UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

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The Susceptibility of Mast Cells for Rhinovirus Infection: Implications for Asthma Exacerbations

By

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Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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Mast cells (MCs) are classically involved in the pathogenesis of allergic asthma, however MCs also have a key role in innate immunity with an emerging role in viral immunity. During allergic asthma MCs localise in greater numbers to the bronchial epithelium which is the principal site of human rhinovirus (HRV) infection. HRVs are a major viral trigger of asthma exacerbations via mechanisms that are not completely understood. MCs are susceptible to HRV infection but their role in anti-HRV responses is unknown. HRV infection of the bronchial epithelium triggers the release of the epithelial derived cytokine IL-33, which induces Th2 cytokine release from target cells with MCs being the major target of IL-33 in allergic asthma. I hypothesised that HRV infection induces MC anti-viral responses and also modulates IL-33-dependent Th2 responses in MCs.

The LAD2 human MC line and/or primary human cord blood-derived MCs (CBMCs) were infected with HRV or UV-irradiated HRV (control infection) with or without IFN-β, IFN-γ, IFN-λ or IL-33. Twenty-four hours following HRV infection, anti-viral and Th2 immune responses were assessed by RT-qPCR, MSD, ELISA and flow cytometry. Viral replication and release were determined by RT-qPCR and $TCID_{50}$ assay respectively.

HRV infection induced the expression of IFN-β and IFN-λ and the induction of IFN stimulated genes (ISGs). Despite this MCs were permissive for HRV replication and the release of infectious HRV particles. This was confirmed in CBMCs. To determine the contribution of endogenous anti-viral responses, CBMCs were treated with a type I IFN receptor blocking antibody. Treatment with the blocking antibody failed to significantly increase HRV replication and release suggesting endogenous type I IFN responses were insufficient to protect MCs against HRV infection. Therefore, in order to enhance anti-viral responses, MCs were treated with exogenous IFN-β, IFN-γ or IFN-λ. The induction of ISGs was enhanced by IFN-β and IFN-γ but not by IFN-λ. In addition, IFN-β treatment significantly suppressed viral replication and the release of infectious virus particles. To investigate the impact of HRV on IL-33-mediated Th2 responses, MCs were treated with IL-33 during HRV infection. IL-33 treatment induced a concentration-dependent increase in the release of IL-5 and IL-13 but this was not modulated by HRV. However, IL-33 treatment increased ICAM1 expression, a receptor for HRV entry, and enhanced HRV-mediated induction of IFN-β and ISGs. This resulted in a significant increase in HRV replication but prevented significant release of infectious HRV particles.

These findings show for the first time that MCs mount anti-viral responses to HRV infection and that HRV-induced IFN-β production is enhanced by IL-33 treatment. In severe asthma, which is associated with impaired bronchial epithelial IFN responses and an increase in the localisation of MCs to the bronchial epithelium, MCs may aggravate HRV-induced exacerbations. However, IL-33 released from the epithelium may protect MCs against productive HRV infection. These findings may have important implications in HRV-induced asthmas exacerbations and the impact of novel asthma therapies particularly anti-IL-33.

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Academic Thesis: Declaration of Authorship

I, Charlene Akua Dufie Akoto declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

The Susceptibility of Mast Cells for Rhinovirus Infection: Implications for Asthma Exacerbations

I confirm that:

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Abbreviations

Anti-IFNAR2	Anti-IFN-α/β receptor 2 antibody
AP-1	Activator protein 1
APC	Allophycocyanin
APC	Antigen presenting cell
ASM	Airway smooth muscle
BAL	Bronchoalveolar lavage
BEC	Bronchial epithelial cell
BSA	Bovine serum albumin
CBMC	Cord blood mast cell
CCL	C-C motif chemokine ligand
CD	Cluster of differentiation
CDHR3	Cadherin-related family member 3
CLP	Cecal ligation and puncture
CLRs	C-type lectin receptors
CPA	Carboxypeptidase
CPE	Cytopathic effect
CRAMP	Cathelin-related antimicrobial peptide
CXCL	C-X-C motif chemokine ligand
Cys-LT	Cysteinyl leukotrienes
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMTU	Epithelial mesenchymal trophic unit
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FcεRI	High affinity IgE receptor
FITC	Fluorescein isothiocyanate
FP	Fluticasone propionate
GAPDH	Ghlyceraldehyde-3-phosphate dehydrogenase
GAS	Group A streptococci
GM-CSF	Granulocyte-macrophage colony stimulating factor
GRB2	Growth factor receptor-bound protein 2
HBSS	Hank’s Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus

HKG	Housekeeping gene
HMC-1	Human mast cell line-1
HRP	Horseradish peroxidase
HRV	Human rhinovirus
IAV	Influenza A virus
ICAM1	Intracellular adhesion molecule 1
ICS	Inhaled corticosteroid
IFN	Interferon
IFNAR2	Interferon alpha/beta receptor 2
IFNGR	Interferon gamma receptor
IFNLR	Interferon lambda receptor
Ig	Immunoglobulin
IKK	Inhibitor of NF-κB kinase
IL	Interleukin
IL-1RAP	IL-1 receptor accessory protein
IL1RL1	Interleukin 1 receptor-like 1
ILC2	Type 2 innate lymphoid cell
IP-10	IFN-γ inducible protein 10
IP_3	Inositol-1,4,5-trisphosphate
IRAK	IL-1 receptor-associated kinase
IRES	Internal ribosomal entry site
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF3	Interferon stimulated gene factor 3
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
JNKs	c-Jun N-terminal kinases
KIT	KIT proto-oncogene receptor tyrosine kinase
LABA	Long acting beta agonist
LAD2	Laboratory of allergic diseases 2 mast cell line
LDLR	Low density lipoprotein receptor
LPS	Lipopolysaccharide
LT	Leukotriene
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinase
MC	Mast cell
MCETs	Mast cell extracellular traps
MCP	Monocyte-chemotactic protein
MCP-1	Mast cell protease 1
MC_T	Tryptase mast cells
MC_{TC}	Tryptase and chymase mast cells
MDA5	Melanoma differentiation associated gene 5

MEM	Minimal essential medium
MHC	Major histocompatibility complex
MMC	Mucosal mast cell
mMCP4	Mouse mast cell protease 4
MOI	Multiplicity of infection
MSD	Meso Scale Discovery
Mx	Myxovirus resistance
MyD88	Myeloid differentiation primary response gene 88
NK	Natural killer
NKT	Natural killer T cell
NLR	Nucleotide-binding oligomerization domain-like receptor
NOD	Nucleotide binding and oligomerization domain
NTAL	Non-T-cell activation linker
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PE	Phycoerythrin
PFA	Paraformaldehyde
PG	Prostaglandin
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PNAG	P-nitrophenyl N-acetyl-β-D-glucosamide
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
RANTES	Regulated on activation, normal T cell expressed and secreted
RIG-I	Retinoic acid-inducible gene 1
RLR	Retinoic acid-inducible gene-I-like receptor
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RSV	Respiratory syncytial virus
RT	Room temperature
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RV16	Human rhinovirus 16
SABA	Short acting beta agonists
SCF	Stem cell factor
SH2	Src-homology-2
siRNA	Small interfering RNA
SNP	Small nucleotide polymorphism
ssRNA	Single stranded RNA

STAT	Signal transducers and activators of transcription
$TCID_{50}$	Tissue culture infective dose of 50%
TCR	T cell receptor
TIR	Toll-interleukin-1 receptor
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumour necrosis factor-α
TRAF	TNF receptor-associated factor
TSLP	Thymic stromal lymphopoietin
UBC	Ubiquitin C
UV	Ultraviolet
Viperin	Virus inhibitory protein, endoplasmic reticulum–associated, IFN-inducible
VLDLR	Very low density lipoprotein receptor
VP1-4	Viral protein 1-4
vRNA	Viral RNA
VSV	Vesicular stomatitis virus
WT	Wild type

Chapter 1: Introduction

1.1 Mast Cells

Mast cells (MCs) are bone marrow derived cells of the haematopoietic lineage. They develop from cluster of differentiation (CD)34^+ (haematopoietic stem cell and progenitor marker) and $CD117^+$ (receptor tyrosine kinase KIT) progenitors and are found in vascularised tissues, particularly at surfaces which interface with the external environment such as the skin, gastrointestinal tract and the airways[1]. MC progenitors are released from the bone marrow into the systemic circulation as undifferentiated mononuclear cells and migrate into tissues where they differentiate into mature MCs[2, 3]. A range of MC chemoattractants have been identified and include chemokines (e.g. C-C motif chemokine ligand 5 [CCL5] a.k.a regulated on activation, normal T cell expressed and secreted [RANTES])[4], growth factors (e.g. stem cell factor [SCF])[5], leukotrienes (e.g. leukotriene B_4; LTB_4)[6] and immunoglobulin E (IgE)[7]. The recruitment of MC progenitors likely occurs in response to a regulated combination of these factors and others (e.g. adhesion molecules). SCF (a.k.a. KIT ligand) is the primary growth factor required for MC growth and survival and is expressed by stromal cells in soluble and membrane-bound forms. The particular cytokine profile of the tissue as well as interactions with the tissue matrix and resident cells is responsible for MC maturation[2, 3]. As a result MCs are a heterogeneous population and can vary across organs and tissues giving rise to differences in traits including ultrastructure, protease content, receptor expression, activation and mediator composition[2, 3].

MCs have an abundance of electron-dense secretory granules filled with pre-formed mediators and in response to different stimuli MCs release their granule contents into the extracellular environment i.e. they degranulate. Proteases are highly expressed in MCs and classically define the phenotype of MCs in specific tissues. For example, MCs expressing tryptase only (MC_T) are typically associated with localisation at mucosal surfaces such as the bronchial lamina propria while those expressing tryptase and chymase (MC_{TC}) are typically located within connective tissue such as the skin[3]. Cathepsin G and carboxypeptidase A (CPA) are also expressed by MC_{TC} with CPA3, tryptase and chymase being MC specific proteases[8]. MC secretory granules also contain a number of additional pre-formed mediators including histamine, lysosomal enzymes, cytokines and growth factors,

proteoglycans, non-MC specific proteases and granule membrane-associated proteins[8]. Pre-formed mediators can be released within seconds of MC activation via degranulation but MC activation can also lead to the rapid (within minutes) *de novo* synthesis of lipid-derived mediators, generated from arachidonic acid without the need for transcription. These include prostaglandins (e.g. prostaglandin D_2; PGD_2), leukotriene B_4 (LTB_4) and cysteinyl leukotrienes (cys-LTs, e.g. LTC_4)[8]. Within hours of activation MCs are also able to generate and release a range of cytokines, chemokines and growth factors (e.g. C-X-C motif chemokine ligand 8, CXCL8; interleukin (IL)-4 and granulocyte-macrophage colony stimulating factor, GM-CSF)[3]. MC activation can also lead to the release of mediators without degranulation via alternative pathways including exocytosis and piecemeal degranulation[9, 10].

The physiological role of MCs is not fully understood, however, they are ideally placed at mucosal surfaces to detect and rapidly alert the immune system to environmental signals. Studies into the role of MCs against parasitic infections suggest MCs respond to clear infections by driving Th2 responses including mucus production and eosinophil recruitment through IgE dependent mechanisms[11]. More recent studies have looked into the role of MCs against bacterial and viral pathogens[12-14], however, MCs are classically known as the effector cell type of allergic disorders. As developments in sanitation infrastructure and hygiene practices have meant parasite infections are no longer prevalent in some parts of the world, MC antigen-specific IgE and Th2 responses appear to be misdirected against non-infectious antigens i.e. the hygiene hypothesis.

1.2 Mast Cells and Innate Immunity

The release of MC mediators is key in facilitating MC functions including their roles in innate immunity. A number of MC mediators have roles in the alteration of vascular permeability, tissue remodelling and the recruitment of effectors cells[13]. For instance, mediators including histamine and leukotrienes (LT) increase vascular permeability, while mediators such as MC proteases, tumour necrosis factor (TNF)-α, leukotrienes and chemokines recruit effector cells including natural killer (NK) cells (via CXCL8), neutrophils (via CXCL8, TNF-α, LTB_4) and eosinophils (via CCL11, LTC_4)[12-14]. As well as the classic Th2 cytokines IL-4, IL-5 and IL-13 produced in response to helminth infections, MCs produce Th1 cytokines and chemokines. These include IFN-γ, IL-12, CXCL8 and CXCL10 which promote Th1 cell development and aid in the immune response against intracellular pathogens[15].

MC also produce cathelicidins, a family of antimicrobial peptides which act by pathogen specific disruption of microbial membranes. Human and mouse MCs express LL-37[16] and cathelin-related antimicrobial peptide (CRAMP)[17] respectively. CRAMP is inducible by lipopolysaccharide (LPS) and lipoteichoic acid (LTA) of Gram negative and positive bacteria respectively and has been demonstrated to inhibit the growth of group A streptococci (GAS)[18]. In mice, cathelicidins have also been shown to protect against invasive GAS skin infections and influence neutrophil recruitment[19]. Extracellular MC innate immune mechanisms also include the formation of extracellular traps. Extracellular traps were first described in neutrophils which release granule proteins and DNA forming an extracellular matrix (extracellular trap) able to degrade virulence factors and kill bacteria[20]. MC extracellular traps (MCETs) are formed of components including DNA, histones, tryptase and the anti-microbial peptide LL-37; and inhibit the growth of pathogens including *Streptococcus pyogenes*[21] and *Listeria monocytogenes*[22]. Intracellular innate immune functions of MCs include phagocytosis which has been demonstrated by human cord blood MCs in response to bacteria including *Staphylococcus aureus* and *Escherichia coli* and is associated with a decrease in bacterial viability[23]. However, phagocytosis is not always observed in response to bacterial infection[24] and bacterial uptake may occur without killing and in the case of *S. aureus* can be mediated by the pathogen as a mechanism of immune evasion[25, 26].

MC are also able to directly detect and respond to pathogens via the expression of pathogen recognition receptors (PRRs). PRRs are constructively expressed and recognise conserved microbial features, i.e. pathogen associated molecular patterns (PAMPs) as well as host damage-associated molecular patterns (DAMPs)[27]. MCs express several classes of PRRs including the membrane bound Toll-like receptors (TLRs) and the cytoplasmic receptors nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs)[14]. TLRs are membrane bound receptor molecules and in accordance with the microbial components recognised by each TLR, TLRs are either expressed on the cell surface (TLRs 1, 2, 4, 5, and 6) or within intracellular compartments (TLRs 3, 7, 8, and 9). For example, among other components, TLRs 1, 2, 4 and 6 recognise bacterial lipids (e.g. LPS by TLR4) and TLRs 3, 7 and 8 recognise viral RNA (e.g. single stranded (ss) RNA by TLR7/8). TLRs 1-10 have been identified in humans and MCs have been shown to express TLRs 1-9[9, 28, 29]. Although

TLR expression varies between studies, MCs express a range of TLRs which detect parasitic, bacterial, fungal and viral components[27]. Depending on the activating PAMP, TLR activation triggers MCs secretion of inflammatory cytokines, chemokines, type I IFNs and antimicrobial peptides. For example, activation of TLR4 by LPS results in the selective secretion of GM-CSF and IL-1β not secreted in response to TLR2 activation by peptidoglycan[29]. MCs also express NOD-1 and -2 which are members of the NLR family of PRRs. These receptors recognise bacterial cell wall components leading to the secretion of pro-inflammatory cytokines such as IL-6 and CXCL8[30] and in murine MCs NOD-1, in association with TLR2, activates MC degranulation in response to peptidoglycan[31]. MC expression of the RLR family of cytoplasmic PRRs includes retinoic acid-induced gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) which detect PAMPs within viral RNA. RLR activation induces the transcription of target genes including type I interferon (IFN) and the induction of anti-viral responses[32]. RLR expression is induced by IFN (i.e. they are IFN stimulated genes [ISGs]) and has been demonstrated in MC e.g. type I IFN treatment upregulates the expression of MAD5 and RIG-I in human MCs[33]. Furthermore, human MCs secrete type I IFN, cytokines and chemokines via RIG-I and MDA5 in response to virus infection[34, 35]. MCs also recognise pathogens via c-type lectin receptors (CLRs), a class of carbohydrate-binding receptors of which MCs express dectin-1. MC detection of the fungal cell wall component zymosan results in leukotriene C_4 production[36], a MC mediator which induces vascular permeability, bronchoconstriction and eosinophil recruitment[13].

Overall, MCs are able detect pathogens via multiple receptors which allows them to rapidly initiate immune responses tailored to the activating pathogen.

MCs are classically associated with defence against parasitic infections caused by nematode helminths. Infections caused by parasitic helminths, or worms, generally result in mild disease in the majority of people who become infected, however, in some cases helminth infection results in more serious and potentially life threatening disease. Helminths are diverse and have complex lifecycles, however, the host immune response to infection generally involves Th2 responses with the generation of Th2 cytokines including IL-4, IL-5, IL-13 and IL-10 as well as IgE and the recruitment of Th2 cells, eosinophils and MCs[37]. Protective immune responses to helminth infection include the formation of granulomas which encapsulate parasite eggs preventing their migration and maturation. For example, during *Schistosoma mansoni* infection parasite eggs in the liver trigger granuloma

formation mediated by CD4 T cells specific for antigens released from the eggs. Granulomas are well defined lesions composed of cells including macrophages, eosinophils and lymphocytes surrounded by an extracellular matrix. Granulomas can form in type 1 or type 2 immune environments although in mouse models type 2 responses have been shown to be required to limit potentially lethal inflammation in the liver[38]. However, during chronic murine infection pathological tissue fibrosis can develop as a result of ongoing Th2 inflammation with particular involvement of IL-13[39]. A number of cell types are involved in anti-parasitic immunity, which directly target parasites and/or promote type 2 immune responses. Macrophages are classically activated by Th1 immune cytokines, however they can be alternatively activated by Th2 cytokines and rapidly recruited to sites of parasitic infection[40, 41]. As well as their role in granuloma formation, alternatively activated macrophages have been demonstrated to be important for protection against *Heligmosomoides polygyrus* infection via a mechanism dependent on macrophage arginase-1 and the presence of CD4 T cells and IL-4[42]. Neutrophils are also recruited to sites of parasitic infection and have been shown to play a role, in combination with eosinophils, in the killing of *Strongyloides stercoralis*[43]. However, during *S. mansoni* infection neutrophils are not required for parasite killing[44]. Similarly, the role of eosinophils in anti-parasitic immunity is mixed, for instance, they are suggested to protect against *Strongyloides stercoralis* infection but not *Schistosoma mansoni* infection[43, 45]. MC degranulation has been observed during certain parasitic infections and MC mediators may mediate anti-parasite immunity via a number of mechanisms including MC protease-mediated degradation of structural parasite components[46], the stimulation of intestinal smooth muscle contraction and the regulation of vascular permeability (via protease mediated degradation of tight junctions) which may promote parasite expulsion[47], glycosaminoglycan mediated prevention of parasite attachment and invasion[48, 49] and the recruitment of effector cells such as eosinophils[50]. In mouse models MC anti-parasitic immunity has been demonstrated against *Heligmosomoides polygyrus*, *Trichinella spiralis*, *Strongyloides ratti* and *Strongyloides venezuelensis*, infection with which results in increases in murine mucosal MC numbers which is associated with increased resistance to infection and accelerated parasite clearance[51-54]. In addition, the depletion of SCF or *c-kit* deficiency delays clearance of *Trichinella spiralis* infection from the gut which is associated with a suppression in the expansion of mucosal MCs[55]. However, *c-kit* deficient mice have abnormalities including altered gut motility due to a deficiency of the interstitial

cells of Cajal in the gastrointestinal tract which also express KIT. Therefore MCs may not be solely responsible for the observed anti-helminth responses. In addition, the MC specific protease, mouse mast cell protease 1 (MCP)-1, has been shown be important for the clearance of T*richinella spiralis*, however, mMCP-1 has no effect on the clearance of *Nippostrongylus brasiliensis* from mMCP-1 deficient mice[56]. Overall, there may not be a particular cell type which is indispensable for anti-parasitic immunity but instead anti-parasitic immunity likely relies on the co-ordinated response of a variety of cell types.

As well as roles in anti-parasitic immunity, the type 2 immune responses of MCs are thought to mediate immunity against venoms. For example, the symptoms of type I hypersensitivity reactions including sneezing, coughing, vomiting and diarrhoea have been suggested to provide mechanisms to rapidly expel, neutralise and avoid toxic substances in sensitised individuals[57]. MCs can be activated by venoms resulting in the release of mediators which diminish venom toxicity e.g. via the degradation of venom components[58]. For example, murine MCs incubated with sarafotoxin 6b, a component of Israeli mole viper venom, diminishes venom toxicity and significantly, MCs also minimise the toxicity of whole viper venom[59]. Detoxification is associated with increased survival and is mediated, at least in part, by CPA3. MC-mediated immunity against venoms has also been demonstrated against the whole venom of the Gila monster (a venomous lizard) mediated by the murine MC protease mMCP4[60]. In addition, MCs limit the morbidity and mortality associated with honey bee, diamondback rattlesnake, southern copperhead snake, Deathstalker scorpion and Arizona bark scorpion venom[59, 60]. The position of MCs within the skin, which is a common site of envenomation, means that they are well placed to rapidly detect and degrade toxins and venoms.

The role for MCs in bacterial immunity was first demonstrated in murine models of septic peritonitis and *Klebsiella pneumoniae* lung infection, in which bacterial clearance and survival were impaired in MC-deficient mice compared to wild type animals[61, 62]. The MC deficient mouse models used in these studies were WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ (*Kit*$^{W/W\text{-}v}$) mice which carry a *c-kit* mutation (*Kit*^{W})[63] resulting in a truncated KIT receptor which is not cell surface expressed and another mutation (*Kit*$^{W\text{-}v}$)[64] in the *c-kit* tyrosine kinase domain that significantly reduces KIT's kinase activity. As SCF is an obligate MC growth factor inhibition of KIT signalling depletes MC populations. Further studies in the *Kit*$^{W/W\text{-}v}$ model suggests a protective role for MCs in immunity against a number of Gram-positive and Gram-negative bacteria including *Listeria*

monocytogenes[65] and *Streptococcus pyogenes*[66] as well as *Pseudomonas aeruginosa*[67] and *Helicobacter pylori* respectively[68]. However, *Kit*$^{W/W\text{-}v}$ mice have reduced numbers of basophils and neutrophils and are anaemic and sterile[69]. Another MC deficient model was developed, C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ (a.k.a. Sash mice), which carry an inversion mutation upstream of the *c-kit* start site interrupting its transcription[70]. This model has increased numbers of basophils and neutrophils but is not sterile or anaemic[69] and has also demonstrated a role of MCs in anti-bacterial immunity[71, 72]. KIT is expressed on cells including haematopoietic stem cells, melanocytes, germ cells and interstitial cells of Cajal in the gastrointestinal tract, and as mentioned, *c-kit* deficient mice develop phenotypic abnormalities associated with the expression of *c-kit* outside of the MC compartment[69]. Therefore, an important limitation of *c-kit* deficient mice is that where differences in their responses are observed compared to wild type (WT) animals, the contribution of MCs versus the contribution of off-target phenotypic abnormalities can not be definitively concluded. To overcome this, knock-in models engraft WT or genetically altered MCs that are cultured *in vitro* into *c-kit* deficient mice[69]. Therefore if the introduction of MCs results in a return to the WT phenotype MCs may be concluded to play a role in a given response. However, there are also limitations with this approach, for example, depending on the route of administration and the numbers of MCs introduced, the numbers and anatomical distribution of MCs varies and/or does not match the WT phenotype[69, 73]. Engraftment can also be achieved via bone marrow transplantation, however, this does not selectively replace MCs alone. To overcome the problems of *c-kit*-dependent mouse models, *c-kit*-independent MC deficient models have been developed. These models typically express a Cre-recombinase under the control of MC specific/MC associated promoters namely *Mcpt5*[74] (mouse equivalent of chymase 1) and *Cpa3*[75, 76]. In these models MCs are constitutively knocked out, therefore to nullify potential off-target effects of constitutive MC ablation, inducible knock-out models have been developed in which the diphtheria toxin receptor is under the control of MC specific/MC associated promoters[74, 77]. Once again these models are not without their limitations e.g. CPA3 is expressed in basophils as well as MCs and treatment with diphtheria toxin may have off-target effects. The MC deficient mouse models discussed are also relevant to studies of MC anti-parasitic immune responses discussed above. Although these *c-kit*-independent MC deficient models have been developmed the majority of the investigations demonstrating the role of MCs in anti-bacterial immunity have been demonstrated with *Kit*$^{W/W\text{-}v}$ mice[78].

The action of MCs in anti-bacterial immunity is thought to involve the direct targeting of bacteria via MC mediators and the recruitment and/or modulation of other effector cells. For example, in the initial demonstrations of MC immunity during septic peritonitis (*Klebsiella pneumoniae* and *E. coli* infection), the impaired prognosis of MC-deficient mice was due to diminished neutrophil recruitment resulting from a lack of MC derived TNF-α and leukotrienes[61, 62, 79]. In addition, MCs may promote neutrophil bactericidal activity via MC-derived IL-6[80]. MC proteases also have roles in bacterial immunity, for example, mMCP6 is thought to mediate the release of neutrophil chemoattractants from surrounding cells protecting WT mice during Klebsiella infection compared to mMCP6-deficient mice[81]. In addition, treatment with recombinant tryptases lead to the recruitment of effectors including neutrophils and eosinophils. MCs have also been implicated in human bacterial infection, for instance, MCs have been shown to accumulate in the stomach mucosa of patients with *Helicobacter pylori*-associated gastritis and associated with the accumulation of neutrophils, macrophages and T cells[82]. In addition, t he risk of severe *Shigella* infection is greater in children than in adults and has been suggested to be due in part to the delayed accumulation of MCs to the rectal mucosa of paediatric patients[83]. Although the majority of reports suggest MCs are protective against bacterial infection, some studies demonstrate harmful MC responses. For example, using the cecal ligation and puncture (CLP) model of sepsis in $Kit^{W\text{-}sh/W\text{-}sh}$ mice, MCs were protective during less severe bacterial infection but MC-derived TNF-α mediated detrimental responses during severe infection[84]. This was in agreement with a CLP study using *c-kit*-independent MC-deficient mice in which survival during severe infection was improved compared to controls via a lack of MC-derived IL-4 suppressing macrophage phagocytosis[85]. Although this was in agreement with the previous study, these mice were not subjected to milder infection so that comparison can not be made. The role of MCs in bacterial immunity may require further investigation, particularly using *c-kit*-independent MC-deficient mice and considering parameters such as disease severity.

MCs also play a role in the induction of adaptive immune responses which involve the pathogen/antigen specific immune responses of cells including cytotoxic CD8 T cells and the generation of immunological memory. DCs bridge the gap between innate and adaptive immune responses via the presentation of antigen to naive T cells within draining lymph nodes in the process of T cell differentiation and maturation into antigen-specific effector cells. MCs produce mediators such as TNF-

α, IL-6, and histamine, which promote the migration of DCs into lymph nodes following bacterial peptidoglycan treatment of WT mice compared to MC-deficient mice[86, 87]. In addition, MCs have also been demonstrated to recruit CD4 T cells to lymph nodes via TNF-α[88] which suggests MCs promote DC antigen presentation to CD4 T cells and the induction of adaptive immune responses.

More recently, the role of MCs in viral immunity has also begun to be investigated. Less data exists on the role of MCs in viral immunity but MCs have been shown to release mediators in response to challenge with virus and virus products[12, 13, 89]. One of the viruses studied in relation to MC responses to viral pathogens is dengue virus, an arthropod-borne positive-sense single-stranded (ss) ribonucleic acid (RNA) virus of the *Flaviridae* family. Dengue virus infection can result in asymptomatic or subclinical infection with mild flu-like symptoms but can also result in life threatening dengue haemorrhagic fever and/or dengue shock syndrome for which there are currently no vaccines or specific therapies[90]. In response to dengue virus infection, MCs upregulate anti-viral responses such as the expression of the viral sensors MDA5 and RIG-I, IFNs and ISGs[34, 91]. MCs also produce chemokines including CCL5, CCL3 (a.k.a. macrophage inflammatory protein 1-α [MIP1-α]) and CCL4 (a.k.a. macrophage inflammatory protein 1-β [MIP1-β]) in response to antibody-enhanced dengue virus infection, which are cytokines associated with the recruitment and activation of T cells during infection[92]. Furthermore, MC-deficient mice show increased dengue virus burden as a result of impaired MC-dependent natural killer (NK) and natural killer T (NKT) cell recruitment[91].

MCs also respond to viruses such as vesicular stomatitis virus (VSV), a negative sense ssRNA virus, to which MCs mount anti-viral responses including the upregulation sensors of viral RNA (e.g. MDA5), type I IFNs (IFN-α and IFN-β) and chemokines (e.g. CCL5 and CXCL10 [a.k.a. IFN-γ inducible protein 10, IP-10])[35]. In response to respiratory viruses such as respiratory syncytial virus (RSV) cord blood-derived MCs (CBMCs) also upregulate IFNs and ISGs and secret CXCL10, CCL5 and CCL4 which are associated with NK cell, T cell and monocyte recruitment respectively[93]. NK cells have been also shown to have enhanced activation in response to MCs infected with reovirus[94], a double stranded (ds) RNA virus associated with human upper respiratory tract infections. Furthermore, reovirus as well as influenza virus, RSV and polyinosinic:polycytidylic acid (poly I:C; a synthetic mimic of viral dsRNA), induce IFN release from peripheral-blood derived MCs via

toll-like receptor (TLR) 3 activation[9]. Sendai virus is a murine virus used to model infections with human parainfluenza viruses which cause upper and lower respiratory tract infections[95]. Peripheral-blood derived MCs also respond to sendai virus infection by upregulating type I and type III IFNs and ISGs (Myxovirus resistance protein 1 [MxA] and interferon-induced protein with tetratricopeptide repeats 3 [IFIT3]), RIG-I, MDA5 and TLR3[33]. Rhinovirus infection of MCs has been investigated in the immature MC line, HMC-1, which secretes IL-4, IL-6, CXCL8 (a.k.a. IL-8) and GM-CSF in response to infection. However, in this case cytokine secretion in response to infection only occurs in the presence of a stimulator of MC degranulation (e.g. IgE plus anti-IgE)[96].

These responses to a range of viruses suggest MCs upregulate protective anti-viral immune responses, however, in other instances MC responses are reported to be detrimental. For instance, during murine influenza A virus infection MCs have been demonstrated to be responsible for pulmonary and systemic inflammation via the release a range of cytokines and chemokines mediated by RIG-I[97]. MCs have been implicated with detrimental responses during HIV infection by promoting the infection of $CD4^+$ T cells[98], furthermore, HIV infected progenitor MCs mature into latently infected cells capable of releasing infectious virus[99]. Additionally, MCs are susceptible to antibody-enhanced dengue virus infection to which they can respond with the release of IL-1β and IL-6 able to induce fever, inflammation and shock and modulate inflammatory cell migration[100]. Antibody-enhanced dengue virus infection of MCs can also result in TNF-α mediated endothelial cell activation[101] and in MC-deficient mice dengue virus associated vascular permeability has been shown to be MC dependent[102]. Overall, this suggests the role of MCs in viral immunity is complex and may require pathogen specific investigation.

MCs are well placed to detect and respond to invading pathogens, however, the pathogenic release of MC mediators in response to innocuous substances make MCs key effectors in allergic disorders including allergic asthma[103, 104]. MCs express the high affinity IgE receptor, FcεRI, and in atopic individuals such as those with allergic asthma, FcεRI is bound by allergen specific IgE. As a result individuals become sensitised to allergens, such as Der p 1 found within house dust mite, which are able to trigger the release of MC mediators associated with allergy.

1.3 Mast Cells and Allergic Asthma

Asthma is a complex chronic respiratory disease affecting over 5 million people in the UK and over 300 million people worldwide[105, 106]. It is characterised by airway inflammation, hyper-responsive airway smooth muscle (ASM) and variable and reversible airway obstruction[107]. Airway obstruction occurs as a result of factors including mucus hyper-secretion, mucosal oedema and smooth muscle hyperplasia and results in symptoms including wheeze, chest tightness and shortness of breath[107]. Asthma is heterogeneous and can be described according to demographic, clinical and/or pathophysiological characteristics. Allergic asthma generally has an early onset and is often associated with other allergic disorders such as eczema (atopic dermatitis)[107]. It is a common phenotype defined by the presence of circulating IgE directed against aeroallergens generated when individuals become sensitised to innocuous allergens e.g. found within house dust mite and pollen.

In the generation of an adaptive immune response, antigen is sampled and processed by antigen presenting cells (APCs). APCs present antigen to naive T cells which become activated and go on to mediate their effector functions. In the development of allergic sensitisation, the same pathways result in the generation of anti-IgE immune responses against innocuous antigens. APCs such as DCs, B cells and macrophages detect and phagocytose foreign organisms and allergens delivering them across epithelial barriers for presentation to naive T cells (**Figure 1-1**). Epithelial barriers may also be disrupted by pathogens or environmental stress (e.g. cigarette smoke) and some allergens have protease activity (e.g. Der p 1 of house dust mite) which may facilitate entry via cleavage of epithelial tight junctions[108]. Depending on the source of the antigen, intracellular or extracellular respectively, antigen presentation occurs via major histocompatibility complex (MHC) class I or class II on the surface of APCs presenting to CD8 or CD4 T cells respectively[109] (**Figure 1-1**). However, certain DC subsets can also present exogenous antigen via MHC class I in a process called cross-presentation[110] and endogenous peptides expressed by APCs can be presented by MHC class II[111, 112]. Prior to antigen presentation, proteins (both self and non-self) are degraded into peptides which are loaded onto MHC molecules. APCs engage naïve T cells via interactions between peptide bound MHC molecules on APCs and the T cell receptor (TCR). These interactions are stabilised by CD4 and CD8 expressed on T cells binding to their respective MHC molecules at areas away from the peptide

binding site[113]. The antigen specific interaction between the TCR and the MHC:peptide complex is the first signal in the "three-signal activation model" of T cell activation. The second activating signal is provided by co-stimulatory molecules such as CD80/CD86 on APCs binding to CD28 on T cells. These signals promote (or inhibit) T cell survival and expansion. The binding of cytokines to their receptors on T cells provides the third activating signal driving the differentiation of naïve cells into mature effector and memory cell populations. Naïve CD8+ T cells mature into cytotoxic effector cells under the influence of cytokines including IL-2, IL-12 and type I IFNs secreted by DCs and/or macrophages[114]. Naïve CD4 T cells differentiate into subsets including Th1, Th2 and regulatory T cells which are defined according to their cytokine secretion profile and relates to their effector functions. For example, Th1 cells secrete IFN-γ and TNF-α which can activate macrophages in the killing of intracellular pathogens, whereas Th2 cells typically secrete IL-4, IL-5 and IL-13 which activate responses against extracellular parasites. Th1 development is promoted by IL-12 and IFN-γ whereas Th2 differentiation is driven by IL-4[115]. The activating cytokines are secreted by cells including activated NK cells, T cells, basophils, eosinophils and MCs[116]. In the process of allergic sensitisation to allergen, APCs present allergen-derived peptides to naïve CD4 T cells which in the presence of an early source of IL-4 differentiate into Th2 cells which secrete Th2 cytokines including IL-4 and IL-13, further promoting Th2 differentiation (**Figure 1-1**). Activated Th2 cells engage plasma cells (antibody secreting B cells) via co-stimulatory molecules such as CD40 ligand and CD28 binding with CD40 and CD80/86 on B cells. Co-stimulatory receptor ligation in the presence of IL-4 and IL-13 induces plasma cell immunoglobulin class switching and the secretion of IgE (and IgG)[103, 117, 118] (**Figure 1-1**). IL-4 and IL-13 may also be produced by MCs and basophils. IgE ultimately enters the systemic circulation and binds to cells such as MCs and basophils via FcεRI, the high affinity IgE receptor, thus sensitising the individual to the specific allergen (**Figure 1-1**). Allergen-specific IgE is generated by plasma cells in lymphoid germinal centres but class-switch recombination to IgE may also occur in organs specific to allergic disorders for example, the bronchial[119], intestinal[120] and nasal[121-123] mucosa of individuals with asthma, food allergy and allergic rhinitis respectively. In a sensitised individual, IgE bound to MCs via FcεRI is not complexed with antigen and MCs are not activated. However, re-exposure to the sensitising allergen can result in FcεRI receptor cross-linking which triggers signalling events leading to MC degranulation.

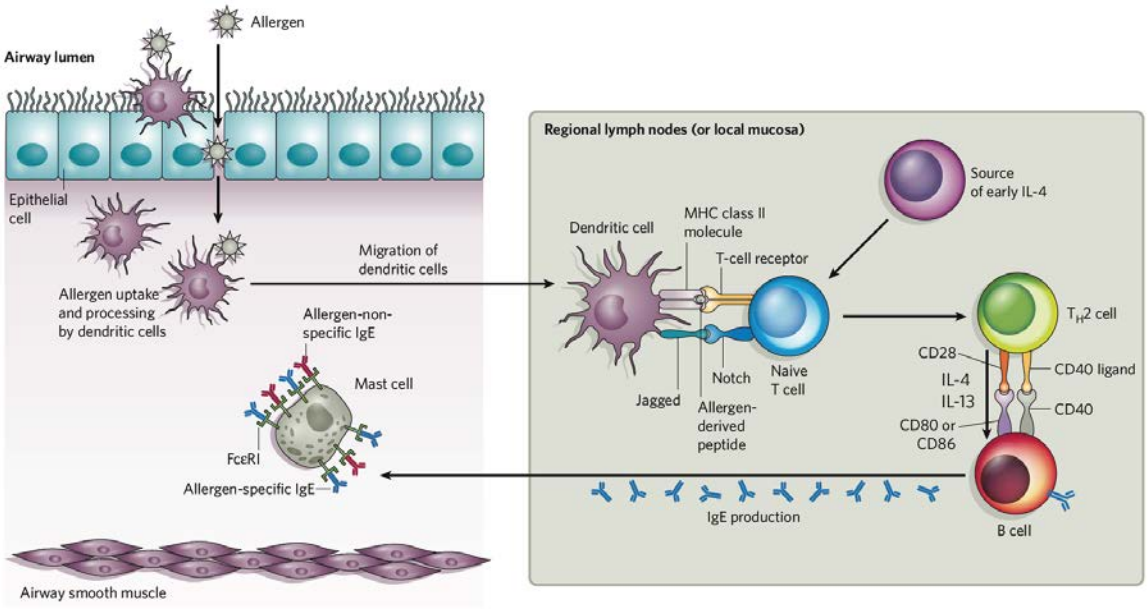


Figure 1-1. Allergen sensitisation. Allergen is sampled by antigen presenting cells and may disrupt epithelial barriers via inherent enzymatic activity e.g. house dust mite protease Der p 1. Antigen presenting cells process and present allergen via MHC class II molecules to naive CD4 T cells which in the presence of IL-4 differentiate into active Th2 cells. Activated Th2 cells secrete IL-4 and IL-13 and engage B cells inducing B cells to undergo immunoglobulin class switching and mature into IgE secreting plasma cells. Allergen-specific IgE enters the circulation and is bound by FcεRI expressing cells such as mast cells completing the process of sensitisation. Figure reprinted by permission from Macmillan Publishers Ltd: Nature (Galli SJ, Tsai M and Piliponsky AM. The development of allergic inflammation), copyright (2008)[117].

The FcεRI receptor is expressed in two isoforms, one isoform which is expressed on MCs and basophils is a tetramer composed of three chains, $\alpha\beta\gamma_2$ whilst the second isoform is a trimer lacking the β chain and expressed on a range of cells including macrophages[124]. The α chain mediates IgE-binding while the β chain mediates signal amplification and both isoforms bind and mediate IgE responses. IgE has a second receptor, FcεRII (CD23), expressed on cells including B cells and epithelial cells where it is involved in IgE-mediated responses via the regulation of IgE synthesis and the transcytosis of IgE and IgE-antigen complexes across epithelial barriers[124, 125]. However, this receptor has a lower affinity for IgE and is termed the low affinity IgE receptor. The dissociation of IgE and FcεRI is very slow with a half-life reported at 2-3 weeks[126]. This defines the high affinity nature of the IgE-FcεRI interaction and also facilitates long term sensitisation. Re-exposure to a sensitising allergen results in receptor cross-linking which occurs when multivalent

antigen (i.e. multiple IgE binding sites) binds and aggregates FcεRI molecules on the surface of MCs initiating their association with lipid rafts[3]. The resulting signalling events are complex[127-129] (**Figure 1-2**) but the key events are briefly described here. FcεRI β and γ chains contain immunoreceptor tyrosine-based activation motifs (ITAMs) within their cytoplasmic domains which are required for effective receptor signalling[3]. ITAMs contain tyrosine residues which are phosphorylated by Lyn kinase (a Src family tyrosine kinase) found within lipid rafts. This creates docking sites for Src-homology-2 (SH2) domain containing proteins including Lyn itself[128]. Syk is recruited to the ITAMs and binds the FcεRI γ chain but not the β chain due to a difference in the ITAM consensus sequence of the FcεRI β chain[3]. Syk is then phosphorylated by Lyn leading to its activation and the phosphorylation of the adaptor molecule NTAL (non-T-cell activation linker) by both Lyn and Syk[129] (**Figure 1-2**). The signalling enzyme phospholipase C γ (PLCγ) is activated downstream of Syk and catalyses the production of inositol-1,4,5-trisphosphate (IP_3) from membrane phospholipids. IP_3 induces Ca^{2+} release from intracellular rough endoplasmic reticulum stores which in turn triggers the influx of extracellular Ca^{2+} via store-operated calcium channels (SOCC). The maintenance of elevated intracellular Ca^{2+} levels is required for degranulation and the release of stored and newly formed MC mediators. Eicosanoid (including leukotrienes and prostaglandins) production is activated via the adaptor molecule growth factor receptor-bound protein 2 (GRB2), which recruits effector molecules including SOS, guanine nucleotide exchange factors which activate the Ras GTPase leading to the activation of mitogen-activated protein kinases (MAPK)[3, 127, 129]. MAPK activation results in phospholipase A_2 activation and subsequent eicosanoid production[129] (**Figure 1-2**).

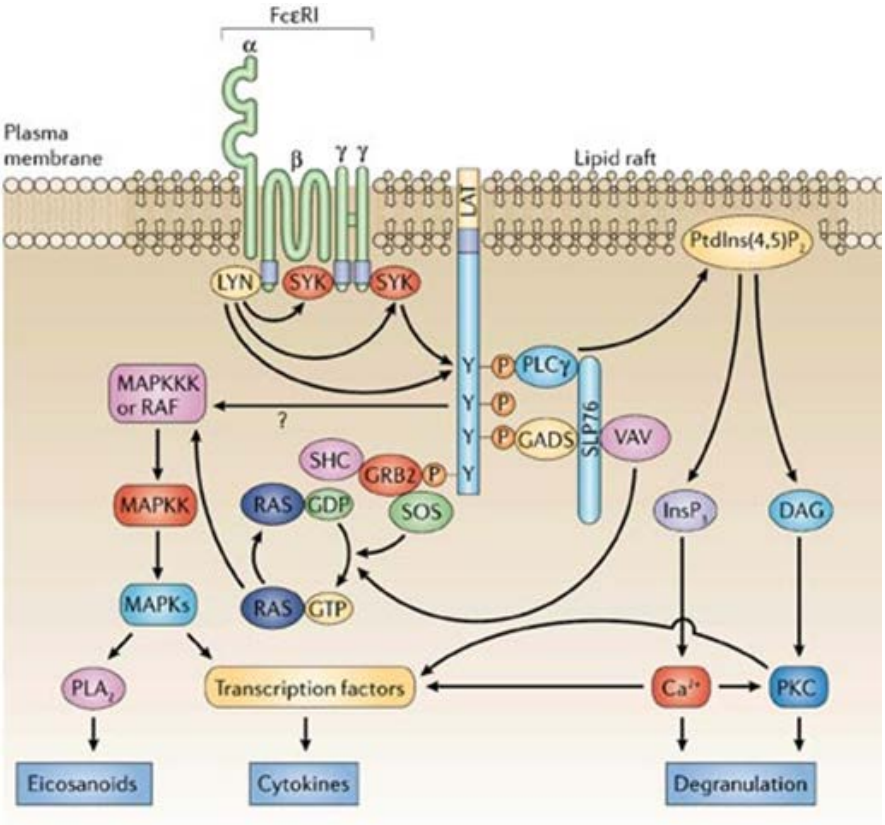


Figure 1-2. FcεRI signalling. Cross-linking of FcεRI by multivalent antigen results in receptor aggregation within lipid rafts (only one receptor is show but multivalent antigen cross-links multiple FCεRI receptors). This brings the receptors' cytoplasmic domains into contact with the kinases Lyn and Syk. Lyn phosphorylates Syk and both kinases phosphorylate the adaptor molecule NTAL (non-T-cell activation linker) which results in the recruitment and activation of signalling and adaptor molecules including phospholipase C γ (PLCγ) and GRB2 (growth factor receptor-bound protein 2). PLCγ signalling leads to the release of intracellular Ca^{2+} stores and the influx of extracellular Ca^{2+} which triggers MC degranulation. GRB2 recruits SOS (Son of Sevenless) proteins which are guanine nucleotide exchange factors which activate the Ras GTPase leading to the activation of mitogen-activated protein kinases (MAPKs). MAPK activation results in phospholipase A_2 activation and subsequent eicosanoid and cytokine production. Figure reprinted by permission from Nature Publishing Group: Nature Reviews Immunology (Gilfillan AM and Tkaczyk C. Integrated signalling pathways for mast-cell activation), copyright (2006)[129].

The binding of antigen-specific IgE to MCs, via FcεRI, prior to antigen binding means only binding of the antigen is required to trigger receptor cross-linking. This facilitates the rapid release of MC mediators in response to IgE and triggers the associated immediate type I hypersensitivity reactions. Furthermore, high levels of IgE can increase MC FcεRI expression, enhancing the sensitivity of cells to activation by IgE[130]. As mentioned, early-phase or type I immediate hypersensitivity reactions are mediated by the release of pre-formed granule associated MC mediators and lipid-derived mediators responsible for the rapid symptoms of early-phase reactions (within seconds to minutes). For instance, histamine, PGD_2 and LTC_4 induce bronchoconstriction, mucosal oedema and mucus hypersecretion which are features of asthmatic airway obstruction[3]. Late phase allergic inflammation develops 2-6 hours after initial exposure and in asthma is typified by increased airway hyper-responsiveness, bronchoconstriction and mucus secretion[117]. Late-phase reactions involve the recruitment and activation of inflammatory cells including Th2 cells, eosinophils and basophils, and MCs are thought to contribute to inflammation via the release of cytokines, chemokines and growth factors[117]. For example, TNF-α, CXCL8 and CCL4 are able to recruit monocytes/macrophages, neutrophils and T cells[3].

MC activation and allergic inflammation are key features of allergic asthma. Another important feature of asthma are exacerbations which are commonly triggered by viral infections[131]. The susceptibility and response of MCs for viral infection may implicate MCs in viral exacerbations of asthma.

1.4 Asthma Exacerbations

Asthma exacerbations are acute deteriorations in respiratory symptoms, such as shortness of breath and wheezing, and are associated with changes in the airways that may perpetuate further exacerbations[132]. Asthma exacerbations cause significant morbidity in patients and pose a burden to healthcare services[132]. Exacerbations typically require additional medical intervention in outpatient services or hospitalisation and have been proposed as a risk factor for accelerated loss of lung function[133]. Exacerbations may be associated with issues such as loss of work productivity and decreased school attendance. In addition, hospitalisation and accident and emergency visits due to exacerbations account for approximately 80% of the direct costs of asthma treatment[134]. In the treatment of asthma, inhaled corticosteroids (ICS) are used as controller therapy to suppress airway inflammation.

They act by suppressing the transcription of genes involved in inflammation including cytokines, adhesion molecules and receptors[135]. They are recommended for daily use to maintain reduced airway inflammation, control symptoms and reduce risk of asthma associated morbidities such as exacerbations and declines in lung function[136]. β_2-agonists are bronchodilators which act by binding to the active site of β_2 adrenergic receptors, which are highly expressed on airway smooth muscle, causing airway smooth muscle relaxation[137]. β_2-agonists may be short acting (SABAs) or long acting (LABAs) with activity lasting for 4 to 6 or approximately 12 hours respectively[137]. β_2-agonists are used as reliever medications to relieve acute symptoms or prevent exercise induced bronchoconstriction and are recommended for use as needed for all individuals with asthma[136]. As asthma severity increases, low or high doses of ICS are introduced which may be combined with one or more controller medication[136]. When asthma symptoms and/or exacerbations persist despite use of high dose controller medications and proper management of modifiable risk factors (e.g. exposure to tobacco smoke) add on therapies may be considered. These include anti-IgE, anti-IL-5 and oral corticosteroids[136], however, in some cases asthma remains uncontrolled despite the highest levels of therapy. Exacerbations are a burden to patients and in severe cases can result in the loss of life. They are often triggered by environmental factors including cigarette smoke, allergens, exercise and respiratory viral infections[107].

As well as being a risk factor for the development of asthma in early life[138-141], human rhinoviruses (HRVs) are the major trigger of viral-induced exacerbations of asthma[142, 143]. HRVs cause the majority of human upper respiratory tract infections and typically result in relatively mild cold-like symptoms in healthy individuals[144]. However, HRVs are associated with more severe lower respiratory tract illness in immunocompromised individuals and those with chronic respiratory diseases such as asthma and chronic obstructive pulmonary disease[145-147]. Currently there are no effective HRV vaccines or anti-viral treatments[144]. The mechanisms underlying HRV-induced asthma exacerbation may involve a damaged bronchial epithelium which facilitates infection of the lower airways[148], impaired anti-viral responses[149], including IFN responses[150, 151] and the host response to HRV aggravating existing inflammation and bronchial hyper-responsiveness[152]. However, the mechanisms of HRV-induced exacerbation are not fully understood.

1.5 Rhinovirus Lifecycle

HRVs are non-enveloped, positive-sense, ssRNA viruses which belong to the *Enterovirus* genus within the *Picornaviridae* family[144, 153]. There are over 100 HRV serotypes formed of three species: HRV-A, HRV-B and the more recently identified HRV-C[154]. HRV-A and HRV-B are divided into major and minor group viruses depending on their cellular receptor usage; major group viruses (the majority of HRV-A and all of HRV-B) use intracellular adhesion molecule 1 (ICAM1) whereas the minor group viruses use the low density lipoprotein receptor (LDLR) family members[144, 153]. The receptor for HRV-C was recently identified as cadherin-related family member 3 (CDHR3) which has also been identified as an asthma susceptibility gene[155, 156]. HRV-A and HRV-B entry occurs via endocytosis or micropinocytosis followed by capsid un-coating and release of the viral genome. This process is triggered by ICAM1 binding or acidification of the endosome in the case of major and minor group viruses respectively. The 7.2 kb HRV genome is translated by host ribosomes into a single polyprotein cleaved by viral proteases into capsid proteins (structural viral proteins; VP1-4 form an icosahedral capsid) and non-structural proteins which include viral proteases and a RNA-dependent RNA polymerase (RDRP) (**Figure 1-3**).

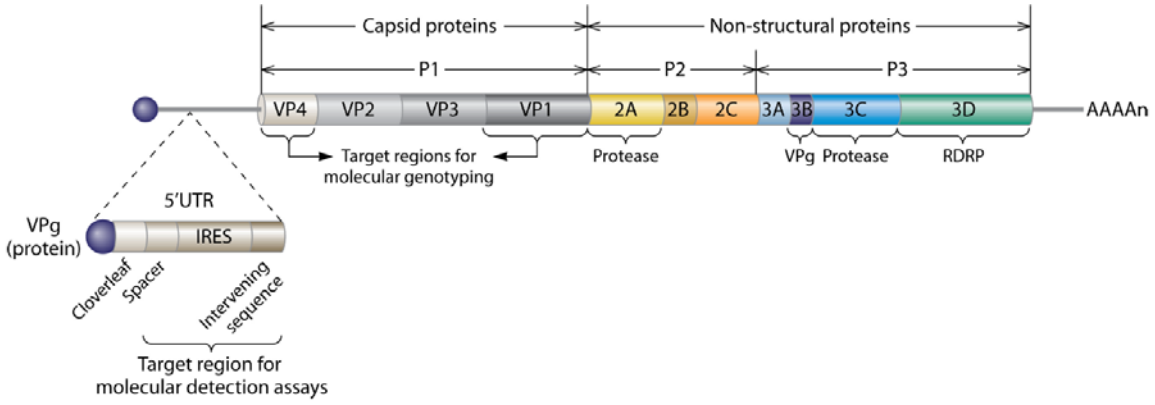


Figure 1-3. HRV genome organisation. The HRV genome is a single open reading frame of ~7.2 Kb. The genome is polyadenylated at the 3' terminal and the 5' terminal is bound by a small viral protein, which primes genome replication and a 5'untranslated region (UTR) which contains an internal ribosomal entry site (IRES) required for translation. The genome is transcribed into a polyprotein which is cleaved by viral proteases into 11 proteins which form the viral capsid or have functions related to replication (non-structural proteins), e.g. proteases and a RNA-dependent RNA polymerase (RDRP). Figure reprinted by permission from American Society for Microbiology: Clinical Microbiology Reviews (Jacobs SE, Lamson DM, St. George K and Walsh TJ. Human Rhinoviruses), copyright (2013)[153].

