IL-12 PE, CD 69 PERCP, CD3 FITC, AND CD4 APC OPTIMIZATION WITH ACTIVATION OF ISOLATED AGENT HEAT-KILLED SONICATED *MYCOBACTERIUM TUBERCULOSIS* BEIJING STRAIN

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ABSTRACT

Infection caused by *Mycobaterium tuberculosis* exists in form of intracellular infection, which leads to lymphocyte activation. CD69 is the first lymphocyte activation marker expressed in Th1 lymphocyte, which follows by IL-12 release. Flow cytometry analysis can identify the subpopulations of lymphocytes and intracellular cytokines such as IL-12, yet precise preparation needs to be done. This research aims to conduct optimization with four color lyse/wash flow cytometry assay system FastImmune™ FACSCalibur examination, with monoclonal antibody IL-12, CD69, CD3, and CD4 in succession uses fluorochrome PE, PerCP, FITC, and APC.To activate the lymphocytes from heparinized whole blood, we used activation agent which derives from isolated heat-killed sonicated *Mycobacterium tuberculosis* Beijing strain. Optimal concentration from the according activation agents is 40 µL. To determine the compensation, BD™ CompBead and blank-cell unstainning are used, but the maximum result showed by blank-cell unstainning.Each monoclonal antibody dosage of IL-12PE, CD69 PerCP, and CD3 FITC is 40 µL, while CD4 APC 5 µL. Total event lymphocyte is determined minimally by 10,000 events. With 18,510 total events and Th gated events quantity are 4,692, the result obtained is IL12-PE has 7.4% gated (347 events); $CD69^+$ perCP/$CD3^+$ FITC 18.2% (850 events); and $CD69^+$ perCP/$CD4^+$ APC 3.9%.

Keywords**:** heat-killed sonicated *Mycobacterium tuberculosis* Beijing strain; IL-12; CD69; flow cytometry

Introduction

Infection caused by *M. tuberculosis* has broad variety of courses, characteristics, and effects in each individual because the victim's immune responses act differently for both responses from nonspecific immune and specific immune. In early stage of infection, nonspecific immune response, which involving macrophages, dendrite cells, NK cells, and lymphocyte T γδ, develops. *Mycobacterium* antigen will be presented via MHC-II to the cell surface and cytokine release follows. Subsequent activities depend on cytokine released, event of macrophage activation, inflammation process, or lymphocyte activation. (Rumende, 2000; vanCrevel R, 2002; Hingley-Wilson *et al*, 2003; Kayser *et al*, 2005; Abbas *et al*, 2007).

Mycobacterium tuberculosis, which lives intracellular inside the macrophages, will induced cellular immune response where T lymphocyte plays main role. T lymphocyte and its subset can be differerentiated based on its protein marker present on molecule surface, called cluster of differentiation (CD). CD3 found in every T lymphocyte and CD4 found in Thelper lymphocytes (Abbas *et al*, 2007; Takeshita, 2007; Paraskevas, 2012). T lymphocytes, with the presence of IL-12, will differentiate to Th1, which plays important roles in immune response for *M. tuberculosis* infection. T lymphocytes heterodimer receptors, *T-cell receptor* (TCR)-α/β and TCR-γ/δ, owns functions in introducing foreign peptide or antigen, which expressed jointly

with MHC by antigen attachment and signal transduction. CD3 inside the CD3/TCR complex acts as activation molecule, transmits signal from cell membrane to the nucleus until T lymphocyte is activated (Kayser *et al*, 2005; Murphy, 2012).CD4 or Th lymphocyte functions as TCR co-receptor to bind MHC II by strengthening the TCR binding target via cytoplasmic part and by elevation initial phase of immunological synapse generation. The according signaling is important in presenting *Mycobacterium* antigen(Kayser, 2005; Pareskevas 2009a, 2009b).

Antigen of *M. tuberculosis* spreads inside the cytoplasm and cell wall, thus induce immune respond, Th lymphocyte recognizes the above immune respond via TCR and MHC-II molecule in macrophage. The infected macrophage secretes IL-2 to activate CD4 T lymphocyte, leads to a variety of cytokine proliferates and differentiates. Macrophage also secretes IL-12 causes Th1 differentiation and together with other cell produce IFN-γ, which activates macrophage to destroy *M. tuberculosis*.Yet, not all mycobacteriumcan be damaged, as a result infection occurs, one of the consequences is hypervirulence of *M. tuberculosis* Beijing strain, which highly capable of growing and surviving inside the infected cells(vanCrevel, 2002; Abbas, 2007; Paraskevas, 2009a; Mestre, 2011).

