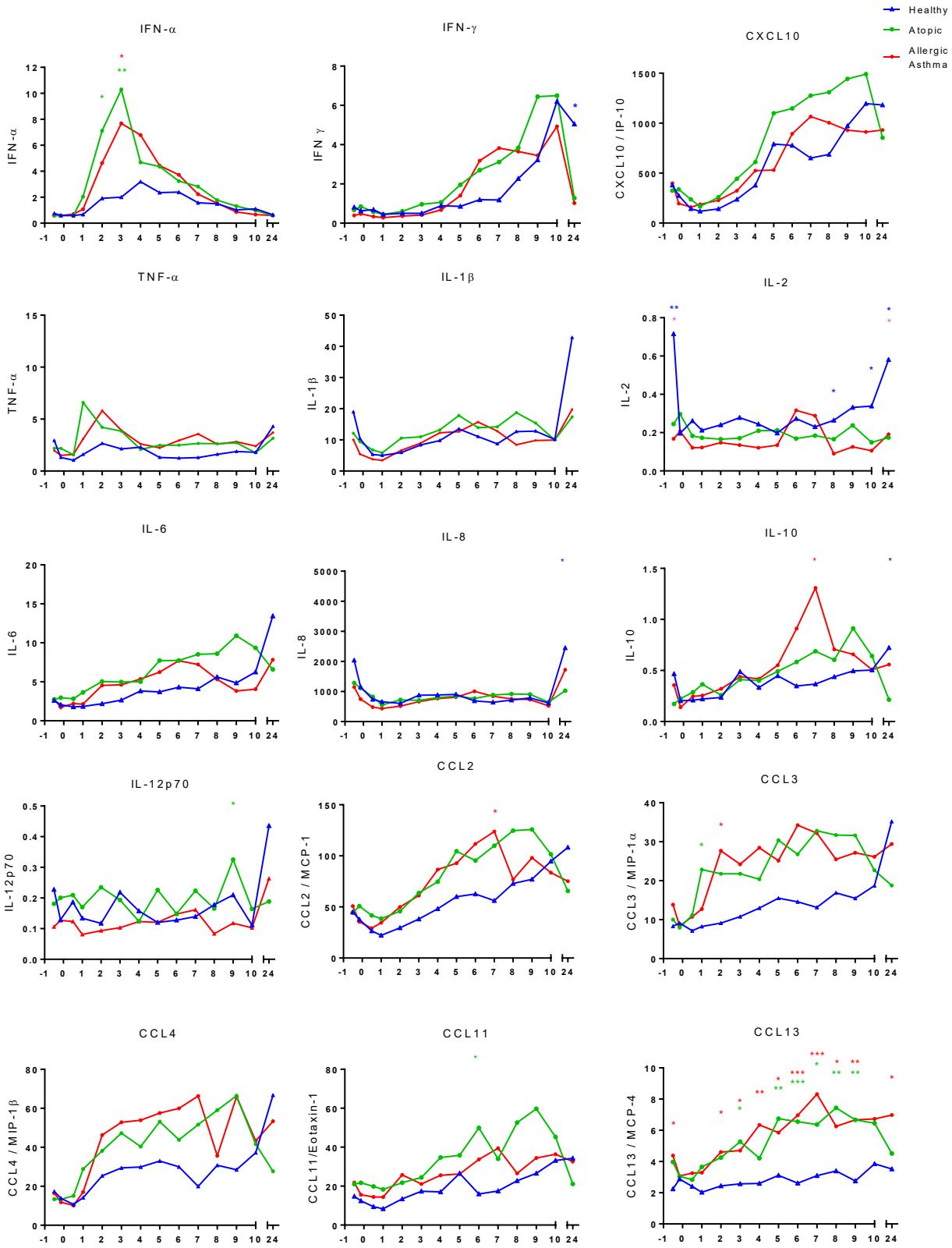


Figure 5.5 Group-specific nasal immune responses after R848 (0.02 µg/kg/100 µL) n=35 (healthy n=12, atopic n=12, allergic asthma n=11). Values expressed as geometric mean with two-way ANOVA comparison performed between groups on log_{10} transformed data at individual time points, *P<0.05, **P<0.01, **P<0.001.

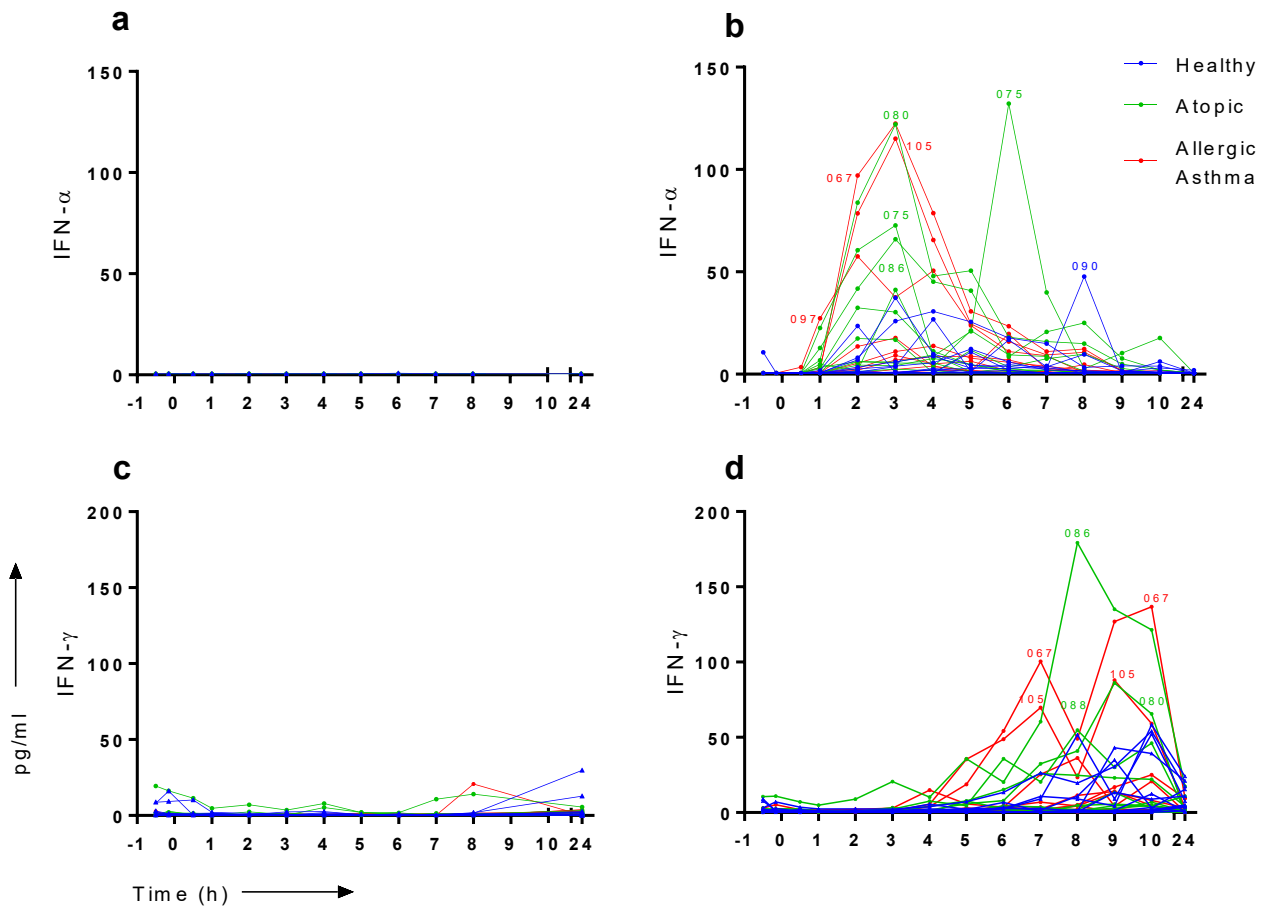


Figure 5.6 Individual nasal IFN responses after (a, c) saline and (b, d) R848 (0.02 µg/kg/100 µL) n=35 (healthy n=12, atopic n=12, allergic asthma n=11), with peak levels of highest responders annotated by subject ID.

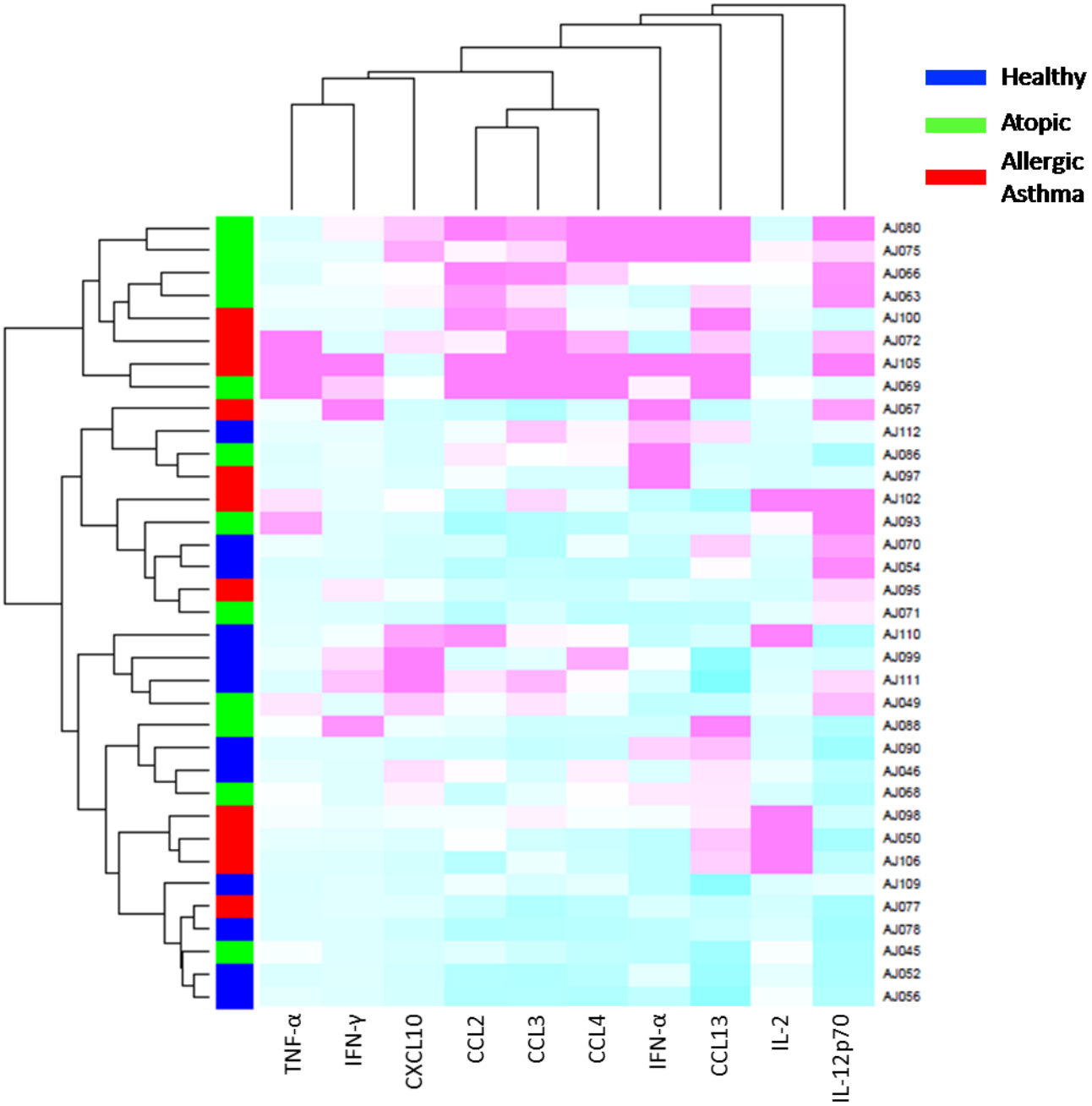


Figure 5.7 Nasal cytokine and chemokine heat map comparing subject groups after R848 Hierarchical clustering of ten cytokine and chemokine mediators significantly induced by R848 (AUC in comparison with saline control), with subject group identified in red-green-blue, using Spearman's correlation and clustered using complete linkage. Generated using R statistical analysis software (version 3.2.2).

5.6.3 Association between baseline atopy and mucosal immune response

To assess the association between markers of atopy and nasal immune responses to R848, mucosal IFN-α and IFN-γ were correlated with baseline blood eosinophils, serum total IgE, timothy grass skin prick diameter and methacholine PC_{20} in atopic and allergic asthma volunteers (Figure 5.8). This demonstrated that baseline blood eosinophil counts were significantly associated with mucosal IFN-α production (r 0.4295, *P* = 0.0408). No other markers of atopy were either positively or negatively correlated with IFN production. The inclusion criteria for this study had permitted atopic volunteers to have a positive methacholine test in the absence of clinical symptoms of asthma. The lack of any correlation between baseline methacholine PC_{20} and mucosal IFN response confirms that the inclusion of a mixed group of atopic volunteers (with and without positive methacholine PC_{20} responses) was not a confounding factor when comparing interferon responses between groups.

The impact of nasal, inhaled and oral corticosteroids on innate immune cell receptor expression and function has been characterized (302, 303). In this study out of 11 volunteers with allergic asthma, six were on inhaled corticosteroids (ICS) with the remaining five taking inhaled beta-agonists only, and no volunteers were taking nasal corticosteroids. The nasal immune response after R848 was compared between volunteers on ICS and those not on ICS (data not shown), and this revealed no significant differences in the induction of IFN- α, IFN γ, IL-4 or IL-13. This suggests that the inhaled route of corticosteroid delivery does not directly modulate nasal mucosal immune responses to R848, although this study was not powered to detect these differences. In contrast, nasal corticosteroid administration may be associated with an alteration in specific cell populations such as pDCs in the nasal mucosa (250), which may result in altered immune mediator production.

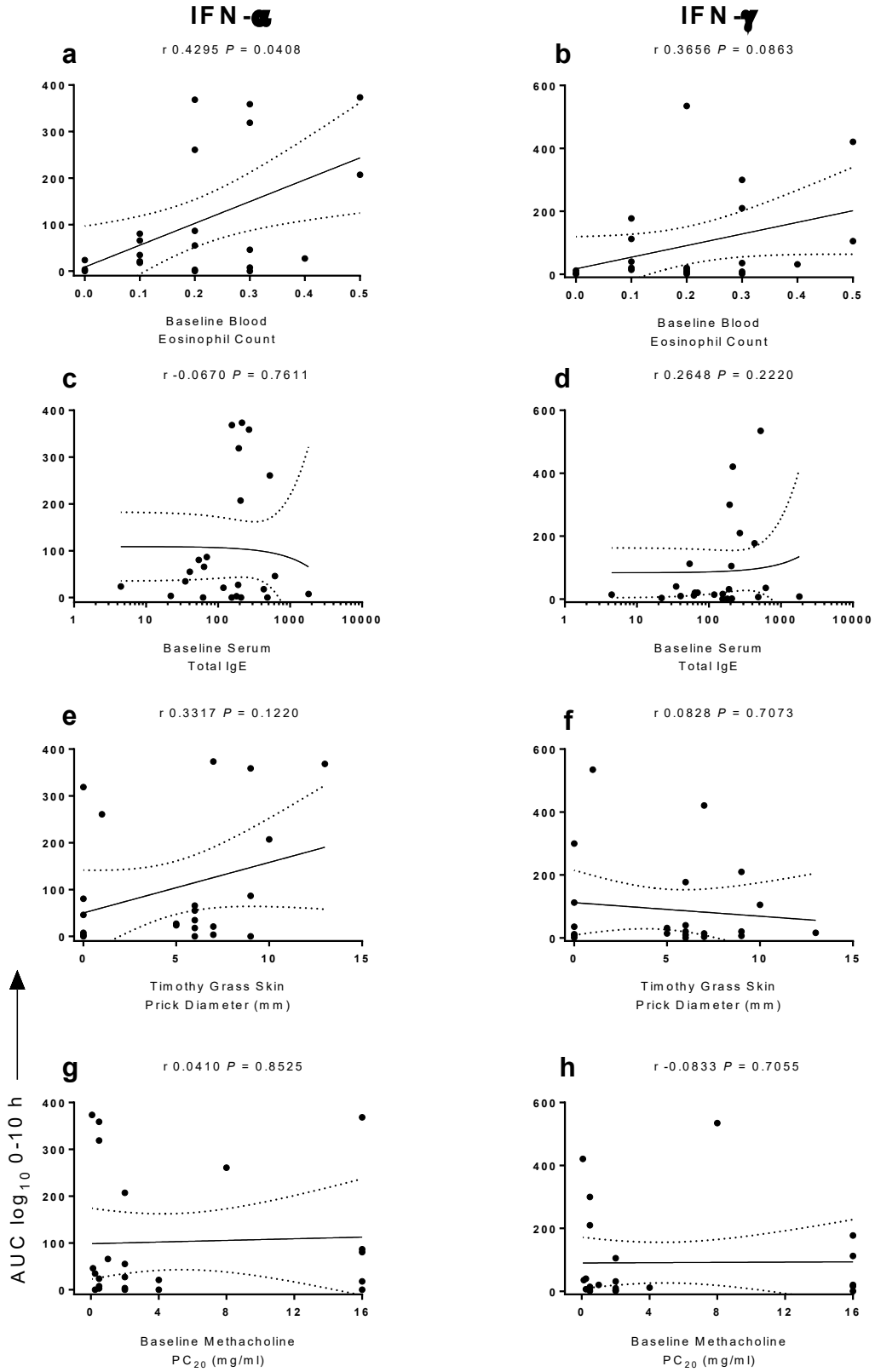


Figure 5.8 Association between baseline atopic features and mucosal IFN response to R848 Mucosal IFN-α and IFN-γ AUC values were correlated with baseline blood eosinophil counts (a, b respectively), baseline total serum IgE (c, d), timothy grass pollen skin prick diameter (e, f) and baseline methacholine PC_{20} values (g, h). Correlations are for volunteers with atopy and asthma only (n=23) and are non-parametric (Spearman's correlation).