A RNA replication complex, comprised of RDRP in association with cellular and viral proteins, forms on cytoplasmic membranes and is the site of RNA synthesis[144, 157]. Negative strand RNA is synthesised as a template for genomic RNA which associates with capsid proteins to form infectious virus particles which are released upon cell lysis. However, picornavirus particles may also be released via non-lytic pathways[158, 159] (**Figure 1-4**).

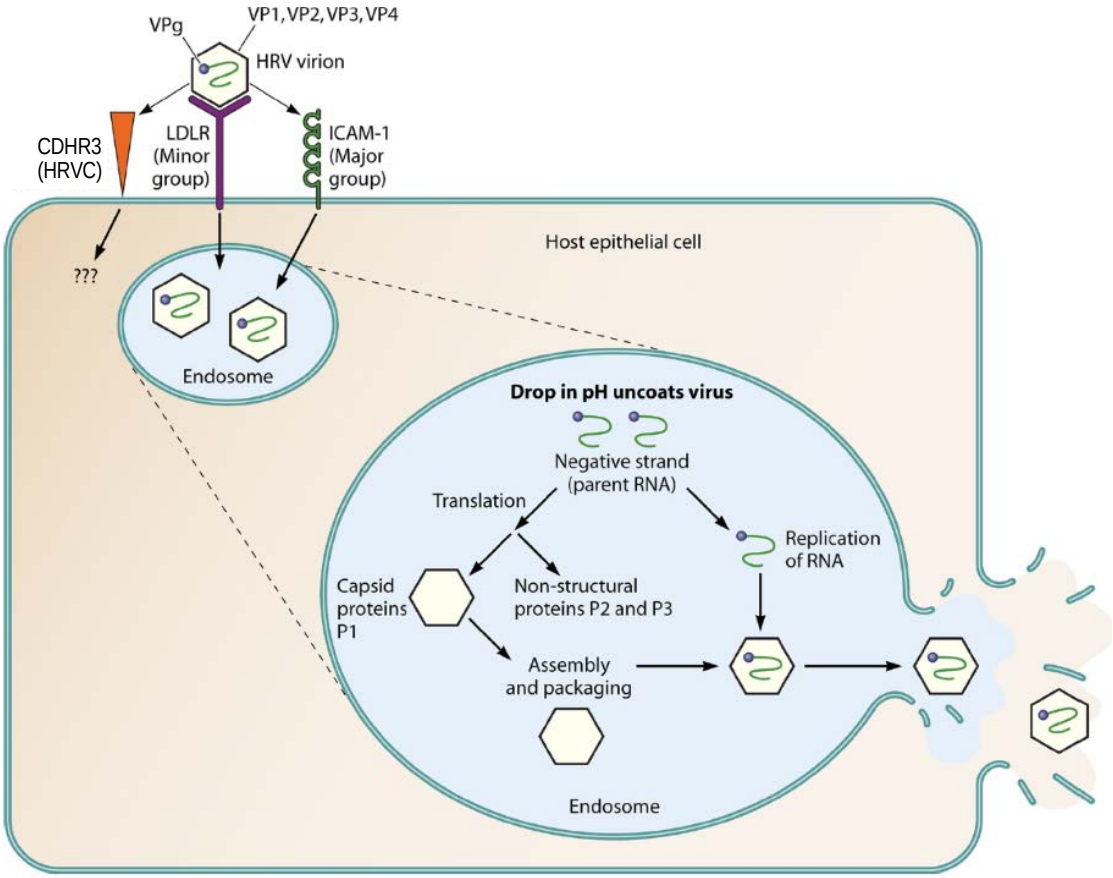


Figure 1-4 HRV replication cycle. The HRV capsid attaches to the cell via ICAM1, LDL receptor family members or CDHR3 in the case of major group HRVs (the majority of HRV-A and all of HRV-B), minor group HRVs (the remaining HRV-A viruses) or HRV-C viruses respectively. Receptor attachment leads to viral entry, capsid un-coating and release of the viral genome which is translated by host ribosomal machinery into a polyprotein precursor. The viral genome is replicated via negative strand RNA templates in complexes associated with cytoplasmic membranes and packaged into capsids. Virus particles exit via cell lysis but may also exit via non-lytic pathways. Figure adapted (HRVC receptor updated from unknown to CDHR3) and reprinted by permission from American Society for Microbiology: Clinical Microbiology Reviews (Jacobs SE, Lamson DM, St. George K and Walsh TJ. Human Rhinoviruses), copyright (2013)[153].

1.6 The Response of the Epithelium to Rhinovirus Infection

The airway epithelium is the primary site of HRV infection and replication as well as the key effector in the initiation of anti-viral host inflammatory responses[160]. HRVs are recognised by the airway epithelium via a number of PRRs. For instance the viral capsid is recognised by TLR2[161] on the epithelial surface while viral RNA is recognised by endosomal TLR3 (dsRNA), TLR7 (ssRNA) and TLR8 (ssRNA)[144, 153, 160, 162] (**Figure 1-5**). Viral RNA is additionally recognised by RLRs, cytoplasmic helicases which include RIG-I and MDA5 (**Figure 1-5**). Receptor engagement results in the activation of IFN regulatory factors (IRFs), a family of transcription factors involved in immune responses as well as haematopoietic differentiation and immune modulation[163]. MDA5 and RIG-I activate IRF3 which induces the initial secretion of IFN-β as well as cytokines including IL-1β, TNFα, IL-6 and CXCL8, recruiting and activating granulocytes, DCs and monocytes to the site of infection[144, 153, 160]. TLR activation similarly activates IFN regulatory factor (IRF)3 and IRF7 as well as NF-κB leading to the release of pro-inflammatory cytokines such as CXCL10 and type I IFNs (IFN-α and IFN-β)[144, 153, 160] (**Figure 1-5**). Type III IFNs (IFN-λ1-3 [a.k.a. IL-28A, IL-28B, IL-29] and IFN-λ4) are also regulated by IRF3 and IRF7. Secreted IFN-β binds to its receptor on infected and neighbouring uninfected cells further inducing its own expression as well as activating IRF7 which in turn amplifies IFN-α and IFN-β[163] (**Figure 1-5**). Furthermore, IFN-β signalling via the janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway and a complex of STAT1/2 and IRF9 (together called Interferon-stimulated gene factor 3, ISGF3) induces the expression of a host of IFN stimulated genes (ISGs)[160]. A number of ISGs have direct anti-viral effects such as degrading viral RNA (e.g. 2'5'-oligoadenylate synthase 1), blocking viral assembly (e.g. MxA) and inhibiting viral exit (e.g. viperin)[164] (**Figure 1-5**). Therefore IFN responses are key in anti-viral immunity.

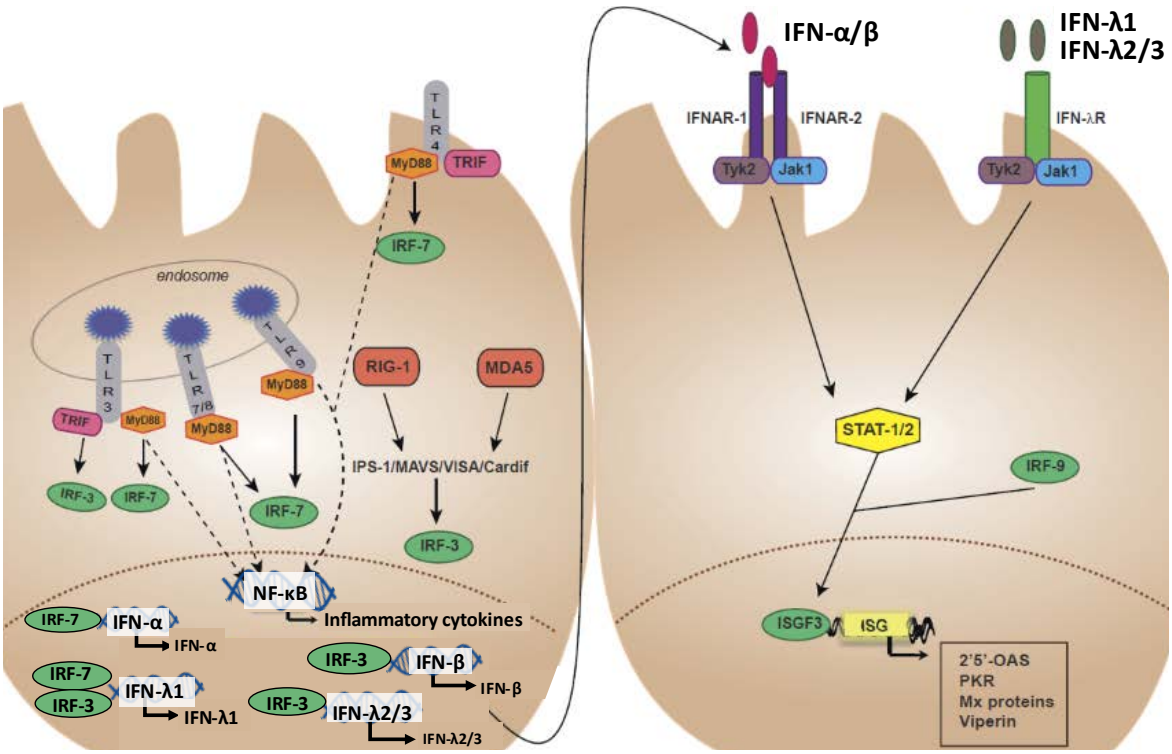


Figure 1-5. Epithelial IFN response to viral infection. The epithelium recognises viral infection via a number of PRRs. TLR3, TLR7/8 recognise dsRNA and ssRNA respectively and activate NF-κB, IRF3 and IRF7 resulting in the induction of pro-inflammatory cytokines and type I and type III IFNs. RIG-I and MDA5 bind cytoplasmic vRNA triggers IRF3 activation via an adaptor protein (called IPS-1, MAVS, VISA, or Cardif) and the transcription of type I and type III IFNs. IFN-β production from infected cells is initially activated by IRF3 and is followed by IFN-β-mediated IRF7 activation which induces late phase type I IFN production. IFN-λ1 is induced by IRF3 and IRF7 and IFN-λ2/3 by IRF7 alone. Secreted type I and type III IFNs bind to their distinct receptors, IFNAR1/2 and IFNLR1/IL10R2 respectively, but both signal via the JAK-STAT pathway activating ISGF3 (a complex of STAT1/2 and IRF9) which induce the transcription of a number of IFN stimulated genes with anti-viral functions including dsRNA-activated protein kinase R (PKR), 2,5-oligoadenylate synthetase (2',5'-OAS), myxovirus resistance proteins (Mx proteins) and viperin. Figure adapted and reprinted by permission from American Society for Microbiology: Clinical Microbiology Reviews (Vareille M, Kieninger E, Edwards MR, Regamey N. The airway epithelium: soldier in the fight against respiratory viruses), copyright (2011)[160].

1.7 The Role of Interferons in Anti-Viral Immunity

As mentioned, IFNs are cytokines with central roles in anti-viral immune responses. In humans, type I IFNs are comprised of IFN-α (13 subtypes), IFN-β, IFN-ε, IFN-κ and IFN-ω which signal via the type I IFN receptor, IFN-α receptor (IFNAR)1/IFNAR2 (**Figure 1-6**). Most cell types are capable of IFN-α/β production however plasmacytoid DCs (pDCs) are specialised for the production of type I IFN during viral infection[165]. Type II IFN (IFN-γ) signals via the IFN-γ receptor complex, formed of IFNGR1/IFNGR2 multimers (**Figure 1-6**). IFN-γ is mainly produced by immune cells including NK cells, NKT cells and CD4 and CD8 T cells but the receptor is widely expressed so most cell types are able to respond. Like type I IFNs, IFN-γ has anti-viral functions but is also involved in immune responses against intracellular bacteria and tumours. IFN-γ also has immunomodulatory functions which are considered particularly important in the generation of adaptive responses. For instance, the promotion of T cell polarisation towards a Th1 phenotype, enhancing NK cell and macrophage activity, B cell class switching and the recruitment of immune cells to sites of infection, including monocytes and T cells via IFN-γ-mediated upregulation of chemokines such as CXCL10, CCL3 (MIP-1α) and CCL5, and adhesion molecules such as ICAM1 and VCAM1[166, 167]. Type III IFNs signal via a receptor composed of IFNLR1 and IL-10R2[168] (**Figure 1-6**). IFNLR1 is specific for type III IFNs and is expressed on epithelial cells and certain immune cells (e.g. pDC)[169] while IL-10R2 is also found in other receptor complexes[168]. Type III IFN expression is induced by most cell types following viral infection[168]. Type I and type III IFNs differ in certain regards, for instance, type III IFNs are particularly involved at protecting epithelial barriers[170] and there are differences in the anti-viral capacities of type I and type III IFNs as well as between type III IFN subsets[168, 171]. However, both groups of IFNs are primarily anti-viral cytokines and despite distinct receptors both signal via the JAK-STAT pathway[164]. IFN receptors are composed of high and low affinity receptor chains which are bound on their cytoplasmic domains by JAKs (JAK1, JAK2, tyrosine kinase 2 [TYK2]). Monomers of type I or type III IFNs bind their respective high affinity IFN receptor chains, IFNAR2 or IFNLR1, which are bound by JAK1 on their cytoplasmic domains. IFN binding triggers the recruitment of the low affinity receptor chain bringing the receptor chains into close proximity and allowing the kinases to undergo auto- and trans-phosphorylation[164] (**Figure 1-6**). Activated JAKs phosphorylate the receptor chains leading to the recruitment and phosphorylation of STAT proteins. Activated STAT1 and STAT2 dimerise and are

bound by IRF9 forming the trimeric complex and transcription factor ISGF3[164]. ISGF3 translocates into the nucleus where it binds consensus sequences and enhances the transcription of ISGs[164] (**Figure 1-6**).

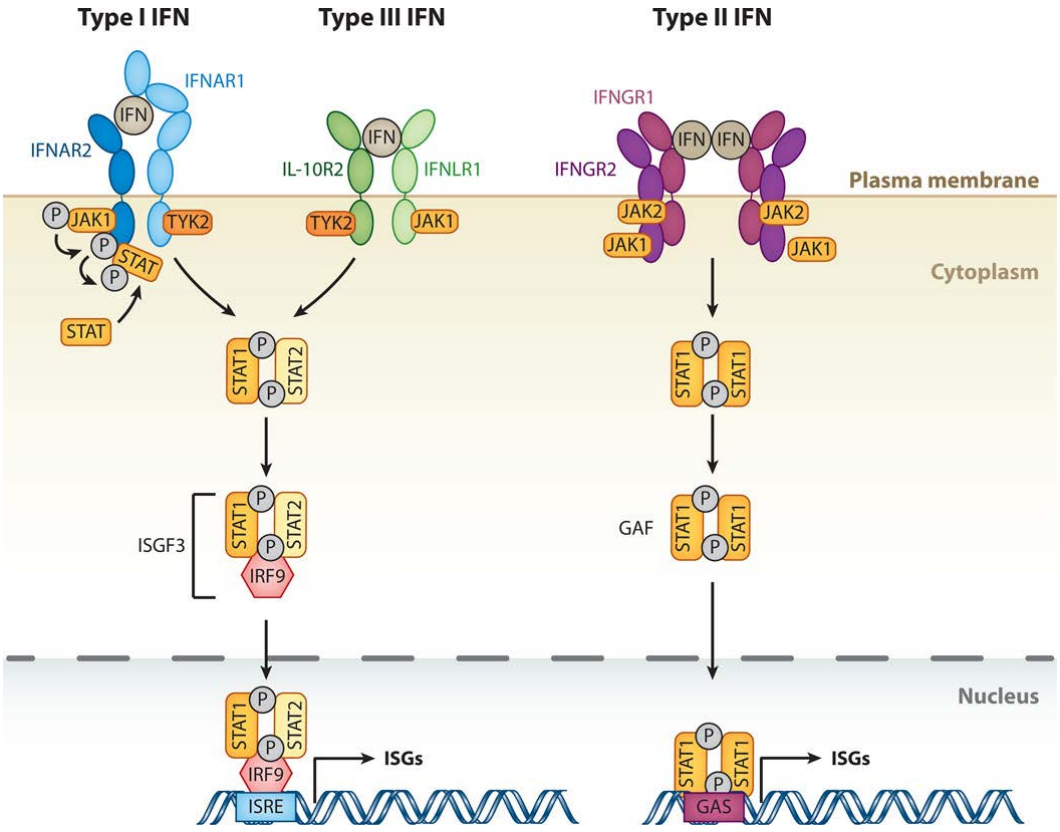


Figure 1-6. IFN signalling. Type I, type II and type III IFNs bind their respective receptors, IFNAR1/2, IFNGR1/2 and IFNLR1/IL10R2 and signal via the JAK-STAT signalling pathway. IFN binding induces receptor oligomerisation bringing the receptor chains, which are bound by JAKs, into close proximity. This activates JAK auto- and transphosporylation and subsequent JAK-mediated phosphorylation of IFN receptor chains. This leads to the recruitment and phosphorylation of STATs. In type I and type III IFN signalling, STAT1 and STAT2 form a complex with IRF9 (Interferon-stimulated gene factor 3, ISGF3) whereas STAT1 forms a homodimer in type II IFN signalling. These complexes translocate to the nucleus where they bind consensus sequences and induce the transcription of IFN stimulated genes (ISGs). Figure reprinted by permission from Annual Reviews: Annual Review of Immunology (Schneider WM, Chevillotte MD, and Rice CM. Interferon-Stimulated Genes: A Complex Web of Host Defenses), copyright (2014)[172].

1.8 IL-33 and Epithelial Derived Cytokines

As well as IFNs the bronchial epithelium is an important source of cytokines. In addition to providing a physical and chemical barrier to the external environment, the epithelium provides an immunological barrier which consists of humoral and cellular mechanisms[173]. Due to the importance of the epithelial barrier, disruptions in barrier function have important consequences, for instance in allergy and asthma. For example, loss-of-function mutations in the gene encoding filaggrin (*FLG*; a protein associated with the keratin cytoskeleton of the epidermis) impairs barrier function resulting in the dry, scaly skin condition ichthyosis vulgaris[174]. Furthermore, *FLG* mutations are associated with the development of allergic disorders including atopic dermatitis and asthma, which may be due to increased transfer of allergens across the skin barrier and a subsequent increase in allergen sensitisation[175]. Disruptions in epithelial barrier functions play a central role in the pathogenesis of asthma. For example, asthma is associated with epithelial fragility, epithelial shedding and impaired junctional proteins thought to be associated with the increased barrier permeability seen in asthma[176, 177].. Cytokines form part of the cellular arm of the immunological barrier via the recruitment and activation of effector cells such as DCs, MCs and T cells[173]. An important set of epithelial derived cytokines are IL-33, IL-25 and thymic stromal lymphopoietin (TSLP). As well as epithelial cells, IL-33, IL-25 and TSLP are produced by cell types including CD4 and CD8 T cells (IL-25, mouse)[178, 179], keratinocytes[180] (TSLP) and macrophages (IL-33, mouse)[181]. IL-33, IL-25 and TSLP are produced prior to the release of the classical Th2 cytokines IL-4, IL-5 and IL-13 and drive Th2 immune responses such as the activation, recruitment and induction of Th2 cytokines from a range of immune and structural cells[182]. Expression of IL-25 and its receptor increases with allergen challenge and IL-25 expression is associated with severity of bronchoconstriction[183]. In addition, expression of IL-33[184] and TSLP [185] is increased in severe asthma and genome wide association studies (GWAS) have demonstrated polymorphisms in the genes encoding TSLP (*TSLP*[186]), as well as IL-33 (*IL33*[187]) and its receptor (*IL1RL1*[186]), are associated with an increased risk of asthma. IL-33 exists in a number of splice variants which are expressed in human airway epithelial cells[188]. These variants show differences in regards to cellular localisation and cytokine activity and a particular splice variant has been demonstrated to be associated with airway type 2 inflammation in asthma[188].

As well as driving Th2 immune responses, IL-33, IL-25 and TSLP are alarmins, which are defined as immunomodulating molecules which are rapidly released from damaged and/or stressed cells following non-programmed cell death[189]. They recruit and activate both innate and adaptive arms of the immune system and promote the reconstruction of damaged tissue to restore homeostasis[189]. IL-33 is released from stressed or damaged cells in a form that is biologically active without the need for further processing. Unlike its IL-1 family member IL-1β, cleavage of IL-33 by caspase-1, or the apoptotic caspases, caspase-3 and -7, leads to IL-33 inactivation. This supports its role as an alarmin which is released in its active form in response to injury or necrosis but inactive when released following programmed cell death. As well as its extracellular functions as a cytokine, IL-33 is also found within the nucleus of cell types including endothelial cells[190, 191] where it associates with heterochromatin and has transcriptional repressor activities[192]. IL-33 has also been demonstrated to bind NF-κB in human HEK293RI cells resulting in an obstruction of NF-κB binding and NF-κB mediated pro-inflammatory responses[193].

Extracellular IL-33 signals via a heterodimeric receptor complex formed of the IL-33 receptor ST2 (Interleukin 1 receptor-like 1, IL1RL1), a member of the IL-1 receptor super family, and the IL-1 receptor accessory protein (IL-1RAP/IL-1RAcP)[194] (**Figure 1-7**). ST2 has multiple splice variants, the membrane bound receptor (ST2) which mediates IL-33 signalling; a soluble form (sST2) which acts as a decoy receptor sequestering free IL-33 and preventing IL-33 signalling; and at least one variant in humans predominantly expressed in the stomach, small intestine and colon[195], however little is known about the function of this variant. ST2 is expressed on a variety of cell types including Th2 T cells, basophils and invariant NKT cells[196] as well as MCs[197, 198]. The intracellular portions of ST2 and IL-1RAP contain cytoplasmic toll-interleukin-1 receptor (TIR) domains which dimerise on IL-33 binding[199, 200].This triggers the recruitment of MyD88, a TIR domain binding adaptor protein and the activation of IL-1R-associated kinase (IRAK)-1/4 followed by the activation of the MAP kinase pathway via TNF receptor-associated factor 6 (TRAF6)[201] (**Figure 1-7**). TRAF6 also activates the NF-κB pathway via activation of the inhibitor of NF-κB kinase (IKK)[201] (**Figure 1-7**). Differential IL-33 pathway activation has been deomonstreated[202] however the mechanisms underlying IL-33 differential signalling are unclear.

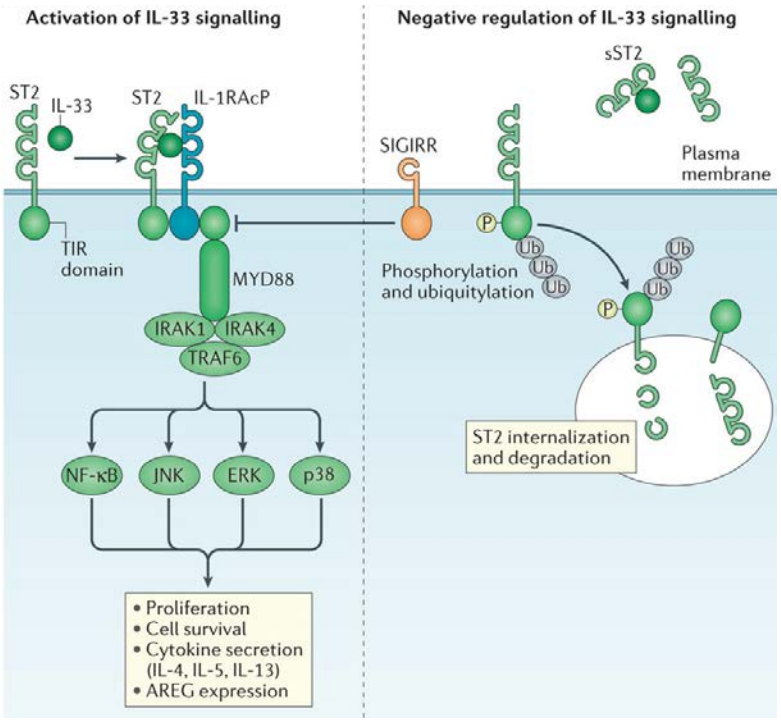


Figure 1-7 IL-33 signalling. IL-33 binds its receptor ST2 which results in a conformational change in the receptor allowing it to interact with its co-receptor IL-1RAcP. Clustering of the receptors brings their TIR domains into close proximity and results in the recruitment of the signalling adaptor MyD88, the kinases IRAK1 and IRAK4 and the adaptor protein TRAF6. Activated TRAF6 results in the activation of NF-κB and MAPK (JNK, ERK, p38) pathways. IL-33 signalling is negatively regulated by a number of mechanisms including single immunoglobulin domain IL-1 receptor-related molecule (SIGIRR) which prevents ST2/IL-1RAcP dimerisation; proteasomal degradation of ST2 and sequestering of IL-33 by sST2. Figure reprinted by permission from Nature Publishing Group: Nature Reviews Immunology (Liew FY, Girard J, Turnquist, HR. Interleukin-33 in health and disease), copyright (2016)[203].

1.9 Mast Cell Distribution and Phenotype in Asthma

Mature MCs are tissue-resident cells normally found in vascularised tissues in close proximity to blood vessels and nerves. For instance, MC_T tend to be found in the bronchial lamina propria while MC_{TC} are more commonly found in connective tissue such as the skin[2]. However, in asthma, MCs have been found to localise to submucosal glands[204], bronchial smooth muscle[205] and the airway epithelium[104], key sites within the asthmatic airway. At these sites, MCs may contribute to asthma pathogenesis in a number of ways, for instance, histamine and leukotrienes induce mucus secretion while IL-13 is implicated in goblet cell hyperplasia[3]. Bronchial smooth muscle MC numbers have been correlated to airway hyper-responsiveness severity[205] and tryptase induces airway epithelial cells to secrete CXCL8 and increase ICAM1 expression[206].

MC localisation has also been implicated in the severity of asthma with the demonstration that MC location as well as phenotype is altered with increasing asthma severity. Balzar *et al*. demonstrated a predominance of MC_{TC} in the airway submucosa and epithelium of severe asthmatics, a phenotype that was rarely seen in the airway epithelium of non-asthmatics but more so in mild asthma[207]. Furthermore, increased proportions of MC_{TC} and BAL fluid PGD_2, which was also elevated in severe asthmatics, were inversely correlated with lung function suggesting MCs were activated and detrimental to asthma severity. Th2 high asthma is an asthma endotype characterised by elevated type 2 inflammation of the airways compared to healthy non-asthmatic controls and is associated with more severe asthma[208]. In a study assessing immune cells, cytokines and inflammatory mediators of individuals with mild-to-severe asthma, MC proteases were found to be elevated in severe asthma, and were increased in both Th2 high atopic and non-atopic asthma[209]. Individuals with Th2 high asthma also demonstrate increased numbers of intraepithelial MCs compared to those with Th2 low asthma or non-asthmatics[210]. In addition, these cells have an altered phenotype expressing tryptase and CPA3 but not chymase and is a MC signature that has also been identified in epithelial brushings of asthmatics patients[211]. The physiological role of this phenotype is unknown but it is associated with bronchoconstriction[212] and eosinophilic asthma[213]. Overall, MCs appear to be adversely associated with asthma severity via a release of their mediators (i.e. are activated) as well as changes in MC phenotype and increased localisation to sites such as the bronchial epithelium.

MCs have been shown to respond to the epithelial-derived Th2 cytokine IL-33[214, 215] and MC functions have been demonstrated to be modulated by the epithelium[216]. Furthermore, in asthma greater numbers of MCs are found within the bronchial epithelium where they increase in number with increasing disease severity[207, 209]. This may suggest that during asthma the impact of IL-33 on MC function may be of particular significance. MCs are susceptible to infection with HRV[96], the major trigger of asthma exacerbations, however investigations into the response of MCs to HRV infection, and in particular MC anti-viral responses, are limited[96, 217]. Type 2 innate lymphoid cells (ILC2s) have been demonstrated to respond to IL-33 released from the epithelium following HRV infection[218]. However, in asthma, MCs as well as basophils and not ILC2s, have been shown to be the cellular targets of IL-33 responsible for type 2 cytokine release[188]. IL-33-induced MC responses have not been investigated in the context of HRV infection. Yet, this is of particular relevance considering the significance of HRV-induced exacerbations of asthma and the increased localisation of MCs at the bronchial epithelium (the major site of HRV infection) during asthma as well as HRV infection[217].

1.10 Hypothesis

HRV infection of MCs induces anti-viral innate immune responses and modulates IL-33-mediated Th2 immune responses.

1.11 Aims

1. Determine the susceptibility and anti-viral response of the mature MC line, LAD2, and primary cord blood-derived MCs to HRV infection.
2. Investigate IFN mediated protection against MC HRV infection.
3. Determine whether MC IL-33-dependent Th2 responses are modulated by HRV infection.

Chapter 2: Materials & Methods

2.1 Materials

Reagent	Manufacture	Product code
2-Mercaptoethanol	Sigma-Aldrich Company Ltd. (Dorset, UK)	M3148
2-Propanol	Sigma-Aldrich Company Ltd. (Dorset, UK)	19516
3,3',5,5'-Tetramethylbenzidine (TMB) solution	eBioscience, Ltd. (Cheshire, UK)	00-4201-56
Amicon Ultra-0.5 centrifugal filter devices	Millipore Ltd. (Hertfordshire, UK)	UFC5003
Bovine Collagen Solution, Type I (3 mg/mL)	Advanced BioMetrix (San Diego, USA)	5005-100ML
Bovine serum albumin	Sigma-Aldrich Company Ltd. (Dorset, UK)	A3059
CCL2 double dye primer	Primer Design (Chandler's Ford, UK)	CCL2 DD-hu-300
CCL2/MCP-1 DuoSet® ELISA (Human)	Bio-Techne Ltd (Abingdon, UK)	DY279
CCL5 double dye primer	Primer Design (Chandler's Ford, UK)	CCL5 DD-hu-300
CD117 PE, clone YB5.B8, mouse IgG_{1K}, anti-human antibody	eBioscience, Ltd. (Cheshire, UK)	12-1179
CD54 FITC, clone RR1/1, mouse IgG_1, anti-human antibody	eBioscience, Ltd. (Cheshire, UK)	BMS108FI
Chloroform	Sigma-Aldrich Company Ltd. (Dorset, UK)	C2432
CMA1 double dye primer	Primer Design (Chandler's Ford, UK)	CMA1 DD-hu-300
Cord blood CD34+ cells, human	Stemcell Technologies (Cambridge, UK)	70008.2
CPA3 double dye primer	Primer Design (Chandler's Ford, UK)	CPA3 DD-hu-300
Crystal violet	Sigma-Aldrich Company Ltd. (Dorset, UK)	C3886
CXCL10 double dye primer	Primer Design (Chandler's Ford, UK)	CXCL10 DD-hu-300
CXCL10/IP-10 DuoSet® ELISA (Human)	Bio-Techne Ltd (Abingdon, UK)	DY266
DDX58 double dye primer	Primer Design (Chandler's Ford, UK)	DDX58 DD-hu-300
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Company Ltd. (Dorset, UK)	D2650
DNA-*free*™ Kit	Thermo Fisher Scientific Inc. (Paisley, UK)	AM1906
DNase I solution	Stemcell Technologies (Cambridge, UK)	07900
Dulbecco's Phosphate Buffered Saline, without calcium chloride and magnesium chloride	Sigma-Aldrich Company Ltd. (Dorset, UK)	D8537
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific Inc. (Paisley, UK)	11960044

EasySep™ human progenitor cell enrichment kit	Stemcell Technologies (Cambridge, UK)	19056
EIF2AK2 double dye primer	Primer Design (Chandler's Ford, UK)	EIF2AK2 DD-hu-300
Ethanol	Sigma-Aldrich Company Ltd. (Dorset, UK)	32221
Ethylenediaminetetraacetic acid (EDTA) solution (0.5 M)	Sigma-Aldrich Company Ltd. (Dorset, UK)	03690
FcεRI APC, clone AER-37, mouse IgG_{2bK}, anti-human antibody	eBioscience, Ltd. (Cheshire, UK)	17-5899
Fetal Bovine Serum (FBS), heat inactivated	Thermo Fisher Scientific Inc. (Paisley, UK)	10500064
Fixable viability dye eFlour 660	Affymetrix	65-0864
Fluticasone propionate	Sigma-Aldrich Company Ltd. (Dorset, UK)	F9428-5MG
Formaldehyde solution	Thermo Fisher Scientific Inc. (Paisley, UK)	F8775
Hank's Balanced Salt Solution (HBSS), without calcium or magnesium	Thermo Fisher Scientific Inc. (Paisley, UK)	14170088
HEPES solution (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (1 M)	Sigma-Aldrich Company Ltd. (Dorset, UK)	H0887
Human Serum	Sigma-Aldrich Company Ltd. (Dorset, UK)	H4522
ICAM1 SYBR® green primer	Primer Design (Chandler's Ford, UK)	ICAM1 SY-hu-600
ICAM-1/CD54 Antibody Monoclonal Mouse IgG1 Clone # BBIG-I1 (11C81), human	R&D Systems, Inc. (Abingdon, UK)	BBA3
IFIH1 (MDA5) double dye primer	Primer Design (Chandler's Ford, UK)	IFIH1 DD-hu-300
IFN-α/β receptor chain 2, clone MMHAR-2 monoclonal anti-human antibody (mouse IgG_{2a})	Pbl Assay Science (New Jersey, USA)	21385-1
IFN-γ protein, recombinant human	Peprotech (London, UK)	300-02
IL29 double dye primer	Primer Design (Chandler's Ford, UK)	IL29 DD-hu-300
IL-29/IL-28B (IFN-lambda 1/3) DuoSet® ELISA (Human)	Bio-Techne Ltd (Abingdon, UK)	DY1598B
IL-3 protein, recombinant human	Peprotech (London, UK)	200-03
IL-33 Protein, recombinant human	Bio-Techne Ltd (Abingdon, UK)	3625-IL
IL-4 protein, recombinant human	Peprotech (London, UK)	200-04
IL5 double dye primer	Primer Design (Chandler's Ford, UK)	IL5 DD-hu-300
IL-6 protein, recombinant human	Peprotech (London, UK)	200-06

Interferon Beta (human, rDNA, glycosylated) recombinant protein	National Institute for Biological Standards and Control (NIBSC) (Hertfordshire, UK)	00/572
Interlukin-29 (Interferon Lambda 1) recombinant protein	National Institute for Biological Standards and Control (NIBSC) (Hertfordshire, UK)	10-176
IRF3 SYBR® green primer	Primer Design (Chandler's Ford, UK)	IRF3 SY-hu-600
IRF7 double dye primer	Primer Design (Chandler's Ford, UK)	IRF7 DD-hu-300
IRF9 double dye primer	Primer Design (Chandler's Ford, UK)	IRF9 DD-hu-300
KIT double dye primer	Primer Design (Chandler's Ford, UK)	KIT DD-hu-300
KITLG double dye primer	Primer Design (Chandler's Ford, UK)	KITLG DD-hu-300
LDL R PE, clone 472413, mouse IgG_1, anti-human antibody	Bio-Techne Ltd (Abingdon, UK)	FAB2148P
L-glutamine	Thermo Fisher Scientific Inc. (Paisley, UK)	25030024
L-glutamine (200mM, 10X)	Life Technologies	25030-081
Magnesium chloride, anhydrous	Sigma-Aldrich Company Ltd. (Dorset, UK)	M8266
MEM GlutaMAX™	Thermo Fisher Scientific Inc. (Paisley, UK)	41090028
MEM Non-Essential Amino Acids Solution (100X)	Thermo Fisher Scientific Inc. (Paisley, UK)	11140035
Mouse IgG_1 FITC monoclonal isotype control, clone P3.6.2.8.1	eBioscience, Ltd. (Cheshire, UK)	11-4714-42
Mouse IgG_{2a} monoclonal isotype control, clone 20102	R&D Systems, Inc. (Abingdon, UK)	MAB003
Mouse $IgG_{2bκ}$ APC monoclonal isotype control, clone eBMG2b	eBioscience, Ltd. (Cheshire, UK)	17-4732
Mouse $IgG_κ$ PE monoclonal isotype control, clone P3.6.2.8.1	eBioscience, Ltd. (Cheshire, UK)	12-4714
MSD 96-well MULTI-ARRAY Human IFN-β assay	Meso Scale Diagnostics, LLC (Maryland, USA)	K151ADB
MSD Human TH1/TH2 7-Plex Tissue Culture Kit	Meso Scale Diagnostics, LLC (Maryland, USA)	K15011B
MX1 double dye primer	Primer Design (Chandler's Ford, UK)	MX1 DD-hu-300
nanoScript 2 Reverse Transcription kit	Primerdesign Ltd (Chandler's Ford, UK)	RT-nanoscript2
Oxoid™ Phosphate Buffered Saline Tablets	Thermo Fisher Scientific Inc. (Paisley, UK)	BR0014G
Paraformaldehyde	TAAB Laboratories Equipment Ltd (Aldermaston, UK)	P001
Penicillin-Streptomycin (5,000 U/mL)	Thermo Fisher Scientific Inc. (Paisley, UK)	15070063
pZerve	Protide Pharmaceuticals (Lake Zurich, USA)	5700
RNeasy mini kit	Qiagen (Manchester, UK)	74104

RPMI 1640 Medium, without glutamine	Thermo Fisher Scientific Inc. (Paisley, UK)	31870025
RSAD2 double dye primer	Primer Design (Chandler's Ford, UK)	RSAD2 DD-hu-300
RV16 double dye primer	Primer Design (Chandler's Ford, UK)	HRV16_1563 DD-hu-300
RV1B double dye primer	Primer Design (Chandler's Ford, UK)	HRV1B_1563 DD-hu-300
Sodium bicarbonate solution	Sigma-Aldrich Company Ltd. (Dorset, UK)	S8761
ST2/IL-33 R PE, polyclonal goat IgG, anti-human antibody	Bio-Techne Ltd (Abingdon, UK)	FAB5231P
Stem cell factor protein, recombinant human	Peprotech (London, UK)	300-07
StemPro-34 serum free media and StemPro-34 Nutrient Supplement	Thermo Fisher Scientific Inc. (Paisley, UK)	10639-011
TLR3 double dye primer	Primer Design (Chandler's Ford, UK)	TLR3 DD-hu-300
TPSB2 double dye primer	Primer Design (Chandler's Ford, UK)	TPSB2 DD-hu-300
TRIzol® Reagent	Thermo Fisher Scientific Inc. (Paisley, UK)	15596018
Trypan Blue solution (0.4%)	Sigma-Aldrich Company Ltd. (Dorset, UK)	93595
Trypsin-EDTA (0.5%)	Thermo Fisher Scientific Inc. (Paisley, UK)	15400054
Tryptose Phosphate Broth solution (29.5 g/L)	Sigma-Aldrich Company Ltd. (Dorset, UK)	T8159
TWEEN® 20	Sigma-Aldrich Company Ltd. (Dorset, UK)	P1379
UBC/GAPDH double dye primers	Primer Design (Chandler's Ford, UK)	HK-PP-hu-900-d-UBC/GAPDH
VLDLR double dye primer	Primer Design (Chandler's Ford, UK)	VLDLR DD-hu-300

2.2 Equipment

Equipment	Manufacturer
BD FACSCalibur™	BD Biosciences (Oxford, UK)
Bio-Rad CFX96 Real-Time system, C1000 Thermal cycler	Bio-Rad Laboratories Ltd. (Hertfordshire, UK)
ChemiDoc™ XRS+ System	BD Biosciences (Oxford, UK)
CL1000 ultraviolet crosslinker	Ultra-Violet Products Ltd (Cambridge, UK)
MESO QuickPlex SQ 120	Meso Scale Diagnostics, LLC (Maryland, USA)
MTX Lab Systems Multiskan Ascent plate reader	Thermo Fisher Scientific Inc. (Paisley, UK)
NanoDrop 1000	Thermo Fisher Scientific Inc. (Paisley, UK)
Mini orbital Shaker	Bibby Scientific Limited (Staffordshire, UK)
Mini see-saw rocker (SSM4)	Bibby Scientific Limited (Staffordshire, UK)
Roller mixer	Bibby Scientific Limited (Staffordshire, UK)
Titramax 100 (vibrating platform shaker)	Heidolph UK (Essex, UK)

2.3 Cell Culture

2.3.1 Mast Cells

2.3.1.1 Laboratory of Allergic Diseases (LAD) 2 Mast Cell Culture

The LAD2 human MC line is a SCF-dependent cell line cultured from a patient with mast cell sarcoma/leukemia[219]. LAD2 MCs contain histamine as well as tryptase and chymase positive granules and express functional FcεRI receptors allowing IgE-dependent degranulation[219].

LAD2 MCs were maintained in StemPro-34® serum free medium supplemented with StemPro®-34 nutrient supplement, L-glutamine (2 mM) and penicillin (100 U/mL)/streptomycin (100 μg/mL) (complete medium) plus SCF (100 ng/mL) in T75 cm^2 non-tissue culture treated flasks. Culture medium was replenished weekly by the addition of an equal volume of medium (hemi-depletion) and grown to a density not exceeding $0.5x10^6$ cells/mL at 37˚C in 5% CO_2 in a humidified atmosphere. To replace the entire medium, cells were pelleted at 200 x *g* for 5 minutes and gently re-suspended in complete medium for continuous culture. Cells were characterised by CD117 (c-KIT, SCF receptor) and FcεRI expression by flow cytometry (**Figure 2-1**), see section 2.7.8 for methodology.

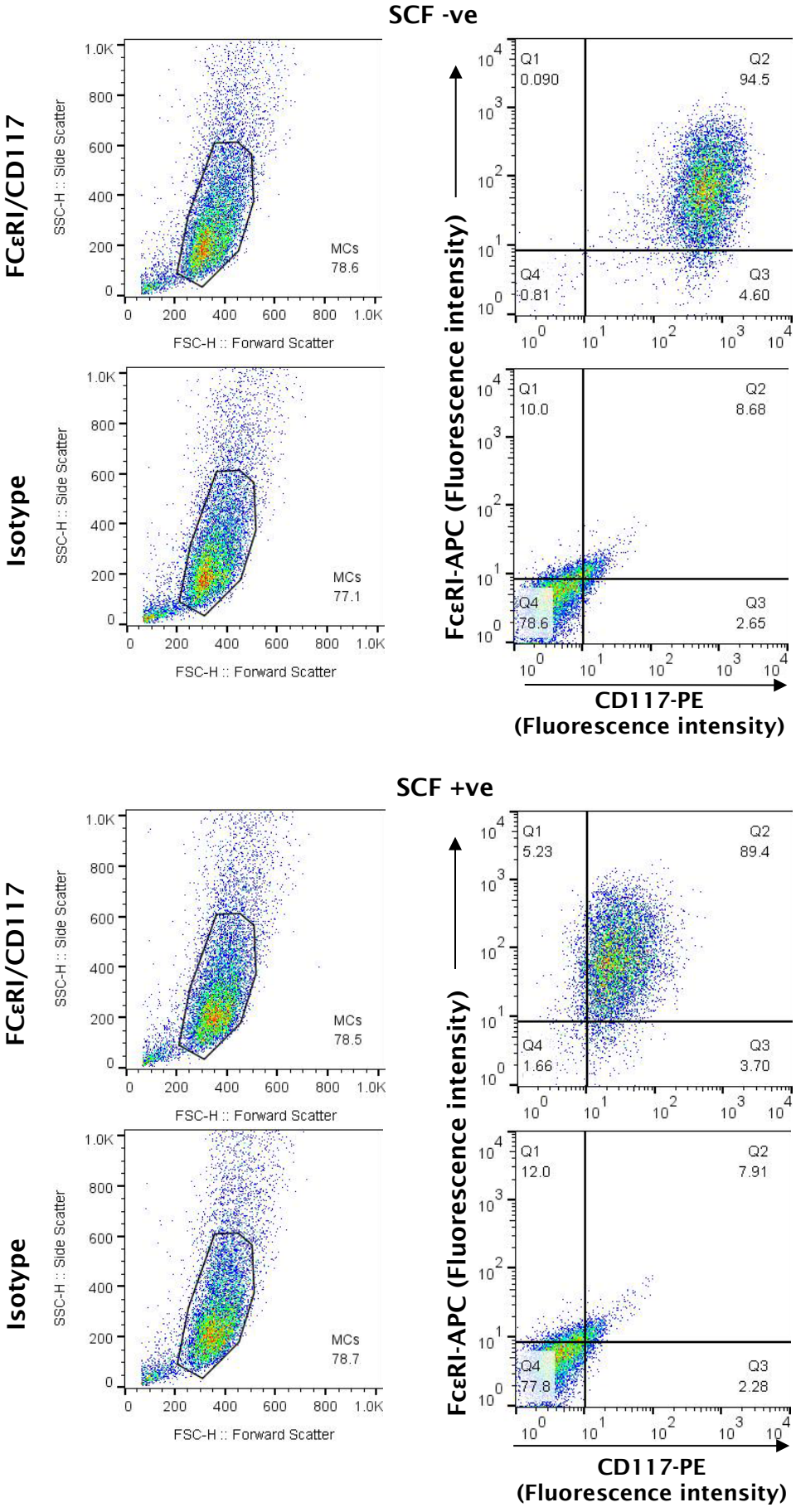

SCF -ve
FCεRI/CD117
Isotype
SSC-H :: Side Scatter
FSC-H :: Forward Scatter
MCs
78.6
MCs
77.1
Q1
0.090
Q2
94.5
Q4
0.81
Q3
4.60
Q1
10.0
Q2
8.68
Q4
78.6
Q3
2.65
FcεRI-APC (Fluorescence intensity)
CD117-PE
(Fluorescence intensity)
SCF +ve
FCεRI/CD117
Isotype
MCs
78.5
MCs
78.7
Q1
5.23
Q2
89.4
Q4
1.66
Q3
3.70
Q1
12.0
Q2
7.91
Q4
77.8
Q3
2.28

Figure 2-1. LAD2 MCs express FcεRI and CD117. LAD2 MCs were cultured with or without 100 ng/mL of SCF overnight and double-stained with FcεRI-APC (mouse $IgG_{2b\kappa}$) and CD117-PE (mouse $IgG_{1\kappa}$,) or equivalent concentrations of isotype antibody. Results shown are representative density plots from flow cytometric analysis. Values in Q1-Q4 represent cell percentage per quadrant. n=1.

CD117 and FcεRI (high affinity IgE receptor) expression by LAD2 MCs was additionally examined by reverse transcription quantitative PCR (RT-qPCR) along with the MC proteases chymase, carboxypeptidase A3 and tryptase as well as the rhinovirus receptors ICAM1 (major group HRVs), very low density lipoprotein receptor (VLDLR) (minor group HRVs) and CDHR3 (HRV-C) (**Table 1**), see section 2.7.4 for methodology.

Gene	Ct value
Proteases	
Chymase A1 (*CMA1)*	26
Carboxypeptidase A3 (*CPA3)*	18
Tryptase β2 (*TPSB2)*	18
FcεRI (*FCER1A)*	24
CD117 (*KIT*)	21
ICAM1	22 (min =22, max =26)
VLDLR	34 (min =33, max =36)
CDHR3	37

Table 1. LAD2 mRNA expression of MC proteases, FcεRI, CD117, ICAM1, VLDLR and CDHR3. mRNA expression was assessed by RT-qPCR. n=1, or n=4 where minimum and maximum Ct values are given.

2.3.1.1.1 Cryopreserving LAD2 Mast Cells

To cryopreserve LAD2 MCs, cells were pelleted at 200 x *g* for 5 min and the supernatant removed leaving behind a small aliquot with which to gently re-suspend the cell pellet. Cells were counted and re-suspended at 1×10^6/mL in pZerve cryopreservation solution containing 200 ng/mL SCF. One mL of the cell suspension was aliquoted per 1.5 mL cryovial and rotated for 30 min at room temperature (RT) on a roller mixer. Cryovials were transferred to an isopropanol based cryo-container (Mr. Frosty™) and placed at -20˚C for an hour and after 20 min cryovials were inverted to re-suspend cells and placed back at -20˚C. After 1

hour at -20˚C the cryo-container was placed at -80˚C for a further hour following which cryovials were stored in liquid nitrogen.

2.3.1.1.2 Thawing Cryopreserved LAD2 Mast Cells

Cryopreserved LAD2 MCs were thawed at room temperature and cell number and viability immediately determined by trypan blue exclusion. The cell volume was made up to 1.6 mL with pZerve cryopreservation solution and 400 µL of LAD2 medium (200 ng/mL SCF) added. Cells were rocked in non-tissue culture treated six well plates at 60 revolutions per minute (rpm) at RT for 6 hours. Every hour cells were mixed gently prior to cell and viability counts and clumps of cells were removed. After 6 hours, 1 mL of LAD2 medium (100 ng/mL SCF) was added to cells which were incubated at 37˚C for 24 hours. After 24 hours a cell and viability count were taken and if at least 80% of the cells were viable culture was continued. The cell medium was replaced by pelleting cells at 200 x *g* for 5 min and re-suspended at 0.1×10^6/mL in LAD2 medium (100 ng/mL SCF) in a T75 cm^2 non-tissue culture treated flask for continuous culture.

2.3.1.2 Primary Mast Cell Culture

Primary human MC culture was previously limited to cells isolated from human tissues, including the skin, lungs and intestine, via tissue digestion followed by cell purification via separation gradients[220]. The isolation of MCs from human tissue is dependent on the availability of fresh tissue specimens and a lack of consistency in tissue sources can result in significant variability between cultures. In addition, isolation procedures can reduce MC viability and cell yield is generally limited to 10^4-10^6 cells per gram of tissue[221]. The development of protocols for the isolation, differentiation and expansion of MC progenitors, derived from cord and peripheral blood, allows the generation of high yields of pure MC cultures. Cord blood contains a higher percentage of CD34+ progenitors compared to peripheral blood, alternatively CD34+ cells are available commercially. In this report, primary human mast cells were cultured from cord blood mononuclear cells or CD34+ cells derived from cord blood.

2.3.1.2.1 Cord Blood Mononuclear Cells

Cord blood was collected under the MRC ethics of Professor Graham Roberts. All procedures were approved by the Southampton and South West Hampshire Research Ethics Committee (Rec code 10/H0502/11) and were undertaken

following written informed consent and samples were fully anonymised. Cord blood mononuclear cells were purified by ficoll-histopaque separation from human cord blood, washed (Roswell Park Memorial Institute (RPMI) medium + 2% HI-FCS) and placed in cryopreservation medium (HI-FBS (85%) + dimethyl sulfoxide [DMSO; 7.5%]) prior to cryopreservation. This technique was performed by Dr Nicole Bedke and Dr Emily Swindle.

2.3.1.2.2 Thawing Cryopreserved Cord Blood Mononuclear Cells

When required, cryopreserved mononuclear cells were thawed rapidly in a 37˚C water bath until a small frozen pellet remained ensuring the temperature of the cells had not risen excessively. Cells were then added drop-wise to 1 mL of cold RPMI 1640 medium then 2 mL of medium added to cells drop-wise over 2 minutes with gentle shaking between additions. A total of 50 mL cold RPMI medium was added to the cells while slowly increasing the rate of addition. Cells were pelleted at 300 x *g* for 10 minutes at 4˚C and washed in 10 mL 2% FBS in Ca^{2+}/Mg^{2+}-free PBS (FBS/PBS). Cells were then incubated with 1 µg/mL DNase I in FBS/PBS for 15 minutes at room temperature. After which, cells were diluted in 9 mL FBS/PBS and at this stage multiple vials from the same donor were pooled. A 15 µL aliquot of cell suspension was taken for a cell viability count then the cells centrifuged at 300 x *g* for 10 minutes at 4˚C prior to the isolation of CD34+ cells.

2.3.1.2.3 Negative Selection of CD34+ Cells from Cord Blood Mononuclear Cells by Magnetic Bead Separation

CD34+ cells were negatively selected following the protocol of the EasySep® human progenitor cell enrichment kit using magnetic bead separation. Unwanted cells are labelled with a cocktail of bi-specific antibody (IgG1) complexes which recognise both cell-surface expressed antigens on unwanted blood cells (CD2, CD3, CD11b, CD11c, CD14, CD16, CD19, CD24, CD56, CD66b and glycophorin A) and dextran on dextran coated magnetic beads. Unwanted cells were then separated using an EasySep® magnet.

