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MAJOR ARTICLE

The Prostanoid 15-Deoxy-$\Delta^{12,14}$-Prostaglangin-J_2 Reduces Lung Inflammation and Protects Mice Against Lethal Influenza Infection

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Background. Growing evidence indicates that influenza pathogenicity relates to altered immune responses and hypercytokinemia. Therefore, dampening the excessive inflammatory response induced after infection might reduce influenza morbidity and mortality.

Methods. Considering this, we investigated the effect of the anti-inflammatory molecule 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) in a mouse model of lethal influenza infection.

Results. Administration of 15d-PGJ_2 on day 1 after infection, but not on day 0, protected 79% of mice against lethal influenza infection. In addition, this treatment considerably reduced the morbidity associated with severe influenza infection. Our results also showed that treatment with 15d-PGJ_2 decreased influenza-induced lung inflammation, as shown by the diminished gene expression of several proinflammatory cytokines and chemokines. Unexpectedly, 15d-PGJ_2 also markedly reduced the viral load in the lungs of infected mice. This could be attributed to maintained type I interferon gene expression levels after treatment. Interestingly, pretreatment of mice with a peroxisome proliferator-activated receptor gamma (PPARγ) antagonist before 15d-PGJ_2 administration completely abrogated its protective effect against influenza infection.

Conclusions. Our results demonstrate for the first time that treatment of mice with 15d-PGJ_2 reduces influenza morbidity and mortality through activation of the PPARγ pathway. PPARγ agonists could thus represent a potential therapeutic avenue for influenza infections.

As highlighted by the 2009 influenza pandemic outbreak, influenza viruses continue to pose a threat to humans. Deaths that occurred during the H5N1 avian influenza outbreak and the 2009 H1N1 pandemic have been highly associated with excessive inflammation leading to acute respiratory distress [1–4]. Indeed, several in vivo and clinical studies have clearly demonstrated that lung infections caused by highly virulent influenza viruses are characterized by excessive inflammation and elevated proinflammatory cytokine and chemokine levels, a phenomenon known as hypercytokinemia or cytokine storm [5–7].

In light of this, the development of treatments targeting the exuberant host immune response after infection instead of the virus itself seems to be an interesting therapeutic avenue [8, 9]. Moreover, such strategies would be independent of circulating strains and obviate the problem of viral mutation or adaptation, and they would nicely complement the existing arsenal of antiviral drugs. Recently, a few in vivo studies using animal models have described the use of immunomodulatory or anti-inflammatory agents to alleviate the severity of influenza infection [8, 10]. Some of these studies gave promising results, whereas others gave mixed results. A report by Budd et al has shown that treatment of mice infected with H2N2 influenza with gemfibrozil, a fibrate and agonist of PPARα, improved survival from 26% to 52% [11]. In

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another study, pretreatment of mice with pioglitazone, a thiazolidinedione and agonist of PPARγ, improved mouse survival to lethal H1N1 infection from 10% to 50% [12]. In a recent study, cyclooxygenase inhibitors used in prophylaxis were shown to be ineffective to reduce mortality of mice infected with a H3N2 virus [13]. Glucocorticoid treatment also failed to reduce the mortality of mice associated with H5N1 infection [14]. Other studies using knockout mice for specific inflammatory genes did not allow the identification of a particular inflammatory gene responsible for the uncontrolled host inflammatory response leading to lung injury. For example, mice that lacked the genes coding for the chemokine CCL2, interleukin 6 (IL-6), or tumor necrosis factor α (TNF-α) were not protected from H5N1 lethal infection [14]. In another study, disruption of genes coding for TNF-α receptors in mice slightly reduced morbidity after H5N1 infection, but mortality was not significantly decreased [15]. Taken together, the literature suggests that inhibition of a single immune mediator is unlikely to improve survival after infection with highly pathogenic influenza virus in contrast with therapies targeting the global inflammatory response.

While searching for alternative treatment targeting the exuberant host immune response during severe influenza infection, we investigated whether 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) may prevent hyperinduction of the inflammatory response and therefore confer protection in a mouse model. The rationale behind this investigation is multifold. First, this cyclopentenone prostaglandin reduces levels of proinflammatory cytokines in vitro as well as in various in vivo studies. Indeed, 15d-PGJ_2 has been shown to inhibit the expression of several inflammatory genes coding for TNF-α, IL-12, IL-6, inducible nitric oxide synthase (iNOS) and the chemokines CXCL10, CXCL8, CCL2, CCL3 and CCL4 in several cell types in vitro [16–20]. Moreover, in vivo studies have shown that 15d-PGJ_2 has protective properties in several animal models of diseases, such as acute lung injury, acute renal failure, acute pancreatitis, sepsis, arthritis, and inflammatory bowel disease [21–26].

