Chapter 4

Vagus nerve activity augments intestinal macrophage phagocytosis via nicotinic acetylcholine receptor alpha4beta2.
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Abstract

**Background and Aims:** The vagus nerve negatively regulates macrophage cytokine production via the release of acetylcholine (ACh) and activation of nicotinic acetylcholine receptors (nAChR). In various models of intestinal inflammation, vagus nerve efferent stimulation ameliorates disease. Given the actively constrained cytokine responses of intestinal macrophages, we explored the effect of nAChR activation on endo- and phagocytosis by macrophages residing in the peritoneal and mucosal compartment.

**Methods:** The phagocytic uptake by intestinal and peritoneal macrophages was measured by FACS analysis, and the nAChR involved was determined by pharmacological blockade, shRNA-assisted gene knockdown and the use of specific nAChR KO mice. The effect of electrical vagus nerve stimulation on epithelial translocation and macrophage uptake of luminal particles was studied in mice.

**Results:** In isolated intestinal and peritoneal macrophages, nAChR activation enhanced endocytosis and phagocytosis. This effect was mediated via stimulated recruitment of GTPase Dynamin-2 to the forming phagocytic cup. These effects involve nAChR α4/β2, rather than nAChR α7. Despite enhanced bacterial uptake, acetylcholine reduced NF-κB activation inflammatory IL10 production. Vagus nerve stimulation in mice altered mucosal immune responses by augmenting epithelial transport and uptake of luminal bacteria by lamina propria macrophages.

**Conclusions:** Acetylcholine enhances phagocytic potential while inhibiting immune reactivity via nAChR α4/β2 in mouse macrophages. Hence, vagus nerve efferent activity may stimulate surveillance in the intestinal mucosa and peritoneal compartment, and pro-inflammatory cytokine production, while stimulating anti-inflammatory IL10 production. Vagus nerve stimulation in mice altered mucosal immune responses by augmenting epithelial transport and uptake of luminal bacteria by lamina propria macrophages.

**Conclusions:** Acetylcholine enhances phagocytic potential while inhibiting immune reactivity via nAChR α4/β2 in mouse macrophages. Hence, vagus nerve efferent activity may stimulate surveillance in the intestinal mucosa and peritoneal compartment.
Introduction
In the gastro-intestinal tract, the vagus nerve regulates motility and digestive functions mostly via the peripheral release of the parasympathetic neurotransmitter acetylcholine (ACh) that activates nicotinic acetylcholine receptors (nAChRs). However, vagal activity has also immune-regulatory properties. While the afferent vagus system is known to regulate the inflammatory response via the hypothalamic pituitary adrenal axis, efferent vagus nerve activity possesses immuno-modulatory potential as well. Borovikova et al (1) have revealed the potency of the vagus nerve to inhibit TNF production by macrophages after systemic endotoxin (2). Peritoneal and PBMC-derived macrophages express nAChRs, and nAChR activation has been shown to inhibit NF-κB transcriptional activity (3;4) and pro-inflammatory cytokine production (4;5). In conjunction, electrical vagus nerve stimulation has been shown to ameliorate disease in animal models of inflammatory conditions such as postoperative ileus (4;6), colitis (7), peritonitis (8;9), and hemorrhage (10). An immune regulating role for the cholinergic nervous system may be particularly evident in intestinal tissue, given the dense cholinergic innervation, and the abundant number of resident macrophages that populate the intestinal mucosa and muscularis externa, of which some closely associate with cholinergic fibers (6).

Cholinergic inhibition of pro-inflammatory cytokine production by macrophages has been firmly established (1;3;6). However, besides an effect on cytokine secretion, the cholinergic nervous system may also affect more professional macrophage functions such as endo- and phagocytosis of bacteria and particles. Especially in the intestinal compartment macrophages may rather function as phagocytes that, along with dendritic cells, form critical effectors in the surveillance of luminal antigens (11;12). Hence, the question arises whether the anti-inflammatory effect of vagus nerve activity in intestinal inflammation solely rests on reduced macrophage cytokine production, or whether the vagus nerve also regulates other macrophage functions important in host defense. This is supported by the observation that vagus nerve activity affects bacterial clearance and mortality in various mouse models of infectious disease (8;13;14). Therefore, we studied the effect of nAChR activation on endo- and phagocytosis by macrophages residing in the peritoneal and mucosal compartment.

We show here that nAChR α4/β2, rather than α7, activation enhances the phagocytic potential in mouse macrophages. Despite enhanced phagocytosis, the activation of NF-κB activity and pro-inflammatory cytokine production is inhibited. In conjunction, we also demonstrate that in mice, electrical stimulation of the vagus nerve increases the epithelial permeability for luminal bacteria and stimulates phagocytosis by F4/80+CD11b+ macrophages residing in the intestinal mucosa. Taken together, our data suggest that vagus nerve activity can enhance macrophage bacterial uptake via activation of the nAChR α4/β2, while reducing inflammatory cell activation.

