Mycoplasma hyopneumoniae does not affect the interferon-related anti-viral response but predisposes the pig to a higher level of inflammation following swine influenza virus infection

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In pigs, influenza A viruses and Mycoplasma hyopneumoniae (Mhp) are major contributors to the porcine respiratory disease complex. Pre-infection with Mhp was previously shown experimentally to exacerbate the clinical outcomes of H1N1 infection during the first week after virus inoculation. In order to better understand the interactions between these pathogens, we aimed to assess very early responses (at 5, 24 and 48 h) after H1N1 infection in pigs pre-infected or not with Mhp. Clinical signs and macroscopic lung lesions were similar in both infected groups at early times post-H1N1 infection; and Mhp pre-infection affected neither the influenza virus replication nor the IFN-induced antiviral responses in the lung. However, it predisposed the animals to a higher inflammatory response to H1N1 infection, as revealed by the massive infiltration of neutrophils and macrophages into the lungs and the increased production of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α). Thus, it seems it is this marked inflammatory state that would play a role in exacerbating the clinical signs subsequent to H1N1 infection.

INTRODUCTION

Influenza A virus (IAV), which belongs to the Orthomyxoviridae family, is a respiratory pathogen that affects human health worldwide, causing seasonal epidemic or occasional pandemic outbreaks. Human influenza infection is generally acute and characterized in its full form by the sudden onset of high fever, coryza, cough, headache, prostration, malaise and inflammation of the upper respiratory tree and trachea (Taubenberger & Morens, 2008). IAV is responsible for a large number of hospitalizations and deaths each year [up to 500 000 deaths per year worldwide (WHO, 2014)]. Morbidity and mortality can be exacerbated by multifactorial mechanisms involving viral, host and bacterial factors (Kash & Taubenberger, 2015). There is an increasing appreciation that many episodes of community-acquired pneumonia related to influenza result from co-infections (McCullers, 2014). Co-infections have an impact on multiple host pathways, and various factors such as the timing between viral and bacterial infections, host states or pathogen strains, may be important for the outcome of the disease. Understanding the interactions between pathogens is essential to be able to adopt better therapeutic strategies in the treatment of severe influenza. It is for this reason that studies...
on the interplay between the virus and other pathogens are expanding. As influenza infections induce damage to the respiratory epithelium, several studies have investigated post-influenza bacterial infections showing that IAV infection facilitates secondary infections with common bacteria and can lead to severe pneumonia that greatly contributes to respiratory distress, enhanced morbidity and mortality (Kash & Taubenberger, 2015). Rarely, these experimental studies are conducted in order to investigate the impact of a pre-existing chronic pulmonary bacterial infection on the influenza.

The knowledge we have so far is mainly based on results obtained in vitro or using animal models such as rodents, ferrets or non-human primates, but these models are not natural hosts of the virus and/or raise ethical questions (especially in primates). Among the variety of mammalian species that are natural hosts for influenza A viruses, pigs are a good model for studying co-infections and severe influenza outcomes. In fact, the pig is considered an excellent animal model in many fields of biomedical research because of the numerous similarities with humans, anatomically, physiologically and immunologically (Meurens et al., 2012; Rajao & Vincent, 2015; Summerfield et al., 2015). Moreover, this species is considered to play an important role in IAV ecology (Crisci et al., 2013). As pigs are susceptible to infection with avian and human influenza viruses, they may be an intermediate host for the adaptation of avian viruses to mammals and a mixing vessel for the generation of reassortant viruses (Van Reeth et al., 2012). On pig farms swine influenza is very common, similar to that observed in humans, but affects the animals all year round, whatever the season. As in humans, the severity of clinical signs in pigs is variable and can be influenced by many factors such as the viral strain, the age and immune status of the infected pig, climatic conditions, the housing type, and also co-infections (Kuntz-Simon et al., 2010; Deblanc et al., 2012, 2013; Fablet et al., 2013). The swine influenza A virus (swIAV) has previously been identified in pig herds as a major pathogen involved in the porcine respiratory disease complex (PRDC), together with Mycoplasma hyopneumoniae (Mhp), another respiratory pathogen widespread on farms (Opriessnig et al., 2011; Fablet et al., 2012). PRDC can be defined as a multifactorial disease of pigs caused by sequential or concurrent infections with several viral or bacterial respiratory pathogens, and is characterized by decreased rate of growth, anorexia, fever, cough and dyspnoea. Under experimental conditions, pre-infection of pigs with Mhp has been shown to exacerbate the outcomes of swIAV infection, i.e. severity of clinical signs during the first week after virus inoculation, virus shedding duration and lung injury (Thacker et al., 2001; Yazawa et al., 2004; Deblanc et al., 2012). It has been hypothesized that the severity of the flu syndrome and reduction in animal performance may vary depending on the level of oxidative stress at the time of swIAV infection, partially explaining how Mhp infection leads to the enhancement of influenza infection outcomes (Deblanc et al., 2013). However, our knowledge of pathogen interactions and the impact of co-infection on host response remains incomplete. For a better understanding, we investigated early immune responses after H1N1 infection in naïve pigs and compared these responses to those observed in pigs already affected by Mhp at the time of flu inoculation. This study provides new knowledge about swIAV-associated PRDC and further shows that the pig species could be as a suitable animal model for studying influenza complications in the context of co-infections.

RESULTS

Mhp pre-infection had no impact on clinical signs and pneumonia lesions induced by H1N1 over the first 48 h post-infection

Specific-pathogen-free (SPF) pigs inoculated with Mhp and H1N1 swIAV 21 days apart (MH1N1 group) or with H1N1 only (H1N1 group) were observed for clinical signs until 48 h post-H1N1 infection (hpi). Clinical data are presented in Table 1.

