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*J Immunol* 2015; 195:2325-2334; Prepublished online 22 July 2015; doi: 10.4049/jimmunol.1400974

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http://www.jimmunol.org/content/suppl/2015/07/22/jimmunol.1400974.DCSupplemental

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Phosphocholine-Modified Macromolecules and Canonical Nicotinic Agonists Inhibit ATP-Induced IL-1β Release

Andreas Hecker,*1 Mira Küllmar,*1 Sigrid Wilker,* Katrin Richter,*† Anna Zakrzewicz,* Srebrena Atanasova,* Verena Mathes,* Thomas Timm,‡ Sabrina Lerner,* Jochen Klein,§ Andreas Kaufmann,¶ Stefan Bauer,*∥ Winfried Padberg,*∥ Sabrina Lerner,*‡ Martin Fronius,†‡ Elke K. H. Schweda,†† Günter Lochnitz,‡ and Veronika Grau*‡,∥

IL-1β is a potent proinflammatory cytokine of the innate immune system that is involved in host defense against infection. However, increased production of IL-1β plays a pathogenic role in various inflammatory diseases, such as rheumatoid arthritis, gout, sepsis, stroke, and transplant rejection. To prevent detrimental collateral damage, IL-1β release is tightly controlled and typically requires two consecutive danger signals. LPS from Gram-negative bacteria is a prototypical first signal inducing pro–IL-1β synthesis, whereas extracellular ATP is a typical second signal sensed by the ATP receptor P2X7 that triggers activation of the NLRP3-containing inflammasome, proteolytic cleavage of pro–IL-1β by caspase-1, and release of mature IL-1β. Mechanisms controlling IL-1β release, even in the presence of both danger signals, are needed to protect from collateral damage and are of therapeutic interest. In this article, we show that acetylcholine, choline, phosphocholine, phosphocholine-modified LPS from Haemophilus influenzae, and phosphocholine-modified protein efficiently inhibit ATP-mediated IL-1β release in human and rat monocytes via nicotinic acetylcholine receptors containing subunits α7, α9, and/or α10. Of note, we identify receptors for phosphocholine-modified macromolecules that are synthesized by microbes and eukaryotic parasites and are well-known modulators of the innate immune system. Our data suggest that an endogenous anti-inflammatory cholinergic control mechanism effectively controls ATP-mediated release of IL-1β and that the same mechanism is used by symbions and misused by parasites to evade innate immune responses of the host. The Journal of Immunology, 2015, 195: 2325–2334.

Interleukin-1β is a multifunctional proinflammatory cytokine of the innate immune system that plays an important role in host defense (1). However, excessive release is hazardous because high systemic levels of IL-1β cause fever, shock, and organ damage. Thus, increased production and release of IL-1β play a pathogenic role in various inflammatory diseases, such as rheumatoid arthritis, sepsis, stroke, and transplant rejection (2). To prevent detrimental collateral damage, IL-1β release typically requires two consecutive danger signals (3, 4). LPS from Gram-negative bacteria is a prototypical first signal inducing pro–IL-1β synthesis. Extracellular ATP is a typical second signal sensed by the ATP receptor P2X7 that triggers activation of the NLRP3-containing inflammasome, proteolytic cleavage of pro–IL-1β by caspase-1, and release of mature IL-1β (3–5). Mechanisms controlling IL-1β release are needed to protect the host from excessive inflammation but are largely unexplored.

Acetylcholine (ACh) was the first neurotransmitter to be identified, but it is also a regulator of the immune system with pro- and anti-inflammatory potential (6, 7). The first data pointing to an anti-inflammatory role of ACh date back to the year 2000, when Borovikova et al. (8) demonstrated that vagus nerve stimulation attenuates the systemic release of TNF in response to LPS. Furthermore, these investigators described the α-conotoxin–sensitive inhibition of the secretion of TNF, IL-1β, IL-6, and IL-18 from LPS-primed human macrophages in vitro (8). The current notion prevails that vagal stimulation triggers neuronal release of norepinephrine from the spleen that stimulates the release of ACh from splenic memory T cells. The released ACh is sensed by splenic macrophages via nicotinic receptors for ACh containing the α7 subunit (CHRNA7) (7, 9), leading to changes in cytokine expression via the Jak2-STAT3 signaling pathway (7, 10). This pathway is suggested to downmodulate LPS-induced expression of pro–IL-1β but not its cleavage or release.

NOTE: Abbreviations used in this article: ACh, acetylcholine; BzATP, 2′(3′)O-(4-benzoylbenzoyl)ATP triethylammonium salt; CHRNA7, nicotinic receptor for ACh containing the α7 subunit; CHRNA9, nicotinic receptor for ACh containing the α9 subunit; CHRNA10, nicotinic receptor for ACh containing the α10 subunit; LDH, lactate dehydrogenase; PC, phosphocholine; PC-BSA, PC-modified BSA; PC-LPS, PC-modified LPS; siRNA, small interfering RNA.
We demonstrated that activated monocytes accumulating in blood vessels of rat renal grafts produce ACh during acute rejection (11, 12). Because monocytes express nicotinic receptors for ACh, endogenously produced ACh might act as an autocrine or paracrine regulator of innate immunity. Interestingly, nicotinic ACh receptors do not function as ligand gated ion channels in primary rat monocytes and alveolar macrophages but rather as metabotropic receptors reducing ATP-induced intracellular calcium signals (11, 13). This led us to speculate that endogenous ACh also inhibits ATP-mediated inflammasome activation.

