TISSUE SPECIFIC PATHOLOGY ASSOCIATED WITH MICRONUTRIENT SUPPLEMENTATION DURING RESPIRATORY SYNCYTIAL VIRUS INFECTION

by

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Abstract

Micronutrients are increasingly appreciated as potent immunomodulators. Long used to 'treat' measles virus (MeV), vitamin A was recently shown to act through RIG-I to up-regulate type 1 interferons. Similar actions are seen with some (mumps virus and CDV) but not all members of the *Paramyxoviridae*. However, children infected with respiratory syncytial virus (RSV), a close relative of MeV, do worse when given pharmacological doses of vitamin A. RSV is known to elicit a pathologic Th2-biased response. Vitamin A also has a strong Th2-deviating influence. Our primary objective was to develop a small animal model of vitamin A deficiency and sufficiency in which we could assess the impact of vitamin A status and supplementation on RSV infection *in vivo.* Such a model will be of great use to characterize the mechanism of action of retinoids in this infection. We succeeded in the development of this model by restricting dietary retinol through 2 generations of BALB/c mice (ie: deficiency state) and introducing novel means to reliably attain a state of consistent vitamin A supplementation (ie: reconstituted & excess states). Preliminary data using this model suggested that there were marked differences in RSV pathology between deficient and sufficient mouse groups. Like the apparent situation in humans, infection in the vitamin A deficient mice was paradoxically less severe than in mice with a positive vitamin A status. This model and the data generated with it may be of particular interest in regions with diets high in vitamin A (North America in particular). Historically, very little attention has been given to possible negative effects of micronutrient 'over-nutrition'. The limited human data and the preliminary data from our new model brings into question whether or not we are

‘priming’ ourselves for more severe RSV infection than would otherwise occur. The data generated in this model may also be highly relevant to guide supplementation efforts in regions of the world that currently have less access to vitamin A.

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Statement of Contribution

Scientific contributions have been made by the following individuals: Erica Gipson, for her help with differential counts; Angela Brewer and Kaitlin Soye (McGill University), for patiently teaching me techniques; Dr. Qutayba Hamid (Meakins Christie Laboratories) for assistance with lung sectioning; and Sylvain Milot (IAF) and Christophe Goncalves (UQAM), for their help with HPLC.

Statement of Originality

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication.

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List of Abbreviations

APC: Antigen presenting cell
ATRA: All-trans retinoic acid
BAL : Bronchioalviolar lavage
CARD: Caspase activation and recruitment domains
CD: Control Diet
CRABP: Cellular retinoic acid binding protein
CRBP: Cellular retinol-binding proteins
DC: Dendritic cell
F: Fusion Protein
FADD: Fas-associated death domain
G: Glycoprotein
GM-CSF: Granulocyte-macrophage colony-stimulating factor
H: Hemaglutanin
HN: Hemaglutanin-neuraminidase
IFN: Interferon
IPS-1: IFN-β promotor stimulator-1
IRF: Interferon regulatory factor
JAK: Janus kinase
JNK: JUN N-terminal kinase
L: Large polymerase
LGP2: Laboratory of Genetics and Physiology 2
M: Matrix protein
MAPK: Mitogen-activated protein kinase
MAVS: Mitochondrial antiviral signalling
MDA5: Melanoma differentiation-associated gene 5
MHC: Major histocompatibility complex
MIP: Macrophage inflammatory protein
MV: Measles virus
MyD88: Myeloid primary response gene 88
N: Nucleocapsid
NOD: Nucleotide-binding oligomerization domain
NS: Non structural
OAS: oligoadenylate synthetase
ORF: Open reading frame
P: Phosphoprotein
PAMP: Pathogen-associated molecular patterns
PFU: Plaque forming units
PRRs: Pattern recognition receptors
RA: Retinoic acid
RANTES: Regulated upon Activation, Normal T-cell Expressed, and Secreted
RAR: Retinoic acid receptor
RBP: Retinol Binding Protein
RE: Retinyl Esters
RIG-I: Retinoic acid-inducible gene-I

RIP-1: Receptor-interacting serine-threonine kinase 1
RLH: Retinoic acid-inducible gene-I-like helicases
RSV: Respiratory syncytial virus
RXR: Retinoid X receptor
SH: Small hydrophobic
STAT: Signal Transducer and Activator of Transcription
STRA6: Stimulated by Retinoic Acid gene 6
TANK: TRAF family member-associated NF-κB activator
T-bet: T-box protein expressed in T cells
TBK1: TANK-binding kinase 1
TCID50: 50% tissue culture infective dose
TICAM: TIR domain containing adaptor molecule-1
TIR: Toll/IL1 receptor
TLR: Toll-like receptor
TRAF: Tumor necrosis factor receptor-associated factor
TRIF: TIR-domain-containing adaptor protein inducing IFN-β
VAD: Vitamin A deficient
VAH: Vitamin A-high
VAN: Vitamin A normalized
LPS: Lipopolysaccharide
CpG: Cytosine-Guanosine

Background and Introduction

History of Pathology and Medicine

In order to understand the concept of disease today, it is helpful to review the evolution and history of disease as a known human affliction. Modern science can be fundamentally understood to have foundations in 'western' natural philosophy. Consistent with that philosophical foundation, even the word physician stems from the Latin—*phisica*, or 'of things relating to nature'. To understand disease according to these philosophers, we must first consider their notions of the world and its constitution; that is— all matter was comprised of four elements. It was the specific combinations of these elements that determined an object's fundamental characteristics. The body too followed this ideology and was governed by four fluid Humors. The humors corresponded to the respective elements that Aristotle (384-322 BCE) described: Air, Water, Earth and Fire. Galen (129-217 CE) suggested that within the body, these elements respectively manifest as Blood, Phlegm, Black bile and Yellow bile. Although the methods used to achieve Humoral balance seem illogical by today's Western standards, disease was understood to be an imbalance of the Humors in the body that could be corrected by re-establishing Humoral equilibrium. This belief structure permitted therapies, such as bloodletting, to gain credibility— for example, 'bleeding' an individual would be a reasonable response to regain equilibrium if it was suspected that the Humors were imbalanced due to excess blood. On occasion, these early physicians were correct: eg haemochromatosis, polycythemia vera or porphyria cutanea tarda, which are all diseases in which accumulated iron or excess blood cells are responsible for

pathology.

It was not until Razi (865-925 CE) that disease was considered to be separate from the human condition and its Humors. In this new 'extrinsic' approach, diseases were caused by particular poisons or afflictions that may or may not be curable by a physician. Razi was the first to recognize allergy and seasonal rhinitis, the first to consider fever a defense mechanism instead of the affliction itself and among the first to recognize his own fallibility as a physician. He was devoted to experimentation and observation as the key to understanding disease. This shift from the body as the inherent source of all disease to discrete 'external' agents allowed for academic and philosophical expansion into disease prevention through interventions such as sterile technique, the concept of microbes and pasteurization.

More recently, though, there has been a shift back from molecular solutions to a more holistic approach to disease prevention. This return to equilibrium: i.e. balancing diet, exercise and lifestyle—posits that in the absence of balance, we may be predisposing ourselves to disease, or more severe manifestations of disease, than would otherwise present in a healthy individual. It is becoming increasingly apparent that host homeostasis and individual immune profiles are often major contributors to both the prevalence and severity of intrinsic and extrinsic disease. Our molecular grasp of disease has certainly enabled breakthroughs in therapeutics (e.g.; antibiotics) and prevention (e.g.; eradication of smallpox); however, by focusing exclusively on the extrinsic model of disease, without understanding how our personal daily activities influence the outcome of illness, we put ourselves at risk for only knowing half of what constitutes 'health'.

Paramyxoviridae

The *Paramyxoviridae* family contains several ubiquitous pathogens that can cause severe disease in both humans and animals (measles virus, mumps virus and respiratory syncytial virus (RSV), Newcastle disease virus, Nipah virus and Sendai virus). These are all enveloped, negative sense RNA viruses containing six to ten genes which typically encode proteins involved in transcriptional regulation and replication (large polymerase, L, and nucleocapsid proteins, N), viral assembly (matrix protein, M) and a variety of proteins used for viral adhesion to and entry into host cells (106).

Typically, viral fusion proteins both bind target receptors and mediate fusion. However, in paramyxoviruses two proteins cooperate to mediate viral entry: an attachment protein (hemaglutanin, H, hemaglutanin-neuraminidase, HN, or glycoprotein, G) and a fusion protein, F. Initial adsorption to the target cell is accomplished by the attachment protein binding sialic acid residues or surface proteins on the target cell. Upon target-adsorption, the F protein initiates virion-cell membrane fusion— thereby releasing the viral contents into the host cell cytoplasm. The release of envelope contents into the cytoplasm generally occurs at the plasma membrane; however, Sendai and Nipah virus are internalized prior to membrane fusion by a mechanism not yet fully understood (106).

Brief History of Measles Virus & Measles Vaccines

Measles virus (MV) is among the most important and virulent human pathogens. Measles was first characterized in the 9th century by the Arab physician Abu Becr as a modification of smallpox that presented with more severe clinical manifestations (18). In 1757, the Scottish physician Home showed that MV was caused by an infectious agent

when he tried to immunize volunteers by blood transfusion from a patient presenting with the early stages of a rash (73). The Danish physician Panum first described the 14-day incubation period for measles, its highly contagious nature and suggested a respiratory route of transmission during his visit to the Faro Islands in 1846 (1).

In 1954, a pediatric fellow, Thomas Peebles, obtained throat swabs and blood samples from school children during a MV outbreak (85). The virus was successfully cultured in primary kidney explants. This first measles vaccine was named after the student from whom the isolate was obtained (David Edmonston) hence the eponymous Edmonston B vaccine strain (85). In parallel with the pursuit of live-attenuated vaccines, vaccines based on wild-type MV, inactivated by either formalin or tween-ether, were also developed. An alum-precipitated, formalin-inactivated, vaccine was licensed in 1963 and used widely in a three-dose regimen across the United States and Canada (51). Recipients developed a modest neutralizing and hemagglutination antibody response with low levels of complement fixing antibodies (51). Antibody titres declined rapidly after immunization and exposure to natural infection after the cursory period of protection resulted in more severe disease, characterized by prolonged fever, unusual lesions on the skin and pneumonitis (51). This syndrome was termed ‘atypical measles’ and its recognition led to the rapid withdrawal of the inactivated vaccines from the market. More or less simultaneously, several successful live-attenuated vaccines were also developed by repeated passage in tissue culture (eg: Schwarz, Moraten, Leningrad, AIK-C) (51). Although these vaccines have been remarkably effective and are still in use today, essentially unchanged, there has recently been a renewed effort to develop vaccines that can be given to infants before six months of age (ie: in the presence of maternal antibody)

and that can be given safely to immunocompromised hosts (51). These include DNA vaccines, recombinant or subunit peptide vaccines and vectored vaccines (51).

Brief History of Respiratory Syncytial Virus (RSV) and RSV Vaccines

Acute bronchiolitis is one of most common serious illnesses of infants and children worldwide and is most often caused by RSV. The specific pathology associated with acute bronchiolitis was first described by J. Eberle in 1850, when he recognized an acute asthma-like attack (his term "catarrhal affection") that included laboured breathing, coughing and wheezing (109). Through the early 20th century, there were a number of outbreaks of severe respiratory disease in which no bacterial agent could be found as the culprit. The disease was eventually attributed to a seasonal viral infection that had typical clinical and pathological characteristics (109). The virus itself was isolated from infected chimpanzees in 1955 and named chimpanzee coryza agent (109). Robert Chanock later found a similar virus in clinical isolates from infants presenting with bronchiolitis and pneumonia (109). The virus was eventually propagated in culture where it produced large multinucleated cells, or syncytia, and was appropriately named: respiratory syncytial virus (109).

Chanock continued his work with RSV, outlining the principal clinical manifestations and epidemiology, including recurrent infections. He also began a close collaboration with Robert Parrott to develop what would become the first formalin-inactivated RSV vaccine. Their pivotal 1969 clinical trial of this vaccine followed orphaned children and infants across the northeastern United States for 34 months post-vaccination (82). During the subsequent peak RSV seasons, 80% of the vaccinated group exposed to wild-type infection required hospitalization, compared to only 5% of the

control group (82). Further, two individuals from the vaccine group died. Post mortem examinations of the infants showed severe bronchopneumonia, hyperinflation and atypical eosinophillic infiltrates (109).

The spectacular failure of this formalin-inactivated RSV vaccine effectively halted RSV vaccine research for close to 30 years and alternative treatments for RSV infection became the principal focus. Prophylactic agents such as bronchodilators, β-agonists, epinephrine, anticholinergic agents and corticosteroids have all been used in an attempt to relieve airway stress during severe infection (109). A number of monoclonal antibodies that target the RSV F protein have also been developed including Palivizumab and Motavizumab. In the last decade, new insights in both basic virology and immunity have led to renewed interest in developing an RSV vaccine. Since 2000, live attenuated, reverse engineered, subunit, vector-based, proteasome-based, nanoparticle and DNA vaccines have all been explored in an effort to find a safe vaccine that can induce long term immunity (35, 109).

Retinoids species and prevalence in the diet

Retinoids are a group of organic chemicals that are essential for survival and can also act as potent immunomodulators. This family includes retinol, retinal, retinoic acid (tretinoin or vitamin A) and isotretinoin, among others. The capacity for *de novo* synthesis of retinols is limited to microorganisms and plants. As such, vitamin A is obtained exclusively from the diet as either preformed vitamin A (animal derived long-chain fatty acid retinyl esters, pharmaceutical supplementation or fortified foods) or plant derived provitamin A carotenoids (such as β-carotene) (61, 78). Retinyl esters (RE) from preformed vitamin A must be hydrolyzed by pancreatic or enterocyte-associated enzymes

before absorption (61). Dietary carotenoids are converted to retinol in the intestinal lumen before they can be absorbed (61). Preformed vitamin A is absorbed at rates of 70-90%, while provitamin A carotenoids are absorbed less efficiently, at rates of 20-50% due to conversion kinetics (78). The richest sources of vitamin A in the North American diet are mammalian and avian liver (4–20 mg retinol/100 g), instant meal-supplement or replacement drinks (3–6 mg/100 g), fortified cereals (0.7–1.5 mg/100 g), and fortified margarines (about 0.8 mg/100 g) (174). Many foods in North America are rich in provitamin A carotenoids include carrots, sweet potatoes, pumpkin, kale, spinach, collards, and squash (5–10 mg effective retinol/100 g) (174). In developed nations, up to 75% of dietary vitamin A is ingested as preformed vitamin A, while in developing nations up to 90% of dietary vitamin A is absorbed from plant sources—outlining a stark difference in socioeconomic and resource influences on vitamin A status (78). These differences can have broad implications on immune function.

Retinoid metabolism

Retinoid uptake in the intestinal lumen is activated by, and dependent on, dietary fat ingestion (61). The luminal presence of dietary fat stimulates the secretion of bile salts and pancreatic enzymes, which provide the end products of fat digestion (monoglycerides and free fatty acids). These dietary lipids are key components of the micells and chylomicrons that ultimately contain and transport dietary retinoids (61). Once hydrolyzed, cellular uptake of retinol is achieved by transporter- and diffusion-mediated processes; however, no definitive protein transporter has been found yet (61). Once inside the enterocyte, free retinol is sequestered and transported by cellular retinol-binding proteins (CRBPs) I, II and III, where CRBP II is exclusively expressed in the gut

lamina and CRBP III in embryos (61, 168). Retinol is then re-esterified with dietary long-chain fatty acids by lecithin-retinol acyltransferase for incorporation into chylomicrons and eventual secretion into the lymphatic system (61). Once in the circulation, the chylomicron remnants containing REs are exclusively taken up by hepatocytes and transferred to perisinusoidal stellate cells (61). Stellate cells typically store 80% of the body's total REs, while the remainder can be found in hepatocyte lipid droplets (15, 150). Circulating retinol is maintained within a strict range (2μM) by stellate cell secretion of retinol complexed with retinol-binding protein (RBP) (168). RBP is a member of the lipocalin protein family that binds to and protects circulating retinol from metabolism (150, 168). Retinol-RBP is reversibly complexed (1:1) with the thyroid-hormone carrier, transthyretin in the bloodstream, which prevents clearance by the kidney before being taken up by target cells (150, 168). The target-cell receptor for this tri-molecular complex was identified in 2007 as the Stimulated by Retinoic Acid gene 6 (STRA6), a retinoid-dependent multitransmembrane receptor protein (86, 127, 168).

Once taken up by the target cell, retinol binds with a cellular retinol binding protein (CRBP) and is directed to further processing by cytosolic enzymes. Retinol has six common biologically active forms: all-trans, 11-cis; 13-cis; 9,13-di-cis; 9-cis and 11,13-di-cis, where all-trans retinoic acid (ATRA) is the predominant and most potent physiological form (168). Post-uptake processing is accomplished in two steps; first, oxidation by retinol dehydrogenase to produce retinal, and then further oxidation by retinaldehyde dehydrogenase to produce retinoic acid (RA) (168). Newly oxidized RA is bound to cellular RA binding protein (CRABP) I and II and is then used for autocrine or

paracrine signaling (168). Both forms of CRABP bind ATRA with a higher affinity than 9-*cis*-RA and RA (120). Regulation of intracellular RA is controlled by synthesis and catabolism rates. Catabolism of RA to its oxidized and inactive metabolites is managed by CYP26 enzyme family members, which hydroxylate either the C4 or C18 position of RA (130).

Retinoid Signaling

The varied biological effects of RA are mediated by its binding to nuclear retinoid receptors. Retinoid receptors contain a DNA binding domain (DBD), which grants sequence or target specificity. Retinoid receptors can be categorized into RA receptors (RARs) and retinoid X receptors (RXRs), which are both further subdivided into α, β and γ isoforms (168). RXRs were originally thought to exclusively bind 9-*cis*-RA with no end effect in mammals, but have since been found to be heterodimerize with several nuclear receptors, including: RAR, thyroid hormone receptors and vitamin D receptors (102, 168). Productive RAR signaling depends on RXR heterodimerization for the competent exposure of ligand and DNA binding domains to their targets (168). Over 500 genes have been recognized as targets of RAR signaling, including CRBP, CRABP and CYP26 regulatory mechanisms, *Hox* gene expression promotors which are critical in foetal development, as well as nervous system, skeletal and genital developmental targets, among others (168).

Biology & Pathology of Respiratory Syncytial Virus

RSV is a negative sense, enveloped RNA virus (~15.2kb genome) of the order *Mononegavirales*, family *Paramyxoviridae* and genus *Pneumoviridae*. The *Pneumoviridae* contain two genera: *Pneumovirinae,* which includes human RSV, bovine

RSV and murine pneumoniavirus; and *Metapneumovirus,* which includes human metapneumovirus and avian metapneumovirus (53). There are two distinct RSV strains, A and B, which differ mostly in their surface glycoproteins. Between these strains, there are a number of substrains with sequence polymorphisms that occur across the genome (Genbank). Replication requires the production of a full-length (positive-sense) antigenome from the negative-sense genomic template, both of which are tightly encapsidated to protect from cellular RNases (8, 29). The RSV genome contains extragenic leader and trailer sequences and ten 3'-5' sequentially transcribed genes that produce separate mRNAs requiring 5'-capping and polyadenylation for function (8). Each viral mRNA contains a highly conserved ribosomal binding site and a single open reading frame (ORF), except for *m2,* which contains the polycistronic *m2-1* and *m2-2* genes (29).

The viral fusion glycoprotein (F) mediates viral penetration into the host cell by enabling envelope-plasma membrane fusion. As part of the replication cycle, the F protein is expressed on the host-cell surface, and can promote fusion between neighboring cells—which causes the characteristic snycytia *in vitro*. RSV F is produced as an inactive precursor, F_0. The signal peptide and an internal furin endoprotease motif of the nascent RSV F_0 are cleaved to produce the active F_1+F_2 (31). The F_1 subunit contains hydrophobic heptad-repeats that promote fusion as an energetically favourable conformational change (30).

The G glycoprotein is a type II transmembrane protein and the major attachment protein for RSV. G has an N-terminal hydrophobic region that serves to anchor it in the viral envelope. The G protein also has a secreted form, which accounts for over 20% of

the total G produced, that uses a second start codon (66). The second G start codon leads to a shortened version that lacks the N-terminal anchor (66). Although the G protein is not necessary for replication, its removal attenuates growth *in vivo* (108). It is heavily glycosylated with host-cell-specific sugar moieties, which may prevent immune recognition (108). The G protein contains a central, conserved, mucin-like domain that was initially thought to confer the major binding function. However, it was later found that this sequence could be deleted without decreasing replication rates *in vitro* or *in vivo* (164). This conserved segment was later found to inhibit NF-κB signaling, thereby dampening the innate immune response (132).

The RSV small hydrophobic (SH) protein is a short integral membrane protein with an N-terminal anchor sequence and extracellular C-terminus (125). Intracellularly, SH homo-oligomerizes to varying degrees: SH0 is a full length unglycosylated species, SHg has a singly N-linked carbohydrate, SHp adds a further polylactosaminoglycan, and SHt is an unglycosylated, truncated, version produced by a downstream start codon (125). SH is not necessary for efficient growth *in vitro* or *in vivo*; and interestingly, deletion provides a moderate growth advantage in certain cell lines with little attenuation *in vivo*—it is thought that the reduction in genome length provides a packaging advantage (125). The actual function of RSV SH remains unknown.

