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## Infectious Bursal Disease Virus: Strains That Differ in Virulence Differentially Modulate the Innate Immune Response to Infection in the Chicken Bursa

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### ABSTRACT

Little is understood about the immune responses involved in the pathogenesis of infectious bursal disease virus (IBDV). Strains of IBDV differ in their virulence: F52/70 is a classical virulent strain (vIBDV), whereas UK661 is a very virulent strain (vvIBDV) that causes greater pathology and earlier mortality. The exact causes of clinical disease and death are still unclear. Pro-inflammatory cytokines such as interleukin (IL)-1 $\beta$  and IL-6, produced by activated macrophages, could play a role, as could cytokines produced by T and natural killer (NK) cells, such as interferon (IFN)- $\gamma$ , which stimulate macrophages.

We quantified mRNA transcription in bursal tissue, by real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR), for the type I IFN (IFN- $\alpha$  and IFN- $\beta$ ), pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, and CXCLi2), the anti-inflammatory cytokine transforming growth factor (TGF)- $\beta$ 4, and Th1 cytokines (IFN- $\gamma$ , IL-2 [and the closely related IL-15], IL-12, and IL-18) for the first 5 days after infection of 3-week-old chickens with F52/70 or UK661 and compared these with levels in bursal tissue from uninfected age-matched controls.

Both strains induced a pro-inflammatory response, evidenced by increased mRNA transcription of IL-1 $\beta$ , IL-6, and CXCLi2, and down-regulation of TGF- $\beta$ 4, of similar magnitude and timing. IFN- $\gamma$  mRNA was induced by both strains, although to a greater degree by the vvIBDV strain, indicating that a cell-mediated response is induced. Neither virus initially induced high levels of type I IFN. F52/70 seems to use a “stealth” approach by not inducing the type I IFNs, whereas UK661 down-regulates their expression. This suggests that both viruses modulate the host immune response, although probably by using different mechanisms.

### INTRODUCTION

**I**NFECTIONOUS BURSAL DISEASE VIRUS (IBDV), a small nonenveloped member of the family *Birnaviridae*, is

a bisegmented, double-stranded RNA virus encoding only five proteins (20). Serotype 1 IBDV are highly infectious and cause chronic immunosuppression, morbidity in the form of infectious bursal disease (IBD), and in

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some cases mortality in chickens. A range of serotype 1 pathotypes have been reported, which are classified in increasing order of virulence as mild, intermediate, intermediate plus, classical virulent, very virulent or hyper-virulent, and, in North America, variant strains (31). Over the past two decades, Europe and Asia have seen the emergence of very virulent IBDV (vvIBDV).

IBDV replicates mainly in actively dividing B lymphocytes and thereby causing their destruction, primarily in the bursa of Fabricius but also in the spleen and other lymphoid tissues. The acute phase usually lasts no more than 1 week. Virulent strains of IBDV (vIBDV) rarely cause clinical disease in commercial chicks younger than 2 weeks of age but merely a sustained immunosuppression. However, in older chicks, vIBDV can cause an acute disease (19) characterized by extensive lymphoid pathology, morbidity, and frequently death (26,31). In surviving birds the virus is cleared, bursal follicles become repopulated, and some degree of immunocompetence can return (14,5,36). However, the recovered chickens usually have immunosuppression (36) because of the loss of the developing B cell population in the bursa of Fabricius and a severely diminished antibody repertoire.

Infection with vvIBDV, unlike classical vIBDV strains such as F52/70, can break vaccine protection and high titers of maternally derived antibody, resulting in high rates of mortality in young birds (1,7). The pathological changes caused by infection with vvIBDV and the immune responses evoked are less well understood. van den Berg (31) suggested that vvIBDV causes similar disease signs to those of classical vIBDV, with the same incubation time of 4 days but an exacerbated acute phase. This may be the case in chicks with maternal antibodies against IBDV; but in those lacking maternal antibodies, clinical IBD developed more rapidly and with greater severity (35). Immunohistochemical and flow cytometric analyses of bursa, spleen, and thymus after infection with the vvIBDV strain UK661 revealed discrete distinctions compared to vIBDV. Lymphocytes expressing the B-cell marker Bu-1<sup>+</sup> and immunoglobulin (Ig)M or IgG were all depleted from the bursa, spleen, and thymus, suggesting loss of both immature and mature B lymphocytes. Interestingly, small numbers of Bu-1<sup>+</sup> cells repopulated the bursa after 14 days post-infection (dpi); but few of these expressed IgM or IgG (34).

The mechanisms underlying the pathology caused by vIBDV and those relating to immune clearance of the virus have begun to be elucidated. However, little is understood about the role of cytokines. All immune cells can produce cytokines, and therefore each has the potential to influence responses to IBDV. Pro-inflammatory cytokines such as IL-1 $\beta$  and IL-6, produced by activated macrophages, could play a role. However, there may also be a role for cytokines produced by T and natural killer (NK) cells, such as interferon- $\gamma$  (IFN- $\gamma$ ), that stimulate macrophages.

