Self-maintaining Gut Macrophages Are Essential for Intestinal Homeostasis

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Summary

Macrophages are highly heterogeneous tissue-resident immune cells that perform a variety of tissue-supportive functions. The current paradigm dictates that intestinal macrophages are continuously replaced by incoming monocytes that acquire a pro-inflammatory or tissue-protective signature. Here, we identify a self-maintaining population of macrophages that arise from both embryonic precursors and adult bone marrow-derived monocytes and persists throughout adulthood. Gene expression and imaging studies of self-maintaining macrophages revealed distinct transcriptional profiles that reflect their unique localization, i.e. closely positioned to blood vessels, submucosal and myenteric plexus, Paneth cells and Peyer's patches. Depletion of self-maintaining macrophages resulted in morphological abnormalities in the submucosal vasculature and loss of enteric neurons, leading to vascular leakage, impaired secretion and reduced intestinal motility. These results provide critical insights in intestinal macrophage heterogeneity with respect to longevity, ontogeny and function and demonstrate the strategic role of self-maintaining macrophages in gut homeostasis and intestinal physiology.

Introduction

Tissue-resident macrophages are highly specialized phagocytes that actively contribute to organ homeostasis. For this delicate task, macrophages perform a variety of tissue-specific functions conforming their microenvironment, including the efficient response towards metabolic changes, tissue damage and microbial insults, while carrying out supportive functions to surrounding cells and structures (Davies *et al.*, 2013; Varol, Mildner and Jung, 2015). Consequently, tissue-resident macrophages are extremely heterogeneous in terms of origin and function and possess a unique transcriptome that allows them to fulfil niche-specific functions (Hashimoto, Miller and Merad, 2011; Gautier *et al.*, 2012; Okabe and Medzhitov, 2014). One of the major challenges facing the field is to identify the different subtypes of tissue-resident macrophages and to understand how these cells are tailored to meet the functional demands of the tissue microenvironment in which they reside.

In recent years, our understanding of intestinal macrophage biology has drastically increased. Gut-resident macrophages (gMacs) represent a heterogeneous mix of $CX3CR1^{hi}$-expressing cells that are strategically positioned within the different layers of the intestine. Lamina propria (LP) gMacs actively contribute to host defense and barrier integrity coupled with high phagocytic activity, as well as the constitutive secretion of IL-10 that promotes the maintenance of $FoxP3^{+}$ T regulatory cells (Hadis *et al.*, 2011; Zigmond *et al.*, 2014). However, many other types of macrophages are present within the intestine, including $CD169^{+}$ gMacs positioned near the crypt base of the villi close to lymphoid tissue and gMacs associated with the submucosal plexus, part of the enteric nervous system (ENS) (Asano *et al.*, 2015; Gabanyi *et al.*, 2016). Recently, gMacs expressing Tim-4 and CD4 were characterized within commensal rich areas of the mucosa, although their function remains unclear (Shaw *et al.*, 2018). More distally from the lumen, substantial numbers of gMacs are found in the muscularis externa (ME) that closely interact with the ENS to support intestinal peristalsis (Muller *et al.*, 2014). Extrinsic signaling through the β2 adrenergic receptor allows ME gMacs to upregulate their tissue-protective genetic signature in response to microbial signals (Gabanyi *et al.*, 2016). These findings further indicate the strong heterogeneity of gMacs with distinct phenotypic and functional characteristics, integrated to the specific needs of the microenvironmental niche in which they reside. It is very likely that additional, yet to-be-defined subpopulations of gMacs exist with unique transcriptional profiles.

The current paradigm states that gMacs in the LP are continuously replaced by bone marrow-derived $Ly6C^{hi}$ monocytes. Although primitive progenitors seem to colonize the embryonic gut, these embryonic-derived gMacs quickly disappear around the time of weaning and are replaced by monocytes in a CCR2- dependent manner (Bain *et al.*, 2014). This is in stark contrast to other tissues including the heart and lungs, showing a rather intermixed

macrophage population that comprises of both embryonic progenitor and adult monocyte-derived macrophages, where the contribution of monocytes depends on the tissue context and increases with aging (Gentek, Molawi and Sieweke, 2014; Ginhoux and Guilliams, 2016). In this study, we challenged the current view that gMacs are continuously replaced by bone marrow monocytes after birth. By using a fate mapping approach, we revealed the presence of self-maintaining gMacs that during embryonic life colonize specific niches including vasculature, submucosal and myenteric plexus, Paneth cells and Peyer's patches (PP). During postnatal development, the respective niches are supplemented with monocyte-derived gMacs implying that in adulthood, they are populated by both embryonic and monocyte-derived gMacs. After diphtheria toxin-mediated depletion, self-maintaining gMacs are replaced by circulating monocytes that differentiate into resident CX3CR1+ gMacs. Of note, transcriptional profiling at the single cell level showed that self-maintaining gMacs possess a distinct transcriptional profile that reflects their unique localization. At the functional level, self-maintaining gMacs support vascular architecture and permeability in the LP and regulate neuronal function and intestinal motility in the LP and ME. These results provide critical insights on gMac heterogeneity and how they are functionally adapted to the niche in which they reside.

Results

Fate-Mapping Identifies Self-Maintaining Macrophages in the Intestinal Submucosa and Muscularis Externa

In order to identify distinct gMac populations with different half-lives, we injected 6-weeks old *Cx3cr1*^{CreERT2}*.Rosa26-LSL-YFP* with tamoxifen (TAM) and longitudinally evaluated YFP-labeling in CD64$^+$ gMacs, both in the ME and LP of the ileum (Fig. 1A and Fig. S1). One week post injection, the majority of CD64$^+$ gMacs were YFP$^+$ (ME: 80% ± 3%; LP: 77 ± 6%) (Fig. 1B). Over time, both ME and LP CX3CR1-YFP$^+$ CD64$^+$ gMacs were gradually replaced by YFP$^-$ gMacs, indicating replacement by YFP$^-$ monocytes. However, 20 weeks (ME: 28 ± 1%; LP: 8 ± 1%) and 35 weeks (ME: 22 ± 1%; LP: 7 ± 1%;) post injection, we still observed YFP$^+$ CD64$^+$ gMacs (Fig 1C,D). Using a FACS-based approach, we showed that YFP$^+$ cells are uniquely CD64$^+$ macrophages and no labeling was observed in Ly6C$^+$ monocytes or CD103$^+$ dendritic cells (Fig. 1E). Moreover, no YFP labeling was observed in CD115$^+$ CD117$^-$ CD11b$^+$ Ly6C^{hi} monocyte precursors in the bone marrow and blood at 1 week and 35 weeks post injection (Fig. 1F). Of note, BrdU pulses into 35-week old mice every other day for 9 days labeled 11 ± 1% and 16 ± 2% of YFP$^+$ cells in the LP and ME, respectively (Fig. 1G).

In a next step, we evaluated whether the identified self-maintaining gMac subpopulation colonizes specific niches using immunofluorescent stainings (IF). At 1 and 4 weeks post injection, F4/80$^+$ YFP$^+$ gMacs were detected in all layers of the ileum (Fig. 1H). However, at

35 weeks, the remaining YFP$^+$ gMacs were uniquely observed in the submucosal region of the LP and within the ME (Fig. 1H,I). Together, these data identify a self-maintaining gMacs subset that is maintained without influence of bone marrow monocytes and colonizes distinct niches, i.e. the submucosa and ME, within the GI tract.

Self-Maintaining gMacs Express a Specific Transcriptional Profile Distinct From Monocyte-Replaced gMacs

The distinct localization of monocyte-replaced and self-maintaining gMacs suggests that these two populations might be differentially imprinted by micro-environmental signals to meet with the specific requirements of the tissue they reside in. To investigate differences in molecular signature, we next isolated YFP$^+$ and YFP$^-$ gMacs 35 weeks after TAM injection and compared their transcriptome by RNA-sequencing (bulk RNA-seq). Principle component analysis (PCA) showed segregation of the 4 different gMacs according to their anatomical location (ME vs LP) and longevity (YFP$^+$ vs YFP$^-$) (Fig. 2A).

Lamina Propria gMacs

In the LP, 2422 genes were differentially expressed between YFP$^+$ and YFP$^-$ (≥ 2-fold; ≤ 0.05 FDR) (Fig. 2B). Among the most enriched genes in YFP$^-$ monocyte-replaced LP gMacs were *Ccr2* and *Ly6c2*, two characteristic genes of monocyte-derived macrophages (Fig. 2D). Gene ontology (GO) enrichment for biological process (BP) showed that this subset is involved in innate immune responses, regulation of defense response and phagocytosis (Table S1).

In contrast, YFP$^+$ self-maintaining LP gMacs were characterized by the enriched expression of transcripts such as *Nova1, Chrm2, Efr3b,* genes that are involved in development and tissue support processes, such as angiogenesis, epithelial cell differentiation and positive regulation of neuron differentiation (Fig. 2D, E). The strongest enrichment was seen for cell adhesion, suggesting that YFP$^+$ self-maintaining LP gMacs are a stable cell population that adheres/anchors to structures within the submucosal region. To identify potential surface markers of self-maintaining LP gMacs among the total LP gMacs pool, we loaded the bulk RNA-seq dataset in a GO software program to enrich for surface markers, yielding a total list of 49 genes (https://david.ncifcrf.gov/). We selected *Cd4, Timd4, Cd63* because of their reproducible expression throughout the different YFP$^+$ samples (Fig. S1B). We then evaluated the potential of CD4/CD63 as discriminating cell surface markers between LP gMacs that are self-maintaining or replaced by monocytes using FACS. By preselecting YFP$^+$ gMacs for CD45.2, CD11b, CD64 and a final gate for CD63 and CD4, we could see that the majority of this subset belonged to P2 (CD63$^+$/CD4$^+$, 79%), whereas a minor fraction belonged to P3 (CD63$^+$/CD4$^-$, 16%), P1 (CD63$^-$/CD4$^-$, 2%) and P4 (CD63$^-$/CD4$^+$, 3%) (Fig. 2F). In contrast, monocyte-replaced YFP$^-$ gMacs were mainly P1 (44%) but could also be detected in P2 (25%),

P3 (21%) and P4 (22%). We also confirmed increased expression of *Tim4d*, *Cd63* and *Cd4* in self-maintaining YFP$^+$ LP gMacs and *Chil3* and *Ccr2* in monocyte-replaced YFP$^-$ LP gMacs by qPCR (Fig. 2I).

