

## REVIEW

**Origin and fate of hematopoietic stem precursor cells in the leech *Hirudo medicinalis*****A Grimaldi***Department of Biotechnology and Life Sciences, University of Insubria, Via J. H. Dunant 3, 21100 Varese, Italy**Accepted July 12, 2016***Abstract**

The hematopoietic process by which blood cells are formed has been intensely studied for over a century using several model systems. An increasing amount of evidence shows that hematopoiesis, angiogenesis, immune response and the regulating these processes (*i.e.*, cytokines) are highly conserved across taxonomic groups. Over the last decade, the leech *Hirudo medicinalis*, given its simple anatomy and its repertoire of less varied cell types when compared to vertebrates, has been proposed as a powerful model for studying basic steps of hematopoiesis and immune responses. Here, I provide a broad overview of *H. medicinalis* hematopoiesis and I highlight the benefits of using leech as a model.

**Key Words:** leech; hematopoietic precursor; stem cells; VEGF; hematopoiesis

**Introduction**

In vertebrates, hematopoiesis occurs throughout embryonal, fetal and/or adult life and has been evolutionarily conserved.

Primitive hematopoiesis in vertebrates first occurs in the extraembryonic yolk sac, where aggregates of cells, forming “blood islands”, give rise to both precursors of endothelial cells (localized at the periphery of the cellular aggregates) and hematopoietic stem precursor cells (HSPCs) (localized at the central part of the cluster) (Choi *et al.*, 1998; Saha *et al.*, 2004). Both hematopoietic and vascular progenitors derive from a common precursor of mesodermal origin, known as hemangioblast (Baron, 2003; Bailey and Fleming, 2003). A second round of hematopoiesis, termed definitive hematopoiesis, occurs in the mesodermal aorta/gonad/ mesonephros (AGM) region of the embryo and gives rise to cells that will seed the fetal liver and the bone marrow in mammals and from which all mature blood cell types of adult organism derive (Dzierzak, 1999; Matsuoka *et al.*, 2001).

In adult life, HSPCs primarily reside in the bone marrow, but during homeostasis and/or under stress conditions they circulate in blood and lymph, reaching other hematopoietic and non-hematopoietic organs (Massberg *et al.*, 2007; Si *et al.*, 2010). Thus, injury, infections and inflammation can all induce stem cell niches outside the bone

marrow (Martinez-Agosto *et al.*, 2016), which, under specific conditions, can support HSPCs proliferation and differentiation. In particular, in sites of tissue inflammation several cell types, including activated endothelial cells, fibroblasts, macrophages, and other innate immune cells, provide a wide range of hematopoietic growth factors required for HSPCs homing, retention, and function (Granick *et al.*, 2012). Since HSPCs circulate in the body periphery, express pattern recognition receptors (PRR) and respond to conserved microbial and viral molecular patterns, it has been hypothesized that they represent cells actively involved in innate immune and inflammatory responses (Nagai *et al.*, 2006). PRR are signaling receptors that recognize conserved pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs) in order to trigger immune responses. Indeed, upon detection of PAMPs, HSPCs preferentially differentiate towards mature myeloid immune cells to fight infection (de Luca *et al.*, 2009). These evidences suggest that HSPCs not only represent a key source of leukocytes in the bone marrow, but also play a crucial role in peripheral tissues, where they act as a source of both mature effectors for immediate local leukocyte needs and innate immune cells, which orchestrate inflammatory responses and mediate tissue repair (Granick *et al.*, 2012).

In the last decade, a large body of evidence has demonstrated the occurrence of significant similarities in hematopoiesis, angiogenesis and immune response among vertebrates and various invertebrate phyla (Hartenstein, 2006; Hartenstein and Mandal 2006). In particular, the host defense in *Hirudo medicinalis* (Annelida, Hirudinea) is extremely

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efficient and relies exclusively on innate immunity, which provides a strong nonspecific response characterized by the proliferation and migration of immune cells towards the sites of infection. In response to surgical wounds, grafts or injections of stimulating factors (*i.e.*, LPS, Gram-positive bacteria, cytokines and growth factors), HSPCs are massively recruited from the bloodstream towards sites of inflammation, where they differentiate into mature leukocyte cells with migratory and phagocytic capacity (de Eguileor *et al.*, 1999; 2000a, b, 2004; Tettamanti *et al.*, 2003a, b; 2006; Grimaldi *et al.*, 2006, 2010; Schorn *et al.*, 2015a, b). These myeloid lineage-derived cells, such as macrophages, granulocytes and natural killer cells (NK), perform different types of responses in relation to the antigen size. Indeed, small dimensions antigens are phagocytized, whereas larger ones induce both cytotoxic responses and encapsulation. Interesting, immune challenges and surgical lesions in leeches not only give rise to immune responses based on the same process of cell lineage commitment, proliferation, differentiation and migration (Schikorski *et al.* 2008) involved in tissue regeneration, but are also regulated by the same key molecules, such as cytokines and growth factors (Schorn *et al.*, 2015a, b; Tettamanti *et al.*, 2006). Given its simple anatomy and the less varied repertoire of cell types with respect to vertebrates, *H. medicinalis* displays a clear and easily detectable pattern of responses evoked by different stimuli and for this reason, it represents a powerful model system for studying the basic steps of both hematopoiesis and immune response (Grimaldi *et al.*, 2006; Schikorski *et al.*, 2009).

In this review, I will describe the hematopoietic process of *H. medicinalis* immune competent cells. Specifically, I will depict the hematopoietic environments representing their site of origin, the various HSPCs-derived cell types and the cellular responses and the regulating factors that are engaged in these processes.

