Research Article

Bioactivity of Benzo[a]pyrene against the expression of $CD4^+TNF\alpha$, $CD8^+$ IFNŸ, and $CD4^+IL1^+$ in mice (*Mus Musculus* L) after paramyxovirus measles vaccine administration

Suwoyo Suwoyo[1], Muhaimin Rifa'i[2]*, Widodo Widodo[2], Muhammad Sasmito Djati[2]

ABSTRACT

Background: Benzo[a]pyrene is one of the external factors that reduce immunity. The disease arises by the accumulation of benzo[a]pyrene in DNA, which forms benzo[a]pyrene and aryl hydrocarbon receptor (AhR bonds). The bond of benzo[a] pyrene and AhR could affect the differentiation of immune cells then induce an immunosuppressive effect. **Objective:** This research aims to identify the bioactivity of benzo[a]pyrene toward the expression of $CD4^+$ tumor necrosis factor (TNF)α, $CD8^+$ IFNŸ, and $CD4^+$ interleukin (IL)1^+ in mice treated with a paramyxovirus vaccine. **Materials and Methods:** This research was conducted using a split-plot design. The bioactivity analysis of benzo[a]pyrene toward immune cells expression in mice (*Mus musculus* L) was analyzed by administration of benzo[a]pyrene at 20 mg/kg BW through intramuscular injection 2 times/week for 4 weeks. The expression of $CD4^+$ TNF-α^+, $CD8^+$ IFN-$Ÿ^+$, and $CD4^+$IL-1^+ was analyzed using flow cytometry. **Results:** Group with vaccine and benzo[a]pyrene exposure at week 4 showed that TNFα was repressed while IFNŸ was elevated. Furthermore, the modest regulatory function of $CD4^+$ was activated. **Conclusion:** The exposure of benzo[a]pyrene to the mice (*M. musculus* L) with dose 20 mg/kg BW for 2 times/weeks exhibited the immune cell repression on $CD4^+TNF\alpha^+$ and immune cell activation on $CD8^+IFNŸ^+$, while $CD4^+$IL-1^+ at week 4.

KEY WORDS: Benzo[a]pyrene, $CD4^+$ interleukin1^+, $CD4^+$ Tumor necrosis factor α, $CD8^+$IFNŸ, Measles vaccine (paramyxovirus)

INTRODUCTION

Measles is an acute and contagious viral disease characterized by three stages, the incubation stage, the prodromal stage, and the eruption stage. The disease affects children[1], and the incidence rate of measles in Indonesia is relatively high, with 12,943 measles cases reported in 2014, rising from 11,521 cases in 2013. An unusual incidence of measles in East Java has been reported with about 41 incidents with 187 cases; Banten and South Sumatra had 14 rare incidents of measles.[2]

The implementation of immunization for children has reached 90% of the targets. This high achievement of vaccination should be followed by the decline in the number and incidence of diseases. However, a portion of children in Indonesia could not obtain immunization.[2]

In general, during the growing phase of children, they tend to experience more than 100 kinds of infectious diseases, with causal agents such as viruses, bacteria, and other parasites, before becoming an adult. Infectious diseases tend to occur in persons with a lowered immune system. This low state of immunity, especially in children, can be triggered by various factors. One is exposure to high levels of pollution of soil, water, and air in daily life[1] as well as in foods consumed.

The Indonesian eating culture is dominated by the consumption of food that is smoked, partially burned, or baked. This practice is getting more common, including in children. The use of such foods needs to be monitored carefully since they can contain benzo[a]

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[1]Department of Environmental Science, Doctoral Program of Environmental Science, Brawijaya University, Jalan Veteran, Malang, East Java 65145, Indonesia, [2]Department of Biology, Faculty of Mathematics and Life Sciences, Brawijaya University, Jalan Veteran, Malang, East Java 65145, Indonesia

