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VETERINARY IMMUNOLOGY AND IMMUNOPATHOLOGY: Short Communication

Oral administration of bovine lactoferrin upregulates neutrophil functions in a dog with familial β2-integrin-related neutrophil dysfunction

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Abstract

Lactoferrin, a glycoprotein present in neutrophils and exocrine secretions, plays important roles in host defense. Administration of bovine lactoferrin has been reported to modulate various neutrophil functions. We found a mixed-breed male dog with novel familial neutrophil dysfunction. The disorder was caused by a decrease of β2-integrin expression encoding CD18 without mutation. Antibiotics therapy alone didn't influence a series of neutrophil functions in the same dog. We examined the effects of oral administration of bovine lactoferrin on the neutrophil function and clinical symptoms in the same dog. Oral chronic administration of bovine lactoferrin increased neutrophilic β2-integrin gene expression comparable to normal dogs, followed by the upregulation of surface CD18 expression. Concurrently, the superoxide production, phagocytic activity and adherence that were β2-integrin-related neutrophil functions increased to normal canine levels. The chronic inflammation from bacterial upper respiratory infections and pneumonia was also alleviated in the dog. Our results indicate that oral treatment with bovine lactoferrin increases neutrophil β2-integrin transcript level, leading to the upregulation of neutrophil functions and improvement of clinical symptoms in the dog with familial neutrophil dysfunction.

Keywords: bovine lactoferrin; integrins; CD11b/CD18; familial neutrophil dysfunction; superoxide production

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1. Introduction

Neutrophils constitute the first line of host defense against microorganisms. Recurrent infections from a young age are associated with inherited neutrophil dysfunction. Congenital neutrophil dysfunction has been reported in dogs, for example, persistent neutropenia in border collies (Allan et al., 1996), canine leukocyte adhesion deficiency (CLAD) in Red and White Irish setters (Kijas et al., 1999) and β2-integrin-related neutrophil dysfunction in mixed-breed dogs (Kobayashi et al., 2009). The only definitive therapy is hematopoietic stem cell transplantation for chronic granulomatous disease (CGD), human and canine LAD (Bauer et al., 2005, Elhasid and Rowe, 2010, Seger, 2010). Recent successes in treating CLAD have demonstrated the therapeutic potential of stem cell gene therapy (Bauer et al, 2006 and 2007). However, these therapies have still some problems such as the need for a matched donor, transplant rejection and the risk of integration near oncogenes by virus receptors. Cytokines including granulocyte colony-stimulating factor (G-CSF) and IFN-γ are also used as one of supportive treatments for CGD and congenital neutropenia (Roy-Ghanta et al., 2010). The cytokine therapy for long duration, however, has a risk of causing adverse effects. Especially, there are only a few products of homogenous cytokines used in veterinary medicine such as canine and feline IFN. Thus, the repeated treatment of anti-human cytokine, example for G-CSF, will stimulate the production of antibody against the heterologous protein in dogs and cats so that the use of G-CSF is limited for a short duration.

Lactoferrin, an 80 kDa iron-binding glycoprotein, is one of the primary host defense systems against infection. It is produced by neutrophils and exocrine glands,

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and is present in neutrophil secondary granules and exocrine secretions (Ward et al., 2002). Its receptors have been found on neutrophils, mononuclear cells and brush-border cells (Spik et al., 1994). Synthesized CD11b/CD18 on neutrophils by stimuli triggered oscillations of cytosolic free Ca^{2+} followed by lactoferrin release and superoxide production in human neutrophils (Neuman et al., 1990, Richter et al., 1990, Suchard et al., 1994). Lactoferrin being released from activated neutrophils contributes to kill microorganisms, and regulates the cell counts and functions of neutrophils (Lönnerdal, and Iyer, 1995, Ward et al., 2005). It has also demonstrated that heterologous lactoferrin has anti-inflammatory and immunomodulatory activities (Kobayashi et al., 2005 and 2008, Yamada et al., 2008). Oral administration of bovine lactoferrin has shown to modulate phagocytic activity, superoxide production or adherence of peripheral neutrophils in healthy or feline immunodeficiency virus (FIV)-positive cats (Sato et al., 1996) and healthy volunteers (Yamauchi et al., 1998). Judging from these reports, administration of heterologous lactoferrin has sufficient potential to influence the performance of peripheral neutrophils.

