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Acetaminophen Attenuates House Dust Mite Induced Allergic Airway Disease in Mice

by

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Abbreviations used:

5-PP:	5-Phenyl-1-Pentyne
ANOVA:	Analysis of variance
APAP:	N-acetyl-p-aminophenol
BAL:	Bronchoalveolar lavage
CBR:	Cannabinoid receptor
CYP:	Cytochrome P450
ETS:	Environmental tobacco smoke
GSH:	Glutathione (reduced)
HDM:	House dust mite
IL:	Interleukin
LPS:	Lipopolysaccharide
NAPQI:	N-acetyl-p-benzoquinone-imine
Nqo1:	NAD(P)H dehydrogenase, quinone 1
Nrf2:	Nuclear factor erythroid 2-related factor 2
OVA:	Ovalbumin
PG:	Prostaglandin
SD:	Standard Deviation
SEM:	Standard Error of the Mean

Section Assignment: Inflammation, Immunopharmacology, and Asthma

Abstract

Epidemiological evidence suggests that APAP may play a role in the pathogenesis of asthma, likely through pro-oxidant mechanisms. However, no studies have investigated the direct effects of APAP on the development of allergic inflammation. To determine the likelihood of a causal relationship between APAP and asthma pathogenesis, we explored the effects of APAP on inflammatory responses in a murine house dust mite (HDM) model of allergic airway disease. We hypothesized that APAP would enhance the development of HDM induced allergic inflammation. The HDM model consisted of once daily intranasal instillations for up to two weeks with APAP or vehicle administration 1 hour prior to HDM during either week 1 or 2. Primary assessment of inflammation included bronchoalveolar lavage (BAL), cytokine expression in lung tissue, and histopathology. Contrary to our hypothesis, the effects of HDM treatment were substantially diminished in APAP treated groups compared to controls. APAP treated groups had markedly reduced airway inflammation: including decreased inflammatory cells in the BAL fluid, lower cytokine expression in lung tissue, and less perivascular and peribronchiolar immune cell infiltration. The anti-inflammatory effect of APAP was not abrogated by an inhibitor of cytochrome P450 metabolism, suggesting that the effect was due to the parent compound or a non-CYP450 generated metabolite. Taken together, our studies do not support the biological plausibility of the "APAP hypothesis" that APAP use may contribute to the causation of asthma. Importantly, we suggest the mechanism by which APAP modulates airway inflammation may provide novel therapeutic targets for asthma.

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Introduction

The past three decades have witnessed a worldwide epidemic-scale increase in asthma prevalence, the cause(s) of which are unknown. The increase in asthma prevalence occurred concomitantly with the expansion in acetaminophen (APAP, *n*-acetyl-*para*-aminophenol) use following identification, in the early 1980s, of a suspected Reyes Syndrome risk from aspirin. The association between APAP use and asthma has prompted the "APAP hypothesis," that APAP contributes to the increase in asthma, likely through its pro-oxidant effects.

Multiple large epidemiological studies have found a strong association between APAP use and asthma prevalence in children and adults (Barr et al., 2004; Beasley et al., 2011; Etminan et al., 2009). However, due to inherent limitations of epidemiological data, a causal relationship between APAP and asthma has not been established. One such limitation is the possibility of confounding by indication; for example, APAP is often used to treat fever caused by infections, and certain respiratory tract infections are independent risk factors for asthma (Heintze and Petersen, 2013). Supporting this explanation are several studies that found adjusting for respiratory infections diminished the association (Lowe et al., 2010; Sordillo et al., 2015).

Despite evidence supporting confounding factors, uncertainty and the near ubiquitous use of APAP make the APAP-asthma association worthy of further investigation. The most developed of several proposed mechanisms for the association is that APAP induced oxidative stress promotes the development of an asthmatic phenotype. Oxidative stress is a hallmark of asthma and thought to be important in asthma pathogenesis. Markers of oxidant levels are higher in people with asthma and polymorphisms in antioxidant genes such as glutathione transferase are asthma risk factors (Holguin, 2013; Riedl and Nel, 2008). However, asthma is an inflammatory disease and oxidative

stress may be a result as well as a cause of active airway inflammation making difficult full definition of its role (Andreadis et al., 2003; Bhalla et al., 2009; Henricks and Nijkamp, 2001). Animal studies confirm the importance of oxidative stress in asthma, e.g. mice with reduced antioxidant defenses (nuclear factor erythroid 2-related factor 2 (Nrf2) knockout mice) demonstrate enhanced susceptibility to allergic airway disease (Rangasamy et al., 2005; Williams et al., 2008). At sufficient doses APAP causes severe liver toxicity primarily through oxidative stress caused by its reactive metabolite, N-acetyl-P-benzoquinone-imine (NAPQI) (McGill and Jaeschke, 2013). Hepatotoxic doses of APAP have been associated with extrahepatic GSH loss and tissue injury in the nose, and lung (Gu et al., 2005; Hart et al., 1995).

The effects of APAP at high doses in the liver and airways suggest an oxidative stress based mechanism is plausible. Recent work in our laboratory focused on the potential for lower, non-hepatotoxic, doses of APAP to cause oxidative stress in the airways. At near-therapeutic doses, APAP causes airway GSH loss, airway cellular oxidative stress response pathway induction, and potentiates acute airway responses to environmental tobacco smoke (ETS) (Smith et al., 2016). That APAP acts as a pro-oxidant in the respiratory tract suggests it could promote the development of asthma similar to other oxidant asthma risk factors such as ETS, ozone, and diesel exhaust (Gilmour et al., 2006). Our previous studies focused on the acute pro-oxidant effects of APAP on the airways. While they support a proposed mechanism for the APAP-asthma association which may involve oxidative stress and the potentiation of the effects of ETS, they do not provide direct evidence linking APAP treatment with the development of future allergic disease. Thus the overarching goal of the current study was to investigate the effects of APAP on allergic responses of the airways in a murine HDM model of allergic airway disease. We hypothesized that APAP administration would enhance the development of allergic airway disease caused by HDM, similar

to the enhancement of HDM induced inflammation observed with other pro-oxidants such as ETS (Lanckacker et al., 2013; Mitchell et al., 2012).

Interestingly, our initial studies indicated that APAP did not enhance the response to HDM antigen, rather the opposite effect was observed. APAP effectively blocked the response to HDM. The approach of the experiments featured in this work then became to characterize and document the unexpected attenuation of the HDM response by APAP through measurements of airway inflammatory cell influx, cytokine mRNA profiles, HDM specific serum immunoglobulin, and airway histological presentation.

Materials and Methods

Experimental Approach: A HDM model of allergic airway disease was chosen for its environmental and human relevance as allergies to HDM are highly prevalent in humans, especially among those with asthma (Boulet et al., 1997; Calderon et al., 2014). The response to HDM in mice exhibits several of the principle features of allergic disease in humans, and importantly HDM produces a respiratory allergic response in mice that has been extensively characterized (Cates et al., 2004; Cates et al., 2007; Johnson et al., 2004; Maes et al., 2010). Immunological tolerance in HDM models has been shown to occur after long-term exposure (Bracken et al., 2015). Therefore, our studies were limited to 2 weeks in duration and designed to examine changes in the early response to both minimal and maximal doses of HDM.

Mouse models of asthma have been defined as having two main phases, an early allergic sensitization phase, followed by a challenge phase (Maes et al., 2010). The timing of co-exposures to other pro-oxidants such as ETS (e.g. during sensitization vs. challenge) has been shown to produce pleiotropic disease outcomes in asthma models (Botelho et al., 2011; Lanckacker et al., 2013; Maes et al., 2010). Therefore, in our experiments investigating the effects of APAP on the response to HDM, APAP is given during either the first or the second week of the HDM model exposure to account for the possibility of differential effects during earlier vs later phases of the model. A 100 mg/kg dose of APAP is used in this study based on dose-response relationships described in our previous work (Smith et al., 2016). This dose causes significant GSH loss throughout the respiratory tract at 1 hr, but, importantly, elicits peak blood APAP levels of 35 µg/ml, which are only slightly higher than therapeutic levels (5-20 µg/ml).

Mice and dosing. Experiments were performed on 9 to 12 week old female C57Bl/6J mice obtained from The Jackson Laboratory. Mice were housed in American Association for Accreditation of Laboratory Animal Care-accredited facilities at the University of Connecticut under standard environmental conditions (12-h light-dark cycle at 23°C). Mice were housed over hardwood shavings (Sani-Chip Dry, P. J. Murphy Forest Products). Food (Lab Diet; PMI Nutrition International) and tap water were provided *ad libitum*. All animal studies were reviewed and received prior approval by the University of Connecticut Institutional Animal Care and Use Committee (protocol #: A12-013).

