

Effect of Infectious Bronchitis Virus (IBV) on Lymphocyte Proliferation and T-Cell Expression of Viral Antigen in Experimentally Infected Chickens

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ABSTRACT

Background: Infectious bronchitis virus (IBV) is a major viral pathogen of poultry that causes significant economic losses worldwide. Although cell-mediated immunity is essential for controlling IBV infection, the roles of CD4⁺ and CD8⁺ T lymphocytes and viral persistence in circulating lymphocytes remain incompletely understood. **Objective:** To investigate changes in circulating CD4⁺ and CD8⁺ T-lymphocyte populations, detect IBV antigen in peripheral blood mononuclear cells (PBMCs), and evaluate the effect of Cyclosporine A (CSA)-induced immunosuppression on viral persistence. **Materials and Methods:** Seventy-five specific pathogen-free chicks were divided into infected (n = 50) and control (n = 25) groups. PBMCs were collected at different time points after IBV infection and CSA treatment. Flow cytometry was used to quantify CD4⁺, CD8⁺, and IBV antigen-positive cells. An in vitro lymphocyte infection assay was performed, and viral replication was confirmed by embryonated egg inoculation and RT-PCR. **Results:** IBV antigen was detected in circulating PBMCs during acute infection, with peak expression on day 7 post-infection (P < 0.005). CD4⁺ T lymphocytes increased significantly on days 14 and 21, whereas CD8⁺ T lymphocytes increased markedly from day 10 onward, indicating their important role in viral clearance. CSA treatment significantly reduced both CD4⁺ and CD8⁺ T-cell populations and resulted in more than 60% of lymphocytes expressing IBV antigen, suggesting viral persistence or reactivation. IBV also replicated successfully in isolated lymphocytes in vitro. **Conclusion:** CD8⁺ T lymphocytes are critical for controlling acute IBV infection, while CD4⁺ T cells support adaptive immune responses. Immunosuppression enhances viral persistence, and lymphocytes may serve as a reservoir for IBV. These findings provide valuable insights for improving IBV vaccination and disease-control strategies.

Keywords: Infectious bronchitis virus; CD4⁺ T lymphocytes; CD8⁺ T lymphocytes; Cyclosporine A; Flow cytometry; PBMCs; Viral persistence; Chickens.

INTRODUCTION

Infectious bronchitis virus (IBV) infection is considered one of the severely contagious respiratory diseases in chickens that cause severe illness, especially in very

young chicks. Signs of IBV infection are usually, coughing, sneezing and nasal discharge (1, 2). IBV infection also affects egg production and might result in abnormal eggs formation (3). Moreover, certain IBV

strains might cause nephritis or enteric disease (4). IBV vaccines are available but couldn't ensure effective infection control as outbreaks of this disease has previously been reported even in vaccinated flocks. Therefore, IBV is one of the economic concerns to poultry industry (5).

Cell mediated immunity (CMI), particularly Cytotoxic T lymphocytes (CTL) play a vital major role in controlling many viral infections in chicken including IBV infection. Most of CTL have been found to be CD8+ T lymphocytes subtype, however limited immunity has been provoked by CD4+ T lymphocytes subtype. In case of CD8+ CTL, the peptide presented to the T cells receptors (TCR) is the major histocompatibility (MHC) class I antigen that is responsible for optimal activation and function of CTL and successful interaction between the CTL and the antigen presenting cells (APC) (6). On the other hand, for CD4+ CTL, the peptide presented to the TCR is the major histocompatibility (MHC) class II (6).

Virus-specific CD8⁺ CTL cells have been proven to be important for resolution of infection and elimination of viral shedding (7, 8). moreover, previous studies stated that cell mediated immunity through CTL elicited in response to inactivated viral vaccines, plays an important role in protection and outbreak control (9, 10). Tripp and Anderson (11) showed that a formalin-inactivated respiratory syncytial virus (RSV) vaccine induced MHC class II CTL subtype in mice. There is also evidence that an inactivated influenza virus vaccine may induce MHC class I CTL subtype (12).

Chickens infected with IBV virus develop highest CTL responses, leading to 80% lysis of target cells, at day post inoculation (pi) (13). Specific CTL activity began to decrease after viral loads peaked in tissues after 8 days (13). Peiris et al. (14) stated that primary CD8⁺ CTL collected at 10 days pi are important in controlling acute IBV infection when they were taken from the spleens of infected chickens at 3-6 weeks pi, and transferred to non-infected chicks, they gave excellent protection against clinical infection.

The present study was planned to investigate the changes in circulating lymphocyte populations and to detect the possible presence of IBV antigens in these cells in chickens following primary infection (acute stage) and during treatment with the immunosuppressant drug, Cyclosporine A (CSA). Moreover, an *invitro* experiment was conducted to investigate the ability of IBV to infect isolated lymphocytes.

Aim of the Study

- The present study aimed to investigate the effect of Infectious Bronchitis Virus (IBV) infection on circulating CD4⁺ and CD8⁺ T-lymphocyte populations and to determine the presence of viral antigen in peripheral blood mononuclear cells

during acute infection and following Cyclosporine A-induced immunosuppression in experimentally infected chickens.

