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Review

Sir Marc Armand Ruffer and Giulio Bizzozero: the first reports on efferocytosis

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ABSTRACT

Sir Marc Armand Ruffer, a physician and a bacteriologist, well-known for his discoveries in paleopathology, and Giulio Bizzozero, an Italian pathologist, famous for his work on platelets, have made significant contributions in the field of phagocytosis. They both reported among the very first descriptions of efferocytosis. *J. Leukoc. Biol.* 93: 39–43; 2013.

Introduction

Progress made in the sciences has always been accompanied by the coining of neologisms, either to name a new discovery or to rename an old phenomenon. They may influence the course of history, as was the case for the term "interferon", coined by Isaacs and Lindenmann in 1957. They were considered the discoverers of IFN [1] until we were reminded of the work of Nagano and Kojima, published in 1954, and their discovery of a "*facteur inhibiteur*" [2, 3]. The use of neologisms may nowadays be far more popular than expected when proposed for the first time, as was the case for the word "cytokine", coined in 1974 by the Nobel Prize winner Stanley Cohen [4, 5]. They can bring phenomena studied over decades into the spotlight, such as the word "inflammasome", coined in 2002 by the late Jürg Tschopp [6], to describe the actors and the cascade of events involved in the production of IL-1β [7]. Sometimes, their etymology may be poorly chosen, such as the word "interleukin" to define cytokines allowing interaction between leukocytes, as these mediators are the universal language of all of our cells and not only leukocytes. However, this was absolutely not the case for the word "efferocytosis", wisely proposed by Aimée deCathelineau and Peter Henson in 2003 [8]. They derived their neologism from the latin prefix "effero", meaning to take away, to put away, to carry to the grave, or to bury. Indeed, this word was coined to define the phagocytosis of apoptotic or dead cells. Removal of apoptotic or dead or senescent cells and microorganism is performed by professional phagocytic cells (macrophages, DCs). In the case of *Caenorhabditis elegans*, which misses professional phagocytic cells, dead cells are engulfed by neighboring cells of differing types. The phenomenon is intrinsically linked with embryonic development, and Gordon and his colleagues [9], demonstrating the early appearance of macrophages in murine embryo, suggested that macrophages contribute in shaping developing organs. Some authors postulated that the primary function of phagocytes was the removal of apoptotic cells during development and that recognition of micro-organisms evolved as a later adaptive function [10]. The dying cells expose several "eat-me" signals tentatively called "apoptotic cell-associated membrane patterns" [10]. Their recognition by phagocytic cells is mediated by different receptors [11], including the PRRs involved in the clearance of bacteria. Some receptors, such as Croquemort (French word for pallbearer), are specifically expressed by embryonic macrophages of Drosophila [12]. In *C. elegans*, several genes are involved in the phagocytosis of corpses [13], and homologs [14] or analogs [15] have been identified in humans.

METCHNIKOFF'S VIEWS ON EFFEROCYTOSIS

However, when was the observation reported for the first time? Who should be considered as the father of efferocytosis? In September 2011, the *Journal of Leukocyte Biology* displayed on its cover a portrait of Elie Metchnikoff, the father of phagocytosis, and a cartoon of all of the ligands on apoptotic cells and the receptors involved in the binding and clearance of apoptotic cells by phagocytes [11, 16]. This cover suggested to us that there was a link between Metchnikoff and the discoverer of efferocytosis. Had Metchnikoff considered this concept? In his "Lectures on the Comparative Pathology of Inflammation", given in 1891 and published in 1892 [17], Metchnikoff indicated that phagocytosis could occur without inflammation and provided an example taken from embryology:

> "*The metamorphosis of Batrachians is accompanied by an absorption of the larval organs—tail and gills. This is effected very rapidly (in a few days) by the aid of phagocytes, which devour all the tissues no longer required by the animal. And yet in this case there are no signs of inflammation, the phagocytes of the tissues in question being quite sufficient by themselves to carry out the work of absorption which is evidently a much easier task for them and requires much less activity on their part than does the struggle with parasites. In mammals, the real* **'scavengers'**, *that is to say the phagocytes which carry out the work of absorption, are the macrophages, especially the mononuclear leukocytes [...] It is much more natural*

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to assume that the emigration is a reaction on the part of the organism and that in the conflict many of the principal combatants, the microphages, perish on the field of battle. Absorption afterwards ensues and is carried out by another variety of phagocytes."

