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This information is current as of March 19, 2012

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J Immunol; Prepublished online 12 March 2012;

doi:10.4049/jimmunol.1101873

<http://www.jimmunol.org/content/early/2012/03/12/jimmunol.1101873>

Supplementary Data	http://www.jimmunol.org/content/suppl/2012/03/13/jimmunol.1101873.DC1.html
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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Prostaglandin E₂ Produced by the Lung Augments the Effector Phase of Allergic Inflammation

Rachel J. Church,* Leigh A. Jania,[†] and Beverly H. Koller*^{*,†,‡}

Elevated PGE₂ is a hallmark of most inflammatory lesions. This lipid mediator can induce the cardinal signs of inflammation, and the beneficial actions of nonsteroidal anti-inflammatory drugs are attributed to inhibition of cyclooxygenase (COX)-1 and COX-2, enzymes essential in the biosynthesis of PGE₂ from arachidonic acid. However, both clinical studies and rodent models suggest that, in the asthmatic lung, PGE₂ acts to restrain the immune response and limit physiological change secondary to inflammation. To directly address the role of PGE₂ in the lung, we examined the development of disease in mice lacking microsomal PGE₂ synthase-1 (mPGES1), which converts COX-1/COX-2-derived PGH₂ to PGE₂. We show that mPGES1 determines PGE₂ levels in the naive lung and is required for increases in PGE₂ after OVA-induced allergy. Although 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 PGE₂ does not impair induction of a Th2 response. Furthermore, mPGES1^{-/-} mice expressing a transgenic OVA-specific TCR are also protected, indicating that PGE₂ acts primarily after challenge with inhaled Ag. PGE₂ 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 PGE₂ produced by populations of cells native to the lung contributes to the effector phase of some allergic responses. *The Journal of Immunology*, 2012, 188: 000–000.

Prostanoids are a family of bioactive lipid mediators produced in almost every cell type by the actions of PG-endoperoxide synthases (cyclooxygenase [COX]) on arachidonic acid (AA). Synthesis is initialized when phospholipase A₂ releases AA from membrane phospholipids in response to a diverse range of stimuli. The AA is then catalyzed to PGH₂ 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 thought to be responsible for basal levels of prostanoid production, whereas COX-2 expression is generally undetectable in most tissues under homeostatic conditions and upregulated in response to inflammatory stimuli (2), although exceptions have been noted. For example, COX-1 expression increases 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). PGH₂ generated by either COX-1 or COX-2 is subsequently converted into a family of related molecules: PGD₂, PGE₂, PGF_{2α}, prosta-

cyclin (PGI₂), and thromboxane A₂ 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). PGE₂ binds with a high affinity to four receptors, E prostanoid (EP) 1–4, all of which are expressed in the lung (6, 7).

PGE₂ 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 with healthy individuals, and this enhancement can be positively correlated with disease severity (8–10). However, elevated synthesis of PGE₂ may reflect an attempt by the organism to limit ongoing inflammation and protect the airways from collateral damage. Certainly, PGE₂-mediated pathways capable of limiting inflammation and restoring homeostasis in the lung have been identified. PGE₂ is a potent smooth muscle relaxant and through the EP₂ 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). PGE₂ can induce ion secretion and thus alter the composition of the airway surface liquid, facilitating mucociliary clearance (20). Furthermore, exogenous PGE₂ can limit T cell proliferation and Th1-type cytokine release from LPS-stimulated macrophages through stimulation of the EP₂ and EP₄ receptors, respectively (21). In addition to its capacity to downregulate proinflammatory cytokine release from immune cells, PGE₂ can also stimulate release of IL-10, a cytokine generally thought to be protective in the immune system (22).

Perhaps the strongest indication that PGE₂ might function to limit allergic inflammation in the lung comes from animal studies conducted using pharmacological and genetic approaches to limit PG synthesis in a model of 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 wild-type

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Received for publication June 24, 2011. Accepted for publication February 6, 2012.

This work was supported by National Institutes of Health Grants R01 HL068141 and U19 A1077437 (to B.H.K.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: 129, 129S6/SvEv; AA, arachidonic acid; AHR, airway hyperresponsiveness; alum, aluminum hydroxide; B6, C57BL/6; BALF, bronchoalveolar lavage fluid; COX, cyclooxygenase; Der f, house dust mite Ag; EP, E prostanoid; F, filial generation; KO, knockout; Mch, methacholine chloride; mPGES1, microsomal PGE₂ synthase-1; mPGES2, microsomal PGE₂ synthase-2; NSAID, nonsteroidal anti-inflammatory; OT-II, OVA-specific TCR-transgenic; WT, wild-type.

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(WT) animals (23). Consistent with this, treatment of mice with indomethacin, a nonsteroidal anti-inflammatory drug (NSAID) that 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 PG(s) responsible for limiting the allergic response. Another study, however, reported increased allergic inflammation in mice lacking EP₃ receptors (26), suggesting that loss of PGE₂ is at least partly responsible for heightened inflammation observed in COX-deficient and NSAID-treated animals.

PGE₂ production occurs through the metabolism of PGH₂ by the microsomal PGE₂ synthase-1 (mPGES1) (5). Although initially two additional enzymes, cytosolic PGE₂ synthase and microsomal PGE₂ synthase-2 (mPGES2), were thought to be capable of this enzymatic conversion, studies using mutant mouse lines carrying mutations in the genes for 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 PGE₂ (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).

In this study, we elucidate the contribution of mPGES1-derived PGE₂ 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 PGE₂ produced by mPGES1 expressed by resident airway cells and PGE₂ 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 National Institutes of Health Guide for the Care and Use of Laboratory Animals and 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 and 12 wk of age. C57BL/6 (B6) (backcrossed >10 generations) COX-1^{−/−}, B6 × 129S6/SvEv (129) filial generation (F1) 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 Laboratory (Bar Harbor, ME). 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 (alum; Sigma-Aldrich). One week following the second injection, experimental mice were challenged for 5 consecutive d (days 21–25) with an aerosol instillation of 1% OVA in 0.9% NaCl for 1 h each day. Control animals were challenged with 0.9% NaCl only. Twenty-four 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). RBCs were lysed in lysis buffer (4.1 g

NH₂Cl, 0.5 g KHCO₃, and 100 μl 0.5M EDTA dissolved in 500 ml distilled H₂O), and splenocytes were washed twice in PBS. Cells were plated at a density of 2.5 × 10⁶ cells/ml in RPMI 1640 media enriched with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 0.29 μg/ml L-glutamine. OVA was added to splenocyte cultures at concentrations ranging from 0–100 μg/ml. After incubation for 72 h 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 pentobarbital 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 pancuronium bromide. Forced oscillatory mechanics were measured every 10 s for 3 min. These measurements allowed for the assessment of airway resistance in the central airways as well as in tissue damping. Increasing doses (between 0 and 50 mg/ml) of methacholine chloride (Mch) were administered to animals through a nebulizer to measure 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 1-ml aliquots of HBSS (Life Technologies). A total of 100 μl BALF was reserved for cell count analysis, and total cell counts were measured by hemacytometer. Differential cell counts were collected using cytospin preparations stained with Hema 3 and Fast Green. All remaining BALF was centrifuged to remove cells and stored at −80°C for immunoassay.

