The vagus nerve as a modulator of intestinal inflammation
van der Zanden, Esmerij
Stimulation of the vagus nerve attenuates macrophage activity by activating the JAK-2-STAT-3 signaling pathway

Wouter J. de Jonge
Esmerij P. van der Zanden
Frans O. Thé
Maarten F. Bijlsma
David J. van Westerloo
Roelof J. Bennink
Hans-Rudolf Berthoud
Satoshi Uematsu
Shizuo Akira
René MJGJ. van den Wijngaard
Guy EE. Boeckxstaens

ABSTRACT

Acetylcholine released by efferent vagus nerves inhibits macrophage activation. Here we show that the anti-inflammatory action of nicotinic receptor activation in peritoneal macrophages was associated with activation of the transcription factor STAT3. STAT3 was phosphorylated by the tyrosine kinase Jak2 that was recruited to the alpha7 subunit of the nicotinic acetylcholine receptor. The anti-inflammatory effect of nicotine required the ability of phosphorylated STAT3 to bind and transactivate its DNA response elements. In a mouse model of intestinal manipulation, stimulation of the vagus nerve ameliorated surgery-induced inflammation and postoperative ileus by activating STAT3 in intestinal macrophages. We conclude that the vagal anti-inflammatory pathway acts by alpha7 subunit-mediated Jak2-STAT3 activation.
INTRODUCTION

The innate immune response has been increasingly recognized as being under substantial neuronal control. For example, acetylcholine or nicotine effectively attenuates the activation of macrophages. This so-called ‘cholinergic anti-inflammatory pathway’ is characterized by a nicotine dose-dependent decrease in the production of proinflammatory mediators, including high-mobility group box 1 proteins, tumor necrosis factor (TNF), interleukin 1β (IL-1β), IL-6 and IL-18, by macrophages stimulated with endotoxin. Consistently, stimulation of the efferent vagus nerve dampens macrophage activation in rodent models of endotoxemia and shock. Two nicotinic acetylcholine receptor (nAChR) subtypes are involved in the nicotine-induced decrease in proinflammatory cytokine production by stimulated human and mouse macrophages: the α7 homopentamer expressed by monocyte-derived human and mouse macrophages, and the α4β2 heteropentamer expressed by alveolar macrophages. Activation of the α7 homopentamer nAChR inhibits transactivational activity of the transcription factor NF-κB p65. However, the subcellular mechanism explaining the deactivating effect of acetylcholine on macrophages has remained unknown.

Here we evaluated the involvement of the transcription factor STAT3 in this process, because STAT3 is a potential negative regulator of inflammatory responses. STAT3 and the tyrosine kinase Jak2, which phosphorylates STAT3, are required for both IL-6 receptor (IL-6R) and IL-10R signaling. IL-6 contributes to the progression of many inflammatory diseases, whereas IL-10 is an anti-inflammatory cytokine that suppresses the activation of macrophages. IL-6R signaling is inhibited by the Src homology 2 domain protein SOCS3, whose expression is induced by STAT3 activation. SOCS3 binds to the glycoprotein 130 (gp130) subunit of the IL-6R, leading to inhibited activation of STAT3 by IL-6R ligands. Consistent with that finding, in LPS-stimulated macrophages deficient in SOCS3, IL-6R ligands induce a sustained STAT3 activation, which leads to the reduced production of proinflammatory cytokines such as TNF.

Here we demonstrate that nicotine exerts its anti-inflammatory effect on peritoneal macrophages via Jak2 and STAT3 signaling in vitro and in vivo. In isolated peritoneal macrophages, nicotine activated nAChRs, leading to phosphorylation of STAT3 via Jak2. Jak2 was recruited to the α7 subunit of the nAChR and was phosphorylated after nicotine binding. We further studied the effect of cholinergic inhibition of macrophage activity in vivo on the occurrence of post-surgical intestinal inflammation in a mouse model of postoperative ileus. Postoperative ileus is characterized by general hypomotility of the gastrointestinal tract and delayed gastric emptying and is a pathological condition commonly noted after abdominal surgery with intestinal manipulation. This condition is the result of inflammation of the intestinal muscularis due to activation of resident macrophages that are triggered...
by bowel manipulation\textsuperscript{12}. We show here that perioperative stimulation of the vagus nerve prevented manipulation-induced inflammation of the intestinal muscularis externa and ameliorated postoperative ileus. The effectiveness of stimulation of the vagus nerve in reducing intestinal inflammation depended on STAT3 activation in macrophages in the intestinal muscularis. Hence, our data demonstrate the molecular pathway responsible for cholinergic inhibition of macrophage activation and suggest that stimulation of the vagus nerve or administration of cholinergic agents may be effective anti-inflammatory therapy for the treatment of postoperative ileus and other inflammatory diseases.

**MATERIALS AND METHODS**

**Reagents and antibodies.** Nicotine, Hexamethonium, α-Bungarotoxin, Methyllycaconitine citrate, (+)-Tubocurarine chloride hydrate, dihydro-β-erythroidine, AG490, cyclohexamin, actinomycin-D, and monoclonal rat anti-β2 were from Sigma-Aldrich. Polyonal rabbit antibodies against Jak-2, phosphorylated Jak-2 (PY\textsuperscript{1007-8} Jak-2), Socs-3 and α7 were obtained from Abcam (Cambridge, UK), goat polyclonal anti-actin, rabbit polyclonal anti-Stat-1, and anti-Stat-3 were from Santa Cruz Biotechnology (Santa Cruz, California), and rabbit polyclonal against phosphorylated Stat-1 and Stat-3 (PY\textsuperscript{705}) were from Cell Signaling Technology (Beverly, Maryland). **ELISAs for** IL-6, IL-10, MIP-1α, MIP-2 and TNF were from R&D Systems, (Minneapolis, Michigan).