All chemicals were obtained from Sigma Aldrich, unless otherwise indicated, and were of USP grade, or better. APAP (PubChem 1983) dissolved in 37°C saline (10 mg/ml) was administered via i.p. injection. The cytochrome P450 inhibitor 5-phenyl-1-pentyne (5PP, GFS Chemicals) (PubChem 74573) was given ip at a dose of 100 mg/kg (10 mg/ml in olive oil) 1 hr prior to APAP treatment (Morris, 2013). For euthanasia, mice were anesthetized by urethane injection (1.3 g/kg i.p.) followed by exsanguination through the abdominal aorta or cardiac puncture.

Murine Model of HDM Antigen-induced Allergic Airway Disease. Based on the exposure protocol of Bracken et al. (2015), equal parts *Dermatophagoides pteronyssinus (Der. p.)* and *Dermatophagoides farinae (Der. f.)* lyophilized HDM extracts (Greer Laboratories) were resuspended in phosphate-buffered saline (PBS) at concentrations from 12.5 to 200 ng dry weight/µL. The lyophilized HDM extract mixture contained 3,131 endotoxin units/mg of HDM, and 3.32 % *Der. p./Der. f.,* and the same lot number was used throughout the experiments. The HDM suspension was delivered in the morning between 9:00 and 12:00AM by intranasal instillation in a single 50 µL volume while the mice were lightly anesthetized with isoflurane (2.5%).

Bronchoalveolar Lavage and Tissue Collection. Following euthanasia, lungs were lavaged *in situ* with PBS (3 x 1 mL lavages). BAL fluid cells were pelleted at 2000 x g for 5 minutes, and lavage supernatant was collected and frozen at -20ºC for total protein determination. Total protein was measured by the Lowry method (Lowry et al., 1951). Cell pellets were resuspended in PBS containing 2% bovine serum albumin (BSA). Total cell counts were obtained with a hemocytometer and Turk's solution. Slides of BAL cells were prepared by cytocentrifugation and stained with the Protocol Hema 3 system (Fisher Healthcare). Differential white blood cell counts were performed on a minimum of 200 cells. No eosinophils, neutrophils, or lymphocytes were observed in the vehicle control and APAP groups that received HDM vehicle instillations. Lungs collected for qRT-PCR analysis were inflated with and stored in aqueous RNA stabilization buffer containing saturating ammonium sulfate, 20 mM EDTA, 25mM sodium citrate, at pH 5.2. When collected, blood was obtained via cardiac puncture from anesthetized mice and spun at 1000 x g for 10 minutes to obtain serum.

Quantitative Real Time-PCR. Total RNA was isolated from mouse lung tissue homogenates using an RNeasy kit from Qiagen. 1 µg of total RNA was used for first strand cDNA synthesis by an iScript cDNA synthesis kit (Bio-rad). RT-PCR was performed using SYBR Green as an indicator with an Applied Biosystems 7500 Fast Real-Time PCR System. PCR reactions contained 10 ng of cDNA (4µl), 500 nM of each primer (1µl total), and 5 µl of 2x SYBR Green PCR Master mix (10µl total volume). The PCR was carried out according to the manufacturer's recommended thermal cycling protocol. β-Actin was used as the internal reference control mRNA. Results are represented as the fold change in expression of target genes over control calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primer sequences (Table 2.) were designed with the Life Technologies OligoPerfect™ designer, or obtained from Harvard Primer Bank (Harvard

Medical School), and synthesized by Invitrogen (Life Technologies). The genes selected for qRT-PCR analysis and the functional basis for their selection are provided in Table 1.

Serum Immunoglobulin Measurement. Detection of serum HDM specific IgE and IgG was performed as described previously (Bracken et al., 2015). Briefly, Nunc MaxiSorp, flat-bottom plates (Thermo Scientific) were coated with 10, or 2 μg/ml of HDM extract in sodium bicarbonate buffer (pH 9.5), for IgE or IgG respectively. Blocking was performed with BD OptEIA assay diluent (BD Biosciences). Serum samples were added for the IgE assay in 2-fold serial dilutions (1/20-1/2,560) and for the IgG assay in 10-fold serial dilutions (1/20-1/200,000,000). For antibodies, biotin-SP-conjugated goat anti-mouse IgE (Southern Biotech) and biotin-SP-conjugated goat anti-mouse IgG Fcγ subclass 1-specific (Jackson ImmunoResearch) were used followed by streptavidin-HRP (BD Biosciences). Development was performed with a BD OptEIA TMB substrate reagent set (BD Biosciences), and reaction were quenched with 1M phosphoric acid. Absorbance measurements at 450 and 570 nm were made with a Tecan Safire 2 microplate reader.

Histology. Following euthanasia, lungs not subject to lavage were removed, inflated and stored in 4% buffered formalin. Formalin fixed lungs were then processed by the Connecticut Veterinary Medical Diagnostic Center using standard techniques. Briefly, paraffin embedded lung sections were stained with hematoxylin and eosin (H&E) and periodic acid-schiff (PAS). Lung sections were examined qualitatively via light microscopy and representative images of each treatment group were taken.

Statistical Analysis. Data are reported as mean ± SEM unless otherwise indicated in the text. Data were analyzed with Graphpad Prism software (Graphpad Software, Inc.). Statistically outlying data points were excluded *a priori* if they were greater than 3x the SD of the group. Data were compared by one-way ANOVA followed by the Newman-Keuls post-hoc test for multiple comparisons. When appropriate data were log transformed to correct for heteroscedasticity. A p-value less than 0.05 was considered statistically significant.

Results

HDM Dose-Response. Initial studies were aimed at defining the dose response relationships for HDM in order to delineate both a minimally and maximally effective dose. Based on previous studies 5 dose levels ranging from 0.625μg to 10μg were used (Bracken et al., 2015). Mice were administered HDM intranasally once daily for two weeks with a 2 day rest period in between weeks 1 and 2. Mice were euthanized on day 12, 24 hrs following the last dose of HDM (Supplemental Figure 1A). HDM produced a dose-dependent increase in the number of airway inflammatory cells as measured by the presence of increased eosinophils, neutrophils, and lymphocytes in the BAL fluid (Supplemental Figures 1 B-D). The numbers of these cells in control mice groups were negligible, accounting for <0.5% of the cells present. Macrophage numbers were significantly higher than control at HDM doses of 5 μg or higher (Supplemental Figure 1E). No consistent increase in BAL cellularity was observed with the 0.625 μg dose. The 1.25 μg dose was the lowest dose that caused a consistent, marked inflammatory response as indicated by statistically significant increases in eosinophils, neutrophils and lymphocytes. Macrophages were increased to 1.4-fold of control at this dose, but the difference was not statistically significant. The response to 10 μg was no higher than that to 5 μg suggesting these were maximally effective doses relative to inflammatory cell influx in this model.

APAP Attenuates the HDM-Inflammatory Response. To examine the effect of APAP on airway inflammation induced by minimally (1.25 μg) and maximally (10 μg) effective doses of HDM, mice were treated daily with APAP, or vehicle (control) one hour prior to HDM. APAP was administered at a non-hepatotoxic dose of 100 mg/kg based on our previous studies (Smith et al., 2016) which indicated that at this dose and time (1 hr) APAP causes depletion of the antioxidant GSH throughout the respiratory tract and potentiates the effects of another oxidant, ETS. APAP

was administered during either week 1 or week 2 of the HDM model and mice were euthanized on day 12 (Figure 1A). APAP was administered for no more than 5 days as this is the maximum recommended duration of treatment for children (Tylenol® Package Insert 2015). As observed previously (see Supplemental Figure 1), both the 1.25 and 10 µg doses of HDM resulted in significant influx of inflammatory cells in the BAL. Inflammatory cell levels in control and APAP groups were negligible. The eosinophilic, neutrophilic, and lymphocytic inflammatory cell response to the 1.25µg dose of HDM was significantly attenuated in animals treated with APAP during week 2 (Figure 1B). Macrophage levels averaged 1.6-2.8-fold over control in HDM (1.25 µg) and HDM (1.25 µg)-APAP groups but no statistical difference was detected among these groups. The attenuation of the HDM response was less pronounced at the 10µg dose level, and was less consistent across all cell types; however, significantly fewer eosinophils were observed in the APAP treated animals at this HDM dose (Figure 1B). For both HDM dose groups, APAP was more effective at attenuating the inflammatory response when given in week 2 than week 1. No significant differences were observed between the control and APAP groups with respect to macrophage numbers (Figure 1B). HDM at both doses significantly elevated BAL supernatant protein levels, a response blocked by APAP treatment during week 2 (Figure 1C).

