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Macrophages Associated with the Intrinsic and Extrinsic Autonomic Innervation of the Rat Gastrointestinal Tract

Robert J. Phillips and Terry L. Powley

Department of Psychological Sciences, Purdue University, West Lafayette, Indiana 47907-2081

Abstract

Interactions between macrophages and the autonomic innervation of gastrointestinal (GI) tract smooth muscle have received little experimental attention. To better understand this relationship, immunohistochemistry was performed on GI whole mounts from rats at three ages. The phenotypes, morphologies, and distributions of gut macrophages are consistent with the cells performing extensive housekeeping functions in the smooth muscle layers. Specifically, a dense population of macrophages was located throughout the muscle wall where they were distributed among the muscle fibers and along the vasculature. Macrophages were also associated with ganglia and connectives of the myenteric plexus and with the sympathetic innervation. Additionally, these cells were in tight registration with the dendrites and axons of the myenteric neurons as well as the varicosities along the length of the sympathetic axons, suggestive of a contribution by the macrophages to the homeostasis of both synapses and contacts between the various elements of the enteric circuitry. Similarly, macrophages were involved in the presumed elimination of neuropathies as indicated by their association with dystrophic neurons and neurites which are located throughout the myenteric plexus and smooth muscle wall of aged rats. Importantly, the patterns of macrophage-neuron interactions in the gut paralleled the much more extensively characterized interactions of macrophages (i.e., microglia) and neurons in the CNS. The present observations in the PNS as well as extrapolations from homologous microglia in the CNS suggest that GI macrophages play significant roles in maintaining the nervous system of the gut in the face of wear and tear, disease, and aging.

Keywords

aged; alpha-synuclein; CD163; myenteric; MHCII; muscularis; resident macrophage; sympathetic

INTRODUCTION

Macrophages in the gastrointestinal (GI) tract comprise the body's single largest population of mononuclear phagocytes (Lee et al., 1985; Smith et al., 2011) and serve as the innate immune system of the gut (Mikkelsen, 1995; Smith et al., 2005). One subpopulation of GI macrophages is distributed within the mucosa and is associated with the epithelial interface with exogenous materials and nutrients in the lumen. These macrophages have been

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Corresponding Author: Robert J. Phillips, Purdue University, Department of Psychological Sciences, 703 Third St, West Lafayette, IN 47907-2081, Phone: 765-494-6268, Fax: 765-496-1264, rphillip@psych.purdue.edu.

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extensively studied in terms of their participation in colitis, IBD, inflammation and other pathogenic insults to gastrointestinal homeostasis (Wehner et al., 2007; Bauer, 2008).

Another subset of GI macrophages is located in the muscle wall of the GI tract (Kalff et al., 1998; Mikkelsen et al., 1985; Mikkelsen et al., 2011). Examinations of these macrophages in the smooth muscle have identified concentrations of the phagocytes associated with three layers: the serosal layer, the interface between the longitudinal and circular muscle layers where the myenteric plexus is located, and the level of the deep muscular plexus (Mikkelsen, 1995; Ozaki et al., 2004; Mikkelsen et al., 2011). Assessments of these smooth muscle macrophages have focused largely on their contributions to post-operative ileus and disturbances in motility associated with inflammation and disease (Torihashi et al., 2000; Bauer, 2008). Complementing this clinical focus, structural examinations of the macrophages in the gut muscle wall have for the most part concentrated on the relations between the phagocytes and the interstitial cells of Cajal (Mikkelsen, 1995; Kinoshita et al., 2007).

In contrast, far less attention has been given to interactions of gut macrophages with the intrinsic and extrinsic neural networks in the wall of the GI tract. In limited light microscopic observations, macrophages have been described in proximity to the myenteric plexus (Mikkelsen, 1995; Mikkelsen et al., 2008), and, in ultrastructural observations, elements of macrophages have been observed in proximity to bundles of neural processes (e.g., Mikkelsen, 1995; Ruhl et al., 1995) as well as within myenteric ganglia (Kinoshita et al., 2007; Mikkelsen et al., 2004). Except for such general observations, though, little is yet known about the structural relationships of macrophages and the autonomic circuitry in the GI tract.

Two sets of recent experimental observations, however, underscore the need for more thorough analyses of interactions between gut macrophages and the autonomic innervation of the GI tract. One set of observations reflects the changing understanding of the diversity and heterogeneity of macrophage functions. In early assessments of macrophages, the cells were considered simply either “resident” or “activated.” *Resident macrophages* were taken to be relatively inactive and stationary, whereas *activated macrophages* were viewed as mobilized to attack exogenous pathogens and to initiate immune responses to those pathogens. This limited view suggested, in effect, that the macrophages interactions with the autonomic network in the gut might be confined to infections or inflammation. More recently, though, it has been recognized that resident macrophages are dynamic, with highly motile processes (Raivich, 2005), and that there are multiple different specialized activational phenotypes (Colton and Wilcock, 2010; Mosser and Edwards, 2008; Town et al., 2005). Furthermore, it has been established that in some “alternative activation” conditions macrophages undertake a variety of neuroprotective and “non-immune” housekeeping functions such as removing neuronal and other endogenous cellular debris (Nimmerjahn et al., 2005; Birge and Ucker, 2008; Kono and Rock, 2008; Neumann et al., 2009). When appropriately activated for such housekeeping operations, CNS macrophages (i.e., microglia) have been shown to play critical roles in neuroprotection, inflammation resolution, and repair as well as in neurodegeneration (Colton and Wilcock, 2010; Graeber and Streit, 2010; Perry and O’Connor, 2010), and PNS macrophages might very well play similar roles in the periphery, including the gut.

The second set of observations that underscores the relevance and need for more thorough examinations of macrophages associated with the innervation of the GI tract relates to the potential role(s) the cells might have, specifically in neural remodeling and aging. Our laboratory group (Phillips and Powley, 2007; Phillips et al., 2010) as well as a number of other laboratories (Wade and Cowen, 2004; Abalo et al., 2005; Camilleri et al., 2008) have

delineated, for example, some of the neuronal losses, dystrophies and remodeling that occurs in the autonomic innervation of the gut with aging. Such neuropathic changes would be expected to elicit phagocytosis and other housekeeping operations of macrophages in the proximity of the intrinsic and extrinsic circuitry in the GI tract. Furthermore, as has been proposed in the case of the CNS microglia and other macrophages (Graeber and Streit, 2010; Ma et al., 2003; Plackett et al., 2004; Streit, 2006; Streit et al., 2009), losses of macrophage number and/or efficacy might contribute to a loss of supportive housekeeping functions, a weakening of neuronal homeostasis, and an accumulation of disordered and dystrophic autonomic neurons innervating the GI tract.

