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Activation of human macrophages by bacterial components relieves the restriction on replication of an interferon-inducing Parainfluenza Virus 5 (PIV5) P/V mutant

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Abstract

Macrophages regulate immune responses during many viral infections, and can be a major determinant of pathogenesis, virus replication and immune response to infection. Here, we have addressed the question of the outcome of infection of primary human macrophages with parainfluenza virus 5 (PIV5) and a PIV5 mutant (P/V-CPI-) that is unable to counteract interferon (IFN) responses. In cultures of naïve monocyte-derived macrophages (MDMs), WT PIV5 established a highly productive infection, whereas the P/V-CPI- mutant was restricted for replication in MDMs by IFN-beta. Restricted replication in vitro was relieved in MDM that had been activated by prior exposure to heat killed Gram positive bacteria, including *Listeria monocytogenes*, *Streptococcus pyogenes*, and *Bacillus anthracis*. Enhanced replication of the P/V mutant in MDM previously activated by bacterial components correlated with a reduced ability to produce IFN-beta in response to virus infection, whereas IFN signaling was intact. Activated MDM were found to upregulate the synthesis of IRAK-M, which has been previously shown to negatively regulate factors involved in TLR signaling and IFN-beta production. We discuss these results in terms of the implications for mixed bacteria-virus infections and for the use of live RNA virus vectors that have been engineered to be attenuated for IFN sensitivity.

Keywords

Parainfluenza virus; macrophage; innate immunity

1. INTRODUCTION

Many viruses infect professional antigen presenting cells (APC) such as dendritic cells (DC) and macrophages, two cell types that play a critical role in coordinating innate and adaptive immune responses [1,2]. This pathogen-APC interaction is particularly important for immune responses to viruses, since the outcome of whether the virus suppresses antiviral responses in APC or whether the APC suppresses the virus infection can be an important determinant of the subsequent adaptive immune response [2,3]. In this study, we have

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addressed the question of the outcome of infection of primary human macrophages with parainfluenza virus 5 (PIV5) and a PIV5 mutant that is unable to counteract type I interferon (IFN) responses.

Macrophages are specialized APC that recognize, phagocytose and destroy pathogens, and can be an important source of antiviral cytokines [6]. In some cases, macrophages have been shown to be indispensable for control of viral infections [4,5]. While DC are thought to be more potent stimulators of T cell activation, macrophages are also capable of expressing high levels of costimulatory molecules such as CD80 and CD86 and presenting microbial peptides to lymphocytes [2,5]. When activated by exposure to pathogens, macrophages are also capable of secreting cytokines such as interleukin-6 (IL-6), TNF-alpha, and IFN-beta. However, in some cases these activated macrophages subsequently become refractory to further stimulation, due at least in part to the upregulation of inhibitory molecules that dampen potential hyperactive responses [7,8].

Macrophages can differ in their response to virus infection depending on the particular virus, becoming either activated or suppressed by virus infection. For example, macrophages are activated following infection with Epstein Barr virus [9] and respiratory syncytial virus (RSV; 10). By contrast, macrophage functions can be inhibited by other viruses such as HIV [11], Hepatitis A virus [12], and Human Cytomegalovirus (HCMV; 13). In addition, it has recently been shown that the outcome of virus infection of APC can differ significantly between naïve APC and cells that are activated by prior exposure to bacterial components [14,15,16,17]. Here, we demonstrate that this difference between infections of naïve versus activated human macrophages also applies to an IFN-sensitive parainfluenza virus.

PIV5 (formerly called Simian Virus 5, SV5) is a prototype member of the parainfluenza virus family. We have previously shown that WT PIV5 establishes a highly productive infection of primary human DC, and DC functions such as upregulation of costimulatory molecules and activation of T cells are inhibited in vitro by PIV5 infection [18]. PIV5 inhibition of host cell responses is due largely to the viral V protein, a multifunctional protein encoded in the viral P/V gene [19]. PIV5 V protein targets the host cell protein STAT1 (signal transducer and activator of transcription 1) for degradation [20], resulting in a block in IFN signaling. V protein also blocks activation of the IFN-beta promoter through targeted inhibition of the RNA helicase mda-5 [21]. Thus, during infection of epithelial- and fibroblast-like cells, WT PIV blocks both the production of IFN and IFN signaling.