Briefly, cord blood mononuclear cells were re-suspended at $5x10^7$/mL in a 5 mL polystyrene tube and incubated at RT for 15 minutes with the monoclonal antibody cocktail (50 µL/mL). The EasySep® magnetic nanoparticles were added (50 µL/mL) and incubated with the cell suspension for 15 minutes at RT after which the suspension was made up to a total volume of 2.5 mL with 2% FBS/1 mM EDTA in

PBS. The cell suspension was placed in the EasySep® magnet for 10 minutes then the desired unlabelled cells collected by upturning the tube held within the magnet. The remaining cells were re-suspended in 2.5 mL 2% FBS/1 mM EDTA in PBS and placed in the magnet for a second round of separation to increase recovery. The fractions were pooled and a 15 µL aliquot taken for a cell count. Cells were pelleted at 300 x *g* for 10 minutes at RT then re-suspended at $5x10^4$/mL in complete StemPro medium plus IL-3 (30 ng/mL), IL-6 (100 ng/mL) and SCF (100 ng/mL) (cord blood mononuclear cell medium + IL3) to promote the growth of MC progenitors. Cells were plated in a non-treated six well plate and incubated at 37˚C with 5% CO_2.

2.3.1.2.4 Cord Blood Derived CD34+ Cells

The selection and expansion of CD34+ MCs from cryopreserved cord blood mononuclear cells, after several attempts, was unsuccessful. Therefore, primary human cord-blood derived CD34+ progenitor cells were purchased from Stemcell Technologies. These cells were previously isolated from cord blood mononuclear cells by positive immunomagnetic selection and cryopreserved.

2.3.1.2.5 Thawing Cryopreserved Cord Blood Derived CD34+ Cells from Stemcell Technologies

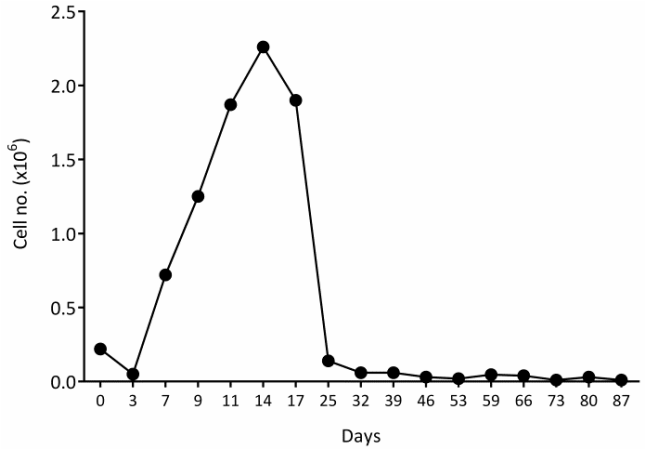
Cryopreserved CD34+ cells were quickly defrosted in a water bath at 37˚C until a small frozen cell pellet remained and a cell count performed. Cells were rinsed with 1 mL complete StemPro® medium (wash medium) added drop-wise in a 50 mL tube with gentle swirling (all further washes were conducted in this manner). A further 20 mL wash medium was added before pelleting at 300 x *g* for 10 minutes at RT. Cells were treated with 100 µg/mL DNase I in 1 mL wash medium for 15 minutes at RT then washed. Cells were re-suspended at $5x10^4$/mL in cord blood mononuclear cell medium + IL-3 (30 ng/mL, first week only) then plated in a non-treated six well plate and incubated at 37˚C with 5% CO_2.

2.3.1.2.6 Expansion of CD34+ Cells

CD34+ progenitor cells (purchased or isolated using negative selection) expand rapidly during the first few weeks in culture and particularly during the first week. Cell density was monitored closely during this period to promote maximal expansion with minimal cell death. After the expansion phase, cell numbers drop as non-mast cell committed progenitors die and around week 4 cell numbers

plateau with cultures formed predominantly of CD34+ mast cell progenitors (**Figure 2-2**).

A



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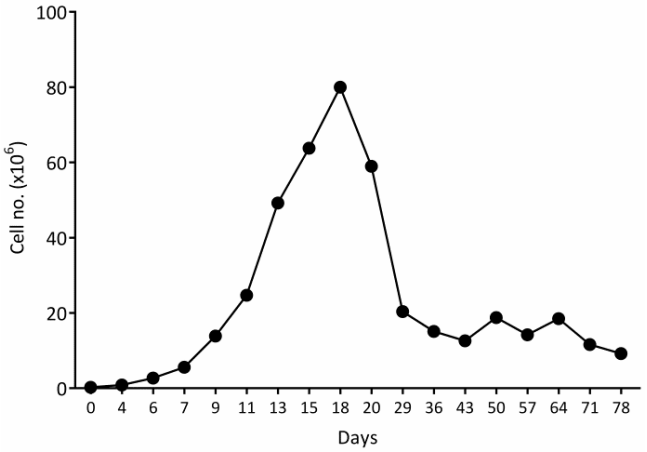


Figure 2-2. Expansion of cord blood derived CD34+ cells. CD34+ cord blood derived cells were cultured for 11-12 weeks and the number of viable cells monitored by trypan blue exclusion. **(A)** CD34+ cells isolated from cord blood mononuclear cells by magnetic bead separation (negative selection) (donor CS0019). 0.22×10^6 cells at day 0. **(B)** Pre-isolated cord blood derived CD34+ cells (purchased from Stemcell Technologies). 0.26×10^6 cells at day 0.

During the first week of culture cells were checked daily and split, as required, by hemi-depletion with cord blood mononuclear cell medium + IL-3 (30 ng/mL, first week only). During this time cells were passaged from a six well plate to T75cm^2 flasks. On day 7 and 14 cells were split by hemi-depletion and from day 21 (or earlier depending on cell debris) the entire culture medium was replaced weekly by

pelleting cells at 200 x *g* for 5 minutes at RT. Cells were cultured and passaged for 8-12 weeks and maintained at a density not exceeding 0.4x10^6/mL (**Figure 2-2**). After 8 weeks, CD34+ cord blood-derived cells were characterised by flow cytometry according to their expression of CD117 and FcεRI indicative of MCs (i.e. cord blood-derived MCs [CMBCs]). ICAM1 expression was also assessed and LAD2 MCs used as a positive control (**Figure 2-3**; see section 2.7.8 for methodology). CBMCs expressed lower levels of all three receptors, particularly FcεRI and ICAM1 compared to LAD2 MCs.

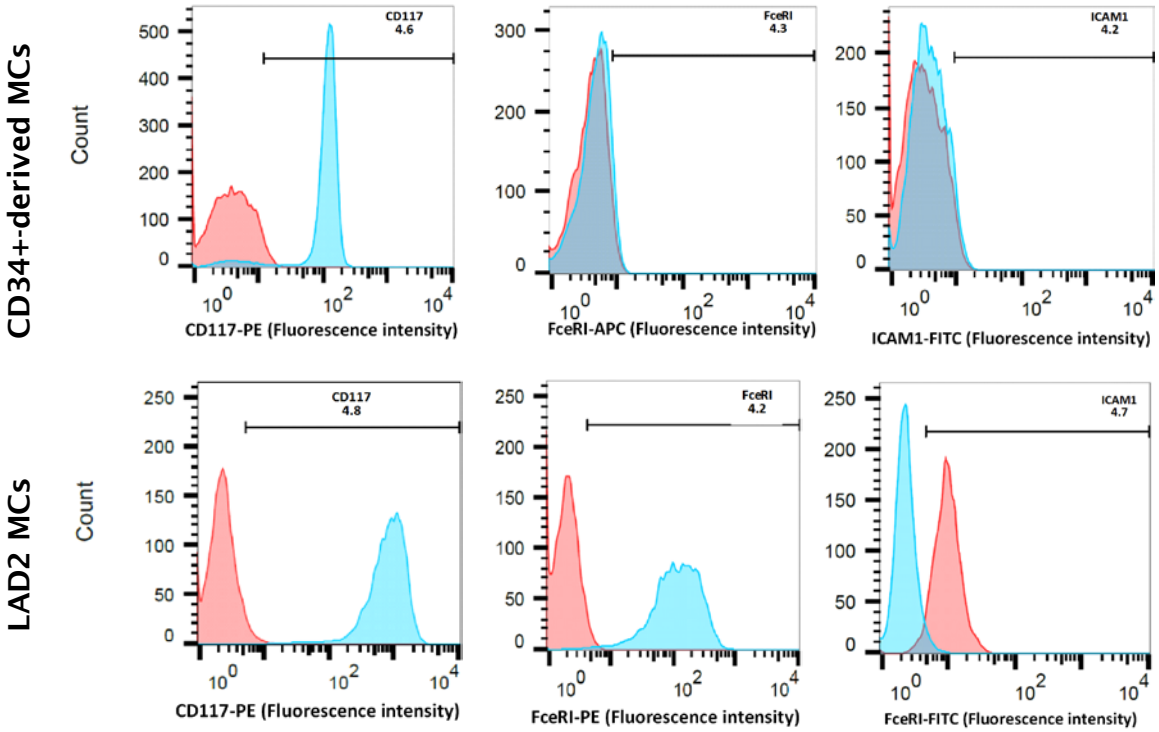


Figure 2-3. CD117, FcεRI and ICAM1 expression on CD34+ cord blood derived MCs or LAD2 MCs. MCs were stained with CD117-PE (mouse IgG1, κ, 0.1 µg/100 µl), FcεRI-APC (mouse IgG2b, κ; 0.025 µg/100 µl) or ICAM1-FITC (mouse IgG1, κ, 0.065 µg/100 µL) or equivalent concentrations of isotype antibody. n=1. Red, isotype; blue, receptor.

2.3.2 Human Bronchial Epithelial (16HBE14o−) Cell Culture

The 16HBE14o− cell line (16HBE) is a SV40 large T-antigen transformed human bronchial epithelial cell (BEC) line that forms tight junctions and is used as a model of a polarised bronchial epithelium[222].

16HBE cells were maintained in minimal essential medium (MEM) GlutaMax supplemented with FBS (10% (v/v), 2% for starvation media) and penicillin (100 U/mL)/streptomycin (100 µg/mL). Cells were cultured on collagen I (30 µg/ml) coated tissue culture plates and flasks (incubated with collagen I for 30 minutes at 37˚C and excess removed). Medium was replaced every two days or replaced with starvation media overnight prior to use in experiments. Cells were grown at 37˚C in 5% CO_2 in a humidified atmosphere and passaged once 80% confluent.

2.3.3 HeLa Cell Culture

HeLa cells are an epithelial cell line originally cultured from a cervical tumour biopsy taken from Henrietta Lacks[223, 224]. The H1-HeLa and Ohio HeLa sublines[225, 226] are susceptible to rhinovirus infection and were used for passaging and titrating rhinovirus respectively.

H1-HeLa cells were maintained in MEM Glutamax plus FBS (10% (v/v)), non-essential amino acids (1% (v/v)) and penicillin (100 U/mL)/streptomycin (100 µg/mL). Ohio HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) plus L-glutamine (2 mM), FBS (10 (v/v), 2% for starvation media) and penicillin (100 U/mL)/streptomycin (100 µg/mL). Medium was replaced every two days and cells cultured at 37˚C in 5% CO_2 in a humidified atmosphere and passaged once 80% confluent.

2.4 Rhinovirus Amplification

The human rhinoviruses (HRVs), RV16 and RV1B, were amplified in H1-HeLa cells. Once H1-HeLa cells were 80-90% confluent they were infected with HRV and infection media (MEM Glutamax supplemented with 4% (v/v) FBS, 1% (v/v) non-essential amino acids, 100 U/mL penicillin/100 µg/mL streptomycin, 16 mM HEPES, 0.12% (v/v) $NaHCO_3$, 0.118% (v/v) tryptose and 0.3 mM $MgCl_2$) according to the ratios in **Table 2**. Cells were treated for 1 hour whilst shaking (35 rpm) at RT then incubated at 33˚C (humidified, 5% CO_2) until cytopathic effects (CPEs) were observed in at least 50% of cells (typically 14 to 18 hours). Cells were lysed by three rounds of freezing and thawing at -80˚C/RT and cell debris pelleted by centrifuging at 1,556 × *g* for 7 minutes at RT. HRV stocks were amplified in several rounds of passage from one T75 flask to one T175 flask followed by seven T75 flasks to finally seven T175 flasks with three freeze/thaw steps before each round of

amplification. Following the last round of amplification supernatants were passed through a 0.22 μm sterile filter then stored at -80˚C in 1.5 mL aliquots. The tissue culture infective does 50% ($TCID_{50}$)/mL of the virus stock was determined after each passage. Master RV16 and RV1B stocks were also stored in liquid nitrogen.

For use in control infections HRV was inactivated by exposure to 1,200 mJ/cm^2 UV-light in a UV cross-linker on ice for 50 minutes which inactivated HRV replication.

	Ratio HRV stock: infection media	
Virus	T75 cm^2 flasks	T175 cm^2 flasks
RV16	1:1	3:5
RV1B	1:3	1:3

Table 2. Ratio of HRV stock to infection media used for HRV amplification. RV16 or RV1B stocks were amplified by incubating H1-HeLa cells with HRV and infection media.

2.5 Tissue Culture Infective Dose 50% ($TCID_{50}$) Assay

$TCID_{50}$ assay is an end-point dilution assay used to measure virus titre[227]. Serial dilutions of virus stock or supernatants are added to replicate wells of Ohio HeLa cells and virus titre determined by the number of wells with at least 50% cytopathic effect (CPE).

Briefly, $0.2x10^6$ Ohio HeLa cells were seeded per well of a 96-well plate and incubated for 3 hours at 37˚C after which the medium was replaced with 180 μL of 2% FBS (v/v) Ohio HeLa medium. Virus stocks or cell-free supernatants were added to cells in a 10-fold serial dilution (20 μl supernatant or virus stock) in 6 replicates. UV-HRV or no virus controls were plated in duplicate wells undiluted. Plates were rocked for 1 hour at 36 rpm at RT then incubated for 96 hours at 37˚C. CPE was visualised by staining monolayers with crystal violet solution (0.13% (w/v) crystal violet, 1.825% (v/v) formaldehyde, 5% ethanol (v/v), 90% PBS (v/v); 50 μL/well) for 30 minutes in the dark. Excess crystal violet was removed by gently rinsing plates by immersing in tap water followed by air drying. The number of wells where at least 50% of the monolayer had been lysed (i.e. 50% CPE) was used to calculate $TCID_{50}$/mL according to the Spearman-Karber Method[227-229]:

Log10 $TCID_{50}$ = l-[d(s-0.5)]

Where l = Log_{10} of lowest dilution

d = Log_{10} of difference of stepwise dilutions

s = (1/n) x (r) where n=wells per dilution and r = no of wells with cytopathic effects ≥50%

Previously generated RV16 and RV1B stocks had titres of 5.56 and 4.93x10^6 $TCID_{50}$/mL respectively. A further RV16 stock was generated with a titre of 459x10^6 $TCID_{50}$/mL. The new RV16 stock was equated to the previous RV16 stock by infecting LAD2 MCs with both stocks in parallel and determining viral copy number by RT-qPCR (see section 2.7.4 for methodology) before calculating how much of the new stock was required to achieve an equivalent infection (**Figure 2-4**). To achieve the desired multiplicity of infection (MOI) of 0.3, 3 and 7.5 observed with the stock of 5.56x10^6 $TCID_{50}$/mL required an MOI of 7.5, 50 and 125 (by extrapolation) with the stock of 459x10^6 $TCID_{50}$/mL. This was performed in order to directly compare MC responses between the two stocks.

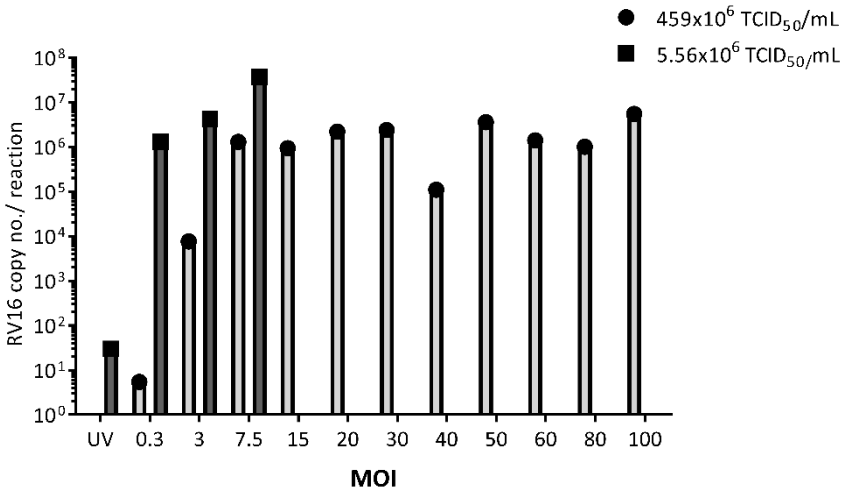


Figure 2-4. Parallel infection of LAD2 MCs with two titres of RV16 stock. LAD2 MCs were infected in parallel at MOI 0.3, 3 or 7.5 with RV16 stocks of 5.56x10^6 $TCID_{50}$/mL and 459x10^6 $TCID_{50}$/mL. Cells were also infected with 459x10^6 $TCID_{50}$/mL stock up to MOI 100. UV-RV16 was used at the highest MOI (7.5 or 100) of the respective RV16 stocks. Cell pellets were collected 24 hours after infection for viral RNA (copy number) determined by RT-qPCR. n=1.

2.6 Cell Treatments

2.6.1 Rhinovirus Infection

LAD2 MCs ($0.5x10^6$ cells/mL in 6 well plates) or CBMCs ($0.25x10^6$ cells/0.5 mL in 24 well plates, equivalent to $0.5x10^6$/mL) were infected with RV16 or RV1B at a MOI of 0.3, 3 or 7.5. Controls were UV irradiated rhinovirus (UV-HRV; MOI 7.5; an infectious but non-replicating control), HRV infection medium or LAD2 complete medium (at equivalent volumes to MOI 7.5). Cells were incubated with virus or control medium for an hour at RT whilst rocking (36 rpm) after which cells were pelleted (200 x *g*, 5 min) and washed with complete medium to remove excess virus. Finally, MCs were re-suspended in complete medium (0.25×10^6/mL or 0.5×10^6/mL, LAD2 MCs and CBMCs respectively) and incubated at 37˚C. As a positive control, 16HBE cells ($0.5x10^6$ cells/mL in 6 well plates) were incubated overnight with 16HBE starvation medium then infected with RV16 or UV-RV16 for 1 hour as above. After an hour cells were washed and then incubated 2 mL of starvation media. MC and 16HBE cell cultures were incubated at 37˚C for a total of 24 hours.

2.6.2 Interferon Treatment

IFN stocks were initially prepared from lyophilised or freeze-dried protein as follows: IFN-β (40,000 IU/mL equivalent to 200 ng/mL; freeze-dried), IFN-γ (2×10^6 IU/mL equivalent to 100 μg/mL; lyophilized) and IFN-λ1 (5,000 IU/mL equivalent to 500 ng/mL; freeze-dried) were reconstituted in a total volume of 1 mL of sterile distilled water. Initially the stocks were dissolved in 0.5 mL water and transferred to a microcentrifuge tube then the stock vials rinsed with a further 0.5 mL of water which was added to the first solution (1 mL total volume). IFN stocks were divided into aliquots and stored at -80˚C.

LAD2 MCs ($0.5\text{-}2x10^6$ cells/mL) were treated with 1, 10 or 100 IU/mL of IFN-β (0.005, 0.05 or 0.5 ng/mL), IFN-γ (0.05, 0.5 or 5 ng/mL) or IFN-λ1 (0.1, 1 or 10 ng/mL) (see **Table 3** for detailed preparation of IFN) in duplicate and incubated at 37˚C for 24-72 hours. As a positive control, 16HBE cells were treated with 100 IU/mL IFN-β, IFN-γ or IFN-λ1 for 4 or 24 hours. LAD2 MCs or CBMCs ($0.5x10^6$ cells/mL) were also treated with IFN-β, IFN-γ or IFN-λ1 (100 IU/mL each) for 24 hours prior to or at the

point of RV16 infection. Cells were maintained in IFN medium throughout the duration of the experiments.

IFN-β (stock = 40,000 IU/mL equivalent to 200 ng/mL)				
Final conc.	**Starting conc.**	**Dilution factor**	**IFN**	**LAD2 medium**
100 IU/mL (0.5 ng/mL)	200 IU/mL	1/200 of 40,000 IU/mL = 200 IU/mL	6 µL (40,000 IU/mL)	1,194 µL
10 IU/mL (0.05 ng/mL)	20 IU/mL	1/10 of 200 IU/mL = 20 IU/mL	120 µL (200 IU/mL)	1,080 µL
1IU/mL (0.005 ng/mL)	2 IU/mL	1/10 of 20 IU/mL = 2 IU/mL	120 µL (20 IU/mL)	1,080 µL

IFN-γ (stock = 2×10^6 IU/mL equivalent to 100 µg/mL)				
Final conc.	**Starting conc.**	**Dilution factor**	**IFN**	**LAD2 medium**
100 IU/mL (5 ng/mL)	200 IU/mL	1/100 of 2×10^6 IU/mL = 2×10^4 U/mL 1/100 of 2×10^4 IU/mL = 200 IU/mL	4 µL (2×10^6 IU/mL) 12 µL (2×10^4 U/mL)	396 µL 1,188 µL
10 IU/mL (0.5 ng/mL)	20 IU/mL	1/10 of 200 IU/mL = 20 IU/mL	120 µL (200 IU/mL)	1,080 µL
1IU/mL (0.05 ng/mL)	2 IU/mL	1/10 of 20 IU/mL = 2 IU/mL	120 µL (20 IU/mL)	1,080 µL

IFN-λ (stock = 5,000 IU/mL equivalent to 500 ng/mL)				
Final conc.	**Starting conc.**	**Dilution factor**	**IFN**	**LAD2 medium**
100 IU/mL (10 ng/mL)	200 IU/mL	1/25 of 5,000 IU/mL = 200 IU/mL	48 µL (5,000 IU/mL)	1,152 µL
10 IU/mL (1 ng/mL)	20 IU/mL	1/10 of 200 IU/mL = 20 IU/mL	120 µL (200 IU/mL)	1,080 µL
1IU/mL (0.1 ng/mL)	2 IU/mL	1/10 of 20 IU/mL = 2 IU/mL	120 µL (20 IU/mL)	1,080 µL

Table 3. Preparation of working stock concentrations of IFN-β, IFN-γ, IFN-λ. Stock concentrations of IFNs were diluted to a starting concentration of 200, 20 or 2 IU/mL by diluting IFN stocks with complete LAD2 medium. Starting IFN concentrations were diluted 1:1 with cell suspensions to give a final concentration of 100, 10 or 1 IU/mL.

2.6.3 Blocking Endogenous and Exogenous Type I IFN

Endogenous type I IFN and exogenous IFN-β responses were inhibited in MCs using an anti-IFN-α/β receptor 2 antibody (anti-IFNAR2; blocking antibody). Anti-IFN-α/β stock (500 µg/mL; frozen) was prepared by thawing under a cold tap and

monoclonal mouse IgG_{2a} isotype control (500 μg/mL; lyophilised) prepared by reconstituting the preparation in 1 mL sterile PBS. Both antibodies were divided into 5 μL aliquots and stored at -80˚C until required. Antibodies were prepared at a starting concentration of 2 or 4 μg/mL in 1 mL complete LAD2 medium with SCF and diluted 1:1 with cell suspensions to give a final concentration of 1 or 2 μg/mL.

LAD2 MCs or CBMCs ($0.5x10^6$ cells/mL) were pre-treated with anti-IFNAR2 antibody (1 or 2 μg/mL), with or without 100 IU/mL IFN-β 1 hour prior to RV16 infection (MOI 1.2-7.5) or UV-RV16 as a control for infection. Cells were also treated with IgG_{2a} isotype antibody as a control for antibody treatment. Cells were maintained in anti-IFNAR2 or IgG_{2a} antibody containing medium throughout the experiments.

2.6.4 IL-33 and TSLP Treatment

Recombinant human IL-33 stock (10 μg; lyophilised) was prepared by reconstitution in 1 mL sterile PBS containing 0.1% bovine serum albumin (BSA). Aliquots were stored at -20˚C. A working concentration of 20 ng/mL of IL-33 was prepared in LAD2 medium with SCF, which was diluted to give stocks of 10 and 2 ng/mL IL-33. These IL-33 stocks (2, 10 and 20 ng/mL) were diluted 1:1 with cell suspensions to give a final IL-33 concentration of 1, 5 and 10 ng/mL.

LAD2 MCs or CBMCs (1×10^6/mL) were treated with IL-33 at 1, 5 or 10 ng/mL for 24 hours. LAD2 MCs were also treated with 10 ng/mL TSLP alone or in combination with 1 or 10 ng/mL IL-33 for 24 hours. Following cytokine treatment cells and cell free supernatants were collected for receptor and protein expression respectively. Alternatively, following IL-33 treatment alone cells were infected with RV16 (MOI 7.5), RV1B (MOI 3) or UV-HRV controls and cell pellets and cell free supernatants collected 24 hours post infection for RT-qPCR and protein expression respectively.

2.6.5 IL-33 and Steroid Treatment

Fluticasone propionate stock (5 mg, 500.6 g/mol; powder) was reconstituted in 1 mL DMSO to a final molarity of 10 mM and aliquots were stored at -20˚C. A working concentration of 5,000 nM was prepared in LAD2 medium with SCF, which was diluted to give a second working stock of 500 nM. Ten μL of each stock was added to cell suspensions in a total volume of 500 μL (1 in 50 dilution) to give a final concentration of 10 and 100 nM fluticasone propionate. DMSO was diluted in the same proportions and used as a vehicle control.

LAD2 MCs were treated with 10 ng/mL IL-33 alone or in combination with fluticasone propionate at 10 or 100 nM for 24 or 48 hours following which samples were collected for protein and receptor expression.

2.6.6 Blocking ICAM1

Human anti-ICAM1 antibody (100 μg) was reconstituted in sterile PBS to a final concentration of 500 μg/mL and aliquots were stored at -80˚C. Ten μL of the stock was added to cell suspensions in a total volume of 500 μL (1 in 50 dilution) to give a final concentration of 10 μg/mL.

LAD2 MCs or CBMCs (1×10^6/mL) were treated with 10 ng/mL IL-33 and after 23 hours cells were treated with an anti-ICAM1 antibody or IgG_{2a} isotype control (10 μg/mL each). After 1 hour of antibody pre-treatment and 24 hours of IL-33 pre-treatment, cells were infected with RV16 or UV-RV16 (MOI 7.5). Cells were maintained in IL-33/antibody media as required throughout the experiments.

2.6.7 Cell Viability

Cell viability and cell number were determined at the end of the specified time-points by trypan blue exclusion. In this method live cells with intact membranes resist staining allowing total and live cell counts to be taken. Cell counts were made with a haemocytometer counting up to 100 cells within the counting area.

MC viability in response to RV16 infection was also assessed with a fixable viability dye (eFlour®660) and flow cytometry. At the end of the infection protocol 0.2×10^6 cells per condition (UV-HRV, HRV, heat-killed positive control) were collected then washed and re-suspended in 0.5 mL LAD2 medium. As a positive control, MCs were heat killed at 60˚C in a water bath for 10 minutes whilst the remaining cells were kept at 37˚C. Cells were washed twice in PBS (without $CaCl_2$, $MgCl_2$) and each condition split into two aliquots for incubation with and without viability dye. Cells were incubated with viability dye or PBS at a 1 in 1000 dilution for 30 minutes at 4˚C in the dark as per manufacturer's instructions then washed with FACS buffer (0.5% (v/v) BSA, 2 mM EDTA in PBS). Cells were fixed by re-suspension in paraformaldehyde (PFA; 4% (w/v)) for 15 minutes at RT then washed with FACS buffer and finally re-suspended in 300 μL FACS buffer. Heat killed cells were used to determine the dead cell population. Fixed and non-fixed cells were compared to investigate the impact of PFA fixation (**Figure 2-5**).

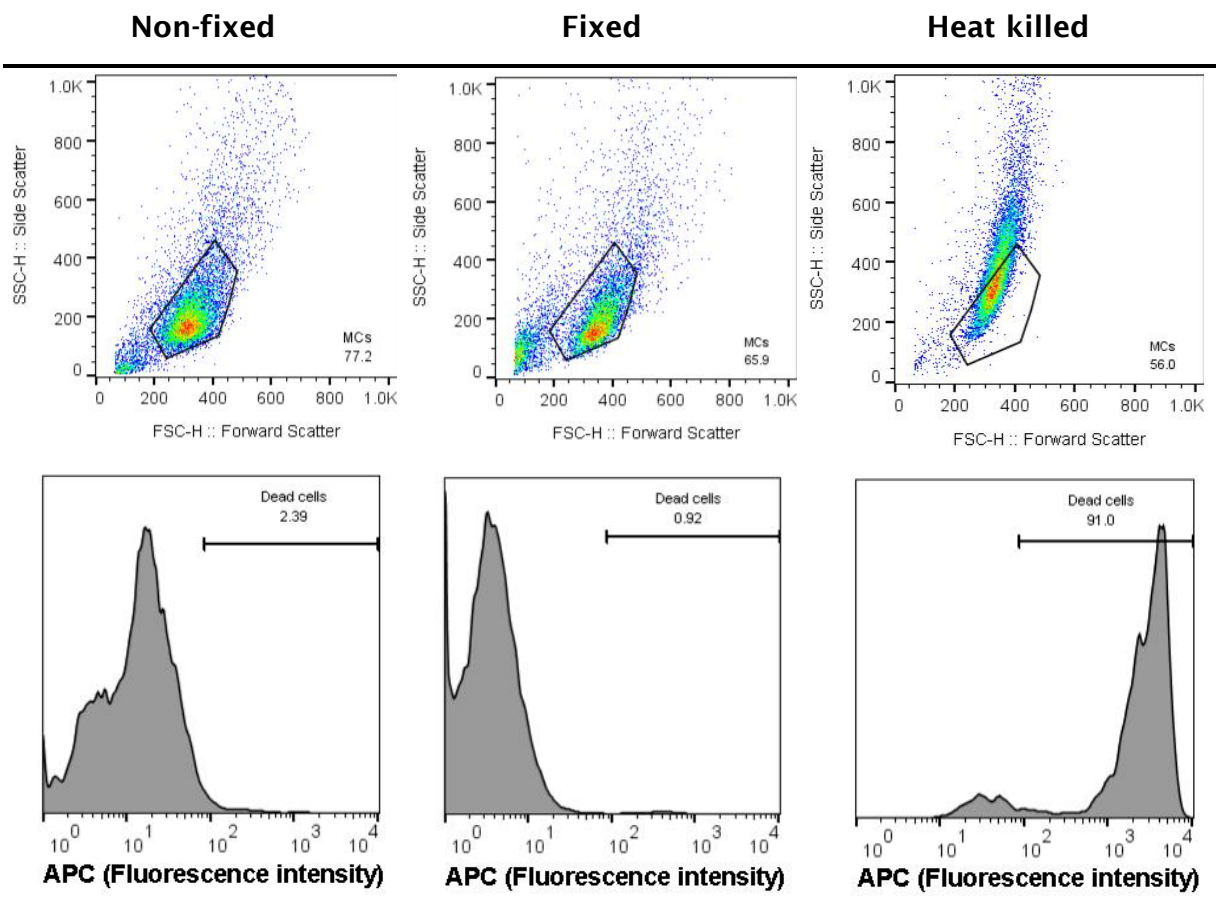


Figure 2-5. LAD2 MC viability determined via a fluorescent viability dye. LAD2 MCs were heat killed (60˚C water bath, 10 minutes), fixed (4% PFA, 15 minutes) or untreated (non-fixed) then stained with a fixable viability dye (eFlour®660). Histograms represent the "MCs" gate. Data are representative of heat killed n=5 and fixed and untreated n=1.

2.6.8 Sample Collection

After the specified time-points, cells were centrifuged at 200 x *g* and cell-free supernatants and cell pellets collected. Cells were collected for RNA used to analyse changes in gene expression by RT-qPCR or cell surface protein expression which was determined by flow cytometry. Supernatants were collected for the determination of protein release by ELISA or MSD assay as well as the quantification of infectious HRV particles by $TCID_{50}$ assay. Supernatants were stored at -80˚C and cell pellets were stored at -80˚ with Trizol® reagent or Qiagen RNeasy® mini kit denaturing buffer for RNA extraction until required. LAD2, CBMC and 16HBE RV16 MOI 0.3–7.5 cell-free supernatants used in ELISA and MSD assay were concentrated (4x) via 3 KDa ultrafiltration centrifugal filter units by centrifuging supernatant (up to 500 µL) at 14,000 x *g* for 10 minutes.

2.7 Procedures

2.7.1 RNA Isolation

Total cellular RNA was isolated by Trizol® reagent or Qiagen RNeasy® mini kit. Cell pellets (suspension cells) or monolayers were washed with Hank's Balanced Salt Solution (HBSS) then lysed with Trizol® reagent or RNeasy® lysis buffer (Buffer RLT, a denaturing guanidine-thiocyanate containing buffer).

The RNeasy® kit was used according to the manufacturer's instructions. Cells were lysed and homogenised by the denaturing buffer and mixed with an equal volume of ethanol. The sample was applied to a spin column containing a silica membrane to which total RNA was bound allowing contaminants to be removed by washing with guanidine and ethanol containing buffers. Finally RNA was eluted twice into a single volume of 30 µl of RNase-free water and immediately DNase treated.

Cells lysed with Trizol® reagent (up to 500 µL) were homogenised by pipetting thoroughly. Chloroform was added (20% of the initial Trizol® volume) and mixed by shaking samples vigorously by hand (15 seconds) to separate the homogenate into RNA and DNA containing phases. Samples were incubated at RT for 10 minutes and centrifuged at 13,500 x *g* for 15 minutes at 4°C. The top RNA-containing aqueous phase was removed, without disturbing the underlying interphase, into a fresh tube and mixed with an equal volume of ice cold isopropanol to precipitate the RNA. RNase-free glycogen (10 µg), an inert co-precipitant of RNA, was added to increase RNA recovery and aid visualisation of the RNA pellet. Samples were vortexed briefly and centrifuged at 13,500 x *g* for 30 minutes at 4°C. Isopropanol was removed and the RNA pellet washed with an equal volume of 75% ethanol to the starting Trizol volume and centrifuged at 7,500 x g for 5 minutes at 4°C. Finally, ethanol was removed and the pellet allowed to air dry after which samples were immediately DNase treated.

2.7.2 DNase Treatment and RNA Quantification

RNA from both extraction methods was DNase treated with a DNA-*free*™ Kit to remove potential contaminating genomic DNA. Samples were treated according to the manufacturer's instructions. Briefly, samples were incubated with DNase I (2 Units/µL) and DNase I buffer (100 mM tris-HCl pH 7.5, 25 mM $MgCl_2$, 5 mM $CaCl_2$; 0.1X volume) for 1 hour in a 37°C water bath. Samples were incubated for 2 minutes

at RT with DNase inactivation reagent (0.25X volume) during which time samples were mixed 2-3 times to re-disperse the inactivation reagent. Finally, samples were centrifuged at 10,000 × *g* for 2 minutes. The total reaction volume was 20 μL. RNA (ng/μL) was quantified with a NanoDrop spectrophotometer which also detailed A260/A280 (nucleic acid/protein) and A260/A230 (nucleic acid/non-protein) ratios which are indicators of sample purity.

2.7.3 cDNA preparation

Up to 1 μg of RNA was reverse transcribed into cDNA in a two-step process where random nonamers and poly T primers were first annealed to the RNA template at 65˚C for 5 minutes in a thermal cycler. Following this samples were immediately cooled on ice to prevent secondary structure formation within the RNA strands. In a second step RNA was reverse transcribed by the moloney murine leukemia virus (MMLV) reverse transcriptase (5 μL; 250 units/μL), with MMLV reverse transcriptase 5X buffer by heating at 37˚C for 10 minutes then 42˚C for 60 minutes. cDNA (50 ng/uL) was diluted to 5 ng/μL in distilled deionised water (dd-H_2O) and stored at -20˚C until required for use in RT-qPCR.

2.7.4 Reverse Transcription-Quantitative Polymerase Chain Reaction

RT-qPCR was performed with probe/primer sets or SYBR® green primers for genes of interest, the human housekeeping genes (HKGs) ghlyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ubiquitin C (UBC) and the RV16 and RV1B genome (**Table 4, Table 5 & Table 6**).

qPCR reaction component	Per reaction	
PrecisionPLUS mastermix	5 μL	
Primer (150 nM)	0.5 μL	
cDNA (12.5 ng)	2.5 μL	
RNase/DNAse free water	2 μL	
Final volume	10 μL	
HRV copy number		
Standard 1	5×10^5 /reaction	2×10^5 /μL
Standard 2	5×10^4 /reaction	2×10^4 /μL
Standard 3	5×10^3 /reaction	2×10^3 /μL
Standard 4	5×10^2 /reaction	2×10^2 /μL
Standard 5	5×10^1 /reaction	2×10^1 /μL
Standard 6	5×10^0 /reaction	2×10^0 /μL

Table 4. RT-qPCR reaction mixes and HRV standard curve. RT-qPCR reagents for reactions using either SYBR® green or probe primer sets and virus copy number of HRV standards.

	Step	Time	Temp.
	Enzyme activation	2 min	95˚C
X40 cycles	Denaturation	10 sec	95˚C
	Data collection	60 sec	60˚C
	Melt curve	5 sec	60˚C to 95˚C, 0.5˚C increments

Table 5. RT-qPCR amplification conditions. Thermal cycler programme for the amplification for qPCR reactions using either SYBR® green or probe primer sets.

	Primer sequence		
Gene	**Sense primer**	**Anti-sense primer**	**Probe sequence**
CCL2	ACCGAGAGGCTGAGACTAAC	AATGAAGGTGGCTGCTATGAG	ACATCCAATTCTCAAACTGAAGCTCGCACT
CCL5	AACCCAGCAGTCGTCTTTGTC	AGCAAGCAGAAACAGGCAAT	CCCGAAAGAACCGCCAAGTGTGTGCCAACTTCGGG
CDHR3	GAGTGGGACAGGTGCGAGC	GTTGTTGGTGGCCTGGATTCT	
CMA1	GTGCTGACGGCTGCTCAT	GAAGTGTTATATTTTGGATGACGGAAT	TCTGCCATGTGTCTTCTTCCTCTGTTATGTT
CPA3	ATATCCCAGGCAGGCACAG	ATCCAATTTTAATACGAGAGACCATTT	TCTTTTCAGTCCAAGCCACAATCTTTTCCCA
CXCL10	CAGAGGAACCTCCAGTCTCAG	GGTACTCCTTGAATGCCACTTA	ACTGCGATTCTGATTTGCTGCCTTATCTTTCTGTCGCAGT
DDX58 (RIG-I)	TTCTCTTGATGCGTCAGTGATA	CCGTGATTCCACTTTCCTGAA	CTTGCTCCAGTTCCTCCAGATTGTGTTTGACGAGCAAG
EIF2AK2 (PRK)	GCGGTCTTCAGAATCAACATC	GGTATGTATTAAGTTCCTCCATGAA	TATCACCAGCCATTTCTTCTTCCCGTATCCTAGGTGATA
FCER1A	CTGTGTGTAGCCTTACTGTTCT	CATGGAGGGTTCAAGGAGAC	TTTCTGAGGGACTGCTAACACGCCATCTG
ICAM1	CCTATGGCAACGACTCCTTC	TCTCCTGGCTCTGGTTCC	
IFIH1 (MDA5)	GTCTCGTCACCAATGAAATAGC	TTATACATCATCTTCTCTCGGAAATC	CGATAACTCCTGAACCACTGTGAGCAACCAAGTTATCG
IL5	CCCACAGAAATTCCCACAAGT	CTCAGAGTCTCATTGGCTATCAG	ACCTTGGCACTGCTTTCTACTCATCGAACT
IL13	AGGCACACTTCTTCTTGGTCT	GAGTCTCTGAACCCTTGGCT	AGCCACAGTCTTCCCCAATCCCCAACGGTGGCT
IL29	ATGGGAACCTGTGTCTGAGAA	GGGTGAGAGGAAATAAATTAAGGAA	CCAACCCACCCTGAGTCCACCTGACACCCCGGTTGG
IRF3	CAAAGAAGGGTTGCGTTTAGC	GTGTCTGGCTGGGAAAAGTC	
IRF7	GCGACAGGAGCCCTTACC	GCCCTCTCAGGAGCCAAG	ATAACACCTGACCGCCACCTAACTGCCGGTGTTAT
IRF9	CGATTGACCTGTCCTCTTTGTG	ATTAGCCTTGAGTTCTCCACCA	CCGTGATAATCGTGTCCTGAAAATCCTCGCA
KIT	TCCATTTATGTGTTTGTTAGAGATCC	ACCAGCGTGTCGTTGTCTT	TTTCCTTGTTGACCGCTCCTTGTATGGG
KITLG	GCTTTCCATTCTAACTTAATCTTTGT	TTCATGCCCTTTTGTGTCACTA	TTGCCATGTTCTACAACCGTAAGATGACAGT
MX1	CCCCAGTAATGTGGACATCG	ACCTTGTCTTCAGTTCCTTTGT	CGTCAAGATTCCGATGGTCCTGTCTCCCTCTTGACG
RSAD2 (viperin)	GATGAGACCAAAGAGGAGGAAG	TGGCGAGTGAAGTGATAGTTG	TCCTCTGCCCACCACCCCAACCA
TLR3	GTGTGAAAGTATTGCCTGGTTTGT	ATGATAGTGAGGTGGAGTGTTGC	ACGAGACCCATACCAACATCCCTGAGC
TPSB2	GGCGATGTGGACAATGATGAG	CGTCGTCTCCCGTGTAGG	CCTCCCACCGCCATTTCCTCTGAAGCA
VLDLR	GACGTGTGCTGAATCTGACTT	ACTGTTCTGGGCTTTCATCTG	ACCATCTTCGCAGTCAGGATCTCCATCACA
RV16	GAGAGGTTAAGAACTTGATTGAA	CTAATTTTGTTTGTGGTGATAGAG	CCAATAAATAGCACACAGTCTAACATTGGC
RV1B	TGGGTGTTGTACTCTGTTATTCC	TTGCCTACTATTGGTCTTGTGTT	TCCCTCCCTCCCCATCCTTTTACGTAAC
GAPDH	Accession number – NM_002046, Anchor nucleotide – 1087, Context sequence length – 142		
UBC	Accession number – NM_021009, Anchor nucleotide – 452, Context sequence length – 192		

Table 6. RT-qPCR primer sequences. Sense and anti-sense primer sequences for commercial Taqman® and SYBR® green primers. Probe sequences are included for probe primer sets. Primers for the amplification of GAPDH and UBC are not provided by the manufacture but they do provide accession number, anchor nucleotide and amplicon length.

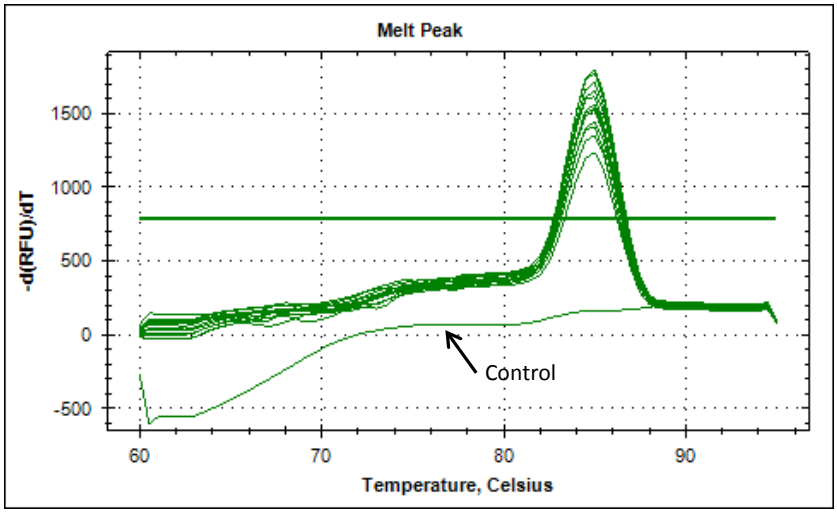
SYBR® green is a fluorescent dye which intercalates with dsDNA in a process which increases the dye's fluorescence. As more PCR product is generated the fluorescence signal intensifies, however, intercalation of the dye is non-sequence specific[230]. A dissociation or melt curve plots the change in fluorescence as PCR products dissociate (or melt) into ssDNA as they are heated and is used to assess reaction specificity. Different PCR products have different melting characteristics and a plot of change in fluorescence/change in temperature plotted against temperature produces a single peak if the primers have amplified a single PCR product and multiple peaks if not (**Figure 2-6 A**). Hydrolysis probes (e.g. Taqman®) are sequence specific oligonucleotides which are bound by a fluorescent reporter and a quencher molecule at their 5' and 3' end respectively. The probe binds downstream of the primer and is cleaved by the polymerase during the PCR reaction thereby separating the reporter and quencher. This relieves the suppressive effect of the quencher on the fluorescence of the reporter therefore fluorescence increases as more product is generated[230]. This is demonstrated in a plot of the change in fluorescence with cycle number (**Figure 2-6 B**). Reactions were performed in duplicate and gene of interest expression was normalised to the geometric means of GAPDH and UBC. Fold change in gene expression was calculated relative to controls according to the ΔΔCt method[231]:

Fold change in gene of interest = $2^{-\Delta\Delta Ct}$

Where $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{control}$ and ΔCt = Ct – HKG geometric mean

Rhinovirus RNA copy number was quantified from a standard curve (10-fold serial dilution) of known copies of the rhinovirus genome (**Table 4**).

A



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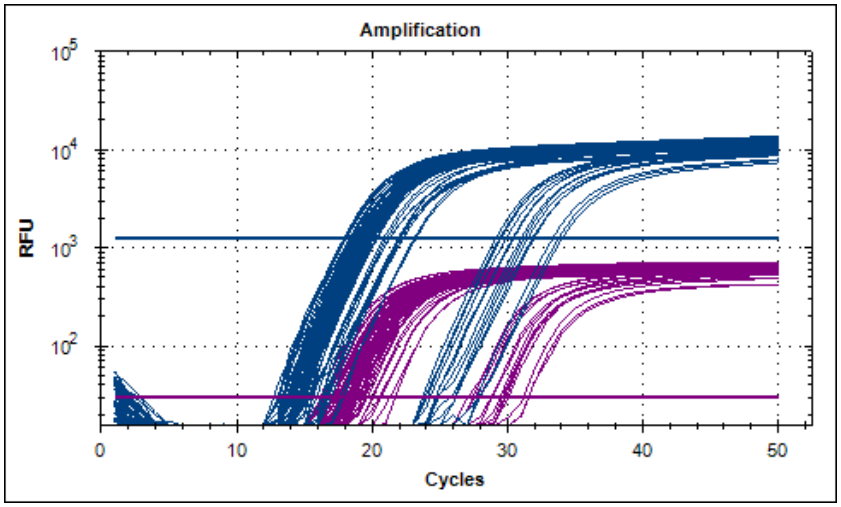


Figure 2-6. RT-qPCR melt curves and amplification plots. LAD2 MC cDNA was amplified by qPCR. A representative RT-qPCR **(A)** melt curve (SYBR® green *OAS1* primer), [change in fluorescence/change in temperature] plotted against temperature and **(B)** amplification plot (*GAPDH* (blue) and *UBC* (purple) HKGs probe primers), fluorescence plotted against cycle number. RFU, relative fluorescence intensity.

2.7.5 ELISA

Enzyme linked immunosorbent assay (ELISA) is an assay which uses antibodies to detect and quantify specific analytes, typically proteins, within a sample (**Figure 2-7**). Analytes of interest are first immobilised and depending on the ELISA format, analytes are bound via a capture antibody or are adsorbed directly onto the plate. Detection occurs via an enzyme-linked detection antibody which with the addition of substrate catalyses a reaction which produces a detectable signal such as a

colorimetric change. This signal is proportional to the amount of target analyte in a sample which is calculated from a standard curve generated from known concentrations of the target of interest.

ELISA was used to quantify IFN-λ, CXCL10 and CCL2 from LAD2 and 16HBE cells in cell-free supernatants of virally infected and/or IFN treated cells. These were according to manufacturer's instructions with some modifications including use of half the recommended volumes of capture and detection antibodies and samples and standards.

Maxisorp® 96 well plates were coated with capture antibody (50 μL) and incubated overnight at RT. The capture antibody solution was aspirated and plates were washed with 0.05% Tween20 in 1X PBS (wash buffer) (3X) prior to blocking for 1 hour with 1% BSA in 1X PBS (reagent diluent). Plates were aspirated and washed (3X) before standards and samples (diluted in reagent diluent as required) were added to plates (50 μL per well). The range of the standards was as follows: IFN-λ 1/3 (4,000 – 62.5 pg/mL), CXCL10 (2,000 – 31.25 pg/mL) and CCL2 (1,000 – 15.63 pg/mL) forming a seven point standard curve via 2-fold serial dilutions (**Figure 2-8**). The plates containing samples and standards were then incubated at 4˚C overnight. After aspirating and washing (3X), plates were incubated with biotinylated detection antibody (50 μL) for 2 hours at RT. Following incubation (and aspiration and washing) plates were incubated with streptavidin-horseradish peroxidase (HRP; 50 μL) for 20 minutes at RT in the dark. At the end of the incubation, plates were aspirated and washed (3X) then incubated with 3,3',5,5'-tetramethylbenzidine (TMB; 50 μL), a chromogenic substrate of HRP which changes from a pale blue to a deep blue solution when oxidised by HRP. Plates were incubated for 20 minutes at RT in the dark or until adequate development of the standards. Sulphuric acid solution (1M H_2SO_4; 25 μL) was added to terminate the colorimetric reaction and the optical density read immediately at a wavelength of 450 nm (absorbance of TMB with sulphuric acid) and 570 nm (corrects for non-specific absorbance from the plate) with a microplate reader. A standard curve was generated and was used to determine the concentration of each analyte in unknown cell-free supernatants.

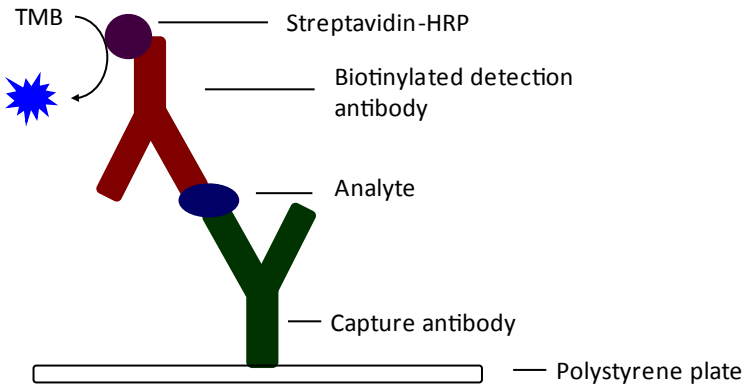


Figure 2-7. ELISA schematic (direct sandwich ELISA). Capture antibody is coated onto polystyrene plates and binds analytes of interest contained within supernatants. Analytes are subsequently bound by biotinylated detection antibodies which bind streptavidin-HRP (via streptavidin). Streptavidin-HRP (via HRP) catalyses the oxidation of TMB generating a deep blue colour the absorbance of which is detected by a plate reader and is proportional to the amount of analyte in the sample. HRP, horseradish peroxidase; TMB, 3,3',5,5'-tetramethylbenzidine.

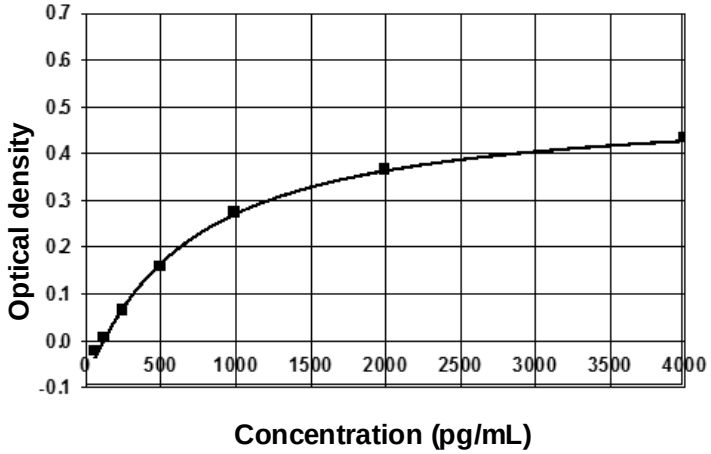


Figure 2-8. ELISA standard curve. An example of a seven point standard curve of an IFN-λ 1/3 ELISA fit according to a four parameter logistic regression.

