

Conference Paper

Toll-Like Receptors (TLRs) Play Role in Adaptive Immunity in Rabbits Immunized by *Sarcoptes scabiei* Proteins

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Abstract

Sarcoptic mange is one of the most economically important diseases in goats in Indonesia, and increasing number of cases of treatment failure is being reported because of drug resistance. Nowadays, it is considered as an emerging/re-emerging parasitic disease that threatens human and animal health globally. Toll-Like Receptors (TLR) is a receptor which plays role in innate immunity due to microbial infection. TLR-5 is a receptor that can recognize ligand produced by bacterial component, and TLR-7 is involved in the recognition of ligand that similar to ssRNA virus. This research aims are to detect *Sarcoptes scabiei* antigenic protein which can induce cellular immune response in rabbit as adaptive immunity with TLR-5 and TLR-7 as marker. This research was performed in several stages: isolation and identification *S. scabiei* from scabies infected goats; extraction of soluble protein *S. scabiei* mite, the rabbit immunization by inoculating protein antigen *S. scabiei* with dosage of 500µg, repeated five times as booster in two weeks, the examination of TLR-5 and TLR-7 expression using direct immunofluorescence technique. The result of cellular immune response is shown by TLR-5 and TLR-7 expression in rabbit T lymphocytes which appear yellow to green fluorescence color using fluorescence microscope. The amount of fluorescence T lymphocytes showed a significant difference ($p < 0.05$) between control and various boosters, and significantly increased in 3rd booster or 42 days post immunization. The conclusion showed protein from *S. scabiei* mites taken from goat contains ligands, acting as receptors which involve in pathogen associated molecular pattern (PAMP) that can induce adaptive immunity and recognized by TLR-5 and TLR-7. It shows that TLR is not only involved in innate immunity but also in adaptive immunity, and can be used as alternative adjuvant development.

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Keywords: *Sarcoptes scabiei*, TLR- 5, TLR-7, goats, rabbits.

1. Introduction

Scabies is an endemic disease, but occasionally outbreaks can be occurred and attacks most of cattle. Sarcoptic mange is one of the most economically important diseases in goats in Indonesia, and increasing number of cases of treatment failure is being reported because of drug resistance (1). In Japan, mange outbreaks was observed in raccoon dogs with high morbidity and mortality, and the debilitated animals caused by *S.scabiei* were often sent to veterinary hospitals and wildlife rescue facilities (2). Pathogenesis of scabies disease is related to the host immune response after invaded by mites *S.scabiei*, it has been known that *S.scabiei* mite will penetrate the skin until reach the stratum corneum, then suck the lymph fluid and consume epidermis cells for its survival (3). Mites protein is known as antigen, when antigen enters the body it will activate lymphocyte B cells to produce immunoglobulin. The *S. scabiei* var. *caprae* protein with molecular weight of 57.3 kDa was the specific antigenic protein that had high level of sensitivity and specificity, it can induce antibody both humoral and cellular (4). Presently it is considered as an emerging/re-emerging parasitic disease that threatens human and animal healthy globally (5). TLR is a membrane protein that helps receptor recognition pattern to various molecules derivatives from microbes and stimulate innate immunity due to microbe molecules exposure. TLR is known to be a recognition receptor which involves in pathogen associated molecular pattern (PAMP) recognized by pattern recognition molecules (PRMs) and a phagocytes development system in recognizing pathogen which can be stimulated any time to respond as inflammatory system. TLR stimulation through microbial product initiates signaling pathways which activates not only innate immunity but also adaptive immunity, and according to research result held by some researchers showed that TLR can also activate T cells which play role in adaptive immunity (6). It has been known that TLR in lymphocytes act as essential signal to regulate lymphocytes activation and proliferation, produce antibody and regulate antigen presentation (6,7, 8). The latest research result showed that TLR signal is capable as determiner in naive T cells toward the Th1 and Th2 response (9,10).

2. Material and Methods

2.1. Isolation and identification of *S. scabiei* mites from goat

S.scabiei mites was isolated from domestic goats that showed clinical signs of scabies, such as thickening of the skin, crust formation, hair loss on the area around eyes, ears,

mouth and legs. The area of the skin that has crust was scraped, and the scraping result was put on object glass and given drops of 10% KOH, then mites were observed under microscope using magnification of 40x. The mites identification based on key identification from Soulsby (11). The selected Sarcoptes was washed by centrifugation at 3000 rpm for 10 minutes, this step was performed at least three times to get the result free of dirty materials that still carried in scraping process. The deposit result would be formed as pellets and kept in freezer at minus 80°C, to be processed into soluble protein (4).

