JPET Fast Forward. Published on September 19, 2008 as DOI: 10.1124/jpet.108.140848

JPET #140848

Comparison of cigarette smoke-induced acute inflammation in multiple strains of mice and the effect of an MMP inhibitor on these responses

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JPET #140848

Running title: Smoking-induced acute inflammation in four mouse strains

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Number of pages: 34

Number of tables: 0

Number of figures: 13

Number of references: 20

Number of words in abstract: 250

Number of words in introduction: 732

Number of words in discussion: 1496

Abbreviations:

COPD Chronic obstructive pulmonary disease

MMP Matrix metalloproteinase

CS Cigarette smoke

CSE Cigarette smoke exposure

BAL Bronchoalveolar lavage

p.o. Per os (by mouth)

i.n. Intranasal

b.i.d Bis in die (twice a day)

Section Assignment: Inflammation, Immunopharmacology, and Asthma

Abstract

The activities of proteases in the lung, specifically matrix metalloproteinases (MMPs), have been implicated in driving the inflammation and lung destruction observed in smokers with COPD. Here, our aims were to compare the acute response to cigarette smoke exposure (CSE) in 4 mouse strains to identify common and distinguishing features and to assess the effect of a MMP inhibitor on this response. To do this we exposed mice (BALB/C, C57BL/6, A/J, or 129/Sv) to whole-body CSE (1h/d) for 3d. CSE induced dose- and time-dependent increases in neutrophils and KC levels in the airways of all strains; however the proportion of the neutrophilia differed amongst strains. In the two most contrasting strains, BALB/C and C57BL/6, we examined MMP gene expression and found only small changes apart from MMP-12, which was highly expressed in both strains. Both strains were then treated with a broad spectrum MMP inhibitor, PKF242-484 (0.5-10 mg/kg) either per os (p.o.) or intranasally (i.n.) 1h before and 5h after CSE for 3d. PKF242-484 dose-dependently reduced neutrophilia in BALB/C mice when dosed p.o. ($p<0.01$) or i.n. ($p<0.01$), but had no clear effect in C57BL/6 by either route. PKF242-484 reduced BAL macrophages when dosed i.n. ($p<0.05$), but had no dose-dependent effect when dosed p.o. in both strains. These data suggests the inflammation induced by CSE is similar, but not identical, in different mouse strains. Additionally, the ability of broad spectrum MMP inhibitors to inhibit smoke-induced acute neutrophil inflammation is strain-dependent, while its ability to limit macrophage infiltration may be route dependent.

Introduction

Chronic obstructive pulmonary disease (COPD) is a smoking-related syndrome that is currently the 5th leading cause of death worldwide and is projected to become the 3rd leading cause of death by the year 2020. COPD has been defined as "airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious gases and particles" (Pauwels et al., 2001). Cigarette smoke (CS) elicits a repetitive inflammatory insult that is believed to, through the actions of mediators such as proteinases, lead to structural and functional changes in the lung (i.e., emphysema, airway remodeling and airflow obstruction). Current anti-inflammatory approaches (e.g., steroids) have not been successful in treating this condition, suggesting that there is something unusual about the inflammatory response to CS exposure (CSE) that, at present, is not understood.

As CS is the principle etiological factor for developing COPD (responsible for > 80% of all cases), it has been used to develop preclinical *in vivo* models to investigate prospective disease mechanisms and evaluate the efficacy of candidate compounds. Most investigators have focused on using models of chronic CSE in mice. Some of these studies have demonstrated that there are significant differences in the responses amongst the different strains of mice (Triantafillopoulos et al., 2004; Cavarra et al., 2001). Understanding these differences may provide insights into potential (genetic) factors that influence COPD susceptibility (only ~25% of smokers are believed to develop COPD); however, understanding the common features of this response may also help us identify central mechanisms underlying the inflammatory response induced by CSE. Further, identifying compounds that inhibit CSE-induced inflammation in multiple strains implies a greater probability for it working in other

species, such as man. Two groups have characterized the changes which occur after short-term (< 1 week) CSE in multiple strains of mice (Vlahos et al., 2006; Yao et al., 2008). These studies have demonstrated that there are some differences in the degree of the response amongst the strains; however, the mechanistic consequences of these differences have not been investigated.

Therefore, we had two aims for the studies reported here. First, we intended to characterize the kinetics of CSE-induced acute inflammation in multiple strains of mice (which had not previously been done) to identify common and distinct features of the response amongst the different strains. The strains chosen for these studies are commonly used in other models of lung inflammation and were a mix of those studied previously (Vlahos et al., 2006; Yao et al., 2008). The second aim was to determine whether strains which differed in their response to cigarette smoke responded differently to pharmacological intervention. We specifically focused on one mechanism that has been shown to be important to both the acute and chronic response to CSE – the activity of matrix metalloproteinases (MMPs). MMPs are believed to drive the inflammation and pathological changes associated with CSE through their ability to activate latent cytokines and degrade components of the lung matrix (Churg et al., 2003; Churg et al., 2002; Houghton et al., 2006). MMP-9 and MMP-12, in particular, have been implicated in COPD as the expression of these proteinases are elevated in the lungs of COPD patients and appear to localize to areas of the lung that are diseased (Segura-Valdez et al., 2000; Grumelli et al., 2004). In mice, MMP-12 has been shown to be important for mediating CSE-induced acute inflammation by activating latent, membrane-bound TNF-α (Churg et al., 2003) and generating chemotactic matrix fragments (Houghton et al., 2006; Churg et al., 2002). In addition, mice deficient for MMP-12 have less inflammation and appear to be

completely protected from developing emphysema after chronic CSE (Hautamaki et al., 1997). These data suggest that MMP-12 is central to the response to CSE in mice; however, most of these studies were conducted in a single strain. Thus, we assessed the anti-inflammatory efficacy of a broad spectrum MMP inhibitor, PKF242-484 (Trifilieff et al., 2002), in a mouse model of short-term CSE-induced acute inflammation using the two strains whose responses differed most in this system.

In the data reported here, we demonstrate that the principle acute response to CSE shared by each strain is an increase in airway neutrophilia. There are, however, some disparities in the degree of this response amongst the strains and we also show that PKF242-484 has different anti-inflammatory effects in the two strains in which it was tested.

Methods

Materials. BALB/C and C57BL/6 mice were obtained from Charles River UK. A/J and 129/Sv mice were obtained from Harlan UK. PKF242-484 [(2S,3R)-N4-((S)-2,2-Dimethyl-1-methylcarbamoyl-propyl)-N1-hydroxy-2-hydroxymethyl-3-(4-methoxy-phenyl)-succinamide] (Trifilieff et al., 2002) was made in-house (Novartis Institutes for BioMedical Research, Basel, Switzerland). University of Kentucky Research Cigarettes (brand 1R3F) were obtained from the University of Kentucky (Louisville, KY, USA). All ELISAs were purchased from R&D Systems UK.

Animal Maintenance Conditions. Female (BALB/C, C57BL/6, 129/Sv, A/J) mice (16-20 g) were housed in rooms maintained at constant temperature (21 ± 2°C) and humidity (55 ± 15%) with a 12 h light cycle and 15 - 20 air changes per h. Ten animals were housed per cage containing 2 nest packs filled with grade 6 sawdust (Datesand, Manchester, UK), nesting material (Enviro-Dri, Lillico, UK), maxi fun tunnels and Aspen chew blocks (Lillico, UK) to provide environmental enrichment. Animals were allowed food, RM1 Pellets, (SDS UK Ltd.) and water ad libitum.

Statement on Animal Welfare. Studies described herein were performed under a Project License issued by the United Kingdom Home Office and protocols were approved by the Local Ethical Review Process at Novartis Institutes for BioMedical Research, Horsham.

Cigarette smoke exposure methodology. CSE was performed as described by Stevenson and colleagues (Stevenson et al., 2005). Briefly, CS was delivered in 40 mL puffs of smoke every 60 seconds with fresh air being pumped in for the remaining time. The smoke was generated using 1R3F Research Cigarettes (University of Kentucky, Louisville, KY) and was drawn into the chambers via a peristaltic pump.

This exposure regimen generated 481 mg/m3 of total suspended particulate mass in the exposure chambers. This was measured by dividing the difference of the mass of exposed and unexposed Whatman glass microfibre filters by the volume of air sampled from the chambers through the filter beginning at the 12th minute of the exposure period for a total of 3 minutes. Sham, age- and sex-matched control animals were exposed to air only in the same manner for 50 minutes, which was the same duration of exposure for animals exposed to 5 cigarettes (i.e., approximately 10 minutes per cigarette used during the exposure period). This regimen was repeated for 3 consecutive days.

CSE dose-response studies. Female mice from 4 strains (BALB/C, C57BL/6, 129/Sv, and A/J) were exposed to 2, 3, 4 or 5 cigarettes per exposure period and sham controls were exposed to room-air only as described above. Animals were sacrificed with an overdose of terminal anesthetic (sodium pentobarbitone 200 mg i.p.) followed by exsanguination 24 hours after the last exposure.

CSE-induced inflammation time course studies. Female mice from 4 strains (BALB/C, C57BL/6, 129/Sv, and A/J) were exposed to 5 cigarettes per exposure period and sham controls were exposed to room-air only as described above. Animals were sacrificed with an overdose of terminal anesthetic (sodium pentobarbitone 200 mg i.p.) followed by exsanguination 3, 6, 24, 48, and 72 hours after the last exposure. There were sham time-matched controls for each time point.

Evaluating the effect of PKF242-484 on CSE-induced inflammation. Animals (female, BALB/C or C57BL/6) were dosed with either vehicle or compound, PKF242-484 (0.5 - 10 mg/kg), 1 hour before and 5 hours after each sham or smoke exposure (5 cigarettes for BALB/C and 4 cigarettes for C57BL/6 per exposure period). After the preliminary dose-response and time course studies, a small number

of C57/BL6 mice began to show some signs of distress during the exposure period in subsequent studies; therefore, we chose to reduce the duration of the exposures (to 40 minutes) for both smoke- and sham-exposed C57/BL6 mice during the compound studies to ensure their exposure level was not too high. Two routes of administration were evaluated, intranasal (i.n.) and per os (p.o.). For i.n. studies, animals were treated with 0.025 mL of compound or vehicle (PBS) under isofluorane/oxygen/nitrous oxide anesthesia. For p.o. studies, animals were administered 0.2 mL of either compound or vehicle (PEG 300). There was one exception – the study where C57/BL6 mice were dosed with 0.5, 1 and 5 mg/kg PKF242-484 – where the dosing volume for compound and vehicle was 0.1 mL.

Preparation of bronchoalveolar lavage (BAL) fluid and lung tissue leukocytes. Following exsanguination, the lungs were lavaged using a cannula inserted into the trachea and instilling the lungs with 4 x 0.3 mL aliquots of sterile PBS. All aliquots were combined for individual mice. Cytospins were prepared for differential staining. The cells were then spun down (300*g)* and resuspended in 0.5 mL methyl violet fixative to perform total cell counts using a hemocytometer. Differential cell counts were performed using standard morphological criteria on Hema-Gurr stained cytospins (300 cells/sample) (Merck, Poole, UK). Leukocyte numbers were determined by multiplying the percentage of each leukocyte subpopulation with the total number of cells for each sample and expressed as cells/mL for BAL cells.