5.6.4 Association between R848 dose and mucosal immune response

There were no significant differences between subject groups in the mean doses of R848 being administered nasally (table 5.1). However, the previous phase of the study using a significantly higher R848 dose of 10 µg, induced potent nasal IFN responses. To help determine whether nasal immune responses also varied with the very small differences in dosage between subjects in this latter phase of the study (due to the dose being based on subject weight at 0.02 µg/kg/100 µL), individual R848 doses were correlated with individual (AUC) nasal IFN-α and IFN-γ responses. This showed that R848 dosage (when calculated by body weight) was not associated with variation in IFN responses and was therefore not a confounding factor when comparing immune responses between different subject groups (figure 5.9).

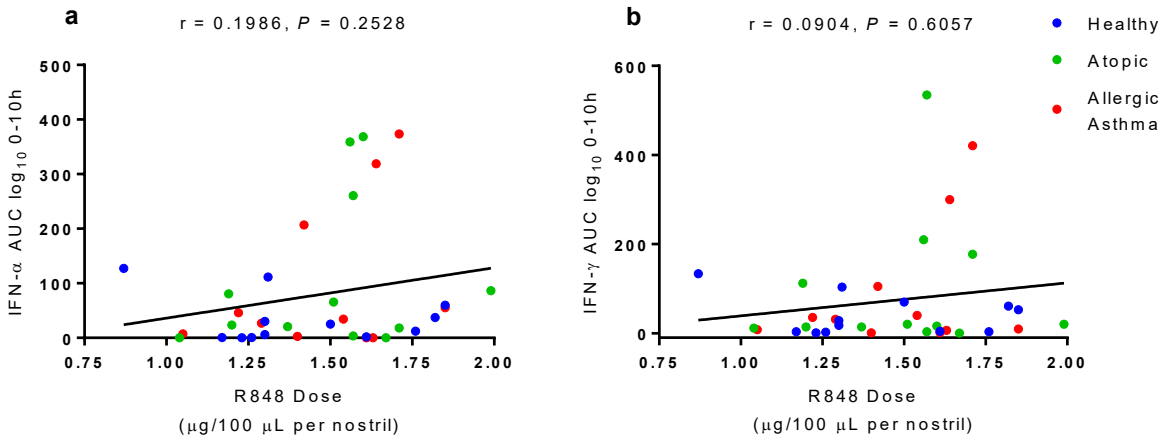


Figure 5.9 Association between nasal R848 administration dose and mucosal IFN response Mucosal (a) IFN-α and (b) IFN-γ AUC values were correlated with the R848 dose administered. Correlations are for all volunteers (healthy n=12, atopic n=12, allergic asthma n=11) and are non-parametric (Spearman's correlation).

5.7 Systemic immune response

5.7.1 Peripheral white cell differential response

A full blood count was collected at 0 h, 4 h, 8 h and 24 h after R848 challenge. Whole group data (figure 5.10) showed a significant reduction in lymphocyte counts at 24 h when compared to baseline (mean difference -0.3 $x10^9$/L, 95% CI -0.5 to -0.1, *P* = 0.0011). R848 caused a reduction in peripheral eosinophil counts at 4 h (-0.05 $x10^9$/L, 95% CI -0.08 to -0.02, *P* = 0.0012), 8 h (-0.04 $x10^9$/L, -0.075 to -0.005, *P* = 0.0229) and 24 h (-0.03 $x10^9$/L, -0.054 to -0.003, *P* = 0.0273). There were no differences at all time-points in neutrophils and total white cell counts.

Interestingly, group-specific analysis (figure 5.10) demonstrated that lymphopenia and eosinopenia after R848 was restricted to volunteers with atopy and allergic asthma with no significant differences in cell count being observed in healthy individuals (although there was a trend towards lymphopenia at 24 h). Compared to baseline, at 24 h there was a significant drop in lymphocytes in volunteers with atopy (mean difference 0.3 $x10^9$/L, 95% CI 0.05 to 0.59, *P* = 0.0219) and asthma (0.3 $x10^9$/L, 95% CI 0.05 to 0.50, *P* = 0.0147). Significant eosinopenia was also recorded at 4 h in volunteers with atopy (0.07 $x10^9$/L, 95% CI 0.005 to 0.129, *P* = 0.0329) and asthma (0.08 $x10^9$/L, 95% CI 0.15 to 0.149, *P* = 0.0142).

5.7.2 Systemic immune mediator response

Serum was collected at 0 h, 4 h, 8 h and 24 h after R848 challenge. An identical MSD assay panel to that used to assess nasal immune mediators was used to measure systemic immune mediator responses (figure 5.11). Whilst there was a significant reduction in serum CCL2 at all time-points and a reduction in IL-6 at 4 h compared to baseline, R848 did not cause an increase or decrease in the serum levels of any other IFNs, cytokines or chemokines.

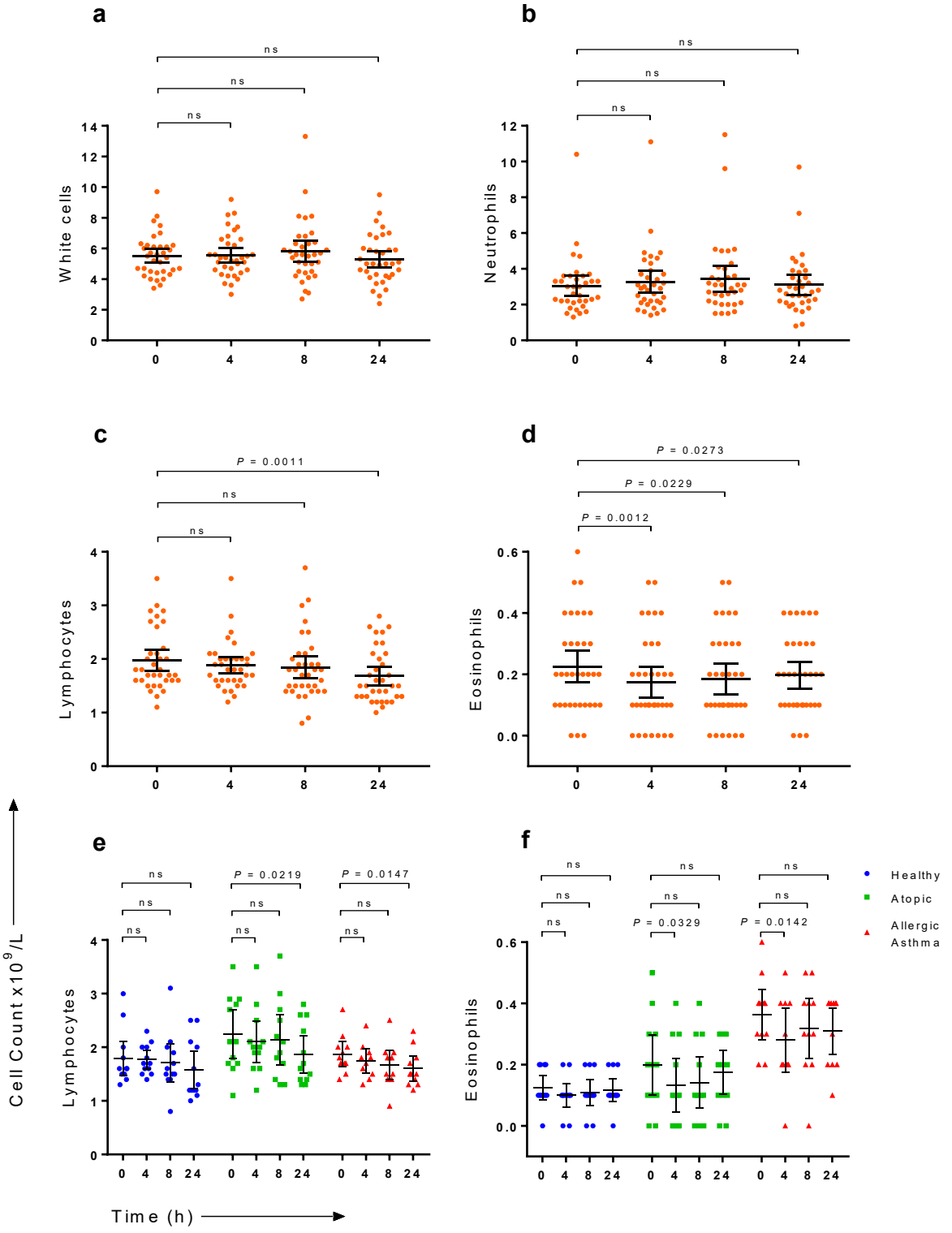


Figure 5.10 Differential peripheral white cell count after R848 (0.02 µg/kg/100 µL). Data shown for all volunteers (a-d; n=35) and by group (e, f; healthy n=12, atopic n=12, allergic asthma n=11). Error bars represent mean and 95% CI, comparison versus baseline by repeated measures one-way ANOVA and Dunnett's multiple comparison test.

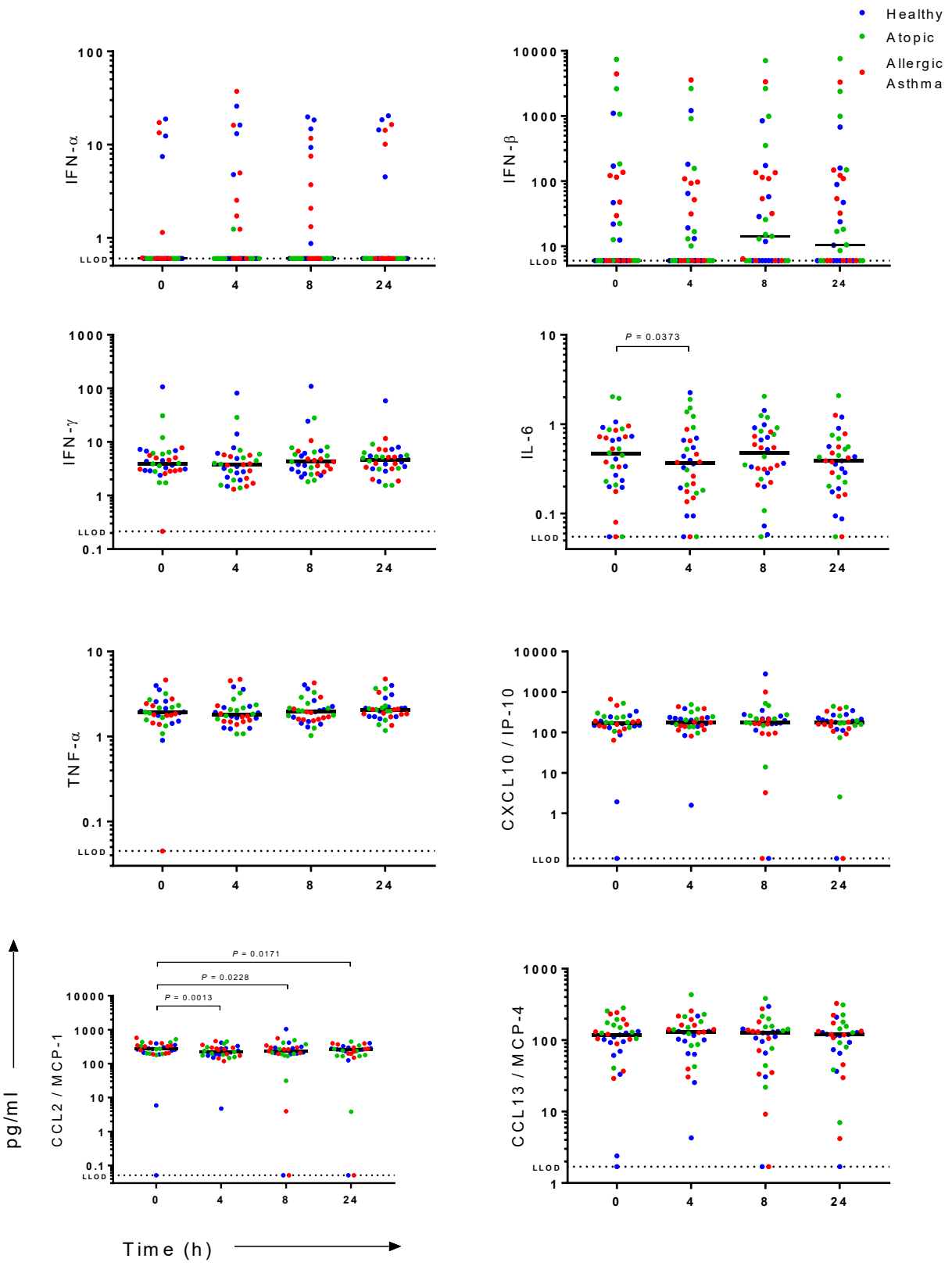


Figure 5.11 Systemic immune mediator response after R848 (0.02 µg/kg/100 µL) Error bar represents grand median, statistical analysis comparing all individuals to baseline values was performed using Friedman test and Dunn's multiple comparison, n=35. Abbreviations, LLOD lower limit of detection.

5.8 Nasal mucosal transcriptomics

5.8.1 Baseline differences

Expression analysis of 43 genes was performed on 70 nasal mucosal curettage samples collected eight hours after challenge (saline n=35, R848 n=35). Baseline ΔCT values after saline challenge were compared between groups (healthy n=12, atopic n=12, allergic asthma n=11) using the false discovery rate as determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 5% (figure 5.12). This revealed that only KRT5 and SCGB1A1 were differentially expressed between groups eight hours after saline challenge. KRT5 expression was reduced in volunteers with atopy compared to those with allergic asthma and those who were healthy (mean difference -0.89ΔCT, *P* = 0.0318 and -1.42ΔCT, *P* = 0.0008 respectively). SCGB1A1 had significantly reduced expression in individuals with atopy and allergic asthma compared to healthy volunteers (mean difference -3.84ΔCT, *P* <0.0001 and -4.66ΔCT, *P* <0.0001 respectively).

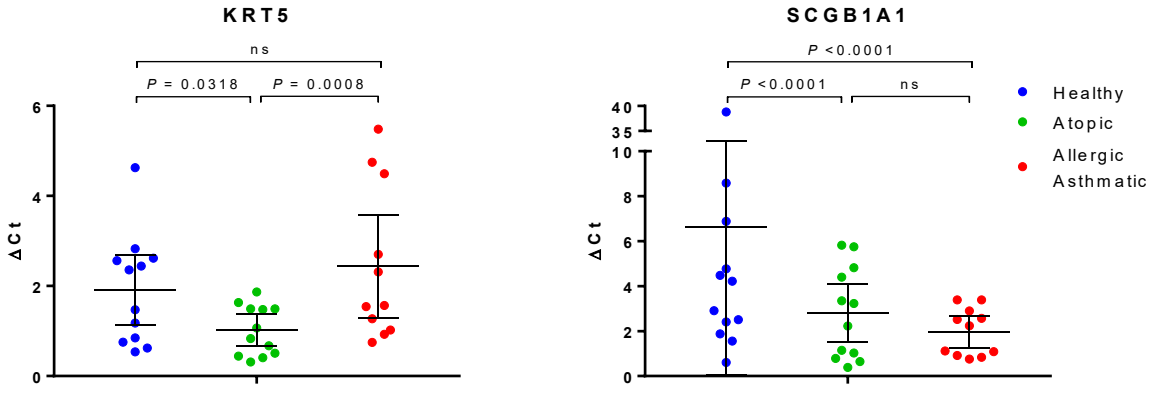


Figure 5.12 Differentially expressed genes between groups at baseline ΔCT values calculated from nasal mucosal samples taken eight hours after saline challenge. Comparison between groups (healthy n=12, atopic n-12, asthma n=11) using the false discovery rate as determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 5%, identified two differentially expressed genes. Error bars represent mean + 95% CI.

KRT5 is a cytokeratin gene that characterizes basal epithelial cells (191). Nasal expression of KRT5 has been associated with asthma status independently of atopy (122) and may explain why there was higher expression in volunteers with allergic asthma compared to those with atopy alone. To assess whether baseline nasal KRT5 expression was associated with subsequent immune activation by R848, baseline KRT5 expression was correlated with nasal immune mediators. This showed no correlation with any mediators in any subject group other than a weak negative correlation with IL-8 in volunteers with allergic asthma (r -0.65, P = 0.0368).

Secretoglobins are a family of proteins expressed by basal secretory cells, and SCGB1A1 (also known as CCSP, CC10 and CC16) is a nonmucous secretory cell-associated protein with anti-inflammatory and immunomodulatory properties (304). SCGB1A1 is a potent inhibitor of phospholipase A_2 (PLA_2), an important regulatory enzyme in the synthesis of prostaglandins and leukotrienes (305) and has *in vitro* activity against IFN-γ production and its ability to stimulate phagocytosis (306). The direct *in vivo* physiological role in humans is less well characterized but in an equine model, airway SCGB1A1 expression has been shown to reduce neutrophil chemotaxis (304). Levels of SCGB1A1 are significantly reduced in the BAL of patients with both refractory and non-refractory asthma compared to healthy controls (307, 308). It is reduced in individuals with asthma with a longer duration of disease (>10 years) (309) and genetic polymorphisms have been shown to be associated with airway hyperreactivity and asthma status (310, 311). SCGB1A1-positive epithelial cells expression is reduced in the small airways of subjects with asthma and is negatively correlated with numbers of T cells and mast cells but not eosinophils (309). Reduced SCGB1A1 protein levels have also been noted in nasal secretions from individuals with persistent allergic rhinitis (312). Reduced mRNA expression is associated with an increase in total numbers of infiltrating cells in the nasal mucosa and has been reported in sinonasal disease (313). It is worth noting that SCGB1A1 epithelial expression is likely to have local variation within the airways (314) and therefore nasal and lower airway mucosal expression

may differ. This study confirms the finding that at baseline volunteers with atopy and allergic asthma express lower levels of nasal mucosal SCGB1A1 compared to healthy volunteers.

