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Interstitial cells of Cajal, macrophages and mast cells in the gut musculature: morphology, distribution, spatial and possible functional interactions

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Abstract

Interstitial cells of Cajal (ICC) are recognized as pacemaker cells for gastrointestinal movement and are suggested to be mediators of neuromuscular transmission. Intestinal motility disturbances are often associated with a reduced number of ICC and/or ultrastructural damage, sometimes associated with immune cells. Macrophages and mast cells in the intestinal muscularis externa of rodents can be found in close spatial contact with ICC. Macrophages are a constant and regularly distributed cell population in the serosa and at the level of Auerbach's plexus (AP). In human colon, ICC are in close contact with macrophages at the level of AP, suggesting functional interaction. It has therefore been proposed that ICC and macrophages interact. Macrophages and mast cells are considered to play important roles in the innate immune defence by producing pro-inflammatory mediators during classical activation, which may in itself result in damage to the tissue. They also take part in alternative activation which is associated with anti-inflammatory mediators, tissue remodelling and homeostasis, cancer, helminth infections and immunophenotype switch. ICC become damaged under various circumstances – surgical resection, possibly post-operative ileus in rodents – where innate activation takes place, and in helminth infections – where alternative activation takes place. During alternative activation the muscularis macrophage can switch phenotype resulting in up-regulation of F4/80 and the mannose receptor. In more chronic conditions such as Crohn's disease and achalasia, ICC and mast cells develop close spatial contacts and piecemeal degranulation is possibly triggered.

Keywords: interstitial cells of Cajal • macrophages • mast cells • intestine • muscularis • classical activation • alternative activation • immunohistochemistry • ultrastructure

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Introduction

Interstitial cells of Cajal (ICC) are recognized as pacemaker cells for gastrointestinal movement [1, 2] and are suggested to be mediators of neuromuscular transmission [3]. Macrophages in the muscularis externa of mice are in close spatial contact with the ICC and create a constant and regularly distributed cell population. It would therefore seem legitimate to propose that macrophages and ICC functionally interact [4–7]. When my review on macrophages in the normal mammalian muscularis externa was published in 1995 [6], there were only a few other publications on these cells. Since then studies on ICC function, distribution and morphology have flourished, and disturbances in gastrointestinal motility have been related to pathologies associated with ICC. It has been suggested that immune cells, *e.g.* macrophages and mast cells, influence and affect the ICC and interact with enteric nerves. Reports on several diseases and on certain animal models have noted damage and a decrease in the number of ICC, as well as an increase in the number of macrophages and mast cells in achalasia [8], diabetic gastroparesis [9], Crohn's disease (CD) [10], ulcerative colitis (UC) [11], animal models for inflammatory bowel disease [12], gut resection [13] and helminth infections [14, 15]. Finally, both macrophages and mast cells seem to be involved in post-operative ileus and motility disturbance, see reviews [16–18].

This report will review studies on macrophages and mast cells, their distribution, morphology and spatial relationships with ICC in the normal gastrointestinal tract of rodents and human beings as well as their activation states, polarization and possible interactions during pathological conditions.

Identification of the cells

Immunohistochemistry and subsequent light microscopy of sections and whole mounts provide a comprehensive overview of the cells regarding their localization, morphology and densities in the tissue. Their relationship to other cells and structures can be visualized with double-staining techniques. When evaluating pathological tissue one should bear in mind that macrophages and possibly mast cells are able to switch from one activation state to another – and thereby change their immunophenotype and function [19, 20].

Electron microscopy and to a certain extent immuno-electron microscopy, are clearly the best methods to establish the identity of ICC, macrophages and mast cells, their exact location in the muscularis, as well as possible activation states and pathological changes. As spatial associations in biological systems are often indicative of functional interactions, it is important to evaluate the spatial relationships between the cells: the distance between them and the presence of gap junctions, peg-and-socket junctions and close appositions. However, it is a most laborious method and demands great skill and knowledge.

ICC

Both ICC and mast cells depend on stem cell factor (SCF) for their development and express the protooncogene *c-kit* which encodes a receptor tyrosine kinase (KIT). Antibodies towards KIT (CD117) are the main markers for ICC organization and distribution [1, 2, 21]. In addition, numerous immunohistochemical markers have been reported to stain ICC, see review [22].

Macrophages

Activation

Macrophages can be identified as resting-tissue macrophages, several subgroups have been identified within the same tissue, and activated macrophages [23]. For the last decade or so, studies have mainly focused on activated macrophages and their different activation states, *i.e.* classically activated macrophages (M1) and alternatively activated macrophages (M2), the type of activation being dependent on their microenvironment, see reviews [24–26].

Classical activated macrophages (M1) develop in response to concomitant stimulation by interferon (IFN)-γ and microbial products, such as lipopolysaccharide (LPS). IFN-γ is mainly secreted by TH1 and $CD8^+$ cytotoxic lymphocytes, NK cells and professional antigen-presenting cells. The stimulation of macrophages results in the production of pro-inflammatory cytokines and nitric oxide which can be associated with deleterious effects in the tissue environment [25]. Classically activated macrophages secrete pro-inflammatory cytokines, such as interleukin (IL)-β, IL-12, IL-15, IL-18, tumour necrosis factor (TNF)-α, the chemokines CCL15, CCL20, CXCL9, CXCL10, CXCL13 and display elevated expression levels of MHCII (class II major histocompatibility complex) and the co-stimulatory molecules CD80 and CD86. Functionally they are characterized by enhanced endocytic functions and an enhanced ability to kill intracellular pathogens. The increased bactericidal activity is mediated by several mechanisms that include restriction of iron and other nutrients for microorganisms, acidification of the phagosome, synthesis of reactive oxygen intermediates, and release of nitric oxide due to inducible nitric oxide synthase (iNOS) activity [25]. The activation is mediated by a combination of transcription factors such as signal transducer and activator of transcription, nuclear factor-κB (NF-κB) and mitogen-activated protein kinases [26]. Furthermore, macrophages can undergo innate activation, after exposure to LPS, flagellin, bacterial DNA, viral RNA as well as cellular debris, through germline encoded pattern recognition receptors, such as toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD) receptors [25, 27]. This activation phenotype resembles to a certain extent M1 activation and is characterized by the secretion of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, and an increased expression of co-stimulatory molecules [25].