**Cell culture and transient transfection.** Resident peritoneal macrophages were harvested from Balb/C mice by flushing the peritoneal cavity with 5 mL of ice-cold Hank’s Balanced Salt Solution containing 10 U/mL heparin. Peritoneal cells (1\times10^6 per cm\textsuperscript{2}) were plated in RPMI medium supplemented with 10% FCS and macrophages were left to adhere for 2 h in a humidified atmosphere at 37 °C with 5% CO\textsubscript{2}. Cells were washed and remaining macrophages were left for 16-20 h. Subsequently, cells were pre-incubated with the appropriate concentration of nicotine for 15 min, followed by LPS (1-100 ng/mL) challenge for 3 h. NachR blockers were added 30 min before nicotine, and no toxicity was observed after 4 h incubation of any blocker as assessed by tryphan blue exclusion test. Cells were lysed 30 min following nicotine/LPS exposure for immunoblotting. Transfection of peritoneal macrophages was performed using Effectine reagent (Qiagen, Cambridge, UK) according to the manufacturer’s instructions. A CMV driven Renilla luciferase reporter plasmid was co-transfected to assess transfection efficiency. The pCAGGS-neo expression vectors encoding wild type hemagluttinin (HA)-tagged Stat-3, or the dominant negative mutant HA-Stat-3D cDNA\textsuperscript{17} were kindly provided by Drs I. Touw and T. Hirano. In HA-Stat-3D, glutamic acids 434 and 435 were replaced by alanines\textsuperscript{17}. Following
transfection, cells were selected using neomycin (2.0 mg/mL; Sigma-Aldrich) for 16 h, washed, and treated with nicotine-LPS 24 h after transfection. Transfection was verified by immunoblotting using HRP-tagged anti-HA rabbit polyclonal antibodies (Abcam). For siRNA transfection, a siRNA oligonucleotide specific to Socs-3 (ID 160220; Ambion) was transfected using RNAiFect (Qiagen) according to the manufacturer's instructions. A FITC labeled control random RNA oligonucleotide (Ambion) was co-transfected to allow optimisation of transfection efficiency.

**Immunoblotting.** Cells were scraped in 50 µL of ice-cold lysis buffer containing 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, and 0.1% SDS. Samples were taken up in 50 µL sample buffer (125 mM Tris-HCl pH 6.8, 2% SDS, 10% β2-mercaptoethanol, 10% glycerol, and 0.5 mg/mL bromophenol blue), loaded onto SDS-PAGE gels and blotted onto PVDF membranes (Millipore). Membranes were blocked in TBS/0.1% Tween-20 (TBST) containing 5% non-fat dry milk and incubated overnight with appropriate antibodies in TBST/1% BSA. HRP-conjugated secondary antibodies were visualized using Lumilite plus (Boehringer-Mannheim, Germany).

**Immunoprecipitation.** Peritoneal macrophages (1*10^6 per cm^2) were scraped in lysis buffer (20 mM Tris-HCl pH 7.6, 2.5 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% sodiumdeoxycholate, 10% glycerol, 1 mM Na_3VO_4, 50 mM NaF, 1 µg/mL aprotinin, 1 µg/mL leupeptine, and 1 mM PMSF), sonicated for 10 s and centrifuged at 14000 x g at 4 °C for 20 min. Lysates, preabsorbed with 20 µL Protein A/G (Sigma-Aldrich), were incubated overnight with the appropriate antibodies, and immunoprecipitated with 40 µL of Protein A/G. Alternatively, immunoprecipitation was carried out using the TrueBlot™ system (eBioscience, San Diego, CA) according to the manufacturer's instructions. Immunoprecipitates were recovered by centrifugation, washed in ice-cold wash buffer (TBS, 0.1% Triton X-100, 1 mM PMSF), taken up in sample buffer (125 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, and 0.5 mg/mL bromophenol blue), and immunoblotted as described above.

**Surgical procedures.** Mice (female BalB/C) were used at 15-20 weeks of age. IL-6 and IL-10 deficient mice and their respective C57Bl wild types were obtained from Jackson Laboratories (Maine, USA). LysM-Cre Stat-3^fl/fl^ and Stat-3^fl/fl^ mice were maintained at Osaka University, Japan. Abdominal surgery with intestinal manipulation was performed as described elsewhere 12. Mice (n=10-12) were divided in 4 groups: 1-undergoing control surgery of only laparotomy (L), 2-laparotomy followed by intestinal manipulation (IM) combined with sham preparation of the cervical area, or 3-L- or 4-IM in combination with electrical stimulation of the vagus nerve. IM consisted of manipulation from the distal duodenum to the cecum during 5 min using sterile moist cotton applicators. At 3 or 24 h after surgery, mice were killed by cervical dislocation. Small intestine was removed, flushed, and fixed in ice-cold 100% ethanol.
for preparation of whole mounts. Small intestinal muscularis strips were prepared by pinning freshly isolated intestinal segments in ice-cold PBS and removal of mucosa facing upwards. Muscle strips were snap-frozen in liquid nitrogen until analysis.