Time Course of APAP's Effect on HDM response. To determine the time course of the effects of APAP on the HDM response, we performed an experiment in which groups of mice treated with HDM, or APAP (week 2) and HDM were euthanized on each day of the second week of the HDM model. The lungs of euthanized mice were lavaged and lung tissue samples were collected for determination of mRNA levels of selected genes by qRT-PCR. The genes included the TH2 cytokines *IL-4, IL-5 and IL-13,* the pro-inflammatory genes *Mip2* and *iNos,* genes associated

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with allergic airway disease, *CaSR* and *Muc5AC*, and genes associated with APAP bioactivation and oxidative stress response, *Cyp2E1* and *Nqo1*.

HDM alone induced a progressive increase in lavage inflammatory cell numbers during week 2 of this model (Figure 2). The eosinophil, neutrophil, and lymphocyte cellular influx caused by HDM was absent in mice treated with APAP from days 9 to 11 (Figure 2A-C). Macrophage levels were increased by approximately 2-fold by HDM and this was also attenuated by APAP (Figure 2D); however based on enlarged, highly vacuolated cytoplasm, activated macrophages were present in both the HDM and HDM+APAP groups. A slight, but not statistically significant increase in cell influx was observed on day 12 in the HDM+APAP group (Figure 2A-D). A significant increase in BAL fluid protein concentration was observed during week 2 of HDM treatment that was absent in the HDM+APAP treated group (Figure 2E). BAL protein levels between APAP alone exposure and control were not significantly different. Data from these groups were pooled for analysis. The cell counts and BAL supernatant protein levels on day 12 repeat the results of our previous experiments (See Figure 1B-D). With respect to the time course of the inflammatory response, the attenuating effects of APAP were fully apparent by day 10 of the model, the first day in which increased inflammatory cells were observed. (Figure 2A, B, and C).

Based on the inflammatory cell influx response data, lung tissues from animals euthanized on days 8, 10 and 12 were analyzed for gene expression. These represent days prior to an inflammatory cell influx (day 8), the first day in which a consistent increased in inflammatory cells occurs (day 10) and the last day of the model (day 12). For no genes were the expression levels in APAP treated mice different from control, therefore, the APAP alone and control groups were pooled before analysis. Several, but not all of the examined genes were significantly induced by HDM. Specifically, HDM resulted in 2-fold or more, statistically significant, induction of the TH2

cytokines *Il-4* and *Il-5*, the mucus forming *Muc5AC* gene, and the pro-inflammatory cytokine *Mip2* (Figure 3A-D). The effects of APAP on gene expression were apparent on day 12 of the models. Specifically, the expression of *Il-4* and *Mip2* was significantly lower in groups treated with APAP. The *Il-5* and *Muc5AC* responses were also attenuated by APAP but the effect did not attain statistical significance ($p=0.08$ and $p=0.053$, respectively). Notably, APAP was without effect on expression of any of these genes on day 10. The genes *Il-13, CaSR, iNos, Nqo1* (Supplemental Figure 2), and *Cyp2E1* (Figure 3E) were not significantly elevated over control levels by HDM and the degree of change was less than 1.5-fold in all cases. APAP itself did not alter the expression of these genes (Supplemental Figure 2). Interestingly, the expression of *Cyp2E1* was significantly lower than control in mice treated with HDM on day 12 of the model, a response prevented by APAP (Figure 3E).

Effect of CYP450 inhibition. To determine whether the attenuation of the HDM induced inflammatory response was due to the parent APAP molecule or a CYP450 metabolite, we performed a separate experiment in which mice were treated with the CYP450 inhibitor, 5-phenyl-1-pentyne (5PP). Were a CYP450 metabolite involved it would be anticipated that the attenuating effects of APAP would be absent in the 5PP treated mice. 5PP was administered 1 hr prior to APAP based on our previous studies (Morris, 2013; Smith et al., 2016) in which it was shown that 5PP at this dosage blocked the airway oxidant response to APAP. In the current study, 5-PP was administered for a maximum of two days (on day 8, and 9) to minimize the potential for toxicity, and maintain effective CYP inhibition. Mice were euthanized on day 10 as this is the first day of the model in which the attenuating effects of APAP on inflammation were observed (See Figure 2A-C). The effect of APAP is apparent in the 5PP treated groups with respect to decreased eosinophils and neutrophils (Figure 4A and B). For neither cell type was the response different

between the APAP and 5-PP APAP treated groups. 5PP alone may have caused a decrease in inflammation (Figure 4A and B). This decrease, however, was not statistically significant. In both the APAP and 5PP-APAP treated groups there are decreased lymphocytes, although no statistically significant differences were found (Figure 4C). Macrophage levels were increased to approximately 2-fold of control by HDM and fewer macrophages were observed in the APAP treated group, a response which was not different from the 5PP-APAP group (Figure 4D).

Histological Effects. To confirm the results of previous endpoints, a qualitative histological assessment of lungs from HDM exposed mice with or without APAP treatment, and controls was performed. No inflammation was apparent in vehicle- or APAP-treated control mice (Figures 5A and B). A focal and moderate degree of lung inflammation was present in HDM exposed mice (Figure 5C). Macrophages, and polymorphonuclear cells were observed in perivascular and peribronchiolar clusters distributed throughout the majority of the lung lobes. Fewer clusters of inflammatory cells were observed in APAP treated groups, and some lobes did not have any indication of inflammation (Figure 5D). Both HDM, and APAP+HDM groups had increased small airway PAS staining indicative of enhanced mucus production (Figures 5G and H). The degree of PAS staining appeared to be somewhat reduced in the APAP+HDM groups (Figure 5H). No evidence of increased PAS staining was observed in the control groups (Figures 5E and F).

Effect of APAP on HDM Specific Serum Immunoglobulin. HDM-specific IgE and IgG1 was not detectable in sera of mice on day 12 of the model in any treatment group (control, APAP, HDM or HDM+APAP) (Data not shown).

Discussion

The epidemiological evidence suggesting APAP use may influence the pathogenesis of asthma is controversial and the plausibility of the model has not been tested in animal models. Therefore, the objective of this study was to evaluate the effects of APAP in a murine HDM model of allergic airway disease. HDM produced a robust and dose dependent increase in inflammation as measured by increased airway eosinophilia, and neutrophilia similar to that observed by other investigators using HDM models (Bracken et al., 2015; Cates et al., 2004). An approximately 2-fold increase in airway macrophages was consistently observed in our model, however the change was not uniformly detected as statistically significant. Previous studies observed a maximal response at a 25 µg dose (Cates et al., 2004). The response to HDM in our model peaked at a 10 µg dose, likely influenced by a difference in the day on which BAL was performed (day 12 in our model, compared to day 15 in the Cates *et al.*, 2004 study), and perhaps differences in HDM extract composition (LPS, and protein levels). In preliminary studies, using 1.25 µg and 10 µg doses of HDM, neutrophil and eosinophil numbers were lower on day 15 than day 12-signaling some resolution of inflammation over two days without exposure. Therefore, BAL was performed on day 12 for the majority of these studies to examine the progression of inflammation.

We hypothesized that APAP would enhance the response to HDM based on our previous studies which indicated that APAP was an airway pro-oxidant (Smith et al., 2016). Unexpectedly, APAP caused a marked attenuation of the HDM response. A recently published report has shown that, in mice, maternal exposure to APAP during pregnancy and lactation did not enhance HDM induced allergic airway disease in offspring (Lee et al., 2015). Our study is also consistent with a lack of causality between APAP and asthma as proposed by Heintze and Petersen (2013).

Moreover, our results suggest APAP may actually have a therapeutic role relative to asthma pathogenesis.

