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Determining the effect of benzo[a]pyrene exposures toward innate and adaptive immunity profiles in post-measles vaccination mouse model

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ABSTRACT

Measles is an infectious disease caused by a virus of the genus Morbillivirus and is considered as global health problem. Several causes have been postulated that affect the success rate of vaccination, including benzo[a]pyrene exposure which widely distributed in air pollution. This study aims to evaluate the effect of BaP exposure to mouse immunity after measles vaccination. Several immunological parameters observed in this study were the expression of $CD4^+TNF\text{-}\alpha^+$, $CD4^+IFN\text{-}\gamma^+$, $CD4^+IFN\text{-}\alpha^+$, $CD8^+IFN\text{-}\alpha^+$, $CD11b^+IL17^+$, $B220^+CD25^+$, and the ratio of $CD4^+$:$B220^+$ from benzo[a]pyrene-exposured BALB/c mouse post-measles vaccination. Each sample was analyzed using a flow cytometry analysis. According to our findings, we found that benzo[a] pyrene interferes several parameters of immune system in measles-vaccinated mice. These findings suggested that the pollutant compounds especially benzo[a]pyrene can suppress the success rate of vaccination.

Keywords: adaptive immunity, benzo[a]pyrene, innate immunity, measles, vaccination

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INTRODUCTION

Measles is an infectious disease caused by a virus of the genus Morbillivirus. Initial infection with measles affects the respiratory and lymphatic systems. The measles virus replicates and enters the bloodstream[1]. Furthermore, the measles virus will then replicate and flow into the bloodstream. In the circulation, viruses infect erythrocytes and leukocytes. Infected leukocytes diminish lymphocytes and exacerbate immunosuppression[2]. Measles also cause secondary infections such as pneumonia, respiratory tract infections, and inflammation of the brain. A report showed that secondary infection has high possibility causing deaths[3]. According to Widagdo, about 90% of measles patients have a history of contact with other patients. Transmission of this disease can be through large droplets from the respiratory tract, but also through small droplets such as through inhaled air[4].

A prominent strategy to reduce the incidence of measles is expanding the vaccination program among society. Vaccination is an activity to induce specific antigens in the body to trigger a specific immune response[5]. Generally, the measles vaccination can be divided into a single or a combination vaccine administration. Measles vaccine can induce body protection for a long time and the very common measles vaccine that widely used is a live attenuated vaccine[2].

The success rate of vaccination can be influenced by internal and external factors. The success rate of the measles vaccination depends on the presence of maternal inhibitory antibodies, the maturity of the immune system of the vaccine recipient, and the dose of vaccine administered to the patient[6]. In addition, external factors such as pollution are also thought to affect the success rate of vaccination by reducing antibody responses to the vaccine[7]. One of the pollutant compounds contained in vehicle exhaust gas, cigarette smoke, and the residue of organic materials combustion such as benzo[a]pyrene (BaP). Research has shown that BaP can affect the immune system[8]. As widely known, the immune system components such as lymphocytes, macrophages, or pro-inflammatory cytokines have an important role in the immune response after vaccination. For an instance, the TNF-α activity post-vaccination is critical for regulating the cascade of pro-inflammatory cytokine production and recruitment of immunocompetent cells. Therefore, from above explanation we aim to evaluate the effect of BaP exposure to mouse innate and adaptive immunity after measles vaccination.

METHODOLOGY

Sample preparation and treatment groups

This study was occupied about 24 pathogen-free female BALB/c mice aged 2-week-old which obtained from LPPM Gadjah Mada University. In this study, the treatment groups were vehicle (Veh.), mice injected with BaP (B), mice injected with vaccine (V), and mice injected with both vaccine and BaP (VB). The measles vaccine used in this present study is a live attenuated type of measles vaccine. Each dose of vaccine contains dry measles virus, kanamycin sulfate and erythromycin (Bio Farma, Indonesia). The dose of measles vaccine injected was 20 mg/kg BW. Similarly, the dose of BaP injected was 20 mg/kg BW. This study has been evaluated by Research Ethics Commission of Brawijaya University with ethical clearance no. 930-KEP-UB.

Treatment procedures

The experimental treatment of mice was carried out after a week acclimatization process. The injection of BaP was carried out every 3 days for 5 weeks (9 times injection), except for the vehicle treatment and the measles vaccine. Measles vaccine injection was carried out once at the beginning of the treatment through subcutaneous approach. On the other hand, BaP injection was performed intraperitoneally after finishing vaccination procedures (Figure 1).