Thus, the present experiment was undertaken to provide a more detailed morphological examination of macrophage-neuron relationships in the muscle wall of the GI tract. For a group of adult rats, multiple double labeling immunohistochemical series of whole mounts in which macrophages and different populations of the autonomic circuitry were prepared from stomach to colon. In addition, similar sets of whole mounts were prepared from middle-aged and aged rats in which a higher incidence of neural dystrophies and reorganizational profiles would be observed. This initial survey of different sites along the GI tract and different ages of animals was undertaken to explore the possibility that macrophages might be implicated in the changes of the gut innervation associated with aging as well as autonomic disorders and to provide preliminary observations that might help guide the planning of more systematic and quantitative evaluations of key features of macrophage-neuron interactions in the GI tract.

MATERIALS AND METHODS

Animals

Virgin male Fischer 344 (F344; n = 30) and Brown Norway (BN; n = 12) rats were purchased from the National Institute on Aging colonies maintained, respectively, by Taconic Farms (Germantown, NY) and Harlan Laboratory (Indianapolis, IN). The rats represented three ages: 3–9 (F344, n = 12), 16 (F344, n = 6), and 20–24 (F344, n = 12; BN, n = 12) months, referred to in the text, respectively, as adult, middle-aged, and aged. Middle-aged and aged rats were sampled to provide tissue containing degenerating sympathetic nerves (Phillips et al., 2006) and misfolded protein (Phillips et al., 2009).

Rats were housed (n = 2/cage) in polypropylene cages containing either sterilized Tek-Fresh bedding (Harlan Teklad, Madison, WI) or Alpha-dri (Cincinnati Lab Supply, Cincinnati, OH) in a room kept at 20–22 °C on a 12:12 hour light:dark schedule. Group housing, nylabones and polycarbonate tunnels (Bio Serv, Frenchtown, NJ) provided environmental enrichment to minimize stress (Nadon, 2004). Solid chow (NIH-31; Harlan Teklad) and tap water were available ad libitum. Conditions in the AAALAC-approved colony approximated the housing, husbandry, and barrier conditions at the National Institutes of Aging colonies, but did not provide a specific pathogen-free environment. Nonetheless, to ensure the exclusion from the study of rats with age-associated pathologies, each animal's health status (body weight, food intake; malocclusions, chromodacryorrhea, tumors, fecal texture, etc.) was monitored on a routine basis. In addition, to eliminate the possibility of the presence of known murine pathogens, the colony room was maintained under the Purdue University veterinary monitoring and surveillance program through the use of sentinels that were rotated through the colony room every three months followed by examination for parasites and serum testing for evidence of antiviral antibodies. All procedures were conducted in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publications NO. 80-23), revised 1996, and approved by the Purdue University Animal Care and Use Committee.

Fixation Protocol and Whole Mount Preparation

Rats were weighed, killed with a lethal dose of sodium pentobarbital (180 mg/kg, i.p.), and perfused through the left ventricle of the heart with 200 ml of 0.01 M PBS followed by 400 ml of Zamboni's fixative. Prior to removal of the organs of interest, a gross inspection for an enlarged spleen, the presence of white bumps on the lung or liver, and especially, tumors associated with the GI tract was conducted. The stomach, small intestine, and large intestine from healthy aged rats were sampled based on the criteria of Hebel and Stromberg (1976). Intestinal whole mounts consisted of 3 cm segments from the duodenum (the first 8 cm anal to the pyloric sphincter), jejunum (typically the middle third), ileum (the first 10 cm oral to the ileocaecal junction), proximal colon (the 6–8 cm of tissue distal to the cecum), and distal colon (6–8 cm of tissue starting approximately 2–3 cm proximal to the pelvic brim). Whole mounts were further fixed overnight in the same fixative, and then the mucosa and submucosa were removed. Dissection of the smooth muscle wall, including the myenteric plexus, was varied for each whole mount by removing the circular muscle from small regions. After dissection, the whole mounts contained regions of intact muscularis and adjacent areas with only the longitudinal muscle and adhering myenteric plexus attached, thus, providing better differentiation between the circular and longitudinal muscle and myenteric plexus within the same whole mount.

Immunoperoxidase Staining Protocol

Double staining of free-floating whole mounts consisted of rinses in 0.1 M phosphate-buffered saline (PBS; pH 7.4), 30 min soak in methanol:H₂O₂ (4:1) to inhibit endogenous peroxidase activity, rinses in PBS, 4 d soak in normal serum block, 24 h soak in the first primary (primary antibodies and dilutions are provided in Table 1), rinses in PBST (PBS + 0.3% Triton X-100), 2 h soak in biotinylated secondary (1:500, see below for specifics), rinses in PBS, 1 h incubation in ABC (PK-6100; Vector laboratories), rinses in PBS, 3 min reaction with diaminobenzidine (DAB; D5637; Sigma-Aldrich, St Louis, MO) and H₂O₂ in tris buffered saline, rinses in dH₂O, rinses in PBS, 15 min Avidin block, rinses in PBS, 15 min Biotin block, rinses in PBS, overnight incubation in normal serum block, 24 h soak in the second primary (either CD163 or major histocompatibility complex class II [MHCII], see below for description of the macrophage antibodies and Table 1 for the primary dilutions), rinses in PBST, 2 h soak in biotinylated secondary (1:500), rinses in PBS, 1 h incubation in ABC, rinses in PBS, 5 min reaction with Vector SG Peroxidase Substrate Kit (SK-4700; Vector Laboratories, Inc.) in PBS (working solution mixed according to the manufacturer's instructions immediately prior to use), and, finally, rinses in dH₂O. Every step was done at room temperature with gentle agitation on a shaker table. Stained whole mounts were mounted on gelatin-coated slides, air-dried overnight, dehydrated in an ascending series of alcohols, cleared in two xylene steps, and coverslipped with Cytoseal XYL (Richard-Allen Scientific, Kalamazoo, MI).

Normal serum block consisted of 5% normal serum (either horse or goat, depending on the host species of the secondary), 2% bovine serum albumin (BSA), and 0.08% Na Azide in 0.5% PBST. Primaries were diluted with 0.3% PBST containing 2% normal serum, 2% BSA, and 0.08% Na Azide. Depending on the host species of the primary, either biotinylated anti-mouse IgG, rat absorbed, raised in horse (1:500; BA-2001; Vector Laboratories, Inc., Burlingame, CA) or biotinylated anti-rabbit IgG, raised in goat (1:500; 111-065-144; Jackson Immuno Research, West Grove, PA) was used. Finally, secondaries were diluted with 0.3% PBST containing 2% normal serum and 2% BSA.

