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The Phylogeny, Ontogeny, and Organ-specific Differentiation of Macrophages in the Developing Intestine

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Abstract

Macrophages are large highly motile phagocytic leukocytes that appear early during embryonic development and have been conserved during evolution. The developmental roles of macrophages were first described nearly a century ago, at about the time these cells were being identified as central effectors in phagocytosis and elimination of microbes. Since then, we have made considerable progress in understanding the development of various subsets of macrophages and the diverse roles these cells play in both physiology and disease. This article reviews the phylogeny and the ontogeny of macrophages with a particular focus on the gastrointestinal tract, and the role of these mucosal macrophages in immune surveillance, innate immunity, homeostasis, tissue remodeling, angiogenesis, and repair of damaged tissues. We also discuss the importance of these macrophages in the inflammatory changes in neonatal necrotizing enterocolitis (NEC). This article presents a combination of our own peer-reviewed clinical and preclinical studies, with an extensive review of the literature using the databases PubMed, EMBASE, and Scopus. Macrophages were first described at the beginning of the previous century by Paul Ehrlich and Ilya Metchnikoff as important mediators of innate immunity.¹ The name “macrophages” or “big eaters” came from the Greek words, “makros” or large, and “phagein” or eat.² Macrophages are large cells with an irregular cell shape, oval- or kidney-shaped nucleus, cytoplasmic vesicles, central nucleus, and high cytoplasm-to-nucleus ratio.³ These cells are highly phagocytic and motile, and modulate immune responses by releasing various mediators.⁴ The term mononuclear phagocyte includes lineage-committed bone marrow precursors, circulating monocytes, resident macrophages, and dendritic cells (DCs).⁵ In this review, we have focused on the macrophage lineage as it expands and matures first, *in utero*, and plays an important role in the innate immune responses of newborn infants.

Keywords

Blood counts; Inflammation; Macrophages; Monocytes; Organ injury; Signaling

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Evolution of Macrophages

Macrophages have Developed in a Conserved Process through Evolution

Macrophages can phagocytose smaller organisms, foreign materials, and cellular debris.^{6,7} This ability to ingest particles larger than 0.4 μm in diameter has followed a recognizable pattern across evolution, be it in unicellular Protists such as the soil-living amoebae *Dictyostelium discoideum* and protozoans such as *Trichomonas vaginalis*, or in macrophages in multicellular eukaryotes.^{8,9} Phagocytic cells in invertebrate species are known by other names such as amebocytes, celomocytes, or hemocytes, but each shows morphological similarities with the macrophages of vertebrates (Flowchart 1).^{10,11} These similarities extend to the molecular level with the expression of proteins containing the scavenger receptor cysteine-rich (SRCR) domains.^{6,12}

In diploblastic animals, the cellular endo- and ectoderm are separated by a gelatinous matrix (mesoglea) that contains motile amebocytes, which ingest and digest food caught by enterocytes and then transport the nutrients to the other cells.⁸ These cells also promote innate immunity with pattern-recognition receptors (PRRs) and pore-forming proteins such as the macrophage-expressed gene 1 protein (Mpeg1).¹³

In triploblasts, which represent the next stage in evolution, the middle layer is composed of mesodermal cells instead of the mesoglea but it contains similar phagocytes.¹⁴ The two subclasses, the Protostomia and the Deuterostomia, are named based on sequence of the development of the gastrointestinal tract openings.¹⁵ Vertebrates are a subclass of this group.¹⁶

Macrophage-like Cells in Triploblastic Prostomes with a Celomatic Cavity

In celomatic animals, an inner mesenteric layer holds the gut in the central cavity, and this promotes somatic growth.¹⁰ The circulatory system further helps in increasing body size through efficient diffusion of gases and nutrients, and the removal of metabolic waste products.¹⁷ This vascular system contains circulating macrophage-like defense cells known as the celomocytes/hemocytes. Similar cells, the plasmatocytes, have been identified in the corresponding ontogenic stage in developing fruit flies (*Drosophila melanogaster*).¹⁸

Blood vascular systems may be structured in one of two principal designs: open or closed.¹⁷ In the open circulatory system of arthropods and noncephalopod molluscs, the circulating hemolymph empties from a contractile heart and major supply vessels into the hemocele body cavity where it directly bathes the organs. Annelids like the earthworm, cephalopods, and nonvertebrate chordates have a closed circulatory system, where the intestinal surface is in contact with soil microorganisms.¹⁹ There are two freely circulating subpopulations of phagocytes, the autofluorescent eleocytes and amebocytes celomocytes.²⁰ Amebocytes, not eleocytes, express PRRs and the Toll-like receptor (TLR) signaling pathway.²¹ These cells also express the oxidative stress-related superoxide dismutase and the antimicrobials lysozyme and lumbricin.²²

Macrophage-like Cells in Deuterostomes such as the Chordates and its Constituent Vertebrates

Deuterostomes include the subclasses Chordata and Echinodermata (Flowchart 1).²³ One of the chordate subphyla is comprised of the vertebrates. In the vertebrate intestine, the mononuclear phagocyte system has three cellular lineages: monocytes, macrophages, and DCs,²⁴ that are released from the bone marrow.^{25,26} The other phylum, Echinodermata, also contains circulating macrophage-like phagocytes.^{27–31}

In zebrafish, embryonic macrophages migrate from the mesoderm into the spleen and gut.^{32,33} The macrophages express tumor necrosis factor (TNF) to regulate the expression of mediators such as interferon regulatory factor 8 (IRF8), the complement Clq genes, and the G-protein-coupled receptor 35 and shape the gut microbiota. Macrophages also promote intestinal lymphangiogenesis through the vascular endothelial growth factors.

Amphibian macrophages promote immune defenses, homeostasis, and tissue remodeling.³⁴ Chemokines such as the CXC ligand 12 (CXCL12) stimulate granulocyte/macrophage precursors to migrate from the liver to the bone marrow.³⁵ Macrophage differentiation is controlled through binding of the colony-stimulating factor-1 (CSF-1) to its cognate CSF-1 receptor (CSF-1R) on committed macrophage precursors. IL-34 also binds CSF-1R and promotes differentiation into morphologically and functionally distinct macrophages.³⁶

In reptiles, macrophages are seen in the lamina propria but can migrate into the subepithelial lymphoid aggregates.¹⁰ Birds also have a well-developed mucosal immune system.³⁷ The gut lamina propria contains innate immune cells such as macrophages, although the differences with DCs have not been reported in detail. These cells express many PRRs. Early-life microbial colonization is critical for immunological maturation. Macrophages are involved in antigen uptake and protect against invading pathogens.^{38,39}

Mammals show macrophage maturation in patterns that resemble those in humans (as described below). Obviously, there are important species-specific differences in the timing in gestation (day of pregnancy) and the relative importance of specific genes or genetic isoforms.

Development of Macrophages in Humans

Macrophage development has three different phases during the embryonic, fetal, and neonatal period (Flowchart 2).