Although 15d-PGJ_2 can engage cell surface receptors, its cellular effects seem to be mediated principally by modulation of intracellular signaling pathways. Among them, the nuclear factor-kappa B (NF-κB) and the PPARγ pathways represent its main targets. Indeed, 15d-PGJ_2 is a potent inhibitor of the inhibitory kappa B (IκB) kinase and it also directly prevents NF-κB binding to DNA, therefore reducing the inflammatory response induced by the NF-κB cascade [27–30]. Furthermore, 15d-PGJ_2 also stimulates the PPARγ nuclear receptor, which has anti-inflammatory effects mainly through transrepression of inflammatory genes [31].

In this study, we demonstrate that 15d-PGJ_2 significantly decreases severe influenza morbidity and mortality through the reduction of the exaggerated lung proinflammatory response. We clearly demonstrate that activation of the PPARγ pathway was necessary for the protective effects of this prostaglandin. Specifically, we show that 15d-PGJ_2 treatment leads to reduced cytokine and chemokine expression in the lungs of infected mice as well as to reduced viral titers. Therefore, our results suggest that targeting PPARγ may have significant therapeutic value in patients with complications associated with severe influenza infection.

MATERIALS AND METHODS

Mice and Viruses

C57BL/6 mice, 6–8 weeks old, were purchased from Charles River Laboratories and housed in specific pathogen-free conditions at the animal care facility of the Faculty of Medicine and Health Sciences of the Université de Sherbrooke. All experiments were approved by the institutional Animal Ethics Committee. Original viral stocks of the mouse-adapted A/PR/8/34 (H1N1) virus (PR8) were kindly provided by Dr David Topham (University of Rochester Medical Center, Rochester, NY).

Infection and Treatment of Mice

For influenza infection, anesthetized mice were infected intranasally with 1×10^3 plaque-forming units of PR8 virus in 30 μL of phosphate-buffered saline (PBS). Mice were weighed daily, and those that lost ≥30% of their original body weight were euthanized in accordance with the guidelines of Canadian Council on Animal Care. 15d-PGJ_2 was purchased from Cayman Chemical. Mice were treated with 15d-PGJ_2 (250 μg/kg) by subcutaneous injection, and 15d-PGJ_2 was diluted in 100 μL of sterile endotoxin-free PBS. Treatment was initiated either at the time of infection (day 0) or 24 hours after the infection (day 1), and continued daily for a total of 7 days. In some experiments, the PPARγ antagonist 2-chloro-5-nitro-*N*-phenylbenzamide (GW9662; Cayman Chemical) was subcutaneously injected 4 hours before 15d-PGJ_2 treatment.

Lung Viral Titer Determination

Serial 10-fold dilutions of clarified lung homogenates were prepared in incomplete Eagle's minimal essential medium (EMEM) (containing 0.1% bovine serum albumin instead of fetal bovine serum) and were titered on Madin-Darby canine kidney (MDCK) cells according to standard viral plaque assays. Briefly, confluent cells were exposed to lung supernatant dilutions for 1 hour to allow virus adsorption. Cells were then washed, and a semifluid medium containing Avicel RC-581 (FMC BioPolymer), incomplete EMEM, and 1 μg/mL Tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich) was added to the cells. Cells were incubated for 48 hours, and viral plaques were revealed with 2% crystal violet after Carnoy fixation.

In Vitro Antiviral Assay

MDCK and Calu-3 cells were infected with PR8 for 1 hour and treated with 15d-PGJ_2 at different times during experiments. After a 48-hour incubation period, viral replication was determined as described above. Additional details for these assays are available in the Supplementary Materials.

Real-time Polymerase Chain Reaction Analyses

For each experimental condition, total RNA was extracted from lung homogenates using Trizol reagent according to the manufacturer's protocol (Invitrogen); 1 μg of the resulting RNA was then reverse transcribed using random decamers (Ambion) and the Omniscript reverse-transcriptase kit (Qiagen). Duplicate real-time polymerase chain reactions for each sample were performed with the iQ SYBR Green Supermix (Bio-Rad) in a volume of 20 μL containing 50 ng of complementary DNA and 200 nmol/L of forward and reverse primers in a Rotorgene 6000 instrument (Corbett Research). Primer sequences are detailed in the Supplementary Materials. Reaction conditions were as follows with varying hybridization temperature according to the primer set used: 95°C for 5 minutes, followed by multiple cycles (95°C for 20 seconds, 55°C–60°C for 25 seconds, 72°C for 30 seconds). Amplification plots were generated using the Rotorgene 6000 Application software version 1.7 (Corbett Research), and fold induction was calculated using the $2^{-\Delta\Delta^{Ct}}$ method and using 18S expression for normalization [32].

Statistical Analyses

Results were represented as means ± standard errors of the mean. Weight loss data and cytokine gene expression results for each group were compared using 1-way analysis of variance followed by Tukey's multiple comparison test. Survival data were compared using Kaplan Meier curves followed by the log-rank (Mantel-Cox) test using GraphPad Prism 5 software (GraphPad Software). Mean time to death and hazard ratios were calculated using the PASW Statistics software, version 18.0 (SPSS).

