

# Ontogeny of ramified CD45+ cells in avian embryo and in the ganglia of the enteric nervous system

Doctoral theses

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## **1. Introduction**

During vertebrate development, hematopoiesis, which is the process of proliferation, asymmetric self renewal and differentiation of hematopoietic stem cells (HSC), is responsible for blood cell formation. This process occurs at anatomically distinct sites, starting in extra-embryonic mesoderm (primitive hematopoiesis) and continues in the dorsal aorta of the embryo, ultimately seeding the para-aortic mesenchyme and fetal liver, after which HSC colonize the primary lymphoid organs and the bone marrow (definitive hematopoiesis). The avian embryo has been an exceptional model system for investigating hematopoiesis for over a century. Since the original demonstration, in chick-quail chimeric embryos, which the definitive HSC originate from an intra-embryonic source, namely from the ventral wall of the dorsal aorta and replace the extra-embryonically located yolk-sac-derived HSC, numerous research laboratories have used this model organism to reveal the origin and fate of embryonic HSC.

In Amniotes, the highly glycosylated cell surface protein CD34 and the common leukocyte antigen CD45 (a transmembrane glycoprotein with phosphotyrosine function) are considered to be pan-HSC markers. Notably, CD45 is not expressed on erythrocytes and endothelial cells, whereas CD34 expression is heterogeneous and is expressed by endothelial cells. Similar to mammals, the CD34 gene in chick is expressed by differentiating chicken HSC cells but no avian CD34-specific antibody has been reported. Therefore, in avian embryo, as in mouse and human, CD45 is considered the most specific cell surface marker of the hematopoietic lineage.

In avian embryos, as in mammalian embryos, HSC appear first in the extra-embryonic yolk-sac blood islands and predominantly generate a transient wave of erythroid and thrombocytic cells. The use of chick-quail chimeras and QH1 monoclonal antibody, which specifically labels quail endothelial and hematopoietic cells has demonstrated that, in addition to the erythroid-thrombocytic lineage, the yolk sac can also generate circulating endothelial cells and primitive macrophages. However, experimental evidence strongly supports that yolk-sac-derived myeloid cells invade the neural tube to differentiate into microglia and migrate to the ectoderm to give rise to epidermal dendritic cells but the contribution of yolk-sac-derived cells to the developing lymphoid organs is still a matter of debate.

The endothelial-associated intra-aortic clusters develop between E3–4 in chicken embryo, protrude into the lumen of the aorta and give rise to definitive HSC. The newly generated

aorta-associated HSCs are either released into the circulation or ingress into the mesenchyme of the dorsal mesentery forming the next place for aorta-associated hematopoiesis, the para-aortic foci. The CD45<sup>+</sup> pool of chick HSCs expands in the para-aortic region between E6 and E8 and disappears a few days before the onset of primary lymphoid organ development. Although chicken HSCs can be easily identified by CD45 expression, a detailed distribution pattern and immunophenotypic derivatives of this cell type have not been described.

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. The CNS includes a third population of cells, referred to as microglia, which are highly ramified cells first described by del Rio-Hortega. Although microglia are considered glial cells, they derive from hematopoietic, not neural crest precursors and are capable of antigen presentation. Multiple roles for microglia have been demonstrated in the developing and mature CNS, including contributing to learning-dependent synapse formation, phagocytosis and neuroprotection during inflammation and ischemia, synaptic pruning, and participation in crosstalk with neurons through fractalkine (FKN) and its receptor, CX3CR1. In the ENS, however, no cells corresponding to microglia have been identified. Recently, a CSF1R<sup>+</sup>/CX3CR1<sup>+</sup>/CD11b<sup>+</sup>/MHCII<sup>+</sup> macrophage population in the muscularis externa layer (muscularis macrophages, MMs) was found closely opposed to enteric ganglia in rodents. These cells appear to play a role in neuro-immune crosstalk between the mucosa-associated lymphatic tissue of the gut and the ENS. Further, the fractalkine receptor, CX3CR1, is uniquely expressed on intestinal macrophages and microglia, and not on other tissue macrophages. 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.

In my doctoral theses we demonstrate the first time a thorough analysis of the spatiotemporal appearance, colonisation and differentiation of embryonic CD45<sup>+</sup> cells, demonstrating cellular morphology and immunophenotypes as well. During the characterisation of embryonic CD45<sup>+</sup> cells we acknowledged the presence of a ramified macrophage population in the wall of the developing gut tube, close to the myenteric plexus. This observation raises the possibility that in the ganglia of the ENS in addition to neurons and glial cells of neural crest origin, a third population of cells is also present.

