The role of IL-1 and MyD88-dependent signaling in rhinoviral infection

Clare A. Stokes, Saila Ismail, Emily P. Dick[‡], Julie A. Bennett, Sebastian L. Johnston[†], Michael R. Edwards[†], Ian Sabroe[#], Lisa C. Parker*[#]

Academic Unit of Respiratory Medicine, School of Medicine and Biomedical Sciences, University of Sheffield, Sheffield, UK. [†]Department of Respiratory Medicine, National Heart and Lung Institute, Wright Fleming Institute of Infection and Immunity and MRC and Asthma UK Centre in Allergic Mechanisms of Asthma, Imperial College London, London, UK.

[#] L.C.P. and I.S. are joint senior authors for this work.

[‡]E.P.D. present address: Wolfson Centre for Stem Cells, Tissue Engineering and Modelling (STEM), Centre for Biomolecular Sciences, University Park, The University of Nottingham, Nottingham, UK.

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*Address for correspondence:
Dr L.C. Parker, Academic Unit of Respiratory Medicine, School of Medicine and Biomedical Sciences, University of Sheffield, L Floor, Royal Hallamshire Hospital, Sheffield, UK, S10 2JF. Tel +44 (0)114 271 3452. Fax: +44 (0) 114 226 8898.
Email l.c.parker@sheffield.ac.uk

Abstract

Rhinoviral infection is an important trigger of acute inflammatory exacerbations in patients with underlying airway disease. We have previously established that IL-1β is central in the communication between epithelial cells and monocytes during the initiation of inflammation. In this study we explored the roles of IL-1β and its signaling pathways in the responses of airway cells to rhinovirus-1B (RV-1B), and further determined how responses to RV-1B were modified in a model of bacterial coinfection. Our results revealed that IL-1β dramatically potentiated RV-1B-induced proinflammatory responses, and whilst monocytes did not directly amplify responses to RV-1B alone, they played an important role in the responses observed within our coinfection model. MyD88 is the essential signaling adapter for IL-1β and most Toll-like receptors. To examine the role of MyD88 in more detail, we created stable MyD88 knockdown epithelial cells using shRNA targeted to MyD88. We determined that IL-1β/MyD88 plays a role in regulating RV-1B replication and the inflammatory response to viral infection of airways cells. These results identify central roles for IL-1β and its signaling pathways in the production of CXCL8, a potent neutrophil chemoattractant, in viral infection. Thus IL-1β is a viable target for controlling the neutrophilia that is often found in inflammatory airways disease and is exacerbated by viral infection of the airways.

Introduction

The incidence and prevalence of asthma and chronic obstructive pulmonary disease (COPD) have increased substantially in recent decades, with acute exacerbations contributing considerably to the healthcare and economic burden generated by these conditions. Human rhinoviruses (RV) represent a frequent trigger of acute inflammatory exacerbations in patients with underlying airway disease (23). RV are non-enveloped, positive, single-stranded RNA viruses of the *Picornaviridae* family, and can be divided into major (RV-A) or minor (RV-B) group strains as determined by their recognition via intracellular adhesion molecule-1 (ICAM-1) or the low-density lipoprotein (LDL) receptor respectively. A new and distinct group of RV (RV-C) have recently been identified (32). Viral dsRNA produced during RV replication are recognized by the host pattern recognition receptors TLR3, melanoma differentiation-associated gene (MDA)-5 and retinoic acid inducible gene (RIG-I) (56, 63). Rhinoviral infection of only a small proportion of airway epithelial cells induces the production of an array of cytokines and chemokines, which mediate the recruitment of immune cells to the airways and potentiate airway inflammation (53).

There is increasing evidence that monocytes may play important roles in driving the inflammation commonly seen in RV-induced acute exacerbations of airways disease. Monocytes and macrophages express high levels of both ICAM-1 and the LDL receptor, and RV exposure evokes the release of inflammatory molecules from both cell types (17, 26, 57). Initial studies suggested that whilst monocytic cells were able to internalize RV, viral replication did not take place (17, 20, 26). In contrast, recent work indicates that limited replication can occur resulting in early induction of type I and III interferons (IFN) (9, 30, 33).

We have previously developed models of inflammation examining the cooperative signaling between monocytes and various tissue cells including epithelial cells, endothelial cells, vascular or airway smooth muscle, that we believe are crucial to effective airway responses to pathogens (5, 37, 38, 44, 49, 50, 64). We have reported that IL-1β plays a major role in the communication between monocytes and tissue cells, and in the initiation of inflammation in response to stimuli modeling predominantly bacterial, but also to some extent viral, infection (5, 37, 38, 44). In particular, activation of monocytes by agonists of TLR4 or TLR5 induces IL-1β release, which is essential for activation of tissue cells (5, 38). IL-1β also potentiates airway cell responses to the synthetic dsRNA mimic, poly(I:C), enhancing proinflammatory cytokine release and ICAM-1 expression (37). This suggests that communication between airway epithelial cells and monocytic cells is likely to be important in managing the response to RV infection. However, the role of IL-1β in RV infection remains to be fully explored and the contribution of monocytes in airway responses to respiratory viruses remains uncertain.

Whilst respiratory viruses are most frequently associated with acute exacerbations of asthma, respiratory bacterial infections can also give rise to these episodes, and can aggravate symptoms following viral infections of the respiratory tract (41). Coinfections with viral and bacterial pathogens are common within the airways of asthmatic and COPD patients (35, 66). RV infection of epithelial cells enhances bacterial adherence and internalization (22, 46, 62), whilst bacterial infection augments ICAM-1 expression on epithelial cells, enhancing inflammation induced by RV (16, 52). Additionally, products of tissue damage such as HMGB1 may be able to activate TLR4 signaling in a manner analogous to LPS (55), and thus multiple signaling pathways are likely to regulate airway responses to infectious stimuli. Our work would predict important roles for IL-1β and cooperative signaling between monocytes and tissue cells in these

responses, but this major component of the innate response to airways infection has not been directly studied.

We determined that IL-1β dramatically potentiated RV-induced proinflammatory responses. Disruption of MyD88 dependent signaling within epithelial cells resulted in impaired CXCL8 production and increased viral replication in response to RV infection. Monocytes did not directly amplify responses to RV alone, but played an important role in the responses seen in coinfection models. These results reveal for the first time that specific epithelial cell responses to rhinovirus are modulated by IL-1β signaling via MyD88, and support IL-1β as a therapeutic target to reduce RV-induced airway inflammation, especially during RV-triggered acute exacerbations in patients with underlying airway disease.

Materials and Methods

Cell and viral culture

All cells were grown in a humidified incubator at 37°C with 5% CO_2. The immortalized epithelial cell line BEAS-2B epithelial cells (American Type Culture Collection [ATCC], LGC Standards, Teddington, UK) were maintained in RPMI 1640 containing 2mM L-glutamine, supplemented with 10% FCS, 100U/ml penicillin, and 100µg/ml streptomycin (cell culture reagents, Invitrogen, Paisley, UK; FCS [endotoxin levels <0.5 EU/ml], Promocell, Heidelberg, Germany). Human rhinovirus minor group serotype 1B (RV-1B) was grown in Ohio HeLa cells (European Collection of Cell Cultures [ECACC], Sigma-Aldrich, Paisley, UK) and stocks prepared as HeLa lysates on average $1x10^7$ $TCID_{50}$/ml (24, 43). Neutralization using serotype specific antibody (ATCC) was carried out to confirm RV-1B identity. UV inactivation and filtration of virus was performed as previously described (25, 43).

