Doctoral thesis

Pathological study of the producing reaction of α-defensin against pathogenic microorganisms in eosinophils

好酸球における、病原微生物に対する α-ディフェンシン産生反応の病理学的研究

The United Graduate School of Veterinary Science

Yamaguchi University

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AFIA KHATUN

Thesis submitted to the United Graduate School of Veterinary Science at Yamaguchi University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

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Blood eosinophilia and infiltration of eosinophils into tissue are common in helminth infection and allergic diseases that is usually associated with Th2 immunity. On the other hand, bacterial infections are associated with Th1 immune response as protective immunity and have mutual inhibition effect against Th2. But unlike other bacterial infections, mycobacterial infection is known to induce eosinophilia, but the reason was completely unknown. Recently, human eosinophils stimulated by mycobacterium have been shown to produce α-defensin (a potent antimicrobial peptide) using molecular biological technique, but no studies have been done on α-defensin production in infiltrating eosinophils in tissue. Also, production of α-defensin in animal eosinophils, other than humans, is not known. In this study, at first, I examined α-defensin protein production in mouse eosinophils, infiltrating during helminth infection, using Zamboni fixation and immunohistochemical method. Most of eosinophils infiltrating the

intestinal mucosa during helminth infection showed immunopositivity for α-defensin. In helminth infection, expression level of α-defensin mRNA was 50 fold that in the control, While the number of Paneth cells was doubled, eosinophils number increased markedly. These results suggested that eosinophils are important producer of α-defensin and that α-defensin might be involved in defensive mechanisms against helminths. Next, I established mycobacterial infection showing blood eosinophilia (like human mycobacterial infection) and detected infiltration of α-defensin producing eosinophils that to surrounded mycobacteria in the liver and spleen, indicating direct involvement of eosinophil derived α-defensin against mycobacteria as host defense. Both experimental models used in this study are good model to study host defense mechanism through α-defensin via eosinophils. Mycobacteria infected mouse model is good to study human and animal mycobacterial disease.

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GENERAL INTRODUCTION AND BACK GROUND

1. Eosinophil reactions

Eosinophil reactions caused by a wide variety of disease that include parasite infection, allergy, tumor and autoimmune disease, and are induced by Th2 immunity (Klion and Nutman, 2004; Kovalszki and Weller, 2016). On the other hands, bacterial infection induces Th1 immune response, and mutual inhibition system exists between Th1 and Th2 immune systems (Costalonga et al., 2009). Unlike other bacterial infections, mycobacterial infection is known to induce eosinophilia and infiltration of eosinophils into the infectious lesion in humans (Flores et al., 1983; Wright et al., 1983). In addition, eosinophil recruitment into lesion of infectious site also found in Johne's disease caused by Mycobacterium avium subspecies (Castro et al., 1991; D'Avila et al., 2007; Monif and Williams, 2015). However, the role of eosinophil during these infections was completely unknow. A recent study showed that eosinophils stimulated by Mycobacterium bovis BCG has been shown to produce α-defensin, which is most strong inhibitors of mycobacteria (Driss et al., 2009). However, this study demonstrated that eosinophils collected from the vaccine recipient produce α-defensin using molecular biological techniques (Driss et al.,

2009), there was no study showing α-defensin production in infiltrating eosinophils in tissue within our knowledge.One of the reasons is that there is no optimal model in laboratory animals for mycobacterial infection such as no eosinophilia.

2. Effects of α-defensin

Antimicrobial peptides (AMPs) are ancient and naturally occurring antibiotics in innate immune responses in a variety of organisms (Shin and Jo, 2011). Once in a target microbial membrane, the peptide kills target cells through diverse mechanisms (Izadpanah and Gallo, 2005). Defensins are one of major epidermal AMPs and are produced by granulocytes and mucosal epithelial cells (Izadpanah and Gallo, 2005; Lisitsyn et al., 2012). There are three distinct subfamilies of defensins, α, β and θ-defensins (Lisitsyn et al., 2012).

α-Dfensin are small molecular weight broad spectrum microbicides, secreted by leukocytes and intestinal Paneth cells of mammals. (Izadpanah and Gallo, 2005; Lisitsyn et al., 2012). α-defensins achieve disruption of target microbial cell membrane by several mechanisms including pore formation or a detergent like solubilization (Izadpanah and Gallo, 2005; Ouellette, 1997). In human four α-defensin peptides expressed by neutrophils

and two enteric α-defensins are secreted by small intestinal Paneth cells (Dong et al., 2016). Unlike human neutrophils, murine neutrophils do not express α-defensin (Eisenhauer and Lehrer, 1992; Ouellette, 2011). Murine Paneth cells in small intestinal crypts secrete α-defensin termed as cryptdin (Ayabe et al., 2002; Ouellette, 2011). To date six mouse α-defensins variants, cryptdins 1-6 have been purified from mouse small intestine (Eisenhauer et al., 1992). Among all, cryptdin 4 (Crp4) gene is highly conserved mouse α-defensin gene and have most potent bactericidal activity (Jing et al., 2004; Ouellette et al., 1999).

It has been shown that mice transfected with human α-defensin have acquired resistance to *Salmonella* spp. (Ouellette, 2005; Salzman et al., 2003). In addition, murine α-defensin also has been shown to have bactericidal effect to *Escherichia coli* (Ouellette et al., 1994), *Staphylococcus aureus*, *Listeria monocytogenes*, etc. (Masuda et al., 2011).

α-Defensin has effects not only on bacteria but also on fungi, protozoa and viruses (Lisitsyn et al., 2012; Ouellette, 2005; Zasloff, 2002). Furthermore, it has been also shown to act against helminths (Gallin et al., 1995; Gutierrez-Pena et al., 1996; Kamal et al., 2001; Magalhaes et al., 2008). Human α-defensin clearly show antimicrobial activity against Mycobacterium tuberculosis by increasing the permeability of the mycobacterial

cell envelop (Dong et al., 2016). In addition to direct antimicrobial activity α-defensin into the early lesion, act as chemotactic factor (Dong et al., 2016).

3. Animal models of eosinophilia

Several animal models were reported to exhibit eosinophilia and used for studying the eosinophil (Castro et al., 1991; D'Avila et al., 2007; Daly et al., 1999). Each of these models have the advantages and disadvantages in terms of purpose of investigation. Previous studies reported that both helminth and mycobacteria infected mouse models induced eosinophil infiltration into infectious lesions (Castro et al., 1991; Shin et al., 1997), however, mycobacteria infected mouse model showed relatively small amount of eosinophil infiltration into the tissues (Castro et al., 1991; D'Avila et al., 2007).

3.1 **helminth infected mouse model** (Figure. 1)

Helminth infected mouse model has been reported to show remarkable eosinophilia and tissue infiltration of eosinophils especially in nematode infection to digestive tract (Yantiss, 2015). Eosinophil infiltrative reaction is stronger and widely dispersed in helminth infected model compared to other models showing eosinophil infiltration (Shin et al., 1997; Yantiss, 2015).

3.2 mycobacteria infected mouse model (Figure. 2)

Mycobacterium avium can infect mice and cause eosinophil infiltration into infectious lesion (Castro et al., 1991; D'Avila et al., 2007). To our knowledge, there is no report of eosinophilia in this model, however, it is possible to use the higher concentration of bacteria to infect than the concentration used in these models (Castro et al., 1991; Power et al., 1998).

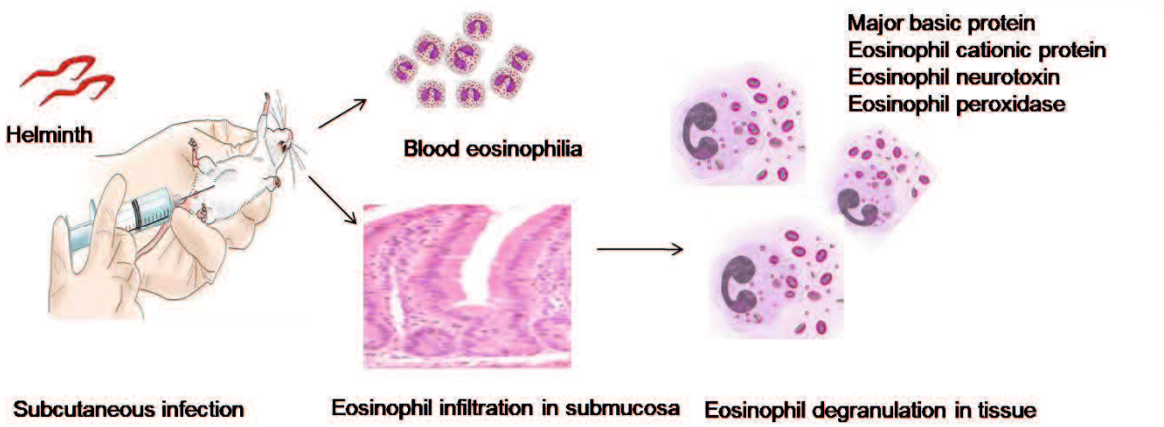

Figure 1. Schematic diagram shows helminth infection to mouse induce blood eosinophilia and infiltration of eosinophils in intestinal submucosa and eosinophil degranulation.

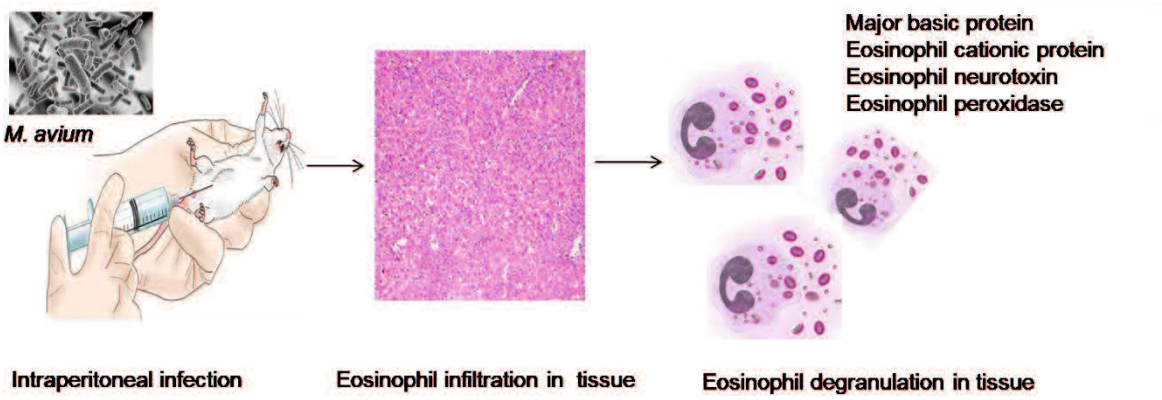

Figure 2. Schematic diagram shows mycobacterial infection to mouse induce infiltration of eosinophils in tissue and eosinophil degranulation.

OBJECTIVE AND STRUCTURE OF THE THESIS

To investigate, whether murine eosinophil express α-defensin (antimicrobial peptide), similar to human eosinophils, against pathogenic microorganisms as host response, helminth infected mouse model and mycobacterium infected mouse model were examined. We also aimed to established models for future study and understanding relationship between eosinophilia and α-defensin production.

In chapter 1: To investigate, whether murine eosinophil produce α-defensin against helminth, using *Nippostrongylus brasiliensis* (Nb) infected mouse model, histopathological analysis, immunofluorescent analysis and real-time PCR in the duodenum were performed, which was the infection site of Nb.

In chapter 2: To investigate whether eosinophils reacting to mycobacteria producing α-defensin in mice and to make good model to study human mycobacterial disease, using *Mycobacterium avium* infected mouse model, histopathological and immunofluorescent analysis in the liver were performed.

OBJECTIVE

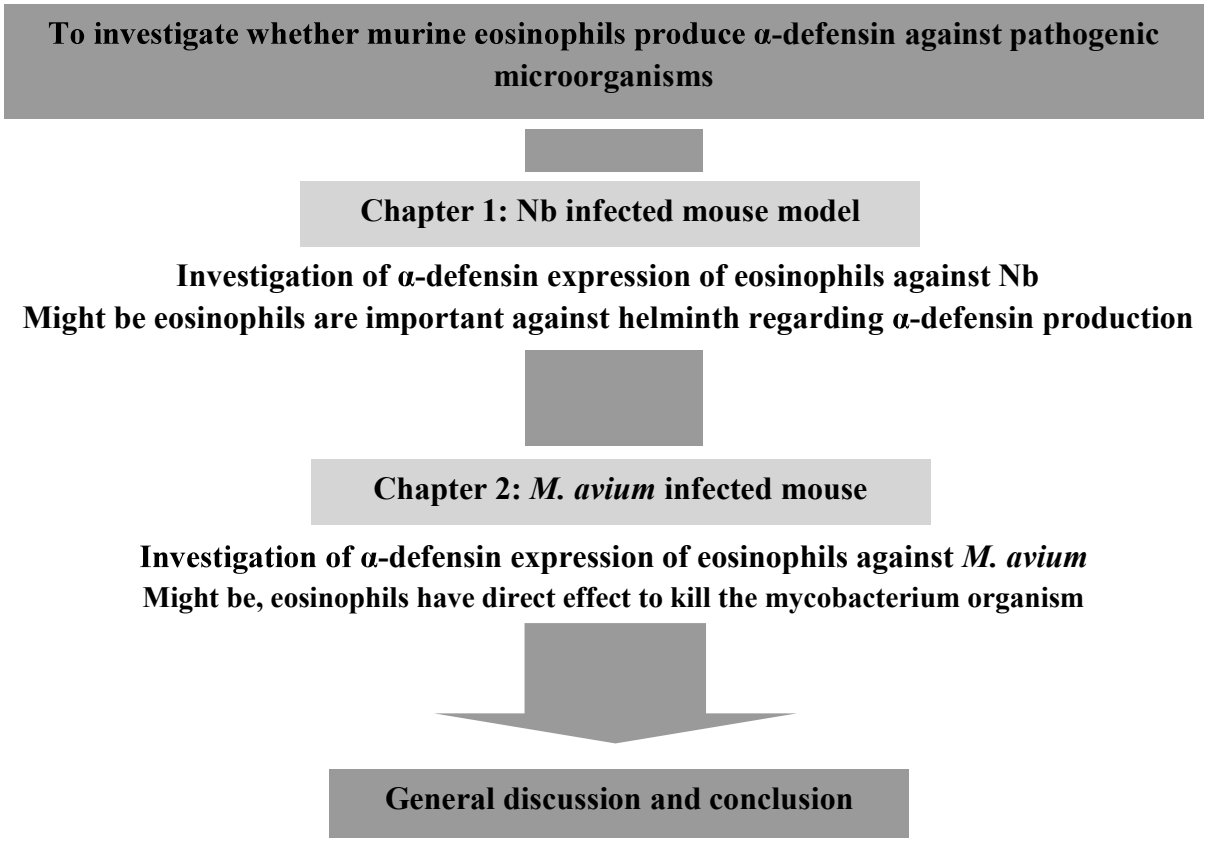

Figure: Structure of the thesis

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications.

