

# Activated prostaglandin D<sub>2</sub> receptors on macrophages enhance neutrophil recruitment into the lung

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**Background:** Prostaglandin (PG) D<sub>2</sub> is an early-phase mediator in inflammation, but its action and the roles of the 2 D-type prostanoid receptors (DPs) DP<sub>1</sub> and DP<sub>2</sub> (also called chemoattractant receptor–homologous molecule expressed on T<sub>H</sub>2 cells) in regulating macrophages have not been elucidated to date.

**Objective:** We investigated the role of PGD<sub>2</sub> 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, Ca<sup>2+</sup> 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 DP<sub>1</sub>, DP<sub>2</sub>, or both receptors on human macrophages induced strong intracellular Ca<sup>2+</sup> flux, cytokine release, and migration of macrophages. In a murine model of LPS-induced pulmonary inflammation, activation of each PGD<sub>2</sub> receptor resulted in aggravated airway neutrophilia, tissue myeloperoxidase activity, cytokine contents, and decreased lung compliance. Selective depletion of alveolar macrophages abolished the PGD<sub>2</sub>-enhanced inflammatory response. Activation of PGD<sub>2</sub> receptors on human macrophages enhanced the migratory capacity and prolonged the survival of neutrophils *in vitro*. In human lung tissue specimens both DP<sub>1</sub> and DP<sub>2</sub> receptors were located on alveolar macrophages along with hematopoietic PG synthase, the rate-limiting enzyme of PGD<sub>2</sub> synthesis.

**Conclusion:** For the first time, our results show that PGD<sub>2</sub> 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<sub>H</sub>2 cells, prostaglandin D<sub>2</sub>, hematopoietic prostaglandin D synthase, macrophages, pulmonary inflammation, neutrophils

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**Abbreviations used**

ALI:	Acute lung injury
ARDS:	Acute respiratory distress syndrome
BAL:	Bronchoalveolar lavage
DK-PGD <sub>2</sub> :	13,14-Dihydro-15-keto prostaglandin D <sub>2</sub>
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.<sup>10</sup> In asthmatic patients induction of hematopoietic prostaglandin D synthase (HPGDS), the rate-limiting enzyme of PGD<sub>2</sub> synthesis, was observed in the epithelial compartment,<sup>11</sup> and PGD<sub>2</sub> levels in bronchoalveolar lavage (BAL) fluid correlated positively with the severity of the disease.<sup>12</sup> DP<sub>2</sub>/chemoattractant receptor–homologous molecule expressed on T<sub>H</sub>2 cell antagonists was found to have some effects in allergic rhinitis,<sup>13</sup> allergic conjunctivitis,<sup>14</sup> eosinophilic esophagitis,<sup>15</sup> and bronchial asthma.<sup>16,17</sup>

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- $\alpha$ , IL-1 $\beta$ , and IL-6, their role in inflammation is pivotal.<sup>18</sup> 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,<sup>19</sup> subsequent edema formation, and dysfunction of the involved cell types, including epithelial cells,<sup>20</sup> endothelial cells,<sup>21</sup> and macrophages.<sup>22–24</sup> Several studies have made it clear that macrophages orchestrate neutrophilic infiltration and thus strongly modulate the outcome of ARDS.<sup>23–27</sup>

Stimulated by the dominant physiologic role of PGD<sub>2</sub> in the lung, we hypothesized that PGD<sub>2</sub> might govern disease activity and progression by acting on lung macrophages. Previous studies revealed anti-inflammatory effects of the PGD<sub>2</sub> metabolite 15d-PGJ<sub>2</sub> acting through peroxisome proliferator–activated receptor  $\gamma$  in RAW 264.7 macrophages<sup>28</sup> and demonstrated the expression of PGD<sub>2</sub> receptors on human monocytes,<sup>29</sup> whereas the role of PGD<sub>2</sub> 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](http://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.<sup>30</sup>

**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 Ca<sup>2+</sup> imaging**

Macrophages were loaded with 2  $\mu$ 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.<sup>31</sup>

**Monocyte Ca<sup>2+</sup> flux**

Ca<sup>2+</sup> flux was measured by means of flow cytometry, as previously described.<sup>30</sup>

**Neutrophil apoptosis**

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

**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  $\mu$ L of fixative solution, and enumerated by means of flow cytometric analysis.<sup>33</sup> Migration of human MDMs was assessed by using Transwell inserts, as described previously.<sup>30</sup>

**Flow cytometric staining**

The following antibodies and concentrations were used: DP<sub>2</sub> (20  $\mu$ g/mL), DP<sub>1</sub> (20  $\mu$ g/mL), anti-mouse MHC class II (2.5  $\mu$ g/mL), anti-mouse Siglec-F (5  $\mu$ g/mL), anti-mouse CD3e (5  $\mu$ g/mL), anti-mouse B220 (2  $\mu$ g/mL), and anti-mouse CD11c (2  $\mu$ 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.<sup>34</sup>