In our model, APAP was more effective at blocking the HDM response at the 1.25µg HDM dose level, than the 10µg HDM dose level suggesting that the attenuation might be overwhelmed at higher antigen burdens. APAP was more effective during week 2 of the HDM model despite the fact that mice had been previously exposed/sensitized to antigen for a week. This suggests that APAP has a greater effect on the exacerbation compared to the induction of allergic inflammation. The inflammatory endpoints we measured, airway eosinophilia and neutrophilia in particular, are the result of differentially regulated processes (Lambrecht and Hammad, 2015). Therefore, the lack of an increase in any of these cell types, or in BAL protein, after APAP treatment is suggestive of a general anti-inflammatory effect. This is unexpected because APAP is thought to have weak anti-inflammatory effects (Graham et al., 2013). Furthermore, on day 12 APAP blocked the attenuating effect of HDM on *Cyp2E1* expression, a gene that is not directly related to the HDM inflammatory response, but is known to decrease in other allergic airway disease models (Stoilov et al., 2006).

Mild to moderate peribronchiolar and perivascular inflammation was observed in HDM-treated mice. Clusters of inflammatory cells were absent or less apparent in APAP treated mice, confirming the decreased BAL inflammatory cell response of APAP treated mice. The degree of inflammation observed in this study was less than that observed in our previous studies which used a higher dose of HDM (Bracken et al., 2015). However, a similar perivascular and peribronchiolar clustering pattern was observed in both studies. Neither HDM-specific IgG1 nor IgE was detected in sera of HDM treated mice. Bracken reports low levels of these antibodies on day 15 of their model that used 25 µg intranasal instillation of HDM (5 days per week). The lack of IgG1 and

IgE may be due to the shorter time period (12 vs 15 days) and lower HDM dosage (10 vs 25 µg) in the current study.

The gene expression analyses were intended to determine specific gene expression patterns caused by both HDM and the combination of HDM and APAP treatment. *Il-4*, *Il-5*, and *Il-13* were chosen as they are primary effector cytokines of the Th2-type allergic response (Lambrecht and Hammad, 2015). Associated with both allergic and non-allergic inflammatory responses, *Muc5AC, Mip2, and iNos* are effectors of increased mucus glycoprotein production, neutrophil influx, and airway and vascular smooth muscle tone, respectively (Coleman, 2002; Evans et al., 2015; Hamid et al., 1993; Wolpe et al., 1989). *Cyp2E1* is one of the primary cytochrome P450 enzymes responsible for the biotransformation of APAP into NAPQI, while *Nqo1* is involved in the detoxification of NAPQI (Hinson et al., 2010; Moffit et al., 2007). Importantly, APAP appears to have attenuated the increase in all genes that were increased by HDM. This was indicated by statistically significant decreases in *Il-4*, and *Mip2*, and slight decreases in *Il-5* (p=0.08) and *Muc5AC* (p=0.053) on day 12. The slight decrease in *Muc5AC* gene expression did not translate to a marked difference mucus in the qualitative histological assessment perhaps due to the timing of *Muc5AC* induction relative to tissue collection or the influence of other mucin proteins such as *Muc5b* which were not examined. No other clear changes were observed except a decrease in *Cyp2E1* with HDM, which was reversed by APAP on day 12.

CaSR may be a gatekeeper of airway hyperresponsiveness and inflammation in allergic asthma (Yarova et al., 2015). Yarova et al. observed that *CaSR* mRNA and protein expression were elevated in asthmatic humans, and mice and treatment with CaSR antagonists blocked the characteristic features of asthma in their mixed allergen (combination of OVA, and extracts of HDM, *Alternaria*, and *Aspergillus*) model. We did not observe any significant changes in *CaSR*

expression with HDM exposure in our studies. Additionally APAP attenuated the response to HDM without producing any alteration in *CaSR* expression. These results suggest that CaSR may not be the key to inflammation in all models of asthma, and may not be critical to HDM allergen sensitization.

The mechanism of APAP's effect on the response to HDM is unknown. That 5PP did not block the effect of APAP suggests the effect is not due to a CYP metabolite, but more likely the parent compound or a (non-CYP metabolite). This explains the apparent discordance between the current results and our previous studies in which the acute pro-oxidant effects of APAP were blocked by 5PP (Smith et al., 2016). It is possible that the repeat dosing paradigm in the current study has an anti-oxidant effect in the airways through upregulation of NRF2 (O'Connor et al., 2014). However, this seems unlikely because the NRF2 mediated gene *Nqo1* was not induced throughout the time course. Our previous study demonstrated an acute pro-oxidant interaction between APAP and ETS. Perhaps rather than promoting asthma pathogenesis directly, APAP may predispose individuals to other pro-oxidant asthma causative factors such as ETS (Smith et al., 2016). The potential for APAP to potentiate the effects of other airborne oxidants such as ETS was not investigated here, and therefore remains an important consideration.

There were no differences in cytokine expression, or BAL protein levels between the HDM and APAP+HDM groups on day 10, a time at which significant changes in BAL cellularity were observed. Thus, the changes in gene expression lagged the changes in cellularity suggesting that the cytokine expression following APAP treatment is most likely an effect rather than the cause of the decreased inflammation by APAP. A possible mechanism, is that APAP could be influencing the trafficking of immune cells. This concept is supported by an apparent differential effect of APAP on resident versus recruited cells. Specifically, APAP exerted profound effects on cells that

are largely recruited to the lungs (eosinophils, neutrophils, lymphocytes), whereas lesser effects were seen on resident cells. Macrophage numbers were decreased by APAP, but the effect was not as great as for eosinophils or neutrophils and did not consistently achieve statistical significance. Similarly, the effect of APAP on mucus cell metaplasia appeared to be of lesser magnitude. Decreased immune cell migration could be caused by activation of the cannabinoid receptor system by APAP; either by diverting metabolism of arachidonic acid to endocannabinoids through inhibition of PG synthesis, or through a direct effect of its recently discovered metabolite and anandamide analogue, AM404 (Graham et al., 2013). Cannabinoid receptor activity has been shown to decrease the recruitment of immune cells (Lunn et al., 2006). Activation of the cannabinoid system also enhances immune cell apoptosis in mice (Rieder et al., 2010). This effect may also be involved in the attenuation of the HDM model by APAP. However, inflammatory cell numbers were never increased in the APAP treated group suggesting that an effect on cell trafficking into the lungs is more likely than an enhanced apoptosis of cells within the lungs.

In conclusion, we provide direct biological data which do not support the APAP hypothesis in asthma, a result in agreement with recent epidemiological and animal studies. It is possible the association between APAP use and asthma can be explained by confounding factors. Importantly, our results indicate that HDM-induced allergic airway disease is markedly attenuated by APAP. Indicating the possibility of a novel therapeutic effect of APAP. More studies will be needed to confirm these results in other models of airway inflammation and asthma, and to determine the mechanism of the observed effects. The mechanistic insights provided by the modulation of allergic airway disease by APAP may provide novel information on the pathogenesis of asthma, and potential novel therapeutic targets.

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Author Contributions

Participated in research design: Smith, Thrall, Cloutier, Manautou, and Morris.

Conducted experiments: Smith.

Performed data analysis: Smith.

Wrote or contributed to the writing of the manuscript: Smith and Morris.

References

Andreadis AA, Hazen SL, Comhair SA, Erzurum SC. (2003) Oxidative and nitrosative events in asthma. *Free Radic Biol Med* **35**:213-225.

Barr RG, Wentowski CC, Curhan GC, Somers SC, Stampfer MJ, Schwartz J, Speizer FE, Camargo CA,Jr. (2004) Prospective study of acetaminophen use and newly diagnosed asthma among women. *Am J Respir Crit Care Med* **169**:836-841.

Beasley RW, Clayton TO, Crane J, Lai CK, Montefort SR, Mutius E, Stewart AW, ISAAC Phase Three Study Group. (2011) Acetaminophen use and risk of asthma, rhinoconjunctivitis, and eczema in adolescents: International study of asthma and allergies in childhood phase three. *Am J Respir Crit Care Med* **183**:171-178.

Bhalla DK, Hirata F, Rishi AK, Gairola CG. (2009) Cigarette smoke, inflammation, and lung injury: A mechanistic perspective. *J Toxicol Environ Health B Crit Rev* **12**:45-64.

Botelho FM, Llop-Guevara A, Trimble NJ, Nikota JK, Bauer CM, Lambert KN, Kianpour S, Jordana M, Stampfli MR. (2011) Cigarette smoke differentially affects eosinophilia and remodeling in a model of house dust mite asthma. *Am J Respir Cell Mol Biol* **45**:753-760.

Boulet LP, Turcotte H, Laprise C, Lavertu C, Bedard PM, Lavoie A, Hebert J. (1997) Comparative degree and type of sensitization to common indoor and outdoor allergens in subjects with allergic rhinitis and/or asthma. *Clin Exp Allergy* **27**:52-59.

