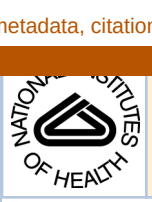



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# $PGE_2$ produced by the lung augments the effector phase of allergic inflammation

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## Abstract

Elevated $PGE_2$ is a hallmark of most inflammatory lesions. This lipid mediator can induce the cardinal signs of inflammation, and the beneficial actions of non-steroidal anti-inflammatory drugs are attributed to inhibition of cyclooxygenase COX-1 and COX-2, enzymes essential in the biosynthesis of $PGE_2$ from arachidonic acid. However, both clinical studies and rodent models suggest that, in the asthmatic lung, $PGE_2$ acts to restrain the immune response and limit physiological change secondary to inflammation. To directly address the role of $PGE_2$ in the lung, we examined the development of disease in mice lacking microsomal prostaglandin E synthase 1 (mPGES1), which converts COX-1/COX-2 derived $PGH_2$ to $PGE_2$. We show that mPGES1 determines $PGE_2$ levels in the naïve lung and is required for increases in $PGE_2$ after ovalbumin (OVA) induced allergy. While loss of either COX-1 or COX-2 increases the disease severity, surprisingly mPGES1 −/− mice show reduced inflammation. However, an increase in serum IgE is still observed in the mPGES1 −/− mice, suggesting that loss of $PGE_2$ does not impair induction of a $T_H2$ response. Furthermore, mPGES1 −/− mice expressing a transgenic OVA-specific T cell receptor are also protected, indicating that $PGE_2$ acts primarily after challenge with inhaled antigen. $PGE_2$ produced by the lung plays the critical role in this response, as loss of lung mPGES1 is sufficient to protect against disease. Together this supports a model in which mPGES1-dependent $PGE_2$ produced by populations of cells native to the lung contributes to the effector phase of some allergic responses.

## Introduction

Prostanoids are a family of bioactive lipid mediators produced in almost every cell type by the actions of prostaglandin-endoperoxide synthases (cyclooxygenase, COX) on arachidonic acid (AA). Synthesis is initialized when phospholipase $A_2$ releases AA from membrane phospholipids in response to a diverse range of stimuli. The AA is then catalyzed to prostaglandin $H_2$ ($PGH_2$) by one of two isoforms of the Cox enzyme, COX-1 or COX-2 (1, 2) COX-1 is constitutively expressed by most cell types and is thought to be responsible for basal levels of prostanoid production while COX-2 expression is generally undetectable in most tissues under homeostatic conditions and is upregulated in response to inflammatory stimuli (2), although exceptions have been noted. For example, COX-1 expression increases

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dramatically in the lactating mammary gland (3) and COX-2 expression can easily be detected in the healthy lung of both mice and humans (4, 5). $PGH_2$ generated by either COX-1 or COX-2 is subsequently converted into a family of related molecules: prostaglandin $D_2$ ($PGD_2$), prostaglandin $E_2$ ($PGE_2$), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin ($PGI_2$), and thromboxane $A_2$ ($TXA_2$) by pathway specific synthases. These lipids mediate their actions through the selective binding to G-coupled protein receptors, each with a unique but overlapping pattern of expression (1, 2). $PGE_2$ binds with a high affinity to four receptors, E prostanoid (EP) 1–4, all of which are expressed in the lung (6, 7).

$PGE_2$ levels are elevated during most inflammatory responses and therefore, not surprisingly, augmented levels of this lipid mediator have been reported in the induced sputum from asthmatics compared to healthy individuals and this enhancement can be positively correlated with disease severity (8–10). However, elevated synthesis of $PGE_2$ may reflect an attempt by the organism to limit ongoing inflammation and protect the airways from collateral damage. Certainly, $PGE_2$ mediated pathways capable of limiting inflammation and restoring homeostasis in the lung have been identified. $PGE_2$ is a potent smooth muscle relaxant and through the $EP_2$ receptor can limit constriction of the airways (11). Indeed, administration of this mediator into the airways ameliorates airway hyperresponsiveness (AHR) caused by several bronchoconstrictive agents in humans and animals (12–17) and attenuates aspirin-induced and exercise-induced bronchoconstriction (18, 19). $PGE_2$ can induce ion secretion and thus alter the composition of the airway surface liquid, facilitating mucociliary clearance (20). Furthermore, exogenous $PGE_2$ can limit T cell proliferation and $T_H1$ type cytokine release from LPS-stimulated macrophages through stimulation of the $EP_2$ and $EP_4$ receptors, respectively (21). In addition to its capacity to down-regulate pro-inflammatory cytokine release from immune cells, $PGE_2$ can also stimulate release of IL-10, a cytokine generally thought to be protective in the immune system (22).

Perhaps the strongest indication that $PGE_2$ might function to limit allergic inflammation in the lung comes from animal studies conducted using pharmacological and genetic approaches to limit prostaglandin synthesis in a model of ovalbumin (OVA) induced lung allergy. Mice of mixed genetic background and lacking either COX-1 or COX-2 were reported to develop far more severe disease than wildtype animals (23). Consistent with this, treatment of mice with indomethacin, a non-steroidal anti-inflammatory drug (NSAID) which suppresses the actions of both COX -1 and COX -2, or alternatively, with Cox-specific NSAIDs induced elevated eosinophilia and IL-13 production in the lung (24, 25); although these approaches did not allow for the identification of the specific prostaglandin(s) responsible for limiting the allergic response. Another study, however, reported increased allergic inflammation in mice lacking $EP_3$ receptors (26), suggesting that loss of $PGE_2$ is at least partly responsible for heightened inflammation observed in COX-deficient and NSAID treated animals.

$PGE_2$ production occurs through the metabolism of $PGH_2$ by the microsomal $PGE_2$ synthase-1 (mPGES1) (5). While initially two additional enzymes, cytosolic $PGE_2$ synthase (cPGES) and microsomal $PGE_2$ synthase-2 (mPGES2) were thought to be capable of this enzymatic conversion, studies using mutant mouse lines carrying mutations in the genes for these synthases, *mPges1*, *cPges/p23* or *mPges2* respectively, have failed to support this *in vivo* function for any product other than mPGES1 (27–29). mPGES1 is expressed in many tissues and cell types of both humans and animals, including in the lung and leukocytes (5, 30, 31). Similar to COX-2, the expression of mPGES1 increases dramatically in response to inflammatory mediators suggesting coupling with this enzyme; however, evidence demonstrates that mPGES1 can couple with both COX-1 and COX-2 to synthesize $PGE_2$ (5, 29, 30, 32). Furthermore, studies using mice lacking mPGES1 in models of pain

nociception, rheumatoid arthritis, atherogenesis, and abdominal aortic aneurysm provide evidence that this synthase contributes to the pathogenesis of both acute and chronic inflammation (29, 33–35).

Here we elucidate the contribution of mPGES1-derived $PGE_2$ in the development of allergic lung disease, a mouse model of asthma. First, using congenic mouse lines lacking either COX-1 or COX-2, we confirm the role of this pathway in our model. We then evaluate the role of $PGE_2$ produced by mPGES1 expressed by resident airway cells and $PGE_2$ released from recruited inflammatory cells in this allergic response.

## Materials and Methods

### Experimental Animals

All experiments were conducted in accordance with standard guidelines as defined by the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee guidelines of the University of North Carolina at Chapel Hill. Experiments were carried out on age and sex matched mice between 8–12 weeks of age. C57BL/6 (B6) (backcrossed >10 generations) COX-1 −/−, B6 x 129S6/SvEv (129) fililal generation (F)1 COX-2 −/−, and B6 (backcrossed >10 generations) mPGES1 −/− mice were generated as previously described (29, 36, 37). B6 OVA-Specific TCR-Transgenic (OT-II) mice were purchased from The Jackson Laboratories (Bar Harbor, Ma). OT-II mice were crossed to mPGES1 −/− mice to generate OT-II/mPGES1 −/− animals.