Antibodies to CD163 and MHCII were used for the immunophenotypic characterizations of macrophages because of the quality as well as the relative completeness of the labeling they provide. Specifically, the mouse monoclonal anti-rat CD163 antibody recognizes the plasma

membrane glycoprotein receptor CD163 (also known as ED2) which is a member of the scavenger receptor cystein-rich superfamily class B that is highly expressed on resident tissue macrophages, and can function as a macrophage receptor for bacteria that, during bacterial infection, acts as an innate immune sensor and inducer of local inflammation (Polfliet et al., 2006; Fabrik et al., 2009). Whereas, the mouse monoclonal anti-rat MHCII antibody recognizes a monomorphic determinant of the rat I-A antigen that is found in only a few specialized cell types, all of which are professional antigen-presenting cells, and includes macrophages, B cells and dendritic cells (Pabst and Bernhardt, 2010; Rescigno, 2010).

Tracings

As needed to facilitate wide-field evaluations of low-contrast or lightly stained elements (e.g., fine filopodia-like processes), images of the macrophages were captured using high power objectives (e.g., 63× oil or 100× oil objectives), opened in Photoshop CS5 (Adobe Systems, San Jose, CA) on a Wacom Tablet (Cintiq; Wacom, Vancouver, WA), enlarged 300–500%, and then traced as high contrast silhouettes onto a transparent overlay using the pen tool and function. The accuracy of the silhouette-like tracings was verified during and after the tracing process using the transparency slider. Tracings of the macrophages were then reduced or enlarged to the desired magnification without any loss in resolution.

Brightfield Image Capture, Image Post-processing, and Preparation of Figures

Brightfield photomicrographs were acquired using a Leica DM microscope fitted with a Spot Flex camera controlled using Spot Software (V4.7 Advanced Plus; Diagnostic Instruments, Sterling Heights, MI). Focus stacking (Gulbins and Gulbins, 2009) was used to maximize the depth of field of images taken from thick GI whole mounts (indicated in the figure legend when applicable to an image). Specifically, single all-in-focus images representing the thickness of the tissue containing the elements of interest were generated using Helicon Focus Pro X64 (Version 5.1.23; HeliconSoft Ltd., Kharkov, Ukraine) by merging a series of shots taken at different focal distances along the z-axis with slightly overlapping regions of focus. All-in-focus images and single planes of focus are stated as such in the appropriate figure legend(s). Photoshop CS5 (Adobe Systems) was used to: (1) organize the layout of the figures; (2) adjust color, hue, evenness of illumination, brightness, contrast, and sharpness of images; (3) remove artifact [done sparingly and stated in the appropriate figure legend]; and (4) apply text and scale bars. The goal of these adjustments was to generate final images which accurately conveyed our observations of the nervous innervation of the GI tract and not faithful reproductions of artifact.

RESULTS

Immunolabeling Macrophage Phenotypes in Smooth Muscle Whole Mounts

Whole mounts were collected from rats without clinical or pathological signs of disease, inflammation, infection or stress. Macrophages immunoreactive for CD163 formed dense, uniformly distributed populations of cells at the level of the serosa, myenteric plexus, and deep muscular plexus (Fig. 1A). These CD163+ cells were located in close proximity to each other and were spatially distributed so that individual cells rarely overlapped. Often, in adult rats, CD163+ macrophages had relatively smooth, tapering processes with few spines, spurs, filopodia or swellings, however, CD163+ cells with morphological features similar to those of MHCII+ cells (i.e., with nodules and thread-like tendrils along their primary processes) were identified, often in whole mounts from aged rats, and frequently in association with neuropathies and protein aggregates; see below.

In contrast, the MHCII⁺ macrophages exhibited distinctly different distributions and morphologies. The antibody towards MHCII labeled clusters of macrophages within the same three layers of the smooth muscle as did the CD163 antibody; however, in contrast to the regularly spaced and distributed CD163⁺ cells pattern, the density of the MHCII⁺ macrophages was highly variable (Fig. 1B). Specifically, the density of MHCII⁺ cells ranged from areas with only a few immunoreactive cells to others with tightly packed dense clusters. The variability in packing density of MHCII⁺ cells did not appear to follow an oral-to-anal gradient or a mesentery to anti-mesentery preference, but rather, the pattern was consistent with increased macrophage presence at focal points or acutely disturbed hotspots (perhaps--see below--undergoing local neuronal or other cellular remodeling) in the smooth muscle whole mounts. At sites of highest packing density, MHCII⁺ cells were likely to overlap and aggregate, apparently coming into direct contact with one another.

Monocytes

Monocytes were round, without processes (Fig 2A,E), and had different morphologies depending upon their immunohistochemical phenotype. Monocytes stained for CD163 had a prominent, unstained nucleus surrounded by granular cytoplasm (Fig. 2A–D), while monocytes immunoreactive for MHCII were, in comparison, typically smaller with a poorly defined nuclei and cytoplasm (Fig. 2E,F).

Macrophage Morphology was Diverse

The morphology of macrophages was dynamic and diverse. Cells immunoreactive for CD163, often consisted of smooth primary processes radiating from a centrally located soma (Fig. 3A), although some CD163⁺ cells (Fig. 3B) did have structural features similar to MHCII⁺ cells. Specifically, short, thread-like tendrils and bulbous nodules localized to the surface of the cell (Fig. 3B–G). More dramatic structural extensions (Fig. 3H,I) consisting of long, thick processes projecting away from the cell body into the nearby environment with thread-like tendrils of varying lengths and thicknesses (Fig 4A–C), also occurred in both phenotypes. The presence of tendrils, protrusions, and nodules was not restricted to just the leading edge of the primary processes, but rather possible anywhere along the cell's surface, including the soma (Fig. 4B,D) and length of the primary processes (Fig. 4A–C).

Macrophages were in Close Registration with Myenteric Neurons

Macrophages immunoreactive for CD163 (Fig. 5A) and MHCII outlined the ganglia and connectives of the myenteric plexus. In some cases, the primary process of a macrophage was in close apposition with a neuron (Fig. 5B) while in other instances the point of contact was limited to the short, thread-like tendrils originating from the macrophage (Fig. 5C). Points of contact between CD163⁺ (Fig. 5B,C) and MHCII⁺ (Fig. 5D) cells was determined by light microscopy using a 100× oil objective.

The total population of myenteric neurons consists of nitrergic and cholinergic neurons, two mutually exclusive phenotypes. Both phenotypes regularly appeared to be contacted by macrophages (NOS⁺: Fig. 6A; CB⁺: Fig. 6B,C,E). The pan-neuronal antibody HuC/D (Phillips et al., 2004a) and a marker for nitrergic neurons (i.e., labeling for NOS) only filled the neuronal cell body and axon, making it impractical to observe potential macrophage contacts on their dendrites, whereas the antibody to CB, which is a marker for a subpopulation of cholinergic neurons, occasionally filled the dendrites of a small subpopulation of CB⁺ neurons. The dendrites of these well labeled CB⁺ neurons surrounded the perimeter of the cell body, and then flattened out as they extended away from the soma exhibiting a wavy or saw-toothed appearance as the result of irregularities along their perimeter. The degree of the relationship between macrophages and dendrites of these cholinergic myenteric neurons ranged from subtle (Fig. 6B) to dramatic (Fig. 6C,E), with the

latter consisting of the processes of macrophages conforming tightly to the surfaces of CB+ dendrites (Fig. 6C,E) in a lock-and-key configuration (Fig. 6D) as the process melded to the contour of the dendrite (Fig. 6F–H).

