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In vitro rapid clearance of infectious bursal disease virus in peripheral blood mononuclear cells of chicken lines divergent for antibody response might be related to the enhanced expression of proinflammatory cytokines

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ABSTRACT

Infectious bursal disease (IBD) is an acute and highly contagious viral disease of young chickens caused by infectious bursal disease virus (IBDV). An effective way to control IBDV would be to breed chickens with a reduced susceptibility to IBDV infection. In the present work, we used chickens selected for high and low specific responses to sheep red blood cells (SRBC) (H and L, respectively) to assess the susceptibility of differential immune competent animals to IBDV infection. The peripheral blood mononuclear cells (PBMCs) of high SRBC line (HL) and low SRBC line (LL) were infected with IBDV and viral RNA loads were determined at different time post-IBDV infection. Chicken orthologues of the T helper 1 (Th1) cytokines, interferon-γ (IFN-γ) and interleukin-2 (IL-2); a Th2 cytokine, IL-10; a pro inflammatory cytokine, IL-6; the CCL chemokines, chCCLi2, chCCLi4 and chCCLi7; colony stimulating factor, GM-CSF; and a anti-inflammatory cytokine, transforming growth factor β-2 (TGFβ-2) were quantified. The expression of chCCLi2, chCCLi4 and chCCLi7 was significantly higher in L line as compared to H line. However, in H line the viral RNA loads were significantly lower than in L line. Therefore, the upregulated chemokines might be associated with the susceptibility to IBDV. The expression of IFN-γ, IL-2 and IL-6 was significantly higher in H line as compared to L line. We assume that the higher proinflammatory cytokines expression in H line might be related to the rapid clearance of virus from PBMCs. Significantly higher levels of IL-10 and TGFβ-2 mRNAs in L line might be related to the pathogenesis of IBDV. In conclusion, selection for antibody responses appears to influence the expression profiles of chemokines and cytokines against IBDV. Further, the selection for high SRBC response might improve the immuno-competence of chickens against IBDV.
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1. Introduction

Poultry farming has established as an industry in India and registered a phenomenal growth during last 3–4 decades. As a result, India ranks fourth largest producer of poultry eggs and fifth largest producer of poultry meat in the world. Worldwide poultry is producing 40% of consumed animal products. However, diseases are one of the major threats inflicting great economic losses to poultry industry. It is therefore important that poultry production and products processing should be free from pathogenic organisms. Infectious bursal disease (IBD) (Gumboro disease) has constituted a serious problem for the poultry industry. It is an acute and highly contagious viral disease of young chicken, caused by infectious bursal disease virus (IBDV) (Muller et al., 1979). IBDV is a non-enveloped, double stranded RNA virus that has bi-segmented genome, segment A (3.2 kb) and B (2.9 kb), encoding five proteins and belongs to the genus *Avibirnavirus* of family *Birnaviridae*, which specifically targets dividing IgM-bearing B cells, especially those in the bursa of Fabricius and other immune organs (Hirai et al., 1981; Rodenberg et al., 1994). Two serotypes of IBDV have been identified but only strains of serotype 1 are pathogenic for chickens (McFerran et al., 1980; Lukert and Saif, 1997). Based on virulence, serotype 1 strains are classified as classical, intermediate, virulent and very or hyper virulent (van den Berg et al., 2000).

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Direct losses of poultry are linked to specific mortality because of recent 're-emergence' of the IBDV in the form of antigenic variants and hypervirulent strains. The indirect economic impact of the disease is also considerable, due to virus-induced immunosuppression and/or potential interactions between IBDV and other viruses, bacteria or parasites (Balamurugan and Kataria, 2006). These indirect losses are due to secondary infections, growth retardation and poor immune response to vaccines (Kibenge et al., 1988). Moreover, the increased use of antibiotics against secondary infections constitutes a growing public health concern (Eldaghayes et al., 2006).

Exploitation of available chicken genetic resistance to pathogens has been adjudged as best strategy for the control and prevention of disease. Many studies on infectious diseases in birds provided evidence for genetic differences in susceptibility (Fadly and Witter, 1986; Girard-Santosuosso et al., 1998; Ignjatovic et al., 2003). Comparisons between inbred lines of chickens show large differences in susceptibility of different genotypes to IBD. Some lines show little or no mortality and limited damage to bursal lymphocytes, while the BrL (Brown Leghorn) line in particular showed high levels of mortality and extreme destruction of the bursa which suggested that resistance might be due to a very small number of genes (Bumstead et al., 1993) but not major histocompatibility complex (MHC) (Hudson et al., 2002). Furthermore, transcriptional profiling of genetically resistant and sensitive birds against IBD revealed that a more rapid inflammatory response and more-extensive p53-related induction of apoptosis in the target B cells might limit viral replication and consequent pathology in resistant birds (Ruby et al., 2006).

