Veterinary World, EISSN: 2231-0916
Available at www.veterinaryworld.org/Vol.12/October-2019/3.pdf

RESEARCH ARTICLE
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Immune response evaluation in *Balb/c* mice after crude extract of *Anisakis typica* sensitization

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Received: 12-06-2019, **Accepted:** 27-08-2019, **Published online:** 04-10-2019

doi: 10.14202/vetworld.2019.1529-1534 **How to cite this article:** Haryadi L, Suprayitno E, Aulanni'am A, Hariati AM (2019) Immune response evaluation in *Balb/c* mice after crude extract of *Anisakis typica* sensitization, *Veterinary World*, 12(10): 1529-1534.

Abstract

Background and Aim: *Anisakis* is a global challenge for a fish product which may lead to a decrease in economic value and consumers' preference. Skipjack (*Katsuwonus pelamis*) in Kupang, Nusa Tenggara Timur, Indonesia, have important economic value for local fisheries. *Anisakis typica* is one of the *Anisakis* species which potent to induce an allergic reaction. However, the study about *A. typica* involved in the dendritic cells (DCs), T helper 1 (Th_1), T helper 2 (Th_2), and regulatory T cells (Tregs) is still limited. This study aimed to analyze the dynamic changed of the immune system including DCs, $CD4^+$ T cells, and Tregs after 1 week of *A. typica* sensitization.

Materials and Methods: Twenty-four male *Balb/C* mice were randomly divided into four groups (n=6), mice treated with crude *A. typica* extract (CAE) 50, 75, and 100 mg/kg BW, respectively. CAE was given orally per day for a week. At the end of the experiment, the animals were sacrificed and the spleen was collected. DCs were labeled as $CD11c^+$ interleukin-6^+ (IL-6^+); $CD4^+$ T cells were distinguished as Th_1 ($CD4^+$ interferon-γ^+ [IFN-γ^+]) and Th_2 ($CD4^+$ IL-4^+ and $CD4^+$ IL-5^+); Tregs were labeled as $CD4^+CD25^+CD62L^+$. The expression of each cell was determined by flow cytometry.

Results: Our result described that CAE elicits $CD11c^+$ IL-6^+, $CD4^+$ IFN-γ^+, $CD4^+$ IL-4^+, and $CD4^+$ IL-5^+ and reduces $CD4^+CD25^+CD62L^+$ significantly ($p<0.05$) in dose-dependent manner in mice after *A. typica* infection.

Conclusion: The Th_1/Th_2 ratio after *A. typica* crude extract treatment exhibits a mixed pattern rather than the classical model allergy to food antigens. Our study is expected as a basic understanding of the changes in immune response after *A. typic*a infection.

Keywords: allergy, dendritic cells, inflammation, nematode, regulatory T cells.

Introduction

Food safety and food security due to food-borne infections are gaining interest in the past decade [1,2]. Anisakiasis, the zoonotic disease caused by nematode larvae of the genus *Anisakis* is considered as one of the most important biohazards in the fish products [3]. The previous study reported that *Anisakis* spp. were found in commercially fish, particularly anchovies (*Engraulis encrasicolus*), sardines (*Sardina pilchardus*), European hake (*Merluccius merluccius*), whiting (*Merlangius merlangus*), chub mackerel (*Scomber japonicus*), and Atlantic bluefin tuna (*Thunnus thynnus*) [4].

Anisakis spp. have a complex life cycle and reach maturation in the third stage (L3). Marine mammals are a final host to complete its life cycle [5]. A human can accidentally be infected by *Anisakis* after consumed the raw or half-cooked fish meats, which is strongly associated with acute gastrointestinal (GI) symptoms [6] and allergen reaction [7]. Both live and death larva of *Anisakis* could induce the allergic reaction due to its thermal- and pepsin-resistant properties [5,8]. Interestingly, the simple prepared crude extract of *Anisakis* is enough to induce the allergic reaction [9].

