University of Alberta

Respiratory Syncytial Virus infection biases the immune response in favor of Th2: the role of Indoleamine 2, 3-dioxygenase

By

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Dedication

This work is dedicated to my wife Mitra Rafati, whose infinite support and tireless sacrifice has made it possible; and my precious daughter, Elina, for her patience and understanding during those long years that I was focused to finish my Ph.D.

I thank my parents, father Ayoub Ajamian and mother Fatemeh Moazzen, who believe in the pursuit of academic excellence, for their direction and encouragement.

Abstract

Infants that develop severe bronchiolitis due to Respiratory Syncytial Virus (RSV) are at increased risk of developing asthma later in life. To begin my studies *in vitro*, purified RSV stock was needed. My first study established the critical steps in RSV purification to determine a procedure that ensures the removal of potential contaminating pro-inflammatory mediators in viral preparations. Using polyethylene glycol and ultracentrifugation through various sucrose gradient concentrations, we collected samples at all steps of purification to determine the RSV titer, total protein (µg/mL) and pro-inflammatory cytokines (ELISA). We analyzed the efficacy of each step and determined that, regardless of optimal purification methods employed, CCL5, a bioactive chemokine in allergic inflammation, persisted in virus preparations and co-purified with RSV. This conclusion is important for research on RSV or allergic diseases.

In the second part of the study, the basis of the association between RSV and the development of allergic inflammation was investigated**.** The tryptophan catabolizing enzyme indoleamine 2, 3-dioxygenase (IDO) induces apoptosis of Th1, but not Th2 cells, which may contribute to immune responses associated with allergy and asthma. I hypothesized that RSV induces IDO in human dendritic cells, which results in a Th2-biased immune profile. Human peripheral blood monocytes from healthy adult donors were isolated, differentiated to dendritic cells (moDC), *in vitro,* and infected with RSV. RSV induced IDO activity and this effect was inhibited by Palivizumab, UV-inactivation and Ribavarin. Inhibition of endosomal TLR function with chloroquine did not block

IDO activity. The signal transduction cascade for RSV-induced IDO activity was initiated by intracellular pattern recognition receptors (i.e., RIG-I related) via NF-κB and p38 MAPK pathways. In a transwell system, co-culture of RSV infected moDC with activated T-cells suppressed t-bet (a Th1-associated factor) but not GATA3 (Th2 regulator) expression. Inhibition of IDO activity with the competitive inhibitor, 1-methyl tryptophan, blocked this effect on t-bet expression. In conclusion, this study showed that RSV induced the expression and bioactivity of IDO in moDC, in a virus replication-dependant fashion. My study suggests that RSV has the capacity to play a role in increasing Th2-type response via IDO.

Preface

This thesis has been written in the “Paper Format” of University of Alberta and references were quoted following the style of The Journal of Immunology.

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List of Symbols, Nomenclature, or Abbreviations:

AA: Amino Acid

CCL5: Chemokine (C-C motif) ligand 5

CTL: Cytotoxic T lymphocyte

DMEM: Dulbecco's Modified Eagle Medium

FI-RSV: Formalin-inactivated Respiratory Syncytial Virus

GAG: Glycosaminoglycan

GFP: Green Fluorescence Protein

ICAM-1: Inter-Cellular Adhesion Molecule 1

ICD-9: International Classification of Diseases, Ninth Revision

IDO: Indoleamine 2,3-Dioxygenase

KYN: Kynurenines

MAB: Monoclonal antibody

moDC: Monocyte Derived Dendritic Cells

MOI: Multiplicity of Infection

NK cells: Natural Killer cells

PEG: Poly Ethylene Glycol

PFU: Plaque Forming Unit

PIC: Polyinosine-polycytidylic acid

PRRs: Pattern recognition receptors

RANTES: Regulated upon Activation, Normal T-cell Expressed, and Secreted

RIG-I: Retinoic Acid-inducible Gene-I

RPMI: Roswell Park Memorial Institute medium

RSV: Respiratory Syncytial Virus

TLR: Toll-like receptor

Chapter 1- Introduction

Asthma and its significance

The term asthma originated from a Greek word meaning “panting”. Asthma is a common chronic airways inflammatory disease with episodic symptoms including wheezing, coughing, shortness of breath and chest tightness, mostly at night. It is characterized by recurring episodes of symptoms with variable intensity. Typically the bronchospasm and airflow obstruction is reversible either spontaneously or following appropriate intervention (1).

Based on epidemiological evidences, asthma is a serious public health problem with over 100 million patients world-wide (2). As of 2010, around 300 million people worldwide have asthma and the prevalence is growing (3). Although the geographical distribution of childhood asthma is extremely uneven, the prevalence of asthma seems to be highest among Western societies (4). Indeed, asthma is the most common chronic disease of childhood in industrialized countries (5, 6). In Canada, it has been reported that the prevalence among children aged less than 14 years increased from 2.5% in 1978 to 11.2 % in 1995; the current prevalence remains high (7). Canada has one of the highest prevalence rates of asthma in the world. Regrettably, there is close to 40% risk for developing asthma in a child born in Canada (8). As such, asthma represents a major economic and social burden in terms of healthcare expenses (9). The total cost of asthma in Canada for all ages was estimated in 1990 to be more than $500 million (8). Although mortality rate from asthma has gradually declined in Canada since 1990, the disease still may kill about 10 Canadians per week (10). Unlike most other chronic diseases, asthma tends to start at a younger age. As a result, the life time risk of developing asthma is “front loaded” leading to more years of affected life compared to other conditions (e.g. diabetes or heart diseases). This makes asthma one of the most critical illnesses in terms of potential detriment to quality of life.

Allergic inflammation and the role of Th1/Th2 cell balance:

Allergic asthma is characterized by airway infiltration by inflammatory cells including eosinophils, mast cells and basophils. The activation of inflammatory cells and their recruitment to bronchial tissues is mediated in part through cytokines produced by T helper cells. T helper cells are a subclass of T lymphocytes characterized by expression of CD4 ($CD4^+$, $CD8^-$) and are engaged in the regulation and modulation of adaptive immune responses. Based on their cytokine production, T cells have been classically subdivided into Th1 and Th2 subclasses (11, 12). Th1 cells are a key cell phenotype in fighting viral and bacterial infections. They also are prevalent in auto-immune disease and in association with tissue necrosis. Th1 cells produce IFN-γ and IL-2 and help in the generation of cytotoxic T cells. Th2 cells, on the other hand are associated with the spurious allergic-type reaction and immune reaction to parasitic helminth infections. They produce IL-4, IL-5 and IL-13 among other cytokines. Cytokines produced by Th2 cells are known to be involved in switching the isotype of immunoglobulin produced by B cells from IgG to Ig-E subclasses. IL-5 is also known to be a terminal differentiator of eosinophils in the bone marrow and an activator of mature eosinophils. Together with IL-13 and granulocyte/monocyte-colony stimulating factor (GM-CSF), IL-5 influences the process of eosinophil development in the bone marrow and together with the chemokine, eotaxin, promotes their mobilization from bone marrow to peripheral blood and eventual recruitment to sites of inflammation (13). Both eosinophils and basophils, two important effecter cells in allergy, differentiate from a common CD34+ myelocytic progenitor. Expression of IL-5 receptor in the progenitor is a sign of its commitment to become an eosinophil (14). Another Th-2 cytokine, IL4, also plays chemotactic role on eosinophils.

It should be noted that the distinction between these two subtypes of Th lymphocytes was originally described in the mouse (12, 15). However, there is compelling evidence available for a role of these two groups of cytokines in pathogenesis of many diseases including allergic asthma in humans. While the Th1-Th2 paradigm may seem "simplistic" within the grand scheme of immunological regulation of asthma, there is evidence of a bias towards a Th2-type response in the immune profile of asthmatic patients. The mechanisms regulating this imbalance remain poorly understood.

Gene and environment interactions: The etiology of asthma is believed to be multi-factorial with a combination of genetic predisposition and environmental factors. However, the interactions between these two major factors are complex and subject of ongoing intense studies. In a review by Ober C. and Hoffjan S. in 2006, almost 500 papers on gene association with asthma were reviewed. The authors acknowledged that at least 25 genes were associated with an asthmatic or atopic phenotype in various populations (16). Many of the genes described are related to the immune system and various inflammatory responses. No standalone, independent gene with direct link to asthma was identified.

The Hygiene Hypothesis and allergic disease

The incidence and prevalence of asthma in developed countries and Western societies has increased considerably in the last few decades. This trend has been similar in other allergic diseases including hay fever (17) and childhood eczema (18). It was so noticeable that hay fever was called a "post industrial revolution epidemic" (17). In 1989, Strachan (19) provided evidence that household size was an important factor in the incidence of allergic disease in England and Wales. He suggested that "declining family size and higher standards of personal cleanliness have reduced the opportunity for cross infection in young families accompanied with more widespread clinical expression of atopic disease" (19). At the time this

novel but speculative explanation for the apparent increase in prevalence of allergic disease was contrary to the notion that viral infections, especially respiratory ones, increase atopic diseases. This explanation was became known as the "hygiene hypothesis" and was the subject of intense reviews and editorials during 90s (20, 21). One decade later, the hygiene hypothesis was reviewed by the original author (22) who further explained that "the hypothesis, is considered immunologically plausible and is consistent with the epidemiological features of atopy, however, an inverse association between infection and atopy has not been confirmed directly by epidemiological studies and the available data are either inconsistent or inconclusive" (22). These inconsistencies between history of infection and future development of asthma became more challenging in terms of the hygiene hypothesis. It was suggested that "asthma shows a different epidemiological pattern than indicators of atopy" (22). Pearce *et al.* in a meta-analysis suggested that there is only a weak and inconsistent association between the prevalence of asthma and the prevalence of atopy and less than 50% of cases of asthma are atopic (23). These findings conclude there is an inconsistency that exists between hygiene hypothesis and the development of asthma.

In this thesis, I will explore the mechanism by which another environmental factor may contribute to the genesis and development of asthma. This factor is thought to play an important role in asthma etiology, *i.e.* the Respiratory Syncytial virus.

Allergic asthma following RSV bronchiolitis

Epidemiological evidence

RSV is the cause of 70% of all cases of bronchiolitis (24). The risk of bronchiolitis peaks around two to six months of age (25). There is a strong epidemiological link between viral infection of the lower respiratory tract and subsequent development of asthma and allergy in children (26, 27). Many studies

by different groups from different regions in the world have proposed an association between severe RSV bronchiolitis in infancy with later development of allergic asthma (28, 29). Despite differences in study designs related to timing or severity of infection, these studies share a common message, cumulatively substantiating the evidence for the link between RSV and allergic asthma (table 1-1). A few decades ago, Pullan ***et. al.*** reported that lower respiratory RSV infections are accompanied by impaired lung function at age 10 (30). In a birth cohort study, the Tucson Children's Respiratory Study (Stein ***et. al.***) reported that early RSV disease increased the risk of frequent wheeze in children up to age 11 but not 13 (31). Neither study reported an increase in atopic status following RSV infection.

Another investigation linking RSV and asthma was a prospective study from Sweden (28, 32-34). Sigurs ***et. al.*** followed children with severe RSV bronchiolitis in first year of life for more than 18 years and published their findings at different ages of the children as they grew (time points of 3, 7 ,13 years and the more recent at age 18). Hospitalization was considered as a sign of severity. The study demonstrated that prevalence of asthma and recurrent wheezing and also allergic sensitization were higher in the index group (RSV) compared with matched control cohorts. At age 18, which is beyond childhood, 33% had current asthma compared with only 7% of controls. The RSV group also showed increased prevalence of allergy to animal dander or house dust mite, when evaluated by a skin prick test (41% vs. 14%). Interestingly a family history of atopy or asthma did not prove to be a risk factor for development of asthma in RSV vs. controls groups. In RSV group itself, those with a parental history of asthma had a higher prevalence of allergic disease or asthma compared with children without such history (32). Cumulatively, the study showed that the only important risk factor for the development of "current asthma"/recurrent wheeze was severe RSV disease. Similar to this study, another prospective investigation proposed that severe RSV bronchiolitis is accompanied by higher IgE and subsequent development of allergic asthma (35). It was suggested that RSV

Study	*Design*	*Subjects with RSV(Total)*	*Link*	*Ref.*
Pullan *et. al.* (1982)	R	130 (241)	No	(30)
Schauer *et. al.* (2002)	P	42 (126)	Yes	(35)
Singleton *et. al.* (2003)	R	95 (208)	Yes	(36)
Stein *et. al.* (1999)	P	207 (888)	Yes	(31)
Korppi *et. al.* (2004)	P	36 (81)	No	(37)
Henderson *et. al.* (2005)	R	150 (13,971)	Yes	(38)
Carroll *et. al.* (2009)	R	12,916 (90,341)	Yes	(39)
Escobar *et. al.* (2010)	R	1,181 (71,102)	Yes	(40)
Sigurs *et. al.* (1995, 2000,2005,2010)	P	46 (138)	Yes	(28, 32-34, 41)

Table 1-1- Summary of studies that focus on the association between early childhood RSV disease and the development of asthma/recurrent wheezing later in life; Retrospective cohort, R; Prospective cohort, P. (Adapted from Ref. (42))

infection, in first year of life, can influence the Th1/Th2 pattern of immune response and sensitization to allergens in the context of the development of a chronic asthmatic phenotype (43).

It should be emphasized that the timing of RSV infection appears to be of critical importance. RSV infections follow a seasonal pattern, usually peaking in the winter time(44). Wu *et. al.* have shown that, in the northern hemisphere, children born 4 months before the seasonal prevalence peak of RSV bronchiolitis had a higher risk of developing childhood asthma (44). In addition to human epidemiological observations, animal models of RSV infections suggest that the immune response is affected by the timing of when a neonate encounters the virus (25). Mice were infected at different time points (day 1 or 1, 4, or 8 weeks of age). All groups were re-infected at 12 weeks of age. Animals infected earlier showed a more prominent inflammatory response following re-infection, including eosinophil recruitment and a Th-2 type response. In contrast, delayed priming with viral infection led to a less severe inflammation during re-infection (25).

These studies suggested that there is a "window of susceptibility" or "opportunity" during which both the immune system and respiratory system continue to grow and develop (45). Goulding *et. al.* proposed that the initial antigenic exposure (i.e. viral infection) of the lung will leave an "innate imprinting" in which the cellular composition and its immunological environment may alter in a way that affect all future challenges (46).

Increased severity of bronchiolitis increases the risk of asthma development: There are studies suggesting that the severity of the response to infection may also be important. In a large retrospective birth cohort study in Tennessee, Carroll *et. al.* investigated whether the association between infant bronchiolitis and the morbidity of early childhood asthma was quantitatively dependent on the severity of bronchiolitis (39). Using the International Classification of Diseases, Version 9

(ICD-9), the authors classified more than 90,000 otherwise healthy infants into four categories. They were “no visit”, “outpatient bronchiolitis”, “emergency department” and “hospitalized bronchiolitis”. The study suggested that the relationship between severity of bronchiolitis and the risk of early childhood asthma (4.5 to 5.5 yrs old) followed “a dose-response” pattern with hospitalized infants having the highest risk. This pattern was not modified by a familial predisposition to asthma (39).

Possible mechanisms

Early infection versus late infection: *In vitro* and *in vivo* based studies to understand the mechanism of early versus late viral infections, showed that infection of rat lungs by Sendai virus induced more profound anatomical damage in the neonates (5 day old) than in older mice (25 day old) (47). This phenomenon may contribute to airway damage or remodeling may last beyond the infection period.

Are RSV proteins allergy inducing: From a biological perspective, RSV infection induces release of type-I interferons, IL-12, IL-18, CCL2, and a panel of different chemokines including CCL5 (RANTES), CCL4 (macrophage inflammatory protein-1β), and eotaxin (CCL11, 24 and 26) which have a role in allergic reactions (48-50). In murine models, various RSV proteins induced different phenotypes of Th1 or Th2 immune responses involving relevant cytokines. Mouse lymphocytes primed with the G protein of RSV were stimulated *in vitro* and released Th2-like cytokines. This was not the case for RSV F protein (51, 52). Thus, one possibility could be that individuals respond differently to distinct RSV proteins while others are more prone to develop a Th2-type response after they encounter RSV G-protein (53).

RSV alters immune responses: In mice, antigen-specific Th1 immune responses to viral infection can lead to antigen-non-specific Th2 type of inflammation

associated with allergy (54). The authors adoptively transferred Ag-specific Th2 cells to naïve mice then challenged them with the specific Ag. This challenge *inefficiently* recruited Th2 cells to the site of challenge (lung). However when the study adoptively co-transferred both Th1 and Th2 cells specific for different antigens, challenge of mice with antigens specific for Th1 cells resulted in the recruitment of Th2 cells to the lung, although the cells were not specific to the challenged antigen. In summary, these findings suggest that Th1-type responses specific to respiratory viral infections can enhance a Th2-type disease by the recruitment of Ag-non-specific Th2 cells to the site of inflammation(54). These findings also imply that Th1 and Th2 responses are not exclusively counter-regulatory.

It has also been shown in a murine model that interaction between viral infection and allergen exposure during infancy is essential in forming an asthmatic Th2-biased phenotype (55). Siegle ***et. al.*** infected neonatal mice with pneumonia virus of mice (PVM), which mimics severe RSV disease of human neonates. After recovery, they sensitized animals with intranasal ovalbumin. Challenge of animals with ovalbumin resulted in a Th2-type immune response with high levels of IgE, elevated levels of Th2- cytokines including IL-4, IL-5 and IL-13, and allergic airway inflammation with recruitment of eosinophils. These findings were observed only in animals that had neonatal PVM infections followed by antigen sensitization and chronic challenge (55).

Eosinophils appear to have a unique role in RSV-induced airway hyper-reactivity (56-60). Infants with RSV-bronchiolitis have higher levels of eosinophil cationic protein (ECP) in their nasopharyngeal secretions (58, 59). Schwarze ***et. al.*** used IL-4, IL5 and IFN-γ KO models of mice and concluded that mice lacking IL-5 will not develop airway eosinophilia and hyperresponsiveness following RSV infection. They suggested an essential role for this Th2-type cytokine in post-RSV hyper-reactivity of airway(56).

Despite these studies providing strong epidemiological evidence for the association between RSV bronchiolitis and subsequent asthma/allergy, there remains a debate whether this link is truly causative or if there was a predisposition to asthma even before RSV infection. Thus, the allergic bias preceded infection, and the severe response to RSV may be the result of such a bias (61). For example, Adamko ***et. al.*** suggested that previous sensitization with a non-viral antigen can induce an allergic inflammatory response to a viral infection (62). They sensitized ***Guinea pigs*** using intra-peritoneal injections of ovalbumin and 3 weeks later animals were infected with the parainfluenza virus. The viral infection caused airway hyper-responsiveness (AHR), and in OVA-sensitized animals, the AHR could be blocked using an anti-IL5 antibody or an antibody against eosinophil major basic protein (MBP). Anti-IL5 depletes eosinophils while anti-MBP prevented the function of MBP. Overall, it is also likely that an altered epithelial barrier or altered immune response may predispose an infant to a more severe lower respiratory track disease.

Whether the link between RSV bronchiolitis and asthma/wheezing is causative or not, this association has attracted attention to the prevention of asthma (24). There is evidence that being genetically predisposed alone may not proceed to a full asthma phenotype without an environmental factor including severe lower respiratory infections (63). Stein and Martinez (63) suggested that the answer to this enduring question can only be provided by a "randomized, placebo-controlled clinical trial in which the prevention of lower respiratory RSV illness is associated with decreased incidence of subsequent asthma"(63). There is only one study thus far that comes close to this goal. In a prospective multicenter, cohort study in 27 centers in Europe and Canada, Simoes ***et. al.*** investigated whether administration of palivizumab (that reduces RSV infection rates) to premature infants would have a protective effect on subsequent recurring wheeze in infants (64). This study suggested that prophylaxis against RSV reduced the risk of recurrent wheeze in an atopy-independent mechanism (64). Genetic background or

environmental factors (viral infection) are not mutually exclusive and they may have a co-operative role in developing the final outcome (42).

Virology of RSV and the challenges surrounding its prevention

RSV biology and pathophysiology: RSV was first isolated in 1956. Human Respiratory Syncytial Virus (hRSV) is an enveloped single-stranded negative-sense RNA virus (ssRNA(-)) belonging to *Pneumovirus* genus of the *pneumovirinae* subfamily in *Paramyxoviridae* family. By definition, *Paramyxoviridae* viruses have a protein that facilitates virus-host cell membrane fusion (F protein). There are two subtypes defined for RSV: A and B, which are differentiated largely by variations within the G protein. Subtype A is more predominant and virulent compared to subtype B. RSV is a spherical virus with less than 350 nm in diameter, but pleiomorphism and filamentous forms are common. Structurally, RSV contains a lipid bilayer envelope that originates from the host cell in which the virus grow and from which it is released (figure 1-1). This envelope surrounds a nucleocapsid containing a viral genome plus a few proteins. Upon infection, RSV's ssRNA genome replicates in the cytosol of the host cell. Three surface glycoproteins (fusion [F], small hydrophobic [SH] and major attachment [G]) are scattered in the envelope, and together with nonstructural-1 and 2 (NS1 and NS2), matrix (M), large (L), SH, nucleoprotein (N), phosphoprotein (P), M2-1 and M2-2, the RSV genome encodes 11 different proteins (65). NS1 and NS2 are not found in the virion, however, they can be detected in cells infected with RSV. Except for these two proteins, all other proteins are structural that function during viral replication. The F and the G proteins are important intermediates during viral infection and penetration. Indeed, pathogenesis of RSV is strongly dependent on the function of these proteins. The G protein mediates attachment of virus to host cell while the F protein facilitates fusion of virion with the host cell membrane allowing entry. RSV is known for its ability to form syncytia (hence its name) and F protein is

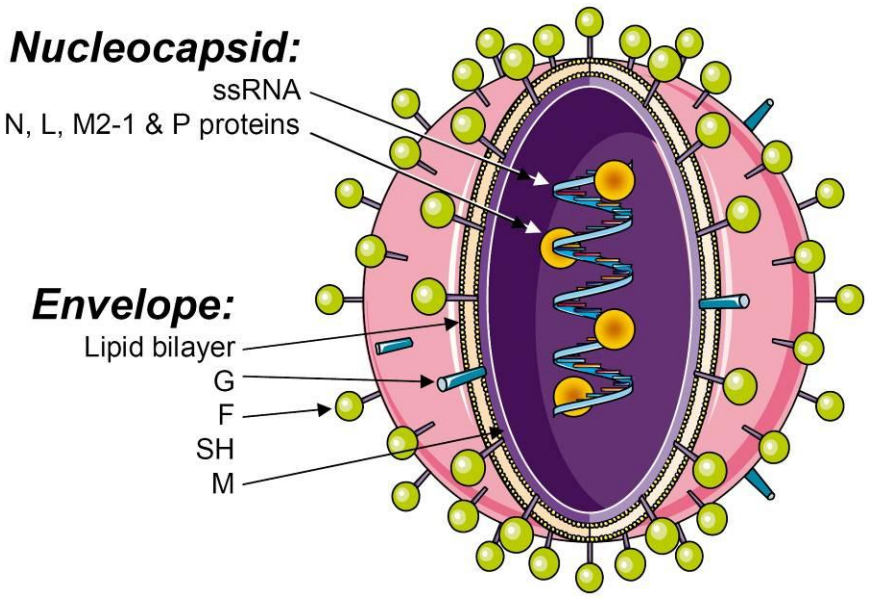

Figure 1-1- This picture shows a schematic view of RSV structure. NS1 and NS2 proteins are not depicted, as they are only detected in cells infected with RSV.

believed to have unique role in this. Upon formation of syncytium, virus can transmit from cell to cell. Infectious and mature RSV particles consist of ribonucleoproteins, which form as ssRNA (the viral genome), N, P and L proteins.

The airway mucosa is the main target of RSV infection. The precise receptor for RSV on the cell surface has not been identified but there are a number of possibilities. It is known that heparin-like glycosaminoglycans on the surface of cells interact with G proteins (66, 67). It is believed that the pattern recognition receptor, TLR4 (68-70), and CD14 (69) are important cell surface markers that mediate infection with RSV. Inter-cellular adhesion molecule-1 (ICAM-1) has also been shown to play a role in viral F protein binding to cell membrane (71).

