Virulent Newcastle disease virus elicits a strong innate immune response in chickens

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Newcastle disease virus (NDV) is an avian paramyxovirus that causes significant economic losses to the poultry industry worldwide. There is limited knowledge about the avian immune response to infection with virulent NDVs, and how this response may contribute to disease. In this study, pathogenesis and the transcriptional host response of chickens to a virulent NDV strain that rapidly causes 100% mortality was characterized. Using microarrays, a strong transcriptional host response was observed in spleens at early times after infection with the induction of groups of genes involved in innate antiviral and pro-inflammatory responses. There were multiple genes induced at 48 h post-infection including: type I and II interferons (IFNs), several cytokines and chemokines, IFN effectors and inducible nitric oxide synthase (iNOS). The increased transcription of nitric oxide synthase was confirmed by immunohistochemistry for iNOS in spleens and measured levels of nitric oxide in serum. In vitro experiments showed strong induction of the key host response genes, alpha IFN, beta interferon, and interleukin 1β and interleukin 6, in splenic leukocytes at 6 h post-infection in comparison to a non-virulent NDV. The robust host response to virulent NDV, in conjunction with severe pathological damage observed, is somewhat surprising considering that all NDV encode a gene, V, which functions as a suppressor of class I IFNs. Taken together, these results suggest that the host response itself may contribute to the pathogenesis of this highly virulent strain in chickens.

INTRODUCTION

Newcastle disease virus (NDV) is an avian paramyxovirus that causes significant economic damage to the poultry industry. NDV is a negative-stranded RNA virus with a 15 kb genome that contains six genes: nucleoprotein (NP), phosphoprotein (P) (RNA editing of the P gene can also result in expression of V and W), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN) and polymerase (L). Over 200 avian species are naturally or experimentally susceptible to NDV. Isolates are of a single serotype, but have a wide range of naturally occurring pathogenicities from avirulent (lentogen), to mildly virulent (mesogen) and highly virulent (velogen) (Alexander & Senne, 2008).

While the highly virulent strains of NDV are not normally found in poultry in the USA, an outbreak of Newcastle disease (ND) in the South-western USA in 2002 required extensive culling and surveillance for control and resulted in a loss of millions of dollars (Yu et al., 2001). This outbreak was caused by a highly virulent isolate that typically causes 100% mortality in susceptible poultry 3–6 days post-infection (p.i.) (Wakamatsu et al., 2006a). Virulent NDV is endemic in a large number of countries around the world, and there are reports of disease occurring in vaccinated birds (Yu et al., 2001). In addition, some NDV strains are now becoming particularly virulent in avian species, e.g. geese, which had generally been resistant to clinical disease with virulent NDV infections (Liu et al., 2003).

The host innate immune response to virus infection is an immediate reaction designed to retard virus growth and aid the host in developing specific protection from the adaptive immune responses. Research has shown that, in chicken cells, NDV can induce nitric oxide (NO) in vitro in heterophils (Sick et al., 2000) and peripheral blood mononuclear cells (Ahmed et al., 2007), alpha (IFN-α) and beta (IFN-β) interferon mRNA in macrophages (Sick et al., 1998) and gamma interferon (IFN-γ) mRNA in peripheral blood mononuclear cells (Ahmed et al., 2007). Yet this existing knowledge of the in vitro response to NDV
is insufficient to understand the nature of the host response to virulent NDV in vivo or to understand the underlying mechanisms of pathogenesis. Despite decades of research characterizing pathogenesis of different isolates, little is known about the underlying molecular mechanisms of disease caused by NDV. Here, we report a global analysis of the host response to infection in vivo with a virulent NDV strain from the 2002 outbreak in California (NDV-CA02), and characterize the early host response of splenocytes.

RESULTS

NDV-CA02 induces a robust host response in spleens in vivo

To measure the early host response to NDV in vivo, microarray experiments using complete chicken genome array slides were performed on spleens from specific-pathogen-free (SPF) chickens inoculated bilaterally intranasal and into the conjunctiva with an embryo infectious doses (EID<sub>50</sub>) per 0.1 ml dose of 10<sup>6.3</sup> NDV-CA02 or 0.1 ml of PBS control. The host gene expression profiles in the spleens of birds inoculated with PBS were compared with NDV-CA02 at both 1 and 2 days p.i. (five birds per treatment per time point). Spleens were chosen for analysis because these organs are known to be infected early and are lymphoid in nature. At 1 day p.i., the microarray analysis identified 125 genes that were induced by NDV-CA02 at least twofold, and 65 genes that were repressed at least twofold out of 6772 genes with significant data (P<0.05). By 2 days p.i., out of 6317 genes with significant data (P<0.05), 704 were induced at least twofold and 405 were repressed at least twofold (complete results presented in Supplementary Table S1, available in JGV Online). For certain genes, the microarray analysis generated no data. This occurs when appropriate probes representing genes are not present on the array or when the raw hybridization of the fluorescently labelled cDNA to the probe is too weak to produce a reliable signal. Of the significantly induced or repressed genes detected, genes specifically involved in the host inflammatory response, the innate antiviral response and well-characterized cytokines were chosen for further discussion in this study.

