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Intr ganglionic macrophages: a new population of cells in the enteric ganglia**Running Title:** Intra-ganglionic macrophages in the ENS

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Abstract

The enteric nervous system (ENS) shares embryological, morphological, neurochemical, and functional features with the CNS. In addition to neurons and glia, the CNS includes a third component, microglia, which are functionally and immunophenotypically similar to macrophages, but a similar cell type has not previously been identified in enteric ganglia. In this study we identify a population of macrophages in the enteric ganglia, intermingling with the neurons and glia. These intraganglionic macrophages (IMs) are highly ramified and express the hematopoietic marker CD45, MHC class II antigen, and chB6, a marker specific for B cells and microglia in avians. These IMs do not express antigens typically associated with T cells or dendritic cells. The CD45+/ChB6+/MHCII+ signature supports a hematopoietic origin and this was confirmed using intestinal chimeras in GFP-transgenic chick embryos. The presence of GFP+/CD45+ cells in the intestinal graft's ENS confirms that IMs residing within enteric ganglia have a hematopoietic origin. IMs are also found in the ganglia of CSF1R^{GFP} chicken and CX3CR1^{GFP} mice. Based on the expression pattern and location of IMs in avians and rodents, we conclude that they represent a novel non-neural crest-derived microglia-like cell population within the enteric ganglia.

Introduction

The CNS and the ENS are both of neuroectodermal origin, although the ENS is formed by enteric neural crest cells (ENCCs) which migrate away from the neuroectoderm to give rise to the intrinsic neurons and glia of the intestinal tract (Nagy and Goldstein, 2016). The CNS includes a third population of cells, referred to as microglia, which are a highly ramified cell first described by del Rio-Hortega (Rio-Hortega, 1919). While microglia are considered glial cells, they derive from hematopoietic, not neural crest precursors and are capable of antigen presentation (Hickey and Kimura, 1988). Multiple roles for microglia have been demonstrated in the developing (Squarzoni et al. 2014, Frost and Schafer, 2016), and mature CNS, including contributing to learning-dependent synapse formation (Parkhurst et al., 2013), phagocytosis and neuroprotection during inflammation and ischemia (Fu et al., 2014, Szalay et al., 2016), synaptic pruning (Ji et al., 2013), and participation in crosstalk with neurons through fractalkine (FKN) and its receptor, CX3CR1 (Sheridan et al., 2013, Elmore et al., 2014). In the ENS, however, no cells corresponding to microglia have been identified.

An early electron microscopy study suggested the existence of a non-neuroectodermal ramified cell type within the ENS of adult rabbit colon (Komuro et al., 1982). Similar ramified cells expressing MHC class II (Kulkarni et al., 2017), and macrophage markers, including CD163, CD11b, CSF1R, Iba1, and F4/80 (Mikkelsen 1995; Mikkelsen et al. 2004, Orandle et al., 2007; Grover et al., 2011; Phillips and Powley, 2012; Giovangiulio et al., 2015; Yuan and Taché, 2017; Avetisyan et al., 2018) have been identified closely associated with enteric ganglia and its connecting fibers in the rodent intestine. Recently, a CSF1R+/CX3CR1+/CD11b+/MHCII+ macrophage population in the muscularis externa layer (myenteric macrophages, MMs) was found closely apposed to enteric ganglia. These cells appear to play a role in neuro-immune crosstalk between the mucosa-associated lymphatic tissue of the gut and the ENS (Muller et al. 2014). Another group found that gut macrophages exhibit different gene expression patterns depending on their proximity to the lumen: lamina propria macrophages (LpMs) are proinflammatory, while MMs are tissue-protective (Gabanyi et al. 2016). MMs were also shown to phagocytose apoptotic enteric neurons (Kulkarni et al. 2017), a role that resembles the “scavenger” activity of CNS microglia. Genomic hierarchical clustering, based on a 39-gene macrophage signature, revealed that intestinal macrophages show the strongest relationship to CNS microglia (Gautiar et al. 2012; Butovsky et al. 2013). Further, the fractalkine receptor, CX3CR1, is uniquely expressed on intestinal macrophages and microglia, and not on other tissue macrophages (Bain & Mowat 2014). While the presence of MMs has been described, the existence of an intraganglionic population of macrophages and its embryologic origin has not been previously reported

Methods

Animals

Fertilized White Leghorn chicken eggs were obtained from commercial breeders and maintained at 38°C in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (HH) tables (Hamburger and Hamilton, 1951) or the number of embryonic days (E). Transgenic green fluorescent protein (GFP)-expressing chicken eggs were provided by the courtesy of Prof. Helen Sang, The Roslin Institute, University of Edinburgh (McGrew et al. 2004). Colony stimulating factor 1 receptor-GFP (CSF1R^{GFP}) chicken were obtained from The Roslin Institute (Balic et al., 2014). CX3CR1^{GFP} transgenic mice were kindly provided by Dr. Hans-Christian Reinecker, Massachusetts General Hospital, Boston. The design and

condition of the animal experiments were approved by the Animal Ethical Committee of Semmelweis University, Budapest, Hungary.

Histological procedures

For light microscopy, the tissue blocks were placed in 4% buffered glutaraldehyde for 24 hours, followed by dehydration in ethanol and embedding in a Polybed/Araldite 6500 mixture (Polysciences). The 1- μ m-thick semithin sections were stained with toluidine blue as described (Nagy and Oláh, 2007). For haematoxylin-eosin staining, specimens were fixed in buffered formalin and embedded in paraffin.

Immunocytochemistry

Samples were fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 1 hour, rinsed with PBS, and infiltrated with 15% sucrose/PBS overnight at 4°C. The medium was changed to 7.5% gelatin containing 15% sucrose at 37°C for 1–2 hr, and the tissues rapidly frozen at -60°C in isopentane (Sigma). Frozen sections were cut at 12 μ m, collected on poly-L-lysine-coated slides (Sigma), and stained by immunocytochemistry as previously described (Nagy et al. 2004). The primary antibodies used are listed in Table 1. Frozen sections were incubated with primary antibodies for 45 minutes, followed by biotinylated goat anti-mouse IgG (Vector Labs, Burlingame, CA) and avidin-biotinylated peroxidase complex (Vectastain Elite ABC kit, Vector Labs). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (Sigma) for 10 minutes. The binding sites of the primary antibodies were visualized by 4-chloro-1-naphthol (Sigma).

