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ORIGINAL ARTICLE

High levels of virus replication and an intense inflammatory response contribute to the severe pathology in lymphoid tissues caused by Newcastle disease virus genotype VIId

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Abstract Some strains of Newcastle disease virus (NDV) genotype VIId cause more-severe tissue damage in lymphoid organs compared to other virulent strains. In this study, we aim to define the mechanism of this distinct pathological manifestation of genotype VII viruses. Pathology, virus replication, and the innate immune response in lymphoid tissues of chickens infected with two genotype VIId NDV strains (JS5/05 and JS3/05), genotype IX NDV F48E8 and genotype IV NDV Herts/33, were compared. Histopathologic examination showed that JS5/05 and JS3/05 produced more-severe lesions in the spleen and thymus, but these four virulent strains caused comparable mild lesions in the bursa. In addition, JS3/05 and JS5/05 replicated at significantly higher levels in the lymphatic organs than F48E8 and Herts/33. A microarray assay performed on the spleens of chickens infected with JS5/05 or Herts/33 revealed that JS5/05 elicited a more potent inflammatory response by increasing the number and expression levels of activated genes. Moreover, cytokine gene expression profiling showed that JS5/05 and JS3/05 induced a stronger cytokine response in lymphoid tissues compared to F48E8 and Herts/33. Taken together, our results indicate that the severe pathology in immune organs caused by genotype VIId NDV strains is associated with high levels of virus replication and an intense inflammatory response.

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Introduction

The severity of disease caused by Newcastle disease virus (NDV) infection depends on multiple factors, including host species, age, immune status, the dose, the route of exposure, and environment conditions, but the most important factor is the virulence of the infecting strain [1]. Virulent NDV strains usually cause acute lethal infection with profound clinical signs and severe necro-hemorrhagic lesions in the lymphoid and digestive system, whereas lentogenic strains produce mild or inapparent respiratory infections [1].

However, different virulent NDV strains vary in the pathological manifestations they induce, despite having high intracerebral pathogenicity index (ICPI) values and a fusion (F) protein cleavage site associated with high virulence. Merino et al. have demonstrated that a genotype V strain, Quail2006, produces clinical signs and microscopic lesions in lymphatic tissues that are more severe than those produced by another genotype V strain, Chicken2000 [14]. Susta et al. have shown that the genotype VIId strain Long Bien causes more-severe lesions in the lymphoid organs compared to virulent genotype I strain Australia [19]. In addition, a previous study indicated that genotype VIId strains of different host origin can induce increased lesion severity in the spleen compared to genotype IV strain Herts/33 [24]. Ecco et al. have shown that genotype VIId strain ZJ1 and genotype V strain CA02 produce more-

severe clinical signs and pathological outcomes in the spleen compared to other virulent strains (Australia, Turkey ND and Texas GB) [3]. These findings, combined with previous results [19, 23], confirm that neither the ICPI nor the F protein cleavage site is sufficient to fully predict the clinicopathological outcome induced by virulent viruses.

Genotype VIId NDV is dominant in Asia and poses a great threat to the poultry industry [2, 12, 13, 15, 25]. The aforementioned studies have consistently shown that viruses of this genotype can produce severe damage in lymphoid organs compared to other virulent strains. More importantly, Qin et al. and Yu et al. have performed cross-protection studies showing that the La Sota vaccine provides poor protection against some genotype VIId NDV isolates [15, 26]. In addition, some studies have shown that the homologous vaccine can provide good protection and inhibit virus shedding after challenge with genotype VII NDV [6, 7]. These findings indicate that genotype VIId NDV can still disseminate efficiently in chickens vaccinated with conventional vaccines and thus may have a potential to injure the lymphoid tissues and impair the immune response. In this study, we aim to determine the mechanism of the more-severe pathological damage in the lymphocytic tissues caused by genotype VII NDV strains.

Based on contrasting pathological changes in the spleen between genotype VIId and genotype IV NDV [24], we selected two genotype VIId strains (JS3/05 and JS5/05) and two representative strains of early genotypes, including genotype IX NDV F48E8 and genotype IV NDV Herts/33. The ICPI values of these strain are 1.88 (JS3/05), 1.88 (JS5/05), 1.90 (F48E8) and 2.0 (Herts/33). In this paper, we report that genotype VII NDV strains JS3/05 and JS5/05 produce more-severe tissue damage in chicken lymphoid organs compared to Herts/33 and F48E8 and that high levels of virus replication and a strong innate immune response contribute to the greater pathological damage induced by genotype VII NDV strains.

Materials and methods

Ethics statement

All animal experiments were approved by the Jiangsu Administrative Committee for Laboratory Animals (Permission number: SYXK-SU-2007-0005) and complied with the guidelines of Jiangsu Laboratory Animal Welfare and Ethics of Jiangsu Administrative Committee of Laboratory Animals.

Viruses and antibodies

Four virulent NDV strains of different genotypes, including genotype VIId strains JS3/05 and JS5/05 [24], genotype IX strain F48E8, and genotype IV strain Herts/33, were used in this study. These viruses were plaque-purified three times in chicken embryo fibroblasts (CEFs). Viruses were propagated in 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs. Infectious allantoic fluids were harvested and stored at −80 °C. Virus titers were measured as 50 % embryo infectious dose (EID_{50}) using the Reed and Muench method [17]. All experiments involving live viruses were conducted in a biosafety level-3 (BSL-3) facility. mAb 6B1 against the hemagglutinin-neuraminidase (HN) protein of genotype VIId NDV strain ZJ1 was prepared previously [5]. The secondary antibody goat anti-mouse IgG-FITC (SothernBiotech, Birmingham, USA) was used in the indirect immunofluorescence assay (IFA).

