

# PEG-fDAO Reduces Lung Inflammation in Chronic Granulomatous Disease Mice Post Challenge with Nonviable *Candida Albicans*

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## Research Article

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# Abstract

We previously reported that polyethylene glycol-conjugated recombinant porcine D-amino acid oxidase (PEG-pDAO) could supply reactive oxygen species (ROS) to defective NADPH oxidase in neutrophils of patients with chronic granulomatous disease (CGD), and neutrophils regain bactericidal activity *in vitro*. In the present study, we employed an *in vivo* nonviable *Candida albicans* (nCA)-induced lung inflammation model using gp91-phox knockout CGD mice and novel PEG conjugates of *Fusarium spp.* D-amino acid oxidase (PEG-fDAO), rather than PEG-pDAO. Using three experimentation strategies with the *in vivo* lung inflammation model, the mouse body weight, lung weight, and lung pathology were evaluated to confirm the efficacy of ROS-generating enzyme replacement therapy with PEG-fDAO. The lung weight and pathological findings were significantly ameliorated by the administration of PEG-fDAO followed by intraperitoneal injection of D-phenylalanine or D-proline. These data suggest that PEG-fDAO with the function of targeted delivery to the nCA-induced inflammation site is applicable in the treatment of inflammation in CGD *in vivo*.

## Introduction

Chronic granulomatous disease (CGD) is a primary immunodeficiency characterized by the inability of phagocytes to produce reactive oxygen species (ROS) due to a defect in the NADPH oxidase complex. Clinically, CGD patients frequently suffer from bacterial infections and excessive inflammatory disorders, such as CGD-associated bowel inflammation and sterile granulomas. ROS generation is crucial for the phagocytic killing of ingested microbes [1] and inflammasome signaling processes, such as the

Caspase-1 activation platform for cytokine production [2–4], the Keap1-Nrf2 pathway for antioxidative stress [5], and efferocytosis through phosphatidylserine and its receptor [6].

Anti-inflammatory cytokine therapies (anti-interleukin (IL)-1 antibody, etc.) have been proven to alleviate excessive cytokine production in an *in vivo* CGD mouse model and clinical studies on severe colitis [4]. In addition, a PPAR $\gamma$  agonist (pioglitazone) has been confirmed to enhance mitochondrial ROS production and partially restore host defense in a CGD mouse model [6]. Bone marrow transplantation or gene therapy with a lentiviral vector and myeloid-specific promoter has been proposed as a successful curative therapy [7]. Although bone marrow transplantation and gene therapy are thought to be theoretically curative treatments, residual pathogenic components, live or dead, lead to severe vital reactions, such as inflammation and/or organ damage, in CGD patients. Unprecedented antibiotic treatments are pathogen-specific remedies for the removal of pathogens. Potent pathogen-specific antibiotic treatments can kill pathogens, but may not prevent the excessive inflammation that can persist due to the presence of residual materials of killed pathogens, such as fungal cell wall  $\beta$ -glucan. Moreover, persistent chronic inflammation in patients with CGD also leads to impairment of hematopoietic stem cell functions [8].

To overcome these limitations, we aimed to develop a novel enzyme replacement therapy with D-amino acid oxidase (DAO) for supplying H<sub>2</sub>O<sub>2</sub> *in vivo*. DAO is a flavo-enzyme that selectively catalyzes the

oxidative deamination of D-amino acids, leading to the generation of the corresponding imino acid and H<sub>2</sub>O<sub>2</sub>. We previously reported that DAO restores the bactericidal activity of ROS-deficient neutrophils by supplying H<sub>2</sub>O<sub>2</sub> *in vitro* [9]. The advantages of using fungal DAO are easy availability of this enzyme and more potent selective activity compared with that of mouse or human DAO. [10]. This phenomenon is called the enhanced permeability and retention effect [11].

In this study, a novel polyethylene glycol-conjugated *Fusarium spp.* DAO (PEG-fDAO) was characterized and evaluated for its *in vivo* anti-inflammatory activity in a lung inflammation model induced with nonviable *Candida albicans* (nCA) using gp91-phox knockout CGD mice. Lack of ROS production has been shown to result in high levels of proinflammatory mediators (IL-1b, tumor necrosis factor- $\alpha$ , and keratinocyte chemoattractant) through the inflammasome activation system (dectin-1 receptor) in neutrophils and macrophages [3]. In this model, viable fungi are not always a prerequisite for the development of inflammation in CGD mice [12].

Therefore, using this model, we propose that oxidase replacement therapy with the novel PEG-fDAO could be applicable in the treatment of inflammation *in vivo*.

## Materials And Methods

### Reagents

DAO isolated from *Fusarium spp.* (fDAO) was a kind gift from Ikeda Food Research Co., Ltd, Minami-ku Kumamoto, Japan. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and H<sub>2</sub>DCFDA (2',7'-dichlorodihydrofluorescein diacetate) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Molecular Probes (Eugene, OR, USA), respectively. H<sub>2</sub>O<sub>2</sub> and tert-butyl hydroperoxide (t-BuOOH) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). D-Phenylalanine, D-proline, and rhodamine B isothiocyanate were purchased from Wako Pure Chemical Industries, Ltd.

Succinimidyl-activated PEG (SUNBRIGHT MEGC-50HS-PEG), with an average molecular size of 5250 Da, was purchased from NOF Corp., Tokyo, Japan. All other reagents were of reagent grade and used without further purification.

