Title: Macrophages in the gut: masters in multitasking

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45 word abstract:

Macrophages in the gastrointestinal tract occupy different anatomic niches where they are exposed to a variety of environmental cues and perform niche-specific functions. In this review we describe the function of these subpopulations under homeostatic conditions and how they respond to challenges.

- **Summary**

The gastrointestinal tract has the important task to absorb nutrients, a complex process that requires an intact barrier allowing the passage of nutrients, but that simultaneously protects the host against invading microorganisms. To maintain and regulate intestinal homeostasis the gut is equipped with one of the largest populations of macrophages in the body. Here, we will discuss our current understanding of intestinal macrophage heterogeneity and describe their main functions in the different anatomical niches of the gut during steady state. In addition, their role in inflammatory conditions such as infection, inflammatory bowel disease and postoperative ileus are discussed, highlighting the roles of macrophages in immune defense. To conclude, we describe the interaction between macrophages and the enteric nervous system during development and adulthood and highlight their contribution to neurodegeneration in the context of aging and diabetes.

Introduction

Macrophages were first identified by Elie Metchnikoff in the early 1880s as cells that swarm to an injury site and phagocytose particulate materials. Subsequent studies have shown that tissue resident macrophages in different organs have similar morphological and functional characteristics, suggestive of a common progenitor. In the 1970s, van Furth therefore introduced the concept of the "mononuclear phagocyte system", proposing that all tissue macrophages derive from circulating monocytes that migrate into tissues to undergo further differentiation. However, in the past decade, the advent of fate-mapping technologies and parabiosis experiments has allowed for precise identification of tissue macrophage origin, uncovering that many tissue-resident macrophage populations are largely, if not exclusively, seeded during embryogenesis and are able to self-maintain locally throughout adulthood. Seminal studies further defined that during embryogenesis, macrophage precursors seed the different organs in three independent and subsequent waves of colonization deriving from either yolk sac-derived, fetal liver-derived and definitive bone marrow-derived hematopoiesis (Ginhoux et al., 2010; Gomez Perdiguero et al., 2015; Guilliams et al., 2013; Hoeffel et al., 2015; Yona et al., 2013). Tissue-resident macrophages are thus only replenished by circulating haematopoietic monocytes under specific conditions, such as depletion or disappearance over time. Thus, tissue macrophages in different organs represent a heterogeneous pool of macrophages with embryonic and haematopoietic origin (reviewed in (Ginhoux and Guilliams, 2016)).

The crucial importance of macrophages as major gatekeepers of tissue homeostasis is now well-established (Viola and Boeckxstaens, 2021). Macrophages rapidly adapt their function by sensing the surrounding micro-environment and acquiring a specific phenotype based on the microanatomical niche they occupy (Bleriot et al., 2020). This particularly applies to complex heterogeneous organs such as the gastro-intestinal (GI) tract.

The GI tract has the important task to orchestrate (I) the digestion and absorption of nutrients, (II) the protection of our body against intraluminal bacteria and, at the same time (III) the maintenance of a tolerogenic environment towards dietary and commensal antigens. Intestinal macrophages actively support homeostasis in the GI tract by performing a unique mix of diverse, and seemingly discordant duties. For example, recent studies suggest that muscularis macrophages can promote GI motility by supporting enteric neurons (De Schepper et al., 2018), while mucosal macrophages are tasked with supporting oral tolerance towards food antigens, while also eliminating pathogens that breach the epithelial barrier (Chang et al., 2013). As such, functionally distinct macrophage populations are found in different anatomical niches ranging from the epithelium, vascular plexus, intestinal crypts, secondary lymphoid organs, muscularis externa and the enteric nervous system.

In this review, we will first discuss the role of macrophages in the different intestinal microanatomical niches during homeostatic conditions. Subsequently, we will describe how these specific populations respond to challenges to ultimately orchestrate the return to homeostasis. We will discuss inflammatory challenges, as well as neuroimmune interactions and age-related changes that occur during development and aging.

Subepithelial macrophages: instructors of oral tolerance

The intestinal epithelium is composed of a single layer of polarized columnar cells that is continuously exposed to innocent and harmful microorganisms. To protect the host against invading pathogens, the entire length of the GI tract is highly enriched for tissue-resident macrophages, some of which closely interact with the epithelium (Chieppa et al., 2006). Positioned just underneath the epithelial monolayer, subepithelial macrophages are the first line of defense against invading pathogens, and as such are (I) highly phagocytic and (II) have strong bactericidal properties (Bain et al., 2013; Smythies et al., 2005). Interestingly, despite their constant exposure to and phagocytosis of microbiota and their byproducts, these cells do not generate an inflammatory response due to their unresponsive phenotype, a phenomenon defined as inflammatory anergy (Smythies et al., 2005). This phenotype is mainly induced by the tolerogenic environment of the lamina propria (the first intestinal layer from the lumen), conferred by high levels of interleukin-10 (IL-10) released by tissue resident $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cells (reviewed in (Barnes and Powrie, 2009)) and epithelial mediators such as IL-33 and IL-25 (Perez et al., 2020; Rivollier et al., 2012; Seo et al., 2017; Ueda et al., 2010; Wang et al., 2019). Of interest, subepithelial macrophages play a key role in the maintenance of this tolerogenic environment by contributing to the generation of IL-10 producing Treg cells. Moreover, transcriptional analysis of macrophages containing apoptotic bodies derived from epithelial cells reveals that efferocytosis (i.e. the phagocytosis of apoptotic bodies) also contributes to the repression of pro-inflammatory pathways (Cummings et al., 2016). Clearance of apoptotic epithelial cells by subepithelial macrophages is also vital for the maintenance of a healthy epithelium as evidenced by macrophage depletion experiments, where impaired clearance of apoptotic bodies led to epithelial disruption and colitis (Huynh et al., 2013; Sauter et al., 2014).

The peculiar combination of inflammatory anergy and high phagocytic capacity allows subepithelial macrophages to be key players in instructing T cell-mediated tolerance towards intraluminal antigens (Chang et al., 2013). It has been shown that subepithelial macrophages can sample harmless intraluminal antigens via transepithelial dendrites that extend into the intestinal lumen (Chieppa et al., 2006; Chikina et al., 2020; Niess et al., 2005). These protrusions towards the luminal space are dependent on the sensing of microbiota-derived metabolites such as lactate and pyruvate (Kim et al., 2018; Morita et al., 2019) and allow for phagocytosis of luminal content. The trapped antigens are subsequently transferred to $CD103^+$ dendritic cells (DCs) in the absence of inflammatory stimulation. DCs then migrate to the mesenteric lymph nodes for presentation of the processed antigens, which in turn will lead to the induction of antigen specific Treg cells. Treg cells then home to the gut where they expand and maintain oral tolerance via IL-10, thus perpetrating the tolerogenic macrophage-Treg cell loop (Hadis et al., 2011; Mazzini et al., 2014; Schulz et al., 2009).

Subepithelial macrophages require high energy expenditure and are constantly exposed to noxious agents from the lumen, and are thus short-lived, requiring constant replenishment by CCR2-dependent circulating progenitors (Bain et al., 2014; Bogunovic et al., 2009). Upon extravasation, $CCR2^+$ $Ly6C^+$ monocytes follow a well-defined path of maturation in which cells progressively lose the expression of the chemokine receptor CCR2 and Ly6C, while acquiring major histocompatibility complex class II molecules (MHCII), CD64 and CX3CR1 expression (Tamoutounour et al., 2012). This well-defined “waterfall” of differentiation takes place over

approximately one week (Kinnebrew et al., 2012; Schridde et al., 2017). Comparable to their murine counterpart, $CD14^+$ $CCR2^+$ human monocytes progressively lose CCR2 expression and upregulate the integrin CD11b and HLA-DR upon extravasation in the lamina propria (Bain et al., 2013; Bujko et al., 2018). Both in humans and mice, the driving force leading behind the constant recruitment and differentiation of circulating monocytes is the constant exposure to microbial-derived products (Collins and Bercik, 2009; Schridde et al., 2017) as evidenced by reduced recruitment and macrophage turnover in germ-free or antibiotic-treated mice (Bain et al., 2014; Niess and Adler, 2010; Shaw et al., 2018). Given the central role of the microbiota in regulating intestinal macrophage populations, it is not surprising that the GI tract harbors regional differences in terms of immune infiltrate. In fact, the load of enteric flora increases from the small intestine to the colon, and is accompanied by changes in its composition (Martinez-Guryn et al., 2019). Correlating with this increased bacterial colonization, the terminal segments of the gut are infiltrated by higher numbers of macrophages (Bernardo et al., 2018). Moreover, monocyte adoptive-transfer experiments show that, when extravasating to the intestinal tissue, differentiating cells upregulate different gene sets depending on the location of their egress (Gross-Vered et al., 2020). In line with these observations, transepithelial projections of macrophages aimed to impede absorption of noxious molecules by epithelial cells have only been observed in the distal colon (Chikina et al., 2020).

Blood vessel-associated macrophages: the gatekeepers of the intestines

The host requires protection from invading microorganisms not only at the level of the mucosal department, but it also requires protection from distribution of pathogens to the systemic circulation. To this end, endothelial cells are supported by a dense network of blood vessel-associated macrophages that establish a so-called gut-vascular barrier (Honda et al., 2020; Spadoni et al., 2015). Vasculature-associated macrophages in the villi are continuously replaced by circulating $CCR2^+$ cells and rely on differentiation of monocytes via NR4A1. This transcription factor is induced by mediators derived from the microbiome as the density and distribution of perivascular macrophages in the villi is significantly impaired in germ-free and antibiotic-treated mice (Honda et al., 2020; Kang et al., 2020). Notably, in the latter condition, the gut-vascular barrier is compromised resulting in increased systemic dissemination of intestinal bacteria during infection and increased tumor metastasis (Bertocchi et al., 2021; Honda et al., 2020).

