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Article type : Letter to the Editor

To the Editor:

Transgenic mice overexpressing the PGE₂ receptor EP₂ on mast cells exhibit a protective phenotype in a model of allergic asthma

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Pharmacological inhibition of airway mast cell (MC) activity has received considerable attention as a plausible anti-asthma strategy. Activation of the MC prostaglandin E₂ (PGE₂) receptor E prostanoid 2 (EP₂) may drive this potentially therapeutic mechanism. Indeed, specific EP₂ activation inhibits both murine¹ and human² airway MC activity *in vitro*. Furthermore, EP₂ agonism-mediated reduction of airway pathology has been linked to restricted MC activity in human³ and in murine⁴ models, and we reported up-regulation of PGE₂ and of EP₂ mRNA, but not EP₁, EP₃, and EP₄, upon exposure of mice to house dust mite (HDM)⁵. In light of these data, we hypothesized that the EP₂-mediated improvement observed in mouse models of asthma was a consequence of reduced MC activity, and that the PGE₂-EP₂-MC axis acts as an endogenous protective mechanism⁶. To bridge this knowledge gap, we have created transgenic (TG) mice overexpressing the EP₂ receptor on MCs and have studied the response of mice sensitized to HDM aeroallergens (protocol in Supp. Fig. 1). To generate the TG colony, a construct containing the MC-specific *Cma1* promoter and the EP₂ receptor coding region (*Ptger2*) was microinjected into C57BL/6OlaHsd zygotes. The TG mice were backcrossed to obtain mice overexpressing EP₂ on a BALB/c genetic background (see details in the Repository Material and Suppl. Figs. 2–4). All animals were bred at the Animal Facility of the UAB, and the procedures were approved by the Ethics Committee for Animal

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Research. To our knowledge, this is the first report of a mouse line constitutively overexpressing EP₂ selectively on MCs.

To assess the impact of MC-specific EP₂ overexpression on airway MC activity, murine chemokine mouse MC protease 1 (mMCP1) levels were measured in lung homogenates of mice sensitized to HDM aeroallergens. Non-sensitized wild type (WT) and TG animals both had virtually no MC activity (Fig. 1a), which reflected the equivalent baseline airway MC numbers (Supp. Fig. 4a). mMCP1 production increased in HDM-exposed mice of both genotypes. However, mMCP1 upregulation was significantly lower in sensitized TG than in sensitized WT animals (Fig. 1a). Therefore, EP₂ overexpression reduced HDM-induced MC activation *in vivo*. To confirm that this effect was specifically driven by EP₂ activation, mice were pre-treated with the selective EP₂ antagonist PF-04418948 prior to sensitization. In both TG and WT animals, EP₂ antagonism fostered a further increase of HDM-induced MC activity, along the lines of our recent observation in murine lung MCs *in vitro*¹. Interestingly, the difference in HDM-induced MC activity observed between TG and WT mice was abrogated after blockade of the EP₂ receptor. All in all, this suggests that EP₂ receptor overexpression on MCs leads to increased endogenous PGE₂ and EP₂ interactions, which restrict HDM-induced MC activity.

To analyze the consequences of MC inhibition, HDM-induced airway inflammation and reactivity were assessed. Inflammation was quantitated through a differential inflammatory cell count in bronchoalveolar lavage suspensions (Fig. 1b). Prominent bronchovascular eosinophilic infiltration developed in mice exposed to HDM. Mimicking the MC activity pattern, the eosinophilia was lower in TG mice than in WT animals (although the difference did not achieve statistical significance). The lower eosinophilic infiltration was paralleled by a trend towards a higher number of macrophages, whereas neutrophil and lymphocyte counts remained equivalent between TG and WT mice (Supp. Fig. 5). Also paralleling the effect observed on MCs, both TG and WT animals exhibited a further dose-dependent increase of inflammatory infiltrate in mice pre-treated with EP₂ antagonist. However, EP₂ antagonism did not fully reverse the improvement of the inflammatory process driven by EP₂ overexpression. Analyzed in the context of previous data where reduced bronchovascular inflammation was demonstrated in HDM-sensitized mice treated with a selective EP₂ agonist⁴, the current data support the view that an endogenous PGE₂-EP₂-MC compensatory mechanism may attenuate HDM-driven eosinophilic inflammation. Finally, invasive plethysmography was used to measure airway reactivity to methacholine (Fig. 2). Non-sensitized TG and WT animals exhibited no difference in baseline airway constriction upon increasing doses of methacholine (Fig. 2a). HDM-sensitized WT animals developed the expected level of airway hyperresponsiveness (AHR) after a 10-day exposure to HDM aeroallergens. Indeed, as per ANOVA, lung resistance (R_L) was significantly higher in

sensitized than in non-sensitized WT mice. By contrast, the methacholine dose-response curve of sensitized TG animals overexpressing EP₂ on MCs showed no induction of AHR upon exposure to HDM. Indeed, contrary to WT animals, the R_L of sensitized and non-sensitized TG mice did not differ. This finding agrees with our previous observations after exogenous EP₂ activation⁴. Pretreatment of mice with the EP₂ antagonist PF-0418948 (Fig. 2b) abrogated the beneficial effect exerted by EP₂ overexpression on HDM-induced airway reactivity. Contrary to mice that were not pretreated with PF-0418948, both TG and WT animals exhibited similar levels of AHR after methacholine treatment in the presence of 2.5 mg/kg of the antagonist.

We have shown that MC-specific EP₂ overexpression protects from HDM aeroallergen-induced airway pathology in a murine asthma model known to be mediated by IgE. A direct connection between reduced MC activity and improvement of airway inflammation and reactivity has been demonstrated. The finding that EP₂ antagonism worsens HDM-induced harmful effects in WT animals, and reverses the benefit exerted by EP₂ overexpression, supports the hypothesis that the PGE₂–EP₂–MC axis acts as a natural defensive barrier. This is consistent with the recent observation that PGE₂ protects from the development of anaphylaxis and MC hyperresponsiveness⁷. It also builds on our hypothesis of compensatory PGE₂ and EP₂ upregulation in mice exposed to HDM⁵ and on the preventive effect exerted by exogenous EP₂ activation⁴. In a recent consensus report⁸ the European Academy of Allergy and Clinical Immunology (EAACI) Task Force discussed the need to further research the role of eicosanoids in asthma and allergic diseases. The authors specifically mention the bronchoprotective and anti-inflammatory properties of PGE₂ in the lungs, remind us of its ability to reduce MC activity, and request further studies with selective EP₁₋₄ analogues. By using an alternative approach, we hope to have contributed to the link between eicosanoid biology and asthma pathophysiology. Further characterization of the cellular and molecular events involved in the PGE₂–EP₂–MC axis in the newly created C.B6-Tg (*Cma1-Ptger2*) mice will undoubtedly unravel new anti-asthma therapeutic target candidates and offer future research opportunities.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

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Accepted Article

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CONTRIBUTION STATEMENT

Urbano A: Induction of mice's sensitization and conduction of airway hyperreactivity and inflammation studies, as well as mast cell activity assessment. Data analysis and interpretation. Manuscript writing.

Plaza J : Contribution to early stage airway hyperreactivity and inflammation studies in HDM-sensitized mice, as well as mast cell activity assessment.

Turon S: Transgenic mice colony generation and handling.

Pujol A: Transgenic mice colony generation and handling. Manuscript revision.

Costa-Farré C: Mice handling for airway hyperreactivity studies.

Marco A: Assessment of airway inflammation, and interpretation.

Picado C: Study design and data interpretation, and manuscript revision.