Objectives

- To evaluate changes in circulating CD4⁺ and CD8⁺ T-lymphocyte populations following experimental IBV infection.
- To detect the expression of IBV antigen in peripheral blood mononuclear cells using flow cytometry.
- To assess the effect of Cyclosporine A-induced immunosuppression on T-lymphocyte populations and viral antigen expression.
- To investigate the ability of IBV to infect and replicate in isolated chicken lymphocytes under in vitro conditions.

MATERIALS AND METHODS

Experimental design

Seventy-five, one-day-old, SPF chicks were divided into two groups, fifty chicks were considered as the infected group that was inoculated intranasally and intraocularly with a total of 100µl of M41 IBV (4.9 log10) and twenty-five chicks were considered as the control group. Both groups were maintained in a physically separated isolation pen. Peripheral blood samples were collected in heparin-coated tubes on 0, 3, 7, 10, 14, 21 and 28 days pi and were processed for flow cytometry. After 35 days, both infected and non-infected chickens were divided into two subgroups one of which was injected with CSA every three days and considered as CSA treated group and the other subgroup was kept non-treated. Blood samples were then collected in heparinized tubes and processed for flow cytometry on days 0, 3, 7, 10, 12 and 15 post CSA drug administrations (i.e days 35, 38, 42, 45, 47 and 50 post virus inoculation).

Table 1. Experimental Design of the Study

Group	No. of Birds	Treatment	Sampling Days
Control	25	Non-infected	0, 3, 7, 10, 14, 21, 28
IBV Infected	50	M41 IBV	0, 3, 7, 10, 14, 21, 28
CSA Treated	Subgroups	Cyclosporine A	35, 38, 42, 45, 47, 50

Sample Size Justification

The sample size was determined based on previous experimental studies evaluating immune responses following Infectious Bronchitis Virus infection in chickens. A total of seventy-five specific pathogen-free (SPF) chicks was considered adequate to detect significant differences in lymphocyte populations and viral antigen expression between experimental groups while maintaining sufficient statistical power.

Mononuclear cells collection and separation from blood samples

Peripheral blood samples were collected in heparinized tubes at days 0, 3, 7, 10, 14, 21 and 28-post infection and on days 0, 3, 7, 10, 12 and 15 post CSA administration to be used for flow cytometry analysis. Peripheral blood mononuclear cells were separated from heparinised blood samples, by density gradient centrifugation (Pharmacia Fin Chemicals, Uppsala, Sweden). Cell viability was determined by the trypan blue exclusion technique and then re-suspended in RPMI medium to give a concentration of 1×10^7 viable cells/ ml.

Monoclonal antibodies

The monoclonal antibodies (MAb) Av29, Av27 and A13/38 prepared in mice against the avian CD4+, CD8+ cells and IBV respectively were kindly provided by Dr T.F. Davison, the Institute for Animal Health, Compton, Newbury, Berks. The optimal dilution of each antibody was determined by means of a checkerboard titration.

immunofluorescence staining of peripheral mononuclear cells expressing viral antigen

For detection of avian IBV antigens, mononuclear cells were fixed and stained according to the method described by Woldehiwet and Hussin (15) with some modifications. Briefly, the peripheral blood mononuclear cells (PBMC) were fixed with freshly prepared 1% paraformaldehyde in PBS, for 2-3 minutes then washed twice, firstly in PBS containing 10% normal rabbit serum (PBS-NRS) and secondly with 1 ml of PBS-NRS containing 0.5% saponin (PBS-NRS-SP) (Sigma). Saponin was used to permeabilize cell membranes for better expression of antigens following this step, cells were stained with an optimal dilution of anti-chicken IBV MAb for 45 minutes at 37°C. Stained cells were washed with PBS-NRS-SP three times and incubated with optimally diluted FITC-labelled swine anti-mouse IgG (Sigma) for 45 minutes at 37°C and washed again three times. Finally, the cells were resuspended in 500 ml of PBS containing 3% formalin and 2% glucose and stored in the dark at 4°C until analysed by flow cytometry.

Immunofluorescence staining of CD4⁺ and CD8⁺ CTL

Approximately 1×10^6 cells of isolated mononuclear cells were incubated with optimally diluted monoclonal antibodies at 4°C for 45 minutes and washed 3 times with PBS containing 0.1 % sodium azide and 0.5 % bovine serum albumen (BSA). The cells were re-suspended in 100µl of PBS/BSA and incubated with 25µl of FITC-conjugate swine anti-mouse IgG antibodies for 45 minutes, at 37°C, washed three times with PBS/BSA and finally re-suspended in 500µl of PBS containing 3% formalin and 2 % glucose. and stored in the dark at 4°C until analysed by flow cytometry.

Flow cytometry analysis of CD4⁺, CD8⁺ CTL and IBV expressing lymphocytes

Stained cells were analysed by flow cytometry using a FACS 420 fluorescence activated cell sorter. The machine was standardized using glutaraldehyde-fixed chicken red blood cells and a minimum of 10,000 cells were analysed using logarithmic amplifiers. The number of positive cells was defined as those expressing a higher degree of fluorescence than the cells stained by the second antibody, using the 481 nm band of an argon ion laser at 300mW. The lymphocytes expressing avian IBV antigens were estimated by subtracting the percent fluorescence in the same sample stained with second antibody only.

