The Impact of Vitamin D on Innate Immune Responsiveness to Pattern Recognition Receptor Stimulation in Humans

by

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Abstract

Objective: Study the effects of vitamin D on viral driven innate immune responses, by looking at differences in cytokine production, receptor expression, and endogenous vitamin D levels.

Methods: Primary peripheral blood mononuclear cells (PBMC) and epithelial cells (EC) were cultured in the presence of viral ligands and vitamin D. Enzyme-linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (RT-PCR) were used to determine cytokine production and mRNA expression.

Results: PBMC stimulated with toll-like receptor 4 ligand (TLR4L), but not viral TLR8L, led to decreased pro- and anti-inflammatory cytokine production in the presence of $1,25(OH)_2D_3$. RIG-like receptor (RLR) activation, on the other hand, in primary EC exhibited decreased pro-inflammatory cytokine production in the presence of vitamin D.

Conclusions: Our findings are among the first to show differences between bacterial and viral driven innate immune responses in the presence of vitamin D. As responsiveness in RLR activated primary EC was altered in the presence of vitamin D, our data reveal the importance of studying the immune system as a whole.

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Dedications

I would like to dedicate this thesis to my husband, parents and sister. Thank-you all for you continuous support, especially Jaron who listened to countless research presentations and always provided positive feedback. I love you all! You too Rupes!

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List of Abbreviations

7-DHC: 7-dehydrocholesterol

AHR: Airway hyperresponsiveness

AMP: Anti-microbial peptide

APC: Antigen presenting cell

APOBEC: Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like

ATP: Adenosine-triphosphate

BMI: Body mass index

CARD: Caspase recruitment domain

CAPPS: Canadian Asthma and Allergy Prevention Program

CBMC: Cord blood mononuclear cells

CCL: Chemokine (C-C motif) ligand

CD: Cluster of differentiation

CD: Crohn`s Disease

CHILD: Canadian Healthy Infant Longitudinal Development Study

CM: Culture media

CpG: Cytosine-phosphate-guanine

CRP: C-reactive protein

CTLA-4: Cytotoxic T lymphocyte antigen-4

CXCL: Chemokine (C-X-C motif) ligand

CYP2R1: Cytochrome P450, family 2, subfamily R, polypeptide 1

CYP24a1: Cytochrome P450, family 24, subfamily A, polypeptide 1

CYP27a1: Cytochrome P450, family 27, subfamily A, polypeptide 1

CYP27b1: Cytochrome P450, family 27, subfamily B, polypeptide 1

DAMP: Danger associated molecular pattern

DBP: Vitamin D binding protein

DC: Dendritic cells

dsRNA: Double-stranded ribonucleic acid

EC: Epithelial cells

ELISA: Enzyme-linked immuno sorbent assay

EMCV: Encephalomyocarditis picornavirus

FEV_1: Forced expiratory volume in 1 second

FGF-23: Fibroblast growth factor-23

Foxp3: Forkhead box P3

FVC: Forced vital capacitance

G-CSF: Granulocyte colony stimulating factor

HAEC: Human airway epithelial cells

HBD: Human beta defensin

hCAP: Human cathelicidin AMP gene

HCV: Hepatitis C virus

HIV: Human immunodeficiency virus

IBD: Inflammatory bowel disease

IFI: Interferon alpha inducible protein

IFIT: Interferon-inducible protein with tetratricopeptide repeats

IFN: Interferon

Ig: Immunoglobin

IκB: NF-κB inhibitor

IL: Interleukin

iNOS: Inducible nitric oxide synthase

IRF: Interferon regulatory factor

ISG: Interferon stimulated gene

ISRE: Interferon stimulated response element

LBP: Lipopolysaccharide binding protein

LGP2: Laboratory of genetics and physiology 2

LPS: Lipopolysaccharide

LTRI: Lower tract respiratory infections

MAPK: Mitogen activated protein kinases

MAVS: Mitochondrial antiviral-signalling protein

MD-2: Lymphocyte antigen 96

MDA-5: Melanoma differentiation-associated gene 5

MDP: Muramyl dipeptide

MHC: Major histocompatibility complex

MKP-1: MAPK phosphatase-1

MMP: Matrix metalloproteinases

Mx1: Myovirus resistance 1

MyD88: Myeloid differentiation primary response gene

NA: Not available

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NHANES III: Third National Health and Nutrition Examination Survey

NK: Natural Killer

NLR: NOD-like receptor

NOD: Nucleotide-binding oligomerization domain-containing protein

OAS: 2',5'-oligoadenylate synthase

OVA: Ovalbumin

PAMP: Pattern associated molecular pattern

PBMC: Peripheral blood mononuclear cells

PC20: Provocative concentration causing a 20% drop in FEV_1

PKR: Protein kinase R

pNPP: p-nitrophenyl phosphate

Poly(I:C): Polyinosinic:polycytidylic acid

PRR: Pattern recognition receptors

PTH: Parathyroid hormone

RIG-I: Retinoic acid inducible gene 1

RLR: RIG-like receptor

RNaseL: Ribonuclease L

RORγt: Retinoic-acid-receptor-related orphan receptor gamma

RPMI: Roswell Park Memorial Institute

RSV: Respiratory syncytial virus

RT-PCR: Real-time polymerase chain reaction

RXR: Retinoid X receptor

SAAP: Streptavidin alkaline phosphatase

SEM: Standard error of the mean

ssRNA: Single-stranded ribonucleic acid

STING: Stimulator of interferon genes protein

sTNFRII: Soluble TNF receptor 2

TCID: Tissue culture infectious dose

TGF-β: Transforming growth factor-β

Th1: T-helper cell type 1

Th2: T-helper cell type 2

Th17: T-helper cell type 17

TIL: Reovirus strain type 1 lang

TIR: Toll-interleukin 1 receptor

TIRAP: Toll-interleukin 1 receptor domain containing adaptor protein

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TRAM: TRIF related adaptor molecule

Tregs: Regulatory T cell

TRIF: TIR-domain-containing adaptor inducing IFN-β

TSLP: Thymic stromal lymphopoietin

VDR: Vitamin D receptor

VDRE: Vitamin D response element

VSV: Vesicular stomatitis virus

Section 1: Pattern Recognition Receptors

1.1 Overview of Innate Immunity

Our knowledge of the innate immune system has increased dramatically over the last two decades. Before the discovery of Toll-like receptors (TLRs) in the mid 1990s our understanding of the innate immune system was that it non-specifically recognized pathogens. However, over a decade ago papers showed that the Toll/NF-κB pathway is conserved from *Drosophila* flies to humans, where the human version of Toll can induce an innate immune response (1,2). Although the notion of pattern recognition receptors (PRRs) binding to specific pathogen associated molecular patterns (PAMPs) had been proposed previously (3), the discovery of TLRs confirmed the concept of PRRs thereby leading to increased research in the field of innate immunity (4-6).

Currently recognized PRRs include TLRs, Nod-like receptors (NLRs), RIG-like receptors (RLRs), C-type lectin receptors and cytosolic DNA sensing receptors, such as RNA polymerase III, DAI and AIM2 (7). These receptors are all located in different areas of the cell or are secreted into blood. They function as innate immune receptors in the recognition of PAMPs (5) and are found on most innate immune cells, including monocytes, macrophages, dendritic cells (DC), neutrophils and epithelial cells (EC), and even on some adaptive cells including B cells and T cells (8-11). Besides their ability to recognize molecular patterns on pathogens, they also respond to cellular stress, as well as danger associated molecular patterns (DAMPs) and are therefore a vital component of the immune system (7,12). PRRs work collectively to recognize foreign organisms and

elicit an appropriate immune response, however it is important to note different PRR generally recognize distinct PAMPs (12). This feature in combination with their ability to form complex lipid rafts, gives the innate immune system more specificity and usually allows an appropriate immune response to be generated (13).

The innate immune response can either clear an infection or pave the way for an adaptive immune response, which is highly specific. Depending on the PRR that has been activated, cytokines, interferon (IFN), cathelicidins, chemokines and many other molecules and proteins can be synthesized or released by the stimulated cell (8). If this activated cell happens to be an antigen presenting cell (APC) (ie: macrophage, DC or innate B cell), the cell will process the foreign antigen and present it to adaptive cells (T cells and B cells) as a foreign peptide through the major histocompatibility complex (MHC) molecules (reviewed in (14,15)).

Cytokines, which are released by activated and resting cells and consist largely of interleukins (IL) and chemokines, are released by cells for the purpose of proliferation, differentiation, cell recruitment/trafficking, cell survival and effector function (16). In terms of an innate immune response, macrophages and DC release cytokines to initiate, direct and maintain an inflammatory or anti-inflammatory response (17). Innate pro-inflammatory cytokines including IL-8 (CXCL-8), Tumor Necrosis Factor-α (TNF-α), IL-1, IL-12 and others are involved in producing this inflammatory response, whereas other cytokines such as IL-10 and IL-1R antagonist attempt to suppress and regulate the response (18). IFNs are responsible for generating an anti-viral response if they are Type

I (IFN-α, IFN-β) or Type III (IFN-$\lambda_{1,2,3}$) IFNs, or a T helper cell 1 (Th1) inflammatory response if they are Type II (IFN-γ) (19). Chemokines, which are another family of cytokines, that are smaller in structure; as reviewed by Mortier et al (20), guide leukocyte subsets to the site of infection and are responsible for coordinating the homing and recirculation of lymphocytes. Among their cell trafficking ability, many chemokines are also involved in tissue extravasation, inhibition of angiogenesis, and modulating leukocyte response during infection (20-22). Therefore, depending on the ligand that activates the innate cell different types of cytokines and chemokines can be released in an attempt to generate an appropriate response.

More recently in the field of innate immunity, the production of anti-microbial peptides (AMPs) released from innate immune cells has been of great interest. The two main families of AMPs are defensins and cathelicidins, which are classified based on their structure and function (23,24). There are several types of defensins in humans, but only one cathelicidin known as LL-37. Both defensins and cathelicidins are cationic peptides, meaning they are positively charged, which helps them attract to negatively charged bacterial membranes (25). AMPs can directly kill bacteria by interacting with gram-negative lipopolysaccharide (LPS) or gram-positive teichoic acids thus resulting in bacterial outer membrane disruption (26). Although little evidence exists indicating that AMPs can directly kill viruses, they have been shown to decrease viral titers and inflammatory cytokines in influenza and human immunodeficiency virus (HIV) infected cells (27,28).

As the innate immune system is responsible for initial recognition and activation to bacterial and viral ligands, below I review the key characteristics of PRRs, in particular those that are examined in the conduct of our research.

1.2 TLRs

TLRs are a group of PRRs. These receptors are type I transmembrane proteins whose extracellular domain is comprised of leucine-rich repeats that recognize PAMPs (29). Each TLR contains a toll-interleukin 1 receptor (TIR) domain that binds adaptor proteins to facilitate downstream signal transduction (30). To date 10 functional TLRs have been documented in humans and 12 in mice, with TLR1-9 conserved in both species. Several mouse knock out studies, as reviewed by Akira et al, (31) show that mice lacking specific TLRs demonstrate the importance of having all TLR receptors, as each recognizes different PAMPs which then elicits a different functional immune response. Interestingly, TLRs can also dimerize forming a combinatorial TLR that can generate a different immunological response than if it were to recognize a PAMP alone (21). Therefore the different TLRs, as well as the different heterodimer combinations that can be made by these receptors, gives the innate immune system the ability to recognize a wider range of pathogens more specifically than was once understood. TLRs recognize different types of PAMPs including, lipids, lipoproteins, proteins, and nucleic acids from a variety of different microbes such as bacteria, viruses, parasites and fungi (32). TLRs are either located on the cell surface, where they recognize extracellular PAMPs, or in endosomes, where they recognize PAMPs that have been endocytosed by

the plasma membrane of the cell. The specific cellular location of TLRs is important as different pathogens invade and disrupt cellular processes differently, therefore depending on the location of the TLR and the ligand it binds different downstream signalling transduction and functional outcomes can occur (33).

1.21 Cell Surface TLRs

TLRs can be separated into those that are located on the surface of the cell, embedded in the plasma membrane, and those that are located in endosomes. TLR1, 2, 4, 5 and 6 are all located on the cell surface and tend to recognize microbial membrane components such as lipids, lipoproteins and proteins (34). TLR4 is one of the most widely studied TLRs, as it binds to the gram-negative bacterial outer membrane component LPS which is responsible for septic shock. The TLR4 receptor forms a complex with lymphocyte antigen-96 (MD2), requiring lymphocyte binding protein (LBP) and cluster of differentiation-14 (CD-14) as well, where both aid in the recognition of LPS (35). Once bound to LPS the TLR4 receptor complex recruits adaptor proteins in order to initiate signal transduction. Activation of the TLR4 pathway can initiate early phase activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) where TIR domain containing adaptor protein (TIRAP) and myeloid differentiation primary response 88 (MyD88) are recruited to the TLR4 complex as adaptor proteins and bind to the TIR domain; this in turn elicits the MyD88 dependent pathway that gives rise to inflammatory cytokine production(29,36). However, TLR4 activation can also initiate late phase activation of NF-κB where the TLR4 complex is internalized inside an

endosome, here TIR domain containing adaptor-inducing interferon-β (TRIF) and TRIF related adaptor molecule (TRAM) are the adaptor proteins which bind to TLR4 TIR domain and elicit the IFN and late NF-κB pathway through the MyD88 independent pathway (34,37). Therefore, even though TLR4 mainly recognizes LPS, it can also elicit the IFN pathway to initiate an anti-viral response. Literature indicates that respiratory syncytial virus (RSV) interacts with TLR4 in combination with other receptors (38). Here it has been shown that RSV-F binds TLR4 and CD14 to induce cytokine responses (39). However it is important to note that this finding is controversial, as others have found opposing results (40,41). TLR2 and TLR5 are other surface receptors belonging to the TLR family, which are responsible for the recognition of peptidoglycan and flagellin, respectively.

1.22 Endosomal TLRs

Endosomal TLRs, including TLR 3, 7, 8 and 9, recognize various forms of nucleic acids and therefore are localized in intracellular compartments. Like surface TLRs, intracellular TLRs have extracellular leucine-rich repeats and a cytoplasmic TIR domain that bind adaptor proteins which elicit signal transduction (42).

TLR3 is widely expressed in innate immune cells with the exception of neutrophils and plasmacytoid DC (10). This receptor interacts with double-stranded RNA (dsRNA) which is generated by most viruses during infection (31,43). TLR3 was first discovered in 2001 where it was shown to contribute to type I IFN production in macrophages when activated by the synthetic dsRNA analogue polyinosinic:polycytidylic acid (poly(I:C)) (44).

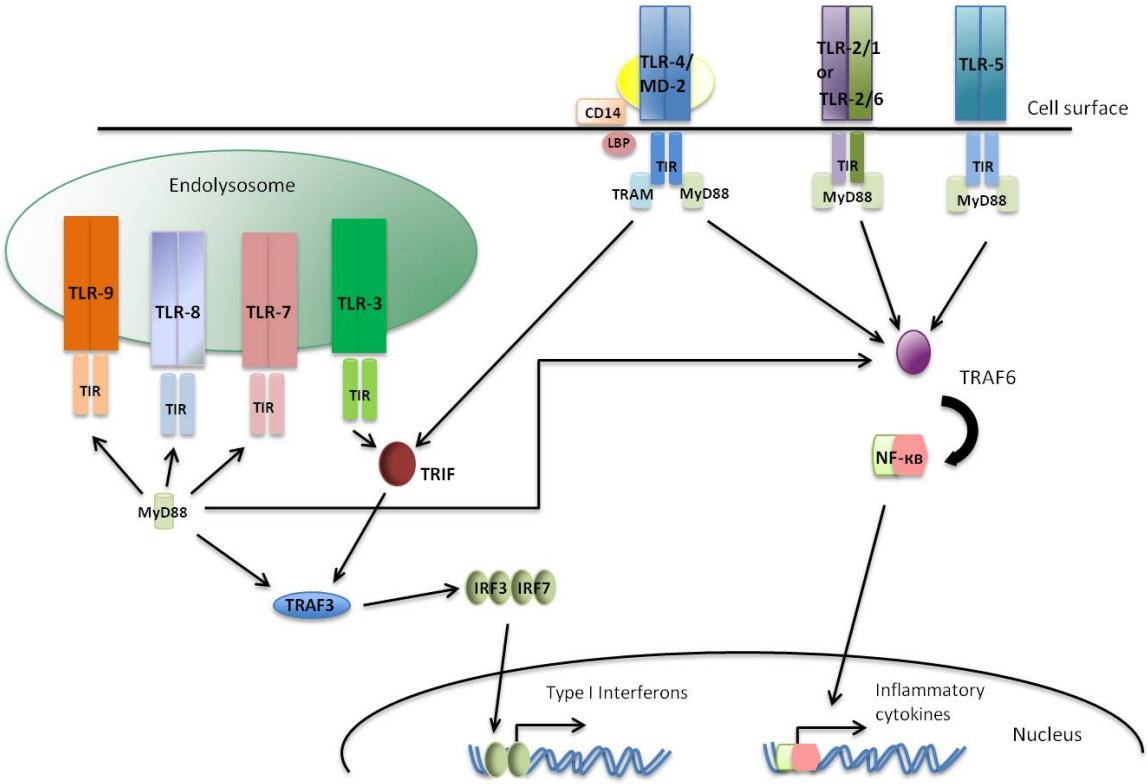
Upon activation, TRIF binds to the TIR domain of TLR3 which activates NF-κB and IFN regulatory factor-3 (IRF3) to produce inflammatory cytokines and type I IFNs respectively (5,34). Recent studies have shown that TLR3 can also bind single-stranded RNA (ssRNA) in the form of polyinosinic acid to elicit an innate immune response and therefore is an important PRR of the innate immune system (45).

ssRNA is recognized by TLR7 and TLR8, which are both endosomal receptors. Prior to activation TLR7/8 resides in the endoplasmic reticulum, after uptake of RNA these receptors are delivered to endolysosomes where they recognize the endocytosed foreign RNA (46). This recognition recruits the adaptor protein MyD88 to interact with the receptor TIR domain, signal transduction leads to the nuclear translocation of NF-κB and IRF7 followed by subsequent production of inflammatory cytokines and type I IFNs (42). Although these receptors are similar in structure and can function together they do have differences. For instance TLR7 is highly expressed on DC whereas TLR8 is highest in monocytes (34). Short dsRNA will sometimes preferentially activate TLR7 as opposed to TLR8 (47), and peripheral blood mononuclear cells (PBMC) stimulated with TLR8 agonist 3M-002 produce more IFN-γ (type II IFN) compared to stimulation with TLR7 agonist 3M-001 (48). It has also been shown that TLR7 activation preferentially increases IFN-α, whereas TLR8 activation increases TNF-α, IL-12p40/70, IL-6 and IL-8, suggesting that TLR8 may be a better activator of NF-κB than TLR7 (49,50). However, these receptors can also respond quite similar, as one study showed that human PBMC stimulated with TLR7 agonist, TLR8 agonist or TLR7/8 agonist, gave similar results in terms of type I and type III IFN production at 24 hours (51). Thus, although there may be differences

between receptors, most agonists target both receptors to a certain degree and therefore when discussing activation researchers tend to group the two receptors together.

1.3 RLRs

The innate immune system recognizes viral nucleic acids through multiple mechanisms, including RLRs and TLRs, often engaging both simultaneously in the presence of a virus to ensure protection (52). RLRs are expressed by most innate immune cells and are found in the cytosol where they recognize viral dsRNA. Monocytes, DC, EC (53), NK cells (reviewed by (54)), neutrophils (55), and even eosinophils (56) have been shown to express RLRs. Retinoic acid inducible gene 1 (RIG-I), melanoma differentiation-associated protein 5 (MDA-5) and laboratory of genetics and physiology 2 (LGP2) are members of the RLR family and are all characterized by the presence of an adenosine triphosphate (ATP) containing DEAD box helicase and therefore are a family of DEx/H box RNA helicases (57). Similarly, all three RLRs contain a C-terminal regulatory domain (RD) that senses dsRNA. RIG-I and MDA-5 also have a caspase activation and recruitment domain (CARD) located at their N-terminus that facilitates downstream signalling, whereas LGP2 does not (58). Upon activation, RIG-I and MDA-5 interact with the adaptor protein mitochondrial antiviral-signalling protein (MAVS), which is bound to mitochondrial or peroxisomal membranes (59). This interaction causes downstream activation of NF-κB and IRF 3 and 7, which leads to the production of inflammatory cytokines and IFNs (reviewed in(60)).



Artwork by Natascha Fitch

Figure 1- Overview: Toll-like receptor activation leads to Type I IFN and pro- and anti-inflammatory cytokine gene transcription

Although RIG- I and MDA-5 have similar structures they tend to recognize different types of dsRNA. RIG-I for instance has been shown to interact with short dsRNA up to 1kb in length, whereas MDA-5 detects longer dsRNA molecules that are over 2kb (61,62). Although RIG-I is most noted for its ability to bind dsRNA, recent studies have shown that RIG-I binds the 5'-triphosphate portion of RNA and thus can also bind ssRNA as well (63). Therefore, it is not surprising that some viruses are recognized primarily by RIG-I (influenza, vesicular stomatitis virus (VSV)), whereas others are recognized by MDA-5 (encephalomyocarditis picornavirus (EMCV)). Several studies have been conducted using the influenza virus, and have showed that knocking-out or silencing RIG-I decreases IFN production thereby increasing viral titers whereas silencing MDA-5 has no effect on IFN production (64,65). Interestingly, some viruses like West Nile are recognized by both receptors indicating that RLRs have some degree of specificity and react differently depending on the virus (66-68).

LGP2, an RLR that does not contain a CARD domain, plays a highly controversial role in the innate immune system. Unlike RIG-I and MDA-5, LGP2 appears to be more of a regulatory receptor for RIG-I. Early culture experiments looking into the functional role of LGP2 suggested that it acted as a negative regulator, because it could bind dsRNA but not activate downstream events and it appeared to inhibit RIG-I activation (69,70). However, a more recent study by Satoh et al, showed that mice lacking LGP2 required this receptor for RIG-I and MDA-5 antiviral response, particularly in response to picornaviridae virus where it was essential for a type I IFN response (71). As other current research has showed that LGP2 can act as a negative regulator in the case of

paramyxoviruses, it is now understood that LGP2 can act as both a positive and negative regulator depending on the virus encountered (52).

Section 2: Vitamin D

2.1 Mechanism of Action

Vitamin D is considered a pro-hormone, more so than a vitamin, whose precursor is located in the skin (72). Upon exposure to sunlight the vitamin D precursor 7-dehydrocholesterol (7-DHC) is cleaved by UV rays into pre-vitamin D_3 which then spontaneous converts into vitamin D_3 (reviewed in (73,74)). This form of vitamin D then enters circulation where it binds to the vitamin D binding protein (DBP), as do other vitamin D metabolites, and travels to the liver. Here vitamin D_3 is hydroxylated by the enzyme cytochrome P450, family 2, subfamily R, polypeptide 1 (CYP2R1) or cytochrome P450, family 27, subfamily A, polypeptide 1 (CYP27A1) to give the inactive form of vitamin D 25(OH)D_3 (as reviewed by (75)). This form of vitamin D then enters circulation where it also binds to DBP, and travels to the kidneys or immune cells where it is hydroxylated for the second time by the enzyme cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27b1) to give the active form of vitamin D 1,25$(OH)_2D_3$, also known as calcitriol (as reviewed by (76)). Once inside the cells the active form of vitamin D binds to the ligand binding domain of the vitamin D receptor (VDR), this induces heterodimerization with the retinoid X receptor (RXR) (as reviewed by (75)). This complex then binds to vitamin D response elements (VDRE) which are specific sequences located in the proximal promoter region of genes; depending on the gene,

the binding of this complex can either recruit activators or repressors to activate or repress gene transcription (as reviewed by (77,78)). For instance IL-10, IL-2 and IL-12B have been shown to have several vitamin D binding sites located within their genes which can lead to their enhancement or reduction in the presence of exogenous $1,25(OH)_2D_3$ (79,80). Carlberg et al, performed a recent genome wide analysis of the VDR showing that monocytes alone had 2340 VDR binding sites and that only 32% of these sites are the typical DR3 type response element (81).

2.2 Vitamin D is Regulated by Several Vitamin D Associated Proteins

2.21 CYP24a1 and CYP27b1

Interestingly, vitamin D has a variety of ways to regulate itself to ensure that it is not being overly produced. One such way is through the regulation of the active form of vitamin D $1,25(OH)_2D_3$ by the enzyme CYP27b1 and cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24a1). CYP27b1 is expressed in cells from many different tissues as well as the kidneys (82). Its function is to convert $25(OH)D_3$ into $1,25(OH)_2D_3$. Although it has been shown that *in vitro*, a variety of cells can locally convert $25(OH)D_3$ to $1,25(OH)_2D_3$ by way of CYP27b1, the relevance of these events *in vivo* remains uncertain (83). To date, only the placenta and macrophages in a disease-activated state have been shown to produce enough CYP27b1 gene products to contribute to circulating $1,25(OH)_2D_3$ levels (84). Therefore, the purpose of CYP27b1 is to regulate the amount of $1,25(OH)_2D_3$ that is produced, and only in a highly stimulated environment is this enough to mediate an innate immune response (84). CYP27b1 is positively

regulated by calcium, parathyroid hormone (PTH), calcitonin, growth hormone, insulin-like growth factor, and in macrophages by immunoactivator stimulation, whereas it is negatively regulated by phosphate, fibroblast growth factor-23 (FGF-23) and $1,25(OH)_2D_3$ (reviewed in (72)). Therefore, CYP27b1 converts the inactive form of vitamin D to active, and is itself highly regulated to ensure overproduction of $1,25(OH)_2D_3$ does not occur.

The enzyme CYP24a1, on the other hand, is produced in the mitochondria and converts the active ($1,25(OH)_2D_3$) form of vitamin D to $24,25(OH)_2D_3$ or $1,24,25(OH)_2D_3$ which are then catabolized into other vitamin D products (76,85). It can also convert the inactive form ($25(OH)D_3$) into 24-hydroxylated products and is therefore important in vitamin D degradation (86). CYP24a1 is often referred to as the vitamin D target gene 24-hydroxylase because it is a measure of vitamin D activity, the more $1,25(OH)_2D_3$ in a given system the more CYP24a1 expressed (87). This was confirmed when analysis of the CYP24a1 gene revealed a VDRE in the upstream promoter suggesting that CYP24a1 protects the cell from excessive vitamin D (86). Masuda et al found that mice deficient in CYP24a1 had increased $1,25(OH)_2D_3$ and $25(OH)D_3$ levels compared to CYP24a1 sufficient mice (88). Therefore, this enzyme works as a way to regulate the amount of free floating $1,25(OH)_2D_3$.

2.22 Vitamin D Binding Protein

DBP is a protein belonging to the albumin, α-fetoprotein, α-albumin/afamin gene family encoded on chromosome 4 (89). Many different tissues express DBP; however,

the majority of it is expressed and secreted by the liver (90). DBP binds to various plasma vitamin D metabolites and brings them to tissues such as the liver, kidneys or immune cells (91). There are three major genetic variations of DBP (GC1F, GC1S and GC2) and interestingly distribution among different ethnic groups is apparent (92). GCIF is the most abundant form and is found in those of African descent, whereas GCIS and GC2 dominate the Asian and European populations with very few African ethnic groups having the GC2 genotype (53). These three DBP variants exhibit different affinities for $1,25(OH)_2D_3$ and $25(OH)D_3$, and are also found in human serum at different abundances; for instance GCIF has a high affinity for $1,25(OH)_2D_3$ /$25(OH)D_3$ and is found in high abundance in human sera, whereas GC2 has a low affinity for vitamin D metabolites and is found in relatively low levels in serum (93). Chun et al showed that knocking out DBP resulted in increased cathelicidin production in $1,25(OH)_2D_3$ and $25(OH)D_3$ treated monocytes and that treatment of these cells with exogenous DBP caused a decrease in $1,25(OH)_2D_3$ -and $25(OH)D_3$- induced cathelicidin and CYP24a1 levels (94). Therefore it is thought that individuals who live in the north have low affinity GC1S and GC2 forms thereby allowing vitamin D to float freely, whereas individuals who have darker skin have the GC1F form of DBP and thus have less free floating vitamin D (93). This form of regulation allows for individuals who are less exposed to sunlight (northern) to get more $25(OH)D_3$ and $1,25(OH)_2D_3$, whereas individuals who are continuously exposed to sunlight get less. Thus, DBP helps to regulate the amount of inactive/active vitamin D that is getting to tissues and may be one of the reasons for the difference in vitamin D nutritional status amongst different ethnicities.

2.23 Vitamin D Receptor and Vitamin D Response Elements

$1,25(OH)_2D_3$ bound with VDR and RXR binds to VDRE and influences gene expression in thousands of genes (78,95). Some of these genes include those that encode for cytokines, chemokines, transcription factor or enzymes involved in signalling pathways and therefore active vitamin D can modulate an immune response by influencing the level of gene expression (91). However there are other genes that the VDR complex influences which can directly deactivate $1,25(OH)_2D_3$ such as CYP24a1. Wu et al found that wild-type mice infected with *Salmonella typhimurium* had increased VDR protein compared to wild-type mice treated with control. Additionally they also found that CaCO2 BBE cells treated with $1,25(OH)_2D_3$ had increased VDR as well (96). These results indicate that $1,25(OH)_2D_3$ has mechanisms to regulate itself, thereby ensuring that over responsiveness does not occur.

2.24 PTH, Calcium and FGF23

PTH is a hormone secreted by the parathyroid gland in response to a decrease in blood ionized calcium. In bone PTH causes bone resorption, which indirectly causes a release of calcium. However, in the kidney PTH promotes calcium retention and inhibits phosphate reabsorption in kidney tubules, thereby promoting the formation of active vitamin D (97). PTH increases CYP27b1 expression and therefore favours the formation of active vitamin D (98). Although PTH favours active vitamin D formation, $1,25(OH)_2D_3$ and other vitamin D metabolites actually reduce PTH expression (99-101). Here studies have shown that deficient levels of serum vitamin D ($25(OH)D_3$) correlate with high

levels of PTH whereas sufficient serum vitamin D levels correlate with lower serum PTH (102). Thereby indicating that vitamin D, calcium and PTH are in balance with one another.

FGF-23 is a protein that promotes renal phosphate secretion. $1,25(OH)_2D_3$ has been shown to increase FGF-23 in bone, however FGF-23 suppresses CYP27b1 and promotes the expression of CYP24a1 thereby regulating the amount of active vitamin D being produced (91,103).

2.3 Vitamin D and Its Impact on Innate Immunity

Vitamin D deficiency has been known as the cause of childhood rickets for more than two century (72); however its potential as an immune regulator was not seriously considered until a paper was published in 1983. Here Provvedini et al discovered a receptor in PBMC which bound ^3H-$1,25(OH)_2D_3$ with high affinity and specificity (104). It was the discovery of this receptor, known as VDR, in immune cells that spawned the question of the importance of vitamin D in immunity. Although the knowledge of VDR in immune cells was a breakthrough in the field of vitamin D, little was understood regarding the implications of this finding.

2.31 Vitamin D and Antimicrobial Peptide Production

In 2006 a group in Los Angeles found that monocytes activated through TLR2/1 resulted in upregulation of VDR and CYP27b1, which in turn led to an increase in the sole human cathelicidin AMP, LL-37, when cells were stimulated with $1,25(OH)_2D_3$ (105). A similar study a few years later showed that although hCAP, the gene that encodes for

the pre-pro-protein of cathelicidin LL-37, was not correlated with circulating vitamin D levels, individuals who were deficient in vitamin D had reduced hCAP expression after TLR2/1 challenge (106). These results were particularly promising for the treatment of *M. tuberculosis* as this bacteria activates TLR2/1 pathway. Apart from monocytes other cells such as EC (107-109) and keratinocytes (110,111) also show an increase in LL-37 production in the presence of exogenous vitamin D. Evidence exists that this type of response occurs in neutrophils as well, however the significance of this observation is yet to be determined as there is no evidence these cells express CYP27b1 (111). These findings indicated that circulating vitamin D is important in immunological responses to bacterial ligand stimulation in multiple innate immune cells. As individuals who are deficient in vitamin D are not able to mount as large of an AMP response as those who are sufficient in vitamin D, lots of emphasis in the field of vitamin D has been directed towards understanding the role vitamin D plays in the production of AMP.

2.32 Vitamin D and Cytokine Production

Apart from its effect on AMP production, vitamin D has been shown to alter cytokine and chemokine production patterns under PRR activation. Treatment of cells with *M. tuberculosis* or LPS and $1,25(OH)_2D_3$ decreases IL-6, CXCL-8 (112-114), TNF-α (114-116), IL-12p40 and IFN-γ levels in healthy adults (113). $1,25(OH)_2D_3$ can reduce matrix metalloproteinases (MMP-7, MMP-9) in antigen activated PBMC from healthy adults as well (117). Interestingly, the effect of vitamin D on IL-10 cytokine production has been an issue of debate. Here several publications have shown that antigen stimulated cells produce more IL-10 in the presence of $1,25(OH)_2D_3$ (118,119), whereas

others indicate a decrease in IL-10 (113). This contradiction in data may be due to differences in kinetics, as a study by Matilainen et al found that IL-10 expression decreased in the first 24 hours and then began to increase (79,80). Differences in cell models is another possible explanation as another study found an increase in IL-10 production in the presence of $1,25(OH)_2D_3$ when cells were stimulated with beads and a decrease in IL-10 when using monocytes (120). According to these results $1,25(OH)_2D_3$ has immunosuppressive properties and therefore may be beneficial in the treatment of inflammatory diseases. However, it is important to note that not all cells act the same in the presence of $1,25(OH)_2D_3$, as Gambhir et al found that DC only suppressed pro-inflammatory cytokine production when they were pre-treated with $1,25(OH)_2D_3$ (121).

