# Resident macrophages activated by lipopolysaccharide (LPS) suppress muscle tension and initiate inflammatory response in the gastrointestinal muscle layer

Shigeko Torihashi, Hiroshi Ozaki*,Masatoshi Hori*, Muneto Kita*, Sachiyo Ohota, Hideaki Karaki*
Department of Anatomy, Nagoya University School of Medicine
Tsurumai Nagoya 466-8550 Japan
Phone:+81-52-744-2002
Fax:+81-52-744-2013
e-mail:storiha@tsuru.med.nagoya-u.ac.jp
* Department of Veterinary Pharmacology, Graduate School of Agriculture and Life Science, The University of Tokyo113-8657 Japan

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Correspondence: Dr. Shigeko Torihashi

## Abstract

A great number of macrophages is found to be evenly distributed in the muscle layer of the gastrointestinal tract. We investigated their effects on smooth muscle contraction and the initiation of immune reactions such as inflammatory responses. Macrophages were demonstrated by uptake of FITC-dextran and their ultrastructural features were elucidated by electronmicroscopy. Muscle layers of rats' ileums were incubated with lipopolysaccharide (LPS) for 4 to 8 h and the force of smooth muscle contraction was measured. The induction effect of inducible nitric oxide synthase on macrophages was then checked by immunohistochemistry. The expression of Main Histocompatible Complex (MHC) class II was also examined. Macrophages in the muscle layer were confirmed as resident macrophages and were different from a population of dendritic cells. After incubation with LPS, macrophages begun to express iNOS and produced NO, and it reduced smooth muscle contraction. iNOS immunopositive cells increased in a time dependent manner. Macrophages also began to express MHC class II. The total number of macrophages did not alter after incubation. Results indicate that resident macrophages in the muscle layer induced iNOS as an inflammatory reaction, affected smooth muscle contraction, and initiated immune response in the smooth muscle layer of the gastrointestinal tract, when activated by LPS.

## Introduction

Gastrointestinal tracts are the first line of defense against from pathogens out side the body, and from a protective sheath against bacterial translocation and endotoxins. In the mucosa, immunologically potent cells including macrophages are prominent and take part in well-developed immune responses. It is thought that these macrophages are closely related to inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (Collins 1996). Recently, macrophages in the muscle layer have been reported as a distinct population from mucosa macrophages in mammalian intestine. These macrophages are regularly distributed in subserosa, at the level of the myenteric plexus and inside of the muscle layer (at the level of the deep muscular plexus in rodent small intestines) (Mikkelsen et al. 1985; Mikkelsen et al. 1988; Mikkelsen and Rumessen 1992; Mikkelsen 1995). Although their unique distribution and great number imply on important role in the muscle layer, the function of macrophages is remains to be clarified and their pathological effects on the GI tract have not yet been demonstrated.

It has been attracted considerable attention that inflammation at the mucosa affects and suppresses intestinal motility in animal models, and in humans in Crohn's disease (Collins 1996; Myers et al. 1997). In addition to neural elements, inflammatory mediators are supposed to be involved in this motility disorder. Eskandari et al. reported lipopolysaccharide (LPS) activated macrophages in the muscle layer and suppressed circular smooth muscle contraction (Eskandari et al. 1997). They confirmed intraperitoneal administration of LPS reduced spontaneous circular muscle activity *in vivo* as a potential inflammatory response. It was also found that macrophages in the muscle layer had latent ability to induce nitric oxide synthase (NOS) and produced nitric oxide (NO) under the dosage of LPS given by Schroeder and coworkers (1997). These data strongly suggest that macrophages in the muscle layer induce NOS and produce NO which

reduces circular muscle contractions, when activated by LPS.

In the present study, we established an experimental protocol to activate macrophages and measure the mechanical contraction of the muscle layer after incubation with LPS *in vitro.* We then investigated the mechanisms of the relaxation of the muscle layer and induction of the immune response, and in what way macrophages were involved in these mechanisms. Our data strongly suggest macrophages in the muscle layer play an important role in the connection between inflammation of the mucosa and the motility of the GI tract.