***Corresponding author:** Muhaimin Rifa'i, Department of Biology, Faculty of Mathematics and Life Sciences, Brawijaya University, Jalan Veteran, Malang, East Java 65145, Indonesia. E-mail: rifa123@ub.ac.id

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pyrene compounds. Benzo[a]pyrene compounds that enter the body can be carcinogenic, immunotoxic, and immunosuppressive.[3] In terms of the latter, benzo[a]pyrene represses the development of various immunocompetent cells, especially T cells and B cells.[4] It has been reported, for instance, that benzo[a] pyrene level of smoked fish can reach about 97.2 ppm, exceeding the set threshold of server name indication, which only 0.005 ppm.[5] Benzo[a]pyrene exposure can also be attained by both direct and indirect exposure from the environment. It can enter the body through the respiratory tract and skin as well as the digestive system; benzo[a]pyrene enters through the respiratory tract through air pollution.[6]

The level of the effect caused by pollutants is dependent on the amount of exposure and the vulnerability of the host. Exposure to air pollutants can affect any part of the body and is not only restricted to the respiratory tract. Moreover, the particulate size of contaminants also determines the anatomic location of pollutant deposits and their effects on surrounding tissues. For example, fine particulate matter (PM) with a size of <1 μm can be easily absorbed into the systemic blood vessels. The ultrafine PM can cause procoagulant effects that disrupt blood circulation and facilitates the spread of pollutants throughout the body.[7]

PM levels are usually higher as the level of benzo[a] pyrene in the air increases. The hydrophobic character of benzo[a]pyrene makes it challenging to be excreted from the body so that it easily accumulates in tissues. By having a similar structure to the nucleic bases, the benzo[a]pyrene is also easily inserted into DNA strands. Therefore, it can block the interleukin-1 (IL-1) gene product and cause abnormal chemical induction for cell functions.[8] This situation triggers the lowered immune response to various infectious diseases, including measles.

Therefore, this research aims to identify the bioactivity of benzo[a]pyrene toward the expression of $CD4^+$ tumor necrosis factor (TNF)α, $CD8^+$ IFNŸ, and $CD4^+IL1^+$ in mice treated with a paramyxovirus vaccine.

MATERIALS AND METHODS

The research was conducted for 3 months in the Laboratory of Animal Structure and Development, Department of Biology, Faculty of Mathematics and Natural Science, Brawijaya University, Malang, Indonesia. The research obtained ethical approval No: 1012-KEP-UB on February 6, 2018, from Animal Care and Use Committee, Brawijaya University.

Research Procedure

The research design used was a split-plot design. Forty female mice with BALB/C strain were adapted for the environment for 2 weeks. Mice were divided by random sampling into four groups as follows. Group P1 consisted of ten mice without vaccine exposure. Then, five mice were observed for 2 weeks, while another five mice were observed for 4 weeks. Group P2 consisted of ten mice with measles vaccine exposure. Five mice were observed for 2 weeks, while another five mice were observed for 4 weeks. Group P3 consisted of ten mice with benzo[a]pyrene exposure by intramuscular injection. They were injected with a dosage of about 20 mg/kg BW for 2 times/week. Five mice were observed for 2 weeks, while another five mice were observed for 4 weeks. Group P4 consisted of ten mice with a measles vaccine treatment, which was then injected with benzo[a]pyrene by intramuscular with a specific dosage about 20 mg/kg BW for 2 times/week. Five mice were observed for 2 weeks, while another five mice were observed for 4 weeks. At the end of the determined week, mice from each treatment were sacrificed to collect their spleens for staining with antibody. Then, the expression of immune cells ($CD4^+$TNFα, $CD8^+$ IFNŸ, and $CD4^+IL1^+$) of mice in the spleen was measured by flow cytometry using the BD Fluorescence-activated cell sorting Calibur apparatus.