Thus, as each cell type responds to vitamin D differently it is important to look at how PBMC (ie the circulating immune repertoire as a whole) respond to exogenous vitamin D. B cells, T cells, NK cells, NK T cells, monocytes and multiple DC subsets are all found in PBMC, each at different concentrations in different individuals. Each cell subset may respond differently to vitamin D. As our goal is to examine the <u>*net*</u> responsiveness of an individual's repertoire to exogenous vitamin D supplementation, and to control for the degree of variation that exists within clinically homogeneous (healthy/asymptomatic) but inherently diverse human populations, we focussed on examination of the repertoire as a whole—whole PBMC. We also note that while cell lines can be an invaluable tool, it is well recognized that it is important to examine the affects of vitamin D on primary cells, as cell lines (ie transformed cells) can often give different results than do fresh cells isolated directly *ex vivo*.

2.33 Vitamin D Influences Intracellular Signalling Pathways: From Receptor Expression to Transcription Factor Function

Besides its ability to alter the extracellular environment through AMP and cytokine production, vitamin D has been shown to alter various PRRs, costimulatory and antigen presenting abilities, as well as intracellular enzyme/transcription factor functioning. In terms of PRR expression, $1,25(OH)_2D_3$has been shown to reduce TLR4 and TLR2 expression in PBMC (122,123), and decrease TLR9 in monocytes (124). Although this apparent decrease seems widespread amongst PRRs, other TLRs such as TLR3 remain unchanged in the presence of $1,25(OH)_2D_3$ (124) and others still such as nucleotide-binding oligomerization domain-containing protein 2 (NOD2) are increased in the presence of $1,25(OH)_2D_3$ (111). Equils et al conducted a similar study looking at the effects of vitamin D and LPS on endothelial cells and found that although vitamin D inhibited IL-6 and CXCL-8 cytokine levels, it was unable to alter TLR4 expression (125) suggesting that the function of vitamin D differs depending on the phenotype of cell.

In terms of costimulatory receptors $1,25(OH)_2D_3$ has been shown to reduce CD80/86 (119), but increase CD14 levels (124,126) in monocytes. Other important antigen receptors including dectin-1 and mannose receptor expression were also found to be reduced on PBMC after active vitamin D treatment (114). Even more interesting is the fact that $1,25(OH)_2D_3$ has been shown to directly affect the expression of intracellular enzymes such as inducible nitric oxide synthase (iNOS) (127) and mitogen activated protein kinase (MAPK) phosphate 1 (116), which contribute to many important cellular processes. As MAPK phosphatase-1 (MKP-1) is part of the MAP Kinase

pathway, it was hypothesized by Zhang et al that $1,25(OH)_2D_3$ was suppressing pro-inflammatory cytokine production through its interaction with this molecule. Their studies led to the discovery of a potential VDRE close to the MKP-1 gene and further studies in MKP-1 deficient mice showed that treatment of LPS stimulated MKP-1 deficient cells with vitamin D did not lead to pro-inflammatory cytokine suppression like the wild-type cells (116). Although these results bore significance others have shown that cells stimulated with $1,25(OH)_2D_3$ have increased NF-κB inhibitor-α (IκBα) expression compared to control cells, claiming that enhancing this inhibitor prevents NF-κB from translocating to the nucleus, therefore reducing transcription of these inflammatory genes (128). Still others have shown that the vitamin D receptor can actually bind to the p65 subunit of NF-κB and prevent it from translocating to the nucleus in epithelial cells (96) and that the p105 and p50 subunits are decreased in the presence of $1,25(OH)_2D_3$ in B cells (129). All these results, summarized in **Figure 2**, confirm that vitamin D is a complex hormone capable of altering many innate immunological pathways.

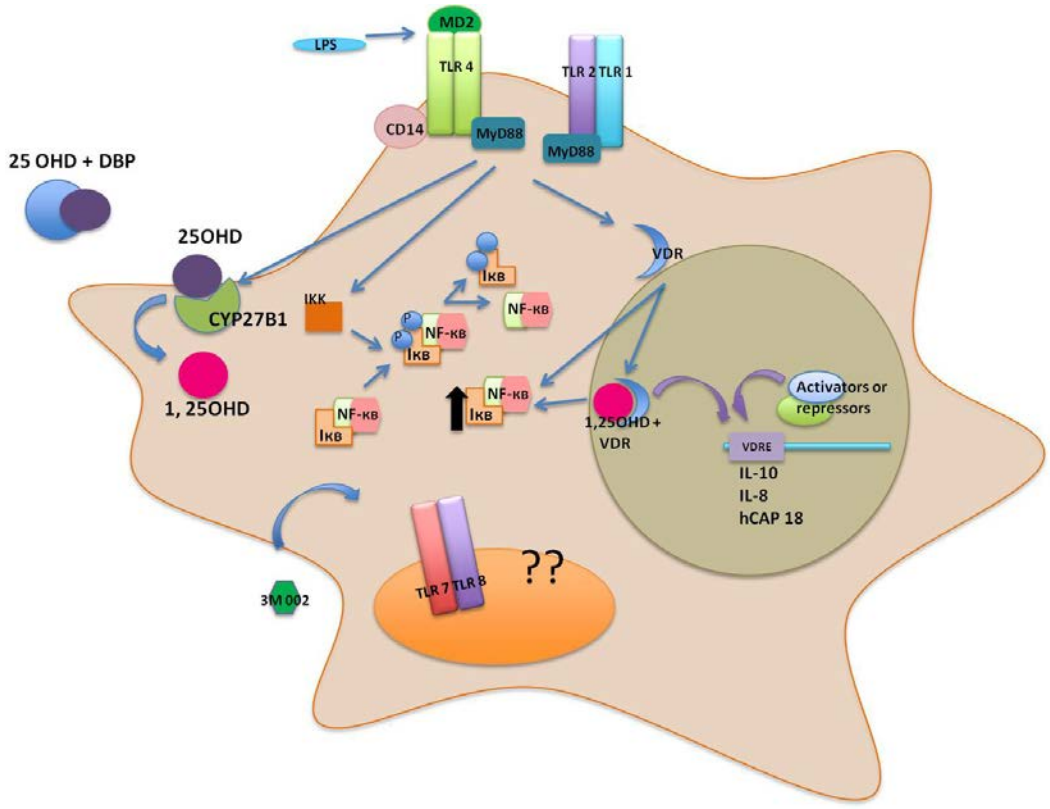
2.4 Vitamin D and Adaptive Immunity

Although Vitamin D is highly involved in regulating innate immune responses, it has also been shown to exert its properties on adaptive functions as well. One of the earlier reports linking vitamin D with the adaptive immune system showed that $1,25(OH)_2D_3$ inhibits T-cell proliferation (130,131). Further studies showed that T-cells only express VDR when activated and addition of $1,25(OH)_2D_3$ to these activated cells

results in downregulation of IL-2 expression (132). Since these studies it has become clear that vitamin D regulates many different cytokines and acts on subsets of T-cells differently. Here studies have indicated that vitamin D can inhibit Th1 cytokines, such as IFN-γ, while increasing T helper 2 (Th2) cytokines, such as IL-5, IL-4 and IL-13 (133,134). However, the Th1:Th2 observation in terms of vitamin D is controversial as others have shown that PBMC stimulated with *M. Tuberculosis* have reduced IL-5 production in the presence of vitamin D (113). Further studies in mice show similar results that ovalbumin (OVA) sensitized mice receiving vitamin D treatment have reduced IL-5 and IL-13 production (135,136).

Interestingly other T cell helper subsets, such as T helper cell 17 (Th17) and regulatory T cells (Tregs) are also affected by the presence of vitamin D. Here studies have shown that vitamin D can inhibit RAR-related orphan receptor gamma of thymus (RORyt), IL-17A, IL-17F in CD4+ T cells thereby impairing their development into Th17 cells (137-139). In terms of Tregs, studies have shown that $1,25(OH)_2D_3$ enhances the production of IL-10 producing Tregs (140-142). Forkhead box p3 (Foxp3), cytotoxic T-lymphocyte antigen 4 (CTLA4) and transforming growth factor-β (TGF-β), molecules associated with Tregs, are also increased in CD4+ T cells that have been stimulated with exogenous vitamin D (118,120). Little information exists on the effect of active vitamin D on CD8+ cytotoxic T cells and B cells. That said, a recent study showed that CD8+ T cells from healthy subjects showed decreased IFN-γ and TNF-α production in the presence of $1,25(OH)_2D_3$ (143). Moreover, B cell studies indicate that vitamin D induces their IL-10

Vitamin D and monocyte immune response



Artwork by Natascha Fitch

Figure 2- Overview: Vitamin D interacts with downstream signalling molecules to alter gene expression.

production and that in the presence of a stimulus VDR production is enhanced and NF-κB phosphorylation reduced (129,144). Thus, vitamin D displays immunomodulatory effects on CD8+ T cells and B cells in addition to its numerous effects on CD4+ T cells.

Section 3: Vitamin D and Disease

3.1 Vitamin D Deficiency in the Population

As the active form of vitamin D has a very short half-life of 4-6 hours, the inactive form with a half-life of a couple of weeks is generally the form that is used to measure vitamin D nutritional status (145). Until recently, vitamin D sufficiency was determined simply by the presence or absence of rickets; however observations by a researcher in the late 1990s showed that serum $25(OH)D_3$ inversely correlates with PTH up to 75nM (146). Therefore, vitamin D sufficiency is now defined as $25(OH)D_3$ levels exceeding 75nM or 30 ng/mL, thereby stating that insufficient vitamin D levels are those below 75 nM and deficient levels are those lower than 50nM of $25(OH)D_3$ (75,147).

According to the Third National Health and Nutrition Examination Survey (NHANES III), which examined over 23,000 individuals between the years of 1988-1994 living in the United States, $25(OH)D_3$ levels are slightly higher in men (78 nM) than women (73 nM) and are lowest in non-Hispanic blacks (49 nM) and highest in non-Hispanic whites (79 nM). Moreover, this survey found that vitamin D levels decrease with age showing that individuals between the ages of 20-29 tend to have vitamin D levels near 81 nM whereas those over 70 have levels closer to 67 nM (148).

Similar studies to NHANES, but on a smaller scale, have been conducted recently to determine serum vitamin D levels among different ages focusing on early development. Here it was observed that teens and adults have similar $25(OH)D_3$ levels and that adolescent males have increased levels over females and non-Hispanic Blacks have lower levels than non-Hispanic whites (149). Children between the ages of 2 and 13 were also found to have similar vitamin D levels to that of teens and adults; however, it was observed that older children 9-13 tend to have higher rates of vitamin D deficiency than that of younger children (150). Interestingly studies have shown that the average infant is born with sufficient vitamin D serum levels and that this same observation is apparent at 4 months as well. However, deficiency rates differ between birth and 4 months post-birth with a larger proportion of newborns experiencing vitamin D deficiency versus those at 4 months of age (151).

There has been a large focus as of late on supplementing people with vitamin D_3 or $25(OH)D_3$, both of which are metabolites in the vitamin D pathway, to determine whether the incidence of vitamin D deficiency can change with supplementation. Here some data indicates that taking vitamin D_3 does not augment $25(OH)D_3$ levels (152). However, others have shown that supplementing elderly individuals with vitamin D_3 does increase $25(OH)D_3$, yet supplementing with $25(OH)D_3$ directly is much more potent at increasing serum $25(OH)D_3$ levels than the former (153,154). In terms of newborns, it was shown that infants supplemented with vitamin D via formula or a dropper had lower vitamin D deficiency rates compared to those that were not supplemented (151),

indicating that vitamin D supplementation may be necessary in those with deficient vitamin D levels.

As the body requires sunlight to cleave 7-DHC to pre-vitamin D_3, it is not surprising that circulating vitamin D levels within the population undergo seasonal variation. Here several studies have shown that 25(OH)D_3 levels are lowest during the winter months, followed by increased levels during the spring and autumn months peaking during the summer (114,115,149,155,156). According to literature taking vitamin D_3 supplementation during the winter months reduces the rate of vitamin D deficiency and insufficiency (157,158).

Unfortunately there remains a gap in literature with regards to the amount of vitamin D actually being used in these individuals. As the inactive form of vitamin D must be converted to the active form to be of use it is difficult to link together the importance of having high serum 25(OH)D_3 levels in terms of an innate immune response. This may be the reason for the inconsistency between supplementation and immune responsiveness. Therefore, looking at the direct impact of exogenous vitamin D on innate immune cells is of utter importance, as it is with this knowledge that we can bridge the gap between circulating inactive vitamin D levels and the ability to amount an immune response.

3.2 Vitamin D Deficiency and Risk of Respiratory Tract Infections

3.21 Epidemiological Evidence

According to literature a large degree of discrepancy exists between the occurrence of viral infections within the population and the prevalence of vitamin D deficiency. Here some epidemiological data suggests that circulating vitamin D levels negatively correlate with the prevalence of respiratory infections (159-162). However, these results are not very convincing as some show only weak significance and others lack significance altogether (163-165). Influenza based studies have shown similar results in that vitamin D supplementation appears to reduce the risk of Influenza A, however when this was examined with influenza B no correlation was observed (166).

Recent emphasis has been placed on the role vitamin D plays in chronic viral infections such as HIV and hepatitis C virus (HCV). Here research indicates that women with CD4+ T cell counts below that of 500 are more likely to have vitamin D deficiency, however no difference in mean 25(OH)D levels were observed between HIV negative and HIV positive women (167,168). In terms of HCV infection, current research shows that HCV positive individuals have lower active and circulating vitamin D levels than healthy adults. Interestingly, HCV infected patients with higher active vitamin D levels have lower viral loads and Th17 cytokines than individuals lower vitamin D status (169,170).

Unfortunately these epidemiological results are inconclusive and do not promote or disprove a connection between vitamin D deficiency and increased viral infections. As viral infections can cause serious infectious diseases or respiratory problems it is pivotal we understand the role vitamin D plays in the innate immune system especially in terms of viral infections. Therefore, we have identified the need to focus on the functional outcomes of adding exogenous vitamin D to innate immune cells, particularly in the presence of viral activation.

3.22 Vitamin D Lowers Risk of Infection by Respiratory Syncytial Virus

RSV is the leading cause of respiratory illness in young adults and infants worldwide (171). Several studies on cord circulating 25(OH)D_3 levels have shown that infants with deficient vitamin D levels (below that of 50 nM) have a much higher risk of contracting RSV than infants with sufficient levels (172,173).

RSV is a virus recognized by PRRs located on DC, macrophages, EC, eosinophils and neutrophils. As this virus is ssRNA negative sense both single stranded and double stranded products are formed, thereby allow the virus to interact with a larger portion of the innate immune system (reviewed by (174)). Here studies have shown that RSV interacts with a variety of TLRs including TLR2 and TLR4 (38,175-177). Polymorphisms within these receptors may increase susceptibility to RSV infection in infants and adults (178-180). PRR engagement with RSV results in the upregulation of a variety of pro-inflammatory and anti-inflammatory cytokine and chemokines, including IFN-γ, IL-6, CCL-5, IL-1B and IL-10 (177,181-183). Besides TLRs, RSV in combination with muramyl

dipeptide (MDP) has been shown to induce a type I IFN response greater than if RSV were infecting cells alone (184).

Studies using cord blood indicate that RSV infected infants express less TLR8 and TNF-α than healthy controls and that this decrease is most severe during acute infection as opposed to convalescent (185). Murine neonatal models further this notion by claiming that IFN-γ is downregulated in early life, here studies have shown that pretreating mice with cytosine-phosphate-guanine (CpG) or IFN-γ increases their IFN-γ response thereby decreasing viral infection. Interestingly these studies also show that pretreatment with IL-4 dampens IFN-γ thereby worsening RSV infection, but that pretreating with CpG decreases IL-4 and IL-5 (186,187).

As EC are the first cells activated upon viral infections, it is not surprising that these cells have been shown to convert the inactive form of vitamin D to active (188). Furthermore, addition of exogenous active vitamin D has been shown to decrease cytokines and chemokines in EC activated by RSV while maintaining normal levels of these cytokines when stimulated by the IFN pathway (128). In addition, other studies have shown that EC can produce AMPs (189). Here cells infected with RSV have been shown to increase human beta defensin-2 (HBD-2), which in turn reduces RSV titers (190). Studies on vitamin D show that addition of active or inactive vitamin D to culture increases AMP production by immune cells (refer to section 2.31 for further explanation) thereby reducing RSV infection.

Although the *in vitro* cell culture literature suggests a positive role for vitamin D in the prevention and treatment of viral infections the *in vivo* data is less convincing. Here studies using calves infected with RSV show higher pro-inflammatory cytokine levels with higher vitamin D circulating concentrations rather than lower (127,191). Thus, these varying results in literature need to be addressed, and further research is required to help understand these discrepancies. As several reports indicate that the severity of RSV infection in children and adults may be linked to polymorphisms in the VDR, it is important we understand the immunomodulatory properties of vitamin D in order to best treat those needed (192,193).

3.3 Vitamin D Deficiency and Increased Risk of Autoimmunity

3.21 Asthma and Allergic Disease

Epidemiological evidence concerning vitamin D deficiency and the prevalence of asthma has contradictory results. Some studies show that children with asthma have a higher prevalence of vitamin D deficiency and insufficiency compared to healthy children (194,195). Although vitamin D levels in these individuals are inversely correlated with total immunoglobulin (Ig) E, eosinophil count and in some cases forced expiratory volume in 1 second/forced vital capacity (FEV_1/FVC) (196,197), adult studies show no difference between asthmatics and non-asthmatics vitamin D levels (198).

As maternal 25(OH)D_3 status is strongly correlated to that of the neonate (199), the association between sufficient maternal vitamin D status during pregnancy and the risk of asthma in the neonate is of debate. Here epidemiological data indicates an

inverse correlation between 25(OH)D levels in infants or mothers and decreased respiratory tract infections in neonates at 3 months (200,201). Although similar studies find that 25(OH)D_3 levels in infants are inversely correlated with risk of wheeze (201), maternal vitamin D status shows no association with risk of infant wheeze and no general association with asthma development (200,202).

Many asthmatic individuals take corticosteroids in order to control exacerbations, studies focusing on this area of treatment have shown improved lung function and provocative concentration causing a 20% drop in FEV_1 (PC20) values with higher 25(OH)D_3 levels in corticoid treated patients (203,204). Th2 cytokines are also lowered in vitamin D treated patients compared to those treated with cortisol alone (205,206).

Murine models have shown insight into the asthma-vitamin D debate. Here OVA sensitized mouse models indicate that vitamin D supplementation prior to OVA challenge results in decreased Th2 cytokines, decreased eosinophil count, decreased airway hyperresponsiveness (AHR) to methacholine challenge and increased IL-10 production (135,136,207).

As asthma is one of the leading childhood illnesses worldwide it is necessary to develop better methods for disease control, especially with 5-10 percent of asthmatic individuals being steroid-resistant (208). Although the general consensus in literature is that vitamin D supplementation reduces asthma severity (209), others argue that vitamin D may worsen the disease state (210).The discrepancy in literature is in part the result of a lack of research, as little focus has been directed towards freshly isolated

human asthmatic cells and exogenous supplementation of $1,25(OH)_2D_3$. This aspect is crucial, as murine models are of a different species and epidemiological models focus on inactive vitamin D supplementation. Interestingly, vitamin D levels have been shown to be significantly lower in severe therapy-resistant asthmatic patients when compared to moderate asthmatic individuals (211). Therefore, the phenotype of the patients may be extremely important when considering the benefits of vitamin D supplementation as well.

3.22 Crohn's Disease and Diabetes

Crohn's disease (CD) and diabetes are two autoimmune diseases that have been associated with vitamin D deficiency. Studies on serum vitamin D levels have shown that individuals with CD and type II diabetes have high rates of vitamin D deficiency (212,213). As diabetes is correlated with body mass index (BMI) and Crohn's disease with AMP, interest has increased regarding whether these diseases have a link to vitamin D deficiency.

Serum cytokine levels in CD patients show that vitamin D insufficiency correlates with lower IL-10. However, supplementation with vitamin D appears less conclusive, as some report altered cytokine levels during therapy and others report none (214-217). Studies as of late have focused more intently on the connection between NOD2 mutations in CD patients and how this impacts AMP production rather than cytokine production. Here studies show that stimulation of cells from CD patients with vitamin D

and MDP enhances AMP production when compared to cells stimulated with MDP alone (111).

Individuals with diabetes have increased pro-inflammatory cytokine levels *in vitro* upon stimulation with PRR as compared to healthy controls, and addition of exogenous vitamin D has been shown to decreased these levels to those of healthy adults (218). As type 2 diabetes is thought to be linked with obesity, the affects of vitamin D supplementation on overweight individuals is of great interest. Here studies remain inconclusive, as supplementation in overweight individuals did not show alterations in cytokine production (219,220), yet studies on adipocytes indicate that vitamin D decreases inflammation (221).

Thesis Overview

Population health studies demonstrate that insufficient/deficient vitamin D status is strongly associated with increased upper respiratory tract infections, such as influenza, RSV and the common cold. As vitamin D deficiency is widespread in Canada, it is imperative we understand the role vitamin D plays in innate immune responses to viral infections. Current studies focus on the epidemiological link between vitamin D nutritional status and viral infection prevalence, however, beyond its important roles in AMP regulation, little is understood about the effects of exogenous vitamin D on innate immune responsiveness in the presence of cell activation, and upon stimulation by viral ligands in particular. Therefore, this study, with specific focus on immunoregulatory cytokines and chemokines, focuses on two types of PRRs, TLRs and RLRs, which

recognize viral ligands, with the aim of determining whether exogenous vitamin D has an impact on viral driven innate immune responsiveness.

It is well established that exogenous active vitamin D improves innate immune responses to *M. tuberculosis* infection by decreasing pro-inflammatory cytokine production and increasing anti-inflammatory and AMP production. Therefore, the objectives of this research were:

1) To determine whether exogenous $1,25(OH)_2D_3$ is able to alter viral driven innate immune responses in a similar manner to that of bacterial (ie. LPS and *M. tuberculosis*).
2) Compare the ability for exogenous $1,25(OH)_2D_3$ to modulate cytokine production in neonates versus adults
3) Understand whether endogenous $25(OH)D_3$ levels are able to predict innate immune cytokine responsiveness
4) To determine whether exogenous vitamin D is able to modulate innate immune responses to RLR activation
5) Examine vitamin D supplementation in multiple cell types to better understand the overall potential for vitamin D to regulate innate immune responses to viral infections

Materials and Methods

Volunteers

This study was approved by the University of Manitoba, Biomedical Research Ethics Board for the use of human samples in research. In collaboration with Dr. A Becker and his team, written informed consent was obtained from adult participants, or consent was given by parents of those under 18 years of age, who were recruited for the Canadian Healthy Infant Longitudinal Development (CHILD) study. This nation-wide cohort was established for the purpose of studying normal immune development in Canadian babies in the hopes of determining some of the many factors that control development of allergies and asthma in childhood. Adolescents enrolled in the Canadian Asthma and Allergy Prevention Program (CAPPS) were also recruited for the purpose of this thesis. All participants recruited for this thesis work were in good health and did not have a prior history of allergy or asthma.

Table 1. Study participant information

Subject Types	Ages (yrs)	Number of Females	Number of Males	Total Number of Subjects
Seniors	60+	NA (not available)	NA	7
Adults	18+	84	48	132
Teens	16-18	24	31	55
Children	1-3	19	14	33
Cords	Full term neonates	12	10	22

Peripheral Blood Mononuclear Cell (PBMC) Isolation

20-30 mL of peripheral blood was obtained from healthy adults. Whole blood was centrifuged at 500g for 10 minutes to separate plasma from cells. Blood samples were then diluted 1:1 with 0.85% saline solution and layered onto Ficoll-Paque^{TM}PLUS (GE-Healthcare Bio Sciences, Uppsala, Sweden). Samples were then centrifuged for 30 minutes at 600g to allow separation of PBMC from red blood cells and granulocytes. PBMC, which forms a "buffy" coat layer between the remaining plasma and the Ficoll, were removed using a pasture pipette and resuspended in 0.85% saline. Isolated PBMC were then centrifuged at 250g for 10 minutes to wash the cell pellet. A repeat saline wash was then performed to ensure no residual contaminants. Isolated PBMC were resuspended in 2 mL of culture media (CM) (Roswell Park Memorial Institute (RPMI)-1640 medium, 10% fetal calf serum, 1% 200 mM L-glutamine, 1% antibiotic-antimycotic and 55 µM 2mercaptoethanol). Cells were counted using a BioRad TC10 automated cell counter to determine cell concentration.

Preparation of Tissue Culture Reagents

Preparation of 1,25(OH)$_2$D$_3$

50 µg of 1α,25-dihydroxyvitamin D_3 (Enzo Life Sciences, Plymouth Pennsylvania) was resuspended in 1 mL of ethanol. 50 µL of the 50 µg/mL solution of 1α,25-dihydroxyvitamin D_3 was used to test the sensitivity of this molecule to light. Here 50 µL of ethanol was added to 2 wells and 50 µL of 1,25$(OH)_2D_3$ was added to a third well, the absorbance was then measured at 264 nm. The samples were measured after 10, 20, 30

and 40 minutes exposure to dim light at room temperature. At 0 minutes and 10 minutes the absorbance of $1,25(OH)_2D_3$ was 0.098, when measured again at 20 minutes the absorbance dropped to 0.077 **Appendix A**.

The 50 µL/mL solution of $1,25(OH)_2D_3$ was diluted in CM to give $1,25(OH)_2D_3$ at 9.0×10^{-9} M, 9.0×10^{-8} M and 9.0×10^{-7} M. Aliquots of 1 mL were then made for each of the three concentration of $1,25(OH)_2D_3$ to avoid repeated freeze-thaw cycles and stored in the -80°C freezer until required.

Preparation of $25(OH)D_3$

1 mg of 25-hydroxyvitamin D (Enzo Life Sciences, Plymouth Pennsylvania) was dissolved in 1 mL of 100% ethanol to give a concentration of 1 mg/mL. 50 µL of the 1 mg/mL solution was used to test the sensitivity of this molecule to light. Here 50 µL of ethanol was added to two wells and 50 µL of $25(OH)D_3$ vitamin D was added to the third well, the results were measured at 265 nm. The samples were measured after 10, 20, and 30 minutes exposure to dim light at room temperature. Here it was noticed that there was a small change in absorbance within the first 20 minutes exposure to light, which shows that the $25(OH)D_3$ is not as sensitive to light as the $1,25(OH)_2D_3$.

Once the experiment was complete the 1 mg/mL of $25(OH)D_3$ was diluted in CM to give three concentrations of $25(OH)D_3$, 9.0×10^{-8} M, 9.0×10^{-7} M and 9.0×10^{-6} M. Aliquots were stored as previously mentioned.

Preparation of Respiratory Syncytial Virus and Reovirus

RSV strain Long was provided by Dr. Renee Douville of the University of Manitoba. Briefly, virus was cultured on HEp-2 cells and incubated at 37°C for a maximum of 21 days. RSV strain was then titrated by the quantal assay tissue culture infectious dose ($TCID_{50}$) and live virus was used in tissue culture as described (179). Viral titers of $10^{4.9}TCID_{50}$/mL were used and all virus samples were only thawed once.

Reovirus strain type 1 Lang (TIL) was also provided by Dr. Renee Douville. Briefly, viral cultures were grown in L929 cultures as described (222). Aliquots of the virus were then inactivated and titers determined by plaque assay. Virus stocks were aliquotted at a concentration of 2 $x10^7$ pfu/mL for tissue culture use.

Primary Cell Culture

Isolated PBMC were plated at 350,000 cells per well in 96 well U-bottom plates in triplicate **Table 1**. Cultures were stimulated with CM, 3.6 ng/mL LPS, 1.75 ug/mL 3M 002, 250 ng/mL Poly (I:C)/Lyovec, long strain RSV or TIL Reovirus with/without 1, 10 or 100 nM $1,25(OH)_2D_3$ **Table 2**. RSV was used at a concentration of $10^{4.9}TCID_{50}$/mL as described (179) and Reovirus at a concentration of 2 $x10^7$ pfu/mL. In some experiments $25(OH)D_3$ was used at a concentration of 10, 100 or 1000 nM instead of $1,25(OH)_2D_3$. All cultures were incubated at 37°C in a 5% carbon dioxide environment for 24 hours. Supernatants from cultured cells were then used for cytokine analysis and PBMC isolates were used for real-time polymerase chain reaction (RT-PCR) expression analysis.

In cases where RSV and Reovirus were used in culture, virus was rapidly thawed at 37°C upon PBMC isolation and added directly to plates at the time of cell culture. All vitamin D metabolites were thawed following PBMC isolation and added to cell culture last under light controlled conditions (ie. with lights off in biological safety cabinet in tissue culture area); this in an effort to ensure degradation of reagents was minimal **Appendix A**. All other reagents used were added to culture plates prior to PBMC isolation and were frozen; plates were then thawed when needed.

Table 2. Antigen and vitamin D concentrations used in cell culture

Stimulus	Concentration	Company Purchased
Control Medium	NA	In House
Lipopolysaccharide (LPS) Ultra-Pure *E. Coli* 0111:B4	3.6 ng/mL	Invivogen, Dan Diego California
3M 002 (CL075)	1.75 µg/mL	Invivogen, San Diego California
Poly (I:C)/Lyovec (PL)	250 ng/mL	Invivogen, San Diego California
Respiratory Syncytial Virus (RSV)	$10^{4.9}TCID_{50}$/mL	Titered by Dr. Yan Li and Dr. Nathalie Bastien, Canadian Centre for Human and Animal Health, Winnipeg, Manitoba
Reovirus	2 $x10^{7}$ pfu/mL	Titered by Dr. Kevin Coombs, Dept. Medical Microbiology, University of Manitoba
1,25$(OH)_2D_3$ (active VitD)	1, 10, 100 nM	Enzo Life Sciences, Plymouth Pennsylvania
25$(OH)D_3$ (inactive VitD)	10, 100, 1000 nM	Enzo Life Sciences, Plymouth Pennsylvania

Human Cytokine Enzyme-Linked Immuno Sorbent Assay (ELISA)

Capture and biotinylated detection antibodies, as well as standards, were purchased from Peptrotech and Biolegend for cytokine measurement. They were used as previously described on tissue cultured supernatants (223). Briefly, 96 well plates were coated with 50 µL of coating buffer and sealed in a moisture box overnight at 4°C. Coating buffer was then removed the following day and 75 µL of blocking buffer was added; plates were then incubated with blocking buffer for 2 hours at 37°C. After blocking, plates were washed 4x using an automated plate washer and 50 µL of dilution buffer was added to all plate wells using a multidrop dispenser. Samples were then added to the plates in triplicate at varying dilutions (ex: CXCL-8 added at 1/25 or 1/50) depending on the cytokine being measured or the stimulus condition. Samples were then titrated down the plate 4x doing a 2 fold dilution each time. Standards were added to all plates as well and titrated down the plate 8x doing a 2 fold dilution each time. Following sample and standard addition plates were stored overnight in a sealed moisture box at 4°C.

50 µL of detection antibody in dilution buffer was added to all plates the following day; here plates were washed 4x using the automated washer and antibody was added to plates using the multidrop dispenser. Plates were then stored in a moisture box overnight at 4°C.

Development was performed the following day where plates were washed 4x and 50 µL of Streptavidin-alkaline phosphatase (SAAP) in dilution buffer was added to the

wells. Plates were then incubated for 45 minutes at 37°C and washed 4x. Diethanolamine and $MgCl_2$ in distilled water (known as substrate buffer) was used to dissolve p-nitrophenyl phosphate (pNPP) tablets; this pNPP solution was then added to all wells and plates were incubated at 37°C until detection was observed (varies depending on cytokine). Plates were then read using a spectra MAX 190 microplate spectrophotometer from Molecular Devices at 405-690 nm. A 4-parameter curve fit was used to construct a standard curve from the eight standard serial dilutions using softmaxPro 5.4.1 software. Concentration of the samples was then determined using this curve.

RNA Isolation and RT-PCR

Total RNA was extracted from one million PBMC samples using RNeasy Plus Mini Kit (Qiagen). Samples consisted of PBMC stimulated according to **Table 2**. Once extracted, RNA was quantified using a GE nanovue plus from healthcare lifesciences and 100 ng of RNA was reverse transcribed into cDNA using a QuantiTect reverse transcription kit (Qiagen). RT- PCR was performed using the Light Cycler 480 and SYBR Green. Targets assessed were TLR4, TLR8, CYP27b1, CYP24a1, VDR, hCAP, RIG-I, MDA-5, LGP2, IFNα, IFNβ, $IFN\lambda_1$, $IFN\lambda_{2,3}$, ISG56, ISG15, IL-1Ra, sTNFRII, IL-10, IL-6, CXCL-10 and 18s **Table 3**. Standards were made for each primer set using human universal RNA and data was normalized to 18s ratios of samples, all this in an effort to increase accuracy and quality control. Samples were repeated on different days to determine reproducibility; all this was done prior to assessment of target genes **Table 4**.