All Mhp-infected pigs developed coughing from 10 days after Mhp inoculation. H1N1 infection itself did not induce coughing, as no cough was noted in the H1N1 group, as in the Control group, and did not influence the average frequency of coughing in the MH1N1 group. All pigs had normal rectal temperature (39±1°C) during the first three weeks of the experiment. At 24 hpi, a peak of hyperthermia was observed in both infected groups and, at 48 hpi, temperatures returned to normal. In addition, all infected pigs showed a significant increase (P<0.05) in the respiratory rate at 24 hpi and a similar reduction in feed consumption at 48 hpi in both groups. This decrease in food consumption induced a significant reduction (P<0.05) in daily mean weight gain (MWG) and even weight loss in both infected groups.

For lung examination and subsequent analyses, three pigs from each group were sacrificed at 5, 24 and 48 hpi. At 5 hpi, pigs in the H1N1 group had no macroscopic lesions, whereas lesions of pneumonia were observed in the cardiac lobes in the MH1N1 group (data not shown). At 24 hpi, lesions reached all pulmonary lobes similarly in the infected groups, but were not extensive. At 48 hpi, an increase in lesion size was observed in both infected groups and the calculated mean scores were identical (mean score of 12.7/28). No lesion of pneumonia was observed in the Control group.

Mhp pre-infected pigs developed more severe microscopic lung lesions, and earlier, than H1N1 single-infected pigs

To study the inflammatory reaction at the local level, tissue samples from each lung lobe obtained at 5, 24 and 48 hpi were submitted to histological examination. At 5 hpi, few or no lesions were observed in H1N1 pigs, unlike MH1N1 pigs that showed severe microscopic lesions already present in the apical and cardiac lobes (Table 2). At 24 and 48 hpi, inflammatory lesions were extensive in the H1N1 group and healthy tissue was no longer apparent on sections from
the apical and cardiac lobes. No inflammatory lesions were observed in the diaphragmatic lobes in the H1N1 group, whereas in the MH1N1 group this lobe started being affected at 24 hpi and was totally inflamed at 48 hpi, with a large influx of neutrophils (data not shown).

**Mhp pre-infection did not affect viral excretion and multiplication in the lung over the first 48 h post-H1N1 infection**

Detection and quantification of pathogens were performed on nasal secretions and/or on the lungs in the time-course of the experiment. No difference was observed in virus shedding between the infected groups. The viral genome was detected from 24 hpi in nasal swabs in two out of three pigs in both groups (data not shown). At 48 hpi, all nasal swabs from the H1N1 group and two out of three from the MH1N1 group were found to be positive. However, matrix (M) gene copy numbers were still very low in nasal secretions at these early times post-infection and not comparable between groups, as they were below the quantification limit threshold of the quantitative RT-PCR (<2.10^3 copies of M gene).

In the lungs, the virus replicated and disseminated in the organ similarly in the H1N1 and MH1N1 groups (Fig. 1).

The highest gene copy numbers were obtained at 24 hpi in the cardiac lobes, but no significant differences were observed between lung lobes at a given time, or during the course of the experiment in a given lobe.

Mhp replicated to the same extent in all lobes. However, a significant decrease in bacterial genetic load was highlighted between 24 and 48 hpi in the apical, cardiac and diaphragmatic lobes (Fig. 1).

**Mhp pre-infected pigs exhibited an earlier important influx of CD163+ cells in cardiac lobes**

Immunofluorescent staining of the viral nucleoprotein (NP) and CD163+ cells was performed on the cardiac lobes to assess viral multiplication and macrophage/monocyte influx, respectively. Staining of NP and CD163+ cells from the Control group showed negative and constant results, respectively, over the time course of the experiment. As a result, only sections from samples taken at 24 hpi are reported (Fig. 2). Staining of NP showed very few infected cells in both the H1N1 and MH1N1 groups at 5 hpi, while large numbers were then similarly recorded at 24 and 48 hpi. At 5 hpi, CD163+ cells (which correspond to a subset of monocytes and pulmonary macrophages) were observed at similar frequency in the Control and H1N1 groups, but appeared in

**Table 1. Clinical data after H1N1 infection**

Mean (±standard deviation) of frequencies of coughing, rectal temperatures and respiratory rates at 5, 24 and 48 hpi, feed intakes at 48 hpi and daily MWG calculated over the 48 hpi in H1N1, MH1N1 and Control groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Frequency of coughing (pig⁻¹ 15 min⁻¹)</th>
<th>Rectal temperature (°C)</th>
<th>Respiratory rate (breaths pig⁻¹ min⁻¹)</th>
<th>Feed intake (kg pig⁻¹) at 48 hpi</th>
<th>Daily MWG (kg) over the 48 hpi</th>
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<tbody>
<tr>
<td></td>
<td>5 hpi 24 hpi 48 hpi</td>
<td>5 hpi 24 hpi 48 hpi</td>
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<tr>
<td>H1N1</td>
<td>0 0 0</td>
<td>39.6±0.2 40.8±0.2* 39.6±0.4</td>
<td>38±5 85±16* 44±0</td>
<td>0.45</td>
<td>−0.2±0.3*</td>
</tr>
<tr>
<td>MH1N1</td>
<td>1.3 1.9 1.8</td>
<td>39.4±0.2 40.6±0.1* 39.6±0.2</td>
<td>40±9 72±21* 45±4</td>
<td>0.48</td>
<td>−0.1±0.5*</td>
</tr>
<tr>
<td>Control</td>
<td>0 0 0</td>
<td>39.6±0.1 39.6±0.1 39.5±0.1</td>
<td>30±2 33±2 35±4</td>
<td>1.47</td>
<td>0.5±0.5</td>
</tr>
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</table>

*indicates that group is significantly different from Control group (P<0.05).

**Table 2. Microscopic lesions at 5, 24 and 48 hpi in apical, cardiac and diaphragmatic lobes (right lung)**

The extent of inflammatory lesions was scored between 0 and 4, as explained in Methods. Two tissue samples were examined per pig (two animals examined per group) and the average score was calculated for each pig.