Monocyte preparation

Peripheral venous blood was taken from healthy volunteers, with written informed consent, in accordance with a protocol approved by South Sheffield Local Research Ethics Committee. Peripheral blood mononuclear cells (PBMCs) were enriched by centrifugation over OptiPrep™ (Axis Shield, Oslo, Norway) density gradients. Monocytes were further purified by negative magnetic selection using Monocyte Isolation Kit II (Miltenyi Biotec, Audurn, CA, USA) to a typical mean purity of 90 ± 1.185 (mean ± SEM) $CD14^+$ cells.

Stimulation of cells with synthetic agonists

BEAS-2B cells were seeded in 24 well plates and grown to 80% confluence (cells seeded 24–48h before use to attain this confluency). At the time of stimulation cells were washed with phosphate buffered saline (PBS) and cell culture media replaced.

Cells were stimulated with the indicated concentrations of TLR agonists (purified LPS from *Escherichia coli* serotype R515 from Alexis, Nottingham, UK; and the synthetic analogue of double-stranded RNA, polyinosinic-polycytidylic acid (poly(I:C)), InvivoGen, Toulouse, France) or cytokines (recombinant TNFα and IL-1β, PeproTech EC, London, UK) in the presence or absence of 10μg/ml IL-1ra. Cocultures of BEAS-2B epithelial cells and monocytes were created with the addition of 5,000 monocytes/well to 80–90% confluent BEAS-2B cells. Monoculture controls were included in all experiments. Each experiment was conducted multiple times using separate monocyte donors and BEAS-2B cell culture passages. Stimulated cells were incubated at 37°C and supernatants harvested at 24h and stored at -80°C until required.

Infection of cells with RV-1B

BEAS-2B epithelial cells were seeded in 12 well plates and grown to 95% confluence, then placed in RPMI 1640 + 2% FCS (infection media) overnight. Cells were infected with RV-1B at the indicated multiplicity of infections (MOI) for 1h at room temperature with shaking. Virus was then removed and replaced with 1ml infection medium. Cells were incubated at 37°C for 8, 24, or 48h. Cell supernatants or lysates were harvested and stored at -80°C until required.

Stimulation of cells with RV-1B and IL-1β or LPS

BEAS-2B epithelial cells were infected with RV-1B as described above. After removal of the virus 1ml of infection medium was added, containing IL-1β (0.1ng/ml) or LPS (1ng/ml) where required. Cocultures of BEAS-2B cells and monocytes were created with the addition of 9,500 monocytes/well following infection with RV-1B. Monoculture controls were included in all experiments. Each experiment was conducted multiple times using separate monocyte donors and BEAS-2B cell culture passages. Cells were incubated at 37°C for 24h. Cell supernatants were harvested and stored at -80°C until

required.

Virus CPE assay

Subconfluent Ohio HeLa cells in 96 well plates were exposed to serial dilutions of infectious supernatants. Development of a cytopathic effect (CPE) was visualized after 4 days. Assays were performed in eight replicate wells and endpoint titres were defined by the highest dilution at which CPE was observed in 50% of the wells ($TCID_{50}$).

Quantitative PCR

RNA was extracted (RNeasy Mini Kit, Qiagen, Crawley, UK) according to the manufacturers instructions, followed by DNase I digestion of contaminating DNA (DNase [RNase free], Ambion, Warrington, UK). cDNA was synthesised using the high capacity cDNA reverse transcriptase kit (Applied Biosystems, Warrington, UK) from 1μg total RNA as directed by the manufacturer. Quantitative PCR (qPCR) was carried out using primers and probes (Sigma-Aldrich) specific for RV and 18S rRNA as previously described (9), and TaqMan® gene expression assay probe set specific for MyD88 (Hs00182082_m1; Applied Biosystems). Reactions consisted of 10μl 2 x PCR Master Mix (Eurogentec, Southampton, UK), 1μl 20 x probe set for MyD88, 300nM of each primer for 18S rRNA, and 900nM of each primer specific for RV; 18S rRNA and RV probes were used at 175nM. One microlitre of cDNA (18S diluted 1/1000) was made up to 20μl with nuclease-free water. Reactions were carried out using an ABI7900 Automated TaqMan (Applied Biosystems), with cycles consisting of 50°C for 2 min, 94°C for 10 min and 45 cycles of 94°C for 15s, 60°C for 15s.

Quantification of cytokines by ELISA

Cell-free supernatants were collected and stored at -80°C until use. CXCL8, CCL5, CXCL10, IL-1α and IL-1β proteins were quantified by ELISA, using matched Ab pairs

from R&D systems (Abingdon, UK) at previously optimized concentrations (37). The detection limits were 62.5, 312.5, 125, 62.5 and 78.125 pg/ml respectively. Samples containing levels below the limit of detection (LD) value were assigned the LD value.

Protein detection by Western Blot analysis

Cell lysates were prepared from approximately 500,000 BEAS-2B cells and western blot analysis was conducted as previously described (45). Samples were probed with anti-MyD88 (1:500; Cell Signaling Technology, Danvers, MA, USA) or anti-actin (1:10,000; Sigma-Aldrich), all detected using an HRP-coupled anti-rabbit secondary Ab (1:2,000: Cell Signaling Technology) and Amersham ECL™ western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). Densitometric analysis was carried out using NIH image 1.62 analysis software (California, USA).

Lentiviral vector construction

Two single-stranded DNA oligonucleotides targeting MyD88 were designed using BLOCK-IT™ RNAi designer (Invitrogen), one encoding the target shRNA and the other its complement. These oligonucleotides were annealed together and ligated into the pENTR™/H1/TO vector according to the manufacturers instructions (BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit, Invitrogen) to generate an entry clone. Knockdown capability was assessed. The entry clone was then used to carry out a recombination reaction with the pLenti4/BLOCK-iT™-DEST vector, following the manufacturers instructions (BLOCK-iT™ Inducible H1 Lentiviral RNAi System, Invitrogen) to generate an expression clone, which was then packaged into Lentivirus using the Virapower packaging mix (Invitrogen). Lentiviruses were concentrated and purified using tangential flow filtration (Minimate™; PAL Corp., Hampshire, UK), and titred in HT1080 cells or by p24 enzyme-linked immunosorbent assay (ELISA) (Cell Biolabs, San Diego, CA).

Lentiviral vector transduction

BEAS-2B cells were seeded in 24 well plates, grown to 85% confluence, and transduced with MyD88 specific shRNA expressing lentivirus or shRNA transduction particles targeted at IRF3 (HCLNV-NM_001571; Sigma-Aldrich), at MOI of 1 or 5 particles/cell in medium containing 6 μg/mL polybrene (Sigma-Aldrich) for 24h. Virus was removed and fresh growth medium added to the cells and further incubated for 24h. Zeocin™ [MyD88 (Invitrogen)] or Puromycin [IRF3 (Sigma-Aldrich)] was then added to the cells in order to select for clones containing stably integrated lentivirus, which then underwent cell expansion. Cells were subsequently single cell cloned, and individual clones were expanded and assessed for gene knockdown. The creation of stable knockdown cell lines took place over a period of months, and thus our results are unlikely to be caused by the initial transfection process. Moreover, additional control cells created by stably transfecting BEAS-2B cells with a GFP-overexpression plasmid (12) showed no difference from untransfected controls (data not shown).