We have previously engineered a recombinant PIV5 mutant (P/V-CPI-) to encode six amino acid substitutions in the P/V gene in the background of an otherwise WT PIV5 genome [22]. These P/V gene substitutions converted WT PIV5 into a mutant that failed to target STAT1 for degradation, and the P/V-CPI- virus was also a potent inducer of IFN-beta and proinflammatory cytokines [22]. The immunostimulatory properties and IFN sensitivity of the P/V-CPI- mutant makes it an attractive virus for our current development of PIV5-based therapeutic vectors [18,23,24]. Consistent with this effort, P/V-CPI- is restricted for replication in primary cultures of human DC, but infected DC are activated and are better than WT PIV5-infected DC at inducing T cell proliferation [18].

Given that a virus can differ significantly in its interactions with and replication within naïve versus activated cells and between DC and macrophages [16,25,26], we have tested the outcome of infection of primary human macrophages with the PIV5 P/V mutant. Here we demonstrate that replication of P/V-CPI- is restricted in naïve human macrophages by IFN-beta production, but this restricted replication is relieved in MDM that are activated by components from Gram positive bacteria. Our results have implications for mixed bacteria-

virus infections and for the potential use of live RNA virus vectors that are engineered to be attenuated for IFN sensitivity.

2. MATERIALS AND METHODS

2.1 Cells, bacteria and viruses

Immature peripheral blood mononuclear cells (PBMCs) were derived from whole human blood as described previously [18]. Briefly, PBMC were isolated from randomly selected donors by standard density gradient centrifugation on Ficoll-Hypaque. CD14⁺ monocytes were isolated by positive selection using CD14⁺ microbeads (Miltenyi-Biotec). Generally, the isolated cells were found to be $\geq 95\%$ CD14⁺ by flow cytometry. Monocyte derived macrophages were generated as described previously [13]. Briefly, enriched CD14⁺ monocytes were cultured for 6 days in RPMI media (Lonza Inc.) containing 20 ng/ml MCSF (Invitrogen) supplemented with 10% FBS, and 1% each of L-glutamine, penicillin/streptomycin, nonessential amino acids, 1M HEPES, and sodium pyruvate (Lonza). After 3 days in culture, half of the media was replaced, and additional MCSF was added.

WT PIV5 and P/V-CPI- both expressing GFP were recovered as described previously [22] from a cDNA plasmid kindly provided by Robert Lamb (Northwestern University) and Biao He (University of Georgia). WT PIV5 and P/V-CPI- stocks were grown in MDBK and Vero cells, respectively.

Streptococcus pyogenes (MGAS5005 strain) was cultured as described previously [46] in Todd-Hewitt broth (Becton-Dickinson) supplemented with 2% yeast extract (Fisher Scientific). *Bacillus anthracis* (Sterne strain) was cultured as described previously in brain-heart infusion broth (BHI; Becton-Dickinson) [47]. Bacteria were killed by immersion in a water bath at 80°C for 75 min. Alternatively, heat-killed *Listeria monocytogenes* was obtained commercially (Invivogen) and was used according to manufacturer's instructions.

2.2 Western Blotting

For Western Blotting, six well dishes of cells were infected as described in the figure legends. At the indicated time point, the cells were washed with PBS and lysed in 1% SDS. Protein concentration was determined by BCA (Pierce Chemicals) and equivalent amounts of protein were analyzed by Western Blotting with rabbit antiserum against the PIV5 NP, P or M proteins [22] or against IRAK-M (Abcam) and actin (clone AC-74; Sigma). Blots were visualized with horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence (Pierce Chemicals).

2.3 Enzyme-linked immunosorbent assays (ELISAs) and IFN signaling assays

Levels of immunoreactive IL-6 (BD Opt EIA, BD Biosciences) or IFN-beta (PBL Biomedical Laboratories) in extracellular media were quantified by ELISA according to manufacturer's protocols. To allow for comparison between experiments, cytokine levels were normalized to 10⁶ cells, determined at the time of infection.

