Immune response in the duck intestine following infection with low-pathogenic avian influenza viruses or stimulation with a Toll-like receptor 7 agonist administered orally

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This study analysed the immune response in the intestinal tract of ducks infected with low-pathogenic avian influenza viruses compared with ducks treated orally with R848, a synthetic Toll-like receptor 7 (TLR7) agonist. Influenza virus infection induced a type I interferon (IFN)-dependent immune response characterized by the expression of Mx transcripts in the ileum at levels that were proportional to viral load. Mx transcripts were detected in differentiated enterocytes from influenza virus-infected ducks. By contrast, in R848-treated ducks, Mx transcripts were detected solely in intraepithelial round cells of haematopoietic origin. An increase was detected in the number of intraepithelial TLR7-positive cells and intraepithelial IFN-α-producing cells in influenza virus-infected ducks, albeit to a lower level than in R848-treated ducks. IFN-γ expression was also upregulated in the intestine of influenza virus-infected and R848-treated ducks. Finally, interleukin (IL)-1β and IL-8 transcripts were expressed at high levels in R848-treated ducks but were not increased in influenza virus-infected ducks. These findings suggest that a type I IFN-mediated immune response in enterocytes and the activation of IFN-γ-secreting cells contribute to the control of influenza virus replication in the duck intestine.

INTRODUCTION

Ducks are frequently infected with avian influenza viruses and have been shown to shed viruses belonging to multiple subtypes (Olsen et al., 2006). They represent an important reservoir species of avian influenza viruses. These viruses can occasionally be transmitted from ducks to other bird species, such as gallinaceous poultry, or to mammals, including humans (Taubenberger & Kash, 2010). Ducks thus have a central role in the epidemiology of influenza virus infection.

The vast majority of viruses isolated from ducks are low-pathogenic avian influenza (LPAI) viruses (Munster et al., 2007). LPAI viruses replicate asymptomatically in epithelial cells from the duck intestine and are excreted in the faeces (Kida et al., 1980; Webster et al., 1978). Highly pathogenic avian influenza (HPAI) viruses have also been isolated from ducks (De Marco et al., 2005; Sturm-Ramirez et al., 2005). Interestingly, whilst abrupt death is the usual outcome of HPAI virus infection in gallinaceous poultry, HPAI virus-infected ducks usually present only mild clinical signs and recover from infection (Perkins & Swayne, 2003; Wood et al., 1995). Asian HPAI H5N1 viruses from the clade 2.2 genotype represent an exception, as they have been described as being lethal to ducks (Kim et al., 2008). However, lethality is mainly observed and reproduced experimentally in young ducks (Löndt et al., 2010; Pantin-Jackwood et al., 2007). By contrast, older ducks are able to survive infection with the Asian HPAI H5N1 viruses from the clade 2.2 genotype, suggesting that ducks have the ability to control the replication of the most virulent HPAI viruses. Importantly, HPAI viruses have a similar tropism in poultry and ducks, and comparative analyses have revealed that viral titres found in ducks are lower than in chickens (Perkins & Swayne, 2003; Wood et al., 1995). These observations suggest that ducks have developed specific immune mechanisms enabling them to control influenza virus replication efficiently.

In this study, we analysed the immune response in the intestinal tract, which is the main site of LPAI virus replication in ducks. We showed that ducks developed an innate immune response to influenza virus characterized by the expression of Mx, a type I interferon (IFN)-induced gene transcript, in enterocytes, which represent the main target cell of LPAI viruses in vivo. Interestingly, Mx...
transcript levels were proportional to viral load in the ileum. The immune response to influenza virus was associated with the activation or recruitment of alpha interferon (IFN-α) and Toll-like receptor 7 (TLR7)-positive cells, as well as of IFN-γ-producing cells at the site of infection.