Divergent selection based on high and low antibody response against sheep red blood cells (SRBCs) (nonpathogenic antigen) is an immuno-competent trait (Yunis et al., 2002), which is considered an indicator of humoral immune response or B cell response. It has been reported that divergent selection for sheep RBC (SRBC) affects humoral immune response to IBD (Parmentier et al., 1996). However, further information is lacking on the effect of genetic selection for SRBC responses on susceptibility/resistance to IBD infection. Recent studies suggested that cytokines play a major role in differential immune responses to various antigens in chicken lines divergently selected for either high (H) or low (L) SRBC antibody responses (Hangalapura et al., 2006). Moreover, cytokines are identified as key molecules involved in the susceptibility or resistance to various pathogens like *Salmonella enteritidis and Enterococcus gallinarum* (Swaggerty et al., 2004, 2006; Kaiser et al., 2006; Singh et al., 2012).

In IBDV infection, the proinflammatory cytokines (interleukin-IL-1, IL-6), Th1 cytokine (interferon-IFN-γ) and chemokines (MGF, chCXCLi2) were found upregulated, whereas the anti-inflammatory cytokine, transforming growth factor (TGF) β-4 was downregulated (Kim et al., 1998). Moreover, IBDV infection suppresses transcription of both IFN-α and IFN-γ in peripheral blood leukocytes (Ragland et al., 2002). By contrast, IFN-γ expression was increased in the bursa of fabricious after infection with IBDV (Rautenschlein et al., 2003; Eldaghayes et al., 2006).

However, little information is available concerning the role of cytokines in IBDV genetic resistance. In the present investigation, two immunodivergent chicken lines, selected on the basis of high and low antibody response against SRBC antigen, H and L lines respectively, were assessed for the genetic resistance against IBDV. The peripheral blood mononuclear cells (PBMCs) from H and L line were challenged with IBDV and the viral RNA loads were also quantified at different time intervals post IBDV infection. Chicken orthologues Th1 cytokines, IFN-γ and IL-2; a Th2 cytokine, IL-10; a pro-inflammatory cytokine, IL-6; the chemokines, chCCLi2, chCCLi4 and chCCLi7; colony stimulating factor, GM-CSF; and a anti-inflammatory cytokine, TGF-β2 were quantified in PBMCs from chicken lines divergent for SRBC response (H and L lines) post IBDV challenge.

2. Materials and methods

2.1. Genetic background of experimental birds

This experiment was performed in accordance with the rules of the Animal Ethics and Monitoring Committee of the Central Avian Research Institute (CARI) Izatnagar, India (Registration No. 452/01/AB/CPCSEA). White plumaged Synthetic Broiler Dam Line (SDL) is a parent line of broilers chicken, which has been developed from synthetic base population at experimental broiler farm, CARI, Izatnagar in the year 1989–90. Six elite crosses available in the country and pure dam line (IR-3) were used for developing the base population of SDL. These SDL broiler birds were maintained at experimental broiler farm of CARI, Izatnagar, Bareilly, India, were divergently selected for response to SRBC and individuals of F4 generation were utilized in present study. For divergent selection, antibody response to SRBC was measured by Haemagglutination (HA) test at day 5 post i.v. administration of SRBC suspension in 4–5 weeks old birds (Siegal and Gross, 1980; Saxena et al., 1997). High responding males and females were used in full sib mating plan for generation of H line and similarly low responding males and females were used to produce L line in each generation. In the present study, 5 weeks old, ten extreme responder birds each from the H and L line, with an average SRBC agglutination titer of 15.2 and 1.35, respectively, were used. All the birds were reared on deep litter system in brooder houses with temperature maintained at 25–27 °C and atmospheric relative humidity as 60 ± 5% with free access to feed and water. The birds were vaccinated against Newcastle disease, Marek's disease and infectious bronchitis, except infectious bursal disease as per standard protocols. All the birds used in this study were five week old and negative for serum IBDV antibodies as tested by ELISA test using IDEXX-ELISA kit ((FlockCheck∗) procured from M/s IDEXX Laboratories.

2.2. Virus

Intermediate (Georgia) strain of IBD virus was procured from M/s Indovax Pvt. Ltd., Hisar, India by Dr. A.K. Tiwari, Division of Veterinary Biotechnology, Indian Veterinary Research Institute, Izatnagar, India who kindly provided the virus strain for this study.

2.3. Peripheral blood mononuclear cells (PBMCs) culture

PBMCs were separated from the ten extreme responder birds each from the H and L line at 5 week of age using Histopaque-1.077 (Sigma Diagnostics Inc., St. Louis, MO, USA) as described earlier (Sundaresan et al., 2005) briefly 2 ml of heparinzed venous blood was obtained under experimental conditions, layered on 2 ml Histopaque and centrifuged at 800*g* for 15 min. Mononuclear cells at the interphase were collected and washed tree times with RPMI 1640 medium (Sigma). Washed cells were assessed for the viability using trypan blue (0.4% solution, Invitrogen) staining and pooled per line. This pool was distributed then into three aliquots per line containing 2×10^6 cells per ml in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; Sigma), 2 mM L-glutamine (Sigma), 2 mM L-arginine (Sigma), penicillin (1000 IU/ml; Sigma). Each aliquot was plated in 24 well tissue culture plate (2×10^6 cells per well) as triplicate for different time intervals like 0, 0.5, 1, 3, 6, 12, 24, and 48 h post IBDV challenge. In each triplicate, two wells were induced with IBDV Intermediate (Gerogia) strain at a multiplicity of infection of 0.1. The remaining well was kept as un-induced control under 5% CO_2 tension at 41 °C

in a humidified chamber. Induced PBMCs culture along with uninduced controls of both the lines was harvested at various time points (0, 0.5, 1, 3, 6, 12, 24, and 48 h post IBDV challenge) by gentle friction with a rubber policeman and flushing with media. Harvested cells were collected in 1.5 ml eppendorf tubes and centrifuged at 5000*g* for 1 min. After centrifugation the pellet was dissolved in denaturing solution for RNA isolation.