To test the hypothesis of whether reduced nasal mucosal SCGB1A1 gene expression is associated with increased immune activation, baseline expression was assessed for its relationship with nasal immune mediators after R848 challenge (table 5.3). This demonstrated that in volunteers with atopy, SCG1AB1 expression had a strong negative correlation with R848-induced production of IFN-γ, IL-4, CCL2, CCL3, CCL4, CCL17 and CCL22. In particular, SCGB1A1 was very strongly negatively correlated with CCL3 (*r* -0.9161, 95% CI -0.9774 to -0.7121, *P* <0.0001) and CCL17 ((*r* -0.8182, 95% CI --0.9492 to -0.4452, *P* <0.0019). In contrast, healthy volunteers had a positive correlation between baseline SCG1AB1 expression and IL-2 and CCL22 levels, whilst there was no relationship between SCG1AB1 expression and immune activation in volunteers with allergic asthma. Therefore, whilst baseline SCGB1A1 is reduced in volunteers with atopy and allergic asthma relative to healthy volunteers, a negative correlation with innate immune activation was only seen in those with atopy alone.

Immune Mediator	Healthy n=12		Atopy n=12		Allergic Asthma n=11	
	Correlation *R*	*P* Value	Correlation *R*	*P* Value	Correlation *R*	*P* Value
IFN-α	0.5594	0.0628	-0.5315	0.0794	-0.0182	0.9618
IFN-β	-0.2817	0.3745	0.3287	0.2973	0.2000	0.5574
IFN-γ	0.5594	0.0628	**-0.6783**	**0.0185**	-0.0818	0.8179
CXCL10/IP-10	0.1888	0.5577	-0.5455	0.0708	0.0364	0.9241
TNF-α	0.3706	0.2367	0.3986	0.2010	0.2000	0.5574
IL-1β	-0.3007	0.3424	-0.3147	0.3194	0.0091	0.9895
IL-2	**0.7273**	**0.0096**	-0.1049	0.7493	-0.3303	0.3176
IL-4	0.1261	0.1261	**-0.6573**	**0.0238**	0.4000	0.2250
IL-6	-0.0840	0.8004	-0.3706	0.2367	0.3273	0.3269
IL-8	-0.0559	0.8692	-0.09091	0.7830	-0.1909	0.5765
IL-10	0.5594	0.0628	-0.6853	**0.0170**	0.0546	0.8812
IL-12p70	-0.2168	0.4990	-0.4755	0.1215	0.5513	0.0828
IL-13	-0.0490	0.8861	-0.3566	0.2560	0.3636	0.2731
CCL2/MCP-1	0.3147	0.3194	**-0.7343**	**0.0087**	-0.0634	0.8603
CCL3/MIP-1α	0.3497	0.2662	**-0.9161**	**<0.0001**	-0.0546	0.8812
CCL4/MIP-1β	0.4615	0.1340	**-0.6154**	**0.0373**	-0.1818	0.5950
CCL11/eotaxin-1	0.3217	0.3085	-0.4545	0.1404	-0.1686	0.6185
CCL13/MCP-4	-0.2517	0.4303	-0.5315	0.0794	0.1091	0.7545
CCL17/TARC	0.5315	0.0794	**-0.8182**	**0.0019**	0.0727	0.8385
CCL22/MDC	**0.7622**	**0.0055**	**-0.7203**	**0.0106**	0.0364	0.9241
CCL26/eotaxin-3*	0.5035	0.0989	-0.2657	0.4042	0.2167	0.5809

Table 5.3 Correlation between baseline nasal mucosal SCG1AB1 gene expression and nasal immune response to R848 Baseline SCG1AB1 expression correlated with AUC of log_{10} transformed immune mediator values 0-10 hours after R848 using Spearmans correlation. Statistically significant values highlighted in red, *n=33.

5.8.2 Saline versus R848

Differentially expressed genes between groups after R848 challenge versus saline (ΔΔCT) were compared using the same statistical approach described above (table 5.4). This identified 14 genes that were differentially expressed after R848 with upregulation of IFIT3, OAS2, IRF7, MX1, MYD88, DDX58, STAT1, SOCS1, TLR3, TLR7, KRT5 and CLEC4C, and downregulation of IFNAR1 and ADGRG1.

This confirmed along with the immune mediator data that R848 potently induces IFNs and ISGs. Whilst several ISGs have been discovered, only a few have had their antiviral activity characterized. The majority of genes upregulated by R848 are ISGs that are known to have a critical role in type 1 IFN pathways and play a key role in anti-viral immunity (16, 144). IFIT3, MX1 and OAS2 are upregulated In the acute phase after influenza infection (256). IFIT proteins have been shown to recognise viral RNA and inhibit viral translation and replication (249). OAS2 is part of a family of four OAS genes that act to cleave and degrade viral RNA in the cytosol (315). The transcription factors IRF7 and STAT1 are known to form a central axis of transcriptional regulation after influenza infection (316). IRF7 rather than IRF3 drives the IFN response after influenza A infection in airway epithelia (317) and an *in vivo* study of natural influenza infection in humans revealed that inherited IRF7 deficiency can lead to susceptibility to severe influenza infection (53). IRF7 production after HRV infection of PBMCs has shown to be highly dependent on pDCs (318).

MXI gene encodes MxA protein that is localised in the cytoplasm and is predominantly produced by Type I and III IFNs (319). Monocytes from older adults demonstrate reduced MxA expression after infection with IAV compared to monocytes from younger individuals, and a congenic mouse model suggests that this age-related defect in innate immunity is likely to enhance susceptibility to secondary bacterial infection in older individuals (320). MyD88 is a constitutively expressed adapter protein for all TLRs except TLR3 and deficiency leads to abolishing of most TLR mediated signalling in leukocytes and fibroblasts (321). MyD88 deficiency in humans can lead to delayed CRP induction and fever (322), but susceptibility to infection is restricted to a narrow range of pyogenic bacteria with normal

resistance to viruses (323). SOCS1 expression is known to be rapidly upregulated by type 1 IFNs to enhance inhibitory feedback by reducing IFNAR1 surface expression, and its production is only maintained with continuously high levels of IFN-α (17, 324). The induction of TLR3 and DDX58 (which codes for the cytosolic nucleic sensor RIG-I) suggests that whilst R848 acts as a ssRNA analogue, it enhances generalized cellular responsiveness to viruses including those composed of dsRNA.

CLEC4C (also known as BDCA2) is a C-type lectin uniquely expressed on pDCs (28). pDCs secrete large amounts of type 1 IFNs to generate an antiviral state in infected and bystander cells, activate NK cells and promote a host of adaptive immune responses (28). Its expression in nasal mucosal biopsy tissue has also been shown to be altered depending on the atopic status of individuals along with the presence of acute and chronic inflammation (250–252, 325). The upregulation of CLEC4C suggests that R848 induces pDCs in the nasal mucosa, which can be detected at eight hours and may provide a mechanism for the increased production of nasal IFN-α.

ADGRG1 (also known as GPR56) is a marker for NK cells that is known to be upregulated after acute influenza infection (256). Given that NK cells are a well-known source of IFN-γ (326), a mediator that peaked at eight hours after R848, and at which point the nasal curettage samples were taken, it was surprising to note a downregulation of this gene. This finding would need further investigation by enumerating NK cells in the nasal mucosa but it is pertinent to note that there were no changes in the other NK cell-associated markers KLRD1 and NCAM1. The downregulation of IFNAR eight hours after R848, and several hours after the initial induction of IFN-α, demonstrates an appropriate negative feedback mechanism to regulate IFN responses.

Gene	*P*-value	Saline ΔCT (Mean)	R848 ΔCT (Mean)	Difference	Standard error of difference	*Q*-value	Upregulation or downregulation by R848?
IFIT3	<0.0001	0.3678	3.5640	3.1960	0.6411	<0.0001	Upregulation
OAS2	<0.0001	0.1473	0.6393	0.4920	0.1005	<0.0001	Upregulation
IRF7	<0.0001	0.0597	0.2950	0.2353	0.0484	<0.0001	Upregulation
MX1	<0.0001	0.7182	3.9400	3.2220	0.6654	<0.0001	Upregulation
MYD88	<0.0001	0.2729	0.4383	0.1654	0.0361	0.0001	Upregulation
DDX58	<0.0001	0.1127	0.8995	0.7868	0.1884	0.0005	Upregulation
STAT1	0.0001	0.9773	2.2450	1.2670	0.3087	0.0005	Upregulation
IFNAR1	0.0008	0.4778	0.3723	-0.1055	0.0299	0.0030	Downregulation
ADGRG1	0.0009	0.6089	0.4720	-0.1369	0.0392	0.0030	Downregulation
SOCS1	0.0010	0.0117	0.0764	0.0647	0.0189	0.0031	Upregulation
TLR3	0.0011	0.0841	0.1657	0.0816	0.0239	0.0031	Upregulation
TLR7	0.0022	0.0087	0.0147	0.0061	0.0019	0.0057	Upregulation
KRT5	0.0067	1.7680	2.9550	1.1870	0.4246	0.0158	Upregulation
CLEC4C	0.0071	0.0025	0.0053	0.0028	0.0010	0.0158	Upregulation
MME	0.1278	0.1039	0.0685	-0.0354	0.0230	0.2667	N/A
IRF3	0.1366	0.1659	0.1484	-0.0176	0.0117	0.2674	N/A
CXCR2	0.1524	0.0871	0.0500	-0.0371	0.0256	0.2710	N/A
MPO	0.1558	0.0004	0.0006	0.0002	0.0001	0.2710	N/A
MMP12	0.1959	0.0167	0.0126	-0.0041	0.0032	0.2928	N/A
LGALS12	0.1977	0.0035	0.0025	-0.0010	0.0008	0.2928	N/A
CLCA1	0.2115	0.0741	0.0537	-0.0204	0.0162	0.2928	N/A
POSTN	0.2204	0.0712	0.0271	-0.0441	0.0357	0.2928	N/A
SIGLEC8	0.2262	0.0005	0.0003	-0.0002	0.0002	0.2928	N/A
KLRD1	0.2325	0.0152	0.0193	0.0041	0.0034	0.2928	N/A
IL33	0.2405	0.3017	0.3600	0.0583	0.0492	0.2928	N/A
TLR8	0.2450	0.0487	0.0351	-0.0135	0.0115	0.2928	N/A
NFKB1	0.2525	0.2710	0.2897	0.0187	0.0162	0.2928	N/A
FCER1A	0.2686	0.0011	0.0002	-0.0009	0.0008	0.2971	N/A
CHIT1	0.2752	0.0004	0.0001	-0.0003	0.0003	0.2971	N/A
TSLP	0.2864	0.0168	0.0211	0.0043	0.0040	0.2989	N/A
FOXJ1	0.3055	0.6028	0.4483	-0.1545	0.1496	0.3076	N/A
NRP1	0.3144	0.0950	0.0632	-0.0318	0.0314	0.3076	N/A
CD1C	0.4565	0.0768	0.0681	-0.0087	0.0116	0.4331	N/A
IRAK3	0.5029	0.3536	0.3275	-0.0261	0.0388	0.4632	N/A
CD163	0.5334	0.0911	0.0809	-0.0103	0.0164	0.4771	N/A
SCGB1A1	0.6157	3.8540	3.2650	-0.5887	1.1670	0.5222	N/A
CLC	0.6171	0.0178	0.0135	-0.0043	0.0086	0.5222	N/A
TNFAIP3	0.6484	0.4285	0.4054	-0.0231	0.0504	0.5343	N/A
IL25	0.6977	0.0007	0.0006	-0.0001	0.0003	0.5601	N/A
NCAM1	0.8180	0.0008	0.0008	0.0000	0.0002	0.6146	N/A
THBD	0.8196	0.0799	0.0770	-0.0029	0.0127	0.6146	N/A
KIT	0.8244	0.1209	0.1169	-0.0039	0.0176	0.6146	N/A
TPSAB1	0.9481	0.0714	0.0731	0.0018	0.0272	0.6904	N/A

Table 5.4 Comparison of nasal curettage gene expression after saline and R848 43 genes analysed from 35 volunteers, eight hours after saline and R848 challenge. ΔCT values compared using false discovery rate determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 5%. Each row analysed individually, without assuming a consistent SD. Values displayed in order of significance.

5.8.3 Group-specific differences

Subsequently, ΔΔCT values were compared between groups using the same statistical approach described above, which identified three key ISGs (IFIT3, DDX58 and MX1) that were differentially expressed between groups after R848 versus saline challenge (figure 5.13). Compared to healthy individuals and those with atopy, volunteers with allergic asthma had enhanced expression of IFIT3 (mean difference 10.45ΔΔCT, *P* = 0.0002 and 14.82ΔΔCT, *P* <0.0001 respectively), DDX58 (mean difference 8.26ΔΔCT, *P* = 0.0033 and 7.53ΔΔCT, *P* = 0.0073 respectively) and MX1 (mean difference 6.93ΔΔCT, *P* = 0.0135 and 7.39ΔΔCT, *P* = 0.0084 respectively). This therefore demonstrated that subjects with allergic asthma had exaggerated ISG responses after R848 compared to those without asthma.

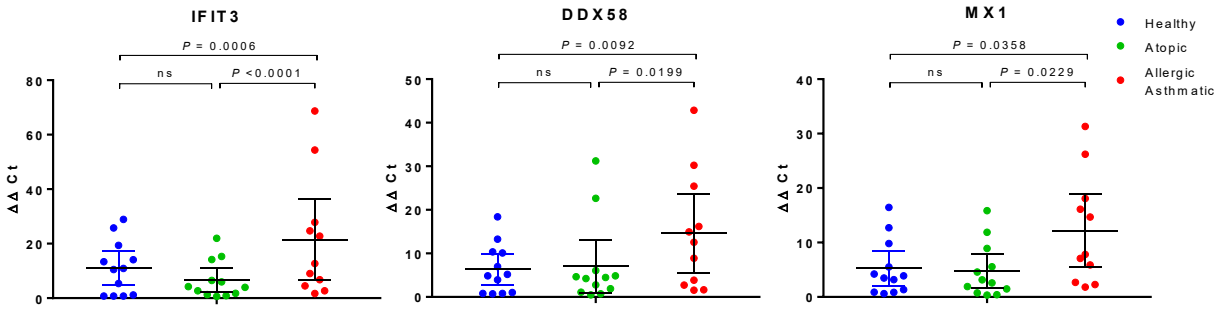


Figure 5.13 Group comparison of differentially expressed genes induced by R848 ΔΔCT values calculated for R848 compared to saline. Comparison of ΔΔCT between groups (healthy n=12, atopic n-12, asthma n=11) using the false discovery rate as determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 5%, identified three differentially expressed genes. Error bars represent mean + 95% CI.

5.8.4 Combined protein and gene expression analysis

The most significant immune mediators and differentially expressed genes were then assessed in combination to investigate whether there are correlated patterns of induction of potential immune signalling pathways after R848 challenge (figure 5.14). This demonstrated that the ISGs strongly correlated with each other, especially IFIT3, DDX58 and IRF7 (r >0.95). Although having differing kinetics, IFN-α and IFN-γ induction correlated with each other. Additionally, a strong positive correlation was seen between the chemokines CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β) and CXCL10 (IP-10). CCL3 additionally correlated with IFN-α and IFN-γ production. An especially interesting finding was the identification that CCL13 (MCP-4) positively correlated with IFIT3, DDX58 and IRF7, highlighting a potentially key role for CCL13 in the induction of ISGs.

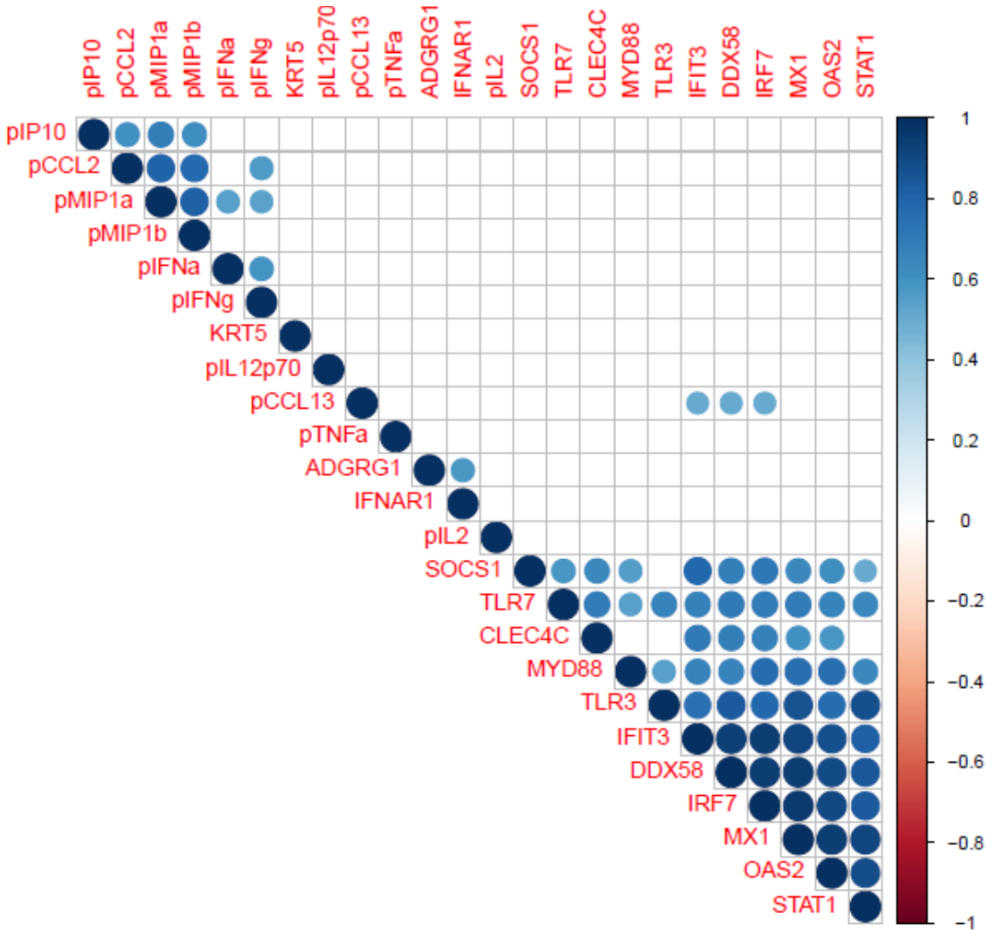


Figure 5.14 Correlogram of nasal mediators and mucosal gene expression after R848 challenge Blank squares denote insignificant (P > .05) correlations, and colour denotes Spearman *R* value. Proteins denoted by a preceding letter "p".