Torres R: Experiments supervision and support in transgenic mice colony generation. Data analysis and interpretation. Manuscript revision.

de Mora F: Study design, experiments supervision, and data analysis and interpretation. Manuscript writing.

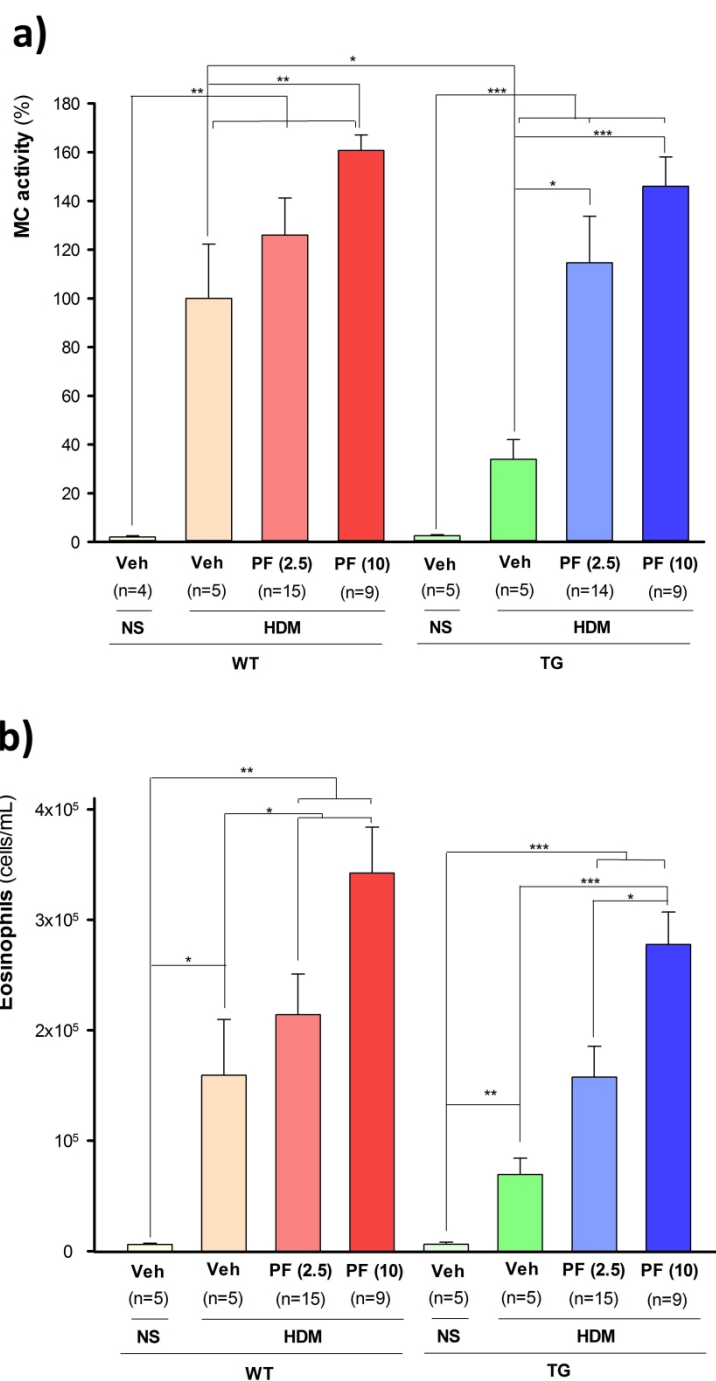


Figure 1. (a) HDM-induced airway MC activity in mice overexpressing EP₂. Mast cell (MC) activity was assessed by measuring mMCP1 concentration in lung homogenates normalized by total protein. WT mice exposed to HDM exhibit a significant increase in MC activity. The level of MC activation increased further in the presence of an E prostanooid receptor 2 (EP₂) antagonist (PF) at both 2.5 and 10 mg/kg. TG mice overexpressing EP₂ on MC exhibited a less pronounced increase of MC activity. Pretreatment with an EP₂ antagonist counteracted the reduced MC activity in TG mice, resulting in activity levels that were

essentially equivalent between both genotypes (TG and WT). **(b) HDM-induced eosinophilia in mice overexpressing EP₂.** Airway inflammation was assessed by a differential cell count in bronchoalveolar lavage suspensions (see the full inflammatory cell count in Supp. Fig. 5). WT mice exposed to HDM exhibited significant eosinophilia, which further increased in the presence of an EP₂ antagonist at both 2.5 and 10 mg/kg. In TG mice overexpressing EP₂ on MC, eosinophil recruitment was attenuated. After pretreatment with the EP₂ antagonist, the effect of EP₂ overexpression on eosinophilia was abrogated. Results are expressed as mean $\pm$ SEM. Statistical significance was evaluated using a two-way analysis of variance (ANOVA) with a Bonferroni post hoc test. *p<0.05, **p<0.01, and ***p<0.001. HDM (House dust mite-sensitized); MC (Mast cell); NS (Non-sensitized); PF (PF-0441848); TG (Transgenic); Veh (Vehicle); WT (Wild type).

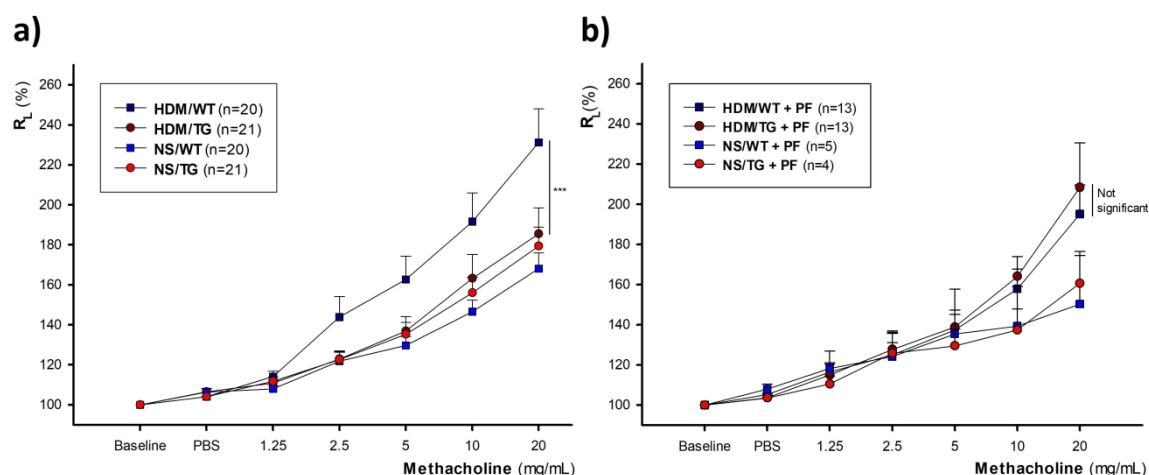


Figure 2. HDM-induced airway reactivity in mice overexpressing EP₂. Airway reactivity was assessed by means of invasive plethysmography and expressed as a percentage of baseline lung resistance (R_L). **(a) Airway resistance in WT and TG mice exposed to HDM.** Sensitization to HDM aeroallergens induced a significant increase in airway reactivity to methacholine (i.e., airway hyperresponsiveness [AHR]) in WT mice (HDM/WT), while sensitized TG mice overexpressing E prostanoïd receptor 2 (EP₂) (HDM/TG) did not develop AHR. **(b) Airway reactivity after pretreatment with an EP₂ antagonist.** In the presence of the EP₂ antagonist (PF), both sensitized WT (HDM/WT+PF) and sensitized transgenic (HDM/TG+PF) mice developed AHR. Indeed, contrary to what is observed in Fig. 2a, there was no significant difference between HDM/WT and HDM/TG when the mice were pre-treated with 2.5 mg/kg of PF. Results are expressed as mean ± SEM. Statistical significance was evaluated using a two-way analysis of variance (ANOVA) with a Bonferroni post hoc test. Statistically significant differences were found between baseline reactivity (NS mice) and HDM-sensitized mice. HDM (House dust mite-sensitized); R_L (Lung resistance); NS (Non-sensitized); PF (PF-0441848); TG (Transgenic); WT (Wild type).