RESULTS

Therapeutic Efficacy of 15d-PGJ_2 Against Severe H1N1 Infection in Mice

In view of its potent anti-inflammatory activity, we sought to investigate whether 15d-PGJ_2 could protect mice from a lethal influenza infection. To evaluate the therapeutic efficacy of 15d-PGJ_2 against a highly virulent H1N1 virus, female C57BL/6 mice were infected with 1×10^3 plaque-forming units of influenza A/PR/8/34 H1N1 (PR8). Groups of mice were either mock-treated (PBS) or treated with 15d-PGJ_2 (250 μg/kg/d) once daily for 7 days after infection, with treatment starting at the time of infection, on day 0 (15d-PGJ_2 d0–d7), or 24 hours after infection, on day 1 (15d-PGJ_2 d1–d7) with 15d-PGJ_2. Mice were monitored daily for 14 days for weight change and survival. Mice infected and treated from day 1 to day 7 after infection with 15d-PGJ_2 (15d-PGJ_2 d1–d7) showed significantly enhanced survival (79.16%) compared with mock-treated mice (13.7%) (Figure 1*A*). Weight loss was also significantly reduced in 15d-PGJ_2-treated mice compared with mock-treated controls, as shown by an average weight loss of 2.22% on day 5 for the treatment group versus 15.88% for the PBS group (Table 1 and Figure 1*B*). Subsequent to lethal influenza challenge, 86.37% of mock-treated mice succumbed to infection, with a mean time to death of ~9 days. Interestingly, mice that were treated on the same day as they were infected (15d-PGJ_2 d0–d7) showed no significant change in survival compared with the mock-treated group (20% of survivors in the group treated from day 0 vs

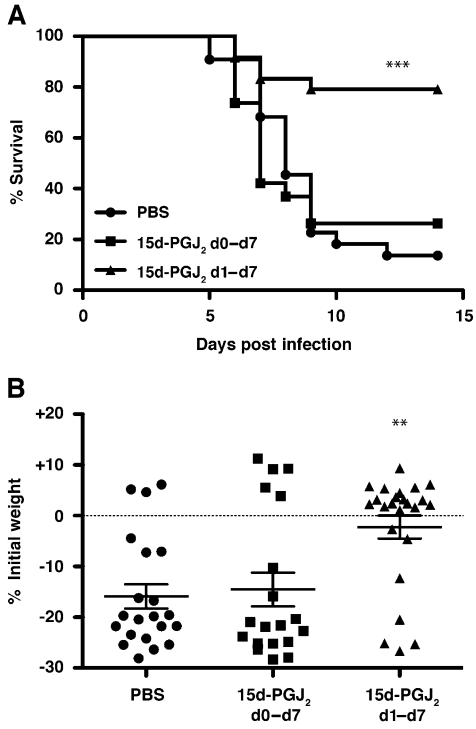

Figure 1. Reduced morbidity and mortality in mice treated with 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ2) after lethal infection. Female C57BL/6 mice were anesthetized and infected through intranasal instillation with 1×10^3 plaque-forming units (PFU) of influenza A/PR/8/34 (PR8) virus. Mice were treated daily until day 7 of infection with 15d-PGJ_2 (250 μg/kg administered subcutaneously), starting either on day 0 (ie, at time of infection) (15d-PGJ_2 d0–d7) or on day 1 (24 hours after infection) (15d-PGJ_2 d1–d7) or mock-treated with phosphate-buffered saline (PBS). *A*, Kaplan–Meier survival analysis comparing mice infected with 1×10^3 PFU of PR8 and treated or not. By log-rank test, $^{***}P < .0001$ for comparison between PBS and 15d-PGJ_2 d1–d7 treatment; the difference between the PBS and the 15d-PGJ_2 d0–d7 groups was not significant. Survival was monitored daily for 14 days ($n \geq 20$ per group). *B*, Weight change was monitored during the same period and is presented here as mean weight change on day 5 after infection ($n \geq 20$ per group); $^{**}P < .01$.

Table 1. Morbidity and Survival in Mice Lethally Infected With A/PR/8/34 (H1N1) Virus (PR8) and Treated With 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2)

Treatment[a]	Mean Body Weight Change on Day 5 After Infection, Mean ± SEM (95% CI), %	Survivors/Total, No. (%)[b]	Time to Death, Mean ± SEM (95% CI), d[c]	Hazard Ratio for Death
PBS	−15.88 ± 2.38 (−20.83 to −10.92)	3/22 (13.63)	8.81 ± 0.55 (7.67–9.97)	1.00
15d-PGJ_2 d0–d7	−14.54 ± 3.31[d] (−21.48 to −7.59)	5/20 (20.00)[d]	8.85 ± 0.72[d] (7.35–10.35)	1.02
15d-PGJ_2 d1–d7	−2.22 ± 2.24[e] (−6.85 to 2.41)	19/24 (79.16)[f]	12.54 ± 0.60[f] (11.30–13.79)	0.18[g]

Abbreviations: CI, confidence interval; PBS, phosphate-buffered saline; SEM, standard error of the mean.