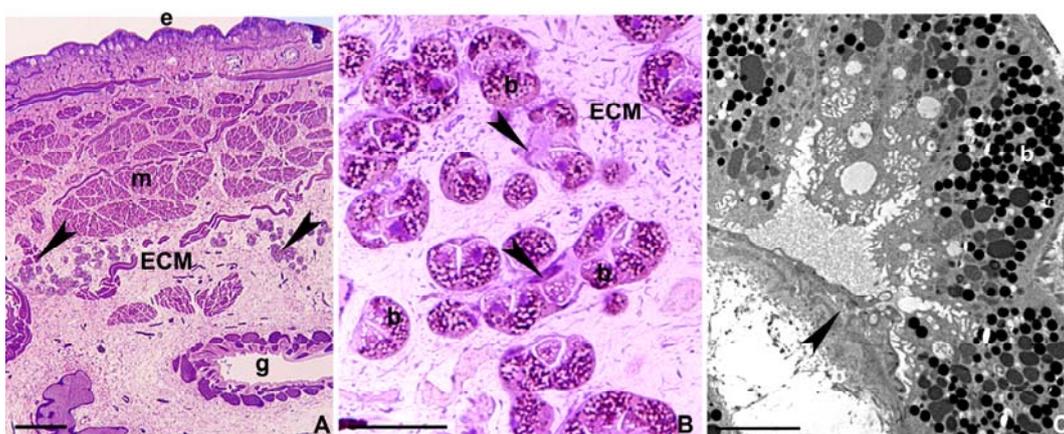
#### The leech body organization

*H. medicinalis* shows a parenchymatous body characterized by a relatively simple anatomy. Underneath the cuticle and the epithelium, muscles are organized in three layers of fibers circularly, obliquely and longitudinally disposed. In each layers, muscles are organized in fields which are in close contact to each other, surrounded by a scant extracellular matrix. Within the entire thickness of the muscular sac, blood vessels are almost absent (de Eguileor *et al.*, 2003, 2004; Tettamanti *et al.*, 2004). The musculocutaneous sac is separated from the digestive tube by a thick layer of loose connective tissue, containing the botryoidal tissue located close to the digestive system (Fig. 1A).

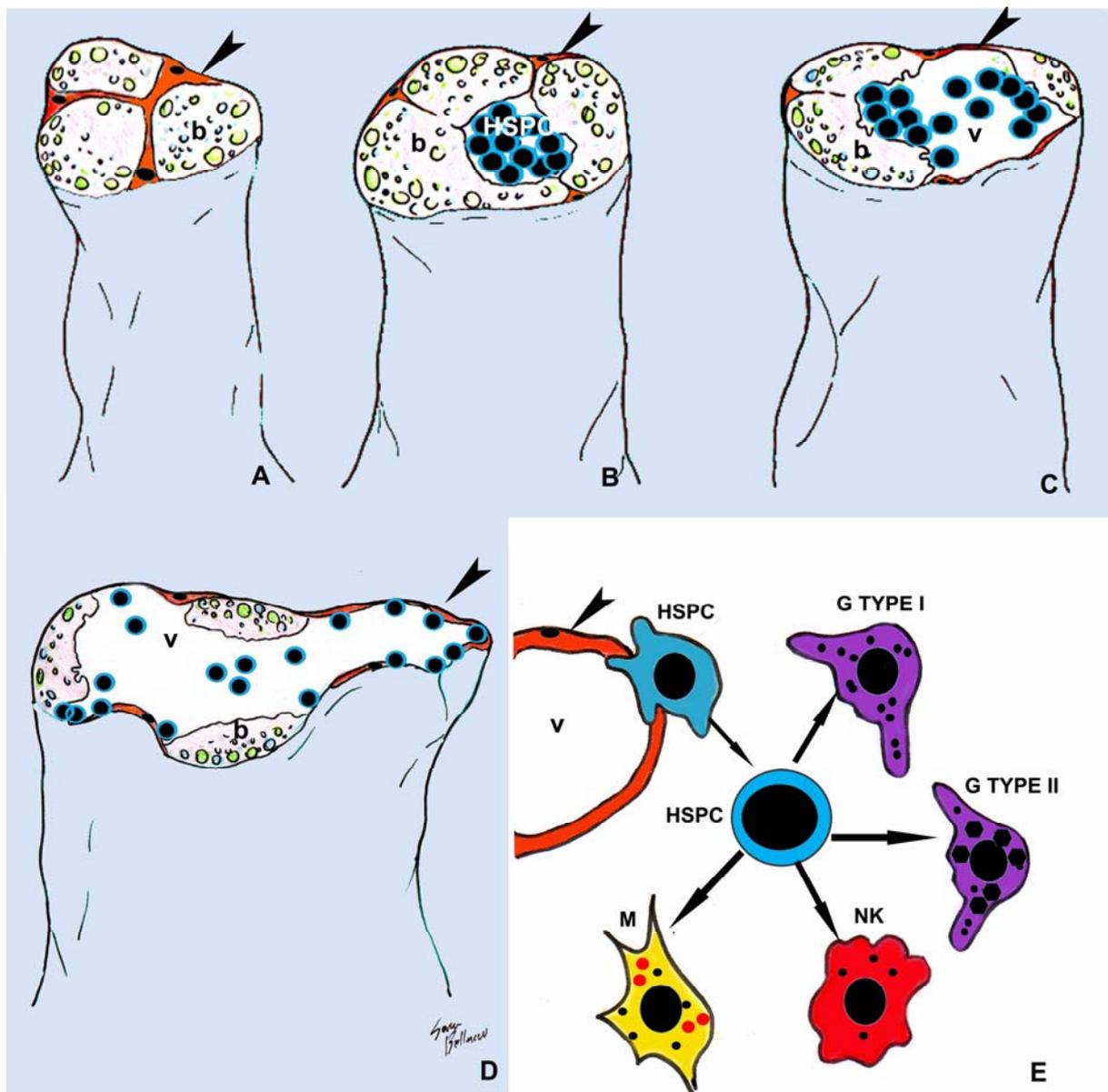
#### The botryoidal tissue and the origin of *H. medicinalis* HSPC

In leeches, immune competent cells have a mesodermal origin (Grimaldi *et al.*, 2006) and to date the botryoidal tissue has been defined as the hematopoietic organ from which myeloid lineage-derived leucocytes arise. This tissue is surprisingly versatile, since it not only displays myelo/erythroid and storage functions (Fischer *et al.*, 1976; Sawyer, 1986; de Eguileor *et al.*, 2001b), but is also involved in hematopoietic cells production (Grimaldi *et al.*, 2006) and angiogenesis (de Eguileor *et al.*, 2001a, b).