PGE₂ and cytokine production

Levels of cytokines and PGE₂ 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 × 10⁷ cells/ml in the presence of 100 μg/ml OVA. After 72 h, 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, 1 mM CaCl₂, and 1 mM MgCl₂ 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 Systems), and IL-17a (eBioscience). For quantification of PGE₂, lung tissue was pulverized and then homogenized in 1× PBS/1 mM EDTA and 10 μM indomethacin. Lipids were purified through octadecyl C₁₈ 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 Ab (clone R35-72; BD Pharmingen), blocked with 1% BSA/PBS, and then incubated with IgE standard (BD Pharmingen) or serum followed by biotinylated rat anti-mouse IgE (clone R35-118; BD Pharmingen). Detection was carried out using streptavidin-HRP (BD Pharmingen) and hydrogen peroxide/2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). Absorbance at 405 nm was measured.

Bone marrow chimera generation

Recipient mice were exposed to 5 grays irradiation from a Cesium γ-irradiator at 0 and 3 h. 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 wk animals were sensitized and challenged with OVA.

Statistical analysis

Statistical analysis was performed using Prism 4 (GraphPad). Comparisons of the mean were made by F test, Student *t* test, or ANOVA followed by the Tukey-Kramer honestly significant difference post hoc test as necessary. Data are shown as mean ± SEM. Differences with *p* < 0.05 were considered statistically significant.

Results

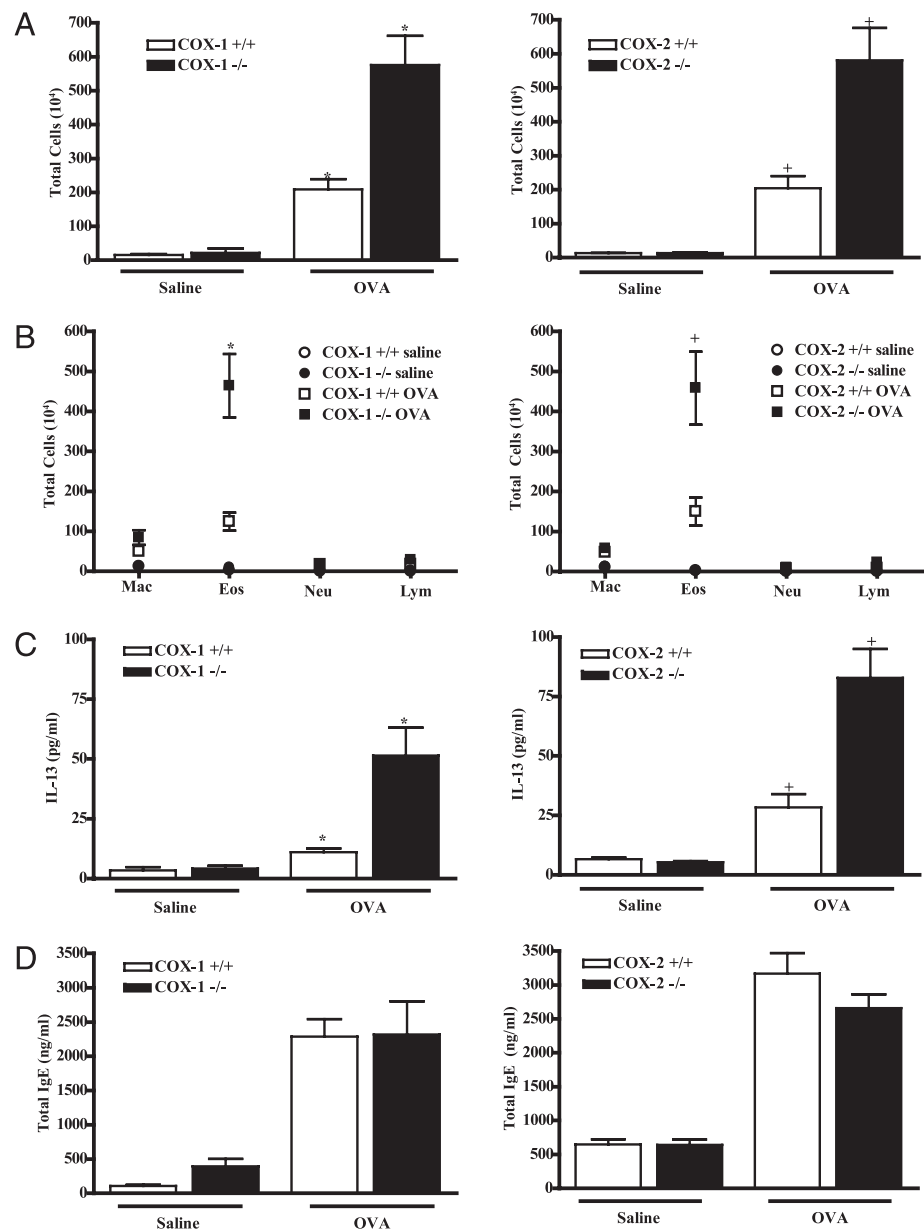
Allergic asthma in the COX-1 and COX-2-deficient mice

We first verified, using congenic mouse lines, the contribution of prostanooids to allergic lung disease induced by sensitization and challenge with OVA Ag. 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 used > 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 coisogenic 129 genetic background and the second congenic line on the B6 genetic background. The 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 in this study. Allergic airway disease was induced through sensitization by an i.p. injection of OVA emulsified in alum and challenged with re-

peated aerosols of Ag. Inflammation was assessed 24 h after the final Ag challenge.

As expected, our immunization protocol induced a robust cellular influx in B6 WT mice (COX-1^{+/-}) compared with 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 Ag challenge was marked by heightened levels of eosinophils, typical of Th2-type allergic responses (Fig. 1B). Characteristic of this type of response, IL-13 levels were elevated in the BALF of WT mice with allergic lung disease compared with 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 WT control, was surprisingly similar in the two lines. Elevated IgE levels were observed in both B6 COX-1^{+/-} and B6/129 F1 COX-2^{+/-} WT

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 Ag. Twenty-four hours after the final challenge serum IgE, AHR (Supplemental Fig. 1A, 1B) 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 with 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 WT 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 WT 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 three times, and COX-2 experiments were conducted twice. Data from one experiment are 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.



