**Streptococcus pneumoniae** inhibits purinergic signaling and promotes purinergic receptor P2Y$_2$ internalization in alveolar epithelial cells

Bacterial pneumonia is a global health challenge that causes up to 2 million deaths each year. Purinergic signaling plays a pivotal role in healthy alveolar epithelium. Here, we used flophore-based analysis and live-cell calcium imaging to address the question of whether the bacterial pathogen *Streptococcus pneumoniae* directly interferes with purinergic signaling in alveolar epithelial cells. Disturbed purinergic signaling might result in pathophysiologic changes like edema formation and atelectasis, which are commonly seen in bacterial pneumonia. Purine receptors are mainly activated by ATP, mediating a cytosolic calcium response. We found that this purinergic receptor P2Y$_2$-mediated response is suppressed in the presence of *S. pneumoniae* in A549 and isolated primary human alveolar cells in a temperature-dependent manner. Downstream inositol 3-phosphate (IP$_3$) signaling appeared to be unaffected, as calcium signaling via protease-activated receptor 2 remained unaltered. *S. pneumoniae*-induced suppression of the P2Y$_2$-mediated calcium response depended on the P2Y$_2$ phosphorylation sites Ser-243, Thr-344, and Ser-356, which are involved in receptor desensitization and internalization. Spinning-disk live-cell imaging revealed that *S. pneumoniae* induces P2Y$_2$ translocation into the cytosol. In conclusion, our results show that *S. pneumoniae* directly inhibits purinergic signaling by inducing P2Y$_2$ phosphorylation and internalization, resulting in the suppression of the calcium response of alveolar epithelial cells to ATP, thereby affecting cellular integrity and function.

Like virtually all eukaryotic tissues, the alveolar epithelium expresses purine receptors (P2Rs). These receptors are physiologically activated by extracellular nucleotides, particularly ATP. ATP and UTP are constantly secreted by adjacent cells, although lower levels of UTP are secreted than ATP, and these nucleotides function as paracrine mediators (2, 3). P2Rs are divided into two families, P2Y and P2X (P2YRs and P2XRs). P2XRs comprise seven subgroups (numbered 1–7) that form a membrane-spanning pore and function as ion channels upon activation. P2YRs are seven-transmembrane domain G protein–coupled receptors (GPCRs) and consist of eight subgroups (numbered 1, 2, 4, 6, and 11–14). Agonist-binding activates $G_i$, $G_{q/11}$, and $G_s$ signaling through PKC and IP$_3$ pathways. Consequently, P2R activation by ATP usually elicits an increase in the cytosolic calcium concentration [Ca$^{2+}$]$_{cyt}$. P2Y$_2$ seems to be predominantly expressed on the alveolar epithelium and has been detected on immortalized and isolated primary human alveolar epithelial cell (AEC) lines (4–6). Purine receptors have key functions in regulating surfactant synthesis, cell growth and integrity, cytoskeleton reorganization, and fluid reabsorption in the alveolar epithelium and contribute to inflammatory processes and immune responses (7–14).

In the course of an infection, AECs increase their secretion of ATP. Compared with basal extracellular ATP concentrations, which are estimated to be in the low nanomolar range, infection and other causes of cellular perturbation and stress can lead to a distinct increase up to 100 mM (15, 16). ATP then constitutes a danger-associated molecular pattern (DAMP) and induces host immune responses, including the release of interleukins (2, 17). Pathogens react by developing strategies to bypass the ATP/P2R-mediated defense mechanism. For example, *Porphyromonas gingivalis* scavenges ATP, preventing P2X$_7$-mediated apoptosis of gingival epithelial cells (18). *Leishmania amazonensis* utilizes a similar approach to inhibit macrophage

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The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Figs. S1–S4.

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2 The abbreviations used are: P2R, purine receptor; GPCR, G-protein–coupled receptor; IP$_3$, inositol 3-phosphate; PKC, protein kinase C; AEC, alveolar epithelial cell; MOI, multiplicity of infection; PEG-SOD, pegylated superoxide dismutase; LTG, Lysotracker Green; huBEC, primary human bronchial epithelial cell; CPS, capsule polysaccharide; ROS, reactive oxygen species; PAR 2, protease-activated receptor type 2; KGF, keratinocyte growth factor; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; ANOVA, analysis of variance; piAEC, primary AEC; HuAEpi cells, human AECs.
cytolysis (19) and the respiratory syncytial virus interferes with ATP signaling, leading to a disruption of alveolar fluid clearance (20).