RESULTS

Histopathological analysis and detection of viral antigen in the ileum

We recently showed that the C-terminal domain of the non-structural NS1 protein of influenza A virus regulates virus growth in the duck intestine (Soubies et al., 2010a). Using LPAI viruses rescued by reverse genetics, we observed that an H7N1 virus containing NS1 with a C-terminal RSKV domain (RSKV virus) replicated to higher levels than an H7N1 virus containing NS1 with a C-terminal ESEV domain (ESEV virus), reaching significantly higher titres at 6 days post-infection (p.i.) in the ileum (Soubies et al., 2010a). In the present study, we used samples taken at 1 and 6 days p.i. to study the intestinal immune response. No viral RNA could be detected by quantitative RT-PCR (RT-qPCR) in the ileal (Fig. 1a) or colonic (data not shown) mucosa of ESEV and RSKV virus-infected ducks at day 1 p.i. By contrast, viral RNA was detected in the ileal mucosa at day 6 p.i., and the level of viral RNA was higher in RSKV virus-infected ducks than in ESEV virus-infected ducks (Fig. 1a). Infected cells were more abundant in the ileum than in the colon, and viral antigen was detected exclusively in differentiated enterocytes (Fig. 1b). We also treated a group of ducks orally with R848, a TLR7/8 agonist (Hemmi et al., 2002; Jurk et al., 2002), which has been shown to trigger an intense innate immune response and inflammation in the intestine after oral or intra-colonic administration in mammals (Karlsson et al., 2008; Pockros et al., 2007; Yrlid et al., 2006). Ducks express a functional TLR7 receptor but lack a functional TLR8 receptor (MacDonald et al., 2008).

Histological analysis performed on the ileum revealed that ESEV and RSKV virus-infected ducks had mild enteritis characterized by moderate infiltrates of heterophils and mononuclear cells in the lamina propria and rare foci of necrotic epithelial cells (Fig. 1c, d). By contrast, the ileum of R848-treated ducks had moderate necrotizing enteritis, characterized by marked infiltrates of heterophils and few foci of epithelial necrosis (Fig. 1c, d). Histological scoring of inflammation and necrosis confirmed that the lesions were significantly higher in R848-treated animals than in animals infected with the ESEV and RSKV viruses (Fig. 1e).

Analysis of the type I IFN immune response

We first quantified Mx transcription. Mx is a type I IFN-induced gene and is a good indicator of the level of type I IFN produced in situ (Bazzigher et al., 1993; Holzinger et al., 2007; Sommereyns et al., 2008). As shown previously (Soubies et al., 2010a), we detected significantly higher levels of Mx transcripts in the ileum of the RSKV virus-infected ducks compared with ESEV virus-infected ducks at days 1 and 6 p.i. (Fig. 2a). In order to study how the viral load influenced the immune response in the duck intestine, we plotted the levels of Mx transcripts against viral load in the ileum at day 6 p.i. We observed a significant correlation between Mx transcript level and viral load (Fig. 2b). Mx transcripts were also significantly increased in R848-treated ducks, indicating that R848 treatment led to type I IFN synthesis in the duck intestine (Fig. 2a). The levels of Mx transcripts did not differ significantly between RSKV virus-infected ducks and R848-treated ducks.

In order to study which cells expressed Mx transcripts, we analysed the pattern of Mx transcript expression by in situ hybridization (ISH) at day 6 p.i. In non-infected mock-treated ducks, as well as in ESEV virus-infected ducks, an Mx signal was present in a few intraepithelial round cells that were not typically lying on a basement membrane. The morphology and topology of these cells were thus reminiscent of cells of haematopoietic origin (Fig. 2c). The same cells were labelled with the Mx probe in RSKV virus-infected ducks and were found to be more abundant in R848-treated ducks. In addition, we observed an Mx signal in differentiated enterocytes of RSKV virus-infected ducks (Fig. 2c). Thus, even though the global levels of Mx did not differ significantly between RSKV virus-infected ducks and R848-treated ducks, the pattern of Mx expression was different.

