

Activated prostaglandin D₂ receptors on macrophages enhance neutrophil recruitment into the lung

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Background: Prostaglandin (PG) D₂ is an early-phase mediator in inflammation, but its action and the roles of the 2 D-type prostanoid receptors (DPs) DP₁ and DP₂ (also called chemoattractant receptor–homologous molecule expressed on T_H2 cells) in regulating macrophages have not been elucidated to date.

Objective: We investigated the role of PGD₂ receptors on primary human macrophages, as well as primary murine lung macrophages, and their ability to influence neutrophil action *in vitro* and *in vivo*.

Methods: *In vitro* studies, including migration, Ca²⁺ flux, and cytokine secretion, were conducted with primary human monocyte-derived macrophages and neutrophils and freshly isolated murine alveolar and pulmonary interstitial macrophages. *In vivo* pulmonary inflammation was assessed in male BALB/c mice.

Results: Activation of DP₁, DP₂, or both receptors on human macrophages induced strong intracellular Ca²⁺ flux, cytokine release, and migration of macrophages. In a murine model of LPS-induced pulmonary inflammation, activation of each PGD₂ receptor resulted in aggravated airway neutrophilia, tissue myeloperoxidase activity, cytokine contents, and decreased lung compliance. Selective depletion of alveolar macrophages abolished the PGD₂-enhanced inflammatory response.

Activation of PGD₂ receptors on human macrophages enhanced the migratory capacity and prolonged the survival of neutrophils *in vitro*. In human lung tissue specimens both DP₁ and DP₂ receptors were located on alveolar macrophages along with hematopoietic PGD synthase, the rate-limiting enzyme of PGD₂ synthesis.

Conclusion: For the first time, our results show that PGD₂ markedly augments disease activity through its ability to enhance the proinflammatory actions of macrophages and subsequent neutrophil activation. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: D-type prostanoid receptor 1, D-type prostanoid receptor 2/chemoattractant receptor–homologous molecule expressed on T_H2 cells, prostaglandin D₂, hematopoietic prostaglandin D synthase, macrophages, pulmonary inflammation, neutrophils

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Prostaglandin (PG) D₂, a lipid mediator from the arachidonic acid/COX pathway, has been shown to play complex and often opposing roles in the development and resolution of inflammation, which can be attributed to differential activation of its receptors. PGD₂ activates 2 G protein–coupled receptors, the D-type prostanoid receptors (DPs) DP₁ and DP₂, with the latter also known as chemoattractant receptor–homologous molecule expressed on T_H2 cells.¹ At higher concentrations, PGD₂ can also signal through the thromboxane A₂ receptor.^{2,3} Although PGD₂ exerts similar binding affinities toward the DP₁ and DP₂ receptors, its metabolites, formed rapidly by enzymatic and nonenzymatic pathways, can differentially induce DP₂-mediated effects.¹

In patients with allergic diseases, the role of PGD₂ has mostly been associated with its release from activated mast cells and induction of vasodilation. More recently, however, PGD₂ has been found to promote additional proinflammatory responses through activation of DP₂ receptors reflected by increased eosinophilic infiltration into the lungs and skin of mice.^{4,5} Consequently, DP₂ antagonists were shown to ameliorate eosinophilic pulmonary inflammation in murine ovalbumin-induced⁶ and house dust mite–induced⁷ models, a rat *Alternaria* species–induced model,⁸ and the setting of chronic allergic skin inflammation.⁹ Moreover, the DP₂ antagonist CAY10471 ameliorated weight

Abbreviations used

ALI:	Acute lung injury
ARDS:	Acute respiratory distress syndrome
BAL:	Bronchoalveolar lavage
DK-PGD ₂ :	13,14-Dihydro-15-keto prostaglandin D ₂
DP:	D-type prostanoid receptor
HPGDS:	Hematopoietic prostaglandin D synthase
KC:	Keratinocyte-derived chemokine
MDM:	Monocyte-derived macrophages
MPO:	Myeloperoxidase
PG:	Prostaglandin
TX:	Thromboxane

loss and intestinal inflammation in a dextran sodium sulfate–induced colitis model in mice.¹⁰ In asthmatic patients induction of hematopoietic prostaglandin D synthase (HPGDS), the rate-limiting enzyme of PGD₂ synthesis, was observed in the epithelial compartment,¹¹ and PGD₂ levels in bronchoalveolar lavage (BAL) fluid correlated positively with the severity of the disease.¹² DP₂/chemoattractant receptor–homologous molecule expressed on T_H2 cell antagonists was found to have some effects in allergic rhinitis,¹³ allergic conjunctivitis,¹⁴ eosinophilic esophagitis,¹⁵ and bronchial asthma.^{16,17}

Macrophages are essential in pulmonary inflammatory diseases by maintaining tissue homeostasis and mounting rapid responses to exogenous and endogenous stimuli. Because they are the main source of proinflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, their role in inflammation is pivotal.¹⁸ Acute lung injury (ALI) or its more severe clinical manifestation, acute respiratory distress syndrome (ARDS), is a pulmonary inflammatory disease that can lead to respiratory failure. Pulmonary complications in this disease are mainly attributed to rapid neutrophil infiltration into the alveolar space,¹⁹ subsequent edema formation, and dysfunction of the involved cell types, including epithelial cells,²⁰ endothelial cells,²¹ and macrophages.^{22–24} Several studies have made it clear that macrophages orchestrate neutrophilic infiltration and thus strongly modulate the outcome of ARDS.^{23–27}

Stimulated by the dominant physiologic role of PGD₂ in the lung, we hypothesized that PGD₂ might govern disease activity and progression by acting on lung macrophages. Previous studies revealed anti-inflammatory effects of the PGD₂ metabolite 15d-PGJ₂ acting through peroxisome proliferator–activated receptor γ in RAW 264.7 macrophages²⁸ and demonstrated the expression of PGD₂ receptors on human monocytes,²⁹ whereas the role of PGD₂ in regulation of macrophage function has not been assessed yet.

METHODS

Detailed description of ethical permits, materials, and procedures is provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Isolation of peripheral blood polymorphonuclear neutrophils and PBMCs

Human peripheral blood polymorphonuclear cells and PBMCs were isolated from healthy donors independent of sex and age, as described previously.³⁰

Differentiation from monocytes to macrophages

Human peripheral blood monocytes isolated from healthy donors were differentiated to human monocyte-derived macrophages (MDMs) for 7 to

10 days with 20 ng/mL human recombinant macrophage colony-stimulating factor.

Live cell fluorescent Ca²⁺ imaging

Macrophages were loaded with 2 μ Mol/L Fura-2/AM. Fluorescence images were obtained with alternate excitation at 340 and 380 nm, and emitted light was collected at 510 nm. Intracellular calcium levels were calculated, as previously described.³¹

Monocyte Ca²⁺ flux

Ca²⁺ flux was measured by means of flow cytometry, as previously described.³⁰

Neutrophil apoptosis

Neutrophil survival was assessed by using Annexin V/propidium iodide staining, as described previously.³²

Neutrophil and macrophage chemotaxis

Neutrophils were placed in the upper compartment of a Transwell chamber (Corning, Inc, New York, NY) in the absence or presence of MDMs. After 1 hour, neutrophils that migrated to the bottom well were collected, suspended in 150 μ L of fixative solution, and enumerated by means of flow cytometric analysis.³³ Migration of human MDMs was assessed by using Transwell inserts, as described previously.³⁰

Flow cytometric staining

The following antibodies and concentrations were used: DP₂ (20 μ g/mL), DP₁ (20 μ g/mL), anti-mouse MHC class II (2.5 μ g/mL), anti-mouse Siglec-F (5 μ g/mL), anti-mouse CD3e (5 μ g/mL), anti-mouse B220 (2 μ g/mL), and anti-mouse CD11c (2 μ g/mL) antibodies.

LPS-induced lung injury

Pulmonary inflammation was induced in 8- to 10-week-old BALB/c mice by means of intranasal application of 1 mg/kg LPS. Agonists or antagonists were applied 24 hours before LPS application subcutaneously every 12 hours. Unless stated otherwise, mice were killed 4 hours after LPS administration.

Myeloperoxidase assay

Myeloperoxidase (MPO) activity was determined, as described previously.³⁴

BAL protein content

BAL protein concentrations were measured by using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, Ill), according to the manufacturer's protocol.

Vascular permeability in lung tissue was assessed by using Evans blue dye extravasation after 60 minutes of circulation. Evans blue protein leak was determined, as described in the [Methods](#) section in this article's Online Repository.

Murine lung histology

Paraffin-embedded murine lungs were cut (5- μ m sections), deparaffinized, and immunostained with anti-Ly6G and hematoxylin.

Isolation of murine alveolar and interstitial macrophages

Alveolar and interstitial macrophages were isolated from BAL fluid, as described in the [Methods](#) section in this article's Online Repository.

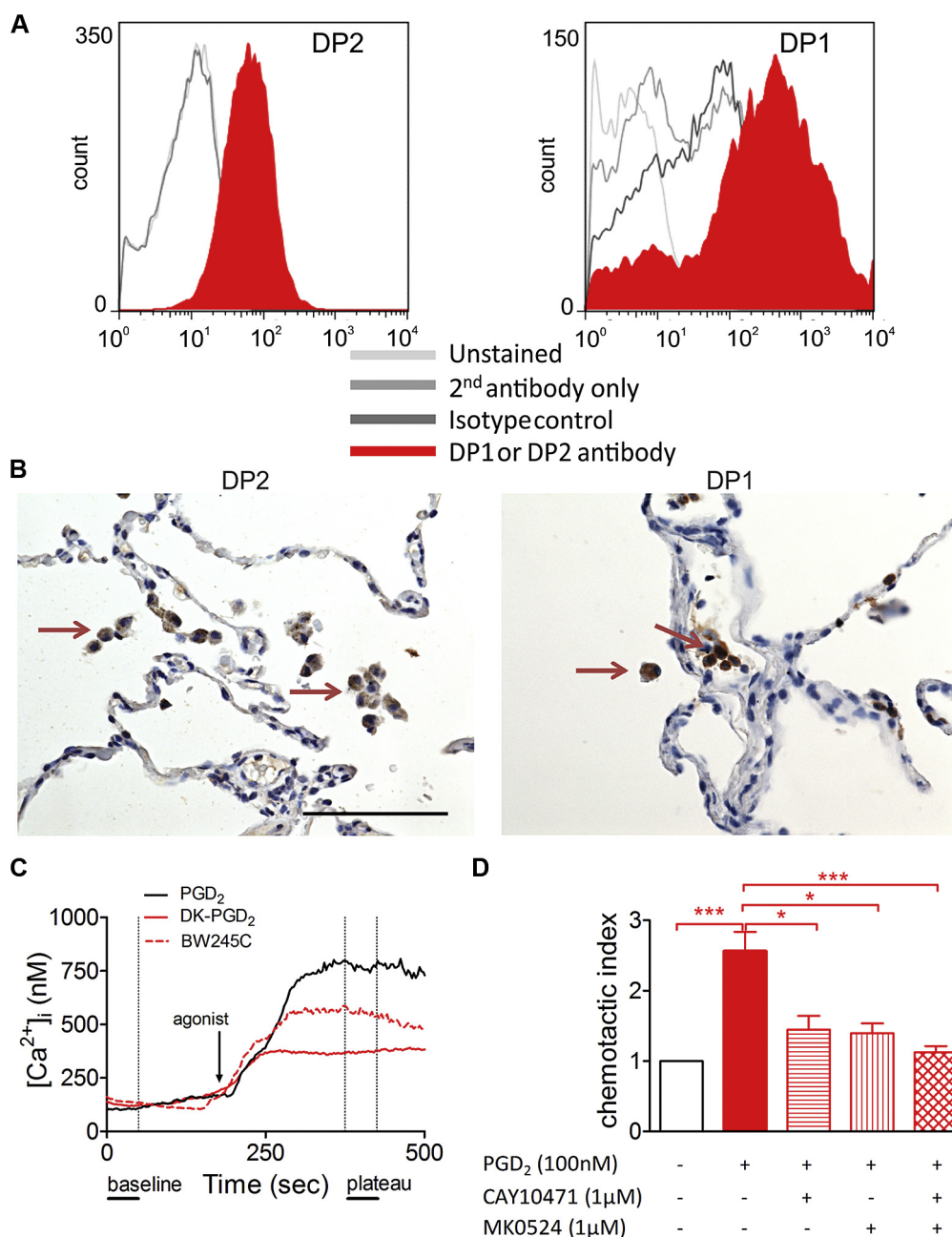


FIG 1. PGD₂ receptors DP₁ and DP₂ are expressed on macrophages and induce Ca²⁺ flux and migration. **A**, Flow cytometric histograms of DP₂ and DP₁ staining (filled histograms) on MDMs, respectively. **B**, Immunohistochemistry of healthy human lung tissue showing DP₂- and DP₁-positive alveolar macrophages (arrows). **C**, Representative Ca²⁺ responses of MDMs over time. **D**, MDM migration toward PGD₂ is blocked by DP₁- and DP₂-specific antagonists (n = 4-5). *P < .05 and ***P < .001.