Chicken experiment

Seventy-two 4-week-old SPF White Leghorn (*Gallus gallus domesticus*) chickens were equally divided into four groups. Chickens in each group were inoculated intranasally and intraconjunctivally with 10^6 EID_{50} of JS3/05, JS5/05, F48E8 or Herts/33. An additional eight birds were mock-infected with PBS. Chickens were monitored daily for clinical symptoms. At 12, 24, 48 and 72 h postinfection (pi), three chickens per group were killed and used for gross pathology observation and tissue collection (spleen, thymus and bursa). At 48 and 72 h pi, gross pathological changes were scored based on the following standards, and average values for three birds are shown: spleen (−, normal; +, atrophy without necrosis; ++, enlarged and mottled; +++, mild to moderate necrosis; ++++, severe necrosis), thymus (−, normal; +, mild hemorrhage; ++, moderate hemorrhage; +++, severe hemorrhage), bursa (−, normal; +, edematous; ++, atrophy). Relative expression of three cytokine genes, including interferon (IFN)-β, interleukin (IL)-1β and IL-18, in samples harvested at 12, 24 and 48 h pi was determined using quantitative real-time PCR (RT-qPCR). Tissues collected at 48 and 72 h pi were also processed for histopathology assessment. The remaining six chickens in each group were monitored for mortality, and clinical signs were scored daily: 0 for normal, 1 for sick (depression, decreased food-intake and respiratory symptoms), 2 for paralysis or twitching, 3 for prostration, and 4 for dead.

Histopathology

Tissues were fixed in 10 % neutral buffered formalin. All samples were routinely dehydrated, embedded into paraffin and cut into 3-μm sections for hematoxylin and eosin (HE) staining. Tissue sections were observed and histopathological changes were scored as described elsewhere [20], and

average values for three birds are presented: −, normal; +, mild to moderate hyperplasia of lymphocytes; ++, mild lymphocytic depletion; +++, moderate (<50 %) lymphocyte depletion, histiocytic accumulation and necrosis; ++++, severe (>50 %) lymphocytic depletion, histiocytosis and extensive necrosis.

Microarray analysis

To identify the host response specific to genotype VIId NDV infection, the spleens were collected from JS5/05- or Herts/33- or mock-infected chickens (n = 3) at 48 h pi for microarray analysis.

Separate microarrays were run for each experimental sample using Affymetrix Chicken Genome GeneChips (Affymetrix, Santa Clara, CA, US). Global gene expression in infected spleens was compared to mock control RNA from three uninfected birds. Total RNA was extracted using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) and examined using an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, USA). The total RNA was further purified using an RNeasy Micro Kit (QIAGEN, GmBH, Germany) and an RNase-Free DNase Set (QIAGEN). The RNA was then amplified, labeled and purified using a GeneChip 3'IVT Express Kit (Affymetrix) to obtain biotin-labeled cRNA. Array hybridization and washing were performed using the Affymetrix system including GeneChip Hybridization, Wash and Stain Kit, Hybridization Oven 645 and Fluidics Station 450. Slides were scanned using a GeneChip Scanner 3000, and raw data were normalized by the MAS 5.0 algorithm, Gene Spring Software 11.0 (Agilent Technologies).

T-test and Significant Analysis of Microarray (SAM) were used to identify genes that were significantly differentially expressed (SDE) ($p < 0.05$ and >2-fold change). For bio-function and pathway analysis, files containing significantly differentially expressed genes were uploaded into the Ingenuity Pathways Analysis (IPA) platform (Ingenuity Systems, Redwood, CA, US).

Biological function and canonical pathway analysis was performed for all genes that were associated with biological functions in the Ingenuity Pathways Knowledge Base. Fisher's exact test was used to calculate a *p*-value determining the probability that each biological function assigned to the data set was due to chance alone.

Virus load

Tissue samples were homogenized in PBS, and virus titers were measured in CEFs. Briefly, the cleared tissue homogenates were serially 10-fold diluted and used to inoculate CEFs. At 96 h pi, cells were fixed using methanol/acetone (50:50) and incubated with the first antibody mAb 6B1 at 37 °C for 1 h to determine the presence of virus. The cells were then washed three times with PBS and incubated with the secondary antibody, goat anti-mouse IgG-FITC (SouthernBiotech, Birmingham, USA) at 37 °C for 1 h. The virus titer was determined as $TCID_{50}$ per gram using the Reed and Muench method [17].