Next, we evaluated the levels of type I IFN synthesis in vivo. Unfortunately, the sequences of duck IFN-β and IFN-α are unknown. We therefore focused on the analysis of duck IFN-α transcription (Schultz et al., 1995). We did not detect any significant upregulation of IFN-α transcripts by RT-qPCR in the ileum of ESEV or RSKV virus-infected ducks at day 1 or 6 p.i. (Fig. 3a). By contrast, IFN-α transcript levels were significantly increased in R848-treated ducks (Fig. 3a). We next performed ISH to identify the source of IFN-α at day 6 p.i. Rare IFN-α-positive intraepithelial round cells, not typically lying on a basement membrane, were detected by ISH in the ileum of mock-infected animals and ESEV virus-infected ducks (Fig. 3b). Based on their morphology and topology, these cells were identified as haematopoietic cells. RSKV virus-infected ducks exhibited a stronger IFN-α signal, detected in a higher number of intraepithelial round cells, compared with mock-infected animals and ESEV virus-infected ducks (Fig. 3b). In R848-treated ducks, the IFN-α signal was also increased compared with mock-infected animals and ESEV virus-infected ducks, and was detected in cells with the same morphology and topology as in RSKV virus-infected animals (Fig. 3b). Taken together, these results indicated that IFN-α-secreting cells are either activated or recruited to the mucosa of RSKV virus-infected ducks and R848-treated ducks but not of ESEV virus-infected ducks.
Fig. 1. Analysis of virus replication and histopathological changes following infection with LPAI viruses or treatment with R848. (a) Viral RNA level in the ileum. Viral RNA levels were determined by RT-qPCR and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. Each shaded square represents an ESEV virus-infected duck and each filled triangle represents an RSKV virus-infected duck. The dotted line corresponds to the detection limit for viral RNA in the ileum. (b) Immunohistochemical anti-nucleoprotein (NP) staining of formalin-fixed ileum sections collected at day 6 p.i. Infected enterocytes at the tip of the villi are stained in brown in ESEV and RSKV virus-infected ducks. Haematoxylin counterstaining. Bars, 10 μm. (c) Formalin-fixed ileum sections collected at day 6 p.i. Mock, no lesion; ESEV, mild enteritis; RSKV, mild enteritis; R848, moderate enteritis. Haematoxylin and eosin (H&E) stained. Bars, 50 μm. (d) Magnification of the inset shown in (c). Bars, 12.5 μm. (e) Histological scoring of inflammation and necrosis in H&E-stained ileum sections. *, P<0.05; **, P<0.01; ***, P<0.001 by Student’s unpaired t-test.
Fig. 2. Analysis of Mx expression. (a) RT-qPCR analysis of Mx expression in the ileum. Mx levels were normalized to GAPDH levels. Results are expressed as means ± SEM from five mock-treated animals, three animals in each virus-infected group and five R848-treated animals. (b) Correlation between Mx expression and viral load in the ileum at day 6 p.i. Each shaded square represents an ESEV virus-infected duck and each filled triangle represents an RSKV virus-infected duck. Spearman's correlation test: r = 0.8857; P < 0.05. (c) Localization of Mx-expressing cells in the ileum. ISH with an Mx probe was performed on formalin-fixed ileal sections from mock-treated, infected (day 6 p.i.) and R848-treated animals. ISH-positive cells are stained dark purple. Arrowheads indicate Mx-positive cells, which are further magnified fourfold in the inset. Haematoxylin counterstaining. Bars, 50 μm. **, P < 0.01; ***, P < 0.001 by Student’s unpaired t-test.