Depletion of murine lung macrophages

Three hundred micrograms (in 60 μL volume) of clodronate or control liposomes were intranasally applied to mice 24 hours before LPS challenge.³⁵

Cytokine measurements

Cytokine levels were determined by using either a multianalyte immunoassay (Bender Medsystems, Vienna, Austria), ELISA (PeproTech, Rocky Hill, NJ), or the ProcartaPlex Mouse Cytokine Kit (eBioscience, San Diego, Calif).

Immunohistochemistry of human lung tissue

Human paraffin-embedded lung samples were stained with anti-DP₂ (1:200), anti-DP₁ (1:100), or anti-HPGDS (1:200) antibodies.

Lipid mediator analysis

PGD₂, PGE₂, TXB₂, 12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid, and 6-keto-PGF_{1α} were analyzed by using liquid chromatography–tandem mass spectrometry, as previously published³⁶ and as described in the [Methods](#) section in this article's Online Repository.

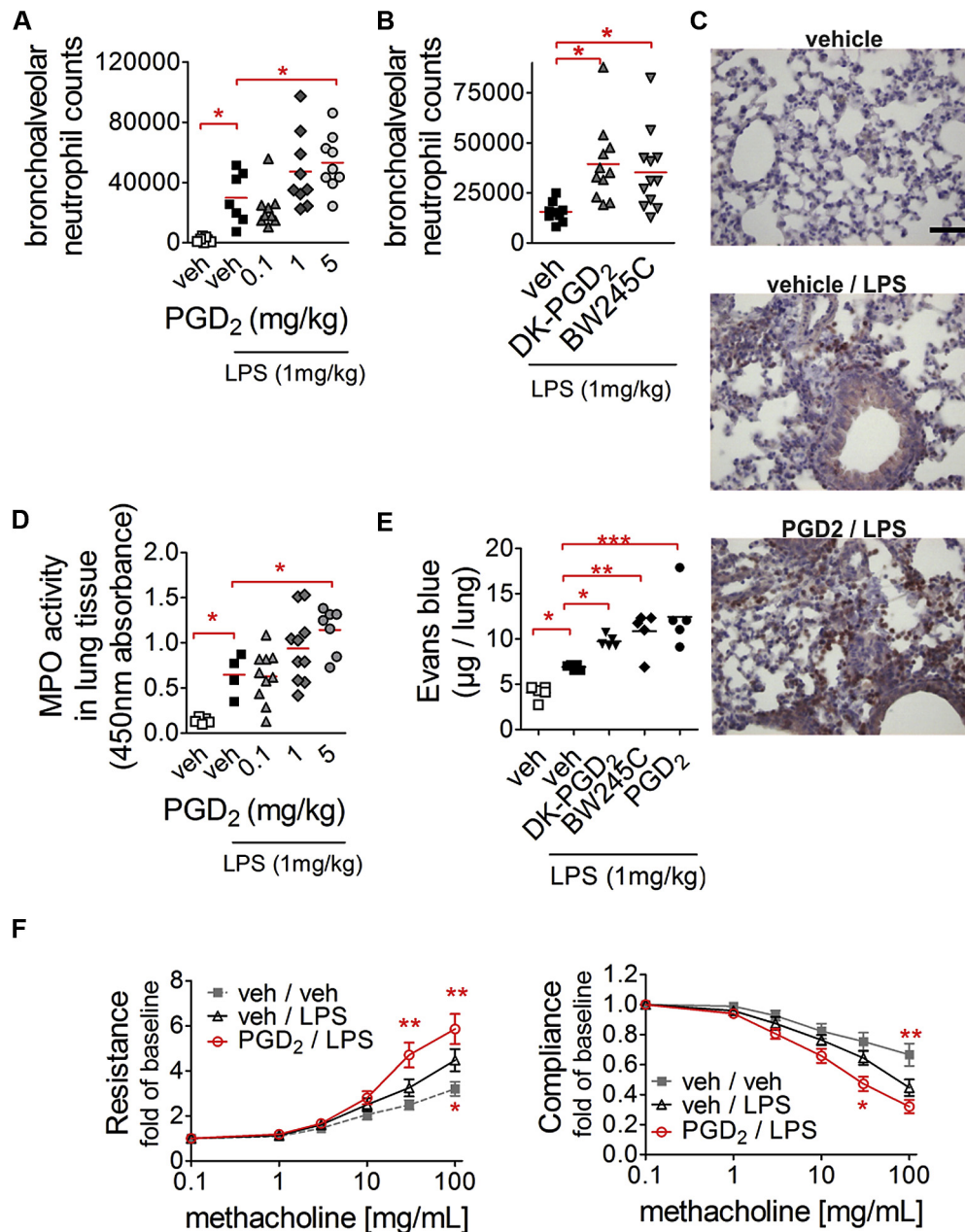


FIG 2. PGD₂ acting through DP₁ and DP₂ promotes neutrophil influx into lungs and aggravates airway hyperreactivity. **A** and **B**, Neutrophil infiltration is increased in animals pretreated with PGD₂ (Fig 2, **A**) or DP₁- and DP₂-selective agonists (Fig 2, **B**). **C-F**, PGD₂ pretreatment increases Ly6G-positive neutrophil infiltration in the peribronchial and alveolar space (representative pictures, scale bar = 50 μm; Fig 2, **C**), MPO activity (Fig 2, **D**), Evans blue dye extravasation (Fig 2, **E**), and airway hyperreactivity (vs vehicle/LPS; Fig 2, **F**). **P* < .05, ***P* < .01, and ****P* < .001.

Measurement of murine lung function

Decreased lung compliance caused by pulmonary edema and atelectasis is a hallmark of human ALI/ARDS and a preferred readout in mouse models.³⁷ Therefore mouse lung function was determined by using the flexiVent system (SCIREQ, Montreal, Quebec, Canada), as described previously.³⁸

Statistical analysis

Data were analyzed by using either the Student *t* test, 1-way ANOVA, or 2-way ANOVA, followed by the Bonferroni or Dunnett posttest, with GraphPad Prism software (version 5.04; GraphPad Software, La Jolla, Calif). *In vitro*

experiments were performed in duplicates, with *n* numbers indicating biological replicates with cells from different donors. *P* values of less than .05 were considered significant.

RESULTS

DP₁ and DP₂ are expressed on human macrophages and induce Ca²⁺ signaling

We first set out to elucidate whether PGD₂ receptors are expressed on human monocytes and macrophages and found that

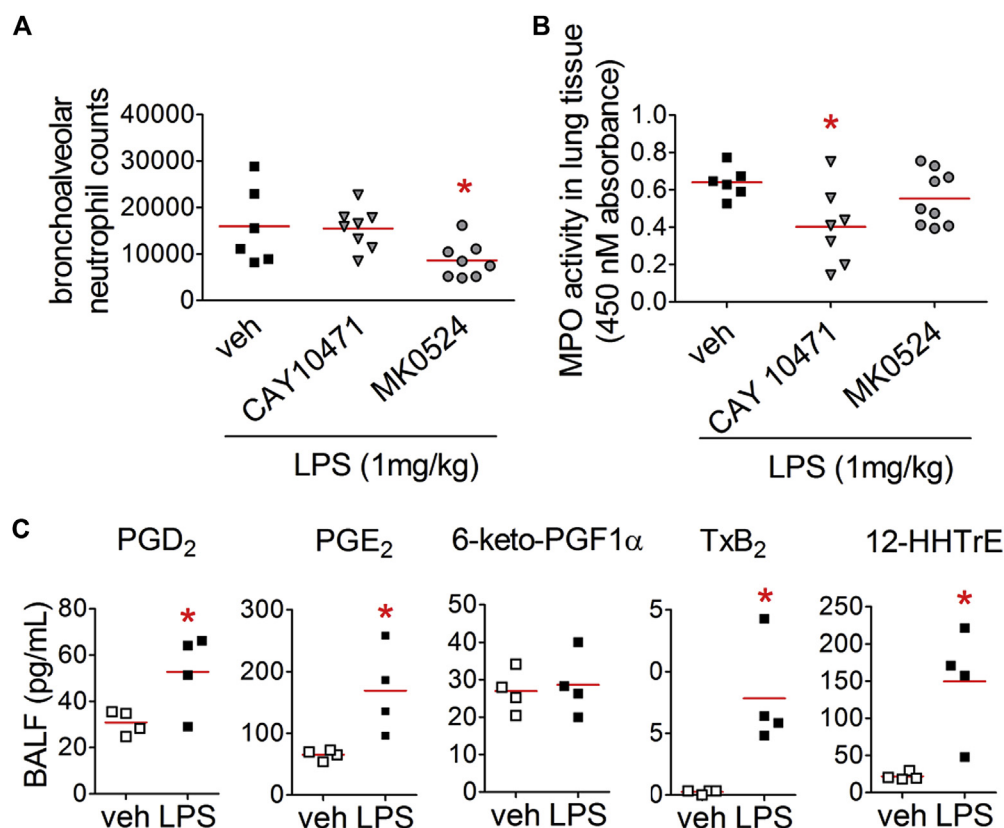


FIG 3. Blocking of endogenous PGD₂ ameliorates LPS-induced neutrophil influx into the alveolar space and pulmonary tissue. **A** and **B**, DP₁ antagonist reduced neutrophil counts in the bronchoalveolar space (Fig 3, A), whereas DP₂ antagonist reduced MPO activity (Fig 3, B; n = 6-9). *P < .05 versus vehicle. **C**, Lipid mediators in BAL fluid 4 hours after vehicle or LPS treatment were quantified by using liquid chromatography-tandem mass spectrometry (n = 4). HHTrE, 12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid. *P < .05 versus vehicle.

both cell types express DP₁ and DP₂ on their cell surface (Fig 1, A, and see Fig E1 in this article's Online Repository at www.jacionline.org). Interestingly, expression of both receptors increased during differentiation of MDMs, with DP₂ expression being more abundant than DP₁ expression in both monocytes and macrophages (see Fig E1). Next, we probed for DP₁ and DP₂ expression in human lung tissue with different pathologies (see Table E1 in this article's Online Repository at www.jacionline.org). Both were highly and consistently expressed on human alveolar macrophages (Fig 1, B) because 60% to 75% of alveolar macrophages were positively stained for both DP₁ and DP₂ independent of the underlying disease (see Figs E2 and E3 in this article's Online Repository at www.jacionline.org). This expression pattern suggested a possible role for the PGD₂-DP₁-DP₂ axis in the modulation of macrophage biology in patients with pulmonary diseases.

Then we sought to determine the biological activities of DP₁ and DP₂ *in vitro*. Ca²⁺ imaging revealed that superfusion of human MDMs with 1 μ M/L PGD₂ led to a strong release of intracellular calcium (Fig 1, C, and see Fig E4, A, in this article's Online Repository at www.jacionline.org), whereas human monocytes showed no Ca²⁺ response to PGD₂ stimulation (see Fig E5 in this article's Online Repository at www.jacionline.org). Both DP₂- and DP₁-selective agonists (13,14-dihydro-15-keto PGD₂, and BW245C, respectively; 1 μ M/L) were likewise

able to elicit Ca²⁺ flux, although to a lesser extent than PGD₂ (Fig 1, C, and see Fig E4, A). In agreement with this observation, blockade of either DP₂ (CAY10471, 10 μ M/L) or DP₁ (MK0524, 10 μ M/L) slightly decreased, whereas simultaneous antagonism of both receptors abolished the PGD₂-induced Ca²⁺ signal (see Fig E4, B and C). Ca²⁺ release induced by both PGD₂ and 13,14-dihydro-15-keto prostaglandin D₂ (DK-PGD₂) was prevented by overnight incubation with pertussis toxin (100 ng/mL), indicating involvement of G α i heterotrimers in this process (see Fig E4, D and E).