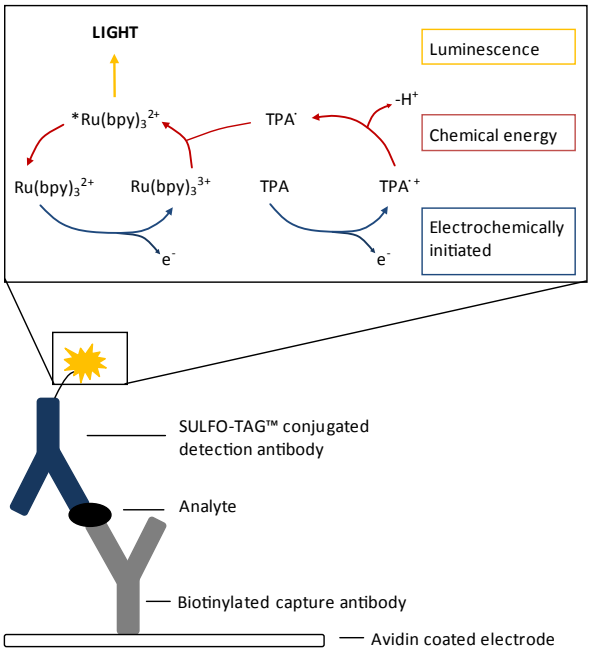
2.7.6 Multiplex Assay Kits (Meso Scale Discovery)

MSD immunoassays use antibody capture and detection methods to quantify specific analytes in biological samples. Detection occurs via detection antibodies which are conjugated to electrochemiluminescent tags which catalyse a light generating reaction (**Figure 2-9 A**). Each well of an MSD plate contains an avidin-coated carbon electrode which is bound by biotinylated capture antibody. The capture antibody subsequently binds a specific analyte e.g. IFN-β, which is bound by the electrochemiluminescent (SULFO-TAG™) detection antibody. The read buffer provides a chemical environment in which the detection antibody generates light upon electrical stimulation from the plate reader. The plate reader detects the emitted light and light intensity is used to quantify analyte concentration (**Figure 2-9 A**). MSD immunoassays are generally more sensitive than standard ELISA kits and have a broader dynamic range. For example, the Bio-Techne IFN-β ELISA has a detection range of 50 – 4,000 pg/mL compared to 24 – 100,000 pg/mL for the MDS assay. In addition MSD assays have a 3.5 hour protocol compared to up to three days of a standard ELISA.

IL-4, IL-5 and IL-13 (part of a 7-plex assay) and IFN-β (single-plex assay) were detected by MSD immunoassays according to the manufacturer's protocol. Plates for the 7-plex assay including IL-4, IL-5 and IL-13 came pre-coated with capture antibody and were blocked with 150 µL 1% (w/v) milk powder in PBS for 1 hour at RT with shaking (750 rpm). Plates were washed 3X with PBS and 25 µL sample or calibrator added per well and incubated for 2 hours at RT with shaking. IL-4, IL-5 and IL-13 standards ranged from 10,000 pg/mL – 2.4 pg/mL and a seven-point standard curve was generated by a 4-fold dilution series (**Figure 2-9 B**). After 2 hours 25 µL electrochemiluminescent (SULFO-TAG™) detection antibody (1X) was added per well and incubated for 2 hours at RT with shaking. For the IFN-β assay plates were washed 3X with PBS then coated with 20 µL biotinylated capture antibody (1X) for an hour at RT with shaking (750 rpm). Following this, plates were washed 3X with PBS and incubated with the electrochemiluminescent (SULFO-TAG™) detection antibody (1X; 20 µL) and 50 µL of sample or standards for two hours at RT while shaking. The top standard of 100,000 pg/mL was diluted by a 4-fold serial dilution to the lowest standard of 24 pg/mL generating a seven point standard curve. Following incubation with the detection antibody, plates were washed 3X with PBS (+ 0.05% Tween®20 for 7-plex plates) and 150 µL of 1X (2X for 7-plex

plates) read buffer in deionised water was added per well. Plates were read on the MESO QuickPlex SQ 120 instrument. A standard curve was generated, which was fit according to a four parameter logistic regression, and was used to determine the concentration of each analyte in unknown cell-free supernatants.

A



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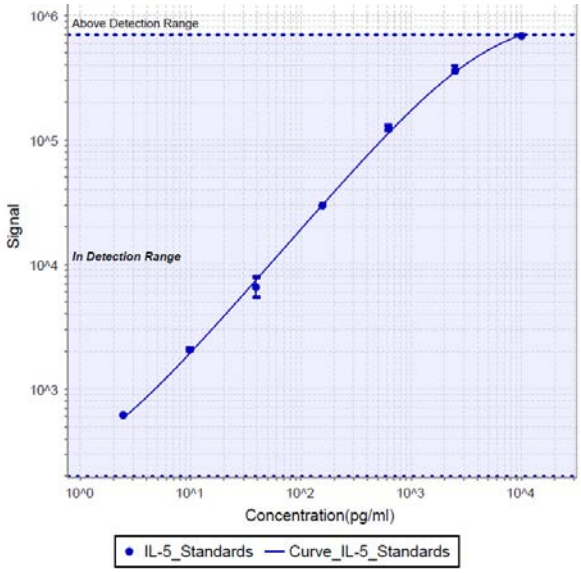


Figure 2-9. MSD assay standard curve and electrochimiluminescent detection. MSD assay was used to quantify IFN-β, IL-4, IL-5 and IL-13 in cell free supernatants from MCs and BECs. **(A)** Schematic of analyte detection and the electrochemical light generating reaction mediated by SULFO-TAG™. Biotinylated capture antibody is bound to an avidin coated electrode in the bottom of each well. The analyte is bound by the capture antibody and an electrochemiluminescent (SULFO-TAG™) detection antibody. In the appropriate electrochemical environment the detection antibody emits light. **(B)** A representative IL-5 standard curve demonstrating standards and detection range. $Ru(bpy)_3$, tris-2,2′-bipyridylruthenium, TPA, tripropylamine, *, excited state, ·, free radical.

2.7.7 B-hexosaminidase Release Assay

In MCs, the β-hexosaminidase enzyme is mostly stored in MC granules therefore its release is determined as a measure of MC degranulation. The assay was performed by Dr Emily Swindle. Briefly, MCs (0.2-1×10^6/mL) were sensitised with monomeric human IgE (0.5 μg/mL) overnight (≥ 18 hours) after which cells were washed 3X with 10 mL HEPES buffer [pH 7.4; HEPES (10 mM), NaCl (137 mM), KCl (2.7 mM), $Na_2HPO_4 \cdot 7H_2O$ (0.4 mM), glucose (5.6 mM), $CaCl_2 \cdot 2H_2O$ (1.8mM), $MgSO_4 \cdot 7H_2O$ (1.3mM), bovine serum albumin (BSA) (0.04% (v/v)] warmed at 37˚C. Cells were re-suspended at 10,000 cells/90 μL (0.11×10^6/mL) in HEPES buffer and 90 μL added per well to a 96 well plate. Cells were incubated for 10 minutes at 37˚C before the addition of the stimuli (10 μL) prepared as a 10X stock in HEPES buffer: human anti-IgE (1 μg/mL), ionomycin (calcium ionophore, 1 μM), HRV or HEPES buffer alone at 37˚C for 30 minutes. Meanwhile, a solution of the β-hexosaminidase substrate, p-nitrophenyl N-acetyl-β-D-glucosamide (PNAG; 3.5 mg/mL) dissolved in citrate buffer [pH 4.5; citric acid (40 mM), $Na_2HPO_4 \cdot 7H_2O$ (20 mM)] was prepared and 100 μL added per well to a 96-well plate and stored at 4˚C until use. After 30 minutes stimulation cells were centrifuged at 450 x *g*, at 4 ˚C for 5 min and 50 μL of supernatant removed and added to the PNAG-containing plate. The remaining cells and supernatants were mixed with 150 μL Trition-X 100 solution (0.1%) and 50 μL of the resulting lysate also added to the PNAG plate before incubation at 37˚C for 90 minutes. The reaction was terminated by the addition of 100 μL glycine solution (0.4 M) causing a colour change from clear to yellow then the absorbance at 405 nm (reference filter 620 nm) read on a plate reader. B-hexosaminidase release was determined according to the following equation:

$$\% \text{ release} = 100 \times (\text{supernatant}) / (\text{supernatant} + \text{lysate content})$$

$$= 100 \times (2 \times \text{supernatant}) / (\text{supernatant}/2) + (4 \times \text{lysate})$$

2.7.8 Flow Cytometry

Mast cell surface receptor expression (**Figure 2-1**) was determined by flow cytometry. Cells were washed with 5 mL ice cold FACS buffer (0.5% (v/v) BSA, 2 mM EDTA in PBS) and pelleted at 200 x *g* for 5 minutes at 4˚C. Following this cells were

blocked with 10% (v/v) heat inactivated human serum containing 2 mM EDTA in PBS at 1×10^6 cells/mL for 20 minutes on ice. Cells were washed with FACS buffer and re-suspended at 100,000/80 μL of FACS buffer (1.25×10^6/mL) and stained with fluorescently labelled receptor antibodies or isotype controls (20 μL) for 30 minutes on ice protected from light. Antibody concentrations are detailed in **Table 7**. Cells were washed with 3 mL FACS buffer and re-suspended in 100 – 300 μL FACS buffer and kept on ice until analysed by flow cytometry. Flow cytometry was performed using the BD FACSCalibur cell analyser and data analysed in FlowJo software (version 7.6.5, Oregon, USA).

Target-conjugate	Stock concentration (μg/100 uL)	Working concentration (μg/100 uL)	Dilution	Antibody vol./100 μL	Viability dye (APC)
Receptor antibodies					
CD117 (c-KIT)-PE (mouse $IgG_{1\kappa}$)	5	0.1	1 in 50	2 μL	--
FcεR1-APC (mouse $IgG_{2b\kappa}$)	2.5	0.025	1 in 100	1 μL	--
ICAM1 (CD54)-FITC (mouse IgG_1)	1.3	0.065	1 in 20	5 μL	1 in 1000
TSLP-PE (mouse $IgG_{1\kappa}$)	10	0.5	1 in 20	5 μL	1 in 1000
ST2 (IL1R4)-PE (goat IgG)	2.5	0.25	1 in 10	10 μL	1 in 1000
LDLR-PE (mouse IgG_1)	2.5	0.25	1 in 10	10 μL	1 in 1000
Isotype antibodies					
PE (mouse $IgG_{1\kappa}$)	10	Stock antibodies diluted to the same concentration and used at the same volume as respective receptor antibodies			1 in 1000
APC (mouse $IgG_{2b\kappa}$)	20				1 in 1000
FITC (mouse IgG_1)	20				1 in 1000
PE (goat IgG)	2.5				1 in 1000

Table 7. Flow cytometry antibody concentrations. Stock and working concentrations of fluorescent antibodies used for flow cytometric analysis of MC receptor expression. Cells were co-stained with CD117-PE and FCεRI-APC and therefore the viability dye (APC) was not added to these samples.

2.7.9 Statistical Analysis

Data were analysed for normality and determined to be non-parametric i.e. not normally distributed. Paired non-parametric data were analysed by Friedman repeated measures one-way ANOVA by ranks with Dunn's correction for multiple

comparisons or Wilcoxon signed rank test for matched pair comparisons. Unpaired non-parametric data were analysed with Kruskal-Wallis one-way ANOVA with Dunn's correction for multiple comparisons or Mann-Whitney ranked sum test for matched pair comparisons. Data are presented as scatter dot plots with or without bars. Normalised data were analysed by Student's *t*-test and represent the mean. All data were analysed using GraphPad Prism (GraphPad Software, Inc. CA, USA) and $p \leq 0.05$ was considered statistically significant.

Chapter 3: The Replication and Release of Major and Minor Group Rhinovirus from the LAD2 MC Line and Primary Human Mast Cells

3.1 Introduction

MCs express a range of PRRs including those for the detection of viral products such as TLR3, TLR7[9, 33, 232], MDA5 and RIG-I[33-35]. These facilitate the detection of invading pathogens at mucosal surfaces which interface with the external environment, such as the airways. MCs have been demonstrated to respond to viruses such as the respiratory viruses RSV[93] and influenza by the release of cytokines and chemokines including type I and type III IFNs[9, 33, 35, 93]. However, depending on the particular pathogen MC responses to parasites, bacteria and viruses may not always be beneficial to the host[12, 97]. HRVs are a cause of the common cold but infections have serious implications in asthma. For instance, HRV-associated wheezing in infancy is a risk factor for persistent childhood wheeze and the development of asthma[138-141], in addition, infections with HRV trigger the majority of asthma viral-induced exacerbations[142, 143]. The increased localisation of MCs at the bronchial epithelium[104], which is the major site of HRV replication, suggests MCs may play a role in immune responses during HRV infection. The immature MC line, HMC-1 has been demonstrated to release infectious virus particles following infection with a major group rhinovirus (RV14)[96], although cytokine release was only demonstrated when degranulation was induced. This work aims to extend these observations by investigating the susceptibility of a mature MC line and primary MCs to HRV infection and examine HRV-induced anti-viral responses.

3.1.1 Hypothesis

Mast cells mount anti-viral and pro-inflammatory responses to HRV infection and are susceptible to the replication and release of infectious HRV particles.

3.1.2 Aims

1. Determine the susceptibility of LAD2 MCs and primary MCs to virus replication and release following infection with a major or minor group HRV, RV16 or RV1B respectively, at increasing MOIs.
2. Investigate the anti-viral and pro-inflammatory response of MCs to RV16 and RV1B infection.

3.1.3 Objectives

1. Determine HRV replication by RT-qPCR for viral RNA in cell pellets and the release of infectious HRV particles in cell-free supernatants by $TCID_{50}$ assay.
2. Determine the induction and upregulation of anti-viral and pro-inflammatory mediators including TLRs, RLRs, type I and type III IFNs, ISGs and pro-inflammatory cytokines and chemokines, by RT-qPCR and ELISA.

3.2 Results

3.2.1 The LAD2 Human MC Line Mounts an Innate Immune Response to Rhinovirus Infection

To investigate the responses of MCs to HRV infection LAD2 MCs were treated with HRV at an MOI of 0.3, 3, 7.5 or UV-HRV (MOI 7.5) as a non-replicating control for 1 hour. Twenty-four hours following infection immune responses were assessed by RT-qPCR and ELISA. LAD2 MCs responded to RV16 and RV1B treatment (respective major and minor group HRVs) with statistically significant MOI-dependent increases in the transcription of the type I and type III IFNs, *IFNB1* and *IFNL1* respectively (**Figure 3-1 A**). This was accompanied by a trend for increased release of IFN-λ and IFN-β protein (IFN-β was measured in RV16 supernatants only) as detected by ELISA (**Figure 3-1 B**).

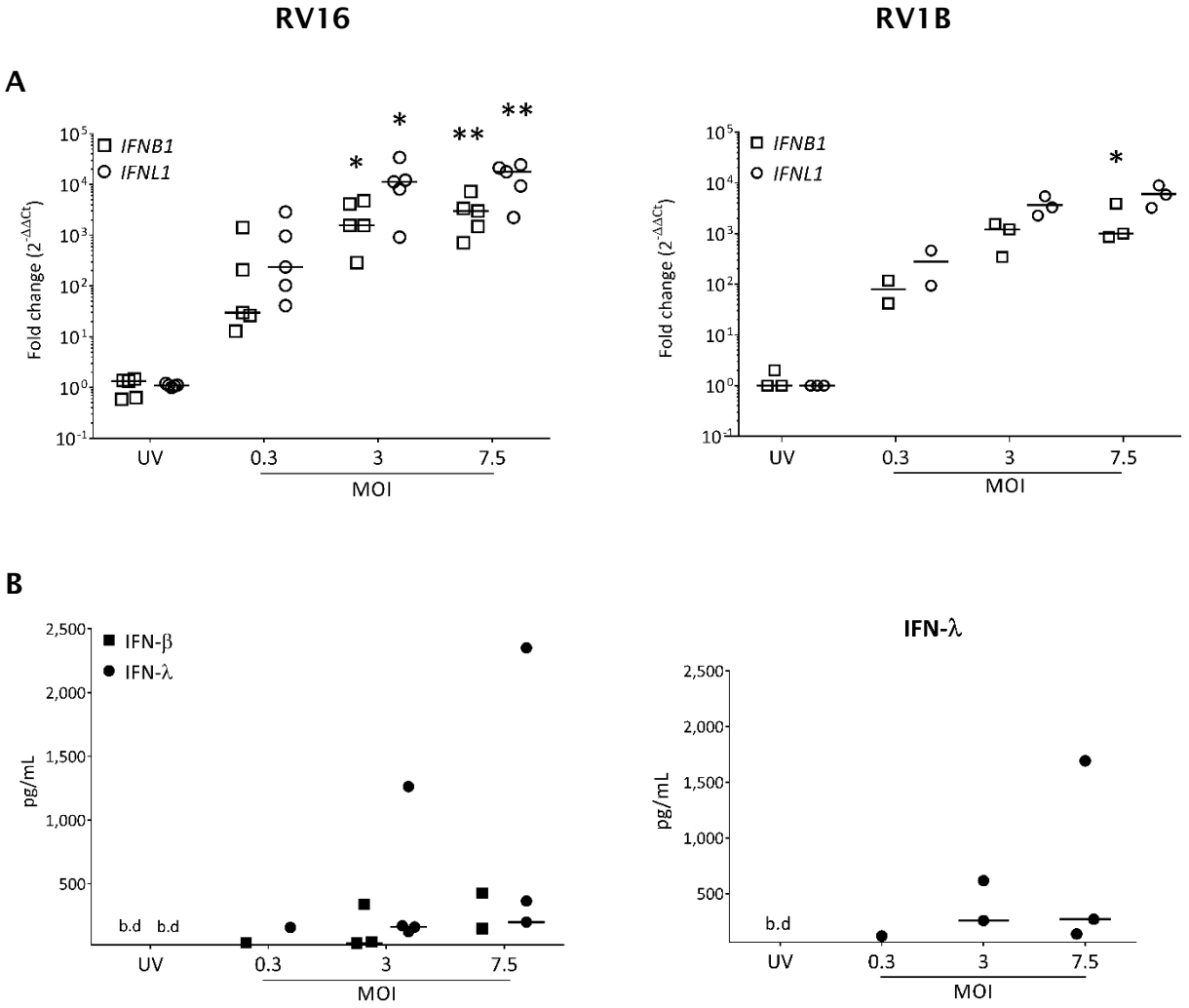
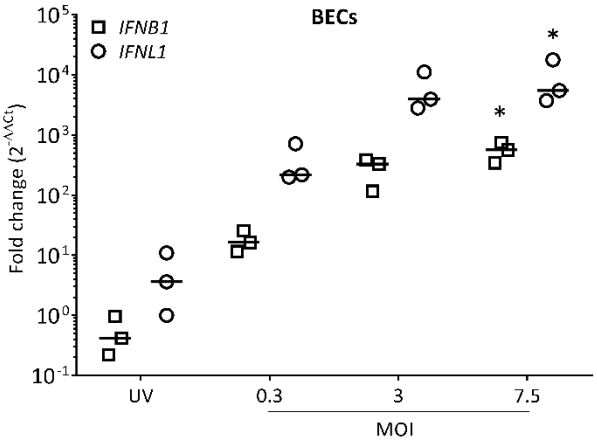


Figure 3-1. IFN response of LAD2 MCs to rhinovirus exposure. LAD2 MCs were exposed to RV16 or RV1B at MOI 0.3, 3 or 7.5 or UV-HRV at MOI 7.5 (control). Cell pellets and cell-free supernatants were collected 24 hours post infection for gene and protein expression respectively. **(A)** RV16- or RV1B-induced *IFNB1* and *IFNL1* gene expression was determined by RT-qPCR, n=5 (RV16), n=2 (MOI 0.3) or 3 (RV1B). **(B)** RV16- or RV1B-induced IFN-β and IFN-λ protein expression was determined by ELISA, n=5 (RV16), n=3 (RV1B). Cell-free supernatants were concentrated (4x) for ELISA. Bars at median. *$p < 0.05$, **$p < 0.01$ versus UV-HRV, one-way ANOVA (Friedman test).

There was minimal mRNA or protein expression with UV-HRV indicating virus replication was required to induce these IFN responses. The bronchial epithelium is the main site of HRV replication so the 16HBE bronchial epithelial cell (BEC) line was exposed to RV16 as a positive control. RV16 also induced IFN expression in

BECs (**Figure 3-2**). In control experiments RV16 did not induce LAD2 MC degranulation (**Figure 3-3**).

A



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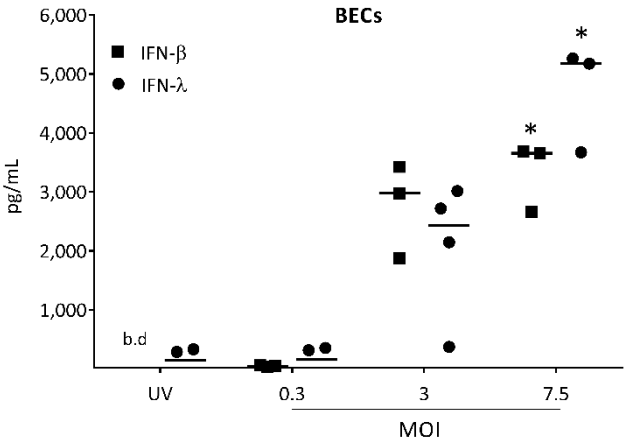

Figure 3-2. IFN response of bronchial epithelial cells to rhinovirus exposure. BECs were exposed to RV16 at MOI 0.3, 3 or 7.5 or UV-HRV MOI 7.5 (control) in the same experiments as LAD2 HRV infections as a positive control. Cell pellets and cell-free supernatants were collected 24 hours post infection for gene and protein expression respectively. **(A)** RV16-induced *IFNB1* and *IFNL1* gene expression was determined by RT-qPCR, n=3. **(B)** RV16-induced IFN-β (n=3) and IFN-λ protein production (n=3 [MOI 7.5] or n=4) was determined by ELISA. Cell-free supernatants used in IFN-β ELISA were concentrated (4x). Bars at median. *$p < 0.05$ versus UV-RV16, one-way ANOVA (Friedman test [paired data] or Kruskal-Wallis test [non-paired data]).

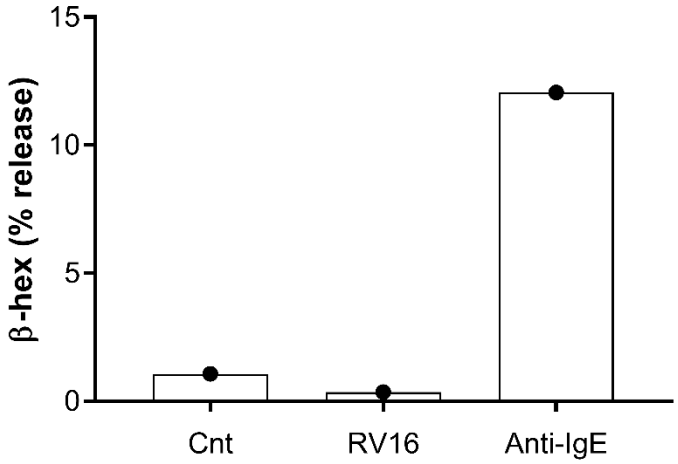

Figure 3-3. Rhinovirus does not induce LAD2 MC degranulation. LAD2 MCs were sensitised with 500 ng/mL human myeloma IgE overnight then treated with RV16 MOI 1, 10 μg/mL anti-IgE as a positive control or medium alone (cnt) for 1 hour. Degranulation was determined according to β-hexosaminidase release into cell-free supernatants. Results are percent β-hexosaminidase release and are representative of one experiment performed in triplicate.

In parallel with HRV-mediated IFN expression there were also statistically significant increases in the mRNA expression of a range of ISGs in response to RV16 and RV1B (**Figure 3-4**). There were MOI-dependent inductions in the sensors of viral RNA *TLR3,* MDA5 (*IFIH1*) and RIG-I (*DDX58*); IFN regulatory factors (IRFs), *IRF7* and *IRF9* and anti-viral effectors *MX1*, *OAS1* and viperin (*RSAD2*) (**Figure 3-4**). In addition, the inflammatory mediators *CXCL10, CCL5* and TNF-α (*TNF*) were also induced (**Figure 3-4**). Responses were similar to those observed in BECs exposed to RV16 (**Figure 3-5**) and in all cases inductions were dependent on viral replication. There were no changes in the mRNA expression of the ISGs PKR (*EIF2AK2*) and *IRF3*, the cytokines *CCL2*, *TSLP* and *IL33* or the HRV receptors *ICAM1* and *VLDLR* in MCs in response to HRV (Supplementary **Table A.1**. & **Table A.2**.).

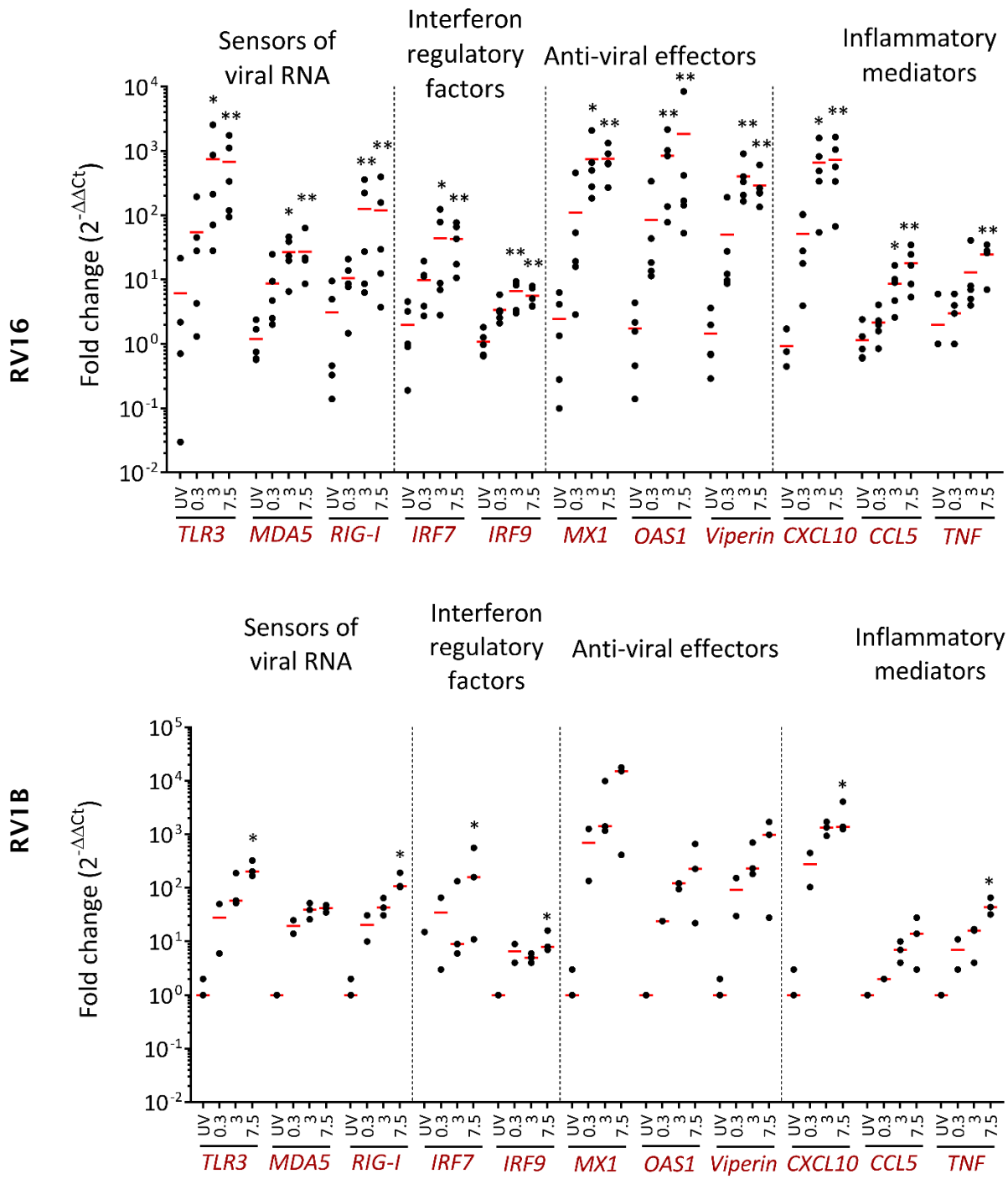

Figure 3-4. IFN stimulated gene and inflammatory mediator mRNA expression in LAD2 MCs exposed to rhinovirus. LAD2 MCs were exposed to RV16 or RV1B at an MOI of 0.3, 3 or 7.5 or UV-RV MOI 7.5 (control) and cell pellets collected 24 hours post infection. *TLR3*, MDA5 (*IFIH1*), RIGI-I (*DDX58*), *IRF7, IRF9*, *MX1*, *OAS1*, viperin (*RSAD2*), *CXCL10*, *CCL5* and *TNF* gene expression was determined by RT-qPCR. RV16: n=4 (*TLR3* and *TNF* UV-RV16) or n=5, RV1B: n=2 (MOI 0.3) or n=3. Bars at median. *$p < 0.05$, **$p < 0.01$, versus UV-HRV, one-way ANOVA (Friedman test [paired data] or Kruskal-Wallis test [non-paired data]).

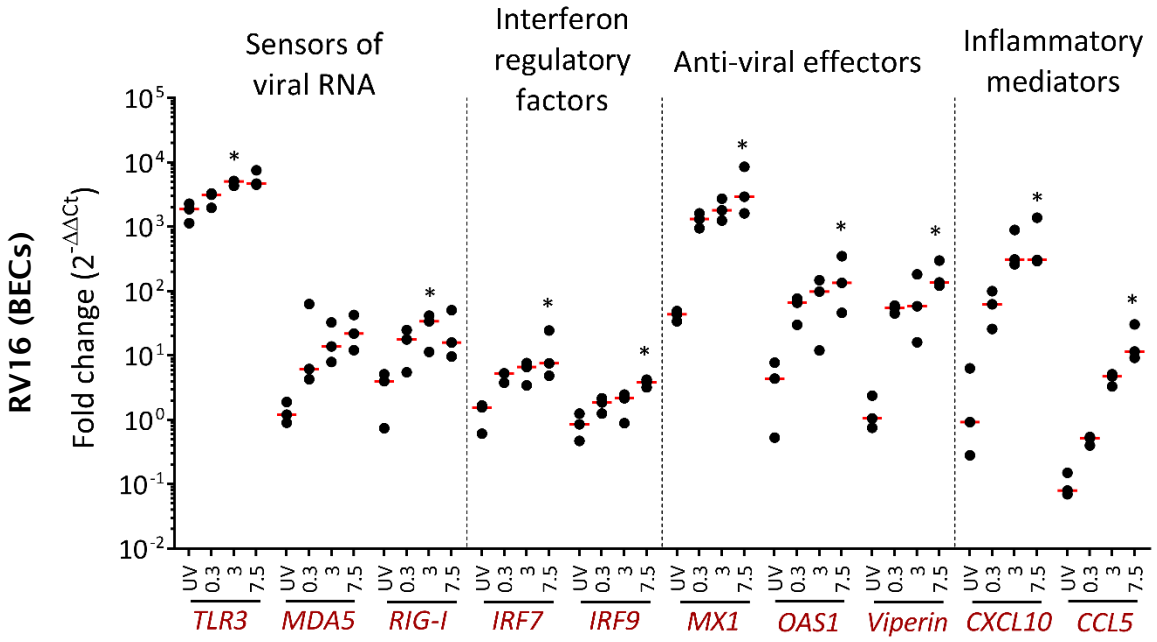

Figure 3-5 IFN stimulated gene and inflammatory mediator mRNA expression in bronchial epithelial cells exposed to RV16. BECS were exposed to RV16 at an MOI of 0.3, 3 or 7.5 or UV-RV MOI 7.5 (control) in the same experiments as LAD2 HRV infection as a positive control. Cell pellets were collected 24 hours post infection. *TLR3*, MDA5 (*IFIH1*), RIGI-I (*DDX58*), *IRF7, IRF9*, *MX1*, *OAS1*, viperin (*RSAD2*), *CXCL10*, *CCL5, TNF* gene expression were determined by RT-qPCR. n=3. Bars at median. *p < 0.05 versus UV-RV16, one-way ANOVA (Friedman test).

3.2.2 The LAD2 MC Line Is Permissive For Rhinovirus Replication and the Release of Infectious Virus Particles

The observed responses of LAD2 MCs to HRV were dependent on viral replication, therefore viral replication in MCs was assessed by RT-qPCR for the viral genome and compared to infection of BECs. HRV treatment caused a statistically significant MOI-dependent increase in RV16 and RV1B RNA compared to UV-HRV controls (**Figure 3-6 A**). This demonstration of viral replication led to an investigation into whether LAD2 MCs, like BECs, were susceptible for the release of viable virus particles. This was confirmed by $TCID_{50}$ assay which demonstrated MCs released significant levels of viable RV16 and RV1B particles, which increased with increasing MOI (**Figure 3-6 B**). The level of RV16 infection observed in LAD2 MCs was comparable to that of BECs tested in the same experiments although virus release tended to be greater in LAD2 MCS (**Figure 3-6 C**).

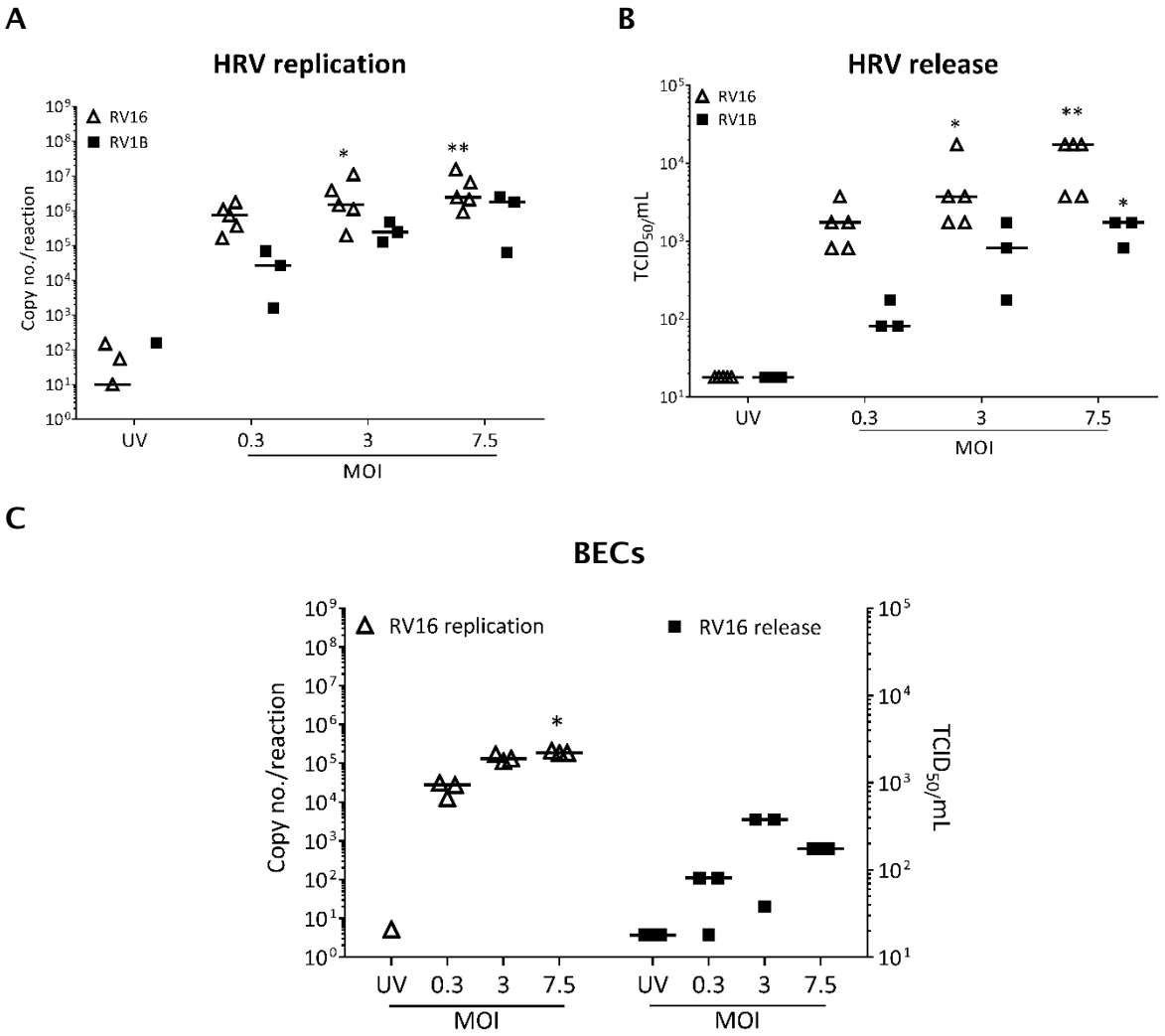

Figure 3-6. Replication and release of infectious rhinovirus particles from LAD2 MCs and bronchial epithelial cells. LAD2 MCs were exposed to RV16 or RV1B at MOI 0.3, 3 or 7.5 or UV-RV MOI 7.5 (control). Cell pellets and cell-free supernatants were collected 24 hours post infection to assay viral RNA and release of infectious virus particles by RT-qPCR and $TCID_{50}$ assay respectively. BECs were exposed to RV16 in the same experiments as a positive control. **(A)** RV16 or RV1B viral RNA in LAD2 MCs, n=5 (RV16), n=3 (RV1B). **(B)** Infectious RV16 or RV1B particles released by LAD2 MCs, n=5 (RV16), n=3 (RV1B). **(C)** RV16 RNA and release of infectious RV16 particles from BECs, n=3. Bars at median. * $p < 0.05$, ** $p < 0.01$ versus UV-HRV, one-way ANOVA (Friedman test).

3.2.3 MC Viability Is Unaffected By HRV Infection

The viability and cell number of LAD2 MCs following HRV infection was determined using the trypan blue exclusion method or a fluorescent viability due and flow cytometry. Trypan blue exclusion showed no significant differences in cell viability or cell number 24 hours post infection (**Figure 3-7**).

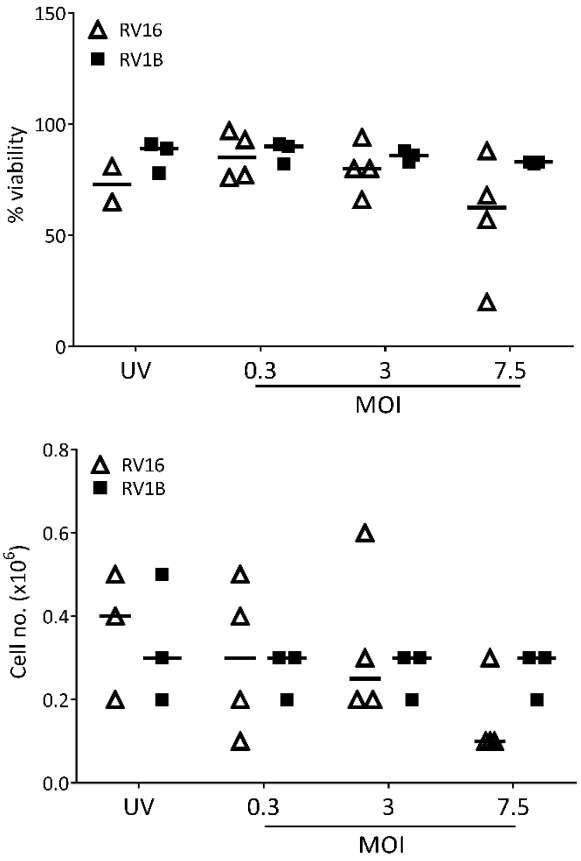

Figure 3-7. LAD2 MC viability and cell number following rhinovirus infection. LAD2 MCs were exposed to RV16 or RV1B at MOI 0.3, 3 or 7.5 or UV-HRV MOI 7.5. Cell viability and cell number were determined by trypan blue exclusion 24 hours post infection. RV16 n=2 (UV) or n=4, RV1B n=3. Bars at median.

Preliminary experiments using the fluorescent viability dye revealed no difference in the percentage of live/dead cells between UV-RV16 and RV16 infected cells and no increase in cell death over time (8 or 24 hours) (**Figure 3-8 A**) compared to a positive control of heat-killed LAD2 MCs (**Figure 3-8 B**).

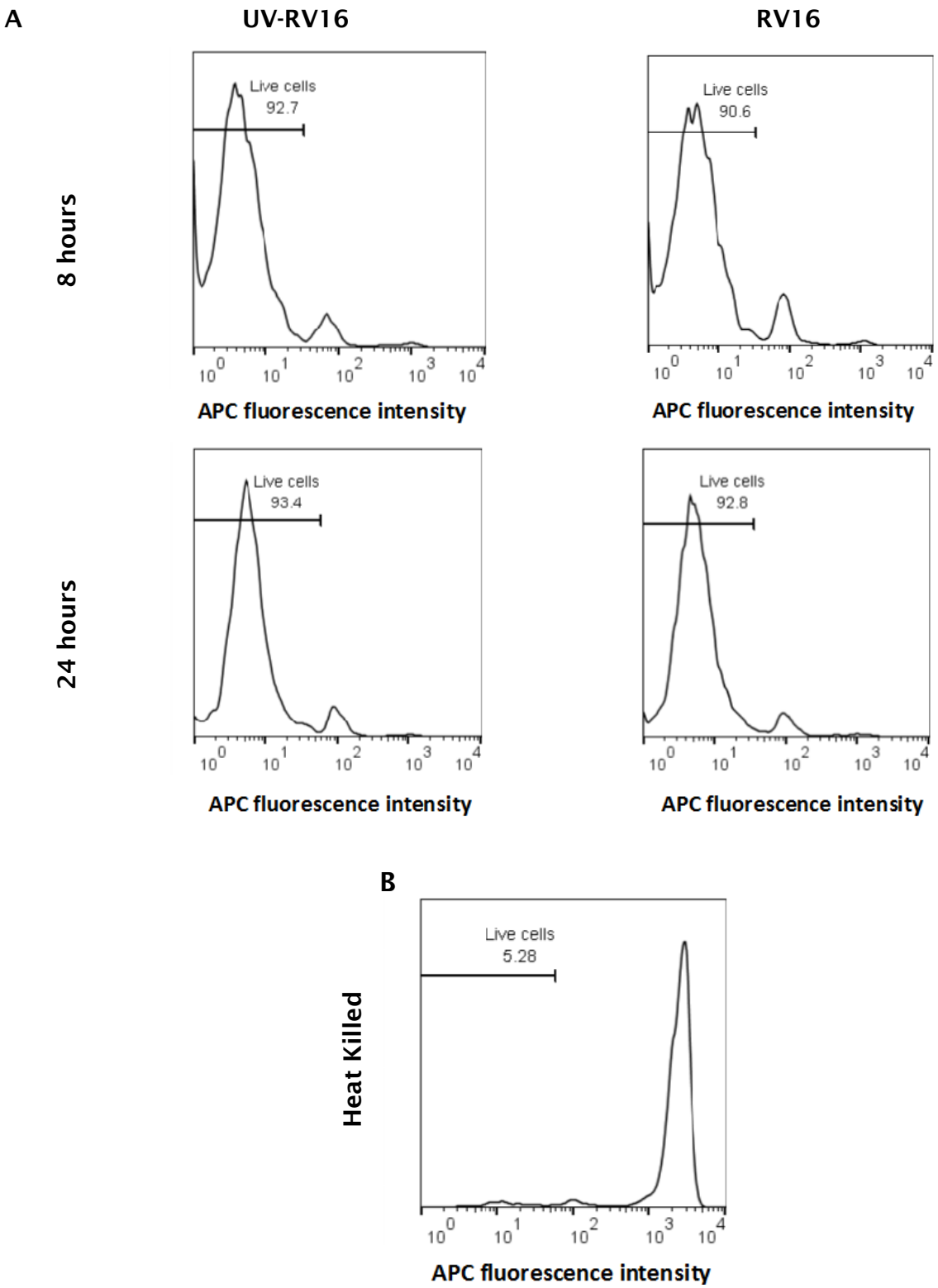

Figure 3-8. LAD2 MC viability following HRV infection. LAD2 MCs were exposed to RV16 MOI 1.2 or UV-HRV MOI 1.2 for 8 or 24 hours then stained with a fluorescent (APC) dye for dead cells and fixed (4% PFA). Fluorescence was measured by flow cytometry. **(A)** Histograms of LAD2 MCs infected with UV-RV16 or RV16 for 8 or 24 hours. **(B)** Histogram of heat killed LAD2 MCs (60˚C 10 minutes) as a positive control for cell death. Gates indicate percentage of live cells. Histograms are representative of n=2 UV-RV16/RV16 experiments and n=5 heat killed experiments.

3.2.4 Primary human MCs Mount an Innate Immune Response to RV16 Infection

Having demonstrated a range of responses of LAD2 MCs to HRV infection, the response of primary human MCs to RV16 was next investigated. CBMCs were exposed to RV16 at MOI 0.3, 3 or 7.5 or UV-RV16 MOI 7.5 for 24 hours, following which IFN responses were assessed by RT-qPCR and ELISA. CBMCs upregulated *IFNB1* and *IFNL1* transcripts which was confirmed at the protein level at the highest MOI for IFN-λ (**Figure 3-9 A**). IFN-β protein was also induced at the highest MOI but did not reach statistical significance (**Figure 3-9 A**). There was also a statistically significant upregulation of RV16 replication-dependent induction of the anti-viral ISGs *OAS1* and *MX1* (**Figure 3-9 B**). Next RV16 replication and virus release was investigated. CBMCs were also found to be permissive for the replication and release of infectious RV16 particles, confirmed by an increase in RV16 RNA and the release of infectious virus particles by RT-qPCR and $TCID_{50}$ assay respectively (**Figure 3-9 C**). As was the case with LAD2 MCs, trypan blue exclusion demonstrated CBMC viability was unaffected by RV16 infection (**Figure 3-10**).

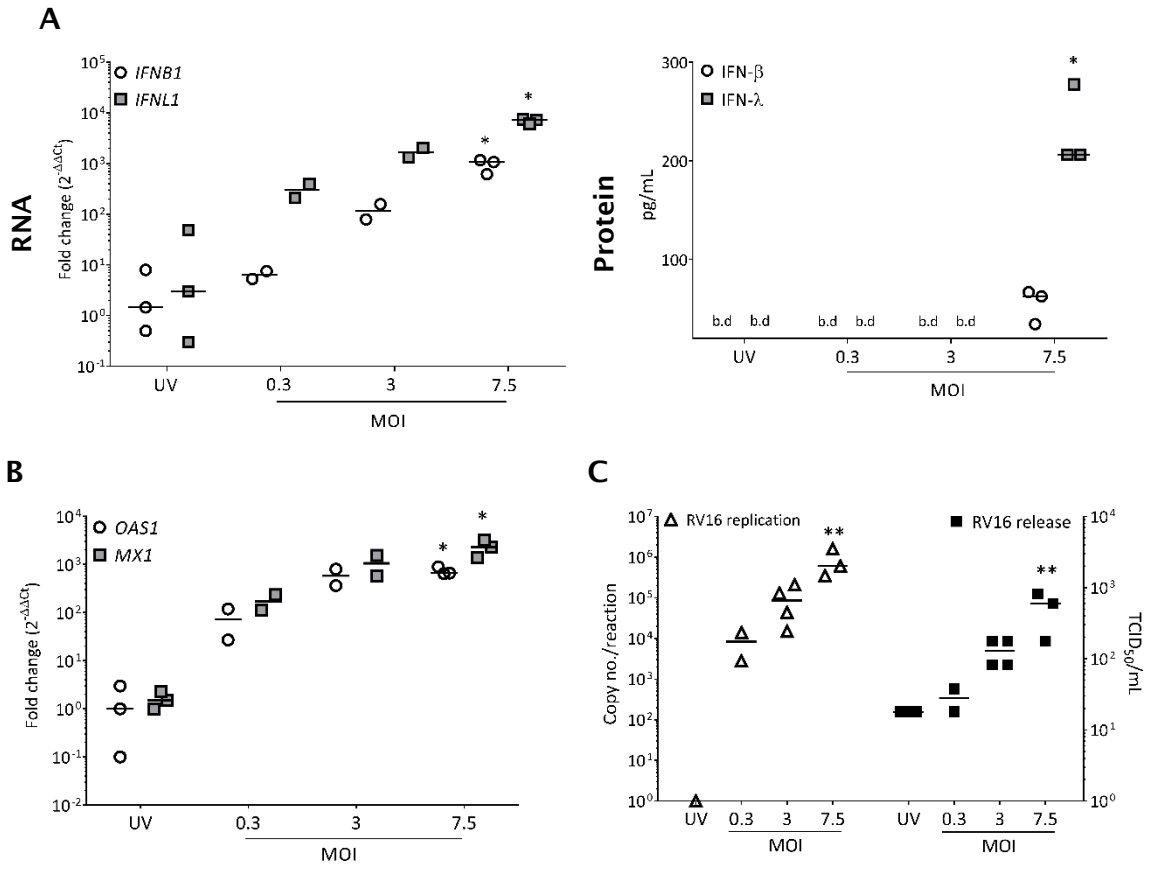

Figure 3-9. Innate immune responses and release of infectious RV16 from CBMCs. CBMCs were exposed to RV16 at MOI 0.3, 3 and 7.5 or UV-RV16 MOI 7.5 (control). Cell pellets and cell-free supernatants were collected 24 hours post infection to assay mRNA (RT-qPCR), protein (ELISA) and infectious virus particles ($TCID_{50}$ assay). **(A)** IFN-β (IFNB1) and IFN-λ (IFNL1) RNA and protein expression, n=2 (MOI 0.3, 3), n=3 (UV, MOI 7.5). **(B)** *OAS1* and *MX1* mRNA, n=2 (MOI 0.3, 3), n=3 (UV, MOI 7.5). Cell-free supernatants were concentrated (4x) for ELISA. **(C)** RV16 RNA and release of infectious RV16 particles, n=2 (MOI 0.3), n=3 (MOI 7.5), n=4 (UV, MOI 3). Bars at median. * $p < 0.05$, ** $p < 0.01$. **(A, B)** UV-RV16 vs MOI 7.5 Mann-Whitney test, **(C)** one-way ANOVA (Kruskal-Wallis test).

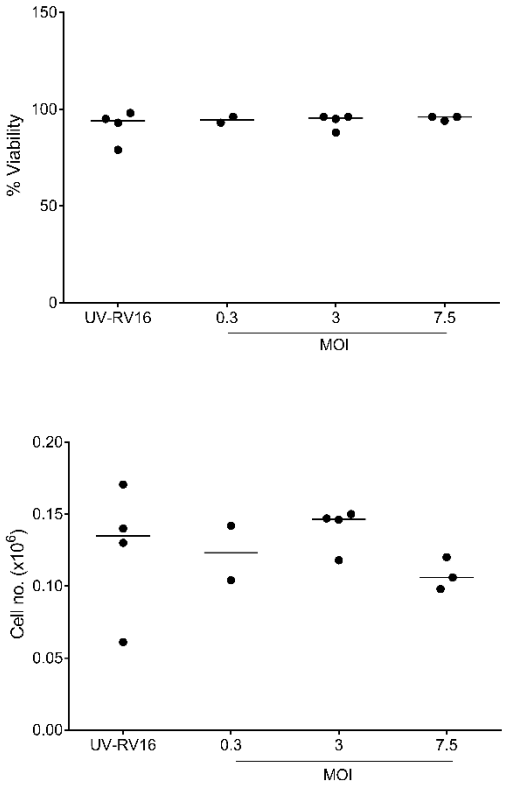

Figure 3-10. CBMC viability and cell number following RV16 infection. CBMCs were exposed to RV16 at MOI 0.3, 3 or 7.5 or UV-RV16 MOI 7.5. Cell viability and cell numer were determined by trypan blue exclusion 24 hours post infection. n=2 (MOI 0.3), n=3 (MOI 7.5), n=4 (UV, MOI 3). Bars at median.

3.3 Discussion

HRV infection is the major trigger of asthma exacerbations[142, 143] which are a significant source of asthma morbidity via mechanisms that are not fully understood. The role of MCs in viral immunity is unclear, however, MCs increase in numbers at the asthmatic bronchial epithelium following HRV infection[217] and with increasing asthma severity[207, 210] suggesting a potential role of MCs in HRV-induced exacerbations. In this investigation, infection of human MCs with either a major or minor group HRV triggered responses including the upregulation of both type I and type III IFNs and ISGs.

In response to HRV infection, MCs were observed to upregulate immune responses including: type I and type III IFNs, IFN-β and IFN-λ respectively; PRRs (e.g. *MDA5*), ISGs (e.g. *OAS1*) and chemokines (e.g. *CCL5*). The production of these mediators of innate anti-viral responses, which increased with HRV infection, aptly demonstrates the role of MCs in pathogen recognition and innate immunity. The production of type I IFNs, to which MCs can also respond, has also been observed by MCs in response to dengue virus[34], IAV[9] and RSV[9]. Type III IFN, which is generated following viral infection by many different cell types[151, 233, 234], has been shown to be expressed and released by MCs in response to infection with sendai virus[33] as well as reovirus[94]. Although MCs do not express the type III IFN receptor[33] the secretion of IFN-λ may help protect epithelial cells, which do express IFN-λ receptors[235], from viral infection. ISGs are activated via IFN signalling but IFN-independent ISG activation has also been demonstrated to occur, for instance via TLR activation[27]. MCs express TLR3, which in response to viral dsRNA (including replication intermediates of ssRNA viruses[236]) and poly I:C can activate MC anti-viral responses[9]. Therefore, ISGs may be induced by IFNs as well as the activation of TLRs during HRV infection. MCs respond to viruses including dengue virus, reovirus and RSV with the release of cytokines and chemokines which are speculated or have been demonstrated to recruit inflammatory cells thought to aid in the clearance of infection[92, 93, 237]. For instance, reovirus induces CXCL8 release from MCs which recruits NK cells[237] and CCL3, CCL4 and CCL5 which recruit a subset of T cells[238] *in vitro*. MCs are also responsible for the recruitment of NK and NKT cells *in vivo* during dengue virus infection of mice [91]. Therefore MCs may drive the recruitment of effector cells including T cells, NK cells and DCs via chemokines including CXCL10 and CCL5 released in response to HRV infection. However, an important

consideration is the role these cells may play. Recruitment of inflammatory cells may aid in viral clearance, however, an influx of inflammatory cells could also contribute to asthma pathology. Which scenario MC cytokines may contribute to requires further investigation.