Statistical analysis

All data are presented as means ± SEM (where appropriate) of at least three independent experiments on separate monocyte donors (11 donors used in total) and BEAS-2B cell culture passages. Data were analyzed using the statistical tests stated, with ANOVA and the indicated post test used for multiple comparisons. Data were analyzed using Prism (version 5, GraphPad).

Results

Monocytes do not potentiate epithelial cell responses to RV-1B infection

We have extensive evidence that monocytes play a crucial role in amplifying epithelial cell responses to bacterial agonists (5, 37, 44), whilst their role in rhinoviral exacerbations has only recently been explored (30). In our first experiments we therefore wished to determine if monocytes would amplify CXCL8 and CCL5 release from BEAS-2B epithelial cells infected with the primary human pathogen RV-1B. CXCL8 was chosen as it is the principal mediator responsible for neutrophil recruitment, and hence is highly relevant to the pathology of asthma and COPD. Its generation is also indicative of activation of classical NF-κB and MAPK dependent proinflammatory signaling. CCL5 was selected since it is a key IFN-stimulated gene (ISG) product generated downstream of IFN-α/β/λ production, which acts to co-ordinate the innate and adaptive immune responses to eliminate viral infections from the host, and has also been linked to asthma susceptibility (31). We found that whilst increasing MOIs of RV-1B caused the expected release of CXCL8 (Fig. 1A) and CCL5 (Fig. 1B) from BEAS-2B epithelial cells, the release of these cytokines was not modulated by the presence of monocytes (Fig. 1A,B). Monocytes have been shown to interact with rhinoviruses (9, 26, 29, 33), and we determined that active RV-1B was released from the epithelial cells by 8h post-infection (Supplemental Fig. 1), thus confirming that the monocytes were exposed to RV-1B for more than 16h of the 24h total incubation period shown in Figure 1.

Co-activation of viral and bacterial signaling pathways potentiates epithelial cell proinflammatory responses

Coinfections with viral and bacterial pathogens are common within the airways of asthmatic and COPD patients (35, 66). Thus we explored whether a model of airway coinfection/multiple TLR signaling would reveal increased inflammatory responses

when monocytes were present. We have previously modeled signaling pathway interactions by stimulating cells with combinations of poly(I:C) (a dsRNA viral mimic) and LPS, and we found that this combination potentiated the release of CXCL8 from cocultures of BEAS-2B epithelial cells and PBMCs (37).

In keeping with our previous data (37), the presence of monocytes notably increased BEAS-2B epithelial cell CXCL8 production in response to LPS alone, or dual poly(I:C) and LPS stimulation (Fig. 2A). The presence of monocytes also resulted in a modest enhancement of CCL5 generation in response to combined stimulation with poly(I:C) and LPS (Fig. 2B). We next sought to determine whether monocytes would amplify RV-1B-infected epithelial cell responses to LPS. Again the presence of monocytes did not increase the cytokine response of epithelial cells to RV-1B alone, but CXCL8 production was substantially potentiated when RV-1B-infected cells were treated with LPS in the presence of monocytes (Fig. 2C). In contrast, no significant difference in CCL5 release was detected in the presence of monocytes, whether or not LPS was added to the cultures (Fig. 2D). Of note, incubation of monocytes alone with any agonist combination caused no detectable cytokine release at the low numbers used in our coculture model (data not shown). Similar patterns of responses were seen over a range of poly(I:C) concentrations (1, 10, 100 μg/ml; Fig. 2A,B and Supplemental Fig. 2) and RV-1B MOIs (MOI 0.6, 1.5, 3; Fig. 2C,D and Supplemental Fig. 3).

Our observations demonstrate that cooperative actions of epithelial cells and monocytes can regulate the proinflammatory environment in response to TLR4 engagement, and that this combination of cells and stimuli can amplify the response to viral infection. We therefore wanted to identify the specific factor(s) responsible for the monocyte-dependent enhancement of CXCL8 release during co-infections.

IL-1β potentiates epithelial cell responses to RV-1B infection.

We believe that IL-1β signaling is a key early proinflammatory stimulus in airways inflammation, since monocyte-derived IL-1β is required for effective induction of inflammation in response to TLR agonists in a range of airway tissue cells (5, 37, 38, 44, 64). Furthermore, we have previously shown that IL-1β potentiates cytokine release from epithelial cells stimulated with poly(I:C) (37). Thus, we investigated whether the direct addition of this apical cytokine would potentiate responses to RV-1B in airway epithelial cells. When a submaximal concentration of IL-1β (0.1ng/ml; Fig. 5A) was added to virally-infected cells (immediately following the initial infection) a marked increase in CXCL8 release was observed (Fig. 3A). In contrast, CCL5 production from RV-1B-infected epithelial cells was significantly, though modestly, inhibited when IL-1β was also present (Fig. 3B). No significant differences in CXCL10 release were observed (Fig. 3C).

shRNA suppression of MyD88 expression in BEAS-2B epithelial cells.

In order to dissect the effects and roles of IL-1β signaling on RV-1B epithelial cell infection in more detail we used lentiviral delivery to create a stable knockdown of MyD88, the IL-1R1 signaling adapter. The stable knockdown line exhibited significantly lower MyD88 mRNA (71% mean reduction, Fig. 4A) levels compared to wild-type (WT) cells. Figure 4B shows a representative blot of MyD88 protein levels, which were significantly reduced in MyD88 knockdown cells compared to WT ($p<0.05$; $n=14$). Of note MyD88 is present at low levels even in WT cells, and blots required prolonged exposure (1h) to detect the protein in all cells. These data confirm that shRNAs are expressed long-term in the BEAS-2B cell line resulting in stable reduction of MyD88 expression; cells are henceforth referred to as $MyD88^{KD}$. The $MyD88^{KD}$ stable line (initially frozen in bulk, with each aliquot used over approximately 12 passages in parallel with WT BEAS-2B cells) exhibited a stable phenotype over >1 year usage. IRF3 is a key transcription factor involved in the induction of IFNs in response to TLR3 and

RLR signaling. IRF3 signaling is strongly implicated in responses to viral infection or poly(I:C) (27) but is not thought to be involved in responses to IL-1β. We therefore generated a second stable cell line in which IRF3 was knocked down by shRNA in order to create an additional control for the MyD88^{KD} line. IRF3 mRNA and protein knockdown was confirmed as described above (data not shown) and cells are in future referred to as IRF3^{KD}.

MyD88^{KD} and IRF3^{KD} epithelial cells exhibit selective defects in responses to proinflammatory stimuli.

MyD88 is the essential adapter for IL-1β signaling (13). Figure 5A shows that MyD88^{KD} cells produced significantly less CXCL8 than WT cells in response to IL-1β stimulation, whereas IRF3^{KD} cells showed no difference in CXCL8 production when compared to WT cells. In contrast, IRF3^{KD} cells showed preserved responses to IL-1β but marked defects in their responses to poly(I:C) (Fig. 5B, E). Both IRF3^{KD} and MyD88^{KD} cells retained normal responses to TNFα (Fig. 5C), whose signaling occurs independently of these components (11). The process of creating stable lines did not alter the ability of cells to respond to subsequent viral agonists, since CXCL8 and CCL5 production from MyD88^{KD} cells in response to poly(I:C) stimulus was comparable to that from WT cells (Fig. 5B, E).

Cellular communication between epithelial cells and monocytes requires MyD88 expression.