The matrix (M) protein is an essential packaging component that accumulates at the host cell plasma membrane by interactions with the cytosolic tail of F during particle formation (65, 165). The M protein is also thought to promote host translational shut-off (47).

The RSV nucleocapsid (N) protein, phosphoprotein (P), large (L) protein and M2 ORF 1 (M2-1) are all necessary for productive RNA synthesis and replication (28). The N protein binds tightly to genomic and antigenomic RNA to resist cellular RNAse activity (9). N is maintained in an open and soluble conformation, ready for nucleocapsid assembly, by association with P— without which N forms inclusion bodies (9). P also acts as part of the polymerase complex, but requires heavy phosphorylation for proper function (38). RSV L protein contains six putative polymerase motifs and a nucleotide-binding domain responsible for the blunt-end capping of viral mRNA (96, 156). Finally, M2-1 is an essential transcription factor that promotes polymerase read-through; in its absence, the viral polymerase prematurely terminates transcription, leading to gross inconsistencies in viral protein ratios (28). Although the RSV M2-2 is not essential, suggested by an infectious and productive deletion strain, it is thought to play a role in shifting between transcription-oriented and replication-oriented RNA production for packaging (14, 166).

The RSV non-structural (NS) proteins 1 and 2 are produced in limited quantities, but play an important role in immune evasion. Deletion of NS1 or NS2 attenuates viral growth *in vitro* and *in vivo* (163, 166). These proteins inhibit the production of interferon (IFN) α and β by preventing phosphorylation, and therefore activation, of interferon regulatory factor (IRF) 3— a crucial amplifying step in interferon induction (153, 154). NS1 downregulates the proliferation of Th17 cell subsets and attenuates dendritic cell (DC) signaling to dampen the immune response. NS2 inhibits type 1 interferon production by targeting JAK/STAT signaling partner STAT for proteasomal degradation (136).

RSV Replication

RSV is largely transmitted by fomite deposition, contaminated with airway secretions, onto the apical surface of the upper and lower respiratory epithelium. Initial binding to a target cell is mediated by G protein binding to long, unbranched, extracellular glycosaminoglycans containing sialic acid, such as heparan sulfate and chondroitin sulfate B (59, 162). RSV F protein-mediated envelope-host fusion is facilitated by conformational change. M-N interactions dissolve as the viral-envelope contents are released into the host-cell cytoplasm, where replication is carried out. Regulation of RNA synthesis is controlled by several *cis* acting elements to ensure that RNA levels and types are synthesized. The RSV polymerase complex is composed of N, P, L and the transcription anti-terminator M2-1. The PL complex typically binds N-RNA at a conserved 3' entry site, where transcription initiates and proceeds in the 5' direction (8). The polymerase complex responds to c*is* acting start and stop sequences within the viral genome to trigger transcription start and stop along the genome— producing positive sense capped and polyadenylated transcripts (8). At each of the intergenic regions, the polymerase terminates and reinitiates transcription at the subsequent start site. The rate of gene transcription is linked to gene sequence, such that 3' proximal genes are transcribed more frequently than 5' proximal genes as a function polymerase release (53). Excluding the low transcription of NS1 and NS2, the gene transcription gradient in RSV correspond to genic sequence: *ns1, ns2, n, p, m, sh, g, f, m2-1/2, l* (53). Differences in the efficiency of the particular start sites also affect the likelihood of readthrough or release (60, 111).

RSV-origin proteins and mRNA can be detected four hours post-infection and plateau 14-18 hours post-infection (14). This perceived transcriptional halt is likely due to the accumulation of the M2-1 and M2-2 proteins, which promote full-length antigenome transcription (14). This halt defines a shift from protein production to genome replication; however, progeny begin to be released at 10-12 hours post infection and peak at 24 (14). In an individual cell, progeny release declines as cells deteriorate by 30-48 hours post-infection (53).

As with all enveloped viruses, RSV virions are formed by budding from a host envelope. Protein-protein interactions mediate co-localization and organization of a number of mature viral components along the inner leaflet of the host-cell membrane. The RSV genome is encapsidated by N, forming a helical nucleocapsid, which allows P and L proteins to bind (106). M proteins promote ribonucleoprotein (genome, N, L and P) localization to the cell membrane by bridging their interaction with the cytosolic surface of viral glycoprotein clusters in lipid rafts (53, 106). Budding is accomplished both by lateral packing pressure of the virus glycoprotein insertion into the envelope and host-cell cytoskeleton activity. Any perturbation of either the actin or microtubule network impairs budding and therefore viral proliferation (106). Recently it was suggested that myosin motors are actually responsible for budding and final egress from the apical surface of target cells (145).

Pathology

In vitro

RSV has a wide cellular tropism and is able to establish a productive infection in any cell type that expresses sialic acid residues, but is most productive in human

epithelial cells (53). In non-polarized cells, cytopathic effects manifest as syncytia with ruffled cellular borders (53). Polarized human airway epithelial cells grown on a basement membrane show very few signs of syncytia (191). In polarized cells, the F protein is exclusively expressed on the apical surface, which limits fusion of neighbouring cells (191). Up to 90% of progeny virus can remain cell-associated in tissue culture systems, though these virions can be released by sonication or repeated freeze-thaw cycles (53). Unsurprisingly, microarray analysis of infected cells shows altered expression of over 1300 host genes (170, 192).

In vivo: animal models and human infection

Although there is no animal reservoir, RSV can infect a wide range of animal species following intranasal inoculation. These include cotton rats, mice, ferrets, guinea pigs, hamsters, marmosets, cattle, ovids and several nonhuman primates; however, chimpanzees singularly recapitulate the human disease state (12, 20). In inbred mice, there is considerable variability between strains, with up to a 100-fold differences in viral replication rates (20). Typically cotton-rats and BALB/c mice are used in modeling the disease, though, the severity of the infection is reduced compared to primates and only physically manifests with a high titre inoculum (20, 53). *In situ* hybridization of infected cotton rat lung tissue at peak virus replication shows only sparse signs of infection (116).

RSV is among the most infectious viruses that affect humans. As noted above, infection is spread primarily by fomites contaminated by respiratory secretions (53), rather than by aerosol (55). Typically, virus is introduced onto upper respiratory mucosal surfaces and spreads to the lower respiratory tract by inhalation of secretions (53). Incubation of infection and disease onset is between four and five days, when titres reach

greater than 10^5 plaque forming units (PFU) in both upper and lower respiratory tract secretions (81, 187). Viral clearance usually coincides with clinical recovery, although there have been reports of viral persistence in immunosuppressed individuals with virus detected >100 days after initial infection (149).

RSV-induced pathology in infants most often results in edemic bronchiolitis or pneumonia with early influx of lymphocytes, plasma cells and macrophages. Viral entry and egress causes impaired cilliary function and inflammation promotes excessive mucous secretion (53). Along with immune infiltrates and cell debris, these issues compound to obstruct the infantile alveolar spaces and bronchioles, potentially leading to emphysema or airway collapse (53, 70). It has recently been suggested that cell debris, as a result of apoptotic epithelial sloughing, is one of the main reasons for the clinical manifestations of RSV (109). Pneumonia manifests with mononuclear cell infiltration into interalveolar walls, leading to edema of the pulmonary tissues and fluid leakage in alveolar spaces. In severe RSV infection, lower respiratory tract infection is often confined to a discrete number of foci—occasionally with low levels of antigen, suggesting an immunologic basis for severe disease (46, 121).

Visible cellular pathology arising from RSV infection in a host is limited. The strict localization for the RSV F protein to the apical surface of respiratory epithelial cells reduces the number of syncytia *in vivo.* Host metabolism and macromolecular processes are largely unaffected, but apoptosis develops slowly as a stress response to viral RNA protein expression and membrane destabilization from viral egress (53). Cellular damage is only a major concern for immunosuppressed individuals, in whom RSV infection can be fatal as a result of both increased and prolonged replication (104, 186). In

immunosuppressed cotton rats, viral dissemination outside of the respiratory system has been demonstrated (186).

Epidemiology and genetic susceptibility

Since the 1960s, RSV has been recognized as a serious pediatric respiratory disease. During a 13 year surveillance program for respiratory disease in Washington D.C., RSV was found in 43% of bronchiolitis and 25% of pneumonia cases. Overall RSV was detected in 23.3% of infants and children that presented with any respiratory tract disease (88). In an Arizona study, researchers found that 39.5% of all symptomatic respiratory infections within the first year of life were caused by RSV (71). RSV infections have a 1-3% rate of hospitalization (up to 10% in First Nations populations) (143). In the United States and Canada, RSV is estimated to cause between 73000-123000 and 6000-13000 hospitalizations in children under two years of age, respectively. However, estimates of infection rates and at risk populations are ill-defined, making accurate disease projections difficult (143, 151). Serological studies indicate that nearly the entire population has been exposed to RSV by two years of age (64). RSV is not exclusively a pediatric disease however. In healthy adults, reinfection by experimental challenge at 2, 4, 8, 14, 20 and 26 months after natural infection led to symptomatic infection of approximately 25% of the tested group at each time point (57).

Whether or not there is a genetic predisposition to serious RSV infection is controversial. Genetic disorders leading to diseases such as cystic fibrosis or severe immunodeficiency indisputably increase the risk associated with RSV infection. However, there is also significant evidence suggesting a more nuanced genetic susceptibility in animals and humans (16, 72, 107, 134). Polymorphisms in genes

associated with immune function are more likely to influence disease progression; for example, promotional polymorphisms which increase expression of Th2 cytokines Interleukin (IL)-4, IL-5 and IL-13, among others, are highly correlated with increased severity during RSV infection (27, 68, 69, 107). Other genetic susceptibility loci that have been suggested include the fractalkine CXC3, inflammatory mediator RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted), IL-8, IL-10, and various cell adhesion molecules (4).

Morbidity and Mortality

RSV-associated morbidity is affected by several factors including socioeconomic standing, prematurity, genetic predisposition (including race) and local environment. As such, hospitalization rates vary depending on region and can exceed 250 per 1000 in certain populations; typically however, RSV causes 1-20 hospitalizations per 1000 children in developed countries (48, 83, 88). There are no precise accounts of deaths attributable to RSV, but mortality is uncommon in developed countries. In 1985, there were an estimated 4500 deaths from RSV infection in the United States, but by 2003, this number had dropped to fewer than 100 per year in large part due to the introduction of monoclonal antibody prophylaxis for premature infants (151, 169). A recent study examining virus-attributable death in England between 1989 and 2000 found that RSV consistently caused more deaths than influenza (11). While impressive advances have been made in treating pediatric RSV, improvements are needed for treatment of the elderly in whom mortality can exceed 17000 deaths per year in the United States alone (169). The relationship between age and RSV-associated mortality seen in developed

countries is essentially the reverse of that observed in developing nations, where pediatric mortality predominates (53).

Immunity to RSV

The morbidity and mortality associated with RSV is the result of a highly complex, virus-induced, pro-inflammatory immune response leading to airway damage. Upon infection, RSV stimulates lung resident leukocytes and airway epithelial cells to release chemokines and cytokines (190). Circulating leukocytes are recruited to the infected lung where they add to the cytokine pool, further promoting chemokine release and chemotaxis. Cytokines, chemokines, epithelial cells, DCs, eosinophils, macrophages and neutrophils are all implicated in the RSV-induced immune response.

Role of Innate Immunity in RSV Infection

The innate immune system has evolved to recognize pathogens via pattern recognition receptors (PRRs), which allow the host to respond to a range of pathogens without previous exposure. Three classes of these PRRs are of particular interest in the case of viral detection and subsequent immune activation: nucleotide-binding oligomerization domain (NOD)-like receptors, Toll-like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I)-like helicases (RLHs). Of these three, the TLRs and RLHs appear to be the most influential in RSV infection.

TLRs are type-1 transmembrane proteins that are widely expressed across tissue types, and constitutively expressed on epithelial and endothelial cells. However, their expression pattern is heavily dependent on cell type and activation state (89). They typically bind conserved patterns that are necessary for pathogen survival (pathogen-associated molecular patterns, or PAMPs), such as bacteria-specific carbohydrates and

peptidoglycans, viral nucleic acids, lipoproteins and fungal glucans (79). TLR signalling stimulates anti-viral cytokine production and release to initiate the adaptive immune response (190). There are 10 known TLRs which can react to both endogenous and exogenous ligands; TLR1 and 2 and 6 bind lipopeptides as heterodimers, TLR3 binds double stranded (ds) RNA structures, TLR4 binds lipopolysaccharide (LPS) and RSV F protein, TLR5 binds bacterial flagellin, TLR7 and 8 bind specific single stranded (ss) RNA sequences, TLR9 binds unmethylated cytosine-guanosine (CpG) repeats and the TLR10 ligand remains unknown (40, 89, 94, 152). There is a clear relationship between RSV infection and the upregulation of TLR3 and TLR4 during human disease, while other TLRs (2, 6, 7 and 9) are mainly associated with murine models (45, 89, 142).

For viral replication, RSV requires a full length positive sense RNA template for genome synthesis, and therefore produces dsRNA intermediates. TLR3 is one of the most abundant TLR isoforms expressed in the airway epithelium (95). The intermediary dsRNA is a ligand for TLR3, RIG-I and melanoma differentiation-associated gene-5 (MDA-5) (89). Studies using small interfering RNA-mediated RIG-I silencing have shown that TLR3 is dependent on RIG-I signalling for expression—and suggest a temporal relationship between RIG-I and TLR3 for immune activation (95). Once expressed and activated by RSV dsRNA, TLR3 signals nearly exclusively through TICAM (TIR (Toll/IL1 receptor) domain containing adaptor molecule-1), activating IRF3, NF-κB and AP-1 transcription factors (89). These induce type 1 interferons (IFN-α, IFN-β, IFN-θ, IFN-ε, and IFN-κ), cytokine and chemokine production and DC maturation (89).

TLR4 is expressed as a surface receptor on peripheral leucocytes such as neutrophils and macrophages, and to a lesser extent on epithelial cells to prevent hyperactive responses to its other ligand— LPS (89) (40). Upon activation by RSV F protein, TLR4 can follow two signalling pathways to produce IL-6 and IL-8-like cytokines or type 1 interferons (IFN-α and IFN-β) (89). The first pathway acts through MyD88 (myeloid primary response gene 88) and TIRAP signalling by activating JUN N-terminal kinase (JNK), and mitogen-activated protein kinase (MAPK) pathways to activate NF-κB (89). The second pathway signals through MyD88, TRAM and TRIF (TIR-domain-containing adaptor protein inducing IFN-β), activating NF-κB and IRF3 and 7 to produce type 1 IFNs and apoptotic stimulators (89) (123).

RLHs (RIG-I, MDA5, and Laboratory of Genetics and Physiology 2 (LGP2)) are a family of RNA sensor-helicases that participate in an array of biological processes including, but not limited to, transcription, translation, mRNA splicing, ribosome synthesis and decay (95). RIG-I and MDA5 each contain two C-terminus proximal caspase recruitment domains (CARDs), a central DRxD/H-Box helicase domain and a C-terminal repressor domain (10). LGP2 has no CARD and therefore cannot transduce CARD-dependent signals, but instead is thought to be a repressor of RIG-I mediated signalling by heterodimerization via their homologous self-inhibition domains (10). LGP2 inhibition is thought to act by sequestering viral dsRNA from productive RIG-I recognition or by blockade of RIG-I-dependent downstream signalling molecules (10, 90, 141, 178, 189). However, LGP2 may also act to upregulate signalling by unwinding ribonucleoprotein complexes, exposing the naked ligand for binding and subsequent signalling through RIG-I (146). It was initially thought that RIG-I could recognize a

wide range of viral RNAs by binding ssRNA containing terminal 5′-triphosphate (5′ppp) residues (10). However, recently it was shown that 5'ppp ssRNA was unable to activate RIG-I; instead, the optimal ligand was found to be a 20bp blunt-ended 5'ppp in a proposed panhandle structure (147). All RNA polymerases, cellular and viral, produce 5'ppps during transcription; however, cellular mRNA capping mechanisms truncate these into monophosphates—producing a clear delineation between self and non-self RNA species (119). The untruncated caps explain the ability of RIG-I to effectively signal during infection with certain dsDNA viruses (Epstein-Barr virus, Orthoreovirus and Rotavirus) (10). RNA species length is a further determinant for receptor binding affinity, in that longer RNA species containing polyinosinic-polycytidylicacid (poly(I:C)) are MDA5 ligands, while shorter poly(I:C) sequences bind RIG-I (84).

RIG-I signalling is dependent on ubiquitination by the tripartite motif (TRIM) E3 ubiquitin ligase (161). RIG-I signals through two pathways: the mitochondrial antiviral signalling (MAVS) pathway and the tumor necrosis factor receptor-associated factor (TRAF)-mediated IRF activation (10). The MAVS pathway is specific for double stranded DNA viruses, so for the purposes of the current work, only the TRAF pathway will be outlined. Upon appropriate RSV-RNA stimulation, RIG-I associates with mitochondrial IPS-1 (IFN-β promotor stimulator-1) through their CARD homology (10). IPS-1 was found to associate with the polyubiquitinated TNF (tumor necrossis factor)-receptor associated factor (TRAF)-3 (161). TRAF-3 recruits a complex of three proteins: TRAF family member-associated NF-κB activator (TANK), NAK-associated protein 1 (NAP1) and similar to NAP1 TBK1 adaptor (SINTBAD) (161). These then recruit TANK-binding kinase 1 (TBK1) and inducible IκB kinase (IKK-i), which are responsible

IRF3 and 7 phosphorylation, dimerization and activation to produce type 1 IFNs (10, 161). IPS-1-TRADD also has affinity for the fas-associated death domain (FADD) protein, which interacts with RIP-1 (receptor-interacting serine-threonine kinase 1) through their death domains and TRAF3/6 (10). FADD and RIP-1 then signal through NF-κB to produce inflammatory mediators (10).

Type 1 IFNs, IFN-α and β in particular, are critical to the early anti-viral response. Type I IFNs are broadly expressed, but strictly regulated, across cellular types and upregulate a number of antiviral agents (176). Among the antiviral effectors produced are dsRNA-activated protein kinase R (PKR), lactoferrin and 2',5'-oligoadenylate synthetase (OAS) which ultimately mediate antiviral activity by inhibiting various stages of viral replication (176). PKR phosphorylates a number of cellular proteins, such as eIf-2 (translation arrest) and Fas/Fas-ligand dependent pathways, thereby promoting apoptosis and nitric oxide production (176). Lactoferrin prevents viral entry of both DNA and RNA viruses by physically binding the viral fusion proteins or by blocking cellular receptors (144). 2',5'-OAS leads to the cleavage of cellular and viral ssRNAs (176).

Under the influence of type 1 IFNs, epithelial and endothelial cells heavily shape the local innate immune environment by cytokine and chemokine synthesis. Neutrophils are recruited via TLR4-IL-8 production; IL-5 recruits eosinophils by inducing the production of granulocyte-macrophage colony-stimulating factor (GM-CSF), eotaxin and RANTES; natural killer cells and macrophages are recruited by macrophage inflammatory protein-1 α (MIP-1 α), IFN-α/β and IL-1β (176). Epithelial and endothelial

cells are also the primary driving force behind chemotaxis of conventional, marrow-born, DCs via MIP3-α—and therefore initiate the establishment of adaptive immunity (180).

Role of Adaptive Immunity in RSV Infection

The adaptive immune response plays the central role in recovering from and attenuating future RSV infection. The airway epithelium regulates adaptive immunity primarily through interactions with DCs, T and B cells. Epithelial derived MIP3-α constantly recruits conventional DCs (cDCs) from the bone marrow to epithelial sites, regardless of inflammatory state (180). Upon recognition of foreign antigen, cDCs undergo maturation (mDC), where they downregulate epithelial chemotactic receptors and express the CCR7 receptor, driving them towards the local draining lymphnode (180). Within the lymph node, mDCs express and present antigen to CD8+ and CD4+ T cells in the context of major histocompatibility complex (MHC) class I and II (180, 182). Antigen specific T cells appear in the airways between 6 and 7 days post-infection via Th susbset-specific chemotaxis; RANTES, IP-10 recruit Th1 cells, while IL-1β recruits Th2 cell migration in mice(180). Antibody production coincides with T cell infiltrates and peaks at 3-4 weeks after initial infection (182). Infection typically results in anti-RSV G and F IgM, IgG, IgA and occasionally IgE (182).

Preterm infants are among the highest risk groups for severe RSV infection (53). Infants begin to acquire maternal IgG1 between 17 (10% of maternal IgG levels) and 32 (50% of maternal IgG levels) weeks of gestation (175). In infants carried to term, high titres of maternal RSV-neutralizing antibodies are associated with relatively low risk of severe RSV disease (109). Breast milk also contains neutralizing antibodies but these antibodies generally do not have access to pulmonary tissues. The birth of preterm

infants before adoptive transfer of maternal IgG1 puts them at particularly high risk for respiratory infections. Upon exposure to wild-type RSV, high antibody titres against F and G proteins are produced in infants with waning maternal antibody levels. However, antibodies generated during these responses are less effective than their maternal counterparts, possibly due to the immunologic immaturity of the infants (115). In general, studies show that secreted antibodies play an important role in resistance to reinfection, but these effects are short-lived. Consecutive reinfection induces higher titres of anti-RSV IgA which, in turn, are correlated with reduced clinical manifestations and a better prognosis for recovery (64). Immunocompromised children and adults are at risk of developing serious disease after infection and often continue to shed RSV for many months (56).