**Materials and Methods**

Wister rats of either sex, and 3-8 weeks old (100-200g) were used. Four animals were anesthetized and given injections of FITC-dextran M.W.70.000 (0.25mg / g; Molecular Probes, Eugene, Oregon) from tail vein. Rats were fixed 2 days after injections and processed for conventional electronmicroscopy and immunohistochemistry. The use and treatment of animals followed the *Guide to Animal Use and Care* of the Nagoya University School of Medicine. For electronmicroscopy, rats were perfused with a fixative of 2.5% glutaraldehyde in 0.05M cacodylate buffer pH7.4 containing 1.25mM $CaCl_2$ and 3% sucrose. Dissected ileum were post-fixed with 1% $OsO_4$, stained *en bloc* with saturated uranyl acetate, dehydrated through graded ethanol, and embedded in Epon 812. Ultrathin sections were examined with a Hitachi electron microscope (H-7100, Hitachi Japan). For immunohistochemistry, animals were fixed with 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4, and ileums were dissected and muscle layers were processed for whole mount preparation. Samples were incubated overnight at 4°C with anti-iNOS antibody (1:1000; Transduction Lab., Lexington, KY), anti-rat resident macrophage antibody (ED2, 1:500; Serotec Oxford England), or anti-Ia (Major Histocompatibility Complex class II) antibody (OX6, 1:500; Serotec). They were then treated by Vector Stain ABC Kit (Vector, Burlingame CA) and detected with Texas red-conjugated streptavidine (1:100, Vector). Samples were examined with a confocal laser scanning microscope (MRC-1024, Bio-Rad, Hercules, CA). To check the specificity of immunohistochemistry, tissues in which primary antibodies were omitted from the initial incubation were also prepared.

Twelve rats were stunned and killed. Ileums were then dissected into 2-3 cm long segments, cut open along the mesenteric attachment, and the mucosa and submucosa were removed from specimens. The remaining muscle layers were pinned out serosal side up on silgard plates and incubated in tissue culture medium (mM): NaCl 136.9, KCl 5.4, $MgCl_2$

1.0, $NaHCO_3$ 23.8 and glucose 5.5, containing 100μg/ml of lipopolysaccharide (LPS; Sigma B5) for 4 or 8 h at 37ºC. Control tissues were treated in the same manner without LPS and other tissues were also prepared before incubation as another set of controls. For mechanical tension recording, strips of muscle layer (2mm X 7mm) were cut off from incubated tissues. The force of contraction was measured isometrically with a force displacement transducer (model TB611T Nihon Kohden, Tokyo Japan). Besides mechanical recording, remaining tissue was processed for immunohistochemistry using antibodies as described above. Treatment of 0.1% diaminobenzidine tetrahydrochloride with 0.02% $H_2O_2$ for detection, immunopositive cells were counted and tabulated (per $mm^2$) in each sample (n=4). Anti-iNOS antibody and ED2 were treated at the same time for double staining, and they were detected with Texas red-conjugated streptavidine and fluorescein isothiocyanate (FITC)-conjugated IgG, respectively. Colocalization was analyzed with the confocal microscope.

## Results

### *1. Ultrastructural features and properties of resident macrophages in the intact tissue*

Resident macrophages were distributed throughout the gastrointestinal (GI) tract. They were located mainly under the serosa, at the level of the myenteric plexus and around the deep muscular plexus (DMP). Their ultrastructural feature was different from both interstitial cells of Cajal and fibroblasts. Resident macrophages had well developed cell processes, lysosomes, golgi area, dense bodies, tubular vesicles and coated vesicles (Fig. 1A). After injection of FITC-dextran, macrophages contained dextran particles in their phagosomes. They sometimes appeared similar to the light vesicles reported in mice injected with FITC-dextran (Fig. 1B). Confocal microscopy demonstrated a three dimensional distribution of resident macrophages which had uptaken FITC-dextran. They

were distributed almost evenly in the muscle coat. Most of them had a multipolar configuration, however, some of them located close to the muscle layer were bipolar cells running parallel to the neighboring muscle fiber (Fig. 2A, B). Immunostaining with anti-resident macrophage antibody (ED2) showed their cellular outline clearly (Fig. 2D). Almost all FITC-dextran uptaking cells were ED2 positive (Fig. 2C, D, E). However, only a few cells expressed immunoreactivity for Ia (OX6; Fig. 2F, G, H).