BAL fluid and tissue cytokine analysis. After collection, aliquots of cell-free BAL fluid and lung tissue were frozen in liquid N_2 and stored at -80°C. Tissues were further processed prior to cytokine analysis by homogenizing approximately 250 mg of tissue in 2 mL of ice-cold homogenization buffer (saline with 1 mg/mL protease inhibitor cocktail [Roche]). Homogenates were spun at 800*g* and the supernatants

along with cell-free BAL fluid were analyzed for cytokine levels by enzyme-linked immunosorbent assay (ELISA). KC (mouse GRO-α homologue) was measured using ELISA Duo-Sets from R&D Systems (Abingdon, UK). Tissue homogenate supernatant protein levels were measured using the Bio-Rad Protein assay (Bio-Rad Laboratories, Hertfordshire, UK) and cytokine values were normalized against protein levels for individual homogenate samples. Bronchoalveolar lavage fluid protein levels were below the level of detection of the assay.

Examining CSE-induced MMP gene expression in lung tissue. Total RNA was isolated from ~25mg of lung tissue using the RNeasy mini RNA isolation kit (QIAGEN, Crawley, UK) according to the manufacturer's instructions. First strand cDNA was prepared from total RNA using the reagents and protocol provided in the first strand cDNA synthesis kit (Roche Molecular Biochemicals, Lewes, UK). For quantitative PCR (TaqMan Analysis), messenger RNA levels in total RNA samples were measured by TaqMan RT-PCR. Inventoried TaqMan gene expression assays for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), MMP-9, MMP-12, MMP-8, MMP-2, Tissue inhibitor of metalloprotease 1 (TIMP1) and TIMP2 were purchased from Applied Biosystems (Foster City, CA). Quantitative RT-PCR reactions were performed in triplicate in 20 µl final volumes and contained final concentrations 1× TaqMan Universal PCR master mix with 10 ng of the target cDNA preparation in each reaction. Experiments were performed using an ABI PRISM 7900 sequence detector (Applied Biosystems) and analyzed using ABI PRISM 7900 Sequence Detection System software. Amplification conditions were as follows: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The data were quantitated by extrapolation from the standard curve, normalized to GAPDH, and the mean ± S.E.M. was plotted.

Statistical Analysis. All data are presented as Mean ± Standard Error of Mean (SEM). For CSE dose-response studies, a Kruskal-Wallis test with a post-hoc Dunn's multiple comparison test was used. For time course studies, a Student's t-test was used comparing all smoke-exposed animals to their corresponding time-matched sham-exposed controls. For the compound dose-response studies, a one-way ANOVA with Dunnett correction for multiple comparisons was used. A P value of less than 0.05 was considered significant.

Results

CSE-induced changes in BAL inflammatory cell numbers. CSE-induced a predominantly neutrophilic inflammation in the lungs of all 4 strains of mice in a dose-dependent fashion (figures 1A-D). The greatest numbers of infiltrating neutrophils were recovered in the BAL fluid of BALB/C mice (figure 1A) ($p < 0.01$). There were similar dose-dependent changes, but slightly fewer neutrophils recovered in the BAL of A/J mice (figure 1C). The fewest numbers of neutrophils were recovered in the BAL of 129/Sv mice (figure 1D) and C57BL/6 mice (figure 1B). There were no changes in macrophages and lymphocytes recovered in the lavage fluid in any strain (data not shown). Proportionally, the BALB/C mice had the greatest percentage of neutrophils in the BAL fluid (60 $\pm$ 3% after 5 cigarettes) and C57BL/6 had the lowest (27 $\pm$ 3%).

CSE-induced changes in BAL neutrophil chemokine levels. CSE also increased the neutrophil chemokine, KC (murine GRO-α homologue) in a dose-dependent fashion in the BAL fluid of all 4 strains (figures 2A-D). The greatest levels achieved were in 129/Sv mice in the group exposed to 4 cigarettes ($p < 0.001$) (figure 2D). However, this was the only dose in 129/Sv mice to have increased levels of KC in the BAL. BALB/C and A/J mice (figure 2A & C, respectively) had increases in BAL KC levels in the groups exposed to 3, 4 and 5 cigarettes. Again, the overall lowest levels measured were in C57BL/6 mice (figure 2B).

CSE-induced changes in lung tissue neutrophil chemokine levels. CSE also increased KC levels in the lung tissue in a dose-dependent fashion in all 4 strains of mice (figure 3A-D). Similar levels measured were in BALB/C and 129/Sv mice in the groups exposed to 4 cigarettes ($p < 0.05$ for BALB/C and $p < 0.001$ for 129/Sv) (figures 3A

& D, respectively). The levels in BALB/C were only marginally less than the A/J and 129/Sv groups exposed to 5 cigarettes. Again, the lowest levels measured were in C57BL/6 mice (figure 3C).

Time-dependent changes in lung KC levels after 3 days of CSE. As 5 cigarettes elicited the greatest and most consistent response across all 4 strains, it was the dose of CS chosen for studies investigating the kinetics of the inflammatory response. The levels of KC in the lung tissue were increased at maximum levels at the earliest time point examined in BALB/C and A/J mice (figures 4A & C, respectively). In 129/Sv mice the levels were increased at 3 hours and peaked 24 hours after the final exposure (figure 4D). The maximum concentrations of KC measured were similar in BALB/C, A/J, and 129/Sv mice and levels began to subside after 24 hours, returning to control levels by 72 hours. The levels in C57BL/6 mice followed a similar pattern as the 129/Sv mice, but failed to reach significance at any time point examined (figure 4B).

Time-dependent changes in BAL fluid KC levels after 3 days of CSE. Levels of KC appeared to peak slightly later in the BAL fluid than they did in the lung tissue. In BALB/C mice, levels of KC in the BAL fluid were increased at 3 hours ($p < 0.01$) and reached maximum levels 6 hours ($p < 0.001$) after the final exposure (figure 5A). In the 3 other strains, the levels were increased at 3 hours and peaked 24 hours after the final exposure (figures 5B-D). The levels began to subside after 24 hours, returning to control levels by 72 hours in all four strains. The maximum concentration of KC measured was greatest in BALB/C and lowest in C57BL/6.

Time-dependent changes in BAL neutrophil numbers after 3 days of CSE. The number of neutrophils recovered in the BAL fluid appeared to follow and peak slightly later than the neutrophil chemokines. In all four strains of mice, the number of neutrophils recovered in the BAL fluid was increased at 3 hours and reached

maximum levels 24 hours after the final exposure (figures 6A-D). The levels began to subside after 24 hours, but stayed elevated in BALB/C and C57BL/6 mice up to 72 hours after the final exposure (figures 6A & B, respectively). They returned to control levels by 72 hours in A/J and 129/Sv mice (figures 6C & D, respectively). The greatest number of neutrophils recovered was in the BAL fluid of BALB/C and the lowest in C57BL/6 mice. Similarly, the BALB/C mice had the greatest overall percentage of neutrophils in the lavage fluid (63 $\pm$ 3%) and C57BL/6 mice had the lowest (13 $\pm$ 3%) 24 hours after CSE.

Time-dependent changes in BAL macrophage numbers after 3 days of CSE. In BALB/C ($P < 0.001$) and A/J ($p < 0.05$) mice, the number of macrophages recovered in the BAL fluid was only increased 72 hours after the final exposure (figures 7A and C, respectively). Interestingly, the number of macrophages recovered in the BAL fluid of C57BL/6 mice was increased at 3 and 6 hours after the final exposure ($p < 0.05$) (figure 7B). In addition, C57BL/6 mice had the highest proportion of macrophages in the BAL fluid 3 and 6 hours after the 3rd exposure to CS (84 $\pm$ 2% and 83 $\pm$ 3%, respectively), while BALB/C mice had the lowest (36 $\pm$ 4% and 38 $\pm$ 3%, respectively).

Time-dependent changes in BAL lymphocyte numbers after 3 days of CSE. The number of lymphocytes recovered in the BAL fluid of all 4 strains was much lower than the numbers of neutrophils and macrophages recovered (figures 6-8). In BALB/C, C57BL/6 and 129/Sv mice, the number of lymphocytes increased marginally at the earliest time point examined and was only significant in BALB/C mice ($p < 0.05$) (figures 8A, B & D, respectively). In BALB/C and C57BL/6, the number of lymphocytes recovered in the BAL fluid were increased 24 hours and remained elevated up to 72 hours after the final exposure (figures 8A & B,

respectively). Lymphocyte numbers were also elevated 48 and 72 hours after exposure in A/J mice (figures 8C). The number of lymphocytes recovered in the BAL of 129/Sv mice was variable and was not significant at any time point (figures 8D).

Time-dependent changes in matrix metalloproteinase expression in lung tissue after 3 days of CSE. Investigations into the expression of matrix metalloproteinases after an acute (3 day) exposure to cigarette smoke were conducted in BALB/C and C57BL/6 mice. BALB/C mice were chosen because the inflammatory changes consistently appeared to be the greatest in this strain. The reason for using C57BL/6 was that the inflammation induced by smoke in these mice appeared to be different to the other 3 strains – i.e., a greater component of the inflammation coming from macrophages in this strain versus neutrophils in the 3 other strains.

There were no consistent changes in the expression of MMP-9 in the lung tissue after 3 days of CSE, although there were small decreases in C57BL/6 mice at some time points (figures 9A & B). In general, the expression of MMP-9 was higher in C57BL/6 compared to BALB/c mice. MMP-12 was increased in both strains ($p < 0.001$) in a time-dependent fashion and to similar levels relative to GAPDH (figures 9C & D).

In general, the expression of MMP-2 was reduced in both strains after smoke ($p < 0.01$) (figures 10A & B), with the reduction appearing to be greater in BALB/C mice. In addition, there appeared to be greater expression of MMP-2 in BALB/C versus C57BL/6 mice. MMP-8 expression was increased at 3, 48 and 72 hours after exposure in C57BL/6 mice ($p < 0.05$) (figure 10C). In BALB/C mice, MMP-8 levels were only increased 3 hours after exposure ($p < 0.01$) (figure 10D), and subsequently returned to control levels.

CSE increased TIMP-1 expression in both C57BL/6 and BALB/C mice (figures 11A & B, respectively). TIMP-1 expression levels were greater and consistently up-regulated in the lungs of BALB/C mice between 3 ($p < 0.01$) and 48 ($p < 0.01$) hours after CSE. In general, the expression of TIMP-2 was reduced in both strains after CSE (figures 11C & D). The decrease in TIMP-2 expression was more consistent in BALB/C mice, with levels reduced 3, 6 and 48 hours after CSE.

Effect of twice-daily, prophylactic, oral administration of PKF242-484 on CSE-induced inflammation. In BALB/C mice, 3 days of CSE induced an increase in BAL neutrophil numbers ($p < 0.01$) (figure 12A). PKF242-484 dose-dependently reduced the number of neutrophils recovered in the lavage fluid. The greatest level of inhibition was achieved at the 10 mg/kg dose where there was a 46% reduction in BAL neutrophils ($p < 0.01$). There were no significant changes in macrophages except for an increase in the number recovered from the group dosed with 10 mg/kg PKF242-484 relative to sham vehicle controls (figure 12B).