2.2. Rabbits Immunization

This research used six rabbits were treated as per animal welfare concept (five freedoms) and given health examination based on both clinical symptoms and laboratory tests (approved by Ethical Committee, Faculty of Veterinary Medicine, Universitas Airlangga, No: 630-KE). Rabbits which are used as experimental animals have to comply inclusion factor. While for exclusion factor criteria, the rabbit should be originated from scabies endemic farm. Each of experimental rabbits injected by 500 µg *S.scabiei* protein and added adjuvant complete (Sigma, USA) with ratio 1:1. Every two weeks the injection was performed with the same protein with the dosage 500 µg each and added adjuvant incomplete (Sigma, USA), booster was performed 5 times in 2 weeks. Prior to the first injection, about 5 ml rabbit blood was taken for TLR-5 and TLR-7 examination as preliminary data (control) and whole blood examination were conducted in the end of first booster until the fifth booster (4).

2.3. Immunofluorescence Technique for TLR-5 and TLR-7 examination

For the examination of TLR-5 and TLR-7, the isolation of PBMC in T cells was performed based on Boyum method (1968) with some modifications (12), as these following procedures: 5 ml whole blood and washed with 10% PBS, centrifuged at 1600 rpm and temperature 10°C for 10 minutes, next the undercoat to be put on Ficoll isopaque. The mixture contained blood and Ficoll isopaque is centrifuged at 1600 rpm and temperature 10°C for 10 minutes. The resultant buffy coat is separated and washed with PBS. TLR-5 and TLR-7 examination was performed as these following stages, buffy coat and 300 µl MEM incubated at 37°C for an hour, the solution is fixated by absolute methanol, and blocked by PBS and 1% serum for 15 minutes, next the solution is washed using PBS and was added first antibody of Monoclonal Antibody TLR5- FITC labeled (IMGENEX

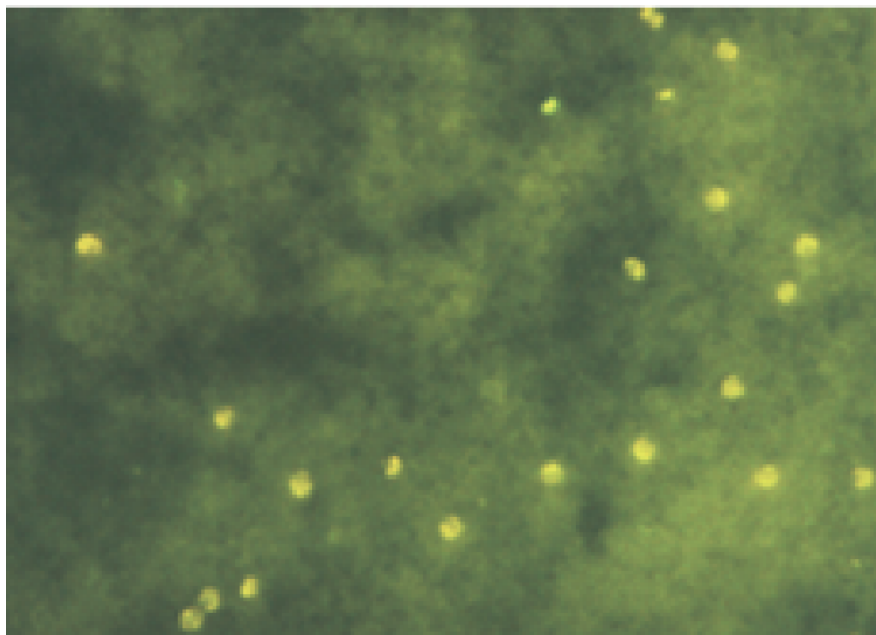


Figure 1: TLR-5 expression in Rabbit T cells visualized by FITC (400x).