**Electric stimulation of the vagal nerve.** Stimulation of the vagus nerve was essentially performed as described previously. The left cervical nerve was prepared free from the carotid artery and ligated with 6-0 silk suture. The distal part of the ligated nerve trunk was placed between a bipolar platinum electrode unit. In part of the experiments, the vagus nerve was transected, and the distal part stimulated. Voltage stimuli (5Hz, 2ms, 1 or 5 V) were applied for 5 min before-, and 15 min following the intestinal manipulation protocol described above. In sham VNS control mice the cervical skin was opened and left for 20 min. covered by moist gaze.

**Local hexamethonium application.** Local blockade of nicotinic receptors in the ileum was performed as follows: in anaesthetized mice (n=7) a midline laparotomy was performed, and 6 cm of ileum proximal to the cecum was carefully externalized and placed in a sterile preheated tube. The segment was continuously flushed with a preheated (37 °C) solution of hexamethonium (10^{-4} M in 0.9% NaCl), or vehicle for 20 min. Temperature of intestinal tissue was monitored using a thermal probe. Leakage of hexamethonium solution into the peritoneal cavity was strictly avoided. After incubation, the hexamethonium solution was removed, the ileal segment was washed three times with 0.9% NaCl, and included in the manipulation protocol.

**Measurement of gastric emptying.** Gastric emptying of a semi-liquid, non-caloric test meal (0.5% methylcellulose) containing 10Mq 99Tc was determined by scintigraphic imaging as described previously.

**Quantification of leukocyte accumulation at the intestinal muscularis.** Myeloperoxidase (MPO) activity in ileal muscularis tissue was assayed as a measure of leukocyte infiltration as described. Whole mounts of ethanol-fixed ileal muscularis were prepared and stained for MPO activity as described.

**RT-PCR.** Total RNA from tissue was isolated using Trizol (Invitrogen, Carlsbad, CA), treated with Dnase, and reverse transcribed. The resulting cDNA (0.5 ng) was subjected to Light Cycler PCR (CYBR Green Fast start polymerase; Roche, Mannheim, Germany) for 40 cycles. Primers used were: TNF: As 5-AAAGCATGATCCGCGACGT-3 and Sen 5-TGCCACAAGCAGGAATGAGAA-3; MIP-2: As 5-AGTGAACTGCGCTGTCAATGC-3 and Sen 5-GCAAACAAGTTTTTGACCGCCCT-3; Socs-3 As 5-ACCTTTCTATCCGCGACAG-3 and Sen 5'-TCACCAGCTTGAGTACACAG-3'; and GAPDH As 5'-ATGTGTCCGTGTGCTCAATGC-3 and Sen 5'-ATGCCTGCTTTCCACCTTCT-3'. PCR products were quantified using a linear regression method on the Log(fluorescence) per cycle number data, and expressed as percentage of GAPDH transcripts for
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each sample. For qualification, resulting PCR products were analyzed on an ethidium bromide-stained 2.5% agarose gel.

**Immunohistochemistry.** For double-labeling of macrophages and cholinergic fibers, rats were anesthetized with pentobarbital sodium (90mg/kg, ip) and transcardially perfused with heparinized saline (20 U/ml), followed by ice-cold 4% phosphate-buffered (pH 7.4) paraformaldehyde. Gastric and intestinal tissue were extracted and postfixed in the same fixative for a minimum of 2 hr. Tissue was cryoprotected overnight in 18% sucrose and 0.05% sodium azide in 0.01M phosphate-buffered saline (PBS). Twenty-micron flat-sections and 25 µm cross-sections of corpus and mid ileum were cryostat cut and processed in PBS. Sections were pretreated with 0.5% sodium borohydride in PBS and subsequently blocked in donkey normal serum. Monoclonal mouse anti-rat ED2 (Serotec, Raleigh, NC), and polyclonal goat anti-Vesicular Acetylcholine Transporter (VACHT; Chemicon, Temecula, CA) were diluted in 0.1% gelatin and 0.05% sodium azide in PBS with 0.5% Triton X-100 (PBST) and incubated for 20 hr at room temperature or for 48 hr at 4°C. Secondary antibodies used were Cy-3-conjugated donkey anti-mouse (Jackson ImmunoResearch, West Grove, PA) for ED2 and Cy-2-conjugated donkey anti-goat (Jackson ImmunoResearch, West Grove, PA) for VACHT in PBST. Sections were mounted in 100% glycerol with the addition of 5% n-propyl gallate as an antifade agent.

In vivo labeling of mouse phagocytes was performed by i.p. injection of 20 µg Alexa546-labeled Dextran particles (Mw 10,000; Molecular Probes, Sunnydale, CA) 24 h before surgery. One hour following surgery, anaesthetized mice were perfused with 10 mL of ice-cold 0.9% NaCl containing 1mM Na3VO4, followed by 20 mL of ice-cold 4 % formaldehyde solution, pH 7.4. Intestinal tissue was isolated, fixed overnight in 4% formaldehyde, dehydrated and embedded in paraffin. Six mm sections were cut and immunostained for PY705-Stat-3 using polyclonal rabbit antibodies (Cell Signaling Technologies) and biotin-labeled anti-rabbit antibodies according to the manufacturer's instructions. Biotin was visualized using 3-amino-9-ethyl carbazole (Sigma, St Louis, MO) as chromogen, followed by counterstaining using haematoxylin. Alternatively, Alexa488-streptavidin (Molecular probes) with DaPi nuclear counterstain was used for analysis by confocal microscopy.