Methods
Reagents and antibodies.
(-)-Nicotine, (+)-Nicotine, Acetylcholine, α-Bugurotoxin, Methylcaconitine, 2,2,6,6- T tetramethylpiperidin-4-y methyl-2-pyrrolidinylisoxazole, Zymosan A from S. cerevisiae were from Sigma-Aldrich (Zwijndrecht, the Netherlands). Polyclonal antibodies against phosphorylated and unphosphorylated Akt and P38, and anti-NFkB-p65 were from Cell Signalling. Anti-α4 nAChR was purchased from Santa Cruz Biotechnology; anti-β2 nAChR subunit (MAb clone 270) and anti-Dynamin-2 (clone 3457) were from Abcam (Cambridge, UK). Dil-AcLDL was obtained from (Invitrogen). Rat anti mouse monoclonal CD11b, F4/80 and CD11c were
purchased from BDBiosciences (Franklin Lakes, NJ). FITC (Sigma-Aldrich), labelling of 1x10^9 heat-killed E. faecium was performed in 0.1 M NaHCO3 pH9.0 for 1h at 37°C (in shaking waterbath). heptanoate, Dihydro-β-erythroidine,

**Mammalian cell culture.**
Macrophages from an immortalized spleen macrophage cell line (Mf4/4(15)) were cultured in RPMI 1640/10% FCS. Primary peritoneal cells (PMF) from BALB/C, C57/Bl6 WT mice, nAChR alpha7/-/- or nAChRbeta2/-/- mice (kindly provided by Dr. G.LaRosa and Dr. U. Maskos respectively) were collected by lavage 4 days after ip. injection of 1ml of Biogel solution and cultured in OptiMEM (Invitrogen).

**Phagocytosis assays.**
For phagocytosis assays, cells were plated to 80 % confluence in 24 well suspension plates and pretreated with cholinergic agonists for 20min. Subsequently, cells were challenged with FITC-labeled Zymosan particles (20 particles/cell) for 10min or other time-points where indicated. Cells were washed by PBS, final washing was in PBS/EDTA/lidocaine and cells were harvested by scraping. After fixation in 2% PFA, bead uptake was determined by flow cytometry (FACS Calibur, Becton Dickinson). Fc Receptor-mediated phagocytosis of RBC. Sheep RBC (300 μl of a 10% suspension; Cappel/ICN) were opsonized with 1:1000 diluted rabbit-anti-sheep RBC IgG (Cappel/ICN). Opsonized RBCs were allowed to bind to Mf4/4 or peritoneal macrophages at a 1/80 cell to RBC ratio at 37°C. After 15min, slides were exposed to ice-cold water for 15 sec to lyse RBCs that were not internalized, washed in PBS and fixed.

**Cell stimulations**
Peritoneal macrophages were incubated with nicotinic agonists at the concentration indicated for 45 minutes and stimulated with Zymosan (10 particles/cell) for 6hrs. After treatment, the medium was removed; the cells washed three times with ice-cold PBS, and medium and cell lysates were measured by ELISA.

**RT-PCR of total RNA**
To determine nAChR expression on MF4/4 cells and PMF, total RNA was isolated using the RNeasy Mini kit (Qiagen), treated with DNase, and reverse transcribed. The resulting cDNA was subjected to Light Cycler polymerase chain reaction (Roche) for 35 cycles. Primer sequences are provided in supplementary data.

**Immunostaining and imaging**
Immune-histochemistry on intestinal sections was performed as described earlier(6). For confocal microscopy, MF4/4 were grown on glass slides (Nuncbrand), pretreated with nicotine, challenged with FITC-labeled Zymosan and phagocytosis was allowed for 5min. For a detailed description of the protocols and antibodies used, see supplementary methods.

**shRNA transfection**
2x10^6 MF4/4 cells were transfected by electroporation with 2 μg shRNA expression plasmid (psilencerTM-CMV4.1neo; Ambion, Austin, TX) according to the manufacturer’s instructions (570 V, 50 μs, Amaza; Gaithersburg, MD). shRNA sequences are given in the supplementary method section.
Immunoblotting
Immunoblotting was performed as previously described(6). Plasma membrane was extracted using the cell compartment kit (Qiagen), according to the manufacturer's instructions.

Isolation of lamina propria macrophages
Lamina propria macrophages were isolated after neutral protease digestion (a modified protocol as described previously(16)) and Magnetic bead-assisted Cell Sorting (MACS; Miltenyi Biotec Inc., Auburn, CA). A detailed description of the procedure is provided in the supplementary methods. After isolation, cells were taken up in RPMI/1%FCS at 107 cells/mL, left for 2 h, allowed to phagocytose FITC labeled heat-killed E.faecium at 37°C during 1h, finally washed and analyzed by FACS.

In vivo uptake assay and electrical vagus nerve stimulation
Animals. Male C57/Bl6 mice (Harlan Nederland, Horst, The Netherlands), 12 to 15 weeks old, were kept under environmentally controlled conditions (light on from 8:00 AM till 8:00 PM; water and rodent non-purified diet ad libitum; temperature 20 C-22 C; 55% humidity). Experiments were performed according to the guidelines of the Ethical Animal Research Committee of the University of Amsterdam. Mice were used after a 7 days adaptation period.

Electrical vagus nerve stimulation
Mice were anesthetized by i.p. injection of a mixture of Fentanyl Citrate / Fluanisone (Hypnorm; Janssen, Beerse, Belgium) and Midazolam (Dormicum; Roche, Mijdrecht, The Netherlands). VNS was performed as described previously (6). In short: the right cervical vagal branch was prepared free from the carotid artery and ligated with 6-0 silk suture. The part distal from the ligation was attached to an electrode and 5V stimuli with a frequency of 5Hz, duration of 2ms (10) were applied for 5 min. In sham mice the cervical skin was opened and left for 30 min. covered by moist gauze. After a 30 min. recovery period, the in vivo uptake assay was initiated as described below.