Fig. 3. Analysis of IFN-α expression. (a) RT-qPCR analysis of IFN-α expression in the ileum. IFN-α levels were normalized to GAPDH levels. Results are expressed as means ± SEM from five mock-treated animals, three animals in each virus-infected group and five R848-treated animals. (b) Localization of IFN-α-expressing cells in the ileum. ISH with an IFN-α probe was performed on formalin-fixed ileal sections from mock-treated, infected (day 6 p.i.) and R848-treated animals. ISH-positive cells are stained dark purple. Arrowheads indicate IFN-α-positive cells, which are further magnified fourfold in the inset. Haematoxylin counterstaining. Bars, 50 μm. *, P < 0.05 by Student’s unpaired t-test.
We analysed the level and pattern of TLR7 expression, which, in mammals, is expressed mainly by specialized immune cells, such as plasmacytoid dendritic cells (Ito et al., 2002). These cells are able to secrete large amounts of IFN-α in response to TLR7 stimulation with ssRNA or chemical agonists such as R848 (Heil et al., 2004; Ito et al., 2002). No significant difference was observed between groups when we measured TLR7 mRNA levels by RT-qPCR (Fig. 4a). By contrast, we observed a pronounced increase in the number of TLR7-positive cells detected by ISH in RSKV virus-infected ducks at day 6 p.i. and R848-treated ducks but not in ESEV virus-infected ducks (Fig. 4b). In R848-treated animals, TLR7 staining was observed in haematopoietic cells, which appeared as round cells located in the intraepithelial compartment (Fig. 4b). RSKV virus-infected ducks exhibited the same pattern of TLR7 staining, albeit in fewer cells than R848-treated animals. Thus, our results indicated that TLR7-positive cells are activated or recruited to the ileal mucosa and thus could contribute to the early immune response in RSKV virus-infected ducks and R848-treated ducks.

Analysis of IFN-γ synthesis

In order to test whether viral infection triggers the activation of a Th1-mediated immune response or the activation of NK cells in the duck intestine, we measured duck IFN-γ synthesis (Schultz & Chisari, 1999). No difference in IFN-γ transcript levels was observed between infected ducks and mock-infected animals at day 1 p.i. (Fig. 5a). At day 6 p.i., no difference in IFN-γ transcript levels was observed between ESEV virus-infected ducks and mock-infected animals (Fig. 5a). However, IFN-γ transcription was significantly increased at day 6 p.i. in RSKV virus-infected ducks compared with ESEV virus-infected ducks and mock-infected animals (Fig. 5a). The level of IFN-γ transcripts was further increased in R848-treated animals (Fig. 5a). No IFN-γ staining was detected by ISH in ESEV or RSKV virus-infected ducks, whilst a few IFN-γ-positive round cells were located predominantly in the vicinity of the ileal crypts of R848-treated animals (Fig. 5b).

Analysis of interleukin (IL)-1β and IL-8 synthesis

The histological lesions observed in influenza virus-infected ducks and R848-treated ducks prompted us to analyse expression of the pro-inflammatory cytokine IL-1β and the chemokine IL-8 (Wu et al., 2007, 2008). No difference in IL-1β (Fig. 6a) or IL-8 transcript levels (Fig. 6b) was observed between ESEV or RSKV virus-infected ducks and mock-infected animals at day 1 or 6 p.i. By contrast, in R848-treated ducks, IL-1β transcripts were significantly increased in the ileum (Fig. 6a). We also detected significantly higher levels of IL-8 transcripts in the ileum of R848-treated ducks (Fig. 6b).

DISCUSSION

The immune response of ducks to influenza virus has, as yet, been poorly studied. In this study, we combined RT-qPCR and ISH to analyse the expression of a subset of duck genes implicated in the host immune response to pathogens. We showed that infected ducks developed a type I IFN-mediated immune response to influenza viruses that was associated with mild inflammation in the ileum and with rare necrotic foci. This contrasted with R848-treated ducks, which developed a type I IFN-mediated immune response with moderate inflammation characterized by a marked recruitment of heterophils in the ileal mucosa and few foci of epithelial necrosis. The immunopathology observed in R848-treated ducks was associated with high levels of IL-1β and IL-8. These transcripts were not upregulated in influenza virus-infected ducks. Interestingly, Adams et al. (2009) showed recently that chicken peripheral blood mononuclear cells infected with an avian...
H1N9 virus upregulated IL-1β transcripts, whilst duck peripheral blood mononuclear cells infected with the same virus did not upregulate IL-1β transcripts (Adams et al., 2009). This result suggests that the immune response to influenza virus could differ between chickens and ducks. Whether differential induction of pro-inflammatory cytokines contributes to the difference in the severity of symptoms observed between ducks and chickens infected with influenza viruses remains unknown. Comparative analyses of the innate immune response of ducks and chickens infected with LPAI and HPAI viruses are needed to support this hypothesis.