<table>
<thead>
<tr>
<th></th>
<th>H1N1 group</th>
<th>MH1N1 group</th>
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<tbody>
<tr>
<td></td>
<td>5 hpi 24 hpi 48 hpi</td>
<td>5 hpi 24 hpi 48 hpi</td>
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<tr>
<td>Apical lobe</td>
<td></td>
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</tr>
<tr>
<td>Pig 1</td>
<td>0 2.5 3</td>
<td>3 4 3.5</td>
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<tr>
<td>Pig 2</td>
<td>1 3 4</td>
<td>3 1 2</td>
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<tr>
<td>Cardiac lobe</td>
<td></td>
<td></td>
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<tr>
<td>Pig 1</td>
<td>0 3 4</td>
<td>4 4 4</td>
</tr>
<tr>
<td>Pig 2</td>
<td>2 3 4</td>
<td>4 4 4</td>
</tr>
<tr>
<td>Diaphragmatic lobe</td>
<td>0 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Pig 1</td>
<td>0 0 1</td>
<td>0 0 2.5</td>
</tr>
<tr>
<td>Pig 2</td>
<td>0 0 1</td>
<td>0 0 4</td>
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</table>
greater numbers in the MH1N1 group. CD163 staining did not highlight any change in monocyte/macrophage numbers in any group at 24 hpi compared with 5 hpi. However, at 48 hpi, the numbers of CD163+ cells increased in the H1N1 group compared with 24 hpi, reaching similar levels to those observed in the MH1N1 group. Merged immunostaining did not show double-stained cells.

Co-infection led to a greater influx of neutrophils in lungs at 24 and 48 h post-H1N1 infection

Phenotypic analysis of immune cells was performed on bronchoalveolar lavage fluid (BALF) collected at necropsy. H1N1 infection alone did not have any impact on the CD172+ cell percentage in BALF (Fig. 3). By contrast, Mhp/H1N1 co-infection induced a decrease in the myeloid cell population at 24 hpi. Thus, 80.3±6.4 % of CD172+ cells were detected in the MH1N1 group versus 95.1±2.7 % in the Control group.

Interestingly, at 5 and 24 hpi, the antigen-presenting cells proportions (CD172+ CD80/86+ double-positive cells) and CD163+ cells were much lower in the MH1N1 group than in both the Control and H1N1 groups. Conversely, the proportion of myeloid cells not CD172+ CD80/86− were more important in the MH1N1 group. Thus, significant differences were observed between both infected groups at 24 hpi. However, at 48 hpi the numbers of CD163+ and CD172+ CD80/86+ cells increased while the number of CD172+ CD80/86− cells decreased in the MH1N1 group, reaching the levels measured for these cell populations in the H1N1 group.

H1N1 infection also induced an increase in the neutrophil proportion (CD172+ SWC8+ double-positive cells) in both infected groups, but more so in the MH1N1 group. Thus, at 48 hpi, the percentage of neutrophils in BALF was 13.5±5.3 % in the MH1N1 group versus 3.7±3.3 % in the H1N1 group, and 0.5±0.2 % in the Control group, leading to a significant difference between MH1N1 and the other two groups.

Additional production of cytokines in bronchoalveolar lavage fluids from co-infected pigs

To evaluate the impact of Mhp/H1N1 co-infection on the production of proteins related to inflammation, concentrations of haptoglobin and cytokines were measured in sera and BALF, respectively. H1N1 infection induced a gradual increase in haptoglobin concentrations in sera sampled in the H1N1 and MH1N1 groups from 5 to 48 hpi (Fig. 4). At 48 hpi, both infected groups were significantly different from the Control group, with a significantly higher concentration in the H1N1 group than in the MH1N1 group.

In cell-free BALF, an increase in IL-6, IFN-α and IL-1β concentrations was detected in the H1N1 group at 24 hpi (Fig. 4). Given the high individual variations observed, a trend of significant differences between the Control and H1N1 groups only was demonstrated (0.05<P<0.1). Viral infection did not induce any variation in IL-12 and IFN-γ concentrations (data not shown). Pre-infection with Mhp did not influence concentrations of IFN-α, IL-12 and IFN-γ significantly, as compared with those measured in the control groups.
H1N1 group. However, IL-6 concentrations were significantly higher at 5 hpi ($P=0.03$) in the MH1N1 group and concentrations of IL-1$\beta$ and TNF-\(\alpha\) also tended to be different at 5 hpi ($P=0.06$).

**H1N1 infection induced an IFN response in lung that was not impacted by Mhp**

To evaluate the local inflammatory and antiviral responses at a molecular level, the expression of different transcripts was

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**Fig. 2.** Immunostaining of cardiac lobes. Cryosections of samples from cardiac lobes of Control, H1N1 and MH1N1 pigs at 5, 24 and 48 hpi were prepared and stained using an anti-NP antibody (green) for the detection of H1N1, and an anti-CD163 antibody (red) for the detection of infiltrated monocytes and macrophages. Cell nuclei were stained by Hoechst solution (blue). For Control group, pictures obtained at 5, 24 and 48 hpi were similar and only pictures at 24 hpi are presented in the figure. Objective: $\times 10$, scale bar: 500 $\mu$m.
**Fig. 3.** Phenotypic analysis of cells from BALF. Proportion of immune cells in BALF of H1N1 (black), MH1N1 (light grey) and Control (dark grey) pigs at 5, 24 and 48 hpi. For Control group, only cells at 48 hpi were analysed. Results are expressed as percentage (±standard deviation) of cells expressing the molecule among the total population of viable cells in BALF. * indicates that group is significantly different from Control group at 48 hpi (*P* < 0.05); ** indicates that group is significantly different from H1N1 group at the same time point post-infection and also different from the Control group at 48 hpi (*P* < 0.05).
quantified in lung tissues. Expression of transcripts involved in cytokine production and IFN response was assessed in the cardiac (Figs 5 and 6), apical and diaphragmatic (data not shown) lobes, respectively. Temporal profiles obtained in the cardiac lobes were similar to those obtained in the others, and were shown to be representative of regulations observed in the whole lung. Neither H1N1 infection nor Mhp/H1N1 co-infection induced any change in the expression levels of IFN-α, TNF-α and IL-1β mRNA, but a tendency of increased IL-12 mRNA level was observed in both infected groups at 24 hpi ($P=0.057$) (Fig. 5). It also appeared that, unlike the H1N1 group, the expression of the IL-6 transcript in the MH1N1 group at 24 hpi was significantly different ($P<0.05$) from the Control group.