Also blood vessels in the submucous vascular plexus are surrounded and supported by resident macrophages (De Schepper et al., 2018). In particular, we recently described a long-lived, self-maintaining population of CX3CR1+ macrophages characterized by the expression of genes involved in angiogenesis such as *Hif1a, Mmp14, Adamdec1* and *Rgs1*. Targeted depletion of these long-lived, resident $CX3CR1^+$ cells led to increased vascular leakage, indicating that these cells play a critical role in the maintenance of vascular integrity. Of note, while this population is clearly able to self-maintain, the precise origin of these and other intestinal macrophage populations remains still elusive. Indeed, the fate-mapping experiments performed in the intestine were performed at timepoints later than definitive hematopoiesis, thus not allowing for distinction between yolk sac, fetal liver or definitive

hematopoiesis derived macrophages (De Schepper et al., 2018; Honda et al., 2020). Finally, a population of CD169^{+} macrophages can be found located in the deeper margin of the lamina propria (Asano et al., 2018; Asano et al., 2015; Hiemstra et al., 2014). Based on surface marker expression, these CD169+ macrophages strongly resemble vascular-associated macrophages in the spleen (Asano et al., 2018; Asano et al., 2015; Tamoutounour et al., 2012). CD169^{+} macrophages display a strong phagocytic capacity and play a role in sensing and clearing circulating antigens (Asano et al., 2018; Asano et al., 2015; Kikuchi et al., 2018), and may therefore represent important gatekeepers regulating the intestinal inflammatory response (Kikuchi et al., 2018). Of interest, these cells are the main source of chemoattractant cues responsible for the recruitment of inflammatory cells from the circulation during colitis (Asano et al., 2015).

Enteric neuron-associated macrophages: the microglia of the gut

The digestion and absorption of food requires the fine coordination of many biological processes, including secretion and intestinal motility. To finely orchestrate different processes the GI tract is equipped with its own nervous system, i.e. the enteric nervous system (ENS), which controls intestinal function largely independently of brain or spinal cord input. The ENS is organized in two plexi: the submucous plexus located in the submucosa and the myenteric plexus located between the circular and longitudinal muscle layers of the muscularis externa.

While macrophages residing in the lamina propria display a classic, ameboid morphology, resident macrophages that are located in close proximity to the enteric plexi and within the muscle layers of the gut display a completely different morphology (De Schepper et al., 2018; Gabanyi et al., 2016). Macrophages located alongside the muscular fibers appear as elongated and bipolar, while those in the myenteric plane have a ramified morphology with long dendrites that remain static over time. Functionally, bipolar macrophages are important regulators of intestinal contractility through paracrine release of PGE2 that stimulates motility in a neuron-independent fashion (Luo et al., 2018), while ramified macrophages closely interact with neurons as evidenced by studies by our group and others (De Schepper et al., 2018; Gabanyi et al., 2016). We have recently demonstrated that the population of macrophages residing in close proximity to enteric neurons (neuron-associated macrophages) is long-lived and self-maintaining (De Schepper et al., 2018). Transcriptomic analysis reveals that these long-lived neuron-associated macrophages express several canonical microglia-like transcripts such as *Trem2, Tmem119, P2ry12* and *Olfml3*, a signature now known to be shared by several peripheral nerve-associated macrophage populations (Wang et al., 2020). Functionally, these neuron-associated macrophages are indispensable for neuronal survival: specific depletion of the long-lived population induced caspase3-dependent neuronal loss which ultimately leads to altered peristalsis and reduced intestinal secretion (De Schepper et al., 2018). These results suggest that this subpopulation may be involved in the pathogenesis of neurodegeneration of the ENS, as observed for example in aging and diabetes (Choi et al., 2008; Choi et al., 2010; Cipriani et al., 2018).

Concurrently, myenteric neurons are a major source of Csf1, thereby maintaining the local macrophage population (Drokhlyansky et al., 2020; Gabanyi et al., 2016; Muller et al., 2014). Moreover, macrophage-neuron crosstalk extends beyond their reciprocal induction of survival as many signaling interactions between these two cell types contribute to their respective functional outcome. For example, macrophages can stimulate neuronal activity in a BMP2-dependent fashion, regulating peristalsis (Muller et al., 2014). Conversely, activation of extrinsic sympathetic neurons during infection enhances the tissue-protective programs of macrophages via their β2 adrenergic receptors (Gabanyi et al., 2016).

Despite the growing body of evidence defining the presence and roles of a murine long-lived neuron-associated macrophage population, their presence in the human gut remains elusive. Flow cytometric profiling of human intestinal macrophages has identified 4 different populations based on the expression of surface markers such as CD14, CD11b, HLA-DR and CD11c (Bujko et al., 2018). Taking advantage of partial HLA mismatch during organ transplantation, the authors reveal that $CD14^+CD11b^+HLA\text{-}DR^+CD206^+$ macrophages have a significantly slower replacement by circulating monocytes when compared to the other three populations. Interestingly, these long-lived macrophages are located in the deeper layers of the intestine, where enteric neuronal soma are present. Despite this, direct evidence identifying the human neuron-associated population is still missing, mainly as none of the identified populations appear to possess the murine microglia-like core signature (De Schepper et al., 2018; Domanska et al., 2022; Wang et al., 2020). To overcome this, a recent transcriptomic study has used a specific cell–cell communication algorithms and gene network analysis to identify the $PMP22^+$ muscularis macrophage subpopulation as neuron-associated. Of note, PMP22 has been described to be a cell-adhesion molecule expressed by oligodendrocytes that allow their physical interaction with neuronal fibers. As such, PMP22 could be used also by intestinal macrophages to mediate their interaction with enteric neurons (Domanska et al., 2022). Clearly, more research is required to identify and characterize the human neuron-associated macrophage population.

In addition to neuron-associated and blood vessel-associated macrophages, we identified two smaller subpopulations that are long-lived; those residing close to Paneth cells in the intestinal crypts and a subpopulation in Peyer's patches. Insight into their function however is rather limited to date. Of interest though, macrophages associated with the crypt epithelium have been shown to be crucial to maintain and guide the correct differentiation of epithelial stem cells. Macrophage depletion by antiCSF1R antibody injection indeed reduced epithelial stem cell density and altered Paneth and goblet cell differentiation (Sehgal et al., 2018). Moreover, these pericryptal cells play a key role in the resolving phase that follows epithelial damage by mediating epithelial wound healing (cfr. challenge section) (Seno et al., 2009). The last important microanatomical location in the intestinal mucosa is represented by the secondary lymphoid organs, i.e. Peyer's patches. Given the complexity and specificity of the mononuclear system of Peyer's patches, we refer to other excellent reviews on this topic (Da Silva et al., 2017; Wagner et al., 2018).

Macrophages during infection

The mammalian digestive tract is home to trillions of microbes, archaea, protozoa, fungi, and viruses. Their density and composition changes along the GI tract to perform region-specific functions. Symbiotic interactions between the microbiota and host are required to maintain tissue homeostasis and prevent colonization of pathogens that damage the mucosal epithelium, enter the tissue and cause gastroenteritis, diarrhea and, in the worst-case scenario, sepsis and death. Indeed, infection-related intestinal disease still remains a global health problem, with significant morbidity and mortality, especially in developing countries and in infants (Khan et al., 2021).

Especially the gram-negative Enterobacteriacacae family including Citrobacter, Escherichia coli, Salmonella and Shigella, induce severe diarrhea with or without systemic disease (Reis and Horn, 2010). Unlike extracellular pathogens (such as enteropathogenic Escherichia coli [EPEC] and C. rodentium) which do not readily invade host cells, intracellular pathogens have evolved an efficient mechanism to enter and transverse the host epithelial cell (i.e. Salmonella and Shigella) (reviewed in (Cossart and Sansonetti, 2004)) to subsequently be taken up by the underlying immune cells such as subepithelial macrophages (Reis and Horn, 2010) in which they replicate (Beuzon et al., 2000; Bulgin et al., 2009; Fernandez-Prada et al., 2000).

Throughout this invasion and replication process, the innate immune system is not oblivious to bacterial pathogens. In fact, they are quickly alerted to their presence by intra- and extracellular pathogen-associated molecular patterns (PAMP)s which bind to specific pattern recognition receptors (PRR)s. This PRRs stimulation leads to the activation of the interferon regulatory factor-3 (IRF3), NF-κβ or mitogen-activated protein kinase (MAPK) signaling pathways resulting in the production of type I IFN and pro-inflammatory cytokines, ultimately leading to clearance of the invading pathogens (Kawasaki and Kawai, 2014; Locati et al., 2020).

