Jurnal Veteriner
pISSN: 1411-8327; eISSN: 2477-5665
Terakreditasi Nasional SK. No. 15/XI/Dirjen Dikti/2011

Juni 2016 Vol. 17 No. 2 : 197-204
DOI: 10.19087/jveteriner.2016.17.2.197
online pada http://ojs.unud.ac.id/php.index/jvet

# Lymphocytes Subpopulation in Peripheral Blood and Spleen of Village Chickens Recognized by Monoclonal Antibodies

**(***SUBPOPULASI LIMFOSIT PADA DARAH TEPI DAN LIMPA AYAM KAMPUNG YANG DIKENALI OLEH ANTIBODI MONOCLONAL***)**

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**Abstract**

Lymphocytes play important role in host defence system against pathogenic agents both in mammalian and avian species. Monoclonal antibodies (mAbs) have been widely used to identify lymphocytes subpopulation in a host based on their surface cluster differentiation (CD) markers. Currently, mAbs against lymphocytes surface markers of village chickens have been produced by fusion of myeloma with lymphocytes derived from spleen of mice immune to chicken lymphocytes. In two fusion experiments, 623 clones of hybridomas were produced and four (BG4, CB1, DB2 and BB2) of which secreted mAbs against chickens lymphocyte surface molecules. Two mAbs (BG4 and DB2) recognized protein of 32 kDa, one mAb (CB1) recognized protein of 64 kDa, and one mAb was unable to recognize any protein of chicken lymphocyte surface molecule. Three mAbs recognized lymphocyte subpopulation in spleen and peripheral blood of village chickens. In peripheral blood, mAbs BG4, CB1 and DB2 recognized lymphocytes subpopulation with the percentages of 11.2%, 21.4% and 7.4% respectively. In spleen those three mAbs recognized lymphocytes subpopulations at the percentages of 38.2%, 51.54% and 31.5% respectively. Based on those result, it is very likely that mAbs BG4 and DB2 recognized CD4 molecule and mAb CB1 recognized CD8 molecule of village chickens lymphocytes.

Key words: CD8+, CD4+, lymphocytes, chickens, monoclonal, antibodies

**Abstrak**

Limfosit berperan amat penting dalam system pertahanan tubuh inang terhadap patogen , baik pada hewan mamalia maupun unggas. Antibodi monoklonal (AbMo) telah dipakai secara luas untuk mengidentifikasi subpopulasi limfosit berdasarkan protein khas permukaannya yang disebut molekul *cluster differentiation* (CD). Antibodi monoclonal terhadap molekul permukaan limfosit telah dibuat dengan cara fusi sel myeloma dengan limfosit asal linpa mencit yang kebal terhadap limfosit ayam kampung. Dari dua fusi, dihasilkan sebanyak 623 klon hibridoma dan empat klon di antaranya (BG4, CB1, DB2, dan BB2) menghasilkan AbMo terhadap molekul permukaan limfosit ayam kampung. Dua AbMo (BG4 dan DB2) mengenali protein 32 kDa, satu AbMo (CB1) mengenali protein 64 kDa, dan satu mAb tidak bereaksi dengan protein permukaan limfosit pada uji Western blotting. Tiga AbMo kemudian dipakai dalam uji imunositokimia untuk melacak subpopulasi limfosit yang dikenali oleh AbMo. Pada darah tepi, AbMo BG4, CB1, dan DB2 mengenali suppopulasi limfosit dengan persentase berturut-turut 11,2%, 21,4%, dan 7.4%. Dalam limpa, ketiga AbMo tersebut mengenali limfosit dengan persentase berturut-turut 38,2%, 51,54%, dan 31,5% . Dari hasil Western blotting dan imnositokimia, tampak BG4 dan DB2 mengenali molekul CD4 danAbMo CB1 mengenali molekul CD8 pada permukaan limfosit ayam kampung.