Upon infection, RSV replicates in the epithelial cells resulting in cytopathic changes to the cells. Infected cells will produce potent inflammatory cytokines and chemokines, the latter recruit immune cells to the site of infection. It has been shown that RSV infection activates genes inducible via NFκB, a transcription factor, a central mediator in viral induced inflammatory response (72-77). Messenger RNA (mRNA) is conventionally considered as having positive sense. Negative sense RNA viruses including RSV, use the host cell machinery to translate their genetic codes to viral protein sequences. They utilize their own "RNA-dependent RNA polymerase" to prepare a positive sense anti-genomic copy of their genomic RNA. During the course of ssRNA virus replication, dsRNA and RNAs with 5`-triphosphate ends develop in infected cells. This latter product is different from host mRNA, because the 5` ends of host RNAs are typically "capped".

The innate immune system distinguishes between viral and host RNAs through pattern recognition receptors (PRR). For example, the existence of dsRNA is sensed as abnormal, since host cells do not generate dsRNA. Two categories are defined for dsRNA based on their size. It is believed that short dsRNA (<1Kb) are

detected mainly by RIG-I. On the other hand, it is through MDA5 that long dsRNAs (>2 kb) are recognized (78, 79).

Another subgroup of PRRs is represented by TLRs. It has been shown that interaction of RSV with TLR4, independent of viral replication, has a role in activation of the inducible NFκB pathway (72). The importance of TLR4 in innate immune response to RSV has been shown in different studies (68-70). TLR3, another member of the TLR family, has also been reported to play role in immune responses to RSV (80, 81). The role of Pattern Recognition receptors in RSV infection will be discussed further in Chapter 3.

While a vaccine for influenza is part of the seasonal immunization routine, decades of research has not yielded any vaccine for RSV. The latter is transmitted from person to person with activity that typically peaks in Winter seasons. However, there is substantial regional and yearly variability for its onset (65). Despite high levels of maternally transferred specific serum antibodies (82), fifty percent of infants are infected in the first year of life and all by age three. RSV infection usually manifests as mild cold symptoms in young children and adults, while in the first year of life about 30-70 % develop bronchiolitis or pneumonia (83). Although many viruses can precipitate bronchiolitis, RSV has been shown to be responsible for 50-90% of infant bronchiolitis cases (84). Re-infection with RSV is common throughout life, suggesting that protective immunity is incomplete. As acute respiratory tract disease is the primary cause of mortality due to infectious disease, RSV is considered as one of the most important targets for vaccine development. Although RSV disease is a huge burden on health system, five decades of rigorous research has failed to generate an effective and cheap strategy to prevent RSV infection. It is estimated that the health burden of RSV is much higher than that caused by the influenza virus or rotavirus (85). As such, there is a great need to develop prevention strategies for RSV bronchiolitis.

Passive immunoprophylaxis of RSV

One method of protection against infection by RSV via the immune system is through the transfer of neutralizing antibodies from another person to the immature immune system of the infant. In this strategy, the immune system of the host does not play an active role in protection, and as such it is "passive" protection. This naturally happens against many infectious pathogens, when neutralizing antibodies enter fetus circulation from pregnant mothers. Studies done in cotton rats determined the effective dose of neutralizing antibodies for the prevention of RSV infection; these produced a frame work for the subsequent development of commercially available anti-RSV immunoglobulin (86). RespiGam, a human immunoglobulin for intravenous administration was licensed in 1996. RespiGam was extracted from the blood of human donors. Parenteral infusions of RespiGam on a monthly basis in high risk infants reduced the frequency of disease (87). While it has disadvantages, such as the high protein content and the need for large volume administration to achieve effective dose, it was the only option available in high risk cases to prevent RSV disease. Other disadvantages include the presence of antibodies specific to other pathogens in RespiGam, which could interfere with the function of other childhood vaccines.

To address these shortcomings, recombinant humanized monoclonal antibodies against RSV were developed (88). MEDI-493 or Palivizumab® is a monoclonal antibody specific to the F protein, which competently neutralizes it. It is at least 50 fold more potent than RespiGam. This allowed a reduced amount of immunoglobulin to be administered to high risk infants (premature infants, infants with chronic lung disease or major congenital heart disease). Despite the advantages of Palivizuamb as the only commercially available anti-RSV MAB (monoclonal antibody), it has not alleviated the need to find an effective vaccine, as cost-benefit analysis and financial burden of humanized anti-RSV MAB continues to be the subject of an ongoing debate among pediatric specialists (89, 90).

Active immunoprophylaxis:

Boosting the immune system to become involved actively in defense against RSV infection is a goal for researchers over more than 5 decades. Indeed, great efforts have been made to produce an RSV vaccine. Initial attempts during the 1960s (91-93) with a formalin-inactivated RSV (FI-RSV) failed dramatically with fatal outcomes. Vaccinated children experienced a more severe disease following exposure to wild-type RSVs compared with un-immunized controls; sadly, two infants died. This disastrous outcome of immunization hampered future research in the field and made researchers more cautious despite the recognized need of to develop a vaccine for this virus. The enhanced RSV disease is believed to result from a combination of (1) the lack of cellular immune response, (2) insufficient levels of RSV-neutralizing antibodies in serum, and (3) the induction of a disproportionate Th2-biased immune response that include lung eosinophilia and related cytokines and chemokines such as IL-4, IL-5 and MIP-1α (94). As a result, there is currently no licensed RSV vaccine.

The exact mechanism that resulted in an enhanced disease remains to be determined. The use of concentrated, formalin inactivated and alum-adjuvant boosted vaccine (FI-RSV) did not protect against RSV infection. Autopsies of two fatalities showed that RSV replication was present (95, 96). FI-RSV induced a different pattern of serum antibody response in comparison to what naturally happens in an RSV infection. FI-RSV induced a strong antibody response that efficiently bound to RSV antigens. However, it could not prevent infectivity. This could be explained to some extent by the denaturation process of formalin inactivation, a process that changed those antigenic epitopes of RSV, which induce protection naturally. Thus, no protective antibody was induced by FI-RSV, suggesting that induced antibodies orchestrate an immune response which is neither beneficiary to the host nor protective (97). Experimental animal models immunized with FI-RSV showed an increased Th2-type response, as depletion of

IL-4 prior to RSV challenge abrogated disease enhancement (98, 99). *In vitro,* studies also demonstrated an increased peripheral blood lymphocytes proliferative response to RSV antigens in of immunized subjects in compared with control groups (100). These studies provided evidence for an altered cellular immunity response following FI-RSV vaccination. In a more recent review, it was suggested that both Th1 and Th2 CD4, as well as CD8 T cell responses may contribute to RSV vaccine-enhanced disease (100). The review suggested that prevention of RSV-enhanced disease may need a well balanced immune response to a vaccine candidate; such a "well balanced" response was not defined more precisely by the authors.

In animal studies, use of purified RSV proteins for immunization (obtained from RSV-infected cell lines or from recombinant sources) has also resulted in enhanced RSV disease (101, 102). The enhancement phenomenon did not happen after natural infection or after immunization with vectors expressing RSV antigens (103). This dissimilarity in disease following natural infection versus immunization with RSV proteins may be explained in part by current understanding of the process of antigen presentation within the immune system. Immune reaction during a natural RSV infection (or following immunization by vectors expressing RSV antigens) involves production of RSV proteins inside cells that results in antigen presentation within the context of a MHC class I system, while denatured proteins (FI-RSV) or purified RSV proteins may have a different course in being presented with MHC class II system in antigen presenting cells. Viral antigens expressed in the context of MHC-I system usually elicit a cytotoxic T lymphocyte (CTL) and/or natural killer cell (NK) immune response (Figure 1-2). The pertinent anti-viral IFN-γ production during a classic CTL/NK immune response would favor a Th1 biased reaction, different from the Th2 biased immune reaction associated with a FI-RSV enhanced disease.

Another approach would be to apply a live attenuated RSV vaccine in this population. Unfortunately, clinical observations indicate that a live RSV that is

sufficiently attenuated to be safe for the pediatric population, may turn out to be over attenuated and thus not beneficial or immunogenic in individuals with immune memory to RSV(104).

It is interesting to note that FI-RSV-enhanced infection did not occur in mice previously primed with live RSV but rather only in the absence of RSV priming (103). In other words, RSV enhanced disease only occurs if priming with FI-RSV happens as an initial immunization in life. This observation suggested that a vaccine consisting of RSV antigenic protein may be harmless in older children and adults who would have had natural exposure to RSV. This may indicate that an RSV antigenic protein vaccine may be beneficiary in enhancing immunity in older individuals (87, 103). Although developing a safe vaccine has not yet been achieved for infants, production of a less attenuated but more virulent RSV vaccine that is quite safe in adults appears to be likely. Such a vaccine could only be used for elderly people and young adults. Individuals who have seen RSV before and who are at high risk for developing serious RSV disease (*i.e.* due to underlying pulmonary or cardiac disease or due to advanced age) could benefit from this vaccine.

Another approach in these target populations (individuals with previous exposure to RSV) would be utilization of purified RSV protein vaccines. This vaccine, despite its similarity to FI-RSV, appears to be safe in RSV experienced individuals. A purified F protein (PFP) derived from RSV-infected cell culture and mixed with alum as an adjuvant has been tested extensively in adults, children (with and without underlying disease), and the elderly (105, 106). These studies revealed a moderate, well tolerated immune response in vaccinated elderly. Later a new generation of this vaccine (PFP-3) was tested in children with cystic fibrosis. This provided evidence for protection against RSV in cystic fibrosis patients(107); regardless, the evidence was not universally well received (87).

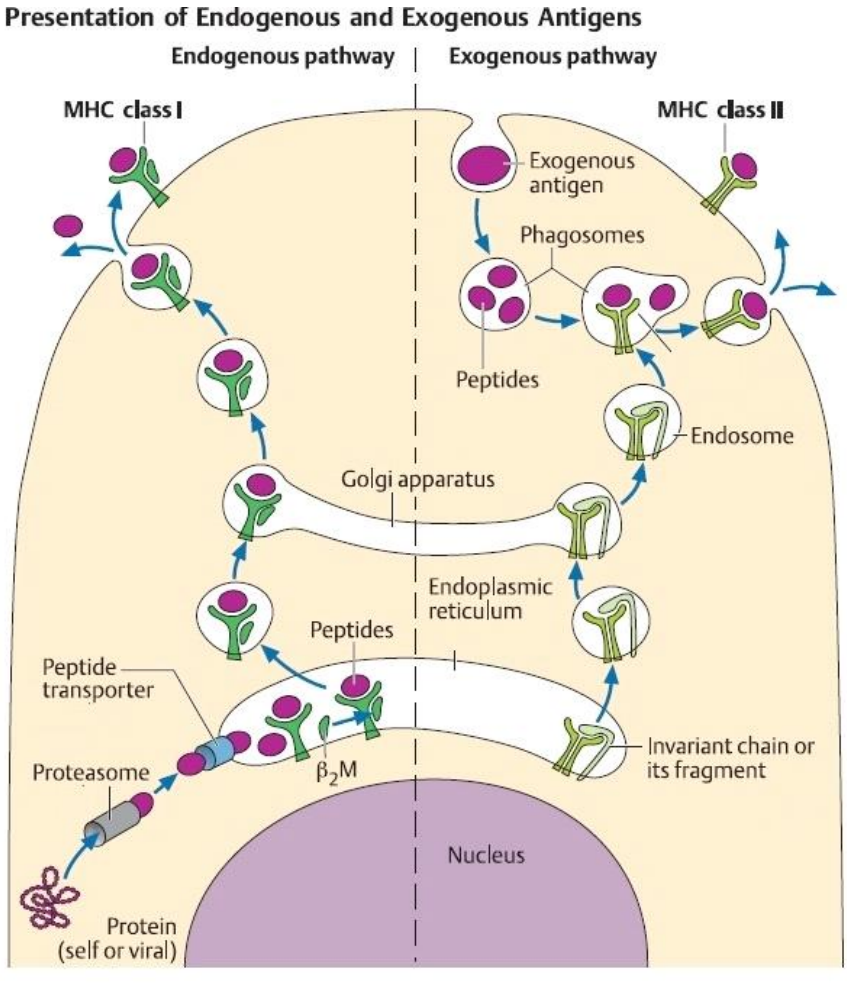

Figure 1-2. Endogenous antigen peptides (self or RSV) synthesized within a cell (left side) are bound to MHC-I molecules within the endoplasmic reticulum and eventually are presented on the cell surface. Antigenic proteins taken up from exogenous sources (like FI-RSV) are cleaved into peptides within phagosomes. Eventually, they are presented in the context of MHC-II system (Adapted from Kayser medical Microbiology, 2005, Thieme).

Indoleamine 2,3-dioxygenase (IDO) and tryptophan catabolism: a link between RSV and asthma

Tryptophan 2,3-dioxygenase (TDO) (EC 1.13.11.11) was described in 1936 as an enzyme that catalyzes the conversion of tryptophan to formyl-kynurenine(108). In mammals, TDO is only found in the liver. Patients suffering from a range of diseases, including tuberculosis and a few malignancies, excreted high levels of tryptophan metabolites in the urine, however, TDO activity was not increased in the liver of patients.

IDO as an alternative enzyme in the oxidative catabolism of tryptophan was first described in 1963 by Hayaishi *et. al.* (108, 109). IDO and TDO represent a small family of haem enzymes, where TDO is found both in eukaryotes and prokaryotes, while functional IDO has only been reported in eukaryotes(110, 111). After the report of Munn *et. al.* (1998) regarding the role of tryptophan and IDO in prevention of fetal rejection (112), IDO fell under intense spotlight in many labs. IDO metabolic pathways became a hot topic of study as reflected in Medline where more than 1300 articles were published since 1998. The goal of discovering a true role for this enzyme in pathology of the diseases went as far as a researcher injecting himself with quinolinic acid (a tryptophan catabolite via IDO) to provide evidence for a role of IDO in eosinophilic fasciitis (113). Part of the reason for this enthusiasm is in the capacity of IDO pathway to fit in various paradoxical concepts and as a potential candidate to answer a number of questions that could not be explained otherwise. It appears that tryptophan/IDO pathway may have a fundamental role in biology, since it has been conserved during 600 million years of evolution as a "jack-of-all-trades"(114).

Tryptophan is the least common essential amino acid and has a unique role in mammalian cell function. Protein biosynthesis and consequently cell growth is

dependent on the availability of tryptophan (115). Tryptophan hydroxylase converts tryptophan to serotonin (5-hydroxytryptamine) which is a neurotransmitter, while tryptophan catabolism through IDO pathway results in production of different catabolites (figure 1-3). It is known that 99% of dietary intake of tryptophan will enter the IDO pathway(116). TDO and IDO are the key enzymes in the rate-limiting step of tryptophan catabolism, with the TDO being only found in liver while IDO is ubiquitously expressed (at least at low levels of mRNA) and present in many extra-hepatic organs, especially in the placenta and lungs (117). IDO is not usually constitutively *active* in immune cells, but it has been suggested that eosinophils constitutively express IDO although IFN-γ can boost its activity (118). A distinguished but small subset of dendritic cells is also capable of expressing IDO constitutively in mice, but may not be functionally active (119). Constitutive expression of an enzyme which catabolizes an essential amino acid can have potential toxic effects unless it is closely regulated. As such, the enzymatic activity of IDO and its expression at protein levels appears to be highly regulated (114, 116) Following IDO activity, concentration of tryptophan can reach very low nanomolar levels (120) thus imposing a condition of starvation in susceptible cells and pathogens (121).

IDO is inducible by type I and II of interferons (specially IFN-γ), several microbial components (LPS, dsRNA) and soluble and membrane-bound forms of cytotoxic T lymphocytes-associated antigen 4 (CTLA-4) and prostaglandins (122-125). In contrast, TDO is not inducible immunologically (114). TGF-β (126) and IL-4 (127) were shown to inhibit IDO induction in fibroblasts and monocytes, respectively.

IDO induction will also result in production of “neurotoxic”, “immunocytotoxic” or “immune-active” kynurenines (KYN) (128). Tryptophan depletion and/or these products have been suggested to be key factors in different physiological and pathological conditions. This includes the modulation of the balance between

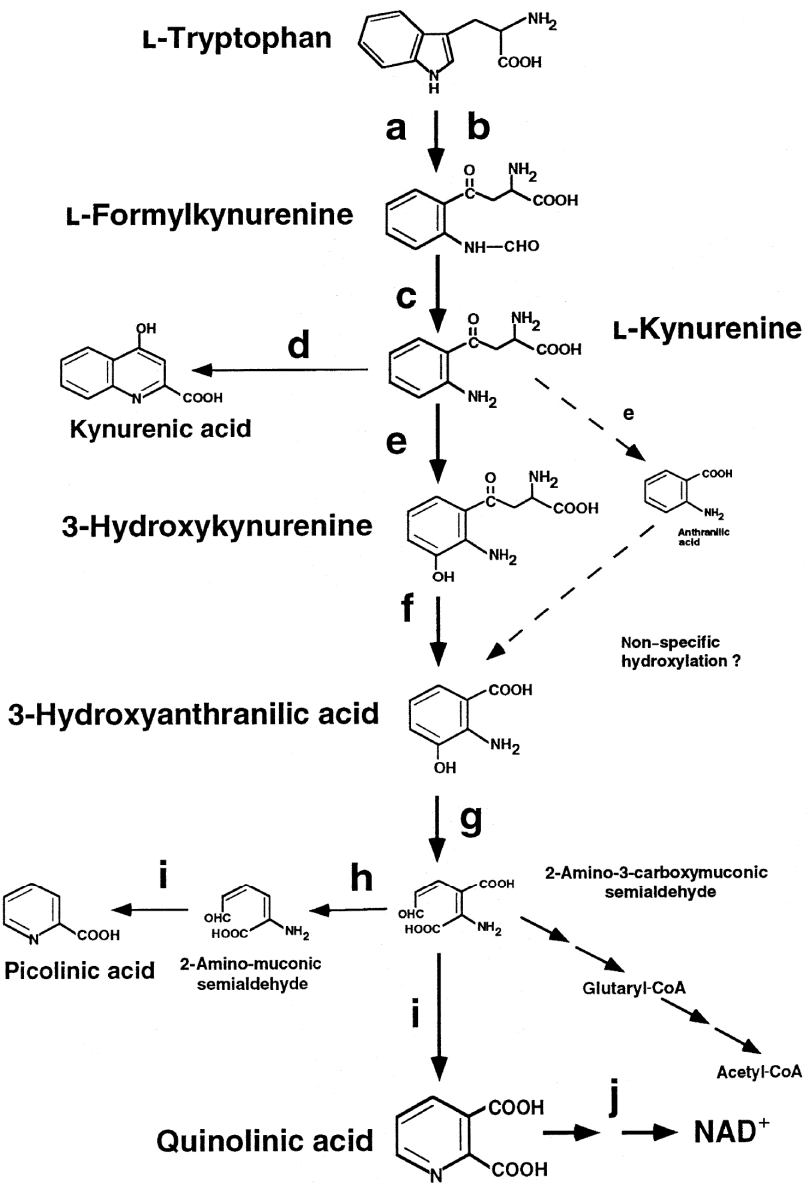

Figure 1-3- simplified metabolic pathway of tryptophan which leads to the production of different catabolites and the enzymes involved. IDO (a) is the rate-limiting step. (a) Indoleamine 2,3-dioxygenase, EC 1.13.11.17; (b) tryptophan 2,3-dioxygenase, EC1.13.11.11; (c) kynurenine formamidase; (d) kynurenine aminotransferase (2-oxoglutarate aminotransferase); (e) kynurenine 3-hydroxylase; (f) kynureninase; (g) 3-hydroxyanthranilate 3,4-dioxygenase; (h) picolinic carboxylase; (i) non-enzymic cyclization; (j)quinolinic acid phosphoribosyltransferase [source: Heyes et. al. (129)].

Th1-Th2 type responses. It has been shown that IDO activity induces apoptosis in proliferating T cells, preferentially in Th1 versus Th2 cells (130-132).

Lee *et. al.* argued that the inhibitory role of tryptophan depletion on proliferation of T cells can not be completely explained by the suppression of protein synthesis (133). The rationale for their argument originated from the scarcity of tryptophan within the context of cell proteins (~1%) in comparison to isoleucine and leucine which together account for 10% of amino acids in protein structure. The authors provided evidence in a murine model that depletion of isoleuicine/leucine to levels much lower than tryptophan, despite accounting for 10% of amino acids of cell proteins, failed to arrest cell cycle progression. They postulated that T cells are sensitive to free tryptophan concentration at a critical point during cell cycle progression (133).

As mentioned above, protection of the fetus from T cell mediated allogenic rejection by the mother's immune system has been suggested to be, to some extent, the result of IDO expression in human syncytiotrophoblast of the placenta (112, 134, 135). IFN-γ-induced IDO activity has been shown to have anti-proliferative function on cells, reversible by the addition of exogenous tryptophan in a time- and dose-dependent fashion (136).

In addition there are pathological conditions that IDO-mediated tryptophan degradation is increased. These include viral, bacterial and parasitic infections, malignancy, autoimmune diseases, and neurodegenerative disorders. Human immunodeficiency virus (HIV) infection induces IDO in an interferon-independent fashion and infection inhibits CD4+ T-cell proliferation (137, 138). It has been demonstrated that this type of induction also occurs in hepatitis C (139), influenza (140, 141) and herpes simplex viral infections (142). Steptococcal toxic TE
ck-like syndrome (143), *Borrelia burgdorferi* (the agent of Lyme disease) (144) and very recently *Mycobacterium leprae* (agent of Leprosi) (145) are examples of bacterial infections with their pathogenesis associated with IDO. Patients with

Patients with acute Lyme neuroborreliosis had *L*-kynurenine detected in their cerebrospinal fluid (144); antibiotic treatments returned the values to normal levels.

It has been reported that IDO induction is a defense mechanism of immune system in which tryptophan depletion inhibits the growth of tryptophan-sensitive intra-cellular parasites such as *Toxoplasma gondii* (146, 147). *Leishmania* infection stimulated IDO expression; and IDO inhibitors have been suggested as a potential new approach in treatment of leishmaniasis (148). Higher serum KYN/tryptophan concentration ratio is observed in few malignant diseases including adult T-cell leukemia (149) and colorectal cancer (150), and now for more than two decades, the induction of IDO has been considered as a mechanism for anti-tumor activity of IFN-γ (151). Use of IDO-expressing monocyte-derived DCs is considered as a modality in immunotherapy for cancer (152). Nonetheless, there remains controversy around the inhibition of IDO and its probable beneficial role in tumor chemotherapy (153). Interestingly, IL-18 and IL-12 have the ability to augment IDO activity in the presence of activated lymphocytes in human osteosarcoma cell lines (HOS and MG-63); this augmentation was independent of IFN-γ (154). In 1989, IL-12 was defined as an activator of NK cells released mostly by antigen presenting cells (155). It is believed that in mice, and suggested in humans, the source of IFN-γ during RSV infection is NK cells (156). The pleotropic cytokine IFN-γ exerts its physiological functions via different routes, a key one of which is IDO.

IDO activity in dendritic cells: Western blot and flow cytometry analyses have provided evidence that human DCs can constitutively express IDO. This, however, does not indicate functional activity unless CD80/CD86 receptors on the DCs are being ligated with CTLA4/CD28 expressed on T cells. IDO activation also occurs in DCs following treatment with IFN-γ (157). Likewise, it has been shown that all splenic DCs in the mouse express IDO protein constitutively (analysed by Western blot), though only $CD8\alpha^+$ fraction of these cells showed

tryptophan catabolizing characteristics following IFN-γ treatment (158). These observations have important implications in the context of the methodology used in our experiments. Detection of IDO based on Western blot or flow cytometry would not necessarily mean that IDO is functionally induced following a treatment, including after RSV infection. Assessment of IDO induction following RSV infection would best be addressed by the measurement of the end product of IDO activity i.e. KYN (kynurenines) as catabolyites of the enzyme. Similarly, elevated values of IDO mRNA my not accurately reflect its functional induction (figure 1-4).