In this study, we examine the interaction between *S. pneumoniae* (*Spn*) and purinergic signaling in AECs. We used an *in vitro* infection model of isolated primary AECs and A549 cells that were exposed to *Spn* strain D39. Physiologically, AECs exhibit a distinct P2Y₂-mediated calcium response when stimulated with ATP. We detected a pronounced suppression of this ATP-induced reaction in isolated primary AECs and A549 cells following an incubation with *S. pneumoniae*. Live-cell imaging with a CFP-tagged P2Y₂ revealed that pneumococcal-induced P2Y₂ internalization might be a potential mechanism underlying this phenomenon.

**Results**

*S. pneumoniae* inhibits the ATP-induced calcium response in AECs

Isolated primary rat AECs II were identified by LysoTracker Green (LTG) staining, which labeled lamellar bodies, a characteristic marker of this respective cell type. AECs II were stimulated with ATP and exhibited a sustained increase in the intracellular calcium concentration \([\text{Ca}^{2+}]_{\text{cyt}}\) as evidenced by a significant increase in the Fura-2 ratio. Although the maximum amplitude of the ATP-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) peak differed between cell lines, this reaction was almost identical in human A549 adenocarcinoma alveolar epithelial cells, primary human bronchial epithelial cells (huBECs), immortalized rat pulmonary epithelial L2 cells, and rat alveolar epithelial type 1-like R3/1 cells (Fig. 1).

Confluent isolated primary rat AECs II were then inoculated with either an encapsulated or a decapsulated strain of *S. pneumoniae* D39 at an MOI of 100 (Fig. S1). Under physiological conditions, the capsule polysaccharide (CPS) offers protection from opsonization and phagocytosis and is required for pneumococcal colonization. *S. pneumoniae* must form close contacts with host cells to complete the conversion from coloniza-
response was not altered compared with PEG-SOD–untreated AECs in this experiment (Fig. S3).

**Incubation of AECs with Pseudomonas aeruginosa has no effect on purinergic calcium signaling**

A549 cells were incubated for 2 h with *P. aeruginosa* at an MOI of 100, following the same protocol as for *S. pneumoniae* (Fig. 4). AECs were then stimulated with 100 μM ATP. However, the ATP-induced calcium response was not affected by this pathogen.

**Inhibition of purinergic calcium signaling is only induced by live *S. pneumoniae***

The pneumococcus-induced inhibition of the ATP-mediated increase in \([Ca^{2+}]_{cyt}\) in AECs was only observed if AECs...
were incubated with live bacteria. Heat inactivation of pneumococci at 65 °C for 15 min significantly attenuated the phenomenon described above (Fig. 5).

**S. pneumoniae inhibited the calcium response mediated by the GPCR P2R, but signaling mediated by the GPCR PAR was unaltered**

The GPCR protease-activated receptor type 2 (PAR 2) is activated by trypsin. Upon activation, it elicits an IP3-mediated calcium response, sharing a common final pathway with P2YR. PAR 2 is expressed in the alveolar epithelium and on A549 cells (25, 26). Under control conditions, trypsin stimulation led to an immediate, explicit calcium response in A549 cells. This response remained unaltered, even after A549 cells were preincubated with *S. pneumoniae* for 2 h (Fig. 6).

**The effect of the pneumococcus on the ATP-induced calcium response depends substantially on the temperature**

A549 cells were incubated with *S. pneumoniae* for 2 h at 37 °C, as described above, and compared with A549 cells that were subjected to the same procedure at 20 °C. Following ATP stimulation, the calcium response was only inhibited in AECs that were incubated with *S. pneumoniae* at 37 °C. In contrast, in AECs that were incubated at 20 °C, a distinct increase in [Ca2+]cyt persisted upon ATP stimulation. In these cells, the incubation with *S. pneumoniae* had no effect on the ATP-induced calcium response (Fig. 7).

**Mutation of three N-terminal phosphorylation sites in P2Y2 attenuates the *S. pneumoniae*-evoked inhibition of the ATP-induced calcium response in AECs**