5.9 Nasal mucosal immunohistology

Nasal curettage samples were also collected for immunohistology. These represented fragile and very small cross-sections of tissue that were fixed in minimal volumes of paraformaldehyde, which required careful handling to minimise cell dispersal. Although virtually all samples collected contained epithelial cells, the architecture of the nasal mucosal tissue did not always survive the processing techniques and ranged from suboptimal to excellent quality, with most samples being either good or excellent (figures 5.15a-c). Samples taken from mixed saline and R848 challenges were then labelled with anti-TLR7, anti-TLR8 and anti-IFN-γ antibodies (figure 5.16). This demonstrated the ability to identify intraepithelial expression of proteins in very small tissue specimens obtained using nasal curettage.

Given the significant induction of IFN-γ after R848 in nasal mucosal fluid between four and eight hours, nasal curettage samples taken eight hours after saline and R848 challenge were compared for tissue expression of IFN-γ, which showed a generalised background increase in expression in the epithelium (figure 5.17). The immunostaining intensity for IFN-γ on nasal epithelium was semi-quantitatively scored in a blinded manner and compared eight hours after saline and R848, which confirmed a quantitative increase in IFN-γ producing cells after R848 (figure 5.18). There were insufficient samples available for immunohistology to allow comparison of different subject groups. Epithelial cells are a well-characterized source of both type I and III IFNs (6, 93), whilst IFN-γ production is typically mediated by NK and NKT cells (326). It was not possible to clearly identify the cellular sources of IFN-γ production, which may have been due to several possibilities including (i) the technique of nasal curettage obtaining cells predominantly from the epithelial layer rather than the underlying lamina propria, (ii) the reduced frequency of IFN-γ producing cells relative to the epithelial cells and (iii) difficulty for the cells in surviving the fixation and embedding process. Cell-specific markers could be utilised for better characterization in future studies and flow cytometry offers a potentially superior technique to quantify and phenotype cells from nasal curettage tissue (75, 138).

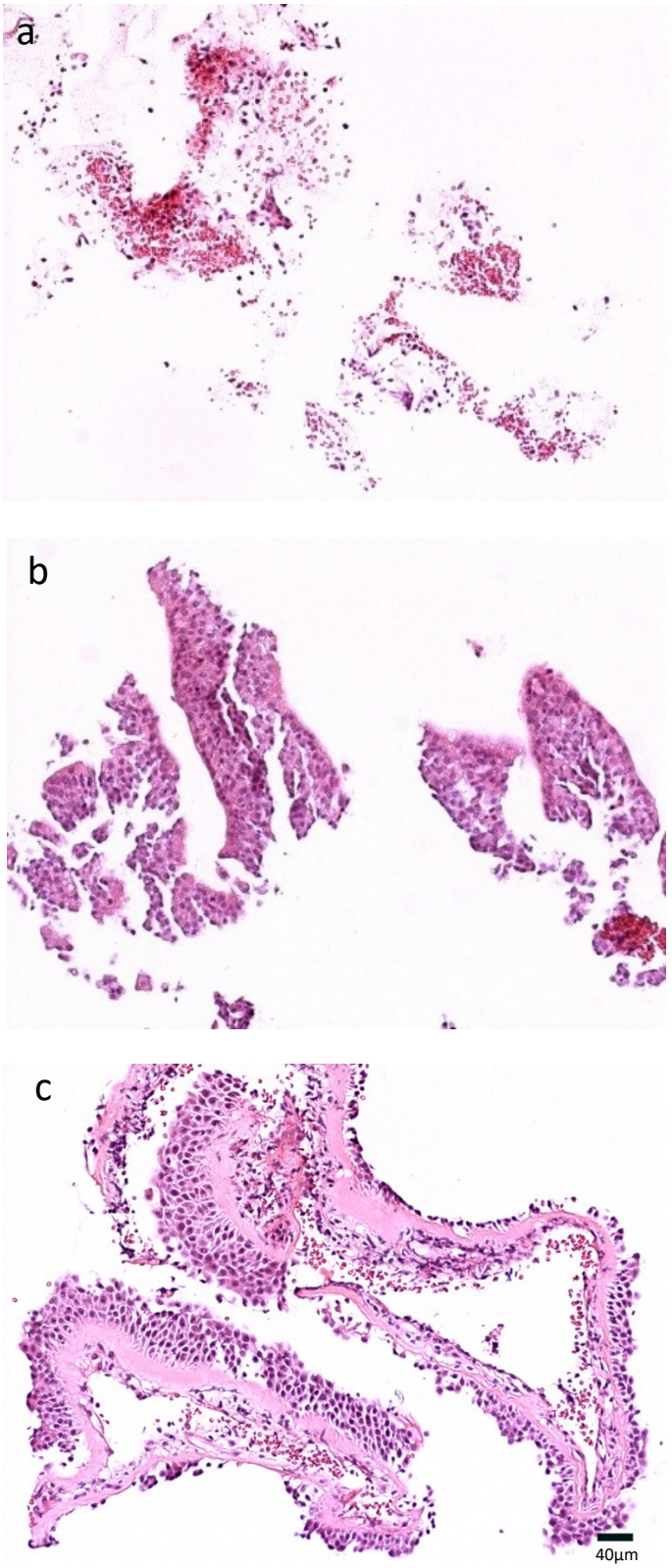


Figure 5.15 Quality of nasal mucosal tissue samples available for histology After fixation, embedding and H&E staining, the quality of samples ranged from (a) suboptimal, (b) good to (c) excellent with most samples being either good to excellent.

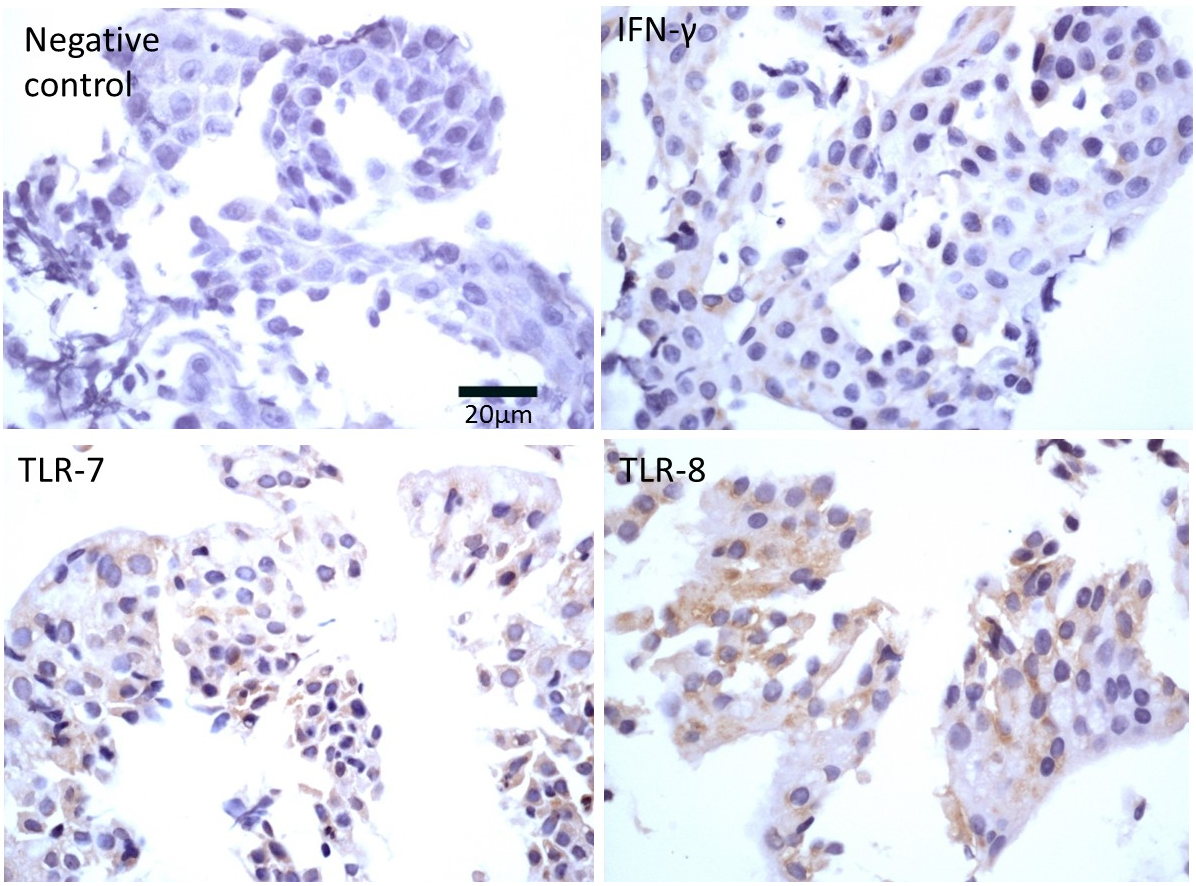


Figure 5.16 Immunohistology staining for IFN-γ, TLR7 and TLR8 in nasal curettage samples The paraffin sections of nasal curettage were dewaxed, incubated with peroxidase blocking solution and then incubated overnight at 4°C with either non-specific rabbit IgG polyclonal-isotype control, anti-IFN-γ, anti-TLR7 or anti-TLR8 monoclonal antibody. The sections were incubated with EnVision System-HRP labelled polymer goat anti-rabbit secondary antibody solution for 30 minutes. After washing, sections were incubated with chromogen diaminobenzidine (DAB) liquid and peroxide buffer and stained antigen sites were detected as a brown product. Representative samples from mixed saline and R848 challenge.

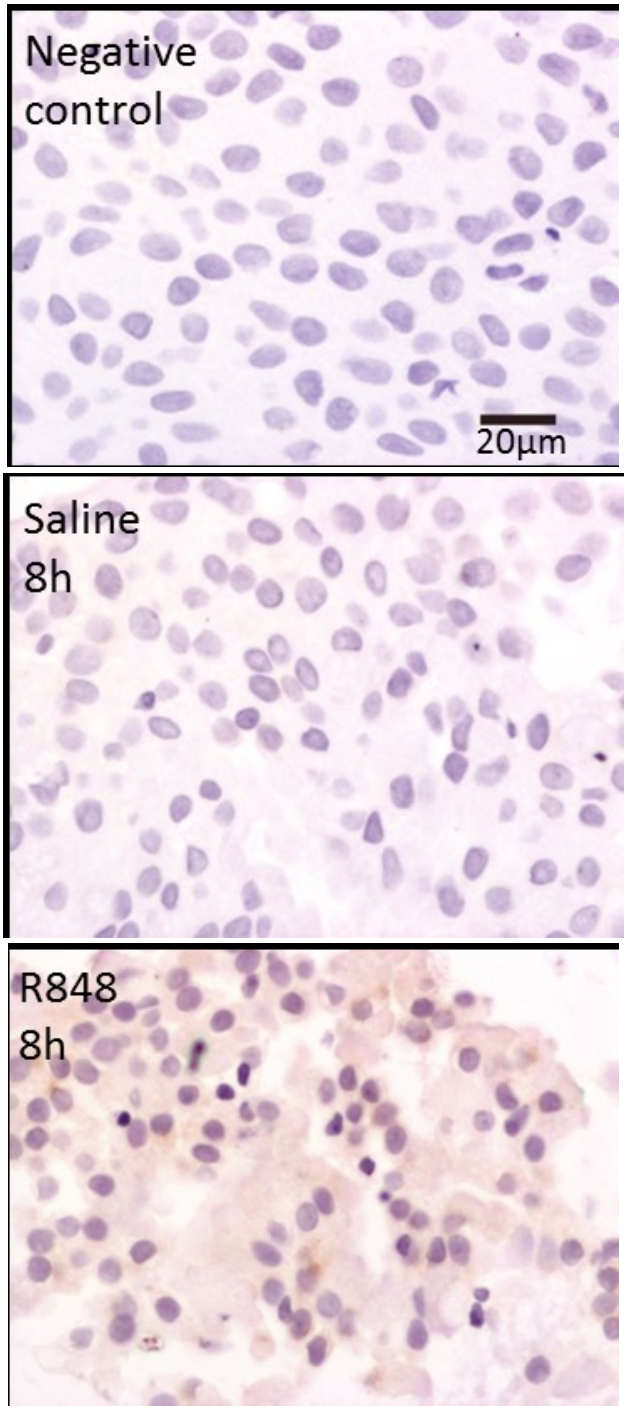


Figure 5.17 Immunohistology staining for IFN-γ in nasal curettage samples after saline and R848 The paraffin sections of nasal curettage were dewaxed, incubated with peroxidase blocking solution and then incubated overnight at 4°C with either non-specific rabbit IgG polyclonal-isotype control or anti-IFN-γ monoclonal antibody. The sections were incubated with EnVision System-HRP labelled polymer goat anti-rabbit secondary antibody solution for 30 minutes. After washing, sections were incubated with chromogen diaminobenzidine (DAB) liquid and peroxide buffer and stained antigen sites were detected as a brown product.

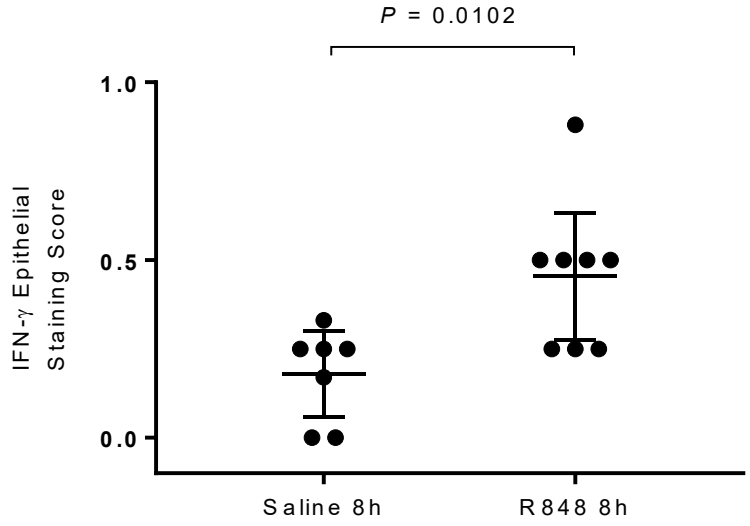


Figure 5.18 Comparison of IFN-γ epithelial staining scores The immunostaining intensity for IFN-γ on nasal epithelium was semi-quantitatively scored in a blinded manner ranging from 0-3 (0-negative, 1-weak, 2-moderate and 3-strong staining. Several fields at 400x magnification were scored to cover all epithelial areas from each biopsy. An average of scores was taken to represent entire epithelial staining intensity of two to three biopsies from each subject. Statistical comparison using paired t-test. Error bars represent mean and 95% CI.

5.10 Discussion

Nasal R848 administration at a mean dose of 1.5µg/100 µL/nostril was well tolerated by all volunteers without significant symptoms. It did not lead to any significant increase in local nasal or systemic clinical responses and did not cause detectable systemic effects on blood biomarkers of inflammation. R848 induced a nasal mucosal lining fluid IFN response with distinct kinetics: an early rise in IFN-α (peak 3 h) and a later rise in IFN-γ and CXCL10 (peak 8-10 h). It caused minimal proinflammatory IL-1β, IL-12p70 and TNF-α responses, but led to significant production of the chemokines CCL2, CCL3, CCL4 and CCL13. Nasal R848 challenge led to an early (4 h) decrease in peripheral eosinophil count and a later (24 h) decrease in lymphocytes that was restricted to volunteers with allergic rhinitis and allergic asthma. Baseline eosinophils in volunteers with atopy and allergic asthma correlated positively with mucosal IFN-α production. There was significantly lower expression of the anti-inflammatory secretoglobin gene SCGB1A1 in volunteers with atopy and asthma relative to healthy volunteers, and in volunteers with atopy this was strongly negatively correlated with subsequent R848-induced production of several immune mediators, and in particular CCL3. R848 induced several ISGs that are known to have a critical role in type 1 IFN pathways and play a key role in anti-viral immunity (16, 144). Specifically, R848 led to enhanced mucosal ISG expression (IFIT3, DDX58 and MX1) in volunteers with allergic asthma relative to those who were healthy or had atopy. These results indicate that nasal R848 challenge is a safe method of inducing mucosal innate immune responses and that individuals with allergic rhinitis and allergic asthma have a heightened sensitivity to TLR7/8 agonists, which leads to significantly enhanced innate immune activation relative to healthy non-atopic subjects.