Reagents used in this research are: FITC anti-mouse CD4 (Clone H129, Biolegend), PE anti-mouse CD8a (Clone: 53-6.7, Biolegend), PE anti-mouse/rat TNF-α (Clone: TN3-19.12, Biolegend), PE/Cy7 anti-mouse IFN-Ÿ (Clone XMG 1.2, Biolegend), PerCP anti-mouse/human IL-1 (Clone: 11n92, LS Bio), Perm Wash Buffer (Catalog: 421002, #/size 100 ml, Biolegend), and Fixation Buffer (Catalog: 420801, #/size 100 ml, Biolegend).

Statistical Analysis

To find out the cell expression in mice (*Mus musculus* L), which analyzed using flow cytometry. Then, the data between groups were completed using statistics by SPSS 18 software for windows using the one-way ANOVA test. If homogeneous data, then continued by least significance different test with $P = 0.05$. If $P > 0.05$ then there is no real difference between treatment, whereas if $P < 0.05$.

RESULTS

Expression of $CD4^+$ TNF-α^+

As shown in Figure 1, the expression of $CD4^+TNF\alpha^+$ is about 0.2% within the group that was exposed to benzo[a]pyrene at week 2. This result is considered a difference as compared to the control group. This result demonstrates that benzo[a]pyrene is one of the PAHs that can induce repress regulation of $TNF\alpha^+$.

Based on LSD testing, it was found that the control group alone had different immunity values compared to the other groups [Table 1].

From the results of the LSD analysis above the control group and the vaccine group, there are differences. There is a difference between the vaccine group and the benzo [a] pyrene group and the benzo [a] pyrene group of vaccines. This result is considered significant different compared to the control group with $P = 0.036 < \alpha$.

Groups of mice that were treated with the vaccine showed the expression of $CD4^+TNF\alpha^+$ of about 0.24%. This expression was not significantly different compared to the control group with $P = 0.05$. The different expression levels of $CD4^+TNF\alpha^+$ indicated that the vaccine had the immunosuppressive ability. This effect demonstrated low secretion of $TNF\alpha^+$ as the main cytokine of an acute inflammatory response.

Exposure of benzo[a]pyrene to the group of mice with measles vaccine (paramiksovirus) showed similar results to the group with vaccine only. This expression demonstrated that exposure to benzo[a]pyrene had an effect as immunosuppressor and repressed the regulation of $TNF\alpha^+$. This statement is supported by the research conducted by Fanali *et al*.[9]

The expression of $CD4^+TNF\alpha^+$ at week 4 showed no significant difference between mice with benzo[a] pyrene exposure and the control with $P = 0.755 > \alpha$. This expression demonstrated that benzo[a]pyrene is one of PAHs that can repress the regulation of $TNF\alpha^+$.

The group of mice treated with the vaccine showed expression of $CD4^+TNF\alpha^+$ about 3.02%. This expression is considered to have significant different compared to the group with benzo[a] pyrene exposure with $P = 0.015 < \alpha$; this occurs since the paramyxovirus vaccine contained attenuated virus, which induces an acute inflammatory response in the body.

The expression of $CD4^+TNF\alpha^+$ within the group of mice with the vaccine is significantly different compared to the group of mice with vaccine plus benzo[a]pyrene exposure with $P = 0.08 < \alpha$. The expression of $CD4^+TNF\alpha^+$ in mice with vaccine and benzo[a]pyrene exposure was 1.43%. This expression shows that benzo[a]pyrene can suppress $TNF\alpha^+$ regulation. The infection response due to vaccines is indicated by $TNF\alpha^+$ expression, which was repressed by benzo[a]pyrene. This response is supported by the study conducted by Holladay and Smith.[10]