Strikingly, numerous bacteria and eukaryotic parasites colonizing mammals express cell wall components and secretory macromolecules modified with phosphocholine (PC), a molecule with structural similarity to ACh. PC-modified macromolecules exert strong anti-inflammatory effects on innate and adaptive immunity and help to evade the immune system of the host (14, 15). For example, extracellular bacteria chronically colonizing the human upper respiratory tract, such as the pathogen *H. influenzae*, carry PC-modifications on their LPS (16). PC-modified LPS (PC-LPS) is a virulence factor that depends on genes encoded in the *licl* operon (15); however, the exact molecular mechanism of its anti-inflammatory action is still elusive.

In this study, we tested the hypotheses that cholinergic agonists inhibit ATP signaling in monocytes and limit the release of mature IL-1β into the circulation and that PC-modified macromolecules produced by pathogens also inhibit IL-1β release by triggering the suggested anti-inflammatory mechanism. We propose that this powerful endogenous pathway that protects the host from IL-1β-induced damage can be used by pathogens to evade host immunity.

Materials and Methods

*Mononuclear blood leukocytes from experimental rat renal allografts*

Experimental animals received humane care according to the National Institute’s of Health’s *Guide for the Care and Use of Laboratory Animals*. Animal experiments were approved by the local committee at the Regierungspräsidium Giessen, Hesse, Germany (permit number G120/10 Nr. 23/2008). Orthotopic transplantation of allogeneic rat Dark Agouti kidneys to Lewis recipients, perfusion of graft blood vessels to isolate intravascular graft leukocytes, and Percoll gradient centrifugation to enrich for mononuclear leukocytes were performed 2 d after transplantation, as described previously (17). A total of 5 × 10⁶ mononuclear cells was incubated for 3 h in 24-well plates at 37˚C, 5% CO₂, in RPMI 1640 supplemented with 10% FCS (FCS gold) and 2 mM L-glutamine (all from PAA Laboratories, Colbe, Germany). Thereafter, 2(3’)-O-(4-benzoylbenzoyl)ATP triethylammonium salt (BzATP; 100 μM; Sigma-Aldrich, Taufkirchen, Germany) was added in the presence or absence of ACh chloride (10 or 100 μM), nicotine (100 μM), and cholinesterase from *Electrophorus electricus* (1 U/ml) (all from Sigma-Aldrich). Supernatants were collected and stored at −20˚C before cytokines and lactate dehydrogenase (LDH) were measured.

*U937 cells*

The human histiocytic lymphoma cell line U937 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and maintained in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine. Cells in the log-phase of growth were transferred to 24-well plates (1 × 10⁶ cells/ml and per well) and primed with 1 μg/ml LPS from *Escherichia coli* (L2654; Sigma-Aldrich) for 5 h. Thereafter, BzATP (100 μM) or nigericin (50 μM; Sigma-Aldrich) was added for 30 min in the presence or absence of different concentrations of cholinergic agonists and antagonists. ACh chloride, choline chloride, PC chloride calcium salt tetrahydrate, mecamylamine hydrochloride, nicotine, and strychnine hydrochloride were obtained from Sigma-Aldrich, and α-bungarotoxin was obtained from Toctris Bioscience (Bristol, U.K.). Unless otherwise stated, the following concentrations were used: ACh 100 μM, mecamylamine 100 μM, nicotine 100 μM, strychnine 10 μM, and α-bungarotoxin 1 μM. Supernatants were collected 30 min after the addition of BzATP or nigericin and stored at −20˚C to measure IL-1β and LDH. IL-1β, IL-6, and TNF-α were measured in the supernatants by Quantikine Immunoassays (R&D Systems, Minneapolis, MN).

*Human PBMCs*

Studies on primary human cells were approved by the local ethics committee of the University of Giessen (No. 81/13). PBMCs were freshly isolated from heparinized blood of healthy nonsmoking male volunteers by LeucoSep gradients (Greiner Bio-One, Frickenhausen, Germany). A total of 5 × 10⁹ PBMC/0.5 ml/well was incubated for 3 h in 24-well plates in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine. Thereafter, nonadherent cells were removed, cell culture medium was replaced, and BzATP (100 μM) was added in the presence or absence of nicotine (100 μM) and ACh (10 μM), and incubated for 30 min. Supernatants were collected and stored at −20˚C before measurement of IL-1β and LDH.

*Human monocytes*

Blood was obtained from healthy donors and either separated directly by gradient centrifugation using LeucoSep gradients or pulsed with 0.5 ng LPS/ml before separation. Thereafter, monocytes were purified by positive selection using Dynabeads CD14 (Invitrogen, Karlsruhe, Germany), according to the supplier’s instructions. Isolated monocytes were cultivated as described for PBMCs, with the exception that the medium was not replaced after 3 h of culture. Thereafter BzATP, nicotine, ACh, choline, and PC were added for 30 min, as described.