Many genes associated with an early innate host response were induced by NDV-CA02 at 1 day p.i., including the pro-inflammatory cytokine interleukin 6 (IL-6), chemokine macrophage inflammatory protein-3 alpha (MIP-3α), myxovirus resistance (Mx), lysozyme, interferon-induced protein with tetratricopeptide repeats 5 (IFIT-5), interferon-stimulated gene (ISG)12-2, and melanoma differentiation associated protein-5 (MDA-5) and IFN-γ precursor (Table 1).

Changes in mRNA expression at 1 day p.i. of four early innate response genes were confirmed by quantitative RT-PCR on spleens from infected chickens (Supplementary Fig. S1, available in JGV Online). By 2 days p.i., each of these genes were further upregulated. In addition, several other markers of the innate immune response that were not induced at 1 day p.i. were upregulated at 2 days p.i., such as, iNOS, the pro-inflammatory cytokines IL-1β, IL-18, IL-8 and IFN-γ. The fact that expression of a subset of genes associated with an early host response increased from 1–2 days p.i. concomitant with an increase in virus titres in the spleen is evidence that these times are early in the infection. Further changes after 2 days p.i. were not measured in this study because they could be complicated by the host’s transition from an innate to an adaptive immune response.

In addition to upregulating Mx, NDV-CA02 induced expression of the antiviral IFN effector genes protein kinase R (PKR) and 2’–5’-oligoadenylate synthetase (OAS) (Table 1). Also induced were numerous cytokines: K203, ah221, CXCL13/BCA-1, CCL21, MIP-3α and MIP-1β. Several of these cytokines are chemokines, most notably MIP-3α and MIP-1β that function to set up later cell-mediated responses by recruiting effector leukocytes like neutrophils. Finally, many genes that were significantly induced by NDV-CA02 infection are part of the innate response signalling processes: regulator of G-protein signalling 1 (ADORA), suppressor of cytokine signalling (SOCS)-1 and -3, N-myc and STAT4 interactor (signal transducer and activator of transcription), STAT4 and IFN regulatory factors (IRF) 1, 7 and 10 (Table 1).

NDV-CA02 infection in vivo induces a strong iNOS response

With evidence from microarrays that NDV-CA02 was inducing iNOS expression in spleens, immunohistochemistry (IHC) for iNOS was done. Fig. 1(a), left panel shows that intense immunoreactivity for iNOS was seen in spleens of NDV-CA02-infected birds at 3 days p.i. The immunostaining is most intense in areas where lymphocytic depletion is most severe and, not surprisingly, the accumulated fixed macrophages are positive for iNOS antigen. Spleen tissue sections from PBS-inoculated birds demonstrated no positive signal (Fig. 1a, right panel). Also, an assay to measure the product of active iNOS, NO, was done (Fig. 1b). Serum from NDV-CA02-infected birds were assayed for nitrates (the assay converts NO and nitrates to stable nitrites) at 1, 2 and 3 days p.i. and compared with PBS-inoculated bird serum. NDV-CA02 induced NO production in serum as early as 2 days p.i. with greater than a twofold induction that increased to over fourfold by day 3.