REPOSITORY MATERIAL:

Letter to the Editor (submitted manuscript)

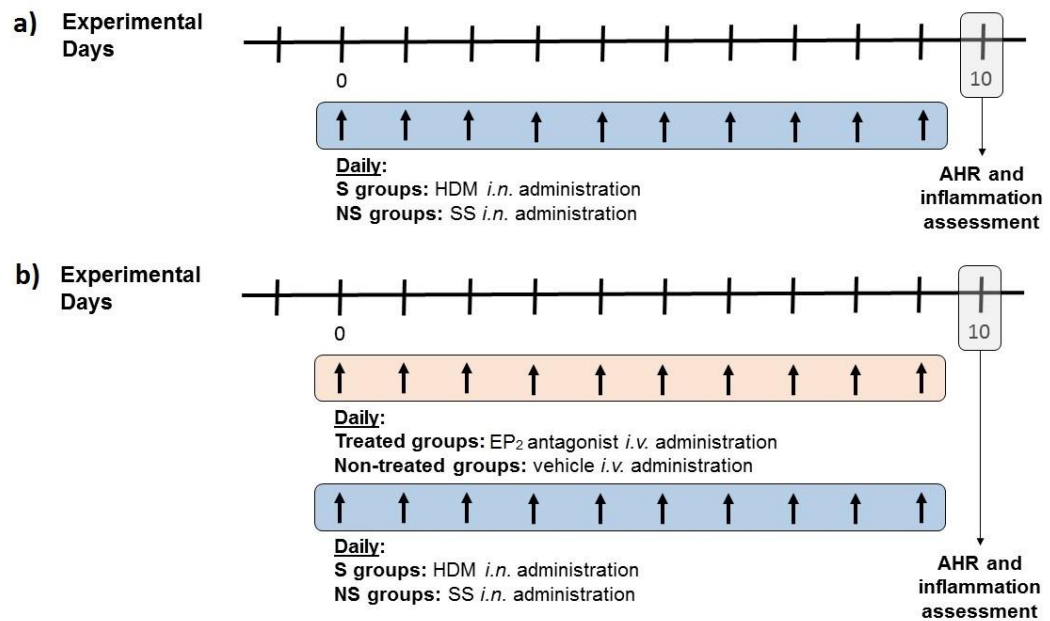
Transgenic mice overexpressing the PGE₂ receptor EP₂ on mast cells exhibit a protective phenotype in a model of allergic asthma

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As additional supporting information we provide (6 pages):

1. Sensitization to HDM, and treatment with EP₂ antagonist: **Supplementary Figure 1**
2. Creation of transgenic mice overexpressing EP₂ on mast cells and outcome: **(a)** Text, and **(b)** Figures: **Supplementary Figures 2, 3 and 4**
3. Differential inflammatory cell count: **Supplementary Figure 5**

1. Sensitization to HDM, and treatment with EP₂ antagonist.



Supplementary Figure 1. Experimental protocols. (a) Sensitization: Sensitization to HDM aeroallergens was achieved through nasal exposure (*i.n.*) to an HDM extract (lipopolysaccharide content <0.5 EU/dose) from Alk-abelló (25 µg/mouse) for 10 consecutive days (days 0 to 9). Control groups received *i.n.* saline solution (SS); **(b) EP₂ antagonist treatment:** the selective EP₂ antagonist PF-04418948 was administrated intravenously (*i.v.*) one hour before exposure to either HDM extract or SS. AHR (Airway hyperresponsiveness); HDM (House dust mite); S (Sensitized); SS (Saline solution); NS (Non-sensitized).

2. Creation of transgenic mice overexpressing EP₂ on mast cells and outcome.

a) Text

Transgenic plasmid

To generate transgenic mice overexpressing the EP₂ receptor selectively on the MC surface, a DNA construct with the MC-specific α -Chymase promoter (Cma1 promoter) and the EP₂ receptor coding region (Ptger2) was created. The α -Chymase promoter region was obtained from a plasmid donated by Dr. Thomas Hehlhans (*Institut für Immunologie, Universität Regensburg, Germany*). The coding sequence of Ptger2 was obtained by codon optimized synthesis ("GeneART™ Gene Synthesis" by Thermo Fisher Scientific, Waltham, MA, USA), was linked to a Simian vacuolating virus 40 (SV40) polyA sequence, and was inserted at the HindIII site of the plasmid containing the promoter (**Supplementary Figure 2**).

Construct injection and BALB/c transgenic mice generation

The Cma1/Ptger2 construct was inserted by pronuclear microinjection into C57BL/6OlaHsd zygotes. Micromanipulated embryos were transferred into ICR pseudo-pregnant females who carried out the pregnancy. Genotype analysis performed by Southern blot (**Supplementary Figure 3**) indicated that 10 out of 37 animals had successfully inserted the transgene into their genomes and were transgenic founders (F₀). Each founder was mated with a wild type (WT) C57BL/6OlaHsd mouse, and transgenic F₁ generations were obtained from 6 out of the 10 founders.

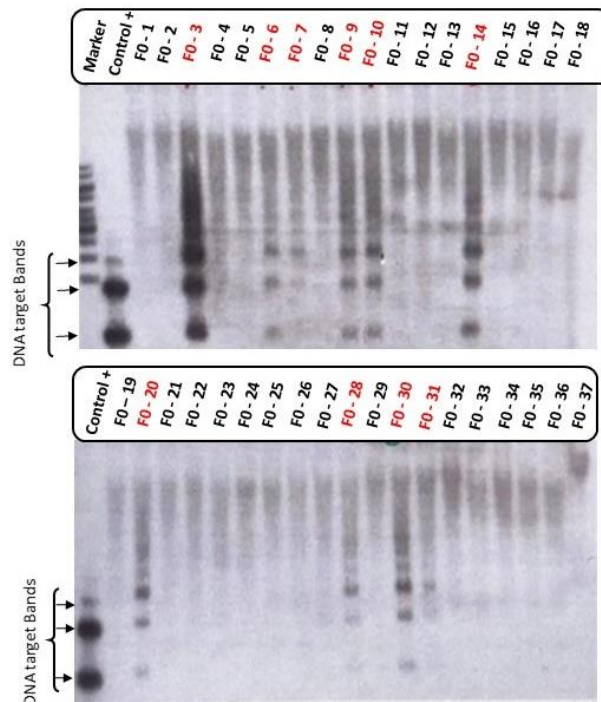
The C.B6-Tg (Cma1-Ptger2) transgenic lines with the highest levels of transgene expression were preselected on the basis of the EP₂ protein expression determined by western blot in pulmonary tissue homogenates. Results were calculated as a ratio of EP₂ to CPA3, a constitutive MC protein that indicates the number of pulmonary MCs present. Two newly created mouse lines, L3 and L4, exhibited robust overexpression and were selected on that basis (**Supplementary Figure 4**). The TG mouse line with the highest ratio, L3, was selected to perform experiments. Given that BALB/c mice are more prone to develop an atopic phenotype, C57BL/6OlaHsd transgenic mice were backcrossed a minimum of seven times with BALB/c WT animals, to attain over 95% of that genetic background.

As shown by RT-PCR, approximately 50% of offspring carry the transgene, revealing that it is inherited following Mendel's genetic laws. Animals from the same breed not expressing the transgene were used as WT controls within each experiment.

