Conference Paper

# *Mus musculus* (Linnaeus, 1758) Immune Responses Caused by *Escherichia coli* (Migula, 1895) Infection

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## Abstract

*Escherichia coli* (Migula, 1895) is a negative gram bacteria which have an ability to cause some deseases, such as meningitis, urinary tract infection and digestive tract infection. Lipopolysaccharide (LPS) in its cell wall induces immune responses in many ways. The objective of this study was to investigate the effect of *E. coli* infection on lymphocyte numbers and spleen weight in mice [*Mus musculus* (Linnaeus, 1758)]. Twenty five male mice were grouped into negative control, positive control group treated with PBS, and three experimental groups injected intraperitonially with *E. coli* $1.5 \times 10^3$cfu/mL,$1.5 \times 10^5$cfu/mL or $1.5 \times 10^7$cfu/mL respectively. About 5 d after the injection, the spleen were collected. Spleen were weighed using digital balance, number of lymphocyte were counted using hemocytometer. *E. coli* infection significantly increase both weight of the spleen and number lymphocyte. In conclusion, mice responded to *E. coli* infection by increasing the number of its lymphocyte.

**Keywords:** *Escerechia coli* (Migula, 1895); immune respone; infection; *Mus musculus* (Linnaeus, 1758).

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## 1. Introduction

Vertebrate immune system consist of various molecules that recognize and response to infection agent with complex mechanism [1]. Strange infection agent such as bacteria, viruses, and fungus [2]. *Escherechia coli* (Migula, 1895) is a negative gram bacteria which have Lipopolysaccharide (LPS) in its outer cell membrane. The LPS is a potential molecule which cause inflammation reaction [3]. *E. coli* cause the death of 2 million people per year for intraintestinal nor extra intestinal disease [4] such as meningitis, urinary tract infection and digestive tract infection [5, 6].

*E. coli* can be found in soil or water contaminated feses. It also can live in digestive tract vertebrate [4, 5] such as small intestine and large intestine. These bacteria abundant in facultative anaerobic environmental conditions [6]. LPS composed 10% of

the overall weight of the cell wall components of bacteria. One *E. coli* cell containing almost $3.5 \times 10^6$ molecules of LPS with around 10 kDa molecular weight [7].

The immune response occurs because of an antigen infected the body. There are two mechanisms of the body to block the antigen, i.e. (1) innate immunity, the first line of defense against antigens, the characteristic is not specific to against the antigens, quick respond but lasted a short time and there is no immunological memory and (2) adaptive immunity, an immune response that is specific to each antigen, progress has been slow but persist for a long time and have immunological memory [8]. The body will respond if there is an infection pathogen. The form of response is the proliferation and differentiation of lymphocytes to become cells that able to react with the pathogen. Increased T lymphocytes and B lymphocyte antigen exposure indicates that stimulates lymphocytes to proliferate rapidly [9].

Lymphocytes most commonly found in the lymph nodes, but can also be found in specialized lymphoid tissues, such as spleen, sub mucosal digestive tract area, thymus, and bone marrow [10]. The spleen may represent lymphatic organs associated with the immune response. The spleen works to filter the blood and trap antigens involved blood stream so that it can respond to systemic infection [11]. Spleen structure allows it to eliminate microorganisms [12]. The spleen consists of two large compartment, i.e. white pulp (containing lymphocytes T and B) and red pulp (containing blood) [12–14].

Research on the effects of *E. coli* infection on the immune response of mice interesting to study as it relates to health. The purpose of this study were (1) the effect of *E. coli* bacterial infection of the mice's spleen weight and (2) the effect of *E. coli* bacterial infections of the number of lymphocytes in the mice's spleen [*Mus musculus* (Linnaeus, 1758)].

## 2. Material and Methods

### 2.1. Mice and infection

Animal tests used were male mice strain BALB/C, aged 6 wk to 7 wk, 20 g to 32 g weight as much as 25 animals obtained from Malang Murine Farm Singosari Malang. *E. coli* bacteria cultures used were obtained from the Laboratory of Microbiology University of Malang. The variables in this study, are: (a) The independent variables such as the number of *E. coli* bacteria were injected into the intra peritoneal cavity of mice, which is $1.5 \times 10^3$ cfu/mL, $1.5 \times 10^5$ cfu/mL and $1.5 \times 10^7$ cfu/mL, (b) the dependent variable in the form of spleen weight and number of lymphocytes in the spleen of mice and (c) the control variables of the lighting cycle, the type of pellet and the type, weight and age of mice .