Despite mounting anti-viral responses to HRV infection, both LAD2 MCs and CBMCs were permissive for infection with HRV with increases in HRV replication and the release of viable virus particles with increasing MOI. The demonstration of HRV infection was in agreement with Hosoda *et al*. who demonstrated the permissiveness of an immature MC line (HMC-1) for the replication and release of a major group virus (RV14)[96]. Viral infection of MCs resulting in the release of viable virus particles is in contrast to infection of MCs by other viruses including reovirus, RSV and IAV. These viruses have been demonstrated to infect human MCs and induce innate immune responses, however, this occurs with little or no release of virus progeny determined for example via plaque assays of infected cell supernatants[93, 237, 239]. While CBMCs are susceptible for the replication of dengue virus with the release of infectious virus particles, this process is antibody-mediated i.e. requires sub-neutralising concentrations of dengue immune sera to enhance infection[92, 240].

The HRV-C species is more often associated with asthma exacerbations and acute respiratory illness in hospitalised children and is a cause of more severe exacerbations than HRV-A or HRV-B[241 242]. In addition, a CDHR3 polymorphism has been associated with an increased risk of severe childhood asthma[156], however, in this investigation MCs have been demonstrated not to express CDHR3. The lack of MC CDHR3 expression could be due to the LAD2 cell line or the immaturity of the CBMCs. To investigate this, CDHR3 expression may be dertermined in CBMCs matured via IL-4, IgE or serum treatment or in peripheral blood-derived MCs, which are more mature than CBMCs. However, only fully differentiated cultures of human airway epithelial cells have been shown to express CDHR3[243, 244] (compared to undifferentiated monolayers and cell lines) and CDHR3 expression appears to be limited to the bronchus and nasopharynx (protein) and fallopian tubes (RNA)[245]. Therefore, MCs may be unlikely to express CDHR3 or be susceptible for HRV-C infection. Regardless of CDHR3 expression MCs are susceptible for HRV-A (major and minor group HRVs) infection which are also an important trigger of HRV-induced asthma exacerbations in adults and children[246, 247].

HRV release from epithelial cells typically occurs via cell lysis[153] but in this investigation HRV shedding from either LAD2 MCs or primary cells was not associated with significant cell death. Non-lytic viral exit has been reported for other picornaviruses such as poliovirus[248, 249] and may be the mechanism by which viable HRV is released from MCs[250]. However, it is also possible that the infection and lysis of a small number of cells was responsible for the observed HRV release. To investigate this in a different model, Bird *et al.*[248] employed single-cell analysis and time-lapse microscopy. Briefly, the hepatocyte cell line Huh7-A-1 was sparsely infected with a poliovirus variant expressing a fluorescent protein allowing the visualisation of virus spread as the fluorescent protein accumulated during viral translation. During non-lytic virus spread, poliovirus translation was detected in neighbouring cells without lysis of the donor cell and this process was shown to involve the autophagy pathway. Aggregation of fluorescently tagged LC3, which becomes membrane-associated with the initiation of autophagy, preceded viral replication and viral spread and stimulation of autophagy enhanced poliovirus spread[248]. These techniques may be applied to HRV infection of MCs which would require the generation of a HRV variant expressing a fluorescent protein as well as a MC line expressing a fluorescently tagged autophagy associated protein.

HRV replication and release increased with increasing MOI demonstrating MCs were susceptible for HRV replication. However, this was determined at a single time-point. Infection of MCs at a single MOI and determination of HRV copy number and virus release over time would provide a clearer indication of HRV replication in MCs. An increase in HRV copy number and virus release over time would indicate virus replication. MC degranulation was not observed following HRV infection which is in accordance with the general response of MCs to microbial infection. Although MC degranulation has been reported in some instances of dengue virus infection[91], during bacterial as well as viral infections[9, 33, 35, 92] MCs are able to release mediators including cytokines and chemokines in the absence of degranulation[9, 78, 81, 106,251].

The localisation of MCs to the bronchial epithelium during asthma means MCs may come into contact with HRV during infection of this principal site of HRV replication. The permissiveness of MCs for productive HRV infection suggests they are likely to become infected if contact with HRV is made and go on to release viable HRV particles. Importantly, the lack of significant cell death suggests MCs may act as viral reservoirs shedding HRV and contributing to HRV-induced asthma exacerbations. This may be particularly relevant in HRV-induced asthma

exacerbations of individuals with severe asthma where MCs[207] and their mediators[209] are both increased.

3.3.1 Conclusion

The susceptibility of MCs for the release of infectious HRV particles, despite anti-viral immune responses, suggests MCs may have a detrimental role in HRV-induced asthma exacerbations.

Chapter 4: The Protective Effect of IFN-β Against HRV Infection of Mast Cells

4.1 Introduction

IFN responses are key to effective anti-viral immune responses as demonstrated in IFN receptor knockout mice which, have increased susceptibility to viral infection and impaired induction of anti-viral responses[169, 252]. Diminished anti-viral IFN responses have been demonstrated in asthma and may explain the increased frequency and severity of lower respiratory tract infections[253]. IFN-β production from asthmatic BECs is reduced following HRV infection compared to non-asthmatic controls[150], furthermore, IFN deficiency is associated with diminished HRV-induced apoptosis and increased virus titres. Other studies have failed to detect impaired IFN production from asthmatic BECs[254, 255] but evidence suggests that IFN deficiency may be most evident in severe asthma. For instance, the impaired induction of IFN-λ and IFN-β from asthmatic BECs following HRV infection has been demonstrated in both adults[151, 256] and children[257, 258] and is associated with increased asthma severity. Whereas in mild well controlled asthma IFN-β and IFN-λ release in response to HRV is not impaired compared to non-asthmatics[259]. Impaired asthmatic IFN responses are not limited to BECs. Type I and type III IFN deficiencies are also evident in asthmatic bronchoalveolar lavage (BAL) macrophages[151, 260] and peripheral blood mononuclear cells[256]. In further support of the role of impaired IFN responses in HRV infection, exogenous IFN treatment has been effective at reducing the increased HRV replication seen in asthmatic cells[150, 261, 262]. Furthermore, inhaled IFN-β has been shown to be particularly effective in moderating viral exacerbations in difficult-to-treat asthmatics[263]. The permissiveness of MCs for productive HRV infection demonstrated in chapter 3 suggests that endogenous IFN responses may be insufficient to protect MCs against HRV infection. Therefore, exogenous IFN may also be effective at enhancing MC anti-viral responses, such as the induction of ISGs, which protect MCs against HRV infection.

4.1.1 Hypothesis

Endogenous MC IFN responses are insufficient to protect against HRV infection. Treatment with exogenous IFN will potentially protect MCs against HRV replication and the release of infectious virus particles by the induction of IFN-stimulated genes.

4.1.2 Aims

1. Investigate the potential protective effect of exogenous IFN against HRV infection of MCs.
2. Investigate the impact of endogenous type I IFN on HRV infection of MCs.

4.1.3 Objectives

1. Treat LAD2 MCs and CBMCs with exogenous IFN-β, IFN-γ or IFN-λ in the presence and absence of RV16 infection and determine ISG induction and RV16 replication by RT-qPCR and the release of infectious RV16 particles by $TCID_{50}$ assay.
2. Treat CBMCs with a type I IFN blocking antibody and determine IFN-dependent responses, such as ISG induction, and RV16 replication by RT-qPCR and the release of infectious RV16 particles by $TCID_{50}$ assay.

4.2 Results

4.2.1 Time Course of LAD2 MC IFN-Mediated Immune Responses

In preliminary experiments, LAD2 MCs were treated with 1, 10 or 100 IU/mL IFN-β, IFN-γ or IFN-λ over 4, 8 or 24 hours and the induction of anti-viral immune responses was determined by RT-qPCR. These responses included the induction of IFNs, PRRs, anti-inflammatory cytokines and anti-viral effectors which were greatest with 100 IU/mL of IFN (**Figure 4-1**). IFN-β treatment resulted in the greatest inductions at each time-point, followed by IFN-γ then IFN-λ and overall inductions were greatest at 4 hours. MCs are not know to express the IFN-λ receptor, however, at 4 hours, and more so at 8 hours, there were inductions in certain genes including *IFNL1*. The inductions may be due to factors such as contamination from IFN-β or IFN-γ but as the experiment has an n=1 further experimental replicates are required to determine this. From these experiments it was determined that cells would be treated with 100 IU/mL of IFN-β, IFN-γ or IFN-λ for 24 hours to observe changes in gene expression as well as protein production.

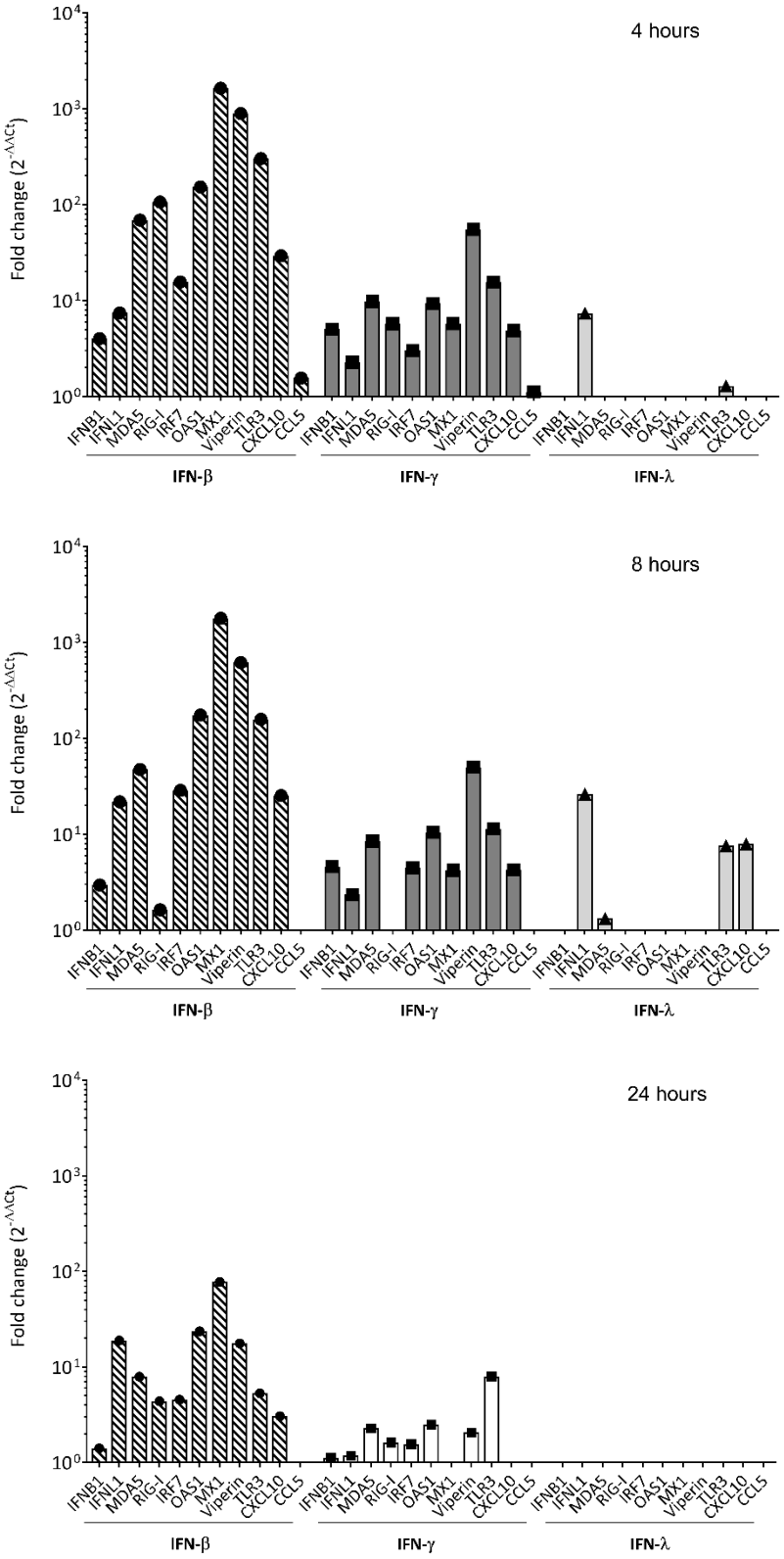

Figure 4-1. Type I, type II and type III IFN-induced responses of LAD2 MCs. LAD2 MCs were treated with IFN-β, IFN-γ or IFN-λ (100 IU/mL each) for 4, 8 or 24 hours. Gene induction was analysed by RT-qPCR for the following genes: *IFNB1*, *IFNL1*, MDA5 (*IFIH1*), RIG-I (*DDX58*), *IRF7*, *OAS1*, *MX1*, Viperin (*RSAD2*), *TLR3*, *CXCL10* and *CCL5*. n=1.

4.2.2 The Impact of IFN stimulation on LAD2 MC Responses to RV16 Infection

LAD2 MCs were treated with type I, II or III IFNs for 24 hours prior to RV16 infection and the induction of the ISGs *MDA5*, *MX1* and *OAS1* was determined by RT-qPCR. In the absence of replicating virus (vehicle control and UV-RV16) IFN-β induced significant increases in ISG expression, which was not observed with IFN-γ or IFN-λ treatment (**Figure 4-2**). During RV16 infection, IFN-β pre-treatment did not further enhance ISG responses whereas IFN-γ pre-treatment caused a significant increase in RV16-induced *MDA5* and trends for increased *MX1* and *OAS1* expression (**Figure 4-2**). Cells were unresponsive to IFN-λ and although one replicate was more responsive.

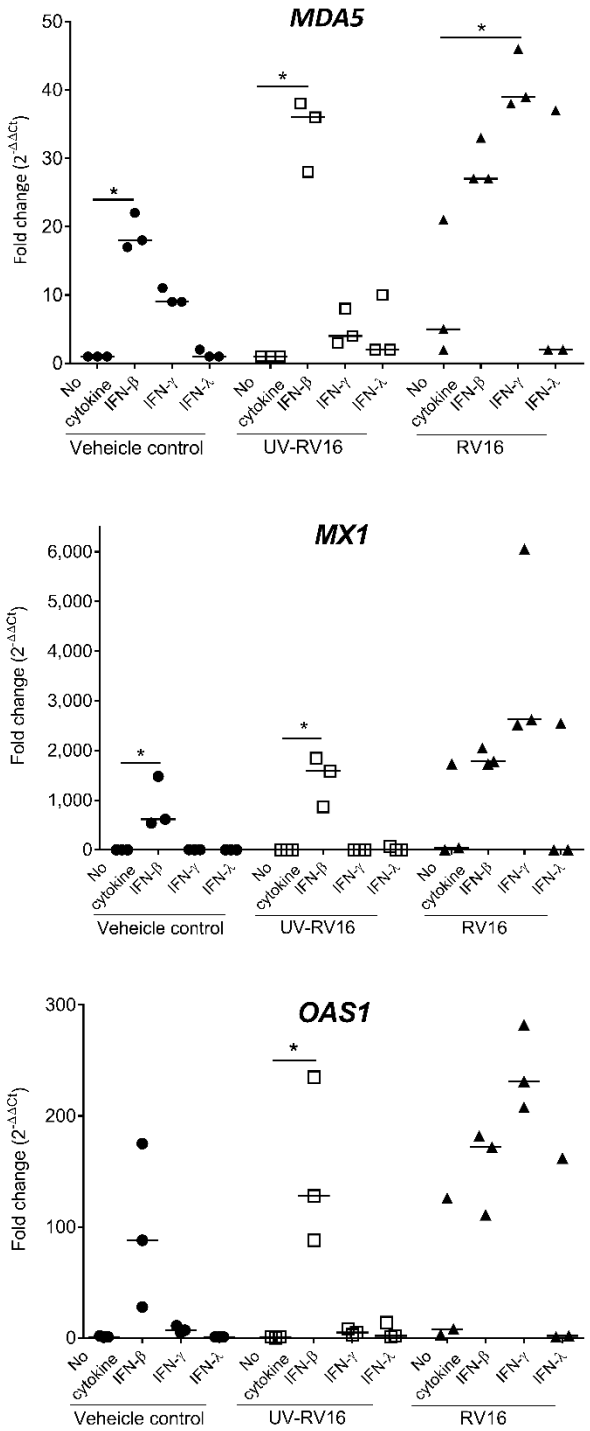

Figure 4-2. The induction of anti-viral genes in response to IFN pre-treatment and RV16 infection of LAD2 MCs. LAD2 MCs were treated with IFN-β, IFN-γ or IFN-λ (100 IU/mL) for 24 hours prior to RV16 or UV-RV16 infection (MOI 7.5). Cell pellets were collected for expression of MDA5 (*IFIH1*), *MX1* and *OAS1* by RT-qPCR 24 hours post infection. n=3. Bars at median, * $p \leq 0.05$, one-way ANOVA (Friedman test).

The effect of IFNs on RV16 replication was next investigated. IFN-β pre-treatment resulted in a statistically significant decrease in RV16 copy number and a trend for suppressed virus release (**Figure 4-3**) demonstrating that IFN-β-induced immune responses were protective against RV16 infection. Pre-treatment with IFN-γ and IFN-λ showed trends for decreased viral replication, however, this was not statistically significant and neither IFN suppressed the release of infectious virus particles (**Figure 4-3**).

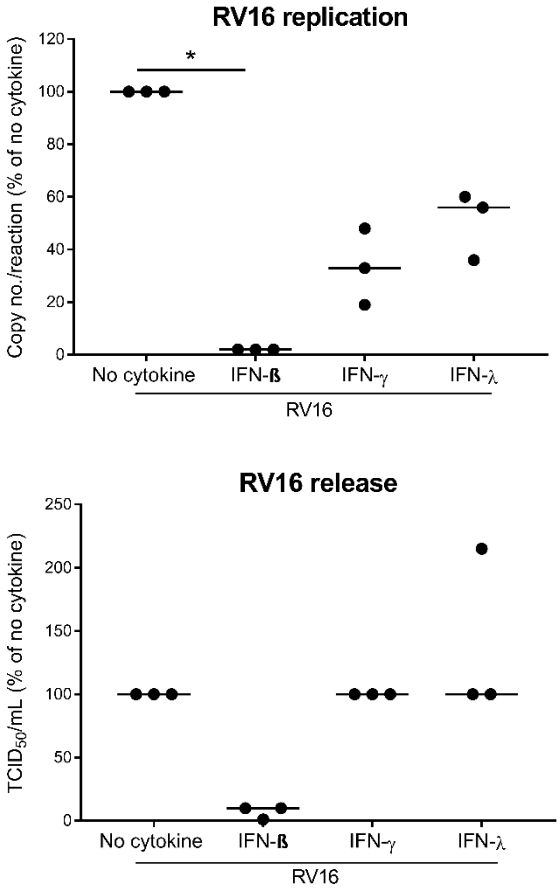

Figure 4-3. The impact of IFN pre-treatment on RV16 replication and release from LAD2 MCs. LAD2 MCs were pre-treated with IFN-β, IFN-γ or IFN-λ (100 IU/mL each) for 24 hours prior to RV16 or UV-RV16 infection (MOI 7.5). Cell pellets and cell-free supernatants were collected 24 hours post infection. Viral RNA was assayed in cell pellets by RT-qPCR and infectious virus particles determined in cell free supernatants by $TCID_{50}$ assay. Results are a percentage of no cytokine control. n=3. Bars at median, * $p \leq 0.05$, one-way ANOVA (Friedman test).

4.2.3 The Impact of Exogenous IFN on RV16 Infection of CBMCs

Having demonstrated the protective effect of IFN treatment against RV16 infection of LAD2 MCs, CBMCs were similarly treated with type I, II or III IFNs and anti-viral responses determined. CBMCs were treated with 100 IU/mL IFN-β, IFN-γ or IFN-λ at the point of RV16 infection (MOI 7.5). Cells responded to IFN-β with statistically significantly increased expression of *IFNB1* and *IFNL1* mRNA above that observed with RV16 alone (**Figure 4-4 A**). However, there was no induction of the ISGs *MX1* or *OSA1* (**Figure 4-4 B**). The effect of IFN-γ appeared to be minimal suggesting IFN-β was the principal IFN driving the observed type I and type III IFN responses. This was supported by the upregulation of IFN-λ protein by IFN-β to levels above that with RV16 alone (**Figure 4-4 C**).

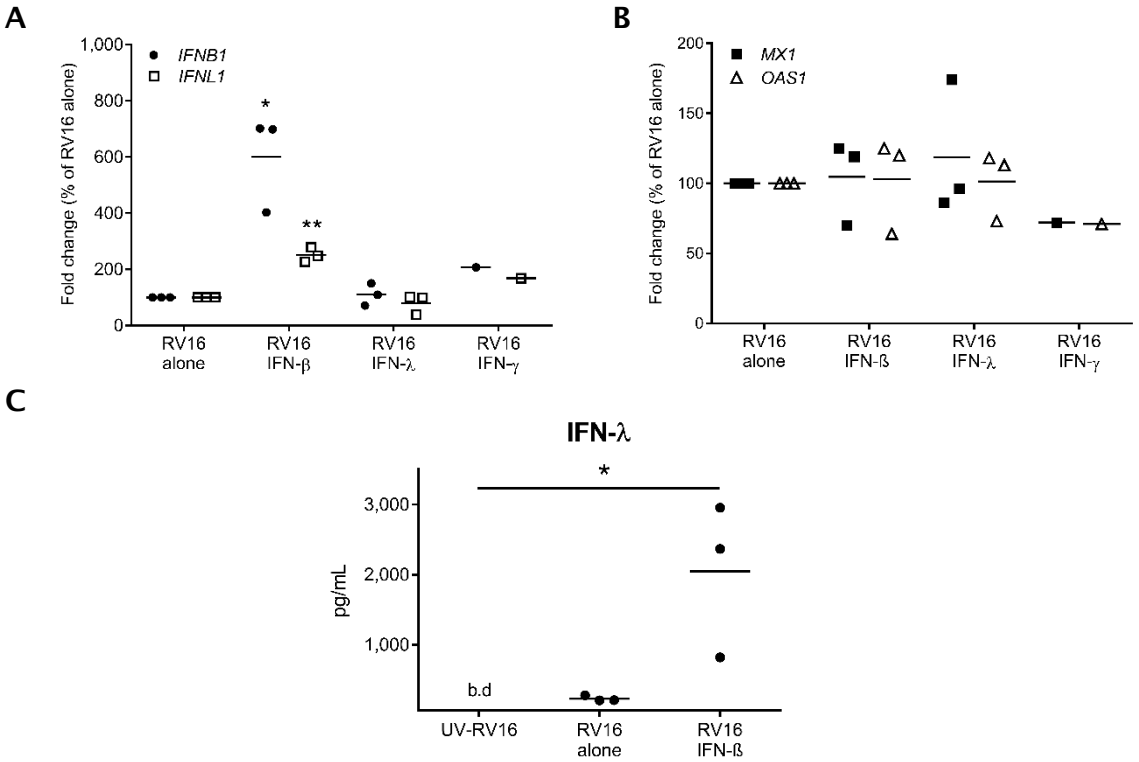

Figure 4-4. The impact of exogenous IFN treatment on the anti-viral responses of CBMCs to RV16 infection. CBMCs were treated with or without IFN-β, IFN-γ or IFN-λ (100 IU/mL) at the time of RV16 infection (MOI 7.5). Cell pellets and cell-free supernatants were collected 24 hours post infection for gene and protein expression which were assayed by RT-qPCR and ELISA respectively. **(A)** *IFNB1* and *IFNL1* mRNA induction. **(B)** *MX1* and *OAS1* mRNA induction. **(C)** IFN-λ protein expression. n=3 or n=1 (RV16/IFN-γ). Bars at mean. **(A)** and **(B)** are a percentage of RV16 alone. * $p \leq 0.05$, ** $p \leq 0.01$ versus RV16 alone, paired *t*-test.

The effect of IFNs was next determined on viral replication. Exogenous IFN-β, but not IFN-λ, protected cells against RV16 infection by significantly suppressing RV16 copy number and the release of infectious RV16 particles (**Figure 4-5**). IFN-γ also appears to have protected cells against RV16 replication and release (**Figure 4-5**), however, this was a preliminary experiment (n=1) and requires further experimental replicates.

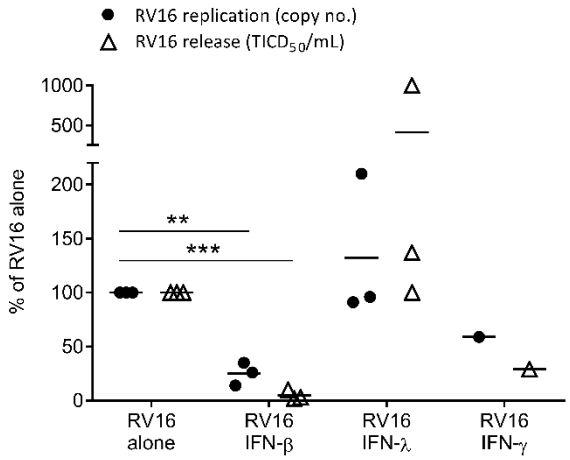

Figure 4-5. RV16 replication and release from CBMCs following exogenous IFN treatment during infection. CBMCs were treated with IFN-β, IFN-γ or IFN-λ (100 IU/mL) at the point of RV16 infection (MOI 7.5). Cell pellets and cell-free supernatants were collected 24 hours post infection for viral RNA (copy number) and RV16 release, which were assayed by RT-qPCR and $TCID_{50}$ assay respectively. n=3 or n=1 (RV16/IFN-γ). Results are a percentage of RV16 alone. Bars at mean. ** $p \leq 0.01$ versus RV16, *** $p \leq 0.001$ versus RV16 alone, paired *t*-test.

CBMCs were treated with RV16 plus IFN-β in the presence of a type I IFN blocking antibody, anti-IFNAR2, or isotype control. Prior to this, the concentration of anti-IFNAR2 was optimised in LAD2 MCs. LAD2 MCs were pre-treated for 1 hour with 1 or 2 μg/mL anti-IFNAR2 or an IgG_{2a} isotype control followed by RV16 infection (MOI 1.2) in the presence of IFN-β (100 IU/mL). Exogenous IFN-β-mediated suppression of RV16 replication was blocked by 1 μg/mL anti-IFNAR2, which increased RV16 copy number from 8.2×10^3 (IgG_{2a} control) to 1.3×10^6 (anti-IFNAR2) copies/reaction, similar to infection with RV16 alone (1.1×10^6 copies/reaction) (**Figure 4-6 A**).

However, 2 μg/mL anti-IFNAR2 appeared not to block the exogenous IFN-β-mediated suppression in HRV replication (**Figure 4-6 A**). Although anti-IFNAR2 increased infectious virus release, neither antibody concentration reversed the suppression of IFN-β to that of infection with RV16 alone (**Figure 4-6 B**). Transcription of the ISG *MX1* was effectively blocked by both concentrations of antibody but at the higher concentration of 2 μg/mL the isotype antibody also appeared to have a suppressive effect (**Figure 4-6 C**). Furthermore, 1 μg/mL of anti-IFNAR2 (150 kDa) was equivalent to 6.7 nM and well in excess of 100 IU/mL of IFN-β (22 kDa) equivalent to 22.7 pM. Therefore, from these data a concentration of 1 μg/mL of anti-IFNAR2 was used in subsequent experiments.

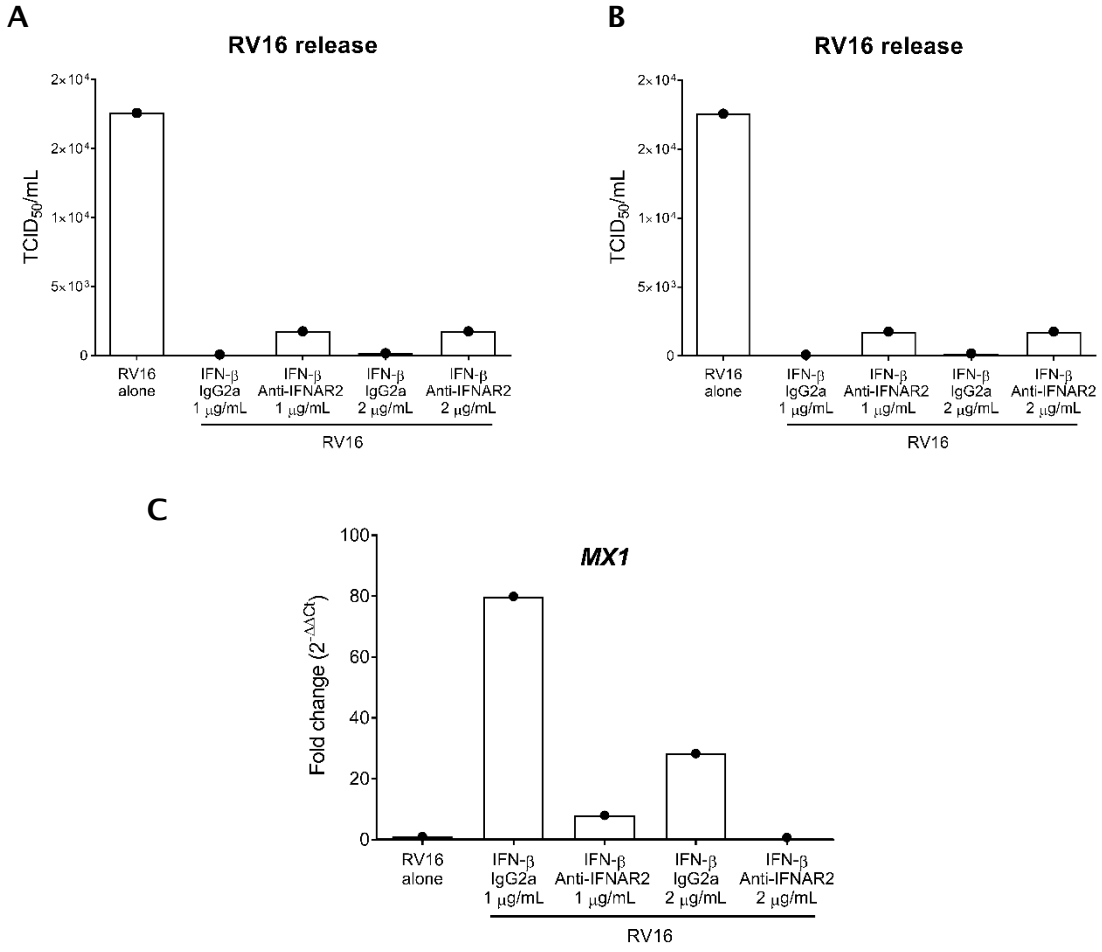

Figure 4-6. Anti-IFNAR2 blockade of exogenous IFN-β-mediated response of LAD2 MCs to RV16 infection. LAD2 MCs were pre-treated for 1 hour with 1 or 2 μg/mL anti-IFNAR2, IgG_{2a} isotype control or un-treated prior to RV16 infection (MOI 1.2). Cell pellets and cell-free supernatants were collected 24 hours post infection for mRNA (RT-qPCR) and infectious virus particles ($TCID_{50}$ assay). **(A)** RV16 copy number. **(B)** Release of infectious RV16 particles. **(C)** *MX1* mRNA induction. n=1. Anti-IFNAR2, anti-IFN-α/β receptor 2 antibody.

CBMCs treated with RV16 plus IFN-β upregulated *IFNB1* and *IFNL1* compared to RV16 alone and this was associated with a suppression in RV16 replication and release (**Figure 4-7**). In the presence of anti-IFNAR2 the induction of IFNs and suppression of viral infection mediated by IFN-β was reversed (**Figure 4-7**), which confirms the antibody effectively inhibited type I IFN signalling.

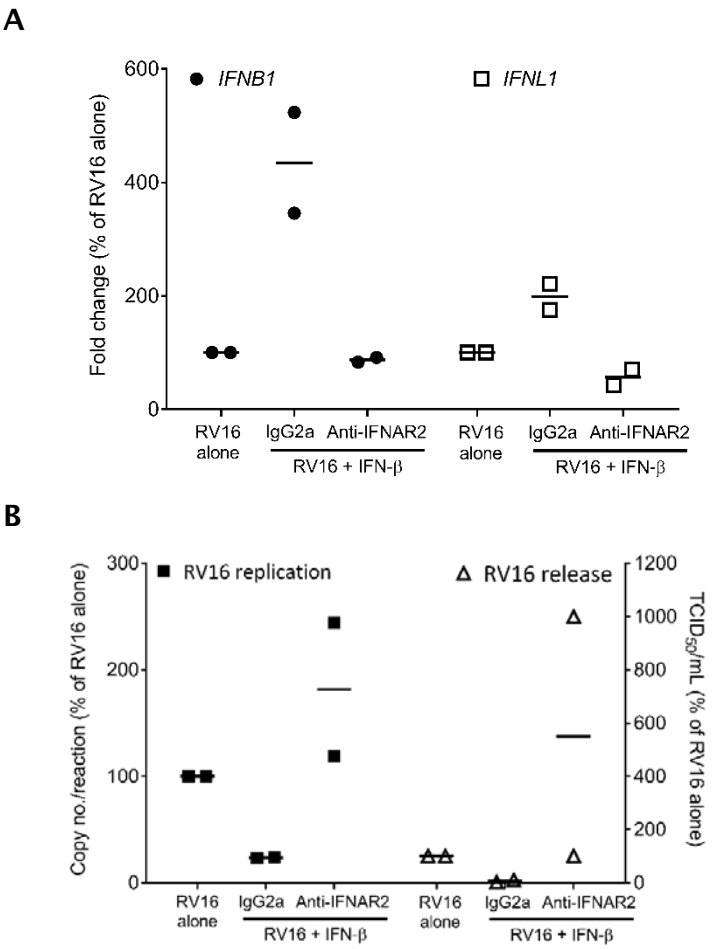

Figure 4-7. Type I IFN receptor blockade during RV16 infection. CBMCs were treated with 100 IU/mL IFN-β plus 1 hour pre-treatment with anti-IFNAR2 antibody or IgG_{2a} isotype antibody (1 μg/mL each) prior to RV16 infection. Cell pellets and cell free supernatants were collected 24 hours post infection for gene expression and RV16 release, which were assayed by RT-qPCR and TCID50 assay respectively. **(A)** *IFNB1* and *IFNL1* mRNA induction. **(B)** RV16 RNA (copy number) and release of infectious virus particles. Percentage of RV16 alone, bars at median, n=2.

4.2.4 The Impact of Blocking Endogenous IFN on CBMC Responses to RV16 Infection

As shown in chapter 3, despite the induction of anti-viral responses against HRV infection, MCs were permissive for the release of infectious HRV particles. As IFNs are critical for effective anti-viral responses, MC HRV replication and release could be explained by low levels of endogenous IFN-β. To test the role of endogenous type I IFN mediated protection during RV16 infection, CBMCs were treated with anti-IFNAR2 or IgG_{2a} isotype control prior to RV16 infection. Anti-IFNAR2 treatment resulted in a significant reduction in RV16-dependent expression of both IFN (*IFNB1* and *IFNL1*) and ISG (*MX1* and *OAS1*) induction compared to RV16 infection alone (**Figure 4-8 A-B**). While anti-IFNAR2 treatment also increased viral replication and the release of infectious virus particles, the trend was not statistically significant (**Figure 4-8 C**). These data suggest that there is a minimal protective effect of endogenous type I IFN against HRV replication in CBMCs.

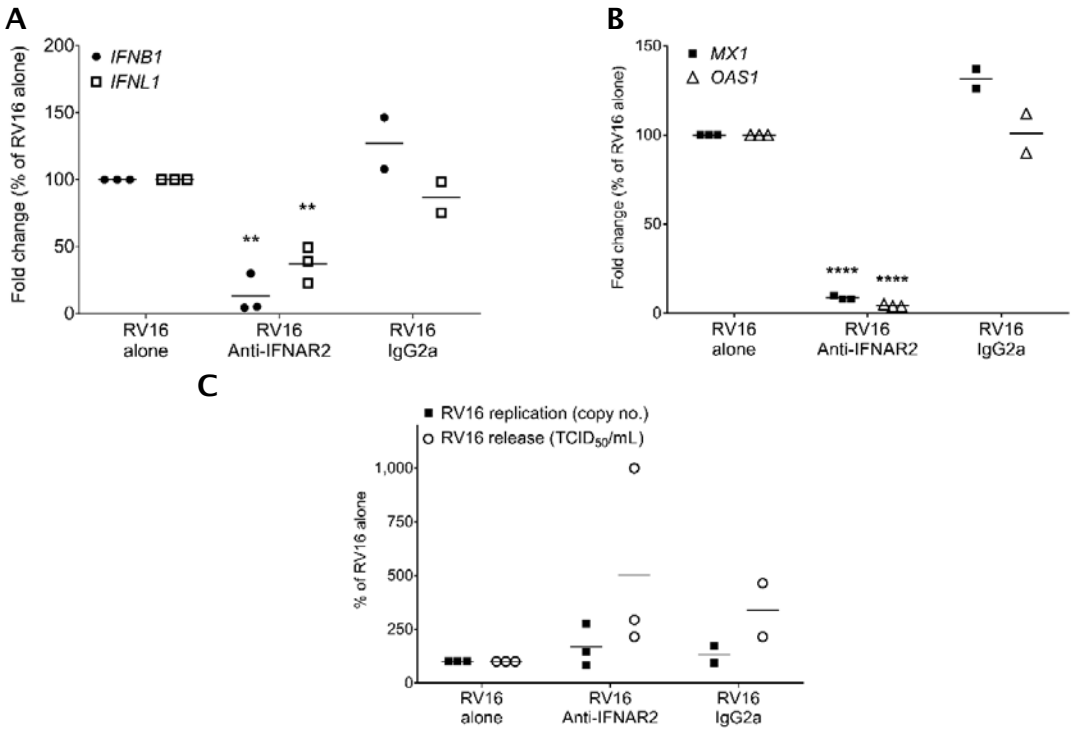

Figure 4-8. CBMC type I IFN receptor blockade during RV16 infection. CBMCs were treated with anti-IFNAR2 or IgG2a isotype (1 μg/mL) 1 hour prior to RV16 or UV-RV16 infection (MOI 7.5). Twenty-four hours post infection cell pellets were collected for mRNA (RT-qPCR) and cell free supernatants were collected for infectious RV16 particles ($TCID_{50}$ assay). **(A)** *IFNB1* and *IFNL1* mRNA induction. **(B)** *MX1* and *OAS1* mRNA induction. **(C)** RV16 RNA and release of infectious particles. Results are a percentage of RV16 alone. Mean ± SD. n=3 (RV16 alone, anti-IFNAR2), n=2 (IgG_{2a}). $^{**}p < 0.01$, $^{***}\ p \leq 0.001$ versus RV16 alone, paired *t*-test.

4.3 Discussion

Effective anti-viral responses require type I and type III IFNs but despite upregulating both IFN types in response to HRV infection, MCs were permissive for viral replication and the production of infectious virus particles. Exogenous IFN-β, but not IFN-λ, protected MCs against HRV infection, preventing the release of infectious virus particles. Together this implied that the endogenous immune response was insufficient to limit HRV replication, and consistent with this, blockade of endogenous type I IFN only minimally increased RV16 replication and release.

LAD2 MCs were treated with IFN-β, IFN-γ or IFN-λ for 4, 8 or 24 hours or pre-treated with IFN for 24 hours followed by UV-RV16 or RV16 infection. In the absence of replicating virus IFN-β, followed by IFN-γ, caused the greatest inductions in ISGs. In CBMCs IFN-β treatment upregulated *IFNB1* and *IFNL1* mRNA expression which was confirmed at the protein level for IFN-λ. IFN-β primed MCs against HRV infection by upregulating ISGs prior to infection whereas IFN-γ pre-treatment appeared to prime cells via a mechanism whereby ISGs were only significantly induced in the presence of viral replication. In fact, ISG induction by LAD2 MCs during RV16 infection was greatest following IFN-γ pre-treatment. The requirement of a dual signal of IFN-γ and replicating virus may be a mechanism of controlling MC inflammatory responses, ensuring MC responses are only triggered by IFN-β as well as IFN-γ during active viral replication. IFN-γ-mediated MC immunity has also been demonstrated during *S. aureus* infection where IFN-γ was demonstrated to enhance bacterial killing, via the generation of reactive oxygen species, and the secretion of the pro-inflammatory cytokines CXCL8 and GM-CSF[264].

MCs express type I and type II IFN receptors but are not known to express the type III receptor[33] whose expression is mainly limited to cells of epithelial origin as well as DCs[265]. Despite this, there was an upregulation in *IFNL1*, *TLR3* and *CXCL10* gene expression in LAD2 MCs treated with IFN-λ. IFN-λ pre-treatment of MCs prior to RV16 infection generally did not result in the upregulation of ISGs, however, in one replicate (n=3 total) MCs appeared to upregulate ISGs in response to IFN-λ. In addition, there was a trend for decreased RV16 replication in LAD2 MCs with IFN-λ treatment. MC IFNLR expression was not determined in this investigation but could be examined by flow cytometry of MCs at baseline and in response to type I and type III IFN or treatment with poly I:C or virus infection to investigate possible

induced receptor expression. However, the observed responses of MCs to IFN-λ were not as consistent as the responses to IFN-β or IFN-γ. For instance, LAD2 MCs pre-treated with IFN prior to RV16 infection showed no induction in ISGs with IFN-λ alone (vehicle control). Furthermore, CBMCs did not upregulate *IFNL1*, *IFNB1* or ISGs in response to IFN-λ. Therefore, the responses of LAD2 MCs to IFN-λ could be down to the contamination of cultures with IFN-β or IFN-γ media and needs to be investigated with further experimental replicates.

In both LAD2 MCs and CBMCs the IFN-β-mediated upregulation of *IFNB1*, *IFNL1* and ISGs resulted in a suppression in viral replication and a suppression in the release of infectious RV16 particles. IFN-γ also appeared to provide protection against MC HRV infection (n=1). In CBMCs, the gene expression of the ISGs *MX1* and *OAS1* in the presence of IFN-β was comparable to the control of RV16 infection alone. This suggests IFN-β-mediated suppression of HRV replication and release is mediated by ISGs other than *MX1* or *OAS1*; alternatively, peaks in *MX1* and *OAS1* gene expression may have occurred at earlier time-points and returned to baseline by 24 hours. CBMCs were also treated with IFN-β plus an IFNAR2 blocking antibody. To optimise the concentration of antibody LAD2 MCs were treated with IFN-β and 1 or 2 μg/mL of the blocking antibody. While 1 μg/mL of the antibody reversed the IFN-β-mediated suppression of RV16 replication and suppressed IFN-β induction of the ISG *MX1*, other responses to the antibody were not as expected. For instance, at 2 μg/mL the blocking antibody did not block the impact of IFN-β on RV16 replication but did block IFN-β-induced *MX1*, however, at this concentration the isotype control also appeared to have a suppressive effect on *MX1*. Furthermore, the IFN-β-mediated suppression of RV16 release was not reversed to that of infection with RV16 alone with either antibody concentration. The effect of the blocking antibody on *MX1* would suggest that the lack of an impact on RV16 replication at this concentration was not a true response. However, at 2 μg/mL the antibody may be bound by Fc receptors on the surface of MCs preventing the antibody from effectively blocking IFNAR2. The anomalies in the results could be due to the behaviour of the antibody at different concentrations but to determine which responses are true and not simply anomalous or due experimental error, the experiment needs to be repeated. Despite this, CBMCs treated with IFN-β and 1 μg/mL IFNAR2 antibody responded as would be expected and confirmed IFN-β-mediated protection.

To investigate the contribution of endogenous type I IFN in the defence against HRV infection, CBMCs were pre-treated with the IFNAR2 antibody prior to RV16 infection. Treatment with the blocking antibody resulted in a significant suppression in *IFNB1* and *IFNL1* as well as *MX1* and *OAS1* gene expression. However, the blocking antibody had no impact on viral replication and caused an increase in viral shedding which was not significant. This suggested that the protection provided by endogenously generated type I IFN was limited in its capacity to supress productive HRV infection. It is possible that peaks in virus infection occurred at an earlier time-point, particularly considering the rapid response of type I IFNs to virus infection. On the other hand, increases in RV16 infection in the absence of type I IFN may have been more apparent at later time-points following the accumulation of virus. To investigate this, the experiment may be repeated over a time-course of 6 to 48 hours and viral replication and released determined for each time-point.

In asthma, BECs[150, 151], macrophages[151, 260] and dendritic cells[266] have been reported to have impaired IFN responses to viral infection, in addition, MCs are disordered in asthma in that they localise to areas associated with asthma pathogenesis, for instance, the bronchial epithelium[104]. The bronchial epithelium is the major site of HRV infection and replication where MCs increase with increasing asthma severity[207, 210]. In addition, impaired bronchial epithelial IFN responses, which this investigation has partly modelled in that low levels of endogenous MC type I IFN responses resulted in a trend for increased HRV infection, are also associated with severe asthma[151, 258]. Therefore, the demonstration that exogenous IFN-β effectively protects MCs from HRV infection is significant and may help to further understand the mechanism of protection provided by inhaled IFN-β, which has been particularly effective in difficult-to-treat asthmatics[263]. Furthermore, CBMCs were treated with IFN at the time of infection; nevertheless IFN-β was protective against HRV infection and may demonstrate the effectiveness of IFN-β as either a 'prophylactic' or a 'therapy' against HRV infection.

4.3.1 Conclusion

Exogenous IFN-β treatment protects MCs against HRV replication and release of infectious virus particle but the endogenous type I IFN response is not sufficient to suppress productive HRV infection.

Chapter 5: IL-33-Mediated Enhancement of the Anti-Viral Response of Mast Cells to RV16 Infection

5.1 Introduction

IL-33 has been associated with asthma through the identification of *IL33* and *ST2* as asthma susceptibility genes[267], in addition, IL-33 has been associated with an asthma phonotype in murine models *in vivo*. For instance, mice treated with IL-33 develop airway hyper responsiveness and goblet cell metaplasia via the induction of IL-4, IL-5 and IL-13 in the lung[268]. In humans, IL-33 expression increases with disease severity in bronchial biopsies of adults with asthma compared to non-asthmatic controls[184]. Likewise, endobronchial biopsies of paediatric patients with severe therapy-resistant asthma show an increased expression of IL-33[269]. In these paediatric patients, bronchial IL-33 expression is associated with increased reticular basement membrane thickness. Mucosal MC numbers have also been linked to reticular basement membrane thickness in infants and adversely predicts inhaled corticosteroid use[270]. These observations may suggest a link between MCs, IL-33 and the development of asthma in early life.

MCs express ST2, the receptor for IL-33, to which human MCs respond with increased survival, maturation and cytokine production, including IL-13 and IL-5[214, 215, 271]. MC responses to IL-33 are enhanced by TSLP[215], which MCs respond to in combination with other factors including IL-1 and TNF[272]. In a mouse model of asthma, IL-33 treatment of sensitised animals was shown to increase the activation of lung MCs and increase airway hyperresponsiveness, lung inflammation and remodelling[273]; thus linking allergic sensitisation, MCs, IL-33 and asthma.

The airway smooth muscle (ASM) plays a key role bronchoconstriction of the airways in asthma. In asthma the ASM expresses elevated levels of IL-33[274] as well as an increase in the localisation of MCs[205]. ASM IL-33 expression correlates with airway hyper-responsiveness and MC-ASM co-cultures demonstrate MCs increase ASM contraction via IL-33[275]. This suggests IL-33 may mediate increases in airway hyper-responsiveness/bronchoconstriction in asthma via MCs. For instance, in an allergic mouse model, MCs were implicated in allergen-induced bronchoconstriction via IL-33-induced MC serotonin production[276].

IL-33 is also implicated in HRV-associated asthma pathogenesis. IL-33 expression is increased in the airway epithelium of HRV infected mice (as is TSLP) where it enhances HRV-induced mucus metaplasia and airway hyperresponsiveness[277]. Similarly, during experimental HRV infection, airway IL-33 was increased in individuals with asthma and associated with IL-5 and IL-13 secretion as well as

exacerbation severity and viral load[218]. Although ILC2s treated with IL-33 containing conditioned media of BECs challenged with HRV produced IL-5 and IL-13[218], the response of MCs to the conditioned media was not investigated. The MC response is particularly relevant considering MCs (and basophils), rather than ILC2s, are the major responders to IL-33 during allergic asthma[188].

As discussed, MCs respond to IL-33 with Th2 responses that are relevant to the pathogenesis of asthma, however, MC responses to IL-33 have not been investigated in the context of HRV infection. This is relevant as HRV is the major trigger of viral exacerbations of asthma and both IL-33[277, 278] and MCs[217] have been implicated in HRV infection. Furthermore, IL-33 mediated MC responses are enhanced by factors including other cytokines[215, 272] and FcεRI activation[214], therefore, HRV infection may similarly enhance IL-33 mediated MC responses.

5.1.1 Hypothesis

IL-33 induces MC Th2 responses, which are modulated by HRV infection.

5.1.2 Aims

1. Characterise the response of MCs to IL-33
2. Investigate HRV-mediated modulation of MC responses to IL-33 and TSLP

5.1.3 Objectives

1. Treat LAD2 MCs and CBMCs with increasing concentrations of IL-33 and determine:
    a. IL-33-dependedent IL-4, IL-5 and IL-13 release by ELISA
    b. IL-33-dependent ST2, TSLPR and ICAM1 receptor expression by flow cytometry
2. Infect LAD2 or CBMCs with HRV in the presence of IL-33 and determine:
    a. IL-33-dependent IL-4, IL-5 and IL-13 release by ELISA
    b. The induction anti-viral (IFN and ISGs) and pro-inflammatory responses by RT-qPCR
    c. Viral replication and release by RT-qPCR and $TCID_{50}$ assay respectively

5.2 Results

5.2.1 IL-33 Induces Mast Cell Th2 Cytokine Release

MCs have been demonstrated to release Th2 cytokines in response to IL-33 but not in response to TSLP[214, 215, 271, 272]. In order to confirm these observations in the current model, LAD2 MCs were treated with IL-33 or TSLP each at 10 ng/mL. After 24 hours IL-4, IL-5 and IL-13 release into cell free supernatants was determined by MSD assay.

IL-33 but not TSLP induced significant increases in the secretion of IL-4, IL-5 and IL-13 (**Figure 5-1**) confirming previous reports[123-125, 179].

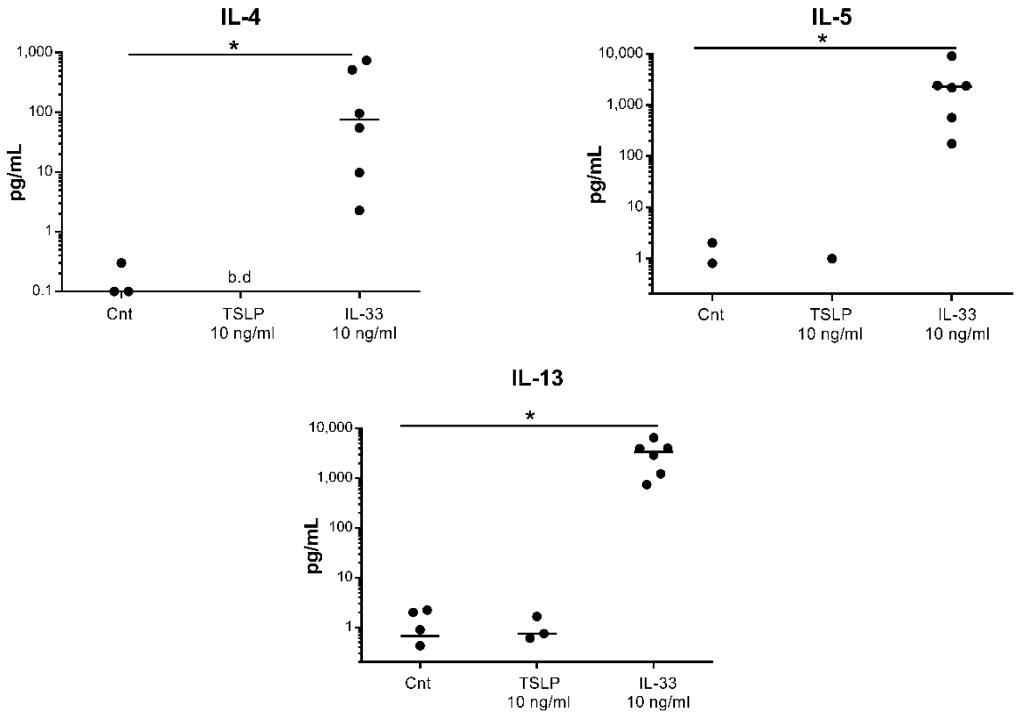

Figure 5-1. Th2 cytokine release by LAD2 MCs following TSLP or IL-33 treatment. LAD2 MCs were treated with TSLP or IL 33 (10 ng/mL each) or no cytokine (control) for 24 hours. Secretion of IL-4 **(A)**, IL-5 **(B)** and IL-13 **(C)** was measured in cell free supernatants by MSD assay. n=5 control and IL-33 and n=3 TSLP. Bars at median. * $p \leq 0.05$, one-way ANOVA (Kruskal-Wallis test). b.d, below detection.