Khatun, A., Sakurai, M., Okada, K., Sakai, Y., and Morimoto, M. (2018). Detection of alpha-defensin in eosinophils in helminth-infected mouse model. *J Vet Med Sci*. (in press)

Khatun, A., Sakurai, M., Okada, K., Sakai, Y., and Morimoto, M. (2018). Mycobacterial infection induces eosinophilia and production of alpha-defensin by eosinophils in mice. *J Vet Med Sci*.(in press)

CHAPTER 1

Detection of α-defensin in eosinophils in helminth-infected mouse model

ABSTRACT

α-defensin is a potent antimicrobial peptide secreted from intestinal mucosal epithelial cells, such as Paneth cells, and affects not only bacteria but also parasites and fungi. Recently, human eosinophils have also been shown to produce α-defensin, but no studies have been done on other animals. In this study, we attempted to detect α-defensin protein in mouse eosinophils infiltrating the intestinal mucosa during a helminth infection using Zamboni fixation and immunohistochemistry. Most of the eosinophils infiltrating the intestinal mucosa during helminth infection were positive for α-defensin. The expression level of α-defensin mRNA was 50 fold that in the control. Meanwhile, the number of Paneth cells was doubled, and their α-defensin fluorescence intensity was increased. These results suggested that eosinophils are also important producers of α-defensin, such as Paneth cells in mice, and that α-defensin produced from eosinophils might be involved in defensive mechanisms against helminths. Moreover, the experimental system used in this study is a good model to study the generation of α-defensin by eosinophils.

INTRODUCTION

Eosinophilia can be caused by a wide variety of diseases, including parasite infection, allergy, tumor, autoimmune disease, and is known to be induced by Th2-mediated immune reactions (Furuta et al., 2014; Klion and Nutman, 2004; Kovalszki and Weller, 2016; Spencer and Weller, 2010). Usually, bacterial infection is associated with Th1 immune responses as protective immunity, is characterized by production of gamma interferon, and exhibits mutual inhibitory effects against Th2 (Atarashi et al., 2017; Costalonga et al., 2009; Huang et al., 1999). However, unlike other bacterial infections, mycobacterial infection is known to induce eosinophilia that is usually associated with Th2 immunity (Kirman et al., 2000). A recent study reported that, in humans, eosinophils, activated against mycobacteria, produced α-defensin (Driss et al., 2009) and antibacterial cationic peptides, which are most strong inhibitors for mycobacteria (Dong et al., 2016). However, it is still unknown whether eosinophils produced α-defensin in other animals.

Several types of defensins are found both in human and mouse; human defensins are released from neutrophils and epithelial cells during bacterial infection (Dong et al., 2016; Lisitsyn et al., 2012). Human α-defensin homologue has also been identified in mice (cryptdin 4) (Ouellette et al., 1999). It has already been shown that mice,

transfected with human α-defensin, have acquired resistance to *Salmonella spp.* (Ouellette, 2005; Salzman et al., 2003a; Salzman et al., 2003b). In addition, murine α-defensin has also been shown to exhibit resistance to *Escherichia coli* (Ouellette et al., 1994), *Staphylococcus aureus*, *Listeria monocytogenes*, etc. (Masuda et al., 2011). α-defensin acts not only against bacteria but also against fungi, protozoa, and viruses (Lisitsyn et al., 2012; Llenado et al., 2009; Ouellette, 2005; Zasloff, 2002); furthermore, it has also been shown to act against helminths (Gallin et al., 1995; Gutierrez-Pena et al., 1996; Kamal et al., 2001; Magalhaes et al., 2008).

It has been previously reported that Paneth cells are crucial for mouse α-defensin production (Kamal et al., 2001; Lisitsyn et al., 2012; Salzman et al., 2003a), because unlike human neutrophils, murine neutrophils do not express α-defensin (Eisenhauer and Lehrer, 1992; Ouellette, 2011). Therefore, most of the researches related to α-defensin are limited to Paneth cells in mice. Paneth cells are distributed along the length of the small intestine but are most abundant in the jejunum and ileum (Salzman et al., 2003b). Paneth cells are predominantly increased during helminth infection (Kamal et al., 2002; Shin et al., 1997) and have been speculated to contribute to elimination of parasites via α-defensin production (Gallin et al., 1995; Magalhaes et al., 2008). Many previous researches have studied the direct action of defensin against helminths (Gallin et al.,

1995; Magalhaes et al., 2008) and there have been few studies on *in vivo* kinetics and activation mechanisms. In the previous studies related to α-defensin production by Paneth cells, researchers used neutral buffered formalin for sample fixation for immunohistochemistry (Ayabe et al., 2002; Ouellette et al., 1999; Wehkamp et al., 2006). However, Zamboni fixative solution is more suitable for fixing small size molecules and soluble substances, including α-defensin, compared to buffered formalin, because of rapid penetration property (Accinni et al., 1974). If we can detect α-defensin production by murine eosinophils (similar to human eosinophils) (Driss et al., 2009), eosinophils might be recognized as important cells, similar to Paneth cells, producing α-defensin in mice.

Using Nb infected mouse models, we examined α-defensin expression using immunofluorescent analysis and real-time PCR in the duodenum, which was the infection site of Nb.

MATERIALS AND METHODS

Animal

Pathogen free female ICR mice (Clea Japan, Tokyo, Japan) were fed with autoclaved food (MF; Oriental Yeast, Tokyo, Japan) and tap water ad libitum. All animals were handled according to the regulations for animal welfare of Yamaguchi University (Permit number: 223).

Parasitological technique

Nb maintained by serial passage in Sprague Dawley rats as previously described (Morimoto et al., 2006) and infective stage larvae were recovered from feces by using a modified Baermann apparatus, and were washed (Morimoto et al., 2006). Mice were infected by subcutaneous injection of 800 infective larvae.

Blood eosinophil count and intestinal tissue preparation

Mice were anesthetized with mixture of Xylazine and ketamine and killed by

exsanguination at day 9 after nematode infection. Blood was drawn from the heart using a heparinized syringe. The number of eosinophils in blood was counted after staining with Hinkelman's solution, as reported previously (Morimoto et al., 1998). Duodenum, jejunum and ileum were thoroughly observed and fixed in Zamboni's solution (Rieger et al., 2013) at 4 °C overnight.

Histopathology and immunofluorescence

Fixed intestinal samples were routinely processed and embedded in paraffin. Then, they were cut into 2 μm-thick sections. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin. Paneth cell number was counted in hematoxylin and eosin stained intestinal sections (characterized by pyramidal shape with apical located eosinophilic granules at crypt base) and expressed as number per 10 villus crypt units (VCU). The sections were examined using a microscope (BX53 microscope with DP73 camera, Olympus Corporation, Tokyo, Japan).

As duodenum was main infection site for Nb, only duodenum part was choose for immunofluorescence analysis and rest of experimental procedures. For double immunofluorescence staining assay, duodenum sections were deparaffinized and

subjected to antigen retrieval by treating with 0.5 % trypsin for 30 min. Then, the sections were blocked by 5 % skim milk with albumin, obtained from Bovine Serum Cohn Fraction V, pH 7.0, (Wako Pure chemical Industries Ltd, Osaka, Japan) in PBS for 30 min at room temperature to avoid non-specific reactions. For detection of α-defensin production by eosinophils, sections were incubated with rabbit anti-mouse eosinophil cationic protein (ECP) IgG antibody (1:400, Aviscera bioscience, Inc., Santa Clara, CA, USA) for 1 h at room temperature and washed, followed by incubation with Alexa Flour 488 conjugated goat anti-rabbit IgG (1:200, Life technologies, Eugene, OR, USA). After washing, sections were incubated with α-defensin 4 (R-19) polyclonal antibody (Ayabe et al., 2002; Maemoto et al., 2004) (1:50, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) over night at 4 °C and washed, followed by incubation with Alexa Fluor 555 donkey anti goat IgG (H&L) antibody (1:200, Abcam, Cambridge, UK) for 1 h at room temperature. After washing, specimens were mounted with glycerol. Among all mice α-defensin, α-defensin 4 is unique and the most potent one [17, 34], so we chose it for our study. For detection of α-defensin-positive enteroendocrine cells, sections were incubated with α-defensin 4 (R-19) polyclonal antibody (Santa Cruz Biotechnology, Inc.) overnight at 4 °C, followed by incubation with Alexa Fluor 488 donkey anti goat IgG (H&L) antibody (1:200, Abcam) for 1 h at room temperature. Then,

after washing, the sections were incubated with sheep anti-human chromogranin A antibody (1:50, Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 1 h at room temperature followed by incubation with Alexa Fluor 555 donkey Anti sheep IgG H&L (Abcam) for 1 h at room temperature. After washing, the specimens were mounted. All observations were performed using a fluorescence microscope (BX53 fluorescence microscope with DP73 camera, Olympus Corporation) equipped with suitable filter set (red filter with excitation range of 530-550 nm and an emission range of 575 nm, and green filter with excitation range of 470-495 nm and an emission range of 510 nm). Images were analyzed using GIMP software (version 2.8).

RNA extraction and reverse-transcription (RT)

Thirty milligrams of duodenum of the control group and infected group were used for RNA extraction according to the manual of RNeasy plus mini kit (Qiagen, Tokyo, Japan). Next, 12.5 μl of total isolated RNA was mixed with 5x RT buffer, dNTP, 0.1 M DTT, random primers (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and RNasin (Promega, Wisconsin, USA), and total volume was kept at 24.5 µl. Samples were incubated at 70 °C for 5 min. Then, 0.5 µl SuperScript III (Invitrogen) was added to

reach total volume at 25 μl and incubated at 37 °C for 60 min, followed by incubation at 95 °C for 5 min, and on ice for 5 min to generate cDNA.

Real time PCR

One microgram of cDNA sample was amplified by TaqMan®Gene expression assay for murine α-defensin (Mm00651736_g1 Defa4, Applied Biosystems, Tokyo, Japan) using a Step One^{TM} Real-time PCR System (Applied Biosystems). For amplification, the protocol followed was: 50 °C for 2 min; 95 °C for 10 min; 95 °C for 15 sec, 60 °C for 1 min cycle was repeated for 50 times. For the quantification of the α-defensin 4 mRNA, 18s rRNA (Mm03928990_g1 18S, Applied Biosystems, Tokyo, Japan) was used as housekeeping gene and α-defensin 4 expression was normalized against the value of 18s rRNA.

Fluorescent intensity analysis

Using fluorescent image for cell counting (Fig. 6), fluorescent intensity of α-defensin was quantified for eosinophils, Paneth cells, and enteroendocrine cells. The images were

analyzed. in the software BZ-II analyzer (Keyence, Osaka, Japan). For each cell type, 20 cells were randomly selected per mice and maximum fluorescent intensity was measured.

Statistical analysis

All data were expressed as mean ± standard error (SEM). Statistical significance was determined at $p < 0.05$ following the Student's t test. For the comparison of the α-defensin 4 mRNA expressions, Mann-Whitney *U* test was used, because the expression was represented in logarithmic number and there was no normal distribution for it.

RESULTS

Eosinophil reaction during Nb infection in mice

The number of eosinophils in the blood was significantly higher in infected group (91.5 ± 24.4) than in control group (11.0 ± 2.5). (Fig. 1). In the duodenum, infiltration of eosinophils in the mucosal lamina propria around the epithelial cells of crypts lined in tubular form was observed by immunofluorescence in the infected group, and it was significantly increased compared to the control group (Fig. 2, Table 1).

Epithelial cell reaction during Nb infection in mice

In control group, in duodenum several Paneth cells existed in the bottom of crypts and they had small number of eosinophilic granules in the cytoplasm (Fig. 3a). On the other hand, the Paneth cells became filled with more prominent eosinophilic granules in the infected group (Fig. 3b). The number of Paneth cells increased significantly throughout the intestine. In duodenum the number of Paneth cells (number/10 villus crypt units (VCU)) for control and infected group were 15.4 ± 1.6 and 35.3 ± 2.1, respectively; that is, a significant increase in the number of Paneth cells was observed

(Table 1).

Production of α-defensin by infiltrated eosinophils

To investigate whether eosinophil produced α-defensin, we conducted a double immunofluorescence staining using antibodies specific for eosinophil cationic protein (ECP), a matrix protein associated with specific granules in the eosinophils [43], and α-defensin 4 protein. Immunoreactivity against ECP and α-defensin 4 protein almost co-localized, and more than 82 % of increased eosinophils were positive for α-defensin in the Nb infected group (Fig. 5). However, the α-defensin-positive eosinophils were not found in the specimens in the control group; in addition, no co-localization was found in the control group (Fig. 4, Table 1).

Production of α-defensin by epithelial cells

To detect α-defensin-positive epithelial cells, we conducted immunofluorescence staining using antibodies specific for α-defensin 4 protein and chromogranin A, marker of enteroendocrine cells. Paneth cells were identified from their site of existence and morphological characteristics. Besides eosinophils, some percentages of Paneth cells

and chromogranin A-positive enteroendocrine cells also showed positivity for α-defensin (Fig. 6 and 7, Table 1). The number of α-defensin-positive Paneth cells doubled after infection, and thus, an increase in the fluorescence intensity was observed (Fig. 6, Table 1). On the other hand, there was no change in the number of α-defensin-positive enteroendocrine cells or their fluorescence intensity before and after infection (Table 1 and 2).

