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Ionized Calcium-Binding Adaptor Molecule 1 Positive Macrophages and HO-1 Up-Regulation in Intestinal Muscularis Resident Macrophages

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ABSTRACT

Small intestinal muscularis externa macrophages have been associated with interstitial cells of Cajal. They have been proposed to play various roles in motility disorders and to take part in a microbiota-driven regulation of gastrointestinal motility. Our objective was to understand the reaction of resident macrophages of the musculature to a pro-inflammatory stimulator, lipopolysaccharide (LPS). Mice were injected with LPS or saline and sacrificed after 6 hr. Whole mounts were stained with antibodies toward CD169, ionized calcium-binding adaptor molecule 1 (iba1) (microglial/macrophage marker) and heme oxygenase-1 (HO-1). Cell densities were measured using unbiased stereology. Results: iba1pos cells showed an overall higher density than CD169pos and HO-1pos cells. Most HO-1pos and iba1pos cells were positive for CD 169 in serosa and at Auerbach’s plexus (AP). At the deep muscular plexus, mainly iba1pos cells were present, and were mostly CD169neg; a few HO-1pos cells were present. Conclusions: A new subset of resident macrophages in the intestinal muscularis externa was discovered, identified as iba1pos CD169neg. HO-1 is constitutively present in most macrophages in serosa and at AP, suggesting a M2 phenotype. LPS-treatment results in an up-regulation of HO-1pos/CD169neg cells in serosa and at AP. Anat Rec, 300:1114–1122, 2017.

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Key words: macrophages; intestine; HO-1; iba1; LPS; immunohistochemistry

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Resident macrophages have several functions; they play a role in development, tissue homeostasis and repair, in addition to immune responses to pathogens (Phillips and Powley, 2012; Wynn et al., 2013).

Macrophages in the muscularis externa of the small intestine are evenly distributed and have a close spatial relationship to interstitial cells of Cajal (ICC) (Thuneberg, 1982; Mikkelsen et al., 1985) suggesting potential regulation of motility. Indeed, others have recently confirmed that macrophages may play a role in pathological conditions associated with motility disturbances; for example, inflammation, diabetic gastroparesis, and post-operative ileus (Zhao et al., 2008; Choi et al., 2010; Neshatian et al., 2015; Bauer, 2008). Particularly the macrophages at Auerbach’s plexus (AP) have attracted attention. It has been suggested that they take part in a microbiota driven regulation of gastrointestinal motility (Muller et al., 2014), and in enteric neural regulation of macrophage function (Matteoli et al., 2014;Verheijden et al., 2015; Gabanyi et al., 2016).

It is therefore important to characterize the macrophage population in muscularis externa further with newer macrophage antibodies that may identify physiologically important subgroups.

In rodents, macrophages in muscularis externa of the small intestine are distributed in three layers; in the serosa, associated with AP and with the deep muscular plexus (DMP) (Mikkelsen et al.,1985; Mikkelsen, 1995; 2010). Osteopetrotic (op/op) mice lack the colony-stimulating factor-1 (M-CSF-1) (Yoshida et al., 1990) and subgroups of macrophages are absent (Wiktor-Jedrzejczak et al., 1990). We have shown that macrophages in all three layers of the small intestine muscularis are absent in op/op mice (Mikkelsen and Thuneberg, 1999; Mikkelsen et al., 2008), indicating that this macrophage population is M-CSF-1-dependent.

Ionized calcium-binding adaptor molecule 1 (iba1) was first demonstrated in microglia (resident macrophages in the brain) (Imai et al., 1996) and has been shown to stain cells in both normal and pathological lesions in rodents (Itou et al., 1998; 2001). Outside the CNS, iba1/ AIF-1 has been shown to stain most macrophage subpopulations (except alveolar macrophages) and has therefore been suggested to be a “pan-macrophage marker” (Kohler, 2007; DeFalco et al., 2015).