In C57BL/6 mice, 3 days of CSE also increased BAL neutrophil numbers ($p < 0.01$) (figure 12C). PKF242-484 dosed in the same manner, but at slightly different doses, had no effect on neutrophil numbers in C57BL/6 mice. CSE did increase BAL macrophage numbers in C57BL/6 mice ($p < 0.01$) and PKF242-484 only reduced the numbers of macrophages recovered in the group receiving the lowest dose (0.5 mg/kg; $p < 0.05$) and trended towards increasing at higher doses (figure 12D).

Effect of twice-daily, prophylactic, intranasal administration of PKF242-484 on CSE-induced inflammation. In BALB/C mice, 3 days of CSE induced an increase in BAL neutrophil numbers ($p < 0.01$) (figure 13A). PKF242-484 dose-dependently reduced the number of neutrophils recovered in the lavage fluid. The greatest level of inhibition was achieved at the 10 mg/kg dose where there was a 62% reduction in

BAL neutrophils ($p < 0.01$). CSE trended toward increasing BAL macrophage numbers, but did not achieve significance ($p = 0.07$). PKF242-484 at 10 mg/kg did reduce in macrophage numbers compared to the CSE-vehicle control group ($p < 0.05$) (figure 13B).

In C57BL/6 mice, 3 days of CSE also increased BAL neutrophil numbers ($p < 0.01$) (figure 13C). PKF242-484 dosed i.n. had no dose-dependent effect on neutrophil numbers in C57BL/6 mice, but there was a significant reduction in the group receiving 3 mg/kg. In a second study, which was a repeat of this same experiment, PKF242-484 had no effect on BAL neutrophil numbers at any dose tested (data not shown). CSE did increase BAL macrophage numbers in C57BL/6 mice ($p < 0.01$). PKF242-484 at 10 mg/kg reduced these numbers of BAL macrophages back to control levels ($p < 0.05$) (figure 13D).

Discussion

The predominant change in response to short-term (3 days) CSE was an increase in neutrophil numbers in the BAL fluid and neutrophil chemokines in both BAL fluid and the lung tissue. The kinetics of the inflammation saw neutrophils peak 24 hours after the third CSE. Neutrophil infiltration was preceded or paralleled by increases in KC levels, suggesting this may be a primary mechanism for driving the acute response to CSE. This conclusion is supported by previous reports showing CXCR2 antagonists attenuate the acute inflammatory response to CSE in both C57BL/6 mice (Thatcher et al., 2005) and Sprague-Dawley rats (Stevenson et al., 2005). After BAL neutrophil numbers peaked, increases in BAL lymphocytes and macrophages followed, the exception being in C57BL/6 mice where macrophage numbers increased immediately after CSE. Generally, this pattern of neutrophil infiltration followed by macrophage and lymphocyte influx agrees with the kinetics of most models of acute neutrophilia in both the airways (Corteling et al., 2002) as well as other organs, such as the skin (MacKay et al., 1985).

Overall, the types of changes in response to CSE were similar amongst the strains, although the degree of the changes did vary. Generally, BALB/C mice consistently had the greatest response to CSE (i.e., proportion and numbers of neutrophils and levels of KC in the BAL fluid), while C57BL/6 mice consistently had the smallest response. These data are consistent with observations reported by Vlahos and colleagues (Vlahos et al., 2006). In addition, these observations about the different degree of inflammation amongst these strains are similar to those made from other models of acute lung inflammation such as the LPS (Corteling et al., 2002) and allergen-challenge (Trifileff et al., 2000) models. Our data does not correlate entirely with that of Yao and colleagues who reported that C57/BL6 mice were the most

responsive to CSE compared to other strains including A/J mice and 129/Sv mice, the latter being the least responsive amongst the strains tested in their study (Yao et al., 2008). Our exposure levels were comparable to those used by Yao and colleagues as was the degree of inflammation in C57/BL6 mice; thus, the responses of A/J and 129/Sv mice to CSE are the main discrepancies between the two studies. The reason for this difference is unknown at present. Because responses in BALB/C and C57BL/6 mice differed most in our model system, we studied whether there were mechanistic differences by which CSE induced inflammation in the lungs of these two strains. In addition, because studies investigating the effects of CSE in mice are typically conducted using C57BL/6 mice, it is important to identify mechanisms that may be either strain-dependent or –independent.

The activities of MMPs are believed to be central to mediating CSE-induced inflammation and airspace enlargement. Specifically, the activity of MMP-12 was shown to be required for the development of chronic CSE-induced inflammation and emphysema in mice (Hautamaki et al, 1997). MMP-12 activity has also been shown to be important for driving the acute response to cigarette smoke (Churg et al., 2003; Leclerc et al., 2006; Le Quement et al., 2008) and a broad spectrum MMP inhibitor has been shown to inhibit CSE-induced neutrophilia (Churg et al., 2002) in C57BL/6 mice, which contradicts our data. In this previous study, Churg and colleagues reported that oral administration of the broad spectrum MMP inhibitor, RS113456 (100 mg/kg), inhibited neutrophilia, but not macrophage infiltration after a single CSE; however, it was also acknowledged by the authors that the anti-inflammatory effect of RS113456 may not have been mediated through the compound's inhibition of MMPs but rather through the ability of this compound to directly impact on neutrophil migration which was demonstrated by the same group in another model of

lung inflammation (Churg et al., 2001). If that is the case, our observations (using a potent, broad spectrum MMP inhibitor) would be consistent with and clarify their results – i.e., no effect of a broad spectrum inhibitor (administered p.o.) on inflammatory cell (neutrophil and macrophage) infiltration in C57BL/6 mice. This does conflict, however, with other reports of reduced neutrophil infiltration in MMP-12-deficient mice (C57/BL6 background) (Leclerc et al., 2006, Churg et al., 2003) and after treatment with a specific MMP-12 inhibitor in C57BL/6 mice after acute CSE (Le Quement et al., 2008). One possible explanation for this discrepancy is that there are different effects using a broad spectrum MMP inhibitor versus the selective inhibition of MMP-12. In addition, these studies (Leclerc et al., 2006; Le Quement et al., 2008) reported a greater degree of inflammation in the C57/BL6 mice after CSE than we observed (> 2-fold greater), which may also account for the differences. The reason for the different level of acute inflammation is not clear, but one possibility is different levels of cigarette smoke exposure.

PKF242-484 administered p.o. and i.n. reduced CSE-induced neutrophilia in BALB/C mice where the neutrophil response was more robust. The differences in inflammatory cell profiles suggest that there was a different milieu of MMPs in the lungs after acute CSE in BALB/C versus C57BL/6 mice. We found limited evidence of this when looking at the expression of MMPs/TIMPs at the message level (MMP-2, MMP-8 and TIMP-1), but the most consistent change in both strains was a time-dependent increase in MMP-12 message levels. Others have characterized MMP activity levels by zymography after short-term CSE (Seagrave et al., 2004) and its likely that the activities of MMP-2, -8 and -9 are up-regulated in this model due to their release from infiltrating inflammatory cells (i.e., not regulated transcritptionally). The fact there were greater numbers of inflammatory cells in the lungs in BALB/C

mice may explain why these mice were more sensitive to the effects of an MMP inhibitor. This premise is supported somewhat by the greater relative levels of MMP message expression in BALB/C versus C57BL/6 mice after CSEs. How PKF242-484 reduced the neutrophilia in the BALB/C mice is unclear. We did not observe any clear effects on CSE-mediated increases in cytokine (IL-1β) or chemokine (KC, MIP-2) production in response to PKF242-484 treatment. Another possible explanation for the compound's anti-inflammatory effects is that it reduced the production of chemotactic matrix fragments such as Pro-Gly-Pro (PGP), which works through the CXCR2 receptor to drive neutrophilia (Weathington et al., 2006).

Interestingly, PKF242-484 was only effective at reducing macrophage infiltration at the top dose (10 mg/kg) in both strains when dosed i.n,, but had no effect when dosed p.o. At present we cannot explain this anomaly; however, one possibility is systemic administration of PKF242-484 could not achieve high enough levels in the lung to inactivate MMPs involved in mediating macrophage recruitment to the lung. These data would suggest that inhaled delivery may be an attractive approach for the development of a MMP inhibitor for the treatment of COPD. This may not only enhance the anti-inflammatory attributes of MMP inhibition, but may also limit the associated systemic (musculoskeletal) toxicities associated with such compounds (Belvisi and Bottomley, 2003).

An advantage to this model is its relative short duration when compared to more chronic models that take on average 6 months from beginning to end. Pemberton and colleagues tested a broad spectrum MMP inhibitor, ilomilast, in a chronic (C57BL/6) CSE model and demonstrated that dosed (i.n.) prophylactically and therapeutically the compound effectively attenuated the inflammation and emphysema induced by CSE (Pemberton et al., 2005). These data suggest that the

acute model is not only a shorter model, but may also be predictive of the effects of a compound in a more chronic inflammatory setting.

In summary, CSE induces dose- and time-dependent inflammatory changes in all strains of mice examined; however the extent and timing of some of these changes differ amongst the strains. We evaluated the effects of a broad spectrum MMP inhibitor in the two most contrasting strains, BALB/C and C57BL/6. MMP inhibition attenuated CSE-induced neutrophilia in the strain with the more prominent lung neutrophil inflammation, BALB/C, but had no effect on neutrophils in C57BL/6 mice. This disparity may be due to the contrasting levels of inflammatory cells in the lungs, thus providing a greater window to see the effect in BALB/C mice. Another possibility is that the different degree of inflammation could also change the relative contributions of various MMPs to the response. MMP inhibition did not affect macrophage numbers in either strain when dosed systemically, but did when dosed topically, suggesting high levels of PKF242-484 were necessary in the lung in order to reduce BAL macrophage numbers.

These data suggest that this system can be used as a robust model to evaluate the efficacy of prospective candidate compounds being developed for the treatment of COPD. The data also highlight the importance of testing compounds in multiple strains (or species) in order to understand which strain (or species) will be the most appropriate (mechanistically) for evaluating the *in vivo* efficacy of test compounds with respect to drug discovery. In addition, understanding these strain-dependent differences may also be important for understanding factors that influence the susceptibility of some smokers for developing COPD, while others do not.

Acknowledgements

The authors would like to thank Dr. Chris Poll, Dr. Alex Trifilieff and Dr. Mark Birrell for their careful review of this manuscript.

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Footnotes

Dr. Stevenson's salary during the preparation of this manuscript was supported by a Capacity Building Award in Integrative Mammalian Biology funded by the BBSRC, BPS, HEFCE, KTN, and MRC.

Legends for Figures

Figure 1. Dose-response of CSE-induced BAL neutrophil infiltration. CSE induces a dose-dependent increase in the number of BAL neutrophils in BALB/C (A), C57BL/6 (B), A/J (C), and 129/Sv (D) mice. Data plotted as the mean + sem with an n = 10 for each group. Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) was determined versus sham control group.