Kinetics of α-defensin 4 mRNA expression in duodenum

Using comparative Ct method, the values of α-defensin 4 mRNA expression level in infected group relative to control were calculated. The expression level of α-defensin 4 mRNA in Nb infected mice was significantly higher than that in control mice (50-fold increase) (Fig.8).

Fluorescent intensity analysis of α-defensin

The fluorescent intensity of α-defensin was not detected in eosinophils in control group. However, high fluorescent intensities of α-defensin were observed in Paneth cells and enteroendocrine cells (Tables 1 and 2). In addition, high fluorescent intensity of

α-defensin was detected in infiltrating eosinophils in Nb infected group. The fluorescent intensity of α-defensin in Paneth cells was higher in Nb infected group compared with control group, and intensity of α-defensin was stronger compared with that of eosinophils. However, the fluorescent intensity of α-defensin in enteroendocrine cells did not change significantly compared with the control group (Table 2).

DISCUSSION

The results of the immunohistochemical analysis done in this study showed, for the first time, that the tissue infiltrating eosinophils produce α-defensin (Fig. 5), and that these eosinophils reacted with host defense immune Th2 responses against helminth Nb in murine model (Gerbe et al., 2016; Lawrence et al., 1996).

Several studies have shown that number of Paneth cells increased significantly during intestinal helminth infection (Kamal et al., 2002; Shin et al., 1997) and α-defensin production by activated Paneth cells has been detected using immunohistochemical methods (Ayabe et al., 2002; Ouellette et al., 1999). In this study, we also observed increase in the number of α-defensin-positive Paneth cells throughout the intestine and increase in the fluorescent intensity of the α-defensin in duodenum. However, detection of eosinophil-derived α-defensin has not been reported, and murine neutrophils do not express α-defensin (Eisenhauer and Lehrer, 1992; Ouellette, 2011), therefore, Paneth cells were speculated to be the only important cells which produce α-defensin in mice against helminth infection. However, previous studies used ordinary fixing solutions, such as neutral buffered formalin, for the samples subjected to immunohistochemistry (Ayabe et

al., 2002; Ouellette et al., 1999). We also tried the ordinary fixation methods using formalin and paraformaldehyde, but we could not detect α-defensin in eosinophils (data not shown). Finally, further experiments revealed that Zamboni fixation made it possible to detect α-defensin in eosinophils. Zamboni fixative contains phosphate buffered picric acid and formaldehyde, is very stable, and provides good general fixation with rapid penetration and optimal preservation and stabilization of cellular proteins (Accinni et al., 1974). These facts suggested that the eosinophil secretory granules are easier to degranulate than the Paneth cell granules, and the ordinary fixation methods were not enough to keep α-defensin within eosinophils. Therefore, Zamboni fixation is recommended for future α-defensin research in the intestine rather than using ordinary formalin fixation.

In this study, α-defensin mRNA expression in the duodenum of helminth-infected mice upregulated significantly and showed 50-fold increase compared to mice without helminth infection (Fig. 8). Infiltration of α-defensin-positive eosinophils into the submucosal tissue was prominent (Figs. 2b, 5); however, there was only a 2-fold increase in the number of Paneth cells and the α-defensin-positive fluorescence intensity strengthened too (Table 1 and 2). There were no significant changes in enteroendocrine cell number and their fluorescence before and after infection (Table 1 and 2). These

facts indicated that eosinophil-derived α-defensin contributed a considerable part in the increased α-defensin production due to infection.

Marked eosinophilia is well documented in Nb-infected mice associated with nematode expulsion (Kamal et al., 2002; Shin et al., 1997). Previous evidence indicated that eosinophil and its granule proteins participated in host resistance to helminths; *in vitro* experiments showed that eosinophils can kill a wide range of helminth species and are considered highly toxic for schistosomula, *Trichinella spiralis*, and *Trypanosoma cruzi* (Daly et al., 1999). Secreted proteins have been speculated to be important in mediating the anti-helminthic action of eosinophils, and these proteins include major basic protein-1, major basic protein-2, eosinophil peroxidase, ECP, and eosinophil-derived neurotoxin (Acharya and Ackerman, 2014; Gleich et al., 1993). Major basic protein-1 is highly toxic to mammalian cells *in vitro*, and can damage helminths by disrupting the lipid bilayer membrane or altering the activity of enzymes within tissues (Gleich et al., 1993). ECP caused membrane disruption of helminth parasites by non-ion selective membrane pore formation (Acharya and Ackerman, 2014). Eosinophil peroxidase exerted cytotoxic effects as a cationic toxin, being able to eliminate parasites by lipid peroxidation (Acharya and Ackerman, 2014). In addition to these eosinophil-secreted proteins, α-defensin also has been reported to have anti-helminthic effects (Gallin et al., 1995; Gutierrez-Pena et al.,

1996). Therefore, eosinophils, similar to Paneth cells, may play an important role in helminth exclusion via α-defensin (Elphick and Mahida, 2005). Because several proteins in the eosinophil granules have anti-helminthic action, it is difficult to compare their activities. Moreover, the anti-helminthic effects of these proteins may differ depending on the type of helminth (Acharya and Ackerman, 2014; Gleich et al., 1993).

In this study, α-defensin was detected in Paneth cells, eosinophils, and endocrine cells, but their fluorescence intensities were different (Table 2). Since, each cell produces different class of proteins (Acharya and Ackerman, 2014; Gleich et al., 1993; Ouellette, 2005), it might be possible that they may differ in their ability to produce α-defensin. In addition, α-defensin was detected in eosinophils for the first time in this study using Zamboni fixative solution; however, it may still have been insufficient to fix all the α-defensin in eosinophils. In addition, Paneth cells and enteroendocrine cells produced α-defensin in non-infected control groups. α-defensin eliminated the pathogen but did not kill commensal bacteria (Nakamura et al., 2016); α-defensin derived from Paneth cells and endocrine cells may keep enteric microbiota in health. However, more research is needed to further elucidate the role of α-defensin.

We also showed that enteroendocrine cells produced α-defensin. However, α-defensin from enteroendocrine cells seemed to have no anti-helminthic role, because the α-defensin-positive cell number and the fluorescence intensity of α-defensin of enteroendocrine cells did not change after the helminth infection. Therefore, α-defensin produced by enteroendocrine cell may not be important for the antiparasitic effect. Enteroendocrine cells are known as cells secreting more than 20 peptide hormones for digestion (Worthington, 2015). α-defensin also plays a role in regulating the immune system (Lisitsyn et al., 2012; Ouellette, 2005). Thus, these data suggested that enteroendocrine cells may be involved in the regulation of the intestinal environment.

In the current study, we have shown, for the first time, that eosinophils produce α-defensin, so future research is expected to elucidate the anti-helminthic action of eosinophils via α-defensin production. In addition, Nb infection is well known to activate Th2 immune response (Gerbe et al., 2016; Lawrence et al., 1996) and as Nb infection increased α-defensin production, we speculated that Th2 upregulation was associated with the production of antimicrobial peptides in mice.

In conclusion, this was the first study which revealed the production of α-defensin in murine eosinophils. α-defensin expressing eosinophils played an important role in

helminth exclusion in Nb-infected mouse model. This animal model is well known as a model to activate Th2 immune response (Gerbe et al., 2016; Lawrence et al., 1996). Therefore, mice infected with Nb could be a useful animal model for the study of α-defensin mechanisms with respect to helminth expulsion and to elucidate the relationship between eosinophilia and α-defensin production.

FIGURE AND FIGURE LEGENDS

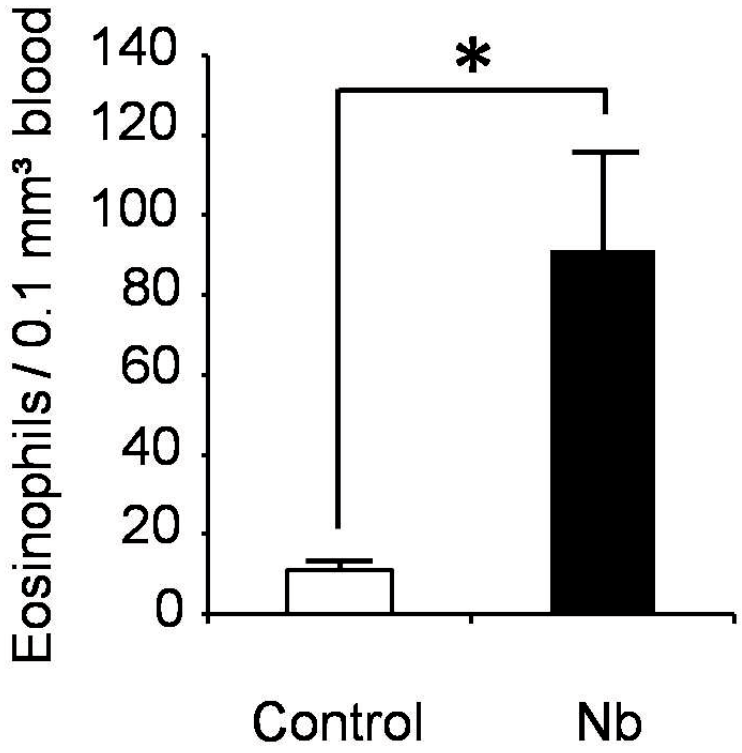

Fig. 1. Blood eosinophil count in control and infected mice. Each group consisted of 5 mice. Data are shown as mean ± SEM. *Significantly different from the values of control mice ($P<0.05$).

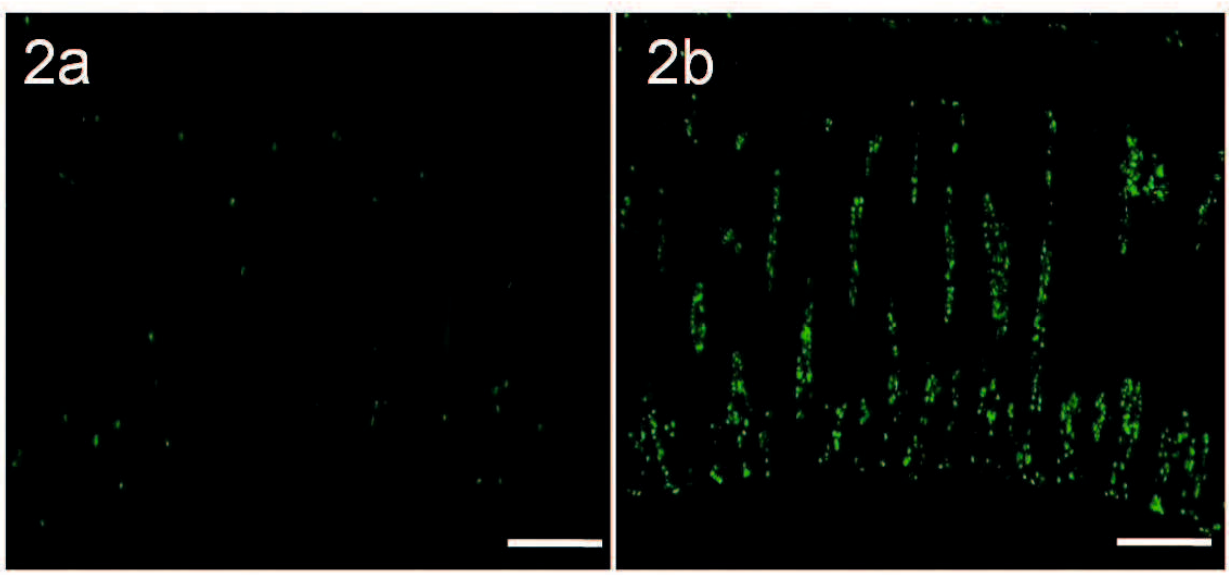

Fig. 2. Immunofluorescence analysis for ECP. (a) A few ECP-positive eosinophils in duodenum of control mice. (b) Numerous ECP-positive eosinophils in duodenum of infected mice. Scale bar = 200μm.

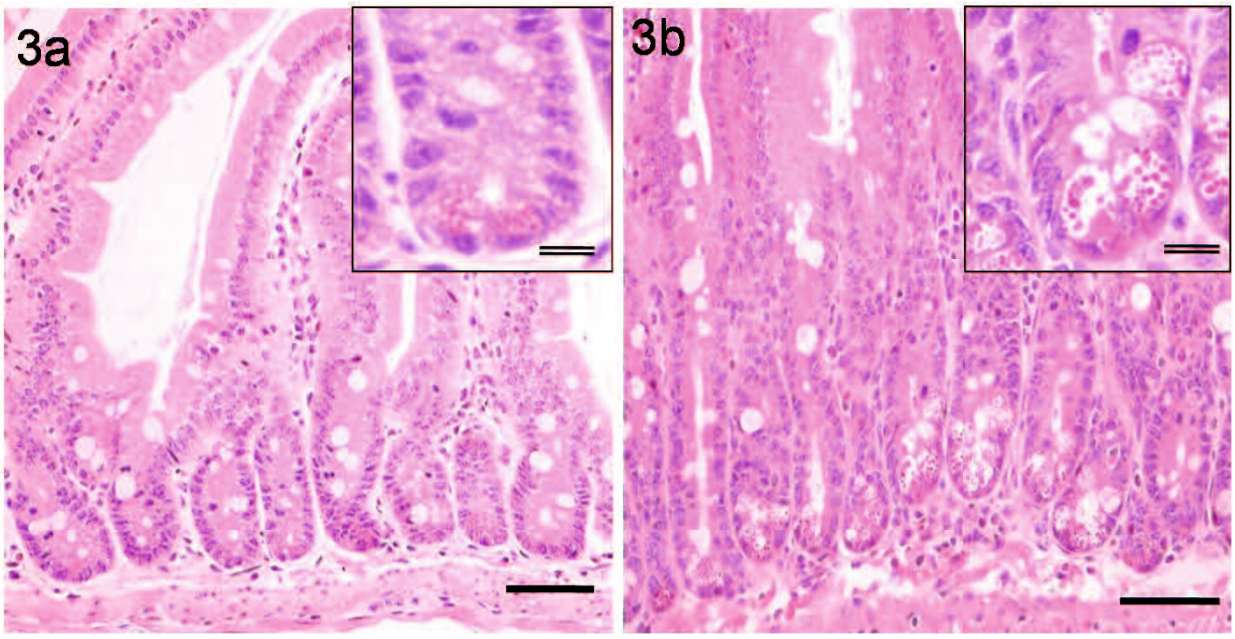

Fig. 3. Histological detection of Paneth cell. Hematoxylin and eosin (HE) staining (a) A couple of Paneth cells in crypt bottom of duodenum of control mice. (b) Paneth cells increased in infected duodenum than control duodenum. Cytoplasm of Paneth cells increase in volume with marked prominent eosinophilic granule in infected duodenum. Scale bars (single line) = 100 μm, scale bar (double lines) = 20 μm.