Mouse studies have been used extensively to study RSV adaptive immunity. In the BALB/c model, natural killer (NK) cells migrate to the lung within the first two days of infection followed by pulmonary CD8+ cytotoxic T lymphocytes (CTL). Several days later, one can measure secretory then systemic antibody production (53). Both NK and CTLs are necessary for effective clearance of RSV. Depletion of either of these lymphocyte sub-populations leads to increased pathology and viral persistence (50, 105). Although B cell depletion does not affect viral clearance, B cells are primarily responsible for preventing re-infection (49). Active production of RSV-specific IgA is associated with decreased viral shedding and recovery; however, this effect is less pronounced in animal models (105).

Th1 vs Th2 in RSV Infection

T helper cell balance and polarization represents the management of a highly heterogeneous pool of CD4+ T cells to tailor the local and systemic immune activity to best respond to a pathogen that the immune system perceives as a threat. Several T lymphocyte subsets have been characterized, including Th1, Th2, Th9, Tregulatory and Th17 cells, which each have different (although at times ill-defined) target ranges or rolls. For our purposes however, a simple model that focuses primarily on the Th1 and Th2 subsets should be highly informative. Naïve Th cell differentiation is influenced by both genetic and environmental factors during antigen recognition in the context of an antigen presenting cell (APC). These include: T cell receptor recognition and ligation of presented antigen, co-stimulation, cytokine environment and post-activation proliferation (139). Th1 differentiation is dependent on early release of IL-12 by APCs and activation of STAT4 through the IL-12 receptor (137). Ultimately the T-box protein expressed in T cells (T-bet) promotes the expression of the characteristic Th1 cytokine profile including IL-2, TNF-β and IFN-γ (110, 124). Transduction of T-bet into polarized Th2 cells is sufficient to suppress Th2 cytokine production and convert them into competent IFN-γ-producing Th1 cells (139). In contrast, effective Th2 cell development requires early autocrine IL-4 stimulation, activating STAT6 and subsequently c-Maf, but more importantly—GATA-3, that suppresses IFN-γ expression (110, 124). Differentiation promotes the expression of the prototypical Th2 cytokines: IL-4, IL-5 and IL-13 (137). The cytokine environment necessary to elicit an effective immune response is a complex and highly nuanced series of signalling cascades. For instance, depending on the circumstance, either CD4+ or CD8+ T cells can be experimentally manipulated to

simultaneously or exclusively express IFN-γ and IL-4, IL-17 and IL-9 or IL-17 and IFN-γ, all of which are classically recognized to have opposing effects (13, 33, 63, 124, 177, 184).

Cellular damage caused by RSV is only a minor component of the disease state. The host immune system plays a significant role in disease outcome and tissue damage. The balance between CD4+ Th1 and Th2 lymphocytes, in particular, is a strong determinant in protective versus pathogenic responses to RSV infection. In retrospect, the initial attempt to introduce a formalin-inactivated vaccine appears to have induced a Th2 biased response to RSV antigens upon natural infection, which led to enhanced pathology in many of the vaccinated children (82). The response to natural infection with wild type virus in neonates and infants also tends to be Th2 biased, showing elevated IL-4, IL-5 and IL-13 with decreased IL-12 and IFN-γ. However, reinfection after natural infection does not show disease enhancement (2). Peripheral blood mononuclear cells from children with acute RSV infection cultured *in vitro* in the presence of a mitogen show that IFN-γ was highly suppressed, while IL-4 levels remained largely unchanged leading to heavily skewing IFN-γ:IL-4 ratios (11). Further, RT-PCR analysis of IFN-γ mRNA in lymphocytes from acute RSV-infected children reveals that significantly less IFN-γ is produced than in those with milder clinical manifestations (11). Comparison studies between Influenza A and RSV immunologic responses show that RSV infection polarizes serum cytokines towards a Th2 profile: IL-4, IL-5, IL-13 (11). Older children and adults tend to develop a more balanced response (7). In studies exploiting recombinant vaccinia constructs, it has been shown that the G protein is, at least partially responsible for the Th2-biased response (3). More recent studies have revealed a 14

amino-acid peptide from the G protein sequence drives this Th2-polarizing effect. Conversely, F-targeted antibody responses bias towards Th1 subset development (3, 11). It has also been noted that the RSV G protein does not elicit a significant CTL response, and that the Th2 imbalance might be a product of this inactivity (74, 155).

Role of T regulatory and Th17 Cells in RSV Infection

Both T regulatory cells (Treg) and Th17 cells have recently been suggested to play a role in the immune response during RSV infection. In 2010, Cusi *et al*. suggested for the first time that Treg-induced immunosuppression is increased in aged individuals, producing a measurable Th2 bias that is absent in younger individuals and that is associated with a decline of RSV specific $CD8^+$ memory cell populations (34). These authors propose that this shift in balance may be responsible for the increased susceptibility of the elderly to severe RSV infection (34). Tregs are generally implicated in the dampening of aggressive $CD8^+$ action during viral infections, which may enhance the natural Th2 biasing nature of RSV (43). Interestingly however, Tregs appear to act as a double-edged sword in RSV infection—depletion of Tregs in mice paradoxically leads to increased disease severity caused by immune hyperactivity (43). Recent data also suggests a role for Th17 cells which are strongly regulated by NS1 (113). When an NS1-knockout strain of RSV was loaded into DCs and infused into naïve mice, the resulting Th17 response showed increased activity and proliferation, suggesting that NS1 normally downregulates the function of these pro-inflammatory cells (113). Th17s have been shown to protect mice from vaccinia virus and influenza virus infection through an IFN-γ independent neutrophil recruitment mechanism (113).

Overall Immune balance in RSV Infection

The respiratory epithelium is a large mucosal surface that is constantly exposed to wide variety of environmental antigens and pathogens. To avoid chronic inflammation, the respiratory mucosa has developed mechanisms to discern benign versus pathogen-born antigens (43). A careful balance is maintained between immune ignorance, where immune activation thresholds are dampened, and immune sensitivity to generate a highly controlled inflammatory humoral response (43). When this system is working as it should, once an activation threshold is reached, the immune system creates a balanced response that is able to clear the invading pathogens while limiting damage to the surrounding tissues caused by immune effectors (43). Failure to achieve this balance can lead either to loss of control of the invading organism or to immune-mediated pathology. RSV appears to exploit this delicately balanced system to prolong infection by using both immune misdirection (above) and evasion.

Immune evasion in RSV Infection

RSV appears to be a remarkably difficult target for the human immune system to confront. For example, this virus has the ability to infect despite the presence of competent neutralizing antibodies; permitting infection of infants with normal maternal antibody titres (53). Further, the ability of RSV to consistently reinfect regardless of age represents a failure of the body to establish effective immunological memory. While there is evidence that suggests this may be a result of dominant subgroups in a given RSV season, there are also data suggesting that antigenic diversity plays only a minor role in evasion (58, 129). The ability of RSV to shift the Th1-Th2 balance outlined above is a critical strategy of this virus to evade the immune response but it is certainly not the only

mechanism. For example, the restricted respiratory tissue-tropism of RSV may be considered a 'privileged site', in that the secretory IgA response at mucosal surfaces is relatively short lived (months) compared to the more stable serum IgG response (years). IgA may also be less efficient at neutralizing and opsonizing respiratory pathogens, which attack the apical surface of the respiratory epithelium (53). The heavy glycosylation of F and G may also sterically hinder antibody binding—thereby preventing opsonization. Further, the prevalence of secretory G protein (up to 80% of all G in supernatants from RSV-infected cultures) likely provides a decoy from antibody recognition as well as promoting Th2 polarization by out-competing F for antigenic recognition and eventual antibody production (66). NS1 and NS2 are also known to directly inhibit type 1 IFN responses by preventing IRF3 phosphorylation (153, 154) and may also have a role in directing Th subset differentiation (113). Lastly, RSV F protein has been shown to inhibit T cell proliferation *in vitro* by an as yet unknown mechanism (133).

Treatment of RSV Infection

The treatment of severe RSV disease is currently largely supportive, including mucous removal, administration of humidified oxygen, hydration and mechanical ventilation. Further treatments aimed at controlling immunity and inflammation have not shown much promise, but may be more effective in severely immunocompromised individuals (91). Indicators suggesting the need for hospitalization are hypoxia, respiratory distress and dehydration— if left unchecked infants are at risk for developing apnea (53). Bronchodialators and steroids have been studied extensively in emergency settings, but have failed to produce more than transient improvements (44, 62, 91). The effects of

antiviral treatment on severe RSV are ambiguous. Approved by the FDA in 1986, ribavarin is a mutagenic guanosine analog that inhibits viral polymerase and 5'capping to reduce productive replication after genomic incorporation (179). Ribavarin is administered as an aerosol via ventilator for 6-18 hours a day for 3-7 days (53, 179). Initial trials included a very limited number of subjects, but showed slight beneficial effects such as decreased viral shedding and clinical severity scores (179). However, follow up studies have found that ribavarin has little to no effect on the course of RSV recovery (53, 91). In contrast to the dismal state of options for active RSV therapy, prophylaxis against RSV has been a major medical success. For prophylaxis, the RSV F-specific humanized mouse monoclonal-antibody Palivizumab (Synagis™) is administered intramuscularly (15mg/kg) once a month (91). Early studies demonstrated a significant benefit, particularly for preterm infants with underlying respiratory disorders (52, 91). Palivizumab was approved in 1998 for use in high-risk infants. Unfortunately, this drug is extremely expensive and cost-benefit analyses have led to the imposition of strict guidelines for its use, limiting availability to preterm infants born between 32 and 35 weeks gestation (91, 92). These infants are given up to three doses and prophylaxis is halted at 90 days post-delivery (92). The second-generation monoclonal antibody, motavizumab, was developed by affinity maturation of palivizumab (91). However, motavizumab showed no particular decrease in mean hospitalization time over palivizumab (22, 188).

Measles and Vitamin A

Micronutrient supplementation with vitamin A has been identified as a highly effective treatment for acute measles infection (183). Two pharmacological doses of

200,000 IU of water-soluble retinol can significantly reduce morbidity and mortality associated with measles infection (36). Several possible mechanisms have been suggested for this beneficial effect including improved epithelial repair and optimal immune cell function. In this context, the Ward and Miller laboratories at McGill have recently shown that retinol 'treatments' may also act by upregulating RIG-I and therefore RIG-I inducible type 1 IFNs that promote and antiviral state in non-infected bystander cells. These cells are effectively sensitized to prevent viral propagation and spread (172, 173). Further, this group also demonstrated that the retinol metabolite, ATRA, was 5-10fold better than retinol at increasing type 1 IFNs *in* vitro (173).

Vitamin A as an Anti-viral in other Viral Infections

The success in reducing measles pathology spurred efforts to use retinol supplementation as a therapeutic agent for other viral infections, particularly those caused by negative-sense RNA viruses including other members of the Paramyxoviridae family. The Miller and Ward laboratories have recently shown that replication of vaccine strain and some (but not all) wild-type isolates of mumps virus can be significantly suppressed using ATRA *in vitro* (Soye. *et al*, submitted). In this mumps model, ATRA supplementation appears to act in a similar fashion to that seen in measles infection; ie: ATRA acts through IRF-1 and RIG-I to produce a broad-spectrum antiviral state and bystander effect (Soye, *et al.*, submitted) (Soye and Trottier, *et al.*, under revision). Canine distemper virus (CDV), another member of the paramyxoviridae family, has also been exploited by this group and Dr V von Messling to assess retinoid actions in vivo (138). Infected ferrets treated with 30mg of retinol did not display typical signs of CDV

infection (fever, conjunctivitis, cough, coryza, and diarrhea) although they did develop a mild rash (138).

The success of retinoids as a therapeutic agent is not restricted to paramyxoviruses. Although the most recent data suggests a complex role for vitamin A supplements in both HIV infection and mother-to-child HIV transmission, a large body of evidence has shown that vitamin A status and supplements can influence HIV biology both *in vitro* (increased replication) and *in vivo* (more rapid disease progression and increased mortality in some groups) (87, 185). There is some consistent evidence for pathogen clearance upon retinol supplementation during rubella infection, where the robustness of rubella-specific antibody responses were found to be highly correlated to single nucleotide polymorphisms in *RIG-I* (126). Vitamin A supplements have also been shown to markedly decrease gastrointestinal symptoms in Mexican children infected with norovirus (97-99). Paradoxically however, these less-symptomatic children had higher gut viral titres and shed virus for longer. Most interesting for the current work, Stephensen and his colleagues have published a series of papers exploring the effects of vitamin A deficiency and supplements in mouse models of influenza (32, 157). While they report generally decreased symptoms with better vitamin A status, it is very interesting that vitamin A appears to strongly promote Th2 deviation of the immune response to an exclusively respiratory virus (32).

The evidence of vitamin A impact on RSV infection is derived exclusively from human studies. A recent meta-analysis of many large vitamin A field trials in developing world children strongly suggests that such supplements are not beneficial and may even cause harm in respiratory infections (122). Targeted retinoid therapy in children

admitted to hospital with severe RSV infection also suggest that retinoids may cause harm rather than benefits in this situation: either exacerbating the disease or prolonging recovery time (17, 37).

Retinoids and T cell Biology

Retinoid immunomodulation extends well beyond innate immunity, and for nearly 20 years, retinoids have been recognized as agents that can shift Th cell balance (23). More specifically, retinoid status is linked to Th1-Th2 polarization, where a vitamin A deficient state promotes a Th1-biased response to infection, and conversely vitamin A supplementation biases immune responses towards a Th2-type patterns *in vivo* and *in vitro* (21, 39, 67, 76, 80, 103, 112, 131, 135, 148). ATRA and 9cRA can affect Th balance by acting on monocytes and naïve CD4/8+ T cells (80). ATRA decreases IL-12 production in activated macrophages, which is a key component for Th1 polarization (80). *In vitro* TNF-α levels were also seen to drop after ATRA treatment of activated macrophages, while upregulating IL-10 secretion (131). Treating immature CD4+ or CD8+ T cells with RA *in vitro* during Th1 cytokine stimulation by IL-12 and IL-2 prevented the development of a Th1 cytokine profile; conversely, treatment of T cells with RA and Th2-cytokine stimulation by IL-2 and IL-4 enhanced the shift to a Th2-type subset compared to controls (131). *In vitro*, retinoids can directly inhibit the polarization of Th1 cells from stimulated naïve precursors; however, promotion of Th2 subsets is time sensitive— if retinoids are applied parallel to TCR stimulation Th2 development is suppressed (80). Further, acting through RAR/RXR, both ATRA and 9-*cis*-RA inhibit IFN-γ production while promoting IL-4 production in primed CD4+ T cells (76). The opposite effect is seen when DCs are treated with retinoids, where treatment of immature

DCs caused MHCII upregulation and IL-12 secretion, which then biases T cell subsets towards the Th1 subset (131). These dramatic Th2 polarizing effects are also seen in mouse models of asthma, where a vitamin A deficient diet decreased serum IgE and local IL-4 and IL-5 levels in bronchiolar lavage fluids (148). In the same study, high dietary intake of vitamin A increased IgE and exacerbated airway hyperresponsiveness in mice (148).

Retinoids also act to shift the Treg/Th17 balance towards a Treg-biased environment (112). Retinoic acid acts by promoting TGF-β inhibition of IL-6, the principal inducer of Th17 cells (112). This shift would reduce proinflammatory processes and dampen the Th1 response, which is consistent with findings in aged populations, where uncontrolled expansion of Treg populations biases Thelper cell responses towards a Th2-type response (34). All of these respective effects support our hypothesis that retinoids create a pathologic microenvironment during RSV infection that exacerbates an already dangerous disease for millions around the world.

Central hypothesis

The goal of this project is to develop a small animal model to characterize the possibility of a malignant interaction between retinoids and RSV infection. Reasons for the potential disease exacerbation are currently unknown; however, we suspect a pathologic synergy occurs between the Th2 biasing nature of RSV in human and mouse hosts (the 'extrinsic' factor) and tissue-specific Th2-biasing effects of retinoids in the lung (the 'intrinsic' factor). This animal model will include mice in four different conditions: normal vitamin A status (CD), vitamin A deficient (VAD), a vitamin A normalized group (VAN) and a supplemented group (VAS). Based upon our central

hypothesis, we expect to see less severe manifestations of RSV infection in VAD mice compared to the control animals (CD) and possibly enhanced disease in the supplemented group. According to this hypothesis, the manifestations of RSV infection in the VAN mice should be comparable what is observed in the CD group. The first comparison (ie: VAS vs CD) may be particularly relevant in the context of RSV disease in developed countries, where both societal and individual 'overnutrition' and micronutrient supplementation are common.

Methods

Overview

To compare the pathology of RSV infection by vitamin A status, I developed a model in BALB/c mice bred to be either vitamin A deficient (VAD), vitamin A replete (CD and VAN) or bred under high vitamin A conditions (VAH). The vitamin A status of these groups was validated by measuring liver retinol concentrations by HPLC. The severity of infection was characterized by morbidity (ie: behavioural changes) and weight loss. The immune status of the lung tissues prior to infection as well as the response to infection were assessed by characterizing the mononuclear cells present in broncho-alveolar lavage (BAL) by cytospin as well as cytokine profiling prior to infection and at intervals during infection. Sera were collected at all time points which will be used to measure IgG1, IgG2 and total IgG antibody titres directed against RSV antigens by enzyme linked immunosorbant assay (ELISA). Lung histopathologic assessment is currently being examined in paraffin-embedded tissue sections collected at sacrifice. Virologic outcomes included total lung viral load assessed by 50% tissue culture infective dose (TCID50). In addition to the mouse model of RSV infection, we also developed an *in vitro* model based on RSV infection of primary human respiratory epithelial cells *in vitro* in preparation for molecular and mechanistic studies.

Studies on VAH animals is ongoing; therefore, no data is provided for this experimental group.

In vitro

Viral Growth

Vero green monkey kidney cells (ATCC#CCL-81™) were seeded into Corning T175 culture plates in EMEM (Wisent corporation) supplemented with 5% fetal bovine serum (FBS), 1% HEPES (Wisent) and 50µg/mL gentamicin (Wisent). The cells were allowed to grow to 90% confluency at 37°C, 5% CO_2, and infected with RSV (ATCC# VR-26™) at a multiplicity of infection (MOI) of 0.001 and grown in EMEM supplemented with 3% FBS, 1% HEPES and 50µg/mL gentamicin. After four days, post-infection (p.i.) supernatants were collected, passed through a 0.45µm Durapore Stericup™ (Milipore Corporation) and stored in liquid nitrogen until further used for testing.

Viral Titration

Viral titres were determined by 50% tissue culture infectious dose ($TCID_{50}$), which is defined as the dose at which 50% of the cells in a monolayer are infected. Vero cells were seeded at $1.5x10^6$cells/mL in flat-bottom 96-well MICROTEST™ tissue culture plates (Becton-Dickinson Labware). Serially diluted (log_{10}) RSV samples were added to quadruplicate wells and were incubated for five days at 37°C, 5% CO_2. Wells presenting with syncytia were recorded and titers were calculated using the Karber method (54).

Primary Explants

Normal human small airway epithelial cells (ATCC#PCS-301-010) were seeded at $5x10^3$cells/mL into T75 Primaria™ tissue culture flasks (BD) using an airway epithelial cell basal medium (ATCC PCS-300-030) supplemented with a small airway epithelial cell growth kit (ATCC PCS-301-040) and 50µg/mL gentamicin (Wisent). Cells were

allowed to grow to 70-80% confluency at 37°C, 5% CO_2, then subcultured, re-seeding at $5x10^3$cells/mL. Cells were divided into four experimental groups: Mock, Dimethyl sulfoxide (DMSO: 15µL), ATRA (10^{-7}M in DMSO), DMSO-RSV (MOI 0.001) and ATRA-RSV (MOI 0.001); and were seeded in triplicate at $1.5x10^6$cells/mL in flat-bottom Multiwell™ Primaria™ 6-well plates. Experimental groups were infected at an MOI of 0.001. Supernatants were collected five days p.i. and titers were determined by $TCID_{50}$.

In vivo

All animal procedures were approved by the McGill University Animal Care and Use Committee (protocol# 5857).