Our previous study showed that the first recognized cases in mixed-breed dogs with familial neutrophil dysfunction (Kobayashi et al., 2009). The defect of neutrophil function was caused by downregulation of β2-integrin transcript level without mutation. We examined the effects of oral chronic administration of bovine lactoferrin on the neutrophil function in one of the same dogs in this report.

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2. **Materials and methods**

2.1. Dogs

Heparinized peripheral blood sample was obtained from a mixed-breed 6-year-old male dog and eight healthy beagles (five males and two females, 2–6 years old). The healthy beagles were used as healthy controls for all neutrophil functions. The healthy dogs did not treat with any antibiotics.

The mixed-breed dog with familial neutrophil dysfunction was one of the same dogs that we reported previously (Kobayashi et al., 2009). Briefly, the mixed-breed littermates had suffered recurrent chronic bacterial infections from puppyhood, which was refractive to antibiotics or IFN-γ therapy. At initial presentation to our Veterinary Teaching Hospital, the male dog had recurrent severe upper respiratory bacterial infections, oculo-nasal mucopurulent discharge, pneumonia and severe bilateral corneal opacity. The affected dog treated with antibiotics showed the disorders of neutrophil function.

2.2. Oral administration of bovine lactoferrin

Bovine lactoferrin (40mg/kg/day, twice a day) was administered orally with antibiotics for 140 days. It is a highly pure lyophilized powder derived from cow's milk. The powder is light red-pink in color and virtually odorless and tasteless.

2.3. Neutrophil functions

Each measurement of neutrophil function was performed as described in our

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previous report (Kobayashi et al., 2009). The healthy beagles were used as healthy controls for all neutrophil functions. The results of all neutrophil functions except superoxide production, phagocytic activity and adherence in the male dog with neutrophil dysfunction shown are representative of two independent experiments. Preliminary examination indicated that the oral administration with bovine lactoferrin for 28 days decreased slightly expression of CD18 and adherence, and increased slightly phagocytic activity and superoxide production in a healthy dog. All results were expressed as the mean value and min-max range in parentheses.

2.3.1. Isolation of canine peripheral neutrophils

Neutrophils (4.5-5.5 x 10^6 cells/ml) were isolated from 10 ml of blood using dextran sedimentation and Ficoll-conray density-gradient separation. The viability of isolated PMN was determined by 0.2% trypan blue staining (> 95%).

2.3.2 Real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Because the male dog with neutrophil dysfunction received antibiotics therapy had a low transcript level of CD11b and β2-integrin, the real-time RT-PCR analysis was carried out with SYBR Green I as described in our previous report (Kobayashi et al., 2009). Briefly, total RNA from isolated neutrophils (5 x 10^6 cells) was extracted and was eluted in a final volume of 30 μl RNase-free water. Spectrophotometer determined the high RNA yield and purity. The complementary deoxyribonucleic acid (cDNA) was synthesized from total RNA (0.17 μg) of isolated neutrophils. Amplification of canine CD11b, β2-integrin, lactoferrin and β-actin mRNA was performed by 1cycle of 2 min at

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50 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C, 30 s at 62 °C, 40 s at 72 °C. Expression levels were quantified in duplicate by means of real-time RT-PCR. Cycle threshold values for genes of interest were normalized to β-actin and used to calculate the relative quantity of mRNA expression. The primer sequences used were described in our previous report (Kobayashi et al., 2009).

2.3.3 Surface expression of adhesion molecules on leukocytes

Because the male dog with neutrophil disorder showed a decrease in surface expression of CD11b/CD18 molecules, the expression of CD11b and CD18 was analyzed by a whole blood flow cytometric method as described in our previous report (Kobayashi et al., 2009). Briefly, after staining with FITC-labeled anti-CD11b and CD18, cells were resuspended in 0.5% paraformaldehyde in PBS. Analysis gate for neutrophils was expressed as mean fluorescence intensity (MFI) on a log-scale analyzing 10000 cells per sample as follows: MFI = (Geo mean of target antibody – geo mean of negative control) / geo mean of negative control.