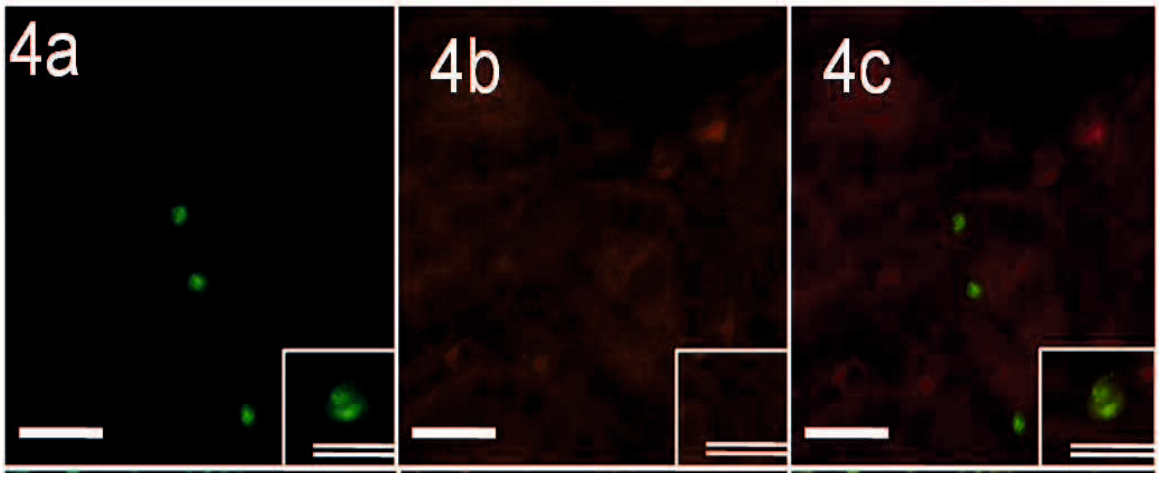

Fig. 4. Double immunofluorescence analysis for ECP (a) and α-defensin 4 (b) in control mice. (c, merged) A few ECP-positive eosinophils in duodenum without α-defensin 4 expression. Scale bars (single line) = 50 μm, scale bar (double lines) = 20 μm.

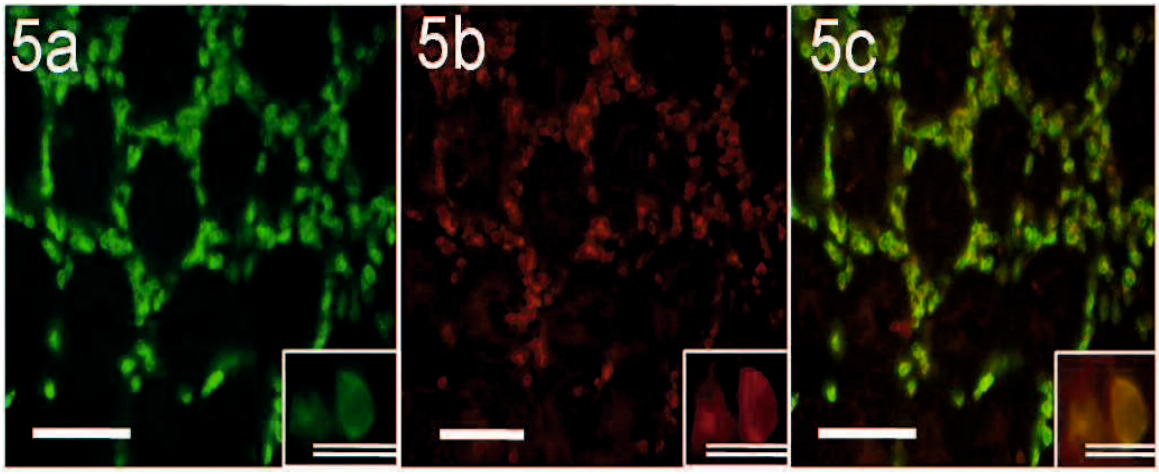

Fig. 5. Double immunofluorescence analysis for ECP (a) and α-defensin 4 (b) in infected mice. (c, merged) Almost all ECP-positive eosinophils in duodenum express α-defensin 4. Scale bars (single line) = 50 μm, scale bar (double lines) = 20 μm.

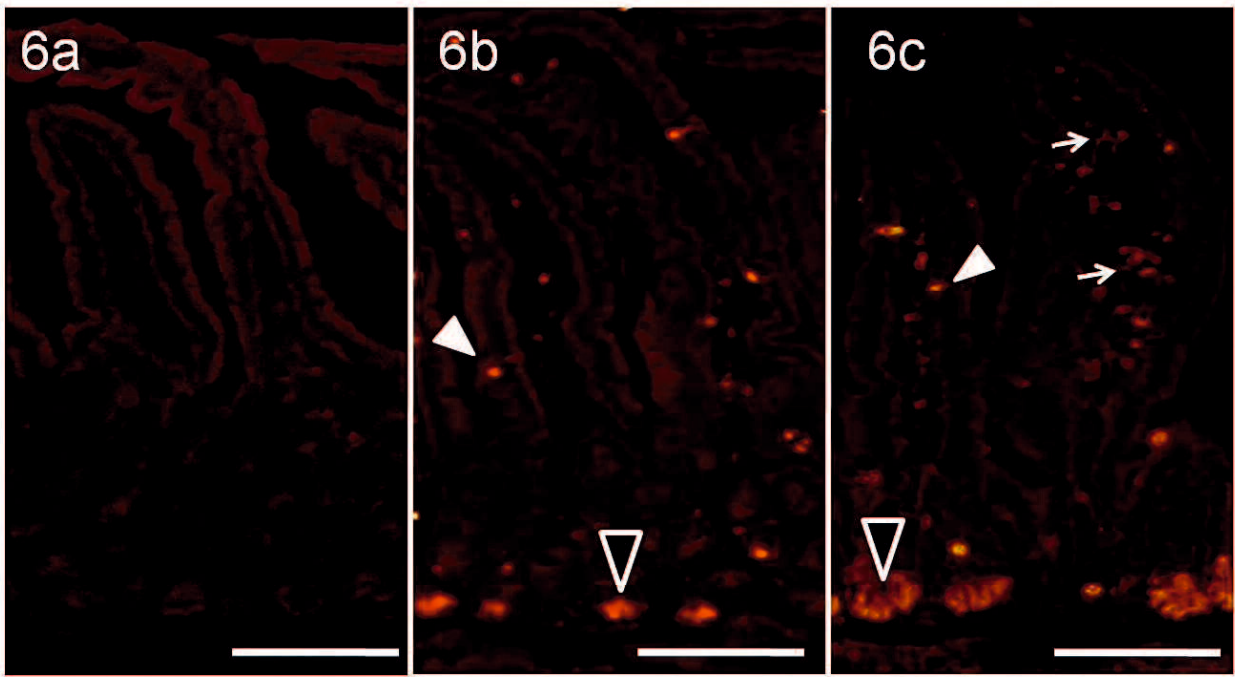

Fig. 6. Immunofluorescence analysis for α-defensin 4. (a) Isotype control. (b) A couple of α-defensin 4-positive Paneth cells (black arrowhead) and enteroendocrine cells (white arrowhead) in duodenum of control mice. (c) Numerous α-defensin 4-positive eosinophils(arrows)with a couple of α-defensin 4-positive Paneth cells (black arrowhead)and enteroendocrine cells (white arrowhead)in duodenum of infected mice. Scale bar = 200μm.

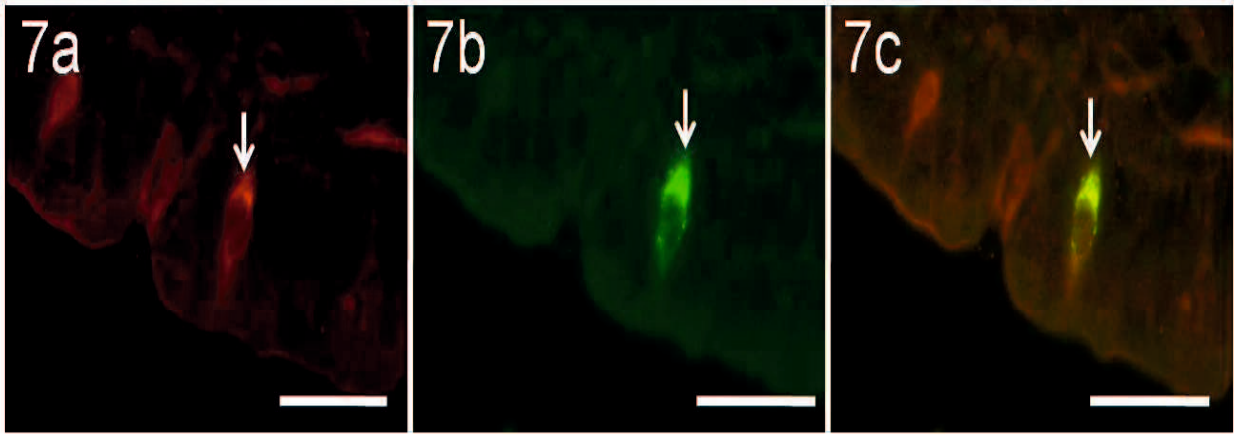

Fig. 7. Double immunofluorescence analysis for chromogranin A (a) and α-defensin 4 (b) in infected mice. (c, merged) Apart of chromogranin A-positive enteroendocrine cells in duodenum express α-defensin 4 (arrow). Scale bar = 20μm.

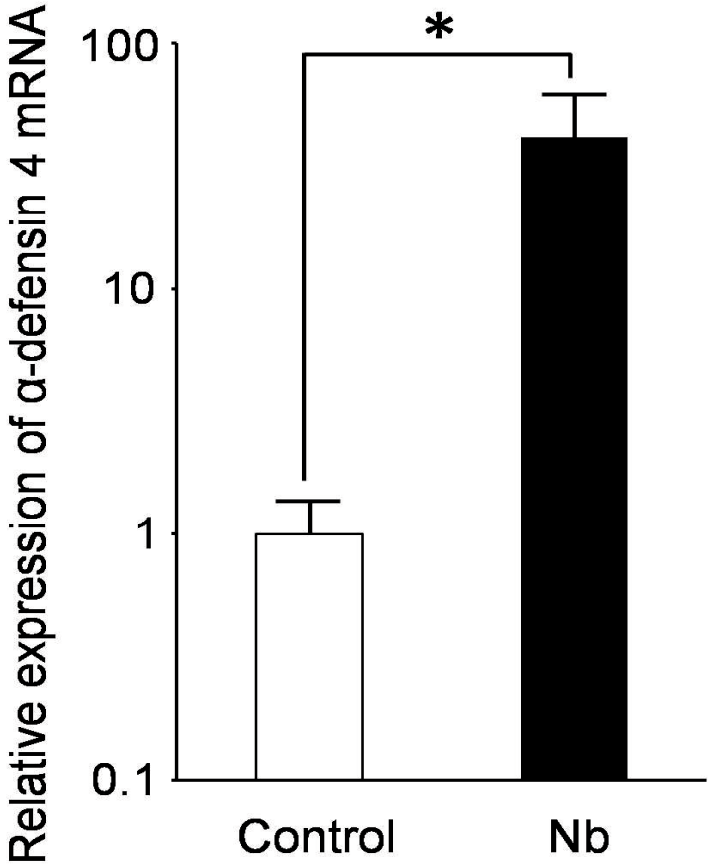

Fig. 8. The effect of Nb infection on α-defensin 4 mRNA expression levels in duodenum. Each group consisted of 5 mice. Data were expressed relative to control non-infected group. *Significantly different from the values of control mice (P<0.05).

Table 1. Number of Duodenal α-defensin-positive cells in control and *Nippostrongylus brasiliensis* infected group.

Cell type	Control			Nb		
	Cell number	α-Defensin positive cell number	Percentage of α-defensin positive cells number	Cell number	α-Defensin positive cell number	Percentage of α-defensin positive cells number
Eosinophil	28.0±0.6	0±0	0±0	532.0±12.0[a)]	450.6±13.3[a)]	81.7±9.4
Paneth cell	15.4±1.6	7.6±0.9	49.1±1.2	35.3±2.1[a)]	17.8±2.7[b)]	48.1±1.2
Enteroendocr -ine cell	36.2±2.8	25.4±3.0	72.2±9.4	45.6±7.1	29.6±1.9	68.7±6.2

Data are presented as the mean ±SEM of 5 mice in each group. Significantly different from the values of control mice a) $P<0.05$, b) $P<0.01$. Cells number express as per 10 VCU. (VCU: Villus crypt units).

Table 2. Measurement of intensity level of α-defensin positive signals in duodenal tissue for control and *Nippostrongylus brasiliensis* infected group.

Cell type	Control	Nb
Eosinophil	0	171.1±10.60[b)]
Paneth cell	141.57±22.84	219.72±17.12[a)]
Enteroendocrine cell	220.3±2.98	223.26±7.02

Data are presented as mean ± SEM of 5 mice in each group. Significantly different from the values of control mice. a) $P<0.05$, b) $P<0.01$.

CHAPTER 2

Mycobacterial infection induces eosinophilia and production of α-defensin by eosinophils in mice

ABSTRACT

It has been well known in humans that eosinophil infiltration into the site of inflammation and eosinophilia occur in mycobacterial infections. However, the role of eosinophils against the mycobacterium is unclear. We showed in previous study that *in situ* mouse eosinophils infiltrated into tissues produce α-defensin, which is the strong anti-bacterial peptide. We investigated in this study whether eosinophils reacting to mycobacteria producing α defensin in mice and to investigate whether it can be used as a model.

