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Hj. Sri Iswati
Anwar Ma'ruf
Dina Sunyowati
Ignatius Nalarkusumamurti Sutirto

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Implementation of Climate Change Agreement to Meet Sustainable Development Goals (ICPSUAS 2017)

Antigenic Protein of *Leucocytozoon caulleryi* schizont Inducing Cellular Immune Response: TLR-2 and CD4 as Marker

Nunuk Dyah Retno Lastuti[1a)]
Endang Suprihati[1)]
[1)] Department of Parasitology, Faculty of Veterinary Medicine Universitas Airlangga, Surabaya
[a)] Corresponding Author: nunuk_dyah@yahoo.com

Dony Chrismanto[2)]
[2)]Study Program of Animal Health, Faculty of Vocation Universitas Airlangga, Surabaya
Anwar Ma'ruf [3)]
[3)] Department of Basic Medical Science Universitas Airlangga, Surabaya

Abstract: Leucocytozoonosis is caused by *Leucocytozoon caulleryi* and is responsible for death in chickens by bleeding. Leucocytozoonosis is an endemic disease in Indonesia and incidences have been reported in several regions in East and Central Java. The financial losses caused by this disease include growth disorders in chick, decreased egg production, higher mortality rate and also a higher production cost. This research aims to detect TLR-2 and CD4 expression as a cellular immune response in rabbits immunised by the protein of *L.caulleryi* schizont. It is needed as a preliminary research study for molecular vaccine development which is considerably effective when it comes to preventing leucocytozoonosis occurance in Indonesia. This research study was performed in several stages i.e. **the** isolation of *L.caulleryi* schizont from a chicken liver infected with leucocytozoonosis to be based on the clinical signs observed, microscopic examination, and the pathological changes in the other chicken organs. The purification of the soluble protein of *L.caulleryi* schizont including the immunisation of the rabbits. Each of the experimental rabbits was injected with 500 µg of *L.caulleryi* schizont protein and added adjuvant complete with a ratio of 1:1. Every two weeks the injection was performed with the same protein with a dosage of 500 µg each and an added adjuvant that was incomplete (the booster was performed 5 times in 2 weeks). The examination of the cellular immune response of CD4 and TLR-2 expression in the rabbits' T cells using an immunocytochemistry method visualised by fluorescein isothiocyanate. The examination of the results was done by immunocytochemistry showing TLR-2 and CD4 expression as yellow to green fluorescent colour, mainly in the 5th booster where the activation of the CD4 co-receptor and TLR-2 occurred. The conclusion shows that the antigenic protein of *L.caulleryi* schizont has the ability to induce a cellular immune response through the co-receptors CD4 and TLR-2 in the rabbits' T cells as the preliminary research in the sub-unit vaccine development for leucocytozoonosis in chickens.

KEY WORD: *antigenic protein, Leucocytozoon caulleryi, schizont, TLR-2, CD4.*

I. INTRODUCTION

Leucocytozoonosis is one of the diseases caused by *Leucocytozoon caulleryi* in poultry, which is transmitted by flies Culicoides sp. or Simulium sp. Leucocytozoonosis is an endemic disease in Indonesia and incidences have been reported in several regions in East and Central Java. Leucocytozoon parasites infect a large number of avian hosts, including the domestic chicken, and causes a significant economical loss to the poultry industry (1). The financial losses impacted by this disease include growth disorders in chicks, decreased egg production, higher mortality rate and also a higher production cost (2, 3, 4, 6). The occurrence of leucocytozoonosis in broiler is between 7-40%, while the mortality rate in chicks is about 7-50% and in layer is about 2-60% respectively (7). The clinical signs observed in chickens are green faeces, depression, a loss of appetite, vomiting blood, paralysis and death due to bleeding (8). In order to overcome leucocytozoonosis in chickens, farmers have carried out the dispensing of medications, eradicating flies using insecticides, and improving the water irrigation in the area around the henhouse, but the latter method is less efficient due to the rapid growth of flies. Based on the vaccine developed by Onaga *et al* (1999), chickens can be protected from *L.caulleryi* infection by giving them a second generation schizont extract and blood serum containing an antigen (5). The weakness of this live vaccine administration is due to the possibility of infection because the parasites may become pathogens when the host's condition is weakened. Molecular vaccine development is considerably more effective when it comes to preventing leucocytozoonosis occurance in

Indonesia. Based on the phylogenetic analysis of Cytochrom B *Leucocytozoon* spp in broilers, it was shown that the *L.caulleryi* from various endemic regions is highly homologous (>95%) (6). Referring to the problems above, a preliminary research about the rabbits' immune response induced by *L. caulleryi* schizont protein with TLR-2 and CD4 as marker to explore the cellular immune response is necessary.