Figure 2. Dose-response of CSE-induced BAL KC levels. CSE induces a dose-dependent increase in the KC in the BAL fluid of BALB/C (A), C57BL/6 (B), A/J (C), and 129/Sv (D) mice. Data plotted as the mean + sem with an n = 10 for each group. Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) was determined versus sham control group.

Figure 3. Dose-response of CSE-induced lung tissue KC levels. CSE induces a dose-dependent increase in tissue KC levels (measured using the supernatant of lung tissue homogenates) from BALB/C (A), C57BL/6 (B), A/J (C), and 129/Sv (D) mice. Data plotted as the mean + sem with an n = 10 for each group. Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) was determined versus sham control group.

Figure 4. Time course of lung tissue KC levels after 3 days of CSE. CSE induced an immediate increase in KC tissue levels (measured using the supernatant of lung tissue homogenates) from BALB/C (A), C57BL/6 (B), A/J (C), and 129/Sv (D) mice. Data from smoke-exposed mice are represented by black squares connected by solid black lines. Data from sham controls were represented by gray triangles connected by

gray hashed lines. Data plotted as the mean ± sem with a n = 10 for each group. Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) was determined versus sham control group.

Figure 5. Time course of KC levels in BAL fluid after 3 days of CSE. CSE induced a time-dependent increase in KC levels in the BAL fluid of BALB/C (A), C57BL/6 (B), A/J (C), and 129/Sv (D) mice. Data from smoke-exposed mice are represented by black squares connected by solid black lines. Data from sham controls were represented by gray triangles connected by gray hashed lines. Data plotted as the mean ± sem with a n = 5 for each group. Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) was determined versus sham control group.

Figure 6. Time course of neutrophil infiltration after 3 days of CSE. CSE induced a time-dependent increase in neutrophil numbers recovered in the BAL fluid of BALB/C (A), C57BL/6 (B), A/J (C), and 129/Sv (D) mice. Data from smoke-exposed mice are represented by black squares connected by solid black lines. Data from sham controls were represented by gray triangles connected by gray hashed lines. Data plotted as the mean ± sem with a n = 10 for each group. Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) was determined versus sham control group.

Figure 7. Time course of macrophage infiltration after 3 days of CSE. CSE induced a time-dependent increase in macrophage numbers recovered in the BAL fluid of BALB/C (A), C57BL/6 (B), A/J (C), and 129/Sv (D) mice. Data from smoke-exposed mice are represented by black squares connected by solid black lines.

Data from sham controls were represented by gray triangles connected by gray hashed lines. Data plotted as the mean ± sem with a n = 10 for each group. Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) was determined versus sham control group.

Figure 8. Time course of lymphocyte infiltration after 3 days of CSE. CSE induced a time-dependent increase in lymphocyte numbers recovered in the BAL fluid of BALB/C (A), C57BL/6 (B), A/J (C), and 129/Sv (D) mice. Data from smoke-exposed mice are represented by black squares connected by solid black lines. Data from sham controls were represented by gray triangles connected by gray hashed lines. Data plotted as the mean ± sem with a n = 10 for each group. Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) was determined versus sham control group.

Figure 9. Time course of MMP-9 and MMP-12 gene expression after 3 days of CSE

CSE induced a time-dependent increase in MMP-9 and MMP-12 expression in the lungs of C57BL/6 (A and C, respectively), BALB/C (B and D, respectively) mice. Data from smoke-exposed mice are represented by black squares connected by solid lines. Data from sham controls are represented by gray triangles connected by hashed lines. Data plotted as the mean ± sem with a n = 10 for each group. Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) was determined versus sham control group.

Figure 10. Time course of MMP-2 and MMP-8 gene expression after 3 days of CSE

CSE induced changes in MMP-2 and MMP-8 expression levels in the lungs of C57BL/6 (A and C, respectively) and BALB/C (B and D, respectively) mice. Data from smoke-exposed mice are represented by black squares connected by solid lines. Data from sham controls are represented by gray diamonds connected by hashed lines. Data plotted as the mean $\pm$ sem with a n = 10 for each group. Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) was determined versus sham control group.

Figure 11. Time course of TIMP-1 and TIMP-2 gene expression after 3 days of CSE

CSE induced changes in TIMP-1 and TIMP-2 expression levels in the lungs of C57BL/6 (A and C, respectively) and BALB/C (B and D, respectively) mice. Data from smoke-exposed mice are represented by black squares connected by solid black lines. Data from sham controls are represented by gray triangles connected by hashed lines. Data plotted as the mean $\pm$ sem with a n = 10 for each group. Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) was determined versus sham control group.

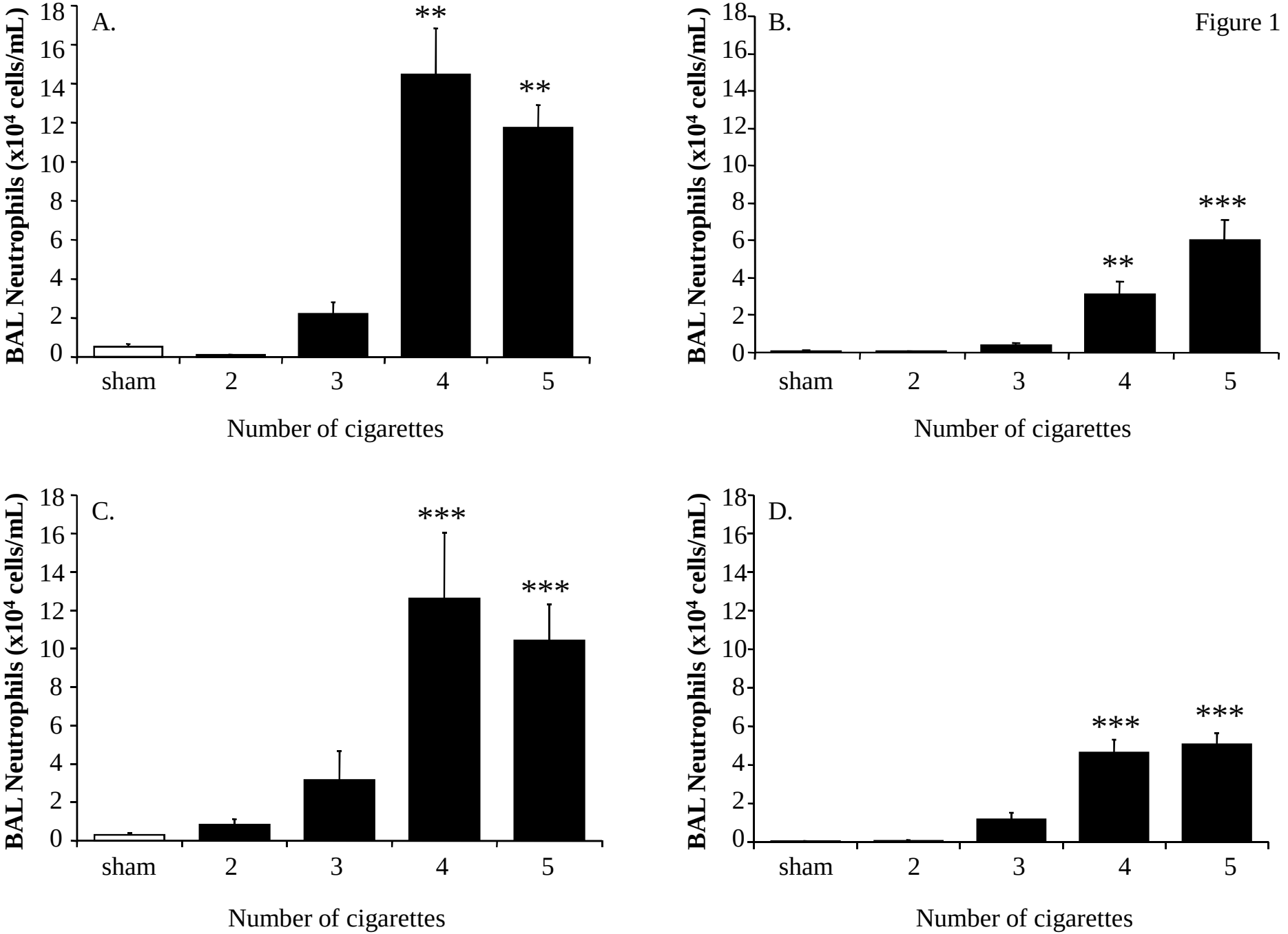
Figure 12. Effect of PKF242-484 dosed p.o., b.i.d. on CSE-induced inflammation

The effect of PKF242-484 (p.o.) on CSE-induced neutrophil and macrophage infiltration in BALB/C (A and B, respectively) and C57BL/6 (C and D, respectively) mice. Data plotted as the mean + sem with a n = 10 for each group. Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) was determined versus smoke-exposed vehicle control group.

Figure 13. Effect of PKF242-484 dosed i.n., b.i.d. on CSE-induced inflammation

The effect of PKF242-484 (i.n.) on CSE-induced neutrophil and macrophage infiltration in BALB/C (A and B, respectively) and C57BL/6 (C and D, respectively) mice. Data plotted as the mean + sem with a n = 10 for each group. Significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) was determined versus smoke-exposed vehicle control group.