The rapid production of IFN-α in the nasal mucosa within one hour of R848 challenge (figure 5.6) suggests the presence of resident epithelial and immune cells that are responsible for its production. Whilst TLR7 is weakly expressed on the apical surface of human tracheal mucosa, the predominant IFN that is produced by epithelial cells in response to TLR3 but not TLR7 agonists is IFN-λ (93), a

mediator that was undetectable in the current study. In contrast, pDCs possess TLR7 and have been reported to produce 200-1000 times more IFN-α than other cells after viral stimulation (327), effecting a profound impact on immune responses despite constituting only 0.4% of total PBMCs (318). The later production of IFN-γ, beginning after five hours (figure 5.6) suggests that its source may be from immune cells that migrate either from the lamina propria or the peripheral circulation to the mucosa. IFN-γ production is typically mediated by NK and NKT cells as part of the innate immune response and CD4 Th1 and CD8 cytotoxic T-lymphocyte effector cells as part of the antigen-specific immune response and has pleotropic effects (85, 326).

The role of interferons in determining susceptibility to viral infections and in particular to HRV has been extensively studied but with conflicting results (85). Interferon deficiency has been described in cultured bronchial epithelial cells from individuals with asthma following HRV infection (86–88) although this finding has not always been reproducible (328), and robust IFN-γ and IFN-λ responses have been found in children with asthma (89, 90). PBMC responses to the TLR7 agonist imiquimod in adolescents with asthma demonstrated reduced IP-10 and MxA protein and OAS mRNA expression when compared to healthy non-atopic controls (329). In contrast, R848 stimulation of PBMCs from adults with asthma display no difference in TLR responsiveness, rather they have enhanced production of IFN-β in BECs compared to healthy controls (330). It may be the case that IFN deficiency occurs in a subgroup or separate endophenotype of asthma (85). However, recently a study utilising live HRV challenge has demonstrated enhanced activation of several innate immune mediators including IFN-γ and IFN-λ in the respiratory tract *in vivo* amongst volunteers with asthma compared to those who were healthy (196). The results of the current study confirm that in the nasal mucosa of atopic and mild allergic asthma individuals, there is an increase in IFN production relative to healthy volunteers in response to a ssRNA analogue. Additionally, unlike in viral challenge studies, these responses are not confounded by *in vivo* viral load titres.

The increased expression of CLEC4C eight hours after challenge suggests that pDCs may proliferate in the nasal mucosa in response to R848, which may be a potentially advantageous finding for the development of TLR-conjugated vaccines (331, 332). Whilst CLE4C expression was not significantly different at eight hours between allergic and healthy volunteers, the exaggerated IFN-α response in the former suggest an important role for pDCs in mediating anti-viral responses in individuals with allergy. Individuals with atopy and asthma have increased circulating populations of all DC subsets compared to healthy controls (333) and allergen challenge enhances DC trafficking to the airways (334). Airway DCs have been implicated with a critical role in inducing Th2 immunity in response to allergen, with the molecular cross-talk between barrier epithelial cells and lung DCs further promoting Th2-mediated inflammation (21). However, the role of DCs in response to infectious pathogenic stimuli in the setting of allergy and asthma is less well characterized. The high-affinity IgE receptor, FcεRIα has enhanced expression on pDCs from subjects with asthma (335). Cross-linking of this receptor reduces the ability of pDCs to release IFN-α, with secretion inversely correlated to serum IgE levels (336), suggesting that allergic individuals may have deficient host IFN responses. Furthermore, omalizumab (anti-IgE) administration in children with asthma has been shown to reduce exacerbations when administered prior to returning to school in September (337). These authors collected PBMCs from children before and after omalizumab administration and stimulated them with HRV, which demonstrated an increase in IFN-α levels post-treatment. However, a mechanism to explain how anti-IgE therapy restores IFN-α in PBMCs after HRV exposure is not clear. Additionally, there may be other anti-viral immune mediators or chemokines that contribute to improved exacerbation rates. Indeed, it has been recently shown in another study utilising omalizumab that markers of type-2 inflammation identified individuals more likely to respond, whereas in contrast, high blood ISG expression was not associated with a reduction in exacerbations in response to omalizumab (338). The response to viruses other than HRV is also unknown. The results of the current *in vivo*

study indicate an enhanced mucosal IFN-α response amongst volunteers with atopy and mild allergic asthma volunteers after R848 challenge. This was not correlated with baseline total serum IgE or skin prick reactivity, indeed baseline eosinophils actually correlated positively with enhanced mucosal IFN responses.

The regulatory mechanisms involved in the migration of leukocytes is a complex process, which involve multiple chemokines. A role for CCL3 in regulating eosinophil influx and activation is suggested by the identification of increased nasal levels of MBP, a toxic cationic molecule expressed by activated eosinophils, alongside elevated CCL3 levels in the nasal mucosa of children experiencing virus-induced asthma exacerbation (339). CCL13 was the only chemokine that was significantly increased at baseline volunteers with atopy and allergic asthma individuals and particularly exaggerated responses were seen after R848 challenge compared to healthy volunteers. Additionally, CCL13 was found to be correlated with the induction of the ISGs IFIT3, DDX58 and IRF7, suggesting it has a key role in the interferon response. CCL13 is a chemoattractant for eosinophils, monocytes and T-lymphocytes and nasal CCL13 levels have been correlated with the presence of macrophages in natural viral infection (340). CCL13 is upregulated in the lower airway mucosa of individuals with asthma (341) and increased serum levels occur during acute asthma exacerbations (342). Elevated CCL13 expression in the epithelium and submucosa of bronchial biopsies taken from subjects with allergic asthma is also associated with eosinophils (341). In the current study, a significant reduction in peripheral eosinophils and lymphocytes was seen only in volunteers with atopy and allergic asthma. The differences seen in the timing of the reduction in peripheral cell counts may also provide clues as to when these cells undergo chemotaxis. The drop at 4 h in peripheral eosinophil counts coupled with the early production of CCL2, CCL3, CCL4 and in particular CCL13 in volunteers with atopy and allergic asthma, supports the notion that eosinophil trafficking from peripheral sites to the nasal mucosa may be occurring early on in the immune response to R848. The later drop (24 h) in peripheral lymphocyte counts was coupled

with the persistence of CXCL10 at 24 h. Type 1 IFNs have been shown to play a key role in regulating lymphocyte recirculation (290) and CXCL10 levels correlate with number of lymphocytes in airway secretions after HRV challenge (343), which suggests that lymphocytes may also undergo trafficking to mucosal sites in response to microbial or TLR agonist challenge. However, these results are hypothesis-generating and the chemotaxis of immune cells to the nasal mucosa requires more definitive investigation.

Collectively, the induction of a wide range of IFNs, chemokines and ISGs indicates that an anti-viral state is induced in the nasal mucosa after R848 administration, highlighting the usefulness of the R848 nasal challenge method as a tool to study innate immunity. It also shows that host factors can significantly influence immune responses and interferon related gene expression. In this case, it demonstrates that patients with atopy and allergic asthma have heightened innate immune activation relative to healthy non-atopic volunteers highlighting a key role for atopy in determining responses to viral analogues.

6 Discussion

6.1 Key findings

6.1.1 Poly(I:C) and poly-ICLC fail to reliably induce mucosal immune responses

Nasal administration of the dsRNA analogues poly(I:C) and poly-ICLC in a panel of healthy volunteers and those with allergic rhinitis was well tolerated but did not reliably elicit mucosal IFNs and cytokines in a dose dependent manner, despite poly(I:C) inducing the release of IFN-γ and IL-6 in PBMCs. This suggests that either the host environment unique to the mucosa or the delivery method of the challenge agent may influence the bioavailability of poly(I:C) and poly-ICLC within cells. Poly-(I:C) and poly-ICLC are far larger molecules than R848 and would require active uptake to enter the cytosol or endosome to activate PRRs. By comparison R848 is lipid soluble and is likely to pass directly into cells. In cell culture, poly-(I:C) can be taken up in this manner and has been shown to cause the release of proinflammatory cytokines and the induction of several ISGs (192, 344). The airway mucosa however, may represent a more hostile environment than cell cultures, with the presence of increased concentrations of RNases that rapidly degrade unshielded nucleic acids (345). This can be overcome to an extent by particulate delivery systems including liposomal envelopes that aim to mediate uptake in to cells (278). In marked contrast to these negative findings in humans, several *in vivo* murine studies involving the application of poly(I:C) to the respiratory mucosa have shown an increase in innate immune activation, T cell recruitment to the airways, increased mucus production and protection from lethal influenza challenge (264, 346, 347). Although TLR3 mRNA expression is induced by type 1 IFNs in both mice and humans (348), there are species-specific differences in its tissue expression. For example, LPS strongly induces TLR3 expression in murine macrophages and DCs with a lack of effect noted in human cells (349). Additionally, the sequences of the promoter regions of the gene encoding TLR3 are different between species (350). These factors may help understand the differences observed in the mucosal immune response to TLR3 agonists between mice and humans, and suggests significant limitations when translating findings from animal models (350).

6.1.2 Both high and low dose R848 induce immune responses but the lower dose has improved clinical tolerability

Nasal administration of R848 at a dose of 10 µg / 100 µL per nostril in nine healthy volunteers and those with allergic rhinitis was sufficient to induce significant changes in immune mediator release from the nasal mucosa. A single volunteer receiving a higher dose of 100 µg / 100 µL per nostril developed very potent mucosal IFN and pro-inflammatory cytokine responses indicating a dose response to R848. One-third of volunteers had flu-like symptoms, and one individual in particular experienced significant flu-like symptoms associated with neutrophilia and lymphopenia. This prompted a revision of the R848 dose so it would be based on body weight, resulting in an approximately 5-10 fold lower dose. Subsequently, administration at a mean dose of 1.5µg/100 µL/nostril was well tolerated by 35 volunteers, without any significant increase in local nasal or systemic clinical responses or rise in serum inflammatory markers. R848 induced an early increase in IFN-α levels with a later induction of IFN-γ and CXCL10, with a sustained production of the chemokines CCL2, CCL3, CCL4 and CCL13.

6.1.3 Nasal curettage can be used to characterize the molecular basis of the heterogeneity seen between volunteers

Utilizing the technique of nasal curettage to collect small amounts of mucosal tissue for assessment of gene expression was successful in identifying 14 genes that were differentially expressed after R848 challenge. These genes were predominantly composed of ISGs, which are critical effectors of the antiviral response. In addition to identifying several ISGs, the method was also sensitive enough to demonstrate a downregulation of IFNAR. This was to be expected due the known feedback mechanisms that regulate type 1 IFN signalling, which includes endocytosis of IFNAR within minutes of activation by type 1 IFN, as well as SOCS1 and USP18 mediated inhibitory feedback (17). The clinical importance of IFNAR in mediating anti-viral immunity via type I IFNs is demonstrated by a recent phase IIb placebo-controlled trial of Anifrolumab, an IFNAR-blocking antibody that prevents signalling by all

type I IFNs. Intravenous administration of the drug compared to placebo in patients with systemic lupus erythematosus (SLE, a condition in which autoimmunity is partially driven by chronic type 1 IFN activation), led to increased rates of nasopharyngitis (11.4% vs 4%), bronchitis (8.6% vs 4%) and influenza (7.6% vs 2%) (351).

R848 is known to activate both TLR7 and TLR8, however it is likely that the dose used for *in vitro* and *in vivo* studies may influence the nature of the immune response. In this study, R848 given at doses of 10 μg/100 μL and 100 μg/100 μL induced significant mixed IFN and proinflammatory responses. In contrast, a dose of 1.5 μg/100 μL induced a predominantly IFN response with relatively limited production of proinflammatory mediators. Increased mucosal gene expression of TLR7 (and not TLR8) and the transcription factor IRF7 (and not NFκB) suggests that R848 at a dose of 1.5 μg/100 μL may exert its effects through TLR7 via an IRF7-dependent mechanism. This suggests a critical role for pDCs (which express TLR7) compared to cDCs (which predominantly express TLR8). However, several other transcription factors that likely play a key role in IFN induction, were not measured in this study and may also alter intracellular signalling to modulate the immune response.

6.1.4 Atopy and asthma have a key role in determining immune responses to R848

Intriguingly, volunteers with atopy and allergic asthma demonstrated significantly exaggerated nasal mucosal IFN and chemokine responses compared to healthy volunteers with associated reductions in circulating eosinophil and lymphocyte populations. Volunteers with asthma had increased R848-induced expression of the ISGs IFIT3, DDX58 and MX1 relative to atopic and healthy volunteers. In subjects with atopy, reduced baseline nasal mucosal expression of the anti-inflammatory secretoglobin SCGB1A1 had a strong negative correlation with subsequent innate immune activation by R848, in particular with CCL2. Given that SCGB1A1 levels are reduced in volunteers with allergic rhinitis and negatively correlate with lymphocyte infiltration (312, 313), this may partially provide a

mechanism by which volunteers with atopy are unable to dampen the initial release of mucosal cytokines and chemokines. However, the lack of a similar finding in those with allergic asthma suggest that there are likely to be other pathways that regulate the degree of inflammatory response.

The earlier and augmented IFN and chemokine response seen in volunteers with atopy and allergic asthma suggest that their nasal mucosa is very responsive to potential invasion by ssRNA viruses such as influenza. The H1N1 influenza pandemic (2009-2011) was characterized by a remarkably increased preponderance of inpatients with asthma, making up between 25-31% of hospitalized cases worldwide, although paradoxically they also experienced less severe outcomes with earlier discharge (352–354). Earlier presentation to hospital (≤4 days from symptom onset) in patients with asthma compared to those without asthma has been associated with a decreased likelihood of severe outcomes (352). The results of a comprehensive screen of mucosal and systemic immune responses in patients hospitalized with severe influenza demonstrates that patients with asthma have equivalent mucosal IFN and enhanced serum IFN-α responses compared to those without asthma (355). The results of the current study suggest that individuals with asthma have early and exaggerated innate immune activation in response to R848 compared to healthy controls. This could provide a mechanism by which asthma exacerbations that are caused by influenza, may induce early mucosal immune responses that lead to clinical symptoms, particularly in the lower airways such as wheeze and prompt patients to seek medical attention at an earlier stage.

However, the question arises as to whether exaggerated IFN and chemokine responses are beneficial or detrimental in determining the host response to viral triggers, especially in those with asthma? In murine models, IFN-γ has been shown to act in concert with IL-13 to induce smooth muscle contraction and promote Th2 cell homing to the lungs (356, 357). An observational study of influenza infection in a mixed group of children with and without asthma suggest that an innate immune profile

characterized by increased nasal IFN-α, CCL7 (MCP3) and IL-10 is predictive of progression to severe disease independently of viral load (358). Live HRV challenge results in significantly increased IFN-γ, CXCL10, CXCL11 and type-2 immune responses in the upper and lower airways of subjects with asthma, who are also more symptomatic and have a bigger decline in lung function relative to healthy volunteers (196). In the current study, the nasal route of delivery of R848 at the 0.02 µg/kg/100 µL dose did not induce a significant increase in correlates of nasal obstruction or changes in FEV1, so immune mediator or ISG responses could not be correlated with clinical symptoms. However, in the steady (unchallenged) state, ISG expression in the bronchial mucosa as well as blood has been found to be increased in subjects with asthma relative to healthy volunteers (338). Increased ISG expression was associated with worse lung function, and this association was stronger than that between type-2 inflammation and lung function (338). Therefore, exaggerated IFN and inflammatory responses in the steady state as well as in response to a viral trigger may have the potential to lead to detrimental host responses. Large cohort studies with well-characterized subjects such as the Severe Asthma Research Programme (SARP)(359) and Unbiased BIOmarkers in PREDiction of respiratory disease outcomes (U-BIOPRED)(360) have the potential to identify how underlying asthma endophenotypes influence susceptibility to infection and predict treatment response.

6.1.5 Implications

A large body of research has been focussed on obtaining cells and tissue (primary bronchial epithelial cells, nasal epithelial cells, alveolar macrophages and PBMCs) from healthy volunteers and those with diseases such as asthma, which are then stimulated *ex vivo* with respiratory viruses or viral RNA analogues. This approach has the advantage of being able to deliver a pre-defined standardized dose of viral inoculum or viral RNA analogue. Any subsequent differences detected in gene transcripts or release of protein mediators can therefore be attributed to fundamental differences in the characteristics of the cell being studied. However, based on these findings inferences are often

subsequently made about differences that might exist in host disease characteristics *in vivo*, which can be problematic for a variety of reasons. For example, a deficiency of IFNs in one cell type (e.g. epithelial cells) may not be representative of IFN production in other cells and therefore the relative contribution to the overall immune response may be difficult to quantify. The interaction between cell types, such as those between epithelial cells and plasmacytoid dendritic cells, can be difficult to study *ex vivo*. The nasal and airway lumen also have an array of other host defence features such as anti-microbial peptides, ciliary kinesis and the presence of mucous, that all play important roles in defending against foreign pathogens (11, 12, 361). The role of the microbiome has also become increasingly recognised in modulating immune responses against viruses with research highlighting both protective and detrimental effects (56, 362–364). All these factors underline the complex nature of mucosal immune responses occurring in a viral infection, and suggest that studying single cell types in isolation *ex vivo* have limitations in fully recapitulating *in vivo* immune responses. Novel techniques to better model the human airway are being developed that incorporate multiple cell types as well as organising cells and tissue structurally in an organ-specific manner (141, 365–368). However, the main challenge for these methods in being physiologically relevant is the ability to accurately mimic the complex and dynamic immunological networks that exist *in vivo*. Human challenge models have transformed our ability to better appreciate the early immune responses that occur after administering various stimuli. In this study, the ability to safely administer a TLR agonist nasally at a standardized dose to human volunteers and measure their mucosal immune responses *in vivo* allows observations to be unbiased and the findings more directly translatable to human disease.

