



A research article

**The Effect of Stress, BCG Vaccination,
and Combination of Stress-BCG Vaccination to The Ability of
Macrophage to Bind with CD4+ T Cells in BALB/c Mice**

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ABSTRACT

Background. BCG has been recognized as potentiator for cellular immune system, while stress has been reported to suppress it.

Objective. This research was emphasized on the effect of electric foot shock as stressor and BCG vaccination in cellular immune alteration through the evaluation of CD4+ T cells bound to macrophage.

Methods. This research adopted Laboratory Experimental and Post-Test Only Group Design. The 24 female BALB/c mice (6-8 week old with average weight of 21.88 ± 1.75 grams) were obtained from PUSVETMA (Pusat Veterinaria Farma Surabaya). All mice were then divided into four groups and received standard lab diet daily. The first group (control group=C) received no other additional treatment. The second group (BCG group=B) received intraperitoneal injection of 0.1 cc BCG on day 1 and 11. The third group (electric foot shock group=E) received electric foot shock (EFS) with increasing amount and length of sessions for ten days. The fourth group received intraperitoneal injection of 0.1 cc BCG on day 1 and day 11 along with electric foot shock with increasing amount and length of sessions for ten days (EFS+BCG group=EB). At day 21 all groups received intravenous injection of 10^4 live *Listeria monocytogenes* ($LD_{50}=2 \times 10^5$ bacteria) and terminated on day 26.

Results. The result showed significant differences in the amount of CD4+ T cells bound to macrophage ($p < 0.05$) among the groups. The lowest number of binding was found in group E. The significant difference was found between group BE and both group C and E ($p < 0.05$).

Conclusion. It can be concluded that stress caused suppression of the macrophage ability to bind with CD4+ T cells, while the BCG caused its enhancement. The BCG vaccination may prevent the decrease of macrophage ability to bind with CD4+ T cells in BALB/c mice caused by stress.

Keywords: Stress, BCG, CD4+ T cells binding

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Introduction

BCG is a mutant strain of *Mycobacterium bovis*, isolated by Calmette and Guerin through culture in glycerol potato bile. Subculture of the original isolation is used for both vaccination and cancer therapy.^{1,2,3,4}

BCG can alter few components of the immune system, change several types of cells and either stimulates or inhibits of the immune system depending on how it is utilized.^{5,6} BCG is made of weakened intracellular bacteria

and therefore used to stimulate cellular immunity in macrophage.^{3,7} BCG in low dose stimulate type 1 response, while higher dose stimulate mixed response of both type 1 and type 2 immunity. BCG inoculation through respiratory tract stimulates protein which enhances production of type 1 cytokine such as IL-12, IFN- γ , and TNF- α .⁸ BCG vaccination enhances functions of macrophage, T cells, B cells and NK cells.⁹

In vitro administration of BCG by Demangel *et al.* accelerated the maturation of dendritic cells shown by increase of MHC II antigen expression, costimulator molecule CD 80 and CD 86. mRNA synthesis of IL-1, IL-6, IL-12, IL-10 and IL-1 receptor antagonist. BCG given intrathecal to mice stimulated potent response of T cell and IFN- γ towards Mycobacterial antigen in mediastinal lymph nodes which protected from aerosol infection of *Mycobacterium tuberculosis*.⁴

Stress, ignited by a stressor, is a result of threat to our adaptive capacity which interferes with our body's dynamic or homeostatic balance.^{10,11,12,13} In animal testing, it's concluded that stress may disrupt the cellular immune function, specifically the decrease of lymphocyte proliferation, decrease of macrophage phagocytic activity, change of Th1 and Th2 balance, cytokine secretion and expression of cytokine receptors. Stress may also modulate the MHC II expression, a vital process in macrophage function as antigen presenting cells (APC).^{10,14,15}

Stress caused by unpredictable and unavoidable electric foot shock (EFS) was proven to decrease lymphocytic activity, increase corticosteroid level, and increase the vulnerability to viral infection such as coxsackie B, herpes simplex and poliomyelitis.¹³

Listeria monocytogenes is a gram-positive, facultative intracellular bacteria. It is capable to survive inside macrophage, showing defence against macrophage's bactericidal mechanism.¹⁴⁻¹⁹ *L. monocytogenes* infection is a well-established model for studying host resistance to infection.²¹ Innate immunity is critical for the early control of infection. Neutrophils accumulate quickly within 24 h after infection at infectious foci and phagocytose extracellular bacteria, along with tissue-fixed macrophages (e.g., Kupffer cells) and infiltrating monocytes/macrophages. Upon phagocytosis of bacteria, the macrophages become activated and produce a

variety of cytokines (including pro-inflammatory cytokines IL-6, IL-1, and TNF- α), chemokines, and NO; activate NK cells (innate responses) via IL-12; and begin to activate CD4⁺ T cells (adaptive responses) through MHC II and IL-12. NK cells and differentiated CD4⁺ T cells are major sources of IFN- γ , which can fully activate macrophages to become effective killers. In addition, CD4⁺ T cells can activate CD8⁺ T cells to become cytotoxic T cells and kill infected cells. Thus, CD4⁺ T cell activation is critical in the initiation of adaptive immune response. Macrophages, CD4⁺ T cells, and CD8⁺ T cells are the major effector cells during host defence against *L. monocytogenes*.¹⁴