Table 3. Forward and reverse primer sequences for quantitative real-time PCR

Target Gene	Forward Primer Sequence	Reverse Primer Sequence
VDR	5'-GACCTGGTCAGTTAC AGCATCCAA-3'	5'-CCTCAATGGCACTTGACTT CAGCA-3'
CYP27b1	5'-TTACAAGTTCGGACTG GAAGGCATCG-3'	5'-ATGGTCAACAGCGTGGA CACAAAC-3'
CYP24a1	—ORDERED FROM QIAGEN—sequence unavailable	
TLR4	5'-AGTTGAACGAATGGAA TGTGCA -3'	5'-CTGAGGACCGACACACC AATG -3'
TLR8	5'-GCCTTGTGATGGTGGTGC TTCAAT-3'	5'-TCCAGCACCTTCAGATG AGGCATA-3'
RIG-1	5'-GCAGAGGCCGGC ATGAC-3'	5'-TGTAGGTAGGGTCCAGG GTCTTC -3'
hCAP	5'-TATAGATGGCATCAA CCACCAGCGGTC-3'	5'-GGACACTGTCTCCT TCA CTGT-3'
MDA-5	5'-TGAAGTAGGAGTCA AAGCCCACCA -3'	5'-ATCCAGACCTTCTTCTG CCACTGT -3'
LGP2	5'-GCAAACAGTACAACCTCTG CCACA -3'	5'-TCTCCAGGTGGTCATGGA TTTGGT -3'
IFN-α	5'-GCTGAATGACTTGGAAGC CTGTGT-3'	5'-ATTTCTGCTCTGACAAC CTCCCAG-3'
IFN-β	5'-TGGCACAACAGGTAG TAGGCGA -3'	5'-TGGAGAAGCACAACAGGA GAGCAA -3'
IFN-λ_1	5'-TGGTGACTTTGGTGCT AGGCTT -3'	5'-TTGAGTGACTCTTCCAA GGCGT -3'
IFN-$\lambda_{2,3}$	5'-AGTGCTGACCGTGA CTGGA -3'	5'-ACAGGGACTTGAACTGGG CTATGT -3'
ISG-15	5'-AGCTCCATGTCGGTGTCAG -3'	5'-GAAGGTCAGCCAGAACAGGT -3'
ISG-56	5'-AAAGGTGCTTGA AGTGGACCCT -3'	5'-TCTGGATTTAAGCGG ACAGCCT -3'
IL-1Ra	5'-TGTCCTGTGTCAAGTCTG GTGATG -3'	5'-CTTGTCCTGCTTTCTGTT CTCGCT -3'
sTNF-RII	—ORDERED FROM QIAGEN—sequence unavailable	
IL-10	5'-CGTTTCAGAGCCATGAGG ATGCTT -3'	5'-CAGTGCCAAGGTCTCTTT CACCAA -3'
IL-6	5'-AGCCACTCACCTCTTCAG AACGAA -3'	5'-CAGTGCCTCTTTGCTGCTT TCACA -3'
CXCL-10	5'-AGCTGCTACTACTCCTGTA GGAAGGT -3'	5'-TGGAAGATGGGAAAGGT GAGGGAA -3'
18s	5'-AAAGGAATTGACGGAAG GGCACCA -3'	5'-ACCAGACAAATCGCTCC A CCAACT -3'

Table 4. Reproducibility of RT-PCR analysis of mRNA expression

Subject/Sample	Day 1	Day 2	Percentage Ratio (Mean ± SEM)
*1YrM 40031 CM	470	576	81.6 (523 ± 53)
1YrM 40031 CM-7	1444	1481	97.5 (1463 ± 18.5)
1YrM 40031 LPS	10404	10351	100.5 (10378 ± 26.5)
1YrM 40031 LPS-7	21588	20585	104.9 (21087 ± 501.5)
1YrM 40031 3M	12702	12144	104.6 (12423 ± 279)
1YrM 40031 3M-7	27438	26964	101.8 (27201 ± 237)
F 40348 CM	1092	1231	88.7 (1162 ± 69.5)
F 40348 CM-7	1078	1238	87.1 (1158 ± 80)

* 1YrM = Adult Females, F = Adult Males, Numerical value = subject number, CM = culture media, -7 = 100 nM $1,25(OH)_2D_3$, 3M = 1.75 ug/mL 3M 002

Mass Spectrometry

Plasma from adult whole blood was aliquotted into 1.5 milliliter centrifuge tubes containing 20 µL of sodium azide (NaN_3) and frozen in -20°C until further use. Samples were subsequently thawed and centrifuged. 0.75 mL of samples were then aliquotted into tubes and shipped to Lawrence Fisher at the Children's Hospital of Eastern Ontario in Ottawa Ontario. Here the concentration of $25(OH)D_3$ in the adult plasma samples was determined in nM using liquid chromatography tandem mass spectrometry.

Statistical Analysis

Graph Pad Prism 5.0 was used to determine the significance between conditions. The D'Agostino and Pearson normality test was applied to data to determine whether data was normally distributed. Most data did not display normal distribution and thus a two-tailed wilcoxon matched pairs test (paired, non-parametric) was used to determine significance. In cases where data was normally distributed a paired t test was used. Mann-whitney and unpaired t-test were also used to analyze some data sets. For plasma samples analyzed for $25(OH)D_3$ concentration correlation and linear regression analysis were also done. Significance was defined by a p value of <0.05. Data is represented as median or mean $\pm$ standard error of the mean (SEM).

CHAPTER 1

Vitamin D Inhibits Human Innate Inflammatory Cytokine Production in the Presence of Bacterial PRR Stimulation, but not Viral

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Chapter 1 is based on a draft of a paper that is currently in preparation

Abstract

Rationale: Population health studies demonstrate that insufficient and deficient Vitamin D status is strongly associated with increased upper respiratory tract infections. As low vitamin D status is widespread amongst Canadians, it is imperative we understand the role vitamin D plays in the innate and adaptive immune systems.

Objectives: Our focus here is the human innate immune system. Our goal is to determine the effects of active vitamin D ($1,25(OH)_2D_3$) on human cytokine responses elicited by bacterial or viral innate immune activation of PBMC. Moreover, we will further our understanding of the mechanisms by which vitamin D regulates innate immune responses.

Methods: PBMC, from asymptomic, healthy adult donors were isolated and cultured with bacterial stimuli LPS or viral stimuli 3M 002 with/without a titration of active or inactive vitamin D. ELISA and RT-PCR were used to determine cytokine responses and mRNA expression, respectively.

Results: Adult PBMC stimulated with TLR4 ligand (TLR4L) LPS and exogenous $1,25(OH)_2D_3$ exhibited decreased pro-inflammatory cytokine production; whereas cultures stimulated with a viral ligand specific for TLR8 did not exhibit any alterations in cytokine production patterns in the presence of active vitamin D. Further examination into the mechanism of action revealed decreased receptor expression of both TLR4 and TLR8 in the presence of exogenous vitamin D. Interestingly, the vitamin D activating

enzyme (CYP27b1) was more abundantly expressed in TLR8L stimulated cells and the vitamin D deactivating enzyme (CYP24a1) less expressed in cells exposed to TLR4L. Comparison between vitamin D receptor (VDR) expression in PBMC stimulated with TLR8L versus TLR4L revealed decreased VDR in cells exposed to the synthetic viral ligand.

Conclusions: The data argue that exogenous $1,25(OH)_2D_3$ has potent immune regulatory effects on cells stimulated with TLR4L, but not TLR8L. Our molecular studies show that TLR8L stimulated cells differ in their expression of VDR and vitamin D related enzymes when compared to TLR4L stimulated cells. Therefore, future studies will focus on understanding these observed differences between bacterial and viral ligands in response to $1,25(OH)_2D_3$.

Funding: CIHR, CRC.

Results

Vitamin D Decreases Pro-inflammatory and Anti-inflammatory Cytokine Production in Adult PBMC Stimulated with LPS

Previous studies have shown that active vitamin D has the ability to reduce inflammatory cytokine production in the presence of bacterial stimuli (116,218). However, the effects of vitamin D on anti-inflammatory cytokine production are not so concrete; here previous studies show that depending on the stimuli $1,25(OH)_2D_3$ can either promote or dampen IL-10 cytokine production (113,123). Therefore to study the impact of active vitamin D on cytokine production patterns in PBMC exposed to bacterial and viral ligands, our first objective was to confirm the findings that $1,25(OH)_2D_3$ decreased pro-inflammatory cytokine production in the presence of LPS. Fresh PBMC were cultured with exogenous $1,25(OH)_2D_3$ in the presence or absence of TLR4 ligand LPS. Our data show that PBMC stimulated with LPS experience enhanced CCL-2 and CXCL-8 production compared to unstimulated cells. Further stimulation of these LPS activated cells with $1,25(OH)_2D_3$ showed significant decreases in CCL-2 and CXCL-8 cytokine production levels **Figure 4A.** These results were observed in a dose dependent manner suggesting that the active form of vitamin D has the ability to dramatically reduce pro-inflammatory cytokine production and that this response can be controlled.

As the effects of vitamin D on anti-inflammatory cytokine production appear to vary with the stimuli used, we wanted to determine the effect of vitamin D on IL-10

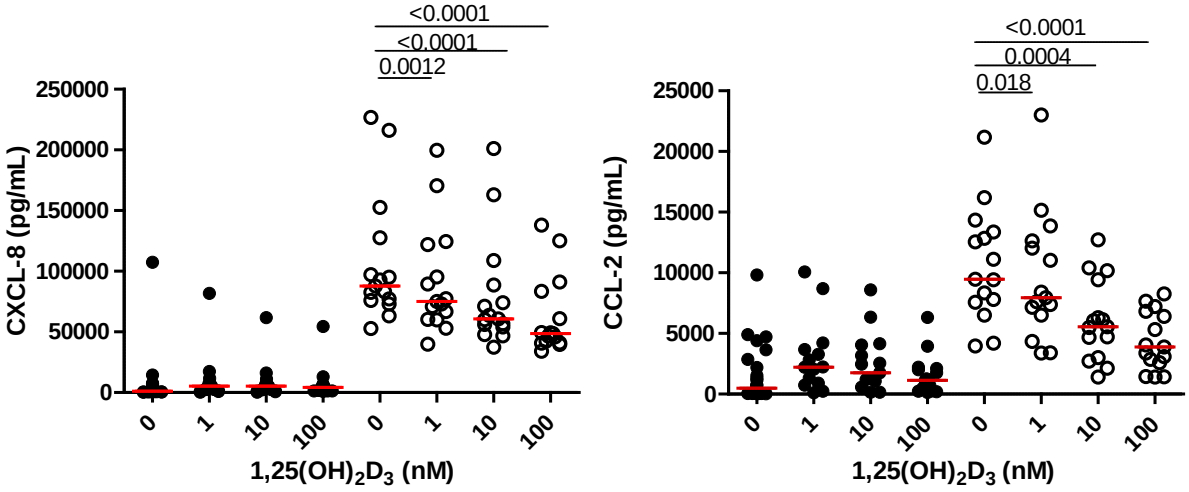

Figure 4A: Active vitamin D decreases pro-inflammatory cytokine production in TLR4 activated cells. PBMC were cultured for 24 hours with CM or 3.6 ng/mL LPS with/without 1, 10, or 100 nM of 1,25$(OH)_2D_3$. (n=15, filled circles represent resting cells and empty circles represent cells stimulated with LPS). Red bar representes the median.

cytokine production in our model upon stimulation with LPS. Here the data shows that stimulation of PBMC with LPS reduces IL-10 cytokine production in the presence of LPS **Figure 4B**. To confirm this trend of decreased cytokine production in the presence of vitamin D and LPS, we decided to test whether the global cytokine IL-6 was decreased under these conditions. Our results show that the same trend of decreased cytokine production was observed when PBMC were stimulated with 1,25$(OH)_2D_3$ and LPS **Figure 4C.** These results, in combination with those of the data for pro-inflammatory cytokine production, suggest that vitamin D can regulate innate cytokine responses upon activation by bacterial PRR ligation.

Vitamin D Fails to Alter Innate Cytokine Responses to TLR8 Stimulation in Primary PBMC

As active vitamin D decreases inflammatory cytokine production in the presence of bacterial PRR activation to LPS, we decided to test whether active vitamin D has the ability to modulate cytokine responses in the presence of a viral ligand. Due to the mounting evidence that increased vitmain D status correlates with decreased upper respiratory tract infections, we wanted to examine the effects of vitamin D on PBMC stimulated with 3M 002, a synthetic TLR8L ligand. Our decision for using 3M 002 as our main viral stimuli throughout these experiments was because 3M 002 preferentially stimulates TLR8 as opposed to TLR7, and has been shown to induce a strong pro-inflammatory cytokine response upon activation in monocytes (49,224). The similarity between TLR8 activation and TLR4 activation, thereby allowed more of a comparison

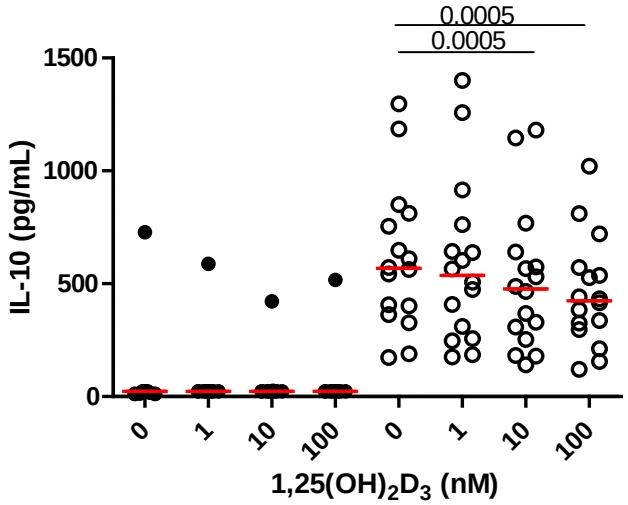

Figure 4B: Active vitamin D decreases anti-inflammatory cytokine production in TLR4 activated cells. PBMC were cultured for 24 hours with CM or 3.6 ng/mL LPS with/without 1, 10, or 100 nM of $1,25(OH)_2D_3$. (n=15, filled circles represent resting cells and empty circles represent cells stimulated with LPS). Red bar represented the median.

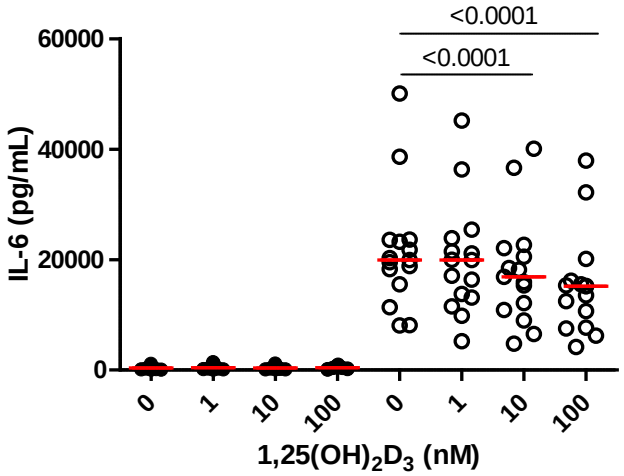

Figure 4C: Active vitamin D decreases global cytokine production in TLR4 activated cells. PBMC were cultured for 24 hours with CM or 3.6 ng/mL LPS with/without 1, 10, or 100 nM of $1,25(OH)_2D_3$. (n=15, filled circles represent resting cells and empty circles represent cells stimulated with LPS). Red bar represented the median.

between the two stimuli. As few studies have examined the effects of exogenous active vitamin D on virally stimulated cells, our experimental design proved novel. Our results show that stimulation of PBMC with 3M 002 leads to statistically enhanced CCL-2 and CXCL-8 production, yet addition of exogenous vitamin D did not reduce pro-inflammatory cytokine production **Figure 5A**. When these experiments were repeated to measure anti-inflammatory and global cytokine production, the same results were observed that vitamin failed to reduce cytokine levels in PBMC stimulated with TLR8L **Figure 5B,C**. These results proved very interesting and suggest that vitamin D may regulate a bacterial driven inflammatory cytokine response different than that of a viral.

Vitamin D Fails to Reduce Pro-inflammatory and Anti-inflammatory Production in the Presence of Intact Infectious Virus RSV

As 3M 002 is a synthetic ligand that is designed to specifically induce one virally associated receptor, we decided to examine whether this observed difference between TLR8L and TLR4L cytokine response to vitamin D was prevalent when PBMC were stimulated with an intact virus. RSV has been shown to severely impact newborns and individuals with asthma, whereas healthy adults are rarely seriously affected by this virus. RSV interacts with a variety of receptors including TLR4, RIG-I as well as TLR7/8 receptors in PBMC (38) and therefore is a great virus to test the effects of vitamin D. Thus, we decided to look at PBMC stimulated with RSV in the presence or absence of vitamin D and examine pro-inflammatory and anti-inflammatory cytokine production. Our results showed that RSV has the ability to stimulate intense pro-inflammatory (CCL-2) and anti-inflammatory (IL-10) cytokine production when compared to unstimulated

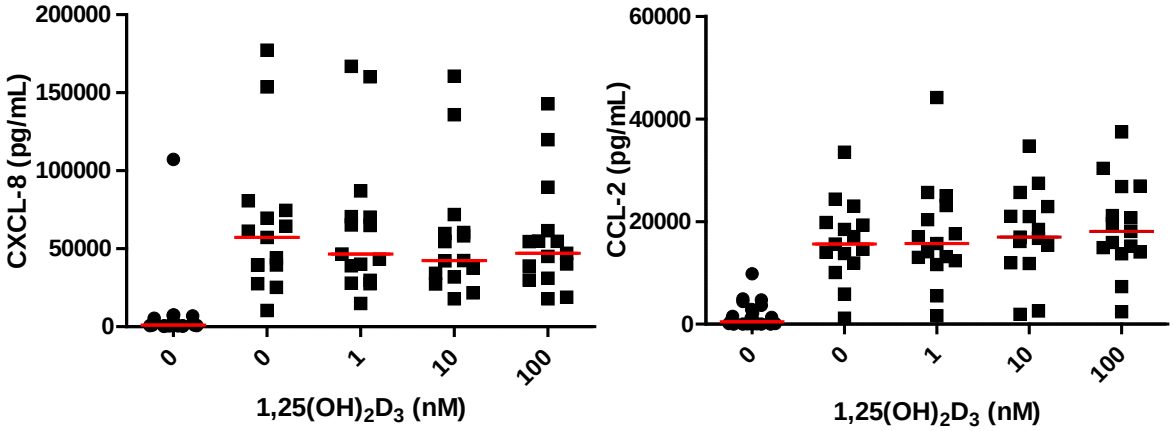

Figure 5A: Active vitamin D does not alter pro-inflammatory cytokine production in TLR8 activated cells. PBMC were cultured for 24 hours with CM or 1.75 µg/mL 3M 002 with/without 1, 10, or 100 nM of 1,25$(OH)_2D_3$. (n=15, filled circles represent resting cells and full squares represent cells stimulated with 3M 002). Red bar represented the median.

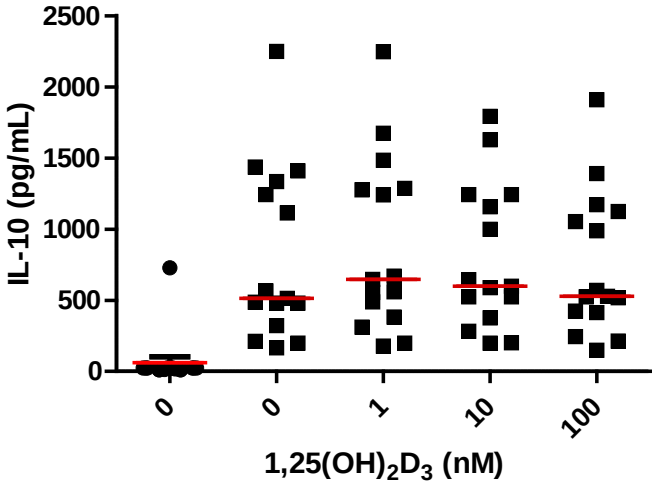

Figure 5B: Active vitamin D does not alter anti-inflammatory cytokine production in TLR8 activated cells. PBMC were cultured for 24 hours with CM or 1.75 µg/mL 3M 002 with/without 1, 10, or 100 nM of $1,25(OH)_2D_3$. (n=15, filled circles represent resting cells and full squares represent cells stimulated with 3M 002). Red bar represented the median.

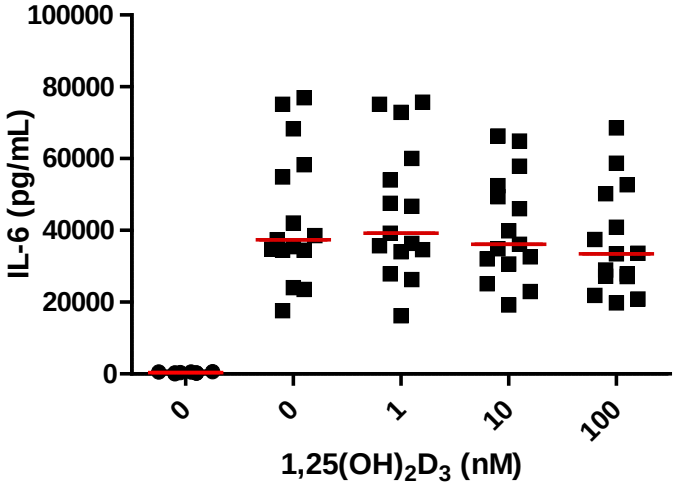

Figure 5C: Active vitamin D does not alter global cytokine production in TLR8 activated cells. PBMC were cultured for 24 hours with CM or 1.75 µg/mL 3M 002 with/without 1, 10, or 100 nM of $1,25(OH)_2D_3$. (n=15, filled circles represent resting cells and full squares represent cells stimulated with 3M 002). Red bar represented the median.

cells, and that simultaneous simulation with active vitamin D does not reduce either cytokine response **Figure 6**. These results demonstrate that vitamin D has different mechanisms of action in the presence of bacterial stimuli as compared to viral. Therefore, taking these results into account, it appears that vitamin D may not be important in regulating viral induced inflammatory cytokine production.

Cathelicidin Gene hCAP-18 is Downregulated in the Presence of LPS and 3M 002

AMPs are involved in the protection against foreign microbes and cell recruitment to the site of inflammation. Several studies have previously shown that LL-37 and human beta-defensins are upregulated in the presence of active vitamin D, and some even suggest that this phenomenon occurs in TLR activated cells (105,106,225). Therefore, our objective was to determine whether or not hCAP was induced in cells stimulated with LPS or 3M 002 in the presence of active or inactive vitamin D. Here our hypothesis was that cells stimulated with LPS and vitamin D would induce hCAP expression whereas cells stimulated with 3M 002 and vitamin D would not.

According to the results unstimulated cells expressed more hCAP than LPS or 3M 002 stimulated cells **Figure 7**. When stimulated and unstimulated cells were treated with $25(OH)D_3$ little difference was observed from control conditions that were absent of $25(OH)D_3$, however, when cells were treated with $1,25(OH)_2D_3$ a significance increase in hCAP expression was observed in all conditions treated with $1,25(OH)_2D_3$ compared

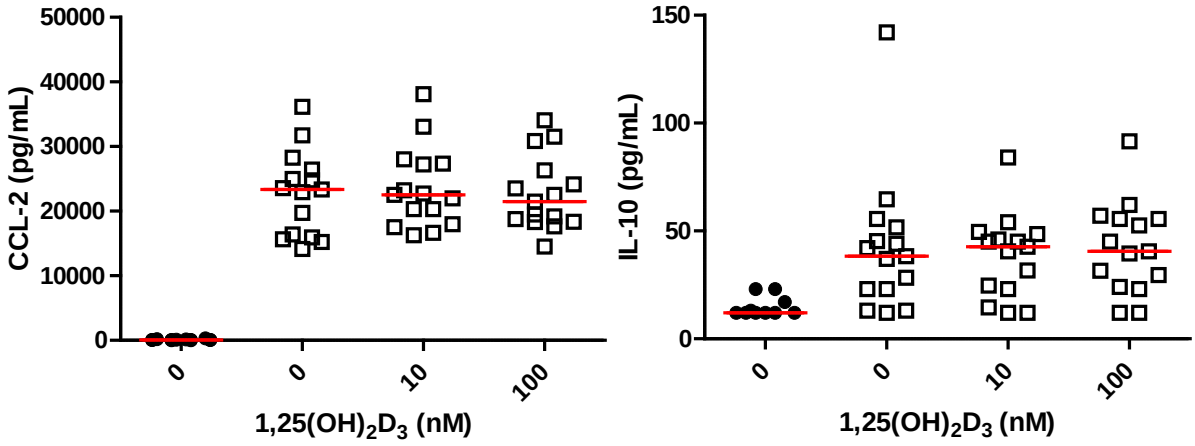

Figure 6: Active vitamin D does not alter inflammatory cytokine production in cells stimulated with RSV. PBMC were cultured for 24 hours with CM or RSV with/without 1, 10, or 100 nM of 1,25$(OH)_2D_3$. (n=15, filled circles represent resting cells and empty squares represent cells stimulated with RSV). Red bar represented the median.

to controls that were not. A closer look at this data revealed that LPS cells produced more hCAP than 3M 002 stimulated cells.

Collectively these results show that cells (both stimulated and unstimulated) in the presence of active vitamin D significantly increase hCAP expression. However, when cells are activated by LPS hCAP is downregulated, and further downregulation is observed in the presence of 3M 002. None the less, these results proved interesting as we hypothesized that exogenous active vitamin D added to PBMC stimulated with 3M 002 would have no effect in comparison to PBMC stimulated with 3M 002 alone. Therefore, this data suggests that even though PBMC stimulated with 3M 002 are unable to decrease cytokine production levels in the presence of exogenous vitamin D , perhaps vitamin D is being used by these cells to modulate another response.

Vitamin D Inhibits TLR4 and TLR8 Receptor Expression in PBMC in the Presence or Absence of Stimulation

As active vitamin D reduces inflammatory cytokine production in bacterial stimulated cells, but not viral stimulated cells, the mechanism by which this phenomenon occurs became our next priority. We began by looking at TLR4 and TLR8 receptor expression in adult PBMC after stimulation with bacterial LPS or viral 3M 002. Here our hypothesis was that active vitamin D decreases innate inflammatory and anti-inflammatory cytokine response in LPS stimulated cells by decreasing TLR4 expression.

As LPS induces TLR4 activation, which leads to downstream signalling, our thoughts were that a downregulation in this receptor would cause decreased

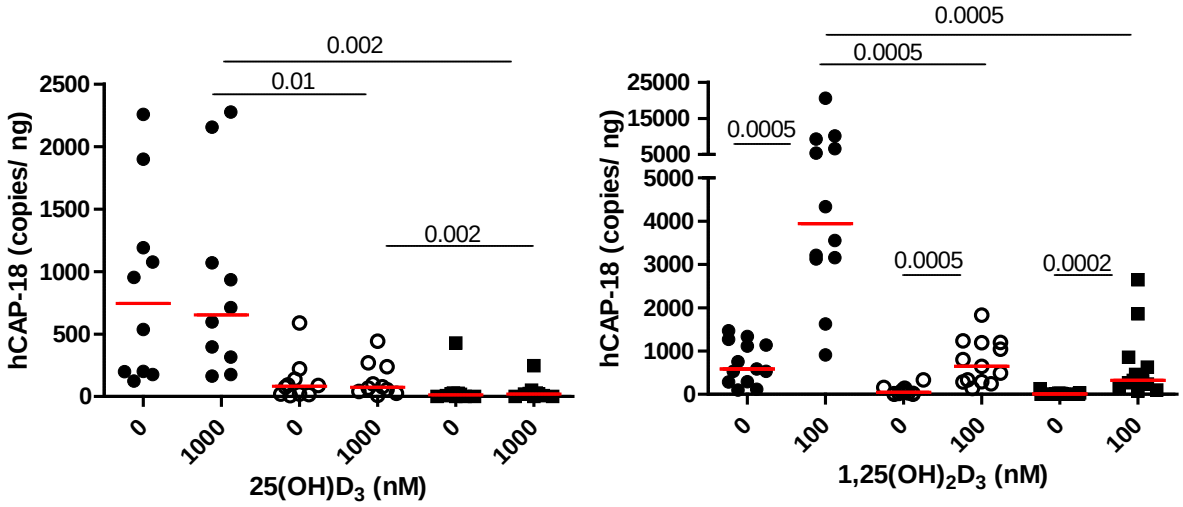

Figure 7: hCAP expression in active and inactive vitamin D stimulated cells. PBMC were cultured for 24 hours with CM, 3.6 ng/mL LPS or 1.75 µg/mL 3M 002 with/without 1000 nM of $25(OH)D_3$ or 100 nM of $1,25(OH)_2D_3$. (n=10-13, filled circles represent resting cells, empty circles represent cells stimulated with LPS and full squares represent cells stimulated with 3M 002). Red bar represented the median.

inflammatory cytokine release. Here the data shows that unstimulated cells constitutively express high levels of TLR4 and that upon $1,25(OH)_2D_3$ stimulation these cells downregulate TLR4 expression. Furthermore, in PBMC stimulated with LPS TLR4 expression was lower than in unstimulated cells, and cells stimulated with both LPS and active vitamin D had even lower TLR4 expression **Figure 8**.

As the TLR4 expression results agreed with our initial hypothesis that $1,25(OH)_2D_3$ would decrease receptor expression levels, this led to the hypothesis that PBMC stimulated with 3M002 and vitamin D would express TLR8 to the same degree as cells stimulated with 3M 002 alone. Here addition of active vitamin D to unstimulated cells resulted in downregulation of TLR8 expression compared to the control, and stimulation of cells with viral ligand 3M 002 and active vitamin D led to reduced TLR8 expression compared to cells stimulated with viral ligand alone **Figure 8**. These results indicate that active vitamin D reduces both TLR4 and TLR8 receptor expression in the presence or absence of ligand stimulation. Thus, as exogenous vitamin D is able to alter both TLR4 and TLR8 receptor expression levels it appears that the difference in cytokine production patterns between LPS and 3M 002 stimulated PBMC in the presence of $1,25(OH)_2D_3$ is not merely the result of receptor expression differences.

Vitamin D Activating and Deactivating Enzyme Expression Levels Differ in TLR8L Compared to TLR4L Stimulated PBMC

$1,25(OH)_2D_3$ is regulated by a variety of enzymes, proteins and hormones. Two of the most important enzymes in the vitamin D pathway that aid in this area are CYP27b1,

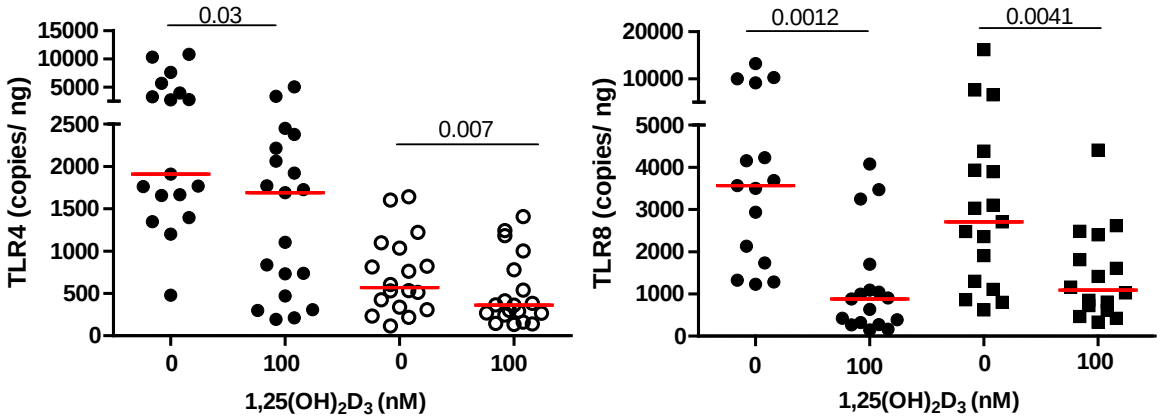

Figure 8: Active vitamin D decreases TLR4 and TLR8 receptor expression. PBMC were cultured for 24 hours with CM, 3.6 ng/mL LPS or 1.75 ug/mL 3M 002 with/without 100 nM of $1,25(OH)_2D_3$. (n=13-19, filled circles represent resting cells, empty circles represent cells stimulated with LPS and full squares represent cells stimulated with 3M 002). Red bar represented the median.

which converts 25(OH)D_3 into 1,25(OH)$_2D_3$ and CYP24a1, which converts 1,25(OH)$_2D_3$ to 24-hydroxylated products. As alterations in these enzymes could account for the differences observed between bacterial and viral driven cytokine production in the presence of exogenous vitamin D, we proceeded to determine whether these two enzymes were differentially expressed in PBMC stimulated with LPS or 3M 002.

