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Responses of Human Mast Cells and Epithelial Cells Following Exposure to Influenza A Virus

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Abstract

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As a part of innate immune defense, the role of mast cells during viral replication has been incompletely understood. In this study, we characterized and compared the responses of the human mast cell line, LAD2, and human lung epithelial cell line, Calu-3, against three influenza A virus strains; A/PR/8/34 (H1N1), A/WS/33 (H1N1) and A/HK/8/68 (H3N2). We found that there were strain-dependent mast cell responses, and different profiles of cytokine, chemokine and antiviral gene expression between the two cell types. All three strains did not induce histamine or β-hexosaminidase release in LAD2. A/HK/8/68 induced release of prostaglandin D2 in LAD2, whereas A/PR/8/34 and A/WS/33 did not. We found that, among those examined, only CCL4 (by A/PR/8/34) was statistically significantly released from LAD2 cells. Furthermore, there was increased mRNA expression of viral recognition receptors (RIG-I and MDA5) and antiviral protein, viperin, but levels and kinetics of the expression were different among the cell types, as well as by the strains examined. Our findings highlight the variability in innate response to different strains of influenza A virus in two human cell types, indicating that further investigation is needed to understand better the role of mast cells and epithelial cells in innate immunity against influenza A viruses.

Keywords: Mast cells, Epithelial cells, Influenza A virus, Host response

1. Introduction
Influenza A virus (FluA) causes one of the most common respiratory diseases. Despite advances in vaccine development and other health care improvements regarding infectious diseases, influenza affects millions of individuals annually and pandemics, such as that of H1N1 in 2009, remain a major concern. FluA primarily infects and replicates within airway epithelial cells (EC), but host defences against influenza involve many cell types, including monocytes (Bussfeld et al., 1998), dendritic cells (Hao et al., 2008), eosinophils (Samarasinghe et al., 2017) and neutrophils (Wang et al., 2008). By investigating the response of different cell types to FluA infection, we can gain a better understanding of the mechanisms of host resistance and viral clearance.

The role of mast cells (MC) in anti-viral immunity is emerging, but still incompletely understood. MC are enriched near mucosal surfaces, a prime location to respond to infections, and thus help initiate and boost host defences (Galli et al., 1999; Galli and Tsai, 2008). Beyond their classical role in allergic inflammation, evidence has emerged that MC play critical roles in bacterial, fungal and viral infections, as well as protection against helminths (Galli et al., 1999; Galli and Tsai, 2008; Marshall and Jawdat, 2004; Moon et al., 2010; Rao and Brown, 2008; Ryan and Fernando, 2009). MC can be activated through IgE-dependent or IgE-independent mechanisms, resulting in the release of mediators stored in granules, as well as newly synthesized cytokines, chemokines and arachidonic acid metabolites that may be important in viral pathogenesis and host defences. For example, respiratory syncytial virus (Shirato and Taguchi, 2009), vesicular stomatitis virus (Marshall and Jawdat, 2004) and dengue virus (Furuta et al., 2012; King et al., 2002; King et al., 2000) induce various cytokine, chemokine and anti-viral gene responses in MC. The close proximity of MC to EC, the primary host cell for FluA replication, may help enable MC responses to FluA infections. Indeed, MC play an important role in FluA infection in mice, where MC deficiency reduces respiratory pathology and inflammatory mediator levels in
bronchoalveolar lavage fluid compared to normal mice (Graham et al., 2013). However, the complexity of responses of murine MC to FluA exposure may also be confounded by variations in virulence of FluA strains. Graham et al. showed that the level of IL-6 release from murine MC was dependent on FluA strain used (Graham et al., 2013). Thus, single strain disease models may be insufficient to investigate host defenses against FluA infections. Instead, using multiple strains of FluA may result in a more comprehensive understanding of host defenses.

The murine MC inflammatory response to FluA is mediated by RIG-I signaling (Graham et al., 2013), but another viral recognition receptor, MDA5, has also been implicated in FluA infections of other cell types in other species (Husser et al., 2011; Le Goffic et al., 2007; Siren et al., 2006). As many studies are done with murine MC and given that there are differences between human and murine MC, investigating human MC responses after FluA exposure may provide insights into the role of MC in FluA pathogenesis in humans. To understand the response of human MC to FluA, it is essential to investigate various virus recognition receptors and associated antiviral genes. Because we previously showed that human MC undergo a restricted productive FluA infection, despite evidence of viral protein production (Marcet et al., 2013), human MC may have antiviral mechanisms that affect FluA assembly and/or release. As human MC responses to FluA infection have been poorly characterized and studies have focused upon one FluA strain, it is essential to investigate responses to different FluA strains to understand the role of MC in FluA pathogenesis. In this study, we examined human MC responses using three different strains, FluA/PR/8/34 which we studied before (Marcet et al., 2013), and FluA/Wilson-Smith/33 and FluA/Hong Kong/8/68 which had been shown to induce strong mediator release from mouse MC (Graham et al., 2013). We establish that there are FluA strain-dependent human MC responses
and that these differ from those of EC. The strain-dependent human MC responses suggest that FluA strains may affect different signalling pathways resulting in variations in MC responses.

