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IL-4 Enhances Interferon Production by Virus-Infected Human Mast Cells

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Keywords: Innate immunity, Respiratory Syncytial Virus, Reovirus, Influenza, Asthma, Anti-viral immunity

30 **Capsule Summary**

31 IL-4 treatment of human mast cells enhanced IFN- α 2 and IFN- λ 1 production in response to
32 several respiratory viral infections. This suggests an unforeseen role for IL-4 in promoting innate
33 immunity to viral infection.

To the Editors,

It is well recognised that individuals with allergic diseases such as asthma and atopic dermatitis clear some viral infections poorly compared to healthy subjects^{1, 2}. Respiratory viral infections including respiratory syncytial virus (RSV), influenza A virus (IAV), and rhinovirus can promote asthma exacerbations and are associated with increased morbidity and mortality. Type I (α/β) and type III (λ) interferons (IFNs) are critical for antiviral defence, inhibit viral replication, and mobilise host defense mechanisms. Deficient IFN production in allergic individuals has been associated with impaired antiviral responses^{3, 4}. Some authors have reported that T_H2 -associated mediators, such as IL-4, IL-13, or IgE binding to FcεRI, critically downregulate anti-viral mechanisms and IFN production in virus infected plasmacytoid dendritic cells and epithelial cells⁵

Mast cells are both key players in allergic disease and critical sentinel cells for host defence against infection. They have been demonstrated to respond to a variety of viral infections, including common respiratory viruses, through the production of mediators including substantial amounts of a wide range of IFNs, usually without degranulation⁶⁻⁸. These responses can be evaluated *in vitro* using primary cultured human mast cells such as cord blood-derived human mast cells. We hypothesised that mast cells may modify their IFN expression in the context of an IL-4 or IL-13 rich microenvironment. The impact of IL-4 or IL-13 treatment on human mast cell IFN responses to viral infection was therefore examined.

In initial studies, human mast cells were pretreated for 48h with IL-4, then infected with the dsRNA virus, reovirus. The levels of production of IFN- α 2 and IFN- λ 1 in response to these

infections were assessed. Notably, IL-4 pre-treatment significantly enhanced the IFN responses of mast cells following viral infection (Fig 1A). These responses were dose-dependent (Fig 1B) and consistent across multiple human donors, although there was some variation in the magnitude of effect between individuals. The ability of IL-4 to modify the IFN responses to the ssRNA viruses RSV and IAV was also examined, focussing on IFN- α 2 since IFN- λ 1 was not substantially induced by these infections in mast cells. The IFN- α 2 response to both viruses was significantly enhanced by IL-4 pre-treatment of mast cells (Fig 1C, D). Notably, for both RSV and reovirus infection, the observed increase in IFN responses took place in the absence of evidence of enhanced levels of mast cell viral infection. In contrast, elevated IAV gene expression was observed following IL-4 treatment of mast cells (Fig E1 in online repository).

In view of these findings, the ability of both human epithelial cells and peripheral blood monocytes to modify their IFN response to viral infection following IL-4 treatment was examined. Peripheral blood monocyte production of IFN- α 2 and IFN- λ 1 or Calu-3 cell production of IFN- λ 1 in response to viral infection was neither enhanced nor inhibited by prior IL-4 treatment (Fig E2 A, B).

Since decreased IFN production could be mediated by alterations in RNA sensor expression, the impact of IL-4 treatment of mast cells on selected RNA sensors was also examined (supplementary Table 1). The expression levels of most of these were unchanged, with only a modest increase in TLR3 mRNA observed. However, this was not accompanied by increased

responsiveness to transfection with the TLR3 ligand high molecular weight poly(I:C) (Fig E2 C, D).