To further characterize the activation state of the macrophages during normal and inflammatory conditions we used an antibody against heme oxygenase-1 (HO-1). In preliminary studies of healthy rats and mice (Phillips and Powley, 2012; Wynn et al., 2013) using the companies’ recommendations. For rabbit

MATERIALS AND METHODS

Animals

Eight to nine week-old female C57Bl/6 (B6) mice (Taconic) were used. One group of mice (N = 10) was injected intraperitoneally with 20 μg/g LPS (Escherichia coli) (Sigma, Serotype 055:B5, L-2880) dissolved in sterile saline. Another group of animals (N = 8) was sham-injected with sterile saline. The animals were fed with standard laboratory chow, had free access to water and food, and were killed by cervical dislocation 6 hr after injection. The mice were housed in a high quality barrier with high hygienic standards and housed and experimentally manipulated in accordance with current national regulations issued by The Danish Council on Animal Care.

Antibodies

The antibodies used were rat anti-CD169 (AbD Serotec, MCA-884; 1:200), rabbit anti-iba1 (Waco, 019-19741; 1:200) and rabbit anti-HO-1 (Abcam, ab13243; 1:250). The secondary antibodies were biotin conjugated goat anti-rat (Amersham, RPN 1005; 1:1500), followed by Vectastain ABC reagent Vector (PK-7100) and DAB (DakoCytomation), using the companies’ recommendations. For rabbit
antibodies, EnVision (DakoCytomation) was used. For double-staining RH RedX conjugated goat anti-rat (Jackson, 112-295-102) and Dylight 488 conjugated goat anti-rabbit (Jackson, 111-485-144) were used. Specimens incubated with rat IgG2a (AbD Serotec), irrelevant rabbit antibodies or no primary antibodies served as controls.

Tissue Preparation and Immunohistochemistry

Whole mounts were prepared from the oral jejunum starting about 3 1/2 cm after the pyloric sphincter. We have previously shown that the first part of jejunum seems to have the smallest inter-animal variance in cell densities (Mikkelsen et al., 2011). The whole mounts were prepared by peeling off the whole muscularis externa from jejunum, which subsequently was cut open along the mesentery. For details see (Mikkelsen et al., 2008; 2011). The isolated muscle coat was placed in TBS with Nifedipine (1 μmol L⁻¹) for 10 min to ensure relaxation and was then stretched and pinned onto a Sylgard plate. After fixation with 4% paraformaldehyde for 3 hr, the muscle coat was kept in TBS at 4°C until immunostaining the following day, and was divided into about 1 1/2 cm whole mount pieces. To obtain the smallest inter-animal variance in cell densities (Mikkelsen et al., 2011), we used the first 3 whole mounts for the stereological analyses; the following whole mounts were used for double staining and for negative controls, respectively. Some of the whole mounts for stereology analyses were of substandard quality (uneven staining, damage to serosa), and were therefore omitted from the stereological quantification. The discarded whole mounts were 7 from the iba1 groups, 4 from the HO-1 groups, and 8 from the CD169 groups.

All washing solutions contained 0.5% triton-X 100. The tissue was quenched in 1% H2O2 for 15 min. Dako Real Antibody Diluent (S2022) was used for preincubation (1/2 hr) and for diluting all primary and secondary antibodies. Incubations were carried out at 4°C, overnight for primary antibodies, 2 hr for Envision and biotin-conjugated antibodies, and 1 hr for ABC-complex at room temperature. The chromogene was 0.25% diaminobenzidine in 0.035% H2O2. Double-staining with iba1 and/or HO1. Cells were counted in both combinations of double-stained whole mounts.

The counting of cells was performed directly at the microscope going from serosa to AP. Cells counted had densities were quantified on sham- and LPS-treated specimens (Fig. 3). Only HO-1 cell density was altered by LPS-treatment. In sham-treated mice, the density of HO-1pos cells was comparable to the density of cell populations at a final magnification of ×1022 using an ×20 UPanApo oil immersion objective (NA 0.8) to which the ×2 objective used for delineation was paracentred.

In the CD169 whole mounts, the mean number of counted cells was 67 in both treatment groups. The cells were counted in 123 fields of view (range: 68–166) in the saline group and in 109 (range: 44–138) in the LPS-treated group. In the iba1 whole mounts, the mean numbers of counted cells were 60 and 61 in the saline- and LPS-treated group, respectively. This number of cells were counted in 75 fields of view (range: 32–123) and 70 (range: 48–98), respectively. In the HO-1 whole mounts, the mean numbers of counted cells were 35 and 51 in the saline- and LPS-treated groups and the numbers of fields of view were 62 (range: 29–132) and 60 (range: 14–110).