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 Ig isotype.

AHR following challenge with 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 and tissue damping. 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 (Supplemental Fig. 1A, 1B).

Contribution of mPGES1 to PGE₂ production in the naive and inflamed lung

The ability to catalyze the conversion of PGH₂ to PGE₂ has been assigned to three distinct enzymes: mPGES1, mPGES2, and cytosolic PGE₂ synthase (5, 41, 42). However, studies using mutant mouse lines have shown *in vivo* alteration of PGE₂ levels only in the mPGES1 mice (29, 43). We therefore first determined whether PGE₂ 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 PGE₂ were determined by enzyme immunoassay (Fig. 2). PGE₂ 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 PGE₂ in this organ. PGE₂ levels were substantially potentiated in the lungs of WT 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 COX-2-deficient animals.

Impact of PGE₂ on allergic lung inflammation

PGE₂ levels in the lung increase dramatically after allergic lung disease, and this increase is absent in both the COX-1- and COX-2-deficient mice (Supplemental Fig. 2), supporting the hypothesis

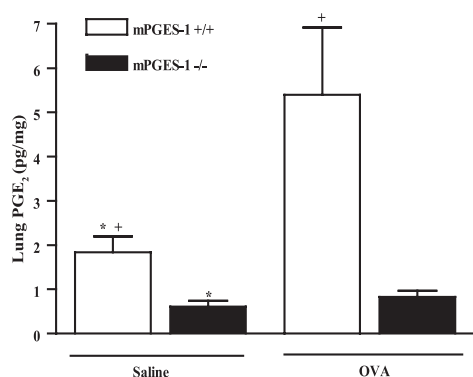


FIGURE 2. PGE₂ production by the naive and allergic mPGES1^{-/-} lungs. PGE₂ levels were measured in lung homogenate prepared from naive mice or after sensitization, and challenged with OVA. In the naive lung, concentrations of PGE₂ are significantly attenuated in mPGES1^{-/-} mice relative to WT mice (**p* < 0.02). PGE₂ levels are increased significantly in lungs collected from mice sensitized and challenged with OVA (**p* < 0.05). In contrast, no significant increase in PGE₂ is observed in mPGES1^{-/-} mice. PGE₂ quantifications were made for two independent cohorts. Data represent one experiment. mPGES1: ^{+/+} saline, *n* = 5; ^{-/-} saline, *n* = 5; ^{+/+} OVA, *n* = 7; ^{-/-} OVA, *n* = 11.

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 with OVA-challenged WT animals (Fig. 3A, 3B). A significant decrease in IL-13 production was observed in the BALF of the mPGES1^{-/-} mice compared with WT 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 (44); 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 Th2 cytokine between the inflamed lungs of WT 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 Ag 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 (Supplemental Fig. 3).

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 WT animals or in the mPGES1^{-/-} line (Supplemental Fig. 1C).

Effect of PGE₂ on lung inflammation in mice carrying a transgenic OVA-specific TCR

The observation that IL-4 and IgE concentrations were unaffected by endogenous levels of PGE₂ suggests that proinflammatory actions mediated by this prostanoid occur subsequent to the sensitization phase of Ag. To explore this, we examined the induction of IgE and the response of splenocytes to Ag in sensitized animals prior to challenge with aerosolized Ag (Supplemental Fig. 3). No difference was observed in serum IgE between WT and mPGES1^{-/-} mice. Splenocytes isolated from WT 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 Ag, but rather the response of exposure of the lungs in sensitized animals to Ag.

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 TCR specific to OVA (45), and thus, exposure of the airways to this Ag 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 nonatopic allergic lung disease (46). 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 WT mice (Fig. 4A). Transgenic mPGES1^{-/-} animals showed a significant attenuation in

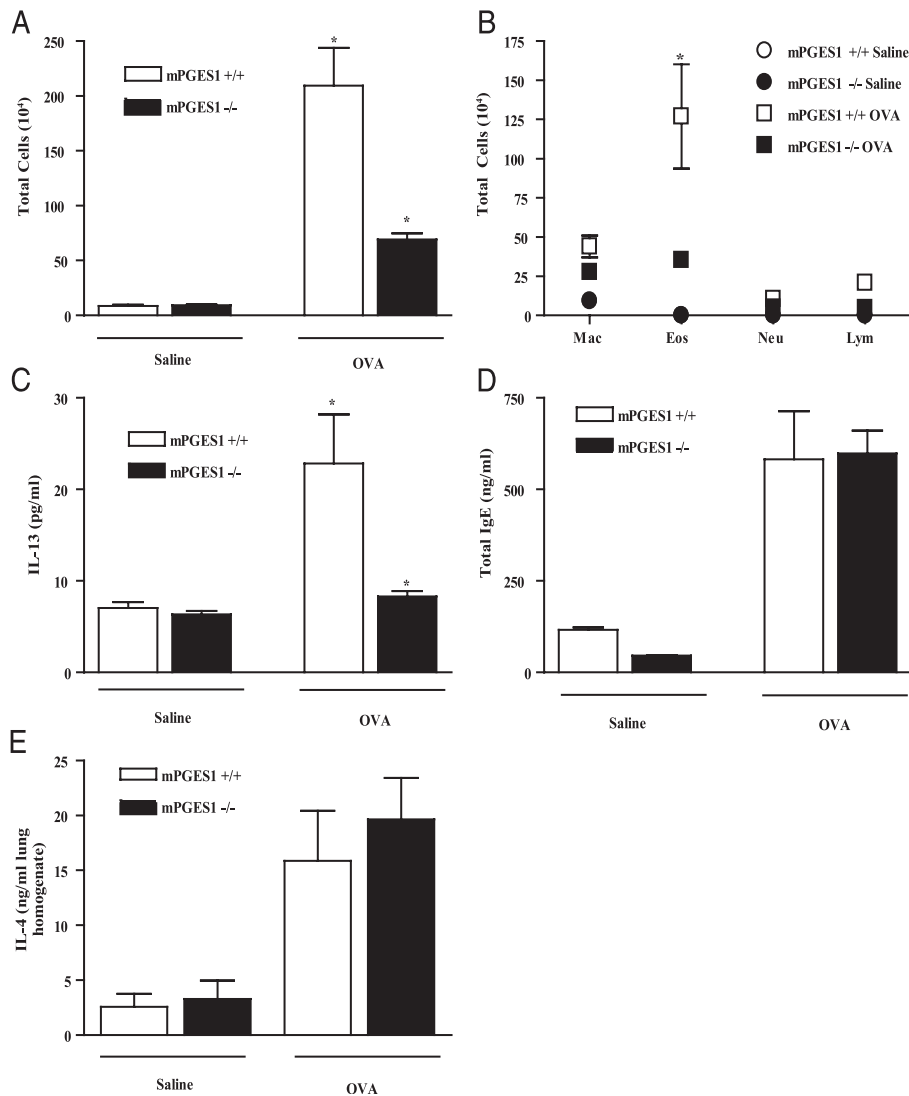


FIGURE 3. Inflammatory response in mPGES1^{-/-} mice sensitized and challenged with OVA. **(A)** OVA sensitization and challenge leads to an increase in total cells present in the BALF of WT mice. Although increased numbers of cells are also observed in BALF collected from mPGES1^{-/-} animals, the total cell count was significantly reduced compared with similarly treated WT 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 with 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 WT and mPGES1^{-/-} animals; however, higher levels are observed in the samples collected from OVA-treated WT 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 WT 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 with samples prepared from saline treated cohorts. Experiments for (A)–(D) were conducted three times independently, and data from one experiment are 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, $n = 5$.

