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REVIEW

Elie Metchnikoff and the multidisciplinary link novelty among Zoology, Embryology and Innate Immunity

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Abstract

Elie Metchnikoff was a Russian scientist known as the pioneer of innate immunity. In particular, he was awarded the Nobel Prize for discovering the process of phagocytosis and its significance in the development and disease. Here, we endeavor to demonstrate the enduring fascination of his scientific research, in particular the experiment involving the first observation of a macrophage reaction in the sea star. This applies to both adult and larvae immunity studies. Recent work on sea star larval cellular immunity and adult immune systems using modern expansions of molecular and cellular techniques shows that it is a continually exciting research field that cannot just be consigned to history. Finally, aspects of human scientific roles - from the zoologist to embryological experiences to the father of innate immunity - can teach us much about the oft-neglected added value of multidisciplinary knowledge and integration in animal science research.

Key Words: Metchnikoff, Phagocytosis, zoology, immunobiology, sea star larvae

Introduction

In 1882, Elie Metchnikoff (Ilia Mechnikov, 15 May 1845 - 15 July 1916) pioneered the study of cellular immunology and initiated the scientific process that led him to the discovery of phagocytosis. Although the phenomenon of endocytosis by leukocytes (more related to pinocytosis) had already been described 30 years before, Metchnikoff clarified the defensive function of phagocytosis, highlighting its importance in immunity where it is a nodal point of the immunity network. He devoted much of his life to studying the different aspects of phagocytosis and related immunological phenomena. It took 25 years of hard work for the theory of phagocytosis to be recognized: the first experimentally based theory in immunology. This struggle finally paid off in 1908, when the Nobel laureate was awarded to Metchnikoff, together with Paul Ehrlich, who had developed the methods for standardization of antibody activity in immune sera (Gordon, 2008).

In line with his zoological interest and scientific education, he adopted an evolutionary and comparative approach to understand the animal biology of different organisms, from protozoan to exotic primates. However, he normally preferred simple organisms, convinced that they "affording as they do infinitely simpler and more primitive conditions than those in man and vertebrata, really furnish the key to the comprehension of the complex pathological phenomena which are of special interest in medical science".

Despite his weak eyesight, he used light microscopy and scientific illustration to considerable effect (Metschnikoff, 1884) to investigate the early development of germ layers, and focused on the biological processes of development (Racine, 2014).

Metchnikoff displayed a passionate interest in science and natural history from an early age, and was thus encouraged by his mother to pursue a scientific career in the life sciences. While at the University in Kharkov (1863-1865), he published his first scientific paper on the histology of *Vorticella*, a genus of protozoa, and dedicated himself to studying marine life on the small island of Helgoland in the North Sea (1864).

He was greatly influenced by Charles Darwin's theory of evolution and his scientific works and Darwinism inspired theories.

Interest in invertebrate marine organisms and their development accompanied Elie Metchnikoff on

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his travels around Europe, including the Baltics, Germany and the Mediterranean area including Naples and Messina. During this period, he made his first scientific discovery of the sexual and asexual alternation of generations in nematodes and subsequently identified intracellular digestion in planarians; this study influenced his later work. In Naples, he had been working on a doctoral thesis with Kowalensky, who described the tailed larval form in ascidians containing a dorsal neural tube. Only then did zoologists begin to realize that the tunicate should be placed within the chordate. Elie Metchnikoff returned to St. Petersburg to complete doctoral studies on the embryonic development of the cuttlefish of genus *Sepiola* and crustaceans such as *Nebalia* (Racine, 2014). Metchnikoff's objective was a comparative study of the development of germ layers in vertebrate and invertebrate embryos. Specifically, he sought to establish a common link in the evolution of vertebrates and invertebrates, and to confirm Darwin's theory of the animal common ancestor. He was appointed at Imperial Novorossiya University of Odessa Professor of Zoology and Comparative Anatomy at only 25 years of age.