Figure 1



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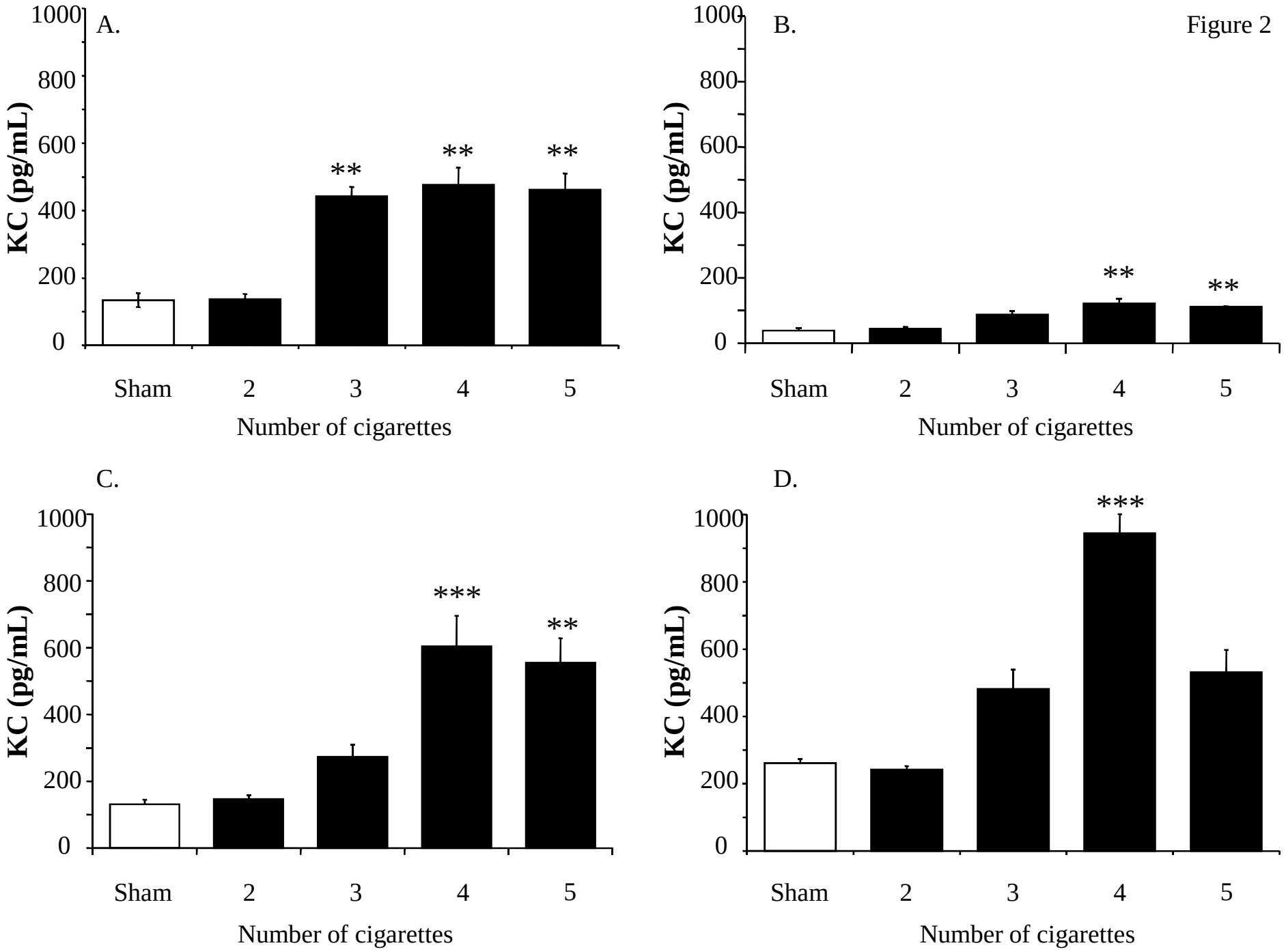

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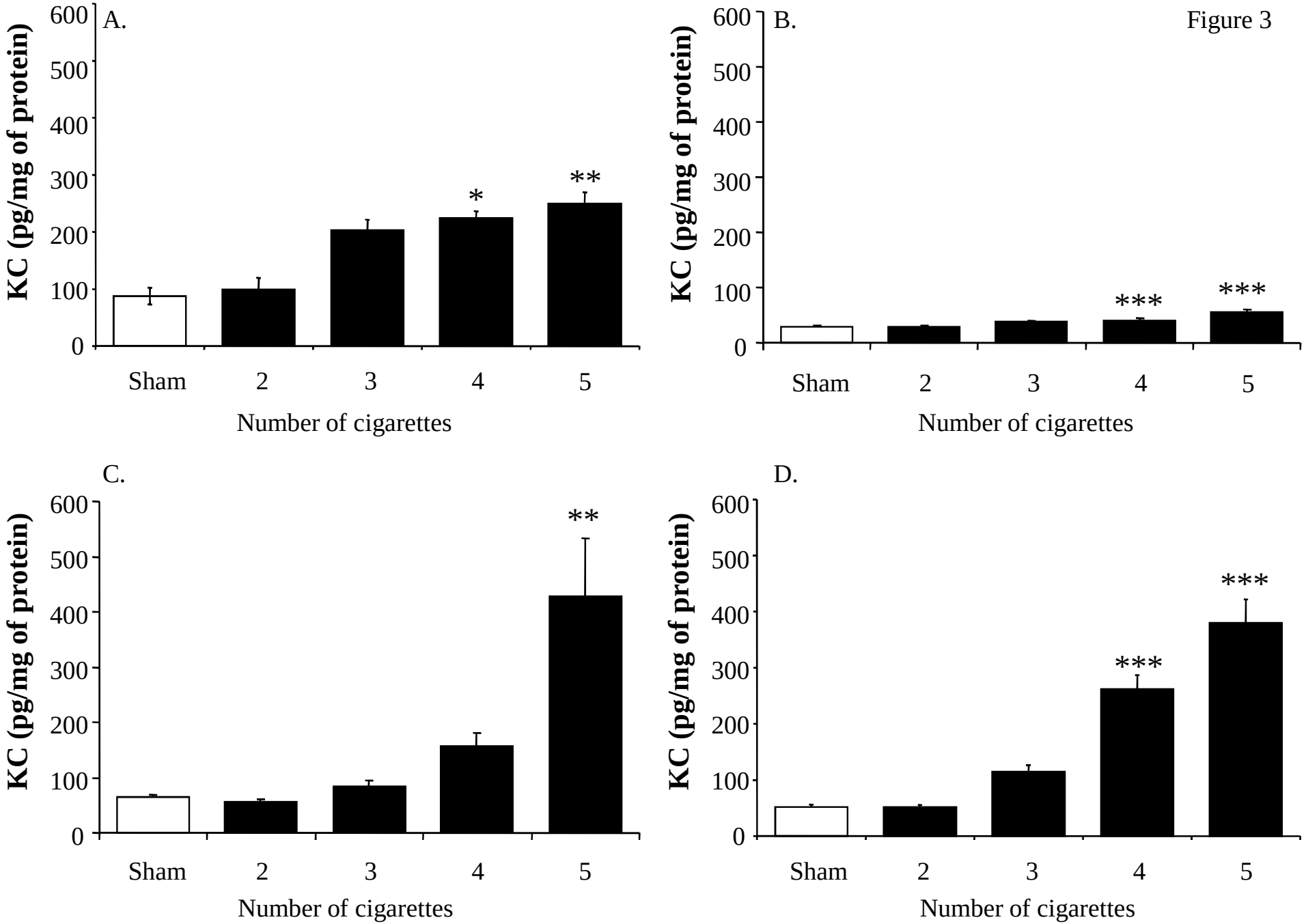

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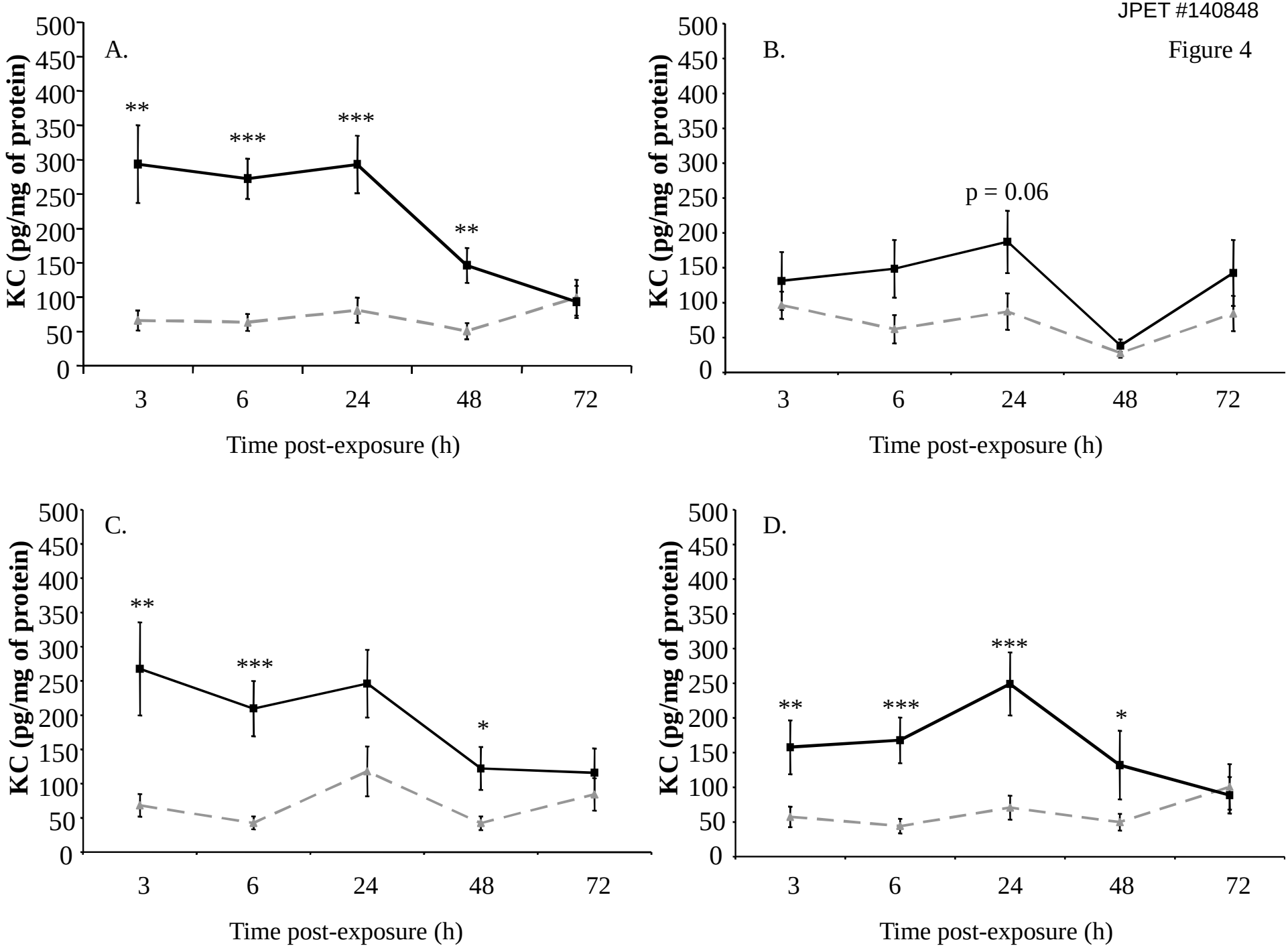