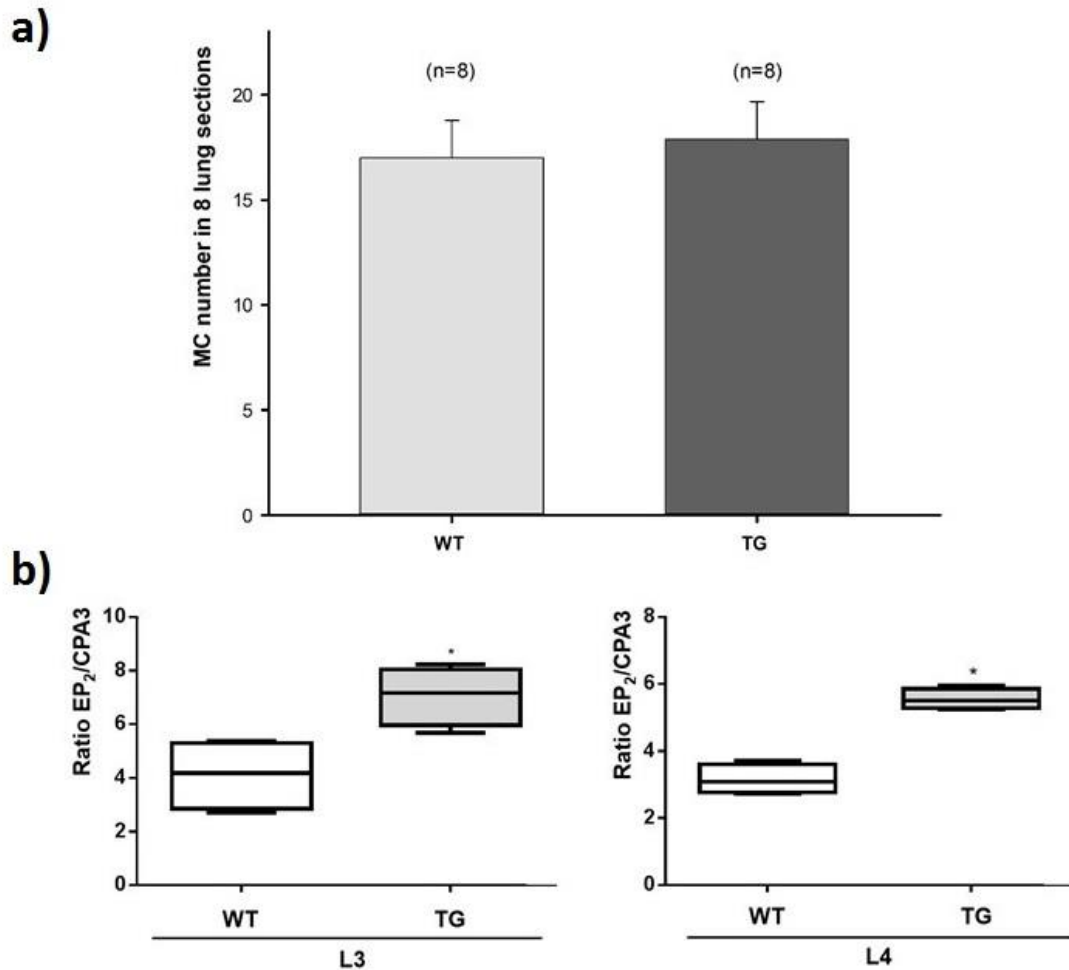
b) Figures



Supplementary Figure 2. Transgene α -Chymase-Ptger2-SV40PA construct. The construct bore three parts: (1) α -Chymase promotor region, (2) cDNA of the mouse EP₂ gene (Ptger2), and (3) the polyA SV40 sequence. The transgene contained HindIII and NotI restriction sites to ensure a correct insertion of the fragment in the vector. cDNA (complementary DNA); HindIII (Restriction site for the type II HindIII restriction enzyme produced by *Haemophilus influenzae*); NotI (Restriction site for the type II NotI restriction enzyme produced by *Nocardia otitidis*); SV40 polyA (Simian vacuolating virus 40 polyA sequence).



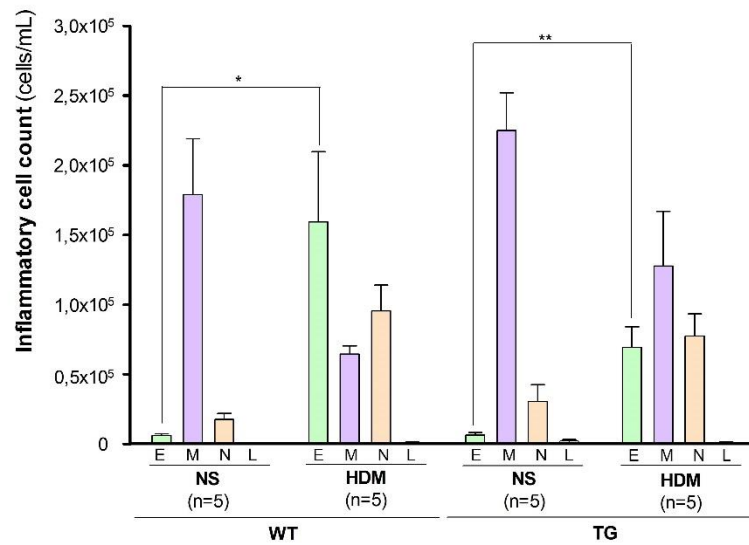
Supplementary Figure 3. Autoradiography of the Southern blot membrane. Results of the F₀ (founding) generation. In red, animals bearing the specific sequence of the target DNA that have integrated the EP₂ cDNA into their genome.



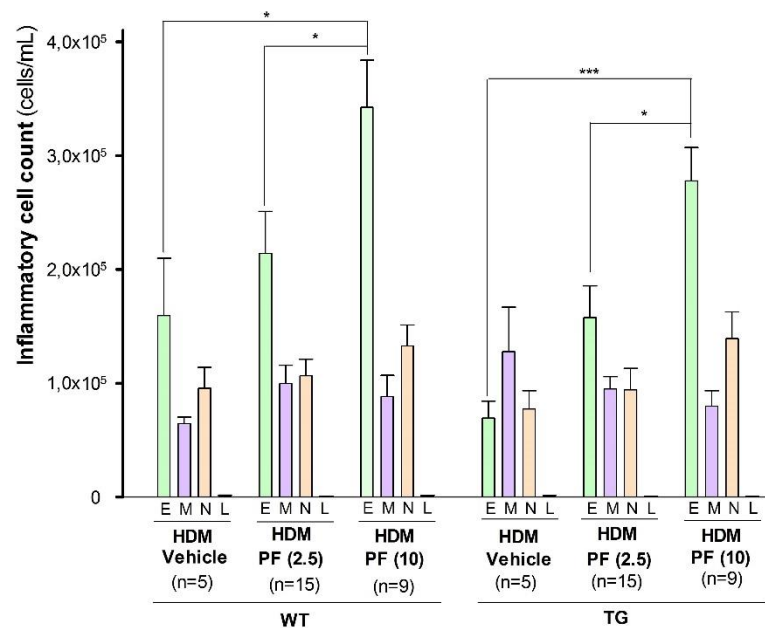
Supplementary Figure 4. (a) Baseline airway mast cell number in lung histological preparations. The number of mast cells was counted in a total of 8 sections per lung (i.e., per animal), 8 animals per group (n=8). A blind count was performed by two researchers, and the number per section was calculated as the average of the duplicate analysis. The baseline number of mast cells is the same in WT and TG mice. **(b) Baseline protein expression of EP₂ receptor in lung tissue of WT and TG mice.** Densitometric assessment of the EP₂ baseline expression in lungs from mouse lines L3 and L4. The results are expressed as EP₂/CPA3 ratio (both normalized with the constitutive protein β -actin). CPA3 is a constitutive MC protein that correlates with the number of pulmonary MCs. Results represent means with the 25 and 75 percentiles of the EP₂/CPA3 ratio (*p-value<0.05, **p-value<0.01, and ***p-value<0.001). CPA3 (Carboxypeptidase A3); EP₂ (E prostanoid receptor 2); L3 (mouse line 3); L4 (mouse line 4); TG (Transgenic); WT (Wild type).