Development of *In Vivo* Model

Breeding, Supplementation and Treatment

Eight breeding cages were established as 1:2 ratio, male to female, from 24 BALB/c (Jackson Laboratories). Breeders were separated into four diet groups: control diet (CD), vitamin A deficient (VAD), ATRA supplemented (vitamin A high, VAH) and vitamn A normalized (VAN). CD and VAH breeders were maintained on the 8604 Teklad Rodent Diet (12.6 IU/g) (Harlan Teklad). VAD and VAN breeders were maintained on the AIN-93G vitamin A deficient diet (0 IU/g) (TestDiet). After experimentation with a number of ways to provide supplemental vitamin A reliably in mice, we developed a silastic metered dose delivery system. To our knowledge, this is the first use of this technique to provide a reliable vitamin supplement over a prolonged period in small rodents. VAH females had a 1cm SIL-TEC™ silastic tubing (Technical Products Inc.) (0.062" internal diameter 0.125" external diameter) implant containing 60mg of ATRA surgically inserted

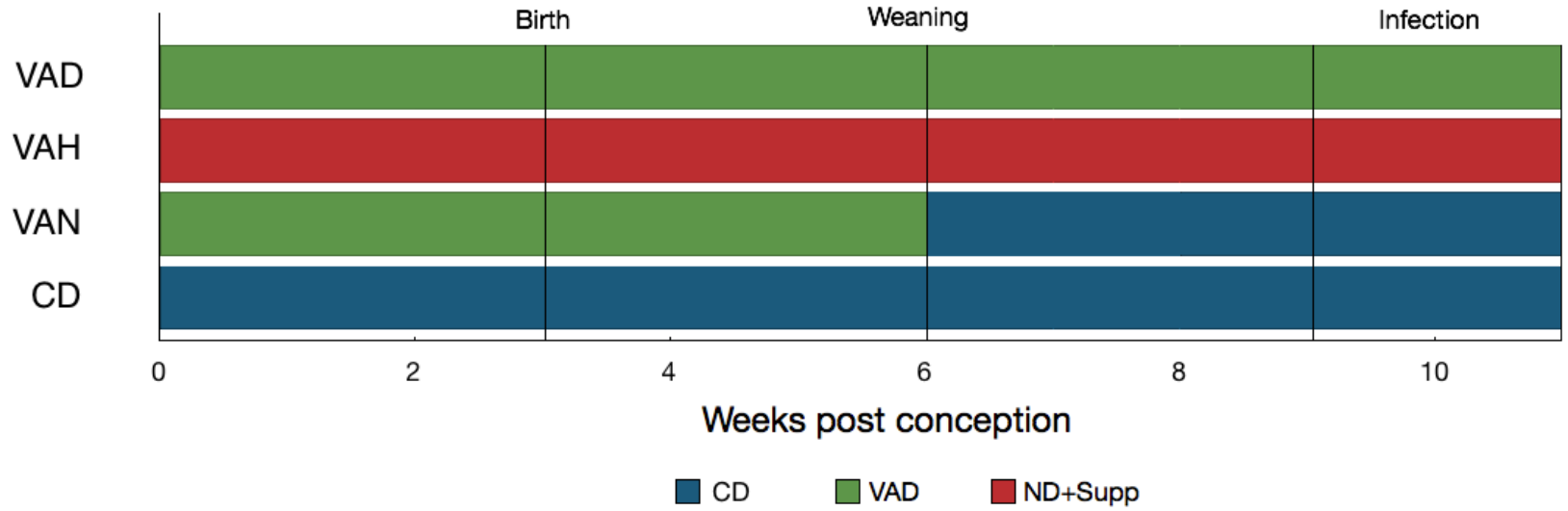
between their scapulae. Implants were sealed with Silicone A adhesive (Bluestar). VAH offspring had silastic implants inserted upon weaning, controls for VAH had empty silastic implants inserted upon weaning. Surgical details will follow. CD, VAN and VAH offspring were maintained on 8604 Teklad Rodent Diet (Harlan Teklad) after weaning. VAD offspring were maintained on AIN-93G vitamin A deficient diet after weaning. See Figure 1 for breeding and maintenance outline.

Surgical procedure

VAH-weaned experimental mice and VAH female breeders were anesthetized with isofluorane and subcutaneously injected between their scapulae with Carprofen (5µL/g). The area was shaved and sterilized with ethanol (70%) and dovidine™ (10% povidone-iodine, Laboratoire Atlas Inc.). A 5mm incision was made at the base of the skull and tunneled 1-1.5cm between scapulae using forceps. The sterile implant was inserted and the wound was treated with a drop of lidocaine-bupivacaine solution. The incision was closed using Vetbond™ (3M Company) skin adhesive. Mice were allowed to recover in a 37˚C recovery-incubator for six hours before being introduced back into their cages.

<u>Figure 1.</u> Mouse Groups are bred to achieve different vitamin A statuses in offspring.

Vitamin A Deficient (VAD) mice were bred and maintained on a diet containing no vitamin A (0 IU/g). Supplemented (vitamin A high, VAH) mothers received a silastic tubing implant (60mg ATRA) and offspring had implant inserted upon weaning, all VAH mice were maintained on the control diet (12.6 IU/g vitamin A). Vitamin A normalized (VAN) mice were bred by VAD mothers and maintained on the control diet after weaning. Control diet (CD) mice were bred and maintained on the control, normal rodent, diet.



Infection

A total of 144 mice were used for this study, with n=6 per experimental group (Table. 1). Mice were anesthetized with Isofluorane (2 minutes) and intranasally infected with 50µL of RSV ($TCID_{50}$ $5x10^6$). Controls were similarly anesthetized and intranasally inoculated with cell-free media (EMEM, 3% FBS, 1% HEPES).

Animal procedures and sample collection

Mice were monitored and weighed daily following infection. On the terminal day of each study (days 2, 7 and 14 p.i.) mice were euthanized by CO_2 asphyxyation and exsanguinated by cardiac puncture. Serum samples were obtained from blood by centrifugation, aliquoted and stored at -20˚C until further use. Lung secretions were collected by bronchialveolar lavage (BAL), by exposing and incising the trachea and inserting a 22 gauge BD Insyte catheter (BD). Catheters were fixed by a suture loop and a Luer-Lok™ tip syringe (BD) containing 800µL of protease inhibitor cocktail (SIGMAFAST™ protease inhibitor tablets for general use, Sigma-Alderich Inc.) diluted in sterile 4˚C PBS (Wisent). Wash fluid was slowly injected into the lungs and aspirated a total of three times, then collected with approximately 80% fluid recovery and kept at 4˚C until further processing. The right lung was excised, fixed in a 4% paraformaldehyde solution and stored at 4˚C. The left lung was excised, homogenized for 30 seconds with a Polytron homogenizer (Kinematica) and diluted in equal parts SIGMAFAST™ protease inhibitor cocktail. The samples were centrifuged, the supernatants were aliquoted and stored at -80˚C. Mouse livers were also collected and stored at -20˚C until further use.

Table 1. Description of experimental Groups.

Six to eight week old BALB/c mice were infected and weighed daily throughout the infective course. Study termination was performed on days 2, 7 and 14. Groups were Control Diet (CD), Vitamin A Deficient (VAD), Vitamin A Normalized (VAN) and ATRA supplemented (Vitamin A High, VAH).

	Experimental Endpoint					
Experimental Group	Day 2 +RSV	Day 2 -RSV	Day 7 +RSV	Day 7 -RSV	Day 14 +RSV	Day 14 -RSV
CD	6	6	6	6	6	6
VAD	6	6	6	6	6	6
VAN	6	6	6	6	6	6
VAH	6	6	6	6	6	6

Cytospin

Cells present in the BAL were identified by cytospin, cell staining and manual differential counts. BAL samples were centrifuged (8000g for 8 minutes), the supernatants were aliquoted and stored at -80˚C. The cell pellets were resuspended in EMEM supplemented with 5% FBS, 1% HEPES and 50µg/mL gentamicin. Cells were counted by trypan blue exclusion and further diluted to $1x10^5$cells/mL when possible. Shandon single cytofunnels™ (Thermo Scientific) were prepared with Superfrost™ microscope slides (Fisher Scientific) in a Shandon Cytospin (Thermo Scientific) and BAL cell-suspensions were added to individual cytofunnels. Samples were centrifuged, allowed to air dry and fixed in 95% methanol (Sigma-Alderich Inc.) for 30 minutes. Slides were then stained with Diff-Quick™ (Dade Behring) and analyzed for cell populations based on staining and morphology. A total of 200 cells were counted on each slide.

Viral Titre

Viral titres from BAL fluid were determined by $TCID_{50}$ (as above).

Retinol levels

Liver retinol levels were determined by HPLC analysis. Samples were shielded from direct light at all times. Equal volumes of anhydrous ethanol (American Chemicals) containing 1g/L butylated hydroxytoluene (American Chemicals) were added to liver samples, after which samples were saponified with potassium hydroxide (American Chemicals) for four hours at 70˚C. Samples were diluted in ddH_2O and Vitamin A was extracted three times with HPLC grade n-hexane (American Chemicals). Solutes were concentrated by evaporation and

reconstituted in 1mL n-hexane. Samples were washed with methanol (American Chemicals), evaporated under nitrogen gas, then resuspended in methanol:CH_3N (American Chemicals) and filtered before being transported to the Armand Frappier HPLC core facility for analysis using a 15cm Zorbax XDB column. Retinol was detected at 320nm from 20µL of purified sample. Sample retinol was eluted with two solutions, water and acetonitrile, containing 2 mmol/L of ammonium acetate (pH 7.4). The initial flow composition was 20% water, 80% acetonitrile for 0.5 min, increasing to 100% acetonitrile after 5 min, then maintained for 10 min at a pressure of 400 μL/min. Retention time was generally at 13.2 minutes and the calibration curve (5µM-100µM) was straight.

Lung Morphology

Fixed lungs were frozen at -20˚C in disposable base molds (Marivac) filled with Tissue-tek Optimal Cutting Temperature compound (Sakura). Frozen samples were then sectioned at 11µm in a Shandon Crytome SME (Thermo Scientific) and placed on Superfrost™ microscope slides (Fisher Scientific). Sections were then stained with Hematoxylin/Eosin or Hansel's Stain and examined at low (10x) and high magnification (40x & 100x) for differences in morphology. On average, at least 20 high power fields were examined on each slide. In some experiments, one lung was harvested for histopathology and possibly immunohistopathology. At the time of writing, these samples have been sent for sectioning. They will initially be examined for gross morphologic changes (eg: gross lung architecture as well as cellular infiltrates). Depending upon these results, further studies may include assessment of the infiltrating cells and *in situ* cytokine analyses. These

studies will be performed with assistance from Dr Q Hamid (Meakins Christie Laboratories).

Antibody determination by ELISA

Total serum RSV-specific total IgG as well as IgG1 and IgG2 levels were determined by enzyme-linked immunosorbant assay (ELISA) in 14 day infected mice. 96-well U-bottom plates (Greiner Bio-One) were coated over night at 4˚C with RSV Long strain whole protein isolate (15µg/mL) (Fitzgerald Industries International) in carbonate-bicarbonate buffer. Plates were washed four times with PBS-Tween 20 (0.05%) (PBS-T) blocked for 90 minutes at 37˚C with 2% milk in PBS-T. Plates were washed four times with PBS-T and serum samples were diluted 1:5 in blocking buffer, then added to the wells and incubated for two hours at 37˚C. Plates were washed four times in PBS-T and a horseradish peroxidase-conjugated primary antibody (goat anti-mouse IgG_1 (SouthernBiotech) or goat anti-mouse IgG_2 (SouthernBiotech)) was added to the wells and incubated for 30 minutes at 37˚C. See below for standard and control wells. Plates were washed five times with PBS-T and 37˚C TMB/E substrate (Millipore) was added to the wells. Plates were allowed to incubate at 37˚C for 15 minutes. The reaction was stopped with 50µL of 0.5M H_2SO_4 (Fluka Analytical) and the optical densities at 450 nm were read immediately on an EL-800 Universal Microplate Reader (Bio-Tek Instruments Inc.).

Antibody titres were calculated based on standard curves run on each plate with purified Mouse IgG_1 κ Isotype Control (BD Biosciences) and Purified Mouse IgG_{2a} κ Isotype Control (BD Biosciences) as coating antigen, respectively.

Negative control wells were coated with empty coating buffer. Antibody studies are ongoing; therefore, no data are included in the results.

Albumin determination by ELISA

Total BAL albumin levels were determined by ELISA in four- and seven-day infected mice. 96-well U-bottom plates (Greiner Bio-One) were coated for one hour at room temperature with a goat anti-mouse albumin antibody (Bethyl Laboratories Inc) in carbonate-bicarbonate buffer. Plates were washed five times (50mM Tris, 0.14M NaCl, Tween 20, pH8.0) and blocked for 30 minutes at 37˚C with 1% BSA (Wisent) (50mM Tris, 0.14M NaCl). Plates were washed five times and samples were diluted 1:50 in blocking buffer with Tween 20. Samples were then added to the wells and incubated for one hour at room temperature. Plates were washed five times and a goat anti-mouse albumin, HRP-linked, detection antibody (Bethyl Laboratories Inc), in sample diluent, was added and incubated for one hour at room temperature. Plates were washed five times and 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Millipore) was added and left to develop for 15 minutes. The reaction was stopped with H_2SO_4 (0.18M, Fluka Analytical) and the optical densities at 450 nm were read immediately on an EL-800 Universal Microplate Reader (Bio-Tek Instruments Inc.). Albumin level determination is ongoing; therefore, no data are included in the results.

Signaling Profile

BAL cytokine and chemokine profiles were determined by multiplex ELISA (Quansys Biosciences). An eight point standard curve (1:3 serial dilutions) was

produced using the provided antigen standards. All samples (Neat-undiluted, 1:10, 1:50) and standards were diluted in Sample Diluent (Quansys Biosciences). Samples and standards were added to a 96-well multiplex plate (Quansys Biosciences) containing capture antibodies for IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, MCP-1, IFN-γ, TNF-α, MIP-1α, GMCSF and RANTES. The plates were covered and agitated at room temperature for one hour on a shaker. The plates were washed three times with Wash Buffer (Quansys Biosciences) and the Detection Mix (Quansys Biosciences) was added. The plates were covered and agitated at room temperature for one hour on a shaker. Plates were washed three times with Wash Buffer and the Streptavidin HRP Mix (Quansys Biosciences) was added to the wells. The plates were covered and agitated at room temperature for 15 minutes. The plates were then washed with six times with Wash Buffer, the Substrate Mix (Quansys Biosciences) was added and plates were imaged immediately using Q-View™ imager (Quansys Biosciences). Signal intensities were determined by Q-View™ software

Results

Development of the Mouse Model

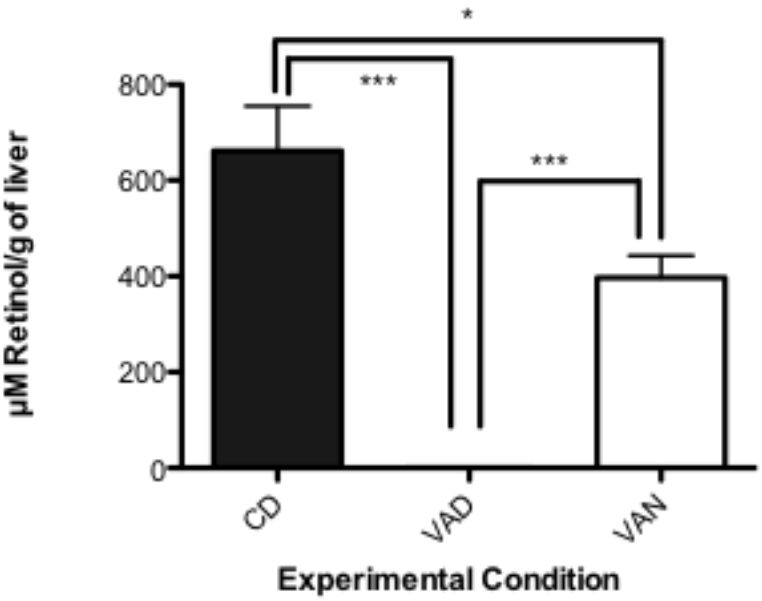
None of the animals in this program showed any outward signs of illness and gained weight normally regardless of their group assignment. Neither the VAD nor VAH females appeared to have any trouble with fertility (no significant differences in litter sizes or birth weights), breastfeeding or behavioural changes (data not shown). All progeny weighed between 10 and 13g when weaned, again with no significant differences between groups. Similarly, all of the mice used in these studies manifest no deformation or outward signs of illness prior to infections (e.g. blindness or hair loss) regardless of their group assignment. Weight gain in all of the groups increased in parallel prior to infection.

Confirmation of vitamin A status in deficient and normalized mice.

The liver is the primary storage location for retinoids. As shown in Figure 2, CD mice had a liver-retinol concentration of 661.0 mol/g liver, which is significantly more retinol stored in their liver than mice from both VAD mice ($p<0.0001$), which contained 397.5 mol/g liver, and VAN ($p=0.01$), which had lower concentrations of liver-retinol than the detection limit of the apparatus (3µM). VAN mice had also stored more retinol in their liver than VAD groups ($p<0.0001$).

<u>Figure 2.</u> Liver retinol levels are dependent on treatment during breeding.

Control diet mice were bred and maintained on a normal rodent diet (4IU/g vitamin A). VAD mice were bred on a vitamin A deficient diet (0IU/g vitamin A) and VAN mice were bred vitamin A deficient and placed on the control diet upon weaning. Vitamin A was solublized from liver samples by potassium hydroxide sapponification and purified by hexane extraction. Data are shown as the mean ± SD (*p<0.05, ***p<0.001).

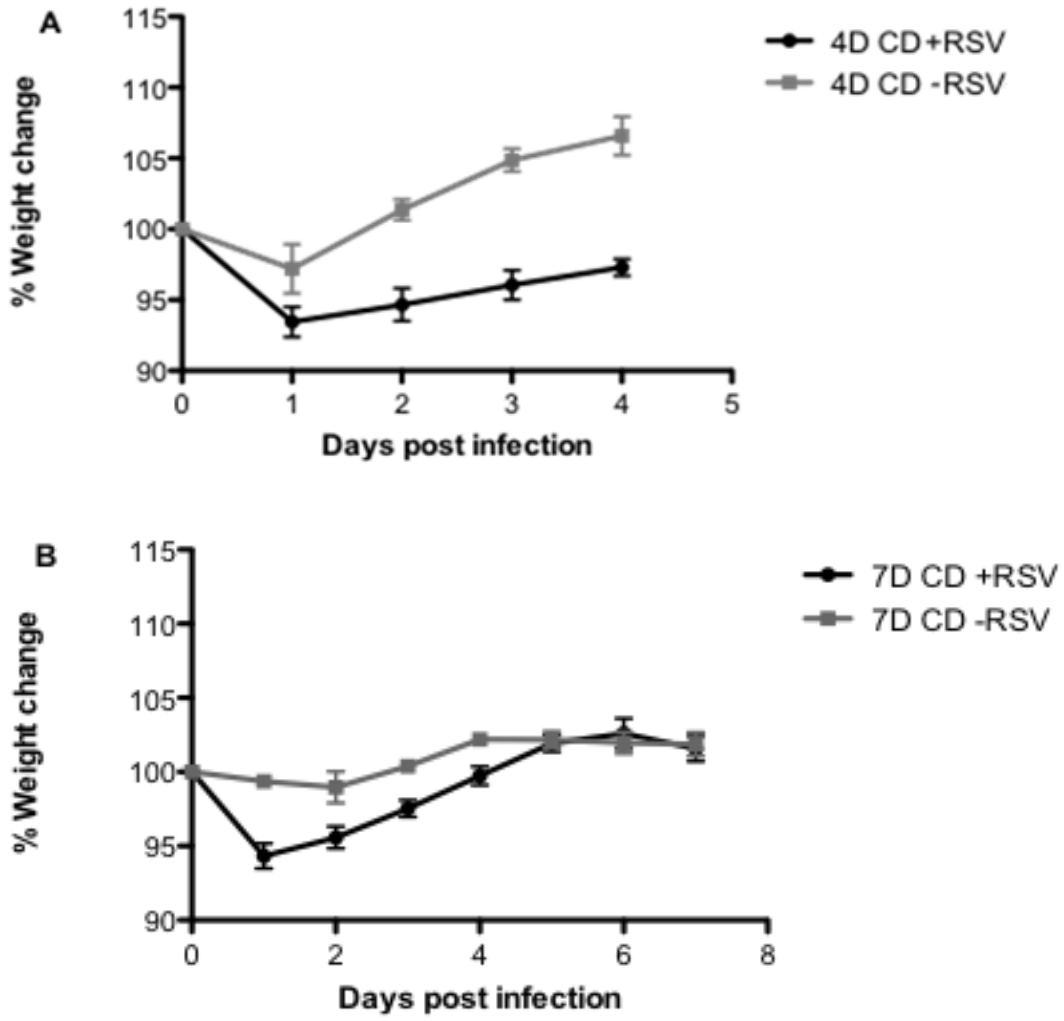


BALB/c mice are infected by RSV.

Weight loss is a quantitative measure of the severity of RSV infection in BALB/c mice. Intranasal administration of RSV established a productive infection in these mice, with classic behavioural changes including hunched posture, increased nesting and reduced grooming. Using a 2(time) by 2(diet-group) repeated measures analysis of variance, weight loss was compared between the CD group and those that that were either mock infected or infected under four dietary conditions (CD, VAD, VAN and VAH). There was significant weight loss ($p=0.047$ and $p=0.003$) for both four- and seven-day infections, respectively, indicating disease burden. As shown in Figures 3A and 3B, mice generally recovered 100% of their original weight by day four p.i.

<u>Figure 3.</u> RSV infection in CD mice.

Control diet mice were bred and maintained on a normal rodent diet (4IU/g vitamin A). In two separate studies, weights were monitored over a four (A) or seven (B) day study period prior to sacrifice. Mice were infected with either 50µL of $5x10^7$ TCID50/mL RSV (RSV+) or cell free supernatant (RSV-). Data are shown as the mean ± s.e.m. Statistical analyses were performed by grouped two-way ANOVA.