2.3.4. Neutrophil adherence

Because the neutrophils in the male dog had abnormality through a mild decrease of adherence, neutrophil adherence to nylon fibers was examined according to the method of Nagahata et al. (1993). Total and differential neutrophil counts were performed before and after neutrophils suspension was allowed to percolate through the nylon fiber columns by gravity flow. Briefly, neutrophil suspension (5 x 10^5 cells) containing 10% autologous plasma was incubated for 10 min at 37 °C and then was

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applied to a preincubated nylon wool fiber column. After percolating through the nylon fiber at room temperature, neutrophil counts were performed. Neutrophil adherence was calculated from the formula: percentage of neutrophil adherence = (1 - counts of effluent neutrophil/counts of initial neutrophil) x 100.

2.3.5. Neutrophil phagocytic activity

Because the neutrophils in the male dog showed a mild decrease of non-specific phagocytic activity, neutrophil phagocytic activity was measured by a whole blood flow cytometric method as described in our previous report (Kobayashi et al., 2009). Briefly, whole blood and non-opsonized microspheres were incubated for 30 min at 37 ºC and then PBS with 3 mM EDTA□2Na was added. After hemolysis, the cells were resuspended in 0.5% paraformaldehyde in PBS. Phagocytic activity expressed as percentage of the total neutrophil population ingesting fluorescent microspheres.

2.3.6. Neutrophil superoxide production

The previous our study also showed that neutrophils from two littermates had a marked reduction in serum-opsonized zymosan (OZ)-stimulated superoxide production. The production of superoxide was measured by chemiluminescence with luminol as described in our previous report (Kobayashi et al., 2009) The chemiluminescence was measured with a luminometer (Luminescencer-PSN, ATTO Co., Tokyo, Japan) at intervals of 2 s for total 30 min at 37 ºC.

3. Results and Discussion

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3.1 The effect of bovine lactoferrin treatment on clinical findings

At initial presentation to our Veterinary Teaching Hospital, the male dog with neutrophil dysfunction had recurrent severe respiratory bacterial infections as described in our previous report (Kobayashi et al., 2009). Despite symptomatic therapies such as fluid therapy, nebulization and administration of antibiotics for the first 2 weeks, clinical symptoms of the same dog did not improve. Twenty days after additional oral administration of bovine lactoferrin, oculo-nasal mucopurulent discharge appreciably decreased. And the symptoms of upper respiratory bacterial infections and bilateral corneal opacity were gradually improved. There was no difficulty in giving the dog lactoferrin. Finally, the dog stopped cough from pneumonia and was released from nasal obstruction 50-day lactoferrin treatment. And the dog had kept been in a comparative lull for the period of bovine lactoferrin administration. However, his owner failed to give the dog bovine lactoferrin for 2 weeks. The dog gradually developed bacterial upper respiratory infections. Bilateral oculo-nasal mucopurulent discharge was recurred on 140-day treatment after 14 day-suspension of lactoferrin. We also observed the recovery of clinical symptoms of upper respiratory bacterial infections in another female littermate with familial neutrophil dysfunction by oral treatment with bovine lactoferrin. The absorption and transportation kinetics of orally administered bovine lactoferrin still remain unclear. It has widely accepted that orally administered bovine lactoferrin is absorbed through the intestinal epithelium cells mediated by pathway of lactoferrin receptors, endocytosis or M cells in Peyer's patch. Takeuchi et al. (2004) demonstrated that intraduodenally administered bovine lactoferrin was transported into blood circulation via the thoracic duct lymph fluid in adult rats. This finding indicated

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that the adult body could absorb orally administered heterologous lactoferrin. In addition, oral administration of bovine lactoferrin was reported to modulate functions of peripheral neutrophils in cats and human (Sato et al., 1996, Yamauchi et al., 1998). Therefore, it is possible that bovine lactoferrin and its derived peptides may be absorbed by the intestinal tract mucosal and influence the functions of peripheral blood neutrophils in the dogs with familial neutrophil dysfunction.