The allergic reaction due to *Anisakis* has been reported to elicit the host immune response which is characterized by T helper 2 (Th_2) response predominantly by secreting cytokines such as interleukin-4 (IL-4) and IL-5 [10]. Furthermore, T helper 1 (Th_1) maturation by dendritic cells (DCs) was suppressed by regulatory T cells [11] which assist Th_2 polarization during helminth infection. This regulatory network results in the decrease of interferon-γ (IFN-γ), the cytokine which secreted by Th_1 [12]. In contrast, the previous study reported that *Anisakis* allergy exhibits a mixed Th_1/Th_2 pattern [13]. Meanwhile, prolonged nematode infection may lead to chronic infection predominantly by Th_1 [14].

Nowadays, there are nine *Anisakis* species which have been confirmed [15]. Among them, *Anisakis*

simplex sensu stricto (s.s) and *Anisakis pegreffii* are the best known caused the allergic reaction and other health problems [16]. However, as far as our knowledge, there is little information about allergen reaction which involved immune cells such as DCs, Th_1, Th_2, and regulatory T cells (Tregs) caused by *Anisakis typica*. *A. typica* have been found parasitizes Delphinidae, Phocoenidae, and Pontoporidae in Atlantic and Indian Oceans and in the Eastern Mediterranean Sea. Surprisingly, *A. typica* also found in Australian and Indonesia [17]. This finding increases the potential risk for anisakiasis frequency in Southeast Asia, mainly in Indonesia.

This study aimed to evaluate the immune response underlying host after *A. typica* infection. The present study is expected as a basic understanding of *A. typica* accidental infection due to marine products and, subsequently, develops the intervention strategies.

Materials and Methods

Ethical approval

This study was approved by the Ethical Committee of Brawijaya University (approval number 938-KEP-UB).

Animals

Male *Balb/C* mice aged 5 weeks were supplied from the Integrated Research and Testing Laboratory-Unit IV, Gadjah Mada University. Mice were housed in plastic cages for a period of acclimatization. Mice were given food and water *ad libitum* and maintained at room temperature with a 12 h light/dark cycle.

Crude *A. typica* preparation

Skipjack (*Katsuwonus pelamis*) were purchased from the traditional market in Kupang, Nusa Tenggara Timur, Indonesia. *A. typica* was manually harvested from the abdominal cavity of skipjack which naturally parasitized by *A. typica*. *A. typica* was identified by polymerase chain reaction according to Soewarlan *et al*. [18] using NC5 (forward; 5'-GTAGGTGAACCTGCGGAAGATCATT-3') and NC2 (reverse: 5'-TTAGTTTCTTTTCCTCCGCT-3') primer (data not shown). *A. typica* washed with distilled water then stored at 4°C. The crude extract of *A. typica* was made by crushed *A. typica* using porcelain mortar and pestle at 4°C. The protein content of milled *A. typica* then measured using nanodrop spectrophotometer (ND1000). The protein content then considered as a standard to determine the dosage given to animals.

Experimental design

Twenty-four male *Balb/C* mice weight 25 g were randomly and equally divided into four groups (n=6): Normal (unsensitized mice) and mice treated with crude *Anisakis* extract (CAE) 50, 75, and 100 mg/kg BW, respectively. Mice were intragastrically challenged per day for 7 days consecutively with CAE, except the normal group. At the 8th day, mice were anesthetized through intraperitoneal injection the combination of ketamine and xylazine (90 mg/kg and 10 mg/kg, respectively) [19] followed by euthanized by cervical dislocation. The spleen was collected and washed 3 times in sterile phosphate-buffered saline (PBS) then crushed into single-cell suspensions. Single-cell suspensions then added with PBS until reached 10 mL and centrifuged at 2500 rpm for 5 min at 10°C. The supernatant then discarded and the pellet was homogenized with 1 mL PBS [20]. Homogenates then divided into several 1.5 mL tubes according to the staining used then centrifuged at 2500 rpm for 5 min at 10°C.