2.4. Extraction of total RNA and cDNA synthesis

Total RNA was isolated from each induced and uninduced PBMCs of H and L line by 'RNAgents- Total RNA isolation system' (Promega, Madison, WI, USA) according to the manufacturer's instructions and eluted into a 20 μl volume of diethylpyrocarbonate (DEPC)-treated water. The concentrations and purities of RNA preparations were determined spectrophotometrically at OD_{260} *versus* OD_{280}. The possible traces of genomic DNA were removed by treating 5 μg of each RNA samples with 5 U of RNase-free DNase (Biogene, CA, USA) at 37 °C for 1 h. The DNase was subsequently inactivated by incubation at 65 °C for 10 min. Each DNase treated total RNA sample (2 μg) was reverse transcribed with suitable negative and positive controls using the 'RevertAid First strand cDNA synthesis kit' (MBI Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. The resultant cDNA was stored frozen at −20 °C till used. Negative controls were performed using all components except reverse transcriptase. Total RNA from chicken spleen was used in positive controls and for standardizing reaction conditions.

2.5. Quantification of chemokines and cytokines mRNA expression by real time PCR

Real time PCR was performed to estimate the chemokines and cytokines mRNA as described elsewhere (Kaiser et al., 2006) by using Mx3000P™ system (Stratagene). The 3′ and 5′ gene specific primer pairs (Table 1) were designed from the published reports (Sundaresan et al., 2007). An initial validation experiment was conducted to confirm the specificity of primers (data not shown). β-Actin gene was used as a internal control as it was reported to express at constant amounts during IBDV infection (Li et al., 2005). All PCR reactions were performed in optical 96-well reaction plates, in duplicates. The amplification was carried out in 25 μl volume containing 1X QuantiTect SYBR Green PCR master mix (SYBR Green 1 dye, ROX passive reference dye, HotStarTaq DNA polymerase and dNTPs with dUTP in optimized buffer components; QIAGEN GmBH, Germany), at 0.2 μM concentration of each gene-specific primer, and 1 μl of cDNA template. PCR cycling conditions were: initial denaturation of 95 °C for 15 min, followed by 45 cycles of denaturation 95 °C for 30 s; annealing (Table 1) for 30 s and extension 72 °C; 45 s. For each gene of interest, negative and positive controls were included. Recombinant plasmids with insert of chemokines or cytokines were used as a positive control. Negative controls were those samples in which cDNA were not added. For each sample a dissociation curve was generated after completion of amplification and analyzed in comparison to negative and positive controls, to determine the specificity of PCR reaction. Moreover, few amplicons were sequenced to confirm the specificity of primers. Results are expressed in terms of the threshold cycle value (Ct), the cycle at which the change in the reporter dye (DRn) passes the significance threshold. To generate gene-specific standard curves, plasmids containing each of the chemokine or cytokine genes were serially diluted from 10^{-1} to 10^{-5}. To generate standard curve for β-actin, pooled cDNA sample was serially diluted and used. Each RT-PCR experiment contained triplicates of test samples, one no-template-control, and a log10 dilution series. Regression analysis of the standard curve was used to calculate the slopes of the gene specific log10 dilution series.

To convey the inverse relationship between starting template concentration and Ct value, results were expressed and analyzed as a 40 − Ct value. The corrected cytokine mRNA per sample was calculated using the following formula:

$$\text{Adjusted Ct value} = (\text{mean } 40 - \text{Cttarget}) \times (\text{Slopetarget})/(\beta\text{-actin df})(\text{Slope } \beta\text{-actin})$$

where, − Cttarget = the triplicate mean of 40 − Ct value; Slopetarget = the slope from the standard curve regression equation for the target gene; β-actin df = the triplicate mean of β-actin/overall mean for all β-actin values within the experiment; and Slope β-actin = the slope from the standard curve regression equation for the β-actin gene.

2.6. Quantification of IBDV-specific RNA in PBMC culture

The load of IBDV was also quantified in each induced and uninduced PBMCs of both H and L line at 0, 0.5, 1, 3, 6, 12, 24 and 48 h post IBDV induction by real time PCR. Primers specific for VP2 gene (Table 1) of IBDV were designed from the published paper (Li et al., 2005). PCR cycling conditions were: initial denaturation of 95 °C for 15 min, followed by 45 cycles of denaturation 95 °C for 30 s; annealing 52 °C for 30 s and extension 72 °C; 45 s. Results are expressed and analyzed as a 40 − Ct value relative to β-actin. The corrected viral load per sample was calculated using the same formula which was used for quantifying cytokines and chemokines expression.