### OVA Sensitization and Challenge

All mice were sensitized systemically on days 0 and 14 with an i.p injection of 40 μg OVA (Sigma-Aldrich) emulsified in aluminum hydroxide (Sigma-Aldrich). One week following the second injection, experimental mice were challenged for 5 consecutive days (days 21–25) with an aerosol instillation of 1% OVA in 0.9% sodium chloride (NaCl) for 1 hour each day. Control animals were challenged with 0.9% NaCl only. 24 hours after the final challenge, lung mechanics were measured and bronchoalveolar lavage fluid (BALF), serum, whole lungs, and spleens were collected for further analysis. For experiments examining allergic sensitization, animals were immunized as described and harvested on day 21.

### Measurement of Cell Proliferation

Splenocytes were prepared by mechanical dispersion of spleen over a 70 μm cell strainer (BD Falcon). Red blood cells were lysed in lysis buffer (4.1g $NH_2Cl$, 0.5g $KHCO_3$, 100 μl 0.5M EDTA dissolved in 500 ml $dH_20$) and splenocytes were washed twice in PBS. Cells were plated at a density of 2.5 X $10^6$ cells/ml in RPMI 1640 media enriched with 10% FBS, 100 units/ml penicillin, 100 units/ml streptomycin, and .29 μg/ml L-glutamine. OVA was added to splenocyte cultures at concentrations ranging from 0 μg /ml to 100 μg/ml. After incubation for 72 hours at 37°C, cell proliferation was assessed using WST-1 reagent (Roche) according to the manufacturer's instructions.

### Measurement of Airway Mechanics in Intubated Mice

Mechanical ventilation and airway mechanic measurements were conducted as previously described (38). Briefly, mice were anesthetized with 70–90 mg/kg pentabarbitol sodium (American Pharmaceutical Partners, Los Angeles, CA), tracheostomized, and mechanically ventilated with a computer-controlled small-animal ventilator. Once ventilated, mice were paralyzed with 0.8 mg/kg pancaronium bromide. Forced Oscillatory Mechanics (FOM) was measured every 10 seconds for 3 minutes. These measurements allowed for the assessment of airway resistance in the central airways (Rn), as well as in tissue damping (G). Increasing doses (between 0 and 50mg/ml) of methacholine chloride (Mch) were administered to

animals through a nebulizer to measure airway hyperresponsiveness (AHR). These data are presented as percent above baseline.

### BALF Collection and Cell Counts

Following measurements of lung mechanics, mice were humanely euthanized. Lungs were lavaged with five 1ml aliquots of HBSS (Gibco). 100 μl of BALF was reserved for cell count analysis and total cell counts were measured by hemacytometer. Differential cell counts were collected using cytospin preparation stained with Hema 3 and Fast Green. All remaining BALF was centrifuged to remove cells, and stored at −80°C for immunoassay.

### $PGE_2$ and Cytokine Production

Levels of cytokines and $PGE_2$ were determined by immunoassay in BALF, lung tissue homogenate, and/or tissue culture supernatant. To determine cytokine production by stimulated splenocytes, cells were prepared as described above. Cells were cultured at a density of 1 X $10^7$ cells/ml in the presence of 100 μg/ml OVA. After 72 hours, supernatants were collected and stored at −80°C prior to evaluation by ELISA. To determine lung cytokine levels, lungs were flash frozen in liquid nitrogen, weighed, and stored at −80°C. Lung tissue was pulverized and homogenized in buffer containing 150 mM NaCl, 15 mM Tris-HCl, 1mM $CaCl_2$, 1mM $MgCl_2$ supplemented with protease inhibitor (Roche). Values shown represent the total quantity of cytokine or mediator measured divided by tissue weight. Cytokines were determined by ELISA following manufacturer's protocols: IL-13 (R&D Systems), IFN-γ (R&D Systems), IL-4 (R&D) and IL-17a (eBiosciences). For quantification of $PGE_2$, lung tissue was pulverized then homogenized in 1XPBS/1mM EDTA and 10 μM indomethacin. Lipids were purified through octadecyl $C_{18}$ mini columns (Amersham Biosciences for mPGES1; Alltech Associates for COX-1 and COX-2), and prostanoid content was determined using an enzyme immunoassay kit (Assay Designs) according to the manufacturer's instructions. Blood was obtained by cardiac puncture, allowed to coagulate, and centrifuged to isolate serum. IgE levels were determined by immunoassay using 96 well EIA/RIA plates (Costar). Plates were coated with IgE capture antibody (Pharmingen; clone R35-72), blocked with 1% BSA/PBS and then incubated with IgE standard (Pharmingen) or serum followed by biotinylated rat anti-mouse IgE (Pharmingen; clone R35-118). Detection was carried out using streptavidin-horseradish peroxidase (HRP) (Pharmingen) and hydrogen peroxide /2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Absorbance at 405nm was measured.

### Bone Marrow Chimera Generation

Recipient mice were exposed to 5 grays irradiation from a Cesium g-irradiator at 0 and 3 hours. Femurs and tibias were collected from donor mice and flushed with cold PBS to isolate bone marrow. Bone marrow was introduced by tail vein injection into recipient mice immediately following the second round of radiation and after 8 weeks animals were sensitized and challenged with OVA.

### Statistical Analysis

Statistical analysis was performed using Prism 4 (GraphPad Software). Comparisons of the mean were made by F test, Student's t-test or ANOVA followed by Tukey-Kramer's HSD *post hoc* test as necessary. Data are shown as mean ± SEM. Differences with $p<0.05$ were considered statistically significant.

## Results

### Allergic Asthma in the COX-1 and COX-2 deficient mice

We first verified, using congenic mouse lines, the contribution of prostanoids to allergic lung disease induced by sensitization and challenge with OVA antigen. COX-1 −/− B6 congenic mice were generated by >10 crosses between mice carrying a null allele at this locus and commercially purchased B6 mice. COX-2 mice survive poorly on most inbred genetic backgrounds, in part due to a patent ductus arteriosus (39, 40), and thus most experiments assessing COX-2 function have utilized > F2 mice; mice expected to carry a random assortment of 129 and B6 derived alleles. To circumvent this problem, we generated two lines of COX-2 mutant mice, the first line on the co-isogenic 129 genetic background and the second congenic line on the B6 genetic background. 129 COX-2 +/− females were intercrossed with B6 +/− males to generate congenic F1 progeny. The COX-2 −/− and COX-2 +/+ littermates were used in the experiments presented here. Allergic airway disease was induced through sensitization by an i.p. injection of OVA emulsified in aluminum hydroxide and challenged with repeated aerosols of antigen. Inflammation was assessed twenty four hours after the final antigen challenge.

As expected, our immunization protocol induced a robust cellular influx in B6 wildtype mice (COX-1 +/+), compared to saline treated animals (Fig 1A). The F1 OVA-treated controls (COX-2 +/+) displayed a similar pattern of increased cellularity. Surprisingly, the loss of either COX-1 or COX-2 had comparable impacts on recruitment of cells into the airways: in each line, the total number of cells recovered by BALF was about twice that observed in the genetically matched controls. The cellular infiltrate present in the airways following antigen challenge was marked by heightened levels of eosinophils, typical of $T_H2$ type allergic responses (Fig 1B). Characteristic of this type of response, IL-13 levels were elevated in the BALF of wildtype mice with allergic lung disease compared to saline controls (Fig 1C). Loss of either COX-1 or COX-2 led to increased levels of this cytokine in the airways. Again the magnitude of this increase, relative to the wildtype control, was surprisingly similar in the two lines. Elevated IgE levels were observed in both B6 COX-1 +/+ and B6/129 F1 COX-2 +/+ wildtype animals (Fig 1D). However, unlike inflammatory disease in the lung, the loss of neither the COX-1 nor the COX-2 pathway significantly altered levels of this immunoglobulin isotype.