PGD₂ induces migration of macrophages through DP₁ and DP₂ and enhances TNF- α secretion

We next evaluated the chemotactic potential of PGD₂ toward MDMs by using Transwell inserts, followed by enumeration of fluorescently labeled cell nuclei of the migrated cells on the lower surface of the filter (see Fig E6, B). We found that PGD₂ exerted a concentration-dependent chemotactic activity toward MDMs, with the highest responses observed at 100 nmol/L (Fig 1, D, and see Fig E6, A, in this article's Online Repository at www.jacionline.org). This could be partially blocked by either the DP₂- or DP₁-specific antagonist (CAY10471 or MK0524, 1 μ M/L, respectively; Fig 1, D). Inhibition of both receptors completely abolished the migratory

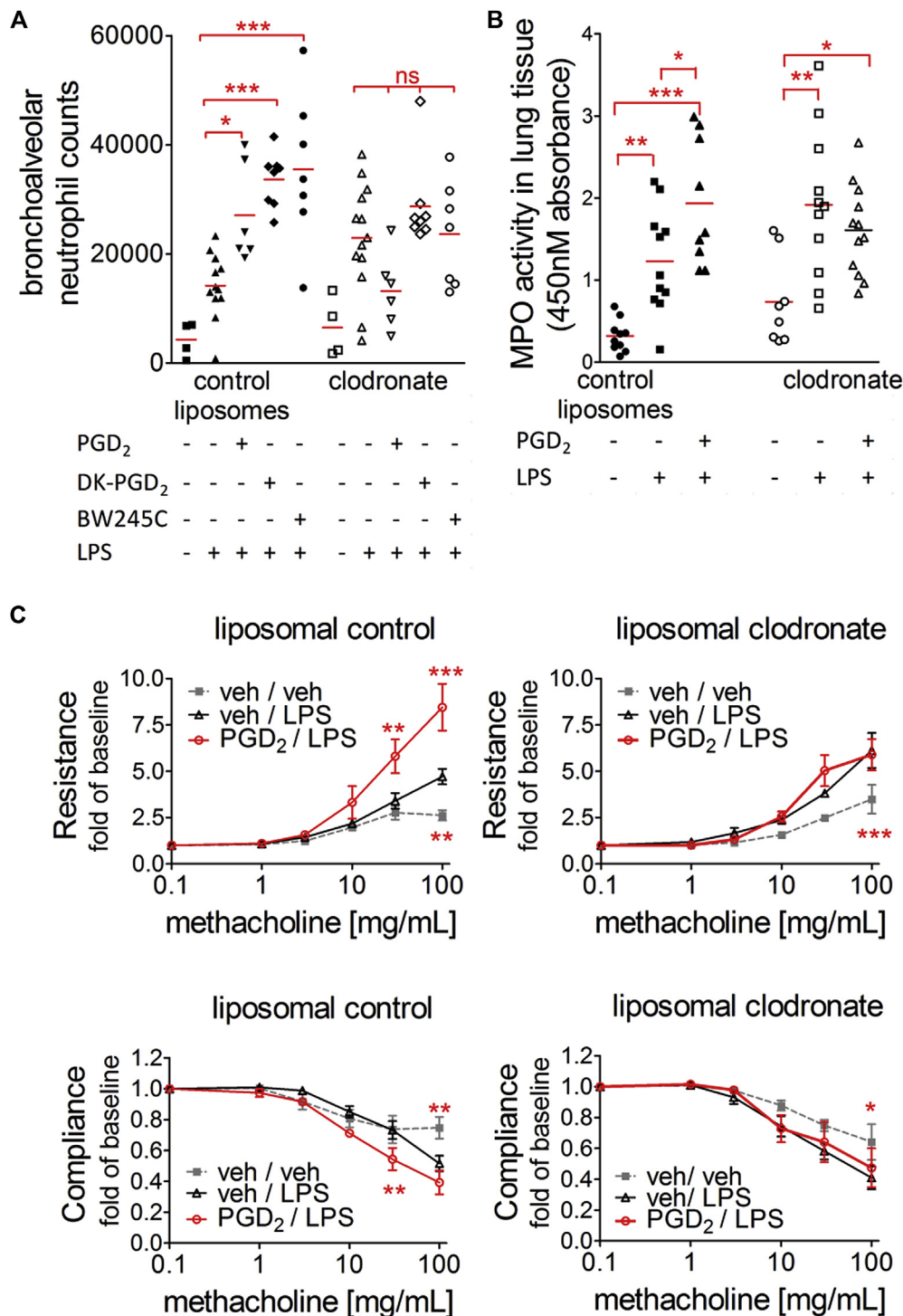


FIG 4. Macrophage depletion prevents the proinflammatory effect of PGD₂ on neutrophil recruitment. **A**, Neutrophil numbers in BAL fluid. **B**, MPO activity in lung tissue. **P* < .05, ***P* < .01, and ****P* < .001. **C**, Macrophage depletion prevents reduction in lung function induced by PGD₂ (n = 6-8). **P* < .05, ***P* < .01, and ****P* < .001 versus vehicle/LPS.

activity of MDMs toward PGD₂ (Fig 1, D). Because PGD₂ was shown to be critical in mediating macrophage migration toward LPS in a murine model,³⁹ we hypothesized that PGD₂ could alter the LPS-induced cytokine secretion from MDMs. Although almost no TNF-α was detectable in the supernatants of vehicle-treated cells, LPS stimulation (100 ng/mL) induced

a strong release of the proinflammatory cytokine. Indeed, this response was markedly enhanced in cells pretreated with PGD₂ or DK-PGD₂ (see Fig E6, C). Although the DP₁-specific agonist BW245C was not able to induce changes in TNF-α secretion, involvement of both DP₁ and DP₂ receptors in the PGD₂ response is still likely because only antagonism of both

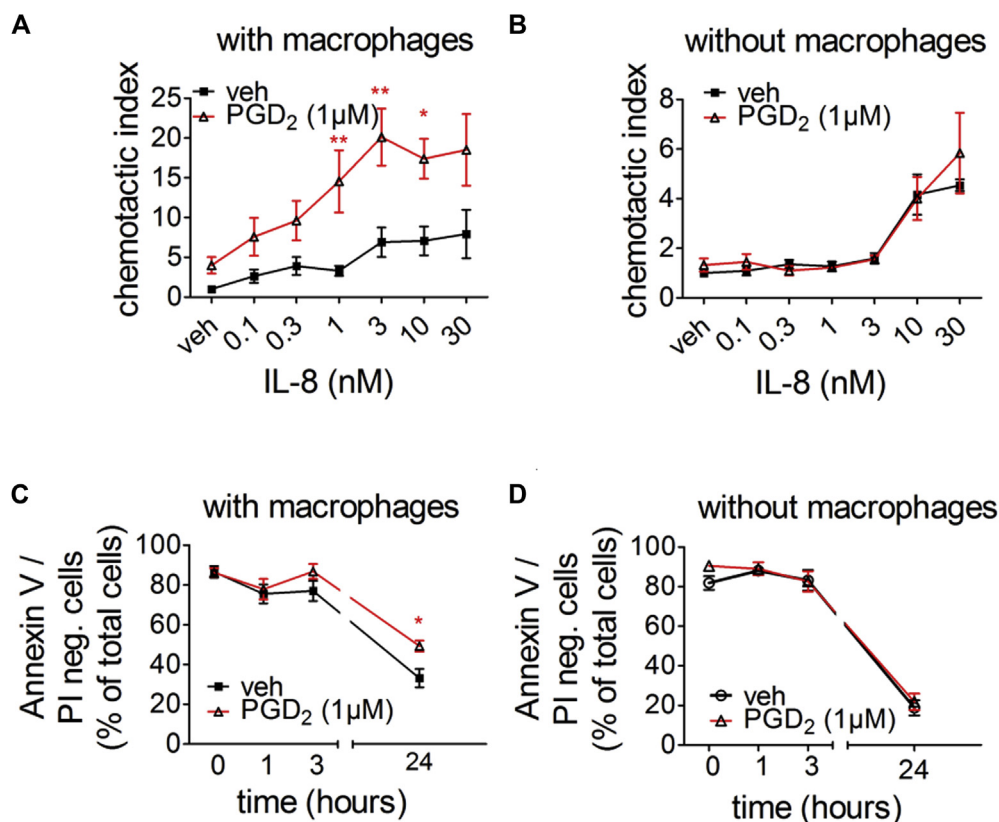


FIG 5. PGD₂ receptor activation on MDMs increases neutrophil migration toward IL-8 (**A** and **B**) and prolongs neutrophil survival (**C** and **D**) *in vitro*. Fig 5, **A**, **C**, and **D**: n = 5-10; Fig 5, **B**: n = 3-5. **P* < .05 and ***P* < .01 versus vehicle. PI, Propidium iodide.

receptors was able to completely block the PGD₂-mediated increase in TNF-α secretion.

Endotoxin-induced lung injury is aggravated by PGD₂

Prompted by these observations, we investigated the effect of PGD₂ in an *in vivo* model of pulmonary inflammation. Here neutrophil infiltration is a main pathogenic feature that essentially depends on macrophage function.²³ LPS significantly increased neutrophil influx into the bronchoalveolar space after 4 hours of treatment. Of particular interest, this effect was almost doubled when PGD₂ (5 mg/kg) was administered subcutaneously before LPS challenge (Fig 2, **A**). Selective DP₁ and DP₂ agonists induced an even more pronounced response; in detail, the selective DP₂ agonist DK-PGD₂ (5 mg/kg) enhanced neutrophil influx by 2.5-fold and the DP₁ agonist BW245C (5 mg/kg) enhanced neutrophil influx by 2.3-fold (Fig 2, **B**). Histologically, the inflammatory state of the lungs was indicated by dense Ly6G-positive neutrophil infiltrates in the bronchiolar region, with disturbed alveolar morphology in LPS-treated animals. When PGD₂ was administered before LPS treatment, even more Ly6G-positive neutrophils were found around the bronchioli accompanied by neutrophil infiltrates in the alveolar space, which was largely absent in vehicle/LPS-treated mice (Fig 2, **C**, and see Fig E7, **A**, in this article's Online Repository at www.jacionline.org). MPO activity, a marker for lung inflammation, was likewise increased depending on the applied dose of PGD₂ (Fig 2, **D**). These observations were

reflected by increased protein content in the BAL fluid in PGD₂-treated animals (see Fig E7, **B**), as well as enhanced Evans blue extravasation in the lung tissue that depended on both DP₁ and DP₂ (Fig 2, **E**). It is noteworthy that the capacity of PGD₂ to increase neutrophil recruitment was sustained after 24 hours (see Fig E7, **C**). Only minor alterations were found in the number of other cell types, such as monocytes, macrophages, lymphocytes, and eosinophils (see Fig E8 in this article's Online Repository at www.jacionline.org). The observation that PGD₂ aggravated pulmonary inflammation was also reflected by a decrease in lung function. In these experiments we found that LPS alone caused hyperresponsiveness to methacholine with respect to airway resistance and compliance (Fig 2, **F**), which was further accentuated after combined treatment of mice with PGD₂ and LPS (Fig 2, **F**). In all experimental readouts, including cell infiltration into the alveolar space, MPO activity, and lung function, PGD₂ treatment in the absence of LPS had no effect (data not shown).