***2. Incubation with Lipopolysaccharide (LPS)***

*Inducible Nitric Oxide Synthase (iNOS) immunoreactivity*

Before incubation, macrophages did not express iNOS immunoreactivity (Fig. 3A). After 4h incubation, some macrophages showed iNOS immunoreactivity and the number of positive cells increased after 8h incubation (Fig. 3B, C). Although iNOS positive cells also appeared in the control tissue incubated without LPS, their population was small (Fig. 3D). Furthermore, we confirmed that cells expressing iNOS immunoreactivity were resident macrophages that were ED2 positive (Fig. 4A, B, C). The numerical data is shown in Figure 5. During incubation with LPS, resident macrophages begin to express iNOS immunoreactivity, and the number of positive cells raised in proportion to incubation time, reaching $185 \pm 109$ cells /$mm^2$ after 8h. On the other hand, in controls incubated in the medium without LPS, most of resident macrophages did not express iNOS immunoreactivity and the population of positive cells was $19.6 \pm 2$ cells /$mm^2$ after 8h (Fig. 5). We counted the number of resident macrophages, i.e., ED2 immunopositive cells, per square millimeter before and after incubation (Fig. 6). The number of resident macrophages was almost same in all samples and was not altered by incubation with or without LPS. The population of ED2 positive cells was $418 \pm 30$ cells /$mm^2$ before incubation, and $408 \pm 33$ cells /$mm^2$ and $443 \pm 12$ cells /$mm^2$ after 8h incubation with and without LPS, respectively.

It was also confirmed that macrophages did not change location and distributed evenly in the muscle layer after incubation with LPS. Macrophages aggregated around neither blood vessels nor lymph vessels.

*Muscle tension recording*

Carbachol ($10^{-8}$ - $10^{-5}$ M) induced graded increases in muscle tension in a concentration-dependent manner. Treatment of the tissue with LPS (100 µg/ml) for 4 h significantly decreased the carbachol-induced responses shifting the concentration-response curve to the right. However, when the muscle strips were treated with L-NMMA (300 µM), the reduced contractions induced by the LPS treatment was completely restored (Fig. 7A). Aminoguanidine, a more selective iNOS inhibitor, showed similar results with L-NMMA (Fig. 7B).

*Expression of Major Histocompatibility Complex ( MHC) class II*

Expression of MHC class II (Ia) was examined with immunohistochemistry using clone OX6 monoclonal antibody. The population of OX6 immunopositive cells increased after incubation with LPS. Before LPS treatment a few resident macrophages expressed OX6 immunoreactivity, this population increased with treatment of LPS in time dependent manner (Fig. 8A, B, C). Differing from iNOS expression, OX6 immunopositive cells increased slightly even when they were incubated in the medium excluding LPS (Fig. 8D). The increase in OX6 immunopositive cells is shown in Figure 9. OX6 positive cells increased from $19 \pm 1$ cells / $mm^2$ to $261 \pm 17$ cells / $mm^2$ with 8h treatment of LPS. Positive cells increased to twice the preincubation level and reached $55 \pm 2$ cells / $mm^2$ after 8h incubation without LPS (Fig. 9).

## Discussion

In the present study, macrophages in the rat muscle coat were well identified by electronmicroscopy as being similar structures to those in mice, humans and other mammals (Mikkelsen 1995). They were shown to be a multipolar cells or bipolar cells lying parallel to neighboring smooth muscle cells. Since these macrophages were stained with antibody clone ED2, it was confirmed that they were resident macrophages, and different from typical dendritic cells that did not express ED2. Mikkelsen and her colleague (Mikkelsen and Thuneberg 1999) mentioned in their report on osteopetrotic (*op/op*) mice, which have a mutation affecting colony-stimulating factor-1 (CSF-1), that macrophages in the muscle layer disappeared, though dendritic cells were not affected. They indicated that the macrophages which had disappeared from the muscle layer were not the dendritic cell type. Our observations of the rat muscle layer also support this concept.