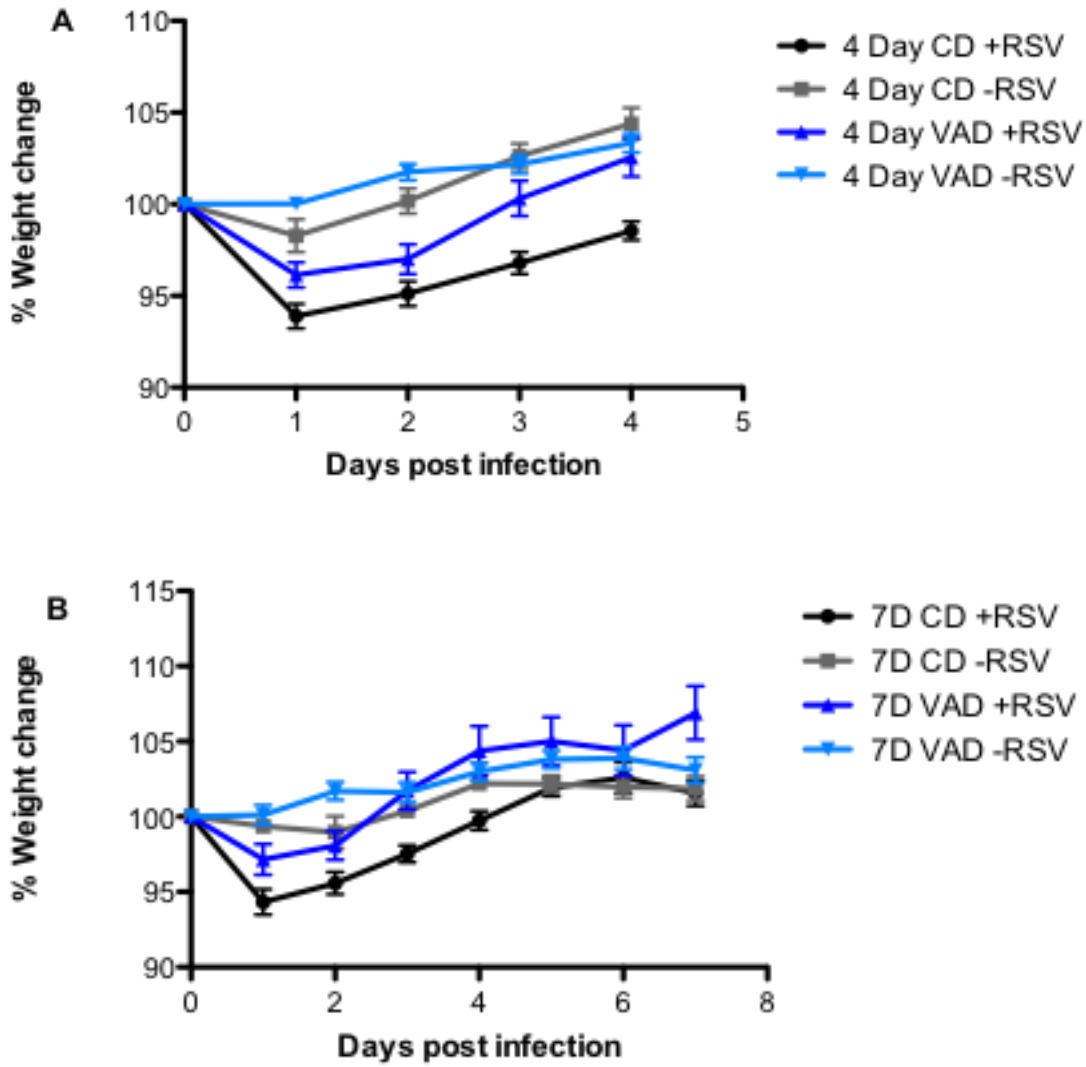


Vitamin A deficiency reduces disease burden and expedites recovery time as measured by weight change.

Our central hypothesis predicted that vitamin A deficiency would modulate disease severity in infected mice. Vitamin A deficiency in BALB/c mice did indeed reduce the extent of weight loss compared to CD animals during both four- and seven-day infections ($p=0.0359$ and $p=0.00018$, respectively) as depicted in Figures 4A and 4B. Further, VAD mice recovered more quickly from RSV infection than vitamin A sufficient animals.

<u>Figure 4.</u> Vitamin A deficiency modulates weight loss during RSV infection.

VAD mice were bred and maintained on a vitamin A deficient diet (0IU/g vitamin A). In two separate studies, weights were monitored over either a four (A) or seven (B) day study period before animals were sacrificed. Mice were infected with either 50µL of $5.6x10^7$ TCID50/mL RSV (RSV+) or cell free supernatant (RSV-). Data are shown as the mean ± s.e.m. Statistical analyses were performed by grouped two-way ANOVA.



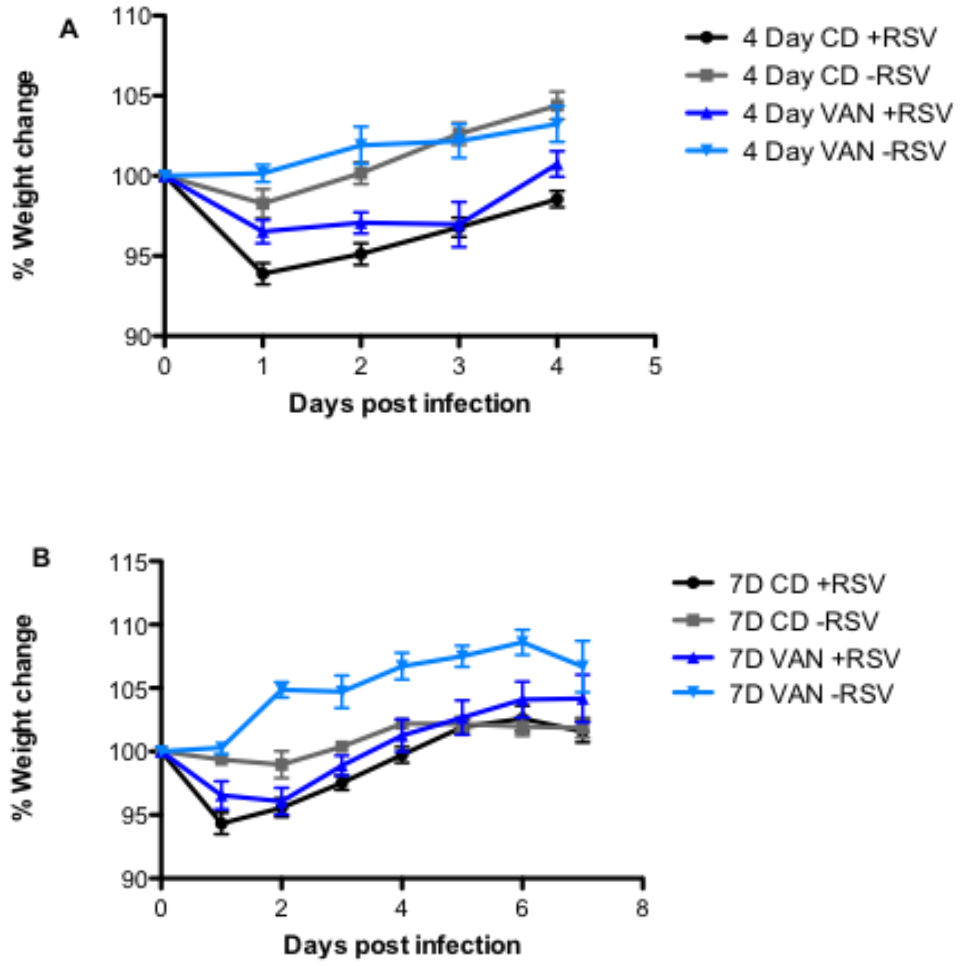
Although there were slight differences in the absolute weight loss and kinetics of weight recovery in these two experiments ($p=0.034$ for the kinetics of recovery at day 4 between the studies), the data were consistent with our hypothesis overall. These results strongly suggest that 'normal' vitamin A status may exacerbate the pathology associated with RSV infection and, conversely, that VAD may protect against severe disease.

Vitamin A normalized mice respond similarly to CD mice during RSV infection.

There was no statistical difference in weight loss between CD and VAN mice for four- and seven-day experimental infections, showing that they act similarly during RSV infection ($p=0.114$ and $p=0.213$ compared to CD, respectively) (fig. 5A and fig. 5B). Consistent with our assumption that re-introduction of vitamin A to the diet would exacerbate RSV pathology; preliminary data from a four-day infection suggest that VAN mice were more severely affected by RSV infection than VAD mice ($p=0.019$). VAN mice sacrificed at seven days p.i. also manifest more severe RSV infection than VAD counterparts, as seen by weight loss and gain over the infective course ($p=0.016$). These results suggest that there is a direct causal link between vitamin A status and increased morbidity, and not a developmental difference from breeding on a vitamin A deficient diet.

<u>Figure 5.</u> Vitamin A normalized mice respond similarly during infection to mice bred on the control diet.

VAN mice were bred on a vitamin A deficient (0IU/g vitamin A) diet and maintained the control diet (4IU/g vitamin A) after weaning. In two separate experiments, weights were monitored over either a four (A) and seven (B) day study period prior to sacrifice. Mice were infected with either 50µL of 5.6x107 TCID50/mL RSV (RSV+) or cell free supernatant (RSV-). Data are shown as the mean ± s.e.m. Statistical analyses were performed by grouped two-way ANNOVA.



BAL Cytokine levels – Multiplex Quansys system.

For cytokine analysis we used the Quansys Biosciences multiplex ELISA. The Quansys ELISA is a powerful tool for determining local immune context and systemic signaling environments. However, this system has never been used before to assess cytokine levels on BAL fluids, to our knowledge, and had not been optimized for BAL from mice. Without guidance from prior experience, we encountered significant difficulties in generating reproducible data. The data shown in Table 2 are preliminary trials from seven-day CD infections, using different volumes (2mL, 1mL and 800µL for trials 1, 2 and 3, respectively) to collect the BALs. In our experience, the data generated from any dilution of the BAL samples produced highly inconsistent results, occasionally with one to three orders of magnitude difference in BAL-cytokine concentration between replicates. Samples from four- and seven-day VAD experimental controls and infections were also analyzed, and produced similar inconsistencies in their results (data not shown). Specifically, we were unable to document whether or not BAL fluids had a cytokine profile suggestive of a Th1-bias in the VAD group or a Th2 bias in the VAN and VAH groups at baseline. There was a general tendency for higher pro-inflammatory and Th1 cytokine concentrations in BAL fluids of all infected animals (data not shown). We hope that these difficulties will be resolved by focusing on total lung homogenates rather than BAL samples in future experiments.

<u>Table 2.</u> There were no significant differences in BAL cytokine and chemokine environment between infected and uninfected mice.

CD mice were infected with either 50µL of $5x10^7$ TCID50/mL RSV (RSV+) or cell free supernatant (RSV-). BAL was collected at upon day-seven sacrifice. All samples were analyzed by Quansys Biosystems multiplex ELISA. Samples were analyzed as neat (undiluted), 1:2 and 1:50 dilutions. All values represent sample means. Trial 3 represents the mean of neat data only. All values are in pg/mL. Statistical analyses were performed by non-parametric t-test. *p<0.01

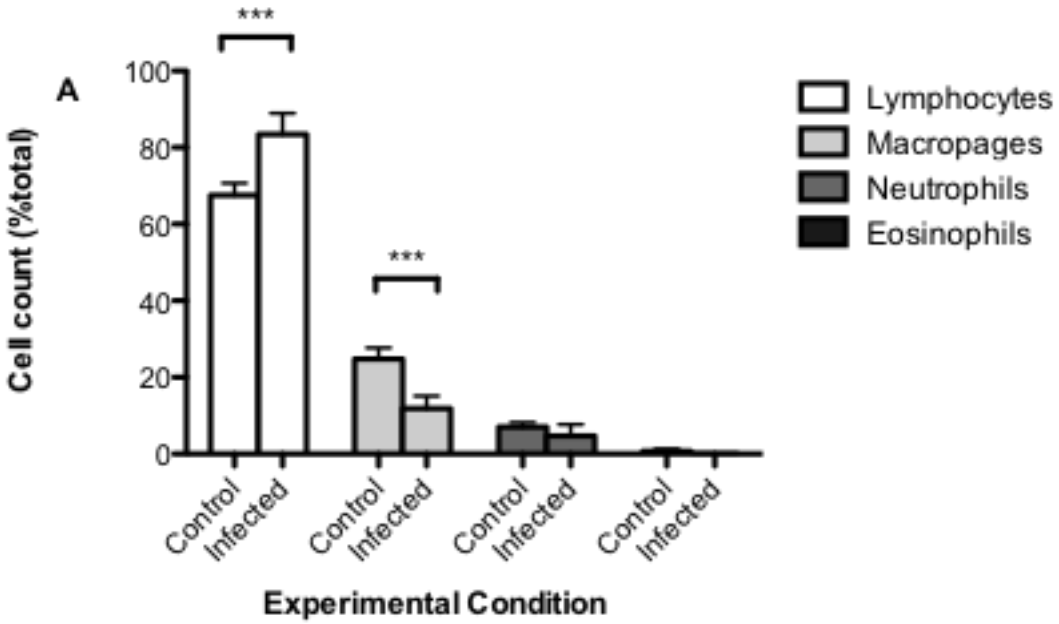
	Trial 1 (n=5)		Trial 2 (n=3)		Trial 3 (n=3)	
	CD RSV+	CD RSV-	CD RSV+	CD RSV-	CD RSV+	CD RSV-
			Th0, Th1-like and pro-inflammatory cytokines			
INF-γ	0	0	91.4	6.84	120.04	129.52
TNF-α	4.2	12.42	2.4	8.02	3.99	4.73
IL-2	0	0	0.07	2.54	4.29	1.72
IL-1α	0	0	0.86	0	1.86	1.76
GM-CSF	0	0	0	0	0	0
IL-1β	81.6	0	0	0.98	9.04*	1.51*
IL-3	0	0	0	0.56	0.55	1.18
IL-12p40	28.7	78.98	14.5	16.43	11.81	5.61
IL-6	1.08	0	2.34	1.98	5.54	10.09
			Th2-like and regulatory cytokines			
IL-4	2.4	0	0	0	0.42	0
IL-5	0	0	0	0.87	1.83	3.33
IL10	1.36	0	1.93	1.44	2.94	1.18
IL-17	0	0	0	3.3	3.68	13.72
			Chemokines			
MIP-1α	0	0	0.76	9.31	15.22	19.69
MCP-1	37	0	28.6	21.45	31.7	23.7
RANTES	13.02	4.4	22.1	44.5	39.5	46.04

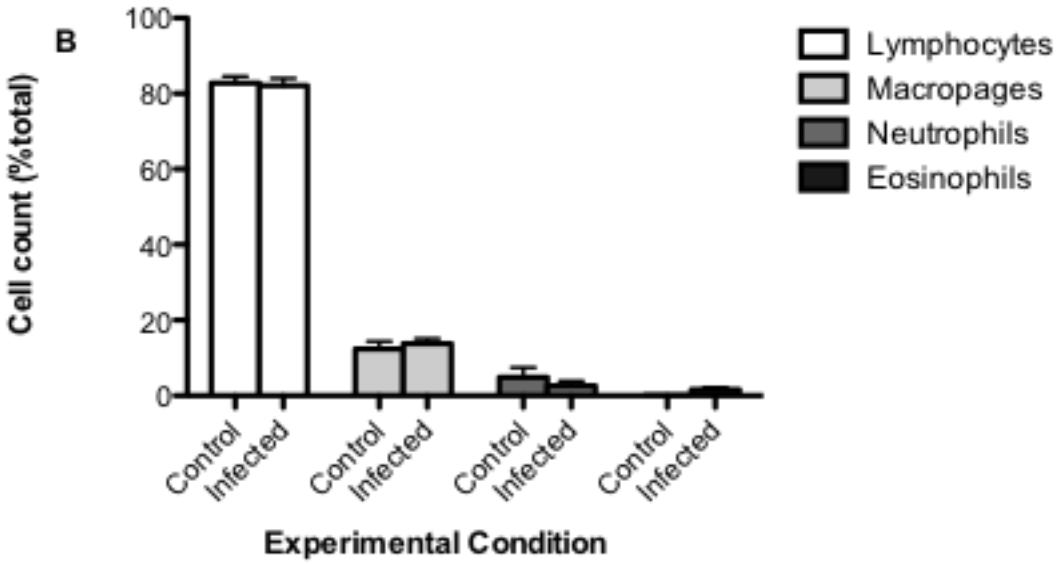
Preliminary BAL differential cell counts show little difference between experimental groups.

Differential cell counts from mice sacrificed from seven-day experimental groups showed very little difference between infected and control both within (CD, VAN and VAD, infected and uninfected respectively) and without (CD, VAN and VAD, collectively) treatment conditions (figure 6). CD mice were the only group that showed a difference in cell-infiltrate ratios before and after infection, where lymphocyte and macrophage levels were inversely proportional to one another across infected and uninfected mouse groups (fig. 6A). CD mice also started with significantly more macrophages ($p=0.0002$) than either VAN or VAD mice, which then declined after infection. VAN and VAD groups showed little change in cellular ratios either at baseline or following infection, and cell-types in the BAL were generally quite similar in all experimental groups. Mice from VAN and CD groups both contained eosinophils in their BALs either before or after infection, while VAD mice had no eosinophils.

<u>Figure 6.</u> BAL cell populations pre- and post-RSV infection.

Bronchioalveolar lavage was taken from seven-day RSV infected and uninfected mice from CD (A), VAN (B) and VAD (C) experimental groups. The cellular component was collected by centrifugation, cytospun onto slides and stained using DiffQuick. Cells were then examined 100x magnification. A differential count was done from four mice per condition, counting 200 cells from each sample. Data are shown as mean ± standard deviation. Statistics were performed by two-tailed t-test.




B
Cell count (%total)
100
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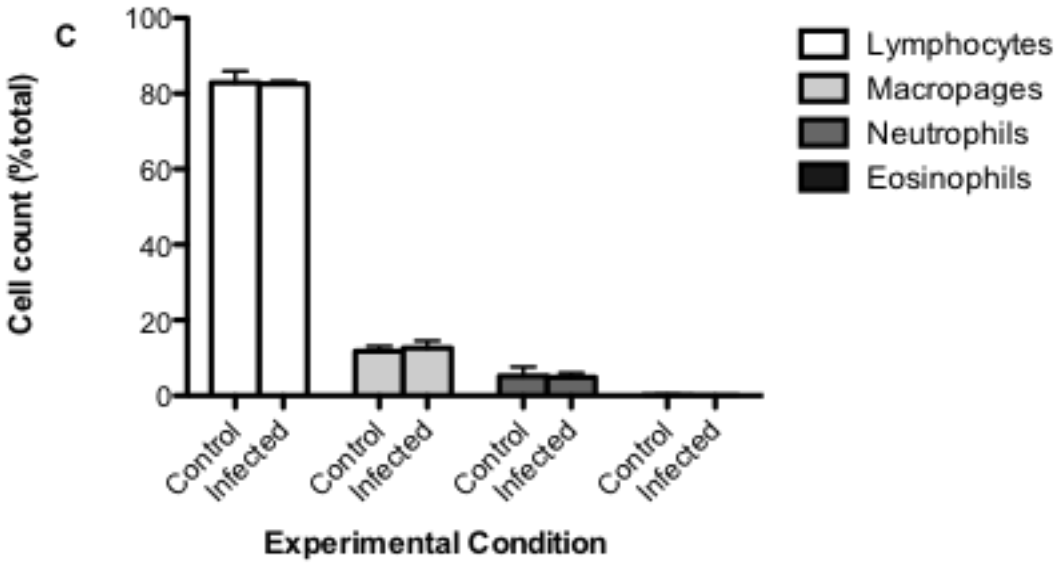
Lymphocytes
Macropages
Neutrophils
Eosinophils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Cell count (%total)
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Control
Infected
Experimental Condition
Lymphocytes
Macropages
Neutrophils
Eosinophils

Discussion

The nature of RSV makes it a difficult disease to control. It is highly pervasive and sufficiently avirulent in healthy individuals that controlling or quarantining those infected would waste time and resources. However, the prevalence of individuals at risk for developing severe RSV infection (1-20/1000, and at times up to 250/1000) has made the need for an effective treatment regimen a growing concern (48, 88). Furthermore, these numbers focus primarily on neonatal and early infant disease, rather than looking at RSV-related morbidity and mortality throughout the lifespan. The spread of other significant viral pathogens has been limited by effective vaccination courses (e.g., measles, polio, mumps and influenza). However, despite the >50 years of effort in the production of RSV vaccines (i.e. inactivated virus preparations, live attenuated and genetically engineered viruses, purified RSV protein subunit vaccine preparations, vector-based vaccine candidates and DNA-based vaccines), these endeavours have generated no marketable products (114). There has been considerable success in controlling RSV infection in neonates with the monoclonal antibody Palivizumab (91, 92). The cost per treatment, however, is extremely high and as a result, completely unaffordable in all but the richest nations. Palivizumab treatment costs range from $1600-$2500 per 15mg/kg dose, with a recommended five doses per RSV season for high-risk individuals (122). Since antibody-based treatment only provides transient protection after treatment (ie: passive immunity), these are difficulty costs to justify for an individual or healthcare system.