2. Materials and Methods

2.1. Cell Culture

The human MC line, LAD2 (Laboratory of Allergic Diseases 2, generously provided by Dr. D.D. Metcalfe and A. Kirshenbaum, National Institutes of Health, Bethesda, MD, USA), was cultured as described (Radinger et al., 2010). The human lung adenocarcinoma EC line, Calu-3 (ATCC® HTB-55; American Type Cell Culture, Manassas, VA, USA), was cultured with minimum essential medium and Earle’s salts supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies, Carlsbad, CA, USA).

2.2. Influenza Virus Strains

Flu A/Puerto Rico/8/34 virus strain (H1N1), propagated in duck eggs, was obtained from Dr. K. P. Kane from the University of Alberta (Edmonton, AB, Canada). FluA/Wilson-Smith/33 (H1N1) (ATCC® VR-1520) and FluA/Hong Kong/8/68 (H3N2) (ATCC® VR-1679) were obtained from ATCC and propagated with the help of Dr. Katharine Magor (University of Alberta) in chicken eggs. Virus stock titre was measured by plaque assay using MDCK cells: 3.7x10^4 pfu/mL/10^6 cells for A/PR/8/34, 4.2x10^5 pfu/mL/10^6 cells for A/WS/33, and 2.1x10^5 pfu/mL/10^6 cells for A/HK/8/68, which are 20 hemagglutination unit (HAU)/mL/10^6 cells equivalent. UV inactivation of each virus strain was performed using a UV lamp (ENF-280C, 450 µW/cm^2 at 15 cm, Spectroline, Westbury, NY, USA) at 254 nm, placed 20 cm from the sample for 20 min. We
previously confirmed UV inactivation using A/PR/8/34 with positive hemagglutination, negative hemadsorption, negative Flu A polymerase B1 (PB1) mRNA expression, and negative Flu A protein expression by western blot using rabbit antiserum against A/PR/8/34 in Calu-3, LAD2 and human peripheral blood-derived mast cells (Marcet et al., 2013).

2.3. β-hexosaminidase (β-hex) and Histamine Measurements

β-hex assay was performed in HEPES-buffered Tyrode’s solution (HTB) as previously described with modifications (Moon et al., 2014). Briefly, LAD2 cells (1 × 10^5/200 µL) were incubated with FluA virus (1.0 MOI, 5.0 MOI) and UV inactivated (UVI)-FluA (1.0 MOI) for 30 min at 37°C. Cells were centrifuged (5 min, 300 g) after incubation and supernatants were collected. Cell pellets were resuspended in HTB and lysed by three freeze-thaw cycles using liquid nitrogen and sonication. Results are expressed as % secretion using the following formula: 
\[
\frac{[\text{β-hex in supernatant}]}{[\text{β-hex in supernatant} + \text{β-hex in cell pellet}]} \times 100
\]
Histamine was measured by an automated fluorometric histamine assay as previously described (Arizmendi-Puga et al., 2006). Chicken allantoic fluid (virus propagation medium) was used as a negative control and A23187 (Sigma Aldrich, St. Louis, MO, USA) was used as a positive control for MC activation and these controls produced results as expected.

2.4. Influenza A Virus Exposure and Virus Quantification

LAD2 cells were seeded at 5 × 10^5 cells/mL in 2 mL of complete culture media (StemPro-34 SFM with 100 ng/mL human stem cell factor) in 6-well tissue culture plates (Corning Inc., Corning, NY, USA) and rested for 1 hr prior to FluA exposure. Calu-3 were plated 24 hr prior to FluA exposure to allow formation of monolayers at 1.0 x 10^6 cells/well in 6-well plates. Cells
were exposed to FluA virus (1.0 or 5.0 MOI) or UV-inactivated FluA (1.0 or 5.0 MOI) for 1 hr to allow virus adsorption. Cells were then washed three times with Hanks’ balanced salt solution (HBSS, Life Technologies) before culture in fresh media. No FluA was detected in third wash supernatant by hemagglutination assay. Hemagglutination assay using human RBC was performed to quantify viral particles in samples because there was no difference between hemagglutination assay and hemadsorption assay (infectious virus production) results as previously described (Marcet et al., 2013).