Eskandari et al. (1998) reported that LPS activated resident macrophages through CD14 (LPS receptor) and resulted in the suppression of circular muscle contraction in the rat mid-jejunum. They injected LPS intraperitoneally to examine the effects *in vivo*, and then measured muscle contraction after killing the animals at various times (1, 3, 6, 12, and 24h). The present investigation directly demonstrated that LPS induced iNOS in macrophages and produced NO there, which led to the relaxation of smooth muscle. Control tissue untreated by LPS had few cells that expressed iNOS immunoreactivity. In contrast tissue incubated with LPS contained a great number of iNOS expressing cells by whole mount immunohistochemistry. It was also confirmed by double staining with anti-iNOS and ED2 antibodies that iNOS expressing cells were macrophages. The number of iNOS expressing cells increased in time dependent manner. The total number of macrophages was not altered by either treatment with LPS or incubation without LPS. All of these data indicate that iNOS positive macrophages originated in the muscle layer and

did not come from or go to the outside of the muscle layer. Treatment with LPS activated and induced iNOS in resident macrophages distributed in the muscle layer. This conclusion was also confirmed by iNOS RT-PCR (unpublished data). The percentage of iNOS expressing macrophages was estimated from the total number of ED2 positive cells and iNOS expressing cells determined by whole mount immunohistochemistry. After 8h incubation with LPS, more than 40% of macrophages expressed iNOS immunoreactivity. LPS quickly induced iNOS in the macrophages. LPS mediated NO production in a mouse macrophage cell line, as reported by Schroeder et al (Schroeder *et al* 1997). They suggested CD14 coupled to G protein and protein kinase C (PKC) to initiate iNOS gene transcriptions, however, little is known of the signal transduction pathway that expresses iNOS in macrophages (Schroeder *et al* 1997).

Incubation with LPS caused suppression of circular muscle contraction. In immunohistochemical analysis, iNOS immunoreactivity was detected in the macrophages but not in the smooth muscle. Thus, NO originated from macrophages in this case, and the NO from macrophages reduced contraction of the smooth muscle. This is demonstrated in the tension recordings (Fig. 7). Carbachol, an acetylcholine agonist, caused contraction of smooth muscle at concentrations of $10^{-8}$ M to $10^{-5}$M. The effective concentration, however, was shifted to a higher amount of carbachol when the tissue had been incubated with LPS. The contraction force was recovered by LPS treatment accompanied by L-NMMA, a NOS antagonist. A more selective iNOS antagonist, aminoguanidine, confirmed the results above. Taken together, NO produced by macrophages with iNOS suppressed contraction of the smooth muscle. In the case of an inflammatory disease such as Crohn's disease, the intestinal motility is suppressed (Annese et al. 1997). It is strongly suggested that inflammation at the mucosa activated not only macrophages, initiating the immune response in the mucosa, but also macrophages in the muscle layer, suppressing muscle

contraction. This may help to understand the disturbance of motility in inflammatory disorders.

Macrophages also began to express Major Histocompatibility Complex (MHC) class II (Ia) following treatment with LPS. Before incubation, only a few macrophages were Ia positive. The number of Ia positive cells in macrophages increased, depending on the incubation time. After 8h incubation with LPS, the number grew to more than 250 / $mm^2$, ten times higher than the level in non incubated tissue, although the number of positive cells slightly increased under incubation without LPS. During the experiments, the total number of macrophages did not alter and there was no suggestion of exchange of the macrophage population. This indicates that macrophages that had not expressed Ia became to exhibit it. Inflammatory stimuli induce accumulation of MHC class II complexes on the cell membrane (Cella et al. 1997). LPS induced resident macrophages to express Ia, which activates antigen coupling to T lymphocytes. In the choroid, LPS affected macrophages as an endotoxin to exploit Ia, and induced uveitis (Yang and Kijlstra 1997). In our case, macrophages activated by LPS initiated immune response in the muscle layer. There are several reports suggesting that Ia positive dendritic cells migrate as their phenotype maturation, or translocate to present antigen to local lymph nodes (Matsuno et al. 1996; Kudo et al. 1997; Matsuno et al. 1997; Sato,Tetsuji 1998). In the present study, however, Ia positive cells did not show any sign of migration and settled within the muscle layer. It is possible that they couple and activate T cells in the muscle layer, as reported by Cicalese et al. (1997).

In conclusion, our findings confirmed that LPS activated macrophages in the muscle layer to induce NOS, and its product NO reduced muscle tension. Furthermore, activated macrophages emphasized antigen presentation and initiated immune responses in the muscle layer. The immune cells induced in the muscle layer also produced cytokines

and may have affected muscle contraction. Thus, resident macrophages in the muscle layer play a key role in gastrointestinal motility and immune response under pathological conditions.