To examine the requirement for extended IL-4 pre-treatment and potentially for IL-4-induced maturation, mast cells were exposed to IL-4 at the time of viral infection. Such acute IL-4 treatment had a similar enhancing impact on IFN production as 48h IL-4 pre-exposure (Fig 1A). IL-4 exerts its activities by interacting with either type I (IL-4R α and γ C subunits) or type II (IL-4R α and IL-13R α 1) IL-4 receptor complexes. Since the latter receptor complex is shared with IL-13, these cytokines often induce similar biologic effects. We therefore examined the receptor complex mediating IL-4 enhanced IFN responses to viral infection. Antibody blockade of either the IL-4R α or the γ C chain, or pharmacological inhibition of STAT6 with AS1517499 resulted in loss of IL-4 enhanced IFN responses to viral infection (Fig 2 A-B). In contrast, inhibition of IRS-2 did not significantly modify the response to IL-4 (IFN- λ 1, pg/ml; n=6. Reovirus: 1337 $\pm$ 378.6 vs Reovirus + IL-4: 1998 $\pm$ 518.1. Reovirus + NT-157: 1181 $\pm$ 363.5 vs Reovirus + NT-157 + IL-4: 1672 $\pm$ 466.4 pg/ml). Taken together, these data suggest that the action of IL-4 in enhancing mast cell IFN- α 2 and IFN- λ 1 responses to viral infection occurs through the type I IL-4 receptor. To further define the potential role of the type II IL-4 receptor, mast cells were pretreated with IL-13 in place of IL-4. While enhanced STAT6 phosphorylation was detected in response to IL-13, no significant enhancement of IFN responses to viral infection was observed (Fig E3 A, B).

To further consider the impact of allergic disease on mast cell responses to viral infection we also evaluated the impact of IgE-mediated activation of IL-4 primed mast cells on IFN- α 2 and

IFN- λ 1 responses to viral infection. Notably, concurrent IgE-mediated activation increased the IFN- α 2 and IFN- λ 1 responses of mast cells to viral infection by 1.5- – 53-fold (Fig 2 C).

Overall, these data demonstrate that unlike its established impact on bronchial epithelial cells⁵ and plasmacytoid dendritic cells⁹, IL-4 treatment of mast cells induces an increased type I and type III IFN response to mucosal viral infection. This response is mediated by the type I IL-4 receptor and does not occur in response to IL-13. These findings suggest that care should be taken in considering potential local impacts of IL-4 or IgE-blockade on viral host responses at mast cell-rich sites. The optimal size, nature and longevity of the local IFN responses necessary to combat mucosal viral infection while avoiding damaging inflammatory responses remains poorly defined, especially in the context of asthma. Our data demonstrate novel mast cell and IL-4-dependent processes which could substantially modify such responses to viral infection in allergic individuals.

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Figure Legends.

Figure 1. Human mast cells were primed with 10 ng/ml or indicated concentration of IL-4, A-D) or left unstimulated (Media) for 48h. Mast cells were then exposed to reovirus, A, B and E), IAV C), or RSV D), and cultured in the presence or absence (Media) of 10 ng/ml IL-4 A, C-E) or as indicated B for 24 hours. IFN- λ 1 and IFN- α 2 production were determined by ELISA and Luminex, respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2. Mast cells were pre-treated for 1.5 hours with antibodies A) specific for human IL-4R α , human γ C or a mouse IgG2a isotype control. A STAT6 inhibitor (A51517499) was pre-incubated, B) with cells for 1 hour. Mast cells were sensitized, C) with human IgE in the presence of IL-4 for 4 days. Mast cells were then infected with reovirus A-C) and cultured in the presence or absence of IL-4 (acute treatment A-B), antibodies or chemical inhibitors, as indicated, for 24h for IFN- λ 1 and IFN- α 2 analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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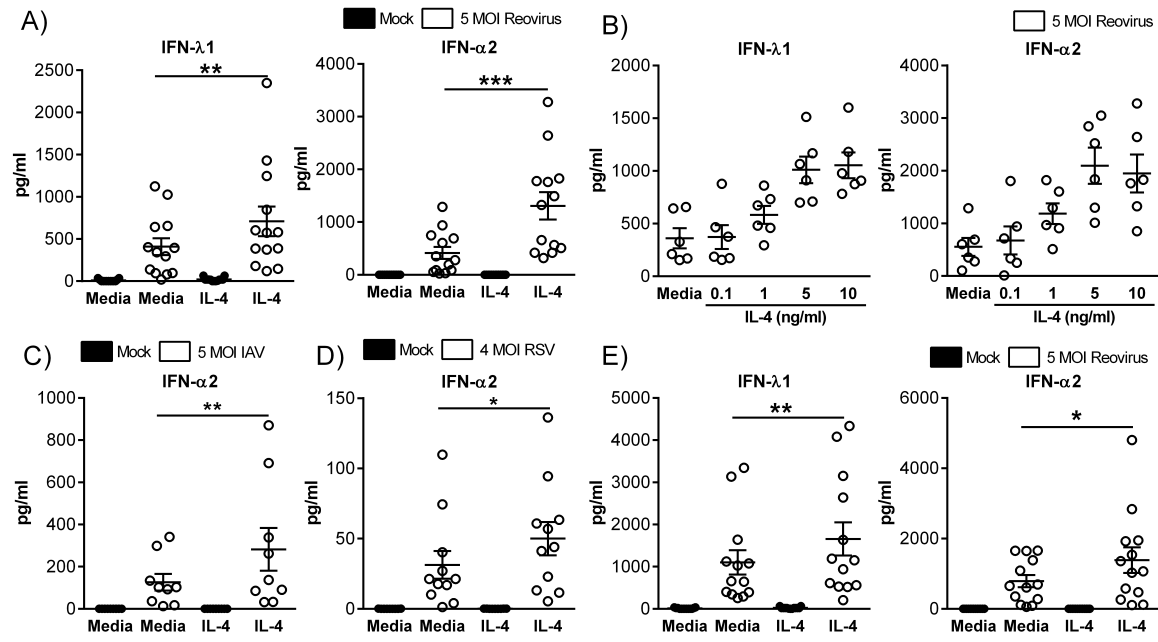