**FIGURE 1.** Endogenous ACh inhibits BzATP-induced release of IL-1β. Mononuclear leukocytes were isolated by intensive single-organ perfusion and Percoll density gradient centrifugation from the blood vessels of rat renal allografts on day 2 after transplantation in the Dark Agouti to Lewis rat strain combination. (A) Expression of pro–IL-1β and ACh receptor mRNA was investigated by real-time RT-PCR, and products were separated in agarose gels. The mRNA of pro–IL-1β, CHRNA7, CHRNA9, and CHRNA10 was detected, whereas the nonfunctional receptor CHRFAM7A was absent from mononuclear leukocytes and skin. In negative controls, in which cDNA was replaced by water, no product was obtained. Signals seen in the lower part of the gel are primer dimers. (B) BzATP only induced a modest increase in IL-1β release. When BzATP was combined with cholinesterase (ChE, 1 U/ml), release of IL-1β increased significantly. Data are presented as individual data points. *p ≤ 0.05 versus BzATP alone, Wilcoxon signed-rank test.
and IL-1β, IL-6, and TNF-α were measured in the supernatant. The purity of the isolated monocytes was evaluated by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA) using FITC-labeled mAb M5E2 to CD14 (BioLegend, San Diego, CA); monocyte purity was >75%.

**Cell viability**

To test for cell viability, LDH released from dead cells was measured at the end of the experiments in cell culture supernatants by a Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI), according to the supplier’s instructions, and compared with the total content of LDH in lysed cells. Irrespective of the reagents applied, LDH values typically remained <6% for U937 cells and <10% when primary cells were used.

![Figure 2](http://www.jimmunol.org/)

**RNA isolation and real-time RT-PCR**

Total RNA was extracted using the QIAGEN RNeasy Miniprep Kit (QIAGEN, Hilden, Germany), according to the manufacturer’s instructions. RNA samples were reverse transcribed using M-MLV Reverse Transcriptase (Promega, Mannheim, Germany). Real-time PCR was performed in an ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using Platinum SYBR Green qPCR Super Mix-UDG (Invitrogen). Signals were normalized to porphobilinogen deaminase. Primer pairs are shown in Supplemental Table I. Negative controls were included in each experiment, where the cDNA was replaced by water. The specificity of the PCR was confirmed by sequencing (SeqLab, Göttingen, Germany) and by separation in a 1.5% agarose gel. Gene expression was normalized to the housekeeping gene...
porphobilinogen deaminase and analyzed using the 2^−ΔΔCt method, and the mean values of the controls were set to one arbitrary unit.

**Transfection of siRNA**

The expression of CHRNA7, the nicotinic receptor for ACh containing the α9 subunit (CHRN9), and the nicotinic receptor for ACh containing the α10 subunit (CHRNA10) in U937 cells was reduced using small interfering RNA (siRNA) technology. Cells were transfected with ON-TARGETplus human CHRNA7, CHRNA9, or CHRNA10 siRNA SMARTpool or with negative control ON-TARGETplus Non-targeting Control Pool (Thermo Fisher Scientific, Schwerte, Germany) to control for nonspecific gene inhibition. Cells were transfected with 30 pmol SMARTpool or with negative control ON-TARGETplus Non-targeting ON-TARGETplus human CHRNA7, CHRNA9, or CHRNA10 siRNA small interfering RNA (siRNA) technology. Cells were transfected with 30 pmol siRNA/1 × 10^6 cells using Amaxa Cell Line Nucleofector Kit C and Nucleofector II Device (both from Lonza Cologne, Cologne, Germany), according to the manufacturer’s instructions. The siRNA-mediated downregulation of the target gene was assessed by real-time RT-PCR 6 h after transfection, according to the protocol provided by Lonza. Experiments on the release of IL-1β were performed 48 h after transfection.

**Whole-cell patch-clamp recordings**

For electrophysiological experiments, U937 cells were placed in coated (poly-1-lysine) cell culture dishes (Nunc, Roskilde, Denmark) containing bath solution and incubated for 5 h with LPS (1 μg/ml) at 37°C. Bath solution contained 5.4 mM KCl, 120 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES (4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid), and 25 mM glucose (pH 7.4). After incubation with LPS, dishes were mounted on an inverted microscope (Axiovert; Zeiss, Göttingen, Germany), and whole-cell recordings were performed at room temperature. Borosilicate glass capillaries (outer diameter 1.6 mm; Hilgenberg, Malsfeld, Germany) were pulled to patch pipettes with a resistance of 2–4 MΩ by an automated puller (Zeitz, Augsburg, Germany). Pipettes were filled with 120 mM KCl, 1 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, 11 mM EGTA, and 20 mM glucose (pH 7.3).

Membrane potential was clamped to −60 mV, and the resulting transmembrane currents were amplified with an EPC 9 amplifier and acquired via an ITC-16 interface with Pulse software (both from HEKA, Lambrecht, Germany). BzATP (100 μM), nicotine (100 μM), and mecamylamine (100 μM) were dissolved in bath solution and applied via a pressure-driven microperfusion system.

**Purification and characterization of PC-LPS**

Purification and characterization of LPS from the various strains followed a standard protocol (18). Briefly, LPS was isolated from bacterial cells using a phenol/chloroform/light-petroleum method and purified by subsequent ultracentrifugation. Detailed structural knowledge of LPS was obtained using high-field nuclear magnetic resonance and electrospray ionization–mass spectrometry techniques, along with composition and linkage analyses on O-deacylated LPS and oligosaccharide samples.