For inhibition of IFN-beta, neutralizing antibodies against IFN-beta or TNF-alpha (Millipore) were added to cell cultures at a concentration of 5 ug/ml immediately following infection as described previously [18].

For IFN signaling assays, cells were treated with 10³ IU of universal type I IFN (Millipore) over night, before being infected at an moi of 3 with VSV Orsay (the kind gift of Dr. Douglas Lyles). Cell viability was measured using a CellTiter96AQueous One Solution cell proliferation reagent (Promega) according to manufacturer's instructions.

2.4 Microscopy and fluorescence activated cell sorting (FACS)

Microscopy was carried out as described previously [22]. Images were captured using a Qimaging digital camera and processed using QCapture software. Exposure times were manually set to be constant between samples.

For flow cytometry, MDMs were removed from the dish using Accutase (Millipore), pelleted at 1300 rpm, and resuspended in PBS containing 2% FBS. Cells were analyzed on a FACSCalibur (BD Biosciences) to detect GFP expression or after surface staining using a PE-conjugated anti-CD80 monoclonal antibody (BD Pharmingen, San Diego, CA) as described previously [18] FACS data was collected and analyzed using CellQuest Pro software.

3. RESULTS

3.1 The P/V-CPI- mutant is restricted for growth in primary human macrophages due to the presence of IFN-beta

Replication of WT PIV5 and P/V-CPI- was analyzed in primary human macrophages. CD14⁺ monocytes were isolated from human PBMC and cultured for six days in the presence of MCSF. At that time, >90% were found to be CD14⁺, CD11b⁺, and CD64⁺ by flow cytometry, a characteristic marker profile of monocyte-derived macrophages (MDM, 14). Cells were infected at an moi of 10 with WT PIV5 and/or the P/V-CPI- mutant, both of which express green fluorescence protein (GFP) as an additional gene between HN and L [22]. At 18 hours post infection (h pi), infection was assayed by microscopy and western blotting with antibodies to PIV5 NP and P. As shown in Fig. 1A, primary human MDM were susceptible to infection with WT PIV5, evidenced by the large number of GFP-positive cells. By contrast, cells infected with P/V-CPI- displayed far fewer GFP-positive cells. The level of GFP-positive cells was not higher in MDM infected at increasing mois up to 200 (data not shown). The low level of infection by P/V-CPI- was supported by results from western blotting for viral proteins. As shown in Fig. 1B, MDM infected with WT PIV5 displayed much higher levels of viral protein compared to cells infected with P/V-CPI-.

In epithelial and fibroblast cell lines, we have shown that P/V-CPI- virus is a potent inducer of type I IFN [22,24], and that growth of P/V-CPI- is partially sensitive to IFN [27]. As shown in Fig. 1C, MDM infected with P/V-CPI- secreted higher levels of IFN-beta than cells infected with WT PIV5. To determine the role of secreted IFN-beta on susceptibility of MDM to P/V-CPI-, primary human MDMs were infected with P/V-CPI- at an moi of 25, and media were supplemented with PBS as a control, or with a neutralizing antibody against IFN-beta or TNF-alpha as a control. At 18 h pi, cells were visualized by microscopy to detect GFP expression. As shown in Fig. 1D, P/V-CPI- infected cells treated with a neutralizing antibody against IFN-beta display increased numbers of GFP-positive cells compared to mock or control antibody treated cells.

Taken together, these results indicate that P/V-CPI- is restricted for infection of primary human MDM, this restriction cannot be overcome by increasing moi, and that IFN-beta secreted in response to P/V-CPI- infection is at least partially responsible for restricted replication.