Our previous work has shown that complex networks exist between monocytes and many tissue cell types, and that these cells communicate to amplify inflammatory responses via the release of IL-1β (5, 37, 38, 44, 64). Such communication facilitates and enhances responses to innate immune stimuli. This communication is particularly evident in responses to TLR4 agonists, since the expression of this receptor is limited in

epithelial cells, but is also evident in response to TLR5 agonists, despite clear evidence of functional TLR5 in epithelial cells (5, 70).

Accordingly, we tested the ability of WT and MyD88^{KD} cells to respond to LPS, or IL-1β itself, in the presence or absence of monocytes. In keeping with previous data, monocytes were required to initiate CXCL8 production in response to LPS (Fig. 6A). We now provide further support for data showing that IL-1β signaling is crucial to these responses, since lack of MyD88 expression within epithelial cells markedly reduced CXCL8 release in response to LPS, achieving an equivalent reduction in CXCL8 release to exogenous IL-1ra addition (Fig. 6A). We also reveal that epithelial cell responses to IL-1β are potentiated by monocytes (Fig. 6B) and confirm that MyD88 is the crucial adapter for IL-1β-dependent signaling (Fig. 6B).

RV-1B infection triggers MyD88-dependent signaling pathways that can regulate RV-1B replication.

Most research to date has focused on the roles of the IFN response systems in rhinoviral infections. We wished to investigate whether MyD88-dependent signaling also plays a biological role in rhinoviral infections since IL-1β, and therefore MyD88, plays such a major role in amplifying the proinflammatory response to RV-1B (Fig. 3A). We found that RV-1B infection in MyD88^{KD} cells resulted in significantly less CXCL8 release compared to WT cells at the higher MOIs tested (MOI 1.5 and 3; Fig. 7A). These observations demonstrate for the first time that MyD88-dependent signaling mechanisms play an important role in epithelial cell responses to RV-1B infection.

To explore whether MyD88 signaling was occurring in an autocrine manner, as a consequence of IL-1β release in response to RV-1B infection, we measured IL-1α and IL-1β levels in the supernatant 24h after RV-1B infection. We found that both IL-1α and IL-1β were below the detection limit of their ELISA, 62.5 and 78.125 pg/ml respectively

(data not shown). In our assays, IL-1β potently amplifies RV-1B signaling at very low concentrations (see Fig. 3A, where 100 pg/ml IL-1β causes marked CXCL8 generation). Therefore to further probe if IL-1 signaling was implicated in our experiments, we exploited the specific antagonist of IL-1R1, IL-1ra. Addition of IL-1ra immediately following RV-1B infection of epithelial cells caused a significant decrease in CXCL8 production to RV-1B at an MOI of 3 (Fig. 7B), similar to that produced in MyD88^{KD} cells (Fig. 7A), but did not alter cytokine production at lower viral infectivities (Fig. 7B). These data suggest that autocrine release of IL-1 from RV-1B-infected cells does contribute to the resulting inflammatory response to viral infections, particularly when viral infections are severe (higher infectivities).

Given the critical role of IL-1β signaling in damage/danger responses we postulated that MyD88^{KD} would have more far reaching consequences on RV-1B infection, and thus determined its effect on RV-1B replication. WT and MyD88^{KD} cells were infected with RV-1B (MOI 0.6, 1.5, 3), and intracellular levels of RV-1B RNA determined 24h post infection by qPCR. As expected, no viral RNA was detected within uninfected cells and RV-1B RNA gradually increased with increasing MOI of virus in WT cells (Fig. 7C). Importantly, elevated levels of viral RNA were detected in MyD88^{KD} cells compared to WT cells, which reached statistical significance at RV-1B MOI 3 (Fig. 7C), correlating with the MOI at which exogenous inhibition of IL-1β using IL-1ra also had the greatest impact (Fig. 7B). The release of infective RV-1B from the MyD88^{KD} epithelial cells did not significantly differ from WT cells at 8 or 24h post-infection (data not shown) but was augmented at 48h, again reaching statistical significance at RV-1B MOI 3 (Fig. 7D). The difference between viral copy number and $TCID_{50}$ is in keeping with results of other groups (65), and is probably a result of two factors. Firstly, $TCID_{50}$ underestimates the number of viral copies measured by qPCR, potentially by several orders of magnitude

(51). Secondly, the $TCID_{50}$ in Figure 7D represents viral particles released into the supernatant, and not the total cellular viral content measured by qPCR.

Discussion

The airways are frequently exposed to coinfections with more than one pathogen, particularly in conditions such as COPD. Even during infection with a single pathogen, such as RV-1B, multiple inflammatory pathways may be activated by the pathogen or independently through inhalation of environmental levels of endotoxin (5), or the release of endogenous mediators of tissue damage such as IL-1 species or HMGB1 (14). Additionally, monocytes and macrophages are present in the airways of patients with lung disease and are likely to contribute to the response to pathogens (36, 61). Thus, determining how pathogens induce inflammation requires modeling of potentially complicated interactions between cell types and signaling pathways. In this study we establish that monocytes play an important role in responses to dual stimulation with RV-1B and the TLR4 agonist LPS, and begin to define the potential of IL-1β to act as a major player during the exacerbations that commonly afflict asthmatic and COPD patients. In particular we identify IL-1β as the driving force controlling release of CXCL8, a potent neutrophil chemoattractant, and thus anticipate IL-1β may be a viable target for controlling the neutrophilia that is often implicated in airways disease. Finally, we determine that IL-1β/MyD88 plays a role in regulating RV-1B replication and the inflammatory response to viral infection of the airways.

The vast majority of studies into rhinoviral infections focus on airway epithelial cells, since they are the primary sites of replication, and are known to release a variety of inflammatory mediators to combat infection (42, 53). In this study we utilised the BEAS-2B epithelial cell line because they closely resemble bronchial epithelial cells by electron microscopy, express keratin, form tight junctions, and produce mucin (40, 47). The BEAS-2B cell line has also been used extensively for the study of rhinoviral infections in epithelial cells, and the findings using this model system have been verified in primary cells (28, 63, 67). We have previously provided strong evidence that effective

tissue cell responses to TLR agonists, including those acting on TLR4, require monocyte-derived IL-1β to initiate inflammation (5, 37, 38, 44, 64). However, synthetic dsRNAs are less efficacious activators of monocytes (37), and typically act directly on tissue cells via TLR3 and the RLRs (27). Monocytes have been shown to interact with rhinoviruses (9, 26, 29, 33), and we hypothesized that they would amplify responses to RV-1B. Contrary to our initial expectation, our data reveal that whilst monocytes are exposed to active RV-1B released from the BEAS-2B epithelial cells, they do not amplify RV-1B-induced cytokine (CXCL8, CCL5) production from epithelial cells.

Of note, one group recently showed that release of CXCL10 from epithelial cells in response to RV-16, a major group virus, was augmented in a monocytic-cell dependent manner (30). However, this study used a different monocyte purification protocol that generated a population including CD16+ monocytes, and performed coculture experiments with an epithelial medium that contained alternative supplements including low amounts of hydrocortisone. Furthermore, the work of Korpi-Steiner et al allowed monocytes to interact with epithelial cells overnight before stimulation, allowing potential for cellular differentiation to occur (30). In contrast, we found that RV-1B-induced CXCL10 release is modestly decreased in the presence of monocytes (data not shown). Thus, it is feasible that in some circumstances monocytes may amplify responses to RV, but our data indicate that the relative amplification of responses to RV by monocytes is considerably more evident when models are designed to explore the contexts of coinfection.