2.5. Arachidonic Acid Metabolite, Cytokine and Chemokine Measurements

Prostaglandin (PG) D2 release was measured using a commercially available enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI, USA) after exposing LAD2 to FluA for 30 min. Human cytokine/chemokine array performed by Eve Technologies (Calgary, AB, Canada) was used for screening differential release of cytokines and chemokines between LAD2 and Calu-3. GM-CSF, Flt-3L, CCL-3, CCL-4, CCL-5, CXCL-10, IFN-λ1, IFN-λ2 and IFN-λ3 release was measured using commercially available enzyme-linked immunosorbent assays (ELISAs) according to the supplier’s protocols (R&D Systems, Minneapolis, MN, USA).

2.6. RNA Extraction and Quantitative Real-time Polymerase Chain Reaction

Total RNA from LAD2 and Calu-3 cells was prepared using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to supplier’s protocols. Reverse transcription was performed using SuperScript III First-Strand Synthesis SuperMix (Life Technologies). SYBR green (Life Technologies) was used for qPCR with the following primers: Viperin, forward 5’- TGG TGA GGT TCT GCA AAG TAG-3’, reverse 5’- GTC ACA GGA GAT AGC GAG AAT G-3’ (Xu et
al., 2017); MAVS, forward 5’-TGC CGT TTG CTG AAG ACA A-3’, reverse 5’-TTC GTC CGC GAG ATC AAC T-3’ (Sasai et al., 2006); GAPDH, forward 5’-CTG AGA ACG GGA AGC TTG TCA-3’, reverse 5’-GCA AAT GAG CCC CAG CCT T-3’ (Bredo et al., 2015); MDA-5, forward 5’-TGG TCT CAC CAA TGA AA-3’, reverse 5’-CTC CTG AAC CAC TGT GAG CA-3’ (Asdonk et al., 2016); and RIG-I, forward 5’-TGG CAT ATT GAC TGG ACG TG-3’, reverse 5’-CAC TGG CTT TGA ATG CAT CC-3’. Forty-three cycles of qPCR were performed using 95°C for denaturation (15 seconds) and 60°C for annealing/extension (1 min). qPCR results were analyzed using ∆∆CT method and relative quantification using human GAPDH as a housekeeping gene (Duta et al., 2006; Gilchrist and Befus, 2008).

2.7. Statistical Analysis
Statistical analysis was performed using GraphPad Prism v8.1.1 (GraphPad Software Inc., La Jolla, CA, USA) where applicable and described in figure legends. Data shown are mean ± SEM. A p value <0.05 was considered significant.

3. Results
3.1. Release of FluA from LAD2 and Calu-3 After Exposure to Different Influenza A Virus Strains
We previously showed that the viability of Calu-3 was not different between A/PR/8/34 infection and mock infection. In LAD2 cells, viability was 75% at 5 days post infection and had decreased to 50% at 8 days of post infection. Comparable levels of vRNA, cRNA and mRNA for hemagglutinin, PB1 mRNA level and Flu A protein expression, using rabbit antiserum against A/PR/8/34, were observed in LAD2 and Calu-3 after A/PR/8/34 infection, although viral
progeny were not detected in LAD2. (Marcet et al., 2013). Based on these observations, we investigated FluA release and mediator release from human MC line, LAD2, and compared it to FluA release from human EC line, Calu-3, up to 5 days of post infection. We determined that LAD2 released low levels of FluA (A/PR/8/34, 995 ± 71 HAU/mL; A/WS/33, 604 ± 36 HAU/mL; A/HK/8/68, 568 ± 36 HAU/mL) on Day 5 when compared to Calu-3 (A/PR/8/34, 47573 ± 3387 HAU/mL; A/WS/33, 15928 ± 1138 HAU/mL; A/HK/8/68, 37413 ± 3387 HAU/mL) for all three strains (p < 0.001) (Figure 1a-c). Although we cannot compare viral yields from three different virus infections (Figure 1a vs b vs c) because we used 100 HAU/mL of virus for inoculation, the message from Figure 1 is consistent with our previous finding (Marcet et al., 2013) that LAD2 MC released few, if any, virus progeny in total (ie., active and inactive as of hemagglutination assay) at least up to 5 days post infection with all three strains tested. By contrast, all three strains of virus actively replicated and released virus in Calu-3 epithelial cells. To match infectivity of different virus strains, we used MOI rather than HAU for following experiments.

3.2. Short-term Mediator Release from LAD2 After Exposure to Different Strains of Influenza A Virus

We investigated LAD2 responses to different FluA strains (A/PR/8/34, A/WS/33, A/HK/8/68) by measuring short-term (30 min post-exposure) release of β-hex, histamine and PGD2. None of three strains induced significant β-hex or histamine release in LAD2 (Figure 2a, b). Neither A/PR/8/34 nor A/WS/33 induced significant prostaglandin D2 release compared to mock treatment. However, A/HK/8/68 induced 629.1 ± 51.7 pg/mL prostaglandin D2 release at 5 MOI
(p <0.05) (Figure 2c). These results suggest that release of MC-derived prostaglandin D2 induced by FluA exposure varies among different strains of FluA.