Blocking of endogenous PGD₂ reduces neutrophilic infiltration into the lungs

Mice were pretreated with the PGD₂ receptor antagonists MK0524 or CAY10471 (5 mg/kg) followed by LPS challenge to delineate the pathogenic role of endogenous PGD₂. Analysis of BAL cells revealed that the DP₁ antagonist MK0524 reduced endotoxin-induced neutrophilia (Fig 3, **A**), whereas the DP₂ antagonist CAY10471 reduced MPO activity (Fig 3, **B**). Next,

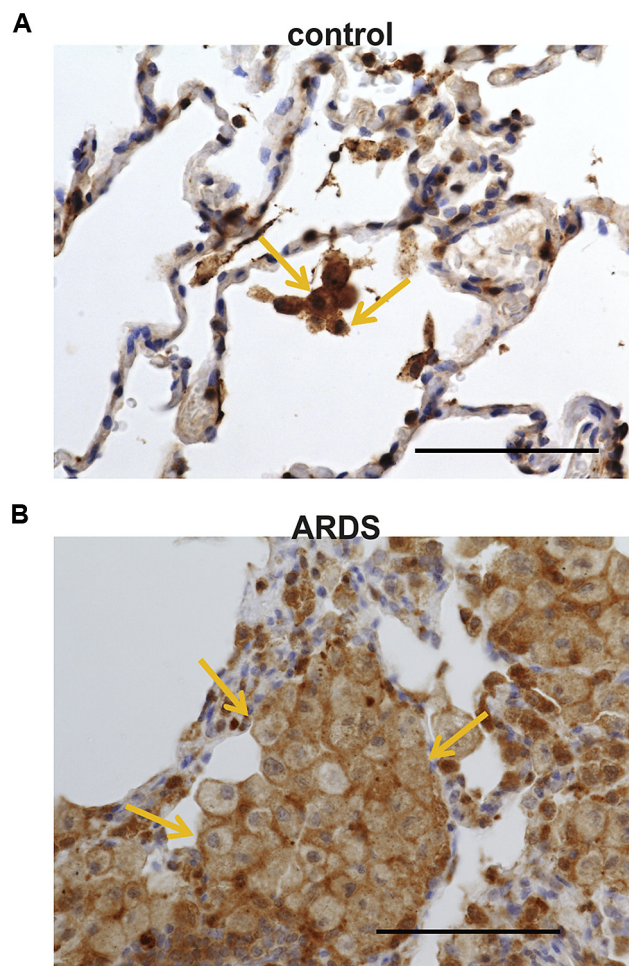


FIG 6. Increased numbers of HPGDS-expressing cells in lungs of patients with ARDS. Representative immunohistochemical staining of human lung samples showing positive cells for HPGDS (brown) in a control subject (A) and a patient with ARDS (B). Stainings are representative pictures of 5 patients and control subjects. Note the high amount of HPGDS found in alveolar macrophages. Bar = 100 μ m.

we compared lipid mediator levels in the BAL fluid of vehicle- and LPS-treated animals and found that there was a marked increase in PGD₂ levels in the latter (Fig 3, C). In agreement with previous findings, PGE₂ levels also increased, as did TXB₂ and 12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid levels. We could not detect any significant changes in PGI₂ levels (estimated through its metabolite 6-keto-PGF_{1 α}).

PGD₂ treatment enhances proinflammatory cytokine release *in vivo* and *in vitro*

In addition to prostanooids, increased keratinocyte-derived chemokine (KC) and monocyte chemoattractant protein 1 levels were observed in the cell-free BAL fluid of LPS-treated mice. Importantly, the increase in levels of these chemokines was significantly stronger in PGD₂-treated animals (see Fig E9, A, in this article's Online Repository at www.jacionline.org). To determine whether macrophages were responsible for the increased release of these chemokines, we isolated alveolar and interstitial macrophages and mimicked the *in vivo* model. Both alveolar and

interstitial pulmonary macrophages increased secretion of the neutrophil chemoattractant KC when pretreated with PGD₂ (see Fig E9, B and C), implicating macrophages as the cell type responsible for the enhanced neutrophil recruitment *in vivo*. Interestingly, monocyte chemoattractant protein 1 secretion by alveolar and interstitial macrophages was influenced by neither LPS nor PGD₂ treatment (see Fig E9, B and C).

Macrophage depletion prevents the increased inflammatory response mediated by PGD₂

To highlight the role of macrophages in the PGD₂-induced enhancement of pulmonary inflammation, we next selectively reduced alveolar macrophage counts in the pulmonary LPS model. Intranasal application of 300 μ g of clodronate led to a marked reduction of macrophage counts by approximately 70% throughout the groups (see Fig E10 in this article's Online Repository at www.jacionline.org). This dose was chosen to avoid proinflammatory responses caused by clodronate and/or liposomes themselves, which were observed at higher doses (data not shown). In animals that had received control liposomes, LPS treatment led to neutrophil recruitment into the bronchoalveolar space, which was again further enhanced when the animals were pretreated with PGD₂ or any of the specific agonists for DP₁ and DP₂. After reduction of alveolar macrophage counts, LPS still enhanced neutrophil counts in the lungs, suggesting that 30% residual macrophages were sufficient to induce lung neutrophilia, but no further increase in neutrophil counts was seen in PGD₂-, DK-PGD₂-, or BW245C-treated animals (Fig 4, A, and see Fig E11 in this article's Online Repository at www.jacionline.org). Concomitant to decreased alveolar neutrophil counts, the PGD₂-induced increase in MPO activity, as well as compromised lung function, were reversed by alveolar macrophage reduction (Fig 4, B and C).

PGD₂ receptor activation on macrophages modulates neutrophil function

In vitro neutrophil migration assays were performed in the presence of human MDMs treated with vehicle or PGD₂ to further clarify the mechanisms through which PGD₂ receptor activation on macrophages augmented the LPS-induced neutrophil recruitment. In this coculture experiment PGD₂-treated macrophages enhanced the migration of neutrophils when compared with basal neutrophil migration in the presence of untreated MDMs (Fig 5, A). This effect did not depend on IL-8 concentrations because PGD₂ treatment of MDMs enhanced the basal migratory capacity and IL-8-induced migration of neutrophils alike. Importantly, neutrophil migration was unaffected by PGD₂ when macrophages were absent (Fig 5, B). Therefore PGD₂ does not exert its effect through direct stimulation of neutrophils but rather through a macrophage-dependent pathway. In addition to migration, we monitored neutrophil survival in further coculture experiments. MDMs grown on 48-well plates were treated with vehicle or PGD₂ 24 hours before neutrophils were added. Neutrophil apoptosis was monitored over 24 hours. The portion of viable neutrophils (Annexin V/propidium iodide negative) was higher when macrophages were treated with PGD₂ (Fig 5, C). Importantly, this effect was not due to enhanced macrophage phagocytosis of apoptotic/necrotic neutrophils because neutrophil numbers did not change throughout the experiment (see Fig

E12 in this article's Online Repository at www.jacionline.org). Furthermore, neutrophils cultivated without macrophages did not react to PGD₂ and showed the same survival rate as in the presence of vehicle (Fig 5, D). These data show that PGD₂ receptor activation on macrophages profoundly influences neutrophil function by enhancing their migratory capacity and survival.

Cells expressing hematopoietic PGD₂ synthase are abundant in lungs of patients with ARDS

We finally examined whether levels of HPGDS, the rate-limiting enzyme responsible for the production of PGD₂, differed in patients with ARDS and control subjects. Indeed, immunohistochemistry revealed that, compared with control lung samples (Fig 6, A), there was a clear increase in the number of cells highly expressing HPGDS in patients with ARDS (Fig 6, B). Moreover, although 50% to 60% of macrophages, as identified by means of morphology, were positive for HPGDS, this value increased to 85% to 95% in lung sections from patients with ARDS.

DISCUSSION

In this study we propose a novel role for PGD₂ and its corresponding receptors in pulmonary inflammation. Here, for the first time, we show that human macrophages express active DP₁ and DP₂, which are associated with alterations in cytokine profiles and migratory responses, factors that contribute greatly to inflammation in pulmonary diseases. First, we confirmed basal expression of PGD₂ receptors on monocytes,²⁹ but we also showed an upregulation that was more pronounced in the expression of DP₂ than DP₁ during differentiation to macrophages. Furthermore, we were able to demonstrate that both PGD₂ receptors are functional on human macrophages: not only did macrophages themselves migrate toward PGD₂, but the PG also stimulated the interaction of macrophages with neutrophils, thereby supporting neutrophil migration and survival. This finding might be explained by the increased production of cytokines after PGD₂ stimulation of macrophages prompted by DP₁/DP₂-mediated Ca²⁺ flux.

Previous reports suggested that DP₁- and DP₂-mediated actions oppose each other, being anti-inflammatory and proinflammatory, respectively.^{10,40} Such divergent actions of PGD₂ have not been observed here because we could show that on human MDMs, selective activation of both receptors increases intracellular free Ca²⁺ levels and induces migration. Furthermore, these responses were only partially inhibited by either selective antagonist, and only blockade of DP₁ along with DP₂ was sufficient to abolish the PGD₂-induced activation of macrophages. In this respect PGD₂ receptors on macrophages differed from other cell types, such as eosinophils, where these receptors engage in a crosstalk and both are needed for the complete functional response,⁴¹ and bronchial epithelial cells, which express DP₂ but not DP₁.¹¹

We also observed DP₁ and DP₂ expression on human macrophages in the lung, both in the healthy state and in several pathologic conditions, from organizing pneumonia to diffuse alveolar damage. Moreover, it was mainly the macrophages themselves that were positive for the PGD₂-synthesizing enzyme HPGDS in the lungs of patients with ARDS. Although upregulation of HPGDS has up to now mostly been described as a consequence of increased numbers of epithelial and submucosal mast cells,⁴²

our finding proposes a new important role for PGD₂ in the regulation of macrophages in the lung.

Next, we investigated the biological role of PGD₂ in lung pathophysiology *in vivo* and used a murine, LPS-induced lung injury model that relies largely on functional macrophages.^{22,23} As predicted by our *in vitro* data, systemic application of PGD₂, acting through both DP₁ and DP₂, aggravated LPS-induced lung pathology in several ways by (1) increasing neutrophil influx into the airways, (2) promoting MPO activity in lung tissue, (3) increasing cytokine levels, and (4) impairing lung function. Thus PGD₂ enhances neutrophilic inflammatory responses in the presence of disease-activating triggers. In parallel with increased levels of PGD₂ in the BAL fluid of LPS-treated animals, TXB₂ levels also increased. This upregulation of the TX pathway can further substantiate our finding of DP₂ activation in pulmonary inflammation because we previously identified the TX metabolite 11-dehydro-TXB₂ as a full agonist for DP₂.⁴³ Finally, we provided evidence that these PGD₂ responses essentially depended on macrophages because murine alveolar macrophages were a rich source of the neutrophil chemoattractant KC and macrophage depletion reversed the ability of PGD₂ to enhance lung inflammation. Two types of macrophages can be found in the lungs: alveolar and interstitial macrophages. Both are long-lived resident cells that orchestrate tissue homeostasis and can react rapidly to endogenous and exogenous stimuli, thus forming a first line of defense,²² and both seemed to respond to PGD₂ when KC secretion was analyzed. Human cell studies, which we conducted in parallel, identified 2 novel mechanisms of how PGD₂ receptor activation on macrophages can regulate neutrophil function, namely enhanced migratory capacity and survival of the latter. A recent study showed that activated T_H2 cells in response to PGD₂ treatment react with secretion of IL-6 and GM-CSF at levels that are able to modulate neutrophil functions.⁴⁴ Our data strongly suggest that this mechanism is not restricted to T_H2 cells but also applies to macrophages. One could speculate that type 2 activated macrophages would be even more susceptible in this respect.

Finally, using the same mouse model, we could also demonstrate a clear proinflammatory role of endogenous PGD₂. Blocking of endogenous PGD₂ either by DP₁- or DP₂-specific antagonists ameliorated the inflammatory response, although in a differential manner. On the one hand, the DP₁-specific antagonist MK0524 decreased neutrophilia in the bronchoalveolar space. On the other hand, the DP₂-specific antagonist CAY10471 markedly reduced MPO activity measured in lung tissue. One reason for that discrepancy might be that here we are looking at 2 different lung compartments. Neutrophils migrate from the blood through the interstitium into the alveolar space, and the 2 receptors might play different roles in this process, such as DP₂ regulating neutrophil recruitment from the blood to the tissue and DP₁ promoting alveolar evasion of neutrophils. Along with the increase in PGD₂ levels in the BAL fluid, these observations clearly highlight endogenous PGD₂ in the development of experimental pulmonary inflammation.

Recently, it was shown that mice lacking the DP₁ receptor display aggravated neutrophil influx and increased mortality in experimental ALI.⁴⁵ Although this study seemingly contradicts our results, several experimental details make a direct comparison difficult. First, the authors used almost 4 times higher doses of LPS and observed the ensuing effects for 3 days. Second, DP₁ was knocked out unconditionally, so that mice might have experienced compensational mechanisms, such as an upregulation of

DP₂ receptors, thereby driving inflammation. A significant advantage of our study is the use of pharmacologic approaches, which lends our findings clear translational potential toward clinical application.

Collectively, we propose that the PGD₂-DP₁-DP₂ axis on macrophages is of pivotal importance in regulating inflammatory responses, and thus also in tissue damage, by triggering and maintaining a proinflammatory environment. Given the involvement of both PGD₂ receptors in macrophage regulation, recently developed dual DP₁/DP₂ antagonists⁴⁶ appear to be a promising approach to treating distinct inflammatory diseases that involve macrophage activation and neutrophil accumulation in the lung.