TSLP has been reported to induce Th2 cytokine release when used in combination with other factors including IL-33[215, 272]. Therefore, LAD2 MCs were treated with TSLP in combination with IL-33 to investigate whether the combination could stimulate

a Th2 response. Unlike previously reported, IL-33 did not enhance the effect of TSLP on Th2 cytokine secretion above that of IL-33 alone (**Figure 5-2**). This was the case when IL-33 was used at either 1 or 10 ng/mL in combination with 10 ng/mL of TSLP (**Figure 5-2**).

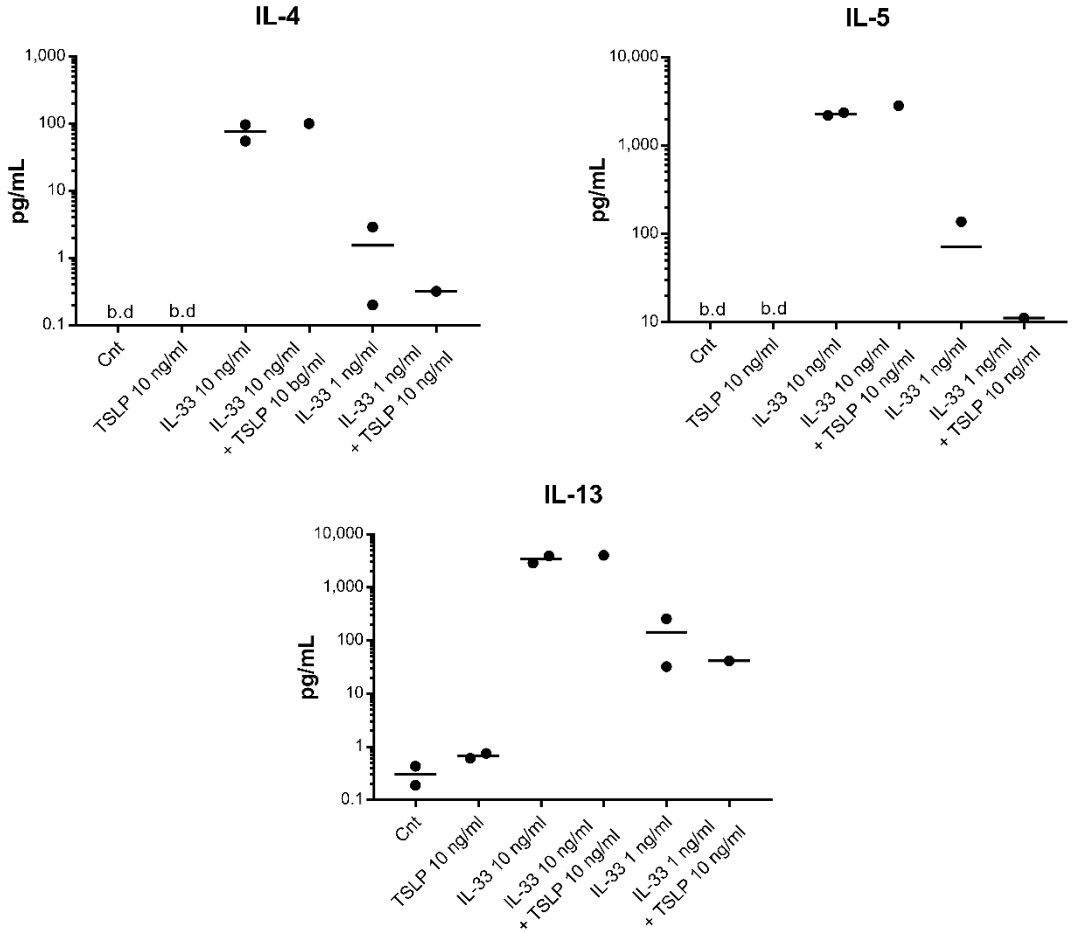

Figure 5-2. Th2 cytokine release from LAD2 MCs following IL-33 treatment alone or in combination with TSLP. LAD2 MCs were treated with 10 ng/mL TSLP and/or IL-33 at 1 or 10 ng/mL or no cytokine (control) for 24 hours. Following this, IL-4 **(A)**, IL-5 **(B)** and IL-13 **(C)** were measured in cell free supernatants by MSD assay. n=2: control, 1 or 10 ng/mL IL-33, 10 ng/mL TSLP and n=1: IL-33 (1 or 10 ng/mL) + 10 ng/mL TSLP. b.d, below detection.

As significant cytokine release was induced by IL-33 and not by TSLP, investigations were continued with IL-33 alone. LAD2 MCs treated with IL-33 at 1, 5 or 10 ng/mL showed a concentration dependent increase in IL-4, IL-5 and IL-13 release which was significant at the highest concentration of IL-33 (**Figure 5-3**).

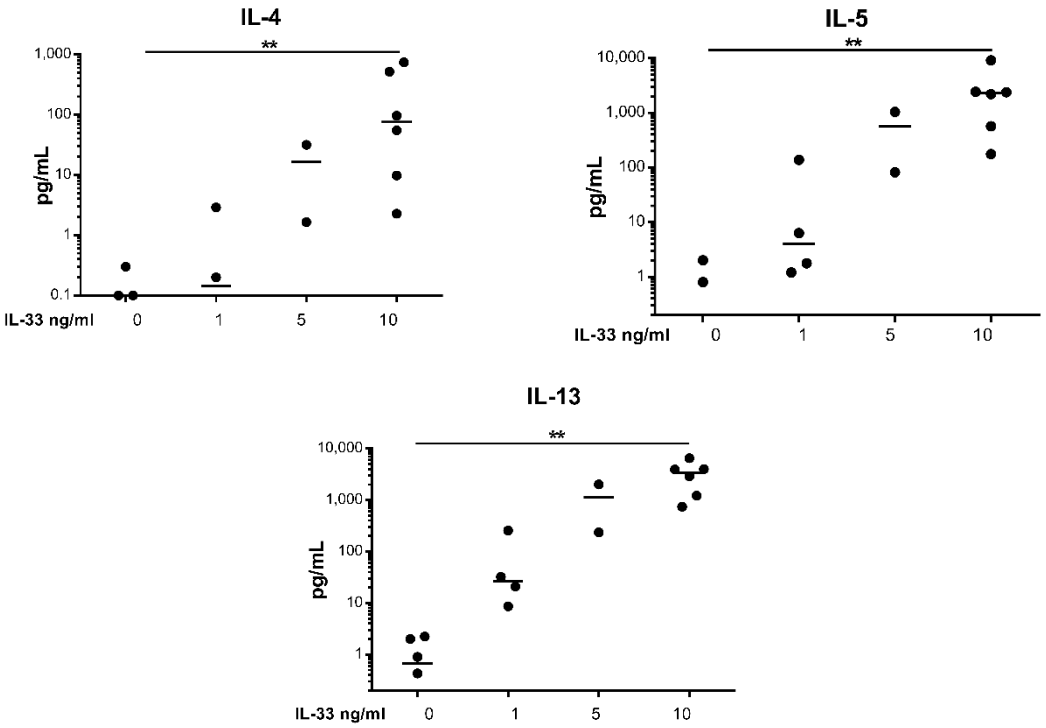

Figure 5-3. Th2 cytokine release from LAD2 MCs treated with increasing concentrations of IL-33. LAD2 MCs were treated with 1, 5 or 10 ng/mL IL-33 or no cytokine for 24 hours following which, IL-4 **(A)**, IL-5 **(B)** and IL-13 **(C)** were measured in cell free supernatants by MSD assay. n=5 IL-33 0 (control) and 10 ng/mL, n=4 1 ng/mL, n=2 5 ng/mL. Bars at median. ** $p \leq 0.01$, one-way ANOVA (Kruskal-Wallis test).

Further characterisation of the responses of LAD2 MCs to 10 ng/mL of IL-33 revealed a time-dependent increase in IL-4, IL-5 and IL-13 release at 48 compared to 24 hours (**Figure 5-4 A-C**). The steroid sensitivity of IL-33-dependent Th2 responses was also investigated. Cells were treated with IL-33 in combination with the steroid fluticasone propionate at 10 or 100 nM for 24 and 48 hours. Cytokine secretion with and without steroid treatment was greater at 48 hours and IL-33-dependent IL-4, IL-5 and IL-13 secretion was steroid sensitive at both time-points, compared to IL-33 treatment alone (**Figure 5-4 D-F**).

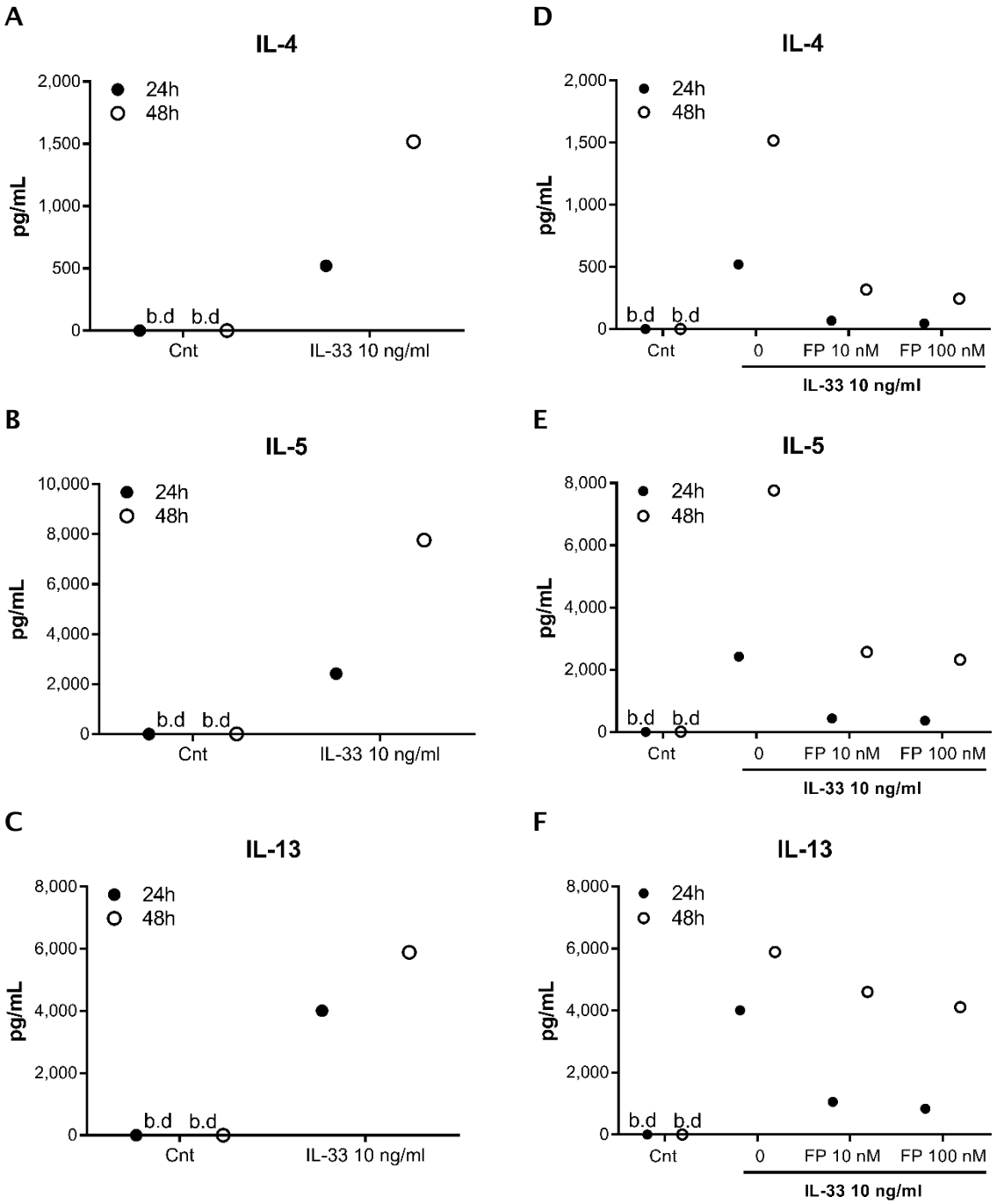

Figure 5-4. Time course and steroid sensitivity of IL-33-dependent Th2 cytokine release by LAD2 MCs. LAD2 MCs were treated with 10 ng/mL IL-33 in the absence or presence of 10 or 100 nM fluticasone propionate (FP). Twenty-four or 48 hours following treatment IL-4 **(A, D)**, IL-5 **(B, E)** and IL-13 **(C, F)** were measured in cell free supernatants by MSD assay. n=1. b.d, below detection.

5.2.2 IL-33 Upregulates the Expression of ST2 and ICAM1 on Mast Cells

The responses of MCs to IL-33 were also investigated in regards to the modulation of MC receptor expression. LAD2 MCs were treated with 1, 5 or 10 ng/mL of IL-33 for 24 hours and receptor expression analysed by flow cytometry. IL-33 induced a concentration-dependent increase in the cell surface expression of ST2 and ICAM1 which was significant at the highest concentration of IL-33 (**Figure 5-5 A-B**). There was also an IL-33-dependent trend for increased TSLPR expression (**Figure 5-5 C**). Furthermore, TSLPR was expressed at baseline suggesting the unresponsiveness of MCs to TSLP treatment (as determined by Th2 cytokine responses) was not due to a lack of receptor expression (**Figure 5-5 C**).

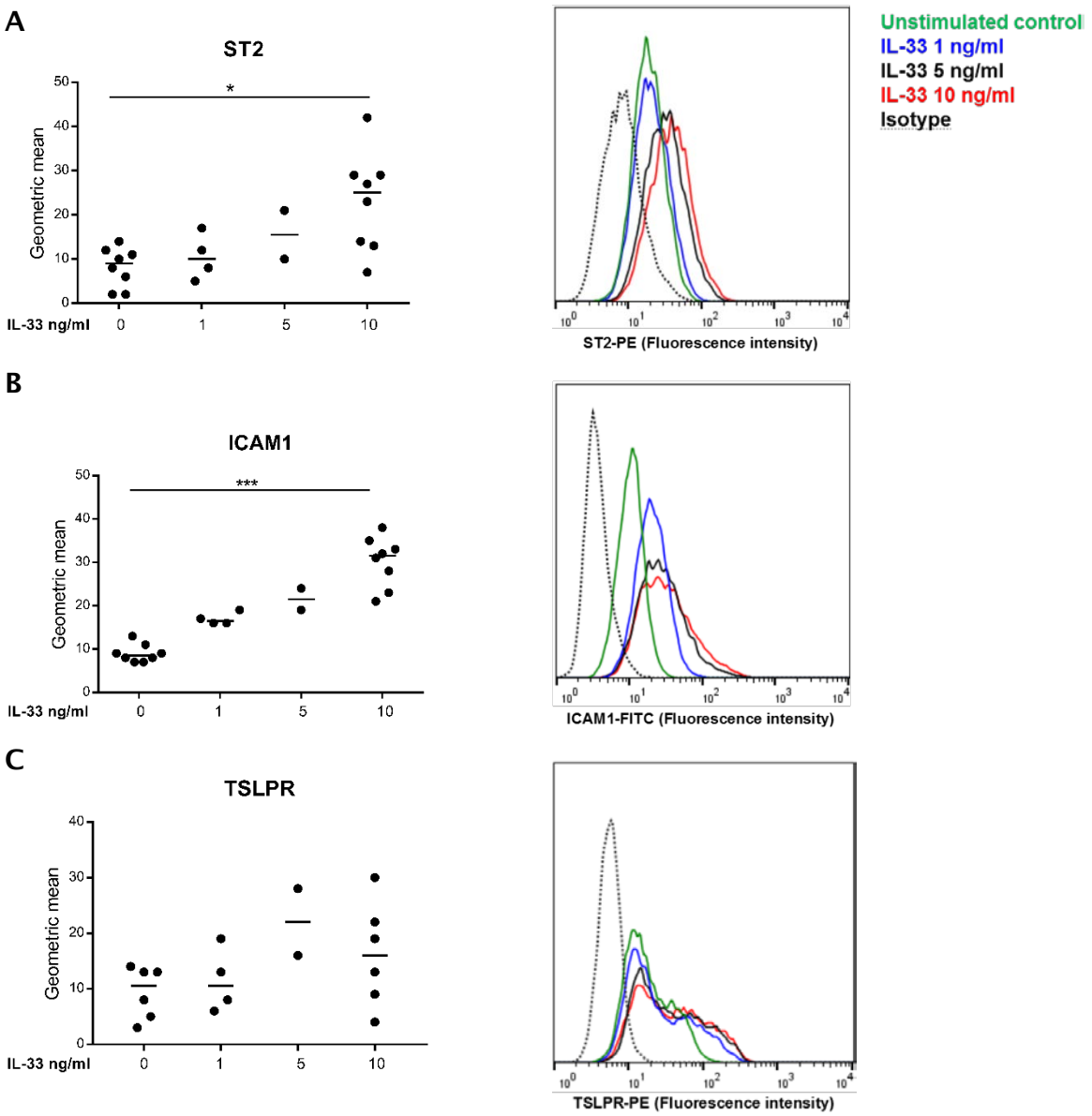

Figure 5-5. IL-33 upregulates ST2, ICAM1 and TSLPR expression on the surface of LAD2 MCs. LAD2 MCs were treated with IL-33 at 1 (blue), 5 (black) or 10 (red) ng/mL or no cytokine (green, unstimulated control) for 24 hours. ST2 **(A)**, ICAM1 **(B)** and TSLPR **(C)** expression were determined by flow cytometry. n=6-8 control (IL-33 0 ng/mL) and IL-33 10 ng/mL, n=4 IL-33 1 ng/mL, n=2 IL-33 5 ng/mL. Bars at median, * $p \leq 0.05$, *** $p \leq 0.001$, one-way ANOVA (Kruskal-Wallis test) compared to unstimulated control. Representative histograms, isotype control (dotted line).

A time-course of IL-33 treatment at 24 and 48 hours revealed IL-33-mediated ST2 and ICAM1 expression was greatest at 24 hours (**Figure 5-6**). By 48 hours IL-33-dependent ST2 expression had returned to baseline (**Figure 5-6**) whereas ICAM1 expression remained increased compared to control (**Figure 5-6**). To investigate

the steroid sensitivity of IL-33-dependent ST2 and ICAM1 expression LAD2 MCs were treated with IL-33 in combination with fluticasone propionate at 10 or 100 nM for 24 and 48 hours. ST2 expression was unaffected by fluticasone propionate treatment whereas ICAM1 expression was suppressed at both time points. At 48 hours, 10 nM fluticasone propionate appeared to suppress ICAM1 expression to levels below the control (**Figure 5-6**).

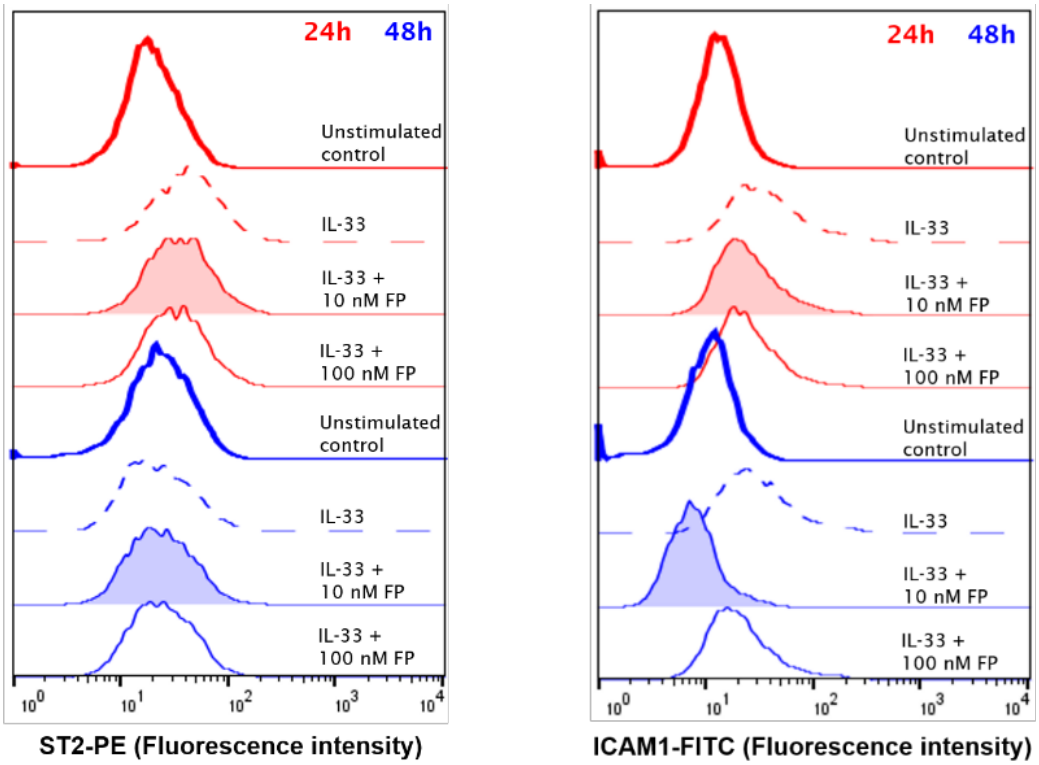

Figure 5-6. IL-33-dependent ST2 and ICAM1 expression on LAD2 MCs at 24 & 48 hours. LAD2 MCs were treated with 10 ng/mL IL-33 in the absence or presence of 10 or 100 nM fluticasone propionate (FP) for 24 (red) or 48 (blue) hours. ST2 and ICAM1 cell surface expression were assayed by flow cytometry. n=1.

5.2.3 IL-33-Dependent Th2 Cytokine Release is not Enhanced by RV16 Infection

IL-33 dependent activation of MC responses can be enhanced in combination with factors including FcεRI activation[214], TSLP[215], the neuropeptide substance P[279] and the complement factor C5a[280]. Furthermore, the IL-33-dependent increase in ICAM1 expression (the receptor for major group HRVs including RV16), raised the question of whether MC responses to IL-33 are modulated by RV16 infection and whether RV16 infection is increased by IL-33. To investigate this, LAD2 MCs were pre-treated with IL-33 at 1, 5 or 10 ng/mL for 24 hours followed by infection with RV16 or UV-RV16 (non-replicating control) at an MOI of 7.5 for 24 hours.

RT-qPCR for *IL5* and *IL13* gene expression demonstrated an IL-33 concentration dependent increase in *IL5* and *IL13* transcripts (**Figure 5-7**). However, there were no differences in gene induction by UV-RV16 compared to RV16 infection, suggesting infection with replicating RV16 has no impact on IL-33-mediated *IL5* and *IL13* gene expression.

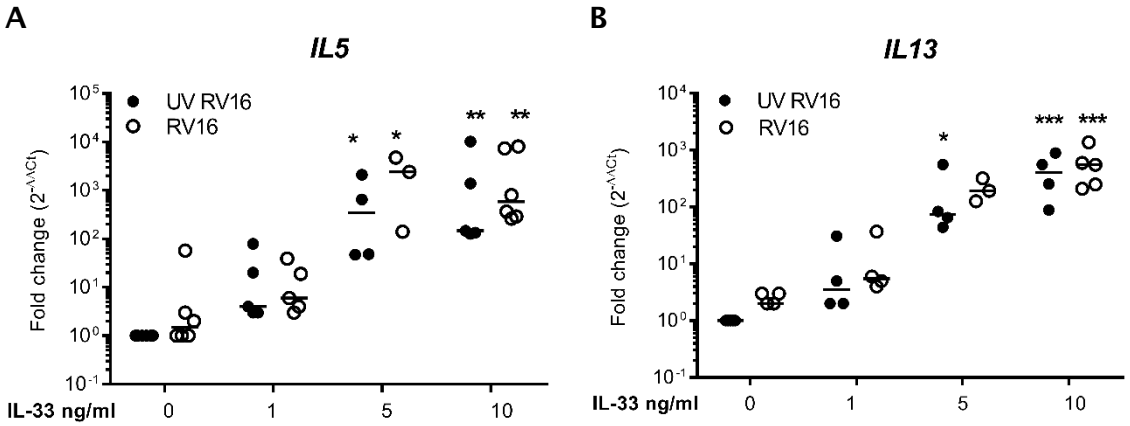

Figure 5-7. IL-33 and RV16-mediated Th2 cytokine gene expression. LAD2 MCs were pre-treated with IL-33 at 1, 5 or 10 ng/mL or no cytokine for 24 hours prior to infection with UV-RV16 (control) or RV16 MOI 7.5.for 24 hours. *IL5* **(A)** and *IL13* **(B)** gene expression was assayed by RT-qPCR. n=6 (A) or n=5 (B) IL-33 0 ng/mL, n=5 (A) or n=4 (B) 1 ng/mL, n=4 UV-RV16 or n=3 RV16 5 ng/mL, n=5 (A) or n=4 (B) UV-RV16 10 ng/mL and n=6 (A) or n=5 (B) RV16 10 ng/mL. Bars at median. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ versus respective no cytokine control, one-way ANOVA (Kruskal-Wallis test).

An investigation into Th2 cytokine secretion revealed a similar IL-33 concentration dependent increase in IL-4, IL-5 and IL-13 release (**Figure 5-8**) but as with gene induction, there was no difference in cytokine release between UV-RV16 and RV16 infection.

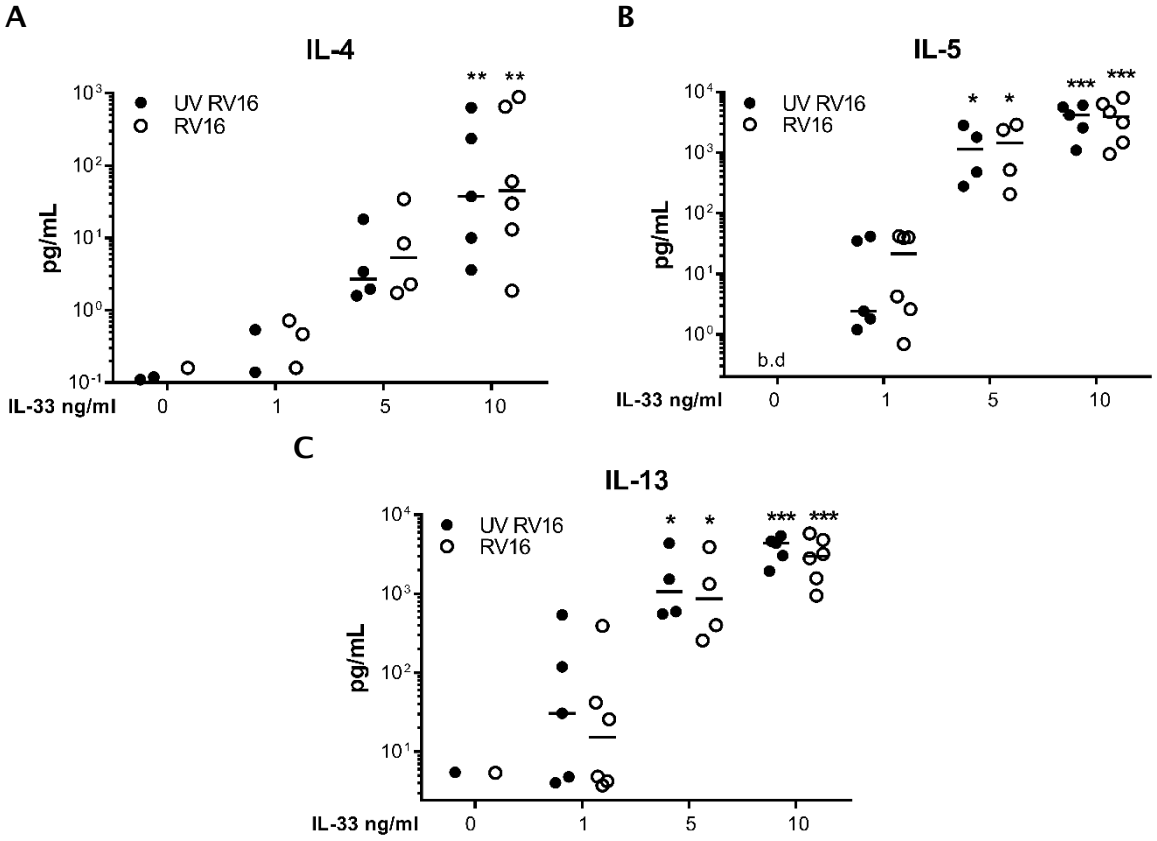

Figure 5-8. IL-33 and RV16-mediated Th2 cytokine secretion. LAD2 MCs were pre-treated with IL-33 at 1, 5 or 10 ng/mL or with no cytokine for 24 hours prior to infection with UV-RV16 or RV16 MOI 7.5 for 24 hours. IL-4 **(A)**, IL-5 **(B)** and IL-13 **(C)** protein were measured in cell free supernatants by MSD assay. n=5 1 and 10 ng/mL IL-33, n=4 5 ng/mL, n=6 no cytokine. Bars at median. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ versus respective no cytokine control, one-way ANOVA (Kruskal-Wallis test).

5.2.4 IL-33 Enhances the Replication of RV16 in Mast Cells

RV16 infection did not modulate the Th2 cytokine response of LAD2 MCs to IL-33, however, IL-33 may have influenced the response of MCs to RV16 infection particularly in light of the IL-33-mediated increase in the cell surface expression of ICAM1. To investigate this, RV16 replication was determined in LAD2 MCs pre-treated for 24 hours with or without IL-33 at 1, 5 and 10 ng/mL and infected with RV16 or UV-RV16 MOI 7.5 for 24 hours. RT-qPCR assay for the viral genome revealed a statistically significant increase in RV16 replication with 10 ng/mL of IL-33 (**Figure 5-9 A**). Virus release was investigated by $TCID_{50}$ assay and despite the increase in viral replication there was not a statistically significant increase in RV16 release with IL-33 treatment (**Figure 5-9 B**).

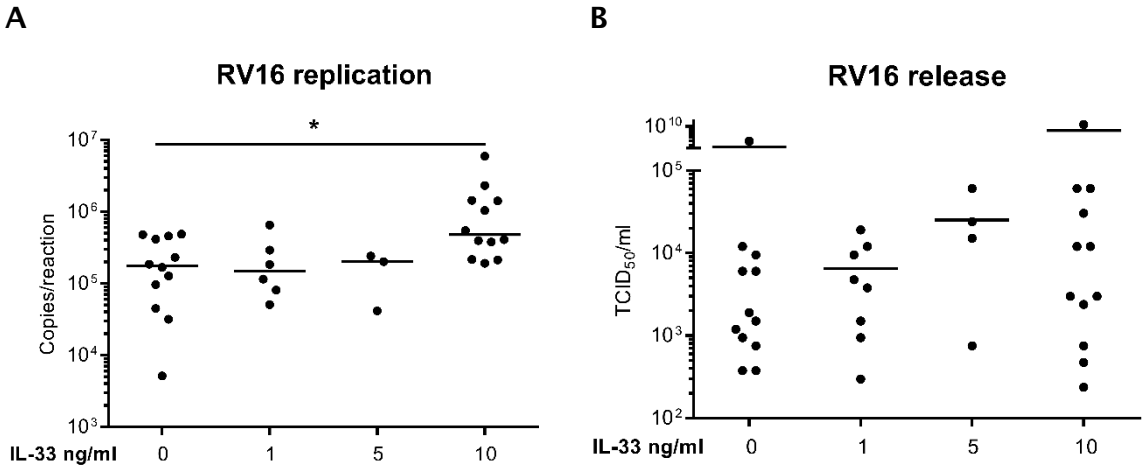

Figure 5-9. RV16 replication and release from LAD2 MCs with IL-33 treatment. LAD2 MCs were pre-treated with IL-33 at 1, 5 or 10 ng/mL or with no cytokine for 24 hours prior to infection with RV16 at MOI 7.5 for 24 hours. RV16 replication was determined by RT-qPCR for the viral genome **(A)** and virus release by $TCID_{50}$ assay for infectious virus particles in cell free supernatants **(B)**. n=12 IL-33 0 ng/mL, n=6 (A) or n=8 1 ng/mL, n=3 (A) or n=4 (B) 5 ng/mL, n=12 10 ng/mL. Bars at median. * $p \leq 0.05$, one-way ANOVA (Kruskal-Wallis test).

5.2.5 IL-33 does not Enhance the Infection of LAD2 Mast Cells by the Minor Group Virus RV1B

As MCs are susceptible for infection by both major and minor group HRVs the susceptibility of LAD2 MCs for IL-33-enhanced replication of RV1B, a minor group HRV, was also investigated. Flow cytometric analysis revealed 24 hours of IL-33 treatment at 1 and 10 ng/mL induced a concentration dependent increase in the expression of LDLR (**Figure 5-10**), a receptor for minor group viruses including RV1B.

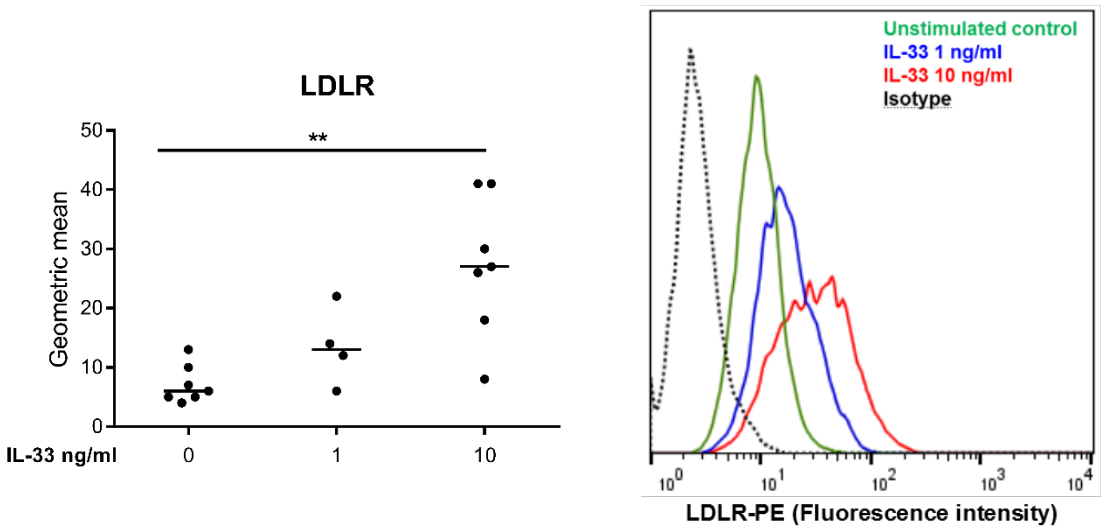

Figure 5-10. IL-33-dependent LDLR expression on LAD2 MCs. LAD2 MCs were treated with 1 (blue) or 10 (red) ng/mL IL-33 or no cytokine (green, unstimulated control) for 24 hours after which LDLR expression was determined by flow cytometry. n=7 control (IL-33 0 ng/mL) and IL-33 10 ng/mL or n=4 IL-33 1 ng/mL. Bars at median, ** $p \leq 0.01$, one-way ANOVA (Kruskal-Wallis test).

This IL-33 dependent increase in receptor expression suggested that IL-33 would similarly enhance RV1B infection of LAD2 MCs. To investigate this, LAD2 MCs were pre-treated with 1 or 10 ng/mL IL-33 or no cytokine for 24 hours then infected with the minor group virus RV1B (MOI 3) for 24 hours. RT-qPCR for the viral genome revealed no increase in RV1B replication with IL-33 treatment at 1 or 10 ng/mL (**Figure 5-11 A**) and in accordance with this RV1B release was also unaffected by IL-33 treatment (**Figure 5-11 B**).

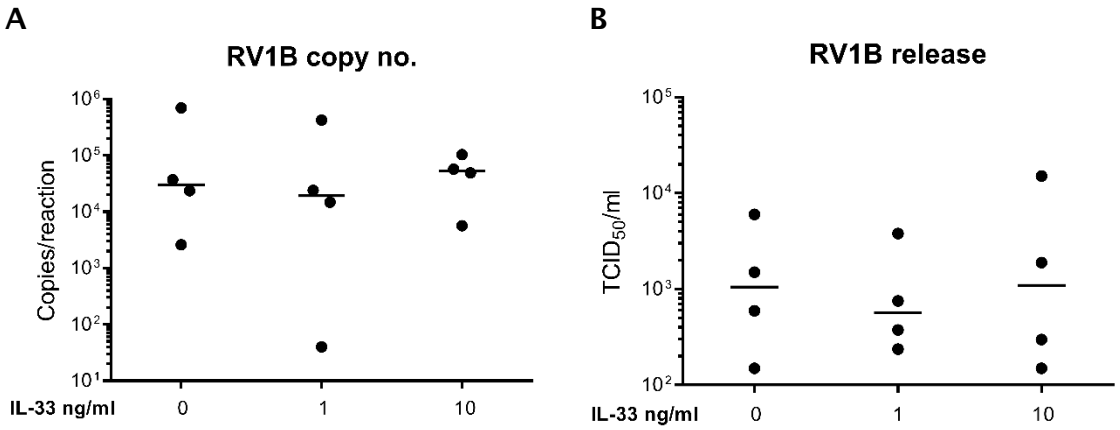

Figure 5-11. RV1B replication and release from LAD2 MCs with IL-33 treatment. LAD2 MCs were pre-treated with 1 or 10 ng/mL IL-33 or no cytokine for 24h then infected with RV1B MOI 3. RV1B replication was determined by RT-qPCR for the viral genome **(A)** and virus release by $TCID_{50}$ assay for infectious virus particles in cell free supernatants **(B)**. n=4. Bars at median.

5.2.6 IL-33 Enhances the Anti-Viral and Inflammatory Response of Mast Cells to RV16 Infection

In light of the modulation of IL-33 on RV16 replication, the influence of IL-33 on the anti-viral and pro-inflammatory response of MCs to RV16 infection was investigated. RT-qPCR showed that, as previously demonstrated, in the absence of IL-33 RV16 but not UV-RV16 infection caused inductions in IFN (*IFNB1*and *IFNL1*) and ISG (*MDA5* and *OAS1*) gene expression (**Figure 5-12**). In the presence of IL-33 UV-RV16 infection was able to induce significant IFN and ISG responses although these responses were not concentration-dependent (**Figure 5-12 A-D**). As with UV-RV16 infection, IFN and ISG responses to RV16 infection underwent statistically significant increases with IL-33 pre-treatment (**Figure 5-12**).

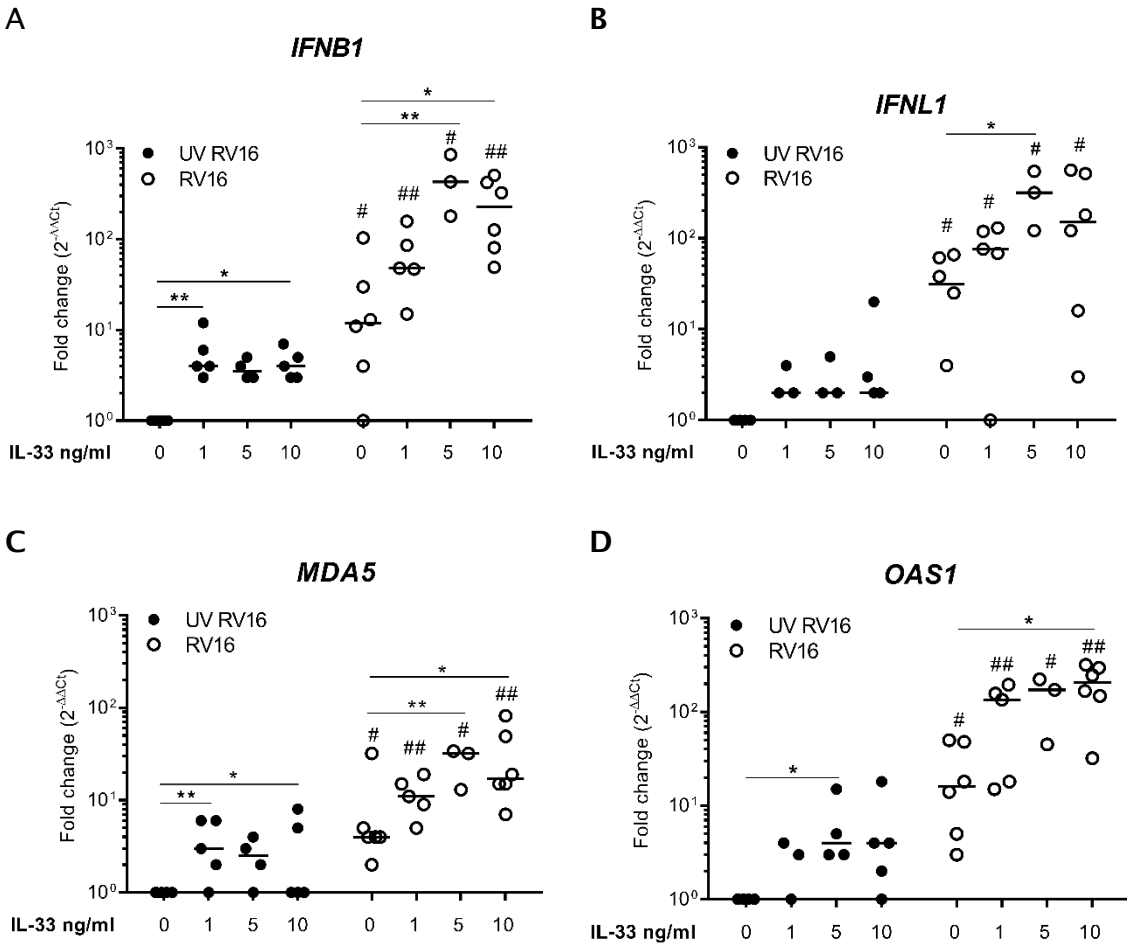

Figure 5-12. IL-33 and RV16-dependent interferon and interferon stimulated gene expression. LAD2 MCs were pre-treated with 1, 5 or 10 ng/mL IL-33 or no cytokine for 24h then infected with UV-RV16 or RV16 MOI 7.5. Interferon [*IFNB1* **(A)**, *IFNL1* **(B)**] and interferon stimulated gene [*MDA5* **(C)**, *OAS1* **(D)**] expression was determined by RT-qPCR. n=6 IL-33 0 ng/mL, n=5 1 ng/mL, n=4 UV-RV16 or n=3 RV16 5 ng/mL, n=5 UV-RV16 or n=6 RV16 10 ng/mL. Bars at median. * $p \leq 0.05$, ** $p \leq 0.01$ versus respective no cytokine control, one-way ANOVA (Kruskal-Wallis test). # $p \leq 0.05$, ## $p \leq 0.01$ UV-RV16 versus RV16, Wilcoxon test (paired data) or Mann-Whitney test (unpaired data).

These response were concentration-dependent and were significantly (statistically) enhanced compared to UV-RV16. In addition to IFN and ISG responses, *CCL5*, *CXCL10* and IL-6 pro-inflammatory responses were also enhanced by IL-33 compared to UV-RV16 or RV16 infection alone (**Figure 5-13**). Also, with 10 ng/mL IL-33 RV16 induction of *CXCL10* and secreted IL-6 were significantly enhanced compared to UV-RV16 infection (**Figure 5-13 B, D**).

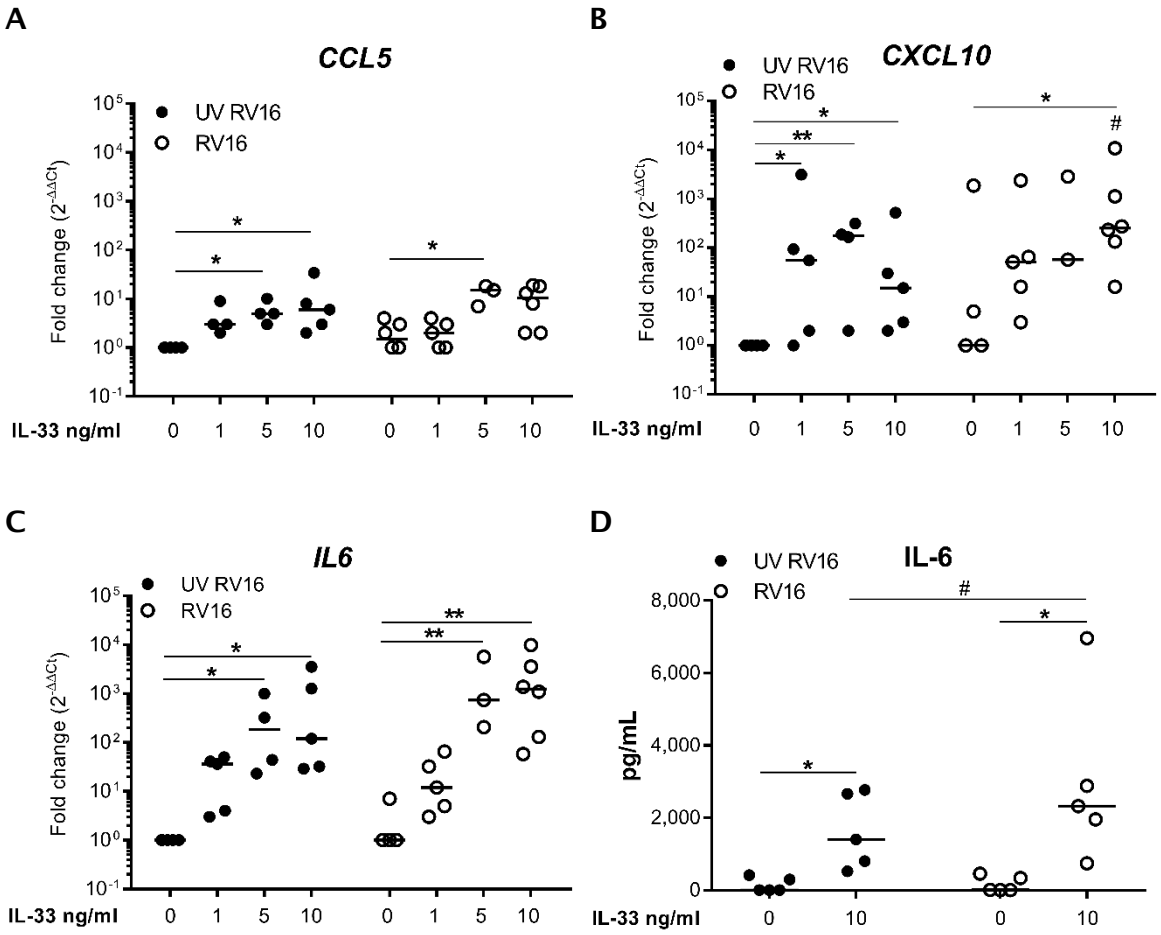

Figure 5-13. IL-33 and RV16-dependent pro-inflammatory cytokine expression. LAD2 MCs were pre-treated for 24 hours with no cytokine or 1, 5 or 10 ng/mL IL-33 prior to infection with UV-RV16 or RV16 MOI 7.5 for 24 hours. *CCL5* **(A)**, *CXCL10* **(B)** and *IL6* **(C)** gene expression was determined by RT-qPCR. n=6 IL-33 0 ng/mL, n=5 1 ng/mL, UV- n=4 RV16 or n=3 RV16 5 ng/mL, n=5 UV-RV16 or n=6 RV16 10 ng/mL. IL-6 protein was determined in cell free supernatants by MSD assay **(D)**, n=5. Bars at median. * $p \leq 0.05$, ** $p \leq 0.01$ versus respective no cytokine control, one-way ANOVA (Kruskal-Wallis test), # $p \leq 0.05$ UV-RV16 versus RV16, Wilcoxon test (paired data) or Mann-Whitney test (un-paired data).

5.2.7 Blockade of ICAM1 Abolishes IL-33-Dependent RV16 Replication

IL-33 mediates an increase in RV16 replication which was associated with an IL-33 –dependent increase in anti-viral and pro-inflammatory responses. To confirm that these responses occurred via the IL-33-dependent increase in ICAM1 expression, LAD2 MCs were pre-treated with IL-33 for a total of 24 hours. One hour prior to RV16 or UV-RV16 infection (MOI 7.5), cells were incubated with an ICAM1 blocking antibody (anti-ICAM1) or isotype control and samples collected 24 hours post infection. Treatment with the ICAM1 blocking antibody significantly reduced RV16 replication in the presence of IL-33, compared to the isotype control (**Figure 5-14 A**), and caused a complete blockade in the release of infectious RV16 particles (**Figure 5-14 B**). RV16-induced IFN-β production was enhanced by IL-33 and in the presence of the ICAM1 blocking antibody, IFN-β release was reduced to levels below the detection of the assay (**Figure 5-14 C**).

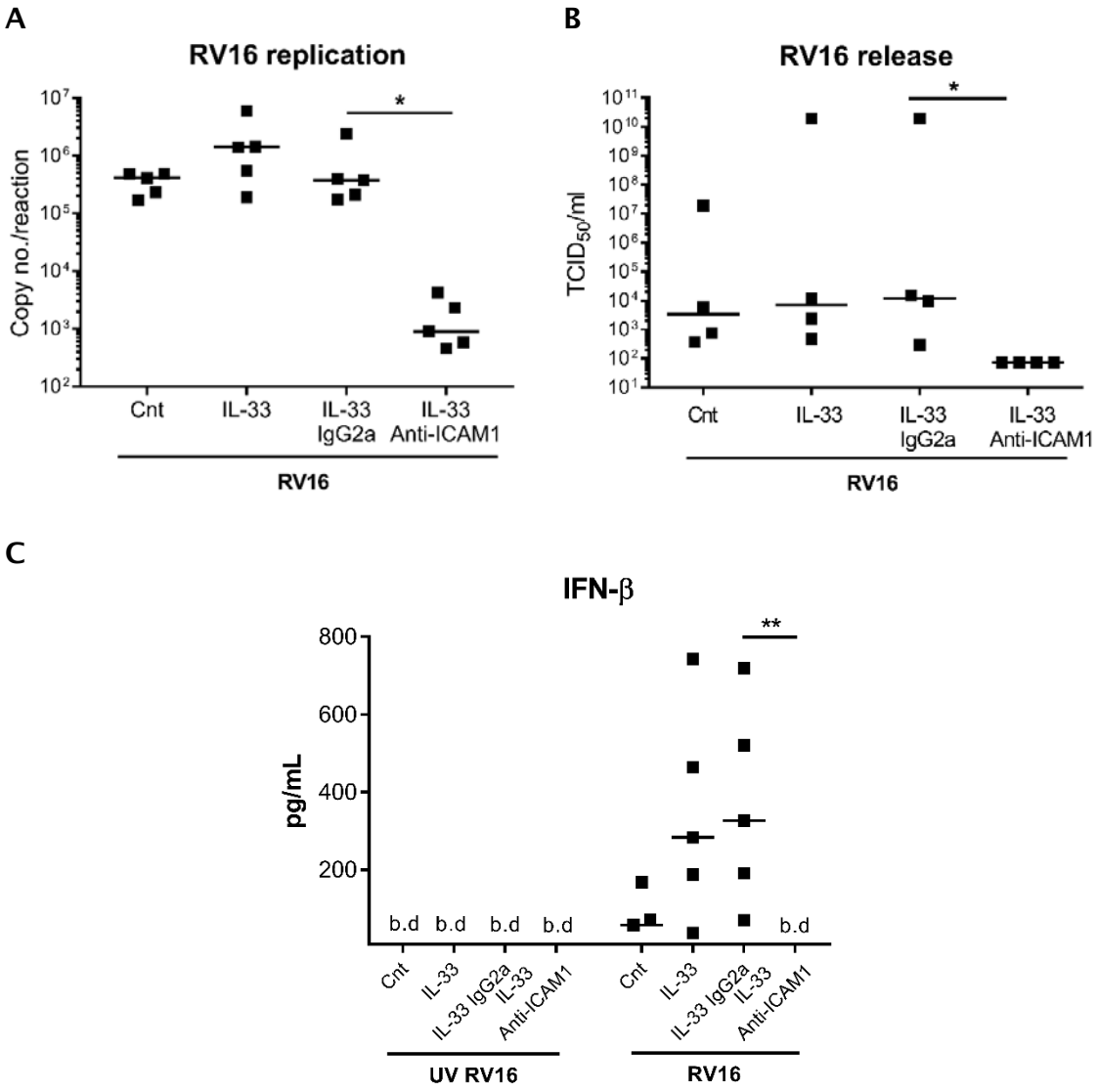

Figure 5-14. RV16 replication and release and IFN-β secretion following IL-33 treatment and ICAM1 blockade. LAD2 MCs were pre-treated for 24 hours with or without IL-33 10 ng/mL prior to infection with UV-RV16 or RV16 MOI 7.5. One hour prior to infection cells were incubated with anti-ICAM1 antibody or an IgG2a isotype control (each at 10 μg/mL). Twenty-four hours following infection RV16 replication was determined by RT-qPCR for the viral genome **(A)** and RV16 release by $TCID_{50}$ assay for infectious virus particles in cell free supernatants **(B)**. IFN-β protein was measured in cell free supernatants by MSD assay **(C)**. n=5, bars at median. * $p \leq 0.05$, ** $p \leq 0.01$, one-way ANOVA (Friedman test). b.d, below detection.