In vivo uptake.
Surgical procedures were performed under sterile conditions. Mice underwent a laparotomy and an ileum segment 3-10 cm proximal from the caecum was opened and its lumen rinsed with pre-warmed (37°C) oxygenated Krebs buffer. The ileum segment was filled with 1-2 mL of buffer containing FITC-labeled E.faecium bacteria and Alexa546-labeled Dextran particles (10,000Mw, Mol. Probes), and clamped at both sides. After 30 min. the clamped intestinal segment was removed, washed in PBS and processed for immune-histochemistry.

Bacterial translocation. Mice were killed 18 h after surgery and mesenteric lymph nodes (MLN) were harvested under aseptic conditions. Lymph nodes were weighed, placed in a tube containing 300 μl of ice-cold Luria-Bertani (LB) broth, homogenized with a sterile grinder and plated onto blood agar plates in aerobic and anaerobic conditions. After 48 h of incubation at 37°C, the number of colony forming units (CFU) per g lymph node was assessed.

Ussing chamber experiments
Segments of tissue of the distal small intestine were opened, cut and immersed Kreb's buffer (115 mM NaCl, 1.25 mM CaCl2, 1.2 mM MgCl2, 2.0 mM KH2PO4, and 25 mM NaHCO3 at pH 7.35). Within 15 minutes the tissue was mounted in Ussing chambers (World Precision Instruments, Berlin, Germany) with serosal and mucosal areas exposed to 2 ml of circulating oxygenated Krebs buffer (containing 10 mM glucose) maintained at 37°C. Ie (in μA/cm²), a measure of net active ion transport, was recorded by a computer connected a voltage-clamp system. After 15 minutes, HRP was added to the luminal buffer at a final concentration of 10μM.
Samples (300μl) were taken at the serosal side and replaced with fresh buffer. After 30 minutes, nicotine (1μM), Carbachol (1 μM) or buffer was added to the serosal side. The enzymatic activity of HRP was measured using tetramethyl-benzidine (TMB) (Biosource Europe, Nivelles, Belgium) as a substrate.

**Statistics**
Statistical analysis was performed using SPSS 12.02 software (SPSS Inc. Chicago, Ill, USA). The Friedman’s two-way analysis of variance was used to explore multiple dependent value assays. If the Friedman’s analysis was significant, individual values compared to the 0 nM concentration were tested with a Mann-Whitney U test. P-values <0.05 were considered statistically significant and results were expressed as mean ± SEM.

**Results**

**Cholinergic agonists enhance phagocytosis in macrophages.**
We first tested whether ACh, the main neurotransmitter of the vagus nerve, or nicotine, that activates nAChRs, affected macrophage endo- and phagocytosis. In PMF (Fig. 1A-D), and spleen macrophage Mf4/4 cells (not shown), ACh as well as nicotine significantly (p<0.05) enhanced phagocytosis in a time-dependent (Fig. 1B) and dose-dependent (Fig. 1C) manner as analyzed by FACS and fluorescence microscopy. The enhanced phagocytosis induced by nicotine was not based on enhanced particle binding to the cells, as no increase in phagocytosis was observed when cells were incubated on ice instead of 37°C (Fig. 1A, B).

To assess whether the cholinergic stimulation of phagocytosis was receptor pathway specific, we compared the effect of nicotine on the uptake of heat-killed E.faecium, opsonised sheep red blood cells (RBC), Zymosan, and on the endocytosis of acetylated low-density lipoprotein (AcLDL) by PMF (Fig. 1D) and Mf4/4 macrophages (not shown). Zymosan uptake depends on Dectin-1, IgG-opsonized RBC serve as cargo for Fc receptor-mediated phagocytosis, while AcLDL endocytosis is dependent on the scavenger receptor binding. However, nicotine stimulated uptake via all these pathways to a similar extent, indicating that nicotinic enhancement of phagocytosis involves a general pathway in phagocytosis rather than a specific effect on receptor expression. Moreover, further analysis confirmed that expression of Dectin-1, CD11b/CD18, or scavenger receptor A was not affected in peritoneal macrophages after a 6h treatment with 1μM of nicotine (results not shown).
Cholinergic agonists stimulate phagocytosis via the α4/β2 nAChR rather than α7 nAChR.

We next sought to establish which nAChR mediated the cholinergic effects on macrophage phagocytosis. The nAChR α7 has been implicated in the inhibition of cytokine production and NF-κB activity by nicotinic receptor activation in macrophages (a.o. in (4;5) ). In accordance with these results, nicotine inhibited the activation of NF-κB induced by Zymosan ((4) and results not shown), inhibited Zymosan–induced TNFα and MIP-2 production and induced IL-10 production (Fig. 2A). This effect was not only blocked by nAChRα7 blockers Bungarotoxin (Bgt) and Methyllycaconitine (MLA), but also by nAChR α4/β2 antagonist dihydro-β-erythroidine (DHβE) (Fig. 2B). In PMF of nAChRα7 knock-out mice, nicotine failed to reduce TNF production, confirming earlier reports ((5) and results not shown).
Surprisingly, specific blockers of α7 nAChRs failed to block the nicotinic increase in phagocytosis in peritoneal macrophages, in fact, MLA pretreatment further augmented nicotinic stimulation of phagocytosis. On the other hand, 2,2,6,6-tetramethylpiperidin-4-yl heptanoate (TMPH), a blocker of nAChR with low affinity for the α7 nAChR (17) blocked the nicotinic stimulation of phagocytosis (Fig. 3A, right panel). Subsequent analysis indicated that specific α4/β2 nAChR blocker DHβE almost completely counteracted the effect of nicotine on phagocytosis (Fig. 3A). In agreement with these results, an agonist for α7 nAChRs (AR-R17779) failed to induce phagocytosis, while a specific agonist of α4/β2 nAChR, 3-Methyl-5-[(2S)-1-methyl-2-pyrrolidinyl]isoxazole (ABT418) stimulated phagocytosis, albeit with less potency compared to nicotine (Fig. 3B). These results demonstrate that cholinergic stimulation of phagocytosis is mediated via nAChR α4β2 rather than α7 nAChRs.