Bracken SJ, Adami AJ, Szczepanek SM, Ehsan M, Natarajan P, Guernsey LA, Shahriari N, Rafti E, Matson AP, Schramm CM, Thrall RS. (2015) Long-term exposure to house dust mite leads to the suppression of allergic airway disease despite persistent lung inflammation. *Int Arch Allergy Immunol* **166**:243-258.

Calderon MA, Linneberg A, Kleine-Tebbe J, De Blay F, Hernandez Fernandez de Rojas D, Virchow JC, Demoly P. (2014) Respiratory allergy caused by house dust mites: What do we really know? *J Allergy Clin Immunol* .

Cates EC, Fattouh R, Johnson JR, Llop-Guevara A, Jordana M. (2007) Modeling responses to respiratory house dust mite exposure. *Contrib Microbiol* **14**:42-67.

Cates EC, Fattouh R, Wattie J, Inman MD, Goncharova S, Coyle AJ, Gutierrez-Ramos JC, Jordana M. (2004) Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *J Immunol* **173**:6384-6392.

Coleman JW. (2002) Nitric oxide: A regulator of mast cell activation and mast cell-mediated inflammation. *Clin Exp Immunol* **129**:4-10.

Etminan M, Sadatsafavi M, Jafari S, Doyle-Waters M, Aminzadeh K, Fitzgerald JM. (2009) Acetaminophen use and the risk of asthma in children and adults: A systematic review and metaanalysis. *Chest* **136**:1316-1323.

Evans CM, Raclawska DS, Ttofali F, Liptzin DR, Fletcher AA, Harper DN, McGing MA, McElwee MM, Williams OW, Sanchez E, Roy MG, Kindrachuk KN, Wynn TA, Eltzschig HK, Blackburn MR, Tuvim MJ, Janssen WJ, Schwartz DA, Dickey BF. (2015) The polymeric mucin Muc5ac is required for allergic airway hyperreactivity. *Nat Commun* **6**:6281.

Gilmour MI, Jaakkola MS, London SJ, Nel AE, Rogers CA. (2006) How exposure to environmental tobacco smoke, outdoor air pollutants, and increased pollen burdens influences the incidence of asthma. *Environ Health Perspect* **114**:627-633.

Graham GG, Davies MJ, Day RO, Mohamudally A, Scott KF. (2013) The modern pharmacology of paracetamol: Therapeutic actions, mechanism of action, metabolism, toxicity and recent pharmacological findings. *Inflammopharmacology* **21**:201-232.

Gu J, Cui H, Behr M, Zhang L, Zhang QY, Yang W, Hinson JA, Ding X. (2005) In vivo mechanisms of tissue-selective drug toxicity: Effects of liver-specific knockout of the NADPH-cytochrome P450 reductase gene on acetaminophen toxicity in kidney, lung, and nasal mucosa. *Mol Pharmacol* **67**:623-630.

Hamid Q, Springall DR, Riveros-Moreno V, Chanez P, Howarth P, Redington A, Bousquet J, Godard P, Holgate S, Polak JM. (1993) Induction of nitric oxide synthase in asthma. *Lancet* **342**:1510-1513.

Hart SG, Cartun RW, Wyand DS, Khairallah EA, Cohen SD. (1995) Immunohistochemical localization of acetaminophen in target tissues of the CD-1 mouse: Correspondence of covalent binding with toxicity. *Fundam Appl Toxicol* **24**:260-274.

Heintze K and Petersen KU. (2013) The case of drug causation of childhood asthma: Antibiotics and paracetamol. *Eur J Clin Pharmacol* **69**:1197-1209.

Henricks PA and Nijkamp FP. (2001) Reactive oxygen species as mediators in asthma. *Pulm Pharmacol Ther* **14**:409-420.

Hinson JA, Roberts DW, James LP. (2010) Mechanisms of acetaminophen-induced liver necrosis. *Handb Exp Pharmacol* **(196):369-405. doi**:369-405.

Holguin F. (2013) Oxidative stress in airway diseases. *Ann Am Thorac Soc* **10 Suppl**:S150-7.

Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, Coyle AJ, Gutierrez-Ramos JC, Ellis R, Inman MD, Jordana M. (2004) Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am J Respir Crit Care Med* **169**:378-385.

Lambrecht BN and Hammad H. (2015) The immunology of asthma. *Nat Immunol* **16**:45-56.

Lanckacker EA, Tournoy KG, Hammad H, Holtappels G, Lambrecht BN, Joos GF, Maes T. (2013) Short cigarette smoke exposure facilitates sensitisation and asthma development in mice. *Eur Respir J* **41**:1189-1199.

Lee DC, Walker SA, Byrne AJ, Gregory LG, Buckley J, Bush A, Shaheen SO, Saglani S, Lloyd CM. (2015) Perinatal paracetamol exposure in mice does not affect the development of allergic airways disease in early life. *Thorax* **70**:528-536.

Livak KJ and Schmittgen TD. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* **25**:402-408.

Lowe AJ, Carlin JB, Bennett CM, Hosking CS, Allen KJ, Robertson CF, Axelrad C, Abramson MJ, Hill DJ, Dharmage SC. (2010) Paracetamol use in early life and asthma: Prospective birth cohort study. *Bmj* **341**:c4616.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* **193**:265-275.

Lunn CA, Fine JS, Rojas-Triana A, Jackson JV, Fan X, Kung TT, Gonsiorek W, Schwarz MA, Lavey B, Kozlowski JA, Narula SK, Lundell DJ, Hipkin RW, Bober LA. (2006) A novel cannabinoid peripheral cannabinoid receptor-selective inverse agonist blocks leukocyte recruitment in vivo. *J Pharmacol Exp Ther* **316**:780-788.

Maes T, Provoost S, Lanckacker E, Cataldo D, Vanoirbeek J, Nemery B, Tournoy K, Joos G. (2010) Mouse models to unravel the role of inhaled pollutants on allergic sensitization and airway inflammation. *Respiratory Research* **11**:7.

McGill MR and Jaeschke H. (2013) Metabolism and disposition of acetaminophen: Recent advances in relation to hepatotoxicity and diagnosis. *Pharm Res* **30**:2174-2187.

Mitchell VL, Van Winkle LS, Gershwin LJ. (2012) Environmental tobacco smoke and progesterone alter lung inflammation and mucous metaplasia in a mouse model of allergic airway disease. *Clin Rev Allergy Immunol* **43**:57-68.

Moffit JS, Aleksunes LM, Kardas MJ, Slitt AL, Klaassen CD, Manautou JE. (2007) Role of NAD(P)H:Quinone oxidoreductase 1 in clofibrate-mediated hepatoprotection from acetaminophen. *Toxicology* **230**:197-206.

Morris JB. (2013) Nasal dosimetry of inspired naphthalene vapor in the male and female B6C3F1 mouse. *Toxicology* **309**:66-72.

O'Connor MA, Koza-Taylor P, Campion SN, Aleksunes LM, Gu X, Enayetallah AE, Lawton MP, Manautou JE. (2014) Analysis of changes in hepatic gene expression in a murine model of tolerance to acetaminophen hepatotoxicity (autoprotection). *Toxicol Appl Pharmacol* **274**:156-167.

PubChem 1983. National center for biotechnology information. PubChem compound database; CID=1983, https://Pubchem.ncbi.nlm.nih.gov/compound/1983 (accessed june 30, 2016).

PubChem 74573. National center for biotechnology information. PubChem compound database; CID=74573, https://Pubchem.ncbi.nlm.nih.gov/compound/74573 (accessed june 30, 2016).

Rangasamy T, Guo J, Mitzner WA, Roman J, Singh A, Fryer AD, Yamamoto M, Kensler TW, Tuder RM, Georas SN, Biswal S. (2005) Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. *J Exp Med* **202**:47-59.

Rieder SA, Chauhan A, Singh U, Nagarkatti M, Nagarkatti P. (2010) Cannabinoid-induced apoptosis in immune cells as a pathway to immunosuppression. *Immunobiology* **215**:598-605.

Riedl MA and Nel AE. (2008) Importance of oxidative stress in the pathogenesis and treatment of asthma. *Curr Opin Allergy Clin Immunol* **8**:49-56.

Smith GJ, Cichocki JA, Doughty BJ, Manautou JE, Jordt SE, Morris JB. (2016) Effects of acetaminophen on oxidant and irritant respiratory tract responses to environmental tobacco smoke in female mice. *Environ Health Perspect* **124**:642-650.