**RESULTS**

Nicotine activates STAT3 in macrophages. To study the cellular response of macrophages to nicotinic receptor activation, we isolated peritoneal macrophages from mice and investigated the effect of nicotine on LPS-induced cytokine production. Nicotine reduced the LPS-induced release of TNF, MIP-2 and IL-6 but not IL-10 in a dose-dependent way (fig.1a), consistent with published reports on
Figure 1. Nicotine attenuates peritoneal macrophage activation and induces phosphorylation of STAT3 and SOCS3 expression. (a) ELISA of TNF, MIP-2, IL-6 and IL-10 in the supernatants of peritoneal macrophages stimulated with 100 ng/ml of LPS in vitro in the presence of nicotine (dose, horizontal axes). Data represent mean +/- s.e.m. of four independent experiments in triplicate. (b) Immunoblots for phosphotyrosine-STAT3 (PY-STAT3), STAT3 and SOCS3 in cell lysates of peritoneal macrophages stimulated with 1 ng/ml of LPS (right) or no LPS (left) in the presence of nicotine (concentration, above lanes). Blot is representative of five independent experiments. (c) Immunoblot of phosphorylated STAT3 (PY-STAT3) and STAT3 in cell lysates of peritoneal macrophages stimulated with 100 nM nicotine (time, above lanes). Blot is one representative of three independent experiments. (d) Immunoblot of phosphorylated STAT3 (PY-STAT3), STAT3 and SOCS3 in cell lysates of peritoneal macrophages pretreated with vehicle, actinomycin-D (Act-D) or cycloheximide (CHX) and incubated with saline (-) or 100 nM nicotine (+). Blot is representative of three independent experiments. (e) Immunoblot of phosphorylated STAT1 (PY-STAT1) and STAT1 in peritoneal macrophages incubated with nicotine (concentration, above lanes) and stimulated with 100 ng/ml of interferon-γ (IFN-γ). Actin, loading control.
the anti-inflammatory effect of nicotine on human and mouse monocyte-derived macrophages. Given the crucial function of STAT3 in anti-inflammatory responses, we hypothesized that activation of STAT3 and its gp130-binding regulatory protein SOCS3 may be involved in the anti-inflammatory effect of nicotine. Consistent with that hypothesis, we found that nicotine treatment activated STAT3 as well as SOCS3 in resting and LPS-stimulated primary peritoneal macrophages in a dose- and time-dependent way (fig. 1b,c). Nicotine activated STAT3 directly, as phosphorylation of STAT3 was not affected by the protein synthesis inhibitors actinomycin D and cycloheximide (Fig.1d). In contrast, interferon-γ-induced STAT1 activation was not affected by nicotine (Fig. 1e). Thus, nicotine reduced the production of proinflammatory cytokines and activated STAT3 as well as SOCS3 in stimulated macrophages.

**Deactivation by nicotine requires STAT3 transactivation**

We next sought to determine whether the anti-inflammatory effect of nicotine depended on nuclear transactivation of phosphorylated STAT3. We overexpressed a dominant negative form of STAT3 (STAT3D) in primary peritoneal macrophages. Dimerized STAT3D is altered in its ability to bind DNA response elements and induce transcription of target genes. Nicotine failed to reduce LPS-induced TNF release in LPS-stimulated macrophages transfected with STAT3D but not those transfected with the STAT3 wild-type construct (Fig. 2a). Thus, the nicotine-induced inhibition of TNF release is dependent on STAT3 DNA transactivation. To evaluate whether SOCS3 expression is crucial to the nicotinic anti-inflammatory effect, we abrogated SOCS3 expression in peritoneal macrophages with SOCS3-specific small interfering RNA (Fig. 1b). SOCS3 expression was substantially decreased in response to nicotine (less than 10% of that expressed in control transfected cells), whereas STAT3 activation was not affected (transfection efficiency was more than 90%; Fig. 2b). In macrophages with reduced SOCS3, however, nicotine was still able to decrease endotoxin-induced production of IL-6 (data not shown) and TNF in a concentration-dependent way, although the reduction was less pronounced than that in control transfected cells (Fig. 2c). Thus, blockade of STAT3 transactivation counteracted the anti-inflammatory effects of nicotine, whereas blockade of SOCS3 expression did not. These results indicate that SOCS3 expression is not strictly required for the reduction in macrophage TNF release by nicotine.

**STAT3 phosphorylation depends on α7 nAChR activation**

To determine whether STAT3 activation by nicotine was mediated by nAChR, we pretreated cells with nAChR antagonists. The nonselective antagonists hexamethonium and d-tubocurarine prevented the STAT3 phosphorylation induced by nicotine (Fig. 3a). In addition, the α7 nAChR–selective antagonists α-bungarotoxin...
and methyllycaconitine blocked the nicotine-induced STAT3 activation (Fig. 3a).
A prominent function for the α7 receptor in nicotine-induced deactivation of
macrophages corroborates published reports on human and mouse monocyte-derived
macrophage cultures3,4. The selective non-α7 nAChR antagonist dihydro-β-
erythroidine did not affect nicotine-induced STAT3 activation (data not shown).
Blocking nAChR also counteracted the attenuation of proinflammatory mediator
release by nicotine in activated macrophages. Hexamethonium, d-tubocurarine and
methyllycaconitine prevented the reduction in endotoxin-induced release of IL-6 (Fig.
3b) and MIP-2 (data not shown) by nicotine in a dose-dependent way. Hexamethonium
effective dose leading to 50% inhibition (ED50), 6.46 +/-2.90 nM) was more potent
than methyllycaconitine (ED50, 24.0 +/-3.4 nM) and was far more potent than
d-tubocurarine (ED50, 0.80 +/-0.23 +/-M) in attenuating the inhibition of IL-6 release
(Fig. 3b). The high ED50 for d-tubocurarine is probably due to its low affinity for α7
nAChRs18 and is in line with its modest inhibitory effect on STAT3 activation by
nicotine (Fig. 3a). In addition to methyllycaconitine, α-bungarotoxin abolished IL-6
reduction by nicotine. However, exposure of the cells to α-bungarotoxin decreased
IL-6 production in the presence and absence of nicotine, which compromised adequate
determination of its ED50 (data not shown). Thus, STAT3 activation is dependent on
the activation of nAChRs by nicotine, most likely exclusive through activation of the
α7 nAChR subunit.