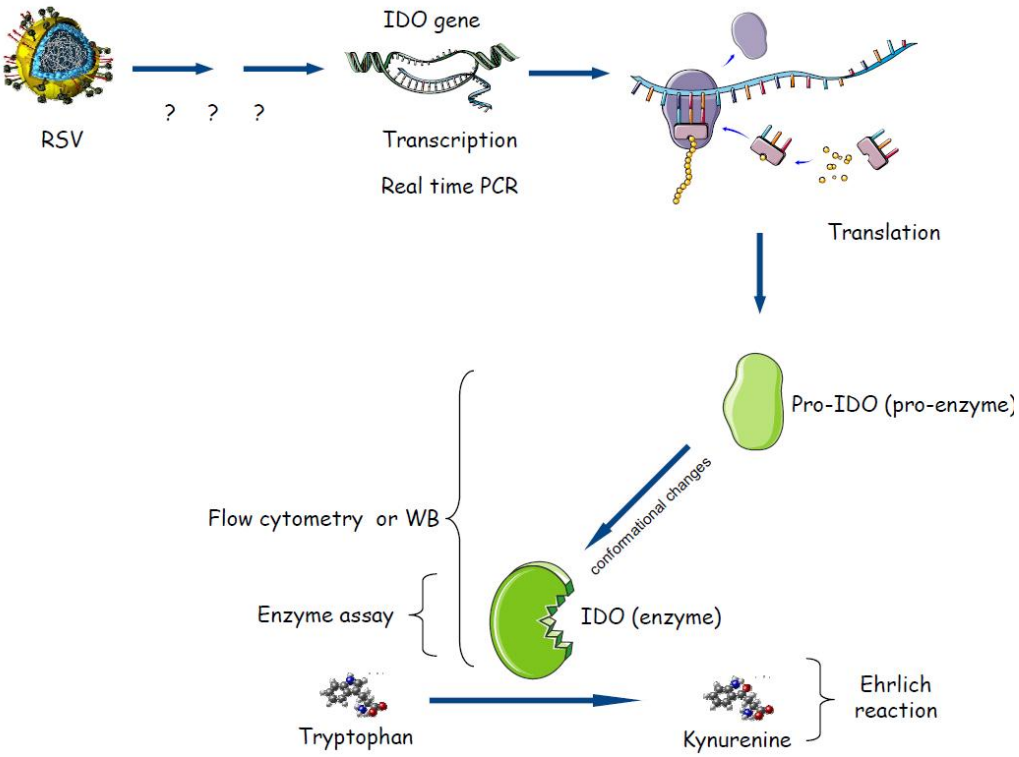

Figure 1-4- From a methodological stand point, induction of IDO can be evaluated at different steps. Transcription of IDO gene will lead to mRNA which can be detected by real-time PCR. These mRNAs will in turn determine the sequence of amino acids in the peptides forming the IDO pro-enzyme. Conformational changes (in addition of other complex mechanisms) will convert the pro-enzyme to an active form. Both pro-enzyme (biologically inactive) and the active form of IDO can be detected by flow cytometry or Western blot. However, detection of pro-enzyme does not reflect the true bioactivity of the enzyme, as there are situations that IDO is constitutively expressed in cells while in the absence of any bioactivity (see text). The gold standard remains to be the enzymatic assay or detection of end-products of the enzymatic activity, i.e. Kynureniens. In my studies, I chose the latter option.

Indoleamine 2, 3-dioxygenase (IDO) and its role in Th1-Th2 imbalance

Activation of IDO has a wide range of immune modulatory effect (159). It is known that IDO has an immunosuppressive role by inhibition of proliferation in CD4, CD8 and NK cells. Recently a sizable body of research has focused on the role of IDO in modulation of the immune system, inducing an imbalance between Th1 and Th2 cells. It has been shown that IDO activity and the production of kynurenines, induce apoptosis in proliferating T cells, preferentially in Th1 versus Th2 cells (130, 131). Thus, Th2 dominance seen in atopic disease may be the result of increased Th1 apoptosis (160). Xu *et. al.* have suggested that the immunosuppressive role of IDO was described mostly in models with a Th1 dominant response (132). However, the role of the kynurenine pathway has not been well understood in Th2 dominant responses. These authors proposed that IDO pathway acts as a negative feedback loop in Th1-type responses, while IDO may even promote up-regulation of Th2 immune responses (132). As will be discussed in chapter three, the proposed negative feedback loop on Th1 cells is in line with our findings.

Rationale and general hypothesis

RSV infection of DC induces IDO activity and a Th2 imbalance.

A better analysis of the nature of the link between RSV and asthma development would have valuable application in asthma prevention. As noted, infants with severe RSV infection appear to be at increased risk of developing asthma later in life. As part of an anti-viral response, RSV infection induces interferons including IFN-γ. Although RSV is not a very potent inducer of IFN-γ compared to some other respiratory viruses, the severe clinical picture and the timing of RSV infection, places RSV in a unique position. Infection of innate immune cells by RSV will directly (or indirectly through cytokines) increase IDO activity with a subsequent Th2 imbalance in the immune response. Thus, my ***hypothesis*** is that RSV can infect human DC. This infection will induce IDO activity. Such RSV induced activation of IDO will modulate the immune response towards a Th2 phenotype (figure 1-5). There are several basic points in this hypothesis that need to be established first, including the question of IDO inducibilty by RSV. To address these, I approached the hypothesis by defining the following 3 specific aims.

Specific aims

My *in vitro* experiments will address the following goals:

Aim 1- To determine whether RSV can infect human DC and induces IDO activity.

Aim 2- To determine the mechanism of IDO activation by RSV.

Aim 3- To determine whether RSV-induced IDO activity can result in a decrease in Th1/Th2 ratio in favor of a Th2 profile.

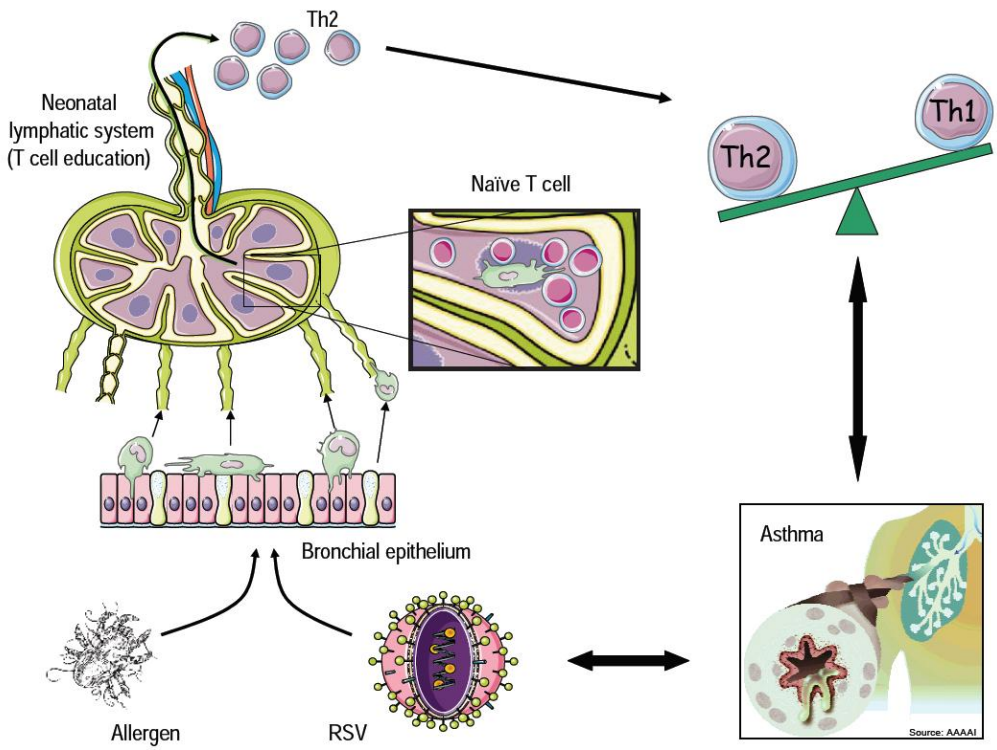

Figure 1-5- Co-exposure of the immune system to RSV and allergens will result in the development of a Th2-type immune response against the allergen. This effect is hypothesized to occur through the effect of immune-modulating enzyme, IDO. Activation of IDO in RSV infected cells promotes a microenvironment in lymphatic tissues of neonates that will favor apoptosis of Th1 cells, but not Th2.

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Chapter 2- CCL5 Persists in RSV Stocks Following Sucrose Gradient Purification

Introduction

Respiratory Syncytial Virus (RSV) is the most important cause of lower respiratory tract infection during infancy and childhood. Despite high levels of maternally transferred specific serum antibodies (1), 50% of children are infected in first year of life and all by age three.

Research on RSV requires preparation of a purified RSV stock, which can be a challenging task to perform the first time. Although there are studies outlining basic RSV purification procedures (2-5), to our knowledge, no studies exist that address the efficacy of the purification procedure. Specifically, there are no studies confirming the removal of potential contaminating pro-inflammatory mediators generated by the host cell infected by RSV during virus preparation in cell culture. The presence of such contaminants could give rise to false experimental results and others have shown concern for the presence of such contaminants in their virus stock (6).

Our objective was to establish the critical steps in the methodology of RSV purification. In addition, we established the fact that despite what may be considered as a reasonable purification method, the chemokine CCL5 (RANTES), was not removed from the virus preparation. CCL5 is recognized for its potent chemotactic effect in recruiting allergic inflammatory cells (7). Compared to other common airway viruses, RSV has been strongly associated with allergic inflammatory responses (8). We suggest that our data shed new light on our understanding of the association between RSV and allergic inflammation.

Materials and Methods

Cell culture and virus propagation: Mycoplasma-free HEp-2 cells (1.5×10^7 cells, ATCC CCL-23) were seeded on polystyrene tissue culture flasks (225 cm^2) 36-48 hours before the start of experiments. Cells were grown in complete DMEM

containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/L penicillin, 100 mg/L streptomycin and 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada). Recombinant enhanced green fluorescent protein-expressing Respiratory Syncytial Virus(rgRSV) (9) was used in some experiments (a generous gift from Drs. Peter L. Collins and Mark E. Peeples, The Ohio State University College of Medicine). A non-fluorescent, Respiratory Syncytial Virus strain A2 (ATCC VR-1540) (hRSV) was also used in place of the rgRSV. The use of the rgRSV improved our ability to quantify RSV using a plaque assay compared to non-fluorescent RSV. HEp-2 cell monolayers (at 50-70% confluence) were infected with a multiplicity of infection (MOI) of 0.1 to 0.2, previously suggested as an optimal concentration to avoid "defective interfering particles" (10). After one hour of incubation on a slow moving rocker at room temperature (RT) followed by one hour incubation at 37°C and 5% CO_2, cells were nourished with fresh complete medium containing 10% FBS and kept in incubator. After 24 hrs, 50% of the medium was replaced with fresh medium containing 2% FBS.

RSV harvesting: Cells were checked regularly for signs of RSV infection by fluorescence microscopy and/or syncytia formation. Cells were harvested after 48-72 hours when more than 50% of cells were detached from the cell culture flasks due to cytopathic effects. Using a cell scraper, the remaining attached cells were collected and pooled with the virus-rich supernatant.

RSV purification: To purify virus, we optimized a protocol based on previous original work (2, 3) (figure 2-1). All steps of the purification were carried out on ice or under cold conditions (4ºC) and, except for centrifugation, all work was performed in a biosafety hood under sterile conditions. The mixture of supernatant and cell debris was vortexed in a 50 mL conical tube for one minute and centrifuged at 3200g for 5 min. The virus-rich supernatant was collected and kept on ice. One aliquot was stored at -80 °C for cytokine/protein analysis (**sample A**) while a second aliquot was mixed with equivalent amount of 40%

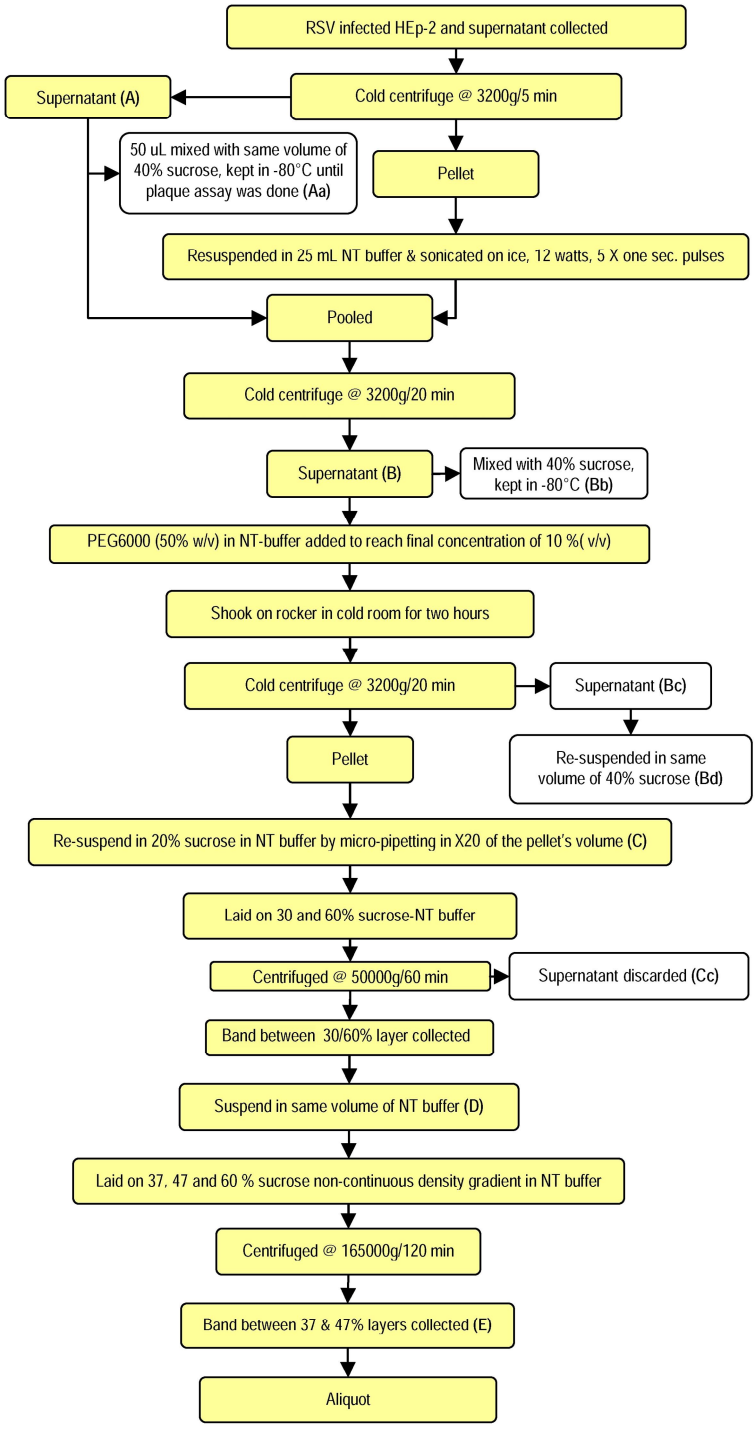

Figure 2-1. Purification of Respiratory Syncytial Virus using PEG concentration (×1) and Sucrose Density Gradient Ultracentrifugation steps (×2). Samples A, B, Bc, C, D and E collected for total protein and cytokine quantifications, while samples Aa, Bb, Bd, C,D and E were used for virus plaque assay.

sucrose (w/w) in NT buffer (150mM NaCl, 50 mM Tris-HCL, pH 7.5) and stored (-80 $^{\circ}$C) for virus quantification. Sucrose (a minimum of 20% concentration) was needed to ensure virus stability until virus titration assays were complete.

To increase virus titres, we detached cell-associated virus using sonication. The pellet was resuspended in 25 mL of NT buffer, which was sonicated at 12 watts for five consecutive 1-second pulses, after one minute of vigorous vortexing. We chose 12 watts based on a comparison of 8, 12 and 16 watts in a separate experiment (virus yield was 3.54×10^5 vs. 1.22×10^6 vs. 2.34×10^5 PFU/ml respectively, n=1). The sonicated cell suspension was remixed with the cell supernatant. To remove larger pieces of cell debris, the mixture was centrifuged (3200g for 20 min) and the supernatant separated from the pellet (**sample B**). Virus particles in the supernatant were pulled out of solution and concentrated using polyethylene glycol 6000 (PEG6000, Sigma-Aldrich) in NT buffer (50% w/v) followed by centrifugation (3200g for 20 min, temp). The pellet was diluted in NT buffer containing 20% sucrose (w/w) up to 20× of its original volume (**sample C**).

To remove all the contaminants with less than 1.13 g/cm^{3}density from the solution, the sample was layered on 30% sucrose (w/w) and ultracentrifuged (17000 RPM for 60 minutes, 4 ºC, SW40Ti swinging bucket rotor, Beckman XL-90 ultracentrifuge). This method can yield a dense pellet that is difficult to resuspend. To avoid this, we layered 1 mL of 60% sucrose in NT-buffer at the bottom of the ultracentrifuge tube to form a high-density cushion. An opaque band formed between 30% and 60% layer, which was collected and diluted with a similar volume of NT-buffer (with no added sucrose) (**sample D**).

Finally, this mixture was layered on top of a discontinuous gradient of 37, 47 and 60% sucrose in NT-buffer (corresponding to 1.16, 1.21 and 1.29 g/cm^3 density) and was centrifuged for two hours at 165,000g (40,000 RPM, 4 ºC, SW40Ti,

Beckman XL-90 ultracentrifuge). These densities were chosen due to high concurrence of RSV particles expected between 1.16 and 1.21 g/cm^3 levels (3, 4, 11). As predicted, a distinct visible band formed in the interface between 37% and 47%. This was collected and saved as the final virus stock (**sample E**) (figure 2-2).

RSV quantification: HEp-2 cells were seeded in 24-well plates (80,000 cells per well) 24 hr earlier, resulting in the desired 70% confluent monolayer (37ºC, 5% CO2) for the day of experiment. HEp-2 cells were chosen over Vero cells since they appear to form larger syncytial plaques needed for quantification (12). Ten-fold serial dilutions of the various sample conditions (A, B, C, D, E) were prepared in DMEM with 2% FBS and distributed in triplicate in wells with 70% confluent monolayer. Inoculated cells were incubated 1 hour on a rocker at RT and 1 hour at 37°C (5% CO_2). Infected cell supernatants were removed and the infected cells were topped up with complete DMEM containing 2% Carboxy Methyl Cellulose. After 24 to 48 hrs (37°C,5% CO_2), bright green spots were counted by fluorescent microscopy; each spot representing one Plaque Forming Unit (PFU). To confirm the accuracy of the fluorescent counts, plates were kept for up to seven days (37°C, 5% CO_2) allowing the typical syncytial plaques to mature. Cells were fixed (1% Gluteraldehyde, 1 hour at RT). Monolayers were stained with crystal violet (0.0375% w/v in water, 5 minutes). Syncytial plaques (seen as clearings without violet staining) were counted using 5× magnification.

Protein quantification: Protein concentration in various steps of purification was measured using "BCA Protein Assay Kit" (Pierce Biotechnology, Rockford, IL, USA). Before measuring protein content, "Compat-Able Protein Assay Preparation Reagent Kit" (Pierce Biotechnology) was used to eliminate interfering substances including sucrose and PEG from samples.

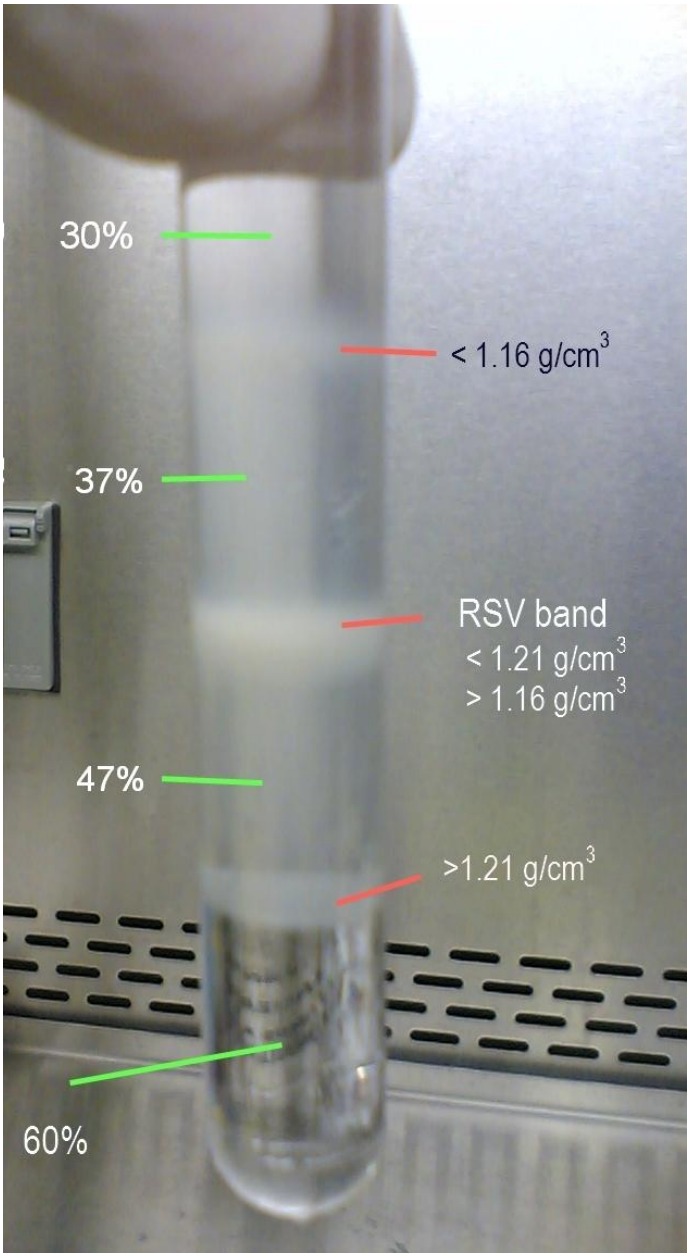

Figure 2-2. Shown is the image of final step in sucrose gradient ultracentrifugation of RSV preparation. The collected RSV-rich band is between 37% and 47% of sucrose (w/w) that corresponds to 1.16 and 1.21 g/cm^3 of mass density, respectively.

Cytokine/chemokine measurement: To verify that our modified protocol removed potential known cytokines and/or chemokines from the virus stock, we used commercially available cytokine testing service based on multi-array ELISA (Aushon Biosystems, Woburn, USA) to measure CCL5 (RANTES), TNF, and IFN-α. Samples were diluted ×10 in PBS containing 0.1% Human Serum Albumin to eliminate potential interference from sucrose or PEG in these measurements. We further confirmed the CCL5 concentration in sample E using a single-analyte ELISA assay (R&D Systems, Minneapolis, USA).

Western blot analysis for CCL5 protein: Our ELISA results in both the multiple array and the single-analyte assay indicated the presence of CCL5 in our purified RSV stocks (Sample E). We sought to determine whether this was possibly an error in the ELISA assay; possibly a false positive reading due to cross reaction with RSV proteins. Aliquots of **Sample E (**20 µl of 1:5 dilution) were loaded per gel lane and run through "Any KD Mini-PROTEAN Precast gel" (Bio-Rad, Mississauga, ON, Canada). Recombinant human CCL5 (R&D Systems, Minneapolis, USA, 10 ng/lane) was used as positive control for the assay. Loading buffer contained 5% β-mercaptoethanol (120 mM final concentration) and both samples and standards were denatured for 5 minutes at 95ºC before loading on to the gel. Following transfer of separated proteins, nitrocellulose membranes were blocked overnight (4°C) with Odyssey Blocking Buffer (Licor, NB, USA) and then incubated (1 hour, RT) with mouse anti-RSV Fusion protein (Millipore, Billerica, MA, USA) and goat anti-human CCL5 (R&D Systems, Minneapolis, MN, USA). Next, the membrane was probed for 45 minutes (RT) using donkey IRDye800-labeled anti-mouse IgG and donkey IRDye700Dx-labeled anti-goat IgG (Rockland Immunochemicals, Gilbertsville, PA, USA) antibodies and scanned for infrared signal using the Odyssey Imaging System (LI-COR Biosciences, Lincoln, USA). Precision Plus Protein Standars (Bio rad) was used to estimate the molecular weight of apparent bands.