Alternatively activated macrophages (M2) are activated in the presence of IL-4 and IL-13, and a phenotype vital to the immune

response to parasites is triggered. Helminth infection serves as a prototypic disease [24]. Macrophages exposed to IL-4 express the mannose receptor (CD206) and their secretory capacity includes IL-10, increased arginase activity and decreased nitric oxide production. Other stimuli are glucocorticoids, IL-10 and immune complexes. Alternatively activated macrophages have – apart from their role in parasite infection – been associated with tissue remodelling, wound healing, fibrosis, as well as tumorigenesis [25]. IL-10, hemin and 15-deoxy-δ 12,14 prostaglandin J2 appear to be able to exert an anti-inflammatory effect *via* induction of the heme-catabolizing enzyme heme oxygenase-1 (HO-1) in macrophages, see review [28]. Alternative activation seems to be both adaptive and innate in origin. IL-4 is produced by $CD4^+$ TH2 and $CD8^+$ T cells, NK cells, basophils, eosinophils and mast cells [24]. Since the IL-4/IL-13 cascades also seem to cross-talk with TLRs and IFN pathways – and since there may be many different degrees of activation – a different classification system for macrophages has been suggested based on three different homeostatic activities – host defence, wound healing and immune regulation [26]. Also, macrophages are able to switch from one activation state to another in a reversible manner, suggesting that a given cell may participate sequentially in both the induction and the resolution of inflammation [19, 20].

Identification

Mouse macrophages can be marked with several rat monoclonal antibodies. F4/80 is directed towards a plasma membrane glycoprotein and has been identified as a maturation marker for monocytes, eosinophils, subsets of dendritic cells and most mature macrophages [29], but it may also be implicated in immunological tolerance [30]. F4/80 stains macrophages in the muscularis externa faintly, when using conventional fixatives and immunohistochemistry; therefore special fixation and enhancement techniques are recommended [31]. CD169 is an antibody towards sialoadhesin, a lectin which is expressed by some macrophages in lymphoid organs [32], but also by muscularis externa macrophages [31, 33]. Antibodies towards class A scavenger receptor (CD204) work well on macrophages in NMRI mice [34], but not in C57Bl/6 mice [35]. In our first immunohistochemical studies we also used antibodies towards M1/70 (CD11b) and MHCII [36].

In rat muscularis externa, macrophages can be marked with several monoclonal antibodies: ED1 antibodies directed towards macrosialin (CD68) have been described as being restricted mainly to cells of the monocyte–macrophage lineage; ED2 antibodies directed towards the macrophage haemoglobin scavenger receptor CD163 which is present on most macrophage subsets; ED3 are directed towards CD169 [37]. In macrophages, macrosialin is mainly localized in lysosomes and endosomes [38]. MHCII expressing cells have also been reported [39, 40].

In human muscularis externa, macrophages have been demonstrated with monoclonal antibodies towards CD68, CD11b and CD163 [41, 42].

Modes of activation: Inducible iNOS and cyclooxygenase-2 (COX-2) expressions in macrophages are associated with classical activation and a pro-inflammatory response (*i.e.* LPS administration), see reviews [24, 26]. Up-regulation of the mannose receptor (CD206) is a distinctive marker of IL-4 activated macrophages and alternative activation [43]. In addition, up-regulation of the F4/80 antigen has been associated with alternative activation where macrophages seem to be involved in induction of regulatory T cells [20], see review [44]. MHCII immunoreactivity is expressed by B lymphocytes, dendritic cells and some macrophages and monocytes, and seems to be regulated both *via* classical and alternative activation [25].

Tracers can be used to study the endocytic capacity and distribution of macrophages. In our first study we used fluorescein-labelled dextran (FITC-D) combined with fluorescence and electron microscopy [5]. This method is also convenient combined with macrophage antibodies [36] as well as for identifying cells with activation markers. However, intraperitoneal injection with FITC-D results in COX-2 expression – which suggests innate activation – but also release of prostaglandin E_2 and reduced muscle contractility [45].

Mast cells

Activation

Functional activity of mast cells is mostly associated with degranulation and the release of proteases, heparin, histamine, serotonin as in allergies and asthma. However recent studies in mice and rats, and to some extent also in human beings, have shown that mast cells play critical roles in both innate and adaptive immunity. Like classically activated macrophages they express TLRs and, when activated, release pro-inflammatory cytokines (TNF-α and IL-6) and other mediators that recruit neutrophils and eosinophils to the site of infection, see reviews [46, 47]. Moreover, similar to alternatively activated macrophages, IL-4 induction changes the cytokine profile released by mast cells by decreasing the production of pro-inflammatory cytokines such as TNF-α and IL-6, and increasing the production of TH2 cytokines, such as IL-3, IL-5 and IL-13 (like in macrophages the activation state can switch in a reversible manner) [48]. Anti-inflammatory cytokine IL-10 can be produced by mast cells which – in an autocrine manner – can regulate themselves [49].

Identification

By immunohistochemistry mast cells can be stained with antibodies towards KIT, but also with antibodies towards the content of their granules, *i.e.* tryptase, chymase or chymotryptase. In addition, after activation, IL-4 immunoreactivity can be localized to mast cells [50].

Using conventional histochemistry mast cells are large and recognized by basophilic granules in their cytoplasm. As the granules contain negatively charged sulphated proteoglycans (heparin) they become metachromatic when stained with toluidine blue. They can also be stained with Alcian blue, which stains carboxyl and sulphate groups, but it is probably less specific. Activation of

mast cells results in degranulation and exocytosis of the content into the surroundings, although at times only cytokines are released. Piecemeal degranulation is typified by variable losses of the granule content, see review [51].

Cell distribution

ICC in rodent gastrointestinal tract

The general organization of ICC networks in the rodent gastrointestinal tract was illustrated schematically by Hanani *et al.* [52] based on ultrastructural and KIT immunohistochemical data from a large number of reviews. ICC are in contact with each other and form networks in four locations: (1) ICC with a stellate morphology are located in the serosa (ICC-SS); (2) between the longitudinal (LM) and circular (CM) muscle layers at the level of Auerbach's plexus (ICC-AP) where they form a network around the ganglia; (3) in the small intestine, where bipolar-shaped ICC are located at the level of the deep muscular plexus between the inner and outer circular muscle layers (ICC-DMP); (4) in the stomach and colon, where they are located at the border between the submucosa and the circular muscle and are called ICC-SM in the stomach [53, 54] and ICC-SMP in the colon [55]. In the oesophagus, stomach and colon, solitary bipolar ICC are seen in the circular and longitudinal muscle layers, ICC-CM and ICC-LM, respectively, or combined: ICC-IM. The oesophagus and fundus of the stomach are completely devoid of ICC at the level of AP [3, 53], but ICC-AP are present in the corpus, antrum and pylorus [3], in the small intestine [1, 2] and colon [56].