5.2.8 IL-33 Modulation of RV16 Infection of CBMCs

Previous comparison of the LAD2 MC line to primary MCs (CBMCs) demonstrated LAD2 MCs behaved similarly to the primary MCs and therefore were a good model for RV16/RV1B infection of MCs. Similarly, CBMCs were treated with 10 ng/mL IL-33 for 24 hours with and without 24 hour RV16 infection in order to compare the IL-33 dependent responses of primary MCs and the LAD2 cell line.

Similarly to LAD2 MCs and as has been previously demonstrated[214, 215, 271, 272] IL-33 enhanced the production of IL-4, IL-5 and IL-13 by CBMCs (**Figure 5-15 A-C**). However, CBMCs released lower concentrations of cytokine compared to LAD2 MCs (**Figure 5-3**), with IL-33-induced IL-4 barely above the lower detection limit of the assay (**Figure 5-15 A**). An examination of receptor expression showed that as with LAD2 MCs, IL-33 induced CBMC expression of ST2, ICAM1 and LDLR (**Figure 5-15 D-F**). While the expression of ST2 and LDLR were lower than that of LAD2 MCs (**Figure 5-5 A, Figure 5-10** respectively), the magnitude of IL-33-induced ICAM1 expression (according to geometric means) was greater in CBMCs (**Figure 5-5 B, Figure 5-15 E**).

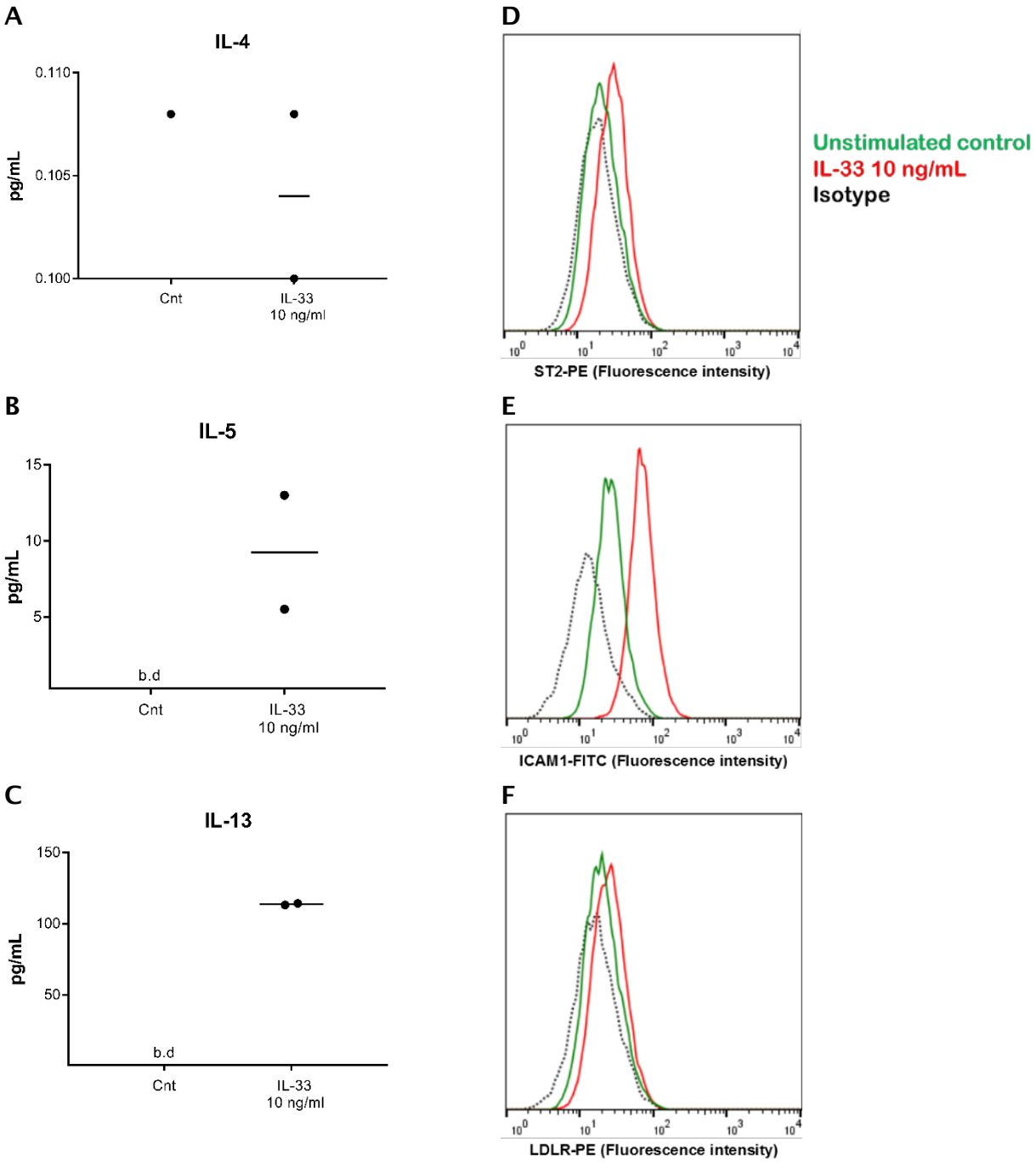

Figure 5-15. IL-33-mediated induction of Th2 cytokine release and ST2, ICAM1 and LDLR expression on CBMC. CBMCs were treated with 10 ng/mL IL-33 or no cytokine for 24 hours following which IL-4 **(A)**, IL-5 **(B)** and IL-13 **(C)** were measured in cell free supernatants by MSD assay. ST2 **(D)**, ICAM1 **(E)** and LDLR **(F)** expression were determined by flow cytometry. n=2. Bars at median.

The steroid sensitivity of CBMC responses were also investigated and as with LAD2 MCs IL-33-induced IL-5 and IL-13 responses were steroid sensitive (IL-4 was below the limit of detection of the assay) (**Figure 5-16 A-C**). LDLR expression was down

regulated by steroid treatment but unlike LAD2 MCs, ST2 and ICAM1 were not steroid sensitive at 24 hours (**Figure 5-6 C-D, Figure 5-16 D-F**).

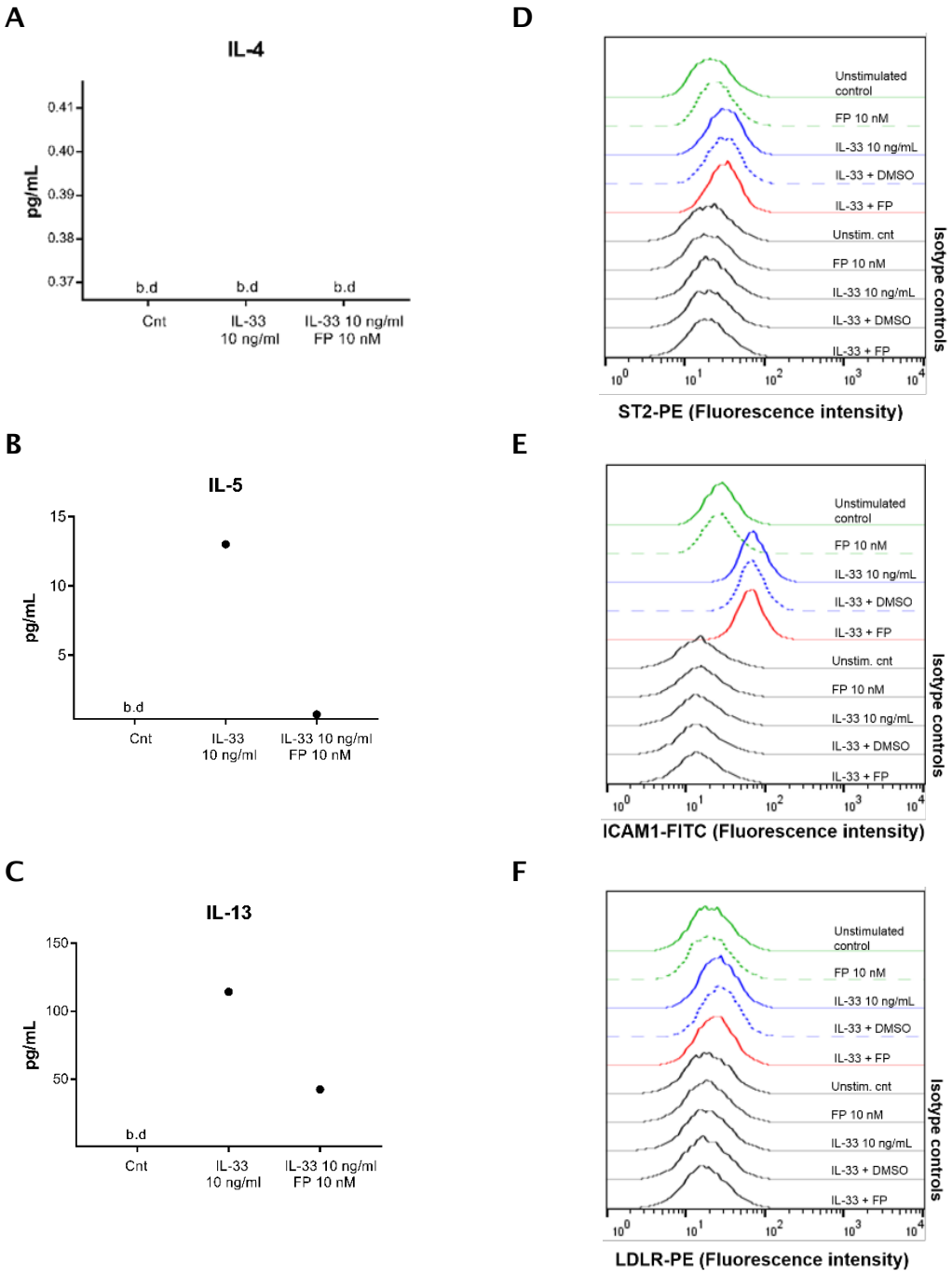

Figure 5-16. Steroid sensitivity of IL-33-dependent Th2 cytokine secretion and receptor expression on CBMCs. CBMCs were treated with 10 ng/mL IL-33 with or without 10 nM fluticasone propionate (FP) or not treated (control) for 24 hours. IL-4 **(A)**, IL-5 **(B)** and IL-13 **(C)** were measured in cell free supernatants by MSD assay. ST2 **(D)**, ICAM1 **(E)** and LDLR **(F)** cell surface expression was determined by flow cytometry. n=1.

Next the effect of IL-33 on Th2 cytokine secretion in the presence of RV16 was investigated. The IL-33-induced Th2 cytokine response of LAD2 MC was not modulated by RV16 infection but CBMCs showed a trend for greater IL-33-dependent IL-5 and IL-13 secretion with RV16 infection compared to UV-RV16 (**Figure 5-17**).

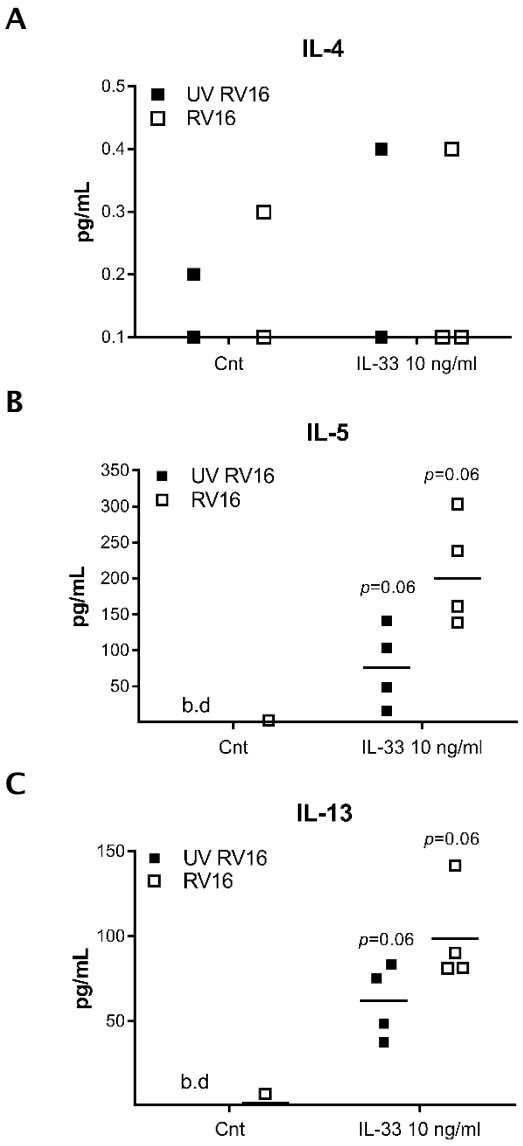

Figure 5-17. CBMC IL-33 and RV16-mediated Th2 cytokine secretion. CBMCs were pre-treated with IL-33 10 ng/mL or no cytokine for 24h then infected with UV-RV16 or RV16 MOI 7.5 for 24 hours. IL-4 **(A)**, IL-5 **(B)** and IL-13 **(C)** protein were measured in cell free supernatants by MSD assay. n=4, Bars at median. Wilcoxon test IL-33/ (UV)-RV16 versus (UV)-RV16 alone.

RV16 replication in CBMC was also enhanced by IL-33 although this did not reach statistical significance (**Figure 5-18 A**) and there was no increase in RV16 release with IL-33 treatment (**Figure 5-18 B**). Similarly to LAD2 MCs, the trend for increased RV16 replication was associated with a trend for increased IL-33-dependent IFN-β secretion compared to RV16 infection alone (**Figure 5-18 C**).

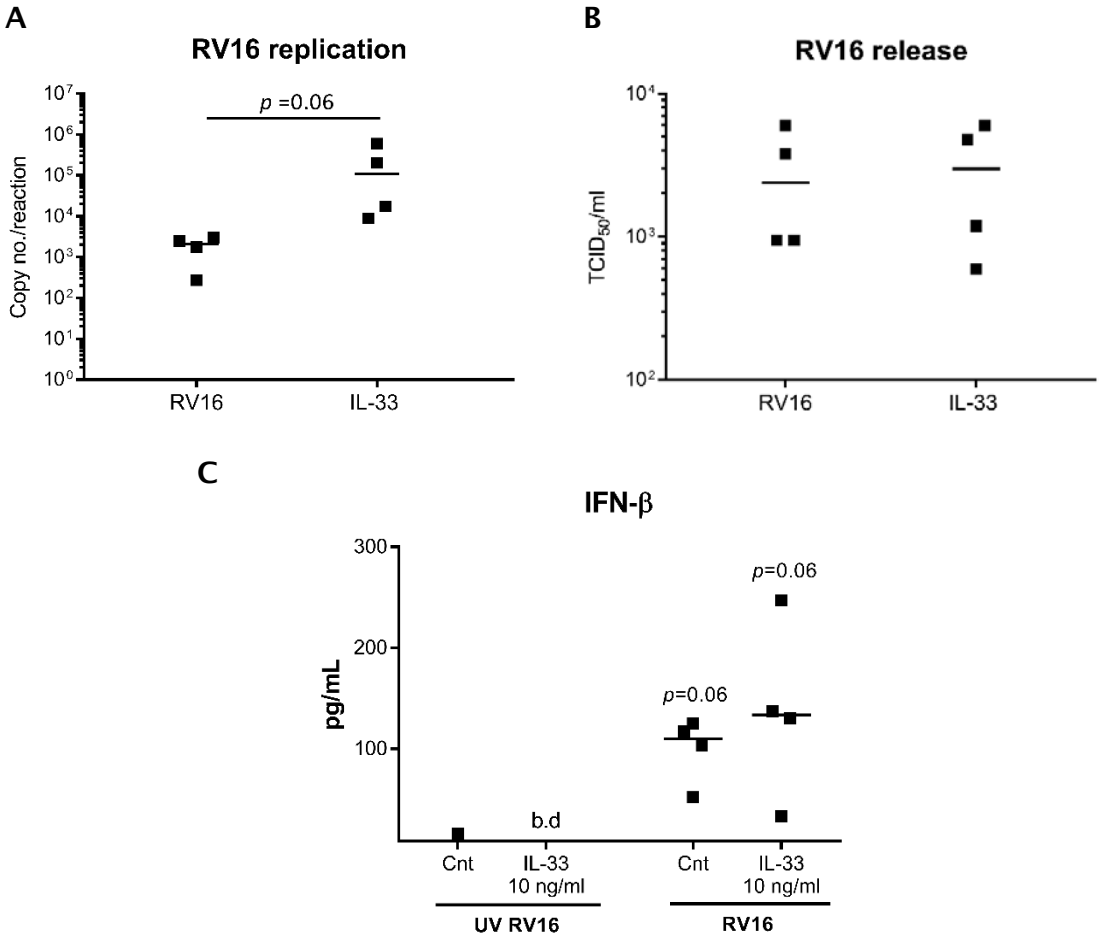

Figure 5-18. CBMC RV16 replication and release and IFN-β production in response to IL-33. CBMCs were pre-treated with 10 ng/mL IL-33 or no cytokine for 24 hours then infected with RV16 MOI 7.5 for a further 24 hours. RV16 replication was determined by RT-qPCR for the viral genome **(A)** and RV16 release by $TCID_{50}$ assay for infectious virus particles in cell free supernatants **(B)**. IFN-β protein was measured in cell free supernatants by MSD assay **(C)**. n=4, bars at median. Wilcoxon test (t-test).

As with LAD2 MCs, blockade of ICAM1inhibited RV16 replication and release as well as IFN-β secretion (**Figure 5-19 C**).

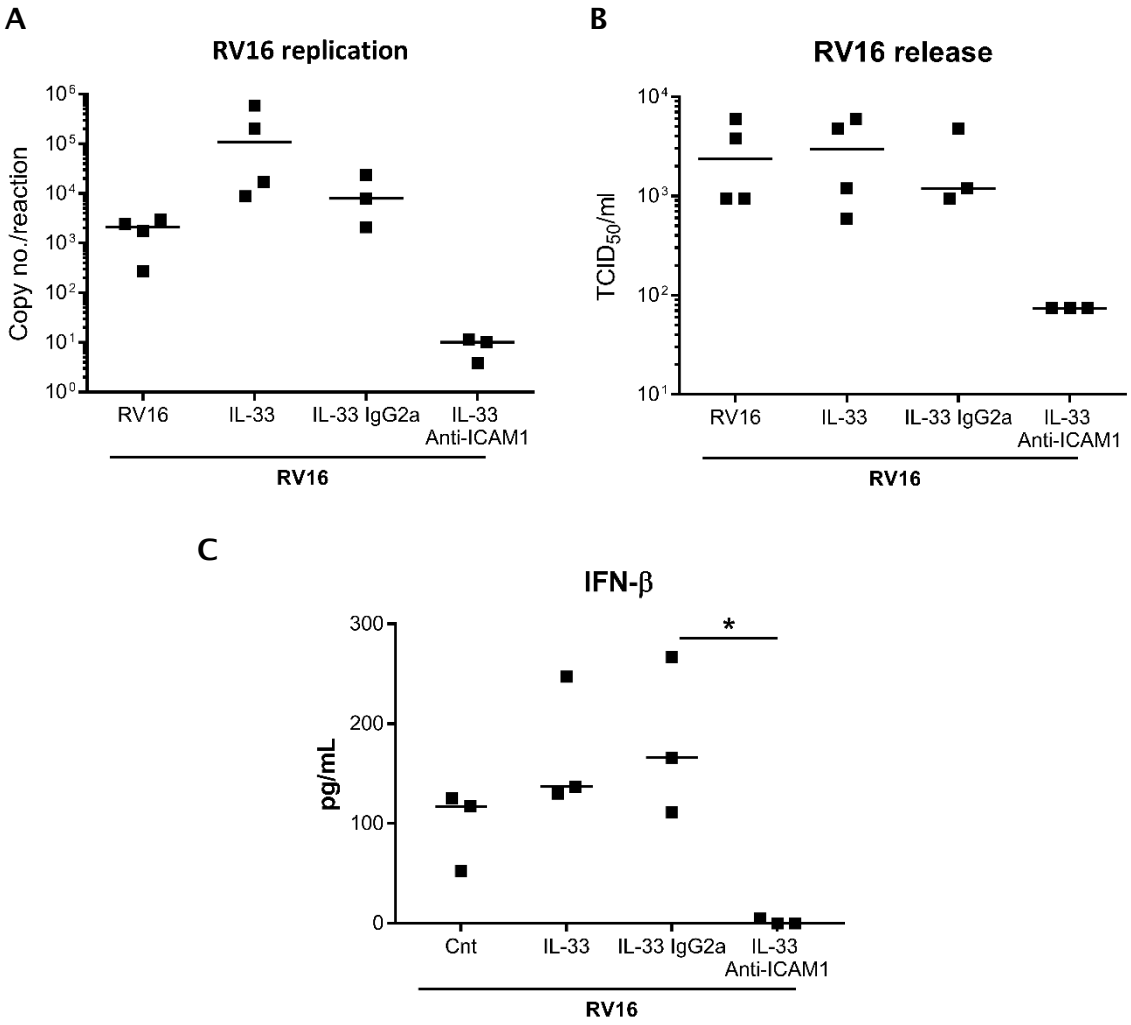

Figure 5-19. CBMC RV16 replication and release and IFN-β production following IL-33 treatment and ICAM1 blockade. CBMCs were pre-treated for 24 hours with or without 10 ng/mL IL-33 alone or in combination with anti-ICAM1 antibody (anti-ICAM1) or an IgG2a isotype control (each at 10 μg/mL). Cells were infected with UV-RV16 or RV16 MOI 7.5 for 24 hours. RV16 replication was determined by RT-qPCR for the viral genome and **(A)** RV16 release by $TCID_{50}$ assay for infectious virus particles in cell free supernatants **(B)**. IFN-β protein was measured in cell free supernatants by MDS assay **(C)**. n=4 UV-RV16 and RV16 with and without IL-33, n=3 UV-RV16/RV16 IL-33 IgG2a or IL-33 anti-ICAM1. Bars at median. * $p \leq 0.05$, one-way ANOVA (Kruskal-Wallis test).

5.3 Discussion

IL-33 is an important cytokine in the induction of Th2 responses from a range of cell types including MCs[198, 215, 271]. Single nucleotide polymorphisms (SNPs) in *IL33*[187] and its receptor *IL1RL1*[186] (ST2) have implicated the cytokine in the development of asthma. Furthermore, IL-33 expression has been demonstrated in association with HRV infection in humans and mice[218, 277], linking IL-33 expression with the major viral trigger of asthma exacerbations. IL-33 is an epithelial derived cytokine released from the epithelium in response to triggers including HRV[218] to which MCs are susceptible to infection[96, 281]. As MCs are susceptible to both HRV infection and IL-33-mediated stimulation, it is possible that these activators modulate each other. In this investigation, human MCs showed a concentration-dependent increase in IL-33-mediated Th2 responses, although these responses were not modulated by RV16 infection. However, in a novel finding IL-33 was found to enhance the anti-viral response of MCs to RV16 infection via an increase in ICAM1. This lead to an increase in RV16 replication but prevented a statistically significant increase in virus release.

In accordance with previous reports, IL-33 but not TSLP treatment induced MC secretion of the Th2 cytokines IL-4, IL-5 and IL-13[198, 215, 272]. Cytokine release increased in an IL-33 concentration dependent manner and in a preliminary investigation IL-4, IL-5 and IL-13 release was greater at 48 hours compared to 24 hours. Unlike previously reported[215], TSLP did not enhance the effects of IL-33-induced Th2 cytokine secretion which may be due to differences in the response of primary MCs versus the LAD2 MC line (used here). In further characterisation of MC responses to IL-33, flow cytometric analysis revealed a concentration-dependent increase in ST2 expression which suggests IL-33 increases MC responsiveness to itself, although receptor expression was back down by 48 hours. IL-33 has also been suggested to increase ST2 expression in murine Treg cells in which the recruitment of GATA3 to the *st2* promoter is enhanced in the presence of IL-33 and also associated with increased binding of RNA polymerase II at the promoter site[282]. TSLPR was also induced by IL-33 demonstrating the lack of a Th2 cytokine response to TSLP was not due to poor receptor expression. IL-33-induced ICAM1 expression has been demonstrated in endothelial cells[283, 284] and murine MCs[285] but this is the first demonstration of IL-33 dependent increases in human MC ICAM1 expression. Furthermore, ICAM1 expression was shown to be maintained at 48 hours. Despite evidence demonstrating IL-33-dependent MC

responses are augmented in combination with other stimuli[214, 215, 272] and the IL-33 dependent increase in its receptor, RV16 infection did not enhance IL-33 mediated Th2 secretion. As a control cells were infected with UV-irradiated RV16, an infectious but non-replicating control. Therefore it is possible that cytokine responses are augmented by infection with either replicating or non-replicating RV16 but a control of no virus would be needed to determine this.

RV16 did not modulate IL-33-dependent MC responses but in line with the IL-33-dependent increase in ICAM1 expression, RV16 replication showed a statistically significant increase in response to IL-33 treatment. In a study investigating HRV-induced type 2 inflammation in asthma, IL-33 was correlated with increased viral load[218]. The authors did not investigate the involvement of MCs, however, it is possible that IL-33 enhanced HRV replication in MCs may have contributed to the increase in viral load. As well as the epithelium, cell types including macrophages[286, 287] and DCs[288], which may come into contract with the epithelium during infection, are susceptible to HRV infection and it would be of interest to understand the impact of IL-33 on their susceptibility to RV16 infection. Unexpectedly, the IL-33-dependent increase in RV16 replication was not associated with a significant increase in the release of infectious RV16 particles. However, IL-33 was also found to enhance IFN and ISG anti-viral responses to RV16 which likely suppressed RV16 infection enough to prevent significant release of infectious virus particles. Pro-inflammatory mediators were also induced, with replication-dependent inductions in *CXCL10* and IL-6 suggesting even in the absence of productive infection IL-33 augments responses which impact cellular anti-viral responses as well as the recruitment of cell types such as T cells, neutrophils and monocytes. IL-33 mediated anti-viral responses have also been demonstrated in BECs, which were shown to increase HRV-induced CXCL10 production with IL-33 treatment[289]. IL-33-mediated CXCL10 production was confirmed by blockade of IRAK1, ST2 or IL-33 which decreased CXCL10 expression. However, ST2 in macrophages is not required for CXCL10 expression and does not increase IFN expression, therefore, the anti-viral IL-33 response may be cell type specific. For instance, in a mouse model of early-life allergen and virus exposure, IL-33 was shown to negatively regulate anti-viral responses[290]. Cockroach extract-induced-IL-33 increased viral load, IL-13 production and airway smooth muscle growth but suppressed IFN-α, IFN-β and ISGs in response to infection with pneumonia virus of mice[290]. The IL-33 mediated suppression in anti-viral responses was suggested to occur via an IL-33-dependent

suppression in IRAK1 in pDCs and further demonstrates the need for cell and pathogen specific investigations into the anti-viral functions of IL-33. IL-33 enhanced MC IFN-β responses to HRV infection and in chapter 2 MCs were demonstrated to produce IFN-λ in response to HRV infection in greater concentrations than IFN-β. IL-33-mediated IFN-λ responses were not investigated, however, this would be of interest as an IL-33 mediated increase in MC IFN-λ may represent a mechanism whereby MCs respond to signals of epithelial damage by producing type III IFN to augment the anti-viral responses of the epithelium. If so this may be particularly important in severe asthma where bronchial IFN responses are impaired but IL-33 expression is increased.

Since MCs are also susceptible for infection by RV1B, a minor group HRV, IL-33-mediated enhancement of RV1B infection was also investigated. Although there was a concentration dependent increase in the expression of LDLR, the receptor for RV1B, this was not associated with an increase in either RV1B replication or release. RV16 and RV1B infections were carried out at MOI 7.5 and 3 respectively, which may explain the difference in IL-33-mediated replication. The IL-33 mediated increase in RV16 replication was associated with an enhancement of anti-viral responses; since IL-33 did not enhance RV1B replication this suggests RV1B anti-viral responses were similarly unaffected by IL-33 treatment but this would need to be confirmed.

Similarly to the LAD2 MC line, CBMCs produced IL-5 and IL-13 and upregulated ST2, ICAM1 and LDLR in response to IL-33. However, unlike LAD2 MCs, IL-4 production was negligible and IL-33 induced lower concentrations of IL-5 and IL-13 and smaller increases in ST2 and LDLR expression, although the upregulation of ICAM1 was greater on CBMCs. A notable difference to LAD2 MCs was the trend of a RV16-dependent enhancement of IL-5 and IL-13 production compared to UV-RV16, which may have been a result of the greater increase in IL-33-induced ICAM1 expression. Differences in levels of cytokine production may be due to the immature nature of CBMCs compared to LAD2 MCs, in addition, CBMCs may have been less responsive to IL-33 due to lower levels of ST2 expression. MC tryptase and chymase are able to cleave IL-33 increasing its potency and activity on cells including MCs[291, 292]. If IL-33/RV16 treatment cause MC protease release, the activity and response to even low concentrations of IL-33 may be greatly amplified. Therefore, it may be relevant to investigate the presence of MC proteases in IL-33/RV16 treated MC supernatants. IL-33 also enhanced CBMC RV16 replication, but not virus release, and increased

RV16-induced IFN-β. The IL-33-dependent increase in IL-5 and IL-13, RV16 replication and IFN-β production neared but did not reach statistical significance (p=0.06). However, the differences in each case were evident suggesting the experiments were underpowered (n=4).

Although LAD2 and CBMC Th2 cytokine responses were steroid sensitive, IL-13 was suppressed to a lesser extent. This may be due to a difference in the transcriptional regulation of IL-13 in MCs such that it has a reduced reliance on NF-κB compared to IL-4 and IL-15.

In the context of impaired bronchial IFN responses, MCs may be more susceptible to HRV infection, particularly in severe asthma. However, this investigation has shown that IL-33, which is released from the epithelium in response to stress including HRV, may protect MCs by augmenting type I IFN responses. Furthermore, IL-33 expression increases in the asthmatic bronchial epithelium with disease severity. This could suggest that in severe disease MCs are protected from HRV infection by IL-33 released from the epithelium. However, unlike IFN-β, IL-33 treatment alone was unable to induce anti-viral responses and exogenous IFN-β treatment had a greater inhibitory effect on HRV replication and release from MCs than IL-33. This demonstrates that if IFN responses are impaired IL-33 is able to provide a level of protection against HRV infection but IFN plays a bigger role in the protection of MCs against HRV infection.

In both LAD2 and CBMCs RV16 replication and release and IFN-β production, including that mediated by IL-33, was inhibited by ICAM1 blockade. Together these data suggest that during RV16 infection of MCs, IL-33 upregulates ICAM1 expression resulting in increased viral replication and an increase in replication-dependent anti-viral responses which supress significant virus release. Previously, MCs were demonstrated to be susceptible to HRV infection and suggested to act as reservoirs for viral infection despite HRV-induced anti-viral responses. However, the current data suggests that in the context of the epithelium, MC anti-viral responses, including IFN-β which was demonstrated to protect MCs against HRV infection, are enhanced and productive RV16 infection dampened. Therefore, IL-33 paradoxically enhances MC anti-viral responses by enhancing RV16 infection.

Chapter 6: Final Discussion

6.1 Summary

Asthma is a chronic respiratory disease in which MCs are an important effector cell and HRVs are a common trigger of exacerbations via mechanisms that are not fully understood. In allergic asthma, MCs localise in increasing numbers to the bronchial epithelium (**Figure 6-1**) which is also the principal site of HRV infection. This suggests that *in vivo* MCs may be susceptible to HRV infection during infection of the epithelium, potentially implicating them in HRV-induced asthma exacerbations. Furthermore, MCs are susceptible to HRV infection *in vitro* and have been shown to respond to viral infection, however, their role in anti-viral immunity, and specifically anti-HRV responses, remains unclear. Therefore, this investigation examined the anti-viral response of MCs to HRV infection.

MCs mounted anti-viral responses including the induction of IFNs and ISGs, however, unlike infection with other viruses, MCs were permissive for productive HRV infection (**Figure 6-1**). The mechanisms of productive versus non-productive MC viral infection are unknown but likely involve a combination of host and viral factors. In severe asthma bronchial epithelial IFN responses are impaired and inhaled IFN-β is in development as a therapy to boost IFN responses and prevent HRV-induced exacerbations. Treatment with exogenous IFN-β protected MCs against infection (**Figure 6-1**) and suggests an inhaled IFN-β therapy would be beneficial in protecting MCs against productive HRV infection, which potentially contributes to HRV-induced exacerbations. Th2 responses play a key role in asthma pathogenesis and IL-33 is an epithelial-derived cytokine which induces Th2 responses in target cells. In allergic asthma, MCs are the major target of IL-33 which has been shown to be released from the epithelium during experimental HRV challenge. Therefore the impact of HRV on IL-33-induced MC Th2 responses was investigated. HRV infection did not modulate MC Th2 responses, however, IL-33 enhanced the induction of IFN-β and ISGs in response to HRV infection and prevented statistically significant virus release (**Figure 6-1**). This suggests that in the context of the MC microenvironment *in vivo,* MCs may be protected from productive HRV infection by signals including IL-33. This may be particularly important in severe asthma where bronchial IFN responses are impaired and may have consequences on the implementation of anti-IL-33 asthma therapies.

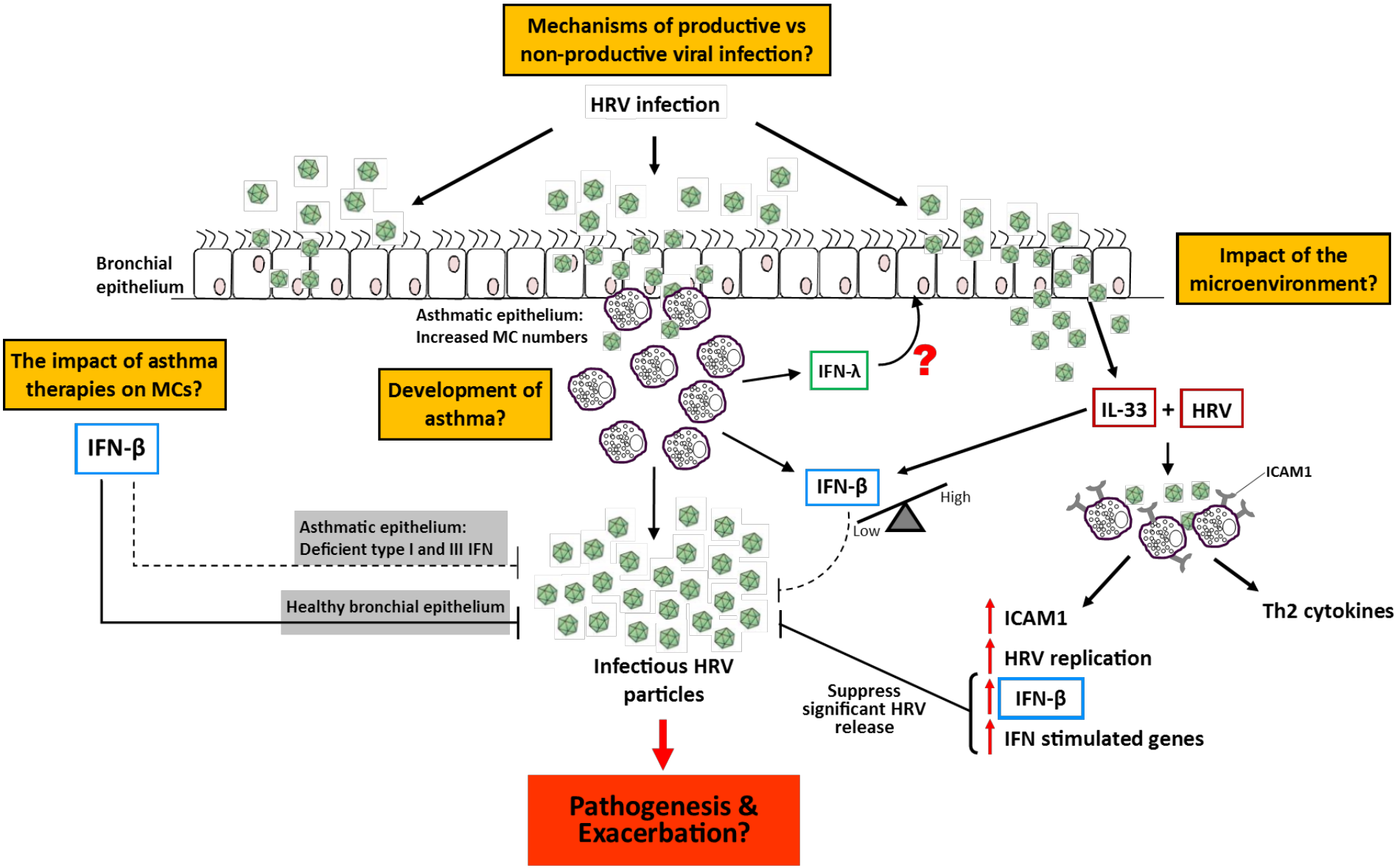
Mechanisms of productive vs non-productive viral infection?
HRV infection
Bronchial epithelium
Impact of the microenvironment?
Asthmatic epithelium: Increased MC numbers
The impact of asthma therapies on MCs?
Development of asthma?
IFN-λ
?
IL-33 + HRV
IFN-β
IFN-β
High
Low
ICAM1
Asthmatic epithelium: Deficient type I and III IFN
Healthy bronchial epithelium
Infectious HRV particles
Th2 cytokines
ICAM1
HRV replication
IFN-β
IFN stimulated genes
Suppress significant HRV release
Pathogenesis & Exacerbation?

Figure 6-1. Schematic summary of the principal findings of this investigation and their potential implications. MCs are permissive for productive HRV infection which may implicate them in the pathogenesis and exacerbation of asthma. Productive HRV infection appears to occur as a result of insufficient HRV-induced type I IFN production, and in support of this, exogenous IFN-β enhanced MC anti-viral responses and prevented productive HRV infection. This suggests inhaled IFN-β therapy may act in part by protecting MCs from HRV infection. IL-33 may be released form the epithelium in response to HRV infection and induces MC Th2 cytokine release. HRV infection does not modulate IL-33-mediated Th2 cytokine release, however, IL-33 increases MC ICAM1 expression and HRV replication resulting in enhanced IFN-β and ISG responses. IL-33 enhancement of anti-viral responses prevents significant HRV release. This suggests that in the context of the epithelium IL-33 may provide protection against MC HRV infection.

6.2 Viral infection

Unlike the infection of MCs by viruses including IAV[239], RSV[93] and reovirus[237], HRV infection results in the release of infectious virus particles. The mechanism behind this appears to a lack of a robust endogenous IFN-β response. This is indicated by the minimal impact of blocking endogenous type I IFN on HRV infection, the protective effect of exogenous IFN-β and the IL-33-mediated suppression of HRV infection via an enhancement of IFN-β responses. Furthermore, statistically significant secretion of type I IFNs (IFN-α) has been demonstrated by MCs in response to reovirus[94] and RSV infection[93], and type I IFN secreted by MCs in response to dengue virus infection has been shown to protect uninfected cells from infection[34]. Therefore, if robust type I IFN responses are normally generated in response to virus infection of MCs, there remains the question of how HRV suppress these responses.

RIG-I and MDA5 mediate IFN signalling via the adaptor protein, IFN-β promoter stimulator 1 (IPS-1) (also known as MAVS, VISA, or Cardiff) resulting in the activation of IRF3 and IFN-β transcription (**Figure 1-5**). HRV can disrupt the detection of viral RNA via the degradation MDA5[293] as well as RIG-I[294] which would result in suppressed IFN signalling. Although MDA5 rather than RIG-I has been demonstrated to detect picornavirus RNA[295], it has been suggested that RIG-I, which detects 5′-triphosphate RNA not present within the picornavirus genome, may also detect picornaviruses via the recognition of non-5′-triphosphate motifs[296]. HRV has been shown to disrupt IFN signalling by the suppression of IRF3 dimerisation and nuclear accumulation[297] as well as the cleavage of IPS-1 by the HRV proteases $2A^{pro}$ and $3C^{pro}$ [298]. To investigate HRV-mediated IFN suppression in MCs, western blotting could be used to detect intact versus potentially cleaved MDA5 and RIG-I over a time course of HRV or UV-HRV infection and treatment with a vehicle control. An increase in cleavage products would suggest HRV-mediated degradation. The generation of and infection with HRV mutants lacking $2A^{pro}$ and $3C^{pro}$ protease activity compared to infection with WT HRV controls may indicate a mechanism of potential degradation. In response to HRV infection, *IRF3* expression was not altered (Supplementary **Table A.1**.) which may have been a result of IRF3 inactivation by HRV. IRF3 activity depends on its phosphorylation, dimerisation and nuclear translocation. Similarly to above, a time-course of HRV infection followed

by analysis of phosphorylated versus un-phosphorylated IRF3 as well as dimerisation of IRF3 (according to protein size) by western blotting, may indicate HRV-induced inactivation. Vesicular stomatitis virus infection of A549 epithelial cells results in IRF3 phosphorylation, dimerisation and nuclear localisation so this may be used as a positive control[297]. Similarly to HRV, RSV is able to inhibit type I IFN production via the non-structural proteins NS1 and NS2 which inhibit IRF3 activation and promote the degradation of STAT2 required for type I IFN signalling[299] (**Figure 1-6**). Despite this, RSV infection of MCs is associated with IFN production and a lack of viral shedding[93]. This may suggest theses mechanisms are subverted by MCs during RSV infection. The mechanism of productive HRV infection needs to be investigated specifically in MCs and is likely to involve a combination of host as well as viral factors. Understanding the mechanisms of productive versus non-productive MC viral infection may provide insights into the mechanisms of MC anti-viral immunity including mechanisms of viral immune evasion in MCs.

An important question is whether MCs are susceptible to HRV infection *in vivo*. The location of MCs at the bronchial epithelium[104] suggests they may come into contact with HRV during infection of the airway, and particularly during asthma which is associated with an increase in bronchial epithelial MC numbers[207, 209]. However, HRV infection of MCs *in vivo* has not been established. The susceptibility of MCs for HRV infection *in vivo* could be investigated with experimental HRV challenge of non-asthmatics versus asthmatics. Ideally bronchial biopsies would be taken over the course of infection and the tissue fixed and sectioned then stained for MCs. *In situ* hybridisation could be used to determine whether hybridisation of RNA probes for HRV co-localise with MC staining. This would determine HRV infection *in vivo* and indicate whether a difference exists in susceptibility to infection between asthmatics and non-asthmatics. Due to the invasive nature of bronchoscopies and the collection of biopsies, taking bronchial biopsies over the course of infection may not be feasible. Therefore a single time-point may be used according to peaks in virus titre e.g. 3-4 days[218] or samples taken over a time course but from age and condition matched patients rather than from individual patients over time.

6.3 The Airway Microenvironment

In vivo MCs exist in a microenvironment in the context of a mixture of cell types and chemical signals. Signals from the local environment impact factors such as

MC maturation, localisation and survival[3], therefore a limitation of this study has been the study of MCs in a monoculture, which does not completely mimic the complexity of the *in vivo* microenvironment. Although it will be difficult for an *in vitro* culture system to entirely recreate the *in vivo* microenvironment, *in vitro* co-culture techniques may go further in understanding the response of MCs *in vivo*. MCs are found in association with mucosal surfaces including the airways and MCs increase in number at the bronchial epithelium during asthma[104] and following HRV infection[217]. Therefore, the bronchial epithelium would be a relevant cell type to co-culture with MCs, particularly in the context of HRV-associated asthma exacerbations. To more closely model *in vivo* conditions MCs may be cultured with differentiated BECs. Unlike submerged cultures of respiratory cell lines and primary cells, culture of primary BECs at an air-liquid interface (ALI) mimics the conditions found in the airway and induces mucociliary differentiation[300]. BECs are seeded onto cell culture inserts (apical compartment) with a permeable membrane via which cells are fed with culture media via the basal compartment. In this system BECs could be co-cultured with MCs in the basal compartment and the co-culture used to interrogate MC and BEC responses to HRV infection in co-culture versus monoculture. For example, BECs could be challenged apically with HRV and a difference in the response of BECs in co-culture would suggest MC-mediated modulation of BEC HRV infection.

In chapter 3 MCs were shown to produce IFN-λ as well as IFN-β in response to HRV infection (**Figure 3-1**). MCs do not express the receptor for IFN-λ suggesting it is produced for the protection of cells including the epithelium. This suggests BECs in co-culture with MCs may be less susceptible to HRV infection. HRV infection of MCs following BEC HRV challenge could also be looked at to investigate whether HRV released from BECs could result in HRV infection of MCs. In addition, the influence of the epithelium on MC HRV infection could be investigated by directly infecting MCs in the basal compartment in the presence and absence of BEC HRV challenge and comparing HRV replication, virus release and anti-viral responses between co-cultures and monocultures.

The epithelium secretes type I IFN in response to HRV infection which could potentially protect MCs from HRV infection. Also, HRV-induced release of IL-33 from BECs may additionally enhance MC anti-viral responses. An influence of the epithelium on MC function has been demonstrated in previous studies. For instance, BECs have been demonstrated to suppress MC degranulation potentially in a

mechanism whereby the epithelium suppresses harmful MC responses[301]. It would be interesting to compare the impact of non-asthmatic BECs versus BECs from patients with mild, moderate and severe asthma, particularly considering the differences in barrier function, IFN and IL-33 expression[151, 176, 177, 184, 256-258]. *In vivo* the epithelium is underlined by a sheath of sub-epithelial fibroblasts embedded within the extracellular matrix which together form an integrated unit termed the epithelial mesenchymal trophic unit (EMTU)[302]. Bidirectional communication within the EMTU regulates process including lung development, repair and inflammation, and in asthma disordered communication within the EMTU contributes to basement membrane thickening associated with airway remodelling[302]. MCs have been shown to interact with and modulate fibroblast function, for example MCs enhance fibroblast contraction in a mechanism involving direct cell-cell contact[303]. In addition, fibroblast are susceptible to productive HRV infection to which they respond with significantly limited IFN-β[262]. Therefore, studies of MCs in co-culture with a model of the EMTU may be particularly relevant to asthma, especially in the context of HRV infection.

A key finding in this investigation was the IL-33 dependent enhancement of MC IFN-β production in response to HRV infection (**Figure 5-14 C**). However, whether this occurs in response to increased RV16 replication following IL-33-dependent increases in ICAM1 expression, or via IL-33 directly inducing IFN production is unclear. IL-33 alone did not induce IFN which could suggest it does not directly signal to induce IFN production. Despite this, it is possible that IL-33 requires additional signals provided by HRV in order to directly induce IFN. IL-33 is an alarmin and in order to drive anti-viral immune responses it may also require signals provided by DAMPs in what may be a mechanism of immune regulation. To investigate the mechanism of IL-33 mediated IFN-β enhancement, MCs could be treated with IFN-β with and without IL-33 to determine whether IL-33 enhanced IFN-β mediated IFN production. Synergy would suggest IL-33 was able to directly signal to induce IFN production. However, as mentioned IL-33 may require a DAMP, therefore, MCs may be pre-treated with IL-33 and infected with poly I:C rather than HRV which would provide the signal of a DAMP but its cellular entry would not depend on ICAM1. Poly I:C signals via TLR3/IRF3 (**Figure 1-6**) and triggers the activation of IFN signalling mediators such as IRF3 and an increase in IFN-β production. These factors should be enhanced if IL-33 directly enhances type I IFN

and suppressed if IL-33 signalling is blocked, e.g. via a block on IRAK1/4 which is at the top of the IL-33 signalling cascade (**Figure 1-7**).

IL-33 is expressed as a number of splice variants and a particular variant, lacking exons 3 and 4 (Δexon 3,4), is associated with allergic asthma[188]. This variant is actively secreted from epithelial cells and may act on MCs *in vivo*, however, the IL-33 variant is reduced in its capacity to induce MC (HMC-1 cell line) CXCL8 release compared to full length IL-33[188]. This suggest there may also be differences in the response of MCs to the Δexon 3,4 variant including responses to HRV infection. This could be investigated by pre-treating MCs with and without full length IL-33 versus the Δexon 3,4 variant prior to infection with HRV and comparing responses including Th2 and anti-viral responses. As the variant is actively secreted from epithelial cells, it may act on MCs prior to IL-33 released from the epithelium in response to triggers including HRV. Therefore, the affinity of the variant versus full length IL-33 for its receptor is relevant in understanding which form of IL-33 and its associated responses dominate at baseline and in the context of HRV infection.

In vivo MCs exist in the context of other cell types and their responses to stimuli are part of an integrated response of the microenvironment. Understanding and modelling this environment will improve the understanding of the function and implication of MC responses *in vivo*.

6.4 The Development of Asthma

Asthmatic airways are characterised by structural changes which include areas of epithelial injury, increased deposition of extracellular matrix, goblet cell hyperplasia, sub-epithelial fibrosis and smooth muscle cell hypertrophy[117, 208], contributing to the narrowing of the airways at baseline. These structural changes are collectively referred to as airway remodelling and can be detected in children with asthma[304]. MCs have been implicated in this process in a study which found mucosal MC numbers in children aged 1 were associated with increased reticular membrane thickness and respiratory morbidity and were predictive of ICS use by age 3[270]. Persistent childhood wheeze, particularly wheeze associated with aeroallergen sensitisation, is a risk factor for the development of asthma[305]. Furthermore, lower respiratory tract infections in the context of aeroallergen sensitisation markedly increases the risk of persistent asthma, particularly in the case of frequent viral lower respiratory tract infections and high levels of circulating

IgE[305]. This suggests that MCs are important in the development of asthma via allergen specific IgE mediated sensitisation. In addition, MCs harbouring HRV infection in infants may contribute to the development, frequency and severity of lower respiratory tract viral infections which are all asthma risk factors[305]. The infant immune system goes through a process of maturation, therefore differences exist between the mature and infant immune responses including differences in infection susceptibility. This raises the question of whether MCs in childhood differ in their susceptibility for and responses to HRV infection. CBMCs are considered an immature MC type while peripheral blood-derived MCs and CBMCs treated with serum are considered to have a more mature phenotype[3, 220]. Therefore, comparison of HRV infection of these cell types may give an insight into paediatric versus mature MC responses. There is heterogeneity in the molecular and secretory profile of CBMCs versus peripheral blood-derived MCs[306] which suggests immature and mature MCs may respond differently to HRV infection. It may be hypothesised that due to the immature nature of the paediatric immune system theses MCs may be more susceptible to productive HRV infection, which could indicate that MCs contribute to the development of asthma by sustaining HRV infection. However, it is also possible that differences in paediatric versus mature MCs may render paediatric MCs resistant to productive HRV infection. It would also be interesting to investigate whether there are difference in the MCs, for example the susceptibility for HRV infection, of children with non-persistent wheeze/no asthma versus those with persistent wheeze/asthma.

6.5 Asthma Therapies

Despite adherence to current asthma treatments, some patients suffer from uncontrolled severe asthma which is an unmet need that is driving the development of new asthma therapies. These therapies will directly or indirectly impact the functions of a number of cell types including MCs. This investigation has demonstrated that although MCs are susceptible for productive HRV infection, they also induce anti-viral responses which are augmented by IFN-β; both exogenous IFN-β and IL-33 induced IFN-β. Therefore, it may be relevant to consider the impact of asthma therapies on pathogenic as well as anti-viral MC responses.

Glucocorticoids are routinely used to suppress airway inflammation in asthma. Upon glucocorticoid binding of the glucocorticoid receptor (GR) located within the cytosol, the receptor is activated and translocated to the nucleus. Here the activated

GR mediates its effects via the direct binding of DNA sequences or indirectly via interactions with other transcription regulators[307]. In human MCs, dexamethasone suppresses IgE-dependent FcεRI expression[308], while this does not result in an inhibition of allergen-induced FcεRI-dependent MC degranulation[308, 309], glucocorticoids can impact other MC functions such as cytokine release. Not all MC cytokine release is steroid sensitive[310], however, dexamethasone suppresses anti-IgE-induced CCL2, CCL7, CXCL3, and CXCL8 expression from human MCs[311] and has also been shown to inhibit FcεRI-dependent expression of GM-CSF and TNF-α[308]. Furthermore, IL-33-mediated cytokine secretion by murine MCs, as well as IL-33 enhancement of IgE-mediated cytokine release from human MCs, is suppressed by dexamethasone treatment[312]. This is in agreement with this investigation which showed that IL-33-mediated Th2 cytokine release and enhancement of ST2 and ICAM1 expression were sensitive to fluticasone propionate treatment (**Figure 5-4** and **Figure 5-5**). While glucocorticoids inhibit MC responses which may be detrimental to asthma pathogenesis, the inhibition of MC chemokine secretion may suppress the recruitment of effector cells to sites of infection and therefore negatively impact MC innate immune functions.

