The vagus nerve as a modulator of intestinal inflammation
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Deciphering STAT3 signaling in cholinergic inhibition of inflammation

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ABSTRACT

We previously reported that stimulation of the vagus nerve attenuates macrophage responses by activating the intracellular Jak2-STAT3 signaling pathway. Here, we further analyzed the potential of STAT3 to modulate TNF responses using STAT silencing strategies, pharmacological blockade, and dominant negative STAT3 constructs. We reveal that deletion of STAT3 using siRNA strategies augments the TNF production by endotoxin, confirming that STAT3 is a negative regulator of the immune response. On the other hand, pharmacological blockade of JAK2-STAT3 phosphorylation attenuates LPS-induced TNF production and NF-kB activation in macrophages. Expression of a mutant form that allows STAT3 tyrosine phosphorylation, but not STAT3 DNA binding, prevents the anti-inflammatory potential of nicotine. STAT3 inhibition specifically prevents the activation of the p65RelA/p50NF-kB1 pathway without affecting alternative other NF-kB signaling routes. These data suggest that nicotinic inhibition of inflammation in macrophages is dependent on STAT3 DNA binding and STAT3 protein, rather than STAT3 phosphorylation. Hypothetically, unphosphorylated STAT3 (U-STAT) can be important in mediating the anti-inflammatory effect of nAChR activation, via binding to NF-kB20 and inhibition of NF-kB-activation of TNF transcription.
INTRODUCTION

Vagal activity has been implicated in the negative regulation of inflammatory reactions via the peripheral release of acetylcholine. Vagus nerve activity either via electrical stimulation or ablation of vagal output, has been shown to affect the disease course in experimental animal models of inflammatory conditions such as sepsis\(^1\), post-operative ileus\(^2\), colitis\(^3\), peritonitis\(^4\) and arthritis\(^5\). This concept is based on the assumption that the vagus nerve, via peripheral release of the neurotransmitter acetylcholine (Ach), can regulate the immune response through activation of nicotinic acetylcholine receptors (nAChR) expressed on immune cells. At present, two nicotinic acetylcholine receptor (nAChR) subtypes have been put forward to be involved in this process: the nAChRa7 homopentamer\(^6\), and the α4β2 hetero pentamer\(^7\).

This so called ‘cholinergic anti-inflammatory pathway’ is characterized by a nicotine dose-dependent decrease in the production of pro-inflammatory mediators, including TNF, MIP2, IL-6 and HMGB1 by macrophages\(^2\). In conjunction, nAChR activation has shown to inhibit NF-κB transcriptional activity\(^8\). Besides an effect on inflammatory properties, the cholinergic nervous system also affects more professional macrophage functions such as endo- and phagocytosis of bacteria and particles\(^7\). Nicotine has already been used in clinical trials for inflammatory disorders such as ulcerative colitis, but the therapeutic potential of this mechanism is hampered by the collateral toxicity of nicotine\(^3\). Identification and specific targeting of the intracellular signaling pathway involved in the anti-inflammatory effect of nAChR activation would increase the translational potential of this mechanism to a great extent.

We previously evaluated the involvement of the janus kinase 2/signal transducer and activator of transcription 3 (JAK2-STAT3) pathway in mediating cholinergic anti-inflammatory effects in macrophages\(^2\). Mice, that specifically lack expression of STAT3 in their in macrophages and neutrophils, are highly susceptible to endotoxin shock and develop chronic enterocolitis\(^10\). Furthermore, production of inflammatory cytokines from STAT3-deficient macrophages is dramatically augmented in response to lipopolysaccharide\(^10\). These findings indicate that STAT3 is a potential negative regulator of inflammation.

In JAK2-STAT3 signaling, cytokine receptor activation induces tyrosine phosphorylation activation of JAK2, which leads to the phosphorylation of STAT3. Upon phosphorylation, STAT3 dimerizes and translocates to the nucleus to bind specific DNA sequences, to activate genes involved in diverse functions such as cell cycle progression, apoptosis and inflammation\(^4\). STAT3 activation is required for both IL-10 receptor (IL-10R) and IL-6 receptor (IL-6R) signaling\(^5\). IL6 receptor signaling is negatively regulated through the suppressor of cytokine signaling protein SOCS3, whose expression is induced by STAT3 activation\(^11,6\). We previously showed that nicotine induced STAT3 phosphorylation and SOCS-3 activation and failed to decrease TNF production in macrophages transfected with an inactive form of
STAT3. Moreover, stimulation of the vagus nerve failed to reduce inflammation in LysM-Stat3fl/fl mice. These data demonstrated that nicotine exerts its anti-inflammatory effect on peritoneal macrophages via Jak2-STAT3 signaling.