AHR following challenge with methacholine (Mch), a potent airway constrictor, was quantified in intubated animals using a computer-controlled small-animal ventilator. This method allows for measurement of resistance in the central airway (Rn) and tissue damping (G). We failed to observe AHR in response to Mch in mice with allergic lung disease. This was also true of the B6/129 F1 mice. Even in the COX-1 and COX-2 mice, which showed elevated levels of inflammation, no difference was observed in the response to methacholine in either parameter (Fig S1A/B).

### Contribution of mPGES1 to $PGE_2$ production in the naïve and inflamed lung

The ability to catalyze the conversion of $PGH_2$ to $PGE_2$ has been assigned to three distinct enzymes, mPGES1, mPGES2 and cPGES (5, 44, 45). However, studies using mutant mouse lines have shown *in vivo* alteration of $PGE_2$ levels only in the mPGES1 mice (29, 46). We therefore first determined whether $PGE_2$ levels are elevated in this model of allergic lung disease and if so, whether this increase is dependent on the mPGES1 synthase. Allergic lung disease was induced in congenic mPGES1 −/− mice and their B6 controls (mPGES1 +/+), as described above. Lungs were harvested and the levels of $PGE_2$ were determined by enzyme immunoassay (Fig 2). $PGE_2$ production could be detected in the lungs of healthy mice exposed to saline. This production was significantly reduced in the lungs obtained

from mPGES1 −/− mice, indicating that mPGES1 is active in the normal lung and contributes to the basal levels of $PGE_2$ in this organ. $PGE_2$ levels were substantially potentiated in the lungs of wildtype mice with allergic lung disease. This increase was entirely dependent on expression of the mPGES1 synthase, indicating that animals lacking this synthase provide an appropriate model for determining whether loss of this COX1/2 downstream pathway contributes to the increased disease observed in the COX-1 and the COX-2 deficient animals.

### Impact of $PGE_2$ on allergic lung inflammation

$PGE_2$ levels in the lung increase dramatically after allergic lung disease and this increase is absent in both the COX-1 and the COX-2 deficient mice (Fig S2), supporting the hypothesis that loss of this prostanoid could contribute to the increased disease observed in both of these mouse lines. To test this hypothesis, mice lacking mPGES1, and their congenic controls, were sensitized and challenged, as previously described. Twenty four hours after the final challenge, the impact of mPGES1 synthase on inflammation of the airways, IgE production and airway mechanics was assessed. Surprisingly, and contrary to our prediction based on analysis with the COX-1/COX-2 deficient mice, mice lacking mPGES1 had significantly less cellular infiltrate and associated eosinophilia in comparison to OVA-challenged wildtype animals (Fig 3A,B). A significant decrease in IL-13 production was observed in the BALF of the mPGES1 −/− mice compared to wildtype control animals (Fig 3C). No difference in serum IgE levels was observed between mPGES1−/− mice and their genetic controls (Fig 3D). IL-4 production is critical for IgE isotype switching (47), therefore we characterized IL-4 concentrations present in lung homogenate following induction of allergy in this model (Fig 3E). No difference was observed in the production of this $T_H2$ cytokine between the inflamed lungs of wildtype and mPGES1 −/− mice. To study this further, an additional experiment was carried out to examine proliferation and cytokine production by splenocytes from immunized and challenged mice. The proliferative response of the mPGES1 −/− cells to antigen did not differ significantly from that of control cultures, nor was a significant difference observed in the production of IFN-γ or IL-17a. Similar to the BALF, a decrease in IL-13 levels was observed in these cultures, although in this case the decreased production by mPGES1 −/− cells did not achieve statistical significance (Fig S3).

Changes in airway mechanics were determined, as previously described. As was the case for the COX-1 and COX-2 deficient cohorts, inflammation and allergic airway disease induced using this immunization protocol did not result in AHR in any parameter, either in the wildtype animals or in the mPGES1 −/− line (Fig S1 C).

### Effect of $PGE_2$ on lung inflammation in mice carrying a transgenic OVA- specific T cell receptor

The observation that IL-4 and IgE concentrations were unaffected by endogenous levels of $PGE_2$ suggests that pro-inflammatory actions mediated by this prostanoid occur subsequent to the sensitization phase of antigen. To explore this, we examined the induction of IgE and the response of splenocytes to antigen in sensitized animals prior to challenge with aerosolized antigen (Fig S3). No difference was observed in serum IgE between wildtype and mPGES1-deficient mice Splenocytes isolated from wildtype and mPGES1 −/− animals a week after booster sensitization demonstrate similar proliferative responses and no difference was observed in the production of IL-13, IFN-γ, or IL-17a between groups. Collectively, these results indicate that the reduced inflammation observed in the mPGES1 −/− lungs is unlikely to reflect alterations in the sensitization of mice to antigen, but rather the response of exposure of the lungs in sensitized animals to antigen.

To explore this further, we determined whether loss of mPGES1 would alter the development of lung inflammation in OT-II mice. These mice carry a transgenic T cell receptor specific to ovalbumin (48) and thus, exposure of the airways to this antigen results in inflammation in non-sensitized animals. In this case, however, the prominent cell type in the BALF is the neutrophil and thus this response is thought to model non-atopic allergic lung disease (49). Mice lacking mPGES1 were crossed to congenic B6 mice carrying the OT-II transgene. As expected, mice lacking the transgene, OT-II (−), showed little inflammation in response to OVA challenge. In contrast, robust cell recruitment was observed in transgenic wildtype mice (Fig 4A). Transgenic mPGES1 −/− animals showed a significant attenuation in cell infiltration compared to wildtype controls. This attenuation reflects significantly fewer granulocytes present in the BALF of the mPGES1-deficient animals (Fig S4). In addition, IL-13 levels were assessed. The levels of this cytokine in the BALF of wildtype animals was low, compared to that measured in OVA/alum sensitized animals, in agreement with the neutrophil-dominated response observed in this model. These levels were further reduced in animals lacking mPGES1, consistent with overall reduced levels of inflammation in the lung of these mice (Fig 4B).

## Individual contributions of lung and recruited inflammatory cells to $PGE_2$ mediated allergic responses

As shown above, mPGES1 contributes to the $PGE_2$ present in the healthy lung. To determine the relative contributions of mPGES1 produced by the lung and that produced by the recruited immune cells to this model of allergic lung disease, we studied the development of allergy in bone marrow chimeras. We first established that the contribution of mPGES1 to the allergic response was not altered in animals undergoing this experimental procedure. The difference in the OVA-induced cellularity of the BALF between wildtype mice irradiated and reconstituted with wildtype marrow (WT→WT) compared to that observed in mPGES1 −/− animals irradiated and reconstituted with autologous marrow (KO→KO) recapitulated the differences observed between these groups in previous experiments (Fig 5 compared to Fig 3A).

We next asked whether $PGE_2$ produced by the immune cells recruited to the lung during allergic inflammation contributes to allergic airway disease. To address this, wildtype mice were irradiated and reconstituted with either wildtype (WT→WT) or mPGES1−/− (KO→WT) bone marrow. Reconstitution of mice with mPGES1-deficient bone marrow had only a modest impact on the inflammatory response; however, no statistically significant changes were seen in total BALF cellularity, eosinophil numbers, IL-13 production or serum total IgE (Fig 6A–D).