In the absence of any effective extrinsic means to prevent or treat RSV, it is even more important to understand extrinsic factors that **can** be controlled as well as intrinsic factors that could potentially influence RSV pathogenesis. For example, studies focusing on environmental impact have identified smoke inhalation and particulate pollution as a risk factor for serious RSV infection (16, 42, 72). Although there has been extensive study of the general influence of diet and nutritional deficiency on the incidence and severity of infectious diseases (75, 167, 173)(WHO), the potential impact of over-nutrition in exacerbating disease has been largely ignored.

Certainly the best example of overnourishment enhancing the risk of severe infection is that of iron overload (181). Two factors contribute to iron's toxicity and potential as an enhancer of microbial pathology: 1) in its redox-active form, it can release free radicals and 2) it is an essential growth factor for most bacteria, protozoa, fungi and all neoplasmic cells (181). In response to iron's direct toxicity, the body has developed strict iron sequestering mechanisms that act to lower free or liganded iron levels (181). If the body's iron content exceeds its capacity to sequesters this micronutrient for any reason (eg: hemolysis, supplements, transfusions), there is a serious risk of overwhelming infection due to a number of 'iron-sensitive organisms (eg: Salmonella species, mycobacteria, Listeria species) (118). Furthermore, even modest amounts of 'excess' iron can hamper adequate immune responses (25). Consistent, overconsumption of iron can also induce non-alcoholic fatty liver disease, due to β-cell sensitivity to iron that sensitize an individual to poor outcomes from hepatitis viruses (181). As

noted above, there are also limited data suggesting that vitamin A may have some negative effects in norovirus and HIV infections as well as influenza and a number of other respiratory diseases (32, 97, 99).

RSV-Balb/c mouse model; successes and limitations

The complexity of a disease like RSV needs to be modeled in an intact biological system (eg: whole organism) rather than more simple *in vitro* representations. To date, few studies have considered the complete spectrum of vitamin A status—i.e. deficient, control, replete and over-nourished. Virtually all work on the potential role of micronutrients in infectious diseases has focused almost on deficiency versus replete states. Without an established deficiency and supplementationmodel we set out to design our own. However, this required considerable effort to produce a stable protocol. The fat-soluble nature of vitamin A means that mice, like humans, have large liver retinol stores. As a result, mice cannot be made deficient over the course of a single generation. In our first approach, we attempted to time the introduction of a vitamin A deficient diet based on copulation date (E14) to reduce the likelihood of deformation during gestation. This strategy was not only labour-intensive but also yielded inconsistent results, as plugs (a hardened semen deposit left in the female mouse's vagina) were not always detectable. In the end, we opted to maintain the first generation females (P1) on vitamin A deficient feed with a return to the control diet (4IU/g vitamin A) two days per month. This 26/2 day schedule was sufficient to ensure essentially normal fertility and fecundity rates in these animals despite very low liver retinol stores (Figure 2). The livers of our control mice (CD)

contained on average 661.0 mol/g retinol. The pups born of our VAD mothers and then maintained on the deficient diet consistently had retinol levels in their livers below the detection limit of the HPLC system used (< 3μM). There was also a significant difference in liver retinol stores between the CD and VAN groups (661.0 mol/g vs 397.5 mol/g; $p < .0001$). However, it is likely that this latter difference is only indicative of length of time spent consuming a diet with 'normal' retinoid content, and does not necessarily reflect circulating levels. In normal circumstances of retinoid intake, serum levels are held constant despite wide variation in liver stores (see above). As a result, liver retinol content does not always reflect circulating levels except in extreme depletion (ie: VAD animals). Furthermore, retinol-binding protein (RBP) is an acute phase protein, and as such, its production and release are altered during infection (6). Interestingly, Aoki *et al.* found that RBP was up-regulated during asthma exacerbations, potentially making retinol more bioavailable and, in the context of our hypothesis, more likely to cause adverse effects during RSV infection (6). In a state of severe deficiency, the amount of available vitamin A free for export should be limited. In future studies, this could be verified by studies of serum retinol.

We initially planned to supplement VAH mice by biweekly injection, two weeks prior to, and throughout, the experimental infections. However, this approach proved to be labour-intensive, subject to 'leakage' of the injected retinoid and stressful for the animals. To address these issues, we experimented with silastic tubing to generate an implant that would deliver a constant low dose

of retinoid over a prolonged period of time. We chose to fill these implants with 65mg of ATRA based on wall and pore size (data on ATRA release in vitro is still being analyzed). Silastic tubing is stable in biological systems, and is frequently used in long-term human applications (e.g. cerebrospinal fluid shunts) (171) so implant-associated toxicity was not thought to be a risk. Implanted mice had some slight skin discolouration (yellowing over the implant) but no other health effects were observed. We believe this to be the first use of this approach to supply a micronutrient supplement in a small animal model of over-nutrition.

Though the success of the model permitted a number of small studies to be performed, midway through my research program, the adequacy of the RSV inoculum became a major issue. Infection of control groups in later experiments showed marked decreases in weight loss and much slower recovery time to 100% normal weight compared to original control infections ($p<0.001$) (data not shown). Before restarting infections, RSV growth was attempted in both Vero and Hela cell lines. Although Vero cells have long been the standard cell line in which RSV is propagated, recent work revealed that these cells can sometimes produce a truncated form of the G protein, which reduces virus competency when grown *in vivo* (93). To more closely mimic natural infection, we attempted to grown new RSV stocks in Hela cells for all future applications. Despite a large literature supporting the use of these cells to grow RSV, viral stocks prepared in Hela cells also proved to be largely unproductive for *in vivo* infection. Hep2, an epithelial-like rat cell line, is widely used with the RSV A2 strain for infection in mice (19, 100, 101). A growth protocol using these cells and the new virus strain

has been optimized and is currently being tested for the infectivity of the virions produced in our mouse model.

It is not clear at this time why so much difficulty was encountered in measuring BAL cytokine levels. Sacrificial points for experimental groups were initially set at 4 and 7 days p.i. Day four was determined to be the peak of virus output in mice, while day seven was determined to be the beginning of recovery in mice (20, 158). Peak virus output, however, does not coincide with peak pathology, which typically occurs between days 1 and 2 p.i. with the inoculum used (158). As a result, day four may effectively 'miss' the innate immune response to virus infection and the associated rapid influx of cells. An earlier sacrificial point would likely show increased chemotactic and signaling molecules in the BAL, thereby providing greater insight into the impact of vitamin A on innate immune effector function during infection. To better characterize the antibody responses generated against RSV infection during micronutrient deficiency and supplementation, a 14-day time point will be added in the next phase of the research for all experimental groups.

High vitamin A status has been shown to bias towards a Th2 polarization and increase eosinophilia in mouse asthma models (21, 39, 67, 112, 148). Conversely, vitamin A deficiency has been shown to decrease associated eosinphillia and promote a Th1-type polarization (21, 39, 67, 76, 80, 103, 112, 131, 135, 148). In our model, there was a clear difference in the severity of RSV disease between the CD (and VAN) and the VAD groups based on weight loss

(figures. 3, 4 and 5). The vitamin A replete mice also demonstrated more behavioural changes (eg: hunching, immobility) than their deficient counterparts (data not shown). Even in the absence of more definitive immunologic data, these 'whole animal' observations strongly support our hypothesis that 'better' vitamin A status has a paradoxically negative effect on RSV.

The mouse lung is the source of a significant portion of the total collectable data (signaling environment, cellular infiltrates, differential morphology, viral titres and pathology); however, a number of concerns arose from the initial experimental design. Issues with cytokine profiling were most apparent (see Table 2). The 800µL wash is likely the lowest amount of fluid that can be used during a BAL to ensure a sufficient volume of sample for further experimentation, particularly considering that the average recovery rate was roughly 80% of the total volume. To compensate for BAL dilution, whole lung homogenates will be analyzed in parallel to BALs in the next phase of research. Quansys multiplex ELISA analysis have historically been used to measure cytokines and chemokines in serum and cell free supernatant, which often have relatively high concentrations of signaling molecules. Both BAL and lung homogenate analysis will be optimized before application to experimental samples.

Differential morphology of the lung was initially attempted using frozen tissue blocks embedded in a glycol solution and frozen at -20˚C before sectioning on a cryotome (data not shown). This method of tissue sectioning is widely used

in neurobiology with impressive and consistent results. Nervous tissue, however, does not share the large surface area and fenestration found in the lung. When frozen, nervous tissues are significantly more solid than lung tissues and permit the preparation of <10µm slices. In the course of this work, we discovered that lung tissue cannot be sectioned as thinly by cryotome without risking perturbation of the respiratory architecture. Furthermore, in sections thicker than ~6µm, it is difficult to see edema and cell infiltrates clearly due to cell stacking and occlusion. Paraffin embedded lung samples have already been sectioned and are currently being analyzed for gross differences in architecture (e.g. fenestration) and signs of inflammation.

Cellular infiltrates, collected by BAL, were stained with both Hematoxylin/Eosin and Hansel stain; however, eosinophils could not be readily identified. According to preliminary staining, DiffQuick appears to be a more suitable stain to facilitate cell-type identification for our purposes. Cellular infiltration during the seven-day infection was largely unvaried (figure. 6 A, B and C). Preliminary results do not show a significant difference between CD and VAN or VAD with respect to cellular subsets in the BAL fluids at day seven p.i, between infected and uninfected groups (except for CD lymphocytes $p=0.007$). Day-seven p.i. marks the beginning stages of the humoral response, which may account for the prevalence of lymphocytes in the airspaces of the lung; however, their presence in the uninfected samples is puzzling. A large body of evidence shows a correlation between severe RSV disease and eosinophilia suggests that our current results may not reflect a more severe lung pathology (140). At two

days post-infection, when innate immunity should dominate, our hypothesis would predict increased eosinophil numbers in the VAH and CD samples, compared to VAD BALs. Sustained IL-5 levels should also occur in the VAH mice infected with RSV, contributing to a sustained eosinophilia in the lung (140). Although the BAL data are preliminary at this stage, the presence of low levels of eosinophils in the BALs from seven-day CD and VAN mice, and their absence in VAD mice, (fig. 6A, 6B and 6C) certainly support the over-arching hypothesis.

We initially planned to measure viral titres by real-time polymerase chain reaction (RTPCR) from both BAL and lung tissue, and this assay was established and validated targeting the F gene (data not shown). RTPCR has been widely used to assess both the presence and burden of viral pathogens. However, in a model where the collective effects of the pathogen and the pharmacological agent (ATRA in this case) are unknown, using a method such as mRNA-based RTPCR may produce misleading results. Intracellular mRNA levels do not necessarily correlate with viral output. Any trend towards enhanced apoptosis might substantially increase the viral titres estimated by by RTPCR. In the next phase of these studies, viral titres will be determined both by TCID50 and RTPCR. Viral titres may or may not be affected by vitamin A status. Certainly, retinoid supplements in norovirus infection alters clinical presentation while paradoxically increasing gut viral loads (97). Although retinoids have a clear Th2 biasing effect, this effect will not necessarily translate into an increase in RSV viral titres. The fact that RSV has evolved to produce a similar shift in Th2-polarization

might suggest that viral output will be increased in VAH mice compared to their VAD counterparts.

Finally, lung damage can be quantified by luminal albumin levels. Damage to any epithelium in the body causes cellular infiltration, and, as a result, often causes the basement membrane to leak—allowing serum albumin to seep out. Albumin concentrations can then be determined by ELISA. This principle has been extensively used in studies examining both respiratory and renal pathologies. In each case, heightened disease (ie: cellular infiltration) correlates with increased albumin in the lavage or urine, respectively (117, 159, 160). Because vitamin A plays important roles in both tissue development and epithelial cell maintenance, we expected to see increased albumin in the BAL fluids of CD and VAH mice compared to levels in VAD mice. To date, the results of these ELISA are not consistent with our hypothesis since both infected and uninfected VAD mice had higher concentrations of albumin into the BAL than their CD counterparts (data not shown). These results are almost certainly due to the crucial role that vitamin A plays in the maintenance of epithelial integrity. Although these data were not helpful to our hypothesis, they are another indication that the deficiency state created in our model was physiologically relevant.

Future research and expectations

The impact of retinoids on the Th1-Th2 balance is fairly well defined (21, 39, 67, 76, 80, 103, 112, 131, 135, 148). Given the impact of its roles, we should be able to predict some of the outcomes of upcoming experiments. The Quansys multiplex ELISA, once optimized, should generate comprehensive differences

VAD, CD and VAH group cytokine profiles. In VAD groups we would expect to see higher levels of Th1 cytokines—IL-12p40, IFN-γ, TNF-α and IL-1β; and the corollary reduction of IL-4, IL-5 and IL-13 levels. These results should be reversed in CD and more drastically reversed in VAH groups.

The continuation of this project should also help clarify the validity of some alternate hypotheses. Another potential result of vitamin A activity, and Th2 bias, is immunologic dampening or impairment. If the inflammatory response is diminished, there would be less damage to the host, and as a result fewer symptoms. However, there would also be less control exerted on the infection, and as a result we would see increased viral output and prolonged shedding. This sort of reduced pathogenicity phenomenon has been observed in cutaneous tuberculosis (77). Sufficient exposure of cutaneous immune cells to ultraviolet light will impair the host's resistance to infection at the irradiation site (77). After tuberculosis inoculation in irradiated mice, researchers recovered significantly more, viable, mycobacteria from the lymphoid organs of irradiated mice than unexposed mice for up to two months post-inoculation (77). Viral titre studies should elucidate the presence of this potential mechanism.

Now that the model is in place, completion of this study will permit in vivo studies of the interaction between vitamin A status and RSV infection for the first time. For example, flow cytometric analysis of lung cell populations during infection in VAD vs VAH could provide a mechanistic understanding of the retinoid immune modulation in this system. It is clear that a number of cell populations may be implicated in the effects of lack or excess of vitamin A (e.g. Th1, Th2, Th17, Treg, DCs, eosinophils, neutrophils and macrophages).

Understanding the role of vitamin A export and circulation during the course of infection may also provide important insight as to whether excess stored vitamin A and/or sustained high serum retinol levels confer elevated risk for pathology.

Perspectives and conclusions

The data generated in the next phase of this work may be particularly relevant with regard to the North American diet and lifestyle. For the general population it is often hard to gauge the true nutritional density and composition of foods, especially considering that the current percent of daily value (PDV) system displayed on food is based on a 1968 estimation of an individual's recommended daily allowance (RDA), instead of the significantly more conservative 2001 suggestion (5, 128). The 1968 data suggest that adults require 1500µg of vitamin A per day, which is roughly twice the current recommended allowance (900µg and 700µg/day for adult males and females, respectively) (128). This discrepancy between the PDV and RDA could potentially place consumers at risk of consistently ingesting close to the upper limit of suggested vitamin A intake (3000µg/day for adults) (128). The discrepancy between PVD and current RDAs is even more problematic in infants and children, for whom the vitamin A RDA is between 300-500µg with a daily upper limit of 600µg/day (128).

There has been a growing trend in fortifying food with preformed vitamin A to enhance marketability in certain populations. Some foods, such as dairy, oil products and cereals, have been fortified for many years; however, more recently, fortification has been expanded to include readily available products such as candy and snack bars (128). One of the more extreme examples is a gumball

containing 1500µg of preformed retinol as retinyl palmitate, which is nearly three to four times the upper intake-limit for children (128). A typical meal replacement bar contains roughly 750µg of preformed retinol, which is over the RDA of an adult woman (128). Furthermore, daily supplementation has become increasingly popular within the last ten years, largely due to the wide misquotation and exaggeration of a 2002 paper, by vitamin manufacturers and lobbyists, recommending a daily multivitamin for adults and two for elderly individuals (41, 128). One multivitamin can contain as much as 1500µg of vitamin A (128). The recent interest in unsaturated fatty acids also has implications in vitamin A consumption. In particular, n-3 unsaturated fats are readily found in and extracted from fish liver—a rich source of retinyl esters (5, 128). These products are rarely the only vitamin A sources for an individual, and since the North American diet is heavily based on dairy and meat as a mainstay in meals, overconsumption of vitamin A in North America if effectively universal.

The intermittent consumption of even large doses of water-soluble vitamins (e.g. vitamin C) likely poses very little risk of substantially altering host biology. Lipid-soluble vitamins, however, are not actively excreted and therefore accumulate in fat stores (primarily stellate cells). Although vitamin A intake at twice the RDA may not physically manifest as hypervitaminosis and ultimately lead to liver cirrhosis, the potential effects on immune responses to ubiquitous respirator pathogens, such as RSV, need to be carefully evaluated (24).

Until a safe and long-term treatment for RSV is developed, we must continue to explore the impact of lifestyle on severe RSV infection. The belief that all fortified foods are beneficial, and even necessary, has been cultivated in

North America for several decades; and as a result, our population has developed a propensity for consuming nutrient dense foods. To return to the ideas posed at the beginning of this thesis, there is likely a fine balance between under and over-nourished states. There is little doubt that, with obesity rates exceeding 50% in several developed countries (26) and the expanding popularity of heavily-fortified foods, that we have pushed the pendulum too far in the direction of over-nutrition. Without a better understanding of what constitutes 'equilibrium' in this context, we run the risk of predisposing ourselves to unnecessary pathology from relatively benign organisms. The model of enhanced RSV disease in vitamin A overnourished mice that we have developed may be a first small step towards regaining some of this essential equilibrium.

References

1. **1846 Report**. Observations made during the epidemic of measles on the Faroe Islands in the year 1846. *Medical Classics* 3: 829-886, 1846.
2. **Adkins B, Leclerc C, and Marshall-Clarke S**. Neonatal adaptive immunity comes of age. *Nat Rev Immunol* 4: 553-564, 2004.
3. **Alwan WH, Kozlowska WJ, and Openshaw PJ**. Distinct types of lung disease caused by functional subsets of antiviral T cells. *J Exp Med* 179: 81-89, 1994.
4. **Amanatidou V, Apostolakis S, and Spandidos DA**. Genetic Diversity of the Host and Severe Respiratory Syncytial Virus-Induced Lower Respiratory Tract Infection. *The Pediatric Infectious Disease Journal* 28: 135-140 110.1097/INF.1090b1013e31818c31818d31817, 2009.
5. **Anderson JJB**. Oversupplementation of Vitamin A and Osteoporotic Fractures in the Elderly: To Supplement or Not to Supplement With Vitamin A. *Journal of Bone and Mineral Research* 17: 1359-1362, 2002.
6. **Aoki T, Matsumoto Y, Hitara K, Ochiai K, Okada M, Ichikawa K, Shibasaki M, Arinami T, Shmazaki R, and Noguchi E.** Expression profiling of genes related to asthma exacerbations. *Clin Exp Allerg 39: 213-221, 2009.*
7. **Aung S, Tang Y-W, and Graham BS**. Interleukin-4 Diminishes CD8+ Respiratory Syncytial Virus-Specific Cytotoxic T-Lymphocyte Activity In Vivo. *J Virol* 73: 8944-8949, 1999.
8. **Barik S**. The structure of the 5' terminal cap of the respiratory syncytial virus mRNA. *J Gen Virol* 74: 485-490, 1993.
9. **Barr J, Chambers P, Pringle CR, and Easton AJ**. Sequence of the major nucleocapsid protein gene of pneumonia virus of mice: sequence comparisons suggest structural homology between nucleocapsid proteins of pneumoviruses, paramyxoviruses, rhabdoviruses and filoviruses. *J Gen Virol* 72: 677-685, 1991.
10. **Barral PM, Sarkar D, Su Z-z, Barber GN, DeSalle R, Racaniello VR, and Fisher PB**. Functions of the cytoplasmic RNA sensors RIG-I and MDA-5: Key regulators of innate immunity. *Pharmacology & Therapeutics* 124: 219-234, 2009.
11. **Becker Y**. Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergy—a review. *Virus Genes* 33: 235-252, 2006.
12. **Bem RA, Domachowske JB, and Rosenberg HF**. Animal models of human respiratory syncytial virus disease. *Am J Physiol Lung Cell Mol Physiol* 2011.