3.2 Stimulation of macrophages with heat killed bacteria enhances the susceptibility of human MDM to P/V-CPI- infection

MDMs generated by culturing with MCSF are considered to be in a naïve state [28]. Macrophages can be activated upon encountering bacterial pathogens, which results in increased levels of cell surface markers such as CD80, and the secretion of pro-

inflammatory cytokines such as IL-6 [2]. Given that the phenotype of activated macrophages differs significantly from naïve cells, we tested the hypothesis that macrophages activated by exposure to stimuli such as bacteria would display enhanced susceptibility to infection with P/V-CPI-. Primary human MDM were stimulated overnight with a panel of heat killed Gram positive bacteria, including *Listeria monocytogenes* (LM), and *Streptococcus pyogenes* (GAS), and *Bacillus anthracis* (BA) and levels of CD80 at 18 h post exposure were determined by flow cytometry. As shown in Fig. 2A for two individual donors, exposure of MDM to heat killed LM (HKLM) or GAS (HKGAS) resulted in more cells expressing high levels of CD80. Similar results were seen with HKBA-treated MDM (data not shown). Likewise, MDM exposed to heat killed LM, GAS, or BA secreted very high levels of IL-6 at 24 h post exposure (Fig. 2B). Importantly, MDM exposed to these heat killed bacteria did not secrete IFN-beta (Fig. 2C), consistent with previous data [29]. These results indicate that MDM exposed to heat killed bacteria are converted from a naïve to an active state.

To determine if the P/V-CPI- mutant was restricted for replication in activated cells, MDM were treated with PBS or exposed to heat killed LM, GAS, or BA for 12 hours. Bacteria were removed, and cells were washed before infection at an moi of 25 with P/V-CPI-. At 18 h pi, cells were examined by microscopy for levels of GFP-positive cells. As shown in Fig. 3A, the number of GFP-positive MDM in cultures stimulated with heat killed LM, GAS, or BA prior to infection was dramatically higher compared to PBS-treated cells. Fig. 3B shows flow cytometric quantification of the percent of GFP-positive cells and mean fluorescence intensity (MFI) for infected cells from a typical donor after treatment with heat killed Gram positive bacteria. Since viral enhancement was observed at relatively equal levels using all Gram positive bacteria tested, subsequent experiments were conducted using commercially available HKLM.

Enhanced viral gene expression in activated MDM was not limited to GFP. This is evident in Fig. 3C, where western blotting of cell extracts from MDM exposed to HKLM prior to infection had increased levels of viral NP, P and M compared to PBS-treated infected macrophages. GFP expression was consistently found to be the highest for cells that were treated with heat killed GAS, but was observed with all Gram positive bacteria tested so far. Enhanced GFP expression was seen following treatment of cells for as little as one hr before P/V-CPI- infection, or by simultaneous addition of virus and HKLM treatment (not shown). Enhanced virus gene expression was not seen when MDM were treated with live bacteria, or when MDM were treated with heat killed Gram negative bacteria (e.g. *Haemophilus influenzae* and *Bordetella pertussis*) or cytokines (e.g. IL-1beta, TNF-alpha; data not shown). Virus output did not differ from cells treated with PBS control or with HKLM (not shown). Taken together, these data indicate that activated macrophages are more susceptible to infection with P/V-CPI- compared to naïve macrophages, and that this activation is specific to stimulation with heat killed Gram positive bacteria prior to viral infection.

3.3 Bacteria-induced enhancement of P/V-CPI- infection is transient

The differences in susceptibility of naïve and activated macrophages to P/V-CPI- infection raised the question of whether this was a permanent or transient property of cells exposed to heat killed bacteria. To test this, MDM were treated with heat killed LM for 3 hrs, bacteria were removed and the cells were washed twice with PBS. MDM were then infected with P/V-CPI- at an moi of 25 at 0, 4, 12, 24, and 30 h post removal of the HKLM. At 18 h pi of each culture, cells were examined for GFP expression by microscopy and FACS. As shown in Fig. 4A for cells from a representative donor, MDM were highly susceptible to infection with P/V-CPI- at 0 and 4 h post removal of HKLM. However, by 12 h post removal of HKLM, the number of cells susceptible to infection with P/V-CPI- had decreased to levels seen with MDM treated with PBS alone. When quantitated by FACS, ~40% of cells were GFP-positive at 0 and 4 h post removal of HKLM, with an MFI of ~25 and 15, respectively.

However, by 12 h post removal of HKLM, less than 10% of cells were found to be GFP-positive, and the MFI of these cells was similar to that of PBS-treated control cells. These results indicate that the susceptibility of MDM to P/V infection that is induced by HKLM treatment is transient in nature, lasting up to 12 hrs post removal of HKLM.