### Animals

CGD mice (gp91-phox knockout) obtained from Dr. Mary C. Dinauer [13] were backcrossed at least 12 times with C57BL/6 mice to ensure similar genetic backgrounds. Experiments on 8–12-week-old CGD C57BL/6 mice were performed according to the guidelines of the Laboratory Protocol of Animal Handling, Sojo University Faculty of Pharmaceutical Sciences. All animals were housed under specific pathogen-free conditions.

## Preparation of nCA

nCA was kindly gifted by Dr. Ohno [14]. Briefly, a C-limiting medium was used to grow *Candida albicans* and cultured at 27 °C with aeration. Viable cells were collected by centrifugation, killed with ethanol, and dried with acetone. The resulting nCA was suspended in phosphate-buffered saline (PBS).

## Preparation of PEG-fDAO

PEGylation of fDAO was conducted as described previously [15]. In brief, to the fDAO solution (2.0 mg/mL protein in 0.1 M sodium bicarbonate), succinimide-activated PEG was added at a 3-fold molar excess of PEG to free the amino groups in fDAO and allowed to react for 1 hour at 4 °C. The reaction mixture containing PEG-fDAO was purified to remove free PEG and other low-molecular-weight reactants by ultrafiltration with a YM-10 membrane (Merck, Darmstadt, Germany) using 0.1 M sodium bicarbonate aqueous solution. PEG-fDAO was stored at -80 °C until further use.

## Evaluation of the enzymatic activities of fDAO and PEG-fDAO

The enzymatic activity of fDAO and PEG-fDAO were determined by a horseradish peroxidase-coupled colorimetric assay with o-dianisidine as the substrate. In this assay, the substrate was reduced, and a color developed with a maximal absorption of 460 nm. D-alanine was used as the substrate for fDAO at a final concentration of 10 mM. The enzymatic reaction was performed at 25 °C in 0.1 M Tris-HCl buffer (pH 8.2), where 1 U of fDAO activity is defined as the rate of formation of 1 μmol of H<sub>2</sub>O<sub>2</sub> per minute. The maximal rate of activity (V<sub>max</sub>) and K<sub>m</sub> for each D-amino acid were calculated by curve fitting of the non-linear plot of reaction rate versus substrate concentration using the gnu plot software.

## Analysis of *in vivo* pharmacokinetics of fDAO and PEG-fDAO using the plasma

For the *in vivo* pharmacokinetics analysis, fDAO or PEG-fDAO (20 U/mL, 0.1 mL/mouse, n = 3) was injected intravenously into C57BL/6 (BALB/cAjcl) mice. Blood was withdrawn from the medial canthus of the eye using a microhematocrit at 0.5, 4, 24, 48, and 72 hour after fDAO or PEG-fDAO administration. Each blood sample was centrifuged and the plasma was obtained in an ice-cold buffer (100 mM Tris-HCl, pH 8.0) containing a mixture of protease inhibitors (1mM PMSF, 10 μg/mL leupeptin, and 2.5 mM EDTA). The DAO activity in the plasma was determined based on the formation of pyruvic acid during the reaction between D-Alanine and DAO, as described previously [15].

## Induction of lung inflammation by nCA aspiration

Mice were anesthetized by intraperitoneal injection with 200 mg/kg of 2,2,2-tribromoethanol (Sigma-Aldrich), as described by Dr. Aratani. CGD mice were challenged with intranasal administration of  $10^7$  nCA cells in a volume of 30  $\mu$ L of PBS, and control mice were administered 30  $\mu$ L of PBS alone [12]. Since we had no information on the time course of *in vivo* production of H<sub>2</sub>O<sub>2</sub> induced by PEG-fDAO with D-amino acids in the nCA-induced lung inflammation model, a short, medium, and long treatment schedule was explored.

## Experiment-1 (Exp-1)

Mice were divided into two groups. The first group was administered nCA intranasally as the positive control (n = 5). The second group was administered nCA intranasally on day 0 of experiment and PEG-fDAO was injected through the tail vein on the second day, followed by intraperitoneal administration of D-phenylalanine (0.1 M, 0.5 mL/mouse) on the third, fourth, and fifth days (n = 5). Mice were sacrificed on the eighth day.

## Experiment-2 (Exp-2)

Mice were divided into three groups. The first group was administered PBS intranasally as the negative control (n = 3). The second group was administered nCA intranasally as the positive control (n = 3). The third group was administered nCA on day 0 and injected with PEG-fDAO (10 U/mL, 0.1 mL/mouse) (n = 3) through the tail vein on the fourth day, followed by intraperitoneal injection of D-phenylalanine (0.1 M, 0.5 mL/mouse) on the fifth, sixth, and seventh days instead of D-proline. Rhodamine-labeled bovine serum albumin (rhodamine-BSA) (10 mg/kg) was injected one day before sacrifice on the 14<sup>th</sup> day. Fluorescent imaging of the excised lungs was conducted using the IVIS Lumina XR (excitation: 555–585 emission: 695–770 nm; PerkinElmer Japan Co., Ltd., Kanagawa, Japan).

## Experiment-3 (Exp-3)

Mice were divided into three groups. The first group was administered PBS intranasally as the negative control (n = 3). The second group was administered nCA intranasally as the positive control (n = 3). The third group was administered nCA on day 0 and injected with PEG-fDAO (10 U/mL, 0.1 mL/mouse) (n = 3) on the ninth day, followed by intraperitoneal injection of D-proline (1 M, 0.5 mL/mouse) on the 10<sup>th</sup>, 11<sup>th</sup>, and 12<sup>th</sup> days. Rhodamine-BSA (10 mg/kg) was injected one day before sacrifice on the 21<sup>st</sup> day. Fluorescent imaging of the excised lungs was performed using the IVIS Lumina XR (PerkinElmer Japan Co., Ltd.).