Here, we further analyze the role of the JAK2-STAT3 pathway in the anti-inflammatory potential of nAChR activation. The immunological implications of the JAK2-STAT3 pathway are analyzed in NF-κB signaling and cytokine production from macrophages. Our results confirm that Jak2-STAT3 signaling is involved in mediating cholinergic anti-inflammatory responses and add to the previous findings in STAT3 conditional knockout mice. However, these data show that this appears to be independent of STAT3 phosphorylation, and suggest there might be a role for unphosphorylated STAT3 in this process.

MATERIALS AND METHODS

**Chemicals and reagents.** AG490, Stattic, LPS and nicotine were purchased from Sigma- Aldrich (Zwijndrecht, the Netherlands). AG490 was dissolved in ethanol stock 20mM; and LPS dissolved in PBS stock 1 mg/ml (GIBCO, Invitrogen, CA). Anti-NFκB-p65, anti-actin and anti-stat3 antibodies were from Cell Signaling and HRP-conjugated anti-rabbit from DakoCytomation.

**Cell Culture.** Murine RAW264.7 cells (ATCC, Middlesex, UK) were grown in RMPI-1640 medium with antibiotics and L-glutamine (Gibco, Breda, The Netherlands), supplemented with 10% heat-inactivated fetal bovine serum at 37ºC in a humidified incubator with 5% CO2.

**Stable transfection.** RAW264.7 macrophages were stably transfected with a NF-κB luciferase reporter construct (Clontech, Mountain View, CA) in which a PDNA3.1(+) derived neomycin resistance TK cassette was inserted (referred to as pNF-κBneoluc). Transfection was performed using electroporation. Briefly, 2*10^6 cells were resuspended in 100µL Nucleofector V reagent (Amaxa Biosystems Inc.) with 2 µg of DNA, mixed and electroporated using the Amaxa nucleofection device according to manufacturer’s instructions. Immediately after transfection, cells were cultured in RPMI 1640/10% FCS o/n. Transfected cells were selected in the culture medium supplemented with 1000ug/ml neomycin for 14 days. Resistant cells were subcloned and clones were cultured up to 20 passages with RPMI containing neomycin.

**Transient transfections.** The pCAGGS-neo expression vectors encoding hemagglutinin-tagged dominant negative mutants STAT3D, STAT3F or the empty expression vector (EV) were provided by I. Touw (Erasmus University, Rotterdam, The Netherlands). RAW264.7 cells were transfected with STAT3F, STAT3D, STAT3EV
reporter constructs using Jet PEI transfection reagent (PolyTransfection), according
to the manufacturer’s instructions. For shRNA transfection, Macrophages were
nucleofected using materials supplied in the Amaza Cell Line Nucleofector Kit V
(Lonza Inc). Briefly, 1 x106 cells were centrifuged and suspended with 100 ul of Cell
Line Nucleofector Solution V, in an Amaza-certified cuvette, using 0.25 to 2 nmol/ml
Non-Targeting DHARMACON siCONTROL (CAT# D-001206-13) or siGENOME
SMART pool STAT3 (Cat# M-003544-00) and the program V-001 (AMAXA
Biosystem nucleofector) for high transfection efficiency.

Cell stimulation. For experiments, cells were transferred to 48-well suspension
plates (Greiner-Bio, Alphen aan de Rijn, The Netherlands) at 2.5 x105 cells/well. After
overnight incubation, the medium was removed and replaced with RPMI containing
1% serum. Cells were pretreated with AG490, statitic or nicotine at the concentrations
indicated for 30 minutes, washed and subsequently stimulated with LPS for 3 h. Medium was harvested three hours after LPS stimulation and TNF levels were
analyzed using the TNF ELISA kit from R&D systems Inc (Abingdon, UK). For NF-kB luciferase measurement, the medium was removed 3 hours after LPS stimulation; the
cells were washed three times with ice-cold PBS and lysed with Passive Lysis Buffer
supplied in the LuciferaseTM Reporter Assay Kit (Promega Corporation, Madison,
WI ), the lysate was assayed for luciferase activity according to the manufacturer’s
instructions. Specific NF-κB protein binding to DNA was analyzed using the TransAM
DNA-Binding ELISA (Active Motif; Cambridge, MA) following the manufacturer’s
instructions.