**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.

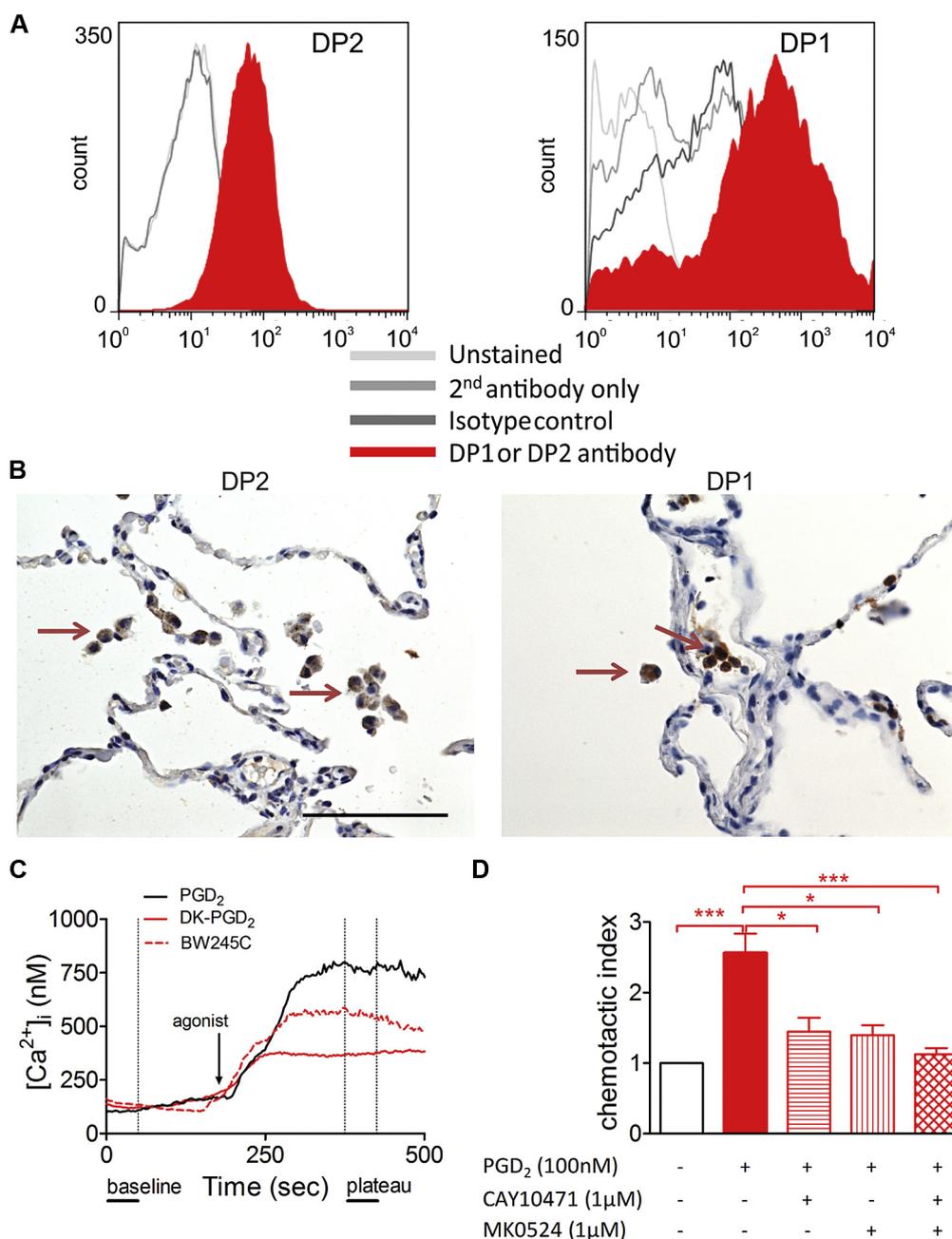
Vascular permeability in lung tissue was assessed by using Evans blue dye extravasation after 60 minutes of circulation. Evans blue protein leak was determined, as described in the [Methods](#) section in this article's Online Repository.

**Murine lung histology**

Paraffin-embedded murine lungs were cut (5- $\mu$ 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.** PGD<sub>2</sub> receptors DP<sub>1</sub> and DP<sub>2</sub> are expressed on macrophages and induce Ca<sup>2+</sup> flux and migration. **A**, Flow cytometric histograms of DP<sub>2</sub> and DP<sub>1</sub> staining (filled histograms) on MDMs, respectively. **B**, Immunohistochemistry of healthy human lung tissue showing DP<sub>2</sub>- and DP<sub>1</sub>-positive alveolar macrophages (arrows). **C**, Representative Ca<sup>2+</sup> responses of MDMs over time. **D**, MDM migration toward PGD<sub>2</sub> is blocked by DP<sub>1</sub>- and DP<sub>2</sub>-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.<sup>35</sup>