This research was aimed to evaluate the differences in the ability of macrophage to bind with CD4⁺ T cells in electric foot shock (EFS)-stimulated mice with BCG vaccination and EFS-stimulated mice without BCG vaccination. The result of this research was expected to provide information about the utilization of BCG vaccine in preventing the decrease of cellular immunity during stress.

Materials and Method

This research adopted Laboratory Experimental and Post-Test Only Group Design. The 24 female BALB/c mice were obtained from PUSVETMA (Pusat Veterinaria Farma Surabaya). Female BALB/c mice were housed six to eight mice per cage with food and water ad libitum. To avoid bias, the following factors were controlled: genetic factor of mice (by using BALB/c mice), age of mice (6-8 weeks), gender (female), weight before experiment, caging (located at the same place with identical cages), cleanliness (similar frequency and quality of cleaning for every mice), ventilation and lighting.

The average weight of mice used is (21.88 \pm 1.75) grams. Homogeneity of samples were tested with Levene test ($p = 0.272$) with the conclusion that the weight of samples were homogenous. Animals were allowed at least 1 week of habituation before used in the experiment. All mice were then divided into four groups and received standard lab diet daily.

The first group (control group=C) received no other additional treatment. The second group (BCG group=B) received intraperitoneal injection of 0.1 cc BCG on day 1 and 11. The third group (electric foot shock

group=E) received electric foot shock with increasing amount and length of sessions for ten days. The fourth group received intraperitoneal injection of 0.1 cc BCG on day 1 and day 11 along with electric foot shock with increasing amount and length of sessions for ten days (EFS+BCG group=EB). On day 21 all groups received intravenous injection of 10^4 live *L. monocytogenes* ($LD_{50}=2 \times 10^5$ bacteria) obtained from Balai Laboratorium Kesehatan Semarang and terminated on day 26.

EFS treatment was given for group E and BE from day 12 to day 21. The mice were put each time within a group of three inside a separate cage equipped with EFS. The EFS was given by having electrical current run through the copper plate on the floor of the EFS cage. The electric current is set at 1-3 mA. Four minute interval was allowed in-between sessions. On the first day, two sessions of four shocks were given. On the second day, 2 sessions of 8 shocks were given. From the third to tenth day of EFS treatment, the shock frequency and amount of session was constantly increased. The mice were treated with EFS for 10 days to allow optimum time for cortisol, the stress hormone, to be released and causing modulation of the immune system.¹⁶

BCG vaccine used was the product of Bio Farma. BCG vaccine was injected intraperitoneally, 0,1 cc per dose. BCG was administered on day 1 of the experiment followed by another booster injection with the same dose 10 days later (day 11). BCG vaccine was selected among many other immunopotentiators that frequently used in researches.

Listeria monocytogenes were obtained from Balai Laboratorium Kesehatan Semarang. *L. monocytogenes* was selected because it is a well-established model of intracellular bacterial infection.¹⁷ $CD4^+$ T cell activation is critical in the initiation of adaptive immune response. And macrophages and $CD4^+$ T cells are the major effector cells during host defence against *L. monocytogenes*, thus showing the activation of cellular immune system involving macrophage as APC.¹⁴

Examination procedure

The procedure was based on Ziegler K modification of Lipscomb MF method. 5×10^5 peritoneal exudate

cells (PEC) in 2 ml of medium was incubated in the temperature of 37°C for 2 hours inside flat based glass tube with round cover slip at the bottom of the glass. Non-adherent cells suspension then moved to a different tube as PEL. Cover slip then washed with PBS so that only adherent cells remained on top of the cover slip. 0.3 ml of 10⁷ heat-killed *L. monocytogenes* (heated for 2 hours at 63°C) were added to the tube. The medium then centrifuged at 800g and 20°C for 5 minutes, continued with 37°C incubation for 1 hour. The tube was washed 3 times with PBS. The tube was then added by 10⁵ PEL from the same mice before being centrifuged at 500g and 4°C for 4 minutes, then the tube was incubated at 37°C for 1 hour. Afterwards, the tube was washed 2 times with PBS. The cover slip was then fixated with methanol and coloured with 20% Giemsa for 20 minutes.¹⁸⁻¹⁹ The amount of CD4+ T cells bound to macrophage, mounted on the object glass, was determined by microscopically counting the CD4+ T cells bound to 100 macrophages.