We observed that exogenous IL-1β caused striking amplification of CXCL8 release in response to RV-1B. Coinfections with viral and bacterial pathogens are common within the airways of asthmatic and COPD patients (35, 66). To explore the potential for coinfections to drive neutrophilic inflammation, we set up models of RV-1B-infected BEAS-2B epithelial cells and monocytes in coculture, stimulated with low concentrations

of LPS. In this coinfection model, we found that monocytes were markedly able to enhance RV-1B-induced CXCL8 release, in a pathway that our data and previous work indicates is likely to involve the in-culture generation of IL-1β by LPS-activated monocytes (5, 37, 38, 44, 64). Clinical studies report that rhinoviral induction of CXCL8 release from bronchial epithelial cells is an important trigger of acute exacerbations (1, 15), and our data indicate how such CXCL8 generation may be strongly activated. It is therefore clear that communication between airway tissue cells and infiltrating monocytes is a key driver of CXCL8 release, and consequently neutrophilic inflammation, and we therefore focused further on the roles of IL-1β and its signaling pathways in viral-induced inflammation.

One potential mechanism of amplification of responses in our coinfection model is IL-1β-induced upregulation of the receptors responsible for viral infection or detection. Indeed, major group HRVs cause the endogenous release of IL-1β, which then acts in an autocrine manner to further potentiate inflammatory responses, for example through upregulation of the HRV major group receptor, ICAM-1 (18, 19, 58). However, we found that whilst IL-1β enhanced CXCL8 release from virally infected epithelial cells, it did not augment virally-induced ISGs, having no impact on CXCL10 production and in fact modestly reducing CCL5 release, implying cooperation of stimuli has selective actions on specific pathways. The recent discovery that MyD88 inhibits IKKε-induced IRF3 activation in response to TLR3 activation, thus restricting IFNβ and CCL5 production (54), may help to explain our findings. In keeping with this concept, the cooperative effects of RV and exogenous TNFα on epithelial cell chemokine production have also been described, an effect attributed to transcriptional changes in CXCL8 promoter activity rather than changes in ICAM-1 expression (39).

To investigate the role of the IL-1β signaling pathway in more detail, we created BEAS-2B cells deficient in MyD88, the adapter protein that is crucial for IL-1R1 signaling.

MyD88^{KD} cells showed significantly impaired responses to IL-1β signaling in monoculture and coculture, but had preserved responses to poly(I:C) and TNFα, demonstrating that we had created a stable cell line with selective impairment of MyD88-dependent signaling. Intriguingly we found that, even in the absence of exogenous addition of IL-1β, MyD88 deficiency led to a reduction in RV-1B-induced CXCL8 release, whilst intracellular viral replication and release of infective virus from cells was concomitantly enhanced. One potential explanation for the observed effects of MyD88 deficiency is that the release of small amounts of IL-1α from virally-infected epithelial cells acts in an autocrine manner to potentiate inflammation. Infection of airway epithelial cells with respiratory syncytial virus or adenovirus type 37 causes the release of IL-1α, which subsequently upregulates expression of cell-surface adhesion molecules including ICAM-1 (3, 4). IL-1α is thought to act either intracellularly, or in a membrane-bound form, unless cells are undergoing necrosis (6, 8, 13). We did not detect free IL-1α or β production from viral-infected BEAS-2B cells, but our data indicate that IL-1 is biologically active at very low concentrations, and thus to probe whether IL-1 generation was relevant we also used the specific antagonist IL-1ra, which inhibited virally-induced CXCL8 release at the highest MOI to a similar degree as MyD88 knockdown. These data show that autocrine release of IL-1 does indeed play a role during more severe viral infection, possibly via the localized release of IL-1α or β, through cell-associated IL-1α, or virally-induced epithelial cell necrosis allowing IL-1α release at levels below detection by ELISA.

There is a wealth of data to suggest that IL-1β levels are enhanced within the airways of patients with COPD or asthma, with further increases detected during acute exacerbations (7, 10, 59). Furthermore, epithelial and monocytic cells (PBMCs and alveolar macrophages) taken from such patients respond to inflammatory stimuli with greater IL-1β production (21, 48, 68). Polymorphisms in the IL1B (IL-1β) and IL1RN (IL-

1ra) are associated with a greater risk of COPD (34) and asthma (69), although the exact correlation remains to be clarified (2). Thus, evidence of the detrimental effects of IL-1β within the airway is established (60). Our work now identifies MyD88 signaling as a valid and potentially important target to limit viral-induced airway inflammation, since its signaling is implicated at two important points: (1) the endogenous response to virus in BEAS-2B cells, and (2) the IL-1β-mediated cooperative signaling seen in models of airway coinfection.

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Figure Legends

Figure 1. Monocytes do not potentiate epithelial cell responses to RV-1B infection.
BEAS-2B cells were grown to 95% confluency in 12 well plates. Cells were infected for 1h with RV-1B at MOI of 0.6, 1.5 and 3. Cocultures were created with the addition of 9500 highly purified $CD14^+$ monocytes. Monoculture controls were also created. After 24h cell free supernatants were generated and amounts of secreted CXCL8 (A) and CCL5 (B) release measured by ELISA. Data shown are mean ± SEM of n=7, with each replicate performed on separate passages of WT cells with freshly prepared monocytes from independent donors.

Figure 2. Co-activation of viral and bacterial signalling pathways potentiates epithelial cell pro-inflammatory responses
BEAS-2B cells were grown to 95% confluency in 12 well plates. Cells were stimulated with media, LPS (0.1ng/ml), Poly(I:C) (1μg/ml), or LPS/Poly(I:C) in combination (A, B). In viral experiments cells were infected for 1h with RV-1B (MOI 3), followed by stimulation with media or LPS (0.1ng/ml) (C, D). Cocultures were created with the addition of 9500 highly purified $CD14^+$ monocytes. Monoculture controls were also created. After 24h cell free supernatants were generated and release of CXCL8 (A, C) or CCL5 (B, D) measured by ELISA. Data shown are mean ± SEM of n=3, with each replicate performed on separate passages of BEAS-2B cells with freshly prepared monocytes from independent donors. Data were analysed by two-way ANOVA with Bonferroni's posttest. Significant differences are indicated by *P<0.05 and ***P<0.001.

Figure 3. IL-1β potentiates epithelial cell responses to RV-1B infection.
BEAS-2B cells were grown to 95% confluency in 12 well plates. Cells were infected for 1h

with RV-1B (MOI 0.6, 1.5, 3), followed by stimulation with IL-1β (0.1ng/ml) for 24h. Cell free supernatants were generated and CXCL8 (A), CCL5 (B), or CXCL10 (C) release measured by ELISA. Data are mean ± SEM of n=4 (A, B) or n=3 (C), with each replicate performed on separate passages of BEAS-2B cells. Data were analysed by two-way ANOVA with Bonferroni's posttest. Significant differences are indicated by **P<0.01 and ***P<0.001 compared to media control at the same MOI.

Figure 4. shRNA suppression of MyD88 expression in BEAS-2B epithelial cells.

BEAS-2B cells were transduced with lentivirus containing a MyD88-targeted shRNA vector, and cultured for 24h. This was followed by antibiotic selection for 10-14 days using puromycin, single cell cloning, and individual colony expansion. Total RNAs were extracted and analysed by quantitative PCR with primers specific to MyD88 and 18srRNA (A). Whole cell lysates were analysed by western blot using antibodies specific to MyD88 and β-actin (B). A representative image of three independent BEAS-2B and $MyD88^{KD}$ passages (P1-P3) are shown (B). Data are presented as mean ± SEM of N=5 (A). Significant differences are indicated by ***P<0.001 for quantitative PCR, as analysed by paired t-test.