## Analysis of the lung pathology

Mice were sacrificed on the eighth day in Exp-1, 14<sup>th</sup> day in Exp-2, or 21<sup>st</sup> day in Exp-3 after nCA administration. The lungs were removed and fixed in 10% buffered formalin. For light microscopy, tissues were fixed overnight, dehydrated in graded ethanol solutions, embedded in paraffin, sectioned at 2- $\mu$ m thickness, and stained with hematoxylin and eosin using standard protocols.

## Statistical analysis

Statistical analysis of the obtained data was performed using a two-tailed unpaired Student's *t*-test. Differences were considered statistically significant at  $p < 0.05$ .

## Results

### Physicochemical characterization of fDAO and PEG-fDAO

Characterization of PEG-fDAO is summarized as follows. The reaction between fDAO and succinimide-PEG resulted in five chain numbers of PEG conjugates on fDAO, which was determined by quantification of the primary amine. The fDAO formed a tetramer (165 kDa) in the physiological solution, and the molecular weight of PEG-fDAO was calculated to be 206 kDa. An increase in the molecular size of fDAO by PEGylation was also confirmed by SDS-PAGE and size exclusion chromatography (data not shown). Enzyme-specific activity of fDAO was  $26 \pm 1.3$  units/mg protein, and its enzyme activity was not altered by PEGylation (PEG-fDAO:  $26 \pm 0.53$  units/mg protein). The  $V_{max}$  and  $K_m$  values of fDAO for each D-type amino acid were almost similar with those of PEG-fDAO.  $V_{max}$  and  $K_m$  values of fDAO and PEG-fDAO towards neutral D-amino acids also showed similar trends at the optimal pH of 8.2, and an inflammatory environmental pH of 6.5 (Supplemental Table 1). The enzymatic activity of PEG-fDAO at pH 6.5 was lower than that at pH 8.2, for all substrates tested. Similar substrate specificity was observed at pH 6.5 and 8.2.

D-phenylalanine showed the highest  $V_{max}$  ( $V_{max} = 18.6 \mu\text{mol H}_2\text{O}_2/\text{min}$  at pH 6.5) and was active even at low substrate concentrations ( $K_m = 0.22 \text{ mM}$  at pH 6.5) among the D-amino acids tested. We employed D-proline in Exp-3 to compare the *in vitro* bactericidal activity<sup>1</sup> with the *in vivo* anti-inflammatory activity ( $V_{max} = 6.4$ ,  $K_m = 5.2$  at pH 6.5). In Exp-1 and Exp-2, D-phenylalanine was selected as the substrate to confirm PEG-fDAO activity *in vivo*.

### *In vivo* pharmacokinetics of fDAO and PEG-fDAO using the plasma

We employed a methodology to exclude denatured DAO proteins with lost enzymatic activity for measuring the enzymatic activity of DAO *in vivo* as in Figure 1. The apparent half-life ( $t_{1/2}$ ) of fDAO ( $n=3$ ) and PEG-fDAO ( $n=7$ ) are statistically  $7.69 \pm 1.36$ , hour,  $33.68 \pm 3.10$  hour, respectively, and T-Test with un-paired samples shows that p-value is 0.000000415. Since the  $t_{1/2}$  of PEG-fDAO appeared to be long

enough, we employed intraperitoneal injection of D-amino acid on three consecutive days post PEG- fDAO administration.

## ***In vivo* experimental findings**

In Exp-1 with an 8-day schedule, there was a marked mean body weight loss in the nCA group ( $-6.18 \pm 2.28$  g) compared with that in the PEG-fDAO experimental group ( $-4.14 \pm 2.74$  g), but the difference was not significant ( $p = 0.236$ ,  $n = 5$ ) (Figure 2A and 2B). The mean lung weight in the nCA group ( $0.562 \pm 0.04$  g) was significantly higher than that in the PEG-fDAO experimental group ( $0.314 \pm 0.095$  g) ( $p = 0.001$ ,  $n = 5$ ) (Figure 3A). Images of the excised lungs are shown in Supplemental Figure 1. Rhodamine-labeled fluorescent imaging was not performed in this experiment.

In Exp-2 with a 14-day schedule and D-phenylalanine substrate, the mean body weight loss in the nCA group ( $-1.6 \pm 0.7$  g) was lower than that in the PEG-fDAO group ( $-2.5 \pm 2.8$  g) ( $p = 0.618$ ,  $n = 3$ ) (Figure 2D and 2E). The mean lung weight in the nCA group ( $0.283 \pm 0.025$  g) was significantly higher than that in the PEG-fDAO group ( $0.236 \pm 0.005$  g) ( $p = 0.035$ ,  $n = 3$ ) (Figure 3B 2/3). Furthermore, the mean lung weight in the control group was  $0.24 \pm 0.01$  g, which was almost the same as that in the PEG-fDAO group ( $p = 0.64$ ,  $n = 3$ ) (Figure 3B 1/3). To determine the inflammatory region in the lung, rhodamine- BSA was intravenously injected, and accumulated rhodamine-BSA in the lung was quantified by measuring the fluorescence intensity. Rhodamine-labeled fluorescence intensity in the excised lungs of mice from the nCA group was brighter and the tissue was more swollen than that observed for the mice in the PEG-fDAO and control groups (Figure 4A). The pathological findings were different in the nCA group; pneumonia and focal accumulation of neutrophils reacting to and phagocytizing eosinophilic filaments were observed in the nCA group (Figure 5C/D), but these were milder in the lung tissues from mice in the PEG-fDAO group (Figure 5E/F).