Figure 1

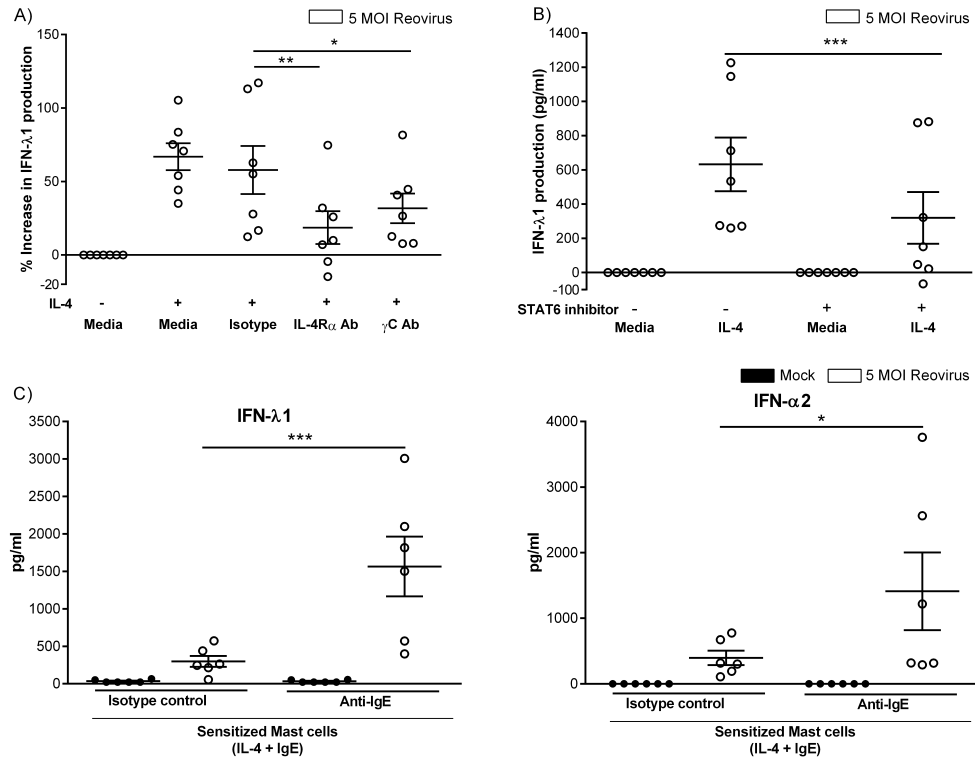


Figure 2

SUPPLEMENTARY MATERIAL**IL-4 Enhances Interferon Production by Virus-Infected Human Mast Cells**

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Supplementary Methods

Study approval

Umbilical cord blood samples and peripheral blood samples were obtained following approval from the Research Ethics Board of the Izaak Walton Killam (IWK) Health Centre, Halifax, Canada. Adult blood donors provided written informed consent.