Macrophage Differentiation in the Embryo

- *Lineage-restricted progenitors in the yolk sac (YS):* Hemocytoblasts with myeloid characteristics are first seen in blood sinuses in the secondary YS on day 18.⁴⁰ On day 19, some large-sized histiocytes, which is a term for tissue-resident macrophages, can be seen.^{41–44} Two distinct macrophage lineages appear at 5 weeks; a larger MHC II-neg fraction appears first in the YS, mesenchyme and the fetal liver, and then in the thymic cortex, lymph nodes, splenic red pulp, and the bone marrow.⁴⁵ A minor population of MHC II⁺ cells can be seen in the liver at 7–8 weeks, lymph nodes at 11–13 weeks, the thymic medulla at 16 weeks, and

then in the gastrointestinal tract. These MHC II⁺ cells then expand gradually.^{45,46} Yolk sac-derived macrophages are independent of the transcriptional factor c-Myb during development, but depend on the transcriptional factor PU.1.⁴⁷

- *Erythro-myeloid progenitors (EMPs)*: On day 25, the YS and the embryo show EMPs developing from the capillary endothelium.⁴⁸ These cells proliferate and differentiate into macrophages by day 30 and then migrate to all embryonic organs except the central nervous system (CNS).⁴⁹
- *Endothelial precursors in the aorta-gonad-mesonephros (AGM) zone*: The vascular endothelium in this zone produces CD34⁺ CD45⁺ hematopoietic stem cells (HSCs),⁵⁰ which differentiate into common myeloid progenitors (CMPs) and then into tissue macrophages either directly or via a monocyte stage. These macrophages migrate to all embryonic organs, except the CNS.⁴⁹
- *Microglial precursors*: Some cluster of differentiation (CD) 45⁺ CD34⁺ myeloid cells located near the dorsal aorta migrate around day 30 into the CNS and differentiate into microglial precursors.^{49,51,52}

Most macrophages in fetal organs develop from EMP and AGM progenitors.^{51,52} Some EMPs in the liver differentiate directly into Kupffer cells without passing through a monocyte stage.⁵³ However, both these EMP-derived lineages are eventually replaced by BM-derived macrophages.

Macrophage Differentiation in the Liver—On day 32, some CD45⁺ CD34⁺ HSCs migrate from the AGM zone to the liver⁵¹ and then differentiate into monocytes and macrophage precursors in the 8–20 weeks period. These cells comprise nearly 70% of all hematopoietic cells in the fetal liver, but then involute during the 20–23 weeks period when the erythroid cell pool begin to expand.⁵⁴ Some of these macrophages may arise from EMPs or from the CD34⁺ CD45⁻ hemogenic endothelial cells that produce CD33⁺ macrophage precursors.

Monocyte development in the bone marrow followed by tissue migration and macrophage differentiation: While some CD34⁺ CD45⁺ HSCs migrate from the AGM zone into the liver, others migrate on the same day into the bone marrow. These cells remain detectable at 1 in 60 CD34⁺ CD45⁻ cells even at 24 weeks' gestation.⁵⁴ In the monocyte–macrophage lineage, the differentiation shows several discernible stages, including the CMPs, granulocyte–monocyte precursors (GMP), common monocyte and DC precursors (MDP), premonocytes (committed monocyte progenitors), and then monocytes.⁵⁵ These are all noticeable by the 7th week of gestation. After birth, the HSCs migrate from the liver to the bone marrow and mature as part of “definitive” hematopoiesis.⁵⁶

There are three subsets of differentiating monocytes, a classification recognized by the nomenclature committee of the International Union of Immunologic Societies:

- *Classical, CD14⁺⁺ CD16⁻ cells* (80–90%), which express CCR2, CD64, and CD62L. These show strong phagocytic activity, rapid responses to TLR ligation,

and express inflammatory cytokines and reactive oxygen species (ROS) to recruit other leukocytes.⁵⁷

In mice, classical monocytes (Ly6C⁺⁺) differentiate into macrophages in most organs⁵⁸ except in the gut, which has its own precursor cells.³ However, these cells are uniform precursors of inflammatory macrophages in disease states in all organs. Some Ly6C⁺⁺ “tissue monocytes” in nonlymphoid organs may serve as effectors without differentiating into macrophages or DC, and present antigens to T-cells.⁵⁹ A subgroup of Ly6C⁺⁺ monocytes released from the marrow may also serve in a diurnal “anticipatory inflammation” regulated by the circadian gene Brain and Muscle ARNT-like 1 (BMAL1), as an innate response to frequently occurring environmental challenges.⁶⁰

- *Non-classical, CD14^{low}CD16⁺ cells* (10%) that lack CCR2, but express the Fc γ receptors CD64 and CD32.⁶¹ These monocytes patrol the blood vessels and respond via a TLR7-triggered pathway to remove senescent endothelial cells.⁶² Some cells extravasate to promote tissue healing, but show limited phagocytic activity and inflammatory responses to bacterial products.^{61,63}

Murine studies show the corresponding Ly6C⁻ cells to be terminally differentiated.⁵⁸ These cells are regulated by the transcription factor Nuclear Receptor Subfamily 4 Group A Member 1 (NR4A1).⁶⁴ Some of these Ly6C⁻ monocytes might directly develop from MDPs in the marrow.⁶⁵ Ly6C⁻ monocytes exhibit a longer half-life of 5–7 days, longer than the 8-hour lifespan of Ly6C⁺ cells.^{66,67} Ly6C⁻ monocytes may be also be seen as terminally differentiated resident macrophages in blood or the “vasculature macrophages,” rather than *bona fide* monocytes.⁶⁸ Indeed, the primary function of these cells seems to be to patrol the vascular endothelium and monitor its integrity.

- *Intermediate cells that express both CD14 and CD16.*⁶⁸ A variable number of such cells express MHC-II, present antigens, and activate T lymphocytes. The exact role of these cells is not clear.

Monocytes are limited to blood, bone marrow, and spleen. These cells first appear in blood during the 5th month of gestation,⁵⁴ but comprise only a small proportion of the cellular lineages until the bone marrow becomes the predominant site of hematopoiesis.⁶⁹ Monocyte concentrations in the blood rise between 22 and 42 weeks; these cells comprise 3–7% of hematopoietic cells at 30 weeks⁷⁰ and there is a relative monocytosis at term with counts between 300 and 3300/ μ L (median 1400/ μ L). After birth, absolute monocyte counts rise during the first 2 weeks and then begin to drop in the 3rd week.^{71–73} After leaving the bone marrow, monocytes circulate for 1–3 days and then move into tissues to differentiate into macrophages or into myeloid dendritic cells (mDCs). Both monocyte-derived macrophages (MDMs) and mDCs are involved in a variety of immune functions such as phagocytosis, antigen presentation and cytokine production.⁷⁴ During the fetal/neonatal period, monocytes frequently develop epigenetic changes such as DNA methylation, microRNA expression, or histone modifications, that alter the immune pathways.⁷⁵ For instance, decreased trimethylation of lysine 4 on histone 3 (H3K4me3) alters the maturation of neonatal monocytes.⁷⁶

In infants, monocytes are important determinants of innate immunity. There are several aspects to consider:

- *Maturity in movement:* By term gestation, monocytes attain considerable maturity in movement needed in trans-endothelial migration, chemotaxis, phagocytosis, and respiratory burst. Unlike neutrophils, cord blood monocytes show adherence, random migration, chemotaxis, phagocytosis, bactericidal activity.⁷⁷
- *Maturity in pathogen elimination:* Neonatal monocytes generate superoxide anion (O²⁻) and hydrogen peroxide at levels comparable to those from adults.^{78–80} Fetal and neonatal monocytes can kill pathogens such as *S. aureus*, *S. epidermidis*, *E. coli*, and *C. albicans* similar to those from adults.^{78,81}
- *Maturity in inflammatory responses:* Neonatal monocytes produce cytokines such IL-1 β , IFN- α and TNF at levels comparable to adults, but not IFN- γ , IL-8, IL-10, and granulocyte–macrophage colony-stimulating factor (GM-CSF). These cells also produce less extracellular proteins such as fibronectin, and bioactive lipids like leukotriene B₄.^{82–86}

As in amphibians and other phyla, monocyte differentiation into macrophages is controlled through binding of the colony-stimulating factor-1 (CSF-1) to its cognate CSF-1 receptor (CSF1-R) on committed monocytes.⁸⁷ IL-34, which is expressed mainly in the skin and the CNS, also binds CSF-1R.⁸⁸ These ligands promote the differentiation of myeloid precursors into morphologically and functionally distinct macrophages.