Characterization of early pathogenesis of NDV-CA02 in vivo

Virulent NDV from the 2002 outbreak in California virus was administered to 15 naive, SPF chickens bilaterally intranasal and into the conjunctiva with a 10<sup>6.3</sup> EID<sub>50</sub> per 0.1 ml dose. Eyelids, caecal tonsils and spleens harvested from five birds at 1, 2 and 3 days p.i. were used to characterize early infection and pathogenesis. These organs
have been shown previously to be sites of early replication for NDV, and all have abundant lymphoid cells (Alexander & Senne, 2008; Wakamatsu et al., 2006b). Results of histopathological evaluation and IHC for virus are presented in Table 2. Overall, there were minimal pathological changes seen histologically at 1 day p.i. The spleen and eyelids both had very mild lymphocyte depletion, while the caecal tonsils did not show lesions. The IHC for NDV at 1 and 2 days p.i. were weakly positive in the spleen of each bird. A mean NDV-CA02 titre of 10^{4.2} EID_{50} per 1 mg tissue (with a range of 10^{3.9}-10^{4.5}) was detected in the spleen by 1 day p.i. and titres increased by 2 days p.i. to 10^{6.8} EID_{50} per 1 mg tissue (with a range of 10^{5.9}-10^{7.1}). By day 2 the disease progressed, and moderate to severe lymphocyte depletion was observed by haematoxylin and eosin (H&E) in the spleens of three birds, and lesions were found mostly in the periarteriolar lymphoid sheaths (Fig. 2a, b). The splenic lesions progressed to severe multifocal to coalescing splenic necrosis and severe lymphocytic depletion by 3 days p.i. (data not shown). The lymphocyte depletion was associated with accumulation of macrophages and scattered heterophils, and the necrosis was characterized by areas of homogeneous amorphous cellular debris especially in the periarteriolar lymphoid sheaths. IHC of the spleens was weakly positive for NDV-CA02-infected birds at 2 days p.i., and nucleoprotein was detected mainly in the macrophages near the periarteriolar lymphoid sheaths. By 3 days p.i., NDV antigen was no longer confined to the periarteriolar region, consistent with dissemination of the virus to the spleen. Virus titres in the spleen remained high with a mean EID_{50} mg^{-1} of tissue of 10^{7.2} (with a range of 10^{5.7}-10^{7.7}).

In the caecal tonsils, the pathological changes were similar but occurred slightly later than those in the spleen.

Table 1. Selected genes significantly upregulated in the spleen during infection with NDV-CA02

<table>
<thead>
<tr>
<th>GenBank best hit</th>
<th>Fold increase (24 h)</th>
<th>Probability (24 h)</th>
<th>Fold increase (48 h)</th>
<th>Probability (48 h)</th>
<th>Gene description</th>
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<td>BU138064</td>
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<td>0.00132</td>
<td>37.9</td>
<td>2.76e-05</td>
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<tr>
<td>X05343</td>
<td>11.35</td>
<td>0.00747</td>
<td>29.03</td>
<td>0.000223</td>
<td>Mature avidin</td>
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<td>AB0885533</td>
<td>18.6</td>
<td>0.00814</td>
<td>25.84</td>
<td>7.87e-06</td>
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<td>BX275538</td>
<td>12.24</td>
<td>2.48E-05</td>
<td>17.22</td>
<td>0.000277</td>
<td>Putative ISG12-2 protein</td>
</tr>
<tr>
<td>CO765511</td>
<td>13.77</td>
<td>0.00832</td>
<td>14.41</td>
<td>0.000934</td>
<td>Lysozyme</td>
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<tr>
<td>AJ309540</td>
<td>5.584</td>
<td>0.014</td>
<td>12.45</td>
<td>0.000489</td>
<td>IL-6 (IFN-β2)</td>
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<tr>
<td>BX933162</td>
<td>9.837</td>
<td>0.00177</td>
<td>12.21</td>
<td>0.00104</td>
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<td>BX929857</td>
<td>8.891</td>
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<td>Y18692</td>
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<td>CR389044</td>
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<td>CR522995</td>
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<td>0.019</td>
<td>6.992</td>
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<td>Y14971</td>
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<td>BX264625</td>
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<td>U46504</td>
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<td>3.233</td>
<td>0.00174</td>
<td>iNOS</td>
</tr>
<tr>
<td>BX935231</td>
<td>0.789</td>
<td>0.699</td>
<td>3.088</td>
<td>0.00266</td>
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<tr>
<td>AB100005</td>
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<td>0.544</td>
<td>2.862</td>
<td>0.00185</td>
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<tr>
<td>BX932486</td>
<td>1.811</td>
<td>0.0276</td>
<td>2.815</td>
<td>2.96e-05</td>
<td>Phorbol-12-myristate-13-acetate-induced protein 1</td>
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<td>0.00997</td>
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<td>IFN-γ precursor</td>
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<td>Y705999</td>
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<td>0.0315</td>
<td>2.611</td>
<td>0.00617</td>
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</tr>
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<td>AF380350</td>
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<td>2.143</td>
<td>0.000023</td>
<td>ADORA</td>
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*24 h data for all genes in the 48 h list are included regardless of fold-change or P-value.
†Raw signal of fluorescent cDNA hybridized to probe was insignificant.

