ASTHMA, CHILDHOOD EXPOSURES AND GENETICS

SHAPE ANTI-VIRAL CYTOKINE RESPONSES

IN HUMANS

by

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THESIS ABSTRACT

Respiratory virus infections are associated with asthma pathogenesis and exacerbations in children and adults. Unfortunately, it remains largely unknown whether innate and adaptive T cell anti-viral immunity differs in allergic disease versus health.

Here, we established a short-term primary cell culture system using human peripheral blood mononuclear cells (PBMC) optimized for measuring immune responses to reovirus, respiratory syncytial virus (RSV) and metapneumovirus (MPV) based on virus-specific cytokine and chemokine production. The prevalence and intensity of innate and adaptive responses in children and adult populations was addressed. Using this *in vitro* model of human anti-viral immunity, we tested our global hypothesis that asthmatics mount anti-viral cytokine responses to respiratory viruses that differ from those of healthy individuals.

MPV and RSV, although both ubiquitous and leading to very high levels of infection, seroconversion and clinically similar presentation in the population, evoke distinct innate and adaptive T cell-dependent cytokine responses. Reovirus induced exceptionally strong IFNγ recall responses concomitant with intense IL-10 production, which were independent of viral replication in PBMC.

Surprisingly, despite Type 1 cytokine production dominated adaptive immune responses in both asthmatic and non-asthmatic individuals, asthmatics exhibited significantly stronger pro-inflammatory IFNγ and IL-10 production towards virus stimulation than non-asthmatic children and adults. Moreover, children with current AHR, regardless of asthmatic status, exhibit a greater frequency and intensity of IFNγ responses towards pneumoviruses than do non-AHR counterparts. Conversely, expression of chemokine CCL5 was substantially weaker in asthmatics, and was further decreased in children with AHR and familial history of asthma. This pattern of enhanced pro-inflammatory and deficient anti-

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viral CCL5 responses towards pneumoviruses in children with markers of symptomatic asthma or AHR may underlie the enhanced sensitivity of these children to experience breathing difficulties following infection with respiratory viruses.

Furthermore, we have clearly demonstrated a gene by environment interaction, whereby ETS exposure in children with familial asthma results in suppressed anti-viral IFNγ and IL-10 production. Therefore, we have attempted to determine whether genetic variation affects the intermediate phenotype of anti-viral immunity, in the population and dependent on clinical status.

In summary, we have demonstrated that asthma, childhood exposures and genetics shape anti-viral cytokine responses in human. These findings have a substantial impact for physicians deciding the contextually appropriate treatment for asthma symptoms in their patients and could have implications for experimentation relating to mechanisms of disease, clinical practice and development of appropriate therapeutics.

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ABBREVIATIONS

APC　　　　Antigen presenting cell

AHR　　　　Airway hyper-responsiveness

B cells　　　Bone marrow-derived lymphocytes

CCL5　　　Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES)

CCL17　　　Thymus and Activation Regulated Chemokine (TARC)

CD4　　　　Glycoprotein predominantly found on the surface of helper T cells

CD8　　　　Glycoprotein predominantly found on the surface of CTLs

CD25　　　α-chain of the interleukin-2 receptor, activation marker

CD69　　　C-type lectin, early activation marker

CD80　　　Costimulatory molecule involved in T cell activation

CD86　　　Costimulatory molecule involved in T cell activation

cDNA　　　DNA copy of RNA template

CTLs　　　Cytotoxic T lymphocytes

CTLA-4 Ig　Fusion protein between the extracellular domain of CTLA-4 and the Fc

　　　　　　　portion of human IgG1

CXCL9　　Monokine induced by IFNγ (MIG)

CXCL10　　IFNγ-inducible protein of 10 kDaltons (IP-10)

CX3CL1　　Fractalkine

CysLT　　　Cysteinyl leukotrienes

ELISA　　　Enzyme-linked immunosorbent assay

EDTA　　　Ethylene diamine tetra acetic acid

ETS　　　　Environmental tobacco smoke

FBS	Fetal bovine serum
FEV$_1$	Forced expiratory volume in 1 second
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ICD-9	International Classification of Diseases, Ninth Revision
ICS	Inhaled corticosteroid
IFNα	Interferon-alpha
IFNβ	Interferon-beta
IFNγ	Interferon-gamma
IFNλ	Interferon-lambda
Ig	Immunoglobulin (M, D, G, E, A)
IL	Interleukin-(1 to 33)
ISAAC	International Study on Asthma and Allergies in Childhood
L1	Reovirus RNA polymerase gene
LPS	Lipopolysaccharide
MHC I	Major histocompatibily antigen class I, endogenous pathway
MHC II	Major histocompatibily antigen class II, exogenous pathway
μg	Microgram
mg	Milligram
ml	Milliliter
ng	Nanogram
NK cells	Natural killer cells
PC$_{20}$	Provocative concentration of methacholine which causes a 20% fall of FEV$_1$
PHA	Phytohemagglutinin mitogen
PSF	Penicillin G sodium, streptomycin sulfate, amphotericin B
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
pg	Picogram
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RPMI	Roswell Park Memorial Institute medium
SAGE	Study of Asthma Genes and the Environment
SEB	Staphylococcal enterotoxin B
SNP	Single nucleotide polymorphism
SPT	Skin prick test
SK	Streptokinase
TCR	T cell receptor
T cells	Thymus-derived lymphocytes
Th1 / Th2	T helper 1 / T helper 2

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Figure 46   Deficient and exaggerated anti-viral cytokine responses in asthma. Modified from model proposed by Gern [349], to reflect anti-viral immune profiles in humans in response to pneumoviruses. Adapted with permission from Lippincott, Williams and Wilkins.

INTRODUCTION

Respiratory Viruses Associate

with the Inception and Exacerbation of Airway Disease

in Asthmatic and Non-Asthmatic Humans

ALLERGIC DISEASE

Prevalence and Social Impact of Allergic Disease

The International Study of Asthma and Allergies in Childhood (ISAAC) was monumental for the appreciation of the worldwide prevalence and distribution of allergic disease [1]. Several global studies demonstrate a steady increase in the prevalence of atopy and asthma between the 1960s and 1990s [2]. Type I hypersensitivity diseases such as allergy, asthma, rhinitis and atopic dermatitis are an ever-growing health concern in industrialized societies. Canada highlights this greater prevalence of atopic disease, when compared to less developed nations. Allergy and asthma are rarely life-threatening, yet their chronicity begets a greater social impact through missed productivity at home, school and work, diet modification and daily activities, anxiety and occasionally depression [3-5]. These altered social patterns result in a multi-million dollar toll on the health care system and the economy. Estimated direct and indirect costs of asthma alone in Canada (1990) were between $504 - $648 million dollars [6]. Apart from medication, the next significant proportion of direct costs stem from urgent care of asthma exacerbations [7]. Therefore

attempts to understand, prevent and treat the triggers of asthma are a major contribution towards reducing the individual and social burden of allergic disease globally.

Manifestation of Allergic Disease

Atopy and Allergy

The traditional immunologic cornerstone of allergic disease is the development of Th2-like responses to innocuous antigens (allergens) resulting in the production of allergen-specific immunoglobulin E (IgE) [8]. This type of response to allergen is termed atopic sensitization, although it does not necessarily result in clinical allergy [9]. Sensitisation to allergens most commonly arises in childhood, although late-onset atopy does occur [9, 10]. Clinical diagnosis of atopic disease is assessed by identification of the offending allergen(s), in association with medical and family history of allergic symptoms. The most common measure of sensitisation to allergen is the skin prick test (SPT), despite indications it may not serve as a consistent marker of symptomatic allergic disease [11-13]. In individuals that demonstrate sensitization by SPT, up to 40% have never shown signs of allergic manifestation [12, 14]. In addition, the size of wheal and flare of a SPT is not a consistent indicator of allergy severity [12]. Local inflammation at the site of a SPT reflects an immediate IgE mediated response to allergen, although many patients may not display current or historical allergic symptoms when routinely exposed to said antigen. These discrepancies represent the heterogeneity among patients: SPT+/non-symptomatic, "classical" SPT+/allergic symptoms, and a rare (largely ignored) group of SPT-/allergic symptoms [12]. Moreover, the widely-used ImmunoCAP method, an *in vitro* measurement of IgE levels towards specific allergens, frequently concurs broadly with SPT results, but demonstrates incongruency in regards to cat, mold and grass allergen sensitization among

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others [15, 16]. Clearly, clinical markers of atopy on their own are not always sufficient to identify an allergic individual, highlighting the rift between measurable sensitisation and allergic manifestations.

Upon allergen exposure, allergic individuals may react with symptoms ranging from mild urticaria to severe anaphylaxis. Immediate symptoms are initiated by allergen crosslinking of IgE receptors bound to the surface of mast cells, basophils or eosinophils. This action leads to a rapid degranulation of effector cells. Release of preformed mediators from within these granules results in an acute inflammatory responses in the surrounding tissue. Local inflammation promotes recruitment and activation of additional leukocytes that propagate the Th2 type inflammatory cascade. Thus, an allergic reaction is characterized by two sets of responses: the acute response that occurs 15-30 minutes after allergen exposure and the late-phase response occurring 4-6 hours later, which can result in prolonged symptoms. Long-lived allergic inflammation due to late-phase responses is associated with the chronic symptoms of atopic asthma, rhinitis and dermatitis. Manifestations of allergic inflammation are varied depending on afflicted tissue, dissemination and severity.

Most people suffer from one or more atopic pathology. It is common to see the allergic triad: atopic dermatitis, allergic rhinitis and atopic asthma in children. Indeed, as many as 80-90% of atopic asthmatics also have allergic rhinitis [17], suggesting allergic inflammation affects the whole respiratory tract. As of late, the united airways' theory has attempted to bridge the link between asthma and rhinitis [18, 19]. Furthermore, otitis media with effusion (OME) is more frequently becoming a recognised inflammatory condition in individuals with allergic disease, suggesting the middle ear should be included in the concept of a united airway [20-24].

Asthma

The underlying pathology of asthmatic lung reveals damaged / repairing epithelium, mucus plugs, chronic inflammation and tissue remodelling [25-28]. These alterations in lung pathology are evident prior to symptomatic asthma, as they have been found in young children and pre-symptomatic individuals at high risk of developing asthma [29-31]. Although airway inflammation has classically been considered the pathologic mechanism of asthma, it would be more accurate to state that inflammation is the main contributor to asthma symptoms [32]. Hallmarks of acute asthma include airway hyper-responsiveness (AHR), variable degree of airway obstruction, mucus overproduction, air trapping and inflammatory cell infiltrates [25, 26]. Alteration of the physiologic structure of the lung (persistent AHR) and acute inflammation (variable or episodic increase in AHR over baseline) both contribute to the degree of AHR during an asthma exacerbation [26, 33].

AHR is a risk factor for the development of asthma, especially in first degree relatives of asthmatics [34-40]. In children, the prevalence of non-asthmatics with AHR ranges from 20 – 40% in population cohort studies [37, 41, 42]. Although the prevalence in adults drops to 9.2% in the general population, 40% of adults with rhinitis but no asthma display AHR, suggesting a clinical march towards symptomatic asthma [43]. However, many children with no history of breathing difficulties display AHR, which may persist for years prior to asthma symptoms (while some never develop asthma) [35].

Atopic status is not considered a compulsory attribute in the asthma phenotype, especially in children [44, 45]. Recent evidence points towards separate genetic risk for atopy and AHR/asthma [39, 45-48]. However, atopy does play a significant role in the severity and persistence of asthma [49]. Earlier studies argued as to the merit of the terms atopic asthma versus non-atopic asthma, and were challenged on the basis of an insufficient repertoire of

allergens for skin prick tests (SPT), in addition to asthma's consistent association with Th2 dependent inflammation and IgE mediated reactions [50, 51]. Moderately elevated serum IgE levels in non-atopic asthmatics may very well be a function of specific antibodies against unidentified allergens. However, more recent studies have demonstrated the heterogeneity of asthma is more likely due to differential susceptibility towards allergic and non-allergic triggers [25, 52]. Indeed, there are multiple triggers of asthma exacerbation (**Table 1** [32]), which are initiated by vastly different immune responses. Thus, children predominantly exhibit an intermittent pattern of episodic symptoms, while fewer demonstrate persistent symptoms related to chronic asthma, as seen in adulthood [53].

Timing of Genetic and Environmental Interactions in Allergy and Asthma

The cause of the increased prevalence of atopic disease in "westernized" countries is currently unknown. Causal hypotheses strive to address fundamental changes in lifestyle and environment over the past century. Theories include increased recognition and diagnosis of allergic diseases, childbearing latter in life, altered diet and physical health, stress, the hygiene hypothesis and environmental pollution and even excessive watching of television [2, 54, 55]. Early in the study of allergic disease, it was suspected that there was a strong genetic component due to increased allergy and asthma propagation within families having a previous history of allergic disease. Twin studies confirmed that the probability of developing an allergic disease increased if one (25-30%) or both parents (50-75%) were atopic, as compared to non-atopic parents (20%) [56]. Nonetheless, despite increased risk with family history, most affected individuals have no familial risk factors for allergic disease [57]. Taken together, genetic predisposition alone is insufficient to result in the manifestation of allergic disease.

Table 1: Common aetiologies of asthma exacerbation from Singh and Busse [32]. Reproduced with permission from BMJ Publishing Group Ltd.

Table 1 Common aetiologies of asthma exacerbations

Virus induced	Rhinovirus (RV)
	Respiratory syncytial virus (RSV)
	Human metapneumovirus (HMV)
	Influenza virus
Bacteria induced	*Mycoplasma pneumoniae*
	Chlamydia pneumoniae
Allergen	Fungi
	Tree, weed and grass pollen
	Indoor allergens
Occupational	Animal exposures
	Chemical exposures
Irritants	Airway pollutants

Genetic contributions by multiple gene polymorphisms may predispose individuals to be more susceptible to environmental influences. Single nucleotide polymorphisms (SNPs) can alter the expression or function of gene products, leading to various outcomes relating to inflammation, anti-microbial immunity and tissue remodelling [56, 58, 59]. In concert, continuous environmental exposures shape our dynamic genetic information through epigenetic regulation [60-62]. Epigenetic modification may have a dramatic impact on gene expression patterns and thus the overall immuno-competence of an individual [63-65].

Many environmental exposures are suspected of initiating allergen sensitisation and asthma pathology: lack of infection or exposure to microbial components, lack of gastrointestinal pro-biotic flora, use of antibiotics, vaccination, pollution, diet, physical health and stress, exposure to environmental tobacco smoke (ETS), allergen exposure and severe or repeated viral respiratory infections [66-71]. While infant airway development is incomplete at birth, inflammatory triggers may disrupt "normal" lung maturation and pilot remodelling processes in genetically susceptible individuals [33]. Pathogenic immunity (towards allergens, viruses or air pollutants), pulmonary vulnerability (smaller airways due to premature birth, ETS or gender) and/or insufficient repair processes may encourage architecturally altered lung growth in early life (**Figure 1**) [72]. After birth, lungs continue to develop to their full capacity, followed by a gradual decline in lung function with age in adulthood [73, 74]. Prospective studies examining lung function in asthmatics and non-asthmatics clearly demonstrate that lung function is relatively fixed, as demonstrated by longitudinal measurement of percent predicted forced expiratory volume in one second (FEV$_1$) in both groups (**Figure 2**) [75], and that any loss in airway function of asthmatics occurred prior to the age of 5 years [75, 76].
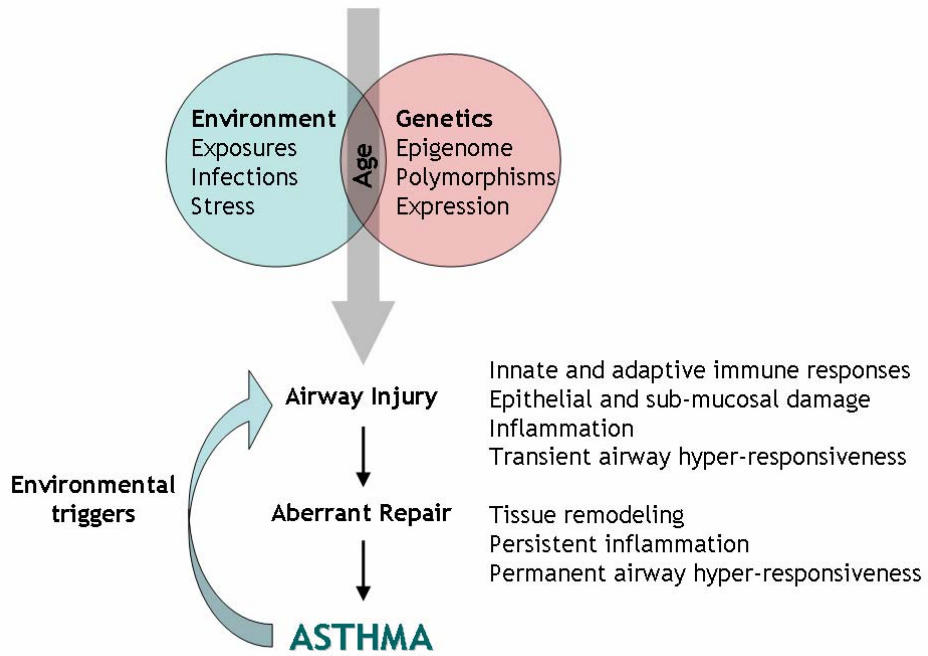
Figure 1: Pathogenesis of childhood asthma. Artwork by R. Douville.
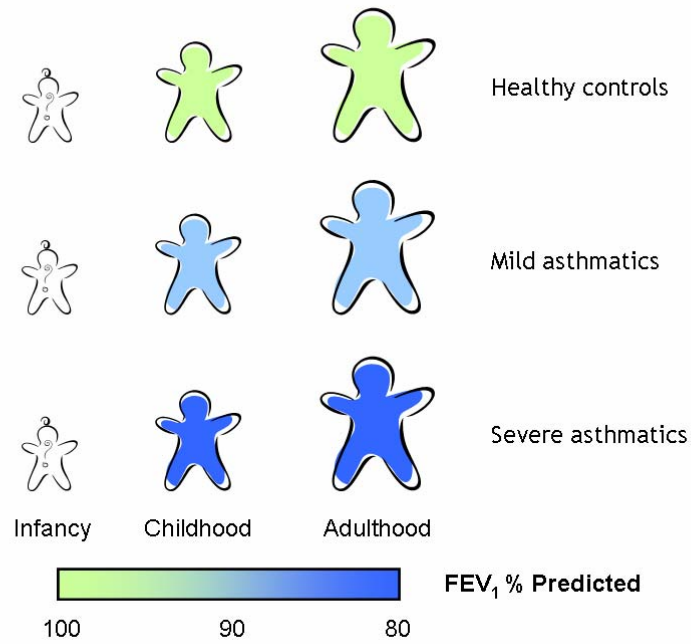
Figure 2: Lung function over time in asthmatics and non-asthmatics. Artwork by R. Douville.

In addition, the prevalence of allergic disease which predominates in young boys, switches post-puberty, resulting in twice the number of women having allergic disease in later life [77]. These findings predict 'critical windows' (in early life and at puberty) for the beginnings of symptomatic allergy and asthma.

Once allergy or asthma have been established, environmental exposures can elicit exacerbation of disease by acting as symptom triggers [33]. In school-aged children, asthma exacerbations are generally associated with sensitisation to aeroallergens and concomitant respiratory infection [77-79]. The risk of hospitalisation increases drastically (OR 19.4 [3.7-101.5]) in children with current respiratory virus infection and high exposure to an allergen for which they are sensitized [80]. Many studies have also demonstrated that asthma hospitalizations in children coincide with degree of air pollution to which they are exposed [81-83]. Fungal allergies (especially sensitivity to *Alternaria*), contribute significantly to seasonal rhinitis and hospitalizations in both children and adults [84]. Taken together, multiple triggers can initiate exacerbation of allergic disease, with respiratory viruses adding synergistic effects alongside allergen exposure.

Development and Maintenance of Immuno-Regulatory Responses in Allergic Disease

Cytokines and chemokines serve as immunomodulatory proteins, which help shape and direct immune responses and lymphocyte repertoires. Embraced by immunologists and other disciplines, the Th1-Th2 paradigm initially proposed by Mosmann and colleagues, has provided a useful framework for categorizing cytokine and chemokine responses from CD4$^+$ T helper cells based on anti-microbial/autoimmune (Th1) and anti-parasitic/allergic (Th2) immunity [85]. With evolution of the paradigm, the CD4$^+$ T cell cytokine profile has expanded

to include cytokines that promote or contribute to Th1 and Th2 differentiation. Polarised cytokine secretion patterns are now dubbed Th1 type (IL-12, IFNγ, CXCL10,…) and Th2 type (IL-4, IL-5, IL-13, CCL17,…), or Type 1 and Type 2 responses, and can display mutually antagonistic effects. Therefore, they provide important information regarding the internal control mediating effector pathways of immunity and allergy [86, 87].

Allergy and asthma are characterised as diseases promoted by the excessive production of Th2 type cytokines and chemokines linked to the production of allergen-specific IgE. Immunologically, it has become clear that allergen-specific cytokine and chemokine responses can be measured in the entire population, not just sensitised subjects [88, 89]. Allergen-specific responses from non-atopic individuals clearly exist, but do not share the cytokine pattern typified by atopic or allergic subjects. In general, non-allergic individuals respond to allergen with demonstrably weaker Th2-bias than allergic subjects do. Thus, the continuum of responses to allergen seen in the general population, from protective to hyper-responsive, may represent a gradual breakdown in one's ability to maintain active tolerance to innocuous antigens. Tolerogenic mechanisms which subvert the expansion of atopic defects are scantily understood, although mounting evidence points towards the action of regulatory T cells [90].

As aforementioned, allergen-specific Th2 type responses and IgE production are conventional markers of atopic disease. The involvement of Th1 polarised cells in initiating and promoting allergic responses is an emerging concept in recent literature [87, 91-94]. Many conventional viewpoints still embrace Th1 type redirection as a means of diminishing Th2-mediated inflammation, despite mounting evidence to the contrary [92, 95-98]. In reality, the observation of Th1 mediators enhancing existing Th2 inflammation ties into well known

concepts about virus-triggered asthma exacerbation. Respiratory RNA viruses have had a long-standing association with asthma and allergic sensitisation, both in terms of inception and exacerbation. Additionally, there is familial susceptibility for the development of severe lower respiratory tract infection (LRTI) in the offspring of asthmatic mothers, suggesting that allergic disease may influence the susceptibility or severity to respiratory infection [99]. Unfortunately, it remains largely unknown whether innate and adaptive T cell anti-viral immunity differs in allergic disease versus health. A broader appreciation of anti-viral immune responses in human with specific clinical outcomes permits us to re-examine the role of respiratory viruses and anti-viral mechanisms in the development and maintenance of allergy and asthma.

INNATE & ADAPTIVE IMMUNITY AGAINST RESPIRATORY RNA VIRUSES

Respiratory virus infection debuts with relatively few infectious particles (hundreds to thousands) being inoculated into the airway epithelium. The epithelial layer is believed to be the primary site of virus replication, although other cells may become infected as propagation occurs. Infection progression increases tissue damage, such as epithelial desquamation, airway oedema and increased mucosal permeability. Deposition of cellular debris and mucus into the airway lumen obstructs airflow, which contributes to wheezing.

Consequently, an acute viral infection forces the host immune system into a race against viral replication. The stakes are high: death is limited to infected cells or death of the host. For host immunity, the up-side is that replicating virus provides a constant supply of antigen, both endogenous and exogenous. There are two main effector arms that combat viral infection; the innate and adaptive systems. Although these players may use different tactics, there is constant communication between both forces. This usually takes the form of antigen presentation, costimulatory molecules and soluble intercellular signals.

Pattern recognition receptors in the innate response to respiratory RNA viruses

Innate immunity is a collection of intrinsic protective mechanisms that aim to recognise pathogens and thwart their attempts to colonize the host. Innate immunity generates no immunological memory. At best, in most viral infections, the innate response reduces the systemic spread of virus. This buys time for the host organism to develop effective adaptive immune responses against the invading microbe. Therefore, innate immunity against respiratory viruses confers protection by way of non-specific viral recognition and clearance. Such a task is in part achieved through the use of pattern

recognition receptors (PRRs) which evolved to identify pathogen-associated molecular patterns (PAMPs) [100], or more precisely defined by the term MAMPs (microbe-associated molecular patterns, which encompass both pathogenic and commensal organisms). MAMPs are produced by microorganisms, and are not native to the host. A general characteristic of MAMPs is their conserved structures ("signatures") which are usually essential survival components shared by a group of microbes. Classic examples of these MAMP danger signals include, but not are limited to: LPS, peptidoglycan, double stranded RNA (dsRNA), flagellin and CpG DNA [101, 102]. Renewed interest in innate immunity surfaced with the discovery of a distinct family of pattern recognition receptors, named Toll-like receptors (TLRs). TLRs involved in recognition of RNA viruses are TLR3 (dsRNA), TLR4 (RSV F protein) and TLR7/TLR8 (ssRNA) [103, 104]. Although TLR recognition of viral products is very well characterised, recent studies have demonstrated that RNA helicase enzymes are essential mediators of innate immunity. Cytoplasmic RNA helicases, such as retinoic acid-inducible gene I (RIG-I; 5'-triphosphate RNA/3pRNA) and melanoma differentiation-associated gene 5 (MDA5), in addition to their negative regulators, such as LGP2, demonstrate specific roles in innate responses to RNA viruses [104, 105].

Using respiratory syncytial virus (RSV) as a prototypic example of virus versus innate host defence, we will examine the role of PRR in the identification and response against RNA viruses and the evasive viral mechanisms of innate immunity. RSV particles contain 3 main components, a lipid envelope, viral proteins and ssRNA. Upon infection, 3pRNA and dsRNA are formed as by-products of viral replication. Taken together, many conserved motifs are available for innate viral recognition of respiratory viruses (**Figure 3**).
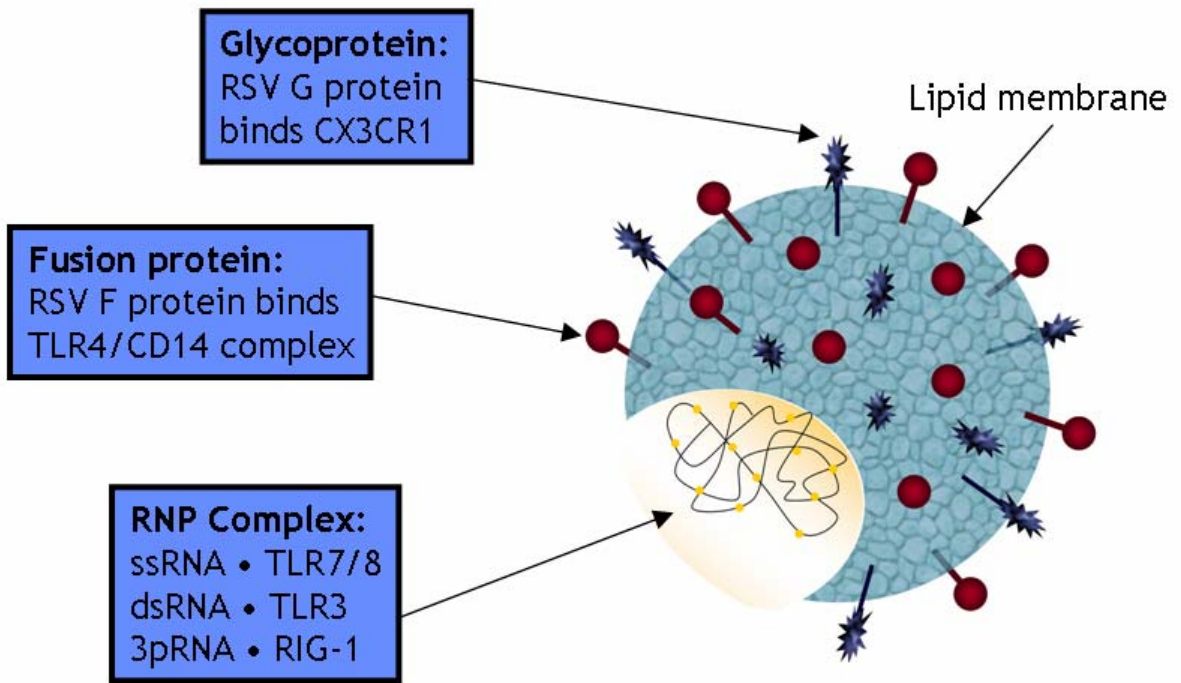
Figure 3: Recognition of RSV viral motifs by the innate immune system. Artwork by R. Douville.

Innate Recognition of Viral Genomes

Recognition of foreign viral RNA genomes is a key component of successful anti-viral innate immunity. This is evidenced by the various cellular locations of RNA-specific PPRs, either cytoplasmic (RIG or MDA5), extra-cellular plasma membrane-bound (TLR3) [106] or endosomal membrane-bound (TLR3 and TLR7/8). RSV is able to avoid TLR7/8 detection by directly entering host cells via membrane fusion [107]. Moreover, RSV infection of plasmacytoid dendritic cells (pDC) can inhibit TLR7 and TLR9 signalling, thus suppressing innate responses against other viruses and bacteria [108]. Taken together, RSV not only avoids the endosomal recognition pathway, but also diminishes its function during infection.

Therefore, cytoplasmic recognition of RSV replication is essential in driving innate type I interferon responses. RIG successfully fills this role in epithelial cells [109]. RIG, but not MDA5, specifically recognizes RSV RNA and induces NF-κB and IRF-3 transcription factors, which prompts inflammatory cytokine production. Early RIG signalling also induces surface TLR3 expression through the action of paracrine IFNβ [109, 110]. This TLR3 up-regulation is essential for epithelial CCL5 and CXCL10 production in late response to infection [109, 111], likely by increasing epithelial sensitization to dsRNA [112]. Interestingly, RSV induced TLR3 signalling does not alter the course of infection, but plays a significant role in maintaining lung homeostasis [113]. TLR3-/- mice demonstrate increased pulmonary Th2 cytokines and mucus production [113]. As RIG is responsible for proper up-regulation of TLR3, impairment in RSV RNA recognition may lead to increased lung pathology during infection. To this effect, RSV non-structural (NS) proteins are known to bind and inhibit IRF-3 signalling and diminish IFNα/β responses [114]. This has a significant effect on RSV infectivity in humans [115]. Hornung *et al.* have demonstrated that pDC IFNα production

varies greatly (with bimodal distribution) between individual human subjects in response RSV, suggesting that the efficiency of viral RNA recognition may play a role in the severity of infection [107]. Unlike paramyxoviruses (RSV), picornaviruses like rhinovirus (RV) are recognised by MDA5 for the induction of type IFNs [116].

The production of IFNα, IFNβ and IFNλ is classically known to elicit a systemic anti-viral state. IFNs activate enzymes which inhibit viral replication, facilitate apoptosis of infected cells, activate natural killer (NK) cells and prime other cells to express IFNs [117-120]. This mechanism has a priming effect for further pathogen recognition. The functions of IFNs are also interlaced with the development of adaptive responses. Maturation of dendritic cells can be induced by IFNs. In fact, recent evidence points to IFNα / IFNβ in promoting a natural adjuvant effect for improved cross priming of exogenous antigens to the MHC I pathway, antibody production and T cell priming [110, 118].

Innate Recognition of Viral Proteins

The impact of RSV fusion (F) protein recognition by the CD14-TLR4 complex in innate immunity remains controversial [103]. Poor design of murine studies (use of TLR4 deficient mice with IL-12R deficiency, use of pneumonia virus of mice (PVM) instead of RSV), results in conflicting claims about TLR4 involvement in RSV immunity and lung pathology [103, 121-124]. More convincing is RSV's ability to up-regulate surface TLR4 expression in monocytes and epithelial cells [125, 126]. Infection of epithelial cells with RSV sensitizes them to increased LPS binding and responsiveness [125]. Epithelial cells are generally unresponsive to LPS stimulation; however RSV pre-sensitization resulted in robust pro-inflammatory cytokine production [125]. Thus, RSV infection may indirectly alter innate immunity in the lung by increasing surface expression of TLR4 (as well as TLR3) and

facilitate bacterial colonisation of airway epithelium [127]. As TLR4 responsiveness has been linked to allergic disease susceptibility and exacerbation [59, 128], this viral induction of TLR4 expression may contribute to asthma pathogenesis.

Likewise, RSV G protein also exhibits an immuno-modulatory effect on the innate immune system. Soluble RSV G protein, secreted from RSV infected cells, has the ability to interfere with lung surfactants, NK cell activation and epithelial cell pro-inflammatory cytokine production.

First, purified RSV G protein can bind to CX3CR1 in the airways, leading to increased surfactant protein D (SP-D) levels, which results in apnea and respiratory distress in uninfected mice [129]. SP-D specifically binds RSV glycoprotein (G) and fusion (F) proteins on whole RSV particles [130]. However, RSV G protein in excess could compete with RSV binding for SP-D and inhibit SP-D mediated phagocytosis of RSV [129]. Despite a role for SP-D in viral clearance from infected lung tissue, soluble RSV G protein counteracts this mechanism, likely leading to increased infection and reduction in respiratory rate of infected individuals.

RSV G protein may also inhibit natural killer (NK) cell activation by mimicking the chemokine CX3CL1 [131]. In a dose dependent manner, membrane bound CX3CL1 on inflamed endothelium enhances IFNγ production by NK cells [132, 133]. CX3CL1 signals also enhance NK cell cytolysis, where these cells perform lysis regardless of target cell expression of MHC I [132]. If soluble RSV G protein saturates NK cell CX3CR1, it would impair NK binding to the membrane bound form required for activation. As over 90% of CD16^{+} NK cells from peripheral blood of healthy adults express CX3CR1, secretion of RSV G protein by infected cells in the inflamed airway may drastically impair the activation and function of NK cells. Although this CX3CL1-antagonism of NK cell inhibition has been

described in other models of infection and autoimmunity, it has yet to be demonstrated in RSV infection.

RSV G protein is also believed to act via the cystein-rich domain (GCRR), independently of CX3CR1 binding, to depress pro-inflammatory cytokine production by airway epithelial cells and macrophages [134, 135]. Interestingly, the suppression of pro-inflammatory cytokines by the GCRR is not limited to innate RSV responses, but can also inhibit LPS-driven cytokine production as well [135]. However, it remains unclear how the GCRR dampens the innate response. In summary, RSV G protein can modulate various components of airway innate immunity through CX3CR1 dependent and independent mechanisms, thus increasing the challenge of the adaptive system to resolve the infection.

Airway microenvironment following innate response to respiratory RNA viruses

As respiratory virus infection occurs within the airway, several cell types may serve as targets of replication (epithelium, neurons, airway smooth muscle, DC, macrophages and infiltrating immune cells) [136-140]. Virally infected structural and immune cells produce inflammatory mediators in response to viral recognition and cellular damage. Cell type-specific innate responses by infected cells initiate both intracellular anti-viral pathways and emit paracrine (and systemic) signals to surrounding cells. This early warning system activates anti-viral pathways and inflammation in adjacent tissue. Thus, the innate anti-viral response within the lung sets the stage for ongoing inflammation, cellular recruitment, and initiation of the adaptive response, repair or chronic inflammatory processes (**Figure 4** [141]).

Structural cells, such as epithelium and neurons, play a central role in directing innate anti-viral immunity [142, 143]. Activation of transcription factors NF-κB in virally infected

epithelial cells leads to release of cytokines (IL-11, IL-1β) and chemokines (IL-6, IL-8, RANTES, MCP-1 and MIP-1α) [144-152]. Adhesion molecules for leukocytes, such as ICAM-1, are also up-regulated in the airways during viral infection [153, 154]. Increases in chemokines, inflammatory mediators and adhesion molecules lead to increased leukocyte trafficking to the lung [155]. Epithelial cells engage anti-viral responses in order to eradicate infection; however, inflammation itself can cause unavoidable tissue damage. Damaged epithelium undergoes extensive repair processes, prior to re-establishing an uninterrupted airway barrier [156].

An often overlooked component of lung immunity is neurogenic inflammation [142]. As shown in **Figure 4**, the lung is innervated by autonomic nerves, which are essential for ASM tone, mucus secretion, microvascular permeability and immune cell trafficking [142]. RSV infection impacts neuroimmune mechanisms, by upregulating tachykinin neuropetide substance P (by unmyelinated C-type sensory afferent nerve fibers below the epithelial surface) and its receptor NK1 (on epithelium, vascular endothelium and T cells) [157]. This heightened stimulation of C-type sensory nerves (which can be further amplified by nerve growth factor (NGF) secreted from RSV-infected epithelium) facilitates airway contraction, neurogenic inflammation and neuro-immune cross-talk [157, 158]. This mechanism likely underlies the increased AHR in humans and animals during pneumovirus infection [159-162].
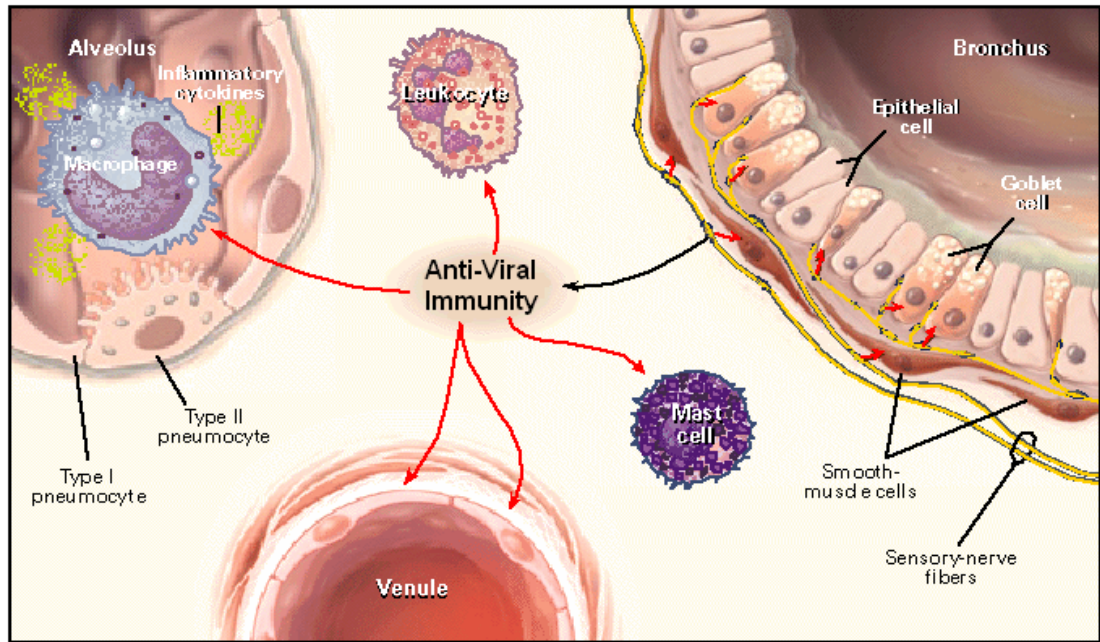
Figure 4: Lung microenvironment is modulated by innate anti-viral immunity. Adapted from Colten and Krause [141] with permission from Massachusetts Medical Society.

Signals from resident airway cells also stimulate activation of innate immune cells. Alveolar macrophages and mast cells that dwell within the lung tissue, and these sentinel cells for innate detection of viruses can become activated during early phases of infection [130, 163, 164]. In addition, neutrophils are the most rapidly recruited and numerous inflammatory cell within RSV infected airways [165]. Although first to arrive in RSV infected airways, neutrophils do not specifically display innate recognition of RSV [166]. Instead, neutrophils become activated by epithelial derived-factors [166], such as surfactant protein D [130]. SP-D specifically binds RSV glycoprotein (G) and fusion (F) proteins, which enhance phagocytosis of RSV by alveolar macrophages and neutrophils [130]. Accordingly, immune cells mediating the early innate response to RSV receive crucial signals from infected tissue for boosting the inflammatory response and inhibiting virus spread by mopping-up debris within the airway. Taken together, the microenvironment created by structural and immune cell innate responses towards infection have a significant impact of the ensuing adaptive response.

Initiation of the adaptive response to respiratory RNA viruses

The adaptive immune system receives critical clues for conduct from the microenvironment created by the innate response to virus. Furthermore, the activation state and costimulatory capacity of antigen-presenting cells (APCs) help direct T cells to engage in viral clearance. For activation, T cells require both antigen-specific recognition signals (mediated by engagement of TcR complex with cognate peptide-MHC on APC) and costimulatory signals. Costimulation acts as an amplification step that helps exceed or inhibit threshold limits for activation in T cells. Innate stimuli can increase the expression of

costimulatory molecules on APCs, which is an important link between the innate and adaptive responses.

Dendritic cells can fulfill the task of APC within the lung, as their networks survey the forefront of the sub-epithelial airway. DC can become infected with respiratory viruses; this initiates innate anti-viral mechanisms and engages the antigen presentation machinery necessary to activate T cells. However, RSV infection of DCs leads to an inability to prime naïve T cells into IFNγ producing effectors [167]. Moreover, this decreased ability to activate CD4$^+$ T cells extends to RSV-specific T cells <u>and</u> T cells specific for other antigens presented by the infected DC [168]. De Graff *et al*. propose that this effect is mediated via an unknown soluble mediator secreted from RSV-infected DCs [168].

T cell-mediated cytokine responses stimulated by virus have the function of i) directing the adaptive and innate effector cells, ii) activating cytolytic cells to clear virally infected host cells, and iii) modulating the intensity of the anti-viral response to avoid pathologic tissue damage, via anti-inflammatory mechanisms such as the production of IL-10. Viral antigen presentation occurs via the major histocompatibility complex (MHC I, endogenous) and MHC II (exogenous) pathways, leading to respective virus-specific populations of CD8$^+$ (CTL) and CD4$^+$ T cells. Upon re-exposure, viral clearance is generally executed via cell-mediated memory responses, whereby high levels of IFNγ are produced as a signal for cellular induction of anti-viral mechanisms. IFNγ is a multi-functional cytokine, with sometimes paradoxical effects, as reviewed in [169]. Enhanced virus detection capacity is mediated by IFNγ, and leads to broad increases in MHC I and II molecule expression. Moreover, IFNγ is also a powerful pro-inflammatory cytokine. As discussed earlier, epithelial cells play an important role in leukocyte recruitment during viral infection. IFNγ in the airways increases virus-induced chemokine production, thus acting as a positive feedback mechanism for increased airway inflammation [170]. It can also promote

superoxide secretion from eosinophils and histamine release from basophils, which would seemingly enhance allergic inflammation. All subjects, atopic / asthmatic / non-atopic, demonstrate Th1 type dominated responses to RNA viruses, with minimal to undetectable levels of Th2 type cytokines [171]. Taken together, this clearly demonstrates the necessity of IFNγ-mediated Type 1 responses in the elimination of respiratory virus infection.

Modulation of host anti-viral mechanisms can be mediated by IL-10. In the balance between protection and pathology of innate and adaptive immune responses to viruses, IL-10 plays a key role in minimizing the duration and intensity of the immune response. As reviewed in [172], IL-10 can limit many steps in the development of immunity, such as macrophage/monocyte activation, TLR4, MHC II and costimulatory molecule expression, cytokine and chemokine synthesis and effector functions like nitric oxide production. Ironically, IL-10 also has stimulatory effects on $CD8^+$ T cells in terms of recruitment, activation and proliferation. Pathogen-specific T cell responses can be restored in "unresponsive", but infected patients with the use of mAbs against IL-10. Taken together, this imparts a specific function for IL-10 in viral infection: promoting targeted killing of infected cells while limiting collateral damage of excessive Th1 type inflammation.

Primary immune encounter with virus also stimulates the production of antiviral antibody responses (seroconversion). Antibodies recognise intact virus or cell-bound viral antigens. Neutralization of viral particles occurs by agglutination, thus preventing further infection of surrounding cells. However, some RNA viruses induce poor antibody responses. Early life infection with RSV does not consistently result in the production of neutralizing antibodies in serum or mucosa [173-176]. Maternal antibodies are thought to contribute to the protection of infants against respiratory disease. High serum levels of

maternally transferred RSV neutralizing antibodies directly correlate with milder RSV illness in infants [177, 178]. Detectable levels of maternal RSV-specific antibodies circulate in the infant at birth and persist upwards of six months [179]. Avidity of infant antibodies is generally poor, especially in response to RSV G protein [180]. Antibody responses wane within months of the primary RSV infection in infants [174], and may contribute to the high re-infection rate. Only re-infection promotes the development of robust humoral responses, whereby passive transfer of maternal antibodies can suppress and delay active humoral responses in infants [181]. Ergo, failure to reach immunologic T cell maturity quickly may result in the inability of infants to mount sufficient anti-viral defences.

Airway pathology resulting from the adaptive response to respiratory RNA viruses

Due to discrepancies in human and murine models, RSV has become known predominantly as a Th2 promoting agent in the literature. This idea has been propagated largely because it is an attractive explanation for the apparent association of RSV with allergy and asthma. However, when one carefully examines experimental research on the pathology and immunology of RSV infection, it becomes clear that classical host anti-viral responses are predominant. Immunological studies of both *in vivo* and *in vitro* models of RSV infection in humans suggest a Th1 type biased response. In adults, PBMC memory responses to RSV are Th1 dominated as measured by increased mRNA and cytokine production [182-184]. Comparison of RSV-specific responses in adult PBMC and cord blood PBMC demonstrate that adults produce marginal, but detectable IL-4 responses to UV inactivated RSV (which may be an underestimate due to the lability of IL-4), whereas the innate pro-inflammatory response to inactivated RSV was more robust in neonates [183]. Otherwise, responses from neonates and adults demonstrate a similar pattern of RSV-

specific cytokine production: increased IFNγ, IL-10, IL-6 and TNF-α, but not IL-5, IL-13 or IL-12. Other publications have made claims like "RSV infection in infants is associated with predominant Th2-like response" [185], however the measurements were derived from phytohemagglutinin (PHA) mitogen stimulated PBMC cultures of RSV infected infants and controls. This does not represent an RSV-specific response, insomuch as it describes the propensity of these infants to respond to polyclonal stimulation. Studies suggesting that RSV fails to induce a Th1 response in PBMC have examined cytokine production after 48 hours of culture and therefore are actually measuring innate immune responses [186]. Complementary to human models of RSV infection, murine models also demonstrate a RSV-specific Th1 response besides being characterised by eosinophil recruitment [187, 188]. IFNγ is the key mediator in the anti-viral response to RSV [189]. Identification of RSV proteins that induce IFNγ have demonstrated that the fusion (F) protein and M2 protein induce strong $CD8^+$ T cell CTL responses, mediated by Th1 type cytokine production [188]. In constrast, the RSV viral attachment (G) protein induces recruitment of $CD4^+$ T cells and mediates eosinophilia in mice [190]. Together, human and murine research confirms that natural infection with RSV elicits a Th1 type memory response. However, the nature of the primary RSV response and anti-viral responses during current RSV infection in humans remains to be elucidated.

Neonatal (but not weanling or adult) mice infected with RSV spontaneously develop long-term airway inflammation, IL-13 in BALF, AHR and subepithelial fibrosis [191, 192]. Culley demonstrated that early infection with RSV leads to more severe disease compared to adult infection [193]. Reinfection of neonatally-infected mice is also more severe and Th2 biased. This suggests that initial age of infection with RSV can shape life-long disease outcome. In human, respiratory virus infection under the age of 3 months, leads to increased

Th2 cytokines in nasopharyngeal secretions compared to older infected children and children without infection [194].

Therefore, if RSV exerts Th2 promoting adjuvant effects, it is not mediated by virus-specific Th2 cytokine production directly. Indirect mechanisms of RSV-mediated Th2 redirection of the immune system have been postulated [195], but have not been confirmed in human models. Murine models however, have demonstrated that RSV infection can amplify inflammation and AHR in allergic mice, although the mechanisms by which this occurs are not necessarily Th2 cytokine driven.

Viral counter-strike on immunity and memory facilitates virus persistence

Immune deviation by viral proteins

As mentioned above, respiratory viruses may target anti-viral pathways in order to subvert innate and adaptive immunity. RSV is particularly proficient, as it targets multiple pathways: expression of non-structural (NS) proteins which inhibit JAK-STAT and IRF3 pathway for type I interferon expression, inhibition of TLR7 and TLR9 signalling pathway, inhibition epithelial cell apoptosis of RSV-infected cells and multiple effects of G protein mimicry of CX3CL1 [196-200].

RSV glycoprotein (G) protein is highly immuno-modulatory, as it mimics the chemokine CX3CL1 (fractalkine) [131]. The G protein can be translated as a membrane bound protein or a secreted form. Roughly, 10% of all G protein in an RSV infected cell is secreted, thus creating a CX3CL1-like gradient. This likely plays a significant role in shaping the infected lung microenvironment, as CX3CL1 and RSV G protein are important

for inflammatory cell trafficking during viral clearance [201, 202]. However, this is a double-edged sword, as the expression of CX3CR1 on various cell types, such as NK cells, monocytes, DCs, T cells and neuronal cells facilitates RSV infection [131, 137, 198].

In addition, RSV G protein acts as a potent chemoattractant for NK cells, mast cells, monocytes and T lymphocytes [201, 203, 204]. It has been reported that soluble CX3CL1, and perhaps RSV G protein, favors Th1 polarized cell recruitment over Th2 cells [205]. Among T cells, soluble CX3CL1 preferentially recruits perforin and granzyme B expressing cytotoxic cells [201].

Structural (neurons and endothelial cells) and antigen-presenting cells (APCs) also utilize CX3CL1 as a selective adhesion molecule [133, 204, 206-209]. Moreover, CX3CL1 can costimulate both innate and adaptive immune responses, via modulation of NK cell and T cell function [207, 210-212]. Indeed, RSV-specific effector T cells are entirely of the CX3CR1$^+$ phenotype [202]. Blocking the adhesive or costimulatory effect of CX3CL1/CX3CR1 can be achieved by the antagonistic effect of soluble CX3CL1 or RSV G protein. Taken together, the secretion of RSV G protein can alter immune cell recruitment, adhesion, and innate and adaptive immune activation during RSV infection.

Incomplete immunity to respiratory viruses leads to symptomatic reinfection

There is strong epidemiological evidence that natural immunity to RSV does not confer long term protection. Re-infection is common among all age groups [213]. This may be partially explained by the fact that re-infection with RSV does not boost T cell immunity [214]. Indeed, the frequency of circulating RSV-specific CD8$^+$ T cells is much lower than that of influenza-specific CD8$^+$ T cells [215]. Murine studies have demonstrated that RSV infection impedes T cell signalling, resulting in impaired development of CD8$^+$ T cell memory [216].

Impairment in recall immunity likely contributes to repeated symptomatic infections. This is perhaps why RSV elicits the weakest known immune responses and most severe infection symptoms of the commonly studied respiratory viruses [217-220].

To complicate matters further, it has been demonstrated that RSV (and MPV) infection lead to low level viral persistence in the airways and circulating peripheral blood mononuclear cells (PBMC) [138, 139, 221-224]. Screening of human PBMC has revealed persistence of RSV RNA (genetic and mRNA) in individuals with and without current RSV infection [139]. This demonstrates that despite anti-viral immunity, these viruses are effectively able to evade the immune system. In murine and guinea pig models, low level replication in the lung occurred despite CTL activity and neutralizing antibodies [223]. It has been postulated that viral persistence may contribute to the pathogenesis of chronic wheezing, AHR and asthma in children who have had a severe infection with RSV [198]. Furthermore, infected PBMC may act as an infectious reservoir, contributing to repeated low-grade infection of lung tissues. It remains speculative whether delayed presence of RSV within immune cells, subsequent to active infection, may also alter immune function. RSV persistence in macrophages can alter cytokine profiles, Fcγ receptors and phagocytosis, which may impact on lung homeostasis [225]. The effect of viral persistence on long-term airway function is not known, nor is the extent of virus persistence in asthmatics versus healthy individuals.