Kata-kata kunci: CD8+, CD4+, limfosit, ayam, kampung, antibodi, monoklonal

## INTRODUCTION

Lymphocytes play important role in the immune system of both mammalian and avian species. Based on their surface cluster differentiation (CD) markers, lymphocytes are grouped into many subsets (subpopulation) and each subset plays specific functions in the immune system. Two lymphocyte subsets that play important role in the immunity against infectious disease are CD4 and $CD8^+$T lymphocytes. $CD4^+$ cells consist mainly of T-helper (Th) and T regulatory (T reg) lymphocytes. Meanwhile, $CD8^+$ T cells consist of mainly T cytotoxic (Tc) cells, although in human, CD8 molecule is also expressed on surface of dendritic cells. Cells expressing CD4 (Th and Treg) and CD8 cells (Tc) play important role in host defence system against intracellular and extracellular pathogens such as virus, and intracellular bacteria and parasites (Koretzky. 2010). Both CD4 $^+$and $CD8^+$ T lymphocytes originate from bone marrow which then migrate into thymus in which they undergo maturation into thymocytes. In this maturation, double CD4+/ CD84+ T cells differentiate into mature naïve T cells either as CD8+/CD4 or CD4+/CD8 T cells. These mature naive T cells then migrate from thymus to peripheral blood and secondary lymphoid organs including spleen (Wang *et al.*, 2012) to be activated in response to antigen exposure.

$CD4^+$ and $CD8^+$ T lymphocytes play important role in both cellular and humoral immune response against pathogens. In cellular immunity, $CD4^+$ Th lymphocytes produce IL-2, a proliferating and activating factor for T lymphocytes and natural killer (NK) cells (Bihl *et al.*, 2010). Activation and secretion of interferon γ(IFN γ) by natural killer cells also dependent upon the help of $CD4^+$ cells (Bihl *et al.*, 2010). IFNγ secretion by NK cells enhances the ability of macrophages in killing pathogens (Herbst *et al.*, 2011). In humoral immune response, the role of $CD4^+$ T lymphocytes is in the production of IL-4 by T-helper 2 cells which is required for the proliferation and maturation of B cells into plasma cells (King and Mohrs, 2009). Treg $CD4^+$ cells which regulate immune system develop outside thymus under sub immunogenic antigen presentation during chronic inflammation (Curotto de Lafaille and Lafaille, 2009). In regulating immune system, Treg cells produces cytokines such as Il-10 which suppress the immune inflammatory response by inhibiting the production of proinflammatory cytokines such Il-1β, Il-6 and TNFα (Couper *et al.*, 2008). Meanwhile, $CD8^+$ cytotoxic T cells play roles in the destruction cells infected by intracellular pathogens in an effort to limit the spread and clearance infectious agents from the infected individual (Nagata and Koide, 2010). In doing such a function, $CD8^+$ cells require help from Th-1 cells which produces IL-2, a cytokine that activates most T cells including CD8+ T cells (Boymen and Sprent, 2012).

Recently, monoclonal antibodies (mAbs) against lymphocyte surface markers of village chickens have been produced and some of which recognized proteins of 32 kDa, while others recognized the protein of the molecular weight of 64 kDa. CD8 molecules in many avian species have been reported to have molecular weight of 32-35 kDa (Li *et al.*, 1999) which is similar to CD8 molecule found in the lymphocytes of mammalian species (33 to 34 kDa) (Giblin *et al.*, 1989). Meanwhile, CD4 molecule of avian species is reported to be around 64 kDa (Lutala, 1998 ) which is slightly larger the molecular weight of $CD4^+$ molecule found in human (55 kDa) (Lynch *et al.*, 2006). The 32 kDa and 64 kDa proteins on the surface of lymphocytes recognized by mAbs produced in this study appear to be CD8 and CD4 proteins respectively. A study was therefore conducted to investigate lymphocyte subpopulation recognized by those mAbs in peripheral blood and spleen of village chickens.

## RESEARCH METHODS

### Preparation of Lymphocytes for Immunization of Mice

Lymphocytes were isolated from spleens collected of village chickens slaughtered for human consumptions. After the removal of the capsules, the spleens were torn and scrapped by using bent 22 G needle. The spleenocytes were passed several times gradually through 18 G, 22 G and 23 G needle in 3 mL syringes. After the disruption into individual cells, the lymphocytes were purified using picoll-paque density gradient centrifugation. The lymphocytes were then washed twice with buffered saline solution (PBS). The purified lymphocytes were stored at -20$^o$C in aliquots.

