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Cotinine-induced convergence of the cholinergic and PI3 kinase-dependent anti-inflammatory pathways in innate immune cells

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Abstract

Nicotine [(*S*)-3-(1-methyl-2-pyrrolidinyl)pyridine] is a major component of tobacco and a highly efficient acetylcholine receptor (nAChR) agonist that triggers the cholinergic anti-inflammatory pathway. We demonstrate that pre-treatment of monocytes with the stable nicotine catabolite, cotinine [(*S*)-1-methyl-5-(3-pyridinyl)-2-pyrrolidinone], dramatically alters the nature of the inflammatory response to Gram negative bacteria by abrogating the production of cytokines that are under the transcriptional control of the NF-κB system (TNF-α, IL-1β, IL-6, IL-12/IL-23 p40) and shifting the response towards an IL-10-dominated anti-inflammatory profile. This anti-inflammatory phenomenon is initiated specifically by engagement of the monocytic α7 nAChR; and is PI3K/GSK-3β-dependent; but NF-κB-independent. These mechanistic insights suggest an ability to exploit convergent, endogenous anti-inflammatory pathway(s) to either up-regulate or down-regulate the production of specific cytokine groups (pro- or anti-inflammatory cytokines) depending on the clinical necessity.
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Keywords: Cholinergic anti-inflammatory pathway; Cotinine; Cytokine; Inflammation; Monocyte; PI3K-dependent anti-inflammatory pathway; Tobacco smoking

1. Introduction

Components of Gram negative bacteria, and particularly lipopolysaccharides (LPS), are potent inducers of the inflammatory response. LPS recruits, activates, and promotes degranulation events in the most numerous inflammatory leukocyte, the neutrophil. LPS and neutrophil degranulation products each recruit monocytes and macrophages to the locus of infection. While neutrophils are, in relative terms, short-lived and transcriptionally quiescent, monocytes/macrophages are longer-lived cells that when stimulated by LPS, and other inflammatory mediators, produce large amounts of pro-inflammatory cytokines, including TNF, IL-1β, IL-6, IL-12/IL-23 p40, IL-18, and HMGB-1 de novo. These macrophage-derived mediators amplify and direct the inflammatory response and link the innate and adaptive immune responses.

The ability to regulate against prolonged or excessive inflammation is critical in preventing the onset of septic shock and the host-mediated damage associated with multiple chronic inflammatory diseases. However, the mechanisms that dictate the establishment of pro- versus anti-inflammatory cytokine-dominated environments are poorly understood. In recent years it has become clear that the nicotinic acetylcholine receptor α7 subunit is a critical regulator of inflammation [1,2]. Acetylcholine, produced by the vagus nerve network, is an endogenous α7 nAChR agonist. Acetylcholine and the exogenous α7 nAChR agonist, nicotine, both suppress the release of TNF-α from activated cells of monocytic lineage and are protective against LPS-mediated toxicity [2–5]. Thus, nAChRs and downstream components of the cholinergic anti-inflammatory pathway present novel therapeutic targets for controlling inflammatory diseases.

However, we are only beginning to understand the mechanisms of action of acetylcholine and nicotine in innate immune suppression. Furthermore, nicotine is rapidly converted into multiple

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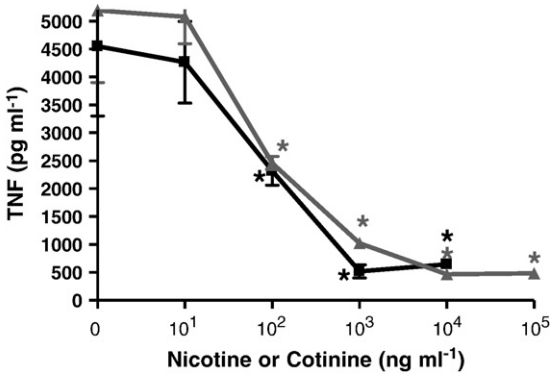

Fig. 1. Human monocytes treated with nicotine or cotinine produce significantly less TNF-α when stimulated with *P. gingivalis*. Monocytes were pre-treated for 2 h with nicotine (triangles) or cotinine (squares) then stimulated with *P. gingivalis* (MOI=10) for 20 h. Cell-free supernatants were harvested by centrifugation and levels of TNF-α were determined by ELISA. The alkaloid suppression of TNF production is dose-dependent. Mean nicotine concentrations in smokers would be expected to be in the region of 30 ng ml^{-1} with mean cotinine levels in smokers in the region of 200–500 ng ml^{-1} [6]. Systemic cotinine levels of 1–15 ng ml^{-1} are found in second-hand smokers [6]. *P. gingivalis* is a Gram negative periodontal pathogen. Similar results are seen with *E. coli* and purified LPS (data not shown). Data are the arithmetic mean±s.d. of 3 experiments. *Indicates statistical significance at $p<0.05$, as compared to cells stimulated with *P. gingivalis* alone. Cotinine (0 to 10^5 ng ml^{-1}) had no statistically significant effect on the viability of primary monocytes, compared to *P. gingivalis* alone.

metabolites in humans. While the pharmacological properties of nicotine have been extensively studied, the major proximate metabolite – cotinine – has received relatively little attention. Yet, cotinine is a much more stable molecule and systemic concentrations approach ten fold that of nicotine [6].