Consistent with the process of phagocytosis, macrophages were observed wrapped around individual neurons (Fig. 7A,B) or even entire ganglia (Fig. 7C,D). It is impossible to ascertain the exact nature of this macrophage-neuron relationship without additional information about the health of the targeted neuron(s); however, it is instructive that cholinergic neurons immunoreactive for calretinin (CR), which disproportionately die during aging, were often targeted (Fig. 7B–D). Macrophages targeting ganglia in this manner consisted of one of two categories: those that lost their characteristic stellate or bipolar morphology as they spread across the neuron engulfing their target (Fig. 7A,B) or those that became more complex in morphology as they spread their processes throughout the ganglion (Fig. 7C,D).

To elaborate, a macrophage would infiltrate a ganglion (infiltration was inferred based on the presence of the macrophage soma within the ganglion instead of its customary location at the perimeter; Fig. 5A) and then insinuate its branching processes throughout the entire ganglion with most—if not all—of the neurons either encircled or contacted (Fig. 7C,D). The processes of these macrophages would branch extensively within the ganglion from longitudinal muscle face to circular muscle face as indicated by their presence at different focal planes (Fig. 7. E,F), and were often extensive in size and complexity (Fig. 7G). Finally, of potential importance is the observation that monocytes were present in the vicinity of ganglia occupied by these large, complex macrophages (Fig. 7D), raising the possibility that additional cells had migrated to the region.

Macrophages Contact Varicosities on Noradrenergic Axons

Sympathetic axons originating from prevertebral ganglia, identified by their noradrenergic phenotype (i.e., immunoreactive for tyrosine hydroxylase; TH), innervated the myenteric plexus, its connectives, and the smooth muscle, as has been previously described (Furness and Costa, 1974; Phillips et al., 2006). Within the myenteric plexus, TH+ axons traveling in the connectives and passing through the ganglia were smooth in appearance, but became varicose upon entering a ganglion. Within a ganglion, individual TH+ axons dotted with small varicosities would branch extensively as they encircled HuC/D+ neurons (Fig. 8A). Additionally, within the same layer of the muscularis as the myenteric plexus, numerous varicose TH+ axons formed a tertiary plexus (Furness and Costa, 1974) with its perimeter delineated by the ganglia and connectives. We observed varicose TH+ axons in close apposition to CD163+ macrophages in a manner structurally similar to the sympathetic innervation of myenteric neurons (compare in Fig. 8 panel A to panel B), both the somata (arrowheads in Fig. 8B) and primary processes (dotted box in Fig. 8B) of macrophages were contacted by (or, conversely, contacted) TH+ varicosities. Cells immunoreactive for MHCII were similarly in close registration with TH+ axons (Fig. 8C–E), running parallel to TH+ axons and contacting numerous varicosities along the length of the axon (Fig. 8E). Of potential importance, macrophages were observed in contact with both markedly swollen varicosities (see inset in panel E of Fig. 8 for an enlarged tracing of a point of contact of a macrophage with a swollen varicosity along the length of an otherwise healthy appearing axon) and normal size varicosities (arrowheads in panel B of Fig. 8).

Aggregated Macrophages

Dense clusters of MHCII+ cells were also observed in the myenteric plexus (Fig. 9) and the smooth muscle wall (Fig. 10) of the GI tract. These aggregates occurred in the stomach, small intestine, and large intestine at every age sampled. There did not appear to be an age-

related increase in the number of aggregates, indicating that they do not accumulate and persist, but rather that they are likely cleared from the tissue. This, along with the “healthy” appearance of the plexus at their location (Fig. 9E,F & 10), is consistent with a limited focal disturbance in otherwise healthy tissue.

These aggregates cells had several defining features: First, they were immunoreactive for MHCII; Second, the surrounding cells had an amoeboid morphology (Fig. 9C–E and 10A,B); Third, at their core, amoeboid cells lost their morphological integrity as they fused together into a large mass (Fig. 10B–E); And finally, the density of MHCII+ macrophages was often reduced in the region immediately surrounding an aggregate (see inset in Fig. 10C), which potentially indicates that as cells migrate to the aggregate, the local population of macrophages is depleted until monocytes can be recruited to the region for replenishment of the local macrophage population.

Fragmented Macrophages

In various tissues, after exhaustion, macrophages fragment into smaller pieces and are eventually cleared from the tissue making way for new cells. Fragmented macrophages occurred in the GI whole mounts in the present study (Fig. 11A–C), and were immunoreactive for CD163 (Fig. 11C–E) and MHCII (Fig. 11A,B). Fragmented macrophages (encircled by dotted lines in Fig. 11A,B) were in close proximity to myenteric neurons (Fig. 11A,B) and noradrenergic axons (Fig. 11D,E). Monocytes were also present in the same location as the fragmented macrophages (Fig. 11A).

The general health of the neurons in panels A and B of Fig. 11 was not assessed, but their appearance is that of healthy neurons; thus, suggesting macrophage fragmentation occurred following cycles of routine cellular housekeeping. In contrast, the noradrenergic axons in panels D and E of Fig. 11 are swollen and tangled, indicative of degeneration or dystrophy. The presence of fragmented macrophages within the tangled web of dystrophic noradrenergic axons presumably signifies their attempt to clear them out of the system. In this example, the fragments are in the same plane of focus as intact macrophages and undamaged axons, and therefore are presumably not a byproduct of damage that occurred during dissection of the tissue in preparation for immunohistochemistry.

Evidence for Phagocytosis of Aggregated Alpha-synuclein by Macrophages

Alpha-synuclein (α -SYNC) is abundant in its normal state throughout the myenteric plexus (Phillips et al., 2008) and has been shown to be prone to fibrillization as evidenced by the presence of α -SYNC+ dystrophic axons and terminals in aged rats (Phillips et al., 2009). In the present study, double staining for α -SYNC and both CD163 and MHCII revealed macrophages in tight registrations with aggregated α -SYNC (Fig. 12A,B,D). The tracing in panel C is an enlargement of the region shown in the dotted box in panel B of Fig. 12 illustrating two small filopodia, or thread-like tendrils, extending from a CD163+ macrophage toward a mass of aggregated α -SYNC. Similarly, phagocytosis is suggested by the dramatic morphology of an MHCII+ cell (shown in panel D of Fig. 12 whose silhouette is shown in panel E) in close proximity to small clumps of aggregated alpha-synuclein protein. Numerous branching filopodia-like extensions can be seen originating from the cell body of the MHCII+ cell and insinuating within the deposits of aggregated α -SYNC.