### 3.3. Influenza A Induced Cytokine and Chemokine Release in LAD2 and Calu-3 Cells

We measured cytokine/chemokine release after infecting human EC, Calu-3, and human MC, LAD2, with different strains of FluA (1.0 MOI). Human cytokine / chemokine 42-plex array results (Supplementary Figure 1) were re-evaluated using ELISA. In LAD2 we found that A/PR/8/34 induced maximum mean releases of 1010.18 ± 306.53 pg/mL of CCL-4 on day 2 (not significant vs UVI), 294.9 ± 74.2 pg/mL of CCL-5 on day 4 and 57.6 ± 17.1 pg/mL of CXCL-10 on day 4 (statistical analyses could not be conducted vs UVI virus treatment for both CCL-5 and CXCL-10 because it was below detection limit)(Figure 3a, d, g). By contrast, in Calu-3, A/PR/8/34 did not induce detectable CCL-4 release, but induced CCL-5 (1077.6 ± 143.3 pg/mL, p<0.05 vs UVI) on day 2 and CXCL-10 (2481.2 ± 95.8 pg/mL, p<0.001 vs UVI) release on day 2 (Figure 3a, d, g).

A/WS/33 did not induce significant release of CCL-4, CCL5 or CCL10 from LAD2 MC (Figure 3b, e, h). However, upon exposure to A/WS/33, Calu-3 released detectable CCL-5 (812.4 ± 55.2 pg/mL, maximum mean release on day 1, not significant vs UVI) and CXCL-10 (1943.5 ± 482.2 pg/mL, maximum mean release on day 1, not significant vs UVI), but release of CCL-4 was undetectable. (Figure 3b, e, h).

A/HK/8/68 did not induce significant CCL4, CCL5 or CCL10 release from LAD2 (Figure 3c, f, i). In Calu-3, A/HK/8/68 induced statistically significant CCL-5 release (maximum mean on day 4; 713.0 ± 104.2 pg/mL, p<0.05 vs UVI), and significant CXCL-10 release on day 2 (1668.0 ±129.3 pg/mL, p<0.001 vs UVI) (Figure 3f, i). No CCL-3 or Flt-3L release was detected in Calu-
3 and LAD2 after FluA infection (Supplementary Figure 2). These results show significant differences in cytokine and chemokine release by MC when compared to EC, suggesting different viral response pathways in the two human cell types, and FluA strain dependency. We also investigated interferon release from LAD2 and Calu-3 after FluA infection. FluA infection induced no statistically significant levels of IFN-α release by LAD2, whereas IFN-β was not detected in LAD2 or Calu-3 supernatants (Supplementary Figure 3). Moreover, Calu-3 released higher levels of IFN-λ1, -λ2, and -λ3 in the presence of each of the three strains of FluA (1.0 MOI) compared to LAD2 (Figure 4a-i). In Calu-3, A/PR/8/34 induced maximum mean release of IFN-λ1 on day 1 (2302.9 ± 117.2 pg/mL, statistical analyses could not be conducted vs UVI), IFN-λ2 on day 2 (6593 ± 582.7 pg/mL, p <0.01 vs UVI) and IFN-λ3 on day 2 (2877.2 ± 415.4 pg/mL; statistical analyses could not be conducted vs UVI). In LAD2, A/PR/8/34 induced maximum mean release of IFN-λ1 on day 2 (138.1 ± 41.7 pg/mL; statistical analyses could not be conducted vs UVI) and IFN-λ3 on day 2 (53.4 ± 12.8 pg/mL, not significant vs UVI), but we could not detect any IFN-λ2 up to day 4. A/WS/33 induced maximum mean releases of IFN-λ1 (2446.15 ± 246.9 pg/mL, statistical analyses could not be conducted vs UVI), IFN-λ2 (7274.7 ± 286.2 pg/mL, statistical analyses could not be conducted vs UVI) on day 1, and IFN-λ3 (2567.2 ± 738.4 pg/mL, not significant vs UVI) on day 2 in Calu-3. Significant IFN-λ3 release by A/WS/33 (2429.2 ± 326.5, p<0.05 vs UVI) was observed on day 4 in Calu-3. In LAD2, neither IFN-λ1 nor IFN-λ2 release was detected by A/WS/33, but IFN-λ3 release was observed (51.5 ± 3.9 pg/mL on day 1, 52.5 ± 7.7 pg/mL on day 2, and 55.6 ± 5.3 pg/mL on day 4; statistical analyses could not be conducted vs UVI). Maximum releases of IFN-λ1 (712.2 ± 81.8 pg/mL, statistical analyses could not be conducted vs UVI), IFN-λ2 (4961.4 ± 297.7 pg/mL, statistical analyses could not be conducted vs UVI), and IFN-λ3 (1647.5 ± 517.3 pg/mL, statistical analyses
could not be conducted vs UVI) were detected on day 2 for Calu-3 when infected with A/HK/8/68. However, like A/WS/33, A/HK/8/68 did not induce detectable IFN-λ1 and IFN-λ2, but induced maximum mean release of 58.7 ± 4.0 pg/mL IFN-λ3 (statistical analyses could not be conducted vs UVI) on day 2 in LAD2. We also tested these cytokine and chemokine releases at lower virus doses (MOI of 0.1 and 0.01). The lower dose showed similar trends but to a lesser extent compared to the MOI of 1 (data not shown).