### Cytokine measurements

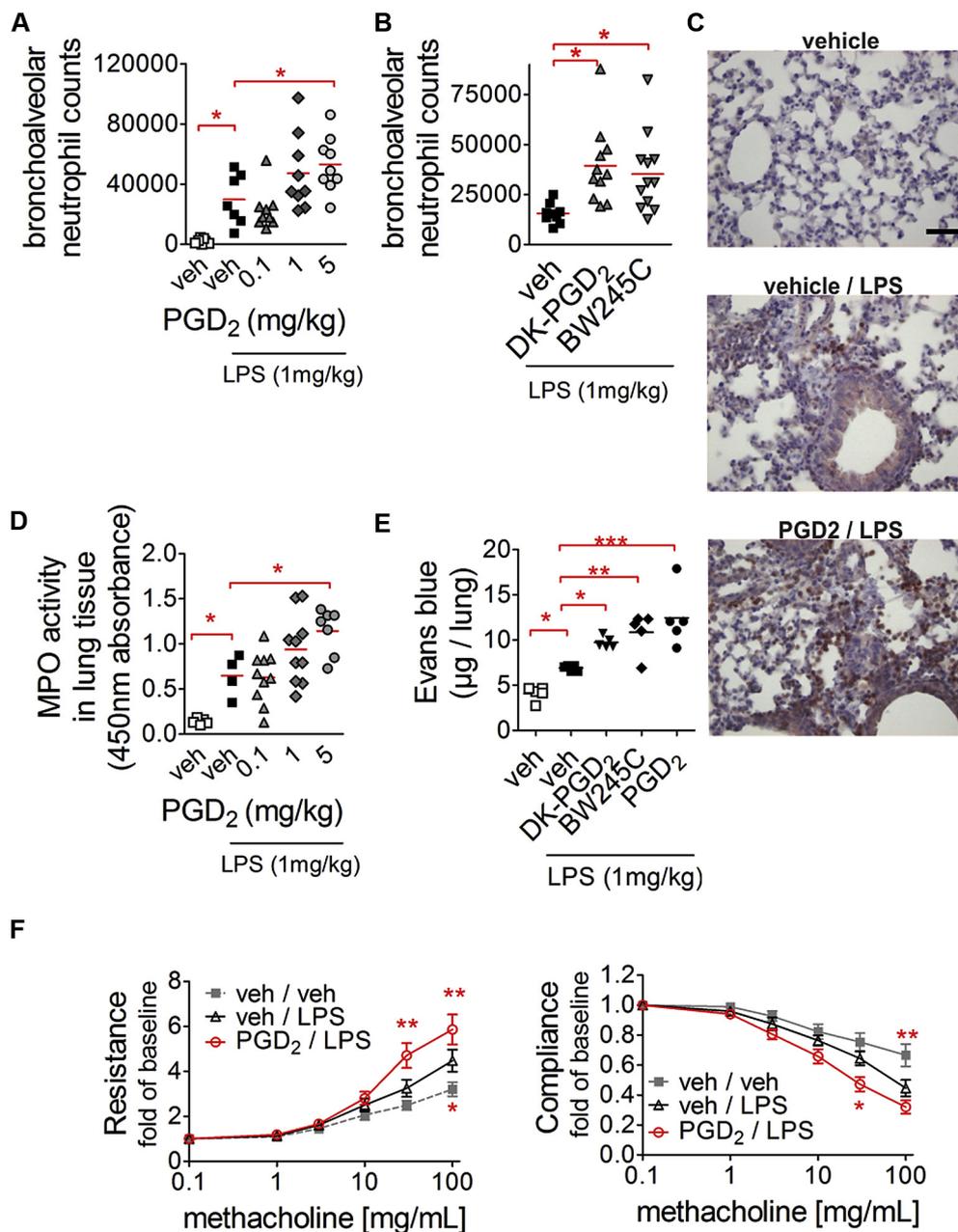
Cytokine levels were determined by using either a multianalyte immunoassay (Bender Medsystems, Vienna, Austria), ELISA (PeproTech, Rocky Hill, NJ), or the ProcartaPlex Mouse Cytokine Kit (eBioscience, San Diego, Calif).

### Immunohistochemistry of human lung tissue

Human paraffin-embedded lung samples were stained with anti-DP<sub>2</sub> (1:200), anti-DP<sub>1</sub> (1:100), or anti-HPGDS (1:200) antibodies.

### Lipid mediator analysis

PGD<sub>2</sub>, PGE<sub>2</sub>, TXB<sub>2</sub>, 12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid, and 6-keto-PGF<sub>1α</sub> were analyzed by using liquid chromatography–tandem mass spectrometry, as previously published<sup>36</sup> and as described in the [Methods](#) section in this article's Online Repository.



**FIG 2.** PGD<sub>2</sub> acting through DP<sub>1</sub> and DP<sub>2</sub> promotes neutrophil influx into lungs and aggravates airway hyperreactivity. **A** and **B**, Neutrophil infiltration is increased in animals pretreated with PGD<sub>2</sub> (Fig 2, **A**) or DP<sub>1</sub>- and DP<sub>2</sub>-selective agonists (Fig 2, **B**). **C-F**, PGD<sub>2</sub> pretreatment increases Ly6G-positive neutrophil infiltration in the peribronchial and alveolar space (representative pictures, scale bar = 50  $\mu$ m; 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.<sup>37</sup> Therefore mouse lung function was determined by using the flexiVent system (SCIREQ, Montreal, Quebec, Canada), as described previously.<sup>38</sup>