3.4 MDM that are activated with heat killed Gram positive bacteria maintain IFN signaling, but produce lower levels of IFN-beta in response to P/V-CPI- infection

The above finding that the P/V-CPI- is restricted for replication in MDM by IFN-beta raised the hypotheses that treatment of MDM with heat killed bacteria altered either the production of IFN or IFN signaling. To determine if the IFN signaling pathway was altered by exposure to heat killed bacteria, we tested whether activated MDM that were treated with IFN were protected from killing by VSV challenge. MDM were treated with either PBS, HKLM, or HKGAS for 3 h as described previously, washed to remove bacteria and then treated with exogenous IFN-beta overnight to induce an anti-viral state. These cells were infected with VSV at an moi of 3, and viability was determined at 24 h pi. As shown in Fig. 5A for two donors, cells that did not receive exogenous IFN had viability that was reduced following VSV infection as expected (black bars). VSV reduced viability to only ~30–40% of control cells, consistent with the previous finding that macrophages generated with MCSF are partially resistant to killing by VSV [30]. Reduced viability was also seen in cells treated with HKLM and HKGAS, consistent with a lack of IFN induction by bacterial treatment. Most importantly, IFN treatment protected cells from VSV-mediated cell killing (hatched bars, Fig. 5A), with increased viability seen in the case of PBS as well as HKLM-treated MDM.

To determine levels of IFN-beta produced by infected MDM that were activated with heat killed Gram positive bacteria, MDM were treated with PBS as a control, or with heat killed LM, GAS or BA and then washed and infected with P/V-CPI- at an moi of 25. At 24 h pi, levels of secreted IFN-beta were assayed by an ELISA. As shown in Fig. 5B for cells from three individual donors, infection of PBS-treated MDM with P/V-CPI- resulted in increased levels of IFN-beta, consistent with results in Fig. 1D above. By contrast, MDM exposed to heat killed bacteria prior to infection with P/V-CPI- produced background levels of IFN-beta similar to that seen in the absence of virus infection. For one sample (donor 1), IFN-beta was induced by infected MDM that had been treated with GAS, but this was not consistently seen for cells from a number of other donors. Taken together, these data are consistent with the proposal that the increased susceptibility of activated MDM to P/V-CPI- is due to decreased production of IFN following virus infection and not due to alterations in IFN signaling.

3.5 Elevated levels of IRAK-M in MDM that are activated with heat killed Gram positive bacteria

In some cases, macrophages activated by bacterial components have been shown to subsequently become refractory to further stimulation, and this is thought to be at least in part from the upregulation of inhibitory molecules that dampen potential hyperactive responses [7,8]. We tested the hypothesis that P/V-CPI- infection of MDM that have been treated with HKLM results in elevated expression of negative regulators of anti-microbial responses. MDM were treated with PBS as a control or with HKLM and then infected at an moi of 25 with P/V-CPI-. Cell extracts were analyzed by western blotting for levels of IRAK-M, an inhibitor of TLR signaling and IFN induction [31]. As shown in Fig. 6 for two separate donors, IRAK-M levels were elevated in the infected MDM that had been activated by prior exposure to HKLM compared to the PBS-treated control. Similar increased expression was seen in the absence of virus infection (Fig 6, right panel). Western blotting showed no differences in levels of other negative regulators of innate immune responses,

including Suppressor of Cytokine Signaling 1 (SOCS-1) or in Traf6 which is targeted for degradation by A20 (data not shown). Given that IRAK-M targets IRAK-1 for inhibition and IRAK-1 can be involved in the induction of IFN-beta [31,41], our data are consistent with a model whereby activation of MDM by bacterial components leads to elevated IRAK-M and a reduction in the capacity of these activated MDM to respond to P/V-CPI- infection.