13. **Beriou Gl, Bradshaw EM, Lozano E, Costantino CM, Hastings WD, Orban T, Elyaman W, Khoury SJ, Kuchroo VK, Baecher-Allan C, and Hafler DA**. TGF-B Induces IL-9 Production from Human Th17 Cells. *The Journal of Immunology* 185: 46-54, 2010.
14. **Bermingham A, and Collins PL**. The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA replication and transcription. *Proceedings of the National Academy of Sciences* 96: 11259-11264, 1999.
15. **Blomhoff R, Holte K, NÊss L, and Berg T**. Newly administered [3H]retinol is transferred from hepatocytes to stellate cells in liver for storage. *Experimental Cell Research* 150: 186-193, 1984.
16. **Bradley JP, Bacharier LB, Bonfiglio J, Schechtman KB, Strunk R, Storch G, and Castro M**. Severity of respiratory syncytial virus bronchiolitis is affected by cigarette smoke exposure and atopy. *Pediatrics* 115: e7-14, 2005.
17. **Bresee JS, Fischer M, Dowell SF, Johnston BD, Biggs VM, Levine RS, Lingappa JR, Keyserling Hl, Petersen KM, Bak JR, Gary Hej, Sowell Al, Rubens CE, and Anderson LJ**. Vitamin A therapy for children with respiratory syncytial virus infection: a multicenter trial in the United States. *The Pediatric Infectious Disease Journal* 15: 777-782, 1996.
18. **Brindley J**. Rhazes A-B. Treatise on the Smallpox and Measles. London: 1748.
19. **Bukreyev A, Murphy BR, and Collins PL**. Respiratory Syncytial Virus Can Tolerate an Intergenic Sequence of at Least 160 Nucleotides with Little Effect on Transcription or Replication In Vitro and In Vivo. *J Virol* 74: 11017-11026, 2000.
20. **Byrd LG, and Prince GA**. Animal Models of Respiratory Syncytial Virus Infection. *Clinical Infectious Diseases* 25: 1363-1368, 1997.
21. **Cantorna M, Nashold F, and Hayes C**. In vitamin A deficiency multiple mechanisms establish a regulatory T helper cell imbalance with excess Th1 and insufficient Th2 function. *The Journal of Immunology* 152: 1515-1522, 1994.
22. **Carbonell-Estrany X, Sim√µes EAF, Dagan R, Hall CB, Harris B, Hultquist M, Connor EM, Losonsky GA, and Group ftMS**. Motavizumab for Prophylaxis of Respiratory Syncytial Virus in High-Risk Children: A Noninferiority Trial. *Pediatrics* 125: e35-e51, 2010.
23. **Carman JA, Pond L, Nashold F, Wassom DL, and Hayes CE**. Immunity to Trichinella spiralis infection in vitamin A-deficient mice. *J Exp Med* 175: 111-120, 1992.
24. **Castano G, Etchart C, and Sookoian S**. Vitamin A toxicity in a physical culturist patient: a case report and review of the literature. *Ann Hepatol* 5: 293-395, 2006.
25. **Cherayil B**. The role of iron in the immune response to bacterial infection. *Immunologic Research* 50: 1-9, 2011.

26. **Chiang DJ, Pritchard MT, and Nagy LE**. Obesity, diabetes mellitus, and liver fibrosis. *Am J Physiol Gastrointest Liver Physiol* 300: G697-702, 2011.
27. **Choi EH, Lee HJ, Yoo T, and Chanock SJ**. A Common Haplotype of Interleukin-4 Gene IL4 Is Associated with Severe Respiratory Syncytial Virus Disease in Korean Children. *Journal of Infectious Diseases* 186: 1207-1211, 2002.
28. **Collins PL, Hill MG, Cristina J, and Grosfeld H**. Transcription elongation factor of respiratory syncytial virus, a nonsegmented negative-strand RNA virus. *Proceedings of the National Academy of Sciences* 93: 81-85, 1996.
29. **Collins PL, Hill MG, and Johnson PR**. The Two Open Reading Frames of the 22K mRNA of Human Respiratory Syncytial Virus: Sequence Comparison of Antigenic Subgroups A and B and Expression in Vitro. *J Gen Virol* 71: 3015-3020, 1990.
30. **Collins PL, Huang YT, and Wertz GW**. Nucleotide sequence of the gene encoding the fusion (F) glycoprotein of human respiratory syncytial virus. *Proceedings of the National Academy of Sciences* 81: 7683-7687, 1984.
31. **Collins PL, and Mottet G**. Oligomerization and post-translational processing of glycoprotein G of human respiratory syncytial virus: altered O-glycosylation in the presence of brefeldin A. *J Gen Virol* 73: 849-863, 1992.
32. **Cui D, Moldoveanu Z, and Stephensen CB**. High-Level Dietary Vitamin A Enhances T-Helper Type 2 Cytokine Production and Secretory Immunoglobulin A Response to Influenza A Virus Infection in BALB/c Mice. *The Journal of Nutrition* 130: 1132-1139, 2000.
33. **Curtis MM, Way SS, and Wilson CB**. IL-23 Promotes the Production of IL-17 by Antigen-Specific CD8 T Cells in the Absence of IL-12 and Type-I Interferons. *The Journal of Immunology* 183: 381-387, 2009.
34. **Cusi M, Martorelli B, Di Genova G, Terrosi C, Campoccia G, and Correale P**. Age related changes in T cell mediated immune response and effector memory to Respiratory Syncytial Virus (RSV) in healthy subjects. *Immunity & Ageing* 7: 14, 2010.
35. **Cyr SL, Jones T, Stoica-Popescu I, Brewer A, Chabot S, Lussier M, Burt D, and Ward BJ**. Intranasal proteosome-based respiratory syncytial virus (RSV) vaccines protect BALB/c mice against challenge without eosinophilia or enhanced pathology. *Vaccine* 25: 5378-5389, 2007.
36. **D'Souza RM, and D'Souza R**. Vitamin A for the Treatment of Children with Measles,ÄîA Systematic Review. *Journal of Tropical Pediatrics* 48: 323-327, 2002.
37. **Dowell SF, Papic Z, Bresee JS, Larra-Eaga C, Mendez M, Sowell AL, Gary Hej, Anderson LJ, And Avenda-Ëo LF**. Treatment of respiratory syncytial virus infection with vitamin A: a randomized, placebo-

controlled trial in Santiago, Chile. *The Pediatric Infectious Disease Journal* 15: 782-786, 1996.
38. **Dupuy LC, Dobson S, Bitko V, and Barik S**. Casein kinase 2-mediated phosphorylation of respiratory syncytial virus phosphoprotein P is essential for the transcription elongation activity of the viral polymerase; phosphorylation by casein kinase 1 occurs mainly at Ser(215) and is without effect. *J Virol* 73: 8384-8392, 1999.
39. **Elias KM, Laurence A, Davidson TS, Stephens G, Kanno Y, Shevach EM, and O'Shea JJ**. Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood* 111: 1013-1020, 2008.
40. **Finberg RW, Wang JP, and Kurt-Jones EA**. Toll like receptors and viruses. *Reviews in Medical Virology* 17: 35-43, 2007.
41. **Fletcher RH, and Fairfield KM**. Vitamins for Chronic Disease Prevention in Adults. *JAMA: The Journal of the American Medical Association* 287: 3127-3129, 2002.
42. **Fukuda K, Hider PN, Epton MJ, Jennings LC, and Kingham SP**. Including viral infection data supports an association between particulate pollution and respiratory admissions. *Aust N Z J Public Health* 35: 163-169, 2011.
43. **Fulton RB, Meyerholz DK, and Varga SM**. Foxp3+ CD4 Regulatory T Cells Limit Pulmonary Immunopathology by Modulating the CD8 T Cell Response during Respiratory Syncytial Virus Infection. *The Journal of Immunology* 185: 2382-2392, 2010.
44. **Gadomski AM, and Bhasale AL**. Bronchodilators for bronchiolitis. *Cochrane Database Syst Rev* 3: CD001266, 2006.
45. **Gagro A, Tominac M, KrŠUloviĆ-HreŠIĆ V, BaĆE A, MatiĆ M, DraŽEnoviĆ V, MlinariĆ-GalinoviĆ G, Kosor E, Gotovac K, BolanČA I, Batinica S, and RabatiĆ S**. Increased Toll-like receptor 4 expression in infants with respiratory syncytial virus bronchiolitis. *Clinical & Experimental Immunology* 135: 267-272, 2004.
46. **Gardner PS, McQuillin J, and Court SD**. Speculation on pathogenesis in death from respiratory syncytial virus infection. *Br Med J* 1: 327-330, 1970.
47. **Ghildyal R, Baulch-Brown C, Mills J, and Meanger J**. The matrix protein of Human respiratory syncytial virus localises to the nucleus of infected cells and inhibits transcription. *Archives of Virology* 148: 1419-1429, 2003.
48. **Glezen WP, Paredes A, Allison JE, Taber LH, and Frank AL**. Risk of respiratory syncytial virus infection for infants from low-income families in relationship to age, sex, ethnic group, and maternal antibody level. *J Pediatr* 98: 708-715, 1981.
49. **Graham BS, Bunton LA, Rowland J, Wright PF, and Karzon DT**. Respiratory syncytial virus infection in anti-mu-treated mice. *J Virol* 65: 4936-4942, 1991.
50. **Graham BS, Bunton LA, Wright PF, and Karzon DT**. Role of T lymphocyte subsets in the pathogenesis of primary infection and

rechallenge with respiratory syncytial virus in mice. *The Journal of Clinical Investigation* 88: 1026-1033, 1991.
51. **Griffin DE, and Pan CH**. Measles: old vaccines, new vaccines. *Curr Top Microbiol Immunol* 330: 191-212, 2009.
52. **Group TI-RS**. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. . *Pediatrics* 102: 531-537, 1998.
53. **Gunasegaran K**. Fields Virology, 5th ed. *Immunol Cell Biol* 80: 314-315, 2006.
54. **Haas CN**. Estimation of microbial densities from dilution count experiments. *Appl Environ Microbiol* 55: 1934-1942, 1989.
55. **Hall CB, Douglas RG, and Geiman JM**. Possible Transmission by Fomites of Respiratory Syncytial Virus. *Journal of Infectious Diseases* 141: 98-102, 1980.
56. **Hall CB, Powell KR, MacDonald NE, Gala CL, Menegus ME, Suffin SC, and Cohen HJ**. Respiratory Syncytial Viral Infection in Children with Compromised Immune Function. *New England Journal of Medicine* 315: 77-81, 1986.
57. **Hall CB, Walsh EE, Long CE, and Schnabel KC**. Immunity to and Frequency of Reinfection with Respiratory Syncytial Virus. *Journal of Infectious Diseases* 163: 693-698, 1991.
58. **Hall CB, Walsh EE, Schnabel KC, Long CE, McConnochie KM, Hildreth SW, and Anderson LJ**. Occurrence of Groups A and B of Respiratory Syncytial Virus over 15 Years: Associated Epidemiologic and Clinical Characteristics in Hospitalized and Ambulatory Children. *Journal of Infectious Diseases* 162: 1283-1290, 1990.
59. **Hallak LK, Spillmann D, Collins PL, and Peeples ME**. Glycosaminoglycan sulfation requirements for respiratory syncytial virus infection. *J Virol* 74: 10508-10513, 2000.
60. **Harmon SB, and Wertz GW**. Transcriptional Termination Modulated by Nucleotides Outside the Characterized Gene End Sequence of Respiratory Syncytial Virus. *Virology* 300: 304-315, 2002.
61. **Harrison E**. Mechanisms of digestion and absorption of dietary vitamin A. *Annual Review in Nutrition* 87-103, 2005.
62. **Hartling L, Wiebe N, Russell K, Patel H, and Klassen TP**. Epinephrine for bronchiolitis. *Cochrane Database Syst Rev* CD003123, 2004.
63. **Hegazy AN, Peine M, Helmstetter C, Panse I, Frˆhlich A, Bergthaler A, Flatz L, Pinschewer DD, Radbruch A, and Lˆhning M**. Interferons Direct Th2 Cell Reprogramming to Generate a Stable GATA-3+/T-bet+ Cell Subset with Combined Th2 and Th1 Cell Functions. *Immunity* 32: 116-128, 2010.
64. **Henderson FW, Collier AM, Clyde WA, and Denny FW**. Respiratory-Syncytial-Virus Infections, Reinfections and Immunity. *New England Journal of Medicine* 300: 530-534, 1979.
65. **Henderson G, Murray J, and Yeo RP**. Sorting of the Respiratory Syncytial Virus Matrix Protein into Detergent-Resistant Structures Is

Dependent on Cell-Surface Expression of the Glycoproteins. *Virology* 300: 244-254, 2002.
66. **Hendricks DA, McIntosh K, and Patterson JL**. Further characterization of the soluble form of the G glycoprotein of respiratory syncytial virus. *J Virol* 62: 2228-2233, 1988.
67. **Hoag KA, Nashold FE, Goverman J, and Hayes CE**. Retinoic Acid Enhances the T Helper 2 Cell Development That Is Essential for Robust Antibody Responses through Its Action on Antigen-Presenting Cells. *The Journal of Nutrition* 132: 3736-3739, 2002.
68. **Hoebee B, Bont L, Rietveld E, van Oosten M, Hodemaekers HM, Nagelkerke NJD, Neijens HJ, Kimpen JLL, and Kimman TG**. Influence of Promoter Variants of Interleukin-10, Interleukin-9, and Tumor Necrosis Factor‚Äì Genes on Respiratory Syncytial Virus Bronchiolitis. *Journal of Infectious Diseases* 189: 239-247, 2004.
69. **Hoebee B, Rietveld E, Bont L, Oosten Mv, Hodemaekers HM, Nagelkerke NJD, Neijens HJ, Kimpen JLL, and Kimman TG**. Association of Severe Respiratory Syncytial Virus Bronchiolitis with Interleukin-4 and Interleukin-4 Receptor Polymorphisms. *Journal of Infectious Diseases* 187: 2-11, 2003.
70. **Hogg JC, Williams J, Richardson JB, Macklem PT, and Thurlbeck WM**. Age as a Factor in the Distribution of Lower-Airway Conductance and in the Pathologic Anatomy of Obstructive Lung Disease. *New England Journal of Medicine* 282: 1283-1287, 1970.
71. **Holberg CJ, Wright AL, Martinez FD, Ray CG, Taussig LM, and Lebowitz MD**. Risk factors for respiratory syncytial virus-associated lower respiratory illnesses in the first year of life. *Am J Epidemiol* 133: 1135-1151, 1991.
72. **Holman RC, Curns AT, Cheek JE, Bresee JS, Singleton RJ, Carver K, and Anderson LJ**. Respiratory syncytial virus hospitalizations among American Indian and Alaska Native infants and the general United States infant population. *Pediatrics* 114: e437-444, 2004.
73. **Home F**. Medical Facts and Experiments. London: Miller H: 1759.
74. **Hussell T, Baldwin CJ, O'Garra A, and Openshaw PJM**. CD8+ T cells control Th2-driven pathology during pulmonary respiratory syncytial virus infection. *European Journal of Immunology* 27: 3341-3349, 1997.
75. **Imdad A, Herzer K, Mayo-Wilson E, Yakoob MY, and Bhutta ZA**. Vitamin A supplementation for preventing morbidity and mortality in children from 6 months to 5 years of age. *Cochrane Database Syst Rev* CD008524, 2010.
76. **Iwata M, Eshima Y, and Kagechika H**. Retinoic acids exert direct effects on T cells to suppress Th1 development and enhance Th2 development via retinoic acid receptors. *International Immunology* 15: 1017-1025, 2003.
77. **Jeevan A, Evans R, Brown EL, and Kripke ML**. Effect of local ultraviolet irradiation on infections of mice with Candida albicans, Mycobacterium bovis BCG, and Schistosoma mansoni. *J Invest Dermatol* 99: 59-64, 1992.

78. **K Penniston ST**. The acute and chronic toxic effects of vitamin A. *American Journal of Clinical Nutrition* 191-201, 2006.
79. **Kaisho T, and Akira S**. Toll-like receptor function and signaling. *Journal of Allergy and Clinical Immunology* 117: 979-987, 2006.
80. **Kang BY, Chung SW, Kim SH, Kang SN, Choe YK, and Kim TS**. Retinoid-mediated inhibition of interleukin-12 production in mouse macrophages suppresses Th1 cytokine profile in CD4+ T cells. *British Journal of Pharmacology* 130: 581-586, 2000.
81. **KAPIKIAN AZ, BELL JA, MASTROTA FM, JOHNSON KM, HUEBNER RJ, and CHANOCK RM**. An outbreak of febrile illness and pneumonia associated with respiratory syncytial virus infection. *American Journal of Epidemiology* 74: 234-248, 1961.
82. **KapikianAZ M, ChanockRM,ShvedoffRA,Stewart CE**. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. *Am J Epidemiol* 89: 405-421, 1969.
83. **Karron RA, Singleton RJ, Bulkow L, Parkinson A, Kruse D, DeSmet I, Indorf C, Petersen KM, Leombruno D, Hurlburt D, Santosham M, and Harrison LH**. Severe Respiratory Syncytial Virus Disease in Alaska Native Children. *Journal of Infectious Diseases* 180: 41-49, 1999.
84. **Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh C-S, Reis e Sousa C, Matsuura Y, Fujita T, and Akira S**. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441: 101-105, 2006.
85. **Katz SL**. ***Chapter 1: John F. Enders and Measles Virus Vaccine—a Reminiscence***. 2009.
86. **Kawaguchi R, Yu J, Honda J, Hu J, Whitelegge J, Ping P, Wiita P, Bok D, and Sun H**. A Membrane Receptor for Retinol Binding Protein Mediates Cellular Uptake of Vitamin A. *Science* 315: 820-825, 2007.
87. **Kawai K, Msamanga G, Manji K, Villamor E, Bosch RJ, Hertzmark E, and Fawzi WW**. Sex differences in the effects of maternal vitamin supplements on mortality and morbidity among children born to HIV-infected women in Tanzania. *British Journal of Nutrition* 103: 1784-1791, 2010.
88. **Kim HW, Arrobio JO, Brandt CD, Jeffries BC, Pyles G, Reid JL, Chanock RM, and Parrott RH**. Epidemiology of respiratory syncytial virus infection in Washington, D.C. I. Importance of the virus in different respiratory tract disease syndromes and temporal distribution of infection. *Am J Epidemiol* 98: 216-225, 1973.
89. **Klouwenberg PK, Tan L, Werkman W, van Bleek GM, and Coenjaerts F**. The Role of Toll-like Receptors in Regulating the Immune Response against Respiratory Syncytial Virus. 29: 531-550, 2009.
90. **Komuro A, and Horvath CM**. RNA- and Virus-Independent Inhibition of Antiviral Signaling by RNA Helicase LGP2. *J Virol* 80: 12332-12342, 2006.

91. **Krilov LR**. Respiratory syncytial virus disease: update on treatment and prevention. *Expert Rev Anti Infect Ther* 9: 27-32, 2011.
92. **Krilov LR, Weiner LB, Yogev R, Fergie J, Katz BZ, Henrickson KJ, and Welliver RC, Sr.** The 2009 COID recommendations for RSV prophylaxis: issues of efficacy, cost, and evidence-based medicine. *Pediatrics* 124: 1682-1684, 2009.
93. **Kwilas S, Liesman RM, Zhang L, Walsh E, Pickles RJ, and Peeples ME**. Respiratory syncytial virus grown in Vero cells contains a truncated attachment protein that alters its infectivity and dependence on glycosaminoglycans. *J Virol* 83: 10710-10718, 2009.
94. **Liu G, and Zhao Y**. Toll-like receptors and immune regulation: their direct and indirect modulation on regulatory CD4+ CD25+ T cells. *Immunology* 122: 149-156, 2007.
95. **Liu P, Jamaluddin M, Li K, Garofalo RP, Casola A, and Brasier AR**. Retinoic Acid-Inducible Gene I Mediates Early Antiviral Response and Toll-Like Receptor 3 Expression in Respiratory Syncytial Virus-Infected Airway Epithelial Cells. *J Virol* 81: 1401-1411, 2007.
96. **Liuzzi M, Mason SW, Cartier M, Lawetz C, McCollum RS, Dansereau N, Bolger G, Lapeyre N, Gaudette Y, Lagace L, Massariol MJ, Do F, Whitehead P, Lamarre L, Scouten E, Bordeleau J, Landry S, Rancourt J, Fazal G, and Simoneau B**. Inhibitors of respiratory syncytial virus replication target cotranscriptional mRNA guanylylation by viral RNA-dependent RNA polymerase. *J Virol* 79: 13105-13115, 2005.
97. **Long KZ, Garcia C, Santos JI, Rosado JL, Hertzmark E, DuPont HL, and Ko G**. Vitamin A Supplementation Has Divergent Effects on Norovirus Infections and Clinical Symptoms among Mexican Children. *Journal of Infectious Diseases* 196: 978-985, 2007.
98. **Long KZ, Rosado JL, DuPont HL, Hertzmark E, and Santos JI**. Supplementation with vitamin A reduces watery diarrhoea and respiratory infections in Mexican children. *British Journal of Nutrition* 97: 337-343, 2007.
99. **Long KZ, Rosado JL, Montoya Y, de Lourdes Solano M, Hertzmark E, DuPont HL, and Santos JI**. Effect of Vitamin A and Zinc Supplementation on Gastrointestinal Parasitic Infections Among Mexican Children. *Pediatrics* 120: e846-e855, 2007.
100. **Lukacs NW, Moore ML, Rudd BD, Berlin AA, Collins RD, Olson SJ, Ho SB, and Peebles Jr RS**. Differential Immune Responses and Pulmonary Pathophysiology Are Induced by Two Different Strains of Respiratory Syncytial Virus. *The American Journal of Pathology* 169: 977-986, 2006.
101. **Maher CF, Hussell T, Blair E, Ring CJA, and Openshaw PJM**. Recombinant respiratory syncytial virus lacking secreted glycoprotein G is attenuated, non-pathogenic but induces protective immunity. *Microbes and Infection* 6: 1049-1055, 2004.
102. **Mangelsdorf DJ, Ong ES, Dyck JA, and Evans RM**. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* 345: 224-229, 1990.