Key messages

- DP₁ and DP₂ are expressed and functionally active on isolated MDMs and pulmonary macrophages.
- PGD₂ receptor activation of macrophages promotes their interaction with neutrophils and enhances neutrophil function.
- The presence of hematopoietic PGD synthase, DP₁, and DP₂ in macrophages highlights a novel role for PGD₂ in pulmonary inflammation.

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Activated prostaglandin D₂ receptors on macrophages enhance neutrophil recruitment into the lung

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Ethical approvals. All procedures involving human subjects were approved by the Institutional Review Board of the Medical University of Graz. Animal studies were approved by the Animal Ethics Committee of the Austrian Federal Ministry of Science and Research (BMWFW-66.010/0046-II/3b/2013) and were carried out in line with the European Community's Council Directive.

Reagents. All laboratory reagents were from Sigma (Vienna, Austria), unless otherwise specified. Assay buffer was made from Dulbecco modified PBS (with 0.9 mmol/L Ca^{2+} and 0.5 mmol/L Mg^{2+} ; Invitrogen, Vienna, Austria), 0.1% BSA, 10 mmol/L HEPES, and 10 mmol/L glucose, pH 7.4. Human recombinant (hr) M-CSF and ELISAs (TNF- α , MCP-1, IL-6 and KC) were from Peprotech (London, UK). PGD₂, dihydro-15-keto-(DK)-PGD₂, BW245C, CAY10471 and MK0524 were purchased from Cayman (Ann Arbor, MI, US). Rabbit anti-goat IgG secondary antibody conjugated with Alexa fluor-647 was from Invitrogen. Monoclonal anti-human CRTH2 (DP₂), anti-mouse CD11c, Siglec F, MHC II, B220, and CD3 ϵ were from BD Biosciences (Vienna, Austria). Anti-human DP₁ antibody was from Santa Cruz Biotechnology (Heidelberg, Germany). CellFix and FACS-Flow were from Becton Dickinson (Vienna, Austria). Fixative solution was prepared by adding 9 mL of distilled water and 30 mL of FACS-Flow to 1 mL of CellFix. F4/80 antibody and ProcartaPlex Mouse Cytokine & Chemokine Detection Kit was from eBioscience (San Diego, US). Clodronate Standard Macrophage Depletion Kit was purchased from Encapsula NanoSciences LLC (Brentwood, TN, US). Vectashield/DAPI was from Vector Laboratories (Peterborough, UK). Drugs were dissolved in ethanol or dimethyl sulfoxide (DMSO) and further diluted in assay buffer to produce a final concentration of the solvents of less than 0.1%.

Isolation of peripheral blood mononuclear cells and differentiation into macrophages. Following informed consent, blood was sampled from healthy donors, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat and cell numbers were determined. Isolated PBMCs were seeded on cell culture plates at a density of 1×10^7 cells/mL in RPMI media supplemented with 0.5% human male AB serum. After allowing monocytes to adhere for two hours at 37°C in a humidified atmosphere containing 5% CO₂, non-adherent cells were removed by gently washing. Remaining adherent monocytes were supplied with RPMI differentiation media containing 20% FCS, 1x

Pen/Strep and 20ng/mL hr M-CSF. Full differentiation was reached after 7 – 10 days as judged by microscopic appearance ¹.

Preparation of human peripheral blood neutrophils. Following blood sampling, preparation of polymorphonuclear leukocytes was achieved by dextran sedimentation to remove erythrocytes followed by Histopaque density gradient centrifugation as described before ².

Neutrophil apoptosis. Neutrophil survival was assessed as described previously ³. Briefly, neutrophils were cultured in RPMI + 1% Pen/Strep in the absence of additional cytokines either alone or in the presence of MDM in 48-well plates for up to 24 hours to determine apoptosis. MDM were either pretreated with vehicle or PGD₂ 24 hours before neutrophils were added. In the absence of MDM, neutrophils were cultured in the presence of vehicle or PGD₂. Flow cytometric analysis revealed non-apoptotic neutrophils as annexin V/PI-negative cells. Staining was performed as advised by the manufacturer (BD Pharmingen, Vienna, Austria).

Neutrophil chemotaxis. Ten thousand neutrophils were placed in the upper compartments of a modified Boyden chamber and were allowed to migrate towards increasing concentrations of IL-8 through a 5μM Transwell insert for 1 hour. When migration was assessed in the presence of macrophages, MDM were seeded on the insert 24 hours before neutrophils were added to allow MDM spreading and adhesion. MDM were either treated with vehicle or PGD₂ for 24 hours before neutrophils were added. After 1 hour neutrophils that had migrated to the bottom well of the insert were collected, resuspended in 150μL fixative solution and cells were enumerated by flow cytometric analysis ⁴.

Live-cell fluorescent Ca²⁺ imaging. MDM were cultured on 25mm-diameter, glass cover slides at 50% confluence, loaded for 40 min with 2μM fura-2/AM, and then washed with Ringer solution. A single glass cover slide was mounted on the stage of a Zeiss 200M inverted epifluorescence microscope coupled to a PolyChrome V monochromator (Till Photonics) light source in a sealed, temperature-controlled RC-21B chamber (Warner Instruments). Fluorescence images were obtained with alternate excitation at 340 and 380nm. Emitted light was collected at 510nm by an Andor Ixon camera. The acquired images were stored and subsequently processed off-line with TillVision software (Till Photonics). During the measurement the cells were perfused with agonist in the

presence of extracellular Ca^{2+} . For antagonist experiments, cells were preincubated with antagonist for 7 min while baseline was recorded followed by agonist treatment. Measurements were made every 3 sec. Background fluorescence was recorded from each cover slip and subtracted before calculation. Maximal and minimal ratio values of the $[\text{Ca}^{2+}]_i$ were determined at the end of each experiment by first treating the cells with 10 μM ionomycin (maximal ratio) and then chelating all free Ca^{2+} with 20mM EGTA (minimal ratio). Cells that did not respond to ionomycin were excluded. $[\text{Ca}^{2+}]_i$ was calculated as described by Grynkiewicz et al ⁵. For data analysis, the basal level of Ca^{2+} was determined as an average value of the first 50 seconds of the curve. Whereas, the agent-induced Ca^{2+} change was determined as the value of the signal plateau (50 seconds averaged in the 375-425 second interval) after subtracting the baseline. This was quantified for each individual cell separately.

Monocyte Ca^{2+} flux. As previously described ², 10^7 PBMCs/mL (10^7 cells/mL) were loaded with 2 μM fluo-3-acetoxymethyl ester Ca^{2+} -sensitive dye in the presence of 0.02% pluronic F-127 for 60 min at room temperature. Monocytes were identified as CD14-positive cells after labeling with anti-CD14 PerCP Ab. Baseline was recorded for 60 sec, and then agonists were applied. Changes in intracellular Ca^{2+} levels were detected by flow cytometry as the increase of fluorescence of fluo-3 in the FL1-channel.

Macrophage migration. Migration of human MDM was assessed as described previously ². Briefly, human MDM (2×10^5 per insert in RPMI 1640 medium containing 5mM HEPES, 1% nonessential amino acids, and 1% sodium pyruvate) were allowed to migrate through an 8 μM filter of a Transwell insert (Corning) for 18 h at 37°C. Cells that had migrated towards the lower part of the insert were fixed with 3.7% paraformaldehyde for 30 min at 4°C. Filters were mounted on microscope slides with DAPI-containing mounting medium and migration was analyzed by Zeiss Axiovert 40 CFL microscope and Zeiss A-Plan 10 $\times$ /0.25 Ph1 lens; five images were acquired per filter using a Hamamatsu ORCA-03G digital camera. Migration was quantified by counting DAPI-labeled nuclei by a person unaware of the respective treatment using ImageJ software (National Institutes of Health, US). The chemotactic index was then calculated as the number of cells that migrated in response to PGD_2 divided by the number of cells that migrated spontaneously towards vehicle.

Flow cytometric staining for DP₁ and DP₂. After blocking with UV block, human peripheral blood monocytes or the corresponding MDM were incubated with rat anti-human Alexa Fluor 647 CRTH2 (DP₂) or goat anti-human DP₁ antibody at 20µg/mL for 30 min at 4°C. For DP₁ staining, cells were then incubated with secondary rabbit anti-goat Alexa Fluor 647 antibody for 30 min 4°C. Finally, cells were taken up in fixative solution and analyzed on a FACSCalibur (Becton Dickinson, Mountain View, CA).

LPS-induced lung injury. Male 6-10 week old BALB/c mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Mice were acclimated for at least 1 week before use. Mice had access to water and food ad libitum and were kept in a 12-hour light-dark cycle. PGD₂, DK-PGD₂, BW245C or their vehicle was injected subcutaneously (s.c.) every 12 hours in a volume of 200µL saline at given concentrations starting 24 hours before LPS challenge. Antagonists, CAY10471 and MK0524, were administered s.c. every 12 hours in a volume of 200 µL saline at given concentrations starting 24 hours before LPS challenge. Mice were slightly anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg) by intraperitoneal injection. LPS was administered intranasally at a dose of 1mg/kg in a volume of 50µL dissolved in saline. Mice were sacrificed by intraperitoneal injection of ketamine (150 mg/kg) and xylazine (15 mg/kg) 4 or 24 hours after LPS challenge and BAL fluid was obtained by intratracheal administration of 3mL of PBS with 1mM EDTA. After centrifugation, supernatants were collected and stored at -70°C for further analysis. Cells were stained with corresponding antibodies. Erythrocytes were lysed and remaining leukocytes were stained with anti-mouse MHC II (2.5µg/mL) anti-mouse Siglec F (5µg/mL), anti-mouse CD3ε (5µg/mL), anti-mouse B220 (2µg/mL) and anti-mouse CD11c (2µg/mL) antibodies. Cells were taken up in 200µL fixative solution and cells were counted for 30 sec on a FACSCalibur flow cytometer. Cell populations were identified with adaptations as described elsewhere ⁶. In detail, lymphocytes were identified as FSC^{low}/SSC^{low}, CD3^{high}/B220^{high} cells and further divided into MHC-II^{neg} T- and MHC-II^{high} B-lymphocytes. FSC^{med}, SSC^{med}, CD3^{neg}/B220^{neg} granulocytes were identified as CCR3^{neg} neutrophils and CCR3^{high} eosinophils. FSC^{high}/SSC^{med} alveolar macrophages were gated as CD11c^{high}/MHC-II^{high} and monocytes were identified as CD11c^{med}/MHC-II^{neg} cells.

Myeloperoxidase (MPO) assay. MPO activity was measured as an indicator for the severity of neutrophilic lung inflammation. Activity was determined as described before ⁷. In brief, frozen lung samples were homogenized in phosphate buffer with 0.5% hexadecyltrimethyl ammonium bromide. After centrifugation (20min, 10000 rpm, 4°C), 10 µl of supernatant was added to 200 µl of 50 mM potassium phosphate buffer (pH 6.0) containing 0.167 mg/mL of O-dianisidine hydrochloride and 0.5 µl of 1% H₂O₂/mL. The kinetics of MPO activity was measured at 460 nm (xMark™, Bio-Rad, Austria).

Evans blue dye extravasation. Evans blue dye (60mg/kg) was injected into the tail vein three hours post intranasal LPS application and allowed to circulate for 60 mins. The lungs were then perfused with PBS containing 5mM EDTA, weighed and homogenized in PBS (1mL/100µg) and incubated with 2 volumes of formamide for 24 hours at 60°C. Following centrifugation supernatants were collected and the absorption of Evans blue was measured at 620nm, corrected for the presence of heme pigments using the following equation: Absorption₆₂₀ (corrected) = Absorption₆₂₀ - (1.426 x Absorption₇₄₀ + 0.030) and calculated against a standard curve ^{8,9}.

Immunohistochemistry of murine lung tissue. Lungs were perfused with PBS-EDTA followed by perfusion with 4% paraformaldehyde. After dissection, lungs were kept in paraformaldehyde for further 24 hours until embedded in paraffin. 4µM sections were cut and deparaffinized. Following heat-mediated antigen retrieval using an antigen unmasking solution (Vector, Vector Labs, Burlingame, CA, USA) sections were incubated in blocking solution (Vectastain ABC Kit, Vector Labs). Sections were incubated with anti-Ly6G (1:1000) over night at 4°C. Horse-raddish conjugated goat anti-rat antibody was used as secondary antibody. Detection was performed using the Vectastain ABC kit and 3-amino-9-ethycarbazole (AEC) and counterstained with hematoxylin. Sections were visually examined with an Olympus BX41 microscope (Olympus) and an Olympus U Plan Apo x40/0.8 lens. Photographs were taken with an Olympus DP50 camera (2776 x 2074 pixels) and further processed with CELL^P software (Olympus) for additional white balance, contrast, and brightness adjustments. Ly6G positive cells were counted in 6 high power fields (40x magnification) per section.