In Exp-3 with a 21-day schedule, there was no marked difference in the mean body weight loss in the nCA group ( $-0.33 \pm 0.68$  g) compared with that in the PEG-fDAO group ( $-0.17 \pm 0.87$  g) ( $p = 0.66$ ,  $n = 3$ ) (Figure 2G and 2F). The mean lung weight in the nCA group ( $0.334 \pm 0.065$  g) was higher than that in the PEG-fDAO group ( $0.230 \pm 0.021$  g), but the difference was not significant ( $p = 0.058$ ,  $n = 3$ ) (Figure 3C). In the control group, the mean lung weight was  $0.156 \pm 0.020$  g. Rhodamine-labeled fluorescence intensity in the excised lungs from mice in the nCA group was brighter and the tissue was more swollen than that noted for mice in the PEG-fDAO and control groups (Figure 4B). The pathological findings were particularly different in the nCA group, showing pneumonia with accumulation of eosinophilic filament-phagocytizing neutrophils, accompanied by granulomatous accumulation of histiocytic cells and lymphoid cells. These pathological changes were apparently mild, with the eosinophilic filaments fragmented and smaller in the tissues from mice in the PEG-fDAO group (Figure 5K/L) compared with that found in mice from the nCA group (Figure 5I/J). Unexpectedly, a small amount of granuloma formation with giant cells was observed in the PEG-fDAO group.

## Discussion

In the present study, our pharmacokinetics analysis revealed that fDAO (165 kDa) was rapidly cleared from the circulation after intravenous infusion ( $t_{1/2} = 7.67 \pm 1.36$  hour ( $n = 3$ )), but PEG-fDAO (206 kDa) was rather stable ( $t_{1/2} = 33.68 \pm 3.10$  hour ( $n = 7$ )) (Fig. 1). Based on these findings, we employed PEG-fDAO administration, followed by administration of D-phenylalanine or D-proline for three days in the nCA-induced lung inflammation model to evaluate the anti-inflammatory effect of PEG-fDAO. According to studies on *Aspergillus* hyphae- [16] and sterile nCA- [12] induced lung inflammation models, sterile fungal cell wall (branched ( $\beta$ -1,3) ( $\beta$ -1,6) glucan)-induced inflammation model with gp91<sup>-/-</sup> CGD mice [17] and zymosan (the same fungal cell wall-derived product)-induced lung inflammation

model with p47<sup>-/-</sup> [18] and p91<sup>-/-</sup> [19] CGD mice, the MAPK signaling pathway (ERK1/2 and NF- $\kappa$ B) involving toll-like receptor 2, dectin-1 receptor and cytokines (IL-1b, tumor necrosis factor- $\alpha$ , IL-17, keratinocyte chemoattractant, and granulocyte colony-stimulating factor) and leukotriene B4 [19] are activated on the second day or earlier post stimulation. The net balance of cytokine and leukotriene B4 expression in the lungs of CGD mice in response to the fungal cell wall component is prone to be in a proinflammatory state, which could promote a continued inflammatory response [16, 19]. Based on these previous findings, we administered PEG-fDAO on the second day, followed by an intraperitoneal injection of D-phenylalanine for three days in Exp-1. Endo et al [12] reported that nCA-treated CGD mice show prominent airway accumulation of inflammatory cells at 3 and 6 days post nCA administration.

Similarly, the nCA-treated CGD mice in Exp-1 showed a prominent loss of body weight and gain of the lung weight. In contrast, nCA-treated CGD mice injected with PEG-fDAO and D-phenylalanine showed a significantly lower loss of body weight and gain of the lung weight (Figs. 2A/B and Fig. 3A) compared with that observed in the control group. These differences were also apparent from a physiological point of view.

Next, to determine the effect of PEG-fDAO over a longer period, as in the *Aspergillus* hyphae-induced lung inflammation model [16], PEG-fDAO was administered on the fourth day in Exp-2 with a 14-day schedule, and the ninth day in Exp-3 with a 21-day schedule. We found the body and lung weight to tend to recover over the experimental time course. In Exp-2, the loss of body weight in the nCA group was approximately the same as that in the PEG-fDAO group ( $p = 0.62$ ) (Fig. 2D/E), but the lung weight gain in the nCA group was significantly higher ( $p = 0.035$ ) than that in the PEG-fDAO group (Fig. 3B). Furthermore, the lung pathology in the nCA group appeared to be stronger than that in the PEG-fDAO group (Fig. 5C/D vs 5E/F). In Exp-3, the loss of body weight in the nCA group was higher than that in the PEG-fDAO group, but the difference was not significant ( $p = 0.66$ ) (Fig. 2G/H). The lung weight gain in the nCA group was not significantly high ( $p = 0.058$ ), but it was higher than that in the PEG-fDAO group (Fig. 3C; 2 vs 3). Although PEG-fDAO seemed effective in ameliorating the physiological changes induced by nCA stimulation, we could not confirm if this effect was significant through these evaluations.