[a] Once-daily administration of 15d-PGJ_2 or vehicle only (PBS) until day 7, starting on the same day as infection (d0–d7) or 24 hours after virus challenge (d1–d7). Infection was with 1×10^3 plaque-forming units of PR8.

[b] Number of survivors relative to total number of mice in group; mice were monitored daily for survival and weight loss for 14 days after challenge.

[c] Mean day of death for all mice in each group; survival was recorded as 14 days for mice that survived the 14-day observation period.

[d] Not statistically significant.

[e] $P < .01$, compared with PBS control group.

[f] $P < .0001$, compared with PBS control group.

[g] $P < .001$, compared with PBS control group.

13.63% in the mock-treated group). Moreover, the mean time to death between these 2 groups was identical (∼9 days). Also, no significant reduction in weight loss (14.54% vs 15.88%) was observed in mice treated from day 0 compared with mock-treated mice. A protective effect for 15d-PGJ_2 was only found when this molecule was administered 1 day after infection. Indeed, administration of 15d-PGJ_2 on day 1 after infection was highly protective compared with an earlier administration (hazard ratio for death, 0.18 vs 1.02; $P < .0001$). This suggests that prophylactic administration might impede a beneficial early antiviral host response.

15d-PGJ_2 Administration Decreases H1N1 Viral Load in Lungs of Infected Mice

Because 15d-PGJ_2 had potent protective properties with regard to weight loss and survival, we asked whether 15d-PGJ_2 had antiviral effects. To assess the effects of 15d-PGJ_2 treatment on H1N1 virus replication, we next determined viral titers in the lungs of mice 3 days after infection, the time at which virus replication in the lungs peaked in untreated mice [33]. Interestingly, starting treatment of mice with 15d-PGJ_2 1 day after infection markedly reduced infectious influenza virus production in the lungs by 3.07-fold at day 3 compared with mock-treated mice (15d-PGJ_2 d1–d3 vs PBS; Figure 2*A*). H1N1 viral replication was also decreased, as represented by an average reduction of 75.14% in influenza polymerase acidic (PA) gene expression (Figure 2*B*; 15d-PGJ_2 d1–d3 vs PBS). Notably, infectious virus load and viral gene expression were not significantly decreased in the lungs of mice treated with 15d-PGJ_2 starting on day 0 (Figure 2*A* and 2*B*; 15d-PGJ_2 d0–d3 vs PBS). These results suggested that 15d-PGJ_2 might have a direct antiviral effect. To test this hypothesis, we performed viral replication assays in MDCK cells and in human airway epithelial cells (Calu-3). These studies showed no in vitro antiviral effect because treatment of cells with 15d-PGJ_2 before, during, or after infection with PR8 virus did not decrease viral plaque formation, even at the highest noncytotoxic concentration of 15d-PGJ_2 (Figure 2*C* and 2*D*).

Impact of 15d-PGJ_2 Treatment on the Inflammatory Response Induced by Influenza Infection

Because a potent inflammatory response is one of the hallmarks of severe influenza infection and is associated with poor outcome in infected individuals, and because high levels of cytokines and chemokines are observed in the lungs of infected animals and humans, we examined whether 15d-PGJ_2 treatment might down-regulate cytokine production in the lungs of infected mice [7, 34]. We performed multiple cytokine real-time polymerase chain reaction analyses from messenger RNA (mRNA) isolated from lungs of mice in the different treatment groups. As illustrated in Figure 3, mRNA expression of cytokines and chemokines in lungs was strongly induced on day 3 after severe infection of mice with the PR8 virus (PBS) compared with uninfected mice (naive). Indeed, mRNA expression of cytokines IL-6, TNF-α, and interferon γ (IFN-γ) was up-regulated 1059-, 64-, and 13-fold, respectively. Expression of chemokines CCL2 (110-fold), CCL3 (82-fold), CCL4 (85-fold), and CXCL10 (1500-fold) in lungs was also strongly induced after influenza infection (Figure 3 and not shown). Starting the treatment of mice with 15d-PGJ_2 1 day after infection markedly reduced influenza-induced cytokine and chemokine gene expression in the lungs of these mice compared with mock-treated mice (15d-PGJ_2 d1–d3 vs PBS). Indeed, our results show a substantial reduction of IL-6 (−53%), TNF-α (−49.5%), CCL2 (−70%), CCL3 (−50%), CCL4 (−54%), and CXCL10 (−41%) gene expression in the lungs of mice of the 15d-PGJ_2 d1–d3 group. Strikingly, no difference was observed for IFN-γ gene expression, suggesting that inflammatory genes were down-regulated