Kim et al. (13) showed that splenic macrophages enhanced expression of type I IFN, chicken myelomonocytic growth factor (MGF, incorrectly described as a chicken homologue of IL-6), and IL-8 after vIBDV infection. Ragland et al. (23) reported that vIBDV infection suppresses transcription of both IFN- $\alpha$  and IFN- $\gamma$  in peripheral blood lymphocytes. By contrast, IFN- $\gamma$  expression was increased in the bursa of Fabricius after infection with vIBDV from 2–5 dpi (24).

We have developed a panel of real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR) assays to quantify the expression of a wide variety of chicken cytokines and chemokines (10,11,12,16,17,22,28). These include the type I IFNs, the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, and CXCLi2 (we recently proposed a new nomenclature for the chicken chemokines (9), and CXCLi2 was previously known as IL-8); the Th1 cytokines IL-2 (and the closely related IL-15), IL-12 $\alpha$ , IL-12 $\beta$ , IL-18 and IFN- $\gamma$ , and the anti-inflammatory cytokine TGF- $\beta$ 4. In this study, we assessed the production of these cytokines in the bursa of Fabricius during the course of infection with both the benchmark vIBDV strain, F52/70, and the benchmark vvIBDV strain, UK661. The aim was to determine which cytokines are produced at the main site of infection, to provide an indication of the type or types of immune response induced, and also to test the hypothesis that strains of IBDV with differing virulence induce different cytokine profiles during the course of infection.

## MATERIALS AND METHODS

**Chickens.** Rhode Island Red (RIR) chicks were obtained from an unvaccinated flock maintained in isolation accommodation at the Institute for Animal Health (Compton, U.K.). The parents were confirmed to be free of antibodies to IBDV, chicken infectious anemia virus, Marek's disease virus, reovirus and a number of other pathogens, so the chicks used in these experiments were deemed to be free of maternal antibodies against IBDV. The experiments met with local ethical guidelines as well as those of the U.K. Home Office.

**Virus.** The vIBDV strain F52/70 (6) and the very virulent IBDV isolate UK661 (3) were used. The titers of both virus stocks were kindly determined by Dr. Adriaan van Loon (Intervet BV, Boxmeer, The Netherlands) as previously described (32). Based on earlier studies using these virus stocks, the following doses of 10<sup>1.7</sup> EID<sub>50</sub> vIBDV strain F52/70 and 10<sup>1.3</sup> EID<sub>50</sub> vvIBDV strain UK661 were selected to cause the same degree of clinical disease and bursal damage, measured as the bursal lesion score in RIR chicks 2–3 weeks of age (27,35).

**Experimental design.** At 3 weeks of age, chicks were selected at random and placed into two groups designated as the infected group and the control group. Because of