Consistent with a previous study by Dinauer [16], which had a 21-day experimental schedule with intratracheal administration of *Aspergillus fumigatus* hyphae to gp91<sup>-/-</sup> mice, many granulomatous formations in our Exp-3 were observed in the pathological lung specimens from mice in the nCA group (Fig. 5I/J) but a few in the those from mice in the PEG-fDAO group (Fig. 5K/L). Granulomatous formations were not apparent in Exp-2, but a few macrophage accumulations around the eosinophilic *Candida* body were found in the pathological lung specimens from mice in the nCA group (Fig. 5C/D

and not in those from mice in the PEG-fDAO group (Fig. 5E/F). In addition, the lung pathology in the nCA group (Fig. 5I/J) was stronger than that observed in the PEG-fDAO group in Exp-3 (Fig. 5K/L). These pathological findings also support the idea that PEG-fDAO administration, followed by D-amino acid treatment, would ameliorate the pathological changes induced by nCA stimulation.

According to the characterization of PEG-fDAO, the  $K_m$  value of D-phenylalanine was approximately 24–40 times lower than that of D-proline, but the blood concentration of D-phenylalanine or D-proline was supposed to be 2–3 times higher than the  $K_m$  value of both D-amino acids. In addition, the  $V_{max}$  of PEG-fDAO for D-phenylalanine was approximately three times higher than that for D-proline (Supplemental Table 1). Since D-phenylalanine has lower solubility than D-proline in aqueous media, bolus injectable D-phenylalanine was 10-fold lower than D-proline (D-phenylalanine: 0.1 M, 0.5 mL/mouse (Exp-1, 2) and D-proline: 1 M, 0.5 mL/mouse (Exp-3)). Therefore, although PEG-fDAO activity was higher towards D-phenylalanine than D-proline, a low dose of D-phenylalanine might result in a therapeutic efficacy comparable to that of D-proline in alleviating lung inflammation. However, more stable conditions and protocols are necessary to confirm the apparent effects of PEG-fDAO observed in this model and develop clinical applications.

Therefore, in these series of experiments, PEG-fDAO administration followed by D-amino acid injection was apparently useful for the treatment of nCA-induced lung inflammation, especially in Exp-1, but also in Exp-2 and Exp-3. Considering the mechanism of the nCA-induced pneumonia model, H<sub>2</sub>O<sub>2</sub> seems to be involved in many inflammatory processes. As Endo et al [12] reported in their 3-day pneumonia model, a low amount of MAPKs in CGD mice may lead to prolonged phosphorylation of ERK1/2. Segal et al [18] showed impaired Nrf2 activity and increased NF- $\kappa$ B activation in zymosan- treated mononuclear cells from X-linked CGD patients. Furthermore, many cytokines are thought to be involved in granuloma formation with giant cells [20] wherein H<sub>2</sub>O<sub>2</sub> is involved. As neutrophil-derived ROS, the targeted delivery of PEG-fDAO could also restore the anti-inflammatory responses by supplying H<sub>2</sub>O<sub>2</sub> to the site of inflammation. Setoguchi et al [21] also reported the therapeutic value of pulmonary granuloma by controlling nitric oxide and ROS.

In conclusion, we prepared PEG-fDAO, characterized its enzymatic properties, and evaluated its anti-inflammatory effect on gp91-phox knockout CGD mice. The features of PEG-fDAO were comparable to those of pDAO in terms of  $V_{max}$  and substrate specificity, but PEG-fDAO was more stable than PEG-pDAO. Moreover, PEG-fDAO exhibited anti-inflammatory effects in CGD mice with nCA-induced lung

inflammation. Nonetheless, a protocol with a more optimized regimen will be necessary to confirm the anti-inflammatory effects of PEG-fDAO.

## Declarations

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## Conflicts of interest/Competing interests

The authors declare no competing financial interests.

## Availability of data and material

For original data, please contact [hnunoi@med.miyazaki-u.ac.jp](mailto:hnunoi@med.miyazaki-u.ac.jp).

## Code availability

Manuscript was written by MacBook Pro (13-inch,2016) with Microsoft R word for Mac (version 16.44), and Microsoft power point for Mac (version 16.44).

## Authors' contributions

Hiroshi Maeda proposed basic concept of PEGylated DAO for the treatment of CGD. Makoto Matsukura and Hiroyuki Nunoi design the research. Hideaki Nakamura prepared materials especially PEG-fDAO. Yasuaki Aratani performed the first experiment with the nCA-induced lung inflammation model. Peiyu Xie, Jun Fang and collaborators performed the other experiments. Peiyu Xie did equal contribution for this work with Hiroyuki Nunoi. Analysis of *in vivo* pharmacokinetics of fDAO and PEG- fDAO using the plasma were performed by Makoto Matsukura and his coworkers. Hiroaki Kataoka conducted pathological

evaluation. Hiroyuki Nuno, Taiki Nisimura and Makoto Matsukura analyzed the data and wrote the first draft of the manuscript. All authors commented on the manuscript and approved it.

## Ethics approval

Experiments on 8–12-week-old CGD C57BL/6 mice were performed according to the guidelines of the Laboratory Protocol of Animal Handling, Sojo University Faculty of Pharmaceutical Sciences. All animals were housed under specific pathogen-free conditions.