Muscularis Externa gMacs

Although the difference between self-maintaining and monocyte-replaced gMacs was less pronounced in the ME (314 DE genes) (Fig. 2C), biological adhesion was also strongly enriched in YFP$^+$ self-maintaining ME gMacs, further supporting the idea that longevity of gMacs coincides with the induction of molecular pathways that enable macrophages to anchor to specific structures (Fig. 2G,H). In addition, YFP$^+$ self-maintaining ME gMacs showed further enrichment for cell-cell adhesion, cytoskeletal anchoring at plasma membrane and neuron development, amongst others (Fig. 2H, Table S2). The enrichment of these terms suggests that self-maintaining ME gMacs mainly associate with neuronal structures within the ME. Indeed, IF for typical enteric neuron (HuC/D) and nerve fiber (βIII-tubulin) markers showed that self-maintaining ME gMacs were only present in close association with myenteric neuronal ganglia, but not in between ganglia nor in the circular muscle (Fig. S2). Although we could validate some of the differentially expressed genes between self-maintaining and monocyte-replaced ME gMacs by qPCR, we could not identify specific surface markers that could discriminate between these two ME subsets (Fig. 2I).

Together, these data show that self-maintaining LP gMacs are transcriptionally distinct from monocyte-replaced gMacs and comprise a heterogeneous pool. In contrast, self-maintaining and monocyte-replaced gMacs residing in the ME have a relatively similar molecular phenotype with minor transcriptional differences.

Distinct Transcriptional Profiles Among Self-Maintaining gMacs Correspond to Colonization of Specific Micro-Anatomical Niches

To further investigate the heterogeneity among self-maintaining LP gMacs, we performed single-cell RNA-seq (scRNA-seq) on YFP$^+$ LP gMacs 35 weeks post TAM injection. Clustering analysis based on the first 8 principal components with a resolution of 0.4 yielded 4 distinct clusters within the self-maintaining LP gMac population (Fig. 3A,B). We next evaluated typical markers expressed by the largest cluster (cluster 0, containing 64% of the cells) and used a published RNA-seq database to evaluate expression of these genes in typical tissue macrophage subsets (Lavin *et al.*, 2014). Interestingly, 26% of the genes enriched in cluster 0 were mainly expressed by CNS microglia, which are considered to represent a very unique subset compared to other tissue macrophages (Fig 3C) (Prinz *et al.*, 2014). Indeed, manual curation of the scRNA-seq dataset showed that cluster 0 expressed many genes that have been reported to be specifically enriched in CNS microglia, including *Fcrls, C1qa, C1qc, Cst3,*

Grn, Mef2a, Gas6, Hexb and *Gpr34* (Gautier *et al.*, 2012). These findings suggest that self-maintaining LP gMacs in cluster 0 are imprinted by neural signals, similar to microglia. Indeed, as self-maintaining gMacs colonize the submucosa, a region within the intestine that is heavily innervated by both intrinsic (enteric) and extrinsic neurons of the autonomic nervous system, it seems plausible that cluster 0 represents a gMac subset interacting with enteric neurons/nerve fibers. To evaluate this, we performed IF for HuC/D and βIII-tubulin expression in whole mount preparations of reporter mice 35 weeks post TAM injection. Notably, the majority of YFP^+ LP gMacs were found in close proximity to enteric neurons and enteric nerve fibers of the submucosal plexus (Fig. 3D), however within the villi no YFP^+ LP gMacs were present (not shown). Moreover, 3-dimensional reconstruction of these gMacs showed physical interaction with neuronal structures in addition to a remarkable ramified morphology, similar to what is observed for microglia (Fig 3D, lower panel).

Next, we performed a similar analysis for cluster 1, 2 and 3. Genes with enriched expression within cluster 1 (examples include *Adamdec1, Dnase1l3, Apol7c*) were mostly expressed by small intestinal (SI) and colonic gMacs, but remarkably, also by splenic macrophages (Fig 3C). The top 5 enriched gene ontology annotations were angiogenesis, positive regulation of cell migration, positive regulation of T-cell proliferation, immune response and regulation of cytokine production (Table S3). Because of the enrichment in angiogenesis and the similarities between red pulp spleen macrophages, known to be in close contact to blood vessels, we next stained whole mount submucosal preparations of reporter mice (35 weeks post TAM injection) for vascular endothelial cadherin (VE-cadherin). We could indeed detect self-maintaining gMacs in close contact with VE-$cadherin^+$ submucosal blood vessels but not with blood vessels in the villi (Fig 3E). To confirm the spatial association of self-maintaining LP gMacs in cluster 0 and 1 with their specific niche, we used an in-situ hybridization approach (RNAscope) to directly examine the mRNA expression of *Fcrls* and *Adamdec1,* top marker genes of cluster 0 and 1 respectively (Fig. 3B). We could detect the presence of self-maintaining gMacs that simultaneously express *Fcrls* and *EYFP* in close contact with βIII-$Tubulin^+$ nerve fibers (Fig. 3F). In addition, probing for *Adamdec1* shows significant overlap with *EYFP*-positive cells in proximity to VE-$Cadherin^+$ blood vessels (Fig. 3G). These results indicate that the unique genetic signature of self-maintaining gMacs can be assigned to the specific niche in which they reside within the LP. Finally, comparing cluster 2 and 3 with other clusters showed strong enrichment for B-cell markers *Cd79a, Cd79b* and *Ms4a1* (cluster 2) and Paneth cell-specific markers *Itln1* and *Defa* genes (cluster 3) (Fig 3B). Pan-macrophage markers including *Cd68*, *Cx3cr1, Fcgr1*, *Adgre1* and *Cd63*, a typical marker for self-maintaining gMacs defined by bulk RNA-seq, were equally expressed throughout different clusters, except for cluster 2. (Fig S3A). This raises the possibility that this cluster could be generated by (a) doublet formation between self-maintaining gMacs and B-cells due to strong

anatomical association (Boisset *et al.*, 2018), or (b) by phagocytosis of B-cells by self-maintaining gMacs (Haimon *et al.*, 2018). To evaluate this, we stained sections of the small intestine of reporter mice 35 weeks post TAM injection for the B-cell marker B220. We indeed observed close apposition of self-maintaining gMacs with B cells in PP and could observe phagocytosis of TUNEL+ B-cells in PP (Fig 3H). Using a similar approach, we were able to demonstrate the close associated between self-maintaining gMacs and Paneth cells at the crypt base (Fig. 3I). To what extent these 2 smaller subsets of gMacs indeed express a unique transcriptome, as cluster 0 and 1, remains however to be further evaluated.

In conclusion, by combining a fate-mapping approach with bulk- and scRNA-seq, we were able to capture the heterogeneity of gMacs and connect the abundance of self-maintaining gMacs to the colonization of specific micro-anatomical niches within the intestinal submucosa in particular the ENS (neuronal-associated self-maintaining gMacs or NASM-gMacs), vasculature (blood vessel associated self-maintaining gMacs or BVASM-gMacs), PP (gut-associated lymphoid tissue associated self-maintaining gMacs or GALT-gMacs) and Paneth cells (Paneth cell associated self-maintaining gMacs or PASM-gMacs).

Embryonic Progenitors and Bone Marrow-Derived Monocytes Contribute to Distinct Micro-Anatomical Niches

To investigate the ontological origins of gMacs within different niches, we first injected pregnant *Cx3cr1*^{CreERT2}*.Rosa26-LSL-YFP* with TAM 8.5 days post conception (E8.5) to label embryonic progenitors. At this time point, embryonic yolk-sac derived progenitors solely give rise to CX3CR1^{+} embryonic macrophages. YFP-labeling of gMacs was checked at date of birth and in 6-week old animals (Fig. 4A). At date of birth, YFP^{+} cells were detected in all different layers of the intestine, similar to what has been published before (Fig. 4B) (Bain *et al.*, 2014). However, at 6 weeks of age, the remaining embryonic-derived macrophages in the LP were indeed only present in proximity to enteric neurons (NASM-gMacs), blood vessels (BVASM-gMacs), PP (GALT-gMacs) and Paneth cells (PASM-gMacs) (Fig. 4C). In addition, embryonic-derived gMacs in the ME could only be detected in close proximity to myenteric neurons (Fig. 4D). In various tissues including the gut, circulating monocytes contribute to the adult macrophage pool. To further examine the contribution of monocytes to the different gut niches during postnatal development, we performed adoptive transfers of *Cx3cr1*$^{+/GFP}$ bone marrow cells into *Cx3cr1*^{CreERT2}*.Rosa26-LSL-TdTomato* pups that had received tamoxifen injection in the first week of life, when gMacs are predominantly of embryonic origin (Bain *et al.*, 2014) (Fig. 4E). Nine weeks after transfer, engrafted GFP^{+} monocytes contributed to all intestinal compartments, predominantly colonizing the villi and submucosa (Fig. 4F). In contrast, TdTomato^{+} gMacs subset was the dominant population in the ME while a significant population was still present in the villi and not replaced by GFP^{+} monocytes (Fig. 4F). Further,

whole mount IF revealed that TdTomato^{+} gMacs were particularly concentrated in the submucosal and myenteric plexus niche and within PP (Fig. 4G). Together, these data show that self-maintaining gMacs in different micro-anatomical niches can arise from both primitive and postnatal haematopoiesis.