Sequence comparison analysis of RANTES and RSV proteins. To further determine whether the CCL5 ELISA was cross reacting with an RSV epitope, we analyzed sequence identities of the 11 RSV proteins compared to human CCL5 (Protein Database at NCBI). The Protein Database at NCBI is a compendium of various information sources including protein sequence as well as translation of DNA/RNA coding sequences (SwissProt, PIR, PRF, PDB, GenBank, RefSeq, TPA). We analyzed RSV proteins NS1 (Protein Database access number NP_056856.1), NS2 (NP_056857.1), Nucleocapsid (gp03, NP_056858.1), Phosphoprotein (gp04, NP_056859.1), Matrix protein (gp05, NP_056860.1), Small Hydrophobic protein (gp06,NP_056861.1), Attachment protein (G-protein, NP_056862.1), Fusion protein (F-protein, NP_056863.1), Matrix protein 2 (gp09, ORF1, NP_056864.1), Matrix protein 2 (gp09, ORF2, NP_056865.1) and Polymerase protein (NP_056866.1). We compared these proteins against CCL5-precursor reported sequence (NP_002976); CCL5 precursor protein comprises both the signal peptide (aminoacids 1-23) and the active peptide (aminoacids 24-91) for CCL5. Thus, the complete sequence of both the precursor and the active forms of CCL5 were compared with RSV proteins. Sequence analysis was done using the online SIM Alignment Tool for protein sequences from the Swiss Institute of Bioinformatics (http://ca.expasy.org/tools/sim-prot.html), based on a previously reported informatic algorithm (13).

Statistical analysis: Each value of *n* represents one purification experiment. Results are reported as average of values ± Standard Error of Mean (mean±SEM) except in few instances where the Median proved to be a better representative. Data were analyzed by the Kruskal-Wallis test with Dunn correction to identify statistical differences between means, using GraphPadPrism version 3.0 (GraphPad Software Inc., La Jolla, CA, USA). P values >0.05 were considered as non-significant.

Results

The concentration of RSV increases with each step of purification: Each step in the protocol was measured for the amount of RSV using a plaque assay. **Sample A** was the initial reference point for virus titre ($2.3\pm1.24 \times 10^7$ PFU/mL) (Table 2-1 and figure 2-3). The addition of cell-associated virus (**sample B**) increased the virus yield by 26.4%±5.9 (figure 2-3, $p<0.05$). Approximately half (52±1.3 %, n=3) of the amount of virus in this large volume of media was precipitated in the pellet of the PEG concentration step. A small amount of RSV was lost through the discarded supernatant of the PEG concentration step (1.6±0.31 %, n=3) (**sample Bc**). Consistent with previous reports, total virus yield continued to drop in the next two steps of the sucrose purification (Figure 2-3). The final recovery in step E was 25±7.3 % of the initial harvest.

The concentration of total protein had a declining trend throughout the purification steps: The amount of total protein was measured in each step of purification. The supernatant of HEp2 cells infected with RSV showed the highest measured value (1310±159 µg/mL; Table 2-1). However, this value was reduced by 50% when measured by the last step of purification (638±60 µg/mL).

Unlike other cytokines, CCL5 cannot be removed using sucrose gradient purification: We determined the quality of our purification method in relation to removal of contaminating proteins (cytokines/chemokines), as measured by ELISA at each step. TNF (49.4±8.4 pg/mL) and IFN-α (7.55±1.0 pg/mL) were detected in supernatant of infected Hep-2 cells (**sample A**), but their levels fell below detection limits following the PEG concentration step (**sample C**). As expected, CCL5 was also present in the **sample A** (15,820±1395 pg/mL, n=3), but despite discarding 99.4 ± 0.39% of the volume in the PEG step (sample Bc), CCL5 levels remained elevated in the pellet. The total amount of CCL5 before the PEG (sample B) was 1624±812 ng (volume multiplied by concentration). Only

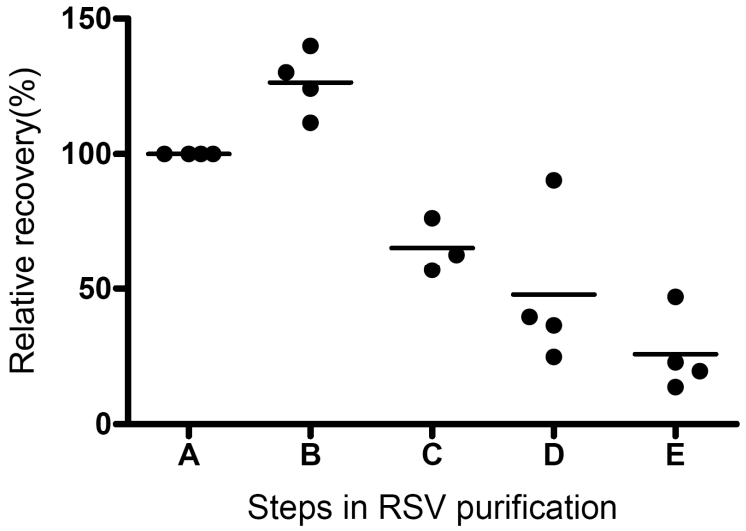

Figure 2-3. Relative recovery of RSV in consecutive steps of the purification procedure. Each dot represents the total amount of virus (pfu) in a given volume (mL) per step of the experiment (total volume collected in mL) × virus titer (pfu/mL). The calculated values of the initial harvests (A) were assigned a normalized ratio of 100%. For downstream purification steps (B to E), the values shown were calculated and normalized compared with the initial harvest (A).

	Steps in viral purification				
	A	B	C	D	E
Relative infectivity [a]	1	0.887	6.90	5.00	5.95
Absolute infectivity ($\times 10^7$)(PFU/mL)[b]	2.298 (±1.24)	2.608 (±1.6)	17.65 (±15.7)	45.68 (±39.8)	27.95 (±23.8)
Protein concentration (μg/mL) [b]	1310 (±159)	1249 (±105)	822 (±100)	441 (±114)	638 (±60)
Infectivity/total protein ($\times 10^4$)(PFU/pg) [a]	0.895	1.13	2.558	15.9	10.7

[a] Numbers represent median of the values for three separate experiments. Infectivity in step A is considered as start point and all others are compared to A.
[b] Numbers represent mean (±SEM) of the values for three separate experiments

Table 2-1- Changes in absolute and relative concentration of RSV particles in different steps of purification.

41.0±10.9 % of this total CCL5 was eliminated in the PEG supernatant (785±526 ng; sample Bc). After resuspending the PEG pellet (a 20 times dilution), CCL5 levels actually increased to an average of 61,784±18,722 pg/mL. Extrapolating these values based on the dilution, the levels of CCL5 in the virus-rich pellet at PEG step were 112 ± 38 fold greater than in the initial harvest (Step A). Despite sucrose purification steps, CCL5 levels remained elevated at 5,301±2,491 pg/mL (sample E).

Sensitivity of Western blot analysis was not enough to detect CCL5 in the RSV stock: We used Western blot analysis to determine whether our ELISA data were correct. Recombinant human CCL5 (10 ng/lane), used in the assay as standard, was detected with a clear band corresponding to about 9 *KD*. F protein of RSV was detected as a band of about 72 *KD* in Sample E. We were not able to see any signal in the predicted size range of CCL5 (figure 2-4A, n=3). To elucidate the detection limit of the in-house anti-CCL5 antibody and Western blot technique, we ran a slot blot analysis of different amount of CCL5. Unfortunately, values below 3 ng/lane are not in the detection limit of Western blot analysis (figure 2-4B).

RANTES and RSV do not share similarities in protein sequence: We considered the possibility that a protein derived from RSV resembles CCL5, thereby generating false positive results in our ELISA-based assays. Our sequence analysis on human CCL5 compared to RSV proteins NS1, NS2, Nucleocapsid (gp03), Phosphoprotein (gp04), Matrix protein (gp05), Small Hydrophobic protein (gp06), Attachment protein (G-protein), Fusion protein (F-protein), Matrix protein 2 (gp09, ORF1 and 2) and Polymerase protein yielded less than 50% sequence homology in any 10-amino acid (aa) length fragments analyzed for any combination.

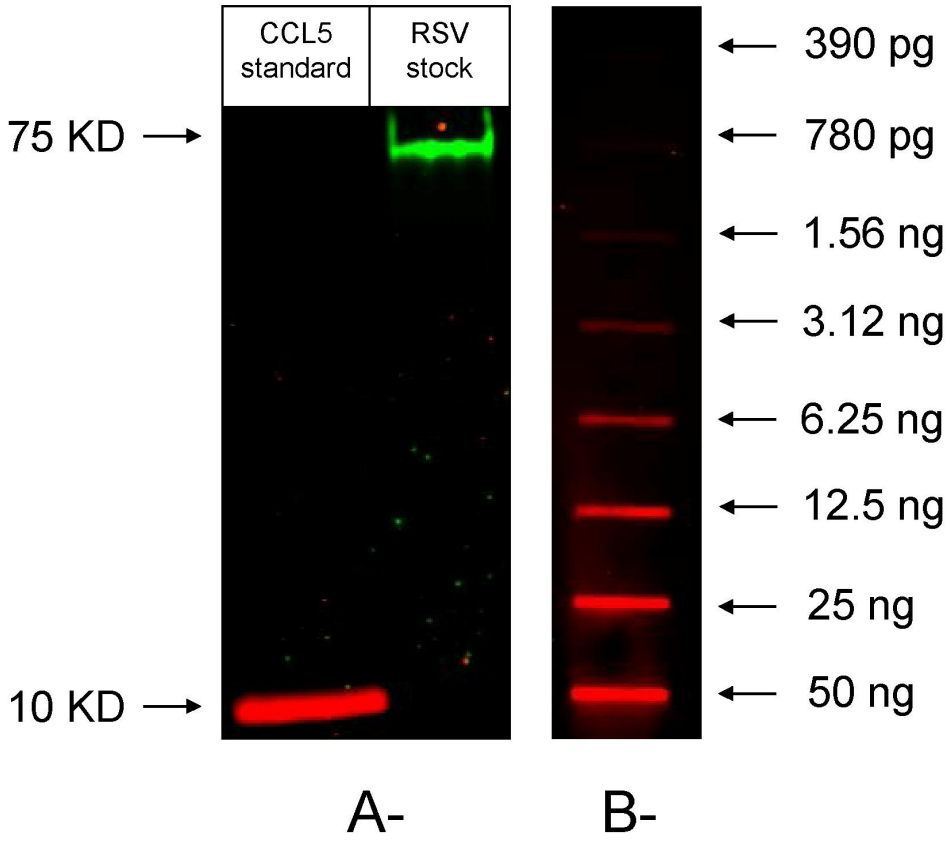

Figure 2-4. Western blot analysis of RSV stock (sample E) for detection of CCL5. A- 20 uL of either 1:5 RSV stock dilution or CCL5 standard (containing 10ng/lane) were loaded to each well. The nitrocellulose membrane was incubated with anti-RSV Fusion protein and goat anti-human CCL5 before scanning for infrared signal using the Odyssey Imaging System. Western blot was not able to detect CCL5 in RSV sample. B- Slot analysis of CCL5: serial dilutions of CCL5 standard were loaded to different slots on a nitrocellulose membrane prior to incubation with anti-CCL5 antibody and subsequent probing with donkey anti-goat IgG (IRDye700Dx). Values of CCL5 less than 3 ng/lane were not detectable by Western blot analysis.

The highest identity with CCL5, found in segments of 10 or more aminoacids were for the Polymerase protein (5 out of 11 non-consecutive aminoacids, 45%) and NS2, Phosphoprotein, Small Hydrophobic protein and G-protein (4 out of 10 non-consecutive aminoacids, 40%). The longest sequence of contiguous aminoacids with 100% identity was 4, and it comprises 2 regions in the Polymerase protein (CCL5 precursor protein aminoacids 51-54, compared with RSV Polymerase protein aminoacids 1668-1671, and CCL5 aminoacids 69-72 to Polymerase aminoacids 1277-1280) and one in the Matrix protein (CCL5 aminoacids 86-89, compared with Matrix protein aminoacids 175-178). In any event, these values were considered too low for non-specific antibody cross-reactivity due to sequence homology.

Discussion

The initial intent of this work was to understand and confirm the importance for each step in the methods of RSV purification. Purification of RSV, using sonication to release undetached viral particles from cell debris had a remarkable effect on virus-enrichment; approximately 25% improvement compared with using a freeze-thaw cycle. This became a critical step in purification, since we started with the highest titer of virus possible. Thereafter, it was inevitable to observe a significant loss of RSV yield during the ensuing purification steps; the longer the procedure, the more virus loss was seen. The largest loss was following the PEG concentration step, used to bind virus and pull it out of the dilute solution into a pellet. While it appears that the PEG did not capture approximately half the virus(sample C), only 1.3 % of the virus was measured in PEG supernatants. Therefore, the loss of half of total RSV at the PEG concentration step does not appear to be a failure of PEG to capture the virus. Further, we observed at each subsequent step of purification approximately 50% loss of virus. Thus, we hypothesize that these drops in virus titer were not due to loss of virus particles, but rather to loss of functional (infectious) virus. Unlike many other viruses, RSV loses function in room temperature, and to a lesser extent in ice-cold conditions. Even at 4°C, there is loss of virus infectivity compared to minus 80° (14). Further, it is well known that for long term storage, RSV needs a stabilizing agent (e.g. sucrose) to prevent loss of virus infectivity (14). Despite having sucrose in Steps D and E, significant losses were observed. Therefore, we suspect that the lability of RSV from thermal damage may play a major role in the loss of virus titre.

Despite the loss of virus titre, the advantage of this purification process is the ability to concentrate RSV into the smallest volume possible. Under typical *in vitro* experimental conditions, it is important to achieve the required multiplicity of infection (MOI) without adding unnecessary medium. Such media could have unwanted factors released from the infected cell line used to propagate the virus. Due to large reductions in volume at each step, the relative and absolute

concentration of viral infectivity increased successively (Table 2-1). As a result, the concentration of total protein also declined (Table 2-1). An important determinant of the quality of virus purification is the net ratio of infectivity to total protein (PFU/pg). We observed the intended goal of combining increasing virus titers with decreasing total protein following each purification step (Table 2-1). This ratio was almost 18 times higher in sample D in comparison to the initial harvest. We believe that the small loss of RSV infectivity in the last step of purification may be due to the dilution of the virus with sucrose, not present in the previous step. This dilution is difficult to avoid if one wants to isolate the entire purified band of virus. Overall, when planning to create a virus stock, it is important to optimize procedures for the highest viral unit per volume, minimizing the inevitable loss of virus titer during purification process. We believe this was accomplished.

We used sucrose as a density gradient rather that iodixanol (4). Given our interest in co-culturing RSV with live immune cells (3), we considered sucrose to be a better alternative, based on the current lack of knowledge as to the immunological effects of iodixanol (15). Although there still could be some concern about the osmotic effect of sucrose, we have calculated that at 1:100 dilution of RSV, the resultant osmolarity in culture media would be within a physiologically normal and acceptable range (20%-sucrose in a 1:100 dilution equals a 6.3 mM increase in osmolarity for a 280-300 mOs/L culture media).

To study efficacy of sucrose gradient purification of RSV in eliminating contaminating pro-inflammatory chemokine/cytokines, we tested TNF, IFN-α, and CCL5 as some examples of a broad range of chemokine/cytokines that may be induced by RSV in HEp-2 cells (16, 17). Our TNF and IFN-α data showed that the PEG concentration step alone was sufficient to remove these cytokines. In contrast, the PEG step not only failed to reduce CCL5 concentration, but in contrast increased the concentration of CCL5 over 100-fold (112 ± 38, n=3) in the virus rich pellet compared to sample B (this number was extrapolated from

readings at sample C). Further, the sucrose steps failed to remove CCL5. It is important to consider that the several steps of dilution, which occurred during the detailed purification process, did not reduce the CCL5 levels. Putting this concept into numbers, the initial harvest was diluted by a factor of 425 ± 19 (n=3) by the last step of purification. Given this, we mathematically predicted that the concentration of CCL5 at step B would decrease by more than 400 fold by step E. Instead, the CCL5 levels were concentrated similar to the RSV. This observation suggests that the majority of CCL5 is not virus-free, but somehow is associated with RSV particles (figure 2-5).

We also considered the possibility that RSV proteins may share sequence homology with CCL5, leading to a false positive result from the ELISA-based assays. In fact, sequences of human CCL5 and RSV proteins showed less than 50% homology in any 10-aminoacid length fragment analyzed for any combination. Considering that the longest sequence of identical contiguous aminoacids was 4, and it involves RSV proteins not associated with the surface of the viral particle (the RNA-dependent RNA-Polymerase and one of the Matrix proteins) these values were considered too low to account for non-specific cross-reactivity due solely to sequence homology. There is a possibility that secondary and tertiary structures, three-dimensional folding and interactions between RSV proteins may result in CCL5-like structures. However, such an event is even more unlikely if we take into account the fact that similar results were obtained in 2 different ELISA kits, which are based on antibodies from different sources. However, we acknowledge that *in silico* sequence analysis alone is insufficient to rule out such possibility.

It is well established that host cell proteins can be incorporated into RSV particles during virus formation (18). This experience is not unique to RSV, as other viruses including human immunodeficiency virus 1 (19) or influenza (20) have shown a similar ability. In a more recent report, almost 25 host cell proteins have

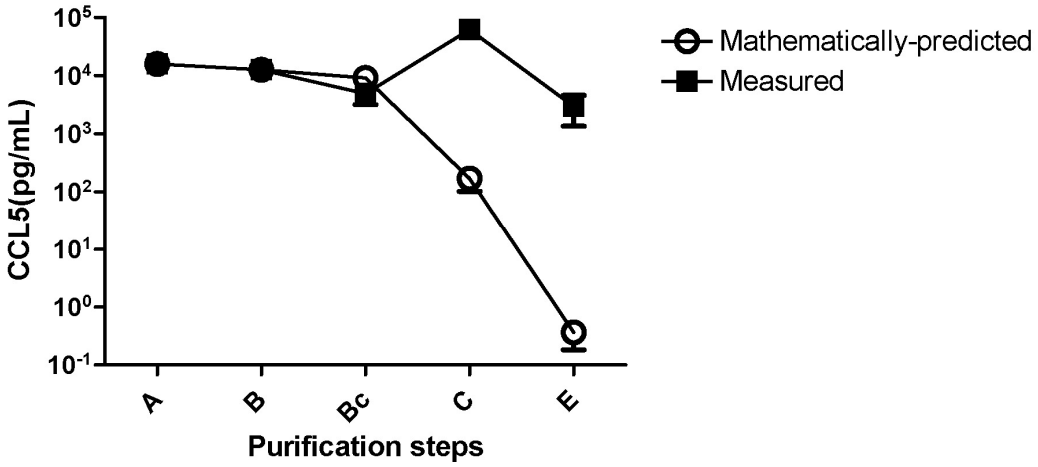

Figure 2-5. Measured concentrations of CCL5 at different purification steps plotted against a mathematically-predicted pattern of free running molecules. Throughout purification, there were several steps to dilute a typical soluble low molecular weight substance (>400×) from step A to step E (dilution factor: 425 ± 19, n=3). If CCL5 behaved like a free-running molecule, the mathematically-predicted concentrations of CCL5 are shown from step B to step E (open circles). In contrast, filled squares show the ***measured*** levels of CCL5 per purification step (n=3). Error bars represent SEM.

been detected in a purified RSV stock (21). Incorporation of host cell proteins span the range from proteins involved in cell signaling (22) to those of cellular skeleton, (e.g. actin) (23). It has been rationalized that those proteins with a role in virus assembly and maturation may get inserted into viral particles or remain attached to viral progeny following release from the host cell (21). During purification of virus, these proteins also become purified, since they form part of the assembled virus. It remains unclear whether CCL5 is a part of the RSV capsule, is attached, or forms multimers that share similar density as that of RSV. To our knowledge, this is the first time that CCL5 is suggested as a host cell protein co-purified with RSV.

It is well known that negatively charged glycosaminoglycans (GAGs) like heparan sulfate on cell membranes play important roles in binding to and entrance of many viruses to target cells (24). RSV is among those viruses where heparin-binding domains on its F and G proteins interact with GAGs on target cells. Blocking these interactions with soluble heparin diminishes RSV infectivity (25-27). It is reported that CCL5 binds with selectivity to GAGs and with highest affinity to heparin (28). Treatment of epithelial cells with recombinant CCL5 (5-20 μg/mL) is reported to inhibit RSV infection *in vitro* (29). Antibodies against CCR1, CCR3, CCR4 or CCR5 (receptors for CCL5), did not inhibit such infection. We speculate that the exogenous CCL5 in the study may have competed with CCL5 present on RSV. Exogenous CCL5 could attach to heparan sulfate on the cell surface, preventing access for the CCL5 on the RSV envelope; this could impair binding of RSV and infectivity. It is reported that the Ectromelia Virus E163 (a murine poxvirus pathogen) counter-acts the anti-viral action of chemokines, by producing a glycoprotein, which blocks GAG-binding epitopes of different chemokines (30). With our suggestion that CCL5 is part of the RSV capsule, the role of CCL5 may be to help RSV attach and enter target cells through CCL5 affinity to heparan sulfate on cell membranes. This novel role for CCL5 incorporated to RSV may be an independent mechanism for RSV

attachment or additional to the role of heparin binding domains on RSV's F and G proteins.

It should be emphasized that despite the failure to remove *all* contaminating CCL5, we did obtain a high infectivity to CCL5 ratio (PFU/pg) (Figure 2-6). This high ratio creates an RSV stock with high MOI but minimal contaminating chemokine. At a 1:100 dilution of the stock, which is a typical dilution in our experimental settings, values of CCL5 are below 100 pg/mL. This would be below the minimum concentration of CCL5 reported with potential effect (31).

In conclusion, our study provides details of the necessary steps required for a standard method of RSV purification using a sucrose gradient. Further and by serendipity, we determined that despite what would be considered reasonable purification methods, a significant level of CCL5 (RANTES) persisted in the virus preparation. We propose, therefore, that researchers using RSV should be aware of this "contamination". Beyond purification, these data may also shed new light on the association of RSV with allergic disease. CCL5 is a critical chemokine in allergic inflammation including chemotaxis of effector cells and T cell activation (32-35). RSV is reported to induce a more allergic like inflammatory response compared to other airway viruses. Though the exact mechanisms are not yet defined, high levels of CCL5 in RSV bronchiolitis infants are associated with increased risk of recurrent wheezing in later childhood (33-36). If RSV and CCL5 are biologically linked, then our data may have significant impact on our understanding of the biology of RSV in the context of allergic airway diseases.

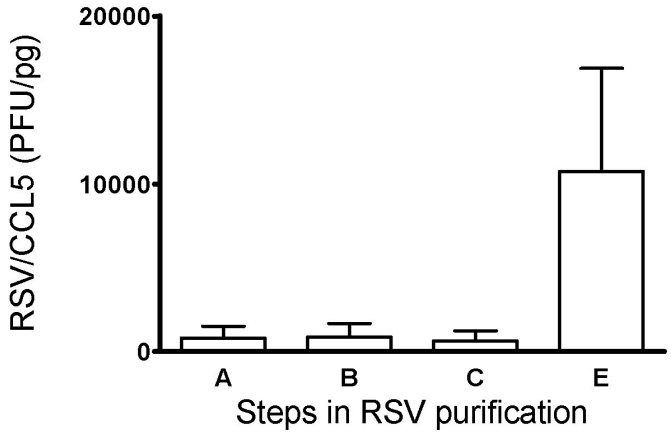

Figure 2-6. Ratios of RSV concentration in different steps (PFU/mL) to CCL5 concentration (pg/mL) in corresponding samples. CCL5 was measured by ELISA at each purification step (n=3). Error bars represent SEM.