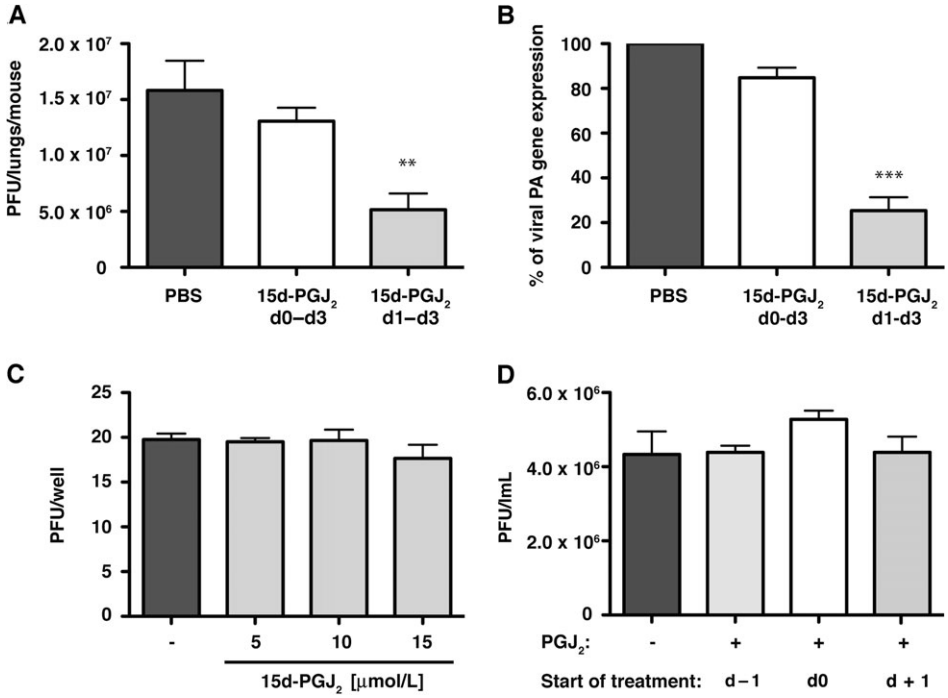

Figure 2. Administration of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) reduces viral titers in the lungs of treated mice but does not have a direct antiviral effect in vitro. Female C57BL/6 mice were anesthetized and infected through intranasal instillation with 1×10^3 plaque-forming units (PFU) of influenza A/PR8/34 virus. Mice were treated subcutaneously daily until day 3 of infection with 15d-PGJ_2 (250 μg/kg), starting either on day 0 of infection (15d-PGJ_2 d0–d3) or day 1 after infection (24 hours after infection; 15d-PGJ_2 d1–d3), or mock-treated with phosphate-buffered saline (PBS). At 3 days after infection mice were killed, and their lungs were dissected and homogenized. *A*, Infectious influenza virus loads in the lungs were measured by standard viral plaque assays in Madin-Darby canine kidney (MDCK) cells. Results are expressed as PFUs per lungs per mouse ($n \geq 6$ for 3 independent experiments); $^{**}P < .01$. *B*, PR8 gene expression was evaluated by analyzing viral PA gene expression using real-time polymerase chain reaction. Results are expressed as the relative percentage of viral PA expression compared with infected and mock-treated mice (PBS) ($n \geq 6$ for 3 independent experiments); $^{***}P < .001$. *C*, Confluent MDCK cells were exposed to 20 PFU/well of A/PR/8/34 (H1N1) for 1 hour and then washed. Avicel semifluid media containing 1 μg/mL TPCK-trypsin and the indicated concentrations of 15d-PGJ_2 were added, and cells were incubated for 48 hours. Plaques were revealed after Carnoy fixation with 2% crystal violet and counted ($n = 6$ for 3 independent experiments). *D*, Confluent Calu-3 cells were either pretreated 24 hours before infection (d-1), treated at the time of infection (d0) or 24 hours after infection (d+1) with 15d-PGJ_2 (10μM). Calu-3 cells were infected with the PR8 virus at a multiplicity of infection of 0.01. Supernatants were collected 48 hours after infection and titered by standard plaque assays for the presence of infectious virus ($n = 9$ for 4 independent experiments).

but that a certain antiviral defense was maintained. As anticipated by the lack of protective effect of 15d-PGJ_2 administered on day 0 of infection, cytokine and chemokine gene expression in the lungs of mice in this treatment group was not significantly reduced compared with mock-treated mice (Figure 3; 15d-PGJ_2 d0–d3 vs PBS).

Because 15d-PGJ_2 treatment starting on day 1 after infection seemed to maintain antiviral IFN-γ gene expression, we sought to determine whether this treatment affected the type I antiviral response. As shown in Figure 3, infection of mice with the PR8 virus triggered a 35- and 1165-fold induction of IFN-α and IFN-β in the lungs of infected mice on day 3 (PBS group) compared with uninfected mice (naive). Our results show no statistically significant reduction of IFN-α and IFN-β on day 3 after infection in lungs of mice treated with 15d-PGJ_2 whether treatment was initiated on day 0 or day 1 after infection compared with mock-treated mice. However, although not significant, a downward trend for IFN-β gene expression was observed when treatment was initiated on day 1 after infection.