Physiological desensitization of GPCRs is induced by agonist overstimulation. Underlying mechanisms include N-terminal phosphorylation through GPCR kinases, as well as arrestin- and clathrin-mediated internalization (27). For P2Y2, three N-terminal phosphorylation sites have been identified as crucial for its desensitization (Ser-243, Thr-344, and Ser-356) (28). The P2Y2 cDNA was cloned into pTagGFP2-N, and the respective phosphorylation sites were deleted by site-directed mutagenesis, creating P2Y2-AAA, to analyze the potential role of P2R phosphorylation in the effect of pneumococcal infection. WT and P2Y2-AAA were transfected into HPMEpC, a commercially available human-derived AEC line that appears to have lost the ability to express endogenous ATP-sensitive P2R, as this line lacked a physiological calcium response, sharing a common final pathway with P2YR.
with P2Y2-WT and P2Y2-AAA. This calcium response was suppressed in P2Y2-WT–transfected huAECs after an incubation with S. pneumoniae. In contrast, S. pneumoniae did not alter the ATP-induced increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) in P2Y2-AAA–transfected huAECs (Fig. 8). Thus, the incubation with pneumococci attenuated the calcium response mediated by P2Y2-WT, but not by P2Y2-AAA. However, all three phosphorylation sites must be mutated to obtain the complete attenuation of the calcium response. In AECs transfected with single P2Y2-S243A mutants, S. pneumoniae was still able to distinctly suppress the ATP-induced calcium response. This effect was further diminished in cells transfected with double P2Y2-S243A/T344A mutants, and the peak effect was only observed when all three phosphorylation sites were mutated (Fig. 9).

**GFP-P2R localization experiments via spinning-disk microscopy display differences between P2Y2-WT and P2Y2-AAA mutants**

A549 cells were transfected with pTagGFP2-N P2Y2-WT and P2Y2-AAA, respectively, and visualized by spinning-disk live-cell microscopy. GFP-P2Y2 constructs were mainly visible along cellular membranes. P2Y2-WT–transfected cells were exposed to ATP for 2 h to trigger P2Y2 internalization induced by agonist overstimulation. P2Y2 internalization was observed as a gradual increase in cytosolic GFP-containing structures, referred to as “vesicles” (Fig. 10). No cytosolic P2Y2 vesicles were observed when P2Y2-AAA–transfected cells were exposed to ATP. This finding was consistent with the hypothesis that mutation of Ser-243, Thr-344, and Ser-356 disrupted P2Y2 internalization. Inoculation of cells with S. pneumoniae elicited a similar gradual increase in P2Y2-GFP vesicles within the cytosol as observed in GFP-P2Y2-WT–transfected cells exposed to ATP. Surprisingly, internalization occurred in both P2Y2-WT– and P2Y2-AAA–transfected cells. Thus, P2Y2 internalization does not solely account for the S. pneumoniae–induced suppression of the ATP-evoked calcium response.

**Discussion**

Interactions between bacteria and their hosts are complex and diverse. Over the centuries, pathogens have adapted to their hosts and specifically target and modify signaling pathways in their favor, whereas invaded organisms have developed just as many options to react to their intruders by switching from normal cellular signaling into a defense mode. Pathophysiological changes occurring during a bacterial infection might emerge as collateral damage to the host inflammatory reaction or are directly induced by the pathogen itself. Many mammalian ATP-associated processes are known to be targeted by invading pathogens (18–20).

The data presented in this study point toward an interaction of S. pneumoniae with purinergic signaling in AECs. The physiological response of AECs to extracellular ATP—a P2R, IP3–mediated increase in \([\text{Ca}^{2+}]_{\text{cyt}}\)—was inhibited following exposure to S. pneumoniae in vitro. This effect was highly reproducible and observed in different immortalized and isolated primary rat AECs. In addition, S. pneumoniae promoted P2Y2 uptake by A549 cells.

ATP is released from cells as a DAMP upon exposure to infectious stimuli (2). Infection of AECs with S. pneumoniae elicits an increase in ATP secretion, resulting in elevated ATP concentrations in the extracellular medium. As the observed S. pneumoniae–induced suppression of the ATP-mediated calcium response resulted from P2Y2R overstimulation caused by high extracellular ATP levels, ATP concentrations in the supernatant of S. pneumoniae–preincubated AECs were determined. The continuous exposure of AECs to ATP concentrations exceeding those determined did not result in overstimulation and quiescent P2R signaling.

The attenuation of the ATP-mediated calcium response was more pronounced in cells infected with capsule-deficient bac-
S. pneumoniae inhibits P2Y<sub>2</sub> signaling

Figure 10. Increase in cytosolic GFP-P2Y<sub>2</sub> levels in A549 cells as visualized using live-cell laser spinning-disk microscopy. Baseline conditions were recorded for 30 min. ATP, exposure to 100 μM ATP after baseline (beige colored boxes); S. pneumoniae, exposure after baseline recordings were collected. n = 5 (per group). A, cytosolic P2Y<sub>2</sub>-WT levels after exposure to ATP (top) or S. pneumoniae (bottom). B, cytosolic P2Y<sub>2</sub>-AAA levels after exposure to ATP (top) or S. pneumoniae (bottom). C, spinning-disk microscopy images of a single P2Y<sub>2</sub>-WT–transfected cell captured over 120 min. Top, cells that were not exposed to S. pneumoniae (ctrl); bottom, cells that were exposed to S. pneumoniae (SP). Interpretation of box plots is as follows: midline, median; top/bottom box limit, third/first quartile, respectively; whiskers, limits 1.5 × interquartile range.