We observed a significantly higher level of Mx transcripts in RSKV virus-infected ducks compared with ESEV virus-infected ducks. As both viruses antagonize type I IFN synthesis similarly (Soubies et al., 2010a), we interpreted this difference as resulting from differences in the level of virus replication. Indeed, we observed a significant correlation between the level of influenza virus replication at day 6 p.i. in the ileum and the level of Mx expression, a type I IFN-stimulated gene. This suggested that the level of type I IFN production correlates with viral load in the duck ileum. A similar correlation between viral load and IFN-α levels has been shown recently in the respiratory tract of chickens infected with an LPAI influenza virus (Reemers et al., 2010). It is well known that influenza viruses antagonize type I IFN synthesis by infected cells through multiple actions of the NS1 protein (Hale et al., 2008; Kochs et al., 2007). In addition, we have shown recently that the NS1 protein of the H7N1 virus used in this study inhibits type I IFN induction in duck cells (Soubies et al., 2010b) and that the H7N1 viruses used in this study are inhibited by pre-treatment of duck cells with type I IFN (Soubies et al., 2010a). Thus, the level of type I IFN induced in vivo by influenza viruses with a functional NS1 protein is probably a consequence of a higher number of viral genome copies detected by the host innate immune system or a higher number of infected cells able to synthesize type I IFN.

In RSKV virus-infected ducks, an Mx signal was detected in differentiated enterocytes, which represent the main target cells for influenza virus in the duck intestine. Although no antiviral activity has been attributed to duck Mx protein so far (Bazzigher et al., 1993), our results showed that the expression of type I IFN-stimulated genes is induced in enterocytes. This type I IFN-induced response could limit influenza virus replication by stimulating the expression of other type I IFN-induced antiviral proteins, which remain to be identified in ducks. Mx staining in differentiated enterocytes was not observed in ESEV virus-infected ducks.

Fig. 5. Analysis of IFN-γ expression. (a) RT-qPCR analysis of IFN-γ expression in the ileum. IFN-γ levels were normalized to GAPDH levels. Results are expressed as means ± SEM from five mock-treated animals, three animals in each virus-infected group and five R848-treated animals. (b) Localization of IFN-γ-expressing cells in the ileum. ISH with a TLR7 probe was performed on formalin-fixed ileal sections from mock-treated, infected (day 6 p.i.) and R848-treated animals. ISH-positive cells are stained dark purple. The arrowhead indicates an IFN-γ-positive cell, which is further magnified fourfold in the inset. Haematoxylin counterstaining. Bars, 50 μm. **, P<0.01; ***, P<0.001 by Student’s unpaired t-test.

Fig. 6. Analysis of IL-1β and IL-8 expression. (a) RT-qPCR analysis of IL-1β expression in the ileum. IL-1β levels were normalized to GAPDH levels. (b) RT-qPCR analysis of IL-8 expression in the ileum. IL-8 levels were normalized to GAPDH levels. Results are expressed as means ± SEM from five mock-treated animals, three animals in each virus-infected group and five R848-treated animals. *, P<0.05 by Student’s unpaired t-test.
probably because of a low level of Mx expression that could not be detected by ISH. We detected similar levels of Mx expression in R848-treated ducks and in RSKV virus-infected ducks. However, the Mx staining pattern detected by ISH differed between infected ducks and ducks stimulated with the chemical TLR7 agonist. In R848-treated ducks, Mx staining was not detected in differentiated enterocytes, whereas bright Mx staining was detected in haematopoietic cells found in the intraepithelial compartment. As Mx staining in differentiated enterocytes was present in RSKV virus-infected animals but absent from R848-treated ducks, we hypothesize that a source of type I IFN specifically triggering Mx expression in enterocytes is present in influenza virus-infected animals but is absent in animals treated with the chemical TLR7 agonist.