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## Figures

### Figure 1

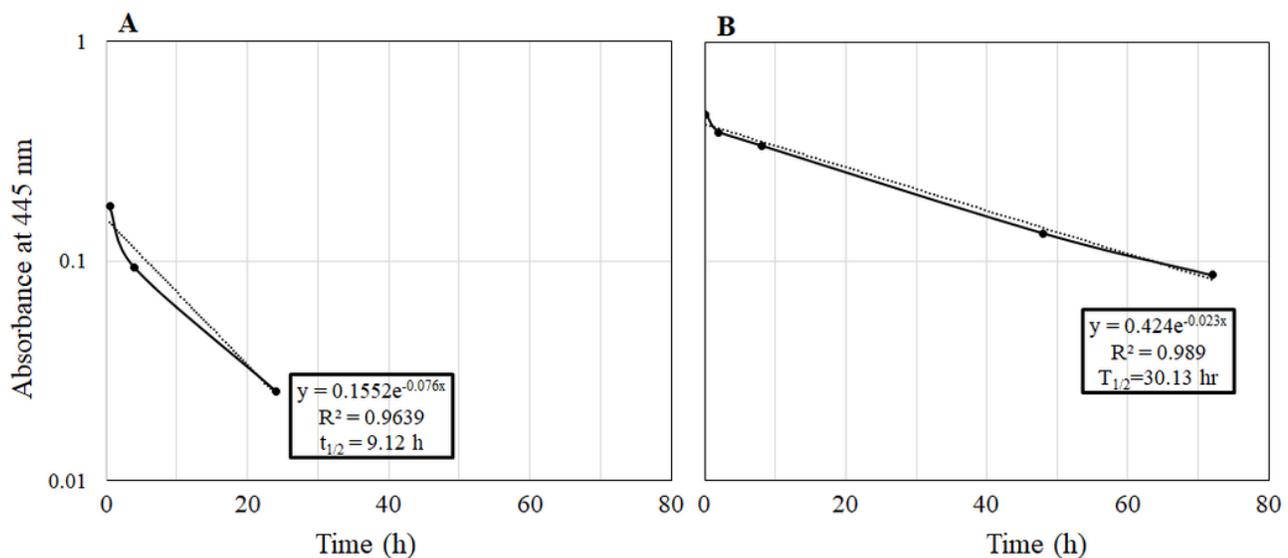
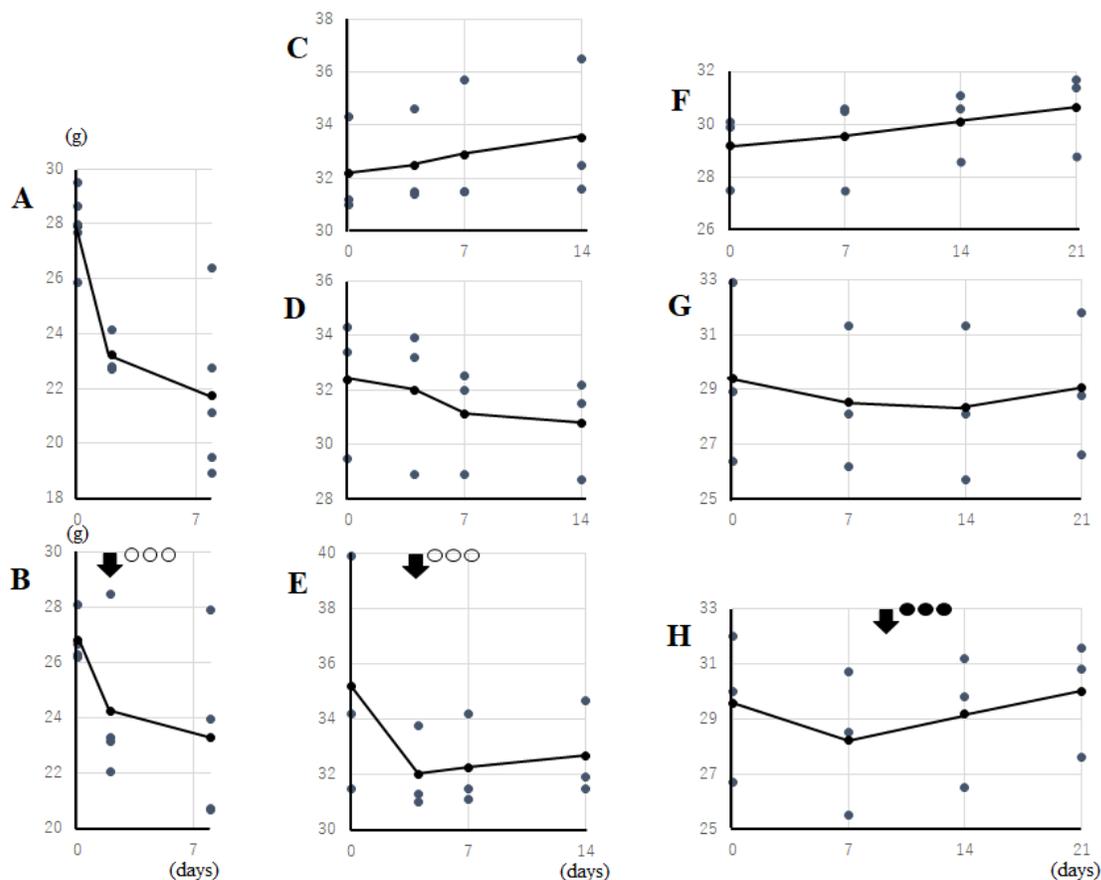