For double immunofluorescence staining the sections were incubated with the first primary antibody at room temperature for 45 min. followed by second primary antibody. Secondary antibodies (Alexa Fluor 594 and 488 conjugated anti-mouse IgG, Alexa Fluor 594 conjugated anti-mouse IgM, Alexa Fluor 594 and 488 conjugated anti-mouse IgG2a, and IgG1, Alexa Fluor 647 and 488 conjugated anti-goat, Alexa Fluor 488 conjugated anti-rabbit from Invitrogen) were used for 1 hour. Sections were covered with aqueous Poly/Mount (Polyscience Inc., Warrington, PA) and examined by using a Nikon Eclipse 80i microscope or

with a Nikon A1R laser-scanning confocal microscope (Nikon Instruments). Images were compiled using Adobe Photoshop.

Chorioallantoic membrane (CAM) transplants

CAM grafts were performed as previously described (Nagy et al., 2007). Briefly, gut segments from E8 chick embryos were dissected and transplanted onto the chorioallantoic membrane (CAM) of E8 GFP expressing chick embryos. During grafting, a small portion of the CAM was gently traumatized by laying a strip of sterile lens paper onto the surface of the epithelium and then removing it immediately. The dissected intestine was placed over the junction of blood vessels on the traumatized area of the CAM and incubated for 9 days. The graft, together with the surrounding CAM, was excised, fixed in 4% buffered formaldehyde and embedded in gelatin. These studies included a total of 21 chimeric experiments coming from three separate series.

Results and Discussion

While characterizing the distribution and ontogeny of CD45⁺ hematopoietic cells in the chick embryo, we noted the presence of a ramified CD45⁺ cell type characteristic of macrophages within the gut mesenchyme (Dóra et al., 2017). Similar to mammals, chicken CD45 is a transmembrane tyrosine phosphatase protein expressed by all cells of hematopoietic lineage, excluding erythrocytes. The cells appeared to be closely associated with the ENS and we therefore sought to further characterize this relationship. At E18, immunofluorescent staining of chick midgut shows the presence of ramified CD45⁺ cells within the nerve of Remak (NoR; Fig. 1A) and within the submucosal and myenteric ganglia (Fig. 1B-D). Enteric neurons were stained with antibody against the neuron-specific marker Hu. This observation suggested the presence of a population of hematopoietic-derived cells within the enteric ganglia, which are thought to contain only neural crest-derived neurons and glia. To confirm the hematopoietic origin of this new cell population, E8 hindgut was grafted onto the CAM of a host E9 GFP-expressing chick embryo in which GFP protein is ubiquitously expressed (Fig. 1E). After 9 days, the grafted hindgut was examined and GFP⁺ cells can be seen throughout the wall of the intestine, including interspersed within the NoR (Fig. 1F) and ganglia (Fig. 1F, G). The GFP⁺ cells present within the myenteric ganglia have

a highly ramified appearance and express CD45 (Fig. 1H, I-I''), consistent with their hematopoietic origin.

We next characterized the immunophenotype of the hematopoietic cells in the enteric ganglia of postnatal animals. Staining of ileum from a 6-week-old chicken reveals the continued presence of CD45⁺ ramified cells in the muscular layer, including within HNK1⁺ myenteric ganglia (Fig. 2A), which also label with antibody against the neuronal markers, Hu and beta-III Tubulin (Tuj-1). Ramified CD45⁺ cells also express MHC type II (Fig. 2B), CSF1R (Fig. 2C), and chB6 (Fig. 2D, E). MHC class II is known to be expressed by avian antigen-presenting cells, including macrophages and dendritic cells and their precursors (Guillemot et al. 1984), (Nagy et al. 2016), and also by microglial cells (Hala et al. 1984). ChB6 recognizes B cells within the avian lymphomyeloid organs (Igyártó et al., 2008; Veromaa et al., 1988) and has also been described on a subset of non-phagocytic, MHC II⁺ myeloid cells in the liver and within the intestinal epithelium (Houssaint, 1987; Weber et al., 2000). Furthermore, chB6 immunoreactivity also marks CD45⁺ microglia within the sensory epithelium of the ear and the pineal gland follicles (O'Halloran and Oesterle, 2004; Mosenson and McNulty, 2006; Bird et al., 2010). ChB6 is a 70-kD homodimer transmembrane protein with a highly glycosylated extracellular domain (Tregaskes et al., 1996). Its function is not entirely known, though it has been reported to control cell survival, apoptosis and adhesion during B cell development (Funk et al., 1997; Bhattacharya et al., 2017). We find chB6 expressed by B cells scattered throughout the gut mucosa (Fig. S1C), microglia in the cerebellum (Fig. S2E), and a population of ramified cells within the enteric ganglia (Fig. 2D, E). Interestingly, the absence of chick B cell-specific EIVE12 or CD1 antigens on the intestinal macrophages (data not shown) suggests that these hematopoietic-derived cells within the enteric ganglia do not represent B cells.

CSF1R is a marker of monocytes and macrophages in the chick (Fig. S1D; Garcia-Morales et al. 2014), as well as microglia (Balic et al., 2014) (Fig. S2D). We find CSF1R expressed not only by the MMs, but also by the CD45⁺/chB6⁺ ramified cells in the enteric ganglia (data not shown). 74.2 antibody labels an unknown cytoplasmic molecule that is highly restricted to phagocytic tissue macrophages (Jeurissen et al., 1992; Nagy et al., 2005). Interestingly, mAb 74.2 (Fig. 2F) and other chick-specific tissue macrophage markers, including 68.2 and KUL01 (data not shown) mark cells outside, but not within, the ganglia. **CD45⁺ IMs co-express chB6 and MHCII (Fig. 2G,H). We also labelled sections of cerebellum, ileum and colon of CSF1R^{GFP} transgenic chicken with Tuj1, CD45, chB6 and**

MHCII antibodies (Fig. 3). CD45 and CSF1R transgene-expressing cells were scattered throughout the gut wall (Fig. 3D-D’), with CSF1R/chB6 double-positive cells clustered within the myenteric ganglia (Fig 3E,F-F’). CSF1R⁺ transgene-expressing cells co-express MHCII (Fig. 3G). In mammals, mast cells were reported in close apposition to the enteric nervous system (Buhner et al., 2012; Wang et al., 2014). Similarly, in chicken colon we observe mast cells near the enteric ganglia (Fig. S3). Together, these results show that avian enteric ganglia include ramified cells with a macrophage- and microglia-specific immunophenotype. These cells are distinct from mast cells and tissue macrophage-like cells, suggesting that these “intraganglionic macrophages” (IMs) represent a novel myeloid cell population.