Viral agents

Viruses used in this study were kindly provided by Dr. Shashi Gujar (type 3 Dearing reovirus; Dalhousie University, Halifax, Canada), Dr. Robert Anderson (RSV long strain; Dalhousie University), Dr. Mark Peebles (rgRSV; Center for Vaccines and Immunity, The Research Institute at Nationwide Children's Hospital, Columbus, OH), and Dr. Craig McCormick (mouse adapted and human pandemic influenza A virus, H1N1 A/CA/07/2009; Dalhousie University).

Cell culture

Cord blood-derived human mast cells were differentiated by long term culture of cord blood-derived mononuclear cells according to Enoksson *et al*¹ following the adaptation described by Portales-Cervantes *et al*². Mast cell purity was $\geq 95\%$ based on c-kit (anti-CD117 antibody) expression. Typically, mast cell cultures were $>99\%$ positive for tryptase and devoid of dendritic cells³. Human monocytes were isolated from healthy donors. Briefly, peripheral blood mononuclear cells were separated by Lympholyte-H cell separation media (Cedarlane, Burlington, Canada) density gradient centrifugation. Monocytes were isolated by positive selection using an EasySep human monocyte enrichment kit (STEMCELL Technologies, Vancouver, Canada) and were cultured in RPMI 1640 (Thermo Fisher Scientific, Mississauga, Canada) supplemented with 10% FBS (Gibco, Gaithersburg, MD) and 10 ng/ml GM-CSF BioLegend (San Diego, CA) prior to virus infection. Calu-3 cells were purchased from ATCC (Manassas, VA) and cultured in Dulbecco's modified Eagle medium/Ham's F-12 nutrient mixture (Thermo Fisher Scientific) supplemented with 10% FBS.

Cell activation

Mast cells, monocytes, and Calu-3 cells were activated with 10 ng/ml IL-4 (BioLegend) at 37°C for up to 48 hours in their corresponding culture medium. Control cells were left non-stimulated. Culture medium was removed, cells were resuspended in plain RPMI, counted and infected with 5 MOI reovirus at 37°C for one hour. Once reovirus was washed off, cells were resuspended in activation medium (culture medium with FCS reduced to 1% for monocytes and Calu-3 cells; RPMI supplemented with 1% FCS, 10 ng/ml SCF, and 100 µg/ml soybean trypsin inhibitor for mast cells) at 1×10^6 cells/ml for 24 hours. IL-4 was added to cultures of IL-4 primed mast cells following infection. In addition to reovirus, IL-4-primed mast cells were exposed to 5 MOI influenza A virus for one hour or 4 MOI RSV for two hours. Where indicated, mast cells were primed with 10 or 50 ng/ml IL-13 following infection with reovirus as mentioned above. For TLR3 activation, IL-4-primed- or control-mast cells were transfected with high molecular weight poly(I:C) (Invivogen, San Diego, CA) following the manufacturer's instructions. Briefly, both Lipofectamine 2000 (Thermo Fisher Scientific) and 10 µg/ml poly(I:C) were mixed at a 1:1 ratio. Mast cells (1×10^6 cells/ml) were cultured in activation medium in the presence of poly(I:C) or Lipofectamine alone (control) at 37°C for 24 hours. For FcεRI cross-linking experiments, mast cells were sensitized with 2.5 µg/ml human IgE (MilliporeSigma, Burlington, MA) in the presence of 10 ng/ml IL-4 for 96 hours at 37°C. Culture medium was removed, cells washed and subsequently infected with reovirus. Following infection, mast cells were resuspended at 1×10^6 cells/ml in activation medium and treated with 2.5 µg/ml anti-human IgE (SouthernBiotech, Birmingham, AL) or IgG1 (BioLegend) antibodies at 37°C for up to 24 hours post-infection.