Figure 5. $MyD88^{KD}$ and $IRF3^{KD}$ epithelial cells exhibit selective defects in responses to pro-inflammatory stimuli.

Wildtype BEAS-2B (WT), $MyD88^{KD}$ or $IRF3^{KD}$ cells were grown to 90-95% confluency in 24-well plates and stimulated with IL-1β (A, D), Poly(I:C) (B, E) or TNFα (C, F) at the indicated concentrations for 24h. Cell free supernatants were generated and CXCL8 (A, B, C) or CCL5 (D, E, F) release measured by ELISA. Data are mean ± SEM of n=4-6, with each replicate performed on separate passages of WT, $MyD88^{KD}$ and $IRF3^{KD}$ cells. Data are analysed by two-way ANOVA, with Bonferroni's posttest. Significant differences are

indicated by *P<0.05, **P<0.01 and ***P<0.001 compared to WT control.

Figure 6. Cellular communication between epithelial cells and monocytes requires MyD88 expression

Wildtype BEAS-2B (WT) or MyD88^{KD} cells were grown to 90-95% confluency in 24-well plates, and cocultures created with the addition of 5000 highly purified CD14^{+} monocytes. Monoculture controls of WT or MyD88^{KD} were also created. Cells were stimulated with (A) LPS (10ng/ml) or (B) IL-1β (10ng/ml) in the presences or absence of IL-1ra (10μg/ml) for 24h. Cell free supernatants were generated and CXCL8 release measured by ELISA. Data shown are mean ± SEM of n=5 with each replicate performed on separate passages of WT or MyD88^{KD} cells, with freshly prepared monocytes from independent donors. Data were analysed by two-way ANOVA and Bonferroni's posttest. Significant differences are indicated by ***P<0.001 or $^{###}$P<0.001 WT monoculture versus WT coculture (IL-1β-stimulated).

Figure 7. RV-1B infection triggers MyD88-dependent signalling pathways

Wildtype BEAS-2B (WT) or MyD88^{KD} cells were grown to 95% confluency in 12 well plates. Cells were infected for 1h with RV-1B at MOI of 0.6, 1.5 and 3, and then incubated for 24h (A-C) or 48h (D). BEAS-2B cells were incubated in the presence of IL-1ra (10μg/ml) for 24h where required (B). Cell free supernatants were generated and amounts of secreted CXCL8 release (A, B) measured by ELISA. For viral replication quantification (C), RNA was extracted from lysates and rhinovirus RNA expression was quantified using TaqMan® PCR, with data presented as the total intracellular viral RNA copies per well. The average number of cells per well at the time of lysis was WT=6.1x10^5±1.9x10^5 and MyD88^{KD}=4.5x10^5±1x10^5 (n=4). Viral particle release into the supernatant (D) was quantified by viral CPE assay and presented as $TCID_{50}$/ml. Data shown are mean ± SEM

of n=7 (A), n=6 (B) or n=4 (C, D) with each replicate performed on separate passages of cells. Data were analysed by two-way ANOVA and Bonferroni's posttest. Significant differences between WT and MyD88^{KD} are indicated by *P<0.05, **P<0.01 and ***P<0.001. Significant differences between IL-1ra treated RV-1B-infected WT cells are indicated by **P<0.01 compared to media control at the same MOI.

Figure 1

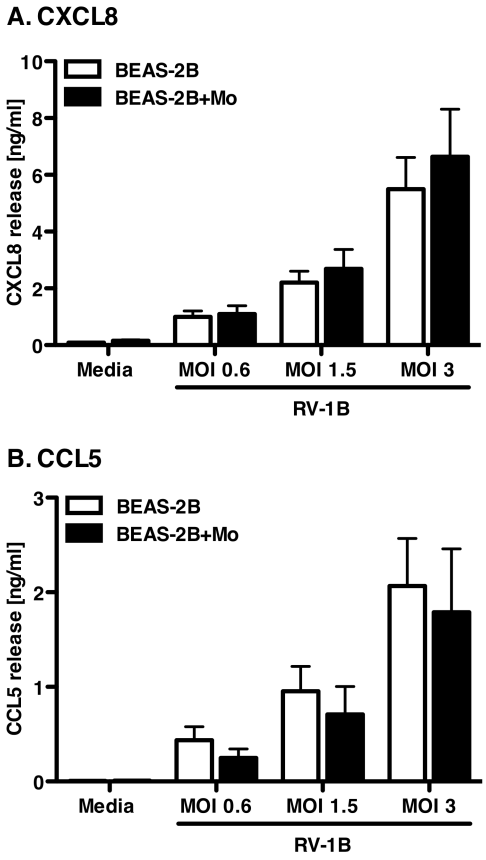

A. CXCL8
BEAS-2B
BEAS-2B+Mo
CXCL8 release [ng/ml]
10
8
6
4
2
0
Media
MOI 0.6
MOI 1.5
MOI 3
RV-1B
B. CCL5
BEAS-2B
BEAS-2B+Mo
CCL5 release [ng/ml]
3
2
1
0
Media
MOI 0.6
MOI 1.5
MOI 3
RV-1B