To determine whether a similar population of IMs is present in mammals, we used CX3CR1^{GFP} transgenic mice, in which GFP labels macrophages and microglia, which are known to express CX3CR1. As shown in Figure 4, CX3CR1^{GFP} and CD11b⁺ cells are present in the adult mouse ileum (Fig. 4A) and colon (Fig. 4B), including in close association with the myenteric ganglia. Wholemount immunostaining reveals that ramified CX3CR1-expressing cells are present within the myenteric ganglia, interdigitating among the Hu⁺ enteric neurons (Fig. 4C). A confocal image of a myenteric ganglion stained with Hu (red) and agrin (purple) clearly shows GFP⁺ cells present both within the ganglia and surrounding them (Fig. 4D, Supplementary video 1). Agrin expression delineates the outer border of the enteric ganglia, as recently shown in mouse and chicken (Nagy et al., 2018). The CX3CR1^{GFP} cells present within the myenteric ganglia have a highly ramified appearance and express CD45 (Fig. 4E-F’), and CD11b (Fig. 4F-F’)

Based on our findings, we conclude that enteric ganglia in both avian and rodents contain, in addition to neural crest-derived neurons and glia, a third population of cells that has not been previously described. These cells are hematopoietic-derived, highly-ramified, and express markers consistent with a macrophage/microglia signature: CD45⁺/MHCII⁺/CSF1R⁺/chB6⁺ in chick (Figs 2,3) and CX3CR1⁺/CD11b⁺ (Fig. 4), and MHC II (Kulkarni et al. 2017) in mice. The immunophenotype of the IMs, however, supports the idea that these cells may represent microglia. Studies of the CNS of chickens revealed that CD45 immunoreactivity identified all microglia, which have a highly ramified morphology (Cuadros et al., 2006; Bird et al., 2010), (Fig. S2B). CX3CR1 and chB6 are expressed on mammalian (Nishiyori et al., 1998) and avian microglia (Fig. S2E), respectively. CSF1R expression is also characteristic of microglia in mice (Elmore et al., 2014; Luo et al., 2017)

and chicken (Balic et al., 2014). In chicken, both the brain and intraganglionic ramified CD45⁺ cells co-express CSF1R and chB6 antigens (Figs 2; S2D,E). Analysis of sections at high magnification revealed that the intraganglionic chB6⁻, and CX3CR1-expressing cells possess a highly ramified morphology characteristic of microglial cells. Based on their morphological characteristics and on the expression of MHC II, CSF1R and chB6 in the absence of tissue macrophage-specific antigens (74.2, 68.2 or KUL01), we propose that the CD45⁺/chB6⁺ IMs are an intraganglionic population of cells distinct from MMs and other gut macrophages. Furthermore, these IMs appear to represent a novel cell type that shares a molecular signature characteristic of microglia, although whether they possess a microglial function in the ENS remains to be determined.

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Disclosures

The authors have no financial or other conflicts of interest to disclose.

Author Contributions

D.D., A.M.G. and N.N. conceived the experiments, participated in their design and wrote the manuscript with contributions from all authors. R.H., C. B., S.B., E.A, T, K., D.D., and N.N. performed the experiments and analyzed the results. A.B. contributed with reagents and transgenic chicken materials. All authors reviewed and approved the final manuscript.

Figure legends

Figure 1.

Hematopoietic-derived cells are present in the enteric ganglia of the developing avian embryo. Immunofluorescent labeling of E18 chick midgut shows ramified hematopoietic-derived CD45⁺ cells interspersed among Hu⁺ enteric neurons in the nerve of Remak (NoR, A) and enteric ganglia (B,C,D). C and D panels are magnified from submucosal and myenteric ganglia seen in panel B. Schematic illustration of CAM transplantation in which E8 hindgut is placed onto the CAM of a 9-day-old GFP⁺ host embryo (E). **After 9 days of incubation, GFP⁺ cells are present within the NoR and myenteric ganglia (F, arrows; outlined ganglia is magnified in G). CD45 staining confirms the presence of GFP⁺ chick-derived hematopoietic cells (H) and endothelial cells (H, arrows) in the grafted hindgut. Graft-derived CD45⁺ cells (H, arrowheads) are also present in the lamina propria. High magnification shows a ramified GFP⁺ cell within an enteric ganglia co-expressing CD45 (I-I’).** mp: myenteric plexus; NoR: nerve of Remak, smp: submucosal plexus

Figure 2.

Intraganglionic hematopoietic-derived cells possess a macrophage signature. CD45 immunostaining of 6-week-old chicken ileum reveals the presence of CD45⁺ ramified cells in the muscular layer, including within HNK1⁺ myenteric ganglia (A, boxed area magnified in inset). MHCII⁺ (B), CSF1R⁺ (C), and chB6⁺ (D,E) cells are also present within the Tuj1⁺ (B-D) myenteric ganglia (dashed line). **Double-labeling show the presence of ramified chB6⁺ cells among the Hu⁺ enteric neurons (E). The tissue macrophage-specific 74.2 antibody stains cells outside, but not within, the ganglia (F). IMs co-express chB6, CD45, and MHCII (G,H), whereas MMs do not express chB6 (G,H).** cm, circular muscle; mp, myenteric plexus; smp, submucosal plexus

Figure 3.

Distribution of chB6⁺/CSF1R⁺ macrophages in CNS and ENS. Confocal analysis was performed on 4 week-old CSF1R^{GFP} chicken cerebellum (A), ileum (B) and colon (C,D). Tuj1-immunoreactive (red) and GFP⁺ (green) cells represent Purkinje cells and microglia, respectively. Inset shows an adjacent section demonstrating that CSF1R-expressing microglial cells (green) colocalise with chB6 staining (red) (A). CSF1R^{GFP} macrophages (green) are present in the mucosal and muscular layers, including within Tuj1⁺ (red) myenteric ganglia

(dashed line) of the ileum (B) and colon (C). **CD45 immunostaining (red) indicates CSF1R^{GFP} and CD45 co-expression in both MM and IM populations (D-D'').** Lamina propria macrophages and MMs express GFP and are not stained by chB6, while IMs are positive for both (E). IMs marked with arrowhead in E are magnified to show co-localization of CSF1R^{GFP} (F) and chB6 (F'). These cells also express MHC II (G). Ep, epithelium

Figure 4.