3.2. The effect of bovine lactoferrin treatment on expression of neutrophil integrins and lactoferrin, and superoxide production

Our previous study showed that antibiotics therapy didn't regulate all neutrophil functions of the male dog (Kobayashi et al., 2009). Real-time RT-PCR analysis revealed that low transcript levels of both CD11b and β2-integrin in neutrophils from the same dog increased to the same levels as those of normal dogs after treatment of bovine lactoferrin (Fig. 1A). The CD11b mRNA level slightly increased from 0.64 (0.39-0.95) to 1.06 (1.02-1.11) in the dog after 54-day bovine lactoferrin treatment [normal dogs, 0.84 (0.47-1.41), n=5]. Concurrently, β2-integrin mRNA level upregulated profoundly from 0.09 (0.054-0.13) to 0.65 (0.52-0.78) in the affected dog after bovine lactoferrin treatment [normal dogs, 0.67 (0.42-0.93), n=5]. However, on day 140 after 14 day-suspension of bovine lactoferrin due to his owner's reasons, the dog showed decreases in CD11b and β2-integrin mRNA expression to pretreatment level, 0.36 (0.30-0.43) and 0.09 (0.06-0.12), respectively. On the other hand, the dog that had normal lactoferrin transcript levels [0.43 (0.42-0.43) versus normal dogs, 0.43 (0.27-0.56), n=5] showed a decrease of the lactoferrin level [0.29 (0.28-0.29)] after 140-

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day treatment. The result indicates that heterologous lactoferrin may affect the secretion of endogenous lactoferrin. A study reported that β2-integrin transcript level was downregulated by overexpression of PKC-zeta (Noti et al., 2001). However, the factors and mechanisms that regulate the transcript expression of β2-integrin in neutrophils are not fully understood. We could not clarify the mechanism of positive modulation of transcript level of β2-integrin by oral treatment with bovine lactoferrin in the dog. It might be possible that neutrophils of the dog may have a defect in transcriptional regulatory mechanism of β2-integrin mRNA including intracellular signaling or in system of lactoferrin release. And bovine lactoferrin might compensate for the defect directly or indirectly. Further experiments will be required to examine the recoverable mechanism in oral treatment with bovine lactoferrin on neutrophil functions in the dog.

Cytometric analysis showed that chronic administration of bovine lactoferrin gradually increased the surface expression of CD18 but not CD11b in the dog (Fig. 1B). On 94-day treatment with bovine lactoferrin, the expression of CD18 molecule was increased by about 146% of pretreatment level in the affected dog (pretreatment, 28.5%; 94 days, 41.72%). However, the expression level didn't increase to normal canine levels [67.41 (58.7-75.3) %, n=5). On day 140 after 14 day-suspension of bovine lactoferrin, the dog showed a slight decrease in CD18 expression from 41.72% to 40.75%. On the contrary, surface expression of CD11b was kept in a low level after bovine lactoferrin treatment [pretreatment, 2.88%; 94 days, 2.97%; 140 days, 2.78%; normal dogs, 6.28 (5.78-7.64) %, n=5]. Noti et al. (2001) demonstrated that change of β2-integrin transcript level resulted in modulation of membrane CD18 expression on neutrophils. Our result suggested that β2-integrin mRNA expression was

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247 increased profoundly by oral administration of bovine lactoferrin and led to an increase
248 of membrane CD18 expression. On the other hand, the slight increased the transcript
249 level of CD11b by treatment with bovine lactoferrin did not result in an increase of
250 membrane CD11b expression. The results suggested that increased expression of
251 membrane integrin may require a significant increase in the gene expression.