Corp), and for TLR-7 using Polyclonal Antibody to TLR7-Atto 488 (IMGENEX Corp). The solution added Foetal Calf Serum 1% and FITC conjugate. The result was examined by fluorescence microscope using magnification 400x, to find out whether any yellow to green fluorescence color from T cells expressing TLR. If there is fluorescence light from T cells showing activated immune response, then the calculation is conducted towards the amount of fluorescent T cells.

3. Results and Discussion

Cellular immune response is shown by the expression of TLR-5 and TLR-7 in rabbit T cells which is marked by the yellow to green fluorescence colour after rabbit immunization up to 5 times booster (Figure 1 and 2).

In addition, the amount of fluorescence T cells was counted for each 20 μ l buffy coat, it was shown the amount of fluorescence T cells is increased in accordance with the treatment from various boosters (booster one up to booster five) as mentioned in table 1 and 2).

In table 1 and 2, the amount of T cells which express TLR-5 and TLR-7 shows a significant difference ($p < 0,05$) between control and various booster (repetations), but between booster 3 (day 42) and booster 4 (day 56) is not significantly different by statistic despite the increasing amount of fluorescence T cells. For TLR-5 the amount of fluorescence T cells in booster 5 (day 70) is highly increased and significantly different

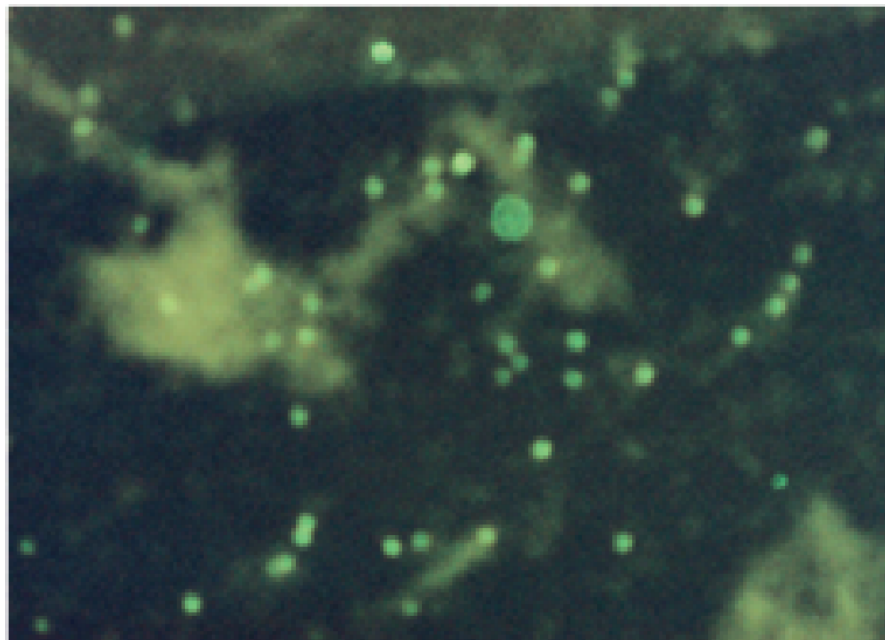


Figure 2: TLR-7 expression in Rabbit T cells visualized by FITC (400x).

TABLE 1: Average amount of T cells which express TLR-5 in rabbit immunized by protein of *S.scabiei* mites.

Immunization Time (day)/repetition	TLR5
1 (kontrol)	18,15 ^a ± 7,175
14 (booster 1)	108,00 ^b ± 14,638
28 (booster 2)	154,20 ^c ± 16,417
42 (booster 3)	189,65 ^d ± 13,256
56 (booster 4)	207,47 ^d ± 13,198
70 (booster 5)	249,65 ^e ± 20,689

*Different superscript in the same column indicated significant difference (p<0,05)

with booster 4 (p<0,05). While for TLR- 7, between booster 3 is significantly different with booster 5 but not significantly different with booster 4.

According the result research, it is shown that antibody TLR-5 and TLR-7 can recognize ligand from protein antigen *S.scabiei var.caprae* mites by stimulating T cells activation, marked by the presence of yellow to green fluorescence color which increased in accordance with the treatment from various boosters, as shown in table 1. Mechanism of cellular immune response activation will be explained below, *S.scabiei* mites posses ligand or pathogen associated molecular pattern (PAMP) which recognized by TLR-5 and TLR-7. Ligands that recognized by TLR is consisted of lipoprotein/lipopolypeptide, flagelin, ssRNA, CpG DNA.