## **2. OBJECTIVES**

1. To identify the first CD45<sup>+</sup> cells of intra- and extra-embryonic tissues before and after they were connected by the vasculature.
2. To characterize the immunophenotype and to map the tissue distribution of CD45<sup>+</sup> HSC during early chicken development.
3. To determine the contribution of yolk-sac-derived HSC to the developing bursa of Fabricius (BF).
4. To characterize the ganglion associated CD45<sup>+</sup> cell population in the avian and mammalian enteric nervous system and to reveal their ontogeny by the use of embryomanipulation techniques.

Using double-immunofluorescence staining, we precisely evaluated the expression of specific hematopoietic and lymphomyeloid markers. We obtained evidence that CD45 antigen was expressed first in the yolk-sac blood island during primitive hematopoiesis. When green fluorescent protein (GFP)-expressing chick embryos were grafted into the yolk sac of normal chick embryos, extra-embryonic derived GFP<sup>+</sup> cells colonized all organ primordia. Moreover, when fragments of GFP<sup>+</sup> yolk sac were recombined with normal embryonic BF and co-cultured on the choriallantoic membrane, ramified GFP<sup>+</sup>CD45<sup>+</sup> cells migrated to the developing lymphoid follicles to differentiate into bursal secretory dendritic cells (BSDC).

Using intestinal chorioallantoic chimeras we showed that enteric ganglia-associated CD45<sup>+</sup> cells are of hematopoietic and not of neural crest origin. During the immunophenotypical characterisation of intraganglionic CD45<sup>+</sup> cells we revealed their macrophage signature, and that they express similar cell surface molecules as microglia.

### **3. METHODS**

#### **3.1 Animals**

Fertilized White Leghorn chicken (*Gallus gallus domesticus*) eggs were obtained from commercial breeders and incubated at 38 °C. Transgenic GFP-expressing chicken eggs were provided by the courtesy of Prof. Helen Sang, The Roslin Institute, University of Edinburgh. Embryos were staged according to the developmental tables of Hamburger and Hamilton (HH) or the number of embryonic days (E). Colony stimulating factor 1 receptor-GFP (CSF1RGFP) chicken were obtained from The Roslin Institute. CX3CR1GFP transgenic mice were kindly provided by Dr. Hans-Christian Reinecker, Massachusetts General Hospital, Boston. The design conditions of the animal experiments were approved by the Animal Ethical Committee of Semmelweis University, Budapest, Hungary.

#### **3.2 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. Frozen sections were cut at 12µm for epifluorescent imaging or 20 µm for confocal microscopy, collected on poly-L-lysine-coated slides, and stained by immunocytochemistry. Frozen sections were incubated with primary antibodies for 45 minutes, followed by biotinylated goat anti-mouse IgG and avidin-biotinylated peroxidase complex. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 minutes. The binding sites of the primary antibodies were visualized by 4-chloro-1-naphthol. 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. Mitotic cells were detected by using rabbit polyclonal antiphospho-histone H3 antibody. Anti-activated caspase-3 antibody was used to detect apoptosis. Cell nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole-dihydrochloride). Sections were covered with aqueous Poly/Mount and examined by using a Nikon Eclipse 80i microscope or with a Nikon A1R laser-scanning confocal microscope. Images were compiled using ImageJ and Adobe Photoshop.

### **3.4 India ink injections and in ovo yolk-sac culture**

To visualize the establishment of the circulation in chicken embryo, a Narishige microinjector device with a 50- $\mu$ l Hamilton syringe was used to inoculate embryos intracardially between stages HH10–13 with 2  $\mu$ l Pelikan India ink diluted 1:10 in PBS (n = 21). For in ovo yolk-sac cultures, 36-h-old (HH10) embryos (at this stage, no anastomosis is present between the yolk-sac blood islands and the vasculature of the embryo) were dissected out from the blastodisc by using Moria Pascheff-Wolff Spring scissors. To ensure the survival of the embryo-ablated yolk sac, the artificial inner margins of the yolk sacs were pinched with fine forceps. As a last step, the pinched margins were sealed with a single parallel cut with Pascheff scissors to prevent leakage of the yolk and the eggs were further incubated for 2 to 5 days at 38 °C. At the end of incubation, the yolk sac was dissected out from the egg, cut into 1 × 1 cm size, fixed in 4 % PFA for 2 h, embedded in gelatin and processed for immunostaining (n = 8).