ICC in human gastrointestinal tract

In the human gastrointestinal tract, our knowledge of the distribution and morphology of ICC is based on KIT immunohistochemistry and electron microscopy and has been reviewed by several authors [22, 57, 58]. In human *oesophagus* ICC-LM and ICC-CM appear as elongated cells within both muscle layers but are absent at the level of AP; at the submucosal border the ICC are in close contact with nerve endings [57, 59]. In the human *stomach* KIT^+ bipolar cells are present in CM and LM of the corpus, but in the antrum they are also present at the level of AP [57, 60]. ICC are also found at the submucosal border, ICC-SM. They lie close to naked nerve terminals and muscle cells, and are more frequent in antrum and corpus than in the fundus [61]. In the human *small intestine*, KIT^+ ICC are present in the longitudinal and circular muscle layers and at the level of AP [57, 62] whereas opinions differ as to whether ICC-DMP are KIT immunoreactive or not. Ultrastructurally ICC-AP have close associations with nerve varicosities [63] and ICC-DMP form contacts with both nitrergic and cholinergic nerves, they also form gap junctions with the outer CM [64]. In the normal *colon* many KIT^+ ICC are distributed regularly in the circular muscle layer. In the taenia of the longitudinal muscle layer, their long axes run parallel to those of the muscle cells, and they embrace the ganglion cells in AP [62]. Ultrastructurally, only ICC at AP [65, 66] and at the submucosal border have been described [11].

In the mouse, slow waves that represent pacemaker activity are generated in ICC-AP in the small intestine [1, 2]; and in ICC-AP and ICC-IM in the stomach [67, 68]. In canine colon the prominent pacemaker is associated with ICC-SMP [69]; in rat colon both ICC-AP and ICC-SMP may be associated with pacemaker activity [55, 70]. Neurotransmission to the muscles is believed to be mediated *via* ICC-IM in oesophagus, in antrum and corpus of the stomach [3], and *via* ICC-DMP in the small intestine [71].

Macrophages in rodent gastrointestinal tract

The morphology and distribution of macrophages in the muscularis externa have been studied in detail in the mouse small intestine, and human small intestine and colon (see review [6]). In the last decade many papers have been published on experimental inflammation in animal models in which it has been suggested that macrophages are involved. However, in contrast to the many studies and reviews on ICC, there are very few studies on macrophage distribution, location and morphology in normal tissue. Macrophages have mostly been reported in control tissue in motility studies on inflammation, or ICC studies associated with various experimental and/or pathological conditions without any reference to the precise location of the macrophage.

Small intestine: In the mouse, macrophages have been described at three levels: (1) in the serosa as bipolar cells with two to three ramifications; (2) at the level of AP where the cells have between three and five ramifications; (3) a few bipolar cells at the level of the DMP between the inner and outer CM. The cell populations at levels (1) and (2) are regularly distributed, but do not create regular networks with cell contacts similar to those of the ICC, but the macrophages are often enveloped by and in close spatial contact with ICC. At the level of AP the macrophages line the vessels as well as the plexus. At the level of the DMP (level 3) the bipolar macrophages are also enveloped by processes of ICC or fibroblast-like cells, but close spatial contacts between macrophages and nerves have not been observed [5, 7, 72] .

Ultrastructurally, macrophages can be characterized by having a centrally situated, deeply indented nucleus and long slender cell processes, they do not have a basal lamina nor do they form specialized contacts with other cells, but they are often enveloped by ICC. In adult conventional mice they harbour several types of vesicles which vary in size, shape, content and membranes [4, 5] (Fig. 1A and B). However, the number, form and type of vesicles seem to be dependant on the activation state of the macrophage.

In germfree mice the macrophages appeared to contain a similar if not higher number of vesicles with electron-dense granular content (lysosomes) and less light vesicles (vacuoles) in the cytoplasm [34] (Fig. 1C and D).

In mice that had been treated with FITC-dextran intravenously, the macrophages contained many dextran-filled vesicles. Notably