Bone Marrow-Derived Macrophages Repopulate the Niche after Depletion of Self-Maintaining gMacs

Having determined the distinct colonization of self-maintaining gMacs in the LP and ME, we next evaluated whether these populations are involved in supporting these micro-anatomical niches to maintain tissue homeostasis. To this end, we adapted our fate-mapping model to a depletion model by crossing *Cx3cr1*^{CreERT2} to *Rosa26-iDTR* mice. After injection of TAM (or oil as control) at 6 weeks of age, *Cx3cr1*^{CreERT2}.*Rosa26-iDTR* mice were aged to 35w and injected with diphtheria toxin (DTx) to specifically deplete the self-maintaining gMac pool and used for analysis 3 and 7 days post DTx injection (Fig 5A). Histological analysis of ileal cross sections confirmed the presence of DTx receptor (DTR)-positive gMacs residing in the submucosa and ME, which were completely depleted by Dtx administration (Fig 5B,C). In line, typical markers for self-maintaining LP (*Timd4, Cd63* and *Cd4*) and ME gMacs (*Pcdhb16* and *Igf2bp3*) were significantly downregulated at 3 and 7 days post DTx injection (Fig. 5D). Depletion of self-maintaining gMacs did not lead to overt inflammation in this timeframe, as measured by gene expression for *Tnf, Il1b, Ccl3 and Ccl7* and the inflammatory infiltration of monocytes, eosinophils or neutrophils by FACS (Fig. S4A,B). To investigate if depleted self-maintaining gMacs are replaced by circulating monocytes, we developed shielded chimeras reconstituted with bone marrow of *Cx3cr1*$^{+/GFP}$.*Ccr2*$^{+/RFP}$ double reporter mice. This approach resulted in 27 ± 4% Ly6C^{hi} blood monocyte chimerism after 6 weeks (Fig. 5E, Fig. S4C). In control conditions, we were unable to detect macrophages derived from donor bone marrow in close proximity to submucosal and myenteric neurons (Fig. 5F), confirming that there is no monocyte contribution to this niche at 35 weeks post-TAM injection. In contrast, depletion of self-maintaining gMacs resulted in the gradual repopulation of CCR2-CX3CR1 double-positive monocytes over the period of 14 days. This indicates that bone marrow-derived monocyte engraftment accounts for niche repopulation in our depletion model.

Self-Maintaining gMacs Support Enteric Neurons and Submucosal Vasculature

Considering that the majority of self-maintaining LP gMacs belong to NASM-gMacs (cluster 0) and BVASM-gMacs (cluster 1), we next explored the effect of self-maintaining gMac depletion on neuronal and blood vessel structures. Neuronal apoptosis was observed by IF for cleaved caspase-3 (CCasp3) and HuC/D, as demonstrated by a significant increase in the number of HuC/D^{+} submucosal and myenteric neurons expressing CCasp3 (Fig. 6A,B). Consistently, we

found a reduction in the density of HuC/D$^+$ enteric neurons within ganglia in both the submucosal and myenteric plexus over the course of 7 days post DTx treatment (Fig. 6B), indicating increased neuronal apoptosis after depletion of self-maintaining gMacs.

We next performed a similar approach for blood vessel morphology and abundance in self-maintaining gMac depleted mice. We observed a pronounced disruption of blood vessel morphology and reduced abundance at 3 and 7 days post DTx injection, which was most notable in the submucosal regions but eventually also affected blood vessels in the mucosal region (Fig. 6C,D). This was associated with notable leakage of microspheres at 3 days post DTx injection into the submucosa region that increased over the course of 7 days after treatment. Vascularization in the ME was not affected, supporting the concept that self-maintaining gMacs in this region are mainly involved in supporting enteric neurons (Fig. S5).

Together, these data show that NASM-gMacs and BVASM-gMacs are involved in maintaining tissue homeostasis by providing support to submucosal and myenteric enteric neurons and submucosal vasculature.

NASM-gMacs Control Neuron-Evoked Secretion, Neurogenic Contractility and Intestinal Transit

Having established the supportive functions of NASM-gMacs, we next evaluated their role in regulating physiological intestinal functions. As submucosal neurons are mainly involved in regulating intestinal secretion, we used Ussing chambers to measure anion secretion upon neuronal stimulation with veratridine, a voltage-gated sodium channel agonist. In self-maintaining gMac depleted mice, the increase in anion secretion upon veratridine treatment was significantly decreased compared to controls, which was blocked upon treatment with tetrodotoxin (TTX), a voltage-gated sodium channel antagonist (Fig. 7A). This shows that NASM-gMacs are essential to maintain neuron-evoked secretion through support of submucosal enteric neurons. Also in the myenteric plexus, we observed differences in neuronal activity in mice depleted of self-maintaining gMacs. Fluo-4 Ca^{2+} imaging of myenteric plexus preparations showed increased mean arbitrary fluorescence counts in myenteric neurons (Fig S6, Movie S1-2), suggesting increased baseline intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) or increased Fluo-4 uptake by myenteric neurons. In addition, the maximum $[Ca^{2+}]_i$ response upon electrical stimulation of interganglionic fiber tracts was significantly reduced, suggestive for alterations in the enteric neuronal network (Fig. 7B). Interestingly, although more myenteric neurons responded to stimulation with the nicotinic acetylcholine receptor agonist diphenylpiperazinium (DMPP) (Fig S6, Movie S3-4), the maximum $[Ca^{2+}]_i$ response upon DMPP stimulation was not changed after self-maintaining gMac depletion (Fig. 7C). To validate the changes in myenteric neuron responses, we next performed muscle strip organ bath experiments and assessed nerve-induced contractile activity. Muscle strips were

electrically stimulated (1–32 Hz, 0.3 ms during 10 s) to activate the release of neurotransmitter from enteric neurons triggering contractions mainly of cholinergic nature. Depletion of self-maintaining gMacs resulted in significant reduction in electrical stimulation-induced contractions at 16 Hz and 32 Hz 3 days post DTx injection (Fig. 7D,E) in self-maintaining gMac depleted muscle strips, while smooth muscle contractility, assessed by administration of KCl, was preserved (Fig. 7F).

We then examined the effect of long-term self-maintaining gMac depletion on intestinal function and homeostasis by a prolonged 7-day administration of DTx. Since niche repopulation primarily occurs through monocyte recruitment (Fig. 5E,F), we simultaneously administrated anti-CCR2 antibodies (MC-21) to TAM- and oil-treated animals. Self-maintaining gMac depleted mice had a significant reduced small intestinal transit and a prolonged total transit time compared to controls (Fig. 7G,H), while stomach emptying, albeit not statistically significant, was delayed (Fig 7I). Consistent with the observed decreased intestinal function, these mice exhibited a reduction in body weight compared to control animals (Fig. 7J).

The above results suggest that self-maintaining ME gMacs control intestinal motility through their support of enteric neurons. To further investigate the consequence of ENS degeneration, we performed *in vivo* MRI analysis to assess intestinal distension (Fig. 7K). Interestingly, we observed a significant increase in colonic distension compared to control animals (Fig 7L), an observation which is most likely due to the loss of enteric neurons. In conclusion, these data indicate the essential role of NASM-gMacs in the LP and myenteric plexus in supporting enteric neurons and, thereby, controlling essential intestinal functions including secretion, motility and homeostasis.

Discussion

Macrophages in the intestine are thought to be continuously replaced by incoming monocytes that mature into pro-inflammatory or tissue-protective macrophages depending on the microenvironment in which they reside. In this study, we show that the macrophage pool in the gastrointestinal tract is composed of distinct subsets with different half-lives, ontogeny and functions. The niche-specific colonization of self-maintaining gMacs coincides with highly specialized functions including support of enteric neurons and submucosal vasculature, B-cell phagocytosis in PP and contacts with Paneth cells. Our data further highlight the complexity of intra-tissue specialization of tissue-resident macrophages in the gut with a strategic role in gut homeostasis and physiology, including secretion and motility.

In recent years, multiple studies have demonstrated that the majority of tissue macrophages arise from embryonic haematopoiesis. Depending on the tissue of residence, they can maintain themselves throughout adulthood, as in the brain (Ginhoux *et al.*, 2010), or will

require constant replenishment from blood monocytes, as in dermis and intestine (Tamoutounour *et al.*, 2013; Bain *et al.*, 2014). Although embryonic macrophages seeded in the gut are rapidly replaced in a microbiota-dependent manner by Ly6C^{hi} monocytes, our findings demonstrate that at least a subpopulation of embryonic progenitors persists within specialized gut niches. During neonatal development, the contribution of bone marrow-derived macrophages to the LP and ME can be likely attributed to niche availability that concurs with organ growth (Guilliams and Scott, 2017). In this respect, it remains to be identified to what extent the underlying ontological origin of gMacs influences their function.

Of note, the distinct micro-anatomical colonization of monocyte-replaced and self-maintaining gMacs coincides with pronounced transcriptional differences which were mainly evident in the LP. Transcriptional heterogeneity among tissue macrophages results from their adaptation to specific tissue environments and reflects a functional polarization governed by local tissue-derived signals (Gautier *et al.*, 2012; Okabe and Medzhitov, 2014). Our data could indicate that ME gMacs are exposed to relatively similar environmental cues independent of their longevity. This suggests that the environmental niche controls gMac signature rather than intrinsic features (Guilliams and Scott, 2017). In contrast, the different regional colonization of monocyte-replaced and self-maintaining gMacs in the LP entails stronger transcriptional divergence, as also supported by pathway enrichment analysis and scRNA-seq. The latter led to the identification of 4 subpopulations with a distinct anatomical localization and functional specialization in the intestinal LP.

Neuronal Niche – NASM gMacs

Within the ME, it is repeatedly shown that macrophages reside in close proximity to enteric neurons and neuronal processes and reciprocally interact with enteric neurons (Mikkelsen, 1995; Cailotto *et al.*, 2012; Muller *et al.*, 2014; Gabanyi *et al.*, 2016). Of note, we collected evidence supporting a similar neuro-supportive macrophage network in the intestinal LP. Although the vast majority of LP gMacs are continuously replenished by highly plastic blood monocytes that gradually differentiate into tolerogenic IL10^{+} macrophages, a minority is self-maintaining and resides in an anatomical niche distant from luminal signals to support submucosal neurons. In this study, we introduced an elegant depletion model where we could evaluate the direct role of this subset in supporting enteric neurons. The strength of this model relies on the specific depletion of a minor population while maintaining phagocytic capacity of other gut macrophages to remove dying NASM gMacs. This approach minimizes the hazard of inducing an immune response after DTx administration due to build-up of cellular debris, which could eventually impact on surrounding cells including neurons. Importantly, we also found that depleted gMacs are efficiently repopulated by bone marrow-derived monocytes,

indicating the plasticity of the niche when depletion occurs. Nevertheless, the functional specialization of the repopulated cells remains to be investigated.

