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*J Immunol* published online 2 June 2010
http://www.jimmunol.org/content/early/2010/06/02/jimmunol.0904042

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**Supplementary Material**  [http://www.jimmunol.org/content/suppl/2010/06/01/jimmunol.0904042.DC1](http://www.jimmunol.org/content/suppl/2010/06/01/jimmunol.0904042.DC1)

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Purinergic Receptor Inhibition Prevents the Development of Smoke-Induced Lung Injury and Emphysema

Sanja Cicko,*1 Monica Lucattelli,†1 Tobias Müller,*1 Marek Lommatzsch,‡ Giovanna De Cunto,‡ Silvia Cardini,† William Sundas,† Melanie Grimm,* Robert Zeiser,§ Thorsten Dürk,* Gernot Zissel,* Jean-Marie Boeynaems,* Stephan Sorichter,* Davide Ferrari,¶ Francesco Di Virgilio,§ J. Christian Virchow,* Giuseppe Lungarella,*1 and Marco Idzko*1

Extracellular ATP acts as a “danger signal” and can induce inflammation by binding to purinergic receptors. Chronic obstructive pulmonary disease is one of the most common inflammatory diseases associated with cigarette smoke inhalation, but the underlying mechanisms are incompletely understood. In this study, we show that endogenous pulmonary ATP levels are increased in a mouse model of smoke-induced acute lung inflammation and emphysema. ATP neutralization or nonspecific P2R-blockade markedly reduced smoke-induced lung inflammation and emphysema. We detected an upregulation of the purinergic receptors subtypes on neutrophils (e.g., P2Y2R), macrophages, and lung tissue from animals with smoke-induced lung inflammation. By using P2Y2R deficient (−/−) animals, we show that ATP induces the recruitment of blood neutrophils to the lungs via P2Y2R. Moreover, P2Y2R deficient animals had a reduced pulmonary inflammation following acute smoke-exposure. A series of experiments with P2Y2R−/− and wild type chimera revealed that P2Y2R expression on hematopoietic cell plays the pivotal role in the observed effect. We demonstrate, for the first time, that endogenous ATP contributes to smoke-induced lung inflammation and then development of emphysema via activation of the purinergic receptor subtypes, such as P2Y2R. The Journal of Immunology, 2010, 185: 000–000.

C hronic obstructive pulmonary disease (COPD) is one of the leading causes of morbidity and mortality worldwide (1). COPD is associated with a pulmonary inflammation and obstruction, particularly of the small airways, and the destruction of lung parenchyma resulting in emphysema (2). Epidemiologically, inhalation of cigarette smoke is the main risk factor for the development of COPD. However, the precise mechanisms that initiate and perpetuate COPD and the underlying inflammation are poorly understood (3). The major effector cells present in the lungs of patients with COPD are neutrophils, macrophages, CD4+ and CD8+ T lymphocytes, which are capable of releasing various mediators (including reactive oxygen metabolites and proinflammatory cytokines), and tissue degrading enzymes (e.g., neutrophil elastase or matrix metalloproteinases) that contribute to tissue destruction, emphysema formation, and chronic inflammation (4, 5).

Recently, extracellular ATP, which activates purinergic receptors belonging to the P2Y family (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) (6, 7) or the P2X family (P2X1–P2X7) (8–10), has gained attention as a mediator of inflammation (11, 12). Under physiologic conditions, extracellular concentrations of ATP are low and tightly regulated by ectonucleotidases, which dephosphorylate ATP to ADP, AMP, and adenosine. However, under conditions such as hypoxia, trauma, infection, or inflammation, extracellular concentrations of ATP are elevated, because of active or passive release from a number of cell types including airway epithelial cells and inflammatory cells (11, 13) and/or because of a concomitant downregulation of nucleotidases (14, 15). ATP modulates mucin secretion and mucociliary clearance in human lungs. The activation of the P2Y2 subtype improves mucociliary clearance and clinical trials using P2Y2 agonists are in progress (16, 17). ATP can also modify the recruitment and function of inflammatory cells, such as neutrophils (18–22), macrophages (23–25), lymphocytes, and dendritic cells (13, 26–29). Finally, we recently demonstrated that endogenous ATP plays a role in the pathogenesis of acute asthmatic airway inflammation, where it has been shown to activate dendritic cells (30).