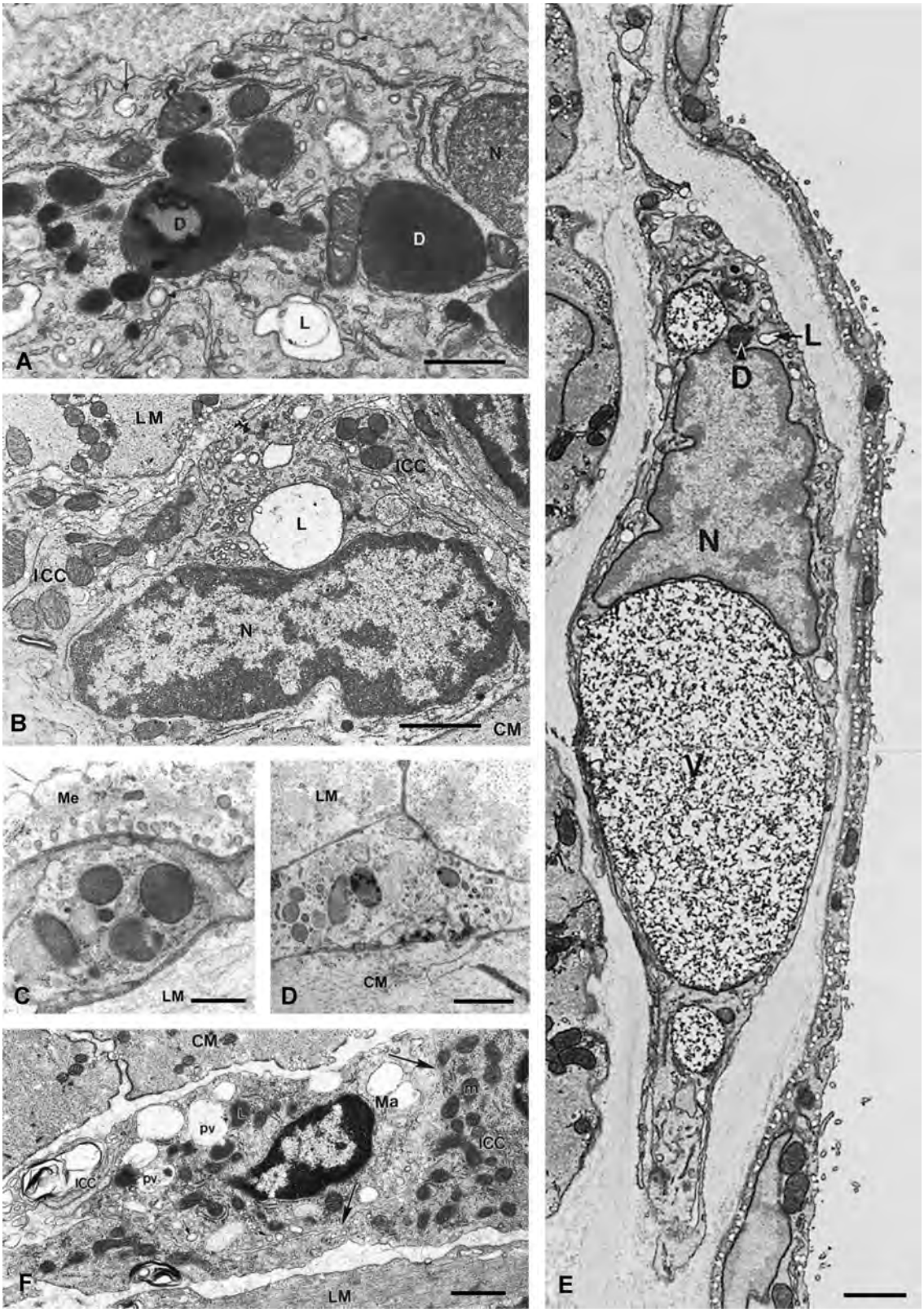

Fig. 1 Muscularis externa from mouse small intestine. (**A**)–(**B**): Conventional mice, (**A**) Serosal macrophage containing dense bodies (lysosomes) (D), light vesicles (L), coated vesicle (arrow head), doughnut-shaped vesicle (arrow), nucleus (N), bar: 0.5 μm. **B**. Macrophage at the level of AP, between the longitudinal muscle layer (LM) and the circular muscle layer (CM), is enveloped by ICC processes (ICC), bar: 1 μm. (**C**)–(**D**): Germfree mice, (**C**) Process of a serosal macrophage between the mesothelium (Me) and the longitudinal muscle layer (LM), bar: 0.5 μm. (**D**) Macrophage between LM and CM, the macrophages in the germfree mice contain mostly dense bodies, bar: 1 μm. (**E**) Dextran injected mouse (after 4 days), serosal macrophage between the mesothelium and the longitudinal muscle layer, the macrophage contain large dextran-filled vacuoles (V), light vesicles (L), dense bodies (D) and nucleus (N), bar: 1 μm. (**F**) Day 15 after infection with *Trichinella spiralis*, a macrophage (Ma), at the level of AP between LM and CM, is enveloped by ICC cytoplasm characterized by the content of mitochondria (m), the cell borders are marked by a large arrow. The macrophage contains phagocytic vesicles (pv) some lysosomes (L) and coated vesicles (small arrow), bar: 1 μm. Reproduced with kind permission from Wiley-Blackwell: (**A**–**C**) [5], Springer: (**D**) and (**E**) [34] and American Society for Investigative Pathology: (**F**) [15].

serosal macrophages, which were not in the vicinity of vessels, contained dextran already after 1 hr. After 1 and 4 days the macrophages contained large dextran-filled vacuoles [5] (Fig. 1E). After intraperitoneal injection mesothelial cells also contained dextran-filled vesicles [73]. This suggests that dextran can be transported from the peritoneal cavity *via* the mesothelium to the serosal macrophages and that these may have a function as sentinels against external exposure.

After 15 days of *Trichinella spiralis* inflammation some macrophages contained numerous large phagosomes and lysosomes [15] (Fig. 1F).

The macrophages distribution and morphology have been demonstrated immunohistochemically with several antibodies: F4/80, CD11b, class A scavenger receptor and recently CD169 [31, 33, 34, 36]. In adult mouse small intestine the macrophages are constitutively MHCII$^+$ [31, 34, 36, 74, 75], but MHC$^-$ in germfree and newborn mice [34]. This suggests a possible up-regulation/activation due to non-pathogenic, commensal bacterial antigens in the chow or the surroundings. In the MHCII$^+$ cell population in mouse muscularis presence of subsets of dendritic cells has also been described [75]. However, in a recent study only one cell population was found, expressing a MHCII^{high}, CD11c^{low} CD103$^{\div}$, CD11b$^+$, F4/80$^+$ phenotype, being derived from monocytes, with a monocyte/macrophage morphology and responsive to M-colony stimulating factor [76]. Thus a subgroup of classical dendritic cells is unlikely to reside in the muscularis externa along with macrophages.

In an immunohistochemical study on rat *small intestine*, macrophages (ED2$^+$ cells) were described within LM, between the muscle layers at the luminal side of the plexus and within CM [39]. However, ultrastructural studies of rat small intestine show macrophages located only between the mesothelial cells and LM [40, 77]. Furthermore, cross-sections as well as double staining with ED2 and KIT together with confocal microscopy confirm that the macrophages are located at the same three levels as in the mouse (Fig. 2). Kalff et al. describe a few ED1$^+$ monocytes in rat muscularis [39]. However, in our unpublished observations we only found ED1$^+$ granules in cells with a ramified morphology located in the serosa, between the muscle layers at AP, and at DMP (Fig. 2). The ED1$^+$ cells therefore seem to be macrophages too. MHCII immunoreactivity seems to depend on the antibody used, as 70% of the macrophages are described to show MHCII immunoreactivity when using OX76 [39], whereas only a few ramified cells show MHCII imunnoreactivity when using OX6 [40]. The structure of mouse and rat small intestinal muscularis externa is well documented. They are almost similar to one another with regard to their muscle layers, nerve plexuses, ICC networks and the location of macrophages. However, there are some differences between the two species in that the structure is simpler in mouse small intestine, where vessels are only found at AP and not in serosa [5], whereas in the rat both LM and serosa contain vessels [77]. In mice, other immune cells (B and T lymphocytes) – apart from an occasional mast cell – are absent [4, 73], but rat muscularis externa is also reported to contain neutrophils, mast cells, T-cells, natural killer cells and dendritic cells [39]. In mouse *colon*, CD 168$^+$ cells were found in serosa and at AP as ramified cells. In addition more rounded cells were present near the mesenterial attachment (our unpublished observations). In proximal rat colon, ED2$^+$ macrophages at the level of AP seem to have the same morphology and distribution as in the small intestine and are ultrastructurally in close spatial contact with ICC and ganglion cells [12, 78]. Some ramified cells were faintly ED1$^+$ [12]. In another ultrastructural study on rat *colon*, macrophages were found at the level of SMP as well as at AP. There is no description of any close contact to ICC or any macrophage-to-nerve relationship [79]. The distribution of macrophages in the *gastric* muscularis externa has not yet been documented in detail. In a study on the pathogenesis of septic ileus, CD169$^+$ macrophages have been described in the circular muscle layer of the murine gastric corpus. These cells seem to be bipolar in shape [33]. In a recent study, another population of F4/80$^+$ macrophages was also described in the muscularis externa of the gastric corpus [80]. The cells look ramified as macrophages at the level of AP in mouse small intestine. These studies suggest that there are macrophages with different morphologies and in various locations in the stomach as well. In a study of the ultrastructure of the circular muscle layer of antrum and corpus, Ishikawa et al. [81] described scattered nerve bundles and free cells (among them eosinophils and macrophages), which were occasionally observed in the interstitium around the nerve bundles. However, there is still a considerable lack of information on these macrophages in the stomach at both light and electron microscopic levels, especially with regard to their relation to ICC, and reports on serosal macrophages seem to be absent.