**PC-modified BSA**

Preparation of PC-modified BSA (PC-BSA) was described previously (19). Briefly, 25 mg (95 μmol) p-aminophenolphosphocholine (Biozol, Eching, Germany) was dissolved in 1 ml ice-cold 1 N hydrochloric acid (Roth, Karlsruhe, Germany). After the addition of 6.5 mg (95 μmol) sodium nitrite (Roth), the mixture was incubated for 10 min under gentle agitation at room temperature. In a second step, 0.5 ml (42.5 μmol) the freshly prepared diazonium phenylphosphorylchlorine was added to a solution of 100 mg (1.5 μmol) BSA (Roth) in 5 ml boric acid (100 mM boric acid [pH 9]), 150 mM NaCl; Merck, Darmstadt, Germany and Roth, respectively) and incubated under gentle agitation on ice for 12 h.

**FIGURE 3.** Nigericin-mediated release of IL-1β from U937 cells is not inhibited by nicotinic stimulation. U937 cells were primed with LPS for 5 h, and nigericin was added for another 30 min to trigger IL-1β release, which was measured by ELISA. Nigericin-induced (Nig) IL-1β release was unimpaired by nicotine (Nic), ACh, choline (Cho), and PC. Statistical analyses were performed by the Kruskal–Wallis test, followed by the Mann–Whitney rank-sum test.

Finally, PC-BSA was desalted using a Nap5-column (GE Healthcare Bio-Sciences, Uppsala, Sweden), according to the manufacturer’s instructions, and stored at −20°C. The efficiency of coupling was quantified after the release of PC upon HF treatment. Choline was measured by HPLC, according to published methods (20). A total of 9 mol PC was incorporated per mol BSA.

PC residues were removed from PC-BSA by treatment with HF. Ten milligrams (150 nmol) of lyophilized PC-BSA was dissolved in 500 μl hydrofluoric acid (48%; Merck) and incubated on ice overnight. The sample was dried under a stream of nitrogen, dissolved in 500 μl water, and lyophilized.

**Western blots**

Human PBMCs were lysed, and protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein (15 μg) and prestained molecular mass standards (Precision Plus Protein Standards, dual color; Bio-Rad, Hercules, CA) were resolved in 15% reducing SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 5% skimmed milk powder and 0.01% Tween-20 in PBS, mouse mAbs to β-actin (A2228; Sigma-Aldrich) were diluted 1:5,000, rabbit Abs to caspase-1 (#2225; Cell Signaling Technology) were diluted 1:20,000 in PBS and Roti-Block (Roth). After washing in PBS, 0.01% Tween-20, HRP-conjugated rabbit anti-mouse Ig and goat anti-rabbit Ig Abs (both from Dako Cytomation, Glostrup, Denmark), diluted 1:20,000 in PBS and 5% skimmed milk powder, whereas polyclonal rabbit Abs to caspase-1 (#2225; Cell Signaling Technology, Danvers, MA) were diluted 1:1,000 in PBS and 2.5% skimmed milk powder. Mouse mAbs to β-actin (A2228; Sigma-Aldrich) were diluted 1:50,000 in PBS and Roti-Block (Roth). After washing in PBS, 0.01% Tween-20, HRP-conjugated rabbit anti-mouse Ig and goat anti-rabbit Ig Abs (both from Dako Cytomation, Glostrup, Denmark), diluted 1:5,000 in PBS, 2.5% skimmed milk powder, and 0.01% Tween-20, were used to detect primary Abs, followed by the chemiluminescent SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL) to detect IL-1β or by the Lumi-Light substrate (Roche, Mannheim, Germany) to detect β-actin. Blots were documented using a digital gel-documentation system (Biozym, Hessisch Oldendorf, Germany).

For the detection of secreted caspase-1 and IL-1β, human PBMCs were stimulated, as described, but in the absence of FCS. Cell culture supernatants were harvested and concentrated by a factor of 10 using Amicon Ultra centrifugal filters (Ultracel 10K; Merck Millipore, Darmstadt, Germany).

**Statistics**

Data were analyzed with SPSS software (Munich, Germany) by the nonparametric Kruskal–Wallis test, followed by the Mann–Whitney rank-sum test; p ≤ 0.05 was considered statistically significant. Data obtained from primary leukocytes were analyzed by the Wilcoxon signed-rank test.

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**Table I. Concentrations of compounds causing an ∼50% inhibition (IC50) of BzATP-mediated release of IL-1β from LPS-primed U937 cells**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50</th>
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<tbody>
<tr>
<td>ACh</td>
<td>1 μM</td>
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<tr>
<td>Nicotine</td>
<td>10 μM</td>
</tr>
<tr>
<td>Choline</td>
<td>10 μM</td>
</tr>
<tr>
<td>PC</td>
<td>10 μM</td>
</tr>
<tr>
<td>PC-LPS RM118</td>
<td>25 nM</td>
</tr>
<tr>
<td>PC-LPS NTH123323</td>
<td>25 nM</td>
</tr>
<tr>
<td>PC-BSA</td>
<td>140 nM</td>
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</table>
Results

Endogenous ACh inhibits IL-1β release

We isolated mononuclear leukocytes from the blood vessels of rat renal allografts. These cells expressed the mRNA of pro–IL-1β, as well as CHRNA7, CHRNA9, and CHRNA10, but not the nonfunctional receptor CHRFAMA7A (Fig. 1A). When stimulated with the P2X7-specific agonist BzATP, these leukocytes release only slightly increased amounts of IL-1β (without BzATP: median, 32 pg/ml; range: 14–56 pg/ml; with BzATP: median, 41 pg/ml; range: 31–71 pg/ml; n = 5 each). However, when BzATP was applied with cholinesterase, an enzyme that efficiently degrades ACh, significantly more IL-1β was detected in the cell culture supernatant (median, 71 pg/ml; range: 44–116 pg/ml, n = 5, p = 0.05 compared with BzATP alone) (Fig. 1B). The results from this first set of experiments supported our hypothesis that endogenous ACh inhibits BzATP-mediated release of IL-1β.