Prompted by these data, we analyzed the expression of α7 and α4/β2 nAChR subunits in intestinal and peritoneal macrophages. Quantitative RT-PCR analyses revealed expression of nAChR α4/β2 transcripts in lamina propria macrophages (LPMF) and PMF, as well as Mf4/4 cells, while we failed to detect α7 transcripts in either of these macrophage types (Fig. 3C). Protein expression of the nAChR β2 and α4 subunits was confirmed in PMF (Fig. 3D) and Mf4/4 macrophages (not shown). To demonstrate further that the cholinergic increase in phagocytosis depended on β2 nAChR expression we transfected β2 shRNA to knock-down β2 nAChR transcripts in Mf4/4 cells (Fig. 4A-C). We confirmed the potency of the shRNA expression vector to reduce β2 nAChR mRNA expression to 10–20% of normal in these cells, while expression of regular housekeeping genes was not affected (Fig. 4C). Nicotine pretreatment enhanced phagocytosis in macrophages transfected with random scrambled shRNA expression vectors, but knockdown of β2 nAChR expression abolished this effect (Fig. 4A-B). In addition, in cells transfected with shRNAs directed to α7 nAChR the nicotinic increase in phagocytosis was unaffected. Finally, nicotine enhanced phagocytosis in peritoneal macrophages from both WT and nAChRa7−/− mice, while it failed to do so in PMF from AChRβ2−/− mice (Fig. 4D). These data demonstrate that cholinergic agonists stimulate macrophage phagocytosis via activation of α4/β2 nAChRs, rather than α7 nAChRs.
Figure 3. Cholinergic agonists stimulate phagocytosis via nAChR α4β2. Left panel, nicotine increases phagocytosis (percentage of cells positive for Zymosan) dose-dependently. Right panel, nicotinic (10-6 M) increase in phagocytosis is counteracted by cholinergic blocker DHβE and 2,2,6,6-Tetramethylpiperidin-4-yl heptanoate hydrochloride (TMPH), but not by NACeR α7 blockers Bgt and MLA. Data shown are average +/- SEM of 3-5 independent experiments in triplicate, asterisks indicate significant differences (P<0.05) compared to vehicle treated group (A). Nicotine, as well as nAChR α4β2 agonist 3-Methyl-5-[(2S)-1-methyl-2-pyrrolidinyl]isoxazole (ABT418) increase phagocytosis of Zymosan, while nAChR α7 agonists AR-R17779 is not effective. Data shown are averages +/- SEM of 3 assays, asterisks indicate significant differences (P<0.05) compared to vehicle treated group (B). RT-PCR showing transcript expression of nAChR α4 and β2 subunits in RNA derived from mouse brain, spleen Mf4/4 macrophages, lamina propria macrophages (LMPF) and PMF (C). Immune-histochemical staining showing expression of α4 or β2 subunit protein (red) in PMF. Dapi counterstain, scale bar: 1 μm (D).
Figure 4. NACHR α4β2 activation enhances macrophage phagocytosis of Zymosan.