Figure 2

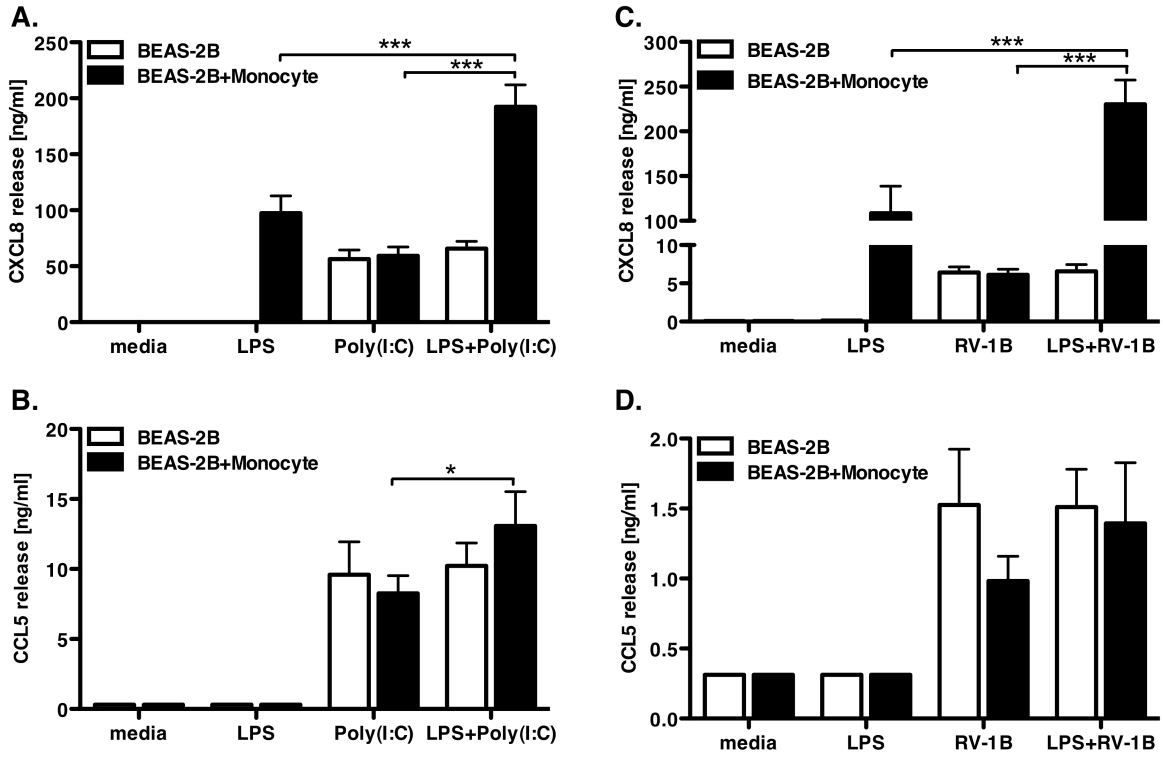

A.
BEAS-2B
BEAS-2B+Monocyte
CXCL8 release [ng/ml]
250
200
150
100
50
0
***
***
media
LPS
Poly(I:C)
LPS+Poly(I:C)
C.
BEAS-2B
BEAS-2B+Monocyte
CXCL8 release [ng/ml]
300
250
200
150
100
10
5
0
***
***
media
LPS
RV-1B
LPS+RV-1B
B.
BEAS-2B
BEAS-2B+Monocyte
CCL5 release [ng/ml]
20
15
10
5
0
*
media
LPS
Poly(I:C)
LPS+Poly(I:C)
D.
BEAS-2B
BEAS-2B+Monocyte
CCL5 release [ng/ml]
2.0
1.5
1.0
0.5
0.0
media
LPS
RV-1B
LPS+RV-1B

Figure 3

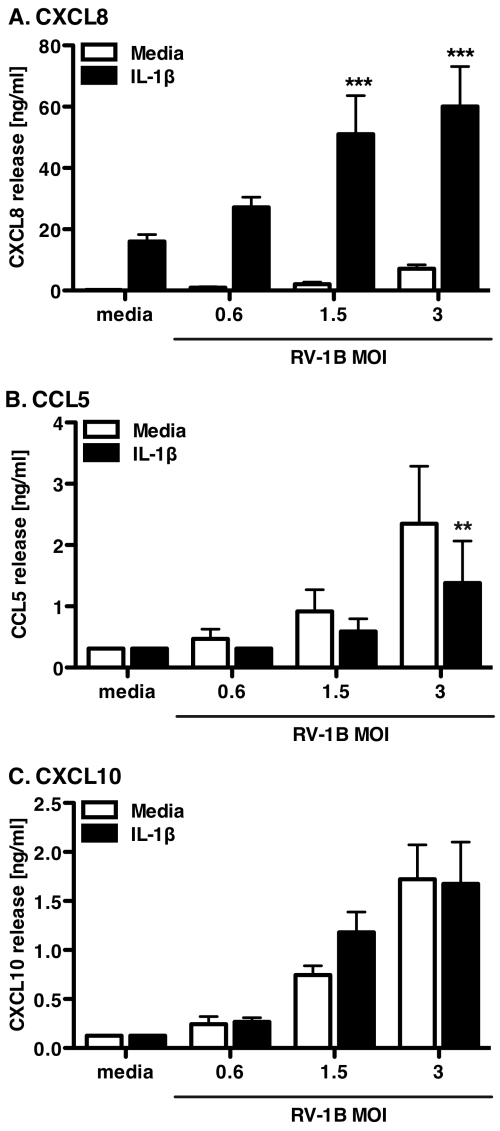

A. CXCL8
Media
IL-1β
CXCL8 release [ng/ml]
80
60
40
20
0
***
***
media
0.6
1.5
3
RV-1B MOI
B. CCL5
Media
IL-1β
CCL5 release [ng/ml]
4
3
2
1
0
**
media
0.6
1.5
3
RV-1B MOI
C. CXCL10
Media
IL-1β
CXCL10 release [ng/ml]
2.5
2.0
1.5
1.0
0.5
0.0
media
0.6
1.5
3
RV-1B MOI

Figure 4

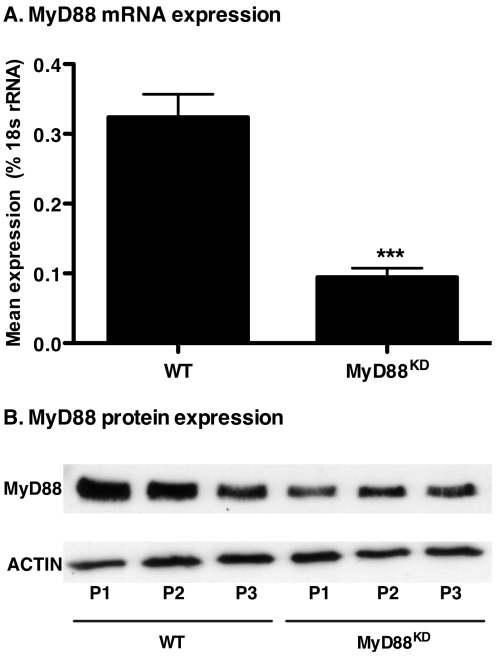

A. MyD88 mRNA expression
Mean expression (% 18s rRNA)
0.4
0.3
0.2
0.1
0.0
***
WT
MyD88KD
B. MyD88 protein expression
MyD88
ACTIN
P1
P2
P3
P1
P2
P3
WT
MyD88KD