Activation agents that has been used to activate T lymphocyte are heat-killed *M. tuberculosis* H37Ra (Sancho *et al*, 2003), heat-killed sonicated *M. tuberculosis* H37Rv, *phytohemaglutinin* (PHA) (Hu *et al*, 2009), heparin-binding hemagglutinin (HBHA) (Place *et al*, 2010), ESAT-6, andstaphylococcal enterotoxin B (SEB), pokeweed mitogen (PWM), phytohemagglutinin (PHA), dan Candida albicans [BD, Cat. No. 337184 and 340365]. Based on spoligotyping, *M. tuberculosis* can be differentiated between Beijing strain and non-Beijing strain. *M. tuberculosis* Beijing strain is more virulence, it cause worse tuberculosis infection, clinically, histopathologically, and radiologically (van-Crevel *et al*, 2001; Pheiffer*et al*, 2005; Parwati, 2010a, 2010b; Soetikno, 2011). In this research, heat-killed sonicated *M. tuberculosis* Beijing strain is used to activate the T lymphocyte.

Flow cytometry is an examination used to identify cellular parameter, surface antigen, internal epitopes, and DNA analysis; as well as intracellular cytokine and sublymphocyte examination. Flow cytometry examination uses lyse or no lyse, wash or no wash, with many fluorochrome or fluorescence, more than 15 types of it has been produced; such as fluorescein isothiocyanate (FITC), allophycocyanin (APC), phycoerythrin (PE), dan peridinin chlorophyl (PerCP) (Haron, 2014). This research aims to conduct optimization *four-color lyse/wash flow cytometry assay* sistem FastImmune™ FACSCalibur, with monoclonal antibodies IL-12PE, CD69 PerCP, CD3 FITC, and CD4 APC examinationused activation agent isolat of heat-killed sonicated *M. tuberculosis* Beijing strain.

Materials and Methods

Blood collection

Heparinized whole blood was taken from vena fossa cubiti of healthy people who were not having diabetes mellitus, HIV, malignancy, pregnancy, and under corticosteroid medication. Examination material of heparinized whole blood must have been analyzed 4 hours prior to sampling time.

Heat-killed sonicated Mycobacterium tuberculosis

Isolate *M. tuberculosis* Beijing strain (isolate no.099) and non-Beijing strain (isolate no. 073) resulted from Ogawa culture that was saved on glycerol 20%, were taken from Clinical Pathology Biology Molecular Laboratory of Hasan Sadikin Hospital, Bandung, Indonesia. The production of this heat-killed sonicated *M. tuberculosis* Beijing strain was done based on compilation of various methods available. Isolate resulted from *M. tuberculosis* Ogawa culture from -80^0C was awaited at room temperature, then heat block 100^0C applied and continued

with sonication. Sonication was applied inside distilled water at temperature of 20^0C and iced water (4^0C) with frequency of 47 kHz. The result from the isolate was diluted with sterile PBS with ratios between isolate and PBS of 1:49 and 1:4. Based on that, isolate was divided into 5 groups, which are group 1 (number 1–3): Beijing strain with ratio of 1:49 and sonification on 20^OC of water, group 2 (number 4–6) Beijing strain with ratio of 1:49 and sonification on 4^OC of iced water, group 3 (number 7–9) non-Beijing strain withratio of 1:49 and sonification on 20^OC of water, group 3 (number 10–12) non-Beijing strain with ratio of 1:49 and sonification on 20^OC of water, and group 5 (number 13–15) Beijing strain with ratio of 1:4 and sonification on 4^OC of iced water. To ensure the existence of protein from result of the heat-killed sonicated, each group was taken 2 for examination of sodium deodesil sulphate polyacrilamida gel electrophoresis (SDS-Page) tris-glycine 15%.

Activation of lymphocytes

Heparinized whole blood that was taken from healthy adult volunteersas much as 500 µL were activated withheat-killed sonicated *M. tuberculosis* Beijing strain. Because of heat-killed sonicated *M. tuberculosis* Beijing strain utilization as activation agent as far as researcher knew had not been done, to know which gave optimal result, was applied with several concentration of 20 µL, 40 µL, 60 µL, 80 µL, and 100 µL. After limfosit T on heparinized whole blood was activated, it was incubated for overnight at 37^OC of temperature. As control, unstimulated-heparinized whole blood was used, while other procedures were the same with activated-heparinized whole blood.