In vivo innate response to Newcastle disease virus

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Table 2. Histological analysis of spleens of NDV-CA02-infected chickens

ND, Not determined.

<table>
<thead>
<tr>
<th>Bird #</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
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<td>H&amp;E</td>
<td>IHC†</td>
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<td>Spleen</td>
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<td>–</td>
<td>+</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>5</td>
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<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Caecal tonsil</td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>5</td>
<td>ND</td>
<td>–</td>
<td>+</td>
</tr>
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</tr>
<tr>
<td>1</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>ND</td>
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</tr>
<tr>
<td>5</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
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</table>

* = Normal; +/−, minimal lymphocytic depletion; +, mild to moderate lymphocytic depletion and increase in histiocytes; ++, moderate to severe lymphocyte depletion, necrosis, histiocytosis; ++++, severe lymphocytic depletion, severe necrosis, haemorrhages, histiocytosis and heterophilic infiltration.

†, Negative; +, scattered-positive cells in the tissue for NDV nucleoprotein (N); ++, moderate amount of viral N detected; ++++, large amount of NDV nucleoprotein detected (N).

Pathological changes in these tissues were also characterized by severe lymphocytic depletion, marked macrophage accumulation and moderate to severe heterophil infiltration by 3 days p.i. (Fig. 2d). IHC revealed moderate staining in the macrophages by day 2, in greater amounts than what was detected at the same time in the spleen. By 3 days p.i., the caecal tonsils were strongly immunopositive for NDV. The areas with severe lymphocyte depletion were those that had

Fig. 1. iNOS response to NDV-CA02 infection in chicken spleen and serum. (a) IHC using anti-iNOS antibody on spleen tissue sections 3 days p.i. Image of spleen section from NDV-CA02-inoculated birds on the left (CA02) and PBS-inoculated birds on the right (PBS). Fast Red chromogen and haematoxylin counter-stain. CA02 image includes ×5 magnified insert. (b) NO levels in the serum of NDV-CA02-infected birds. Serum from each of five NDV-CA02 (CA02)-inoculated birds at each time point was assayed for nitrites by a colorimetric assay and compared with values from serum of PBS-inoculated birds. Hatched bar represents PBS control and solid bar represents NDV-CA02.
the greatest numbers of cells positive for NDV antigen (Fig. 2e) in comparison to the uninfected control (Fig. 2f).

Pathological changes in the eyelids followed a similar pattern of disease progression. At 3 days p.i., lymphocyte depletion, macrophage accumulation, necrosis and heterophil infiltration were predominant. IHC for NDV was mildly positive by 2 days p.i., while at 3 days p.i. the immunoreactivity was intense. The nucleoprotein appeared mainly confined to the macrophage population and some of the muscular cells of the eyelid.

Overall, the lesions observed in all the three organs are consistent with a necrotizing pattern of inflammation, and for all the tissue, the detection of the antigen by IHC was greatest at 3 days p.i. Both the anatomical lesions and the IHC results follow an increasing severity pattern from 1 to 3 days p.i.

**NDV-CA02 infection of chicken splenocytes in vitro induces strong early cytokine response**

To confirm that the most relevant early response genes were induced at early times post-infection, real-time RT-PCR was performed with RNA samples from chicken splenocytes following infection with NDV-CA02 in vitro. Splenocytes were infected with purified NDV-CA02 or LaSota (vaccine strain) at an m.o.i. of 1 and RNA was collected at 6 h p.i. Real-time RT-PCR was done with primers against IFN-α, IFN-γ, IL-1β and IL-6 (Fig. 3). Both type I and type II interferons, IFN-α and IFN-γ, were induced significantly by NDV-CA02, six- and sevenfold, respectively, while LaSota did not induce them over twofold relative to mock (Fig. 3). The pro-inflammatory cytokines, IL-1β and IL-6 were induced to a much greater degree by NDV-CA02, 18- and 35-fold, respectively. LaSota did not induce IL-1β over twofold and IL-6 was only modestly induced sixfold. These results corroborate the microarray and RT-PCR data from spleens in vivo, and show that NDV-CA02 induces a strong cytokine response in chickens.

