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Canine neutrophil dysfunction caused by downregulation of β-2 integrin expression without mutation

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Abstract

Canine leukocyte adhesion deficiency (CLAD) in Irish setters is caused by genetic defects of leukocyte integrin CD18 leading to recurrent bacterial infections. We report clinical features and analysis of neutrophil function from two mixed-breed canine littermates (one female and one male dog) similar to CLAD. The symptoms of pyogenic infection were first recognized at 3 months of age and since then the patients suffered from recurrent bacterial infections. These clinical findings were strongly suggestive of genetic phagocyte dysfunction. Neutrophil function tests revealed a marked reduction of serum-opsonized zymosan-mediated superoxide production in the two littermates. Neutrophils of the male dog revealed impaired integrin-mediated adherence and phagocytic activity, whereas ability of serum opsonization was normal. There was also a profound decrease of surface expression of CD11b/CD18 and β2-integrin transcript level, detected by real-time RT-PCR without missense mutations unlike CLAD. Immunoblot analysis indicated that protein expression of cytochrome b_{558} component gp91^{phox}, the cytosolic components p47^{phox} and p67^{phox} of NADPH oxidase components increased profoundly in the male. Our study suggests that decreased transcriptional levels of β2-integrin without mutations, lead to downregulation of surface expression, resulting in multiple defects in adhesion-related neutrophil functions and consequently, recurrent bacterial infections from puppyhood.

Keywords: Integrins; CD11b/CD18; congenital neutrophil dysfunction; canine leukocyte adhesion deficiency

1. Introduction

Neutrophils have a central role in the first line of host defense against invading microorganisms. Breakdown of neutrophil functions results in a greatly increased susceptibility to severe bacterial and fungal or life-threatening infections in humans and animals. Infants, children and young adults who suffer chronic and recurrent bacterial infection despite adequate numbers of circulating phagocytes and antibiotics therapies should be suspected of genetic defects in phagocyte function. Inherited disorders of neutrophils that cause defects in neutrophil adhesion, migration, and oxidative killing have been reported. These include leukocyte adhesion deficiency (LAD), other disorders of chemotaxis, Chédiak-Higashi syndrome, neutrophil specific granule deficiency, chronic granulomatous disease (CGD) and myeloperoxidase deficiency (Dinauer, 2007). Several studies in dogs have reported that primary neutrophil dysfunctions are pure breed dog-related, including persistent neutropenia in border collies (Allan et al., 1996), the Pelger-Huët anomaly in American foxhounds (Bowles et al., 1979) and canine leukocyte adhesion deficiency (CLAD) in Red and White Irish setters (Kijas et al., 1999).

Reactive oxygen species play an important role in killing microbial pathogens. Respiratory burst is catalyzed by an NAPDH oxidase, which is a multicomponent enzyme consisting of membrane proteins ($gp91^{phox}$ and $p22^{phox}$) and cytosolic proteins ($p47^{phox}$, $p67^{phox}$ and Rac). CGD results from mutations in any one of four genes encoding subunits of neutrophil NADPH oxidase showing defective respiratory burst (Heyworth et al., 2003). The mutations result in an absence or very low levels of the protein expression and NADPH oxidase activity. Decreased CD11b/CD18 expression

also leads to defective respiratory burst due to failure to trigger a protein kinase C (PKC)-mediated phosphorylation and the subsequent production of oxygen radicals generated by NADPH oxidase. The observation has been found in neutrophil-specific granule deficiency and LAD in humans (O'Shea et al., 1985; Gu et al., 2004).

LAD syndromes in humans are due to failure of innate host defenses against bacteria, fungi and other microorganisms resulting from defective adhesion and chemotaxis of leukocytes to sites of microbial invasion. LAD-I is an autosomal recessive disorder characterized by deficiency of three leukocyte integrin β2 subunits. This disorder results from genetic defects in CD18, which is required for stable expression of CD11/CD18 and the defective adherence and migration of leukocytes (Gu et al., 2004). CLAD in Irish setters represents the canine homologue of the severe phenotype of LAD-I (Kijas et al., 1999; Bauer et al., 2004). Mutations in β2-integrin encoding CD18 are unable to dimerize with CD11 subunits and result in decreased, or aberrant surface expression of the CD11/CD18 complex. Leukocyte adhesion molecules of β2 integrin family mediate cell-cell and cell-substrate interactions of neutrophils during their recruitment to sites of inflammation. CD11b/CD18 is not only an adhesion molecule but also the major receptor for opsonic complement fragment, an important trigger for phagocytosis of complement-opsonized microbes (Mazzone and Ricevuti, 1995; Kaufmann et al., 2006). In addition, binding to CD11b/CD18 provides an important co-stimulatory signal for other pathways important for adhesion, degranulation, and activation of reactive oxidant production (Lowell and Berton, 1999). Therefore, multiple defects in adhesion-related neutrophil functions lead to recurrent severe bacterial and fungal infections in LAD-I and CLAD patients. In addition, several

unusual LAD variants showing impaired adhesion-related neutrophil disorders have been described in a small number of patients. Patients with LAD-II, which is caused by mutation in the membrane transporter for fucose, have similar clinical features of LAD-I but exhibit intact leukocyte integrin expression and function (Bunting et al., 2002). LAD-III is characterized by defects in cell signaling that interferes with activation of multiple classes of integrins downstream of G protein-coupled receptors (Etzioni and Alon, 2004). Patients show similar clinical features of LAD-I, but however they have normal expression of β2-integrin in spite of unfunctional β2-integrin subunits. However, according to our knowledge, there are no case reports regarding CLAD variants in the literature.

In this study, we report the first recognized cases of congenital neutrophil dysfunction with clinical features similar to CLAD disorder in mixed-breed dogs. The first symptoms of pyogenic infection were recognized at a very early age in both littermates and since then repeated bacterial infections that were unresponsive to antibiotic therapy occurred. These clinical findings strongly suggested genetic phagocyte function disorders. We suspected fundamental defects in neutrophil function and therefore examined following neutrophil functions in order to differentially diagnose this disease from other canine neutrophil dysfunction including CLAD and CGD: serum opsonized zymosan (OZ) -induced production of superoxide in both dogs, and adherence, phagocytic activity, and ability of serum opsonization in the male dog. Furthermore, neutrophil surface expression of CD11b/CD18, transcript levels of CD11b and β2-integrin encoding CD18, the sequence of β2-integrin cDNA, transcript levels of the neutrophil secondary granules, lactoferrin, and protein expression of NADPH

oxidase components were also examined in the male dog.

2. **Materials and methods**

2.1. Dogs

Peripheral blood samples were obtained from two mixed-breed canine littermates (female, 9 months old and male, 6 years old) at different time periods and eight healthy beagles (five males and two females, 2–6 years old). Case #1 was negative for canine adenovirus and distemper antigens.