Pemeriksaan flow cytometry

IL-12, CD69, CD4, dan CD3 monoclonal antibodies assessment were conducted with flow cytometry FACSCalibur®, four fluorochrome color staining from BD FastImmune Pharmingen™ was used. Stainning of IL-12 with PE mouse anti-human IL-12(BD cat. no. 559329), CD69 withfluorochrome PerCP-Cy5.5 (BD cat. no. 340548), CD4 with fluorochrome APC (BD cat. no. 340443), dan CD3 with fluorochrome FITC (BD cat. no. 349201). Assessment procedure of *four-color lyse/wash flow cytometry assay* FastImmune™ sytem with FACSCalibur was modified from kit insert BD Pharmingen™. T lymphocyte activation in heparinized whole blood was done by heat-killed sonicated *M. tuberculosis* Beijing strain and unstimulatted-heparinized whole blood followed by overnight incubation in 37^oC temperature. In day two, cells were lysed with lysing buffer BD Pharm Lyse™, washed with PBS steril, and permeabilizing solution, then staining with IL-12 PE, CD69 PerCP-Cy5.5, CD3 FITC, and CD4 APC was performed. After staining was finished, data was analyzed and acquisition was done using flow cytometer FACSCalibur®.

Results and Discussion

SDS-Page Examination result from the five groups compare to tris-glycine 15% marker shows the band produced matches with band 42–51 kDa. From 5 experimental groups, which band was formed in group 2 and 5, both derived from Beijing strain and sonicated in iced water 4^OC. Yet, the band formed significantly in group 5, which is heat-killed sonicated *M. tuberculosis*with ratio between isolate Ogawa in glycerol 20% 100 µL and PBS sterile 400 µL. Detail result is shown in Figure 1. Based on the above SDS-Page result, heat-killed sonicated *M. tuberculosis* will be used as activation agent for the following examination, sonication result inside iced water with isolate and PBS ration is 1:4.

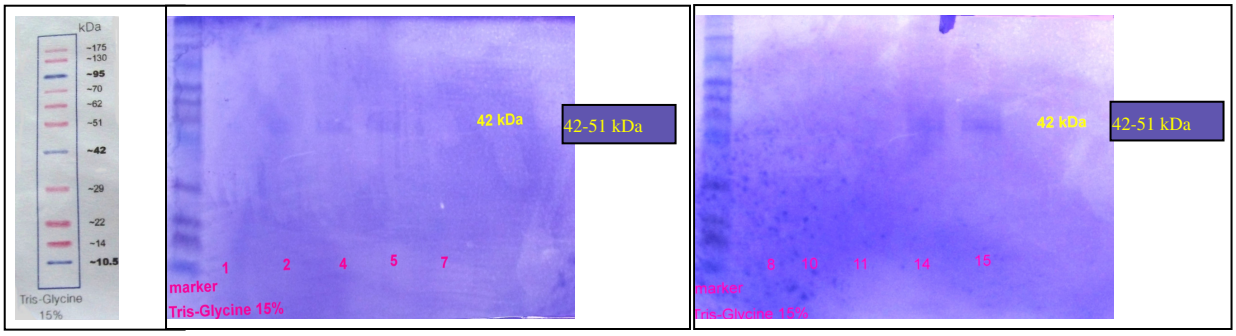

Figure 1. Sodium deodesil sulphatepolyacrilamida gel electrophoresis (SDS-Page) of heat-killed sonicated *M. tuberculosis*.
Marker tris-glycine; 1-2 Beijing strain (ratio isolat:PBS) 1:49, 20^{O}C; 4-5 Beijing strain : PBS 1:49, 4^{O}C; 7-8 non-Beijing strain : PBS 1:49, 20^{O}C; 10-11 non-Beijing strain : PBS 1:49, 4^{O}C; and 14-15 Beijing strain : PBS 1:4, 4^{O}C.

Heat-killed sonicated *M. tuberculosis* Beijing strain result from the previous examination shows in Figure 1. Group 5 result is used to activate T lymphocyte in heparinized whole blood for flow cytometry examination. Examination by using FastImmune system is examination to activate lymphocyte faster compare to other examination to activate the according lymphocyte incubation time needed is 4-19 hours in 37^{O}C to lyse cells. In this research, with sampling time consideration, stability from heparinized whole blood sample, and pre-stainning time, therefore incubation was performed in one night to be analyzed in the next day.

T lymphocyte activation was performed with various concentration of heat-killed sonicated *M. tuberculosis* Beijing strain; 20 µL, 40 µL, 60 µL, 80 µL, and 100 µL. Optimal result showed in 40 µL concentration. Detail data shows in Table 1. Based on the according result, 40 µL of heat-killed sonicated *M. tuberculosis* Beijing strain as the activation agent in T lymphocyte.