Furthermore, intracellular bacterial effector proteins of the type III secretion system (T3SS), a machinery allowing bacteria to invade the intestine, replicate within the cell, evade the immune system and trigger pyroptosis, an inflammatory programmed cell death pathway dependent on caspase-1 (Hersh et al., 1999; Hilbi et al., 1998). In particular, the activation of caspase-1 through for example the NLRP1 and NLRP3 inflammasomes is induced by co-stimulation with microbial products, causing cleavage of the inactive precursors of IL-1β and IL-18 into mature inflammatory cytokines (Fantuzzi and Dinarello, 1999) and consequently rapid cell lysis. The functional relevance of caspase-1, IL-1β and IL-18 is underscored by the observation that respective genetically ablated mice have an increased bacterial burden and succumb earlier than their control littermates (Mattock and Blocker, 2017). The premature demise of infected macrophages, but also epithelial cells, is an intrinsic immune defense strategy to destroy the pathogen's preferred location of replication. Of note, Shigella actually provokes macrophage inflammatory mediated cell death as it is key for their dissemination into the surrounding epithelial cells (Mounier et al., 1992), eventually leading to loss of barrier function and severe mucosal inflammation. When bacteria invade and multiply within host cells, they also induce cellular damage causing the release of DAMPs. In particular, damaged epithelial cells release ATP through hemichannels composed of connexin 26 or 43 (Tran Van Nhieu et al., 2003). The extracellular ATP induces cell-specific activation through PRR, further promoting tissue damage and inflammation (Liu et al., 2017).

Paradoxically, phagocytes can also serve as a Trojan horse to transport pathogens, such as Salmonella, to systemic sites of colonization. To this end, Salmonella prevents its rapid recognition and subsequent macrophage pyroptosis by upregulating T3SS-2 (Lundberg et al., 1999). Studies on T3SS-2 null mutants or downstream effector proteins null mutants showed that T3SS-2 is required for reduced reactive nitrogen species (RNS)- and reactive oxygen species (ROS)-induced killing (Chakravortty et al., 2002; Vazquez-Torres et al., 2000), delayed pyroptosis (van der Velden et al., 2000) and disruption of gut vascular barrier (Spadoni et al., 2015). The Enterobacteriacacae family also circumvents host immune cell responses in other ways, i.e. suppression of IFNγ (Alphonse et al., 2022; Newton et al., 2010), MAPK and NF-κB (de Jong et al., 2016) signaling pathways (Ashida et al., 2015; Jones et al., 2008; Sperandio et al., 2008; Zhuang et al., 2017). Nevertheless, the initial inflammatory response that intracellular pathogens induce to efficiently infect neighboring epithelial cells and immune cells eventually leads to their own destruction. Namely, the recruited monocytes and neutrophils induce pathogen clearance (Cheminay et al., 2004; Dunay et al., 2008), a finding that is supported by clinical data showing that neutropenia is a risk factor for Salmonella bacteremia (Santos et al., 2009). Of note, the involvement of the adaptive immune system in the clearance of Enterobacteriaceae is discussed elsewhere (Ashida et al., 2015; Cummings et al., 2009; Griffin and McSorley, 2011).

While the highly motile mucosal macrophages can directly sense the presence of invading bacteria and its byproducts in addition to distress signals from epithelial cells, the static *muscularis* macrophages are less likely to be immediately alerted by signs of enteric infection due to their relative distance from the lumen (Gabanyi et al., 2016). Yet, as guardians of enteric neurons, it is essential that muscularis macrophages possess mechanisms to shield neuronal processes from infection- or inflammation-induced damage. Mattheis and colleagues have elegantly revealed that Salmonella infection activates extrinsic sympathetic fibers which enhances the tissue-protective phenotype (*Arg1, IL10, Mrc1, Cd163* and *Retnla*) of $CX3CR1^{+}$ muscularis macrophages via norepinephrine-β2 adrenergic receptor signaling (Gabanyi et al., 2016). This signaling pathway protects enteric neurons during bacterial (Salmonella) and protozoan (Toxoplasma Gondii) infection through the production of polyamines. Protective mechanisms notwithstanding, bacterial infection still leads to a significant loss of $VGLUT2^{+}$ excitatory neurons, leading to long-term post-infectious dysmotility (Matheis et al., 2020). Irrespective, these studies clearly show communication between the extrinsic nervous system and muscularis macrophages during bacterial and helminth infections to protect the ENS. To what extent similar mechanisms are at play during viral infections remains however unclear.

Macrophages in Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a debilitating disorder mainly consisting of Crohn's disease (CD) and ulcerative colitis (UC) and is characterized by recurring intermittent inflammation of the GI tract. It is a complex immune-mediated disease that arises in genetically susceptible individuals that display a dysregulated immune response towards the microbiome and other environmental factors (Stakenborg and Boeckxstaens, 2021). Certain IBD susceptibility genes (i.e. *CDH1*, *GNA12*, *C1orf106* and *PTPN2*) cause increased epithelial

cell death and a defective regulation of tight junctions which compromise gut barrier function and enhance the influx of luminal content (Khor et al., 2011). As a result, mucosal immune cells respond to this increased antigen load via activation of PRRs, causing tissue damage. Damaged and dying cells release DAMPs (i.e. S100A8-A100A9 complex, calprotectin, S100A12, HMGB1, and IL-1α and IL-33) that normally reside intracellularly or are sequestrated in the extracellular matrix, which when released promote inflammation (Boyapati et al., 2016). Genetic defects in PRR signaling of intestinal macrophages (i.e. alteration of the human NOD2 and CARD9 loci) lead to improper activation of NF$\kappa\beta$ signaling pathways or NLRP3 inflammasome, causing aberrant inflammatory cytokine production typically observed in IBD (de Lange et al., 2017; Graham and Xavier, 2020). When bacterial and DAMP components have breached the first line of defense (i.e. subepithelial macrophages), they reach the vascular niche. Here, perivascular macrophages attempt to protect the gut-vascular barrier in the villi and submucosa, but are not able to withstand the translocation of luminal content, as evidenced by the presence of endotoxemia detected in serum of IBD patients during active disease (Gardiner et al., 1995; Pastor Rojo et al., 2007). In addition, in a murine model of DSS-induced colitis, vasculature-associated $CD169^{+}$ macrophages act as sentinels and secrete the chemoattractant CCL8 upon PRR activation to orchestrate the recruitment of circulating immune cells. Notably, mucosal biopsies of IBD patients with active disease also express increased levels of CCL8 (Asano et al., 2015; Jones et al., 2018).

This particular inflammatory colitogenic setting in the gut and bloodstream is associated with a significant recruitment of $Ly6C^{hi}$ monocytes which rapidly differentiate into pro-inflammatory effector cells (Bain et al., 2014; Bain et al., 2013). Characteristic in this disease process is the halted differentiation of incoming $CX3CR1^{lo}$ $Ly6C^{hi}$ $MHCII^{lo}$ monocytes (equivalent to $CD14^{hi}$ $CD11c^{hi}$ $CCR2^{+}$ $CX3CR1^{+}$ monocytes in humans) into mature $Ly6C^{neg}$ $MHCII^{hi}$ $CX3CR1^{hi}$ macrophages (equivalent to $CD14^{hi}CD11c^{-}CCR2^{-}CX3CR1^{-}$ macrophages in humans) (Bernardo et al., 2018). In particular, monocyte differentiation is arrested at an immature stage, in which monocytes remain highly responsive to TLR signaling, (Bain et al., 2013; Hausmann et al., 2002) release of high levels of pro-inflammatory mediators (TNFα, IL1β, IL6 and IL12) and display reduced production of IL-10, thus further driving the influx of $Ly6C^{hi}$ monocytes as well as promoting type 1 and type 17 T helper (Th1 cell, Th17 cell) immune responses, which further aggravates tissue damage. The hyporesponsiveness of mature lamina propria $CX3CR1^{hi}$ macrophages, known to retain their anti-inflammatory characteristics also in this proinflammatory environment (Barkhordar, 1989; Weber et al., 2011), may thus be of particularly importance to restore immune regulation and maintenance of epithelial barrier during inflammation. Indeed, diphtheria toxin-targeted depletion of mature macrophages is sufficient to cause epithelial cell death, increased intestinal permeability and the development of colitis (Chikina et al., 2020). Of interest, some studies have interrogated if biological therapies (e.g. anti-TNF agents) commonly used to treat IBD have immunomodulatory effects on intestinal macrophages. In this regard, Dige and colleagues have found that the number of immature $CD14^{+}HLA\text{-}DR^{int}$ macrophages in CD patients is reduced following 4 weeks of anti-TNF adalimumab treatment (Dige et al., 2016). Another study shows that IBD patients responding to anti-TNF infliximab therapy increase the percentage of $CD68^{+}CD206^{+}$ mature macrophages and are capable of reducing T cell proliferation (Vos et al., 2012; Vos et al., 2011). Although these studies did not use identical markers to define macrophage subsets, they indicate that anti-TNF treatment could have the

potential to restore the homeostatic macrophage composition and thus promote mucosal healing in IBD patients.

During an IBD flare, tissue damage and inflammation is not limited to the (sub)mucosa. Also in the *muscularis externa*, morphological and functional changes occur, the most prominent being smooth muscle hypertrophy and plexitis of the ENS. The latter is defined by a significant influx of leukocytes inside and surrounding the enteric ganglia, contributing to neuronal damage, activation of enteric glia, altered peristaltic reflexes and GI dysmotility in models of experimental colitis and IBD patients (Lomax et al., 2005; Spear and Mawe, 2019; Stavely et al., 2020). Notably, histological observation of plexitis in unaffected intestinal regions is emerging as a prognostic tool for CD relapses (Ferrante et al., 2006), suggesting that ENS dysfunction may contribute to chronic intestinal inflammation. In support of this, a recent study by Dora and colleagues describes the existence of a blood-myenteric plexus barrier composed of extracellular matrix (ECM) proteins and enteric glial protrusions reminiscent of the BBB that limits the entry of 4 kDa FITC-dextran into the enteric ganglia. During DSS-induced colitis, this blood-myenteric plexus barrier is compromised in a macrophage-dependent manner, which may be a critical event to initiate enteric neuroinflammation via infiltration of DAMPs, PAMPs and proinflammatory leukocytes observed in plexitis (Dora et al., 2021).