Enteric ganglia in mice contain CX3CR1+ intraganglionic macrophages. CX3CR1^{GFP} cells are present in the region of the myenteric plexus of mouse ileum (A) and colon (B). Wholemout shows the relationship between CX3CR1^{GFP} cells and a myenteric ganglion (C). Confocal image of a myenteric ganglion stained with Hu (red) and agrin (purple) shows the presence of both periganglionic and intraganglionic CX3CR1^{GFP} cells (D). **Agryn marks the outer border of the enteric ganglia. CX3CR1^{GFP} cells present within the ganglia co-express CD45 (E-E'') and CD11b (F-F'').** mp: myenteric plexus; smp: submucosal plexus

Figure 5.

Schematic representation of enteric ganglia-associated macrophages. Smooth muscle cells (pink) are seen surrounding a myenteric ganglion containing enteric neurons (red) and glial cells (magenta) as well as myenteric (MM) and intraganglionic (IM) macrophages (green). IMs are CD45+/MHCII+/CSF1R+/chB6+ in chicken gut and CD45+/MHC-II+/CSF1R+/CX3CR1+/CD11b+/F4/80+ in mice.

Supplemental Figure 1.

Immunostaining of the 6-week-old chicken cecal tonsil. Germinal centers of the lymphoid follicles are marked with asterisks (A). CD45+ lympho-myeloid cells are present throughout the lymphoid tissue and intestinal wall (B). chB6+ B cells are most abundant in the germinal centers, and also occur within the smooth muscle layers (C, arrow). Germinal centers contain CSF1R+ follicular dendritic cells (D). 74.2+ macrophages are scattered through the lamina

propria and smooth muscle (E). The follicular dendritic cell marker 74.3 labels cells in the germinal centers and not in the smooth muscle (F).

Supplemental Figure 2.

Avian cerebellum contains microglia that share immunophenotypic features with intraganglionic macrophages. Sagittal section through the cerebellar cortex of 6-week-old chicken shows GFAP expression in glial cells (A). CD45 immunostaining labels highly ramified microglia throughout the granular layer of cerebellum (B). MHC II (C), CSF1R (D), and chB6 (E) also label chicken microglia. Macrophage-specific 74.2 antibody stains only tissue macrophages (arrows) in the meninges of the cerebellum (F).

Supplemental Figure 3.

Semithin section of 6-week-old chicken colon stained with toluidine blue identifies mast cells (arrow) around, and not within, the myenteric ganglia.

Supplementary video 1:

Image sequence of Z-stack made from 1,1 μm optical sections through adult mouse enteric ganglion. CX3C1R labels intraganglionic macrophages (green). Agrin is expressed in basement membrane of enteric ganglion (blue). Enteric neurons are positive for anti-Hu antibody (red). Cell nuclei were visualized with DAPI (grey).

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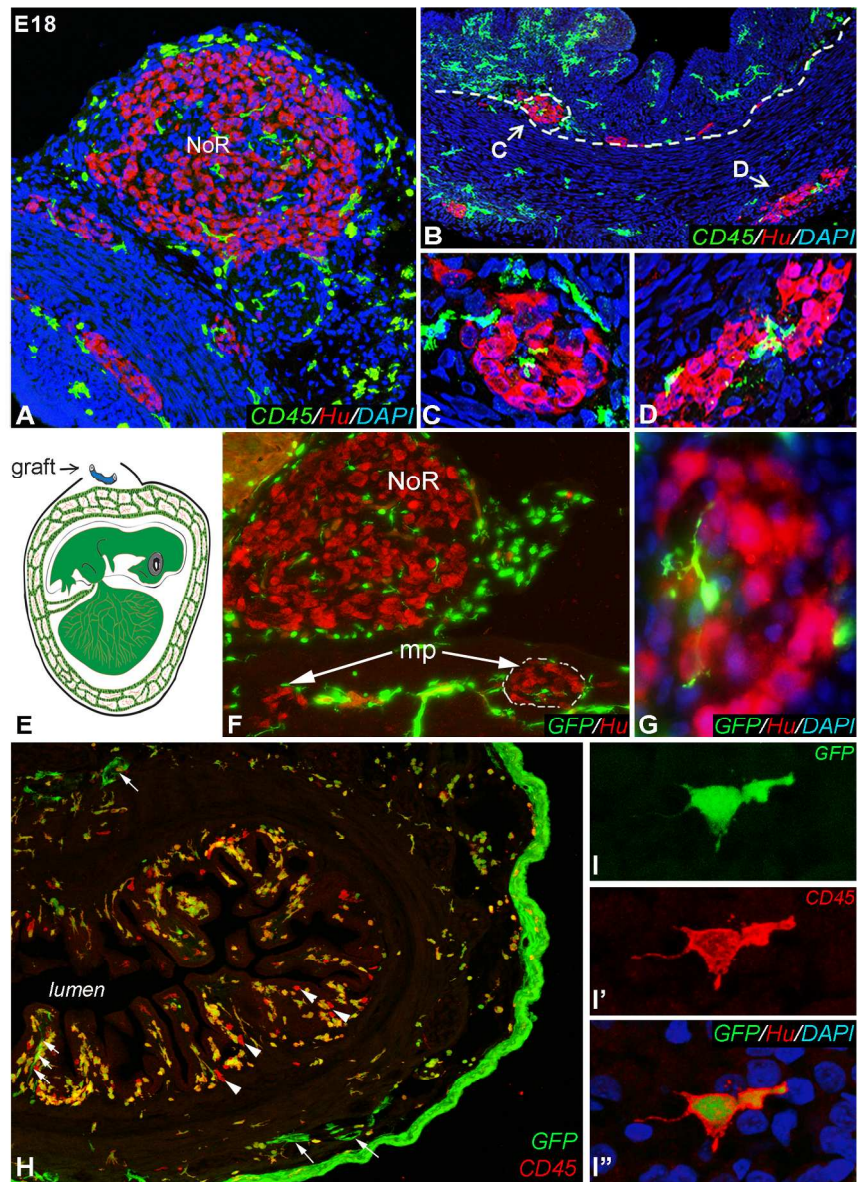


Figure 1

164x226mm (300 x 300 DPI)