The ability to serially sample the nasal mucosa *in vivo* using nasosorption meant that immune mediator production could be measured with a great degree of precision. In particular, the unique kinetics of IFN production was clearly demonstrated with an immediate release and early peak in IFN-α levels, followed by a later production of IFN-γ. In particular, IFN-α has been very difficult to measure

in vivo after live viral challenge (196), which usually employs daily sampling schedules and may therefore miss the earliest innate immune responses. In contrast, hourly nasal sampling after challenge with the TLR agonist R848 was not only able to fully characterize the rise and fall of IFN-α levels but was also sensitive enough to identify important differences in kinetics between allergic and healthy volunteers.

Modern drug development for asthma (including with monoclonal antibodies) have largely relied on the inhaled allergen challenge model as a "proof-of-concept" to identify suitable therapies, which has been important in characterising their *in vivo* mechanism of action (369, 370). However, the failure of large clinical trials of monoclonal antibodies targeted at reducing asthma exacerbations highlights the limitations of inhaled allergen challenge to adequately recapitulate the heterogeneity of asthma pathogenesis (371, 372). In particular, it lacks applicability in those with non-allergic mediated disease, who constitute a significant proportion of individuals with asthma (211). In this context, the development of a practical method of challenging the respiratory mucosa with a viral RNA analogue that is tolerable for healthy volunteers and those with disease, represents an important additional platform for the development of novel therapeutics. Since infective triggers, and in particular viruses can induce asthma exacerbations in individuals with and without allergy, R848 challenge has important applicability for studying immune responses in a wider population. Indeed, the use of R848 challenge can be extended further to study innate immunity in other disease such as COPD, as well as the effects of different host conditions, such as at the extremes of age.

6.1.6 Limitations and improvements

There are limitations in the generalizability of the findings in this study. All respiratory samples were collected from the nasal mucosa and therefore may not represent lower airway inflammation. In subjects with asthma, the lower airways are characterized by increased smooth muscle mass (373),

and human smooth airway muscle cells release cytokines that are involved in promoting eosinophil differentiation (374, 375). The lower airway mucosa may therefore be influenced by release of cytokines dependent on the mesenchymal-epithelial axis, which is not represented in the anterior nasal mucosa. The immune responses presented are those after challenge with a TLR7/8 agonist and may not be representative of effects seen after viral challenge, which are likely to activate several PRRs and interact with the host in multiple ways including perturbations to the microbiome (376). However, it is pertinent to note that live challenge with HRV induces enhanced IFN responses in the nasal mucosa of volunteers with asthma compared to healthy volunteers in a similar manner to the current study (196).

Volunteers with mild asthma utilising either no ICS or low dose ICS were enrolled in this study. Therefore, the findings may not be applicable to individuals with severe asthma, who are characterized by their use of high dose ICS as well as oral corticosteroids, and experience exacerbations on a more frequent basis (211). Whilst volunteers with mild and moderate asthma have exaggerated mucosal immune responses to live HRV challenge (196), other studies have suggested that deficient IFN responses might be seen in volunteers with uncontrolled asthma or in those with more severe disease (91, 328). In this study, the use of inhaled ICS was not associated with immune responses induced by R848 although it was not powered to study this question. However, given the tolerability of R848 in a wide panel of volunteers including those with asthma, nasal challenge with R848 in severe asthma is a feasible option to better understand *in vivo* immune responses to a viral RNA analogue that is independent of viral load.

The administration of nasal R848 caused dose-dependent systemic symptoms in some volunteers. At the higher dose of 10 μg / 100 μL per nostril, one volunteer who had their serum measured after nasal

challenge, developed a rise in IFN-α. However, at the lower dose there was no induction of systemic inflammation in all volunteers. Given the extensive blood supply in the nose, it is possible that some absorption of R848 from the nasal mucosa in to the systemic circulation occurred in volunteers receiving the higher dose, leading to induction of IFNs and systemic symptoms. As this was a pre-clinical challenge study rather than a therapeutic drug trial, systemic levels of R848 were not measured. Efforts have been made to limit systemic absorption from mucosal administration of drugs as evidenced by the successful implementation of an antedrug design of a TLR7 agonist delivered by inhalation (377).

Nasosorption samples were collected at multiple time points and theoretically this may be associated with tissue injury causing the release of damage associated proteins. One study that has assessed the effects of older versions of filter papers and polyurethane foam in unchallenged volunteers, suggest that repeated sampling does not lead to the release of significant amounts of LDH (as a marker of tissue injury) especially when compared to nasal lavage (161). The lack of any immune response seen after saline challenge in almost all volunteers suggests that serial sampling with nasosorption does not induce significant mucosal inflammation.

For this study, in order to sample nasal mucosal lining fluid, a nasosorption device containing a synthetic absorptive matrix was used to assess mediator levels, which effectively characterized differences in immune response between subject groups. Whilst it has also been extensively deployed in nasal challenge with various stimulants (61, 74, 196), it remains to be seen whether other materials may be even more suitable. In particular, an alternative approach could be with the use of polyurethane sponges, which have been utilised in challenges involving cat and grass pollen (170, 171). It has been shown to be better at detecting IL-1β, Il-8 and total IgA compared to nasal lavage (161,

378), and superior to both cellulose based strips and older versions of SAM strips at detecting IL-4, IL-5 and IFN-γ, which may be due to absorption of increased sample volume (172).

Delivery of the nasal challenge was performed using the bidose device, which has been utilised for nasal challenge with allergen and LPS (61, 74). The aerosol that is generated purportedly covers the nasal mucosa surface for effective drug delivery and is designed to meet the regulatory requirements for bioavailability and bioequivalence studies for this purpose (379). However, other methods of administering challenge agents to the nasal mucosa include the use of drops, pipettes, filter discs with adsorbed substances, dry powder insufflation as well as nasal lavage or irrigation (84). It is likely that the use of a bidose spray device is preferable to installation of liquid in to the nasal mucosa using drops or a pipette as these latter methods often lead to a greater loss of fluid from the nasal cavity. Aerosolised delivery of 100 µL volume of challenge agent bilaterally to the nostrils has been adopted as the standard for allergen challenge models (380). Other techniques with the ability to deliver larger volumes of challenge agent to the nasal mucosa may lead to enhanced immune responses. For the nasal challenge experiments with poly(I:C) and poly-ICLC, it remains to be seen whether alternative techniques that deliver increased volumes could have elicited stronger mucosal immune responses.

A microfluidics platform was used to assess regulation of a limited panel of genes after R848 challenge and higher throughput technologies are available. RNAseq is a useful technique to identify global gene expression and is superior than microarray at identifying differentially regulated ISG expression in the mucosa of individuals with asthma (338). However, mRNA expression may not necessarily be predictive of protein secretion and an important facet of the current study was the characterization at serial time points of a large panel of immune mediators, which may have more translational appeal with regards to the development of therapeutics than the assessment of gene expression at limited time-points.

Although mucosal samples collected using nasal curettage identified an upregulation of CLEC4C (a marker for pDCs), in general it was less successful for "cellular footprinting" despite its success in identifying eosinophil associated genes after allergen challenge (74). However, other studies involving the use of flow cytometry on nasal curettage samples have succeeded in assessing overall numbers of T cells and granulocytes in the nasal mucosa (138) and to even isolate rare innate lymphoid cells (ILCs) after allergen challenge (75). Future work may focus on elucidating the cellular source of mucosal interferon and cytokine production after *in vivo* TLR agonist challenge. This could be achieved by employing flow cytometry techniques to enumerate activated DCs and macrophages as identified by the presence of costimulatory molecules such as CD80 and CD86, which are critical for initiating adaptive immunity.

A summary of host nasal mucosal and systemic responses to TLR agonists and in particular R848 is illustrated in figure 6.1.

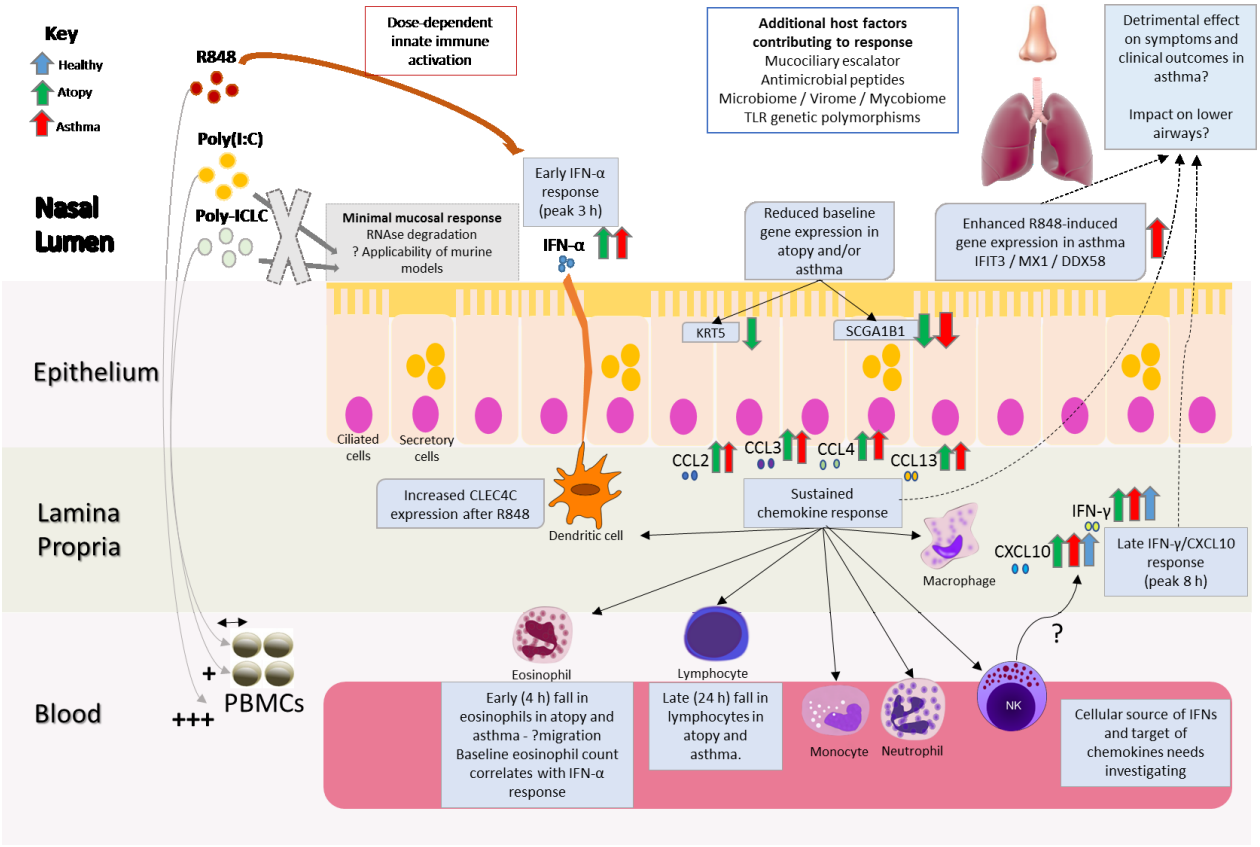


Figure 6.1 Summary of host nasal mucosal and systemic immune responses to TLR agonists Arrows represent relative rather than absolute expression levels. PBMC Peripheral blood mononuclear cells.

6.2 Future research directions

6.2.1 Microbiome analysis

The impact of commensal microbiota on modulating clinical responses has gained significant research interest, with its importance highlighted by the recent discovery that the gut microbiome is more critical than genetic ancestry in predicting glucose and obesity measures (381). The upper respiratory tract has a greater biomass of commensal bacteria than other parts of the respiratory tract and contributes to the composition of microbial populations in the lower airways through mucosal dispersion and micro-aspiration (363). The presence of diverse microbial communities is considered an important component of maintaining a healthy microenvironment (382). In contrast, the airways in volunteers with asthma have a disordered microbiome with a greater prevalence of *Haemophilus* species (65), which in children has also been linked to future susceptibility to viral infections and the development of asthma (383). Viral respiratory tract illness is often complicated by secondary bacterial infections, especially in those with compromised host defences (384). The most famous example of this being the epidemiological and autopsy evidence of severe secondary pneumonia during the 1918-1919 "Spanish" pandemic influenza pandemic (385). Experimental models using HRV challenge in subjects with COPD demonstrates the potential for viruses to subsequently modulate the lung microbiota (376), whilst LPS nasal challenge suggests that baseline mucosal immune tone may be affected by environmental factors such as host microbiota (61). One mechanism may be due to the presence of virus in the nose altering epithelial cell receptor expression allowing any resident bacteria present in the associated mucosal biofilm to become pathogenic (386). In murine models, respiratory (and possibly gut) commensal bacteria play a role in regulating T cell function against acute respiratory viral infections such as influenza (387), whilst lower respiratory tract immune responses to bacterial TLR ligands can be attenuated for at least 6 weeks after infection (388).

In the current study, to investigate the effect of host nasal microbiota on innate immune responses to TLR agonists, additional nasosorption samples were collected from healthy participants and those with atopy and asthma at their screening visit and at serial time points after challenge with saline and R848. The results of the microbiome analysis will be correlated with the atopic status of individuals as well as their immune responses. Additionally, cotton wool swab samples were taken and sent for 16s ribosomal RNA sequencing and will be compared to data obtained from nasosorption to assess the comparability of sampling techniques.

6.2.2 Potential applications for respiratory mucosal delivery of TLR agonists

Whilst allergic rhinitis and asthma are common in the general population, treatments are often non-specific and patients have difficulties with concordance so alternative approaches have been developed. Specific allergen immunotherapy aims to deliver increasing quantities of allergen to promote immunologic tolerance, with the goal of modifying the underlying basis and natural history of allergy mediated disease (389). Successful immunotherapy is characterised by a shift from type-2 to type-1 immune responses as well as the induction of T-regulatory mechanisms mediated by IL-10 and TGF-β (390). TLR agonists can be used to further augment these immune responses and has been the basis for "adjuvanting" allergy vaccines. An early and well-studied example of this is Pollinex Quattro (Allergy Therapeutics, West Sussex, United Kingdom), which consists of glutaraldehyde modified L-tyrosine adsorbed pollen allergens with monophosphoryl lipid (MPL – a TLR4 agonist) and has been shown to safe and efficacious in patients with allergic rhinitis and asthma with durable responses (391). Repeat dose standalone TLR agonists delivered via the intranasal route have also been used for allergen immunotherapy with the purpose of skewing immunity towards a type-1 response. Whilst R848 is a combined TLR7/8 agonist that activates multiple pathways to generate IFN, chemokine and proinflammatory cytokines, in contrast the use of TLR7 agonists aims to induce predominantly IFN rather than proinflammatory cytokine pathways. Intranasal administration of the

TLR7 agonist AZD8848 resulted in a reduction in allergen-induced clinical symptoms and nasal tryptase levels and a repeat dose regimen was tolerable (292). However, when a repeat dose regimen was given via the inhaled route in healthy volunteers, it resulted in systemic IFN production and significant flu-like symptoms (377). This highlights potential site-specific variations in mucosal immune responsiveness, but there are also considerations around the optimal interval between doses (one week in this study) as well as the delivery mechanism of the challenge agent - targeted nasal spray versus more generalised deposition using nebulisation, leading to differences in tissue exposure. A nebulised TLR9 agonist cytidine-phosphorothioate-guanosine oligonucleotide (CPG ODN) AZD1419 induced CXCL10 protein and IFN-inducible genes at 24 h in the sputum of healthy volunteers (392). It also has fewer delayed onset flu-like adverse events than the TLR7 agonist AZD8848, which may have been due to the larger size and greater bioavailability of the drug. A phase 2a trial in eosinophilic mild to moderate asthma is planned (https://clinicaltrials.gov/ct2/show/NCT02898662). To assess whether nasal delivery can modulate lower airway responses, a phase 2 study of a TLR7 agonist GSK-2245035 in volunteers with mild allergic asthma using an 8 weekly dosing schedule will evaluate whether intranasal delivery reduces FEV1 after subsequent bronchial allergen challenge (https://clinicaltrials.gov/ct2/show/NCT02833974). Alternative formulations of TLR agonists including novel delivery methods might also be better tolerated. For example, the compound 3M-052 has a C18 lipid moiety that renders it lipid soluble compared to R848, which is soluble only in aqueous solution. Pre-clinical studies utilising it as a vaccine adjuvant suggests it induces less systemic immune activation relative to R848. The manufactures suggest that the mechanism for this is based on the slow-release of vaccine from the administration site as well as the vaccine acting as a depot site (393, 394).