3. Differential inflammatory cell count.

a)

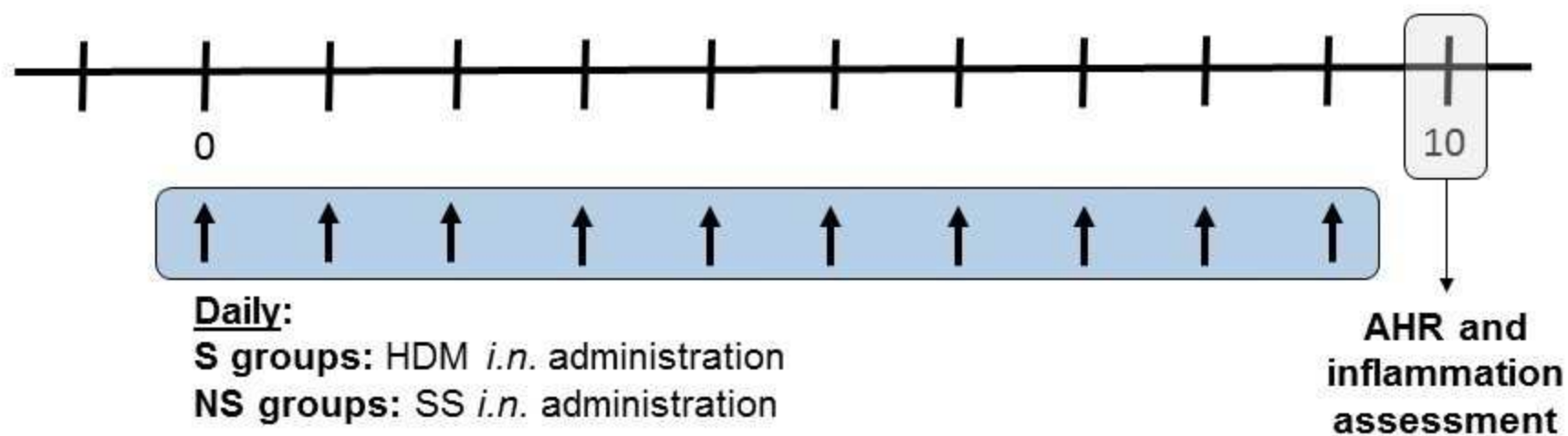


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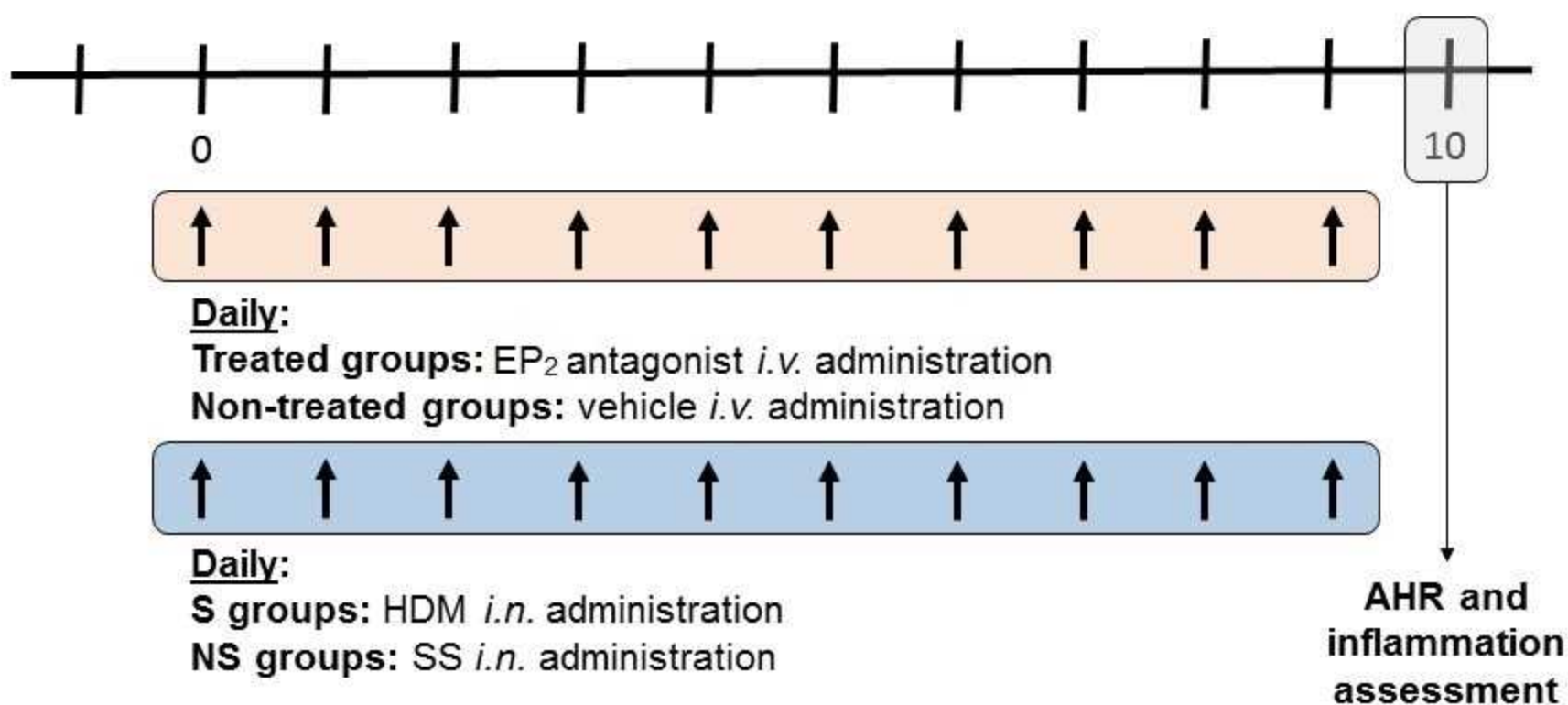


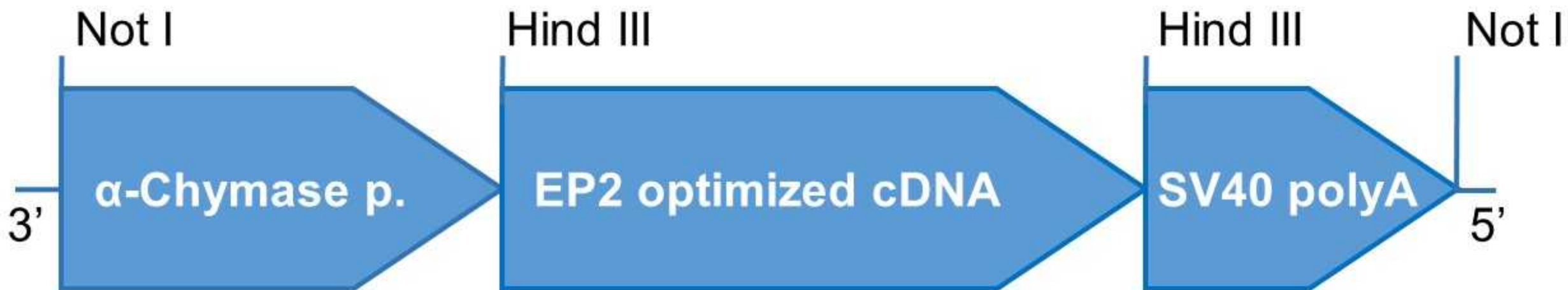
Supplementary Figure 5. Airway inflammation assessment. Differential inflammatory cell count per mL (E, eosinophils; M, macrophages; L, lymphocytes; N, neutrophils). **(a)** Inflammation in HDM-sensitized WT mice and in TG mice overexpressing EP₂. **(b)** Inflammation in HDM-sensitized WT and TG mice pre-treated with EP₂ antagonist PF-04418948. Results represent mean $\pm$ SEM. (*p-value<0.05, **p-value<0.01, and ***p-value<0.001). PF (PF-04418948); HDM (House dust mite); TG (Transgenic); WT (Wild type); NS (Non-sensitized).