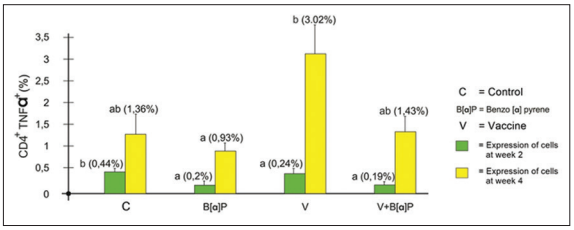

Figure 1: Antigen exposure at week 2 induced the repression of tumor necrosis factor (TNF)α^+ and there was no significant difference among the groups. Moreover, repression of $TNF\alpha^+$ was also demonstrated after exposure to benzo[a]pyrene at week 4. However, there was no different result between the group with vaccine and group with vaccine plus exposure of benzo[a]pyrene. Cells were collected from the spleen and stained with anti-$CD4^+$ and anti-$TNF\alpha^+$. The staining results were analyzed by flow cytometry

Expression of $CD8^+$ IFN-$\ddot{Y}^+$

As shown in Figure 2, mice that were exposed to benzo[a]pyrene had $CD8^+IFN\ddot{Y}^+$ expression of about 75.07% at week 2. This was no difference as compared to the control group. This expression indicates the infection response within that group with $P = 0.674 > \alpha$.

Groups of mice treated with the vaccine showed $CD8^+IFN\ddot{Y}^+$ expression level about 72.4%. This expression was not significantly different as compared to the other groups with $P = 0.951 > \alpha$. Immunization is an administration of attenuated virus. The first contact of measles virus leads to the immunosuppression and prolonged abnormalities of the infected immune cell.[11]

The expression of $CD8^+IFN\ddot{Y}^+$ within a group of mice with vaccine plus benzo[a]pyrene exposure showed about 74.19%. This expression showed no difference in the control and other treatment groups with $P = 0.630 > \alpha$. It showed a response level of the body to the antigen.

The expression of $CD8^+IFN\ddot{Y}^+$ at week 4 within groups of mice with benzo[a]pyrene exposure showed a different result as compared to control. The group of mice with benzo[a]pyrene exposure had $CD8^+IFN\ddot{Y}^+$ expression about 76.65% with $P = 0.021 < \alpha$. Furthermore, the group of mice with vaccine showed no significant difference compared to the control group. Its expression of $CD8^+IFN\ddot{Y}^+$ was 73.11%

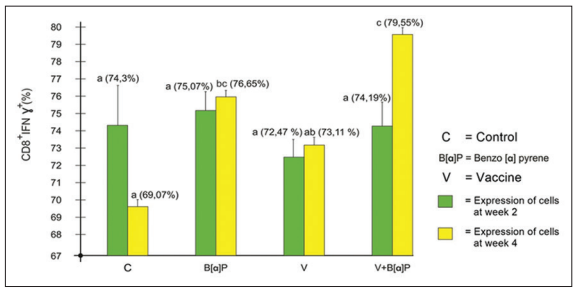

Figure 2: The exposure of antigen at week 2 exhibited a low infection response of IFN$\ddot{Y}$. Furthermore, there was no difference in the results among the groups. After week 4, the exposure of benzo[a]pyrene is detected as an antigen, which induced the expression of IFN$\ddot{Y}$. There is different result, which was demonstrated by group with vaccine treatment and group with vaccine plus benzo[a]pyrene exposure. Cells were obtained from spleen and stained with anti-$CD8^+$ and anti-$IFN\ddot{Y}^+$. Staining was analyzed by flow cytometry

with $P = 0.919 > \alpha$. In the group of mice that treated with vaccine plus benzo[a]pyrene exposure showed differential expression of $CD8^{+}IFN\dot{Y}^{+}$ compared to the group with vaccine only [Table 2].