**DISCUSSION**

The use of a whole chicken genome array (42 000 probes) to analyse the host response to infection in vivo allowed the identification of a large number of genes (704) that are part of the host early innate response to NDV. This included groups of functionally related genes, such as ISGs and pro-inflammatory chemokines, thus providing an additional level of confidence to the results. At 1 and 2 days p.i. *in vivo*, both type I and type II IFN responses were detected using microarrays. The induction of IRF1 and 7 are consistent with a type I IFN response since these proteins are upstream signals in the Toll-like receptor response to viruses that initiate IFN-α and IFN-β production (Au et al., 1998; Parrington et al., 1993). In addition, MDA-5, an RNA helicase that recognizes viral dsRNA and initiates a type I IFN response to many viruses [although reportedly not NDV (Loo et al., 2008)], is induced by NDV-CA02. Downstream effectors of IFN action include several IFN-stimulated-response genes (ISRGs) including, Mx, OAS and PKR that were upregulated following NDV-CA02 infection.

*In vitro* experiments suggest that a pronounced and rapid innate immune response may be induced by virulent...
viruses in splenocytes. Using real-time RT-PCR of RNA isolated from NDV-infected splenocytes, we have demonstrated that the virulent CA-NDV, but not the lentogenic LaSota virus, is capable of rapidly and strongly inducing IFN-γ, IFN-α, IL-1β and IL-6 at 6 h p.i., genes integral to a pro-inflammatory response. Unfortunately, since LaSota does not reach the spleen but only replicates at the site of inoculation (Wakamatsu et al., 2006b, c), parallel in vivo experiments to compare the relative host response to velogenic and lentogenic viruses were not possible. Our results confirm and extend in vitro analysis of gene expression patterns from chicken embryo cells in response to virulent NDV reported previously (Munir et al., 2005). There, the effect of a low m.o.i. (0.01) was analysed throughout a 36 h period, and a significant but late induction of 22 genes was noted (probably as a result of the low m.o.i. used). Upregulated genes included some similar functions to the IFN and pro-inflammatory response that was observed here, including some ISGs, IL-8 and STAT1.

NDV encodes the V protein that blocks various components of IFN pathways. Specifically, the NDV V protein targets STAT1 for degradation and recombinant NDVs that do not express V have drastically lower virulence in vivo (Huang et al., 2003; Wakamatsu et al., 2006a, b, c). STAT1 is a particularly important signalling molecule for ISRG regulation because unlike STAT2, which is targeted by the V proteins of other paramyxoviruses (Parisien et al., 2001), STAT1 is a transactivator for both IFN types I and II pathways (Darnell, 1997). Our data would indicate that at least at the mRNA level, a block of IFN pathways is not occurring with this particular NDV isolate. Here, we show that transcription of the types I and II IFNs and many ISGs are being induced by NDV-CA02, therefore further studies are necessary to determine the expression level and effectiveness of NDV-CA02 V protein during infection in both lymphoid and epithelial cells. In addition to detecting upregulation of IFN and pro-inflammatory genes, we also measured increased iNOS expression in tissues and increased levels of NO in serum. These results are in agreement with the observed upregulation of IFN-γ in microarray since IFN-γ induces iNOS expression (Kapcynski & Kogut, 2008; Koide et al., 1993). Assaying NO levels was particularly valuable because it allowed for confirmation of the microarray data at a functional level, while using a validated commercially available assay. Constitutive low-level expression of NO in the vascular endothelium plays a beneficial role in maintaining blood vessel homeostasis (Palmer et al., 1987), but high levels of NO produced by macrophages in response to pathogens can have toxic effects on the host. NO can indiscriminately modify many chemical groups and result in well-characterized decrease of protein function and cellular processes (Pacher et al., 2007). The nitrite (µM) concentrations measured in NDV-CA02-infected chicken serum are well within its range required for detrimental effect (Shiva et al., 2001). The significant upregulation of iNOS in spleens and NO in serum is a potentially destructive innate response of chickens to NDV infection, but in light of the robust replication and rapid mortality of this virus, it seems quite possible that NO is contributing to mortality not recovery.

Our data are consistent with a strong innate immune response that is unable to protect birds from disease and death, as we observed a clear innate IFN and cytokine response to NDV-CA02 in vivo. In spleens, there was a strong correlation between virus titres, pathological changes, and host innate immune response at 1 and 2 days p.i. The spleens and caecal tonsils of infected birds showed significant lymphoid depletion and infiltration of fixed macrophages by 2 days p.i. that progressed to necrotic lesions. This histopathology provides a good visual correlation to the microarray and PCR assays of cytokine expression levels. The histology and IHC of the spleens demonstrate that the cytokine increases are associated with tissue destruction and increasing quantities of virus.