## CYTOKINE PRODUCTION DURING IBDV INFECTION

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TABLE 1. REAL-TIME QUANTITATIVE RT-PCR PROBES AND PRIMERS

| <i>RNA Target</i>     |                | <i>Probe/primer sequence</i>                       | <i>Accession no.<sup>a</sup></i> | <i>Exon boundaries</i> |
|-----------------------|----------------|--|----------------------------------|------------------------|
| 28S                   | Probe          | (FAM) -AGGACCGCTACGGACCTCCACCA- (TAMRA)            | X59733                           | —                      |
|                       | P <sup>b</sup> | GGCGAAGCCAGAGGAAACT                                |                                  |                        |
|                       | P <sup>c</sup> | GACGACCGATTTGCACGTC                                |                                  |                        |
| IFN- $\alpha$         | Probe          | (FAM) -CTCAACCGGATCCACCGCTACACC- (TAMRA)           | U07868                           | —                      |
|                       | F              | GACAGCCAACGCCAAAGC                                 |                                  |                        |
|                       | R              | GTCGCTGCTGTCCAAGCATT                               |                                  |                        |
| IFN- $\beta$          | Probe          | (FAM) -TTAGCAGCCCACACACTCCAAAACACTG- (TAMRA)       | X92479                           | —                      |
|                       | F              | CCTCCAACACCTCTTCAACATG                             |                                  |                        |
|                       | R              | TGGCGTGCGGTCAAT                                    |                                  |                        |
| IFN- $\gamma$         | Probe          | (FAM) -TGGCCAAGCTCCCGATGAACGA- (TAMRA)             | Y07922                           | 3/4                    |
|                       | F              | GTGAAGAAGGTGAAAGATATCATGGA                         |                                  |                        |
|                       | R              | GCTTTGCGCTGGATTCTCA                                |                                  |                        |
| IL-1 $\beta$          | Probe          | (FAM) -CCACACTGCAGCTGGAGGAAGCC- (TAMRA)            | AJ245728                         | 5/6                    |
|                       | F              | GCTCTACATGTCGTGTGTGATGAG                           |                                  |                        |
|                       | R              | TGTCGATGTCCCGCATGA                                 |                                  |                        |
| IL-2                  | Probe          | (FAM) -ACTGAGACCCAGGAGTGCACCCAGC- (TAMRA)          | AJ009800                         | 2/3                    |
|                       | F              | TTGGAAAATATCAAGAACAAGATTCATC                       |                                  |                        |
|                       | R              | TCCCAGGTAACACTGCAGAGTTT                            |                                  |                        |
| IL-6                  | Probe          | (FAM) -AGGAGAAATGCCTGACGAAGCTCTCCA- (TAMRA)        | AJ309540                         | 4/5                    |
|                       | F              | GCTCGCCGGCTTCGA                                    |                                  |                        |
|                       | R              | GGTAGGTCTGAAAGGCCGAACAG                            |                                  |                        |
| IL-12 $\alpha$        | Probe          | (FAM) -CCAGCGTCCTCTGCTTCTGCCACCTT- (TAMRA)         | AY262751                         | 1/2                    |
|                       | F              | TGGCCGCTGCAAACG                                    |                                  |                        |
|                       | R              | ACCTCTTCAAGGGTGCACCTCA                             |                                  |                        |
| IL-12 $\beta$         | Probe          | (FAM) -CTGAAAAGCTATAAAGAGCCAAGCAAGACGTTCT- (TAMRA) | AJ564201                         | 1/2                    |
|                       | F              | TGGGCAAATGATACGGTCAA                               |                                  |                        |
|                       | R              | CAGAGTAGTTCTTTGCCTCACATTTT                         |                                  |                        |
| IL-15                 | Probe          | (FAM) -AAGTTGCAAATCTTGCATTTCCATTTTCCA- (TAMRA)     | AJ416937                         | 4/5                    |
|                       | F              | TAGGAAGCATGATGTACGGAACAT                           |                                  |                        |
|                       | R              | TTTTTGCTGTTGTGGAATTCAACT                           |                                  |                        |
| IL-18                 | Probe          | (FAM) -CCGCGCCTTCAGCAGGGATG- (TAMRA)               | AJ276026                         | 4/5                    |
|                       | F              | AGGTGAAATCTGGCAGTGGAAAT                            |                                  |                        |
|                       | R              | ACCTGGACGCTGAATGCAA                                |                                  |                        |
| CXCLi2                | Probe          | (FAM) -TCTTTACCAGCGTCTACCTTGCGACA- (TAMRA)         | AJ009800                         | 1/2                    |
|                       | F              | GCCCTCCTCCTGGTTTCAG                                |                                  |                        |
|                       | R              | TGGCACCGCAGCTCATT                                  |                                  |                        |
| TGF- $\beta$ 4        | Probe          | (FAM) -ACCCAAAGGTTATATGGCCAACCTTCTGCAT- (TAMRA)    | M31160                           | 6/7                    |
|                       | F              | AGGATCTGCAGTGGAAAGTGGAT                            |                                  |                        |
|                       | R              | CCCCGGTTGTGTTGGT                                   |                                  |                        |
| IBDV<br>F52/70<br>VP2 | Probe          | (FAM) -TCCCCTGAAGATTGCAGGAGCATTTG- (TAMRA)         | D00869                           | —                      |
|                       | F              | GAGGTGGCCGACCTCAACT                                |                                  |                        |
|                       | R              | AGCCCGGATTATGTCTTTGAAG                             |                                  |                        |
| IBDV<br>UK661<br>VP2  | Probe          | (FAM) -AGCAGCAGCCAACGTGGACCC- (TAMRA)              | X92760                           | —                      |
|                       | F              | ACTCGAGAGCGCCGTCAG                                 |                                  |                        |
|                       | R              | CTGAGCGCAGATTGGAACAG                               |                                  |                        |

<sup>a</sup>Genomic DNA sequence.<sup>b</sup>Forward.<sup>c</sup>Reverse.

the need for contemporaneous sampling of both control and infected chickens, infections with the different viruses were carried out in two separate experiments. In each case, infected birds were housed together in cages in the same filtered-air, positive-pressure isolation room. In the first experiment, birds were infected with  $10^{1.7}$  EID<sub>50</sub> strain F52/70 in a total volume of 100  $\mu$ L by the intranasal route (50  $\mu$ L per nostril). In the second experiment, birds were infected with  $10^{1.3}$  EID<sub>50</sub> strain UK661 in the same volume by the same route. Controls were kept together but in a separate isolation room. Bursal tissue from five infected and three controls was removed into RNALater (Ambion, Huntington, U.K.) at 24 h post-challenge and at 12 h intervals for 3 days thereafter. Samples were stored in RNALater at  $-70^{\circ}\text{C}$  until further processing.