The botryoidal tissue is composed of clusters of cells of mesodermal origin, broadly ranked in two cellular types: botryoidal and endothelial-like cells, linked by desmosome-like junctions and well separated from the connective tissue by a basal lamina. Botryoidal cells are large, roundish or oval and their cytoplasm contained multiple randomly distributed granules of different sizes and staining properties. By contrast, endothelial-like cells display a cytoplasm which is poor in organules and granules, are thin and linked with their neighbors by junctions (Figs 1B, C).



**Fig. 1** Botryoidal tissue. General view at optical microscope of the body of an unlesioned *H. medicinalis* (A). Under the cuticle and epithelia, large layers of muscle tissue (m) are visible. The botryoidal tissue (arrowhead) is localized between muscles and gut (g), is immerse in a loose connective tissue (ECM) and is formed by two type of cells: the large and oval botryoidal cells (b) and smaller and flattened endothelial-like cells (arrowheads). Detail at TEM (C) of botryoidal cells (b) showing a granule-filled cytoplasm and endothelial cells (arrowheads) characterized by smaller size, flattened shape, and by an agranular, electrondense cytoplasm. Bar in A: 100  $\mu$ m; Bar in B: 25  $\mu$ m; Bar in C: 5  $\mu$ m.



**Fig. 2** Drawing representing the proposed model of the hematopoietic and vasculogenesis process in *H. medicinalis*. Left to right: from solid cords of botryoidal tissue cells (A) a vascular lumen (v) is shaped through a dehiscence process (B-D). During this process, in the new vessel cavity (v), lined by endothelial cells (arrowheads) cluster of hematopoietic stem cells (HSPC) become evident (B, C). After crawling between endothelial adjacent cells, the HSPC leave the bloodstream and differentiate into mature leukocyte (E).

The beginning of vasculogenesis, involving the early formation of the vascular system, is characterized by a marked remodeling of the botryoidal tissue. Immediately after the induction of particular stimuli, such as surgical lesions, bacterial infection or the injection of specific growth factors, cells within the botryoidal tissue change their shape from a solid cord of cells to a tubular, pre-vascular structure (Figs 2A-D). Therefore, the diverse functions of botryoidal tissue are related to the varying physiologic demands occurring during the life cycle of leeches.

The process of angiogenesis in *Hirudo* body wall, which is virtually avascular in normal conditions, represents a mechanism for the fast recruitment in the stimulated lesioned area of a large amount of cells involved in immune defense and wound healing. Indeed, concurrently to neovessel formation, groups of closely associated HSPCs become evident in the centre of the immature vessel lumen (Figs 2B, D). As the vessel grows, these circulating precursor cells lose cell-to-cell contacts to move freely within the lumen. Thus, both botryoidal and endothelial cells contribute to

the creation of tissue niches promoting hematopoietic cells maintenance (survival and self-renewal) and regulating precursors cells migration, quiescence and differentiation.

The leech hematopoietic precursors cells show the typical morphology of blast-like cells and, as in vertebrates, are multipotent progenitors that give rise to all types of mature blood cells. As such, they express several surface markers commonly used to identify vertebrate endothelium and myeloid hematopoietic progenitors, in particular the cluster of differentiation CD34, CD117, CD33 and CD45 markers (Wells *et al.*, 1996; Crocker and Varki 2001; Guo *et al.*, 2003; Hildbrand *et al.*, 2004; Kiel *et al.*, 2008; Raaijmakers *et al.*, 2008). These markers have been related to the functional need of maturing cells, and can be up- or down-regulated by different cytokine and growth factors. In particular, CD117, CD33, CD34 and CD45 are typically localized on clustered or isolated precursors within the neo-lumen and in cells adhering to vessel lumen. As in vertebrates, these populations of cells are also positive for the VEGF (Vascular endothelial growth factor) receptors Flk1 and Flt1, confirming their responsiveness to this cytokine (Damert *et al.*, 2002; Tettamanti *et al.*, 2003a).

Most of these HSPCs, conveyed into the lesioned/stimulated area by vessels, adhere to the luminal wall and, after crawling between endothelial adjacent cells, leave the bloodstream, disperse in the surrounding connective tissue and differentiate into mature leukocytes committed to mediate the inflammatory response (Grimaldi *et al.*, 2006; Fig. 2E). As previously reported for vertebrates, several factors play a key role in this interaction, such as L-selectin, talin and vinculin. In particular, leech HSPCs that extravasate are positive for L-selectin, which is a typical marker for hematopoietic cells adhering to the inner vessel wall and destined to leave the blood stream. Subsequently, extravasating HSPCs express talin and vinculin, whose role is to link collagen fibers during migration across the extracellular matrix (Grimaldi *et al.*, 2006).