Figure 5

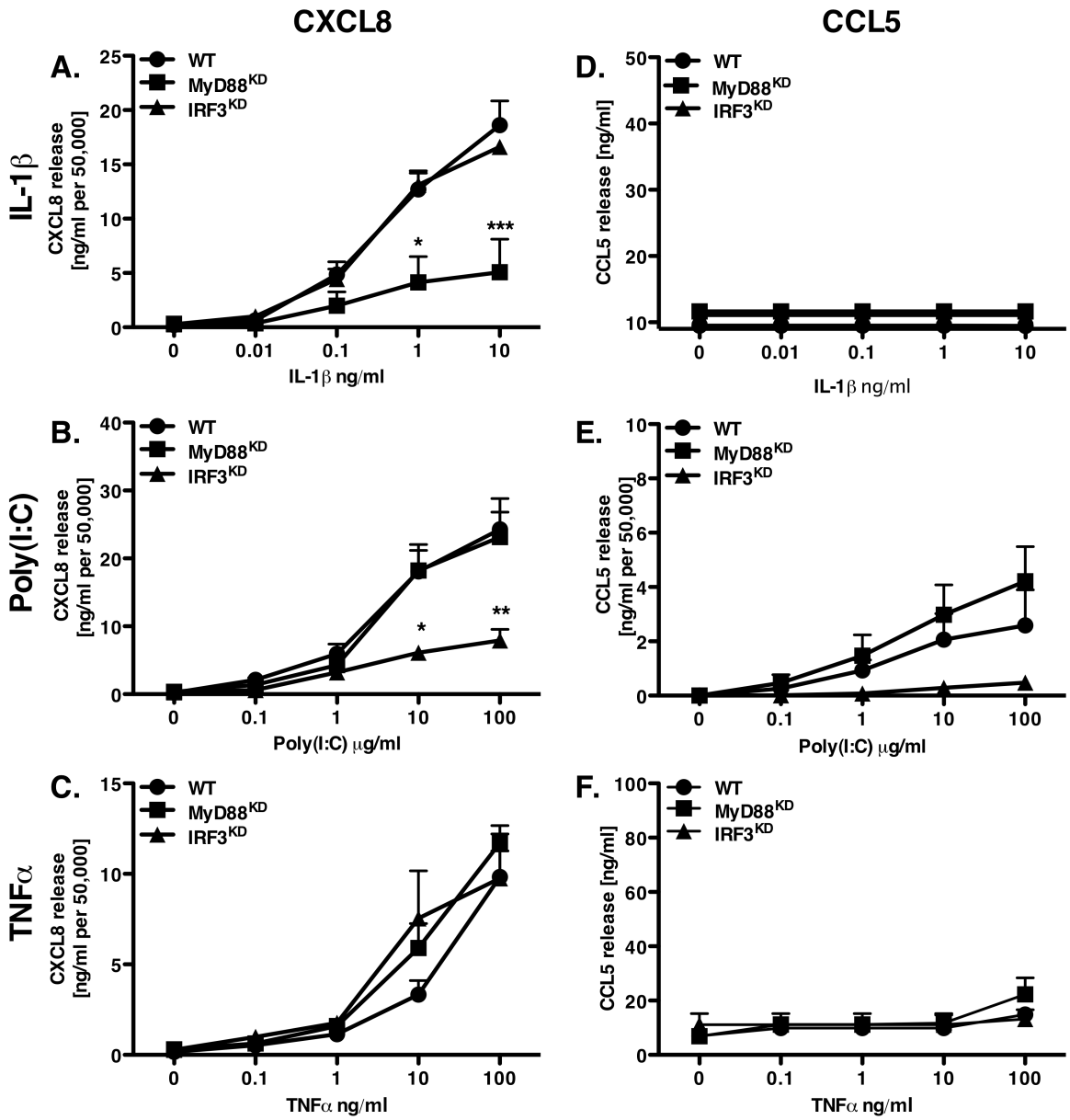

CXCL8
CCL5
A.
D.
B.
E.
C.
F.
WT
MyD88KD
IRF3KD
IL-1β
Poly(I:C)
TNFα
CXCL8 release [ng/ml per 50,000]
CCL5 release [ng/ml]
IL-1β ng/ml
Poly(I:C) μg/ml
TNFα ng/ml
*
**
***

Figure 6

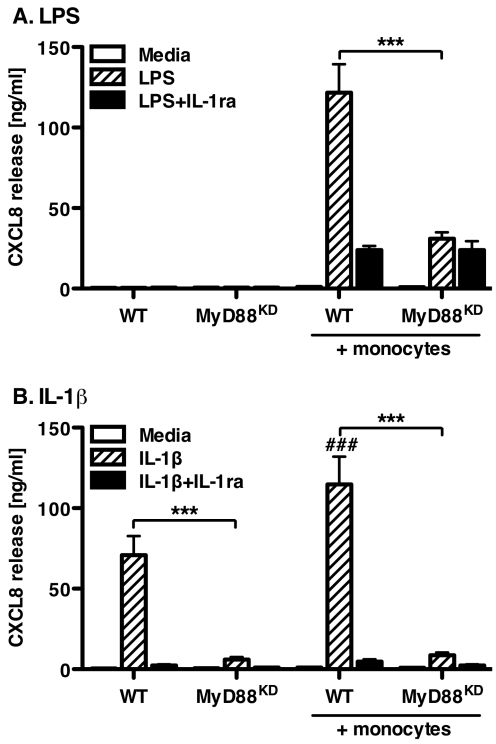
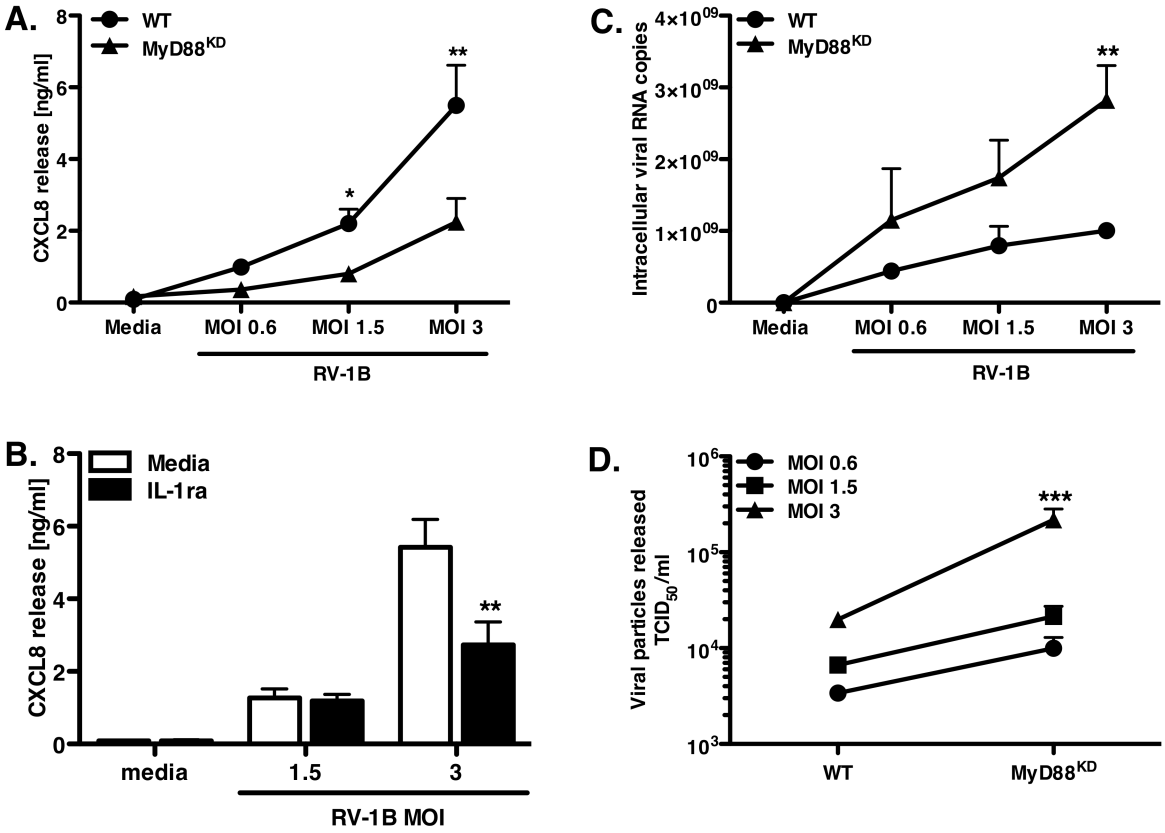

A. LPS
Media
LPS
LPS+IL-1ra
CXCL8 release [ng/ml]
150
100
50
0
***
WT
MyD88KD
WT
MyD88KD
+ monocytes
B. IL-1β
Media
IL-1β
IL-1β+IL-1ra
CXCL8 release [ng/ml]
150
100
50
0
***
###
***
WT
MyD88KD
WT
MyD88KD
+ monocytes

Figure 7


A.
WT
MyD88KD
CXCL8 release [ng/ml]
Media
MOI 0.6
MOI 1.5
MOI 3
RV-1B
C.
Intracellular viral RNA copies
B.
Media
IL-1ra
media
1.5
3
RV-1B MOI
D.
MOI 0.6
MOI 1.5
MOI 3
Viral particles released
TCID50/ml
WT
MyD88KD