Like nicotine, cotinine evokes striatal dopamine release [7]; appears to improve working memory and attention [8]; is neuroprotective [8]; and suppresses the release of free radicals from neutrophils [9]. Thus, cotinine is clearly pharmacologically active. However, cotinine is unlike nicotine in key physiological aspects. Cotinine does not appear to up-regulate the expression of nicotinic receptors in the brain [8]; it may not be vasoactive, as monitored by changes in heart rate, blood pressure, or skin temperature in response to cotinine infusions in humans [10,11]; it is considered non-addictive [12]; and the administration of cotinine to humans at levels as high as 10 times than that attained from cigarette smoking has been shown to be safe [12].

We hypothesized that because of the structural similarities between nicotine (3-(1-methyl-2-pyrrolidinyl) pyridine) and cotinine (1-methyl-5-[3-pyridynl]-2-pyrrolidinone), cotinine would have anti-inflammatory properties.

2. Materials and methods

2.1. Isolation and culture of primary monocytes

Whole human blood was purchased from Lampire Biological Laboratories (Pipersville, PA). Primary monocytes were isolated by an indirect magnetic monocyte isolation kit (Miltenyi Biotec, Auburn, CA) as we have previously reported [13,14]. This procedure routinely results in >95% pure $CD14^+$ cells, as shown by flow cytometry. Human monocytes were cultured at 37 °C and 5% CO_2 atmosphere, in complete RPMI (RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.05 mM 2-mercaptoethanol [Invitrogen Life Technologies, Carlsbad, CA]) plus or minus stimulating agents, as described below.

2.2. Growth of Porphyromonas gingivalis

P. gingivalis ATCC 33277 was grown in modified Gifu anaerobic medium (Nissui Pharmaceutical Company, Tokyo, Japan) under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37 °C.

2.3. Cytokine release by monocytes

Mean nicotine concentrations in smokers would be expected to be in the region of 30 ng ml^{-1} with mean cotinine levels in smokers in the region of 200–500 ng ml^{-1} [6]. Systemic cotinine levels of 1–15 ng ml^{-1} are found in second-hand smokers [6]. Primary monocytes were pre-treated with nicotine (0–100 ng ml^{-1}) or cotinine (0–1000 ng ml^{-1}) then stimulated with the Gram negative periodontal pathogen *P. gingivalis* (MOI=0–10); purified *Escherichia coli* K-12 LPS (0–1 μg ml^{-1}; InVivoGen, San Diego, CA); or purified *P. gingivalis* 33277 LPS for 20 h. Primary monocytes were also pre-treated with cotinine (100 ng ml^{-1}) then stimulated with increasing doses of LPS for 20 h. Cell-free supernatants were harvested by centrifugation and cytokine levels (TNF, IL-1β, IL-6, IL-10, IL-12/IL-23 p40, as appropriate) were determined by ELISA (eBioscience, San Diego, CA or R&D Systems, Minneapolis, MN).

2.4. Expression of α7 nACh receptors

Total primary monocyte cell lysate (40 μg of protein) western blots were probed with an anti-α7AChR specific antibody (Q4A163R, Biodesign International, Saco, ME) recognizing amino acids 493–502 at the C terminus. Promyelocytic HL-60 cells served as a positive control. Proteins recognized by the anti-α7AChR specific antibody were detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

2.5. NF-κB in cotinine-induced inflammatory suppression

THP-1 Blue cells are monocyte-like cells that have been stably transfected with a reporter plasmid expressing secreted embryonic alkaline phosphatase (SEAP) gene under the control of a NF-κB-inducible promoter. THP-1 and THP-1 Blue cells (InVivoGen, San Diego, California) were pre-treated with cotinine (100 ng ml^{-1}) for 2 h then stimulated with *E. coli* LPS (1 μg ml^{-1}) for 24 h. Cell-free supernatants were harvested by centrifugation and relative expression levels of NF-κB and IL-10 were determined by spectrophotometric analysis of SEAP activity and ELISA, respectively.

2.6. The cholinergic anti-inflammatory pathway in cotinine-induced inflammatory suppression

The importance of α7 nAChR in cotinine-induced modulation of cytokine release profiles was assessed using the selective nAChR antagonist, α-bungarotoxin (α-BTX; 2 μg ml^{-1}; Sigma Chemical Company, St. Louis, MO). Of the α-BTX-sensitive nAChRs, monocytes express only α7 nAChR [1,2,15,16].