AIRWAY REPAIR & REMODELING SUBSEQUENT TO VIRUS INFECTION

The extent of viral replication and propagation within the airways determines the amount of tissue damage resulting from infection. The virulence of respiratory pathogens varies, and thus so does the degree of damaged airway epithelium. RV infection results in minimal tissue damage [226], whereas RSV infection results in significant epithelial desquamation and airway injury [157, 192, 227-229]. The more difficult for the immune system to stop infection, the more likely immune mediated pathology will occur. Once immune responses have cleared the infection, repair processes can be engaged to regenerate airway integrity.

RSV and MPV are known to cause airway remodelling in mice [192, 230], although evidence in humans has not been demonstrated experimentally, so much as epidemiologically [231, 232]. This viral remodelling is associated with epithelial injury, persistent mucus production, Clara cell secretory protein-positive airway plugs, myofibroblast thickening and subepithelial fibrosis [192, 230], similar to that seen in asthmatic patients [156]. The mechanisms proposed to cause airway remodelling involve both virus and host-dependent events involved in viral clearance and tissue repair. The epithelial-viral-allergic paradigm [233] or "Hit-and-Run" effect [234], as proposed by Holtzman *et al*., suggest that virus infection can cause changes in epithelial behaviour, some of which may have permanent effects as exemplified in asthma. This viral "reprogramming" involves permanent CCL5, Stat-1 and IRF-1 signalling, despite the lack of IFNγ, which normally engages these pathways [234]. Thus, the inability of asthmatics to turn-off virus-activated pathways in epithelial cells subsequent to infection may contribute to enduring changes in the airway environment, such as AHR and goblet cell metaplasia (which are favourable

devices for viral clearance). Also problematic is the observation that asthmatic individuals display intrinsic defects in their ability to repair damaged epithelium [156], as depicted in **Figure 5**. Chronic epithelial injury leads to release of fibro-proliferative and fibrogenic growth factors that contribute to subepithelial remodelling [156]. Unfortunately, airway remodelling, unlike airway inflammation, is the cornerstone of irreversible AHR [26]. Taken together, virus infection of the airways can initiate immunological and structural events that shape both immediate and long-term airway function. It remains unclear whether the balance of reversible and irreversible airway remodelling contributes to the "outgrowth" of asthma in certain children versus persistent asthmatics.
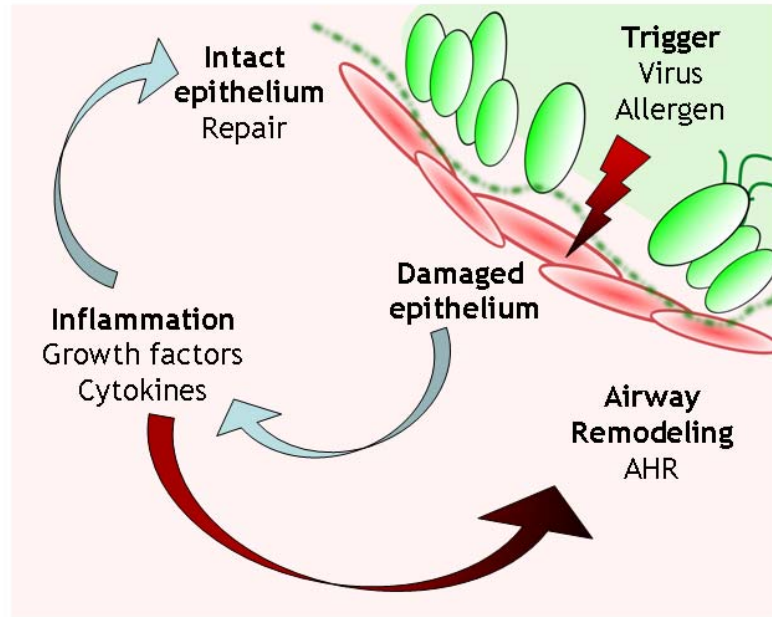
Figure 5: Mechanisms of airway remodelling following virus infection. Artwork by R. Douville.

RESPIRATORY VIRUSES AND THE INCEPTION OF ALLERGIC DISEASE

Prevalence and pathogenesis of respiratory virus infections in childhood

Prevalence of viral respiratory infections in childhood

RNA viruses are the major cause of respiratory illness in all age groups. However, infancy and early childhood claim the most severe respiratory infections. During infancy, the most prevalent type of infectious agents causing lower respiratory tract illness are from the *Paramyxoviridae* virus family: namely Respiratory syncytial virus (RSV), metapneumovirus (MPV) and parainfluenza viruses (PIVs) [235, 236]. Other RNA viruses that can lead to respiratory infection include rhinovirus (RV) and influenza virus [237, 238]. All these pathogens can result in either upper respiratory tract infection (URTI) or lower respiratory tract infections (LRTI) [237, 239, 240]. Serious LRTIs that exhibit increased severity and duration can lead to bronchiolitis (inflammation of the bronchioles in the lower airway) or pneumonia in some individuals. However, severe RSV infection (bronchiolitis) is more predominant (> 2 fold) and occurs at younger ages (mean 5 months versus 13 months) than with RV [241-243]. Human metapneumovirus (MPV), recently discovered in 2001, is a prominent cause of virus-induced hospitalization (14.5%) in infants under 2 years of age [244], especially in severe cases of bronchiolitis [245, 246].

In school-aged children RV, followed closely by RSV, are the most prevalent RNA-based respiratory pathogens causing lower and upper airway infections [243, 247]. Also, children with RSV infection are more likely to wheeze during (but not after) infection than with RV infections (50% versus 25%) [247]. Interestingly, virus infection leading to severe symptoms is increasing in the general population, with more than double the number of RSV

hospitalizations between 1990 and 1999 [248]. Urbanization and increased population density has been proposed as a cause of increased size of RSV epidemics [248].

Clinical outcomes of respiratory virus infection in childhood

Although clinical symptoms of different respiratory infections can be indistinguishable, it has become clear that the outcome and long-term prognosis may be drastically different between diverse types of infections. RSV infection in most children passes as a mere upper respiratory tract infection, although 12-40% of children experience a transient lower respiratory tract infection for reasons unknown. Severe infection in infants can be life threatening with some cases resulting in hospitalization. Indeed, 31 out of 1000 children under the age of 1 are hospitalized due to RSV bronchiolitis per year [249]. Large numbers of hospital admissions usually occur during seasonal outbreaks, between November and April [250]. Symptoms of hospitalized children can include air-trapping, wheezing, hypoxemia, atelectasis, tachypnea and cyanosis [251]. It is hypothesized that RSV induced death may occur by excessive pulmonary oedema, followed by respiratory failure in the infant [252]. The rush to protect newborns from RSV is fuelled in part by an association with subsequently elevated risk of developing asthma and atopic disease [253-257].

Although virus-induced wheeze (and wheeze caused by other heterogeneous triggers) is common in young children, not all wheezing is associated with allergic asthma in later life [52, 258]. Children have been categorised as "persistent wheezers" and "transient wheezers" as shown in **Figure 6** [259], the former being associated with increased serum IgE levels and eosinophilia during acute LRTI in infancy and transient wheezers displaying similar non-atopic characteristics as non-wheezers [260, 261].
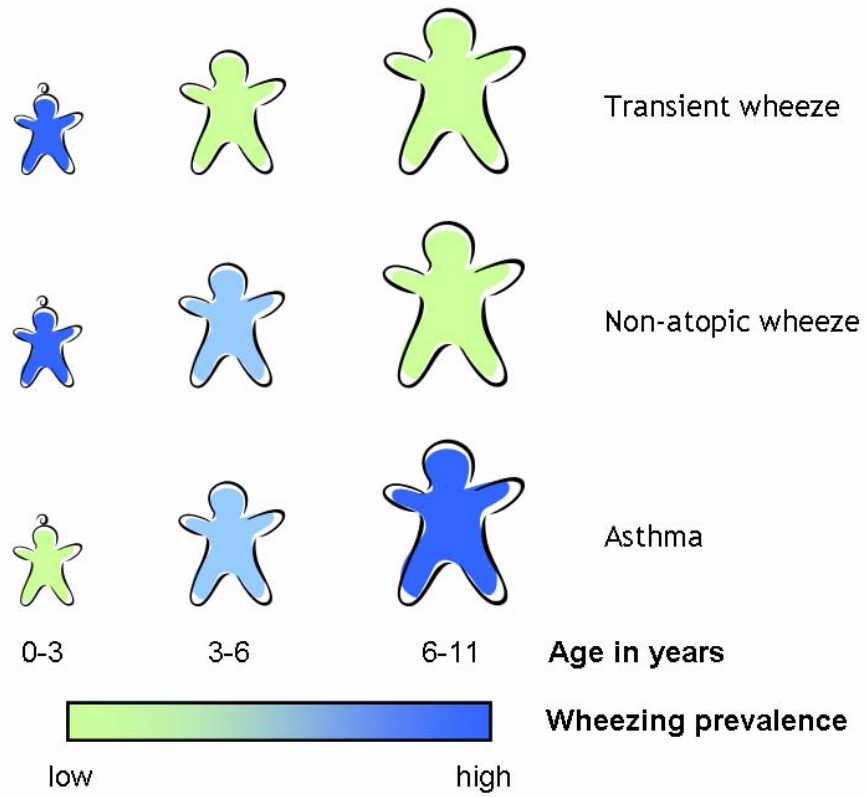
Figure 6: Wheezing phenotypes in children. Artwork by R. Douville.

These wheezing phenotypes are influenced by predisposition towards allergic disease and timing of early life environmental exposures [260], demonstrating the biologic complexity and clinical heterogeneity of asthma.

Epidemiologic evidence for respiratory virus infection initiating allergic disease

The "hygiene hypothesis" proposes that certain early childhood infections can be protective against future development of allergic disease. Stimulation of the immature immune system with Th1 promoting viral or bacterial infections is thought to help skew immune responses away from the Th2 pattern at birth [90]. The popularity of this hypothesis is spurred by supporting data from pro-biotic gastrointestinal models [68], although multiple studies have linked infantile respiratory infections to allergic disease. Therefore, protection or predilection for allergic disease is likely microbe-specific.

The real question (and one that stirs much debate) is whether respiratory virus infection is protective, inductive or has no effect on the development of allergy and asthma [262, 263]. Proxy markers of increased infection, such as daycare attendance, family size and farm life, show a protective (negative) correlation with subsequent development of allergic disease, supporting the hygiene hypothesis [264-268]. On the other hand, when the measurement is specific for respiratory tract infection (parent reporting, physician diagnosis or virological testing), a positive association exists between infection and allergic disease [269-271]. In a prospective study, early life infections (largely respiratory) associate with an increased risk of asthma (odds ratio (OR) 5.6, [3.9-8.0]) [272].

RSV has been in the spotlight in terms of its suspected causative association with asthma and allergic sensitization. RSV bronchiolitis has been linked to recurrent wheezing in later life [273-276]. Approximately 40-50% of children who have had RSV bronchiolitis wheeze repeatedly in childhood display AHR and altered lung function [242, 277-280]. Although "wheeze" is often an inappropriately used surrogate of a possible asthmatic condition in children, recent studies clearly show that severe infantile RSV bronchiolitis is a strong independent risk factor for subsequent physician diagnosed asthma [255, 270, 276, 281]. In the Sigurs study, 47 children hospitalized due to confirmed RSV infection and 93 matched controls were followed prospectively for 13 years [270, 281]. Asthma was defined as a minimum of three episodes of bronchial obstruction verified by a physician. They demonstrate severe RSV infection as the greatest risk factor for the development of wheezing (OR 5.3 [2.2 – 12.5]) and asthma (OR 12.7 [3.4 - 47.1]), even surpassing the effect of bi-parental atopy [270]. The authors concluded that severe RSV infection influences the development of asthma because the risk of RSV bronchiolitis outweighs genetic influences [282].

Infants hospitalised for RV infection are also at significant risk of asthma by school age (OR 4.14 [1.02-16.77] versus RV-negative cases) [276]. Children that were hospitalized for wheeze due to RV infection in the first two years of life had a 10-fold increased risk of asthma in their teenage years [256]. However, wheezing during RV infection is strongly associated with current atopic disease (or high-risk of atopy) in children [247, 283, 284]. This also fits well with the fact that RV bronchiolitis is more responsive to corticosteroid treatment than RSV bronchiolitis [284]. All points considered, RSV infection in infancy demonstrates the strongest relationship with initiation of asthma, whereas wheezy RV infections seem to identify children at high risk of developing AHR or atopic-asthma.

Clinical studies investigating the relationship between RSV bronchiolitis and the development of atopy are controversial and contradictory. Some studies have found an association [270, 285, 286], as where other groups have not [279, 287, 288]. The Tucson study found no association between prior RSV LRTI and atopy in children at age 13 [287]. In terms of aeroallergen sensitisation, results show bi-parental atopy (OR 5.6 [1.7 – 18.4]) is a greater risk factor than severe RSV infection (OR 2.4 [1.1 – 5.5]) [270]. Respiratory virus infection itself may not contribute significantly to the outcome of atopy; however, atopic disease and viral wheezing together are strong predictors of individuals at high risk of developing asthma [289].

Strikingly, bronchiolitis severity is the most important modifier between studies that have found an association between RSV and RV infections and the inception of allergic disease [247, 270, 285, 286], and those who have not [287, 288]. It remains unknown as to why some children develop severe infection with respiratory viruses, whereas others do not. Contributing risk factors for RSV disease severity include genetic predisposition [290, 291], premature birth [292], ETS exposure [291], virus strain [293] and co-infections [217, 241, 245, 294, 295]. This raises the question as to whether severe respiratory virus infection is the initiator, a proxy for a yet unknown causal factor in the development of airway disease or an identifier of children at risk for wheezing and AHR.

Causal mechanisms of respiratory virus induction of allergic disease

Even though the exact mechanisms remain speculative, lung damage from infection and remodelling initiated by the process of tissue repair is believed to contribute to altered lung function in healthy infants who develop severe or repeated viral respiratory infections

[156, 247]. This may occur via ineffective repair and airway remodelling [156, 157, 296], alteration of innate and adaptive immunity [263, 297] and enhancement of allergic disease following viral infection [195]. However, there are no known virus-mediated mechanisms that consistently <u>cause</u> asthma in humans, as opposed to contributing to asthma symptoms.

Enhancing allergic sensitization by viral infection

Increased allergic responses to aeroallergens and AHR during respiratory virus infection is illustrated in animal models [298-301]. A caveat of these murine studies is establishing causation versus exacerbation, as the infection does not occur prior to allergen sensitization and challenge. In addition, the use of adult mice in experimentation may not be reflective of infantile immune function in the face of RSV infection. Neonatal (but not weanling or adult) mice spontaneously develop <u>long-term</u> airway inflammation, IL-13 in BALF, AHR and subepithelial fibrosis [191, 192]. In primary infection, increased IL-13 levels are associated with improved illness, low viral titers and decreased pulmonary IFNγ production [302], suggesting a protective, rather than harmful, role for IL-13 responses upon first encounter with RSV.

If early RSV infection was followed by allergen sensitization and challenge, the airway disease worsened significantly [191]. However, primary infection in later life (adult mice) followed by sensitization and challenge, resulted in protection from allergic response, yet demonstrated weaker but significant AHR [303]. Of note, AHR in RSV infected mice has been shown to be independent of IL-13 production, as opposed to the RSV-enhanced allergic AHR component [304, 305]. This suggests that RSV infection itself can contribute to AHR at any point in life, but may facilitate atopic sensitization and enhance allergic responses only in individuals infected during the neonatal period. Currently, no human *in vitro* models

demonstrate RSV's ability to augment or initiate allergen sensitisation. If RSV can act as a trigger for the development of AHR and allergic sensitization, there remains little evidence (in contrast to speculation) for an RSV-mediated mechanism initiating allergic disease.

RESPIRATORY VIRUSES AND THE EXACERBATION OF ASTHMA

Respiratory virus infection recurrence and severity

RNA viruses are the major cause of respiratory infection in all age groups, with recurrent infections causing symptomatic illness. Estimated health care costs of RSV infections alone are exorbitant due to the prevalence and high rate of re-infection, not only in children but adults and the elderly [177, 213, 220, 306-310]. Children and adults who wheeze with viral infection, or asthmatics, are no more likely to contract virus infection than healthy individuals [247, 311]. However, they most certainly develop more severe symptoms upon infection.

Respiratory viruses are the main triggers of asthma exacerbation

In the natural history of asthma, episodes of exacerbation punctuate stable airway function. These times of increased symptoms are not random events, as most periods of exacerbation follow seasonal cycles associated with increased prevalence of respiratory virus infections in the community [77]. Asthmatics tend to demonstrate more severe and longer lasting LRTI symptoms (but not URTI symptoms) than healthy individuals [311-313]. In children, the most frequent triggers of asthma exacerbation are respiratory viruses, with 65-85% of wheezing and reduced airway function associated with the identification of current viral infection [69, 314, 315]. In adults, viral respiratory tract infection is also a major trigger (45-60% of exacerbations), but to a lesser extent than in children [77, 315-317].

41

The most prevalent viral trigger of exacerbation in asthmatics is rhinoviruses (*Picornaviridae*), accounting for two-thirds of identified viruses [315, 316, 318]. However, many other viral pathogens can cause exacerbation, including RSV, MPV, influenza, coronaviruses and parainfluenza virus.

Mechanisms of virus-triggered asthma exacerbation

Airway exacerbations due to viral infection are likely multi-factorial consequences of host vulnerabilities and virus survival tactics. Contributing and/or conflicting effects comprise initial host airway calibre, extent of tissue damage due to viral replication, pathologic anti-viral immune response, physiologic consequences of infection, repair processes and viral-immune modulation. Therefore, asthma exacerbations and the resulting heterogeneity of symptoms are a composite measure of the underlying interplay between the host response and the invading pathogen. Folkerts *et al*. nicely outline putative mechanisms of virus-induced airway dysfunction [319]. Many pathways contribute to the complex processes underlying the human response to respiratory virus infection. Here, I will describe in depth immune-mediated mechanisms of virus-triggered asthma exacerbation and transient airway dysfunction.

Impairment of innate responses and altered function of epithelial cells in asthma

Epithelial cells are the initial and most susceptible cellular targets of virus invasion. The extent of epithelial damage during virus infection correlates with the clinical severity of exacerbation in asthmatics [320]. Primary bronchial cells from asthmatics support greater (10-fold) RV replication than cells from healthy individuals [120, 321]. Infected epithelium normally

secretes a plethora of inflammatory mediators in response to viral infection. Epithelial cell models of virus infection demonstrate that airway epithelium of asthmatics has impaired ability to mount effective anti-viral immunity through cytokine production, namely type I interferons [25, 321]. In epithelium from asthmatics, RV infection leads to weaker IFNβ and IFNλ mRNA expression and protein secretion than healthy subjects [120, 321]. Similarly, a whole blood model of virus infection demonstrated impaired innate type I interferon (IFNα) production towards RSV and Newcastle disease virus in asthmatics compared to non-asthmatics. Innate IFNs are key regulators of cellular anti-viral networks and negatively correlate with infection severity and airway responsiveness during experimental RV infection [120].

Despite impaired innate IFN production in asthmatic epithelium, virus infection up-regulates several pro-inflammatory mediators [144-152]. Intrinsically, asthmatic epithelium produces altered amounts of cytokines, chemokines, matrix protein and growth factors [30, 322], however there is a paucity of information regarding virus-triggered pro-inflammatory cytokine release in asthmatic and non-asthmatic individuals. In children with asthma exacerbation and evidence of epithelial damage, sputum samples reveal increased levels of IL-8 and neutrophil elastase compared to those individuals without evidence of epithelial damage [323]. It remains unknown whether intrinsic structural and functional differences in asthmatic airway epithelium [30, 324] permit more severe infection with respiratory viruses.

The epithelial-viral-allergic paradigm, as proposed by Holtzman *et al.* [233, 325], states that epithelial cells actively mediate airway immunity and inflammation via host defence mechanisms. In asthmatics, programmed anti-viral networks in airway epithelial cells are active despite lack of overt infection [155, 326, 327]. This aberrant behaviour resembles a persistent anti-viral response, resulting in permanent epithelial proliferation and mucus production. Constant activation of the transcription factor, STAT-1, in asthmatic epithelial

cells (as compared to controls) is thought to promote persistent ICAM-1 expression [327].

Viral persistence in epithelial cells has been suggested as a mechanism for abnormal

"reprogramming", and latency in PBMC has been shown in the case of RSV [138, 139, 198, 221-224].

Neuro-immune interactions amplify innate inflammatory responses in asthma

The desquamation of the epithelial layer also exposes the underlying neural network,

both decreasing the physical barrier function and trophic support. Viral infection or

stimulation of sub-epithelial neural networks can cause bronchoconstriction, increased AHR

and potentially neural remodelling. This may occur via parasympathetic stimulation

(cholinergic) and interfering with inhibitory or excitory non-adrenergic non-cholinergic

neurons (NANCi and NANCe) [157, 158, 319]. The NANCe neurons play an important role in

establishing airway inflammation during virus infection [157]. Sensory C-fibers secretion of

substance P contributes to AHR during viral infection by RSV [328]. Overexpression of

substance P receptor, NK1, occurs on T cells within RSV infected lung [157, 328]. Once

expressed, the NK1 bearing T cells can be easily recruited to the airways upon subsequent

nerve stimulation. Murine asthma models have demonstrated that the substance P-NK1

pathway affects uniquely AHR and mucous production, but not allergen sensitization or

inflammatory response to challenge [329]. Taken together, neuro-immune crosstalk contributes

to the competence of the T cell repertoire responsible for AHR and asthma exacerbation.

Similarly, RSV also enhances the nerve growth factor (NGF) cascade in epithelial

cells, which acts as a positive feedback loop for substance P responsiveness (by increasing

its production and NK1). As NGF is an important growth factor for lung development,

repair and remodelling, virus-induced NGF may contribute towards permanent AHR. It

remains unknown whether neuro-immune pathways are enhanced in asthmatics compared to non-asthmatic individuals, but these mechanisms likely contribute universally to transient AHR during virus infection.

Increased recruitment and altered function of anti-viral T cells in asthma

Due to lacking innate anti-viral activity in asthmatic epithelium and increased pro-inflammatory signals, migrated immune cells are likely to encounter (and have to deal with) greater viral load and tissue damage. Increased recruitment of inflammatory cells is proposed as a mechanism for viral-induced asthma exacerbation. RSV may contribute to inflammation and airway obstruction by non-specifically recruiting large numbers of leukocytes to the area of infection. This hypothesis is further supported by a report of RSV G protein binding to the chemokine receptor CX3CR1 and mimicking its cognate ligand fractalkine [131]. The major effects of fractalkine comprise increased leukocyte-endothelial adhesion and inflammation [133]. Moreover, RSV-specific chemokine induction in lower airway cells, as measured by high-density oligonucleotide arrays, have verified increased expression of Exodus-1, MIP-1α and -1β, RANTES, GRO-α , -β and -γ , IL-8, I-TAC and fractalkine [147]. The recruitment implications are vast: one would expect amplified populations of neutrophils, eosinophils, monocytes, NK cells, T cells and dendritic cells into infected tissue.

In vitro PBMC responses toward RV demonstrate that the responses are Th1 biased in both non-asthmatics and atopic-asthmatic individuals. Atopic-asthmatics also respond by producing IL-4, resulting in a lower IFNγ to IL-4 index [330]. However, these results are potentially biased, as cytokines were measured after 24 hours of RV stimulation, and

therefore are not likely to represent the full response potential of these individuals. The importance of T cell mediated IFNγ production is highlighted in experimental RV infection as decreased Th1 responses to RV in PBMC were associated with increased asthma severity [331]. This is further supported by measuring IFNγ/IL-5 mRNA ratios in sputum samples from experimentally RV-infected asthmatics, where decreased Th1 mRNA was associated with increased symptom severity and slower viral clearance [332]. Thus, severe RV infection typified by asthmatic individuals, is associated with impaired anti-viral immunity, both in terms of innate and adaptive responses. In contrast, it remains unknown whether suppressed type-1 and type-2 interferon is a consistent immune pattern in asthmatics or is specific to RV immunity.

Enhanced tissue remodelling contributes to permanent virus-induced airway dysfunction

IL-11 production due to tissue injury is thought to be a protective mechanism to promote mucosal integrity by down-regulating local antigen-induced inflammation, as discussed in [333]. During viral infections (especially from asthma-associated viruses, RSV, PIV3 and RV), high levels of IL-11 are produced by airway structural cells and resident eosinophils. Unfortunately, IL-11 also demonstrates the propensity to induce airway hyper-responsiveness and remodelling [146]. Loss of epithelial integrity also leads to nitric oxide production that is intended as an antimicrobial agent, but can also have a great impact on airway smooth muscle tone [334, 335].

Synergistic mechanisms of virus infection and allergen exposure in asthma exacerbation

The risk of being hospitalized due to asthma exacerbations increases if sensitized asthmatics who are highly exposed to additional triggers (allergens and/or pollution) have a concomitant viral respiratory infection [336, 337]. Synergy between allergen and viruses in the exacerbation of asthma has been demonstrated not only in humans, but in animal models. Many murine models have demonstrated that respiratory virus infection subsequent to allergen sensitization increases allergic parameters [299, 338-342]. Repeated RSV infections in adult allergen-sensitized mice results in serial boosting of Th2 cytokines and total serum IgE [161, 340, 343]. RSV infection on top of established allergy also coincides with a doubling in AHR (with shift from transient to persistent) and significant airway remodelling compared to allergen alone [192]. RSV infected, dust mite-sensitised mice continued to exhibit increased AHR after each subsequent RSV infection [340]. Taken together, RSV may drive already established atopic responses toward symptomatic airway disease. At this time it remains unclear whether these divergent responses combine to cause disease, or whether an altered anti-viral response in asthmatic and/or atopic individuals' contributes to airway dysfunction during infection with respiratory viruses.

A series of papers by Chaplin have examined the role of Th1 cells in an OVA sensitized murine model of asthma [95, 96, 344]. Using an adoptive transfer system, OVA-specific cells were transferred to naïve mice and subsequently challenged with OVA. Adoptive transfer of OVA-specific Th1 cells could neither prevent nor decrease the established Th2 response generated by OVA challenge. The co-transfer of Th1 cells potentiated an increased inflammatory response in the lung. Surprisingly, efficient

recruitment of Th2 cells to the lung was shown to require not only antigen, but also the presence of Th1 cells. Further experimentation revealed that respiratory viral infection (or Th1 cells specific to a different Ag) increased the recruitment to the lung of OVA-specific Th2 cells and eosinophils <u>without</u> OVA challenge. Increased recruitment potential in the lungs initiated by infection may provide a means to trigger allergen-specific Th2 activation. These studies provide a mechanism by which viral-specific Th1 cells could trigger an increase in asthmatic inflammation. This implies not only the potential for viral asthma exacerbations, but also a way to facilitate initial sensitization to aeroallergens.

In RSV infection, $CD8^+$ T cells appear to be the main force enhancing allergic responses and airway dysfunction in allergen-sensitized animals. Investigation into the mechanism of RSV-induced AHR shows the ability of adoptively transferred $CD8^+$ T cells from RSV infected mice to increase AHR in naïve mice after OVA challenge [345]. Transfer of $CD8^+$ T cells from RSV infected mice leads to increased recruitment of eosinophils to the lung. Depletion of transferred $CD8^+$ T cells prior to OVA sensitisation lead to significantly decreased AHR. Furthermore, these effects were not apparent using $CD4^+$ T cells.

Potential mechanisms for viral exacerbation of allergic-asthma highlight the co-operative effect of anti-viral T cells and effector cells associated with allergic diseases. Atopic asthmatics demonstrate prolonged airway hyper-responsiveness and eosinophilia subsequent to viral infection, whereas control subjects do not [346, 347]. Viral infection leading to IFNγ production by T cells may in turn activate eosinophils and cause increased inflammation. Activated eosinophils release eosinophil cationic protein (ECP) during RSV infection [348], leading to increased inflammation and ASM hyper-reactivity in the airways [146]. Regardless of trigger, both excessive Th1 and Th2 mediated inflammation may cause a flare-

up of asthma symptoms. Alternatively, deficient anti-viral responses during primary and recurrent viral infections may contribute to and augment ongoing allergic inflammation, as described by Gern in **Figure 7** [349].
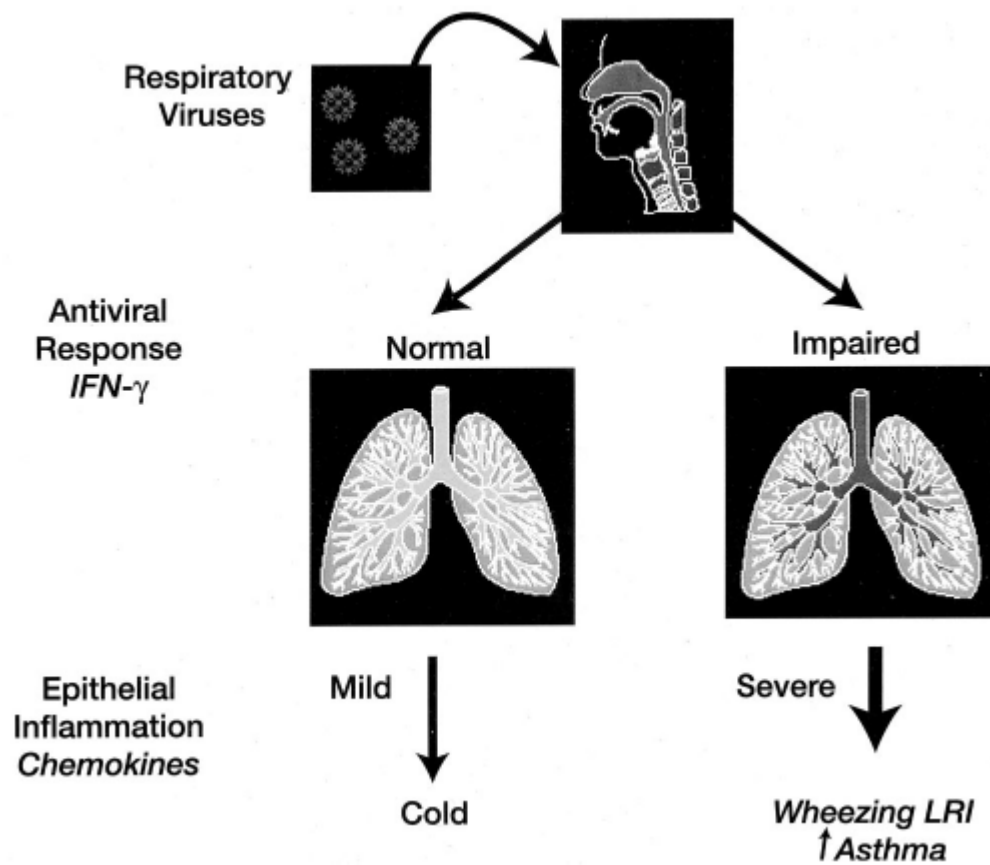
Figure 7: Deficient anti-viral responses contribute to virus-induced wheezing and asthma, as proposed by Gern [349]. Reproduced with permission from Lippincott, Williams and Wilkins.

Treatment of virus-triggered asthma exacerbation

Despite the prescribed daily use of inhaled corticosteroids (ICS) in the preventive treatment of asthma exacerbations, ICS are generally ineffective against virus-induced asthma symptoms [72, 350-355]. In children, ICS decrease exacerbations in persistent asthmatics, yet have no effect in minimizing frequency, duration or severity of episodic virus-induced exacerbations [353]. The addition of long-acting β2 agonist to ICS maintenance therapy did not assist in reducing the number of exacerbations in children uncontrolled on ICS treatment [356]. The use of high dose ICS for acute intervention of virus-induced asthma exacerbations are equally unsuccessful in controlling the progression of severe symptoms requiring urgent care [357-359]. Indeed, ICS are also ineffective treatment in cases of infantile bronchiolitis, although this is common prescription practice [72, 360, 361]. Treatment of bronchiolitis with corticosteroids however, has been linked to decreased onset of wheezing in a 2 year follow-up study [362]. Interestingly, RSV (but not PIV or RV) -infected epithelial cells are resistant to corticosteroid dampening of inflammatory cytokines [363-365]. Corticosteroids may also prolong survival of virus-infected cells and pulmonary neutrophils, leading to increased replication in the lung and airway obstruction [366-368]. Taken together, ICS either as a maintenance therapy or acute intervention should be contra-indicated for the treatment of virus-triggered asthma exacerbation and prevention of asthma onset.

On the other hand, the use of oral corticosteroids (prednisone, dexamethasone) has been shown to be effective in treating severe virus-induced asthma exacerbations, both in children and adults [355, 369]. The use of oral corticosteroids at first sign of respiratory infection prevented hospitalization in children with severe asthma exacerbation [370]. Most convincing is a study emerging from an asthma care program where children with an intermittent pattern of asthma exacerbations were removed from all maintenance therapy and

sent home with inhaled β2 agonists and a supply of oral corticosteroids for use during exacerbations [351, 371]. When compared to urgent medical care in the previous year, these patients demonstrated a >4 fold reduction in urgent care visits and hospitalization. Using this care strategy, these children obtained a similar level of asthma control, compared to persistent asthmatics using maintenance ICS and the same intervention plan [371]. Prompt use of dexamethasone in infants with bronchiolitis can also reduce the need for hospitalization [372]. However, due to the frequency of exacerbations in young asthmatics, physicians may be reluctant to prescribe oral corticosteroids with such frequency, due to adverse effects on development, health and behaviour [373]. Taken together, oral corticosteroids treatment of viral respiratory infections can largely reduce urgent care demands (and expense) of asthmatics.

Thus far, the most effective treatments to alleviate symptoms of viral airway exacerbations due to RSV bronchiolitis have been cysteinyl leukotriene (CysLT) antagonists (Montelukast) or prophylactic anti-RSV antibodies (Palivizumab or RSV-IVIG) [72, 374]. CysLTs are induced during viral infection with RSV, especially in children with a family history of allergic disease [375-378]. When CysLTs are expressed in combination with epidermal growth factor (EGF; induced by epithelial damage), they lead to airway smooth muscle hypertrophy and hyperplasia, key features of airway remodelling [379]. Bisgaard published a double-blind, randomized, placebo controlled study demonstrating the effective symptom reduction in children hospitalized for RSV bronchiolitis who were administered Montelukast treatment [380]. Follow-up studies will be required to determine the long-term effects of Montelukast intervention in the development of wheezing and asthma. The use of Montelukast in asthma may mildly prevent exacerbations, but to date there is no evidence for

the use of CysLT antagonists in prevention or treatment of virus-triggered asthma exacerbations [353, 381].

While CysLT antagonists are relatively inexpensive treatment, the use of palivizumab is generally restricted for use in children at high-risk of severe RSV infections, due to its prohibitive cost [374, 382]. However, passive immuno-prophylaxis with palivizumab significantly reduced RSV hospitalizations among premature infants [383-385].

Anti-viral therapies are emerging as candidate treatments for asthma exacerbation [365, 386, 387], as most cases of exacerbation are associated with a viral trigger. Amazingly, antibiotics, not anti-virals, are commonly prescribed for severe RSV infections and episodic asthma due to presumed risk of bacterial co-infections [351, 388-393]. The development of vaccines or anti-viral medications is complicated by the numerous respiratory viruses (and their serotypes) and their varying modes of action [394-397]. The development of vaccines against RSV has not only proven to be unsuccessful, but also had severe detrimental effects, in the case of the 1960's formalin-inactivated RSV vaccine (enhanced RSV disease post-treatment) [397]. Ribavirin is currently the only anti-viral effective against RSV, although it's effectiveness as a therapy for bronchiolitis is underwhelming [398, 399]. Ribavirin's effectiveness is augmented in combination with montelukast in a murine model of pneumovirus infection [400]. Early use of anti-virals may lessen symptoms of infection and improve long-term outcomes, by minimizing lung injury upon infection with respiratory viruses. Prophylactic anti-viral strategies during periods of airway vulnerability for at-risk infants may also prevent onset of long-term airway dysfunction.

Better prevention (as there are none currently) and treatment strategies of virus-triggered episodic exacerbations of asthma will improve the overall health of most

asthmatics. Important to highlight is that intermittent virus-induced asthma does not mean "mild" asthma, as exacerbations cause significant disruption in daily life. Indeed, this group of asthmatics are the major users of urgent and acute medical care for asthma [314, 317]. Therefore, defining disparity in anti-viral immunity among asthmatics and healthy individuals will help devise improved intervention and maintenance medications for use in asthmatics with virus-triggered exacerbations.

CAN ANTI-VIRAL IMMUNITY IDENTIFY THE ASTHMATIC PHENOTYPE?

"Doctor, is my 9 month old infant with AHR due to viral infection having his first asthma attack?" Apart from risk assessment based on the child and family history, the answer is still "Maybe". To date, there are few predictors of whether children who display virus-induced wheezing in childhood will go on to have asthma in later life [260]. Therefore, exploring putative differences in anti-viral immunity between asthmatic and non-asthmatic individuals may contribute toward improved diagnostic methods and clinical treatments for virus-induced asthma exacerbation and bronchiolitis. Moreover, correlating anti-viral immunity with genetic polymorphisms may reveal predictive genetic markers for asthma versus virus-triggered AHR. Therefore, using respiratory virus-driven immune responses as an intermediate phenotype between genetic predisposition and clinical disease (and accounting for other environmental influences), we will evaluate if there are characteristic immune profiles associated with asthma and health.

Using RNA Viruses as Probes of Immune Responsiveness

To examine differential recall responses to respiratory RNA viruses in atopic and non-atopic subjects, we sought to establish primary, short-term PBMC cultures using virus as a recall antigen. The following RNA viruses were chosen based on similar (and virtually universal) seroconversion prevalence in infancy, as well as detectable humoral responses in adulthood.

Reovirus

This non-enveloped virus is a member of the genus mammalian orthoreoviruses, family *Reoviridae* [401]. Its genome consists of linear double stranded RNA encased in multiple capsids. Many serotypes of reovirus (Type 1 Lang; T1L, Type 3 Dearing; T3D and Type 2 Jones; T2J) have been studied based on the serological differences of their surface proteins. Reoviruses are lytic in nature [402]; however, they are not considered human pathogens due to their inability to evoke symptoms. Infection can occur in both the enteric and respiratory mucosa [403, 404]. Serology indicates that by 5 years of age more than 70% of children have been exposed to reovirus [405]. Ubiquitous and recurrent infection with reovirus provides constant stimulation of the adaptive response. Reovirus has not been associated with allergic disease, nor known to cause asthma exacerbations; however, there are very few epidemiologic or experimental studies examining clinical outcomes or reovirus infection in the general population [406, 407]. To date, there has been extensive research using murine models regarding the innate and adaptive immune response to various reovirus serotypes, predominantly T3D and T1L. The immunoregulatory arm of the adaptive responses to reovirus has largely been unexplored in human models [408].

Respiratory syncytial virus (RSV)

This enveloped virus is a member of the genus pneumovirus, family *Paramyxoviridae*. Respiratory syncytial virus consists of a single negative-sense RNA genome, encoding 10 genes. By the age of two years, virtually all children have been in contact with RSV and passed through their first infection [409]. For unknown reasons, some children develop lower respiratory tract infections (10-40%) as opposed to a simple URTI. Bronchiolitis morbidity is highest in infants under 6 months of age [177, 410]. There is strong epidemiological evidence

that natural immunity to RSV does not confer long term protection. Re-infection is common among all age groups [213]. Yearly outbreaks occur in late winter to early spring. Although a humoral response is present, its effects are short lived and offer only partial protection. After severe bronchiolitis, many children experience recurrent wheezing (prior bronchiolitis 23% versus controls 10% at 6 years of age) and asthma (prior bronchiolitis 38% versus controls 20% at 7 years of age) [255, 273]. The mechanisms behind RSV's ability to induce and exacerbate asthma are currently unidentified. It is known that RSV elicits a Th1 type cytokine and chemokine recall response from both neonate and adult PBMC [183].

Metapneumovirus (MPV)

This enveloped virus consists of a single negative-sense RNA genome encoding 10 genes similar to that of RSV. Human metapneumovirus (MPV) is a recently discovered (2001) member of the *Paramyxoviridae* family. It was first isolated from children with respiratory tract infections in the Netherlands, but since has been identified in the general population in Canada, Japan, Australia, Finland, UK and U.S.A [411-415]. These findings suggest a worldwide distribution. Infections occur predominantly from early winter to mid-spring [415]. Hospital admissions due to severe respiratory tract infection with MPV peak in 3-5 month old infants, as opposed to RSV infections prevalence between 0-2 months [416]. Most are diagnosed as either bronchiolitis and/or pneumonitis [412], with symptoms indistinguishable from other respiratory infections such as RSV [415]. Seropositivity for MPV is approximately 25% for infants under 1 year of age, increasing to 100% by age 5 [411]. Respiratory specimens collected over a 10 year period in Quebec, Canada revealed the overall prevalence of MPV positive cultures to be 7.1% [412]. There are 2 distinct serogroups of MPV, and this is thought to contribute to the re-infection rate [411, 414, 417]. A pivotal finding

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has been MPV's link to severe respiratory bronchiolits requiring hospitalisation; up to 70% of RSV-diagnosed infantile bronchiolitis may be co-infected with MPV [294, 418]. The presence of MPV may be a factor in bronchiolitis disease severity [295]. Several early studies have attempted to determine if MPV is associated with asthma, however the data are contradictory. More recent studies have demonstrated a significant association between MPV infection and childhood asthma exacerbations [318, 419-423]. Using 25 years worth of sample archive, representing 2000 children [236], there was a significant association between MPV and diagnosed asthma. This strengthens the data identifying MPV as a cause of acute expiratory wheezing (9% prevalence) in infants less than 2 years of age [424]. Furthermore, cohort data from ≈600 children suggest that MPV is a stronger trigger of asthma exacerbations than RSV or influenza [422]. Reported statistics of 66.7% of MPV wheezing was directly attributable to asthma exacerbation, rather than bronchiolitis, as compared with 16.7% of wheeze in RSV infections. Interestingly, MPV is also suspected of contributing to the severity of symptoms in SARS-associated coronavirus infection [425], as 12% of WHO confirmed SARS cases tested positive for MPV. Few publications are currently available regarding the profile of MPV-specific recall responses in either humans or animal models [138, 222, 426].

Human Peripheral Blood Mononuclear Cell (PBMC) Model of Allergic Disease

Human models of allergy and asthma are limited due to the difficulties in studying *in vivo* events by way of highly invasive techniques. Consequently, common model systems include the use of clonal cell cultures, methodically differentiated cell cultures, cell lines, primary cell cultures and infrequently the use of tissue explants directly *ex vivo* [427]. The main dilemma is true-to-life replication of the *in vivo* situation; the case of

clonal/differentiated cell cultures is based on forced set of selective pressures that do not necessarily promote the natural maturation or activation states *in situ*. Although cell responses are easily quantifiable in these systems, they may not be representative of proceedings in a more complex cellular environment. Although biopsies represent airway sampling (albeit of the upper airway), they like PBMC sampling, are not a fully accurate measure of the lower airways, including the bronchioles and alveolar regions, which account for the majority of the lung area. Moreover, biopsy studies are limited compared to PBMC, in terms of sample size, number of participants who can be recruited for this more invasive procedure and availability of specific clinical phenotypes (especially healthy individuals). In systems generating less robust responses, such as tissue explants, it is difficult to measure cytokines at the protein level using conventional assays; as a result, mRNA is prominently used as a surrogate marker of extracellular protein levels. Nevertheless, the quantity of mRNA may or may not correlate with protein production due to post-transcriptional regulation. Secreted protein levels are the prefered physiological read-out, although often assays are not sensitive enough to detect minute responses. Unfortunately, this and reliance on T cell clones has propelled the idea of divergent responses (ie. Th1 <u>or</u> Th2), as opposed to <u>biased</u> responses (more Th1 than Th2) which is likely the physiologic outcome.

Proponents of short-term primary peripheral blood mononuclear cell (PBMC) cultures argue that, while an *in vitro* system, it is preferential to monoculture of highly enriched cells, such as cell lines and clones. Besides representing a broader cell population (T cells, B cells, NK cells, NKT cells, DCs, monocytes and trace basophils), it provides a "net" response which depicts the heterogeneity of cellular responses [428]. Assay of culture supernatants using ultra-sensitive enzyme-linked immuno-sorbent assays (ELISAs) enables quantification of cytokines and chemokines induced by a variety of stimulatory conditions.

Classically, primary culture of PBMC has used polyclonal stimulants as a tool to determine immune responsiveness. Overall, antigen and allergen driven PBMC cultures generate minimal cytokine and chemokine responses as compared to polyclonally stimulated cultures. This can, and has, been problematic in terms of cytokine measurement at the protein level using conventional ELISAs. Proper kinetic studies are required to determine optimal cytokine expression and production in the culture system, in order to represent accurately the antigen-driven immune response. Therefore, choice of antigen is also an important consideration in PBMC culture. Stimulation with intact antigen yields a net response that represents multiple responses to its encompassed proteins; yet the same proteins individually can yield different results. Chemical, heat or radiation may also alter an antigen in such a way that it no longer generates the usual response.

Differential cytokine and chemokine biases are demonstrable in PBMC cultures from atopic and non-atopic subjects. Polyclonal stimulation of PBMC with PHA from atopic subjects yields greater production of both Th1 (IFNγ) and Th2 (IL-4, IL-5, GM-CSF) cytokines, as compared with PBMC from non-atopics [429]. In the same system, depressed levels of IL-10 were found in PHA-stimulated PBMC from atopics. The authors conclude that responses from atopic individuals can include concomitant expression of Th1 and Th2 cytokines, contrary to the proposed strict Th2 bias. Using allergen-driven PBMC cultures specific for the allergies of atopic individuals, it has become evident that allergic subjects respond to allergen with preferential production of Th2 cytokines when compared with non-allergic individuals [430]. In contrast, recent evidence has emerged to indicate that atopic children and adults do not display impaired Th1 responses to allergens and viral proteins. Indeed, higher levels of IFNγ induction were seen in atopics compared with non-atopics [431-433]. In fact, together this suggests that atopic individuals have increased responsiveness to

allergen compared to non-atopics, as demonstrated by their ability to mount stronger Th1 and Th2 allergen-specific responses. In conjunction, PBMC from atopic individuals display reduced antigen-specific IL-10 production compared to non-atopic subjects [434]. This suggests impaired IL-10 production in atopics / asthmatics may relate to their excessive responses to allergens and other antigens, as reviewed in [172]. However, there is no clear consensus regarding the predominant immuno-regulatory differences between heathy individuals and those with allergic disease.

Assessing virus-driven cytokine responses as a model of anti-viral immunity in humans

Literature regarding human immune responses to most RNA respiratory viruses predominantly examines either antibody responses or cytopathic effects. However, in humans the T cell responses to these viruses are largely uninvestigated, with the exception of respiratory syncytial virus (RSV). Our working model will characterize the specific innate and recall immune responses to respiratory RNA viruses from PBMC in primary culture. The establishment of a short-term primary cell culture system using adult PBMC will be optimized and immune responses to reovirus, RSV and MPV are to be profiled based on virus-specific cytokine and chemokine production. Prevalence and intensity of innate and adpative responses in children and adult human populations is to be addressed. Using this *in vitro* model of human anti-viral immunity, we will test our global hypothesis that asthmatics mount anti-viral cytokine responses to respiratory viruses that differ from those of healthy individuals.

THESIS OVERVIEW

GLOBAL RATIONALE

Epidemiologic, clinical and pathological data suggests that respiratory viruses contribute to both the inception and exacerbation of asthma. Host immune responses towards these viruses substantially contribute to clinical outcomes during and subsequent to infection. Therefore, we seek to determine whether anti-viral immune responses, as measured by virus-driven immuno-regulatory cytokine produced by *in vitro* culture of PBMC, relate to parameters of disease (AHR and SPT), clinical status (asthma or atopy), epidemiologic markers (ETS and bronchiolitis) and genetic polymorphisms.

GLOBAL HYPOTHESIS

Asthmatics mount anti-viral cytokine responses to respiratory viruses that differ from those of healthy individuals.

THESIS OBJECTIVES

Specific Aim 1: To characterise respiratory virus-driven cytokine responses using viral triggers of asthma exacerbation (RSV and MPV) and ubiquitous asymptomatic virus (reovirus).

Specific Aim 2: To determine patterns of anti-viral immunity in asthmatic and healthy individuals.

Specific Aim 3: To determine if patterns of anti-viral immunity are influenced by environmental and genetic co-factors.

RESEARCH APPROACH & RESULTS

Anti-Viral Immunity in Humans

as Measured by Immuno-Regulatory Cytokines

MATERIALS AND METHODS

Recruitment of adult participants

Ethics approval was obtained from the University of Manitoba Faculty Committee on Use of Human Subjects in Research. In collaboration with Dr. F.E.R. Simons, written informed consent was obtained from 63 healthy adults, 18-45 years of age, with no evidence of current upper or lower respiratory tract infection. Seventeen allergic asthmatic individuals were identified on the basis of 1) a positive epicutaneous test (wheal diameter >6mm than that of the negative control) to an environmental antigen; most frequently to cat antigen (Fel d1 containing standardized cat hair extract at 500 BAU/ml; ALK SQ, Wallingford, CT), house dust mite and/or grass pollen (grass mix 1649- consisting of Kentucky blue grass, orchard grass, redtop grass, and timothy grass; Hollister-Stier, Spokane, WA) and 2) a clear clinical history of allergic asthma. Twenty-five control individuals had no history of asthma and most exhibited negative epicutaneous tests to common environmental antigens. We excluded individuals who were of child-bearing

potential or pregnant, those with medical disorders other than allergy-related problems and those who had received immunotherapy within the preceding year.

Recruitment of child participants

Study approval was obtained from the University of Manitoba Faculty Committee on Use of Human Subjects in Research. In collaboration with Dr. Allan Becker and Dr. Anita Kozyrskyj, written informed parental consent was obtained for 723 children (of which 345 were randomly selected for detailed analysis) within a general population survey case-control cohort. This 1995 birth cohort (SAGE, Study of Allergy Genes and Environment) was created from the provincial health care registry. It identified the 16,320 children born that year in Manitoba. Of these, 2340 children moved out of the province or died over the next seven years and were excluded from the cohort at the time of its construction. A one-page survey was mailed out to the households of all children to inquire about their health and home environment exposure and for permission to be contacted for future study. From the returned surveys, children were stratified according to the presence of asthma (n = 392), to the presence of allergic rhinitis or food allergy (n = 192) or neither (n = 3002). All children in the asthma and allergy strata were invited to participate in the case-control study, as were a random sample (n = 200) from the strata of children with neither condition. Consented children and parents came to the Paediatric Allergy clinic in Winnipeg or mobile Paediatric Allergy clinic in their community (over a 3 year period) for the detailed assessment used to characterize the individuals studied in this publication.

In prior studies [411, 413, 435], 100% of this age group was seropositive for prior RSV and MPV infection, reinforcing the ubiquity of these infections in the general population. These

7-9 year old children had no evidence (parental report) of upper or lower respiratory tract infection within one month of recruitment.