Figure 2. Inhibition of macrophage activation by nicotine requires transactivation of STAT3
but not SOCS3 expression. (a) TNF in the supernatants of peritoneal macrophages transiently
transfected with dominant negative STAT3D, wild-type STAT3 (STAT3 WT)17 or empty vector (Vector),
then incubated with nicotine and stimulated with 10 ng/ml of endotoxin. Values are expressed as the
percent of TNF released without the addition of nicotine for each group. Data are mean +/-s.e.m. of
three independent experiments done in duplicate. *, P < 0.05 (one-way analysis of variance followed by
Dunnett’s multiple comparison test). (b) Immunoblot for phosphorylated STAT3 (PY-STAT3), STAT3 and
SOCS3 in peritoneal macrophages transiently transfected with control oligonucleotide or SOCS3-specific
small interfering RNA (siRNA), then incubated with 100 nM nicotine. Blot is representative of three
independent experiments. (c) TNF in the culture supernatants of peritoneal macrophages transfected with
control oligonucleotide or SOCS3 siRNA, then preincubated with nicotine and stimulated with 10 ng/ml
of LPS. Data are presented as percentage of TNF produced without addition of nicotine for each treatment
group and are the mean +/-s.e.m. of three independent experiments done in duplicate.
The macrophage α7 nAChR recruits Jak2

STAT3 phosphorylation normally requires activity of the cytoplasmic tyrosine kinase Jak2 (ref. 8). Therefore, we investigated whether STAT3 phosphorylation depended on Jak2 activity and whether nAChRs expressed on macrophages recruit Jak2. Phosphorylation of STAT3 after nicotine treatment of peritoneal macrophages was effectively blocked by AG 490, a selective inhibitor of Jak2 phosphorylation\(^{19,20}\) (fig. 4a). In agreement with that finding, nicotine failed to reduce IL-6 release by LPS-stimulated peritoneal macrophages treated with AG 490 (data not shown).

Binding studies have distinguished two main categories of nAChRs based on their affinity for either α-bungarotoxin (α7-containing homopentamers) or nicotine (α4β2 pentamers)\(^{18}\). Because our blocking studies suggested involvement of the α7 nAChR subtype, we analyzed putative associations of α7 with Jak2 (ref. 20) by immunoprecipitation (Fig. 4b). The α7 (56-kilodalton)\(^{21}\) receptor was expressed in primary peritoneal macrophage lysates. Immunoprecipitation of Jak2 from peritoneal

![Image](image.png)

**Figure 3. STAT3 phosphorylation by nicotine is prevented by α7-selective nAChR antagonists.** (a, b) Peritoneal macrophages were pretreated with the nAChR blockers d-tubocurarin (d-TC), α-bungarotoxin (αBgt), hexamethonium (Hexa) or -methyllycaconitine (MLA) and were incubated with nicotine (concentration, above lanes). Lysates were collected for immunoblot of phosphorylated STAT3 (PY-STAT3), STAT3 and actin (a) and IL-6 was measured in supernatants (b). (a) Blots are representative of three independent experiments. (b) Filled squares, hexamethonium; open squares, methyllycaconitine; open circles, d-tubocurarine. Data are presented as the percentage of inhibition of IL-6 release measured without the addition of an nAChR blocker and represent mean values +/- s.e.m. of three independent experiments done in triplicate.
macrophage cell lysates showed a weak association of Jak2 with the α7 receptor after culture in the absence of nicotine. To investigate whether Jak2 is recruited to the nAChR and is phosphorylated after binding of its ligand, we preincubated cells with nicotine. Nicotine exposure increased the amount of α7 nAChR detected in Jak2 and phosphorylated Jak2 immunoprecipitates (fig. 4b). To further demonstrate that Jak2 is phosphorylated after nAChR activation, we pretreated cells with the Jak2 phosphorylation blocker AG 490 before adding nicotine. Cells treated with AG 490 had reduced phosphorylated Jak2 in α7 immunoprecipitates, whereas Jak2 recruitment to the α7 receptor was not affected (Fig. 4b). The latter finding demonstrates that Jak2 is recruited and phosphorylated after nicotine binding.

**Stimulation of the vagus nerve ameliorates inflammation**

We next evaluated whether activation of nAChR on macrophages would attenuate intestinal inflammation *in vivo*. We assessed the effect of stimulation of the vagus nerve on the inflammation that follows intestinal manipulation in our mouse model, because this immune response is associated with the activation of macrophages. We electrically stimulated the left cervical vagus nerve during intestinal manipulation surgery and investigated the effects on muscular inflammation and gastric emptying 24 h later (Fig. 5). Consistent with published findings, intestinal manipulation of mice resulted in a delayed gastric emptying compared with that of mice that underwent only laparotomy, indicative of the development of postoperative ileus (gastric retention after 60 min, 14.5% +/-2.7% for laparotomy and 43.0 +/-6.7% for intestinal manipulation). However, stimulation of the vagus nerve prevented the intestinal manipulation-induced gastroparesis 24 h after surgery (gastric retention, 25.2% +/-3.2%; Fig. 5). Notably, stimulation of the vagus nerve in itself may alter gastric emptying during the vagus stimulation protocol. However, we found that stimulation of the vagus nerve did not affect basal gastric emptying 24 h after surgery (gastric retention, 15.7% +/-3.6%; Fig 5). The last finding demonstrates that normalization of gastric emptying after stimulation of the vagus nerve was not a direct effect on gastric motility but resulted from reduced inflammation of the manipulated bowel segment.