Classification of MDMs—Monocyte-derived macrophages are comprised of two major phenotypes, the classically activated M1 and an alternatively activated, immunoregulatory M2 phenotypes (Flowchart 2).⁸⁹ The M1 and M2 subpopulations could represent distinct differentiation paths of a common precursor, or could represent a maturational pathway where the M1 phenotype transitions to M2 with loss of CD14 and increased expression of CD16 and other markers. Based on murine models with progressive loss of Ly6C, the latter possibility seems more likely.⁹⁰ The two subgroups show different surface markers, tissue localization, function, and intracellular signaling.

- M1 macrophages* respond to cytokines such as TNF and interferon- γ , bacterial lipopolysaccharide (LPS), and the granulocyte–macrophage colony-stimulating factor (GM-CSF). These cells express CD54, CD80, CD86, and CD197.^{91,92} In most situations, M1 macrophages are more efficient at phagocytosis and bactericidal functions;⁹³ *in vitro*, macrophages are activated toward an M1 functional program by infectious microorganism-related molecules and by inflammation-related cytokines TNF or IFN- γ .⁹⁰ M1 macrophages are characterized *in vitro* by an IL-12^{hi}IL-23^{hi}IL-10^{lo} phenotype; are efficient producers of toxic effector molecules (ROS and NO) and inflammatory cytokines (IL-1 β , TNF, IL-6); participate as inducers and effectors in polarized Th1 responses.⁹⁰ These cells show high level expression of the CC chemokine receptor 2 (CCR2) and low levels of the CX3C-chemokine receptor 1

(CX3CR1).⁹⁰ Accordingly, the chemokine CCL2 recruits CD14⁺ human/Ly6C⁺ murine monocytes to inflammatory sites.⁹⁴

Most M1 macrophages die, killed by their own NO production.⁹⁵ In an experimental acute lung injury model, these cells undergo Fas-mediated death, while the resident alveolar cells persist. M1 is likely a terminal differentiation phenotype, but some can undergo phenotype conversion to become tissue-resident macrophages. Macrophage polarization may be both transient and plastic.⁹⁶ The patrolling monocytes respond to the CX3C-chemokine ligand 1 (CX3CL1; human fractalkine/murine neurotactin), a chemokine present both in soluble and membrane-bound forms expressed on endothelial cells and in tissues.⁹⁷

- b.** *M2 macrophages* may respond more strongly to IL-4, IL-10, IL-13, and IL-21, and to glucocorticoids, and express high levels of surface scavenger receptors such as CD163, CD204, and the mannose receptor, CD206.^{91,92} Accordingly, these cells show low-level expression of the CC chemokine receptor 2 (CCR2) and high levels of the CX3C-chemokine receptor 1 (CX3CR1).⁹⁸

M2 macrophages are active in immunoregulation, maintain tissue integrity following injuries and in chronic infections, and promote angiogenesis.⁹³ The M2 macrophages are a relatively heterogeneous group, comprised of at least 5 sub-categories (M2a, M2b, M2c, M2d, and M2f).^{4,99}

In vitro, M2 polarization has been noted in response to the concomitant activation of Fcγ receptors and TLRs, and to exposure to immune complexes and to anti-inflammatory molecules such as IL-10, TGF-β, and glucocorticoids.⁸⁹ M2 cells are characterized by an IL-12^{lo}IL-23^{lo}IL-10^{hi}TGF-β^{hi} phenotype and generally have high levels of scavenger, mannose, and galactose-type receptors.^{58,100} In general, these macrophages take part in polarized Th2 responses, dampening of inflammation, tissue remodeling, angiogenesis, and immunoregulation.¹⁰¹

Tissue-resident macrophages are maintained locally by proliferative self-renewal, and retain an M2-like phenotype, for example, in the peritoneal cavity, brain, and lung.¹⁰² The proliferation rates are low in steady-state conditions, but increase under inflammatory challenges.¹⁰³ Monocyte-derived macrophages may have fates similar to tissue-resident macrophages with maintenance of M2-like phenotypes and a low self-renewal capacity.⁵⁸ A number of cells probably die during inflammation, where the extent of survival possibly depends on the nature and magnitude of the insult.⁵⁸

Generally, monocyte/macrophage development, differentiation, and proliferation are driven by macrophage colony-stimulating factor (CSF-1), GM-CSF, and cytokines such as IL-4 and IL-34.¹⁰⁴ CSF-1 is constitutively produced by mesenchymal cells, and it promotes the maturation of Ly6C⁺ monocytes to Ly6C⁻.¹⁰⁵ CSF-1 also increases macrophage proliferation with a negative feedback loop via macrophage production of CCL2.⁵⁸ It helps maintain the macrophage pool in the gut, kidney, peritoneal cavity, BM and hence in circulation, but not in the liver. It promotes M2 polarization. During inflammation, GM-CSF

is the primary driver of hematopoiesis and it promotes the proliferation of M1-polarized MDMs.¹⁰⁶

Dendritic cells (DCs) are a leukocyte subset specialized for antigen-presenting function. Similar to macrophages, DCs also originate early from a common granulocyte–monocyte–dendritic cell progenitor¹⁰⁷ in the YS, mesenchyme, and the liver at 4–6 weeks of age.¹⁰⁸ DC precursors differentiate into:^{109,110} (1) myeloid DCs (or mDCs), CD11c⁺ cells that express myeloid markers such as CD13, CD33, CD1a-d, and CD11b; and (2) plasmacytoid DCs (or pDCs), CD11c[−] cells with a plasmacytoid morphology with well-developed rough endoplasmic reticulum and Golgi apparatus.¹¹¹ However, DCs mature most after birth¹¹² Neonatal DCs comprise about 0.3% of all mononuclear cells.¹¹³ Compared to adults, neonatal pDCs exhibit low expression of ICAM-1 and MHC antigens and of co-stimulatory molecules CD40, CD80 or CD86, show a less efficient maturational response and immunostimulatory function,^{114,115} Neonatal DCs express, and are less efficient in lymphocyte activation than those from adults.¹¹⁴

Determinants of Macrophage Polarization—In inflamed tissues, different subtypes of macrophages can be seen. The mechanisms are unclear, but such variability in polarization may arise from the presence of conserved lineages (rooted phenotypes), plasticity (possible to shape/mold), or flexibility (possible inter-convertibility). Here are these three possibilities:

1. *Conserved lineages:* resident macrophages derived from YS or hepatic precursors are hypo-inflammatory and retain tissue-protective and reparative functions, whereas MDMs may evolve into different lineages.^{5,116} For instance, human CD14⁺⁺ inflammatory monocytes and murine Ly6C⁺ monocytes may differentiate into M1 macrophages.¹¹⁷ Human CD16⁺ or murine Ly6C[−] monocytes may differentiate into resident tissue cells (if not from transdifferentiation of M1 macrophages, *vide infra*).^{58,118}
2. *Plasticity related to phases of inflammation/specific tissues:* monocytes recruited soon after the onset of inflammation may differentiate into M1 macrophages, and those recruited during disease resolution may develop M2 characteristics.^{119,120} In some tissues such as the intestine, extracellular matrix (ECM) contents such as transforming growth factor (TGF)- β and other mediators such as IL-10 can convert monocytes into hypo- or noninflammatory macrophages.¹²¹
3. *Flexibility related to phases of inflammation/microenvironment:* macrophages may retain the ability to switch from one phenotype to the other depending on the microenvironmental stimuli.¹²² M2 macrophages can be activated into M1 following exposure to TLR ligands or IFN- γ .¹²² M1 macrophages might acquire M2 properties during resolving inflammation with increased sensitivity to ECM components such as TGF- β , although further evidence is needed that these changes do not get interrupted by nitric oxide.¹²³ Another example of such a phenotypic switch is with repeated exposures to LPS, which can induce *endotoxin tolerance* with a global switch in gene expression program.¹²³

Monocytes/Macrophages Can Carry and Present Antigens in Lymphoid Tissue

—Some monocytes that enter inflamed tissues do not differentiate into macrophages, but are able to phagocytose antigen(s) and carry those to naïve T-cells in lymph nodes. Tissue macrophages can also present antigens to the maturing adaptive immune system. M1 MDMs may present antigens and activate/polarize effector Th1 and Th17 cells upon production of IL-12 and IL-23, respectively. The TNF superfamily and the TNF receptor superfamily are likely involved, but not co-stimulators such as CD80, CD86, and CD28. Similarly, M2-like tissue macrophages, which produce TGF- β and express the α V β 8 integrin may be involved in the polarization of regulatory T cells.

Innate Immune Memory^{124–130}

- Tissue-resident macrophages and MDMs can recognize microbial or damage-associated molecules for enhanced recruitment and differentiation of circulating monocytes into M1 macrophages.
- Tissue monocytes are recently described cells that can take up antigens in the tissue and move to lymph nodes, where they are able to present antigens to naïve T-cells.
- After resolution of an acute inflammatory illness, memory macrophages or monocytes may be retained. These cells are functionally programmed by a previously stimulus for either altered cytokine production to optimize the immune response depending on the type/concentration of the immune stimulus.

Gut Macrophages in the Developing Intestine are a Specialized, Hyper-inflammatory Cellular Population

The gastrointestinal tract contains the largest reservoir of macrophages in the body.¹³¹ Macrophages appear in the fetal intestine at 11–12 weeks' gestation, increase rapidly during 12–22 weeks, and then at a slower pace through early childhood.^{44,132–137} These cellular groups are broadly similar in the small intestine and colon,¹⁰ and form a critical part of the innate immune system to encounter luminal bacteria that may breach the epithelium to enter the lamina propria^{112,138,139} Gut macrophages also promote peristaltic movements and promote tolerance for antigens derived from diet and commensal microbiota.

In the neonatal intestine, there are two distinct pools of macrophages, one comprised of mature macrophages that might have been derived from YS precursors, and another that could represent MDMs. During inflammation, the MDM pool enlarges and newly recruited monocytes are seen (Fig. 1). The numerical development of the macrophage pool, unlike that of mucosal lymphoid aggregates, is programmed in the fetal intestine and does not require the presence of dietary or microbial antigens.^{132,140} The gut macrophage pool in the fetus contrasts with the macrophages in the lung; there are very few alveolar macrophages in the fetus and this population expands after birth.^{141–152} However, the functional maturation of gut macrophages continues during early infancy and is influenced by antigens in ingested food and microbiota.

Unlike many other organs, intestinal macrophages have very limited ability to undergo clonal expansion.¹³⁸ The primary mechanism for maintaining the gut macrophage pool is through the recruitment and differentiation of blood monocytes.^{138,139,153} In adults, interleukin-8/CXC ligand 8 (IL-8/CXCL8) and transforming growth factor-beta (TGF- β) recruit monocytes to the intestinal mucosa.¹³⁸ However, developmental limitations in the fetal intestine may preclude these two cytokine systems in effective recruitment of monocytes as macrophage precursors. In the fetus, IL-8 is expressed primarily as a longer, less-potent 77-amino acid isoform unlike the shorter 72-amino acid isomer in the adult.¹⁵⁴ Similarly, TGF- β bioactivity is low in the early-/mid-gestation fetal intestine.¹⁵⁵ Finally, macrophage populations begin to expand in the fetal intestine several weeks before lymphocytes or neutrophils,^{132,133,135} suggesting that the recruitment of monocytes as macrophage precursors may occur via specific chemoattractant(s) other than IL-8/CXCL8, which recruits both neutrophil and macrophage precursors,^{138,156} or TGF- β , which also mobilizes T-lymphocytes.^{138,157}

We have reported that chemerin (the retinoic acid receptor responder-2/RARRES2) might be a key chemoattractant for monocytes in the normally developing fetal intestine. Chemerin is a 16 kDa heparin-binding molecule^{158,159} expressed in fetal IECs beginning at 10–14 weeks with a peak at 20–32 weeks and then gradual diminution to minimal levels towards term.^{160,161} The chemerin promoter contains several CpG islands located in close vicinity of retinoic acid receptor (RAR)- β binding sites;^{162,163} RAR- β is now recognized as an epigenetic regulator with multiple variants, the expression of which is regulated through alternate promoter usage and differential splicing.¹⁶⁴ Interestingly, only monocytes, not neutrophils or lymphocytes, express the chemerin receptor, the chemokine-like receptor-1 (CMKLR1).^{165,166} The developmental importance of chemerin as a monocyte chemoattractant in the intestine is also related to the fact that it is broken by cysteine proteases into fragments that inhibit the responses of inflammatory responses of these cells to bacterial products.¹⁶⁷ In the injured neonatal intestine, macrophage-rich infiltrates are prominent, which contrasts with the pleomorphic leukocyte infiltrates in inflammatory bowel disease or gut inflammation models in adult mice.^{168–173} The chemokine CXCL5 is an important chemoattractant for macrophage precursors to the neonatal intestine.¹⁶⁸

Intestinal macrophages in mammals share some common features. Unlike macrophages in other organs, human gut macrophages express CD14 and CD16 at very low levels.¹⁷⁴ Murine gut macrophages can be identified by the markers F4/80¹⁷⁵ and the fractalkine receptor CX3C receptor 1 (CX3CR1).¹⁷⁶ Gut macrophages typically express CD64 Fc-gamma receptor 1,¹⁷⁷ CD163 (scavenger receptor for free hemoglobin or the hemoglobin–haptoglobin complex),¹⁷⁸ and the myeloid-epithelial-reproductive tyrosine kinase (MerTK).³ In adults, most gut macrophages except those at immune inductive sites such as Peyer’s patches (PPs) typically display anti-inflammatory characteristics.¹³⁹ These properties are still maturing in neonates.¹³⁹ There are four main categories of gut macrophages (Flowchart 3).

- a. *Lamina propria macrophages* (LPMs): These cells are located below the epithelium, around crypts, and near blood vessels in both the small intestine and the colon.^{3,44,168,169,172,173} The primary functions are in host defense, clearance

of dead cells, and maintenance of tissue integrity.³ Mucosal perivascular macrophages also form tight interdigitating connections around the vasculature to prevent bacterial translocation into the blood circulation. These cells express angiogenesis-related genes necessary for the repair and strengthening of the vasculature.