Since the $PGE_2$ produced by recruited immune cells contributed little to the inflammation in the lung, we next addressed the possibility that the primary source of this pro-inflammatory $PGE_2$ was from the lung itself. We evaluated whether mPGES1 −/− animals reconstituted with wildtype bone marrow (WT→KO) would have attenuated allergy compared to animals in which all cells are capable of producing $PGE_2$ (WT→WT). Lung inflammation was attenuated in OVA sensitized and challenged WT→KO animals compared to animals in which both the lung and the bone marrow express mPGES1 (WT→WT). A significant decrease in BALF cellularity was observed, again primarily reflecting reduced recruitment of eosinophils (Fig 7A, B). IL-13 production was also reduced in this group (Fig 7C). These observations suggest that mPGES1 produced by cells in the lung, not recruited leukocytes, contributed to the development of allergic disease in response to ovalbumin. Consistent with the studies reported above, IgE levels were not significantly affected by loss of lung mPGES1 (Fig 7D).

## Discussion

Previous studies have shown that in the absence of COX-1 or COX-2, antigen exposure results in more severe allergic lung disease (23). Using mice lacking mPGES1 synthase, we show that attenuation of $PGE_2$ synthesis in the COX-1 and the COX-2 deficient mice does not account for the increase in disease observed in these mouse lines. In fact, in this model mPGES1 deficient mice showed reduced airway inflammation, indicating that $PGE_2$ enhances this aspect of allergic disease.

The impact of the genetic composition of mouse lines on the development of various aspects of allergic lung disease has been well established (50–53). The majority of the early studies assigning roles for COX-1 and COX-2 metabolites in inflammatory responses were carried out using mice of mixed genetic background, thus the representation of B6 and 129 genes in the COX deficient and control animals can be very different. We therefore first verified that the protection that COX-1 and COX-2 provided in this response could be observed when congenic animals were studied. Consistent with previous work, we report that both COX-1 and COX-2 dependent prostaglandins limit allergic inflammation in the lung. However, we show that both enzymes provided the mice with a similar level of protection. This differs from previous studies in which loss of COX-1 was reported to have a greater role than COX-2 both in production of $PGE_2$ in the naïve and inflamed lung as well as in limiting allergic inflammation (23). Since COX-1 and COX-2 have unique but overlapping patterns of expression and, depending on the cell type, can lead to the preferential production of a particular eicosanoid, this observation suggests that multiple prostaglandins or prostaglandins made by different cells types limit inflammation in this model.

We saw no development of AHR in either the COX-1 or the COX-2 deficient animals. This is not surprising given the genetic background of the mice. AHR is often absent in B6 mice (50, 51, 53). In previous studies, inflammation associated with loss of COX-1 but not COX-2 was reported to result in increased sensitivity to methacholine (23). It is possible that this difference reflected differences in the segregation of 129 and B6 alleles in the two populations. This would be expected, as the closure of the ductus arteriosus in mice lacking $EP_4$ or COX-2 depends on the inheritance of a particular compliment of 129 and B6 alleles (40, 54). In contrast, no such selective pressure would skew inheritance of alleles in the COX-1 population.

Early work suggested that $PGE_2$ could play an important role in regulating the differentiation of mouse B lymphocytes to IgE secreting cells (55). However, this role was not supported by the report that IgE levels were actually higher in the COX-1 and COX-2 antigen treated animals (23). Our study did not observe this increase in the COX-1 and COX-2 deficient animals compared to their genetically matched controls and therefore does not support a role for $PGE_2$ in switching B cells to IgE production. No difference was noted in serum IgE levels between COX-1 −/−, COX-2 −/− or mPGES1 −/− and their control animals after induction of a $T_H2$ response. However, direct comparison of the IgE response of COX deficient animals reported here and those reported previously is difficult for a number of reasons. Not only do our studies utilize congenic mice, the cohort examined here were between 8 to 12 weeks of age while previous studies examined mice that ranged in age from 5 and 9 months. In addition, these studies evaluated IgE levels in the BALF, while we examined serum IgE.

In patients with allergic asthma, inhaled $PGE_2$ is reported to attenuate both the early and late phase response after exposure to antigen (12, 13, 15, 16). $PGE_2$ has also been shown to limit inflammation in animal models of asthma (14, 17, 56). Given this, it seemed likely that the heightened inflammation observed in the COX deficient mice reflected a loss of this

protective prostanoid. Indeed, induction of allergic disease with ovalbumin dramatically increased $PGE_2$ levels in the lung and this augmentation was not observed when COX-1, COX-2, or mPGES1 was absent, suggesting that both enzymes are capable of coupling with mPGES1 to promote prostanoid production during lung inflammation. However, unlike a genetic loss of COX enzymatic activity, a loss of mPGES1 did not result in heightened disease, in fact, quite the opposite; loss of this pathway attenuated the inflammatory response. Our results are consistent with a model in which the primary protective COX dependent eicosanoid is prostacyclin, not $PGE_2$. Mice lacking the I-prostanoid (IP) receptor, specific for prostacyclin, were reported to have more severe allergic inflammation in the lung (57). Prostacyclin, but not $PGE_2$ was also shown to protect against the development of fibrosis in the bleomycin model of idiopathic pulmonary fibrosis (58), suggesting that, at least in the rodent lung, this might be the most important anti-inflammatory prostanoid.

We cannot rule out the possibility that the lack of a protective role for $PGE_2$ in this study is specific to this particular model and immunization protocol. A recent study examining the function of mPGES1 in a house dust mite antigen (Der f)-induced allergic model reported that $PGE_2$ limited vascular changes associated with chronic exposure to antigen while decreased $PGE_2$ had no significant impact on total recruitment of inflammatory cells to the lungs after Der f challenge (59). However, the vascular remodeling which this study showed was enhanced in the mPGES1 −/− mice is associated with chronic models of asthma and is not apparent in the acute model used in our study, preventing extension of this finding to this model of allergic lung disease. In contrast to our findings, decreased $PGE_2$ had no significant impact on recruitment of inflammatory cells to the lungs after Der f challenge. Again this difference might reflect different roles for $PGE_2$ in an acute allergic response, such as that induced by ovalbumin and adjuvant, versus a chronic model established by inhalation of a complex antigen with intrinsic ability to activate the innate immune response. Alternatively, it could reflect the fact that the mPGES1 −/− animals were compared to purchased wildtype B6 mice, whereas both the mPGES1 −/− and wildtype mice used in our studies were bred in the same facility, as studies have highlighted the importance of environmental factors, including the microbiome, in molding the immune response (60–63) and it is possible that some phenotypes reflect such differences in addition to the genetic lesion under study.

Both our findings and the phenotype of mPGES1 −/− animals in the Der f allergic model do not support early reports of heightened inflammation in $EP_3$ −/− mice sensitized and challenged with ovalbumin (26). The reason for this discrepancy is not apparent, however, we have been unable to reproduce this finding using B6 congenic $EP_3$ −/− mice (unpublished data). Furthermore, previous work in our lab has indicated that $PGE_2$, through the $EP_3$ receptor, can promote inflammation by augmenting IgE mediated mast cell degranulation, and in some circumstances $PGE_2$ alone is sufficient to mediate this response in rodents (64, 65). Much of the support for the hypothesis that $PGE_2$ plays a protective role in the lung, limiting inflammation, comes from studies in which exposure of mice to antigen is accompanied by inhalation of $PGE_2$, its stable analog, or a $PGE_2$ receptor preferring antagonist and agonist (17, 56, 66). *In vitro* studies have reinforced this hypothesis, with studies such as those which have shown $PGE_2$ to be effective in limiting migration of eosinophils and increasing production of IL-10 by dendritic cells and naïve T cells (22, 66, 67). However, extrapolating findings from either or both of these types of studies to develop models which predict the contribution of $PGE_2$ to inflammatory responses *in vivo* has proven difficult. Some of this difficulty is related to the fact that very few of the pathways attributed to $PGE_2$ through pharmacological studies with inhaled $PGE_2$ or $PGE_2$ receptor preferring agonists/antagonists are supported by evaluation of mice lacking specific $PGE_2$ receptors or combination of receptors. In some cases, the discrepancies may reflect the effective dose and specificity of the reagents used. For example, early studies assigning anti-