TLR is a membrane protein that helps receptor recognition patterns in response to various molecule derivatives from microbes and stimulated innate immunity due to microbe molecule exposure. TLR is known to be a recognition receptor which is involved in pathogen-associated molecular patterns (PAMP) recognised by pattern recognition molecules (PRMs). A phagocytes development system in recognising pathogens can be stimulated any time to respond as an inflammatory system. TLR stimulation through microbial product initiates the signalling pathways which activate not only the innate immunity but also adaptive immunity (9, 10). CD4+T cells play a central role in immune protection and the B cells to produce antibodies, to induce the macrophages to develop enhanced microbicidal activity, to recruit neutrophils, eosinophils, and basophils to the sites of infection and inflammation, and through their production of cytokines and chemokines (11). As a vaccine kit candidate, it is required to know whether or not the *L.caulleryi* protein can induce either humoral or cellular immune response, because then a favourable immune response and immunogenic protein can be explored. Immunogenic protein has main characteristics such as a heavy molecule weight, homogeny and a complex chemical structure and alienation (13, 15). It is necessary to study whether the results of the immunogenic proteins from a liver containing *L. caulleryi* can be developed for use in a vaccine in the effort to overcome Leucocytozoonosis, for example, with a vaccination program for chickens with a vaccine sub-unit that is safe in its use.

II. METHODS

A. Isolation and identification of L.caulleryi schizont

Schizont *L.caulleryi* isolated from chicken liver infected by leucocytozoonosis is based on the clinical signs observed, microscopic examination and the pathological changes of other chicken organs. Microscopic examination was performed to detect any gametosit stadium developed in eritrosit. Then, further assesment was done on several other organs such as the liver, spleen and intestine to detect the schizont stadium by crushing the organ and a pathological examination. The chicken liver and spleen containing schizont *L.caulleryi* was isolated in 50-100 mg or 0.05 ml cultured wet pellets, with a 2-3 ml 2-Drehydration solution/sample buffer added. Next, the sample was put on ice, sonicated for 30 seconds and cooled down to -80°C for 5 minutes. This treatment was repeated four times. The sample was centrifuged using a microcentrifuge (16.000 x g) for 20-30 minutes at 18-20 °C, and was then taken out from the centrifuge, Supernatan was put into a clean tube and the sample was stored at -80°C.

B. Immunization of rabbits

This research used five rabbits treated as per the animal welfare concept. They were given a health examination based on both clinical symptoms and laboratory tests. All of the animals were handled in strict accordance with Ethical Clearance and the experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine, of the Universitas Airlangga, No: 630-KE. Four-month old naïve rabbits were prepared for the immunisation trial at the laboratory of experimental animals, in the Faculty of Veterinary Medicine at the Universitas Airlangga. Each of the experimental rabbits were injected with 500 µg *L.caulleryii* schizont protein (0.3 ml) with an added Freund adjuvant complete (Sigma, USA) with a ratio of 1:1. The injection was performed every two weeks with the same protein with a dosage of 500 µg each and with the added adjuvant incomplete (Sigma, USA). The immunisation (booster) was performed 5 times in 2 weeks. Prior to the first injection, about 10 ml of rabbit blood was taken for TLR-2 and CD4 examination as preliminary data (control) and whole blood examination was conducted at the end of the first booster until the fifth booster (13, 15).

C. Examination of TLR 2 and CD4 expression using Immunocytochemistry

The principle of immunocytochemistry examination is that it is an immunology technique used to visualise specific proteins or antigens in the cells using the first antibody (**www.abcam.com/index** html). Several stages of the examination will be explained: 1) the blood sample is washed using 10% PBS-T20 five times, and the sample is fixated with 100% methanol (10 minutes) or with paraformaldehyde in PBS pH 7,4 for 15 minutes at room temperature, 2) the sample is washed twice using cold PBS, the sample is incubated for 10 minutes in PBS consisting of 0.1% Triton X-100 or 100 mM digitonin, and then the cells are washed in PBS three times for 5 minutes, 3) the cells are incubated with 1% BSA in PBS-T20 for 30 minutes, and incubated in conjugated antibody TLR 2-FITC labelled (Abcam's RabMab, USA) and CD4-FITC labeled (Abcam's RabMab, USA) and diluted in 1% BSA in PBS-T20 at room temperature for an hour or at night at a temperature of 4°C, 4) the cells are washed three times in PBS (5 minutes for each washing). The results were examined by using a fluorescent microscope using a magnification of 200 times, to find out whether the yellow to green fluorescent colour from the T cells expresses TLR-2 and CD4.

III. RESULTS AND DISCUSSION

The cellular immune response was shown by the expression of TLR-2 and CD4 in the rabbit T cells marked by the yellow to green fluorescent colour after the rabbit immunisation (Figure 1 and Figure 2).

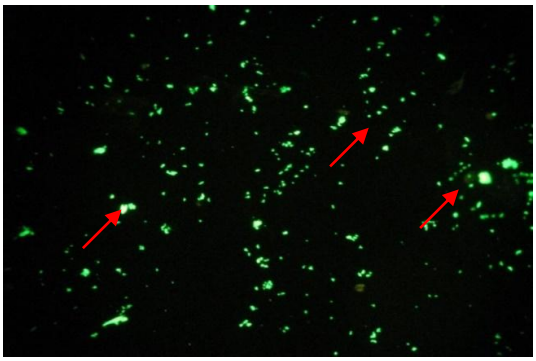

Figure 1. TLR2 expression in rabbit T cells visualized by Fluorescein Isothiocyanate (FITC) (200x magnification).