The self-perpetuating pro-inflammatory milieu that sustains immune cell influx and activation needs to be halted in order to achieve resolution of inflammation. Unlike previously thought, this phase of tissue repair is a tightly controlled and is an active process of overlapping events. In fact, the resolution phase starts immediately following mucosal damage, as epithelial cells adjacent to the lesion lose their columnar polarity and start to proliferate to seal the wound and thus limit the entry of bacteria. Lamina propria macrophages support the regeneration of the epithelial barrier at the level of the pericryptal niche, where they accumulate upon damage. These pericryptal macrophages extend multiple cellular processes to interact with epithelial progenitors, thereby promoting their activation, proliferation and differentiation via Wnt (Cosin-Roger et al., 2016; Quiros et al., 2017), gp130 (Serrano et al., 2019; Taniguchi et al., 2015), TLR4 (Malvin et al., 2012; Riehl et al., 2020) and NOX1 (Leoni et al., 2013) signaling. The importance of pericryptal macrophages following mucosal injury is emphasized by the observation that macrophage-deficient $Csf1^{op/op}$ mice and Csf1r antibody injected macrophage-depleted mice display a delay in epithelial regeneration (Cosin-Roger et al., 2016; Sehgal et al., 2018). In parallel, dead epithelial and inflammatory cells are removed, a process that is crucial to limit secondary necrosis and prevent protraction of mucosal inflammation, as lingering apoptotic cells cause uncontrolled release of DAMPs and enzymes into the extracellular space (Na et al., 2019). To circumvent this, apoptotic cells themselves secrete “find me” signals including lysophosphatidylcholine, sphingosine-1-phosphate, CX3CL1 and nucleotides (ATP and UTP) to attract phagocytes. Once macrophages have migrated towards the apoptotic cells, they engulf the dying cells that express “eat me” signals (annexin-1, phosphatidylserine) at their cell surface (Schett and Neurath, 2018). In addition to limiting damage caused by lingering apoptotic cells, phagocytosis itself appears to promote inflammatory anergy. Indeed, transcriptional profiling of macrophages that phagocytose apoptotic epithelial cells show a general downregulation of genes involved in the immune response. Notably, these macrophages overexpress genes which overlap with 41 susceptibility genes for IBD, clearly indicating that defects in macrophage engulfment, i.e. efferocytosis, may contribute to the lack of mucosal healing observed in IBD (Cummings et

al., 2016). In addition, efferocytosis induces a phenotypic switch from a pro-inflammatory to pro-resolving macrophage. How pro-resolving mediators mediate the resolution of IBD inflammation is discussed in great detail elsewhere (Na et al., 2019).

Finally, for IBD resolution to occur, the typical stasis of macrophage differentiation in an immature state needs to be reversed, but the mechanisms involved remain elusive. Crucial to this resolution may be the restoration of the altered local environment that normally promotes macrophage differentiation (i.e IL10 and TGF**β**) and is now imbalanced by the presence of inflammatory mediators (Schridde et al., 2017). In particular, the levels of IFN***γ*** are significantly increased in the mucosa of IBD patients. This cytokine drives the pro-inflammatory features of $CD14^{hi}$ monocytes and macrophages in human (Kamada et al., 2008). In line, a general deletion of IFNγR1 or its downstream transcription factor STAT1 inhibits the generation of immature $Ly6C^{+}$ $MHCII^{+}$ macrophages (Nakanishi et al., 2018). Moreover, new evidence has also implicated the gut microbiota as a possible inhibitor of monocyte-macrophage differentiation. In fact, antibiotic-treated mice have a delayed differentiation of monocytes to $CX3CR1^{+}$ macrophages compared with non-antibiotic-treated mice. Besides the local microenvironment, circulating monocytes are found to have a more pathogenic phenotype (i.e. high expression of TNFα, iNOS, IL6 and STAT1) compared to healthy controls, which may arise from altered monocyte priming in the bone marrow as observed in infection (Askenase et al., 2015) or even the circulation (i.e. high bacterial serum levels in IBD patients) (Gardiner et al., 1995; Pastor Rojo et al., 2007).

Recurring intermittent inflammation and improper wound healing of the intestine eventually leads to progressive fibrosis and organ damage in IBD patients. Especially, CD patients often have to undergo surgery due to fibrotic complications, such as strictures. Recruited monocytes and immature macrophages have been associated with IBD-induced fibrosis (West et al., 2017), but the mechanisms involved remain poorly understood. A better understanding of the fibrotic consequences of recurrent inflammation has been obtained in murine models of chronic colitis. Progressive rounds of colitis induce the recruitment and accumulation of CCR2-dependent monocytes and fibrocytes, respectively (Kuroda et al., 2019). This, in turn, leads to increased production of metalloproteinases (MMP9, MMP13, TIMP1) and growth factors (TGFβ1, IL-33), leading to stimulation of $CD34^{+}$ mesenchymal stem cells (Stzepourginski et al., 2017; Waddell et al., 2021). Proliferation and differentiation towards the myofibroblast phenotype further promote the generation of a chronic profibrotic environment as these cells can both promote an autocrine feedback loop of stimulation via IL-6, IL-11 TGFβ1-mediated signaling, as well as promote CCL2- and CCL7-mediated inflammatory cell recruitment (Jasso et al., 2022). The progression of these loops of stimulation leads to collagen deposition, resulting in the stiffening and thickening of the submucosa and eventually pathological fibrosis, as reviewed in further detail in (Pasztoi and Ohnmacht, 2022).

Of interest, in CD patients several susceptibility loci associated to macrophages (i.e. NOD2, ATG16L1, IL12p40, IL23R and CX3CR1), are predictors of fibrostenosis (Rieder et al., 2017). Notably, NOD2 deficiency leads to a dysregulated homeostasis of macrophages and fibroblasts. Particularly, $CD14^{+}$ peripheral blood cells of NOD2 mutation carriers cause cells to increase expression of collagen *ex vivo*, a finding that was confirmed in a NOD2-deficient zebrafish model of DSS-induced colitis (Nayar et al., 2021). Moreover, an increased number of $IL36\alpha^{+}$ macrophages lying in close proximity to pericryptal fibroblasts has been reported in

IBD patients, a finding that was linked to the degree of inflammation and occurrence of stenosis (Scheibe et al., 2017; Scheibe et al., 2019). In line with this, Martin et al. have shown that immature macrophages closely interact with activated fibroblasts in the mucosa of CD patients (Martin et al., 2019). These activated fibroblasts express high levels of oncostatin in biopsies of IBD patients, and predict a failure to respond to anti-TNF therapy (West et al., 2017). Further single cell RNA sequencing studies also suggest immunomodulatory roles for oncostatin receptor^{+} intestinal fibroblasts through interactions with inflammatory monocytes (Smillie et al., 2019). Of interest, gp130 (encoded by *Il6st*) inhibition alleviates DSS-induced colitis and fibrosis, most likely via interactions with its ligands oncostatin-M, IL6 or IL11 (Nayar et al., 2021). Nevertheless, future studies are required to further study the macrophage-fibroblast interactions and their involvement in IBD-induced fibrosis.

Besides the fibrotic complications associated with IBD, the recurring intermittent inflammation also increases the risk for developing colorectal cancer (CRC). Colitis-associated cancer occurs in approximately 20% of UC patients opposed to around 8% of CD patients after 30 years of disease (Yuan et al., 2021). The sequence of inflammation–dysplasia–carcinoma in IBD-CRC is distinct from the sporadic normal–adenoma–adenocarcinoma sequence and often predicts a poorer prognosis. The chronic inflammation typically observed in IBD has been proposed to support oxidative stress-induced mutagenesis. In addition, the pro-inflammatory milieu in the gut contributes to cancer development via aberrant TNF-α-dependent NF-$\kappa\beta$ (Greten et al., 2004) and IL-6-dependent STAT3 (Becker et al., 2004) (Grivennikov et al., 2009; Matsumoto et al., 2010) signaling. Even though these key molecular pathways have been identified, the immune cell landscape of IBD-CRC remains to be further elucidated. Nevertheless, growing evidence is supporting an important role for macrophage for CRC development, growth and progression.

Macrophages during non-classical (sterile) challenges - PostOperative Ileus and diabetic gastroparesis

Each patient undergoing abdominal surgery will develop a transient impairment of GI motility, referred to as postoperative ileus (POI). This disorder has a significant impact on patient morbidity as it is characterized by symptoms including nausea, vomiting, absence of defecation and intolerance to solid food. This makes POI the most common complication responsible for prolonged hospitalization following abdominal surgery with an estimated cost of $1.5 billion in the US (Stakenborg et al., 2017).

Light microscopic studies have initially revealed that surgical handling of the intestine induces immediate histopathological changes in the mucosa followed by leukocyte infiltration in the muscularis externa starting 2 hours following tissue damage (Farro et al., 2016; Kalff et al., 1998). Of note, the observed mucosal damage was not accompanied by increased expression of pro-inflammatory mediators, which is in line with the finding that subepithelial macrophages do not become highly inflammatory upon bacterial challenge (De Backer et al., 2009). Only epithelial cells are found to have increased oxidative stress activity peaking 1 hour following gut manipulation (Anup et al., 1999), causing a transient failure of the epithelial barrier. However, bacterial translocation is unlikely to trigger POI, since *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice are not protected against POI (Stoffels et al., 2014). Therefore, most efforts towards unraveling the mechanisms of POI have focused on the muscle layer of the intestine. Bauer and colleagues were the first to discover that surgical damage to the intestine causes a subtle

inflammatory process in the muscularis externa leading to a prolonged inhibition of gut contractility (Kalff et al., 1998). In particular, they found that stellate macrophages residing in close proximity to enteric neurons are activated, as evidenced by increased expression of pro-inflammatory mediators peaking 1.5 hours following surgical insult (Farro et al., 2016). As a result, circulating leukocytes, mainly neutrophils and monocytes, infiltrate the muscle layer, where they produce nitric oxide (NO), further contributing to POI development (Turler et al., 2006). The crucial importance of muscularis macrophages in the pathogenesis of POI has been underscored in macrophage-specific depletion experiments showing that treatment with clodronate liposome abrogates leukocyte recruitment and prevents impairment of GI motility. In line, macrophage-deficient $Csf1^{op/op}$ mice are protected against POI (Wehner et al., 2007).