(A)-(D): Phagocytosis of Zymosan in shRNA transfected Mf4/4 macrophages and in PMF from WT mice, nACHRα7-/- mice or nACHRβ2-/- mice, after pretreatment with nicotine at the concentrations indicated. Cells were transfected with scrambled control shRNA, or shRNA specific for nACHR β2, or α7 as indicated (A). Numbers are given as percentage of cells positive for Zymosan (A), or in (B) as phagocytic index as percentage of positive cells times the number of particles per cell. Asterisks indicate significant difference (P<0.05) compared to vehicle treatment (medium). Transcript levels of nACHR β2, β-actin, and GAPDH in cells transfected with random scrambled shRNA or nACHR β2-directed shRNA (C). Nicotine dose-dependently enhances Zymosan phagocytosis in PMF from both WT mice and nACHRα7-/- mice, but not in PMF from nACHRβ2-/- mice. Data shown are average +/- SEM of 3 independent experiments performed in triplicate. Asterisks indicate significant differences (* P < 0.05) (D).
Nicotine enhances Dynamin-2 recruitment to the phagocytic cup.
To elucidate the underlying cellular mechanism behind the nicotinic stimulation of phagocytosis we analyzed whether nicotine augmented phosphatidylinositol 3-kinase (PI-3K)/Akt activation, known to be required for phagocytosis. Macrophage phagocytosis of Zymosan stimulated PI-3K and Akt phosphorylation, but nicotine did not significantly enhance this process, or phosphorylation of p38 MAP kinase, (Supplementary Fig.1a). In addition, we analyzed whether the enhanced phagocytosis by nAChR activation resulted from induced intracellular calcium fluxes. To this end, we analyzed whether nicotine induced calcium fluxes in freshly isolated peritoneal macrophages using FURA-3 probe assisted imaging. However, although macrophages respond potently to ATP, no calcium flux was observed after exposure of macrophages to nicotine at concentrations ranging from 0.1–25 μM (Supplementary Fig. 1b). Similarly, nicotine enhanced phagocytosis was not associated with enhanced Rac1 GTPase activity (Supplementary Fig. 1c). The polymerization of actin, and the phagocytic process are crucially dependent on the activity and cellular distribution of the large GTPase Dynamin-2, which mediates membrane extension, and formation of the phagocytic cup(18). In neuronal tissue, the nAChR β2 subunit has recently been shown to complex to Dynamin-1(19). We therefore investigated whether nAChR β2 stimulates phagocytosis by affecting the expression and cellular distribution of macrophage Dynamin-2, which is highly homologous to Dynamin-1. To this end we analyzed the distribution of Dynamin-2 protein, and cup formation in PMF after challenge with Zymosan particles. As shown in Fig. 5, Zymosan induced formation of the phagocytic cup in control cells within 5 min. (Fig.5A). Pretreatment with nicotine (1μM) augmented the recruitment of Dynamin-2 protein towards the phagocytic cup and stimulated cup-formation (Fig. 5B-C). This was corroborated by Western analysis of preparations of cell membranes; nicotine pretreatment led to a transient increase in membrane-associated Dynamin-2 protein (Fig. 5D). The enhanced membrane recruitment by nicotine peaked at 5 min, and was no longer observed at 20 min. after Zymosan exposure.
Vagus nerve stimulation enhances luminal uptake by intestinal phagocytes. Next we investigated the effect of vagus nerve stimulation on macrophages residing in the intestinal lamina propria (LPMF). LPMF in human and mouse lack CD14 expression, produce little cytokines, and have a primarily phagocytic function (11). Therefore, we tested the effect of nAChR activation on phagocytosis of E. faecium bacteria by isolated F4/80+CD11c- LPMF (Fig. 6A) that, as shown earlier in Fig. 3C, express the α4β2 nAChR. Furthermore, in analogy to PMF; LPMF pre-treated with nicotine showed increased uptake of E. faecium (Fig. 6B-C) or Zymosan (not shown).
Next, we tested whether VNS affected the mucosal uptake of luminal particles in vivo. To this end, a segment of small intestine was ligated, allowing normal innervation and blood supply, and the effect of VNS on the mucosal uptake of heat-killed *Enterococcus faecium* and Dextran particles administered to the intestinal lumen was studied (Fig. 7A, B). In sham stimulated mice, FITC-labeled *E. faecium* adhered to enterocyte layer but was only scarcely found in the mucosal compartment. Similarly, Dextran particles were endocytosed by enterocytes, but no Dextran uptake was seen in lamina propria phagocytes after sham stimulation. However, VNS at 1 or 5 V stimuli led to enhanced (p=0.04) translocation and uptake of *E. faecium* and Dextran particles by lamina propria phagocytes (Fig. 7A,B). In the mucosa, staining for phagosome marker LAMP-2 partly co-localized with *E. faecium*, indicating that these bacteria were indeed in the phagosome (not shown). To identify the lamina propria cells that had taken up luminal antigen after VNS, we subsequently performed immune-histochemical staining using macrophage markers F4/80 and CD11b and CD11c in sections of VNS-intestinal tissue. These analyses indicated that lamina propria cells that had taken up *E. faecium* were F4/80+ and CD11b+ (Fig. 7C-D). Moreover, most lamina propria phagocytes positive for *E. faecium*, stained negative for dendritic cells marker CD11c (Fig. 7E), indicating that bacteria were taken up by LPMF rather than dendritic cells.

*Figure 6. Nicotine stimulates phagocytosis in lamina propria macrophages.* Nicotine enhances *E. faecium* uptake in F4/80+CD11c− LPMF. FACS analysis of intestinal macrophages. Cells enriched using F4/80 MACS microbeads are CD11c negative (A). Histogram plot showing *E. faecium* FITC positive macrophages that have phagocytized *E. faecium* after 10 min. incubation at 37°C (B). Shaded plot indicates uptake after vehicle, open plot (red line) shows uptake after 1 μM nicotine treatment. Quantification of LPMF positive for FITC-*E. faecium* treated with vehicle (Veh) or nicotine (Nico), incubated on ice or at 37°C where indicated (C). Data are averages +/- SEM; n=3.
The increased uptake of luminal particles after VNS may also be the result of modulation of the epithelial barrier. Enhanced cholinergic activity has been implicated in changes in mucosal barrier function (20;21). To evaluate whether the enhanced mucosal uptake of luminal bacteria after VNS resulted from enhanced epithelial transport, we mounted intestinal tissue in Ussing chambers to measure the flux of HRP passing the bowel wall via para- or transcellular routes. As shown in Fig. 8A, basal flux of HRP was not altered in intestinal tissue derived from mice that had undergone VNS 2h earlier. However, when control tissue was mounted and nicotine, or carbachol (an agonist for cholinergic receptors) was added to the mucosal compartment, a transient (30min.) increase in HRP flux towards the serosal layer was observed (Fig. 8A). No enhanced flux was observed after subsequent washout of nicotine (not shown).
These observations indicate that activation of cholinergic receptors in the intestinal tissue induces a transiently enhanced mucosal passage of luminal bacteria, in agreement with the role of ACh in stress-induced epithelial permeability (22;23).