252 The characteristic finding of the dog was a profound reduced response of the
253 OZ-induced superoxide production (Kobayashi et al., 2009). Chronic oral
254 administration of bovine lactoferrin resulted in a marked increase of superoxide
255 production in the same dog (Fig. 2). The maximum amount of luminescence was
256 increased by about 75% (79055/sec) and 72% (76966/sec) of normal canine level on
257 14-day and 54-day treatment with bovine lactoferrin, respectively. Eventually, the
258 superoxide production increased to the same level of healthy dogs completely after
259 94-day treatment (114775/sec). However, suspension of bovine lactoferrin for 14 days
260 led to a slight decrease of the maximum amount of luminescence to 106807/sec on day
261 140. CD11b/CD18 blockade or Ca^{2+} chelators inhibited both lactoferrin release and
262 superoxide production in human and mouse neutrophils (Nielsen et al., 1997, Mocsai e
263 al., 2002). A recent study using lactoferrin–deficient mice with normal expression of
264 CD18 showed that superoxide production was normal in response to stimulation with
265 opsonized bacteria (Ward et al., 2008). Moreover, CD18-deficient neutrophils from
266 LAD patients were shown to fail to release lactoferrin and produce superoxide in
267 response to OZ or fMLP (Suchard et al., 1994, Bauer et al., 1998). These reports
268 suggest that expression level of CD18 on neutrophils is major requirement for
269 degranulation of lactoferrin and subsequent superoxide production in response to OZ in

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neutrophils. In addition, our *in vitro* study showed that addition of bovine lactoferrin increased superoxide production in feline isolated neutrophils dose-dependently (unpublished data). It was also reported that oral administration of bovine lactoferrin showed a slight increase of neutrophilic phagocytic activity and superoxide production in cats and human (Sato et al., 1996, Yamauchi et al., 1998). Therefore, our results suggested that increased expression of CD18 by oral administration of bovine lactoferrin resulted in upregulation of OZ-induced superoxide production in the dog.

3.3. The effect of bovine lactoferrin treatment on adherence and phagocytic activity

Our previous report demonstrated that the dog's neutrophils exhibited reductions in β2-integrin-related adherence and non-specific phagocytic activity (Kobayashi et al., 2009). As shown in Fig. 3A, the adherence increased to a normal canine level in the same dog after 7-day lactoferrin treatment [pretreatment, 19.30%; 7 days, 35.41%; normal dogs, 31.74 (29.1-34.0) %, n=5]. Thereafter, adherence was decreased to 32.72% on 14 days and 26.5% on 94-day treatment, followed by a slight increase on 140-day treatment (29.6%). As shown in Fig. 3B, the low non-specific phagocytic activity (32.20%) increased to normal level on 14-day treatment [47.64%; normal dogs, 45.15 (43.3-48.6) %, n=5] and 58.35% on 94-day treatment. However, the activity on day 140 showed a slight decrease to 50.48% by suspension of bovine lactoferrin for 14 days. It was demonstrated that oral administration of bovine lactoferrin modulated neutrophilic phagocytic activity in cats and human, suggesting the involvement of expression of adhesion molecules (Sato et al., 1996, Yamauchi et al., 1998). Our results suggested one of the possible mechanisms by which adherence and

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phagocytic activity through membrane integrins may be increased by an upregulation of membrane CD18 expression.

In conclusion, our all results suggest that the upregulation of β2-integrin transcript level by treatment with bovine lactoferrin led to improve integrin-related neutrophil functions and clinical symptoms in the same dog in our previous study (Kobayashi et al., 2009). Therefore, our findings indicate that upregulation of β2-integrin expression is of key importance to restoration of neutrophil function in the case. In addition, suspension of bovine lactoferrin resulted in regression of the neutrophil functions in the dog. It seems likely that the dog with the disorders of neutrophil function and clinical symptoms in this report needs to take lifelong medication of bovine lactoferrin. Regarding long-term administration, bovine lactoferrin has been thought to be one of the dairy foods, because it has been detected in natural cheese and cheese whey, Moreover, 13-week oral repeated administration toxicity study showed that oral administration of bovine lactoferrin at high dose (2000 mg/kg/day) did not cause any adverse effects noted in the general condition of rats (Yamauchi et al., 2000). The observation suggests that heterologous bovine lactoferrin can be safe for chronic oral administration without allergy to lactoferrin. Oral administration with bovine lactoferrin may represent a therapeutic approach to the familial β2-integrin-related neutrophil dysfunction without β2-integrin gene mutation.