cell infiltration compared with WT controls. This attenuation reflects significantly fewer granulocytes present in the BALF of the mPGES1-deficient animals (Supplemental Fig. 4). In addition, IL-13 levels were assessed. The levels of this cytokine in the BALF of WT animals was low compared with 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 PGE₂-mediated allergic responses

As shown above, mPGES1 contributes to the PGE₂ 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 WT mice irradiated and reconstituted with WT marrow (WT→WT) compared with that observed in mPGES1^{-/-} animals irradiated and reconstituted with autologous marrow (knockout [KO]→KO) recapitulated the differences observed between these groups in previous experiments (Figs. 5 compared with 3A).

We next asked whether PGE₂ produced by the immune cells recruited to the lung during allergic inflammation contributes to allergic airway disease. To address this, WT mice were irradiated and reconstituted with either WT (WT→WT) or mPGES1^{-/-} (KO→WT) bone marrow. Reconstitution of mice with mPGES1-

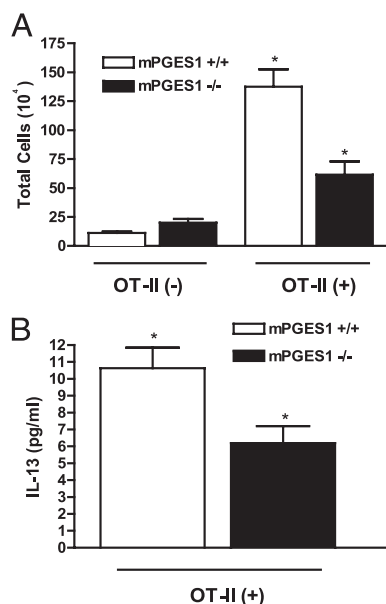


FIGURE 4. OVA-induced allergic inflammation in mPGES1^{-/-} mice carrying an OVA-specific transgene. OT-II mice, mPGES1^{-/-} mice, OT-II/mPGES1^{-/-} mice, and WT animals were challenged with aerosolized OVA, and the development of lung inflammation was assessed. **(A)** BALF was collected 24 h after the final challenge. Ag 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 with 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 with OT-II/mPGES1^{+/+} animals expressing mPGES1 (**p* < 0.02). OT-II experiments were conducted twice, and data from one experiment are 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.

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. 6).

Because the PGE₂ produced by recruited immune cells contributed little to the inflammation in the lung, we next addressed the possibility that the primary source of this proinflammatory PGE₂ was from the lung itself. We evaluated whether mPGES1^{-/-} animals reconstituted with WT bone marrow (WT→KO) would

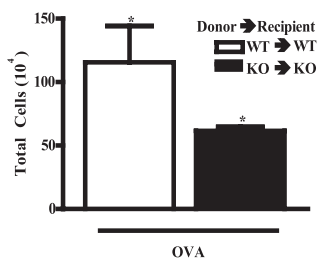


FIGURE 5. Development of allergic inflammation in mPGES1 bone marrow chimeras. WT and mPGES1^{-/-} mice exposed to lethal doses of radiation were reconstituted with bone marrow from autologous donors. Eight weeks after reconstitution, mice were sensitized and challenged with OVA. WT mice reconstituted with WT bone marrow (WT→WT) have significantly elevated cell counts relative to mPGES1^{-/-} mice reconstituted with mPGES1^{-/-} bone marrow (KO→KO). This experiment was conducted once. For WT→WT, *n* = 6; for KO→KO, *n* = 8. **p* = 0.05.

have attenuated allergy compared with animals in which all cells are capable of producing PGE₂ (WT→WT). Lung inflammation was attenuated in OVA-sensitized and challenged WT→KO animals compared with 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, 7B). 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 OVA. 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, Ag exposure results in more severe allergic lung disease (23). Using mice lacking mPGES1 synthase, we show that attenuation of PGE₂ synthesis in the COX-1- and 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 PGE₂ 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 (47–50). 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 PGs 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 PGE₂ in the naive and inflamed lung as well as in limiting allergic inflammation (23). Because 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 PGs or PGs made by different cells types limit inflammation in this model.

We saw no development of AHR in either the COX-1- or COX-2-deficient animals. This is not surprising given the genetic background of the mice. AHR is often absent in B6 mice (47, 48, 50). 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 EP₄ or COX-2 depends on the inheritance of a particular complement of 129 and B6 alleles (40, 51). In contrast, no such selective pressure would skew inheritance of alleles in the COX-1 population.

Early work suggested that PGE₂ could play an important role in regulating the differentiation of mouse B lymphocytes to IgE-secreting cells (52). However, this role was not supported by the report that IgE levels were actually higher in the COX-1 and COX-2 Ag-treated animals (23). Our study did not observe this increase in the COX-1- and COX-2-deficient animals compared with their genetically matched controls and therefore does not support a role for PGE₂ in switching B cells to IgE production. No