The overt degeneration of enteric neurons in both the myenteric and submucosal plexus after depletion of self-maintaining gMacs indicates that NASM gMacs in both anatomical niches perform similar tasks. Loss of the neuro-supportive functions of NASM gMacs nevertheless has a different outcome on gastrointestinal function. Whereas depletion of ME NASM gMacs impacts on muscle contractility due to loss of enteric neurons, loss of neuronal support by LP NASM gMacs leads to abnormalities in neuron-evoked gastrointestinal secretion. Enteric neurons are part of programmed reflex circuits that respond to various stimuli, including changes in luminal content. These sensory signals are relayed via interneurons to motor and secretomotor neurons that install a coordinated response to efficiently modulate motility and secretion (Furness, 2012). Accordingly, neurodegenerative changes in the ENS, as observed in patients with constipation, Parkinson's disease, diabetes mellitus, and chronic intestinal pseudo-obstruction often affect the regulation of ion secretion and/or intestinal motility ultimately leading to aberrant intestinal transit and intestinal dilation (Sellin and Chang, 2008; Preziosi and Emmanuel, 2009). Also in the context of aging, ME gMacs may be crucial players in the process of neurodegeneration in the ENS (Becker *et al.*, 2017). The impact of alterations in the gastrointestinal environment, including changes in microbiota and diet, on the phenotype and function of NASM gMacs should also be further explored. Given the emerging evidence that microbiota can drive phenotypic and functional changes in different tissue macrophages, including ME gMacs (Muller *et al.*, 2014), white adipose tissue macrophages (Suárez-Zamorano *et al.*, 2015) and microglia (Erny *et al.*, 2015; Thion *et al.*, 2018), it seems plausible that this may also hold true for the newly identified gMac subsets.

Blood Vessel Niche – BVASM gMacs

Although macrophages are known to support angiogenesis, mainly in the context of developmental angiogenesis and tumour growth, the role of macrophages in supporting endothelial cells and blood vessel integrity has only been sporadically reported. Blood vessels can regulate macrophage differentiation from recruited monocytes, which in turn promote arteriogenesis and ischemic tissue recovery (Krishnasamy *et al.*, 2017). In the case of ischemic damage, monocyte-derived macrophages indeed actively contribute to the remodeling and healing of damaged arteries and arterioles (Heil and Schaper, 2004). To what extent macrophages influence blood vessel integrity in steady-state conditions remains however unclear. Here, we demonstrate that depletion of the self-maintaining gMac population leads to abnormalities in the submucosal vascular network, and thus are important to maintain vascular supply even during homeostasis. Although one can only speculate, the continuous low-grade inflammation in the intestine implies a constant influx of immune cells in the

intestinal LP, potentially requiring constant remodeling of the submucosal vasculature. The latter would indeed benefit from a stable macrophage population to close inter-endothelial gaps created by immune cells that have left the circulation. The unaffected vascular network in the ME, not characterized by low-grade inflammation and constant immune influx, might indirectly corroborate this hypothesis. In addition to the increased expression of genes involved in angiogenesis, including *Ecm1, Tnfaip2, Anpep, Hif1a, Mmp2, Mmp14*, it was also remarkable that BVASM gMacs in the LP showed increased expression of genes typically expressed by red pulp spleen macrophages. This transcriptional resemblance might further reflect the intimate relationship of this subset with blood vasculature given the role of red pulp spleen macrophages in blood homeostasis by phagocytosing senescent erythrocytes and blood-borne particles (Kurotaki, Uede and Tamura, 2015).

Gut Associated Lymphoid Tissue Niche – GALT gMacs and Paneth Cell Niche – PASM gMacs
Of interest, a small fraction of self-maintaining gMacs are found within PP and in close proximity to Paneth cells. PP are abundantly populated by T- and B-cell zones that continuously monitor the intestinal lumen for both microbiome and food-derived antigens (Reboldi and Cyster, 2016). Accordingly, B-cell turnover in PP is a dynamic process characterized by high apoptotic rates to avoid excessive tissue expansion and to remove nonreactive B-cells. Macrophages could be involved in this delicate process, as indicated by co-culturing experiments demonstrating phagocytosis of PP B-cells by $MHCII^+$ macrophages (Bhogal *et al.*, 2004). Consistently with these findings, we revealed that self-maintaining gMacs phagocytose apoptotic B-cells within PP, as indicated by the engulfment of B-cells and fragmented DNA and the expression of typical B-cell markers within self-maintaining gMacs. This is in line with a recent study of phagocytes within PP, showing the presence of $CD4^+$ self-maintaining macrophages with strong phagocytic activity and anti-bacterial gene signature (Bonnardel *et al.*, 2015). In addition, we also demonstrated close proximity of self-maintaining gMacs to Paneth cells. This finding is in line with a recent publication describing a CSF1R-dependent macrophage population in control of the intestinal stem-cell niche with aberrant Paneth cell differentiation and reduction in intestinal stem cells upon ablation (Sehgal *et al.*, 2018). It should however be noted that both gMac clusters were identified based on the enrichment of B-cell and Paneth cell specific markers, suggesting that the transcriptomic differences are mainly driven by physical interactions of gMacs with these cell types, as has been recently suggested (Boisset *et al.*, 2018). Although these findings provided us with indirect evidence that self-maintaining gMacs colonize B-cell and Paneth-cell rich niches, alternative approaches should be applied to identify specific markers of these subsets. In conclusion, this study revisits the current paradigm of gMac ontogeny and heterogeneity and highlights the importance of evaluating the role of distinct gMac subset in gastrointestinal

function. Future efforts should focus on investigating the presence and phenotypical changes of these subsets in animal models and human biopsy material in different disease settings. These studies will potentially pave the way for macrophage-centered target identification for functional and immune-mediated gastrointestinal disorders.

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Author Contributions

S.D.S. and S.V. designed the study, wrote the manuscript and performed experiments. G.B. led the project, revised the manuscript and provided excellent intellectual input; J.A-L., N.S., M.F.V., I.S., I.V., I.D.C., E.D.G., U.H. and S.S. performed experiments; W.B., E.A.J. performed experiments, provided intellectual input and provided reagents; M.G., P.V.B., B.D.S. and B.S. provided intellectual input; B.B. and D.L. performed scRNA-seq analysis; M.F.V. performed 3D analysis. P.V.B. and I.D. provided functional setups. V.B. supplied mice and M.M. provided reagents.

Declaration of Interests

The authors declare no competing interests

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Figures titles and legends

Figure 1: Fate-Mapping Reveals Self-Maintaining Gut Macrophages That Are Not Replaced By Monocytes

(A) Time scheme illustrating the study protocol of self-maintaining gMac labeling in *Cx3cr1*^{CreERT2}*.Rosa26-LSL-YFP* animals.

(B) Representative FACS plots of the expression of YFP (CX3CR1) on gated live- CD64^{+} MHCII^{+} CD11b^{+} cells isolated from ileal lamina propria (LP) and muscularis externa (ME).

(C) Quantification of YFP-labeling in LP and ME (n=4 mice, mean ± S.E.M).

(D) Absolute numbers of YFP-expressing cells at 1 week and 35 weeks after TAM treatment in LP and ME (n=4 mice from 2 independent experiments, mean ± S.E.M).

(E) FACS plots of monocyte and monocyte progenitors in blood and bone marrow-derived cells at 1 and 35 weeks after TAM treatment (representative of at least n=3 mice from 1 experiment).

(F) Histograms representing the expression of CD64, CD103, Ly6C and FMO controls (grey) in YFP^{+} cells at 35 weeks after TAM treatment (n=4 mice from 2 independent experiments).

(G) Percentage of proliferating BrdU^{+} cells within LP and ME YFP^{+} CD64^{+} cells after 9 days pulse-labeling. (n=4 mice from 1 experiment).

(H) Immunofluorescent images showing the distribution of YFP^{+} cells in cross-sections of small intestine over time. Scale bars represent 100µm. (Images are representative from at least 4 independent experiments).

(I) Quantitative analysis of YFP-expression by F4/80^{+} gMacs over time. (at least 3 sections of a minimum of 2 mice were used, mean ± S.E.M).

See also Figure S1.

Figure 2: Self-Maintaining Gut Macrophages Express a Specific Transcriptional Profile Distinct From Monocyte-Replaced Gut Macrophages

(A-I) Self-maintaining (YFP^+) and monocyte-replaced (YFP^-) gMacs were analyzed by bulk RNA-sequencing (RNA-seq) [data are from 1 experiment, YFP^+ versus YFP^- LP gMacs (n=4 each) and YFP^+ versus YFP^- ME gMacs (n=4 each)].

(A) PCA plot of gene expression by YFP^+ and YFP^- gMacs from LP and ME.

(B and C) Volcano plot showing statistical significance ($-\log_{10}$) against $\log_2$ fold change between YFP^+ and YFP^- gMacs in LP (B) and ME (C). Genes statistically significant (fold change >2, $p<0.05$) in YFP^- gMacs are represented by green symbols, whereas those in YFP^+ gMacs are shown in red.

(D) Heatmap graphical representation of top enriched genes in YFP^- compared to YFP^+ gMacs (left) and YFP^+ compared to YFP^- gMacs (right) from LP.

(E) Visual representation of statistical significance ($-\log_{10}$) against fold enrichment of Gene Ontology (GO) terms (biological processes) associated with enriched genes in YFP^+ compared to YFP^- LP gMacs.

(F) Representative FACS plots and pie charts of CD63 and CD4 expression among YFP^+ and YFP^- gMacs (n=4 mice from 2 independent experiments).

(G) Heatmap graphical representation of top enriched genes in YFP^- compared to YFP^+ gMacs (left) and YFP^+ compared to YFP^- gMacs (right) from ME.

(H) Visual representation of statistical significance ($-\log_{10}$) against fold enrichment of Gene Ontology (GO) terms (biological processes) associated with enriched genes in YFP^+ compared to YFP^- ME gMacs.

(I) Gene expression analysis by qPCR of candidate surface markers identified by RNA-seq analysis in YFP^+ compared to YFP^- gMacs in LP and ME (n=3 mice, log mean ± S.E.M). Data are presented relative to *rpl32* expression. See also Figure S2 and Table S1, 2.

Figure 3: Distinct Transcriptional Profiles Among Self-Maintaining Gut Macrophages Correspond To Colonization Of Specific Anatomical Niches

(A-C) YFP^+ self-maintaining LP gMacs were subjected to single cell RNA-seq (scRNA-seq).

(A) tSNE plot of 697 YFP^+ LP gMacs, color-coded by their associated cluster.

(B) Combined volcano plot showing differentially expressed marker genes (fold change) in each cluster compared to the remaining three clusters (x-axis) and their statistical significance (y-axis, $-\log_{10}$).

(C) Distribution of marker genes identified in cluster 0 (upper chart) or cluster 1 (lower chart) across different tissue-resident macrophages, as identified by the ImmGen dataset.