Figure 1

In vivo pharmacokinetics of fDAO and PEG-fDAO by enzymatic activities using the plasma. The y-axis shows the log of the absorbance at 445 nm, which denotes the production of pyruvic acid from D-alanine by D-amino acid oxidase. In this figure, two typical sets of exponential curve fitting analysis along with the mathematical formula are shown for fDAO (Figure 1A) and PEG-fDAO (Figure 1B) (Microsoft ExcelR). The mathematical calculation for biological half-life ( $x = t_{1/2}$ ) is also shown in the figure. R2 is R squared, which is coefficient of determination that is predictable from the independent variable

## Figure 2



## Figure 2

See the Supplemental Files section for the complete figure caption

# Figure 3

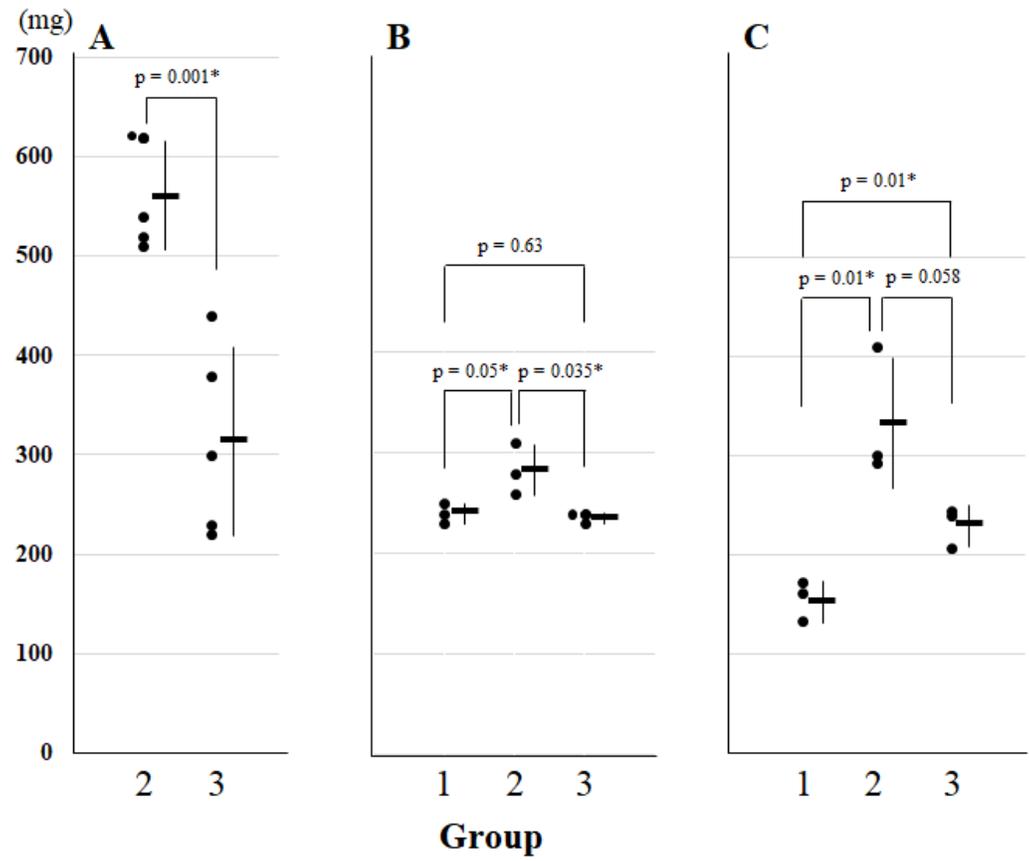
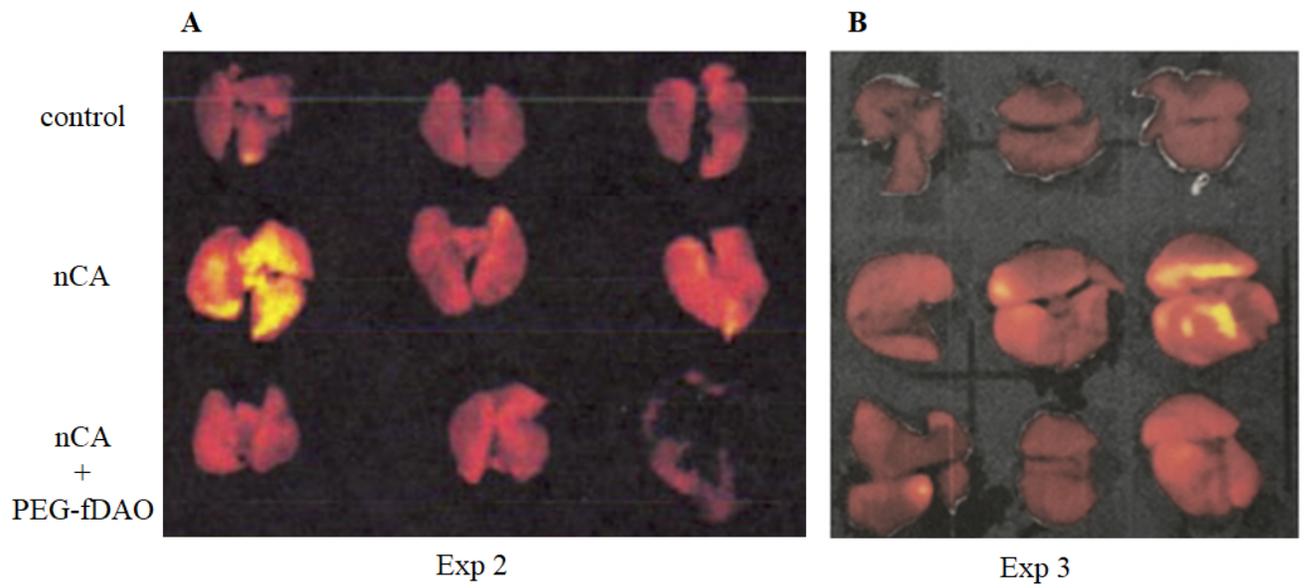