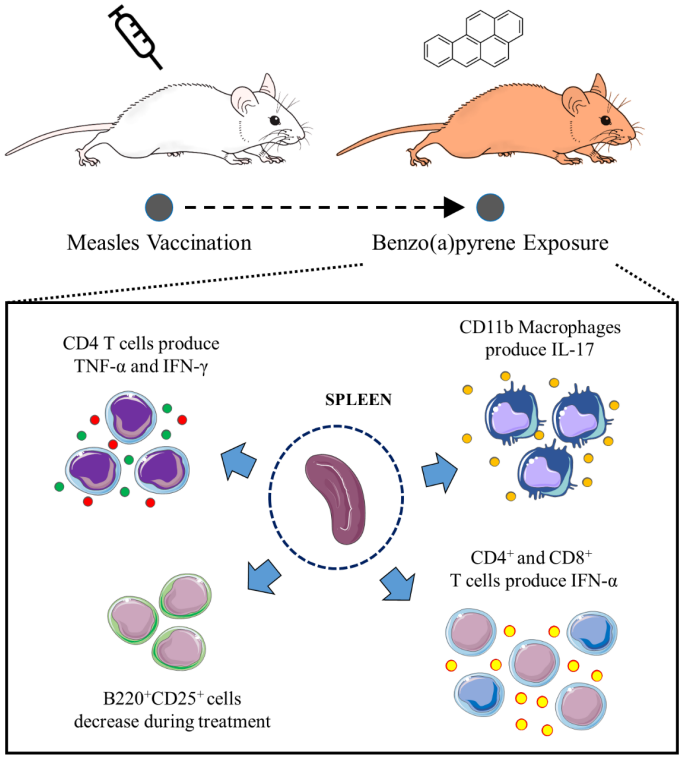

Figure 1. Schematic picture showed how benzo[a]pyrene interferes the measles vaccination effects of the immune system, including the innate and adaptive immunity and the production of cytokines on BALB/c mice.

Isolation of splenocytes and antibody staining

The protocol used for splenocytes and antibody staining was based on our previous studies[9-12]. Spleen organs from each treatment were isolated and washed using phosphate buffer saline to remove fat and debris. The homogenate of sample was put in a propylene tube and stored in an ice box. All homogenates were centrifuged at 2500 rpm for five minutes at 4°C to separate the pellet and supernatant. The pellet was resuspended using 1 ml of PBS. Each sample was put in a microtube for next antibody staining procedures. The sample was then centrifuged at 2500 rpm for five minutes at 4°C. The supernatant portion was discarded, while the pellet portion was added with 50 µl of extracellular antibody. The added antibodies were anti-CD4, anti-CD8, anti-CD11b, and anti-B220 (Biolegend, San Diego). Samples were incubated for 20 minutes in an ice box. Samples containing extracellular antibodies were added with 50 µl of cytofix. The samples were then incubated for 20 minutes in an ice box. Then

500 µl of washperm was added to the sample. The sample was centrifuged again at 2500 rpm for five minutes at 4°C. The supernatant was discarded while the pellet was added with 50 µl of intracellular antibody. The intracellular antibodies used were anti-TNF-α, anti-IFN-α, anti-IFNγ, and anti-IL-17 (Biolegend, San Diego). Samples were incubated for 20 minutes in an ice box.

Flow cytometry, chemical interaction prediction, and data analysis

Stained samples were added with 300 µl of PBS. Each sample was put into a cuvette for analysis using a flow cytometer. Each sample was analyzed using the BD Bioscience FACSCalibur™ flow cytometer (BD Biosciences). Data analysis of flow cytometry results was carried out using the BD CellQuest Pro™ program (BD Biosciences). Furthermore, STITCH database (http://stitch.embl.de/) was used to evaluate the interaction between chemical (benzo[a]pyrene) with certain protein[13,14]. Statistical test was performed by one way ANOVA analysis. The analysis was carried out with SPSS Ver. 20 software. The further tests used were the Tukey-HSD and Games Howell tests with $p<0.05$.