Infection of lymphocytes in vitro

Separation of lymphocytes

Heparinised blood samples were collected from two-week-old uninfected SPF chicks and processed to separate the mononuclear cells by Ficoll-Paque density gradient centrifugation. The peripheral blood mononuclear cells were seeded into tissue culture flasks, rinsed with filtered autologous plasma and incubated at 37°C for 4 hours. At the end of this, the non-adherent cells were gently aspirated by pipette. The process was repeated, and pools were resuspended in RPMI 1640 medium to give a final concentration of 10^5 cells/ml. The non-adherent cells comprised more than 95% lymphocytes as determined by the Diff-Quick staining method (Diff Quick AG, Duding, Switzerland).

Inoculation of lymphocytes and assay of virus growth

Isolated lymphocytes were cultured *In vitro* then infected with 100µl M41 IBV (4.9 log₁₀) and left for adsorption for 3 hours at 37°C. Unabsorbed virus was removed by washing the lymphocyte suspension with warm RPMI medium three times. Cultures were incubated at 37°C in 5% CO₂ for up to 48 hours. Samples were collected at 0, 24, 48 and 72 hours post inoculation, two flasks were used each time. At harvesting, flasks were subjected to three cycles of freezing and thawing to release cell-associated virus. After clarifying the cell suspension by

centrifugation, supernatants were inoculated into the allantoic cavities of SPF fertile eggs after 9-11 days incubation and embryos were examined after 6 days of incubation. In addition to virus isolation, lymphocyte culture suspensions were tested by RT-PCR according to Pohuang et al., (16) using the primer sets of FOR2 (5'-CAG TGT TTG TCA CAC ATT GT-3') and RE2 (5'-CCA TCT GAA AAA TTG CCA GT-3').

Ethical Approval

All experimental procedures involving animals were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of the respective institution and were conducted in accordance with national and international guidelines for the care and use of laboratory animals.

Animal Welfare Statement

All animals were handled humanely throughout the study. Every effort was made to minimize pain, distress, and unnecessary suffering. The study was conducted in accordance with internationally accepted guidelines for the ethical use and care of experimental animals.

Statistical analysis

Statistical analysis was performed using SYSTAT statistical software (Systat Inc., Evanston, IL, USA). Continuous data were expressed as mean ± standard deviation (SD). Comparisons between groups were performed using Student's *t*-test or one-way analysis of variance (ANOVA), followed by appropriate post hoc analysis where applicable. Differences between categorical variables were analysed using the Chi-square test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Clinical disease

In the acute phase clinical signs of IBV virus started within 72 hours pi lasting until days 10 pi. Signs of illness are demonstrated by tracheal rales, coughing, sneezing and nasal discharge. After CSA treatment at 5 weeks pi a small number of birds showed very mild respiratory signs such as faint rales and clear nasal discharge, which was evident after nasal squeezing.

Table 2. Clinical Signs Observed During Acute IBV Infection

Day Post Infection	Clinical Signs	Severity
0	Normal	None
3	Sneezing, nasal discharge	Mild

Day Post Infection	Clinical Signs	Severity
7	Tracheal rales, coughing	Moderate
10	Respiratory signs	Moderate
14	Recovery	Mild
21	Normal	None
28	Normal	None

IBV-antigen expressing mononuclear cells during acute IBV infection

Lymphocytes were apparently carried IBV antigens on all occasions after infection (Figure 1). However, there were background readings in uninfected controls on days 10, 21 and 28. Values from IBV-infected chicks were significantly different (*P* < 0.005) from controls on days 3, 7 and 14, with a major peak on day 7. At this time, 12% of cells were expressing IBV antigen.

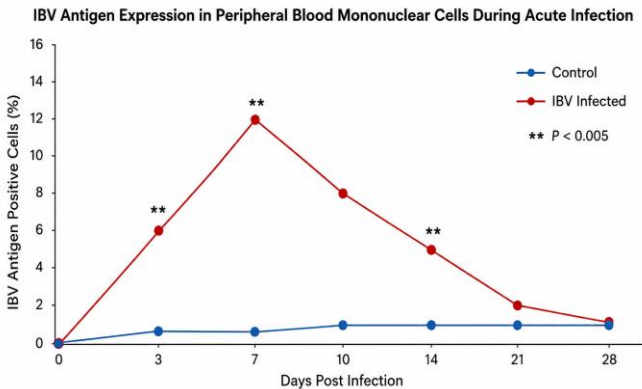


Figure 1. Percentage of IBV antigen-positive peripheral blood mononuclear cells (PBMCs) following experimental IBV infection. IBV antigen expression was detected from day 3 post-infection, reached a maximum level (~12%) on day 7, and gradually declined thereafter. Significant differences compared with control birds were observed on days 3, 7, and 14 (*P* < 0.005).