We next evaluated whether VNS also led to an enhanced drainage of phagocytosed bacteria to mesenteric lymph nodes. To this end, we determined whether VNS increased the number of Colony Forming Units (CFUs) cultured from MLN. As shown in Fig. 8B, stimulation of the vagus nerve led to a significant increase (aerobic p=0.04; anaerobic p=0.018) in the number of CFUs cultured as compared to sham stimulated mice, confirming that stimulation of vagal activity enhances mucosal uptake and drainage of luminal bacteria.

Figure 8. Cholinergic activity reduces the epithelial barrier function.
Translocation of HRP over the intestinal mucosa measured in Ussing chambers (A). Nicotine (1μM) or Carbachol (1μM) added to the chambers increases HRP flux to the serosal compartment. Data are average +/- SEM of 8 preparations from 4 mice. The number of bacteria cultured from mesenteric lymph node (MLN) after cervical stimulation of the vagus nerve (VNS), compared to sham stimulation (B). Data shown are average +/- SEM, n = 5. Asterisks indicate significant differences (*P<0.05; ** P<0.01).
Discussion
The cholinergic anti-inflammatory pathway represents a physiological system to control macrophage activation and inflammation (2). Its working mechanism ultimately involves modulation of STAT3 pathways (6) and prevention of NF-κB p65 transcriptional activity (3;4) following nAChR activation. Efferent vagus nerve signaling has been shown to reduce pro-inflammatory cytokine responses in macrophages stimulated with endotoxin (1). Much less attention has been paid to professional functions of macrophages, i.e. endo- and phagocytosis of bacteria and particles. We show here that the vagus nerve signaling has a dual effect in macrophages; nAChR activation stimulates phagocytosis, while reducing NF-κB activation and inflammatory cytokine production (Supplementary Fig. 2).

Our data demonstrate that vagus activity conveys its anti-inflammatory effect via distinct nAChRs expressed in macrophages. The anti-inflammatory effects of nAChR activation on macrophages have previously been solely attributed to activation of the nAChR α7(5;6). Although previous data imply a role for α7 nAChR in modulation of NF-κB activation(4), we clearly demonstrate that the nAChR α4β2, rather than α7, mediates stimulation of phagocytosis. Matsunaga et al. (24) and others (25) have shown expression of α4β2, and no α7 nAChR in alveolar macrophages. Likewise, we failed to demonstrate α7 nAChR transcripts in intestinal, Mφ/4 spleen or peritoneal macrophages. Further analysis of potential α7 nAChR protein in these macrophages was hampered by our observation that commercially available α7 nAChR antisera are not specific and stain an approximately 57kD protein in brain homogenates from wildtype as well as α7 nAChR –/- mice, in agreement with earlier reports (26). Surprisingly, α7 nAChR blockers were effective in counteracting nicotinic effects on NF-κB activity as well as cytokine production. These data indicate that the selectivity of commonly used blockers αBgt and MLA for α7 nAChR may be questioned, and both blockers have shown to bear affinity for other nAChR subunits, including α1, α6, α9, α10 and β2, as well (27). Alternatively, the β2 nAChR blocker DHβE used in the current study also bears affinity for other nAChRs.

Together, our results indicate that ACh induces phagocytosis via α4β2 nAChR, but seemingly activates different nAChR systems on macrophages to achieve inflammatory anergy.

Our data reveal the potency of vagus nerve activity to stimulate macrophage phagocytosis via the large GTPase Dynamin-2. In macrophages, Dynamin-2 plays a crucial role in exocytosis of endomembranes at the site of phagocytosis, and phagosome formation (28). Our data indicate that the vagal modulation of phagocytosis involves nAChRβ2 mediated modulation of Dynamin-2 activity and cellular distribution, rather than PI3K, Akt, Rac1 activation or calcium signaling pathways. Formation of the phagosome and subsequent phagocytosis is the result of cellular remodeling of actin filaments, a process that requires Dynamin GTPase activity (18). Interestingly, in mouse cholinergic neurons, the nAChR β2 subunit has recently been shown to complex to the GTPase Dynamin-1, which is highly homologous to the macrophage Dynamin-2. Although we do not know how the nAChR β2-mediated cellular redistribution of the Dynamin is brought about, it is tempting to speculate that in macrophages, in analogy to cholinergic neurons, nAChR β2 activation modulates Dynamin-2 protein distribution directly. The recruitment of Dynamin to membrane receptors was initially thought to be mainly required for receptor internalization (29). However, the internalization of nAChRs has been shown not to involve Dynamin (30) and our results demonstrate that ACh mediated Dynamin-2 activation supports the phagocytic potential. The exact cellular mechanism by which nAChR β2 mediates Dynamin-2 cellular distribution is currently under investigation.