According to our results cells stimulated with LPS have increased levels of CYP27b1 compared to unstimulated cells, however, cells stimulated with viral ligand 3M 002 have the highest expression level of CYP27b1 **Figure 9**. In both stimulated conditions, addition of the inactive or active forms of vitamin D did not augment CYP27b1 expression compared to LPS or 3M 002 conditions in the absence of vitamin D. These results suggest that in the presence of viral ligation high levels of CYP27b1 are being generated and therefore active vitamin D should be produced.

The vitamin D deactivating enzyme CYP24a1 was also examined upon the addition of bacterial and viral stimulation in the presence of vitamin D. Here the data shows that addition of active vitamin D leads to increased deactivating enzyme levels. This was most drastically seen in unstimulated PBMC samples where CYP24a1 is not detectable until active vitamin D addition. Interestingly, stimulated cells in the presence of 1,25(OH)$_2D_3$ express less CYP24a1 than that of unstimulated cells, with the 3M 002 condition expressing less CYP24a1 than LPS **Figure 10**. These results in collaboration with those of CYP27b1 expression suggest that 3M 002 stimulated cells possess active vitamin D. Therefore the difference between bacterial and viral driven cytokine

production in the presence of vitamin D is not merely the result of vitamin D degradation or inactivation. In fact these results would cause one to assume that vitamin D is doing more in these cells than that of LPS because less is being deactivated and more is being activated.

TLR8L Downregulates VDR Expression in Primary PBMC

To determine the mechanism by which vitamin D reduces bacterial cytokine responses, but not viral, we decided to examine whether vitamin D has the ability to increase the expression of VDR during TLR4 or TLR8 immune activation. Therefore, our hypothesis was that vitamin D in the presence of TLR4L would cause an increase in VDR expression; however, this increase would not be observed when cells were stimulated with TLR8L and vitamin D. If this were true, then this could help explain the phenomena we were seeing by which vitamin D reduces bacterial produced inflammation and not viral.

Our results showed that VDR expression in PBMC stimulated with bacterial ligand LPS in the presence or absence of vitamin D resulted in similar expression levels to unstimulated PBMC. Interestingly, cells stimulated with 3M 002 downregulated VDR under these conditions compared to cells stimulated with CM or LPS **Figure 11**.

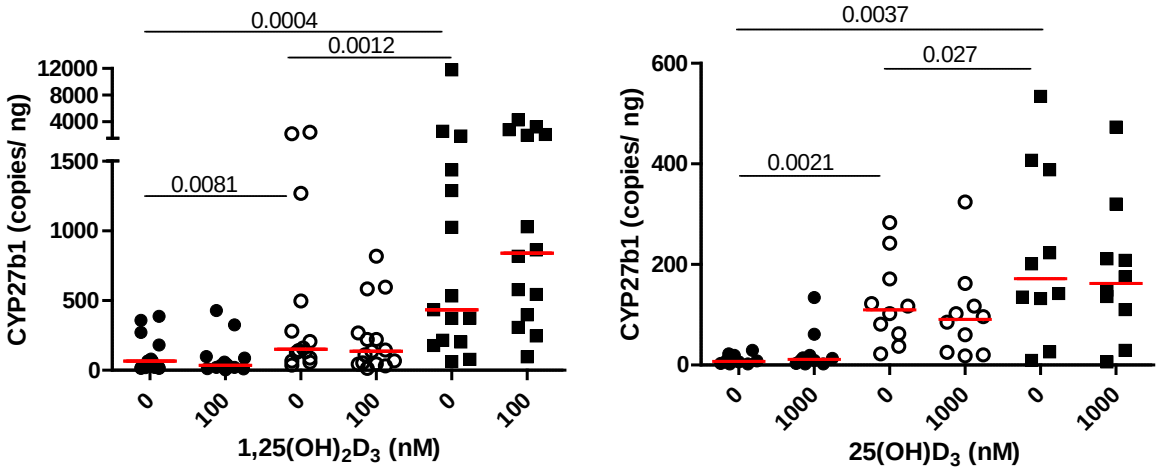

Figure 9: Vitamin D activating enzyme expression is increased in the presence of viral stimulation. PBMC were cultured for 24 hours with CM, 3.6 ng/mL LPS or 1.75 µg/mL 3M 002 with/without 100 nM of $1,25(OH)_2D_3$ or 1000 nM of $25(OH)D_3$. (n=10-15, filled circles represent resting cells, empty circles represent cells stimulated with LPS and full squares represent cells stimulated with 3M 002). Red bar represented the median.

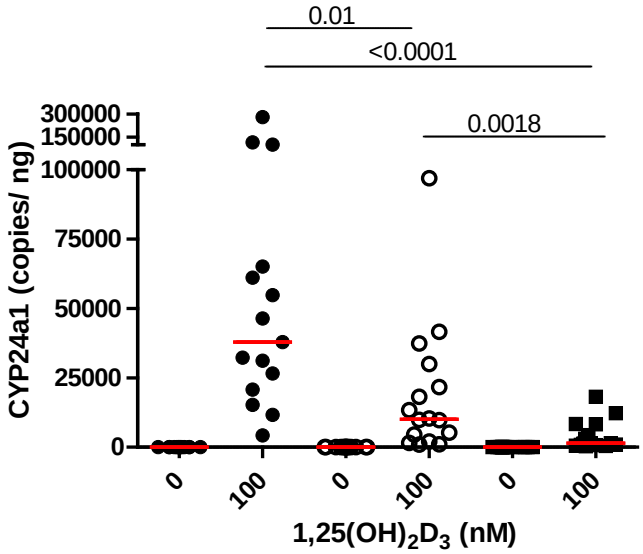

Figure 10: Vitamin D deactivating enzyme expression is decreased in the presence of viral stimulation. PBMC were cultured for 24 hours with CM, 3.6 ng/mL LPS or 1.75 µg/mL 3M 002 with/without 100 nM of $1,25(OH)_2D_3$. (n=15-16, filled circles represent resting cells, empty circles represent cells stimulated with LPS and full squares represent cells stimulated with 3M 002). Red bar represented the median.

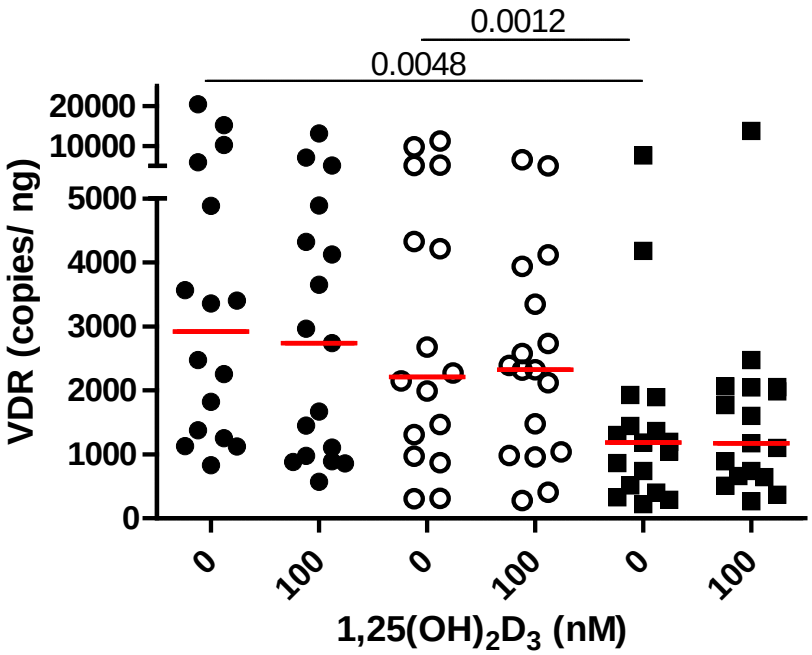

Figure 11: Vitamin D receptor expression is decreased upon viral stimulation. PBMC were cultured for 24 hours with CM, 3.6 ng/mL LPS or 1.75 µg/mL 3M 002 with/without 100 nM of $1,25(OH)_2D_3$. (n=16-17, filled circles represent resting cells, empty circles represent cells stimulated with LPS and full squares represent cells stimulated with 3M 002). Red bar represented the median.

Discussion

Chronic inflammatory disorders including inflammatory bowel disease (IBD), diabetes and allergic asthma are characterized by excessive pro-inflammatory cytokine and chemokine responses which can result in flare ups or exacerbations. According to previous studies the active form of vitamin D has the ability to reduce pro-inflammatory cytokine production in the presence of LPS (116) or *M. tuberculosis* (113,123) while increasing anti-inflammatory cytokine production (123). Therefore, it has been speculated that vitamin D supplementation may be beneficial in individuals with severe inflammation whether that be due to infection or disease.

Current epidemiological and corrleation studies suggest that individuals with higher serum vitamin D levels are less likely to develop viral infections (159). When comparing upper respiratory tract infections or influenza, some researchers have noticed a higher prevalence in individuals with lower circulating vitamin D levels (160,161,166). However, these results remain inconclusive, as other studies have found results less convincing (162,226). Unfortunately, the conclusions drawn from these studies are based upon measurement of the inactive form of vitamin D. As 25(OH)D_3 is merely a metabolite in the vitamin D pathway and must be converted to the active form by the enzyme CYP27b1 in order to be of use, little can be drawn from these studies without additional experiments using the active form of vitamin D. Exacerbations in asthmatic individuals can be induced by viral infections (reviewed in (227)), therefore, it

is imperative we understand the role vitamin D plays in modulating the immune system, especially during viral infections.

Thus, the main objective of this study was to determine the effect of active vitamin D on cytokine production in the presence of PRR activation in an effort to better understand the potential vitamin D plays in the innate and adaptive immune responses to bacterial and viral infections. Our initial findings are similar to that of literature, where it is observed that vitamin D reduces pro-inflammatory cytokine production in the presence of bacterial activated PRR. When PBMC were stimulated with TLR8L 3M 0002 induction of both pro- and anti-inflammatory cytokine production was observed (IL-6, IL-10, CXCL-8 and CCL-2), however, further addition of exogenous $1,25(OH)_2D_3$ to culture did not alter TLR8L driven pro- or anti-inflammatory cytokine production. These results indicate that LPS and 3M 002 tend to activate similar pathways, which result in the upregulation of pro- and anti-inflammatory cytokines, yet in the presence of exogenous vitamin D different results are seen. To confirm whether this phenomena between bacteria and virus was in fact true, we repeated these experiments using an intact virus (RSV). Here the same results were observed that PBMC stimulated with RSV induced pro- and anti-inflammatory cytokine production, but further addition of exogenous vitamin D did not alter cytokine production patterns; thus indicating that active vitamin D does not affect viral driven immune responsiveness in the same manner as that of bacterial.

Although our results proved novel, similar studies in EC have drawn different conclusions showing that stimulating with whole and synthetic viral ligands in the presence of exogenous active vitamin D reduces inflammatory cytokine production in comparison to virus alone (108,109). However, as PBMC home to tissues and lymph nodes, and EC remain stationary, their role in viral recognition is not the same and thus comparison between the two cell types is difficult. These studies also tend use Poly(I:C), a dsRNA ligand for TLR3, to activate EC whereas our studies used 3M 002 which activates TLR8. These receptors stimulate different pathways with TLR3 activating downstream signals independent of MyD88 and TLR8 using MyD88 (reviewed in (5,34)), therefore, the difference seen between PBMC and EC may be due to differential pathway activation. The dosage difference between our two studies is also of importance, as Poly(I:C) was administered to EC at 50 μg/mL whereas our study used 1.75 μg/mL of 3M 002.

As our initial results opposed our hypothesis, and that of the EC literature, our next step was to determine whether this apparent difference between bacterial and viral innate immune responsivenss in the presence of exogenous vitmain D is observed in other inflammatory processes. Here both inactive and active exogenous vitamin D has been shown to augment AMP production in the presence or absence of PRR activation (106). Thus, we decided to determine whether stimulation with 3M 002 and exogenous $1,25(OH)_2D_3$ increased hCAP expression compared to PBMC stimulated with 3M 002 alone. Here we found that cells stimulated with LPS and 3M 002 downregulated hCAP expression compared to unstimulated cells. Interestingly, stimulating cells with vitamin

D under all conditions examined (CM, LPS and 3M 002) led to increased hCAP expression. Thus, it appears that in the presence of exogenous vitamin D 3M 002 stimulated cells augment hCAP expression, however the question as to why vitamin D does not downregulate inflammatory cytokine production in the presence of 3M 002 remains unanswered.

As TLR8L stimulated PBMC were found to modulate hCAP expression, but not pro-inflammatory cytokine production, our next step was to determine whether vitamin D was modulating TLR4 and TLR8 receptor expression. Here stimulating cells with LPS or 3M 002 with vitamin D resulted in decreased TLR4 or TLR8 receptor expression, respectively. These results suggest that vitamin D should have similar effects on LPS and 3M 002 induced inflammation. As vitamin D reduces TLR4 and TLR8 expression, this would most presumably lead to decreased NF-κB transciptional activation as less receptor expression would equate to less receptor signalling. Thus, one would expect pro-inflammatory cytokine production to be downregualted in both LPS and 3M 002 conditions in the presence of exogenous $1,25(OH)_2D_3$. Studies by others have shown similar results in that vitamin D downregulates TLR2 and TLR9 (114,115,122,124), however decreased expression of these receptors also led to reduced cytokine production.

Further analysis was therefore conducted on the mechanism by which vitamin D can modulate hCAP, but not pro-inflammatory cytokine production under 3M 002 stimulated conditions. Here TLR8L stimulated cells were found to have increased

CYP27b1 expression and decreased CYP24a1 expression compared to LPS or unstimulated cells. As CYP27b1 converts inactive vitmain D to active and CYP24a1 converts active vitmain D to inactive, these results would cause one to assume that 3M 002 simulated cells have increased $1,25(OH)_2D_3$ levels in comparison to that of LPS because more vitmain D is being activated and less is being deactivated.

Examination into VDR expression patterns in these cells showed that TLR8L stimulated cells have decreased VDR expression compared to TLR4L or unstimulated cells. These results suggest that in the presence of a viral ligand, such as 3M 002, PBMC downregulate VDR. This could account for the difference observed between bacterial and viral inflammatory cytokine production patterns in the presence of active vitamin D. As vitamin D bound to VDR has been shown to increase IκBα expression (128) as well as directly bind to the p65 subunit of NF-κB (96) thereby preventing translocation of NF-κB into the nucleus, it is possible that there is a lack of VDR binding to vitamin D in 3M 002 stimulated PBMC. If this were true than NF-κB would still translocte to the nucleus thereby promoting transcription of inflammatory genes, as vitamin D/VDR is not preventing translocation.

To summarize, in this chapter we have shown that PBMC stimulated with different PRR ligands do not respond to exogenous active vitamin D the same. Our results are among the first to show that vitmain D influences bacterial stimulated PBMC cytokine production differently than viral. A recent publication by Xiong et al, showed that VDR loss in mice with chronic kidney failure is due to an increase in TNF-α (228).

This study supports our hypothesis that decreased VDR expression can lead to alterations in pro-inflammatory cytokine production. Therefore, although not conclusive, decreased VDR expression in the presence of TLR8 stimulated PBMC compared to unstimulated or TLR4 simtulated PBMC may be one reason why 3M 002 stimulated PBMC did not experience decreased pro- and anti-inflammatory cytokine production in the presence of exogenous vitmain D.

CHAPTER 2

Circulating 25(OH)D_3 Levels may be an Indicator of Innate Immune Responsiveness

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Abstract

Rationale: Vitamin D deficiency rates are prevalent in the population, but tend to be the most common in elderly individuals and children. As vitamin D supplementation has been shown to increase circulating $25(OH)D_3$ levels, it is important to assess the role vitamin D plays in innate immune responsiveness. Current studies that have focused on connecting vitamin D deficiency and immune responsiveness are inconclusive.

Objectives: To confirm deficiency rates and vitamin D levels among the population and determine whether cytokine plasma levels correlate to endogenous $25(OH)D_3$. Furthermore, to assess whether resting or activated cell responsiveness correlates to plasma $25(OH)D_3$ levels. Lastly, to determine whether exogenous vitamin D supplementation is effective in regulating infant innate immune responsiveness.

Methods: Plasma samples from teens and adults were analyzed by liquid chromatography tandem mass spectrometry for $25(OH)D_3$. PBMC and cord blood mononuclear cells (CBMC) were isolated and cultured with bacterial stimuli LPS with/without a titration of active vitamin D. ELISA was used to determine cytokine responses in cultures or plasma samples and RT-PCR was used to measure mRNA expression in PBMC and CBMC.

Results: Plasma $25(OH)D_3$ levels show that teens are lower in vitamin D than adults and have a higher rate of deficiency. Male adults appear to have slightly lower vitamin D levels than females. $25(OH)D_3$ levels are lower in the winter months than the summer

and spring. Interestingly, plasma 25(OH)D_3 levels did not correlate with *in vivo* cytokine levels even though resting PBMC pro-inflammatory cytokine production levels negatively correlated with 25(OH)D_3 status. Analysis of CBMC cytokine responsiveness showed that cells stimulated with LPS were less susceptible to vitamin D modulation than PBMC from adults. Further experiments revealed that VDR expression is similar amongst all ages and is not correlated to 25(OH)D_3 plasma levels.

Conclusions: The data argue that 25(OH)D_3 levels may predict innate immune responsiveness in terms of cytokine production. As cytokine responses in neonates are less susceptible to exogenous vitamin D modulation, future studies may focus on determining why this difference is observed and whether older children experience this same suppression. As VDR expression is consistent across several different age groups, further analysis may be directed towards PRR expression or vitamin D enzymatic activity.

Funding: CIHR, CRC.

Results

Teens Exhibit Lower 25(OH)D_3 Levels than Adults, which is Most Prominent During the Winter Months

25(OH)D_3 is a precursor in the vitamin D pathway that has been shown to be the major circulating vitamin D metabolite, and therefore is the form that is used to measure vitamin D nutritional status. Many epidemiological studies have shown the typical plasma 25(OH)D_3 levels in the adult population, therefore we sought to confirm these levels within our study subjects and to determine whether teens have similar circulating concentrations to those of adults. Here 130 plasma samples consisting of teens from CAPPS and adults from the CHILD study were measured for 25(OH)D_3, and sex and date of sample attainment were taken into consideration for analysis. As was mentioned previously, NHANES III study found that the average 25(OH)D_3 level among the adult American population was 73 nM for women and 78 nM for men (148), we observed similar levels within our adult population with the average concentration being 70 nM. However, when we compared teen 25(OH)D_3 to that of adults we found that teens had significantly lower circulating vitamin D levels with the average concentration being 50 nM **Figure 12A**. Plasma samples from female adults and male adults were then compared and we found that contrary to literature our male population had lower levels of 25(OH)D_3 at 54 nM, whereas our female population had an average of 73 nM which is consistent with literature **Figure 12B.** These results were further examined and the populations stratified based on sufficient (>75 nM), insufficient (51-74 nM) and deficient (< 50 nM) levels of 25(OH)D_3 to determine

deficiency rates within the population. According to **Figure 12C** 57.5 % of teens are deficient of vitamin D and only 8.5 % are sufficient, 40% of male adults are also deficient and 16% of female adults. These results show that vitamin D deficiency is quite prevalent especially amongst teens and male adults. To determine whether $25(OH)D_3$ concentration changes seasonally, plasma samples were separated based on season obtained. Here $25(OH)D_3$ levels were lower in the winter months with an average plasma concentration of 58 nM, as compared to the spring (74 nM) and summer (71 nM) **Figure 13**. This 18-22 percent difference in plasma $25(OH)D_3$ concentration between winter and summer months is consistent with literature (115,229). Therefore, our initial results confirm that endogenous vitamin D levels fluctuate with season; however, the fact that males and teenagers exhibit lower circulating vitamin D levels than females is quite novel.

Systemic Inflammatory Cytokines do not Correlate with Endogenous Vitamin D, yet Resting PBMC Secrete Less Inflammatory Cytokines

As *in vivo* data regarding the connection between innate cytokine responsiveness and vitamin D nutritional status is inconclusive, with some claiming higher $25(OH)D_3$ levels correlate with lower systemic inflammatory cytokines levels, we sought to investigate whether increased circulating vitamin D levels correlated with decreased systemic inflammatory cytokine production amongst our subjects. According to our results increased $25(OH)D_3$ levels do not correlate with decreased pro- or anti-inflammatory systemic cytokine levels **Figure 14**. We decided to pursue this notion further and ask whether or not isolated PBMC from individuals with higher $25(OH)D_3$

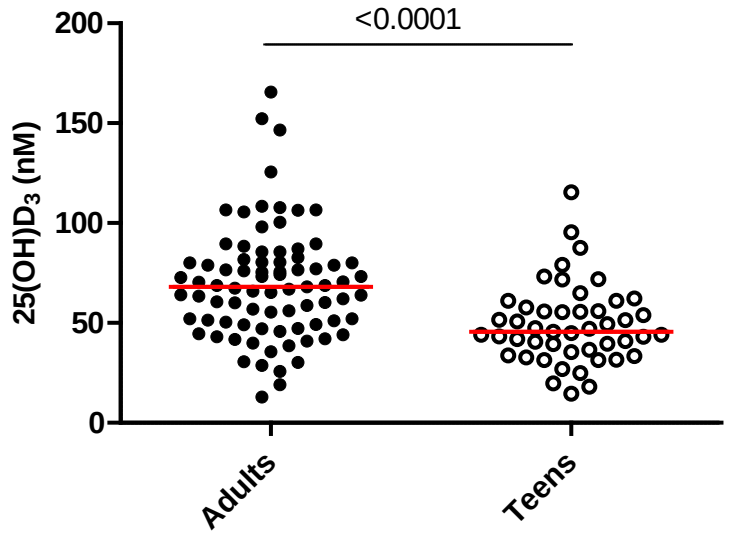

Figure 12A: Teens exhibit lower circulating 25(OH)D_3 than adults. Plasma samples from adults (n = 82) and teens (n =47) were analyzed by mass spectrometry for 25(OH)D_3 concentration (nM). Statistical analysis was performed using Mann Whitney test. Red bar represents the median.

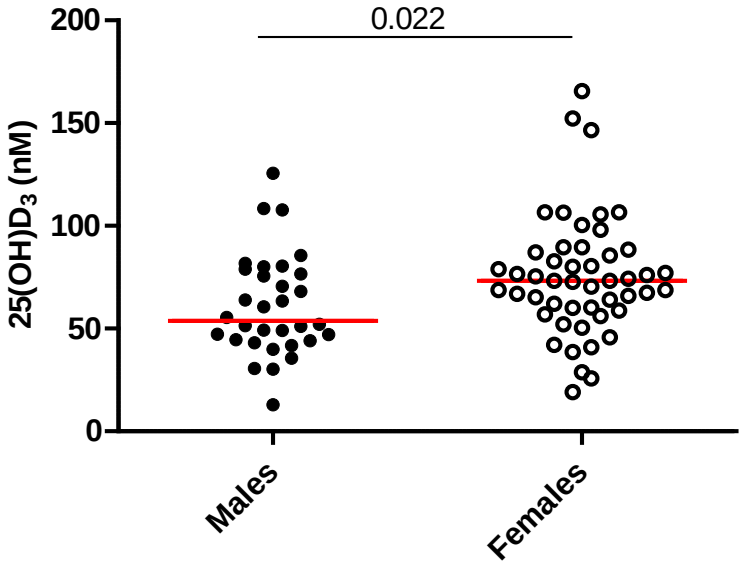

Figure 12B: Males exhibit lower circulating 25(OH)D_3 than females. Plasma samples from adult males (n = 33) and adult females (n =49) were analyzed by mass spectrometry for 25(OH)D_3 concentration (nM). Statistical analysis was performed using Mann Whitney test. Red bar represents the median.

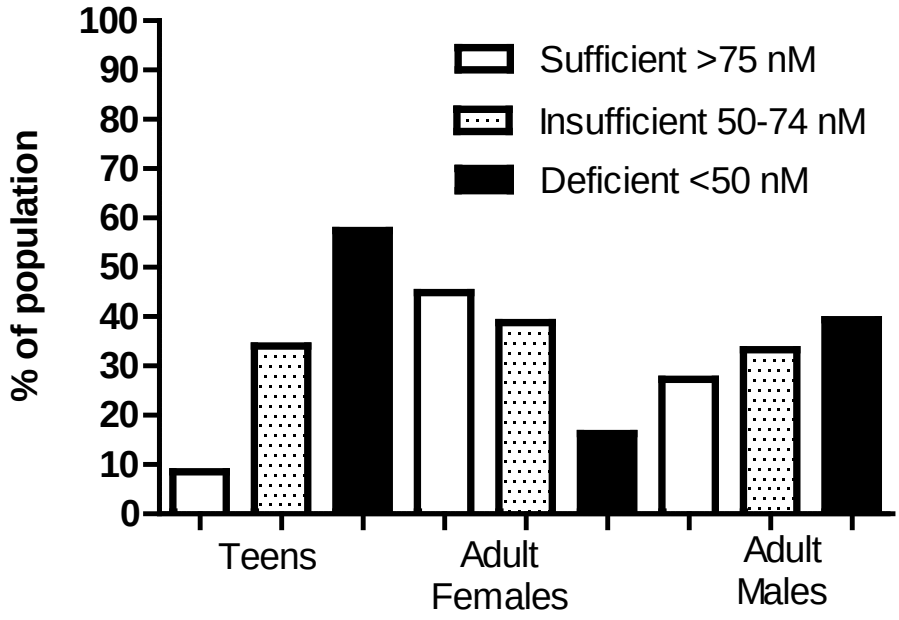

Figure 12C: Teens exhibit highest rate of vitamin D deficiency. Plasma samples from teens (n =47), adult females (n = 49) and adult males (n =33) were analyzed by mass spectrometry for 25(OH)D_3 concentration (nM) and categorized according to 25(OH)D_3 status and age and gender of individual.

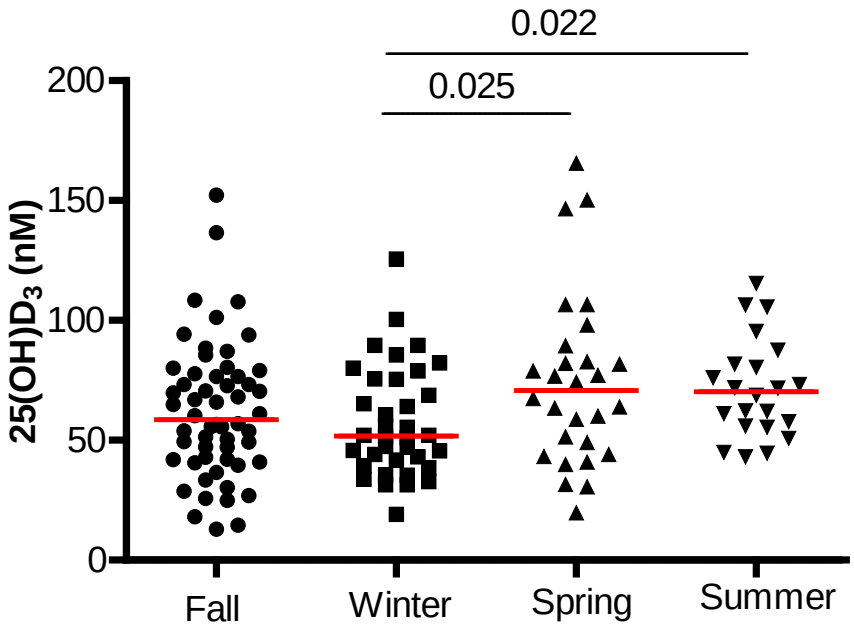

Figure 13: Individuals in the population collectively display lower 25(OH)D_3 levels during winter months. Plasma samples from adults were analyzed by mass spectrometry for 25(OH)D_3 concentration (nM) and samples were separated into season collected (n=143). Statistical analysis was performed using unpaired t-test. Red bar represents the median.

status *in vivo* had lower pro-inflammatory levels compared to those of lower $25(OH)D_3$ status. Here the data shows that resting PBMC from individuals with higher $25(OH)D_3$ tend to produce lower levels of CXCL-8 and CCL-2 **Figure 15**, thereby suggesting that perhaps vitamin D can regulate *in vivo* innate immune responsiveness. As the previous chapter showed that LPS induced inflammatory cytokine responses could be decreased upon the addition of exogenous vitamin D, we thought that perhaps PBMC from individuals with increased endogenous $25(OH)D_3$ would have dampened inflammatory cytokine responses to bacterial stimulation compared to those with lower $25(OH)D_3$. However, our results showed that people with higher $25(OH)D_3$ plasma concentrations have increased CCL-2 and CXCL-8 levels when their PBMC are stimulated with LPS. These results opposed our original hypothesis which stated that increased endogenous $25(OH)D_3$ would correlate with decreased cytokine responsiveness to LPS.

CBMC from Cord Blood are Less Susceptible to Vitamin D Modulation in the Presence of LPS than PBMC from Adults

As our previous studies in adults showed that vitamin D was able to modulate innate cytokine responses to LPS *in vitro*, we sought to repeat these experiments in cord blood CBMC to determine whether exogenous vitamin D suppressed innate cytokine responses in neonates. Here our data shows that addition of exogenous $1,25(OH)_2D_3$ to LPS stimulated CBMC resulted in significant decreases in CCL-2, CXCL-8, IL-6 and IL-10 **Figure 17.** However, comparison between the levels of cytokine inhibition in the presence of exogenous vitamin D in adults versus that of neonates shows that neonates

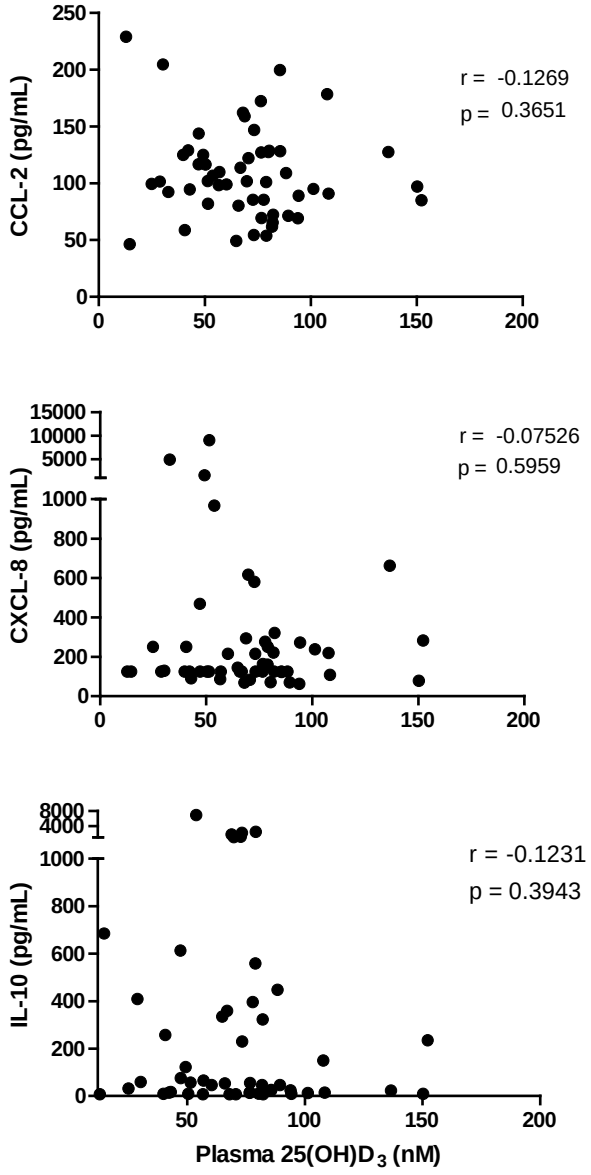

Figure 14: Inflammatory cytokine levels in plasma do not correlate with circulating vitamin D levels. Plasma samples were analyzed by mass spectrometry for 25(OH)D_3 concentration (nM) and by ELISA for IL-10, CXCL-8 and CCL-2 concentrations (pg/mL) (n =50-53). Correlation analysis was performed using Spearman test.