The spleen was chosen for analysis of the early innate host response because it is a lymphoid organ that the virus reaches early in disease (Brown et al., 1999b). However, analysis of the response to infection in the spleen may not be sufficient to completely understand NDV mechanisms of disease or death. The global analysis of the innate response to NDV in vivo and analysis of select innate genes in vitro provide a good overview of the host response; however, the roles of individual cell types in this response cannot be effectively measured in this way. Several major cell types present in spleens (macrophages, heterophils, B-cells and T-cells) appear to be permissive to NDV (Brown et al.,

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**Fig. 3.** Real-time RT-PCR of NDV-CA02 and LaSota vaccine strain-infected chicken splenocytes in vitro. Total RNA isolated from chicken splenocytes infected with NDV-CA02 and LaSota for 6 h was used for real-time RT-PCR using SYBR Green for IFN-α, IFN-γ, IL-1β and IL-6. Results were normalized against 28S rRNA and fold-changes relative to mock-infected samples are shown. Open bar represents LaSota and solid bar represents NDV-CA02 (CA02).
1999a; Dalgaard et al., 2010; Lam, 1996; Lam et al., 1996; von Bulow & Klasen, 1983). Our data highlight the important role that splenocytes have in the host response to NDV. IL-6 and IL-1β were greatly induced in spleens by NDV-CA02 and are two well-characterized early innate response genes of macrophages to viruses through Toll-like receptor activation (Alexopoulou et al., 2001). Additionally, the iNOS induction measured at the transcript and functional (NO) levels is mainly produced by macrophages. Given the proclivity of macrophages to be present at the sites of infection, often harbouring virus (Fig. 1 and Brown et al., 1999b), future analysis of the role of individual leukocyte cell populations in the innate response to NDV are warranted.

The existence of new virulent NDV genotypes throughout the world and the susceptibility of wild avian species are cause for concern over the continued effectiveness of the currently used 50-year-old vaccine. The development of better vaccines and control strategies will require a greater understanding of the mechanisms of pathogenesis. Our report of the first global analysis of the natural host’s response to an avian paramyxovirus in vivo provides basic information needed to extend our understanding of the nature of the virus–host interactions.

**METHODS**

**Viruses.** NDV from chicken isolate California/S0212676/2002 (NDV-CA02) was originally isolated from the field during the 2002–2003 California exotic ND outbreak (Pedersen et al., 2004). This isolate was plaque-purified three times on chicken embryo fibroblasts and amplified by inoculation of SPF 9–11-day-old embryonating chicken eggs (ECE). The pathotype of the plaque-purified isolate was confirmed as a virulent by having a mean death time of 54 h in ECE (<60 being virulent) and intracerebral pathogenicity index of 1.85 (>1.5 being virulent) using standard procedures (Alexander, 1998). The low virulence NDV vaccine virus, LaSota, was received from Lohmann Animal Health International (Gainesville, Ga). For in vitro splenocyte infections, viruses were first purified by ultracentrifugation for 1 h (35 000 g) at 4 °C through a 10% sucrose cushion. Pellets were resuspended in PBS with 1% BSA, and mock infections were done with PBS with 1% BSA. Virus inoculated into birds was harvested from infected allantoic fluid, titrated in ECE and diluted in PBS.

**Birds and eggs.** The source of SPF chickens and ECE was the South-east Poultry Research Laboratory’s SPF White Leghorn chicken flock.

**Cells.** Splenocytes used for in vitro infections and real-time RT-PCR assays were isolated from 2-week-old SPF White Leghorn chickens. Spleens were collected aseptically and placed in sterile 4 °C PBS. Single-cell suspensions were prepared by gently pushing the splenic pulp through a sterile nylon mesh with a pore size of 70 μm (Fisher Scientific). Cells were washed and resuspended in 3 ml cold PBS and then layered over 3 ml Histopaque 1077 (Sigma). The preparations were enriched for leukocytes by centrifugation (450 g) for 30 min at 18 °C. Cells were recovered from the interface, resuspended in cold PBS, and washed twice in 3 ml cold PBS.

**Animal experiment and tissue collection.** Each of 15 chickens was inoculated with a total dose of 10^3.3 50% EID₃₀ in 0.1 ml of virus divided into the right nares (50%) and both eyes (25% each). PBS was used as inocula for non-infected controls. Five infected birds were sampled each day. The five non-infected control birds were sampled only on 1 day p.i. Birds were anaesthetized and blood samples were drawn from the heart. Birds were then euthanized with an intravenous injection of pentobarbitol (Sigma-Aldrich). Immediately post-mortem, eyelid, spleen and caecal tonsils were harvested aseptically for histology, IHC and RNA extraction. Spleens harvested for analysis were cut into three pieces, with one piece of each used for histology, RNA isolation and virus titration. Virus titres of spleens were determined after homogenization in 10% (w/v) PBS. Confirmation that sampled birds were infected with virus was based on virus recovery from oral or cloacal swabs collected before or on the day tissue samples were collected. Oral and cloacal swabs from all birds were positive by haemaggulination assay for NDV-CA02 by 2 and 3 days p.i., respectively.