We postulated that the S. pneumoniae–induced suppression of the ATP-evoked calcium response either resulted from an interaction between the pathogen and membrane-anchored P2Y<sub>2</sub> or an effect of S. pneumoniae on downstream P2Y<sub>2</sub> signaling pathways. The IP<sub>3</sub> cascade downstream of GPCR signaling is known to be disrupted by several bacteria upon infection (e.g., Yersinia spp. or P. aeruginosa [29]). The effect of S. pneumoniae on the protease-activated receptor PAR 2 was examined to investigate a potential impact on downstream purinergic signaling. PAR, which is a member of the GPCR family that shares a common final pathway with P2Y<sub>2</sub>, is activated by proteolytic cleavage and elicits an IP<sub>3</sub>-mediated increase in [Ca<sup>2+</sup>]<sub>cys</sub> upon stimulation (30). To date, four subtypes (PAR 1–4) have been identified, of which PAR 2 is expressed in A549 cells and other AECs and is activated by trypsin (25, 26). PAR 2 induces G<sub>Gq/11</sub> or G<sub>Gi</sub> signaling (31–33). Stimulation of AECs with trypsin evoked a marked calcium response that was not altered by preincubation with S. pneumoniae. Therefore, IP<sub>3</sub> signaling downstream of the GPCR seemed to be unaffected by the S. pneumoniae infection in AECs.

We focused on an indirect mechanism by which P2Y<sub>2</sub> interfered with the pathogen rather than a direct physical interaction to elucidate the nature of the interaction between P2Y<sub>2</sub> and S. pneumoniae. This hypothesis was supported by the observation that S. pneumoniae completely suppressed the ATP-mediated calcium response, indicating that a majority of P2Y<sub>2</sub> was affected. The interaction seemed to depend on the integrity of pneumococcal membrane protein, as heat inactivation, resulting in bacterial lysis, inhibited the described effect. Interestingly, the effect could not be reproduced when cells were exposed to P. aeruginosa, indicating that a mere nonspecific pathogen–cell mechanism was unlikely. Membrane-anchored P2Y<sub>2</sub> distinctively outnumbered attached pneumococci; therefore, an effect resulting from a direct physical interaction between S. pneumoniae and P2Y<sub>2</sub> seemed improbable. In this context, the hypothesis that S. pneumoniae induced P2Y<sub>2</sub>R desensitization through receptor modification was more likely.

GPCR desensitization is a physiological feature that usually follows repeated or prolonged agonist stimulation. It generally occurs through GPCR phosphorylation by G protein–coupled receptor kinases and the subsequent interaction of GPCRs with
arrestins, promoting clathrin-mediated receptor endocytosis (34, 35). However, clathrin-independent mechanisms of GPCR internalization have been described as well (36). Phosphorylation of the cytoplasmic terminus of GPCR is a prevalent mechanism to regulate the numerous functions of these receptors in almost every tissue. As several kinases and multiple phosphorylation sites have currently been identified, researchers have not clearly determined whether a particular phosphorylation pattern or rather the total number of phosphorylation sites per molecule is responsible for the desensitization of the GPCR (37). For P2Y2, three C-terminal phosphorylation sites associated with receptor desensitization and internalization have been described (28). The hypothesis that S. pneumoniae might induce P2Y2R desensitization was supported by the finding that disruption of the ATP-induced calcium response was a highly temperature-dependent process, as has been described for the course of GPCR phosphorylation-mediated desensitization (38, 39). The presence of pneumococci on the surface of the AECs seems to induce a reaction that promotes the desensitization of P2Y2 to its physiological agonist; this reaction likely includes GPCR phosphorylation. Phosphorylation of the host protein is a known mechanism in the course of pathogen–host interactions, as shown for c-Jun N-terminal kinase in promoting release of interleukin-6 and -8 (40) and for STAT1 and STAT3 in the context of interferon signaling (41).

The three phosphorylation sites were mutated and disabled, creating a P2Y2-S243A/T344A/S356A (P2Y2-AAA) variant (28), to investigate whether the suppression of the ATP-induced calcium response was mediated by S. pneumoniae–induced P2Y2 phosphorylation.