LPMs can be derived from the primordial myeloid precursors developing in the intestine, those immigrating from the liver, or from the blood monocytes.¹⁷⁹ The maturation of Ly6c1 lymphocyte antigen 6 complex, locus C1 (Ly6C)^{hi} CCR2 receptor 2 (CCR2)^{hi} CX3CR1^{int} monocytes into Ly6C^{lo} CCR2^{lo} CX3CR1^{hi} macrophages is driven by the microbiota and by the transcription factor nuclear receptor subfamily 4, group A, member 1 (NR4A1).¹⁸⁰ The fractalkine receptor CX3C receptor 1 (CX3CR1) promotes dendrite formation and luminal migration.⁹⁷ At the base of SI and colonic crypts, some LPMs that express sialoadhesion (CD169)⁺ are closely associated with the stem cell niche.¹⁸¹ In the small intestine, these LPMs promote Paneth cell differentiation, maintain the LGR5⁺ stem cell pool, and promote epithelial proliferation. The analogous subset in the colon is not essential to maintain the stem cell niche, but may promote regenerative responses after injury.

- b. *Submucosal macrophages*: These help maintain the submucosal vasculature. LPMs and submucosal macrophages differ in life span, transcriptional programs, and function. These cells are either self-maintaining or are derived from regular differentiation from monocytes.^{3,181} Some submucosal macrophages resemble those in the muscularis externa and are located in close proximity to the neurons and vasculature. These cells comprise a long-lived, self-maintained subset of macrophages.¹⁸¹ Considering the location away from the gut lumen, this pool of macrophages does not seem to need the stimulation from the microbiota and dietary antigens for replenishment by circulating monocytes.
- c. *Muscularis macrophages* are located underneath the submucosal region between circular and longitudinal muscle layers, and are therefore, relatively distant from luminal stimuli. These cells may show either a bipolar (associated with the circular muscles and the deep muscular plexus) or a stellate (in the serosal and myenteric plexus) shape.¹⁸² These are associated with the *muscularis externa* and associated enteric neurons, distant from any luminal stimulation.^{3,183}

The development of muscularis macrophages is ensured by CSF-1 produced by the enteric neurons, and possibly also by endothelial cells or interstitial cells of Cajal.¹⁸⁴ The gene expression profile of these macrophages suggests a role in tissue protection, neuronal development, and intestinal peristalsis. The bone morphogenetic protein (BMP) 2 and prostaglandin E2 (PGE2), which act on enteric neurons and smooth muscles, respectively, are also expressed.¹⁸² Muscularis macrophages may also serve in a neuroprotective role by limiting infection-induced neuronal loss through the adrenergic/arginase 1/polyamines axis¹⁸⁵ and the norepinephrine signaling via β_2 adrenergic receptors (β_2 ARs).¹⁸⁶

- d. *Serosal macrophages*: These cells may be derived from precursors arising in the muscularis, or from peritoneal macrophages adherent to the serosa.¹⁸⁷ The surface markers and properties vary with the degree of inflammation in the peritoneal cavity.

Inflammatory Characteristics of Macrophages in the Developing Intestine—In the adult human intestine, newly recruited monocytes differentiating into macrophages retain avid phagocytic and bactericidal activity but develop inflammatory anergy and tolerance to bacterial products.¹³⁹ These macrophages lose innate response receptors such as CD14, Fc α (CD89), Fc γ (CD64, CD32, CD16), the integrin lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18); and the complement receptors (CR) 3 (CD11b/CD18) and CR4 (CD11c/CD18). These cells no longer produce inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-12, CC receptor ligand 5 (CCL5), and the TNF. This inflammatory downregulation occurs due to the exposure to extracellular matrix (ECM) factors such as TGF- β .

Unlike the gut macrophages in adults, those in the fetus/premature infants are yet to develop complete tolerance to bacterial products and display inflammatory responses upon stimulation. These cells express many of the inflammatory markers listed above (Fig. 2).

There are three main reasons:

1. The developing intestine has a deficiency of TGF- β , particularly its TGF- β ₂ isoform.¹²¹ During NEC, TGF- β expression and bioactivity are further reduced to levels that are even lower than in a normal fetus of a similar gestational age.
2. Macrophages in the premature intestine are resistant to TGF- β and are therefore, intrinsically hyper-inflammatory because of high expression of an inhibitor of TGF- β signaling, the Smad7.¹⁶⁹ The abbreviation Smad refers to the homologies in this protein to the “*Caenorhabditis elegans*” SMA, the “small” worm phenotype and the MAD, the “Mothers against Decapentaplegic family of genes in *Drosophila*. Bacterial products further induce Smad7 expression in neonatal, but not adult, gut macrophages.¹⁶⁹ Smad7 increases inflammatory activity by augmenting LPS-induced NF- κ B activation; it activates the inhibitor of nuclear factor kappa-B kinase subunit beta (IKK- β) expression by attaching to two smad-binding elements in the IKK- β promoter, and consequent acetylation of Lys12 residue on histone 4 (H4K12).¹⁸⁸ Increased Smad7 expression in the developing intestine may be due to a developmental deficiency of the SKI (Sloan-Kettering Institute)-like proto-oncogene (SKIL) oncoprotein, which is a physiological repressor of the Smad7 promoter.^{173,189} Our findings showed Smad7 to be an important negative regulator of TGF- β signaling in the gastrointestinal tract.¹⁹⁰ Smad7 can also suppress TGF- β signaling by competing with the activating Smads, increasing the degradation of the TGF- β receptors, and through epigenetic mechanisms by interacting with histone deacetylases.^{191–193} IKK- β is an essential catalytic subunit of the IKK complex, which includes another catalytic subunit, IKK- α , and a regulatory subunit, IKK- γ .¹⁹⁴ During inflammation, cytokines and bacterial products promote phosphorylation of IKK-

β , which in turn, activates the IKK complex. Activated IKK phosphorylates the I κ Bs, triggering their degradation and thereby releasing the NF- κ B dimers for nuclear translocation. In our study, Smad7 activation of the IKK- β promoter was associated with increased H4K12 acetylation, an epigenetic marker of euchromatin,¹⁹⁵ on the IKK- β nucleosome. H4K12 acetylation may neutralize its electrical charge, leading to structural changes that promote DNA accessibility and interactions with the H2A–H2B dimer in neighboring histones.¹⁹⁶

3. Human milk contains substantial amounts of TGF- β , particularly the isoform TGF- β_2 , but most of it is in a latent, inactive form.¹⁹⁷ A substantial proportion of milk-borne TGF- β_2 is also inactive because it is bound to chondroitin sulfate-containing proteoglycan(s) such as biglycan.¹⁹⁸

Macrophages in immune inductive sites in the developing intestine show some unique features.¹⁹⁹ Peyer's patches first appear at 11 weeks and develop during mid-late gestation.²⁰⁰ At birth, these lymphoid aggregates are structurally complete but "naive", as the germinal centers take a few weeks to develop. The number of PPs in the ileum increases as a function of gestational maturation, and premature infants born prior to 32 weeks' gestation may have only half as many PPs than those born at full term. Lymphoid aggregates in the vermiform appendix may develop only after birth following postnatal bacterial colonization.²⁰⁰