Macrophage Migration through the Musculature

Macrophages migrate through the muscle wall to where they are needed, and this migratory process was potentially evident in the GI whole mounts in our study based upon the density gradient of cells in the smooth muscle wall. Specifically, large numbers of monocytes were observed clustered together (Fig. 13A), and cell density steadily decrease moving away from

the point of highest density of monocytes (Fig. 13B). As the density of the monocytes decreased, the cells transitioned from monocytes to an amoeboid-like morphology (Fig. 13C).

Perivascular Macrophages

Macrophages had a close anatomical relationship to blood vessels within the smooth muscle wall of the GI tract (Fig. 14). Blood vessels were easily identifiable, due to the perivascular location of the macrophages, and double staining for noradrenergic axons that were in tight, overlapping registration with the perivascular macrophages along the length of the blood vessels (Fig. 14A). Macrophages appeared to preferentially accumulate between the muscle sheets and the vessel walls, while avoiding the serosal and mucosal facing walls of the vessels with the density of the macrophages along the length of the perimeter of the blood vessels varying from few (Fig. 14B) to many (Fig. 14C) cells. This was the case for both the CD163+ and MHCII+ cells. The morphology of the perivascular macrophages ranged from monocyte (Fig. 14C) to amoeboid (Fig. 14C), and, finally, to a mature macrophage (Fig. 14A). Cells anatomically associated with blood vessels were always located at a perivascular site, and regardless of phenotype, were never observed within the lumen of the vessel.

DISCUSSION

The present experiment was designed to provide a more complete characterization of the macrophages located in the muscle wall of the GI tract, with a focus on how this population articulates with both intrinsic and extrinsic autonomic networks located in the smooth muscle. Previous examinations of macrophages in other tissues have established that these leucocyte-derived cells not only execute, when activated, innate immune defenses against exogenous pathogens entering the tissues, but that they also perform housekeeping functions related to the normal cellular turnover associated with wear and tear or aging (Streit et al., 2004). Such observations for the most part, however, have not been made in the GI tract. And even those macrophage observations that have examined the GI tract have concentrated primarily on the macrophages found in the mucosal lamina propria, not the population that is associated with the innervation of the smooth muscle wall.

The present observations indicate that the different macrophage morphologies, locations, and immunohistochemical phenotypes in the muscularis are consistent with the inference that gut macrophages in the smooth muscle wall have features--and presumably functions--similar to those seen in such mononuclear phagocytes found in other tissues. Further, the results indicate that macrophages in the muscularis establish (by light microscopic criteria) extensive appositions with both the intrinsic and extrinsic autonomic circuitry, suggesting that macrophages execute their housekeeping and immune functions for the peripheral nervous system circuitry of the gut as well as for muscle and other tissues. Our observations can be considered in terms of several of the functional categories with which macrophages have been associated.

Housekeeping Functions of Muscularis Macrophages

Resident macrophages, in various tissues, and homologous microglia in the CNS, are implicated not only in innate immunological responses, but also in routine and ongoing housekeeping operations of native tissues as well. More particularly, macrophages have been implicated in both (a) digestion and elimination of damaged and apoptotic cells and cellular material that accumulates with normal aging as well as routine wear and tear or damage of the tissues (Birge and Ucker, 2008; Kono and Rock, 2008; Neumann et al., 2009), and (b) maintenance of synaptic homeostasis (Nimmerjahn et al., 2005; Wake et al., 2009). The present observations on the macrophage populations in GI smooth muscle

suggest that both types of cellular housekeeping occur for the autonomic innervation of the gut.

In terms of eliminating native cellular debris, macrophages were commonly observed with their processes extended to contact, and in some cases apparently to engulf, neuronal somata of the myenteric plexus. Notably, macrophages established similar patterns of contact with both the nitrergic and cholinergic subpopulations of the plexus. In addition, macrophage processes were also regularly observed in association with both normal swellings and pathological dystrophies of TH+ or catecholaminergic axons projecting to the muscularis of aging animals. Similarly, macrophages also formed apparent appositions with neuronal swellings and dilations that were α -SYNC+ in these animals. Such morphological patterns and appositions suggest that these macrophages may have been in the initial phases of phagocytic responses, though other types of candidate housekeeping functions cannot be excluded.

Macrophages also contacted apparently healthy myenteric neurons and their neurites. Appositions observed between macrophages and the seemingly viable somata and neurites of autonomic circuitry of the GI tract smooth muscle were also consistent with the conclusion, extrapolated from macrophage-neuron interactions in other tissues, that gut macrophages may contribute to the regulation of synaptic functions. Both the evidence of close appositions between macrophages and neuronal somata and processes and, even more tellingly, our structural observations of macrophages introducing protrusions or pegs or spines into pockets or clefts in neuronal somata and processes in the muscularis are consistent with the ideas that gut macrophages may help regulate neuropeptide, neurotransmitter, or neuromodulator functions at the synapse (Markus et al., 1998; Neuhuber and Tiegs, 2004; Wake et al., 2009) and/or participate in synaptic stripping (Perry and Connor, 2010).

Macrophage Morphology Consistent with Neuro-immune Effector Functions

In housekeeping functions as well as in the classical innate immune functions, macrophages presumably respond to conditions and perturbations in the local GI environment and operate to effect changes in other tissues or to mobilize responses against pathogens. Though, as discussed above, the morphology and distributions of gut macrophages seem consistent with such functions, it is also relevant that in some of the material sampled the structural features observed are consistent with the alternative idea that neurons, in some instances, are directly contacting macrophages and potentially influencing their functional status. In particular, the observations of appositions, including peg-and-socket appositions, formed between macrophages and neural elements in the smooth muscle wall (though discussed above in terms of housekeeping possibilities) are consistent with the idea that neural efferent outflows might, through close association with macrophages, modulate the function(s) of the leucocyte-derived cells.

Such neuro-immune effector function(s) would be consistent with physiological evidence indicating that sympathetic (Markus et al., 1998; Elenkov et al., 2000; Gulbransen et al., 2010) as well as parasympathetic (Borovikova et al., 2000) nervous system efferent outflows influence peripheral immune responses. These observed effects of the autonomic outflow on macrophage functions could, of course, be mediated by indirect effects, though it is also possible in light of the nerve-macrophage appositions found in the muscularis that some of the immune effector function elicited by nerve stimulation, in particular the noradrenergic nerves, result from more direct activation of neuro-immune effects which receives considerable support from the demonstration that macrophages express both α - and β -adrenergic receptors (Elenkov et al., 2000; Grisanti et al., 2011).