When stimulated with ATP, P2Y2-AAA–transfected AECs exhibited a calcium response that did not differ from P2Y2-WT–transfected cells. Interestingly, S. pneumoniae inhibited the ATP-induced calcium response in P2Y2-WT–transfected AECs, but not in P2Y2-AAA–transfected AECs. Thus, S. pneumoniae seems to initiate a process in AECs that involves P2Y2 phosphorylation and leads to the silencing of the physiological calcium response to ATP. In P2Y2, residues 243 and 344 in the third intracellular loop and N-terminal residue 356 seem to be crucial for this process. However, all three phosphorylation sites appeared to be required to maintain the suppression of the calcium response, as the mutation of only one or two of the respective phosphorylation sites only resulted in a partial restoration of the ATP calcium response. A complete restoration of the physiological calcium reaction to ATP was only observed in cells transfected with the P2Y2-AAA variant.

Desensitization of P2YR is usually followed by its internalization; however, internalization is not a precondition for functional silencing, as this process also occurs with membrane-anchored P2Y2 (42, 43). Spinning-disk microscopy of GFP-tagged P2Y2-WT transfected AECs showed a gradual increase in cytosolic P2Y2 levels after stimulation with ATP. As expected, this increase was not observed in P2Y2-AAA–transfected AECs, confirming the hypothesis that the visualized effect indeed corresponded to P2Y2R internalization. Exposure to pneumococci in the absence of ATP led to a similar, although slightly delayed increase in cytosolic GFP-P2Y2 levels. Hence, S. pneumoniae evokes P2Y2 internalization to a similar extent as its natural agonist ATP. Interestingly, this phenomenon was observed in both P2Y2 WT- and AAA-transfected AECs. Therefore, the S. pneumoniae–induced inhibition of the ATP-evoked calcium response and the induction of P2Y2 internalization seem to be mediated by different underlying mechanisms. The ATP-induced calcium reaction was restored by mutagenesis of the P2Y2 phosphorylation sites S243A/T344A/S356A, which, in turn, had no effect on pneumococci-induced P2Y2 internalization. This finding might be explained by a different or more complex P2Y2 phosphorylation pattern induced by S. pneumoniae compared with the pattern induced by ATP overstimulation. On the other hand, phosphorylation by GPK is not mandatory for purine receptor internalization. Other kinases, such as PKA or PKC, might also facilitate P2YR phosphorylation and promote receptor internalization (44, 45). The induction of other signaling pathways by S. pneumoniae leading to phosphorylation-independent P2Y2 internalization must also be considered.

Many pathogens influence host cell homeostasis. By taking advantage of the physiological cellular signaling cascades of the host and modifying them in favor of the pathogen, bacteria adhere to host cells, become internalized, and evade the host immune defense mechanisms (46–48). As calcium is a crucial intracellular messenger regulating various cellular processes, such as gene expression, cytoskeletal rearrangements, cell growth, proliferation, and immune reactions, calcium signaling pathways are frequently targeted by bacteria. S. pneumoniae causes transient increases in [Ca2+]cyt derived from internal calcium stores in host cells (49). Similar observations have been reported for Chlamydia trachomatis, Campylobacter jejuni, and P. aeruginosa (50–52). On one hand, elevated calcium levels seem to be beneficial for bacterial survival and may be mandatory for bacteria to breach cellular membranes, as observed for Listeria monocytogenes or Salmonella Typhimurium (53, 54). In contrast, bacterial internalization into host cells is disturbed if [Ca2+]cyt increase, as described for S. pneumoniae and uropathogenic Escherichia coli (49). Furthermore, [Ca2+]cyt signals induce the expression and secretion of pro-inflammatory genes and mediators. Many pathogens have therefore developed strategies to dampen the immune response by interfering with the [Ca2+]cyt signaling in host cells needed to induce antibacterial defense mechanisms (55–57). Thus, researchers have not conclusively determined whether an increase in [Ca2+]cyt in the course of a bacterial infection is more advantageous for the pathogen or the host. This apparent contradiction can be resolved if the diverse complexity of calcium-mediated effects is considered. In this regard, studies focusing on a particular effect (i.e. cytoskeletal reorganization, inhibition or promotion of phagocytosis, induction of apoptosis, or gene expression) might be a more constructive approach than studies simply examining the increase in [Ca2+]cyt per se. A [Ca2+]cyt elevation represents a potent inducer of all of the aforementioned effects and is involved in almost all cellular processes through various mechanisms (58–61).