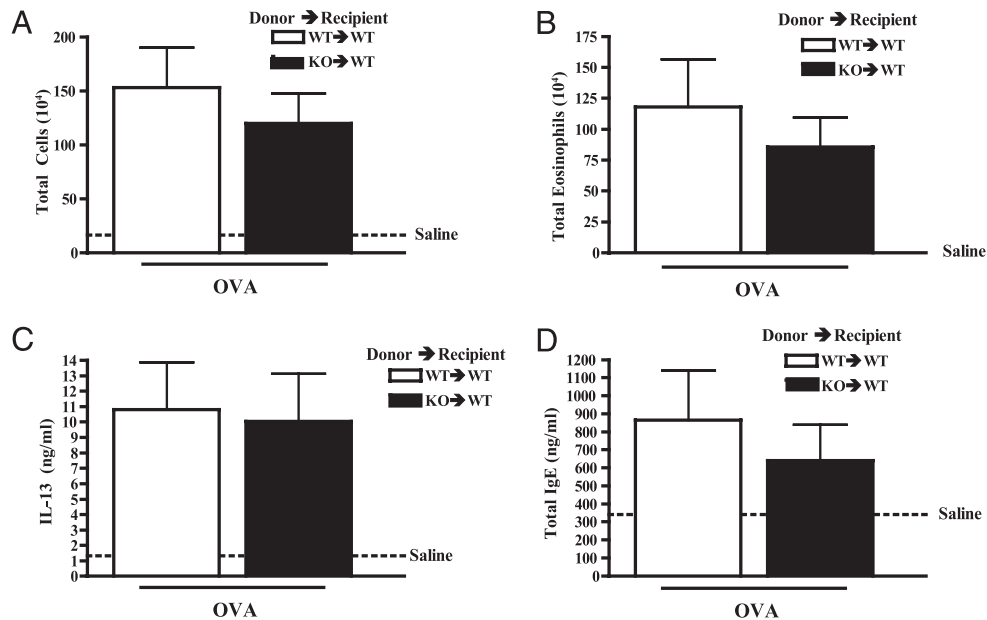
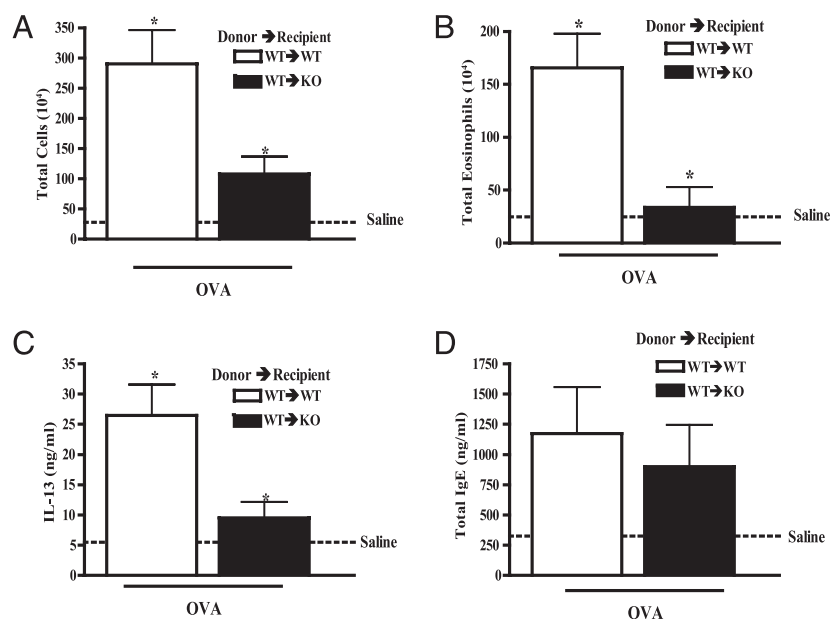


FIGURE 6. Contribution of PGE₂ from bone marrow-derived cell populations to OVA-induced lung inflammation. Lethally irradiated WT mice were reconstituted with either WT bone marrow (WT→WT) or mPGES1^{-/-} bone marrow (KO→WT). **(A)** As expected, an increase in the cellularity of the BALF was observed in samples collected from the animals sensitized and challenged with OVA. 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 WT 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 are shown. For WT→WT: saline, *n* = 3; OVA, *n* = 8; KO→WT: saline, *n* = 2; OVA, *n* = 8.

difference was noted in serum IgE levels among COX-1^{-/-}, COX-2^{-/-}, or mPGES1^{-/-} and their control animals after induction of a Th2 response. However, direct comparison of the IgE response of COX-deficient animals reported in this study and those reported previously is difficult for a number of reasons. Not only do our studies use congenic mice, but also the cohort examined in this study was between 8 and 12 wk of age, whereas previous studies examined mice that ranged in age from 5–9 mo. In addition, these studies evaluated IgE levels in the BALF, whereas we examined serum IgE.

In patients with allergic asthma, inhaled PGE₂ is reported to attenuate both the early- and late-phase response after exposure to Ag (12, 13, 15, 16). PGE₂ has also been shown to limit inflammation in animal models of asthma (14, 17, 53). 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 OVA dramatically increased PGE₂ 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

FIGURE 7. Contribution of PGE₂ produced by radiation-resistant lung populations to allergic inflammation. WT mice or mPGES1^{-/-} mice exposed to lethal doses of radiation were reconstituted with WT 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 WT marrow compared with samples from similarly reconstituted and treated WT animals (WT→WT). **p* < 0.01. **(C)** IL-13 levels are significantly higher in the BALF from OVA-sensitized and -challenged WT→WT mice compared with levels in BALF from similarly treated mPGES1^{-/-} animals that received WT 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 are shown. For WT→WT: saline, *n* = 4; OVA, *n* = 8; WT→KO: saline, *n* = 4; OVA, *n* = 7.



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 PGE₂. Mice lacking the I-prostanoid receptor, specific for prostacyclin, were reported to have more severe allergic inflammation in the lung (54). Prostacyclin, but not PGE₂, was also shown to protect against the development of fibrosis in the bleomycin model of idiopathic pulmonary fibrosis (55), 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 PGE₂ 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 Ag (Der f)-induced allergic model reported that PGE₂ limited vascular changes associated with chronic exposure to Ag, whereas decreased PGE₂ had no significant impact on total recruitment of inflammatory cells to the lungs after Der f challenge (56). However, the vascular remodeling that 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 PGE₂ had no significant impact on recruitment of inflammatory cells to the lungs after Der f challenge. Again, this difference might reflect different roles for PGE₂ in an acute allergic response, such as that induced by OVA and adjuvant versus a chronic model established by inhalation of a complex Ag with intrinsic ability to activate the innate immune response. Alternatively, it could reflect the fact that the mPGES1^{-/-} animals were compared with purchased WT B6 mice, whereas both the mPGES1^{-/-} and WT 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 (57–60), 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 EP₃^{-/-} mice sensitized and challenged with OVA (26). The reason for this discrepancy is not apparent; however, we have been unable to reproduce this finding using B6 congenic EP₃^{-/-} mice (M. Nguyen and B.H. Koller, unpublished observations). Furthermore, previous work in our laboratory has indicated that PGE₂, through the EP₃ receptor, can promote inflammation by augmenting IgE-mediated mast cell degranulation, and, in some circumstances, PGE₂ alone is sufficient to mediate this response in rodents (61, 62).