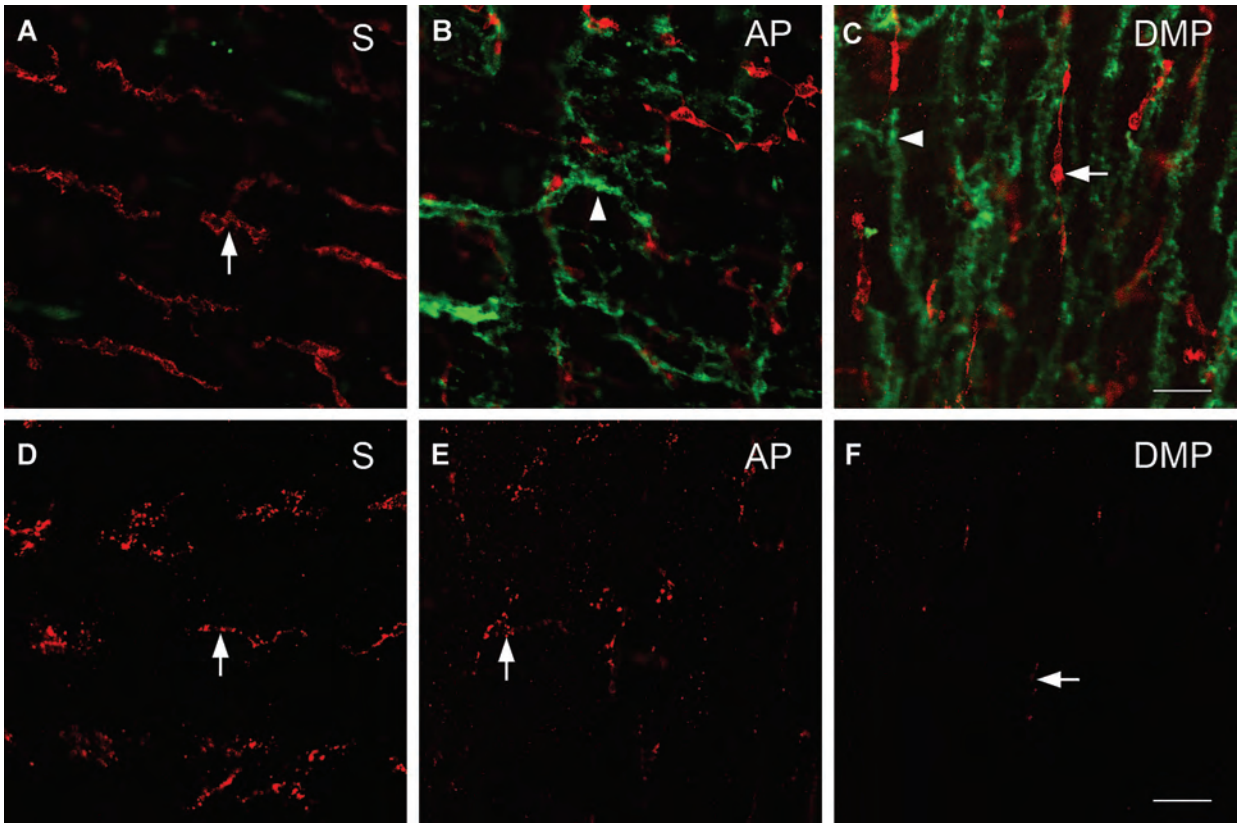

Fig. 2. Whole mounts from rat jejunum. Confocal micrographs. (**A**–**C**) Double staining with ED2 (red) towards macrophages and KIT (green) towards ICC. (**A**) Macrophages in serosa. (**B**) Macrophages at the level of AP and ICC-AP. (**C**) Macrophages at the level of DMP and ICC-DMP. Arrows: macrophages, arrowheads: ICC. (**D**–**F**) ED1 (CD68) stained macrophages display a granular staining pattern and a ramified morphology. (**D**) In serosa. (**E**) At the level of AP. (**F**) At the level of DMP. Bars: 30 μm.

Macrophages in human gastrointestinal tract

Very few studies of the muscularis externa of normal human oesophagus and stomach focus on macrophages. The muscularis of the small and large intestine do have immunoreactive $CD68^+$ and $CD11b^+$ cells which seem to have long cell processes. These cells were found in the serosa, in the muscle layers and in the submucosa. Some of the serosal macrophages appeared to line the longitudinal muscle layer. Macrophages also occurred in septa between the main muscle lamellae, in smaller intralamellar septa between the muscle cells as well as in the lining of AP. Moreover they were located in the connective tissue of the serosa and submucosa. HLA-DR^+ cells (MHCII) were present in the muscle layers and seemed to co-stain with $CD68^+$ and $CD11b^+$ cells, but they were scarce in serosa and submucosa. Accordingly, macrophages in the muscularis differ from macrophages in serosa and submucosa with regard to their immunophenotype [41]. Full-thickness whole mounts of jejunum have been studied, where $CD163^+$ cells were suggested to be located in the septa [42], but they do not seem to have a ramified morphology. Ultrastructurally, human macrophages resemble those of mice and rats [41]. However, in contrast to rodent small intestine, specific relations between macrophages and ICC have only been reported in a recent ultrastructural study on colonic ICC-AP, where macrophages were described as being frequent at the level of AP with intimate and close appositions to ICC-AP. Membrane-to-membrane contacts between macrophages and fibroblast-like cells also seemed to be a characteristic and frequent feature, but contacts to other cell types or nerves are not described [66]. In CM and LM they were encountered as single cells [82]. In the DMP of the small intestine there were many macrophages in close proximity to fibroblast-like cell processes, but they did not form special contacts. In serosa and submucosa the macrophages were surrounded by collagen and elastic fibres; in the septa they were often in close contact with fibroblasts and nerve fibres [41].