2.7. Statistical analysis

The relative amount of mRNA expression of each gene (expressed as adjusted Ct value) was analyzed using least-square analysis (Harvey, 1975), considering a fixed effect model. Sub-class

Table 1
Primers used for real time PCR.

Sr. No.	Genes	Primer sequence	Accession no.	Product size (bp)	Annealing temperature (°C)
1	chCCLi2	5′ATTGCCATCTGCTACCAGACCT 3′ 5′TCAGGTAGCTCTCCATGTCACA 3′	L34553	322	60
2	chCCLi4	5′TGCCGCCCTCTTCCCTCAA 3′ 5′GGTCCCGGCGCTCACTGC 3′	AY037859	247	59
3	chCCLi7	5′CTGGCCGCTCTGCTCCTCCTG 3′ 5′TATCTCCCTCCCTTTCTTGGTCAC 3′	AY037860	189	55
4	IL6	5′CTGCCCAAGGTGACGGAGGAGGAC 3′ 5′GATTGGCGAGGAGGGATTTCTGG 3′	AJ250838	263	52
5	IFNγ	5′ATGACTTGCCAGACTTACAACTTG 3′ 5′TTAGCAATTGCATCTCCTCTGAGA 3′	AJ634956	495	52
6	IL2	5′ATGATGTGCAAAGTACTGATC 3′ 5′TTATTTTTGCAGATATCTCAC 3′	AJ578467	432	50
7	IL10	5′TGCGGGAGCTGAGGGTGAAGTTTG 3′ 5′CGCGGGGCTGGGCTGAGAG 3′	AJ621614	455	61
8	TGF-β2	5′TGCACTGCTATCTCCTGAG 3′ 5′ATTTTGTAAACTTCTTTGGCG 3′	NM_01031045	316	60
9	GM-CSF	5′CTGCGCCCACCACAACATACTCCT 3′ 5′ACGATTCCGCTTTCTTCCTCTGTC 3′	NM_01007078	206	56
10	β-Actin	5′CATCACCATTGGCAATGAGAGG 3′ 5′GCAAGCAGGAGTACGATGAATC 3′	L08165	353	60
11	IBDV VP2	5′CGCTATAGGGCTTGACCCAAAAA 3′ 5′CTCACCCCAGCGACCGTAACGACG 3′	EU114865	552	52

means for effects that showed significant differences were compared by Duncan's multiple-range test (Duncan, 1955), as modified by Kramer (1964). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Chemokine and cytokine expression profiles

The expression of IL-6, GM-CSF, chCCLi2, chCCLi4, chCCLi7, IFN-γ, IL-2, IL-10 and TGF-β2 mRNAs were observed in the control as well as IBDV induced PBMCs of divergent chicken lines selected for SRBCs responses (Fig. 1–3). The expression of chCCLi2 mRNA was up regulated in H and L line post IBDV challenge. Furthermore, the expression was significantly higher in L line at 1 and 3 h (Fig. 1A). The mRNA expression of chCCLi4 was significantly higher in L line at 1 and 24 h, when compared to H line (Fig. 1B). However, the expression of chCCLi7 showed no significant difference in both lines after induction with IBDV (Fig. 1C).

The mRNA expression of IL-6 was significantly higher in H line at 0.5 and 6 h and in L line at 3 and 24 h when compared to respective counterparts (Fig. 2A). A rapid increase in IFN-γ mRNA was observed in both lines immediately after IBDV challenge. However, at 0.5 and 48 h, the expression of IFN-γ mRNA was significantly higher in H line when compared to L line. The expression of IFN-γ mRNA was almost similar in remaining intervals in both H and L lines (Fig. 2B). The expression of IL-2 was significantly higher in H line at initial intervals (0.5–3 h) and higher in L line at later intervals (12–48 h). Moreover, very low or almost negligible mRNA expression was found in all control samples at later intervals (12–48 h) (Fig. 2C).

The expression of IL-10 mRNA was upregulated in both H and L lines post IBDV challenge at all the intervals studied. However, the expression of IL-10 was significantly higher in L line from 0.5 to 12 h and in H line at 24 and 48 h when compared to respective counterparts (Fig. 3A). Chicken TGF-β2 mRNA expression was up regulated in PBMCs of both lines at different time intervals post IBDV challenge. However, its expression was significantly higher in L line at initial intervals (0.5–3 h) when compared to H line (Fig. 3B). The expression of GM-CSF mRNA was up regulated at 3 h in both H and L lines and thereafter it was down regulated significantly at later intervals (24–48 h). However, the down regulation was significantly higher in H line when compared to L line (Fig. 3C).

3.2. Quantification of IBDV-specific RNA

The viral load was assessed in the control as well as IBDV induced PBMCs of divergent chicken lines selected for SRBCs responses (Fig. 4). At initial intervals, 0.5 and 1 h, viral load was almost equal in both H and L lines however, at later intervals L line was showing significantly greater viral load as compared to H line. Maximum viral load was observed at 6 h post IBDV challenge in both H and L lines. After peaking viral load was decreased significantly in both the lines but a rapid clearance of virus was observed in H line after 12 h whereas in L line viral load was detected up to 48 h.