### Statistical analysis

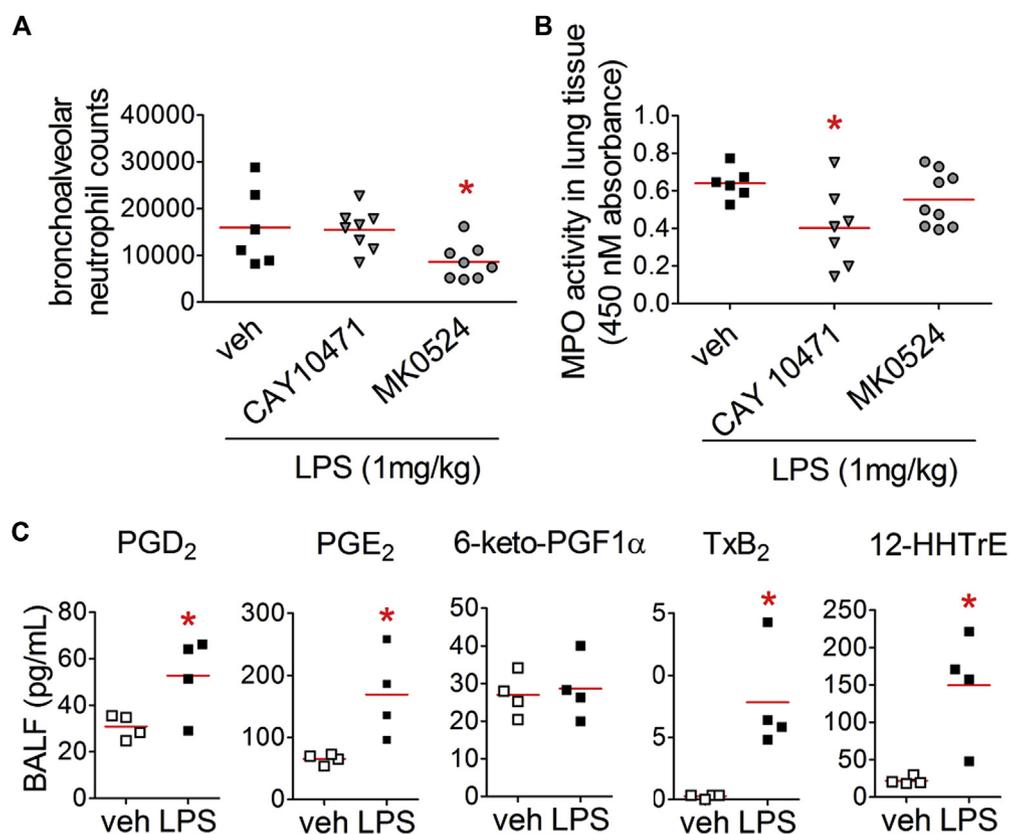
Data were analyzed by using either the Student *t* test, 1-way ANOVA, or 2-way ANOVA, followed by the Bonferroni or Dunnett posttest, with GraphPad Prism software (version 5.04; GraphPad Software, La Jolla, Calif). *In vitro*

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

### DP<sub>1</sub> and DP<sub>2</sub> are expressed on human macrophages and induce Ca<sup>2+</sup> signaling

We first set out to elucidate whether PGD<sub>2</sub> receptors are expressed on human monocytes and macrophages and found that



**FIG 3.** Blocking of endogenous PGD<sub>2</sub> ameliorates LPS-induced neutrophil influx into the alveolar space and pulmonary tissue. **A** and **B**, DP<sub>1</sub> antagonist reduced neutrophil counts in the bronchoalveolar space (Fig 3, A), whereas DP<sub>2</sub> 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.