In the H1N1 group, expression of retinoic acid-inducible gene 1 (RIG-I) mRNA ($P=0.062$), a pattern recognition receptor involved in viral RNA recognition and establishment of IFN response, as well as IFN-β mRNA ($P\leq0.05$), was observed at 24 hpi (Fig. 6). An increase in expression of IFN-stimulated transcripts, i.e. myxovirus resistance 1 (Mx1, $P=0.060$), Mx2 ($P\leq0.05$), 2′,5′-oligoadenylate synthetase (OAS1, $P\leq0.05$) and RNA-dependent protein kinase (PKR, $P=0.057$), was also observed at 24 hpi in this group. In the same way, an increase in the suppressor of cytokine signalling 1 (SOCS1) but not SOCS3 mRNA expression was observed at 24 hpi ($P\leq0.05$). At 48 hpi, all transcripts returned to baseline expression levels. Pre-infection with Mhp had no impact on transcript expression levels that were measured following H1N1 infection, except on Mx2 mRNA, for which a significantly lower level was observed in the MH1N1 group as compared with the H1N1 group at 24 hpi in the cardiac lobes.

The amount of neutrophils and levels of pro-inflammatory cytokines, but not the IFN response, were correlated to the Mhp genome load in lungs

To better evaluate the impact of Mhp on the different parameters representative of the early responses of pigs to H1N1 infection, correlation analyses were performed to establish the links between the variables measured in this study. Correlations between the different quantitative variables measured in the time course of the experiment in all groups, i.e. scores of macroscopic and microscopic pulmonary lesions, Mhp and H1N1 genome loads in the lungs, haptoglobin concentrations in sera, counts of immune cells and concentrations of cytokines in BALFes, as well as relative expressions of transcripts related to IFN and innate immune response in lung tissues, were similar, whatever the lung lobe considered. Results obtained using data from the entire lung are detailed in Table 3. Analyses using data from each lobe individually gave similar results (data not shown). Thus, a correlation was found between pneumonia lesions and all other parameters, excepting the extent of inflammatory areas observed microscopically that was not significantly linked to the IFN response. The viral genetic load was correlated with that of Mhp. It was also correlated with all measured parameters of inflammatory and innate immune response. By contrast, the Mhp level was related neither to haptoglobin and IFN-α concentrations, nor to expression of transcripts involved in the IFN response. The proportions of neutrophils (CD172a SWC8+), CD172a CD80/86+ cells and CD172a CD80/86+ cells correlated with one another and with pulmonary lesions, levels of pathogens and concentrations of IL-6 (except neutrophils), TNF-α and IL1β. It has to be mentioned that correlation coefficients observed for CD172a CD80/86+ cells were negative, possibly due to a markedly higher recruitment of other immune cells (CD172a SWC8+ and CD172a CD80/86+ cells) into the lung or reflecting a migration of these cells to other tissues. Regarding cytokines, IL-6 and IL-1β concentrations were correlated with all parameters, except IL-6, which was not found to be related significantly to the percentage of neutrophils in BALF. IFN-α was linked to IL-6 and IL-1β, as well as mRNA involved in the IFN pathway, but not with Mhp load, TNF concentrations and immune cell counts in BALF. For TNF-α, correlations were observed between its concentration in BALF and pulmonary lesion scores, pathogen levels, IL-6 and IL-1β concentrations and numbers of immune cells, but no link was highlighted with haptoglobin, IFN-α concentrations or mRNA synthesis. In addition to macroscopic lesions and viral genetic load, the detection of transcripts related to the IFN response was itself correlated with concentrations of haptoglobin in serum and IL-6, IL-1β and IFN-α in BALF.

**DISCUSSION**

The purpose of this study was to investigate clinical and immune responses at early time points after H1N1 infection in pigs and to evaluate the impact of a pre-existing chronic pulmonary Mhp infection on these responses. Mhp itself induces a dry, non-productive cough and the pathogen may persist for several weeks. In our experimental model, the H1N1 infection was initiated when the Mhp infection was well established.

H1N1 and MH1N1 animals developed similar clinical signs of flu over the first 48 hpi. In a previous study conducted under the same experimental conditions, but where infected and co-infected pigs were observed for one week after H1N1 infection, MWG reductions were also reported in both infected groups when calculated over the first four days (Deblanc et al., 2012). However, the MWG was positively maintained for H1N1 pigs over these 4 days but found to be negative in Mhp pre-infected pigs. All the above data would suggest that H1N1 itself induces weight loss at early time points post-infection and that Mhp pre-infection does not impact the level of this weight loss but prolongs it over time.

The macroscopic lesions observed only in the lungs of MH1N1 animals at 5 hpi were probably due to Mhp itself, and not H1N1, as a similar macroscopic lesion score was previously obtained in pigs infected only with Mhp 28 days after bacteria inoculation (Deblanc et al., 2012).
H1N1 was then also directly involved in pneumonia as progressive extents of lesions were subsequently observed in both groups, leading to the same scores at 48 hpi. Even though the pre-existence of Mhp did not exacerbate H1N1-induced lesions during the first 2 days, the mycoplasma probably impeded lung recovery because the score observed at 7 days post-infection (dpi) in the MH1N1 group was still as high as that obtained in the present study at 48 hpi, whereas it was reduced by half in the H1N1 group (Deblanc et al., 2012).