**RNA isolation, reverse transcription, labelling and microarray hybridization.** Spleen cell suspensions were generated by pushing freshly isolated tissue through a 70 μM pore-size mesh cup, immediately into Trizol (Invitrogen) and stored at −80 °C after collection of all samples for that day. Samples were later thawed at room temperature and chloroform was added according to the manufacturer’s protocol. Samples were incubated for 10 min on ice. Following centrifugation at 2400 g for 15 min, the aqueous phase was removed, mixed with an equal volume of 70% ethanol, and applied to Qiagen RNEasy midi prep columns. RNA quantity was assessed using a Nanodrop spectrophotometer on the RNA-40 setting and quality was assessed using a nanochip on a Bioanalyser (Agilent Technologies). All RNA samples had an RNA integrity number greater than 8.6 with a mean of 9.7. Total RNA (50–300 ng) was reverse transcribed to cDNA, amplified, and labelled with Cy3 or Cy5 using the Two-colour Linear Amplification kit (Agilent Technologies). Complete chicken genome microarray slides (42 000 probes) were purchased from Agilent and hybridized cRNAs were hybridized for 17 h at 65 °C with Gene Expression Hybridization Solution (Agilent Technologies). Slides were disassembled from their hybridization chambers in Gene Expression Wash Buffer 1 then washed for 1 min in Gene Expression Wash Buffer 1, 1 min in Gene Expression Wash Buffer 2, 1 min in acetonitrile, and 30 s in Stabilization and Drying Solution (Agilent Technologies) following manufacturer’s specifications. RNA from individual NDV-CA02-inoculated animals (n=4) were considered as separate experiments and compared with pooled RNA from the PBS-inoculated animals (n=4 and n=3 for 24 and 48 h experiments, respectively).

**Microarray data collection and analysis.** Differential expression measurements based on simultaneous two-colour hybridizations were performed with a GenePix 4200A scanner using a 5 μm resolution and the GenePix Pro 6.1 data acquisition and analysis software (Axon Instruments; Molecular Devices), GeneSpring GX computer software (Agilent Technologies) was used for all normalization and statistical analysis of the GenePix output. RNA from PBS-inoculated birds was pooled and compared with RNA from individual NDV-CA02-infected birds for a total of four arrays. The intensity ratio of expression for each gene was calculated by dividing the measured infected bird values (test channel) by the intensity of the mock-infected bird values (control channel). All outputs with control channel values of less than 300.0 were not considered. Intensity-dependent normalization was used to reduce the ratios to the residual of the Lowess fit of the intensity-versus-ratio curve. Per-chip normalization was done by creating an artificial-positive control for each sample with the 50th percentile of all measurements across the entire chip. Chips were scanned with high laser strength and low laser strength independently to generate non-saturated data for the maximum number of spots. Individual sample t tests were calculated as the mean of replicate normalized values for a single gene – 1/standard error. This analysis produces low P-values when the mean of replicate normalized values (fold-changes) is significantly different from one and the standard error is small.
Quantitative RT-PCR. In vitro determination of cytokine induction of chicken splenocytes following NDV-Ca02 infection was performed as previously described (Kapczynski & Kogut, 2008). Briefly, isolation of total RNA from infected splenocytes was performed using the Qiagen RNeasy Mini kit following the manufacturer’s recommendations. Standard cycling conditions were used with iScript one-step RT-PCR kit with SYBR Green (Bio-Rad) with annealing temperatures optimized for each primer pair. Each RT-PCR experiment was run in triplicate with a no-template control, test samples and a log_{10} dilution series of standard RNA. Regression analysis of the mean values of three replicate RT-PCRs for the log_{10} diluted RNA was used to generate standard curves. Primer pairs used were (forward, reverse): IFN-α (5'-GAGGCG-CAACGCCAAGCG-3', 5'-CTGTTCGCTTACATACATGCGTUGTG-3'); IFN-γ (5'-GCCAGACTTTACACTTGTTTG-3', 5'-AGAACTGGCAGAGAGAAATGAG-3'); IL-1β (5'-ACAATCTGCTGTTGATAGCA-3', 5'-GCCCTACCTTACCGCGCTG-3'); IL-6 (5'-GCGAGAACACAGATG-GAGATG-3', 5'-GAGGCGCAGGAAAATC-3', 5'-GAGACGCGATTTGGCAGTGACCGT-3').