2.7. The PI3K-dependent anti-inflammatory pathway in cotinine-induced inflammatory suppression

Levels of phosphorylated Akt and phoshorylated GSK-3β following cotinine (0–100 ng ml^{-1}) and/or *E. coli* LPS (1 μg ml^{-1}) treatment were determined by western blot using whole-cell lysates (20 μg) with probing for Akt using a phospho-specific Akt (Ser473) antibody; probing for GSK-3β using a phospho-specific GSK3-β (Ser9; denoted pGSK-3β) antibody; with the blots stripped and re-probed for total p38 to ensure equivalent loading. Antibodies were purchased from Cell Signaling, Beverly, MA. Blots were visualized by enhanced chemiluminescence. The importance of the PI3K-dependent anti-inflammatory pathway in cotinine-induced inflammatory suppression was also established by

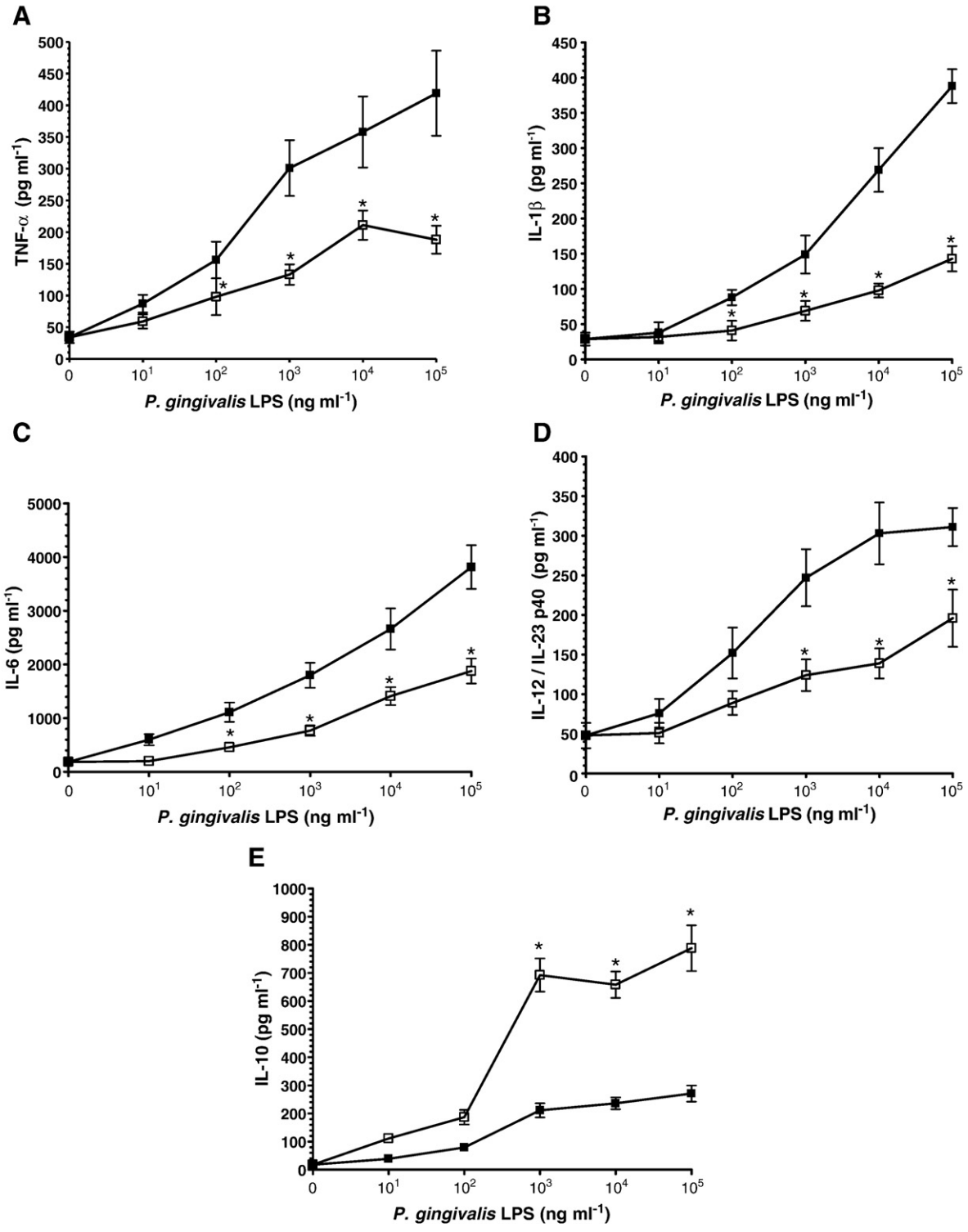

Fig. 2. Cotinine inhibits the production of multiple pro-inflammatory cytokines in *P. gingivalis*-stimulated human monocytes (A–D) but augments IL-10 production (E). (A–E) Control monocytes (closed squares) and monocytes pre-treated with cotinine (100 ng ml^{-1}) for 2 h (open squares) were stimulated with *P. gingivalis* 33277 LPS (0–10^5 ng ml^{-1}) for 24 h. Cell-free supernatants were harvested by centrifugation and levels of pro-inflammatory cytokines (A–E) and IL-10 (E) were determined by ELISA. Data are the arithmetic mean ± s.d. of 3 experiments. Statistical significance was set at $p < 0.05$, and cotinine altered the production of all cytokines tested, as compared to cells stimulated with LPS alone. Cotinine (100 ng ml^{-1}) had no statistically significant effect on the viability of primary monocytes, compared to LPS alone.