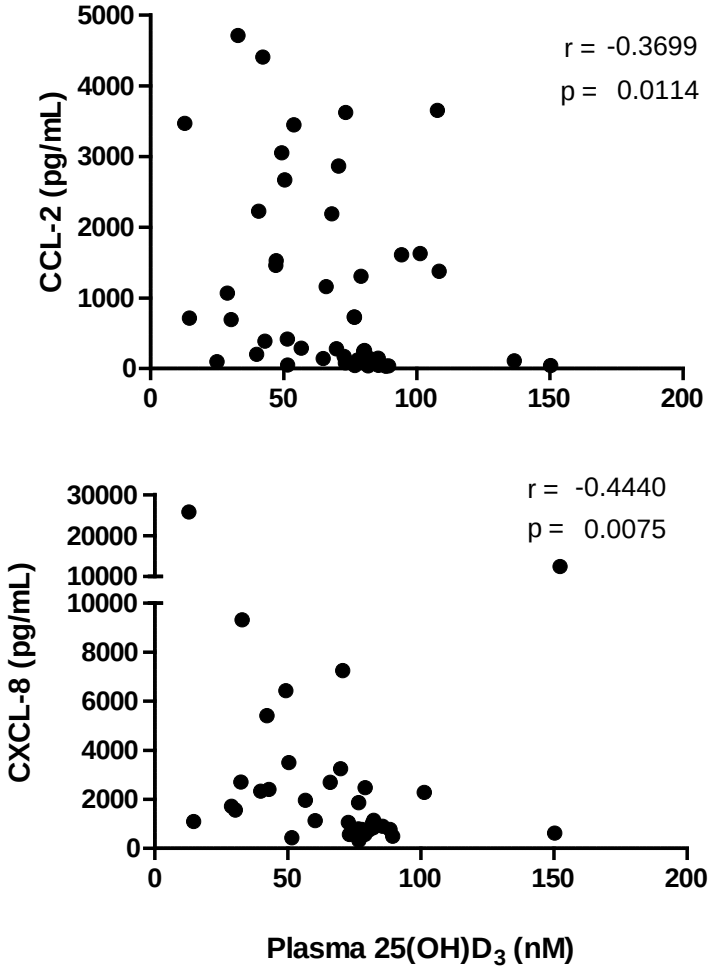

Figure 15: Basal pro-inflammatory cytokine levels in unstimulated PBMC negatively correlate with circulating vitamin D levels. Plasma samples were analyzed by mass spectrometry for 25(OH)D_3. ELISA was used to measure CXCL-8 and CCL-2 concentrations from unstimulated PBMC cultured for 24 hours (n= 35-47). Correlation analysis was performed using Spearman test.

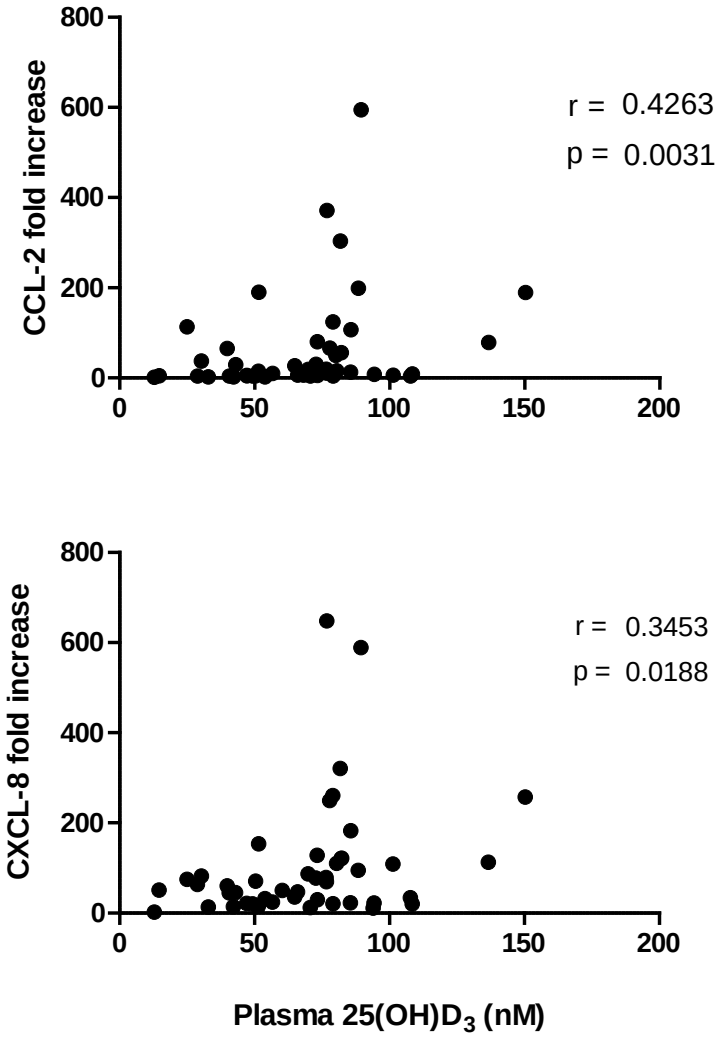

Figure 16: LPS induced pro-inflammatory cytokine production positively correlates with circulating vitamin D levels. Plasma samples were analyzed by mass spectrometry for 25(OH)D_3. ELISA was used to measure CXCL-8 and CCL-2 concentrations from PBMC stimulated with LPS for 24 hours (n=46). Correlation analysis was performed using Spearman test.

are less susceptible to vitamin D modulation than that of adults. This is further evident in **Table 5** where the average percent inhibition for each cytokine in the presence of all three concentrations of exogenous vitamin D is documented, and a comparison between adults and neonates is shown. Here the average neonate experiences a 25% reduction in innate cytokine production in the presence of 100 nM $1,25(OH)_2D_3$, whereas the average adult experiences a 38% reduction. The difference between neonate and adult cytokine suppression in the presence of $1,25(OH)_2D_3$ is most dramatic in the pro-inflammatory chemokine CCL-2 where adults experience on average 57% reduction and neonates only 27% with a p value of 0.0006. Interestingly, comparing the inhibition percentages in IL-10 between neonates and adults shows that both groups experience similar degrees of inhibition in the presence of 100 nM of $1,25(OH)_2D_3$ with neonates producing 23% less cytokine and adults 24% less **Table 5**. These results suggest that exogenous active vitamin D may play a larger role in adult innate immune responsiveness than infant.

Vitamin D Receptor is not Correlated to $25(OH)D_3$ Plasma Concentrations, but is Expressed at a Lower Level in CBMC than PBMC

As endogenous $25(OH)D_3$ was shown to negatively correlate to decreased basal pro-inflammatory cytokine production, our next step was to see if VDR expression in PBMC directly *ex vivo* correlated with vitamin D plasma concentrations. Here our hypothesis was that increased plasma $25(OH)D_3$ would lead to increased VDR expression, which in turn would increased $1,25(OH)_2D_3$/VDR binding thereby suppressing basal pro-

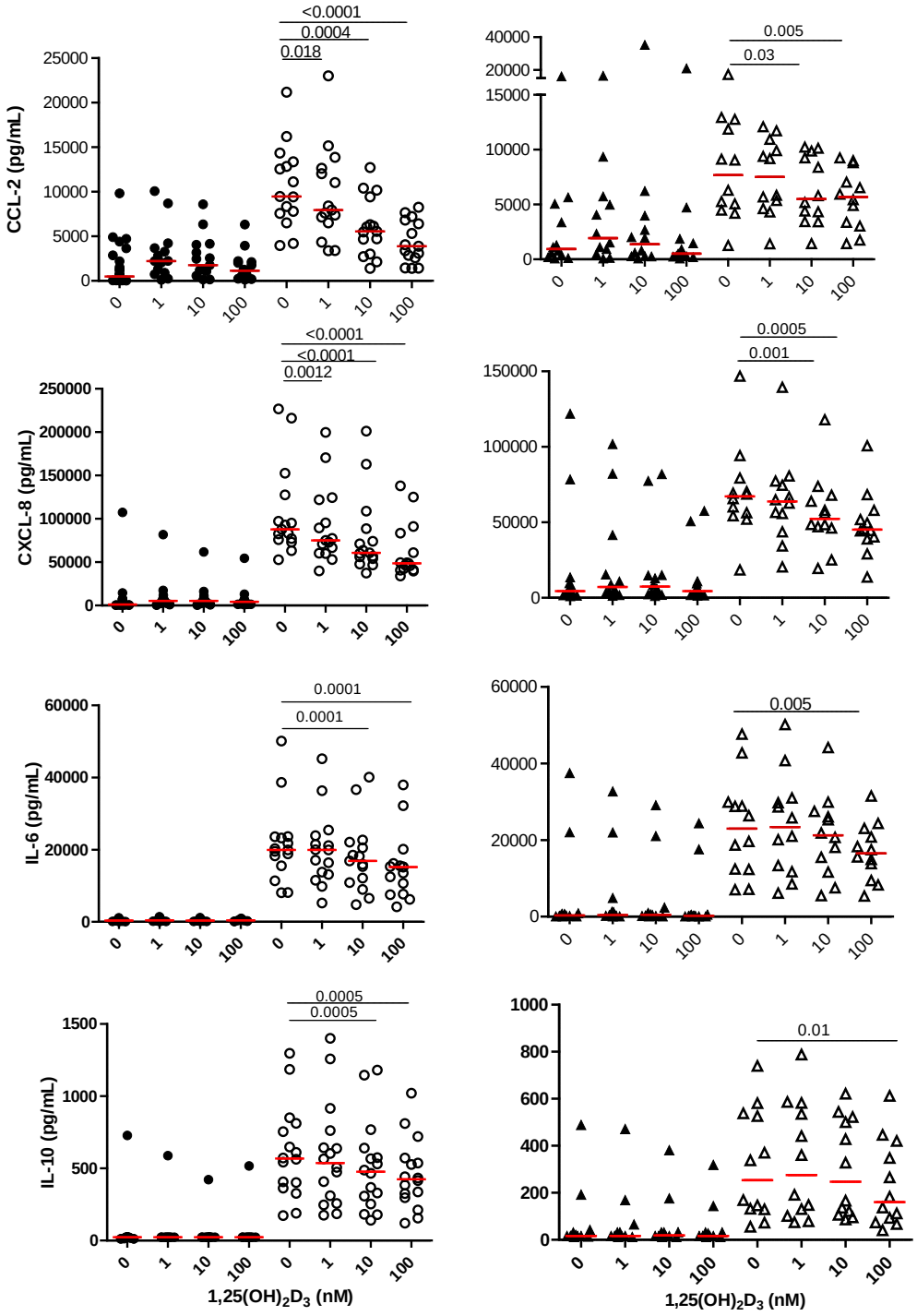

Figure 17: LPS induced inflammatory cytokine production is modulated less in CBMC stimulated with vitamin D than adult PBMC. ELISA was used to measure CCL-2, CXCL-8, IL-6 and IL-10 concentrations from PBMC (circles) and CBMC (triangles) stimulated with LPS (open shapes) or CM (filled shapes) for 24 hours in the presence or absence of varying concentrations of vitamin D (n=12-15). Red bar represents the median.

Table 5. Exogenous vitamin D decreases LPS induced cytokine production in healthy adult and cord blood samples

Cytokines/ Chemokines	% inhibition (10^{-9}M Vitamin D)		% inhibition (10^{-8}M Vitamin D)		% inhibition (10^{-7}M Vitamin D)	
	Adults	***Cords***	***Adults***	***Cords***	***Adults***	***Cords***
CCL-2	12	0	43	17	57	27
CXCL-8	13	1	26	18	41	29
IL-6	6	0	18	6	31	21
IL-10	6	0	18	0	24	23

inflammatory cytokine production. However, according to our data there is no correlation between endogenous $25(OH)D_3$ and increased VDR expression **Figure 18A**.

As CBMC from cord blood was less susceptible to exogenous $1,25(OH)_2D_3$ innate cytokine modulation, we sought to determine whether VDR was expressed at a similar level across different age groups. Since our results from Chapter 2 showed that PBMC stimulated with 3M 002 had lower VDR expression, which may be the reason for the lack of cytokine modulation in PBMC stimulated with active vitamin D and 3M 002, we measured VDR expression in CBMC of neonates and PBMC from 1 year olds, 3 year olds, teens, adults and seniors. Here the data shows that neonates express significantly lower VDR levels when compared to teens and adults **Figure 18B**. These results prove quite novel and suggest that perhaps lower VDR expression leads to the weakened innate cytokine modulation by exogenous vitamin D.

Discussion

According to current literature vitamin D deficiency is prevalent amongst the population especially in colder climates (114,115,149,155,156). As this notion is quite established our results showing the rates of vitamin D deficiency amongst the population were expected to be similar to literature. However, the degree to which the teen population suffers from vitamin D deficiency is quite shocking. Here we found that almost 60 percent of teens were vitamin D deficiency with an average status of 50 nM, whereas other studies have shown that teens elsewhere have 30 percent deficiency

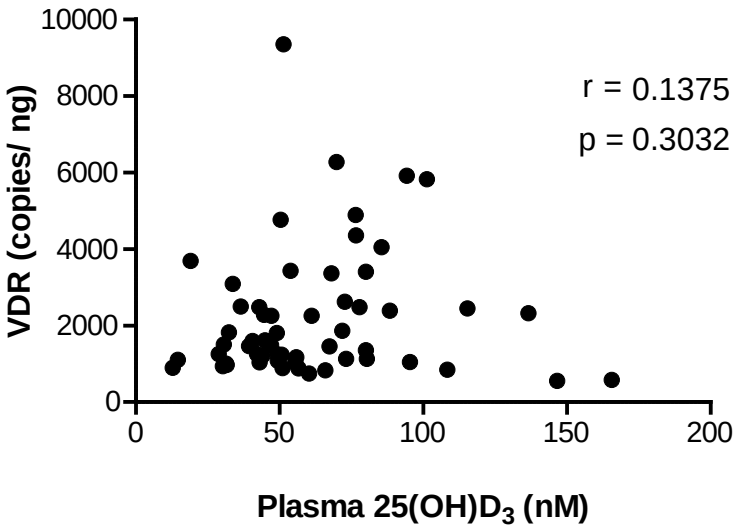

Figure 18A: Endogenous vitamin D plasma levels are not correlated to vitamin D receptor expression in PBMC. Plasma samples were analyzed by mass spectrometry for 25(OH)D_3. RT-PCR was used to measure VDR mRNA levels in PBMC from the same individuals (n=58). Correlation analysis was performed using the Spearman test.

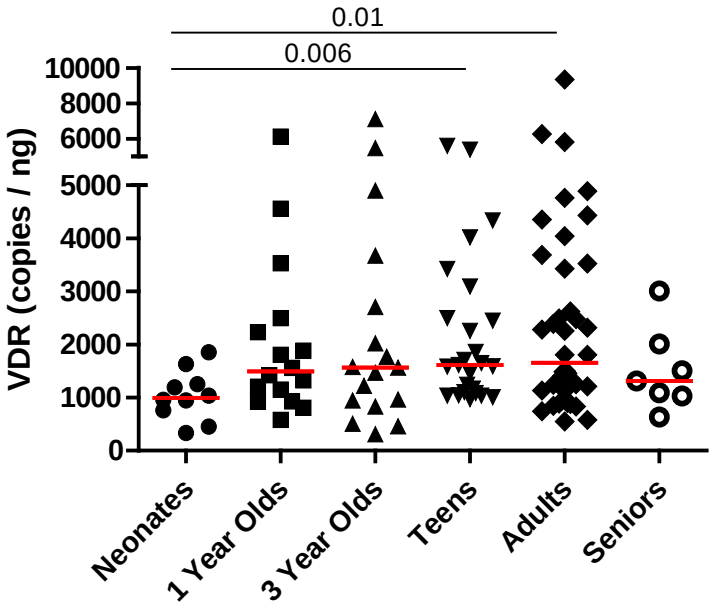

Figure 18B: VDR expression is lower in CBMC from neonates. RT-PCR was used to determine VDR mRNA levels in PBMC or CBMC from individuals (n=115). Statistical analysis was performed using Mann Whitney test. Red bars represent median.

rates with an average status of 73 nM (149). Research that has focus on vitamin D status in Canada indicate that the average teen $25(OH)D_3$ level is 65 nM (230). These results indicate that vitamin D deficiency is common in Canada especially Winnipeg and therefore needs to be addressed and taken seriously.

Studies connecting serum vitamin D levels and immune responsiveness are inconclusive. Some researchers have shown that supplementation can suppress eotaxin, IL-12, CCL-2, CCL-3, yet not CXCL-8, CXCL-10 and CCL-5 (153). However, others have shown that IL-6, CXCL-8, TNF-α (231) and CRP (232) are reduced upon supplementation, yet some show no difference in pro-inflammatory cytokine production (152,210). Our data on endogenous $25(OH)D_3$ levels and plasma innate cytokine responses did not show a correlation, however, we did observe that isolated PBMC produced less CCL-2 and CXCL-8 in individuals with higher vitamin D status. Thus, even though plasma cytokine levels do not correlate with vitamin D nutritional status the amount of pro-inflammatory cytokines being produced by PBMC decreases with increasing $25(OH)D_3$ status indicating that endogenous vitamin D may aid in the regulation or suppression of inflammatory cytokine production.

Khoo et al showed that TLR2 and TLR4 are lower in the summer compared to the winter and that PBMC responsiveness to bacterial ligands are suppressed during the summer months as well, which is thought to be due to increased vitamin D levels (115). Therefore, taking our observations with that of Khoo et al, decreased vitamin D status in the winter may lead to increased PBMC CCL-2 and CXCL-8 production which would be

further augmented in the presence of a bacteria. Interestingly, our results on PBMC responsiveness to LPS stimulation with increased endogenous vitamin D levels showed that individuals with higher vitamin D status have increased responsiveness to LPS than individuals with lower status. These results appear to oppose our previous findings that exogenous vitamin D decreases PBMC responsiveness to LPS.

In terms of neonatal vitamin D status, many studies have shown that maternal vitamin D status strongly correlates with neonatal status and that the same seasonal variations in $25(OH)D_3$ are applicable to infants as well (233,234). However, few studies show whether cord blood mononuclear cells are able to respond to endogenous or exogenous vitamin D. Here one study showed that umbilical cord cells from neonates with increased endogenous vitamin D levels produce significantly more IFN-γ in the presence of LPS in comparison to cells from infants with lower vitamin D status (210). However, when other cytokines or stimuli were compared the authors claimed that no clear pattern was observed between cord $25(OH)D_3$ levels and immunological cytokine responsiveness. Our results show that CBMC are less susceptible to vitamin D modulation in comparison to adult PBMC. Here we observed that in the presence of LPS stimulation addition of 100 nM of exogenous vitamin D was only able to reduce innate cytokine responses in cord blood by 25% whereas adult cytokine responses were reduced 24-57% depending on the cytokine. As several studies have recently shown that lower vitamin D levels in infants correlates with increased risk of allergic disease (233,235), it is important to understand what status of vitamin D is necessary for appropriate neonatal immune development. Our results indicate that infants may

require higher vitamin D levels in order to achieve innate immune regulation similar to that of adults. However, as further research into the difference between cord and adult susceptibility to vitamin D modulation showed that cord blood cells express less VDR than adult PBMC, it is possible that increased neonatal supplementation may not result in increased cytokine inhibition. Therefore, further studies should be directed towards understanding the difference between adult and infant responsiveness to vitamin D and whether increased supplementation during infancy would be beneficial.

To summarize, in this chapter we have shown that vitamin D deficiency is widespread among the Winnipeg population and that sufficient vitamin D status vs insufficient or deficient may aid in pro-inflammatory cytokine regulation. Interestingly, we observed differences in neonatal immune responsiveness to exogenous active vitamin D, which may in part be due to decreased VDR expression in early life. These results may help target populations at risk of vitamin D deficiency and aid in determining appropriate vitamin D serum levels in different age groups.

CHAPTER 3

Poly(I:C)/Lyovec Induces Expression of Viral Associated Genes

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Chapter 3 is based on draft of paper that is currently in preparation

Abstract

Rationale: Viral infections are a major health problem worldwide, yet ways to control these infections are not well understood. PRRs (TLR3, TLR7/8 and RLRs) recognize viral associated molecular patterns, thereby activating an innate antiviral immune response. RLRs are one such group of PRRs that respond to viral nucleic acids, yet little is known about the cytokine profile and gene expression that is associated with this recognition. Current studies focus almost entirely on the type I IFN response that follows RLR activation, however these readouts are far from robust. Thus, to better understand how innate immune cells recognize viral nucleic acids using RLRs it is imperative we look at other cytokines and genes whose expression typically follows viral antigen recongition.

Objectives: To determine which pro-inflammatory and anti-inflammatory genes and cytokines are activated in human PBMC stimulated with synthetic RLR ligand, Poly(I:C)/Lyovec. Furthermore, to understand which cells are the major players in Poly(I:C)/Lyovec recognition by RLRs and whether LGP2 can regulate this recognition.

Methods: PBMC were isolated and cultured with Poly(I:C)/Lyovec for 6-24 hours. ELISA and RT-PCR were used to determine cytokine responses and mRNA expression, respectively. In other experiments CD14 enriched and depleted cells were stimulated and cultured with Poly(I:C)/Lyovec for 24 hours.

Results: Titration experiments for Poly(I:C)Lyovec showed optimal pro-inflammatory cytokine production at 250-300 ng/mL. Kinetics experiments revealed that PBMC

stimulated with Poly(I:C)/Lyovec had maximal increased expression for all virally associated inflammatory/anti-inflammatory genes tested at 24 hours. Monocytes proved necessary for Poly(I:C)/Lyovec recognition by RLRs, whereas isolated lymphocytes were unresponsive. Looking into RLR activation induced by Poly(I:C)/Lyovec in a large cohort of healthy individuals revealed increased expression of multiple RLRs, IFN stimulating genes, IFNs, and anti-inflammatory molecules. Cytokine production analysis in these individuals showed enhanced CCL-2, CCL-8, CXCL-10 and IFN-β as optimal biomarkers of RLR activation.

Conclusions: These results argue that stimulation of PBMC by Poly(I:C)/Lyovec activates a potent anti-viral and inflammatory innate immune response mediated by monocytes. Kinetics and titration studies show that this response is optimal at 24 hours in the presence of 250-300 ng/mL Poly(I:C)/Lyovec. Future studies will assess whether similar innate immune responses upon Poly(I:C)/Lyovec stimulation is apparent in developing infants, and older individuals as well. As viral infections in infants have been linked to increased prevalence and severity of asthma it is imperative we understand innate immune viral mechanisms in developing children.

Results

Poly(I:C)/Lyovec Induces Maximal Expression of Virally Associated Genes and Cytokines After 24 hour and 250 ng/mL Stimulation

RLRs are a type of cytosolic PRR that play a key role in recognizing and initiating active defense mechanisms against viral infection by binding viral dsRNA (236). To study the virally triggered RLR-pathway we utilized a synthetic dsRNA analog of viral RNA, linked to an agent to allow membrane penetration, Poly(I:C)/Lyovec. Although naked Poly(I:C) can also bind to TLR3, Poly(I:C)/Lyovec is specific for RIG-I/MDA-5. Therefore, to begin exploring and identifying optimal markers of viral immune responsiveness, kinetics and concentrations were determined for several viral associated genes and cytokines/chemokines **Figure 19-20**. Here Poly (I:C)/Lyovec was found to induce RLR (RIG-I, MDA-5, LGP2) mRNA expression best at 24 hours **Figure 19**. Other PRRs were also assessed (TLR4 and TLR8) to determine whether Poly (I:C)/Lyovec was selective in RLR induction or if it indirectly (or non-specifically) enhanced their expression; here the results indicated that both TLR4 and TLR8 are not induced upon stimulation with Poly(I:C)/Lyovec **Figure 19B.** IFN stimulated genes (ISG) 15 and 56 were also optimized and found to be optimally enhanced in PBMC stimulated with Poly(I:C)/Lyovec at 24 hours **Figure 19A**. Pro-inflammatory (CXCL-10), anti-inflammatory cytokine/chemokine (IL-1R antagonist) as well as Type I (IFN-α, IFN-β1) and III IFNs (IL-29) were induced upon Poly(I:C)/Lyovec stimulation, all peaking at 24 hours as well **Figure 19A**. However, other pro-inflammatory (IL-6), anti-inflammatory (IL-10, soluble TNF receptor 2 (sTNFRII)) and Type III IFNs (IL-28) were not enhanced upon stimulation with Poly(:IC)/Lyovec **Figure**

19B. These results show that Poly(I:C)/Lyovec stimulation in adult PBMC results in peak expression of innate PRR, Type I and III IFN, pro-inflammatory, IFN signalling molecules, and regulatory gene expression at 24 hours and therefore may be a useful tool in the analysis of viral driven immune responses.

To assess the optimal concentration of Poly(I:C)/Lyovec in primary tissue culture we chose two representative pro-inflammatory cytokines that were found to give a response to this ligand. Results showed that cytokine production was significantly enhanced at most concentrations when PBMC were treated with Poly(I:C)/Lyovec. Here it was noticed that median responses were not substantially different between stimulating conditions of 100 and 300 µg/mL and therefore we chose 250 ng/mL of Poly(I:C)/Lyovec as our concentration for future experiments **Figure 20**.

Monocytes are the Predominate Cell Type Responsible for Anti-viral Response Against Poly(I:C)/Lyovec

A typical PBMC population is comprised of T cells, B cells, monocytes, NK cells and DC. Monocytes, and to a lesser extent DC have been shown to express RLRs, RIG-I and MDA-5 (237). Interestingly, many other cell types, both immune and non-immune, express RLRs including EC (53), NK cells (reviewed by (54)), neutrophils (55), and even eosinophils (56). Thus, to determine whether monocytes are a principal contributor to Poly(I:C)/Lyovec driven production of anti-viral responses, CD14 monocyte enriched and CD14 depleted lymphocyte populations were stimulated with Poly(I:C)/Lyovec for 24 hours and then assessed for CXCL-10 and CCL-8 responses. Here the purity of the

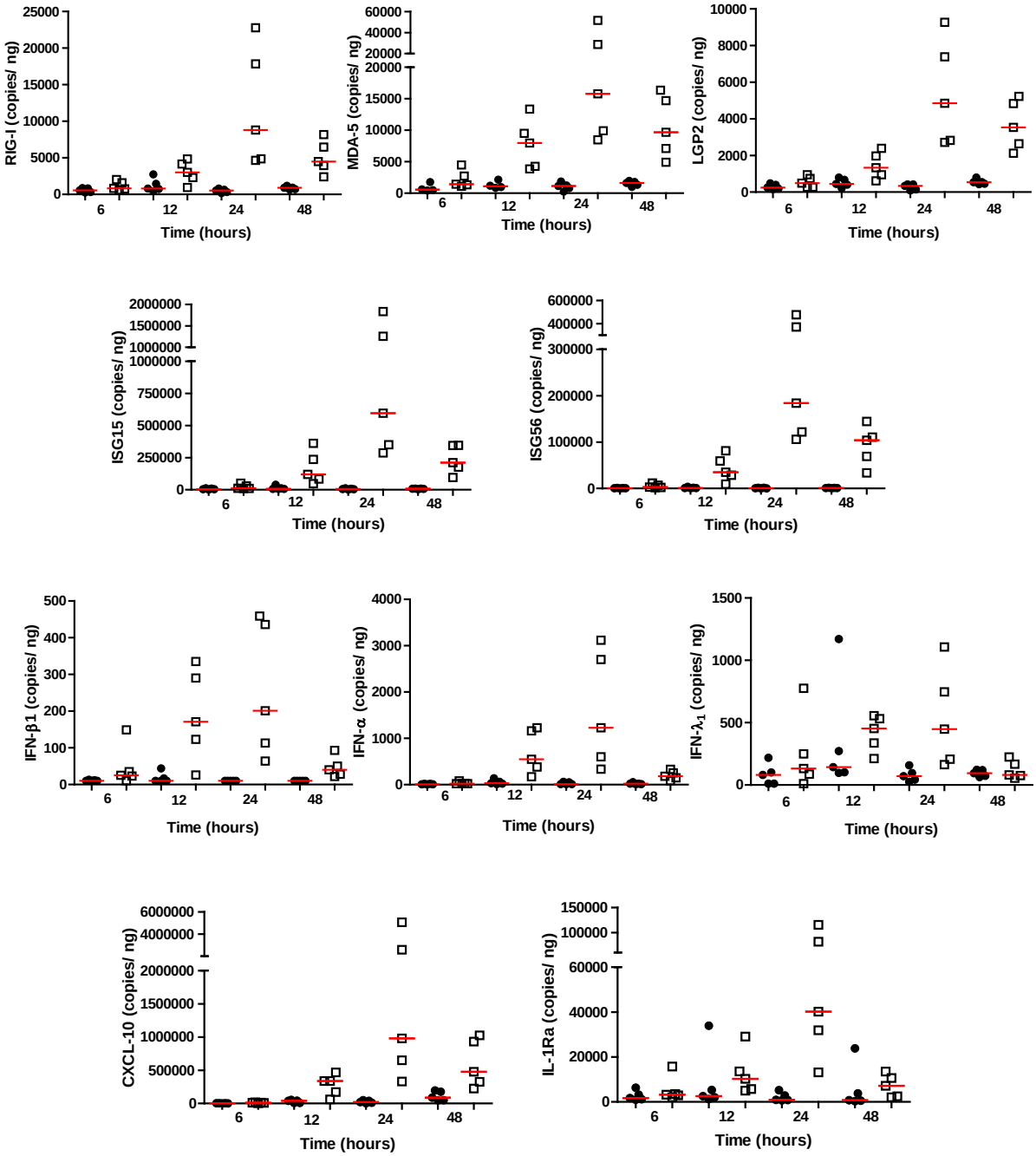

Figure 19A:Poly(I:C)/Lyovec kinetics peaks at 24 hours. PBMC were stimulated with CM and 250 ng/mL Poly(I:C)/Lyovec for 6-48 hr. mRNA expression of RLRs (RIG-I, MDA-5, LGP2), pro-inflammatory cytokines/chemokines (IL-29, CXCL-10, IFN-β1, IFN-α) signalling molecules (ISG15, ISG56) and regulatory mediators (IL-1RA) were determined by RT-PCR. (n=5, filled circles represent cells stimulated with CM, empty squares represent cells stimulated with 250 ng/mL Poly(I:C)/Lyovec). Red bar represents median.

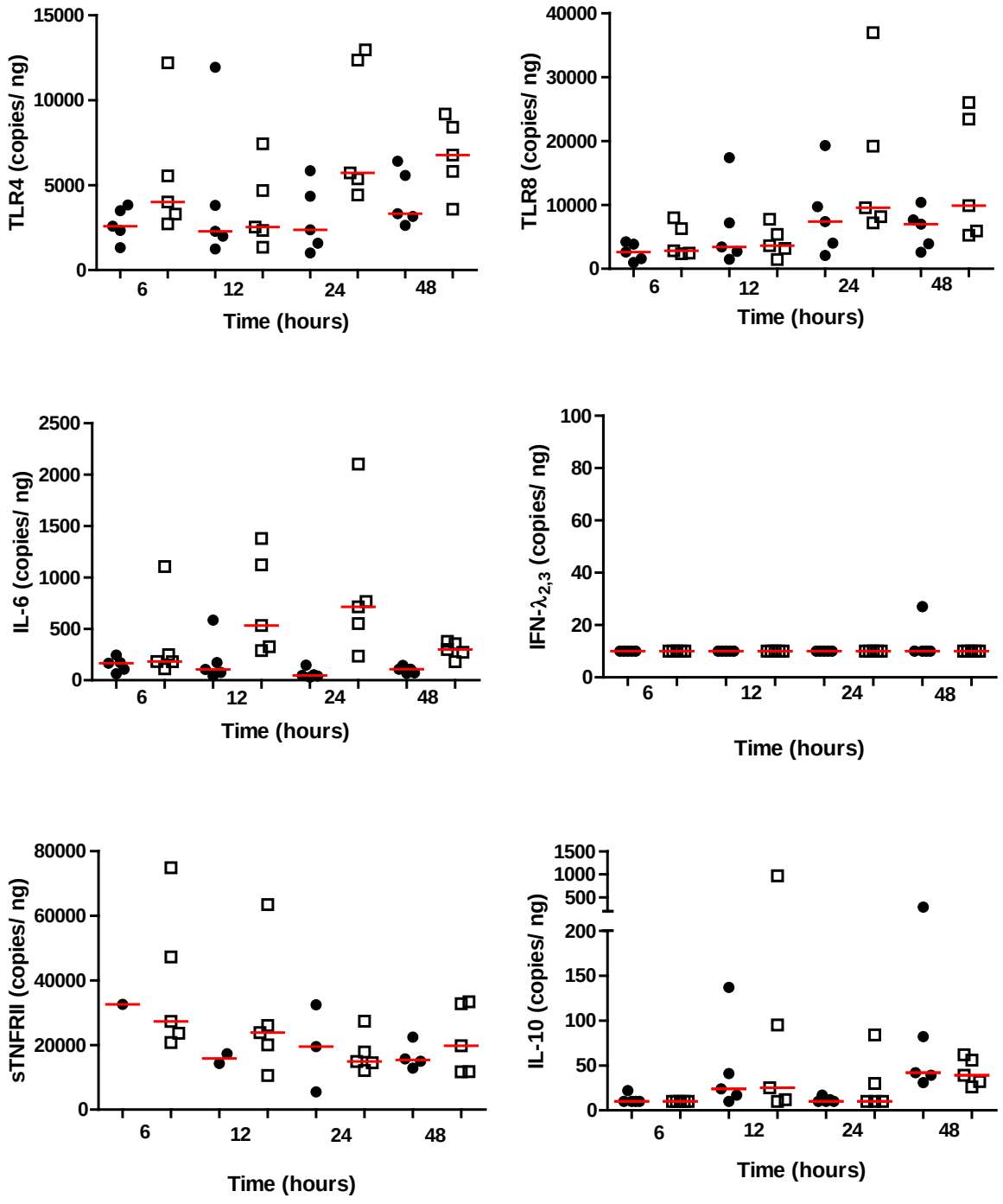

Figure 19B: Poly(I:C)/Lyovec does not enhance all pro- and anti-inflammatory markers. PBMC were stimulated with CM and 250 ng/mL Poly(I:C)/Lyovec for 6-48 hr. mRNA expression of TLRs (TLR4, TLR8), pro-inflammatory cytokines/chemokines (IL-6 and IL-28) and regulatory mediators (IL-10, sTNFRII) were determined by RT-PCR. (n=5, filled circles represent cells stimulated with CM, empty squares represent cells stimulated with 250 ng/mL Poly(I:C)/Lyovec). Red bar represents median.