**Immunization of Mice with Lymphocytes**

Four female mice of seven week-old were immunized approximately with 2 x $10^7$ chicken lymphocytes suspended in 0.5 mL serum-free *Dulbeco modified essential medium* (DMEM) without adjuvant. The immunizations were conducted six times intraperitonelally at weekly interval. Blood from the immunized mice was collected and tested by ELISA to detect the antibody response against the lymphocyte surface molecules. When appropriate immune response of mice against chicken lymphocytes was detected, one mouse was boosted with the same antigen and used for preparation hybridomas.

**Preparation of Hybridomas for the Production of Monoclonal Antibodies**

At three days before fusion, a mouse was boosted by immunization with the same antigen as described above. Lymphocytes were isolated from spleen, washed once with serum-free medium. As many as $10^8$ lymphocytes derived from spleen were fused with 2 x $10^7$ myeloma cells using 45% polyethylene glycole (PEG). The fused cells were cultured in *Dulbeco modified essential medium- hypxanthine amainopterin thymine* (DMEM-HAT) selective media containing 20% *fetal bovine serum* (FBS), *hypxanthine amainopterin thymine*, $10^6$ cells feeder per mL at 37°C incubator with 5% $CO_2$ atmosphere. At day seven, the cells were cultured in DMEM-HAT (DMEM-HAT without aminopterin). When large clones of hybridomas were observed in micro plate wells, the hybridoma media was collected and screened for mAbs by enzyme-linked immunosorbent assay (ELISA). The antibody-secreting hybridomas were the isolated and used for the production of mAbs against lymphocyte surface markers.

**ELISA for Screening MAbs**

ELISA used for screening hybridomas were developed using intact lymphocytes as antigen. Wells of ELISA micro plates were firstly treated with poly-L-lysine for 15 minutes at room temperature and air dried at 37°C. Purified lymphocytes derived from spleens of village chickens were then added to each well, and were incubated at 37°C until dry. The cells in micro plate wells were fixed with methanol for 10 minutes at room temperature, and treated with 3% $H_2O_2$ in PBS for 20 minutes. Micro plate wells were washed twice with PBS and blocked with 5% skim milk for one hour at room temperature. Fifty µL 5% skim milk in PBS containing 0.5% Tween-20 and 50 µL hybdidoma medium were added to each well and incubated for one hour at room temperature. Antimouse IgG-horseradish peroxidase conjugate (KPL USA) diluted 1:1000 in PBS-T was added into each well. TMB substrate and stopping buffer were added into each well and the absorbance of substrate in each well was read by ELISA reader.

**Determination of Proteins Weight Recognized by mAbs**

Purified lymphocytes prepared as above were diluted in sample reducing buffer (SDS 2.3 %, mercaptoethanol 5%, Tris-HCl 0.0625 M. pH. 6.8, glycerol 10%, bromophenol blue 0.001%) and subjected for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins on the gel were transferred onto nitrocellulose membrane using mini transblot cells (Biorad, USA). Transferred protein onto nitrocellulose membrane was the blocked with 5% skim milk in PBS for one hour at 37°C. The membrane was then cut into 5 mm strips and each strip was soaked in hybridoma culture medium diluted 1:10 in Tris buffered saline (TBS) for overnight at 4°C. Antimouse IgG-alkaline phosphatase was the added and incubated for one hour at 37°C. The lymphocyte proteins recognized by mAbs was visualized addition of BCIP/NBT substrate.

**Detection of Lymphocyte Population of Village Chickens Recognized by mAbs**

Ten young adult village chickens purchased from local traditional markets were used in this study. Peripheral blood lymphocytes were isolated by collecting whole blood in tubes containing EDTA as anticoagulant. The blood was then centrifuge at 1000 x g for 10 minutes and the buffy coat layer in the interspace between plasma and red blood cells was collected, diluted 1:5 in PBS, and layered on the top of picoll-paque in 15 mL centrifuge tube for density gradient centrifugation. The tubes were then centrifuged at 1500 x g for 20 minutes. Lymphocytes on the top of ficoll were collected, washed twice with PBS. Smears of lymphocytes were made on poly-L-lysine microscope slide, air dried and fixed with acetone for 10 minutes. Lymphocytes from spleen used for ELISA test were prepared in the same manner as those for immunization of mice

Immonocytochemistry staining technique was used detect lymphocytes recognized by mAbs. Firstly, mAbs diluted 1:10 in 2% skim milk in PBS was added to lymphocyte smear prepared on microscope slides. The cells were then incubated for one hour at room temperature, and

washed twice with PBS. One drop of biotinylated anti-mouse IgG (KPL-USA) was added onto the lymphocytes smear and incubated for 30 minutes at room temperature. Following three times washes as above; one drop of streptavidin-horseradish peroxidase was added onto lymphocyte smears and incubated for 20 minutes at room temperature. The cells were finally washed four times with PBS and diazino-benzidine (DAB) substrate were added. Counterstaining with Meyer Hematoxylin was conducted and the cells were washed twice with tap water. Glycerin was then added and cells were examined under microscope.