coagulatory properties to $PGE_2$ were later shown to reflect the ability of $PGE_2$ at concentrations used in these studies to activate the prostacyclin receptor (68). Thus it is possible that some of the protective actions of inhaled $PGE_2$ and EP receptor agonist are incorrectly assigned to the $PGE_2$ pathway. Carrying out these experiments in mice lacking the IP receptor and or EP receptors should resolve many of these issues. In some cases, inconsistency between results obtained using the various approaches might simply reflect the fact that loss of a $PGE_2$ receptor may have far less consequence for the organism than stimulation of the same pathway, due to compensatory pathways active *in vivo*. For example, stimulation of naïve T cells with $PGE_2$ *in vitro* can inhibit production of a pro-inflammatory cytokines, such as IFN-λ (67), but *in vivo*, the absence of $PGE_2$ does not necessarily lead to altered expression of this cytokine following stimulation (59), emphasizing the point that many other inflammatory mediators, distinct from $PGE_2$, can activate the same downstream pathways to upregulate responses. Inhaled $PGE_2$ through the $EP_2$ receptor limits airway constriction to methacholine (11). However, in mice with inflamed airways, the dose response curve is not shifted to the left in mice lacking $EP_2$ (unpublished data), suggesting that in the inflamed airway other pathways available are capable of regulating airway tone.

Not only were we unable to assign a protective role to $PGE_2$, our studies indicate a novel role for $PGE_2$: in some allergic responses $PGE_2$ acts as a pro-inflammatory mediator, enhancing inflammation in the lung. To further define this pro-inflammatory action of $PGE_2$, we generated bone marrow chimeras, animals in which either the lung or the recruited immune cells were deficient in the enzyme. The results from studies with these animals indicated that $PGE_2$ produced by the lung, rather than from the recruited immune cells, contributed to the inflammatory response. Furthermore, $PGE_2$ does not alter the development of antigen specific T and B cell populations, but rather plays a role either in the expansion of these population after challenge or in the recruitment of the cells to the lung. This interpretation was supported by study of mPGES1 deficient animals carrying an ovalbumin specific transgene. Loss of $PGE_2$ synthesis limited the development of inflammation when these animals were challenged with antigen, implicating $PGE_2$ in the effector phase of this response in the lung. The lack of a role for $PGE_2$ in the sensitizing phase of the allergic response correlates well with the studies of these mice in the Der f allergic model (59). No difference was observed in the repertoire of T cells elicited by this antigen.

We cannot yet identify precise mechanisms by which $PGE_2$ contributes to the inflammatory response in the lung. As discussed above, $PGE_2$ can augment mast cell degranulation *in vitro* and *in vivo* and this action is mediated through the $EP_3$ receptor (64, 65), suggesting that perhaps $PGE_2$ augments inflammation by increasing the release of mediators from these cells. However, the immunization protocol used here is not mast cell dependent (69), making it unlikely that this effector cell contributes substantially to the inflammatory response. $PGE_2$ can also increase vascular permeability and thus increase vascular leakage and formation of inflammatory exudates (70). For example, instillation of $PGE_2$ was reported to increase migration of neutrophils into airways in response to complement exposure (71). This response was attributed to vascular changes as it was attenuated by treatment with a vasoconstrictor. $PGE_2$ has been reported to influence many aspects of epithelial cell physiology, including chemokine and cytokine profiles, release of mucins, ion transport and ciliary beat (72–76). For instance, $PGE_2$ can stimulate the release of IL-6 from many cell types (64, 72, 77, 78) and IL-6 can contribute to inflammation in some allergic models (78, 79). Additional experiments will be required to define precisely the circumstances and the mechanism by which inhibition of $PGE_2$ limits disease in this allergic lung.

In summary, our studies show that loss of mPGES1, the primary enzyme required for production of $PGE_2$ from COX-1 and COX-2 metabolites, is not required for $T_H2$ polarization following sensitization of mice to OVA. However, while $PGE_2$ has largely been considered protective, playing a role in limiting the inflammatory response during the effector phase to inhaled allergens, we show that under some circumstances, this is not the case. Acute inflammation in response to ovalbumin is attenuated in mice with decreased levels of $PGE_2$, both in mice carrying an OVA specific transgene and in mice sensitized by exposure to antigen in the presence of adjuvant. These findings emphasize the complexity of the role for this prostanoid in immune responses and underscore the challenges of targeting $PGE_2$ and its receptors in the treatment of lung diseases.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations used in this paper

| | |
|---|---|
| **129** | 129S6/SvEv |
| **AA** | arachidonic acid |
| **AHR** | airway hyperresponsiveness |
| **B6** | C57BL/6 |
| **BALF** | bronchoalveolar lavage fluid |
| **cPGES** | cytosolic $PGE_2$ synthase |
| **Cox** | cyclooxygenase |
| **EP** | E prostanoid |
| **F** | filial generation |
| **mPGES1** | microsomal $PGE_2$ synthase 1 |
| **mPGES2** | microsomal $PGE_2$ synthase 2 |
| **NSAID** | non-steroidal anti-inflammatory |
| **OVA** | ovalbumin |
| **OT-II** | OVA-specific TCR-transgenic |
| **$PGE_2$** | prostaglandin $E_2$ |
| **$PGH_2$** | prostaglandin $H_2$ |

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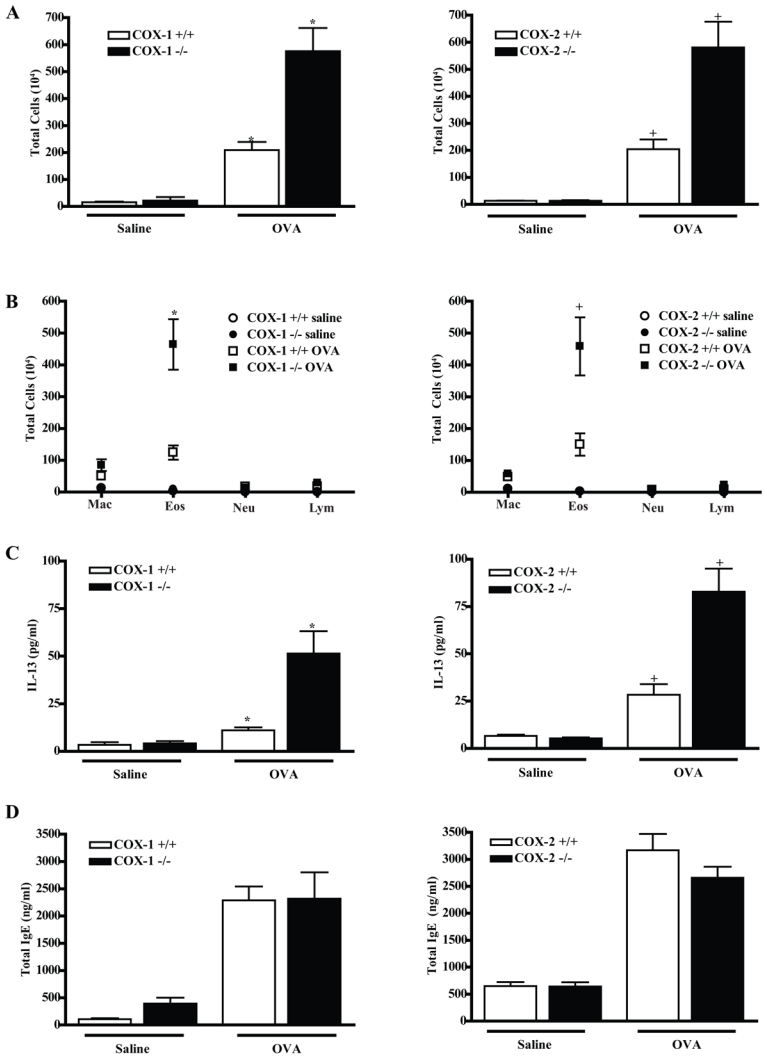