We next analyzed muscularis tissue for granulocytic infiltrates by measuring myeloperoxidase activity in muscularis tissue homogenates and quantifying cellular infiltrates (Supplementary figure 1). The intestinal manipulation–induced inflammation of the muscularis externa in mice that received stimulation of the vagus nerve was reduced in a voltage-dependent way compared with that of mice that received intestinal manipulation plus sham stimulation. Prior vagotomy of the proximal end of the stimulated vagus nerve did not affect these results (data not shown), indicating that the anti-inflammatory effect of stimulation of the vagus nerve was not dependent on the activation of central nuclei, which confirms published
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Figure 4. Nicotine-induced STAT3 phosphorylation occurs through activation of Jak2 that is recruited to the α7 nAChR subunit. (a) Immunoblot of phosphorylated STAT3 (PY-STAT3) and STAT3 in peritoneal macrophages incubated with AG 490 (concentrations, above blots). Blot is representative of three independent experiments. (b) ImmunobLOTS of peritoneal macrophages treated with 1 μM nicotine (lanes 4 and 5) or with 1 μM nicotine plus 100 μM AG 490 (lane 5). Cell lysates were immunoprecipitated (IP) with anti-α7 (top), anti-Jak2 (middle) or anti–phosphorylated Jak2 (PY-Jak2; bottom), followed by immunoblot (IB; antibodies, left margin). Lane 2, coprecipitate in the absence of lysate (negative control). IgH, immunoglobulin heavy chain. Blots are representative of four independent experiments.
reports. We next incubated intestinal segments with the nicotinic receptor blocker hexamethonium before intestinal manipulation combined with stimulation of the vagus nerve. In intestinal segments treated with hexamethonium, stimulation of the vagus nerve failed to prevent inflammation, in contrast to incubation with vehicle (supplementary figure 1), demonstrating that the anti-inflammatory effect of vagus stimulation acted through local activation of nicotinic receptors.

Stimulation of the vagus nerve activates STAT3 in vivo
To further investigate whether macrophages mediated the anti-inflammatory effect of stimulation of the vagus nerve, we analyzed the expression of transcripts of macrophage-derived inflammatory mediators in muscularis tissue 3 h after surgery. Stimulation of the vagus nerve reduced the expression of Cxcl2 mRNA (Fig. 6a,b) and Ccl3 mRNA (data not shown) but did not notably alter the expression of Tnf transcripts in muscularis tissue, confirming earlier reports. However, when we analyzed peritoneal lavage fluid for the presence of macrophage inflammatory mediators 3 h after intestinal manipulation, we found that stimulation of the vagus nerve significantly reduced the secretion of TNF, IL-6, MIP-2 (Fig. 6c) and MIP-1α (data not shown) in the peritoneal cavity. This reduction was not due to enhanced expression of IL-10, as stimulation of the vagus nerve was similarly potent in reducing intestinal manipulation–induced inflammation in IL-10-deficient mice (Fig. 6d). Moreover, the peritoneal IL-10 in wild-type mice did not reach the limit of detection (31 pg/ml) at 1, 3 or 6 h after intestinal manipulation (data not shown). Expression of Socs3 (Fig. 6a) but not Socs1 (data not shown) was increased in muscularis tissue after stimulation of the vagus nerve even in mice that underwent this stimulation without manipulation of the bowel.

Given the short half-life of acetylcholine, cholinergic regulation of macrophage activation most likely requires that cholinergic nerves be in close proximity to intestinal macrophages. To investigate this, we immunohistochemically double-labeled vesicular acetylcholine transporter–positive vagal efferent fibers and macrophages in rat intestinal muscularis tissue. Macrophages were in close proximity to nerve terminals in the myenteric plexus in the ileum (Fig. 7a) and circular muscle of gastric corpus (data not shown). Hence, acetylcholine released from efferent nerve terminals could easily reach macrophages in the nanomolar concentration range.