2.2. Reagents

Dextran 200000 and luminol were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Zymosan A and phorbol 1, 2-myristate 1, 3-acetate (PMA) were from Sigma-Aldrich (St. Louis, MO, USA). Fluoresbrite yellow green carboxylate microspheres (2.0 μm diameter) was from Polysciences, Inc. (Warrington, PA, USA). Fluorescein isothiocyanate conjugate (FITC) labeled anti-human monoclonal antibodies, CD11b and CD18 were from Beckman Coulter, Inc. (Fullerton, CA, USA). FITC-labeled anti-mouse immunoglobulin 1 (IgG1) monoclonal antibody was purchased from Serotec, Ltd. (Oxford, UK). The rabbit anti-gp91^{phox} antibody was from Upstate Millipore Corporation (Billerica, MA, USA), mouse anti-p67^{phox} antibody was from BD Biosciences (Franklin Lakes, NJ, USA). The goat anti-actin antibody and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit anti-p47^{phox} antibody was a kind gift from Babior, The Scripps Research Institute, CA.

137 *2.3. Enzyme staining of peripheral blood smears*

138 Peroxidase staining of peripheral blood smears was performed using an NB-PO
139 staining kit (Muto chemical, Tokyo, Japan) using α-naphthol-brilliant cresyl blue
140 method.

141

142 *2.4. Isolation of canine peripheral neutrophils*

143 Ten milliliters of heparinized whole blood was collected from the cephalic vein,
144 and suspended in an equal volume of Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution
145 (HBSS). The leukocytes were separated by dextran (6% w/v) sedimentation with half its
146 volume of whole blood for 30 min at room temperature. After the supernatant was
147 washed, polymorphonuclear leukocytes (PMNs) were isolated by Ficoll density (1.077)
148 gradient centrifugation at 400 x g for 30 min at 4 ^{o}C. The PMN fraction was harvested
149 and then contaminating erythrocytes were lysed at 4 ^{o}C by 0.83% NH_4Cl solution
150 containing 14.2 mM $NaHCO_3$ and 120 μM EDTA•2Na. After washing, the cells were
151 resuspended in HBSS at 4 ^{o}C. The viability of isolated PMN was determined by 0.2%
152 trypan blue staining (> 95%).

153

154 *2.5. Preparation of OZ and serum-opsonized fluorescent microspheres*

155 Zymosan was suspended in canine sera at a concentration of 5 mg/ml and incubated
156 for 30 min at 37 ^{o}C. After incubation, the suspension was washed twice with HBSS and
157 resuspended in HBSS at a concentration of 5 mg/ml. For preparation of
158 serum-opsonized fluorescent microspheres, the microspheres (2.5 x 10^7) was added to
159 100 μl of canine serum and incubated for 30 min at 37 ^{o}C. After washing with HBSS,

the microspheres were resuspended in HBSS at a concentration of 5 x 10^7 particles/ml.

2.6. Assay of neutrophil superoxide production

The production of superoxide was measured by chemiluminescence with luminol. Seven hundred microliters of HBSS containing 5 x 10^5 neutrophils and 100 μl of 10^{-4} μM luminol was prepared in a tube. The suspension was incubated for 2 min at 37 ^oC. After incubation, neutrophils were activated by adding 200 μl of OZ (5 mg/ml) or 10 μl of PMA (25 μg/ml). The chemiluminescence was measured with a luminometer (Luminescencer-PSN, ATTO Co., Tokyo, Japan) at intervals of 2 s for a total of 30 min at 37 ^oC.

2.7. Measurement of neutrophil phagocytic activity

Neutrophil phagocytosis of fluorescent microspheres was measured by the following whole blood flow cytometric technique. To measure non-specific phagocytic activity, we used non-opsonized microspheres that were suspended in glucose medium (0.1 M NaCl, 30 mM CH_3COONa, 4 mM KCl, 4 mM $CaCl_2$ and 7 mM glucose) at a concentration 5 x 10^7 particles/ml. One hundred microliters of heparinized whole blood, which had been preincubated for 2 min at 37 oC, and 100 μl of non-opsonized (non-specific phagocytosis) or serum opsonized (specific phagocytosis) microspheres were incubated for 30 min at 37 ^oC. For a negative control, 100 μl of heparinized whole blood was incubated at 4 ^oC until hemolysis. Then, phosphate-buffered saline (PBS) containing 3 mM EDTA·2Na was added to the cell suspension (except a negative control) and incubated for 5 min at 4 ^oC. After centrifugation at 260 x g for 5 min at 4

183 °C, contaminating erythrocytes were lysed at 4 °C by 0.83% NH_4Cl solution for 10 min.
184 After washing with PBS containing 3 mM EDTA•2Na, the cells were resuspended in
185 0.5 ml of 0.5% paraformaldehyde in PBS and filtered to remove cell aggregate. The
186 fluorescence of the microspheres in neutrophils was analyzed by flow cytometry
187 (FACScan, BD Biosciences). Analysis gates for neutrophils were established with
188 forward and side scatter profiles. Cell acquisition and data analysis was performed with
189 CELLQuest pro software (BD Biosciences). Phagocytic activity expressed as
190 percentage of the total neutrophil population ingesting fluorescent microspheres. The
191 results of phagocytic activity in case #2 shown are representative of two independent
192 experiments.
193
194 *2.8. Ability of serum opsonization*
195 Two types of serum-opsonized fluorescent microspheres were prepared using serum
196 of case #2 or sera of normal healthy dogs according to above-mentioned methods. One
197 hundred microliters of the serum-opsonized microspheres (5×10^7 particles/ml) and 100
198 μl of isolated neutrophils from healthy dogs (5×10^6 cells/ml) were incubated for 30
199 min at 37 °C. Then, PBS with 3 mM EDTA•2Na was added to the cell suspension and
200 incubated for 5 min at 4 °C. After centrifugation at 260 x g for 5min at 4 °C, cells were
201 resuspended in 500 μl of 0.5% paraformaldehyde in PBS. The phagocytic activity of the
202 neutrophils was analyzed by flow cytometry.
203
204 *2.9. Neutrophil adherence*
205 The adherence of neutrophils to nylon fibers was examined according to the method