Therefore, with a view to his early studies, we consider Metchnikoff as a comparative embryologist who, to explore his first hypothesis on the mesoderm origin of endodermal structures then extended his observations on mesodermal digestive processes to a new theory of immunity. In 1873, Metchnikoff's life was greatly disrupted by the death of his first wife and by a consequent attempt of suicide. He remained absent from scientific life for the next nine years.

The Messina experiments

In 1882, Metchnikoff left Odessa and started up a private research laboratory in Messina, where the turning point in his career took place. Looking at motile cells in a transparent sea star larva, he was struck by a novel idea: to imagine that similar cells could serve as a defense of an organism against dangerous intruders. This idea was the basis for his theory of phagocytosis.

To test his hypothesis, Metchnikoff introduced a rose thorn into the body of a sea star larva, whereupon motile cells rapidly encircled the foreign body. Metchnikoff considered this process to be similar to the inflammatory response found in animals with vascular systems, where white blood cells gather at the site of inflammation. He hypothesized that the mobile cells in sea star larvae, or phagocytes, were the evolutionary ancestors of the mesodermal cells of vertebrate. These cells have a dual role: a primitive digestive function, ingesting particulate nutrients, and a protective function, ingesting foreign materials/invaders and maintaining the integrity of the organism.

These preliminary studies did not garner sufficient appreciation, so Metchnikoff spent much of his time underlaying his phagocytic cell theory with fierce polemics against the seemingly alternative humoralist theory of that time. He devoted much of his subsequent scientific work to developing his theory of phagocytosis in natural immunity.

To support his work and theory, in 1901 he published the treatise "L'immunité dans les Maladies Infectieuses" (Immunity in Infectious Diseases).

Phagocytosis has also been extensively studied from microbiological aspects. Elie Metchnikoff used a series of newly described bacteria in his experiments, including commensal and pathogenic agents such as syphilis and anthrax (Metchnikoff, 1984a,b). Finally, he turned to gut flora and aging, coining the term 'gerontology' (Martin and Gillen, 2013).

From 1895 to 1910, Metchnikoff's cellular phagocytosis theory (cellular versus humoral effectors) became integrated with the development of the humoralist position, with the humoral basis of bactericidal defense starting to define innate versus acquired immune processes.

The novelty of the sea star macrophage after the myth

Since Metchnikoff, sea star larva mesenchyme cells have been extensively used to investigate their morphogenetic functions (Crawford and Chia, 1978; Dan-Sohkawa *et al*., 1980; Crawford and Abed, 1983; Kominami, 1985; Crawford, 1990; Crawford *et al*., 1997; Kaneko *et al.,* 2005). As in echinoids, mesenchyme sea stars cells appear at the tip of archenteron during gastrulation and are widely dispersed throughout the blastocoel (Chia, 1977, Dan-Sohkawa *et al*., 1980). When the embryo reaches the early stage of a bipinnaria larva, the mesenchyme cells amount to 110 cells per individual (Kominami, 1985). These cells change gradually in morphology from a rounded to attenuated shape as they develop cellular processes. Mesenchyme cells sustain their embryonic shape by exerting mechanical tension against the fibrous component of the extracellular matrix (Crawford, 1990; Crawford *et al*., 1997; Kaneko *et al*., 2005). This idea derived primarily from ECM scanning and transmission electron microscopy observations of sea star *Pisaster ochraceus* embryos (Crawford, 1990, Crawford *et al*., 1997). However, since they had only a little experimental evidence available, Crawford and colleagues could only hypothesize that fibrous ECM supports the shape of the body wall, while the mesenchymal cells modify it by changing its length. Afterwards, Kaneko and coworkers (2005) studied the embryogenesis of the sea star *Asterina pectinifera* by using a monoclonal antibody (4H11 Mab), specifically recognizing a fibrous component of embryonic ECM. Through these experiments, they evidenced that 4H11 Mab caused an abnormal distribution of the fibers, and, in turn, an anomalous distribution of mesenchyme cells, in addition to morphological abnormality and delay in the ectoderm and the endoderm.