Expression of $CD4^{+}IL\text{-}1^{+}$

Figure 3 shows that the expression of $CD4^{+}IL\text{-}1^{+}$ at week 2 within the group of mice with benzo[a]pyrene is about 0.36%. This expression is not different than the control group with $P = 0.113 > \alpha$. Administration of benzo[a]pyrene has not been able to repress the regulation function of $CD4^{+}$. It can be explained that the antigen stimulation on the 1st week could not induce the MHC II expression against the viral peptide on the $CD4^{+}$ helper T cells. Therefore, the $IL\text{-}1^{+}$ has not increased and considered lower than the control group.[12]

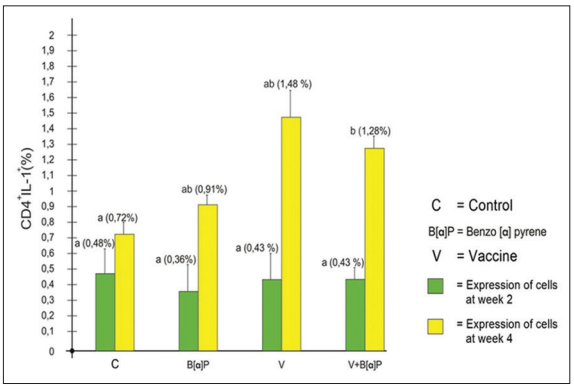

Figure 3: The exposure of benzo[a]pyrene at week 2 showed no significant differences in $CD4^{+}IL\text{-}1^{+}$ expression between all experimental groups. However, the regulation function of $CD4^{+}$ was activated by benzo[a]pyrene exposure at week 4. However, there were no significant differences in $CD4^{+}IL\text{-}1^{+}$ expression between group with vaccine and group with vaccine plus benzo[a]pyrene exposure. Cells were isolated from spleen and stained with anti-$CD4^{+}$ and anti-$IL\text{-}1^{+}$. Then, the staining results were analyzed by flow cytometry

From the results of the LSD test, it can be concluded that there is no difference between each group because the value of $P > 0.05$.

From the results of the LSD test, it can be seen that there is a difference between the vaccine control group and benzo[a]pyrene, between the vaccine group and the vaccine group and benzo[a]pyrene [Table 3].

From the results of the LSD analysis above, there is no difference between groups.

The administration of the vaccine to the mice showed an expression level of $CD4^{+}IL1^{+}$, about 0.43%. This vaccine administration did not show a different result compared to the control group with $P = 0.519 > \alpha$. However, the administration of a vaccine on the 1st week could trigger immunosuppression effect on the infected host cells.[11] Therefore, this response will reduce the main function of $IL\text{-}1^{+}$ as a pro-inflammatory cytokine, which acts as an endogenous pyrogen.

Table 1: Results of CD4+ tumor necrosis factor-α expression analysis

In the 2nd week of treatment		
(I) Groups	**(J) Groups**	***P* value**
Control	Vaccine	0,050
	Benzopirene	0,036
	Vaccine dan Benzopirene	0,034
Vaccine	Control	0,050
	Benzopirene	0,872
	Vaccine dan Benzopirene	0,849
Benzopirene	Control	0,036
	Vaccine	0,872
	Vaccine dan Benzopirene	0,977
Vaccine dan Benzopirene	Control	0,034
	Vaccine	0,849
	Benzopirene	0,977
In the 4th week of treatment		
(I) Groups	**(J) Groups**	***P* value**
Control	Vaccine	0,029
	Benzopirene	0,755
	Vaccine dan Benzopirene	0,568
Vaccine	Control	0,029
	Benzopirene	0,015
	Vaccine dan Benzopirene	0,008
Benzopirene	Control	0,755
	Vaccine	0,015
	Vaccine dan Benzopirene	0,794
Vaccine dan Benzopirene	Control	0,568
	Vaccine	0,008
	Benzopirene	0,794