Intestinal macrophages co-operate with dendritic cells to form the first line of defense in the intestinal mucosa. We show here that vagus nerve activity assists in surveillance of luminal
antigen uptake by inducing a transient increase in epithelial permeability and augmenting the uptake of luminal bacteria by mucosal macrophages. Our data confirm the intimate involvement of the autonomous nervous system in the maintenance of mucosal barrier function (23;31;32). Our current finding of the vagus nerve regulating the mucosal barrier function is in agreement with earlier reports of cholinergic regulation of enterocyte endocytosis and intestinal epithelial permeability (20), although epithelial signaling is thought to be mediated via non-neuronal ACh production (21). The cholinergic modulation of the epithelial barrier function was not explained by altered tight junction protein expression, as levels of occludin and claudin-2 were not affected in intestinal tissue from mice that underwent vagus nerve stimulation (results not shown). A possible alternative explanation for the transient reduction of barrier function after VNS is that stimulation of vagal activity, under normal physiological conditions, leads to a reduction in mean arterial blood pressure, a parameter that is shown to mediate tight junction integrity (33). Hence, although vagal activation preserves barrier function under pathological conditions such as ischemia, via its anti-inflammatory actions, our data indicate that enhanced vagus nerve activity under physiological conditions can allow transient passage of antigens, possibly to assist in routine surveillance. The physiological relevance of this system is currently under investigation.
Reference List


Supplementary Methods:

Suppl. figure 1. Cholinergic increase in phagocytosis does not involve PI3K, Akt or p38 pathways, calcium fluxes, Rac1 GTPase activity. Peritoneal macrophages pretreated with indicated doses of nicotine, allowed to phagocytose Zymosan particles for 5, or 10 min., lysed and analyzed by Western blot. Right graphs: quantification of optical density of phospho-PI3K as compared to PI-3K and phospho-akt, as compared to akt. Grey bars: 5 min. incubation, black bars 10 min. incubation. Gels shown are representative of 3-4 independent analyses (A). Nicotine (1μM) does not elicit intracellular calcium release in peritoneal macrophages. Representative tracing from 5 analyses. ATP added as a positive control, cells were permeabilized using 10% Triton X-100 as indicated (B). Rac1 activity in Mf4/4 cells pretreated with medium (black bars) or nicotine (1mM; white bars) for the time indicated, in the presence or absence of Zymosan, were indicated (C). Data shown are average +/- SEM of 3 independent analyses.
Suppl. Figure 2. Vagal signaling enhances phagocytosis but inhibits inflammatory response.

Vagal nerve activation and the release of acetylcholine drives macrophages towards an inflammatory anergic, but highly phagocytic phenotype; nAChR activation enhances Dynamin-2 GTPase activation and endo-and phagocytosis, whereas the subsequent activation of NF-κB and pro-inflammatory cytokine and chemokines release is inhibited. Both phenomena may involve distinct nAChRs. Nicotinic activation of phagocytosis involves nAChR α4β2, while nicotinic effects on NF-κB activation involve nAChRa7 or alternative nAChR systems. The activation of the STAT3 pathway is a critical intermediate in the cholinergic tolerization of macrophages, as described earlier (6).
Supplementary Methods:

Primer sequences
nAChR β2 sense, 5’- GGGCAGGCACACTATTCTTTC-3’, and antisense, 5’-TCCAATCCTCCCTCACACTC-3’; nAChR α7 sense, 5’-AAATATTGGGTCCTGGTCTCTCCA-3’, and antisense, 5’- GTATGCTGGTGTCGGCAAAGTA-3’; nAChR α4 sense:5’ -GCCTAAGTG- GACCAGGATCA-3’ and antisense 5’-CTC GGGAAGCCAATGAGGAGC-3’.GAPDH sense, 5’-ATGTGTCCGTCGTGGATCTGA-3’, and antisense, 5’-ATGCCTGCTTCACCAC- CTTCT.

Immunostaining and imaging
For confocal microscopy, macrophages (10⁵ cells per well) were left to adhere for 16-20 hrs on glass slides (Nunc, Rochester, NY, USA) in RPMI/10% FCS. After washing, cells were fixed in ice-cold 4% phosphate-buffered (pH 7.4) PFA for 1 h, permeabilized in 0.1 % saponin/2 % BSA in PBS, and incubated with primary antibodies (anti-nAChR α7 antiserum, 1:500; anti-nAChR α4, 1:500; anti-AChR β2, 1/500) at 4ºC for 16-20 hrs. Following washing (3 x 0.1 % saponin/2 % BSA in PBS), phalloidin-TRITC (Molecular Probes) and secondary antibodies (goat-anti-rabbit AlexaFluor546; goat anti-rat AlexaFluor488; Molecular Probes) were applied. After washing with ice-cold PBS pH 7.4, antibodies were visualized using Alexa546-streptavidin (Molecular Probes). Sections were mounted in glycerol mounting medium to which DAPI (10 μg/ml; Molecular Probes) nuclear counter stain was added. Cells were analyzed using a confocal laser scanning microscope LSM510 Meta (Zeiss) and the corresponding software.