This study uses a completely randomized design with treatment, is as follows.

- Control (-): mice not infected (without treatment)
- Control (+): mice were injected with 1 mL of Phosphate Buffer Saline (PBS)
- Treatment 1: mice infected with the bacteria *E. coli* $1.5 \times 10^3$ cfu in 1 mL of PBS
- Treatment 2: mice infected with the bacteria *E. coli* $1.5 \times 10^5$ cfu in 1 mL of PBS

• Treatment 3: mice infected with the bacteria *E. coli* $1.5 \times 10^7$ cfu in 1 mL of PBS

Each of the study groups was repeated 5 times.

Mice were given treatment before first acclimatized for seven days at the animal enclosures Biology Department, State University of Malang. The mice were grouped according to the treatment given to the marking on each repetition with picric acid. Each enclosure consists of two mice. Acclimatization when mice were maintained with the lighting cycle of 12 h of light and 12 h dark.

## 2.2. Preparation of PBS Solution (1 L)

Weigh 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$ and 0.24 g $KH_2PO_4$. All the ingredients are put in a 1 L beaker glass and added with 800 mL of sterile distilled water. The solution is homogenized with a magnetic stirrer. After homogeneous, the solution is added with sterile distilled water to a volume of 1 L. PBS solution medium was transferred to 500 mL bottles and sterilized.

## 2.3. Preparation of E. coli Infection

Taking two ose colonies of *E. coli* on slant Nutrient Agar (NA) media and then dissolving it in 3 mL of PBS solution. Turbidity suspension is then standardized with 0.5 Mc. Farland standards through visual methods. Test tubes containing *E. coli* bacterial suspension was placed parallel to the tube Mc. Farland 0.5 to get the same level of turbidity (Mc. Farland 0.5 equivalent to $1.5 \times 108$ cfu/mL). The suspension is then diluted with PBS solution to obtain a suspension containing *E. coli* bacteria $1.5 \times 10^3$cfu/mL, $1.5 \times 10^5$cfu/mL and $1.5 \times 10^7$ cfu/mL.

## 2.4. Treatment of mice

On the eighth day of acclimatization, mice were infected with *E. coli* bacteria $1.5 \times 10^3$ cfu/mL, $1.5 \times 10^5$ cfu/mL and $1.5 \times 10^7$ cfu/mL in 1 mL intra peritoneal total . At 5 d after treatment, the mice dislocated at neck and then dissected. Spleen of mice is taken, washed with a solution of PBS, counted weighed with a digital balance, put in 2 mL of PBS solution.

## 2.5. Preparation of lymphocyte suspension

Spleen enumerated in 1 ml PBS. Then Homogenates inserted into the microtube and centrifuged speed of 2 500 rpm at 4°C for 5 min. The supernatant was discarded and the pellet re-suspended with 1 mL of PBS. Homogenates were then centrifuged speed of 2 500 rpm at 4°C for 5 min. The supernatant was discarded and the pellet then re-suspended in 1 mL of PBS (stock lymphocytes). Then, stock diluted 100x by taking stock of lymphocyte suspension and added 10 $\mu$L and 990 $\mu$L of PBS solution.

### 2.6. Counting the number of lymphocyte

The number of lymphocyte counted using the hemacytometer with four field observations (room). The average number of lymphocytes per field of observation is calculated by counting the total number of lymphocytes in the four fields of observation divided by four. Cells were observed that are in the box and the cells are in the upper limit and the left while the cells are in the right and lower limits are not counted. Suspension of isolated lymphocytes in PBS solution stained with methylene blue dye solution (volume ratio of methylene blue and the suspension of lymphocytes 1 : 1). For example, 10 $\mu$L of methylene blue and 10 $\mu$L lymphocyte suspensions were homogenized with pippeting. Lymphocyte counts is the living cells stained by the dye methylene blue. The core of lymphocytes large round-shaped.