Viral replication, multiplication and excretion events did not appear to be affected by the presence of Mhp over the 48 hpi. Nevertheless, considering that the viral genome was still present in the diaphragmatic lobes of MH1N1 pigs at 7 dpi, whereas it was not detected at that time in H1N1 pigs (Deblanc et al., 2012), it is suggested that Mhp may play a role in impairing or delaying viral clearance in the lungs.

In line with clinical outcomes and virus multiplication in the lungs, an important inflammatory reaction occurred

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**Fig. 4.** Concentrations of haptoglobin in sera and cytokines in BALF. Mean±standard deviation of haptoglobin levels in sera at 18, 21, 22 and 23 days post-Mhp infection (i.e. 3 days before H1N1 infection, 5, 24 and 48 hpi) and IL-6, IFN-α, TNF-α, IL-1β and IL-12 concentrations in BALF for H1N1 (black), MH1N1 (light grey) and Control (dark grey) groups. * indicates that group is significantly different from the Control group (P<0.05); ** indicates that group is significantly different from the Control and MH1N1 groups (P<0.05); *** indicates that group is significantly different from the H1N1 group (P<0.05).
immediately after the single H1N1 infection. The influx of neutrophils and CD163+ macrophages in the upper lobes once the virus was well established in the lung is in agreement with observations in humans, where these cells were recognized to be responsible for pulmonary lesions in influenza-induced acute respiratory distress syndrome (Short et al., 2014). Mhp pre-infection did not alter the H1N1-dependent haptoglobin concentration in blood but could have favoured the recruitment of neutrophils and macrophages in the lungs. Knowing that Mhp infection is characterized by an infiltration of immune cells into peribronchiolar and adjacent perivascular tissues (Sarradell et al., 2003; Redondo et al., 2009; Thacker & Minion, 2012), it can be assumed that the microscopic lesions and the abundant pulmonary macrophages reported in MH1N1 lungs at 5 hpi would be due to Mhp infection per se.

Individual variations in cytokine and mRNA measurements were observed, demonstrating some heterogeneity in post-infection response among animals and/or illustrating a rapid turnover in protein production and/or expression. In particular, it was not possible to relate any variation in expression of cytokine transcripts in pulmonary tissue to changes in cytokine concentrations in BALF, probably because of the very fast initiation of events after the virus enters the host cells, as well as the short half-life of mRNA. Nevertheless, it can be concluded that the early H1N1-induced inflammation and associated lung lesions described above were supported by the secretion of pro-inflammatory cytokines IL-6 and IL-1β, but not TNF-α. Except for TNF-α, these results are consistent with those of Barbé et al. (2011), who also reported a peak in these pro-inflammatory cytokines in BALF at 24–30 hpi with a similar H1N1 virus. The divergent results for TNF-α could be due to different analytical methods. Other reports have demonstrated TNF-α production in pig lung lysates 3–4 days after influenza infection (Jo et al., 2007; Khatri et al., 2010; Barbé et al., 2011; Pomorska-Mol et al., 2014) and, in humans it was suggested that TNF-α, with IL-8, is part of a second wave of pro-inflammatory cytokines (Hayden et al., 1998). This would also explain why we did not detect TNF-α in BALF from the H1N1 group during the 2 dpi. By contrast, TNF-α was detected together with IL-6 and IL-1β as early as 5 hpi in BALF from the MH1N1 group. In single Mhp infection, it has already been demonstrated that lung expression of IL-6, IL-1β and TNF-α was induced for several weeks (Choi et al., 2006; Thacker & Minion, 2012). Therefore, it can be hypothesized that the pro-inflammatory cytokines detected immediately after the single H1N1 infection.
at 5 hpi in BALF from the MH1N1 group were mostly due to Mhp per se. Surprisingly, TNF-α was no longer detected after 5 hpi in the MH1N1 group, raising questions about a possible negative impact of H1N1 multiplication on Mhp-induced cytokines. Nonetheless, if it exists, this impact would only be transient as in a previous study (Deblanc et al., 2012) TNF-α was detected at 7 dpi in BALF of Mhp-infected pigs (98 pg ml⁻¹) and Mhp/H1N1-co-infected pigs (385 pg ml⁻¹), but not in H1N1-infected pigs (unpublished data).

IFN-related signalling pathways are triggered following influenza infection (Hale et al., 2010; van de Sandt et al., 2012), but only in vitro and ex vivo studies investigated this response after swIAV infection (Delgado-Ortega et al., 2014; Dobrescu et al., 2014; Zhang et al., 2015). We have demonstrated for the first time, to our knowledge, that H1N1 penetration into pulmonary cells induced the RIG-I signalling pathway, followed by up-regulation of IFN-stimulated genes and SOCS1 that is in line with responses also observed in vitro (Delgado-Ortega et al., 2014). By contrast, no significant variation in SOCS3 expression level was evidenced but other reports indicated that the level of SOCS modulation could differ, depending on the influenza virus strain and the infected cell type (Nelli et al., 2012; Ramirez-Martinez et al., 2013). Mhp pre-infection had no impact on the regulation of these genes as confirmed by correlation analyses.