The observed pneumococcal interference with purinergic signaling might have far-reaching effects on cellular integrity.
and function. Although the complexity of pneumococcal infections cannot completely be depicted using in vitro experiments, the consistent inhibition of the ATP-mediated calcium response in different human and rat AEC lines and in isolated primary AECs indicates a robust mechanism that likely contributes to the pathobiology of pneumococcal infections. The effect of this (or: the described) mechanism on the course of infection and cellular damage can only be speculated about. During pneumococcal pneumonia, surfactant breakdown and impaired surfactant synthesis are hallmarks of infection in the alveolar epithelium and contribute to its severity (62, 63). In the healthy lung, surfactant secretion is regulated by purinergic signaling and mediates alveolar collapse, clinically leading to atelectasis. The observed inhibition of the ATP-induced calcium response in different human and rat AEC lines and in isolated primary AECs indicates a robust mechanism that likely contributes to the pathobiology of pneumococcal infections. The effect of this mechanism on the course of infection and cellular damage can only be speculated about. During pneumococcal pneumonia, surfactant breakdown and impaired surfactant synthesis are hallmarks of infection in the alveolar epithelium and contribute to its severity (62, 63). In the healthy lung, surfactant secretion is regulated by purinergic signaling and mediated by ATP in a paracrine manner. Surfactant disruption substantially compromises the alveolar defense barrier and facilitates alveolar collapse, clinically leading to atelectasis. The observed inhibition of the ATP-induced calcium response by S. pneumoniae permits a discussion on whether surfactant disruption in the course of pneumonia might indeed be specifically induced by S. pneumoniae via inhibition of purinergic signaling instead of being nonspecifically caused by inflammation and cell injury. Further research examining the underlying mechanism of the described effect is needed to answer this question and to identify the in vivo impact of this newly described pneumococcal phenomenon.

Experimental procedures

Reagents and kits

Fluorophore—Cal Fura-2/AM and LysoTracker Green were purchased from Invitrogen/Life Technologies, Inc.

Agents and chemicals—ATP, EDTA, EGTA, HEPES, keratinocyte growth factor (KGF), DNase type IV, and trypsin were purchased from Sigma-Aldrich. Elastase grade II was obtained from Worthington.

Kits—The QuikChange II XL mutagenesis kit was purchased from Agilent (Santa Clara, CA), and the EnzChek Direct phospholipase C assay kit, ATP determination kit, and LIVE/DEAD viability/cytotoxicity kit were obtained from Invitrogen/Molecular Probes (Carlsbad, CA). The Plasmid Mini/Maxiprep kit and Omniscript RT kit were purchased from Qiagen (Hilden, Germany).

Plasmids—pTagGFP2-N was purchased from von Evrogen (Moscow, Russia); LeGO-iPuro2 was kindly provided by Dr. Kristoffer Riecken (Department of Stem Cell Transplantation, Center for Oncology, University Medical Center Hamburg, Germany).

Media—All cell culture media and buffers were purchased from Biochrom (Berlin, Germany), except F-12K, which was obtained from LGC (Middlesex, UK), and AEpiCM, which was purchased from ScienCell (Carlsbad, CA). Todd Hewitt broth growth media were purchased from BD Biosciences. The yeast extract was obtained from Fluka Analytical/Sigma-Aldrich, and Columbia Sheepblood Agar was purchased from Oxoid/Thermo Fisher Scientific (Wesel, Germany).

Bacteria

A WT D39 serotype 2 (NCTC 7466) and a capsule-deficient (D39Δcps) strain were used for these experiments (64). The cryopreserved bacteria were seeded on blood-agar plates and grown at 37 °C for 16 h. Clones were then resuspended in THY medium (Todd Hewitt broth with 5% yeast) and grown at 37 °C for 2 h to an optical density at 600 nm of 0.4. The bacteria were harvested and stored in THY medium with 20% glycerol at −80 °C. For strain D39Δcps, agar and media were supplemented with kanamycin. A serial dilution was generated and plated on agar for each new lot to determine the bacterial concentration. For each experiment, a new aliquot of bacteria was slowly thawed and added to the supernatant of the cells at an MOI of 100 as described in the literature (65–68). Cells were incubated at 37 °C in a 5% CO2 atmosphere for 2 h and then subjected to further experiments.