Furukawa and coworkers (2009) characterized the sea star (*A. pectinifera*) larval mesenchyme cells by studying their structural and functional properties. Confocal immunofluorescence, Nomarski, and transmission electron microscopy (TEM) revealed the mesenchyme cells to be responsible for the construction of a dynamic network structure under the body wall; indeed, most of them were typically located under the endodermal and ectodermal wall, while others were dispersed in the blastocoel. In

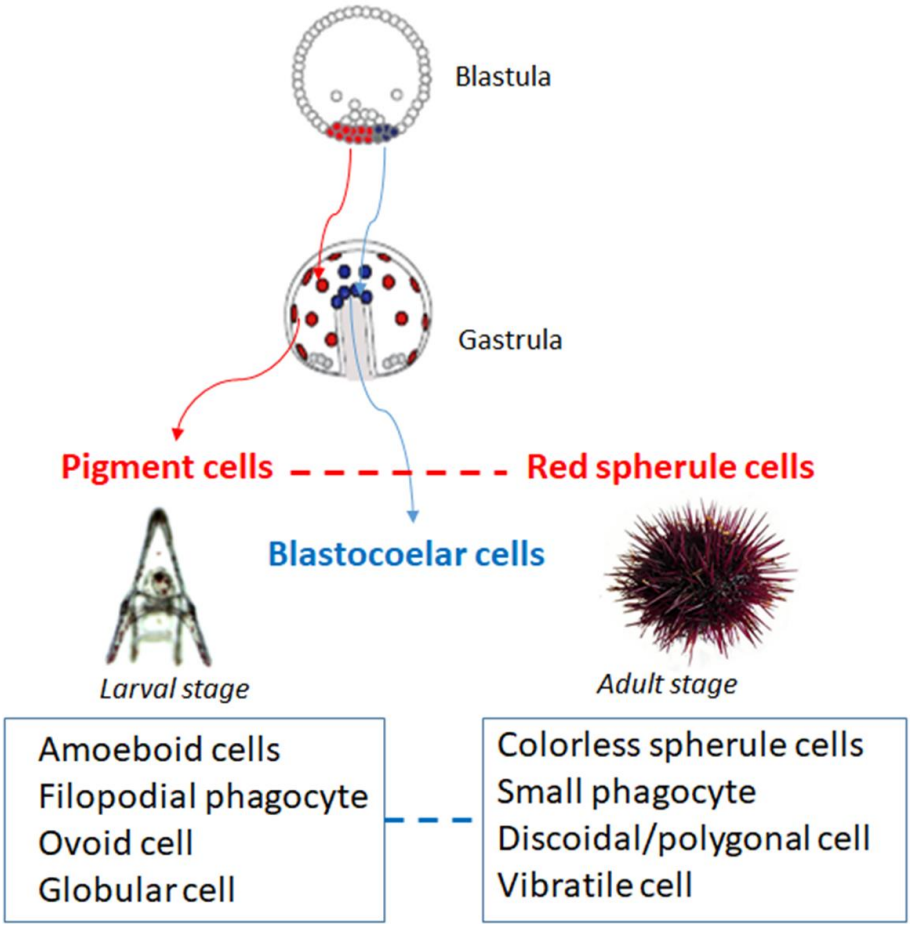

Fig. 1 The echinoderms development and mesenchymal cells in the larval and adult stage. The blastocoelar and pigment precursor cells, differentiated during the blastula stage, are respectively placed in the body cavity and in the ectoderm at the gastrula stage. The pigment precursors become pigment cells and the blastocoelar cells differentiated into four cell types (amoeboid, filopodial, ovoid and globular) in the larvae. In adults, six cell types (red and colorless spherule, small, discoidal, polygonal and vibratile) of mesenchymal cells can be found

particular, the mesenchyme cells tended to distribute unevenly along the ciliary band in the ectodermal wall. However, details of their role in defense activity have been lacking for a long time.