**Fig. 6.** Expression of transcripts involved in IFN response in cardiac lobes. Mean±standard deviation of the relative expression of transcripts involved in the IFN response in cardiac lobes at 5, 24 and 48 hpi in H1N1 (black), MH1N1 (light grey) and Control (dark grey) groups. * indicates that group is significantly different from Control group (P≤0.05); ** indicates that group is significantly different from other two groups (P≤0.05).
Table 3. Correlation analysis for the entire lung

Spearman’s correlation coefficients between total scores of macroscopic and microscopic lesions, means of quantities of pathogens and relative expression of transcripts obtained in the three lobes, haptoglobin in sera, cytokines in BALF and percentage of CD172+ SWC8+ cells, CD172+ CD80/86+ cells and CD172+ CD80/86- cells in BALF. Grey cells indicate that the correlation is significant at \( P \leq 0.05 \).

|                      | Macroscopic lesions | Microscopic lesions | M gene | Mhp genome | Haptoglobin | CD172+ SWC8+ cells | CD172+ CD80/86+ cells | CD172+ CD80/86- cells | IL-6 | IL-1β | TNF-α | IFN-α | IFN-β | IL-6 mRNA | IL-12p35 mRNA | RIG-I mRNA | OAS1 mRNA | PKR mRNA | Mx1 mRNA | Mx2 mRNA | SOCS1 mRNA | SOCS3 mRNA |
|----------------------|---------------------|---------------------|--------|------------|-------------|-------------------|----------------------|-----------------------|------|-------|-------|--------|--------|----------|------------|------------|------------|--------|--------|--------|--------|
| Macroscopic lesions  | 1.000               | 0.476               | 0.736  | 0.682      | 0.439       | 0.735             | 0.632                | 0.703                 | 0.657| 0.654| 0.692| 0.683 | 0.735 | 0.615    | 0.368       | 0.577      | 0.000     | 0.000  | 0.000  | 0.000  | 0.000  |
| Microscopic lesions  |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |
| M gene               | -                   | 1.000               | 0.507  | 0.420      | 0.508       | 0.501             | -0.579               | 0.504                 | 0.441| 0.523| 0.420| 0.331 | 0.351 | 0.511    | 0.297       | 0.335      | 0.293    | 0.328  | 0.393  | 0.251  | 0.140  | 0.221  |
| Mhp genome           |                     |                     | 1.000  | 0.532      | 0.734       | 0.583             | -0.545               | 0.545                 | 0.702| 0.721| 0.473| 0.646 | 0.458 | 0.568    | 0.454       | 0.624      | 0.587    | 0.527  | 0.691  | 0.492  | 0.434  | 0.468  |
| Haptoglobin          |                     |                     |        | -0.545     | 0.583       | -0.545            | 1.000                | -0.286                | 0.633| 0.617| 0.644| 0.516 | 0.220 | 0.396    | 0.288       | 0.285      | 0.301    | 0.355  | 0.167  | 0.155  | 0.312  |        |
| CD172+ SWC8+ cells   |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| CD172+ CD80/86+ cells|                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| CD172+ CD80/86- cells|                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| IL-6                 |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| IL-1β                |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| TNF-α                |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| IFN-α                |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| IFN-β                |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| IL-6 mRNA            |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| IL-12p35 mRNA        |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| RIG-I mRNA           |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| OAS1 mRNA            |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| PKR mRNA             |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| Mx1 mRNA             |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| Mx2 mRNA             |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| SOCS1 mRNA           |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
| SOCS3 mRNA           |                     |                     |        |            |             |                   |                      |                       |      |      |      |       |       |          |             |            |          |        |        |        |        |        |
In conclusion, we have shown that H1N1 infection in the pig induced inflammation characterized by the production of haptoglobin, IL-1β and IL-6, as well as infiltration of macrophages and neutrophils into the lungs, which could cause tissue injury and subsequent clinical signs. In addition, an antiviral response took place via the production of RIG-I- and IFN-induced antiviral proteins. In pigs already affected by Mhp, the inflammatory reaction was more marked and started earlier, with additional production of TNF-α and greater infiltration of macrophages and neutrophils, illustrating additive effects from both pathogens. Whereas Mhp did not seem to influence influenza virus multiplication, bacterial pre-infection appeared to play a role in favouring viral-extended presence in the lungs (Deblanc et al., 2012). To widen our knowledge of Mhp/swIAV co-infection, further investigations on host responses that occur between 2 and 7 dpi, when marked differences in clinical signs and pulmonary lesions are visible, would be needed. Finally, all the above results further suggest the suitability of the pig as a clinical animal model for investigation of severe influenza infections in humans, especially in the case of co-infection. Indeed, although experimental studies with large animals have some disadvantages, the pig is the only natural host of influenza virus exhibiting clinical signs and immune responses similar to those observed in influenza infections in humans. The increasingly broad-based tools and knowledge on this host allow us to consider it as a model that could be used for the development of therapeutic strategies to prevent and treat the severe forms of the disease.

METHODS

Pathogens and animals. Amplification of Mycoplasma hyopneumoniae strain 116 and the European avian-like swine H1N1 virus strain A/Swi/Cotes d’Armor/0231/06, as well as titration of inocula, were performed as previously described (Deblanc et al., 2012).

Twenty-seven SPF Large White pigs were randomly assigned to this study. They were obtained from the experimental pig herd of the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) in Ploufragan (France). They were free of Mhp and swIAV at the beginning of the study on the basis of serological controls obtained from their mothers and rearing conditions, i.e. very strict biosecurity measures, in order to avoid undesirable contamination of the animals (BSL-3).

Experimental design and sample collection. Experiments were performed at the ANSES facilities in accordance with the animal welfare experimentation recommendations drawn up by the Direction Départementale de la Protection des Populations des Côtes d’Armor (ANSES registration number B-22-745-1), under the responsibility of G. Simon (authorization number 22-26).

This animal experiment protocol was approved by the French National Ethics Committee ComEth ANSES/ENVA/UPEC (approval No. 12/06/12-1).