References:

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Chapter 3- Infection of Human Dendritic Cells by Respiratory Syncytial Virus Induces Th2 Imbalance Through Indoleamine 2,3-Dioxygenase

Introduction

Respiratory Syncytial Virus (RSV) is one of the leading causes of lower respiratory tract (LRT) disease during infancy and early childhood. In the United States alone there are up to 125,000 hospitalizations per year due to RSV among children less than 1 year old (1). Despite high levels of maternally transferred specific serum antibodies (2), 50% of infants are infected in the first year of life and all children by age three. RSV usually manifests as mild cold-like symptoms in young children and adults, but 30-70% of infants it can present with a more severe breathing disorder called bronchiolitis (3). Severe RSV bronchiolitis in infancy resulting in hospitalization, is considered a risk factor for the development of allergic asthma later in early adolescence (4). As such, there is interest in preventing or delaying the incidence of severe RSV disease beyond the first 6 months of life in the hopes of diminishing subsequent complications including the likelihood of developing allergic asthma (5).

To determine a potential mechanism for this link between RSV and the development of allergic asthma, we investigated the potential role of the enzyme indoleamine 2,3-dioxygenase (IDO) in promoting an allergic-type, Th2-biased immune response. IDO is the rate-limiting enzyme in the extrahepatic oxidative catabolism of tryptophan. IDO activation both *in vivo* and *in vitro* has been reported to modulate the immune response, including the establishment of a Th1-Th2 imbalance favoring Th2-like responses (6, 7). IDO induces apoptosis in proliferating T cells, preferentially in Th1 versus Th2 cells (6, 7). IDO-induced preferential apoptosis of Th1 leads to a net change in Th1/Th2 ratio. This immune modulation effect could be the result of tryptophan depletion itself and/or production of the tryptophan catabolites, kynurenines, within this milieu (8). The reason why Th1 cells, but not Th2 cells are selectively targeted remains unclear.
It is widely accepted that the dominant immunologic microenvironment in allergic asthma is skewed toward Th2. Human dendritic cells (DC) are important for their

role in antigen presentation(9). DC can be induced to express IDO activity, though this has not been reported for RSV.

It is conceivable that an increase in IDO activity in immune cells during RSV infection could result in a skewed immune response toward Th2. If this IDO activity occurs during a window of opportunity in infancy, when the infant's immune system is learning how to respond to antigens/allergens, this may form the basis for the Th2 polarization observed in infants that develop allergic asthma later in life.

Human dendritic cells (DC) are immunologically important players in viral immunity (9). We approached this with the premise that IDO expression in DC and its bioactivity may be involved in various immunologically-regulated conditions including RSV bronchiolitis and the development of allergic asthma. Classically, induction of IDO in monocyte-derived dendritic cells (moDCs) occurs following stimulation by interferons, especially IFN-γ (10). The pleotropic cytokine, IFN-γ, is produced mainly by the natural killer (NK) lymphocytes and activated T cells as part of their antiviral responses (11). We hypothesized that RSV induces IDO expression and bioactivity in human moDC, *in vitro*. Further, we hypothesized that this activation in turn contributes to a Th2-type response in co-cultured human T cells. In this report, we demonstrate that RSV induces IDO activity in human moDC, and, the activation appears to be RSV-replication dependent, but IFN-γ-independent. Indeed, IDO induction suppressed Th1-like responses in activated human T cells, *in vitro*.

Materials and methods:

Culture media and reagents: Dendritic cells were cultured in RPMI-1640 (Sigma-Aldrich, Oakville, Ontario, Canada) supplemented with 2 mM l-glutamine, 10% FBS, 100 U/L penicillin, and 100 μg/L streptomycin (complete medium). For culture of the THP-1 monocytic cell line (TIB-202™-ATCC®), complete medium was supplemented with 4.5 g/L glucose, 1 mM sodium pyruvate and 0.05 mM 2-mercaptoethanol. For culture of HEp-2 epithelial cell line (CCL-23™-ATCC®), RPMI-1640 in complete medium was replaced with DMEM (Sigma-Aldrich, Oakville, Ontario, Canada) and supplemented with 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. In experiments with virus, media were supplemented with 2% FBS in place of 10% for the first two hours of the incubation time. Recombinant human IL-4, IFNγ, IFNα, TNF and GM-CSF were purchased from R&D Systems (Minneapolis, MN). Ligands of TLR3 and TLR7/8 (PolyIC and R848 respectively) and antagonist of TLR4 receptor, LPS-RS, were purchased from Invivogen (San Diego, CA). Anti-human IFN-γ antibody (Cat. No. AF-285-NA) and monoclonal anti-human TNF (Cat. No. MAB2101) were purchased from R&D Systems (Minneapolis, MN). Mouse anti-human Indoleamine 2,3-dioxygenase monoclonal antibody (clone 10.1, Cat. No. MAB5412) was purchased from Millipore (Billerica, MA) and mouse monoclonal anti-human IFN-α (Cat. No. 21105-1) was purchased from PBL interferonsource (Piscataway, NJ). *L*-tryptophan (Cat. No. T8941), 1-Methyl-*DL*-tryptophan (1MT)(Cat. No. 860646), Menadione (Cat. No. M5625), Ribavirin (Cat. No. R9644), Chloroquine diphosphate (Cat. No. C6628), 4-(Dimethylamino) benzaldehyde (Ehrlich reagent) (Cat. No. D2004) and *L*-Kynurenine (Cat. No. K8625) were obtained from Sigma-Aldrich. Stock solution of menadione (100mM) was prepared in DMSO and kept at -20ºC and in the dark. Stock solution of *L*-Kynurenine (50mM) was prepared in 0.5M HCL and kept in dark. Palivizumab (Synagis®) was provided by MedImmune (Gaithersburg, MD). WST-1 (Cell Proliferation/cytotoxicity reagent) was purchased from Roche Applied Science (Laval, Quebec, Canada).

Cell lines: THP-1 and/or HEp-2 cells were used as a positive control for IDO induction in some experiments. We also used HEp-2 for viral titration (plaque assay), preparation of RSV stock and as a prototype of epithelial cells in the inducibility of IDO by RSV in such cells (see below).

Preparation of primary human DC: Peripheral blood was collected into heparinized tubes by venipuncture from healthy adult donors. All subjects gave written informed consent approved by the University of Alberta, Health Research Ethics Board. After sedimentation of red blood cells in 6% dextran for 30 min at RT, the leukocyte-rich supernatant was collected and subjected to density gradient centrifugation on Ficoll-Paque™ PLUS (Cat. 17-1440-02, GE healthcare, Quebec, Canada). The buffy coat layer containing mononuclear cells was collected on a Ficoll gradient, washed twice and incubated in 6 well plates (about 10^7 cells/well in RPMI-10%FBS) for two hours at 37°C and 5% CO_2. Non-adherent cells were collected and, in some experiments, were enriched using a Human T Cell Enrichment Kit (Cat. No. 19051, Stemcell Technologies, Vancouver, BC, Canada). Remaining adherent cells in 6-well plates from the previous step were washed vigorously with RPMI and incubated for 6 days (37°C, 5% CO_2) with GM-CSF (20ng/ml) and IL-4 (12.5 ng/ml) (12). This regimen differentiated monocytes into myeloid dendritic cells (moDC) with a purity of >95% confirmed by flow cytometry (13).

Preparation of RSV stock and measurement of RSV titre: Briefly, monolayers of mycoplasma-free HEp-2 cell were grown up to 60-70% confluence in T-225 flasks. Cells were infected with either a recombinant enhanced green fluorescent protein (GFP)-expressing Respiratory Syncytial Virus (rgRSV) (14) (a generous gift from Drs. Mark E. Peeples and Peter L. Collins) or human Respiratory Syncytial Virus (hRSV) (strain A-2, ATCC VR-1540) each with MOI of 0.1 for 1 hour incubation at RT and 1 hour at incubator (37°C and 5% CO_2). After inoculation, cells were nourished with fresh complete media (FBS 10%). After 24

hrs, media were changed and infected HEp-2 cells were grown in medium containing 2% FBS. RSV cultures were harvested when more than 60-70% of the HEp-2 monolayer showed signs of cytopathy and syncytia formation. After one step concentration with polyethylene glycol, the harvested material was subjected to two steps of sucrose discontinuous gradient ultracentrifugation. The opaque band formed between 37% and 47% of sucrose (w/w) were collected and kept frozen at -80°C for future use in experiments.

Quantification of RSV by plaque assay: HEp-2 cells were seeded in 24-well plates (80,000 cells per well) the day before, resulting in the desired 70% confluent monolayer (37ºC, 5% CO2) for the day of experiment. Serial dilutions of samples were prepared in DMEM with 2% FBS and distributed in triplicate in wells with 70% confluent monolayer. Inoculated cells were covered with complete DMEM containing 2% Carboxy Methyl Cellulose. After 24 to 48 hrs (37°C, 5% CO2), the presence of bright green spots were counted by fluorescent microscopy; each spot representing one Plaque Forming Unit (PFU).

Infection of moDC with rgRSV: rgRSV (MOI 0.1-20) was added to moDC in RPMI containing 2% FBS (1 hour at RT on a slow rocker; then 1 hour at 37°C, 5% CO_2). Complete medium with 10% FBS was then added to reach the final concentration of 10^6 cells/ml. Following various measurements of different multiplicity of infection (MOI) (0.1-20) we determined the optimized condition of the experiments in terms of infection, IDO induction, and viability of cells in culture. MOI of 1 was chosen. RSV infectivity was blocked with UV-inactivation (UV light for 20 min on ice, 8-watt lamp, located 5 cm above the tube containing the virus), with Palivizumab (a humanized monoclonal antibody directed against an epitope in the F protein of RSV; 200 ug for 10^6 PFU of RSV, 15 min on ice), or with Ribavirin (a competitive inhibitor of viral RNA polymerase, 220 uM).

Measurement of IDO bioactivity: To measure IDO bioactivity, supplemental *L*-tryptophan was added to the medium to a final concentration of 150 μM

(conditioned medium). Culture supernatants of moDCs were collected five days post infection (stored at -20 ºC). Five days was chosen based on data that IFN-γ induces IDO expression in fibroblasts up to 192 hrs (15). Supernatants were de-proteinized with the same volume of 30% trichloroacetic acid in ddH_2O (w/v). Samples were vortexed and centrifuged at 14,000 G for 10 min at 4ºC. Samples were thawed and the kynurenine component of the supernatant was measured by a spectrophotometric method using the Ehrlich reagent at an absorption of 490 nM. Kynurenine concentration values were extrapolated from a standard curve using commercial L-kynurenine (16). All experiments were carried out in duplicates or triplicates.

Flowcytometry: To determine infection with rgRSV, GFP expression was measured in moDC, or HEp-2 cells after fixation with 4% paraformaldehyde on ice for 10 minutes. Cells were washed with cold PBS and resuspended in flow buffer. For cells infected by non-GFP RSV, fixed cells were incubated with mouse anti-RSV Fusion protein (Cat. No. MAB8599, Millipore, Billerica, MA, USA). To evaluate intracellular expression of IDO, cells were fixed with 2% paraformaldehyde and permeabilized using 0.5% saponin in flow buffer. Mouse IgG3 isotype control (R&D, MAB007) and mouse anti-human IDO (Chemicon, MAB5412) were used as primary antibodies. APC-labeled Goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, 115-135-209) was used as secondary antibody (5ug/ml). Samples were read on BD FACSCalibur™ flow cytometer machine. Results were analysed using WinMDI (V2.8).

Assessment of infection by live-cell confocal microscopy: We observed moDCs alone (i.e., without rgRSV) or moDCs incubated with rgRSV for morphology (differential interference contrast (DIC) images) and expression of GFP (excitation and emission wavelengths: 488nm and 520nm, respectively) using a laser-scanning microscope (Olympus FLUOVIEW FV1000 Confocal Microscope and FV10-ASW viewer V1.3b, Olympus, Markham, Ontario, Canada).

Cytokine measurement: Cell supernatants collected one day post infection were kept frozen at -80°C for subsequent measurement of TNF, IFN-γ, IFN-α, CCL-5 (RANTES), IL-12p17, IL-4 and IL-13 levels using a commercially available "Searchlight® Protein Array Analysis" service provided by Pierce Biotechnology (Woburn, MA).

Inhibitors of IDO: 1-Methyl-DL-tryptophan (1MT) is an analogue of tryptophan and a competitive inhibitor of IDO (17). Stock solutions (3.2 mM) of 1-MT were prepared in RPMI (the maximum level of solubility for 1-MT in water is 6 mM (17)). Menadione (2-methyl-1,4-napthoquinone otherwise known as Vitamin K_3) is an alternate inhibitor of IDO with a low micromolar potency (with *in vitro* IC_{50} and LD_{50} values of 28.9 and 126 μM, respectively) (18, 19).

Cell signaling pathways in RSV-induced IDO activation: To evaluate the mechanism of RSV-induced IDO activation, we studied the potential intracellular signaling pathways reported for IDO activation. We used a variety of specific inhibitors from Calbiochem (a Brand of EMD Chemicals Inc., an affiliate of Merck KGaA, Darmstadt, Germany) including a MEK inhibitor I (IC_{50}=12nM; Cat. No. 444937), a MEK inhibitor II (IC_{50}=380nM; Cat. No. 444938), a potent cell-permeable inhibitor of p38 MAP kinase, which has no effect on the activity of the ERK or JNK MAP kinase subgroups (SB202190, (IC_{50} =350nM; Cat. No. 559397), a JNK inhibitor II (SP600125, IC_{50}=40~90nM; Cat. No. 420128), a selective and irreversible inhibitor of IKKα and IKKβ kinase activities that inhibits NF-κB-mediated gene transcription and does not affect the activities of p38 MAP kinase (IKK inhibitor II Wedelolactone; IC_{50} < 10 μM, Cat. No. 401474), an inhibitor of IKKβ (IKK inhibitor III, BMS-345541, IC_{50}=300nM) and appropriate negative controls. To investigate the role of TLR-4 signaling we used an antagonist of TLR-4 receptors, rsLPS (Cat. tlrl-rslp, Invivogen, San Diego, California). To study RIG-I-like receptors we used an inhibitor of TBK1/IKKε, which are downstream signaling kinases in RIG-I/MDA5 induction (BX795; Invivogen, Cat. tlrl-bx7). All inhibitors and vehicles were measured in a dose-

response manner for their cytotoxicity using WST-1 cell cytotoxicity reagent. Working concentrations of the inhibitors were kept well below cytotoxic values.

Transwell assays to measure T cell responses: To determine if RSV can alter T cell responses, we employed a transwell system. A purified population (>95%) of T cells was prepared using the EasySep® Human T Cell Enrichment Kit (Cat. No. 19051, Stemcell Technologies, Vancouver, BC, Canada). T cells were initially activated in a separate plate using anti-CD2/CD3/CD28-loaded microbeads (T Cell Activation/Expansion kit; Cat. No. 130-091-441, Miltenyi Biotech, Auburn, CA). On day 6, some moDCs were infected with RSV in the lower chamber of a 12-well transwell plate (10^6cells/1.2ml) with or without 1MT. After 24 hrs, 8×10^5 of the previously activated T cells were placed in inserts (membrane pore size 0.4 um; Cat. No. 3460, Corning Incorporated, Corning, NY); the inserts were placed in wells with moDC from different experimental pretreatments. The IDO inhibitor, 1MT, was added (final concentration of 320μM) to relevant wells. In some experiments, 8×10^5 of the T cells along with 4×10^5 of anti-CD2/CD3/CD28-loaded microbeads were placed in inserts with or without addition of 1MT (320 μM). After four days, the T cells in inserts were collected. We stained 1×10^6 cells per condition for CD3 (Cat. No. 17-4724-42, ebiosciences), permeabilized for 18-24 hrs in a fixation and permeabilizing buffer (Cat. No. 00-5521-00, ebiosciences) prior to staining to determine the expression of the intra-nuclear transcription factors, t-bet and Gata-3 (Cat. No. 561266 and cat. No. 560077 respectively, BD Biosciences, Mississauga, ON, Canada). Corresponding isotype controls were used according to the manufacture's inter-chromatin (IC) staining protocol originally optimized for the Foxp3 transcription factor. T cells were then assessed by flow cytometry on a FACSCalibur machine. Results were analyzed using WinMDI (V2.8) for the detection of transcription factor expression.

Statistical analysis: Both GraphPad prism® (V.4) and Microsoft Office Excel® (2003) were used for statistical analysis. Paired *t*-test was used to compare two

group experiments. Repeated Measures ANOVA with Bonferroni's Multiple Comparison Test were employed to compare multiple groups. Data are presented as mean ± 1 SEM, unless otherwise specified. A p value of >0.05 was considered non-significant.

Results:

Respiratory Syncytial Virus infects moDC: Primary human moDC were incubated with RSV (MOI 0.1 to 20), both rgRSV and hRSV. Confocal microscopy and flow cytometry of moDC incubated with rgRSV showed that GFP is expressed in the cells, confirming both infection and virus replication (Figure 3-1A and 3-1B respectively). To evaluate the productivity of the infection, moDCs were washed twice following incubation with rgRSV. The supernatants were collected (days 1, 2 and 3) for RSV titration by the plaque assay to investigate presence of progeny viruses. HEp-2 cells treated with the supernatant of infected moDC became infected even at the first time point. The level of infection did not rise over days 2 -3 (Figure 3-1C). Incubation of moDC with viruses that were pre-treated with Palivizumab or UV light did not show infection in Hep-2 cells (Figure 3-1D).

RSV Infection induces IDO activity in moDCs: Five days following infection of moDC with RSV, cell supernatants were collected for evaluation of the kynurenine content. IFN-γ was used as a positive control (1000 IU/ml). Incubation of moDC with RSV (MOI $\geq$ 0.1) induced IDO activity to levels comparable to IFN-γ (Figure 3-2A, $P<0.001$). Palivizumab-treated RSV (MOI=1) did not induce kynurenine release compared with untreated RSV (Figure 3-2B, $P<0.01$). Likewise, UV-inactivated RSV (MOI=1) had IDO activity similar to uninfected moDC (Figure 3-2B). To confirm the IDO specificity of the kynurenine release, we used the IDO inhibitors, 1MT (800μM) and Menadione (10μM). Both inhibitors suppressed kynurenine release in response to RSV and IFN-γ (Figure 3-2C and 3-2D). To further confirm that RSV induces IDO expression, we used flow cytometry to evaluate intracellular IDO expression. Infected cells showed IDO expression compared to isotype control (Figure 3-2E). Considering the importance of TLR4 in the innate immune response to RSV (20-22), we incubated moDC with the TLR4 inhibitor, rsLPS. While pretreatment

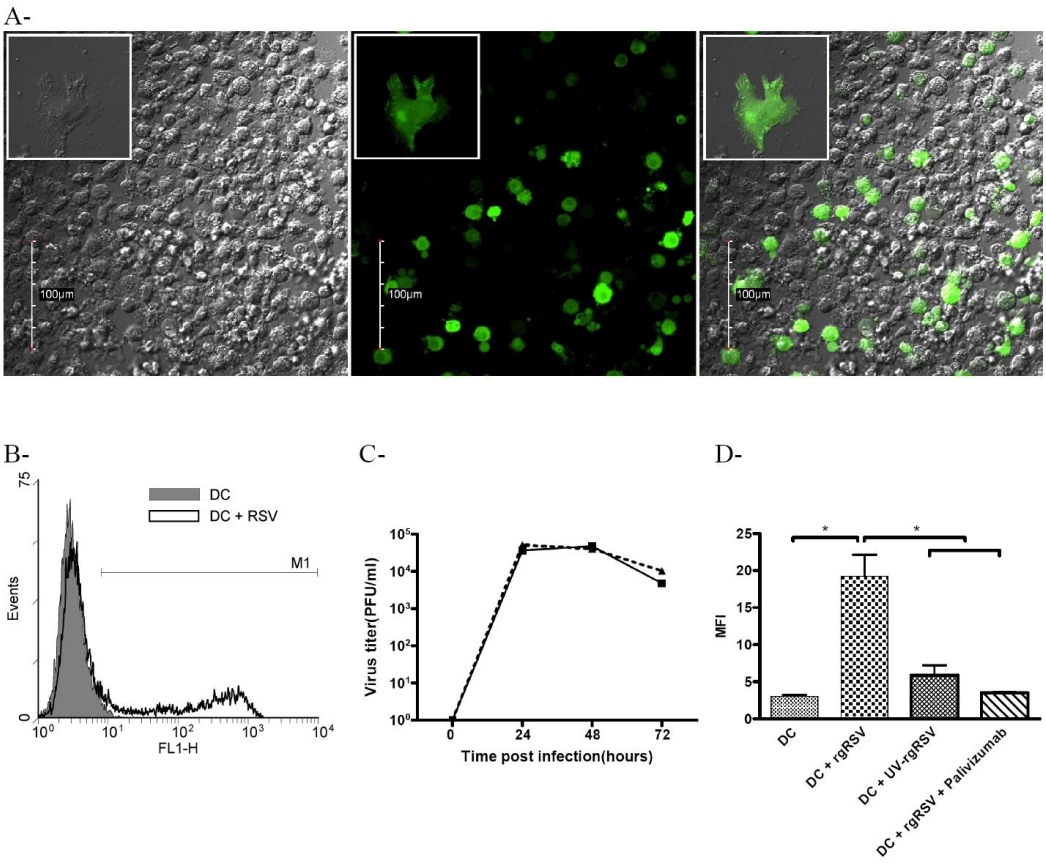

Figure 3-1- RSV (rgRSV) infects human moDC. **A-** Live-cell confocal microscopy images of moDCs incubated with rgRSV at MOI=5 (2 days post infection) show GFP expression (DIC images on the left, fluorescence in the middle, and merged on the right). **B-** Flow cytometry of moDC incubated with rgRSV (MOI=1). **C-** Time-course plaque assays in Hep-2 cells using the supernatant of infected or uninfected moDC with RSV. moDC infection is productive (n=2). **D-** By flow cytometry, Δ Mean Fluorescence Intensity (MFI) of moDCs incubated with UV-inactivated rgRSV or rgRSV blocked with Palivizumab are shown in comparison with un-treated rgRSV (n=3). A and B are representative of three independent experiments.