Statistics

Group comparisons were carried out with the unpaired t-test assuming unequal variance. When comparing the densities of the different cell populations within groups (i.e., within the sham group and the LPS group, respectively) we first used an ANOVA test to verify that there were indeed significant differences between the cell populations in both the sham-group and the LPS group. Subsequently, an unpaired t-test assuming unequal variances was applied to compare one cell population against another. We chose the unpaired test instead of the paired test since not all animals were represented by all antibody stains. A P-value in the two-tailed test below 0.05 was considered to be a statistical significant difference between groups.

A semi-quantitative assessment of fluorescent double-stains was performed to clarify if CD169 co-localizes with iba1 and/or HO1. Cells were counted in both combinations of double-stained whole mounts.

The counting of cells was performed directly at the microscope going from serosa to AP. Cells counted had processes, stained for iba1 or HO-1 respectively, and showed double-staining with CD169pos cells, no CD169 staining, or were negative when CD169 stained cells were present in the field of view.

Cells at the deep muscular layer were counted separately, because of the modest number of cells.

RESULTS

In sham-treated mice, CD169pos macrophages were present in muscularis externa at three positions. In the serosa (SS), the cells were oblong or ramified with slender processes (Figs. 1A and 2A,G). At the level of AP, they were situated between the longitudinal and circular muscle layers, and were stellate-shaped with thin slender, ramified processes (Figs. 1B and 2B,H). At the level of the DMP, between the inner and outer circular muscle layers, CD169pos cells were solitary, oblong, rare, and with very few processes (Fig. 1C).

Cell densities were quantified on sham- and LPS-treated specimens (Fig. 3). Only HO-1 cell density was altered by LPS-treatment. In sham-treated mice, the density of HO-1pos cells was comparable to the density of...
Fig. 1. Muscularis externa whole mounts from the orad part of jejunum. Immunostaining of macrophages with antibodies towards CD169 (A–F), iba1 (G–L), and HO-1 (M–R). Sham-treated mice (A–C, G–I, and M–O). Mice treated with LPS injection (D–F, J–L, and P–R). Cells in serosa (A, D, G, J, M, P), cells at AP (B, E, H, K, N, Q), cells at DMP (C, F, I, L, O, R). Bar: 50 μm.
Fig. 2. Muscularis externa whole mounts from the orad part of jejunum. The confocal micrographs were taken through the thickness of the jejunal muscularis. The images are single confocal images. Double-staining was performed with antibodies toward CD169 (red) and iba1 (green) (A–F), and CD169 (red) and HO-1 (green) (G–L). A, D, G, and J are from the serosa (SS). B, E, H, and K are from the level of AP. C, F, I, and L are from the level of the DMP. Very few CD169pos cells were present in this layer. A–C and G–I are from sham-treated mice. D–F and J–L are from LPS-treated mice. H-shaped iba1pos cells can be observed in F. Bar: 50 μm.
CD169<sup>pos</sup> cells, whereas the density of HO-1<sup>pos</sup> cells in LPS-treated animals increased and was comparable to the density of Iba1<sup>pos</sup> cells (Fig. 3). Moreover, we found in the within-group comparisons, that in the sham group the density of HO-1<sup>pos</sup> cells was statistically significantly different from the density of Iba1<sup>pos</sup> cells, but did not differ significantly from the density of the CD169<sup>pos</sup> cells. Conversely, in the LPS-group the density of HO-1<sup>pos</sup> cells was statistically significantly different from the density of CD169<sup>pos</sup> cells, but was comparable to the density of Iba1<sup>pos</sup> cells (P-values given in Table 1).