In asthma, MC localisation to the bronchial epithelium and ASM but not to the submucosa is suppressed by the use of ICS[313]. This could indicate that ICS diminish the interaction between MCs and the bronchial epithelium minimising their pathogenic responses in asthma and potentially their role in anti-viral immunity during infection of the epithelium. However, as mentioned the epithelium functions with underlying fibroblasts within the integrated network of the EMTU in which there is close communication between fibroblasts and the epithelium and vice versa. MCs can directly interact with fibroblasts and may provide a link between submucosal MCs and the epithelium. Fibroblasts are also susceptible for productive HRV infection, therefore submucosal MCs may also be important in the detection of and response to viral infection of the epithelium. Glucocorticoids inhibit the production of type I IFN and type I IFN mediated signalling[314] suggesting glucocorticoids could also negatively impact MC anti-viral activity by suppressing the availability of IFN within the microenvironment and the ability of MCs to respond to available IFN. For example, glucocorticoid treatment of BECs prior to HRV or IVA infection, suppresses the induction of innate anti-viral responses and is associated with increases in viral replication[315]. In addition, mice treated with steroid develop more severe IAV infection with increased viral load and airway

inflammation[315]. Glucocorticoids have also been shown to mediate immune-enhancing effects, which may be dependent on glucocorticoid dose and the sequence and timing of exposure to inflammatory mediators and steroid[316]. Also, dexamethasone can increase MC chemokine secretion[311] suggesting glucocorticoids could potentially enhance MC immune functions in certain situations. Therefore, it would be relevant to investigate MC HRV infection in the context of glucocorticoid treatment to determine how the balance between suppressive and stimulatory glucocorticoid-mediated responses impacts MC HRV infection. MCs produce and secrete eicosanoids including LCT_4 which can promote bronchoconstriction[13]. In asthma leukotriene receptor antagonists used as an add on therapy in addition to ICS but on their own are less effective at controlling symptoms compared to ICS.

Anti-IgE therapy (omalizumab) is used as an add-on therapy in patients with severe asthma. IgE promotes MC survival and is a MC chemoattractant[3], therefore anti-IgE therapy may decrease MC numbers and alter MC distribution within the airways. MCs are sentinels for infection at sites exposed to the external environment detecting and responding to infection as well as recruiting other effector cells. Therefore, a significant decrease in MC numbers may suppress and/or delay in MC-mediated immune responses. As discussed above, an alteration in MC localisation may also impact MC immune functions. As IgE is a MC chemotactic factor, anti-IgE may influence the migration of MC progenitors from the bone marrow into tissues and impact MC maturation and phenotype which is influenced by the local cytokine milieu.

Anti-IgE reduces the risk of exacerbations[317, 318] and in children and young people anti-IgE has been shown to suppress seasonal exacerbations associated with viral respiratory infections[319]. Anti-IgE suppression of exacerbations may be due to a suppression in MC degranulation and the release of MC mediators, such as histamine and leukotrienes, which contribute to bronchoconstriction. Anti-IgE mediated suppression of seasonal exacerbations (e.g. common cold season) may indicate that allergen sensitisation/IgE activation of MCs increases MC HRV infection. For instance, in allergic individuals, allergen challenge has been shown to induce nasal epithelial ICAM1 expression[320] and IgE cross-linking has also been demonstrated to suppress anti-viral responses including IFN-α and TLR7 in pDCs[321] and IFN-γ in monocytes[322] in response to influenza challenge. This suggests anti-IgE therapy may provide MCs with protection against HRV and viral infection. To

investigate the impact of sensitisation and FcεRI cross-linking on the responses of MCs to HRV infection, MCs may be sensitised with monomeric IgE then infected with HRV/UV-HRV or sensitised then treated with anti-IgE antibody prior to HRV/UV-HRV infection. In each case HRV replication and release and MC anti-viral responses, including IFN and ISG induction, would be compared to HRV infection of MCs in the absences of sensitisation/FcεRI cross-linking.

In addition to current asthma therapies new therapies are being developed and are undergoing clinical trials. These include antibodies directed against IL-4 and IL-13 and their receptors which aim to block their Th2 responses including mucus production, IgE class switching, eosinophil recruitment and smooth muscle proliferation [323]. An anti-TSLP antibody is also in clinical trials. MCs have been reported to respond to TSLP, in combination with IL-33, with the release of cytokines including IL-5, IL-13, GM-CSF, IL-6, and CXCL8[215] as well as IL-10 and CCL1 when treated with TSLP in combination with IL-1 and TNF-α[272]. However, unlike IL-33 treatment, in this investigation MCs were unresponsive to TSLP even when used in combination with IL-33. Anti-IL-33 (ANB020) and anti-ST2 (AMG 282) antibodies are in clinical trials for indications including the treatment of severe asthma. The efficacy of anti-IL-33 therapies in the treatment of asthma has been suggested in a mouse model of allergic airways disease. In this model, anti-IL-33 treatment suppressed serum IgE, lung eosinophilia, concentrations of IL-4, IL-5 and IL-13 in BAL fluid and mucus hypersecretion[324].

The majority of the literature on IL-33 is concerned with its detrimental contribution to Th2 immune responses, however, this investigation has demonstrated that in the context of virus infection IL-33 is able to upregulate MC IFN-β. IFN-β is a key cytokine in the initiation of anti-viral responses and the protection of immune as well as structural cells. *In vivo* bronchial epithelial responses are likely required to fully protect MCs against productive HRV infection but in the case of impaired epithelial IFN responses, IL-33 may provide a second line of defence. Therefore, blockade of IL-33/ST2 may negatively impact MC anti-viral responses. Other reports have also demonstrated IL-33-mediated immune enhancement, for example in BECs[289] as well as in a mouse model of herpes simplex virus encephalitis, where MC deficient mice (*Kit*$^{W/W\text{-}v}$) reconstituted with ST2 deficient MCs had decreased survival rates compared to mice reconstituted with WT MCs[325]. conversely IL-33 has also been reported to mediate immune suppression in sensitised mice[290]. IL-33 immune enhancement versus immune suppression may depend on the cell type or model

used as well as sensitisation and the temporal relationship between IL-33 and other factors. IL-33 is an alarmin i.e. it is released from cells in response to damage and/or stress and as previously discussed, IL-33 exists in a number of splice variants where the Δexon 3,4 variant associated with allergic asthma is actively secreted from the epithelium[188]. The active secretion of this variant and its association with allergic asthma suggests that there may be a difference in the function of IL-33 spice variants. Full length IL-33 may act as an alarmin mediating anti-viral immune responses during infection but the actively and potentially constitutively secreted variant may mediate pathogenic Th2 responses in allergy. This would require anti-IL-33 therapies that targeted disease causing IL-33 variants while maintaining the immune enhancing functions of full length IL-33; furthermore, IL-33 splice variants may potentially be used as biomarkers predictive of the efficacy of anti-IL-33 therapies in individual patients.

Overall, the impact of asthma therapies on MC immune responses may depend on how the therapy impacts MC number, distribution and function and the importance of the contribution of MC anti-viral responses within a particular microenvironment.

6.6 Concluding Remarks

This investigation demonstrates a novel role for MCs and IL-33 in innate immunity against HRV infection and highlights the importance of the MC microenvironment in mediating fully effective MC anti-viral responses. For instance, MCs require type I IFN for the suppression of HRV infection and IL-33 enhances MC anti-viral responses. In the development of novel asthma therapies, the anti-viral functions of MCs and IL-33 pro-immune functions should be taken into account in order to design treatments which inhibit pathogenic responses while maintaining protective immune functions.

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Appendix A: Supplementary data

A.1 Table A.1. RT-qPCR Ct values of LAD2 MCs in response to RV16 infection.

LAD2 MCs were exposed to RV16 at an MOI of 0.3, 3 or 7.5 or UV-HRV MOI 7.5 (control). Cell pellets were collected 24 hours post infection for gene expression by RT-qPCR. n=5.

		LAD2 RV16 median Ct (min-max)			
		UV-HRV	**MOI 0.3**	**MOI 3**	**MOI 7.5**
Sensors of viral RNA	***TLR3***	37 (35-44)	34 (31-38)	31 (28-33)	31 (28-32)
	MAD5 (*IFIH1*)	26 (26-27)	25 (23-26)	22 (22-24)	23 (22-24)
	RIG-I (*DDX58*)	28 (26-31)	25 (25-26)	24 (21-27)	24 (21-25)
IFNs	***IFNB1***	35 (34-35)	30 (26-34)	25 (24-25)	25 (24-25)
	IFNL1	37 (n.d-38)	31 (26-32)	25 (24-26)	24 (24-25)
IRFs	***IRF3***	23 (20-24)	24 (20-25)	24 (20-26)	22 (20-25)
	IRF7	29 (28-31)	27 (26-27)	25 (23-30)	25 (25-26)
	IRF9	27 (26-27)	25 (24-26)	25 (24-26)	25 (24-25)
Anti-viral effectors	***MX1***	30 (30-35)	28 (23-29)	23 (21-25)	22 (19-24)
	OAS1	28 (26-30)	24 (21-25)	20 (18-21)	21 (16-23)
	Viperin (*RSAD2*)	31 (30-32)	29 (26-32)	23 (22-24)	23 (23-25)
	PKR (*EIF2AK2*)	23 (23-27)	24 (23-26)	23 (23-26)	25 (22-25)
Cytokines & Chemokines	***CXCL10***	35 (34-35)	31 (28-32)	27 (25-28)	26 (25-28)
	CCL5	26 (26-27)	26 (25-26)	23 (23-24)	23 (22-24)
	CCL2	25 (23-25)	25 (23-25)	25 (24-27)	25 (25-26)
	TNF	34 (31-36)	33 (31-35)	31 (30-34)	30 (29-31)
	TSLP	33 (30-39)	33 (31-48)	33 (32-35)	33 (32-36)
	IL33	35 (34-46)	35 (34-48)	36 (34-41)	35 (35-36)
HRV Receptors	***ICAM1***	23 (21-24)	23 (22-26)	24 (22-29)	25 (20-35)
	VLDLR	33 (33-36)	34 (33-36)	34 (33-35)	33 (28-36)
	CDHR3	38 (36-39)	38 (36-39)	37 (36-39)	37 (37-39)

A.2 **Table A.2. RT-qPCR Ct values of housekeeping genes, UBC and GAPDH, expressed in LAD2 MCs exposed to RV16.** LAD2 MCs were exposed to RV16 at an MOI of 0.3, 3 or 7.5 or UV-RV16 MOI 7.5 (control). Cell pellets were collected 24 hours post-infection for gene expression by RT-qPCR. Ct values are the geometric means of UBC and GAPDH. n=5. UBC, Ubiquitin C, GAPDH, ghlyceraldehyde-3-phosphate dehydrogenase.

Geometric mean of UBC and GAPDH Ct

	UV-HRV	MOI 0.3	MOI 3	MOI 7.5
	20	20	20	20
	19	19	20	21
	20	21	22	21
	21	21	21	20
	21	21	21	21
Ave Ct	20	20	21	21

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Appendix C: Publications

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ORIGINAL ARTICLE **Basic Mechanisms in Allergic Disease**

Mast cells are permissive for rhinovirus replication: potential implications for asthma exacerbations

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Clinical & Experimental Allergy

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Summary

Background Human rhinoviruses (HRVs) are a major trigger of asthma exacerbations, with the bronchial epithelium being the major site of HRV infection and replication. Mast cells (MCs) play a key role in asthma where their numbers are increased in the bronchial epithelium with increasing disease severity.

Objective In view of the emerging role of MCs in innate immunity and increased localization to the asthmatic bronchial epithelium, we investigated whether HRV infection of MCs generated innate immune responses which were protective against infection.

Methods The LAD2 MC line or primary human cord blood-derived MCs (CBMCs) were infected with HRV or UV-irradiated HRV at increasing multiplicities of infection (MOI) without or with IFN-β or IFN-λ. After 24 h, innate immune responses were assessed by RT-qPCR and IFN protein release by ELISA. Viral replication was determined by RT-qPCR and virion release by $TCID_{50}$ assay.

Results HRV infection of LAD2 MCs induced expression of IFN-β, IFN-λ and IFN-stimulated genes. However, LAD2 MCs were permissive for HRV replication and release of infectious HRV particles. Similar findings were observed with CBMCs. Neutralization of the type I IFN receptor had minimal effects on viral shedding, suggesting that endogenous type I IFN signalling offered limited protection against HRV. However, augmentation of these responses by exogenous IFN-β, but not IFN-λ, protected MCs against HRV infection.

Conclusion and Clinical Relevance MCs are permissive for the replication and release of HRV, which is prevented by exogenous IFN-β treatment. Taken together, these findings suggest a novel mechanism whereby MCs may contribute to HRV-induced asthma exacerbations.

Keywords asthma, interferon, innate immunity, mast cells, virus
Submitted 2 September 2016; revised 28 October 2016; accepted 22 November 2016

Introduction

Asthma is a complex and heterogeneous chronic respiratory disease affecting over 300 million people worldwide [1]. It is characterized by airway inflammation and variable and reversible airway obstruction resulting in symptoms of wheeze, chest tightness and shortness of breath [2]. Human rhinoviruses (HRVs) are a major risk factor for asthma development in early life [3] and are the major cause of viral-induced exacerbations of asthma [4, 5]. There are over 100 serotypes of HRV which fall into three species (HRV-A, HRV-B and HRV-C) that use different receptors to enter their target cell [6]. The majority of HRV-A and all of HRV-B use ICAM-1 (major group), the remaining HRV-A use members of the low-density lipoprotein receptor (LDLR) family (minor group) [7], and the recently discovered HRV-C species uses cadherin-related family member 3 (CDHR3) [8]. A number of cell types are susceptible to HRV infection, including the airway epithelium, which is the principal site of HRV replication [9], and innate immune cells such as macrophages [10, 11] and dendritic cells [12]. These cells detect HRVs via a number of pattern recognition receptors (PRRs), which trigger immune responses including the expression of type I and III IFNs, cytokines and chemokines [13, 14]. IFNs induce a range of IFN-stimulated genes (ISGs) via which they mediate their antiviral activities [15].

Deficiencies in IFN production have been reported in bronchial epithelial cells and bronchoalveolar lavage macrophages from asthmatic subjects [16–18]; however, this may relate to severity of disease [19, 20].

Mast cells (MCs) are tissue-resident innate immune cells found predominantly in vascularized tissues, which interface with the external environment including the skin, gastrointestinal tract and the airways [21]. They are bone marrow-derived haematopoietic cells, which are classically associated with the early-phase allergic reaction in asthma [22]. In asthma, MC numbers are increased in the bronchial epithelium, submucosal glands and bronchial smooth muscle where they have an activated phenotype [23–25]. Recent studies have shown that MC location and phenotype change with increasing asthma severity and are closely related to Th2 biomarkers [26–29]. During experimental HRV infection, MC numbers are increased in the bronchial epithelium of asthmatics [30] putting them in close proximity to the major site of HRV replication where they may contribute to viral immunity during HRV-induced asthma exacerbations.

Aside from their well-established roles in allergic disorders, MCs are ideally placed within the airways to act as sentinels of the immune system and protect the body from invading pathogens. MCs express a range of PRRs including TLRs, retinoic acid-inducible gene (RIG)-I-like family receptors and NOD-like receptors and have roles in immunity to parasite and bacterial infections [31]. Following bacterial exposure, MCs release cytokines and chemokines that recruit and activate effector cells including neutrophils which clear the infection and dendritic cells which induce acquired immune responses [32]. While MCs play a key role in innate immunity towards parasites and bacteria, their role in viral immunity is less clear. In response to dengue virus, MCs release chemokines and cytokines, which recruit NK cells and cytotoxic T cells [33–35], and MCs release IFNs following TLR3 activation, influenza virus, respiratory syncytial virus (RSV) [36] and sendai virus infection (a murine virus used to model human parainfluenza virus infection) [37]. Following RSV infection, cord blood-derived MCs (CBMCs) increase type I IFN expression and release CXCL10 [interferon gamma-induced protein 10 (IP-10)], CCL5 [regulated on activation, normal T cell expressed and secreted (RANTES)] and CCL4 [macrophage inflammatory protein-1β (MIP-1β)], which are associated with NK cell, T cell and monocyte recruitment, respectively [38]. However in mouse models of influenza A virus (IAV) infection, MC-deficient mice are less susceptible to influenza-induced weight loss than MC knock-in mice, suggesting MC activity was detrimental during the infection [39]. Regarding HRV, there is only a single study, which has demonstrated the immature HMC-1 cell line can be infected with HRV14; however, this did not trigger any responses unless the cells were also challenged with other stimuli [40].

In view of the importance of HRV-induced asthma exacerbations and the localization of MCs within asthmatic airways [25], we have investigated the innate immune response of mature LAD2 MCs or primary CBMCs to major and minor group HRV exposure and determined their susceptibility to infection. Exposure of human MCs to HRV induced increases in type I IFNs, ISGs and inflammatory mediators. However, MCs were susceptible to HRV infection and were permissive for viral replication and production of infectious virus particles. Exogenous IFN-β treatment was protective against infection and this may have important consequences in moderate/severe asthma where epithelial IFN responses are impaired [16, 41].

Methods

Reagents

Human IFN-β and IFN-λ1 were purchased from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, UK), and stem cell factor (SCF), IL-6 and IL-3 were purchased from Peprotech (London, UK). Mouse anti-human IFN-α/βR chain 2 antibody (anti-IFNAR2, IgG2a, clone MMHAR2) was purchased from PBL assay science (Piscataway, USA) and mouse IgG2a isotype control was purchased from R&D systems (Abingdon, UK). Unless otherwise stated, all other cell culture medium and reagents were purchased from Thermo Fisher Scientific (Inchinnan, UK).

Cell culture

The human MC line LAD2 [42] was maintained in StemPro®-34 serum-free medium supplemented with SCF (100 ng/mL), L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL). Culture medium was replenished weekly by hemidepletion. Cells were > 99% positive for CD117 and FcεRI expression as determined by flow cytometry.

Human cord blood-derived MCs (CBMCs) were derived from $CD34^+$ cord blood mononuclear cells (Stemcell Technologies, Grenoble, France). $CD34^+$ cells were maintained in StemPro®-34 medium supplemented with IL-3 (30 ng/mL, 1st week only), IL-6 (100 ng/mL) and SCF (100 ng/mL) for a minimum of 8 weeks. CBMCs were > 99% pure by flow cytometric analysis of CD117 expression.

The human bronchial epithelial cell (BEC) line, 16HBE-14o- (16HBE) [43], was maintained in MEM-GlutaMax™ supplemented with FBS (10% v/v), penicillin (100 U/mL) and streptomycin (100 μg/mL) (16HBE

medium) and seeded in six-well plates precoated with collagen (30 μg/mL; Advanced BioMetrix, San Diego, USA) prior to use in experiments.

Human rhinovirus stocks

RV16 (major group) and RV1B (minor group) stocks were generated using HeLa cells as previously described [44]. Virus titres of cell-free supernatant stocks were determined by tissue culture infective dose 50% ($TCID_{50}$)/mL according to the Spearman–Karber method. Controls of UV-irradiated HRV (1200 mJ/cm^2 on ice for 50 min) were included in all experiments.

Human rhinovirus infection and treatment of cells

LAD2 MCs or CBMCs (0.5×10^6 cells/mL) were infected with RV16 or RV1B (multiplicity of infection (MOI) 0.3, 3 or 7.5) or UV-HRV (MOI 7.5) or treated with HRV infection medium [MEM-Glutamax™plus FBS (4% (v/v)), nonessential amino acids (1% (v/v)), penicillin (100 U/mL)/streptomycin (100 μg/mL), HEPES (16 mM), $NaHCO_3$ (0.12% (v/v)), tryptose (0.118% (v/v)) and $MgCl_2$ (0.3 mM)] (IM; mock infection) as a control for 1 h before washing with StemPro®-34 medium to remove excess virus. Cells were then incubated at 37°C and cell-free supernatants and cell pellets harvested at 24 h. Cell viability was determined by trypan blue exclusion. As a positive control, 16HBE cells were infected with RV16 (MOI 0.3, 3 or 7.5), UV-RV16 (MOI 7.5) or HRV infection medium following overnight starvation in 16HBE medium with 2% (v/v) FBS. In selected experiments, cells were treated with IFN-β (100 IU/mL) or IFN-λ1 (100 IU/mL) at the time of RV16 infection or pretreated with anti-IFNAR2 antibody (1 μg/mL) or IgG2a isotype control (1 μg/mL) 1 h prior to RV16 infection.

Real-time quantitative PCR

Total RNA was isolated [Trizol reagent or Qiagen RNeasy mini kit (Manchester, UK)] and treated for genomic DNA contamination prior to quantification and reverse transcription to cDNA (Primerdesign, Southampton, UK). For each quantitative RT-PCR (RT-qPCR), cDNA (12.5 ng) was mixed with PCR master mix containing primer/fluorogenic probes (*IFNB1*, *IFNL1*, interferon regulatory factor-7 (*IRF7*), MX dynamin-like GTPase 1 (*MX1*), melanoma differentiation-associated gene 5 (*MDA5*), *CXCL10*, *CCL5*, RV16, RV1B, cadherin-related family member 3 (*CDHR3*) or the housekeeping genes (HKGs) *GAPDH* and ubiquitin C (*UBC*) for detection of specific amplification products or primer/SYBR green intercalating dye (2′-5′-oligoadenylate synthase 1 (*OAS1*) for detection of double-stranded amplification products as designed by the manufacturer [Primerdesign, Southampton, UK, or Thermo Fisher Scientific (CDHR only)]. All reactions were performed in duplicate for 50 cycles and gene expression analysed using a real-time PCR iCycler (Bio-Rad, Hemel Hempstead, UK). For SYBR green detection-based reactions, melt curves were performed to ensure single PCR product formation. Gene expression was normalized to the geometric means of HKGs and fold changes calculated relative to UV-HRV controls according to the $^{\Delta\Delta}$Ct method and expressed as $2^{-\Delta\Delta Ct}$. Viral RNA copy number was determined against a standard curve of known copies of RV16 or RV1B (Primerdesign, Southampton, UK).

ELISA

IFN-β and IFN-λ protein was quantified in concentrated cell-free supernatants by ELISA according to the manufacturer's instructions (IFN-λ 1/3; R&D systems, Abingdon, UK. IFN-β; MSD, Gaithersburg, MD, USA). Supernatants were concentrated (4×) using 3000 nominal MW limit ultrafiltration units (Merck Millipore, Watford, UK).

Statistical analysis

Paired nonparametric data were analysed with Friedman repeated-measures one-way ANOVA by ranks with Dunn's correction for multiple comparisons or Wilcoxon signed rank test for matched pair comparisons. Unpaired nonparametric data were analysed with Kruskal–Wallis one-way ANOVA with Dunn's correction for multiple comparisons or Mann–Whitney ranked sum test for matched pair comparisons. Data are presented as box and whisker plots showing the median, interquartile range and minimum and maximum values or as floating bars showing median and range. Normalized data were analysed by Student's *t*-test and are presented as mean ± SEM. All data were analysed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). $P \leq 0.05$ was considered statistically significant.

Results

The human mast cell line LAD2 mounts an innate immune response to human rhinovirus infection

To investigate the role of MCs in HRV immunity, LAD2 MCs were exposed to HRV or UV-HRV (as a control) and innate immune responses assessed by RT-qPCR after 24 h. Exposure to the major group virus, RV16, resulted in a significant MOI-dependent increase in mRNA expression of the type I and type III IFNs, *IFNB1* and *IFNL1*, respectively (Fig. 1a). There was also a trend for increased IFN-β and IFN-λ protein release, as detected by ELISA, but this failed to reach statistical

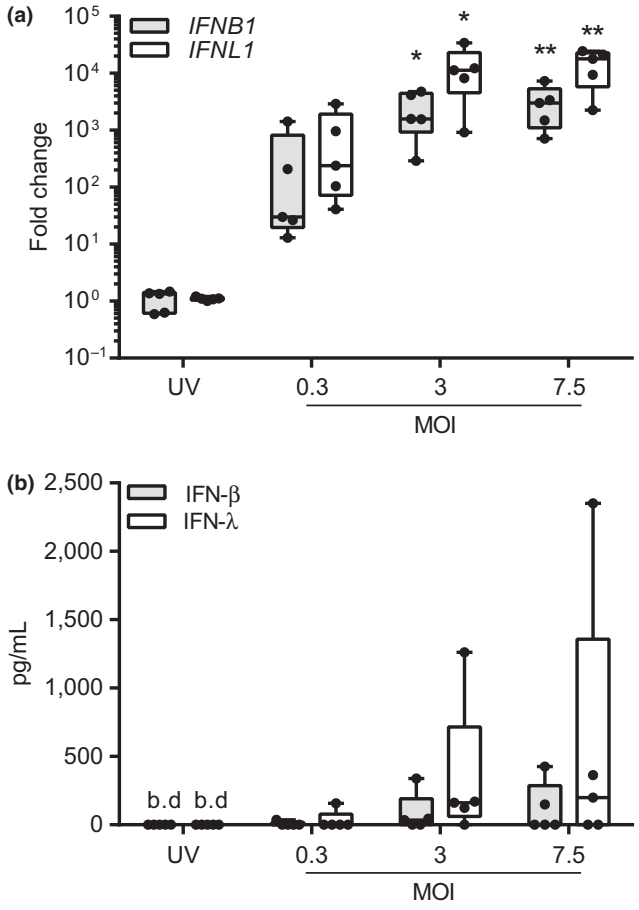

Fig. 1. RV16-induced IFN responses in LAD2 MCs. LAD2 MCs were exposed to RV16 MOI 0.3, 3 or 7.5 or UV-RV16 MOI 7.5 (control). Cell pellets and cell-free supernatants were harvested for gene and protein expression by RT-qPCR and ELISA, respectively. (a) *IFNB1* and *IFNL1* mRNA expression 24 h post-RV16 infection, $n = 5$. (b) IFN-β and IFN-λ protein expression 24 h post-RV16 infection, $n = 5$. Results are box and whisker plots showing the median, interquartile range and min and max values, $*P \leq 0.05$, $**P \leq 0.01$ vs. UV-RV16. MOI, multiplicity of infection. b.d., below limit of detection.

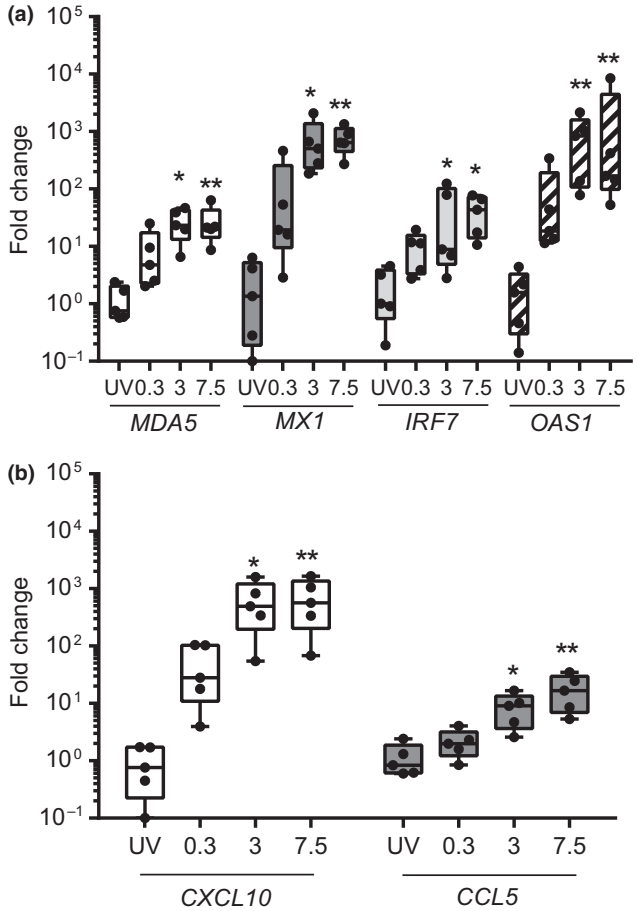

Fig. 2. RV16-induced innate immune responses in LAD2 MCs. LAD2 MCs were exposed to RV16 at MOI 0.3, 3 or 7.5 or UV-RV16 MOI 7.5 (control). Cell pellets were harvested for gene expression by RT-qPCR. (a) mRNA expression of interferon-stimulated genes (*MDA5*, *MX1*, *IRF7* and *OAS1*) and (b) chemokines (*CXCL10* and *CCL5*) 24 h post-RV16 infection. Results are box and whisker plots showing the median, interquartile range and min and max values, $n = 5$, $*P \leq 0.05$, $**P \leq 0.01$ vs. UV-RV16. MOI, multiplicity of infection.

significance (Fig. 1b). There was minimal induction of IFN mRNA or protein with UV-HRV MOI 7.5 [median fold change, *IFNB1*, 1.3 (IQR 0.6–1.4), *IFNL1*, 1.1 (IQR 1.0–1.2)] or mock infection (median fold change, *IFNB1*, 1.2 (IQR 0.9–1.8), *IFNL1*, 2.3 (IQR 0.6–11), data not shown), indicating that virus replication was required to induce the observed responses. In control experiments, RV16 did not induce MC degranulation (Fig. S1). Similar results were obtained with RV1B (minor group virus; Fig. S2a).

In parallel with the upregulation of IFNs, we also observed significant upregulation of antiviral genes following exposure of LAD2 MCs to HRV. This included the MOI-dependent induction of *MDA5*, *MX1*, *IRF7* and *OAS1* following exposure to RV16 (Fig. 2a). Additionally mRNA transcripts for the inflammatory mediators *CXCL10* and *CCL5* were also induced (Fig. 2b). In all cases, induction of ISG transcripts was dependent on viral replication as a lack of induction was observed with mock infection or UV-HRV (MOI 7.5) controls. Similar results were obtained with RV1B (Fig. S2b,c).

The human mast cell line LAD2 is permissive for human rhinovirus replication and releases infectious virus particles

Our data demonstrated that the innate immune responses of LAD2 MCs to HRV was dependent on viral replication as these responses were not observed using the replication deficient UV-HRV control or mock infection media. Therefore, we used RT-qPCR to assess viral copy number in MCs and compared this to HRV-infected BECs, which are the main target for HRV

replication. RV16 exposure resulted in a significant MOI-dependent increase in viral RNA (vRNA) in LAD2 MCs compared with UV-HRV MOI 7.5 [median, 10 copies (IQR 0–103)] or mock infection (median, four copies (IQR 0–65); UV-HRV vs. MOI 3, $P = 0.02$, UV-HRV vs. MOI 7.5, $P = 0.002$) (Fig. 3a). Copies of RV16 RNA in LAD2 MCs exceeded those seen using BECs infected with RV16 in the same experiment. This permissiveness for viral replication prompted us to investigate whether LAD2 MCs, like BECs, had the potential to release infectious virus particles. $TCID_{50}$ assay revealed a significant MOI-dependent increase in the release of infectious RV16 virions from LAD2 MCs [18 $TCID_{50}$/mL for UV-HRV MOI 7.5 compared with 3768 $TCID_{50}$/mL for HRV MOI 3 ($P = 0.03$) and 17 461 $TCID_{50}$/mL for HRV MOI 7.5, ($P = 0.002$)] (Fig. 3b). Similar results were obtained with RV1B (Fig. S2d). Of note, there was no significant difference in cell viability for RV16- or RV1B-infected LAD2 MCs compared with mock-infected control cells (Fig. S3a,b).

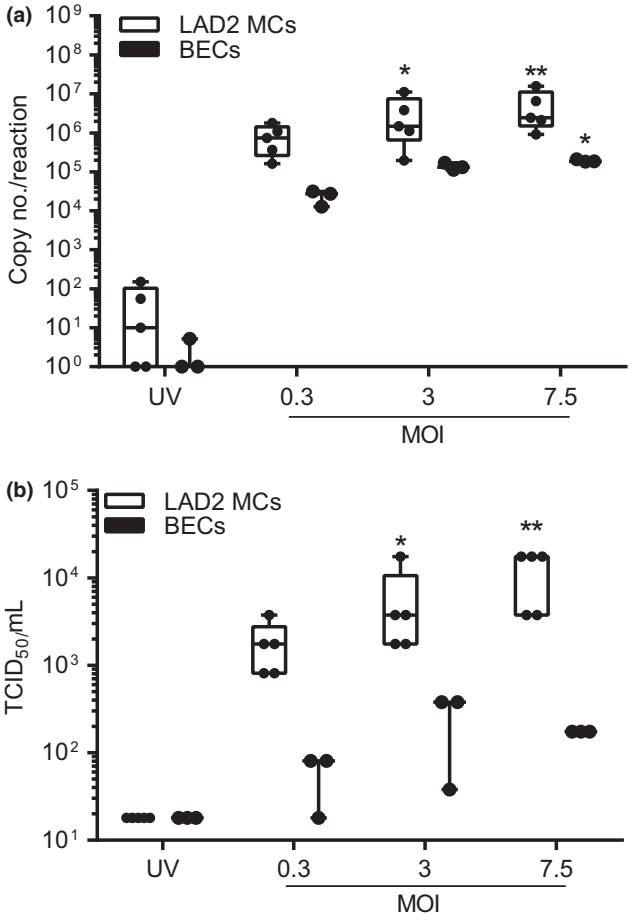

Fig. 3. Comparison of the replication and release of infectious RV16 from LAD2 MCs and bronchial epithelial cells (BECs). LAD2 MCs and BECs were exposed to RV16 MOI 0.3, 3 or 7.5 or UV-RV16 MOI 7.5 (control). Cell pellets and cell-free supernatants were harvested for viral RNA and infectious virus particles by RT-qPCR and $TCID_{50}$ assay, respectively. (a) RV16 copy number and (b) $TCID_{50}$/mL 24 h post-RV16 infection. Results are box and whisker plots showing the median, interquartile range and min and max values, $n = 5$ (LAD2), $n = 3$ (BECs), $^*P \leq 0.05$, $^{**}P \leq 0.01$ vs. UV-RV16. MOI, multiplicity of infection.

Primary human mast cells mount an innate immune response to human rhinovirus infection and also release infectious virus particles

Having established the responses of LAD2 MCs to HRV infection, we next investigated the response of primary human CBMCs to RV16 exposure. CBMCs exposed to RV16 for 24 h upregulated the expression of *IFNB1* and *IFNL1* mRNA transcripts (Fig 4a), which was confirmed at the protein level for IFN-λ but not IFN-β (Fig. 4b). There was also a significant upregulation of the ISGs *MX1* and *OAS1* in RV16-infected CBMCs (Fig. 4c). The upregulation of IFNs and ISGs required replication competent virus as UV-irradiated HRV failed to induce mRNA transcripts (Fig. 4). Crucially, CBMCs were also susceptible for the replication and release of infectious RV16 as observed by a significant increase in vRNA transcripts and virion release (Fig. 4d). As with LAD2 MCs, cell viability was unaffected by RV16 infection (Fig. S3c).

Primary human mast cells are protected from RV16 infection by exogenous IFN-β

Despite the induction of IFNs and antiviral responses, HRV infection of CBMCs still resulted in release of infectious virus particles. We hypothesized that this was due to low levels of endogenous IFN-β protein induced during HRV infection providing inadequate protection. Therefore, to test the extent of protection by endogenous type I IFNs, we pretreated CBMCs with a type I IFN receptor-blocking antibody (anti-IFNAR2) prior to HRV infection. Although this resulted in a significant reduction in HRV-dependent expression of IFN (*IFNB1* and *IFNL1*) and ISG (*OAS1* and *MX1*, data not shown) mRNAs compared with control (Fig 5a), it had minimal effects on vRNA levels and there was only a trend for increased virion release (Fig. 5b). This implied a minimal protective effect of endogenous type I IFN signalling against HRV replication.

We therefore examined whether we could augment antiviral immune responses of CBMCs by the addition of IFNs to the cultures. CBMCs responded to exogenous IFN-β with significantly increased expression of *IFNB1* and *IFNL1* mRNA above that observed with HRV alone (Fig. 5c). In contrast, IFN-λ was without effect suggesting IFN-β is a key driver of type I and type III IFN responses in these cells; this was confirmed by showing up-regulation of IFN-λ protein by IFN-β (Fig. S4a).

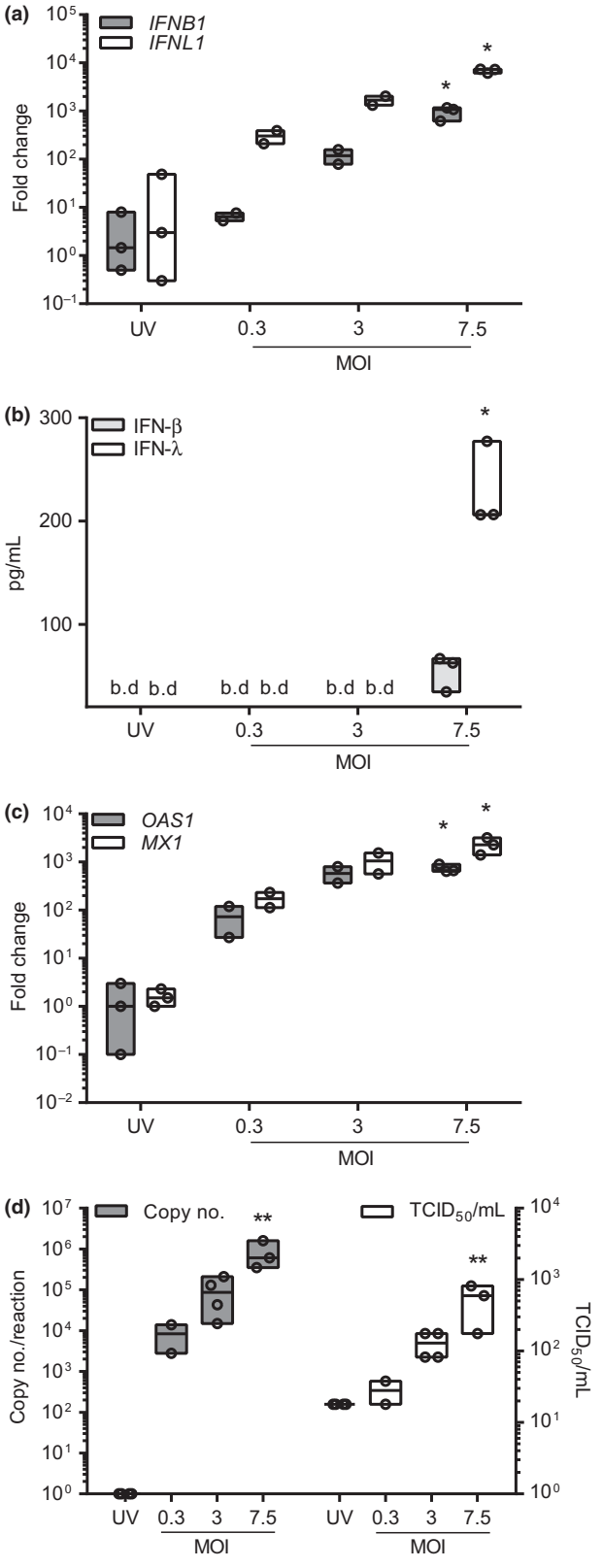

Fig. 4. Innate immune responses and release of infectious RV16 from CBMCs. CBMCs were exposed to RV16 at MOI 0.3, 3 or 7.5 or UV-RV16 MOI 7.5 (control). Twenty-four h post-infection cell pellets were harvested for gene expression and viral RNA by RT-qPCR and cell-free supernatants for protein expression by ELISA and infectious virus particles by $TCID_{50}$ assay. (a) *IFNB1* and *IFNL1* mRNA expression. (b) IFN-β and IFN-λ protein expression. (c) *OAS1* and *MX1* mRNA expression. (d) RV16 copy number and $TCID_{50}$/mL. Results are floating bars representing the median with min and max values, $n = 2–4$, $^*P \leq 0.05$, $^{**}P \leq 0.01$, vs. UV-RV16 ($n = 3–4$). MOI, multiplicity of infection. b.d., below limit of detection.

Most importantly, exogenous IFN-β, but not IFN-λ, protected CBMCs from viral replication and release of infectious virus particles (Fig. 5d). The induction of IFNs and suppression of viral replication mediated by IFN-β was prevented in the presence of anti-IFNAR2 antibody (Fig. S4b,c) confirming the blocking antibody effectively suppressed type I IFN signalling.

Discussion

Viral infections caused by HRVs are a major cause of asthma pathogenesis and exacerbation [4, 5]. MCs localize to the bronchial epithelium in asthma according to disease severity [28] and are recruited to the bronchial epithelium following HRV infection [30]. While MCs are classically associated with early-phase allergic reactions in asthma, their role in viral immunity is unclear. Here, we demonstrate that human MCs exposed to either a major or minor group HRV mount innate immune responses including the induction of type I and III IFNs and ISGs. Despite this, MCs were permissive for viral replication and production of infectious virus particles, suggesting that the endogenous immune response was insufficient to limit HRV replication. Consistent with this, exogenous IFN-β, but not IFN-λ, was sufficient to prevent the release of infective virus particles and protect MCs against HRV infection. The failure of IFN-λ to exert an antiviral immune response suggests that MCs like other hematopoietic cells lack receptors for type III interferons [37] whose expression is mainly restricted to cells of epithelial origin [45].

Despite mounting innate immune responses to HRV infection, MCs were permissive for HRV replication and released infectious viral particles which increased with increasing MOI in both the human MC line and primary CBMCs and confirms a previous study using the immature HMC-1 cell line and HRV14 [40]. However, this study was limited to investigating a single RV14 infection titre (10^4 $TCID_{50}$ U/mL) of unknown MOI and focused on the modulation of PMA/ionomycin- or IgE/anti-IgE-dependent histamine and cytokine release [40]. Our study has expanded the findings of Hosoda *et al.* by demonstrating an MOI-dependent effect on virion release and induction of antiviral and pro-inflammatory responses of the mature LAD2 cell line and primary

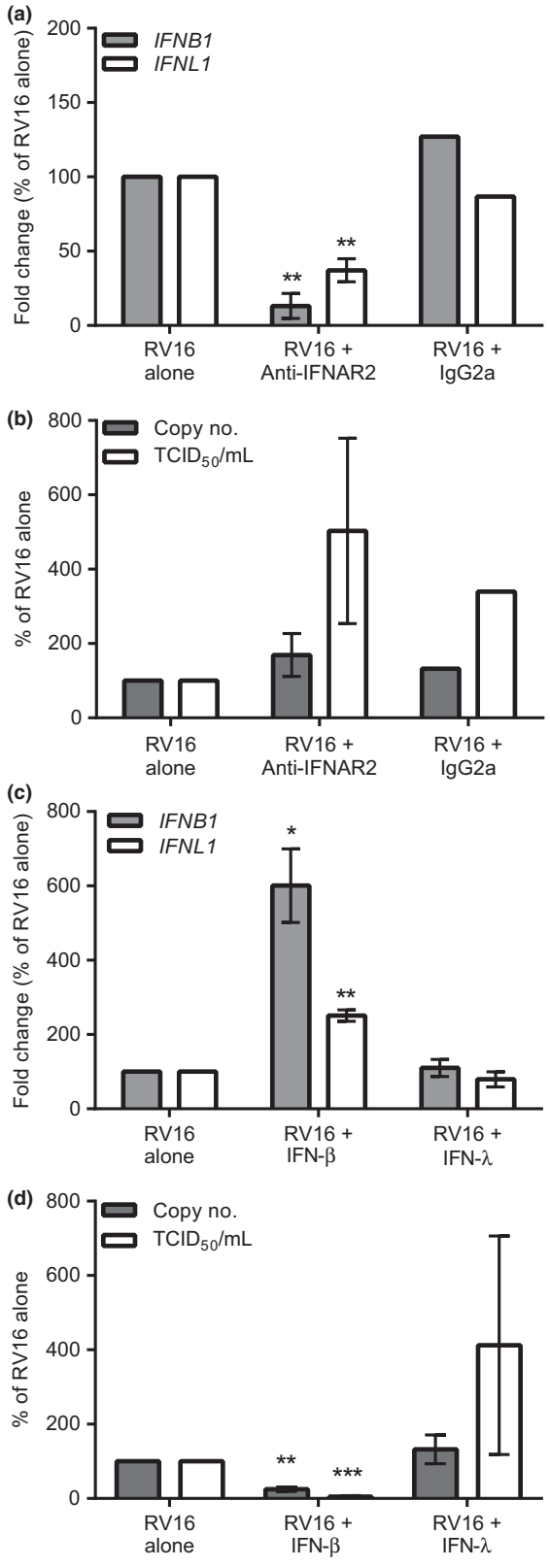

Fig. 5. Type I IFN receptor blockade and the effect of exogenous IFN treatment of CBMCs during RV16 infection. CBMCs were pretreated with anti-IFNAR2 (1 μg/mL) or IgG2a isotype (1 μg/mL) prior to RV16 MOI 7.5 or UV-RV16 MOI 7.5 exposure. Twenty-four h post-infection cell pellets were harvested for gene expression and viral RNA by RT-qPCR and cell-free supernatants were harvested for infectious virus particles by $TCID_{50}$ assay. (a) *IFNB1* and *IFNL1* mRNA expression. (b) RV16 copy number and $TCID_{50}$/mL. CBMCs were exposed to RV16 MOI 7.5 or UV-RV16 MOI 7.5 in the presence or absence of IFN-β (100 IU/mL) or IFN-λ (100 IU/mL). (c) *IFNB1* and *IFNL1* mRNA expression. (d) RV16 copy number and $TCID_{50}$/mL. Results are % of control (RV16 alone) means ± SEM, $n = 2–3$. $^*P \leq 0.05$, $^{**}P \leq 0.01$ vs. control ($n = 3$). Anti-IFNAR2, anti-IFN-α/βR 2 antibody; MOI, multiplicity of infection.

MCs following infection with both a major and minor group HRV. The release of infectious HRV virions by MCs is in contrast to infection of MCs by other respiratory viruses including reovirus, RSV and IAV which are capable of infecting human MCs and inducing innate immune responses; however, there is little or no release of virus progeny [33, 38, 46]. For instance, plaque assays of RSV infected CBMC supernatants confirm a lack of productive RSV infection of CBMCs [38]. It has been shown previously that CBMCs can support replication of dengue virus with release of infectious virus particles; however, this process is antibody dependent [47]. HRV replication and release of infectious virus particles from epithelial cells typically result from lysis [14], but here we show that virus shedding from MCs is not associated with significant cell death. Non-lytic virus shedding has been reported for other picornaviruses including poliovirus [48–50] and may be a mechanism by which infective HRV particles are released from MCs.

As MCs accumulate in the bronchial epithelium in asthma, they have the potential to come into close proximity with HRV during infection of the bronchial epithelium. Our findings that MCs are permissive for HRV replication and that virus shedding was not associated with significant cell death suggest that MCs may act as reservoirs for HRV and this may potentiate HRV-induced asthma exacerbations. This mechanism seems unique among common respiratory viruses and may help to explain the high association of HRV infection and asthma exacerbations, with MCs playing a novel pathological role. This may be particularly relevant in HRV-induced asthma exacerbations of difficult-to-treat severe asthma patients where MC numbers [28] and MC-specific mediators [29] are both increased. During experimental HRV infection of adults, MCs have been demonstrated to accumulate in the bronchial mucosa [30]; however, it is not known whether MCs *in vivo* are susceptible to HRV infection. This question may be addressed by performing *in situ* hybridization on bronchial biopsies taken from subjects following experimental HRV infection to determine whether HRV particles localize within MCs. Alternatively, MCs may be isolated from these biopsies via

laser capture for the detection of vRNA. In children, HRV infection is implicated in the inception of childhood asthma where infection is a major cause of persistent wheeze in infants and is a major risk factor for asthma development in early life [3]. Furthermore, the number of mucosal MCs and reticular basement membrane thickness at age 1 year predicts respiratory morbidity and the use of inhaled corticosteroids at age 3 years [51]. This suggests an important interaction between viral infection and MCs leading to allergic inflammation and the development of asthma in young children. While we demonstrated human MCs are permissive for the replication of major and minor group HRVs, it is unlikely that they would be permissive for HRV-C infection as they do not express CDHR3 (Fig. S5), the cellular receptor for HRV-C [8].

Following HRV infection, MCs upregulated type I and type III IFNs (IFN-β and IFN-λ), PRRs (*MDA5*), ISGs (*IRF7*, *OAS1*, *MX1*) and chemokines (*CXCL10* and *CCL5*). The induction of type I IFNs is likely to be a general response of MCs to virus exposure as these IFNs are also up-regulated with dengue virus [35], IAV [36], RSV [36] and sendai virus [37]. While type III IFN is generated following viral infection by many different cell types [17, 52, 53], reports of virus-dependent type III IFN expression and release by MCs are limited [37]. Although not able to respond to IFN-λ, the release of these antiviral proteins by MCs may help promote antiviral immunity in epithelial cells, which express IFN-λ receptors [54]. Despite the small amounts of IFN-β production, the suppression of *IFNB1* and *IFNL1*, as well as the ISGs *OAS1* and *MX1*, following anti-IFNAR2 treatment of CBMCs, suggests an IFN driven ISG response. ISGs can also be induced via TLR activation [55] and viral dsRNA (including replication intermediates of ssRNA viruses) [56] has been shown to activate MCs via TLR3 [36]. Therefore, HRV-induced IFN as well as TLR activation may contribute to the observed ISG induction. Induction of viral sensors and antiviral genes such as *MDA5*, *MX1*, *OAS1* has also been observed in human MCs infected with dengue virus [35] and vaccinia virus [54], but reports of up-regulation of these genes following infection with respiratory viruses are limited to sendai virus infection, a murine virus used to model human parainfluenza virus infection [37]. Exposure of MCs to many different viruses including dengue virus, reovirus and RSV induces the release of cytokines and chemokines, which are speculated to recruit inflammatory cells to help clear the infection [33, 38, 57]. In response to reovirus, MCs release CXCL8 which recruits NK cells [33] and CCL3-5 which recruit a subsets of T cells [34] *in vitro*. Dengue virus infection of mice also results in MC-dependent recruitment of NK and NKT cells, although the specific mediators involved were not investigated [58]. Therefore, effector cells including T cells, NK cells, and DCs may be recruited via MC-derived chemokines including CXCL10 and CCL5 during HRV infection; however, whether inflammatory cell recruitment would result in viral clearance or contribute to asthma pathology requires further investigation.

Human MCs were highly permissive for HRV infection which appeared to be due to only low-level production of IFN-β. Although we did not measure other type I IFNs, we used a type I IFN receptor-blocking antibody to investigate whether endogenous IFN-β or IFN-α made a substantial contribution to defence against HRV. This showed minimal effects on viral replication and a trend for enhanced shedding of infectious HRV particles confirming limited protection by endogenous type I interferons. Therefore, we investigated whether exogenous IFN-β could boost IFN responses and found significant up-regulation of *IFNB1*, *IFNL1* and an associated suppression of viral replication and release. IFN also up-regulates the expression of RIG-I, MDA5 and TLR3 in MCs suggesting increased detection of HRV by MCs [37]. The demonstration that boosting IFN responses can protect human MCs from HRV infection is of significance, as bronchial epithelial IFN responses following HRV infection are impaired in moderate/severe asthma [16, 17, 19, 20]. In addition, the localization of MCs in the bronchial epithelium increases with asthma severity suggesting that in severe asthma, MCs are at an increased risk of HRV infection and viral shedding. Exogenous IFN-β protects asthmatic primary BECs from HRV infection [16, 59] and inhaled IFN-β has been shown to be particularly effective at reducing symptoms and improving lung function in difficult-to-treat asthmatics during naturally occurring viral respiratory infections [41]. It is currently unknown whether MCs from asthmatic patients have a more severe defect in their IFN response to HRV infection. Nonetheless, our findings suggest that, in addition to protecting the bronchial epithelium against HRV infection, an inhaled IFN-β therapy could protect MCs directly and further boost the innate immune response of the bronchial epithelium by the production of IFN-λ.

In summary, we have shown for the first time that mature LAD2 MCs and primary CBMCs are permissive for the replication and release of HRV, which implicates them in HRV-induced asthma exacerbation. Furthermore, exogenous IFN-β is protective against HRV infection and may be particularly relevant in targeting MCs in severe asthma.

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Conflict of interest

Professor Donna Davies is a cofounder of Synairgen and is paid consultancy fees and also has a patent for the use of inhaled interferon beta therapy for virus-induced exacerbations of asthma and COPD with royalties paid.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. HRV does not induce MC degranulation.
Figure S2. LAD2 MC response to RV1B exposure.
Figure S3. Cell viability following rhinovirus infection of human mast cells.
Figure S4. IFN-b treatment and RV16 exposure of CBMCs and type I IFN receptor blockade.
Figure S5. CDHR3 expression following RV16 infection of LAD2 mast cells.