**Figure 1. Effect of OVA sensitization and challenge on inflammation in congenic COX-1 and COX-2 −/− mice**

Mice were sensitized i.p with OVA and alum and challenged with aerosolized antigen. Twenty-four hours after the final challenge serum IgE, AHR (supplemental data) and lung inflammation were assessed. **A.** OVA exposed animals display an increase in the number of cells present in the BALF. Higher total cell counts are observed in the BALF collected from mice lacking either COX-1 or COX-2 compared to genetically matched controls (*,+ $p<0.001$). **B.** Cell differentials determined for the BALF showed that this increase is due primarily to an increase in the number of eosinophils. A significant increase in eosinophil numbers is observed in the wildtype OVA treated animals and this is further augmented in the COX-1 −/− and COX-2 −/− animals relative to the OVA treated controls (* $p<0.001$, + $p<0.05$). **C.** IL-13 levels in the BALF of the COX-1 −/− and COX-2 −/− mice are also significantly higher than levels measured in the OVA-treated wildtype controls (* $p<0.01$, + $p<0.001$). **D.** OVA sensitization and challenge leads to increased total serum IgE, however, IgE levels do not differ significantly between COX deficient and control animals. (COX-1 experiments were repeated 3 times and COX-2 experiments were conducted twice. Data from one experiment is shown. For COX-1: +/+ saline n=5, −/− saline n=4, +/+ OVA n=8, −/− OVA n=9; for COX-2: +/+ saline n=5, −/− saline n=4, +/+ OVA n=11, −/− OVA n=12)

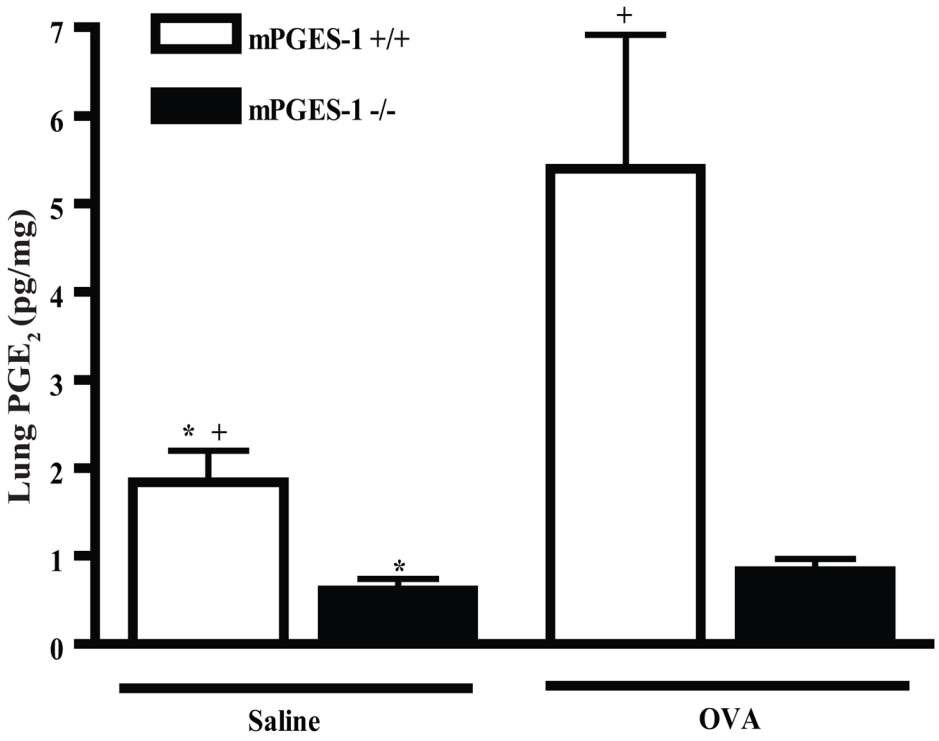


**Figure 2. $PGE_2$production by the naïve and allergic mPGES1 −/− lungs**

$PGE_2$ levels were measured in lung homogenate, prepared from naïve mice or after sensitization and challenge with OVA. In the naïve lung, concentrations of $PGE_2$ are significantly attenuated in mPGES1 −/− mice relative to wildtype mice (* $p<0.02$). $PGE_2$ levels are increased significantly in lungs collected from mice sensitized and challenged with OVA (+ $p<0.05$). In contrast no significant increase in $PGE_2$ is observed in mPGES1 −/− mice. ($PGE_2$ quantifications were made for 2 independent cohorts. Data represents one experiment. mPGES1: +/+ saline n=5, −/− saline n=5, +/+ OVA n=7, −/− OVA n=11)