Clinical assessment of asthma in children

Paediatric allergist assessment was used as the gold standard for diagnosis of asthma among children. Physician diagnosis of asthma was based on history and physical assessment (blinded to skin prick tests and PC_{20}), according to the Canadian Asthma Consensus Guidelines [29]. To aid in diagnosis, a standardized history and physical sheet was used – questions regarding cough with/without colds, wheeze with/without colds, shortness of breath with activities, colds lasting > 2 weeks, response to current medications and the presence of other allergic conditions were elicited. A family history of asthma or personal history of prior/present eczema was established to aid in the diagnosis [30]. Hospital, emergency department and medical visits for breathing difficulty in the past year were noted. Physical examination included examination for allergic facies, chest findings and evidence of atopic dermatitis. All findings were recorded and paediatric allergist diagnoses of current asthma (within the last 12 months) were made. Independently, children were tested to quantify airway hyper-responsiveness (AHR) in response to methacholine testing, as measured by the PC_{20} method of Cockcroft [436]. Atopy was defined as having one or more positive skin prick test for common allergens (tree pollen mix, weed pollen mix, ragweed, grass pollen mix, Alternaria, Cladosporium, Penicillium, house dust mites D. pteronyssinus and D. farinae, cockroach, cat, dog, feathers and peanut). Within this cohort, we examined the following clinical phenotypes: asthmatics with AHR, asthmatics without AHR, non-asthmatics with AHR and non-asthmatics without AHR (**Table 2**).

Table 2: Clinical description of asthmatic and non-asthmatic children.

Clinical description	Asthmatic with AHR	Asthmatic without AHR	Non-asthmatic with AHR	Non-asthmatic without AHR
Children	94	29	109	87
PC_{20}*	1.27 (0.01 - 8)	16+	2.6 (0.01-8)	16+
Age	8 (7-10)	9 (8-10)	8 (7-10)	9 (7-10)
Gender (% Boys)	58.5	58.6	59.6	48.3
Height (cm)*	133.8 (117-161)	138 (122-158)	134 (121-157)	138 (124-159)
Weight (Kg)*	31.3 (21-92)	35 (22-59)	32 (21-67)	35.5 (22-77)
Familial Asthma (%)	50	35	30	31

AHR: Airway hyper-responsiveness

* PC_{20}, height and weight are expressed as median values with range.

Epidemiologic parameters

Familial asthma (in one or more first degree relatives), corticosteroid / rescue medication use within the last 12 months and exposure to environmental tobacco smoke was assessed by questionnaire. Bronchiolitis was defined as an ICD-9 (International Classification of Diseases) diagnosis of "acute bronchitis and bronchiolitis" (466) between birth and age 2, which was identified using the Manitoba Center for Health Policy databases. LRTI was defined as any 466 diagnosis between birth and age 7.

Severe asthma was defined as children who received an anti-inflammatory drug (Singular and/or inhaled corticosteroids) and met at least one of the following criteria: hospitalized in the last year, 5 or more asthma attacks in the last year, missed 1 or more nights sleep or received oral Prednisone in the last year. Moderate asthma was defined as children who received an anti-inflammatory drug but did not meet any of the above criteria or children who did not receive an anti-inflammatory drug but met at least one of the criteria. Mild asthma was defined as children who did not receive an anti-inflammatory drug and did not meet any of the criteria.

Isolation of PBMC

Peripheral blood was collected by venipuncture and treated with 2.7% EDTA (Sigma, St. Louis, MO, U.S.A.) to prevent clotting. Blood samples were stored at room temperature until use. Under sterile conditions, blood was diluted with normal saline (NS, 0.85% NaCl) using a 2:1 ratio. Saline-diluted blood was gently layered on a 3.5ml Ficoll gradient (Histopaque 1077, Sigma, St. Louis, MO, U.S.A.) and centrifuged at 1850 rpm (600 x g for 30 minutes (Eppendorf Centrifuge 5810 R). The gradient centrifugation separated cells based on density, making clear delimitations between layers containing i) platelets and

plasma, ii) the "buffy coat" containing PBMC, iii) Ficoll, and iv) red blood cells and polymorphonuclear cells. Sterile plasma was collected and stored at –20°C. The rest of the plasma layer was discarded. PBMC were carefully suctioned off the Ficoll layer, and subsequently washed twice with 12 ml saline and pelleted (1180 rpm / 250 x g for 10 minutes in Eppendorf Centrifuge 5804). Cells were re-suspended in complete media consisting of RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS), penicillin/streptomycin/fungizome (PSF) and 2-mercaptoethanol (2-ME). The concentration of the cell suspension was determined using duplicate haemocytometer counts. Viability was determined using Trypan blue staining which yielded over 95% viability in all samples. Cells were used immediately for short-term primary culture at 37°C with 5% CO_2. No frozen cells were used in this study.

Generation of paramyxoviruses for cell culture

MPV strain CAN98-75 was cultured on LLC-MK2 cells and RSV strain Long on HEp-2 cells at 37°C in Eagle's MEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 30 μg/ml L-glutamine (Life Technologies). Fetal calf sera (1%) and TPCK trypsin (0.02%) was also added to the RSV and MPV growth media respectively (Sigma). MPV virus isolates were cultured for a maximum of 21 days with weekly media change. Both MPV and RSV strains were titrated by the quantal assay $TCID_{50}$ of Karber, performed in 96-well microtiter plates using tenfold dilutions. Live virus was heat-inactivated for 30 minutes at 56°C, to produce non-infectious virus for culture. All virus samples were aliquoted, flash-frozen and stored at -80°C until used. Virus samples were never freeze-thawed and reused.

Generation of reoviruses for cell culture

Reovirus strains type 1 Lang (T1L) and type 3 Dearing (T3D) are laboratory stocks. They were amplified in mouse L929 monolayer fibroblast cells maintained in Joklik modified MEM supplemented to contain 2.5% FCS (Gibco), 2.5% VSP neonate bovine serum (Biocell, Carson, CA), 2 mM l-glutamine, 100 U of penicillin per ml, 100 μg/mL of streptomycin sulfate and 1 μg/mL of amphotericin B [437]. Large amounts of virus were grown in L929 suspension cultures, extracted with Vertrel-XF®, and purified in cesium chloride gradients as described [438]. Virus bands were harvested, dialyzed extensively against 2X SSC (300mM NaCl, 30mM $C_6H_5Na_3O_7$, pH 7.0), and particle numbers measured by OD_{260} readings. Aliquots of purified virus were inactivated by overnight treatment at pH 12.5, followed by neutralization [439]. Titers of non-inactivated and inactivated viruses were determined by plaque assay on L929 cell monolayers as described [437] to confirm virus inactivation. All virus stocks were then diluted in 2X SSC, aliquotted, and frozen once at -80°C until used. Diluted virus samples were never freeze-thawed and reused.

Primary cell culture

Using complete media, PBMC cell suspensions from each subject were adjusted to give a final concentration of 2.5 x 10^6 cells/ml in culture (5 x 10^5 cells in 0.2ml cultures). Adjusted cell suspensions (100μl) were added directly to virus preparations (100μl). Duplicate cultures were stimulated with media or MPV (live and heat-inactivated), RSV (live and heat-inactivated), reovirus serotype T3D (live and high pH inactivated) and reovirus serotype T1L (live and high pH inactivated), with titer inoculums as described in **Table 3**. Cultures were optimized for maximal cytokine and chemokine production by PBMC for each virus model. Time course and viral concentration were varied to assess

optimal conditions (Cytokine concentration curves increased over time and with increasing viral concentrations used, without reaching a plateau). Based on data obtained in optimization experiments, culture supernatants were harvested following 1 or 6 days culture, the time of peak virus-driven cytokine responses for the cytokines evaluated. Supernatants were collected and frozen at –20°C.

Streptokinase (Aventis Behring, Marburg, Germany) was used at 5000 U/ml as a common recall antigen. In some experiments, anti-CD4 (2μg/ml), -CD8 (4μg/ml), -HLA-DR (2μg/ml), -CD80 (5μg/ml), -CD86 (1μg/ml), isotype controls IgG1 (4μg/ml) and IgG2a (10μg/ml), anti-IL-10 (2μg/ml) (BD Pharmingen, San Diego, CA, U.S.A.), anti-HLA-ABC (10μg/ml) (Immunotech, Marseille, France), CTLA-4 Ig (5 μg/ml, a gift from Dr. P. Nickerson, University of Manitoba) and anti-TLR4 10μg/ml (eBiosciences) were used to evaluate activation requirements. Sterile solutions of these reagents (50μl) were added directly to viral preparation (100μl) and adjusted cell suspension (50μl).

Table 3: Viral Antigen Preparations for PBMC Culture

Virus preparation	Viability	Concentration	Experiment
Reovirus			
Serotype T1L	Live	10^7 pfu/ml (optimal)	All experiments
Serotype T1L	Inactivated		
Serotype T3D	Live		
Serotype T3D	Inactivated		
Graciously provided and titered by Dr. Kevin Coombs, Dept. Medical Microbiology, University of Manitoba.			
RSV			
Strain Long (Lot #1)	Live	$10^{4.4}$ TCID$_{50}$/ml	Adult cohort experiments
	Inactivated		
Strain Long (Lot #2)	Live	$10^{5.7}$ TCID$_{50}$/ml	SAGE cohort experiments
Strain Long (Lot #3)	Live	$10^{6.6}$ TCID$_{50}$/ml	Future experiments
Graciously provided and titered by Dr. Yan Li and Dr. Nathalie Bastien, Canadian Science Centre for Human and Animal Health, Winnipeg, Canada.			
MPV			
Strain CAN98-75 (Lot #1)	Live	$10^{4.3}$ TCID$_{50}$/ml	Adult cohort experiments
	Inactivated		
Strain CAN98-75 (Lot #2)	Live	$10^{4.9}$ TCID$_{50}$/ml	SAGE cohort experiments
Graciously provided and titered by Dr. Yan Li and Dr. Nathalie Bastien, Canadian Science Centre for Human and Animal Health, Winnipeg, Canada.			

Human Cytokine ELISAs

As a safety precaution, culture supernatants were UV irradiated (60 minute exposure at 4cm distance from an FBTI816 transilluminator, Fisher Biotech) to inactivate residual infectious virus prior to ELISA analysis. Preliminary experiments (Data not shown) demonstrated that this had no impact on the sensitivity or precision of the assays used to evaluate cytokine concentrations. Anti-cytokine capture and biotinylated detection antibodies were purchased from BD-Pharmingen (Mississauga, ON, Canada), Endogen (Woburn, MA, U.S.A.), Biolegend (San Diego, CA, U.S.A.) or R&D Systems (Minneapolis, MN, U.S.A.) and recombinant cytokine standards from BD Pharmingen, Endogen or Peprotech (Rocky Hill, NJ, U.S.A.). They were used as previously described [440, 441]. At a minimum, PBMC supernatants from duplicate cultures were assayed. The concentration of cytokine in each supernatant sample was calculated from a minimum of three points falling on the linear portion of titration curves that were calibrated against recombinant cytokine standards serially diluted on each plate (**Table 4**). Standard errors typically ranged from 3-10%.

Table 4: Sensitivity of Cytokine and Chemokine Assays

Cytokine/Chemokine	Range of Detection	Sample Dilutions	Sensitivity
Pro-inflammatory			
IFNα	1000 – 7.8 pg/ml	½ - 1/16	15.6 pg/ml
IL-6	2000 – 15.6 pg/ml	1/10 -1/80	156 pg/ml
IL-11	500 – 3.9 pg/ml	½ - 1/16	7.8 pg/ml
Type 1			
IFNγ	2300 – 18.4 pg/ml	¼ - 1/32	73.6 pg/ml
CXCL9	4000 – 31.2 pg/ml	1/10 – 1/80	312.5 pg/ml
CXCL10	1000 – 7.8 pg/ml	1/10 – 1/80	78 pg/ml
CCL5	500 – 3.9 pg/ml	3/20 – 3/160	26 pg/ml
Type 2			
IL-5	1000 – 7.8 pg/ml	¼ - 1/32	31.3 pg/ml
IL-13	1000 – 7.8 pg/ml	¼ - 1/32	31.3 pg/ml
CCL17	500 – 3.9 pg/ml	1-10 – 1/80	39 pg/ml
Other			
IL-10	1000 – 7.8 pg/ml	¼ - 1/32	31.3 pg/ml

Recombinant MPV protein ELISA

Cell lysates of baculovirus-recombinant MPV nucleocapsid (N) protein (RV473-99/CAN99-80) and control wild-type baculovirus infected insect cells were assayed for protein content using Micro-BCA Protein Assay Kits (Pierce, Rockford, IL, U.S.A.). Using these antigens, an indirect ELISA was constructed to assess plasma antibody levels, as described [442]. Briefly, baculovirus-expressed proteins were diluted to 3 μg/ml in PBS and 100μl coated to polystyrene flat-bottomed microtiter plates (Nunc, Roskilde, Denmark). Plates were washed, blocked and human plasma samples were added at an optimal 1/100 and 1/400 dilution. Positive and negative serum control specimens were included in each assay to ensure reproducibility. Washed plates were subsequently incubated with peroxidase-conjugated anti-human IgG (1:25,000 Pierce) and developed using 1-Step Turbo TMB substrate system (Pierce). To determine seropositivity, each individual's N protein specific IgG response was deemed positive if it was at least 2 fold greater than the mean absorbance reading of the negative controls. In the population studied, the median response was six fold above the threshold of detection.

Flow Cytometry

PBMC were cultured (2.5 x 10^6 cells/ml) in media, staphylococcus enterotoxin B (SEB) (Sigma), infectious MPV or RSV for 6, 12, 18, 24 hours, 3 and 5 days. Washed cells (PBS with 1% BSA, 2mM EDTA) were incubated 30 minutes on ice with fluorochrome-conjugated anti-CD3-PC5, CD4-FITC, CD8-FITC, CD25-PE and CD69-PE and isotype-matched controls (BD Pharmingen or Biolegend). Cells were then washed, resuspended and analyzed using FACS Calibur with acquisition and analysis performed on CellQuest Pro Software (BD Biosciences). The lymphocyte population within total PBMC was gated using

forward and side scatter and CD3-PC5$^+$CD4-FITC$^+$ or CD3-PC5$^+$CD8-FITC$^+$ were selected and analyzed for CD69 and CD25 expression. SEB was used as a positive control for induction of activation markers, assessed as both percentage of activated cells and intensity of marker expression. Also, CD69 and CD25 expression were analyzed as normalized mean fluorescent intensity (percentage of positive cells multiplied by the mean fluorescent intensity), as described in [443].

Real-time PCR

A real-time PCR assay was developed to quantify reovirus genomic RNA using the L1 gene relative to the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) within infected human PBMC and mouse L929 fibroblast cells. Total viral and cellular RNA was extracted from cells using Tri-reagent-chloroform extraction method (Tri reagent, chloroform, isopropanol, and ethanol from Sigma, St. Louis, MO) according to the manufacturer's protocol. RNA pellets were resuspended in DEPC-treated water for 15 minutes at 65°C. A total of 0.68μg of RNA was used for RNase-free DNase I digestion (Pharmingen) prior to cDNA synthesis [444]. RNA was reverse transcribed into cDNA by using SuperscriptII reverse transcriptase (Invitrogen) with unlabeled sequence-specific primers for GAPDH (endogenous control) and reovirus LI gene (target gene, **Table 5**), in a 40-μl reaction mixture. RT-PCR amplification mixtures (25 μl) contained 2.5μl cDNA template, 2x SYBR Green I Master Mix buffer (12.5 μl) (Applied Biosystems) and 0.1μM forward and reverse primer for L1-specific reactions (0.4μM forward and reverse primer for GAPDH-specific reactions, **Table 5**). Reactions were run on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). The cycling conditions consisted of 10 min polymerase activation/DNA melting at 95°C and 42 cycles at 95°C for 15 sec and 61°C for

60 sec. Each assay included (in duplicate): a positive control of reovirus cDNA from infected L929 fibroblast cells, a no-template control, and ½ dilution of each test cDNA. All PCR efficiencies were above 95%. Fluorescence values for L1 gene were normalized to those of GAPDH gene, and expressed as fold-change over control group [445].

Statistical analysis

Associations between antigen-driven responses were determined using 2-tailed Wilcoxon tests (paired, nonparametric data). Normalized data (percent response) were analyzed using a paired t-test. Fisher's exact test was used to compare differences in frequencies of responsive individuals.

Associations between responses in clinical groups were determined using 2-tailed Mann-Whitney tests (nonparametric data). Spearman's test was used to evaluate potential correlations between antigen-driven cytokine responses.

Table 5: Reovirus L1 and human GAPDH sequence-specific primers for Real-Time PCR

Primer	Use	Sequence	Amplicon size (bp)
Reovirus L1 (3829R)	RT	5'-cat ggg tcg tgg tgc gtc caa-3'	N/A
hGAPDH (965R)	RT	5'-gtg gtc tcc tct gac ttc aac ag-3'	N/A
Reovirus L1 (2520F)	PCR	5'-gtg gca gcg gtg gat acg-3'	218
Reovirus L1 (2738R)	PCR	5'-gcc ctc tga tga caa gat gga-3'	
hGAPDH (418F)	PCR	5'-gag aag gct ggg gct cat-3'	129
hGAPDH (547R)	PCR	5'-cag cct caa gat cat cag ca-3'	

CHAPTER 1

Human Metapneumovirus Elicits Weak IFNγ Memory Responses Compared with Respiratory Syncytial Virus

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ABSTRACT

Human metapneumovirus (MPV) is a recently discovered pathogen that causes repeated lower respiratory tract infections beginning in infancy. The prevalence, nature and control of human regulatory responses to MPV are unknown. Here, we develop and optimize systems to evaluate MPV-driven cytokine responses. Using primary culture of human peripheral blood mononuclear cells (PBMC) from previously exposed adults, MPV-stimulated responses were directly compared with those elicited by genetically and clinically similar, respiratory syncytial virus (RSV). Intense IL-6 production was evident following culture with infectious or inactivated RSV. MPV elicited IL-6 responses averaging 3.5 fold more intense ($p < 0.001$). Virus-dependent expression of IL-11, IL-12, IFNα and other innate immunity cytokines differed little between MPV and RSV. When examining adaptive immunity, RSV infection elicited strong IFNγ responses by all 60 adults. In marked contrast, MPV elicited IFNγ in a lower frequency of adults ($p < 0.002$) and at levels averaging six fold weaker ($p < 0.001$). These Th1-dominated responses were CD4, CD8, CD86 dependent, and were closely paralleled by strong virus-driven IL-10 and CCL5 production. For MPV and RSV, Th2 (IL-5, IL-13) responses were sporadic, occurring in 10-40% of the population. Thus, MPV and RSV, although both ubiquitous and leading to very high levels of infection, seroconversion and clinically similar presentation in the population, evoke distinct innate and adaptive T cell-dependent cytokine responses. While both viruses yield Th1-dominated responses with strong IL-10 and CCL5 production, MPV re-stimulation results in markedly more robust IL-6 and significantly weaker adaptive cytokine responses, in both prevalence and intensity, than does RSV.

PREAMBLE

Virus infections are the primary cause of respiratory illness in humans. Of these, RNA viruses such as respiratory syncytial virus (RSV) and human metapneumovirus (MPV) are the most common pathogens associated with lower respiratory tract infections [237, 446]. MPV is a recently discovered member of the *Paramyxoviridae* family [411] that is genetically [447] and clinically [420] similar to RSV. Extensive literature describes human humoral immune responses to RSV [288, 448-451] and MPV [411, 451]. Seropositivity usually develops during early childhood. However, despite humoral immunity, symptomatic re-infection by RSV [309, 435] and MPV [412, 415, 420] occurs throughout life. In marked contrast to our increasing understanding of humoral immune responses to this recently discovered virus, nothing has been published examining the frequency or nature of immune regulation in MPV-specific responses.

RATIONALE AND HYPOTHESIS

Currently, animal models have been utilized to study MPV-driven cytokine and chemokine responses [452-454]. In BALB/c mice, MPV induces weak innate and Th1 responses, with Th2 cytokine production and IL-10 evident late in the course of infection [222, 455]. Very little is known regarding the profile of MPV-dependant cytokine responses in humans [424, 456]. Here, we develop and optimize systems for the first comprehensive immunological study centered on the immune responses of humans to MPV. Using PBMC of healthy adults in short term primary culture, MPV-specific innate and recall immune responses are assessed following *in vitro* re-stimulation, comparing infectious and inactivated virus. ***We hypothesize that despite extensive genetic and clinical similarities***

between RSV and MPV, the immunologic responses they elicit differs in terms of the

prevalence and intensity of anti-viral cytokine production.

RESULTS

Seropositivity for MPV exposure is ubiquitous

Previous studies demonstrate that RSV exposure is ubiquitous and that the vast majority of adults are seropositive, with seroconversion occurring during the first two years of life [220, 435]. To assess the prevalence of MPV seropositivity in this cohort of healthy adults, plasma were tested for antibodies against the nucleocapsid protein of MPV (Data not shown). MPV N protein-specific IgG demonstrated significant increases compared to background in each individual studied (n=63, Wilcoxon p<0.001), indicating that each individual had previously been exposed to MPV and developed an immune response. This finding is in agreement with recent studies indicating a high frequency of MPV seropositivity amongst healthy adults [411, 415, 442].

MPV elicits stronger innate immunity cytokine production than does RSV

To examine the frequency and nature of MPV-driven cytokine production in humans, we developed a short-term, *in vitro* primary culture system using PBMC isolated directly *ex vivo* from healthy adults. PBMC were cultured with infectious vs. heat-inactivated MPV for 24 hours, the time found in preliminary experiments with 5 subjects (Data not shown) to yield maximum production of the innate immunity cytokines of interest. To compare these responses to phylogenetically related RSV, parallel cultures were set up with live RSV at the same titer of infectious virus, and with heat-inactivated RSV. Incubation of PBMC with

LLC-MK2 and Hep-2 cell lysates, as mock infection controls, did not induce detectable cytokine production for any of the readouts examined, demonstrating that cytokine responses elicited by these viruses were antigen-dependent.

Strong IL-6 responses, with median values of ~800 pg/ml, were seen following stimulation with RSV. Similar levels were elicited by infectious and heat-inactivated virus. In contrast to RSV, MPV elicited IL-6 responses that averaged 200-300% more intense (**Figure 8**). These increases were not dependent upon productive viral replication as inactivated virus yielded indistinguishable responses.

In contrast to the robust IL-6 responses seen, only half of participants produced detectable IL-11 responses to either RSV or MPV (**Figure 8**), and between a quarter and third of individuals produced detectable IFNα (Data not shown). For these cytokines, no difference was evident between RSV and MPV driven stimulation. Other cytokines associated with innate immunity that play a role in promoting pro-inflammatory or Th1-like adaptive recall responses, such as IL-12, IL-1β, TNFα, or anti-inflammatory IL-10, were consistently undetectable at 24 hours with either infectious or inactivated RSV or MPV (Data not shown). Thus, based on production of innate immunity associated cytokines in a healthy adult population, MPV elicits similar, or for IL-6, markedly stronger responses than does RSV.
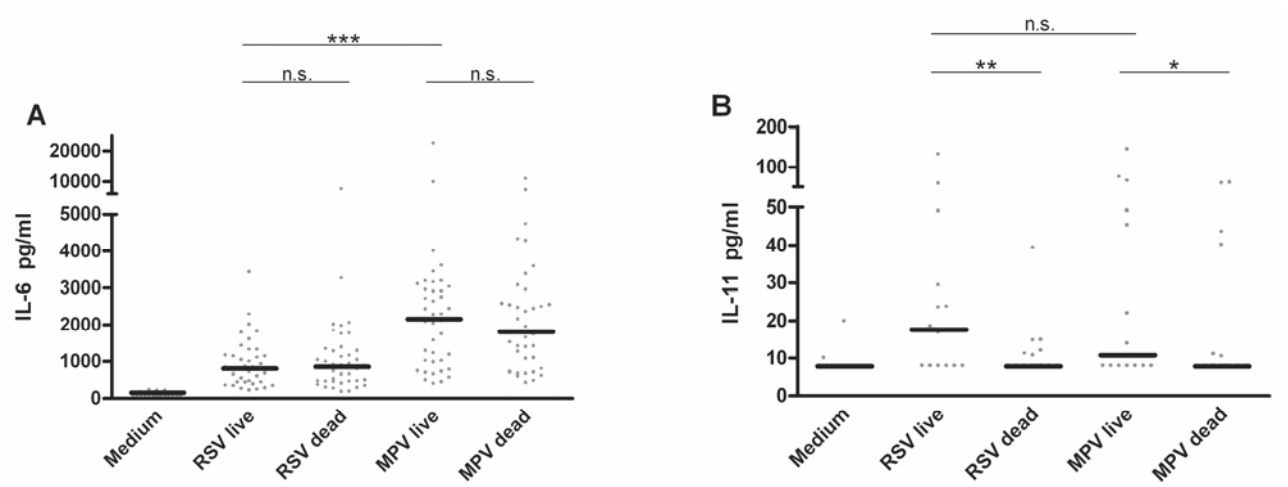
Figure 8: Human metapneumovirus (MPV) induces stronger IL-6 responses than respiratory syncytial virus (RSV). Antigen-dependent innate responses to RSV and MPV were compared to medium alone. IL-6 **(A)** and IL-11 **(B)** were measured by ELISA in supernatants from 24 hour PBMC cultures. Black bars represent median responses from 14-41 healthy adults. P values represent significant differences in cytokine production: (***P<0.001, **P<0.01, *P<0.05).

MPV induced recall responses are distinct from those elicited by RSV

To evaluate adaptive cellular immune responses, fresh primary PBMC were cultured for six days with infectious versus inactivated MPV or RSV. Parallel cultures were established using streptokinase, a ubiquitous bacterial antigen that elicits T cell dependent recall cytokine responses in most adults [457]. As shown in **Figures 9 and 10**, primary culture with streptokinase reveals readily detected type 1 (IFNγ) and type 2 (IL-13) recall responses, demonstrating the sensitivity of this experimental approach.

RSV infection elicited strong IFNγ responses by all 60 adults in short term culture. In marked contrast, stimulation with infectious MPV elicited detectable IFNγ responses in a significantly lower frequency of adults (74% vs. 100%, Fishers $p<0.0002$). Moreover, the median MPV-specific IFNγ response, while clearly substantial (9.6 U/ml, equivalent to 1100 pg/ml WHO standard), was on average, six fold weaker than responses elicited by RSV. IL-13 and IL-5 responses (not shown) were weak in intensity and were detectable in ~10-40% of the population (**Table 6**). Thus, no difference in the intensity or prevalence of type 2 immunity was evident when comparing MPV and RSV stimulation.

Interestingly, Th1 biased memory responses to these viruses were paralleled by RSV-driven IL-10 and CCL5 responses (**Figures 9 and 10**). MPV driven IL-10 production was readily detected, but as seen with IFNγ responses, was on average 2.5 fold lower than RSV elicited responses ($p<0.001$). CCL5 was consistently produced in response to RSV, but was both less frequent ($p<0.0003$, **Table 6**) and markedly weaker ($p<0.001$) in response to MPV stimulation.

Table 6: Prevalence of infectious human metapneumovirus (MPV) and respiratory syncytial virus (RSV)-dependent responses in adults *.

Cytokine	RSV		MPV		*P*
IL-6	41/41	(100)	41/41	(100)	NS
IL-11	7/14	(50)	6/14	(43)	NS
IFNα	8/22	(36)	7/32	(22)	NS
IFNγ	46/46	(100)	34/46	(74)	.0002
IL-10	46/48	(96)	34/48	(71)	.0018
CCL5	26/40	(65)	9/40	(23)	.0003
IL-5	4/48	(8)	10/48	(21)	NS
IL-13	15/48	(31)	18/48	(38)	NS

* Data are frequency (%) of subjects. Fisher's test derived p values.
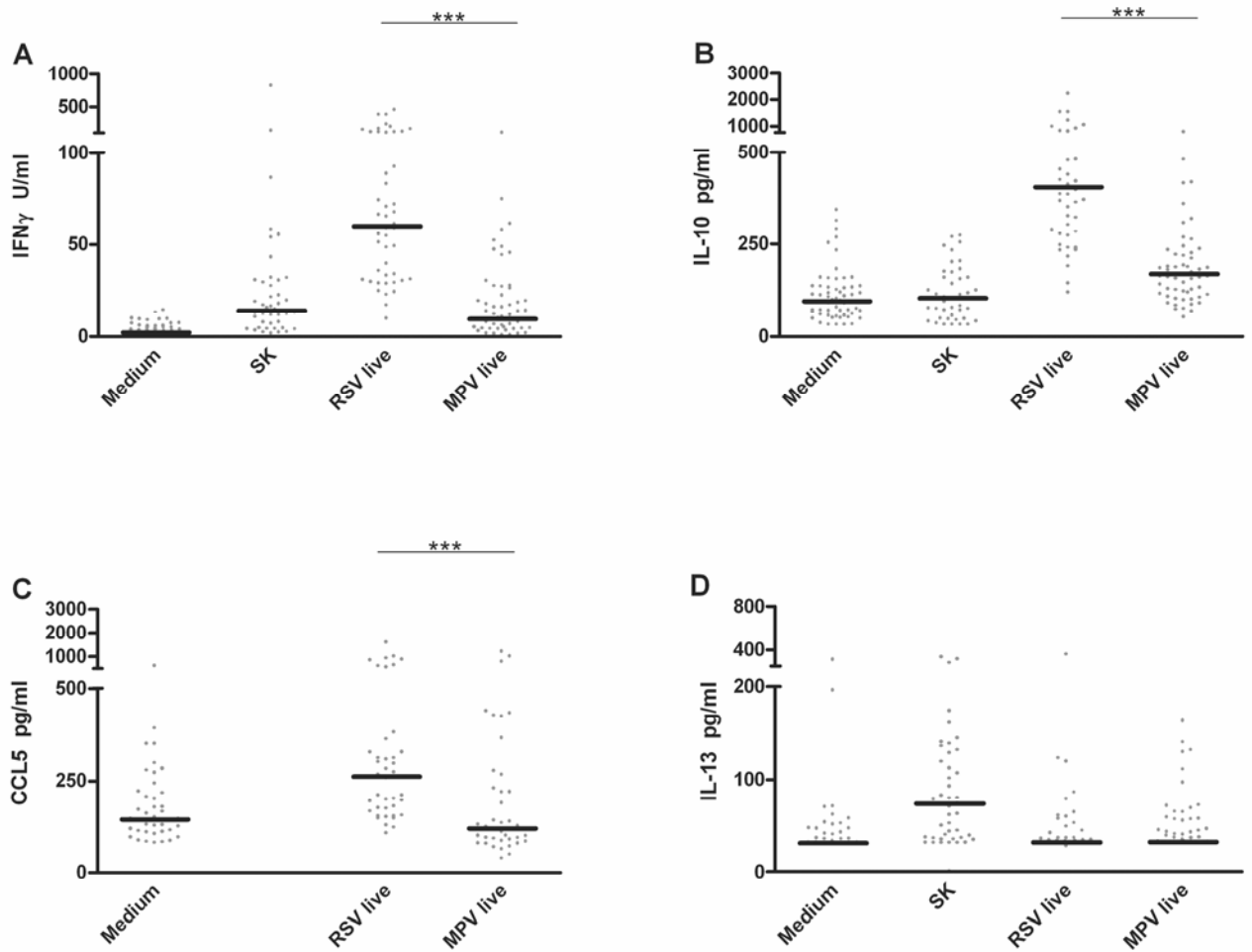
Figure 9: **Infectious human metapneumovirus (MPV) and respiratory syncytial virus (RSV) induce Th1 biased recall responses.** Antigen-dependent recall responses to streptokinase (SK), live RSV and live MPV, as compared to medium alone. IFNγ **(A)**, IL-10 **(B)**, CCL5 **(C)** and IL-13 **(D)** were measured by ELISA in supernatants from 6 day PBMC cultures. Black bars represent median responses from 60 individual healthy adults (▫). (***P<0.001).
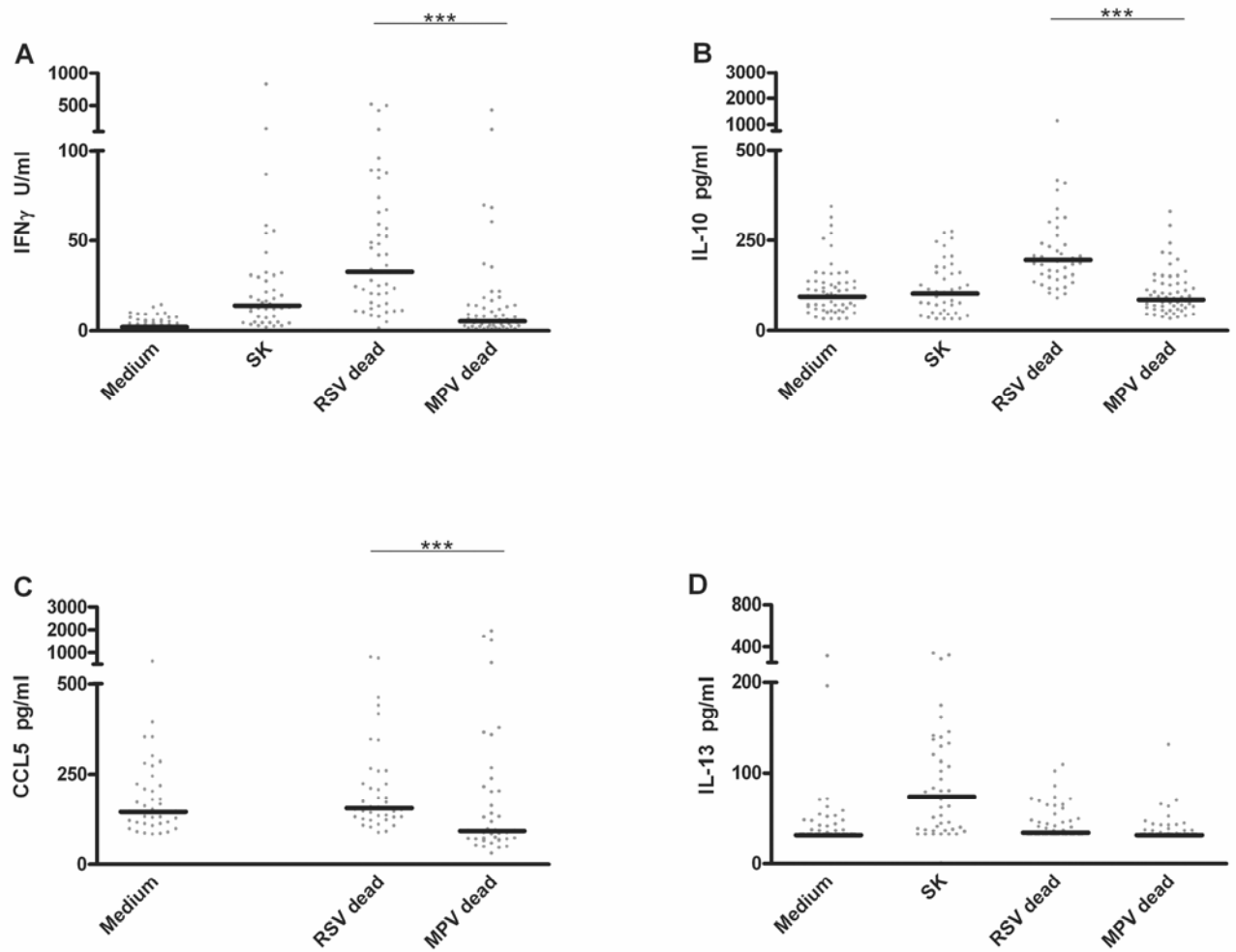
Figure 10: **Inactivated human metapneumovirus (MPV) and respiratory syncytial virus (RSV) induce Th1 biased recall responses.** Antigen-dependent recall responses to streptokinase (SK), dead RSV and dead MPV, as compared to medium alone. IFNγ **(A)**, IL-10 **(B)**, CCL5 **(C)** and IL-13 **(D)** were measured by ELISA in supernatants from 6 day PBMC cultures. Black bars represent median responses from 60 individual healthy adults (∙). (***P<0.001).

Thus, this cohort demonstrated Th1-biased recall responses concomitant with virus dependent IL-10 production to both MPV and RSV. For both viruses, the cytokine responses elicited were most pronounced upon stimulation with live virus. In contrast to its rapid capacity to elicit superior or equivalent innate immune responses, MPV consistently elicited IFNγ, IL-10 and CCL5 in recall responses more weakly than did RSV (**Figure 11**). These increases in MPV stimulated IL-6 production, relative to RSV, and decreases in IFNγ, IL-10 and CCL5 were evident in almost all of the 48 individuals examined.

MPV-driven IFNγ production is mirrored by increased CCL5 and IL-10 production

Studies in IRF-1 deficient mice [458] and in fibroblast cell lines indicate that CCL5 expression is regulated by IFNγ. Here, examination of human immune responses in primary culture to these two physiologically relevant viral stimuli demonstrates that CCL5 production is positively correlated with IFNγ (**Figure 12**).

Given the range of individual responses seen throughout this population, and the fact that immune stimuli ranging from TLR ligands to endogenous and exogenous antigens usually demonstrate inverse production of IL-10 and IFNγ, we also examined the relationship between virus-driven IFNγ and IL-10 production to these respiratory viruses. Surprisingly, a strongly positive correlation between IFNγ and IL-10 levels was evident, with that for MPV-driven responses particularly strong (**Figure 12**, r = 0.73, p<0.001). Thus, individuals demonstrating recall responses to these respiratory viruses show concomitant increases in MPV or RSV dependent IFNγ coincident with proportional IL-10 and CCL5 induction.
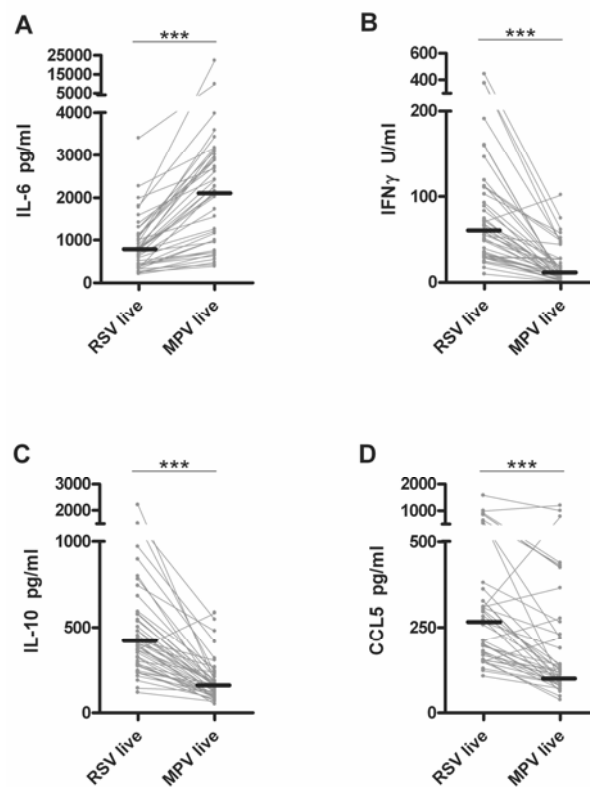
Figure 11: **Consistent differences in the intensity of human metapneumovirus (MPV) dependant responses compared to respiratory syncytial virus (RSV).** IL-6 **(A)**, IFNγ **(B),** IL-10 **(C)** and CCL5 **(D)** were measured by ELISA. Paired RSV and MPV responses from 41-48 healthy adults (•) are shown. P values represent significant increases in cytokine production between RSV and MPV stimulation: (***P<0.001).
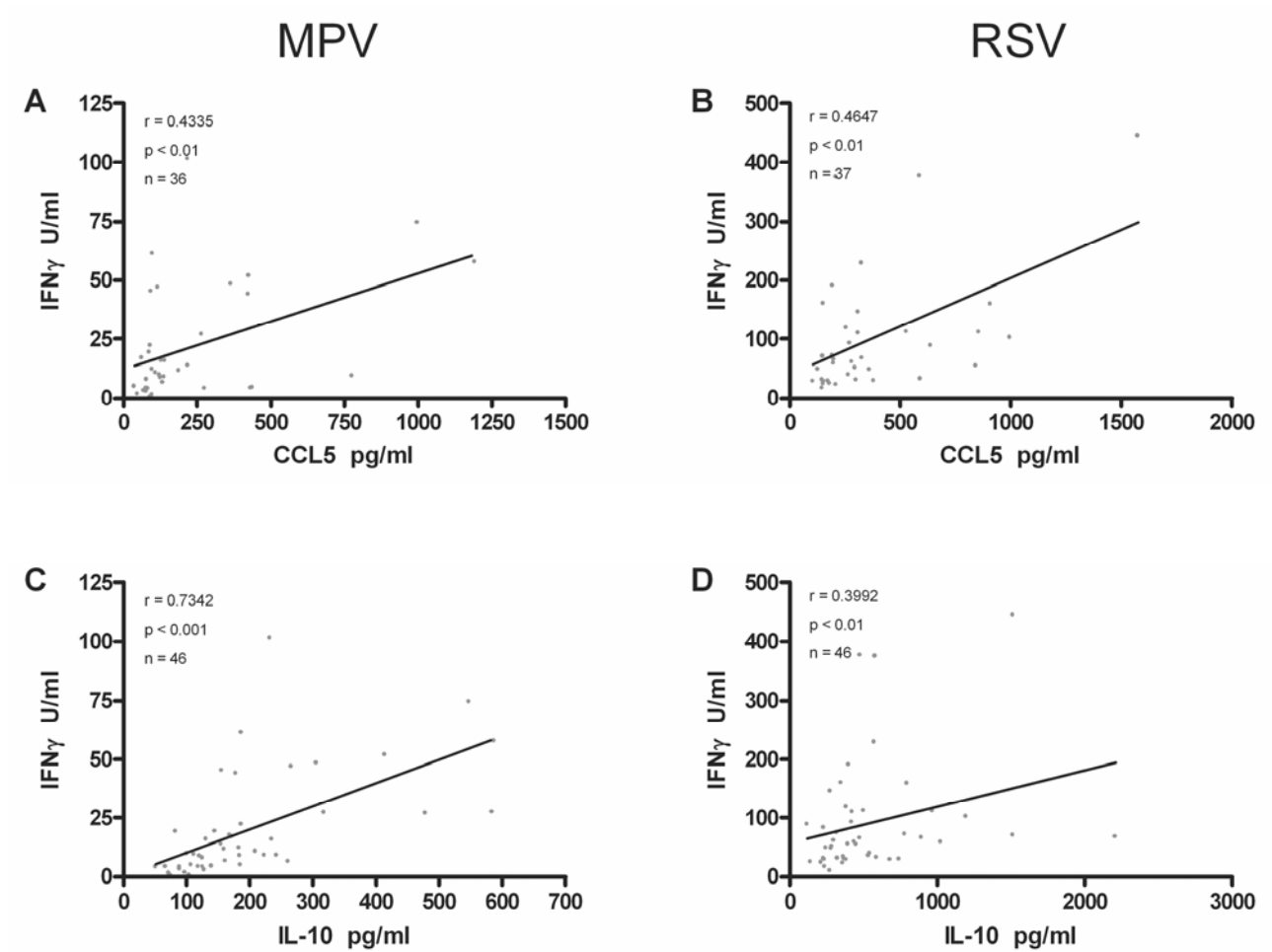
Figure 12:   **IFNγ/CCL5 and IFNγ/IL-10 production during MPV and RSV recall responses**

are positively correlated. Infectious virus dependant IFNγ and CCL5 responses in MPV **(A)** and

RSV **(B)** and IFNγ and IL-10 responses in MPV **(C)** and RSV **(D)** stimulated cultures (Day 6) were

measured by ELISA. Black bars represent best-fit slope (r) of IFNγ/CCL5 or IFNγ/IL-10 pairs

from 46 healthy adults (•). (***P<0.001, **P<0.01).

T cell involvement in MPV- and RSV-Specific Cytokine Production

To examine the role of CD4 and CD8 T cell populations in specific IFNγ responses, PBMC were cultured with live MPV or RSV in the presence of blocking anti-CD4, anti-CD8, anti-MHC class II or class I antibodies (**Figure 13**, panels A, B). As previously, MPV specific responses (median 22 U/ml) were substantially less intense than those elicited by RSV (median 385 U/ml). However, each was dependent on both class I and II antigen presentation and activation of CD4 T cells. CD8 T cells make a substantive contribution to RSV stimulated cytokine production but blocking the CD8 co-receptor had no detectable impact on MPV driven IFNγ production. The potential role of IFNγ production by NK cells was not addressed; however we note that the majority (>80%) of IFNγ induction in both RSV and MPV stimulated PBMC cultures was dependent upon CD4 T cell involvement and that IFNγ levels at day 1 were consistently undetectable (Data not shown).

To assess the requirement for co-stimulation and putatively differential dependence on CD80 versus CD86 in this response, PBMC were virus stimulated in the presence and absence of neutralizing antibodies to CD80, CD86 or with CTLA-4 Ig. Both RSV- and MPV-driven IFNγ production were clearly dependent on co-stimulation, with CD86 playing a pivotal role (**Figure 13**, panels C, D).

In contrast, there was no evidence of T cell requirements for pneumovirus-specific IL-10 production (Data not shown) as neither neutralising antibodies against CD4, CD8, MHC II or MHC I, nor blocking of the CD80/86 costimulatory pathway inhibited MPV- or RSV-driven IL-10 production.
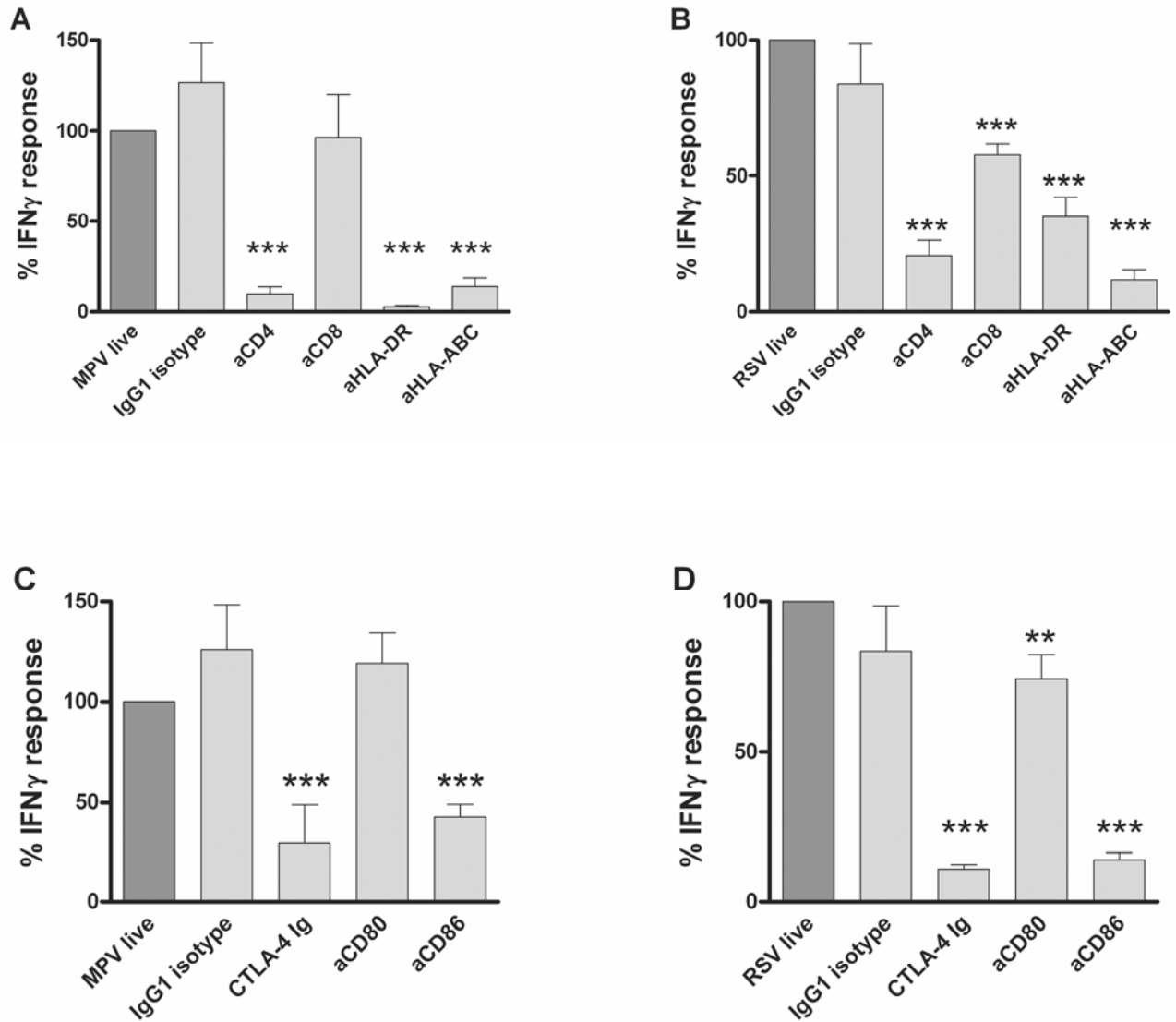
Figure 13: Classical CD4⁺ T cell activation requires CD86 costimulation for human metapneumovirus (MPV) and respiratory syncytial virus (RSV) dependent IFNγ responses. MPV **(A,C)** and RSV **(B,D)** stimulated PBMC were cultured with neutralising antibody to CD4, CD8, HLA-DR or HLA-ABC and with CTLA-4 Ig or neutralizing antibody to CD80 and CD86. Bars represent mean percentage ($\pm$ SEM) of positive control, virus stimulated IFNγ responses from 5 individuals. P values represent significant decreases in percentage of cytokine production compared to viral-driven response: (***$P<0.001$, **$P<0.01$).

MPV demonstrates reduced capacity to activate T cells relative to RSV

To extend the observation that MPV elicits weaker responses than does RSV in healthy adults, virus stimulated PBMC were examined by flow cytometry to assess T cell expression of CD69 as an early marker and CD25 as a late marker of cellular activation. SEB stimulated T cell populations were used as positive controls throughout. $CD4^+$ and $CD8^+$ $CD3^+$ T cells from RSV stimulated cultures exhibited readily detectable, and similar, increases in surface CD69 compared to unstimulated cells at 6-72 hours (Data not shown). Confirming the observations made for cytokine production, MPV induced weaker CD69 expression than did RSV through the time range examined, a finding evident for both CD4 and CD8 populations. Thus, at 12 hours, the time of maximal CD69 expression, an average of 13% $CD69^+CD3^+CD4^+$ T cells were detected in RSV stimulated cultures versus 2% seen in MPV stimulated cultures. In contrast to SEB, RSV and MPV stimulation failed to induce detectable increases in CD25 by either $CD4^+$ or $CD8^+$ T cells.

DISCUSSION

Despite increased understanding of human serological responses to MPV [411, 451], virtually nothing is known of the presence, nature or control of cytokine responses to this virus, or of the relationship between MPV and RSV-driven responses in humans. Here, we demonstrate that these viruses, both ubiquitous and leading to very high levels of infection and seroconversion in the population, elicit distinct innate and T cell-dependent cytokine responses. Primary culture of cells from a substantial cohort of healthy humans reveals that both viruses elicit classical CD4 and CD8 T cell activation that is dependent on antigen-presentation and CD86-mediated costimulation. Among adults, MPV is a consistently

stronger inducer of innate immunity associated IL-6, and a weaker inducer of IFNγ, IL-10 and CCL5 production. These differences in immune responses elicited by MPV versus RSV are evident whether measured by the frequency of responsive individuals or by the intensity of the cytokine response induced by virus exposure.

With the important exception of IL-6, MPV was a weaker inducer of cytokine production (IFNγ, IL-10 and CCL5) than was RSV. Interestingly, Guerrero-Plata *et al.* in a recent detailed analysis of human myeloid and plasmacytoid dendritic cells derived from 4-6 healthy adults also found that these viruses elicit distinct patterns of cytokine responses [459]. Interestingly, MPV driven IL-6 responses by enriched DC were markedly weaker than those obtained upon RSV stimulation [459]. This may be attributable to use of different viral isolates, comparison of isolated DC versus the global PBMC response or to the smaller numbers of subjects examined (n=6 versus n=63 here).