Isolation of murine alveolar macrophages. BAL of 3 mice was pooled, cell number was determined and cells were suspended to 1x10⁶ cell/mL and seeded at a volume of 200µL containing 0.2x10⁶ cells

in 96 well plates ¹⁰. After 1 hour, non-adherent cells were washed away with PBS and remaining alveolar macrophages were supplied with RPMI +10% FBS + 1% Pen/Strep. By examination of their microscopic appearance, these cell preparations contained more than 90% macrophages.

Isolation of murine interstitial pulmonary macrophages. After performing BAL, lungs of 3 BALB/c mice were perfused with PBS-EDTA followed by perfusion with PBS supplemented with 1µg/µL collagenase/dispase (Sigma Aldrich, Vienna, Austria). Lung tissue was minced and incubated with RPMI + 20%FBS + 1µg/µL collagenase/dispase for 60min at 37°C on a shaker. Digested tissues were then poured onto a 60µm cell strainer and washed twice. The resulting pooled cell suspension was then put on ice for 5 min until the particulate matter settled and the supernatant containing isolated cells was taken up. Cells were then washed 3 times, and suspended to 1x10⁶ cell/mL and seeded at a volume of 200µL containing 0.2x10⁶ cells in 96 well plates. After one hour, non-adherent cells were washed away with PBS and remaining interstitial pulmonary macrophages were supplied with RPMI +10% FBS + 1% PS. Based on their microscopic appearance, these cell preparations contained more than 90% macrophages.

Depletion of murine lung macrophages. Mice were slightly anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg) by intraperitoneal injection. Then, mice were intranasally applied 300 µg (in 60 µL volume) of clodronate or control liposomes 24 hours before LPS challenge. This procedure had been shown to deplete up to 84% of alveolar macrophages ¹¹.

Cytokine measurements. Levels of human TNF-α in cell culture supernatants were determined with a multi-analyte immunoassay as recommended (Bender Medsystems, Vienna, Austria). Murine TNF-α, MCP-1, KC and IL-6 were assessed in the BAL fluid of animals using a standard enzyme-linked immunosorbent assay (ELISA) purchased from Peprotech (Rocky Hill, NJ, US). Quantification of cytokines, TNF-α, IL-6, KC, MCP-1 and IL-10, from supernatants of murine alveolar or interstitial lung macrophages was carried out by the ProcartaPlex Mouse Cytokine Kit as described by the manufacturer (eBioscience).

Measurement of murine lung function. Decreased lung compliance due to pulmonary edema and atelectasis is a hallmark of human ALI/ARDS and according to American Thoracic Society guidelines a preferred experimental parameter in mouse models ¹². Therefore, mouse lung function was assessed

using the flexiVent system as described before ¹³. Mice were slightly anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg) by intraperitoneal injection, the trachea was cannulated and mice were then connected to a miniature computerized flexiVent apparatus (SCIREQ, Montreal, QC, Canada). Mice were ventilated with a tidal volume of 10 mL/kg at a rate of 150 breaths/min. Before each experiment, calibration of the FlexiVent device was performed using the tracheal cannula to be used. Nebulized methacholine was used to measure airway responsiveness, and airway resistance as well as lung compliance were recorded.

Immunohistochemistry of human lung tissue. Paraffin-embedded samples of human lung from 5 patients with diffuse alveolar damage (DAD), the histologic correlate to acute respiratory distress syndrome (ARDS) as identified by the American European Consensus Conference definitions ¹⁴, organizing pneumonia, sarcoidosis and respiratory bronchiolitis ¹⁵ as well as 5 healthy control tissues (the latter obtained from resection specimens for trauma) were used. Four μ m sections were cut and deparaffinized, microwaved for 2 $\times$ 5 min cycles in 10 mM citrate buffer, and then processed by ABC method (Vectastain ABC kit; Vector Laboratories), according to the manufacturer's protocol. Sections were incubated with rabbit anti-CRTH2 (DP₂) (1:200; Acris Antibodies) ¹⁶ or rabbit anti-DP₁ (1:100; Cayman Chemicals, Ann Arbor, MI, US) ¹⁷ or HPGDS (1:200, LifeSpan Biosciences, Inc, Seattle, US), visualized with 3-3'-diaminobenzidine and counterstained with hematoxylin. Images were taken with a high-resolution digital camera (Olympus DP 50), processed, and analyzed by Cell^A imaging software (Olympus, Vienna, Austria). Contrast and brightness adjustment was performed.

Lipid mediator analysis. A stable isotope dilution liquid chromatography-mass spectrometry (LC-MS/MS) method was used, as previously reported elsewhere ¹⁸, with slight modifications to quantify the lipid mediators, PGD₂, PGE₂, 6-*keto*-PGF_{1 α} , TBX₂ and 12-HHTrE. Lipid mediator nomenclature provided in Supplementary Table E2. Briefly, BAL fluid samples were mixed with a cocktail of deuterated internal standards (10 μ L) in concentrations according to Supplementary Table E3 and loading onto Waters Oasis HLB solid phase extraction (SPE) cartridges. SPE cartridges were air-dried, and lipid mediators eluted with organic solvent, evaporated under vacuum and reconstituted in 60 μ L of methanol and 10 μ L of water. Following spin filtering, 7.5 μ L were injected onto an Acquity UPLC

with a BEH C18 column (2.1x150 mm, 1.7 μ m, Waters) and analyzed on a Waters Xevo TQS-MS in negative electrospray ionization mode.

SPE extraction. Extraction of analytes was carried out in 3cc/60 mg HLB Oasis SPE cartridges (Waters). Cartridges were conditioned with 2 mL of methanol followed by 2 mL of water. A cocktail of deuterated internal standards (10 μ L; Supplementary Table E3) was added to 750 μ L BALF sample, followed by 10 μ L BHT/EDTA solution (0.2 mg/mL, dissolved in methanol/water 50:50, v/v) and 750 μ L extraction buffer (citric acid: 0.2 M NaH_2PO_4 , pH5.6) and loaded onto the SPE cartridge. Samples were washed with 2 mL of water/methanol (80:20, v/v) and the cartridges were dried in the manifold under vacuum-induced air stream at -30 kPa for 30 min. Analytes were eluted from the columns with 2.5 mL of methanol in cryotubes containing 10 μ L of glycerol 30% in methanol. After evaporation of the eluates under vacuum, samples were resuspended in 60 μ L of methanol. Additionally, 10 μ L of water was added to the samples. Solution was filtered by centrifugation in Amicon Ultrafree-MC, PVDF 0.1 μ m (Millipore) and transferred to autosampler vials with inserts before injection.

UPLC-MS/MS measurement. Liquid chromatography coupled to mass spectrometry (LC-MS/MS) separation and quantification was carried out on a UPLC Acquity-Xevo TQS mass spectrometer system (Waters, Milford, MA). The autosampler and column were kept at 5°C and 60°C , respectively. Injection volume was set to 7.5 μ L. Separation was achieved with an Acquity UPLC BEH C18 (2.1 $\times$ 150 mm, 1.7 μ m, Waters) and a gradient of solvents A (water with 0.1% of acetic acid) and B (acetonitrile/isopropanol 90:10, v/v) at a flow of 0.5 mL min^{-1} . The gradient initiated with 80% of A, which was decreased linearly to 65% at 2.5 min, to 60% at 4.5 min, to 58% at 6 min, to 50% at 8 min, to 35% at 14 min, to 27.5% at 15.50 min and to 0% at 16.60min. The column was then washed with solvent B for 0.9 min and equilibrated to initial conditions. Data was collected on a Waters Xevo TQS-MS mass spectrometer using negative electrospray ionization and scheduled multiple reaction monitoring (MRM) mode. Dwell time was automatically adjusted in order to acquire 20 points per chromatographic peak; capillary voltage was 2.20 kV in negative. Desolvation temperature and gas flow were set according to the instrument recommendations for the chromatographic flow. Detailed MRM transition and chromatographic retention time for each compound is provided in Supplementary Table E4.

Supplementary Figure Legends

Supplementary Figure E1. DP₂ and DP₁ expression is increased during the differentiation of monocyte-derived macrophages. Cell surface receptor expression of **(A)** DP₂ and **(B)** DP₁ receptors was recorded by flow cytometry and is shown as fold increase over isotype control, mean + SEM of independent experiments with 3-5 different donors. (* p<0.05 as determined by Student's t-test).

Supplementary Figure E2. Pulmonary macrophages express DP₂ receptors. Immunohistochemistry stainings of human lung samples derived from indicated pathologies show positive cells for DP₂ (brown). Stainings are representative pictures of 5 patients of each disease. Positive macrophages are indicated by arrows. Bar 100µm.

Supplementary Figure E3. Pulmonary macrophages express DP₁ receptors. Immunohistochemistry staining of human lung samples derived from indicated pathologies show positive cells for DP₁ (brown). Stainings are representative pictures of 5 patients of each disease. Positive macrophages are indicated by arrows. Bar 100µm.

Supplementary Figure E4. PGD₂ induces macrophage Ca²⁺ flux both via DP₂ and DP₁ receptors. **(A)** Activation of DP₂ by selective agonist DK-PGD₂ as well as selective DP₁ receptor activation with BW245C (both 1µM) led to decreased intracellular Ca²⁺ levels as compared to PGD₂ (1µM) control (mean + SEM, n_{experiments}=4-5, *** p<0.001 vs PGD₂ as determined by one-way ANOVA followed by Bonferroni's multiple comparison test). **(B)** Representative tracings and **(C)** mean+SEM notation of Ca²⁺ responses to PGD₂, which were diminished by the DP₂ antagonist CAY10471 and the DP₁ antagonist MK0524 (both 10µM), while blockade of both receptors abolished the Ca²⁺ response to PGD₂ (n_{experiments}=4-5, ** p<0.01 vs PGD₂ as determined by one-way ANOVA followed by Bonferroni's multiple comparison test). **(D)** Representative tracings and **(E)** mean+SEM notation of Ca²⁺ responses to PGD₂, which was inhibited by overnight incubation with 100ng/mL PTX (mean + SEM, *** p<0.001 vs PGD₂ or vs DK-PGD₂ as determined by one-way ANOVA followed by Bonferroni's multiple comparison test).

Intracellular free Ca^{2+} levels were measured by live-cell fluorescent microscopy for at least 20 cells per sample. Baseline was recorded for 3 min, and then agonist (1 μM PGD_2 , 1 μM DK- PGD_2 , or 1 μM BW245C) superfusion was performed. Superfusion of antagonists (CAY10471 or MK0524, 10 μM respectively) or vehicle was started 7 min prior to PGD_2 stimulation. Each data set comprises 3-7 independent experiments, each with an individual donor. Quantification of intracellular Ca^{2+} changes as an increase from baseline ($\Delta[\text{Ca}^{2+}]$) is shown.

Supplementary Figure E5. PGD_2 does not induce Ca^{2+} mobilization in human peripheral blood monocytes. Mononuclear cells were stimulated with vehicle, or the indicated concentrations of MCP-1 or PGD_2 , and changes in intracellular Ca^{2+} levels in CD14-positive monocytes were detected by flow cytometry and are shown as mean $\pm$ SEM percent of baseline from 3 independent experiments, each with an individual donor.

Supplementary Figure E6. PGD_2 modulates human monocyte-derived macrophage (MDM) function by inducing chemotaxis and cytokine release. (A) Human MDM migration towards PGD_2 (concentrations as indicated) was evaluated by DAPI staining of nuclei on the lower surface of the filters. Data are shown as mean $\pm$ SEM, from 3 independent experiments performed in duplicates (** $p < 0.01$ versus vehicle as determined by one-way ANOVA followed by Dunnett's multiple comparison test). **(B)** Representative fluorescent micrographs show migrated MDM on the DAPI-stained filter. Scale bar is 50 μm . **(C)** Human MDM were pretreated with PGD_2 , DK- PGD_2 , BW245C or vehicle for 18 hours and then activated by 100 ng/mL LPS for 24 hours. Antagonists (CAY10471 or MK0524) were incubated 30 min prior to PGD_2 pretreatment. TNF- α in supernatants was detected via ELISA, $n=5$ independent experiments performed in duplicates (* $p < 0.05$, ** $p < 0.01$ compared to veh/LPS and # $p < 0.01$ compared to PGD_2 /LPS as determined by one-way ANOVA followed by Dunnett's comparison test).