Culture supernatants were harvested for cytokine analysis following centrifugation and cells were lysed in RLT buffer (Qiagen, Hilden, Germany) for mRNA analysis.

mRNA Analysis

Total RNA was isolated with an RNeasy Mini kit (Qiagen). cDNA was generated with a QuantiTect Reverse Transcription Kit (Qiagen). Gene expression was analyzed in a CFX96 Real-Time Detection system (Bio-Rad, Hercules, CA) using primers from Bio-Rad (*IFNA2*, *STING*) or Qiagen (*IFNL1*, *TLR3*, *TLR7*, *TLR9*, *PKR*,

IFIH1, *DDX58*) and SsoAdvanced Universal SYBR green supermix (Bio-Rad). Gene expression was normalized to at least two reference genes, *HPRT* and *TBP* (Qiagen). The level of reovirus and IAV infection in human mast cells was analyzed by ddPCR using primer pairs (Sigma, Oakville, Canada) for the S4 and NS1 gene⁴, respectively, in a C1000 touch thermal cycler (Bio-Rad). Data was analyzed in a QX200 droplet reads (Bio-Rad) using Quantasoft software (Bio-Rad).

Cytokine production analysis

IFN- α 2 and IFN- λ 1 were measured using the Bio-Plex Pro-human cytokine assay (Thermo Fisher Scientific) or an ELISA kit from R&D (Minneapolis, MN) respectively, following the manufacturers instructions.

IL-4 signaling blockade

Mast cells were pre-treated with 4 μ g/ml mouse IgG2a (BioLegend), anti-human IL-4R α (R&D) or anti-human common γ chain (γ C; R&D) antibodies for 1.5 hours at 37°C, 5% CO₂. In separate experiments, mast cells were treated with the STAT6 inhibitor AS1517499 (Axon Medchem, Reston, VA) or the IRS 1/2 inhibitor NT-157 (Axon) for one hour at 37°C. Following mast cell pre-treatment with either antibodies or inhibitors, mast cells were set up for reovirus infection as mentioned above. Mast cells were resuspended in activation medium in the presence or absence of 10 ng/ml IL-4 when indicated. Antibodies and chemical inhibitors were re-added to cell culture following infection. Cytokine analysis was performed 24 hours post-infection and represented as % increase in IFN- λ 1 production: (Mast cell IFN production under treatment – Mast cell basal IFN production) / Mast cell basal IFN production * 100. “Treatment” corresponds to: 10 ng/ml IL-4 (IL-4), isotype control + IL-4, anti-IL-4R α antibody + IL-4, or anti- γ C antibody + IL-4; “basal” indicates the absence of IL-4 or antibodies in mast cell cultures previous or post-infection. Alternatively IFN- λ 1 production, represented as IFN- λ 1 production by IL-4-activated reovirus-infected mast cells - IFN- λ 1 production by reovirus-infected mast cells.

FACS analysis

Human mast cells infected with the rgRSV⁵ were fixed in 1% paraformaldehyde. For intracellular staining of non-GFP RSV, cells were manipulated as described by Al-Afif *et al*³. STAT6 phosphorylation was analyzed in

mast cells stimulated in culture medium with 10 ng/ml IL-4 or 10 and 50 ng/ml IL-13 for 30 minutes at 37°C. Cells were fixed with 4% paraformaldehyde following permeabilization in 100% methanol. Cells were resuspended in 1% BSA/PBS and stained with anti-phospho-STAT6 monoclonal antibody (Thermo Fisher Scientific) or a relevant isotype control. Mast cells were analyzed in a FACSCanto II system (BD Biosciences, San Jose, CA). Data was analyzed with FlowJo X software (FlowJo, LLC, Ashland, OR).

Statistical analysis

Distribution of data was examined by Shapiro-Wilk and Kolmogorov-Smirnov tests. Depending on data distribution, repeated-measures ANOVA or Friedman's test were carried out for multiple comparisons with Bonferroni's or Dunn's test on selected pairs used post-hoc test, respectively. Differences were considered significant at $p < 0.05$. Statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software Inc. San Diego, CA) and InStat software (GraphPad Software Inc).

Author contributions

JSM. Initiating and designing research studies, accessing clinical samples, analyzing data, writing the manuscript.

LPC. Designed research studies, performing experiments, analyzing data, writing the manuscript.

OC. Performing experiments, analyzing data.

IDH. Designed experiments, performed experiments, analyzing data, writing the manuscript.

SD. Performing experiments, analyzing data.

CL. Performing experiments, analyzing data.

JG. Performing experiments, analyzing data.

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Supplementary Figure Legends

Fig E1. Human mast cells were primed with 10 ng/ml IL-4 A, C, D or left unstimulated (Media and B) for 48 hours. Mast cells were then exposed to reovirus, IAV or RSV and cultured in the presence or absence (Media) of 10 ng/ml IL-4 for 24 hours. *NS1* and *S4* gene expression were analyzed by ddPCR. RSV level of infection was determined by FACS.