After birth, macrophages in PPs are exposed to more antigens than those in the *Lamina propria*.²⁰¹ These cells do not express the typical macrophage markers such as F4/80 (a glycoprotein marker seen on mature macrophages) and CD64 (IgG Fc Receptor I) in mice and CD163 (high affinity scavenger receptor for the hemoglobin–haptoglobin complex) in humans,²⁰² but most display the apoptotic receptor myeloid-epithelial-reproductive tyrosine kinase (MerTK) and the fractalkine receptor CX3CR1.³ In view of high-level expression of lysozyme, these macrophages labeled as lysozyme-expressing macrophages (LysoMacs).²⁰³ The muscularis and serosal macrophages located below the follicles express CD169 (sialoadhesin, a cell adhesion molecule), unlike those in the PP.¹⁰ The follicular LysoMacs express the phosphatidylserine receptor T-cell membrane protein 4 (TIM-4), whereas subepithelial and upper follicular LysoMacs do not.¹⁰ These factors could comprise one possible mechanistic explanation for the known regional specialization of macrophages inside the PPs.²⁰⁴

Macrophages outside the immune inductive sites such as in the small intestinal *Lamina propria*, both in terms of the number and functional characteristics, might be influenced by dietary factors.²⁰⁵ The impact on the Janus kinases (JAK)-signal transducer and activator of transcription proteins (STAT) pathway, expression of CCL2, and inflammasome activation with increased intestinal permeability, glucose metabolism, and insulin sensitivity may be important.²⁰⁶ Saturated fatty acids, derived from the metabolism of ω -3 PUFAs, activate inflammatory responses through the TLR4–NF- κ B pathway.²⁰⁷ Some macrophages acquire properties that promote the resolution of inflammation induced by dietary antigens.¹²⁶

Colon is functionally important for the absorption of electrolytes and water, and for the management of undigested foodstuffs.²⁰⁸ In the distal colon, macrophages insert balloon-

like protrusions between epithelial cells to sense their microenvironment, although these extensions do not quite extend into the lumen as seen in the small intestine.^{209,210} These protrusions sample the fluids absorbed by epithelial cells to detect toxins and protect by regulating absorption of potentially toxic luminal contents.²¹¹

The macrophages in the colonic lamina propria are continuously exposed to anti-inflammatory microbial metabolites and short-chain fatty acids (SCFAs) such as butyrate,²¹² and produce antimicrobials such as lysozyme, calprotectin, and ROS.²¹³ Butyrate is known to suppress inflammatory signaling by inhibiting histone deacetylase 3.²¹⁴ It also stimulates the goblet cells to produce more organized mucus by changing glycosylation-related gene expression.²¹⁵ These improved mucus layers reduce bacterial translocation and induce physico-chemical changes in the oxygen and pH levels; this feedback loop regulates the inflammatory activity by altering the number and functional diversification of macrophages.^{215–218} Colonic macrophage functioning is also altered by TGF- β and IL-10 signaling. These pathways promote the interaction of Wiskott-Aldrich syndrome protein (WASP) and dedicator of cytokinesis (DOCK) 8, leading to the phosphorylation of STAT3 and the anti-inflammatory polarization of these macrophages.²¹⁹ Dectin-1 activates colonic macrophages, resulting in inflammasome-dependent IL-1 β secretion and monocyte recruitment to the inflamed colon.²²⁰

Gut Macrophages are Key Mediators of Intestinal Inflammation in NEC

Necrotizing enterocolitis is an inflammatory bowel necrosis seen in up to 10% of very-low-birth-weight (VLBW) premature infants born prior to 32 weeks' gestation.²²¹ The mortality rates of NEC continue to be between 20 and 30% all over the world.²²² The pathogenesis of NEC is still unclear, but many risk factors have been identified such as chorioamnionitis, perinatal asphyxia, indomethacin therapy in neonates, formula feedings, use of human milk fortifiers, viral infections, use of feeding thickeners, and severe anemia; anemia may either be a risk factor in itself, or may predispose to NEC following “corrective” red blood cell transfusions.¹⁷¹

Nearly 80% of all intestinal tissue specimens that have been resected for NEC showed inflammatory changes with leukocyte infiltration and edema.^{223–225} Interestingly, despite the rapid progression of intestinal injury, the leukocyte populations were largely comprised of macrophages, not neutrophils.^{224,226} This prominence of macrophages in NEC lesions could be a tissue-specific finding, but also possibly related to the relative immaturity of neutrophils compared to macrophages in premature infants. A few eosinophils were seen in some lesions. The number of lymphocytes did not differ between control areas and NEC lesions, although many recent reports suggest that NEC may alter lymphocyte subsets. The severity of inflammation in NEC is usually graded by the depth of changes, beginning at the mucosa and outward progression, and by the density of leukocyte infiltration. More than half of all NEC lesions show leukocyte infiltrates extending into the submucosa or beyond.

Inflammatory infiltrates can be seen in 70–80% of resected tissue samples.^{168–170,172,200,226,227} These infiltrates were typically comprised of macrophages (70–80% of the leukocytes/high power microscopic field/HPF) and neutrophils (15–20% of the leukocytes/hpf).^{168,224} There was a modest increase in neutrophils (37.9 ± 5.8 cells in

NEC vs. 7.7 ± 1.7 /high-power field in control tissues). The number of lymphocytes usually does not change significantly. A few eosinophils may be seen in the involved areas, and in some infants, inflammatory pseudomembranes and crypt abscesses can also be seen. Pender et al.²²⁸ previously described similar inflammatory changes with macrophage infiltration with NEC. We have described how the pro-inflammatory characteristics of still-maturing intestinal macrophages in the preterm intestine can increase the risk of NEC and refer the reader to the articles listed in the references.^{121,168–173,200,226,229–237} Several genetic and epigenetic factors may also alter the susceptibility to NEC and/or its severity, its clinical and histopathological manifestations, and if surgery is needed, of an adverse postoperative course and outcome.^{227,238}

Conclusions

Recent years have brought considerable progress in our understanding of the heterogeneity and inflammatory immaturity of gut macrophages in premature and full-term newborn infants; particularly in the context of diseases such as NEC. There is a need for continued efforts to understand the maturation of macrophages, and the mechanisms that might derail this process, and the points where timely identification and therapeutic interventions might be of translational importance. There is hope.

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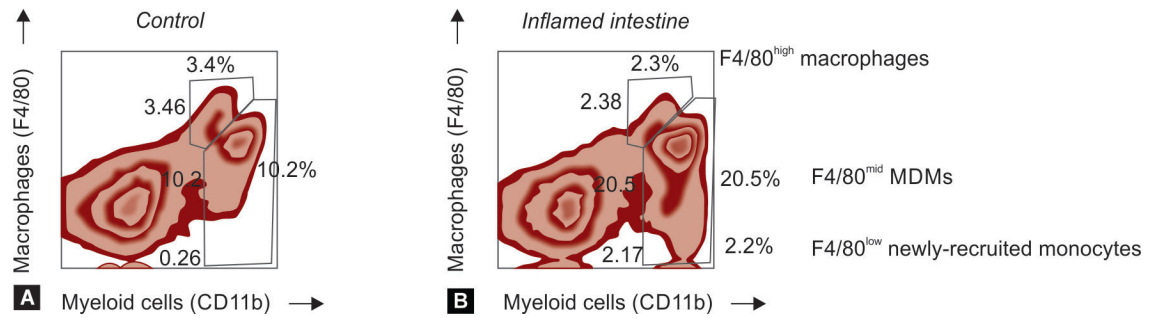
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Key Points

- Macrophages are large highly motile leukocytes with important roles in innate immunity.
- During evolution, many precursors to macrophages, such as amebocytes, coelomocytes, and hemocytes, have been identified.
- In the developing human embryo, macrophage precursors are identified in the yolk sac, the pools of erythro-myeloid progenitors, endothelial precursors, and later in hematopoietic stem cells.
- Macrophages play major roles in tissue homeostasis, innate immunity, inflammation, tissue repair, angiogenesis, and apoptosis of various cellular lineages.
- The differentiation of monocytes into inflammatory M1 and immunoregulatory M2 macrophages is a subject of active investigation.
- The gastrointestinal tract contains the largest reservoir of specialized macrophages. Small intestine and colon contain multiple, distinct macrophage subsets.
- Recognizing the heterogeneity of gut macrophages in premature and term infants raises an exciting possibility that targeted augmentation or depletion of subsets might be therapeutically useful.