#### *Structural and functional characterization of leech mature leukocyte population*

The specific nomenclature of mature leech immunocytes, first established almost 20 years ago by de Eguileor and collaborators (1999), includes macrophages, two types of granulocytes and natural killer cells (NK). These cells, which have been characterized by ultrastructural and immunohistochemistry analyses and are involved in the leech defense system, not only display features and behaviors typical of those found in vertebrates, but also deploy the same molecules used as immune cell markers for vertebrates. Indeed, several reports (de Eguileor *et al.*, 2000a, b; Grimaldi *et al.*, 2004, 2006; Macagno *et al.*, 2010; Schorn *et al.*, 2015a, b) have provided extensive evidence for the occurrence, in leech, of a wide range of CD proteins which are commonly used as immune cell markers, as for mammalian CDs. In vertebrates, the structure and function of many surface markers CD have been deeply investigated and are strictly linked to a specific cell type or to a specific function. For instance, CD45 is a cell

surface glycoprotein that is implicated in integrin-mediated adhesion of macrophages in vertebrates (Roach *et al.* 1997; Zhu *et al.*, 2011; St-Pierre and Ostergaard, 2013) and is thought to play a role in regulating the functional responsiveness of cells to chemoattractants (Roach *et al.* 1997; Mitchell *et al.* 1999).

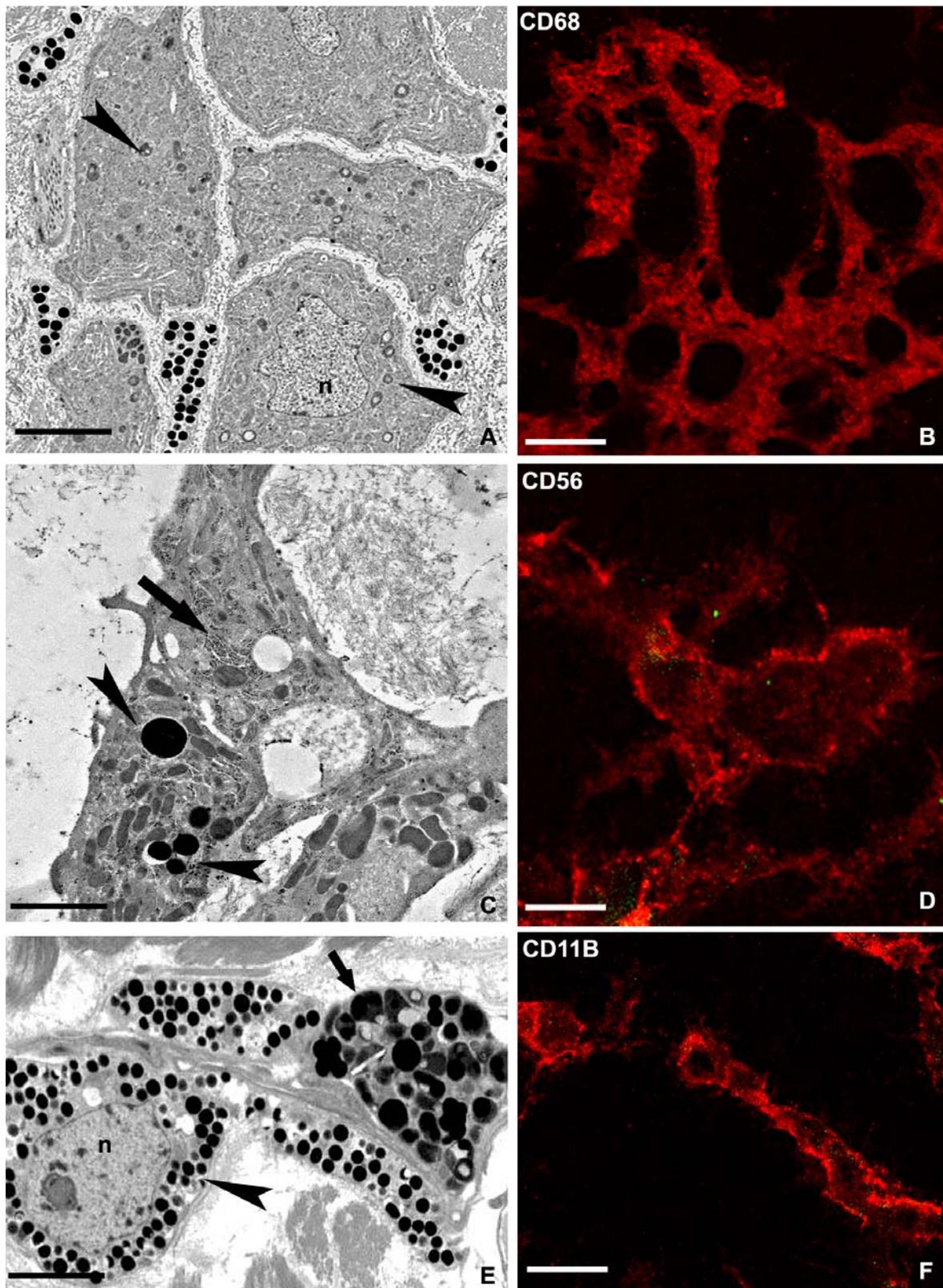
The CD14 and CD68 markers are expressed on cells from the myelomonocytic lineage, including monocytes and macrophages. In particular, CD14 is a glycoprotein receptor for LPS that promotes phagocytic uptake, whereas CD68 is an antigen primarily expressed as an intracytoplasmic molecule in human macrophages. CD11b (Springer, 1990) and CD11c are macrophage and granulocytes-associated proteins (Cabanas and Sanchez-Madrid, 1999) while CD56 and CD57 antigens are expressed by cells with natural killer activity (Robertson *et al.*, 1996).

The use of antibodies raised against mammalian CD antigens to detect immune cells in the leech is also supported by several data from the literature concerning earthworm and sipunculids. By means of cytofluorimetric assays with mouse anti-human monoclonal antibodies, Cossarizza and colleagues (1996) were able to distinguish two types of celomocytes in the annelid *Eisenia foetida*, whereas Blanco and collaborators (1997) identified celomocyte surface antigens cross reacting with anti-human CD14, CD11b and CD11c in sipunculids, a phylum closely related to annelids. The observed cross-reactivity of sipunculid and earthworm leukocytes with anti-human CD markers is a further confirmation that cell recognition and immunodefense system share similar characteristics and effector molecules in leeches and vertebrates.