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404 **Figure captions**

405

406 **Fig. 1.** The effects of oral administration of bovine lactoferrin on transcript level of
407 neutrophil adhesion molecules and surface expression in the affected dog. Mean values
408 and min-max range are shown in controls and the affected dog. (A) The transcript levels
409 of CD11b and β2-integrin before (Pre), 54 days and 140 days after oral administration
410 of bovine lactoferrin were measured by real-time RT-PCR. The administration was
411 resumed on day 140 after 14 days of suspension due to his owner's reasons. The results
412 were expressed as a ratio of CD11b or β2-integrin to β-actin. (B) Surface expression of
413 CD11b and CD18 on neutrophils was quantified by a whole blood flow cytometric assay.
414 The results were expressed as mean fluorescence intensity (MFI).

415

416 **Fig. 2.** The effect of oral administration of bovine lactoferrin on OZ-stimulated
417 superoxide production in the affected dog. Superoxide production in the affected dog
418 was measured by chemiluminescence before (○), 14 (●) and 94 (●) days after oral
419 treatment with bovine lactoferrin. The result of healthy controls is expressed as the
420 mean of five experiments measured (□).

421

422 **Fig. 3.** The effects of oral administration of bovine lactoferrin on neutrophil adherence
423 and phagocytic activity in the affected dog. The result of healthy controls (□) and the
424 affected dog (▲) is expressed as the mean and min-max range. The administration was
425 resumed on day 140 after 14 days of suspension due to his owner's reasons. (A)
426 Neutrophil adherence was measured by the nylon fiber adherence assay. The results

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were expressed as percentage of neutrophil adherence to nylon fibers. (B) Non-specific phagocytic activity of neutrophils was measured by a whole blood flow cytometric assay using non-opsonized fluorescent microspheres. Phagocytic activity expressed as percentage of the total neutrophil population ingesting fluorescent microspheres.

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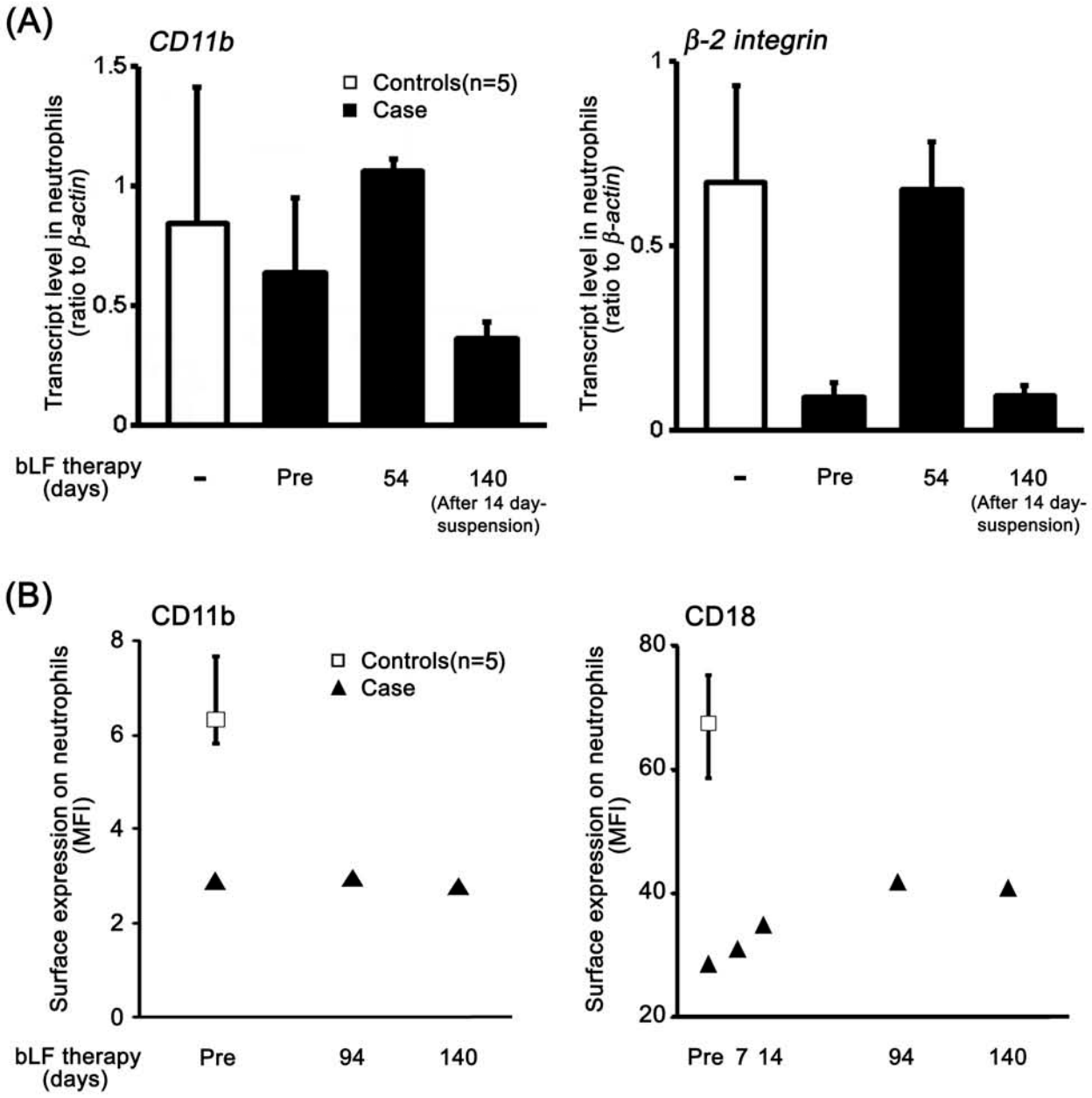