206 of Nagahata et al. (1993). One milliliter of neutrophil suspension (5 x 10^6 cells/ml)
207 containing 10% autologous plasma was incubated for 10 min at 37 ^oC and then was
208 applied to a nylon wool fiber column (50 mg, Polysciences, Inc., Warrington, PA, USA),
209 which was preincubated for 60 min at 37 ^oC. Neutrophil counts were performed after
210 samples were allowed to percolate through the nylon fiber at room temperature.
211 Neutrophil adherence was calculated from the formula: Percentage of neutrophil
212 adherence = (1 – counts of effluent neutrophil / counts of initial neutrophil) x 100. The
213 results of case #2 shown are representative of two independent experiments.
214
215 *2.10. Surface expression of adhesion molecules on leukocytes*
216 In order to activate the cells, heparinized whole blood was preincubated for 2 min
217 at 37 ^oC. For surface marker staining, 100 μl of heparinized whole blood was added into
218 sterile plastic tubes containing 20 μl of one of the following monoclonal antibodies:
219 FITC-labeled anti-CD11b and CD18. The tubes were then incubated for 30 min at room
220 temperature in the dark. Erythrocytes were lysed by 0.83% NH_4Cl solution at 4 ^oC for
221 10 min, followed by a wash in PBS with 3 mM EDTA•2Na and centrifuged at 260 x g
222 for 5 min at 4 ^oC. Cells were resuspended in 500 μl of 0.5% paraformaldehyde in PBS,
223 followed by filtration with a mesh. A negative control incorporating FITC-labeled
224 anti-mouse IgG1 monoclonal antibody was run with each sample. Analysis gates for
225 neutrophils or lymphocytes were expressed as mean fluorescence intensity (MFI) on a
226 log-scale analyzing 10000 cells per sample as follows: MFI = (Geo mean of target
227 antibody – geo mean of negative control) / geo mean of negative control. Flow
228 cytometric analyses of case #2 shown are representative of two independent

229 experiments.

230

231 *2.11. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis of*
232 *expression of neutrophil CD11b, β2-integrin and lactoferrin messenger ribonucleic acid*
233 *(mRNA)*

234 Total RNA from isolated neutrophils (5 x 10^6 cells) was extracted according to the
235 manufacturer's protocol (RNeasy Mini Kit, Qiagen GmbH, Hilden, Germany). The
236 purified RNA was eluted in a final volume of 30 μl RNase-free water and stored at
237 –80^{o}C until complementary deoxyribonucleic acid (cDNA) synthesis. The cDNA was
238 synthesized from total RNA (0.17 μg) using a high capacity cDNA reverse transcription
239 kit (Applied Biosystems, Foster City, CA, USA). The reverse transcription was carried
240 out by subsequent incubation for 10 min at 25 ^{o}C, 120 min at 37 ^{o}C, 5 s at 85 ^{o}C. The
241 cDNA was stored at –80 ^{o}C until use. Amplification of canine CD11b, β2-integrin,
242 lactoferrin and β-actin mRNA was performed by real-time RT-PCR using the 7300
243 Real-Time PCR System (Applied Biosystems) with SYBR Green I as the detection
244 format. Amplification was carried out in a total volume 25 μl containing 0.8 x Power
245 SYBR Green PCR Master Mix (Applied Biosystems), 200 nM each primer and 1 μl (β
246 -actin) or 2 μl of cDNA. The reaction was performed by 1cycle of 2 min at 50 ^{o}C, 10
247 min at 95 ^{o}C and 40 cycles of 15 s at 95 ^{o}C, 30 s at 62 ^{o}C, 40 s at 72 ^{o}C. Expression
248 levels were quantified in duplicate by means of real-time RT-PCR. Cycle threshold
249 values for genes of interest were normalized to β-actin and used to calculate the relative
250 quantity of mRNA expression.

251 The nucleotide sequences of all canine PCR primers and their respective amplified

products are as follows: CD11b (GenBank accession no. XM_547048, position 2591-2651, length 61 bp), forward and reverse: 5'–GAGTCTGACGATTCCACTAATG–3' and 5'–GTTTATGCTGCAGCTGCTA–3', β2-integrin (GenBank accession no. AF181965, position 282-343, length 62 bp), CGCAGAAAGTGACGCTCTAC and CCGGAAGGTCACATTGAA, lactoferrin (GenBank accession no. DQ338567, position 3-277, length 275 bp), CAGGCTGGAACATCCC and GTTCCCTCCGTTTGTGTTC, β-actin (GenBank accession no. AF021873, position 456-641, length 186 bp), CATGGACTCTGGGGATGG and TCCTGATGTCACGCACGA. These primer sets were based on deposited cDNA sequences (GenBank database). β-actin was used as an internal control. Real-time PCR results of case #2 shown are representative of at least two independent experiments.

2.12. Sequencing of neutrophil β2-integrin mRNA

To examine all sequences of neutrophil β2-integrin cDNA, 5 primer sets (forward and reverse,) were designed as follows: β2-integrin set1 (GenBank accession no. AF181965, position 6-993, 988 bp), TGCTCACCCTGGAGGGTCTGCTCTT and ATGACCTCG GTGAGCTTCTCATAGG, β2-integrin set2 (position 121-1148, 1028 bp), CCAGAAGCTGAACTTCACTGGGCTA and CGTTACTGCAGAAGGAGTCATAGGT, β2-integrin set3 (position 969-1655, 687 bp), CCTATGAGAAGCTCACCGAGGTCAT and T G C A G G A G CCC C G A A CT TT A, β2-integrin set4 (position 1124-1958, 835 bp), ACCTATGACTCCTTCTGCAGTAACG and TCTCCGGGGGTTTGCTCAGCAG,

β2-integrin set5 (position 1862-2325, 464 bp), ACCTGTGCCCAGTGCCTGA and CTCAGCCAGCGCCGTCTCCGCCGA. The amplification was performed using a Platinum Taq DNA Polymerase High Fidelity kit (Invitrogen, Carlsbad, CA, USA). Four microliters of cDNA were added to a reaction mixture with the final concentration of 1 x High Fidelity PCR Buffer, 0.2 μM each primer, 3% DMSO, 2 mM $MgSO_4$, 0.2 mM dNTP mixture and 0.02 U/μl Taq polymerase (Platinum Taq High Fidelity) in a final volume of 12 μl. The PCR profile used was 2 min at 94 ^oC for the first cycle, 30 s at 94 ^oC, 45 s at 60 ^oC and 90 s at 68 ^oC for 40 cycles and 7 min at 68 ^oC for a cycle. Each PCR product was cloned into pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen) and transformed into a competent E. coli cell (DH5α). The plasmids were purified from the bacterial solutions originating from four colonies by a QIAprep spin miniprep kit (QIAGEN), followed by sequencing reaction with BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing analysis was performed by ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). The resulting nucleotide sequences were analyzed using genetic information processing software, GENETYX-WIN Ver. 5 (Software development, Tokyo, Japan) and free software, Chromas Lite.

2.13. Western blotting of NADPH oxidase components

Neutrophils (5 x 10^6 cells) were incubated with 60 μl of lysis buffer [1% Triton X-100, 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM $Na_2P_2O_7$, 2 mM EDTA, 50 mM NaF, 10% (v/v) glycerol, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF)] for 30 min at 4 ^oC. Cells were sonicated on

ice using two 30 s bursts, followed by centrifugation at 20000 x g for 30 min at 4 ^{o}C. The supernatant was resuspended in Lammli's sample buffer and separated by SDS-PAGE. Then, the gel (10%, p47^{phox}, p67^{phox} and actin; 7.5%, gp91^{phox}) was transferred to a nitrocellulose membrane. For detection of NADPH oxidase components, rabbit anti-gp91^{phox}, rabbit anti-p47^{phox}, mouse anti-p67^{phox} was used at a dilution of 1:2000, 1:3000 and 1:5000, respectively. Goat anti-actin antibody was used as an internal control at a dilution of 1:2000. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence detection kit (PerkinElmer Life and Analysis Science, Inc., Waltham, MA, USA). The results were evaluated as a ratio of NADPH oxidase component to β-actin.