PPARγ Activation is Involved in the Protective Effect of 15d-PGJ_2 Against Severe Influenza Infection

The PPARγ pathway has been reported to mediate some of the anti-inflammatory effects of 15d-PGJ_2 [31]. Therefore, we next evaluated whether this pathway was involved in the protection conferred by 15d-PGJ_2 after H1N1 infection. To do so, mice were pretreated with a PPARγ antagonist, GW9662, daily 4 hours before 15d-PGJ_2 administration to prevent the potential activation of this pathway. A Kaplan-Meier survival analysis convincingly demonstrated that the PPARγ pathway was essential for the effects of 15d-PGJ_2 because PPARγ antagonist pretreatment completely abolished the protection provided by 15d-PGJ_2 treatment (Figure 4*A*; GW9662 plus

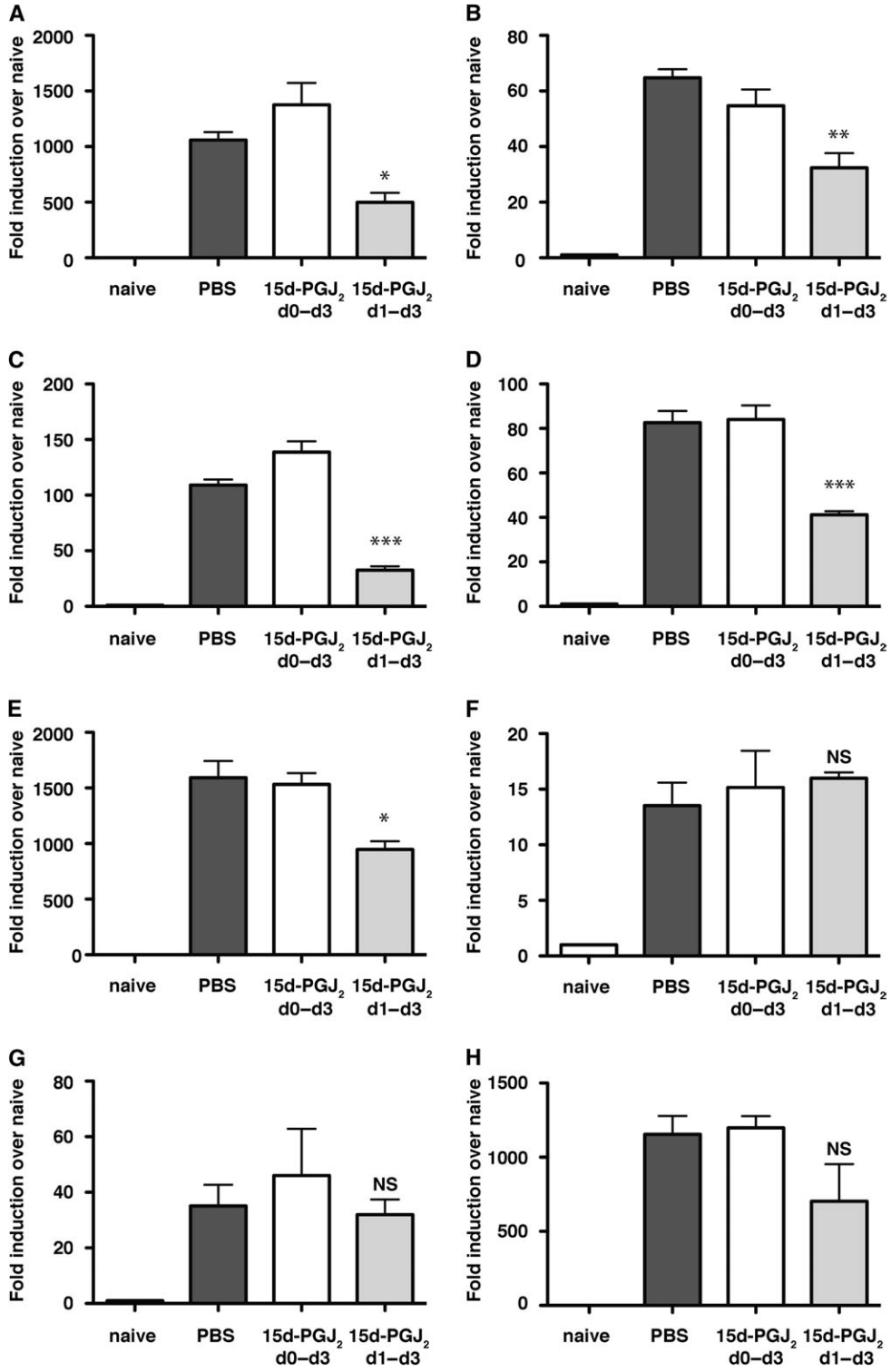

Figure 3. Administration of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) reduces inflammatory cytokine gene expression but not type I interferon (IFN) gene expression in mice after severe H1N1 infection. At 3 days after infection, mice were euthanized, and their lungs were dissected and homogenized in Trizol RNA extraction reagent. RNA was extracted and real-time polymerase chain reaction was performed with specific primers to quantify gene expression of interleukin 6 (*A*), tumor necrosis factor α (*B*), CCL2 (*C*), CCL3 (*D*), CXCL10 (*E*), IFN-γ (*F*), IFN-α (*G*), and IFN-β (*H*) in the lungs of mice from the different treatment groups. Data shown are *n*-fold changes of gene expression relative to uninfected mice, after normalization to expression of ribosomal 18S RNA for each sample, and represent means for ≥4 mice per group. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$; 15d-PGJ_2 d0–d3 or d1–d3, daily 15d-PGJ_2 treatment from day 0 (day of infection) or day 1 (24 hours after infection) to 3 days after infection. Abbreviations: NS, not statistically significant; PBS, phosphate-buffered saline.