Cell staining and flow cytometry analysis

The supernatant then discarded and the pellet stained according to: (1) To determine Th cells, cells surface was stained with fluorescein isothiocyanate (FITC) anti-mouse CD4 (BioLegend, clone: GK1.5), (2) regulatory T cells were identified by a combination of cell surface antibodies of FITC anti-mouse CD4 (BioLegend, clone: GK1.5), phycoerythrin (PE) anti-mouse CD25 (BioLegend, clone: 3C7), and PE-Cyanine5 (PE-Cy5) anti-mouse CD62L (BioLegend, clone: MEL-14), and (3) DCs were identified by cell surface antibodies FITC anti-mouse CD11c (BioLegend, clone: N418). Briefly, 50 µL of Cytofix/Cytoperm Buffer (BioLegend, cat no: 420801) was resuspended in pellet for 20 min in dark condition at 4°C. Then, homogenates were added with 300 µL wash-perm (BioLegend, cat. no.: 421002) and centrifuged at 2500 rpm at 4°C for 5 min. Supernatant was discarded, and the pellet was stained with intracellular staining (50 µL) of PE anti-mouse IL-6 (BioLegend, clone: MP5-20F3) which combined with CD11c. Pellet which previously stained with anti mouse-CD4 were stained with PE-Cy7 anti-mouse IL-4 (BioLegend, clone: 11B11) and PE-anti-mouse IL-5 (BioLegend, clone: TRFK5) to identified T_h2 Besides, pellet which previously stained with anti mouse-CD4 were stained with PE anti-mouse IFN-γ (BioLegend, clone: XMG1.2) to identified T_h1 . Data were obtained using FACSCalibur™ (BD Biosciences, San Jose, CA, USA). A total of 10,000 cell events were collected for each sample. The cell suspensions for each sample were collected immediately with low or medium flow rate. The single-cell populations were gated according to the staining used for further analysis. Data analysis was conducted using software BD CellQuest Pro™ (BD Biosciences, San Jose, CA, USA).

Statistical analysis

Statistical analyses were performed using Microsoft Excel 2016. All data were expressed as mean±standard deviation. $p<0.05$ was determined using one-way ANOVA followed by Duncan's multiple range test.

Results

Anisakis treatment elicits DCs activation and decline regulatory T cell

Dose-dependent increase DCs in mice treated with *A. typica* crude extract (Figure-1a). The degree of $CD11c^+$ $IL\text{-}6^+$ was increasing significantly ($p<0.05$) in treated mice compared with normal mice (Figure-1c). In contrast, regulatory T cells expression ($CD4^+CD25^+CD62L^+$) was decline significantly ($p<0.05$) in treated mice compared with normal mice (Figure-1b). The increase of $CD11c^+$ $IL\text{-}6^+$ and the decrease of $CD4^+CD25^+CD62L^+$ showed a dose-dependent manner after *A. typica* crude extract treatment (Figure-1c).

Anisakis treatment enhance Th_1/Th_2 ratio in sensitized mice

To determine how the immune response shifted after *A. typica* crude extract treatment, Th_1- and Th_2-related cytokine productions were analyzed in the spleen (Figure-2a). Our result suggests that Th_2 ($CD4^+IL\text{-}4^+$ and $CD4^+IL\text{-}5^+$) was increased significantly ($p<0.05$) after *A. typica* crude extract treatment. In addition, $CD4^+$ IFN-γ^+ (Th_1) also increases significantly ($p<0.05$) after *A. typica* crude extract treatment. Interestingly, $CD4^+$ $IL\text{-}4^+$ and $CD4^+$ $IL\text{-}5^+$ expression were higher in a low dose of *A. typica* crude extract (Figure-2b). Surprisingly, our present study resulting in a higher expression of $CD4^+$ IFN-γ^+ compared to $CD4^+$ $IL\text{-}4^+$ and $CD4^+$ $IL\text{-}5^+$ expression.