The morphological characteristics and functions of each of these immune cells are described in detail below, including molecular markers with which they have subsequently been associated.

#### *Macrophage-like cells*

Phagocytosis is a primordial aspect of innate immunity and is highly conserved in all metazoan. In *H. medicinalis*, macrophages-like cells resemble the mammalian monocyte/macrophage lineage cells and represent the cell population primarily involved in non-self recognition. Characterization of these cells was achieved by ultrastructural analysis, acid phosphatase reaction and immunohistochemistry using polyclonal antibodies directed against the human macrophage and leukocytes markers CD68 and CD45 (Schorn *et al.*, 2014). These cells are characterized by pseudopodia and numerous phagolysosomes in their cytoplasm (Fig. 3A). Both ultrastructural morphological analyses and acid phosphatase reaction positivity confirmed that activated macrophages migrating towards non self-producing tissues or towards injured or grafted areas display a strong phagocytic activity (de Eguileor *et al.*, 2000a, b; Girardello *et al.*, 2015a, b; Schorn *et al.*, 2015a, b). Numerous CD68<sup>+</sup> and CD45<sup>+</sup> macrophage-like cells are also recruited in animals injected with LPS or with the pathogen bacterium *Micrococcus nishinomiyaensis* (Fig. 3B). As in mammals, immune challenge in leech body wall induces the migration and accumulation at the



**Fig. 3** TEM (A, C, E) and immunohistochemical analyses of *H. medicinalis* mature leukocytes (B, D, F). Macrophage-like cells (A, B) are characterized by ruffled surfaces, phagolysosomes in the cytoplasm (arrowheads) and by the expression of the specific macrophages marker CD68 (B). NK-like cells show in their cytoplasm a well-developed endoplasmic reticulum (arrow), numerous dense granules (arrowheads) and express the specific superficial marker CD56 (D). Granulocytes of type I (arrowhead in E) and of type II (arrow in E) are characterized by a large number of electron dense granules in their cytoplasm and expressed the CD11b marker (F). Bar in A, C: 6  $\mu$ m; Bar in B, D, F: 10  $\mu$ m; Bar in E: 2  $\mu$ m.

injury site of macrophage-like cells with phagocytic (de Eguileor *et al.*, 2000a, b; Schorn *et al.*, 2015a, b) and antimicrobial activity and the ability to produce proteins and peptides, such as *Hm*-theromacin and destabilase (Schikorski *et al.*, 2008; Hildebrandt and Lemke, 2011).

Phagocytosis of foreign living (yeast, bacteria) and non-living material (sulfate spheres, nanomaterial such as multiwall carbon nanotubes MWCNTs) represent an effective and simple mechanism to engulf and eliminate non-self foreign bodies. When antigens penetrating into the body are of small dimension they are simply phagocytized, while when foreign bodies are too cumbersome to be phagocytized (*i.e.*, group of spheres, aggregates of nanomaterial such as multiwall carbon nanotubes and/or parasites), they are encapsulated and afterward melanized (de Eguileor *et al.*, 2000a; Girardello *et al.*, 2015b). An interesting outcome of recent studies has highlighted that, during the encapsulation process, leech macrophages produce a large amount of amyloid fibrils, which are used as barrier to restrain non-self-material. Indeed, several reports describing and characterizing the morpho-functional events sustaining the amyloid fibril assembly in relation to melanin production have underlined the relationship between melanin synthesis and the production of amyloid fibrils to template the pigment. Therefore, amyloidogenesis has been proposed as a fundamental detoxifying event (Grimaldi *et al.*, 2012a) that also acquired physiological additional functions during evolution by packaging melanin and driving the pigment towards a non self-invader, both in vertebrates (Fowler *et al.*, 2006) and in invertebrates (Falabella *et al.*, 2012; Grimaldi *et al.*, 2012b).

#### *The NK-like cells*

Leech NK-like cells are involved in host defense against bacterial and viral infections, in resistance to parasites and in the regulation of hematopoietic cell growth and differentiation. They are similar to those described in vertebrates (Vivier *et al.*, 2008), in other invertebrates annelids such as oligochetes (Cossarizza *et al.*, 1996; Quaglino *et al.*, 1996), Polychetes (Porchet-Henneré *et al.*, 1992) and molluscs (Franceschi *et al.*, 1991). These data strongly support the hypothesis that a primitive natural killer cell-like activity appeared early in phylogenesis and the same function has been preserved throughout phylogenetic development. These cells are characterized by a low nuclear/cytoplasmic ratio and their cytoplasm displays a well-developed endoplasmic reticulum, numerous mitochondria, and dense granules (Fig. 3C). Activated NK cells express CD56 and CD57 (Fig 3D) on their surface and have cytolytic activity. When these cells come in close contact with the membrane of target cells, they exocytose the granules present in their cytoplasm in order to carry out their cytolytic role (de Eguileor *et al.*, 1999, 2000a, b).

#### *Granulocytes*

Cells belonging to this class contain dense cytoplasmic granules and are characterized by the expression of the two surface markers CD11b<sup>+</sup> and CD11c<sup>+</sup> (Figs 3E, F). Based on size and shape of