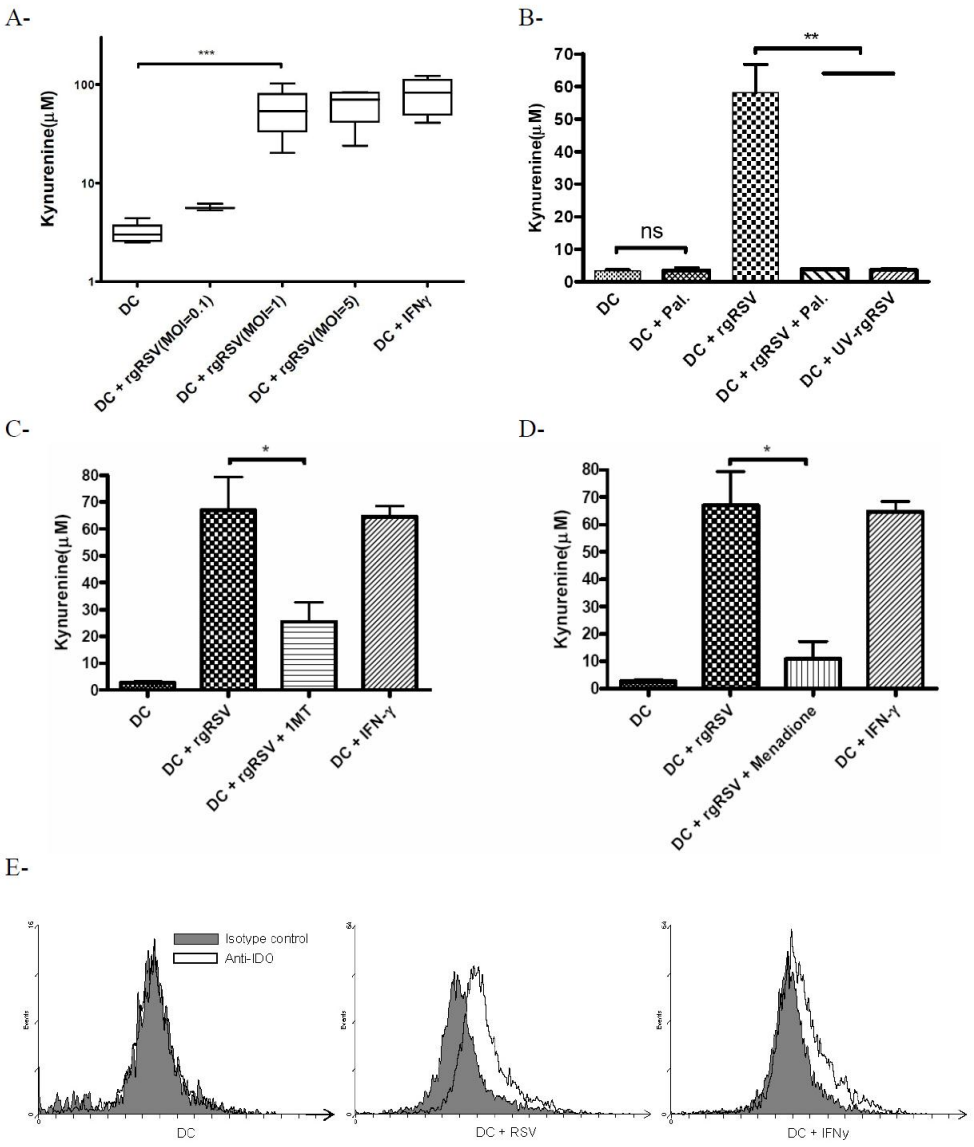

Figure 3-2- RSV induces IDO activation. **A-** Infection of moDC with rgRSV (at different MOIs) induced kynurenine release in culture media (n=9) 5 days post infection. IFNγ was used as a positive control in all experiments (1000 IU/ml). **B-** UV-inactivation or Palivizumab blockade of rgRSV suppressed virus-induced IDO induction in moDCs (n=4). **C and D-** Inhibition of IDO with the specific competitive inhibitor, 1-MT (800µM, n=3), and menadione (10µM, n=2). **E-** Flow cytometry of rgRSV-infected moDCs with an anti-IDO monoclonal antibody confirmed induction of the enzyme one-day post infection (histograms are representative of two independent experiments). Error bars show SEM.

with rsLPS inhibited RSV-induced kynurenine release compared with untreated moDC, flow cytometry of rsLPS-treated moDC also showed negligible expression of RSV GFP. Thus, inhibition of RSV-induced kynurenine release by rsLPS was secondary to the blockade of RSV infection and not IDO inhibition.

We also determined whether IDO activity could be induced in epithelial and monocyte cell lines. Infection of both HEp-2 and THP-1 was confirmed with flow cytometry. HEp-2 monolayers were incubated with RSV and IFN-γ. The latter induced kynurenine release (140±6 μM), while RSV or sham treatment had no effect (28.5±11.8 and 10.8±2.7 respectively, n=3). Similarly, in the monocytic cell line, THP-1, treatment with IFN-γ generated kynurenine (94.98 ± 16.54, n=4) while cells infected with RSV (even at a high MOI of 10) or sham treatment had no effect (4.46 ± 0.03 and 4.38 ± 0.01, respectively; n=4).

Induction of IDO requires viral replication: To investigate the role of viral replication in IDO activation, we used Ribavirin, a nucleoside antimetabolite that blocks RSV nucleic acid synthesis through the inhibition of the enzyme "RNA dependent RNA polymerase". Ribavirin (>220 μM) suppressed kynurenine release (72.1±9.1 vs. 41.6±11.2, figure 3-3A), but did not inhibit IFN-γ-induced kynurenine release compared to IFN-γ alone (78.7±12 vs. 72.3±15, respectively; n=3). This suggested that the Ribavarin effect on IDO was not due to moDC toxicity. Further, this dose of Ribavirin did not appear to have cytotoxic effects on moDC as their ability to metabolize WST-1 was unaffected (n=3). We also incubated moDC with higher doses of UV-inactivated RSV to investigate whether loading moDC culture with more RSV antigenic proteins initiated the IDO induction. Adding 20 times more (in terms of MOI value) UV-inactivated RSV in comparison to live RSV did not induce IDO, further confirming that RSV antigenic epitopes had no role in IDO activation (Figure 3-3B; n=3).

The role of cytokines or chemokines in IDO induction: It is possible that RSV replication induces a factor from the moDC, which may act in an autocrine

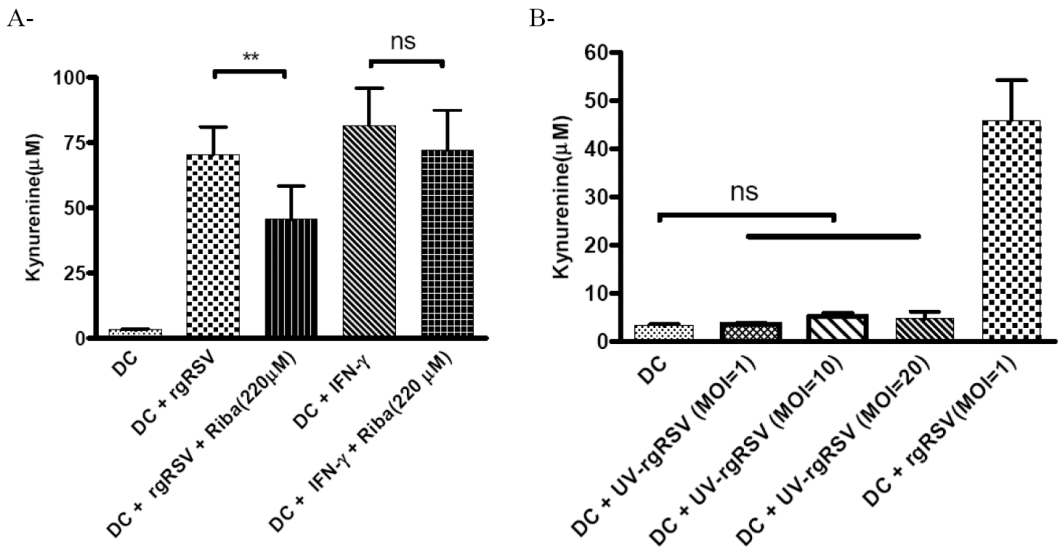

Figure 3-3- Induction of IDO by RSV is dependent on viral replication and needs active infection with the virus. **A-** Blocking replication of RSV by Ribavirn suppresses RSV-induced kynurenine release (n=5). **B-** Incubating moDC with doses up to MOI=20 of UV-inactivated RSV did not result in kynurenine release (n=3). Error bars show SEM.

fashion to induce IDO activation. IFN-γ, as a member of type II interferon family, is one of the most potent inducers of IDO (23). IFN-α, as a type I interferon, is also able to induce IDO but modestly (24). The presence of IFN-α and IFN-γ were negligible in RSV treated moDC similar to uninfected controls (Figure 3-4; 2.00 ± 0.73 vs. 14.10 ± 4.94 pg/ml and 2.52 ± 0.39 vs. 13.15 ± 3.67pg/ml, respectively).

In keeping with these data, neutralizing monoclonal antibodies against human IFN-α and IFN-γ did not block RSV-induced IDO activation (n=6). Anti-IFN-γ (10μg/ml) inhibited the positive control of exogenous IFN-γ (Figure 3-5; n=4). In contrast, TNF release was seen in RSV infected moDC compared to controls (194 ± 58 vs. 9.9 ± 2.0; n=7). TNF alone did not induce kynurenine release even at high doses (up to 200 ng/ml; n=3; Figure 3-6A). In contrast, TNF augmented RSV-induced IDO activity (n=6) and anti-TNF treated moDC (1 or 10 μg/ml) did not generate the same concentrations of kynurenines in response to RSV compared to untreated moDC (15.3 ± 1.9 vs. 89.6 ± 8.9) or matching isotype controls (Figure 3-6B; n=7).

We also measured release of other cytokines and chemokines (CCL5/ RANTES, IL12p17, IL4, IL13 and IL10). Only CCL-5 showed significant release when comparing uninfected to infected supernatants (87 ± 38 vs. 7074 ± 2426, respectively; Figure 3-4; n=8; $p<0.05$).

RSV-induced IDO activation appears to be independent of intracellular TLR3: Since virus replication appeared to be essential for the induction of IDO activation, we determined the potential intracellular site where this stimulation may occur. TLR3 is an intracellular endosomal receptor for double-stranded RNA. RSV is a single-stranded RNA virus. During RSV replication, double stranded RNA is also formed inside the RSV-infected host cell. Incubation of human moDC with the ligand for TLR3, PIC (10 μg/ml), induced kynurenine release (Figure 3-7).

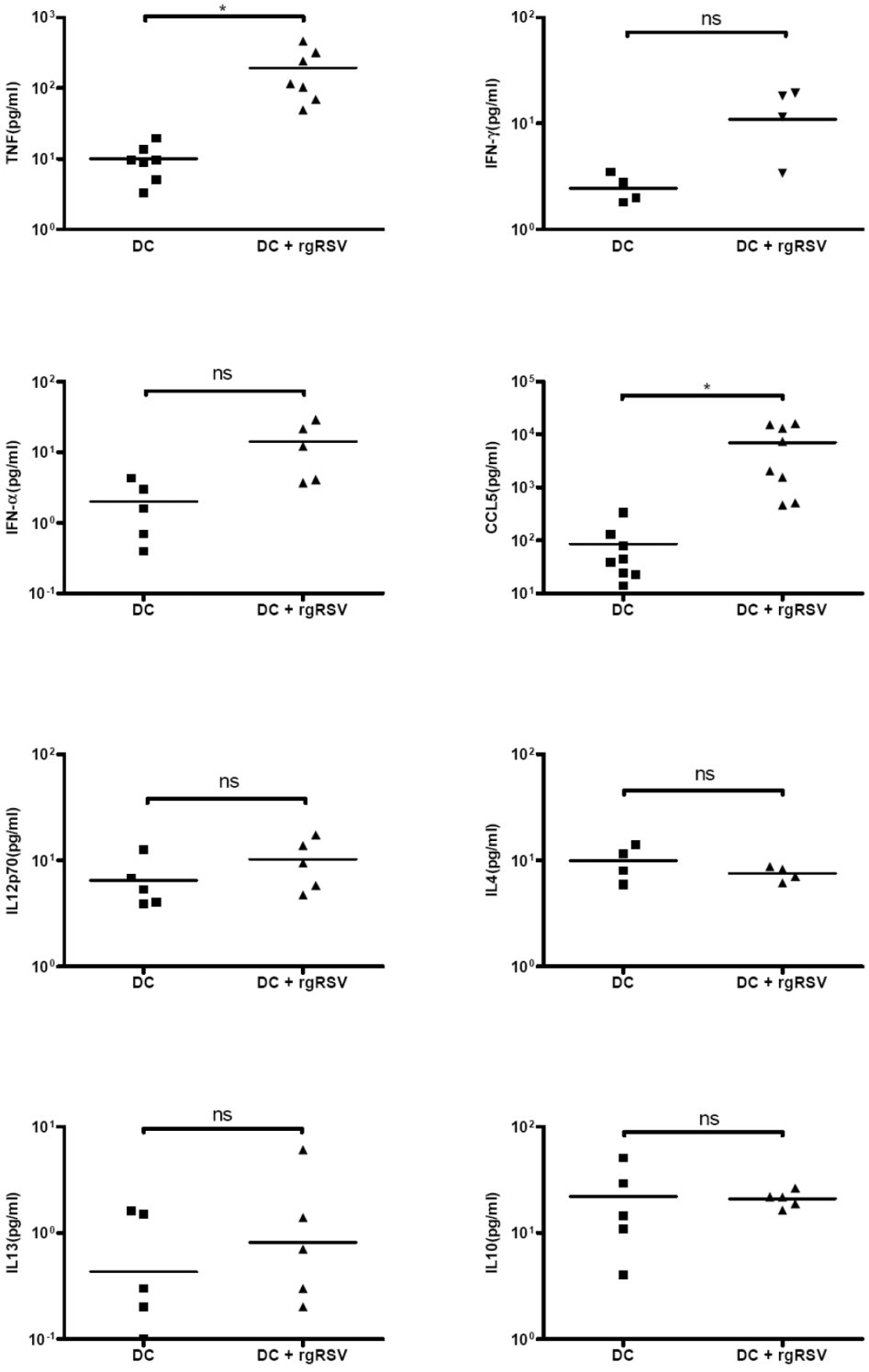

Figure 3-4- Cytokine release in supernatant of moDC infected with rgRSV with MOI=1 (n=5~8). Error bars indicate SEM.

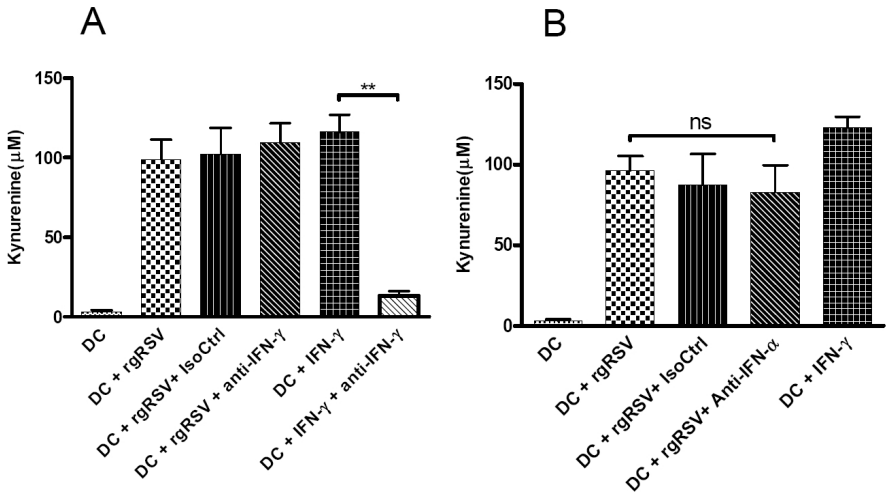

Figure 3-5- **A and B**, Neutralizing monoclonal antibodies against IFN-α and IFN-γ in supernatant (10μg/ml) did not affect the RSV-induced IDO activity in moDC following RSV infection, suggesting the lack of a role in this induction (n=6). Anti-IFN-γ was able to counteract the effect of exogenous IFN-γ in a control experiment (10μg/ml) (n=4). Error bars indicate SEM.

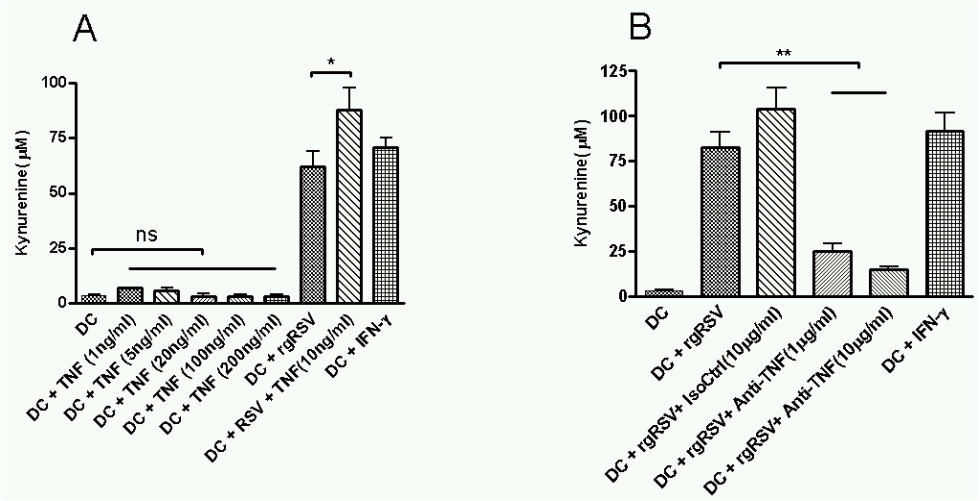

Figure 3-6- Role of TNF in RSV induced IDO activity. **A-** TNF did not induce IDO in human moDC regardless of the doses used (n=3). RSV-induced IDO activity in human moDC was increased by TNF (n=6) B- Addition of a neutralizing monoclonal antibody against TNF to RSV-infected moDC culture medium (10µg/ml) suppressed kynurenine release compared to isotype control (n=7). Error bars indicate SEM.

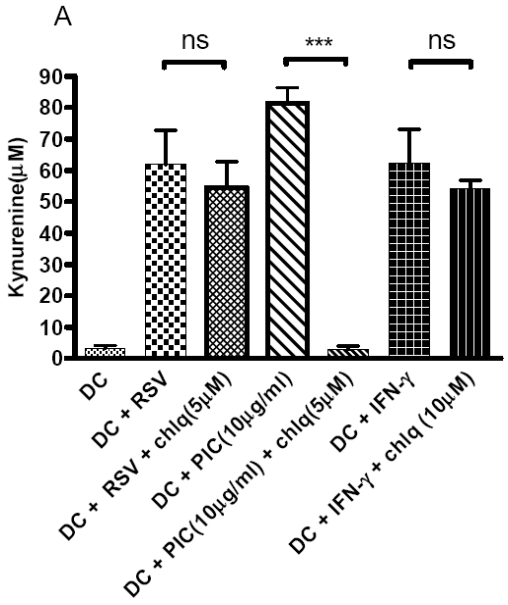

Figure 3-7- Evidence for independence of IDO induction by RSV from TLR-3. **A-** Incubation of moDC with Poly-IC (PIC), the ligand for TLR-3, induced kynurenine generation that was blocked with Chloroquine (Chlq). Addition of Chlq to RSV-infected moDC did not inhibit RSV-induced IDO activity (n=5). Error bars indicate SEM

Chloroquine accumulates in the endosomes where the TLR3 receptor is located and blocks its function (25-27). Chloroquine (5 μM) suppressed PIC-induced kynurenine release but did not inhibit its presence in supernatants of IFN-γ treated or RSV-infected moDC (Figure 3-7, n=5).

Cytoplasmic signaling pathways involved in RSV-induced IDO activity: RIG-I is a cytoplasmic receptor for single stranded RNA (28). RIG-I signals through IKKε/TBK1 which can be inhibited by BX795(29). Addition of BX795 (10 μM), to culture supernatants of RSV-infected moDCs suppressed induction of IDO expression compared to untreated RSV infected moDC (24.0± 2.7 vs. 50.4 ±6.8, respectively; Figure 3-8).

Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and its associated pathways plays an important role in IDO activation (30). BMS-345541, the inhibitor of IKKβ within the NFκB pathway, inhibited kynurenine release compared to untreated RSV infected moDC (40.4±8.8 vs. 73.9±11.3, respectively, n=8; Figure 3-9).

SB202190, the inhibitor of p38-MAPK, (300nM and 3 μM) also blocked kynurenine release (54.7±8.7 and 15.5±1.5, respectively) when compared with untreated RSV-infected moDC (103.3±10.3). Other potential factors in signaling including IKKα of the NFκB pathway (Wedelolactone, 30μM), JNK-MAPK (SP600125, 1μM) and MEK/ERK-MAPK (MEK inhibitor I, 120 nM; MEK inhibitor II, 4 μM) did not appear to be involved in RSV/IDO-mediated kynurenine generation; no inhibition was observed (Figure 3-9). A negative control for each inhibitor was either a specific control provided by the manufacturer or based on the concentration of diluent used for that inhibitor.

Co-culture of RSV-infected moDC with activated T cells suppresses T-bet expression through an IDO-dependent mechanism: We determined whether RSV infection of moDC can influence the bias of the immune response towards

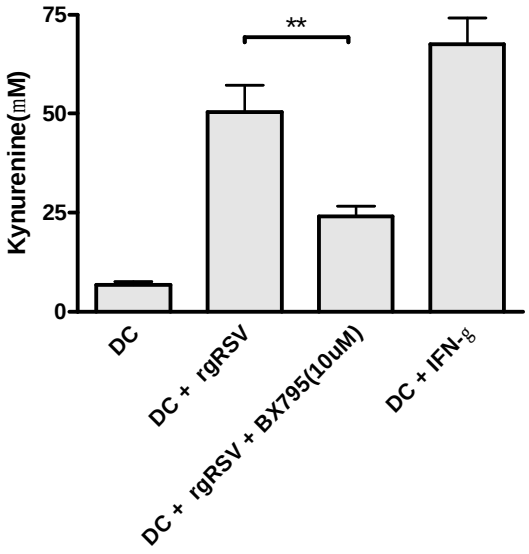

Figure 3-8- Inhibition of downstream signaling pathway of RIG-I-like receptors (TBK1/IKKε) suppressed IDO induction by RSV in moDCs (n=4). Error bars indicate SEM.

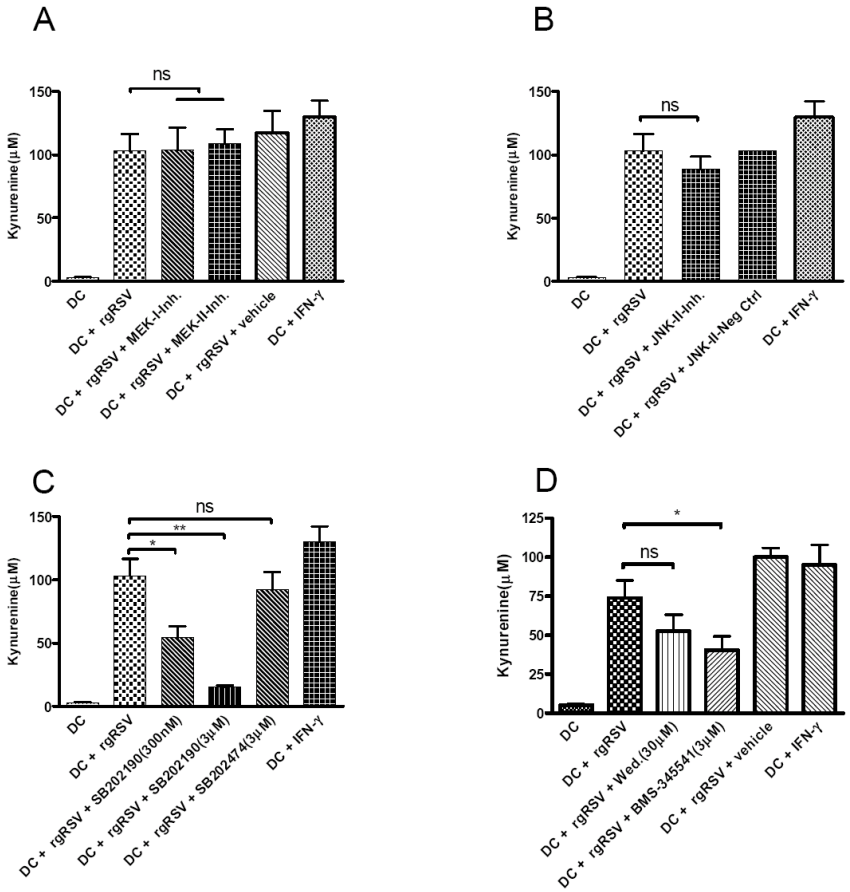

Figure 3-9- Role of down-stream signaling pathways in RSV-induced IDO activity. **A and B**- Inhibition of MEK-I, MEK-II and JNK of the MAPK family signaling pathway did not affect RSV-induced IDO activity (n=4). **C**- Inhibition of MAPK-P38 of the MAPK family with SB202474 suppressed IDO activation by RSV in a dose response pattern compared to negative control (n=4). **D**- IKK Inhibitor III (BMS-345541), the inhibitor of IKKβ of the NFκB signaling pathway, suppressed RSV-induced IDO activity when compared with controls (n=9).

favoring a Th2 response via IDO. We measured the changes in T cell expression of two important transcription factors in Th1 and Th2 immune responses, T-bet and GATA3, respectively. We separated moDC and T cells using a transwell system. To simulate a T cell immune response, T cells were first stimulated by anti CD2/CD3/CD28 beads. They were then added to the inserts in the presence or absence of infected moDC in the lower compartment of the wells. Expression of T-bet and GATA3 were assessed by flow cytometry in CD3+ cells. In contracts to uninfected controls, RSV infection of moDC diminished T-bet expression ($12.3\% \pm 2.9$ vs. $4.8\% \pm 1.8$, $p<0.05$). This effect was not seen in the presence of IDO inhibitor, 1MT. Expression of GATA3 was not affected by the infection (Figure 3-10).