Cells

Isolation and culture of AECs from adult male Sprague-Dawley rats was performed using procedures described previously (69, 70). All animal experiments were reviewed and approved by the local ethics committee and the responsible local government authorities of Hamburg, Germany. Briefly, rats were anesthetized by an injection of esketamine and xylazine (Ketanest S (Pfizer, New York) and Rompun 2% (Bayer, Leverkusen, Germany)). Thereafter, their lungs were surgically exposed and perfused three times with 10 ml of F-12K medium supplemented with 25 mM HEPES. The trachea was cannulated, and lungs were lavaged five times with 10 ml of a phosphate-buffered salt solution containing 5 mM EDTA and 5 mM EGTA. Afterward, the lungs were explanted and incubated with warm F-12K medium enriched with 50 mM HEPES and 4.5 units/ml elastase grade II at 37 °C for 30 min. After dissecting the large airways, the lungs were transferred into 20 ml of F-12K medium containing 25 mM HEPES, 20% fetal bovine serum (FBS), and 100 μg/ml DNase, quickly minced, and incubated at 4 °C for 10 min. Subsequently, the solution was mixed by end-over-end rotation for 4 min and successively filtered through 1-, 2-, and 4-ply cotton gauze, followed by filtration through nylon mesh (100 and 40 μm). Upon centrifugation of the cell suspension at 150 × g for 10 min and resuspension in 20 ml of warm DMEM, the cells were applied to plates coated with rat IgG (Sigma-Aldrich) for 1 h at 37 °C. The nonadherent cells were removed, collected in sterile tubes, centrifuged twice at 150 × g for 10 min, and resuspended in 3 ml of culture medium (DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 10 ng/ml keratinocyte growth factor). After determining the cell count and viability, cells were plated on collagen-coated coverslips and incubated at 37 °C in a 5% CO2 atmosphere. Keratinocyte growth factor was added to the culture medium to slow the dedifferentiation of type II to type I AECs in vitro; this growth factor leads to the maintenance of AEC type II–like expression of surfactant protein C and prevents the expression of aquaporin 5, an AEC type I–specific marker, for up to 7 days in vitro (71). Laminar bodies were labeled with the fluorophore LTG to distinguish between piAECs type I and II. As these organelles only occur in type II cells, LTG–positive cells were therefore defined as type II AECs. The purity of the resulting type II cell population was ~70–80%.
A549 cells are derived from human lung adenocarcinoma (72) (DSMZ, Braunschweig, Germany) and were cultivated in F-12K medium supplemented with 10% FBS. HPAEpiCs are commercially available isolated human pulmonary AECs and were purchased from ScienCell (Carlsbad, CA). These cells were cultured in a specific growth medium provided by the same company. huBECs were purchased from ATCC® (Manassas, VA) (PCS-300-010™) and grown in specific medium from the same company. L2 (ATCC®, CCL-149™) and R3/1 rat alveolar epithelial cells were provided by the laboratory of Thea Koch (University of Dresden) and cultured in DMEM supplemented with 10% FBS (73–75). Growth media were supplemented with 1% penicillin/streptomycin. All cells were grown in T75 flasks under sterile conditions at 37 °C in a 5% CO₂ incubator to semi-confluence. Cells were passaged with trypsin, and the digestion was terminated with FBS. All cells were regularly checked for contaminations and were free of mycoplasma during the experiments.

Cloning of mutated P2Y₂

The human P2Y₂ mRNA was isolated from A549 cells, purified, reverse-transcribed to cDNAs, and amplified (RNeasy Plus Mini Kit (Qiagen), Omniscript RT kit (Qiagen), and Phusion High Fidelity (New England Biolabs)). The DNA was sequenced (Eurofins MWG, Ebersberg, Germany) and found to be homologous to the human P2Y₂ DNA (NCBI GenBank™ no. 176072). The P2Y₂ cDNA was cloned into pTagGFP2-N, creating P2Y₂-pTagGFP2-N. Site-directed mutagenesis was performed to delete each P2Y₂ phosphorylation site (mutagenesis primers: S243A forward (GCCAACGCGCAAGCGCTGGCGACCA) and reverse (TGGTGGACGGCCTTGGCGTGGC)); T344A forward (CGCAGATCGAGACAGTGA-CAATCGAGAGA) and reverse (TCCTCTCAGTGACTGCTTCATGTGGATCTCCG); S356A forward (GATGTGTTGGGCGCGACCCAGA-CCCACT), and reverse (CGCCCTAGATGCTCCAGCGCTGCAACACATC). Successful mutagenesis was confirmed by sequencing.