**Sample processing.** Bursal tissue (20–60 mg) was homogenized and total RNA was then prepared using the RNeasy mini kit (Qiagen, Crawley, U.K.), following the manufacturer's instructions. Purified RNA was eluted in 50  $\mu$ L RNase-free water and stored at  $-70^{\circ}\text{C}$ .

**Detection and quantification of cytokine mRNA.** Cytokine mRNA levels in infected and control samples were quantified using a well-described method (10,11,12,16,17,22,28). IBDV RNA (genome) in infected and control samples was also quantified. Triplicate samples were assayed for each experiment, and each sample was also assayed in triplicate.

Primers and probes were designed using the Primer Express software program (Applied Biosystems, Foster City, CA); details are given in Table 1. For all cytokines, either a primer or probe was designed from the sequence of the relevant genes to lie across intron:exon boundaries. All probes were labeled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5' end and the quencher N, N, N, N'-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end.

Real-time quantitative RT-PCR was performed using the Reverse Transcriptase qPCR Master Mix RT-PCR kit (Eurogentec, Seraing, Belgium). Amplification and detection of specific products were performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with the following cycle profile: one cycle of  $50^{\circ}\text{C}$  for 2 min,  $60^{\circ}\text{C}$  for 30 min, and  $95^{\circ}\text{C}$  for 5 min; and 40 cycles of  $94^{\circ}\text{C}$  for 20 sec and  $59^{\circ}\text{C}$  for 1 min. Quantification was based on the increased fluorescence detected based on hydrolysis of the target-specific probes by the 5'-exonuclease activity of the *rTth* DNA polymerase during PCR amplification. The passive reference dye 6-carboxy-rhodamine, which is not involved in amplification, was used for normalization of the reporter signal. Results are expressed in terms of the threshold cycle value (Ct), the cycle at which the change in the reporter dye passes a significance threshold ( $\Delta R_n$ ).

To account for variation in sampling and RNA preparation, the Ct values for cytokine-specific product for each

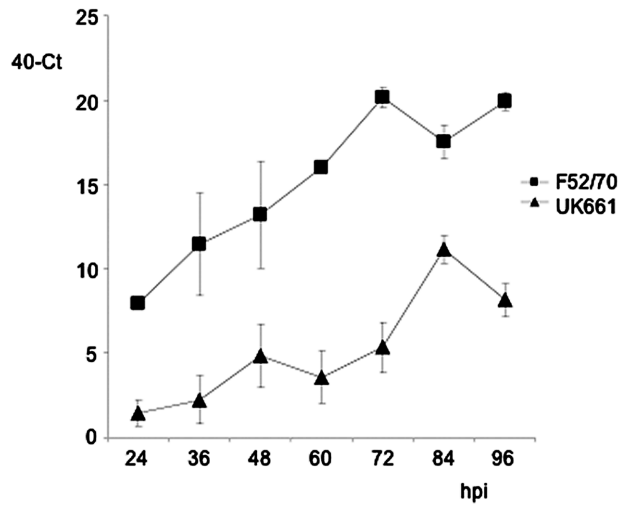
sample were standardized using the Ct value of 28S rRNA product for the same sample. To normalize RNA levels between samples within an experiment, the mean Ct value for 28S rRNA-specific product was calculated by pooling values from all samples in that experiment. Tube-to-tube variations in 28S rRNA Ct values about the experimental mean were calculated. The slope of the 28S rRNA  $\log_{10}$  dilution series regression line was used to calculate differences in input total RNA. Using the slopes of the respective cytokine or IBDV  $\log_{10}$  dilution series regression lines, the difference in input total RNA, as represented by the 28S rRNA, was then used to adjust cytokine-specific Ct values, as follows: Corrected Ct value =  $\text{Ct} + (\text{Nt}-\text{Ct}') * \text{S}/\text{S}'$ , where Ct = mean sample Ct, Nt = experimental 28S mean, Ct' = mean 28S of sample, S = cytokine / IBDV slope, and S' = 28S slope. Results were then expressed as 40-Ct values.

**Statistical analysis.** Analysis of mean values between multiple groups was carried out using one-way analysis of variance. Where a level of significance was found, a Tukey test was then conducted on the sample data to determine the relative difference between the means within each set of data. When there were only two groups of data, means were compared using a paired Student *t* test.

## RESULTS

**Changes in IBDV load in the bursa during the course of infection.** After infection with either vIBDV F52/70 or vvIBDV UK661, viral load (Fig. 1) increased, reaching a maximum at 72 h post-infection (hpi) in the case of F52/70 and 84 hpi in the case of vvIBDV. For vIBDV F52/70 infection, viral load was constant between 72 and 96 hpi, whereas for vvIBDV UK661 infection, viral load had decreased by 96 hpi. Viral load in bursal tissue was greater at all time points after infection with vIBDV F52/70 infection ( $10^{1.7}$  EID<sub>50</sub>) compared to infection with vvIBDV UK661 ( $10^{1.3}$  EID<sub>50</sub>).