Interestingly, the English translation, published in 1893 [18], introduced the word "scavenger", where Metchnikoff used the French word "balayeur" ("sweeper"). As a former embryologist, Metchnikoff focused on the ability of phagocytes to devour other cells during embryonic development. Metchnikoff did, however, address the process now known as efferocytosis during inflammation, offering a simple description:

"*[...] many phagocytes perish and are engulfed by other phagocytes, as can be seen in every case a few days after the onset of the inflammation.*"

This sentence is accompanied by a figure showing phagocytes enclosing other phagocytes from the fin of a *Bombinator* tadpole. However, in his book on immunity in infectious diseases, published a few years later in 1901 [19], Metchnikoff is more eloquent about the topic. Describing the fate of red blood cells injected in the peritoneal cavity of guinea pigs, he wrote:

"*Even in the absorption of red blood cells spread into the peritoneal cavity of the same animal species, a certain number of these cells does not directly pass into the circulation but are first ingested by the amoeboid elements [...]. In inflammatory exudates, leukocytes also become the prey of their congeners. Engulfed white blood cells can be recognized for some time in the interior of other leukocytes, but they soon disintegrate and eventually disappear completely.*"

As a former zoologist, Metchnikoff described all types of digestion observed among invertebrates.

"*As for changes that ingested elements undergo inside amoeboid cells, they must be compared to intracellular digestion. When studied in parallel, changes of particles ingested by amoebae, and those experienced by cells encompassed in the process of resorption, there is a striking analogy.*"

This passage was then followed by a long description on the digestion process in flatworms, particularly planarians. Most surprisingly, however, he never referred to the works that one of his pupils had published in 1890. This pupil was Sir Marc Armand Ruffer. Before recalling his work and his discoveries, let's first review the amazing life of this great scientist [20, 21].

THE AMAZING LIFE OF SIR MARC ARMAND RUFFER

Marc Armand Ruffer was born in Lyon, France, in 1859 and was the son of a banker named Baron Alphonse Jacques de Ruffer and Caroline, a woman of German origin. He received his primary education in Paris and spent some time in Germany. He then went to Brasenose College, Oxford, before joining the University College in London where he received his Bachelor of Medicine and Surgery in 1887 and Doctor of Medicine in 1889. He returned to Paris and had the good fortune to study at the brand new Institut Pasteur under the supervision of both Louis Pasteur (1822–1896) and Elie Metchnikoff (1845–1916). He also joined the Paris University of Medicine and worked with Albert Charrin (1856–1907) in Prof. Charles Bouchard's (1837–1915) laboratory in the Latin Quarter. Upon returning to London in 1891, he was appointed as the first director of the British Institute of Preventive Medicine, now known as the Lister Institute. There, he worked on diphtheria, and while preparing the newly developed diphtheritic antiserum, he developed the disease. Ruffer was seriously ill and so badly affected by paralysis that he resigned his directorship. He went to convalesce in Egypt to enjoy a climate greatly different from the London weather. There, he not only recovered but became professor of bacteriology in the Cairo Medical School in 1896 at the age of 37. He later became president of the Sanitary, Maritime, and Quarantine Council of Egypt and contributed to the elimination of plague and cholera from the country by quarantining pilgrims making their way to and from Mecca. At the outbreak of the first World War, he became head of the Red Cross in Egypt. Having helped to curb the rampant epidemics of enteric infection among troops in Thessaloniki, on April 15, 1917, he boarded the SS Arcadian on a trip from Thessaloniki to Alexandria with a company of 1335 troops and crew. In the Southern Aegean sea, 26 miles northeast of Milo, the ship was torpedoed by the German submarine UC74, commanded by "kapitänleutnant", Whilhem Marschall (1886–1976). The vessel sank in 6 min with the loss of 277 lives, including Ruffer, who gave his own life to save that of a fellow passenger.