2.14. Interval estimation

For analyses of each assay of neutrophil function, a two-sided 95 % confidence interval (CI) on the mean was calculated.

3. Results

3.1. Medical history, clinical and hematological findings in dogs

Two of five canine mixed-breed littermates were presented with recurrent bacterial infections with oculo-nasal mucopurulent discharge from puppyhood, which were refractive to antibiotics. The symptoms were recognized within two puppies after 3 months of age when the protective effect of maternal immunoglobulin has been lost (12–15 weeks of age). Since then they suffered from repeated bacterial infections from which they never completely recovered. Their medical histories strongly suggested genetic neutrophil function disorders. However, their parents and other three littermates remained asymptomatic. The two littermates were examined at different time periods as follows; at initial presentation to our Veterinary Teaching Hospital, the female dog (case #1) was 9 months old and showed signs of lethargy, anorexia and pyrexia. She had recurrent conjunctivitis, rhinitis and severe bilateral corneal opacity and occasional epistaxis. She had repeated bacterial infections with poor response to supportive therapies. Six years later, the male littermate (case #2) was presented with recurrent upper respiratory bacterial infections, oculo-nasal mucopurulent discharge and a productive cough complicated by pneumonia. His condition was very poor and he had pyrexia and severe bilateral corneal opacity as was case in case #1. The number of total leukocytes in peripheral blood from case #1 and #2 was higher (14000/μl and 22600/μl, respectively) than healthy dogs (laboratory specific control value 6000-17000/μl). Both cases had hypoalbuminemia and hyperglobulinemia. The concentrations of albumin in serum from case #1 and #2 were 1.42g/dl and 1.84g/dl, respectively, indicating marked

335 decreases compared to the normal dogs (3.0-4.0g/dl), whereas that of globulin was
336 higher in both cases (4.98g/dl and 4.38g/dl, respectively) than that in normal dogs
337 (3.0-4.0g/dl). They also showed severe reduction in the red blood cell count. During the
338 first 2 weeks, symptomatic therapies such as fluid therapy, nebulization and
339 administration of antibiotics were carried out. However, they showed a poor response
340 to therapy.

341 According to the owner's report, the female dog was later killed in a traffic accident
342 at the age of 3 years and the male dog went missing at 7 years of age, and therefore lost
343 to further follow-up.

344

345 *3.2. Neutrophil counts and morphology*

346 The numbers of total neutrophils in peripheral blood from case #1 and #2 were
347 7000/μl and 16500/μl, respectively (laboratory specific control value 3000-11500/μl).
348 Case #1 showed that the percentage of segmented neutrophil was 46% and that of band
349 neutrophil was 4%. Neutrophils from case #2 had 73% of segmented neutrophils and
350 9% of band neutrophils. Light microscopic examination showed that there was no
351 abnormally hyposegmentation and large granules in Giemsa-stained neutrophils from
352 the affected dogs. When stained with peroxidase, small positively-stained granules were
353 seen in the cytoplasm of neutrophils affected as well as healthy dogs.

354

355 *3.3. Production of superoxide of neutrophils*

356 In view of the fact that the medical history was suggestive of genetic neutrophil
357 dysfunction, further examinations were carried out to establish neutrophil function. We

first examined the production of superoxide in canine neutrophils stimulated with OZ by chemiluminescence with luminol. Superoxide production is evoked by activation of NADPH oxidase mediated by OZ stimulation through plasma membrane receptors. As shown in Fig. 1, there were profound decreases of the OZ-induced superoxide production with an inadequate peak in both cases compared with normal dogs. The maximum amount of luminescence attenuated to about 20% in case #1 and 29.5% in case #2 of normal canine levels (case #1, 21336/sec; case #2, 31228/sec; normal dogs, 105990 ± 27763/sec, n=5, 95% CI 76854 to 136729). When neutrophils stimulated with PMA, which directly activates PKC pathway, however, the value of maximum amount of luminescence in neutrophils from case #2 was comparable to that in normal dogs (case #2, 167760/sec; normal dogs, 175800 ± 821/sec). These observations suggest that some abnormalities may exist in the pathway between plasma membrane receptors and PKC. Henceforth, case #2 was further detailed examination of neutrophil functions.

3.4. Phagocytic activity and ability of serum opsonization

To examine phagocytic activity of neutrophils from case #2, we used non-opsonized or serum-opsonized fluorescent particles. As shown in Fig. 2A, non-specific phagocytic activity of neutrophils from case #2 was lower than that from controls (n=5). About 32.20 ± 1.68% of neutrophils from case #2 phagocytosed the non-opsonized particles, whereas 45.15 ± 2.16% of normal canine neutrophils ingested them (95% CI 42.47 to 47.85). In addition, specific phagocytic activity in case #2 was lower than that in healthy dogs (Fig. 2A). Case #2 showed that the specific phagocytic activity was 5.74 ± 1.35% using healthy canine sera-opsonized particles and 10.02 ±

2.54% using autologous serum-opsonized ones, whereas the activity using healthy sera-opsonized ones in healthy dogs was 15.95 ± 2.71% (n=5, 95% CI 12.59 to 19.31). To examine the ability of serum opsonization in case #2, phagocytic activities of normal canine neutrophils were measured by using fluorescent particles opsonized with canine serum of case #2 or sera of normal dogs for phagocytic stimulation. The capacity of serum opsonization of case #2 resulted in 16.08 ± 2.07% phagocytic neutrophils, and was comparable to that of normal dogs (15.95 ± 2.71%, n=5).

3.5. Adherence of neutrophils

The patient's neutrophils exhibited reductions in adherence to nylon fibers. As shown in Fig. 2B, the adhesion of neutrophils was 19.30 ± 0.01% in case #2, a lower rate than in normal canine neutrophils (31.74 ± 2.41%, n=5, 95% CI 28.75 to 34.73).