Mast cells in rodent gastrointestinal tract

Mast cells are mainly found in the lamina propria of the intestine, but have also been observed in the epithelium, in the submucosal

layer and in serosa covering the peritoneal cavity. General studies of the distribution of mast cells in the normal rodent intestinal muscularis externa are lacking, and mast cells are only mentioned in connection with studies on other cell types or in models of inflammation. In general, rats are considered to have more mast cells than mice, and Wistar rats to have more than Sprague-Dawley rats [83]. In the *stomach* Alcian-blue$^+$ mast cells have been described in the submucosal layer of antrum and corpus, but very few were observed in the muscle layer [81]. In the muscularis externa of mouse *small intestine*, mast cells were extremely rare at both light and electron microscopical levels [4]. Significant differences have been reported between various murine strains: the mucosa, submucosa and muscularis externa of C57/B6 mice have hardly any mast cells, whereas several have been observed in NMRI mouse tissue stained with toluidine blue (our unpublished observation) and with an unspecific avidin stain in rat muscularis externa [39]. Descriptions of mast cells in the muscularis externa of *colon* are lacking.

Mast cells in human gastrointestinal tract

Information on the normal distribution and morphology of mast cells in human muscularis externa is scarce and is mostly derived from descriptions of control (non-pathological) tissues in various studies on pathological tissue. However, KIT$^+$ mast cells were present in the muscularis externa and submucosa in the intestine of 17–19- week-old foetuses [84]. The *oesophagus* exhibited a low density of mast cells around the blood vessels at the level of AP and in the septa, but mast cells were rarely apparent within the muscle layers [8]. There is no information on mast cells in the muscularis externa of the *stomach*. In the *small intestine* and *colon*, scattered mast cells were found in both LM and CM when stained with toluidine blue as well as with a double staining with KIT/tryptase antibodies. Most metachromatic and tryptase/KIT$^+$ cells resided in the inner circular muscle layer [62]. The same distribution has been observed in the ileum [10]. Hagger et al. [85] described mast cells in all layers of muscularis externa of colon and rectum.

Inflammation

In several animal models and intestinal diseases with motility disturbances, ICC, macrophages and mast cells have been suggested to be implicated.

Models of inflammation

Intestinal inflammation leads to disturbances in motility involving both increased and decreased smooth muscle contractility [86]. In cases of peritonitis, surgical anastomosis, experimental obstruction, surgical manipulation and the administration of trinitrobenzene, smooth muscle contractility decreases. These inflammations are mostly associated with products of the TH1 response, particularly IL-1 and IL-6, and with an up-regulation of iNOS. Smooth muscle hypercontractility is associated with nematode inflammations and products of the TH2 response, IL-4 and IL-13 [87, 88].

LPS administration

LPS-administration to mice resulted in delayed gastric emptying and intestinal transit as well as an iNOS up-regulation in some macrophages (about 8%) both in the stomach, where they seem to be located in the circular muscle layer, and in the ileum, where they probably are located at the level of AP [33]. In *op*/*op* mice which lack certain macrophage subtypes, *e.g.* macrophages in the muscularis externa, LPS induced VCAM-1 activation of the vessels, a modest influx of neutrophilic granulocytes, and iNOS activation in a cell type other than macrophages [31]. In both control and *op/op* mice KIT immunocytochemistry confirmed that the ICC network at AP in the small intestine remained intact (our unpublished information). Eskandari et al. administered LPS to rats which led to a suppression of jejunal circular smooth muscle activity and to iNOS induction [89]. In an earlier study on LPS administration to rats, a significant infiltration of neutrophils, mast cells, and monocytes into the muscularis externa was described [90]. Unfortunately, none of the studies on LPS includes an ultrastructural description of the muscle layers, ICC, nerves or spatial relationships to macrophages and other immune cells. Regarding the activation of endothelial cells and the neutrophil influx in *op*/*op* mice, it is well known that LPS also activates TLRs on mast cells, mesothelial cells and endothelial cells as well as on macrophages [91–93].

Surgical anastomosis

Five hours after ileal end-to-end anastomosis have been performed, the KIT$^+$ ICC networks were disrupted at the level of AP and at DMP at the resection site, but recovered gradually above and below it. Ultrastructurally, ICC were absent up to 1–2 cm from the resection site and were distorted up to 2–5 cm from the site. In addition, the number of neutrophils and macrophages was greatly increased. Electrophysiologically, there was a loss of slow waves and phasic contractions near the site of resection. Twenty-four hours after surgery the number of neutrophils and macrophages declined again to near control levels, slow-wave activity showed signs of partial recovery and the KIT immunochemistry of ICC networks at AP and DMP was largely restored at all sites. All these events have been confirmed ultrastructurally [13]. In a similar model using iNOS knockout (iNOS$^{-/-}$) mice or animals treated with iNOS inhibitors, the ICC networks and pacemaking functions were protected. Damaged cells and depressed motility were only apparent at the anastomotic site [94]. It was suggested that the post-operative damage consists of two components: a direct local component, where the cells are damaged by the surgical procedures (the site of anastomosis), and a second

more extensive component where cells, particularly ICC, are negatively affected by the inflammatory responses. In this model there was no significant effect on the resting membrane potentials of smooth muscle cells, which suggests that the loss of slow waves is not caused by a general effect of inflammatory mediators on the resting conductances of the muscle cells [94] In this model cellular debris seems to activate the innate immune response [27] and thereby numerous intracellular signalling pathways that lead to iNOS induction, COX-2 up-regulation and the production of pro-inflammatory cytokines and chemokines, all of which cause damage to the tissue (ICC?) in the vicinity of the incision.

Ileal obstruction

Ileal obstruction in rats resulted in dysmotility, an increased number of macrophages (ED2- and $CD14^+$ cells) at AP, disruption of the network of KIT^+ cells, as well as increased TNF-α mRNA, but no apparent leucocyte infiltration into the muscularis externa [95]. The endothelin ET_B receptor-deficient rat, which is used as a model for long-segment Hirschsprung's disease, displayed distended intestines proximal to a constricted aganglionic part and has abnormal intestinal flora. Neutrophilic infiltration was rarely seen in the muscularis externa, but IL-1β and IL-6 mRNA levels were increased and spontaneous phasic contractions became irregular. This was combined with damage to ICC revealed both by immunohistochemistry and electron microscopy. Furthermore, the number of $ED2^+$ and CD-14 cells was increased, and – on the ultrastructural level – there was an abundance of macrophages in serosa, at AP and in DMP, and close associations between ICC and macrophages at AP [96].