### **3.5 Chick-quail parabiosis**

During the experiment we orientated the 24 hour chick parabiotic embryo to provide the largest possible attachment surface for the quail parabiotic embryo placed next to it, in a common egg-shell. We incubated the eggs at 38°C degree in our laboratory. From the 12 incubated parabiotic embryonic pairs, after 3 days 7 were alive, after 5 days 3, after 7 days 2 parabiotic pair were viable, that means 17% survival rate.

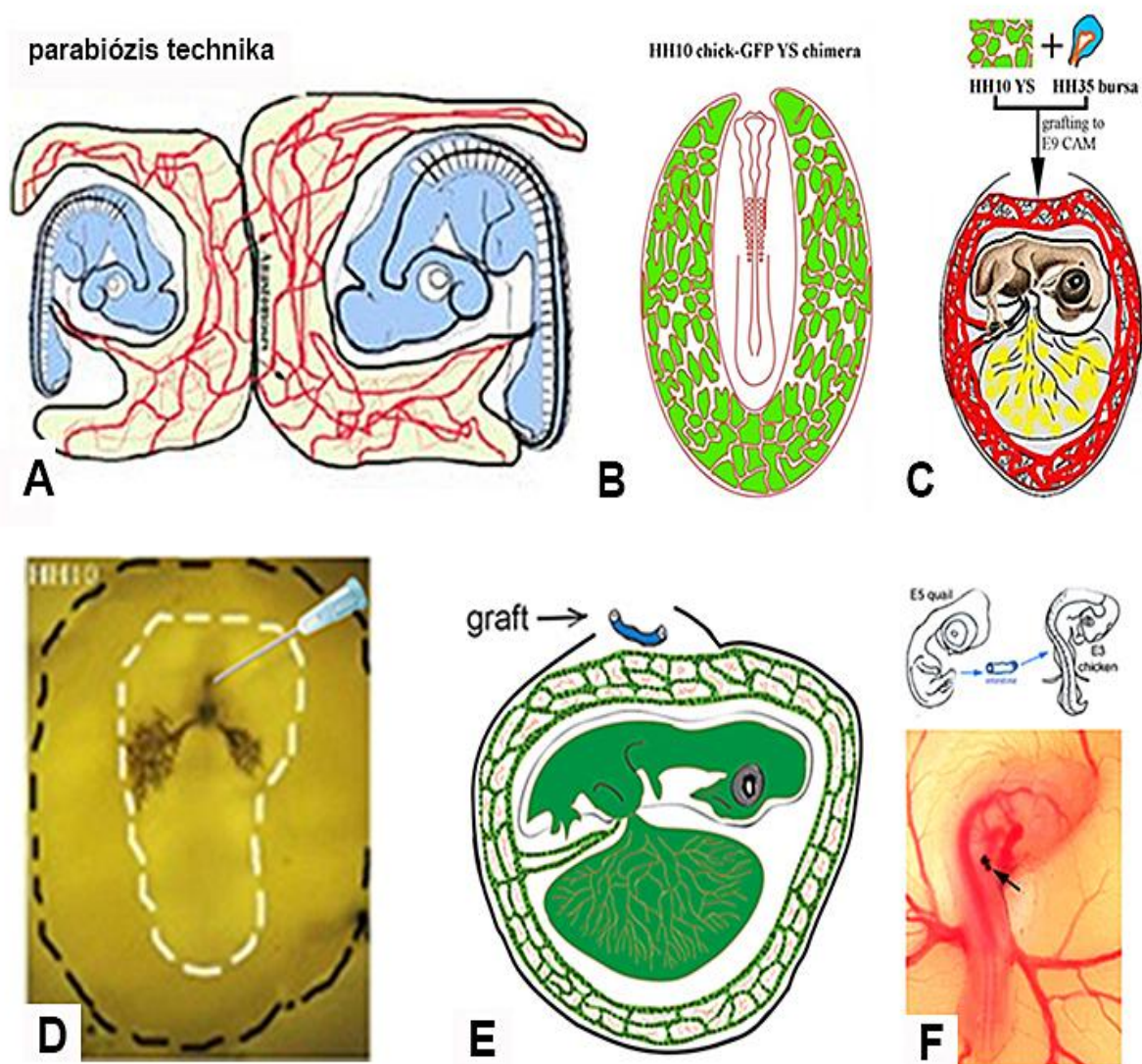
### **3.6 Yolk-sac chimera**

During the experiment chick (White leghorn) and GFP-chick eggs were incubated horizontally for 35–40 h at 38 °C until they developed to stage HH10. After the proper staging of the embryo, the vitelline membrane was removed from the surface by using a tungsten needle. The central area of the exposed blastoderm of a GFP-chick embryo was dissected out and replaced with a non-GFP chick embryo. To ensure the adherence between the associated donor and host blastodisc, free margins were stitched with horizontal cuts by using Pascheff scissors. After the grafting step, eggs were returned to the incubator (n = 32) and allowed to develop for up to an additional 72 h, once circulatory connections had formed through the suture, and the GFP+ yolk-sac-derived cells had colonized the embryo. Yolk-sac chimeras were fixed at E5 (n = 6) and immunostained. To follow the contribution of yolk-sac-derived cells to later stages and in particular to the B-cell-specific primary lymphoid organ, namely the BF, the E9 chicken bursa (stage before B cell colonization) was recombined with

fragments of GFP-chick yolk sac cultured 48 h after embryo ablation at stage HH10. The recombinants were grafted onto the E9 choriallantoic membrane (CAM) for 7 days (n = 24).

### **3.9 Chick-quail coelomic explants**

To generate quail-chick chimeras, the hindgut was removed from E8 quail embryos and were transplanted into the coelomic cavity of E3 chick embryos. During the experiment the vitelline membrane and amnion were carefully opened under the right wing bud. A longitudinal incision was made between the wing bud and heart by using a sterile tungsten needle. The isolated hindgut, labeled with sterile charcoal, was introduced into the coelomic cavity of the host avian embryo by using a blunt-end glass needle. The eggs were closed with adhesive tape and allowed to develop at 38°C for 14 days. The total number of chimeras produced was 8. The survival rate for the chimeras was 75%.



☛ **Figure 1.** Schematic presentation of embryomanipulational techniques. **A)** chick-quail parabiosis **B)** GFP yolk-sac chimera **C)** *in vitro* bursa Fabricii – GFP yolk sac recombination and CAM culture **D)** india ink injections and *in ovo* yolk-sac culture **E)** GFP-CAM culture of embryonic intestinal explant **E)** chick-quail coelomic explant

### **3.11 Chorioallantoic membrane (CAM) transplants**

During the experiment, 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.