Cytokine gene expression profiling

Based on the microarray results, RT-qPCR was performed to confirm the expression of IFN-β, IL-1β and IL-18 genes in lymphoid tissues. Primers for IFN-β, IL-18 and β-actin genes have been described previously [8, 21]. Primer sequences for IL-1β are as follows: 5′-GCTCTACATGT CGTGTGTGATGAG-3′ (forward) and 5′-TGTCGATGTC CCGCATGA-3′ (reverse). Total RNA was isolated from tissues using TRIzol Reagent (Life Technologies). One microgram (μg) of total RNA per sample was treated with DNase I (Fermentas, Maryland, USA) and reverse transcribed into cDNA using 300 U of RevertAid Premium Reverse Transcriptase (Fermentas) and 100 μM random hexamer primer (Fermentas) in the presence of RNase inhibitor (Fermentas) at 50 °C for 60 min. The RT-qPCR reaction mixture contained 2 μl of cDNA, 200 nM (final concentration) each primer and 10 μl of 2× SYBR Green PCR Master Mix (Takara, Shiga, Japan). PCR reactions were performed in triplicate using an ABI Prism 7300 system (Applied Biosystems, Foster City, CA) with the following cycle profile: 1 cycle at 95 °C for 5 s followed by 40 cycles at 95 °C for 30 s and 60 °C for 31 s. One cycle of melting curve analysis was performed for all reactions to verify product specificity. Relative levels of gene expression were normalized to a housekeeping gene, β-actin. The $2^{-\Delta\Delta CT}$ method was used to determine the fold change of gene expression levels.

Statistical analysis

Differences in the expression level of cytokine genes and virus load among chickens infected with JS3/05, JS5/05, F48E8 and Herts/33 were analyzed using one-way analysis of variance (ANOVA) followed by the LSD (least significant difference) multiple-comparison test. A *p*-value of <0.05 was considered significant.

Microarray data accession number

Microarray data have been deposited in the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/) under the accession number GSE40100.

Results

Pathology and histopathology

As shown in Fig. 1a, all infected chickens started to show signs of disease at 3 days postinfection (dpi), with depression, reluctance to move, and diarrhea. Clinical symptoms progressed to marked depression, open-mouthed breathing, severe diarrhea, and paralysis by 4 dpi. Chickens began to die at 4 dpi, and paralyzed animals that were unable to eat or drink were killed humanely and recorded as dead. All birds succumbed to infection by 5 dpi (Fig. 1b).

Gross pathology was observed when the chickens died (Table 1). At 2 dpi, in chickens infected with various strains, spleens were enlarged and mottled, and thymuses were mildly reddened. At 3 dpi, JS3/05 or JS5/05 caused the most-pronounced gross lesions in the spleen, which were characterized by widespread foci of necrosis on both the capsular and cut surfaces. Enlarged and mottled spleens were also found in F48E8-infected birds, whereas Herts/33-infected chickens had markedly atrophic spleens. It is notable that no necrosis foci were detected in the spleens of F48E8- and Herts/33-infected birds. Mild to moderate hemorrhage was found in the thymus. At the late stage of infection (4 dpi), the dying birds were also euthanized and examined for gross changes. The key finding was that the spleens of chickens infected with JS3/05 or JS5/05 began to decrease in size, and severe necrotic lesions were still visually detected. The spleens of chickens infected with F48E8 or Herts/33 had similar pathological changes on day 3 pi. In addition, all of the viruses tested caused the size of the bursa to increase due to edema at 3 dpi, but the bursa began to atrophy at 4 dpi. There were no abnormal findings in mock-infected chickens.

Table 1 Histological analysis of lymphoid tissues of NDV-infected chickens

Virus		Spleen		Thymus		Bursa	
		2 dpi[a]	3 dpi	2 dpi	3 dpi	2 dpi	3 dpi
JS3/05	Gross[b]	+++	++++	+	+++	–	+
	Histologic[c]	++	++++	+	+++	–	++
JS5/05	Gross	++	++++	+	+++	–	+
	Histologic	+	++++	+	+++	–	++
F48E8	Gross	++	+++	+	++	–	+
	Histologic	–	++	–	+	–	+
Herts/33	Gross	++	+	+	+	–	+
	Histologic	+	+	+	+	–	+

[a] dpi: day postinfection

[b] Tissues were visually inspected during necropsy and gross pathology scores were assigned to different tissues as follows: Spleen: -, normal; +, atrophy without necrosis; ++, enlarged and mottled; +++, mild to moderate necrosis; ++++, severe necrosis. Thymus: –, normal; +, mild hemorrhage; ++, moderate hemorrhage; +++, severe hemorrhage. Bursa: –, normal; +, edematous; ++, atrophy. Average values of three birds were shown

[c] Tissue sections were observed, and histopathological changes were scored as follows: –, normal; +, mild to moderate hyperplasia of lymphocytes; ++, mild lymphocytic depletion; +++, moderate (<50 %) lymphocyte depletion, histiocytic accumulation and necrosis; ++++, severe (>50 %) lymphocytic depletion, histiocytosis and extensive necrosis. Average values for three birds are shown

The results of histological scoring (Table 1) showed that there were minimal histological changes at 2 dpi. At 3 dpi, JS3/05 and JS5/05 induced the most-severe histological changes in the spleen, including severe lymphoid depletion and necrosis, marked necrotic debris, and infiltration of macrophages (Fig. 2d and g). F48E8 caused moderate lymphoid depletion and necrosis in the spleen, and only mild lymphocyte depletion and necrosis was observed in the spleens of Hert/33-infected chickens (Fig. 2j and m). In

