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

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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, an anti-bacterial peptide. We investigated in this study whether eosinophils reacting to mycobacteria produce α-defensin in mice and 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.

KEY WORDS: α-defensin, animal model, eosinophil, mycobacteria

Bacterial infections are known to activate host Th1 immune responses [2]. On the other hand, it is known that Th2 immune reaction is activated by allergy and parasite infection and is associated with eosinophilia [11, 21]. It has been also well known in humans that eosinophil infiltration into the site of inflammation and eosinophilia occur in mycobacterial infections [10, 13, 16], 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 that contributed to attack Mycobacterium [6]. 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 [9]. Originally, α-defensin has been identified as an effective antimicrobial substance against bacterium [5, 20], it is known that α-defensin is effective not only for bacteria but also for parasites [8, 9]. Even in animal, eosinophil infiltration also found in Johne's disease caused by *Mycobacterium avium* subspecies [13]. In addition, it has been reported that eosinophil infiltration into tissues occurs also in experimental mycobacterial infection in mice [3, 4]. 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.

Forty pathogen free female 8 weeks old C57BL/6JJc1 mice were purchased from Clea Japan (Tokyo, Japan). Animals were kept at a constant temperature ($24 \pm 1°C$) and humidity ($60 \pm 10\%$) under 12/12 hr light/dark cycle with free access to autoclaved food (Clea Japan) and water. All animals were handled under the regulations for animal welfare of Yamaguchi University. 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, U.S.A.) 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 1.0×10^7cells (low) in 0.5 m*l*. The vehicle with same volume was injected in control animals. Samples of 5 mice in each group were taken at day 10 and 30 post-infection. Mice were euthanized with ketamine and xylazine and then blood was drawn from heart using a heparinized syringe. The number of eosinophils in heart blood was counted after stain with Hinkelman's solution, as reported previously [14]. Liver samples were collected aseptically and fixed in Zamboni's solution [18] at 4°C overnight. Zamboni's solution-fixed liver samples were routinely processed and embedded in paraffin, then cut into 2 μm-thick sections. Sections were deparaffinized, rehydrated and

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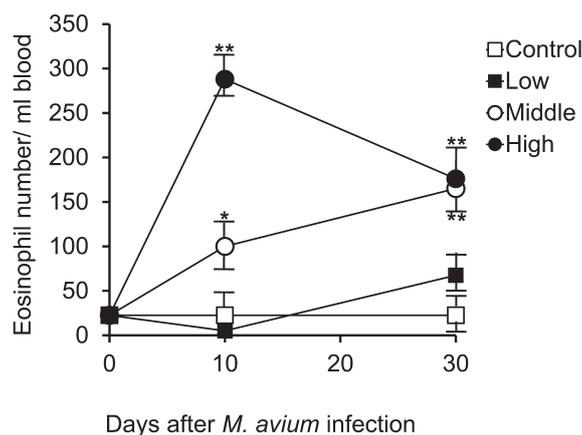
Fig. 1. Blood eosinophilia in different doses of *Mycobacterium avium* infected C57BL/6JJc1 mice. Each group consisted of 5 mice. Data are shown as mean ± SEM. *Significantly different from the values of control mice ($P<0.05$).

stained with hematoxylin and eosin (HE) stain.

For double immunofluorescence (IF) stain, 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, U.S.A.) at room temperature for 1 hr followed by, incubation with secondary antibody Alexa Flour 488 conjugated goat anti-rabbit IgG (1:200 dilution, Life technologies, Eugene, OR, U.S.A.) at room temperature for 1 hr. Then, α-defensin 4 (R-19) polyclonal antibody (1:50, Santa Cruz Biotechnology, Inc., Dallas, TX, U.S.A.) [12] was reacted over night at 4°C followed by incubation with Alexa Fluor 555 donkey anti goat IgG (H&L) antibody (1:200, Abcam, Cambridge, U.K.) for 1 hr at room temperature. Specimens were washed, mounted with glycerine and observed by using a fluorescence microscope. ECP-positive eosinophils and ECP/α-defensin-double-positive cells were counted. At least 10 high power fields (× 40 objective) were counted for each mouse. When combining ECP immunofluorescence and auramine-rhodamine (AR) stain, the coverslips were removed after immunofluorescence stain and the slides were washed and then stained with AR. AR stain was performed with AR stain TB Fluorescent Stain Kit T (Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.) 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 [19]. All data were expressed as mean ± standard error of mean (SEM). Statistical significance was determined with the Student *t*-test, and $P<0.05$ was considered as significant.