**Figs 1A and B:**

Intestinal macrophage populations in neonatal mice. We used flow cytometry to examine intestinal lysates from mouse pups with normal intestine and from others with intestinal inflammation. Macrophages were identified as the cells expressing the myeloid marker CD11b and the macrophage marker, F4/80. The normal intestine showed two distinct pools of macrophages, one of mature macrophages that expressed F4/80 at high levels and likely represents YS-derived cells. There was a second F4/80^{mid} subset consistent with MDMs. During inflammation, the MDM pool got enlarged. Some newly recruited F4/80^{low} monocytes were also seen (expressed high levels of the monocyte marker Ly6C; not shown)

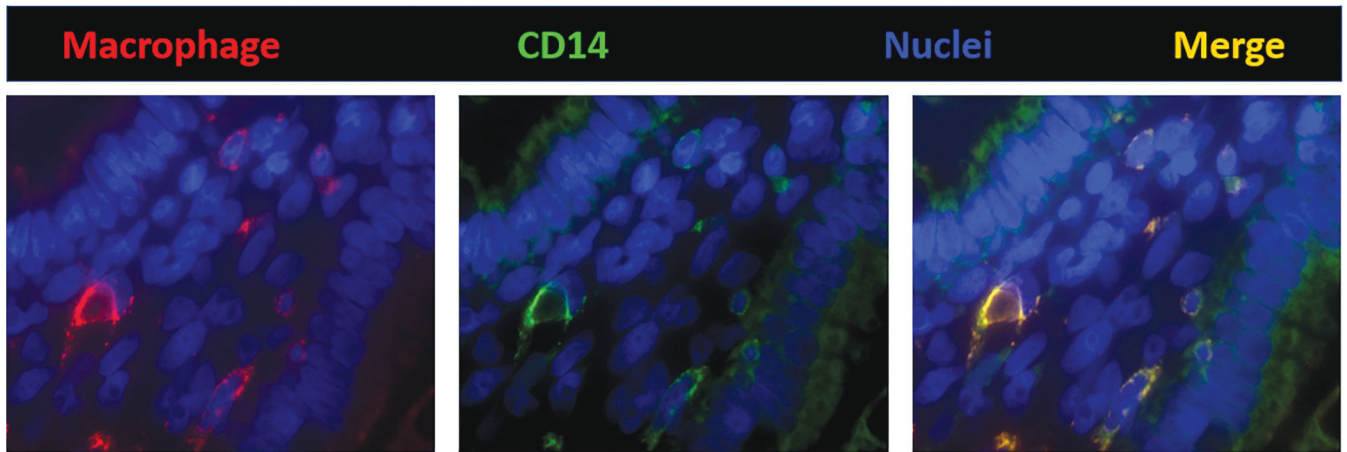
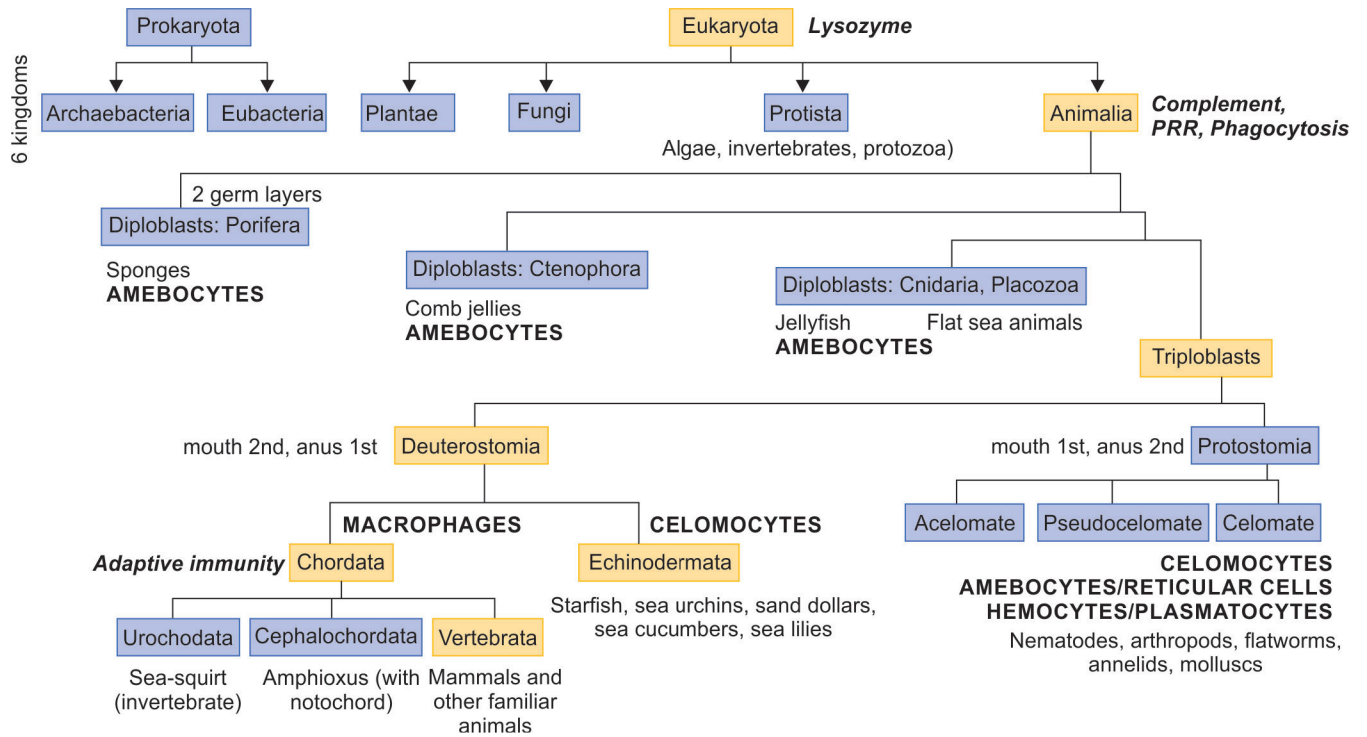