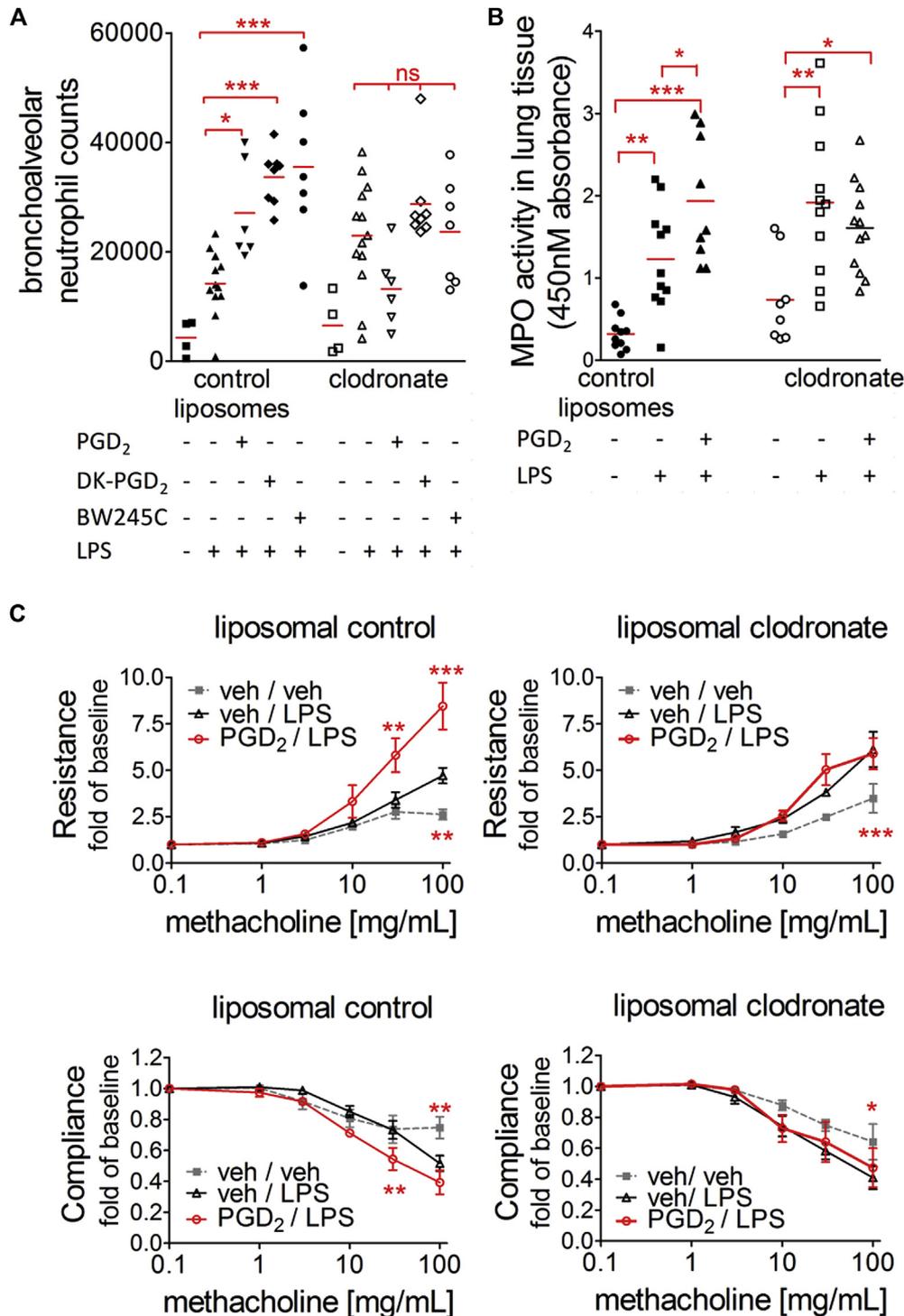
both cell types express DP<sub>1</sub> and DP<sub>2</sub> on their cell surface (Fig 1, A, and see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Interestingly, expression of both receptors increased during differentiation of MDMs, with DP<sub>2</sub> expression being more abundant than DP<sub>1</sub> expression in both monocytes and macrophages (see Fig E1). Next, we probed for DP<sub>1</sub> and DP<sub>2</sub> expression in human lung tissue with different pathologies (see Table E1 in this article's Online Repository at [www.jacionline.org](http://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 DP<sub>1</sub> and DP<sub>2</sub> independent of the underlying disease (see Figs E2 and E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). This expression pattern suggested a possible role for the PGD<sub>2</sub>-DP<sub>1</sub>-DP<sub>2</sub> axis in the modulation of macrophage biology in patients with pulmonary diseases.

Then we sought to determine the biological activities of DP<sub>1</sub> and DP<sub>2</sub> *in vitro*. Ca<sup>2+</sup> imaging revealed that superfusion of human MDMs with 1  $\mu$ mol/L PGD<sub>2</sub> 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](http://www.jacionline.org)), whereas human monocytes showed no Ca<sup>2+</sup> response to PGD<sub>2</sub> stimulation (see Fig E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Both DP<sub>2</sub>- and DP<sub>1</sub>-selective agonists (13,14-dihydro-15-keto PGD<sub>2</sub>, and BW245C, respectively; 1  $\mu$ mol/L) were likewise

able to elicit Ca<sup>2+</sup> flux, although to a lesser extent than PGD<sub>2</sub> (Fig 1, C, and see Fig E4, A). In agreement with this observation, blockade of either DP<sub>2</sub> (CAY10471, 10  $\mu$ mol/L) or DP<sub>1</sub> (MK0524, 10  $\mu$ mol/L) slightly decreased, whereas simultaneous antagonism of both receptors abolished the PGD<sub>2</sub>-induced Ca<sup>2+</sup> signal (see Fig E4, B and C). Ca<sup>2+</sup> release induced by both PGD<sub>2</sub> and 13,14-dihydro-15-keto prostaglandin D<sub>2</sub> (DK-PGD<sub>2</sub>) was prevented by overnight incubation with pertussis toxin (100 ng/mL), indicating involvement of G $\alpha$ i heterotrimers in this process (see Fig E4, D and E).

### PGD<sub>2</sub> induces migration of macrophages through DP<sub>1</sub> and DP<sub>2</sub> and enhances TNF- $\alpha$ secretion