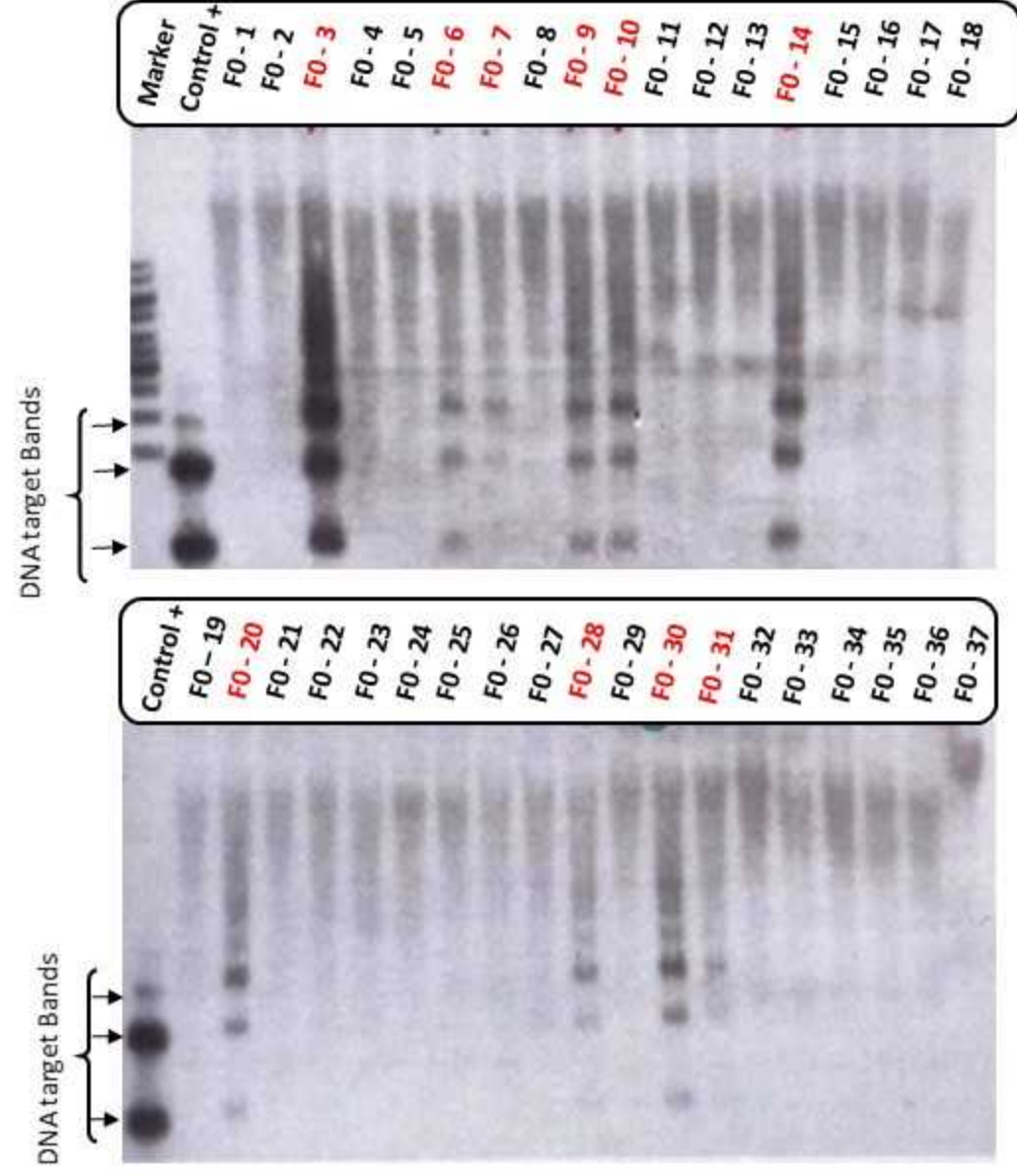
a) Experimental Days