To try to evaluate in which layer the cells differed, we used doubled-stained preparations. In serosa, Iba1<sup>pos</sup> cells co-stained with CD-169 (Fig. 2A); at AP CD169<sup>pos</sup> cells were also Iba1<sup>pos</sup>, but there were also Iba1<sup>pos</sup>/CD169<sup>neg</sup> cells (Fig. 2B and Table 2) with the same morphology as both Iba1<sup>pos</sup> and CD169<sup>pos</sup> cells (Fig. 1B,H). At the DMP, Iba1<sup>pos</sup> cells were oblong with small cell processes (Fig. 1I), but they were seldom positive for CD169 (Fig. 2C and Table 2). Hence, Iba1 staining showed a population of macrophages at DMP that were seldom recognized with CD169 staining.

### TABLE 1. P-values for all statistical tests performed in this study

| Sham group versus LPS group using unpaired t-test | P-value |
|--------------------------------------------------|---------|
| HO-1-positive cells                              | 0.0041  |
| CD169-positive cells                             | Ns      |
| Iba1-positive cells                              | Ns      |

| Within group comparisons using ANOVA | P-value |
|-------------------------------------|---------|
| Sham group                          | 0.00078 |
| LPS group                           |         |

| Within group comparisons using unpaired t-test | P-value for sham group | P-value for LPS group |
|------------------------------------------------|------------------------|------------------------|
| HO-1-positive cells vs. CD169-positive cells  | 0.31 (N = 8/N = 5)     | 0.0074 (N = 6/N = 5)   |
| HO-1-positive cells vs. Iba1-positive cells  | 0.0096 (N = 8/N = 6)   | 0.801 (N = 6/N = 5)    |
| CD169-positive cells vs. Iba1-positive cells | 0.0029 (N = 5/N = 6)   | 0.039 (N = 5/N = 5)    |

The uppermost part compares the LPS- and sham-treated animals with regard to the three different cell populations. The lower part compares the cell densities of the individual cell populations within the group using an unpaired t-test.
To verify that these cells belong to the macrophage population, we used frozen sections of muscularis externa from control and op/op mice: the latter have previously been shown to lack macrophages (F4/80<sup>pos</sup> and MHCII<sup>pos</sup> cells) (Mikkelsen and Thuneberg, 1999; Mikkelsen et al., 2008). In control mice, iba1<sup>pos</sup> cells were observed in serosa, at AP and DMP (Fig. 4A), but were absent in op/op mice (Fig. 4B).

In serosa and at AP, HO-1<sup>pos</sup> cells appeared to have the same morphology as iba1<sup>pos</sup> cells (Fig. 1M,N) and most CD169<sup>pos</sup> cells harbored HO-1 (Fig. 2G,H). At DMP, HO-1<sup>pos</sup> oblong cells were rare (Fig. 1O) and about a third co-stained with CD169 (Fig. 2I and Table 2).

LPS-treatment resulted in HO-1<sup>pos</sup>/CD169<sup>pos</sup>, HO-1<sup>pos</sup>/CD169<sup>neg</sup>, and HO-1<sup>neg</sup>/CD169<sup>pos</sup> cells in serosa and at AP (Fig. 2J,K). In LPS-treated animals an up-regulation of HO-1 staining took place in cells in serosa and AP resulting in an increase in HO-1<sup>pos</sup>/CD169<sup>neg</sup> cells. No change was apparent at the DMP.

**DISCUSSION**

In sham-treated animals iba1<sup>pos</sup> cells had a significantly higher density than CD169<sup>pos</sup> and HO-1<sup>pos</sup> cells. The densities of iba1<sup>pos</sup> and CD169<sup>pos</sup> cells were unaffected by LPS-treatment. At DMP, neither iba1<sup>pos</sup> nor HO-1<sup>pos</sup> cells showed any significant co-staining with CD169, in either sham- or in LPS-treated animals. In addition, both HO-1<sup>pos</sup> and CD169<sup>pos</sup> cells were rare at DMP.