IBV-antigen expressing mononuclear cells during CSA immunosuppression

After 35 days of our experiment, half of the non-infected group was treated with CSA and the other half remained untreated. The percentage of IBV expressing mononuclear cells of non-infected group with or without CSA immunosuppression was shown in figure 2A. Apart from low levels of background staining, there was no evidence of IBV in these cells. On the same approach, half of the IBV infected group was treated with CSA and the other half remained untreated. The percentage of IBV expressing mononuclear cells of IBV infected chicken with or without CSA immunosuppression was shown in figure 2B. On day 7 after the start of CSA treatment, more than 60% of the lymphocytes were expressing viral

antigen. On all other occasions, viral expression was very low or absent and not significantly different from background staining.

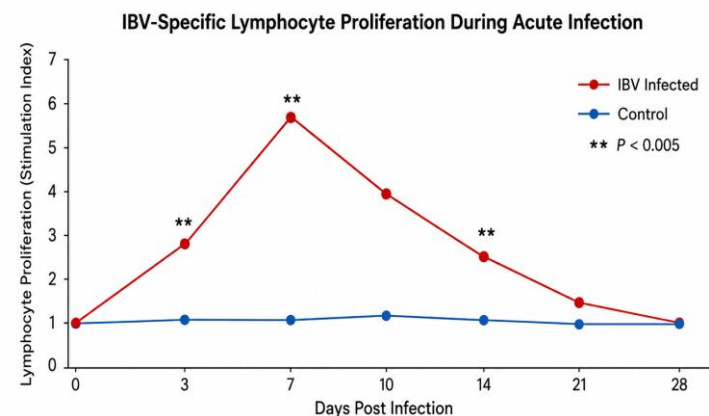


Figure 2. IBV-specific lymphocyte proliferation (stimulation index) in peripheral blood mononuclear cells (PBMCs) during experimental infection. A significant increase in lymphocyte proliferation was observed from day 3 post-infection, with a peak on day 7, followed by a gradual decline. Significant differences compared with control birds were observed on days 3, 7, and 14 ($P < 0.005$).

CD4⁺ and CD 8⁺ lymphocytes percentage during IBV acute infection

Control non-infected chickens did not show any variation in lymphocyte population at any stage but a day-to-day variation in the total lymphocyte was observed in IBV-infected group. Figure 3A, shows the proportions of CD4⁺ lymphocytes in peripheral blood of IBV infected and non-infected chicken. In controls, non-infected group, levels remained low (9-12%) until day 28, where the percentage increased to approximately 40%. IBV infected chicken showed a gradual increase in CD4⁺ T-cells, but the values were only significantly higher than controls on days 14 and 21Pi. CD8⁺T-lymphocyte also remained at similar levels (between 14-20%) between days 0 and 21 pi in non-infected group as shown in figure 3B. on the other hand, the proportion of CD8⁺ T lymphocytes from IBV-infected birds increased to between 35-40% from 10 days Pi onwards and the values were significantly higher than controls on days 10, 14 and 21, but not on the day 28 Pi.

Table 3. Summary of Changes in CD4⁺ and CD8⁺ T-Lymphocyte Populations During Acute IBV Infection

Parameter	Control Group	IBV-Infected Group	Statistical Observation
CD4 ⁺ T lymphocytes	Remained relatively stable (9–12%) until day 28	Gradual increase after infection; significantly higher on days 14 and 21 post-infection	Significant increase on days 14 and 21 ($P < 0.05$)
CD8 ⁺ T lymphocytes	Maintained between	Increased markedly	Significant increase on

Parameter	Control Group	IBV-Infected Group	Statistical Observation
	14–20% during most of the observation period	from day 10 onwards, reaching approximately 35–40%	days 10, 14 and 21 ($P < 0.05$)
Overall immune response	Stable	Enhanced cell-mediated immune response following IBV infection	CD8 ⁺ response predominated during acute infection

CD4⁺ And CD8⁺ lymphocytes percentage during CSA immunosuppression

After 35 days of the experiment, half of both the non-infected group and the IBV infected group were treated with CSA and the other halves remained untreated. The percentage of CD4⁺ T-lymphocytes of the non-infected group with or without CSA immunosuppression was shown in figure 4A. CSA appeared to consistently cause a reduction in CD4⁺ lymphocytes percentage, although the differences were significant only on days 0 and 7 (post treatment). The very low levels on day 10 are difficult to explain. Figure 4B. shows the percentage of CD4⁺ T-lymphocytes in IBV-infected chickens with or without CSA immunosuppression. Values in CSA-treated birds were always lower than in untreated birds, but differences were significant only on days 3, 12 and 15 (post treatment). For both groups, levels rose after treatment from the day 7, then there was a gradual rise from 10 days to 15 days. Figure 4C. shows the percentage of CD8⁺ T-lymphocytes of the non-infected group with or without CSA immunosuppression. CSA treatment again reduced the levels of CD8⁺ T-cells in control chickens after treatment with CSA on all occasions tested, but the differences were significant only on days 12 and 15 after the start of CSA treatment. Finally, the percentage of CD8⁺ T-lymphocytes in blood of infected chickens with or without CSA immunosuppression is shown in Figure 4D. The overall pattern is very much like that of CD4⁺ T-cells, with levels for treated birds being lower than those from untreated birds and there was a rise after onset of CSA treatment followed by a reduction on day 10 followed by another gradual increase. Differences between treated and untreated values were significant on days 3, 10 and 15.