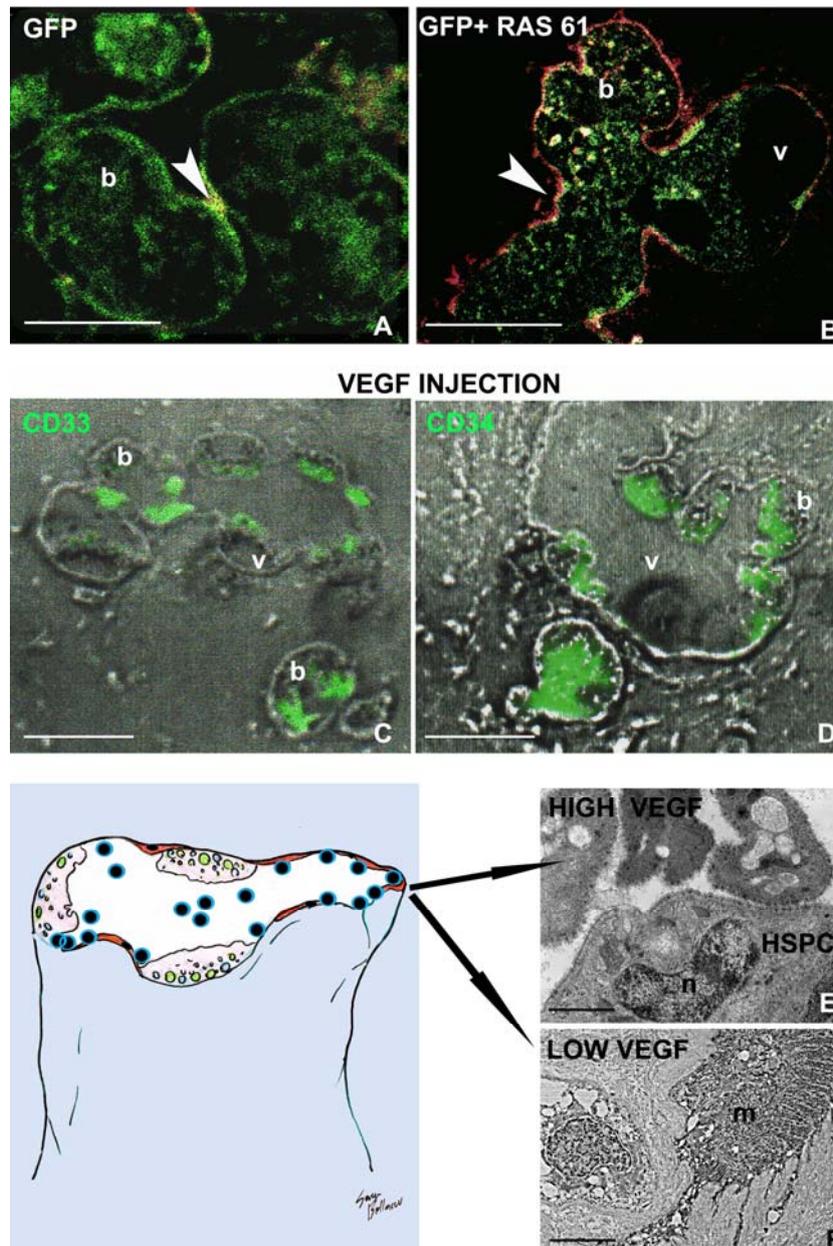
their granules, two different granulocytic types have been identified: granulocytes type I and granulocytes type II. These cells show a different behavior in relation to the dimension and shape of their granules. Granulocytes type I, characterized by small and round granules, can respond to several stimuli by degranulation when a large dose of bacteria (*Escherichia coli*) is injected in the leech body wall. Granulocytes of type II, with large irregularly shaped granules showing complementary profiles, are activated when large antigens cross the epidermis (de Eguileor *et al.*, 1999, 2000b).

#### *Control of hematopoiesis and vasculogenesis*

Hematopoiesis, neovascularization and leukocytes differentiation are complex multistep processes, spatially and temporally harmonized and triggered by extracellular signals conveyed by extracellular matrix components and soluble cytokines. Strikingly, several similarities have been demonstrated between the hematopoietic process in *H. medicinalis* and vertebrates by several investigations, thus highlighting the conserved function of the underlying signaling pathways and transcription factors regulating proliferation, differentiation and lineage commitment (Tettamanti *et al.*, 2006; de Grimaldi *et al.*, 2008, 2009, 2010). Previous studies in leech have indeed demonstrated the importance of a number of cytokines, including VEGF (vascular endothelial growth factor), EGF (endothelial growth factor), bFGF (basic fibroblast growth factor), GM-CSF (granulocyte macrophage colony-stimulating factor) and TGF- $\beta$  (transforming growth factor), during angiogenesis and associated hematopoiesis (Tettamanti *et al.*, 2003a, b, 2006; Grimaldi *et al.*, 2006). An interesting outcome of these investigations showed that injection in leeches of human VEGF, bFGF or GM-CSF promoted vascular growth, immunocytes migration and differentiation. In this review, I have added to the quoted data about VEGF, the new discoveries concerning vasculogenesis, hematopoiesis, and muscle tissue regeneration.

#### *VEGF and RAS have different role in vasculogenesis and hematopoietic processes*

In vertebrates, VEGF mediates its biological effects by binding to a family of transmembrane tyrosine kinase receptors, mainly VEGFR-1 and VEGFR-2 (Neufeld *et al.*, 1999). VEGF is a potent proangiogenic molecule and a mitogen/survival factor or a chemoattractant for endothelial cells (Koch *et al.*, 1994; Gerber and Ferrara, 2003; Wang *et al.*, 2008) under physiological and pathological conditions. Moreover, VEGF165 (the most abundant and biologically active isoform) is a potent chemoattractant for CD34 positive cells and it is critical for the migration of CD34<sup>+</sup> hematopoietic stem cells from the bone marrow into a tumor mass (Lee *et al.*, 2006). In leeches, the VEGF165 isoform is synthesized and released in the extracellular matrix by muscle tissue and its activity is mediated by binding to specific VEGF-like receptors (Flt-1/VEGFR-1 and FLK-1/VEGFR-2 or KDR) expressed on endothelial-like cells (Perletti *et al.*, 2003; de Eguileor *et al.*, 2004).