Post-operative ileus

For the last decade, macrophages in the muscularis externa have been thought to play a role in post-operative ileus – another inflammatory model, see reviews [16–18]. In the most common post-operative ileus model, the gut is manipulated by gently brushing the entire intestine with moistened cotton swab-sticks. This initiates a general hypomobility of the entire gastrointestinal tract. It has two phases: immediately after surgery various neuronal inhibitory pathways are activated for a short period of time [18]; a later phase consists of a recruitment of monocytes, neutrophilic granulocytes and mast cells into the muscularis [97, 98]. This inflammatory infiltrate is thought to activate spinal pathways resulting in gastric retention and delayed intestinal transit time [99]. The infiltrate and the hypomobility are reduced when using various models where the innate immune defence is inhibited, *i.e.* $COX\text{-}2^{-/-}$ mice, $iNOS^{-/-}$ mice, MAP kinases or selective inhibitors of these or other enzymes from the TLR pathway, but also where muscularis macrophages are reduced or lacking as in macrophage-depleted rats or in mutant *op*/*op* mice [16–18]. Mast cells also play a role in post-operative ileus. Murine mast cells in the peritoneal fluid became activated within minutes of the manipulation, as measured by an increase in mast cell proteases. Furthermore, inflammation, delayed gastric emptying, and degranulation were prevented when pre-treating with mast cell stabilizers (ketotifen or doxantrazole). In W/W^v mutant mice – which lack mast cells – the manipulation did not elicit significant leucocyte recruitment; reconstitution in W/W^v mice with bone marrow-derived mast cells from wild-type mice resulted in the restoration of the inflammation [100]. It appears that in order to achieve a full inflammatory response, both macrophages and mast cells have to be present. Activation of the cholinergic anti-inflammatory pathway by electrical stimulation of the vagus nerve also inhibits intestinal inflammation and gastric emptying in this manipulation model [101]. Since *in vitro* studies have shown that macrophages have nicotinic receptors, stimulation of the vagus nerve has been considered to prevent the infiltrate by preventing macrophage activation, but cholinergic nerves in the muscularis externa most likely belong to the enteric nervous system, though the vagus nerve traverse the muscle layers. Also, the spleen seems to play a role in effectuating the anti-inflammatory effects of the vagus nerve, since electric stimulation of the vagus nerve failed to attenuate serum TNF levels in endotoxin-treated splenectomized mice, see review [102].

As many mesothelial cells in the serosa will become damaged during manipulation, and murine peritoneal mesothelial cells constitutively express TLR4, CD14 and MD-2 [92], innate activation of the TLR4 on mesothelial cells as well as on serosal macrophages may very well initiate inflammation in the serosa followed by an amplification from macrophages at AP and mast cells. However, as the serosa is devoid of nerves [5], any possible macrophage inhibition *via* nerves must take place at AP or DMP. Many factors seem to be involved in the pathophysiology of post-operative ileus, but in spite of the severe motility disturbances we know very little about the morphological effect, at the ultrastructural level, on ICC, the adjacent nerves, smooth muscle cells, but also on the immune cells and mesothelial cells.

Helminth infections

Helminth infections induce a TH2 response, where macrophages can be alternatively activated by the TH2 cytokines IL-4 and IL-13; in addition IL-10, arginase 1 and the mannose receptor (CD206) are up-regulated [24]. Enteric nematode infection is characterized by hypermotility and smooth muscle hyperplasia in muscularis externa [103] and an increased infiltration of immune cells: macrophages, lymphocytes, eosinophils and mast cells [77, 104, 105]. A recent study on *Nippostrongylus brasiliensis* infection found an increase in the number of infiltrated macrophages as well as in an up-regulation of. mannose receptor (CD206) and arginase activity [106], markers usually associated with alternative macrophage activation [24]. Not only did $CD206^+$ cells appear in the mucosa and muscularis externa after 5 days, but the F4/80 immunoreactivity of the cells seemed to be up-regulated as it was absent or hardly discernable in muscularis externa and mucosa of control tissue. This is in accord with previous studies using another nematode, *Trichinella spiralis* where F4/80 immunoreactivity in the muscularis externa was strong after 6 and 15 days, respectively, but was absent or weak in control tissue, after

28 days and 40 days when the inflammation had resolved [15, 107]. In both studies the substantial increase of cells was probably due to an invasion of monocytes, macrophages and eosinophils. However, in these studies the densities of F4/80$^+$ macrophages in the control tissue appeared much lower when compared to specimens subjected to Tyramid amplification [31], indicating that, apart from the substantial increase in the number of cells, the F4/80 is up-regulated during this response. This is consistent with the fact that F4/80 is implicated in immune tolerance through the generation of regulatory T lymphocytes (Treg) and a cell-to-cell contact may result in an increased surface expression of F4/80 [30, 44]. In *Trichinella spiralis* infection, mast cells seem to play a role, in addition they can change granule protease phenotype during the inflammation [105]. Ultrastructural studies of *Trichinella spiralis* infection in mice [14] and *Nippostrongylus brasiliensis* infection in rats [108] revealed severe damage to ICC-DMP, nerve varicosities and loss of gap junctions. Further away from the infection – at the level of AP – the ICC network evaluated by KIT immunohistochemistry looked unaffected in the *Trichinella spiralis* infected mice. However, ultrastructurally there was selective and patchy damage to ICC after 1–3 days and even more severe damage after 15 days [15]. In *Nippostrongylus brasiliensis* infected rats KIT immunohistochemistry at AP was weak after 14 and 30 days, but ultrastructurally the cells looked normal. Macrophages were in close spatial contact with both ICC-AP and ICC-DMP in both infections [14, 108], and in *Trichinella spiralis* infection many lymphocytes were present [15].

Inflammatory bowel disease

The inflammatory bowel diseases CD and UC are characterized by chronic intestinal inflammation. The prevailing hypothesis on the pathogenesis of these diseases is that they are the product of a dysfunction in the regulation of the immune response to commensal antigens (bacterial products) in a genetically susceptible host [109]. Only the mucosa has been extensively studied in both CD and UC and it exhibits altered lymphokine secretion profiles [110]. CD tissue produces an increased amount of the TH1 cytokine INF-γ and decreased amounts of the TH2 cytokines IL-4 and IL-5, whereas UC produces an increased amount of IL-5, a normal amount of INF-γ, and a decreased amount of IL-4 [111]. Altered intestinal motility has been described for both CD and UC. The inflammation in CD is transmural and involves a large number of mast cells [112]. KIT immunoreactivity in the small intestine was decreased in ICC-IM and at AP [10, 113]. Ultrastructurally, all ICC subtypes can be damaged, but most frequently at AP. Mast cells were abundant in the muscularis externa and made frequent membrane-to-membrane contacts with all types of injured ICC, but possibly not with intact ICC. Some mast cells exhibited piecemeal degranulation, which can be interpreted as beneficial to ICC – because of their capacity to secrete the growth factors IL-9 and SCF [10] – or damaging to ICC because piecemeal degranulation is also associated with histamine secretion [114] and possibly other pro-inflammatory mediators. ICC-macrophage contacts were far less frequent than ICC-mast cell contacts. However, nerve structures appeared to be most severely damaged, but intimate contact between nerve structures and inflammatory cells were rare [10]. In involved areas of UC patients, KIT immunohistochemistry was reported to show a significant increase in ICC-IM compared to controls [115]. Whereas ultrastructural changes have only been observed in ICC-SMP and were restricted to specific areas within the cell, nerve terminals often appeared swollen and macrophages were often encountered close to ICC-SMP and nerves. Mast cells and eosinophils were occasionally seen, but lymphocytes were scarce [11].