Dynamin-2 immunohistochemistry. MF4/4 cells were grown o/n on 8 well glass slides (nuncbrand) to a 80% confluence, washed and pretreated with nicotine (1μM) for 20 min. Slides were incubated on ice before FITC-labeled-Zymosan (5p/cell) was added and glasses were centrifuged for 2 min. at 400 g at 4ºC to synchronize phagosomes. Phagocytosis was allowed for 5 min. at 37ºC. Subsequently, cells were washed with ice-cold PBS (3x) and fixed in 2% paraformaldehyde for 30 min on ice. Following washing (PBS 3x), cells were blocked (10% normal goat serum (NGS) in PBS) and incubated with primary antibody (anti-dynamin 2 (abcam), 1:600 in PBS with 1% NGS and 0.01% Triton X-100) o/n at 4oC. Dynamin-2 was visualized using biotinylated secondary antibody and alexa647 conjugated strepavidin (Invitrogen). F-actin was visualized by pre-incubation with phalloidin-TRITC (Sigma). Slides were analyzed using confocal laser scanning microscope LSM510 Meta (Zeiss) and the corresponding software. Dynamin recruitment to the phagocytic cup was scored by analysis of 80-100 cells in a blinded fashion.

siRNA sequences
nAChR α7, top: 5’-GATCCTGCTAAAGTGACCAGGATTTCAGAGA ATCCTGGTCC ACTTAGGCATT-3’, bottom:AGCTTAATGCGCTAAGTGACCAGGATTTCCTCTTGAA ATCCTGGTCCACTTAGGCAG; nAChR β2, top: 5’-GATCCGCCTGAGGATTA TTGGTCCCTTTTAAATGCGCTAAGTGACCAGGATTTCCTCTTGAA ATCCTGGTCCACTTAGGCAG.

Measurement of cellular calcium flux
Peritoneal cells were harvested and resuspended in HBSS with CaCl₂ and MgCl₂ supplemented with 0.5% BSA and 10 mM Hepes (HBSS/BSA) at room temperature to a cell density of 10⁷ cells/ml. The cell suspension was incubated with 2 μM Fura-PE3/AM (EMD) for 45 min at room temperature. The cells were then washed twice and resuspended in HBSS/BSA (3 x 10⁷
cells/ml). 100 μl of cell suspension (3 x 10^6 cells) was added to a cuvette containing 400 μl of prewarmed (37°C) HBSS/BSA and placed in a luminescence spectrometer (LS55; PerkinElmer). After a period of at least 60 s, fluorescence measurements were recorded at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. 10 μl of agonist was added under constant stirring at appropriate intervals. At the end of each experiment, 10 μl 10% Triton X-100 was added to determine maximum calcium flux.

Rac activity assays

Cells were treated with ACh or nicotine for 30 min. and lysed in ice-cold lysis buffer containing 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 0.1% SDS, 0.5% deoxycholate, 10% glycerol, 1 mM Na3VO4, 50 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF. For measuring p21Rac GTP-loading we employed a 96-well plate coated with RBD domain of Rho-family effector proteins (POSH), based on the method described in United States Patent 20070020687. The active GTP-bound form of Rac, but not the inactive GDP-bound form, from a biological sample will bind to the plate. Per well 1 μg of protein cell extract of the relevant experimental condition is loaded and after 1 hr of incubation plates are washed and incubated with an anti-Rac antibody followed by a secondary HRP-lapelled antibody and coloring reaction according to routine ELISA procedures. GTP-γ-S-loaded p21Rac serves as a positive control.

Isolation Lamina Propria MF

Jejunum and ileum tissue from Balb/C male mice was isolated, opened along the mesenteric border, and rinsed in ice-cold Ca²⁺- and Mg²⁺-free PBS. Mesentery and Peyer’s patches were removed, remaining intestine was washed in HBSS containing DTT to remove residual mucus and than in HBSS/0.125 M EDTA plus 10 mM β-mercaptoethanol to remove the epithelium. Tissue was minced and treated with the neutral protease collagenase (0.5 mg/ml; type IV; with <0.01 ng/ml endotoxin, Sigma-Aldrich) in RPMI/2.5% FCS for 45 min. The cell suspension was filtered, centrifuged, and resuspended in 40% Percoll solution in PBS pH 4.6. Lamia propria macrophages were enriched by a 40%/60% Percoll centrifugation at 400 g for 30 min. Cells were taken up in MACS buffer (0.5% BSA, 1 mM EDTA) at 2x10⁶ cells/mL and incubated with anti-F4/80 rat monoclonal antibody (1μg/10⁷ cells; BMA Biomedicals, August, Switzerland) for 15 min, washed and incubated with goat anti-rat microbead-conjugated antiserum (1 μg/10⁷ cells; Miltenyi Biotec Inc.) on ice for 15 min. After washing, labeled cells were taken up in 1 mL MACS buffer, loaded onto a pre-cooled MACS MS column according to the manufacturers’ instructions. The resulting macrophage fractions taken up in RPMI/1%FCS at 107 cells/mL, left for 2 h, and then allowed to phagocytose FITC labeled heat-killed E. faecium at 37°C during 1h, washed and analyzed by FACS.

NF-κB activity assays

Mφ4/4(34) cells were co-transfected with NF-κB luciferase and CMV renilla reporter constructs (Clontech, Mountain View, CA) using Jet PEI (PolyTransfection), according to the manufacturer's instructions. For transfection of 2x10⁵ cells, 0.5μg of NF-κB-luc and 5ng CMV Renilla luciferase construct were suspended in 75μL of 150 mM sterile NaCl solution. After 16hrs, cells were washed and pre-treated with nicotinic agonists for 45 minutes, followed by Zymosan (5p/cell) stimulation for 6hrs. After treatment, cells were washed three times with ice-cold PBS and lysed with Passive Lysis Buffer supplied in the Dual Luciferase™ Reporter Assay Kit (Promega) and the lysate was assayed for luciferase activity according to the manufacturer’s instructions.