Much of the support for the hypothesis that PGE₂ plays a protective role in the lung, limiting inflammation, comes from studies in which exposure of mice to Ag is accompanied by inhalation of PGE₂, its stable analog, or a PGE₂ receptor preferring antagonist and agonist (17, 53, 63). In vitro studies have reinforced this hypothesis, with studies such as those which have shown PGE₂ to be effective in limiting migration of eosinophils and increasing production of IL-10 by dendritic cells and naive T cells (22, 63, 64). However, extrapolating findings from either or both of these types of studies to develop models that predict the contribution of PGE₂ 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 PGE₂ through pharmacological studies with inhaled PGE₂ or PGE₂ receptor preferring agonists/

antagonists are supported by evaluation of mice lacking specific PGE₂ receptors or a combination of receptors. In some cases, the discrepancies may reflect the effective dose and specificity of the reagents used. For example, early studies assigning anticoagulatory properties to PGE₂ were later shown to reflect the ability of PGE₂ at concentrations used in these studies to activate the prostacyclin receptor (65). Thus, it is possible that some of the protective actions of inhaled PGE₂ and EP receptor agonist are incorrectly assigned to the PGE₂ pathway. Carrying out these experiments in mice lacking the I-prostanoid receptor and/or EP receptors should resolve many of these issues. In some cases, inconsistency among results obtained using the various approaches might simply reflect the fact that loss of a PGE₂ 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 naive T cells with PGE₂ in vitro can inhibit production of a proinflammatory cytokine, such as IFN- γ (64), but in vivo, the absence of PGE₂ does not necessarily lead to altered expression of this cytokine following stimulation (56), emphasizing the point that many other inflammatory mediators, distinct from PGE₂, can activate the same downstream pathways to upregulate responses. Inhaled PGE₂ through the EP₂ 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 EP₂ (J.M. Hartney and B.H. Koller, unpublished observations), 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 PGE₂, but also our studies indicate a novel role for PGE₂: in some allergic responses, PGE₂ acts as a proinflammatory mediator, enhancing inflammation in the lung. To further define this proinflammatory action of PGE₂, 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 PGE₂ produced by the lung, rather than from the recruited immune cells, contributed to the inflammatory response. Furthermore, PGE₂ does not alter the development of Ag-specific T and B cell populations, but rather plays a role either in the expansion of these populations after challenge or in the recruitment of the cells to the lung. This interpretation was supported by study of mPGES1-deficient animals carrying an OVA-specific transgene. Loss of PGE₂ synthesis limited the development of inflammation when these animals were challenged with Ag, implicating PGE₂ in the effector phase of this response in the lung. The lack of a role for PGE₂ in the sensitizing phase of the allergic response correlates well with the studies of these mice in the Der f allergic model (56). No difference was observed in the repertoire of T cells elicited by this Ag.

We cannot yet identify precise mechanisms by which PGE₂ contributes to the inflammatory response in the lung. As discussed above, PGE₂ can augment mast cell degranulation in vitro and in vivo, and this action is mediated through the EP₃ receptor (61, 62), suggesting that perhaps PGE₂ augments inflammation by increasing the release of mediators from these cells. However, the immunization protocol used in this study is not mast cell dependent (66), making it unlikely that this effector cell contributes substantially to the inflammatory response. PGE₂ can also increase vascular permeability and thus increase vascular leakage and formation of inflammatory exudates (67). For example, instillation of PGE₂ was reported to increase migration of neutrophils into airways in response to complement exposure (68). This response was attributed to vascular changes, as it was attenuated by treatment with a vasoconstrictor. PGE₂ has been reported to influence

many aspects of epithelial cell physiology, including chemokine and cytokine profiles, release of mucins, ion transport, and ciliary beat (69–73). For instance, PGE₂ can stimulate the release of IL-6 from many cell types (61, 69, 74, 75) and IL-6 can contribute to inflammation in some allergic models (75, 76). Additional experiments will be required to define precisely the circumstances and the mechanism by which inhibition of PGE₂ limits disease in this allergic lung.

In summary, our studies show that loss of mPGES1, the primary enzyme required for production of PGE₂ from COX-1 and COX-2 metabolites, is not required for Th2 polarization following sensitization of mice to OVA. However, although PGE₂ 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 OVA is attenuated in mice with decreased levels of PGE₂, both in mice carrying an OVA-specific transgene and in mice sensitized by exposure to Ag 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 PGE₂ and its receptors in the treatment of lung diseases.

Acknowledgments

We thank Dr. Coy Allen for assistance with airway response data, Rebecca Parrish for genotyping, and Ben Buck for assistance with statistical analysis.

Disclosures

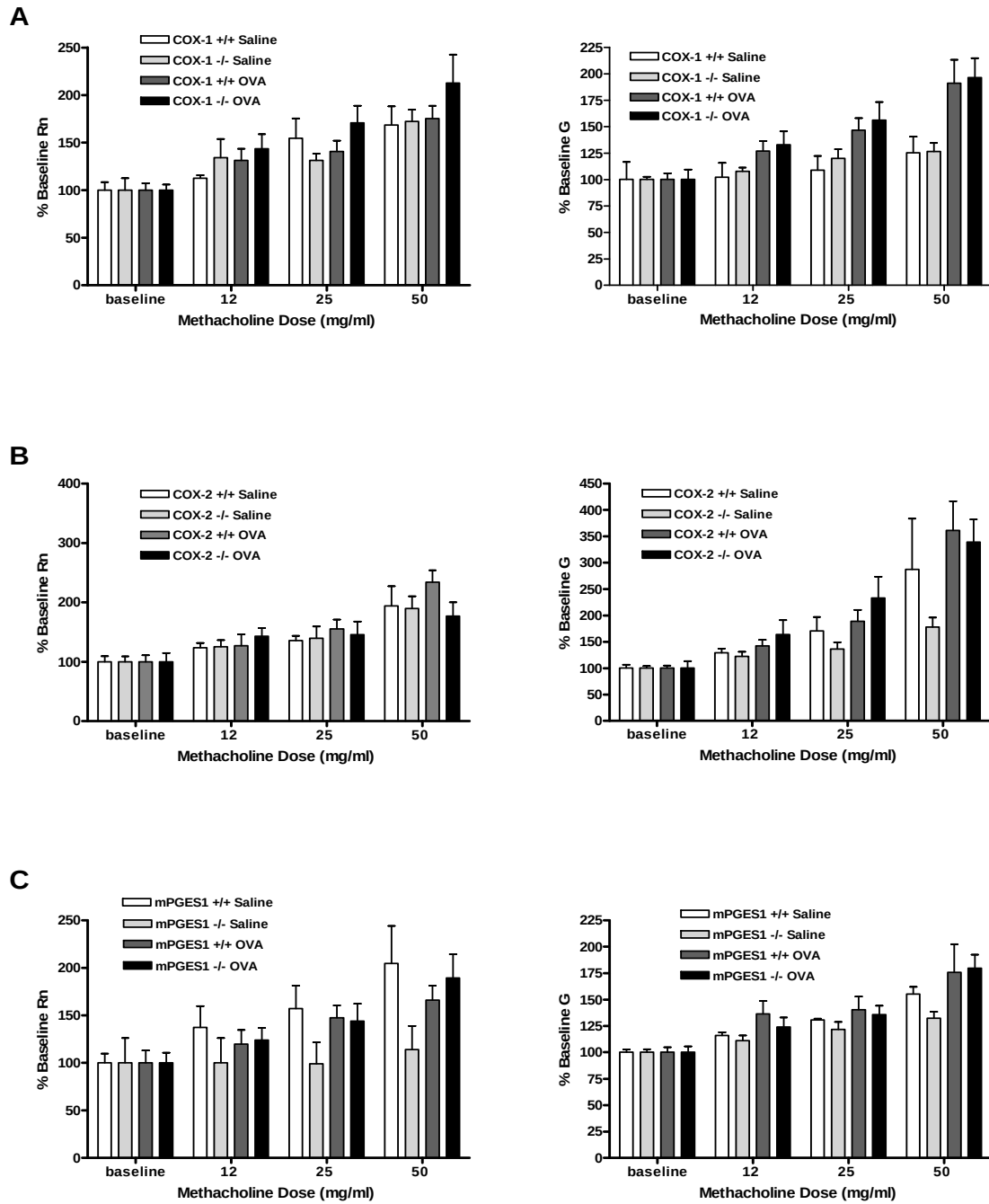
The authors have no financial conflicts of interest.