In this study, we provide in vivo evidence that ATP is linked to the pathogenesis of smoke-induced lung emphysema. We demonstrate that ATP levels are elevated in the lungs of mice with smoke-induced pulmonary inflammation and emphysema and that these ATP levels correlate with pulmonary neutrophilia. During the preparation of this article, Mortaz et al. (31) reported increased ATP levels in the bronchoalveolar lavage fluid (BALF) of animals with emphysema, suggesting that ATP might be involved in the pathogenesis of COPD. However, the effects of increased ATP levels in the
development of lung emphysema are still unknown. In this study, we show for the first time that neutralizing intrapulmonary ATP levels or blocking airway P2 receptors inhibits smoke-induced lung inflammation and confers protection from the development of emphysema. Moreover, in animals with smoke-induced lung injury we observed a specific upregulation of the P2Y2R on blood and lung neutrophils and macrophages. The upregulation of the P2Y2R on neutrophils is of pathogenetic relevance, because ATP induces migration of neutrophils into the lungs of naive mice by this receptor subtype. Using P2Y2R deficient (−/−) and wild type (wt) chimera animals, we provide evidence that the expression of P2Y2R on hematopoietic cells contributes to the observed effects.

Materials and Methods

Mice

C57Bl/6 mice (6–8 wk old) were purchased from Charles River Laboratories (Calco, Italy) or were bred at the animal facilities at the University Hospital of Freiburg or at the University of Siena. P2Y2 receptor deficient mice (P2Y2−/−) on a C57Bl/6 background were generated as previously described (32). All experiments were performed according to institutional guidelines of the animal ethics committee of the Italian or German governments, respectively.

Generation of bone marrow chimera

WT or P2Y2R−/− recipients (both C57BL/6) were given 5 × 105 wt or P2Y2R−/− bone marrow cells (C57BL6/i) iv. after lethal irradiation with 900 cGy (2 × 450 cGy). The following donor–recipient pairs were combined: wt → wt, P2Y2R−/− → wt (hematopoietic system: P2Y2R−/−), wt → P2Y2R−/− (nonhematopoietic system: P2Y2R−/−), P2Y2R−/− → P2Y2R−/−.

Lung inflammation induced by acute smoke exposure

Mice were exposed to whole smoke of five cigarettes (commercial Virginia filter cigarettes [Marlboro Red, Philip Morris, Munich, Germany]; 12 mg tar, 0.9 mg nicotine) or room air for 20 min on three consecutive days in specially designed Makrolon cages (Tecniplast, Buguggiate, Italy), as previously described (33). The smoke was produced by burning a cigarette and was introduced into the chamber with the airflow generated by a mechanical ventilator (7025 Rodent Ventilator, Ugo Basile; Biological Research Instruments, Comerio, Italy), at a rate of 250 ml/min. A second mechanical ventilator was used to provide room air for dilution (1:8) of the smoke stream.

In some experiments, intratracheal treatment was performed prior to the smoke exposure: animals were anesthetized by ip. injection of ketamine-xylazine and received an intratracheal injection of the indicated compounds in a total volume of 80 μl. One hour after the last smoke challenge, mice were sacrificed and bronchoalveolar lavage (BAL) was performed.

To study the effect of the P2R-antagonist in the ongoing smoke-induced lung inflammation, mice were exposed to smoke on the days 1–3. Next, they were randomized to receive either vehicle or P2R-antagonists (suramin or pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid [PPADS]) intratracheally 30 min before each of a series of three smoke or air challenges given at days 4–6. At days 3–6, 1 h after the last smoke or air challenge, mice were killed and the BALF was collected.

Chronic exposure to cigarette smoke

The methodology for chronic smoke exposure has been described previously (33). Mice were exposed to either the smoke of three cigarettes per day, 5 per week, or room air (controls) for 4, 6, or 7 mo, respectively. When indicated, animals received an oral treatment by gavage with either suramin (1.6 mg/kg) or PPADS (0.6 mg/kg) 45 min prior to smoke exposure. At the end of the respective time period, animals were sacrificed; BALF was performed, followed by lung resection and fixation in formalin for histologic analysis.

Migration assay in vivo

To study neutrophil migration to the lungs, ATP, the stable analog ATPγS, or vehicle were administered intratracheally to wt and P2Y2R−/− mice. Twenty-four to 36 h after injection, the total number of neutrophils in the BALF was analyzed by FACs.

BAL in animals

BALF was collected by cannulating the trachea under deep pentobarbital anesthesia and washing the lung three times with 1 ml PBS containing 0.1 mM EDTA, as described previously (30). Total cell numbers were counted, and differential cell counts were performed on cytopsin preparations after staining with Diff-Quick (Medion Diagnostics, Dudingen, Switzerland). Differential cell counts were made on >200 cells using standard morphologic criteria and FACS analysis.