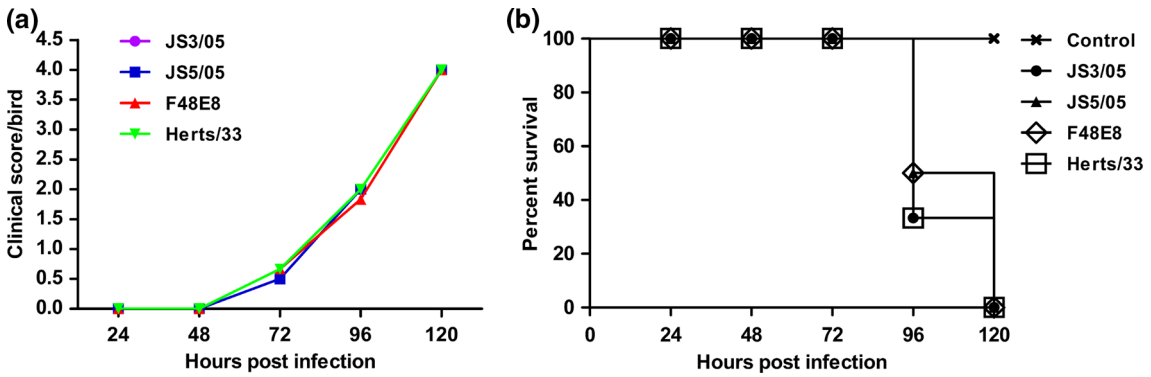

Fig. 1 Clinical scores and survival curve of chickens infected with different NDV strains. (a) Clinical scores of chickens. Six birds were monitored daily and scored as follows: 0 for normal, 1 for sick (depression, decreased food intake, and respiratory symptoms), 2 for paralysis or twitching, 3 for prostration and 4 for dead. The mean scores of each group are shown for each day. (b) Survival curve of infected chickens. Live birds with severe clinical symptoms such as paralysis or prostration that were unable to eat and drink were recorded as dead. The survival curve was plotted using the Kaplan–Meier method

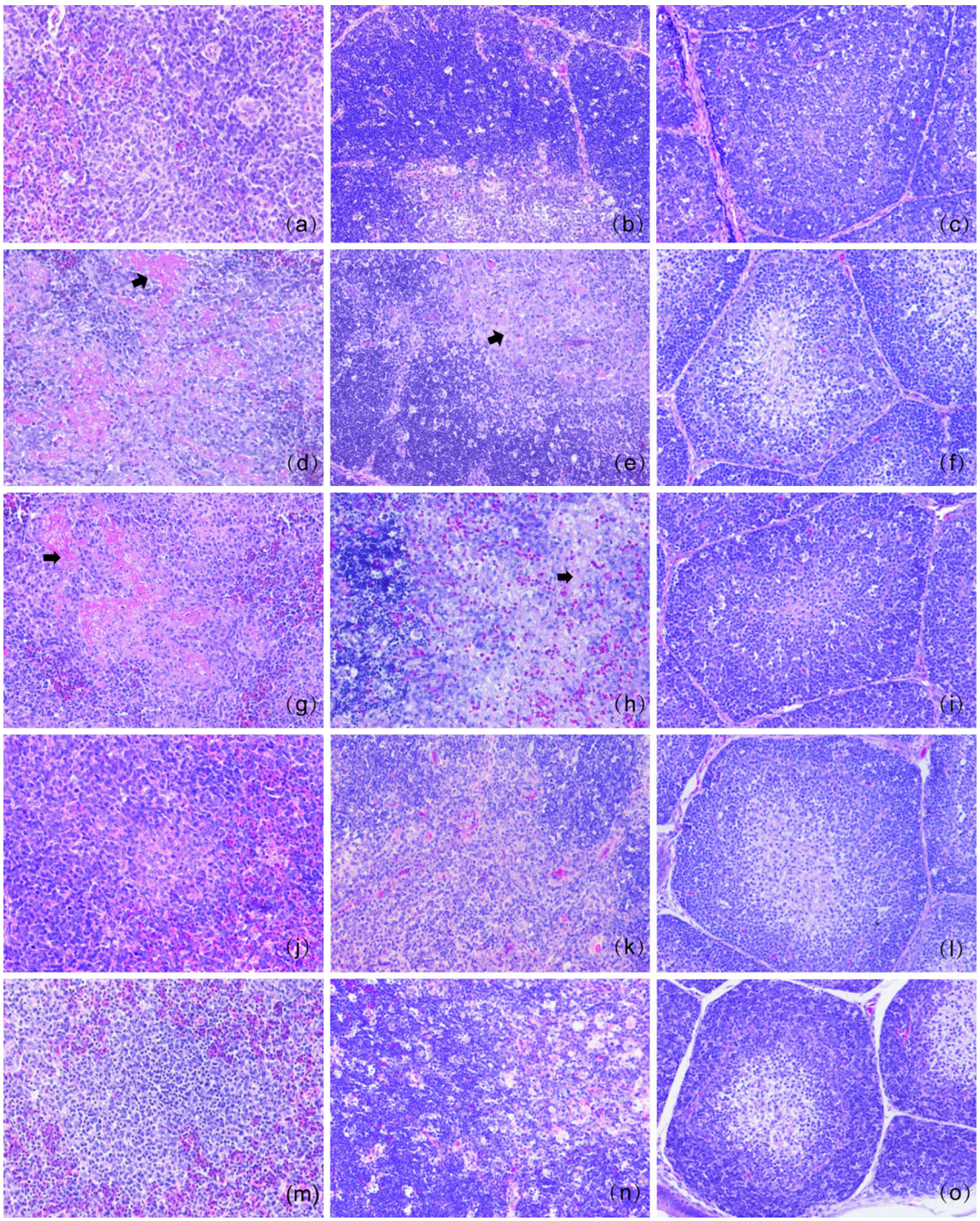

Fig. 2 Histological changes in lymphoid tissues caused by JS3/05, JS5/05, F48E8 and Herts/33. Photomicrographs of tissue sections (×200) in the left, middle and right rows show histological findings in the spleen, thymus and bursa, respectively. (a–c) PBS control. (d–f) JS3/05. (g–i) JS5/05. (j–l) F48E8. (m–o) Herts/33. JS3/05 and JS5/05 produced similar histologic changes, including severe lymphoid depletion and necrosis (arrow) and accumulation of macrophages in the spleen (d and g) and thymus (e and h). F48E8 and Herts/33 induced mild to moderate lymphocyte depletion in the spleen (j and m) and thymus (k and n). All strains caused comparable mild lesions in the bursa (f, i, l and o). No abnormal findings were found in mock-infected chickens (a, b and c)