pharmacological inhibition of PI3K and GSK-3β using the PI3K inhibitors LY294002 (25 μM; Calbiochem, San Diego, CA) and wortmannin (100 nM, Calbiochem); and the GSK-3β inhibitor SB216763 (6 μM; Sigma). Activated PI3K produces PIP3 that, in turn, activates downstream signaling components of the PI3K pathway. Therefore, cells were also analyzed for cotinine-induced PI3K activation by assaying the ability of PI3K to produce PIP3. Levels of PIP3 were

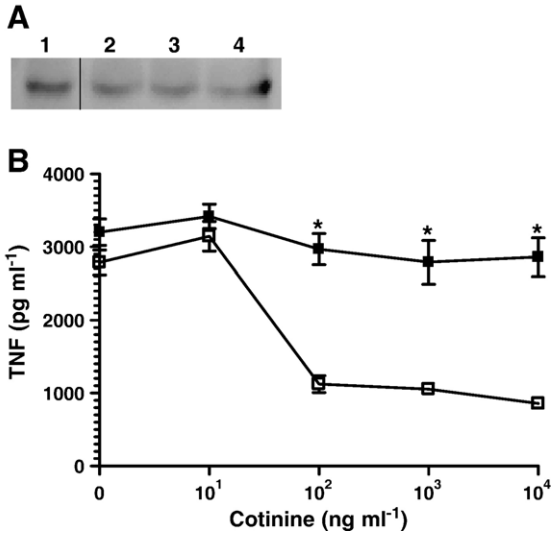

Fig. 3. α-Bungarotoxin treatment of α7AChR-expressing human monocytes abrogates the anti-inflammatory effects of cotinine. (A) Total cell lysate (40 μg of protein) blots of primary monocytes (Lanes 2–4) and a positive control (promyelocytic HL-60 cells; Lane 1) were probed with an anti-α7AChR specific antibody (Q4A163R, Biodesign Intl.) recognizing amino acids 493–502 at the C terminus. Monocytes expressed α7 nACh receptors with relative molecular masses of 55 kDa. (B) Control monocytes (open squares) and monocytes pre-treated for 30 min with the α7 nACHR antagonist α-BTX (2 μg ml^{-1}, closed squares) were incubated for 2 h with various concentrations of cotinine. Cells were then stimulated with *P. gingivalis* (MOI = 10) for 20 h. Cell-free supernatants were harvested by centrifugation and levels of TNF were determined by ELISA. Data are the arithmetic mean ± s.d. of 3 experiments. *Indicates statistical significance at $p < 0.05$, as compared to control cells.

determined by ELISA using a competitive inhibition assay (Echelon Bioscience, Salt Lake City, UT). The specific PI3K inhibitor LY294002 was used at 25 μM and served as a control to block PI3K activity.

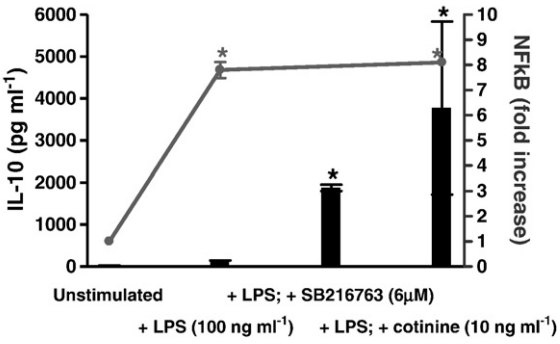

Fig. 4. The anti-inflammatory potential of cotinine is a GSK-3β-dependent but NF-κB-independent phenomenon. THP-1 Blue cells are stably transfected with a reporter plasmid expressing secreted embryonic alkaline phosphatase (SEAP) gene under the control of a NF-κB-inducible promoter. THP-1 and THP-1 Blue cells were pre-treated with cotinine (100 ng ml^{-1}) for 2 h then stimulated with LPS (1 μg ml^{-1}) for 24 h. Cell-free supernatants were harvested by centrifugation and relative expression levels of SEAP (reflecting NF-κB; THP-1 Blue; circles) and IL-10 (THP-1; bars) were determined by spectrophotometric analysis of SEAP activity and ELISA, respectively. Here we also show that the GSK-3β inhibitor – SB216763 – enhances IL-10 production in response to LPS, but it is less potent than even low doses of cotinine (10 ng ml^{-1}; $p < 0.05$). Induction of NF-κB on stimulation with LPS is not influenced by cotinine treatment. All data are the arithmetic mean ± s.d. of 3 experiments. *$p < 0.05$; compared to unstimulated cells. Cotinine (100 ng ml^{-1}) had no statistically significant effect on the viability of THP-1 or THP-1 Blue cells compared to LPS alone.