4. DISCUSSION

Macrophages are an important cell type that links both innate and adaptive immunity and these cells are targeted for infection by a number of important human pathogens. Given the dramatic differences in replication of the parainfluenza virus mutant P/V-CPI- in human epithelial cells [22] versus immature DC [18], it was important to determine the susceptibility of primary human macrophages to P/V-CPI-. Our results indicate that the P/V-CPI- mutant is restricted for replication in naïve MDMs due to the induction of IFN-beta. Our most striking finding came from our work demonstrating that the restriction on P/V-CPI- replication can be transiently alleviated by prior activation of MDMs with heat killed Gram positive bacteria. Increased susceptibility of the MDM that are exposed to Gram positive bacteria is linked to their limited ability to produce IFN-beta in response to infection with P/V-CPI-, while their ability to respond to exogenous IFN-beta remains intact. As detailed below, these data suggest a model whereby exposure to Gram positive bacteria transiently suppresses the ability of MDM to mount an IFN response that normally restricts P/V-CPI- replication.

Our results here with restricted replication of P/V-CPI- in MDM contrast sharply with previous results in epithelial cells. While replication of P/V-CPI- in epithelial cells is sensitive to added IFN, the main effect of IFN is a slight delay and lower overall titers [27]. However, under high moi conditions P/V-CPI- replicates in epithelial cells faster and to higher levels than WT PIV5 [22]. By contrast, our results shown here indicate that P/V-CPI- replication in primary human macrophages is strongly restricted such that very few GFP-positive cells are seen during high moi infection. Thus, the strength of antiviral responses or the landscape of IFN-inducible genes may differ considerably between epithelial cells which are largely permissive to P/V-CPI- and MDM which strongly restrict replication. Consistent with this idea, Newcastle Disease Virus (NDV) has been shown to be restricted for growth in human macrophages and this correlated with high levels of antiviral gene products such as RIG-I, IRF-3 and IRF-7 [32].

It is very likely that the ability of IFN to block detectable GFP expression in infected MDM reflects a restriction at the level of secondary viral transcription, since this is the step in the virus lifecycle that gives rise to abundant and detectable viral gene expression [19]. The step in PIV5 replication that is blocked by IFN is not known, however since IFN induction requires viral RNA synthesis [22], it is unlikely that the restriction is due to altered binding the sialic acid receptors. Previous work has shown that addition of IFN to Vero cells that were infected with a related P/V mutant (CPI-) substantially changed the steepness of the gradient of secondary viral RNA transcription, with a high “fall off” of transcription at the P-M junction and longer poly A tails on M mRNA [33]. This raises the hypothesis that the rapid IFN response induced by P/V-CPI- restricts replication in MDMs at least in part by altering the processivity of the viral polymerase and the steepness of the 3’-5’ gradient of transcription. Consistent with this proposal, our western blotting (Fig. 3C) shows a greater effect of MDM activation on enhancing the expression of 3’ distal genes (e.g. M protein) versus 3’ proximal genes (e.g. NP).

Our most striking finding is that P/V-CPI- replication is no longer restricted in MDM that have been activated by exposure to a range of heat killed Gram positive bacteria such as

Listeria monocytogenes (LM), *Streptococcus pyogenes* (GAS), and *Bacillus anthracis* (BA). The ability of cells to change susceptibility to virus infection after exposure to bacterial components has been reported previously for HIV infection of macrophages [15,16,17]. Similarly, recent work from Nguyen et al. [14] has shown that RSV replication is enhanced when immature DC or primary epithelial cells are treated with the TLR2 agonist Pam3CSK. Our results with the parainfluenza virus PIV5 differ substantially from these other reports in several regards. First, MDM infection by P/V-CPI- is not enhanced by pretreatment with individual TLR agonists as shown for HIV infections (data not shown) [15,34]. Secondly, enhancement of P/V-CPI- replication was found to be dependent on prior treatment of MDM with heat killed bacteria, and this contrasts with enhanced RSV replication following simultaneous addition of virus and stimulant [14]. Finally, enhancement of P/V replication is seen following HKLM treatment of immature DC (not shown) and naïve MDM, but not in epithelial cell lines such as A549 cells. Thus, the mechanism behind enhanced P/V-CPI- replication appears to have different characteristics compared to other previously described systems.