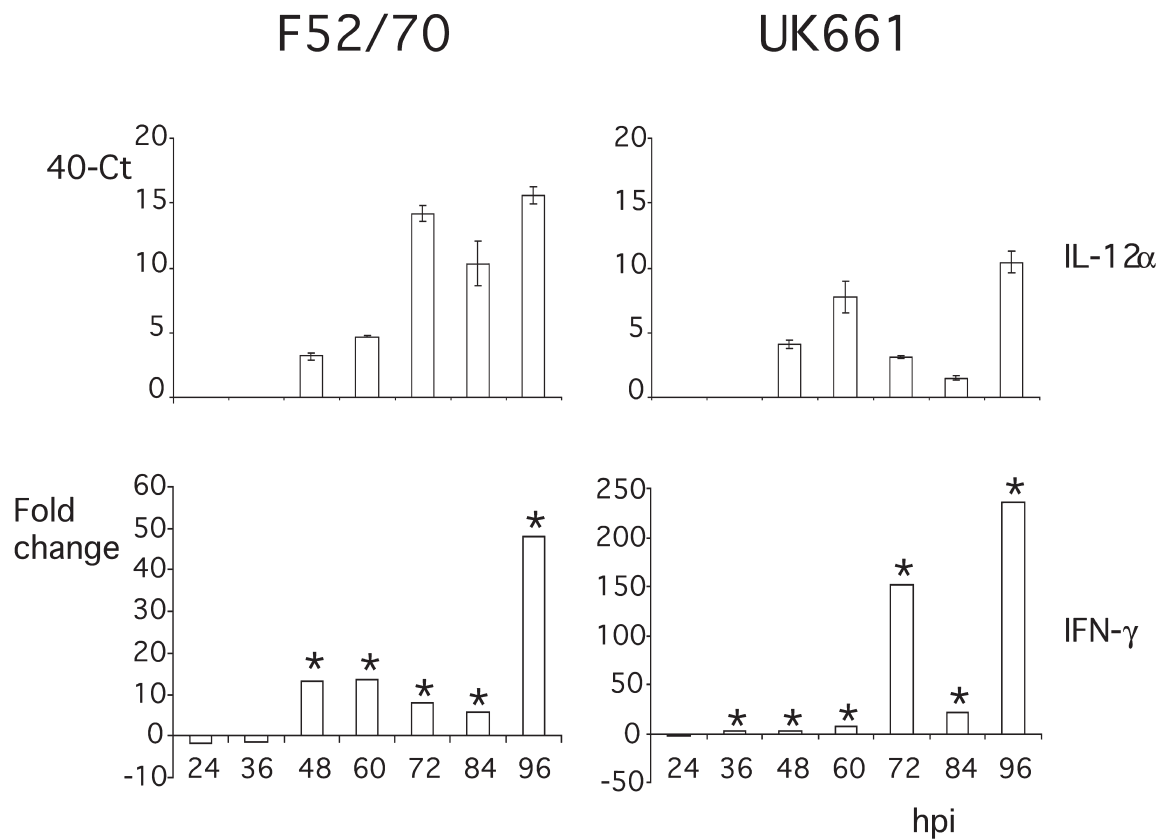
**IBDV infection induces IFN- $\gamma$  expression in the bursa of Fabricius, which increases during the course of infection.** IL-2 mRNA expression was not detectable in bursal tissue from either the infected chicks or the uninfected controls. IL-15 mRNA expression (data not shown) was essentially unaltered in bursal tissue from infected birds compared to controls. IFN- $\gamma$  mRNA expression (Fig. 2) was up-regulated in bursal tissue from infected chicks compared to levels in controls, from 36 hpi (UK661) and 48 hpi (F52/70) for the duration of the experiment. Interestingly, higher levels of IFN- $\gamma$  mRNA were detected for UK661 infection (note different scale) than F52/70 infection. However, this increased IFN- $\gamma$  mRNA expression was not driven by increased expression of IL-12 $\beta$  or IL-18 mRNA in bursal tissue, as levels in infected birds were not significantly increased above those in controls (data not shown). However, IL-12 $\alpha$  mRNA expression was up-regulated in



**FIG. 1.** Bursal load of infectious bursal disease virus (IBDV) (40-Ct) at various times after infection with either virulent (F52/70) or very virulent (UK661) IBDV. hpi = hours post-infection.

bursal tissue from infected chicks from 48 hpi for the duration of the experiment with both strains of virus (Fig. 2). IL-12 $\alpha$  mRNA expression was not detected in bursal tissue from control birds, and therefore these data are presented as 40-Ct values and not as fold change.