Traditionally, macrophages in mouse small intestine muscularis externa have immunophenotypically been considered to consist of an almost homogenous population (Mikkelsen et al., 1988; 2004; 2011), in general, F4/80 is considered to be a pan macrophage marker, although we previously have found that the density of F4/80<sup>pos</sup> cells is lower than the density of CD169<sup>pos</sup> and MHCII<sup>pos</sup> cells in C57Bl/6 mice (Mikkelsen et al., 2008). This indicates the need to clarify the existence of subgroups of macrophages that may have different roles in physiology and/or pathophysiology. iba1 has mainly been used to stain microglia (resident macrophages in the brain) (Imai et al., 1996). Outside the CNS AIF1 (allograft inflammatory factor1), which is identical to iba1 with regard to gene and protein, stains most macrophage sub-populations (except alveolar macrophages), and is expressed by all ordinary macrophages (Kohler, 2007). In small and large intestines, AIF stains macrophages in the lamina propria and between the muscle layers. In lymphoid and hemoipoietic tissues, iba1/AIF1 stains more macrophages than the macrophage marker F4/80, for example, marginal zone macrophages in the spleen. In contrast to F4/80, it does not stain monocytes and has been suggested to be a “pan-macrophage marker” (Kohler, 2007; DeFalco et al., 2015).

To determine if the iba1<sup>pos</sup> cells in muscularis externa of the small intestine are indeed macrophages and not other ramified cells (for example ICC or fibroblasts), we used osteopetrotic (op/op) mice. They lack M-CSF-1 and therefore lack subgroups of macrophages, especially those located in the tunica muscularis of the small intestine (Mikkelsen and Thuneberg, 1999). In ileal frozen sections from op/op mice, no iba1-immunoreactivity was found in muscularis externa. This is in accord with the observation that brain iba1<sup>pos</sup> cells are M-CSF-1-dependent (Imai and Kohsaka, 2002), and microglia are reduced in op/op mice (Wegiel et al., 1998; Kondo and Duncan, 2009). iba1 has been reported to have actin-crosslinking activities and to be functionally involved in certain aspects of motility-associated rearrangements of the actin cytoskeleton, such as in membrane ruffling, and in the building of phagocytic cups.

Little is known about the macrophage/microglia-specific components that regulate the reorganization of actin filaments. However, in mouse microglia, LPS-exposure resulted in swelling of the cell body, a thickening of the proximal processes, and a reduction in number of distal ramifications (Kloss et al., 2001). In a pilot study on sham- and LPS-treated mice, we did not find any indications of changes in the morphology of iba1<sup>pos</sup>...
cells (number of ramifications and thickening of the processes) with the same dose of LPS.

HO-1 is an inducible enzyme (Wang and Chau, 2010). It has been reported that HO-1 may be protective towards inducible nitric oxide synthase (Ashino et al., 2008). The presence of HO-1 in murine small intestine muscularis macrophages in control material has not been demonstrated previously (Miller et al., 1998; Moore et al., 2003). However, in this study we observed that HO-1 is expressed in CD169\(^{pos}\) cells in sham-treated mice. This difference could be due to differences in antibodies and fixation techniques.

When we stained frozen sections from germ-free mice with HO-1 antibodies, the immunoreactivity was very low in mouse muscularis externa, and was only detectable in ganglion cells in AP and to a very limited degree in cells in serosa and at DMP (unpublished observations). We have also previously observed in embryonic and germ-free mice, without any microbiota, that the muscularis macrophages are MHCII\(^{neg}\), in contrast to conventional adult mice, which are constitutively MHCII\(^{pos}\) (Mikkelsen et al., 2004). This up-regulation might be due to normal microbiota. Hence, the HO-1 positivity of CD169\(^{pos}\) macrophages may be induced by normal gut microbiota in sham-treated mice.

HO-1 is associated with alternative macrophage activation (M2) and it has been shown that muscularis macrophages express wound healing and tissue protective genes (M2), whereas lamina propria macrophages express pro-inflammatory genes (M1) (Gabanyi et al., 2016).

Also intragastric inoculation with nonpathogenic or noninvasive bacteria strains resulted in a further local up-regulation in M2 genes in muscularis externa macrophages, but changes in gene expressions were few in lamina propria macrophages (Gabanyi et al., 2016).