## RESULT AND DISCUSSION

### Mice Immune Response and Establishment of Hybridomas

In this study, four mice were immunized with chicken lymphocytes in the preparation of murine immune spleenocytes for use in the production of hybridomas. Following six times immunization at weekly interval, all mice showed antibody titer of $10^{17}$-$10^{18}$ against chicken lymphocytes. Two mice were used in two fusion experiments and as many as 623 hybridomas were produced. Four out of the 623 hybridomas produced mAbs against chicken lymphocytes surface molecules (Table 1).

### Characteristics of mAbs Against Lymphocyte Surface Antigens

In this study, five mAbs against lymphocytes surface antigen were produced and they were designated as BG4, CB1, DB2, and BC2. The isotypes of the mAbs were IgG1 (CB1, BB2), IgG2a (BG4), IgM/IgG1 (DB2)(Table 1). In Western blotting assay, two mAbs (BG4, DB2) recognized lymphocytes protein of 64 kDa and one mAb (CB1) recognized protein of 32 Kda (Figure 1). One mAb was unable to recognize lymphocyte protein in western blotting assay.

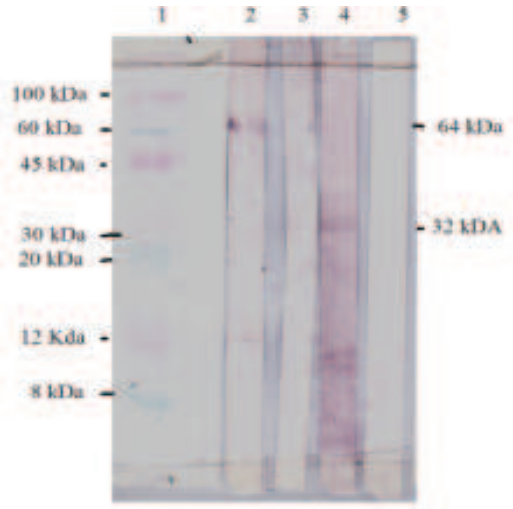


Figure 1. Proteins of chicken lymphocytes recognized by monoclonal antibodies. Lane 1: Molecular weight markers, Lane 2-5: mAbs BG4, DB2, CB1 and BB2.

Table 1. Immune response of mice and mAbs-secreting hybridomas produced by fusion of myelomas and spleenocytes of immunized mice

| Fusion | Mice antibody titer | Total hybridomas | mAbs secreting hybridomas |
|---|---|---|---|
| I | $10^{17}$ | 298 | 1 |
| II | $10^{18}$ | 335 | 3 |
| Total | 623 | 4 | |

Table 2. Characteristics of monoclonal antibodies against lymphocytes surface molecules of Village chickens

| mAbs | Isotypes | Reactive Protein | Peripheral blood | Spleen |
|---|---|---|---|---|
| BG4 | IgG2a | 64 kDa | +++ | +++ |
| CB1 | IgG1 | 32 kDa | +++ | +++ |
| DB2 | IgM/IgG1 | 64 Kda | +++ | +++ |
| BB2 | IgG1 | ND | ++ | ++ |

ND: not determined

**Lymphocyte Subpopulation Recognized by mAbs**

In study ficoll-paque purified lymphocytes derived from peripheral blood and spleen of village chickens were used. In immunocytocehmistry staining, mAb BG1 recognized lymphocytes derived from peripheral blood and spleen with the percentages respectively of 11.4% and 38.2% of the total mononuclear cells. Using same staining method, the percentage of lymphocytes subpopulation recognized by mAb CB1 was higher than recognized by mAb BG4 (Figure 3) which were 21.7% and 51.3% respectively in peripheral blood, and spleen. Meanwhile, the percentages of lymphocytes subpopulation in peripheral blood and spleen recognized by mAb DB2 were 7.5% and 31% respectively (Table 3, Figure 2).