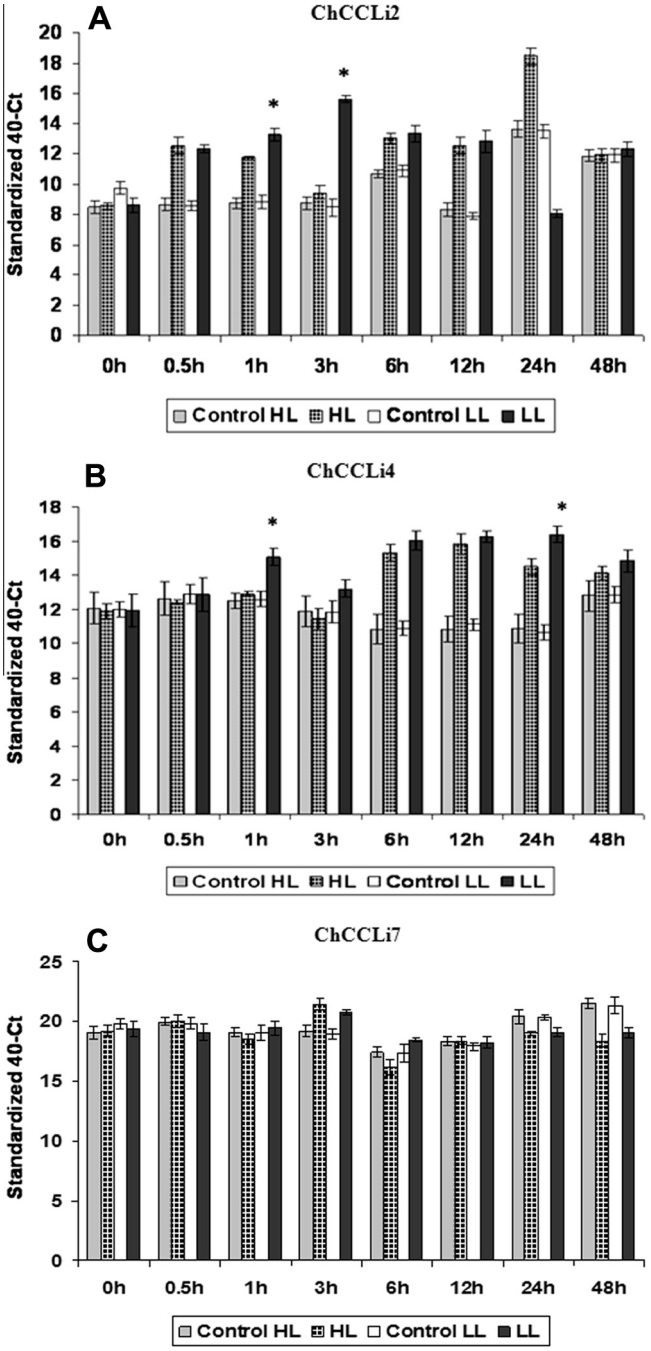

Fig. 1. Chemokines mRNA expression in peripheral blood mononuclear cells (PBMCs) of HL and LL line chickens with or without IBDV infection. (A) chCCLi2, (B) chCCLi4 and (C) chCCLi7. The bars represent standardized values for chemokines mRNA levels subtracted from 40 (negative end point) and corrected for variation in input RNA measured by β-actin levels. Gray bars: H line uninduced control (control HL); Checked bars: H line IBDV induced culture (HL); White bars: L line uninduced control (control LL); Black bars: L line IBDV induced culture (LL). As values are subtracted from negative end point, higher values represent higher levels of cytokine mRNA levels. The result represents the mean ± SE of three independent experiment from one pooled PBMCs of 10 birds per line at each time interval. Asterisks indicate statistical significant ($P < 0.05$) differences in mRNA expression between HL and LL lines at a particular time point.

4. Discussion

Infectious bursal disease virus (IBDV) is an important immunosuppressive virus of chickens (Sharma et al., 2000). Previous studies have indicated that IBDV pathogenesis may vary with the genetic background of the chicken (Ruby et al., 2006; Aricibasi et al., 2010). IBDV induces a strong pro-inflammatory response in bursal macrophages, evidenced by increased mRNA transcription of IL-1, IL-6, IL-18, IFN-γ and CXCLi2, and down-regulation of TGF-β (Palmquist et al., 2006; Eldaghayes et al., 2006; Rauw et al., 2007). However, little is known about the immune responses induced by IBDV in PBMCs and alteration of immune responses