We showed that mycobacterial infection induced blood eosinophilia and infiltration of α-defensin producing eosinophils that to surround mycobacteria at the site of infection. These findings were usually seen during human mycobacterial infection. We established a good model to study host defense mechanism against mycobacteria through α-defensin via eosinophils.

INTRODUCTION

Bacterial infections are known to activate host Th1 immune responses (Atarashi et al., 2017; Costalonga et al., 2009; Huang et al., 1999). On the other hand, it is known that Th2 immune reaction is activated by allergy and parasite infection and is associated with eosinophilia (Furuta et al., 2014; Kovalszki and Weller, 2016; Simon and Simon, 2007). However, It has been also well known in humans that eosinophil infiltration into the site of inflammation and eosinophilia occur in mycobacterial infections (Flores et al., 1983; Monif and Williams, 2015; Wright et al., 1983), but the reason was completely unknown. There is only a report showing that eosinophils produce α-defensin, an antimicrobial peptide, in mycobacterium infected patients, and this study suggested that eosinophilia increased α-defensin production and attacked Mycobacterium (Driss et al., 2009). However, this study demonstrated that eosinophils collected from the patients produce α-defensin using molecular biological techniques, there was no study showing α-defensin production in infiltrating eosinophils. So then, there is no study on the defense mechanism against Mycobacterium of α-defensin via eosinophils.

In our previous study, it was shown that *in situ* mouse eosinophils infiltrated into tissues

produce α-defensin in response to helminth infection (Khatun et al., 2018). Originally, α-defensin has been identified as an effective antimicrobial substance against bacterium (Dong et al., 2016; Shin and Jo, 2011), it is known that α-defensin is effective not only for bacteria but also for parasites (Gallin et al., 1995; Gutierrez-Pena et al., 1996; Kamal et al., 2001).Even in animal, eosinophil infiltration also found in Johne's disease caused by *Mycobacterium avium subspecies*(Monif and Williams, 2015; Sharif et al., 2013; Smeed et al., 2007). In addition, it has been reported that eosinophil infiltration into tissues occurs also in experimental mycobacterial infection in mice (Castro et al., 1991; D'Avila et al., 2007). The purpose of this study is to investigate whether eosinophils reacting to mycobacteria producing α-defensin in mice and to investigate whether it can be used as a model.

MATERIALS AND METHODS

Animal

Forty pathogen free female 8 weeks old C57BL/6JJc1 mice were purchased from Clea Japan (Tokyo, Japan). Animals were kept at a constant temperature (24±1°C) and humidity (60±10%) under 12/12 h light/dark cycle with free access to autoclaved food (Clea Japan) and water. All experiments were carried out with the approved by the animal research committee in accordance with the regulations for animal welfare of Yamaguchi University.

Mycobacterium infection

Animals were randomly divided into control and 3 infected groups. *M. avium* strain 104 was grown at 37°C in Middlebrook 7H9 medium (Difco, Detroit, MI, USA) supplemented with 10.0% albumin-dextrose-catalase (ADC) and 0.05% Tween80, and intraperitoneally administered with following three different doses:1.0×10^9 (high), 1.0×10^8 (middle), and 1.0×10^7 (low) in 0.5 ml. The vehicle with same volume was injected in control animals.

Blood eosinophil count and tissue sampling

Samples of 5 mice in each group were taken at day 10 post-infection and the remains were taken at day 30. Mice were euthanized with ketamine and Xylazine and then blood was drawn from heart using a heparinized syringe. Immediately, blood sample was mixed with Hinkelman's solution to stain eosinophils and the number of eosinophils were counted under microscopy according with the method reported previously (Morimoto et al., 1998). Liver and spleen samples were collected aseptically and fixed in Zamboni's solution (Rieger et al., 2013) at 4˚C overnight. Spleens were used for an index of mycobacterial infections (Flores et al., 1983; Monif and Williams, 2015; Wright et al., 13).

Histopathology and immunofluorescence

Zamboni's solution-fixed liver samples were routinely processed and embedded in paraffin. Samples were cut into 2 μm-thick sections. Sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (HE) stain for a general pathological investigation.

For double immunofluorescence staining sections were deparaffinized, subjected to antigen retrieval with trypsin treatment (0.05%) at 37˚C for 30 min and blocked by 5% skim milk in Phosphate buffer saline (PBS) with Albumin from Bovine Serum Cohn Fraction V (Wako Pure chemical Industries Ltd, Osaka, Japan) at room temperature for 30 min. The sections were then incubated with rabbit anti-mouse eosinophil cationic protein (ECP) IgG antibody (1:400, Aviscera bioscience, Inc., Santa Clara, CA, USA) at room temperature for 1 hr. Sections were washed in PBS, incubated with secondary antibody Alexa Flour 488 conjugated goat anti-rabbit IgG (1:200, Life technologies, Eugene, OR, USA) at room temperature for 1 hr. Then, the sections were washed again and α-defensin 4 (R-19) polyclonal antibody (1:50, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) (Ayabe et al., 2002; Maemoto et al., 2004) was reacted over night at 4ºC. After washing, sections were incubated with Alexa Fluor 555 donkey anti goat IgG (H&L) antibody (1:200, Abcam, Cambridge, UK) for 1 hr at room temperature. Specimens were washed, mounted with glycerine and observed by using a fluorescence microscope (Olympus BX53 fluorescence microscope attached to an Olympus DP73 camera, Olympus Corporation, Tokyo, Japan) equipped with suitable filter set (Red filter with excitation range of 530-550 nm and an emission range of 575 nm, and green filter with excitation range of 470-495 nm and an emission range of 510 nm). ECP-positive eosinophils and ECP/α-defensin-double-

positive cells were counted. At least 10 high power fields (×40objective) were counted for each mouse.

When combining ECP immunofluorescence and Auramine-Rhodamine (AR) staining, the coverslips were removed after immunofluorescence stain and the slides were washed for 15 min in running water and then stained with AR. AR stain was performed with AR stain TB Fluorescent Stain Kit T (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) according to manufacturer's protocol. In brief, sections were stained with 1:20 dilution of TB Auramine-Rhodamine T at room temperature for 30 min and then decolorized with TB decolorizer TM (BD) at room temperature for 5 min. The sections were again mounted and photographed by using same fluorescent microscope. Optimization of IF and AR was done as described previously (Ryan et al., 2010).

Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). Statistical significance was determined with the Student t test. $P< 0.05$ was considered as significant.

RESULTS

Blood eosinophilia

In our study, blood eosinophils were significantly increased with high and middle dose infection of *M. avium* (Fig. 1). Also, blood eosinophils tended to increase with low dose infection. In high dose infection group, blood eosinophils were acutely increased at day 10 post infection, and mildly decreased at day 30 post infection. On the other hand, blood eosinophils continued to increase gradually in middle and low dose infection group.

Histopathology

In high dose group, diffuse infiltration of inflammatory cells was found in the liver parenchyma (Fig. 2b, 2c). At day 10 post-infection, inflammatory cells included small number of eosinophils (Fig. 3b), while a lot of eosinophils were found at day 30. Also, eosinophil clusters scattered at day 30 (Fig. 3c). In mice with low and middle dose infection, the histopathological changes were similar but weaker than high dose group.

Eosinophil accumulation against mycobacterium and α-defensin expression in eosinophils

To investigate whether eosinophil recruited at the site of *M. avium* infection, we conducted Combined ECP immunofluorescence staining with antibodies specific for ECP (localized in the specific granules of eosinophils) (Venge et al., 1999) and AR staining for detection of mycobacterium organisms. In the liver specimens of mice with high dose infection at day 30, AR staining-positive mycobacterium organisms were surrounded by ECP-positive eosinophils, clearly indicating eosinophil reaction to *Mycobacterium* (Fig.4). Immunoreactivity against ECP revealed large cluster of intact eosinophils and degranulated eosinophils existed in the lesions.

To detect α-defensin expression in eosinophils, we performed a double immunofluorescence staining with antibodies specific for ECP and α-defensin 4 protein. In the liver specimens of infected mice, almost ECP-positive eosinophils showed expression of α-defensin (Fig. 6). More than 90% of infiltrated eosinophils were positive for α-defensin in infected groups (Table 1), while, eosinophils in the liver of control group did not show immunopositivity for α-defensin (Fig. 5b, Table 1).

DISCUSSION

In the present study, we showed that mycobacterial infection induced blood eosinophilia and infiltration of α-defensin producing eosinophils that to surround mycobacteria at the site of infection. These results indicated that eosinophils react against *Mycobacterium* to kill bacterial organisms with α-defensin. Our observation confirms previous reports of eosinophil recruitment to sites of infection due to mycobacterial infection (Castro et al., 1991; D'Avila et al., 2007; Lasco et al., 2004). In addition, it is the first report as our knowledge that blood eosinophilia in mice induced by *M. avium* infection similar in human mycobacterial infection (Kirman et al., 2000; Pfeffer et al., 2017). Eosinophil infiltrative reaction against *Mycobacterium* is commonly reported in human and animal mycobacterial diseases and experimental animals. Human patients with mycobacterial infection frequently show peripheral eosinophilia (Pfeffer et al., 2017) and eosinophil recruitment into infected lesions (Castro et al., 1991; D'Avila et al., 2007; Kirman et al., 2000). Eosinophil aggregation also has been reported in Johne's disease that is caused by *Mycobacterium avium subsp.* (Monif and Williams, 2015). However, the mechanism involved in eosinophil roles against mycobacteria have not been investigated sufficiently.

α-Defensin expression in eosinophils were indicated in our previous study using animal model of intestinal helminth infection, however, the eosinophils reacted to helminth infection were limited in submucosal region without direct contact to helminth [18]. The present study demonstrates that, α-defensin producing eosinophils infiltrated and surrounded mycobacteria. These findings suggested that eosinophils have a direct effect to kill the mycobacterium organisms through α-defensin. Defensins are large family of antimicrobial peptides and α-defensin expression of epithelial cells and Paneth cells of the small intestine is well known in mice (Ayabe et al., 2002; Dong et al., 2016; Ouellette, 2005; Shin and Jo, 2011). *In vitro* study revealed that α-defensin directly induced mycobacterial lysis via increasing cell membrane permeability (Dong et al., 2016; Mendez-Samperio, 2008). Human α-defensin 1 (HNP-1) kills *M. avium-intracellulare* at the optimal pH *in vitro*(Ogata et al., 1992), and also, HNP-1 improved clearance of bacilli from the lung, liver and spleen of mice with *M. tuberculosis* infection (Sharma et al., 2001). Our study showed more than 90% of infiltrated eosinophil expressed α-defensin at the sites of infection, indicating α-defensin expressing eosinophils play an important role against mycobacterial infection. In the secretory granule of eosinophils, cytotoxic proteins, cytokines and chemokines are stored (Acharya and Ackerman, 2014; Driss et al., 2009; Gleich et al., 1993), and are released for antimycobacterial actions (D'Avila et al., 2007).

Eosinophil granules contains cytotoxic proteins such as eosinophil cationic protein (ECP), major basic protein-1, major basic protein-2, eosinophil peroxidase, eosinophil-derived neurotoxin etc. (Acharya and Ackerman, 2014; Driss et al., 2009; Gleich et al., 1993). An *in vitro* study using human eosinophils revealed that eosinophils stimulated with live *Mycobacterium bovis* BCG expressed both ECP and α-defensin, and a synergistically inhibited mycobacterial growth (Driss et al., 2009). Therefore, eosinophil-derived α-defensin might contribute to kill mycobacterial organisms together with eosinophil cytotoxic proteins.

The blood eosinophilia observed in our experimental model had different patterns according to the dose of mycobacterium. While blood eosinophilia occurred acutely and sustained till day 30 post infection in animals with high dose administration, animals with middle dose administration showed the gradual mild increase of eosinophils in the blood. Animals with low dose infection tended to increase eosinophils but there were no significant differences with controls. The difference of eosinophilia would be a result of induction of Th2 immunity, because eosinophilia is closely link to Th2 immunity (Simon and Simon, 2007; Spencer and Weller, 2010). High dose of mycobacterium infection has been reported to induce Th2 immune reaction easily than low dose administration (Power et al., 1998). Therefore, in our study, high dose would induce strong Th2 immune reaction

and resulted in acute and sustained eosinophilia. On the other hands, Th2 immune reaction has been reported in the chronic infection of mycobacterium (Jiao et al., 2003). Also, in our animal models with low and middle dose infection, Th2 immunity would be gradually activated, resulting mild and gradual eosinophilia. From our previous report, Th2 immune reaction would be strongly related with α-defensin expression in eosinophils (Khatun et al., 2018). To study α-defensin expression in eosinophils, high dose is recommended for making animal model.

In conclusion, our study has clearly revealed that murine eosinophils recruited against mycobacterial infection produce α-defensin. With other cytotoxic proteins, α-defensin might contribute to attack mycobacteria. We established a good model to study host defense mechanism against mycobacteria through α-defensin via eosinophils. This model would be helpful for better understanding of human and animal mycobacterial diseases including tuberculosis and Johne's disease.