Figure E2. Calu-3 epithelial cells A), and human peripheral monocytes B) were primed with 10 ng/ml IL-4 or left unstimulated (Media) for 48 hours followed by infection with reovirus. IFN production was analyzed 24 hours post-infection. Mast cells were cultured in the presence or absence (Media) of IL-4 for 48 hours. TLR3

gene expression was analyzed by qPCR, C). Non-primed (Media) or IL-4 primed mast cells were treated with Lipofectamine (LPF) or poly(I:C) for 24 hours. *IFNA2* and *IFNL1* gene expression was determined by qPCR D). * denotes $p < 0.05$

Figure E3. Mast cells were stimulated with 10 ng/ml IL-4, 10 or 50 ng/ml IL-13, or left unstimulated (Media) for 30 minutes at 37 °C and then analyzed by FACS for phospho-STAT6 (blue histogram) expression A). Mast cells were primed with 10 ng/ml IL-4, IL-13, or left unstimulated for 48 hours following by infection with reovirus. IFN production was analyzed 24 hours post-infection B). ** denotes $p < 0.01$

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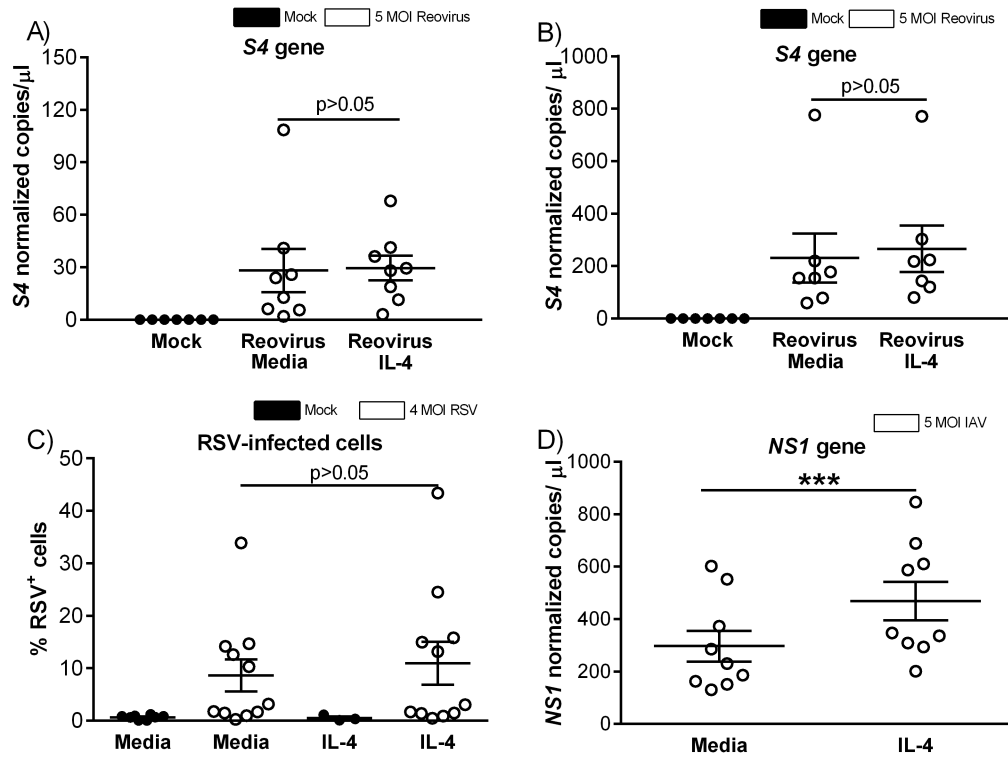