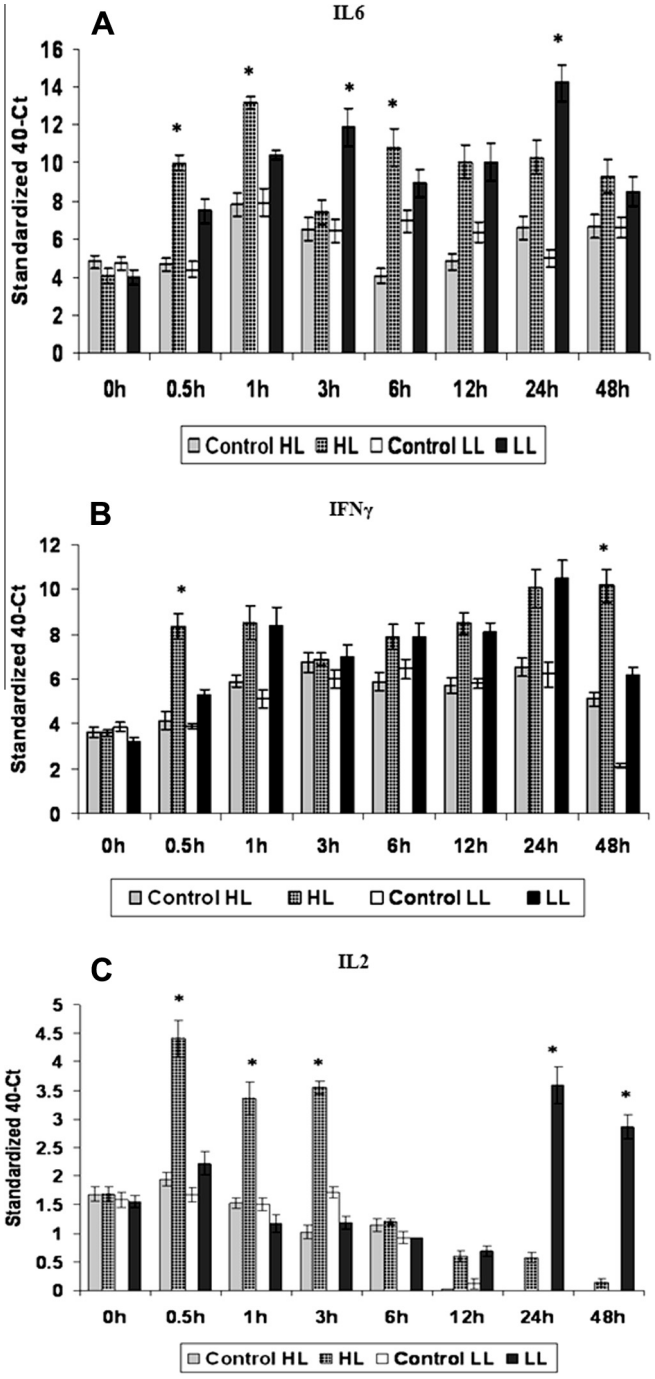

Fig. 2. Proinflammatory and Th1 cytokine mRNA expression in peripheral blood mononuclear cells (PBMCs) of HL and LL line chickens with or without IBDV infection. (A) IL6, (B) IFNγ and (C) IL2. The bars represent standardized values for cytokine mRNA levels subtracted from 40 (negative end point) and corrected for variation in input RNA measured by β-actin levels. Gray bars: H line uninduced control (control HL); Checked bars: H line IBDV induced culture (HL); White bars: L line uninduced control (control LL); Black bars: L line IBDV induced culture (LL). As values are subtracted from negative end point, higher values represent higher levels of cytokine mRNA levels. The result represents the mean ± SE of three independent experiment from one pooled PBMCs of 10 birds per line at each time interval. Asterisks indicate statistical significant ($P < 0.05$) differences in mRNA expression between HL and LL lines at a particular time point.