Isolation of blood neutrophils

Blood neutrophils from smoke- and air-exposed animals were isolated as previously reported (21).

Flow cytometry and sorting

After counting and washing, BALF cells were stained for 30 min with anti-MHC class-II (macrophages/DC), anti-7/4 (Calget) FITC (neutrophils; eBioscience, San Diego, CA) in PBS containing 0.5% BSA and 0.01% sodium azide. Differential cell counts were analyzed by flow cytometry, as described previously (30, 34).

For the sorting of BAL neutrophils and BAL macrophages for real-time PCR, BALF cells were stained for 30 min with anti-CD68 PE (macrophages), anti-7/4/Calget FITC (neutrophils), anti-CD3 and anti-CD19 cy-chrome (lymphocytes), and anti-I-Ad/I-Ed APC (macrophages/DCs) in PBS containing 0.5% BSA and 0.01% sodium azide. In all experiments, dead cells were excluded from analysis using propidium iodide. Analysis was performed on a FACSScalibur flow cytometer (BD Biosciences, San Jose, CA) using Cellquest version 3.3 (BD Biosciences) and FlowJo version 3.3

FIGURE 1. Functional analysis of BALF-ATP concentrations following acute smoke-induced lung inflammation. A, Male C57BL/6 mice were left either untreated or exposed to the smoke of five cigarettes on three consecutive days. One hour after the last smoke exposure, the animals were killed and BALF was collected and analyzed for its ATP content. Data from one representative experiment of four are shown. Data are shown as mean ± SEM. n = 5 mice in each group. ***p < 0.001. B, Correlation between BALF-ATP levels and BALF-neutrophils in animals with acute smoke-induced lung inflammation (r = 0.85; p = 0.00019). Data from three experiments with n = 5 mice in each group are shown. C, Male C57BL/6 mice were left either untreated or exposed to the smoke of five cigarettes on three consecutive days. Thirty minutes before smoke exposure, animals received an intratracheal injection of vehicle, denatured apyrase, or apyrase (4 U/ml; 80 μl). One hour after the last smoke exposure, the animals were killed and BALF was collected and analyzed for the number and distribution of cells and (D) cytokine content. Data from one representative experiment of four are shown. Data are shown as mean ± SEM. n = 5 mice in each group. *p < 0.001, vehicle–air exposed versus vehicle or denatured apyrase–smoke-exposed animals; *p < 0.01, vehicle or denatured-apyrase–treated versus apyrase-treated smoke-exposed animals.
6.4.7 (TreeStar, Ashland, OR) software. The purity of the sorted BAL neutrophils was >98% and was <98% for the BAL macrophages.

**ATP measurements in BAL fluid**

To measure ATP concentrations in BALF in mice, fresh BALF supernatants were used, according to manufacturer’s instructions (ATPlite Assay; PerkinElmer, Wellesley, MA), but without the cell lysis step to avoid any contaminating intracellular ATP, as described previously (30).

**Cytokine measurements**

Cytokine concentrations in BALF were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s recommendations.

**Real-time RT-PCR**

Total RNA was isolated from cell pellets or homogenized lung tissue, using the RNeasy Mini-Kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations. Reverse transcription was performed using Stratascript reverse transcriptase (Stratagene, La Jolla, CA) and random primers (Invitrogen, Karlsruhe, Germany). Quantitative PCR was performed with Taqman Universal PCR Mastermix (Applied Biosystems, Foster City, CA) and preformulated primers and probe mixes (Assay on Demand; Applied Biosystems). PCR conditions were 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 60°C for 1 min using a thermal cycler (iCycler; Bio-Rad, Hercules, CA). PCR amplification of the housekeeping gene encoding GADPH was performed during each run for each sample to allow normalization between samples.
Lung morphology and morphometry

At 4 and 7 mo after chronic exposure to room air or cigarette smoke, 8–12 animals of each group were sacrificed and the lungs were fixed intratracheally with formalin (5%) at a pressure of 20 cm H2O. Lung volume was measured by water displacement. All lungs were then dehydrated, cleared in toluene, and embedded under vacuum in paraffin. Two 7-μm transversal sections were made and stained with H&E. Two pathologists blinded to the exposure protocol performed morphologic and morphometric evaluation. Morphometric assessment included determination of the mean linear intercept (Lm), which represents the average size of air space (alveolar ducts, alveolar sacs, and alveoli), and of the internal surface area of the lungs (ISA). Lm is the length of a test line placed over histologic slides of the lung, divided by the number of times the line crosses alveolar walls (not surfaces). Fields with bronchi, large bronchioli, or blood vessels were excluded from the measurements. Lm values were used to calculate the ISA, necessary for evaluating the degree of emphysema, from the equation 4VLm, where V is the postfixation lung volume. For the determination of the Lm for each pair of lungs, 40 histologic fields were evaluated both vertically and horizontally. Examination of this many fields meant that practically the entire lung area was evaluated.