To verify that the enhanced SOCS3 expression reflected increased STAT3 activation in vivo, we immunohistochemically analyzed intestinal tissues for the presence of phosphorylated STAT3 in mice that underwent control laparotomy surgery, intestinal manipulation alone or intestinal manipulation plus stimulation of the vagus nerve (Fig. 7b,c). We found phosphorylated STAT3–positive nuclei in mice that underwent control laparotomy (Fig. 7b). Intestinal manipulation resulted in the appearance of phosphorylated STAT3–positive cells adhering to the serosal site of the bowel wall,
Supplementary Figure 1 Vagal nerve stimulation reduces recruitment of inflammatory infiltrates to the intestinal muscularis by activating peripheral nicotinic acetylcholine receptors. MPO activity measured in intestinal muscularis tissue homogenates isolated 24 h after surgery with IM. VNS with 5V, but not 1V, stimulus prevents the increased muscularis MPO activity elicited by IM. Asterisks indicate significant differences in MPO activity in intestinal muscularis tissue from L control and IM VNS5V determined by one-way ANOVA followed by Dunnett’s multiple comparison test. Data represent mean ± SEM of 10-15 mice (a). MPO-activity containing cells were stained in whole mount preparations of intestinal muscularis (b and c) prepared 24 hrs post-operatively. Mice underwent IM with sham VNS (IM Sham), or IM combined with VNS using 1, or 5 V pulses (IM VNS1V, and IM VNS5V) (b). Mice were pretreated with hexamethonium (100μM; Hexa) or vehicle and underwent Laparotomy (L) with VNS (L VNS5V) or IM with VNS5V (c). MPO-positive cells were counted in five consecutive microscopic fields of whole mount preparations of the indicated groups. Asterisks indicate significant differences (P<0.05) from (left graph) L control and (right graph) IM VNS5V groups using one-way ANOVA followed by Dunnett’s multiple comparison test. Data represent mean ± SEM of 5-8 mice.
most probably granulocytes and monocytes recruited to the peritoneal compartment as a result of tissue trauma inflicted by the intestinal manipulation procedure. However, when stimulation of the vagus nerve was applied, we noted phosphorylated STAT3-positive nuclei in cells between longitudinal and circular muscle layers surrounding the myenteric plexus. To identify the cellular source of the phosphorylated STAT3-positive nuclei, we labeled tissue phagocytes in vivo by pretreating mice with Alexa 546-labeled dextran particles (molecular weight, 10,000). This procedure labels F4/80 antigen-positive macrophages populating the intestinal muscularis25. Most of phosphorylated STAT3-positive nuclei in intestinal tissue of mice that had undergone stimulation of the vagus nerve localized together with cells that had taken up Alexa 546-labeled dextran particles, indicating that these phosphorylated STAT3-positive nuclei represented macrophages (Fig. 7c). These observations corroborate our in vitro findings on the function of STAT3 in the cholinergic inhibition of tissue macrophages and are in line with our proposed function of the network of resident intestinal macrophages26 as the inflammatory cells targeted by stimulation of the vagus nerve.

To further demonstrate that the cholinergic anti-inflammatory pathway critically depends on STAT3 activation in vivo, we studied the inflammatory response to intestinal manipulation in mice specifically deficient in STAT3 in their myeloid cell lineage (called ‘LysM-Stat3fl/−’ mice here). LysM-Stat3fl/− mice lack STAT3 in their macrophages and granulocytes6. In Stat3fl/+ control mice as well as in LysM-Stat3fl/− mice, intestinal manipulation led to increased peritoneal IL-6 (Fig. 8a) as well as massive inflammatory infiltrates in the manipulated muscularis tissue (Fig. 5).
Figure 6. Vagal stimulation reduces intestinal manipulation-induced proinflammatory mediator expression and release in vivo, independent of IL-10 production. (a,b) Real-time PCR for macrophage proinflammatory mediators (a, left margin; b, above graphs) of RNA isolated from intestinal muscularis strips prepared 3 h after the following procedures: control laparotomy surgery plus sham stimulation of the vagus nerve (L sham); control laparotomy surgery plus stimulation of the vagus nerve with 5-V pulses (L VNS); surgery with intestinal manipulation plus sham stimulation of the vagus nerve (IM sham); or surgery with intestinal manipulation plus stimulation of the vagus nerve with 5-V pulses (IM VNS). (a) No RT, no reverse transcriptase added to reaction (to control for nonspecific amplification); bp, base pairs. (b) Quantification of data and normalization of results to the expression of GAPDH. (c) Release of macrophage proinflammatory mediators into peritoneal lavage fluid obtained 3 h after treatment of mice with the procedures described in a,b. (d) IL-6 in peritoneal cavities (left) and myeloperoxidase-positive cells intestinal muscularis tissues (right) of IL-10-deficient mice (open bars) and their wild-type counterparts (filled bars) after treatment with the procedures described in a,b. Right, myeloperoxidase-positive cells were quantified in whole-mount preparations of intestinal muscularis tissue isolated 24 h after the procedures. *, P < 0.05, compared with the respective control laparotomy surgery group (one-way ANOVA followed by Dunnett’s multiple comparison test (b,c) or Mann Whitney U test (d)). Data represent mean +/-s.e.m. of five to eight mice. ND, not detectable.
Figure 7. Stimulation of the vagus nerve activates STAT3 in intestinal macrophages in muscularis tissue. Cholinergic nerve fibers are in close anatomical apposition to macrophages in small intestine. (a) Confocal microscopy of macrophages (ED2; red) and cholinergic nerve fibers (vesicular acetylcholine transporter; green) around the myenteric plexus of rat ileum. Arrows indicate close anatomical appositions of varicose cholinergic nerve fibers and macrophages at the perimeter of myenteric ganglia and the tertiary plexus outside the ganglia (arrowheads). Scale bar, 10 μm. (b) Mouse ileum sections stained for phosphorylated STAT3 1 h after control laparotomy surgery (L sham), intestinal manipulation (IM sham) or intestinal manipulation combined with stimulation of the vagus nerve (IM VNS). Transverse section of a complete ileal villus of a control mouse (control laparotomy). SM, submucosa; CM, circular muscle layer; LM, longitudinal muscle layer; MP, myenteric plexus. Arrowheads indicate phosphorylated STAT3–positive nuclei. Scale bar, 20 μm (40 μm for left image). (c) Phosphorylated STAT3–positive nuclei (green) in mouse ileum 1 h after intestinal manipulation plus stimulation of the vagus nerve, visualized by confocal microscopy. Arrowheads indicate colocalization of phosphorylated STAT3 nuclei (PYSTAT3; green) with phagocytes prelabeled by prior injection of Alexa 546–labeled dextran particles (red). Nuclear counterstain is 4,6-diamidino-2-phenylindole (DaPi; blue). Inset, enlarged macrophage showing dextran particles and STAT3 immunoreactivity. Scale bar, 20 μm (10 μm for boxed area). Experiments are representative of three independent incubations in three mice per group.
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8b). Notably, however, stimulation of the vagus nerve reduced peritoneal IL-6 and intestinal inflammation in Stat3fl/+ control mice but failed to do so in LysM-Stat3fl/- mice. These data support the critical function of STAT3 activation in the cholinergic anti-inflammatory pathway in vivo.