The mechanisms leading to the muscularis macrophage activation remain a conundrum: possible trigger for macrophage activation following surgical trauma is the presence of DAMPs in the extracellular environment. Indeed, ATP activation via a p38-dependent MAPK pathway triggers cytokine release and gliosis in enteric glia. P2X2 antagonism prevents ATP-induced gliosis, muscularis inflammation and protects against post-surgical dysmotility (Schneider et al., 2021). Similarly, mice pretreated with IL-1α antibodies and *Il1r1*$^{-/-}$ mice are protected against POI (Schneider et al., 2021; Stoffels et al., 2014). In the muscularis externa, IL1-R1 is mainly expressed by enteric glia, indicating that interactions between DAMPs, enteric glia and macrophages could trigger the early inflammatory phase of POI (Stoffels et al., 2014). Besides intracellular DAMPs, ECM components are additionally considered DAMPs. In fact, degradation of the ECM due to surgical damage has been shown to contribute to muscular inflammation during POI (Moore et al., 2011). Indeed, the fragmentation of high molecular weight hyaluronan into low molecular weight hyaluronan is well-known to stimulate pro-inflammatory processes via TLR signaling pathway (Petrey and de la Motte, 2014). Nevertheless, more in depth studies are required to identify mechanisms that are responsible for initial macrophage activation, which is the main orchestrator of the inflammatory processes in POI.

The resolution of muscular inflammation following surgical damage has been a quite neglected topic of POI research. It is however essential to curtail inflammation and restore intestinal homeostasis (Schett and Neurath, 2018). A first step towards tissue resolution depends on halting neutrophil influx into the inflamed tissue. This mainly occurs via a class-switch from the production of pro-inflammatory (such as PGE2) to pro-resolving PUFA-derived lipid mediators such as maresin-1, lipoxin B4, protectin 1, protectin DX and resolvin D1 and 2 (Stein et al., 2016). 12/15-lipoxygenase (ALOX15), the enzyme responsible for the synthesis of these pro-resolving lipid mediators, is highly expressed by monocyte-derived macrophages that are recruited to the post-surgical intestinal muscularis. Particularly, protectin DX has been shown to be potent in halting the influx of leukocytes and preventing POI (Stein et al., 2016). The importance of monocyte-derived macrophages in the resolution phase during POI is further underscored by the fact that *Ccr2*$^{-/-}$ mice have a delayed recovery of GI motility following surgical damage (Farro et al., 2017). Yet, the molecules underlying the switch from pro-inflammatory to pro-resolving macrophages remain to be identified. Besides the $CCR2^{+}$ monocytes, $F4/80^{hi}$ $GATA6^{+}$ large peritoneal macrophages might represent an alternative route to resolve intestinal inflammation. In particular, Honda and colleagues found that large peritoneal macrophages are rapidly recruited to sites of intestinal damage in response to ATP release by damaged serosal cells and subsequent CD44-hyaluronan

interactions. Here, they contribute to the removal of damaged cells, revascularization and collagen deposition, thereby promoting tissue repair (Honda et al., 2021).

Not only transient, but also sustained alterations in resident macrophages in the context of systemic disease, infection or inflammation can affect gut function. Loss of enteric neurons and interstitial cells of Cajal (ICCs, or the pacemaker cell of the gut) has been reported in the stomach and in the colon of both rodent models of diabetes and patients (reviewed in (Chandrasekharan and Srinivasan, 2007)). These widespread alterations to enteric neurons and ICCs commonly manifest with vomiting, constipation, diarrhea, and most notably, gastroparesis in patients (reviewed at: (Marathe et al., 2000)). While the role of macrophages in diabetic neuropathy in the colon is still somewhat unexplored, there is growing evidence that macrophages may play a key role in the development of diabetic gastroparesis. Studies in rodent models of diabetes have uncovered shifts in macrophage populations in the gastric muscularis externa leading to gastroparesis. Diabetes results in a reduction in anti-inflammatory $CD206^+$ macrophages, a finding accompanied by the upregulation of pro-inflammatory genes including *IL-6* and *iNOS*, and down-regulation of anti-inflammatory genes such as *HO-1*, *Arg1* and *Fizz1* (Cipriani et al., 2018). A similar reduction in $CD206^+$ macrophages was reported in the antrum of patients with diabetic gastroparesis, positively correlating with loss of ICC (Bernard et al., 2014; Grover et al., 2017). Intriguingly, in rodent models, loss of $CD206^+$ macrophages in the gastric muscularis externa causes increased oxidative stress and increased pro-inflammatory cytokine expression, leading to a loss of neuronal nitric oxide synthase-expressing neurons and the development of delayed gastric emptying (Choi et al., 2008; Choi et al., 2010). In line, mice that lack muscularis macrophages ($Csf1^{op/op}$ mice) are protected from the development of gastroparesis after diabetes induction; conversely, reconstitution of the macrophage pool in diabetic $Csf1^{op/op}$ mice led to development of delayed gastric emptying and ICC damage (Cipriani et al., 2018; Cipriani et al., 2016). Taken together, these findings suggest that macrophages play a critical role in the development of gastroparesis in diabetes, however further studies are warranted in order to clarify the mechanisms involved and how these cells could be targeted for therapeutic intervention.

<u>A different kind of challenge: macrophage vs time</u>

While macrophage function is clearly imprinted by multiple factors, increasing evidence suggests that developmental programming may also play a critical role. Indeed, macrophages in the kidney, heart and, of course, in the brain, display a significant developmental plasticity, adapting function, phenotype and often transcriptome to the age-specific requirements of the tissue (Cahill et al., 2021; Matcovitch-Natan et al., 2016; Munro et al., 2019). The most striking example of such developmental plasticity is certainly microglia, whose functions shift from synaptic pruning during early postnatal development, to homeostatic functions in adulthood, and finally to disease-associated phenotypes in aging (reviewed in (Matsudaira and Prinz, 2022)). In the intestine, little is known about early life macrophage function and how this may differ to adulthood, although evidence is starting to emerge that intestinal macrophages display extensive developmental plasticity and play a critical role in shaping the neonatal gut. In the mucosa, macrophage-derived insulin-like growth factor 1 (IGF-1) is critical for mucosal microvascular development early in life, and protects the neonatal intestine during necrotizing enterocolitis, suggesting that macrophages first support endothelial sprouting early in life, while then switching to a role of endothelial support in adult life (De

Schepper et al., 2019; Honda et al., 2020; Yan et al., 2022). Similarly, in the muscularis externa, we recently described a maturation switch in which muscularis macrophages are responsible for the refinement of the developing ENS during postnatal development, while then adopting to a neuro-supportive function in adulthood, a functional transition that is accompanied by significant transcriptional shifts (manuscript under peer-review; (Francesca et al., 2022)). While the factors triggering such developmental (re)programming remain to elucidated, more effort should be invested in defining how macrophages may contribute to the correct organization and development of the neonatal intestine, and define how perturbations of such perinatal macrophage functions may impact intestinal function and disease in adulthood.

In an analogous manner to postnatal development, aging may also represent a period of functional and phenotypic transition of intestinal macrophages, although very little is known about the impact of aging on intestinal macrophages. Indeed, while age-related changes in function and phenotype have been reported for circulating monocytes that replenish the intestinal macrophage pool, how these changes correlate to impaired intestinal function in aging remains to be elucidated (De Maeyer and Chambers, 2021). In mice, aging is accompanied by increased intestinal permeability and systemic dissemination of bacterial products, however it is unclear whether impaired clearance by intestinal macrophages drives this dissemination, or whether intestinal macrophages are merely overwhelmed as commensals and pathogens breach an increasingly leaky barrier (Thevaranjan et al., 2017). In addition, while cytokine production by mucosal macrophages appears to change with age, further studies are required to determine whether this is due to cell-intrinsic dysregulation or a mounting response to tissue 'inflamm-aging' (Jeong et al., 2017).

In addition to perturbations to the tolerogenic population of macrophages residing in the lamina propria, aging may also impact specialized macrophage subsets that have been identified within the intestinal layers. Notably, as neuron-associated macrophages are critical for the function and the survival of enteric neurons, it is intriguing to speculate a role of macrophages in age-related enteric neurodegeneration. Enteric neuropathy in aging is subject of somewhat conflicting reports, with age-related neurodegeneration or neuronal remodeling reported in rodents and humans in both submucosal and myenteric plexuses, however the extent of damage and the neuronal populations affected varies greatly between studies (reviewed in (Chandramowlishwaran et al., 2020)). In rodents, age-related neuronal loss in the myenteric plexus and increased transit time may be due to phenotypic changes in muscularis macrophages, correlate with a downregulation of the transcription factor FoxO3 (Becker et al., 2018). Similarly, within the gastrointestinal mucosa, age-related perturbations in the microvasculature are correlated with changes in the local macrophage pool, however whether these changes reflect dysfunction of blood-vessel associated macrophages specifically, or reflect the consequences of persistent low-grade inflammation in the tissue itself remains to be elucidated (Jeong et al., 2017). Given the increasing life-expectancy of the world's population, and the consequent rise in prevalence of age-related intestinal dysfunction, further research to broaden our understanding of age-related changes in macrophage function is urgently needed.