## **4. RESULTS**

### **4.1 Emergence of CD45-immunoreactive cells in the early chick embryo**

Although CD45 is the only known cell surface marker expressed on the early chick HSC, a detailed analysis of its developmental expression pattern has not been described. In this study, we examined by immunohistochemistry the embryonic appearance and tissue distribution of CD45<sup>+</sup> cells from E2 (onset of chick hematopoiesis) to E10 (hematopoietic colonization of the primary lymphoid organs) stage in the chick embryo. Expression of CD45 was first detected at HH13, when two types of cells were identified: round CD45<sup>+</sup> cells observed in the lumen of the blood islands and elongated CD45<sup>+</sup> cells situated in the mesenchyme between the blood islands. No CD45-immunoreactive cells were found in the embryo proper. By HH15-16 (50–56 hours of development), following the onset of circulation between extra-embryonic and intra-embryonic compartments, the first round CD45<sup>+</sup> cells were detected inside the embryonic blood vessels. Starting at HH15, a second CD45<sup>+</sup> cell type was identified within the blood islands, where a subset of round CD45<sup>+</sup> cells co-expressed CD51/61 chick thrombocytic marker. In addition to circulating cells, CD45 antigen was also expressed by a large number of ramified cells with a slender cell body and long thin processes. These ramified cells were concentrated in the cranial mesenchyme around the neuroepithelium. CD45<sup>+</sup> cells with elongated and ramified morphology were also present



outside the vasculature in the trunk mesenchyme as early as HH17. Round CD45 cells were attached to the ventral side of the aortic endothelium, forming intra-aortic clusters previously reported to represent sites of definitive HSC emergence in the chick embryo. Approximately 12 h later, at HH21, the majority of CD45 cells had become scattered throughout the embryonic mesenchyme and showed a ramified morphology.

#### **4.2 Differentiation of CD45+ cells in early hematopoietic and primary lymphomyeloid organs**

By E5 (HH26), the rapidly increasing CD45+ ramified cells were dispersed around the notochord and neural tube, occasionally appearing in the neuroepithelium and surface ectoderm. Numerous highly ramified CD45-immunoreactive cells could be detected in the developing liver, pancreas and heart, in the mesenchyme surrounding the gut epithelium and in the limb bud mesenchyme. Immunohistochemistry of MHC II showed the presence of single cells lying in the mesenchyme around the notochord and having a similar morphology to ramified CD45+ cells. Staining with Gr12 mAb (chick granulocyte marker) revealed a group of amoeboid-shaped cells in the limb bud and in the mesenchyme surrounding the aorta. Cells with a similar morphology were immunoreactive for the macrophage-specific Lep100 (LAMP1 lysosome associated membrane protein 1) but negative for other macrophage markers, such as 74.2 and KUL01 (MRP1 mannose receptor protein 1). Acid-phosphatase histochemistry confirmed the macrophage identity of these CD45+Gr12+Lep100+ cells. At E5, the round CD45+ cells were mostly found as circulating cells inside blood vessels and represented either CD45+CD51/61+ thrombocytes or undifferentiated CD45+CD51/61-HSCs.