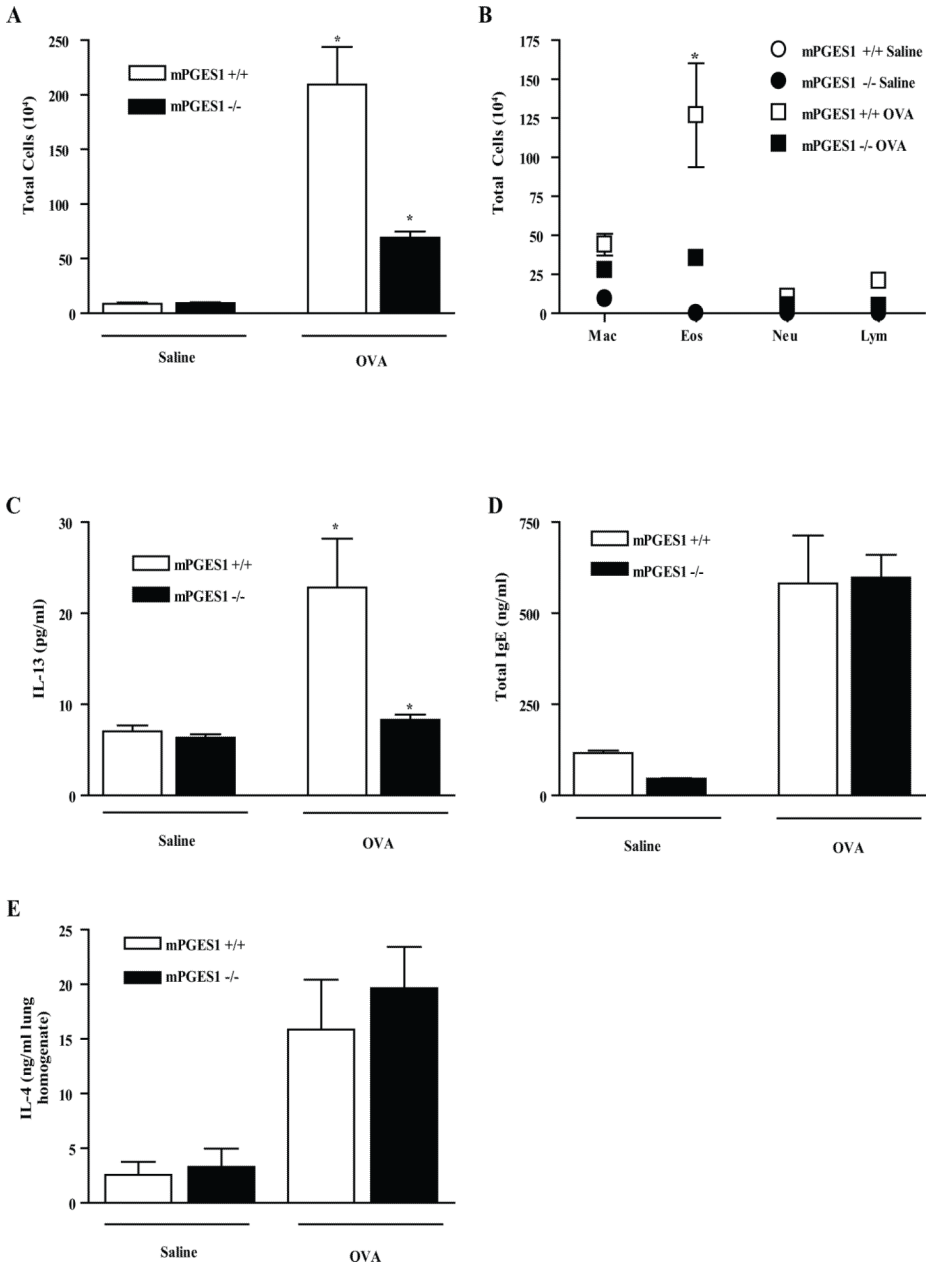


**Figure 3. Inflammatory response in mPGES1 −/− mice sensitized and challenged with OVA**

**A.** OVA sensitization and challenge leads to an increase in total cells present in the BALF of wildtype mice. While increased numbers of cells are also observed in BALF collected from mPGES1 −/− animals, the total cell count was significantly reduced compared to similarly treated wildtype control animals (* $p<0.001$). **B.** The decrease in the cellularity of the BALF of the mPGES1 −/− mice correlates with a significant decrease in the number of eosinophils in the BALF of these animals compared to the numbers present in the BALF from the control animals (* $p<0.001$). **C.** IL-13 levels in the BALF collected from OVA sensitized and challenged animals are significantly higher than those measure in saline treated cohorts, both wildtype and mPGES−/− animals, however, higher levels are observed in the samples collected from OVA treated wild type animals relative to levels in BALF from mPGES1 −/− animals (*$p<0.05$). **D.** OVA sensitization and challenge results in an increase in total

serum IgE of a similar magnitude in wildtype and mPGES1 −/− animals. **E.** IL-4 levels in whole lung homogenates do not differ significantly between samples prepared from mPGES1−/− mice and controls. As expected both groups showed levels elevated in comparison to samples prepared from saline treated cohorts. (Experiments for A–D were conducted 3 times independently and data from one experiment is shown. Data for E was generated once. For A–D: mPGES1 +/+ saline n=4, −/− saline n=3, +/+ OVA n=10, −/− OVA n=11; For E: mPGES1 +/+ saline n=2, −/− saline n=3, +/+ OVA n=5, −/− OVA n=5)

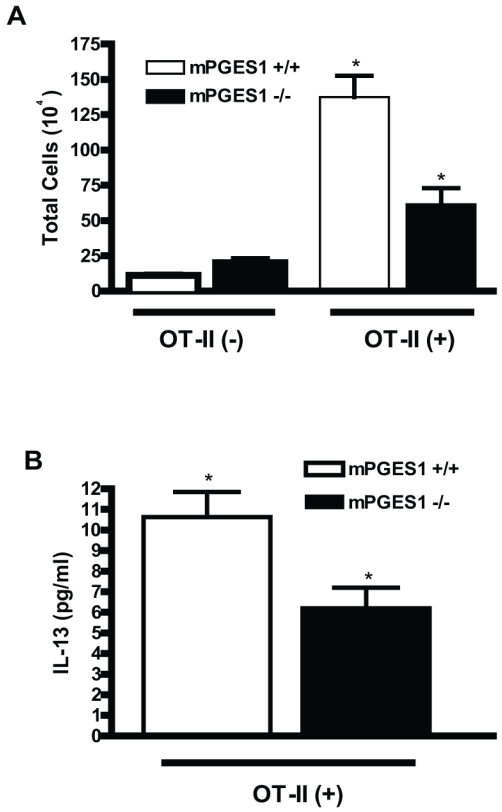


**Figure 4. OVA induced allergic inflammation in mPGES1−/− mice carrying an OVA-specific transgene**

OT-II mice, mPGES1−/− mice, OT-II/mPGES1−/− mice and wild type animals were challenged with aerosolized OVA and the development of lung inflammation was assessed. **A**. BALF was collected 24 hours after the final challenge. Antigen challenge results in an increase in the number of cells in the BALF of both OT-II expressing cohorts, however, the total number of cells is significantly lower in the BALF from the OT-II/ mPGES1 −/− animals compared to OT-II/mPGES1 +/+ mice. (* $p < 0.001$) **B.** BALF IL-13 levels are also significantly lower in samples collected from OT-II/mPGES1 −/− animals compared to OT-II/mPGES1 +/+ animals expressing mPGES1 ($p < 0.02$). (OT-II experiments were conducted twice and data from one experiment is shown. For **A**: mPGES1 +/+ n=3, mPGES1 −/− n=4, OT-II/ mPGES1 +/+ n=12, OT-II/mPGES1 −/− n=12; For **B**, OT-II/ mPGES1 +/+ n=7, OT-II/mPGES1 −/− n=7)