Finally, recent research in organs including the lung and the skin have raised the novel hypothesis that macrophages may be 'trained' by prior inflammatory events, which then affects later responses to related and unrelated immune challenges (Aegerter et al., 2020; Feuerstein et al., 2020; Guilliams and Svedberg, 2021). While the notion that macrophages can be 'trained' (otherwise referred to as memory, adaptive innate, or innate immune memory) by prior exposure to micro-organisms is not new, only recently have we truly begun to appreciate the implications of such innate training on the function and phenotype of tissue-resident macrophages (Kurtz and Franz, 2003; Locati et al., 2020; Netea et al., 2016). In the gut, infections with helminth *S. venezuelensis*, or bacteria *Y. pseudotubercolusis*, can induce a long-lasting tolerogenic and neuroprotective phenotype in muscularis macrophages via the upregulation of Arginase-1, which leads to neuroprotection in a subsequent infection (Ahrends et al., 2021). The notion of such training, a form of an 'innate memory', has a particular relevance when applied to the gut, as the gut hosts a plethora of inflammatory events in the lifetime of most human individuals. In line, when comparing mice bought from a pet shop to mice housed under pathogen-free conditions, a neuroprotective profile is observed in muscularis macrophages, suggesting that free-living animals may benefit from the constant exposure to pathogens colonizing the gastrointestinal tract as a mechanism of establishing tissue tolerance (Ahrends et al., 2021). Furthermore, it has been speculated that engrafting monocyte-derived macrophages that replace resident macrophages during inflammation may retain more plasticity than their resident counterparts, and may potentially perpetuate states of inflammation due to their inflammatory training (Guilliams and Svedberg, 2021). Given the extensive influx of monocytes in inflammatory conditions of the bowel, including IBD and enteric infections, it will be interesting to see how these findings can be expanded to other intestinal infections and disorders, and whether macrophage training and subsequent memory is indeed always beneficial or whether it can prove detrimental in conditions of chronic inflammation.

Concluding remarks

In recent years the field of intestinal macrophage biology has made major advances in terms of our understanding of macrophage heterogeneity, the role of the niche in determining macrophage phenotype and the roles of the various intestinal macrophage subsets in health and disease. However, despite the progress, key questions remain unanswered. In particular, it is striking that the ontogeny of intestinal macrophages has not yet clearly been defined, especially as it is as yet unclear whether ontogeny itself may play a role in macrophage phenotype and function. Furthermore, as macrophages display a striking degree of plasticity and adaptation, further effort is required to elucidate the role of specialized macrophage subsets in the initiation, progression and resolution of inflammation and infection. Finally, the recent advances regarding the role of macrophages in the support and maintenance of the enteric nervous system open the doors for further investigation on how to harness the therapeutic potential of these cells in the context of neurodegenerative disorders of the GI tract, which represent a huge socio-economic burden for the societies of today.

Table 1. Murine intestinal macrophage populations overview.

Population	Classic Mf functions	Specific functions	Ontogeny
Subepithelial Mf	● Highly phagocytic ● Highly bactericidal	● Inflammatory anergy ● Apoptotic bodies clearance ● Luminal antigen sampling	● Short-lived ● Microbiota dependent
Blood vessels associated		● Blood-gut barrier component ● Prevent bacterial dissemination ● Prevent metastasis	● Short-lived ● microbiota dependent ● NR4A1-dependent
Submucosal vascular plxus associated	● CD169+ ● Inflammatory cell recruitment	● Blood-gut barrier component ● Regulation of permeability	● Long-lived ● Self-maintaining
Bipolar muscularis macrophages		● Regulation of muscle contractility through PGE2 production	● Unclear ontogeny
neuron associate stellate muscularis macrophages		● Microglia-like marker expression ● Indispensable for neuronal survival ● Neuronal crosstalk through BMP2/CSF1	● Long-lived ● Self-maintaining
Paneth-associated macrophages		● Regulate epithelial stem cell numbers and differentiation ● Regulate goblet cell density ● Regulate epithelial wound healing	● Long-lived ● Self-maintaining
Peyer's patches associated		● TimD4+ ● CD4+	● Long-lived

Table 2. human intestinal macrophage populations overview as proposed by cited studies.

study	population	markers	characteristics	location
Bujko JEM 2018	Mf1	CD11b hi, CD64+, MerTK+, CD163+, CD115+, CX3CR1+, CD206-, CD1c-, CD103-, CCR2+, Calprotectin+	replaced within 3 weeks highly phagocytic, proinflammatory	Crypts?

			cytokine production upon stimulation	
	Mf2	CD11b+, CD64+, MerTK+, CD163med, CD115+, CX3CR1med, CD206+, CD1c+, CD103+, CCR2+, Calprotectin+	replaced within 3 weeks	Crypts?
	Mf3	CD11b-, CD64low, MerTK+, CD163+, CD115+, CX3CR1-, CD206+, CD1c-, CD103-, CCR2-, Calprotectin-	replaced between 6 and 52 weeks	villi
	Mf4	CD11b+, CD64low, MerTK+, CD163+, CD115low, CX3CR1low, CD206+, CD1c-, CD103-, CCR2-, Calprotectin-	replaced between 6 and 52 weeks BMP2+ low phagocytic	submucosa
Domanska Jem 2022	LpM	C1q+		Villi
	LpM	Calprotectin+		Crypts
	LpM	Lyve1+		Submucosa
	MM	Colec12+ Lyve1+	mRNA expression of PMP22	Neuronal fibers/Blood vessels
Bernardo Muc Imm 2018	CX3CR1 High	CD64+, CD14+, HLA-DRmed, CCR2+, CD40+, CD206+, CD163+	High IL-1b production	
	CX3CR1 med	CD64+, CD14+, HLA-DR+, CCR2-, CD40med, CD206+, CD163+		
	CX3CR1 low	CD64+, CD14+, HLA-DR+, CCR2-, CD40-, CD206+, CD163+	High IL-10 production	

- **Figure Legends**

Figure 1

(A) In the murine intestinal tissue, macrophages (green) reside throughout all the different intestinal layers. In the mucosa macrophages are short lived (light green). Conversely, in the deeper layers of the intestines long-lived, self-maintaining macrophages are present (dark green). (B) Sub-epithelial macrophages dispose of apoptotic epithelial cells. Moreover, sub-epithelial macrophages sample luminal content and transfer sampled antigens to CD103+ DCs. These DCs then migrate to the mesenteric lymph nodes where they mediate CD4+ Treg cell maturation. Tregs subsequently migrate back to the intestinal tissue where they maintain the tolerogenic environment through IL-10 secretion. (C) In the villi, short-lived, monocyte-derived macrophages (light green) wrap around the blood vessels and maintain the integrity of the gut-vascular barrier and prevent bacterial dissemination. The driving force for monocyte recruitment and differentiation are microbiota-derived byproducts as dysbiosis leads to reduced macrophage coverage and increased dissemination of pathogens. (D) Crypt-associated macrophages are long-lived (dark green) and regulate epithelial stem-cell differentiation and goblet-cell numbers. (E) In the submucosal vascular plexus, long-lived macrophages (dark green) regulate blood vessel permeability. (F) Smooth muscle-associated macrophages regulate muscle contractility through paracrine secretion of PGE2. (G) In the myenteric plexus, neuron-associated macrophages are maintained via neuron-derived CSF1 secretion. Conversely, macrophages regulate neuronal function through paracrine secretion of BMP2. Moreover, (H) neuron-associated macrophages are required for enteric neuron survival.

Figure 2

In experimental models of colitis increased intestinal permeability and epithelial cell death cause PAMPs translocation and DAMPs release. Subepithelial macrophages and vascular-associated macrophages are activated via PRR inducing the release of pro-inflammatory mediators (e.g. CCL8) which recruit leukocytes (i.e. monocytes and neutrophils) from the bloodstream. While at the steady-state recruited CCR2+ monocytes differentiate into mature MHCII+ Ly6C- macrophages, during colitis flares, the monocyte-to-macrophage differentiation is halted to an immature state; differentiating monocytes remain highly responsive to danger signals and exacerbate the inflammation by releasing vast amounts of pro-inflammatory mediators. In the muscular layer, inflammation is characterized by a degraded blood-myenteric barrier (BMB) and influx of leukocytes inside and surrounding the enteric ganglia (i.e. plexitis). During resolution pericryptal macrophages promote mucosal healing via increased stem cell proliferation. In parallel, apoptotic epithelial and immune cells are cleared by nearby macrophages, inducing a phenotypic switch from a pro-inflammatory to pro-resolving macrophage. Recurrent inflammatory flares lead to the failure of the pro-resolving pathways and to chronic inflammation and fibrosis. Experimental models of chronic colitis display an altered villi structure and a thickening of the submucosal layer due to fibroblast activation. Pro-resolving macrophage-derived TGFb1 induces the differentiation and proliferation of CD34+ fibrocytes towards the myofibroblast phenotype. Myofibroblasts in turn generate a self-maintaining feedback loop of autocrine TGFb1, IL-6 and IL-11 stimulation. Myofibroblast activation leads to collagen deposition and fibrosis of the intestinal