Immunization of mice and screening of hybridomas appeared to be two most important factors determining the success in the production of mAbs against chicken lymphocyte CD markers. In this study, mice used for their production of mAbs were chicken lymphocytes derived from spleen without being emulsified in adjuvant, but they were capable of inducing a reasonably good antibody response in mice as the titer of antibody against chicken lymphocytes surface marker was above $2^{17}$. Such immune response is required in order to obtain hybridomas stably secreting mAbs against lymphocytes CD markers. The ability of an antigen to induce good immune response in a host is determined many factors and one of which is its foreignness to the host (Khan, 2009).

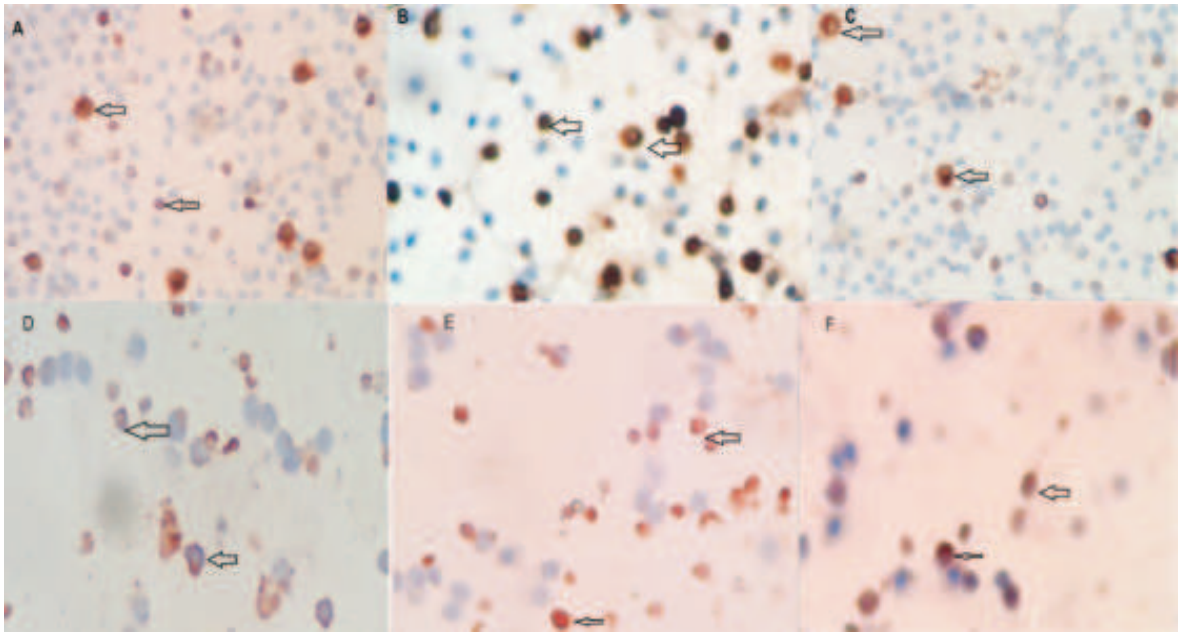


Figure 2. Lymhocytes subpopulation in peripheral blood and spleen of village chickens recognized by monoclonal antibodies. Peripheral blood lymphocytes (A, B and C), spleen lymphocytes (D, E and F). mAbs BG4 (A and D), CB1 (B and E), DB2 (C and F). Positive cells (arrow)

Table 3. The percentages of lymphocytes recognized by 3 mAbs in peripheral blood and the spleen of village chickens

| mAb | MW | Istoype | Peripheral blood | Spleen |
|---|---|---|---|---|
| BG4 | 64 kDa | IgG2a | 11.2% | 38.2% |
| CB1 | 32 kDa | IgG1 | 21.4% | 51.54% |
| DB2 | 64 kDa | IgM/IgG1 | 7.4% | 31.5% |

mAb: monoclonal antibody. MW: molecular weight

Chicken and mouse are two species which are antigenically distant species in animal Kingdom and cells including lymphocytes from the two species are therefore expected to contain many proteins which foreign to each other. The use of chicken's spleen cells for immunization of mice is expected to induce a good immune response.