Nicotinic ACh receptor activation interferes with BzATP-induced release of IL-1β

In the next set of experiments, we used the human monocytic cell line U937. We primed these cells with LPS from E. coli, which resulted in increased pro–IL-1β mRNA levels (Supplemental Fig. 1A). As expected, LPS-primed U937 cells released IL-1β into the culture medium in response to treatment with BzATP for 30 min. However, application of ACh together with BzATP inhibited IL-1β secretion in a dose-dependent manner, whereas IL-6 and TNF-α levels in the supernatant were not reduced (Fig. 2A, data not shown). Of note, the viability of the cells was not impaired (Supplemental Table II).

Similar to ACh, choline and nicotine dose dependently inhibited the release of IL-1β in response to BzATP (IC50 ∼1 μM for ACh, IC50 ∼10 μM for nicotine and choline) (Fig. 2A, Table I). These results suggested the involvement of CHRNA7 homomers or receptors containing CHRNA9 (21, 22). In agreement with the results published by Chernyavsky et al. (23), mRNAs for CHRNA7, CHRNA9, and CHRNA10 were detected in unprimed and LPS-primed U937 cells. In addition, the inactive duplicate CHRFAMA7A was readily detectable (Supplemental Fig. 1B).

Mecamylamine, α-bungarotoxin, and strychnine antagonized the inhibitory effect of nicotine (Fig. 2B). ACh, and choline (data not shown), indicating an involvement of CHRNA7, CHRNA9, and/or CHRNA10. To further corroborate the role of these receptors, we reduced their expression in U937 cells by siRNA (Supplemental Fig. 2A, 2B), which reduced the effect of nicotine by ∼50% in all cases, whereas cells treated with control siRNA behaved like untreated cells (Fig. 2C, Supplemental Fig. 2C). Specific gene silencing was confirmed by real-time RT-PCR, which revealed the expected reduced expression of CHRNA9 and CHRNA10 (Supplemental Fig. 2A, 2B). However, the basal mRNA expression of CHRNA7 was too low for quantification. None of the siRNA treatments resulted in an unspecific downregulation of other receptors (Supplemental Fig. 2A, 2B). In view of the concerns raised with respect to the specificity of nicotinic receptor Abs (24), we refrained from attempts to monitor ACh receptor protein expression.

Nicotinic agonists inhibit BzATP-induced ion fluxes

We used the pore-forming toxin to investigate whether the BzATP-induced signaling cascade is inhibited upstream of the formation of
a functional inflammasome complex. Nigericin activates the inflammasome independent of BzATP nigericin and resulted in the release of IL-1β from LPS-primed U937 cells. Nigericin-induced release of IL-1β was unaffected by nicotine, Ach, and choline (Fig. 3).

To investigate the effect of nicotine on ATP-induced ion currents, patch-clamp experiments were performed in LPS-primed U937 cells. As expected, application of BzATP reproducibly induced ion currents (Fig. 4A), which were completely inhibited by nicotine (Fig. 4B). Mecamylamine, in turn, antagonized the effect of nicotine (Fig. 4C); however, nicotine alone did not induce currents.

**PC and PC-modified macromolecules inhibit BzATP-induced IL-1β release**

We tested whether PC-modified macromolecules also inhibit the release of IL-1β in response to BzATP. PC-LPS from two *H. influenzae* strains, RM118 and NTHi23323, dose dependently and most efficiently inhibited the BzATP-induced release of IL-1β (IC50 ∼ 0.1 μg/ml, corresponding to ∼25 nM PC-LPS) (Fig. 5A, Table I). The inhibitory effect of both PC-LPS variants (1 μg/ml) was antagonized by mecamylamine (Mec), α-bungarotoxin (α-Bun), and strychnine (Stry). Nigericin-induced IL-1β release was not influenced by PC-LPS. *p* ≤ 0.05 versus C3, Kruskal–Wallis test, followed by the Mann–Whitney rank-sum test.