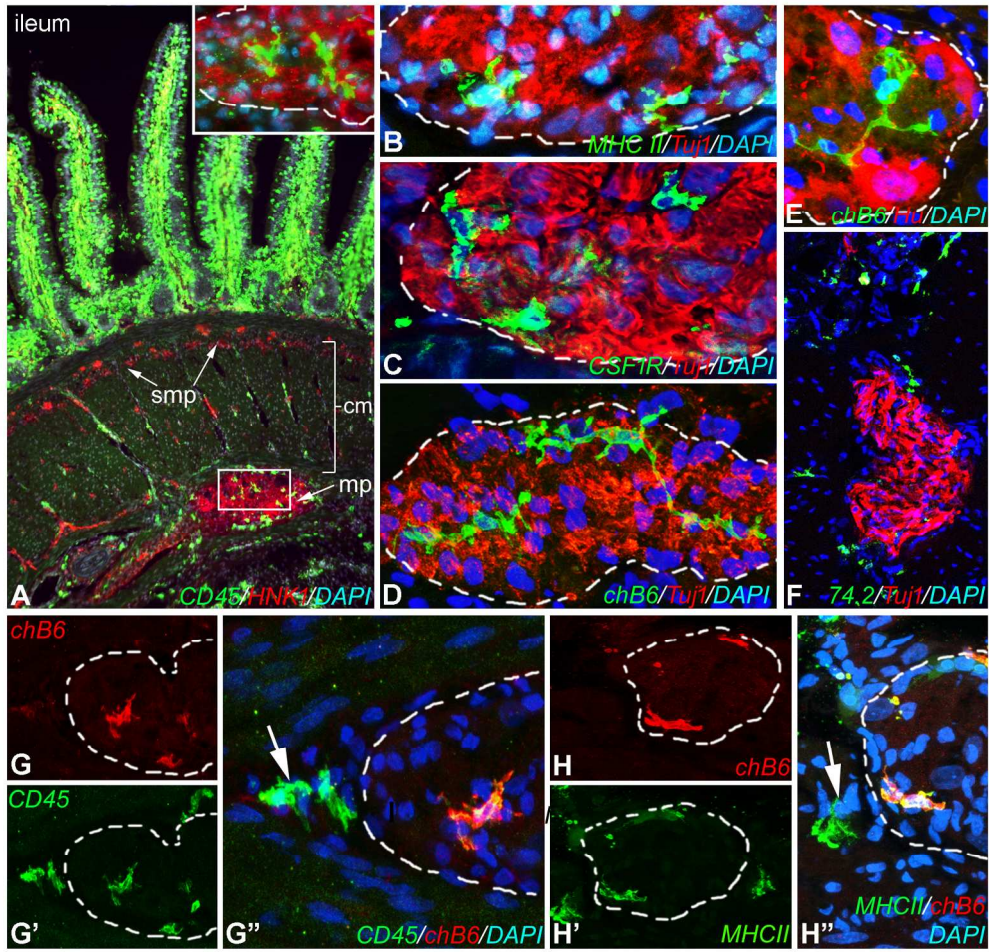


Figure 2

175x169mm (300 x 300 DPI)