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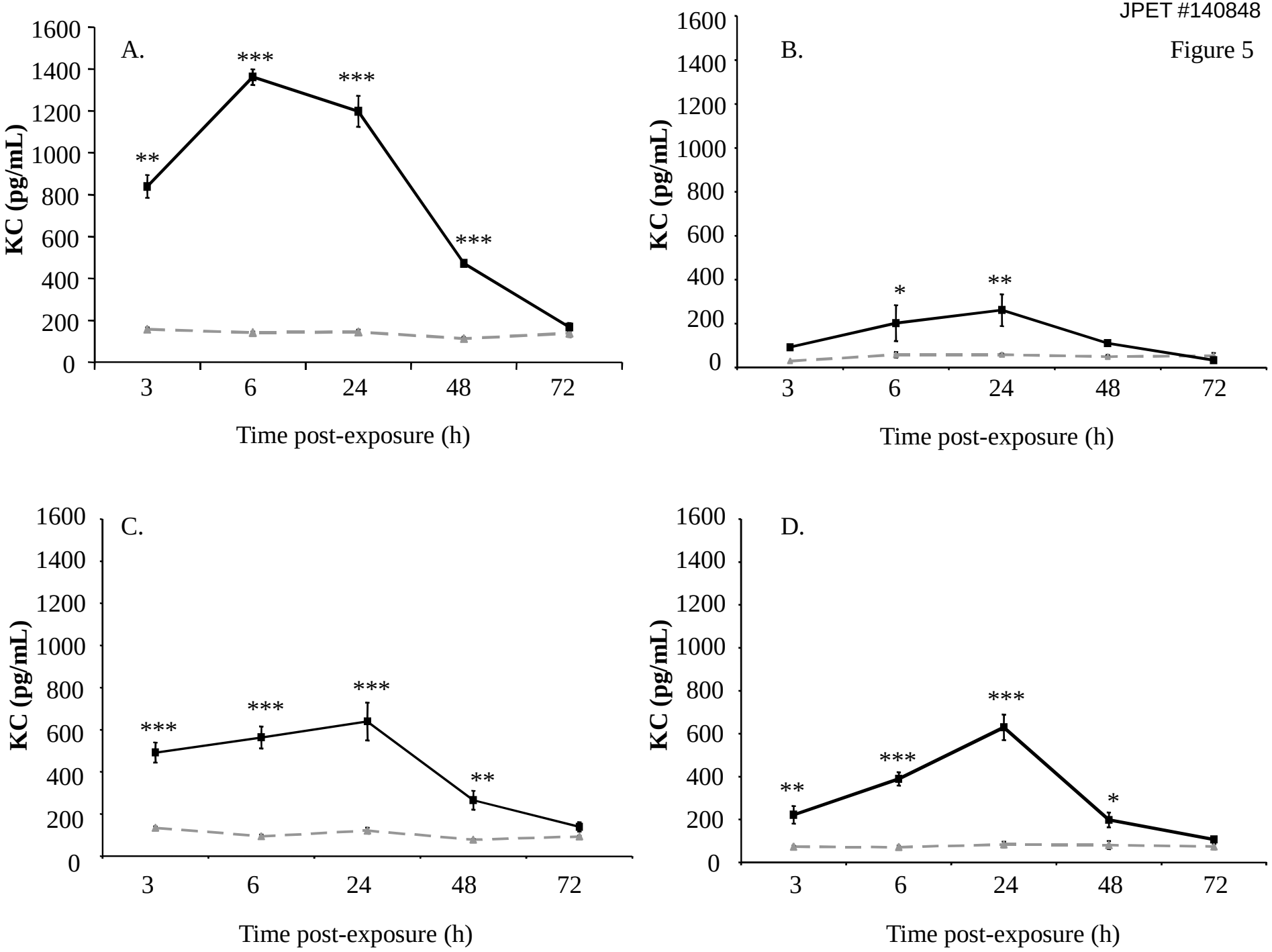

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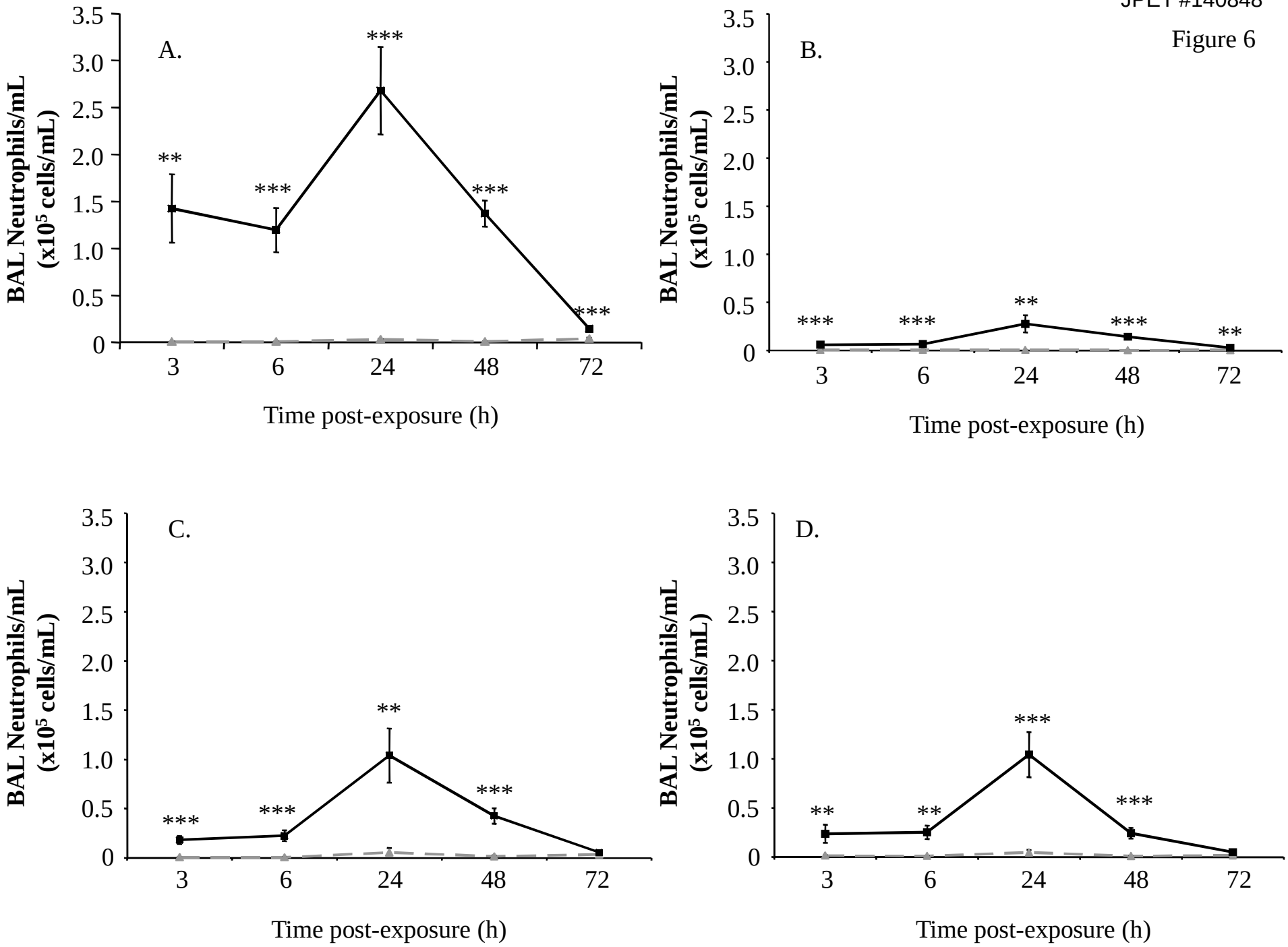

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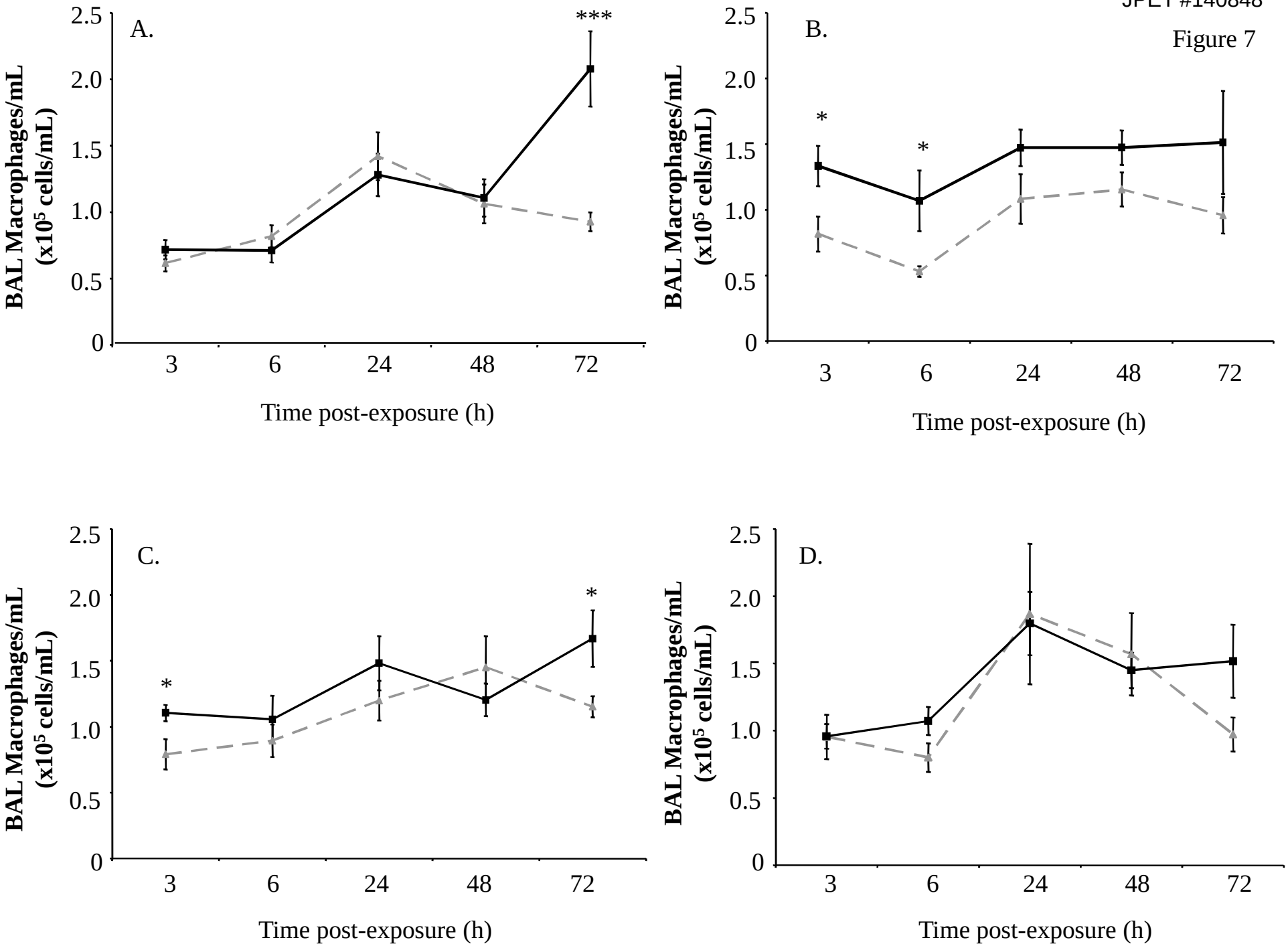