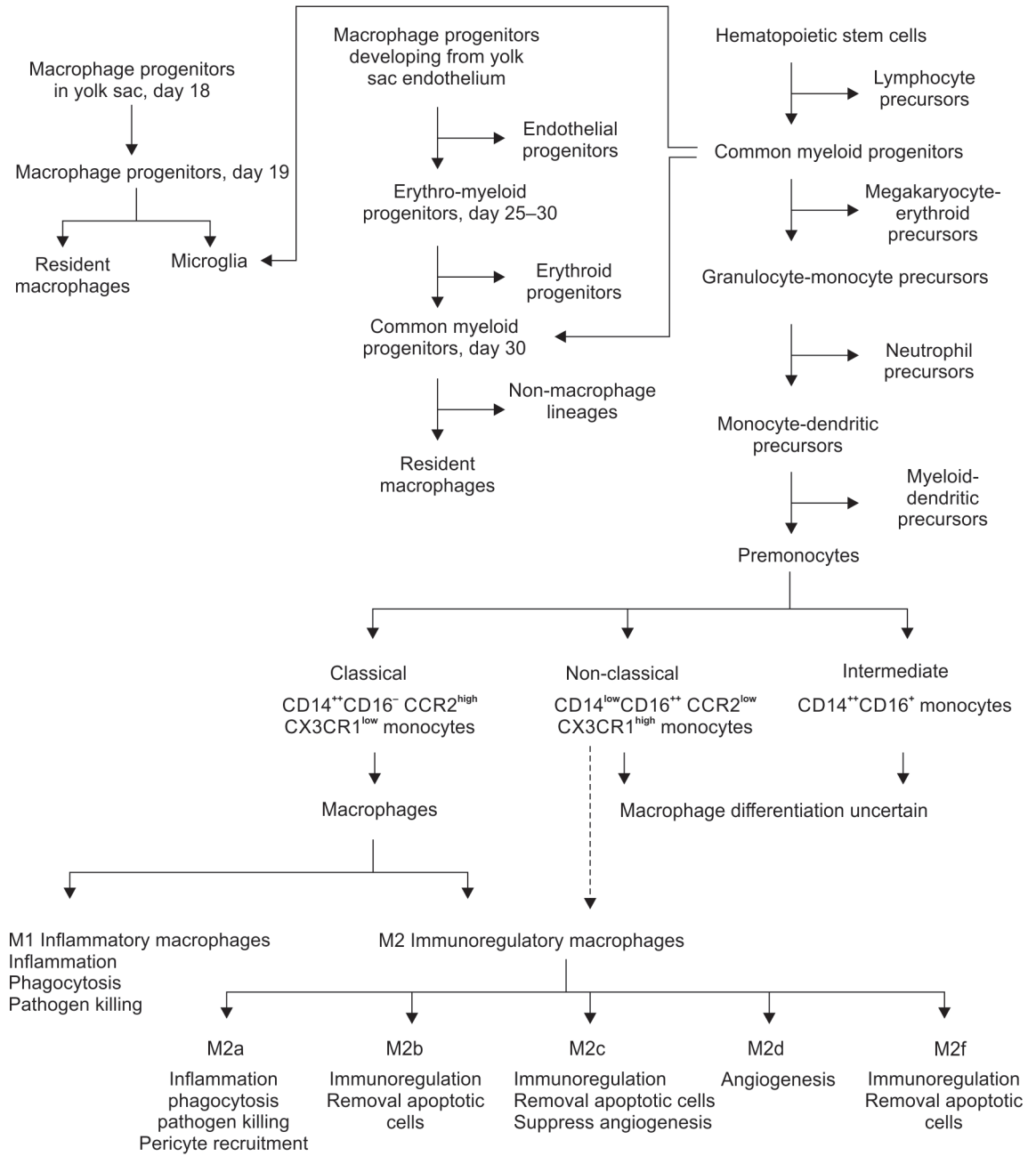
Fig. 2:

Fluorescence photomicrograph (1000 \times) of a villus in preterm (26 weeks) human intestine shows that macrophages (HAM56, red) express CD14 (green). CD14 is an important mediator in the bacterial lipopolysaccharide (LPS)-stimulated signaling pathways. These findings are important because macrophages in the adult human intestine do not express CD14 and are unresponsive to LPS. Nuclei are stained blue (4',6-diamidino-2-phenylindole, or DAPI, is a fluorescent stain that binds adenine–thymine-rich regions in DNA)



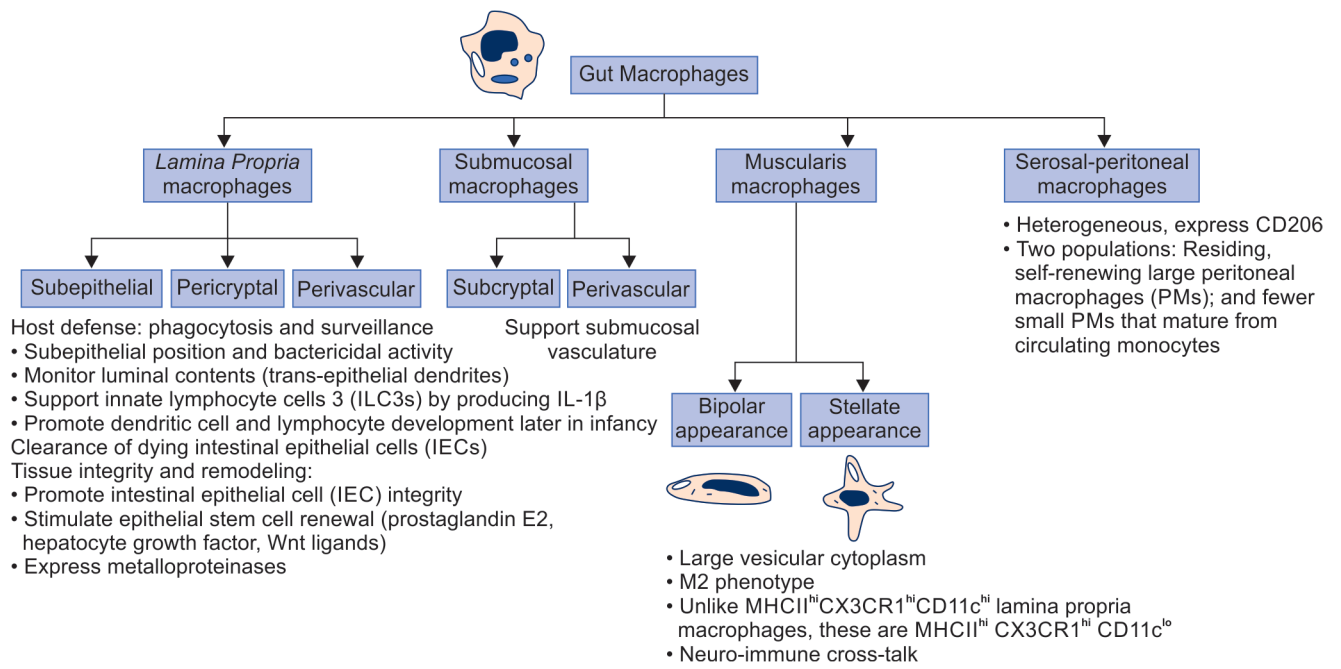
Flowchart 1:

Phylogeny of macrophages. Schematic figure shows the development of macrophage-like cells and macrophages across evolution. The orange-colored boxes with eukaryota, animalia, triploblasts, deuterostomia, chordata, and vertebrata traces the evolution of vertebrate animals such as humans. The green font indicates key events in the development of immunity. Upper-case, red-font labels show the evolution of phagocytes. For each category of animals, one or more representative organisms are listed below in black font



Flowchart 2:

Ontogeny of gut macrophages. Schematic figure shows the development of the three major categories of macrophages in the human embryo, from progenitors in the yolk sac (YS), from the YS endothelium, and from the hematopoietic stem cells (HSCs). A subset of the HSCs evolves into monocytes, and current understanding suggests the classical CD14⁺⁺ monocytes differentiate into the M1 inflammatory and M2 immunoregulatory macrophages. There is a possibility that the CD14^{low}CD16 monocytes may (also) evolve into the immunoregulatory macrophages, which is shown as a broken arrow. Increasing information suggests that the M2 macrophages may be a heterogeneous group comprised of multiple subsets



Flowchart 3:

Classification of gut macrophages by location. Schematic shows the location, classification by location or shape, and the best-known function