Achalasia

Achalasia is a primary oesophageal motor disorder characterized by the absence of oesophageal peristalsis and abnormal relaxation of the lower oesophageal sphincter. The primary morphological feature is a time-dependent degeneration of ganglion cells and nerves at AP. It has recently been suggested that achalasia is due to an immune-inflammatory reaction [116] triggered by herpes virus [117], but an autoimmune background has also been suggested [116]. The infiltrate at AP consists primarily of T lymphocytes, many of these are resting or activated cytotoxic T cells [116]. In a recent study of the muscle layers, nitrergic nerves disappeared during the first three years of the disease, but ICC-IM – which might form functional units with them – did not decline in number during the first two years. Ultrastructurally, cell damage was most severe in neural structures, and membrane-to-membrane communications between nerve varicosities and ICC-IM were less common. Damage to ICC-IM had a patchy distribution, and not all ICC-IM showed ultrastructural damage. Mast cells which were the predominant immune cells in the muscle layers, made frequent membrane-to-membrane contacts with normal and injured ICC-IM and piecemeal degranulation seemed to occur [8]. It was suggested that the mast cell-ICC-IM contact may be responsible for their relatively long survival due to the fact that mast cells can secrete SCF and IL-9 [8].

Diabetes mellitus

NOD/LtJ mice

Patients with diabetes mellitus sometimes show gastrointestinal dysmotility, degeneration and decrease of neuronal structures and of KIT$^+$ ICC [9]. NOD/LtJ mice serve as a model for human type 1 diabetes, as they are susceptible to the spontaneous development of T-cell–mediated autoimmune insulitis. At 6 months of age, the diabetic mice developed delayed gastric emptying and a substantial loss of ICC-IM in the distal antrum. In addition ICC-AP were either completely missing or severely reduced in number in many areas. In the distal half of the corpus only ICC-AP were decreased

in number. The normally close associations between ICC and enteric nerve terminals were infrequent and there were no detectable ultrastructural defects in enteric neurons or in smooth muscle cells in the antrum or fundus of the diabetic mice; only ICC seemed to be damaged. Immune cells are not mentioned [67], but neuronal nitric oxide synthase (nNOS) was down-regulated in this model [80]. A recent study on this diabetic mouse model showed that HO-1 was up-regulated in the early stages and was seen in gastric macrophages, ostensibly at the level of AP, double labelled with F4/80. HO-1 remained up-regulated in all mice resistant to the development of delayed gastric emptying. In contrast, HO-1 was down-regulated in all mice that develop delayed gastric emptying. KIT and nNOS expression were also decreased in these mice. The induction of endogenous HO-1 activity with hemin (the complex of ferric iron with protoporphyrin IX, derived from haemoglobin) restored KIT and nNOS expression and normalized gastric emptying completely in all mice [80]. It has been suggested that HO-1 expression in macrophages could be related to a switch to alternative activation, where they play an anti-inflammatory role and protect ICC from damage by countering the effects of increased oxidative stress associated with diabetes [80].

STZ-DM rats

Streptozotocin-induced diabetes (STZ-DM) in rats, based on the destruction of β-cells, (also a model of type 1 diabetes) resulted in delayed gastric emptying and loss of ICC-IM and ICC-SMP as well as associated enteric nerves in the antrum of the stomach, whereas the density of ICC-AP in antrum was unaffected. Ultrastructurally there was damage to both the ICC-IM and ICC-AP in the antrum. Furthermore, the enteric nerves in the fundus displayed degenerative changes [118]. There was no noticeable ultrastructural evidence of an increase in the number of lymphocytes and macrophages in the musculature of STZ-DM stomach compared to control tissues. Mast cells, which are rarely seen within the muscle layers of control rats were, in this model, in very close spatial contact with injured ICC and enteric nerves in the diabetic antrum [118].

Conclusions

Macrophages in the intestinal muscularis externa of rodents can be found in close spatial contact with ICC. In human colon, ICC are in close contact with macrophages at the level of AP suggesting functional interaction. Mast cells are seldom present in control rodent muscularis externa. Temporary ICC damage occurs during innate activation (surgical resection and possibly post-operative ileus), but also during helminth infections which are associated with alternative activation, and where an up-regulation of F4/80 and the mannose receptor on macrophages seems to take place. The immunohistochemistry of macrophages has been studied in numerous experimental mouse and rat models of inflammation. These studies describe macrophages as well as activation markers in the muscle layers, but few studies have focused on distinguishing the macrophages in the different layers from one another. Since there are several subgroups of macrophages, the macrophages in the three locations may well have different functions. Macrophages in the serosa might be sentinels against bacteria or injury to the peritoneum and might initiate an innate inflammatory response. Macrophages at AP, and possibly at DMP might play trophic and scavenger roles and take part in wound healing and protection of ICC and/or nerves. In CD the number of ICC is decreased and the damaged ICC form close membrane-to-membrane contacts with mast cells; furthermore piecemeal degranulation takes place. This process may be associated with either mast cell secretion of growth factors and /or anti-inflammatory cytokines, or with secretion of pro-inflammatory substances (cytokines, proteases). Also, in achalasia and in a STZ-1 model, ICC and mast cells form close spatial contacts and piecemeal degranulation seems to be triggered. In an autoimmune diabetes-1 mouse model, macrophages which express HO-1 seem to play a role in protecting against the development of delayed gastric emptying, possibly by protecting ICC and/or nerves. However, HO-1 expression in macrophages in combination with unaffected ICC has not yet been demonstrated. Further studies are needed to establish the role of both macrophages and mast cells in acute and chronic intestinal inflammations, notably if they have a positive influence on ICC and nerves in chronic conditions.

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