Most fascinating is the fate that brought Sir Marc Armand Ruffer to Egypt made him a renowned pioneer of modern paleopathology. From 1908, he contributed to the British archaeological survey of Nubia, collaborating with Sir Grafton Elliot Smith (1871–1937), an Australian Egyptologist and anthropologist, and Frederic Jones Wood (1879–1954), a British anatomist and anthropologist. He made many important and fundamental discoveries. In 1910, he discovered and identified *Shistosoma hematobium bilharzia*-calcified eggs in the kidneys of Egyptian mummies dating from 1250 to 1000 BC, revealing for the first time the existence of schistosomiasis in ancient Egypt [22]. He also reported the presence of bacteria in the kidneys of mummified bodies, although he could not describe their exact nature. In addition, he was the first to identify inflammatory diseases of the joints, thereby describing the presence of arthritis and spondylitis in an atypically young population [23]. In his study of Ramses II (1298–1232 BC), he diagnosed atherosclerosis by calcification of the arteries [24]. He also described Pott's disease in another Egyptian mummy, opening the debate about the occurrence of tuberculosis in ancient times [25]. After his death, his wife gathered a record of his past and unpublished studies [26]. All his great pioneering works were later confirmed by other paleopathologists using far more sophisticated technical and molecular approaches.

THE WORK OF SIR MARC ARMAND RUFFER IN PARIS

During his Parisian stay (1889–1890), Ruffer was a pupil of Pasteur and Metchnikoff. As testified by a letter from Pasteur, Ruffer, fluent in many languages, was of great help in translating Pasteur's texts into English [27]. Ruffer never published as a coauthor with the 1908 Nobel Prize co-winner; however, he coauthored papers with Albert Charrin, professor and chair of General and Comparative Pathology at the Collège de France. In particular, two fascinating reports were published. One is devoted to the effect of nerves on local immune responses to pyocyanic bacillus (*Pseudomonas aeruginosa*) [28]. The authors report that a section of sciatic nerve favors the infectious process. They made a similar observation regarding a portion of the pneumogastric nerve (or vagus nerve) after tracheal infection. This is likely one of the very first reports in neuroimmunology and is reminiscent of the key contribution of Kevin Tracey's group on the control of inflammation by the vagus nerve [29]. The other key contribution of Ruffer and Charrin is the discovery that the filtered culture of pyocyanic bacillus could induce fever in rabbits in the absence of live or dead bacteria [30]. Of particular note, this report was published 3 years before Pfeiffer developed his concept of endotoxin [31, 32] and five years before Eugenio Centanni coined the work "pyrotoxina". Most fascinating is that the authors suggested, although they did not provide evidence, that fever was the consequence of the activation of macrophages. This hypothesis was put forth well before the discovery of endogenous pyrogen and IL-1 [33].

THE WORK OF RUFFER ON PHAGOCYTOSIS AND EFFEROCYTOSIS

Ruffer also published several papers on phagocytosis on his own. In his 1890 report on the destruction of micro-organisms during the process of inflammation [34], he thanks Charrin without mentioning him:

> "*I have to thank here the staff of the Laboratoire de Pathologie Générale in Paris for providing me with the necessary material and animals, although I alone am responsible for the views expressed in this paper.*"

In this paper, accompanied by nice pencil drawings made by his wife, Lady Alice Mary Ruffer, the elder daughter of Captain Greenfield of the Royal Artillery, he analyzed LNs and abcesses after injection of *Clostridium chauvoei* into the skin of guinea pigs. He described the recruitment of "amoeboid cells" at the site of inoculation and their capacity to engulf and kill a large number of bacilli. He paid tribute to the first report of Metchnikoff on phagocytosis and also wrote in agreement with his mentor's thoughts on inflammation:

> "*Is the inflammatory process going on at the seat of inoculation a protective process or not? The answer to that question is, as we shall see, in the affirmative.*"

In his paper published on the phagocytic cells of the digestive tract [35], in which he reports his studies on macrophages and neutrophils in tonsils and Peyer's patches of rabbits, guinea pigs, and dogs, Ruffer thanks Metchnikoff:

> "*I must be allowed here to thank M. Metchnikoff, under whose direction these investigations have been carried out at the Pasteur Institute in Paris. M. Metchnikoff has not only examined most of my slides, but he, with the greatest kindness, has placed his vast technical and literary knowledge daily at my disposal. To M. Pasteur my best thanks are due for kindly allowing me to work during many months in the institute which has been raised to him during his lifetime as a token of respect by a grateful humanity.*"

This latter sentence suggests that he also was taught by Louis Pasteur, as testified on December 6, 1889, in a lecture he gave at the Royal Society of Arts in London on "Rabies and Its Preventive Treatment". After the meeting, Ruffer sent a dispatch to Pasteur: "*London. Meeting grand success. The votes on all resolution were unanimous in a milieu of enthusiastic applause for M. Pasteur and his methods*" [27]. In 1893, for Louis Pasteur's Jubilee, he wrote a paper for the Bristish Medical Journal entitled: "Visit to the Institut Pasteur in Paris by an Old Student", in which he detailed the works performed in the different units of the institute. Surprisingly, he did not sign this text [36].