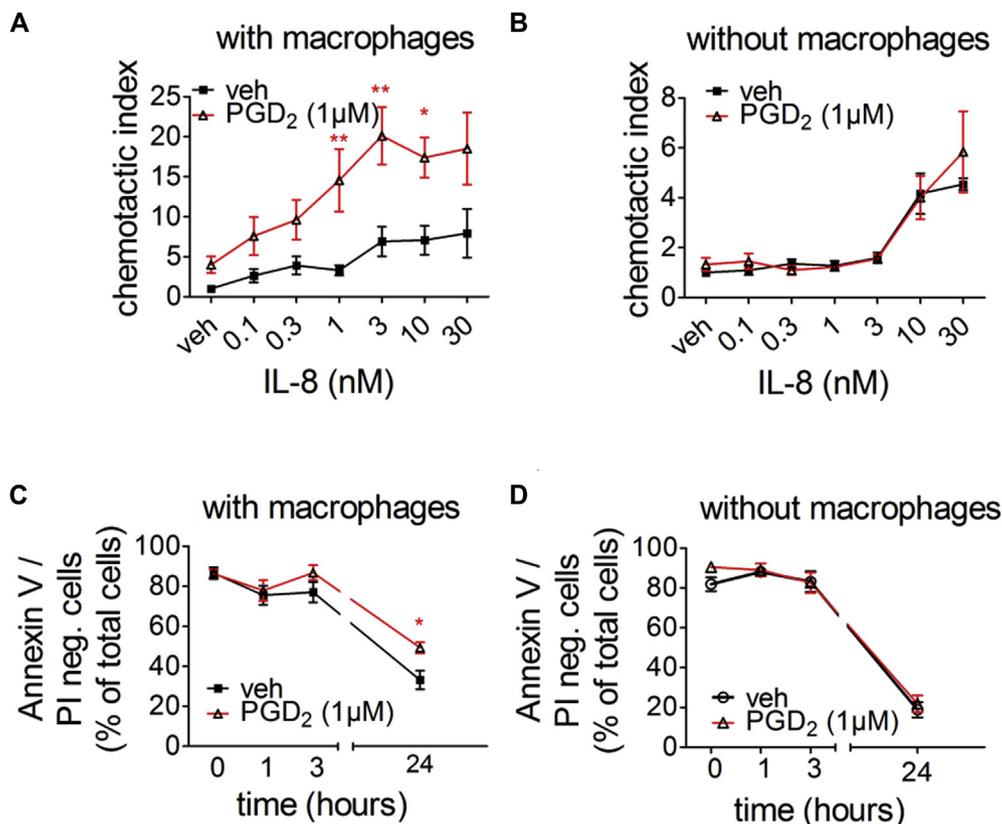
We next evaluated the chemotactic potential of PGD<sub>2</sub> toward MDMs by using Transwell inserts, followed by enumeration of fluorescently labeled cell nuclei of the migrated cells on the lower surface of the filter (see Fig E6, B). We found that PGD<sub>2</sub> exerted a concentration-dependent 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](http://www.jacionline.org)). This could be partially blocked by either the DP<sub>2</sub>- or DP<sub>1</sub>-specific antagonist (CAY10471 or MK0524, 1  $\mu$ mol/L, respectively; Fig 1, D). Inhibition of both receptors completely abolished the migratory



**FIG 4.** Macrophage depletion prevents the proinflammatory effect of PGD<sub>2</sub> 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 PGD<sub>2</sub> (n = 6-8). \**P* < .05, \*\**P* < .01, and \*\*\**P* < .001 versus vehicle/LPS.

activity of MDMs toward PGD<sub>2</sub> (Fig 1, D). Because PGD<sub>2</sub> was shown to be critical in mediating macrophage migration toward LPS in a murine model,<sup>39</sup> we hypothesized that PGD<sub>2</sub> could alter the LPS-induced cytokine secretion from MDMs. Although almost no TNF- $\alpha$  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 PGD<sub>2</sub> or DK-PGD<sub>2</sub> (see Fig E6, C). Although the DP<sub>1</sub>-specific agonist BW245C was not able to induce changes in TNF- $\alpha$  secretion, involvement of both DP<sub>1</sub> and DP<sub>2</sub> receptors in the PGD<sub>2</sub> response is still likely because only antagonism of both



**FIG 5.** PGD<sub>2</sub> 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 PGD<sub>2</sub>-mediated increase in TNF- $\alpha$  secretion.

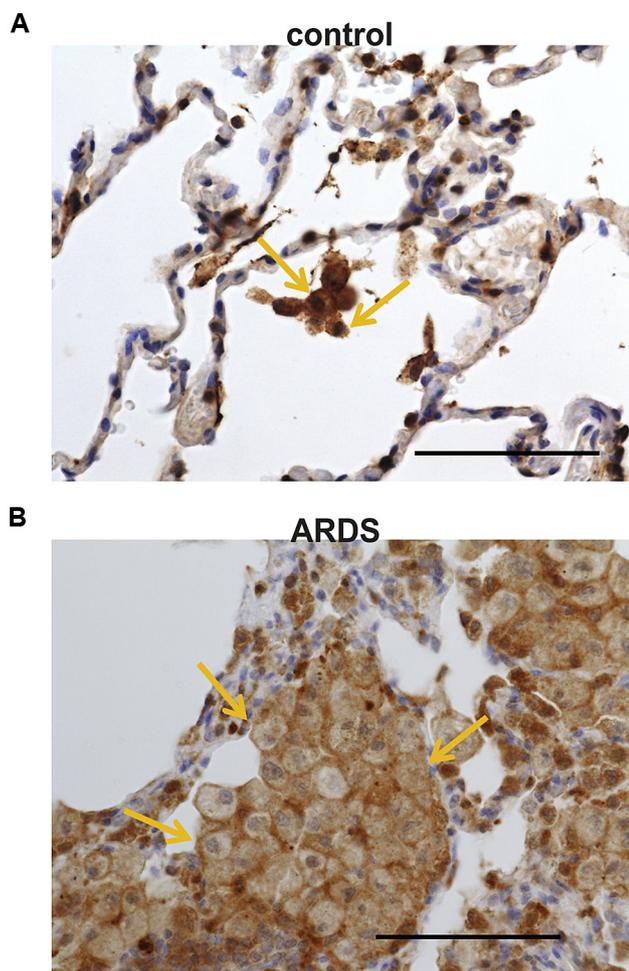
### Endotoxin-induced lung injury is aggravated by PGD<sub>2</sub>