FIGURE AND FIGURE LEGENDS

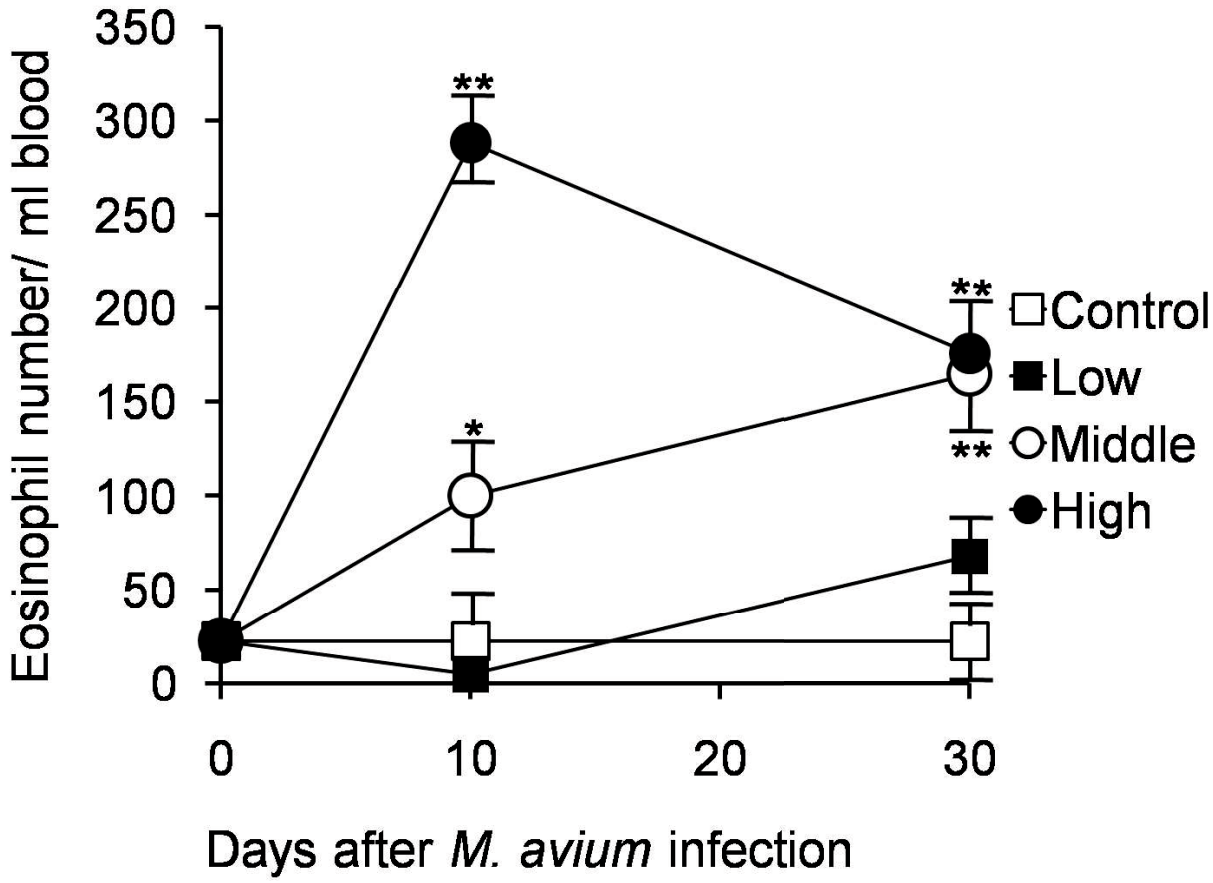

Fig. 1. Blood eosinophilia in different doses of *Mycobacterium avium* infected C57BL/6JJc1 mice. Each group consisted of 5 mice. Data are shown as mean ± SEM. *Significantly different from the values of control mice (P<0.05).

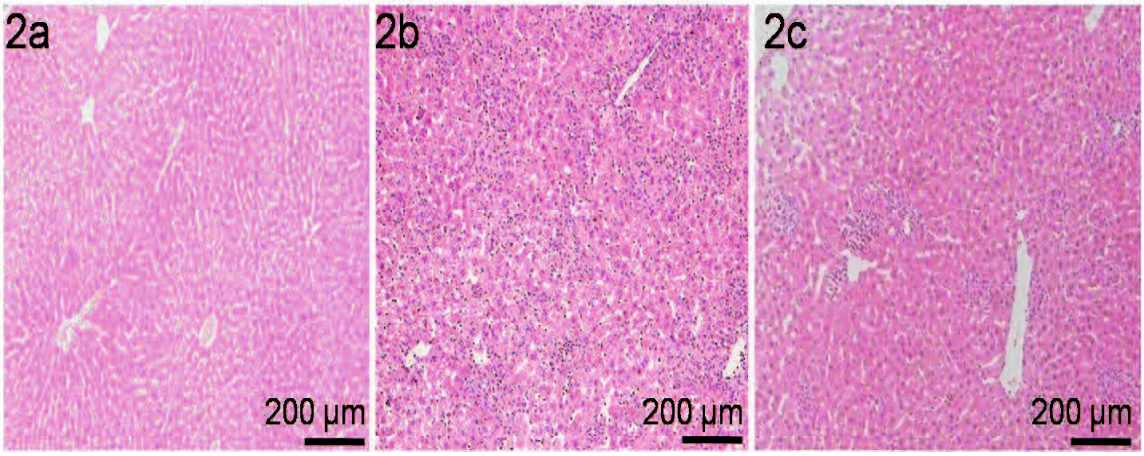

Fig. 2. Histological analysis of inflammatory cells infiltration in liver parenchyma of control (a) and high dose *Mycobacterium avium* infected mice (b and c). Hematoxylin and eosin (HE). (a) No inflammatory cells infiltration. (b) Diffuse infiltration of inflammatory cells at day 10. (c) Diffuse infiltration of inflammatory cells at day 30. Scale bar = 200 μm.

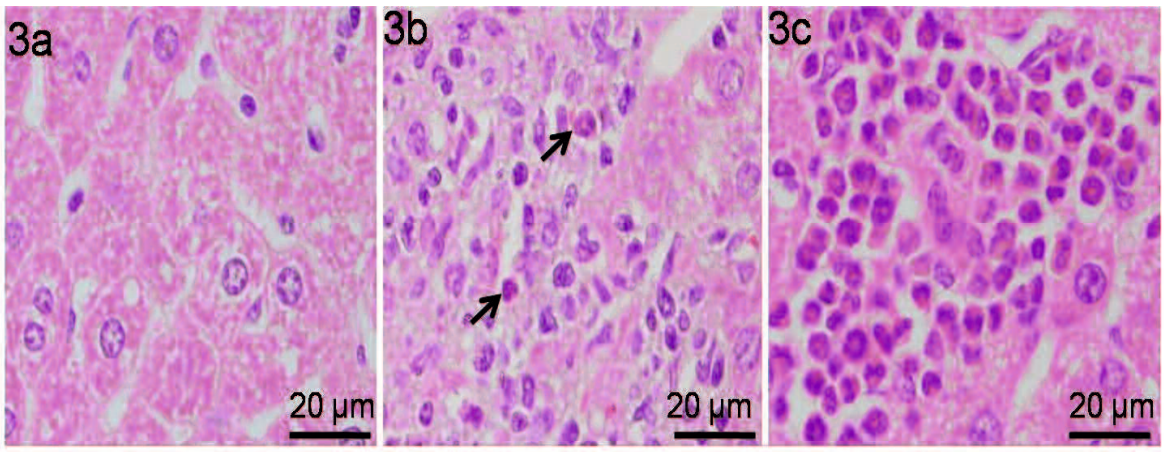

Fig. 3. Histological detection of eosinophils in inflammatory lesions in liver parenchyma of control (a) and high dose *Mycobacterium avium* infected mice (b and c). Hematoxylin and eosin (HE). (a) No eosinophils infiltration. (b) Inflammatory lesions contain lymphocytes, macrophages and small number of eosinophils at day 10. Arrows indicate eosinophils. (c) A lot of eosinophils making clusters noted at day 30. Scale bar = 20 μm.

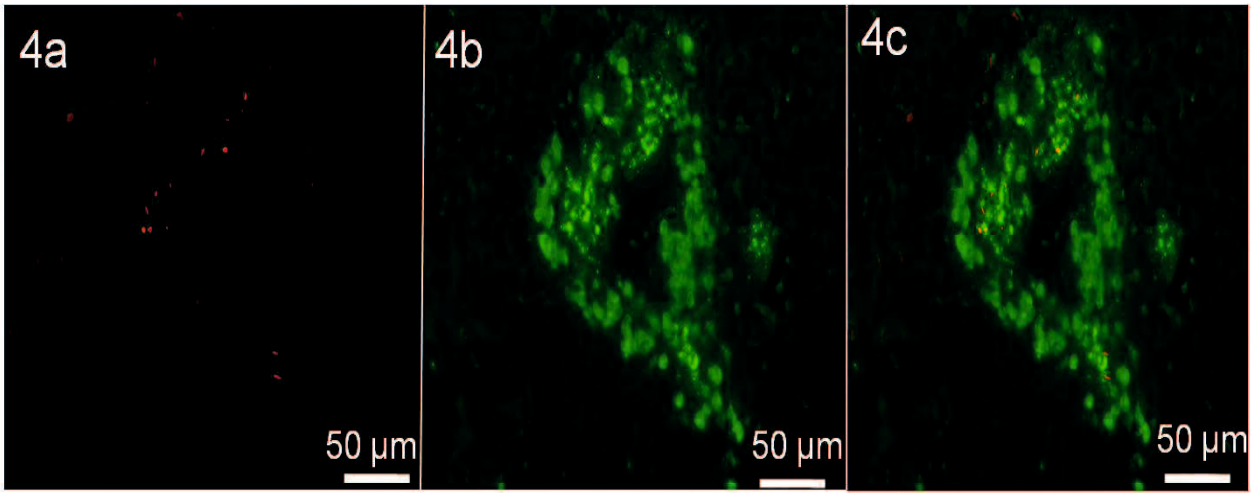

Fig.4. Combined immunofluorescence analysis for auramine-rhodamine (AR) stain (red) (a) and ECP (green) (b) in high dose *Mycobacterium avium* infected mice. (c, merged) eosinophil cluster and degranulated eosinophils existed surrounding *Mycobacterium avium* bacteria in liver tissue at day 30. Scale bar = 50 μm.

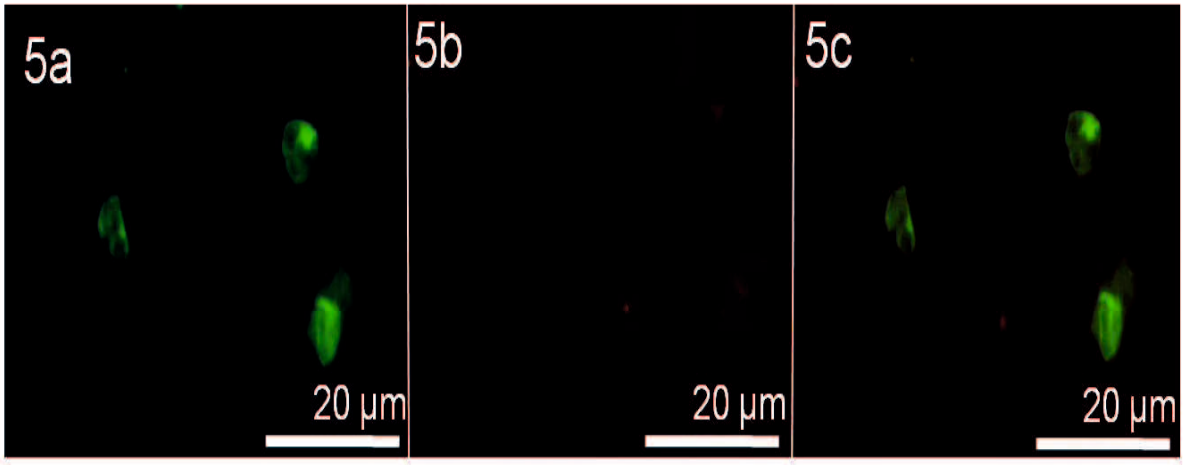

Fig. 5. Double immunofluorescence analysis for ECP (a) and α-defensin 4 (b) in control mice. (c, merged) ECP-positive eosinophils in liver without α-defensin 4 expression. Scale bar = 20 μm.

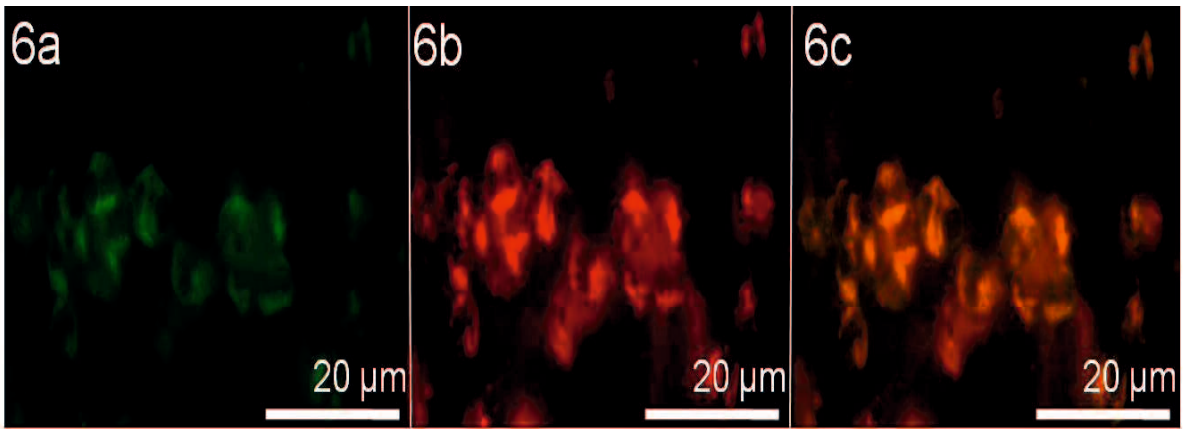

Fig. 6. Double immunofluorescence analysis for ECP (a) and α-defensin 4 (b) in high dose *Mycobacterium avium* infected mice. (c, merged) ECP-positive eosinophils in liver express α-defensin 4. Scale bar = 20 μm.

Table 1. α-Defensin positive eosinophils number counting from liver of control and *M. avium* infected group.

	Eosinophil[b)]	α-defensin positive cells	Percentage[c)]
Control	2.72±0.19	0.00±0.00	0.00±0.00
Infected	58.40±7.12[a)]	53.14±6.84[a)]	90.43±3.63

Data are presented as the mean ± SEM of 5 mice in each group.

Significantly different from the values of control mice a) $P<0.01$.

b) ECP positive cells were regarded as eosinophils.

c) Percentage of α-defensin positive eosinophils in total eosinophils.

GENERAL DISCUSSION AND CONCLUSION

In the first chapter, results of immunohistochemical analysis showed, for the first time, that the tissue infiltrating eosinophils produce α-defensin, and these eosinophils reacted by host defense immune Th2 responses against helminth Nb in murine model (Figure. 1).