Sordillo JE, Scirica CV, Rifas-Shiman SL, Gillman MW, Bunyavanich S, Camargo CA,Jr, Weiss ST, Gold DR, Litonjua AA. (2015) Prenatal and infant exposure to acetaminophen and ibuprofen and the risk for wheeze and asthma in children. *J Allergy Clin Immunol* **135**:441-448.

Stoilov I, Krueger W, Mankowski D, Guernsey L, Kaur A, Glynn J, Thrall RS. (2006) The cytochromes P450 (CYP) response to allergic inflammation of the lung. *Arch Biochem Biophys* **456**:30-38.

Williams MA, Rangasamy T, Bauer SM, Killedar S, Karp M, Kensler TW, Yamamoto M, Breysse P, Biswal S, Georas SN. (2008) Disruption of the transcription factor Nrf2 promotes pro-oxidative dendritic cells that stimulate Th2-like immunoresponsiveness upon activation by ambient particulate matter. *J Immunol* **181**:4545-4559.

Wolpe SD, Sherry B, Juers D, Davatelis G, Yurt RW, Cerami A. (1989) Identification and characterization of macrophage inflammatory protein 2. *Proc Natl Acad Sci U S A* **86**:612-616.

Yarova PL, Stewart AL, Sathish V, Britt RD,Jr, Thompson MA, P Lowe AP, Freeman M, Aravamudan B, Kita H, Brennan SC, Schepelmann M, Davies T, Yung S, Cholisoh Z, Kidd EJ, Ford WR, Broadley KJ, Rietdorf K, Chang W, Bin Khayat ME, Ward DT, Corrigan CJ, T Ward JP, Kemp PJ, Pabelick CM, Prakash YS, Riccardi D. (2015) Calcium-sensing receptor antagonists abrogate airway hyperresponsiveness and inflammation in allergic asthma. *Sci Transl Med* **7**:284ra60.

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Figure Legends

Figure 1. APAP attenuates the inflammatory response to HDM. (A) HDM model timeline with APAP administration either for 5 days during week 1, or 4 days during week 2. Mice were administered either a 1.25 or 10μg dose of HDM in either of the weeks of the 2 week protocol. Mice were euthanized and BAL was performed on day 12. Black arrows above the timeline represent days mice received doses of HDM and or APAP. (B) BAL cells of mice administered either vehicle control (HDM Control), APAP during week 1 (HDM+APAP Wk1), or APAP during week 2 (HDM+APAP Wk2), and a 1.25 μg or 10 μg dose of HDM (i.n.). Differential cell count data are presented as mean cells/ml $\pm$ SEM (n=5 mice/group, except for HDM+APAP Wk2 1.25 μg where n=4). (C) Protein levels represented as mean percent of control $\pm$ SEM in BAL fluid supernatants (n=5 mice/group). The black line at y=100% represents the mean of the pooled control values (n=10 mice/group) and the dotted lines represent $\pm$ the SEM. Controls averaged 116.6 $\pm$ 4.0 μg/mL total protein. Data were analyzed by ANOVA followed by Newman-Keuls test. Separate analyses were performed for the 1.25 μg and 10 μg dose groups. Groups with differing superscripts differ from each other at the $p<0.05$ level. All data are representative of two independent experiments.

Figure 2. Time course of the effect of APAP on the HDM induced inflammatory response. Mice were administered APAP during week 2 (days 8-11), along with a 1.25 μg dose of HDM following the same dosing regimen as in Figure 2A. BAL fluid was collected from separate groups of mice during each day of week 2. (A-D) BAL cell data are presented as mean cells/ml $\pm$ SEM (n=4-6 mice/group). (* $p<0.05$ compared to HDM; ANOVA with Newman-Keuls test). No inflammatory cell types were observed in control groups. (E) Protein levels in BAL fluid supernatants represented as mean percent of control $\pm$ SEM (n=5 mice/group). The vehicle control and APAP

alone groups were not significantly different, therefore they were pooled to form the control group (black line at y=100%, n=10 mice/group). Controls averaged 113.1 ± 4.2 µg/mL total protein. Dotted lines represent control mean ± SEM. Data were analyzed by ANOVA followed by Newman-Keuls test; line and bars with differing superscripts differ from each other at the $p<0.05$ level. All data are representative of two independent experiments.

Figure 3. Time courses of gene induction in the lung for selected genes modulated by HDM and APAP. (A) *Il-4*, (B) *Il-5*, (C) *Muc5AC*, (D) *Mip2* (E) *Cyp2E1*. Lung tissue was collected from groups of mice on days 8, 10, and 12 of the time course experiment. For all genes analyzed, the vehicle control and APAP alone groups did not differ significantly, therefore they were combined to form pooled control group for statistical analysis. Individual gene names are indicated at the top of each figure, and data are presented as mean fold increase ± SEM, over pooled control (black line at y=1). Dotted line represents control mean ± SEM. Line and bars with differing superscripts differ at the $p<0.05$ level (ANOVA with Newman-keuls test). Data are representative of two independent experiments (n=4-6 mice/group).

Figure 4. Effect of CYP450 inhibition on attenuation of HDM response by APAP. Treatment of animals with 5-PP one hour prior to APAP treatment began once per day on day 8 and ended on day 9. BAL was performed on day 10. A 1.25 µg dose of HDM was used. (A-D) Differential cell count data are presented as mean cells/ml ± SEM (n=4-6 mice/group). Bars with differing superscripts are differ at the $p<0.05$ level (ANOVA with Newman-Keuls test). No inflammatory cell types were observed in control groups and data are representative of two independent experiments.

Figure 5. HDM-exposed mice demonstrate moderate perivascular/peribronchiolar inflammation which is largely absent in APAP-treated mice. Lungs were fixed in formalin, sectioned, and stained with H&E or PAS. (A-D) Arrows indicate examples of perivascular/peribronchiolar inflammation. H&E. x200. (E-H) Arrows indicate examples of mucus production by airway goblet cells. PAS x200. Images are representative of lungs from each group indicated above each image column, and two independent experiments (n=3 mice/group).

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Tables

Table 1. Mouse gene names and functions for qRT-PCR analysis.

Gene	*Name*	*Function*	*Reference*
Il-4	Interleukin 4	Th2 type cytokine response	(Lambrecht and Hammad, 2015)
Il-5	Interleukin 5	Th2 type cytokine response	(Lambrecht and Hammad, 2015)
Il-13	Interleukin 13	Th2 type cytokine response	(Lambrecht and Hammad, 2015)
CaSR	Calcium sensing receptor	Airway inflammation, hyperresponsiveness	(Yarova et al., 2015)
Muc5AC	Mucin 5AC	Airway mucus gel formation	(Evans et al., 2015)
Mip2	Macrophage inflammatory protein 2	Neutrophil chemotactic factor	(Wolpe et al., 1989)
iNos	Inducible nitric oxide synthase	Mast cell activation, smooth muscle tone	(Coleman, 2002)
Cyp2E1	Cytochrome P450, family 2, subfamily E	APAP metabolism to NAPQI	(Hinson et al., 2010)
Nqo1	NAD(P)H dehydrogenase, quinone 1	Detoxification of NAPQI	(Moffit et al., 2007)

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Table 2. Mouse primer sequences for qRT-PCR (listed 5'-3')

Gene	***Forward***	***Reverse***
β-Actin	GCAACGAGCGGTTCCG	CCCAAGAAGGAAGGCTGGA
Il-4	CGAGCTCACTCTCTGTGGTG	TGAACGAGGTCACAGGAGAA
Il-5	CTCTGTTGACAAGCAATGAGACG	TCTTCAGTATGTCTAGCCCCTG
Il-13	CCTGGCTCTTGCTTGCCTT	GGTCTTGTGTGATGTTGCTCA
CaSR	AGCAGGTGACCTTCGATGAGT	ACTTCCTTGAACACAATGGAGC
Muc5AC	CTGTGACATTATCCCATAAGCCC	AAGGGGTATAGCTGGCCTGA
Mip2	CCAACCACCAGGCTACAGG	GCGTCACACTCAAGCTCTG
iNos	TGAAGAAAACCCCTTGTGCT	TTCTGTGCTGTCCCAGTGAG
Cyp2E1	GGGACATTCCTGTGTTCCAG	CTTAGGGAAAACCTCCGCAC
Nqo1	TTTAGGGTCGTCTTGGCAAC	GTCTTCTCTGAATGGGCCAG