RESULTS and DISCUSSION

Effect of BaP exposures on $CD4^+TNF\text{-}\alpha^+$ expression

The relative number of $CD4^+TNF\text{-}\alpha^+$ molecules in the BaP group did not have a significant difference with the vehicle group (Figure 2). Oppositely, the research conducted by Lu et al. showed that BaP exposure increases TNF-α production and SIRT1[15]. SIRT1 is a regulatory protein that plays a role in the control of inflammation. SIRT1 protects cells from chronic inflammation by regulating NF-κB activity[16]. Activation of Nf-κB could enhance the production of pro-inflammatory cytokines including IL-1β and TNF-α[17].

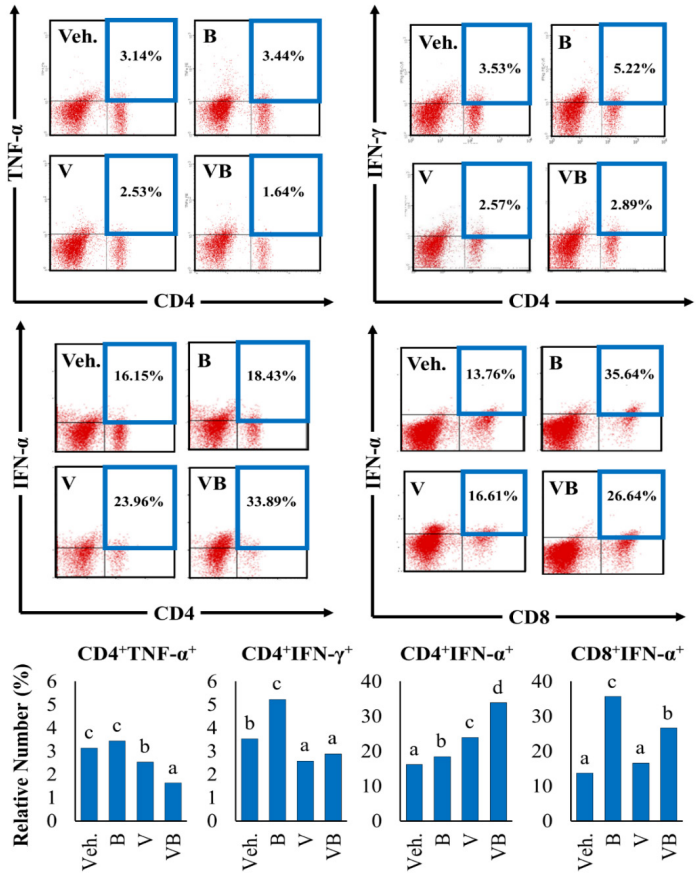

Figure 2. Relative number of $CD4^+TNF\text{-}\alpha^+$, $CD4^+IFN\text{-}\gamma^+$, $CD4^+IFN\text{-}\alpha$+, $CD8^+IFN\text{-}\alpha^+$ on experimental mice. Different number on graph showed the significant difference among experimental group ($p<0.05$).

The treatment groups were vehicle (Veh.), mice injected with BaP (B), mice injected with vaccine (V), and mice injected with both vaccine and BaP (VB).

Furthermore, vaccine group showed that the relative number of $CD4^+TNF\text{-}\alpha^+$ was significantly lower than the vehicle group. Research conducted by Ovsyannikova et al. stated that the administration of the vaccine increases in the amount of TNF-α[18]. The increase in TNF-α is one of the stages of the body's response when an antigen in the form of vaccine is inserted until it finally succeeds in forming specific antibodies. The decrease in $CD4^+TNF\text{-}\alpha^+$ production in the vaccine treatment when compared to the control could be caused by several possibilities. In general, the TNF-α cytokine would experience a significant increase after the second dose of measles vaccine was given.

BaP administration to vaccinated-mice caused a decrease in the relative number of $CD4^+TNF\text{-}\alpha^+$ molecules. BaP is a chemical compound that can cause cell damage and can be converted into metabolites which have immunosuppressant activity. BaP metabolites are immunosuppressant that leading in DNA mutations and DNA damage. In addition, decreased TNF-α production might occurs due to inhibition of CD4 T cell proliferation[19]. Similarly, the decrease in $CD4^+TNF\text{-}\alpha^+$ in VB treatment group could also be caused by an increase in the number of regulatory T cells. Regulatory T cells can produce TGF-β which can suppress the production of pro-inflammatory cytokines[20].