**Changes in pro- and anti-inflammatory cytokine mRNA levels during the course of infection indicate an inflammatory response occurs in the bursa of Fabricius.** After infection with both vIBDV F52/70 and vvIBDV UK661, there was an increase in expression in bursal tissue of mRNA for the pro-inflammatory cytokines IL-1 $\beta$  and IL-6 and the pro-inflammatory chemokine CXCLi2 (Fig. 3), indicating that IBDV infection results in an inflammatory response in the bursa. The magnitude of this response was similar for both strains of virus. Consistent with this, expression of the anti-inflammatory cytokine TGF- $\beta$ 4 was down-regulated in bursal tissue from infected birds compared to uninfected controls, from 48–60 hpi (Fig. 3), and to a greater degree after F52/70 infection than UK661 infection.

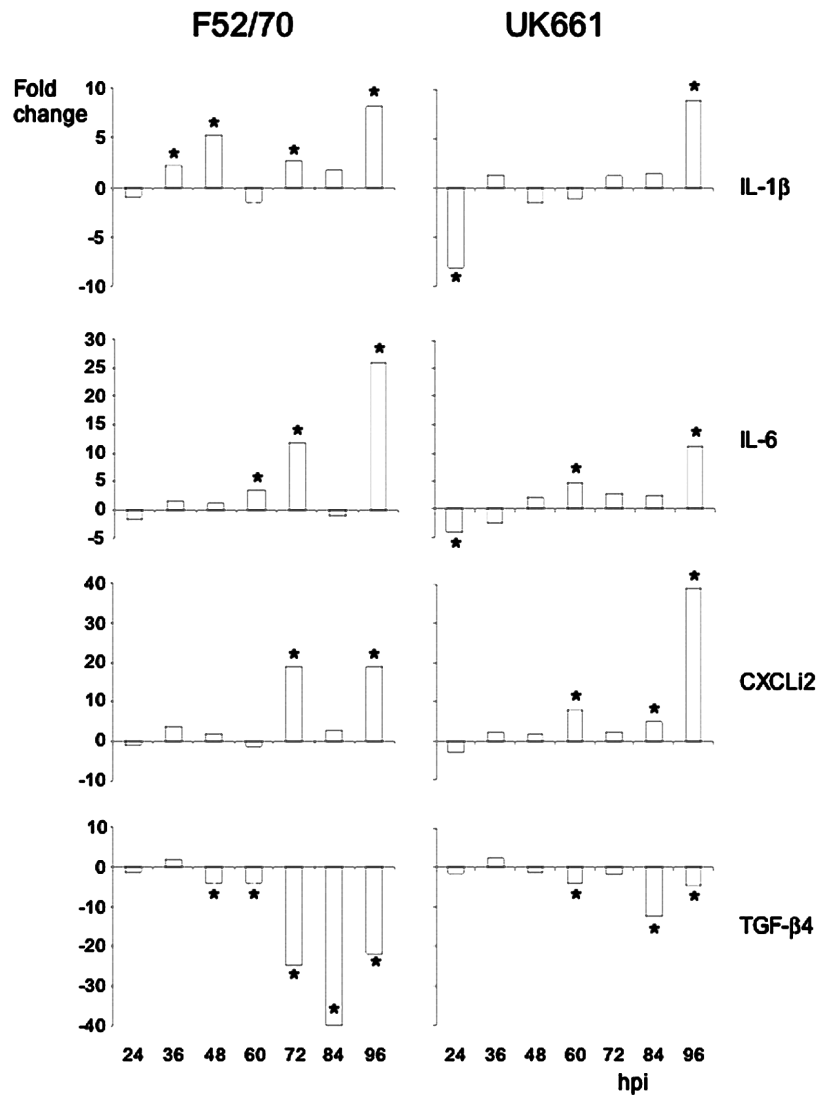


**FIG. 2.** Quantification of interleukin (IL)-12 $\alpha$  and interferon (IFN)- $\gamma$  in bursal cells after infectious bursal disease virus (IBDV) infection, expressed as 40-Ct (IL-12 $\alpha$ ) or fold-change in cytokine mRNA levels in infected birds, compared to those from age-matched, uninfected controls (IFN- $\gamma$ ). hpi = hours post-infection. \*Statistically significantly different from uninfected controls at  $p < 0.05$ .