Prompted by these observations, we investigated the effect of PGD<sub>2</sub> in an *in vivo* model of pulmonary inflammation. Here neutrophil infiltration is a main pathogenic feature that essentially depends on macrophage function.<sup>23</sup> LPS significantly increased neutrophil influx into the bronchoalveolar space after 4 hours of treatment. Of particular interest, this effect was almost doubled when PGD<sub>2</sub> (5 mg/kg) was administered subcutaneously before LPS challenge (Fig 2, A). Selective DP<sub>1</sub> and DP<sub>2</sub> agonists induced an even more pronounced response; in detail, the selective DP<sub>2</sub> agonist DK-PGD<sub>2</sub> (5 mg/kg) enhanced neutrophil influx by 2.5-fold and the DP<sub>1</sub> 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 PGD<sub>2</sub> 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](http://www.jacionline.org)). MPO activity, a marker for lung inflammation, was likewise increased depending on the applied dose of PGD<sub>2</sub> (Fig 2, D). These observations were

reflected by increased protein content in the BAL fluid in PGD<sub>2</sub>-treated animals (see Fig E7, B), as well as enhanced Evans blue extravasation in the lung tissue that depended on both DP<sub>1</sub> and DP<sub>2</sub> (Fig 2, E). It is noteworthy that the capacity of PGD<sub>2</sub> 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](http://www.jacionline.org)). The observation that PGD<sub>2</sub> 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 PGD<sub>2</sub> and LPS (Fig 2, F). In all experimental readouts, including cell infiltration into the alveolar space, MPO activity, and lung function, PGD<sub>2</sub> treatment in the absence of LPS had no effect (data not shown).

### Blocking of endogenous PGD<sub>2</sub> reduces neutrophilic infiltration into the lungs

Mice were pretreated with the PGD<sub>2</sub> receptor antagonists MK0524 or CAY10471 (5 mg/kg) followed by LPS challenge to delineate the pathogenic role of endogenous PGD<sub>2</sub>. Analysis of BAL cells revealed that the DP<sub>1</sub> antagonist MK0524 reduced endotoxin-induced neutrophilia (Fig 3, A), whereas the DP<sub>2</sub> 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  $\mu$ 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 PGD<sub>2</sub> levels in the latter (Fig 3, C). In agreement with previous findings, PGE<sub>2</sub> levels also increased, as did TXB<sub>2</sub> and 12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid levels. We could not detect any significant changes in PGI<sub>2</sub> levels (estimated through its metabolite 6-keto-PGF<sub>1 $\alpha$</sub> ).

### PGD<sub>2</sub> treatment enhances proinflammatory cytokine release *in vivo* and *in vitro*

In addition to prostanoids, 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 PGD<sub>2</sub>-treated animals (see Fig E9, A, in this article's Online Repository at [www.jacionline.org](http://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 PGD<sub>2</sub> (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 PGD<sub>2</sub> treatment (see Fig E9, B and C).

### Macrophage depletion prevents the increased inflammatory response mediated by PGD<sub>2</sub>

To highlight the role of macrophages in the PGD<sub>2</sub>-induced enhancement of pulmonary inflammation, we next selectively reduced alveolar macrophage counts in the pulmonary LPS model. Intranasal application of 300  $\mu$ 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](http://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 PGD<sub>2</sub> or any of the specific agonists for DP<sub>1</sub> and DP<sub>2</sub>. 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 PGD<sub>2</sub>-, DK-PGD<sub>2</sub>-, or BW245C-treated animals (Fig 4, A, and see Fig E11 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Concomitant to decreased alveolar neutrophil counts, the PGD<sub>2</sub>-induced increase in MPO activity, as well as compromised lung function, were reversed by alveolar macrophage reduction (Fig 4, B and C).

### PGD<sub>2</sub> receptor activation on macrophages modulates neutrophil function

*In vitro* neutrophil migration assays were performed in the presence of human MDMs treated with vehicle or PGD<sub>2</sub> to further clarify the mechanisms through which PGD<sub>2</sub> receptor activation on macrophages augmented the LPS-induced neutrophil recruitment. In this coculture experiment PGD<sub>2</sub>-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 PGD<sub>2</sub> treatment of MDMs enhanced the basal migratory capacity and IL-8-induced migration of neutrophils alike. Importantly, neutrophil migration was unaffected by PGD<sub>2</sub> when macrophages were absent (Fig 5, B). Therefore PGD<sub>2</sub> 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 PGD<sub>2</sub> 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 PGD<sub>2</sub> (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](http://www.jacionline.org)). Furthermore, neutrophils cultivated without macrophages did not react to PGD<sub>2</sub> and showed the same survival rate as in the presence of vehicle (Fig 5, D). These data show that PGD<sub>2</sub> receptor activation on macrophages profoundly influences neutrophil function by enhancing their migratory capacity and survival.