The immunohistochemical analysis for the detection of CD4+ T cells, IFN-γ, and IL-13–producing T cells, was performed as reported below. Sections were incubated with 3% BSA for 30 min at room temperature, to block nonspecific Ab binding, and then exposed to rat monoclonal anti-mouse CD4-Ab (Abcam, Cambridge, U.K.), hamster monoclonal anti-mouse IFN-γ Ab (R&D Systems) or goat Ab to mouse IL-13 (R&D Systems) overnight. Next, sections were rinsed and incubated with goat polyclonal anti-rat biotinylated IgG (Abcam), or with mouse anti-hamster biotinylated IgG (BD Pharmingen, Franklin Lakes, NJ) for the detection of CD4 and IFN-γ or with anti-goat IgG (Sigma-Aldrich, Milan, Italy) for IL-13. The staining was revealed by adding Streptavidin-HRP (BD Pharmingen, Bucinasso, Italy) for CD4 and IFN-γ or peroxidase-peroxidase complex (Sigma-Aldrich) prepared from goat serum for IL-13 respectively. Detection was accomplished by incubation in diaminobenzidine freshly dissolved in 0.03% H2O2 in 50 mM Tris/HCl (pH 7.6). As negative controls for the immunostaining, the primary Ab was replaced with nonimmunized serum.

The volume fractions of the immunopositive cells were determined by point counting, using a grid with 48 points, a ×20 objective and a computer screen for a final magnification of ×580. Twenty fields were examined for each pair of lungs, for a total of 960 points. Next, the number of stained points counting, using a grid with 48 points, a

To better reflect the natural course of COPD, we used a model of chronic smoke-induced lung inflammation characterized by the development of pulmonary emphysema. ATP concentrations were increased in BALF from animals chronically exposed to cigarette smoke-induced lung inflammation (Fig. 2A). Because repeated anesthesia (3 d/wk for 4 mo) for intratracheal treatment would have been fatal for the animals, oral treatment with the antagonists suramin and PPADS was used. Prior to this experiment, the relative potency of intratracheal versus oral treatment in the acute model of smoke-induced lung inflammation had to be compared. Oral treatment required higher concentrations of suramin and PPADS to reach a comparable reduction in lung inflammation (Supplemental Fig. 1). Lungs of mice exposed to room air for 4 and 7 mo showed normal parenchyma and airways (Fig. 3Ba, 3Bc), whereas mice exposed for 4 and 7 mo to cigarette smoke showed foci of emphysema disseminated throughout the lung parenchyma (Fig. 3Bb, 3Bd). Oral treatment with suramin (1.6 mg/kg) or PPADS (0.6 mg/kg) prevented the development of emphysema in cigarette smoke-exposed mice. These mice showed normal lung architecture (Fig. 3Be, 3Bf). Morphometric assessment was performed as previously described (36). The results of the various groups are given in Table I. After 4 and 7 mo of smoke exposure, the average interalveolar distance (i.e., Lm) was significantly increased, and the ISA was significantly decreased compared with the respective air-exposed group. These effects were not seen after suramin or PPADS treatment. The reduction of lung inflammation after treatment with suramin or PPADS was accompanied by lower intrapulmonary ATP levels (data not shown). In addition, we also analyzed the number of CD4-positive cells and the T cells producing IL-13 and IFN-γ in the lungs of the animals exposed to cigarette smoke and treated or not with PPADS or suramin. As shown in Table II, the CD4+ T lymphocytes, which were mainly found perivascularly, in the airways and in the lymphoid follicles were increased in the lungs of the mice exposed to cigarette smoke by 670% (p < 0.05).Treatment of smoke-exposed animals with suramin and PPADS significantly

P2R-inhibition prevents the development of emphysema in a model of chronic smoke-induced lung inflammation

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reduced the increase in CD4+ cell by 50.7 and 47.8% (p < 0.05), respectively. As expected, chronic smoke exposure led to an increase by 700% of IFN-\(\gamma\)-positive cells (e.g., CD4+, CD8+, plasmacytoid dendritic cells, B220+ cells, B cells) and by 666% of IL-13–positive cells (a cytokine produced by CD4+, NK, mast cells, macrophages) compared with air-exposed animals. Treatment with PPADS or suramin during chronic smoke exposure significantly decreased the number of IFN-\(\gamma\)–positive cells by 58.6% and 54%, respectively (p < 0.05), and the number of IL-13–producing cells by 45 and or 30%, respectively (p < 0.05).