the thymus, JS3/05 and JS5/05 produced moderate lymphocyte depletion and necrosis and histiocytic accumulation (Fig. 2e and h). F48E8 and Herts/33 infection resulted in similar histologic lesions that were less intense than those with JS5/05 and JS3/05 (Fig. 2k and n). In addition, all viruses caused comparable lesions characterized by

mild to moderate hyperplasia or lymphoid depletion and necrosis mainly in the medullary areas of follicles in the bursa (Fig. 2f, i, l and o). No histologic changes were found in mock-infected chickens (Fig. 2a, b and c).

These results indicate that these four viruses from different genotypes cause similar clinical signs and high mortality in chickens, but they differ in their lesion severity in lymphoid tissues. JS5/05 and JS3/05 produced more-severe tissue lesions in the spleen and thymus when compared to F48E8 and Herts/33.

Virus replication

To understand the basis of the differences in the severity of pathology, we examined virus replication in lymphoid tissues. Only JS3/05 and JS5/05 successfully established an early infection, whereas F48E8 and Herts/33 showed delayed replication. We detected virus replication of JS3/05 and JS5/05 in the spleen at 24 h pi (Fig. 3a), and the presence of JS3/05 in the bursa (12 and 24 h pi) (Fig. 3c). At 48 h pi, JS3/05 and JS5/05 maintained replication in the spleen, thymus and bursa, but replication of F48E8 or Herts/33 was still not detectable in these tissues (Fig. 3). At 72 h pi, virus titers of JS3/05 and JS5/05 were significantly ($p < 0.01$) higher than those of F48E8 and Herts/33 in all organs examined (Fig. 3).

These results suggest that JS3/05 and JS5/05 replicate more efficiently in lymphoid tissues than their two counterparts.

Global transcriptional response in the spleen

To evaluate the contribution of the host response to tissue pathology, we performed microarray analysis on the spleens taken from JS5/05- or Herts/33-infected chickens at 2 dpi. These two strains were selected because pathological data from a previous report [24] and this study show that they induce contrasting pathological changes in the spleen. The transcriptomic data showed that a total of 886 genes exhibited greater than twofold upregulation in JS5/05-affected spleens relative to mock-infected spleens ($p < 0.05$). In contrast, only 674 SDE genes were detected for Herts/33, and 30 % of these genes were downregulated (Fig. 4a). These findings showed that JS5/05 strongly affected the early host transcriptional response. Using the IPA software, we found that the majority of these genes were grouped into the bio-function categories of inflammatory response, cell death, hematological system development and function, and tissue morphology (Fig. 4b). Notably, the largest number of gene expression differences elicited by the two viruses was associated with the inflammatory response. Due to the differences in virus load in the spleens used for microarray, overrepresentation of host genes in the course of JS5/05 infection may reflect the presence of more viral genetic material.

Characterization of inflammatory response based on the microarray data

To analyze the inflammatory response in greater depth, we next analyzed the expression patterns of inflammatory-response-related genes. The expression profiles of selected inflammatory genes are listed in Table 2. The most striking finding was that chickens infected with JS5/05 mounted an overactive type I IFN response compared to those infected with Herts/33. The expression level of IFN-β was greatly increased (177.1-fold) during JS5/05 infection but unchanged during Herts/33 infection. IFN-α induction was much stronger in JS5/05-infected chickens than in the corresponding Herts/33-infected birds. In response to JS5/05 infection, the expression profiles of two IFN-inducible genes, interferon-inducible protein p78 (MX1) and interferon alpha-inducible protein 27-like 2 (IFI27L2), closely followed the relative expression of type I IFNs in chickens infected with both viruses. Moreover, expression values of numerous pro-inflammatory cytokine genes, including IL-6, IFN-γ, IL-1β, IL-18, chemokine (C-C motif) ligand (CCL)-4 and CCL-19, were upregulated to much higher levels by JS5/05 than by Herts/33.

Next, we performed pathway analysis of the SDE genes. We found that the identified pathways were similar between JS5/05 and Herts/33, with significant *p*-values for all the well-known signaling pathways, including triggering receptor expressed on myeloid cells (TREM1) signaling, pattern recognition receptors (PRRs) involved in recognition of bacteria and viruses, JAK-family kinases involved in IL-6-type cytokine signaling, and acute-phase response signaling (Online Resource 1 and 2). The enrichment of genes associated with these pathways suggests that the difference in the pathological manifestation between these two strains is not due to an intrinsic difference in the innate immune response. In contrast, a larger proportion of genes related to the innate immune pathways, such as TREM1 signaling and PRRs involved in recognition of pathogens, were upregulated to higher levels by JS5/05 compared to those regulated by Herts/33 (Table 2). This quantitative increase in the responsiveness of these innate pathways may be indicative of ongoing virus replication of JS5/05, representing a mechanism by which a robust inflammatory response can be perpetuated.