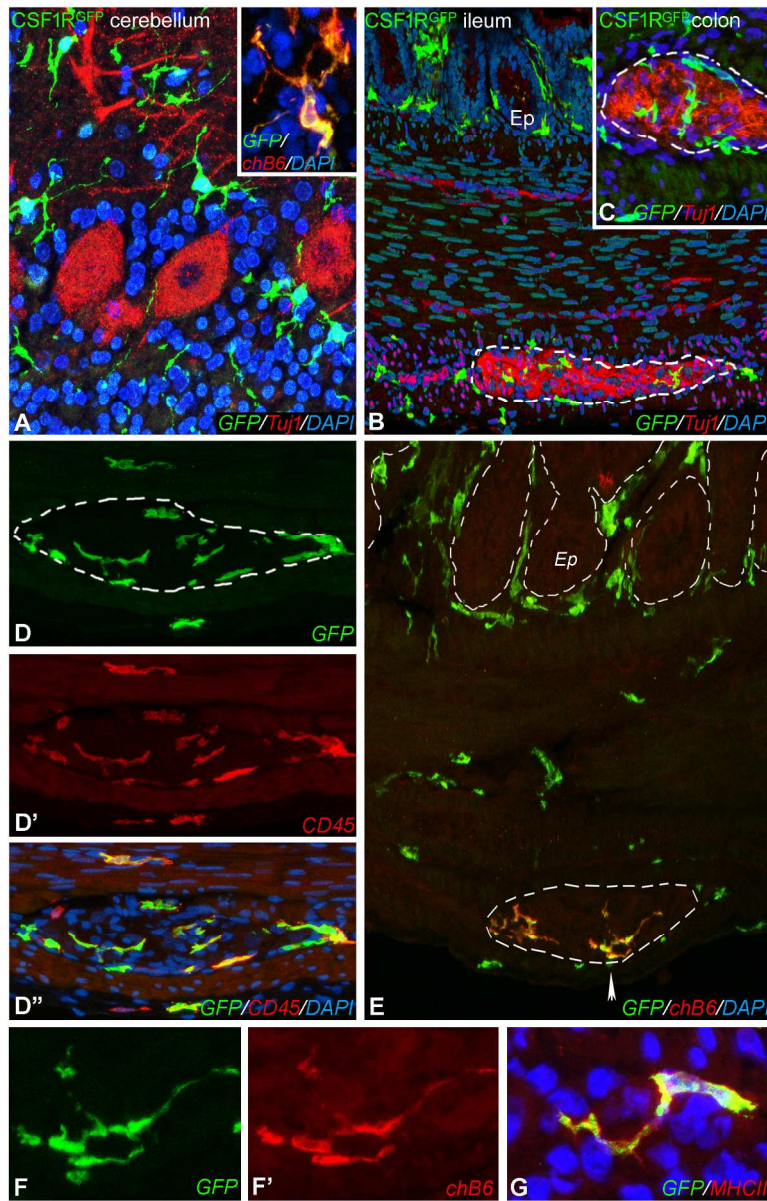


Figure 3

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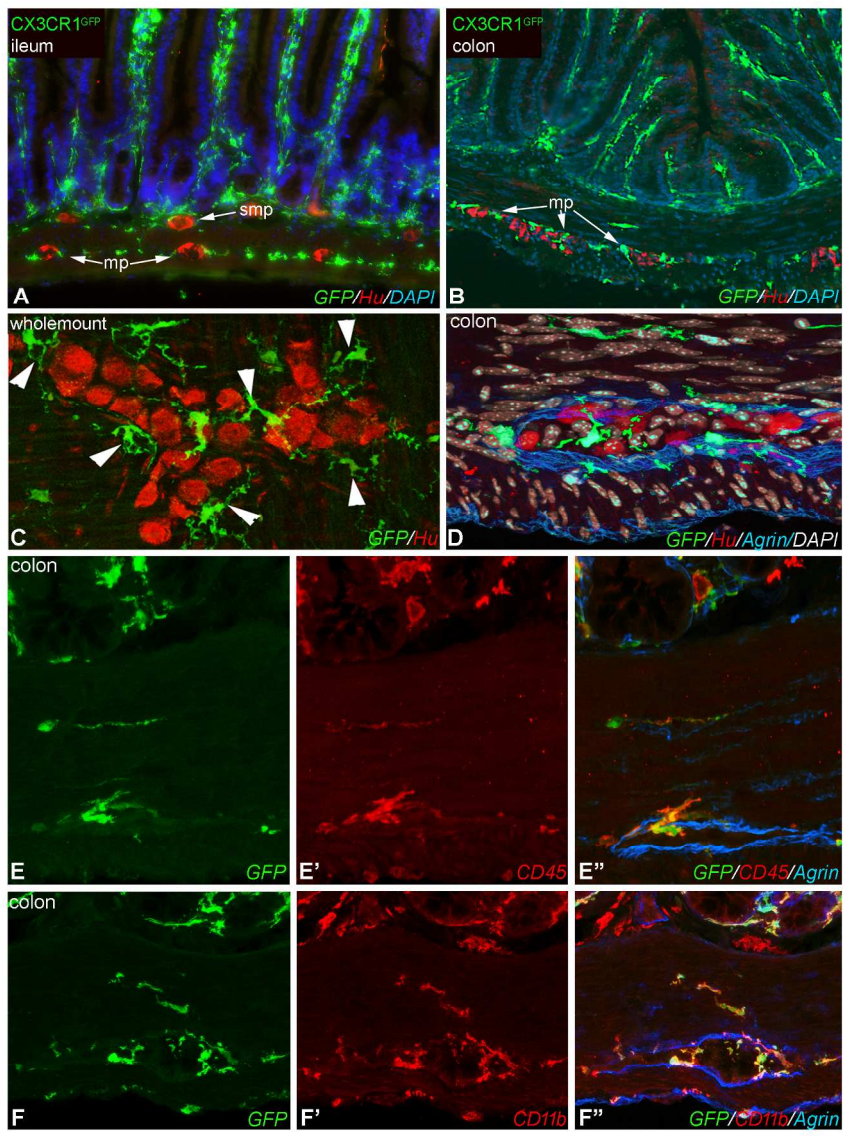


Figure 4

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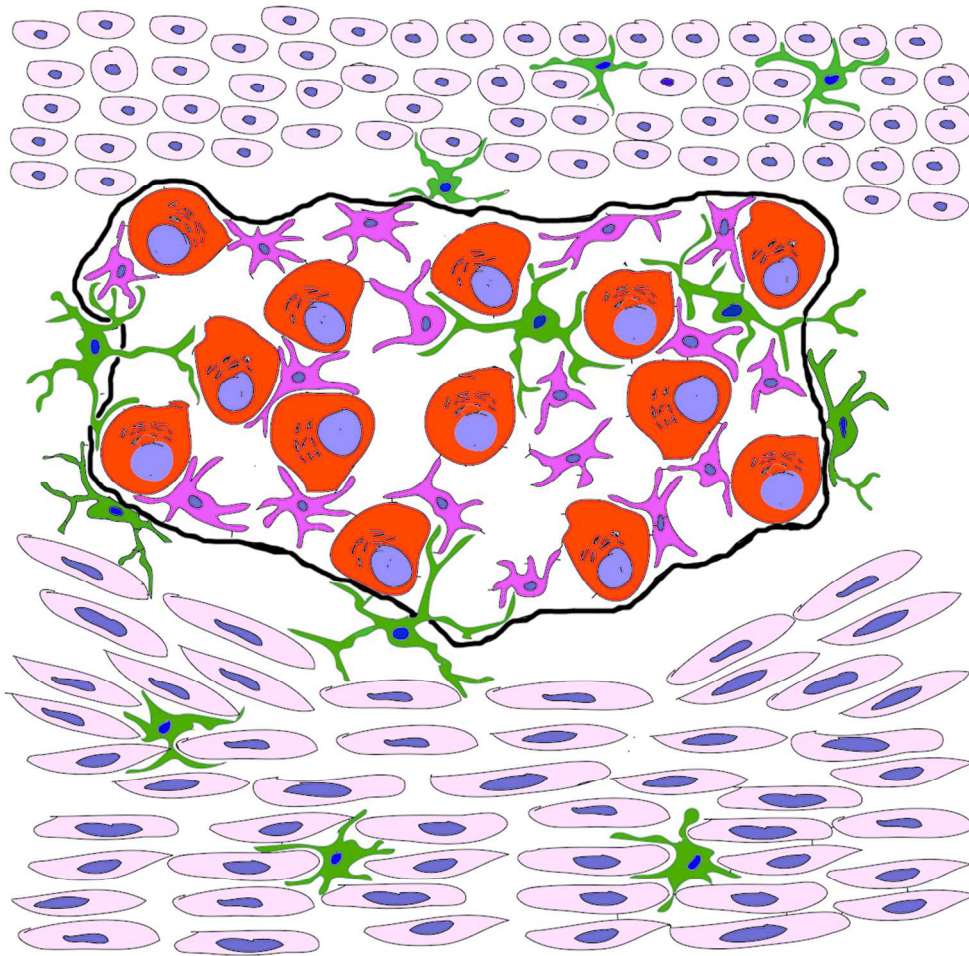


Figure 5

99x98mm (300 x 300 DPI)