Fig 1. Kobayashi et al.

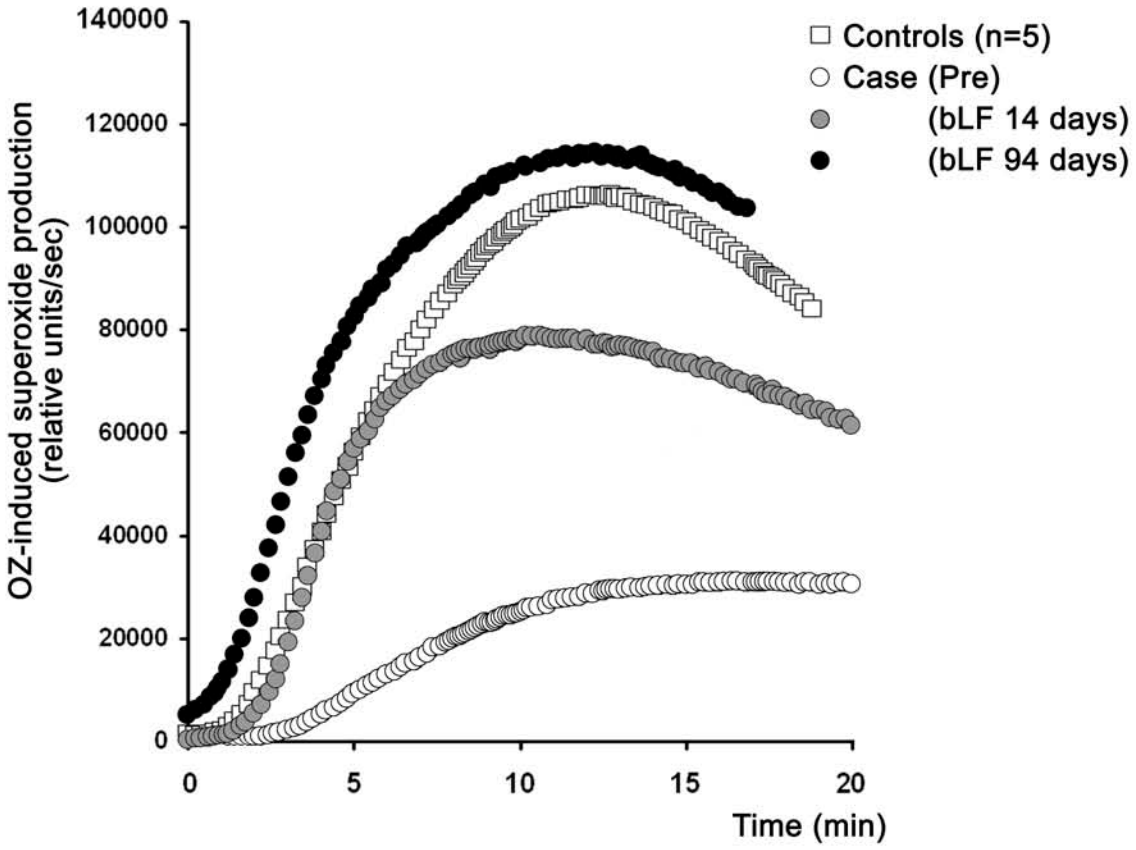