3.4. Upregulation of Antiviral Genes in Mast Cells and Epithelial Cells After Influenza A Exposure

We assessed the effect of the three strains of FluA (at 1.0 MOI) on the expression of selected anti-viral genes in Calu-3 and LAD2 cells. RIG-I mRNA was significantly (p<0.01) upregulated relative to GAPDH on day 1 (97.2 ± 4.6 fold, p<0.05) and 3 (20.7 ± 1.5 fold, p<0.05) by A/PR/8/34 exposure compared to its UVI virus treatments (22.3 ± 12.2 fold and 8.60 ± 2.3 fold, respectively) in LAD2 (Figure 5a). In addition, RIG-I expression induced by 1.0 MOI A/PR/8/34 exposure in LAD2 was significantly (p<0.01) greater than in Calu-3 (day 1, p <0.01) (Figure 5a). A/WS/33 mediated RIG-I mRNA expression in Calu-3 was not significantly elevated compared to that in LAD2, although it showed significant enhancement on day 4 (22.6 ± 0.4 fold) compared to UVI virus treatment (4.8 ± 1.4 fold) (p<0.05) (Figure 5b). A/HK/8/68 upregulated RIG-I mRNA expression in LAD2 (p<0.001) and Calu-3 (p<0.05), compared to its UVI virus treatments (Figure 5c).

A/PR/8/34 and A/HK/8/68 significantly upregulated MDA5 mRNA expression in LAD2 and Calu-3 compared to their UVI virus treatments (Figure 5d, f). A/WS/33 did not significantly affect MDA5 mRNA expression in LAD2 up to day 4 or Calu-3 up to day 2, but significantly
increased MDA5 mRNA expression at day 4 in Calu-3. MDA5 expression in Calu-3 was significantly higher compared to that in LAD2 following exposure to A/WS/33 (p<0.01) and A/HK/8/68 (p<0.05) (Figure 5e, f). Interestingly, FluA virus did not upregulate viperin mRNA expression in LAD2 by A/WS/33, whereas A/PR/8/34 (p<0.01) and A/HK/8/68 (p<0.05) upregulated viperin mRNA expression on days 2 and 1 compared to their UVI virus treatment, respectively (Figure 5g, i). Significantly higher levels of viperin mRNA expression were detected in Calu-3 compared to LAD2 by all three strains (Figure 5g-i). FluA infection did not induce MAVS mRNA expression in either Calu-3 or LAD2 compared to UVI virus treatments for all three strains tested (Supplementary Figure 4).

4. Discussion

Resident MC are located near EC in the lungs, allowing them to contact and respond to respiratory viral infections (Galli et al., 1999; Marshall and Jawdat, 2004; Rao and Brown, 2008; Ryan and Fernando, 2009; Vliagoftis and Befus, 2005). Porcine MC infected with A/California/07/2009 (H1N1) (Lee et al., 2017) and murine bone marrow-cultured MC infected with A/WS/33 (H1N1) (Graham et al., 2013) release histamine. In this study, we examined the response of human MC to different strains of FluA using LAD2 cells. We demonstrated that human MC show limited release of viral progeny of all three FluA strains tested (Figure 1) by hemagglutination assay which measures both active and inactive virus particles, and responses to FluA exposure are strain-dependent (Figures 2-5). When investigating β-hex and histamine release in LAD2, we found that all three FluA strains did not induce significant levels of histamine or β-hex release up to 5.0 MOI. However, using 5.0 MOI, PGD2 secretion in LAD2 was induced by A/HK/8/68 (Figure 2). Our results differ from those seen with murine (Graham...
et al., 2013) and porcine MC (Lee et al., 2017), perhaps because of differences in viral strain, dose, and species origin of the MC. Thus, differences in viral strain, host species and mediator response uncover diverse immunological responses of MC to FluA.