ISH against IFN-α detected a higher signal in RSKV virus-infected ducks and in R848-treated ducks compared with mock-infected and ESEV virus-infected ducks. An IFN-α signal was detected in haematopoietic cells present in the intraepithelial compartment. As these IFN-α-positive cells had the same morphology and topology in RSKV virus-infected and R848-treated ducks, they are unlikely to be responsible for the induction of Mx expression in enterocytes, which was only observed in influenza virus-infected ducks and not in R848-treated ducks. Thus, other type I IFN subtypes undetected by the IFN-α probe used in our study could stimulate Mx expression in the enterocytes of RSKV virus-infected ducks. The retinoic acid inducible gene I protein (RIG-1), which has recently been shown to be expressed and functional in ducks, could trigger IFN-β synthesis in infected enterocytes (Barber et al., 2010). IFN-β could in turn stimulate Mx expression in enterocytes in an autocrine or paracrine fashion. IFN-α has recently been shown to be expressed in chickens (Karpala et al., 2008) and has been shown to trigger an antiviral response in epithelial cells from the mouse respiratory and gastrointestinal tract (Mordstein et al., 2010). Thus, duck IFN-α could contribute to the induction of an antiviral state in enterocytes. Unfortunately, we could not analyse their expression because the sequences of IFN-β and IFN-α remain to be identified in ducks. In addition, we cannot exclude the possibility that other IFN-α subtypes exist in ducks, as in mammals (van Pesch et al., 2004), and that we may have failed to detect specific IFN-α subtypes with the riboprobe used for ISH and with the primers used for the RT-qPCR. We detected upregulation of IFN-α expression when analysed by ISH in RSKV virus-infected ducks, whereas no upregulation was detected by RT-qPCR in the same animals. The discrepancy between ISH and RT-qPCR indicates that the primers used for RT-qPCR could be strictly IFN-α subtype specific, whilst the riboprobe used for the ISH could detect one or a few IFN-α subtypes. Alternatively, the number of IFN-α-expressing cells could be too low to produce an upregulation detectable by RT-qPCR from whole ileal section lysates, whilst nevertheless being detectable by ISH as rare IFN-α-positive cells.

We observed an increase in the number of TLR7-positive cells detected by ISH in the ileum of RSKV virus-infected ducks and R848-treated ducks. The lack of suitable antibodies precludes further phenotypic characterization of the TLR7-positive cells based on the expression of specific antigens, but, based on their morphology, these cells corresponded to haematopoietic cells present in the intraepithelial compartment. Interestingly, cells stained for IFN-α had the same morphology and topology, indicating that a subset of these cells could be positive for both TLR7 and IFN-α. Thus, TLR7-positive cells could contribute to the type I IFN-mediated immune response to influenza virus in the duct intestine. In mammals, macrophages or plasmacytoid dendritic cells expressing TLR7 have been shown to be major type I IFN producers and to contribute largely to the innate immune response against negative-strand RNA viruses in the lung (Kumagai et al., 2007; Smit et al., 2006). In chickens, TLR7 mRNA has been shown to be expressed to high levels in B cells and in various subsets of T cells (Iqbal et al., 2005). Further studies are required to characterize the cellular pattern of TLR7 expression in ducks and to test the contribution of TLR7-positive cells to the antiviral innate immune response.

Our results showed that IFN-γ transcripts are upregulated in the ileum of RSKV virus-infected ducks, indicating that T cells or NK cells are activated as early as 6 days p.i. Thus, in addition to a type I IFN immune response, a cell-mediated immune response is activated during influenza virus infection. Taken together, our results suggest that a type I IFN-mediated immune response, associated with a cell-mediated immune response, could allow ducks to efficiently control influenza virus replication in the intestine.