3.6. Flow cytometric analysis of surface expression of CD11b and CD18 molecules on leukocytes

Due to the fact that neutrophils from case #2 showed decreases in adherence, OZ-mediated superoxide production and phagocytic activity through membrane receptors, we examined the expression of adhesion molecules CD11b and CD18 on neutrophils. Youssef et al. (1995) demonstrated that temperature (37 ^{o}C) activated neutrophils and increased the expression of cell adhesion molecules on neutrophils. We also observed the same phenomenon in the preliminary examination. Therefore, whole blood was preincubated at 37 ^{o}C before surface marker staining. Flow cytometric analysis revealed mild deficiency of the CD11b/CD18 leukocyte surface expression in

404 case #2 (Fig. 3A). On neutrophils from case #2, CD11b was expressed at about 45.9%
405 of normal canine levels (case #2, 2.88 ± 0.13%; normal dogs, 6.28 ± 0.77%, n=5, 95%
406 CI 5.33 to 7.23) and CD18 was approximately 42.3% of healthy levels (case #2, 28.5 ±
407 0.07%; normal dogs, 67.41 ± 6.51%, n=5, 95% CI 59.33 to 75.49). Furthermore, CD18
408 expression on the patient's peripheral blood lymphocytes was also lower than normal
409 levels (case #2, 35.96 ± 0.16%; normal dogs, 48.94 ± 4.64%, n=5, 95% CI 43.17 to
410 54.7).
411
412 *3.7 Real-time RT-PCR analysis of expression of Neutrophil CD11b, β2-integrin and*
413 *lactoferrin mRNA*
414 Because the level of RNA plays a key role in regulating the expression of many
415 proteins, we studied the RNA levels of neutrophil CD11b and β2-integrin genes
416 encoding CD18 in case #2. Additionally, the gene expression of the neutrophil
417 secondary granules, lactoferrin, showing antibacterial activity, was examined on
418 whether specific granules deficiency exists or not. Real-time RT-PCR analysis showed
419 that transcript levels of β2-integrin gene in neutrophils from case #2 were much lower
420 than those in normal dogs (Fig. 3B). The CD11b mRNA expressed at about 76.2% of
421 normal levels (0.64 ± 0.28 versus normal controls 0.84 ± 0.39, n=5, 95% CI 0.43 to
422 1.25) and β2-integrin mRNA expressed at about 13.4% of control values (0.09 ± 0.05
423 versus normal controls 0.67 ± 0.22, n=5, 95% CI 0.41 to 0.94). On the other hand, case
424 #2 had lactoferrin transcript levels (0.43 ± 0.01) in neutrophils comparable to normal
425 healthy controls (0.43 ± 0.12, n=5).
426

3.8. Analysis of β2-integrin sequence data

To investigate whether any mutations in the β2-integrin gene were detected in neutrophils from case #2 as well as CLAD-affected dogs, we examined the sequence of β2-integrin gene in neutrophils from case #2 and normal control dogs. The β2-integrin cDNA sequences from the dog showed a single nucleotide C to T transversion at position 279, codon 93 (Fig. 3C). However, it did not lead to a change in amino acid. Therefore, this result indicates that the sequence of the β2-integrin cDNA in case #2 differs from that of CLAD.

3.9. Immunoblot analysis of NADPH oxidase components

We isolated the cytosol fraction from unstimulated neutrophils in case #2 and evaluated the expression of proteins for NADPH oxidase components by western blot analysis in order to rule out CGD. As shown in Fig. 4, immunoblot analysis revealed that the protein expression of the cytochrome b_{558} component $gp91^{phox}$ increased in neutrophils from case #2 compared to normal dogs. In addition, there were marked increases in the protein expressions of the cytosolic components $p47^{phox}$ and $p67^{phox}$ of NADPH oxidase components in neutrophils from case #2. Judging from the overexpressions of NADPH oxidase components, neutrophils dysfunction of case #2 is unlikely to be caused by CGD.

4. Discussion

In the present study, we identified two canine mixed breed littermates (one 9-month-old female and 6-month-old male) that had suffered recurrent bacterial infections from a young age despite adequate numbers of circulating neutrophils and antibiotic treatment. A genetic background of neutrophil dysfunction was strongly suggested by the medical histories. Neutrophil function tests revealed that neutrophils from two littermates, during antibiotic therapy, showed a marked reduction in superoxide production in response to OZ, stimulating membrane receptors-mediated phagocytosis and PKC activation. The evidence that the 9-month-old puppy still had defective superoxide production implicated a relationship between genetic neutrophil dysfunction and susceptibility to infection. Furthermore, the neutrophils in the male dog showed abnormalities through mild decreases of neutrophil adherence to nylon fibers, phagocytic activity against non-opsonized and serum-opsonized particles. Capacity of serum opsonization in the male dog was normal, indicating normal function of serum immunoglobulin and complement proteins. In addition, we observed that superoxide production stimulated with PMA, which is a direct stimulant of PKC and activates NADPH oxidase, was normal in the male dog. We also found that protein overexpressions of the cytochrome b_{558} component $gp91^{phox}$, cytosolic components $p47^{phox}$ and $p67^{phox}$ of NADPH oxidase components in neutrophils from the male dog. This finding was in disagreement with other CGD studies in humans, which reported that mutations in the genes encoding subunits of neutrophil NADPH oxidase result in an absence or very low levels of the protein expression and defective in superoxide

production (Heyworth et al., 2003). Judging from our findings, this neutrophil disorder of the affected dog differs from CGD. When treated with OZ or PMA, neutrophils have been shown to react in different ways during the respiratory burst process. It is well recognized that OZ activates the cell through a mechanism that binds membrane receptors, followed by stimulation of PKC. OZ has been known to generate complement C5 that reacts with C5 receptor (CD88) on neutrophils. In addition, several reports indicated that OZ-stimulated respiratory burst and phagocytosis, which was inhibited by anti-CD11b/CD18 monoclonal antibody and decrease in CD11b/CD18-deficient neutrophils (Nagahata et al., 1993, Xia et al., 1999). The observation suggests that the CD11b/CD18-dependent pathway is in some part involved in the process of OZ-stimulated respiratory burst. A direct activation of PKC, bypassing signaling through membrane-receptor, via neutrophil stimulation with PMA is known to stimulate NADPH oxidase. In this study, OZ-stimulated superoxide production was significantly reduced, whereas PMA-induced superoxide production was normal. Because both stimuli share a common pathway of downstream activation of PKC, it is unlikely that NADPH oxidase is dysfunctional but that increased levels of its components are the cause of the lesion. It may be that the overexpression of NADPH components is probably a consequence of other failures. Until now, very little work has focused on the correlation between levels of NADPH oxidase components and expression level of CD11b/CD18. A recent study showed that neutrophils from patients with CGD had lower expression levels of CD11b/CD18 (Hartl et al., 2008). However, inhibition of NADPH oxidase did not affect CD11b/CD18 expression on neutrophils from healthy subjects. From these observations, it is unlikely that NADPH oxidase directly modulates

493 the expression of CD11b/CD18 on neutrophils. We however could not clarify the reason
494 for overexpression of NADPH oxidase components and the possible correlation
495 between this observation and the decreased CD11b/CD18 expression in present paper.
496 Therefore, the deficiency of superoxide production in response to OZ but not PMA in
497 littermates suggests that there may be any abnormalities in membrane receptor-mediated
498 pathways.