Ruffer was a strong supporter and admirer of Metchnikoff. In 1892, in a debate at the Pathologic Society of London, he strongly argued against Prof. B. Sanderson and Dr. E. Klein, who would not accept Metchnikoff's view on phagocytes and were strong defenders of the humoral theory [37]. Although he did not deny the role of humoral immunity reported by Emil von Behring and Shibasaburo Kitasato, he strongly advocated the key role of macrophages and phagocytosis in immunity:

> "*Should anyone meet a dead lion and find a lamb inside, he, knowing the habits of the lion would not conclude that the lamb had taken refuge in that. True, after a surfeit of lamb, the lion might die of indigestion but the chance of the lamb ever getting out alive would be very small.*"

In discussing the power of amoeboid cells to take up and destroy micro-organisms, he stated that:

> "*Neither Metchnikoff nor any of his pupils have ever said that it was the only cause of natural or artificial resistance.*"

In the laboratories of the Royal College of Physicians of London and the Royal College of Surgeons of England, he further confirmed in the rabbit the observation he had made in guinea pigs, namely, that the fluid at the site of inoculation of the bacilli contained chemical substances that served as attractants to leukocytes [38]. While he considered that those substances were derived solely from bacteria, he had unknowingly also observed the action of chemokines.

His description of efferocytosis was published twice in 1889. In a paper devoted to the destruction of micro-organisms by amoeboid cells, he wrote [39]:

> "*Many macrophages, as I shall now always call these large cells, contain in their interior distinct and unmistakeable microphages* [microphages being the term used by Metchnikoff to describe PMN phagocytes], *sometimes in the interior of a vacuole. The nuclei of some of these intracellular microphages may still appear to be normal, while others show signs of degeneration, and look as if they were being digested in the interior of the macrophages. In that case they present the following appearances. In the first stage of disintegration the nuclei become irregular or triradiate even. Later on, they lose the property of staining with the usual coloring-matters and become a yellowish dirty brown color, the protoplasm swelling up at the same time. The nuclei of the intracellular microphages finally disappear, and nothing is left in the interior of the macrophage but a large yellow body, which, itself becoming vacuolated, is slowly digested, until it is represented merely by small round, irregular masses of dirty-yellow protoplasm. Sometimes, however, the protoplasm of the cell is digested before the nucleus, and when that is the case, fragments of nuclei are left scattered through the protoplasm of the macrophage.*"

In his work on the phagocytes of the digestive tract, in which he thanked Metchnikoff for his support, he provides a series of drawings of macrophages found in Peyer's patches and tonsils. These cells contain microphages in various stages of destruction and are most probably the very first images of efferocytosis ever published [35]. Interestingly, although the samples were taken from normal rabbits, he noticed that macrophages frequently, simultaneously contained digested bacilli. Among the conclusions of his paper, he stated:

> "*Macrophages are able to swallow microphages (leukocytes) and to destroy and digest them.*"

Therefore, it appears that efferocytosis was observed and reported by this clairvoyant and great scientist whose fame in paleopathology has masked his insightful studies on phagocytosis conducted under the tutelage of Metchnikoff.

GIULIO BIZZOZERO'S RENOWNED WORKS

However, Ruffer was not the first scientist to describe efferocytosis. In fact, Metchnikoff, in his book on immunity in infectious diseases, published in 1901 [19], paid tribute to Giulio Bizzozero for his discovery that amoeboid cells could engulf pus globules. Giulio Bizzozero is best known by hematologists [40] and microbiologists rather than by immunologists. However, Rudolf Virchow called Bizzozero one of the glories of his century [41]. He was born in Varese (Lombardy), Italy, in 1846 and died in 1901 in Torino from a severe pneumonia. He developed a passion for microscopic observation at a very young age. After graduating with a degree in medicine in Pavia, Bizzozero started his academic career in Pavia before moving as a professor to the University of Torino, where he founded the Institute of General Pathology. Among his pupils was the great Camillo Golgi (1843–1926). In 1882, Bizzozero published the first comprehensive study on platelets and their role in thrombosis [42]. Despite that Max Schultze (1825–1874) published the first accurate description of platelets in 1865 [43], and even earlier, Alfred Donné (1801–1878) observed them in 1842 [44], it is Bizzozero's name that is associated with studies on platelets in flow conditions and the relationship among platelet adhesion, aggregation, and subsequent fibrin formation and deposition [45]. In addition, he published several pioneering works on the origin of red and white blood cells from the bone marrow. His other great discovery was the presence of spirilla living in the acidic environment of the stomach of dogs, in other words, describing the bacterium *Helicobacter* [46].