All cell culture fluids were also found to be positive for IBV by RT-PCR as a 400-bp S1 gene fragment was amplified in all tested cell culture fluids (Figure 5).

Table 4. Effect of Cyclosporine A on T-Lymphocyte Populations and IBV Antigen Expression

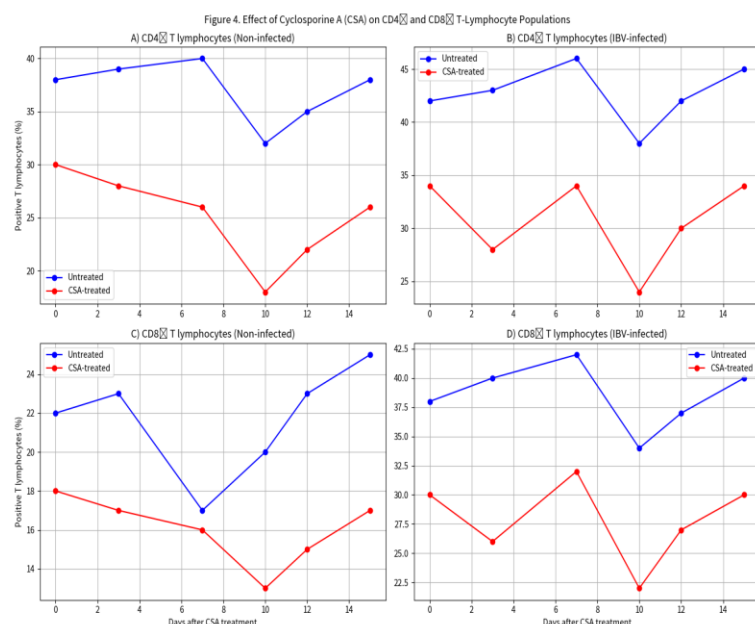
Parameter	Untreated Birds	CSA-Treated Birds	Observation
CD4 ⁺ T lymphocytes	Higher circulating levels	Markedly reduced following CSA treatment	Significant reduction at selected time points
CD8 ⁺ T lymphocytes	Maintained higher levels	Consistently lower than untreated birds	Significant reduction after CSA administration
IBV antigen-positive PBMCs	Very low or absent after acute phase	Marked increase, with >60% positive lymphocytes on day 7 after CSA treatment	Suggests viral persistence/reactivation during immunosuppression
Overall immune status	Normal cellular immunity	Suppressed cellular immune response	CSA effectively induced immunosuppression

DISCUSSION

Cell mediated immunity, particularly Cytotoxic T lymphocytes, plays a vital major role in controlling many viral infections in chicken. But the role of CD4⁺ and CD8⁺ cytotoxic T-lymphocytes in the immunopathogenesis of IBV infection in chickens is not well elucidated. Recently, it has become well established that CD8⁺ CTL rather than CD4⁺ cells are the critical population for the control of IBV infection (17).

In the present study, we examined peripheral blood T lymphocytes rather than spleen T lymphocytes while it is not known how this could affect the outcome of the experiment. During the acute phase of the infection, CD4⁺ cells in control groups remained in stable numbers until there was a marked significant increase in day 28. The increase in CD4⁺ lymphocytes in chickens after 28 days of age could be linked to the maturation of the immune system and the development of gut-associated lymphoid tissue (GALT). Chickens' immune system matures, particularly the thymus, and becomes more efficient at responding to pathogens around 28 days of age. Thymic maturation, responsible for T cell maturation, peaks after 28 days (18, 19).

Exposure to bacteria, viruses, and parasites stimulates the immune system, increasing the number of CD4⁺ lymphocytes essential for coordinating immune responses during infection (20). CD4⁺ cell numbers in infected birds in the acute phase increased from day 10 and continued to rise until day 28. These helper T-cells have the function of processing exogenous antigen in conjunction with MHC class II. The activation of CD4⁺ cells is critical for initiation of an effective immune response (21). It is noted that, the increased CD4⁺ cells numbers were corresponding with the end of virus persistence in the tissues as the CD4⁺ cells playing a crucial role in viral clearance, which probably indicated processing of viral antigen and the onset of the humoral immune response. They help activate CD8⁺ T cells, B cells, and macrophages, and produce cytokines that enhance antiviral immunity. However, chickens have a unique lymphoid organ called the bursa of Fabricius, which is crucial for B cell development. Studies have shown that CD4⁺ cells are important for clearing various viruses in chickens (18, 19). On the other hand, CD8⁺ cells are crucial for eliminating virus-infected cells and undergo developmental changes in the early stages of life. In this study, the CD8⁺ cytotoxic lymphocytes numbers remained constant in non-infected control birds until there was a marked significant increase on day 21. Chickens often show a significant increase in CD8⁺ cytotoxic lymphocyte numbers around day 21 due to the maturation