In the developing chick embryo, CD45+ round cells that were initially present as intra-aortic clusters at E3, ingressed ventrally to establish a new site of intraembryonic hematopoiesis, the para-aortic foci. CD45 immunoreactivity in the para-aortic foci was first detected in cross-sections at E6. Large populations of the round CD45+ cells were diffusely spread in the dorsal mesentery between the aorta and esophagus down to the mesonephros. CD51/61+ cells were also detected in the para-aortic region. Double-immunofluorescence demonstrated that  $49.9 \pm 19.6\%$  of CD45 cells in the para-aortic foci stained with the thrombocytic lineage marker CD51/61. No CD3 (pan-T cell marker) or chB6 (pan-B cell marker) expression was observed in this region.

While investigating the immunophenotype of CD45<sup>+</sup> para-aortic foci, we also observed CD45<sup>+</sup> cell aggregates present caudal to the mesonephros, where CD45<sup>+</sup> cells formed a complete ring around the coeliac artery. At E8, a well-developed CD45<sup>+</sup> periarterial hematopoietic sheath was found around the coeliac artery, possibly representing a new hematopoietic focus. At E8, ramified CD45<sup>+</sup> cells populated virtually all tissues including the neuroepithelium. Highly ramified CD45<sup>+</sup> cells occasionally occurred in the epidermis possibly representing precursors for Langerhans cells. Many CD45<sup>+</sup> ramified cells around the neural tube expressed MHC II ( $23.5 \pm 12.6$  %) but were negative for classic macrophage markers, including Grl2<sup>+</sup> and Lep100<sup>+</sup>, which were frequently observed in the mesenchyme around the aorta, gut and interdigital area of the limb buds. These cells produced acid phosphatase and had an amoeboid morphology. In the limb bud, uniformly distributed ramified CD45<sup>+</sup> cells were clearly distinct from the amoeboid-shaped CD45<sup>+</sup> cell grouped in the interdigital mesenchyme. Immunohistochemistry revealed a close association between Lep100<sup>+</sup> macrophages and areas rich in caspase-3 expression, marking the interdigital regions of the chick limb bud. From E8 to E10, the number of CD45<sup>+</sup> ramified cells steadily increased throughout the embryo in a pattern similar to that observed at earlier stages.

At this stage, the emerging lymphomyeloid organs (spleen, thymus, BF) were also colonized by CD45<sup>+</sup> cells. Spleen primordium started to develop as an appendage dorsal to the duodenum and dorsal pancreatic bud at day E4 and contained rare round-shaped CD45<sup>+</sup> cells, even at this early stage. By E10, dispersed CD45<sup>+</sup> cells were identified in large numbers throughout the splenic mesenchyme. The staining of consecutive sections showed that many round CD45<sup>+</sup> cells co-expressed B-cell-specific chB6 antigen, including in the mesenchyme around the coeliac artery. At E10, CD51/61<sup>+</sup>thrombocytes were also present in the developing spleen, as were MHC II<sup>+</sup> cells with a ramified morphology. The thymic epithelial rudiment arose from pharyngeal endoderm at E5 and was colonized by circulating CD45<sup>+</sup> HSC starting at HH29. At E10, the thymic primordium formed near the jugular vein and vagus nerve and contained mostly round CD45<sup>+</sup> cells. These round cells expressed CD3 (pan-T cell marker), whereas ramified cells expressed MHC II. The BF is the primary lymphoid organ for B cell development. The BF primordium develops at E5 as an epithelial diverticulum from the ectodermal part of the cloacal plate and is surrounded by the tailbud mesenchyme. CD45<sup>+</sup> cells were first observed at E8 when a few round and ramified cells appeared in the loose mesenchyme of the BF. At E10, three distinct cell types were observed in the BF by CD45 immunofluorescence: (1) a few round CD45<sup>+</sup>chB6<sup>+</sup> B cells were evenly

scattered in the mesenchyme; (2) small aggregates of round CD45+ Lep100 macrophages were concentrated in the proximal part of the BF mesenchyme; (3) CD45+MHC II+ cells with a stellate morphology were seen grouped under the BF epithelium.