Macrophages in PNS and CNS Use Common Housekeeping Operations

A major impetus for the present experiment was the dearth of information about macrophage-neuron interactions for the autonomic circuitry of the gut. This absence of information stands in contrast to the extensive literature on macrophage- (i.e., microglia-) neuron interactions in the CNS, where the implications of neuroprotective and neurodegenerative processes in the classic neural diseases are well recognized. Indeed, in a recent review of the CNS macrophages, Graeber and Streit (2010) estimated that 10,000 papers on microglia have been published. This was confirmed in a recent search by us using the US National Library of Medicine and National Institutes of Health PubMed database which resulted in 10,262 papers after we searched for “Macrophage AND Brain” while only 73 papers and 298 papers resulted from our searches, respectively, for “Macrophage AND Myenteric” and “Macrophage and Submucosa”. The close similarities between what we have observed in the present experiment and what has been characterized for microglia-neuron interactions in the CNS should have important experimental and clinical implications. If macrophages in the central and the peripheral nervous systems do display common reactions and execute corresponding housekeeping operations, then extrapolations from the extensive literature on microglia-neuron interactions may help define questions and set priorities for investigations of macrophage-neuron analyses in the PNS, and, similarly, such extrapolations may provide information to improve our understanding of macrophage-neuron interactions in different gut diseases and disorders as well as in normal processes such as aging.

Macrophages Interact with the Aging Innervation of the GI tract: Significant Roles in the Age-Related Decline in Function in the Elderly?

Age-related changes in different tissues have been correlated with alterations in immune function (Plackett et al., 2004; Neumann et al., 2009) and, even more specifically, with changes in macrophage physiology (Conde and Streit, 2006; Moore et al., 2007). In light of such evidence, one impetus for the present experiment followed from previous observations establishing that the innervation of the GI tract suffers losses with aging (Phillips and Powley, 2001, 2007; Phillips et al., 2010) that appear to be correlated with--and perhaps causally related to--the conspicuous and well documented functional GI impairments in the elderly (Talley et al., 1992; Majumdar et al., 1997). The structural losses associated with aging involve progressive myenteric neuron losses (Phillips and Powley, 2001; Mattson et al., 2006; Phillips and Powley, 2007), specifically cholinergic neuron death with concurrent sparing of nitrergic neurons (Phillips et al., 2003; Abalo et al., 2005), parallel losses of enteric glia cells (Phillips et al., 2004b), and progressive accumulations of dystrophic neurites (Phillips et al., 2006) and misfolded proteins (Phillips et al., 2009) throughout the GI smooth muscle. The lack of a general characterization of the macrophage population in the aging GI smooth muscle, however, has limited the prospect for evaluating the role(s) of macrophages in these aging processes of the gut, and so one rationale for the present observations was to provide the groundwork and/or fundamental understanding necessary for the design of future studies examining the interaction of gut macrophages with the age-related changes previously observed for the intrinsic and extrinsic innervations of the GI tract.

Furthermore, taking into consideration the different activation states of macrophages (i.e., classical activation and alternative activation), these monocyte-derived phagocytes might be involved not only in the degenerative changes with age, but conversely, also the processes that limit age-related losses. For example, the dystrophic and swollen neurites that accumulate with age might reflect age-related slowing in the rate at which monocytes differentiate to replace exhausted macrophages, impairments in the ability of macrophages to stem microbial invasions, or decreases in phagocytotic processing of neurites subject to

dystrophic changes and challenges. With so little information available to date on the macrophages found in association with the autonomic innervation of the aging gut, however, such hypotheses could not be evaluated. Thus, the present observations were collected from animals of different ages that reflect the entire lifespan of the species in order to obtain preliminary information relevant for future examinations of mechanisms of aging of the autonomic innervation of the GI tract.

Similarly, given the scope and qualitative nature of the observations in the present study, only general conclusions can be drawn about the interaction(s) between the aging autonomic nervous innervations of the GI tract and macrophages, and, clearly, quantitative analyses of larger samples are needed. Nonetheless, by casting a wide net, we were able to establish two general conclusions that are worth further investigation. First, both CD163+ and MHCII+ cells had similar features at all ages examined. This suggests that any age-related losses of macrophage function might be incremental and quantitative rather than categorical and that a full assessment of the roles of GI macrophages in aging will need to include quantitative analyses of both macrophage phenotypes. Second, in light of the neuronal losses and dystrophic neurite accumulations that characterize aging of the innervation of the gut, it is informative that macrophages engulf, apparently preparatory to phagocytosis, the somata of myenteric neurons and neurites observed in the connectives of the myenteric plexus as well as in the muscle layers. More particularly, it is instructive, again in light of the previously established pattern of aging of the autonomic nervous system in the GI tract, that macrophages were seen in close apposition with cholinergic neurons (which are selectively targeted for age-related cell death), dystrophic TH+ neurites, and deposits of α -SYN protein. These three age-related disturbances are thought to be candidate mechanisms for some of the age-related declines in function evidenced in the elderly (Phillips and Powley, 2007, 2010), specifically the increased incidence with advancing age of motility- or transit-related problems, including delays in gastric emptying and longer intestinal transit time with associated fecal stasis (Bouras and Tangelos, 2009; Salles, 2009; Thompson, 2009; Wiskur et al., 2010). A hypothesis that incorporates the idea that macrophages located in the GI tract interact with the aging autonomic nervous system to produce disturbances in smooth muscle function is particularly parsimonious and consistent with the observation(s) in young-adult rats that these same macrophages play a key role in disturbances in GI motility under pathological conditions (Eskandari et al., 1997; Won et al., 2006; Sato et al., 2007).

In summary, the profiles of GI smooth muscle macrophages observed in the adult, middle-aged, and aged rats are consistent with the hypothesis that macrophages interact with the aging autonomic nervous system, potentially contributing to the degenerative changes--and ultimately loss of function--associated with age.

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Figure 1.

Macrophages immunoreactive for CD163 and major histocompatibility complex class II (MHCII) are present in the smooth muscle wall of the gastrointestinal (GI) tract. Macrophages labeled with the antibody to CD163 (A; blue/grey cells) are uniformly distributed throughout the smooth muscle. In contrast, cells labeled with an antibody to MHCII (B; blue/grey cells) are distributed in a highly variable or patchy pattern. The disparate densities of the two phenotypes are illustrated in the two low power montages, each consisting of four images, double labeled for the panneuronal marker human neuronal protein HuC/HuD (HuC/D; brown neurons) and either CD163 (A) or MHCII (B). The two montages are from whole mounts of the small intestine of adult rats and illustrate

macrophage distribution patterns in the first third of the rat's lifespan. Scale bars = 100 μm in A and B.

Figure 2.