In our study, blood eosinophils were significantly increased with high and middle dose infection of *M. avium* (Fig. 1). 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. In high dose group, diffuse infiltration of inflammatory cells was found in the liver parenchyma (Fig. 2b, 2c). At day 10 post-infection, inflammatory lesion mainly composed of macrophages, lymphocytes and small number of eosinophils (Fig. 3b). On the other hand, eosinophils were main inflammatory cell at day 30 and found to make clusters (Fig. 3c). In mice with low and middle dose infection, the histopathological changes were similar but weaker than high dose group. To investigate whether eosinophil recruited at the site of *M. avium* infection, we conducted combined ECP immunofluorescence stain and AR stain. With high dose infection at day 30, AR stain-positive mycobacterium organisms were surrounded by ECP-positive eosinophils, clearly indicating eosinophil reaction to *M. avium* (Fig. 4). Immunoreactivity against ECP revealed large cluster of intact eosinophils and degranulated eosinophils existed in the lesions. Double immunofluorescence stain with antibodies specific for ECP and α-defensin 4 protein revealed almost all ECP-positive eosinophils showed expression of α-defensin in liver (Fig. 6). More than 90% of infiltrated eosinophils were positive for α-defensin in infected group (Table 1), while in control group eosinophils did not show immunopositivity for α-defensin (Fig. 5b, Table 1).

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 produced α-defensin to kill bacterial organisms in response to Mycobacterium. Our observation confirms previous reports of eosinophil recruitment to sites of infection due to mycobacterial infection [3, 4]. 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 [10, 16]. Eosinophil infiltrative reaction against *Mycobacterium* is commonly reported in human and animal mycobacterial diseases and experimental animals [3, 4, 10, 11, 13]. However, the mechanism involved in eosinophil roles against mycobacteria has not been investigated sufficiently.

α-Defensin expression in eosinophils were indicated in our previous study using animal model of intestinal helminth infection,
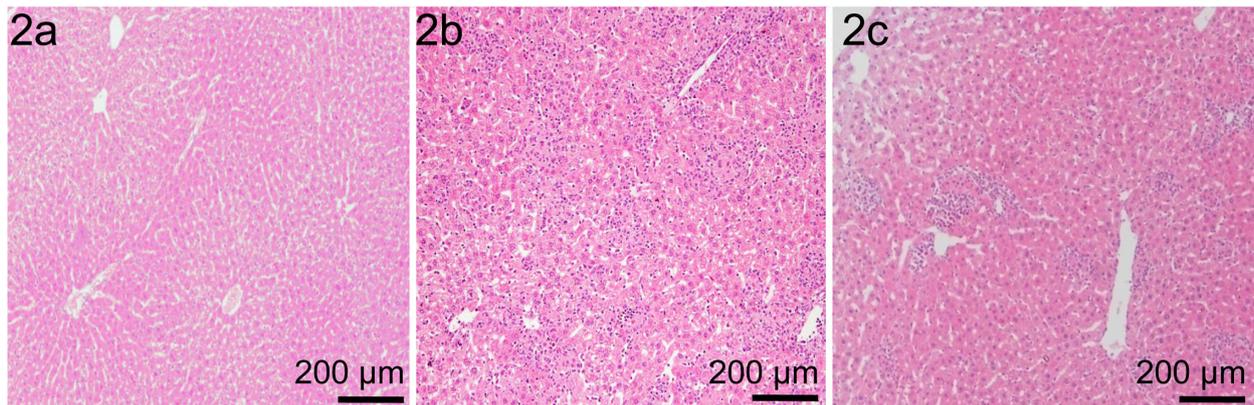
Fig. 2. Histological analysis of inflammatory cells infiltration in liver parenchyma of control (a) and high dose *Mycobacterium avium* infected mice (b and c). Hematoxylin and eosin (HE). (a) No inflammatory cells infiltration. (b) Diffuse infiltration of inflammatory cells at day 10. (c) Diffuse infiltration of inflammatory cells at day 30. Scale bars=200 μm.
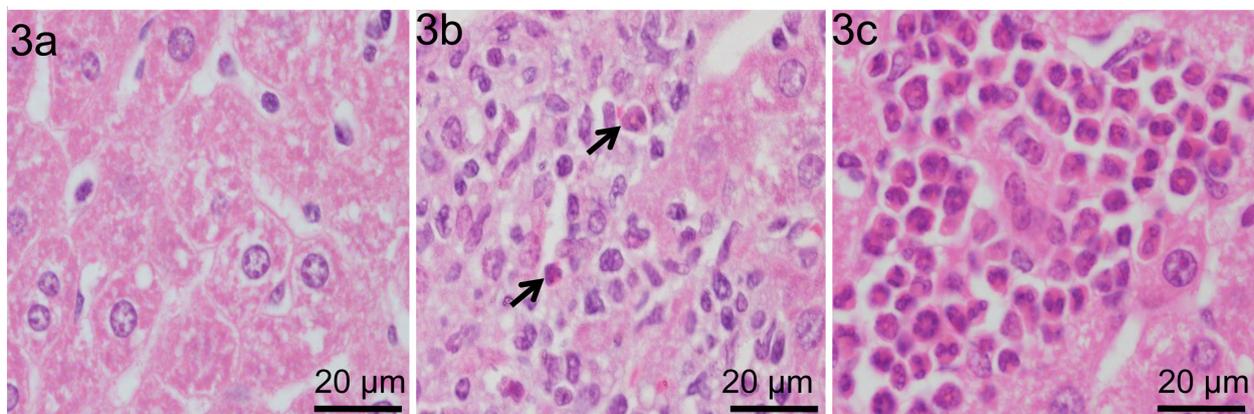