After establishing that human MC are activated by different strains of FluA, we investigated production of selected cytokines and chemokines that may play a role in the responses of MC and EC to FluA. After initial human cytokine / chemokine array screening (Supplementary Figure 1) and validation of the results using ELISA, we found that A/PR/8/34 induced statistically significant levels of CCL-4 release in LAD2. Furthermore, CCL-4 release was not detected from Calu-3 when exposed to the three FluA strains (Figure 3a-c). CCL-5, which attracts T cells, eosinophils and basophils; and CXCL-10, which attracts T cells, NK cells, dendritic cells and monocytes, were released by Calu-3 after FluA infection, but -none or only limited amounts were detected from LAD2 (Figure 3d-i). Lee et al. has demonstrated that CXCL-10 mRNA is upregulated at 12 hr post infection with FluA in porcine MC (Lee et al., 2017), but our results do not show increased release of CXCL-10 protein from LAD2 up to 4 days post-FluA exposure. In contrast to LAD2, Calu-3 released CXCL-10 after FluA exposure, further demonstrating different cell types vary in their response to FluA infection (figure 3g-i).

Thus, there are clear differences in cytokine and chemokine release between human MC and EC after FluA exposure, demonstrating that FluA elicits a diverse response in different cell types, and that recognition and response to infection may involve distinct signaling pathways in different cell types.

Despite type I interferons being an indicator of FluA-infection in EC (Ronni et al., 1997) and monocytes (Hofmann et al., 1997), we did not detect significant IFN-α or IFN-β release in LAD2 (Supplementary Figure 3). Low amount of type I IFN release in LAD2 are consistent with a
recent report that the murine MC line, P815, does not produce much IFN-α or IFN-β upon FluA infection (Meng et al., 2017). We investigated the release of type III IFN; IFN-λ1, -λ2 and -λ3. Our results show that all three FluA strains induced the release of IFN-λ1, -λ2 and -λ3 in Calu-3, but none or limited release in LAD2 (Figure 4). This suggests that LAD2, a human MC, is not a major source of IFN during the first 4 days of FluA infection. However, it is unknown whether LAD2 responds to exogenous IFNs during FluA infections and activate IFN-dependent pathways. Hence, further studies of IFN-stimulated gene response in FluA infected MC are warranted.

Our study also showed that antiviral gene regulation differs significantly between Calu-3 and LAD2. RIG-I is a cytosolic receptor that recognizes FluA and docks on its adaptor MAVS to initiate IRF3- and NFKB-dependent signaling (Graham et al., 2013; Kato et al., 2006; Kulka et al., 2004; Le Goffic et al., 2007). It was shown previously with FluA that RIG-I/MAVS interaction is involved in mouse MC cytokine, chemokine and leukotriene production (Graham et al., 2013). In this study, RIG-I (two out of three strains tested) (Figure 5a-c), but not MAVS (Supplementary Figure 4) mRNA expression was upregulated in LAD2 after FluA exposure, suggesting that RIG-I signaling may play a major role in detecting the three different strains of FluA. RIG-I expression induced by A/PR/8/34 in Calu-3 was significantly lower than that in LAD2 (Figure 5a) but not following A/WS/33 or A/HK/8/68 exposures (Figure 5b, c). This emphasizes the diversity in antiviral responses that different viral strains may elicit in different host cells. MDA5 is another antiviral gene associated with viral recognition and host defense which also uses MAVS as an adaptor protein. Our results showed that both Calu-3 (in all strains tested) and LAD2 (two out of three strains tested) respond to FluA infection by upregulating MDA5 mRNA expression (Figure 5d-f). Additional investigations are warranted about the role...
of MDA5 and RIG-I in antiviral defenses and to understand whether a MAVS-independent pathway is involved with host signaling in MC infected with selected viral strains.

Viperin can interfere with FluA release in other cell types (Tan et al., 2012; Wang et al., 2007), and could also be involved in depressed FluA release in human MC. It is an antiviral protein that disrupts modifications of plasma membrane fluidity which in turn depress FluA budding and release. Our previous study showed FluA transcription, replication and protein synthesis occur in LAD2, but viral release was limited (Marcet et al., 2013). Based on this we initially hypothesized that the limited release of Flu A in MC is related to viperin expression (ie., higher expression in MC compare to EC). Although we found that A/PR/8/34 and A/HK/8/68 exposure induced viperin mRNA expression in LAD2, we observed higher expression of viperin mRNA in Calu-3 than in LAD2 following exposure to all three strains of FluA which did not support our hypothesis (Figure 5g-i). Because of this result, together with the results of no significant IFN release from Flu A infected LAD2, we did not conduct additional experiments such as IFN signaling, including STAT1 activation. We observed that human MC express other IFN-inducible genes, notably PKR, MxA, eNOS, ISG15, p56 mRNA expression by A/PR/8/34 in LAD2, and MxA protein expression in Calu-3 and LAD2 (Marcet, 2010). Therefore, further studies are needed to investigate the mechanism(s) that MC use to interfere with FluA replication.