Taken together, these findings suggest that the degree of the innate response may be associated with the severity of tissue damage.

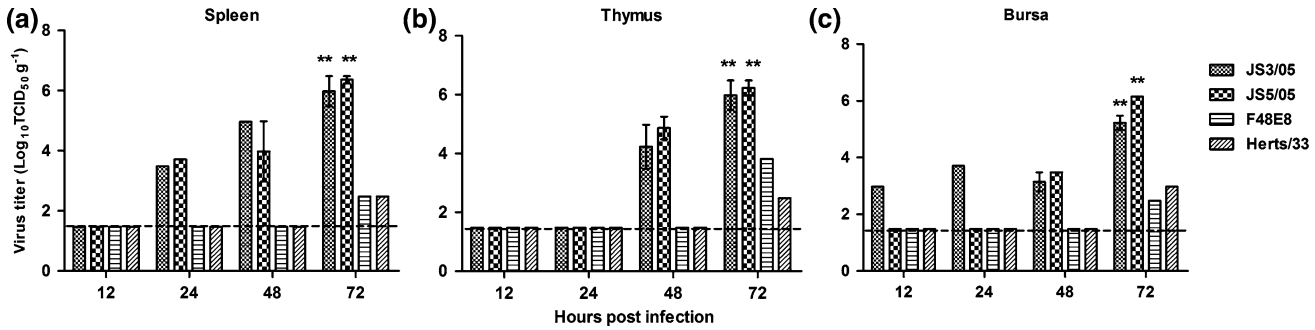

Fig. 3 Virus load in chicken lymphoid tissues. (a) Spleen. (b) Thymus. (c) Bursa. At each time point, three birds were sacrificed, and spleens, thymuses and bursas were collected. Virus titers were determined in CEFs and are presented as average mean titers (log_{10} $TCID_{50}$ g^{-1}). Error bars show the standard error of the mean (SEM). Virus titers were analyzed using one-way ANOVA followed by LSD multiple-comparison test. A double asterisk (**) indicates a significant difference between two genotype VIId NDV isolates (JS3/05 and JS5/05) and F48E8 or Herts/33 ($p < 0.01$). The dotted lines indicate the lower limit of virus detection

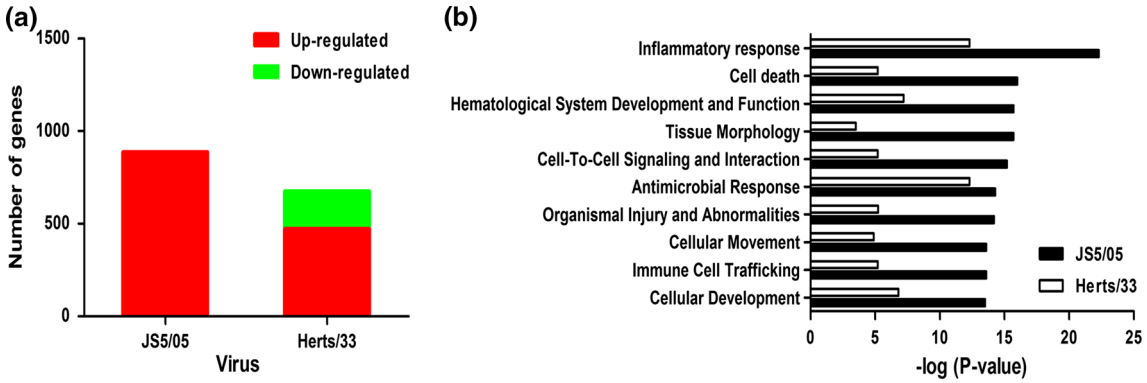

Fig. 4 Differentially expressed genes (SDE) after virus infection and bio-function analysis. (a) Distribution of SDE genes during infection with JS5/05 or Herts/33 relative to mock-infected chickens. (b) Functional categories of differentially regulated genes in the spleen of chickens infected with JS5/05 or Herts/33

Cytokine gene expression in lymphoid tissues

Because microarray data suggest that the inflammatory response is associated with the severity of tissue damage, we next compared the transcriptional cytokine response in lymphoid tissues infected with JS3/05, JS5/05, F48E8 or Herts/33 using RT-qPCR.

Overall, JS3/05 and JS5/05 upregulated expression levels of IFN-β, IL-1β and IL-18 in the spleen and thymus compared to F48E8 and Herts/33 (Fig. 5). In the spleen, expression of cytokine genes in JS3/05- and JS5/05-infected chickens increased at a stepwise pattern and peaked at 48 h pi. In contrast, no marked increase in cytokine gene expression was observed for birds infected with F48E8 or Herts/33 during the detection period. In addition, at the early stage of infection (12 and 24 h pi), mRNA levels of cytokine genes in the thymus were comparable. At 48 h pi, JS5/05 or JS3/05 infection resulted in increased expression of these cytokine genes to higher levels compared to F48E8 or Herts/33. Moreover, these four viruses induced comparable mild cytokine responses in the bursa (Fig. 5).

These results indicate that JS3/05 and JS5/05 trigger a more potent cytokine response in the spleen and thymus compared to F48E8 and Herts/33.