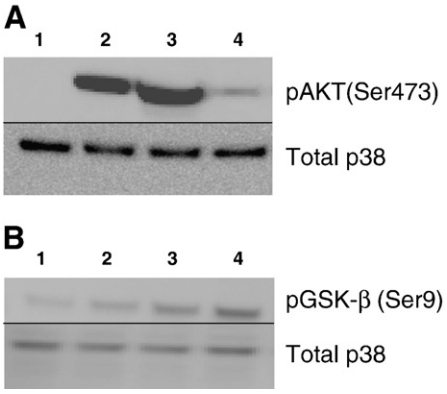

Fig. 5. Cotinine (A) enhances Akt signaling and (B) augments levels of phosphorylated (Ser9) GSK3-β in *P. gingivalis*-stimulated monocytes. (A) Monocytes were pre-treated for 2 h with cotinine then stimulated for 60 min with LPS (1 mg ml^{-1}). Western blot was performed using whole-cell lysates (20 μg) with probing for Akt using a phospho-specific Akt (Ser473) antibody. Blot was stripped and re-probed for total p38 to ensure equivalent loading. Lane 1: Non-stimulated; Lane 2: LPS only; Lane 3: LPS + cotinine (100 ng ml^{-1}), Lane 4: LPS + cotinine + wortmannin (a PI3K inhibitor, 100 nM). Data are representative of three experiments. Similar results were seen with 60 min stimulations with *P. gingivalis* (MOI = 10; data not shown). (B) Monocytes were pre-treated for 2 h with cotinine then stimulated for 60 min with LPS (1 mg ml^{-1}). Western blot was performed using whole-cell lysates (20 μg) with probing for GSK-3β using a phospho-specific GSK3-β (Ser9; denoted pGSK3) antibody. Blot was stripped and re-probed for total p38 to ensure equivalent loading. Lane 1: Non-stimulated; Lane 2: LPS only; Lane 3: LPS + cotinine (10 ng ml^{-1}), Lane 4: LPS + cotinine (100 ng ml^{-1}). Data are representative of three experiments. Similar results were seen with 60 min stimulations with *P. gingivalis* (MOI = 10; data not shown).

2.8. Convergence of the cholinergic and PI3 kinase-dependent anti-inflammatory pathways during cotinine-induced inflammatory suppression

Interactions between the cholinergic and PI3 kinase-dependent anti-inflammatory pathways were examined by assessing the efficacy of α-BTX (2 μg ml^{-1}) to block activation of the PI3K pathway. PIP3 levels were monitored by ELISA following 60 min with LPS (1 μg ml^{-1}) in the presence and absence of cotinine (0–100 ng ml^{-1}).

2.9. Statistical approaches

Statistical significance between groups was evaluated by ANOVA and the Tukey multiple-comparison test using the InStat program (GraphPad Software, San Diego, CA). Differences between groups were considered significant at the level of $p < 0.05$.

3. Results

3.1. Cotinine inhibits TNF release

Cotinine is equally as effective as nicotine at inhibiting TNF release (Fig. 1). Indeed cotinine blocks more than 80% of bacteria-induced TNF release from monocytes at a dose of 100 ng ml^{-1} (Fig. 1), a level commonly observed in cigarette smokers [6].

3.2. Cotinine promotes an anti-inflammatory phenotype

Cotinine also dramatically suppresses the release of multiple pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and the common