103. **Massacesi L, Castigli E, Vergelli M, Olivotto J, Abbamondi AL, Sarlo F, and Amaducci L**. Immunosuppressive activity of 13-cis-retinoic acid and prevention of experimental autoimmune encephalomyelitis in rats. *The Journal of Clinical Investigation* 88: 1331-1337, 1991.
104. **McCann S, Byrne JL, Rovira M, Shaw P, Ribaud P, Sica S, Volin L, Olavarria E, Mackinnon S, Trabasso P, VanLint MT, Ljungman P, Ward K, Browne P, Gratwohl A, Widmer AF, and Cordonnier C**. Outbreaks of infectious diseases in stem cell transplant units: a silent cause of death for patients and transplant programmes. *Bone Marrow Transplant* 33: 519-529, 2004.
105. **McIntosh K, Masters HB, Orr I, Chao RK, and Barkin RM**. The Immunologic Response to Infection with Respiratory Syncytial Virus in Infants. *Journal of Infectious Diseases* 138: 24-32, 1978.
106. **Megan S. Harrison TS, Anthony P. Schmitt**. Paramyxovirus assembly and budding: Building particles that transmit infections. *The International Journal of Biochemistry & Cell Biology* 42: 1416-1429, 2010.
107. **Meissner HC**. Selected populations at increased risk from respiratory syncytial virus infection. *The Pediatric Infectious Disease Journal* 22: S40-S45, 2003.
108. **Melero J, Garcia-Barreno B, Martinez I, Pringle C, and Cane P**. Antigenic structure, evolution and immunobiology of human respiratory syncytial virus attachment (G) protein. *J Gen Virol* 78: 2411-2418, 1997.
109. **Melvin Wright**. Respiratory Syncytial Virus Prevention and Therapy: Past, Present, and Future. *Pediatric Pulmonology* 46: 324-347, 2011.
110. **Miller SA, and Weinmann AS**. Molecular mechanisms by which T-bet regulates T-helper cell commitment. *Immunological Reviews* 238: 233-246, 2010.
111. **Moudy RM, Sullender WM, and Wertz GW**. Variations in intergenic region sequences of Human respiratory syncytial virus clinical isolates: analysis of effects on transcriptional regulation. *Virology* 327: 121-133, 2004.
112. **Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, and Cheroutre H**. Reciprocal TH17 and Regulatory T Cell Differentiation Mediated by Retinoic Acid. *Science* 317: 256-260, 2007.
113. **Munir S, Hillyer P, Le Nou√´n C, Buchholz UJ, Rabin RL, Collins PL, and Bukreyev A**. Respiratory Syncytial Virus Interferon Antagonist NS1 Protein Suppresses and Skews the Human T Lymphocyte Response. *PLoS Pathog* 7: e1001336, 2011.
114. **Murata Y**. Respiratory syncytial virus vaccine development. *Clin Lab Med* 29: 725-739, 2009.
115. **Murphy BR, Alling DW, Snyder MH, Walsh EE, Prince GA, Chanock RM, Hemming VG, Rodriguez WJ, Kim HW, and Graham BS**. Effect of age and preexisting antibody on serum antibody response of infants and children to the F and G glycoproteins during respiratory syncytial virus infection. *J Clin Microbiol* 24: 894-898, 1986.

116. **Murphy BR, Prince GA, Lawrence LA, Croen KD, and Collins PL**. Detection of respiratory syncytial virus (RSV) infected cells by in situ hybridization in the lungs of cotton rats immunized with formalin-inactivated virus or purified RSV F and G glycoprotein subunit vaccine and challenged with RSV. *Virus Research* 16: 153-162, 1990.
117. **Mwandumba HC, Bertel Squire S, White SA, Nyirenda MH, Kampondeni SD, Rhoades ER, Zijlstra EE, Molyneux ME, and Russell DG**. Association between sputum smear status and local immune responses at the site of disease in HIV-infected patients with pulmonary tuberculosis. *Tuberculosis* 88: 58-63, 2008.
118. **Nairz M, Schroll A, Sonnweber T, and Weiss G**. The struggle for iron – a metal at the host–pathogen interface. *Cellular Microbiology* 12: 1691-1702, 2010.
119. **Nallagatla SR TR, Bevilacqua PC**. A brilliant disguise for self RNA: 5'-end and internal modifications of primary transcripts suppress elements of innate immunity. *RNA biol* 5: 140-144, 2008.
120. **Napoli JL**. Biochemical Pathways of Retinoid Transport, Metabolism, and Signal Transduction. *Clinical Immunology and Immunopathology* 80: S52-S62, 1996.
121. **Neilson KA, and Yunis EJ**. Demonstration of respiratory syncytial virus in an autopsy series. *Pediatr Pathol* 10: 491-502, 1990.
122. **Ni J, Wei J, and Wu T**. Vitamin A for non-measles pneumonia in children. *Cochrane Database Syst Rev* CD003700, 2005.
123. **O'Neill LAJ, and Bowie AG**. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol* 7: 353-364, 2007.
124. **O'Shea JJ, and Paul WE**. Mechanisms Underlying Lineage Commitment and Plasticity of Helper CD4+ T Cells. *Science* 327: 1098-1102, 2010.
125. **Olmsted RA, and Collins PL**. The 1A protein of respiratory syncytial virus is an integral membrane protein present as multiple, structurally distinct species. *J Virol* 63: 2019-2029, 1989.
126. **Ovsyannikova IG, Haralambieva IH, Dhiman N, O'Byrne MM, Pankratz VS, Jacobson RM, and Poland GA**. Polymorphisms in the Vitamin A Receptor and Innate Immunity Genes Influence the Antibody Response to Rubella Vaccination. *Journal of Infectious Diseases* 201: 207-213, 2010.
127. **Pasutto F, Sticht H, Hammersen G, Gillessen-Kaesbach G, FitzPatrick DR, N¸rnberg G, Brasch F, Schirmer-Zimmermann H, Tolmie JL, Chitayat D, Houge G, Fern·ndez-Martìnez L, Keating S, Mortier G, Hennekam RCM, von der Wense A, Slavotinek A, Meinecke P, Bitoun P, Becker C, N¸rnberg P, Reis A, and Rauch A**. Mutations in STRA6 Cause a Broad Spectrum of Malformations Including Anophthalmia, Congenital Heart Defects, Diaphragmatic Hernia, Alveolar Capillary Dysplasia, Lung Hypoplasia, and Mental Retardation. *The American Journal of Human Genetics* 80: 550-560, 2007.

128. **Penniston KL, and Tanumihardjo SA**. Vitamin A in dietary supplements and fortified foods: Too much of a good thing? *Journal of the American Dietetic Association* 103: 1185-1187, 2003.
129. **Peret T, Hall C, Schnabel K, Golub J, and Anderson L**. Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. *J Gen Virol* 79: 2221-2229, 1998.
130. **Petkovich PM**. Retinoic acid metabolism. *Journal of the American Academy of Dermatology* 45: S136-S142, 2001.
131. **Pino-Lagos K, Benson MJ, and Noelle RJ**. Retinoic acid in the immune system. *Ann N Y Acad Sci* 1143: 170-187, 2008.
132. **Polack FP, Irusta PM, Hoffman SJ, Schiatti MP, Melendi GA, Delgado MF, Laham FR, Thumar B, Hendry RM, Melero JA, Karron RA, Collins PL, and Kleeberger SR**. The cysteine-rich region of respiratory syncytial virus attachment protein inhibits innate immunity elicited by the virus and endotoxin. *Proceedings of the National Academy of Sciences of the United States of America* 102: 8996-9001, 2005.
133. **Preston FM, Beier PL, and Pope JH**. Infectious Respiratory Syncytial Virus (RSV) Effectively Inhibits the Proliferative T Cell Response to Inactivated RSV In Vitro. *Journal of Infectious Diseases* 165: 819-825, 1992.
134. **Prince GA, Horswood RL, Berndt J, Suffin SC, and Chanock RM**. Respiratory syncytial virus infection in inbred mice. *Infect Immun* 26: 764-766, 1979.
135. **Racke M, Burnett D, Pak S, Albert P, Cannella B, Raine C, McFarlin D, and Scott D**. Retinoid treatment of experimental allergic encephalomyelitis. IL-4 production correlates with improved disease course. *The Journal of Immunology* 154: 450-458, 1995.
136. **Ramaswamy M, Shi L, Monick MM, Hunninghake GW, and Look DC**. Specific Inhibition of Type I Interferon Signal Transduction by Respiratory Syncytial Virus. *Am J Respir Cell Mol Biol* 30: 893-900, 2004.
137. **Rengarajan J, Szabo SJ, and Glimcher LH**. Transcriptional regulation of Th1/Th2 polarization. *Immunology Today* 21: 479-483, 2000.
138. **Rodeheffer C, Von Messling V, Milot S, Lepine Fo, Manges AR, and Ward BJ**. Disease Manifestations of Canine Distemper Virus Infection in Ferrets Are Modulated by Vitamin A Status. *The Journal of Nutrition* 137: 1916-1922, 2007.
139. **Romagnani S**. Immunologic influences on allergy and the TH1/TH2 balance. *Journal of Allergy and Clinical Immunology* 113: 395-400, 2004.
140. **Rosenberg HF, Dyer KD, and Domachowske JB**. Respiratory viruses and eosinophils: exploring the connections. *Antiviral Res* 83: 1-9, 2009.
141. **Rothenfusser S, Goutagny N, DiPerna G, Gong M, Monks BG, Schoenemeyer A, Yamamoto M, Akira S, and Fitzgerald KA**. The RNA Helicase Lgp2 Inhibits TLR-Independent Sensing of Viral Replication by Retinoic Acid-Inducible Gene-I. *The Journal of Immunology* 175: 5260-5268, 2005.

142. **Rudd BD, Smit JJ, Flavell RA, Alexopoulou L, Schaller MA, Gruber A, Berlin AA, and Lukacs NW**. Deletion of TLR3 Alters the Pulmonary Immune Environment and Mucus Production during Respiratory Syncytial Virus Infection. *The Journal of Immunology* 176: 1937-1942, 2006.
143. **Sampalis JS**. Morbidity and mortality after RSV-associated hospitalizations among premature Canadian infants. *The Journal of Pediatrics* 143: 150-156, 2003.
144. **Sano H, Nagai K, Tsutsumi H, and Kuroki Y**. Lactoferrin and surfactant protein A exhibit distinct binding specificity to F protein and differently modulate respiratory syncytial virus infection. *European Journal of Immunology* 33: 2894-2902, 2003.
145. **Santangelo PJ, and Bao G**. Dynamics of filamentous viral RNPs prior to egress. *Nucleic Acids Research* 35: 3602-3611, 2007.
146. **Satoh T, Kato H, Kumagai Y, Yoneyama M, Sato S, Matsushita K, Tsujimura T, Fujita T, Akira S, and Takeuchi O**. LGP2 is a positive regulator of RIG-I, and MDA5-mediated antiviral responses. *Proceedings of the National Academy of Sciences* 107: 1512-1517, 2010.
147. **Schmidt A, Schwerd T, Hamm W, Hellmuth JC, Cui S, Wenzel M, Hoffmann FS, Michallet M-C, Besch R, Hopfner K-P, Endres S, and Rothenfusser S**. 5'-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proceedings of the National Academy of Sciences* 106: 12067-12072, 2009.
148. **Schuster GU, Kenyon NJ, and Stephensen CB**. Vitamin A deficiency decreases and high dietary vitamin A increases disease severity in the mouse model of asthma. *J Immunol* 180: 1834-1842, 2008.
149. **Schwarze J, O'Donnell DR, Rohwedder A, and Openshaw PJM**. Latency and Persistence of Respiratory Syncytial Virus Despite T Cell Immunity. *Am J Respir Crit Care Med* 169: 801-805, 2004.
150. **Senoo H, Kojima N, and Sato M**. Vitamin A[hyphen (true graphic)]Storing Cells (Stellate Cells). In: *Vitamins & Hormones*, edited by Gerald LAcademic Press, 2007, p. 131-159.
151. **Shay DK, Holman RC, Roosevelt GE, Clarke MJ, and Anderson LJ**. Bronchiolitis-Associated Mortality and Estimates of Respiratory Syncytial Virus Associated Deaths among US Children, 1979,Äì1997. *Journal of Infectious Diseases* 183: 16-22, 2001.
152. **Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SLR, Cookson BT, and Aderem A**. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nat Immunol* 4: 1247-1253, 2003.
153. **Spann KM, Tran KC, Chi B, Rabin RL, and Collins PL**. Suppression of the induction of alpha, beta, and lambda interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected]. *J Virol* 78: 4363-4369, 2004.
154. **Spann KM, Tran KC, and Collins PL**. Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon

regulatory factor 3, NF-kappaB, and proinflammatory cytokines. *J Virol* 79: 5353-5362, 2005.
155. **Srikiatkhachorn A, and Braciale TJ**. Virus-specific CD8+ T Lymphocytes Downregulate T Helper Cell Type 2 Cytokine Secretion and Pulmonary Eosinophilia during Experimental Murine Respiratory Syncytial Virus Infection. *The Journal of Experimental Medicine* 186: 421-432, 1997.
156. **Stec DS, Hill MG, 3rd, and Collins PL**. Sequence analysis of the polymerase L gene of human respiratory syncytial virus and predicted phylogeny of nonsegmented negative-strand viruses. *Virology* 183: 273-287, 1991.
157. **Stephensen CB, Blount SR, Schoeb TR, and Park JY**. Vitamin A Deficiency Impairs Some Aspects of the Host Response to Influenza A Virus Infection in BALB/c Mice. *The Journal of Nutrition* 123: 823-833, 1993.
158. **Stokes KL, Chi MH, Sakamoto K, Newcomb DC, Currier MG, Huckabee MM, Lee S, Goleniewska K, Pretto C, Williams JV, Hotard A, Sherrill TP, Peebles RS, Jr, and Moore ML**. Differential Pathogenesis of Respiratory Syncytial Virus Clinical Isolates in BALB/c Mice. *J Virol* 85: 5782-5793, 2011.
159. **Sundaram N, Bennett M, Wilhelm J, Kim MO, Atweh G, Devarajan P, and Malik P**. Biomarkers for early detection of sickle nephropathy. *Am J Hematol* 2011.
160. **Sung JH, Ji JH, Park JD, Song MY, Song KS, Ryu HR, Yun JU, Jeon KS, Jeong J, Han BS, Chung YH, Chang HK, Lee JH, Kim DW, Kelman BJ, and Yu IJ**. Subchronic inhalation toxicity of gold nanoparticles. *Part Fibre Toxicol* 8: 16, 2011.
161. **Takeuchi O, and Akira S**. MDA5/RIG-I and virus recognition. *Curr Opin Immunol* 20: 17-22, 2008.
162. **Techaarpornkul S, Collins PL, and Peeples ME**. Respiratory Syncytial Virus with the Fusion Protein as Its only Viral Glycoprotein Is Less Dependent on Cellular Glycosaminoglycans for Attachment than Complete Virus. *Virology* 294: 296-304, 2002.
163. **Teng MN, and Collins PL**. Altered growth characteristics of recombinant respiratory syncytial viruses which do not produce NS2 protein. *J Virol* 73: 466-473, 1999.
164. **Teng MN, and Collins PL**. The central conserved cystine noose of the attachment G protein of human respiratory syncytial virus is not required for efficient viral infection in vitro or in vivo. *J Virol* 76: 6164-6171, 2002.
165. **Teng MN, and Collins PL**. Identification of the respiratory syncytial virus proteins required for formation and passage of helper-dependent infectious particles. *J Virol* 72: 5707-5716, 1998.
166. **Teng MN, Whitehead SS, Bermingham A, St Claire M, Elkins WR, Murphy BR, and Collins PL**. Recombinant respiratory syncytial virus that does not express the NS1 or M2-2 protein is highly attenuated and immunogenic in chimpanzees. *J Virol* 74: 9317-9321, 2000.

167. **Thacher TD, and Clarke BL**. Vitamin D insufficiency. *Mayo Clin Proc* 86: 50-60, 2011.
168. **Theodosiou M, Laudet V, and Schubert M**. From carrot to clinic: an overview of the retinoic acid signaling pathway. *Cellular and Molecular Life Sciences* 67: 1423-1445, 2010.
169. **Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ, and Fukuda K**. Mortality Associated With Influenza and Respiratory Syncytial Virus in the United States. *JAMA: The Journal of the American Medical Association* 289: 179-186, 2003.
170. **Tian B, Zhang Y, Luxon BA, Garofalo RP, Casola A, Sinha M, and Brasier AR**. Identification of NF-{kappa}B-Dependent Gene Networks in Respiratory Syncytial Virus-Infected Cells. *J Virol* 76: 6800-6814, 2002.
171. **Tomes DJ, Hellbusch LC, and Alberts LR**. Stretching and breaking characteristics of cerebrospinal fluid shunt tubing. *Journal of Neurosurgery* 98: 578-583, 2003.
172. **Trottier C, Chabot S, Mann KK, Colombo M, Chatterjee A, Miller Jr WH, and Ward BJ**. Retinoids inhibit measles virus in vitro via nuclear retinoid receptor signaling pathways. *Antiviral Research* 80: 45-53, 2008.
173. **Trottier C, Colombo M, Mann KK, Miller WH, and Ward BJ**. Retinoids inhibit measles virus through a type I IFN-dependent bystander effect. *The FASEB Journal* 23: 3203-3212, 2009.
174. **US Department of Agriculture**. Nutrient Database for Standard Reference, Release 17. Nutrient Data Laboratory Homepage, 2004.
175. **van den Berg JP, Westerbeek EAM, van der Klis FRM, Berbers GAM, and van Elburg RM**. Transplacental transport of IgG antibodies to preterm infants: A review of the literature. *Early Human Development* 87: 67-72, 2011.
176. **Vareille M, Kieninger E, Edwards MR, and Regamey N**. The Airway Epithelium: Soldier in the Fight against Respiratory Viruses. *Clin Microbiol Rev* 24: 210-229, 2011.
177. **Veldhoen M, Uyttenhove C, van Snick J, Helmby H, Westendorf A, Buer J, Martin B, Wilhelm C, and Stockinger B**. Transforming growth factor-[beta] 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* 9: 1341-1346, 2008.
178. **Venkataraman T, Valdes M, Elsby R, Kakuta S, Caceres G, Saijo S, Iwakura Y, and Barber GN**. Loss of DExD/H Box RNA Helicase LGP2 Manifests Disparate Antiviral Responses. *The Journal of Immunology* 178: 6444-6455, 2007.
179. **Ventre K, and Randolph A**. Ribavirin for respiratory syncytial virus infection of the lower respiratory tract in infants and young children. *Cochrane Database Syst Rev* CD000181, 2004.
180. **Wareing MD, Lyon AB, Lu B, Gerard C, and Sarawar SR**. Chemokine expression during the development and resolution of a pulmonary leukocyte response to influenza A virus infection in mice. *Journal of Leukocyte Biology* 76: 886-895, 2004.

181. **Weinberg ED**. The hazards of iron loading. *Metallomics* 2: 732-740, 2010.
182. **Welliver RC, Sr.** The immune response to respiratory syncytial virus infection: friend or foe? *Clin Rev Allergy Immunol* 34: 163-173, 2008.
183. **WHO**. Vitamin A for Measles. *Weekly Epidemiol Rec* 19: 133-134, 1987.
184. **Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, Basham B, Smith K, Chen T, Morel F, Lecron J-C, Kastelein RA, Cua DJ, McClanahan TK, Bowman EP, and de Waal Malefyt R**. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 8: 950-957, 2007.
185. **Wiysonge CS, Shey M, Kongnyuy EJ, Sterne JA, and Brocklehurst P**. Vitamin A supplementation for reducing the risk of mother-to-child transmission of HIV infection. *Cochrane Database Syst Rev* CD003648, 2011.
186. **Wong DT, Rosenband M, Hovey K, and Ogra PL**. Respiratory syncytial virus infection in immunosuppressed animals: implications in human infection. *J Med Virol* 17: 359-370, 1985.
187. **Wright PF, Gruber WC, Peters M, Reed G, Zhu Y, Robinson F, Coleman-Dockery S, and Graham BS**. Illness Severity, Viral Shedding, and Antibody Responses in Infants Hospitalized with Bronchiolitis Caused by Respiratory Syncytial Virus. *Journal of Infectious Diseases* 185: 1011-1018, 2002.
188. **Wu H, Pfarr DS, Johnson S, Brewah YA, Woods RM, Patel NK, White WI, Young JF, and Kiener PA**. Development of Motavizumab, an Ultra-potent Antibody for the Prevention of Respiratory Syncytial Virus Infection in the Upper and Lower Respiratory Tract. *Journal of Molecular Biology* 368: 652-665, 2007.
189. **Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo Y-M, Gale M, Akira S, Yonehara S, Kato A, and Fujita T**. Shared and Unique Functions of the DExD/H-Box Helicases RIG-I, MDA5, and LGP2 in Antiviral Innate Immunity. *The Journal of Immunology* 175: 2851-2858, 2005.
190. **Zeng R, Li C, Li N, Wei L, and Cui Y**. The role of cytokines and chemokines in severe respiratory syncytial virus infection and subsequent asthma. *Cytokine* 53: 1-7, 2011.
191. **Zhang L, Peeples ME, Boucher RC, Collins PL, and Pickles RJ**. Respiratory Syncytial Virus Infection of Human Airway Epithelial Cells Is Polarized, Specific to Ciliated Cells, and without Obvious Cytopathology. *J Virol* 76: 5654-5666, 2002.
192. **Zhang Y, Luxon BA, Casola A, Garofalo RP, Jamaluddin M, and Brasier AR**. Expression of Respiratory Syncytial Virus-Induced Chemokine Gene Networks in Lower Airway Epithelial Cells Revealed by cDNA Microarrays. *J Virol* 75: 9044-9058, 2001.

Appendix