Table 2: Results of CD8⁺IFNŸ expression analysis

In the 2nd week of treatment		
(I) Groups	**(J) Groups**	***P* value**
Control	Vaccine	0,630
	Benzopirene	0,674
	Vaccine dan Benzopirene	0,410
Vaccine	Control	0,630
	Benzopirene	0,951
	Vaccine dan Benzopirene	0,726
Benzopirene	Control	0,674
	Vaccine	0,951
	Vaccine dan Benzopirene	0,681
Vaccine dan Benzopirene	Control	0,410
	Vaccine	0,726
	Benzopirene	0,681
In the 4th week of treatment		
(I) Groups	**(J) Groups**	***P* value**
Control	Vaccine	0,919
	Benzopirene	0,021
	Vaccine dan Benzopirene	0,047
Vaccine	Control	0,919
	Benzopirene	0,017
	Vaccine dan Benzopirene	0,038
Benzopirene	Control	0,021
	Vaccine	0,017
	Vaccine dan Benzopirene	0,702
Vaccine dan Benzopirene	Control	0,047
	Vaccine	0,038
	Benzopirene	0,702

Furthermore, the expression of $CD4^+IL\text{-}1^+$ within a group of mice with vaccine plus benzo[a]pyrene exposure is about 0.43%. The response shown by this group is similar to the group of mice with vaccine treatment only. It demonstrated that the exposure of benzo[a]pyrene in the 1st week has not been able to increase the MHC II expression against $CD4^+$ T helper cells so the $IL\text{-}1^+$ level is lower than the control group.[13]

The expression of $CD4^+IL1^+$ after week 4 showed a lack of significant differences between the control and the mice with benzo[a]pyrene exposure. The expression of $CD4^+IL1^+$ within a group of mice with benzo[a] pyrene exposure is 0.91% with $P = 0.161 > \alpha$. The exposure conducted at week 4 triggered the infection, as indicated by increased expression of $CD4^+IL\text{-}1^+$.

The group of mice with the vaccine showed the expression of $CD4^+IL\text{-}1^+$, about 1.48%. However, it is exhibited no significant difference compared with other groups. While there is no significant difference between vaccine group compared to benzo[a]pyrene group with $P = 0.980 > \alpha$ and between vaccine group and vaccine plus benzo [a]pyrene with $P = 0.980 > \alpha$. A higher express the ion of $CD4^+IL1^+$ indicates the reaction of the body against the virus.

The expression of $CD4^+IL\text{-}1^+$ within the group of mice with vaccine plus benzo[a]pyrene exposure showed no significant difference compared to the group of mice with vaccine only. The expression of $CD4^+IL\text{-}1^+$ within mice with vaccine plus benzo[a]pyrene is 1.28% with $P = 0.980 > \alpha$. This benzo[a]pyrene exposure will activate the regulation function of $CD4^+$. This expression showed that the individuals were protected against the inflammation due to vaccines and benzo[a] pyrene exposure. The $IL\text{-}1^+$ acts as a pro-inflammatory molecule to fight the infection. $IL\text{-}1^+$ increases the expression of adhesion molecules on endothelial cells, which mediate the migration of immunocompetent cells, such as macrophages, lymphocytes, and other cells toward the infection area.[14]

DISCUSSION

Based on the results of this study, benzo[a]pyrene is one of the PAHs that can induce repress regulation of $TNF\alpha^+$. The hematopoietic effect of benzene[a] pyrene on fetuses is explained by the fact that benzo[a]pyrene can reduce the percentage of $CD4^+$ thymocyte, thymic hypocellularity, and inhibit the maturation process of normal thymocytes.[10] This response is in accord with research reported by Fanali *et al*.[9] Their research observed the effects of benzo[a]pyrene within the blood and liver of *Physalaemus cuvieri* and *Leptodactylus fuscus*. Based on the results of these studies, benzo[a]pyrene appears to affect the integrity of several organs, tissues, and cell function. Thus, they concluded, benzo[a]pyrene appears to be hepatotoxic, genotoxic, and immunotoxic.