### **4.3 Yolk-sac-derived CD45+ cells colonize chick embryo**

Since yolk-sac hematopoietic progenitor cells differentiate into various cell types, we hypothesized that the slightly ramified and elongated CD45+ cells observed in the HH13 yolk sac gave rise to intra-embryonic CD45+MHC II+ ramified cells. To test this, we cultured de-embryonated HH10 stage yolk sac *in ovo*. At this stage, the blood circulation between the embryo and extra-embryonic yolk sac has not yet been established. We confirmed this by India ink injection into the heart tube. Yolk sac cultures were generated by surgical ablation of the India-ink-injected HH10 chick embryo from the egg. By day 3, well-developed blood islands highly packed with aggregated peroxidase expressing erythrocytes formed throughout the extra-embryonic mesoderm. The yolk sac was then dissected from the egg and subsequently prepared sections were stained with phosphohistone-H3, showing that cell proliferation occurred continuously during the *in ovo* culture period, whereas we found no evidence of caspase-3 immunoreactivity. CD45 immunostaining of the cultured yolk sac revealed that morphologically two types of cells developed within the isolated extra-embryonic yolk-sac mesenchyme: round CD45 cells found primarily in the enlarged blood islands and ramified CD45 cells distributed uniformly in the extra-embryonic mesenchyme. Some of the round CD45+ cells expressed CD51/61, whereas some ramified cells expressed MHC II.

To test whether the yolk sac was the source of the intra-embryonic ramified CD45+ cells, we generated yolk-sac chimeras by using two different methodologies. In the first experiment, we surgically ablated the embryo at the HH10 stage from GFP-expressing chicken embryo and replaced it with an age-matched non-GFP embryo. GFP-labeled yolk-sac-derived cells fully colonized the grafted embryo and showed strong CD45 expression. GFP+ cells were uniformly scattered in the intraembryonic mesenchyme, including the neuroepithelium. All GFP+ cells were labeled with CD45 and their distribution pattern was similar to that of CD45+ ramified cells in the normal embryo. Additional staining to characterize the immunophenotype of the GFP+ cells within the embryo revealed that both MHC II and CD51/61 were strongly expressed. Thrombocyte-specific CD51/61 stained only round GFP+ cells and did not label the ramified cells, whereas MHC II marked only the ramified cells.

GFP+ cells with amoeboid morphology were also seen in the chimeras but were restricted to specific areas, such as the subaortic mesenchyme and expressed Lep100. As expected, CD45+ HSC in the intra-aortic ridge did not express GFP, since they were generated intra-embryonically.

#### **4.4 Yolk-sac origin of bursal secretory dendritic cells (BSDC)**

To determine the colonization and differentiation pattern of yolk-sac-derived cells at later stages and, in particular, during development of the primary lymphoid organs, fragments of yolk sac from HH10 GFP+ embryo were recombined with normal E9 chick embryo BF and co-cultured on E9 chick CAM. After 7 days, well-developed CD45+ lymphoid follicles formed in cultured chick BF developed similarly to those *in vivo*. Donor-derived GFP+ cells were observed throughout the BF, especially in the forming follicles. All the follicular GFP+ cells had typical ramified morphology and expressed CD45. To characterize the GFP+ cells further, staining with various antibodies was performed. chB6 staining showed that GFP+ cells were not B lymphocytes. GFP+ cells were also MHC II+, but MHC II was present in all the cells in the lymphoid follicles. Therefore, we used mAb 74.3, a specific and late marker of avian dendritic cells and found intense immunoreactivity in most GFP+ cells, confirming that the BSDC were derived from transplanted yolk-sac cells. The yolk-sac/BF recombinants contained chimeric lymphoid follicles in which some 74.3+ cells were GFP+, and others were GFP-. This occurred because both the GFP-derived yolk sac and the host bursa provided 74.3+ BSDC to the graft.