Vaccinating against respiratory infections remains the most effective method of preventing infection. However, vaccines often need updating and along with increasing rates of anti-microbial resistance, alternative immunomodulatory prophylactic approaches such as adjuvantation with TLR agonists has

gained significant research interest (395). Adjuvanted influenza vaccines delivered via the respiratory mucosal route has been the focus of several pre-clinical studies. 3M have developed an adjuvanted H5N1 vaccine with the TLR7/8 agonist 3M-052. They have demonstrated it to be protective against lethal challenge with H5N1 in mice and ferrets, and have interestingly shown that adjuvanting with R848 does not reduce lung viral tires post immunization (393). Martha A. Alexander-Miller's group (Wake Forest, North Carolina) have generated an inactivated H1N1 virus conjugated with an amine derivative of R848 and tested immunogenicity in African Green Monkey infants. They have shown increases in virus specific IgG and IgM and augmentation of IFN-g producing T cell responses with no adverse events (396). In the clinical setting, topical application of imiquimod cream (a TLR7 agonist) prior to injection with intradermal trivalent influenza vaccine has been shown to improve serum antibody titres compared to placebo and represents a particularly useful approach in elderly patients (397, 398). The field of oncology has witnessed the introduction of an important new therapeutic approach with the advent of immune checkpoint inhibitors. These are immunotherapeutic antibodies that target co-stimulatory and co-inhibitory receptors on T cells, and aim to activate T cells against cancer cells (e.g. via programmed death receptor 1, PD-1) or reverse tumour-induced T cell inhibition (e.g. via cytotoxic T-lymphocyte antigen 4, CTLA-4) (399). TLR ligands can be combined with these agents to enhance antigen presentation by dendritic cells (399). 3M-052 has been shown to potentiate checkpoint blockade therapy with anti-CTLA-4 and anti- PD-1 antibodies in a pre-clinical model of melanoma (400). The combination of R848 with checkpoint inhibitors also has dramatic effects in murine solid tumours (401). Given the success of anti-PD-1 ab in non-small cell lung cancer in humans (402), it may be feasible to target lung cancers with checkpoint inhibitors that could be adjuvanted with TLR agonists, with systemic delivery compared to the inhaled route. It is important to acknowledge that whilst TLR adjuvants represent an exciting and novel approach to target infections and cancer, they would be required to be manufactured to GMP standards with extensive toxicology testing and carefully conducted first-in-human studies (232).

6.3 Conclusions

I. Precision serial mucosal sampling methods using nasosorption and curettage can be utilised to determine nasal innate responses *in vivo*, including difficult to measure mediators such as IFN-α, and characterize the molecular basis of the immune response by identifying differential changes in ISG expression after TLR agonist challenge between subject groups.

II. The dsRNA analogues poly(I:C) and poly-ICLC fail to reliably induce mucosal innate immunity

III. The TLR7/8 agonist R848 successfully induces innate immune responses and can delineate the unique kinetics of mediator production, with good tolerability at a dose 1.5 μg/100 μL/nostril.

IV. Volunteers with allergic rhinitis and mild allergic asthma demonstrate significantly exaggerated mucosal innate immune activation to R848 compared to healthy volunteers, and those with allergic asthma have enhanced interferon-related gene expression, highlighting a critical role for atopy in determining immune responses to viral RNA analogues.

Nasal TLR agonist administration in combination with mucosal sampling techniques can be utilised to extend this body of work to study innate immunity in a range of host conditions, as well as explore the potential for TLR agonists as stand-alone therapeutics for modulating responses in allergy-mediated disease and as vaccine adjuvants against important respiratory infections such as influenza. The field of innate immunity has rapidly advanced since Charles Janeway's revolutionary concepts and the discovery of pattern recognition receptors, and is likely to witness further ground-breaking research. Indeed, the entire notion of innate immunity being non-specific is being challenged by the finding that monocytes, macrophages and NK cells have the ability to mount enhanced responses upon reinfection even in the absence of lymphocytes – so called "trained immunity", which is likely to occur via epigenetic reprogramming on innate immune cells (403). This emphasises the importance of possessing cutting-edge tools such as those highlighted in this body of work to permit in-depth studies of the immune mechanisms of disease for the development of novel therapeutics and vaccines.

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SCREENING Case Report Form TLR AGONIST PART B
Subject Group Code: RESIQUIMOD
Study Name: Nasal Challenge with Microbial Constituents & Allergen
Study Number: 13SM1837

Subject ID Number AJ☐☐☐
Subject Initials ☐☐☐

SCREENING VISIT Day -7 (2-3 hours duration)

1. INFORMED CONSENT

Has the subject read the patient information sheet and given consent? Yes ☐ No ☐

Date of Consent: ☐☐/☐☐/☐☐ Version Number ☐

DEMOGRAPHIC DETAILS

2. GENDER Male ☐ Female ☐ **3. ETHNICITY (see separate chart)** _____

4. DOB ☐☐/☐☐/☐☐ **5. Age** ☐☐

6. HEIGHT ☐.☐☐ Metres **7. WEIGHT** ☐☐☐.☐ Kilograms **8. BMI** ☐☐

SOCIAL AND SMOKING HISTORY

9. SMOKER? Current ☐ Ex ☐ Never ☐ **Cigs per day** ☐☐ **Stopped** ☐☐☐☐

Pack Years ☐☐

10. ALCOHOL? Number of units per week ☐☐☐

11. PAST MEDICAL HISTORY

Asthma? Y ☐ N☐ If yes, ACQ score _____ Age onset Asthma _____

History of Hay fever? Y ☐ N☐ Eczema? Y ☐ N☐ Parental Asthma?_____
Sinus Infections? Y ☐ N☐ Previous Nasal Surgery? Y ☐ N☐
Viral Infections within the last 14 days? /LRTI in the last 28 days? Y ☐ N☐ Pets at home? _____

MEDICAL CONDITION	YES/NO	COMMENTS
Resp	Y ☐ N☐	
CVS	Y ☐ N☐	
GI	Y ☐ N☐	
GU	Y ☐ N☐	
CNS	Y ☐ N☐	
Endocrine	Y ☐ N☐	
MSK	Y ☐ N☐	
Skin	Y ☐ N☐	

GENERAL COMMENTS

Completed by: _____ Date Completed: ☐☐/☐☐/☐☐

SCREENING Case Report Form TLR AGONIST PART B
Subject Group Code: RESIQUIMOD
Study Name: Nasal Challenge with Microbial Constituents & Allergen
Study Number: 13SM1837

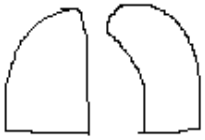
Subject ID Number AJ☐☐☐
Subject Initials ☐☐☐

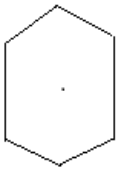
12. DRUG HISTORY			
Medication	Dose	Frequency	Route

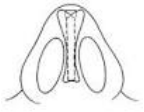
Any inhalers?	Y ☐ N ☐ (If so frequency of use)
Drug Allergies? Details:	Y ☐ N ☐
Any prescribed anti-inflammatory drug therapy?	Y ☐ N ☐
Any Antihistamines?	Y ☐ N ☐ (need to be stopped 48 hours prior to skin prick tests)

13. CLINICAL EXAMINATION

GENERAL







NASAL EXAMINATION: *Polyps?* Y ☐ N ☐ *Mucosal abnormalities?* Y ☐ N ☐
Major Septal Deviation? Y ☐ N ☐ *Hypertrophy turbinates?* Y ☐ N ☐

Completed by: _____ Date Completed: ☐☐/☐☐/☐☐

If found, please return to Dr Akhilesh Jha, ICRRU, Mint Wing Entrance C, St Mary's Hospital, London, W2 1NY ✆ 020331 25744

SCREENING Case Report Form TLR AGONIST PART B
Subject Group Code: RESIQUIMOD
Study Name: Nasal Challenge with Microbial Constituents & Allergen
Study Number: 13SM1837

Subject ID Number AJ☐☐☐
Subject Initials ☐☐☐

14. OBSERVATIONS				
Temperature (°C)	Respiratory Rate (breaths per minute)	Oxygen Saturations (SpO2)	Pulse (beats per minute)	Blood Pressure (mmHg) (after 3 mins sitting)

15. INVESTIGATIONS (ECG / CXR / URINE)		
ECG Normal? Y ☐ N☐ Details:	**CXR** (if available) normal? Y ☐ N☐ Details:	**Urine** Dipstick: Pregnancy Test (if applicable) Pos ☐ Neg ☐

16. SKIN PRICK TESTING (positive response = raised wheal of diameter ≥3mm larger than negative control)					
ALLERGEN	**RESPONSE Dimension (mm x mm)**	**ALLERGEN**	**RESPONSE Dimension (mm x mm)**	**ALLERGEN**	**RESPONSE Dimension (mm x mm)**
Negative Control		HDM		Cat Hair	
6 Grass mix		Aspergillus		Dog Hair	
Timothy Grass Pollen		Tree Mix (x3)		Histamine Control	

17. BLOODS (Request on Cerner – can print out results and enclose in CRF)
FBC ☐ (Mauve Bottle) LFT ☐ U&E ☐ CRP ☐ Total IgE ☐ (**2 x** Yellow Bottle) PAXgene **DNA** sample (blue top) if consented

18. NASAL SAMPLING
Nasal Curettage X2 RIGHT Nostril ☐ Stored in:_____ Nasal Microbiome Swab x1 LEFT Nostril ☐

Completed by: _____ Date Completed: ☐☐/☐☐/☐☐

19. BASELINE SPIROMETRY To be completed prior to Methacholine challenge. If FEV_1 is below 70% do not continue				
	FEV_1	**FVC**	**PEF**	**FEV1/FVC Ratio**
CALCULATED 20% REDUCTION (If FEV_1 falls below this **stop the test immediately)**	**BASELINE FEV_1 X 0.8 =**			

20. METHACHOLINE CHALLENGE		
DOSE	**FEV_1 post 1 minute**	**FEV_1 post 3 minutes**
0.03mg/ml		
0.06mg/ml		
0.12mg/ml		
0.24mg/ml		
0.48mg/ml		
1mg/ml		
2mg/ml		
4mg/ml		
8mg/ml		
16mg/ml (If required)		

CHECKLIST

Patient Meets Inclusion/Exclusion Criteria? **Y ☐ N☐** **If not why?**_____

Letter sent to GP? Y ☐ N☐ Copy of consent form and PIS given to patient? Y ☐ N☐
Subject Identification Code List Completed? Y ☐ N☐ Enrolment log completed? Y ☐ N☐
Bloods taken? Y ☐ N☐ Room booked? Y ☐ N☐

Next Visit Scheduled: ☐☐/☐☐/☐☐

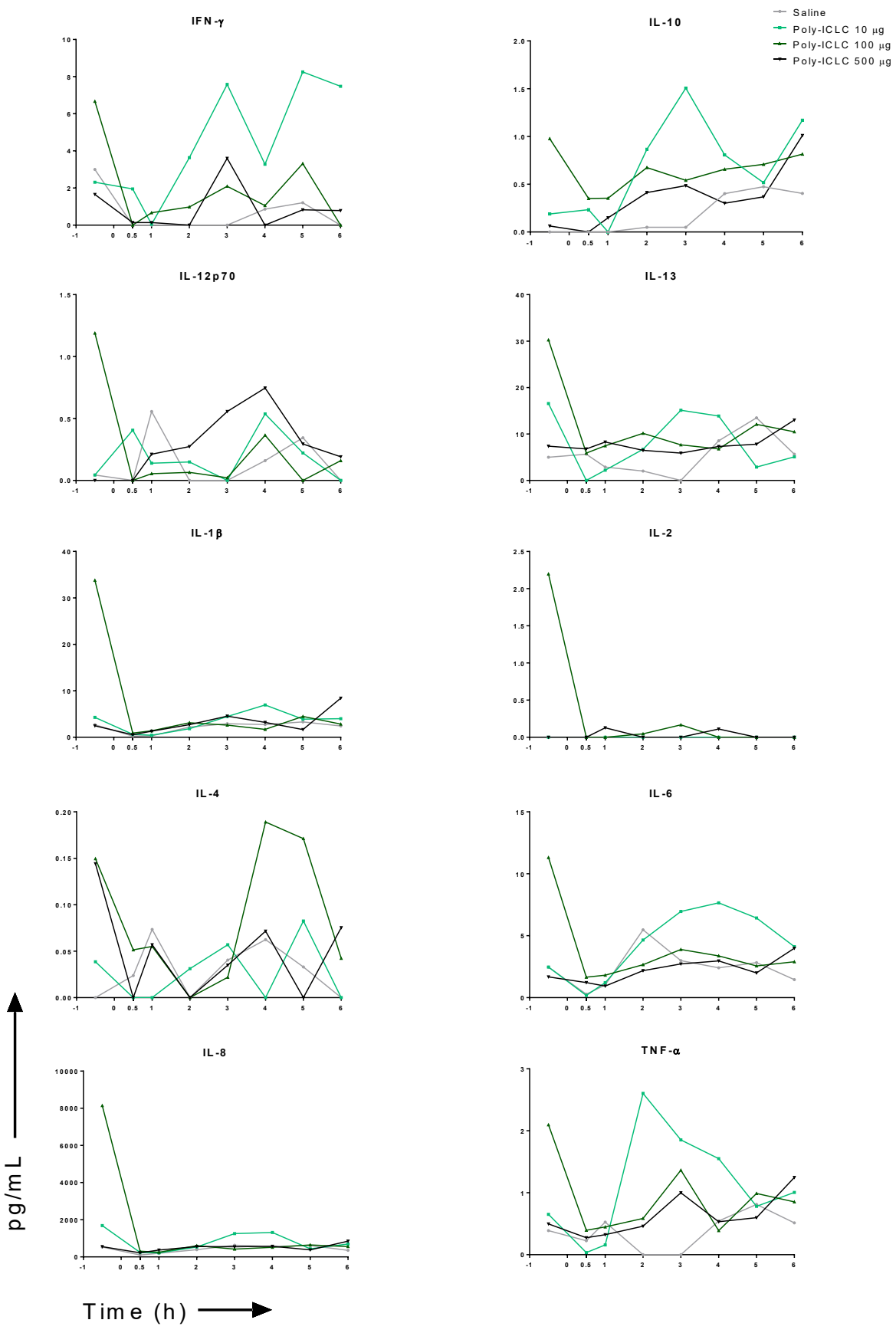
COMMENTS

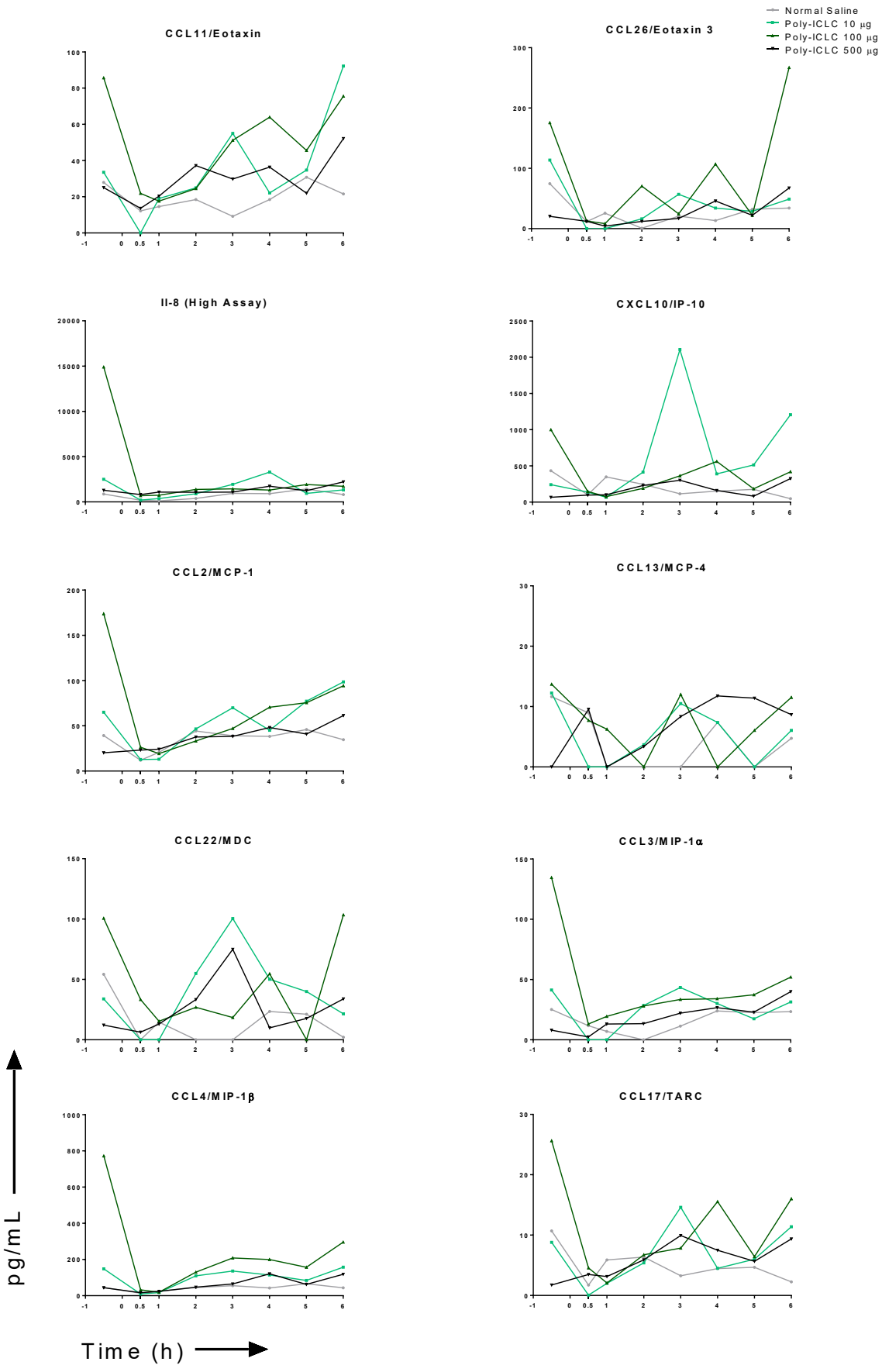
Completed by: _____ Date Completed: ☐☐/☐☐/☐☐