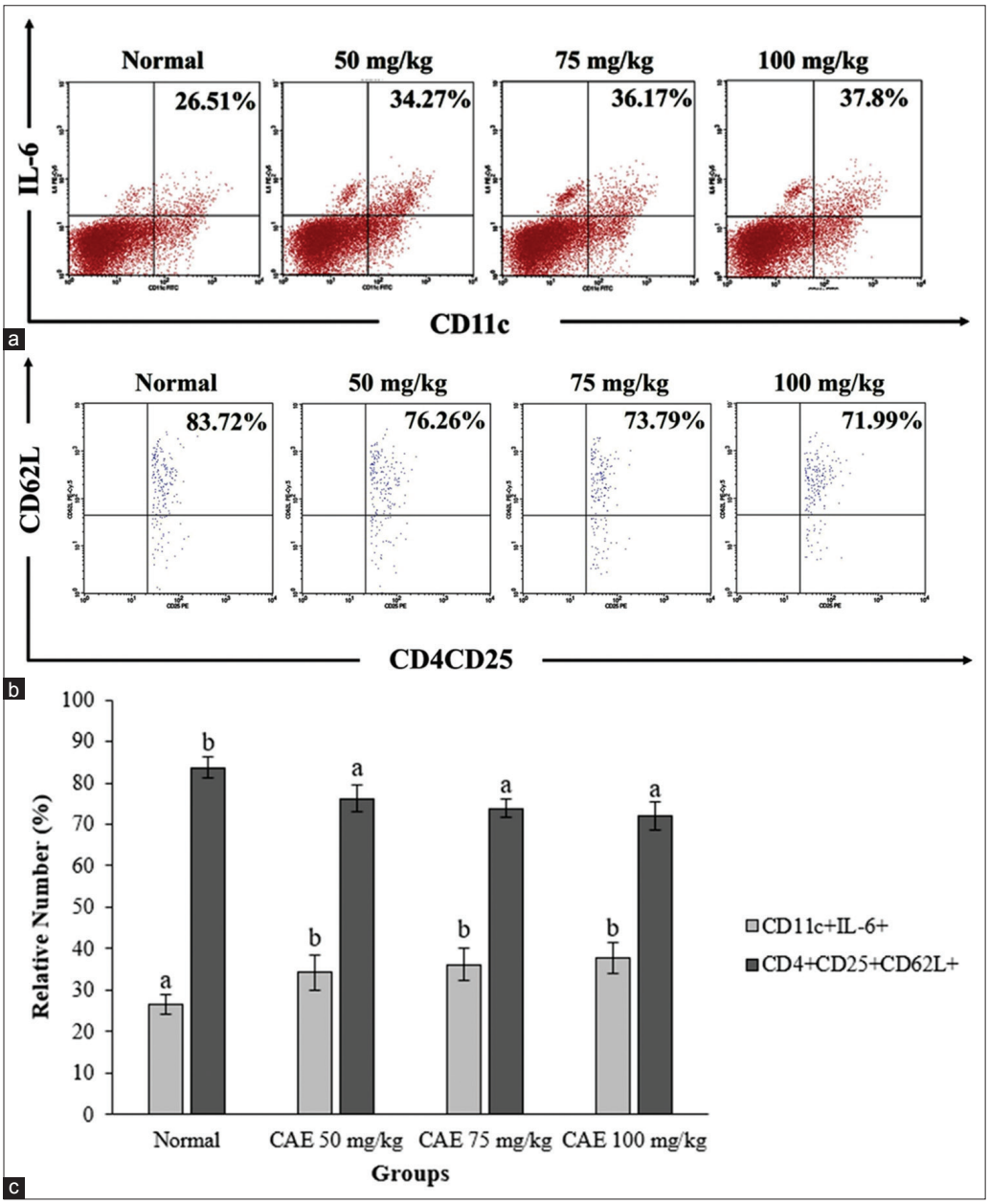

Figure-1: Flow cytometry analysis exhibited dendritic cells and regulatory T cells. (a) $CD11c^+$ interleukin-6^+ ($IL\text{-}6^+$) expressions on splenocytes, (b) $CD4^+CD25^+CD62L^+$ expressions on splenocytes, and (c) $CD11c^+$ $IL\text{-}6^+$ and $CD4^+CD25^+CD62L^+$ expressions were represented as mean±standard deviation (n=6 for each group). The different letter on the chart was considered significantly different for each group ($p<0.05$) and vice versa based on Duncan's multiple range test. CAE=Crude *Anisakis* extract.