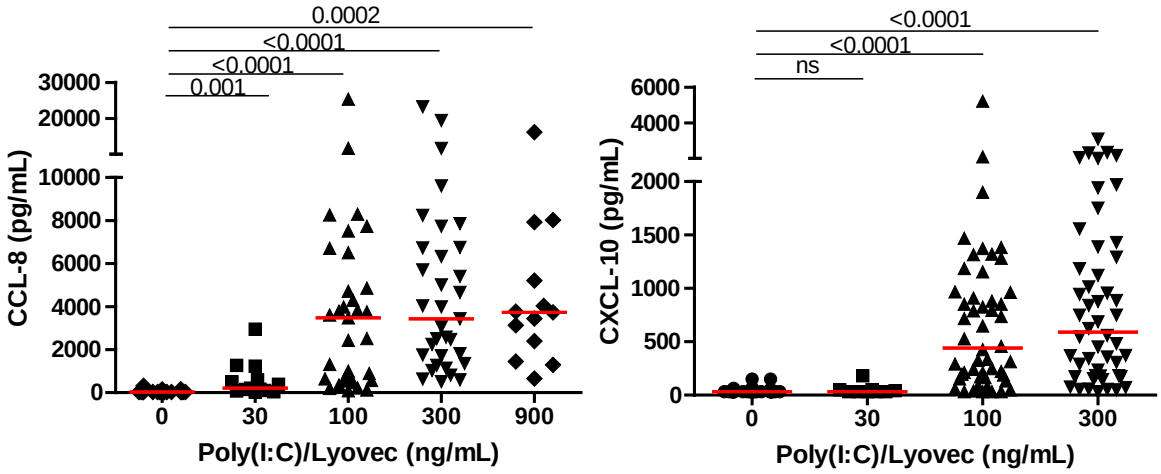

Figure 20:Poly(I:C)/Lyovec primary tissue culture concentration optimization. PBMC were stimulated with 0, 30, 100, 300 or 900 ng/mL Poly(I:C)/Lyovec for 24 hr. ELISA was used to determine CCL-8 and CXCL-10 in tissue culture supernatants (n=13-33). Done by Sara Courtis. Red bar represents median.

monocyte population that was isolated from PBMC, was 90%.

Our results show that CD14+ monocyte are responsible for the production of CXCL-10 and CCL-8 upon activation with Poly(I:C)/Lyovec **Figure 21**. As the CD14 depleted group was unable to elicit a CXCL-10 or CCL-8 cytokine response, these results confirmed our suspicion that monocytes, as opposed to B and T cells, are the main cell type responsible for production of these cytokines upon RLR stimulation with Poly(I:C)/Lyovec. Collectively, our results suggests that the peak expression of RLRs, IFN stimulated genes, IFNs, pro-inflammatory and anti-inflammatory cytokine production seen at 24 hours is mainly observed in the monocyte population as opposed to lymphocyte.

Microarray Analysis of Poly(I:C)/Lyovec Induced Gene Expression in Adult PBMC

Genome-wide microarray expression analysis was performed on three individuals, examining the 24 hour time-point of Poly(I:C)/Lyovec's induced PBMC transcriptome. While we investigated 30,000 genes, for this report we focused our analysis on viral defense response associated genes, as highlighted in **Table 6** and **Figure 22**. Gene expression profiling showed increased expression of various viral associated genes when PBMC were stimulated with Poly(I:C)/Lyovec. According to the data in **Table 6** CCL-8, interferon alpha inducible protein (IFI) 6, interferon-induced protein with tetratricopeptide repeats (IFIT) 1 and 3, 2',5'-oligoadenylate synthase (OAS) 1 and 3, apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) 3A, CXCL-10

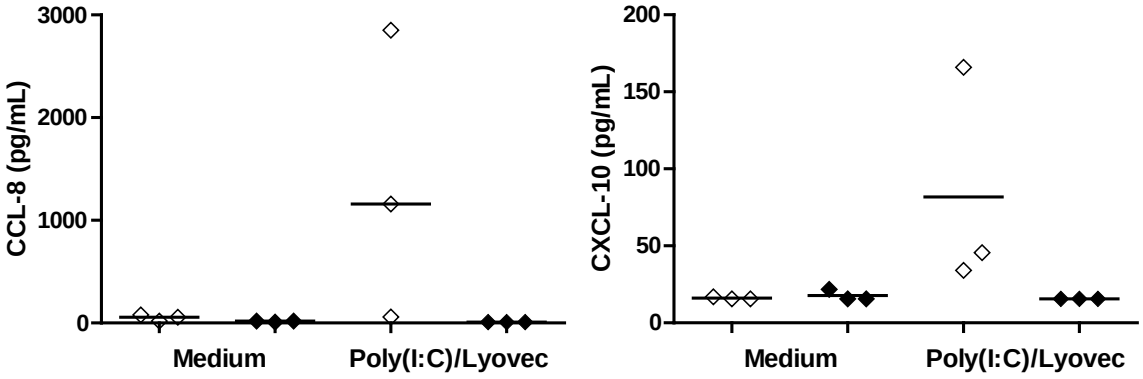

Figure 21: CD14+ monocytes are necessary for poly(I:C)/Lyovec driven pro-inflammatory responses. CD14 enriched monocytes (white) and CD14 depleted lymphocytes (black) were stimulated with Poly(I:C)/Lyovec (250 ng/mL) for 24hr. CCL8 and CXCL10 were quantified in tissue culture supernatants by ELISA (n=3). Done by Larisa Lotoski. Black bar represent median.

Table 6: Microarray analysis of Top 10 genes induced by Poly(I:C)/Lyovec

Gene	Fold increase
CCL-8	5.59
IFI6	5.51
IFIT1	5.36
IFIT3	5.31
ISG15	5.15
OAS1	4.60
OAS3	4.12
APOBEC3A	4.03
CXCL-10	4.01
MX1	3.94

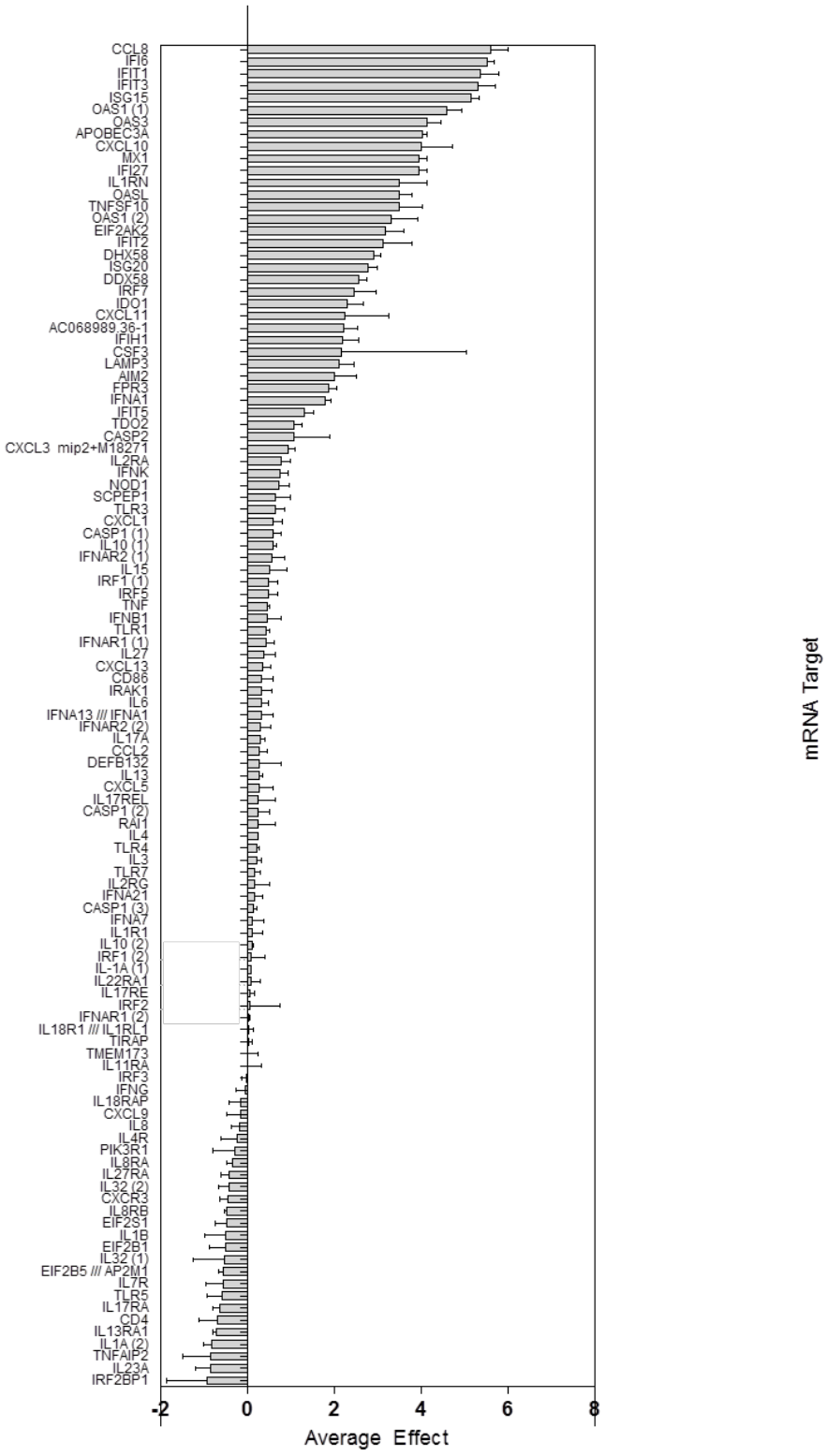

Figure 22: Poly(I:C)/Lyovec driven mRNA expression by microarray analysis. PBMC were stimulated with CM and Poly(I:C)/Lyovec for 24 hr. Mean ± SEM are shown (n=3). Done by Larisa Lotoski.

and myovirus resistance 1 (MX1) were the top genes induced in PBMC in the presence of Poly(I:C)/Lyovec. Most of these genes are IFN-inducible genes meaning that they are expressed in the presence of IFN to help generate an antiviral response; these include IFI6, IFIT1, IFIT3 and ISG15. Mx1 and APOBEC3A are also upregulated in the presence of IFN, where Mx1 is involved in trapping viral components (reviewed in (238)). OAS 1 and 3, which are also IFN inducible genes, were found to be upregulated in the presence of Poly(I:C)/Lyovec and are known for their involvement in the activation of ribonuclease L (RNaseL), which mediates RNA degradation (reviewed in (238)). CCL-8 and CXCL-10 were also found to be among top genes induced in the presence of Poly(I:C)/Lyovec, and this was also observed at the protein level in our previous experiments.

Poly(I:C)/Lyovec Enhances Expression of Virally Associated Genes and Anti-viral Proteins in Adult Population

Following the comprehensive microarray analysis, select pro-inflammatory, Type I and III IFN-family members, and regulatory mediators were examined by RT-PCR and ELISA in greater detail and in a larger population. As most studies that focus on RLR activation tend to be directed towards understanding receptor function and recognition, few studies examine the impact of RLR activation on primary cell responses (beyond type I IFNs and ISG), and virtually never in a heterogeneous human population. The data in **Figure 19** showed enhanced expression of innate immune responses in adult PBMC upon stimulation with Poly(I:C)/Lyovec, thus we wanted to determine how this phenomena translated to a larger population of individuals. Here RIG-I, MDA-5 and LGP2 were highly upregulated in adult PBMC after stimulation with Poly(I:C)/Lyovec **Figure**

23A. To assess whether there was enhancement of the IFN pathway after RLR stimulation, we examined two IFN inducible genes as well as Type I and Type III IFNs in adult PBMC. After stimulation with this synthetic viral ligand, ISG15 and ISG56 were highly upregulated in adult PBMC as was Type I and Type III IFNs **Figure 23B**. Further examination into cytokine responses emitted by adult PBMC revealed that the pro-inflammatory chemokines CCL-2, CCL-8 and CXCL-10 are significantly increased by Poly(I:C)/Lyovec, whereas CCL-5 is not **Figure 23C**. As anti-inflammatory cytokines play an important role in regulating an inflammatory response, we decided to examine whether stimulation of PBMC with Poly(I:C)/Lyovec enhanced this arm of the innate immune system. Here IL-1R antagonist expression was dramatically increased in response to stimulation with the synthetic viral ligand **Figure 23D**. However, when the regulatory mediators IL-10 and sTNFRII were examined for increased expression in the presence of Poly(I:C)/Lyovec, no difference was observed between stimulated and resting cells. These results indicate that Poly(I:C)/Lyovec is able to enhance all RLRs thereby eliciting an anti-viral, in terms of an IFN and inflammatory, response. However, Poly(I:C)/Lyovec does not augment CCL-5, which is a chemokine that is characterized as anti-viral, nor IL-6 or CXCL-8, which are innate pro-inflammatory cytokines. Therefore, Poly(I:C)/Lyovec may be a good indicator of RLR immune function, as it selectively upregulates some makers of viral and inflammatory response, but not others.

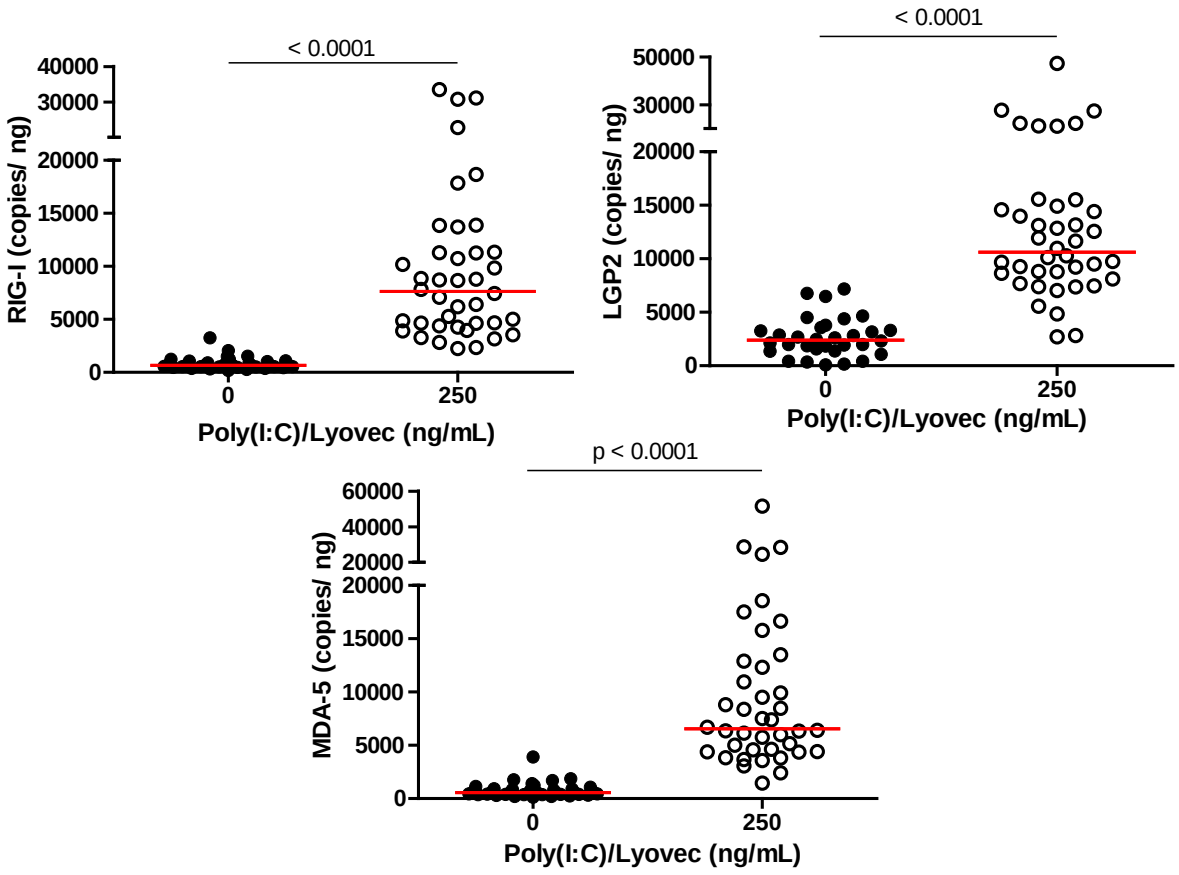

Figure 23A: RIGI/MDA-5 activation results in an upregulation of PRRs. mRNA expression of RLRs (RIG-I, MDA-5, LGP2) were determined by RT-PCR. (n=34-40, filled circles represent cells stimulated with CM, empty circles represent cells stimulated with 250 ng/mL Poly(I:C)/Lyovec). Red bar represents median.

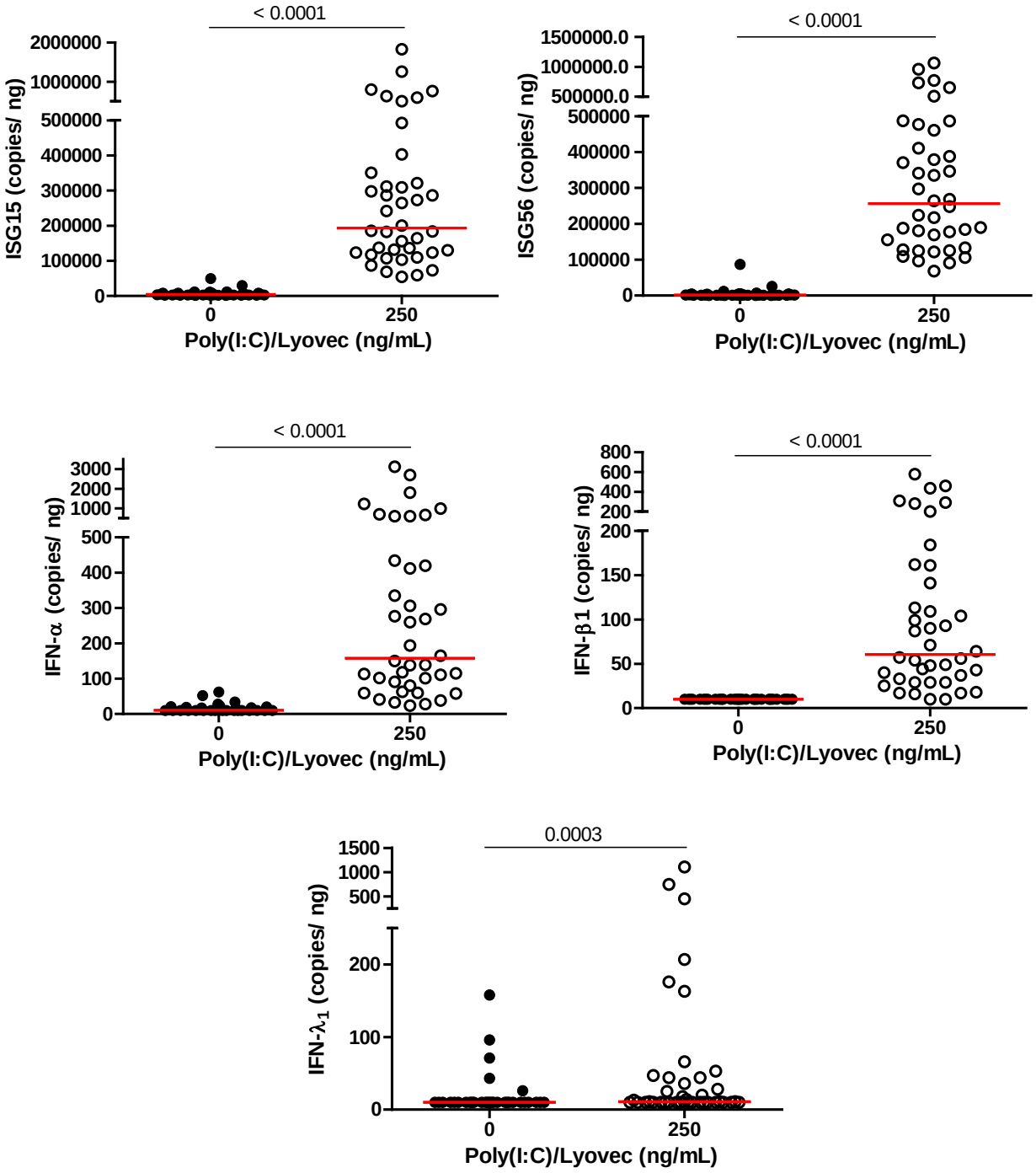

Figure 23B: RIGI/MDA-5 activation results in an upregulation of pro-inflammatory signalling molecules and type I/III IFNs. mRNA expression of ISG15, ISG56, IFN-α, IFN-β1 and IFN-λ_1 were determined by RT-PCR. (n=34-40, full circles represent cells stimulated with CM, empty circles represent cells stimulated with 250 ng/mL Poly(I:C)/Lyovec). Red bar represents median.

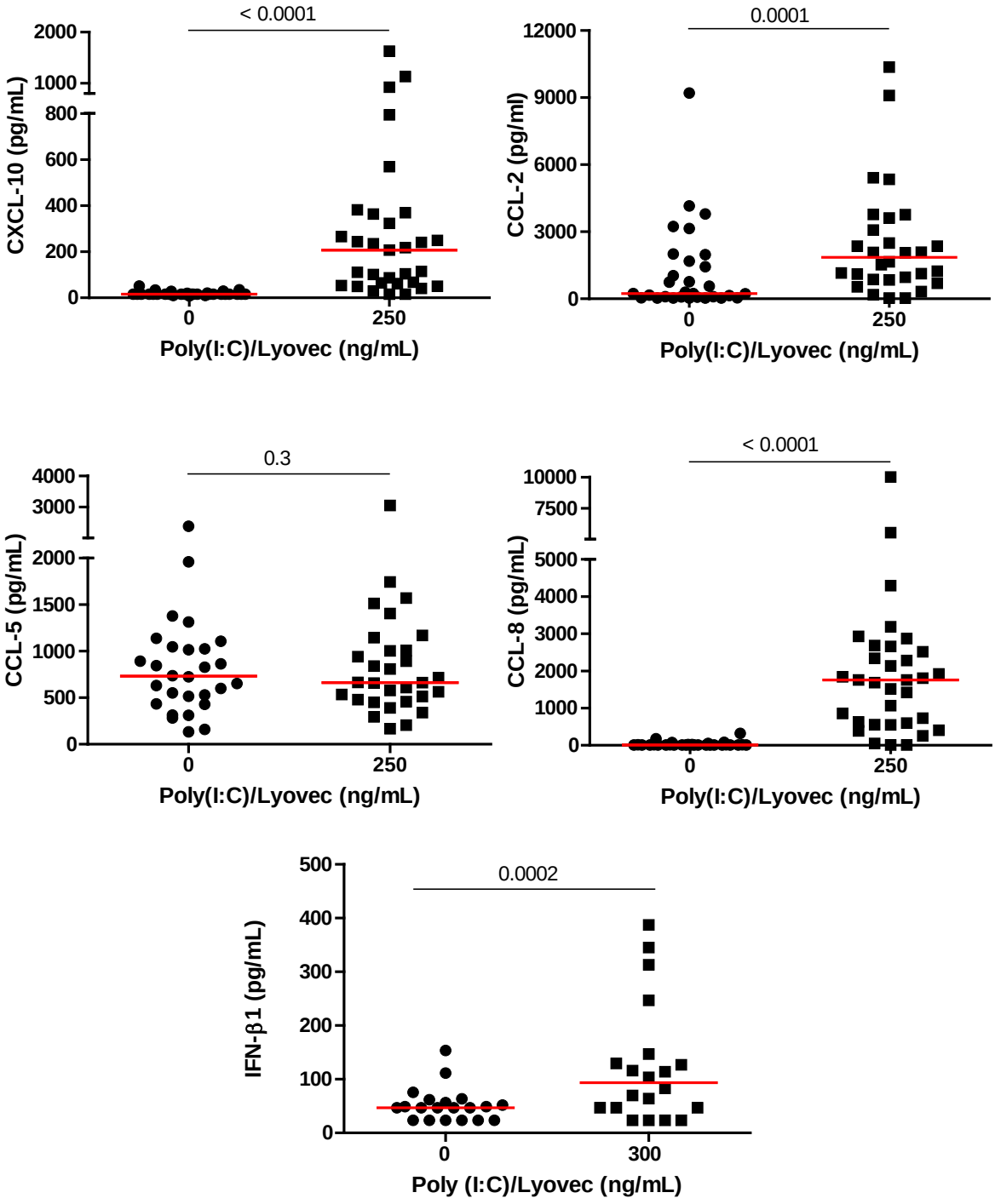

Figure 23C: RIGI/MDA-5 activation results in an upregulation of pro-inflammatory cytokines. Protein levels of CXCL-10, CCL-8, CCL-5, CCL-2 and IFN-β were measured in supernatants of tissue cultured cells by ELISA (n=20-30, filled circles represent cells stimulated with CM, full squares represent cells stimulated with 250 ng/mL Poly(I:C)/Lyovec). Done by Larisa Lotoski and Sara Courtis. Red bar represents median.

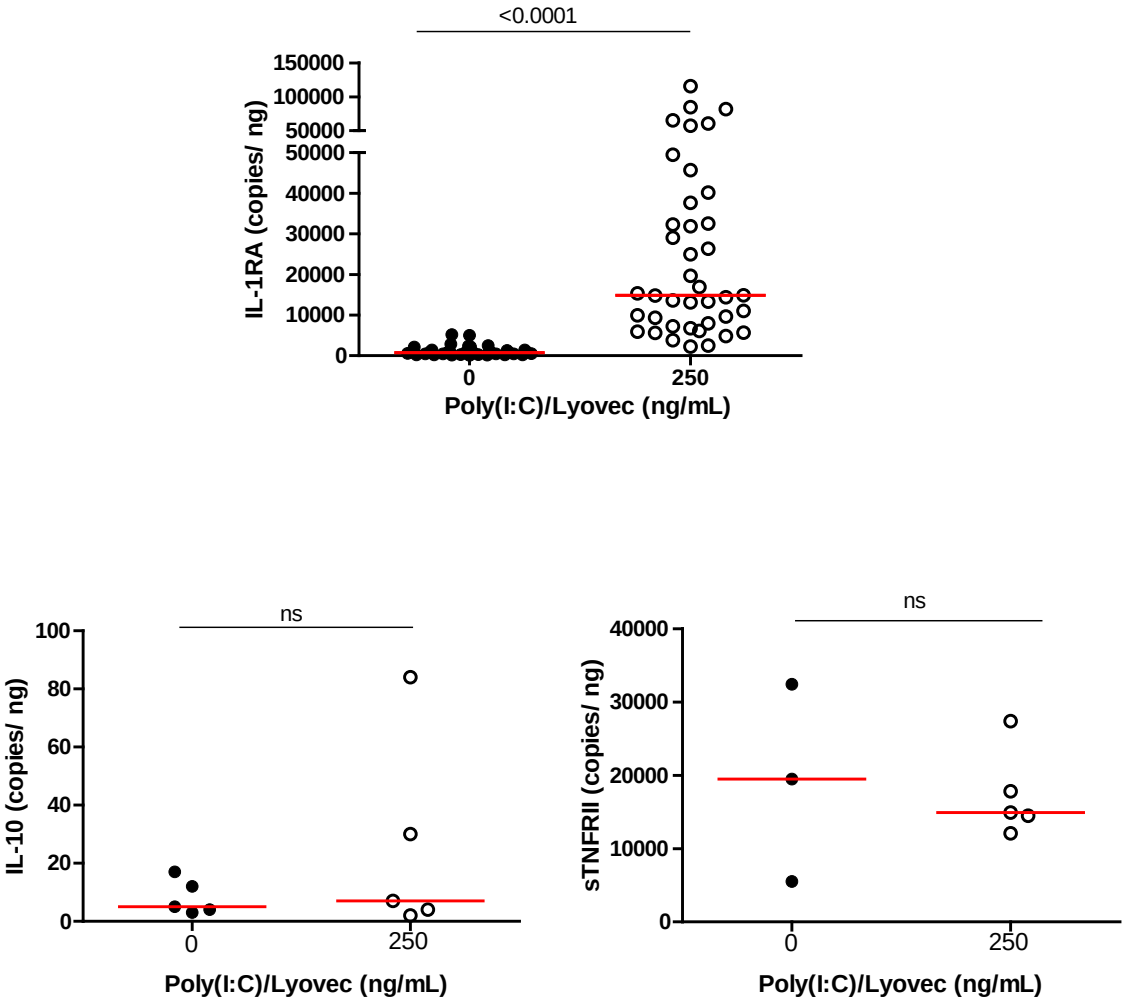

Figure 23D: RIGI/MDA-5 activation results in an upregulation of regulatory IL-1RA.mRNA expression of IL-1Ra, IL-10 and sTNFRII were determined by RT-PCR. (n=37-40 for IL-1Ra or 3-5 for IL-10 and sTNFRII, filled circles represent cells stimulated with CM, empty circles represent cells stimulated with 250 ng/mL Poly(I:C)/Lyovec). Red bar represents median.

Poly(I:C)/Lyovec Enhanced Expression of Anti-viral Mediators is not Dependant on PKR Activation

As we determined that PBMC stimulated with Poly(I:C)/Lyovec induces expression of viral associated genes and proteins, we wanted to determine whether this phenomena was the result of Poly(I:C)/Lyovec activating the RIG-I/MDA-5 pathway or whether protein kinase R (PKR) was also involved. As PKR is a cytosolic kinase that has been shown to bind dsRNA, such as Poly(I:C), we suspected that it may play a role in Poly(I:C)/Lyovec mediated antiviral response (239). To assess this issue a PKR inhibitor was added to PBMC cultured with Poly(I:C)/Lyovec, 5'ppp-dsRNA and naked Poly(I:C). CXCL-10 cytokine production was measured as a readout for antiviral mediated activity. Our results indicate that in the absence of PKR PBMC stimulated with Poly(I:C)/Lyovec do not have reduced CXCL-10 production. As CXCL-10 is not dependent on PKR activation by Poly(I:C)/Lyovec, these results show that Poly(I:C)/Lyovec is interacting with another receptor to induce cytokine production **Figure 24**. Interestingly, our data also shows that PBMC stimulated with naked Poly(I:C) produce significantly less CXCL-10 in the absence of PKR. These results further confirm that our inhibitor is working and show that transfection of Poly(I:C) is necessary for RLR activation.

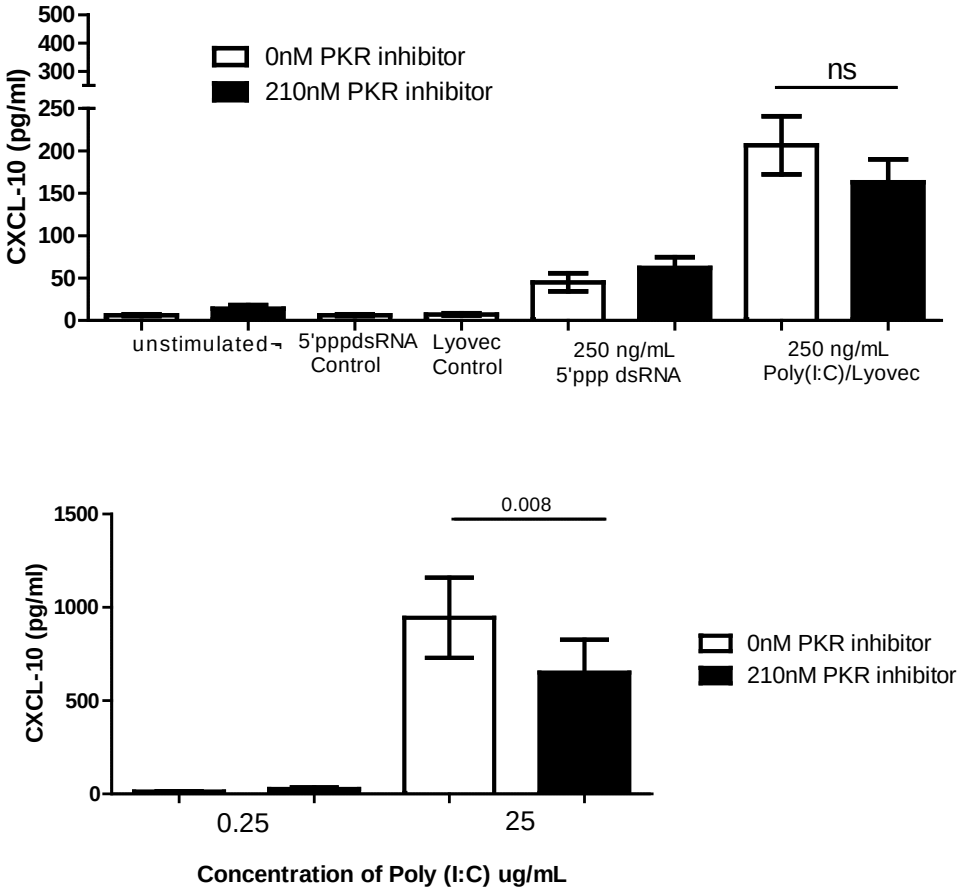

Figure 24: Inhibition of PKR does not alter Poly(I:C)/Lyovec induced CXCL-10 production. Protein levels of CXCL-10 were measured in supernatants of tissue cultured cells by ELISA. Here cells were stimulated with 5'ppp-dsRNA, Poly (I:C)/Lyovec or Poly (I:C) with/without PKR inhibitor (n=9). Mean ± SEM was used to represent data.