The administration of antigen in the form of a vaccine caused the activation of CD4 T cells. Activated CD4 T cells had a higher number of AhRs protein when compared to inactivated CD4 T cells[21]. AhR protein is a BaP receptor for initiating the production of CYP1B1 or CYP1A1 enzymes. These enzymes are involved in the metabolism of BaP to produce metabolites that are immunosuppressant. This fact is in line with our *in silico* finding (Figure 3) about the possible interaction between BaP and other type of target protein such as CYP1A2, CYP1A1, AHR, CYP1B1, TREX1, CYP4B1, CYP4X1, CYP2C8, CYP2J2, and CYP2D6. According to the predicted functional partner score, there are three proteins with the highest value including CYP1A2, CYP1A1, and AHR. The above prediction might confirm that BaP might have the closest influence with these proteins. Furthermore, the predicted proteins were involved in several pathways related to the BaP induction, including metabolism of xenobiotics by cytochrome P450, chemical carcinogenesis, serotonergic synapse, and others (Table 1). To the greater extend, the prediction also showed BaP included in several biological activity including small molecule metabolic process, oxidation-reduction process, cellular catabolic process, cellular lipid metabolic process, monocarboxylic acid metabolic process, and others (Figure 4).

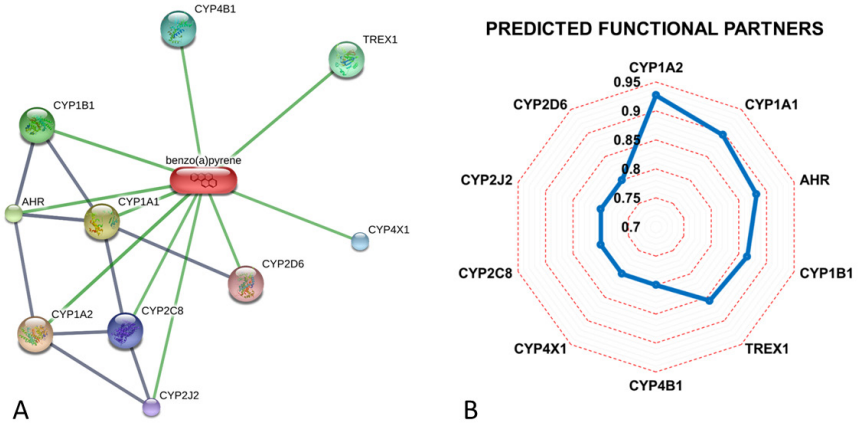

Figure 3. (A) The interaction among benzo[a]pyrene and possible targeted protein that include or affected by benzo[a]pyrene. (B) The predicted functional partners involved within the interaction with benzo[a]pyrene.

Table 1. The list of KEGG enrichment related to the target proteins which involved in the interaction with benzo[a]pyrene

No	Pathway Description	False Discovery Rate	Genes Involved
1	Metabolism of xenobiotics by cytochrome P450	6.19E-06	CYP1A1,CYP1A2,CYP1B1,CYP2D6
2	Chemical carcinogenesis	6.19E-06	CYP1A1,CYP1A2,CYP1B1,CYP2C8
3	Serotonergic synapse	2.34E-05	CYP2C8,CYP2D6,CYP2J2,CYP4X1
4	Linoleic acid metabolism	2.70E-05	CYP1A2,CYP2C8,CYP2J2
5	Tryptophan metabolism	6.00E-05	CYP1A1,CYP1A2,CYP1B1
6	Ovarian steroidogenesis	0.000107	CYP1A1,CYP1B1,CYP2J2
7	Steroid hormone biosynthesis	0.000109	CYP1A1,CYP1A2,CYP1B1
8	Retinol metabolism	0.000126	CYP1A1,CYP1A2,CYP2C8
9	Drug metabolism - cytochrome P450	0.000144	CYP1A2,CYP2C8,CYP2D6
10	Arachidonic acid metabolism	0.0136	CYP2C8,CYP2J2