Replication of IBV in lymphocytes in vitro

Supernatants from IBV-inoculated lymphocytes collected at 24, 48 and 72 hours after inoculation all induced typical effects of IBV in chick embryos, i.e. dwarfing and curling.

of their immune system, and the development of GALT (18, 19). The CD8⁺ cells are crucial for eliminating virus-infected cells and undergo developmental changes in the early stages of life. In case of IBV-infected birds, CD8⁺ cells numbers significantly increased in proportions from day 10 pi compared to uninfected controls. This probably corresponded to the activity initiated by these lymphocytes to limit prolonged infection (13). Exposure to environmental antigens stimulates the immune system, leading to an increase in CD8⁺ cells essential for coordinating immune responses against these antigens (18, 19). These findings well correlated with the peak of IBV antigen in the peripheral lymphocytes at 7 days pi. Because of the sampling, this peak could have ranged from between days 4 and 9 and to some degree reflect the virus activity in the tissues.

As expected, CSA is a potent immunosuppressant and its treatment induced a significant reduction in the circulating T-lymphocytes, which lasted throughout the period of observation. These results agreed with Dhinakar Raj and Jones (22) who found that CSA immunosuppression caused a functionally effective reduction in the cytotoxic T-cell response to the T-cell mitogen Concanavalin A, but not to the T- and B-cell mitogen pokeweed. CSA inhibits the activation of T cells, particularly CD4⁺ helper T cells, by interfering with the production of cytokines like IL-2 (23). The flow cytometric analysis indicated a similar reduction in the number of circulating CD4⁺ and CD8⁺ population in CSA treated birds compared to untreated birds and this result agreed with reports from Kim et al. (24) and Rautenschlein et al. (25). The reasons for the increase in CD4⁺ and CD8⁺ cells up to 7 days after the start of CSA treatment and then the fall at 10 days in uninfected chicks in both treated and untreated groups CD4⁺ and CD8⁺ T cells are key players in the adaptive immune system. The observed changes in CD4⁺ and CD8⁺ cell numbers in chicks treated with CSA could be attributed to transient immune activation, stress response, cytokine release, and homeostatic response. Studies in avian immunology have shown that CSA can have complex effects on T cell populations. The subsequent fall in CSA's dominant immunosuppressive effect is due to the decrease in new T cell production, depletion of activated cells, and suppression of cytokine production (23). Moreover, the striking fall in CD4⁺ cells after 10 days in the IBV infected birds was almost certainly related to immune activity in processing antigen. For CD8⁺ cytotoxic lymphocytes, the fall indicated activity involved in curtailing infection and approximated to the time when virus was detected in the blood lymphocytes by flow cytometry. In view of the persistence of virus in the several other tissues examined until day 10, it was perhaps surprising that the peak in the lymphocytes was on day 7

and the percentage of infected cells at this time was much greater than on days 3 or 10 pi.

It is worthwhile to carefully examine the relative time of the virus found in peripheral blood lymphocytes vs the virus found in kidney (although in the same experiment). The development of 60% of lymphocytes staining for IBV antigen seven days following the commencement of therapy was the most notable aspect of the CSA treatment period. This is equivalent to 42 days of age. Only once, on day 46, or 11 days after the CSA therapy began, was a virus found (in the kidney) following the CSA treatment. This occurred four days after the virus first appeared in the lymphocytes. But the sampling period was 35 days prior to 46 days. This was because, in earlier research (18), the virus initially surfaced 12 days after the beginning.

Nonetheless, the apparent discrepancy of results (42 days in lymphocytes, 46 days in kidney) poses the question as to which one shows reactivated virus first. Because of the late sampling in tissues, it is possible that virus was in the kidney before day 46.

However, another possibility is that the kidney after all, is not the site of IBV persistence, as indicated by previous work (22). Perhaps it resides in a tissue rich in lymphocytes such as the bone marrow or spleen. The bursa of Fabricius, an important lymphoid tissue, was examined, but no persistent virus was found there.

Future studies could perhaps benefit from these findings and persistent virus should be sought in other lymphoid tissues. Also, the identity of the lymphocytes bearing viral antigen should be determined by double staining. One thing is clear, it is not easy to predict the details of persistence after short-term studies.

CONCLUSION

The present study demonstrated that Infectious Bronchitis Virus (IBV) infection induces significant alterations in circulating T-lymphocyte populations in experimentally infected chickens. A marked increase in CD8⁺ cytotoxic T lymphocytes during the acute phase of infection highlights their essential role in controlling viral replication and facilitating viral clearance, whereas CD4⁺ T lymphocytes appear to contribute primarily to immune regulation and the development of adaptive immunity. Cyclosporine A-induced immunosuppression resulted in a significant reduction in circulating CD4⁺ and CD8⁺ T-cell populations and was associated with increased expression of IBV antigen in peripheral blood mononuclear cells, suggesting viral persistence and possible reactivation under immunosuppressive conditions. Furthermore, successful in vitro infection of isolated lymphocytes confirmed that these cells can support IBV replication and may serve as a potential reservoir for viral persistence. Overall, the findings

provide valuable insights into the immunopathogenesis of IBV infection and may contribute to the development of improved vaccination strategies and immunomodulatory approaches for the effective control of infectious bronchitis in poultry.