**METHODS**

*Viruses and in vivo experiments.* The LPAI virus A/Turkey/Italy/977/1999 (H7N1) was used for reverse genetics to rescue a wild-type virus that contained NS1 with a C-terminal ESEV motif (ESEV virus) and RSKV virus, as described previously (Soubies et al., 2010a). All animals used in these experiments were treated according to EEC recommendations for animal welfare and were under the supervision of the local INRA Ethics Committee. Two-week-old Pekin ducks (Anas platyrhynchos domesticus) were obtained from a commercial hatchery of controlled sanitary status (Couvoir de la Seigneuriete, Vieillevigne, France). We verified that the animals had no antibodies against influenza virus prior to inoculation by performing a haemagglutination inhibition assay with a homologous H7N1 virus and by using a commercial Influenza H7 Antibody Competition ELISA kit and a commercial Influenza A NP Antibody Competition ELISA kit (ID Screen; ID-Vet). Ducks were infected with 10^5 p.f.u. virus via the intracoanal cleft route and the oral route, as described previously (Soubies et al., 2010a). R848 (Alexis Biochemicals) was administered orally at a dose of 5 mg kg^-1. Animals were euthanized by intravenous injection of T61 (Intervet) and tissue samples were collected during necropsy. Samples from infected ducks were collected at day 6 p.i. Samples from R848-treated and mock-treated animals were collected at 14 h post-treatment.

*Histological analysis and immunohistochemistry.* After 48 h of fixation in 10% neutral buffered formalin, tissues were routinely
processed and embedded in paraffin. Sections were cut at 3 μm and stained with H&E. Viral staining was performed with a mouse mAb directed against NP (Argene), diluted at 1:50, incubated overnight at 4 °C. A goat anti-mouse HRP-conjugated polyclonal serum (Dak) was used as the secondary antibody. Diaminobenzidine was used as the substrate chromagen, and slides were counterstained with haematoxylin.

Histopathological analysis was carried out by a veterinary pathologist who was blinded to the experimental conditions. The extent of inflammation in the ileum was graded as follows: 0, no increase in cellularity in the lamina propria; 1, scattered groups of inflammatory cells in the lamina propria; 2, thickening of the lamina propria and separation of crypts by more than five strata of inflammatory cells; 3, diffuse and severe thickening of the lamina propria and separation of crypts by more than ten strata of inflammatory cells. The extent of necrosis in the ileum was graded as follows: 0, normal mucosa; 1, focal epithelial cell desquamation; 2, more marked epithelial injury with eroded areas; 3, ulceration of the epithelium. The histological score was presented as the sum of the inflammatory and necrotic scores and was described as follows: mild, 1 ≤ score ≤ 2; moderate, 3 ≤ score ≤ 4; marked, 5 ≤ score ≤ 6.

RNA extraction and qPCR. RNA from ileal segments conserved in TRIzol reagent (Invitrogen) was extracted following the manufacturer’s instructions. For each sample, 5 μg RNA was further purified using a Nucleospin RNA II kit (Clontech). Reverse transcription was performed using a SuperScript II Reverse Transcriptase kit (Invitrogen) and Random Primers (Invitrogen) following the manufacturer's protocol. Quantitative PCR was performed in a final volume of 25 μl containing 100 ng cDNA, 400 nM each primer and 12.5 μl iTaq SYBR Green Supermix with ROX (Bio-Rad). The primers used were listed in Supplementary Table S1 (available in JGV Online). Quantitative PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the following program: initial denaturation for 2 min at 95 °C, followed by 40 cycles of 15 s at 98 °C and 1 min at 60 °C, and a melt-curve analysis. To rule out genomic contamination, control PCRs were performed in the absence of reverse transcriptase. For quantification, we used the DART-PCR version 1 software developed by Peirson et al. (2003) and processed duplicate or triplicate samples as recommended by the authors. Quantification using this software takes into account the efficiency of PCR for each primer set and for each sample. The starting fluorescence, R0, which is proportional to the starting template quantity, is calculated for each primer pair from duplicate or triplicate samples as recommended by the authors. For relative quantification of the target genes, each set of primer pairs was used to detect the expression in response to H1N1 low pathogenic avian influenza virus infection in chicken and Pekin duck peripheral blood mononuclear cells. Mol Immunol 46, 1744–1749.

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