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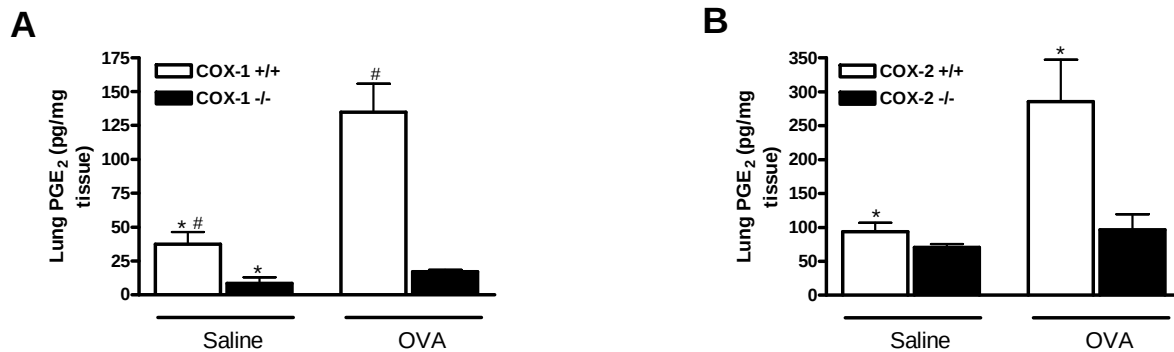
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Figure S1



Supplemental Figure 1. Measurements of airway mechanics in COX-1 -/-, COX-2 -/-, and

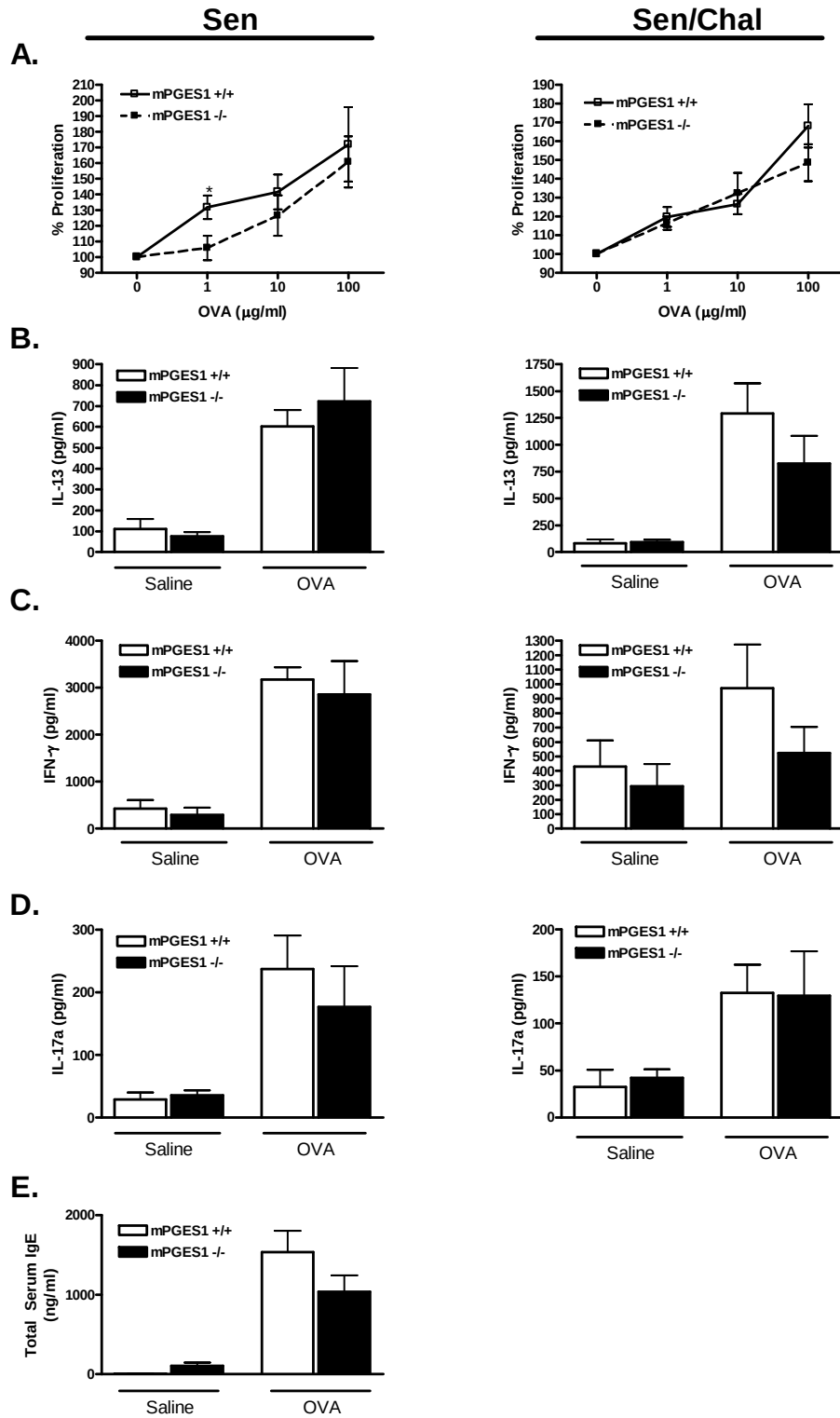
mPGES1 -/- mice Airway mechanics were assessed in COX-1 -/- mice and their congenic controls **(A)** COX-2 -/- mice and their congenic controls **(B)** and mPGES1 -/- mice and their controls **(C)**. Anesthetized and intubated mice, attached to a computer-controlled small-animal ventilator, were exposed to increasing concentrations of aerosolized Mch and central airway resistance (R_n) and tissue damping (G) were assessed. No significant differences were observed between any groups at any concentration for either parameter. (AHR measurements were made on two independent cohorts for COX-1, COX-2, and mPGES1. COX-1 data representative of 2 independent experiments: +/+ saline n=7, -/- saline n=8, +/+ OVA n=15, -/- OVA n=13; for COX-2: +/+ saline n=5, -/- saline n=4, +/+ OVA n=11, -/- OVA n=12; for mPGES1: +/+ saline n=4, -/- saline n=4, +/+ OVA n=9, -/- OVA n=11)