(D-E) Representative confocal images (top, middle) and 3D-reconstruction (bottom) of YFP^+ self-maintaining gMacs (green). Arrowheads indicate contacts between YFP^+ self-maintaining gMacs and tissue structures. Scale bars represent 100 µm for whole mounts, 20 µm for cross-sections and 3D images.

(D) Distribution of YFP$^+$ gMacs in whole mount submucosal plexus (left panels and bottom) and intestinal cross sections (right panels) stained with anti-HuC/D (red, upper left) and anti-βIII-Tubulin antibodies (red) and DAPI (blue). NASM-gMacs, neuron-associated self-maintaining gMacs. Images are representative of at least 4 independent experiments.
(E) Distribution of YFP$^+$ gMacs in intestinal cross sections stained with anti-VE Cadherin (magenta). Images are representative of at least 4 independent experiments. BVASM, blood vessel-associated self-maintaining gMacs.
(F-G) RNAscope in situ hybridization with probes against *EYFP* and *Fcrls* (grey), a marker gene for cluster 0 (F), or *Adamdec1*, a marker gene for cluster 1 (G). Sequential co-labeling with antibodies against βIII-Tubulin (F) and VE-Cadherin (G). Arrowheads indicate double-positive cells in close contact to tissue structures. Nuclei marked by DAPI. Scale bars represent 10 µm. Images are from 1 experiment.
(H) Distribution of YFP$^+$ gMacs in Peyers patches (PP) stained with anti-B220 (red), anti-TUNEL (grey) and DAPI (blue). Arrowheads indicate phagocytosis of apoptotic cells (TUNEL) in PP. GALT, gut-associated lymphoid tissue self-maintaining gMacs.
(I) YFP$^+$ gMacs in intestinal cross sections stained with anti-Lysozyme (blue). PASM, Paneth-associated self-maintaining gMacs. Scale bars represent 100 µm for whole mounts, 20 µm for cross-sections and 3D image.
See also Figure S3 and Table S3.

Figure 4: Embryonic Progenitors and Bone Marrow-Derived Monocytes Contribute to Distinct Micro-Anatomical Niches

(A) Scheme illustrating the irreversible YFP-labeling of *Cx3cr1*$^+$ embryonic precursors in pregnant *Cx3cr1*^{CreERT2}.*Rosa26-LSL-YFP* mice.
(B) Confocal images of YFP$^+$ embryonic gMacs (green) in intestinal cross-sections (left) and whole mount LP and ME from ileum (middle, right) at postnatal day 1. Scale bars represent 100 µm. Images are representative of 2 independent experiments.
(C) Distribution of YFP$^+$ embryonic-derived gMacs in ileal cross sections at 6 weeks of age stained with anti-βIII-Tubulin (red), anti-VE Cadherin (magenta), anti-B220 (red) or anti-Lysozyme (cyan) antibodies and DAPI (blue). Scale bars represent 20 µm. Arrowheads indicate contacts between YFP$^+$ embryonic macrophages and tissue structures. NASM-gMacs, neuron-associated self-maintaining gMacs. BVASM, blood vessel-associated self-maintaining gMacs. GALT, gut-associated lymphoid tissue self-maintaining gMacs. PASM, Paneth-associated self-maintaining gMacs
(D) YFP$^+$ embryonic-derived gMacs in ileal cross sections (left) and whole mount myenteric plexus of the ME (right) stained for anti-βIII-Tubulin (left) or anti-HuC/D antibodies (right) and DAPI (blue). Scale bars represent 50 µm for cross sections and 20 µm for whole mount

images. Arrowheads indicate contacts between YFP$^+$ embryonic gMacs and tissue structures. Images are representative of 2 independent experiments.

(E) Scheme illustrating adoptive transfer of *Cx3cr1*$^{+/GFP}$ bone marrow cells to *Cx3cr1*^{CreERT2}.*Rosa26-LSL-TdTomato* neonates.

(F) Distribution of neonatal TdTomato$^+$ (grey) and GFP$^+$ (green) gMacs in ileal cross sections (upper panel). Scale bars represent 100 µm. Quantitative analysis of GFP- and TdTomato-expression by F4/80$^+$ gMacs. (at least 3 sections of a minimum of 4 mice were used, mean ± S.E.M).

(G) Representative whole mount images stained for anti-HUC/D or anti-B220. Scale bars represent 50 µm. Quantitative analysis of GFP- and TdTomato-expression by F4/80$^+$ gMacs. (at least 3 sections of a minimum of 4 mice were used, mean ± S.E.M).

Figure 5: Bone Marrow-Derived Macrophages Repopulate the Niche after Depletion of Self-Maintaining gMacs

(A-D) Self-maintaining gMacs were depleted by administering diphtheria toxin (DTx) to *Cx3cr1*^{CreERT2}.*Rosa26-iDTR* mice at 35 weeks after TAM or oil treatment.

(A) Scheme for the irreversible expression of DTx receptor (DTR) in CX3CR1$^+$ gMacs.

(B) Distribution of DTR$^+$ gMacs (grey) in ileal cross sections. Scale bars represent 100 µm. Images are representative of 3 independent experiment with 3 mice in each group.

(C) Representative whole mount images stained with anti-HuC/D (red), F4/80 (magenta) and DTR. Scale bars represent 100 µm.

(D) Gene expression analysis by qPCR of surface markers (see figure 2) in LP and ME (n=6 mice from 2 independent experiments). Data were analyzed by Kruskal-Wallis test and are shown as mean ± S.E.M. Data are presented relative to *rpl32* expression.

(E) Shielded chimers were generated in *Cx3cr1*^{CreERT2}.*Rosa26-iDTR* mice at 35 weeks after TAM treatment and reconstituted with BM cells from *Cx3cr1*$^{+/GFP}$.*Ccr2*$^{+/RFP}$ mice.

(F) Representative whole mount images stained with anti-HuC/D (upper panel) and DTR (lower panel). Scale bars represent 50 µm. Images are representative of 1 independent experiment with 4 mice in each group.

See also Figure S4.

Figure 6: Self-Maintaining Gut Macrophages Support Enteric Neurons and Submucosal Vasculature

(A-F) Self-maintaining gMacs were depleted by DTx administration and analyses were performed at different time points after DTx administration.

(A) Representative whole mount images of myenteric plexus stained with anti-HuC/D (red) and anti-cleaved caspase 3 antibodies (grey). Scale bars represent 10 µm.

(B) Quantification of cleaved-caspase 3 (CCasp3) immunoreactive neurons relative to the total number of neurons within different ganglia of submucosal and myenteric plexus of the ileum. Absolute quantification of total numbers HuC/D^{+} neurons within different ganglia of submucosal and myenteric plexus (n=7 mice from 2 independent experiments, with 4 ganglia counted per submucosal and myenteric plexus). Data were analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test and are shown as mean ± S.E.M.

(C) Representative confocal images of ileal sections (upper) and whole mount submucosa (lower) stained with anti-VE-Cadherin antibodies (magenta). Scale bars represent 100 µm.

(D) Quantification of vessel surface (µm^2) and means of VE-Cadherin fluorescence intensity in arbitrary fluorescence units (A.F.U.)(n=5 mice from 2 independent experiments). Data were analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test and are shown as mean ± S.E.M.

(E) Representative confocal images of lectin perfusion (green) and microsphere leakage (red) in whole mount submucosa. Scale bars represent 100 µm.

(F) Quantification of vascular leakage of microspheres (particles per µm^2). Data were analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test and are shown as mean ± S.E.M. See also Figure S5.

Figure 7: NASM-gMacs Control Neuron-Evoked Secretion, Neurogenic Contractility and Intestinal Transit

(A) Ileal LP-submucosa preparations from self-maintaining gMac depleted versus control *Cx3cr1*^{CreERT2}*.Rosa26-iDTR* mice were subjected to Ussing chambers. Graph represents veratridine-evoked (30µM) short-circuit current (I^{sc}) responses recorded over a period of 60 min before tetradotoxine (TTX, 100nM) administration. Area under the curve shows I^{sc} responses to veratridine (n=8 mice from 2 independent experiments). Data were analyzed by Mann-Whitney test and are shown as mean ± S.E.M.

(B-C) Ileal myenteric plexus preparations from self-maintaining gMac depleted versus control *Cx3cr1*^{CreERT2}*.Rosa26-iDTR* mice were loaded with intracellular calcium probe Fluo4.

(B) Baseline Fluo-4 fluorescence and maximum peak amplitude (F_i/F0) in response to electrical pulse train stimulation (20 Hz). See also Figure S6 and Video S1-4.

(C) Percentage of responding neurons and maximum peak amplitude (F_i/F0) in response to nicotinic agonist 1,1 dimethyl-4-phenylpiperazinium iodide (DMPP) (n=11 mice with a total of 110 neurons in control and 128 neurons in DTx-treated mice analyzed). Data were analyzed by unpaired t-test and shown as mean ± S.E.M.

(D, E, F) Ileal muscle strip preparations isolated from self-maintaining gMac depleted versus control *Cx3cr1*^{CreERT2}*.Rosa26-iDTR* mice were tested for their longitudinal contractile responses to different stimuli (each dataset included 6 mice).

(D) Representative tracings of neural contractile responses of muscle strips in response to electric field stimulation (EFS) (1-32 Hz).
(E) Maximum tension in response to EFS. (n=6 mice in each group from 3 independent experiments). Data were analyzed by two-way anova test with multiple comparison test and shown as mean ± S.E.M.
(F) Representative tracings of myogenic contractile responses induced by KCl (60 mM). Bars represent the force (mN/mm^2) of the contractile responses to KCl. (mean ± S.E.M.).
(G-L) *Cx3cr1*^{CreERT2}.*Rosa26-iDTR* mice were treated with DTx and MC-21 (anti-CCR2) for 7 days at 35 weeks after TAM or oil treatment (n=11 mice in each group from 1 experiment). Data were analyzed by unpaired t-test and shown as mean ± S.E.M.
(G) Geometrical center of fluorescence in the gastrointestinal segments 1.5 hours after oral gavage.
(H) Total gastrointestinal transit time to expel feces containing carmine red dye.
(I) Stomach emptying measured after oral gavage with rhodamine B conjugated dextran
(J) Percentage of initial body weight.
(K)Representative T_2-weighted MRI coronal (left) and axial (right) images. Arrowhead indicates colon.
(L) Quantification of colonic distension in diameter (mm). Data were analyzed by unpaired t-test and shown as mean ± S.E.M

Supplemental Information Titles and Legends

Figure S1: Gating Strategy For Self-Maintaining Gut Macrophages and Heat Maps For Identification of Surface Markers, Related To Figure 1

(A) Representative FACS plots showing gating strategy for self-maintaining LP and ME gMacs in TAM or oil-treated *Cx3cr1*^{CreERT2}.*Rosa26-LSL-YFP* animals. Self-maintaining gMacs are gated as live CD45.2$^+$ CD11c$^{lo/hi}$CD11b$^+$ MHCII$^+$ CD64$^+$ CX3CR1(YFP)^{hi} cells.
(B) Heatmap graphical representation of top surface marker candidates in YFP$^+$ compared to YFP$^-$ LP gMacs of *Cx3cr1*^{CreERT2}.*Rosa26-LSL-YFP* mice at 35 weeks after TAM treatment, as determined by RNA-seq. Surface marker candidates were determined using DAVID.