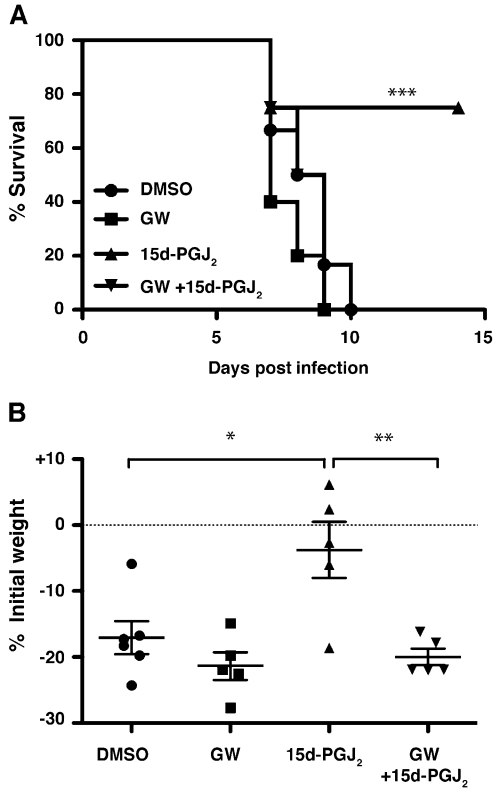

Figure 4. Effects of PPARγ antagonist GW9662 (GW) on 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2)-induced protection of influenza-infected mice. Female C57BL/6 mice were anesthetized with Avertin (240 mg/kg) and infected through intranasal instillation with A/PR/8/34. Mice were injected subcutaneously with GW9662 or mock-treated with dimethyl sulfoxide (DMSO) 4 hours before daily treatment with 15d-PGJ_2 (250 μg/kg) starting on day 1 after infection (24 hours after infection) until day 7 after infection. *A,* Survival was monitored daily for 14 days ($n \geq 5$ per group); $^{***}P < .001$. *B,* Weight loss was monitored during the same period and is presented here as the mean weight loss on day 5 after infection ($n \geq 5$ per group); $^{*}P < .05$; $^{**}P < .01$.

15d-PGJ_2 vs 15d-PGJ_2 alone). Accordingly, an increase in morbidity was observed in mice pretreated with the PPARγ antagonist and treated with 15d-PGJ_2 compared with 15d-PGJ_2 treatment alone. Indeed, the mean weight loss observed in the GW9662 plus 15d-PGJ_2 group reached similar levels compared with mock-treated mice (Figure 4*B*). Although the difference was not statistically significant, the treatment with PPARγ antagonist alone (GW9662) seemed to reduce the time to death of mice by almost 1 day (0.733 day) compared with vehicule (dimethyl sulfoxide)-treated mice.

DISCUSSION

Because excessive cytokine production has been proposed to contribute to influenza pathogenicity, we investigated the therapeutic potential of the anti-inflammatory molecule 15d-PGJ_2 against severe influenza infection. Using a mouse model of severe H1N1 influenza infection, our results convincingly demonstrate that administering 15d-PGJ_2 to mice on days 1–7 after infection significantly improves disease outcome as shown by diminished weight loss and increased survival of treated-mice. This treatment was also accompanied by a considerable reduction in gene expression of several cytokines and chemokines in the lungs of mice, including many of those that have been associated with severe disease in animals or humans infected with H5N1, 1918 H1N1 pandemic, and 2009 H1N1 pandemic viruses [1, 2, 7, 35]. Indeed, influenza treatment with 15d-PGJ_2 from day 1 to day 7 led to decreased gene expression of IL-6, TNF-α, CCL3, CCL2, and CXCL10 in the lungs of treated-mice compared with those of untreated mice. Obviously, lung inflammation is a necessary process to counteract the invading influenza virus. Therefore, careful considerations must be taken to prevent a complete elimination of beneficial immune responses. In this perspective, it is interesting to note that although gene expression of the majority of the inflammatory cytokines and chemokines analyzed was reduced by nearly half with 15d-PGJ_2 treatment from day 1 to day 7, there still was an appreciable expression level of these mediators in the lungs of protected-mice. Thus, the residual cytokine production combined with the unaffected IFN response probably contributed to eliminate influenza viruses and promoted survival.