Discussion

Some genotype VIId viruses can produce more-severe tissue destruction in immune organs than other virulent strains, which may suppress the immune response of the poultry flock. We investigated the mechanism of the more-severe damage in the lymphocytic tissues caused by genotype VII NDVs. In this study, we have demonstrated that genotype VIId strains (JS3/05 and JS5/05) caused more-severe lesions in the spleen and thymus than F48E8 and Herts/33. Microarray analysis showed that JS5/05 elicited a more potent inflammatory response by increasing the number and level of activated genes. In addition, JS3/

Table 2 Partial list of inflammatory genes regulated by JS5/05 or Herts/33

Gene symbol	JS5/05		Herts/33		Gene description
	FC[a]	*p*-value	FC	*p*-value	
BCL2A1	4.4	4.31E−03	2.8	6.51E−06	BCL2-related protein A1
CCBP2	2.7	2.01E−02	2.3	2.72E−02	Chemokine binding protein 2
CCL1	3.9	4.77E−03	–[b]	–	Chemokine (C-C motif) ligand 1
CCL19	4.6	3.24E−03	3.7	2.75E−03	Chemokine (C-C motif) ligand 19
CCL20	3.5	8.14E−03	–	–	Chemokine (C-C motif) ligand 20
CCL4	47.9	5.01E−04	4.7	2.47E−02	Chemokine (C-C motif) ligand 4
CSF2RA	3.6	1.77E−04	2.4	1.25E−02	Colony stimulating factor 2 receptor
IFNA	82.5	1.05E−04	10.7	1.52E−02	Interferon alpha
IFNB	177.1	1.80E−03	–	–	Interferon beta
IFNG	32.1	1.06E−06	16.0	5.17E−04	Interferon gamma
IL1B	14.5	1.59E−04	3.6	2.18E−02	Interleukin 1 beta
IL18	9.9	1.75E−04	5.1	9.29E−05	Interleukin 18
IL6	109.3	2.09E−02	25.1	4.21E−02	Interleukin 6
IL6ST	4.0	2.71E−02	3.1	4.41E−02	Interleukin 6 signal transducer
IL8	5.6	8.38E−03	–	–	Interleukin 8
IRF1	3.8	3.62E−04	2.4	1.88E−03	Interferon regulatory factor 1
IRF7	4.3	8.94E−04	3.0	4.53E−03	Interferon regulatory factor 7
LGALS3	10.4	7.54E−03	4.4	2.30E−02	Lectin, galactoside-binding, soluble, 3
MDA5	19.3	1.18E−04	7.2	1.99E−03	Interferon induced with helicase C domain 1
MST1	5.0	5.87E−04	2.5	3.70E−02	Macrophage stimulating 1
MX1	39.4	6.45E−04	29.1	7.78E−04	Interferon-inducible protein p78
IFI27L2	17.8	8.92E−04	10.2	4.80E−03	Interferon alpha-inducible protein 27-like 2
NMI	5.8	7.66E−05	3.9	1.66E−03	N-myc (and STAT) interactor
PLA2G4A	3.5	2.85E−05	2.3	7.44E−04	Phospholipase A2, group IVA
RIPK2	3.3	1.20E−03	2.5	6.56E−03	Receptor-interacting serine-threonine kinase 2
SOCS1	8.0	1.21E−04	4.6	3.03E−03	Suppressor of cytokine signaling 1
SOCS3	8.6	2.94E−04	3.1	1.41E−02	Suppressor of cytokine signaling 3
STAT3	2.8	6.62E−05	–	–	Signal transducer and activator of transcription 3
TLR3	4.7	4.13E−03	2.8	6.49E−03	Toll-like receptor 3
TLR4	2.8	2.05E−04	2.2	6.34E−03	Toll-like receptor 4
TLR15	3.8	3.34E−03	–	–	Toll-like receptor 15

[a] FC: fold change in expression levels relative to those of uninfected chickens

[b] –: the gene was not significantly differentially expressed

05 and JS5/05 replicated at significantly higher levels and elicited a stronger cytokine response in lymphoid tissues when compared to F48E8 and Herts/33. Our findings indicate that the prominent tissue damage in the immune system caused by JS5/05 and JS3/05 is associated with the high virus replication and intense inflammatory response.

Virulent NDV strains usually cause severe clinical disease and high mortality in chickens. However, different virulent NDV strains display distinct histopathologic manifestations. In this study, we determined that four tested strains of different genotypes induced similar clinical signs and high mortality in chickens, whereas two genotype VIId NDV strains, JS5/05 and JS3/05, caused more-severe pathology characterized by marked lymphocyte depletion and necrosis in the spleen and thymus when compared to F48E8 and Herts/33 (Table 1, Fig. 1 and 2). It is notable that viruses of other genotypes, such as genotype V strain CA02, also exhibit the ability to cause severe tissue damage in lymphoid organs [3, 18, 22]. We focused on genotype VIId NDV because this genotype is endemic in Asia, where intense vaccination has already been implemented for decades. However, cross-protection studies have shown that the conventional vaccine La Sota provides poor protection against some genotype VIId NDV isolates [15, 26]. In this case, genotype VIId viruses may have the potential to replicate in poultry flocks vaccinated with the