499 We next examined not only surface expression but also mRNA level of neutrophil
500 adhesion molecules CD11b/CD18 in the male. Flow cytometric analysis revealed a
501 decrease in surface expression of both CD11b and CD18 molecules on neutrophils. In
502 addition, real-time RT-PCR analysis showed that transcript levels of β2-integrin
503 encoding CD18 in neutrophils from the male were much lower than those in normal
504 dogs, whereas CD11b transcript level showed a slight downregulation. A study with a
505 cDNA clone for β-subunit mRNA indicated that surface expression of CD11b/CD18
506 closely paralleled the levels of mRNA expression in granulocytic cells, and that the
507 mRNA level seemed to be an important determinant of CD11b/CD18 surface expression
508 (Hickstein et al., 1988). Our data suggest that decreased mRNA levels of CD11b/CD18,
509 especially β2-integrin, should result in decreased membrane expression of these
510 proteins on neutrophils from the male. In addition, we examined all neutrophil functions
511 including expression of CD11b/CD18 in four related puppies (one male and three
512 females, 2.5 months old) of case # 1 and #2. Their mother was one of the siblings of
513 case #1 / #2, with the same father. The puppies had never exhibited abnormal clinical
514 findings since birth, whereas case #1 and #2 suffered bacterial infections from 3 months
515 of age. We found that the related puppies did not reveal any disorders of neutrophil

functions compared with four 2-month-old healthy puppies (Data not shown). It is likely that abnormal clinical symptoms are associated with disorders of neutrophil dysfunctions in the colony. Moreover, several reports regarding the relationship between chronic bacterial infections and neutrophil functions indicated that patients with chronic and severe bacterial infections showed an increase in CD11b and decreased CD18 expression on canine neutrophils, and normal opsonized-zymosan stimulated superoxide production, adhesion and phagocytic activity in human neutrophils. Our findings however were not in agreement with these reports.

The clinical features and neutrophil dysfunction in association with decreased surface expression of CD11b/CD18 exhibited by the male dog, is similar to CLAD in Irish setters and LAD-I in humans with severe recurrent bacterial infections (Kishimoto et al., 1987; Allende et al., 2000; Bauer et al., 2004). Sequence analysis of CLAD CD18 alleles has identified a single nucleotide G-to-C transversion at position 107, which leads to a replacement of cysteine by serine at residue 36 (Kijas et al., 1999). To determine whether the neutrophils had CD18 mutations, we examined sequence of cDNA of β2-integrin gene. Sequence analysis revealed that there was no missense mutation detected in β2-integrin gene. The lack of a genetic defect in the β2 subunit excluded the diagnosis of classical CLAD. Thus, these findings suggest that the decreases of CD18 at both protein and transcript levels without mutations should result in the abnormalities in adhesion-related neutrophil function included adherence, phagocytic activity and OZ-induced superoxide production in this case. Unfortunately, we could not examine other neutrophil function except for OZ-induced superoxide production in the female puppy, because she died. From the findings that she showed

539 the same abnormal clinical symptoms from puppyhood and defective OZ-induced
540 superoxide production as the male, it is conceivable to assume that the same pathology
541 in neutrophil functions also existed in the female.

542 Studies regarding neutrophil-specific granule deficiency have reported that
543 neutrophils from patients were found to be defective in granular CD11b/CD18 and did
544 not form clusters on CD11b/CD18-modified cell surfaces (O'Shea et al., 1985; Petty et
545 al., 1987). In addition, neutrophils treated with a stimulator of granule release have
546 demonstrated numerous increases of CD11b/CD18 clusters on the plasma membrane
547 (Petty et al., 1987). These reports indicate that formation of CD11b/CD18 membrane
548 domains requires the participation of specific granules during immune recognition and
549 these domains are formed by fusion of lysosome containing CD11b/CD18-bearing
550 specific granules at local sites of adhesion. Our study showed that the transcript level of
551 lactoferrin was normal in the male, and so we excluded the lactoferrin deficiency, which
552 results in severe chemotactic defects leading to recurrent bacterial and fungal infections
553 of primarily the skin and lungs. However, it may be possible that decreased
554 CD11b/CD18 expression can result from disorder of posttranscriptional regulation of
555 specific granules in the affected dog.

556 Several studies have reported that integrin function in adhesion is related to cellular
557 activation through inside-out and outside-in signaling. For example, neutrophil
558 CD11b/CD18-mediated adhesion was blocked by p38 mitogen-activated protein kinase
559 inhibitor (SB203580) in LPS-stimulated human neutrophils (Detmers et al., 1998) and
560 was regulated by Syk-kinase or phospholipase D1 in human neutrophils (Willeke et al.,
561 2003; Iyer et al., 2006). Furthermore, recent studies demonstrated a few LAD variants

562 with similar clinical features of mild LAD-I, which have no mutations in the integrin β
563 subunit genes. Neutrophils with LAD-III have been shown to have normal surface
564 expression of β2-integrin, but be defective in cell signaling that interferes with
565 activation of multiple classes of integrins downstream of G protein-coupled receptors
566 (Kuijpers et al., 1997; Etzioni and Alon, 2004). Defects in intracellular signaling
567 essential for integrin activation results in lack of regulation of integrin clustering and
568 essential components of integrin-mediated adhesion (McDowall et al., 2003).
569 Neutrophil adhesion-related dysfunction in present study seems not to be consistent
570 with LAD variants and classical CLAD patients. It is speculated that adhesion-related
571 disorders with a decreased expression of CD11b/CD18 mRNA may be associated with
572 inactivation of intracellular events after stimulation. However we could not clarify the
573 relationship between CD11b/CD18 expression and intracellular factor(s) in neutrophils
574 from the affected dog. Further studies will be required to investigate the mechanism of
575 downregulation of CD11b/CD18 transcriptional level in neutrophils.

576 In conclusion, the medical history of recurrent bacterial infections from puppyhood
577 (3 months old) in littermates and the results of neutrophil function tests, suggested that
578 the increased susceptibility to bacterial infections is caused by congenital neutrophil
579 adhesion-related dysfunction. To our knowledge, this is the first case reported of canine
580 congenital neutrophil dysfunction in a mixed-breed dog. Furthermore, our study
581 suggests that decreased transcriptional levels of CD18 without mutations, which lead to
582 downregulation of the surface expression, should result in multiple defects in
583 adhesion-related neutrophil functions with a different pathophysiology from CLAD. It
584 is hoped that this study will give further insights into the mechanism of regulation of

integrin expression at transcriptional level in neutrophils.

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672 **Figure captions**

673

674 **Fig. 1.** Superoxide production in OZ-stimulated canine neutrophils in littermates (□,
675 case #1; ■, case #2) with recurrent infections compared to normal healthy dogs (○).
676 Superoxide production was measured by chemiluminescence as described in the text.
677 The result of healthy controls is expressed as the mean of five experiments.