Use of dead RSV and MPV consistently demonstrates weaker cytokine responses (p<0.05 to <0.001) than does exposure to live virus. While we did not quantify the intensity of productive infection to these two viruses in culture across this population, the finding that stronger responses are observed upon active infection is consistent with prior observations [183, 459]. Guerrero-Plata's finding that RSV's ability to productively infect myeloid dendritic cells is linked with the increased strength of anti-viral cytokine responses, underlines the role played by infection [138]. At the same time, the overall pattern of responses seen for either virus (pro- versus anti-inflammatory; Th1 versus Th2) does not differ markedly following exposure to live or dead virus.

Two additional studies examine non-serological responses of humans to MPV infection. The first assessed levels of inflammatory cytokines in nasopharyngeal aspirates from 10 infants currently infected with MPV in comparison to cytokine levels in historic controls of RSV-infection [424]. They concluded that the pattern of local chemokine

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production seen with MPV differed from that seen for RSV. Laham *et al.,* comparing RSV infected neonates with MPV infected neonates, demonstrated similar clinical manifestations during the course of active infection and, in general, higher levels of cytokine production in respiratory secretions of RSV infected children [456]. Here, using 63 adults with histories but no clinical signs of ongoing MPV or RSV infection, primary culture of virus stimulated PBMC demonstrates that MPV is a clearly superior stimulus for IL-6 production relative to RSV.

It remains unclear why MPV's capacity to elicit stronger IL-6 responses, and similar production of most other innate cytokines, is paralleled here by markedly weaker IFNγ (and IL-10, CCL5) production than RSV. Several mechanisms of IFNγ inhibition have been described in RSV infection, such as inhibitory viral proteins, regulatory effects on dendritic cells, and inhibition of immune memory [167, 216, 460-462]. RSV nonstructural (NS1 and NS2) proteins can inhibit production of type 1 interferons (IFNα/IFNβ), and also have key roles in down-regulating activation of antigen presenting cells [460, 462, 463], thereby potentially diminishing T cell dependent anti-viral responses. One obvious disparity between the *Pneumovirinae* species is that MPV's viral genome does not code for these NS proteins [411]. This initially led us to anticipate that MPV-driven IFNγ responses would be stronger, not weaker, than those elicited by RSV. Given that MPV differs from RSV in lacking NS proteins, yet the IFNγ responses seen are weaker, the difference is clearly not attributable to this mechanism.

Alternately, the weak anti-MPV recall response, relative to those seen with many other respiratory viruses, may be attributable to the initial pattern of innate immunity that develops. MPV is far superior to RSV at inducing IL-6. IL-6 has been shown to inhibit Th1 differentiation and suppress IFNγ expression by direct effects on T cells (reviewed in [464]).

Naïve T cells primed in the context of IL-6 are unable to differentiate into high IFNγ producing effector cells [465]. Thus, we speculate that during natural MPV infection *in vivo*, MPV-specific T cells may be primed in a milieu that leads to weaker primary and recall anti-viral responses than are seen for RSV, a weaker inducer of IL-6 synthesis. Such studies are best approached in longitudinal analyses of deliberate infection of mice or human models [466].

Weaker Th1 responses may reflect lower frequencies of responding memory cells in individuals, as suggested by the substantial decrease in the frequency of individuals in the population with detectable IFNγ recall responses when comparing MPV to RSV. We tested whether this difference was related to activation of T cells by these viruses, however no significant expression of activation markers on CD4 and CD8 T cells were detectable in MPV or RSV stimulated cultures. Minimal differences in frequency of CD69^{+} T cells were observed in RSV versus MPV stimulation, but CD25^{+} T cell frequency did not significantly differ. Others have found that circulating RSV-specific CD8 T cells are found at a lower frequency than influenza-specific cells [215], and their IFNγ recall responses are substantially lower [218]. This may be partially explained by the fact that natural reinfection by RSV does not lead to long term expansion of the RSV-specific T cell compartment in humans [214]. Murine models of RSV infection also demonstrate impaired development and sustainability of memory CD8 T cells [216]. It is presently unclear if the lower MPV dependent IFNγ, IL-10 and CCL5 responses seen reflect a lower prevalence of virus exposure in the community, hence fewer booster infections, or conversely, if lower numbers of clinical reinfections [415, 420, 421], are attributable to IFNγ, IL-10 and CCL5 independent mechanisms of host resistance. In this study, the novel finding of positive correlation between IFNγ/IL-10 and IFNγ/CCL5 levels in both RSV and MPV stimulated PBMC suggests a mechanism governing the overall strength of anti-viral response occurs in each individual.

The relevance of virus-stimulated cytokine production to clinical outcome is made explicit by strong associations between decreased levels of IFNγ and IL-10 production with increased severity of RSV infection [467-469]. Similarly, it will be important to determine the extent to which MPV's weaker capacity to induce IFNγ and IL-10 *in vitro* may relate to symptom severity and clinical outcomes during infection. Direct comparison of laboratory confirmed MPV, RSV and influenza infections in families demonstrates that despite a lower prevalence of infection, MPV is associated with increased wheezing, asthma exacerbations, medical visits and absenteeism [421]. Thus, our data suggests a possible immunologic explanation for the greater symptoms and socioeconomic impact of MPV infection compared to RSV and influenza infections [421]. Moreover, weak anti-viral responses to MPV may be a contributing factor to the epidemiologic observations of increased asthma exacerbations. Consequently, we recently initiated studies to investigate putative differences in the nature and intensity of MPV versus RSV stimulated recall response in individuals with different clinical phenotypes.

The present study provides the first comprehensive overview of the cell-mediated immune response to MPV in humans. Despite extensive genetic and clinical similarities between these viruses, the immune response to MPV is characterized by stronger IL-6 and lower frequency, weaker intensity, IFNγ, IL-10 and CCL5 cytokine production in the population than is seen to RSV. These data demonstrate that the recall immune response to MPV is more limited that that of other typical respiratory viruses, and suggest a mechanism that may underlie the life-long, typically symptomatic, re-infection with this virus [412, 415, 420].

CONCLUDING REMARKS

This publication sets the stage to examine putative differences in anti-viral immunity in a variety of clinical and genetic sub-groupings. Having established a human model of acute viral infection with MPV and RSV allows hypothesis testing using a validated and reliable measure of anti-viral immunity. Specifically, we will use this technique to assess whether asthmatics mount dissimilar (in terms of frequency, intensity and Th1/Th2 skewing) responses to pneumoviruses than healthy individuals.

CHAPTER 2

Adult Asthmatics Display Exaggerated IFNγ Responses to Human Metapneumovirus and Respiratory Syncytial Virus

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ABSTRACT

Human metapneumovirus (MPV) and respiratory syncytial virus (RSV) are RNA viruses associated with lower respiratory tract infections. Regular symptomatic re-infection and sequelae are common, particularly in individuals with compromised pulmonary function. Our understanding of virus-dependent cytokine responses and potential differences between allergic asthmatic and non-asthmatics is limited. To test our hypothesis that adults with mild allergic asthma, the most common form of this disease, exhibit distinct pro-inflammatory responses, we utilized acute *in vitro* infection of fresh peripheral blood mononuclear cells. For both viruses, production of innate immunity associated IL-6 and IL-10 was indistinguishable in the two populations. Type 1 cytokine production dominated adaptive immune responses in both asthmatic and non-asthmatic individuals. Surprisingly, asthmatics exhibited stronger pro-inflammatory IFNγ production to MPV than non-asthmatic adults ($p = 0.01$), with a similar, but statistically non-significant trend in the RSV-stimulated response. Neutralizing IL-10 did not enhance the intensity of IFNγ responses, demonstrating that this pro-inflammatory bias is not counter-regulated by IL-10. Finally, anti-TLR4 blocked LPS but not RSV-driven cytokine production. Collectively, the data demonstrate that asthmatics are characterized by markedly stronger pro-inflammatory IFNγ responses to pneumoviruses than their non-asthmatic counterparts. This distinctive pattern of viral immunity may contribute to the enhanced sensitivity of asthmatics during respiratory virus infection.

MPV and RSV are speculated to play an important role in asthma pathogenesis [281, 419]. The consequences of infection are more severe in certain groups, such as allergic asthmatic individuals, where viral respiratory infections are strongly associated with exacerbation of asthma symptoms [77, 314, 419, 470]. Extensive studies in murine infection models demonstrate the essential role of T cells in pneumovirus-mediated airway inflammation and hyper-responsiveness [230, 471, 472]. In contrast, the human immune response to MPV has only recently begun to be characterized [138, 473]. Given that respiratory viruses are the major cause of asthma exacerbations [474], it is important to assess how, if at all, innate and T cell-dependent cytokine responses may be dysregulated in asthmatic humans compared to non-asthmatic controls.

RATIONALE AND HYPOTHESIS

No evaluation of T cell-dependent immunity against MPV in adult asthmatic and non-asthmatic humans has been published. Intrinsic differences in the way asthmatics develop and express immunity to respiratory virus infection have been speculated to contribute to viral triggering of asthma exacerbation, but our knowledge of the nature of immune responses in currently uninfected, asthmatic and healthy human populations is extremely limited. Therefore, using *in vitro* systems that employ acute viral infection, that we initially developed to assess MPV and RSV-specific cytokine responses in the general population [473], we examined virus-specific responses in adults with and without allergist diagnosed allergic asthma. To test our ***hypothesis that adults with mild allergic asthma, the most common form of this disease, would demonstrate a pattern of pro-inflammatory cytokine production distinct from that elaborated by non-asthmatics during responses***

against pneumoviruses, and to compare our findings with results obtained in murine models of RSV and MPV infection *in vivo*, [222, 475, 476], we evaluated production of key virus-induced cytokines (IL-6, IFNγ, IL-13 and IL-10) reflecting innate and adaptive immunity.

RESULTS

Innate immunity towards pneumoviruses in allergic asthmatic adults are characterised by IL-6 responses similar to those seen in healthy controls.

Using a model of acute viral infection of PBMC obtained from RSV and MPV seropositive individuals who did not report any viral respiratory infections in the preceeding month [473], strong IL-6 responses were seen following stimulation with either MPV or RSV. For both MPV and RSV, the intensity of the response seen in adult asthmatics and non-asthmatic individuals was not significantly different between populations with different clinical phenotypes (**Figure 14**, $p > 0.1$).

Both human and murine models [473, 475, 476], of pneumovirus-specific infection demonstrate substantive IL-10 production. As seen for IL-6, no differences in MPV or RSV stimulated IL-10 production were detectable when comparing allergic-asthmatic with non-asthmatic adults. Thus, each virus stimulates substantive innate immune responses that are of comparable intensity in allergic asthmatic and healthy adults upon acute viral infection of PBMC.
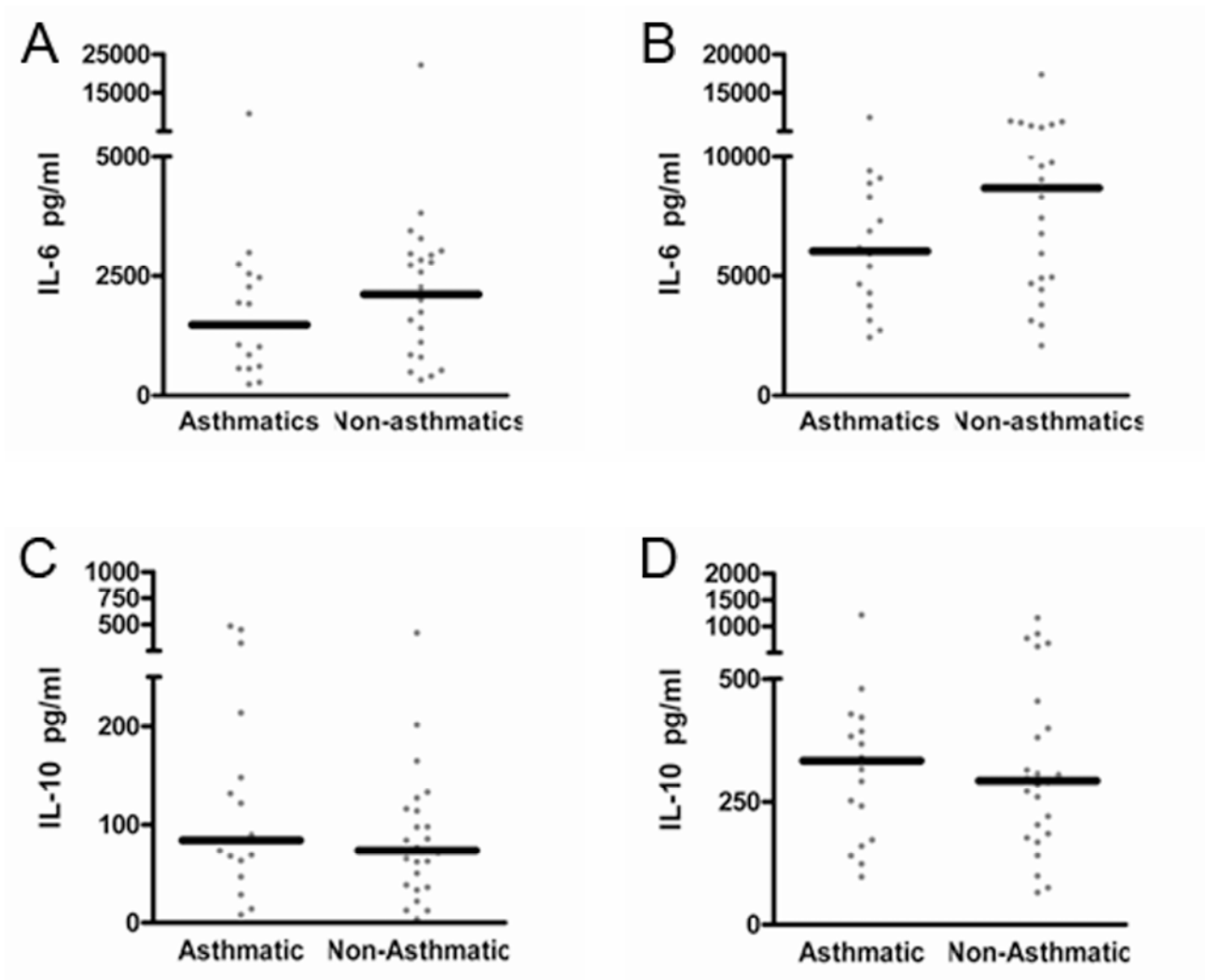
Figure 14: Allergic asthmatic and non-asthmatic adults generate similar IL-6 and IL-10 responses to both human metapneumovirus (MPV) and to respiratory syncytial virus (RSV). IL-6 was measured by ELISA in supernatants from 24 hour MPV **(A)** and RSV **(B)** stimulated PBMC cultures (p>0.05). IL-10 was measured by ELISA in supernatants from 6 day MPV **(C)** and RSV **(D)** stimulated PBMC cultures (p>0.05). Black bars represent median responses, each from an individual adult (•).

There are controversial results regarding the elaboration of Th1 versus Th2 cytokine production in response to pneumoviruses [409, 477], likely as a consequence of the range of experimental approaches used to assess immune capacity in such individuals. These vary from more direct stimulation with live or inactivated virus to more indirect measurements of immunity (i.e., mitogen driven) during RSV infection. Studies of immune capacity during naturally acquired infection are further complicated by the impossibility of determining the size or timing of the infectious dose encountered. Here, to assess RSV and MPV-specific adaptive immunity, we evaluated cytokine production stimulated by acute *in vitro* virus infection of PBMC from previously virus infected, but currently healthy, individuals using IFNγ as a marker of Th1-like anti-viral immunity and IL-13 as a prototypical marker of Th2 biased responses. **Figure 15** demonstrates immune responses of 17 asthmatic and 25 non-asthmatic adults in response to MPV or RSV infection. All individuals produced readily quantified, usually intense, IFNγ responses. IL-13 production was much more limited with less than half of each population generating detectable IL-13 responses, typically at low levels. Thus, in response to either MPV or RSV, both asthmatics and non-asthmatic individuals exhibited markedly type 1 immunity dominated cytokine responses. Surprisingly, asthmatic individuals exhibited markedly stronger pro-inflammatory IFNγ responses towards MPV than did non-asthmatic adults (median response 1575 pg/ml versus 733 pg/ml, p = 0.01). Increased IFNγ responses towards RSV were also observed in the asthmatic population, but these did not reach statistical significance in this size population. PBMC from asthmatic individuals demonstrated marginally decreased IL-13 responses towards RSV compared to the non-asthmatic population (**Figure 15**, p = 0.04). This further

indicates how allergic-asthmatic individuals exhibit enhanced Th1 bias towards respiratory virus infection compared to non-asthmatic individuals.

Notwithstanding the finding that IL-10 responses were indistinguishable in asthmatic and non-asthmatic populations (**Figure 14**), we speculated that the alterations in IFNγ responses were secondary to IL-10 production. However, use of neutralizing anti-IL-10 monoclonal antibodies to block the activity of IL-10 generated in MPV cultures did not influence the intensity of IFNγ responses (p > 0.2, **Figure 16**). These data argue that impaired IL-10 responsiveness was not responsible for the increased Th1 bias demonstrated in the virus-specific response generated by allergic asthmatic individuals.

Pneumovirus-driven IL-6 and IFNγ production are not mediated through virus-dependent TLR4 stimulation

TLR4 is an important component of the receptor for lipopolysaccharide (LPS). RSV fusion (F) protein has also been suggested as a ligand for this innate pattern recognition receptor [103]. Stimulation of human monocytes with RSV F protein was reported to stimulate strong IL-6 production [103]. We therefore determined whether blockade of TLR4 signalling in LPS- or RSV-stimulated PBMC cultures using anti-TLR4 monoclonal antibodies would alter the pattern or intensity of cytokine responses seen upon virus infection (**Figure 17**). Neutralizing antibodies directed at TLR4 block LPS driven responses. In marked contrast, TLR4 blockade had no effect on the intensity of RSV-driven IL-6 production (**Figure 17B**). Similarly, addition of anti-TLR4 monoclonal antibodies did not affect IFNγ production in response to RSV stimulation (Data not shown). These data argue that immune responses in human PBMC driven by RSV are TLR4 independent.
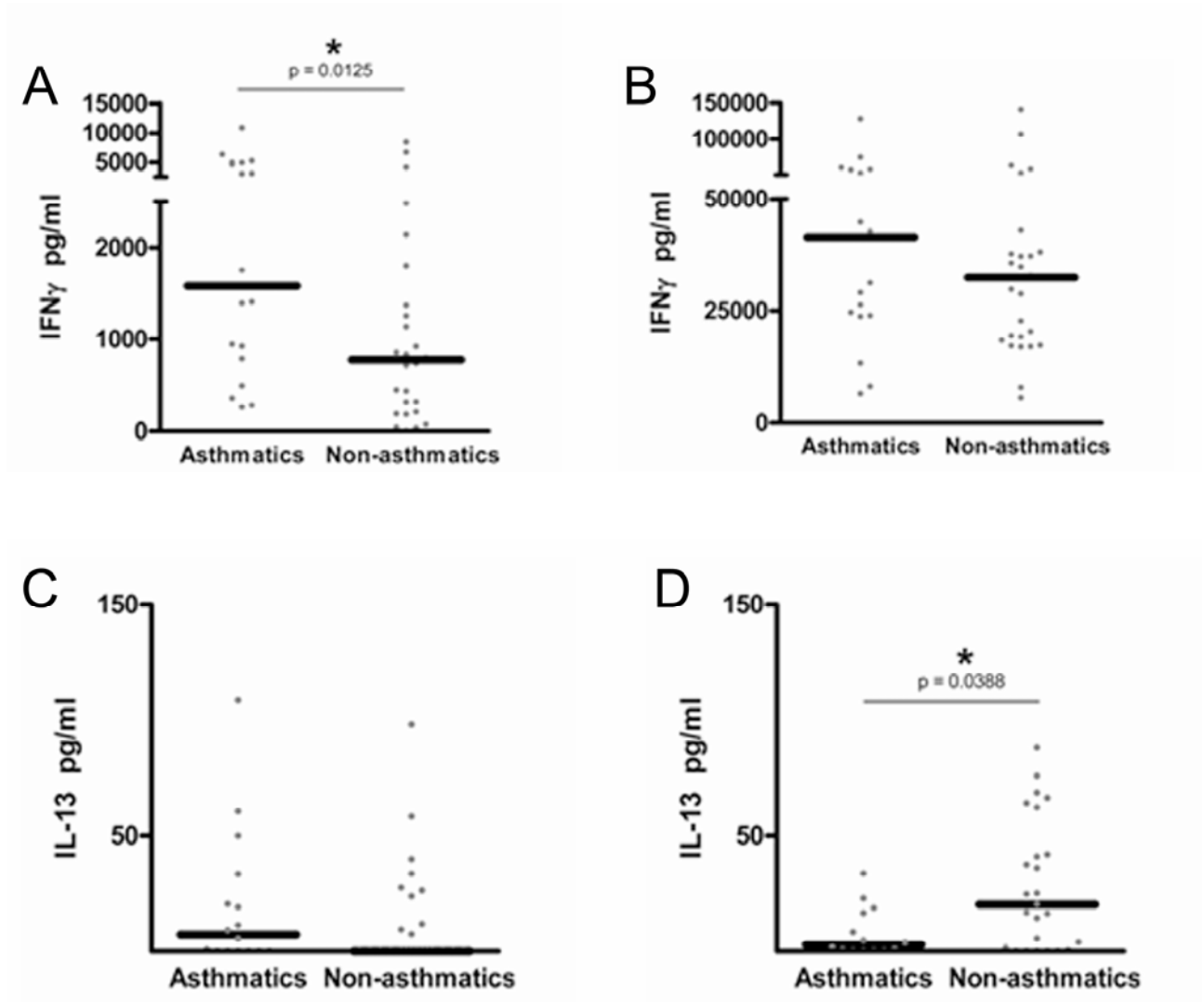
Figure 15: Adult asthmatics display exaggerated IFNγ responses to respiratory viruses. IFNγ **(A, B)** and IL-13 **(C, D)** were measured by ELISA in supernatants from 6 day MPV **(A, C)** and RSV **(B, D)** stimulated PBMC cultures. Black bars represent median responses, with individual values from each adult shown (•). (*P<0.05).
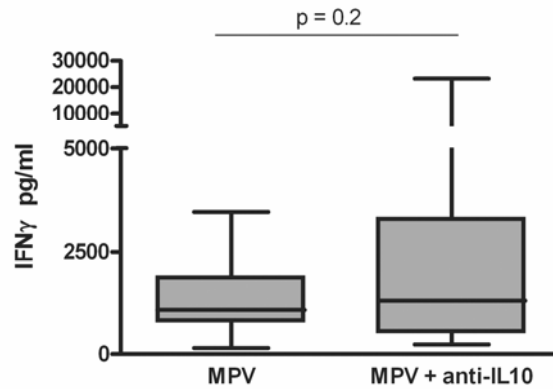
Figure 16: Blocking the activity of IL-10 generated in MPV cultures does not influence the intensity of IFNγ responses. MPV stimulated PBMC were cultured with neutralizing antibody to IL-10. Bars represent median and range of virus stimulated IFNγ responses from 10 individuals.
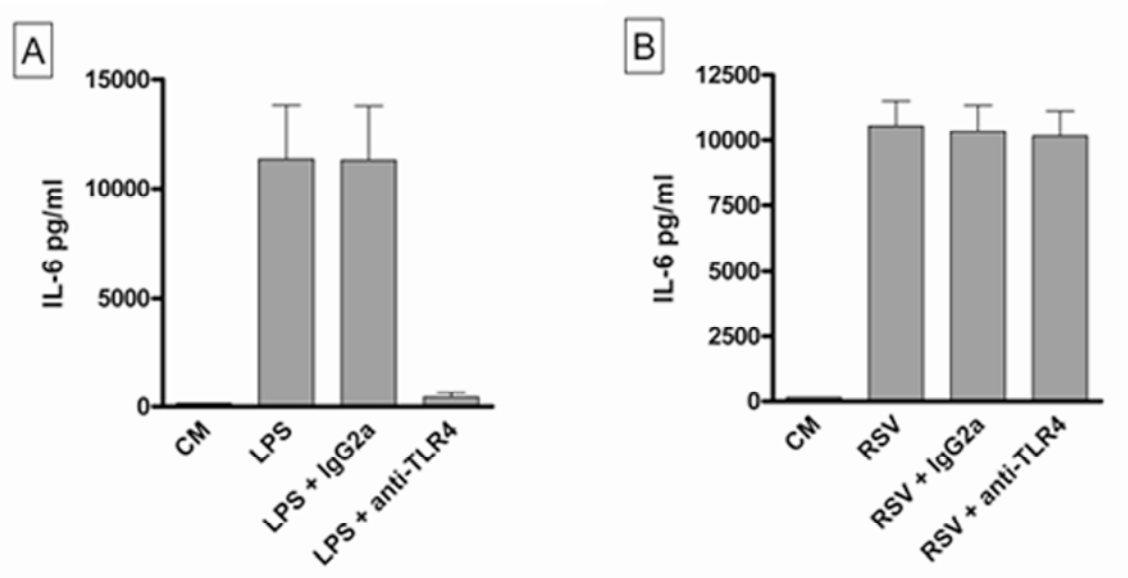
Figure 17: Anti-TLR4 monoclonal antibodies blocks LPS-driven but not RSV-stimulated IL-6 responses. LPS (A) and RSV **(B)** elicited IL-6 was measured by ELISA in supernatants from 24 hour PBMC cultures. Bars represent mean responses with SEM from 6 adults.

DISCUSSION

Despite extensive epidemiologic evidence linking respiratory virus infection to poor clinical outcomes and increased exacerbations of asthma, the cellular and molecular mechanisms by which viruses cause differential clinical responses remain undetermined. Here, we assessed MPV and RSV-specific immuno-regulatory responses in adults with and without current allergic asthma. We demonstrate that asthmatics have a markedly greater frequency and intensity of pro-inflammatory IFNγ responses towards pneumoviruses than do non-asthmatic counterparts. This strong Th1-like bias is not counter-regulated by IL-10, despite concomitant IL-10 production. We suggest that this pattern of viral immunity may contribute to the enhanced sensitivity of asthmatics to experience asthma exacerbation and AHR following infection with such respiratory viruses. Furthermore, we demonstrate that this pattern of substantial innate immunity and intense Th1 responses to RSV was not dependent upon TLR4-mediated stimulation, in contrast with previously published evidence that TLR4 plays a key role in activating immune responses towards RSV [103].

While allergic diseases and asthma were initially described as diseases stemming from excessive "Th2-like" gene expression, it is now recognized that the chronic inflammatory immune responses associated with persistent asthma include a much broader range of cytokine production. Allergic airway inflammation in *D. farinae*-sensitized mice is significantly enhanced by recurrent and low-grade RSV infections [161, 343]. Recurrent experimental respiratory syncytial virus infections in allergen-sensitized mice have been demonstrated to lead to persistent airway inflammation and hyper-responsiveness [298, 340]. The immune mechanisms underlying such effects remain unclear. Importantly, the absence of, or blocking of, IL-13 expression in these animal models of virus-induced asthma pathogenesis or exacerbation, had no effect on airway hyper-responsiveness [304]. Those murine data, taken

with the minimal, and irregular IL-13 responses that are evoked upon pneumovirus infection of human PBMC reported in this manuscript, underline the prominence of pro-inflammatory IFNγ, rather than virus enhanced "Th2-like" cytokine production, in the responses which characterize the response of allergic asthmatic adults to respiratory virus infection.

Multiple other pathways contribute to the complex processes underlying the human response to respiratory virus infection. Epithelial cell models of virus infection demonstrate that asthmatic epithelium has an impaired ability to mount effective anti-viral immunity through cytokine production, namely type I interferons (IFNβ) [25, 321]. It remains unknown whether intrinsic differences in asthmatic epithelium function [30] permit more severe infection with respiratory viruses. However, asthmatic epithelium sustains increased viral replication relative to that of healthy individuals [321]. Similarly, a whole blood model of virus infection demonstrated impaired innate type I interferon (IFNα) production towards RSV and Newcastle disease virus in adult asthmatics compared to non-asthmatic individuals. In contrast, here we describe for the first time, increased adaptive type II interferon (IFNγ) production in response to RSV and MPV in adult allergic-asthmatics compared to non-asthmatic individuals. Whether this increased IFNγ production is a compensatory mechanism for decreased innate interferons remains unknown. Thus, both the respiratory epithelium and leukocyte derived immune responses have important regulatory roles that are likely to contribute to the clinical outcome that results following virus infection. The disease syndrome that results following respiratory virus infection is likely a consequence both of the direct harmful effects of the virus itself and of immunopathology that results from the host immune response via production of inflammatory mediators, cytokines, and chemokines. This leads to the hypothesis that the undesirable consequences of respiratory virus infections seen in asthmatic individuals may be due more to the immune-mediated disease responses of pro-inflammatory cytokines, such as the enhanced IFNγ demonstrated

above, than to virus-induced pathology. If proven so, this would have obvious therapeutic implications for clinical management of such overly exuberant, and ultimately undesirable, immune responses in asthmatic individuals.

The role of TLR signaling in respiratory virus infection is an area of much recent focus. Several TLR are likely to play a key role in innate and ultimately adaptive immune capacity [59, 104]. Murine studies strongly suggested that TLR4 plays a key role in shaping the intensity of immune responsiveness to RSV infection [103, 122] both in *in vivo* infection models utilizing TLR4-deficient mice and in *in vitro* analyses of RSV-F protein-mediated activation of monocytes. In contrast, our data obtained with adult human populations demonstrates that while blocking TLR4 function abrogates the activity of the potent TLR4 ligand LPS, RSV-mediated activation of cytokine production was unchanged in frequency or intensity. Whether this reflects species-specific differential roles of TLR4 function or is attributable to other experimental variables [124], remains to be determined.

In summary, this is the first report to demonstrate that T cell dependent anti-viral IFNγ responses are markedly enhanced in allergic asthmatics compared to non-asthmatic adults. This finding elucidates a mechanism through which bias in the way the immune system of asthmatics responds to respiratory viruses may lead to enhanced inflammation and contribute to increased symptoms upon reinfection [421].

CONCLUDING REMARKS

These results demonstrate that adult asthmatics demonstrate increased Th1 responses towards pneumoviruses compared with non-asthmatic individuals. As this result is clearly novel and contrary to most widely accepted literature [195], we sought to replicate this result in another clinical cohort (SAGE: Study of Asthma Genes and the Environment), one which

has the advantage of extensive epidemiological records, clinical description and measurement of airway function to aid in the diagnosis of asthma.

CHAPTER 3

Asthmatic Children Display Weaker CCL5 and Stronger IFNγ Responses to Human Metapneumovirus Than Do Healthy Individuals

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ABSTRACT

Human metapneumovirus (MPV) is an important respiratory pathogen, causing significant disease burden in children and the elderly. MPV infection has been associated with both asthma pathogenesis and exacerbations in children and adults. The nature of human immuno-regulatory responses of asthmatic individuals to virus reinfection is unknown. Here we compare MPV-dependent immuno-regulatory responses in asthmatic and healthy paediatric populations that are stimulated by acute re-exposure to this common respiratory virus. Using MPV infection of fresh PBMC, we quantified MPV-specific cytokine and chemokine production in over 150 children with and without current asthma. Asthmatic children exhibited a greater frequency and intensity of both Th1 and IL-10 responses towards MPV than did non-asthmatic counterparts ($p<0.001$). Conversely, expression of Th1-immunity associated chemokine CCL5 was substantially weaker. While non-asthmatic children exhibit a negative correlation between CCL5 and IFNγ anti-viral responses ($p<0.001$), asthmatic children exhibit dysregulated responses. This pattern is enhanced in children with familial histories of asthma, suggesting a hereditary component for the altered anti-viral immune profile. Thus, asthmatic children exhibit regulatory responses upon MPV re-exposure that are distinct from those in non-asthmatic children. This profile of intense Th1/IL-10 responses paralleled by deficient CCL5 production may underlie the enhanced sensitivity of asthmatics to this respiratory virus.

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PREAMBLE

Viral respiratory infections are strongly associated with exacerbation of asthma symptoms [470]. There is building consensus that MPV infection enhances asthma symptoms; including wheeze, rhinitis, cough and air-trapping [418, 420, 421, 478]. MPV infection results in exacerbation in both children and adults [419-423] and can result in symptoms severe enough to require hospitalisation [419]. Taken together, these studies indicate that MPV infection within the community is an important cause of symptomatic respiratory illness in all age groups, and has particular impact within the asthmatic population.

RATIONALE AND HYPOTHESIS

To date, there has been no characterization of T cell-dependent immunity against MPV in asthmatic and non-asthmatic children. Intrinsic differences in the way asthmatics develop and express immunity to respiratory virus infection may contribute to viral triggering of asthma exacerbation. Therefore, we assessed MPV-specific responses in children with and without current asthma, using a well established model of acute viral reinfection [473]. Consistent with murine models of MPV primary infection [222, 475], we evaluated key anti-viral cytokine responses (IFNγ, CXCL10, CCL5 and IL-10) in asthmatic and non-asthmatic individuals, *based on the hypothesis that asthmatic children would elicit distinct biases in cytokine production elaborated during responses against MPV compared to healthy children.*

RESULTS

Asthmatic children display stronger Th1 biased immunity towards human metapneumovirus than do non-asthmatic individuals

As cytokine responses to allergens in allergic and asthmatic individuals show marked Th2 bias, we hypothesised that this skewing may also affect viral immunity in asthmatic individuals. Here, we used a PBMC model of acute MPV infection [473] to measure anti-viral cytokine responses in over 75 allergist-diagnosed asthmatic and 75 non-asthmatic children. MPV-driven responses were characterised by quantifying IFNγ, CXCL10, CCL5 and IL-10 production. Undetectable levels of Th2 cytokine (IL-13, IL-5) production to MPV were found in over 95% of individuals (Data not shown). Contrary to our initial hypothesis, we observed a strongly increased frequency and strength of Th1 biased immune responses to MPV in asthmatic individuals compared with non-asthmatics (**Figure 18**). On average, asthmatic children exhibited a 1.4 fold increase in MPV-driven IFNγ production compared to non-asthmatics (median response 935 pg/ml versus 665 pg/ml, p<0.001). A similar Th1 chemokine pattern was evident, with asthmatics displaying increased CXCL10 (median 1.7 fold) responses compared to non-asthmatics.

Asthmatics display stronger IL-10 responses towards human metapneumovirus than do healthy individuals

As described in human and murine models [473, 475], concomitant IL-10 production is characteristic of MPV-specific immunity. As seen above, substantially stronger MPV-driven IL-10 production was evident among asthmatic (median 110 pg/ml) compared to non-asthmatic (median 33 pg/ml, p<0.001) children (**Figure 19A**).
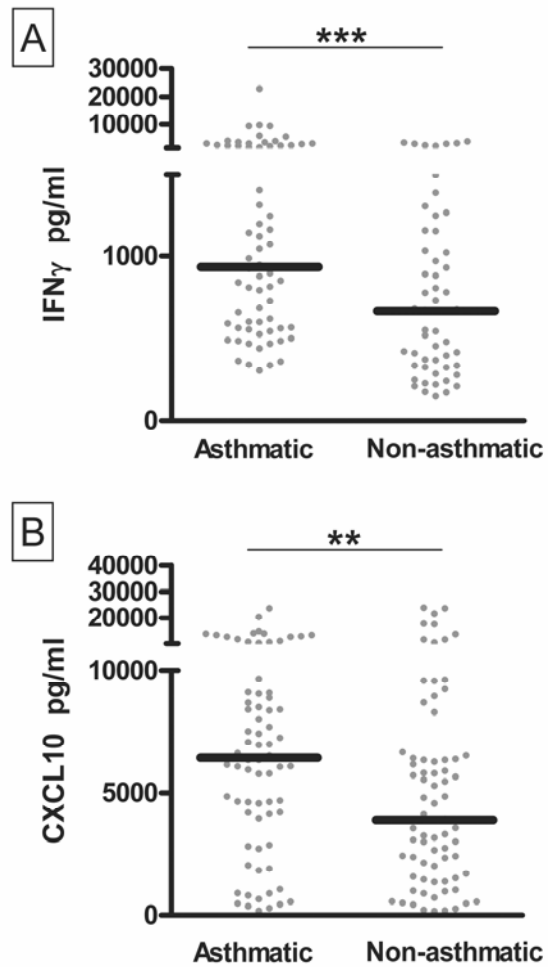
Figure 18: Stronger Th1 biased immunity towards human metapneumovirus (MPV) in asthmatics compared to non-asthmatics. MPV-dependent responses were measured in fresh PBMC obtained from over 150 children. IFNγ **(A)** and CXCL10 **(B)** were measured by ELISA in supernatants from 6-day cultures. Black bars represent median responses, each from an individual child (•). (**P<0.01) (***P<0.001).
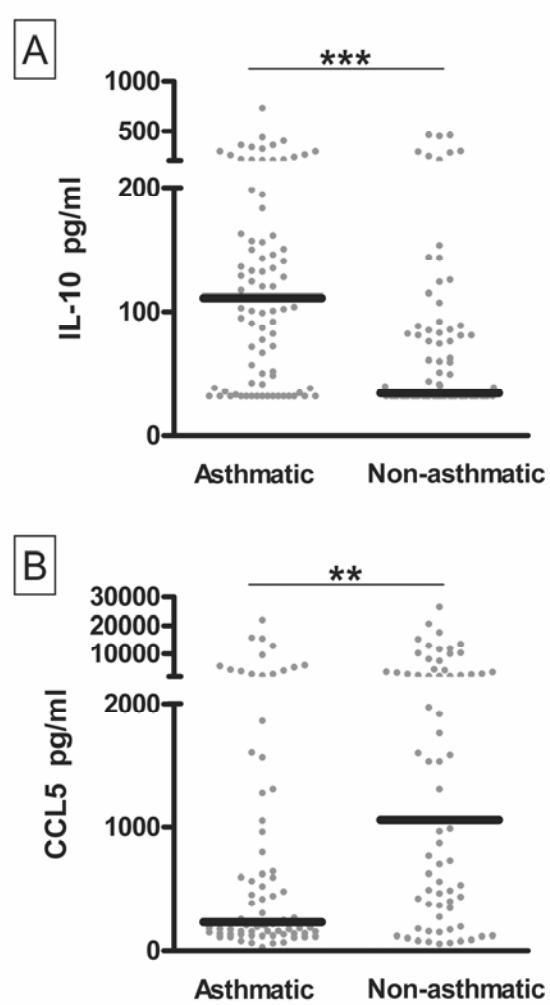
Figure 19: Stronger IL-10 and weaker CCL5 responses towards human metapneumovirus (MPV) in asthmatic compared to non-asthmatic children. MPV-dependent responses in were measured in children. IL-10 **(A)** and CCL5 **(B)** was measured by ELISA in supernatants from 6-day PBMC cultures. Black bars represent median responses, each from an individual child (•). (**P<0.01) (***P<0.001).

Asthmatics display weaker CCL5 responses towards human metapneumovirus than do healthy individuals

CCL5 exhibits a significant impact on the long-term outcome of AHR, both in allergen and virus-induced lung pathology [479, 480]. Increased levels of CCL5, a chemokine often associated with Th1-biased immunity, are protective against airway hyper-reactivity and mucus production [480], in addition to facilitating activation of cytotoxic T cells required for viral clearance [481-483]. Here IFNγ production in response to MPV infection was accompanied by readily quantified CCL5 production in both populations. However, in contrast to Th1 cytokine production, which was markedly stronger in asthmatic individuals, we observed substantially weaker MPV-driven CCL5 responses among asthmatic (median 230 pg/ml) compared to non-asthmatic (median 1060 pg/ml) children (**Figure 19B**).

IFNγ and CCL5 responses are negatively correlated in non-asthmatic, but not in asthmatic individuals

Since Th1 type cytokines and chemokines, as well as IL-10, were produced in response to MPV restimulation *in vitro*, we sought to determine the relationship between cytokine induction and the extent to which expression of classically defined Th1-associated immunity differed in asthmatic and non-asthmatic children. As expected [427], Th1 chemokine CXCL10 and IFNγ were strongly and similarly correlated in both asthmatic and non-asthmatic populations (Spearman r = 0.64 and 0.81; p < 0.001, respectively. Data not shown.). MPV-stimulated IL-10 production was positively correlated with IFNγ responses and this was also similarly evident in both groups (**Figure 20A and 20B**). In stark contrast, CCL5, although often viewed as a Th1 chemokine [484], was negatively correlated with IFNγ production in non-asthmatic children. Interestingly, expression of these two cytokines was

independently regulated in asthmatic individuals (**Figure 20D**, Spearman r = -0.50, p<0.001 among non-asthmatic children; r = -0.12, p>0.05 among asthmatics). Collectively, these data argue for defective, and differential, immune regulation in the immuno-regulatory cytokine expression during MPV-driven responses in asthmatics versus control children.

Weak CCL5 responses contribute to skewed anti-viral immunity in asthmatics

Having identified clear differences in the absolute intensities of cytokine production elicited upon MPV re-exposure in asthmatic and non-asthmatic individuals, we sought to determine the relative balance of cytokine responses in these populations. Despite the increased intensity of both IFNγ and IL-10 production that occurred in asthmatics, the relative ratio of IFNγ:IL-10 production was similar in both asthmatic and non-asthmatic populations (**Figure 21A**). In contrast, IFNγ:CCL5 and IL-10:CCL5 ratios revealed a significant bias of asthmatic individuals towards substantially weaker CCL5 production and elevated IFNγ and IL-10 (**Figure 21, B and C**). This 12.3 fold difference in the IL-10:CCL5 ratio and 4 fold difference in IFNγ:CCL5 ratio of asthmatics versus non-asthmatics identifies impaired MPV-dependent CCL5 production and the altered balance of immune expression as a characteristic feature of anti-viral immunity in asthmatics.
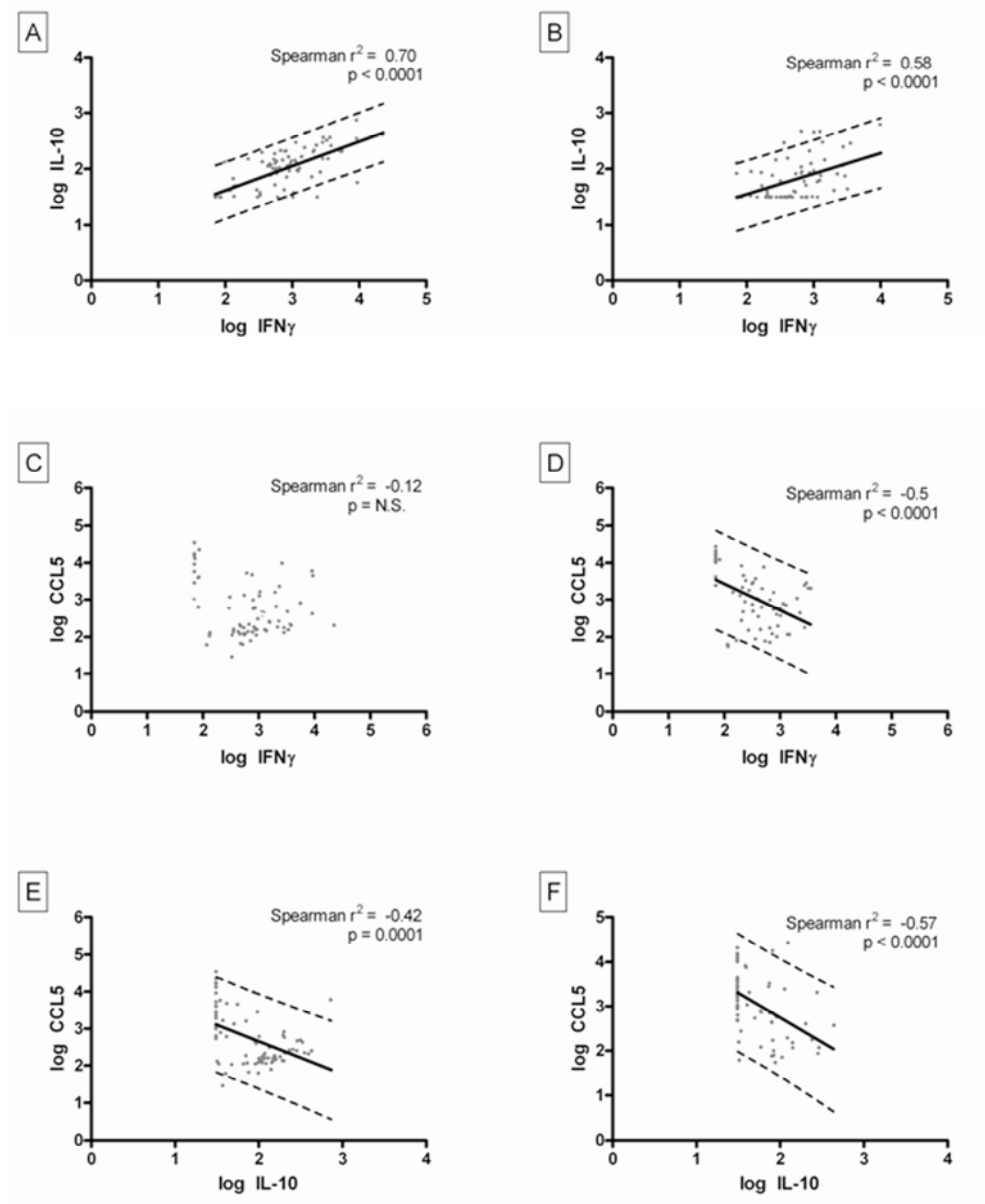
Figure 20: IFNγ and CCL5 MPV-driven responses are not correlated in asthmatic compared to non-asthmatic children. Correlation of IFNγ:IL-10, IFNγ:CCL5 and IL-10:CCL5 responses towards MPV in asthmatic **(A, C, E)** and non-asthmatic children **(B, D, F)**. IFNγ, IL-10, CCL5 were measured by ELISA in supernatants from 6-day PBMC cultures. Black lines represent linear regression of responses, each from an individual child (•). Spearman derived correlations and p values.
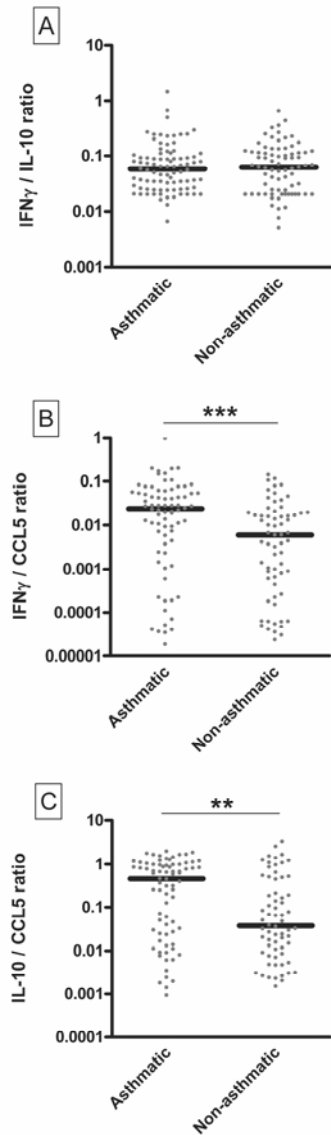
Figure 21: Weak CCL5 production skews pattern of anti-viral immunity in asthmatics. Ratio of MPV-dependent recall responses in children, IFNγ:IL-10 **(A)**, IFNγ:CCL5 **(B)**, IL-10:CCL5 **(C)** are expressed based on cytokine production measured by ELISA in supernatants from 6-day PBMC cultures. Black bars represent median ratios values, each from an individual child (•). (**P<0.01) (***P<0.001).

Children with a familial history of asthma display skewed MPV immunity with increased IFNγ and IL-10 and decreased CCL5 responses.

As stronger Th1 biased and IL-10 production was observed concomitant with weaker CCL5 production among asthmatic individuals in response to MPV, we hypothesized that this pattern of immunity was influenced by genetic predisposition. Therefore, asthmatic and non-asthmatic groups were stratified based upon familial asthma, defined here as having a parent or sibling with current asthma. This segregation revealed that asthmatic children with a familial history of asthma exhibit greater IFNγ/CCL5 skewing of anti-MPV immunity than do asthmatic children from a family without first degree relatives exhibiting asthma (**Figure 22**). This analysis demonstrates that independent of familial asthma, children affected by current asthma generate increased IFNγ and weaker CCL5 responses towards MPV than do their non-asthmatic counterparts. However, the intensity of these changes is strongly influenced by familial asthma with median two fold increases in IFNγ and 7.6 fold decreases in CCL5 production in response to MPV observed in asthmatic children with familial histories of asthma compared to non-asthmatic children without familial asthma (IFNγ; $p<0.001$ and CCL5; $p<0.01$). CXCL10 production was predicted by current asthma in the child, with no demonstrable influence of familial asthma (**Figure 22B**).

Examining IL-10 production, the asthmatic population consistently demonstrates increased IL-10 production relative to non-asthmatic counterparts, but as for IFNγ and CCL5 responses, IL-10 production is also significantly influenced by a familial history of asthma (**Figure 22D**, $p<0.05$, 1.5 fold increase).
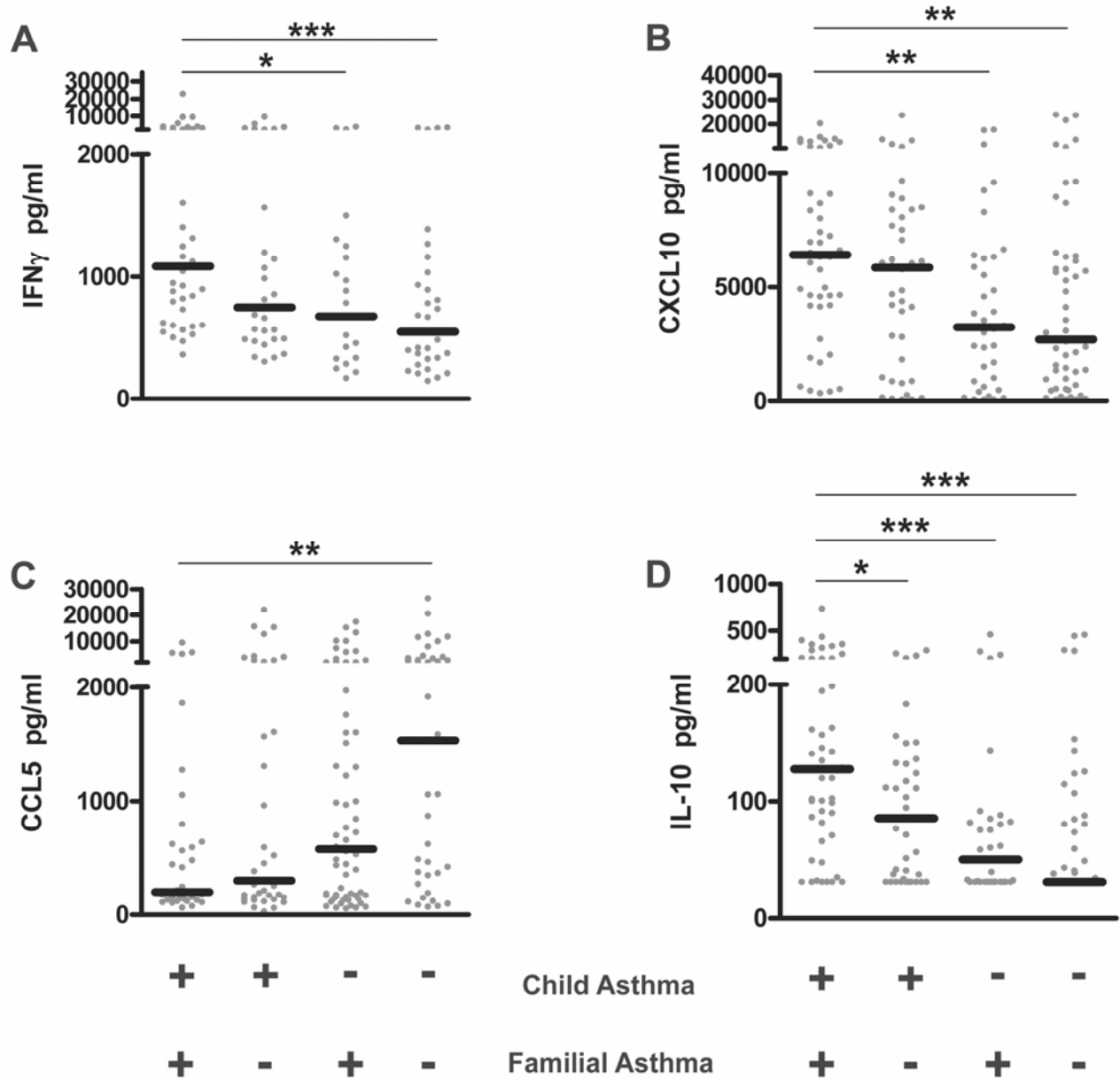
Figure 22: **Familial asthma predisposes children towards stronger IFNγ and IL-10 and weaker CCL5 anti-viral responses.** MPV-dependent recall responses in children, IFNγ **(A)**, CXCL10 **(B)**, CCL5 **(C)** and IL-10 **(D)** were measured by ELISA in supernatants from 6-day PBMC cultures. Black bars represent median responses, each from an individual child (•). (*P<0.05) (**P<0.01) (***P<0.001).