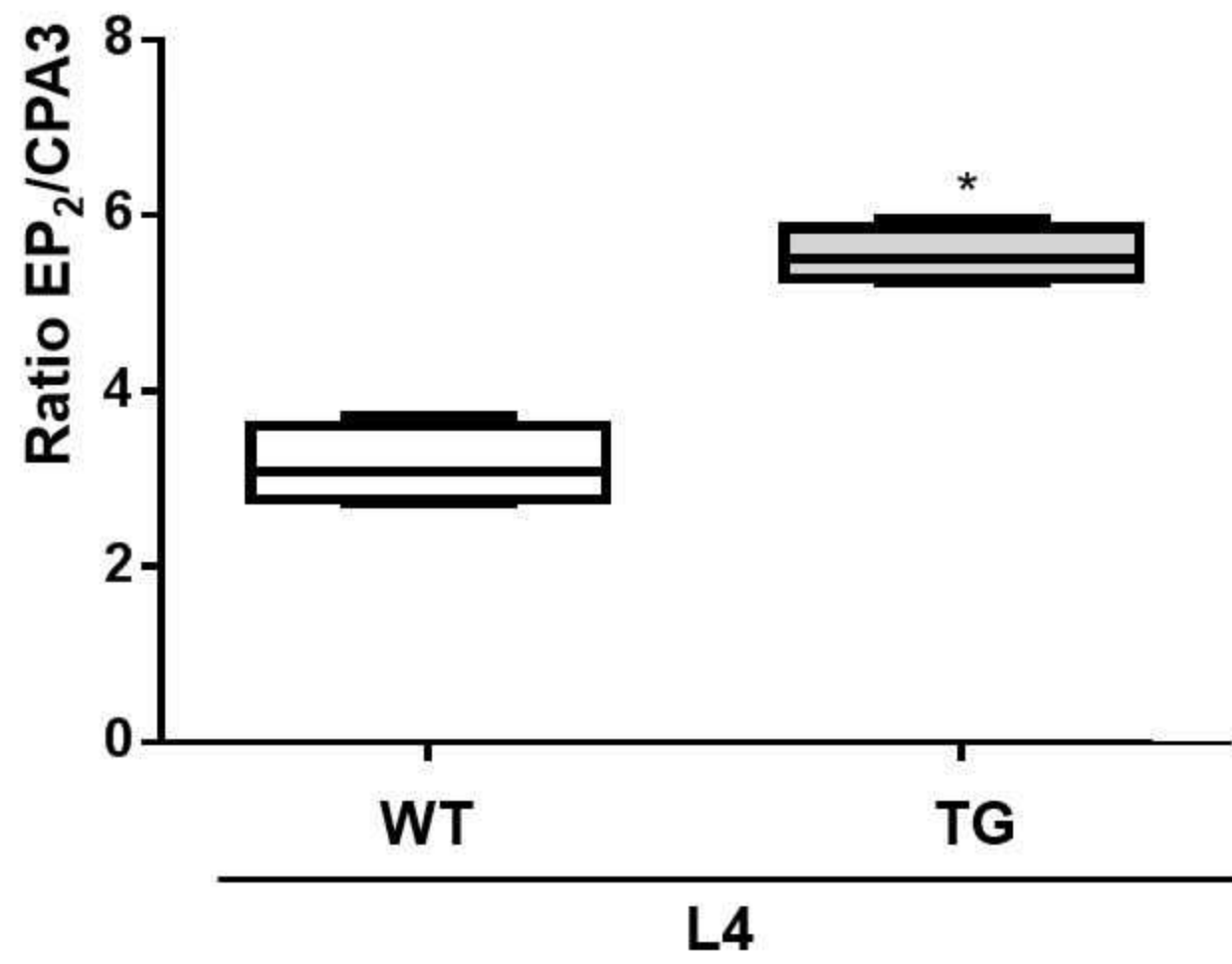
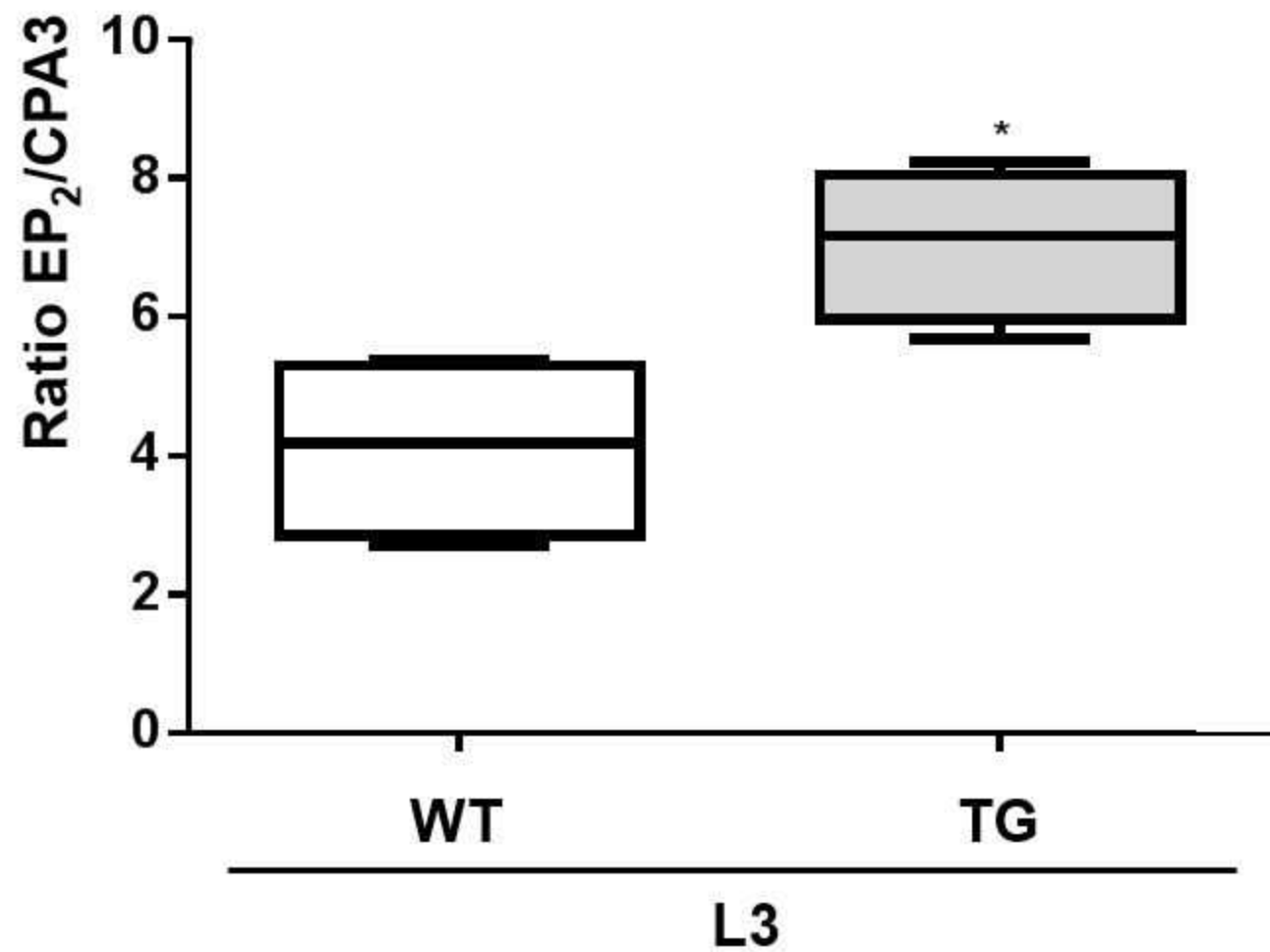