Strengths of the Study

This study provides a comprehensive evaluation of the cellular immune response to Infectious Bronchitis Virus infection in experimentally infected chickens. The use of flow cytometry enabled accurate quantification of CD4⁺ and CD8⁺ T-lymphocyte populations as well as detection of viral antigen expression in peripheral blood mononuclear cells. In addition, the inclusion of Cyclosporine A-induced immunosuppression and in vitro lymphocyte infection experiments provided novel insights into viral persistence, immune suppression, and the possible role of circulating lymphocytes as reservoirs for IBV. The combination of immunological, virological, and molecular approaches enhances the scientific strength and clinical relevance of the study.

Limitations of the Study

The present study has several limitations. It was conducted using a relatively limited number of experimental animals at a single research facility, which may restrict the generalizability of the findings. Long-term persistence of IBV beyond the study period was not investigated. Cytokine profiling and gene expression analysis of immune-related markers were not performed, which could have provided additional information regarding host immune responses. Furthermore, only one IBV strain was evaluated, and therefore the findings may not be directly applicable to other circulating genotypes.

Clinical Implications

The findings of this study improve the understanding of host cellular immune responses during Infectious Bronchitis Virus infection and emphasize the critical role of CD8⁺ cytotoxic T lymphocytes in viral clearance. Recognition of viral persistence in circulating lymphocytes during immunosuppression may assist in developing improved vaccination schedules, immunotherapeutic interventions, and disease control strategies for commercial poultry flocks. These findings may also contribute to better management of recurrent or persistent IBV infections under field conditions.

Future Scope

Future investigations should include multicentre studies involving different breeds of chickens and multiple IBV genotypes to validate the present findings. Long-term follow-up studies are required to determine the duration of viral persistence in lymphoid tissues. Further research involving cytokine profiling, transcriptomic analysis, immunophenotyping, and advanced molecular techniques would provide a better understanding of the mechanisms

underlying immune regulation and viral persistence. Evaluation of novel vaccine strategies targeting cellular immunity may also improve protection against emerging IBV variants.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

Author Contributions

Elhafi A. Giuma¹: Conceptualization, study design, experimental investigation, data collection, formal analysis, manuscript drafting, and revision.

Rabia A. Elzlitni²: Methodology, flow cytometry analysis, laboratory investigations, data validation, and manuscript review.

R. C. Jones²: Study supervision, experimental design, scientific guidance, data interpretation, critical revision of the manuscript, and final approval.

Samah Mosaad³: Molecular investigations, RT-PCR analysis, data interpretation, and manuscript editing.

Basma Salama⁴: Biochemical and molecular analyses, validation of experimental findings, and manuscript review.

Shimaa ElBaz⁵: Sample processing, laboratory investigations, data collection, and technical support.

Sahar Abd El Rahman³: Study supervision, data interpretation, manuscript review, and final approval of the manuscript.

Dr. Idress Hamad Attitalla⁶: Conceptualization, project administration, overall supervision, resources, critical manuscript revision, correspondence, and final approval of the manuscript.

All authors contributed substantially to the work, reviewed the manuscript critically for important intellectual content, and approved the final version for publication.

Data Availability Statement

The datasets generated and analysed during the current study are available from the corresponding author upon reasonable request.

Abbreviation	Full Form
IBV	Infectious Bronchitis Virus
CSA	Cyclosporine A
CTL	Cytotoxic T Lymphocyte
PBMC	Peripheral Blood Mononuclear Cell
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SPF	Specific Pathogen-Free
FITC	Fluorescein Isothiocyanate
PBS	Phosphate Buffered Saline
RPMI	Roswell Park Memorial Institute Medium
MHC	Major Histocompatibility Complex
APC	Antigen Presenting Cell
CMI	Cell-Mediated Immunity

REFERENCES

1. Alexander D, Gough R, Pattison M. A long-term study of the pathogenesis of infection of fowls with three strains of avian infectious bronchitis virus. *Research in veterinary science*. 1978;24(2):228-33.
2. Schalk AF, Hawn MC. An apparently new respiratory disease of baby chicks. 1931.
3. Crinion R, Hofstad M. Pathogenicity of four serotypes of avian infectious bronchitis virus for the oviduct of young chickens of various ages. *Avian Diseases*. 1972;351-63.
4. Lucio B, Fabricant J. Tissue tropism of three cloacal isolates and Massachusetts strain of infectious bronchitis virus. *Avian Diseases*. 1990;865-70.
5. Cook J, Davison T, Huggins M, McLaughlan P. Effect of in ovo bursectomy on the course of an infectious bronchitis virus infection in line C White Leghorn chickens. *Archives of virology*. 1991;118:225-34.
6. Townsend A, Bodmer H. Antigen recognition by class I-restricted T lymphocytes. *Annual review of immunology*. 1989;7:601-24.
7. Lin Y, Askonas BA. Biological properties of an influenza A virus-specific killer T cell clone. Inhibition of virus replication in vivo and induction of delayed-type hypersensitivity reactions. *The*

Journal of experimental medicine. 1981;154(2):225-34.