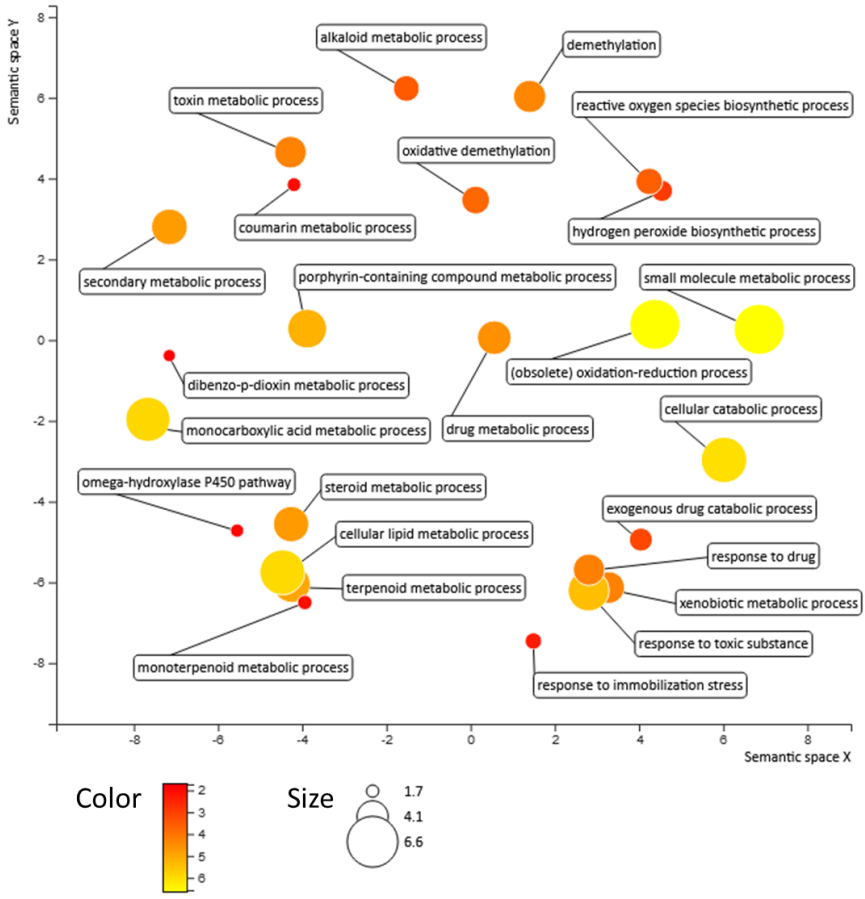

Figure 4. Possible biological process related to the target proteins which involved in the interaction with benzo[a]pyrene

Effect of BaP exposures on $CD4^+IFN\text{-}\gamma^+$ expression

In this present study, the BaP increase the relative number of $CD4^+IFN\text{-}\gamma^+$ (Figure 2). This is probably because CD4 T cells are still able to carry out repair mechanisms against DNA damage caused by BaP metabolites. On the other hand, the production of IFN-γ was lower in the vaccine treatment compared to the vehicle group.

Vaccination will stimulate the immune system to increase immunity against incoming pathogens. Research conducted by Ovsyannikova et al. stated that administering measles vaccine to experimental animals caused an increase in the production of pro-inflammatory cytokines, including IFN-γ[22]. The immune

system has a time limit of activation against the injected measles virus antigen. Antibody production, which indicates the last stage of the immune response to measles virus antigen, occurred on day 35 after vaccination[23]. Meanwhile, the maximum production of IFN-γ as a pro-inflammatory cytokine occurred on day 12 post-vaccination[24].

Furthermore, the results showed that the relative number of $CD4^+IFN\text{-}\gamma^+$ expression did not have a significant difference between the vaccine and BaP-vaccine treatments. In general, BaP administration led to an increase in post-vaccination IFN-γ production. Research conducted by Hur et al. stated that BaP can trigger FasL gene transcription in macrophages. FasL is one of the proteins involved in cell apoptosis[25].

As research conducted by Wu et al. showed that BaP is also a combustion derived particulate matter group that has the potential to damage macrophage cells located in mucosal areas[26]. The IFN-γ is one of the pro-inflammatory cytokines that play a role in macrophage activation[27]. Therefore, increasing the amount of IFN-γ is one of the body's defense mechanisms to balance the number of macrophages in the body.

IFN-γ and TNF-α are pro-inflammatory cytokines that work together in macrophage activation[28-30]. When the production of TNF-α by CD4 T cells decreases, then CD4 T cells will produce more IFN-γ to compensate for the decreased production of TNF-α. In addition, one other function of IFN-γ is to increase the amount of TNF-α[31,32]. Increasing the amount of IFN-γ becomes important to meet the body's need for TNF-α which has decreased due to BaP. Continuous exposure to BaP causes the increasing the expression of $CD4^+IFN\text{-}\gamma^+$. The continuous production of pro-inflammatory cytokines will activate immunocompetent cells. Activated immune cells will cause chronic inflammatory reaction.