Helminth infected mouse model is not suitable for study of direct effect of eosinophil derived α-defensin on pathogen. In our study, eosinophil infiltrative reaction was limited to submucosal region of intestine without direct contact to helminth. These findings were consistent with several previous studies (Daly et al., 1999; Shin et al., 1997). Although infiltrated eosinophils in submucosal region expressed α-defensin, I could not estimate the direct effect of eosinophil infiltration on intestinal stage of helminth via α-defensin.
Nb larvae migrate from the skin to the lungs, where they undergo a molt and maturation phase en route to the small intestine (Daly et al., 1999). In lung, helminth larvae are present in the parenchyma and there would be a possibility to detect direct contact of eosinophil and helminth organisms. On the other hand, eosinophil reaction against helminth must take time because proliferation, migration form blood and activation in lung would be needed (Anthony et al., 2007; Daly et al., 1999). Nb is present in the lung temporally and

eosinophil might not react in time. A previous *in vivo* study indicated IL-5 (crucial Th2 cytokine for induction of eosinophilia) transgenic mice had increased resident-eosinophils in lung that resulted in trapping of helminth larvae, while normal mice could not trap larvae and most of helminth migrate to the intestine (Daly et al., 1999). These data suggested that eosinophils in the lung of normal mice takes time to react against Nb. Therefore, by using Nb infected mouse model, a lot of difficulty would remain to examine the direct effect of eosinophil derived α-defensin against *N. brasiliensis* larvae.

This study showed that eosinophil express α-defensin against helminth infection. Also, a previous study showed, eosinophils expressed α-defensins, localized within the eosinophil crystalloid granules (Driss et al., 2009). Eosinophil have cytoplasmic granules that contains MBP, ECP, EDN and eosinophil peroxidase (Acharya and Ackerman, 2014; Venge et al., 1999).Cytotoxic granule proteins secreted by eosinophils have been speculated to be important in mediating the anti-helminthic action (Klion and Nutman, 2004).In addition, it has been previously reported that human α-defensin have anti-helminthic effect (Magalhaes et al., 2008). Like other cytotoxic granule proteins in eosinophil, α-defensin also secreted from eosinophil and have anti-helminthic effect. The anti- helminthic effects of these proteins may differ depending on the type of helminth. Although it is hard to identify the detailed mechanism of each cytotoxic granule protein

regarding anti-helminthic action, α-defensin would have an important role in eosinophil reaction against helminth infection.

In the second chapter, I established mycobacterium infected mice model that was closely similar to human case. In the model, blood eosinophilia and infiltration of α-defensin producing eosinophils that to surround mycobacteria at the site of infection was demonstrated in mycobacterium infected mouse model (Figure. 2).

Our study showed that eosinophils clearly producedα-defensin against mycobacterial infection in mice and α-defensin producing eosinophils surrounded mycobacteria at the site of infection, indicatingα-defensin have direct effect on mycobacterium. An *in vitro* study reported that α-defensin was induced in eosinophils by stimulation with mycobacteria and showed a synergistic effect with ECP on mycobacterial growth inhibition (Driss et al., 2009). There is also possibility that eosinophil derived α-defensin has synergistic effect with other eosinophil granule proteins against bacteria but to date investigation is not sufficient. For examples, EPO has been reported to have lytic activity against *M. tuberculosis*(Hogan et al., 2013). ECP possesses antimicrobial activity against both gram-negative and gram-positive bacteria(Driss et al., 2009). In addition to previous studies, our study suggested that α-defensin produced by eosinophils participates in the reaction against

mycobacterial infection. For further study to investigate individual and synergistic effects of α-defensin, it would be useful to make α-defensin-knockout animal model.

The anti-mycobacterial ability of eosinophils challenges the concept that vaccination with BCG and resistance against acute tuberculosis is dependent on host ability to generate Th1 immunity (Driss et al., 2009).The last World Health Organization-sponsored trial, carried out between 1968 and 1971, led to the conclusion that BCG vaccination had no overall protective effect against tuberculosis (Power et al., 1998). Eosinophil reaction to mycobacterium infection observed in our study represented the Tn2 immune reaction, because eosinophils often are intimately linked with Th2 immunity (Spencer and Weller, 2010). Unlike BCG vaccination, mycobacterial infection had been reported to induce Th1 and Th2 immune reactions in human case and animal models (Driss et al., 2009; Power et al., 1998). Further investigations about Th2 immune reaction, particularly about eosinophil reaction, would fill the lack of BCG vaccination, leading to improved vaccine technique.

Helminth infected mouse model and mycobacterium infected mouse model, both animal models have some benefit and limitation. In this study, two types of model were established to study eosinophilia-related mechanism. Helminth infected mouse model, in

which eosinophils do not directly come in contact with the pathogens and eosinophil infiltrative reaction limited to warm burden site that this mainly intestine. On the other hand, in mycobacterium infected model infiltrative eosinophils directly come in contact with the pathogens, facilitated study on direct effect of eosinophils on pathogens. In helminth infected model no systemic infection has been achieved, so eosinophil infiltrative reaction to other organs are not so prominent like the adult worm burden site. In contrast mycobacterium infected model achieved systemic infection resulting disseminated eosinophil infiltrative reaction to other organs including liver, spleen, lymphnode etc. While time requirement for helminth infected model to achieve maximum eosinophil infiltrative reaction is less, only 10 days was enough to get the desired eosinophil infiltrative reaction, mycobacteria infected model requires as long as 1 month to achieve maximum eosinophil infiltrative reaction. Previously mouse infected with Nb model was used for the study of eosinophils mechanism related study against helminths (Daly et al., 1999; Shin et al., 1997). Mouse infected with *M avium* model was reported to use for the study of immune effector mechanism during mycobacterial infections and to study rapid chemotaxis and phagocytosed property of eosinophils against mycobacteria (Castro et al., 1991; Haug et al., 2013). Both helminth and mycobacterium infected model is useful to

investigate eosinophil reaction, however, researchers should choose one according to the purpose of study.

This study recommended Zamboni fixative for future α-defensin research and for other water-soluble, small peptides research in the tissue. Through my study, Zamboni fixative made it possible to detect α-defensin in eosinophil. Zamboni fixative contains phosphate buffered picric acid and formaldehyde, is very stable, and provides good general fixation with rapid penetration and optimal preservation and stabilization of cellular proteins (Accinni et al., 1974). In my study, I also tried ordinary fixation methods using formalin and paraformaldehyde, but I could not detect α-defensin in eosinophils. These facts suggested that ordinary fixation methods were not enough to keep α-defensin with in eosinophils. In one previous study, Zamboni fixative was used for detection of eosinophil granule protein EPO, a small molecule contained in eosinophil granule, in ear skin biopsies of mice (Lee et al., 2015). There are several kinds of small molecular weight water soluble peptides other than α-defensin (Izadpanah and Gallo, 2005). Zamboni fixative might help for easy detection of these types of proteins into tissue.

In conclusion, this study revealed α-defensin productive reaction of eosinophil in both helminth and mycobacterium infected models. These models are good for study of

eosinophil derived α-defensin mechanism against microorganisms. Both models facilitate *in situ* detection of α-defensin in eosinophils and elucidate relationship between eosinophilia and α-defensin production. Mycobacterium infected mouse model is a good model to study eosinophil reaction in human and animal mycobacterial diseases including tuberculosis and Johne's disease.

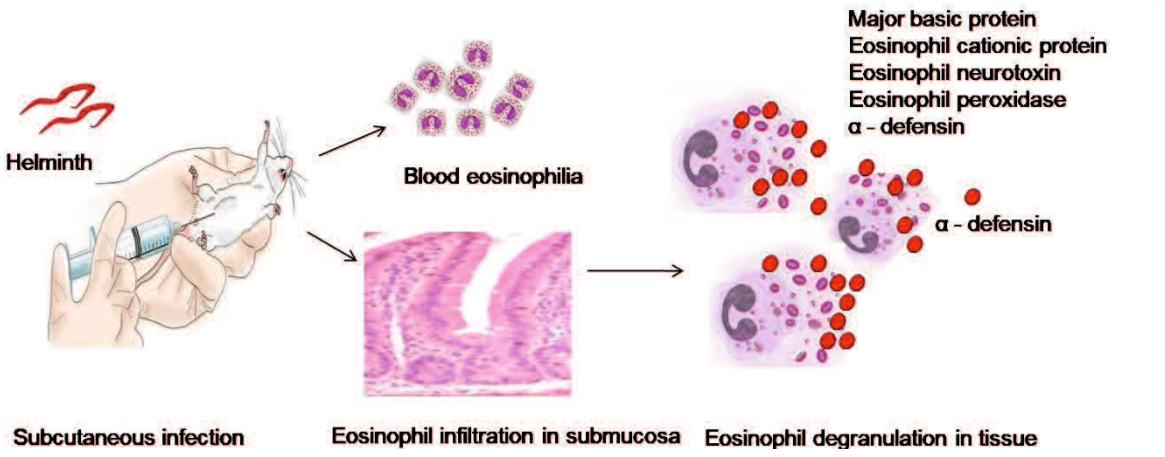

Figure 1. Schematic diagram shows helminth infection to mouse induce blood eosinophilia and infiltration of eosinophils in intestinal submucosa and infiltrated eosinophil produce α-defensin.

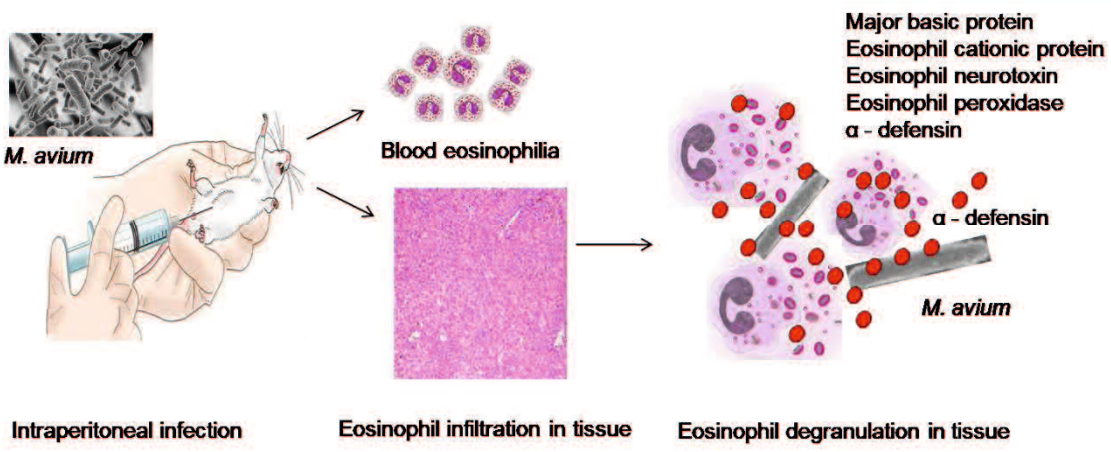

Figure 2. Schematic diagram shows mycobacterial infection to mouse induce blood eosinophilia and infiltration of eosinophils in tissue and infiltrated eosinophils that to surround mycobacteria produce α-defensin.

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REFERENCES

Accinni, L., Hsu, K. C., Spiele, H., and De Martino, C. (1974). Picric acid-formaldehyde fixation for immunoferritin studies. *Histochemistry* **42**, 257-64.

Acharya, K. R., and Ackerman, S. J. (2014). Eosinophil granule proteins: form and function. *J Biol Chem* **289**, 17406-15.

Anthony, R. M., Rutitzky, L. I., Urban, J. F., Jr., Stadecker, M. J., and Gause, W. C. (2007). Protective immune mechanisms in helminth infection. *Nat Rev Immunol* **7**, 975-87.

Atarashi, K., Suda, W., Luo, C., Kawaguchi, T., Motoo, I., Narushima, S., Kiguchi, Y., Yasuma, K., Watanabe, E., Tanoue, T., Thaiss, C. A., Sato, M., Toyooka, K., Said, H. S., Yamagami, H., Rice, S. A., Gevers, D., Johnson, R. C., Segre, J. A., Chen, K., Kolls, J. K., Elinav, E., Morita, H., Xavier, R. J., Hattori, M., and Honda, K. (2017). Ectopic colonization of oral bacteria in the intestine drives TH1 cell induction and inflammation. *Science* **358**, 359-365.

Ayabe, T., Satchell, D. P., Pesendorfer, P., Tanabe, H., Wilson, C. L., Hagen, S. J., and Ouellette, A. J. (2002). Activation of Paneth cell alpha-defensins in mouse small intestine. *J Biol Chem* **277**, 5219-28.

Castro, A. G., Esaguy, N., Macedo, P. M., Aguas, A. P., and Silva, M. T. (1991). Live but not heat-killed mycobacteria cause rapid chemotaxis of large numbers of eosinophils in vivo and are ingested by the attracted granulocytes. *Infect Immun* **59**, 3009-14.

Costalonga, M., Cleary, P. P., Fischer, L. A., and Zhao, Z. (2009). Intranasal bacteria induce Th1 but not Treg or Th2. *Mucosal Immunol* **2**, 85-95.

D'Avila, H., Almeida, P. E., Roque, N. R., Castro-Faria-Neto, H. C., and Bozza, P. T. (2007). Toll-like receptor-2-mediated C-C chemokine receptor 3 and eotaxin-driven eosinophil influx induced by Mycobacterium bovis BCG pleurisy. *Infect Immun* **75**, 1507-11.

Daly, C. M., Mayrhofer, G., and Dent, L. A. (1999). Trapping and immobilization of Nippostrongylus brasiliensis larvae at the site of inoculation in primary infections of interleukin-5 transgenic mice. *Infect Immun* **67**, 5315-23.

Dong, H., Lv, Y., Zhao, D., Barrow, P., and Zhou, X. (2016). Defensins: The Case for Their Use against Mycobacterial Infections. *J Immunol Res* **2016**, 7515687.

Driss, V., Legrand, F., Hermann, E., Loiseau, S., Guerardel, Y., Kremer, L., Adam, E., Woerly, G., Dombrowicz, D., and Capron, M. (2009). TLR2-dependent eosinophil interactions with mycobacteria: role of alpha-defensins. *Blood* **113**, 3235-44.