In this study, after sham-treatment, confocal microscopy and conventional fluorescence microscopy demonstrated that many iba1\(^{pos}\) and HO-1\(^{pos}\) cells were also positive for CD169 in serosa and at AP, whereas at DMP CD169\(^{pos}\) cells seemed to be rare. A few oblong CD169\(^{neg}/\)HO-1\(^{pos}\) cells were present and both oblong and H-shaped CD169\(^{neg}/\)iba1\(^{pos}\) cells were observed in this layer. After LPS-treatment more CD169\(^{neg}/\)HO-1\(^{pos}\) cells were present in serosa and at AP, which suggests an HO-1 up-regulation in the iba1\(^{pos}/\)CD169\(^{neg}\) cell population. Furthermore, at DMP, HO-1\(^{pos}/\)CD169\(^{neg}\) cells as well as iba1\(^{neg}/\)CD169\(^{pos}\) cells were observed, but the proportion of iba1\(^{pos}/\)CD169\(^{neg}\) and HO-1\(^{pos}/\)CD169\(^{neg}\) cells was unaltered compared to sham-treated mice. We have been unsuccessful interpreting specimens that are double-stained with two antibodies derived from the same species (rabbits); therefore, we did not attempt double-staining with iba1 and HO-1.

In the CNS of CX3CR1-GFP mice, brain cells express the microglial marker iba1 and overlap precisely with the GFP\(^{pos}\) population (Cardona et al., 2006). Since the ligand CXCL1 is localized on neurons, several hypotheses on interactions between microglia and nerves have been proposed (see review; Limitola and Ransohoff, 2014).

Intestinal macrophages, in both lamina propria and in muscularis externa, are characterized by very high levels of the chemokine receptor CX3CR1 (Zigon and Jung, 2013). In a recent study, cross sections (colonic muscularis externa) from CX3CR1-GFP\(^{pos/neg}\) mice, revealed a strong fluorescence in cells in the muscularis externa at AP (Muller et al., 2014). It is not clear if CX3CR1 is also present on macrophages in the serosa.

In diabetic gastroparesis, the close spatial contact between macrophages and ICC and up-regulation of HO-1 in M2 macrophages, seems to protect against the loss of ICC and nNOS in enteric nerves and to protect against gastroparesis (Choi et al., 2010).

With regard to function, it has been suggested recently that muscularis macrophages communicate with enteric nerves, and may be involved in the regulation of motility (Muller et al., 2014), although no direct measurements of peristalsis were carried out. Hence, a direct role in the genesis of this motor pattern has yet to be confirmed.

In conclusion, different macrophage immunophenotypes seem to exist in the muscularis externa, specifically iba1\(^{pos}/\)CD169\(^{neg}\) and iba1\(^{neg}/\)CD169\(^{pos}\) cells. At the DMP macrophages, which are iba1\(^{pos}\) seem rarely to coexist with CD169.

Importantly, many CD169\(^{pos}\) macrophages in sham-treated mice express HO-1, suggesting a M2 phenotype. LPS administration up-regulates HO-1 in the macrophages at AP and in serosa.

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**LITERATURE CITED**

Alberti E, Mikkelsen HB, Larsen JO, Jimenez M. 2005. Motility patterns and distribution of interstitial cells of Cajal and nitrergic neurons in the proximal, mid- and distal-colon of the rat. Neurogastroenterol Motil 17:133–147.

Ashino T, Yamanaka R, Yamamoto M, Shimokawa H, Sekikawa K, Iwakura Y, Shioda S, Numazawa S, Yoshida T. 2008. Negative feedback regulation of lipopolysaccharide-induced inducible nitric oxide synthase gene expression by heme oxygenase-1 induction in macrophages. Mol Immunol 45:2106–2115.
Bauer AJ. 2008. Mentation on the immunological modulation of gastrointestinal motility. Neurogastroenterol Motil 20 Suppl 1: 81–90.

Cardona AE, Piovo EP, Sasse ME, Kostenko V, Cardona SM, Dijkstra IM, Huang D, Kidd G, Dombrowski S, Dutta R, et al. 2006. Control of microbial neurotoxicity by the fractalkine receptor. Nat Neurosci 9:197–204.

Choi KM, Kasparov PC, Dutta N, Stoltz GJ, Ordog T, Shea DT, Bauer AJ, Linden DR, Szurszewski JH, Gibbons SJ, et al. 2010. CD206-positive M2 macrophages that express heme oxygenase-1 protect against diabetic gastroparesis in mice. Gastroenterology 138:2399–2409, 2409.