Supplementary Figure E7. PGD_2 aggravates LPS-induced lung inflammation in mice. Animals were pretreated s.c. with PGD_2 at the indicated doses every 12 hours the day before intranasal LPS administration (1 mg/kg). After 4 hours, BAL was performed, cell populations were analyzed by flow cytometry, and lung tissue was collected. **(A)** Quantitation of immunohistochemistry of Ly6G-stained

neutrophils in lung tissue (cf. Fig. 2C) per 6 high power fields. Data is presented as mean + SEM (n=4, ** p<0.01 as determined by one-way ANOVA followed by Bonferroni's multiple comparison test).

(B) PGD₂ treatment increases the protein concentration in the BAL fluid (n=8-13, * p<0.05 as determined by one-way ANOVA followed by Bonferroni's multiple comparison test). **(C)** Increased neutrophilic influx persisted also at 24 hours post LPS (n=4-9, *** p<0.001, vs vehicle, two-way ANOVA followed by Bonferroni's multiple comparison test).

Supplementary Figure E8. Effect of PGD₂ receptor activation on bronchoalveolar counts of monocytes, macrophages, lymphocytes and eosinophils in mice. Animals were pretreated with PGD₂, DK-PGD₂ and BW-245C twice every 12 hours before intranasal LPS administration. After 4 hours, BAL was performed, and **(A)** monocytes, **(B)** macrophages, **(C)** lymphocytes, and **(D)** eosinophils were enumerated by flow cytometry as stated in the Methods (n=7-12, * p<0.05, ** p<0.01 as determined by one-way ANOVA followed by Bonferroni's multiple comparison test).

Supplementary Figure E9. PGD₂ enhances keratinocyte-derived chemokine (KC) secretion from pulmonary macrophages. **(A)** KC and MCP-1 concentrations were increased in BAL fluid of PGD₂-treated animals 4 hours after LPS challenge (n=6-12; * p<0.05 compared to veh/LPS, ** p<0.01 compared to veh as determined by one-way ANOVA followed by Dunnett's post test). PGD₂ treatment also stimulated the *in-vitro* cytokine secretion of **(B)** murine alveolar and **(C)** murine interstitial pulmonary macrophages as determined by bead-based ELISA (n= 3-5 independent experiments performed in triplicates; #p<0.05 compared to veh/LPS as determined by one-way ANOVA followed by Dunnett's post test).

Supplementary Figure E10. Macrophage numbers are reduced after clodronate application. Mice received 300µg of clodronate in 60µL liposomes or 60µL control liposomes intranasally. Macrophage numbers are from the same animals as in Fig. 4A with pooled control and clodronate liposome groups. (n= 32-38; *** p<0.001, compared to control liposomes as determined by student's t-test).

Supplementary Figure E11. Neutrophil counts in the BAL fluid after treatment with control liposomes or clodronate-loaded liposomes. Same as in Figure 4A, but neutrophil counts are normalized to the mean neutrophil counts of the veh / LPS treated mice in the control liposome and clodronate-treated group, respectively (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by one-way ANOVA followed by Bonferroni's multiple comparison test).

Supplementary Figure E12. Neutrophil numbers are not affected by PGD₂-treatment of co-cultured macrophages. Neutrophil counts in the same samples as in Fig. 5C were determined per 60 sec using flow cytometry (n=5).

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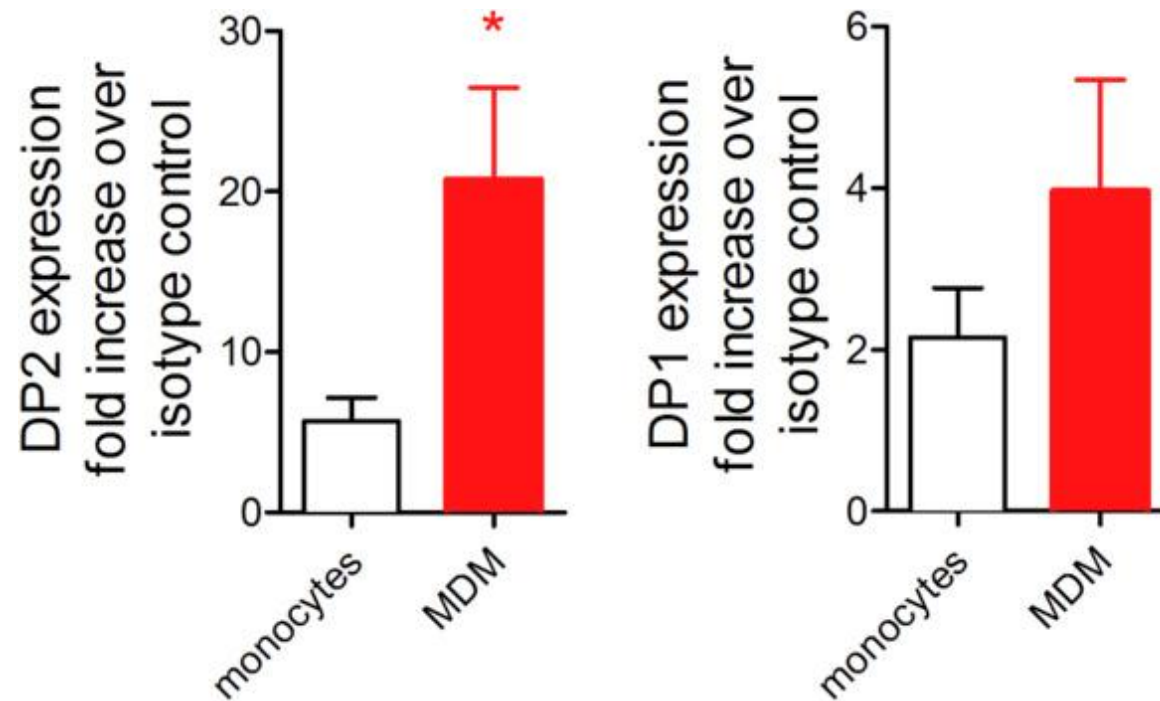


Fig E1.

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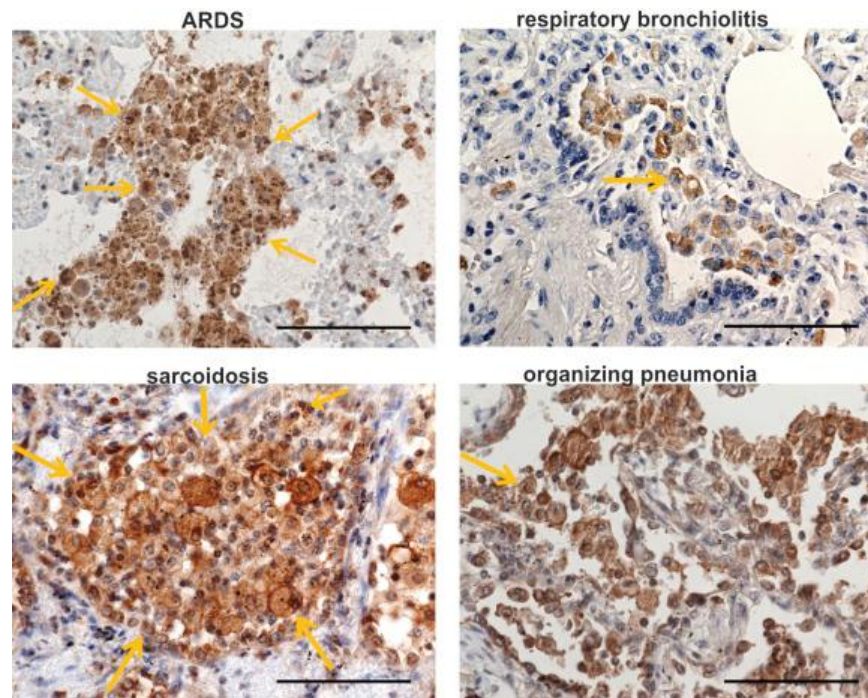


Fig E2.

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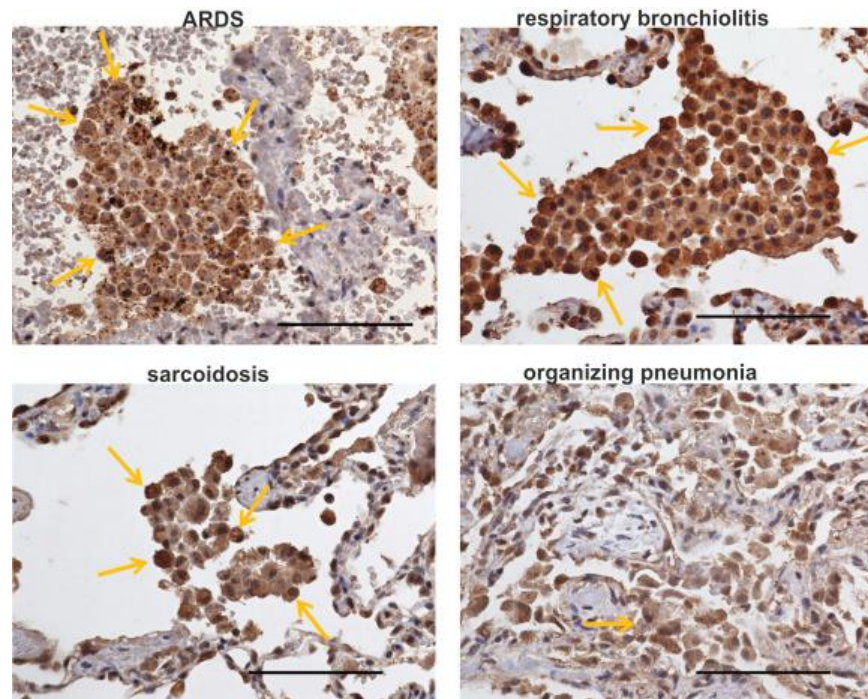


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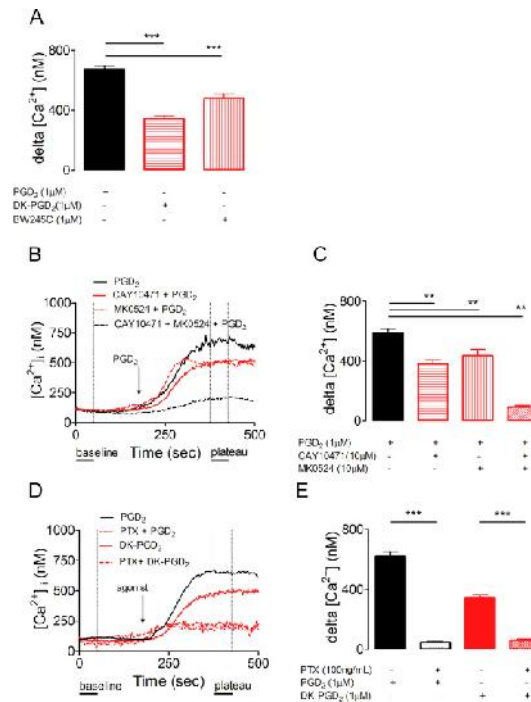


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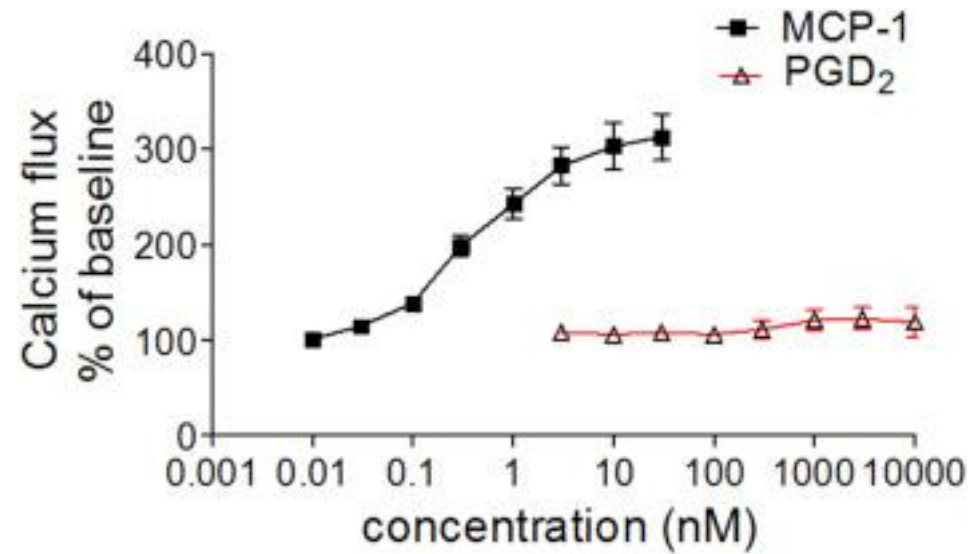