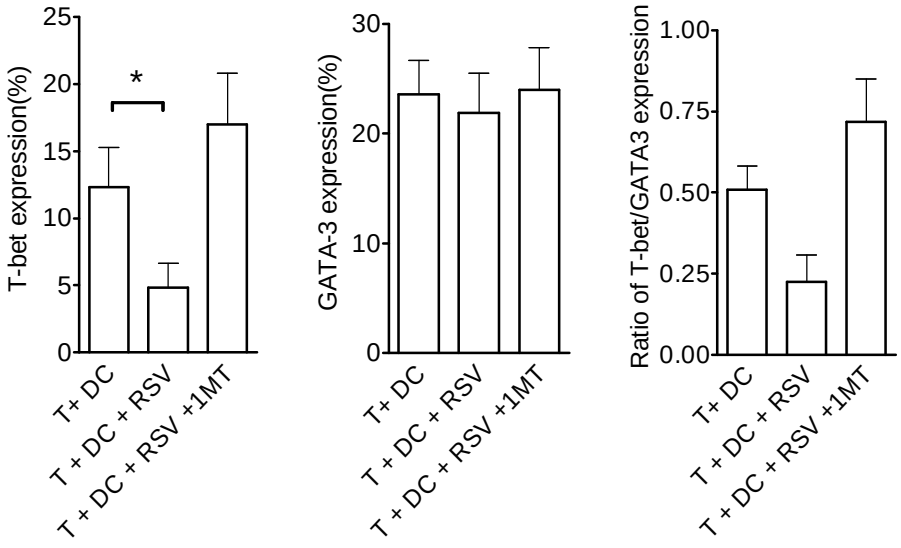

Figure 3-10- RSV infection skewed the immune response toward a Th2 phenotype in an *in vitro* model: **A**- Co-culture of activated T-cell with RSV-moDCs suppressed T-bet expression. This suppression was blocked in the presence of the inhibitor of IDO, 1MT (n=5). **B**- Expression of GATA3 was not affected in this co-culture model (n=5). **C**- Ratio of t-bet/GATA-3 is compared in different conditions of the co-culture model, showing a shift toward Th2 in RSV infection, which is dependent on IDO activity (n=5). Error bars indicate SEM.

Discussion:

Epidemiological studies have clearly illustrated a strong association between RSV bronchiolitis in early infancy and development of allergic asthma in adolescence (4) and up to early adulthood (31). It is possible appears that viral infection acts in synergy with allergen exposure (32, 33). In a mouse model, it was shown that allergen exposure and early-life viral infection interacted to induce a picture of the asthmatic phenotype in adult mice (33). The nature and mechanism of these synergistic interactions remain poorly understood (34). Keeping in mind that the synergy in RSV bronchiolitis and allergen exposure occurs during a window of opportunity in the early life of a child when immunity to the environment is developing (35). We hypothesized that RSV infection provides a milieu for allergens to develop a Th2-type response rather than Th1-typeand this occurs through IDO. Our interest in the potential role of IDO in RSV-induced asthma development post infancy originated from appreciating capacity of IDO to contribute to Th2-biased immune responses (6, 7, 36, 37). IDO is a key enzyme in the rate-limiting step of oxidative catabolism of tryptophan, one of the least available essential amino acids. IDO is expressed in many extra-hepatic organs, with a major presence in the placenta and lungs (38). With the exception of eosinophils (37), IDO is not usually constitutively active in immune cells. Under inflammatory conditions, IDO becomes expressed and activated in antigen presenting cells including DCs (39-43). A distinguished but small subset of dendritic cells is able to express IDO constitutively in mice, though it may not be functionally active (44).

Tryptophan is an essential amino acid and has a distinctive role in the interface between metabolic pathways and immunity. Tryptophan presence is necessary for protein synthesis and without its intracellular presence in sufficient amounts, living cells fail to perform their biological functions. Unlike other amino acids, the concentration of free serum tryptophan is less than 100 µmol/l (typically

between 30-70 µmol/l (45)), lower than most "non-essential" amino acid concentrations in serum (50 µM to 1 mM) (46).

IDO activity generates an important family of biological compounds called kynurenines. IDO converts tryptophan into *N*-formylkynurenine, which is converted subsequently to L-kynurenine by the enzyme formamidase (16, 47). In a milieu where immune cells express IDO activity and most tryptophan is catabolized to kynurenines, the concentration of tryptophan becomes depleted to very low nanomolar levels (48) imposing a condition of starvation for susceptible cells and pathogens (49). It appears that antigen presenting cells can survive this condition by a specific low affinity transporter system that uptakes membrane-impermeable tryptophan to the intracellular space (48). This is not the case for other immune cells including CD4+ cells (15).

Thus, the ability of IDO to induce a Th2-imbalance may result from tryptophan depletion itself and/or production of the tryptophan catabolites, kynurenines, within this milieu (8). IDO-induced tryptophan deprivation and/or production of "immunocytotoxic or immunoactive" kynurenines (50) is suggested as a key factor in different physiologic and pathologic conditions, including the modulation of the imbalance between Th1-Th2 T cell responses. It has been shown that IDO activity induces apoptosis in proliferating T cells, that targets Th1 preferentially while allowing Th2 cells to proliferate (6, 7, 51). The precise mechanism(s) regulating this process is as yet unknown. It is known that the dominant immunologic milieu in allergic asthma is skewed towards a Th2 profile. The ability of IDO to skew the immune response to Th2 places it as a unique candidate for immune regulation in allergic conditions. Our hypothesis was that RSV plays a role in the imbalance between Th1 and Th2 responses if RSV through a direct effect could induce IDO activity in immune cells including DC.
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A secondary goal of this study was to evaluate whether RSV infection may induce IDO activity in moDC through IFN-γ. Initially, we were surprised to discover that interferons had little to do with the induction of IDO by RSV. IFN-γ is a potent stimulator of IDO and is produced during viral infection(52). IFN-γ, as an antiviral Th1-like cytokine, exhibits a protective role in virus infection restricting viral proliferation, however, it also has a role in the generation of airway obstruction (53). Interestingly, it has been shown that cells producing IFN-γ may play a role in RSV-induced wheezing (54). Mice lacking IFN-γ (both anti-IFN-γ-treated mice and IFN-γ-KO mice) show a lesser degree of airway obstruction, even while exhibiting strong airway inflammation than relevant controls. In mice, overproduction of IFN-γ appears to be linked temporarily with virus-induced wheezing (55) while an IFN-γ-inducing factor, IL-18, has been shown to have the ability to enhance airway eosinophilia and increase serum IgE, Th2 cytokines, and allergic sensitization (56). The mechanisms for this type of relationship between IFN- γ and Th2-like responses is yet to be defined, however, we believe that this occurs through IDO, tryptophan catabolism and kynurenine generation. Our data point in this direction and may help explain this paradox.

For RSV to cause bronchiolitis, it would first have to infect epithelial cells that line the bronchial tree. It is interesting that while IFN-γ treatment of an epithelial cell line (HEp-2 cells) had the ability to induce kynurenine production, RSV infection failed to do so. Since moDCs are critical cells that link innate and adaptive immune responses, including a central role in immune response to RSV (57, 58), we focused on moDCs, instead of epithelial cells, for our *in vitro* study to pursue our hypothesis. Unlike epithelial cells, RSV infection induced a significant IDO activity directly in moDCs.

To elaborate the mechanisms involved in the induction of IDO, we studied aspects of RSV infection where stimulation might occur. In most conditions, an intracellular signaling cascade starts with an extracellular stimulus that binds to its receptor on target cells. We investigated possible interactions between components of RSV with a receptor on the cell surface. Blocking RSV binding to the cell surface using the monoclonal antibody, Palivizumab, or rs-LPS, both inhibited infection and IDO bioactivity. We also incubated moDCs with higher doses of UV-inactivated RSV to determine whether loading them with more RSV antigenic proteins could initiate IDO induction. Addition of high concentrations of UV-inactivated RSV did not induce IDO; this confirms the unlikelihood of a role for antigenic epitopes of RSV in IDO induction. If the induction was simply the result of a ligand-receptor interaction, over-loading moDCs with higher doses of RSV proteins would have induced kynurenine release in supernatants. Furthermore, blocking RSV nucleic acid synthesis with Ribavarin suppressed IDO activation, which is interesting since Ribavirin did not block IFN-γ-induced IDO induction. Thus, we concluded that both active infection and viral replication are required for RSV-induced IDO bioactivity.

Although RSV infection is reported to induce phosphorylation and translocation of NF-κB to the cell nucleus (59, 60), the signaling pathway for this induction remains unclear. Pattern recognition receptors (PRRs) are a group of receptors expressed by cells of the innate immune system or non-immune cells. They are

programmed to recognize molecular patterns associated with microbial pathogens or cellular stress. PRRs can be membrane-bound (e.g., TLRs) or cytoplasmic (e.g., RIG-like receptors). Upon recognition, they signal through intracellular signal transduction pathways. For the purpose of this study, we focused on the role of PRRs that have been previously reported to play a role in RSV immunity. Virus replication was essential for the induction of IDO activation in our study and dsRNA is produced during viral replication. Toll-like receptor-3 (TLR3) is located in lysosomes and, upon recognition of viral dsRNA, will induce activation of NF-κB (61). Our observation that PIC, the ligand for TLR3, induces a strong release of kynurenine from moDC is in agreement with another report showing that PIC is able to induce IDO in astrocytes in the central nervous system (30). We used the lysosomotropic agent, chloroquine, to block the function of lysosomal TLRs, including TLR3. Chloroquine is a 4-aminoquinoline drug known for its use in malaria treatment. With a pKa of 8.1, chloroquine is membrane permeable at physiological pH. Upon entrance to lysosome and at its acidic pH, chloroquine becomes protonated and membrane impermeable. This leads to a quantitative "trapping" of chloroquine within the lysosome (26, 62, 63). Recent studies on the mechanism of chloroquine uptake have provided evidence to support the involvement of a carrier-mediated specific transporter on cell membranes (64). Whatever the mechanism, this process of accumulation, interferes with normal function of intracellular TLRs, *i.e.*, TLR3/7/8/9 (27). The inability of chloroquine to inhibit RSV-induced IDO in our study suggests that RSV does not utilize TLR3 as a mechanism in IDO induction. We did not test TLR7 or TLR8 with R848 as a ligand in our model, as a previous report demonstrated that these TLRs do not induce IDO in DCs (65). Nonetheless, the lack of inhibition by chloroquine also applies to endosomal TLR7 and TLR8, rendering their role in RSV-induced IDO activity unlikely.

Another important TLR in RSV pathogenesis is TLR4. The fusion (F) protein of RSV interacts and signals through TLR4/CD14 in human monocytes and TLR4 is important for RSV infection (20-22). LPS binds to the CD14/TLR4/MD2 receptor

complex and LPS induces IDO activation independent of IFN-γ (66-68) in moDC and macrophages (69, 70). To determine if RSV was acting like LPS, we targeted TLR4 and blocked it using rsLPS and anti-TLR4 blocking antibodies. While rsLPS and anti-TLR4 antibodies inhibited RSV-induced IDO activation, moDC incubated with these inhibitors appeared to have much lower RSV infection. We, therefore, were unable to confirm a role for TLR4-receptor in the inhibition of IDO activity beyond that of moDC infection.

"Retinoic acid-inducible gene-I" or RIG-I is another PRRs involved in RSV pathogenesis (71, 72), which recognizes dsRNA in the cytosol of infected cells (28, 73, 74). This recognition signals activation of IKK-related protein kinases, IKKε and TBK1, leading to phosphorylation of interferon regulatory factors 3 and 7 (IRF3/7) (75). Ultimately, the latter upregulates transcription of interferon α and β genes and other IFN-stimulated genes(75). We investigated the role of RIG-I using BX795, an inhibitor of the catalytic activity of TBK1/IKKε (29, 76). Incubation of RSV-infected moDC with the inhibitor of TBK1/IKKε suppressed IDO activity, suggesting a potential role for RIG-I in cytoplasmic detection of RSV-derived nucleic acid. It also suggests that the RIG-I receptor and TBK1/IKKε may play a role in IDO induction. Unfortunately, directly blocking RIG-I in human moDC was beyond our lab's capabilities.

A further mechanistic pathway was considered, namely, through the autocrine action of inflammatory cytokines. Infected moDCs have the capacity to release auto- and paracrine cytokines that may also target neighboring un-infected and infected cells. RSV-induced release of IFN-α or IFN-γ was low; we did not observe an effect on RSV-induced kynurenine release using specific blocking anti-IFN-α and IFN-γ antibodies. These antibodies were added separately to culture media of RSV-infected moDC immediately following their incubation with the virus (10μg/ml). In contrast to interferons, TNF was present in the supernatant of RSV-infected moDC. TNF has an important role in the pathogenesis of RSV (77, 78) and TNF is reported to synergistically increase

IFN-γ-induced IDO activity (79-82). In line with previous studies (69), TNF was not able to induce IDO, regardless of the high pharmacological ranges used. Interestingly, anti-TNF blocking antibodies significantly decreased RSV-induce IDO activity while the addition of exogenous TNF enhanced RSV-induction of kynuremine release. Blocking TNF did not *completely* abrogated RSV-induced IDO activation, which excludes the possibility of an exclusive role for TNF, but rather suggests an additive effect. We believe that TNF can play an important role in IDO induction in moDCs following infection by RSV, although the mechanism is not yet clear. While we did not pursue the mechanism of TNF synergy with RSV, one possible mechanism is that initial infection of moDCs with RSV induces release of TNF. In parallel, intracellular replication of RSV may stimulate intracellular receptors involved in IDO induction, including RIG-I. Whether in autocrine or paracrine fashions, the released TNF may bind to its cell membrane receptor resulting in amplification of intracellular signals already in play (Figure 3-11, Table 3-1). The chemokine, CCL5 (RANTES), was also increased following infection. There are no data associating CCL5 with IDO activation, and we did pursue a role for CCL5 in our model.

To further define possible targets playing a role in RSV-induced IDO activity, we tried to inhibit potential bottle-necks of different and highly prospective intracellular signaling pathways. For this purpose, we included inhibitors for subgroups of the MAPK family (c-Jun-N-terminal kinase (JNK), p38 and MEK). Use of SP600125, an inhibitor of JNK (83), at up to 20 times of its IC_{50} did not

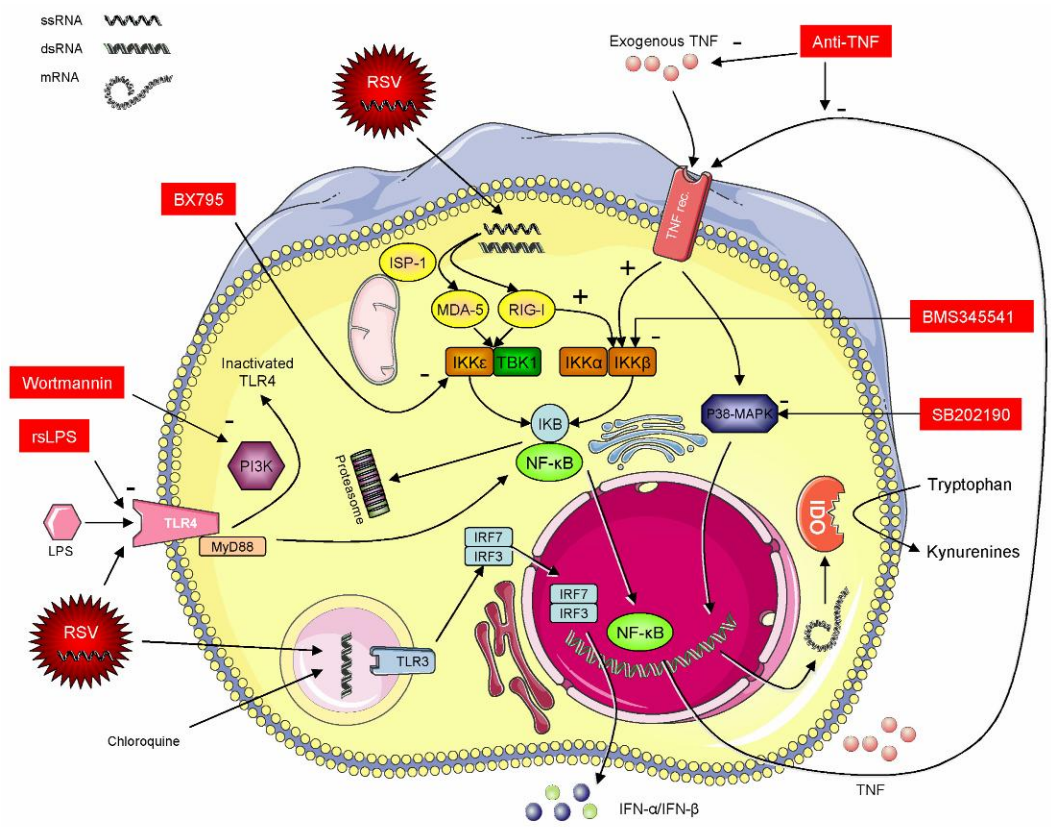

Figure 3-11- A schematic diagram showing signaling pathways that may be involved in RSV-induced IDO induction in moDC. The inhibitors that induced measurable effects are placed in red boxes along with the site of their action.

Group	*Subgroup*	*Inhibitor*	*Target*	*Inhibitory effect on IDO*
MAPK Family	MEK/ERK	MEK inhibitor I	MEK	no
		MEK inhibitor II	MEK1	no
	P38	SB202190	P38 MAPK	yes
	JNK	JNK inhibitor II	JNK	no
NF-κB		IKK inhibitor II(Wedelolactone)	IKKα	no
		IKK inhibitor III(BMS-345541)	IKKβ	yes
PRRs	RIG-I/MDA5	BX795	IKKε/TBK1	yes
	TLR3/7/9	Chloroquine	TLR3	no
	TLR4	rsLPS/anti-TLR4	TLR4 receptor	no
Cytokines	IFN-γ	anti-IFN-γ	Extracellular IFN-γ	no
	IFN-α	anti-IFN-α	Extracellular IFN-α	no
	TNF	anti-TNF	Extracellular TNF	yes

Table 3- 1- Summary of the effects of different inhibitors on RSV-induced IDO activity in human moDC.

suppress the activity of IDO. Inhibitors of MEK/ERK also did not exert any suppressive effect on IDO induction. In contract, SB202190, a potent inhibitor of p38 MAP kinase, inhibited RSV-induced IDO activity. LPS, TLRs and viral infection (84) are considered general stimuli of p38 MAP kinase. According to the manufacturers, SB202190 has no effect on the activity of the ERK or JNK MAP kinase subgroups. Our data suggest that p38 MAPK has a definitive role in the induction of IDO. It remains to be determined whether RSV replication (and its ssRNA/dsRNA products) can directly stimulate p38 MAPK pathway, or if the effect is mediated by up-stream stimulation of p38 MAPK through PRRs (i.e. RIG-I). Inhibition of p38 MAPK with SB202190 inhibits TNF accumulation in IFN-γ-stimulated macrophages (85). While IFN-γ did not play a role in RSV-induction of IDO, TNF did. We speculate that one of the mechanisms by which p38 MAPK mediates IDO activity is through TNF production in moDC in response to RSV.

Expression of many genes involved in immune responses, including IDO, is regulated by NF-κB-associated transcription factors. In homeostasis, NF-κB transcription factors are tied to inhibitory IκB proteins in the cytosol. IKK-α and IKK-β are part of the IκB kinase complex that plays an important role in regulating the NF-κB transcription factor. Upon cell stimulation, IKK-α or IKK-β induce IκB proteins phosphorylation with the phosphorylated IκB degrading through proteasomal proteolysis. This degradation allows NF-κB-associated transcription factors to translocate to the nucleus to express the target genes (75). BMS-345541 (IKK Inhibitor III) is an inhibitor of IKKβ (IKK-2) and it is significantly more selective for IKKβ over the other three members of the IKK subfamily (IKKα, IKKε andTBK1) (76). It is also known that BMS-345541 does not inhibit a panel of over 10 unrelated protein kinases even at high concentrations (76). In this study, we inhibited both IKK-α and IKK-β with their specific inhibitors. Only IKK-β was shown to be involved in RSV-induced IDO activation. Interestingly, it is believed that IKK-β is also essential for the signaling cascade of TNF (75). Thus, IKK-β may be playing a double role in IDO

induction and as a mediator both for RIG-I and TNF signaling. Figure 3-11 summarizes schematically our proposed intracellular signaling pathway for RSV-induced activation of IDO.

Our findings show that co-culture of activated T cells with RSV-infected moDCs reduce the number of cells that express T-bet. This may result in a decrease in Th1 cell numbers that may be available to respond to an antigenic stimulus and lower the Th1/Th2 ratio when compared with uninfected control groups. T-bet is a transcription factor specific to Th1 cells that controls the expression of the characteristic Th1 cytokine, IFN-γ. The reduction in the number of T-bet-expressing cells was not due to interference by any cell marker expression or contact with infected moDCs, since this was tested in a transwell system in which moDCs and T-cells had no direct contact. The inhibiting factor may be a soluble agent in the medium, able to pass through 0.4 μM pores in a transwell membrane with the potential to reach T cells in the upper chamber of the inserts. We measured IFN-γ, IL-12, IL-4 and IL-13 in RSV treated moDC but found negligible levels, rendering them unlikely candidates. The inhibitory effect on T-bet and the change in Th1/Th2 ratio was reversed in the presence of 1-MT, the inhibitor we used to competitively inhibit IDO function. Furthermore, IDO-activated moDC may deplete the medium of tryptophan, including the medium for the T cells in upper chamber inserts. Overall, whether the effect on T cells was through kynurenine-induced cytotoxicity or depletion of tryptophan is yet to be determined.

In conclusion, our study shows, for the first time, that RSV infection can directly induce IDO activation in primary human moDC, *in vitro*. Such IDO induction was dependent on RSV replication. This induction was also further enhanced by TNF. NF-κB (IKK-β) and p38-MAPK pathways appear to be critical intracellular pathways for IDO activation, possibly through recognition of RSV replication by intracellular PRRs (e.g., RIG-I/MDA5). Many known inducers of IDO, including IFN-γ, did not play a role in this induction. Co-culture of RSV-infected moDC

inhibited the Th1-like phenotype of T cells. Thus, our data confirmed our hypothesis that RSV can play a role in the biasing of an immune response towards a Th2 pattern. As the infant's immune system is being educated by exposure to various antigens, having a Th-2 like stimulus may be a factor for a skewing towards an allergic asthma phenotype. Prevention of severe RSV infection may also contribute to a decrease the incidence of allergic asthma. Based on our findings, therefore, it is tempting to speculate that inhibitors of IDO may play an important, but hitherto unexplored role in dissociating probable causative links between RSV bronchiolitis and the subsequent development of asthma. Such therapeutic modalities of IDO inhibitors are the subject of extensive current studies(86).

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Chapter 4- General Discussions and Conclusions

I started my work on this project in January 2005. At that time, a parallel project was ongoing in our lab in which the role of IDO in human eosinophils and its subsequent generation of kynurenines were being assessed. This was in the context of the pathogenesis of eosinophilic allergic asthma. These studies provided an environment conducive to furthering the study of the role of IDO in immune regulation. This was combined with access to knowledgeable colleagues and faculty as educational resources on the topic of IDO, within our lab. Sufficient expertise was available in the lab to cover many aspects and potential questions that might rise during my graduate studies work on the current project. Except for the purification of RSV stock, which was entirely novel and driven by my propensity for engineering-based enterprises, all other aspects of my study in this thesis was influenced by the lab's milieu and provided me with a tremendous learning experience.

RSV Purification and the importance of RANTES: Purification of RSV emerged as an essential element in my project, since much of the data generated during the first two years of my graduate studies turned out to be unreliable likely due to impurities in my viral preparations. Following discussions with my supervisors, it was agreed that working with purified RSV stock was essential before proceeding with our main hypothesis-driven study.