Table 3: The results of the analysis of $CD4^+IL1$ expression

In the 2nd week of treatment		
(I) Groups	**(J) Groups**	***P* value**
Control	Vaccine	0,519
	Benzopirene	0,113
	Vaccine dan Benzopirene	0,140
Vaccine	Control	0,519
	Benzopirene	0,033
	Vaccine dan Benzopirene	0,042
Benzopirene	Control	0,113
	Vaccine	0,033
	Vaccine dan Benzopirene	0,900
Vaccine dan Benzopirene	Control	0,140
	Vaccine	0,042
	Benzopirene	0,900
In the 4th week of treatment		
(I) Groups	**(J) Groups**	***P* value**
Control	Vaccine	0,919
	Benzopirene	0,021
	Vaccine dan Benzopirene	0,047
Vaccine	Control	0,919
	Benzopirene	0,017
	Vaccine dan Benzopirene	0,038
Benzopirene	Control	0,021
	Vaccine	0,017
	Vaccine dan Benzopirene	0,702
Vaccine dan Benzopirene	Control	0,047
	Vaccine	0,038
	Benzopirene	0,702

Karp[11] stated that the measles virus induces immunosuppression to the cellular immune response within an infected host. The ability of the measles virus to prevent monocytes/macrophages to produce IL-12 is an indication of abnormalities both *in vitro* and *in vivo*. The cellular receptor of the measles virus is CD-46, which can inhibit the production of IL-12. The downregulation of IL-12 mediated by CD-46 is a specific example of the strong inhibitory control of IL-12 production. It is influenced by phagocytic receptors and complemented in cells that presented antigens.[11]

TNF is the main cytokine within the acute inflammatory process. It produced by macrophages and acts as an endothelial cell activator. The recognition of pathogens triggers the inflammation process through the complement pathway. One of the substances produced by the complement reaction is complement 5a (C5a). This C5a release the granules containing histamine and $TNF\alpha^+$ as an essential molecule within the inflammatory process.[14] The main sources of $TNF\alpha^+$ are mononuclear phagocytes cells, antigen-activated T cells, Natural killer cells, and mast cells. Moreover, lipopolysaccharide is a stimulator of macrophages to secrete $TNF\alpha^+$. With low levels, $TNF\alpha^+$ works against leukocytes and endothelium for inducing acute inflammation while, with moderate levels, $TNF\alpha^+$ plays a role in systemic inflammation. Then, within high levels, $TNF\alpha^+$ causes pathological abnormalities of septic shock.[13]

Furthermore, interferon is a protein produced by individuals infected by pathogens or parasites. Tumor cells are one of the factors triggering interferon synthesis. The infected cells will immediately produce and secrete interferon.[14] Benzo[a]pyrene induces cell damage and increasing cell mast density due to the inflammatory response. These cells respond to non-specific inflammation.[9] The effect of type I interferon is for protection against viral infections and increases cellular immunity to intracellular microbes.[13]

The molecule $IL\text{-}1^+$ is a pro-inflammatory agent that produced to encounter the infection. It produced by macrophages, monocytes, fibroblasts, dendritic cells, B cells, NK cells, microglia, and epithelial cells.[14] CD4+ is a transmembrane protein that has a function as a receptor on helper T cells, when T cells recognize the antigen complex, the MHC II $CD4^+$ acts as coreceptor to support the signal transduction so that T cells are activated. $IL\text{-}1^+$ is a mediator of inflammatory that is response to the infection and other stimuli. Moreover, it is also pro-inflammatory cytokine that acts as an endogenous pyrogen. Other effects of $IL\text{-}1^+$ including cell proliferation, differentiation cells, and the function of immunocompetent cells in both innate and specific immune systems.[12]

CONCLUSION

The exposure of benzo[a]pyrene to the mice (*M. musculus* L) with dose 20 mg/kg BW for

2 times/weeks exhibited the immune cell repression on $CD4^+TNF\alpha^+$ and immune cell activation on $CD8^+IFN\ddot{Y}^+$, $CD4^+IL\text{-}1^+$ at week 4. The protection of children from benzo[a]pyrene protection could be conducted by reducing the consumption of food that has undergone incomplete combustion processes, or direct contact of food to air, soil, and water pollution containing benzo[a]pyrene.

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