Overall, we observed strain and cell type dependent responses, and therefore we could not make a simple generalized conclusion from this study. However FluA strain dependent responses of MC mediator release are consistent with those reported by others (Graham et al., 2013). The results we showed here are based on matching inoculation dose (MOI) and cell numbers. Therefore, whether our observations are due to cell type specific-, virus strain dependent-
infectivity needs to be assessed to understand better virus strain- and cell type-specific host responses.

In conclusion, our results combined with other studies (Graham et al., 2013; Lee et al., 2017) demonstrate that MC may play different roles in viral infections of various species and tissues. In addition, given that there are variations in MC phenotype depending on factors such as species, age, anatomical site, and microenvironmental conditions, our results from LAD2 cells must be replicated with primary MC. Although much remains to be learned about the role of MC in viral immunity and pathogenesis, our findings contribute to a better understanding of the complexity of MC responses against Flu A infection in humans.

5. Acknowledgement

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Charles Scriver MD/PhD Studentship Award from CIHR. K.N. received a studentship award from TLA.

7. Author Contributions

All authors reviewed and approved the final manuscript. K.N. contributed to the study concept and design, performed laboratory experiments, contributed to data analysis and drafted the manuscript. J.R. performed laboratory experiments, contributed to data analysis and manuscript preparation. C.S. performed laboratory experiments and sample preparation. C.T.M., H.V. and A.D.B. contributed to the study concept and design. T.C.M. contributed to the study concept and design, performed laboratory experiments and contributed to data analysis.

8. Figure Legends

Fig. 1. Virus Release in LAD2 and Calu-3 After Exposure to Three Strains of Influenza A Virus. FluA release in LAD2 and Calu-3 cells after infection with 100 HAU/mL/10^6 cells A/PR/8/34 (a), A/WS/33 (b), and A/HK/8/68 (c) was measured by hemagglutination assay in triplicate. Each infection was done in duplicate. The mean values of each infection from three independent infections (n=3) were used for the analysis. ### p<0.001 between LAD2 and Calu-3 by two-way ANOVA with the Geisser-Greenhouse correction; * p <0.05 and ** p <0.01 by two-way ANOVA with the Geisser-Greenhouse correction followed by the Bonferroni post-test (n=3).

Fig. 2. Selective Mediator Release in LAD2 After Exposure to Three Strains of Influenza A Virus; A/PR/8/34, A/WS/33 and A/HK/8/68. Thirty min after exposure of up to 5.0 MOI of
FluA to MC, β-hex (a, n=4-19), histamine (b, n=3) and PGD2 (c, n=3) were measured as described in methods. Each infection was done in duplicate. Mock values were subtracted from the mean values of each infection in the number of independent infections indicated in the figure. All three virus strains did not affect β-hex nor histamine release, but A/HK/8/68 at MOI of 5 induced statistically significant levels of PGD2 release. Statistical analyses were performed using the mean values before subtracting mock values [4.10 ± 0.58 % (a, n=19), 36.70 ± 11.60 pg/mL (b, n=3), and 65.13 ± 15.51 pg/mL (c, n=3)]. * p <0.05 compared to mock treatments in each virus type by mixed-effects analysis with the Geisser-Greenhouse correction (a) or one-way ANOVA with the Geisser-Greenhouse correction followed by the Bonferroni post-test (b, c). A21387 and biotin-IgE/streptavidin were used as positive controls. #p<0.05, ###p <0.001 compared to mock treatments by two-tailed paired t-test.

Fig. 3. Cytokine and Chemokine Release from LAD2 and Calu-3 After Exposure to Three Strains of Influenza A Virus. FluA (1.0 MOI) and UV-inactivated FluA (1.0 MOI) of the three strains were used to infect LAD2 and Calu-3 cells. CCL-4 (a-c), CCL-5 (d-f), and CXCL-10 (g-i) release was measured 1, 2 and 4 days post-FluA infection by ELISAs in duplicate. Four independent infections were performed in duplicate (n=4). In LAD2, repeated measures two-way ANOVA with the Geisser-Greenhouse correction followed by the Bonferroni post-test could be done in a, b and e, and significant group differences were observed only in a (p <0.01 Mock vs 1 MOI, p <0.01 UVI vs 1 MOI). However, no significant differences were observed within the same post infection days (n=4). # p <0.05 ##p <0.01 and ###p <0.001 compared to respective UVI virus treatments in Calu-3 (d, e, f, g, h, i) by repeated measures two-way ANOVA with the Geisser-Greenhouse correction followed by the Bonferroni post-test (n=4). § p <0.05, §§ p <0.01
and $^{\$\$\$}$ $p < 0.001$ between 1.0 MOI FluA exposed LAD2 and Calu-3 (d, e, g) by repeated measures two-way ANOVA with the Geisser-Greenhouse correction followed by the Bonferroni post-test (n=4). Statistical analyses could not be performed where it is not indicated above because all values in one or more groups were below detection limit. Dotted line denotes detection limit of the ELISAs.