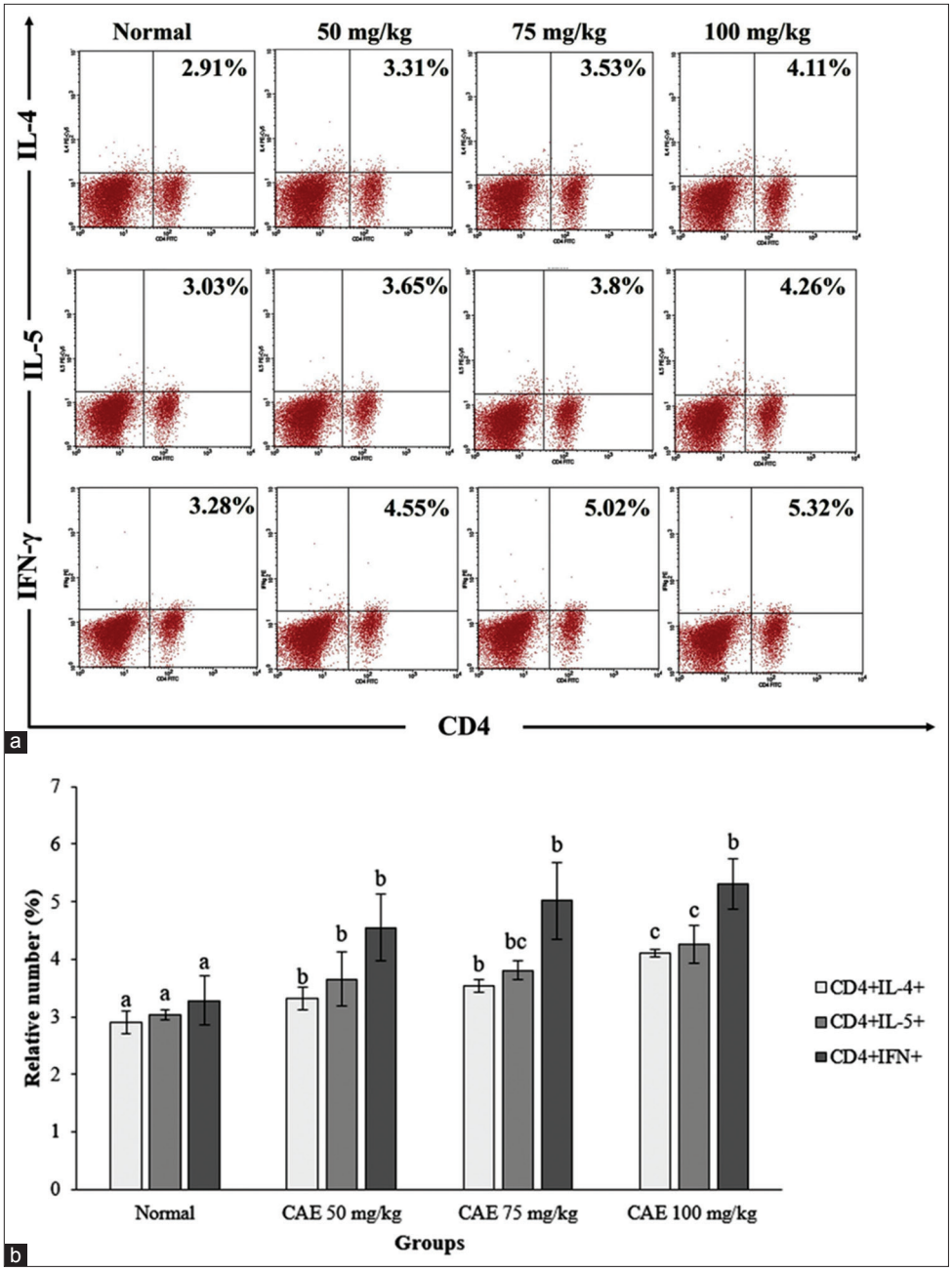

Figure-2: Flow cytometry analysis exhibited T helper 1 and T helper 2 cells. (a) $CD4^+$ interleukin-4^+ (IL-4^+), $CD4^+$ IL-5^+, and $CD4^+$ interferon-γ^+ (IFN-γ^+) expressions on splenocytes and (b) $CD4^+$ IL-4^+, $CD4^+$ IL-5^+, and $CD4^+$ IFN-γ^+ expressions were represented as mean±standard deviation (n=6 for each group). The different letter on the chart was considered significantly different for each group ($p<0.05$) and vice versa based on Duncan's multiple range test. CAE=Crude *Anisakis* extract.