Table I. Morphometric assessment of the effects of P2 receptor antagonists on the progression of the emphysematous changes caused by cigarette smoke exposure

<table>
<thead>
<tr>
<th>Groups</th>
<th>Exposure/Treatment 0–4 mo</th>
<th>Results at 4 mo</th>
<th>Exposure/Treatment 4–7 mo</th>
<th>Results at 7 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ln ((\mu)m)</td>
<td>ISA ((cm^2))</td>
<td>Ln ((\mu)m)</td>
<td>ISA ((cm^2))</td>
</tr>
<tr>
<td>Air/–</td>
<td>37.2 ± 0.8 (8)</td>
<td>1161 ± 37 (8)</td>
<td>38.6 ± 0.9 (8)</td>
<td>1248 ± 37 (8)</td>
</tr>
<tr>
<td>Air/– Air/Vehicle</td>
<td>40.4 ± 1.2* (8)</td>
<td>1025 ± 41* (8)</td>
<td>42.1 ± 1.2** (8)</td>
<td>1103 ± 51**(8)</td>
</tr>
<tr>
<td>CS/–</td>
<td>39.1 ± 1.4*** (11)</td>
<td>1207 ± 79*** (11)</td>
<td>39.3 ± 1.5*** (12)</td>
<td>1191 ± 74*** (12)</td>
</tr>
</tbody>
</table>

*p < 0.05 versus air exposure (4 mo); **p < 0.05 versus air exposure (7 mo); ***p < 0.05 versus smoke exposure (7 mo).

Air, exposure to room air; CS, cigarette smoke.
Upregulation of purinergic receptors in animals with smoke-induced lung inflammation

To elucidate the regulation of purinergic receptors in acute smoke-induced lung inflammation, we analyzed the expression of purinergic receptor subtypes in blood neutrophils, BALF neutrophils, macrophages, and lung tissue after collection of the BALF. As shown in Fig. 4A, smoke exposure led to an upregulation of the P2X1, P2X4, P2X7 and P2Y2R subtypes in blood neutrophils, and of the P2X7R and P2Y2R subtypes in BALF neutrophils (Fig. 4B). In BALF macrophages, an upregulation could be observed for the P2X7R, P2Y2R and P2Y13R (Fig. 4C). In the lung tissue of animals with smoke-induced lung inflammation, an upregulation of the receptors P2Y4, P2Y6, P2Y13, P2X1, P2X4 and P2X7 was observed (Fig. 4D).

FIGURE 4. Expression of P2R subtypes on blood and BALF neutrophils, alveolar macrophages, and lung tissue in mice with acute smoke-induced lung inflammation. Male C57BL/6 mice were left either untreated or exposed to the smoke of five cigarettes on three consecutive days. One hour after the last smoke exposure, animals were killed and blood and BALF for the isolation of neutrophils and macrophages were collected and RNA was isolated from whole lung tissue. Relative expression of the different P2Y and P2X receptors compared with GAPDH in triplicates were analyzed using quantitative RT-PCR. A, Expression of P2Y and P2XR subtypes on pooled blood neutrophils from 5 per group. B, Expression of P2Y and P2XR subtypes on pooled BALF neutrophils from 10 animals for smoke exposure and 20 animals for air exposure. C, Expression of P2Y and P2XR subtypes on pooled BALF macrophages from 10 animals for smoke exposure and 20 animals for air exposure. D, Expression of P2Y and P2XR subtypes on lung tissue of smoke or air exposed animals. Data from one representative experiment of three are shown. Data are shown as mean ± SEM. n = 5 mice in each group. *p < 0.05.
Effect of ATP on the migration of neutrophils

Based on the observation of a close correlation between neutrophil numbers and ATP concentrations in BALF, we questioned whether ATP can enhance the recruitment of neutrophils into the lungs in vivo. Intratracheal treatment with ATP (100 μM) or non-hydrolysable stable ATP-analog ATPyS (100 μM) significantly enhanced the number of neutrophils in BALF compared with vehicle treatment alone (Fig. 5A).