DISCUSSION

MPV is an important source of morbidity in the general population and may act as a viral trigger of asthma pathogenesis and exacerbation. To date, studies of the cellular and molecular response to virus infection have not shown clear cut qualitative or quantitative differences between asthmatic and non-asthmatic subjects [474]. Such studies of immune responsiveness utilized substantively smaller populations than the current report. We assessed MPV-specific immuno-regulatory responses of PBMC obtained from over 150 children with and without current asthma, finding that asthmatics respond at a greater frequency and intensity with Th1-like IFNγ/CXCL10 and IL-10 responses towards MPV than do their non-asthmatic counterparts. Moreover, CCL5 production, strongly associated with CTL activation [481, 482] and inhibition of allergen-induced inflammation [480], is substantially weaker in asthmatic children. We speculate that this distinctive pattern of viral immunity may contribute to the enhanced sensitivity of these children to experience clinical symptoms, which can include asthma exacerbation and AHR following infection with this respiratory virus.

The mechanisms contributing to the consequences of viral infection are manifold (reviewed in [394, 474, 485]). They include weak type-1 interferon production, aberrant expression of Toll-like receptors and increased viral replication by epithelial cells within the lung [112, 234, 321], in addition to altered *in vivo* innate and dendritic cell function that leads to enhanced allergic sensitization [486-488]. Here, we demonstrate a putative contributory mechanism for the aberrant clinical response of asthmatics, namely exaggerated production of IFNγ and IL-10 in anti-viral responses paralleled by deficient CCL5 immune responses.

Innate immunity of resident lung cells slows the progression of infection; however engagement of the adaptive immune system is usually required for virus clearance from the

lung. CTL responses are required for MPV clearance in murine models, where increased T cell-derived IL-10 production coincides with peak virus replication [475]. Here, we describe increased virus-dependent IL-10 production among asthmatics, which is significantly enhanced in individuals with a familial history of asthma. Grissell *et al.* also report synergistic upregulation of IL-10 production in induced sputum of acutely *in vivo* virally infected asthmatics compared with infected non-asthmatics or not currently infected controls [489]. This increased IL-10 production in asthmatics with virally-triggered exacerbation likely contributes to the observed increased AHR that is evident during infection [330, 490]. Moreover, despite a clear role in viral clearance, excessive IFNγ is associated with wheezing and AHR [187, 409]. In children, increased IFNγ in bronchoalveolar lavage fluid and serum is associated with wheeze and asthma [491, 492]. Here, we observed increased MPV-driven IFNγ and Th1-like chemokine CXCL10 responses in asthmatic children, compared to controls. Interestingly, Roman *et al.* reported reduced Th1-like cytokine production in PBMC obtained from 1-13 month old infants in response to RSV infection [185]. Whether this discrepancy is attributable to the use of a related virus, the age of the children (1 versus 8 years old) or the fact that we examined antigen-driven responses and they utilized the global immune activators phytohemaglutinin and pokeweed mitogen that would stimulate cells of many different specificities, remains to be determined.

Decreased MPV stimulated CCL5 responses were evident in asthmatic children compared to healthy individuals. Impaired CCL5 anti-viral responses in asthmatics reveal a missing component of anti-viral immunity which could explain in part their propensity towards increased virus-triggered asthma exacerbation. The role of CCL5 in AHR remains controversial (reviewed in [479]). Murine models of RSV infection have shown that administering Met-RANTES (a competitive inhibitor of CCR5 and CCR1) decreases T cell infiltration into the lungs and results in significant increases in RSV titer [493], demonstrating

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the importance of CCL5 in immune cell recruitment and anti-viral activity within infected lung tissue. Early CCL5 production from CD8^{+} memory/effector cytotoxic T cells after TCR engagement [481, 494] acts as a positive feedback loop to enhance multiple cytotoxic mechanisms [482, 483, 495]. Thus, CCL5 secretion enhances cytotoxic T cell activation and has been demonstrated to be an important component of anti-viral responses [495, 496]. Here, the finding that these asthmatics respond to MPV with weaker CCL5 production suggests potentially ineffectual activation of cytotoxic T cells. Insufficient activation of cytotoxic T cells in asthmatic individuals would promote delayed viral clearance, and may contribute to the severity of virus-triggered asthma exacerbation. Because of the limited volumes of blood available from these paediatric populations, we were unable to also assess MPV-specific CTL activity.

Immune cell production of CCL5 may influence the spread of infection via additional mechanisms. CCL5 treatment of epithelial cells *in vitro* inhibits RSV infection in a dose-dependent manner [497]. Epithelial cells produce CCL5 upon dsRNA recognition, a response that is further enhanced in the presence of IFNγ [498]. If impaired CCL5 production (and concomitantly exaggerated IFNγ) by immune cells results in sustained infection, the dominant epithelial CCL5 response would mask the underlying immune deficiency as tissue injury progressed. Clinically, CCL5 is overly expressed in lung tissue and the BAL fluid of asthmatic individuals compared to healthy individuals [499, 500]. In addition, virus-induced asthma exacerbations are associated with CCL5 in airway secretions [150, 501]. Despite these clinical markers in asthmatics, here we demonstrate markedly impaired ability to secrete CCL5 by immune cells during anti-viral responses. Future murine models of CCL5 deficiency in specific cell types would best address the role of immune cell versus epithelial cell derived CCL5 in the development or exacerbation of AHR.

The observation that familial asthma confers increased risk of weak CCL5 MPV-driven responses, and concomitantly exaggerated IFNγ and IL-10 production, leads us to speculate that CCL5 haplotypes promoting weak CCL5 production by immune cells may be more frequent in asthmatic individuals. A functional CCL5 SNP (In1.1C/T) has been shown to regulate the transcription upon stimulation of T cells and monocytes [502]. Future studies will examine the relationship of intermediate phenotypes (CCL5 production; effectiveness of anti-viral immunity) and genetic polymorphisms in the CCL5 haplotypes of asthmatic and non-asthmatic individuals.

There are several limitations of the present study. Given the recognized large diversity in human immune reponsiveness, hence the requirement to achieve larger sample sizes, we employed PBMC rather than lung derived cells. Secondly multiple additional pathways contribute to the complex processes underlying the human response to respiratory virus infection. Epithelial cell models of virus infection demonstrate that asthmatic epithelium has impaired ability to mount effective anti-viral immunity through cytokine production, namely type I interferons (IFNβ) [25, 321]. It remains unknown whether intrinsic differences in asthmatic epithelium function [30] permit more severe infection with respiratory viruses. However, asthmatic epithelium sustains increased viral replication relative to that of healthy individuals [321]. Similarly, a whole blood model of virus infection demonstrated impaired innate type I interferon (IFNα) production towards RSV and Newcastle disease virus in adult asthmatics compared to non-asthmatic individuals [297]. In contrast, here we describe for the first time, increased adaptive interferon (IFNγ) production in response to MPV in asthmatics compared to non-asthmatic children. Whether this increased IFNγ production is a compensatory mechanism for decreased innate interferons remains unknown. Thus, both the respiratory epithelium and leukocyte derived immune responses have

important regulatory roles that are likely to contribute to the clinical outcome that results following virus infection.

In summary, this is the first report to demonstrate that immune cell-derived anti-viral CCL5 responses are severely impaired in asthmatics compared to non-asthmatic humans. This finding elucidates how bias in the way the immune system of asthmatics respond to respiratory viruses may lead to impaired viral clearance and contribute to increased symptoms upon reinfection [421]. As anti-viral therapies are emerging as candidate treatments for asthma exacerbation [365, 386], the demonstration of impaired ability to induce viral clearance in asthmatics may help promote the use and development of novel therapeutics to minimize symptoms and lung injury upon infection with respiratory viruses, such as MPV. Moreover, we speculate that altered immune regulation of anti-viral CCL5 responses in asthmatics may be evidence of a heritable immune response that predisposes children to develop asthma (as well as exacerbate established disease) when triggered by MPV. The early use of effective anti-virals in high-risk children in early life may even prevent (or delay) the development of asthma, as severe MPV infection with wheezing has been suggested as a potential indicator of children who will develop long-term alterations in lung function [419-421, 424, 503].

CONCLUDING REMARKS

This is the first report in humans to demonstrate dysregulated CCL5 anti-viral immunity in asthmatics compared to healthy individuals. The effect of familial asthma also suggests a genetic bias towards weak CCL5 responses against respiratory viruses. Therefore, this paper further confirms our initial hypothesis that aberrant anti-viral immunity

exists in asthmatic individuals, which may predispose them towards airway exacerbation upon infection. However, this may be true for pathogenic viruses causing symptomatic infection, but raises the question whether this holds when examining anti-viral immunity towards non-pathogenic viruses, such as reovirus.

CHAPTER 4

Reovirus serotypes elicit distinctive

patterns of recall immunity[1]

AND

Atopic-Asthmatic Adults Display Stronger Th1

Biased Immunity towards Reovirus Serotype T1L

Than Do Healthy Individuals[2]

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Reovirus serotypes elicit distinctive patterns of recall immunity

ABSTRACT

Orthoreoviruses (reovirus) are ubiquitous infectious agents that infect cells in mammalian respiratory and enteric tracts. The frequency and nature of human cellular immunity, in particular immuno-regulatory responses against reovirus, are unknown. Here we establish systems to determine reovirus-induced cytokine and chemokine recall responses in 44 healthy adults using primary culture of virus infected peripheral blood mononuclear cells (PBMC) with two major reovirus serotypes, type 1 Lang (T1L) and type 3 Dearing (T3D). Reovirus induced exceptionally strong CD4 and CD8 T cell dependent IFNγ recall responses concomitant with intense IL-10 production. These responses were elicited independently of viral replication in PBMC. Surprisingly, paired analysis of responses of subjects to these two common serotypes revealed that while both elicit Th1-type dominated immunity, T3D-driven responses were two to ten fold weaker than those elicited by T1L. Recall responses evoked by these viral serotypes differed markedly in their mechanism of regulation. While T3D IL-10 and IFNγ responses were CD4 and CD8 dependent and blocked by interfering with CD86 costimulation but not CD80, T1L responses were consistently CD28:CD80/86 independent. Thus, despite extensive genetic and morphologic similarities between reovirus serotypes, the nature and intensity of the human recall responses as well as the control mechanisms regulating them are clearly distinct.

PREAMBLE

Mammalian orthoreoviruses (reovirus) are ubiquitous infectious agents found in untreated water and raw sewage, frequently at the same levels as *Escherichia coli* [504, 505]. Transmitted by fecal-oral routes, these viruses commonly infect respiratory and enteric tracts. Despite causing disease in many mammalian species, reovirus is not considered a human pathogen due to the absence of obvious symptoms or known clinical impact during natural or experimental infection [506, 507]. In pediatric populations, serological testing reveals 50 to 70% prevalence of anti-reovirus antibodies [405, 506, 508]. Amongst adults, seropositivity approaches 100% [509, 510].

Three morphologically similar groups of reovirus have been described based on genetic divergence and antigenic properties among serotypes. These occur mainly in the gene coding for the outer capsid $\sigma1$ protein [511, 512]. For each serotype, the genetics and morphology have been extensively studied: type 1 Lang (T1L), type 2 Jones (T2J) and type 3 Dearing (T3D). Murine studies argue that immunity to reovirus is not serotype-specific [513], as major components of B cell immunity are cross-reactive between serotypes. In addition, cross-reactive CD8 T cell epitopes exist in the $\sigma1$ protein, supplementing other cross-reactive epitopes between serotypes [511, 513, 514]. Immune protection from primary infection is associated with development of a classical Th1 biased response, mediated by both CD4 and CD8 T cells [404, 515, 516].

RATIONALE AND HYPOTHESIS

Reovirus-based therapeutic strategies for anti-cancer treatment are currently in clinical trials [517-522], however the human cell-mediated immune response to reovirus

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infection has not been examined. The prevalence or nature of human cytokine and chemokine responses elicited by reovirus exposure is largely unknown [408]. Here, we develop and optimize systems enabling us to examine, for the first time, human reovirus-specific recall immune responses in short term primary culture directly *ex vivo*. We hypothesize that virus-driven cytokine and chemokine production to two major reovirus serotypes, T3D and T1L, will elicit similar responses in humans, in terms of both the nature and intensity of anti-viral cytokine production.

RESULTS

Reovirus does not replicate in PBMC

To evaluate if reovirus-driven cytokine responses were associated with viral replication in our system, we determined reovirus end-point titers and expression of viral RNA in our PBMC cultures. In time course experiments, ranging from time 0, 24 hours, 3 days and 6 days after reovirus T1L and T3D culture with PBMC, no increase in progeny virus was detectable using viral titer methods. More specifically, 5 to 10 fold fewer viruses were detected at day 6 than were initially inoculated at time 0. To determine whether virus replication occurred without secretion of infectious viral particles, we assessed viral genome replication by real-time PCR. Quantification of reovirus genomic RNA using the L1 gene (viral polymerase) relative to the cellular endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured in reovirus-infected PBMC compared to infected mouse L929 fibroblast cells, which are permissive for productive reovirus replication and serve as a positive control. In time course experiments, ranging from 1 hour, 24 hours, 3 days and 6 days after reovirus T1L and T3D culture with PBMC, an overall decrease in viral L1 RNA was detected at 3 and 6 days after culture when compared with 1 hour of culture.

Taken together, these results show that reovirus, both T1L and T3D serotypes, can enter but are unable to replicate within PBMC. Therefore, reovirus-driven cytokine production in PBMC is not dependent on viral replication, unlike the monocytic cell line THP-1 [408].

Reovirus-specific cytokine production

To examine the frequency and nature of reovirus-driven cytokine production, we developed a short-term, *in vitro* primary culture system using PBMC isolated directly *ex vivo* from healthy adults. PBMC were cultured with infectious and inactivated T3D for 6 days, the time point found in preliminary experiments with 5 subjects (Data not shown) to yield maximum production of the recall immunity cytokines and chemokines of interest. To compare these responses to another prototypic reovirus serotype, parallel cultures were simultaneously set up using blood from the same individuals stimulated with the same titer of infectious T1L. As a positive control of immune responsiveness, PBMC were cultured with streptokinase, a ubiquitous bacterial antigen that elicits T cell dependent recall cytokine and chemokine responses in most adults.

As seen in **Figure 23**, primary culture with streptokinase revealed readily detected type 1 (IFNγ, CXCL9, CXCL10) and type 2 (IL-5, IL-13, CCL17) recall responses in short term primary culture for most of the individuals tested, demonstrating the sensitivity of this approach. Very strong IFNγ responses with median values of ~77 U/ml (8900 pg/ml, based on the WHO standard) were seen following stimulation with infectious T1L. T3D-elicited median IFNγ responses in these paired cultures were substantially less intense (**Figure 23**, $p<0.001$). Weaker T3D stimulated IFNγ responses were evident in 29 of 44 individuals examined (**Figure 24**).

Simultaneous comparison of live vs non-infectious T3D revealed that inactivated virus was markedly weaker in its capacity to elicit IFNγ production, as inactivation decreased IFNγ responses by ~90%.

Other cytokines characteristic of a Th1 biased immune response were also evident. Approximately half the population demonstrated detectable CXCL10 responses to either inactivated or infectious T3D (**Table 7, Figure 23**). In contrast, only a quarter of individuals produced detectable levels of this chemokine following stimulation with infectious T1L (Fishers, *P* = .02). Significant differences in the intensity of CXCL10 production were also evident between T3D and T1L driven responses, with T3D responses stronger in most cases (**Figure 24**). CXCL9 production was consistently decreased in the presence of infectious reovirus. However when inactivated T3D was used, significant CXCL9 responses were evident in the majority of the population (**Figure 23**).

Th2-immunity associated reovirus responses were sporadically detectable, with roughly a third of the population exhibiting IL-13 recall responses, and much lower frequencies of IL-5 or CCL17 production (**Table 7**). There was no evidence of differences in intensity or prevalence of type 2 immunity associated cytokine and chemokine production following T3D versus T1L stimulation. Thus, human reovirus recall responses are dominated by strong Th1-like responses, with minimal Th2-biased activation.
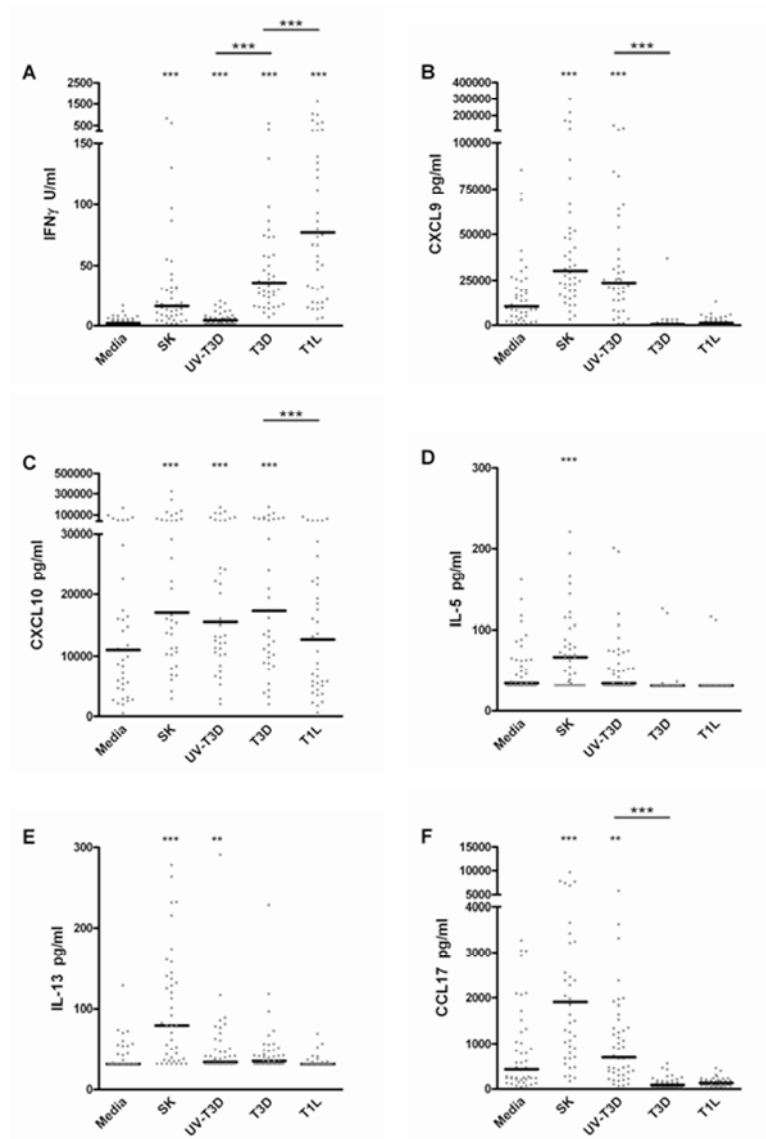
Figure 23: **Reovirus serotypes T3D and T1L induce Th1 biased recall responses.** Antigen-dependent recall responses to streptokinase (SK), pH-inactivated T3D (pH-T3D), T3D and T1L as compared to medium alone. IFNγ **(A)**, CXCL9 **(B)**, CXCL10 **(C)**, IL-5 **(D)**, IL-13 **(E)** and CCL17 **(F)** were measured by ELISA in supernatants from 6 day PBMC cultures. Black bars represent median responses from 44 individual healthy adults (•). P values represent significant differences in cytokine production (media versus stimulated cultures or as indicated): (***$P<.001$, **$P<.01$).
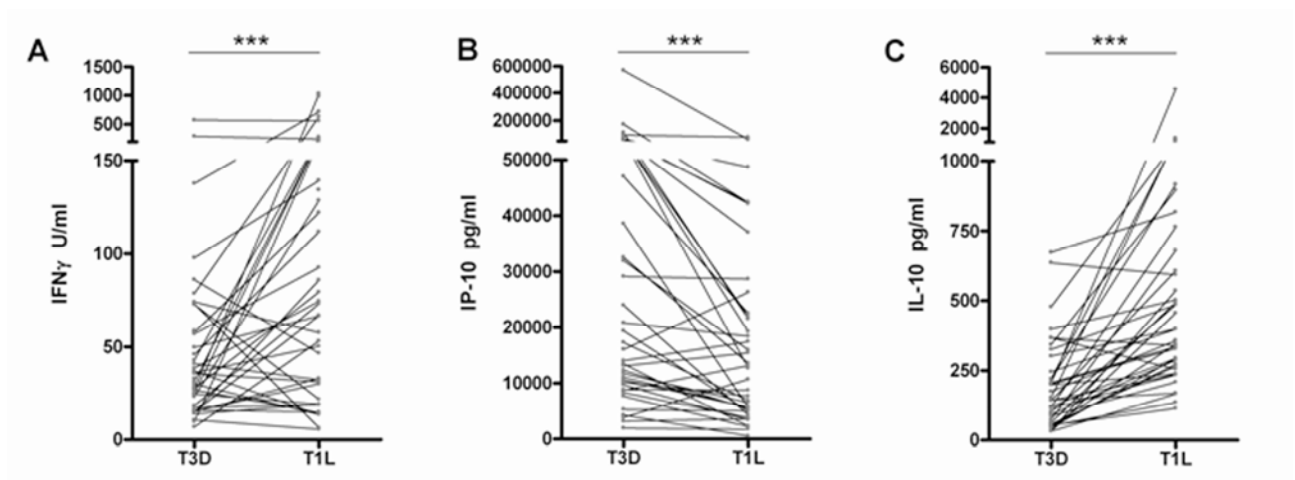
Figure 24: **T3D consistently elicits weaker IFNγ and IL-10 responses compared to T1L.** IFNγ **(A)**, CXCL10 **(B)** and IL-10 **(C)** were measured by ELISA. *P* values represent significant differences in cytokine production following T3D versus T1L stimulation in 44 individuals stimulated with the two serotypes in paired cultures. Black bars represent median responses (***$P<.001$).

Table 7:   **Prevalence of reovirus serotype T3D and T1L dependent responses in healthy adults*.**

Cytokine	T3D		T1L		*P*
IFNγ	44/44	(100)	44/44	(100)	NS
CXCL9	1/44	(2.3)	2/44	(4.5)	NS
CXCL10	21/39	(54)	10/39	(26)	.02
IL-5	1/44	(2.3)	0/44	(0)	NS
IL-13	16/44	(36)	7/44	(16)	NS
CCL17	2/44	(4.5)	4/44	(9)	NS
IL-10	32/39	(82)	39/39	(100)	.0116

* Data are frequency (%) of subjects. Fisher's test derived *P* values.

Interestingly, the Th1 biased memory response was consistently paralleled by expression of very strong reovirus-driven IL-10 responses (**Figure 24**). Stimulation with infectious T3D elicited detectable IL-10 responses in a lower frequency of adults than T1L (82% vs. 100%, Fishers P = .0116). The median T1L-specific IL-10 response was intense (400 pg/ml), and on average three-fold stronger than that elicited by T3D. IL-10 production in response to inactivated T3D was readily detected, but at substantially lower levels and frequency (49%) than responses seen with live virus (Data not shown).

IL-10 is conventionally described as having opposing effects to IFNγ, therefore we sought to determine the relationship between virus-driven IFNγ and IL10 production. Contrary to expectation, a strongly positive correlation between IFNγ and IL-10 levels was seen upon T3D-stimulation (**Figure 25A**). In contrast, no correlation was evident between the stronger and more prevalent IL-10 response and IFNγ production in T1L stimulated cultures.

Thus, as a whole, humans demonstrate strongly Th1-biased recall responses to reovirus that are most pronounced upon live virus stimulation, weak Th2-associated immunity and strong virus dependent IL-10 responses. The T3D serotype consistently elicits weaker induction of IFNγ and IL-10 in recall responses than does T1L stimulation.
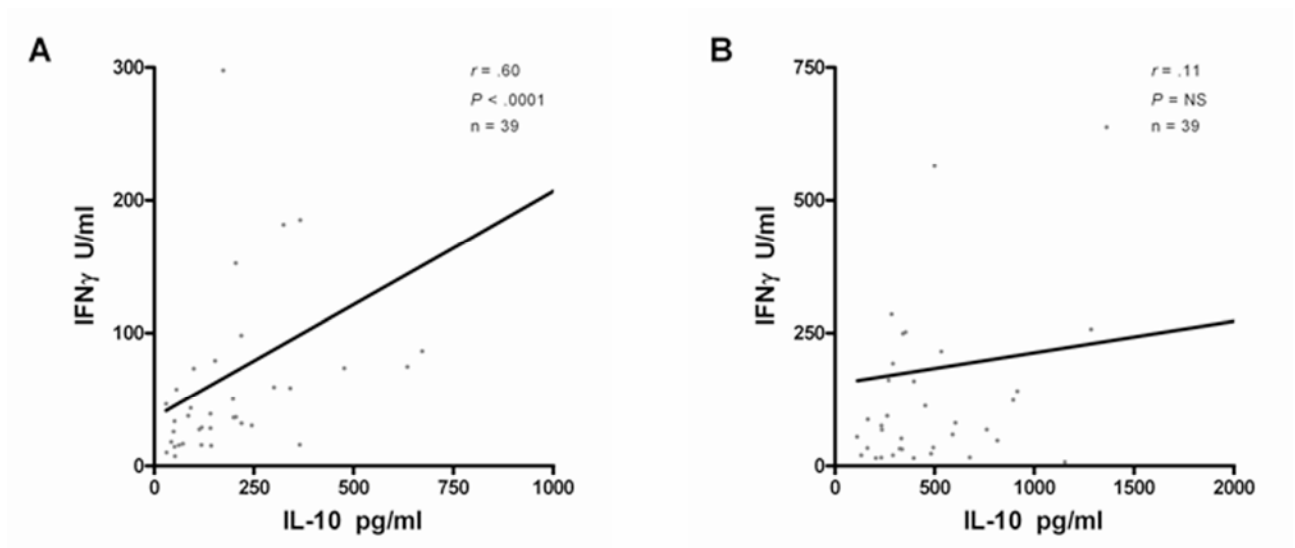
Figure 25: **Positive correlation between T3D dependent IFNγ and IL-10 responses is not seen with T1L serotype.** Correlation between virus specific IFNγ and IL-10 in T3D **(A)** and T1L **(B)** stimulated cultures. Black bars represent best-fit slope (*r*) of IFNγ/IL-10 pairs from 39 individual healthy adults (•). (****P*<.001, ***P*<.01)

Costimulatory requirements of T cell mediated reovirus-dependent cytokine production

To assess the role of CD4 and CD8 T cell populations in reovirus-specific recall responses, PBMC were cultured with live T3D or T1L in the presence of blocking anti-CD4, anti-CD8, anti-MHC class II or class I antibodies (**Figures 26-27**) or isotype control antibodies. As described previously, T3D specific responses were less intense than those elicited by T1L. IFNγ responses to both serotypes were dependent on both class I and II antigen presentation and activation of CD4 and CD8 T cells (**Figure 26**). In marked contrast, IL-10 in T3D stimulated cultures was T cell dependent, but there was no evidence of T cell requirements for T1L driven IL-10 production (**Figure 27**).

To better understand the mechanism underlying reovirus-driven recall responses, we assessed the requirement for costimulation and putatively differential dependence on CD80 versus CD86 costimulation. PBMC were virus stimulated in the presence and absence of neutralizing antibodies to CD80, CD86 or with CTLA-4 Ig. CD86-dependant costimulation is responsible for the majority of T3D stimulated IFNγ and IL-10 production, but consistently had no detectable impact on T1L driven cytokine production (**Figures 26C-D and 27C-D**). Neither response was impacted by blocking CD80 dependent costimulation. Thus, costimulatory requirements for virus induced T cell dependent cytokine production are markedly different between reovirus serotypes.
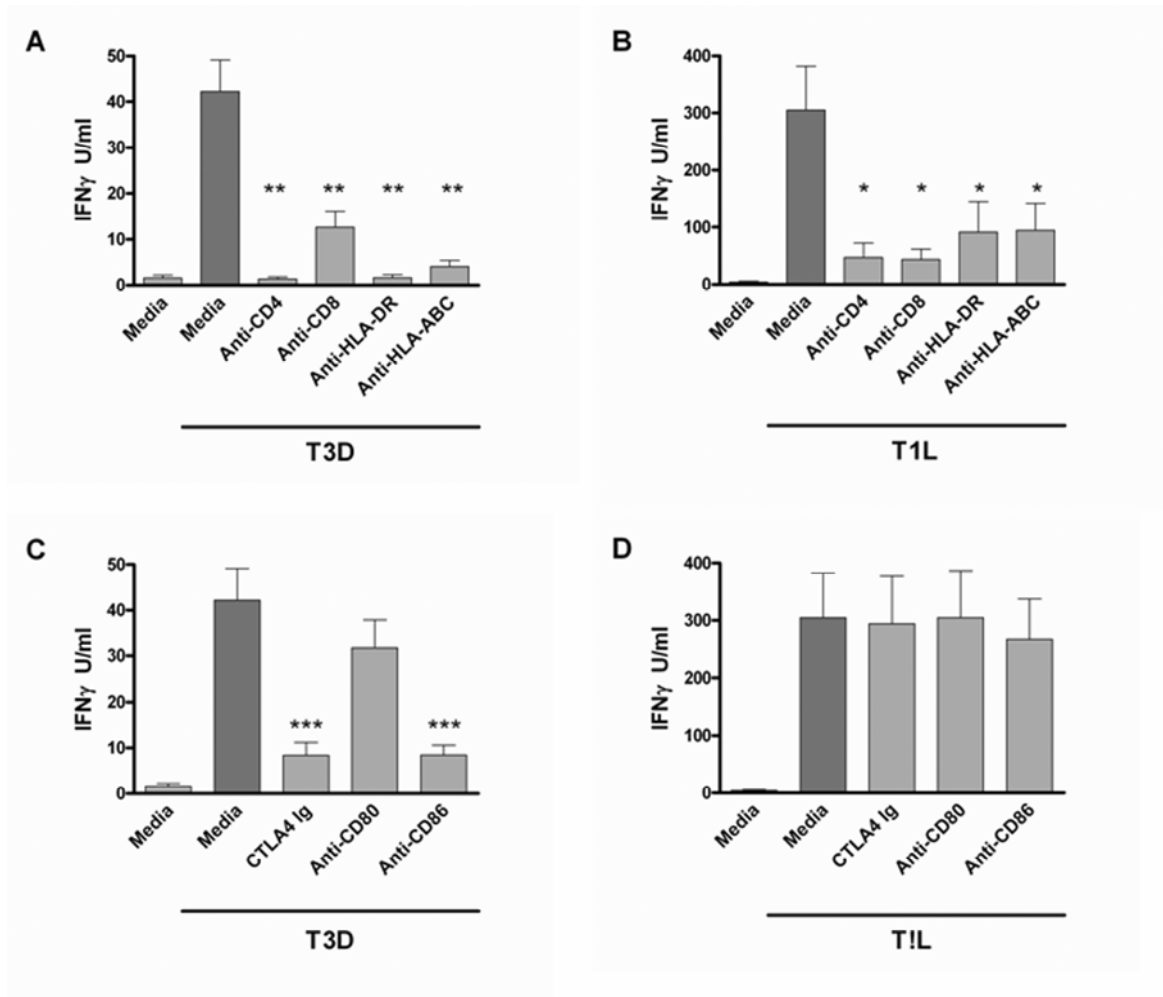
Figure 26: **Classical CD4$^+$ T cell activation for virus dependent IFNγ responses is dependent upon CD86 costimulation in T3D, but not T1L infected cultures.** T3D **(A)** and T1L **(B)** stimulated PBMC were cultured with neutralizing antibody to CD4, CD8, HLA-DR and HLA-ABC. T3D **(C)** and T1L **(D)** infected PBMC were cultured with CTLA-4 Ig or neutralizing antibody to CD80 and CD86. Bars represent mean IFNγ response and standard error from 6 healthy adults. *P* values represent significant changes in cytokine production compared to virus-driven response: (***P<.001, **P<.01, *P<.05).
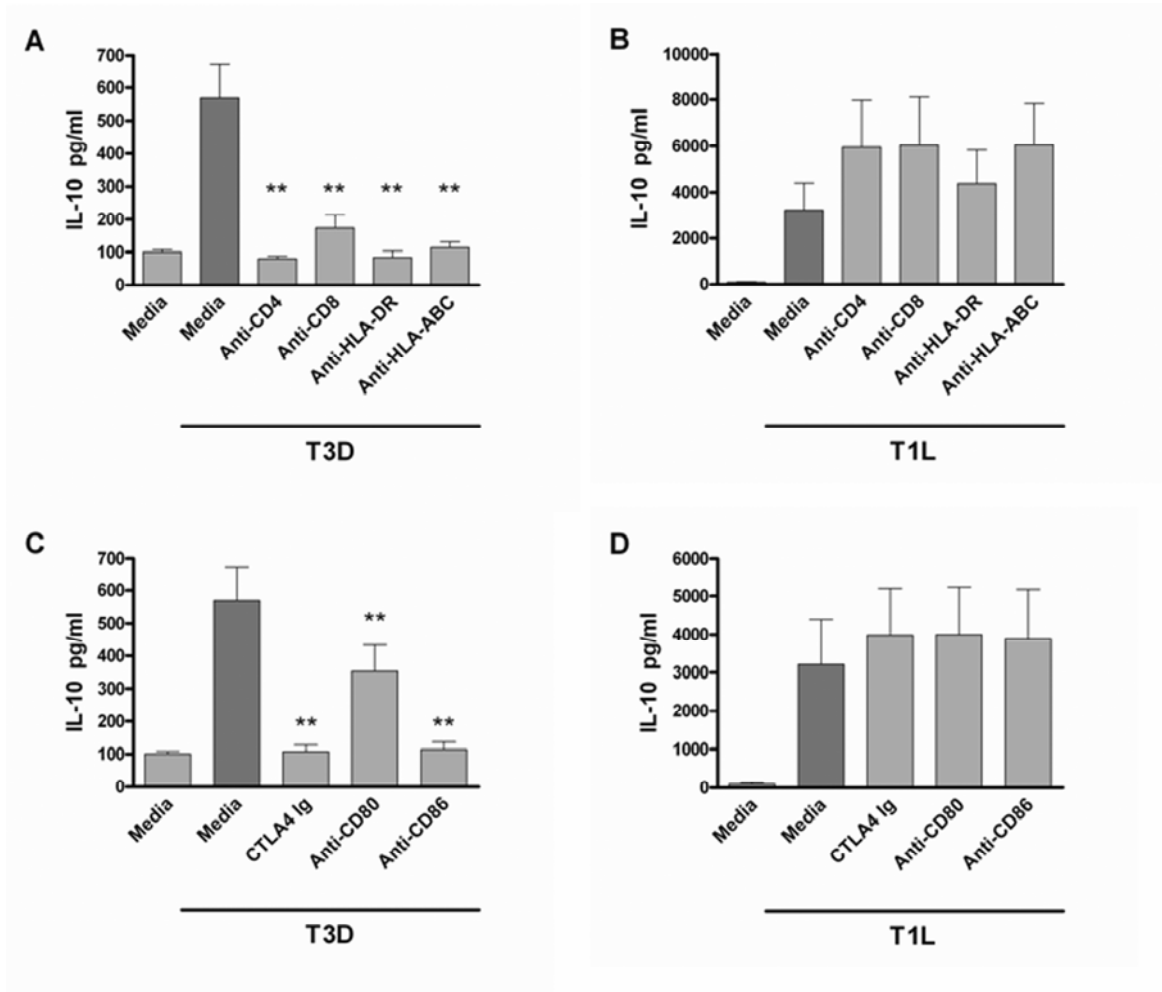
Figure 27: Classical CD4⁺ T cell activation for virus dependent IL-10 responses is dependent upon CD86 costimulation only in T3D stimulated cultures. T3D **(A)** and T1L **(B)** stimulated PBMC were cultured with neutralizing antibody to CD4, CD8, HLA-DR and HLA-ABC. T3D **(C)** and T1L **(D)** infected PBMC were cultured with CTLA-4 Ig or neutralizing antibody to CD80 and CD86. Bars represent mean IL-10 response and standard error from 6 healthy adults. *P* values represent significant decreases in cytokine production compared to viral-driven response: (****P<0.01).

DISCUSSION

In contrast to increased understanding of human serological responses to reovirus [405, 506, 508, 509], no information is available on the presence or nature of the human immuno-regulatory cytokine response to this ubiquitous virus. Here, we demonstrate that T3D and T1L serotypes, both shown in epidemiologic studies to lead to high levels of infection, seroconversion and reinfection in the general population, elicit clearly distinct T cell-dependent cytokine responses, which are independent of viral replication. Both viruses require classical CD4 and CD8 T cell activation for IFNγ induction; however, clearly different costimulatory pathways are involved in activation of reovirus specific memory responses to the two serotypes. Among adults, T3D is a consistently stronger inducer of chemokine CXCL10, and a weaker inducer of both IFNγ and IL-10 production. These differential immune responses elicited by T3D versus T1L are similarly evident whether measured by the frequency of responding individuals or by the intensity of the cytokine response induced.

In murine models of reovirus infection, the protective anti-viral immune response is viewed as a Th1 biased immune response mediated by both CD4 and CD8 cells [515, 516, 523, 524]. CD8 T cell cytotoxicity is dependent on CD4 T cell priming, as depleting MHC II antigen presenting cells diminishes CD8 T cell responses [525]. Here, we demonstrate that a Th1 biased recall response dominates human immunity against reovirus re-exposure or re-infection. Induction of IFNγ by either reovirus serotype is dependent on interaction with cells expressing CD4 and CD8, as demonstrated by experimental blocking of these TcR coreceptors and their respective MHC ligands. Whether this reflects cooperation between CD4 and CD8 T cells as seen in many viral responses, or acquisition of CD4 by CD8 T cells upon activation [523, 524, 526], is currently under investigation. Intracellular IFNγ staining of

CD4$^+$ versus CD8$^+$ cells in reovirus stimulated cultures was technically inadequate to reliably distinguish the phenotype of IFNγ producing cells, likely due to the very low frequency of antigen-specific cells within PBMC of healthy individuals (Data not shown).

Surprisingly, costimulation requirements for T3D and T1L driven IFNγ responses differ markedly. T3D driven IFNγ production is dependent upon CD86 mediated costimulation and independent of CD80. Similarly, while IL-10 production from other viral stimuli may or may not be dependent on CD28-CD80/CD86 pathways [527, 528], T3D driven IL-10 production is highly dependant on CD86, and independent of CD80 costimulation. This observation is similar to the differential use of CD80 versus CD86 seen in influenza infection where virus-specific IFNγ production is dependent on CD86 and not on CD80 [527].

Strikingly, blocking the classical CD80/86 pathway with antibodies to CD80, CD86 or using CTLA-4 Ig consistently failed to inhibit either T1L driven IFNγ or IL-10 responses, indicating a distinctive pathway of regulation for T3D versus T1L serotypes. Although up-regulation of costimulatory molecules such as CD80 and CD86 on antigen presenting cells can be induced by dsRNA via multiple pathways [529], the functional impact of these costimulatory molecules is clearly distinct in T1L versus T3D stimulated responses. Indeed, recent studies indicating that T1L does not directly activate murine DC [530, 531] may actually reflect a lack of use of the CD28-CD80/CD86 pathway in T1L-specific T cell activation and resulting IFNγ production, rather than an inability of reovirus to initiate DC maturation and T cell activation.

Differential dependence of type specific immune responses on costimulation has been identified for influenza. H$_1$N$_1$ induces CD4 T cell dependent CTL responses that require CD86 costimulation while H$_2$N$_2$ elicits CTL responses independent of CD80/CD86 costimulation [532]. Whether reovirus T1L responses are costimulation independent or are

independent of the CD28 pathway but reliant upon alternative costimulatory pathways such as ICOS, CD40, OX40 or 41BB [533-538], remains to be determined. Regardless, both reovirus and influenza provide examples where induction of T cell dependent cytokine responses is differentially regulated between viral serotypes. The clinical impact of this finding is currently under investigation.

The generally innocuous clinical impact perceived upon natural reovirus infection, combined with recent observations that reovirus preferentially kills tumor cells while sparing normal cells, raises possibilities for therapeutic use of this virus [539-541]. Its efficacy in killing disseminated tumors in rodent models argues for its potential in systemic treatment of metastases, either by direct oncolytic activities, or by potentiating protective immune responses against tumor antigens. To date, the great majority of published work has been with T3D, the serotype which in our hands elicits markedly weaker responses. Phase I and II clinical trials are underway [518, 519, 539]. The marked differences in human recall responses demonstrated in this manuscript suggest the importance of evaluating different serotypes to maximize therapeutic potential, as each yields quite different immune responses in humans.

In summary, this study is a novel contribution to our understanding of the immuno-regulatory cytokine response to reovirus in humans. Despite extensive genetic and morphologic similarities between reovirus serotypes T3D and T1L, the control mechanisms and resulting responses elicited by each virus serotype are unique. T3D is characterized by T cell dependent IFNγ and IL-10 cytokine production that clearly requires CD86 mediated costimulation. In contrast, T1L elicits consistently stronger IFNγ and IL-10 responses, both of which are independent of classical CD28:CD80/86 costimulatory requirements. This research impacts the emerging use of reovirus as an anti-cancer agent and expands the

immunological information base that will be needed to construct appropriate and safe therapies.

CONCLUDING REMARKS

Having established a human model of acute viral infection with reovirus, we will assess whether asthmatics mount dissimilar (in terms of frequency, intensity and Th1/Th2 skewing) responses to T1L and T3D than healthy individuals, as seen in the case of anti-pneumovirus immunity.

Atopic-Asthmatic Adults Display Stronger Th1 Biased Immunity towards Reovirus Serotype T1L Than Do Healthy Individuals

PREAMBLE

Our previous studies have demonstrated altered patterns of cytokine production in response to viral stimulation with asthmatogenic pneumovirus that are dependent on the clinical status of the individuals examined. We observed, both in adults and in children, stronger Th1 type cytokine and juxtaposed IL-10 production in asthmatics compared to healthy individuals. However, it remains unclear whether this clinically dependent pattern of immunity is specific to pneumovirus immunity or applies to all viral stimulation.

RATIONALE AND HYPOTHESIS

Anti-viral cytokine production in PBMC is dependent on innate recognition of viral motifs and antigen presentation of both MHC I and MHC II viral peptides that activate T cells. Pneumovirus and reovirus infection results in similar cytoplasmic MAMPs, namely 3pRNA and dsRNA, which initiate innate responses and all individuals have detectable T cell responses specific for these viruses. ***Thus, we speculate that intrinsic differences in anti-viral immunity between asthmatic and non-asthmatic individuals will likely be detected using the reovirus model.***

RESULTS

Atopic-Asthmatic Adults Display Stronger Th1 Biased Immunity towards Reovirus Serotype T1L Than Do Healthy Individuals

As previously described for anti-viral immunity towards pneumoviruses, we observed a strongly increased frequency and strength of Th1 biased immune responses to T1L in atopic-asthmatic adults compared with non-asthmatics (**Figure 28A**). On average, atopic-asthmatics exhibited a 630% fold increase in T1L-driven IFNγ production compared to non-asthmatics (median response 15800 pg/ml versus 2500 pg/ml, p<0.01). In contrast, T3D stimulation revealed no significant differences in responses between atopic-asthmatics and healthy individuals (**Figure 28B**). With either T1L or T3D stimulation, atopic-asthmatics displayed similar IL-10 responses compared to non-asthmatic adults (**Figure 28 C and D**).

DISCUSSION

Using reovirus as a tool to detect differences in virus-driven cytokine responses between adult atopic-asthmatics and non-asthmatics, we observed a substantial increase in Th1 type responses specific to T1L stimulation among asthmatics. This bias towards excessive IFNγ production is reminiscent of pneumovirus-dependent responses in asthmatic adults and children. This demonstrates that asthmatics intrinsically and readily mount increased anti-viral IFNγ responses which are independent of the species of respiratory RNA virus. Overall, this argues for a common alteration in viral recognition, APC costimulation or gene expression among asthmatic individuals, which augments their capacity to respond to viral stimulation with T cell-dependent IFNγ.
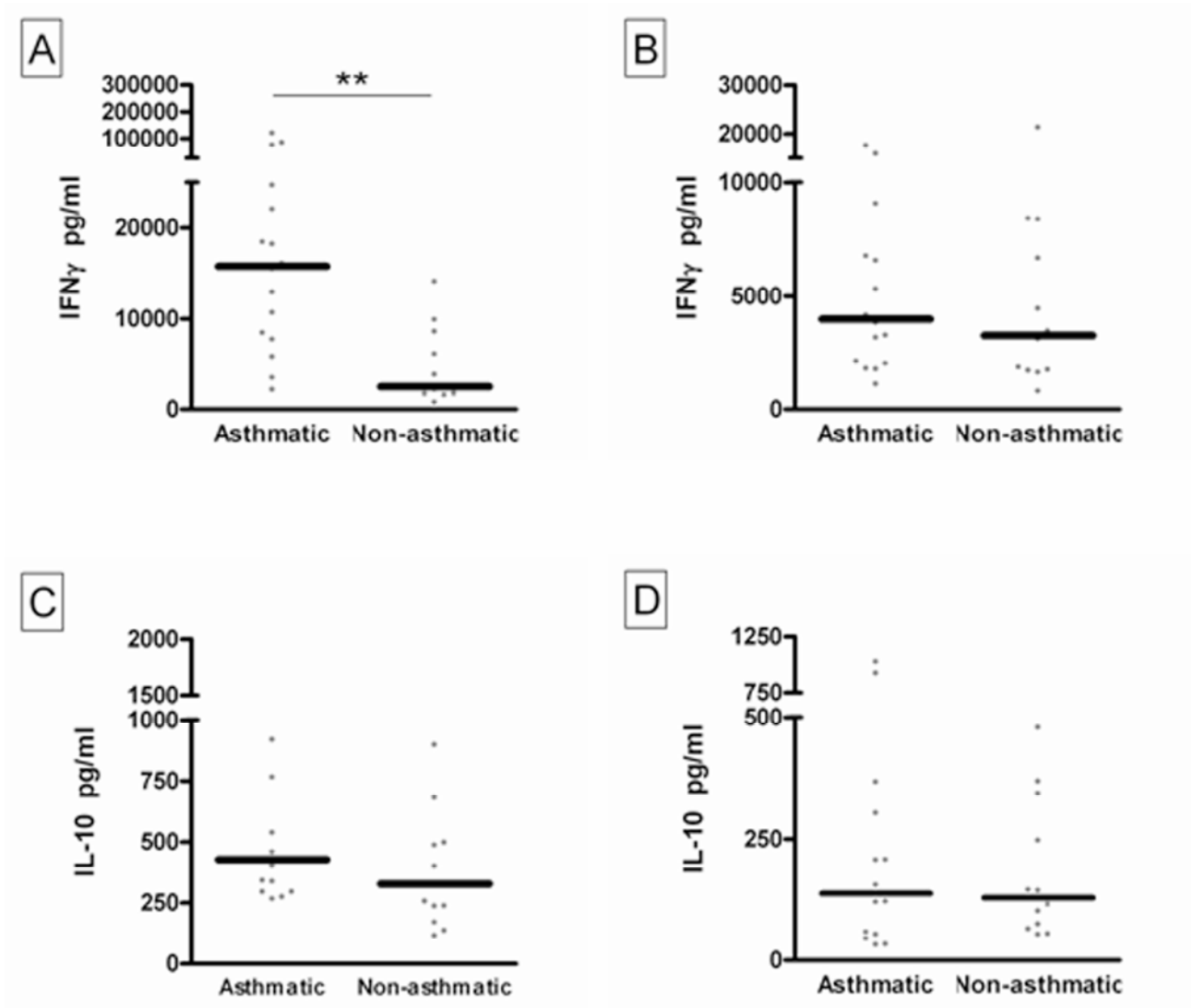
Figure 28: Adult asthmatics display exaggerated IFNγ in responses to reovirus serotype T1L.

IFNγ **(A, B)** and IL-10 **(C, D)** was measured by ELISA in supernatants from 6 day T1L **(A, C)** and T3D **(B, D)** stimulated PBMC cultures. Black bars represent median responses, each from an individual adult (•). (**P<0.01).

Inexplicably, T3D stimulation did not reveal any differences in virus-induced cytokine production between clinical groups. This difference may be due to alternative costimulatory requirements for T cell activation between serotypes. Alternatively, T1L may reveal differences between clinical phenotypes (as do pneumoviruses) due to the source of IL-10 production. Despite similar IL-10 production in both atopic-asthmatic and non-asthmatic adults, immune responsiveness towards APC-derived IL-10 (in T1L, RSV and MPV stimulated cultures) versus T cell-dependent IL-10 production that is characteristic of T3D-driven responses, may differ between clinical populations. Further study is required to identify the role of APC versus T cell-derived IL-10 production in the induction of human anti-viral responses.

Thus, the asthma-dependent pattern of anti-viral immunity is not only specific to pneumoviruses, but also reovirus serotype T1L, suggesting asthmatics display stronger IFNγ responses towards pathogenic and non-pathogenic respiratory viruses. This may contribute towards increased immuno-pathology, despite minimal health risk to the asthmatic individual.

CONCLUDING REMARKS

Here we have demonstrated that intrinsic mechanisms in asthmatics, not virus-specific effects, likely contribute to differential patterns of global anti-viral immunity. The increased propensity of asthmatics to mount excessive Th1 type responses compared to non-asthmatics may be related to asthma pathology, environmental exposures or genetic background. To further explore these possibilities, the nature of anti-viral cytokine responses will be assess according to clinical parameters of disease, environmental exposure identified by epidemiologic assessment and genetic polymorphisms.