Discussion

Marine food consumption, especially fish, is quite popular globally due to its nutritional content. *Anisakis* is one of the important biohazards in fishery products which may lead to a rejection by consumers and economic losses to the fish industry [17,21]. Nowadays, among nine *Anisakis* species, *A. simplex* (s.s) and *Anisakis pegreffii* are best known for causing infection in human. However, there is little information about *A. typica* causing infection. *A. typica* challenge in our research would greatly improve knowledge of anisakiasis besides *A. simplex* (s.s) and *A. pegreffii* epidemiology.

Our result suggests that the expression of $CD11c^+$ (DCs) was increased after *A. typica* treatment. DCs have a responsibility to present antigen then elicited immune response during parasite infection. Macrophage, other antigen-presenting cells have reported to secrete IL-6 through toll-like receptors activation and elicit Th_2 polarization after antigens, native carbohydrates derived from metacestode larvae parasites sensitization [22]. These results are in line with our study which suggests that *A. typica* challenge induces DCs maturation to secrete IL-6 as a pro-inflammatory cytokine. Another study reported that *in vitro* crude extract *A. pegreffii* elicits DCs to develop immune response by increase CCL3, CXCL4,

CCL4, and granulocyte-macrophage colony-stimulating factor. DCs maturation in lymph node provokes pro-inflammatory IL-6 secretion and participates in Th_2 differentiation [23].

Tregs are known to work synchronize with Th_2 in the early phase of infection through TGF-β signaling. Furthermore, Tregs elicit Th_2 response counter worm infection [24] by attracting eosinophils, mast cells, basophils, and production of IgE [25]. Our study suggests that after 1 week challenged by *A. typica,* there is reduce of naive Tregs population. The previous study reported that *A. simplex* challenge represents the balanced between Th_1/Th_2 responses [13]. IL-4 secretion triggers IgE production by B-lymphocyte, while IL-5 involved in eosinophilic production under anisakiasis [26,27]. Elevated Th_1-mediated response during anisakiasis was associated with Th_{17} activation [28] and severe GI symptoms, which displays the clinical manifestation in patients [29]. Furthermore, one of DCs subset ($CD11c^{mid}CD45RB^{high}$) reported to activate $CD4^+$ T cells to secrete the high levels of both IFN-γ and IL-4 in nematode-infected mice [30].

Conclusion

We have evaluated the immune profile after *A. typica* challenged, which have generated new possibilities to understand the role of *A. typica* after infected mice. In our present study, *A. typica* infection exhibits a mixed Th_1/Th_2 pattern which more skewed to the pro-inflammatory state than the classical model of an allergic reaction to food antigens. Further studies are required to understanding the molecular mechanism of *A. typica* infection which may imply the human allergic reaction during parasite infection. Further experiments are needed to explain the detailed mechanism of *Anisakis* infection. More appropriate experiments such as different route of administration and sample preparation are expected to complete the detailed *Anisakis* infection mechanism.

Authors' Contributions

Concept: AA, ES, and AMH; Design: LH; Supervision: AA, ES, and AMH; Resources: LH; Materials: LH; Data collection and/or processing: LH; Analysis and/or interpretation: LH, AA, ES, and AMH; Literature search: LH; Writing manuscript: LH; Critical review: AA, ES, and AMH. All authors read and approved the final manuscript.

Acknowledgments

The authors acknowledge the Ministry of Marine Affairs and Fisheries Republic of Indonesia for research funding (grant no. 113/SJ/KP.532/III/2016), The authors also thankful to Fish Quarantine and Inspection Agency (FQIA) of Kupang, and INBIO-Indonesia to assisting the research and for the helpful discussion.

Competing Interests

The authors declare that they have no competing interests.

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