Monocytes and amoeboid-like cells immunoreactive for CD163 and MHCII are present in the smooth muscle of the GI tract. (A) Monocytes immunoreactive for CD163 have an unstained central nucleus surrounded by heavily stained, granular cytoplasm. (B) Four CD163+ cells with amoeboid-like morphology. (C) A dense cluster of monocyte/amoeboid-like cells and a single bipolar CD163+ macrophage, located above the cells, with two opposing primary processes originating from a centrally located soma. (D) Two CD163+ cells with multiple rudimentary branching processes projecting from their respective somata. In contrast to CD163 labeled monocytes (as in A), MHCII+ monocytes (E) are small and spherical in shape with poorly defined nuclei and cytoplasm. (E) Five monocytes stained for MHCII (blue/grey) surround a single HuC/D+ neuron (brown) in the myenteric plexus. (F) Two MHCII+ monocytes (blue/grey), with poorly developed branching filamentous processes, in close proximity to HuC/D+ myenteric neurons (brown). Focus stacking was used to create an extended depth of field in panel F. Scale bar = 10 μ m in F (applies to A–F).

Figure 3.

The morphology of macrophages is dynamic and diverse. (A) A single macrophage immunoreactive for CD163 with relatively smooth branching primary processes and blunt or bulbous endings at the tips of its processes. (B–D) Three bipolar macrophages, one immunoreactive for CD163 (B), and two for MHCII (C,D) with numerous thread-like filopodia and nodular outgrowths protruding from the cell's surface. (E) A stellate macrophage with a morphology similar to the cells in panels B–D. Numerous nodules or spines (F; arrowheads) and thread-like (G) filopodia protrude from the branching processes of the macrophage. Spines and filopodia were often observed on or near the cell body (B–G), but also were found on the primary processes extending away from the macrophage

soma (H,I). Two macrophages are shown in panels H and I with multiple long, thick processes that project away from the cell's body into the surrounding smooth muscle. Images are from whole mounts of the stomach (B) and small intestine (A, C–I) of adult (C–G,I) and aged (A,B,H) rats stained for CD163 (A,B) or MHCII (C–I) demonstrating that macrophages are dynamic and diverse across the lifespan of the rat. Focus stacking was used to create an extended depth of field in panels C–I. Scale bars = 10 μ m except for insets in panel E. Scale bars = 5 μ m in both insets in Panel E.

Figure 4.

Thin thread-like filaments and filopodia-like tendrils, of varying lengths and thicknesses, are a common feature of macrophages. (A) A single thread-like filament, approximately 120 μm in length (from arrowhead to arrowhead), projects away from the cell body of a macrophage. (B) Multiple tendrils at opposing poles of a macrophage's soma. (C) Filopodia-like tendrils, located at the tip of the processes of a bipolar macrophage, project away from the cell. Enlarged tracings of the tips of the opposing primary process, highlight the presence of multiple interwoven tendrils. (D) Interwoven, thread-like filaments or filopodia-like tendrils were observed not only along macrophage processes, but also originating from the somata. Images are from whole mounts of the small intestine of adult (A,C,D) and aged (B) rats,

demonstrating the presence of these structures regardless of the rat's age. Macrophages in panels A–D were stained with the antibody to MHCII. Focus stacking was used to create an extended depth of field in panels A–D. Scale bars = 10 μm for all of the panels except for the scale bars in the enlarged tracings of the macrophages in panels C and D where the scale bars = 5 μm .

Figure 5.

Macrophages are in tight apposition with neurons in the myenteric plexus. (A) Numerous macrophages at the level of the myenteric plexus outline the perimeter of a ganglion. (B–D) The processes of macrophages conform to the surface of the neurons they contact. Images are from whole mounts of the jejunum of aged rats. Whole mounts were double-labeled for HuC/D (neurons; brown) and either CD163 (A–C; blue/grey) or MHCII (D; blue/grey). Focus stacking was used to create an extended depth of field in panel A. Scale bars = 20 μ m in A; 10 μ m in D (applies to B–D).

Figure 6.

Macrophages within the myenteric plexus, viewed at high power (100× oil objective), appeared to make contact with axons and the dendrites of neurons. (A) Axons immunoreactive for nitric oxide synthase (NOS; brown) are in close association with an MHCII+ macrophage (blue/grey). The inset shows the soma of a macrophage in close apposition with a NOS+ axon. (B) Soma and processes of a CD163+ macrophage run parallel to the dendrites of a neuron immunoreactive for the calcium binding protein calbindin (CB; brown). The primary processes of the macrophage in panel B do not make contact with the neuron, but, in the inset, a small tendril originates from the soma of the macrophage and contacts the dendrites of the neuron. (C,E) The processes of two CD163+

macrophages interdigitate with the dendrites of CB⁺ neurons. (D,F) Enlarged tracings of regions of contact from panels C and E highlight the lock-and-key relationship between macrophages and dendrites. (G,H) An enlargement of two regions from the tracing in panel F. Images are from whole mounts of jejunums from adult (B,E–H) and aged (A,C,D) rats. Focus stacking was used to create an extended depth of field in panels A–C, and E. Scale bars = 10 μm in A–C and E; 5 μm in insets in A,B and D,F; 2.5 μm in G,H.

Figure 7.

Macrophage contacts with myenteric neurons varied from selective (i.e., individual neurons within a ganglion) to extensive (most or all of the neurons within a ganglion contacted). (A) The pole of a single HuC/D+ neuron (brown) is surrounded by an MHCII+ macrophage (blue/grey). (B) Similar targeting of a neuron immunoreactive for the calcium binding protein calretinin (CR; brown) is carried out by an MHCII+ macrophage. (C) Calretinin-positive (brown) and -negative (unstained) neurons are encircled by multiple processes from a single macrophage with its soma located at the center of the ganglion. (D) An MHCII+ macrophage within a ganglion (five CR+ neurons) with its extensive processes spread throughout the entire ganglion. Several MHCII+ monocytes are in close proximity to the

ganglion shown in panel D. (E,F) Different planes of focus of the macrophage in panel D illustrates the soma of the macrophage located on top of the CR+ neurons with its extensively branching processes extending throughout the depth of the ganglion. (G) An enlarged tracing of the macrophage seen in D emphasizes its size and complexity. Images are from whole mounts of the small intestine of adult (A) and aged (B–D) rats demonstrating that this interaction occurs across the lifespan of the rat. Focus stacking was used to create an extended depth of field in panels B–F. Scale bars = 10 μ m; B applies to C; F applies to E.

Figure 8.