Immunoblotting. Immunoblotting was performed routinely as described2. Nuclear
and cytosol extractions were prepared using the NucBuster Protein Extraction Kit
(Novagem), according to the manufacturer’s instructions.

Statistical Analyses. All data in the figures and text are expressed as mean ± standard
error (SEM). Statistical analyses were performed using the non-parametric Mann-
Whitney U test. A probability value (P) of less than 0.05 was considered significant.

RESULTS

Previous studies reveal that the anti-inflammatory potential of nicotine requires
STAT3 activation by α7nAChR2. First, we analyzed the specificity of this pathway
by using the α7nAChR-knockout and littermate wild-type mice. Nicotine inhibits
LPS-induced serum TNF levels in both wild-type and α7nAChR-knockout mice by
over 50% with a statistically similar efficiency (Fig.1A). These results suggest that the
immunomodulating actions of nicotine might not be exclusively mediated by the α7nAChR, but that other nAChRs may also play a role.

The transcription factor STAT3 is considered a potential negative regulator of the inflammatory response. Studies reveal that inhibition of STAT3 expression enhanced cytokine production in STAT3-deficient macrophages and in STAT3 conditional knockout mice. We performed similar strategy silencing STAT3 expression by using small interference siRNA. In agreement with previous studies, STAT3 silencing...
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in peritoneal RAW macrophages enhances LPS-induced TNF responses significantly (Fig. 1B). These results confirm that STAT3 is a negative regulator of the immune response.

**Pharmacological inhibition of the JAK2-STAT3 pathway blunts NF-κB activation**

Cytokine receptor activation induces tyrosine phosphorylation and subsequent activation of JAK2, leading to phosphorylation of STAT3. This is demonstrated in figure 2a, as endotoxin induced STAT3 phosphorylation in tyrosine, which is proportional to endotoxin concentration (Fig. 2A). To assess the role of JAK2-STAT3 signaling in modulating the LPS-induced inflammatory response in macrophages, we pharmacologically blocked JAK2 activation using the tyrosine kinase inhibitor AG490. The efficiency of the JAK2 inhibitor was confirmed with STAT3 phosphorylation. JAK2 inhibition with AG490 prevents LPS-induced STAT3 tyrosine (Y705) phosphorylation in a concentration dependent manner without affecting the STAT3 serine (S727) phosphorylation (Fig. 2B). In figure 2C, it is shown that JAK2 inhibition using AG490, blunts LPS-induced NF-κB transactivation in a dose-dependent way at a maxima activity of ~50% inhibition at 25µM. In line, JAK2 inhibition with AG490 reduces LPS-induced TNF production in RAW264.7 cells (data not shown). These results demonstrate that pharmacological inhibition of the JAK2-STAT3 signaling pathway reduces inflammatory responses in macrophages.

**STAT3 phosphorylation is not involved in cholinergic modulation of TNF release**

Above data reveal that deletion of STAT3 using siRNA strategies augments the TNF production by endotoxin. On the other hand, pharmacological blockade of JAK2-STAT3 signaling attenuates LPS-induced TNF production and NF-κB activation in macrophages. We previously reported that the JAK2-STAT3 pathway is involved in mediating the anti-inflammatory action of nicotinic receptor activation in peritoneal macrophages. Next, we sought to determine whether nAChR activation modulates the immune response via STAT3 phosphorylation, or via STAT3 DNA binding. To establish the role of STAT3 in the immune-modulatory actions of nicotine, we used the dominant negative constructs STAT3F, STAT3D and the empty vector. RAW264.7 cells were transfected with the mutant forms STAT3D or STAT3F, or with the empty vector as a control. STAT3F has the tyrosine 705 mutated by phenylalanine to prevent STAT3 tyrosine phosphorylation and subsequent dimerization, whereas STAT3D has the glutamic residues 434 and 435 replaced by alanine to allow tyrosine phosphorylation and dimerization but preventing STAT3 binding to DNA. Nicotine significantly reduced TNF production in peritoneal macrophages transfected with STAT3F or Empty Vector (Fig. 3). However, this anti-inflammatory
Figure 2. (A) RAW264.7 cells were treated with LPS for three hours, and STAT3 tyrosine (Y705) and serine (S727) phosphorylation were analyzed by Western-blot. Total STAT3 protein was used as a loading control. (B) JAK2 inhibition by AG490 prevents LPS-induced STAT3 tyrosine (Y705) phosphorylation in a concentration dependent manner. The upper panel shows a representative Western-blot, while the lower panel represents the densitometric data of three different experiments represented in mean ± STD. (C) RAW264.7 cells transfected with the NF-kB gene reporter were pretreated with different concentrations of the JAK2 inhibitor AG490 30 minutes prior to 3 hrs of 10ng/ml LPS stimulation. NF-kB activity was analyzed by luminescence.
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effect was prevented by transfection with STAT3D (Fig.3). This implies that the anti-inflammatory effect of nAChR activation is dependent on STAT3 DNA binding and the presence of STAT3 protein, rather than STAT3 phosphorylation.