THE WORKS OF GIULIO BIZZOZERO ON PHAGOCYTOSIS AND EFFEROCYTOSIS

In the field of immunology, not only was Bizzozero the first to describe efferocytosis, but he was also the first—10 years before Metchnikoff—to propose that the process of phagocytosis could be protective against infection (ref. [47]; quoted in ref. [41]):

> "*...this fact* [ingestion of infective particles by reticular cells] *is, perhaps, the cause of the stoppage of some infections to the lymphatic glands which are connected to the part covered by the infection through the lymphatic vessels.*"

While Bizzozero speculated about the phagocytosis of microbes, he had actually described the process of efferocytosis. In an experimental model performed in the inflammed anterior chamber of the eye of rabbits, he described the pus [48]:

> "*If one investigates the exudate, it is possible to detect, in addition to the white blood suppurative cells, different bigger cellular elements (30–50 μm) and containing in addition to the nucleus a variable number (from 1 to 20 and more) of round corpuscules, which in terms of morphology and chemical reaction look exactly like the other suppurative cells.*"

He concluded his second report by stating [49]:

> "*In summary, my observation showed the presence of big cells able to engulfe white blood suppurative cells or red blood cells in their contractile protoplams. This represents a way through which the pus or blood is absorbed from the anterior chamber.*"

Furthermore, in his description of erythropoiesis in bone marrow, Bizzozero also showed pictures of macrophages that had ingested erythrocytes [50]. Indeed, engulfment of erythrocytes corresponds to the very first description of phagocytosis. In his review, Thomas Stossel [51] recalled the very first observation made in 1847 by Alexander Ecker (1816–1887), a German anthropologist and anatomist who described the presence of erythrocytes inside rabbit spleen cells [52]. In 1870, Nathanael Lieberkühn (1821–1887), a German physician and anatomist, showed that leukocytes could ingest erythrocytes [53]. In 1882, while Metchnikoff was in Messina, Sir William Osler (1849–1919), a Canadian physician, reported that cells present in the connective-tissue cells of the embryo in red marrow, spleen pulp, or lymphatic glands could contain from one to 10 or 12 red corpuscules [54]. Interestingly, Osler had reported previously in 1875 the presence of coal dust within alveolar macrophages from miners ' lungs [55], an observation already made by Kranid Slavjansky in 1869 [56]. Regarding the first reports on phagocytosis of bacteria, Charles Ambrose recalled reports in Germany and Denmark published in the early 1870s [57]. Few authors, such as Robert Koch (1843–1910), who identified anthrax bacilli in white blood cells, had interpreted their finding as a mean used by bacterial pathogens to invade their host, facilitating the spread of pathogens throughout the body. However, others, such as Peter Ludvig Panum (1820–1885), a Danish physiologist and pathologist, and Joseph G. Richardson (1836–1886), an American physician and microscopist, associated their observations with the elimination of the microbes. In 1881, Alexander Ogston (1844–1929), a British surgeon and bacteriologist, analyzed pus cells [58]. However, his observations, illustrated by accurate drawings, could not allow him to conclude whether bacilli and micrococci were on or inside pus cells. It illustrates the difficulty to interpret correctly what was under the miscroscope.

CONCLUSIONS

In conclusion, the discovery of phagocytosis was closely linked to the description of efferocytosis. Interestingly, both main contributors, Ruffer and Bizzozero, are better recognized for their other scientific achievements in different fields of investigation, demonstrating that open-minded scientists, who carefully observe, describe, and imagine, can be successful, independently of their scientific background. This is most obviously illustrated by the many accomplishments of Metchnikoff, trained as a zoologist but considered the father of cellular innate immunity. As Pasteur (a successful microbiologist trained as a chemist) said, "*The boldest conceptions, the most legitimate speculations, only find an expression and a soul the day they are enshrined by the observation and the experience*".

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