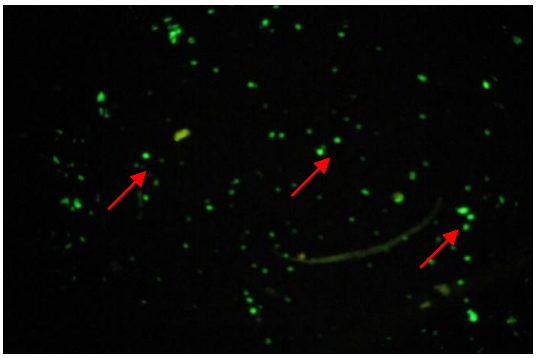

Figure 2. CD4 expression in rabbit T cells visualized by Fluorescein Isothiocyanate (FITC) (200x magnification).

It has been known that the toll-like receptor (TLR) is a receptor that can recognise the ligand from microbes or parasites which are involved in innate immunity. Along with new developments, TLR not only plays a role in innate immunity but also in adaptive immunity (12, 13). The examination results from the immunocytochemistry shows that there is a yellow to green fluorescent colour visualised by fluorescein isothiocyanate (FITC). It shows that the stadium schizont *L.caulleryi* protein has a ligand that is recognised by TLR 2 as a form of signal transduction which actívates and induces a cellular immune response such as the lympocyte T of the rabbits after immunisation. In accordance with the principles of the immunisation method, which is an increasing degree of immunity, it provides protective immunity by inducing a memory response towards a specific pathogen with a non-virulent or non-toxic antigen (14).

According the results of the research, it is shown that the antibody TLR-2 can recognise the ligand from the protein antigen *L.caulleryi* schizont by stimulating T cell activation, marked by the presence of yellow to green fluorescent colour which increased in accordance with the treatment from the various boosters. When the antigen of *L.caulleryi* schizont enters the body, it will be caught by macrophage or dendritic cells and the phagocytes cells will be activated by TLR as a signal transducer. *L.caulleryi* schizont possess a ligand or pathogen-associated molecular pattern (PAMP) which is recognised by TLR-2. Ligands that are recognised by TLR-2 consist of lipoprotein/lipopolypeptide, flagelin, ssRNA and CpG DNA. The schizont of *L.caulleryi* is an intracellular microorganism which contains antigens. When the antigen enters the body, it will be caught by a macrophage and the phagocytes cells will be activated by the TLR as a signalling pathway. The signal produced by TLR will activate the transcription factor NFκB which stimulates cytokine production (10, 16). NFκB activation is initiated by a signal which recruits MyD88 and interacts with the IL-1 receptor associated kinase (IRAK). Autophosphorylation then occurs, separating MyD88 and activating the TNF receptor associated factor 6 (TRAF-6) to activate the IκB kinase (IKK). Activated IKK will activate NFκB to transcript gene IL-12, IL-10, IL-4, TNF-α, IFN-γ. IL-2 roles will increase the cytolytic activity from the cytolytic T lymphocytes. This will also promote Th1 cells development together with CD8 activation in order to produce IL-2 which stimulates the proliferation and differentiation of B cells that will produce antibodies. IL-4 is a cytokine which is produced by subset Th2 from Th cells CD4 that function to induce Th2 cells differentiation and stimulate IgE production. (17, 18).

The main function of CD4 is acting as a transduction signal in antigen recognition and to strengthen the bond between T cells and antigen-presenting cells (APC). APC produces IFN-γ and IL-12 that stimulate the differentiation of CD4+ cells into Th1 which plays a major role in delayed hypersensitivity reactions. CD4+ T-cells produce a protein named IL-4 cytokine which helps B lymphocytes in antibody production and phagocytosis to destroy ingested microbes (11, 17). CD4 molecules as a co-receptor are a surface cell molecule which are expressed by various types of cells in the immune system which were formed by cluster differentiation. The accessory molecule is used as a marker of Th cell activation with B cells and cytotoxic T cells maturation, which is responsible in regulating chronic inflammatory reactions towards antigens through macrophage stimulation. Lymphocytes B activation is marked by a significant increase ($p<0,05$) in the antibody titer of rabbits injected with the protein of *L.caulleryi* schizont (15, 19). As a vaccine kit candidate, the schizont *L.caulleryi* antigen injected in to rabbits needs to induce a cellular immune response, marked by T cell lymphocte activation which expresses TLR 2 in accordance with humoral immune response. There was an antibody (IgG) titre enhancement produced by B lymphoctes (14, 15).

Conclusion

The antigenic protein of *L.caulleryi* schizont has the ability to induce a cellular immune response through the expression of TLR-2 and CD4 in the rabbits' T cells. The TLR-2 signal plays a role in innate immunity, but also in adaptive immunity. The antigenic protein of *L.caulleryi* schizont may contain ligand which acts as a receptor that is involved in pathogen-associated molecular patterns (PAMP). This study is a preliminary research to explore the immunogenic protein which plays a role in immune system activation for future studies in vaccine development to overcome leucocytozoonosis in chickens.

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