CHAPTER 5

Current airway hyper-responsiveness in children predicts enhanced IFNγ responses towards respiratory viruses

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ABSTRACT

Human metapneumovirus (MPV) and respiratory syncytial virus (RSV) are important respiratory pathogens, causing significant disease burden in children. Infection with these viral triggers associates with asthma exacerbations in both children and adults. Sequelae of infection with MPV and RSV include transient wheeze and airway hyper-responsiveness (AHR), however the immunopathology in humans is unknown. Here, we compare MPV and RSV-dependent immuno-regulatory responses stimulated by acute *in vitro* viral re-exposure in asthmatic and healthy paediatric populations displaying varying degrees of AHR. Using viral infection of fresh PBMC, we quantified MPV and RSV-specific cytokine and chemokine production in >150 children with and without current AHR. Children with current AHR, regardless of asthmatic status, exhibit a greater frequency and intensity of IFNγ responses towards MPV than do children without AHR counterparts (p<0.05). Conversely, expression of Th1-immunity-associated chemokine CCL5 is substantially weaker in hyper-responsive asthmatics compared with healthy children (MPV and RSV, p<0.05). In addition, children currently using corticosteroids and demonstrating poorer asthma control exhibit stronger IFNγ and IL-10 production towards pneumoviruses. Atopy did not influence the intensity of anti-viral immunity in asthmatic and healthy paediatric populations. This pattern of enhanced pro-inflammatory response towards pneumoviruses in children with markers of symptomatic asthma or AHR may underlie the enhanced sensitivity of children with AHR to experience breathing difficulties following infection with these respiratory viruses. Deficient anti-viral CCL5 responses in asthmatics may enhance the extent of virus-induced pathology during infection. AHR may be a clinical manifestation of underlying inflammation in both asthmatic AND non-asthmatic children.

PREAMBLE

Viral infections, common in early life, have been implicated with asthma in at least three ways: inception, exacerbation and conversely, protection [542, 543]. Multiple respiratory viruses, including rhinovirus, parainfluenza, respiratory syncytial and most recently human metapneumovirus have been specifically implicated in the pathogenesis of childhood asthma [485, 544]. In addition to the impact of pneumoviruses as triggers of asthma pathogenesis and exacerbation in asthmatics, there is longstanding evidence that they can also induce airway hyper-responsiveness (AHR) in non-asthmatics [159, 160, 545]. Non-asthmatic adults displaying virus-induced wheezing have significantly more respiratory symptoms and lower PC_{20} during colds than those without wheeze, despite exhibiting similar PC_{20} values prior to infection [160]. Viral infection causing paediatric wheezing syndromes has been suggested as a indicator of children who will develop long-term alterations in lung function in the presence or absence of asthma [419-421, 424, 503, 546, 547]. Taken together, these studies indicate that pneumovirus infections within the community are an important cause of symptomatic respiratory illness in all age groups, and can have substantive impact on lung physiology independent of asthmatic status.

RATIONALE AND HYPOTHESIS

Airway inflammation has been thought to be the etiology of airway hyper-responsiveness. As respiratory viruses are the most common triggers of asthma exacerbation and can potentiate airway inflammation and hyper-responsiveness. The relationship between the underlying immuno-regulatory responses to acute virus infection and airway responsiveness (measured here as PC_{20}), as well as the dependence of clinical status

(asthmatic versus non-asthmatic but hyper-responsive) is unclear. *We hypothesise that*
AHR, in addition to asthma (and its severity), will associate with intensity of anti-viral
cytokine production in response to pneumoviruses.

RESULTS

Independent of asthmatic status, children with current airway hyper-responsiveness display
stronger IFNγ responses towards respiratory viruses than do healthy individuals

As we had previously observed that asthmatic adults display increased Th1 biased
responses to MPV compared to healthy individuals, we hypothesised that this skewing may
also affect viral immunity in children displaying current airway hyper-responsiveness (AHR)
[548]. Here, we used a PBMC model of acute MPV and RSV infection [473], to measure anti-
viral cytokine responses in over 200 paediatric allergist-diagnosed children with evidence of
current AHR in comparison to 110 children with normal airway function. Virus-driven
responses were characterised by quantifying IFNγ, CXCL10, CCL5 and IL-10 production
following short-term primary culture with infectious virus. Undetectable levels of Th2-
immunity associated cytokine (IL-13, IL-5) production to MPV were found in over 95% of
individuals (Data not shown). These infrequent and weak Th2 responses were evident in
both asthmatic and non-asthmatic children.

We observed a strongly increased frequency and strength of IFNγ responses to
human metapneumovirus in children with current AHR (regardless of asthmatic status)
compared with children with no evidence of AHR (**Figure 29A**, p<0.01). A similar trend
was observed in RSV-driven IFNγ responses, although it did not reach statistical
significance (**Figure 29B**). The median increase in virus-driven IFNγ production among
children with AHR compared to non-AHR individuals was 170% for MPV and 120% for

RSV. In contrast, production of the Th1-immunity associated chemokine CXCL10, important in cell trafficking and anti-viral immunity [549, 550], did not differ when these populations were stratified solely on the basis of PC20 (**Figure 29, C and D**).

Hyper-responsive asthmatics display the strongest IL-10 and weakest CCL5 responses towards MPV and RSV

To assess the impact of current childhood asthma and its relationship with AHR, these populations were stratified based on asthma with and without AHR and non-asthmatic status with and without AHR (**Figure 30**). Here a trend towards increased virus-driven IFNγ was observed in clinical groups with AHR, regardless of asthmatic status. In contrast, the Th1 chemokine CXCL10 was increased in asthmatics, regardless of AHR, compared to non-asthmatic children (Data not shown, $p<0.05$). In addition to IFNγ and type 1 immunity-associated chemokine CXCL10, we assessed IL-10 and CCL5 production. Substantially stronger virus-driven IL-10 production was evident among hyper-responsive asthmatic (median 110 pg/ml) compared to non-hyper-responsive asthmatic (median 33 pg/ml, $p<0.001$) or non-asthmatic (median 33 pg/ml, $p<0.001$) children (**Figure 30C**). This suggests that the elevated IL-10 production reflects interactions between both AHR and current asthma in the virus-driven response.

Enhanced CCL5 expression has a significant beneficial impact on the long-term outcome of AHR, both in allergen and virus-induced lung pathology [479, 480]. Increased levels of CCL5, a chemokine often associated with Th1-biased immunity, are believed to be protective against airway hyper-reactivity and mucus production [480], in addition to facilitating activation of cytotoxic T cells required for viral clearance [481-483]. Here, in contrast to IFNγ production which was markedly stronger in hyper-responsive individuals, substantially weaker MPV-driven CCL5 responses were evident among hyper-responsive

asthmatic (median 230 pg/ml) compared to non-asthmatic (median 1060 pg/ml) children (**Figure 30, E and F**, $p < 0.05$). Examination of responses in children with only AHR or only asthma suggests that both contribute to the five-fold deficit seen in asthmatics with AHR. The same pattern is evident for both MPV and RSV.

Anti-viral cytokine responses are not correlated with PC$_{20}$ in children with AHR

Methacholine challenge is widely used to aid in the diagnosis of asthma, with PC$_{20}$ values < 8 mg/ml accepted as diagnostic of AHR [26, 43]. However, AHR as measured by PC$_{20}$ values below 8 mg/ml are also observed in approximately 20-50% of children 7-10 years [551-554] who do not have physician-diagnosed asthma (in this study 34.2%). Given that *in vitro* restimulation with MPV and RSV resulted in differential expression of IFNγ, IL-10 and CCL5 in children with AHR, we sought to determine the relationship between cytokine induction and the extent of AHR as measured by PC$_{20}$. Contrary to our expectations [555], no correlation was evident between the degree of AHR and intensity of virus-driven cytokine production for either virus (**Figure 31**). Moreover, stratification of cytokine responses into both asthmatic and non-asthmatic populations revealed no correlation between PC$_{20}$ (or percent predicted FEV$_1$) and anti-viral cytokine production (Data not shown.). This demonstrates that AHR in children defines a pattern of altered anti-viral immunity despite not correlating directly with the severity of AHR.
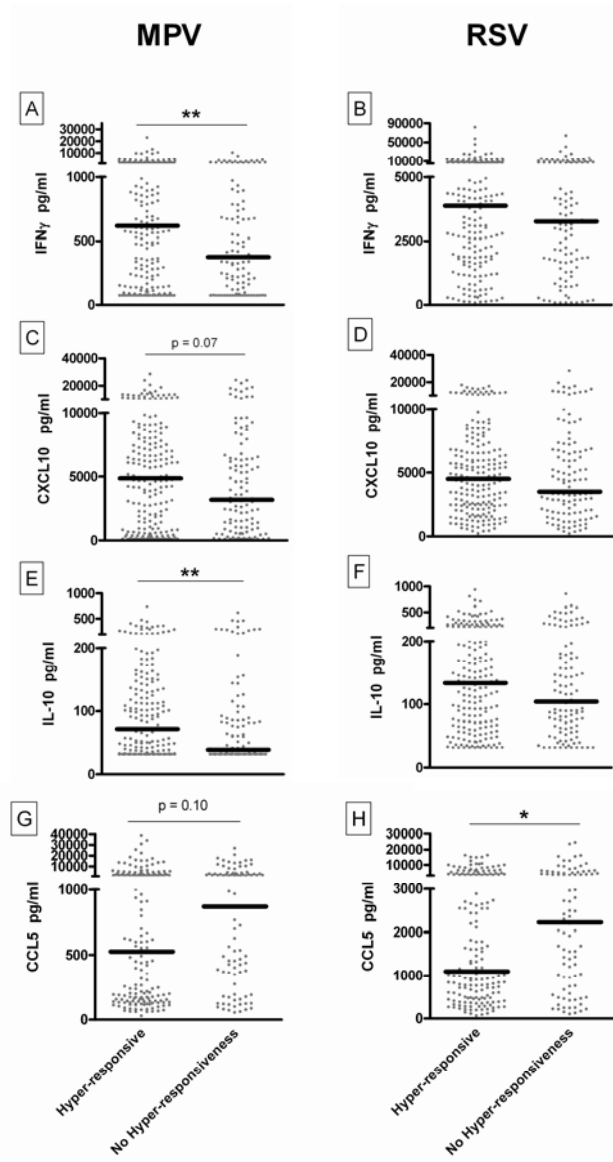
Figure 29: Stronger Th1 biased immunity towards human metapneumovirus (MPV) and respiratory syncytial virus (RSV) in children with airway hyper-responsiveness (AHR). IFNγ **(A, B)** and CXCL10 **(C,D),** IL-10 **(E,F)** and CCL5 **(G,H)** were measured by ELISA in supernatants from 6 day PBMC cultures. Black bars represent median responses, each from an individual child (•). (*P<0.05)(**P<0.01).
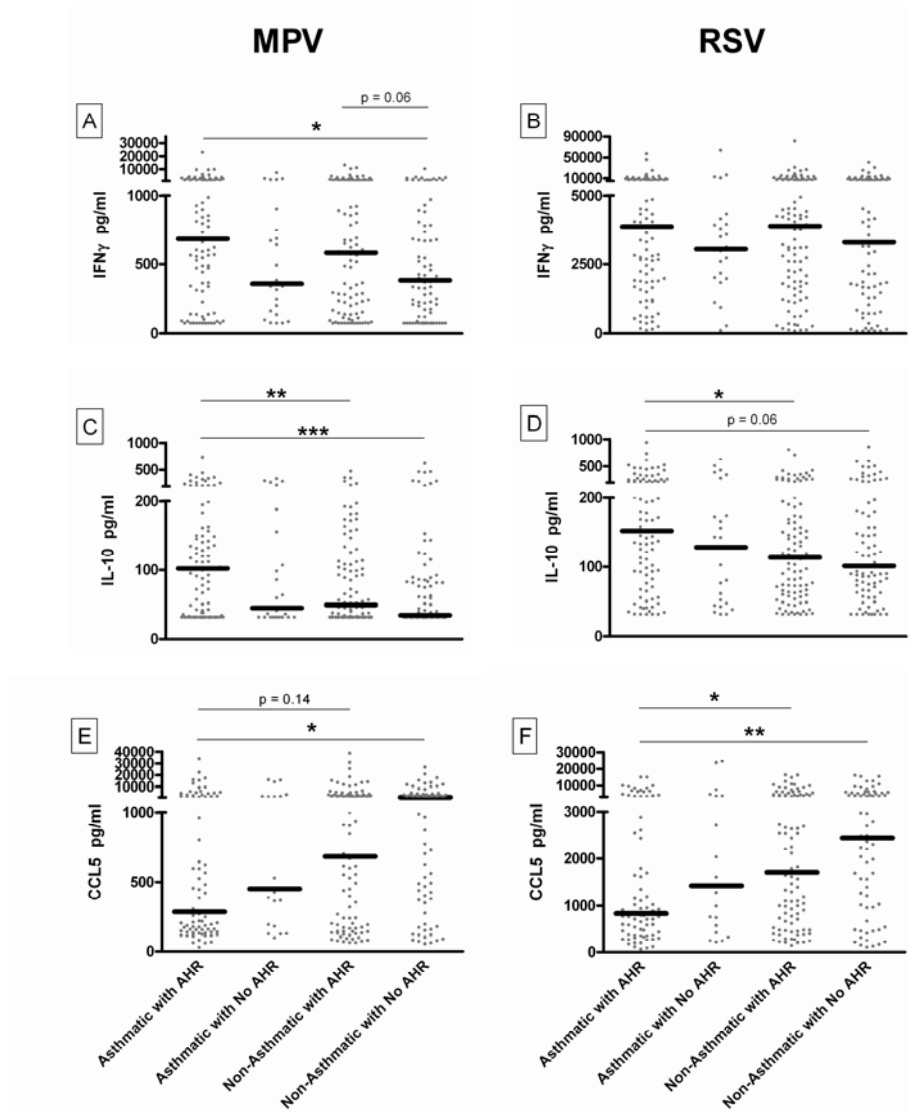
Figure 30: Asthma and airway hyper-responsiveness (AHR) both contribute to increased IL-10 and decreased CCL5 immunity towards human metapneumovirus (MPV) and respiratory syncytial virus (RSV) in children. IFNγ **(A, B)** and IL-10 **(C,D)** and CCL5 **(E,F)** were measured by ELISA in supernatants from 6 day PBMC cultures. Black bars represent median responses, each from an individual child (•). (*P<0.05)(**P<0.01)(***P<0.001).
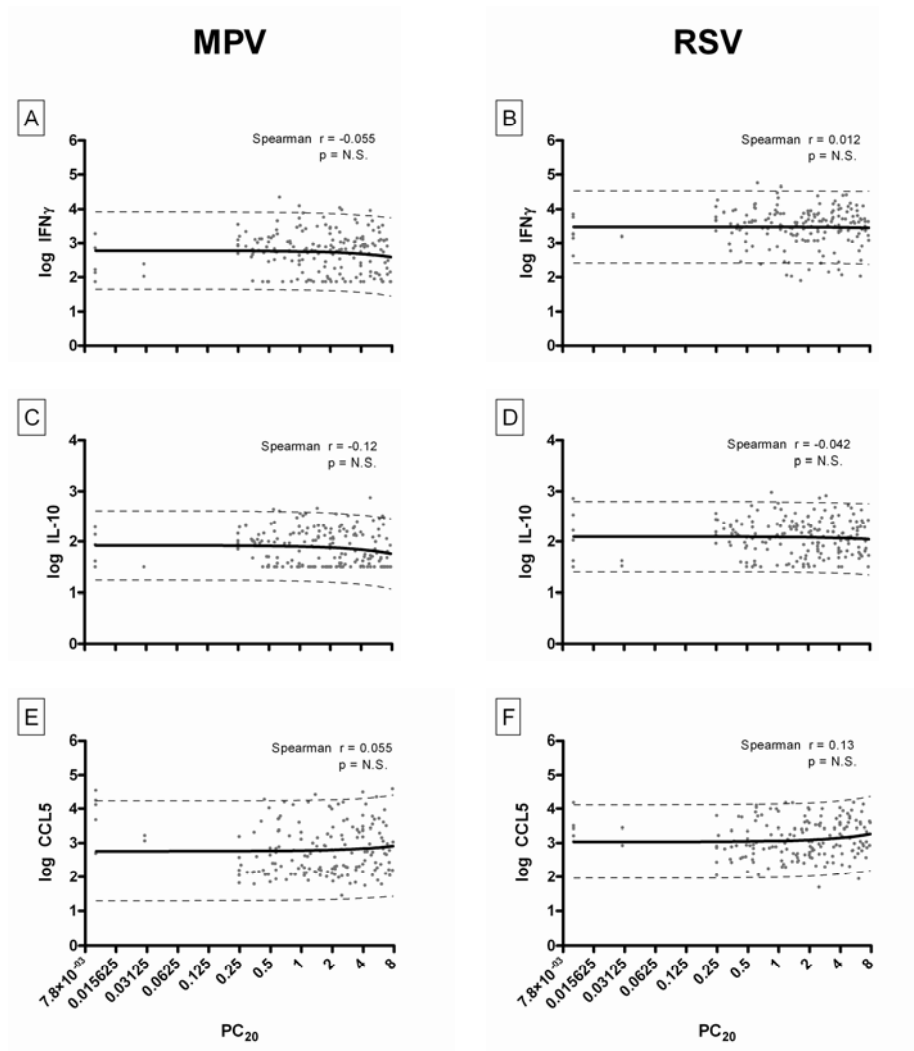
Figure 31: Cytokine virus-driven responses are not correlated with degree of AHR in hyper-responsive children. Correlation of IFNγ, IL-10 and CCL5 responses towards MPV **(A, C, E)** and RSV **(B, D, F)** with provocative concentration of methacholine which causes a 20% fall of FEV_1. IFNγ, IL-10, CCL5 were measured by ELISA in supernatants from 6 day PBMC cultures. Black lines represent linear regression of responses, each from an individual child (•). Spearman derived correlations and p values.

Corticosteroid use predicts enhanced IFNγ and IL-10 and decreased CCL5 anti-viral responses in hyper-responsive children

As an alternative strategy to determine whether airway symptom severity is related to the pattern of anti-pneumovirus immunity, we stratified all children with AHR based on inhaled corticosteroids utilization within the last 12 months. Children with AHR and currently treated with corticosteroids demonstrated elevated production of both IFNγ and IL-10 in response to MPV and RSV (**Figure 32, A-D**). This same pattern was evident when children were further stratified by asthmatic status (**Figure 33**). Taken together, this illustrates that in addition to exaggerated anti-viral responses in children with AHR, children treated with corticosteroids display further enhancement of both IFNγ and IL-10 in response to respiratory viruses.

This is in contrast to CCL5 induction, whereby children currently receiving corticosteroid treatment exhibited markedly suppressed CCL5 responses (**Figure 32, E and F**). As previously described, decreased CCL5 production was associated with asthmatic status. Surprisingly, decreased CCL5 was also associated independently with corticosteroid use in non-asthmatic children with AHR (**Figure 33, E and F**, $p<0.05$ and $p=0.07$ respectively). Consequently, children with AHR who are currently treated with corticosteroids display an identical pattern of anti-viral immunity to that of asthmatic individuals.
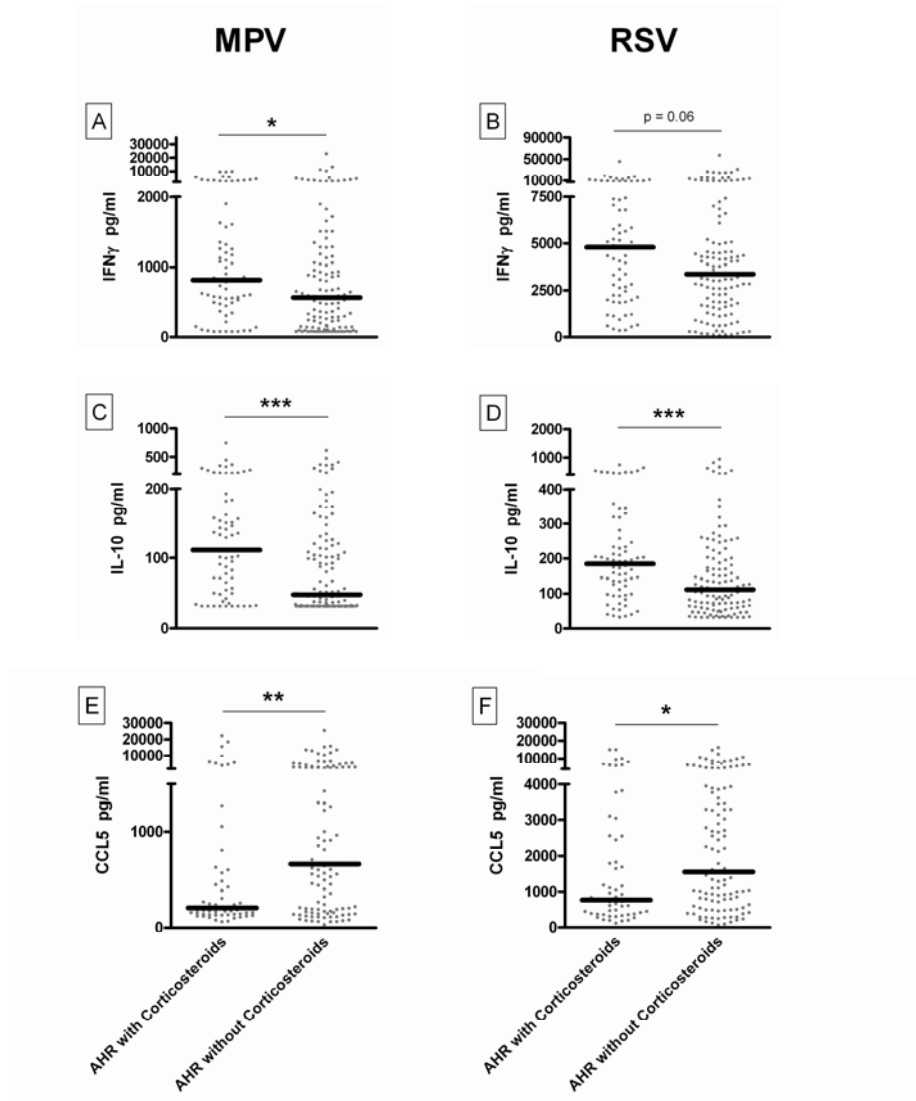
Figure 32: Stronger Th1 biased and IL-10 immunity towards human metapneumovirus (MPV) and respiratory syncytial virus (RSV) in hyper-responsive (AHR) children currently treated with corticosteroid. IFNγ **(A, B)** and IL-10 **(C,D)** and CCL5 **(E,F)** were measured by ELISA in supernatants from 6 day PBMC cultures. Black bars represent median responses, each from an individual child (•). (*P<0.05)(**P<0.01)(***P<0.001).
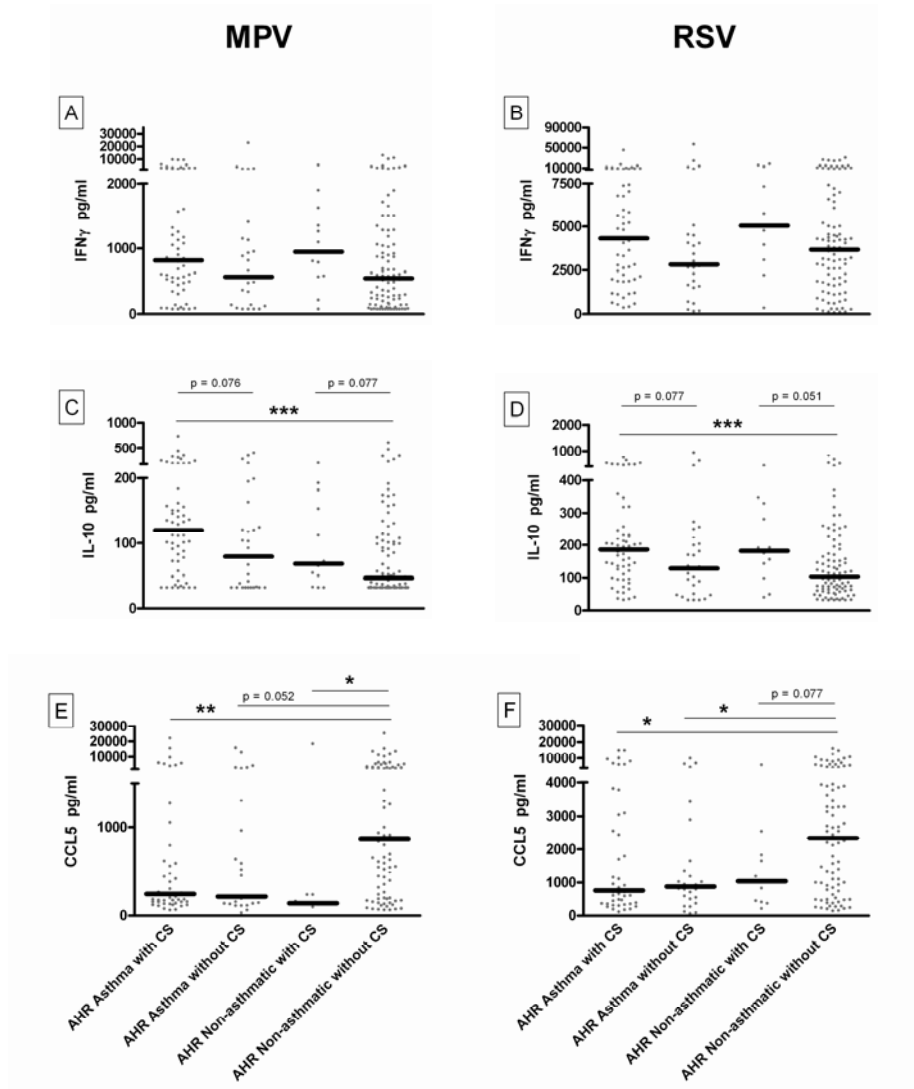
Figure 33: Children with airway hyper-responsiveness (AHR) who are treated with corticosteroids display anti-viral immunity similar to that of asthmatics. IFNγ **(A, B)** and IL-10 **(C,D)** and CCL5 **(E,F)** were measured by ELISA in supernatants from 6 day PBMC cultures. Black bars represent median responses, each from an individual child (•). (*P<0.05)(**P<0.01)(***P<0.001).

Severity of asthma, but not atopy, predicts enhanced IFNγ and IL-10 and decreased CCL5 anti-viral responses

Both airway hyper-responsiveness (**Figure 29**) and corticosteroid use (**Figure 32**) independently predicted increased IFNγ and IL-10, with decreased CCL5 production, in response to respiratory viruses in children. Therefore, we sought to determine whether the overall severity of asthma, or the influence of atopy (as assessed by skin prick test positivity), had the greatest effect on this pattern of anti-viral immunity. Multiple, often conflicting, measures of asthma severity have been reported [556, 557]. To assess asthma severity here, we utilized a composite measure of asthma control, based on reliever and corticosteroid use, hospitalisation, emergency room visits, chronic wheezing and nocturnal asthma symptoms. Using this measure, asthmatic children were stratified into three groups: severe asthma, moderate asthma and mild asthma. **Figure 34**, assessing the relationship between this measure of asthma severity and immune responses to these respiratory viruses, clearly demonstrates that severity of asthma is associated with the strength of IFNγ and IL-10 responses towards MPV and RSV. In contrast, CCL5 production, while strongly influenced by the presence of AHR or corticosteroid usage, does not display a demonstrable relationship with asthma severity. Here, asthmatics regardless of severity display decreased anti-viral CCL5 compared to non-asthmatics (**Figure 34**).

Atopy increases the likelihood of wheezing upon viral infection [271]. The >300 children in this study were stratified based on atopy, defined here as having a positive skin prick test to one or more common allergens. Such analysis, independent of AHR, medication usage or clinical diagnosis of asthma, revealed that the presence of current atopy did not influence the strength of anti-viral immunity towards pneumoviruses (**Figure 35**). Subsequent analyses reinforce this conclusion, demonstrating that atopic status does not

predict increased IFNγ/IL-10 nor decreased CCL5 in any of the clinical subgroups examined (Data not shown).

DISCUSSION

MPV and RSV are important, widespread sources of morbidity in general populations that can induce airway hyper-responsiveness in non-asthmatics and more profound decreases in airway function among asthmatics. Here, we assessed MPV and RSV-specific immuno-regulatory responses in a population of 300 children and subsequently evaluated the contributions of current AHR, corticosteroid usage, as well as asthmatic and atopic status to the immuno-regulatory alterations in virus driven immunity. We demonstrate that children with AHR have both a greater frequency and intensity of IFNγ responses towards pneumoviruses than do non-AHR counterparts, regardless of asthmatic status. Moreover, corticosteroid treated children with AHR demonstrated the strongest anti-viral IFNγ and IL-10 responses. In contrast, concomitant CCL5 production which is strongly associated with CTL activation [481, 482] and inhibition of allergen-induced inflammation [480], is substantially weaker in child asthmatics or AHR children treated with corticosteroids. This pattern of viral immunity may contribute to the enhanced sensitivity of asthmatics or children with current AHR to experience airway exacerbation following infection with these respiratory viruses. Furthermore, we found that this pattern of intense Th1 response and weak CCL5 production in response to pneumoviruses is enhanced in asthmatic children with severe to moderate disease, with no influence of atopy, highlighting the importance of airway-dysfunction for this anti-viral immune profile.
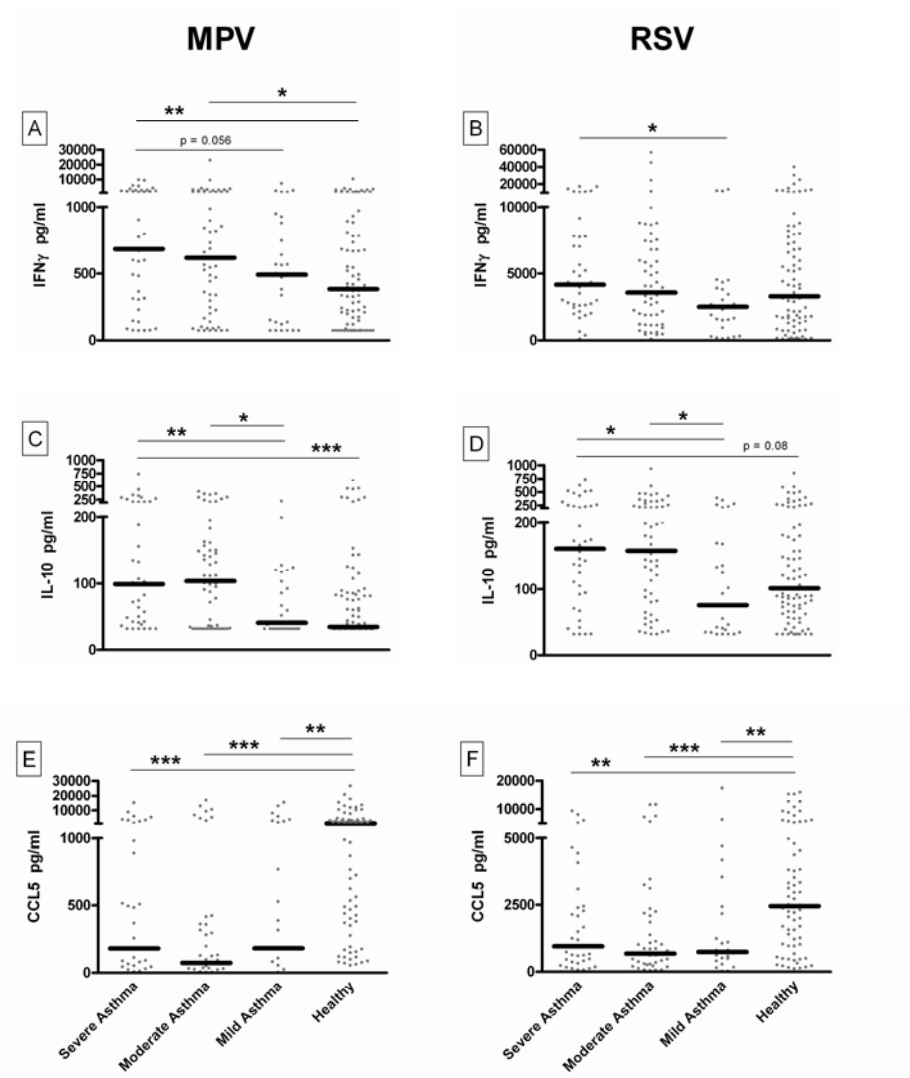
Figure 34: **Severity of asthma influences anti-viral immunity towards human metapneumovirus (MPV) and respiratory syncytial virus (RSV) in asthmatic children.** IFNγ **(A, B)** and IL-10 **(C,D)** and CCL5 **(E,F)** were measured by ELISA in supernatants from 6 day PBMC cultures. Black bars represent median responses, each from an individual child (•). (*P<0.05)(**P<0.01)(***P<0.001).
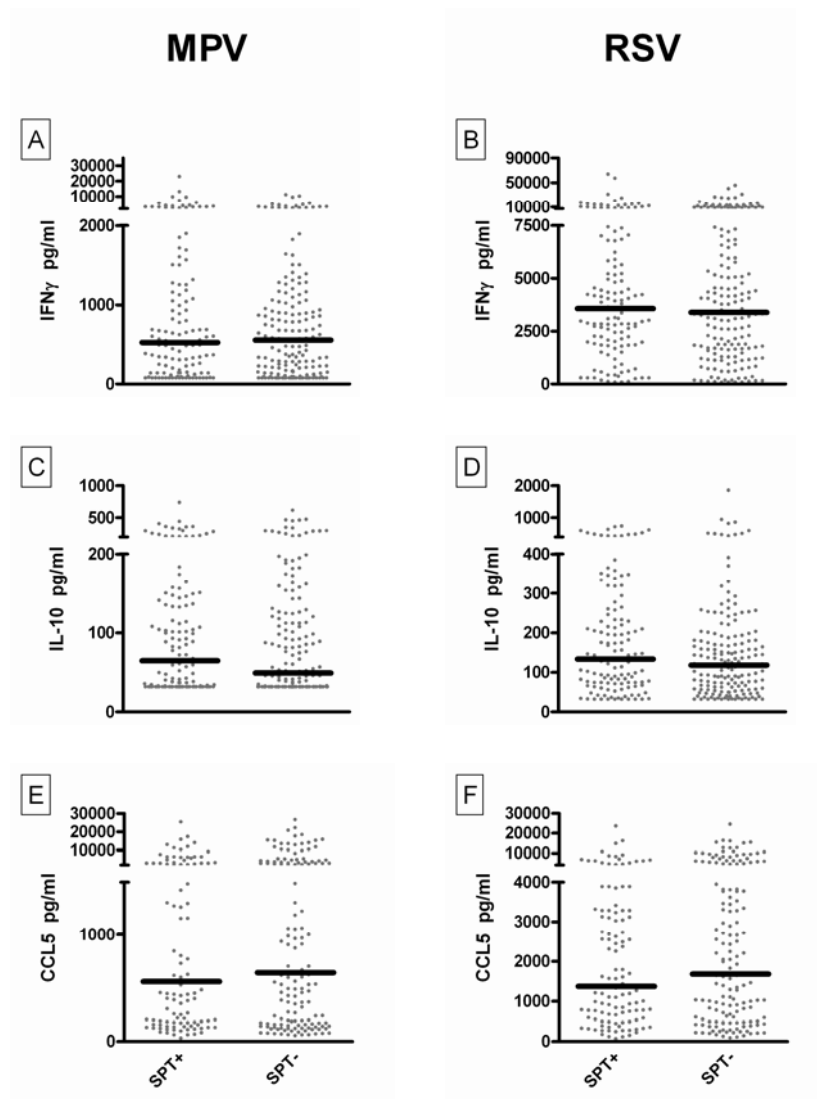
Figure 35: Atopy does not influence anti-viral immunity towards human metapneumovirus (MPV) and respiratory syncytial virus (RSV) children. IFNγ **(A, B)** and IL-10 **(C,D)** and CCL5 **(E,F)** were measured by ELISA in supernatants from 6 day PBMC cultures. Black bars represent median responses, each from an individual child (•), who was either skin prick test positive (SPT+) or skin prick test negative (SPT-).

In murine models, primary high-grade RSV infection or repeated infection resulted in increased severity of allergen-induced airway exacerbation, whereas primary low-grade infection attenuated allergic inflammation [161, 340]. This speaks to the importance of effective viral clearance and memory anti-viral responses in the lung. Here, we demonstrate that immuno-regulatory cytokine production towards pneumoviruses is altered in children with AHR. CCL5 expression is regulated in part by interferon response elements [558], yet despite the observation of increased IFNγ in response to pneumovirus, children with AHR failed to up-regulate CCL5 production to the same extent as healthy children. As T cell-dependent CCL5 induction is essential for viral clearance of RSV infection [493], the failure of children with AHR (and especially asthma) to up-regulate CCL5 production may contribute to increased viral replication in their lungs during infection [479, 493, 497].

Interferons, secreted by either structural lung cells or immune cells, play a crucial role in inhibiting viral replication. Epithelial cell models of virus infection demonstrate that airway epithelium of asthmatics has an impaired ability to mount effective anti-viral immunity through cytokine production, namely type I interferons (IFNβ) [25, 321]. Similarly, a whole blood model of virus infection demonstrated impaired innate type I interferon (IFNα) production towards RSV and Newcastle disease virus in asthmatics compared to non-asthmatics. In contrast, here we describe increased adaptive type II interferon (IFNγ) production in response to RSV and MPV in children with evidence of airway dysfunction compared to healthy individuals. Juntti *et al.* have found that high levels of serum IFNγ production are generally maintained from infancy into childhood, in children with prior severe RSV infection or history of wheezing [492], suggesting increased serum IFNγ is also associated with markers of airway dysfunction in children. Whether this increased IFNγ production is a compensatory mechanism for decreased innate interferons remains unknown. Thus, both intrinsic respiratory epithelium and leukocyte derived immune responses have

important regulatory roles that are likely to contribute to the clinical symptoms of virus infection.

Despite a clear role in viral clearance, excessive IFNγ is associated with wheezing and AHR [187, 409]. In children, increased IFNγ in bronchoalveolar lavage fluid and serum is associated with wheeze (especially those treated with corticosteroids) and asthma [491, 492]. Blocking the effects of IFNγ *in vivo* inhibits the development of AHR, especially in murine chronic challenge models [559-561]. Increased IFNγ may promote virus-induced wheezing by activating eosinophils [562] and inducing leukotriene release [378, 563]. The increased IFNγ in responses to RSV and MPV we observed in children with current airway dysfunction is in contrast to that seen during RV infection, where decreased levels of IFNγ is characteristic of asthmatic anti-viral responses [331]. Taken together, increased IFNγ production during pneumovirus infection can contribute to clinical markers associated with childhood airway hyper-responsiveness, corticosteroid use and asthma severity.

Although IL-10 production is generally described as anti-inflammatory, IL-10 production during viral infection is associated with the development of AHR [489, 564]. During acute viral infection, asthmatics display increased sputum IL-10 levels than non-asthmatic but infected individuals [489, 565]. An *in vitro* PBMC model of rhinovirus infection [330] concur with our findings of increased virus-induced IL-10 production in asthmatic individuals. Murine models reveal a role for IL-10 in the development of AHR, as neutralizing IL-10 abrogates AHR in virus-infected and allergen challenged mice [490, 564]. Increased IL-10 production severely impacts the ongoing innate immune response to respiratory virus [310, 566, 567], and this may contribute to the development of AHR in asthmatics. Although the association of virus-induced IL-10 and asthma remains controversial [568], our data supports a role for virus-induced IL-10 in maintaining airway dysfunction in children.

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There are several limitations of the present study. Given the recognized large diversity in human immune responsiveness, and hence the requirement to achieve larger sample sizes, we employed PBMC rather than lung derived cells. Second, multiple additional pathways contribute to the complex processes underlying the human response to respiratory virus infection. Both the respiratory epithelium and leukocyte-derived immune responses have important regulatory roles that are likely to contribute to the clinical outcome that results following virus infection. Moreover, we did not observe a correlation between PC_{20} and anti-viral cytokine production, although AHR identified children with stronger IFNγ/IL-10 and weaker CCL5 responses. This suggests that AHR in children defines a pattern of altered anti-viral immunity, independent of asthmatic status. The fact that immune responses do not correlate mathematically with the severity of AHR, likely demonstrates the gap between inflammation-provoked transient AHR and persistent AHR due to airway remodelling. Here we used methacholine-induced PC_{20} measurements in children that are reflective of current airway contractility and degree of progressive airway remodelling [26]. Further studies are needed to determine if there is a role for these differential anti-viral responses in transient infection-provoked AHR [77] versus persistent AHR due to virus-induced remodelling processes [156, 157].

Here we demonstrate that children with AHR, both asthmatic and non-asthmatic, produce more IFNγ and IL-10 and less CCL5 in response to respiratory viruses than healthy children. This anti-viral skewing was enhanced with markers of asthma severity, but not atopy, suggesting a relationship with airway function. This finding elucidates how bias in the way the immune system of hyper-responsive children respond to respiratory viruses may lead to impaired viral clearance and contribute to increased symptoms upon reinfection [219, 421]. As anti-viral therapies are emerging as candidate treatments for asthma exacerbation [365,

[386], the identification of AHR in children may be indicative of individuals that could benefit from anti-viral therapy to minimize symptoms and lung injury upon infection with respiratory viruses.

CONCLUDING REMARKS

The children most likely to develop long term AHR and asthma are those with severe bronchiolitis in infancy [270, 273]. Potentially, children with a history of lower respiratory tract infections, such as bronchiolitis in infancy, may present similar alterations in anti-viral immunity as children with asthma and AHR. Therefore, using epidemiologic identification of children with prior bronchiolitis, we sought to determine whether severity of prior respiratory infection associates with intensity of anti-viral immune responses.

CHAPTER 6

Asthma, More so Than Prior Bronchiolitis

or Lower Respiratory Tract Infections,

Predicts Altered Anti-Viral Immunity in Children

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In preparation

ABSTRACT

Viral bronchiolitis has been associated with the development of wheezing syndromes and asthma in children. RSV and MPV are the leading cause of bronchiolitis in infants, and continue to contribute to LRTI throughout life. Sequelae of severe infection with pneumoviruses can lead to transient wheeze and airway hyper-responsiveness (AHR), however the immunopathology in humans is unknown. Here, we compare MPV and RSV-dependent immuno-regulatory responses stimulated by acute *in vitro* re-exposure in asthmatic and healthy paediatric populations with and without prior events of severe lower respiratory tract infection. Using viral infection of fresh PBMC, we quantified MPV and RSV-specific cytokine and chemokine production in >100 children with and without prior bronchiolitis in the first two years of life. Children with prior bronchiolitis in infancy or lower respiratory tract infection in childhood exhibited similar anti-viral responses as children never having had severe respiratory infection. Moreover, only hyper-responsive asthmatic children without prior severe infection exhibit a greater frequency and intensity of IFNγ responses towards pneumoviruses than healthy children (p<0.05). Conversely, expression of CCL5 is substantially weaker in hyper-responsive asthmatics compared with healthy children (MPV and RSV, p<0.05), independent of prior LRTI. In addition, female gender contributes to stronger IFNγ and IL-10, and weaker CCL5, production towards pneumoviruses, especially in children with prior LRTI and asthma. Taken together, this suggests that severity of respiratory infection is not associated with altered anti-viral immunity. However, clinical manifestations following severe respiratory infection, such as AHR and airway immunopathology in asthma, may be associated with increased Th1 type, concomitantly excessive IL-10 and weak CCL5 anti-pneumovirus responses.

PREAMBLE

Viral bronchiolitis has been associated with the development of wheezing syndromes and asthma in children [270, 273]. RSV and MPV are the leading cause of bronchiolitis in infants and continue to contribute to LRTI throughout life. Approximately 40-50% of children who have RSV bronchiolitis and wheeze repeatedly in childhood display AHR and altered lung function [277-279]. However, wheezing in children is generally associated with a heterogeneous set of triggers, not all of which are associated with asthma in later life [52, 258].

RATIONALE AND HYPOTHESIS

It remains unclear whether children with prior bronchiolitis and asthma share a similar immune profile in terms of anti-viral responses towards RSV and MPV; therefore, we sought to assess anti-viral cytokine responses in children with (without) current asthma and/or with (without) a history of prior bronchiolitis in the first two years of life. *We hypothesized that children with prior bronchiolitis, including those who are not currently asthmatic, will respond to respiratory viruses, with increased IFNγ and IL-10 with concomitant weak CCL5 responses, as do asthmatic individuals.*

RESULTS

Asthma, More so Than Prior Bronchiolitis, Predicts Altered Anti-Viral Immunity in Children

Since we had previously observed that asthmatic children with AHR displayed increased Th1 biased responses to respiratory viruses compared to healthy children, we hypothesised that this skewing may also affect viral immunity in children with a prior history of bronchiolitis and/or lower respiratory tract infection (LRTI). Here, we used a

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PBMC model of acute MPV and RSV infection [473] to measure anti-viral cytokine responses in over 100 physician-diagnosed children given a ICD-9 diagnosis code of 466 (Bronchiolitis/Bronchitis) [569] and 150 children with no clinical history of LRTI. Bronchiolitis was defined as a 466 diagnosis in the first two years of life, whereas an LRTI was defined as a 466 diagnosis between birth and time of assessment. Viral-driven responses were characterised by quantifying IFNγ, CXCL10, CCL5 and IL-10 production. In conflict with our initial hypothesis, we observed no detectable difference in frequency or strength of Th1 biased immune responses to respiratory viruses in children with prior bronchiolitis compared with children with no bronchiolitis (**Figure 36, A and B**). Similarly, virus-driven IL-10 and CCL5 production was comparable, regardless of bronchiolitis (**Figure 36C and D**). Stratification based on LRTI, instead of bronchiolitis, revealed identical cytokine responses as well (Data not shown); therefore, to maintain adequate numbers of subjects in our subsequent analysis, we considered the marker of LRTI to be equivalent to that of bronchiolitis.

Intense Th1 and weak CCL5 responses are characteristic of asthmatics, regardless of prior LRTI

We observed differences in the strength of Th1 biased immune responses to respiratory viruses in children without prior bronchiolitis, which were dependent on paediatric-allergist diagnosis of asthma with AHR (**Figure 37, A and B**). Children with AHR asthma also exhibited a 135-220% fold increase in virus-driven IL-10 production compared to non-asthmatic individuals, regardless of bronchiolitis (**Figure 37C and D**, $p < 0.05$).
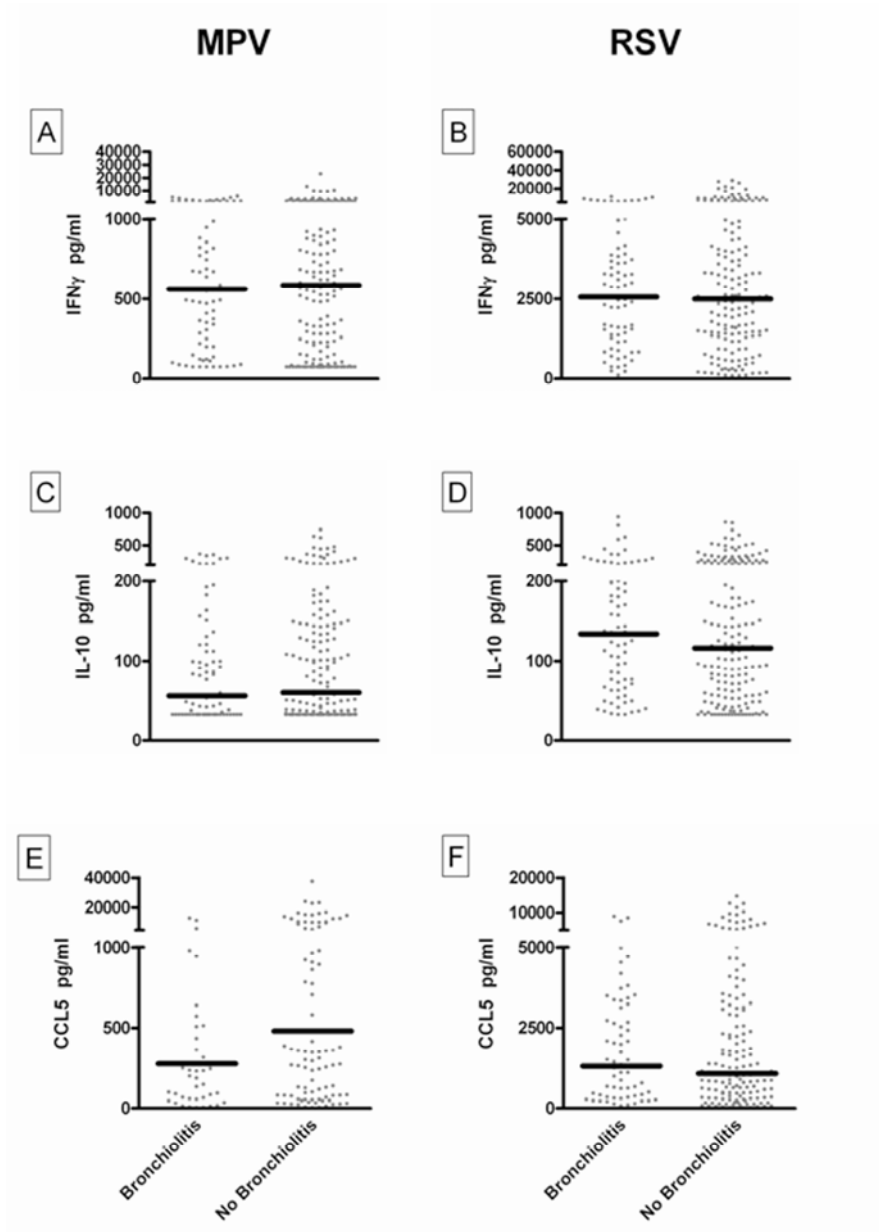
Figure 36: Children with prior bronchiolitis display similar anti-viral responses as children without severe respiratory infections. IFNγ , IL-10 and CCL5 were measured by ELISA in supernatants from 6 day MPV **(A,C,E)** and RSV **(B,D,F)** stimulated PBMC cultures. Black bars represent median responses, each from an individual child (•).
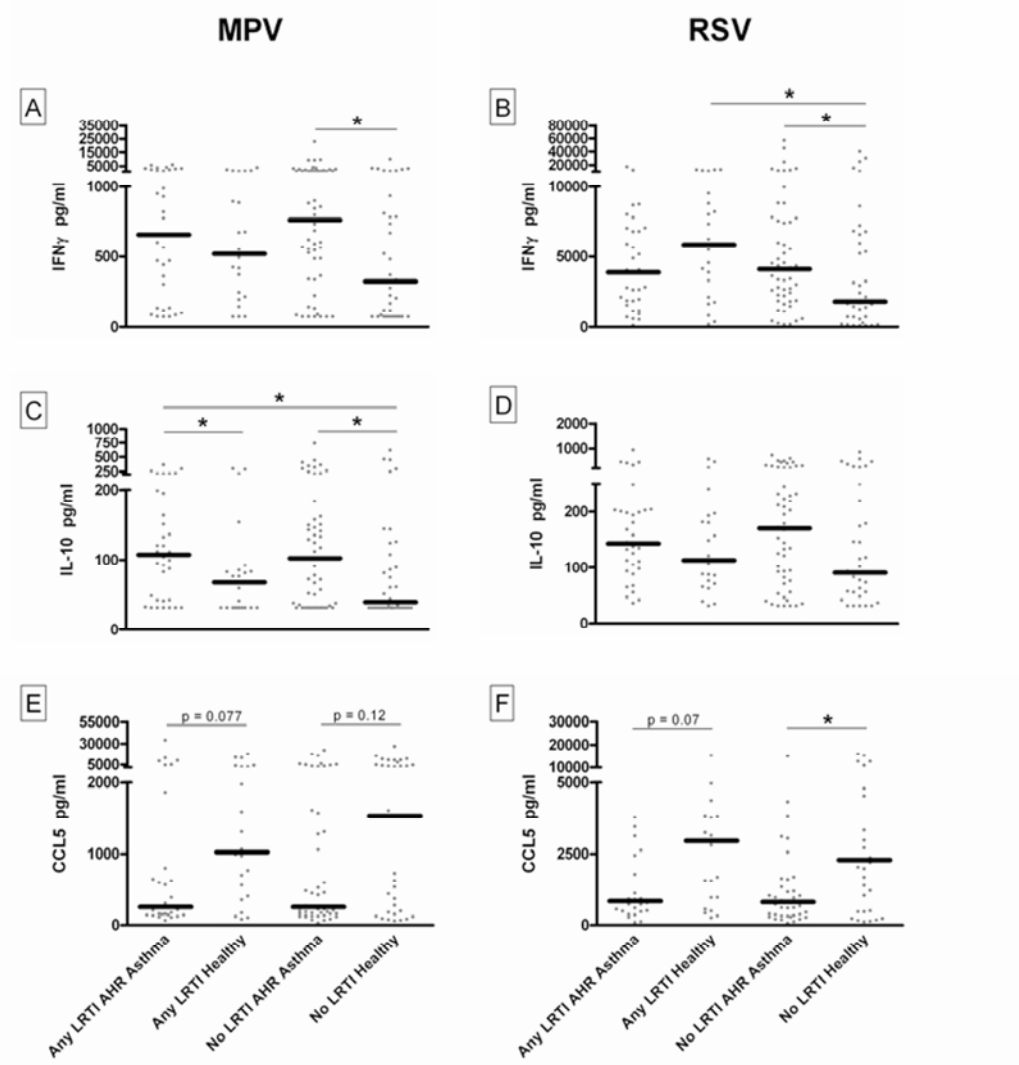
Figure 37: Divergent anti-viral responses are enhanced in children with current asthma with AHR, regardless of prior LRTI. IFNγ, IL-10 and CCL5 were measured by ELISA in supernatants from 6 day MPV **(A,C,E)** and RSV **(B,D,F)** stimulated PBMC cultures. Black bars represent median responses, each from an individual child (•). (*P<0.05).