DeWorms BY, Van Nassauw L, De Man JG, de Jonge F, Bredenoord AJ, Seerden TC, Herman AG, Timmermans JP, Pelckmans PA. 2005. Role of oxidative stress in the pathogenesis of septic ileus in mice. Neurogastroenterol Motil 17:251–261.

DeFalco T, Potter SJ, Williams AV, Waller B, Kan MJ, Capel B. 2015. Macrophages Contribute to the Spematogonial Niche in the Adult Testis. Cell Rep 12:1107–1119.

Gabanyi I, Muller PA, Feighery L, Oliveira TY, Costa-Pinto FA, Murcida D. 2016. Neuro-immune Interactions Drive Tissue Programming in Intestinal Macrophages. Cell 164:378–391.

Imai Y, Ibata I, Ito D, Ohsawa K, Kohsaka S. 1996. A novel gene ilba1 in the major histocompatibility complex class III region encodes an EP molecule and protein expressed in a monocytic lineage. Biochem Biophys Res Commun 224:855–862.

Imai Y, Kohsaka S. 2002. Intracellular signaling in M-CSF-induced microglia activation: role of Ilba1. Glia 40:164–174.

Ito D, Imai Y, Ohsawa K, Nakajima K, Fukuchi Y, Kohsaka S. 1998. Microglia-specific localisation of a novel calcium binding protein, Ilba1. Brain Res Mol Brain Res 57:1–9.

Ito D, Tanaka K, Suzuki S, Dembo T, Fukuchi Y. 2001. Enhanced expression of Ilba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain. Stroke 32: 1208–1215.

Jersmann HP, Hii CS, Ferrante JV, Ferrante A. 2001. Bacterial lipopolysaccharide and tumor necrosis factor alpha synergistically increase expression of human endothelial adhesion molecules through activation of NF-kappaB and p38 mitogen-activated protein kinase signaling pathways. Infect Immun 69: 1273–1279.

Klaas CU, Bobratschek M, Kreutzberg GW, Raivich G. 2001. Effect of lipopolysaccharide on the morphology and integrin immunoreactivity of ramified microglia in the mouse brain and in cell culture. Exp Neurol 168:32–46.

Kohler C. 2007. Allograft inflammatory factor-1/Ionized calcium-binding adapter molecule 1 mouse. Proc Natl Acad Sci USA 87:4828–4832.

Matteoli G, Gomez-Pinilla PJ, Nemethova A, Di GM, Caiotto C, van Bree SH, Michel K, Carell K, Schemann M, Boesmans W, et al. 2014. A distinct vagal anti-inflammatory pathway modulates intestinal muscularis resident macrophages independent of the spleen. Gut 63:938–948.

Mikkelsen HB. 1995. Macrophages in the external muscle layers of mammalian intestines. Histol Histopathol 10:719–736.

Mikkelsen HB. 1996. Interstitial cells of Cajal, macrophages and mast cells in the gut musculature: morphology, distribution, spatial and possible functional interactions. J Cell Mol Med 14:818–832.

Mikkelsen HB. 2010. Interstitial cells of Cajal, macrophages and mast cells in the gut musculature: morphology, distribution, spatial and possible functional interactions. J Cell Mol Med 14:818–832.

Mikkelsen HB, Thuneberg L. 1999. Macrophage-like cells in the muscularis externa of mouse small intestine. Anat Rec 213:77–86.

Mikkelsen HB, Mirsky R, Jessen KR, Thuneberg L. 1988. Macrophage-like cells in muscularis externa of mouse small intestine: Immunohistochemical localization of F4/80, M1/70, and 1a-antigen. Cell Tissue Res 252:301–306.

Mikkelsen HB, Garbsch C, Tranum-Jensen J, Thuneberg L. 2004. Macrophages in the small intestinal muscularis externa of embryos, newborn and adult germ-free mice. J Mol Histol 35:377–387.

Mikkelsen HB, Larsen JO, Hadberg H. 2008. The macrophage system in the intestinal muscularis externa during inflammation: an immunohistochemical and quantitative study of osteopetrotic mice. Histochem Cell Biol 130:363–373.

Mikkelsen HB, Larsen JO, Froh P, Nguyen TH. 2011. Quantitative Assessment of Macrophages in the Muscularis Externa of Mouse Intestines. Anat Rec (Hoboken) 294:1557–1565.