RLR Expression Positively Correlates with Virally Associated Genes and Anti-viral Proteins, yet Correlation with Pro-inflammatory Cytokines are Weak

The role of LGP2 as a regulator of RIG-I is a current topic controversy. Here several studies have shown that LGP2 can positively and negatively regulate RIG-I depending on the virus that activates it (52). Thus, in order to examine the downstream effector molecules and cytokines involved in PBMC activation by Poly(I:C)/Lyovec we decided to further characterize the role LGP2 may play in RIG-I and MDA-5 expression. Here correlation analysis using the Spearman test showed that RIG-I expression in unstimulated PBMC and those stimulated with Poly(I:C)/Lyovec positively correlates with LGP2 and MDA-5 expression. Interestingly, LGP2 expression was not correlated with MDA-5 in unstimulated cells, and only slightly correlated when PBMC were stimulated with Poly (I:C)/Lyovec **Figure 25A**. These findings show that upon RLR activation, RIG-I and LGP2 expression enhances. Although this correlation cannot confirm whether this expression of LGP2 leads to positive or negative regulation of RIG-I, it does show that in the presence of Poly(I:C)/Lyovec increased RIG-I levels equates with increased LGP2 levels. As RLR activation leads to enhanced cytokine production, increased LGP2 with increased RIG-I suggests that LGP2 may be working as a positive regulator. MDA-5, on the other hand, does not appear to be regulated by LGP2 as little correlation between the two was observed.

In order to further characterize the role of LGP2 in the regulation of RIG-I in the presence of Poly(I:C)/Lyovec, we examined several cytokines, chemokines, IFNs and IFN

signalling molecules that we previously showed to be upregulated in PBMC stimulated with Poly(I:C)/Lyovec. Here the data shows that LGP2 positively correlates with IFN-β, IL-1R antagonist, ISG15, ISG56 and CCL-8 **Figure 25B**. Thus, as increased LGP2 expression correlates with increased cytokine and IFN response it appears that LGP2 may positively regulate RIG-I in the presence of Poly(I:C)/Lyovec. However, as most cytokines and IFNs were only weakly correlated to LGP2 the question of whether this is biologically relevant still remains.

Discussion

In this arm of the present study, we demonstrated that stimulation of primary PBMC with the synthetic RIG-I/MDA-5 ligand, Poly (I:C)/Lyovec, leads to enhanced anti-viral and innate inflammatory immune responses. In the presence of a virus, RIG-I has been shown to bind to 5'triphosphate-dsRNA, which then allows for downstream signalling that gives rise to Type I and III IFNs as well as inflammatory cytokines (57). However, the ability for a large cohort of healthy individuals to utilize this pathway in the recognition of dsRNA has not been previously studied.

Our initial experiments showed that optimal kinetics and dosage for the various viral associated genes and inflammatory cytokines of interest in the presence of Poly(I:C)/Lyovec was 250 ng/mL at 24 hours. Interestingly, expression of IFN-β was found to be augmented similarly at 12 and 24 hours. As RIG-I activation has been show to lead to the production of type I IFNs, which then subsequently enhances RIG-I, LGP2, ISG15 and ISG56, it is not surprising that its expression pattern differs slightly from that

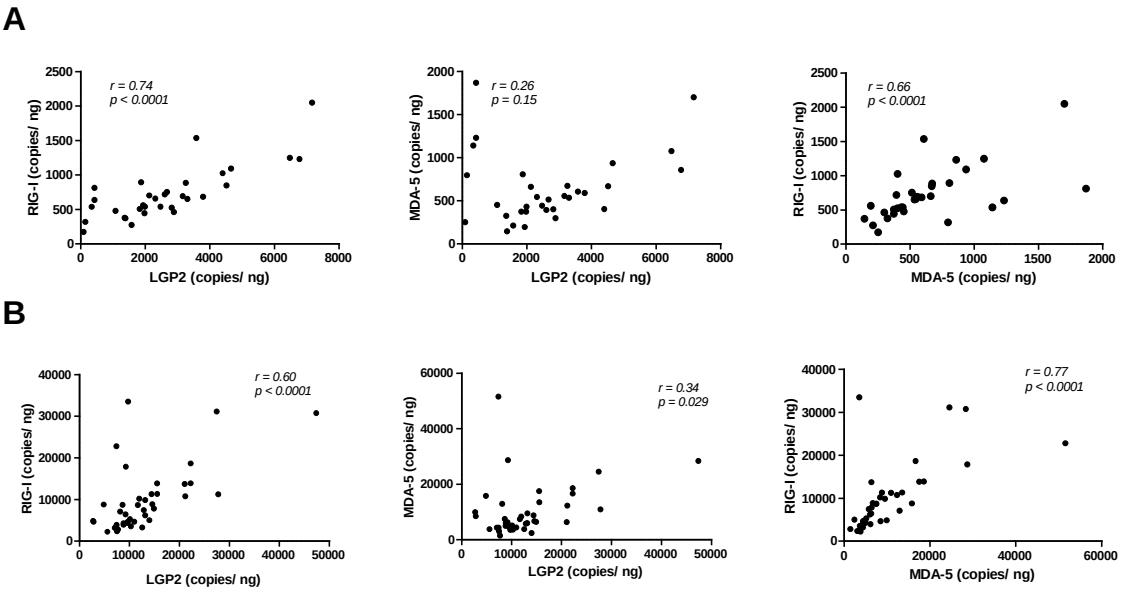

Figure 25A: RIG-I correlates with MDA-5 and LGP2 expression in resting and viral-stimulated conditions. PBMC were stimulated with (A) CM or (B) Poly(I:C)/Lyovec (250 ng/mL) for 24 hr. mRNA expression for RIG-I, MDA-5 and LGP2 was then quantified by RT-PCR. Correlation analysis was performed using the Spearman test (n=32-40). Done by Larisa Lotoski.

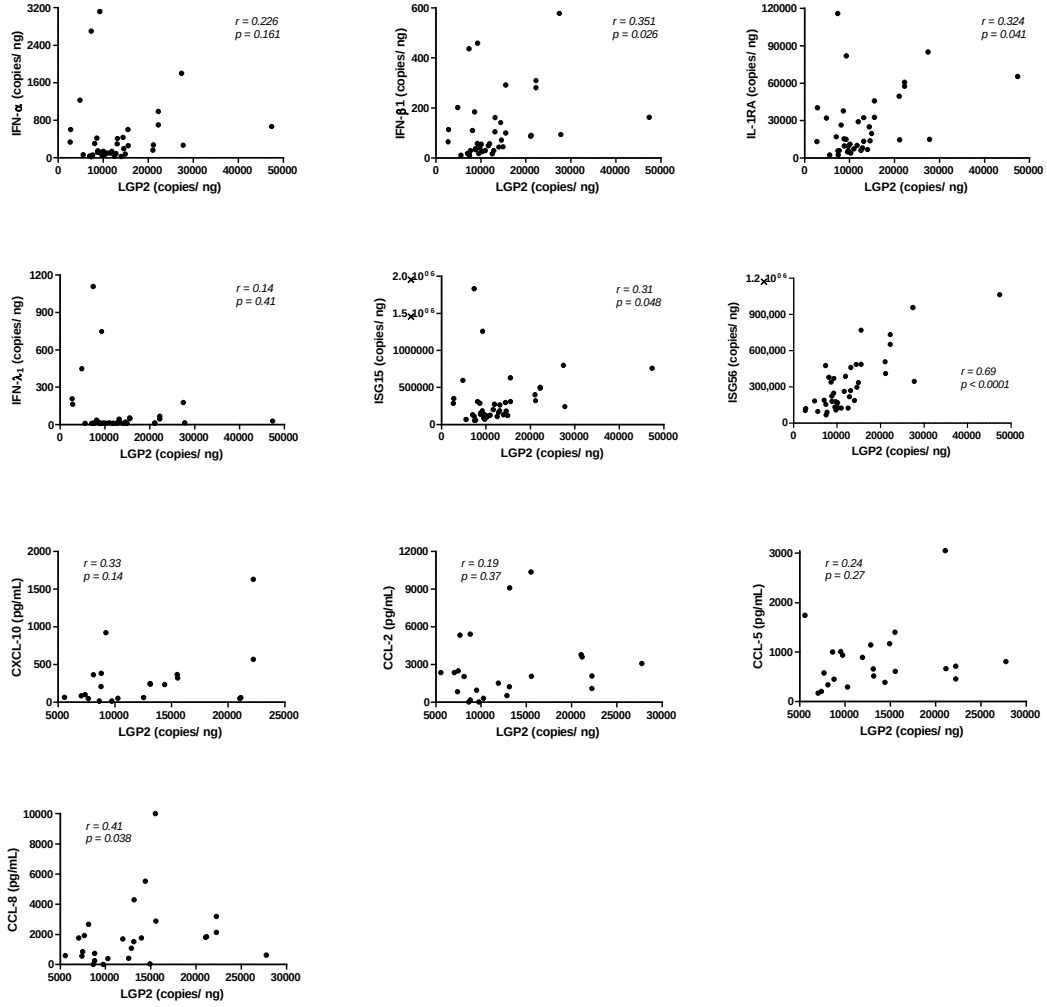

Figure 25B: **Increased viral-driven LGP2 expression is positively associated with selective pro-inflammatory and IFN responses**. PBMC were stimulated with Poly(I:C)/Lyovec for 24 hr. mRNA expression was quantified by RT-PCR. Correlation analysis was performed using the Spearman test (n=21-26). Done by Larisa Lotoski.

other cytokines and anti-viral molecules, as IFN-β is required for their enhancement (240).

Examination into the cell type responsible for the production of inflammatory cytokines, during Poly(I:C)/Lyovec stimulation, showed that CD14 enriched monocytes were the responders to Poly(I:C)/Lyovec at 24 hours. Although RLRs are expressed in a variety of cell types, others have indicated that RLRs play a key role in triggering innate immune signalling in myeloid cells and therefore our results were not altogether surprising (64,237,241). However, the degree to which monocytes responded to Poly(I:C)/Lyovec over that of lymphocytes was quite intriguing, as it appears that monocytes are almost entirely responsible for Poly(I:C)/Lyovec recognition at 24 hours. Thus, further study should be conducted in this area to determine just how important monocyte RLR stimulation is in innate immune recognition to viral pathogens.

For further insight into genes targeted by RLR activation, we performed a microarray analysis on PBMC stimulated with Poly(I:C)/Lyovec. Here several genes that were indicated as inducible by RLR stimulation were confirmed to be highly expressed when tested by RT-PCR. These genes included CCL-8, ISG56 (IFIT1), ISG15, CXCL-10, DHX58 (LGP2) and DDX58 (RIG-I). However, other genes found to be highly inducible such as granulocyte colony-stimulating factor (G-CSF) or slightly inducible such as IL-6, were non-detectable when tested by ELISA. Thus, it may be possible that some targets were missed by ELISA because they were below the detection limit of the assay, or perhaps the mRNA was degraded before being translated into a functional protein and

therefore could not be detected by ELISA. Interestingly some targets found by ELISA at 24 hours, such as CCL-2, were shown to be extremely weak when measured by microarray, further indicating that perhaps some targets were misrepresented by microarray and others misrepresented by ELISA.

Examination into cytokines, chemokines, RLRs, IFNs and IFN stimulated gene expression showed similar results to the kinetics experiment yet on a larger scale. Here it is interesting to note that the expression of LGP2, RIG-I and MDA-5 is relatively similar at 24 hours. As RIG-I and MDA-5 have similar structures it might be expected that they recognize the same types of dsRNA. However, RIG-I has been shown to interact with short dsRNA up to 1kb in length, whereas MDA-5 detects longer dsRNA molecules that are over 2kb (61,62). Thus, as the Poly(I:C)/Lyovec used for our experiments is high molecular weight and ranges from 1.5-8 kilobase pairs, it is most likely to bind to both RIG-I and MDA-5. Other studies conducted on RIG-I and MDA-5 specificity have shown that depending on the virus, some will primarily recognize RIG-I whereas others recognize MDA-5 (64,65). As a result, it is important to understand which viruses are recognized by which receptor, as this could aid in developing a vaccine towards given viruses.

ISG15 and ISG56 were shown to be the most highly upregulated genes in PBMC stimulated with Poly(I:C)/Lyovec. Both of these genes are induced by type I IFNs through IFN-stimulated response elements (ISRE) in their gene promoter regions. ISG15, in particular, has been shown to conjugate to RIG-I thereby negatively regulating its

signalling (240). This form of ISGylation allows RIG-I expression to be balanced so overproduction does not lead to cell exhaustion. ISG56 has been shown to disrupt the interaction between stimulator of IFN genes protein (STING) and MAVS, which inhibits downstream activation of IRF-3 and NF-κB (242). Thus, ISG15 and ISG56 are both negative feedback regulators of RIG-I pathway, and therefore increased RIG-I expression may not directly correlate with downstream RIG-I signalling.

Our results demonstrate that RLR stimulation by Poly(I:C)/Lyovec leads to enhanced IFN and inflammatory cytokine production. As type I IFNs were previously mentioned as mediators of RLR and ISG expression among their ability to mount a vigorous anti-viral immune response, our results of their enhanced expression and secretion upon Poly(I:C)/Lyovec stimulation was not surprising. However, the data indicating increased IL-29 (IFN-λ_1), which is a type III IFN, had only been shown by a few people to be augmented by Poly(I:C)/Lyovec stimulation (241). As type III IFNs are a relatively new group of IFN's, their exact function is not known, however their involvement in anti viral immunity has been shown and it is speculated that they are derived from similar ancestry to type I IFNs (238). Interestingly, Poly(I:C)/Lyovec, although able to enhance IL-29 expression, did not increase IL-28. Recent studies shed some light on this as IL-29 treatment has been shown to increase IL-6, CXCL-8 and IL-10 in PBMC, whereas IL-28 acts more like IL-12 and enhances adaptive immune properties (243,244). Thus, although both IL-29 and IL-28 are extremely homologous they appear to interact differently to generate a distinct immune response.

In terms of inflammatory cytokine production, our results showed that CXCL-10, CCL-2 and CCL-8 secretion were significantly upregulated in PBMC stimulated with Poly(I:C)/Lyovec. As CCL-2 and CCL-8, also commonly known as monocyte chemattractant protein 1 and 2, respectively, their function has mainly been described in terms of monocytes. Although little is known about CCL-8, CCL-2 is produced by many cell types, including EC, endothelial cells, smooth muscle cells and monocytes, and is produced to recruit monocytes, memory T-cells and DC to the site of infection or inflammation (reviewed by (245)). Interestingly, CCL-8 has recently been suggested as an attractant for eosinophil and basophil, as well as the classical monocyte, recruitment. Here studies have found increased CCL-8 levels in intrinsic asthmatics and in the synovium of rheumatoid arthritis patients and that their upregulation is dependent upon NF-κB activation (246,247). CXCL-10, on the other hand, has been shown to be increased during IFN-β treatment and is therefore commonly known as IFN-inducible protein 10 (22,248). CXCL-10 has been studied extensively in transplant immunology, where it is thought to regulate chemotaxis induced allograft rejection (249). Besides its chemotactic abilities, CXCL-10 can also induce apoptosis, cell growth and angiogenesis (250). Thus, even though our results proved that these chemokines were being produced by monocytes, the pathway that induced their production was different and the purpose for their excretion could have been to recruit several different types of cells to the site of inflammation.

Examination into anti-inflammatory cytokine production by PBMC stimulated with Poly(I:C)/Lyovec showed that IL-10 was not upregulated. However, it was found

that IL-1R antagonist, a protein that binds IL-1 receptor thereby preventing IL-1 from binding and eliciting downstream signalling, was significantly upregulated in PBMC stimulated with Poly(I:C)/Lyovec. IL-1 is a potent pro-inflammatory cytokine that is involved in many cellular processes including cell proliferation and differentiation, and is also involved in initiating and sustaining inflammatory processes (reviewed in (18)). Thus, although IL-1R antagonist is not a classical anti-inflammatory cytokine it does possess anti-inflammatory properties. As Poly(I:C)/Lyovec stimulation has been shown to upregulate various pro-inflammatory chemokines it is not surprising that an anti-inflammatory mediator would be required to regulate the inflammatory process to ensure overresponsiveness did not occur. However, it is peculiar that IL-1R antagonist was induced whereas the common IL-10 was not. As IL-1R antagonist is usually secreted by the same cells that secreted IL-1β, one would expect that IL-1R antagonist regulates the effect of IL-1β so as to prevent chronic inflammation that is characteristic of many autoimmune disease (reviewed by (251)). Yet, monocytes transfected with Poly(I:C) are unable to produce mature IL-1β unless they are co-stimulated by some other PAMP (241). Thus, perhaps there is a novel role for IL-1R antagonist in the regulation of RLR activated innate immune responses.

Lastly, correlation analysis was performed on PBMC stimulated with Poly(I:C)/Lyovec to assess whether or not LGP2 plays a positive or negative role in RLR mediated innate immune responses to Poly(I:C)/Lyovec. Here our results show that LGP2 is positively correlated with RIG-I, but not MDA-5, in the presence or absence of Poly(I:C)/Lyovec stimulation. RIG-I activation leads to the translocation of IRF-3 and 7 to

the nucleus thereby promoting the expression of type I IFNs, which subsequently increase the expression of LGP2. Satoh et al, found that although mice lacking LGP2 required this receptor for RIG-I and MDA-5 antiviral response against picornaviridae virus, LGP2 was dispensable when cells were transfected with Poly(I:C) or dsRNA at varying lengths (71). Therefore, the positive correlation observed between RIG-I and LGP2 in our studies does not prove whether LGP2 positively or negatively regulates RIG-I. Looking into the correlation between LGP2 and other markers of anti-viral or inflammatory responses shows strong correlation with ISG56 and weak correlation with ISG15, IFN-β, IL-1R antagonist and CCL-8. ISG15, ISG56 and LGP2 are IFN inducible genes, and are thus increased when IFN is increased. However the fact that CCL-8 and IL-1R antagonist are increased with higher levels of LGP2 suggests it may play a positive role in mediating an inflammatory response.

To summarize, in this chapter we have shown that RLR activation in primary PBMC by Poly(I:C)/Lyovec leads to enhanced inflammatory and anti-viral responses. As previous studies have focused almost exclusively on viral recognition by RLRs, our study showing the immunological response to RLR activation is novel. Here we have confirmed previous findings that RLR activation leads to type I and III production, however, we found that these responses were quite weak. More importantly we identified other markers of pro- (CCL-8) and anti-inflammatory (IL-1R antagonist) responses that were previously unknown. These cytokine responses were more robust than the IFN response, and therefore may be better indicators of RLR activation. Thus, our study is among the first to look at immunological readouts of RLR activation from a diverse

heterogeneous population of primary PBMC. These results prove beneficial as experiments highlighted in chapter 4 use Poly(I:C)/Lyovec as a ligand for RLR activation to determine whether exogenous vitamin D alters this response.

CHAPTER 4

Vitamin D Inhibits Epithelial Innate Inflammatory Cytokine Production in Presence of RIG-like Receptor Activation

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Abstract

Rationale: EC are the first responders to pathogens introduced through the lung, and therefore it is important to understand how epithelial responses to these pathogens can be altered. Vitamin D has been shown to modulate global epithelial cytokine production in the presence of H1N1 and RSV; however, the ability for exogenous vitamin D to modulate responses upon RLR specific activation or to viruses that activate RLRs has not been studied. As many viruses including RSV and H1N1 activate this family of receptors research into this area may help determine the potential for vitamin D to aid in immune responses against viruses.

Objectives: To determine the effects of $1,25(OH)_2D_3$ and $25(OH)D_3$ on cytokine responses elicited by RLR activation of primary and short term lines of human airway epithelial cells (HAEC).

Methods: Minna 6KT cells, a short term epithelial cell preparation, were cultured with LPS, 3M 002 and Poly(I:C)/Lyovec with active or inactive vitamin D. HAEC were cultured with Poly(I:C)/Lyovec in the presence of either active or inactive vitamin D. ELISA and RT-PCR were then used to determine cytokine responses and mRNA expression, respectively, and the capacity of vitamin D to influence such responses.

Results: Minna cells stimulated with Poly(I:C)/Lyovec showed increased pro-inflammatory cytokine production, whereas LPS and 3M 002 did not induce responses for the cytokines we examined. IFN and anti-inflammatory cytokine expression was

upregulated in minna cells stimulated with Poly(I:C)/Lyovec. Thymic stromal lymphopoietin (TSLP) was also upregulated in these cells upon RLR activation. HAEC stimulated with Poly(I:C)/Lyovec showed a panel of cytokine responses which were decreased in the presence of exogenous active ($1,25(OH)_2D_3$) and inactive ($25(OH)D_3$) vitamin D. RLR and IFN expression was also increased in HAEC stimulated with Poly(I:C)/Lyovec, however, addition of exogenous vitamin D to these samples had no effect on the intensity of RLR or IFN expression.

Conclusion: The data argue that exogenous $1,25(OH)_2D_3$ and $25(OH)D_3$ has immunoregulatory effects on epithelial cells in the presence of RLR activation. Interestingly, exogenous vitamin D was not able to modulate IFN production, suggesting it may be pathway specific. Therefore, future studies will focus on increasing the number of subjects, in an effort to determine whether vitamin D can truly modulate epithelial responses to RLR activation.

Results

Minna 6KT Epithelial Cells Increase Viral Associated Genes and Pro-Inflammatory Cytokine Production upon RLR Activation by Poly(I:C)/Lyovec

As our previous studies on RLRs in leukocytes showed increased expression of several viral associated genes as well as pro-/anti-inflammatory genes in the presence of RLR ligand Poly (I:C)/Lyovec we sought to determine whether epithelial cells responded to activation in a similar manner to PBMC. Here stimulation of minna 6KT cells, an immortalized human epithelial cell line derived by Jeremy Hirota of the Department of Physiology, University of British Columbia, showed increased pro-inflammatory cytokine production (CXCL-8, CCL-5 and CXCL-10) when exposed to Poly (I:C)/Lyovec **Figure 26**. Interestingly, stimulation with TLR4 ligand LPS, or TLR8 ligand 3M 002 did not enhance pro-inflammatory cytokine production when compared to CM control. However, RLR activation by Poly (I:C)/Lyovec drastically upregulated CXCL-8, CCL-5 and CXCL-10 at both 250 ng/mL and 2500 ng/mL. For further experiments Poly(I:C)/Lyovec at 250ng/mL was used as opposed to 2500 ng/mL, as the latter concentration resulted in a substantial increase in cell death. Pro-inflammatory cytokines CCL-2 and CCL-8, as well as the anti-inflammatory cytokine IL-10, were also examined in supernatants of minna cells, however these cytokines were non-detectable.

To assess whether RLR activation by Poly(I:C)/Lyovec enhanced viral associated genes and anti-inflammatory molecules RT-PCR was performed on cDNA from minna 6KT cells. Here the data shows that RIG-I, MDA-5 and LGP2 are constitutively expressed

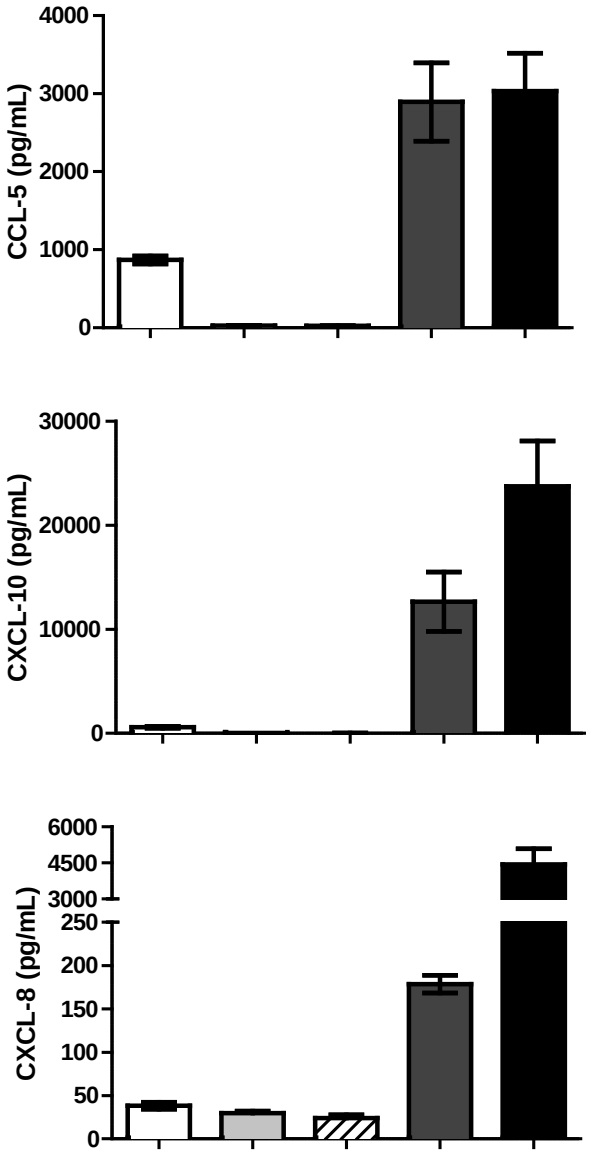

Figure 26: Poly(I:C)/Lyovec induces chemokine production in epithelial cell line. 6KT minna cells were cultured in the presence of CM (white), 2 ug/mL 3M 002 (light grey), 4 ng/mL LPS (striped), 250 (dark grey) or 2500 ng/mL (black) Poly (I:C)/Lyovec for 24 hours. ELISA was used to determine CXCL-8, CCL-5 and CXCL-10 production in cell supernatants (n=3 independent trials). Mean ± SEM represents data.

in minna cells and that stimulation by LPS, 3M 002 or Poly(I:C)/Lyovec does not substantially affect RLR expression levels **Figure 27A.** Examination of IFN expression, however, showed that minna cells stimulated with LPS or 3M 002 did not result in upregulation of IFN-β, IFN-λ_1 or IFN-$\lambda_{2,3}$ yet stimulation by Poly(I:C)/Lyovec led to a dramatic increase in IFN expression compared to CM, LPS or 3M 002 controls **Figure 27B.** Analysis into anti-inflammatory cytokine expression by minna cells was less conclusive compared to the IFN and RLR expression data. Here IL-1R antagonist was constitutively expressed under all conditions tested, but was not further enhanced by Poly(I:C)/Lyovec while sTNFRII was expressed at low levels in control samples but was induced upon Poly(I:C)/Lyovec stimulation **Figure 27C**. Furthermore, IL-10 was previously found to be non detectable in supernatants of minna cells, indicating that although minna cells do express anti-inflammatory molecules their role in anti-viral immunity is not clear. Lastly, we decided to examine whether Poly(I:C)/Lyovec was able to enhance Th2-like cytokine TSLP from minna cells. As epithelial cells have been shown to produce TSLP in the presence of a virus, we sought to determine whether RLR activation would also enhance TSLP expression from EC. Here the data shows that in the presence of Poly(I:C)/Lyovec TSLP is induced from minna cells in comparison to 3M 002, LPS and CM controls **Figure 27D**. Thus, we chose to focus on Poly(I:C)/Lyovec as it stimulated a panel of pro-inflammtory cytokines, type I and III IFNs, as well as anti-inflammatory and Th2-like cytokines.

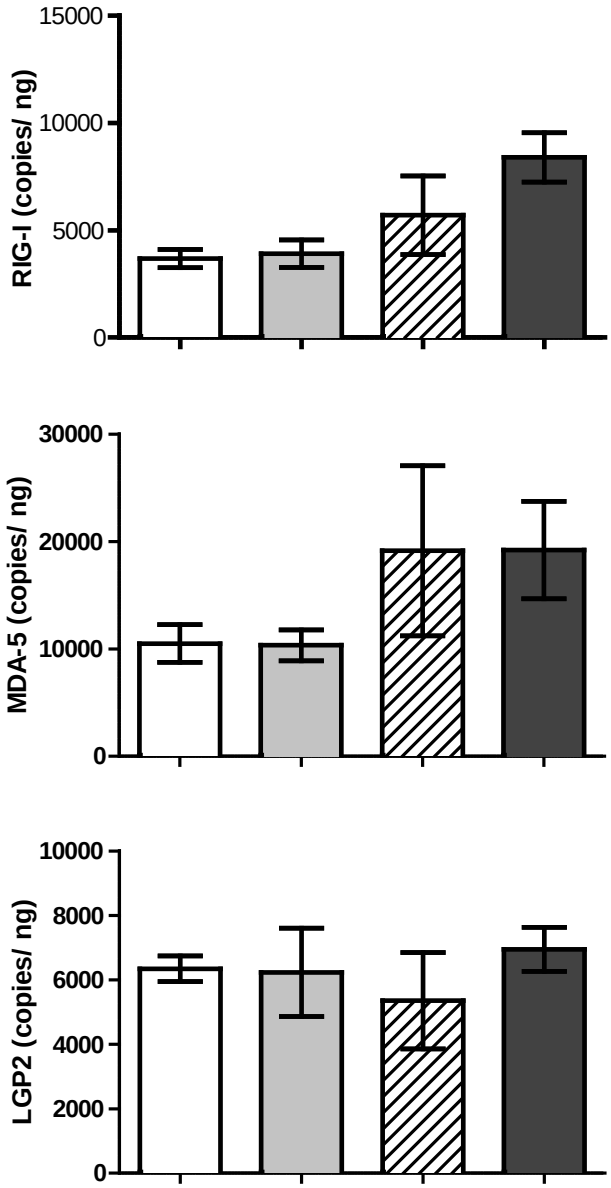

Figure 27A: RLRs are constitutively expressed in EC cell line. 6KT minna cells were cultured in the presence of CM (white), 2 ug/mL 3M 002 (light grey), 4 ng/mL LPS (striped) or 250 (dark grey) Poly (I:C)/Lyovec for 24 hours. RT-PCR was used to determine mRNA expression (n=3 independent trials). Mean ± SEM represents data.

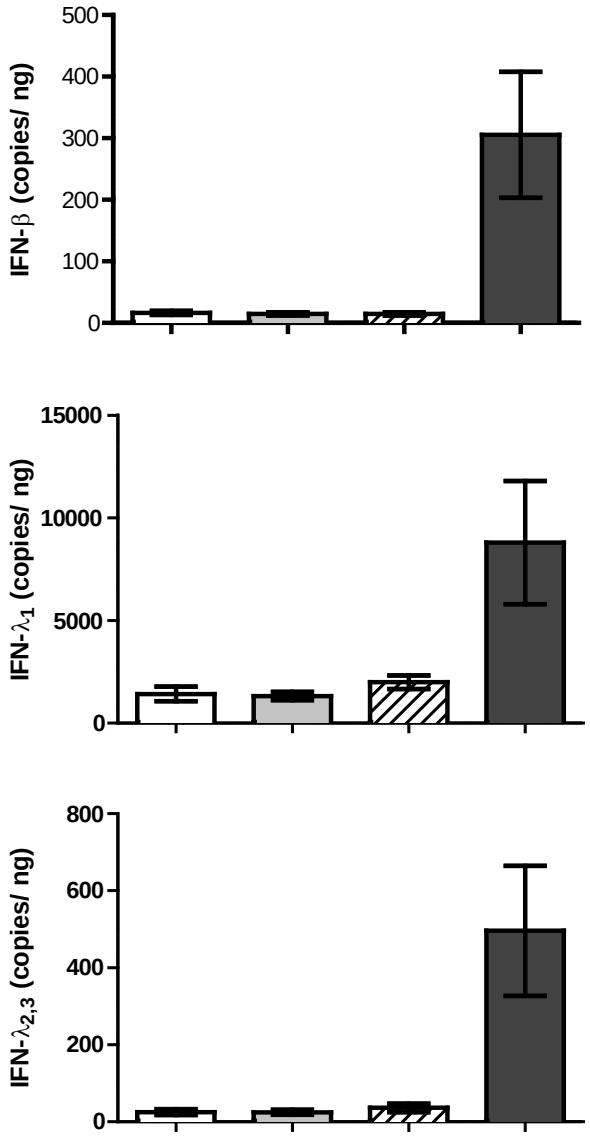

Figure 27B: Poly(I:C)/Lyovec induces IFN expression in EC cell line. 6KT minna cells were cultured in the presence of CM (white), 2 ug/mL 3M 002 (light grey), 4 ng/mL LPS (striped) or 250 (dark grey) Poly (I:C)/Lyovec for 24 hours. RT-PCR was used to determine mRNA expression (n=3 independent trials). Mean ± SEM represents data.