## 3. Results and Discussions

Spleen is the biggest peripheral lymphatic organ which are important on innate and adaptive immune response [15]. The organ can eliminate parasites from circulatory system during infection occure [16]. The immune response parameter of this research are lymphocyte numbers and spleen weight in mice (*Mus musculus*). The result of this research showed that the average of spleen weight of positive control group decreased 0.018 g if compared with negative control group. The *E. coli* infection increase weight of the spleen 0.006 g of $1.5 \times 10^3$ cfu/mL, 0.092 g of $1.5 \times 10^5$ cfu/mL and 0.078 g of $1.5 \times 10^7$cfu/mL. Number of mice's lymphocytes spleen of positive control was increased after infection by by $36 \times 10^6$ cfu. In addition, *E. coli* infection also increased the number of lymphocytes $176.4 \times 10^6$ cfu by 1.5 × 103 cfu/mL, $487.6 \times 10^6$ cfu by $1.5 \times 10^5$cfu/mL and $241.6 \times 10^6$ cfu by 1.5 × 107 cfu/mL.

Based on the statistical analysis one way ANOVA with significantcy level 5% showed that *E. coli* infections significantly effect on spleen weight (sig. 0.012 < sig. Arithmetic 0.05) and the number of spleen lymphocytes (sig. 0.002 < sig. Arithmetic 0.05). *E. coli* infection was increased the number of spleen lymphocytes and mice spleen weight. Infection $1.5 \times 10^5$ cfu/mL *E. coli* in mice are most significant effect on increasing the number of spleen lymphocytes and spleen weight (Table 1).

*E. coli* has a cell wall composed of LPS, which is the most potential molecules that cause inflammation and trigger an inflammatory reaction [3] by initiating cytokine releasing inflammatory mediators [17, 18]. Inflammatory mediators, such as Tumor Necrosis Factor (TNF-$\alpha$), interleukin (IL-4, IL-10, IL-13, IL-1), kimokin, adhesion molecules, Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). LPS stimulates cells through Toll-Like Receptor-4 (TLR-4) which results in producing inflammatory cytokines and increase the regulation of co-stimulatory molecules on APC. Combination signal of antigens, co-stimulatory, and cytokines causes CD4 T cells accumulate in large quantities [19].

1.5 × 105 cfu/mL *E. coli* infection significantly increase the weight of the spleen mice. The spleen consists of white pulp and red pulp surrounded by a capsule of connective tissue. White pulp contains many lymphocytes T and B, when infection occur the wide

| Groups | The Average of Spleen Weight (g) | The Average of Number Spleen Lymphocytes (cell per spleen) |
|---|---|---|
| K (-) | 0.130 ± 0.047[a] | 344.40 ± 54.303[a] |
| K (+) | 0.112 ± 0.028[a] | 380.40 ± 155.173[a] |
| P1 | 0.136 ± 0.039a | 520.80 ± 261.830a |
| **P2** | **0.222 ± 0.059b** | **832.00 ± 144.160b** |
| P3 | 0.208 ± 0.082b | 586.00 ± 192.333a |
| Notes: | | |
| K (-): negative control (mice not infected) | | |
| K (+): positive control (mice injected with 1 ml PBS) | | |
| P1: mice infected with *E. coli* 1.5 × 103 cfu in 1 ml of PBS solution | | |
| P2: mice infected with *E. coli* 1.5 × 105 cfu in 1 ml of PBS solution | | |
| P3: mice infected with *E. coli* 1.5 × 107 cfu in 1 ml of PBS solution | | |

Table 1: The average of number of spleen lymphocytes and spleen weight in mice.

(a) (b)

**Figure** 1: The differences in the size of the spleen of mice (a) infected and (b) uninfected (Self Documentation, 2015).

of white pulp increased due to the extent of cell proliferation. Expansion of the white pulp and red pulp due to an increase in the size of the follicle, resulting in enlarged spleen [16]. The enlarged spleen resulting on spleen weight also increases (Figure 1).