To analyze whether the P2Y₂ receptor is involved, P2Y₂−/− mice were examined. Intratracheal application of the stable non-hydrolyzable ATP analog ATPyS failed to induce a recruitment of neutrophils to the lung P2Y₂−/− animals (Fig. 5B). The non-hydrolyzable ATPyS was used to exclude effects of the ATP-metabolite adenosine on cell migration, as reported previously (20, 21).

Role of P2Y₂R on the hematopoietic system in smoke-induced lung inflammation

To further elucidate the functional relevance of the upregulation of the P2Y₂R in smoke-induced lung inflammation, P2Y₂−/− mice were treated according to the acute smoke-induced lung inflammation model. P2Y₂−/− deficiency was associated with a decrease in smoke-induced lung inflammation (reduced numbers of neutrophils and macrophages; Fig. 6A) and lower levels of ATP, IL-6, IL-1β, KC, IFN-γ, and MIP-2 (Fig. 6B, 6C) in the BALF of these animals.

Finally, to discriminate between the relevance of P2Y₂R expression on the hematopoietic system and nonhematopoietic system, experiments with bone marrow chimera were performed. As shown in Fig. 6D and 6E, wt mice with a lack of P2Y₂R expression in the hematopoietic system displayed a significant decrease in lung inflammation, as evidenced by reduced numbers of neutrophils and macrophages in BALF (Fig. 6D) and reduced BALF concentrations of ATP, IL-6, IL-1β, KC, IFN-γ, and MIP-2 (Fig. 6D). In contrast, P2Y₂R−/− animals reconstituted with a wt hematopoietic system (wt → P2Y₂R−/−) showed no protection against smoke induced lung inflammation.

Discussion

The pathogenesis of smoke-induced lung inflammation and emphysema is still incompletely understood. In this study, we demonstrate that extracellular ATP accumulates in the airways of mice with acute smoke-induced lung inflammation and emphysema. ATP neutralization or unspecific purinergic blockade markedly reduced the inflammatory reaction to cigarette smoke and the development of smoke-induced lung inflammation and emphysema. Mechanistically, we provide evidence that the observed effects might involve the activation of the P2Y₂ receptor on neutrophils and macrophages. Thus, our findings suggest that extracellular ATP, via binding to purinergic receptors, such as P2Y₂R, is involved in the pathogenesis of some of the pathologic features of smoke-induced lung inflammation and emphysema.

In healthy tissues, extracellular ATP levels are regulated by a dynamic balance between ATP release and ATP degradation. ATP release occurs via several tightly controlled mechanisms, such as constitutive or stimulated exocytosis, membrane transporters, or nonselective channels, whereas ATP degradation is due to ubiquitous ectonucleotidases. Under physiologic conditions, the extracellular ATP concentration is kept in the low nanomolar range. However, tissue perturbation, as it occurs in the presence of injury, inflammation or cancer, may cause a massive accumulation of extracellular ATP (up to micromolar levels) (37). We found a strong increase of endobronchial ATP concentrations in mice with smoke-induced lung inflammation. These findings were recently confirmed by another group (31). The underlying reason for this increase in ATP concentrations is currently unclear, because it could be either smoke-induced ATP release or suppression of ATP-degrading ectonucleotidases. Indeed, ectonucleotidases have been shown to be downregulated by reactive oxygen species (ROS), bacterial endotoxin or cytokines, such as TNF-α (15, 38, 39). Of note, cigarette smoke contains high concentrations of ROS (40) and inflammatory cells such activated macrophages and neutrophils can also generate substantial amounts of ROS (41). Alternatively, direct toxic effects of the cigarette smoke on several cell populations could lead to an enhanced release of intracellular ATP into the extracellular space.

It has been hypothesized that the accumulation of extracellular ATP merely reflects damage and does not have pathogenetic implications in inflammation or tissue destruction. However, our data show that ATP neutralization or the inhibition of purinergic receptors can prevent smoke-induced lung inflammation by reducing neutrophil and macrophage infiltration and the release of proinflammatory cytokines, such as IL-1β, MIP-2, KC, IFN-γ, and IL-6 in BALF. A reduction of these parameters was also observed when purinergic signaling was blocked during ongoing inflammation. Thus, we provide evidence that ATP is actively involved in the pathogenesis of several features associated with smoke-induced lung inflammation. This finding is further supported by histomorphologic evidence indicating that inhibition of purinergic receptors interfered with the induction of cigarette smoke-induced emphysema. Interestingly, this effect was still observed in mice with established inflammation and emphysema. It has recently been reported that the lungs of patients with COPD show more CD4⁺ cells and an upregulated expression of Th1 cytokines, such as IFN-γ, and Th2 cytokines, such as IL-13 (3). However, the specific role of the adaptive immune system in the development of the disease is still a matter of discussion. Similarly, the role of the innate immunity in the development of cigarette smoke-induced emphysema has not been clarified. Of interest, P2R-blockade by suramin and PPADS also prevents the development of emphysema in smoke-exposed mice by reducing
the influx into the lungs of the inflammatory cells of both the innate and adaptive immune systems. With all the limitations of such animal models, these data support the hypothesis that purinergic signaling plays a role in the pathogenesis of COPD.