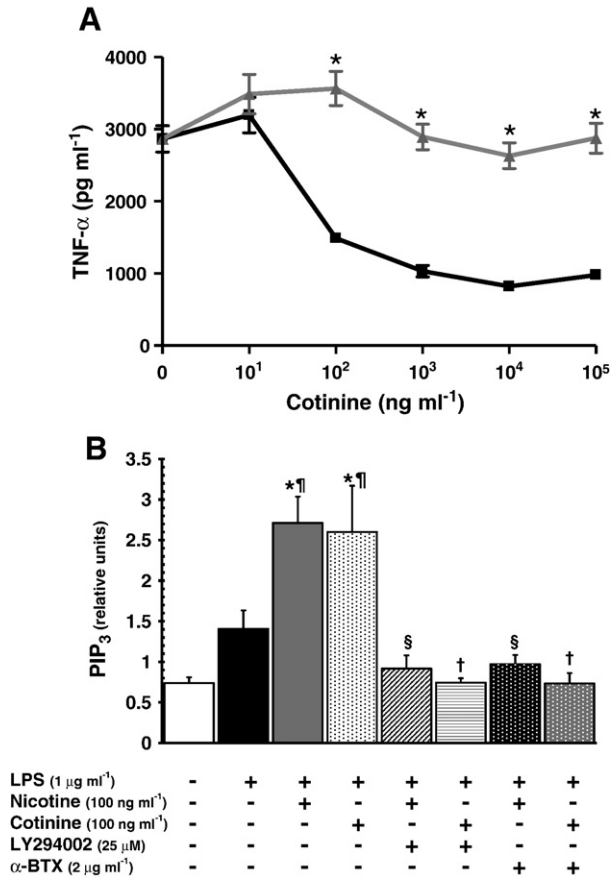

Fig. 6. Innate immune suppression by cotinine requires the α7 nAChR-dependent enhancement of PI3K activation. (A) Inhibition of PI3K using LY294002 abrogates the anti-inflammatory effects of cotinine. Cells were pre-treated with (closed triangles) and without (closed squares) 25 μM of the PI3K inhibitor LY294002 for 30 min followed by 2 h with various concentrations of cotinine. Following 24 h *P. gingivalis*-stimulation (MOI=10), cell-free supernatants were harvested by centrifugation and levels of TNF-α were determined by ELISA. *Indicates statistical significance at $p<0.05$, as compared to cells stimulated with *P. gingivalis* alone (no LY294002). Data are the arithmetic mean±s.d. of 3 experiments. All media used in these experiments contained 0.01% DMSO to act as the organic solvent control used to solubilize LY294002. Similar results are also seen with the alternative PI3K inhibitor, wortmannin (100 nM; data not shown). (B) Cotinine (or nicotine) treated monocytes exhibit increased PIP3 levels when stimulated with LPS in an α7 nAChR-dependent manner. Cells were stimulated for 60 min with 1 μg ml^{-1} of LPS. Cells were analyzed for PI3K activation by the ability of PI3K to produce PIP3. Activated PI3K produces PIP3 that, in turn, activates downstream signaling components of the PI3K pathway. Levels of PIP3 were determined by ELISA using a competitive inhibition assay (Echelon Bioscience). The specific PI3K inhibitor LY294002 was used at 25 μM (cells were pre-treated for 1 h) and served as a control to block PI3K activity. α7 nAchR-dependence was established through the use of the α7 nAChR antagonist, α-bungarotoxin. *Indicates statistical significance at $p<0.05$, as compared to unstimulated control cells. ¶ Indicates statistical significance at $p<0.05$, as compared to cells stimulated with LPS alone. § Indicates statistical significance at $p<0.05$ for cells stimulated with LPS, nicotine and LY294002 (or α-BTX) compared to LPS and nicotine only. † Indicates statistical significance at $p<0.05$ for cells stimulated with LPS, cotinine and LY294002 (or α-BTX) compared to LPS and cotinine only. Data are the arithmetic mean±s.d. of 3 experiments.

IL-12/IL-23 p40 subunit) from monocytes (Fig. 2A–D) while simultaneously enhancing production of the key anti-inflammatory cytokine, IL-10 (Fig. 2E).

3.3. Cotinine-induced inflammatory suppression likely occurs via α7 nAChR

Of the α-bungarotoxin sensitive human nAChRs (α1, α7, and α9), monocytes and macrophages express only functional α7 receptors [1,2,15,16]. The α7 receptor expressed by primary macrophages has a relative molecular mass of 55 kDa by western blot (Fig. 3A), identical to that of the neuronal receptor [17,18]. Cotinine-induced suppression of TNF release was abrogated by pre-treatment with α-bungarotoxin (Fig. 3B), suggesting that the anti-inflammatory activity of cotinine may be mediated via the nicotinic acetylcholine receptor α7 subunit. The importance of α7 nAChR in cotinine-induced immunomodulation is also suggested by the ability of α-bungarotoxin to block cotinine enhancement of IL-10 release (Fig. 7) and to abrogate cotinine-induced alterations to PI3K activity (measured as PIP_3 release; Fig. 6).

3.4. The anti-inflammatory potential of cotinine is a PI3K/GSK-3β-dependent but NF-κB-independent phenomenon

Physiologically relevant doses of cotinine augment IL-10 production in LPS-stimulated monocytes, yet LPS-induced NF-κB is not affected by cotinine (Fig. 4). Therefore, cotinine appears to act as an anti-inflammatory agent in an NF-κB-independent manner. We have previously shown that pharmacological inhibition of GSK-3β enhances IL-10 production in response to LPS [13], and confirm this finding herein (Fig. 4).