**Fig. 4** Cells expressing only GFP (in green) maintained a cord shape (A) and a low signal for polymerized F-actin (in red) is visible in the small endothelial cells (arrowhead) interspersed among botryoidal cells (b). GFP/RAS61 transformed cells (B) show a transformed phenotype and lacunae vessel (v) lined by flattened endothelial (arrowheads) cells highly positive for polymerized F-Actin are visible. After VEGF administration a dehiscence process led the compact cell cords to gradually line new cavities (v) in which HSPC cells proliferate and express the CD33 (C) and CD34 (D) markers (in green). HSPC exposed to a sustained and continuous source of VEGF maintain their undifferentiated phenotype as precursor cells (E). HSPC exposed to a low concentration of VEGF differentiate in muscle fibres (m) (F). n, nucleus. Bars in A, B: 25  $\mu$ m; Bars in C, D: 10  $\mu$ m; Bar in E: 600 nm; Bar in F: 1  $\mu$ m.

The growth of new vessels is characterized by a combination of two distinct events, *i.e.*, an initial vasculogenic step followed by extensive angiogenesis. A VEGF stimulus induces the transformation of the botryoidal tissue into new immature vessels (vasculogenic step) from which new branches and capillaries can further outgrow, following a classic angiogenic pattern (*i.e.*, an extensive development of new vessels from pre-

existing ones) (de Eguileor *et al.*, 2001a). An interesting outcome of recent investigation in leeches highlighted another mechanism regulating vasculogenesis, based on the GTPase Ras protein and MAP/ERK kinase cascade.

In vertebrates the Ras GTPase controls signal transduction pathways (Carpenter 2000; Kranenburg and Moolenaar, 2001) leading to gene expression changes and cell motility through the

MAP/ERK kinase cascade (Ehrhardt *et al.*, 2002). Moreover, it is known that Ras signaling, even if its mode of action is not completely understood, is capable of driving an angiogenic switch in primary endothelial cells in the absence of other angiogenic factors (Meadows *et al.*, 2004). Interestingly, the RAS protein plays an important role in leech vasculogenesis as well. During this process, endothelial cells express RAS and are characterized by marked cellular changes. They become thin, flat, and tapering of these cells allow the definition of a lumen and the increase in diameter and length of new capillary. Moreover, constitutively activated mutant Ras61 expression in stably transfected botryoidal cells causes vessels cavities formation also in total absence of VEGF, whereas cells expressing only GFP show the typical solid cord structure of un-stimulated tissue (Figs 4A-D). Such relevant modification of botryoidal tissue phenotype is due to the cytoskeletal reorganization of endothelial cell. Indeed, Ras/MAP/ERK signalling is directly involved in the morphological modification of botryoidal tissue cells by acting on the polymerization and spatial arrangements of endothelial cell's actin filaments (Grimaldi *et al.*, 2013). Therefore, despite the fact that both botryoidal and endothelial cells cooperate in the formation of new blood vessels, the first step of new vessel formation in leeches, identified as a vasculogenic phase, seems to be mainly regulated by the Ras/MAP/ERK cascade.

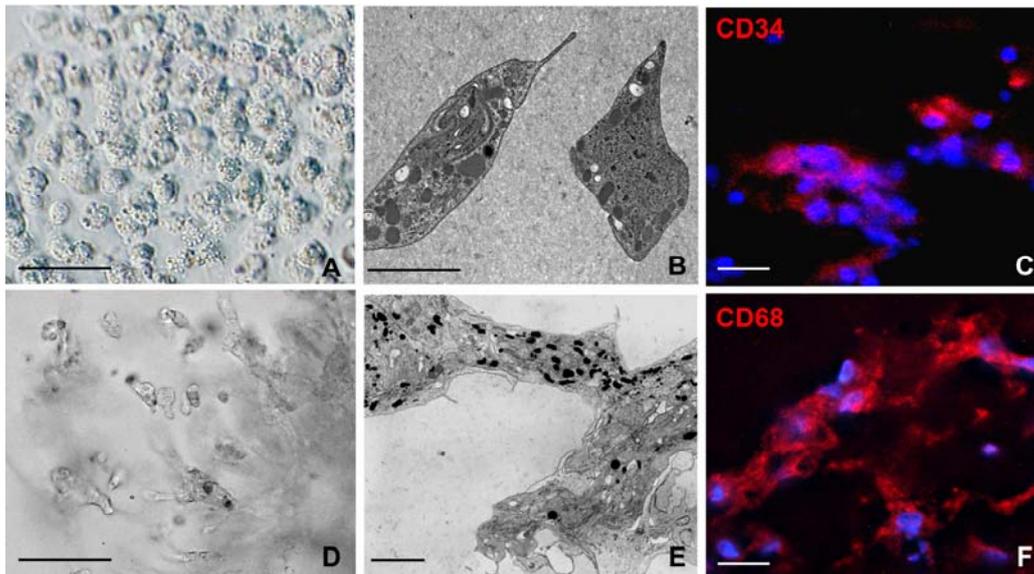
VEGF also play a crucial role in promoting the formation of an extensive vessel network through the entire avascular body wall and in the recruitment of hematopoietic precursors cells. The different pattern of expression of VEGF receptors on endothelial and botryoidal cells could explain the different behavior of these cells to VEGF stimulus (Grimaldi *et al.*, 2006). When VEGF is readily available, such as after injection in the leech body wall, it acts as chemoattractant for endothelial cells and induces the migration and assembly of numerous vessels in the muscular body wall. However, a highly localized VEGF concentration, achieved for instance by implantation of a VEGF-Hydrion pellet within the musculocutaneous sac, induces a massive production of circulating cells, localized in the cavities of neo-vessel lumen and expressing specific markers such as CD33, CD34, CD31, VE-cadherin, c-kit and the VEGF receptors Flt1/VEGFR1 and Flk-1/VEGFR-2 (Tettamanti *et al.*, 2003; Grimaldi *et al.*, 2006). Strikingly, these cells strongly resemble the circulating hematopoietic and endothelial progenitor cells described in vertebrates (Hatzopoulos *et al.*, 1998) and recently a direct correlation has been established between hematopoietic/endothelial precursor cells and muscle regeneration processes (Grimaldi *et al.*, 2009, 2010). Indeed, the hematopoietic precursors cells of *Hirudo*, deriving from the botryoidal tissue and extravasating into the extracellular matrix, not only express the same molecular markers of vertebrates hematopoietic stem/progenitors, but also gives rise to the vessel-associated myoendothelial cells involved in vertebrate muscle