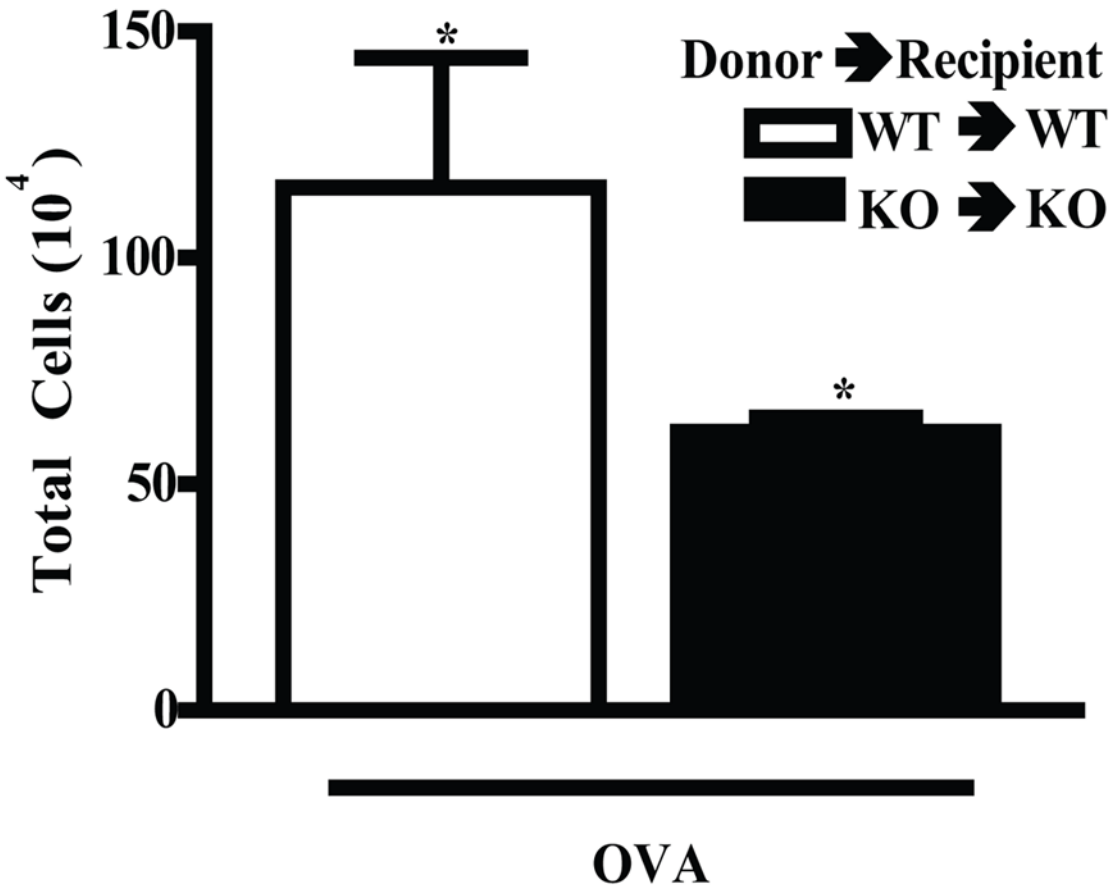


**Figure 5. Development of allergic inflammation in mPGES1 bone marrow chimeras**

**A.** Wildtype and mPGES1 −/− mice exposed to lethal doses of radiation, were reconstituted with bone marrow from autologous donors. Eight weeks after reconstitution, mice were sensitized and challenged with OVA. Wildtype mice reconstituted with wildtype bone marrow (WT→WT) have significantly elevated cell counts relative to mPGES1 −/− mice reconstituted with mPGES1 −/− bone marrow (KO→KO) ($p=0.05$). (This experiment was conducted once. For WT→WT n= 6; For KO→KO n=8)

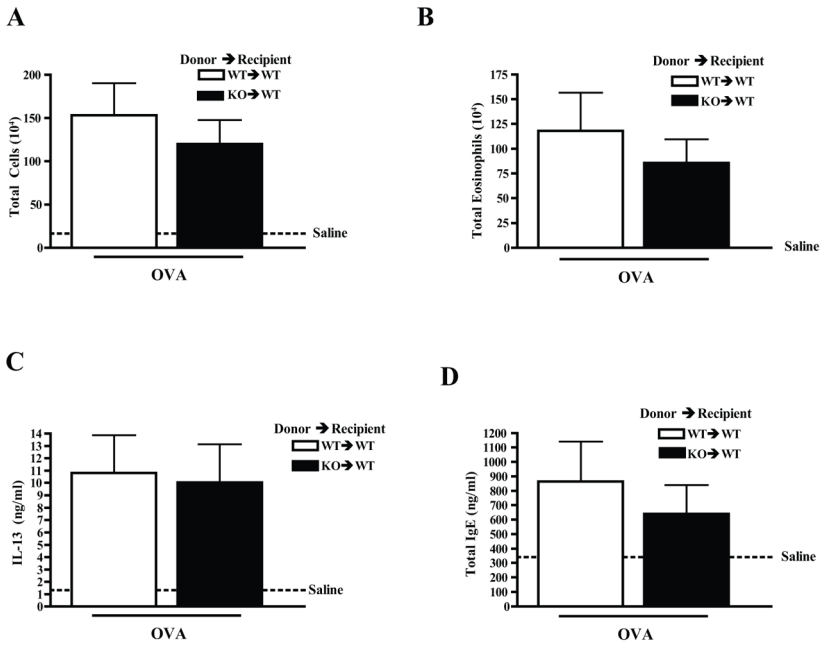


**Figure 6. Contribution of $PGE_2$ from bone marrow derived cell populations to OVA-induced lung inflammation**

Lethally irradiated wildtype mice were reconstituted with either wildtype bone marrow (WT→WT) or mPGES1 −/− bone marrow (KO→WT). **A.** As expected an increase in the cellularity of the BALF was observed in samples collected from the animals sensitized and challenged with ovalbumin. No significant difference was measured in the total number of cells present in the BALF of the two groups (WT→WT versus KO→WT). **B.** Morphological analysis of cell types present in BALF revealed elevated levels of eosinophils in both OVA-treated groups and again the numbers of these cells did not differ significantly between the animals that had received the wild type versus the mPGES1 −/− marrow. **C.** No difference is observed in the level of IL-13 in the BALF of the two OVA-treated groups. **D.** Total serum IgE concentrations are elevated to a similar degree in groups sensitized and challenged with OVA. (This experiment was carried out twice and data from one trial is shown. For WT→WT: saline n=3, OVA n=8; KO→WT: saline n=2, OVA n=8)

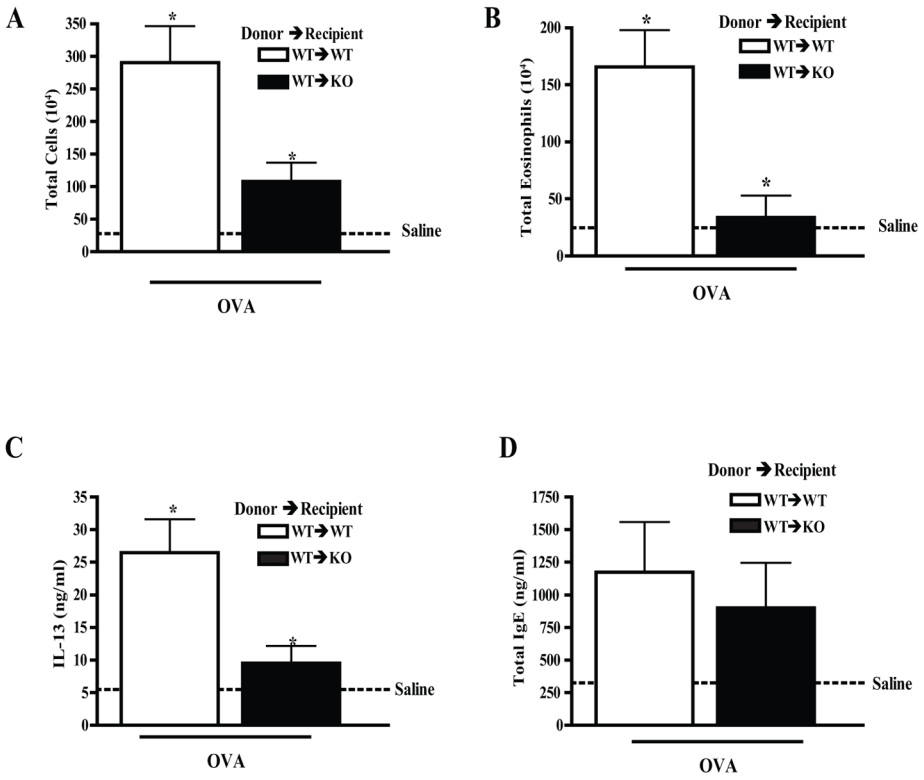


**Figure 7. Contribution of $PGE_2$ produced by radiation resistant lung populations to allergic inflammation**

Wildtype mice or mPGES1 −/− mice exposed to lethal doses of radiation were reconstituted with wildtype bone marrow, (WT→WT) and (WT→KO) respectively. BALF was collected from OVA sensitized and challenged animals and total cell numbers **(A)** and cell differentials **(B)** were determined. A decrease in both the total cell count and the number of eosinophils in the BALF was observed in samples from mPGES1 −/− mice reconstituted with wildtype marrow, compared to samples from similarly reconstituted and treated wildtype animals (WT→WT). (* $p<0.01$). **C.** IL13 levels are significantly higher in the BALF from OVA-sensitized and challenged WT→WT mice compared to levels in BALF from similarly treated mPGES1−/− animals that received wildtype bone marrow ( WT→KO) (*$p<0.05$). **D.** Total serum IgE concentrations were elevated to similar levels following OVA sensitization and challenge in both WT→WT and WT→KO animals. (This experiment was conducted twice and data from one trial is shown. For WT→WT: saline n=4, OVA n=8; WT→KO: saline n=4, OVA n=7)