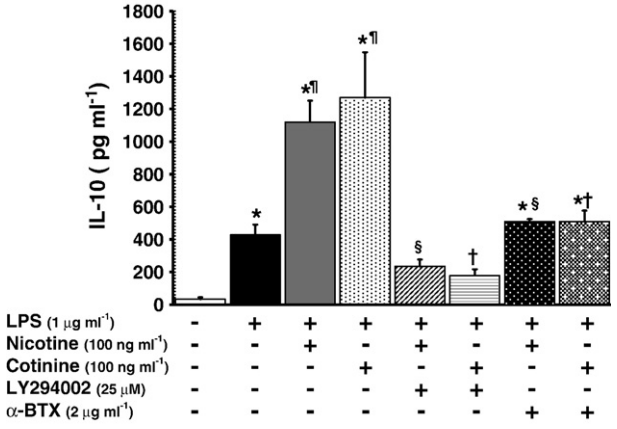

Fig. 7. Enhancement of IL-10 release by cotinine requires the α7 nAChR-dependent enhancement of PI3K activation. Inhibition of PI3K using LY294002 abrogates the anti-inflammatory effects of cotinine and nicotine, as assessed by IL-10 release. Cells were pre-treated with and without the PI3K inhibitor LY294002 (25 μM); and with or without α-bungarotoxin (2 μg ml^{-1}) for 30 min, followed by 2 h with nicotine (100 ng ml^{-1}) or cotinine (100 ng ml^{-1}). Following 24 h LPS stimulation (2 μg ml^{-1}), cell-free supernatants were harvested by centrifugation and levels of IL-10 were determined by ELISA. *Indicates statistical significance at $p<0.05$, as compared to unstimulated control cells. ¶ Indicates statistical significance at $p<0.05$, as compared to cells stimulated with LPS alone. § Indicates statistical significance at $p<0.05$ for cells stimulated with LPS, nicotine and LY294002 (or α-BTX) compared to LPS and nicotine only. † Indicates statistical significance at $p<0.05$ for cells stimulated with LPS, cotinine and LY294002 (or α-BTX) compared to LPS and cotinine only. Data are the arithmetic mean±s.d. of 3 experiments.

However, SB216763 inhibition of GSK-3β is a significantly less potent inducer of IL-10 than cotinine (Fig. 4). To explore this further, we examined the effects of cotinine on the activation state of Akt (Ser473) and GSK3-β (Ser9). As shown in Fig. 5, cotinine enhances levels of phosphorylated (active) Akt in a PI3K-dependendent manner and augments levels of phosphorylated (inactive) GSK3-β in *P. gingivalis*-stimulated monocytes.

3.5. The cholinergic and PI3K anti-inflammatory pathways are convergent on cotinine stimulation

Cotinine potently augments the activation of PI3K and the production of the anti-inflammatory cytokine IL-10. In contrast, blockade of PI3K abrogates the ability of cotinine to suppress the inflammatory response (TNF-α) in *P. gingivalis*-stimulated cells (Figs. 6A and 7). Moreover, inhibition of the α7 nAChR-signaling pathway abrogated the ability of cotinine to enhance PI3K activity (Fig. 6B).

4. Discussion

Borovikova et al have demonstrated that electrical stimulation of the vagus nerve protected against death by LPS toxicity in mice, and showed that acetylcholine significantly attenuated the release of TNF, IL-1β, and IL-6 from macrophages [3]. It is important to note, however, that such vagal protection is abrogated by splenectomy [19]. Specifically it is the α7 nACh receptor subtype that appears key to acetylcholine-induced inflammatory suppression, as we and others have recently reviewed [20–23]. α7 nAChR-deficient mice are not only hypersensitive to LPS, producing high amounts of TNF-α, they also exhibit an exaggerated production of the pro-inflammatory cytokines IL-1β and IL-6 [4,24]. Furthermore, nicotine-dependent suppression of TNF release from primary macrophages is abrogated by α7 AChR-specific antisense oligonucleotides [4], while selective α7 AChR agonists protect against severe sepsis [25]. It should be noted that a limitation of the present study is that we have not confirmed our data, obtained from primary human cells, in vivo.

Functional AChRs are pentameric and are composed of multiple combinations of a possible 16 monomer subtypes (α1–7; α9–10; β1–4; δ; ε; and γ) that exhibit divergent pharmacological behaviors [4,16,26]. nAChR subtype expression by monocytes and macrophages is highly restricted. The α7 receptor expressed by primary macrophages has a relative molecular mass of 55 kDa by western blot, identical to that of the neuronal receptor [17,18]. Of the known AChRs, α7 nAChR exhibits a number of unusual features [27]. First of all, it can assemble and function as a homopentamer [27,28]; the ion channel exhibits high permeability for calcium ions in preference to Na^+ [29]; and it is widely expressed in the central and peripheral nervous system [26] as well as on leukocytes [4]. αBTX is not specific for α7 nAChR. However, of the α-bungarotoxin sensitive human nAChRs (α1, α7, and α9), monocytes and macrophages express only functional α7 receptors [1,2,15,16]. We have been able to use α-bungarotoxin to strongly suggest that cotinine-induced inflammatory suppression occurs through the nicotinic acetylcholine receptor α7 subunit. Definitive confirmation of the role of α7 nAChR will require further experimentation, such as utilizing antisense or silencing RNA techniques.

We extend existing knowledge by demonstrating that cotinine has similar anti-inflammatory properties to its addictive and toxic catabolic precursor, nicotine, showing that cotinine blocks the production of multiple pro-inflammatory cytokines that are known to be under the transcriptional control of NF-κB, including the innate/adaptive immunity bridging cytokine, IL-12/IL-23 p40 [30,31]; while simultaneously augmenting the release of the anti-inflammatory molecule, IL-10; and establishing that these actions of cotinine are likely to be mediated through the α7 nAChR. The suppression of the p40 subunit shared by IL-12 and IL23 is particularly interesting as it is critical for the development of IFN-γ-producing Th1 cells and influences cell-mediated immunity and IgG2a antibody production from B cells [32–35]. Recently, Chen et al have reported that alveolar macrophages from smokers exhibit a reduced pro-inflammatory response (TNF-α, IL-1β, IL-6, IL-8) to TLR-2 and TLR-4 agonists (Pam3Cys and LPS, respectively) compared to cells from non-smokers [36].