Inhibition of smoke-induced lung inflammation by blocking the ATP/P2R pathway could involve an alteration of neutrophil function, because ATP via binding to the P2Y2 receptor has strong chemotactic activity on neutrophils and other inflammatory cells (20, 21). Indirect effects of ATP on chemokines, such as IL-8 (42), might augment the influx of neutrophils into the lungs, as has been shown in a mouse model of sepsis (22, 43). In addition, ATP increases viability of human neutrophils by preventing apoptosis (44). In our study, exogenous administration of ATP to naive mice increases viability of human neutrophils by preventing apoptosis shown in a mouse model of sepsis (22, 43). In addition, ATP increases neutrophil recruitment by ATP activation. Neutrophil recruitment by ATP might be harmful per se, because neutrophils accelerate inflammation and alveolar destruction by secreting proteases, ROS, and proinflammatory cytokines (4, 5). However, ATP has also been shown to directly induce the secretion of ROS, elastase, and LTB4 (19, 47, 48). Thus, the proinflammatory effect of ATP might be due to enhanced neutrophil recruitment and enhanced neutrophil activation.

The hypothesis that P2Y2 receptor signaling contributes to the accumulation of neutrophils in the lungs is supported by our findings of strong upregulation of the P2Y2R expression on blood and BALF neutrophils in mice with smoke-induced lung inflammation. In addition, we demonstrate that P2Y2 receptor-deficient animals have reduced pulmonary inflammation after acute smoke exposure. Experiments with chimera P2Y2R-/- and wt animals revealed that expression of P2Y2R on hematopoietic cells might account for this effect, as P2Y2R-/- reconstituted with wt bone marrow did not show a reduction in smoke-induced lung inflammation compared with wt animals.

Macrophages have been suggested to play a pivotal role in the pathophysiology of COPD and can account for some of the features of the disease (5). ATP is a powerful macrophage stimulant that induces the secretion of cytokines (e.g., IL-6, IL-8, IL-1β, IL-18) of matrix metalloproteinase 9, the predominant elastolytic enzyme

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**FIGURE 6.** Reduced smoke-induced lung inflammation in P2Y2R-deficient animals. A–C: Male P2Y2 receptor−/− mice or wt animals were left either untreated or exposed to the smoke of five cigarettes on three consecutive days. One hour after the last smoke exposure, animals were killed and BALF was analyzed for (A) the number and distribution of cells, (B) ATP, and (C) cytokine content. Data are shown as mean ± SEM; n = 5 mice in each group: *p < 0.001, smoke versus air exposure; †p < 0.05, P2Y2R−/− smoke versus wt smoke. D and E. The different bone marrow chimera animals were exposed to the smoke of five cigarettes on three consecutive days. One hour after the last smoke exposure, animals were killed and BALF was analyzed for (D) the number and distribution of cells and (E) cytokine content. Data are shown as mean ± SEM; n = 5 mice in each group; *p < 0.05 P2Y2R−/− in P2Y2R−/− versus wt in wt; †p < 0.05 P2Y2R−/− in wt versus wt in wt.

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<table>
<thead>
<tr>
<th>Cell Type</th>
<th>P2Y2R+/+ /Air</th>
<th>P2Y2R−/− /Air</th>
<th>P2Y2R−/− /Smoke</th>
<th>P2Y2R+/+ /Smoke</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>480000 ± 12000</td>
<td>320000 ± 8000</td>
<td>160000 ± 4000</td>
<td>100000 ± 2000</td>
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<tr>
<td>Neutrophils</td>
<td>320000 ± 6000</td>
<td>240000 ± 4000</td>
<td>160000 ± 2000</td>
<td>120000 ± 1000</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>P2Y2R+/+ /Air</th>
<th>P2Y2R−/− /Air</th>
<th>P2Y2R−/− /Smoke</th>
<th>P2Y2R+/+ /Smoke</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>IL-1β</td>
<td>200 ± 20</td>
<td>150 ± 15</td>
<td>100 ± 10</td>
<td>75 ± 7</td>
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<tr>
<td>KC</td>
<td>400 ± 40</td>
<td>300 ± 30</td>
<td>200 ± 20</td>
<td>150 ± 15</td>
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<tr>
<td>IL-6</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
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<tr>
<td>MIP-2</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