![FIGURE 5. BzATP-mediated release of IL-1β from LPS-primed U937 cells is inhibited by PC-modified LPS. U937 cells were primed with LPS from *E. coli* for 5 h, and BzATP was added for another 30 min to trigger IL-1β release. Controls: supernatants from untreated cells (C1), from cells primed with LPS (C2), and from LPS-primed cells stimulated with BzATP (C3). (A) PC-LPS from *H. influenzae* strains RM118 (RMPC) and NTHi 1233 (NTHiPC) applied together with BzATP dose dependently inhibited the release of IL-1β. LPS from the corresponding lic1 mutant strains RM7004-Lic1 (RM) and NTHi2333lic1 (NTHi), which lacks PC modification, was ineffective. (B) The inhibitory effect of both PC-LPS variants (1 μg/ml) was antagonized by mecamylamine (Mec), α-bungarotoxin (α-Bun), and strychnine (Stry). (C) Nigericin-induced IL-1β release was not influenced by PC-LPS. *p* ≤ 0.05 versus C3, Kruskal–Wallis test, followed by the Mann–Whitney rank-sum test.](http://www.jimmunol.org/)

Parasitic filarial nematodes secrete PC-modified proteins with anti-inflammatory functions. These proteins can be mimicked by artificial PC-modified proteins (26). We noted an efficient, dose-dependent inhibitory effect on the BzATP-induced release of IL-1β for PC-BSA with an IC50 ∼ 10 μg/ml, corresponding to ∼140 nM PC-BSA (Fig. 6A, Table I). The average PC:BSA stoichiometry was 9:1. Unmodified BSA and PC-BSA pretreated with hydrofluoric acid to remove the PC modification were inactive (Fig. 6B). In addition, free PC (IC50 ∼ 10 μM) inhibited the BzATP-dependent release of IL-1β (Fig. 6A, Table I). The effects of PC, PC-LPS, and PC-BSA were antagonized by mecamylamine, α-bungarotoxin, and strychnine (Figs. 5B, 6A), suggesting an involvement of CHRNA7, CHRNA9, and/or CHRNA10. Again, nigericin-induced release of IL-1β was not impaired by PC (Fig. 3).

**Nicotinic agonists decrease IL-1β release from primary human blood cells**

To confirm that BzATP-mediated release of IL-1β can be inhibited by agonists of CHRNA9 in primary leukocytes, we analyzed PBMCs isolated from healthy human donors. As expected, these cells expressed very low levels of pro–IL-1β mRNA (data not shown). After cell isolation and culture for 3 h, PBMCs expressed the mRNA of pro–IL-1β, CHRNA7, CHRNA9, and CHRNA10 (Supplemental Fig. 1C) and released considerable amounts of IL-1β in response to BzATP (median, 1559 pg/ml, range: 736–7325 pg/ml, n = 5). Addition of ACh or nicotine significantly reduced the release of IL-1β (Fig. 7A). As expected, Western blotting revealed that BzATP reduced the cellular content of pro–IL-1β, and this reduction was attenuated by ACh, nicotine, and PC (Fig. 7B). Mature IL-1β was absent from cell lysates but was detected in cell culture supernatants upon stimulation with BzATP. Release of IL-1β was attenuated in the presence of cholinergic agonists (Fig. 7B, Fig. 8). In the same line, procaspase-1 was detected in cell lysates and active caspase-1 was detected in cell culture.
supernatants in response to BzATP. Similar to IL-1β, activation and release of caspase-1 were sensitive to cholinergic agonists (Fig. 7B).

When monocytes were isolated by positive selection via magnetic beads, they spontaneously released a certain amount of IL-1β (median, 105 pg/ml, range: 22–181 pg/ml, n = 6) within 3.5 h of culture. Stimulation with BzATP for the last 30 min of culture resulted in an additional release of IL-1β (median, 5,088 pg/ml, range: 1,058–14,907 pg/ml, n = 6), which was efficiently reduced by the simultaneous addition of nicotine, ACh, choline, and PC (Fig. 7C). Priming with low-dose LPS (5 ng/ml) further increased the amount of IL-1β released in response to BzATP (median, 9,333 pg/ml, range: 2,864–20,268 pg/ml, n = 6, p = 0.05). Also in LPS-primed monocytes, ACh, PC, and choline reduced the ATP-induced release of IL-1β (p = 0.05), whereas nicotine was only effective in four of six experiments (Fig. 7C). IL-6 and TNF-α were released in similar amounts in the presence or absence of BzATP and nicotinic agonists (Fig. 7C).

Discussion
In this study, we provide evidence for a novel cholinergic mechanism that potently inhibits the ATP-mediated secretion of mature IL-1β from monocytes via CHRNA7, CHRNA9, and/or CHRNA10 (Fig. 8). This mechanism is triggered by canonical endogenous ligands of these receptors, as well as by PC and PC-modified macromolecules produced by bacteria.

Our experiments on activated mononuclear leukocytes isolated from rat renal allografts at the onset of acute rejection already indicated that ACh inhibits BzATP-induced release of IL-1β. We showed previously that mononuclear leukocytes accumulating in the blood vessels of renal allografts produce ACh (11, 12). Therefore, we assumed that endogenous ACh blocks the ATP-induced increased secretion of IL-1β. In fact, addition of cholinesterase, which efficiently destroys endogenous ACh, enabled IL-1β release in response to ATP. This result supported our hypothesis that leukocytic ACh interferes with ATP-mediated secretion of IL-β in vivo.

Because activated leukocytes from experimental rat renal allografts are difficult to obtain and to manipulate, they are inappropriate to elucidate a novel mechanism of action. Therefore, the following experiments were performed on the human monocytic cell line U937 or on primary blood leukocytes. In line with our hypothesis, IL-1β secretion from LPS-primed U937 cells was efficiently inhibited by ACh and nicotine. Because choline also was effective, we hypothesized that CHRNA7 homomers, CHRNA9 homomers, or CHRNA9/CHRNA10 heteromers mediate inhibition. Indeed, mecamylamine, a general nicotinic blocker, as well as α-bungarotoxin and strychnine, antagonists of CHRNA7 and CHRNA9 (18, 27), antagonized cholinergic inhibition of IL-1β release. The pivotal role of CHRNA7, CHRNA9, and CHRNA10 was further corroborated by gene silencing, which significantly attenuated the cholinergic inhibitory effect in all cases. In conclusion, stimulation of the evolutionarily ancient CHRNA7, CHRNA9, and/or CHRNA10 efficiently inhibit ATP-mediated secretion of IL-1β from LPS-primed U937 cells.