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Figure 1

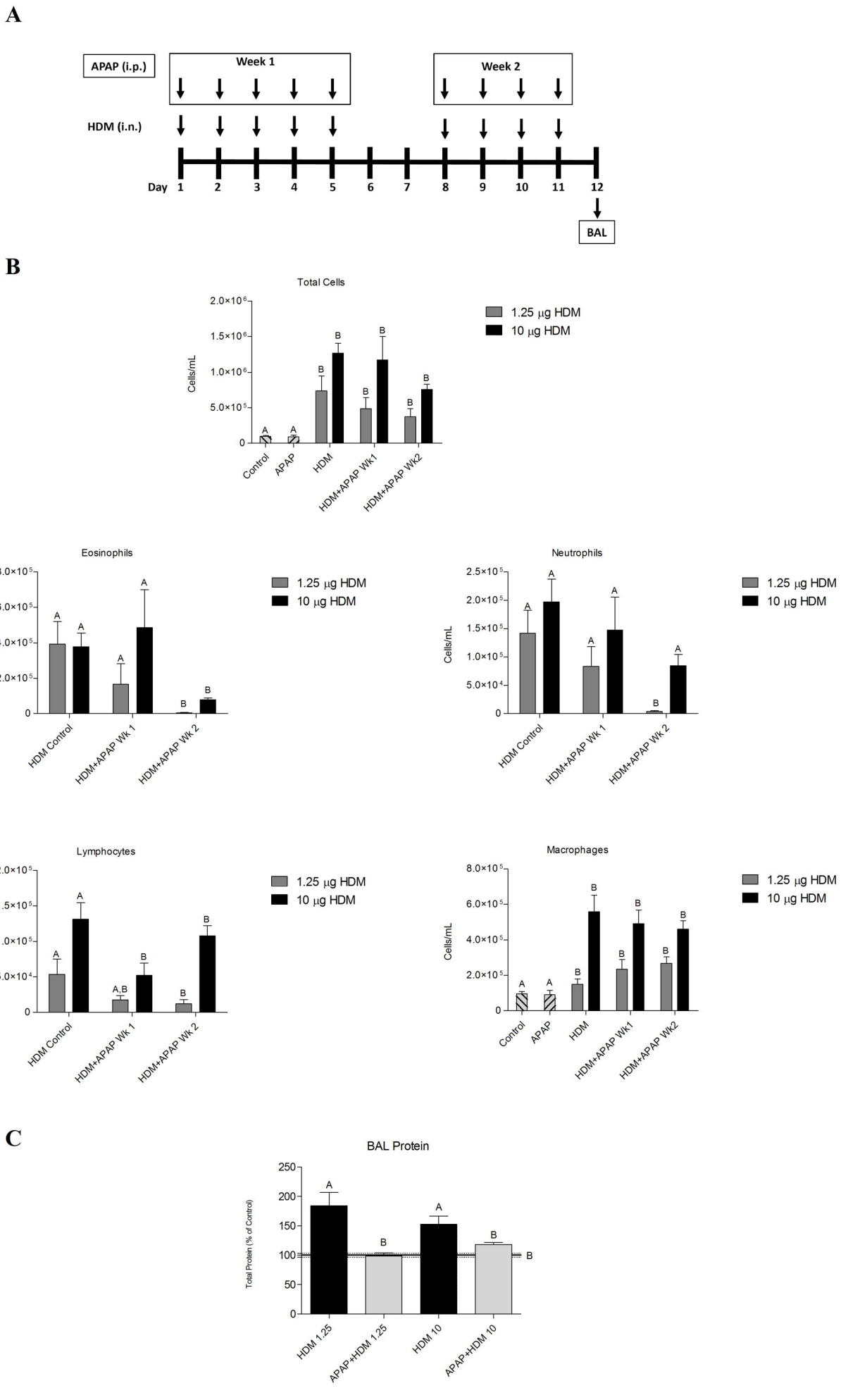

Figure 2

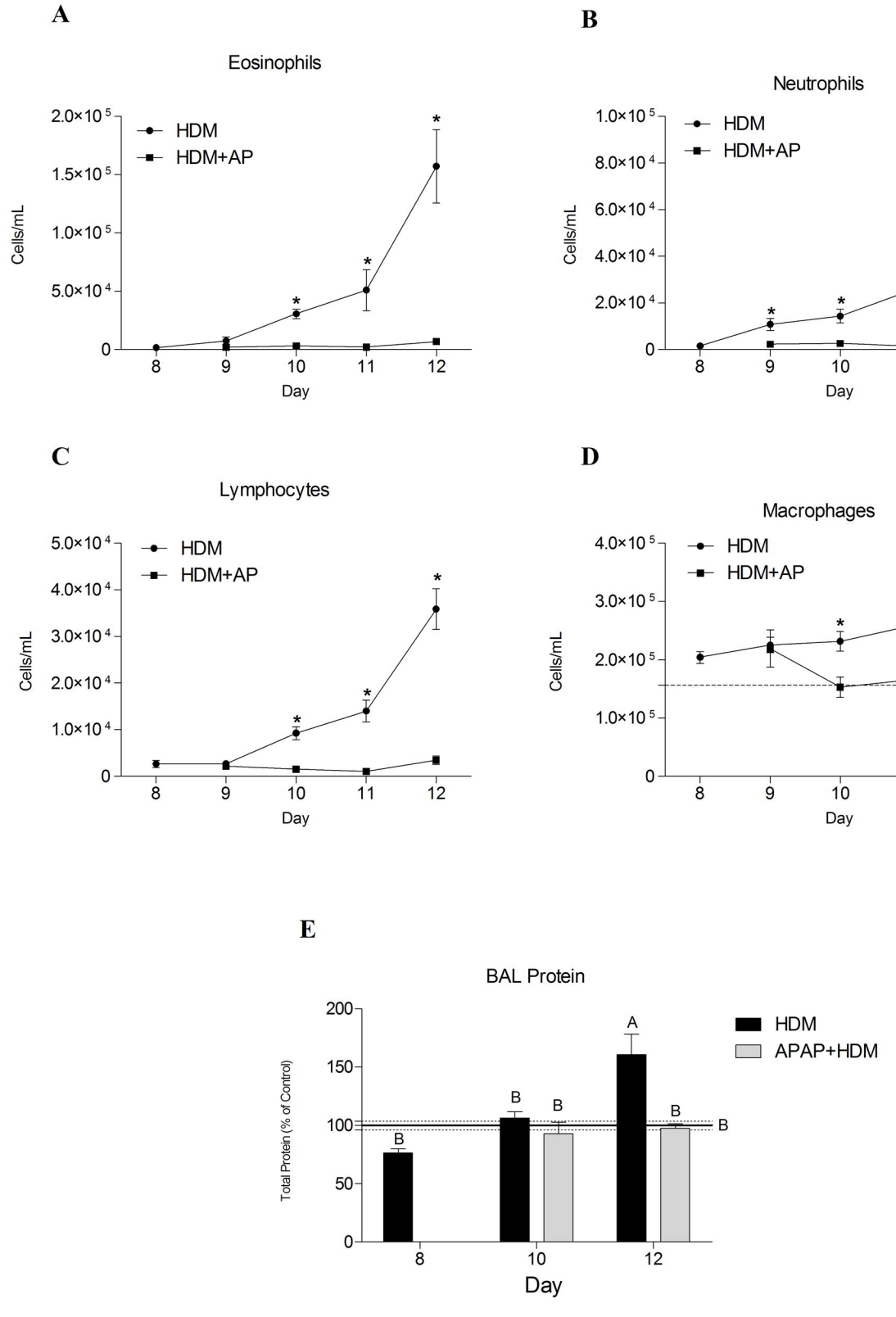
A
Eosinophils
HDM
HDM+AP
Cells/mL
Day
B
Neutrophils
C
Lymphocytes
D
Macrophages
E
BAL Protein
Total Protein (% of Control)
APAP+HDM