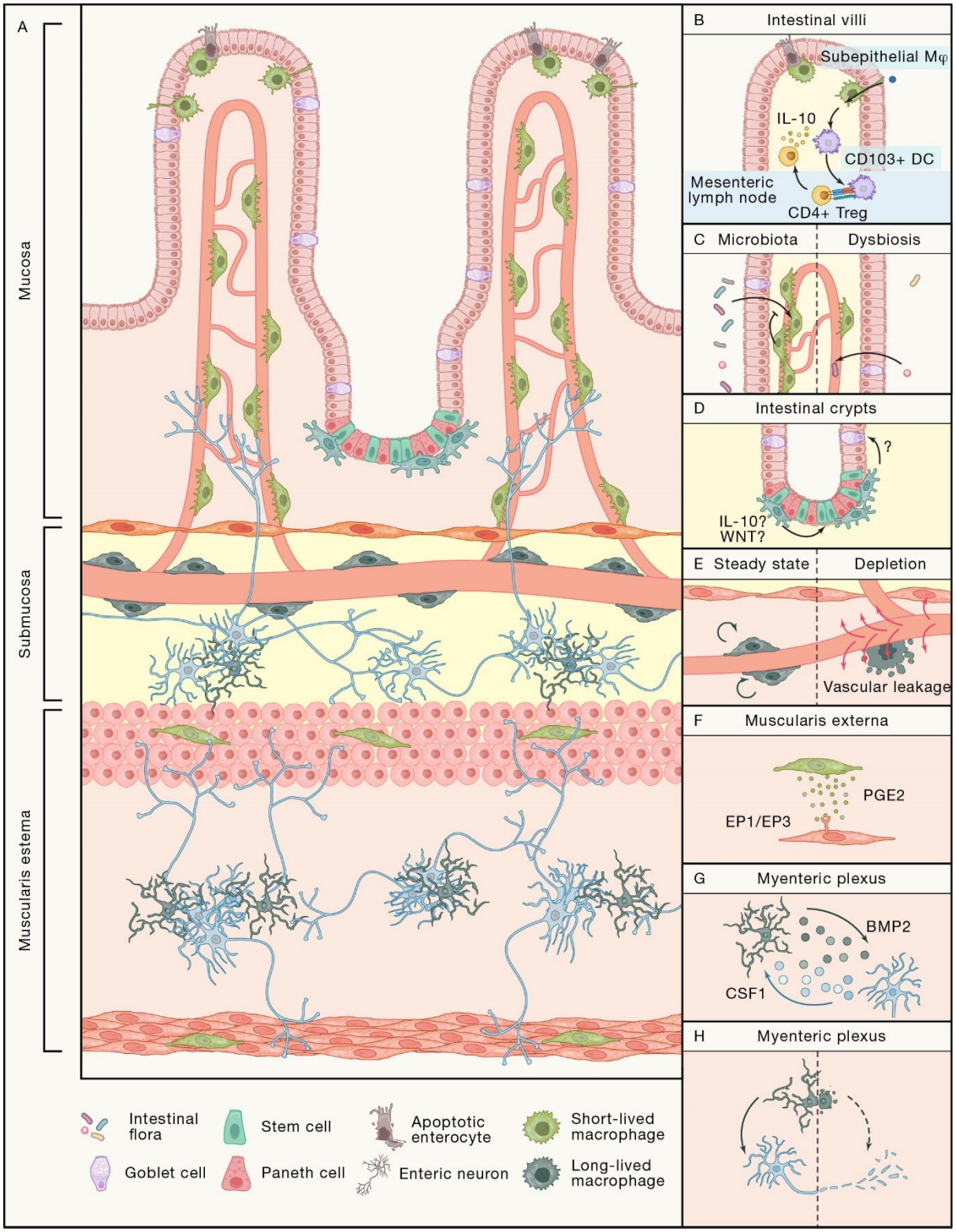
tissue. Furthermore, fibroblast-derived CCL2 promotes circulating monocyte recruitment and the maintenance of a chronic inflammatory microenvironment.