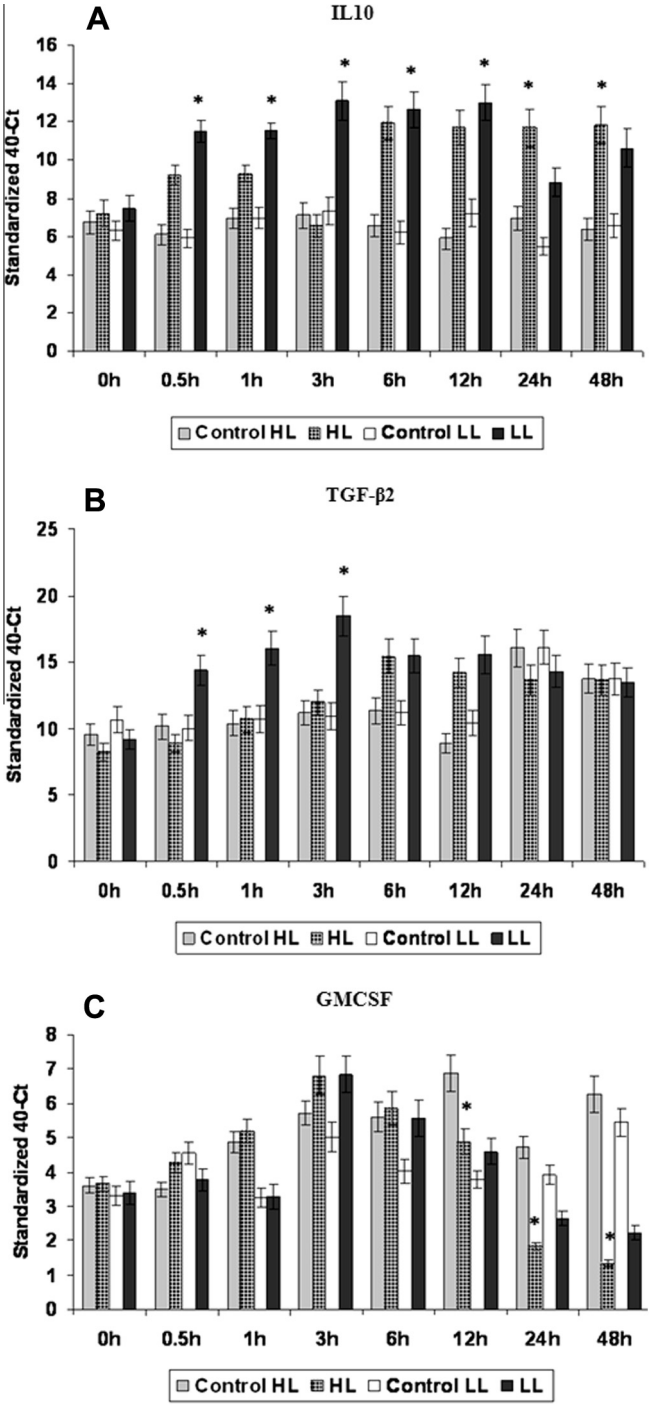

Fig. 3. Th2, anti- inflammatory cytokine and colony stimulating factor mRNA expression in peripheral blood mononuclear cells (PBMCs) of HL and LL line chickens with or without IBDV infection. (A) IL10, (B) TGF-β2 and (C) GMCSF. The bars represent standardized values for cytokine mRNA levels subtracted from 40 (negative end point) and corrected for variation in input RNA measured by β-actin levels. Gray bars: H line uninduced control (control HL); Checked bars: H line IBDV induced culture (HL); White bars: L line uninduced control (control LL); Black bars: L line IBDV induced culture (LL). As values are subtracted from negative end point, higher values represent higher levels of cytokine mRNA levels. The result represents the mean ± SE of three independent experiment from one pooled PBMCs of 10 birds per line at each time interval. Asterisks indicate statistical significant ($P < 0.05$) differences in mRNA expression between HL and LL lines at a particular time point.

by genetic selection for specific antibody responses. The advantages of using PBMCs are (i) PBMCs are easily accessible immune cells and (ii) PBMCs can be harvested from breeder birds without sacrifice (Sundaresan et al., 2005; Hangalapura et al., 2006). In the present study, PBMCs from divergent chicken lines selected for SRBC response were used for studying the genetic influence of cytokine mRNA expression post IBDV challenge under divergent selection.

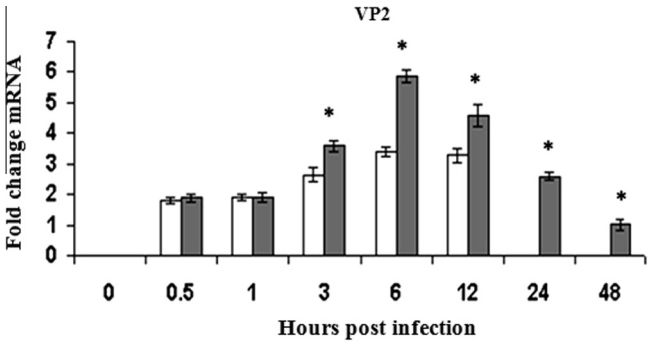

Fig. 4. IBDV-specific RNA loads in peripheral blood mononuclear cells (PBMCs) of HL and LL line chickens with or without IBDV infection. The bars represent standardized values for VP2 mRNA levels subtracted from 40 (negative end point) and corrected for variation in input RNA measured by β-actin levels. White bars: H line IBDV induced culture (HL); Gray bars: L line IBDV induced culture (LL). As values are subtracted from negative end point, higher values represent higher levels of VP2 mRNA levels. The result represents the mean ± SE of three independent experiment from one pooled PBMCs of 10 birds per line at each time interval. Asterisks indicate statistical significant ($P < 0.05$) differences in mRNA expression between HL and LL lines at a particular time point.

The dose of the virus used for *in vitro* challenge was selected based on the initial experiments (data not shown). We found 0.1 moi virus was suitable for the *in vitro* challenge experiments.

Development of immunity to pathogens is determined to a large extent by the timing and relative level of expression of the cytokines (Ulett et al., 2000). Cytokines and chemokines are an integral part of the host immune response in avian species (Eckmann and Kagnoff, 2001). Resistance or susceptibility to most infectious diseases is strongly determined by the balance of cytokines and chemokines produced during infection (van Den Broek et al., 2000). Chemokines are important in the recruitment of immune cells to the site of infection (Hong et al., 2006). In the present study, chicken CC chemokines chCCLi2, chCCLi4 and chCCLi7 were upregulated in PBMCs following IBDV infection. In earlier studies, chCXCLi2 was found to be upregulated post IBDV infection (Eldaghayes et al., 2006). This is the first report suggesting the upregulation of CCL chemokines following IBDV infection in chicken. Here, the expressions of chCCLi2, chCCLi4 and chCCLi7 were significantly higher in L line when compared to H line. However, the viral RNA loads were significantly lower in H line as compared to L line. Earlier studies in *S. enteritidis*-resistant chickens suggested that a higher level of chemokine mRNA expression is related to the Salmonella resistance (Swaggerty et al., 2006). However, recent studies in mice suggested that higher expression of chemokines associated with the disease susceptibility (Yun et al., 2005; Sun et al., 2006). Therefore, we presume that the upregulated chemokines might be associated with the susceptibility to IBDV.