**Fig. 4. Type III Interferon Release in LAD2 and Calu-3 After Exposure to Three Strains of Influenza A Virus.** FluA (1.0 MOI) and UV-inactivated FluA (1.0 MOI) of the three strains were used to infect LAD2 and Calu-3 cells (duplicate per condition per independent infection). IFN-$\lambda_1$ (a-c), IFN-$\lambda_2$ (d-f), and IFN-$\lambda_3$ (g-i) were measured by ELISA in duplicates. Four independent infections were performed (n=4). Statistical analyses were conducted where applicable using repeated measures two-way ANOVA with the Geisser-Greenhouse correction followed by the Bonferroni post-test. No statistical significance was found in LAD2 compared to UVI virus treatments (g) by (n=4). For Calu-3, there was statistically significant release of IFN-$\lambda_3$ (*$p < 0.05$ and **$p < 0.01$) compared to respective UVI virus treatments (d, e, g, h) (n=4). Calu-3 released significantly more IFN-$\lambda$ ($p < 0.05$, $^{\$\$}$ $p < 0.01$ and $^{\$\$\$}$ $p < 0.001$) compared to 1.0 MOI FluA exposed LAD2 (a, g, h, i) (n=4). Statistical analyses could not be performed where it is not indicated above because all values in one or more groups were below levels of detection. Dotted line denotes detection limit of the ELISAs.

**Fig. 5. Antiviral Gene Expression in LAD2 and Calu-3 After Exposure to Three Strains of Influenza A Virus.** Virus was used at 1.0 MOI. UV-inactivated virus was used as control. 
Infection was done in duplicate at each time and total RNA from the duplicate wells was
combined after total RNA isolation. qPCR was conducted in triplicate to determine mRNA expression levels in LAD2 and Calu-3 from four independent experiments (total RNA from two out of four independent experiments were pooled because of limiting amounts for qPCR). GAPDH was used as house-keeping gene to normalize data. Four independent infection experiments were performed and relative mean fold change values compared to mock treatment (no viral exposure) in each qPCR of three total RNA samples (n=3). RIG-I (a-c), MDA5 (d-f) and viperin (g-i) were measured at 1, 2, and 4 days post infection. *p <0.05 and **p <0.01 compared to respective UVI virus treatments in LAD2 by repeated measures two-way ANOVA with the Geisser-Greenhouse correction followed by the Bonferroni post-test (n=3). †p <0.05, ††p <0.01 and †††p <0.001 compared to respective UVI virus treatments in Calu-3 by repeated measures two-way ANOVA with the Geisser-Greenhouse correction followed by the Bonferroni post-test (n=3). §p <0.05, §§p <0.01 and §§§p <0.001 between 1.0 MOI FluA exposed LAD2 and Calu-3 by repeated measures two-way ANOVA with the Geisser-Greenhouse correction followed by the Bonferroni post-test (n=3).

9. References


Figure 1.
Figure 2.
Figure 3.

A/PR/8/34

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A/WS/33

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A/HK/8/68

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Figure 4.

A/PR/8/34

IFN-1.1 (pg/ml) vs Day(s) post infection

IFN-1.2 (pg/ml) vs Day(s) post infection

IFN-1.3 (pg/ml) vs Day(s) post infection

A/WS/33

A/HK/8/68

Day(s) post infection
Figure 5.

A/PR/8/34

- (a): Fold change of RIG-I expression
- (d): Fold change of MDAS expression
- (g): Fold change of Viperin expression

A/WS/33

- (b): Fold change of RIG-I expression
- (e): Fold change of MDAS expression
- (h): Fold change of Viperin expression

A/HK/8/68

- (c): Fold change of RIG-I expression
- (f): Fold change of MDAS expression
- (i): Fold change of Viperin expression

Legend:
- Black: LAD2 (1.0 MOI FluA)
- Light Gray: LAD2 (1.0 MOI UVI-FluA)
- White: Calu-3 (1.0 MOI FluA)
- Gray: Calu-3 (1.0 MOI UVI-FluA)
Highlights

- We examined responses of the human mast cell and lung epithelial cell against three influenza A virus strains.
- Influenza A virus strain-dependent human mast cell responses are described.
- We discussed mast cell responses against influenza A virus are species dependent.
- Variability of response to different strains of influenza A virus in human epithelial cell and mast cell are described.