### Cells expressing hematopoietic PGD<sub>2</sub> 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 PGD<sub>2</sub>, 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 PGD<sub>2</sub> and its corresponding receptors in pulmonary inflammation. Here, for the first time, we show that human macrophages express active DP<sub>1</sub> and DP<sub>2</sub>, 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 PGD<sub>2</sub> receptors on monocytes,<sup>29</sup> but we also showed an upregulation that was more pronounced in the expression of DP<sub>2</sub> than DP<sub>1</sub> during differentiation to macrophages. Furthermore, we were able to demonstrate that both PGD<sub>2</sub> receptors are functional on human macrophages: not only did macrophages themselves migrate toward PGD<sub>2</sub>, 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 PGD<sub>2</sub> stimulation of macrophages prompted by DP<sub>1</sub>/DP<sub>2</sub>-mediated Ca<sup>2+</sup> flux.

Previous reports suggested that DP<sub>1</sub>- and DP<sub>2</sub>-mediated actions oppose each other, being anti-inflammatory and proinflammatory, respectively.<sup>10,40</sup> Such divergent actions of PGD<sub>2</sub> have not been observed here because we could show that on human MDMs, selective activation of both receptors increases intracellular free Ca<sup>2+</sup> levels and induces migration. Furthermore, these responses were only partially inhibited by either selective antagonist, and only blockade of DP<sub>1</sub> along with DP<sub>2</sub> was sufficient to abolish the PGD<sub>2</sub>-induced activation of macrophages. In this respect PGD<sub>2</sub> 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,<sup>41</sup> and bronchial epithelial cells, which express DP<sub>2</sub> but not DP<sub>1</sub>.<sup>11</sup>

We also observed DP<sub>1</sub> and DP<sub>2</sub> 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 PGD<sub>2</sub>-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,<sup>42</sup>

our finding proposes a new important role for PGD<sub>2</sub> in the regulation of macrophages in the lung.

Next, we investigated the biological role of PGD<sub>2</sub> in lung pathophysiology *in vivo* and used a murine, LPS-induced lung injury model that relies largely on functional macrophages.<sup>22,23</sup> As predicted by our *in vitro* data, systemic application of PGD<sub>2</sub>, acting through both DP<sub>1</sub> and DP<sub>2</sub>, 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 PGD<sub>2</sub> enhances neutrophilic inflammatory responses in the presence of disease-activating triggers. In parallel with increased levels of PGD<sub>2</sub> in the BAL fluid of LPS-treated animals, TXB<sub>2</sub> levels also increased. This upregulation of the TX pathway can further substantiate our finding of DP<sub>2</sub> activation in pulmonary inflammation because we previously identified the TX metabolite 11-dehydro-TXB<sub>2</sub> as a full agonist for DP<sub>2</sub>.<sup>43</sup> Finally, we provided evidence that these PGD<sub>2</sub> 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 PGD<sub>2</sub> 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,<sup>22</sup> and both seemed to respond to PGD<sub>2</sub> when KC secretion was analyzed. Human cell studies, which we conducted in parallel, identified 2 novel mechanisms of how PGD<sub>2</sub> receptor activation on macrophages can regulate neutrophil function, namely enhanced migratory capacity and survival of the latter. A recent study showed that activated T<sub>H</sub>2 cells in response to PGD<sub>2</sub> treatment react with secretion of IL-6 and GM-CSF at levels that are able to modulate neutrophil functions.<sup>44</sup> Our data strongly suggest that this mechanism is not restricted to T<sub>H</sub>2 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 PGD<sub>2</sub>. Blocking of endogenous PGD<sub>2</sub> either by DP<sub>1</sub>- or DP<sub>2</sub>-specific antagonists ameliorated the inflammatory response, although in a differential manner. On the one hand, the DP<sub>1</sub>-specific antagonist MK0524 decreased neutrophilia in the bronchoalveolar space. On the other hand, the DP<sub>2</sub>-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 DP<sub>2</sub> regulating neutrophil recruitment from the blood to the tissue and DP<sub>1</sub> promoting alveolar evasion of neutrophils. Along with the increase in PGD<sub>2</sub> levels in the BAL fluid, these observations clearly highlight endogenous PGD<sub>2</sub> in the development of experimental pulmonary inflammation.

Recently, it was shown that mice lacking the DP<sub>1</sub> receptor display aggravated neutrophil influx and increased mortality in experimental ALI.<sup>45</sup> 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, DP<sub>1</sub> was knocked out unconditionally, so that mice might have experienced compensational mechanisms, such as an upregulation of

DP<sub>2</sub> 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 PGD<sub>2</sub>-DP<sub>1</sub>-DP<sub>2</sub> 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 PGD<sub>2</sub> receptors in macrophage regulation, recently developed dual DP<sub>1</sub>/DP<sub>2</sub> antagonists<sup>46</sup> appear to be a promising approach to treating distinct inflammatory diseases that involve macrophage activation and neutrophil accumulation in the lung.

### Key messages

- DP<sub>1</sub> and DP<sub>2</sub> are expressed and functionally active on isolated MDMs and pulmonary macrophages.
- PGD<sub>2</sub> receptor activation of macrophages promotes their interaction with neutrophils and enhances neutrophil function.
- The presence of hematopoietic PGD synthase, DP<sub>1</sub>, and DP<sub>2</sub> in macrophages highlights a novel role for PGD<sub>2</sub> in pulmonary inflammation.

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