678

679 **Fig. 2.** Neutrophil phagocytic activity and adherence in case #2. (A) Non-specific or
680 specific phagocytic activity of neutrophils was measured by a whole blood flow
681 cytometric assay using non-opsonized or serum-opsonized fluorescent microspheres.
682 Phagocytic activity expressed as percentage of the total neutrophil population ingesting
683 fluorescent microspheres. (B) Neutrophil adherence was measured by the nylon fiber
684 adherence assay. The results were expressed as percentage of neutrophil adherence to
685 nylon fibers. The result of healthy controls and case #2 is expressed as the mean ± S.D.
686 The two-sided 95% CI for the mean of each assay in healthy controls was as follows:
687 non-specific phagocytic activity, 42.47 to 47.85, specific phagocytic activity, 12.59 to
688 19.31, adherence, 28.75 to 34.73. All means of case #2 fell out of the 95% CI for the
689 mean of healthy controls.

690

691 **Fig. 3.** Surface expression and transcript level of neutrophil adhesion molecules and
692 sequence of CD18 cDNA in case #2. Mean values ± S.D. are shown in controls and case
693 #2. (A) Surface expression of CD11b and CD18 on neutrophils was quantified by a
694 whole blood flow cytometric assay. The results were expressed as mean fluorescence

695 intensity (MFI). The 95% CI for the mean of CD11b and CD18 expression in healthy
696 controls was 5.33 to 7.23 and 59.33 to 75.49, respectively. Both means of case #2 fell
697 outside of the 95% CI. (B) The transcript levels of CD11b and β2-integrin were
698 measured by real-time RT-PCR. The results were expressed as a ratio of CD11b or
699 β2-integrin to β-actin. The 95% CI for the mean of CD11b and β2-integrin mRNA
700 expression in healthy controls was 0.43 to 1.25 and 0.41 to 0.94, respectively. The mean
701 of β2-integrin in case #2 fell outside of the 95% CI. (C) Sequence analysis of cDNA of
702 β2-integrin gene after TA cloning in neutrophils from case #2 and normal dogs (wild
703 type). CLAD sequence was quoted from a report by Kijas et al. (1999).
704
705 **Fig. 4.** Immunoblot of NADPH oxidase components in canine neutrophils from case #2.
706 Western blots detected by the following primary antibodies: anti-$p47^{phox}$, anti-$p67^{phox}$,
707 anti-$gp91^{phox}$, anti-actin after isolated neutrophils were lysed and the cytosol fraction
708 from unstimulated neutrophils was collected. The results were evaluated as a ratio of the
709 NADPH oxidase component to β-actin.

Figure 1

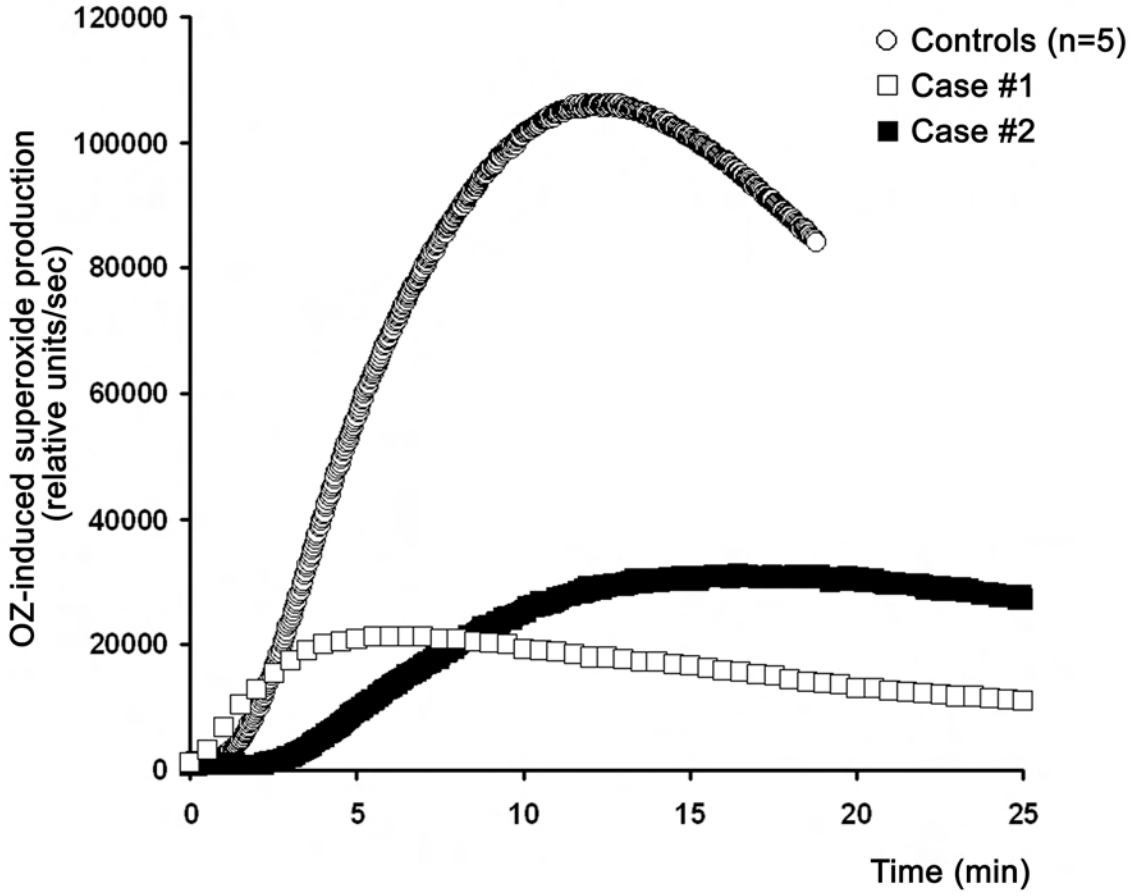


Fig 1. Kobayashi et al.