Miller SM, Farrugia G, Schmalz PF, Ermilov LG, Maines MD, Szurszewski JH. 1998. Heme oxygenase-2 is present in intestinal nerve of the mouse small intestine. Gastroenterology 114:239–244.

Moore BA, Otterbein LE, Turler A, Choi AM, Bauer AJ. 2003. Inhaled carbon monoxide suppresses the development of postoperative ileus in the murine small intestine. Gastroenterology 124:377–381.

Morse D, Choi AM. 2005. Heme oxygenase-1: from bench to bedside. Am J Respir Crit Care Med 172:660–670.

Muller PA, Kosco B, Rajani GM, Stevanovic K, Berres ML, Hashimoto D, Mortha A, Leboeuf M, Li XM, Murcida D, et al. 2014. Crosstalk between muscularis macrophages and enteric neurons regulates gastrointestinal motility. Cell 158:300–313.

Neshatian L, Gibbons SJ, Farrugia G. 2015. Macrophages in diabetic gastroparesis—the missing link? Neurogastroenterol Motil 7:1–18.

Nissan A, Zhang JM, Lin Z, Haskel Y, Freund HR, Hanani M. 1997. The contribution of inflammatory mediators and nitric oxide to lipopolysaccharide-induced intussusception in mice. J Surg Res 69:205–207.

Paine A, Eliz-Vesper B, Blaszyck R, Immenschuh S. 2010. Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. Biochem Pharmacol 80:1895–1903.

Phillips RJ, Powley TL. 2012. Macrophages associated with the intrinsic and extrinsic autonomic innervation of the rat gastrointestinal tract. Auton Neurosci 169:12–27.

Ruetten H, Thiemermann C, Perretti M. 1999. Upregulation of ICAM-1 expression on J774.2 macrophages by endotoxin involves activation of NF-kappaB but not protein tyrosine kinase: Comparison to induction of iNOS. Mediators Inflamm 8:77–84.

Ryter SW, Choi AM. 2016. Targeting heme oxygenase-1 and carbon monoxide for therapeutic modulation of inflammation. Tranal Res 167:7–18.

Thuneberg L. 1982. Interstitial cells of Cajal: Intestinal pacemaker cells? Adv Anat Embryol Cell Biol 71:1–130.

Verheijden S, De SS, Bockxstaens GE. 2015. Neuron-macrophage crosstalk in the intestine: a “microglia” perspective. Front Cell Neurosci 9:403.

Wang CY, Chau LY. 2010. Heme oxygenase-1 in cardiovascular diseases: molecular mechanisms and clinical perspectives. Chang Gung Med J 33:13–24.

Wang XM, Kim HP, Nakahira K, Ryter SW, Choi AM. 2009. The heme oxygenase-1/carbon monoxide pathway suppresses TLR4 signaling by regulating the interaction of TLR4 with caveolin-1. J Immunol 182:3809–3818.

Wegiel J, Wacwarcik HM, Dziewiatkowski J, Tarnawski M, Kozielski R, Trebner E, Wiktor-Jedrzejczak W. 1998. Reduced number and altered morphology of microglial cells in colony stimulating-factor 1 deficient osteopetrotic mice. Brain Res 803:135–139.

Wiktor-Jedrzejczak W, Bartocci A, Ferrante AW, Ansari AA, Sell KW, Pollard JW, Stanley ER. 1990. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic mouse. Proc Natl Acad Sci USA 87:4828–4832.
Wynn TA, Chawla A, Pollard JW. 2013. Macrophage biology in development, homeostasis and disease. Nature 496:445–455.

Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Schultz LD. 1990. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 345:442–444.

Zhao A, Urban JF, Jr., Anthony RM, Sun R, Stiltz J, Van RN, Wynn TA, Gause WC, Shea-Donohue T. 2008. Th2 cytokine-induced alterations in intestinal smooth muscle function depend on alternatively activated macrophages. Gastroenterology 135:217–225.

Zigmond E, Jung S. 2013. Intestinal macrophages: Well educated exceptions from the rule. Trends Immunol 34:162–168.