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**Figure Legends**

Figure 1

Electronmicrographs of resident macrophages

A) Macrophage (MC) at the level of the myenteric plexus havs fine cellular processes, primary lysosomes (asterisks) and a few vesicles suggesting phagosomes (arrows). At the peripheral region of the cytoplasm, coated-vesicles (arrowheads) are also distributed. Macrophages have no basal lamina or electron dense cytoplasm, which are ultrastructural features of the interstitial cells of Cajal. B). After injection of FITC-dextran, macrophages showed a phagosome containing FITC-dextran (asterisk) among their vesicles.

Figure 2

Confocal micrographs of resident macrophages

A) After 2 days injection of FITC-dextran, the muscle layer was illuminated with FITC-dextran that were take into macrophages. Macrophages were distributed almost evenly. Scale bar: 50μm B) The cellular configuration of macrophages is demonstrated by FITC-dextran. An asterisk indicates the nuclear region. Scale bar: 10μm C) Macrophages uptaking FITC-dextran. D) Immunostaining of ED2 antibody in the same area as (C). E). Merged picture of (C) and (D). All macrophages indicated by FITC-dextran express markers of resident macrophages (ED2). F) Macrophages uptaking FITC-dextran. G) Immunostaining of OX6 antibody in the same area as (F). H). Merged picture of (F) and (G). Only a few macrophages are double-positive, indicating Ia (OX6) expression. Scale bar: 10μm in panels from (C) to (H)

Figure 3

Immunohistochemistry of anti-iNOS antibody

A) Before incubation, macrophages did not express iNOS immunoreactivity in the whole mount preparation. B) A small number of iNOS immunoreactive cells appeared after 4h incubation with LPS. C) After 8h incubation, positive cells increased. They were distributed evenly in the muscle layer. D) In tissue incubated for 8h without LPS, only a few positive cells were recognized. They were weakly stained. Scale bar: 100µm in all panels.

Figure 4

Confocal micrographs of iNOS immunopositive macrophages

A) Macrophages containing FITC-dextran in the tissue incubated with LPS .

B) Cells expressing iNOS immunoreactivity in the same area as (A). C) Merged picture of (A) and (B). Not all, but some, macrophages (FITC labeled cells) showed iNOS immunoreactivity, as indicated by asterisks. Scale bar: 10µm in all panels

Figure 5

Number of iNOS expressing macrophages in each incubation condition.

Before incubation, the density of immunopositive cells was 2±1 cells/$mm^2$. The population of positive cells increased in a time dependent manner under the incubation with LPS. Incubation without LPS did not increase the number of positive cells.

Figure 6

Total number of ED2 positive cells in each incubation condition.

The population of macrophages indicated by ED2 immunoreactivity did not change after incubation with or without LPS. The approximate number of macrophages was 400 cells/$mm^2$ in each tissue.

Figure 7

Inhibition of contraction by LPS-treatment and its recovery by iNOS inhibitors in ileal circular smooth muscle.

Tissues were pretreated without (control) or with LPS (100 μg/ml) (+ LPS) for 4 h before starting the experiments. After the incubation, carbachol ($10^{-8}$ – $10^{-5}$ M) was cumulatively added to ileal strip to induce contractions. Tissues were also incubated with L-NMMA (300 μM) (A) or aminoguanidine (300 μM)(B) for 4 h. Values are expressed as percentage of the reference response (72.7 mM KCl) in a fresh tissue (approximately 1 h after the isolation) (n = 9 – 11).

Figure 8

Immunohistochemistry of OX6 antibody

A) Before incubation, there were few OX6 immunopositive cells. B) After 4h incubation with LPS, the number of OX6 positive cells increased. C) They were prominent after 8h incubation with LPS. D) In the tissue incubated without LPS for 8h, a few positive cells appeared. Scale bar: 100μm in all panels

Figure 9

Number of OX6 immunopositive macrophages in each incubation condition.

OX6 positive cells increased in a time dependent manner under incubation with LPS. After 8h the number of positive cells reached more than 260 cells/mm$^2$, though under the incubation without LPS, the number of OX6 immunopositive cells slightly increased.