Figure E1

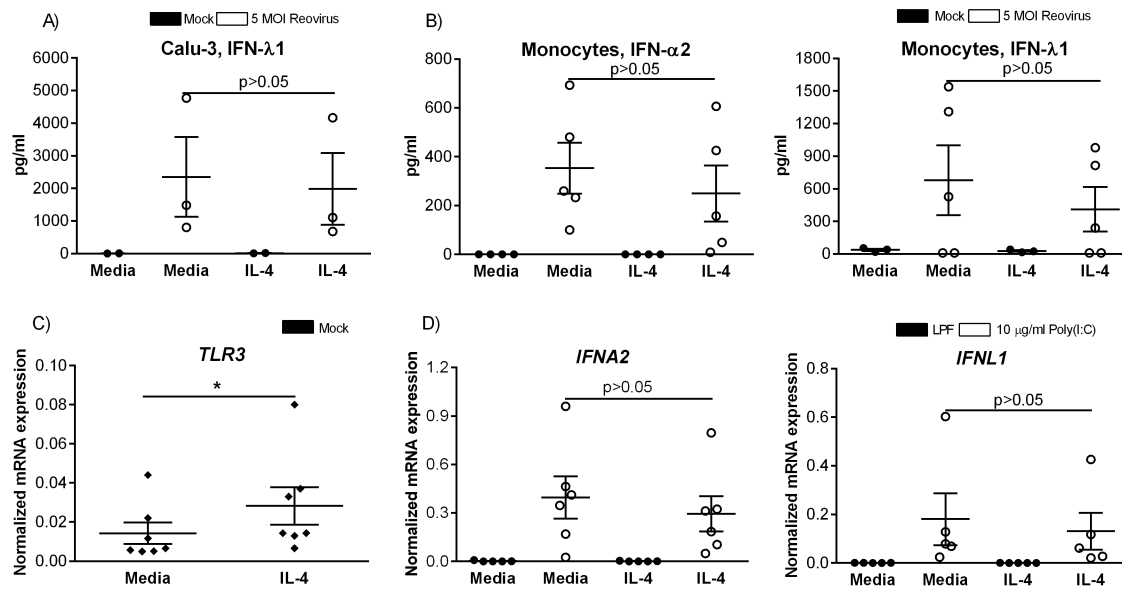
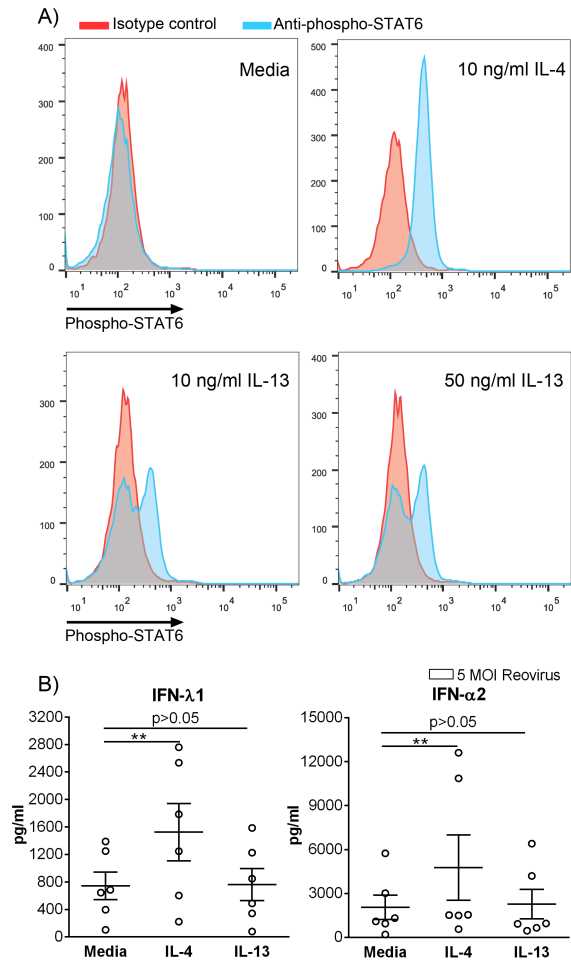


Figure E2



Supplementary Table 1. Viral sensor gene expression in mast cells

Gene	Medium	IL-4 (10 ng/ml)
<i>TLR7</i>	n.d.	n.d.
<i>TLR9</i> ^A	0.047±0.008	0.0048±0.145
<i>STING</i>	2.75±0.326	2.493±0.228
<i>IFIH1</i>	0.119±0.051	0.089±0.031
<i>DDX58</i>	1.38±0.513	1.59±0.625
<i>PKR</i>	1.42±0.335	1.05±0.162

Data shown as mean±SEM. Gene expression normalized to HPRT; n=7; ^An=3; n.d., not detected