In doing so, I also evaluated the quality of our purification by assessing different parameters. By serendipity, it became clear that CCL5 or RANTES could not be purified totally during standard RSV purification procedures. This finding became the overarching purpose of my methodology paper (Chapter 2). As discussed in detail in Chapter 2, CCL5 has a unique role in allergic diseases in the recruitment of allergic inflammatory cells to sites of inflammation (1). As well, RSV has a unique association with allergic immune responses, although the precise mechanism(s) has not been fully elucidated. Unlike other airway viruses, RSV appears to have more allergic immune cells in the airways (2-8). If CCL5 is a

component of the exterior of the RSV envelop, RSV can potentially play a role as a chemokine source in allergic inflammation, which would have significant clinical implications. Thus, I suggested that this novel finding might shed new light on RSV pathogenesis in allergic asthma. This mechanism, independent of its involvement in cell infection within the respiratory tract with its subsequent side effects, may be a typical feature in viral infections. In addition to the clinical implications of this finding, the presence of CCL5 in RSV stocks and the probable inclusion of the chemokine in the viral structure present important potential applications from a purely virological perspective. It has been shown that pretreatment of cells with CCL5 can inhibit level of RSV infection (9). If CCL5 is a component of molecules covering the exterior of the RSV envelop, it likely to potentially play a role in virus attachment to the host cell. Pretreatment of cells with CCL5 would mask those attachment sites and reduce their exposure to CCL5 on the RSV bi-layer envelop. Over all, this project succeeded in defining a protocol for purification of RSV and in elucidating on the fact that CCL5 may serve as a co-purified chemokine with RSV.

Toxicity experiments: Purification of RSV (Chapter 2) contributed vastly to the success of the rest of the study. In reviewing relevant literature on IDO and RSV, the different approaches to pursue the aims defined in chapter 1 were designed and pursued vigorously and meticulously. I am pleased that with a few exceptions, the predefined goals for my project were achieved. In addition, experiments were conducted with a high level of quality and accuracy to ensure repeatability and to allow me to testing a number of avenues that appeared to be missing in IDO literature. Reagents were tested for toxicity before being applied *in vitro* to ensure that the concentrations used in my designed settings did not harm cells, which over time, became clear that such testing was critical. Occasionally, concentrations used in the literature for pure chemistry-based settings without live cells were shown to be toxic to living cells, *in vitro*; for example, as shown in Chapter 3, Ribavarin was toxic at surprisingly and relatively low pharmacological concentrations.

A number of controversial issues were raised in literature regarding the *in vitro* effect of the standard IDO inhibitor, 1-methyl-tryptophan. For this reason, I also tested alternative inhibitors in this study, including Menadione, which performed well as an alternate inhibitor. There was a group of inhibitors generated by researchers at the University of British Columbia, headed by Dr. R.J. Andersen and his colleagues (10, 11). This group generously provided samples of different inhibitors for us to try in our *in vitro* system. These samples included Exiguamine A, Compound 21 and Compound 22. Exiguamine A appeared to inhibit IDO at 100μM, but compound 21 and 22 did not inhibit IDO; in fact, it enhanced IFN-γ induced IDO activity in DCs and THP1 cells. Before I used Exiguamine A in the RSV-IDO induction, I evaluated the inhibitor by WST-1 (Cell Proliferation/cytotoxicity reagent by Roche- see chapter 3) for its safety and non-toxicity to cell culture. Unfortunately, Exiguamine A was toxic (figure 4-1) at the concentrations that coincided with IDO inhibition (100μM). Although some of these inhibitors are suitable for use in pure chemical experiments, they were not appropriate for live cell work.

Over all, this project succeeded in defining a protocol for purification of RSV and in elucidating on the fact that CCL5 may serve as a co-purified chemokine with RSV. I have also provided evidence that RSV can strongly and directly induce IDO activity, an enzyme that has been shown to play a major role in immune regulation.

RSV and IDO: more than just Th2 imbalance: My study focused on the effect of IDO on the Th1/Th2 immune balance in allergic asthma. I suggested that IDO may be a potential player underlying the association that RSV has with long-term changes in immune response to allergens. The induction of IDO activity by RSV could have additional effects beyond the scope of my study. Recent studies have identified many diversified roles for IDO, ranging from its role in malignancies

(12-14) to the pathogenesis of diabetes (15, 16) and lung allograft tolerance (17, 18) .

IDO activity through RSV provides clues within another related area, namely, aspects of the pathogenesis of RSV-related disease. For example, is there a role for RSV-induced IDO in the observed chronic morbidity associated with many asthmatic patients? Relevant animal models have suggested that a respiratory viral infection can persistent thereby activating components of the innate immune response that leads to chronic inflammatory responses in the lung (19). Does the IDO induction play a negative role in propagation of RSV in infected patients, considering that IDO depletes the cells from an essential nutrient? It has been observed that IDO limits intracellular viral replication in astrocytes, thus, suggesting that IDO may act as an innate antiviral protein (20). These are few additional ideas that were not explored in my project but are important to consider. As well, there were experiments, which due to time shortage or lack of facilities, I was unable to pursue fully.

Mechanism for T-bet suppression: We did not fully examine the mechanisms by which RSV-induced IDO activity contributes to the observed T-bet suppression. As discussed in chapter 1, current research suggests that both *tryptophan depletion* and *tryptophan catabolites* (immuno-toxic kynurenines) play important roles independently (21) or together (22). To better study this in my model, I could have added extra-tryptophan to the co-cultures of RSV-infected DCs with T cells in the transwell model. Reversal of T-bet suppression following addition of tryptophan would have suggested the critical nature of tryptophan depletion for T-bet suppression.

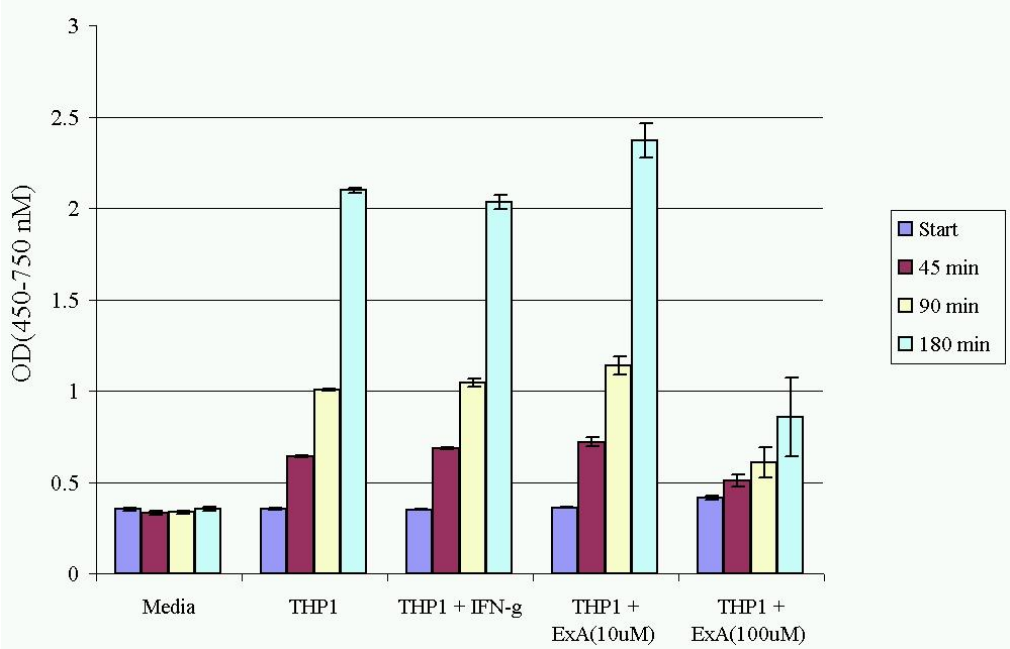

Figure 4-1- WST-1 assay to evaluate toxicity of the inhibitor Exiguamine A (ExA). WST-1 is cleaved into a soluble dye (formazan) by an enzyme found in the mitochondrial respiratory chain which is active only in viable cells. Total activity of this mitochondrial dehydrogenase in a sample is elevated with the increase of viable cells. As the increase of enzyme activity leads to an increase of the production of a dye, quantitation of the dye is correlated directly with the number of metabolically active cells in the medium. This can be quantified by measuring its absorbance using an ELISA reader. The absorbance of the dye solution is in direct proportion to the number of viable cells (source: https://e-labdoc.roche.com/LFR_PublicDocs/ras/11644807001_en_14.pdf). THP1 cells did not convert WST-1 to formazan dye in the presence of Exiguamine A (ExA) at 100µM when compared with THP1 cells alone, with IFN-γ or in the presence of 10µM of ExA.

In addition, we did not study the different catabolites of IDO activity during RSV infection of DCs. We considered all catabolites of tryptophan as a kynurenine family/group. It is now believed that different catabolites of tryptophan may have different immune-modulatory effects (21), with some able to shift cytokine release in iNKT toward a Th2 profile, while the rest without an effect (21). If time had permitted, I would measured each of these catabolites during RSV-infection and determine which one and what concentration were produced compared to other catabolites. Because I did not measure each of the catabolites, I was unable to narrow down the specific catabolite responsible for the observed T-bet suppression in activated T cells during RSV infections. Knowing which catabolites were induced in RSV-infected DC, and by inference which molecule was responsible for the suppression of the T-bet expression, will be the focus of my further studies in this fascinating and exciting study towards the development of relevant therapeutic targets.

Did RSV directly suppress T bet expression? In my transwell model, I co-cultured infected DCs in the lower chamber with T-cells in the upper chamber of inserts. Insert membranes with a pore size of 0.4 μm were used to block cell migration. Thus, DC and T cells did not come in direct contact (lymphocytes are larger than 5 μm and DCs are even larger). The spherical form of RSV varies in size from 0.15 to 0.25 μm in diameter, so the membrane could not act as a blocking membrane for the virus to inhibit migration to the upper chamber. We tried to minimize the chance that RSV in DC chamber could reach the T cells. Free RSV was washed from infected DC before co-culture. Despite this, it is still possible that some RSV could have influenced T cells. In hindsight, we should have added palivizumab to our co-culture system, just before adding T cells to the upper chamber to allow the neutralization of RSV. Using palivizumab, we could have blocked any possible direct effect of the virus on T-bet or GATA-3 expression. Another option would have been to UV-irradiate the media in the lower chamber before adding T cells to the inserts. This means that the entire live virus in the lower chamber would have lost its infectivity. Using palivizumab or UV would

clarify the possible role of live virus in this effect, if any. In addition, while not wholly practical, the best alternative to these solutions would be the use of an ultracentrifuge (50,000g for 1 hour) to precipitate all the viruses and protein and DCs, and then return the supernatants of the ultracentrifugation back to the chambers. This way I would only have soluble proteins and molecules left in the media, including tryptophan or its catabolites. Whether IDO metabolites would be in sufficient concentration would also need to be determined.

Role of IL-17: Only recently, IL17 has attracted the attention of researchers for its role in the pathogenesis of RSV-associated respiratory diseases (23). At the time I started my work and designed my experiments on RSV and IDO, IL17 was not known to be involved. This new and interesting area is worthy of consideration by adding IL17 to the list of potentially important cytokines likely to be involved in IDO function during RSV infection.

The importance of Rhinovirus compared with RSV in the induction of allergic asthma: When I established my hypothesis and started my work, RSV was considered as an important respiratory infection associated with development of asthma or recurrent wheezing later in life. In last few years new studies have emerged linking infection by other respiratory infectious agents and the development of asthma, and associated asthma exacerbations (24-29). For example, a number of researchers suggested that wheezing diseases associated with Rhinovirus (RV) is a stronger predictor of asthma development later in school-age (30) than RSV. It is important to keep in mind that some of the literature that have compared the roles of RV and RSV in the development of a severe acute respiratory illness, have targeted different age groups of children in their studies. For example, RV is reported to cause similar severe hospitalizations in children in comparison to RSV, but with even stronger associations with asthma (31). It is important to note that these populations are usually $\geq$ 24 months old. In my project, all the discussions are based on the role of RSV bronchiolitis in infants, which are less than 12 months old. Thus, while I agree that RV

associated hospitalizations are a strong marker for predicting the development of subsequent asthma, they may be associated only as a marker of future asthma, rather than an active inducer of it. In children beyond 1 year of age, asthma inflammation may have already been established in their airways. In older children, RV is the leading cause of asthma exacerbation, especially in those with an atopic background. It may be that the link of RV with these younger children has similar pathophysiology, and the RV is only a trigger for an inflammatory response that already exists in the airway. In the case of RSV induced bronchiolitis, the children are much younger and often have negligible atopic background or family history of atopy. I, therefore, maintain the view that RSV may be the inciting factor and not just an associated triggering event.

RV and IDO? Regardless, RSV remains the most studied viral disease of childhood, which has been shown to be associated with asthma development. Although new or future findings may provide new and strong evidence that RV (or any other virus) may be more associated with long-term asthma development, it will not be in contradiction with the already established role of RSV in this association. Each of these virus types may play their role independently. Having said that, it would be interesting to follow and investigate the possible role of IDO in RV-infected model and determine whether my findings in RSV infection are reproducible with RV. I co-cultured moDC with RV and measured kynurenine levels in the supernatant. My preliminary data did not provide evidence that RV can induce IDO expression in moDC. It is important to mention that the MOI of RV used in the experiments was ×100 less then that of RSV in our model. Unfortunately, we did not have access to stocks of RV at higher concentrations. Since RV was not the main priority and focus in this project, I did not pursue the role of IDO in RV infections. Evaluating whether RV can induce IDO activity should provide further interpretation of differences that are known to exist between these two viruses. Interestingly, a separate project in our lab, showed that co-culture of RV, but not RSV, with DC in the presence of T cells induced significant release of IFN-γ (Figure 4-2). This finding is in line with

measurements of IFN-γ in supernatants of RSV-infected DC in my experiments (figure 3-4), where IFN-γ was not increased. This is also in agreement with my observation regarding T-bet suppression with RSV. In addition, the difference between RSV and the influenza virus in their ability to induce interferons was studied as early as 1980 (32), where it was shown that RSV was a weak inducer of IFNs in humans. Exploring the mechanisms of this inconsistency between RV and RSV in induction of IDO and IFN-γ, may provide a clue as to their divergent capacity to precipitate an asthmatic phenotype in infected infants.

RSV and IDO in asthma exacerbation: Induction of IDO by RSV may also serve as a mechanism for exacerbations seen during RSV infection in the already asthmatic patient. I have provided evidence that RSV can strongly and directly induce IDO activity, an enzyme that has been shown to play a major role in immune regulation. When an asthmatic patient becomes infected with RSV, this will induce IDO, which can skew the Th1/Th2 imbalance more toward Th2. This balance will favor the release of higher concentrations of Th2-type cytokines (including IL4) and thus enhance the allergic inflammation associated with asthma. This mechanism is not exclusive and, therefore may be paralleled by other mechanisms related to viral infections leading to asthma exacerbation, including mucus production or recruitment and activation of eosinophils and mast cells.

Could latent RSV infection also have a role in immunity: There are a number of studies suggesting prolonged presence of RSV in the lung, while the subject remains clinically asymptomatic (33-36). If true, persistence of RSV following bronchiolitis may have a wide range of effects on the pulmonary system long after the child is asymptomatic. It is open to speculation that the presence of RSV in the cells of the immune system, even in clinically asymptomatic and quiescent states, may induce long-term over-expression of IDO, thereby skewing the immune milieu toward Th2 allergic responses. To put this hypothesis to test, one would need to replicate the experiments that have shown that RSV persists longer

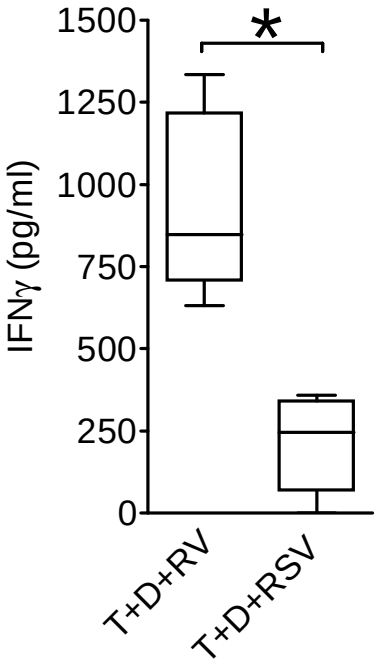

Figure 4-2 – Unpublished data by Ilarraza R. *et. al.* in our lab suggest that RSV and RV are different in their ability to induce IFN-γ in the presence of DC and T cells, with RV being more potent; *=P<0.05, (n=6).

than generally believed and then compare IDO enzymatic activity in lungs of the infected group and compare them with control uninfected animals.

Where the cell signaling pathways seen for RSV merge with those involved in inducing IDO activation: I used different inhibitors to suppress RSV-induced IDO activity. It was beyond the scope of this study to investigate the precise role of these inhibitors in the case of other IDO stimuli, including IFN-γ. Distinguishing parts of signaling pathway that are specific to RSV from those common to other stimuli of IDO can have clinical applications. Considering that inhibition of IDO has been suggested as a therapeutic option in many diseases, and the fact that a clinically safe and effective inhibitor of IDO has not yet been achieved, any alternative method that could affect IDO induction would be a significant therapeutic candidate in IDO-related conditions. Such an approach needs a specific, rather than a nonspecific cell target that may affect other cell functions.

The role of PI3-kinase in IDO activation: To determine if RSV was binding to its receptor, TLR4, I targeted the latter as well as downstream signaling pathways of TLR4. This involved rsLPS experiments and the use of high immunogenic doses of UV-inactivated RSV. In addition, the PI3-kinase inhibitor, wortmannin, reported to have a role in TLR4 downstream signaling in mouse macrophages, was used (37). I determined whether wortmannin ((IC_{50}= 1-10 nM) (Cat. No. 10010591, Cayman Chemical, Ann Arbor, MI) could suppress RSV-induced IDO induction (figure 4-3). Kynurenine release was suppressed by inhibition of PI3K, predicting a role for this pathway in this response; wormannin had no effect on IFN-γ. These data were in direct contrast to my TLR4 inhibitory data and the strong UV-inactivation data. Surprisingly, it has been recently suggested that PI3 kinase activation may have a negative regulatory effect on the TLR system (38, 39). Inhibition of PI3K signaling by wortmannin enhanced TLR signaling (39, 40) and this function of wortmannin is shared also with TLR2/3/4 and TLR9 (40). Considering the controversy that exists regarding the role of PI3K in TLR

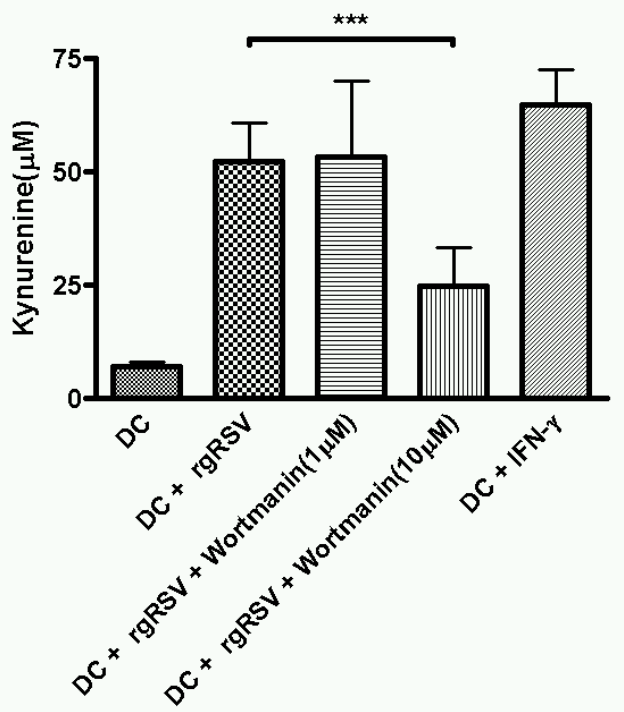

Figure 4-3- IDO induction by RSV is dependent on PI3K. Wortmannin, a specific inhibitor of PI3K was added to RSV-infected DC (10 μM). Kynurenine release was suppressed following PI3K inhibition with no effect on the positive control group (n=4).

signaling, and the disagreement that remains unresolved concerning the specificity and function of wortmannin in TLR signaling inhibition, I believe that further studies are still necessary if we are to publish these data. It is likely that PI3K has a role in PRR in moDC and that inhibition is likely to affect IDO activation through this route.

Experiments with a dendritic cell line prepared from THP-1: In the process of moDC preparation, despite using cell specific magnetic beads, it is not possible to have a 100% pure moDC population. As a result, one criticism that can be raised is that my data may not reflect the results of a pure population of moDCs, but rather the presence of contaminating cells (including T cell) that have the immunological capacity to synthesize and release cytokines as well as other, yet to be determined signals that can stimulate moDC. To address this concern, I used a pure monocyte cell line, THP-1. As discussed in Chapter 3, induction of IDO activity in THP-1 monocytic cell line was observed with IFN-γ stimulation, but high infective doses of RSV did not induce IDO in these cells. I hypothesized that the THP1 cell line may require other factors to induce IDO. I followed a protocol to differentiate the THP1 cell line to a dendritic cell line using IL- 4 (41). Interestingly, unlike undifferentiated THP-1, differentiated THP1 released kynurenine following RSV infection at a somewhat higher MOI (MOI=5; n=1; 4.4 µM in control vs. 42.1 µM in differentiated THP-1). These preliminary findings suggested that the maturation of the monocyte somehow provided a mechanism for RSV to induce IDO. I also confirmed that even in a pure population of DC-like cells, we can observe IDO induction. I did not pursue the protocol further in my experiments, as the protocol required a large concentration of IL-4 to differentiate THP-1 to cells expressing DC features. I suggest that labs with difficulty recruiting donors, this alternative option may be worth considering, although the generated data may have their own limitation because of the use of a cell line.

Presence of lymphocytes augmented RSV-induced IDO release: I found that if DC were infected with RSV in the presence of autologous T cells (T cell to DC ratio, 1:3), kynurenine release was higher compared to moDC alone (45.6 vs. 82.2 respectively; n=2). As lymphocytes are a major source of inflammatory cytokines, including IFN-γ, such a finding was expected. As mentioned in Chapter 3, RSV incubated with purified lymphocytes alone, did not induce the release of kynurenine despite the presence of higher doses of RSV. For this reason, I did not pursue experiments with combined T cells and moDC, as my preliminary data suggested that DCs were playing a more prominent role than T cells in IDO induction following RSV infection.

Alternative method to measure the Th1/Th2 ratio under IDO-induced conditions: The ultimate goal of this project was to determine whether RSV-induced IDO activity could alter (decrease) the Th1/Th2 ratio. To pursue this goal, I performed three different experimental approaches to follow, but eventually chose to follow a more suitable procedure that took into account our expertise and available facilities in the lab (namely, to examine the expression of T-bet and GATA3 by flow cytometry). I considered alternative approaches that can be pursued in the future by other members of the lab. Autologous naïve T helper cells could be isolated and incubated with IL-4 (together with anti-IFN-γ) or IFN-γ and IL-12 (in the presence of anti-IL4) to generate Th2 and Th1 cell lines, respectively, as determined by flow cytometry. Generated Th1 and Th2 cells will then be co-cultured with RSV-infected DCs. The extent of apoptosis in these two subgroups would be measured by flow cytometry using annexin-V, ToPro3 and MitoTracker staining. If my hypothesis is correct, I would expect to observe more apoptosis in Th1 than Th2 cells.

A second alternate method would be to isolate Th1 and Th2 cells (using CRTH2+ staining as a marker for Th2 cells) directly from the peripheral blood of the donors and then allow them to proliferate, *in vitro*. Th1 and Th2 cells would be stimulated using CD2/CD3/CD28 beads during co-culture. Again, I would expect

to see relatively higher apoptosis rates in Th1 cells that are co-cultured with RSV-infected moDCs but not Th2 cells. To confirm the specificity of the influencing enzyme, this process may need to be reversed in the presence of IDO blockers.

In conclusion, I hope that my data convinced you of two major findings; the first is that in spite of developing and following a well-established, carefully-designed and well-explained protocol for the purification of RSV, CCL5 persists as a companion chemokine to its co-purified RSV virus. The other is providing strong evidence that RSV can directly and potently induce IDO activity, an enzyme with an enormous potential impact on immune regulation.

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