In the following experiments, we investigated which step in the ATP-induced signaling cascade is regulated by nicotinic receptor stimulation. A first and sufficient step in ATP-mediated

FIGURE 6. BzATP-mediated release of IL-1β from LPS-primed U937 cells is inhibited by PC-BSA and free PC. U937 cells were primed with LPS from E. coli for 5 h, and BzATP was added for another 30 min to trigger IL-1β release. (A) PC-BSA and free PC dose dependently inhibited BzATP-triggered IL-1β release. The inhibitory effects of PC-BSA (50 µg/ml) and free PC (100 µm) were antagonized by mecamylamine (Mec), α-bungarotoxin (α-Bun), and strychnine (Stry). (B) BSA from the same batch and hydrofluoric acid-treated PC-modified BSA (HF-PC-BSA) did not inhibit BzATP-mediated release of IL-1β. U937 cells were primed with LPS. Addition of BzATP (100 µM) for 30 min resulted in a release of IL-1β, which was measured in the cell culture supernatant. Different concentrations of unmodified BSA and HF-PC-BSA were used. *p < 0.05 versus C3, Kruskal–Wallis test, followed by the Mann–Whitney rank-sum test. C3, supernatants from LPS-primed cells stimulated with BzATP.
maturation of pro–IL-1β is the induction of a K⁺ efflux resulting in a decreased intracellular K⁺ concentration and inflammasome activation (3, 4, 28). Because nigericin, a toxin produced by Streptomyces hygroscopicus, forms pores for K⁺ ions in the cell membrane, it induces NLRP3 inflammasome assembly in the absence of extracellular ATP (29). In fact, ATP-independent, nigericin-induced release of IL-1β was not impaired by stimulation of nicotinic receptors. In neurons, nicotinic ACh receptors physically interact with P2X ATP receptors, resulting in cross-inhibition of ion fluxes (30, 31). Accordingly, patch-clamp experiments using LPS-primed U937 cells directly showed a mecamylamine-sensitive nicotinic inhibition of BzATP-induced ion currents. These findings led to the conclusion that the ATP-mediated signaling is inhibited by nicotinic receptors and that inflammasome assembly triggered by ATP-independent stimuli is unimpaired. However, nicotine alone did not induce ion currents, indicating that nicotinic ACh receptors expressed by U937 cells do not form functional ion channels as was described previously for monocytes and other leukocytes (11, 32, 33). At such unconventional metabotropic nicotinic ACh receptors, antagonists of canonical ionotropic receptors can gain agonist activity (33), which is in line with the present observation that nicotine shares activity with ACh and choline in inhibiting BzATP-induced IL-1β release while acting as an antagonist of ionotropic CHRNA9/CHRNA10 (22). More research is needed to answer the question of how these metabotropic nicotinic receptors are composed, and we do not know whether they form multimers or whether CHRNA7, CHRNA9, and CHRNA10 can be combined together in functional heteromers.

To ensure that cholinergic inhibition of BzATP-mediated IL-1β release also operates in primary human cells, we isolated PBMCs and enriched monocytes from the blood of healthy human volunteers. In line with our data on U937 cells, nicotine, ACh, choline, and PC reduced BzATP-induced release of IL-1β and caspase-1 from primary PBMCs and purified monocytes. ATP-dependent release of caspase-1 from human monocytes was described previously (34). However, after priming of primary monocytes with low-dose LPS, nicotine was inactive in two of six experiments, but the endogenous nicotinic agonists ACh,
choline, and PC were active. These results support the idea that the described mechanism is active in vivo and suggest that it might be an interesting therapeutic target to prevent IL-1β release into the blood. Indeed, a choline-rich diet, which increases blood choline levels, was shown to improve survival in experimental endotoxin shock (35).

The observation that PBMCs and monocytes from healthy human donors express pro–IL-1β after 3 h of culture, but not immediately after isolation, deserves discussion. Most investigators report that release of IL-1β from human monocytes depends on priming (36–38). Our data suggest that cells are primed during gradient centrifugation (39) or adherence to tissue culture dishes (40). Hence, monocytes in the blood of healthy humans are probably devoid of pro–IL-1β. However, during systemic inflammation leading to monocyte activation in vivo, increased blood ATP levels, which are typical of major trauma (41), may cause a substantial release of monocytic IL-1β into the blood. In this situation, specific agonists of CHRNA7, CHRNA9, and CHRNA10 might be useful to prevent shock.