Table 1. The result of T lymphocyte activation with heat-killed sonicated *M. tuberculosis* Beijing strain in various concentration

Konsentrasi (µL)	20	40	60	80	100
% Limfosit teraktivasi*	4.98	5.53	3.72	2.82	2.49

Instrument setting of flow cytometer for *four-color lyse/wash flow cytometry assay*was conducted to be examined with 4 fluorochromes which used monoclonal antibody, before compensation be conducted to determine examination setting. The aim of instrument setting and compensation is to determine the border population of negative cell and positive cells. Instrument setting and compensation is optimal if % gater of negative cells population less than 1%. FSC shows cell size, and SSC shows cell granulity. Instrument setting result is shown in Table 2.

Table 2. Instrument setting with CompBead and cell

MAb-conjugated fluorochrome	Detector	Voltage[1]	Voltage[2]
	FSC	E00	E00
	SSC	370	370
FITC-CD3	FL1	416	531
PE-IL12	FL2	392	650
PerCp Cy5.5_CD69	FL3	523	735
APC-CD4	FL4	733	802

1 instrument setting with compBead, 2 instrument setting with cell

The compensation result for each fluorochrome obtained by using Compbead, were FL1 - 1.7% FL2, FL2 - 20.9% FL1, FL2 - 0.0% FL3, FL3 - 14.7% FL2, FL3 - 0.4% FL4, and FL4 - 7.0% FL3; while compensation with cells in a row were 15.3%, 25.1%, 6.8%, 14.3%, 5.4%, and 0.0%. By using the according instrument setting and compensation, T lymphocyte activation result with 40 µLheat-killed sonicated *M. tuberculosis* Beijing strain is shown in Figure 2.

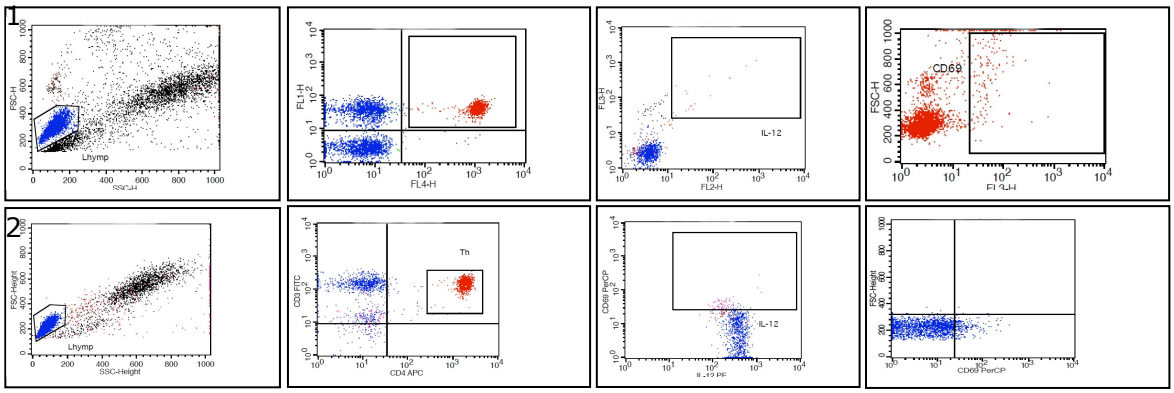

Figure 2. Ekspresi IL-12 dan CD69 on Thelper $CD3^+CD4^+$.
A. instrument setting with compBead, B instrument setting with cell, FL1 FITC-CD3, FL2 PE-IL12, FL3-PerCP Cy5.5-CD69, FL4 APC-CD4

From Figure 2.A, using compBead, % of gated T lymphocyte was 89.33%, IL 12 expression in T helper $CD3^+CD4^+$was 1.77% and CD69 was 3.02%. cells compensation showed IL 12 expression in Thelper $CD3^+CD4^+$was 7.40% and CD69 was 15.52%. Based on the above result, instrument setting that was used is instrument setting with cells. By using instrument setting with cells, compensation for each fluorochrome was followed for monoclonal antibody and unstainning cells. With unstainning cells, negative cells population was obtained, which were $CD3^-$ $IL12^-$, $CD69^-$ $IL12^-$, $CD69^-$ $CD4^-$, and $CD69^-$ $CD3^-$ (Figure 3).