**Figure 3.** Nicotinic inhibition of LPS-induced TNF production is dependent on STAT3 DNA binding, rather than STAT-3 phosphorylation. Peritoneal macrophages were transfected with control plasmid expressing empty vector (EV) STAT3 or the mutant forms expressing STAT3D (3D) or STAT3F (3F). Cell cultures were incubated with nicotine (Nico; 1µM), or vehicle, and stimulated with 10 ng/ml endotoxin for 3 hrs. TNF levels in the supernatants were analyzed by ELISA and values are expressed as the percent of TNF released compared to vehicle. Data are mean ± s.e.m. of three independent experiments done in duplicate. *, P < 0.05

**Nicotine and STAT3 modulate the classical p65RelA/p50NF-kB1 pathway**

To determine at what molecular level nicotine inhibits the NF-κB signaling pathway, we assessed the effect of nicotine on nuclear translocation of p65 subunits in stimulated RAW macrophages. In line with previous data, nicotine reduced LPS-induced NF-κB transactivation in RAW macrophages significantly (Fig.4A). As is shown in figure 4B, LPS stimulation increased translocation of NF-κB p65 to the cell nucleus, which was inhibited by nicotine pretreatment. Next, we analyzed whether the inhibition of STAT3 phosphorylation could modulate NF-kB. STAT3 inhibition with stattic, a well-characterized inhibitor of STAT3 phosphorylation, prevented the LPS-induced activation of p65RelA in peritoneal macrophages transfected with the NF-kB luciferase reporter (Fig 4C). The specificity of this inhibition was evaluated by analyzing the different NF-kB proteins using specific DNA probes for each pathway. LPS enhanced the NF-kB pathway by activating both p65 and p50 (Fig. 4C), and stattic prevented this activation. This inhibition of p65/p50 was specific as stattic failed to affect p52, c-Rel, and RelB.
Figure 4. Nicotine inhibits NF-κB transactivation and p65 translocation to the nucleus. (A) RAW macrophages expressing a NF-κB luciferase reporter construct were incubated with 1µM nicotine 30 min. prior to 100ng/ml LPS stimulation. NF-κB activity was determined after 3hrs LPS using luminescence. (B) Western analyses of p65 in nuclear and cytosolic fractions of RAW cells pretreated with nicotine (1µM) or medium prior to 30min 100ng/ml LPS stimulation. Asterisks indicate significant differences (P < .05) vs LPS. (C) RAW cells were pretreated with static, prior to LPS stimulation, and the specific NF-κB pathways p65RelA, RelB, c-Rel, p52 and p50 were analyzed by using the TransAM DNA-Binding. * represents p<0.01 vs LPS (n=3 experiments; One-way ANOVA with Bonferroni's corrections).
DISCUSSION

The JAK2-STAT3 pathway is an essential modulator of the immune response and plays also an important role in cell proliferation, migration, differentiation and apoptosis. However, the implications of this pathway in inflammatory disorders have remained elusive. We previously reported that the JAK2-STAT3 pathway is involved in mediating the anti-inflammatory action of nicotinic receptor activation in peritoneal macrophages\(^2\). Nicotine induced STAT3 phosphorylation and SOCS-3 activation and did not decrease TNF production in macrophages transfected with an inactive form of STAT3\(^2\). Moreover, stimulation of the vagus nerve failed to reduce inflammation in mice LysM-Stat3\(^{fl/fl}\) mice\(^2\).