PC-modified macromolecules are produced by numerous pro- and eukaryotic pathogens. They enable colonization of the host, most probably by binding to the receptor for platelet activation factor (15). In addition, PC-modified molecules exert pronounced anti-inflammatory functions, the mechanisms of which are still elusive (14). Because inflammasome activation plays a pivotal role in the host defense against numerous pathogens (1), we speculated that the described inhibitory pathway was hijacked by pathogens for immune evasion. Indeed, PC-LPS isolated from two virulent H. influenzae strains was the most efficient agonist, inhibiting BzATP signaling, with an IC₅₀ of ∼10 µM. Chemical binding of PC to macromolecules seemed to potentiate its effectiveness. It is conceivable that bound PC is more stable than its free form and that cellular uptake is prevented. Hence, we describe novel agonists of receptors containing CHRNA7, CHRNA9, and/or CHRNA10. Furthermore, we identified a mechanism that explains, at least in part, the anti-inflammatory effects of PC-modified products of bacterial and eukaryotic parasites. In view of the importance of IL-1β for host defense and its pleiotropic proinflammatory functions (1, 2), we consider this a biologically relevant mechanism by which PC-modified macromolecules modulate inflammation. Because antibiotic resistance is a major clinical problem and bacterial and parasitic infections are still among the leading causes of morbidity and mortality worldwide, the development of specific nicotinic antagonists as antibacterial and anti-parasitic medicaments might be a promising approach.

In conclusion, PC, PC-modified macromolecules, and canonical cholinergic agonists act upon CHRNA7, CHRNA9, and/or CHRNA10 to inhibit ATP-mediated IL-1β release from human and rat mononuclear blood leukocytes. This novel inhibitory pathway is induced by endogenous cholinergic agonists and seems to be efficiently hijacked by bacteria and parasites.

Acknowledgments
We thank Andrea Fischer, Kathrin Petri, Sabine Stumpf, and Laetitia Rabin (all from Justus-Liebig-University Giessen, Department of General and Thoracic Surgery, Laboratory of Experimental Surgery) for excellent technical support and Otto von Holst (Research Center Borstel, Borstel, Germany), Michael Martin (Justus-Liebig-University Giessen Institute for Immunology, Giessen, Germany), Michael Kracht (Justus-Liebig-University Giessen, Institute for Pharmacology, Giessen, Germany), and Marion Meixner (Justus-Liebig-University Giessen, Department of General and Thoracic Surgery, Laboratory of Experimental Surgery) for helpful suggestions. We are grateful to our volunteers for blood donation.

Disclosures
The authors have no financial conflicts of interest.

References


### Supplemental Table S1: Primers used for real-time RT-PCR

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<th>Reverse primer (5'–3')</th>
<th>Product (bp)</th>
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bp, base pairs; CHRNA, nicotinic acetylcholine receptor alpha; PBGD, porphobilinogen deaminase
**Supplemental Table S2: Release of lactate dehydrogenase (LDH)**

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<td>-</td>
<td>3.4 ± 2.2%</td>
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</tr>
<tr>
<td>LPS</td>
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U937 cells were primed with lipopolysaccharide (LPS) from *Escherichia coli* for 5 h. BzATP was added for another 30 min in the presence or absence of nicotinic agonists or variants of LPS from different *Haemophilus influenzae* strains. LDH release into the cell culture supernatant is given as % of the total release. ACh, acetylcholine; NTHi, LPS from the lic1-mutant strain NTHi1233lic1; NTHiPC, PC-modified LPS from *Haemophilus influenzae* strain NTHi 1233; PC, phosphocholine; RM, LPS from the lic1-mutant strain RM7004-Lic1; RMPC, PC-modified LPS from *Haemophilus influenzae* strain RM118.
Fig. S1: Expression of pro-IL-1β and acetylcholine receptor mRNA. (A) U937 cells were incubated for 5 h with 1 µg/ml lipopolysaccharide (LPS) and pro-IL-1β mRNA expression was measured by real-time RT-PCR and compared to untreated control cells. Data are presented as individual data points, bars indicate median, whiskers percentiles 25 and 75; Mann-Whitney rank sum test. (B) Nicotinic acetylcholine receptors CHRNA7, CHRNA9 and CHRNA10 as well as the inactive duplicate CHRFAM7A were detected by real-time RT-PCR. Products obtained by real-time RT-PCR from untreated and LPS-primed U937 were separated in agarose gels. (C) Real-time RT-PCR products from freshly isolated human peripheral blood mononuclear cells (PBMC). In negative controls, cDNA was replaced by water.
Fig. S2: Reduction of CHRNA7, CHRNA9 and CHRNA10 mRNA expression by siRNA transfection. U937 cells were treated with control siRNA (scr) or siRNA targeting nicotinic receptors and the mRNA expression of (A) CHRNA9 and (B) CHRNA10 was measured by real-time RT-PCR 6 h later. The mRNA expression of CHRNA7 was too low for quantification. (C) Control cells without siRNA, transfection with control siRNA (scr) or with siRNA targeting CHRNA9 did not affect the release of IL-1β from unprimed cells, from cells primed with LPS, and from LPS-primed cells stimulated with BzATP. The inhibitory effect of nicotine, however, was blunted in cells treated with siRNA against CHRNA9 but not after transfection of scrambled siRNA. A part of these data is also shown in Figure 2 C. Data are presented as individual data points, bars indicate median, whiskers percentiles 25 and 75; * p ≤ 0.05 (A-B) versus scr siRNA; # p ≤ 0.05 significantly different from respective cells treated with LPS and BzATP but not with nicotine, Mann-Whitney rank sum test (A-B) or Kruskal-Wallis test followed by Mann-Whitney rank sum test (C).