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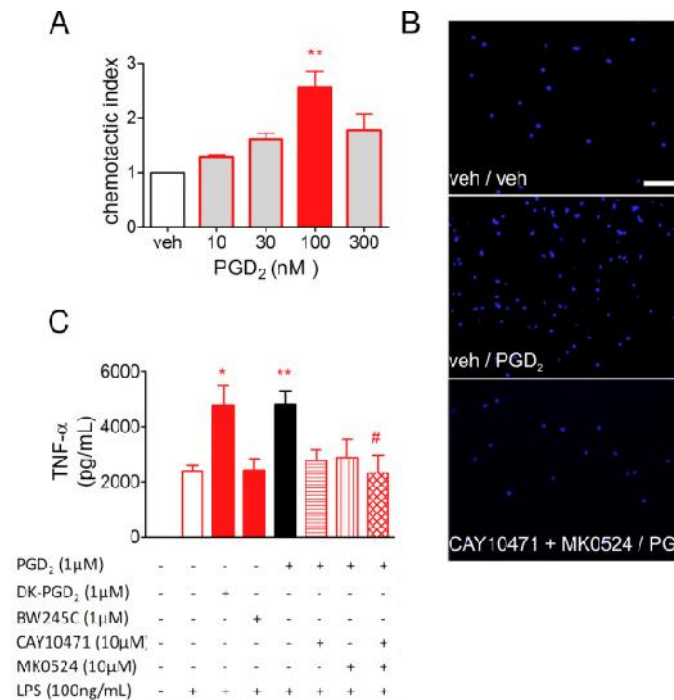


Fig E6.

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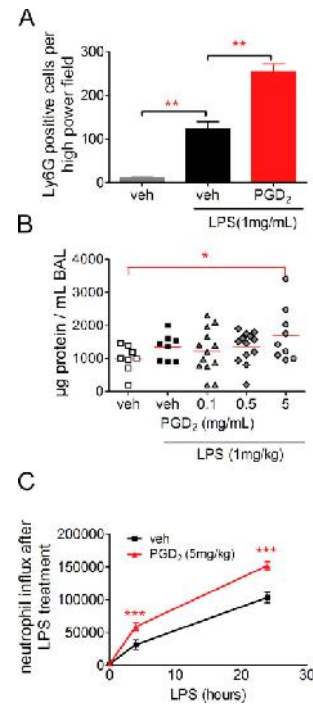


Fig E7.

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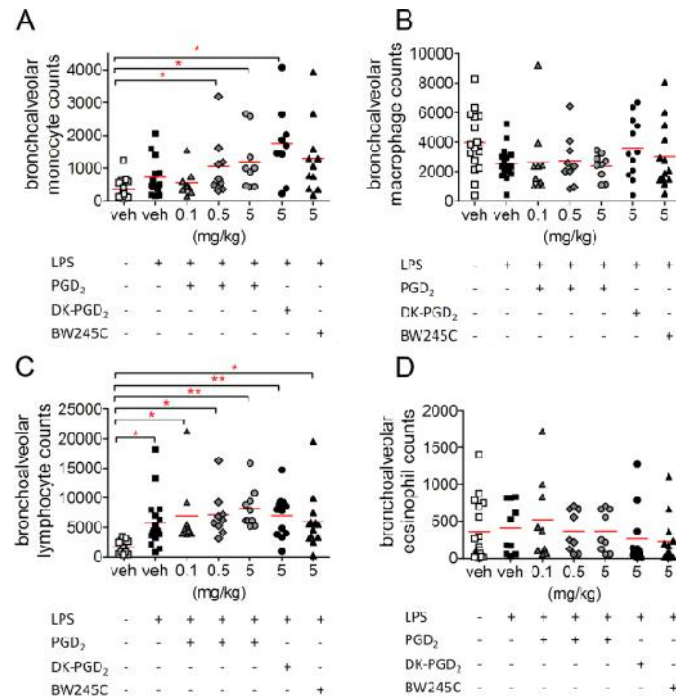


Fig E8.

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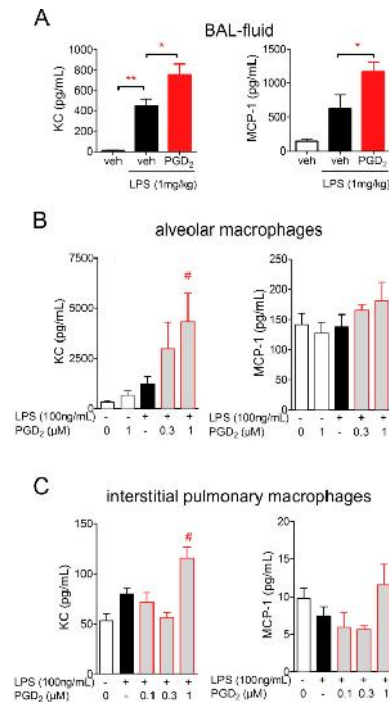


Fig E9.

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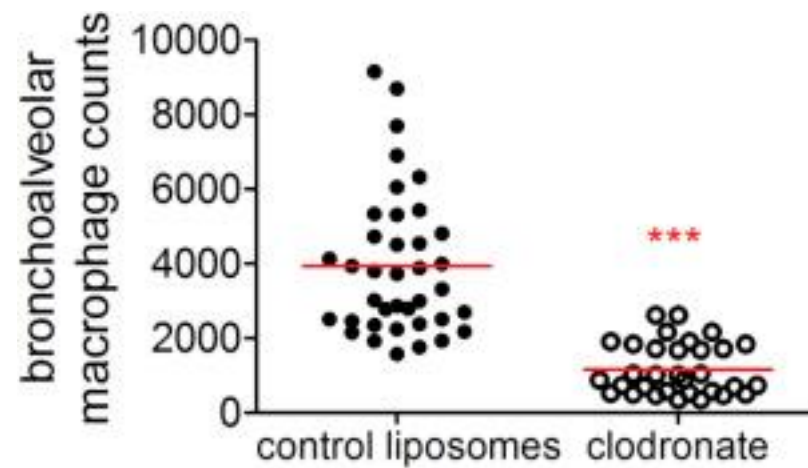


Fig E10.

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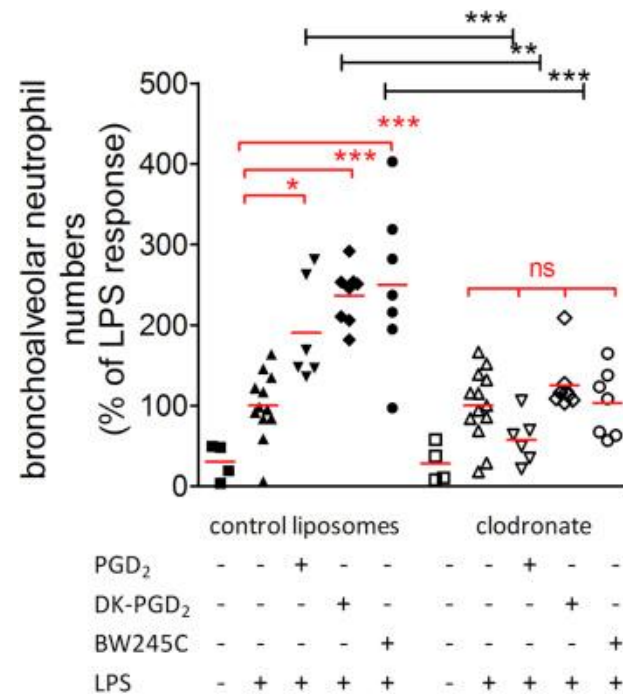


Fig E11.

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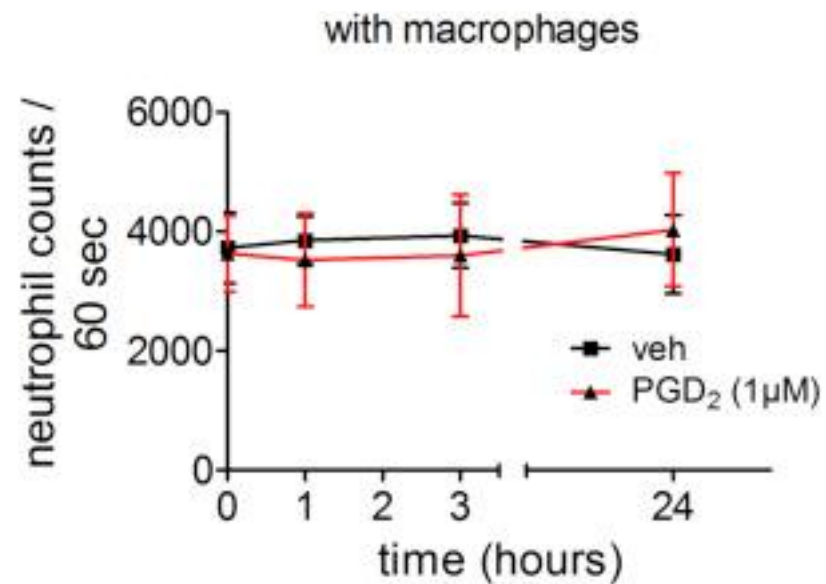


Fig E12.

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Supplementary Table E1. List of lung tissues with corresponding pathologies that were examined for DP2, DP1 and HPGDS expression.

	DP₂		DP₁		HPGDS	
Disease	Number of patient samples	Corresponding picture	Number of patient samples	Corresponding picture	Number of patient samples	Corresponding picture
Control lungs	5	Figure 1B	5	Figure 1B	5	Figure 6A
ARDS	5	Figure E2	5	Figure E3	5	Figure 6B
Respiratory bronchiolitis	5	Figure E2	5	Figure E3	-	
sarcoidosis	5	Figure E2	5	Figure E3	-	
Organizing pneumonia	5	Figure E2	5	Figure E3	-	

Supplementary Table E2. Nomenclature for lipid mediators

Abbreviation	Common name	Systematic name
PGD ₂	prostaglandin D ₂	9 α ,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid
PGE ₂	prostaglandin E ₂ ; dinoprostone	9-oxo-11 α ,15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid
6-keto- PGF1 α	6-keto-prostaglandin F _{1α}	6-oxo-9 α ,11 α ,15S-trihydroxy-prost-13E-en-1-oic acid
TxB ₂	thromboxane B ₂	9 α ,11,15S-trihydroxythromba-5Z,13E-dien-1-oic acid
12-HHTrE	12-HHT	12-hydroxy-5Z,8E,10E-heptadecatrienoic acid

Supplementary Table E3. Calibration levels of the lipid mediators analyzed.

[illegible]

Supplementary Table E4. Analytical characterization of the standards of the lipid mediators.

Chromatographic retention time (RT), parent ion, product ion, the internal standard (IS) assigned to each lipid mediator and the on-column injected linear range are reported.

Lipid mediator	RT (min)	Parent ion [M-H] ⁻ (m/z)	Product ion (m/z)	IS	Linear range (fmol)
PGD ₂	4.87	351.1	271.1	[² H ₄]-PGD ₂	1.7-3951
PGE ₂	4.61	351.2	271.1	[² H ₄]-PGE ₂	1.7-3951
6-keto-PGF _{1α}	3.13	369.2	162.9	[² H ₄]-8-epi-PGF _{2α}	1.6-3759
TxB ₂	3.98 (broad peak)	369.2	168.9	[² H ₄]-TxB ₂	1.6-3780
12-HHTrE	9.29	279.2	179	[² H ₄]-13-HODE	2-10844
[² H ₄]-PGD ₂	4.84	355.2	193.1		
[² H ₄]-PGE ₂	4.58	355.3	275.1		
[² H ₄]-8-epi-PGF _{2α}	3.95	357.2	197		
[² H ₄]-TxB ₂	3.95 (broad peak)	373.2	173		
[² H ₄]-13-HODE	11.27	299.2	198.1		