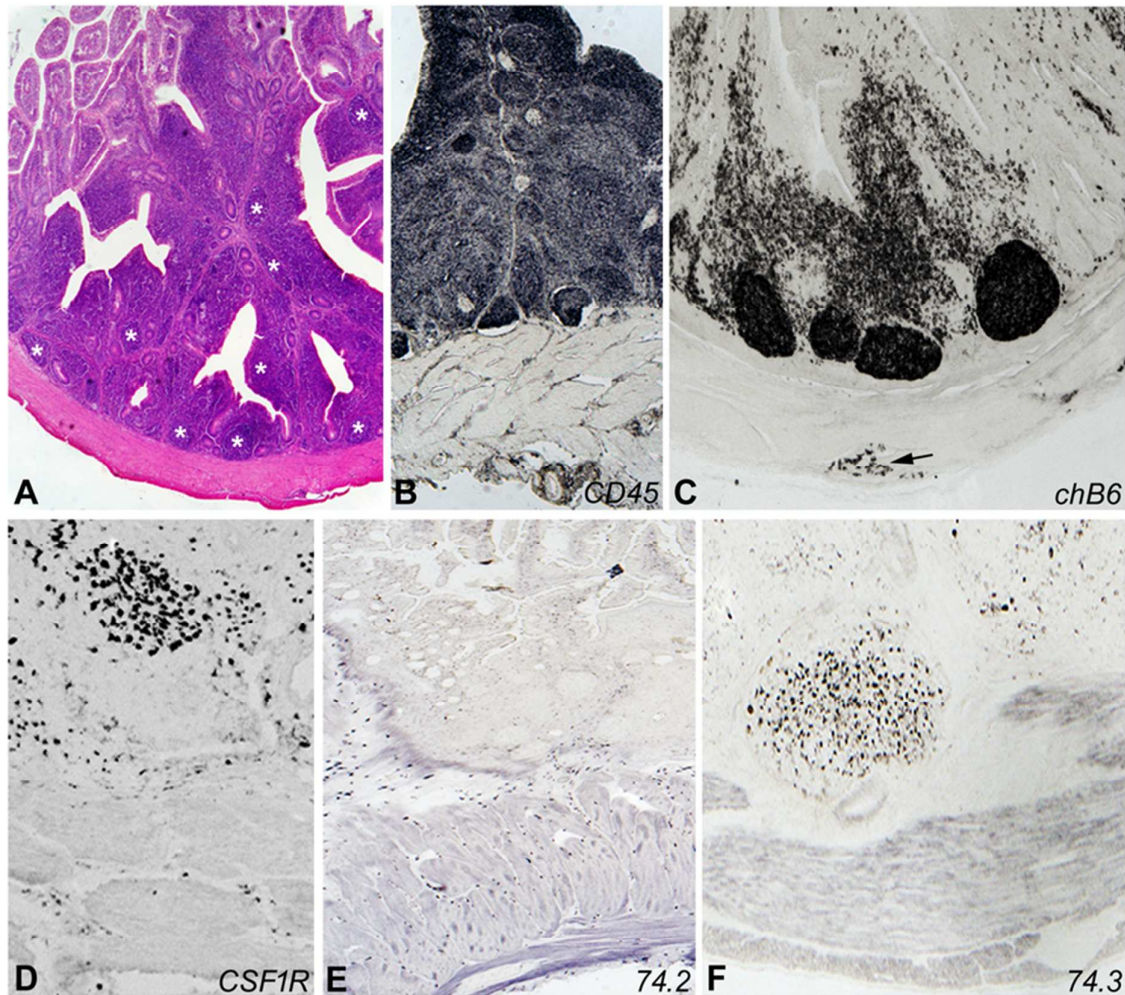


Table 1: Primary antibodies.

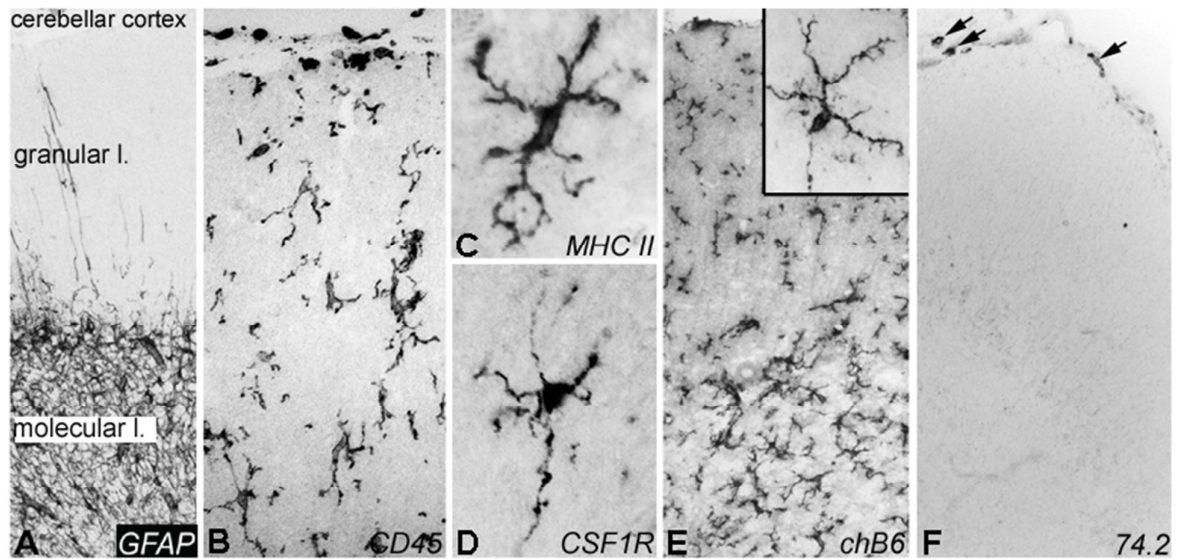
Antibody (clone)	Host	Structure/cells identified	Dilution	Source of antibody, catalogue number
HNK1	Mouse IgM	chick ENCCs	1:50	ThermoFisher (MA5-11605)
Tuj1 (B1195)	Mouse IgG2a	neurons	1:200	Covance (MMS-435P)
HuC/HuD (clone: 16A11)	Mouse IgG2a	neurons	1:100	Invitrogen (A-21271)
CD45 (HIS-C7)	Mouse IgG2a	chicken leukocytes	1:200	ThermoFisher (7500970)
CD45 (30-F11)	Rat IgG2b	mouse leukocytes	1:300	BioLegend (103101)
CD11b (M1/70.15)	Rat IgG2b	mouse macrophages	1:50	Bio-Rad (MCA74GA)
chB6 (BoA1)	Mouse IgG1	avian B-cells, subset of myeloid cells, microglia	1:200	Bio-Rad (MCA5958GA) Igyarto et al., 2008
MHCII (21-1A6)	Mouse IgG1	antigen presenting cells	1:200	Abcam (ab34031)
GFP	Polyclonal rabbit IgG	GFP-expressing cells	1:400	Abcam (ab5450)
8F3	Mouse IgG1	all chicken cells	1:5	Developmental Studies Hybridoma Bank
74.2	Mouse IgG1	chicken tissue macrophages	1:400	ThermoFisher (7500975)
CSF1R (ROS-AV170)	Mouse IgG1	dendritic cells, macrophages, microglia	1:100	Bio-Rad (MCA5956GA)
Agrin (AF550)	Polyclonal goat IgG	mouse extracellular matrix	1:400	R&D Systems (AF550)