Referring to Metchnikoff's idea that during echinoderm gastrulation, mesenchyme cells are capable of phagocytosis almost immediately after reaching the coelomic cavity, Silva (2000) investigated the starting point of phagocytic activity of mesenchymal cells. This study was performed in embryos of the sea urchin *Lytechinus variegatus* by microinjecting the yeast *Saccharomyces cerevisiae* into the blastocoele. Here, secondary mesenchymal cells were first detected phagocytosing injected yeast, through pseudopodia emission and internalization, during the mid-gastrula stage (Silva, 2000). This is the point where mesenchymal cells leave the tip of the gut rudiment. Therefore, the mid-gastrula represents the development stage during which a biological capacity for distinguishing between self and non-self begins to be established in the *L. variegatus* embryo.

In this regard, further data were later derived from injection experiments performed on *A. pectinifera* (Furukawa *et al*., 2009) phagocytic behavior. The mesenchyme cells responded to almost all foreign materials (i.e. polystyrene beads), displaying an active phagocytic function and forming aggregates to eliminate them.

Morphological simplicity and optical transparency of embryonic and larval stages coupled with techniques for transgenesis and gene perturbation in the echinoderms model, as well as the sequenced genome of the purple sea urchin (*Strongylocentrotus purpuratus*) lend additional depth to the field of echinoderm immunity. In particular, sea urchin genome sequencing, carried out a century after the Metchnikoff's discovery, highlighted the genomic complexity of the echinoderms immune system. The availability of this genomic sequence enables us to reconsider Metchnikoff's cellular immunology model using a series of modern molecular tools. Using them, we can investigate fundamental issues of animal immunity, including those shared with vertebrates.

In this context, Furukawa and coworkers (2012) studied the molecular mechanism that regulates

mesenchyme cell dynamics during the immune response to foreign bodies in *A. pectinifera*. In this sea star ApSRCR1 protein, the orthologue of a vertebrate scavenger receptor cysteine-rich-domain-containing protein, serves as an opsonin against bacteria in the larval defense system. ApSRCR1 protein is released extra-cellularly by mesenchyme cells and promotes their phagocytosis ability and aggregate formation. More recently, Ho and coworkers (2016) analyzed the larval immune response in the purple sea urchin. They characterized five distinct larval cell types and defined their role in immune response. The larval response to pathogenic bacteria resulted in changes in gut morphology, cell behavior and alterations in gene expression levels, showing a complexity of reactions that way exceed the morphological simplicity of the larva.

Larval filopodial cells are also responsible for the up-regulation of SpTransformer (SpTrf) genes following immune stimulation (Hirano, 2016). In sea urchin larvae, SpTrf gene expression is restricted to a subset of blastocoelar cells localized in the blastocoel and functioning as the primary larval phagocytes able to protect the host. In the adult, also, the expression of the SpTrf protein seems to be limited to the phagocytes (Smith and Lun, 2017).

Immune cells in larvae and in adult stage

Larvae and adults have different lifestyles, since the echinoderm larval stage does not extend beyond two months, compared with decades spent as adults. The morphologically simple larva is planktotrophic, while the more complex adult feeds primarily on kelp and other benthic algae. Both similarities and differences between the larval and adult immune systems exist, despite larvae and adults sharing the same genomic background (Rast *et al.*, 2006). Currently, we do not know if the few hundred immune cells of larvae give rise to the millions of circulating coelomocytes in the adult. While the adult immune cell types have been identified (Smith and Davidson, 1992; Smith *et al.*, 2010), the larval immune cells are not well characterized. Some larval and adult cells (Fig. 1) show the same morphology and express the same set of genes.