regeneration and are able to produce terminally differentiated muscular cells (Figs 4E, F). Therefore, hematopoietic precursors cells of leech seem to possess the capacity to activate diverse genetic programs in response to peculiar environmental stimulation. When these cells are exposed to a sustained and continuous source of VEGF slowly diffusing from physiological ECM "sinks", they proliferate and principally maintain their undifferentiated phenotype as precursor cells. As the VEGF concentration decreases in the ECM surrounding the muscle fibres, the proliferation stimulus diminishes, permitting these hematopoietic/endothelial precursors cells to enter in a differentiating program instead (Grimaldi *et al.*, 2009, 2010).

#### *Biopolymer matrigel as a tool to isolate leech HSPC*

The isolation and culture of leech hematopoietic precursors offer a unique and accessible tool to study leech hematopoietic and endothelial lineage development (Ballarin *et al.*, 2008; Grimaldi *et al.*, 2008, 2009) and the role of cytokines in the recruitment, commitment and hematopoietic cells differentiation. Recently, a new method has been proposed, based on injection of the biopolymer matrigel (MG) supplemented with different kind of growth factors in the leech body wall. After injection in the leech body wall, the biopolymer form a pellet and the signaling molecules diffusing out from this artificial microenvironment induce the hematopoietic precursors to migrate towards it. When the biopolymer attains a high cell density due to the recruitment of specific cell populations, it can be retrieved and used as a vector to prepare cultures of the infiltrating cells. Thus, unlike standard methods to localize HSPCs in adult tissues, to extract and culture them, the present approach provides a method by which hematopoietic precursor cells move from the tissues in which they reside into the injected biopolymer supplemented with a specific cytokine. The use of biocompatible and bioreabsorbable materials forming a 3D polymer scaffolds thus represents not only a remarkably effective tool for studying and charactering cells involved in leech immune responses, but also represents an easy system to select specific cell populations for *in vitro* culture, expansion and differentiation analyses (Grimaldi *et al.*, 2008, 2009, 2011; Ballarin *et al.*, 2008; Girardello *et al.*, 2015b).

Moreover, the role of several cytokines in the recruitment, commitment and differentiation of hematopoietic cells has been also confirmed by injecting MG supplemented with different kind of growth factors in the leech body wall. A comparative analysis of biopolymer *in vivo*-sorted stem cells indicates that VEGF recruited cells of a hematopoietic/endothelial phenotype expressing CD34, CD117 and the VEGF receptors Flt1/VEGFR1 and Flk-1/VEGFR-2, whereas the cytokines Monocyte chemoattractant protein-1 (MCP-1/CCL2) and the Allograft inflammatory factor-1 (*HmAIF-1*) recruit cells that are of an early myeloid lineage and expressing CD11c, CD14, CD 45, and CD68 (Figs 5A-D).



**Fig. 5** After 1 week *in vivo* the MG supplemented with VEGF or MCP-1 was removed and the cells infiltrating the matrigel sponge were plated out. Cells recruited by VEGF (A) show the blast like phenotype (B) and express the CD34 marker (C), while cells recruited by MCP-1 (D), differentiate in macrophages (E) and express CD68 antigen (F). Bars in A, C, D, F: 10  $\mu\text{m}$ ; Bar in B: 2  $\mu\text{m}$ ; Bar in E: 1  $\mu\text{m}$ .

### Concluding remarks

The main conclusion of the studies reported above is that HSPCs precursor cells in the leech, deriving from the activated botryoidal tissue, seem to share with their vertebrates counterpart most morpho-functional and molecular mechanisms. In leeches, these cells are recruited and activated in response to injury, bacterial injection, or experimental administration of cytokines and contribute to immune response, neovascularization and muscle regeneration. Further, similar to vertebrates these cells are driven toward a leukocyte, endothelial or myogenic, differentiation pathway, as a function of the time course of VEGF release to target cells. Strikingly, as in vertebrates, the key molecules playing pivotal roles for guiding and regulating the hematopoietic cells recruitment, proliferation and differentiation are different from those involved in endothelial cells differentiation and neo vessel formation. In particular, VEGF is mainly involved in promoting stem cell recruitment and proliferation, while the GTPase Ras protein and MAP/ERK kinase cascade, remodeling the endothelial cells cytoskeleton, regulates the new vessel formation. For these reasons, leeches, involving similar cellular mechanisms, can be considered a simple step-by-step model for studying the regulation of vasculo-genesis and hematopoietic cell biology.

Moreover, since investigations on hematopoiesis can greatly help scientists and clinicians to better understand the pathological processes behind blood disorders, cancers, muscle regeneration, HSPCs can be used as a powerful model system for understanding tissue stem cells

and their role in angiogenesis, tissue regeneration and oncogenesis.

Finally, it is well known that numerous factors, *i.e.*, ethical considerations, stricter controls, improvements in animal welfare and housing, have led to a growing number of restrictions in the number of animal species available for experimentation. Significantly, the use of leech as an experimental model for studying hematopoiesis effectively avoids many of these inconveniences. Therefore, although some differences between vertebrates and invertebrates models do exist, *H. medicinalis* can be considered as a new emergent model in hematopoiesis research. In this context, the recently developed biopolymer *in vivo* cell sorting method, which allows the isolation of specific cell populations in relation to the cytokines utilized and the possibility to subsequently culture these cell types, is invaluable for studying HSPCs characteristics.

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