SUPPLEMENTARY MATERIALS**Intraganglionic macrophages: a new population of cells in the enteric ganglia**

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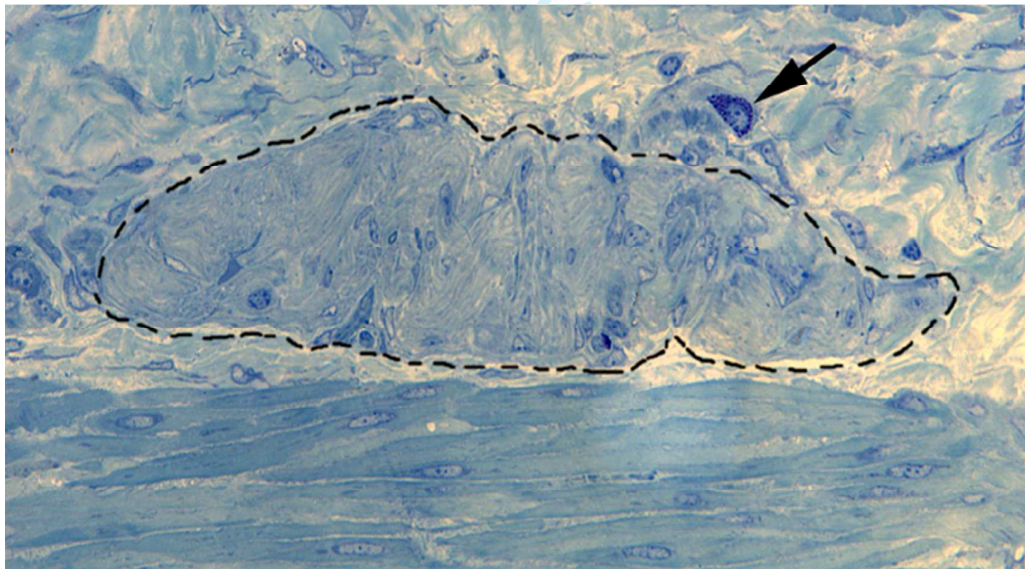
**Supplemental Figure 1.**

Immunostaining of the 6-week-old chicken cecal tonsil. Germinal centers of the lymphoid follicles are marked with asterisks (A). CD45+ lympho-myeloid cells are present throughout the lymphoid tissue and intestinal wall (B). chB6+ B cells are most abundant in the germinal centers, and also occur within the smooth muscle layers (C, arrow). Germinal centers contain CSF1R+ follicular dendritic cells (D). 74.2+ macrophages are scattered through the lamina propria and smooth muscle (E). The follicular dendritic cell marker 74.3 labels cells in the germinal centers and not in the smooth muscle (F).



Supplemental Figure 2.

Avian cerebellum contains microglia that share immunophenotypic features with intraganglionic macrophages. Sagittal section through the cerebellar cortex of 6-week-old chicken shows GFAP expression in glial cells (A). CD45 immunostaining labels highly ramified microglia throughout the granular layer of cerebellum (B). MHC II (C), CSF1R (D), and chB6 (E) also label chicken microglia. Macrophage-specific 74.2 antibody stains only tissue macrophages (arrows) in the meninges of the cerebellum (F).



Supplemental Figure 3.

Semithin section of 6-week-old chicken colon stained with toluidine blue identifies mast cells (arrow) around, and not within, the myenteric ganglia.

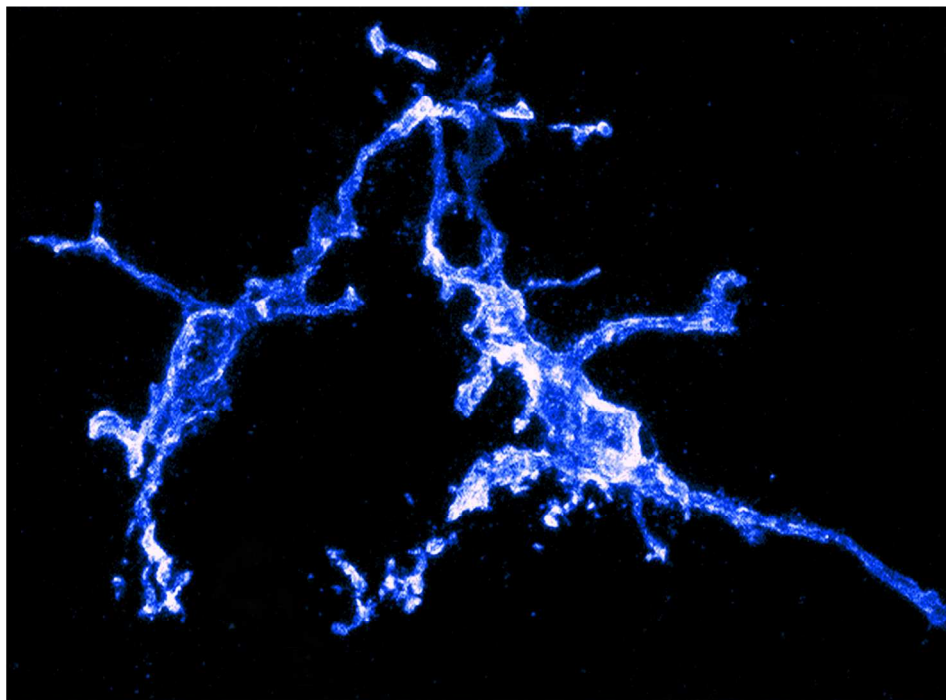
Supplementary video 1:

Image sequence of Z-stack made from 1,1 μ m optical sections through adult mouse enteric ganglion. CX3C1R labels intraganglionic macrophages (green). Agrin is expressed in

basement membrane of enteric ganglion (blue). Enteric neurons are positive for anti-Hu antibody (red). Cell nuclei were visualized with DAPI (grey).

See the video uploaded as separate file.

For Peer Review Only



Cover image suggestion.
Chicken microglia immunostained with chB6 specific antibody.

258x189mm (300 x 300 DPI)

View Only