Eisenhauer, P. B., Harwig, S. S., and Lehrer, R. I. (1992). Cryptdins: antimicrobial defensins of the murine small intestine. *Infect Immun* **60**, 3556-65.

Eisenhauer, P. B., and Lehrer, R. I. (1992). Mouse neutrophils lack defensins. *Infect Immun* **60**, 3446-7.

Elphick, D. A., and Mahida, Y. R. (2005). Paneth cells: their role in innate immunity and inflammatory disease. *Gut* **54**, 1802-9.

Flores, M., Merino-Angulo, J., Tanago, J. G., and Aquirre, C. (1983). Late generalized tuberculosis and eosinophilia. *Arch Intern Med* **143**, 182.

Furuta, G. T., Atkins, F. D., Lee, N. A., and Lee, J. J. (2014). Changing roles of eosinophils in health and disease. *Ann Allergy Asthma Immunol* **113**, 3-8.

Gallin, M. Y., Jacobi, A. B., Buttner, D. W., Schonberger, O., Marti, T., and Erttmann, K. D. (1995). Human autoantibody to defensin: disease association with hyperreactive onchocerciasis (sowda). *J Exp Med* **182**, 41-7.

Gerbe, F., Sidot, E., Smyth, D. J., Ohmoto, M., Matsumoto, I., Dardalhon, V., Cesses, P., Garnier, L., Pouzolles, M., Brulin, B., Bruschi, M., Harcus, Y., Zimmermann, V.

S., Taylor, N., Maizels, R. M., and Jay, P. (2016). Intestinal epithelial tuft cells initiate type 2 mucosal immunity to helminth parasites. *Nature* **529**, 226-30.

Gleich, G. J., Adolphson, C. R., and Leiferman, K. M. (1993). The biology of the eosinophilic leukocyte. *Annu Rev Med* **44**, 85-101.

Gutierrez-Pena, E. J., Knab, J., and Buttner, D. W. (1996). Neutrophil granule proteins: evidence for the participation in the host reaction to skin microfilariae of Onchocerca volvulus after diethylcarbamazine administration. *Parasitology* **113 (Pt 4)**, 403-14.

Haug, M., Awuh, J. A., Steigedal, M., Frengen Kojen, J., Marstad, A., Nordrum, I. S., Halaas, O., and Flo, T. H. (2013). Dynamics of immune effector mechanisms during infection with Mycobacterium avium in C57BL/6 mice. *Immunology* **140**, 232-43.

Hogan, S. P., Waddell, A., and Fulkerson, P. C. (2013). Eosinophils in infection and intestinal immunity. *Curr Opin Gastroenterol* **29**, 7-14.

Huang, L., Krieg, A. M., Eller, N., and Scott, D. E. (1999). Induction and regulation of Th1-inducing cytokines by bacterial DNA, lipopolysaccharide, and heat-inactivated bacteria. *Infect Immun* **67**, 6257-63.

Izadpanah, A., and Gallo, R. L. (2005). Antimicrobial peptides. *J Am Acad Dermatol* **52**, 381-90; quiz 391-2.

Jiao, X., Lo-Man, R., Winter, N., Deriaud, E., Gicquel, B., and Leclerc, C. (2003). The shift of Th1 to Th2 immunodominance associated with the chronicity of Mycobacterium bovis bacille Calmette-Guerin infection does not affect the memory response. *J Immunol* **170**, 1392-8.

Jing, W., Hunter, H. N., Tanabe, H., Ouellette, A. J., and Vogel, H. J. (2004). Solution structure of cryptdin-4, a mouse paneth cell alpha-defensin. *Biochemistry* **43**, 15759-66.

Kamal, M., Dehlawi, M. S., Brunet, L. R., and Wakelin, D. (2002). Paneth and intermediate cell hyperplasia induced in mice by helminth infections. *Parasitology* **125**, 275-81.

Kamal, M., Wakelin, D., Ouellette, A. J., Smith, A., Podolsky, D. K., and Mahida, Y. R. (2001). Mucosal T cells regulate Paneth and intermediate cell numbers in the small intestine of T. spiralis-infected mice. *Clin Exp Immunol* **126**, 117-25.

Khatun, A., Sakurai, M., Okada, K., Sakai, Y., and Morimoto, M. (2018). Detection of alpha-defensin in eosinophils in helminth-infected mouse model. *J Vet Med Sci*.

Kirman, J., Zakaria, Z., McCoy, K., Delahunt, B., and Le Gros, G. (2000). Role of eosinophils in the pathogenesis of Mycobacterium bovis BCG infection in gamma interferon receptor-deficient mice. *Infect Immun* **68**, 2976-8.

Klion, A. D., and Nutman, T. B. (2004). The role of eosinophils in host defense against helminth parasites. *J Allergy Clin Immunol* **113**, 30-7.

Kovalszki, A., and Weller, P. F. (2016). Eosinophilia. *Prim Care* **43**, 607-617.

Lasco, T. M., Turner, O. C., Cassone, L., Sugawara, I., Yamada, H., McMurray, D. N., and Orme, I. M. (2004). Rapid accumulation of eosinophils in lung lesions in guinea pigs infected with Mycobacterium tuberculosis. *Infect Immun* **72**, 1147-9.

Lawrence, R. A., Gray, C. A., Osborne, J., and Maizels, R. M. (1996). Nippostrongylus brasiliensis: cytokine responses and nematode expulsion in normal and IL-4-deficient mice. *Exp Parasitol* **84**, 65-73.

Lee, J. J., Protheroe, C. A., Luo, H., Ochkur, S. I., Scott, G. D., Zellner, K. R., Raish, R. J., Dahl, M. V., Vega, M. L., Conley, O., Condjella, R. M., Kloeber, J. A., Neely, J. L., Patel, Y. S., Maizer, P., Mazzolini, A., Fryer, A. D., Jacoby, N. W., Jacoby, D. B., and Lee, N. A. (2015). Eosinophil-dependent skin innervation and itching following contact toxicant exposure in mice. *J Allergy Clin Immunol* **135**, 477-87.

Lisitsyn, N. A., Bukurova, Y. A., Nikitina, I. G., Krasnov, G. S., Sykulev, Y., and Beresten, S. F. (2012). Enteric alpha defensins in norm and pathology. *Ann Clin Microbiol Antimicrob* **11**, 1.

Llenado, R. A., Weeks, C. S., Cocco, M. J., and Ouellette, A. J. (2009). Electropositive charge in alpha-defensin bactericidal activity: functional effects of Lys-for-Arg substitutions vary with the peptide primary structure. *Infect Immun* **77**, 5035-43.

Maemoto, A., Qu, X., Rosengren, K. J., Tanabe, H., Henschen-Edman, A., Craik, D. J., and Ouellette, A. J. (2004). Functional analysis of the alpha-defensin disulfide array in mouse cryptdin-4. *J Biol Chem* **279**, 44188-96.

Magalhaes, T., Oliveira, I. F., Melo-Santos, M. A., Oliveira, C. M., Lima, C. A., and Ayres, C. F. (2008). Expression of defensin, cecropin, and transferrin in Aedes aegypti (Diptera: Culicidae) infected with Wuchereria bancrofti (Spirurida: Onchocercidae), and the abnormal development of nematodes in the mosquito. *Exp Parasitol* **120**, 364-71.

Masuda, K., Sakai, N., Nakamura, K., Yoshioka, S., and Ayabe, T. (2011). Bactericidal activity of mouse alpha-defensin cryptdin-4 predominantly affects noncommensal bacteria. *J Innate Immun* **3**, 315-26.

Mendez-Samperio, P. (2008). Role of antimicrobial peptides in host defense against mycobacterial infections. *Peptides* **29**, 1836-41.

Monif, G., and Williams, J. E. (2015). "Relationship of intestinal eosinophilia and the acid-fast bacilli in johne's disease."

Morimoto, M., Yamada, M., Arizono, N., and Hayashi, T. (1998). Lactic dehydrogenase virus infection enhances parasite egg production and inhibits eosinophil and mast cell responses in mice infected with the nematode Nippostrongylus brasiliensis. *Immunology* **93**, 540-5.

Morimoto, M., Yamauchi, Y., Shinkawa, S., Ishiwata, K., and Hayashi, T. (2006). Nematode infection in Alymphoplasia (aly) mice: worm species-dependent differential effect of defects of the gut-associated lymphatic tissue system. *Scand J Immunol* **63**, 365-70.

Nakamura, K., Sakuragi, N., Takakuwa, A., and Ayabe, T. (2016). Paneth cell alpha-defensins and enteric microbiota in health and disease. *Biosci Microbiota Food Health* **35**, 57-67.

Ogata, K., Linzer, B. A., Zuberi, R. I., Ganz, T., Lehrer, R. I., and Catanzaro, A. (1992). Activity of defensins from human neutrophilic granulocytes against Mycobacterium avium-Mycobacterium intracellulare. *Infect Immun* **60**, 4720-5.

Ouellette, A. J. (1997). Paneth cells and innate immunity in the crypt microenvironment. *Gastroenterology* **113**, 1779-84.

Ouellette, A. J. (2005). Paneth cell alpha-defensins: peptide mediators of innate immunity in the small intestine. *Springer Semin Immunopathol* **27**, 133-46.

Ouellette, A. J. (2011). Paneth cell alpha-defensins in enteric innate immunity. *Cell Mol Life Sci* **68**, 2215-29.

Ouellette, A. J., Darmoul, D., Tran, D., Huttner, K. M., Yuan, J., and Selsted, M. E. (1999). Peptide localization and gene structure of cryptdin 4, a differentially expressed mouse paneth cell alpha-defensin. *Infect Immun* **67**, 6643-51.

Ouellette, A. J., Hsieh, M. M., Nosek, M. T., Cano-Gauci, D. F., Huttner, K. M., Buick, R. N., and Selsted, M. E. (1994). Mouse Paneth cell defensins: primary structures and antibacterial activities of numerous cryptdin isoforms. *Infect Immun* **62**, 5040-7.

Pfeffer, P. E., Hopkins, S., Cropley, I., Lowe, D. M., and Lipman, M. (2017). An association between pulmonary Mycobacterium avium-intracellulare complex infections and biomarkers of Th2-type inflammation. *Respir Res* **18**, 93.

Power, C. A., Wei, G., and Bretscher, P. A. (1998). Mycobacterial dose defines the Th1/Th2 nature of the immune response independently of whether immunization is

administered by the intravenous, subcutaneous, or intradermal route. *Infect Immun* **66**, 5743-50.

Rieger, J., Twardziok, S., Huenigen, H., Hirschberg, R. M., and Plendl, J. (2013). Porcine intestinal mast cells. Evaluation of different fixatives for histochemical staining techniques considering tissue shrinkage. *Eur J Histochem* **57**, e21.

Ryan, G. J., Hoff, D. R., Driver, E. R., Voskuil, M. I., Gonzalez-Juarrero, M., Basaraba, R. J., Crick, D. C., Spencer, J. S., and Lenaerts, A. J. (2010). Multiple M. tuberculosis phenotypes in mouse and guinea pig lung tissue revealed by a dual-staining approach. *PLoS One* **5**, e11108.

Salzman, N. H., Chou, M. M., de Jong, H., Liu, L., Porter, E. M., and Paterson, Y. (2003a). Enteric salmonella infection inhibits Paneth cell antimicrobial peptide expression. *Infect Immun* **71**, 1109-15.

Salzman, N. H., Ghosh, D., Huttner, K. M., Paterson, Y., and Bevins, C. L. (2003b). Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. *Nature* **422**, 522-6.

Sharif, M. A., Farhat, M. E., Kraim, E. S., Altrabulsi, N. A., Kammon, A. M., Dayhum, A. S., and Eldaghayes, I. M. (2013). Ovine paratuberculosis: a confirmed case of Johne's disease in Libya. *Open Vet J* **3**, 131-4.

Sharma, S., Verma, I., and Khuller, G. K. (2001). Therapeutic potential of human neutrophil peptide 1 against experimental tuberculosis. *Antimicrob Agents Chemother* **45**, 639-40.

Shin, D. M., and Jo, E. K. (2011). Antimicrobial Peptides in Innate Immunity against Mycobacteria. *Immune Netw* **11**, 245-52.

Shin, E. H., Osada, Y., Chai, J. Y., Matsumoto, N., Takatsu, K., and Kojima, S. (1997). Protective roles of eosinophils in Nippostrongylus brasiliensis infection. *Int Arch Allergy Immunol* **114 Suppl 1**, 45-50.

Simon, D., and Simon, H. U. (2007). Eosinophilic disorders. *J Allergy Clin Immunol* **119**, 1291-300; quiz 1301-2.

Smeed, J. A., Watkins, C. A., Rhind, S. M., and Hopkins, J. (2007). Differential cytokine gene expression profiles in the three pathological forms of sheep paratuberculosis. *BMC Vet Res* **3**, 18.

Spencer, L. A., and Weller, P. F. (2010). Eosinophils and Th2 immunity: contemporary insights. *Immunol Cell Biol* **88**, 250-6.

Venge, P., Bystrom, J., Carlson, M., Hakansson, L., Karawacjzyk, M., Peterson, C., Seveus, L., and Trulson, A. (1999). Eosinophil cationic protein (ECP): molecular

and biological properties and the use of ECP as a marker of eosinophil activation in disease. *Clin Exp Allergy* **29**, 1172-86.

Wehkamp, J., Chu, H., Shen, B., Feathers, R. W., Kays, R. J., Lee, S. K., and Bevins, C. L. (2006). Paneth cell antimicrobial peptides: topographical distribution and quantification in human gastrointestinal tissues. *FEBS Lett* **580**, 5344-50.

Worthington, J. J. (2015). The intestinal immunoendocrine axis: novel cross-talk between enteroendocrine cells and the immune system during infection and inflammatory disease. *Biochem Soc Trans* **43**, 727-33.

Wright, J. L., Pare, P. D., Hammond, M., and Donevan, R. E. (1983). Eosinophilic pneumonia and atypical mycobacterial infection. *Am Rev Respir Dis* **127**, 497-9.

Yantiss, R. K. (2015). Eosinophils in the GI tract: how many is too many and what do they mean? *Mod Pathol* **28 Suppl 1**, S7-21.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389-95.