Effect of BaP exposures on IFN-α production by $CD4^+$ and $CD8^+$ T cells

Interestingly, we found the significant differences between all treatment groups. There is an increasing trend in the percentage of IFN-α expression in $CD4^+$ T cells. Meanwhile, the percentage of IFN-α expression by $CD8^+$ T cells in Veh. and V treatment groups did not show any significant difference. However, a significant increase occurred in the treatment groups B and VB (Figure 2).

The increase in IFN-α expression on $CD4^+$ and $CD8^+$ occurred in BaP treatment, either without or in combination with measles vaccine. It is possible that the increase in IFN-α is a response to cell damage caused by BaP injection. The

higher IFN-α production compared to Veh. and V treatment groups aimed to increase the cytotoxicity of cytotoxic T cells. The important reason behind the increase cytotoxic T cells aim to kill damaged cells that have been affected by exposure to BaP. This refers to Li et al. who stated that one of the functions of IFN-α is to increase the cytotoxicity of cytotoxic T cells[33]. Meanwhile, the high $CD8^+IFN\text{-}\alpha^+$ T cells in BaP group were thought to be a form of compensation for the low IFN-α expression in $CD4^+$ T cells.

Meanwhile, when compared with vehicle group, the percentage of $CD4^+IFN\text{-}\alpha^+$ T cells in the vaccine treatment was higher. Similarly, Gibbert et al. stated that IFN-α is one of the signaling proteins produced by the body due to exposure to antigens such as viruses. Meanwhile, the injected vaccine is an attenuated measles virus that has the potential to increase the production of IFN-α in cells[34].

Effect of BaP exposures on $CD11b^+IL\text{-}17^+$ expression

BaP administration to mice significantly increased the production of $CD11b^+IL\text{-}17^+$ (Figure 5). Research conducted by Mohinta et al. stated that BaP can cause an increase in IL-17 gene expression[35]. Measles vaccine administration in experimental animals caused an increase in the relative $CD11b^+IL\text{-}17^+$ count but there was no significant difference with the vehicle group. Research conducted by Nelson et al., stated that IL-17 was produced on day 10, 35, and optimally on day 52 after the entry of measles antigen[36]. The experimental animal surgery in this study was carried out on the 35th day after the measles vaccine injection. Therefore, it is possible that the body has started to produce IL-17 but has not reached the optimal amount.

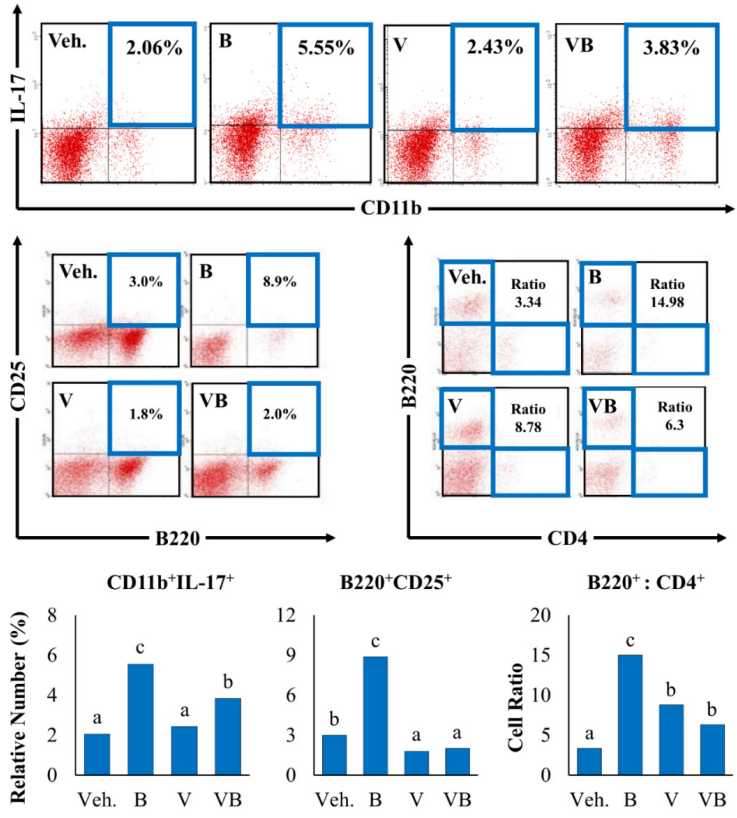

Figure 5. Relative number of CD11b+IL17+, B220+CD25+, and the ratio of CD4+:B220+ on experimental mice. Different number on graph showed the significant difference among experimental group ($p<0.05$). The treatment groups were vehicle (Veh.), mice injected with BaP (B), mice injected with vaccine (V), and mice injected with both vaccine and BaP (VB).