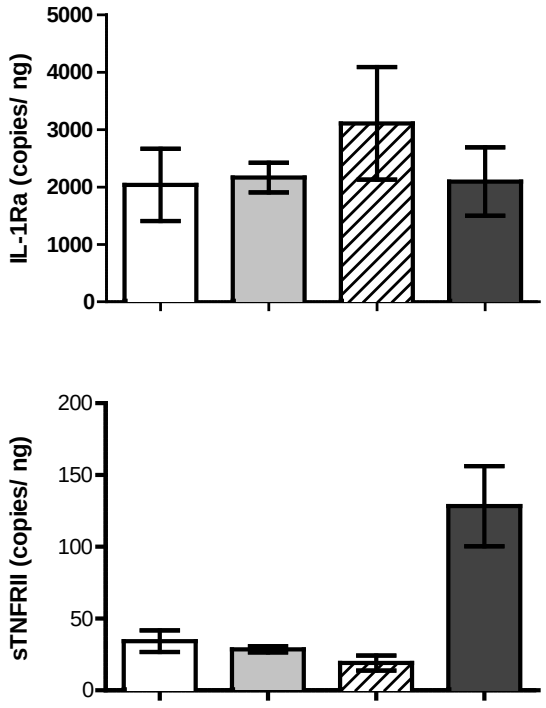

Figure 27C: Anti-inflammatory cytokine expression in EC cell line may be influenced by Poly(I:C)/Lyovec stimulation. 6KT minna cells were cultured in the presence of CM (white), 2 ug/mL 3M 002 (light grey), 4 ng/mL LPS (striped) or 250 (dark grey) Poly (I:C)/Lyovec for 24 hours. RT-PCR was used to determine mRNA expression (n=3 independent trials). Mean $\pm$ SEM represents data.

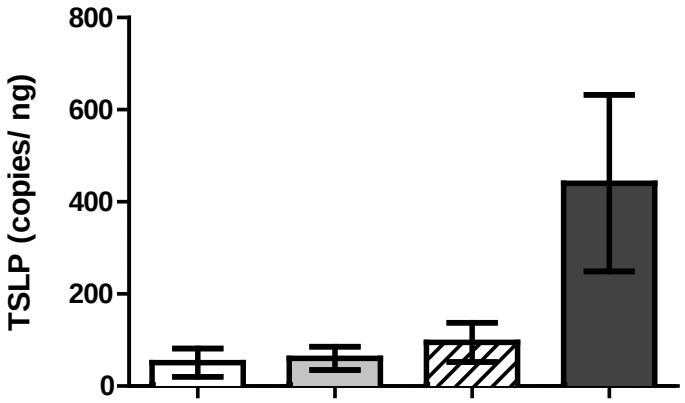

Figure 27D: Th2-like cytokine TSLP is increased in EC cell line upon Poly(I:C)/Lyovec stimulation. 6KT minna cells were cultured in the presence of CM (white), 2 ug/mL 3M 002 (light grey), 4 ng/mL LPS (striped) or 250 (dark grey) Poly (I:C)/Lyovec for 24 hours. RT-PCR was used to determine mRNA expression (n=3 independent trials). Mean ± SEM represents data.

HAEC Stimulated with Poly(I:C)/Lyovec Show Decreased Pro-inflammatory Cytokine Production in the Presence of Vitamin D

As previous studies with PBMC showed that vitamin D was able to modulate innate immune responses to LPS, but not 3M 002, we sought to determine whether HAEC were able to decrease inflammatory cytokine responses in the presence of exogenous vitamin D. Thus, HAEC were obtained from 4 separate individuals and cultured in the presence or absence of Poly(I:C)/Lyovec with/without exogenous active or inactive vitamin D. Here our results indicate that in the presence of Poly(I:C)/Lyovec HAEC increase CCL-5, CXCL-10 and CXCL-8 chemokine production levels **Figure 28.** Subsequent addition of exogenous active vitamin D was shown to decrease CCL-5, CXCL-10 and CXCL-8 chemokine production compared to cells stimulated with Poly(I:C)/Lyovec **Figure 28.** Interestingly, the inactive form of vitamin D reduced CCL-5 and CXCL-10 production levels more than $1,25(OH)_2D_3$, yet did not reduce CXCL-8 levels. These results show that vitamin D has the ability to modulate innate immune responses in EC activated by Poly(I:C)/Lyovec.

Vitamin D does not Alter RLR, IFN or Vitamin D Associated Gene Expression in HAEC Stimulated with Poly(I:C)/Lyovec

Studies using RSV and H1N1 have shown that addition of exogenous vitamin D to infected EC leads to decreased IFN and cytokine production (108,128). Thus, to test whether RLR activation in primary EC leads to altered immune responsiveness in the presence of active vitamin D we began by examining the effects of exogenous

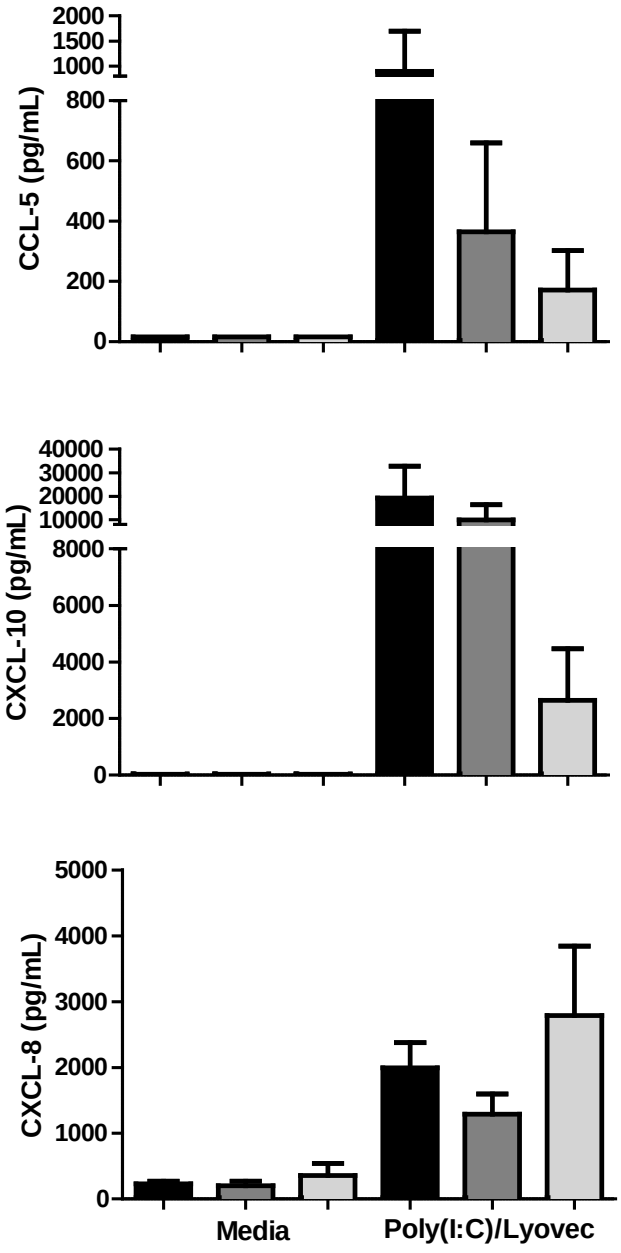

Figure 28: Active and inactive vitamin D decrease pro-inflammatory cytokines induced by RIG-I activation in primary cells. Primary bronchial epithelial cells were cultured in the presence of CM or 250 ng/mL Poly(I:C)/Lyovec with either CM alone (black), 100 nM $1,25(OH)_2D_3$ (dark grey) or 1000nM $25(OH)D_3$ (light grey) for 24 hours. ELISA was used to measure CCL-5, CXCL-10 and CXCL-8 (n=3 separate individuals). Mean ± SEM represents data.

$1,25(OH)_2D_3$ on RLR expression. Here the data shows that in the presence of Poly(I:C)/Lyovec HAEC increase RIG-I and MDA-5 expression, further addition of either active or inactive vitamin D does not alter expression levels **Figure 29A**. As ISG15 and ISG56 are IFN inducible genes, we next decided to test whether active vitamin D was able to decrease either IFN or IFN inducible gene expression upon RLR activation. According to the results HAEC stimulated with Poly(I:C)/Lyovec increased ISG56, ISG15, IFN-β and IFN-$\lambda_{2,3}$ in comparison to CM alone, however, subsequent addition of either $1,25(OH)_2D_3$ or $25(OH)D_3$ was not able to reduce expression levels **Figure 29A**. These results suggest that vitamin D, although able to decrease chemokine production in RLR activated HAEC, is not able to modulate the IFN anti-viral response that is induced upon RLR activation.

As vitamin D is able to decrease pro-inflammatory chemokine responses, but not IFN responses, upon RLR activation in HAEC we sought to determine the expression levels of VDR and its activating (CYP27b1) and deactivating (CYP24a1) enzymes in HAEC stimulated with Poly(I:C)/Lyovec. Here the data shows that regardless of stimulation all conditions tested showed similar levels of VDR and CYP27b1 expression by HAEC, however, as expected CYP24a1 was increased upon addition of vitamin D **Figure 29B**. Interestingly, CYP24a1 expression levels were higher when HAEC were stimulated with 1000 nM of inactive vitamin D as opposed to 100 nM of active vitamin D suggesting a high conversion rate of inactive vitamin D to active vitamin D.

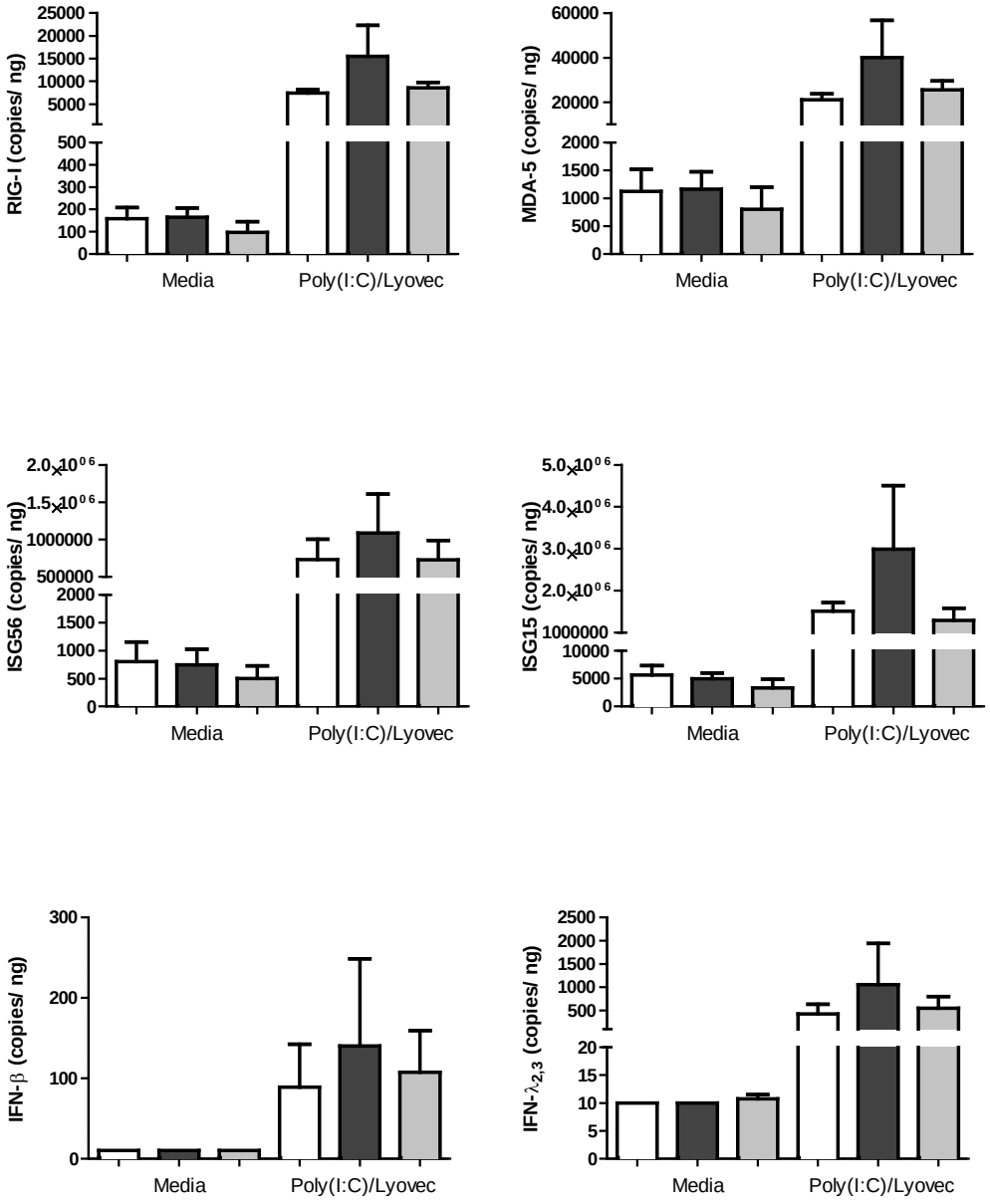

Figure 29A: Poly(I:C)/Lyovec induces expression of anti-viral associated genes in primary bronchial cells, but addition of exogenous vitamin D does not alter expression. Primary bronchial epithelial cells were cultured in the presence of CM or 250 ng/mL Poly (I:C)/Lyovec with either CM alone (white), 100 nM $1,25(OH)_2D_3$ (dark grey) or 1000nM $25(OH)D_3$ (light grey) for 24 hours. RT-PCR was used to measure mRNA expression (n=4 separate individuals). Mean $\pm$ SEM represents data.

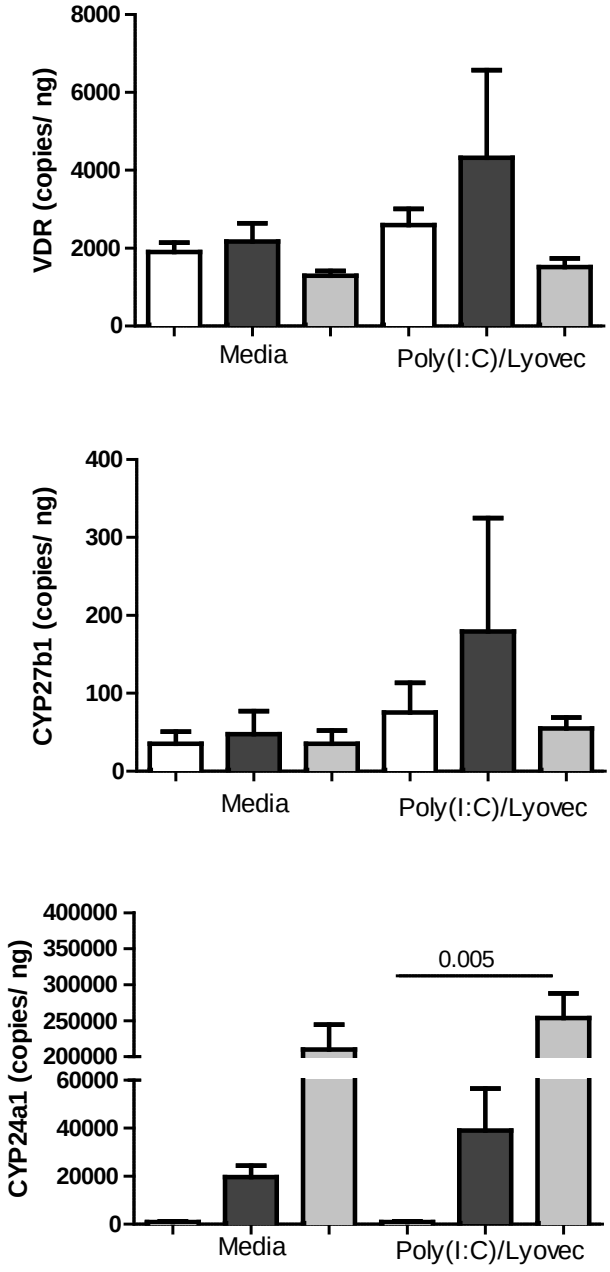

Figure 29B: Vitamin D receptor and enzymes are not enhanced upon Poly(I:C)/Lyovec stimulation. Primary bronchial epithelial cells were cultured in the presence of CM or 250 ng/mL Poly (I:C)/Lyovec with either CM alone (white), 100 nM 1,25$(OH)_2D_3$ (dark grey) or 1000nM 25$(OH)D_3$ (light grey) for 24 hours. RT-PCR was used to measure mRNA expression (n=4 separate individuals). Mean $\pm$ SEM represents data.

Discussion

The airway epithelium is a complex structure that functions as a barrier to foreign antigens that enter the lung, and is involved in cytokine/chemokine production to help recruit cells to areas of insult and modulate immune responses (reviewed by (252)). Viral pathogens are recognized by EC PRRs, which include TLRs and RLRs. Recognition of viral nucleic acids by these receptors leads to downstream signalling followed by upregulation of IFNs and cytokines, which mediate an anti-viral response against the pathogen (reviewed by (253)). More recent studies have shown that EC contain the enzyme CYP27b1, which converts the inactive form of vitamin D to the active form, and utilize the active form of vitamin D to induce AMP production (188).

As previous studies in PBMC have shown that active vitamin D can decrease cytokine production patterns in the presence of bacterial stimuli, we sought to determine whether EC in the presence of a viral stimuli would display decreased anti-viral responses in the presence of exogenous active or inactive vitamin D. Our initial results showed that neither TLR4 ligand LPS nor TLR8 ligand 3M 002 were able to upregulate pro-inflammatory or IFN responses in EC compared to control. Poly(I:C)/Lyovec, a synthetic dsRNA molecule that binds RIG-I/MDA-5, however, drastically upregulated both cytokine production and IFN expression in EC, thereby confirming the notion that RLR activation leads to enhanced IFN response.

Our studies on leukocytes showed increased pro-inflammatory cytokine production, as well as enhanced IFN, RLR and IL-1R antagonist expression in the

presence of Poly(I:C)/Lyovec. In the minna cell line we observed some similarities to our primary leukocyte data in that both CXCL-10 production and IFN expression were upregulated in the presence of Poly(I:C)/Lyovec. However, our HAEC data corresponded much more to the primary leukocyte data than the minna cell line data. Here we observed an increase in RLR, IFN and ISG expression, as well as an increase in CXCL-10 cytokine production. These results further confirm the importance of studying primary cells as opposed to cell lines, as the latter does not always mimic a true response. However, it is worth mentioning that certain responses observed in EC stimulated with Poly(I:C)/Lyovec was not observed in PBMC stimulated with Poly(I:C)/Lyovec. For instance CCL-5 was drastically enhanced in both the minna cells and HAEC in the presence of RLR activation, yet primary PBMC did not increase CCL-5. Similarly, PBMC upon Poly(I:C)/Lyovec stimulation produced CCL-2 and CCL-8, whereas both the minna and HAEC did not produce either of these chemokines. One likely reason for this difference is that CCL-2 and CCL-8 are mainly produced by monocytes and act on monocytes, whereas CCL-5 has been shown to be regulated by IL-10 (254) which was not produced by EC.

According to our vitamin D experiments, HAEC stimulated with Poly(I:C)/Lyovec decrease CCL-5, CXCL-10 and CXCL-8 production in the presence of exogenous active vitamin D. Our results are the first to show that vitamin D decreases pro-inflammatory chemokine production in the presence of RLR activation. Studies using H1N1, which is a virus that has been shown to elicit the RLR pathway, among others, indicate similar results. An EC line infected with H1N1 showed decreased CCL-5 and CXCL-8 production

upon 1,25$(OH)_2D_3$ stimulation, yet IFN-β and ISG15 were only decreased upon pre-treatment with 1,25$(OH)_2D_3$ (108). Interestingly, studies using RSV infected EC show different results, where addition of 1,25$(OH)_2D_3$ decreased IFN and ISG expression in comparison to RSV infected cells in the absence of vitamin D (128). As both RSV and H1N1 have been shown to interact with RIG-I, these results seem opposing. However, the studies using RSV pre-treated the EC with 1,25$(OH)_2D_3$ before infecting them with RSV; as the studies on H1N1 also observed a decrease in IFN and ISG expression when cells were pre-treated with 1,25$(OH)_2D_3$ these results are actually consistent. Therefore, one difference between our study and that of Hansdottir or Khare is that we did not pre-treat our cells with 1,25$(OH)_2D_3$; if we had, we most likely would have seen a decrease in IFN and ISG production in the presence of RLR activation as well.

Our expression results on primary HAEC showed an increase in RLRs, RIG-I and MDA-5, in the presence of Poly(I:C)/Lyovec. These results are consistent with current literature which states that human bronchial epithelial cells constitutively express TLR3, but not RIG-I or MDA-5 (255). Examination into vitamin D associated targets, such as VDR and CYP27b1, showed no difference in expression when HAEC were stimulated with Poly(I:C)/Lyovec or exogenous vitamin D. Schrumpf et al showed similar results in that VDR expression was constitutively expressed, however they found that CYP27b1 was augmented in EC stimulated with IL-13 (256). Our primary PBMC data agrees with Schrumpf et al, as we found that in the presence of 3M 002, and to a less extent LPS, CYP27b1 expression is increased compared to control. Since CYP27b1 expression has

never been tested in the presence of Poly(I:C)/Lyovec, it is possible that RLR activation does not induce expression of this enzyme.

To summarize, the data in this chapter shows that EC respond to Poly(I:C)/Lyovec in a similar manner to that of PBMC. In addition, we also observed that HAEC decrease Poly(I:C)/Lyovec induced pro-inflammatory cytokine production in the presence of exogenous vitamin D. These results, although preliminary, suggest that vitamin D has the potential to regulate innate immune responses to viral stimuli.

General Discussion

Lower tract respiratory infections (LTRI) are the leading cause of hospitalizations each year. In the United States 55,470 cases of influenza were confirmed from September 30,2012 to February 9, 2013 alone (257). Children and seniors are the most susceptible to infection, with 27.9 cases per 1000 children under the age of 5 occurring annually and 547,000 hospitalizations each year in these children due to LTRI (258). Although vaccines are available for some LTRI including influenza, RSV which is responsible for 24% of these hospitalizations has no current vaccine (258).

Epidemiological studies have shown that $25(OH)D_3$ levels below sufficiency (30 ng/mL) are associated with increase acute respiratory infections (159,161,196,259). However, few studies have been conducted on how vitamin D supplementation alters the innate immune system to help improve defenses against viruses that cause these infections. As viruses are complex and interact with several different types of PRR, to fully understand the potential for vitamin D to modulate innate immune responses against viral antigens requires an understanding of how viral PRR pathways can be affected by the presence of $1,25(OH)_2D_3$.

TLRs and RLRs are two types of PRR that recognize viral nucleic acids, therefore, the aim of our study was to look at these receptors in innate immune cells to determine whether exogenous vitamin D supplementation was able to modulate innate cytokine and IFN responses to viral antigens. Our results are the first to show that vitamin D differentially regulates innate cytokine responses in the presence of bacterial ligands in

comparison to viral ligands. Further analysis clarified the notion that vitamin D can regulate innate immune responsiveness to viral antigens; however, the ability for regulation depends on the cell type being examined and the receptor/ligand pathway that is being stimulated. Thus, although vitamin D does have the potential to regulate innate immune responsiveness, it may only do so under certain conditions. Below I will provide a discussion of the importance and context for key findings of this thesis.

Can Exogenous Vitamin D Modulate Bacterial and Viral Innate Immune Responses?

As literature claims that vitamin D may help the immune system to eradicate viruses, our findings that vitamin D alters innate immune responsiveness differently in the presence of a bacterial ligand versus a viral ligand is novel. Looking into the mechanism of why exogenous vitamin D does not cause PBMC to decrease cytokine production in the presence of viral stimulation, showed that a decrease in VDR expression in viral stimulated cells was apparent compared to bacterial or CM control. Although preliminary, these results indicate a potential mechanism for the difference observed between bacterial and viral driven innate cytokine responses in PBMC stimulated with exogenous vitamin D. As our model was limited due to the fact that primary cells are less resilient then cells generated from a cell line, we were unable to determine if decreased VDR was the cause for the apparent difference between bacterial and viral cytokine responses in the presence of exogenous vitamin D. Thus, future studies should determine whether this difference is causative or merely

correlative, as this could help to determine individuals who are less susceptible to vitamin D modulation.

One of the major problems with current epidemiological studies regarding the link between vitamin D and viral infection is that results obtained are only correlative and therefore may not be causative. Even though some studies show a strong association between endogenous vitamin D levels and decreased viral infections, the two may be completely unrelated. That said our studies, although addressing more directly the relationship between vitamin D and the prevalence of viral infections, remove circulating cells from their natural environment and therefore results obtained may not accurately reflect *in vivo* data. A large study, however, was recently conducted showing that vitamin D supplementation did not decrease the prevalence of upper respiratory tract infections (226). These results support the notion that vitamin D may not be beneficial in prevention or treatment of upper respiratory tract infections. Interestingly, basic and epidemiological studies regarding the potential for exogenous vitamin D to help improve/eradicate HIV infection are more promising (27,167,260).

Therefore, our findings show the importance of examining *in vitro* data to complement epidemiological data. As neither method is flawless it is imperative both forms of research be conducted to try and answer questions pertaining to the immune system. Our study is the first to show that exogenous vitamin D has different effects on bacterial stimulated cells versus viral stimulated cells. This finding is particularly important for health research as it demonstrates that vitamin D supplementation may

not be as crucial for the general public as currently believed. Further research should be conducted in this area to help characterize which viruses and viral pathways are and are not modulated by exogenous vitamin D.

Are Endogenous Vitamin D Levels Good Predictors of Innate Immune Responsiveness?

Several studies have shown that individuals with autoimmune diseases, including those with Crohn's disease and diabetes, have decreased circulating 25(OH)D_3 levels (212,213). Therefore, our data showing that higher endogenous vitamin D levels correlate with decreased pro-inflammatory mediators indicate that supplementation may help to suppress undesired inflammatory responses that are characteristic of these diseases. Furthermore, we showed that teens have lower plasma 25(OH)D_3 than adults; as vitamin D deficiency may increase the risk for autoimmune diseases it would be beneficial for these individuals to be on vitamin D supplements to help increase their endogenous 25(OH)D_3 levels.

One limitation to our study on endogenous vitamin D levels was that we were only able to obtain our samples once per individual. Our plasma cytokine data correlated to 25(OH)D_3 results would have been more convincing if we could have determined the impact of seasonality within the same individual. As the human population is heterogeneous, this would have allowed us to make a more direct comparison. Khoo et al published a paper where they did this very comparison and found that multiple pro-inflammatory cytokines and TLRs were decreased during

summer months when vitamin D status was higher compared to winter months (115). This would have allowed us to determine whether endogenous $25(OH)D_3$ had any implication of plasma cytokine levels.

These results further confirm the importance of examining *in vivo* data to complement *in vitro* data. As the two are completely different model systems, their results help to determine whether vitamin D levels are truly beneficial in regulating innate cytokine responses. Future studies should examine cross seasonal cytokine plasma levels in correlation with endogenous vitamin D levels among different ages and clinical phenotypes to determine whether $25(OH)D_3$ is a good predictor of innate immune responsiveness.

Biomarkers of RLR Activation

As of late, translational research has emphasized the importance of biomarker discovery in the diagnosis and treatment of disease. Here some studies have shown that autoimmune diseases, such as SLE, have increased type I IFNs and IFN associated chemokines compared to healthy controls (261-263). Interestingly other autoimmune diseases, such as asthma, have focused on adaptive T cell responses. Examination into the use of cytokine biomarkers in cohorts of asthmatic individuals has proved promising, yet difficult with the increased identification of new clinical phenotypes (264). One of the challenges associated with the discovery of biomarkers is the diversity amongst humans (265). Thus, recognizing the need to broaden the focus from TLR PRR, and the paucity of studies indicating biomarkers of RLR activation we sought to determine the

cytokine and genetic profile of RLR activated PBMC in a diverse heterogeneous population of adults. Our findings suggest that PBMC stimulated with Poly(I:C)/Lyovec, not only have increased IFN response, which has been noted in literature (62,71,237), but also several novel cytokines that have either not been previously studied or studied very little, such as CCL-2, CCL-8, IL-1R antagonist.

These results help to further characterize the innate immune response to ligands that trigger RLR activation. In relation to health studies, these findings are important as many different viruses trigger RLR signalling and therefore an understanding of how the immune system responds in the presence of a virus that triggers this type of receptor is crucial. Furthermore, these results could help in the development of new vaccines against viruses that elicit RLR signalling; this notion is evident in a article that published new insights into RSV vaccines. Here the author stated that “the existence of multiple PRRs that contribute to the host response to RSV infection and, hence, a more comprehensive characterization of the contribution of each to resistance to RSV will ultimately dictate the composition of a successful vaccine against RSV” (266).

Thus, to further characterize the innate immune response to viruses that elicit RLR activation, and how this can either help or hinder viral clearance, is important and future studies should examine RLR capability in different ages, clinical phenotypes and cell types. This in an effort to understand how different individuals respond, or have the potential to respond, to certain viruses.

Does Vitamin D Regulate Immunological Responses the Same in All Cell Types?

Studies regarding the immunological affects of vitamin D are controversial. Depending on the cell type examined exogenous vitamin D can have differential affects on innate immune responses. For instance PBMCs, as shown by us and others, decrease pro-inflammatory cytokine production in the presence of exogenous vitamin D (114-116), whereas DC suppress pro-inflammatory cytokine production only when pre-treated with $1,25(OH)_2D_3$ (121). T cells on the other hand, cannot alter cytokine responses in the presence of $25(OH)D_3$ unless DC are present (267). With regards to EC, although they have been shown to decrease pro-inflammatory cytokine production in the presence of exogenous vitamin D, they also increase TSLP upon vitamin D stimulation (268,269). Thus, we sought to examine the effects of exogenous active and inactive vitamin D on primary HAEC cells to determine whether these cells altered cytokine production or anti-viral gene expression upon RLR activation. Our data suggests that exogenous vitamin D has different effects on different cells of the immune system, as we noted decreased pro-inflammatory cytokine production in the presence of viral ligand activation in HAEC whereas this was not observed in our PBMC studies.

One strength to this study was that we used primary cells, whereas most studies use cell lines, to determine whether exogenous vitamin D modulated innate cytokine responses and anti-viral gene expression patterns upon RLR activation. That said, a limitation to this part of our study was that we had a small number of people. To

strengthen the conclusion that vitamin D modulates EC responses in the presence of RLR activation, a larger sample size would be required.

The results obtained are particularly important for health research as they show that depending on the cell that encounters a particular virus and the pathway/receptors that are activated by the virus, vitamin D may be able to regulate immune responses against the virus encountered. Therefore, further characterization of innate immune responses against viruses is necessary in understanding the potential for exogenous vitamin D to modulate such responses in different cell types.

To summarize, the research presented in this thesis lays the groundwork for understanding the potential for vitamin D to aid in immune regulation. As different antigens were used to induce cellular activation in multiple primary cell types, the role vitamin D plays in innate immune responsiveness becomes more well defined. Due to the plethora of genes regulated by $1,25(OH)_2D_3$ it is important to study how these genes are effected in the presence of antigen stimulation. As viral infections continue to be a major health issue, and treatment for these infections few, the need for prevention and treatment for such infections is great. Our results are the first to show that exogenous vitamin D alters innate immune responsiveness differently in the presence of a bacterial vs viral antigen. Thus, future studies will continue to examine the effects of vitamin D on cells of the immune system in the presence of different viral antigens. Furthermore, the data represented in this thesis suggests differences in neonatal immune responsiveness to exogenous vitamin D in comparison with the adult data; therefore, future studies will

incorporate cells from different age groups as well as different phenotypes with the intent of understanding who are most responsive to exogenous vitamin D modulation and under what conditions.

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Appendix

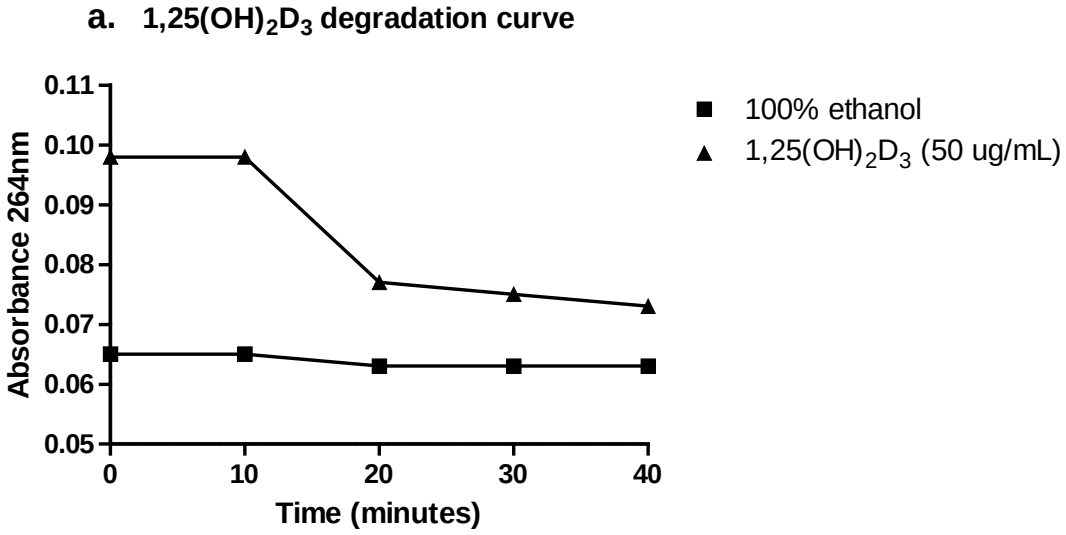

Figure 3: Active vitamin D sensitivity to light.