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The graph shows the distribution of cell numbers and cytokine levels in different groups. The legend indicates the cell type and treatment conditions (air, smoke, wt, and P2Y2R−/−). The data are presented as mean ± SEM, with n = 5 mice in each group.
in patients with COPD (5), and the production of ROS in macrophages (13, 23, 29, 49, 50). Furthermore, extracellular ATP causes the release of IL-8 and IL-6 via activation of P2YR-subtypes from airway epithelial cells (42, 51, 52). Indeed, the involvement of other P2R subtypes in ATP-mediated pulmonary damage is supported by our finding that the administration of the unselective P2R-antagonist PPADS to P2Y<sub>1</sub> mice exposed to cigarette smoke led to a further reduction in smoke-induced pulmonary inflammation (data not shown). Because suramin and PPADS block several purinergic receptors (7, 9), the precise receptors that mediate the observed features of smoke-induced lung inflammation remain to be elucidated. The strong upregulation of the P2X<sub>1</sub>, P2X<sub>4</sub>, P2X<sub>7</sub> and P2Y<sub>6</sub> receptors in the lungs of mice with smoke-induced lung inflammation, and experiments using P2Y<sub>1</sub> mice suggest that this receptor subtype might be involved. Therefore, further studies are needed to identify the specific subtypes of particular relevance in the pathogenesis of COPD to develop selective purinergic receptor antagonists for the treatment or prevention of COPD.

These data suggest that enhanced endogenous ATP concentrations, via activation of purinergic receptors, such as P2Y<sub>2</sub>R, might be causally related to the pathogenesis of smoke-induced lung inflammation and might be a new target for therapy of COPD.

Disclosures

The authors have no financial conflicts of interest.

References


Supporting Table 1: Dose dependency of the anti-inflammatory effect of suramin and PPADS on acute smoke induced lung inflammation in vivo.

<table>
<thead>
<tr>
<th>i.t. application of</th>
<th>Macrophages (cells/BAL)</th>
<th>Neutrophils (cells/BAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>478825 ± 63593</td>
<td>42903 ± 9341</td>
</tr>
<tr>
<td>Suramin 100µM</td>
<td>135135 ± 19090***</td>
<td>7317 ± 1760***</td>
</tr>
<tr>
<td>Suramin 10µM</td>
<td>140588 ± 16603***</td>
<td>6893 ± 1704***</td>
</tr>
<tr>
<td>Suramin 1µM</td>
<td>278556 ± 45796**</td>
<td>22536 ± 4472*</td>
</tr>
<tr>
<td>PPADS 100µM</td>
<td>144469 ± 17156***</td>
<td>7570 ± 1552***</td>
</tr>
<tr>
<td>PPADS 10µM</td>
<td>154818 ± 15079 ***</td>
<td>7461 ± 1119***</td>
</tr>
<tr>
<td>PPADS 1µM</td>
<td>274782 ± 39556**</td>
<td>25149 ± 2863*</td>
</tr>
</tbody>
</table>

Supplementary Table 1: Dose dependency of the anti-inflammatory effect of suramin on smoke induced lung inflammation in vivo. On day 1-3 mice were exposed to smoke or air. 30 min before each exposure, mice received an i.t injection of vehicle or the indicated concentrations of suramin and PPADS. Number of cells in the BAL fluid was analyzed by flow cytometry. n =6-10 mice per group. Data are shown as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 P2R-antagonist treated vs. vehicle treated animals.
Supporting Figure 1

Supplementary Figure 1: The effect of oral or intrapulmonary application of the P2R antagonists Suramin and PPADS on smoke induce lung inflammation. Mice were exposed to cigarette smoke or air on days1-3. 30 min before each smoke exposure mice received an injection of Suramin, PPADS or vehicle via oral gavage or intrapulmonary. One hour after the last smoke exposure the animals were killed and number of cells in the BAL fluid was analyzed by flow cytometry. n =6-8 mice per group. Data are shown as mean±SEM. *P<0.01, #P<0.001 P2R-antagonist treated vs. vehicle treated animals.