CCL5 exhibits a significant impact on the long-term outcome of AHR, both in allergen and virus-induced lung pathology [479, 480]. Increased levels of CCL5, a chemokine often associated with Th1-biased immunity, are protective against airway hyper-reactivity and mucus production [480], in addition to facilitating activation of cytotoxic T cells required for viral clearance [481-483]. Here, we observed substantially weaker MPV-driven CCL5 responses among AHR asthmatics (median 130-275 pg/ml) and children with prior LRTI (median 500 pg/ml) compared to non-asthmatics without a history of LRTI (median 900 pg/ml) (**Figure 37 E**). RSV responses in these clinical groups demonstrated a similar pattern (**Figure 37 F**). This differential expression may suggest that increased CCL5 production may be a key anti-viral cytokine which protects from the development of asthma in children with LRTI.

IL-10/CCL5 ratio is skewed in asthmatics, regardless of prior bronchiolitis

Having identified clear differences in the absolute intensities of cytokine production elicited upon virus re-exposure, we sought to determine the relative balance of cytokine responses in each clinical population. IL-10:CCL5 ratios revealed a significant bias of asthmatic individuals towards substantially weaker CCL5 production and elevated IL-10 in response to pneumoviruses (**Figure 38, A and B**). This 10-20 fold difference in the IL-10:CCL5 ratio of asthmatics versus non-asthmatics identifies impaired virus-dependent CCL5 production and the altered balance of immune expression as a characteristic feature of anti-viral immunity in asthmatics. In addition, a 1.4 fold difference in the IL-10:CCL5 ratio of non-asthmatics with prior LRTI versus non-asthmatics never having had a LRTI, revealed suppressed CCL5 responses in children without asthma, but with a history of LRTI. IFNγ:IL-10 ratios were lower in asthmatics compared to non-asthmatics, with no detectable

effect of LRTI (Data not shown). Taken together, non-asthmatics with no history of prior bronchiolitis or LRTI have the strongest bias towards unsuppressed anti-viral immunity.

Gender influences strength of anti-viral responses in children with prior bronchiolitis.

As boys are more prone to bronchiolitis and severe LRTI in childhood [570], we sought to determine whether gender influences anti-viral cytokine production. **Figure 39** demonstrates gender-specific differences in IFNγ and IL-10 production in asthmatics with prior bronchiolitis, with girls showing increased anti-MPV cytokine responses. Concomitantly weak CCL5 responses were observed in asthmatic girls compared to non-asthmatics ($p < 0.05$). Comparable results were observed in terms of gender biases in RSV-driven responses (Data not shown). Consequently, decreased IFNγ and IL-10 responses in combination with weak CCL5 responses may predispose males with prior bronchiolitis towards more severe infection, as they display meagre innate and T cell-dependent anti-viral cytokine production.
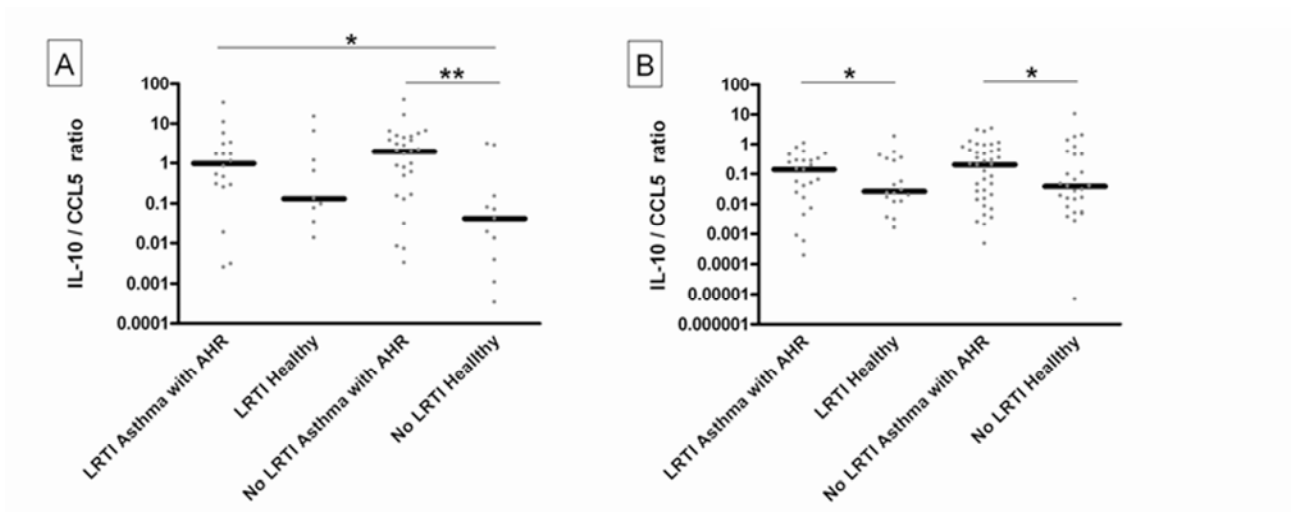
Figure 38: Non-asthmatics with no history of prior LRTI have the strongest bias towards unsuppressed CCL5 responses. IL-10 and CCL5 were measured by ELISA in supernatants from 6 day MPV **(A)** and RSV **(B)** stimulated PBMC cultures. Black bars represent median responses, each from an individual child (•). (*P<0.05) (**P<0.01).
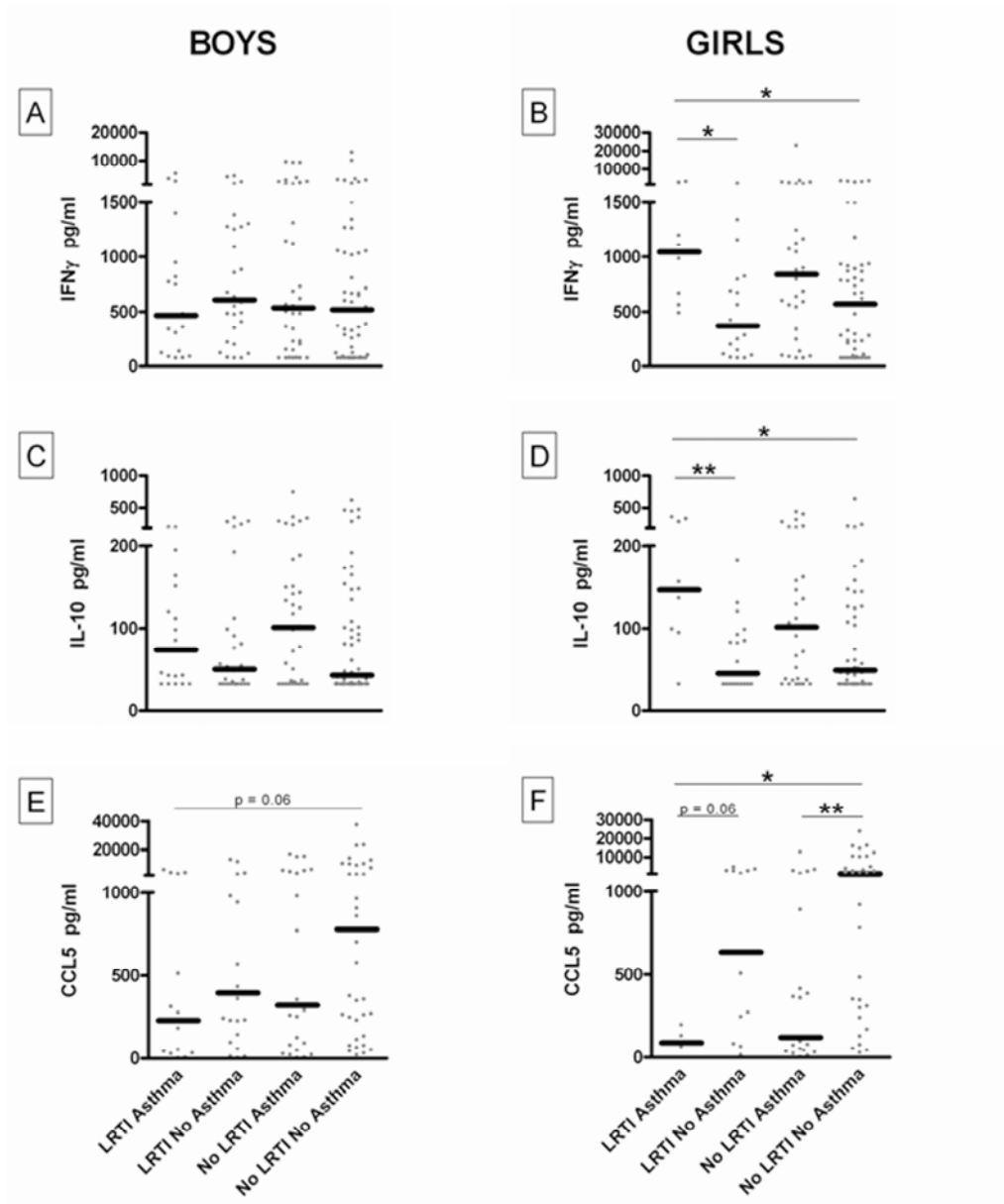
Figure 39: Gender influences strength of anti-viral responses in children with prior LRTI. IFNγ **(A,B)**, IL-10 **(C,D)** and CCL5 **(E,F)** were measured by ELISA in supernatants from 6 day MPV-stimulated PBMC cultures. Black bars represent median responses, each from an individual child (•). (*P<0.05) (**P<0.01).

DISCUSSION

There is much evidence in the literature to suggest that viral infections in early life contribute to inception of asthma and lung injury [157, 192, 255, 270, 276, 281]. Proposed mechanisms include ineffective repair and airway remodelling [156, 157, 296], alteration of innate and adaptive immunity [263, 297] and enhancement of allergic disease following viral infection [195], among others. Therefore, we sought to determine if children with severe bronchiolitis during infancy or LRTI in childhood demonstrate altered virus-driven cytokine responses towards potentially asthmatogenic pneumoviruses. Surprisingly, asthma, more so than prior bronchiolitis or LRTI, predicted altered anti-viral immunity in children. These findings suggest that mechanisms other than anti-viral immune capacity may also be responsible for the severity of bronchiolitis and LRTI.

Some risk factors for bronchiolitis severity include gender [570], exposure to ETS [291, 571], premature birth [572], maternal atopy [99] and genetic background [573, 574]. Severity of infection in RSV bronchiolitis is also associated with decreased levels of IFNγ mRNA in circulating PBMC during acute infection [468]. We observed that boys with prior bronchiolitis have weaker virus-driven IFNγ than girls, which is consistent with boys having more severe courses of RSV infection in infancy than girls [570]. The mechanisms for gender disparity in anti-viral responses and infection severity are yet to be identified.

CONCLUDING REMARKS

Despite the association between severe LRTI and bronchiolitis in infancy and the development of asthma, our results clearly demonstrate that not all children with prior severe respiratory infections exhibit the alterations in anti-viral immunity that are characteristic of asthmatic individuals. This illustrates that asthma, not severity of childhood LRTI, is

associated with immuno-regulatory changes in anti-viral immunity in later life. This may suggest that current childhood anti-viral immunity is not reflective of neonatal immunity or that factors other than immune cell-mediated responses are responsible for severity of pneumovirus infection. Therefore, we further go on to examine whether alternate environmental influences, such as ETS, can influence anti-viral cytokine responses in asthmatics and healthy children.

CHAPTER 7

Exposure to Tobacco Smoke Decreases Inflammatory

Anti-Viral Cytokine Responses in Children with Asthma

or a Familial History of Asthma

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PREAMBLE

Tobacco smoke exposure, whether direct or second-hand, is a risk factor for many diseases, such as cancer, atherosclerosis, asthma, COPD and heart disease [575, 576]. Moreover, tobacco smoke exposure substantially increases the risk of bacterial and viral infections [576]. Suppression of both the innate and adaptive immune system by tobacco smoke inhalation contributes to the susceptibility of infection [576, 577], with nicotine being the most characterized immunosuppressive agent [577] among the 4,500 compounds in environmental tobacco smoke (ETS). ETS-suppression of pro-inflammatory cytokine production may be modulated via epigenetic modification of histones by histone acetyltransferases (HATs) and histone deacetylases (HDACs) [578].

RATIONALE AND HYPOTHESIS

Exposure to ETS in infancy, has been epidemiologically demonstrated to be a risk factor for severe LRTI and the development of asthma [291, 576, 577]. Immunologically, murine models demonstrate that ETS has a substantial effect on immune responses [576, 579]. It remains unknown how early life ETS or continual ETS affects immunity in children. Using a model of anti-viral immunity, we will assess whether ETS influences the intensity of virus-driven cytokine responses in children. ***Here, we hypothesize that current ETS will suppress the production of cytokines in response to RSV and MPV, via differential epigenetic regulation of HDAC and HAT activity.***

RESULTS

ETS exposure suppresses IFNγ and IL-10 responses in asthmatics and healthy children

Exposure to ETS in childhood is commonplace, and is known to increase the frequency of respiratory infection, wheezing and AHR in some children [576, 580, 581]. Yet the full effects of ETS exposure on anti-viral immunity have not been clearly defined. Here, we assessed whether chronic ETS exposure (at birth and continuing to the last 12 months) in asthmatic and non-asthmatic children altered the main anti-viral cytokine IFNγ. As demonstrated by **Figure 40 A**, ETS exposure has a drastic impact on the intensity of MPV-driven IFNγ production ($p<0.001$). This effect is more substantive in asthmatic children (3.5 fold decrease) compared to non-asthmatic children (2 fold decrease). In addition, IL-10 anti-MPV responses were suppressed only in asthmatic children (**Figure 40 B**).

ETS exposure alters IFNγ and IL-10 anti-viral immunity in children with current asthma or a familial history of asthma

To address whether this ETS-mediated suppression of cytokine responses required a susceptible genetic background, we stratified children based on familial asthma. This stratification, as seen in **Figure 41**, revealed that only children with a familial history of asthma are susceptible to anti-viral response suppression by ETS. IFNγ responses from MPV-stimulation of PBMC were similar in children without a history of familial asthma, regardless of ETS exposure. In marked contrast, all children with asthma or a first degree relative with asthma, demonstrated weaker IFNγ responses when exposed to chronic ETS ($p<0.05$).

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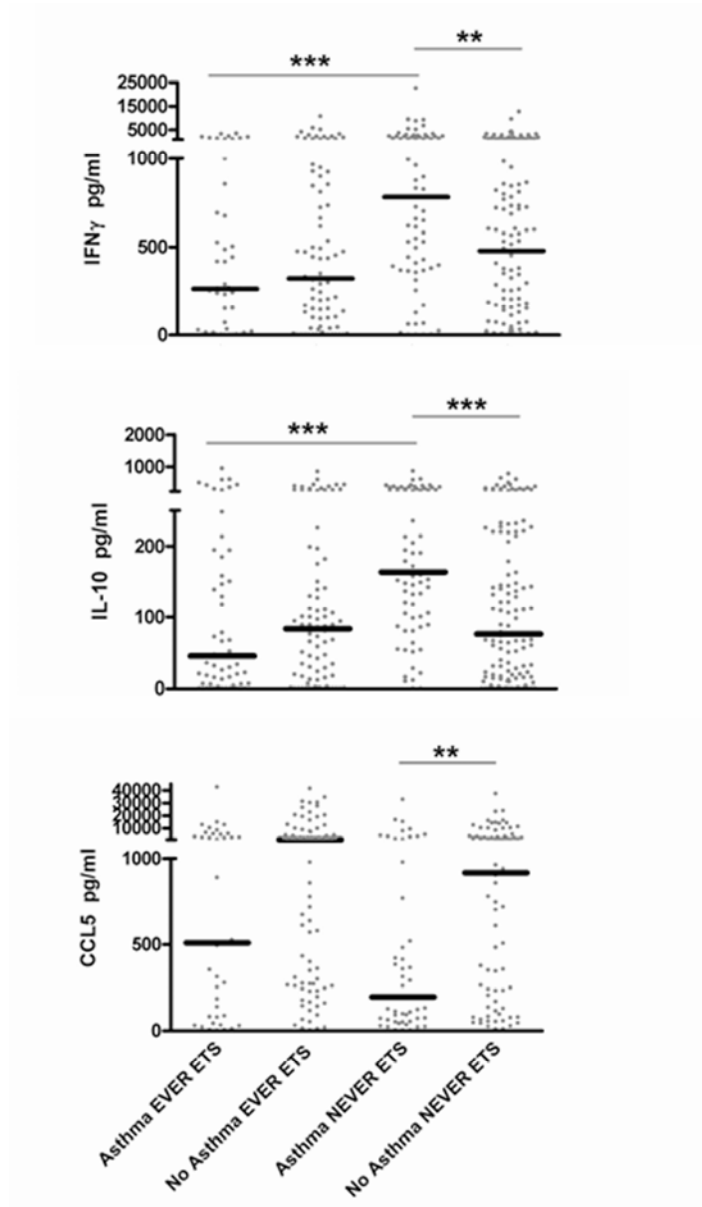
Figure 40: ETS exposure suppresses IFNγ responses in asthmatics and healthy children. IFNγ **(A)** IL-10 **(B)** and CCL5 **(C)** were measured by ELISA in supernatants from 6 day MPV-stimulated PBMC cultures. Black bars represent median responses, each from an individual child (•). (**P<0.01) (***P<0.001).
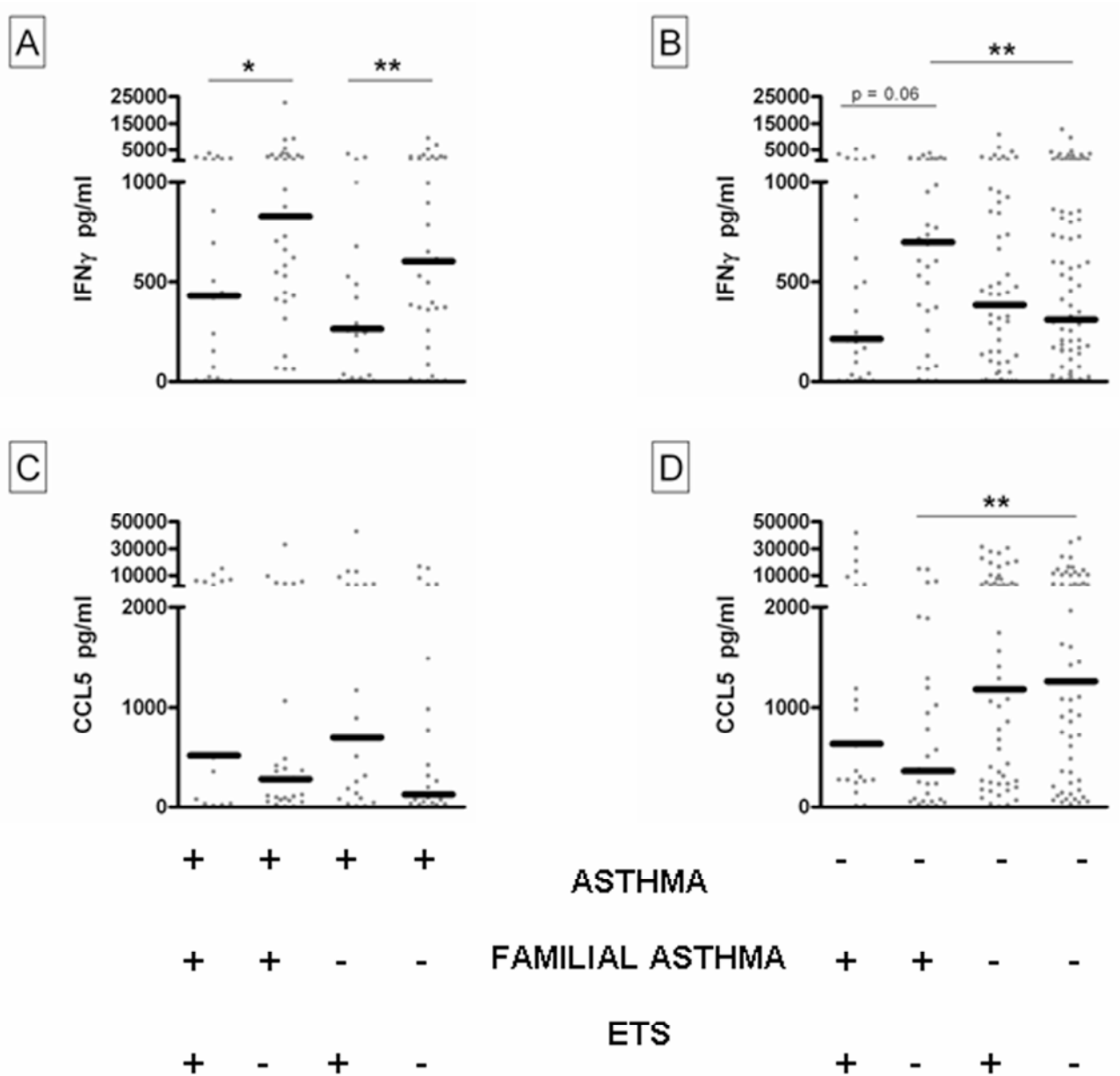
Figure 41: ETS suppresses IFNγ anti-viral responses in children with familial asthma. IFNγ **(A,B)** and CCL5 **(C,D)** were measured by ELISA in supernatants from 6 day MPV-stimulated PBMC cultures. Black bars represent median responses, each from an individual child (•). (*P<0.05) (**P<0.01).

ETS exposure has negligible effects on CCL5 anti-viral immunity compared to familial asthma

Exposure to ETS has been reported to decrease pro-inflammatory cytokine production [576]; however, chronic ETS exposure did not significantly alter CCL5 production in MPV-stimulated (**Figure 40C**) or RSV-stimulated (Data not shown) PBMC. Thus, it is clear that CCL5 anti-viral responses are modulated by genetic heritability (as indicated by the effect of familial asthma), but not ETS exposure (**Figure 41, C and D**).

Association of HDAC and HAT activity with ETS exposure, asthma and anti-viral immunity

To determine whether ETS exposure is modulating anti-viral cytokine production via epigenetic regulation [578, 582, 583] research is currently underway. We are isolating nuclear extracts from PBMC directly *ex vivo* or after 24 hour and 6 day stimulation with RSV and MPV. These nuclear extracts will be assayed for HDAC and HAT activity, and results correlated with anti-viral IFNγ and IL-10 production. This analysis will be performed in stratified populations, namely i) asthmatics with ETS exposure, ii) asthmatics without ETS exposure, iii) non-asthmatics with ETS exposure and familial asthma, iv) non-asthmatics without ETS exposure but with familial asthma and v) non-asthmatics without ETS exposure and no familial asthma. This stratification strategy will allow for determination of dominant effects of either ETS or familial asthma on HDAC and/or HAT activity.

DISCUSSION

Exposure to ETS in children is a substantial risk factor for respiratory and ear infections, however the immunologic mechanisms governing infection susceptibility are

currently unknown. Here we demonstrate that anti-viral immunity can be altered in children who are exposed to ETS; especially when they have current asthma or a familial history of asthma. Specifically, ETS exposure suppresses IFNγ/IL-10 responses in children continually exposed to ETS. In contrast, children with asthma or a familial history of asthma display weak CCL5 responses towards MPV. Thus the combination of asthmatic predisposition and ETS exposure leads to an overall diminished ability to mount anti-viral cytokine responses, potentially increasing the risk for severe or prolonged viral infection.

Anti-viral cytokines, such as IFNγ and CCL5, are essential for activating downstream effector mechanisms involved in viral clearance: activation of cytotoxic T cells which kill infected host cells, activation on intracellular anti-viral machinery (PKR, RNases, Mx proteins) and enhancement of phagocytosis to clear cellular and viral debris [479, 584]. We have previously demonstrated that asthmatics have weak anti-viral CCL5 responses compared to healthy individuals; moreover, ETS exposure additionally suppresses their exaggerated IFNγ responses to weaker levels than healthy children. Healthy children also display weaker IFNγ production when chronically exposed to ETS, although the swing in anti-viral cytokine secretion in not as pronounced as is asthmatics. Interestingly, this decreased IFNγ was only observed in children with asthmatic predisposition, suggesting that ETS exposure itself is not sufficient to alter anti-viral immune responses, but requires a specific heritable predisposition to exert effect. This observation supports the hypothesis that clinical and immunologic endpoints are likely to result from gene-environment interactions [58, 64]. Overall, ETS exposure has a likely detrimental effect on anti-viral pathways by diminishing immune cell-derived IFNγ necessary for viral clearance, as well as previous reports demonstrating impaired pro-inflammatory cytokine induction in epithelial cells [578]. This may have a particularly drastic impact in asthmatics and children with a familial history of

asthma, as they display already impaired anti-viral CCL5 responses, making smoking cessation especially pertinent in these families.

To further determine whether IFNγ/IL-10 supression by ETS may be epigenetically regulated by histone modifications, we proposed to examine putative differences in functional activity of HDAC and HAT in these children. ETS is known to increase histone acetylation and decrease HDAC activity [578, 583], leading to suppression of pro-inflammatory cytokine secretion in epithelial cells [578]; therefore, we hypothesize that children exposed to ETS will show diminished HDAC activity. Increased HAT (or decreased HDAC) activity is associated with increased gene transcription, suggesting that negative regulators of pro-inflammatory cytokine production are enhanced following ETS exposure. Furthermore, asthmatics tend to demonstrate increased HAT activity and decreased HDAC activity, associated with increased severity of asthma [585-587]. Taken together, we propose that the significant down-regulation of exaggerated IFNγ/IL-10 responses in child asthmatics exposed to ETS is associated with enhanced histone acetylation, due to intrinsically increased HAT activity in conjunction with weaker and ETS suppressed HDAC activity.

CONCLUDING REMARKS

Thus, we have clearly demonstrated a gene by environment interaction, whereby ETS exposure in children with familial asthma results in suppressed anti-viral IFNγ and IL-10 production. This demonstrates environmental exposures may alter anti-viral immunity in the context of genetic susceptibility characteristic of individuals with asthma-predisposition. Therefore, we will expand our research to examine candidate genes likely to associate with anti-viral cytokine regulation in asthmatic and non-asthmatic individuals.

CHAPTER 8

TLR4 Genotype Asp299Gly does not Affect Anti-Viral Responses towards Respiratory Viruses

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In preparation

PREAMBLE

The role of TLR4 signaling in respiratory virus infection is an area of much recent focus. Murine studies strongly suggested that TLR4 plays a key role in shaping the intensity of immune responsiveness to RSV infection [103, 122], however other studies have not replicated these results [123, 124, 588]. Our data obtained with whole infectious RSV demonstrates that while blocking TLR4 function abrogates the activity of LPS, RSV-mediated activation of cytokine production does not change in frequency or intensity. Despite the immunologic controversy, TLR4 polymorphisms have been associated with severity of RSV bronchiolitis and asthma.

RATIONALE AND HYPOTHESIS

Tal *et al*. have demonstrated an association between the Asp299Gly and Thr399Ile polymorphism and severe RSV infection [589]; contrarily, an opposite associative trend between severe RSV bronchiolitis and the TLR4 Asp299Gly polymorphism has been demonstrated [590]. Currently, a putative association between severity of RSV infection and TLR4 polymorphism remains inconclusive. Hence, we sought to determine whether the Asp299Gly polymorphism associated with anti-viral immune responses in children who are healthy, asthmatic or had prior bronchiolitis.

RESULTS

Similar RSV-driven cytokine responses in children with TLR4299 Asp/Asp or Asp/Gly genotypes

Despite a putative effect on LPS responsiveness, anti-viral immune responses driven by RSV infection are not affected by TLR4 Asp299Gly polymorphism (**Figure 42**). Analysis of the entire child cohort demonstrates that RSV immunity is similar in both groups regardless of genotype, although the Asp/Gly group trend towards weaker IFNγ responses (**Figure 42A**, p = 0.13).

TLR4 Asp299Gly polymorphism has no effect on RSV-driven cytokine responses in children stratified by asthmatic status or prior bronchiolitis

As demonstrated in **Figures 43 and 44**, stratification of children based on asthma or prior bronchiolitis in infancy, does not reveal any differences in anti-viral cytokine production between individuals with the TLR4299 Asp/Gly genotype or the TLR4299 Gly/Gly genotype.

DISCUSSION

TLR4 Asp299Gly polymorphism has been associated with LPS hypo-responsiveness [591]. Therefore, we sought to determine whether the individuals with the TLR4 Asp/Gly polymorphism differ in RSV-driven immunity compared with the majority of the population carrying the Asp/Asp genotype. Analysis of the entire child cohort demonstrates that RSV immunity is similar in both groups regardless of genotype, although the Asp/Gly group trend towards weaker IFNγ responses.
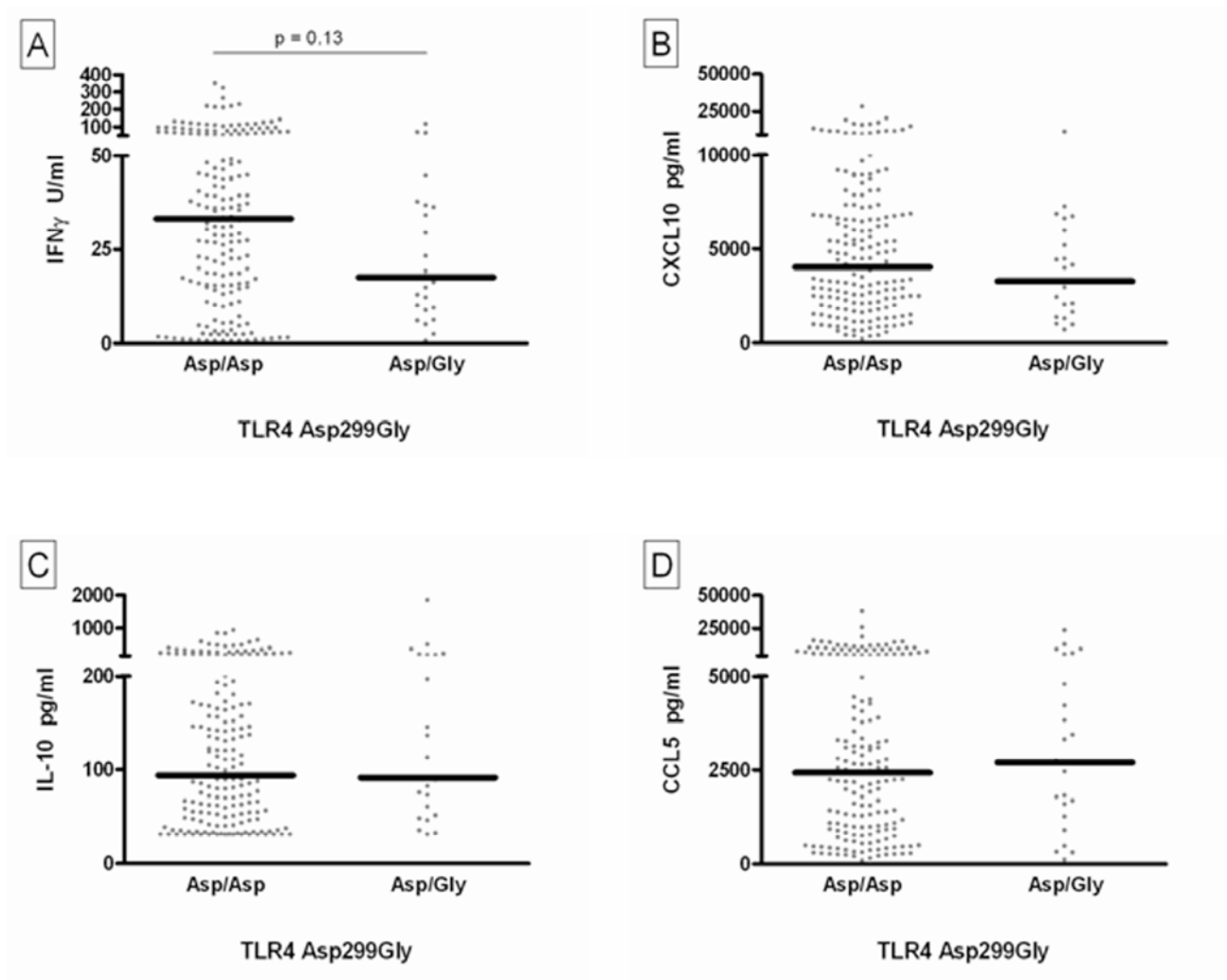
Figure 42: Similar RSV-driven cytokine responses towards respiratory syncytial virus (RSV) in children with TLR4299 Asp/Asp or Asp/Gly genotypes. RSV-dependent responses were measured in children. IFNγ **(A)** , CXCL10 **(B)** , IL-10 **(C)** and CCL5 **(D)** were measured by ELISA in supernatants from 6 day PBMC cultures. Black bars represent median responses, each from an individual child (•).
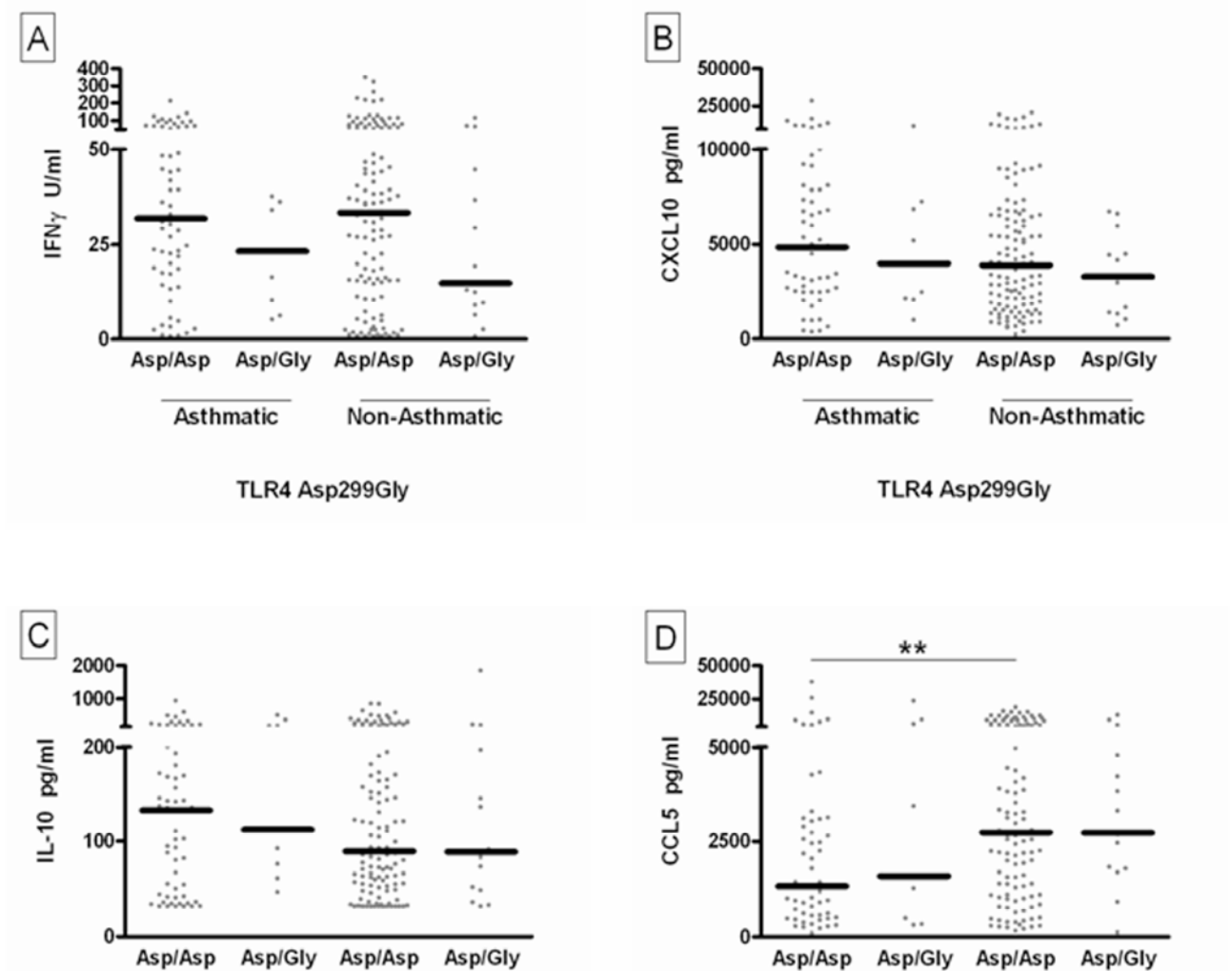
Figure 43: Similar RSV-driven cytokine responses towards respiratory syncytial virus (RSV) in children with TLR4299 Asp/Asp or Asp/Gly genotypes with and without current asthma. RSV-dependent responses were measured in children. IFNγ **(A)** , CXCL10 **(B)** , IL-10 **(C)** and CCL5 **(D)** were measured by ELISA in supernatants from 6 day PBMC cultures. Black bars represent median responses, each from an individual child (•).
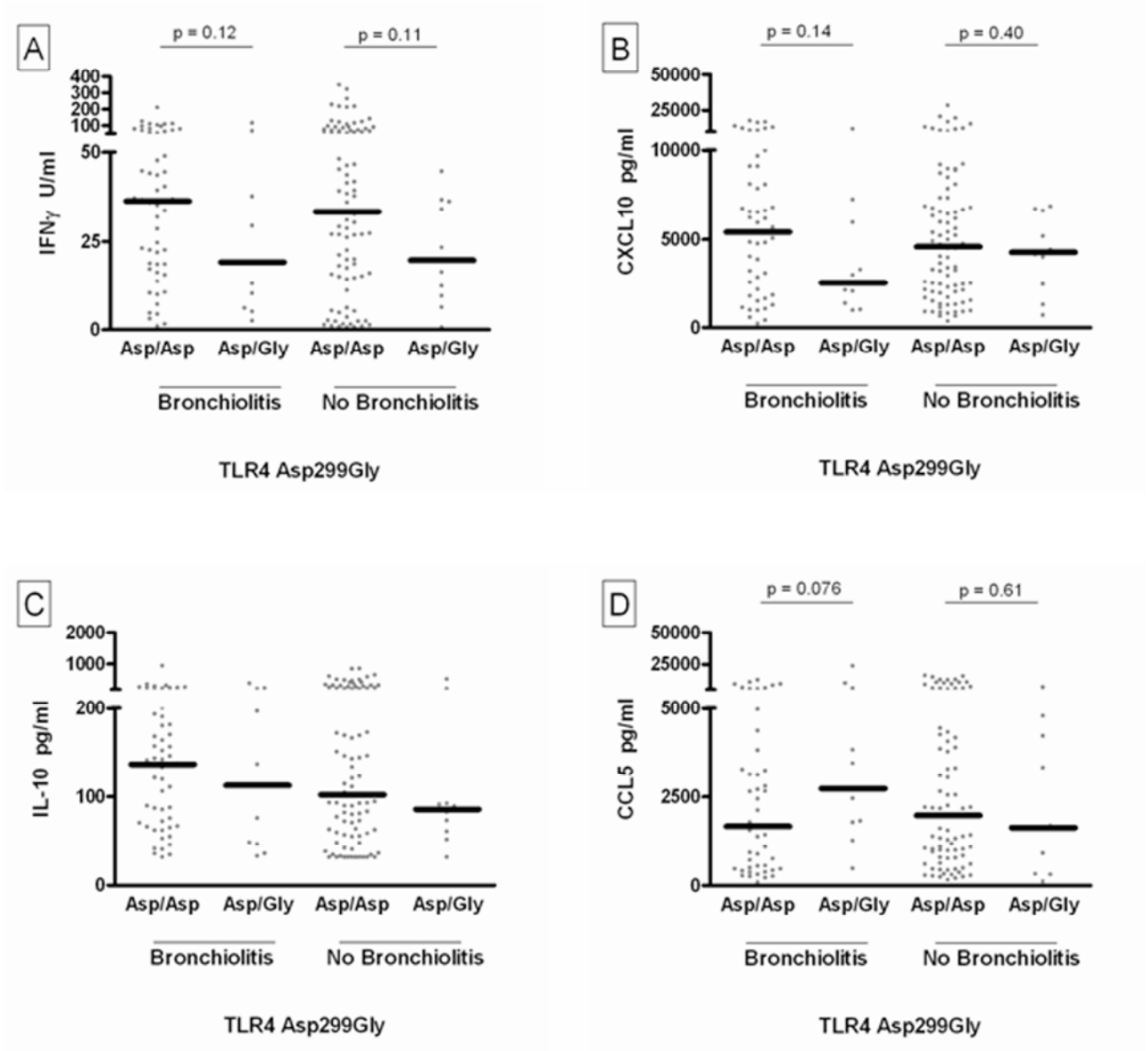
Figure 44: Similar RSV-driven cytokine responses towards respiratory syncytial virus (RSV) in children with TLR4299 Asp/Asp or Asp/Gly genotypes with and without prior bronchiolitis/bronchitis. RSV-dependent responses were measured in children. IFNγ **(A)**, CXCL10 **(B)** , IL-10 **(C)** and CCL5 **(D)** were measured by ELISA in supernatants from 6 day PBMC cultures. Black bars represent median responses, each from an individual child (•).

Further stratification of the cohort based on asthmatic status also revealed similar RSV-driven responses in regards to TLR4 Asp299Gly genotypes. A third independent approach, with stratification based on moderate-severe bronchiolitis/bronchitis in childhood revealed similar RSV-driven responses in regards to TLR4 Asp299Gly genotypes. Therefore, we conclude that individuals with the Asp/Gly genotype demonstrate similar RSV-driven cytokine responses to individuals carrying the Asp/Asp genotype. Overall, the TLR4 Asp299Gly polymorphism has no detectable effect on current RSV immunity in children.

CONCLUDING REMARKS

This study demonstrated no association of TLR4 polymorphism and virus-driven cytokine responses from RSV-stimulated PBMC. This result is not surprising given that the biological plausibility of this association was poor. However, this exercise demonstrates the technique of immuno-genetic association, which will be used in future analysis of candidate genes involved in immune regulation of anti-viral responses.

CHAPTER 9

Associations between Genetic Polymorphisms and Anti-viral Immunity in Clinical Populations

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In preparation

PREAMBLE

The complex nature of arriving at a measurable anti-viral immune response consists of multiple levels of regulation via PRRs, signalling pathways, transcription factors and cytokine production. Polymorphisms, singly or in combination, in any of these effector proteins can potentially have a great impact on the ensuing response.

RATIONALE AND HYPOTHESIS

We have previously demonstrated dysregulated cytokine production in response to viral stimulation in individuals using disease parameters, epidemiologic markers and clinical status. Few studies have identified anti-viral immune responses/mechanisms that could account for the observed differences between asthmatic and healthy individuals. Here we propose that genetic variability, as measured by SNPs or haplotypes, will associate with the intermediate phenotype of anti-viral cytokine production. Previous literature has demonstrated that many SNPs associate with clinical outcomes related to asthma and anti-viral resistance [573, 592, 593]. Most of these have proven difficult to replicate or extend to other populations. Lack of statistical power due to insufficient cohort size and multiple-comparison problems hinder the success of gene association studies [594]. However, the use of intermediate phenotypes, such as measurement of anti-viral immunity, in genetic associations is more likely to identify underlying mechanisms of disease than direct correlation with disease outcomes [595]. *Here, we evaluate if anti-viral immunity against RSV and MPV associates with pre-selected genetic polymorphisms in terms of intensity and frequency of cytokine production, both independently and upon population stratification for disease parameters, epidemiologic markers and clinical status.*

RESULTS

In these experiments, currently underway, data analysis will consist initially of the description of SNP/haplotype frequencies in the cohort, followed by SNP/haplotype associations with intermediate phenotypes (RSV and MPV-driven cytokine production in PBMC), independent of clinical phenotype. Dominant and co-dominant models will be used depending on minor allele frequency in the population. Further stratification will be based on parameters of disease (such as AHR, SPT), epidemiologic markers (such as prior bronchiolitis or repeat childhood infections) and clinical status (such as asthma), and will assess frequency of SNP/haplotypes within each group and evaluate their respective virus-driven cytokine responses for statistical differences in intensity and frequency. At this time, there is a complete data set of anti-viral cytokine responses (n=345), with which to associate each pre-selected SNP as they are genotyped. Candidate genes and SNPs are listed in **Table 8**, with short descriptions of the rationale used for selection.

An example of preliminary results using this technique is demonstrated in **Figure 45**. Here we evaluate CCL5 polymorphisms, rs2107538 A/G, rs4795095 A/G and rs2291229 A/G, using a co-dominant model. Significantly weaker RSV-driven CCL5 responses (both in terms of fold increase and total CCL5, $p<0.05$) are evident in carriers of the A/G genotype compared to the G/G genotype for -403G/A (rs2107538) CCL5 promoter polymorphism. In contrast, this genotype-specific effect was not observed in MPV-driven responses (**Figure 45, A and C**). However, this polymorphism had no significant effect in unstimulated PBMC, as spontaneous CCL5 production was similar in all genotypes (Data not shown). Unfortunately, due to the frequency distribution of the minor allele, some genotypes have insufficient numbers to validate putative differences in cytokine production (ex. 2107538 AA), even in a cohort subset of 345 children.

Table 8: List of candidate genes for immuno-genetic analysis

Gene	Polymorphisms	Frequency of minor allele	Intermediate phenotypes	Population stratifications
ccl5	Int1.1 C/T	10-15% [502]	RSV and MPV-driven CCL5 and IFNγ	Asthma
	-403 G/A	15-20% [596]		Bronchiolitis
	3'222 C/T	7-11% [502]		AHR/ETS

Rationale: CCL5 polymorphisms have been associated with many aspects of allergic disease, such as atopic dermatitis, AHR, atopy and asthma. The CCL5 promoter polymorphism -403A is associated with atopic dermatitis, AHR and asthma [596, 597].

Gene	Polymorphisms	Frequency of minor allele	Intermediate phenotypes	Population stratifications
ccr5	-2554 C/T	35% [598]	RSV and MPV-driven CCL5 and IFNγ	Asthma
	-2459 A/G	45% [598]		Bronchiolitis
	Δ32null	10% [599]		AHR

Rationale: The -2554T and -2459G SNPs are more frequently associated with severe RSV bronchiolitis than in control infants (0.38 vs. 0.33 and 0.48 vs. 0.43, respectively), with a significant transmission distortion of the -2459G allele in affected children [598]. In addition, there is controversial evidence regarding a link between the CCR5 Δ32null allele (found in approximately 10% of European descendants [599]), with reports favouring this mutation as protective against asthma [600-602] and some suggesting no association with asthma susceptibility [603, 604]. Both the Δ32null and -2459G alleles modulate CCR5 expression on T cells [605-608].

Gene	Polymorphisms	Frequency of minor allele	Intermediate phenotypes	Population stratifications
cx3cr1	T280M	30% [609]	RSV and MPV-driven CCL5 and IFNγ	Asthma
	V249I	40% [610]		Bronchiolitis

Rationale: Secretion of the RSV G protein during infection decreases CX3CR1$^+$ T cells (the main cytotoxic effectors in RSV immunity) trafficking to the lung [202]. The genotypes carrying the functional SNP 280M are more predominant in infants diagnosed with severe RSV bronchiolitis compared to controls (0.38 vs. 0.21) [609]. The 249I SNP is also a candidate, as it disrupts receptor affinity for CX3CL1 [610].

Table 8 (continued): List of candidate genes for immuno-genetic analysis

ifng	+874 A/T	10% [611]	RSV and MPV-driven CCL5, CXCL10 and IFNγ	Asthma AHR Bronchiolitis
	Allele 138 (13 CA repeats)	45% [612]		
cxcl10	To be determined	> 10%		
tbx21	c.-7947 A/G	43% [593]		
	-1993 T/C	8% [613]		
irf1	allele 306 (11 GT repeats)	40% [612]		
socs1	−3969C > T	22% [614]		
	−1478CA > del	11% [614]		

Rationale: Increased anti-viral IFNγ and CXCL10 production in asthmatic and children with AHR may associate with polymorphisms in IFNγ [611, 615] and CXCL10 genes as well as their regulators such as t-Bet (asthma and AHR [593, 613]) IRF1 (asthma [612, 616]) and SOCS1 (asthma [614]). In addition, IFNγ gene polymorphisms have been associated with asthma [612, 616], whereas the D4S3042 region (encoding CXCL10) of 4q21 is associated with seasonal allergic rhinitis [617]. Taken together, IFNγ and its regulatory pathways likely play a role in defining patterns immune responsiveness in asthma and viral infection.

il10	-1082 A/G	50% [618]	RSV and MPV-driven IL-10	Asthma
	-592 C/A	38% [618]		Bronchiolitis
	-627 C/A	29% [619]		AHR
	-1117 A/G	50% [620]		Familial asthma
	-3585 T/A	43% [620]		

Rationale: IL-10 promoter polymorphism have been associated with the severity of RSV bronchiolitis [573, 620], although not replicated in all studies [611, 615]. Also, IL-10 polymorphisms are also associated with asthma [592, 618, 619, 621] and increased IL-10 expression [622]. We have previously demonstrated that children with asthma demonstrate increased IL-10 responses to MPV and that this is significantly enhanced in children with a family history of asthma, suggesting a genetic predisposition towards increased anti-viral IL-10 responses.