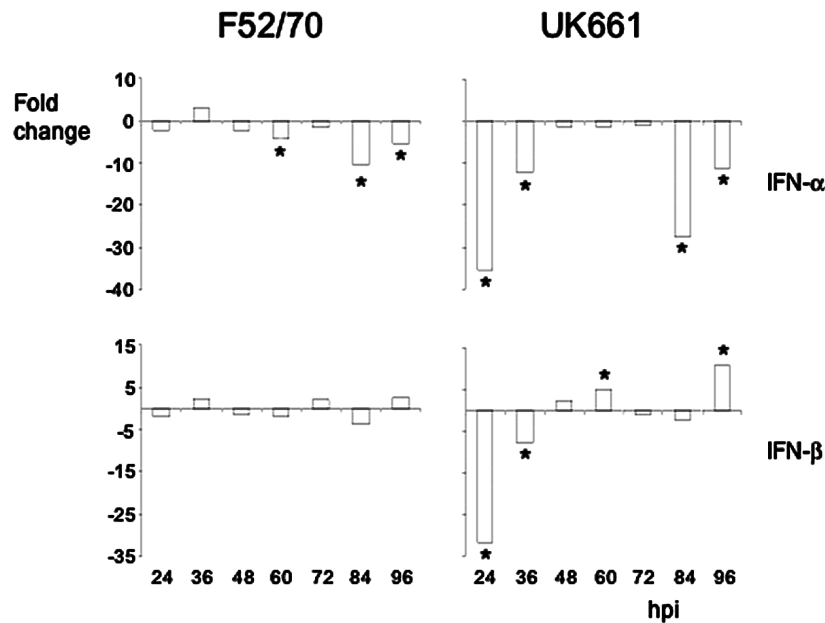
**Strains of IBDV of different virulence modulate the immune response in the bursa of Fabricius by either not inducing or down-regulating type I IFN mRNA expression.** Levels of IFN- $\alpha$  mRNA in bursal tissue from birds infected with vIBDV F52/70 did not differ significantly from levels in uninfected controls for the first 48 hpi. At 60 hpi there was a small but significant ( $p < 0.05$ ) decrease and later decreases at 84 and 96 hpi (Fig. 4). There were no significant changes in the levels of IFN- $\beta$  after infection with vIBDV F52/70. After vvIBDV UK661 infection, expression of both type I IFNs was initially down-regulated but then returned to background levels. In the case of IFN- $\alpha$ , there was again down-regulation from 84 hpi onward, whereas for IFN- $\beta$  there was evidence of significant ( $p < 0.05$ ) up-regulation compared to uninfected controls at 60 and 96 hpi.

## DISCUSSION

IBDV infects chickens by the feco-oral route and initially may replicate in cells of the gut-associated lymphoid tissues (33), although this has yet to be confirmed. IBDV infects bursal B lymphocytes and replication is accompanied by an influx of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (29,14,15), which are activated and express increased levels of an IL-6-like factor and nitric oxide-inducing factor (15). Rodenberg et al. (25) studied changes in populations of lymphocyte subsets within the blood, bursa, spleen, and thymus and found that although the number of IgM<sup>+</sup> cells in the bursa and spleen decreased significantly, the relative proportions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells did not change. Immunohistochemical analysis of chicken lymphoid tissue, after infec-



**FIG. 3.** Quantification of pro-inflammatory (interleukin [IL]-1 $\beta$ , IL-6, and CXCLi2) and anti-inflammatory (transforming growth factor [TGF]- $\beta$ 4) cytokines in bursal cells after infectious bursal disease virus (IBDV) infection, expressed as fold-change in cytokine mRNA levels in infected birds, when compared to those from age-matched, uninfected controls. hpi = hours post-infection. \*Statistically significantly different from uninfected controls at  $p < 0.05$ .



**FIG. 4.** Quantification of type I interferons (IFNs) in bursal cells after infectious bursal disease virus (IBDV) infection, expressed as fold-change in cytokine mRNA levels in infected birds, when compared to those from age-matched, uninfected controls. hpi = hours post-infection. \*Statistically significantly different from uninfected controls at  $p < 0.05$ .

tion with vIBDV, has been used to determine the location of the virus and various leukocyte subsets (21,30,33,36). These studies all indicated an influx of T cells into the bursa, some expressing the activation marker CD25 (IL-2R) (15). Williams and Davison (35) observed a transient influx of macrophages and an influx in CD3<sup>+</sup>, CD4<sup>+</sup>, or CD8<sup>+</sup> lymphocytes into the bursa after infection with vvIBDV. These extended perturbations in CD3<sup>+</sup>, CD4<sup>+</sup> or CD8<sup>+</sup> populations in the spleen and thymus suggest that vvIBDV caused more severe and extensive changes than vIBDV.

The spread of vIBDV and vvIBDV within the bursa is rapid. By 13 hpi, most bursal follicles are positive for the virus; and by 16 hpi, a second and pronounced viremia occurs, with secondary replication in other organs, leading to clinical signs and sometimes death in chicks 3 weeks or more of age. The exact cause of clinical disease and death is still unclear.