Appendix II Realtime RT-PCR Primers for Fluidigm DELTAgene Assays

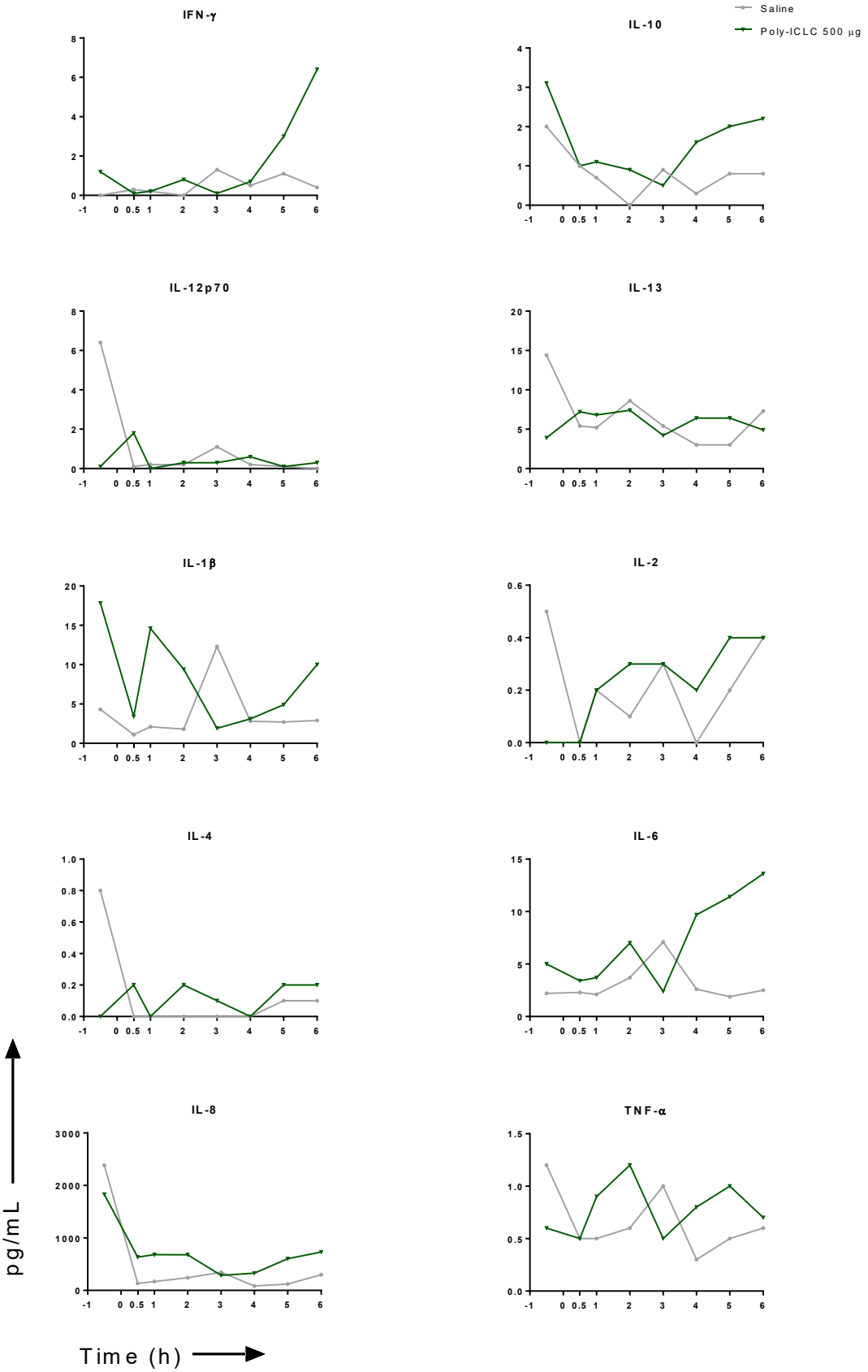
Target	Forward Primer Sequence	Reverse Primer Sequence
ADGRG1	CCAGCTTGTCGTCCTCTACC	GCATGGACCAGTACCAGATGAA
CD163	AGTGCAGAAAACCCCACAA	CAAGGATCCCGACTGCAATA
CD1C	AGGCCAGGACATCATCCTCTA	CCAAGGGCACTATCACTACCAA
CHIT1	GCTGGATACGAGGTGGACAA	CCAAGAGCCATGGAAGTCGTA
CLC	CATCTCAGTGCTGCCAGATA	CACACTTGCACCATCTTCAC
CLCA1	AAGGCTGACTATGTGAGACCAA	GAGGAGTAGACTCAGCAACCA
CLEC4C	CCTGCGTCATGGAAGGAAA	GTCCAAGATTGCATCCCAGTA
CXCR2	ATCGGTGGCCACTCCAATAA	GGTCGCTGGGCTTTTCAC
DDX58	GACTGGACGTGGCAAAACA	CTCCACTGGCTTTGAATGCA
FCER1A	CATCCTCCACCTGTCTACCA	TCATGGACTCCTGGTGCTTA
FOXJ1	ATCACCCTGTCGGCCATCTA	ACAGGTTGTGGCGGATTGAA
GAPDH	GAACGGGAAGCTTGTCATCAA	ATCGCCCCACTTGATTTTGG
HPRT1	GCACTGAATAGAAATAGTGATAGATCC	CTTTCCAGTTAAAGTTGAGAGATCA
IFIT3	ACTGGCAATTGCGATGTACC	GCTCAATGGCCTGCTTCAAA
IFNAR1	AGTGACGCTGTATGTGAGAAAA	ACGGGAGAGCAAATAATGCA
IL25	CAGTGAAGATGGACCCCTCA	AGCCTGTCTGTAGGCTGAC
IL33	TATGAGTCTCAACACCCCTCA	TTGTAGGACTCAGGGTTACCA
IRAK3	CGGGCAAAGTTAAGACCATCA	TGTGGGAGGATCTTCAGCAA
IRF3	ACACCTCTCCGGACACCAA	TGGGGCCAACACCATGTTAC
IRF7	GGCAGAGCCGTACCTGTCA	ACCGTGCGGCCCTTGTA
KIT	GGATTCCCAGAGCCCACAA	ACATCCACTGGCAGTACAGAA
KLRD1	AGCATTTACTCCAGGACCCAAC	TAACAGTTGCACCGGTACCC
KRT5	GAAGCCGAGTCCTGGTATCA	CCTCTGGATCATCCGGTTCA
LGALS12	CACGCTGGGTATATTTGGTGAC	TCTGCTGCCCTCCACAAA
MME	CCTGGACTTGACCTAAATCACA	ACCGCATACTCTGGCCTATA
MMP12	GGATGCACATTTCGATGAGGAC	TCGTGAACAGCAGTGAGGAA
MPO	CATCGGTACCCAGTTCAGGAA	TGCTGCATGCTGAACACAC
MX1	ATGCTACTGTGGCCCAGAAA	GGCGCACCTTCTCCTCATA
MYD88	CTGCAGAGCAAGGAATGTGAC	TGCTGGGGAACTCTTTCTTCA
NCAM1	CTCCCAGTCCATGTACCTTGAA	GGTTCCCCTCCCAAGTGTAC
NFKB1	CTACCTGGTGCCTCTAGTGAAA	ACCTTTGCTGGTCCCACATA
NRP1	ACATGGTGCAGGATTTTCCA	GGTGTTGTGTAGTTCTGGGAA
OAS2	TGGTGAACACCATCTGTGAC	CCATCGGAGTTGCCTCTTAA
POSTN	GCCCTGGTTATATGAGAATGGAA	TGCCCAGAGTGCCATAAAC
SCGB1A1	ACCATGAAACTCGCTGTCAC	GGTTTCGATGACACGCTGAA
SIGLEC8	AGGTGTGACCACGACCAGTA	GCATCTCCTTGGAAGACAGTCA
SOCS1	CATCCGCGTGCACTTTCA	GCTCGAAGAGGCAGTCGAA
STAT1	ATGCTGGCACCAGAACGAA	GCTGGCACAATTGGGTTTCAA
THBD	TGCCACTGCTACCCTAACTAC	TACTCGCAGTTGGCTCTGAA
TLR3	TCTCATGTCCAACTCAATCCA	CAGCTGAACCTGAGTTCCTA
TLR7	TCTTCAACCAGACCTCTACATTCC	AGCCCCAAGGAGTTTGGAAA
TLR8	AGGCTACAGGTCTCTTTCCA	CAGAGGCATCTTTGGTGTCA
TNFAIP3	GAAGCTTGTGGCGCTGAAAA	CCTGAACGCCCCACATGTA
TPSAB1	AGCGAGTGGGCATCGT	CAGAAGTGCATCCAGTATGGG
TSLP	GCCCAGGCTATTCGGAAAC	CGACGCCACAATCCTTGTAA

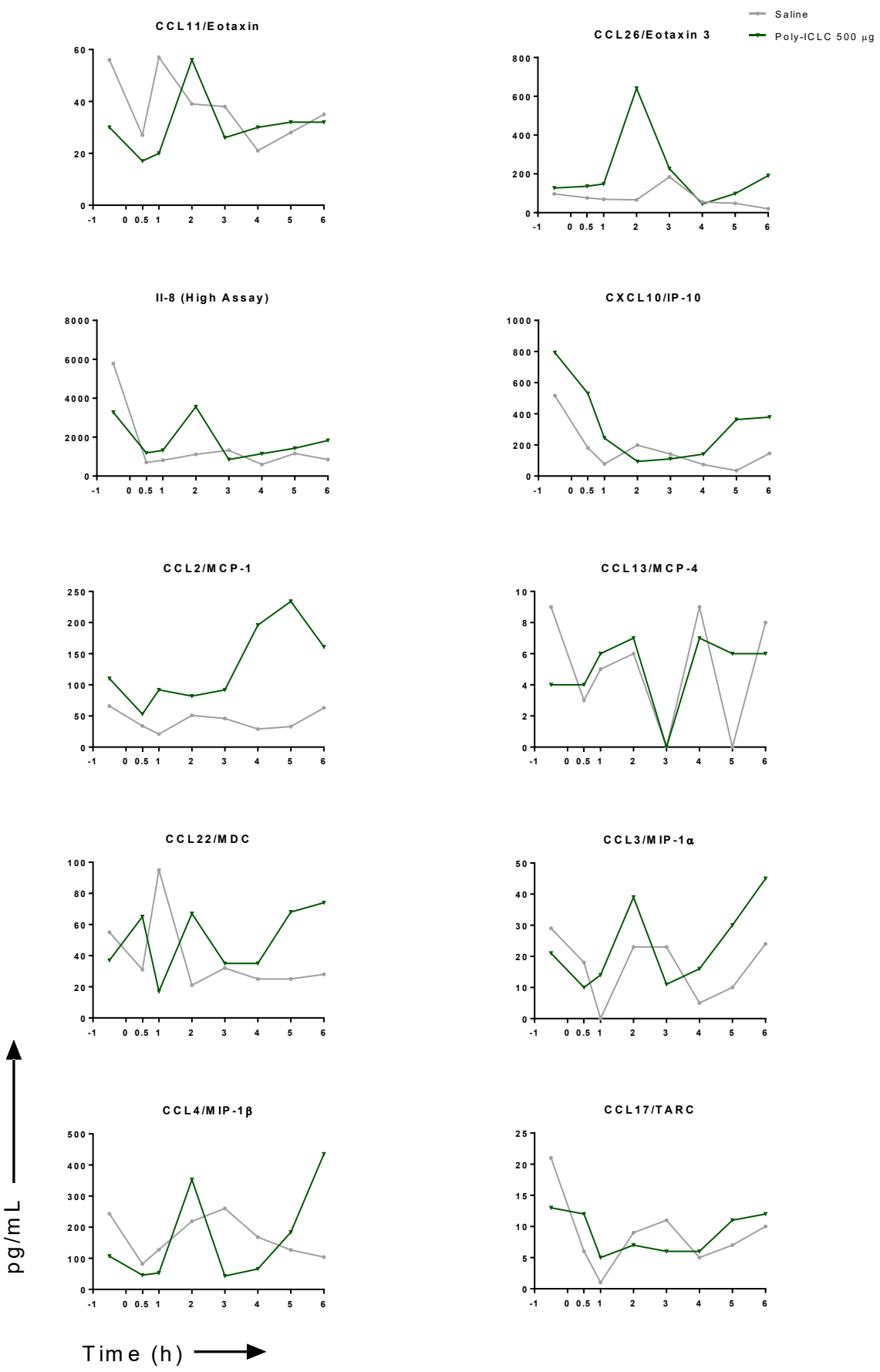
Appendix III Nasal immune profile in single individual after multiple dose poly-ICLC challenge

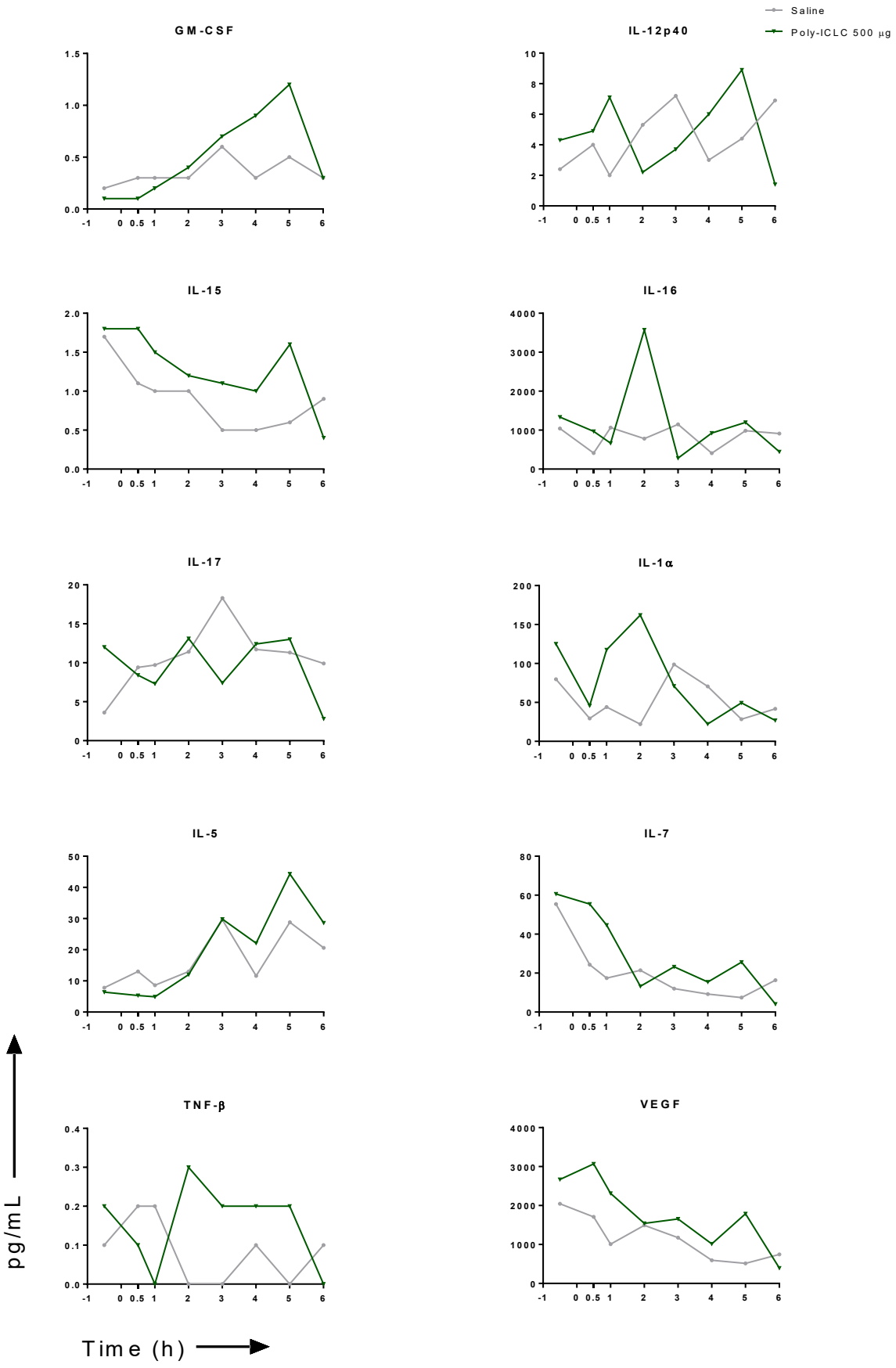



Normal Saline
Poly-ICLC 10 μg
Poly-ICLC 100 μg
Poly-ICLC 500 μg
CCL11/Eotaxin
CCL26/Eotaxin 3
Il-8 (High Assay)
CXCL10/IP-10
CCL2/MCP-1
CCL13/MCP-4
CCL22/MDC
CCL3/MIP-1α
CCL4/MIP-1β
CCL17/TARC
pg/mL
Time (h)

Appendix IV Nasal immune mediator profile of most symptomatic volunteer after poly-ICLC 500 µg challenge




Saline
Poly-ICLC 500 µg
CCL11/Eotaxin
CCL26/Eotaxin 3
Il-8 (High Assay)
CXCL10/IP-10
CCL2/MCP-1
CCL13/MCP-4
CCL22/MDC
CCL3/MIP-1α
CCL4/MIP-1β
CCL17/TARC
pg/mL
Time (h)


Saline
Poly-ICLC 500 µg
GM-CSF
IL-12p40
IL-15
IL-16
IL-17
IL-1α
IL-5
IL-7
TNF-β
VEGF
pg/mL
Time (h)

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Title: The Airway Epithelium: Soldier in the Fight against Respiratory Viruses
Author: Marjolaine Vareille, Elisabeth Kieninger, Michael R. Edwards et al.
Publication: Clinical Microbiology Reviews
Publisher: American Society for Microbiology
Date: Jan 1, 2011

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Title: Toll-Like Receptor Expression and Induction of Type I and Type III Interferons in Primary Airway Epithelial Cells
Author: Ioannis Ioannidis, Fang Ye, Beth McNally et al.
Publication: Journal of Virology
Publisher: American Society for Microbiology
Date: Mar 15, 2013

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