Adult coelomocytes are generally classified into at least six types (Smith *et al.*, 2010) and express homologs of genes involved in the immune response in many animals, including Vertebrates. Indeed, complement factors (Gross *et al.*, 2000), a complex SRCR repertoire (Pancer, 2001) and multigene families of diverse Toll-like receptors (Buckley and Rast, 2012), as well as AIF-1 (Barca *et al.*, 2017) are differentially expressed in coelomocyte subpopulations. Regarding the larval stage, the mesenchymal cells are known to possess immune functions (Smith *et al.*, 2010), being able to recognize and phagocytose bacteria or yeast injected into the body cavity, or blastocoel (Silva, 2000). In particular, in the sea urchin larva, two cell types have been recognized as major immune effectors (Hirano, 2016). These are the filopodial phagocytes, mainly placed in the body cavity, and the pigment cells that are found in the ectoderm. This last cell type is characterized by the red pigment echinochrome A, and is considered responsible for antioxidant, antimicrobial and anti-inflammatory activities (McClendon, 1912; Calestani *et al.*, 2003). The red spherule cells of the adult are morphologically similar to the larval pigment cells. Furthermore, they share the expression of key molecules for Notch signaling (Ransick and Davidson, 2006) and for pigment production (Calestani *et al.*, 2003).

In addition to phagocytic activity, similarities between larval filopodial cells and small adult phagocytes are also evident in the up-regulation of SpTrf genes following immune stimulation (Hirano, 2016). In larvae, blastocoelar cells are the only cell type able to express the SpTrf genes (Smith and Lun, 2017). Conversely, in the adult, these proteins are expressed also in polygonal phagocytes and their strong up-regulation occurs when challenged with PAMPs and heat-killed marine bacteria (Nair *et al.* 2005; Brockton *et al.* 2008; Majeske *et al.* 2013). Regarding the differences, the larval globular cells have not been found in adults, and no equivalents of the adult vibratile cells have been observed in the larva (Ho et al., 2016). For gene expression, a differential pattern has been evidenced among the subfamilies of immune receptors expressed in the two developmental stages, as takes place with Toll-like receptors (Buckley and Rast, 2012). Furthermore, since larval and adult cells express different amounts of effector molecules, a stage-specific gene regulation is made available.

Conclusions

By using invertebrate organisms to make predictions of immune function in other animals, Metchnikoff introduced a new field of biology: comparative immunology. The power of comparative immunology derived from observing invertebrates is evident at both cellular and molecular levels. Indeed, the macrophages are considered a clue to a common evolution of immune and neuroendocrine systems (Ottaviani and Franceschi, 1998). On the other hand, the characterization of several immune active molecules, e.g. lectins (Prokop *et al.*, 1968), antimicrobial peptides (Boman and Hultmark, 1987; Li *et al.*, 2011), Toll-like receptors (Lemaitre *et al.*, 1996; Medzhitov *et al.*, 1997), and RNA interference (Fire *et al.*, 1998) as well as the evidence for molecular variability (Smith *et al.*, 2010), have expanded our understanding of invertebrate immunity and revealed shared features among animals across phylogeny and evolution.

The larval and adult immune cell repertoire similarity unveils new scenarios on the history of animal life, in which the information and adaptation are related in a fitness action that crosses over individual morphology.

Although the implications of Metchnikoff's studies are well known to have characterized the birth and evolution of vertebrate immunity, the reconstruction of Metchnikoff scientific history after so many years, and the recent development of invertebrate immunity constitute some beautiful instructive chapters from a single cell to mammals, including echinoderm larvae (Ballarin and Cammarata, 2016).

Sectorial or confined science areas are often used for aspecialized destination in education,

namely the application of study and project and grant finalization.

Several times over, the story of the “Man Metchnikoff” and its scientific history has clearly shown that multidisciplinary approaches and knowledge add a value that should not be confined within rigid sectoral limits.

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