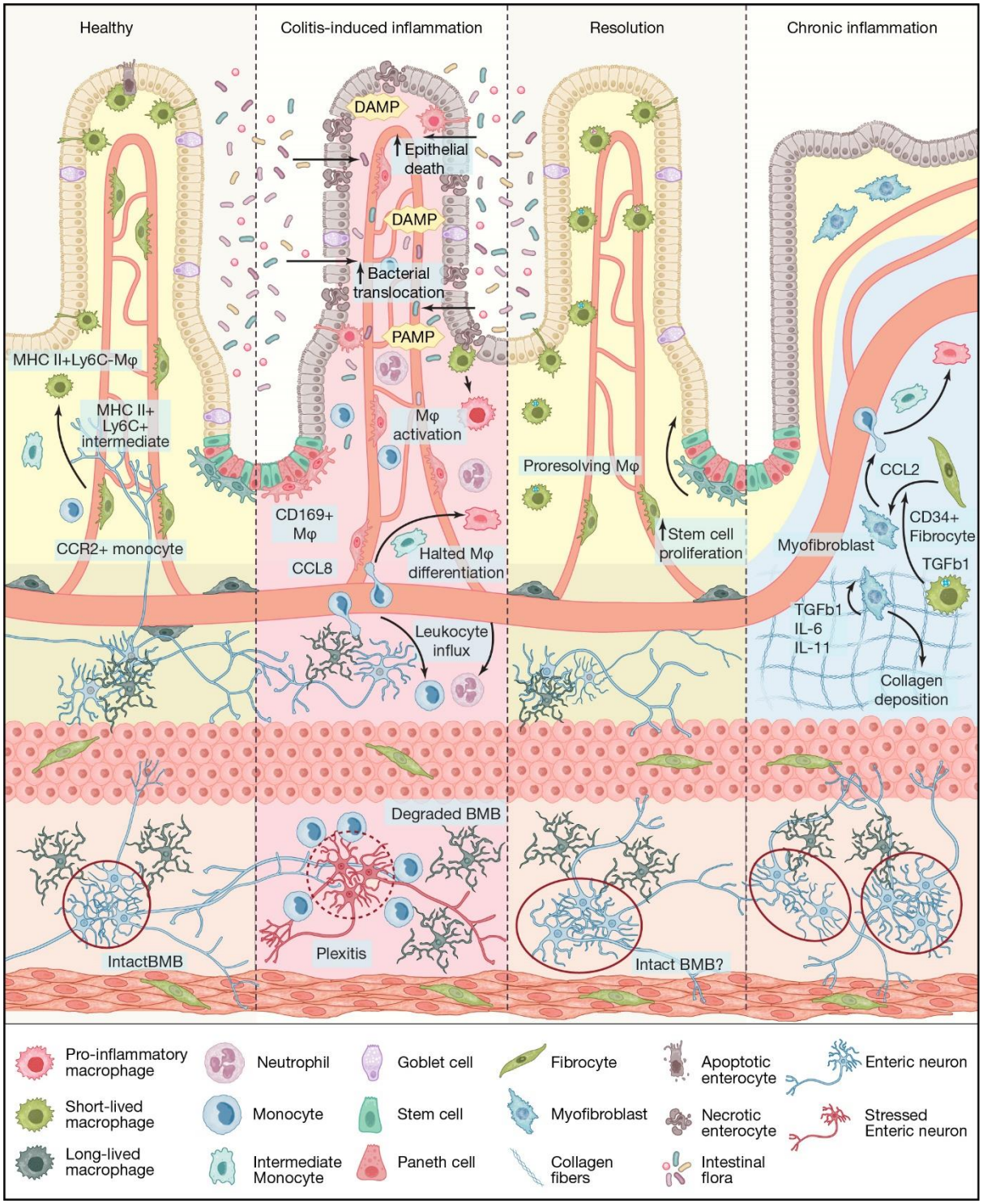
Figure 3

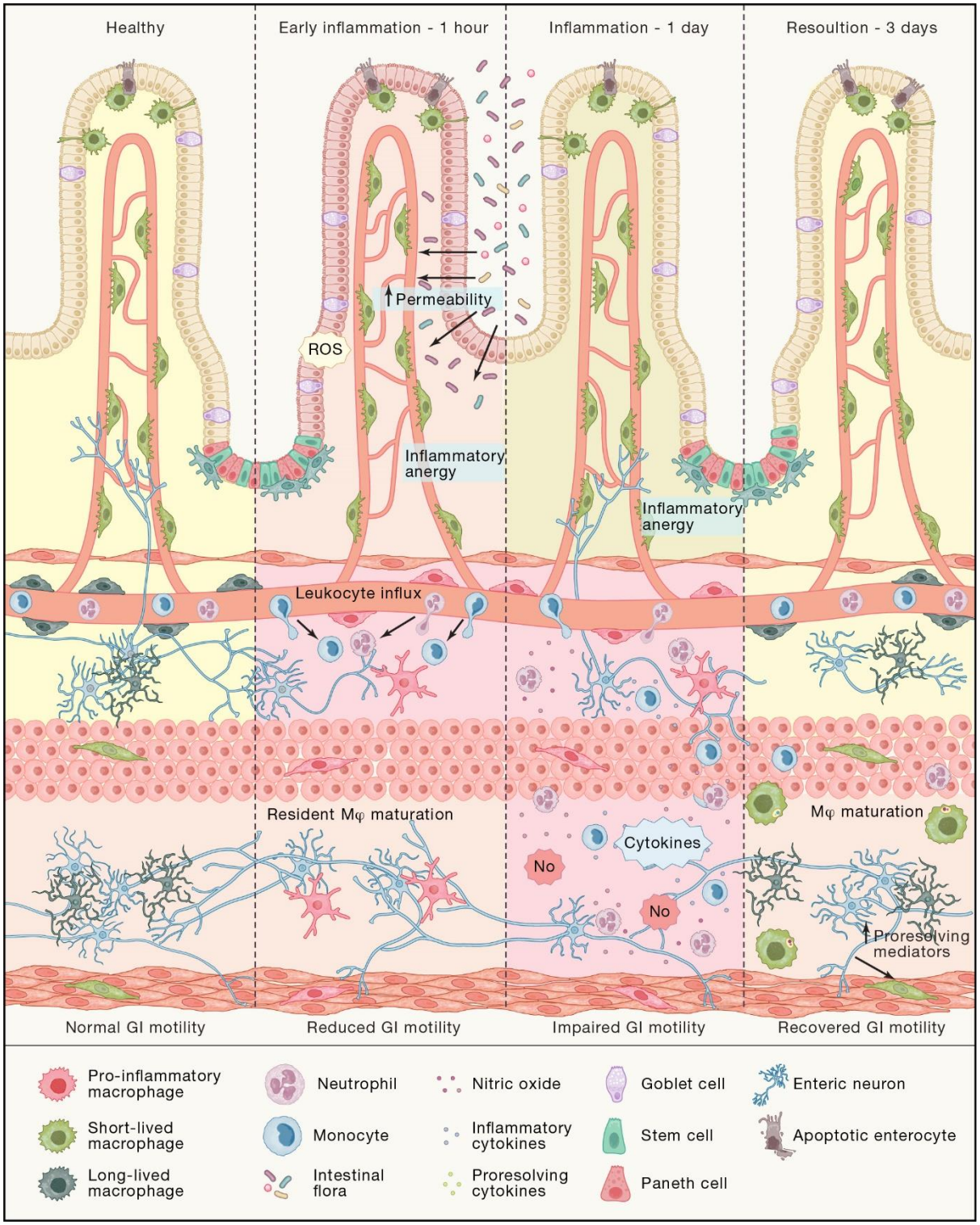
Surgical manipulation of the murine intestine induces reactive oxygen species (ROS) production by the epithelial cells causing a temporary increase in the intestinal permeability. Noteworthy, subepithelial macrophages remain relatively anergic towards the bacterial translocation following intestinal handling. In the muscular layer, however, resident macrophages are activated following surgical handling, leading to the release of pro-inflammatory mediators and influx of circulating leukocytes (i.e. monocytes and neutrophils) already 1 hour after intestinal damage. These recruited leukocytes secrete additional pro-inflammatory cytokines and vast amounts of nitric oxide (NO) eventually causing impaired gastrointestinal motility or postoperative ileus (POI). The resolution of POI is characterized by clearance of apoptotic neutrophils by macrophages, allowing them to switch from a pro-inflammatory to pro-resolving phenotype.

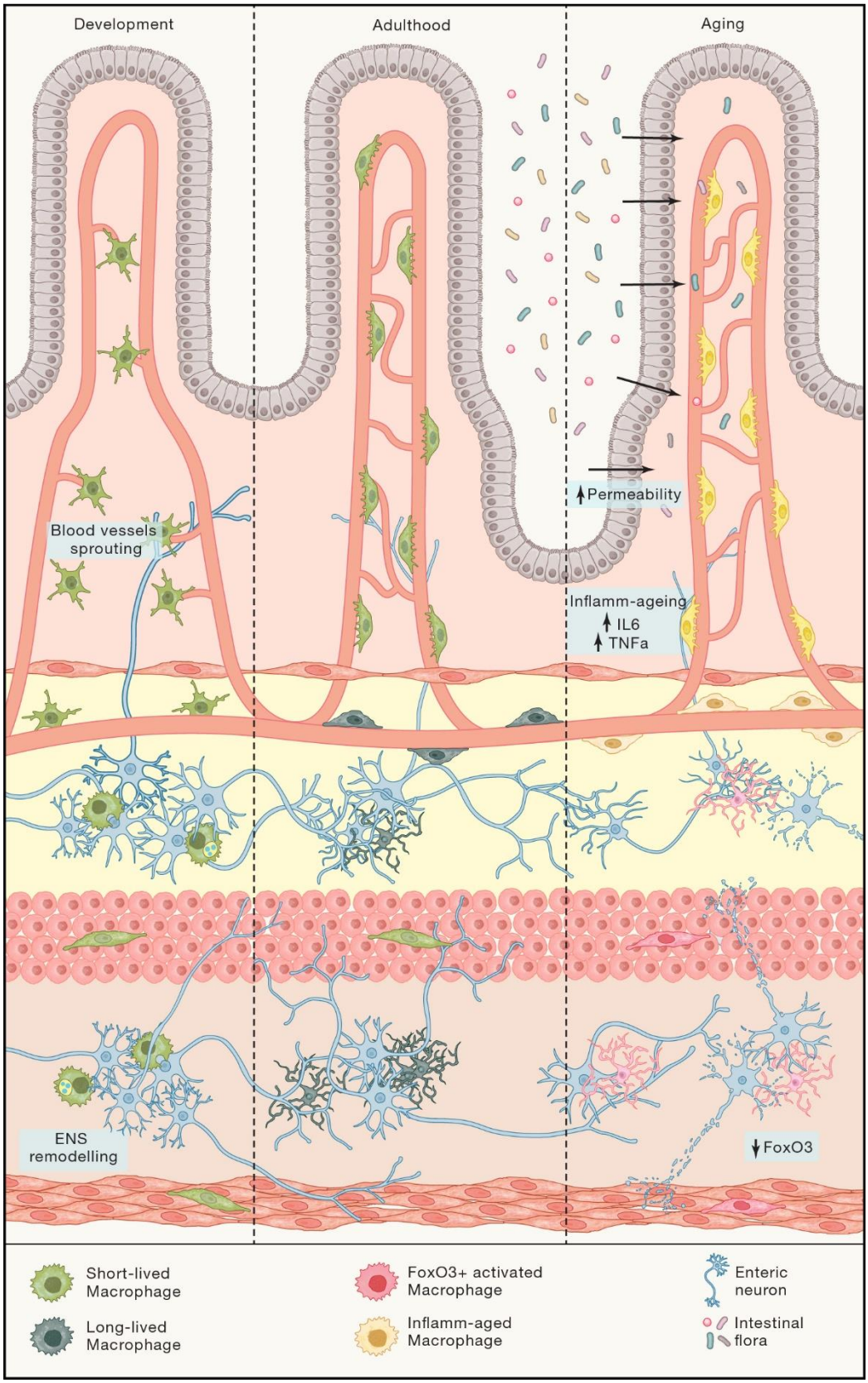
Figure 4

During development, mucosal macrophages are involved in the vascular bed remodeling promoting and regulating blood vessels sprouting. In the deeper intestinal layers, macrophages help refine the neuronal network by phagocytose synaptic material and controlling neuronal numbers. With aging bacterial translocation is increased due to a progressive increase of the epithelial barrier permeability. Concomitantly with the increased bacterial translocation, mucosal macrophages lose their anergic phenotype and produce higher amounts of pro-inflammatory mediators such as IL-6 and TNFa. Similarly, in the deeper intestinal layers, macrophages lose FoxO3 expression and shift their polarization state inducing the progressive loss of enteric neurons.


A
Mucosa
Submucosa
Muscularis estema
B
Intestinal villi
Subepithelial Mφ
IL-10
CD103+ DC
Mesenteric lymph node
CD4+ Treg
C
Microbiota
Dysbiosis
D
Intestinal crypts
?
IL-10?
WNT?
E
Steady state
Depletion
Vascular leakage
F
Muscularis externa
PGE2
EP1/EP3
G
Myenteric plexus
BMP2
CSF1
H
Myenteric plexus
Intestinal flora
Goblet cell
Stem cell
Paneth cell
Apoptotic enterocyte
Enteric neuron
Short-lived macrophage
Long-lived macrophage


Healthy
Colitis-induced inflammation
Resolution
Chronic inflammation
DAMP
Epithelial death
DAMP
Bacterial translocation
PAMP
MHC II+Ly6C-Mφ
MHC II+ Ly6C+ intermediate
Mφ activation
Proresolving Mφ
CCL2
CD34+ Fibrocyte
CD169+ Mφ
CCR2+ monocyte
CCL8
Halted Mφ differentiation
Stem cell proliferation
Myofibroblast
TGFb1
TGFb1 IL-6 IL-11
Leukocyte influx
Collagen deposition
Degraded BMB
IntactBMB
Plexitis
Intact BMB?
Pro-inflammatory macrophage
Short-lived macrophage
Long-lived macrophage
Neutrophil
Monocyte
Intermediate Monocyte
Goblet cell
Stem cell
Paneth cell
Fibrocyte
Myofibroblast
Collagen fibers
Apoptotic enterocyte
Necrotic enterocyte
Intestinal flora
Enteric neuron
Stressed Enteric neuron


Healthy
Early inflammation - 1 hour
Inflammation - 1 day
Resoultion - 3 days
Permeability
ROS
Inflammatory anergy
Inflammatory anergy
Leukocyte influx
Resident Mφ maturation
Mφ maturation
Cytokines
No
No
Proresolving mediators
Normal GI motility
Reduced GI motility
Impaired GI motility
Recovered GI motility
Pro-inflammatory macrophage
Short-lived macrophage
Long-lived macrophage
Neutrophil
Monocyte
Intestinal flora
Nitric oxide
Inflammatory cytokines
Proresolving cytokines
Goblet cell
Stem cell
Paneth cell
Enteric neuron
Apoptotic enterocyte



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