Figure S2: Self-Maintaining Muscularis Externa Gut Macrophages Mainly Colonize Neuronal Structures, Related To Figure 2

(A) Confocal images of YFP$^+$ gMacs (green) in whole mount ME (myenteric plexus and circular muscle layers) stained with anti-βIII-Tubulin and anti-HUC/D antibodies (red) from *Cx3cr1*^{CreERT2}.*Rosa26-LSL-YFP* mice at 1 week and 35 weeks after TAM treatment. Scale bars represent 100 µm.
(B) 3D-reconstruction of YFP$^+$ gMacs (green) in whole mount myenteric plexus stained with anti-βIII-Tubulin and anti-HUC/D antibodies (red) from *Cx3cr1*^{CreERT2}.*Rosa26-LSL-YFP* mice at 35 weeks after TAM treatment. Scale bars represent 20 µm.

Figure S3: Expression of Specific Marker Genes and Pan-Macrophage Markers in Different Clusters of Self-Maintaining Lamina Propria Gut Macrophages, Related To Figure 3

(A) tSNE plot of 697 self-maintaining LP gMacs representing the expression of pan macrophage markers *Cd68*, *Cx3cr1*, *Fcgr1, Adgre1* and bulk RNA-seq identified surface marker *Cd63.*

(B-E) Violin plots of marker gene expression in cluster 0 (B), cluster 1 (C), cluster 2 (D) and cluster 3 (E).

(F) tSNE plot of 697 self-maintaining LP gMacs representing the expression of *Adgre1*

Figure S4: Depletion of Self-Maintaining Macrophages Does Not Lead To Overt Inflammation and Vascularization in the Muscularis Extern, Related To Figure 5

(A) Gene expression analysis by qPCR of inflammatory markers in the intestine at different time points after DTx administration in *Cx3cr1*^{CreERT2}.*Rosa26-iDTR* mice at 35 weeks after TAM treatment (control, 3 days and 7 days) (Data are from 2 experiments with 2 mice each). Data are presented relative to *rpl32* expression.

(B) Graphs showing the percentage of Ly6C^{hi}MHCII^{-}, Ly6C^{+}MHCII^{+}, Ly6C^{-}MHCII^{+}, SiglecF^{+} eosinophils and Ly6G^{+} neutrophils amongst live CD45^{+} cells 3 days after DTx administration. Data are from 1 experiment, 4 mice.

(C) Graph showing percentage of total chimerism amongst blood Ly6C^{hi} monocytes.

Figure S5: Depletion of Self-Maintaining Macrophages Does Not Affect Vascularization in the Muscularis Externa, Related To Figure 6

Confocal images of ME stained with anti-VE Cadherin (magenta) antibodies at different time points after DTx administration in *Cx3cr1*^{CreERT2}.*Rosa26-iDTR* mice (35 weeks after TAM treatment) (control, 3 days and 7 days). Quantification of VE-Cadherin mean fluorescence intensity in arbitrary fluorescence units (A.F.U.) (data are from 2 experiments, 4 mice in total). Data are shown as mean ± S.E.M.

Figure S6: Calcium Imaging of Myenteric Neurons *In Situ*, Related To Figure 7

(A) Representative examples of a Ca^{2+} imaging experiment on a myenteric plexus preparation obtained from small intestine of *Cx3cr1*^{CreERT2}.*Rosa26-iDTR* mice, 35 weeks after TAM treatment (top, control) or oil (bottom, DTx 3 days). Baseline Fluo-4 fluorescence in a myenteric ganglion before electric stimulation (E-Stim) (left). Activity-over-time image (AoT) in which the Ca^{2+} transient amplitude upon electrical pulse train (red: E-stim, 20 Hz, 2 seconds) and nicotinic acetylcholine receptor (green: DMPP, 10 µM, 15 sec) stimulation is color-coded showing responsive neurons (right). Scale bar represent 50 µm.

(B) Ca^{2+} transient profiles of individual myenteric neurons [depicted by the matching arrows in (A)] induced by electrical and DMPP stimulation. See also movies S1-4.

Star Methods

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Guy Boeckxstaens (guy.boeckxstaens@kuleuven.be).

Experimental Model and Subject Details

Mice

All experimental procedures were approved by the Animal Care and Animal Experiments Committee of the Medical Faculty of the KU Leuven. *Cx3cr1*^{CreERT2} and *Cx3cr1*$^{GFP/GFP}$ mice were obtained from Steffen Jung, Weizmann Institute of Science (Yona *et al.*, 2013). *Rosa26-LSL-YFP* mice were obtained from Daniel Richardson, University College London (Srinivas *et al.*, 2001). *Rosa26*-iDTR and *Rosa26-TdTomato* mice were obtained from Jackson Laboratory. *Cx3cr1*$^{+/GFP}$.*Ccr2*$^{+/RFP}$ double reporter mice were generated by breeding *Cx3cr1*$^{GFP/GFP}$ and *Ccr2*$^{RFP/RFP}$ mice. These mice were maintained on C57/BL6 background. All mice used in this study were sex- and age-matched.

Method Details

Lineage Tracing of CX3CR1^{+} Macrophages

For the induction of Cre recombinase to trace CX3CR1^{+} intestinal macrophages, *Cx3cr1*^{CreERT2} mice were crossed to *Rosa26-LSL-YFP* mice and progeny aged 4-6 weeks was treated three times subcutaneously with 4 mg TAM (Sigma-Aldrich)/ 30 g body weight dissolved in corn oil (Sigma-Aldrich), 48h apart. For pulse-labeling experiments in embryos, a timed breeding was set up in *Cx3cr1*^{CreERT2}.*Rosa26-LSL-YFP* mice based on vaginal plug observations: 12pm on the day of plug formation was estimated to be 0.5 days post coitum (dpc). Pregnant *Cx3cr1*^{CreERT2}.*Rosa26-LSL-YFP* females were then treated intraperitoneally at 8.5 dpc with 4 mg TAM and 2 mg progesterone (Sigma-Aldrich)/ 30 g body weight dissolved in corn oil. Since tamoxifen injection during embryonic stage prevents natural birth, offspring was derived from pregnant dams by caesarean section at E18.5 and given to foster mothers.

Depletion of Self-Maintaining CX3CR1^{+} Gut Macrophages

To deplete CX3CR1^{+} intestinal macrophages, *Cx3cr1*^{CreERT2} mice were first crossed to *Rosa26*-iDTR mice and progeny aged 4-6 weeks were treated three times with TAM as described earlier. Self-maintaining macrophages in *Cx3cr1*^{CreERT2}.*Rosa26*-iDTR mice were depleted 35 weeks after TAM treatment by 2 intraperitoneally injections of 300ng diphtheria toxin (DTx, Sigma-Aldrich)/ 30 g body weight.

Chronic Depletion of Self-Maintaining CX3CR1^{+} Gut Macrophages

In order to achieve chronical depletion, *Cx3cr1*^{CreERT2}.*Rosa26*-iDTR mice were treated 35 weeks after TAM or oil treatment with 200ng diphtheria toxin (DTx, Sigma-Aldrich)/ 30 g body

weight every other day for 7 days. To prevent engraftment of circulating monocytes into empty niches, TAM (or oil treated) animals were given daily 20 µg of anti-CCR2 [MC-21, kindly provided by Matthias Mack (University of Regensburg, Regensburg, Germany].

Immunofluorescence

For immunofluorescence (IF), mice were sacrificed by CO_2 overdose and the small intestine was removed and placed in ice-cold HBSS (Gibco) with 5% FBS. The small intestine was flushed with ice-cold PBS to remove luminal contents. For whole mount IF, the intestine was cut open longitudinally before stretching in a Sylgard plate. The ME was carefully removed from the remaining submucosa and LP by gently scraping with forceps and fixed for 20 min in 4% PFA. For IF on intestinal sections, intestinal segments of approximately 2 cm were fixed for 1h in 4% PFA, dehydrated overnight in 30% sucrose and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek). 18 µm-thick cryosections were obtained by cutting on a NX70 Cryostat (Thermo Fisher Scientific). Intestinal whole mount tissues and sections were extensively washed with PBS before permeabilizing in 0.5% Triton X-100 for 1h and blocking for 2h in 2% BSA and 5% Donkey serum in 0.5% Triton X-100 at RT. Subsequently, samples are incubated overnight at 4°C with the primary antibody mix in blocking buffer. Antibodies for IF can be found in the Key Resources Table. Afterwards, samples were washed in PBS and incubated with secondary antibody mix in blocking buffer. Subsequently, samples were washed in PBS and mounted on slides with ProLong Gold Antifade (Thermo Fisher Scientific). Images were collected on a LSM880 multiphoton microscope (Zeiss) and a 20x/0.8 or 40x/1.3 objective (Zeiss) was used. Identical settings were used for all conditions. Images were processed and reconstructed using ImageJ (NIH) or Imaris software (Bitplane).