Table 8 (continued): List of candidate genes for immuno-genetic analysis

Rig	To be determined	Unknown to date	RSV and MPV-driven CCL5, IFNγ and IL-10	Asthma
				Bronchiolitis
				AHR

Rationale: APCs utilise endosomic TLR7 for anti-viral recognition, however RSV strain Long is able to by-pass this mechanism by directly entering the cytosol using the F (fusion) protein [107]. This suggests that cytosolic PRRs, such as RIG, are likely the pathway APCs use to recognize intracellular virus infection. RIG has been shown to be the most important anti-viral PRR for most cell types [116], specifically in the recognition of negative strand ssRNA viruses [623-626], which upon recognition leads to the expression of type1 IFNs. Recently, it has been reported that asthmatics have impaired IFNα2 responses to RSV compared to healthy individuals [297], which may be a function of impaired recognition of viral infection by RIG.

unc-93b	To be determined	Unknown to date	RSV and MPV-driven CCL5, IFNγ and IL-10	Asthma
				Bronchiolitis
				AHR

Rationale: APCs utilise endosomic TLRs to respond to many viral infection. Specifically, optimal TLR3, TLR7, TLR8 and TLR9 signalling requires the transmembrane endoplasmic protein UNC-93b [627, 628]. Mutations in UNC-93b result in immunodeficiency towards virus infection and impaired type 1 IFN and pro-inflammatory cytokine production [629, 630]. Therefore, we hypothesise that functional polymorphism in UNC-93b may associate with impaired anti-viral CCL5 and excessive IFNγ responses in asthmatic individuals.

tlr3	rs3775291	28% [631]	RSV and MPV-driven CCL5 and IFNγ	Asthma
	To be determined	> 10%		Bronchiolitis

Rationale: TLR3 can recognise double stranded RNA generate from viral replication of viruses such as RSV and MPV (refs). Although TLR3 polymorphisms have not been associated with asthma in the Japanese population [631], TLR3 SNPs may be associated with susceptibility to virus infection [632] and maintenance of lung homeostasis following RSV infection [113].
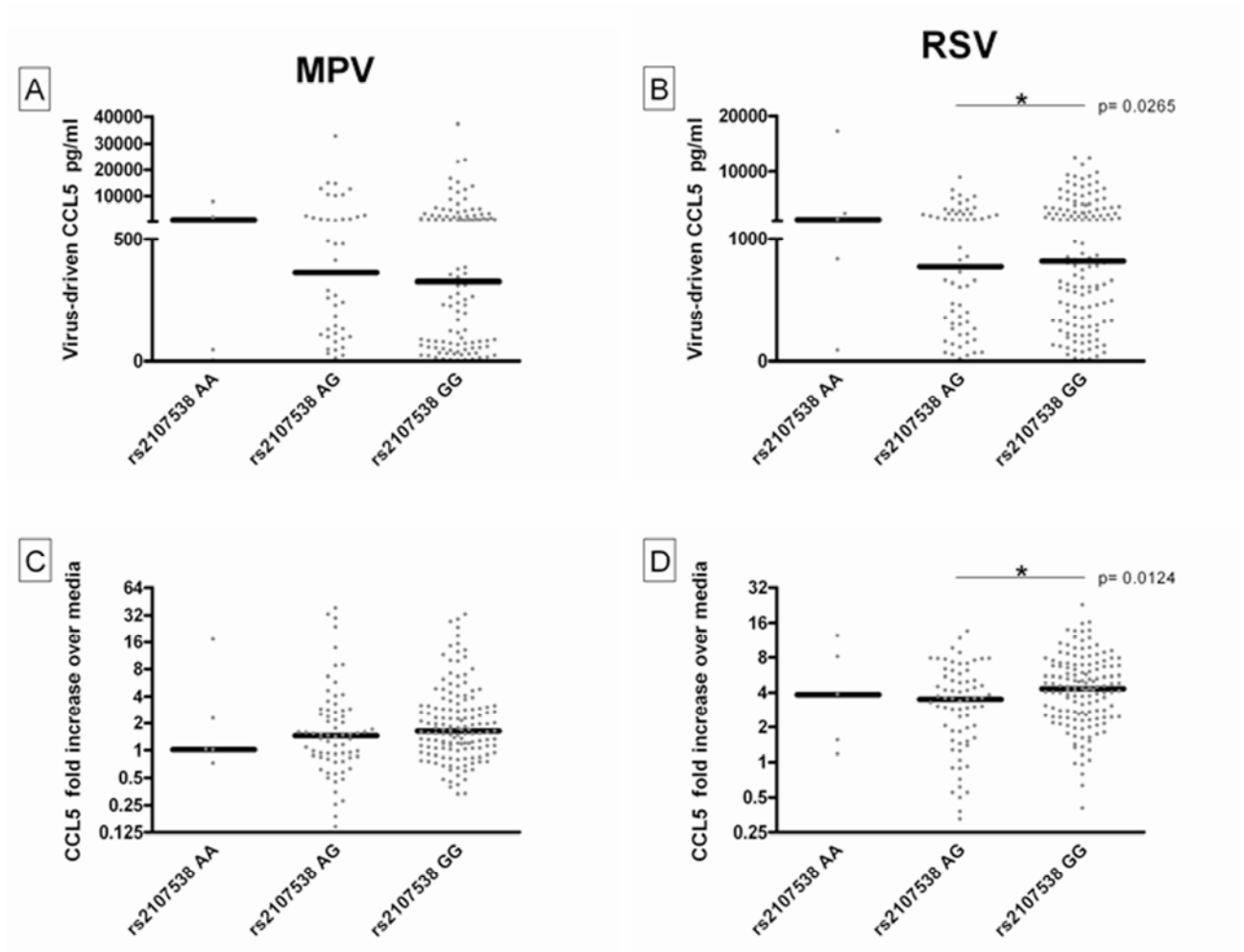
Figure 45: CCL5 promoter polymorphism, -403 A/G (rs2107538), heterozygous AG allele contributes towards decreased CCL5 production in RSV-stimulated PBMC. CCL5 was measured by ELISA in supernatants from 6 day PBMC cultures. Black bars represent median responses, each from an individual child (•).

## CONCLUDING REMARKS

The immuno-genetic collaboration projects are currently in progress. The candidate genes have been identified and genotyping is underway. The large number of intermediate phenotypes available will permit functional analysis of alleles/genotypes with lower frequencies in the general population. Subsequent to future identification of alleles/genotypes with functional effects on anti-viral immunity, in depth studies can be undertaken to address the mechanism and significance of these polymorphisms *in vitro* and *in vivo*.

DISCUSSION

Anti-Viral Immunity is Divergent

in Asthmatic and Non-Asthmatic Humans

VIRUS-SPECIFIC PATTERNS OF ANTI-VIRAL IMMUNITY IN HUMANS

PBMC models of anti-viral immunity detect frequency and intensity of virus-driven cytokine responses

Despite increasing understanding of human serological responses to respiratory viruses [405, 411, 451], there is a dearth of information in regards to the presence, nature or control of innate and T cell-dependent anti-viral cytokine responses against pneumoviruses [138] and reoviruses [408]. Therefore, we initially sought to assess the frequency and intensity of virus-driven cytokine and chemokine responses in the general population. Prototypic strains of respiratory viruses (MPV, RSV and reovirus), all ubiquitous and leading to very high levels of infection and seroconversion in the population, elicit distinct cytokine responses (**Chapter 1 and 4**). It is quite clear that despite equivalent inoculating titers of virus in our *in vitro* PBMC cultures, the strength of anti-viral cytokine production varied according to virus strain, type and current clinical status of the human population evaluated. This variation in individual cytokine responses was often distributed over four orders of

magnitude, demonstrating the amazing diversity of human immune capacity as compared to inbred animals. Nonetheless, within a given individual, their cytokine response to viral antigen was relatively consistent over time, as measured in longitudinal sampling in our adult and child populations. Moreover, the frequency of individuals responding to virus was quantifiable, by determining whether the virus-driven response was more than double (plus standard deviation) that of the basal (media) cytokine induction in our culture system. Taken together, the breadth of variation within human anti-viral responses allows us to dissect putative differences in responsiveness between different clinical, genetic and epidemiologic populations.

Virus-driven responses differ from those elicited by allergen

While allergic diseases and asthma were initially described as diseases stemming from excessive "Th2-like" gene expression, it is now recognized that the chronic inflammatory immune responses associated with persistent asthma involve a much broader range of cytokine production. Claims that allergic or asthmatic individuals consistently exhibit Th2-like bias in their immune profiles are largely based on responses towards polyclonal or allergen stimulation [433, 633], and this conclusion deserves re-examination. When we compare allergen-driven cytokine responses from PBMC [433, 633] versus those elicited by virus-stimulation (**Chapter 1** and [186, 433]), it becomes apparent that allergen elicits predominantly Th2-like biased responses in allergic individuals, whereas strongly Th1-like biased responses are mounted towards respiratory viruses in both allergic and non-allergic populations. Specifically, upon viral stimulation, there are minimal and irregular IL-5 and IL-13 responses by human PBMC and a definite prominence of pro-inflammatory IFNγ. These findings clearly establish that all individuals, regardless of clinical status, mount

typical Th1-like anti-viral immunity rather than virus enhanced Th2-like cytokine production. This should not be a surprising result in itself, as clearly Th1-like responses are required for elimination of viral pathogens during infection (and thus human survival) [187, 634, 635]. Rather it sets the stage for defining anti-viral immunity in asthmatics outside the context of the "Th2 asthma paradigm".

Strengths and limitations of assessing anti-viral immunity using an in vitro model

The study of viral infection *in vivo* in humans, as compared to animal models, is limited by ethical medical practice, especially in high-risk groups such as infants and asthmatics. Most methods of immune measurement require invasive procedures to obtain tissue and extensive medical training. Biopsies, bronchial scrapings, bronchiolar lavage and sputum induction are generally not used for research purposes in large cohorts, especially for study participants outside the safety of a critical care facility. Consequently biopsy studies, which generally are restricted to samples obtained in the first few generations of the lung, tend to exhibit limited sample size. Given the great diversity of human immune responses described above, having adequate sample sizes is essential. For this purpose, the use of peripheral blood obtained by venipuncture, is a well tolerated and safe procedure for use in children (in clinic or in the community), as well as in asthmatics and healthy individuals alike. Although the immune cell component is not directly derived from the end-organ, due to leukocyte recirculation it reflects the diversity and immune responsiveness that may be found within the lungs. The major caveat of using short-term culture of PBMC to represent global patterns of anti-viral immunity is the lack of immune contribution from the structural and biochemical micro-environment within the airway, as the lung milieu may alter the function of immune cells compared to the simplified environment *in vitro*. While we

established that IFNγ production in PBMC was mainly T cell-dependent, the precise cellular sources of cytokines in this model and *in vivo* remains to be determined. In spite of the limitations of this model, it serves as a preferred method to assess differential immune responses in humans, due to the overall technical simplicity and ability to measure various parameters of immunity in large numbers of subjects [636].

Apart from the human model, the viral tools used to assess immune responsiveness must be considered in regard to their infectivity, specificity and physiologic relevance. Here, we used infectious and inactivated whole virus preparations to stimulate PBMC *in vitro*, as opposed to specific viral proteins. The use of intact virus is a more physiologic stimulus than purified viral proteins, as the infection-dependent and independent components of anti-viral immunity can be measured. Nevertheless, the use of infected cell supernatants as a source of non-purified virus had to be validated in terms of the ability to induce detectable immune responses relative to those elicited by uninfected cell supernatant or by *in vitro* culture without added stimulation. These differences in inducible immune responses are attributed to the virus within the supernatants, albeit a component of the stimulatory capacity may be derived from secreted immune mediators produced by the host cells used to grow the virus preparations. In addition, the choice of viral serotype may have a significant impact on the strength and frequency of immune responses (**Chapter 4** and [293]). In all cases, we used prototypic strains for each viral species, so as to best discuss our results within the framework of the current literature, and to allow others to repeat and confirm our findings.

DIVERGENT PATTERNS OF ANTI-VIRAL IMMUNITY IN ASTHMATIC AND NON-ASTHMATIC INDIVIDUALS

A role for IFNγ in airway exacerbations and progression of asthma

A plethora of literature regards allergic disease as a Th2-dominated phenomenon, despite strong evidence to support a role for co-operative Th1 and Th2 cytokines in asthma pathogenesis and maintenance [92, 95-98]. Furthermore, enhancement of Th1 bias or suppression of Th2 cytokines, as a means to redirect established asthma away from Th2 immunity, has consistently failed to improve clinical symptoms of disease. This leads to the currently accepted model that asthma pathogenesis and maintenance is not simply an imbalance of Th2 versus Th1 responses [94, 636]. However, contextual defects in cytokine production, during allergen exposure, infection or healing, may have a significant impact on disease progression and exacerbation.

A main thread in the research of this thesis has been the demonstration that asthmatics exhibit heightened Th1 bias in response to viral stimulation, relative to that seen in healthy individuals. Indeed, we observed that both child and adult asthmatics produce more IFNγ and CXCL10 in response to respiratory viruses (**Chapter 2-4**), compared to non-asthmatics. Moreover, this pattern of exaggerated IFNγ was evident in children with current AHR, but no diagnosis of asthma (**Chapter 5**). As demonstrated in other studies (reviewed in [559]), excess IFNγ may have detrimental effects in both acute asthma exacerbations and progression of chronic asthma. Furthermore, the frequency of IFNγ$^+$ CD4$^+$ T cells significantly correlates with AHR in children [555], suggesting that excess IFNγ secretion may be a useful biomarker of AHR, independent of asthmatic status. It is proposed that genetic polymorphisms in the Th1 polarizing transcription factor, T-bet, may contribute to AHR [593].

Increased IFNγ in asthma has been previously described when measuring the proportion of IFNγ^{+} T cells in peripheral blood, sputum, BALF and biopsies [559, 637-642]. IFNγ-regulated CXCL10 is also increased in BALF and biopsies of asthmatics [643], further supporting a role for Th1 cytokines and chemokines in asthma pathogenesis. Moreover, increases in IFNγ^{+} T cells in sputum are related to the severity of disease [641, 644] and serum IFNγ levels are used as a predictor of oncoming deterioration of lung function in asthmatics [645]. Studies in children have demonstrated an association between polyclonal mitogen PHA-driven IFNγ from PBMC cultures and AHR; however, mitogen-driven IFNγ production was not associated with asthma [636]. For the first time, using physiologically relevant antigens, namely pathogenic and non-pathogenic respiratory viruses, we have demonstrated immuno-epidemiologic associations between excessive IFNγ production and AHR, as well as asthma severity (**Chapter 5**).

The increased propensity of T cells to secrete IFNγ during virus infection likely has multiple consequences for effector cell function and overall inflammation of the airways, which can mechanistically contribute to AHR. Murine experimental models of allergic asthma, both acute and chronic, have demonstrated that activation of Th1 cells exacerbate inflammation and/or contribute to AHR [95-97, 559, 646]. In marked contrast to most acute asthma models [647], development of AHR is independent of Th2 cytokines (IL-4 and IL-13) or Th2-dependent STAT6 signalling in chronic exposure asthma models [646, 648-650]. This indicates that AHR is induced by a Th1 mediator or downstream effector function dependent on Th1 cells. Moreover, virus-induced AHR has also been demonstrated to be independent of IL-13 in adult mice [304], suggesting a shared Th1-dependent mechanism of AHR in both chronic exposure to inhaled allergen and virus-triggered asthma. Specifically, IFNγ production is required for AHR development in cases of inhalant allergen challenge [559, 561, 651, 652], as demonstrated by anti-IFNγ treatment or in IFNγ knock-out mice. Impaired Th1 cell

signalling in mice also reduces AHR development [653], highlighting the importance of T cell-derived IFNγ. In contrast, Koh *et al.* found that increased IFNγ production by stimulated T cells associated with improved peak expiratory flow rates in asthmatics [654], albeit the results are derived using a highly manipulated culture system (T cell enrichment followed by polyclonal and cytokine-driven stimulation), which is not comparable to our antigen-stimulated primary cell cultures, nor *in vivo* models. Taken together, the literature suggests that Th1-biased responses, and specifically IFNγ, have a role in the development and maintenance of AHR and asthma. The counter-argument could be that IFNγ is merely a valid biomarker of disease in humans but has no pathogenic role. Our results clearly demonstrate an association between virus-driven IFNγ, AHR and asthma severity; nonetheless, future studies are necessary to assess a putative causal relationship.

Controversy surrounding IL-10 in airway exacerbations and progression of asthma

The role of IL-10 in asthma remains controversial, as IL-10 is a pleiotropic cytokine involved in many cellular functions: suppression of T cell activation via modulation of APC function, enhancing human $CD8^+$ T cell cytotoxicity, airway smooth muscle contractility and airway homeostasis and remodelling [655, 656].

Airway cells from asthmatics can be stimulated with mitogen to produce more IL-10 than healthy individuals [565]. RV-stimulated PBMC *in vitro* produce more IL-10 than those of non-asthmatic counterparts [330]. Moreover, Grissell *et al.* report up-regulation of IL-10 expression in induced sputum of acutely virus-infected asthmatics compared with infected non-asthmatics or non-infected controls with and without asthma [489]. Similarly, we have demonstrated increased IL-10 production in asthmatics. An original finding is that, counter-intuitively, IL-10 substantially correlates with concomitant IFNγ production in virus-

stimulated culture (**Chapter 1, 3 and 4**). Interestingly, this IL-10 production does not counter-regulate the strong Th1-like bias in pneumovirus infection (**Chapter 2**). Additionally, this heightened IL-10 production is likely to be genetically predisposed in individuals with a family history of asthma (**Chapter 3**). To address this claim, future immuno-genetic studies are ongoing as described in **Chapter 9**.

In vitro stimulated (LPS and IFNγ) monocytes produce substantially more IL-10 in children with wheezing following RSV bronchiolitis in comparison with children without wheeze [657]. Furthermore, longitudinal measurements of monocyte-derived IL-10 correlates with frequency of wheezing episodes [657]. Mice deficient in IL-10 are protected against airway hyper-responsiveness in models of allergic airway disease (Th2 bias) [490, 658, 659] and chronic LPS exposure (Th1 bias) [660]. However, IL-10-deficient allergen-sensitized mice can still develop AHR subsequent to RSV infection [661], demonstrating that virus infection may augment AHR via multiple pathways, especially within the framework of established allergic inflammation. Moreover, in murine models, leukocyte-derived IL-10 is protective against the development of airway fibrosis during chronic LPS challenge [660], despite contributing to increased AHR. Taken together, IL-10 production during viral infection likely contributes to increase AHR, but is likely a homeostatic mechanism to protect against airway remodelling. Increased virus-driven IL-10 production by immune cells in asthmatics, compared to non-asthmatic individuals, may imply that asthmatics sustain a greater degree of cellular or tissue damage during viral infection. Moreover, IL-10 induced AHR may be a physiologic mechanism to clear cellular debris within the infected airways, whereby prolonged AHR following infection may indicate inefficient tissue repair and extended IL-10 production.

Impairment of CCL5 immunity may contribute to inadequate viral clearance, airway exacerbations and progression of asthma

CCL5 acts in concert with IFNγ to promote Th1 type responses and co-activate macrophages during antigen-specific immune responses [484]. Treatment of human monocytes with IFNγ also enhances CCL5 production [662], as CCL5 expression is regulated in part by interferon response elements [558]. An important observation we have made is that despite the increased IFNγ in response to pneumovirus, children with asthma failed to up-regulate CCL5 production to the same extent as healthy children. Moreover, this pattern of weak CCL5 production in response to MPV is enhanced in children with a familial history of asthma, indicating a hereditary component for this anti-viral immune profile. As CCL5 induction is essential for viral clearance of RSV infection [493] and CTL activation [481, 482], failure of children with AHR, and especially those with asthma, to up-regulate monocyte-derived CCL5 production may contribute to increased viral replication in their lungs during infection [479, 493, 497]. In addition, absence of CCL5 signalling in macrophages and epithelial cells renders them vulnerable to apoptosis during virus infection [663]. The resultant decreased viability of macrophages would hamper the removal of pathogens and apoptotic cells following cytotoxic killing of infected cells within the lung. Therefore, we speculate that decreased immune cell production of CCL5 (as observed here in asthmatics compared to healthy individuals) may lead to inadequate debris clearance within infected tissue, leading to prolonged infection and increased inflammation. Therefore, deficient CCL5 anti-viral responses reveal a missing component of anti-viral immunity in asthmatics, which may explain in part their propensity towards increased virus-triggered asthma exacerbation.

The role of CCL5 in AHR remains controversial (reviewed in [479]), but models of chronic allergic airway disease suggest that blockade of CCL5 enhances AHR and goblet

cell number, whereas treatment with CCL5 is associated with decreased AHR [480]. In contrast, Tekkanet et al. reported that blockade of CCL5 in RSV-infected mice suppresses the development of AHR [664]. This apparent discrepancy may be explained by considering the sources of anti-CCL5; in the RSV study, the neutralizing reagent was prepared in-house from whole goat serum containing anti-CCL5 (may contain many other immunomodulatory factors), whereas the allergen challenge model used purified monoclonal anti-CCL5 and showed that administration of an isotype control antibody has no effect on AHR. Future well-designed studies of RSV infection will be needed to determine if CCL5 enhances or inhibits the development of AHR.

However, we can speculate that increased levels of leukocyte-derived CCL5 early in the course of infection may be protective against the development of severe airway symptoms. In RSV infection, murine models have shown that administering Met-RANTES (competitive inhibitor of CCR5 and CCR1) decreases T cells infiltration into the lungs and results in significant increases in RSV titer [493], which is generally correlated with symptom severity [665]. As viral titers increase, infected epithelial cells engage anti-viral mechanisms upon dsRNA recognition (an indicator of productive viral infection) resulting in CCL5 production [111, 136]. If impaired CCL5 production by immune cells results in increased viral load and sustained infection, the dominant epithelial CCL5 response would mask the underlying immune deficiency as tissue injury progressed. More severe tissue damage, despite late attempts of epithelial cells to suppress excessive viral replication, may contribute towards airway remodelling. In support of this hypothesis, virus-induced asthma exacerbations are associated with increased CCL5 in airway secretions [150, 501]. Moreover, clinical markers indicate overly expressed CCL5 in lung tissue and the BAL fluid of asthmatic individuals compared to healthy individuals [499, 500]. This ongoing excessive CCL5 response may actually stem from a markedly impaired secretion of CCL5 by immune cells

from asthmatics during early anti-viral responses. This differential ability of asthmatic leukocytes to respond to virus may well reflect polymorphisms in the CCL5 gene, as Nickel *et al.* have demonstrated that certain SNPs have a substantial impact on leukocyte-derived CCL5 production, but no effect in epithelial cells [596]. Future murine models of CCL5 deficiency in specific cell types would best address the role of immune cells versus epithelial cell derived CCL5 in the development or exacerbation of AHR and airway remodelling.

Anti-viral immunity in the context of established allergic inflammation

When considering our results in the lung environment undergoing allergic inflammation, some additional effector outcomes may play a role in exacerbating disease. Excess IFNγ during eosinophilic inflammation can activate indoleamine 2,3-dioxygenase (IDO). Eosinophil-derived IDO catalyzes the conversion of tryptophan to kynurenines, which can decrease Th1 cell function, leading to impaired proliferation and apoptosis [666]. Activation induced cell death of the IFNγ^{High} Th1 effector compartment may contribute to increased Th2 bias in the T cell repertoire [667]. This collapse of effector (and potentially memory) anti-viral T cell population may further contribute to infection severity and failure to boost T cell memory upon infection [214]. In addition, anti-viral CD8+ T cells can also be coaxed into producing Th2 cytokines, if the inflammatory milieu is Th2 biased [668]. Ineffective anti-viral CTL activation in allergic disease may explain why RSV infection is prolonged in cases of established allergic airway inflammation in mice [634]. Treatment of these mice with exogenous IFNγ decreased allergic inflammation and improved viral clearance, however increased AHR [634], further supporting the idea of an anti-viral and exacerbating role for IFNγ in airway infections.

Interestingly, allergic individuals have been reported to be hypo-responsive to CXCL10 stimulation with allergen stimulation [441]. This impairment would down-regulate the normal Th1 positive feedback loop found in healthy individuals, resulting in decreased IFNγ production. However, here we observed increased anti-viral CXCL10 responses (and IFNγ) in asthmatics, regardless of their atopic status (**Chapter 5**), suggesting that this immuno-regulatory mechanism may differ or have less of an impact on anti-viral immunity compared with allergen-specific responses.

A novel perspective on asthmatic anti-viral immunity

In RSV infection, IFNγ is essential for viral clearance, yet it induces AHR [187]. Therefore, increased IFNγ by asthmatics may well be unavoidable, as it is required to compensate for the impaired innate anti-viral response to pneumovirus infection. Moreover, increased IL-10 production in asthmatics likely contributes to AHR during viral infection [657], yet may be protective against airway remodelling [660]. It would seem that the increased IFNγ/IL-10 in virus-stimulated PBMC from asthmatics (compared to healthy individuals) might reflect an over-compensation for the intrinsic inability to induce type 1 IFNs [297] and CCL5 (**Chapter 3**) or other innate anti-viral pathways (**Figure 46**). Moreover, impaired CCR5 signalling diminishes the capacity of IFNγ to induce downstream effector functions in the lung [669]. These exaggerated or excessive IFNγ and juxtaposed IL-10 responses in asthmatics are probably a physiologic attempt to eradicate infection and restore homeostasis (**Figure 47**). Here, we propose that the altered pattern of anti-viral immunity in asthmatics leads to i) decreased phagocytosis by APC and macrophages in the airway due to impaired CCL5 response, contributing to airway obstruction, ii) a decreased capacity to activate cytotoxic activity in CD8+ T cells (despite strong IFNγ responses) due to impaired CCL5

response, contributing to increased viral replication iii) increased IFNγ/IL-10 responses leading to AHR and increased airway inflammation and repair processes. The overall clinical outcome of this altered pattern of anti-viral immunity likely contributes to airway obstruction, wheezing, AHR and a more prolonged and severe infection.

Similarly, children displaying AHR may display a component of this model, namely exaggerated IFNγ/IL-10 responses, but no impairment in CCL5 production. In this case, increased anti-viral IFNγ/IL-10 may contribute to AHR and wheezing in these children, although the clinical course of infection may be somewhat better than in asthmatics.

The imbalance in leukocyte-derived cytokine production in asthmatics, compared to healthy individuals, may be attributed to both environmental and genetic influences. Therefore, we sought to identify modifiers of anti-viral immunity in children by stratification of clinical populations using epidemiologic markers of environmental exposures and mapping of genetic polymorphisms.
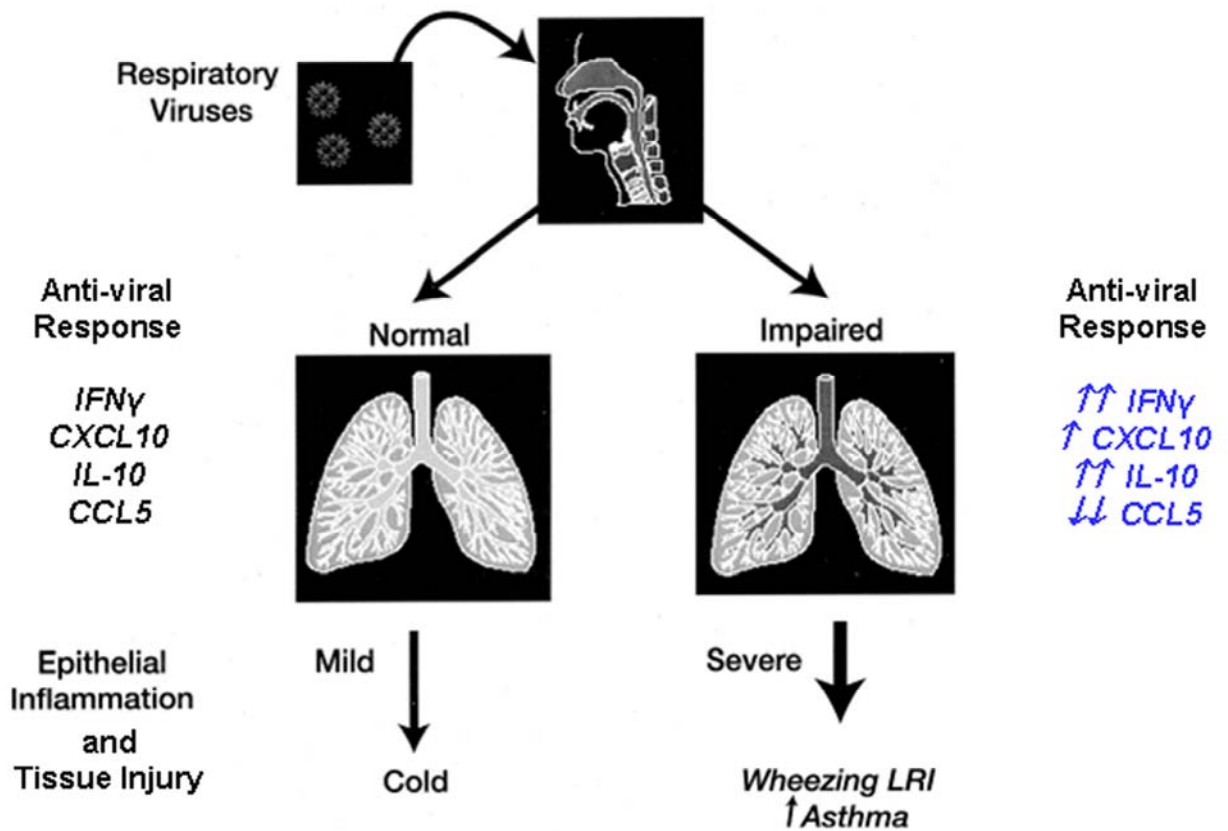
Figure 46: Deficient and exaggerated anti-viral cytokine responses in asthma. Modified from model proposed by Gern [349], to reflect anti-viral immune profiles in humans in response to pneumoviruses. Adapted with permission from Lippincott, Williams and Wilkins.
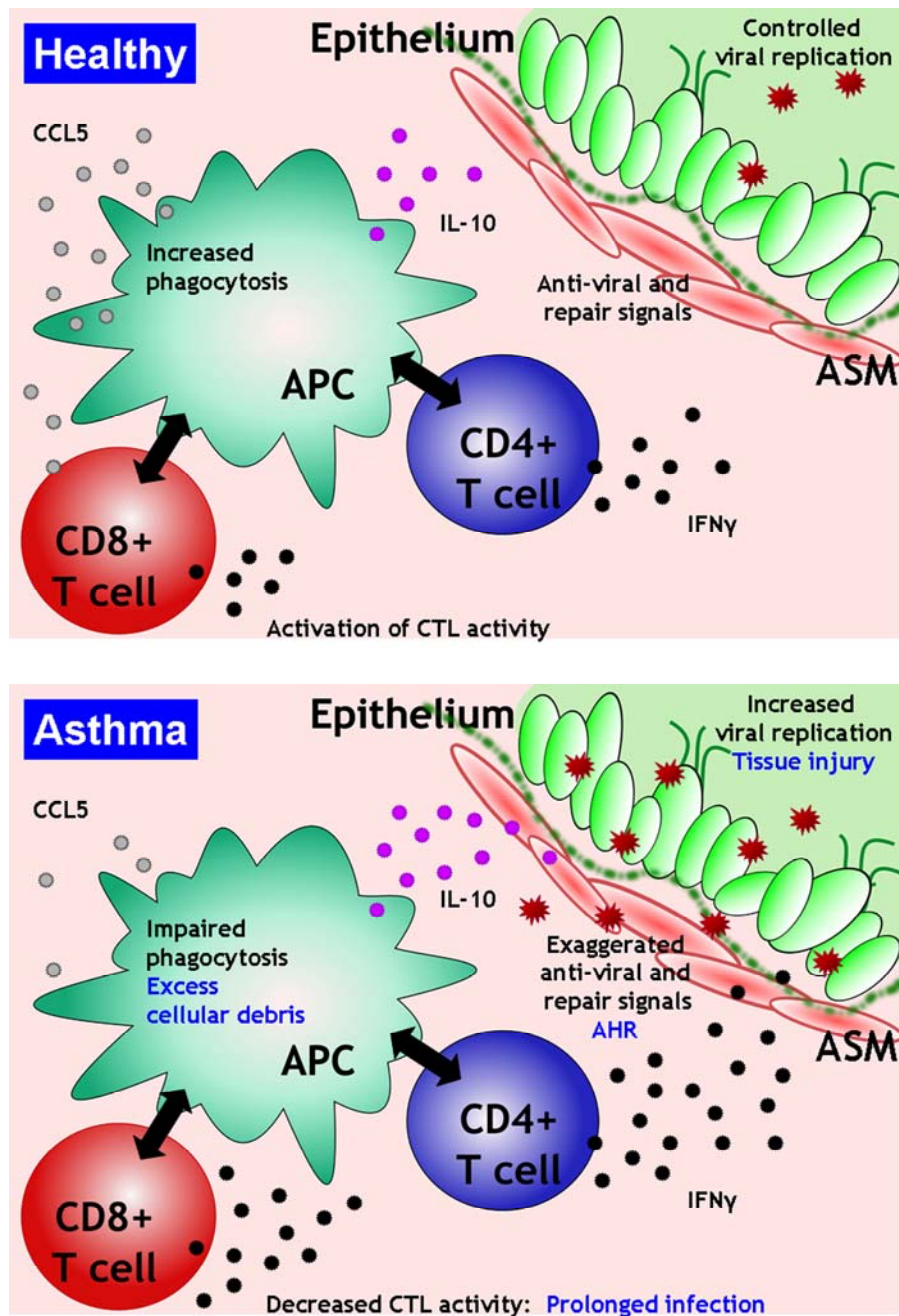
Figure 47: Model of anti-viral immunity in asthmatics and healthy individuals. Artwork by R. Douville.

Strengths and limitations of immuno-clinical associations

Immuno-clinical associations are a valuable research tool, providing accurate and defined clinical diagnosis of individuals. Here, the cohorts were assessed by allergists (the gold standard we used for diagnosis), using standardized criteria for diagnosis of asthma and atopy. Moreover, immunologic readouts must be standardized and reproducible within an individual (which was assessed by comparable readouts in the same individual over time). Finally, stringent measures were used to prevent selection bias in the case-control cohort design; therefore, random sampling of subjects within this cohort is likely to represent the entire population. Taken together, this platform for immuno-clinical associations is an effective method to identify immune parameters within specific clinical populations [636].

One clear advantage of the SAGE study is the large number of children with both clinical and immunologic assessment (n = 345). Subdivision of the study population into target clinical phenotypes produced relatively robust subgroup sizes ranging from 30-110 individuals. This is in contrast to the majority of human studies examining immuno-clinical phenotypes with subgroups between 10-20 individuals [433, 434, 587, 636].

A limitation of this study is the potential for undiagnosed females with an underlying asthmatic phenotype (and thus misclassification of immune responses), as there is a predominance of asthma in boys in this age group [670]. In comparison, young girls are more likely to present with sub-clinical asthma and be diagnosed as AHR or non-asthmatics [671-673], despite an immune profile that may be characteristic of asthmatics. Subsequent longitudinal studies of this cohort are better able to address whether a gender-switch in asthma predominance in puberty leads to more precise measure of asthma immuno-phenotype in this cohort.

INFLUENCE OF ENVIRONMENTAL FACTORS IN THE MAINTENANCE OF ANTI-VIRAL IMMUNITY

Severity of respiratory infections

There is much evidence in the literature to suggest that viral infections in early life contribute to inception of asthma and lung injury [157, 192, 255, 270, 276, 281]. Proposed mechanisms include ineffective repair and airway remodelling [156, 157, 296], alteration of innate and adaptive immunity [263, 297] and enhancement of allergic disease following viral infection [195], among others. Therefore, we sought to determine if children with severe bronchiolitis during infancy or LRTI in childhood demonstrate altered virus-driven cytokine responses towards potentially asthmatogenic pneumoviruses. Surprisingly, asthma, more so than prior bronchiolitis or LRTI, predicted altered anti-viral immunity in children (**Chapter 6**). These findings suggest that mechanisms other than anti-viral immune capacity may also be responsible for the severity of bronchiolitis and LRTI. Some risk factors for bronchiolitis severity include gender [570], exposure to ETS [291, 571], premature birth [572], maternal atopy [99] and genetic background [573, 574].

Severity of infection in RSV bronchiolitis is also associated with decreased levels of IFNγ mRNA in circulating PBMC during acute infection [468]. We observed that boys with prior bronchiolitis have weaker virus-driven IFNγ than girls, which is consistent with boys having more severe courses of RSV infection in infancy than girls [570]. The mechanisms for gender disparity in anti-viral responses and infection severity are yet to be identified.

Environmental tobacco exposure impairs anti-viral immunity in children

Exposure to ETS in children is a substantial risk factor for respiratory and ear infections, however the immunologic mechanisms governing infection susceptibility are currently unknown. Here we demonstrate that IFNγ/IL-10 anti-viral responses are suppressed in children who are exposed to ETS; especially when they have current asthma or a familial history of asthma (**Chapter 7**). The combination of asthmatic predisposition and ETS exposure leads to an overall diminished ability to mount anti-viral cytokine responses, increasing the risk for severe or prolonged viral infection. Chronic ETS exposure itself was not sufficient to alter anti-viral immune responses, but required a specific heritable predisposition to exert effect. This supports the hypothesis that clinical and immunologic endpoints are likely to result from gene-environment interactions [58, 64]. ETS exposure in children is a detriment towards establishing effective anti-viral pathways by diminishing immune cell-derived IFNγ necessary for viral clearance. Moreover, previous reports have demonstrated that ETS extract impairs pro-inflammatory cytokine induction in epithelial cells *in vitro* [578].

We propose that ETS exerts its effect via epigenetic modulation of the histone acetylation:deactylation balance. This balance is already skewed in asthmatic individuals, as described by Su *et al.* (unpublished observations) and [585]. Although there is consensus in the literature that ETS extracts decrease HDAC activity in *in vitro* culture systems, there are conflicting results in regards to induction of proinflammatory cytokine production in cell lines, with epithelial cells demonstrating weaker responses and macrophages showing increased IL-8 production [578, 583]. Moreover, asthmatics tend to demonstrate increased HAT activity and decreased HDAC activity, albeit only in severe asthma [585-587]. Taken together, we propose that the significant down-regulation of exaggerated IFNγ/IL-10 responses in

child asthmatics exposed to ETS is associated with enhanced histone acetylation, due to intrinsically increased HAT activity in conjunction with weaker and ETS suppressed HDAC activity. Increased HAT (or decreased HDAC) activity is associated with increased gene transcription, suggesting that negative regulators of pro-inflammatory cytokine production may be enhanced following ETS exposure.

Overall, our observations reveal that ETS exposure in children diminishes their ability to mount anti-viral IFNγ/IL-10 responses towards pneumoviruses. This was particularly drastic in asthmatics and children with a familial history of asthma, as they display already impaired anti-viral CCL5 responses. This suggests that smoking cessation is especially important in families of asthmatic individuals [674].

Strengths and limitations of immuno-epidemiologic associations

Epidemiologic markers can be useful tools to identify environmental exposures or to track medical histories in individuals. However, these markers, such as ETS, are limited by measurement biases, such as parental / participant recall or willingness to divulge personal information, inability to quantify dose of exposure and unintentional exposure. Similarly, measurement of bronchiolitis in children may be biased by physician misdiagnosis/over-diagnosis/no diagnosis, incorrect classification of medical billing information (ICD-9 code) and lack of database medical records in certain communities. Taken together, one must carefully consider the reliability of epidemiologic markers. Here, we are confident that the presence of chronic ETS exposure in children is reliable, as it is a composite measure of ETS exposure at birth and at 7-9 years of age, and many studies demonstrate that frequency of parental smoking is relatively stable, despite knowledge of heath risks [571, 675, 676]. In contrast, our marker for bronchiolitis (diagnosis of ICD-9 466 before two years of age) is a less

specific and reliable definition [677], as it does not define the microbial cause of the bronchiolitis, uses an arbitrary temporal cut-off for bronchiolitis versus LRTI and inapplicable in first-nations subjects due to lack of database records.

Epidemiologic markers may be just surrogates for other co-exposures or morbidities. In addition, they are always in association with immunologic measures, and cannot address whether there is a causal relationship in retrospective case-control studies (due to temporality error), but are useful to generate hypotheses and inference plausible causal relationships. For example, ETS exposure is associated with suppressed anti-viral responses in children; however, our data cannot address whether ETS caused this altered immune pattern, despite plausible causal mechanisms already reported in the literature [576, 577, 579, 582].

INFLUENCE OF GENETICS IN THE DEVELOPMENT OF ANTI-VIRAL IMMUNITY

The complex nature of arriving at a measurable anti-viral immune response consists of multiple levels of regulation via PRRs, signalling pathways, transcription factors and cytokine production. Polymorphisms, singly or in combination, in any of these effector proteins can potentially have a great impact on the ensuing response.

TLR4 recognition and TLR4 polymorphisms have no effect on anti-RSV immunity

The role of TLR4 signaling in respiratory virus infection is an area of much recent focus. Murine studies stated that TLR4 plays a key role in shaping the intensity of immune responsiveness to RSV infection [103, 122], however other studies have revealed that the results by Kurt-Jones *et al*. were flawed, as they used TLR4-deficient mice that have other immune deficiencies as well [123, 124, 588]. Still, *in vitro* analyses demonstrated that purified RSV-F protein mediated activation of human monocytes [103]; and that this response could be blocked using anti-CD14 (neutralizing the TLR4 co-receptor). In contrast, our data obtained with whole infectious RSV (not baculovirus-expressed viral protein) demonstrates that while blocking TLR4 function abrogates the activity of LPS, RSV-mediated activation of cytokine production does not change in frequency or intensity (**Chapter 2**). These data argue that physiologic exposure by infectious RSV drives immune responses in human PBMC that are TLR4 independent.

As RSV-TLR4 interactions were hypothesized to play a role in directing anti-viral immunity, genetic studies attempted to address whether TLR4 polymorphisms associated with severity of RSV disease. Tal *et al*. demonstrated an association between the

Asp299Gly and Thr399Ile polymorphism and severe RSV infection [589]; contrarily, an opposite associative trend between severe RSV bronchiolitis and the TLR4 Asp299Gly polymorphism has been demonstrated [590]. A closer inspection of the experimental designs reveals that in both studies, the adult controls were not screened for prior history of bronchiolitis. Varying frequencies of severe bronchiolitis in population samples from different ethnic backgrounds and locale may account for the opposing results. Thus, due to insufficient clinical description of subjects and lack of agreement in identifying the susceptibility genotype, a putative association between severity of RSV infection and TLR4 polymorphism remains inconclusive.

TLR4 polymorphisms are suspected of altering immune responsiveness by altering the interaction with co-receptors, such as CD14 [678]. Specifically, the TLR4 Asp299Gly polymorphism has been associated with LPS hypo-responsiveness [591], although this finding is rarely replicated [679-681]. Therefore, Mandelburg's group has attempted to assess if this TLR4 polymorphism associates with immune responsiveness in children with RSV infection [682], however poor study design (use of mitogens, measurement of proliferation and small sample size) undermines the conclusions and overall significance of their observations. Our results demonstrate that the Asp299Gly polymorphism does not affect RSV-specific immunity in PBMC from children (**Chapter 8**). Furthermore, stratification of these immune responses based on current asthma or prior history of bronchiolitis in infancy revealed no association with this TLR4 SNP. In addition, LPS responsiveness is not altered by the Asp299Gly polymorphism in these same children (Lissitsyn *et al*., unpublished observations). Considering the statistical power for detection (n = 210), RSV and LPS responsiveness in PBMC is clearly unaffected by the Asp299Gly polymorphism.

While TLR4 has no apparent direct role in RSV immunity, the ability of RSV infection to up-regulate TLR4 to the surface of epithelial cells and monocytes and sensitize

them to endotoxin may contribute to increased airway inflammation [125, 126]. In this context, TLR4 polymorphism combined with differential LPS exposure at time of infection may contribute to the variable degree of pro-inflammatory cytokine production between individuals. This indirect pathway may contribute to symptom severity during RSV infection. Moreover, this mechanism may account for the controversial association of TLR4 SNPs and asthma severity [59], by increasing the risk of LPS-induced inflammation during RSV infection in asthmatics carrying susceptible haplotypes. Our results demonstrating that asthmatic individuals exhibit impaired CCL5 responses promote this hypothesis, as it has been demonstrated that CCL5 production suppresses TLR4 mediated cytokine production by monocytes [683]. The lack of leukocyte-derived CCL5 in asthmatics may fail to down-regulate RSV-induced TLR4 expression, thus facilitating LPS induced inflammation.

Importance of TLR3 and RIG polymorphisms in regulating anti-viral immunity

Cytosolic PRRs, such as RIG, are likely the recognition pathway used to mount robust IFNα against RSV, MPV and reovirus [116, 623-626]. Recently, it has been reported that asthmatics have impaired IFNα2 responses to RSV compared to healthy individuals [297], which could be a function of impaired recognition of viral infection by RIG. Therefore, it will be interesting to determine whether RIG polymorphisms, some of which likely confer alterations in protein conformation (Turvey, unpublished observations), are responsible for weak anti-viral type 1 IFN and CCL5 production in asthmatics (**Chapter 3** and [297]). Furthermore, if heightened IFNγ production is a compensatory mechanism for impaired innate response, RIG SNPs may associate with the degree of IFNγ induction in infected PBMC cultures. These findings, be they negative or positive associations, would be a novel contribution to the literature as no RIG SNPs have been published to date.

In addition, TLR3 polymorphisms have been characterized, and may also contribute towards differences in anti-viral immunity [632]. However, recent data suggests that RIG and TLR3 polymorphisms are best evaluated together as haplotypes because mechanistically RIG mediates TLR3 responsiveness in models of RSV infection [109].

Importance of cytokine polymorphisms in regulating anti-viral immunity

It would seem logical that cytokine polymorphisms directly modulate anti-viral cytokine production; however, their effects are actually downstream of initial modulators, such as PRRs, signalling molecules and transcription factors. Any functional changes in these initial steps towards cytokine production may synergistically amplify the end outcome of cytokine production, suggesting a combination of polymorphisms may modulate the incoming expression signal for cytokine induction. Despite this apparent logic, cytokine polymorphisms have been repeatedly associated with clinical disease [290, 573, 592, 593, 612, 620]. Therefore, we hypothesised that cytokine SNPs play a role in modulating the ability to mount anti-viral immune responses, and as such, may associate with intermediate phenotypes of immunity (**Chapter 9**).

CCL5 as an example of immuno-genetic association technique validation

The observation that familial asthma confers increased risk towards weak CCL5 virus-driven responses (**Chapter 3**) led us to speculate that CCL5 haplotypes promoting weak CCL5 production by immune cells may be more frequently present in asthmatic individuals. A functional CCL5 SNP (In1.1C/T; 15-20% of the population) has been shown to down-regulate CCL5 transcription upon stimulation of T cells and monocytes [502]. Our

preliminary data demonstrate the -403G/A (rs2107538) heterozygotes produce less anti-viral CCL5 in response to RSV stimulation than the -403G homozygotes (**Chapter 9**). Therefore, future studies will need to examine the relationship of the CCL5 intermediate phenotypes and CCL5 haplotypes of asthmatic and non-asthmatic individuals, as both promoter and intronic polymorphisms may affect CCL5 responses. Moreover, these haplotypes are best studied in populations stratified by clinical status and environmental exposures known to modify HDAC and HAT levels (such as asthma or ETS [578, 585, 587]), as it has been demonstrated that CCL5 responsiveness can be epigenetically regulated in T cells [684].

Strengths and limitations of immuno-genetic associations

Most studies have sought to compare genetic polymorphism with clinical outcomes, while completely overlooking the biologic mechanisms intertwining genes and disease. An important challenge for the community has been the difficulty in reproducing the results of such studies in subsequent cohorts. In contrast, we have attempted to determine first whether genetic variation affects the intermediate phenotype of anti-viral immunity, preceding stratification by clinical status. This three-pronged approach may allow more sensitive detection of the subtle differences that polymorphisms may have in shaping immunity towards respiratory viruses (and potentially other antigen-driven responses as well). These may not individually reach statistical significance when associated with clinical outcome because of the extensive biological pathways between genetic polymorphisms and clinical phenotype. However, identifying haplotypes that lead to large immune deviation in cytokine responses increases the likelihood of association with parameters of disease (such as AHR) and clinical status (such as asthma).

To increase the likelihood that our genotyped polymorphisms will associate with intermediate phenotypes and clinical disease, the selection criteria we used prioritized SNPs by i) effect on expression or protein function, ii) previously identified disease-associated SNPs in the literature, and iii) HapMap [685] SNPs which are in linkage disequilibrium with SNPs in the previous two groups that can serve as a haplotype marker. Moreover, SNPs with population frequencies under 5% were excluded from our analysis, due to lack of statistical power. Despite best efforts to strategically pick candidate SNPs, our results are limited by total number of subjects assayed for both intermediate phenotype and genotype (especially when stratified), cell-type specific effects of some SNPs [596] and unaccounted epigenetic modification of the intermediate phenotype [578, 684], leading to potential for misclassification of haplotype-intermediate phenotype pairs.

GENERAL CONCLUSIONS AND SIGNIFICANCE

The research of this thesis has sought to address the hypothesis that using cytokine production as a measure of anti-viral immunity, divergent patterns of virus-driven responses would be identified in asthmatic and non-asthmatic humans. As expected, both asthmatics and non-asthmatics respond to respiratory viruses; however, there are several biases revealed in the immuno-regulatory cytokine responses in asthmatics compared to healthy counterparts. Having identified these differences, we sought to address clinical parameters, epidemiologic markers and genetic polymorphisms that contribute towards shaping anti-viral immunity in humans. Most importantly, severity of asthma, AHR and corticosteroid use predicts increased IFNγ/IL-10 and concomitantly weak CCL5 anti-viral responses in children, suggesting that immune cell-mediated pathology may contribute to difficulties in airway function following viral respiratory infection. In contrast, children exposed to ETS

demonstrate suppressed IFNγ anti-viral responses, which in concert with decreased CCL5 in asthmatics, may increase the risk of severe or prolonged infection in these children. Finally, we have established a model to identify genetic polymorphisms that associate with aberrant anti-viral response, thus providing a means to use genetic information to predict and identify children at risk of severe virus-induced pathology during and following viral respiratory infection.

KNOWLEDGE TRANSLATION

This research is a significant contribution towards reassessing the tenet of asthma pathogenesis as a Th2-mediated disease. Here, we explored anti-viral immunity, characterized by type-1 dominated immune responses, in asthmatic and non-asthmatic humans. Our results clearly demonstrate that all individuals respond to respiratory viruses with strong Th1-like bias, with little to no detectable Th2-like response (**Chapter 1**), responses quite unlike those of allergen-specific immunity. This highlights that different triggers of asthma exacerbation evoke divergent patterns of immune responses in humans. This finding has substantial impact for physicians deciding the contextually appropriate treatment for asthma symptoms in their patients.

Now that we have identified divergent patterns of anti-viral immunity in asthmatics, which may likely predispose this clinical group towards increased exacerbation upon infection with respiratory viruses, this could have implications for experimentation relating to mechanisms of disease, clinical practice and development of appropriate therapeutics. Specifically, the results support that corticosteroid treatment for asthma may be ineffectual in the context of respiratory virus infection [352, 353, 365], as children treated with corticosteroids still exhibit exaggerated IFNγ and IL-10 responses towards MPV and RSV (Chapter 5). Our

results, as well as that of others [25, 321], indicate that improved innate immunity (IFNα/β and CCL5) and reduction (but not abrogation) of inappropriate or excessive IFNγ/IL-10 responses towards respiratory viruses may be a successful method to treat infection without compromising other parameters of immunity. Alternatively, impairing down-stream effector functions stemming from IFNγ/IL-10-induced AHR, may alleviate acute airway symptoms, while not modulating the underlying leukocyte-mediated immunity (which will eventually clear the virus infection in asthmatics and healthy individuals).

FUTURE DIRECTIONS

The research strategy developed for this thesis was focused on the integration of clinical, epidemiologic and genetic data with the measurement of immune responsiveness. Although this method of data interpretation is extremely valuable for hypothesis generation, it superficially addressed the cellular mechanisms by which these immune responses were regulated and maintained. Further studies are required to establish a causal mechanism for virus-induced asthma phenotypes and more extensive investigation into environmental co-factors and genetic susceptibility in the establishment of airway dysfunction and remodelling.

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