#### **4.5 CD45+ cells of hematopoietic origin in the ganglia of chick and murine ENS**

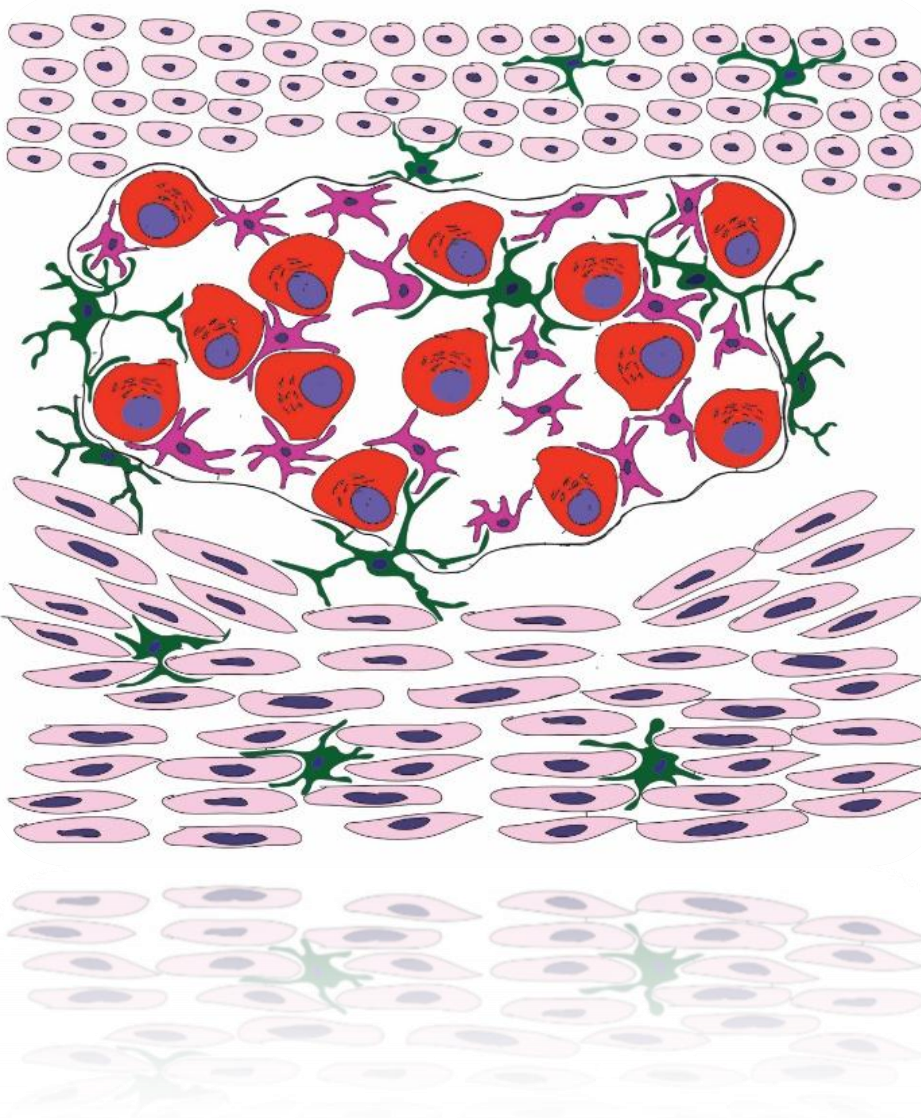
While characterizing the distribution and ontogeny of CD45+ hematopoietic cells in the chick embryo, we noted the presence of a ramified CD45+ cell type characteristic of macrophages within the gut mesenchyme. The cells appeared to be closely associated with the ENS and we therefore sought to further characterize this relationship. At E18, immunofluorescent staining of chick midgut shows the presence of ramified CD45+ cells within the nerve of Remak (NoR) and within the submucosal and myenteric ganglia. 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. 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 and ganglia. The GFP<sup>+</sup> cells present within the myenteric ganglia have a highly ramified appearance and express CD45, 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<sup>+</sup> ramified cells in the muscular layer, including within myenteric ganglia. Ramified CD45<sup>+</sup> cells also express MHC type II, CSF1R and chB6. chB6 recognizes B cells within the avian lymphomyeloid organs and also marks CD45<sup>+</sup> microglia. ChB6 is a 70-kD homodimer transmembrane protein with a highly glycosylated extracellular domain. Its function is not entirely known, though it has been reported to control cell survival, apoptosis and adhesion during B cell development. We find chB6 expressed by B cells scattered throughout the gut mucosa, microglia in the cerebellum, and a population of ramified cells within the enteric ganglia. The absence of chick B cell-specific EIVE12 or CD1 antigens on the intestinal macrophages 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, as well as microglia. We find CSF1R expressed not only by the MMs, but also by the CD45<sup>+</sup>/chB6<sup>+</sup> ramified cells in the enteric ganglia. 74.2 antibody labels an unknown cytoplasmic molecule that is highly restricted to phagocytic tissue macrophages. Interestingly, mAb 74.2 and other chick-specific tissue macrophage markers, including 68.2 and KUL01 mark cells outside, but not within, the ganglia. Together, these results show that avian enteric ganglia include ramified cells with a macrophage- and microglia-specific immunophenotype distinct from the 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<sup>GFP</sup> transgenic mice, in which GFP labels macrophages and microglia, which are known to express CX3CR1. CX3CR1-GFP-expressing cells are present within the myenteric ganglia, interdigitating among the Hu<sup>+</sup> enteric neurons.