Macrophages contact the varicosities on noradrenergic axons. (A) For comparative purposes, we've included an image of a HuC/D+ myenteric neuron (brown) encircled by varicose axons immunoreactive for tyrosine hydroxylase (TH; blue/grey). Varicosities (A; arrowheads) along TH+ axons are thought to be sites of non-synaptic neurotransmitter release. (B) The varicosities of TH+ axons (brown) contact the soma (arrowheads) and process (dotted box) of a CD163+ macrophage (blue/grey). (C–E) Bipolar macrophages immunoreactive for MHCII (blue/grey) are in direct contact with TH+ varicose axons (brown). The inset in panel D is an enlarged tracing of the tip of the macrophage that highlights the two small tendrils (black) along the length of the TH+ axon (brown). The

inset in panel E is an enlarged tracing of the point of contact between the MHCII+ macrophage and varicosities on the TH+ axon. Images are from whole mounts of the duodenum (B), jejunum (A,C,D), and ileum (E) adult (C–E), middle-aged (B), and aged (A) rats. Focus stacking was used to create an extended depth of field in panels A–E. Scale bars = 10 μm in A,C–E; 20 μm in B; 1.5 μm for inset in D; 2.5 μm for inset in E.

Figure 9.

Macrophages often outlined the perimeter of the myenteric plexus without infiltrating the ganglia (A,B); however, ganglia were observed with varying degrees of infiltration by amoeboid-like macrophages (C–F). Macrophages were immunoreactive for either CD163 (A) or MHCII (B–F). The ganglia were visualized by double labeling for α-SYNC (A, C–F) or TH (B). Images were from the whole mounts of the large intestine (A,B) and stomach (C–F) of adult (A,B), and aged (C–F) rats. Scale bars = 25 μm in B (applies to A,B); 50 μm in F (applies to C–F).

Figure 10.

Aggregates or condensations of macrophages, consisting of numerous cells clumped together in large dense masses, occurred in whole mounts stained with the antibody to MHCII (A–E). The loss of morphological definition in an aggregate of MHCII+ macrophages is evident in panel C. The low power (10× objective) inset in panel C illustrates the low density of MHCII+ macrophages in the vicinity of the aggregated macrophages (outlined by the black box). (D,E) Two different focal planes of the smooth muscle wall at the site of an aggregate, illustrate how the core of this particular aggregate of macrophages is located at the level of the deep muscle (D) and not the myenteric plexus (E). The myenteric plexus was identified by double staining for either TH (A,B; brown) or α-

SYNC (C; brown). Myenteric neurons were stained with the antibody to HuC/D (D,E; brown). Images are from whole mounts of the jejunum. Focus stacking was used to create an extended depth of field in panels C–E. Scale bars = 50 μm in B (applies to A,B); 25 μm in C (250 μm in inset); 25 μm in E (applies to D,E).

Figure 11.

Fragmented macrophages were present within the smooth muscle wall of the GI tract. Fragmented macrophages (dotted outline) occurred in close proximity to neurons (A,B; brown) and axons (D,E; brown), and were immunoreactive for both CD163 (C-E; blue/grey) and MHCII (A,B; blue/grey). Neurons (A,B) were labeled with the antibody to HuC/D while axons were labeled with the antibody to TH (D,E). (C) Macrophages with fragmenting processes and intact soma were observed. The TH+ axons in panels D and E are tangled and swollen suggesting reorganization, degeneration, or both. While macrophages in the tangle of TH+ axons, in the upper left of the panel, still maintain some morphological integrity, small CD163+ fragments are all that remain of macrophages in the tangle of TH+ axons in

the bottom right of panel E. Images are from whole mounts of the stomach (D,E) and small intestine (A–C) of middle-aged (D,E) and aged (A–C) rats, ages when plasticity and degeneration of enteric nerves is common. Focus stacking was used to create an extended depth of field in panels A,B,D, and E. Scale bars = 10 μm in C (applies to A–C); 10 μm in D,E.

Figure 12.

Phagocytosis of aggregated alpha-synuclein (α -SYNC) by macrophages in the GI tract of aged rats. (A) The processes of a CD163+ macrophage (blue/grey) contact a large aggregate of α -SYNC (brown). (B) Small thread-like tendrils originating from a CD163+ macrophage (blue/grey) surround an aggregate of α -SYNC (brown). (C) An enlarged tracing of the tendrils in panel B. Panels A–C are from the stomach of an aged rat. (D) An MHCII+ macrophage (blue/grey) with numerous long-thin tendrils within a field of small α -SYNC+ aggregates (arrowheads) in the jejunum of an adult rat. (E) An enlarged tracing of the macrophage in panel D emphasizing the presence of numerous filopodia and tendrils. Aggregated α -SYNC was rare in the adult enteric nervous system (D), but common in the

GI tract of aged (A–C) rats. Focus stacking was used to create an extended depth of field in panels A–C. Scale bars = 10 μm in D (applies to A,B,D); 5 μm in C; 5 μm in E.

Figure 13.

Monocytes and amoeboid-like macrophages (grey/blue) infiltrate the GI musculature and appear, based upon their concentration gradient, to be migrating through the length and width of the small (A,B) and large (C) intestines. Images are from whole mounts stained for MHCII (A,B) and CD163 (C). Whole mounts were double labeled for either α -SYNC (A,B; brown) or TH (C; brown) to visualize the myenteric plexus. Scale bar in C = 125 μ m for A,B; 250 μ m for C.

Figure 14.

The outside perimeter of the blood vessels (BV) located in the smooth muscle wall of the GI tract are well defined by TH+ axons (brown), monocytes, amoeboid-like cells, and macrophages (blue/grey) throughout the GI tract (small intestine: A; large intestine: B–C). Monocytes and macrophages lining the BV are immunoreactive for CD163 (A,C) and MHCII (B). The pattern was the same regardless of age (adult: B,C; middle-aged: A). Panels C is a montage consisting of two low power (20× objective) images. Scale bars = 100 μ m in A; 40 μ m in B; 50 μ m in C.

Table 1

Primary Antibodies

Antibody	Host	Dilution	Manufacturer	Cat. no.
α -SYNC	Mouse	1:5000	BD Transduction (San Jose, CA)	610787
CB	Mouse	1:4000	Sigma (St. Louis, MO)	CD9848
CR	Rabbit	1:10000	Vector Laboratories, Inc. (Burlingame, CA)	VP-RM11
CD163	Mouse	1:1000	AbD Serotec (Oxford, UK)	MCA342R
HuC/D	Mouse	1:5000	Invitrogen Corp. (Carlsbad, CA)	A-21271
MHCII	Mouse	1:4000	AbD Serotec (Oxford, UK)	MCA46R
NOS	Rabbit	1:8000	Santa Cruz Biotechnologies (Santa Cruz, CA)	SC-648
TH	Rabbit	1:4000	Pel Freez Biologicals (Rogers, AK)	P40101-0

Abbreviations: α -SYNC, alpha-synuclein; CB, calbindin; CR, calretinin; HuC/D, human neuronal protein HuC/HuD; MHCII, major histocompatibility complex class II; NOS, neuronal nitric oxide synthase; TH, tyrosine hydroxylase.