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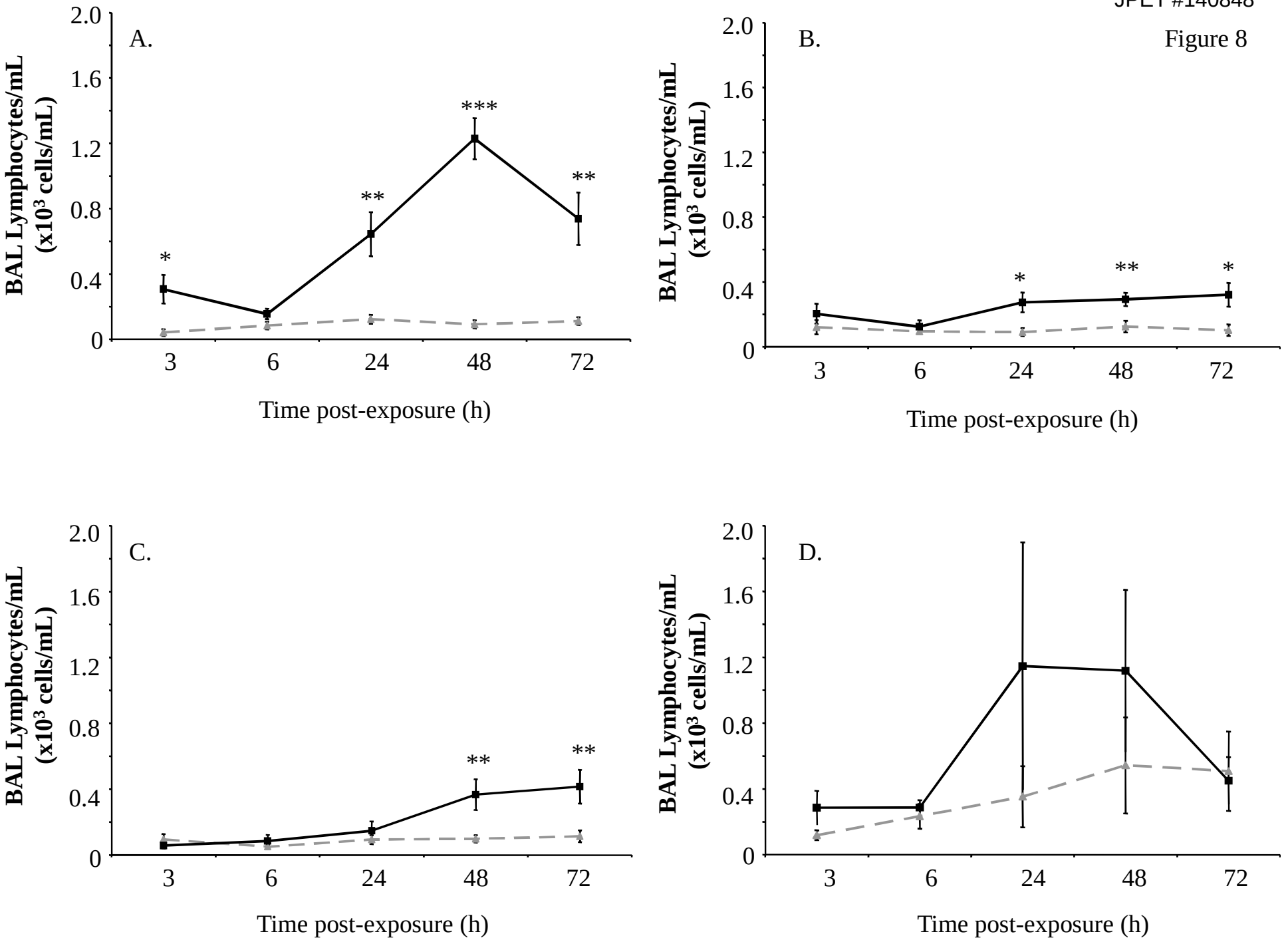

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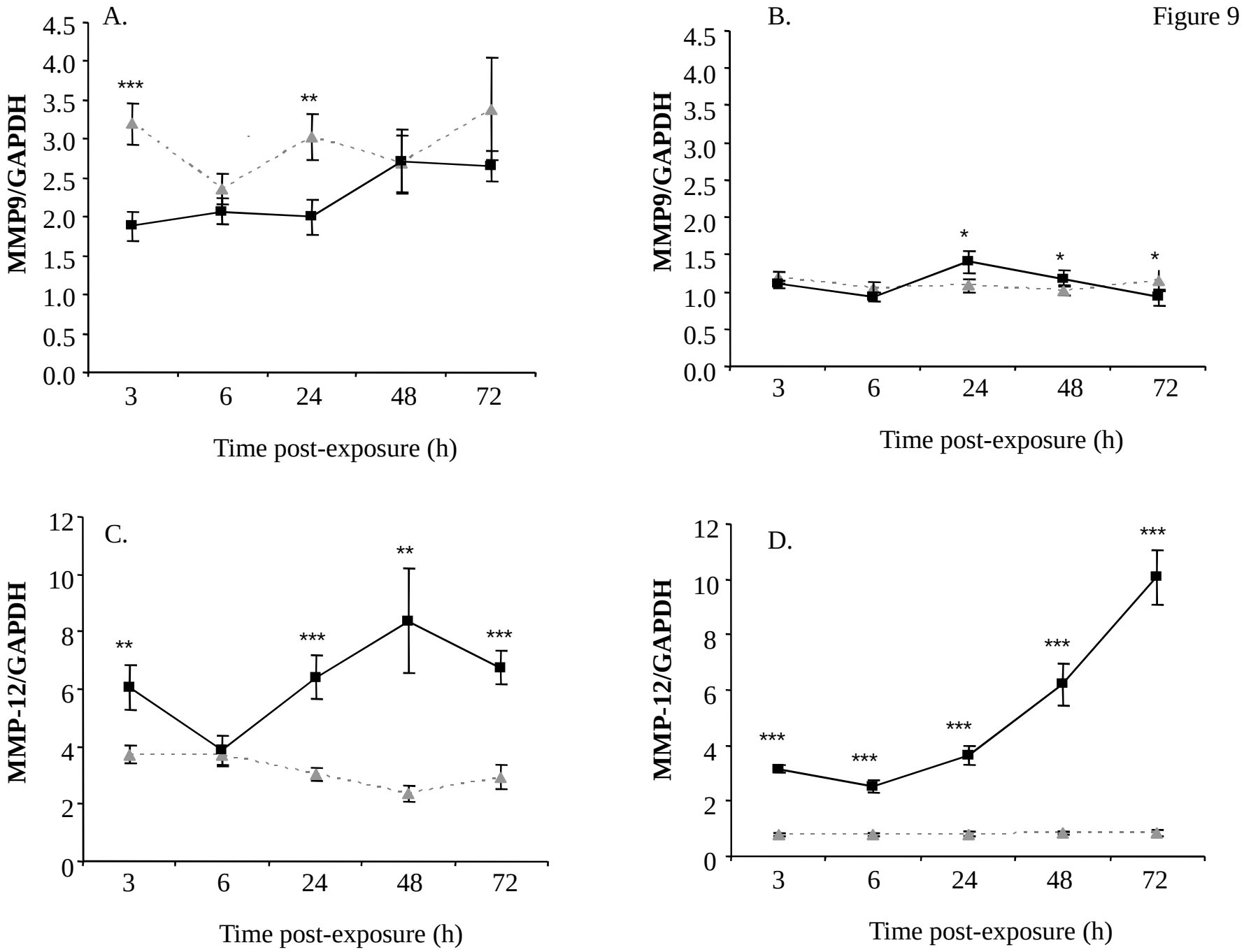

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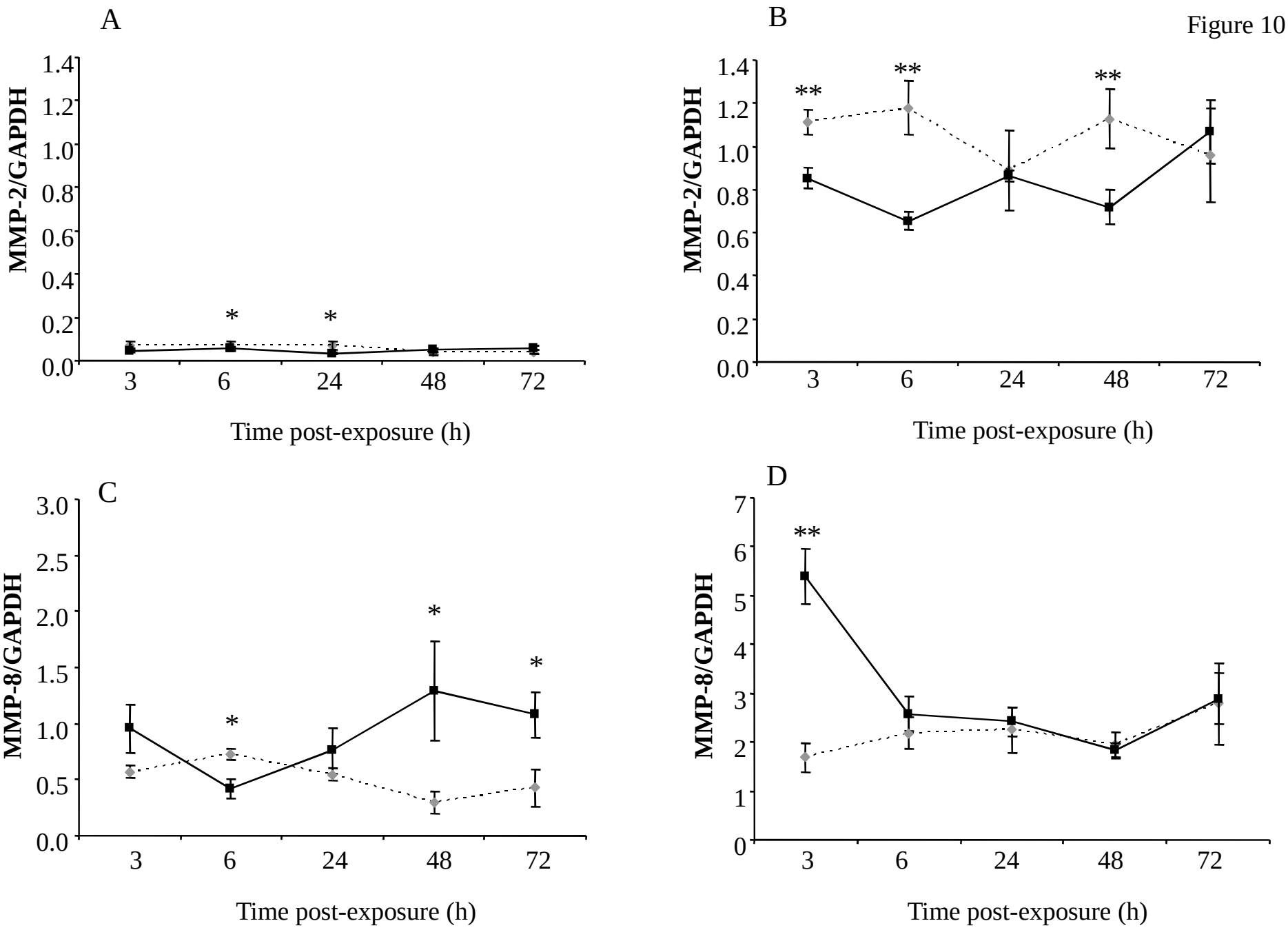