Nine 6-week-old pigs were inoculated intra-tracheally with Mhp 116 (5 × 10^3 colour-changing units in a total of 5 ml) on two consecutive days (D0 and D1), while 18 others were mock-inoculated intra-tracheally with 5 ml of Friis broth medium. Three weeks later, i.e. at D21, all nine Mhp pre-infected animals and nine mock-infected animals were further inoculated intra-tracheally with 5 × 10^5 EID_{50} (embryonic 50% infectious dose) of H1N1 in a total of 5 ml, and were assigned to the M1H1N1 and H1N1 groups, respectively. The last nine mock-infected animals were again mock-inoculated intra-tracheally with 5 ml of allantoic fluid (Control group). Animals were euthanized by an intravenous booster of sodium thiopental (0.6 g of Nesoinal per pig, Sanofi-Aventis, Paris, France), bled and necropsied sequentially at 5, 24 and 48 h post-infection with H1N1 (hpi) (three animals per group at each time point).

At necropsy, macroscopic lesions (purple-red and firm lesions) were estimated visually as previously described (Madec & Kobisch, 1982). Samples of each right lung lobe (apical, cardiac and diaphragmatic) were collected for histopathological examination, quantification of pathogens, immunofluorescent staining and quantification of mRNAs related to host response.

The left lung was flushed with 3 × 20 ml of PBS and the recovered BALF was centrifuged. BALF cells were conserved in liquid nitrogen in fetal bovine serum (Dutscher, Brumath, France) containing 10% DMSO (Sigma, Saint Louis, MO, USA) until flow cytometric analyses, while the BALF cell-free supernatant was stored at −70°C until cytokine quantification.

Histological examination. Two tissue samples of each pulmonary lobe were fixed in 4% neutral buffered formalin, then embedded in paraffin wax, sectioned and stained with hemalum and eosin using standard procedures. Sections were evaluated by light microscopy for histopathological changes. The extent of inflammatory lesions was scored as follows: 0, healthy tissue; 1, no inflammatory parenchyma but development of bronchus-associated lymphoid tissue; 2, inflammatory parenchyma area less extensive than healthy tissue area; 3, inflammatory parenchyma area more extensive than healthy tissue area; and 4, no healthy tissue area. Analyses were conducted at each time point of necropsy and a mean score per lobe was calculated for each animal from the two tissue sample scores.

Detection and quantification of pathogens. Nasal swab supernatants were analysed by influenza-A-specific M gene real-time RT-PCR using the TaqVet Influenza A, INFAP-Swine kit (LSI, Lissieu, France). Five microlitres of RNA was tested using the GoTaq probe One-Step qPCR System (Promega, Madison, WI, USA) in a total volume of 25 µl. Two standard curves were obtained from the amplification (in duplicate) of 10-fold serial dilutions of mRNA obtained from the transcription of the swIAV M gene (2 × 10^9 to 2 × 10^5 copies) and cDNA of the β-actin gene (2 × 10^4 to 2 × 10^2 copies). The primers and probes used for the M gene and β-actin gene amplifications were previously described (Duvigneau et al., 2005; Weingartl et al., 2010). The reverse transcription and amplification steps were performed in an MX3005P qPCR System (Agilent Technologies, Santa Clara, CA, USA) and the thermal program was 45°C for 30 s, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For quantification, the threshold cycles of samples were compared to the standard curves generated by amplification of the known copy numbers of the M and β-actin genes. For normalization, results were expressed as copy numbers of the M gene per 10^5 copies of the β-actin gene.
For Mhp quantification, DNA was extracted from 25 mg of lung sample with a DNeasy Blood and Tissue kit (Qiagen, Courtabeuf, France) and analysed by specific PCR assay as previously described (Marois et al., 2010).

**Frozen lung tissue sections and immunofluorescent staining.** Pieces of cardiac lobe were immersed in Tissue Tek O.C.T Compound (Sakura Finetec, Villeneuve d’Ascq, France), snap-frozen in isopentane/liquid nitrogen and stored at −70 °C. Serial cryosections (8 µm) were cut at −25 °C and fixed on treated glass slides (SuperFrost Plus, Menzel-Gläser, Braunschweig, Germany) in 80 % cold acetone for 10 min. After washing, the slides were stored at −70 °C until staining.

Non-specific binding of antibodies to tissue sections was blocked for 1 h in PBS containing 10 % goat serum and 0.03 % Triton X-100 (both Sigma, Saint Louis, MO, USA). Cells of the monocyte/macrophage lineage were stained indirectly using a mouse monoclonal anti-swine CD163 (clone 2A10/11, dilution 1/50) (BioRad AbD Serotec, Kidlington, UK), followed by a secondary goat anti-mouse IgG1 antibody conjugated to Alexa Fluor 568 (dilution 1/200) (Life Technologies, Carlsbad, CA, USA). The viral NP was revealed with a monoclonal mouse anti-NP antibody (clone IA-52, dilution 1/40) (Argene, Verniols, France) and a secondary goat anti-mouse IgG2a antibody conjugated to Alexa Fluor 488 (dilution 1/200) (Life Technologies, Carlsbad, CA, USA). Controls were incubated with primary isotype control antibodies (mouse IgG1 and IgG2a, Life Technologies, Carlsbad, CA, USA), followed by secondary antibodies. Antibodies were diluted in PBS containing 1 % goat serum and 0.1 % Triton X-100. Primary antibodies were incubated at 4 °C overnight and secondary antibodies at room temperature in the dark. Finally, Hoechst stain solution (Sigma, Saint Louis, MO, USA) was added to sections and microscopy observations were performed with a BX41 microscope (10× objective) (Olympus, Tokyo, Japan) connected to an Exi Aqua Camera (Qimaging, Surrey, Canada) using Image Pro Express 6.0 software (Media Cybernetics, Rockville, MD, USA). Images are representative of at least two different animals.