Lymphocytes are capable of expressing the antigen receptor in very high diversity and its able to recognize foreign substances in very high variable species [14]. Based on the data analysis known that $1.5 \times 10^5$ cfu/mL *E. coli* bacterial infection significantly increase the number of lymphocytes (Figure 2). The increasing of the number lymphocytes caused by lymphocytes proliferation. T and B lymphocyte proliferation are response due to antigen or mitogen stimulation. The proliferation ability was indicated the important role of lymphocytes in the immune system function [20].

The LPS toxicity caused by lipid component while the immunogenicity associated with the polysaccharide component. Immune system stimulation process starts with bacteria that releases LPS into the blood. Complex of LPS binding protein binds to the receptors of Cluster of Differentiation 14 (CD14) plasma proteins, i.e macrophages or types of receptors on endothelial cells. Macrophages produce cytokines, activates the complement cascade and activate the coagulation cascade. LPS activates macrophages to produce interleukin-1 (IL-1) that play a role in triggering the T helper-2 (Th2) to

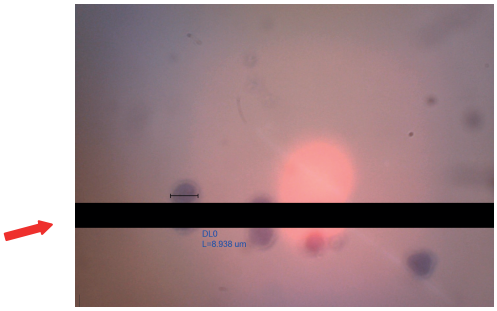

**Figure** 2: The structure of lymphocyte (10 × 40) (showed with red sign) (Self Documentation, 2015).

produce IL-4 and IL-5. The type IL role in triggering B lymphocytes to proliferate and differentiate into plasma cells and produce antibodies [21].

Cytokines play an important role on controlling the proliferation of T lymphocytes. Its activated by regulating expression of cell cycle in early synthesis phase (S) of the cell cycle [22]. Before antigen stimulate it, lymphocytes are in phase Gap 0 (Go) of cell cycle. Stimulation by antigen caused lymphocytes enter to the phase of Gap 1 (G1) of cell cycle. The morphology has been changed into the bigger one (lymphoblasts) containing more RNA (S phase) and then doing cleavage [14]. IL-7 and IL-15 plays role in T lymphocyte homeostasis, IL-15 also plays role in the initiation of T lymphocyte division [22].

The number of lymphocytes in the spleen mice which infected with $1.5 \times 10^7$ cfu/mL *E. coli* decreased if compared with $1.5 \times 10^5$ cfu/mL *E. coli* infection. The decreasing number of lymphocytes might occur as a result of lymphocyte death through apoptosis mechanism. Apoptosis is one of the causes of death due to sepsis lymphocytes [23]. Sepsis is a clinical syndrome resulting from the interaction between the body and infection agent, also the main causes of the death [18]. In apoptosis, nuclei undergoes to condensation and fragmentation, cytoplasm due to vacuolization, and the phagocyte cells occur [14]. Acute infection can lead to sepsis that characterized by activation many pathways of inflammatory [18]. Activation of many pro inflammatory mediators can trigger cell death [24].

Apoptosis of lymphocytes decrease the number of lymphocytes in the circulation system and lymphocytes of spleen white pulp [25]. The death lymphocytes in the spleen located in the area of para folicullar or in the follicle [26]. Apoptosis of lymphocytes danger for the body because its important to fight microorganisms as infection agent [27]. The large death of lymphocyte cause serious problem implications for causing uncontrolled infection and death [24]. There was 33% of mice died caused $1 \times 10^9$ cfu *E. coli* infection and 50% by $1 \times 10^{10}$ cfu *E. coli* infections [28].

## 4. Conclusions

From this study, we conclude that mice responded to *E. coli* infection, i.e. (a) there was an effect of *E. coli* infection on the spleen mice and (b) there was an effect of *E.*

*coli* infection on the number of lymphocytes in the spleen mice. Spleen weight and lymphocyte number significantly increased after infection by 1.5 × 105 cfu/mL *E. coli*.

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