Stable transfection of GFP-P2Y₂

GFP-P2Y₂ was excised from P2Y₂-pTagGFP2-N and cloned into LeGO-iPuro2, creating GFP-P2Y₂-LeGO-iPuro2. This vector was transfected into A549 cells, and transfected cells were selected with puromycin to generate a stable GFP-P2Y₂-expressing cell line. Using an analogous procedure, A549 cells stably transfected with GFP-P2Y₂-S243A (GFP-P2Y₂-SA), GFP-P2Y₂-S243A/T344A (GFP-P2Y₂-AA), GFP-P2Y₂-S243A/T344A/S356A (GFP-P2Y₂-AAA), and GFP (control) were also constructed.

Ca²⁺ imaging

Cells were loaded with 5 μM Fura-2/AM in HEPES buffer (150 mmol/liter Na⁺, 5 mmol/liter K⁺, 1.0 mmol/liter Ca²⁺, 1 mmol/liter Mg²⁺, and 20 mmol/liter HEPES at pH 7.4) and incubated for 30 min under cell culture conditions in the dark. Isolated primary AECs were simultaneously stained with Lyso-Tracker Green, an acidotrophic dye that accumulates in lamellar bodies, which are only present in AEC type II to distinguish AECs type I and II. [Ca²⁺]cyt was only determined in AEC type II. All calcium imaging experiments were performed in HEPES buffer. After measuring baseline calcium concentrations, cells were stimulated with ATP or trypsin without interrupting the real-time imaging. Unless otherwise stated, ATP concentration used was 100 μM (Fig. S4). Calcium concentrations were measured by epifluorescence microscopy (Olympus BX40 microscope, Olympus, Hamburg, Germany) and illumination with a mercury arc lamp directed through 340- and 380-nm interference filters. Cell viability was determined by staining cells with calcein/ethidium homodimer before the termination of each experiment. The images were then recorded and analyzed using MetaFluor ratio imaging software (Molecular Devices, Silicon Valley, CA).

Spinning-disk microscopy

A549 cells were transfected with pTagGFP2-N P2Y₂-WT or P2Y₂-AAA and visualized by spinning-disk live-cell microscopy (Zeiss Axiovert 200M, Carl Zeiss Microscopy, Germany). Ten cells in each well were selected, and their positions were registered. All cells were observed for 30 min under baseline conditions prior to the intervention and 2 h afterward. Three experimental groups were designed for each P2R variant. In the first group, transfected A549 cells were observed for 2 h without the intervention. In the second group, transfected A549 cells were stimulated with ATP. In the last group, transfected A549 cells were exposed to pneumococci.

Statistical analysis

The statistical analysis was performed under the guidance of the Institute of Medical Biometry and Epidemiology, University Medical Center Hamburg-Eppendorf. In calcium measurements, a single experiment contained between 10 and 20 cells and was counted as n = 1. Experiments of a group were always conducted on different days and with different cell passages or isolates. If multiple cells were analyzed in an experiment, values of each cell were included in the statistical analysis. Nonnormally distributed values were logarithmically transformed to enable nonparametric testing. The analysis was performed using paired or unpaired t tests with equal variances for dependent and independent variables or one-way ANOVA, respectively. The results were adjusted using Bonferroni’s method. As each experiment (n) was based on measured values of a variety of single cells, the influences of variations between single experiments and variations between single cells on the total variance of a group were analyzed using the random intercept model. Statistical calculations were performed using the software of the R project for statistical computing. Interpretation of box plots is as follows: midline, median; top/bottom box limit, third/first quartile, respectively; whiskers, limits 1.5 × interquartile range.

Author contributions—C.O. and R.K. conceptualization; C.O., M.K., and R.K. data curation; C.O., F.L., and B.K. formal analysis; C.O., M.K., S.H., and R.K. supervision; C.O. validation; C.O., F.L., B.K., and M.K. investigation; C.O., F.L., and B.K. visualization; C.O., F.L., B.K., M.K., and A.-K.R. methodology; C.O. writing-original draft; C.O. and R.K. project administration; C.O., M.K., A.-K.R., S.H., and R.K. writing-review and editing; B.K. software; S.H. resources.

S. pneumoniae inhibits P2Y₂ signaling

A549 cells were transfected with pTagGFP2-N P2Y₂-WT or P2Y₂-AAA and visualized by spinning-disk live-cell microscopy (Zeiss Axiovert 200M, Carl Zeiss Microscopy, Germany). Ten cells in each well were selected, and their positions were registered. All cells were observed for 30 min under baseline conditions prior to the intervention and 2 h afterward. Three experimental groups were designed for each P2R variant. In the first group, transfected A549 cells were observed for 2 h without the intervention. In the second group, transfected A549 cells were stimulated with ATP. In the last group, transfected A549 cells were exposed to pneumococci.
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