**Flow cytometric analyses.** Frozen BALF-cell samples were rapid thawed at 37 °C and immediately washed in PBS. Then, 10⁶ cells were transferred to 96-well plates, and single or double staining was performed with the following primary monoclonal antibodies or recombinant protein: FITC-coupled anti-swine CD172a, also identified as SWC3a (clone 74-22-15 from Southern Biotechnology, Birmingham, AL, USA), unlabelled anti-swine CD172a (clone 74-22-15A from Washington State University, Pullman, WA, USA), RPE-coupled anti-CD80/86 (fusion protein human CTLA-4 from Ancell Corporation, Stillwater, MN, USA), RPE-coupled anti-swine CD163 (clone 2A10/11) and unlabelled anti-swine SWC8 (clone MIL3) (both from BioRad AbD Serotec, Kidlington, UK). Matched isotype controls for mouse IgG1, IgG2a, IgG2b and IgM were purchased from Life Technologies (Carlsbad, CA, USA). Monoclonal antibodies were added at the concentrations recommended by the manufacturers and cells were allowed to incubate for 30 min in the dark at 4 °C. Samples were washed and immediately analysed or, if necessary, an isotype-specific secondary reagent coupled to PE or FITC (Life Technologies, Carlsbad, CA, USA) was added. Dead cells were excluded by a Cell Viability Solution (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. For each immunostaining, 30,000 events were analysed on an FC500 cytometer and data were analysed with Kaluza Software (both Beckman Coulter, Fullerton, CA, USA). For the control group, only BALF-cell samples from pigs euthanized at 48 hpi H1N1 were analysed.

**Haptoglobin and cytokine measurement.** Haptoglobin was measured in serum using a Phase Range Haptoglobin kit (Tridelta, Maynooth, Ireland) and porcine IL-1β, TNF-α, IL-6, IL-12 and IFN-γ were measured in cell-free BALF using ELISA commercial kits (R&D Systems, Minneapolis, MN, USA). Porcine IFN-α was quantified by an in-house ELISA (Jamin et al., 2006).

**Quantification of mRNA related to IFN response and regulation of innate immunity in lung tissues.** Small pieces of tissue (3 × 3 mm) were selected from apical, cardiac and diaphragmatic lobes and immediately snap-frozen in liquid nitrogen and stored at −80 °C. The frozen tissues were suspended in Trizol reagent (Invitrogen, Cergy-Pontoise, France) with ceramic beads to allow lysis and homogenization of the tissue by a FastPrep FP120 cell disrupter (Qiogene, Illkirch, France). Total RNA was isolated using Nucleospin (Macherey-Nagel Gmbh, Düren, Germany) according to the manufacturer’s recommendations. To minimize sample variations, we used identical amounts of tissue and the quality of RNA from randomly selected samples was assessed by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Massy, France), and RNA integrity numbers were calculated; they were always ≥ 7. Quantitative real-time PCR (qPCR) was performed using cDNA synthesized as previously described (Meuren et al., 2007; Delgado-Ortega et al., 2011). Primers to assess transcript expression have previously been published (Delgado-Ortega et al., 2014; Dobrescu et al., 2014) and were purchased from Eurogentec (Liége, Belgium). The primers can be made available upon request. Diluted cDNA (10⁻x) was combined with primer/probe sets and Mesa Green mix (Eurogentec, Liége, Belgium) according to the manufacturer's recommendations. The qPCR conditions were 98 °C for 30 s, followed by 37 cycles with denaturation at 95 °C for 15 s and annealing/elongation for 30 s. Assays were run on a Bio-Rad chromo 4 (Bio-Rad, Hercules, CA, USA). Samples were normalized internally by simultaneously using the average cycle quantification (Cq) of the most suitable reference genes in each sample to avoid any artefact of variation in the target gene. The three most suitable reference genes were selected among eight commonly used reference genes. The genes included beta-actin, beta-2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase, hydroxymethylbilane synthase, hypoxanthe phosphoribosyltransferase-1, ribosomal protein L19, succinate dehydrogenase complex subunit A and TATA box-binding protein 1. The stability of these reference genes in all selected tissues was assessed using the geNorm application (Vandesompele et al., 2002; Vandesompele et al., 2002; Delgado-Ortega et al., 2014).

**Statistical analysis.** For all data, the non-parametric Kruskal–Wallis test was used to detect differences among groups and P-values were adjusted with Holm’s correction for pairwise comparison. Correlation analyses among pulmonary lesion scores, level of pathogens in the lungs, haptoglobin concentration in sera, numbers of immune cells in BALF and relative expressions of transcripts in the lungs were performed using the spearman rank correlation test. The correlation analyses were performed using data obtained for either one pulmonary lobe (cardiac, apical and diaphragmatic, respectively) or the entire lobe (using global lesion scores, averages of pathogen loads and averages of transcript expressions in the three lobes). In each condition, data from all pigs were included, whatever their time of sampling (5, 24 or 48 hpi) and their infectious status (H1N1, MH1N1 or Control groups). All statistical analyses were performed using R software (version 3.1.3). Differences were considered significant when P-values were less than 0.05. Probability values between 0.05 and 0.10 were considered a trend.

**ACKNOWLEDGEMENTS**

The authors would like to thank colleagues from the ANSES Ploufragan/Plouzané laboratory for their helpful contributions: Roland
Cariolet, André Kéranflec’h, Jean-Marie Guionnet and Yann Baill for animal care and sampling; Stéphane Quéguiner, Nicolas Barbier, Emilie Bonin and Sérénore Hervé for technical help in tissue sampling at necropsy; Séverine Ferré and Anne V. Gautier-Bouchardon for preparing the Mhp inoculum and performing Mhp quantification; and Stéphane Bougeard for her advice on statistical analysis. We also thank Christelle Rossignol and Michel Olivier from INRA for contributing to histological analyses and tissue sampling, respectively. This work has received funding from the European Community’s Seventh Framework Programme (FP7, 2007–2013), Research Infrastructures action, under grant agreement No. FP7-228394 (NADIR project). Remuneration of the ANSES personnel from the unit ‘Service de Production de Porcs Assainis et d’Expermémentation was partially supported by the Conseil Général des Côtes d’Armor. The authors declare that they have no competing interests.

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