What is the bacterial component that serves as an activator of MDM to increase susceptibility to P/V-CPI- infection? MDM susceptibility to P/V-CPI- is not enhanced by exposure to Gram negative bacteria such as *Haemophilus influenzae* and *Bordetella pertussis* (data not shown). This is most likely due to LPS from Gram negative bacteria stimulating type I IFN production through activation of TLR 4 [3,7] and the subsequent induction of an antiviral state in the MDM. By contrast, bacterial lipoprotein signaling through TLR2 does not induce IFN-beta. Bacterial lipoproteins have been identified in multiple species of Gram positive bacteria [35], are released from bacteria when they are damaged or lysed, and have been shown to be present in abundance in preparations of heat killed bacteria. Bacterial lipoproteins have been shown to be capable of activating macrophages and repressing macrophage responses to infection [35]. Work is in progress to identify the bacterial component(s) that activate MDM and enhance P/V-CPI- replication.

What is the mechanism by which MDMs that are activated by bacterial components are more susceptible to P/V-CPI- infection? Several results are not consistent with alterations at an early step in the virus lifecycle such as attachment or fusion at the plasma membrane. First, increasing the moi to 200 did not increase susceptibility. Secondly, neutralization of extracellular IFN increased susceptibility to infection. Since IFN induction by PIV5 is dependent on virus replication [22,27], this supports a mechanism that is post entry. Finally, MDM that are activated by heat killed bacteria secrete less IFN in response to P/V-CPI- infection. Thus, our data support a model whereby activation of MDM by bacterial components renders them less able to mount an IFN response to P/V-CPI- infection.

Our data could be explained by the principle that pathogen-induced signaling is tightly regulated by feedback mechanisms in order to avoid damage to the host through uncontrolled pro-inflammatory cytokine secretion [36]. One method of downregulating antimicrobial cascades involves the production of cellular factors such as IRAK-M which function to negatively regulate signaling [36,37]. Unlike other members of the IRAK family, IRAK-M is expressed exclusively in macrophages, lacks kinase activity and negatively regulates signaling following TLR2, TLR4 and Nod2 stimulation by preventing the phosphorylation of IRAK-1 and its subsequent detachment from MyD88 [31,38–40]. Published data indicate that repeated and prolonged stimulation of Nod2 with MDP, a component of peptidoglycan which is found in Gram positive bacteria, results in upregulation of IRAK-M in primary human macrophages [39]. Additionally, ssRNA generated during paramyxovirus infection has been shown to induce IFN-beta in a Nod2-dependent fashion [41]. Thus, we hypothesize that P/V-CPI- may be detected by Nod2 in primary macrophages, resulting in IFN-beta induction. However, in macrophages that have

been activated by heat killed bacteria, subsequent P/V-CPI- infection provides additional Nod2 stimulation that results in upregulation of IRAK-M, and this feeds back to inhibit further Nod-2 mediated IFN-beta production. In epithelial cell lines, we have previously shown that P/V-CPI- induces IFN-beta through RIG-I and the production of viral dsRNA [42]. There are examples of a link between RIG-I and IRAK-1 in VSV infections of macrophages [43]. Thus, while it is unclear whether detection of P/V-CPI-in primary macrophages is through Nod2 or RIG-I, a role for IRAK-1 in either of these sensing pathways would be consistent with our findings of upregulated IRAK-M, reduced IFN-beta secretion and enhanced P/V-CPI- replication.

Our results have implications for the design of attenuated IFN-sensitive RNA viruses as therapeutic vectors or vaccine candidates [44]. In order to improve safety and reduce virulence, many of RNA virus vectors are being engineered to be sensitive to IFN through alterations to viral antagonists of host cell responses. Our P/V-CPI- mutant which both induces IFN and fails to block IFN signaling is an example of this strategy for attenuation and is being developed as a vaccine vector [18,23] and for oncolytic therapy [24]. Our results with the PIV5 P/V mutant raise the possibility that under some in vivo conditions, viruses that are engineered to be attenuated to improve safety may have enhanced replication and virulence in cells exposed to bacterial components that suppress production of IFN. Clinically, co-infections of the respiratory tract involving Gram positive bacteria and negative strand RNA viruses such as the parainfluenza viruses are well documented [e.g. 45], and our work highlights the need to understand the extent to which one organism attenuates or accentuates replication of the other organism.