Fig 2. Kobayashi et al.

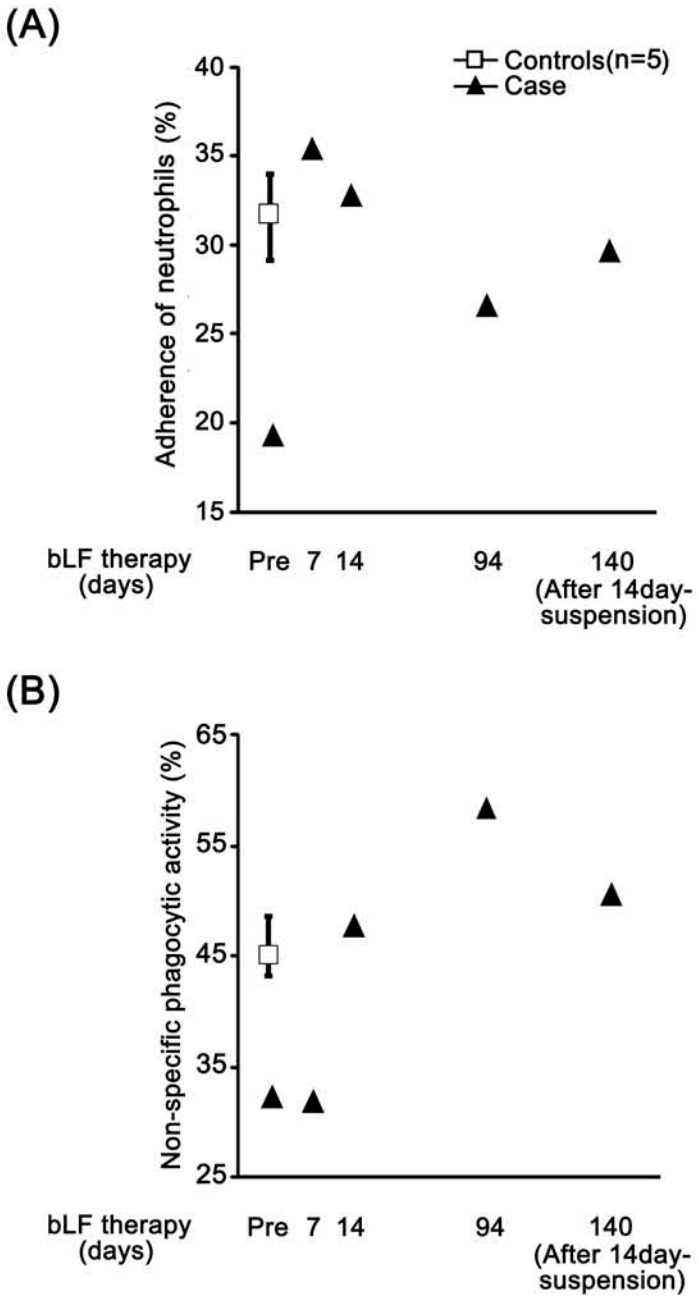

Fig 3. Kobayashi et al.