Fig. 3. Histological detection of eosinophils in inflammatory lesions in liver parenchyma of control (a) and high dose *Mycobacterium avium* infected mice (b and c). Hematoxylin and eosin (HE). (a) No eosinophils infiltration. (b) Inflammatory lesions contain lymphocytes, macrophages and small number of eosinophils at day 10. Arrows indicate eosinophils. (c) A lot of eosinophils making clusters noted at day 30. Scale bars=20 μm.
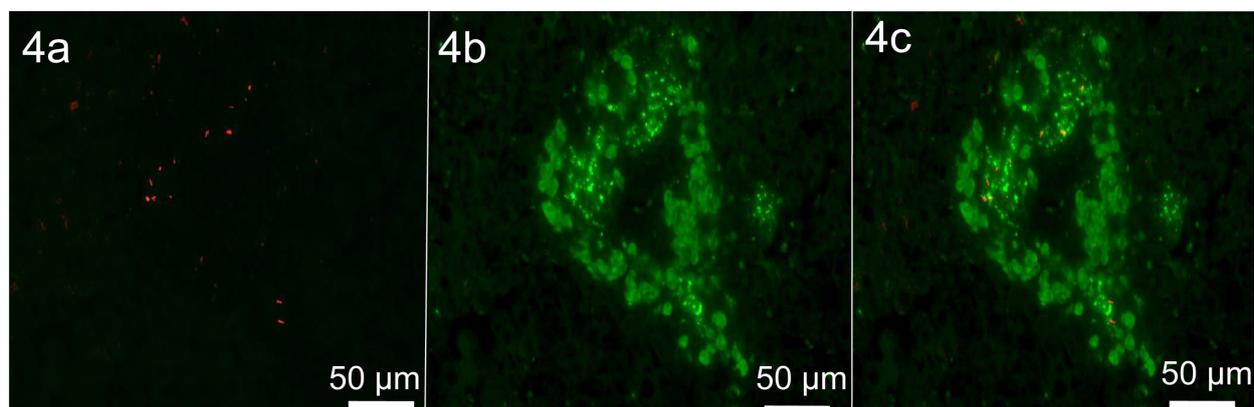
Fig. 4. Combined immunofluorescence analysis for auramine-rhodamine (AR) stain (red) (a) and ECP (green) (b) in high dose *Mycobacterium avium* infected mice. (c, merged) eosinophil cluster and degranulated eosinophils existed surrounding *Mycobacterium avium* bacteria in liver tissue at day 30. Scale bars=50 μm.
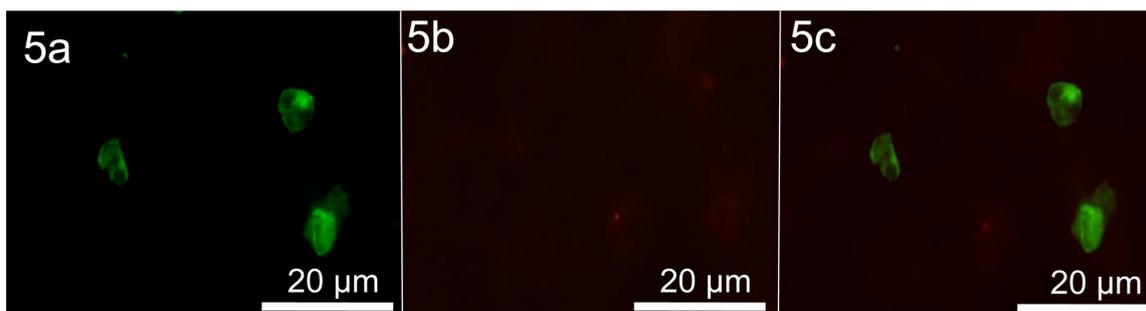
Fig. 5. Double immunofluorescence analysis for ECP (a) and α-defensin 4 (b) in control mice. (c, merged) ECP-positive eosinophils in liver without α-defensin 4 expression.
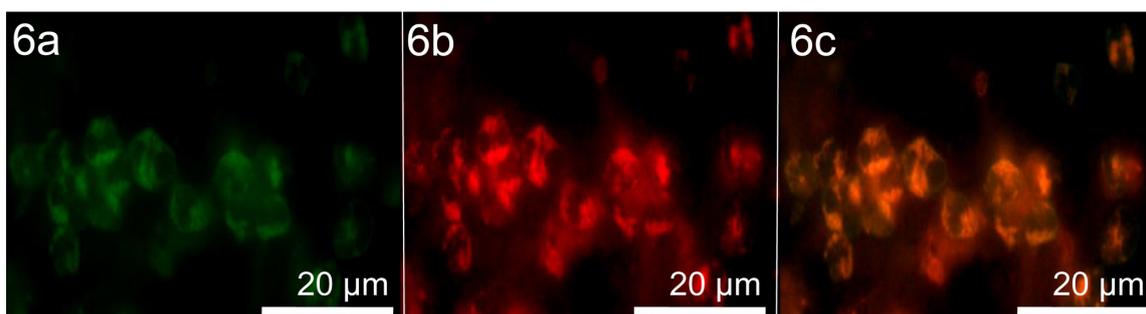
Fig. 6. Double immunofluorescence analysis for ECP (a) and α-defensin 4 (b) in high dose *Mycobacterium avium* infected mice. (c, merged) ECP-positive eosinophils in liver express α-defensin 4. Scale bars=20 μm.

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.

however, the eosinophils reacted to helminth infection were limited in submucosal region without direct contact to helminth [9]. 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 mycobacterial organisms through α-defensin. Defensins are large family of antimicrobial peptides [8, 20]. *In vitro* study revealed that α-defensin directly induced mycobacterial lysis via increasing cell membrane permeability [5]. Human α-defensin 1 (HNP-1) kills *M. avium-intracellulare* at the optimal pH *in vitro* [15]. 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 [1, 6] and are released for antimycobacterial actions [4]. Eosinophil granules contain cytotoxic proteins such as ECP, major basic protein-1, major basic protein-2, eosinophil peroxidase, eosinophil-derived neurotoxin etc. [1, 6]. An *in vitro* study using human eosinophils revealed that eosinophils stimulated with live *Mycobacterium bovis* BCG expressed both ECP and α-defensin, and synergistically inhibited mycobacterial growth [6]. 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. The 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 might be a result of induction of Th2 immunity, because eosinophilia is closely link to Th2 immunity [11, 21]. High dose of mycobacterium infection has been reported to induce Th2 immune reaction easily than low dose administration [17]. 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 [7]. 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 [9]. 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.

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