TABLE 2: Average amount of T cells which express TLR-5 in rabbit immunized by protein of *S.scabiei* mites.

Immunization Time (day)/repetition)	TLR7
1 (kontrol)	17,45 ^a ± 4,837
14 (booster 1)	98,48 ^b ± 15,208
28 (booster 2)	147,48 ^c ± 5,519
42 (booster 3)	199,06 ^d ± 6,128
56 (booster 4)	210,35 ^{de} ± 21,688
70 (booster 5)	229,49 ^e ± 17,989

*Different superscript in the same column indicated significant difference (p<0,05)

S.scabiei mites is an extracellular microorganism which contains antigen, when antigen enter the body it will be caught by macrophage or dendritic cells and phagocytes cells will be activated by TLR as signal transducer. The signal which produced by TLR will activate transcription factor NF κ B which stimulates cytokines production. NF κ B activation initiated by signal which recruits MyD88 and interacts with IL-1 receptor associated kinase (IRAK), then autophosphorylation is occurred, separating MyD88 and activating TNF receptor associated factor 6 (TRAF-6) to activate I κ B kinase (IKK). Activated IKK will activate NF κ B to transcript gene IL-12, IL-10, IL-4, TNF- α , IFN- γ . IL-2 roles will increase cytolytic activity from cytolytic T lymphocytes and promote Th1 cells development together with CD8 activation to produce IL-2 which stimulates proliferation and differentiation of B cells that will produce antibody. IL-4 is a cytokine which produced by subset Th2 from Th cells CD4 that functioned to induce Th2 cells differentiation and stimulate IgE production.

Cellular immune response enhancement will be followed by IgG titre enhancement as humoral immune response (4), either responded by OD value or the increase of antibody titre to the peak at booster 3 but has no significant difference compared to other boosters. It could be caused by the activation of memory cells at day 30 (13). TLR-5 expression in T cells that activated by protein of *S.scabiei* mites means protein ligand contained in *S.scabiei* is recognized by TLR-5 monoclonal antibody, which is shown by the enhancement of activated T cells expression. TLR-5 is a receptor that can recognize ligand produced by bacterial component, flagella which is made from sub unit protein called flagelin. Flagella is a whip-like additional organ, motile and long, arising from basal body on the surface of cells and acts as locomotor, composed by lophotrichous or a group of hair (14). It has been known that *S.scabiei* is equipped by four pairs of legs and ambulacral stem as locomotor and cilia or hairs that grow on the body surface

(11), Cilia is predicted to act as ligand (PAMP) which is recognized by PRR of TLR-5 that functioned to detect microbial infection.

The result is similar to TLR-7 expression that the increasing T cells thoroughly has significant difference ($p < 0,05$) between control and boosters, but between booster 3 and 4 is not significantly different, as well as booster 5. It could be caused by the collaboration between TLR-7 and TLR-5 as signal transduction and competition in activating T cells. The enhancement of TLR-7 expression in T cells specifically booster 3 also followed by the enhancement humoral immune response (showed by the enhancement of antibody titre) significantly in booster 3 and steadily enhance in booster 5. According to some literatures, TLR-7 and TLR-8 is involved in the recognition of ligand that similar to ssRNA virus, other than that TLR-7 in human can recognize imidazoquinoline which is a synthetic mixture to prevent viral infection in genital infection case. It has been reported that TLR-7 in mice can recognize loxoribine mixture which is known as antiviral and antitumor, both the synthetic materials are structurally related to guanosine nucleoside (15). TLR-7 and TLR-9 has been known to induce IL-12 and IFN- α, β which produced by APC through MyD88 dependent pathway and act as therapeutic potency from CpG in preventing diseases that dominated by Th2 cells such as allergy (10). TLR-9 has been developed by some researchers as vaccine adjuvant contains synthetic oligodeoxynucleotide (ODN) to fight infectious diseases, allergies and cancers (16,17).

4. Conclusion

TLR signal is capable as determiner in naive T cells toward the Th1 and Th2 response. It showed that TLR not only plays role in innate immunity but also in adaptive immunity. Antigenic protein of *S. scabies* contains ligand which acts as receptor that involved in pathogen associated molecular pattern (PAMP), this can induce cellular immune response and recognized by TLR-5 and TLR-7 as markers.

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