Statistical Analysis

The data was processed using *SPSS 13.0 for Windows*. Kolmogorov-Smirnov test of normality was used to identify the distribution of data. Further One Way ANOVA was used appropriately resulting in significant difference ($p = 0.000$). Differences between groups were analysed with Bonferroni post hoc test.^{20, 21, 22, 23, 24}

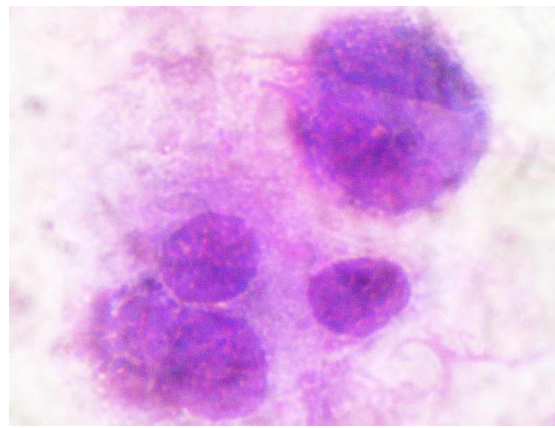
Result

The amount of CD4+ T cells bound to macrophage was determined by microscopically counting the CD4+ T cells bound on 100 macrophages. The amount of CD4+ T cells bound to macrophage is lowest in the group receiving electric foot shock (12.667 ± 5.92) CD4+ T cells/100 macrophages.

Figure 1. Macrophage



Figure 2. CD4+ T cells bound to macrophage



The amount of CD4+ T cells bound to macrophage in EFS group were significantly different compared to Control group ($p = 0.031$), also to BCG group ($p = 0.000$) and EFS+BCG group ($p = 0.000$). There were (127.67 ± 52.74) CD4+ T cells bound to macrophage in the EFS+BCG group, not significantly different from BCG group ($p = 1.000$) but significantly different from Control group ($p = 0.023$). However, the amount of binding in BCG group was also significantly higher than Control group ($p = 0.010$) and EFS group ($p = 0.000$).

Table 1. The amount of CD4+ T cells bound to 100 macrophages

Treatment group	N	Minimum	Maximum	Mean	Standard Deviation
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Control	6	45	88	69	14.52
BCG	6	97	171	134	29.13
Electric foot shock (EFS)	6	5	20	12.67	5.92
EFS+BCG	6	65	187	127.67	52.74

One-Way ANOVA F = 20.032; $p = 0.000$

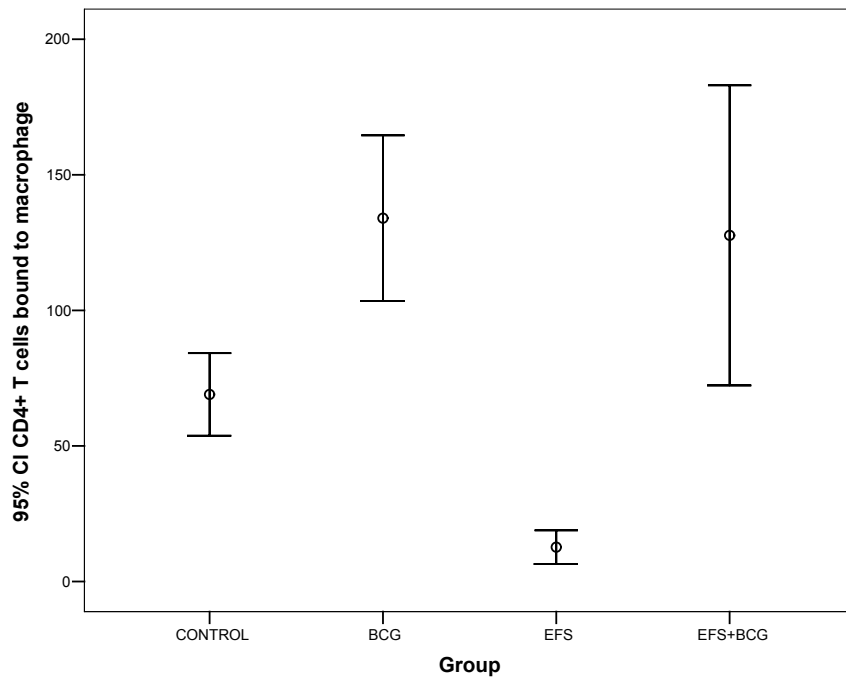
Table 2. The p value of Bonferroni Test for comparison between groups

Bonferroni Test	Control	BCG	EFS	EFS+BCG
Control	-	0.010*	0.031*	0.023*
BCG	0.010*	-	0.000*	1.000
EFS	0.031*	0.000*	-	0.000*
EFS+BCG	0.023*	1.000	0.000*	-

* Significantly different, $p < 0.05$

It is therefore concluded that there were significant difference in the amount of CD4+ T cells bound to macrophage in stressed BALB/c mice with BCG vaccination (EFS+BCG group) compared to stressed BALB/c mice without BCG vaccination (EFS group). The amount of CD4+ T cells bound to macrophage was lower in stressed BALB/c mice without BCG vaccination.