Figure 3

See the Supplemental Files section for the complete figure caption

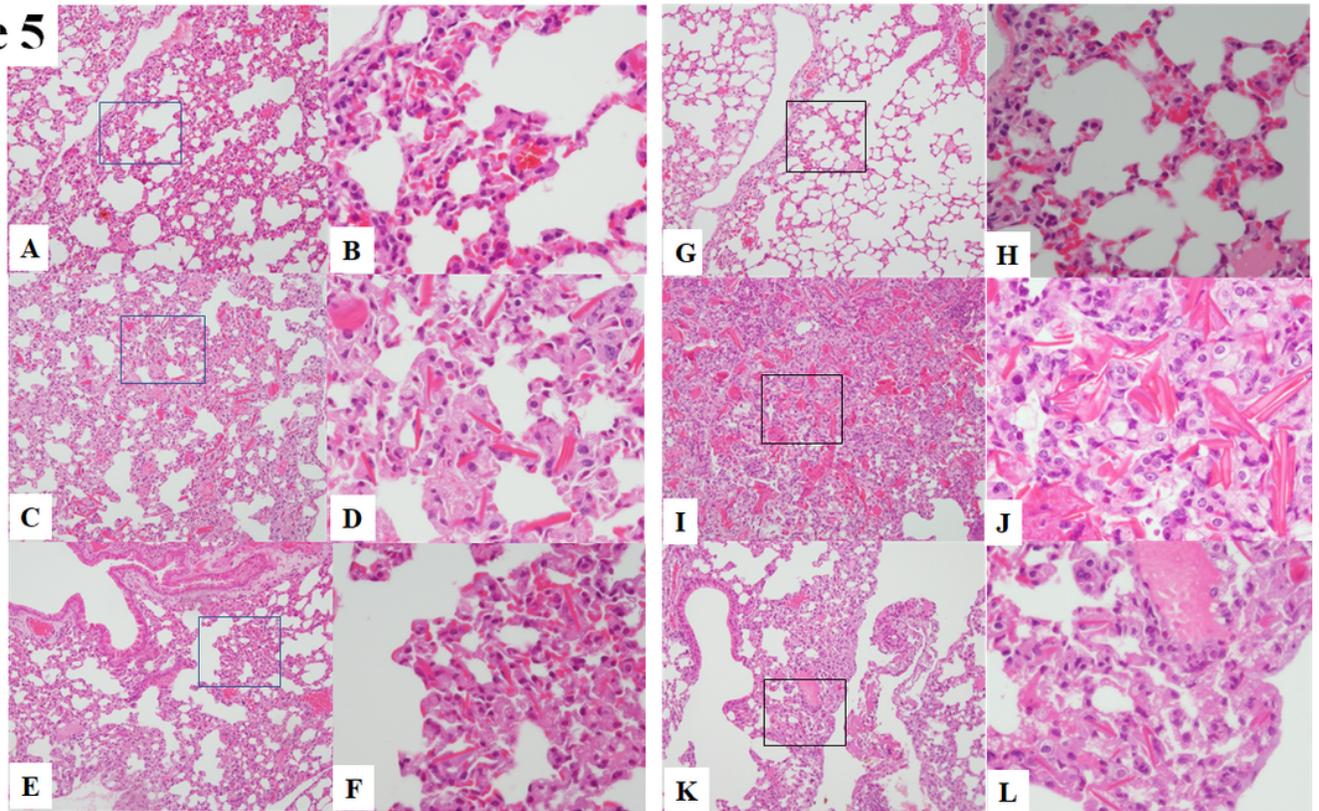
# Figure 4



## Figure 4

Fluorescence imaging of the lung tissues during each experiment. The lung tissues of mice from the control, nCA, and nCA+PEG-fDAO groups in Exp-2 (A) and Exp-3 (B) are shown.

**Figure 5**



**Figure 5**

Pathological findings of the lung tissues in Exp-2 and Exp-3. Lung pathologies in Exp-2 (Figure 5A-F) and Exp-3 (Figure 5G-L) are shown, where A/B and G/H, C/D and I/J, E/F and K/L indicate the control, nCA and PEG-fDAO group, respectively. A/C/E/G/I/K and B/D/F/H/J/L show original magnifications of  $\times 100$  and  $\times 400$ , respectively. The boxed areas are amplified in the right panels. The nCA group showed pneumonia with many eosinophilic filaments and neutrophils phagocytizing the filaments in Exp-2 (Figure 5C/D) and Exp-3 (Figure 5I/J). In addition, a somewhat granulomatous appearance showing a mixed accumulation of neutrophils, macrophages, and lymphoid cells was noted in Exp-3 (Figure 5I/J). The inflammatory changes were milder in the PEG-fDAO group (Figure 5E/F/K/L) than in the nCA group (Figure 5C/D/I/J), showing thin eosinophilic filament-phagocytizing neutrophils in both Exp-2 and Exp-3.

## Supplementary Files

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