The present study reveals that pharmacological inhibition of JAK2-STAT3 activation using AG490 prevents NF-κB transactivation and TNF production in peritoneal macrophages. In contrast, several studies demonstrate that genetic deletion of STAT3 increases inflammatory responses. Stat3 deficient macrophages secrete large amounts of cytokines including TNFa, IL-1, IL-6, and IL-12 in response to inflammatory stimuli. In addition, LysMcre/Stat3\(^{floxed/-}\) mice, which were designed for cell type-specific STAT3 disruption in macrophages and neutrophils, are born normal, but are very susceptible to endotoxemia\(^10\) and sepsis\(^17\), and develop lethal chronic inflammatory bowel disease at \(~\)20 weeks age\(^10\). Two factors appear to contribute to the higher inflammatory responses of these mice. First, STAT3-depletion sustains early inflammatory responses by inhibiting IL-10 signaling. The inhibitory activity of IL-10 on LPS-induced production of pro-inflammatory cytokines from STAT-3 deficient macrophages was completely blocked. Second, nontreated lysMcre/Stat3\(^{floxed/-}\) mice are characterized by macrophages which exhibit an constitutively activated phenotype. Similar to the results in conditional knockout mice, our results with siRNA confirm that STAT3 silencing enhances inflammatory responses.

The finding that pharmacological inhibition of the JAK2-STAT3 pathway reduces inflammation seems to be contradictory to the STAT3 silencing data. Possibly, the JAK2 inhibitor AG490 affects other signaling pathways as well. Indeed, it is shown that AG490 not only inhibits JAK2, but also JAK3 activity, STAT5\(^{a}\) and \(^{b}\) signaling\(^18\). These data suggest that AG490, especially at high dosages, can inhibit other signaling pathways than JAK2-STAT3 signaling alone.

We previously revealed that nicotine exerts its anti-inflammatory effects on macrophages via JAK2 mediated phosphorylation of STAT3. However, the interpretation of these data is not straightforward, because phosphorylation of STAT3 is fluctuating in time. Moreover, it appears that the use of immunoblotting as a readout for STAT3 activation does not correlate well with the response at the level of gene expression\(^8\). Therefore, the use of dominant negative constructs is a more reliable tool to determine the role of STAT3 phosphorylation in mediating the cholinergic anti-inflammatory effect. In RAW264.7 macrophages, we show that
nicotinic reduction of TNF activation does not depend on STAT3 phosphorylation, since nicotine still reduces TNF production in STAT3F transfected cells. In contrast, transfection with STAT3D does prevent the anti-inflammatory potential of nicotine. In STAT3D macrophages, glutamic residues 434 and 435 are replaced by alanine, that way inhibiting the DNA-binding activity of STAT by making dimers unable to bind the target DNA\textsuperscript{19}, while tyrosine phosphorylation and dimerization are allowed. This suggests that nicotinic inhibition of the inflammatory response is dependent on STAT3 DNA binding. However, we clearly showed that nicotine inhibits p65 translocation to the nucleus (Fig. 4B), which makes it more likely that STAT3 exerts its effect in the cytosol, rather than in the nucleus. Moreover, our results indicate that pharmacological inhibition of STAT3 prevents the activation of the p65RelA/p50NF-kB pathway without affecting the other NF-kB proteins including RelB, c-Rel and p52NF-kB2. In normal conditions, after JAK2 phosphorylates STAT3, it dimerizes and translocates into the nucleus. Together, our data indicate a role for unphosphorylated STAT3 (U-STAT) in mediating the anti-inflammatory effect of nicotine. In conjunction with this hypothesis, inhibition of STAT3 tyrosine phosphorylation by using STAT3F constructs can lead to higher levels of unphosphorylated U-STAT3. Recent studies indicate that U-STAT3 can bind to NF-kB in competition with IκBα, ultimately resulting in reduced NF-kB transactivation and cytokine production\textsuperscript{20}. This mechanism is completely abolished with inhibition of STAT3 expression leading to the enhanced activation of NF-kB observed in STAT3-knockout mice. Additional work is required to further explore this role of USTAT3 in the cholinergic anti-inflammatory response.

Identification of intracellular signaling pathways involved in mediating the anti-inflammatory effect of cholinergic agonists is important to specifically target the pathway and avoid toxic side effects. Our results confirm that JAK2-STAT3 signaling is required for nicotine induced reduction in NF-kB activation and cytokine production in peritoneal macrophages. However, STAT3 phosphorylation seems to be of less significance in this process than previously thought.

Furthermore, our results suggest that targeting JAK2-STAT3 signaling may provide pharmacologic advantages for the treatment of inflammatory disorders. However, the JAK2-STAT3 signaling pathway is a complex system which can act both pro-and anti-inflammatory, is tightly regulated and not fully understood yet\textsuperscript{9}. Characteristic examples of different responses are the differential expression of STAT3 isoforms in different cell types. Therefore, further examination is needed to establish the potential of targeting JAK2-STAT3 in restraining inflammation.
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REFERENCES