Tissue Harvest for Single Cell Suspension

Mice were sacrificed by CO_2 overdose and small intestine was removed and placed in ice-cold HBSS (Gibco) with 5% FBS. Tissue was flushed with ice-cold PBS and stretched in a Sylgard plate and ME was carefully removed with forceps. Stripped ME was cut in 1-2 mm pieces and digested in 2 mg/mL Collagenase Type IV (Gibco) in RPMI (Lonza) supplemented with 2% HEPES (Gibco), 2% FBS and DNase (Roche) for 1 h at 37°C with constant stirring. The resulting cell suspension from ME was homogenized using a potter tissue grinder and passed through a 70µm cell strainer (BD Falcon). The remaining piece containing intestinal LP and submucosa was first washed in HBSS (Thermo Fisher Scientific) supplemented with 1mM DTT (Sigma-Aldrich), 1mM EDTA (Invitrogen) and 2% HEPES for 8 min. Afterwards, tissue was digested in 0.85 mg/mL Collagenase Type V (Sigma-Aldrich) in MEMα (Lonza) supplemented with 2% HEPES, β-mercaptoethanol (1:1000, Gibco) and DNase for 30 min at

37°C with constant stirring. Cells were passed through a 70 µm cell strainer (BD Falcon) and then washed twice in ice-cold RMPI to obtain a single cell suspension.

Flow Cytometry and Sorting of Live Cells

Single cell suspensions were blocked with rat anti-mouse CD16/CD32 antibodies (BD Biosciences) (1/200) for 12 min and afterwards incubated with fluorophore-conjugated anti-mouse antibodies at recommended dilutions for 20 min at 4°C. Samples were first excluded of doublets and self-maintaining gMacs were gated as indicated in the results or supplementary figures section. FACS antibodies used can be found in the Key Resources Table. Dead cells were excluded using DAPI stains and wash steps were performed in FACS buffer (PBS, 2% FBS and 0.78mM EDTA). Cells were analyzed on a BD Canto HTS (BD Biosciences) and data was analyzed using FlowJo software (Tree Star Inc.). FACS sorting experiments for gene expression analyses were performed on a BD Aria III (BD Biosciences) and cells were immediately sorted in RLT plus lysis buffer (Qiagen) with beta-mercaptoethanol and placed on dry ice. RNA was isolated using the RNeasy Plus Micro Kit (Qiagen) according to instructions by the manufacturer. For scRNA-seq, cells were sorted in DMEM containing 30% FBS before being pelleted and resuspended in PBS with 0.04% BSA.

BrdU Studies

Mice were injected 1 mg BrdU (APC BrdU Flow Kit, BD Biosciences) intraperitoneally every other day for 9 days. The incorporation of BrdU was assessed by flow cytometry with a BrdU Flow Kit (BD Biosciences).

Quantitative PCR

Cells were lysed in RLT plus lysis buffer and RNA was extracted using the RNeasy Plus Micro Kit, retro-transcribed by qScript cDNA SuperMix (Quanta Biosciences), and quantitative PCR was performed with SYBR Green (Bio-Rad Laboraties) on a LightCycler 96 (Roche). The expression levels of the genes of interest were normalized to the expression levels of the reference gene *rpl32*. For some experiments, isolated RNA was amplified with the Arcturus Riboamp HS PLUS RNA Amplification Kit (Applied Biosystems) to overcome limited amount of recoverable RNA.

Bulk-RNA Sequencing

Total RNA was extracted with the RNeasy Plus Micro Kit as described earlier. Agilent Bioanalyzer was used to assess RNA integrity and only RNA Integrity Numbers ≥ 7 RNA were processed. The SMART-Seq v4 Ultra Low Input RNA kit (Clonentech) was used to amplify and generate cDNA from 10ng of total RNA according to manufacturer instructions. Samples

were sequenced on a NextSeq 500 sequencer (Illumina) using single-end 75 base pair reading. Reads were aligned to the reference genome with STAR 2.4.1d and reads from alignment that are non-primary mappings were removed using samtools 1.1. Gene ontology analysis was performed using the Gene Ontology Consortium website (http://geneontology.org/) and redundant gene ontology terms were removed using REViGO (http://revigo.irb.hr/). Surface marker candidates were determined using DAVID (https://david.ncifcrf.gov/).

scRNA Library Preparation and Sequencing

The FACS isolated cells were immediately counted by a Luna-FL Dual Fluorescence Cell Counter (Logos Biosystems). Single cell libraries were prepared with the Chromium Single Cell 3' V2 Chemistry Library Kit, Gel Bead & Multiplex Kit and Chip Kit from 10X Genomics. The barcoded library was sequenced on an Illumina HiSeq4000 in 25-8-98 paired-end configuration.

scRNA Data Analysis

Raw sequencing reads were demultiplexed, mapped to the mouse reference genome (build mm10) and gene-expression matrices were generated using CellRanger (version 2.0.0). In total 64,139,566 reads were mapped and 905 cells were detected with a sequencing saturation of 92.8%. This gene-expression matrix was further filtered in Seurat 2.0 and cells with less than 200 genes or 1000 unique molecular identifiers (UMIs) were discarded as well as cells with more than 8000 expressed (multiplets) genes and more than 20% of mtRNA, resulting in 697 single cells. After normalization, these cells were clustered using the Seurat workflow based on dimensionality reduction by a PCA of the 1395 most variable genes. The first 8 PCs were used to identify the different clusters in the dataset and to visualize these clusters in a t-Distributed Stochastic Neighbour Embedding (tSNE) plot. Gene ontology analysis was performed using the GeneAnalytics website (https://ga.genecards.org/).

Single-Molecule RNA In Situ Hybridization (RNAscope)

In situ hybridization was performed using the RNAscope Fluorescent Multiplex Assay (ACD Bio) according to the manufacturer's instructions. Intestinal tubes of approximately 2cm were fixed for 1h in 4% PFA, dehydrated overnight in 30% sucrose and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek). 14-16 µm cryosections prepared on Superfrost Plus Slides (Thermo Fisher Scientific) were used for RNAscope assays according to the manufacturer's recommendations. The following paired double-Z oligonucleotide probes for hybridizations were used: *Adamdec1* (target NM_021475.2; bp region 1026 - 2110), EYFP (bp

region 7768 – 8420) and *Fcrls* (target NM_030707.3; bp region 530 – 1682). Bacterial *DapB* probe was used as a negative control. Hybridizations were performed for 2h at 40°C. DAPI was used as a nuclear counterstain. This protocol was adapted to sequentially immunostain with either anti- βIII-T ubulin or anti-VE Cadherin antibodies.

Adoptive Transfer Experiments

Cx3cr1^{CreERT2} mice were crossed to *Rosa26-LSL-TdTomato* mice and neonatal offspring was injected with TAM twice. Total 10 x 10^6 bone marrow cells were obtained from *Cx3cr1*$^{+/GFP}$ mice and intraperitoneally injected in 50 µL PBS four times to 0-21-day old *Cx3cr1*^{CreERT2}*.Rosa26-LSL-TdTomato* mice. Mice were sacrificed 9 weeks later.

Generation of Shielded Bone Marrow Chimeric Mice

TAM- or vehicle (oil)-treated *Cx3cr1*^{CreERT2}*.Rosa26-iDTR* mice were anaesthetized and exposed to a single dose of 9.5 Gy γ-irradiation while abdomen and head were protected by a 12.5 mm lead shield. Animals were subsequently housed in IVC cages supplied with 1% Enrofloxacil (Baytril, Bayer) in drinking water to allow for full recovery. 24 hours after irradiation, animals received 12 x 10^6 bone marrow cells obtained from *Cx3cr1*$^{+/GFP}$*.Ccr2*$^{+/RFP}$ mice via intravenous injection. Bone marrow reconstitution efficiency was analyzed in circulating monocytes by FACS 5 weeks after irradiation on 500 µL blood collected via sub mandibular bleeding. Self-maintaining macrophages were depleted as described earlier and animals were euthanized 6 weeks after irradiation for analysis of chimerism.

Microsphere Vascular Leakage Assay

100 uL of a 1mg/ mL solution of Lectin was injected intravenously and allowed to circulate for 45 min, Thereafter, 50 uL of microspheres (diameter: 10 µM) was injected intravenously before anaesthesia with ketamine and xylazine. Mice were transcardial perfused with saline to remove excess microspheres from the bloodstream followed by 4% PFA. Whole mounts and intestinal tubes were prepared as described earlier and images were obtained using a confocal microscope (microspheres and lectin were excited using the 561 and Argon laser line, respectively).

Ussing Chambers

Mice were sacrificed by cervical dislocation 24h after the last DTx injection and ileum was carefully removed to avoid tissue damage and maintained in bubbled Krebs solution (95% O_2-5% CO_2). The intestinal samples were then pinned in Sylgard plates with LP surface down and ME was gently peeled off with forceps to prepare a LP- submucosal plexus intestinal strip, termed intestinal preparations throughout (Clarke, 2009). Then, intestinal preparations were mounted in Ussing chambers (exposed area 0.0096cm^2) with Krebs solution, glucose-

supplemented at the basolateral side and mannitol-supplemented at the apical side (95% O_2-5%; CO_2, 37°C). Tissues were voltage clamped at 0 mV using an automatic voltage clamp and the short-circuit current (I_{sc}) to keep the 0-mV potential was monitored over time. The transepithelial resistance (TEER) was calculated by Ohm's law. Veratridine was added (30 µM) to the basolateral reservoir to stimulate voltage-sensitive Na^+ intrinsic submucosal neurons (Sheldon *et al.*, 1990). Veratridine-induced changes in I_{sc} were recorded for 60 min, before TTX was added (100nM) to inhibit neurogenic I_{sc} responses. Chambers were connected to a PC with Clamp Software Version 9 (KMSCI) to record I_{sc}.