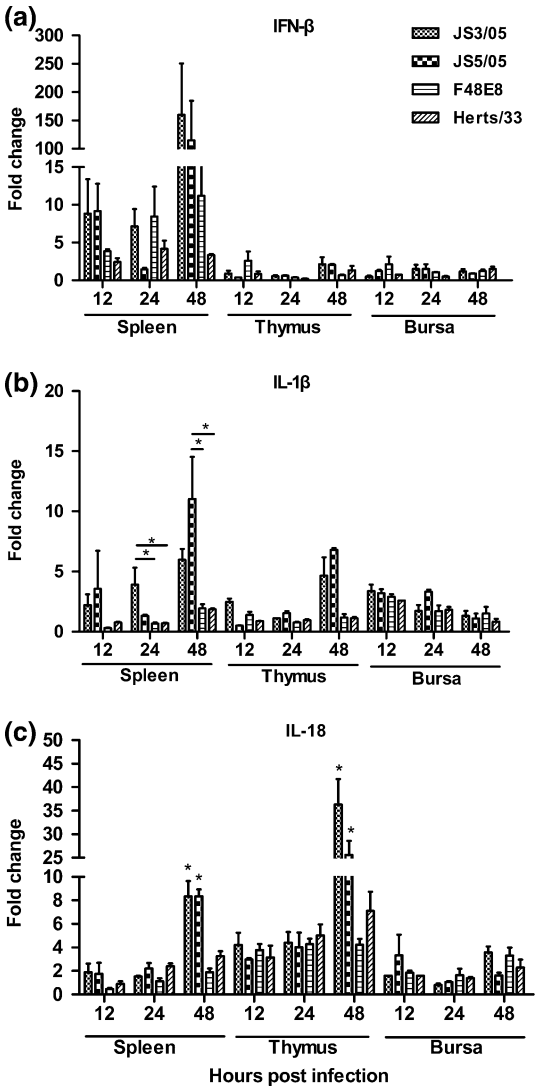

Fig. 5 Expression profiles of representative cytokine genes in lymphoid tissues determined using RT-qPCR. (a) IFN-β. (b) IL-1β. (c) IL-18. The data are the mean fold change ± standard error of the mean (SEM). Expression values of cytokine genes were compared among JS3/05, JS5/05, F48E8 and Herts/33 using one-way ANOVA followed by LSD multiple-comparison test. An asterisk (*) indicates a significant difference at $p < 0.05$

conventional vaccine and suppress the immune response in these birds.

Virus replication is an important determinant of virus pathogenicity. We identified a correlation between the severity of tissue lesions and virus load. The early and sustained replication of JS3/05 and JS5/05 resulted in severe tissue damage characterized by severe lymphocyte depletion and necrosis, whereas the delayed replication of F48E8 and Herts/33 resulted in less pathology (Fig. 3). In lymphoid tissues, a higher rate of NDV replication may result in greater apoptosis and necrosis, which are key causes of histologic changes such as lymphocyte depletion and necrosis [4, 9, 10].

Moreover, the host response also contributes to NDV pathogenicity. Using microarray analysis, we determined that the host response to JS5/05 and Herts/33 had commonalities and differences. Host genes and signaling pathways activated by JS5/05 and Herts/33 were similar (Online Resource 1 and 2), while JS5/05 upregulated more genes related to the innate immune response to higher levels, inducing a more potent inflammatory response compared to Herts/33 (Fig. 4 and Table 2). In addition, RT-qPCR analysis showed that JS3/05 and JS5/05 triggered a stronger cytokine response compared to F48E8 and Herts/33 in the spleen and thymus (Fig. 5). It needs to be pointed out that JS5/05 and JS3/05 had significantly higher virus load in the spleen and thymus than F48E8 and Herts/33 at 48 h pi, when the host response was evaluated in these tissues. Therefore, the difference in the degree of the host response to these two strains correlates to the difference in the efficiency of virus replication in the spleen. However, although the robust host response is not exclusively induced by genotype VII strains, the over-activated host response may exert a critical effect on pathologic manifestations and the disease outcome. Therefore, these results highlight that the degree of the innate immune response to virulent NDVs may be related to the severity of pathology. Moreover, we have previously reported *in vitro* data indicating that JS3/05 and JS5/05 replicate more efficiently and induce a stronger innate immune response and cell death in chicken splenocytes compared to F48E8 and Herts/33, supporting *in vivo* results described in this study [8].

Some recent studies have demonstrated the contribution of the innate immune response to the pathogenicity of NDV [3, 11, 18]. Our results are consistent with these findings. In those studies, NDV strains of varying virulence were used, and the differences in the innate response were mainly attributed to differences in virulence. However, Rasoli et al. have shown that velogenic NDV strains of genotype VII and VIII elicit different lymphocyte responses and cytokine and chemokine profiles in the spleen [16]. Similarly, in this study, we also compared velogenic strains and demonstrated that JS3/05 and JS5/05 induce a more potent cytokine response in lymphoid tissues compared to Herts/33 and F48E8. Therefore, our data, together with those of Rasoli et al., indicate that the level of the host response to virulent NDV strains differs during the course of infection, which may contribute to various disease manifestations. However, different from the GeXP technology applied in the Rasoli et al. study, here, we used microarray analysis to compare the global host response to virulent NDV strains, providing more-comprehensive information on the interaction between the host response and virulent NDV infection.

In conclusion, we have shown that strains JS3/05 and JS5/05, which belong to genotype VIId, cause more-severe

pathology in lymphoid tissues than do strains F48E8 and Herts/33. The high level of virus replication and intense inflammatory response caused by JS3/05 and JS5/05 infection contribute to the severe pathology. Our findings also highlight the fact that differential modulation of the host response by different virulent NDV strains is an important aspect of NDV pathogenesis.

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