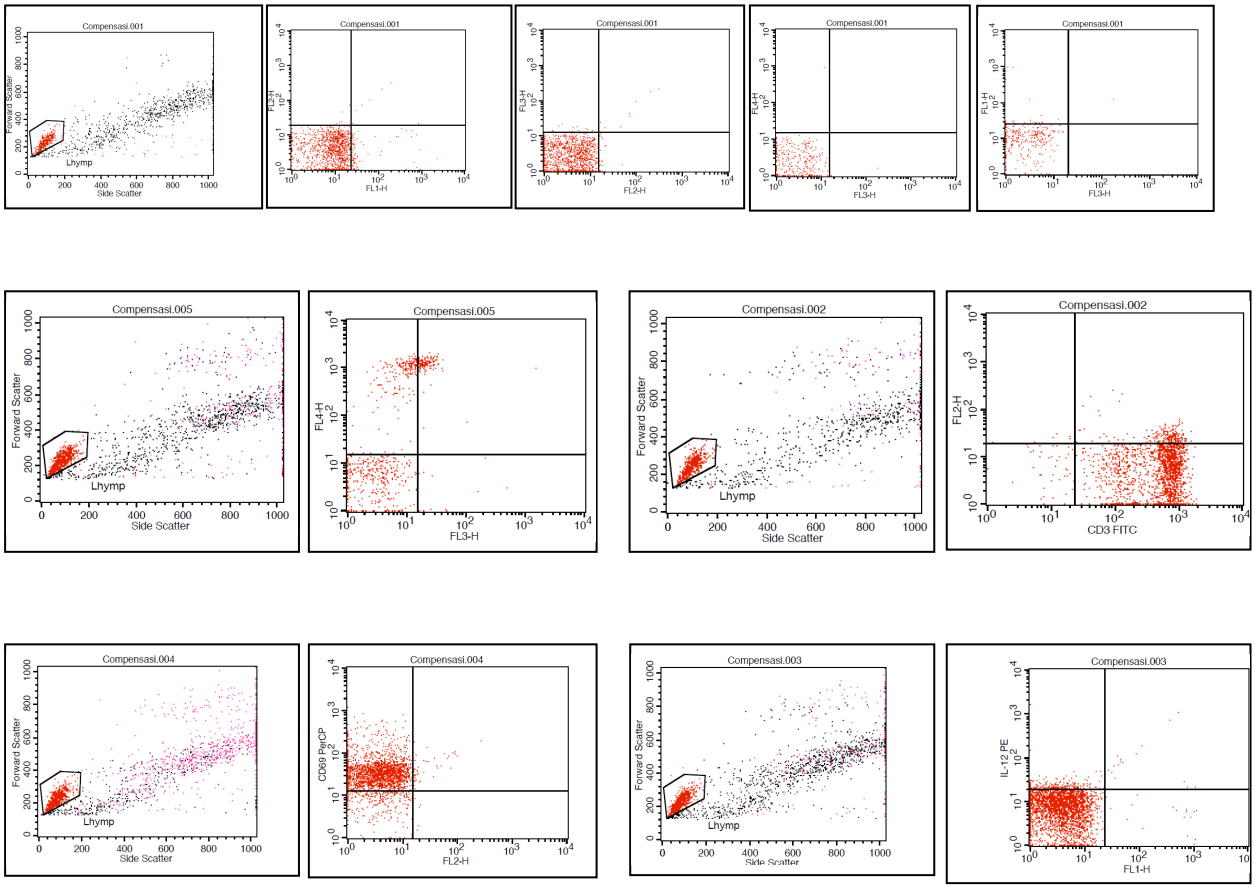

Figure 3. Compensation of unstainning cell (1), compensation of CD4 APC (2), compensation of CD3 FITC (3), compensation of CD69 PerCP Cy5.5 (4), and compensation of IL12 PE (5).

Based on unstainning cell compensation from gatted lymphocytes shows all negative cell population, thus the instrument setting arranged before was able to be used for the following examination template. After positive cell and negative cell population was obtained, compensation for each fluorochrome performance shown in Figure 3 (2-5). With gated-lymphocyte, in APC ($CD4^+$) compensation, $CD4^+CD69^+$cells were found, in FITC ($CD3^+$) compensation, IL 12 was found in $CD3^+$. To overcome the above situation, voltage setting had been conducted and compensation for each fluorochrome, yet same result still occured. CD69 is the earliest marker shows in activated T lymphocyte.

Conclusions

Heat-killed sonicated *M. tuberculosis* Beijing strain could be used as an activation agent to activate lymphocyte. In order to know that T lymphocytes have been activated, it could be shown by CD69 and IL12 on Thelper ($CD3^+CD4^+$) with*four-color lyse/wash flow cytometry assay* FastImmune™ examination system withFACSCalibur which needed setting instrument and optimization from every fluorochrome.

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