Our findings clearly support the notion that morbidity and mortality caused by severe influenza infection are dependent on the excessive host inflammatory response to the virus. These findings are not different from those of other studies that have shown to varying extents that dampening inflammation was beneficial for influenza-infected animals [12, 15, 36–40]. However, unlike in those studies, we cannot rule out the possibility that the reduction in viral titers may have had a significant impact on the disease outcome provided by the treatment. As shown by the results of in vitro experiments, 15d-PGJ_2 did not have a direct inhibitory effect on viral replication in MDCK cells and in human airway epithelial cells (Figure 2*C* and 2*D*). However, treatment of mice with 15d-PGJ_2 led to an important decrease in viral titers at early time points after infection. This is clearly not an event often observed with strategies that target a single inflammatory molecule or overall inflammation [12, 15, 36, 39]. Therefore, this unique characteristic of 15d-PGJ_2 combined with its anti-inflammatory properties could make 15d-PGJ_2 treatment an ideal influenza therapy targeted toward the host and not the virus itself. In this respect, our study supports the recent proposition by Zheng et al that an ideal therapy for severe influenza would be one that targets these 2 clinical features (viral titers and inflammation) of influenza disease [40].

Having established that 15d-PGJ_2 treatment protects mice against lethal influenza challenge, we next investigated which host signaling pathway or pathways was targeted by this molecule.

Because 15d-PGJ_2 has been extensively described as a potential PPARγ agonist, we used a selective PPARγ antagonist to determine whether this pathway was involved in the protective effect of 15d-PGJ_2. Our results demonstrated that PPARγ activation was essential to promote survival of mice in the context of severe influenza infection. To our knowledge, this represents the first direct proof that PPARγ activation protects mice against influenza infection. Although during the completion of this study, a few studies or review articles have proposed that PPARγ activation might protect mice against infections by viruses, our study is the first to provide a direct demonstration in the case of influenza [8, 12, 41–43]. Thus, our study confirms the hypothesis that activation of the PPARγ pathway using agonists is beneficial during severe influenza infection. Although they did show that it was PPARγ-mediated, Aldridge et al recently demonstrated that pioglitazone, a putative PPARγ agonist, decreased mouse morbidity and mortality after lethal influenza challenge [12]. However, in contrast to our study, they used this potential PPARγ agonist in prophylaxis against influenza and not in the context of treatment of an already ongoing disease. Our results showed that a prophylactic administration of 15d-PGJ_2 was not effective to prevent death from lethal influenza challenge. Surprisingly, further experiments performed in our laboratory using rosiglitazone administered at a dose 20 times higher than used for 15d-PGJ_2, on the same day or 1 day after influenza infection showed that this compound was unable to protect mice against lethal influenza challenge (Supplementary Figure 1). Therefore, this raises concerns on the eventual use of thiazolidinediones for the treatment of an ongoing influenza infection. Because thiazolidinediones and 15d-PGJ_2 are known to have PPARγ-dependent and PPARγ-independent actions, these results pinpoint potentially different mechanisms of action for these molecules where PPARγ activation might be involved [44, 45].

Analysis of cell population dynamics in the lungs during influenza infection revealed that 15d-PGJ_2 treatment led to a modest but significant decrease of inflammatory cell recruitment, especially of neutrophils and TNF-α/iNOS-producing dendritic cells (TipDCs) (data not shown). However, inflammatory cells, other than TipDCs, whose cytokine and chemokine production could be decreased still remain to be identified in this mouse model of influenza infection. As the principal target of influenza viruses, airway epithelial cells could also represent one of the targets of 15d-PGJ_2 treatment because they produce inflammatory cytokines in response to infection [46]. In some inflammatory settings, 15d-PGJ_2 treatment of lung epithelial cells was shown to reduce their production of inflammatory cytokines through mechanisms involving PPARγ activation and PPARγ-dependent NF-κB inhibition [47, 48]. In vitro experiments performed in our laboratory effectively revealed that 15d-PGJ_2 was able to activate PPARγ-dependent gene transactivation to a greater extent than rosiglitazone. We have also demonstrated that it inhibited NF-κB–dependent gene transactivation in human airway epithelial cells in a PPARγ-independent manner because PPARγ is activated much after NF-κB in this cell type (Supplementary Figure 2*A* and 2*B* and data not shown). Therefore, it is conceivable that the reduction in cytokine expression observed in our in vivo studies might also be mediated in part through inhibition of the NF-κB pathway.

Collectively, our findings demonstrate that 15d-PGJ_2 treatment through PPARγ activation significantly reduced severe influenza morbidity and mortality. Specifically, we showed that 15d-PGJ_2 treatment leads to reduced inflammatory cytokine and chemokine expression in the lungs of infected mice, maintained antiviral cytokines, and reduced viral titers. Therefore, our results suggest that targeting PPARγ may have significant therapeutic value in patients with complications associated with severe influenza infection. Our findings could lead to a 15d-PGJ_2-based influenza therapy. Further studies are necessary to develop more stable analogues/derivatives of 15d-PGJ_2 and establish the clinical effectiveness of these compounds to treat influenza in humans.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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A. C. designed the research, performed experiments, analyzed the data, and wrote the paper. I. M., C. V., and D. C. performed some experiments. A. M. C designed a portion of the research and revised the manuscript. M. V. R. designed the research, mentored the first author, and wrote the final version of the paper.

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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