Administration of BaP to vaccinated mice significantly increased the relative number of $CD11b^+IL\text{-}17^+$. Research conducted by Mohinta et al. stated that BaP can cause an increase in the expression of the gene encoding IL-17[35]. The relative number of $CD11b^+IL\text{-}17^+$ in VB treatment was lower than in BaP group. This was due to the presence of regulatory T cells. The increase in IL-17 is always accompanied by an increase in regulatory T cells. The relative number of $CD11b^+IL\text{-}17^+$ had a relationship with the number of regulatory T cells and TGF-β production. TGF-β is a cytokine that widely produced by regulatory T cells and plays a role in controlling the number of pro-inflammatory cytokines[20]. The continuous increase in the number of IL-17 has an unfavorable impact because IL-17 is a cytokine that plays a role in controlling autoimmune mechanisms. Autoimmunity is a disorder of control of immune cells and the

production of auto-antibodies that result in damage to the body's own tissues[37]. Excessive IL-17 expression will cause chronic inflammation. The amount of IL-17 in normal measles virus antigen response is always opposite to the amount of IFN-γ. This is because the inducer of IFN-γ, namely IL-12, also acts as an inhibitor of IL-17 production[38].

Effect of BaP exposures on $B220^+CD25^+$ expression

A significant increase of memory B cell was found in BaP group (Figure 5). This is relatively far above the normal limit as reported by Amu et al. that the expression of memory $B220^+CD25^+$ cells in the spleen of healthy mice is about 2% of the total concentration of B cells[39]. The increase in the percentage of $B220^+CD25^+$ expression in BaP group could be due to BaP being able to increase $CD25^+$ expression in B cells through activation of the NF-κB pathway. This is supported by Ba et al. who stated that long-term accumulation of BaP can increase the activity of the NF-κB promoter[40]. Brisslert et al. showed NF-κB pathway blockade can inhibit the production of $CD25^+$ by B cells[41]. Meanwhile, the low $CD25^+$ expression in the VB group was thought to be due to the presence of the injected vaccine increasing the proliferation of B cells.

Effect of BaP exposures on $B220^+$:$CD4^+$ ratio

In this present study we found vehicle group has the lowest $B220^+$:$CD4^+$ ratio. Meanwhile, a significant increase occurred in BaP treatment group. Meanwhile, the VB treatment group experienced a significant decrease compared to BaP group, while vaccine group did not differ significantly from VB group (Figure 5).

The injection of BaP can increase the ratio by decreasing the number of $CD4^+$ T cells. The decrease in the number of $CD4^+$ T cells is related to the nature of BaP as an immunotoxic. Furthermore, BaP suppresses immunity through p53-dependent pathways which modulates signaling in lymphocytes and oxidative stress[42]. Meanwhile, the high ratio in the vaccine treatment was thought to be due to the fact that the vaccination was only carried out once, so that the accumulation of effector cells such as large numbers of $CD4^+$ T cells in the spleen did not occur. This can be explained that the injected antigen is only transient and is unable to induce the accumulation of effector cells in the spleen. According to Sarkander et al., when the antigen is transient, more immune cells will accumulate in the bone marrow[43].

Finally, based on the result, we found that BaP interferes several parameters of immune system in measles-vaccinated mice. These findings suggested that the chemicals compounds such as BaP can suppresses the success rate of vaccination.

STATEMENTS OF ETHICS

The protocols used in this study were following the guide for the care and use of experimental animals and the study passed the institutional ethical clearance by Research Ethics Commission of Brawijaya University with No. 930-KEP-UB.

CONFLICT OF INTEREST STATEMENT

All authors declare there is no conflict of interest.

AUTHOR CONTRIBUTIONS

W.E.P. was involved in data collection and analysis, methodology, and draft preparation. F.R.P.N. and M.I.F. were involved in data collection and analysis & review, and editing. M.R. was involved in study design, funding acquisition, supervision, review, and editing. All authors contributed to revision and approval of the final manuscript.

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