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Figure 1. IFN-beta restricts replication of the P/V-CPI- mutant in primary human macrophages
 A–C) MDM were mock infected or infected at an moi of 10 with WT PIV5 or P/V-CPI- and cells were assayed at 24 h pi by microscopy for GFP expression (panel A), or by western blotting with antibodies specific for the indicated proteins (panel B). Media were assayed by ELISA for levels of IFN-beta (panel C). Cytokine production was normalized to 10^6 MDM. Results are the mean values (with standard error bars) from three samples from a representative donor. D) MDM were infected at an moi of 25 with P/V-CPI- and then incubated in media supplemented with PBS as a control, or with antibody specific for TNF-alpha or IFN-beta. Cells were examined by microscopy at 18 h pi for GFP expression.

Figure 2. Activation of primary human macrophages following treatment with heat killed bacteria

Human MDM were treated overnight with PBS or with 10^8 cfu of heat killed *Listeria monocytogenes* (LM), *Streptococcus pyogenes* (GAS), or *Bacillus anthracis* (BA). At 12 h p exposure, cells were analyzed by flow cytometry for levels of CD80 (panel A). Media were assayed by ELISA for levels of IL-6 (panel B) and IFN-beta (panel C). Cytokine production was normalized to 10^6 MDM. Panels A and B show data from two donors; panel C is a typical result from a representative donor.

Figure 3. The P/V-CPI- mutant is not restricted for replication in primary MDM that have been activated by exposure to Gram positive bacteria

A) GFP expression. Primary human MDM were treated with PBS or 10^8 cfu of heat killed LM, GAS or BA. MDM were washed and infected with P/V-CPI- at an moi of 25. Cells were examined by microscopy at 18 h pi for GFP expression. Results are shown for two independent experiments with two different donors. B) Quantification of GFP-expressing cells. Macrophages treated as described for panel A were analyzed by FACS to determine the number and mean fluorescent intensity (MFI) of GFP positive cells. Results are representative of 3 independent experiments with three different donors. C) Western Blotting. Primary MDM were treated with PBS or 10^8 cfu/mL of heat killed LM for 12 h prior to infection with P/V-CPI- at an moi of 25. Cell lysates were harvested at 18 h pi and analyzed by western blotting with antibodies specific for PIV5 NP, P, and M proteins, and cellular actin.

Figure 4. The increased susceptibility of activated MDM to P/V-CPI- infection is transient
 A) P/V-CPI- replication levels after removal of heat killed LM. Primary human MDM were treated with PBS or 10^8 cfu of heat killed LM for 12 hours, after which the bacteria was removed, cells were washed and replaced in fresh media. At the indicated times post removal of bacteria, MDM were infected with P/V-CPI- at an moi of 25. At 18 h pi with the P/V mutant, cells were examined by microscopy for number of GFP-positive cells. B) FACS analysis to quantify the percentage of GFP-positive cells. Results are representative of two independent experiments with two donors.

Figure 5. MDM that are activated with heat killed Gram positive bacteria maintain IFN signaling, but produce lower levels of IFN-beta in response to P/V-CPI- infection

A) IFN-beta signaling in HKLM-treated MDM. Primary human MDM were treated as described for panel A with PBS, HKLM or HKGAS. Cells were then washed and treated with PBS or 100 IU of IFN-beta for 12 hr. Cells were then infected with VSV at an moi of 3 and cell viability was determined 24 h pi as described in Materials and Methods. Results are the mean values (with standard error bars) from three samples from a representative donor. # $p < 0.006$, and * $p < 0.0002$. B) IFN-beta secretion from activated MDM. Human MDM were treated with PBS or the indicated heat killed bacteria as previously described, and then infected with P/V-CPI- at an moi of 25. Media were analyzed at 24 h pi by ELISA for levels of IFN-beta. Results from four individual donors are shown with the bar indicating mean values. * $p < 0.02$, and ** $p < 0.006$.

Figure 6. IRAK-M is induced to higher levels in P/V-CPI- infected MDM that have been activated by exposure to bacterial components
MDM from two representative donors were treated overnight with PBS or HKLM prior to mock infection or infection with P/V-CPI- at an moi of 25. At 18 h pi, cell lysates were analyzed by western blotting for levels of IRAK-M or actin as a load control.