Here we show that viral load in bursal tissue increased more (between approximately 100- and 1000-fold) throughout the course of infection after inoculation with the vIBDV, F52/70, than with the vvIBDV, UK661. Because UK661 is much more pathogenic than F52/70, the infecting doses of the two IBDV strains had been selected to cause a similar time course of pathogenesis, similar clinical signs, and the same degree of bursal pathology, measured as bursal lesion score (27,35). Earlier work (34) clearly showed that an inoculum containing  $10^{1.7}$  EID<sub>50</sub> UK661 would have resulted in all chicks reaching clinical endpoints by 48 hpi. Therefore, in this experiment chicks were inoculated with  $10^{1.7}$  EID<sub>50</sub> vIBDV strain F52/70 but

only  $10^{1.3}$  EID<sub>50</sub> vvIBDV strain UK661. These data indicate that a higher bursal viral load for vIBDV is necessary to achieve the same degree of clinical disease. It would be useful in the future, however, to compare exactly matched doses to investigate why vvIBDV causes such a marked pathology and early mortality.

As expected, IBDV infection caused a pro-inflammatory cytokine response in the bursa, as evidenced (Fig. 3) by the up-regulation of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, and CXCLi2, and the down-regulation of the anti-inflammatory cytokine TGF- $\beta$ 4. The pro-inflammatory cytokines were induced to similar levels in bursal tissues from birds infected with either virus, whereas F52/70 infection down-regulated TGF- $\beta$ 4 expression to a greater degree than UK661 infection, although this could be related to the higher infecting dose and higher level of IBDV in the bursa.

The observed increase in IFN- $\gamma$  expression (Fig. 2) in infected bursae presumably reflects this inflammatory response and is consistent with earlier published results (8,24), again suggesting that cell-mediated responses are initiated to resolve the infection. The IFN- $\gamma$  up-regulation was presumably driven by IL-12, inasmuch as IL-12 $\alpha$  mRNA was detectable only in bursal tissue from infected birds, whereas IL-12 $\beta$  mRNA was constitutively expressed in all bursal tissues and did not increase after infection with either viral strain. IL-18 (IFN- $\gamma$ -inducing factor) does not seem to play a role, as levels in infected birds were not significantly increased above those in controls (data not shown). Despite the lower infecting dose



and bursal viral load of vvIBDV, UK661 induced far greater expression of IFN- $\gamma$  than infection with F52/70 (~230-fold increase as opposed to a 50-fold increase at 96 hpi), suggesting that UK661 infection induces a stronger cell-mediated inflammatory response.

The lack of increase in type I IFN mRNA in the bursa after IBDV infection was unexpected. Gelb et al. (8) reported that both attenuated and pathogenic strains of IBDV stimulated the production of an antiviral factor, assumed to be IFN, in the bursa of 4-week-old chicks. Although antiviral activity is usually associated with increased levels of the type I IFNs (i.e., IFN- $\alpha$  and IFN- $\beta$ ), there was an increase in IFN- $\beta$  mRNA only at 60 and 96 hpi (Fig. 4). However, chicken IFN- $\gamma$  also has antiviral activity (18).

Our results suggest that IBDV tends to have down-regulatory effects on the type I IFN response. It seems that the two IBDV viruses interfere in the type I response in different ways (Fig. 4). After infection with F52/70, IFN- $\alpha$  mRNA is initially not significantly altered from levels in age-matched uninfected controls, and then levels of IFN- $\alpha$  mRNA are down-regulated from 60 hpi. Expression of IFN- $\beta$  mRNA is not significantly altered from levels in age-matched uninfected controls at any time after infection. After UK661 infection, IFN- $\alpha$  mRNA is initially down-regulated until at least 36 hpi from levels in age-matched uninfected controls. Levels then recover to those in controls, followed by a second down-regulation from 84 hpi onward. IFN- $\beta$  mRNA again is significantly down-regulated from levels in age-matched uninfected controls until at least 36 hpi, but levels thereafter are either the same as in controls or up-regulated. It is clear from these results that infection with F52/70 results initially in the noninduction of type I IFNs, suggestive of a "stealth" approach that allows viral infection to be established. Infection with UK661 initially actively down-regulates type I IFN expression, suggesting active manipulation of the host immune response. This could provide a selective advantage for the vvIBDV strain UK661. The sequence of both F52/70 and UK661 has been fully determined (2,4). However, it is not obvious from the known sequence differences what is responsible for the observed differential regulation of the type I IFN response, or which IBDV protein functions as an immunomodulator, although VP5, which is expressed in IBDV-infected cells and is not essential for viral replication in cell culture (20), may be a candidate.

These results indicate that infection with IBDV induces a pro-inflammatory cytokine response and increases in IFN- $\gamma$ . It was clear that despite the lower infective dose of vvIBDV, strain UK661 induced a stronger IFN- $\gamma$  response. This could in turn drive the cytokine storm or "septic shock syndrome," suggested by van den Berg (31) as a potential killing mechanism of vvIBDV, driving the production of apoptotic mediators such as nitric oxide or tumor necrosis factor- $\alpha$  by macrophages.

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