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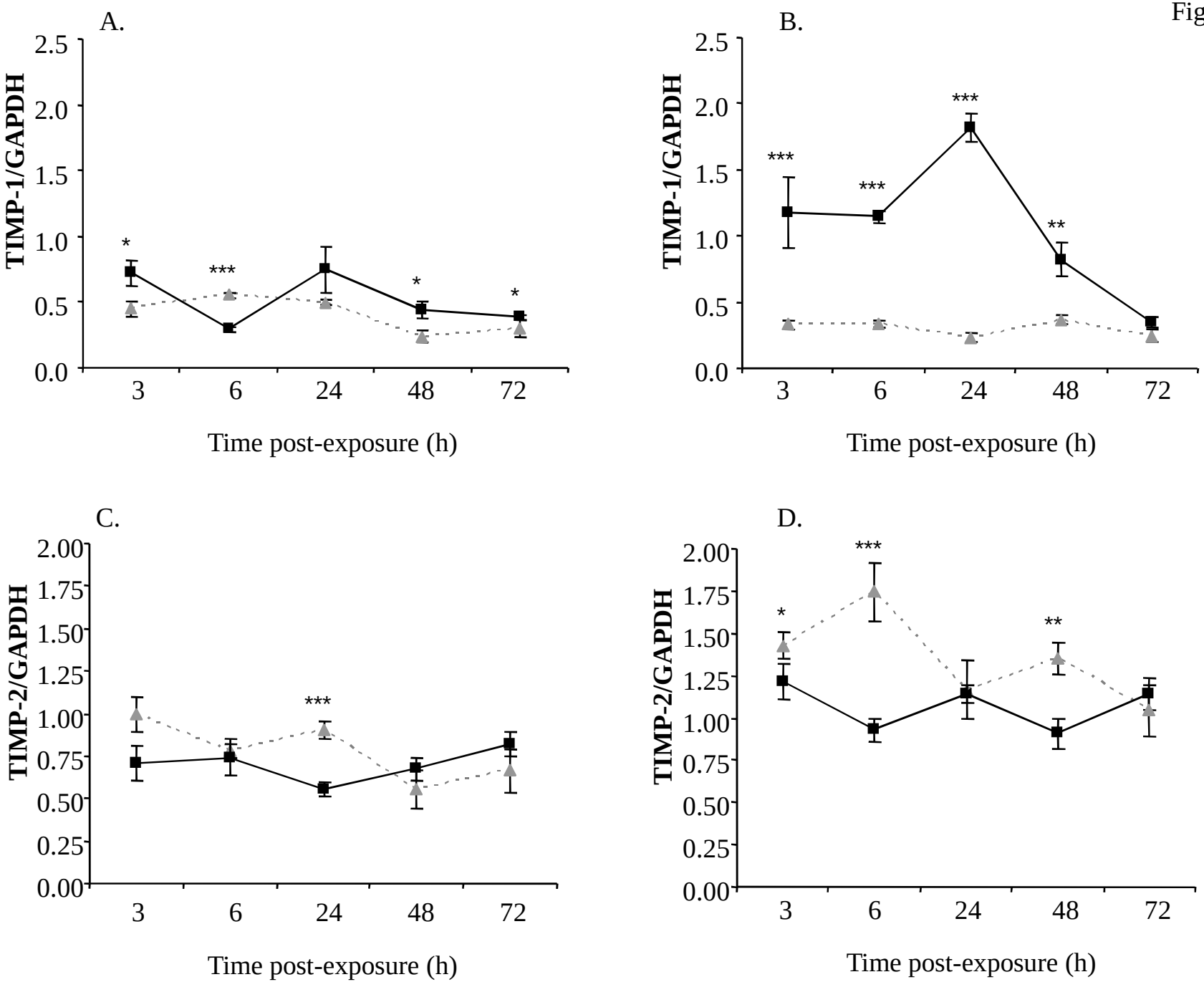

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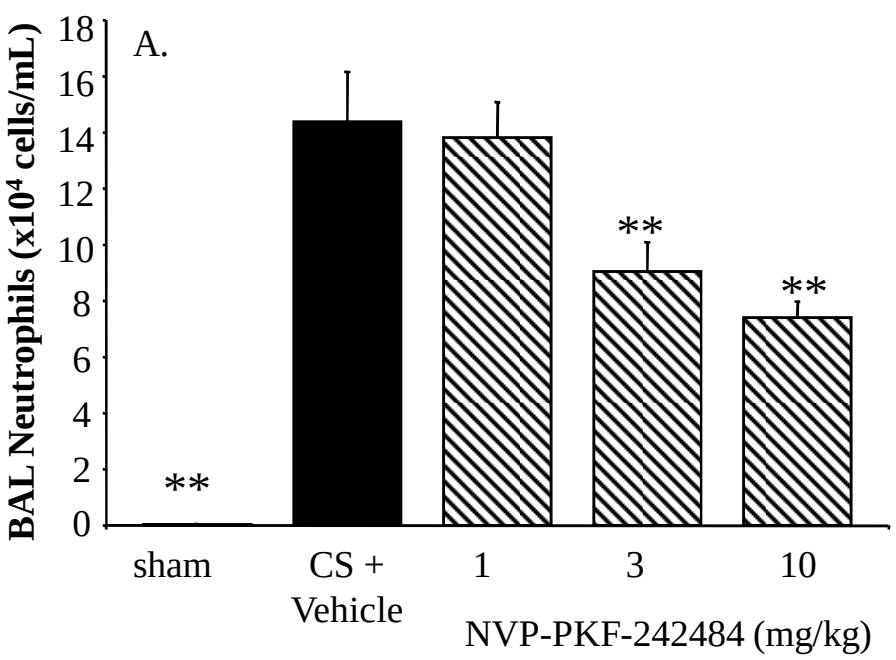

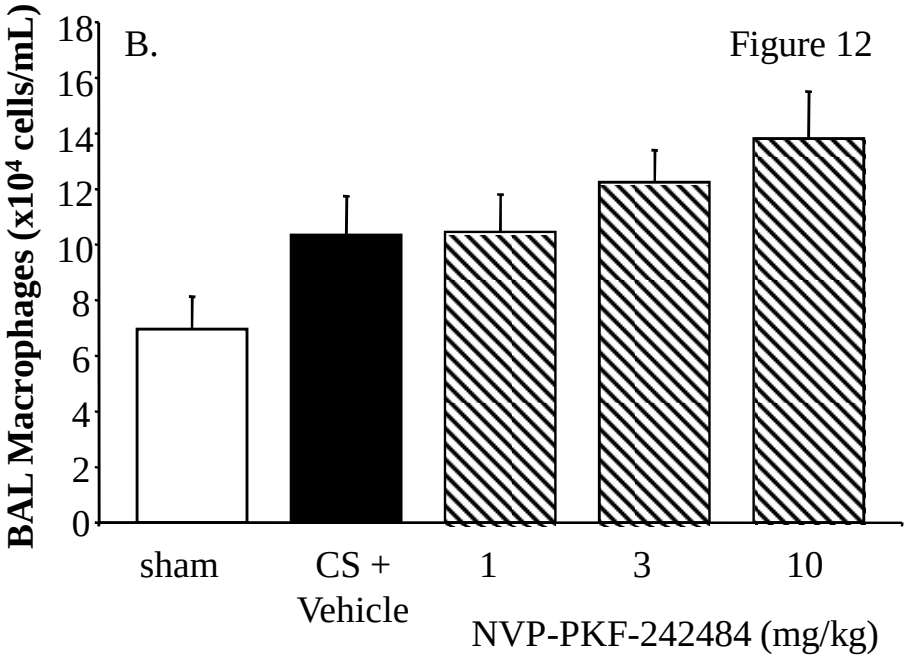

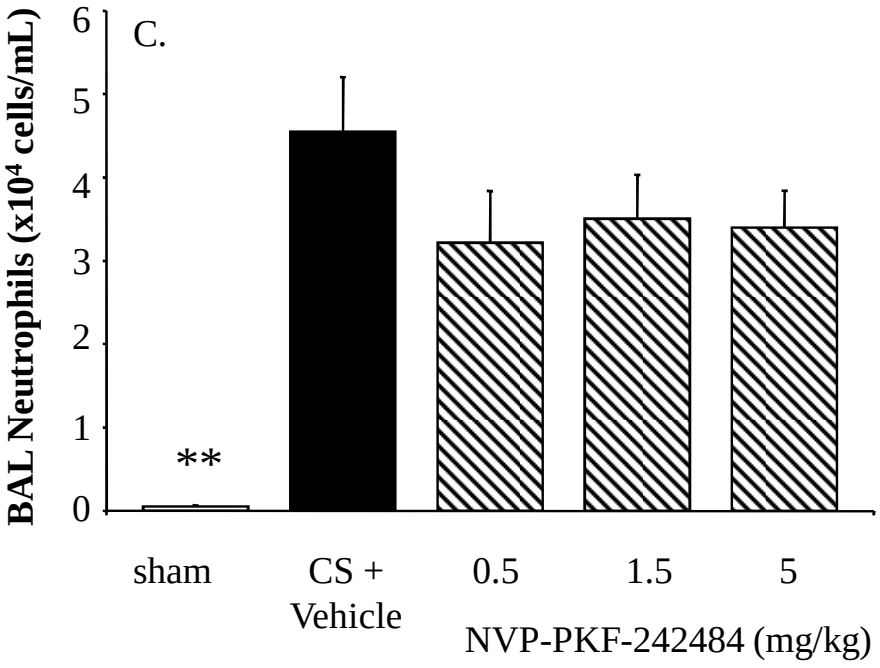

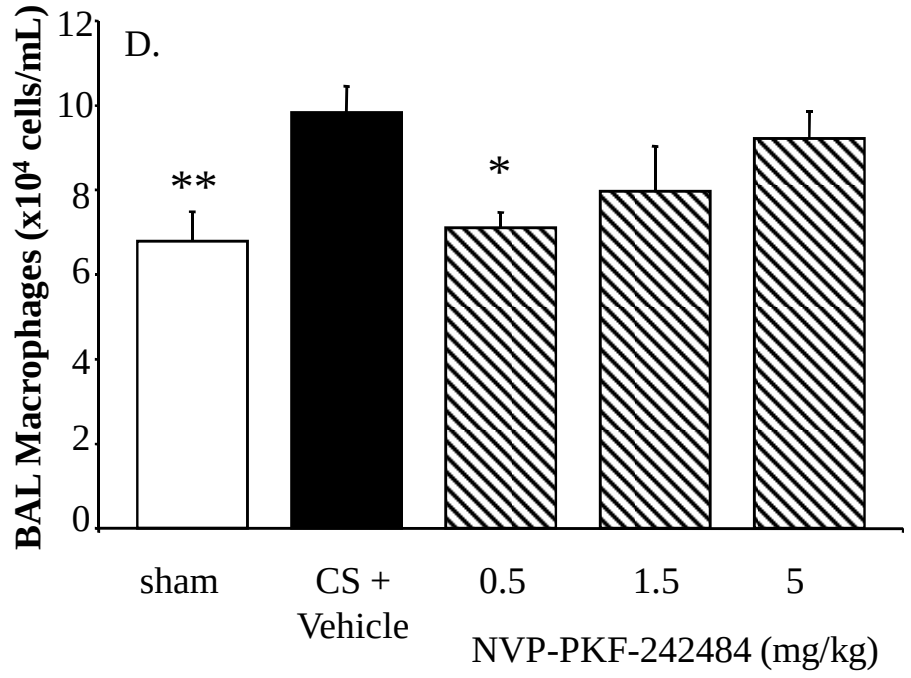

Figure 13