We next set out to address the mechanisms by which cotinine exerts anti-inflammatory effects on innate cells. The NF-κB pathway is critical in the activation of mononuclear phagocytes and in the production of multiple pro-inflammatory cytokines by these cells [30,31]. Limited evidence suggests that nicotine maintains cytoplasmic concentrations of IκB and thus prevents LPS-induced NF-κB activation in monocytes and macrophages, in a dose-dependent manner [3,4,37]. One recent report suggests that smoking inhibits the TLR-2/-4 inducible phosphorylation of IRAK-1 and p38 and also inhibits IκB-α degradation [36]. However, our data shows that cotinine appears to act as an anti-inflammatory agent in an NF-κB-independent manner, as physiologically relevant doses of cotinine augment IL-10 production in LPS-stimulated monocytes, yet LPS-induced NF-κB is not affected by cotinine. Indeed, we demonstrate that cotinine regulates the phosphorylation of GSK3β (Serine 9) via the PI3K-Akt pathway. These data are consistent with previous studies by our laboratory that have shown that GSK3β does not affect the absolute or phosphorylated (activated) levels of NF-κB (p65) but affects the levels of CREB (Ser133) that displaces NF-κB (p65) from the transcriptional complex (CBP) [13]. We have also previously demonstrated that the levels of CREB (Ser133) are important for IL-10 production in TLR-stimulated cells but that the loss of NF-κB (p65) does not affect IL-10 [13].

Once phosphatidylinositol 3 kinase (PI3K) becomes activated, it catalyzes the production of phosphatidylinositol-3,4,5-trisphosphate [PIP3]. The generation of PIP3 subsequently allows for the recruitment and co-localization of phosphoinositide-dependent kinase 1 (PDK1) and the serine/threonine-dependent kinase Akt via their pleckstrin homology domains [38,39]. This co-localization to the plasma membrane allows for the activation of Akt via the phosphorylation of threonine 308 by PDK1 and phosphorylation at serine 473 by a still unidentified kinase called PDK2 [38–40]. Upon dual

phosphorylation, Akt becomes activated and phosphorylates a multitude of downstream targets including the serine phosphorylation that results in the inhibition of glycogen synthase kinase 3 (Ser21, GSK-3α; and Ser9 GSK-3β) [41]. Previous studies from our laboratories have identified that the phosphatidylinositol-3 kinase (PI3K) pathway plays a fundamental role in regulating the host inflammatory response to *P. gingivalis* by negatively regulating IL-12 p40/p70 production while concurrently augmenting IL-10 levels in a toll like receptor (TLR)-dependent manner. Sequential downstream mapping of the PI3K pathway identified that inactivation (by phosphorylation) of the constitutively active serine/threonine kinase, glycogen synthase kinase 3, via Akt, is central to the ability of this pathway to suppress the levels of pro-inflammatory cytokines while augmenting anti-inflammatory cytokine production (IL-10) [13]. Thus, the PI3K pathway can act as a central regulator in modulating the nature (pro- versus anti-inflammatory) and magnitude (absolute levels) of the host inflammatory response. However, at this stage it is important to note that this PI3K anti-inflammatory pathway is normally minimally engaged.

The ability to selectively augment anti-inflammatory cytokine production while concurrently suppressing pro-inflammatory cytokines provides a strong focal point for the elucidation, characterization, and application of potential therapeutic targets that could attenuate or modulate the inflammatory disease process. Thus, we confirmed our previous data that pharmacological inhibition of GSK-3β enhances IL-10 production in response to LPS. However, SB216763 inhibition of GSK-3β is a significantly less potent inducer of IL-10 than even low doses (10 ng ml^{-1}) of cotinine, suggesting a convergence of the cholinergic and GSK-3β-mediated anti-inflammatory pathways. Indeed, cotinine enhances levels of phosphorylated (active) Akt in a PI3K-dependent manner and augments levels of phosphorylated (inactive) GSK3-β in *P. gingivalis*-stimulated monocytes. Inhibition of the α7 nAChR-signaling pathway abrogated the ability of cotinine to enhance PI3K activity. Thus, cotinine augments the activity of PI3K via the α7 nAChR.

In summary, we have shown that, in primary human monocytes, cotinine is a potent anti-inflammatory agent that most likely acts through the α7 nAChR resulting in a convergence of the cholinergic anti-inflammatory pathway and an endogenous PI3K-dependent anti-inflammatory pathway in monocytes. The PI3K-dependent anti-inflammatory pathway in monocytes is normally minimally engaged, but signaling through this PI3K-Akt-GSK-3β route is amplified by cotinine—probably on engagement of α7 nAChR. As our knowledge of these signaling interactions increases, we are likely to identify further attractive anti-inflammatory targets.

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