Figure 3. Error bar of CD4+ T cells bound to macrophage



Discussion

Experimental listeriosis of mice is a convenient model to study anti-microbial immunity in which specific CD4+ T cells activate immunity. In this model, the roles have been established of T cells as mediators of protection, of macrophages as anti-bacterial effectors of T cells mediated immunity, and of IFN- γ as transmitting cytokine between T cells and macrophages.¹⁴ Campbell *et al* demonstrated that surface proteins of *L. monocytogenes* can be presented by macrophage (MHC II) recognized by the body through CD4+ T cells subsets during infection.²⁵

To induce the reaction of CD4+ T cells, macrophages isolated from mice were given heat-killed *L. monocytogenes* in vitro. Heat-killed *L. monocytogenes* acted as extracellular peptides which undergo endocytosis by macrophages as APC. These peptides bound to MHC II as presenting molecules. The presented peptides are then recognized by CD4+ T cells, and can be observed through the binding of CD4+ T cells with macrophages. Mittrucker *et al* reported that population of CD4+ and CD8+ T cells were equal in vivo while in vitro experiments show that 40% of CD4+ T cells responded heat-killed *L. monocytogenes* by releasing IFN- γ (type 1 response).²⁷

In this research, there were significant differences in the amount of CD4+ T cells bound to macrophages in stressed mice with BCG vaccination compared to the stressed mice without BCG vaccination. The amount of CD4+ T cells bound to macrophages was lower in the group of stressed mice without BCG vaccination than the stressed mice with BCG vaccination. The group of stressed BALB/c mice with BCG vaccination showed significantly higher amount of CD4+ T cells bound to macrophage compared to Control group and group of stressed BALB/c mice without BCG vaccination. In this research, the amount of CD4+ T cells bound to macrophage was a part of type 1 immune response (cellular immunity).

The lower number of CD4+ T cells bound to macrophage in the stress group was possibly due to several factors affecting the function of macrophage as APC and the ability of CD4+ T cells to bind with macrophage. This result was similar to previous research reporting that stress in mice caused suppression of *L. monocytogenes*-induced MHC II antigen expression, down regulation of IFN- γ and IL-12, with up-regulation of IL-4 and IL-6. The overall effect is the reduction of CD4+ T cells binding with macrophage and the shift of cellular immunity towards humoral immunity.¹⁷ Schwab *et al.* showed that the average number of cells expressing MHC II in mice decreased consistently with the duration and dosage of stressor.²⁸ Increased plasma corticosterone levels caused by stress have also been associated with decreases in MHC II expression in studies conducted using peritoneal macrophages and splenic B cells.^{15,29}

The higher number of CD4+ T cells bound to macrophage in stress+BCG group compared to stress group was possibly because administration of BCG may increase the function of macrophage, T cells, B cells, and NK cells to produce IL-1.⁹ BCG can enhance lymphocyte proliferation and activate cellular immune response by increasing IL-12, IFN- γ , and TNF.³⁰ BCG vaccine *in vitro* may increase CD4+ T cells, proven by existing CD4+ blast cells in the culture. Peak of lymphocyte T proliferation is within two weeks after vaccination. Djamiatun *et al.* reported that administration of 0,1 cc BCG from Bio Farma with another 0,1 cc as booster 10 days afterwards significantly increased phagocytic ability and killing ability of spleen macrophage against *Staphylococcus aureus*. Previous researches concluded that BCG can increase the response of cellular immune system through the

increase of lymphocyte proliferation, the increase of CD4+ T cells, and the ability of macrophage as APC.^{6, 8, 9}

The result of this research showed that BCG may improve the function of macrophage as APC, possibly by increasing expression of MHC II and its costimulator molecules. There were also possible increase in mRNA synthesis of IL-1, IL-6, IL-12, and IL-10.⁴ Improvement in cellular immunity through BCG administration can be observed from more binding of CD4+ T cells and macrophage in EFS+BCG group than in stress group.

Conclusion

It can be concluded that stress caused by electric foot shock in BALB/c mice decreased the cellular immunity and that BCG vaccination might prevent that decrease by improving the ability of macrophage to bind with CD4+ T cells.

Suggestion

Further study is necessary to determine the mechanisms which underlie the increasing ability of macrophage to bind with CD4+ T cells in BALB/c mice administered with immunopotentiator. This study for instance, may be aimed in examining the balance between the production of IFN- γ and IL-4.

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