Neuronal Calcium Imaging and Analysis

TAM- or vehicle (oil)-treated *Cx3cr1*^{CreERT2}*.Rosa26-iDTR* mice were intraperitoneally injected with DTx as described earlier. Mice were sacrificed by cervical dislocation 24 h after the last DTx injection. Ileum was carefully dissected out and pinned flat in a Sylgard-lined dish filled with Krebs solution, bubbled with 95% O2 - 5% CO2 at RT. The mucosal, submucosal and circular muscle layers were carefully removed to obtain a longitudinal muscle with adherent myenteric plexus preparation which was mounted over a small inox ring, immobilized by a matched rubber O-ring and loaded with 2 µM Fluo-4 AM (Life Technologies) for 30 min at RT in Krebs with 0.01% Kolliphor® EL (Sigma-Aldrich). Myenteric plexus preparations were placed in a recording chamber and constantly superfused with carbogenated Krebs solution (RT) containing 1 µM nifedipine (Sigma-Aldrich) via a local gravity-fed (±1 ml/min) perfusion pipette. Live imaging was performed on an upright Zeiss Axio Examiner.Z1 microscope equipped with a Poly V xenon monochromator (TILL Photonics) and water dipping lens (X20, 1.0 NA, Zeiss). Fluo-4 was excited at 475 nm and captured at 525/50 nm (at 2 Hz) on a Sensicam-QE CCD camera (PCO) using TillVisION (TILL Photonics). Myenteric plexus preparations were stimulated electrically using single (300 µs) or a train of pulses (20 Hz, 2 s, 300 µs) transmitted via a focal electrode (50 µm diameter tungsten wire) placed on an interganglionic connective leading to the selected myenteric ganglia within the field of view. Agonists were diluted in Krebs solution and superfused using the perfusion pipette positioned adjacent to the field of view (1-dimethyl-4-phenylpiperazinium, DMPP: 10 µM, Sigma-Aldrich). Analysis was performed with custom-written routines in Igor Pro (Boesmans *et al.*, 2013). Regions of interest were drawn, after which average Ca^{2+} signal intensity was calculated, normalized to the initial Fluo-4 values and reported as F_i/F_0. Cells or varicosities were considered as responders when the Fluo-4 signal rose above baseline plus 3 times the intrinsic noise (standard deviation) during the recording. The amplitudes of Ca^{2+} transients were measured as the maximum increase in $[Ca^{2+}]_i$ above baseline ($\Delta F_i/F_0$).

Muscle Strip Organ Bath Experiments

TAM- or vehicle (oil)-treated *Cx3cr1*^{CreERT2}.*Rosa26-iDTR* mice were intraperitoneally injected with DTx as described earlier. Mice were sacrificed by cervical dislocation 24 h after the last DTx injection. Freshly obtained intestinal samples were placed in Sylgard plates and maintained in bubbled Krebs solution (95% O_2- 5% CO_2). Ileal segments were then opened along the mesenteric border and pinned flat in a Sylgard-lined dish. Next, the mucosal layer was removed and strips (5 mm x 15 mm) were cut. Strips were suspended along their longitudinal axis in organ bath chambers filled with Krebs buffer at 37°C. After equilibration at optimal stretch (60 mN), electrical field stimulation was applied via two parallel platinum electrodes using a Grass S88 stimulator (EFS, spectra 1-32 Hz, pulse duration 0.3 ms, train 10 s, 8 V). After each EFS spectrum, the muscle strips were washed at least three times every five minutes, before KCl (60 mM) was added to the organ bath to study myogenic receptor-independent contractile responses. Contractions were measured using an isometric force transducer/amplifier (Harvard Apparatus). Data was sampled using the Windaq data acquisition system and a DI-720 box (Dataq Instruments). The neural response obtained in the presence of EFS was calculated as the maximal tension (mN) during the stimulation period. All contractile responses were normalized for the cross-sectional area of the strip (mN/mm^2).

Gastrointestinal Motility Tests in Chronically Depleted Animals

The stomach emptying and small intestinal transit was determined in TAM- or vehicle (oil)-treated *Cx3cr1*^{CreERT2}.*Rosa26-iDTR* mice after 7 days of chronic self-maintaining gMac depletion and MC-21 treatment (as described earlier). In more detail, gastric emptying was measured as the percent of total fluorescence expelled from the stomach 5 min after oral gavage with semiliquid non-absorbable rhodamine B-conjugated dextran (Invitrogen) containing 3% methylcellulose meal (Muller *et al.*, 2014). In parallel, the small intestinal transit was determined by gavaging the mice with non-absorbable liquid fluorescein isothiocyanate (FITC)-labeled dextran (Invitrogen). After 90 minutes, the animals were sacrificed and the contents of stomach, small bowel (divided into 10 segments of equal length), caecum, and colon (3 segments of equal length) were collected and the amount of rhodamine B and FITC in each bowel segment was quantified using a spectrofluorimeter (Ascent Labsystem). The distribution of the fluorescent dextran along the GI tract was calculated using the geometric center (GC): Σ (percent of total fluorescent signal in each segment x the segment number)/100 for quantitative comparison among experimental groups as previously described (Stakenborg *et al.*, 2017). In another set of mice 6% carmine red dye containing 1% methylcellulose meal was administrated by oral gavage after 7 days intraperitoneal injection of 20 µg MC-21 to determine the total gastrointestinal transit time which represents the time required to expel feces containing carmine red dye.

Colonic MR Imaging in Chronically Depleted Animals

For magnetic resonance imaging (MRI), mice were anesthetized with isofluorane (Halocarbon, River Edge, USA) in O2 (4% for induction, 1-1.5% for maintenance). Rectal temperature and respiratory rate were continuously monitored (SAII, Stony Brook, NY, USA), and isoflurane levels were adjusted to maintain a respiratory rate of 80-120 breaths per minute. Rectal temperature was maintained at 36.5-37.5°C. MR images were acquired using a Bruker Biospec 9.4 Tesla small animal MR scanner (Bruker BioSpin, Ettlingen, Germany; horizontal bore, 20 cm) equipped with actively shielded gradients (600 mT/m). Data was acquired using a quadrature radio-frequency resonator (transmit/ receive, inner diameter 7.2 cm, Bruker Biospin). After the acquisition of a localizer scan, the following MRI protocols were acquired: 2D-spin echo sequence (RARE) in axial and coronal orientation. For the axial slices, the following parameters were used: 2 slice packages of 16 axial slices of 1 mm thickness with 1 mm gap placed interleaved and providing coverage of 32 mm in z dimension with no gap and covering a region from the lung to the rectum; effective echo time (TE): 30 ms, repetition time (TR): 2558 ms, RARE factor: 6, 4 averages, FOV: 20 x 30 mm, in plane resolution 0.15mm. For the coronal slices, the following parameters were used: 2 slice packages of 10 coronal slices of 1 mm thickness with 1 mm gap placed interleaved and providing a coverage of 20 mm with no gap, effective TE: 30 ms, TR: 2000 ms, RARE factor: 8, 4 averages, FOV: 60 x 30 mm, in plane resolution 0.15mm. All images were processed using the Paravision 6.0 software (Bruker BioSpin). Colon diameter quantification was performed for each animal on axial images manually.

Quantification and Statistical Analysis

Quantification of Neuronal Ganglia

HuC/D-positive neurons were counted in 4 ganglia per submucosal and myenteric plexus (40x magnification) per animal. A ganglion was defined as a cohesive aggregate of HuC/D$^+$ cells. Extraganglionic cells are not counted.

Quantification of Cleaved-Caspase3

The number of cleaved caspase-3 and HuC/D double-positive neurons were counted in 4 ganglia per submucosal and myenteric plexus (40x magnification) per animal. A ganglion was defined as a cohesive aggregate of HuC/D$^+$ cells. The analyses in control versus self-maintaining gMac depleted mice were blindly conducted.

Quantification of Vascular Leakage

For microsphere particle counting, 4 confocal images were obtained from each whole mount and analysed using ImageJ software. Background intensity tresholds were applied using an ImageJ macro. Afterwards, the number of microsphere particles were counted using a standard particle analysis procedure in ImageJ. All analyses were assessed blindly.

Statistical Analysis

Statistical analysis was performed using Prism 7 (GraphPad Software). Data are represented as mean ± S.E.M and analyzed with the Mann-Whitney test or Kruskal-Wallis test with Dunn's multiple comparisons test. A p-value < 0.05 was considered statistically significant. For neuronal calcium imaging experiments, significance was analyzed using an unpaired two-tailed T-Test. For organ bath experiments, significance between dose- or frequency response curves was determined by two-way repeated ANOVA analysis.

Supplementary video, data and tables

Video S1-2: Ca^{2+} Imaging Recording of Myenteric Neurons *in Situ* After Electrical Field Stimulation, Related to Figure 7

Representative movies showing a myenteric plexus preparation from control (video 1) versus self-maintaining gMac depleted (video 2) *Cx3cr1*^{CreERT2}*.Rosa26-iDTR* mice stimulated by a train of electrical pulses (20 Hz, 2 seconds) transmitted to an interganglionic connective using a focal electrode. Recorded at 2 frames per second, video played at 10 frames per second. Fluo-4 tracings and image projections of this recording are shown in Fig. S6.

Video S3-4: Ca^{2+} Imaging Recording of Myenteric Neurons *in Situ* After DMPP Stimulation, Related to Figure 7

Representative movies showing a myenteric plexus preparation from control (video 3) versus self-maintaining gMac depleted (video 4) *Cx3cr1*^{CreERT2}*.Rosa26-iDTR* mice stimulated by local perfusion of DMPP (10 µM, 15 sec). Recorded at 2 frames per second, video played at 10 frames per second. Fluo-4 tracings and image projections of this recording are shown in Fig. S6.

Table S1: GO Terms, Biological Process and P-values of Self-Maintaining and Monocyte-Replaced YFP^+ and YFP^- LP gMacs as Determined by RNA-seq, Related to Figure 2

Genes were identified by RNA-seq of YFP^+ and YFP^- LP gMacs of *Cx3cr1*^{CreERT2}*.Rosa26-LSL-YFP* mice at 35 weeks after TAM treatment [data are from 1 experiment, YFP^+ versus YFP^- LP gMacs (n=4 each)]. Gene ontology analysis was performed using the Gene Ontology Consortium website (http://geneontology.org/) and redundant gene ontology terms were removed using REViGO.

Table S2: GO terms, Biological Process and P-Values of Self-Maintaining and Monocyte-Replaced YFP^+ and YFP^- ME gMacs as Determined by RNA Seq, Related to Figure 2

Genes were identified by bulk RNA-seq of YFP$^+$ and YFP$^-$ ME gMacs of *Cx3cr1*^{CreERT2}.*Rosa26-LSL-YFP* mice at 35 weeks after TAM treatment [data are from 1 experiment, YFP$^+$ versus YFP$^-$ ME gMacs (n=4 each)]. Gene ontology analysis was performed using the Gene Ontology Consortium website (http://geneontology.org/) and redundant gene ontology terms were removed using REViGO.

Table S3: P-values, Biological Processes and Matched Genes in Different Clusters of Self-Maintaining YFP$^+$ LP gMacs as Determined by scRNA Seq, Related to Figure 3

Genes were identified by scRNA-seq of 700 self-maintaining YFP$^+$ of *Cx3cr1*^{CreERT2}.*Rosa26-LSL-YFP* mice at 35 weeks after TAM treatment. Gene ontology analysis was performed using the GeneAnalytics website (https://ga.genecards.org/) and redundant gene ontology terms were removed using REViGO.