Chicken IL-6 expression is usually indicative of the initiation of an acute-phase response and increased expression of IL-6 creates a population of heterophils that are more capable of eliminating pathogens (Swaggerty et al., 2004; Hong et al., 2006). IFN-γ is a major cytokine mediating resistance to many pathogens (Sadeyen et al., 2004). The expression of IFN-γ was compared in two inbred lines of chickens differing in resistant to the *S. enterica* serover Enteritidis and it was found that significantly low expression of IFN-γ was observed in susceptible birds in comparison to resistant ones (Sadeyen et al., 2004). Furthermore, the local production of IL-2 during Eimeria infection contribute to local proliferation of effector T-cells, including T-cells which are involved in cytotoxic effector mechanisms for the control of infection (Lillehoj, 1989; Hong et al., 2006). In the present study, the expression of IFN-γ, IL-2 and IL-6 were significantly higher in H line as compared to L line, post *in vitro* IBDV challenge. Therefore, these findings suggest that higher proinflammatory cytokines might be related to the rapid clearance of virus from PBMCs.

IL-10 is a pleiotropic cytokine produced mainly by activated macrophages or CD4+ and CD8 + T cells (Groux and Powrie, 1999) and is involved in the control of innate immune reactions and cell-mediated immunity, by preventing the development of Th1 cytokines (Rothwell et al., 2004; Hong et al., 2006). In mammals, disease susceptibility was associated with higher levels of IL-10 expression (Curiel et al., 1998; Ulett et al., 2000; van Den Broek et al., 2000). In Marek's disease infected chickens higher levels of IL-10 expression was observed when compared to vaccine protected birds (Abdul-Careem et al., 2007). In the present study, the expression of IL-10 was significantly higher in L line as compared to H line. Therefore, the results suggest that the higher expression of IL-10 might be associated with the susceptibility to IBDV.

GM-CSF is a cytokine generally produced from activated T cells (both Th1 and Th2), macrophages, endothelial cells, and fibroblasts (Fleetwood et al., 2005). Functionally, chicken GM-CSF was shown to inhibit the synthesis of pro-inflammatory cytokines (including IL-1, TNF-α, and IL-12), thus down-regulating inflammatory (Th1) responses (de Waal Malefyt et al., 1991; Groux and Powrie, 1999; Hong et al., 2006). Here, the expression of GM-CSF mRNA was upregulated initially and down regulated at later stages in both H and L lines. Similarly during Eimeria infection in chicken, the early upregulation of GM-CSF mRNA was observed (Hong et al., 2006). However, there is no report of virus induced suppression of GM-CSF in chicken. In feline coronavirus infection, significantly lower levels of GM-CSF were observed in mesenteric lymph nodes (Kipar et al., 2006). Further, mice challenged with *Mycobacterium tuberculosis* expressed lower GM-CSF (Ordway et al., 2006). In The present study, the down regulation of GM-CSF was significantly higher in H line when compared to L line. Therefore, the findings of the study suggest that down regulation of GM-CSF might be a part of viral pathogenesis and we hypothesize that the lower proinflammatory cytokines expression in L line might be due to the comparatively higher levels of GM-CSF.

TGF-β2 is an important regulator of inflammation, exhibiting pro-inflammatory properties at low concentrations and anti-inflammatory effects at high concentrations (Omer et al., 2000). In chicken, infection with Infectious bursal disease virus strains causes down-regulation of TGF-β4 in bursa (Eldaghayes et al., 2006). However, in the present study, the upregulation of TGF-β2 was observed in PBMCs of chicken. These findings suggest that TGF-β2 might be upregulated to control the inflammation mediated by Th1 cytokines and this upregulation might be the reason for the lower expression of Th1 cytokines in L line. In the present study, the upregulation of TGF-β2 was significantly higher in L line when compared to H line at initial intervals. In human epithelial cells, treatment of TGF-β increased respiratory syncytial virus replication (McCann and Imani, 2007). Therefore, the higher expression of TGF-β2 might be related to the IBDV pathogenesis in chicken and H line might be resistant to IBDV as low level expression of TGF-β2 was observed.

Thus, it was concluded that in chicken, selection for antibody responses appears to influence the expression profiles of chemokines and cytokines post *in vitro* IBDV challenge in PBMCs. Moreover, the rapid clearance of IBDV from PBMCs in H line might be due to the enhanced production of proinflammatory cytokines. Furthermore, selection for high antibody responses to SRBC might improve the immuno-competence of chickens against IBDV.

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