DISCUSSION

The cholinergic anti-inflammatory pathway represents a physiological system for controlling macrophage activation and inflammation in sepsis models. Its working mechanism ultimately involves the prevention of NF-kB p65 activity after α7 nAChR activation, but the exact cellular mechanism has remained unclear. Here we have demonstrated that nicotine acts on macrophages via the recruitment of Jak2 to the α7 nAChR and activation of Jak2, thereby initiating the anti-inflammatory STAT3 and SOCS3 signaling cascade. Notably, recruitment of Jak2 to the α7 nAChR subunit has also been described in neuronal PC12 cells exposed to nicotine, as part of a neuroprotective mechanism against β-amyloid-induced apoptosis. Our results in resident peritoneal macrophages were consistent with our in vivo data, as we found activation of STAT3 in intestinal macrophages in response to stimulation of the vagus nerve in mice, which indicates activation of STAT3 induced by acetylcholine derived from vagal efferents.
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Activation of the STAT3 cascade after nAChR ligation is fully consistent with the observed inhibition of proinflammatory cytokine release by macrophages, because STAT3 is a negative regulator of the inflammatory response\textsuperscript{6,27}. In our studies, the anti-inflammatory effect of nicotine on macrophages required DNA binding and transactivation of STAT3, as nicotine failed to inhibit TNF production in macrophages overexpressing STAT3 altered in its in DNA-binding capacity\textsuperscript{17}. Likewise, activation of STAT3 is required for the anti-inflammatory properties of IL-10 (refs. 8, 28) and the IL-10-induced attenuation of cytokine production and proliferation\textsuperscript{28}. In addition, STAT3 phosphorylation is required for IL-6-induced growth arrest and differentiation\textsuperscript{29}.

SOCS3 specifically disables STAT3 phosphorylation via IL-6R but does not interfere with IL-10R signaling\textsuperscript{9, 10, 30}. Conditional knockout mice specifically lacking SOCS3 in their macrophages (LysM-Socs3\textsuperscript{fl/fl}) show resistance to endotoxemia, explained by the anti-inflammatory effect of sustained STAT3 activation through IL-6R ligands\textsuperscript{10}. Regardless of that finding, our results have indicated that the enhanced expression of SOCS3 did not contribute to the anti-inflammatory effect of nAChR activation, as blockade of SOCS3 expression did not prevent the anti-inflammatory action of nicotine. Hence, the anti-inflammatory effect of cholinergic activation in macrophages rests mainly on enhanced STAT3 rather than SOCS3 activation.

We have shown that STAT3 was activated by nicotine directly and that involvement of enhanced signaling via IL-10R here was unlikely, as we found the macrophage deactivation induced by stimulation of the vagus nerve to be similarly effective in IL-10-deficient mice. Moreover, nicotine-induced STAT3 activation could be prevented by nAChR blockers. Our observations suggest that the molecular route exerting the anti-inflammatory effect of nAChR activation mimics the signaling pathway of IL-10R without the requirement of IL-10 itself. That hypothesis is supported by our finding and those of another study\textsuperscript{2} that, consistent with the action of IL-10 (ref. 31), nicotine does not alter TNF mRNA expression but decreases the release of TNF protein. Furthermore, LysM-Stat3\textsuperscript{fl/fl} mice have a phenotype resembling that of IL-10-deficient mice\textsuperscript{6}. Nicotine-induced inhibition of the release of high-mobility group box 1 in mouse RAW264.7 macrophages is associated with inhibition of NF-κB p65 transcriptional activity\textsuperscript{3}. Our finding that nicotine repressed macrophage activity via STAT3 may very well explain that observation, as IL-10–STAT3 (ref. 28) signaling blocks NF-κB DNA-binding\textsuperscript{32, 33}, possibly through direct interaction of dimerized STAT3 with the p65 subunit\textsuperscript{34}.

We have shown here that recruitment of inflammatory infiltrates induced by bowel manipulation and the resulting symptoms of postoperative ileus were reduced substantially by stimulation of the vagus nerve. Our results have shown strict cholinergic control of macrophage activation in vivo, which may be substantiated by the observation that cholinergic (vesicular acetylcholine transporter–positive) nerve fibers are situated in close proximity to resident macrophages in intestinal myenteric
plexus. At first glance, our data may seem contradictory to the outcome of earlier attempts to treat postoperative ileus using cholinergic agents such as neostigmine, which had only limited success. That lack of efficacy could be explained by the fact that the inflammatory process had already been fully accomplished by the time these agents were administered, leaving the activation of inhibitory neural pathways unaffected. Our results indicate that nicotinic receptor activation before or during surgery prevents postoperative intestinal inflammation and will certainly be a promising strategy for treating postoperative ileus. Notably, vagus nerve stimulators are clinically approved devices for the treatment of epilepsy and depression. In conclusion, we have shown here that inhibition of macrophage activation via the cholinergic anti-inflammatory pathway is brought about via Jak2-STAT3 signaling. Our data may aid in further development of therapeutic strategies for modifying the cholinergic anti-inflammatory pathway to treat various inflammatory conditions.
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REFERENCE LIST

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