Based on our findings, we conclude that enteric ganglia in both avians 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<sup>+</sup>/MHCII<sup>+</sup>/CSF1R<sup>+</sup>/chB6<sup>+</sup> in chick and CX3CR1<sup>+</sup>, F4/80<sup>+</sup>, CD11b<sup>+</sup>, and MHC II in mice. The immunophenotype of the IMs, however, supports the idea that these cells may represent microglia. 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 or KUL01), we propose that the CD45+ 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.



**Figure 2. Schematic illustration of murine and avian enteric ganglia.**

**red:**  
enteric neuron  
**magenta:**  
enteric glia  
**pink:**  
smooth muscle cells  
**green:**  
intraganglionic  
(CD45+MHCII+CSF1R+  
chB6+) and extraganglionic  
(CD45+MHCII+CSF1R+  
KUL01+74.2+) <sup>chick</sup> muscularis  
macrophages

## **5. Conclusions**

- 1.** We demonstrated that extraembryonically two types of CD45<sup>+</sup> cells differentiate: CD45<sup>+</sup> cells with round morphology in the lumen of blood islands, and ramified cells in the extraembryonic mesenchyme. In later stages of development round cells are localised associated to the aorta and its impaired visceral branches, ramified cells are found scattered throughout the embryonic mesenchyme.
- 2.** In addition to the already described intraaortic folds and paraaortic region, we identified a novel hematopoietic locus around the visceral branches of the abdominal aorta, and named it „periarterial hematopoietic sheath”. According to immunostainings of B-cell specific antibodies we hypothesize, that B-cell precursors arise in this anatomical location before the colonisation of lymphoid organs.
- 3.** We established a novel method of embryomanipulation for the culture of the yolk-sac. With combination of parabiosis, *in ovo* yolk-sac culture, yolk-sac chimera techniques and immunocytochemical analysis, we confirmed, that progenitor cells of intraembryonic CD45<sup>+</sup> ramified cells are derived from the yolk-sac, and after migration to the embryo, they colonise the organ primordia, where they differentiate to MHCII expressing dendritic, or „stellate” cells.
- 4.** We revealed the yolk-sac origin of bursal secretory dendritic cells (BSDC) with *in vitro* embryonic bursa Fabricii – yolk sac recombination and *in ovo* chorioallantoic membrane cultures.
- 5.** With intestinal chorioallantoic and coelomic explants we demonstrated, that blood-borne CD45<sup>+</sup> cells colonise the developing enteric ganglia in the chick embryo. These hematopoietic CD45<sup>+</sup> cells later differentiate to ramified cells and express cell surface molecules similar to microglia.
- 6.** We showed that the immunophenotype of intraganglionic and extraganglionic ramified cells are different in the adult chick gut. While CD45, MHC-II, and CSF1R cell surface molecules are expressed in both cell types, the expression of avian B-cell and microglia marker chB6 is specific to intraganglionic cells. In contrast, monocyte-derived tissue macrophage markers 74.2 and KUL-01 are solely expressed in extraganglionic cells.

## **6. Publications of the author**

### **6.1 Publications related to the doctoral theses :**

**Dora, D.**, Fejszák, N., Goldstein, A.M., Minkó, K., Nagy, N., 2017. Ontogeny of ramified CD45 cells in chicken embryo and their contribution to bursal secretory dendritic cells. *Cell and Tissue Research* 368, 353–370. doi:10.1007/s00441-017-2595-y

**IF: 3,043**

**Dora, D.**, Arciero, E., Hotta, R., Barad, C., Bhave, S., Kovacs, T., Balic, A., Goldstein, A.M., Nagy, N., 2018. Intraganglionic macrophages: A new population of cells in the enteric ganglia. *Journal of Anatomy*. doi:10.1111/joa.12863

**IF: 2,479**

### **6.2 Other publications:**

Nagy, N., Barad, C., Hotta, R., Bhave, S., Arciero, E., **Dora, D.**, Goldstein, A.M., 2018. Collagen 18 and agrin are secreted by enteric neural crest cells to remodel their microenvironment and regulate their migration during ENS development., n.d. . *Development*. doi:10.1242/dev.160317

**IF: 5,413**