b) Experimental Days

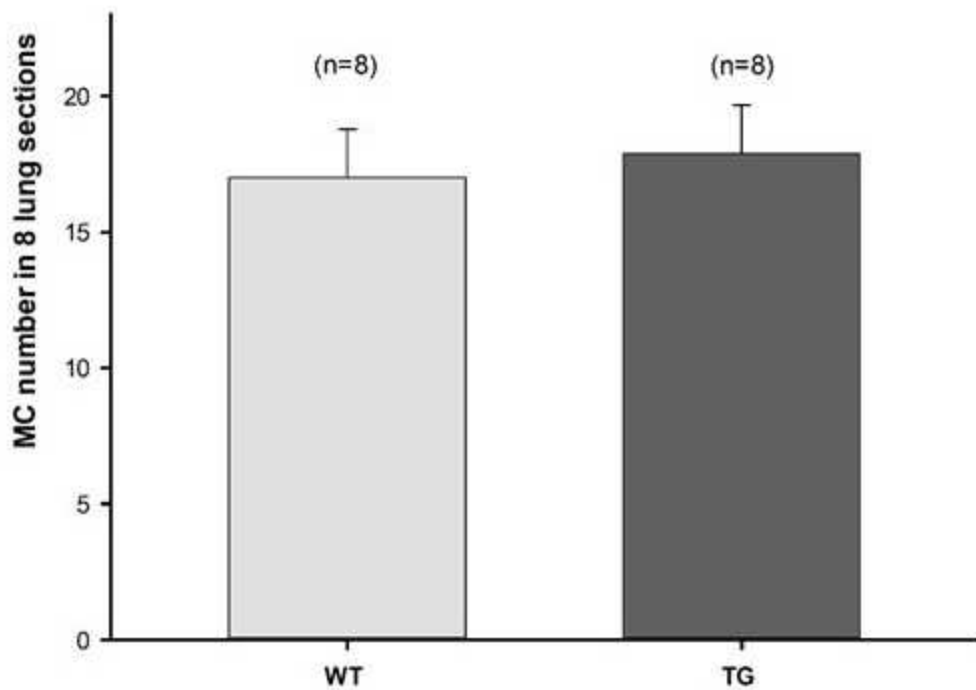




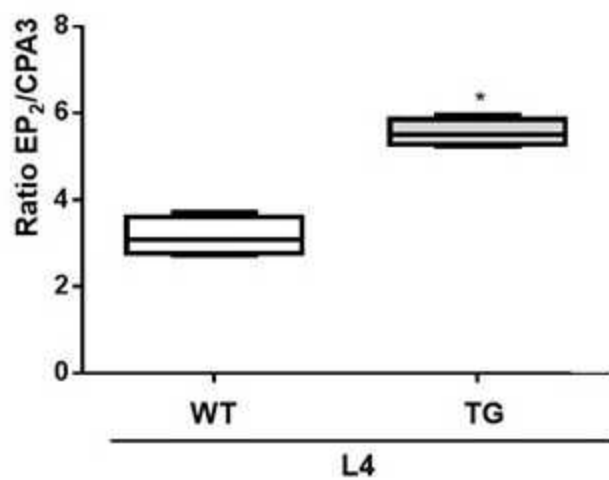
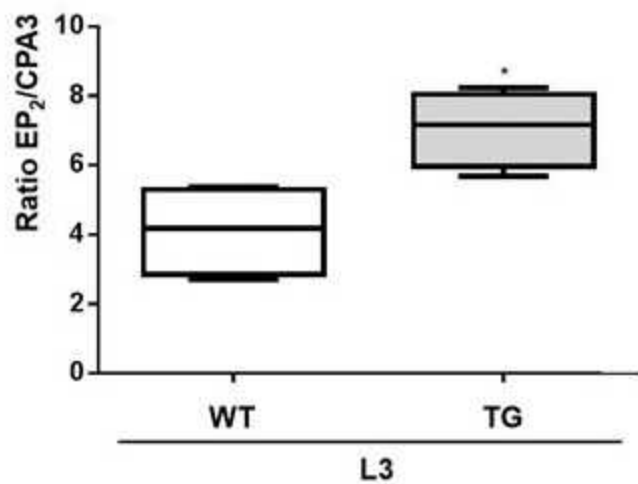


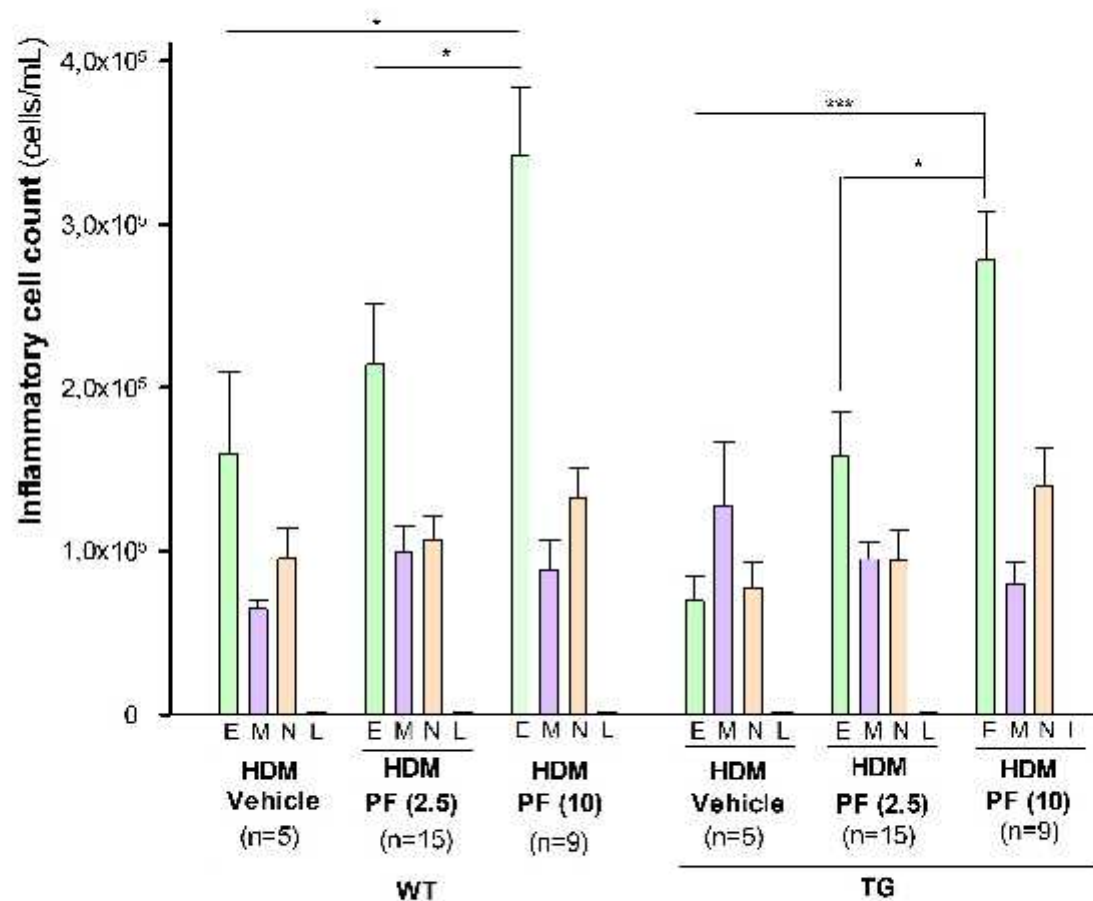


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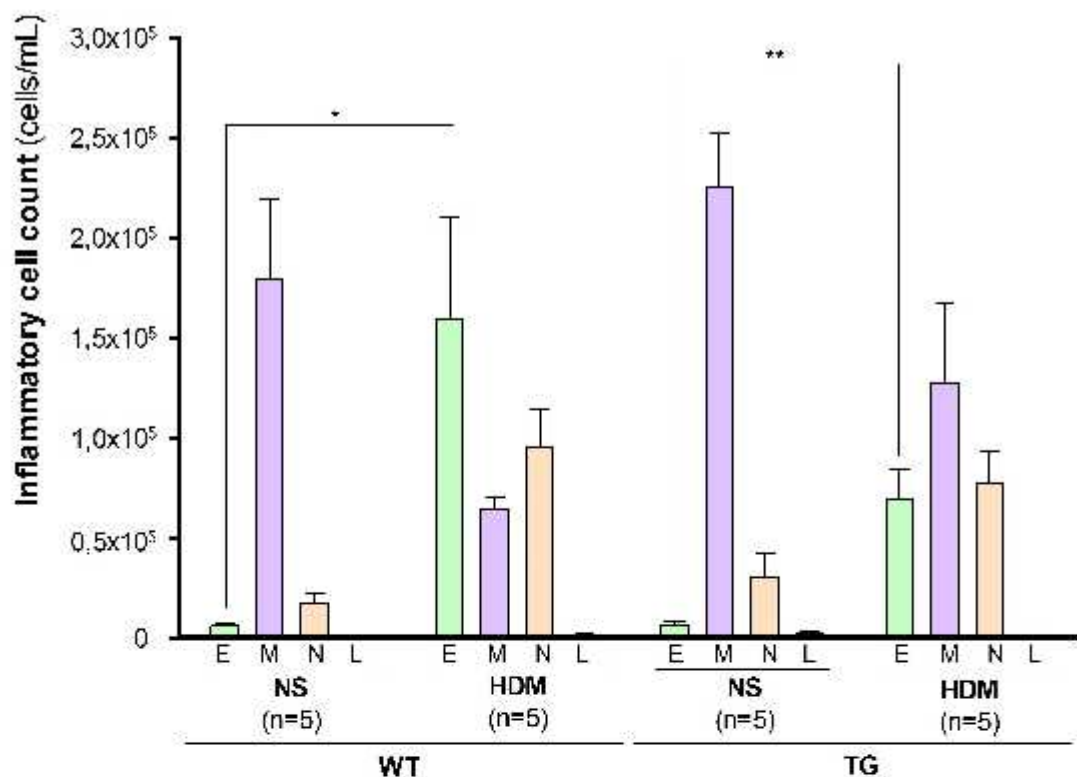


b)





a)



b)