Figure S2

Supplemental Figure 2. Production of PGE₂ in the lungs of COX-1 and COX-2 deficient

animals PGE₂ levels were assessed in lung homogenate obtained from naïve or OVA sensitized and challenged COX-1 -/- (**A**) or COX-2 -/- (**B**) mice and their congenic controls. **A.** The level of PGE₂ measured in the lungs of naïve COX-1 -/- mice is reduced compared to congenic wildtype controls (*p<0.05). Following challenge with antigen in sensitized animals, wildtype mice have substantially elevated concentrations of lung PGE₂ (# p<0.01). No PGE₂ augmentation is observed in the COX-1 -/- inflamed lung. **B.** Naïve COX-2 -/- animals and their congenic controls have similar levels of PGE₂ measured in lung homogenate. Wildtype animals sensitized and challenged with OVA experience a significant enhancement of lung PGE₂ compared to saline treated controls (* p<0.05). No elevation of PGE₂ is measured in the homogenate of inflamed lungs obtained from COX-2 -/- mice. (PGE₂ measurements in the lungs of COX-deficient mice were made once. For COX-1: +/+ saline n=3, -/- saline n=3, +/+ OVA n=5, -/- OVA n=6; For COX-2: +/+ saline n=3, -/- saline n=3, +/+ OVA n=4, -/- OVA n= 6).

Figure S3

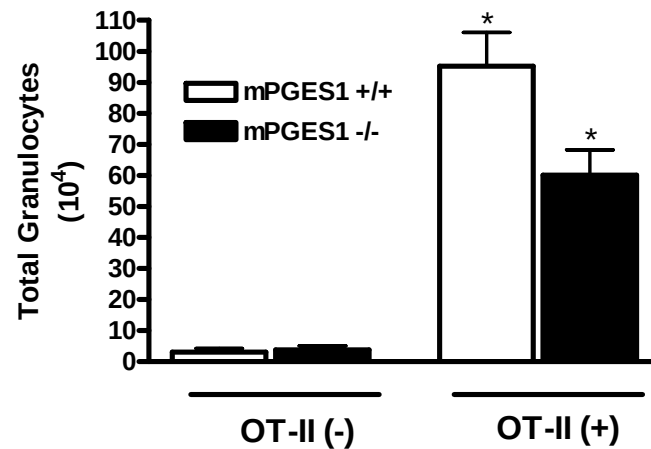


Supplemental Figure 3: Ex vivo mPGES1 splenocyte responses

Wildtype and mPGES1 ^{-/-} animals were sensitized and/or challenged with OVA antigen as described. Briefly, animals were sensitized with OVA in alum on days 0 and 14. On days 21-25 some mice were challenged with OVA and spleens were collected 24 hours later. To examine the cellular response of mice receiving sensitizing OVA only, spleens from some animals were harvested on day 21 prior to challenge. **A.** Splenocytes from sensitized and sensitized/challenged animals were plated in the presence of increasing concentrations of OVA. Following a 72 hour incubation, proliferation was assessed using WST-1 reagent. No significant difference in proliferation was observed between mPGES1-deficient animals and wildtype controls in either sensitized or sensitized/challenged mice. **B-D** Splenocytes were plated in media containing 100 µg/ml OVA. Following a 72 hour incubation, cell supernatants were collected and IL-13 (**B**), IFN-γ (**C**), and IL-17a (**D**) cytokine levels were measured. OVA restimulation resulted in elevated cytokine levels from the splenocytes of both sensitized only and challenged animals. While a reduction was observed in IL-13 levels measured from mPGES1 ^{-/-} sensitized/challenged mice compared to wildtype controls, this difference failed to reach statistical significance. No other differences in supernatant cytokine production were observed for any experimental groups. (**E**) Sensitization with antigen resulted in elevated serum IgE in wildtype and mPGES1 ^{-/-} animals. This augmentation did not differ significantly between groups. (IgE measurements in sensitized only animals and all ex vivo splenocyte experiments were conducted twice. Data from one trial is shown. For sensitized only A: mPGES1: ^{+/+} OVA n=5, mPGES1 ^{-/-} n=7; For sensitized/challenged A: mPGES1 ^{+/+} n=4, mPGES1 ^{-/-} n=4; For sensitized only B and D: mPGES1: ^{+/+} saline n=6, ^{-/-} saline n=6, ^{+/+} OVA n=5, ^{-/-} OVA n=7; For sensitized only C: mPGES1: ^{+/+} saline n=4, ^{-/-} saline n=4, ^{+/+} OVA n=4, ^{-/-} OVA n=4; For

sensitized/challenged B-D: mPGES1: +/+ saline n=4, -/- saline n=4, +/+ OVA n=9, -/- OVA n=7;

For sensitized only E: mPGES1: +/+ saline n=4, -/- saline n=4, +/+ OVA n=6, -/- OVA n=7.)

Figure S4

Supplemental Figure 4. Granulocyte Cell Analysis in OT-II transgenic mPGES1 mice

mPGES1 +/+ and -/- animals carrying an OVA-specific TCR transgene, along with transgene negative controls, were aerosolized for 5 consecutive days with OVA. 24 hours after the final challenge, BALF was collected. Total cell counts were made by hemacytometer analysis and granulocyte content was determined by cytospin preparation followed by staining with Fast Green. Wildtype mice harboring a copy of the transgene show a dramatic elevation in BALF granulocyte content, compared to transgene negative controls. This augmentation was significantly reduced in mPGES1 -/- mice (* p<0.05). (This experiment was repeated 2 times. Data shown represents one trial. mPGES1 +/+ n=4, mPGES1 -/- n=5, OT-II/ mPGES1 +/+ n=16, OT-II/mPGES1 -/- n=9)