NO assay. Blood was drawn into S-monovette Z Serum V 4.5 ml syringes containing the clotting stimulant kaolin (Sarstedt) and centrifuged at 1000 g for 15 min to separate the serum. Serum samples (250 μl each) were applied to pre-rinsed 10 kDa molecular mass cut-off microfuge filters (Millipore) and centrifuged at 5000 g for 40 min. The flow-through was used in the nitric oxide synthase assay kit (Calbiochem) according to the manufacturer’s instructions. Serum from each of five birds for each condition were read and averaged. NO levels were calculated using a standard curve.

Histopathology and IHC. Sample tissues were immediately fixed in 10% neutral buffered formalin for approximately 52 h. All sampled tissues were routinely processed into paraffin blocks, and 3 μm sections were cut onto positively charged slides (Probe On Plus; Fisher Scientific) both for H&E staining and IHC. All tissues were examined histologically, with recording of severity of lesions. For IHC, two infected birds from each sampling day were assayed for the presence of NDV nucleoprotein. For this procedure, after deparaffinization, tissue sections were subjected to antigen retrieval by microwave in citrate buffer solution (Antigen Unmasking Solution; Vector Laboratories) for 10 min at full power. Blocking was performed with a universal blocking reagent (Biogenex) as recommended by the manufacturer. The primary antibodies, polyclonal rabbit anti-NDV nucleoprotein (peptide) (Kapczynski & Kogut, 2008; Kommers et al., 2001) and polyclonal rabbit anti-iNOS (Thermo Scientific Anatomical Pathology) were diluted in 10% normal sheep serum, and incubated at 37 °C for 1 h. The dilutions used were 1:8000 for rabbit anti-nucleoprotein, and 1:75 for rabbit anti-iNOS. After washes, the slides were incubated with an alkaline phosphatase-based polymer system specific for the Fc portion of rabbit IgG (Ultra Vision One; Thermo Scientific Anatomical Pathology). The reaction was revealed with a naphthol-based chromogen, Fast Red (Biogenex). Sections were counterstained lightly with haematoxylin and cover-slipped with Permount for a permanent record.

Quantitative RT-PCR confirmation of mRNA expression changes seen on microarray. RNA was isolated from spleens 24 h p.i. as described above. Changes in mRNA gene expression of IL-6, IFN-γ, iNOS and IFN-β were monitored as changes in SYBR Green fluorescence after amplification with specific primer sets. Primers for amplifying IFN-γ, IL-6, iNOS and IFN-β were derived from previously published sequences (Degen et al., 2005; Kaiser et al., 2003). β-Actin primers were manually selected according to the available nucleotide sequences available in GenBank (accession no. X00182) and properties were verified using the OligoNucleotide Properties Calculator (www.basic.northwestern.edu/biotools/oligocalc.html). The primers were designed to span introns in the genomic DNA to prevent DNA contamination.

All primers were synthesized by Integrated DNA Technologies. Sequences are as follows: β-actin (5'-AGAGGCGCTCCCTGAAAGCACAGGACGACGACGACTTTACACATACATGCGTUGTG-3'), IFN-γ (as above); IL-6, (as above); IFN-β (5'-GTTAGTCTGAAAGGCGGACACAG-3', 5'-TTGTAGTCGGTGTGACCGTCTG-3'); and iNOS (5'-TTGGAACAAAGTGTGTAATATCTTG-3', 5'-CCTGGCCATGCGTACATC-3').

One-step quantitative RT-PCR amplification and detection were performed using a 7500 FAST real-time PCR System (Applied Biosystems) with SYBR Green fluorescence detection of PCR product on 20 μl reaction mixtures containing: 10 μl Power SYBR Green RNA-to-CT 1-Step kit (Applied Biosystems), 500 nmol l^{-1} each of forward and reverse specific primer and 60 ng RNA. Thermocycler conditions were as follows: one cycle at 48 °C for 30 min, one cycle at 95 °C for 15 min, 40 cycles at 95 °C for 15 s and at 58 °C at 30 s. One cycle for dissociation curve for all reactions was added and the melting curve analysed.

Relative changes in gene expression were calculated according to the Pfaffl method (Pfaffl, 2001) using LinRegPCR version 12 (Ramakers et al., 2003) to calculate individual specific primer efficiency by linear regression. All cycle threshold fluorescence (Ct) values were corrected to the mean C_{T} of PBS, mock-infected control for each gene of interest and β-actin was used as the endogenous control.

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