8. MCNEAL MM, Barone KS, RAE MN, WARD RL. Effector functions of antibody and CD8+ cells in resolution of rotavirus infection and protection against reinfection in mice. *Virology*. 1995;214(2):387-97.

9. Christensen JP, Doherty PC, Branum KC, Riberdy JM. Profound protection against respiratory challenge with a lethal H7N7 influenza A virus by increasing the magnitude of CD8+ T-cell memory. *Journal of virology*. 2000;74(24):11690-6.

10. Hegde NR, Srikumaran S. Reverse immunogenetic and polyepitopic approaches for the induction of cell-mediated immunity against bovine viral pathogens. *Animal Health Research Reviews*. 2000;1(2):103-18.

11. Tripp RA, Anderson LJ. Cytotoxic T-lymphocyte precursor frequencies in BALB/c mice after acute respiratory syncytial virus (RSV) infection or immunization with a formalin-inactivated RSV vaccine. *Journal of virology*. 1998;72(11):8971-5.

12. Mbawuike IN, Wyde PR. Induction of CD8+ cytotoxic T cells by immunization with killed influenza virus and effect of cholera toxin B subunit. *Vaccine*. 1993;11(12):1205-13.

13. Seo SH, Collisson EW. Specific cytotoxic T lymphocytes are involved in in vivo clearance of infectious bronchitis virus. *Journal of virology*. 1997;71(7):5173-7.

14. Peiris J, Lai S, Poon L, Guan Y, Yam L, Lim W, et al. Coronavirus as a possible cause of severe acute respiratory syndrome. *The lancet*. 2003;361(9366):1319-25.

15. Woldehiwet Z, Hussin A. Border disease virus antigens in lymphocyte subpopulations in the peripheral blood of persistently infected sheep. *Veterinary Immunology and immunopathology*. 1994;42(2):127-35.

16. Pohuang T, Chansiripornchai N, Tawatsin A, Sasipreeyajan J. Detection and molecular characterization of infectious bronchitis virus isolated from recent outbreaks in broiler flocks in Thailand. *Journal of Veterinary Science*. 2009;10(3):219-23.

17. Collisson EW, Pei J, Dzielawa J, Seo SH. Cytotoxic T lymphocytes are critical in the control of infectious bronchitis virus in poultry. *Developmental & Comparative Immunology*. 2000;24(2-3):187-200.

18. Smith KG, Hunt JL. On the use of spleen mass as a measure of avian immune system strength. *Oecologia*. 2004;138:28-31.
19. Yang Y, Pan C, Zhong R, Pan J. The quantitative models for broiler chicken response to monochromatic, combined, and mixed light-emitting diode light: A meta-analysis. *Poultry science*. 2018;97(6):1980-9.
20. Arstila TP, Vainio O, LASSILA O. Central role of CD4+ T cells in avian immune response. *Poultry science*. 1994;73(7):1019-26.
21. Sharma J. The avian immune system. *Diseases of poultry*. 2003;11:5-16.
22. Raj GD, Jones R. Infectious bronchitis virus: immunopathogenesis of infection in the chicken. *Avian Pathology*. 1997;26(4):677-706.
23. Russell P, Dwivedi P, Davison T. The effects of cyclosporin A and cyclophosphamide on the populations of B and T cells and virus in the Harderian gland of chickens vaccinated with the Hitchner B1 strain of Newcastle disease virus. *Veterinary immunology and immunopathology*. 1997;60(1-2):171-85.
24. Kim I-J, You SK, Kim H, Yeh H-Y, Sharma JM. Characteristics of bursal T lymphocytes induced by infectious bursal disease virus. *Journal of virology*. 2000;74(19):8884-92.
25. Rautenschlein S, Yeh H-Y, Sharma J. The role of T cells in protection by an inactivated infectious bursal disease virus vaccine. *Veterinary immunology and immunopathology*. 2002;89(3-4):159-67.
26. Paramithiotis, G., & Golec, S. (2011). Avian CD4(+) T cells: subsets and functions. *Veterinary Immunology and Immunopathology*, 140(1-2), 1-11.
27. Attitalla, I.H. (2025). *T-Cell Mediated Immunosuppression And Its Impact On Avian Infectious Bronchitis Virus Persistence In Chickens*. SEEJPH Volume XXVI, S1.
28. Elhafi A. Giuma et al. (2025). *Comparative Analysis Of Pathogenesis In Two Strains Of Infectious Bronchitis Virus In Chicks: Implications for Future Control Strategies*.
29. Elhafi A. Giuma et al. (2025). *Exploring the Extended Pathogenesis of Infectious Bronchitis Virus Strain M41: Insights from Expected and Novel Tissue Targets in Chicks*.
30. Attitalla, I.H., et al. (2011). *Modified CTAB method for high quality genomic DNA extraction from medicinal plants*. *Pak J Biol Sci*.
31. Attitalla, I.H., et al. (2004). A rapid molecular method for differentiating two special forms of *Fusarium oxysporum*. *Mycological Research*.