Figure 3

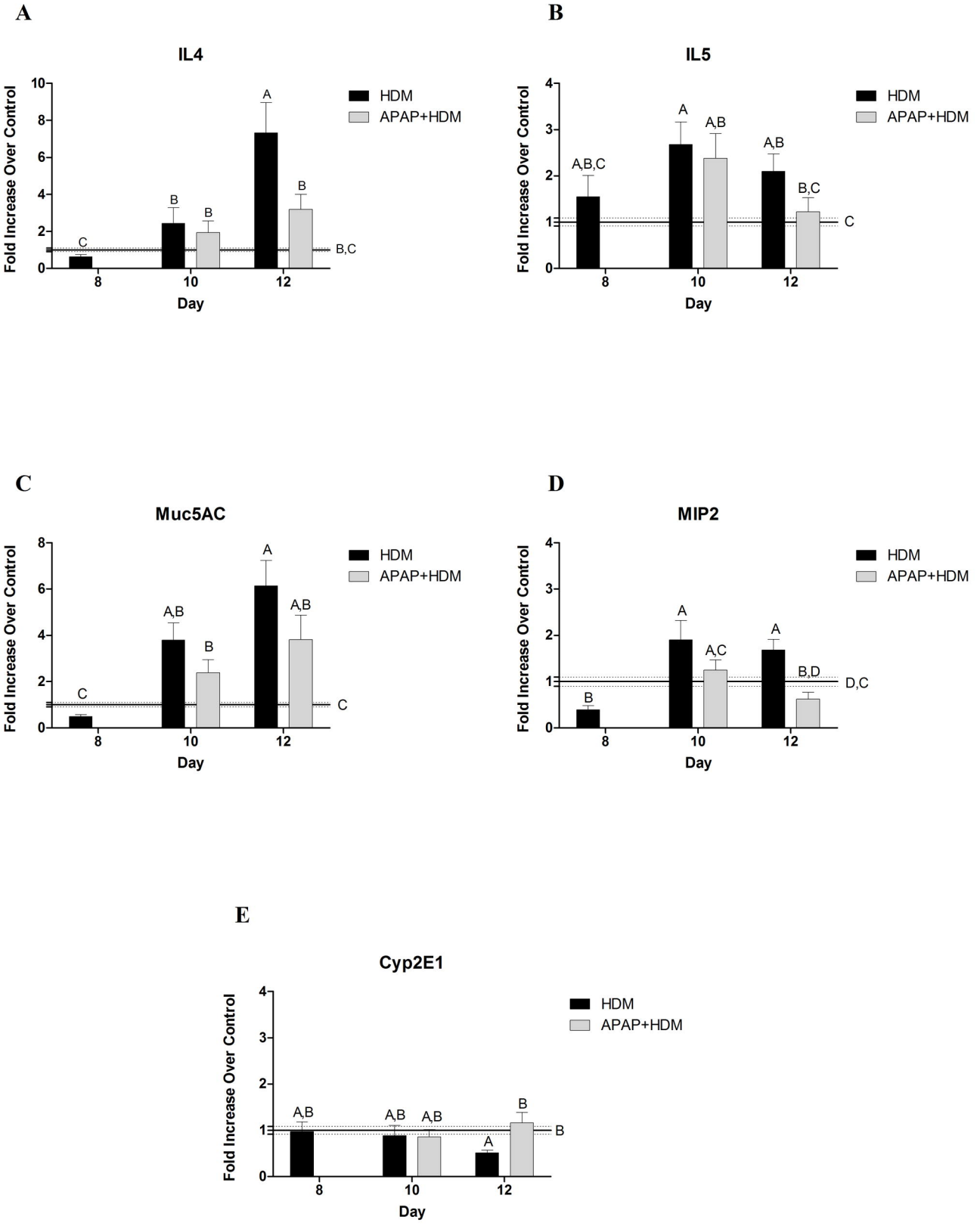
A
IL4
Fold Increase Over Control
Day
HDM
APAP+HDM
C
B
B
A
B
B,C
B
IL5
A,B,C
A
A,B
A,B
B,C
C
C
Muc5AC
A,B
B
A
A,B
C
C
MIP2
B
A
A,C
A
B,D
D,C
E
Cyp2E1
A,B
A,B
A,B
A
B
B

Figure 4

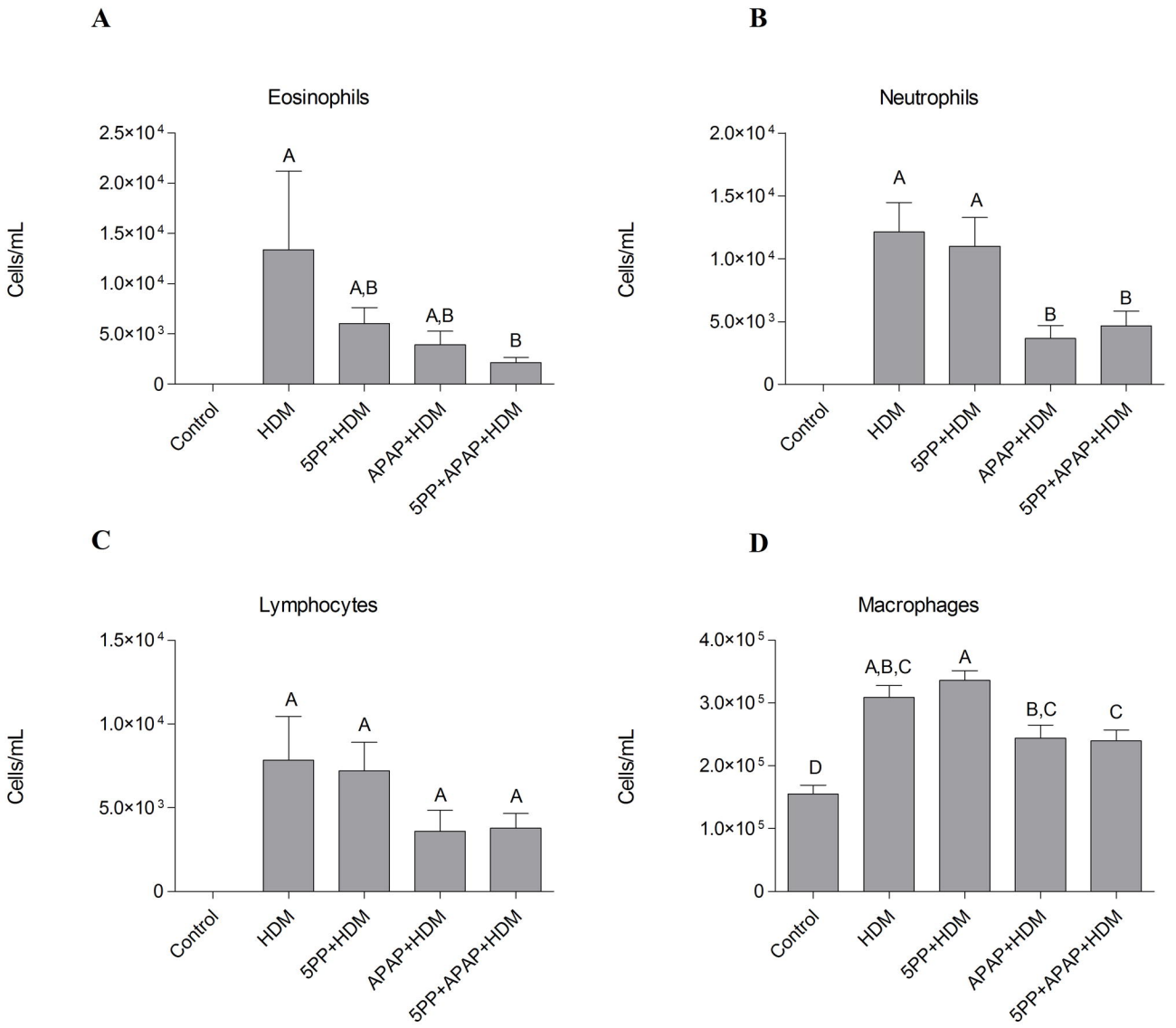
A
Eosinophils
Cells/mL
2.5×10⁴
2.0×10⁴
1.5×10⁴
1.0×10⁴
5.0×10³
0
A
A,B
A,B
B
Control
HDM
5PP+HDM
APAP+HDM
5PP+APAP+HDM
B
Neutrophils
Cells/mL
2.0×10⁴
1.5×10⁴
1.0×10⁴
5.0×10³
0
A
A
B
B
Control
HDM
5PP+HDM
APAP+HDM
5PP+APAP+HDM
C
Lymphocytes
Cells/mL
1.5×10⁴
1.0×10⁴
5.0×10³
0
A
A
A
A
Control
HDM
5PP+HDM
APAP+HDM
5PP+APAP+HDM
D
Macrophages
Cells/mL
4.0×10⁵
3.0×10⁵
2.0×10⁵
1.0×10⁵
0
D
A,B,C
A
B,C
C
Control
HDM
5PP+HDM
APAP+HDM
5PP+APAP+HDM

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Figure 5