Figure 2

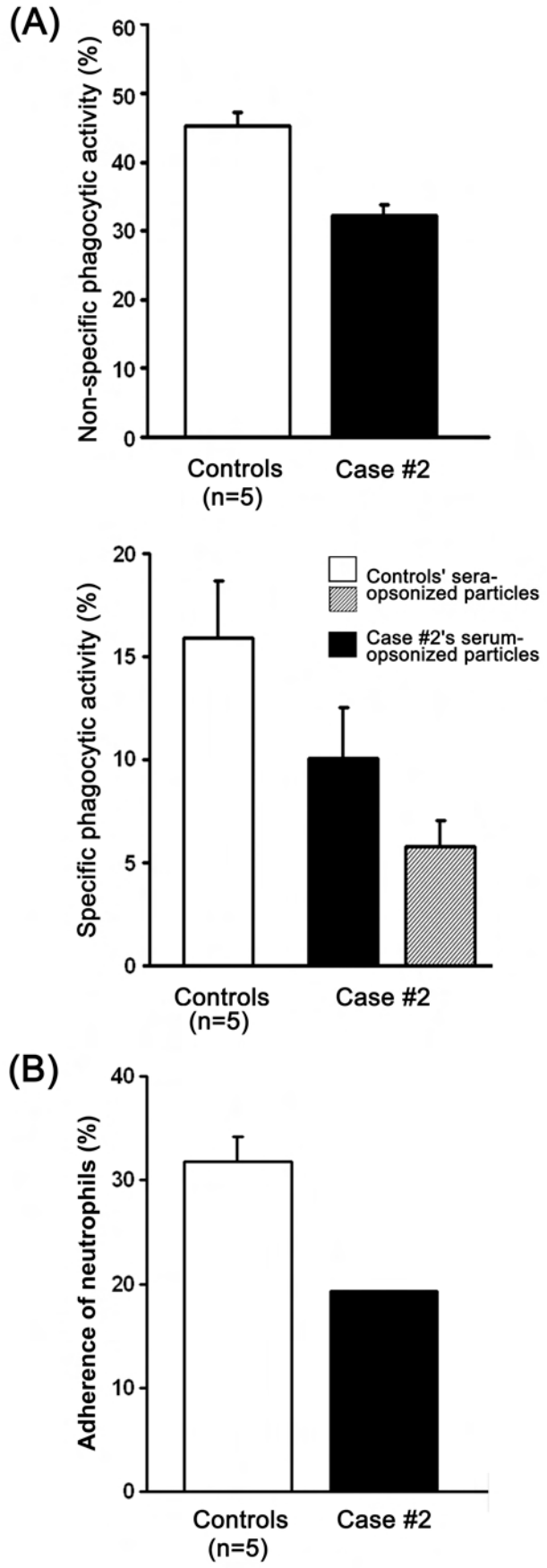
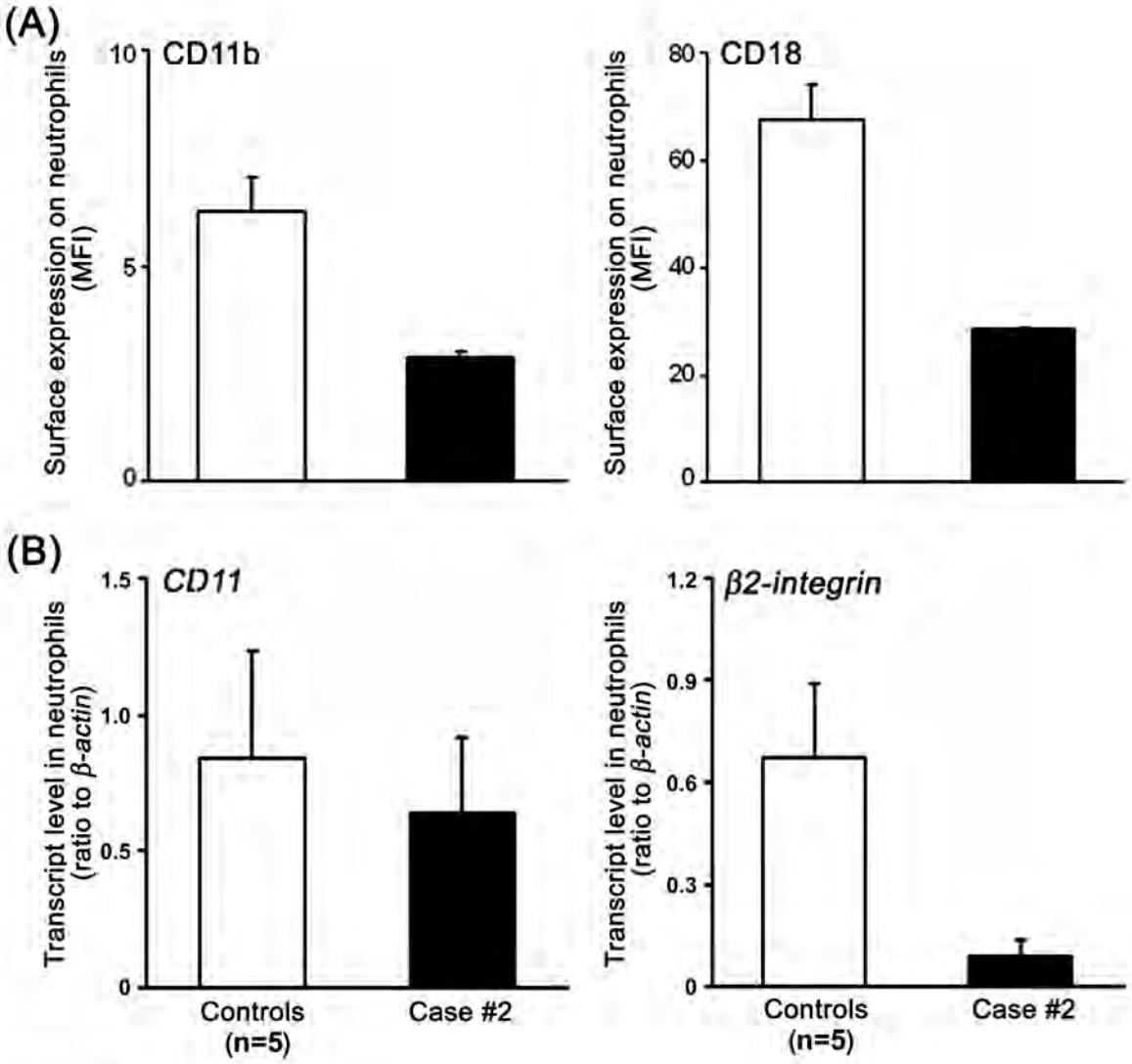


Fig 2. Kobayashi et al.

Figure 3



(C) *β2-integrin* cDNA

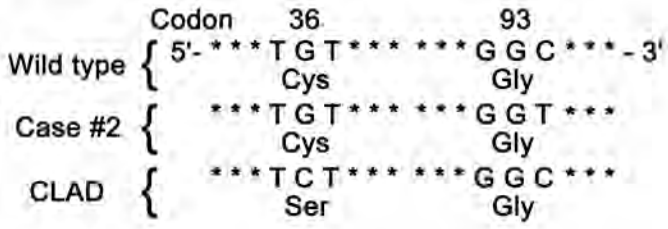


Fig 3. Kobayashi et al.

Figure 4

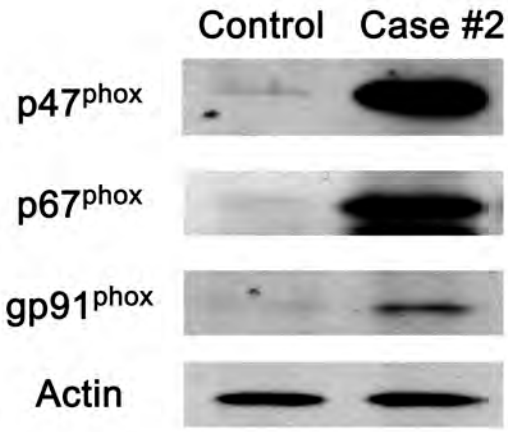


Fig 4. Kobayashi et al.