Another important factor for the success of producing mAbs against surface markers of chickens lymphocytes was the screening test for detecting which hybriidomas produced mAbs against lymphocytes surface marker was also important. In this study, antigen used for ELISA was purified lymphocytes obtained from the spleen of village chickens. They were coated onto ELISA micro plate wells which have previously been treated with poly-L-lysine. The cells were then fixed with methanol and treated with $H_2O_2$ to block endogenous peroxidase that can interfere with enzyme activity used for ELISA. The use of ELISA test for screening mAbs against surface marker protein of lymphocytes was simple and can be used for screening many hybridomas at one times. The use of cells antigen for ELISA test has been used for to detect both intracytoplasmic and cell membrane protein (Bishop and Hwang, 1992)

In Western blotting, the protein recognized by one mAb (BG4) was 64 kDa protein, while two mAbs (CB1 and DB2) recognized protein at the molecular weight of 32 kDa. The 64 kDa and 32 kDa proteins recognized by mAbs (BG4 and DB2) and CB1 were likely to be CD4 and CD8 molecules respectively. In chickens, CD8 molecules found on the surface of lymphocytes has been reported to have molecular weight of 32-34 kDa (Nestor *et al.*, 1999), whereas CD4 molecule has been reported to have molecular weight of around 64 kDa (Luhtala, 1998). It was not yet known, however, whether the CD8 molecule recognized by mAb CB1 was á or â chain as it has been reported that CD8 molecule of avian and mammalian species consist of á and â chains (Gobel *et al.,* 1993).

In peripheral blood and spleen of village chickens, the lymphocyte subpopulation recognized by mAb BG4 was around 11.2% and 38.2% of the total lymphocytes respectively, whereas those recognized by mAb CB1 were 21% and 51.2% of the total lymphocytes. The populations of $CD4^+$ T lymphocytes in peripheral blood of chickens were generally lower than those of CD8 lymphocytes (Kannan *et al.*, 2012). Other workers also reported the population of CD8 cells to be 42-51% of the total T lymphocytes in young adult chickens and 56-62% of the total T lymphocytes in adult chickens, whereas CD4+ T lymphocytes population was 35-41% of the total T cells in young adult and 31-35% of the total T cells in adult chickens (Bridle *et al.*, 2006) . In thymus, subpopulation of CD4 and CD8 cells were reported to consist of double (CD8 and CD4) positive cells, $CD4^+$ $CD^-$ ells and $CD8^+CD4^-$ cell which were respectively around 7%, 11% and 68%. (Vaziry *et al.*, 2006). Circulating T lymphocyte subpopulation including CD8+ and CD4+ T cells have been widely used for the evaluation of the immunocompetence of hosts (including chickens) against many infection (Bacon, 1992).

T lymphocytes in peripheral blood and spleen originate from T progenitor cells of bone marrow which then migrate into thymus for maturation and differentiation into naïve C4+/CD8 (CD4+) or CD8+/CD4 (CD8+). These naïve mature T lymphocytes the migrate into peripheral blood and secondary lymphoid organs such as spleen for activation by antigen (Germain, 2002). In addition to T lymphocytes, peripheral blood and spleen also consist of B and ã/ä cells. B cells also originate from bone marrow and in chicken they migrate to Bursa fabricius for maturation into naïve mature B cells (Tucker *et al.*, 2008).

In chicken thymus, large population (67-78%) of T lymphocytes are double positive cells expressing both CD4 and CD8 molecules. T cells expressing CD8+/CD- and CD4+/CD8- account for 7–12% and 6–7% respectively (Vaziry *et al.*, 2011). In peripheral blood and spleen, lymphocytes consists of both those originate from bursa (B cells) and thymus (Tcells) as well as from other lymphoid organs such as ã/ä T cells. The ratio of CD4+/CD8+ cells in peripheral blood and the spleen has been used widely as the indicator of immune system in many species including chickens. The use of peripheral blood is more practical as they can represent all secondary lymphoid organs and can be examined without sacrificing the animal.

## CONCLUSION

The result of of this study indicate that mAbs BG4 and DB2 recognized CD4 molecule and mAb CB1 recognized CD8 molecule of village chickens lymphocytes.

## SUGGESTION

Further studies are however still required to confirm that the two protein recognized by mAbs were CD4 and CD8 molecules.

## ACKNOLEDGEMENT

I wish to thank all for help in providing village chickens in this study. This work was conducted own self funding.

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