

MINIREVIEW

Historical links between toxinology and immunology

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One sentence summary: Toxinology and immunology are historically linked since their foundation during the 19th century, but there are still strong contemporary bonds.

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ABSTRACT

Research on bacterial toxins is closely linked to the birth of immunology. Our understanding of the interaction of bacterial protein toxins with immune cells has helped to decipher immunopathology, develop preventive and curative treatments for infections, and propose anti-cancer immunotherapies. The link started when Behring and Kitasato demonstrated that serotherapy was effective against ‘the strangling angel’, namely diphtheria, and its dreadful toxin discovered by Roux and Yersin. The antitoxin treatment helped to save thousands of children. Glenny demonstrated the efficacy of the secondary immune response compared to the primary one. Ramon described anatoxins that allowed the elaboration of effective vaccines and discovered the use of adjuvant to boost the antibody response. Similar approaches were later made for the tetanus toxin. Studying antitoxin antibodies Ehrlich demonstrated, for the first time, the transfer of immunity from mother to newborns. In 1989 Marrack and Kappler coined the concept of ‘superantigens’ to characterize protein toxins that induce T-lymphocyte proliferation, and cytokine release by both T-lymphocytes and antigen presenting cells. More recently, immunotoxins have been designed to kill cancer cells targeted by either specific antibodies or cytokines. Finally, the action of IgE antibodies against toxins may explain their persistence through evolution despite their side effect in allergy.

Keywords: allergy; antiserum; cytokines; diphtheria; endotoxin; exotoxin; mitogen; tetanus; vaccine

INTRODUCTION

By the end of the 19th century, the fully entangled foundations of toxinology and immunology had been both laid. The first immunological approaches to protect the host from devastating diseases were aimed at neutralizing snake venoms and microbial toxins. On the one hand, it appeared that the pathology of certain infectious diseases was linked to the secretion of poisons/toxins by some bacteria. On the other hand, the protective serotherapy against diphtheria and tetanus was found to consist of the neutralization of these toxins. The understanding of protective immunity unraveled humoral immunity and introduced the concept of antibodies. These important initial steps ended in the development of vaccines against toxins, which have been rendered non-toxic (anatoxin/toxoid). The historic proximity

between toxinology and immunology is further supported by the identification of some immunopathological mechanisms linking the activation of immune cells by toxins (some of which act as superantigens), and their capacity to induce the release of inflammatory cytokines. The overzealous production of cytokines, known as a cytokine storm, has been associated with the dire consequences of severe toxic shock. Another, end of the 19th century, link between toxins and immunology is the use of toxins as the original cancer immunotherapy. More recently, an evolutionary link between toxins and IgE-dependent immunity has been proposed. In the following sections, I will review some of the key steps that illustrate how toxinology and immunology have been intricately linked for more than 125 years (Fig. 1).

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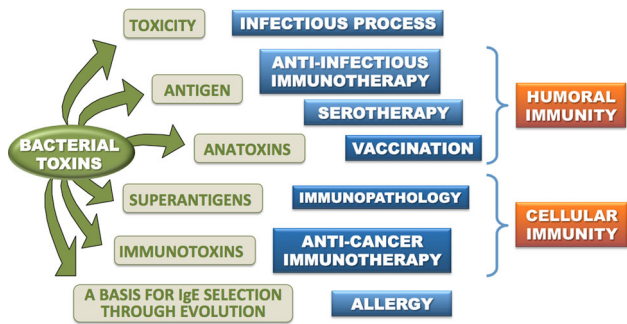


Figure 1. Summary of the links between bacterial protein toxins and the main branches of immunology.

FROM PUTREFACTION TO BACTERIAL POISONS TO TOXINS

The concept of bacterial poisons emerged from observations showing that putrid materials injected into animals were capable of killing them (Table 1). In 1822 Bernard Gaspard (1788–1871), a physician in Châlon/Saone (France), showed that intravenous or intraperitoneal injection of purulent material (pus, or putrid water made from meat or cabbage) could induce death in dogs, sheeps, pigs and even foxes (Gaspard 1822). He further demonstrated that the gases released by purulent material (carbonic acid, hydrogen sulfide and ammonia) were devoid of

any lethal activity. The following year François Magendie (1783–1855), a French physiologist who held the very first chair of experimental physiology at the Collège de France, confirmed Gaspard’s observation. He added that the administration of purulent material per os was not lethal (Magendie 1823). Magendie also attempted to demonstrate that miasma emanating from purulent material could induce death. In 1856 Peter Panum (1820–1885), a Danish pathologist, proposed the concept of a putrid poison (Panum 1856). It took him a few years to admit that his putrid poison could be derived from bacteria (Panum 1874). In 1857 Stéphane Tarnier, a young Parisian medical student, performed an important experiment. He transferred the putrid poison harvested from the peritoneal cavity of a young woman who died from puerperal sepsis into dogs and rabbits and discovered that this led to the death of the animals (Tarnier 1857). On his side Arnold Hiller (1847–1919), a German medical officer and bacteriologist, favored the idea that putrid poisons were a complex chemical mixture made independently of bacteria (Hiller 1876). Attempts to further characterize these poisonous substances ended with the identification of sepsin by Ernst von Bergmann (1836–1907), a Baltic German surgeon (Bergmann and Schmiedeberg 1866). Francesco Selmi (1817–1881), an Italian chemist, coined the term ptomaines to name the cadaveric alkaloids (Selmi 1878). From the ptomaines, putrescine and cadaverine were identified by Ludwig Brieger (1849–1919), a professor of Medicine at Humboldt-Universität in Berlin (Brieger 1885). He considered that the injurious action of bacteria was consecutive to their capacity to release some specific poisons.

Table 1. Historical steps from the concept of putrefaction to the discovery of bacteria, bacterial poisons and toxins.

YEARS	IDENTIFICATION	MAIN CONTRIBUTORS
1822 1823	PUTREFACTION PRODUCTS	Bernard Gaspard (FRA) (1788-1871) François Magendie (FRA) (1783-1855)
1856-1876	PUTRID POISON	Peter L. Panum (DEN) (1820-1885) Arnold Hiller (GER) (1847-1919) Stéphane Tarnier (FRA) (1828-1897)
1868	Sepsin	Ernst von Bergmann (GER) (1836-1907)
1878	PTOMAÏNES	Francesco Selmi (ITA) (1817-1881)
1885	Putrescine & cadaverine	Ludwig Brieger (GER) (1849-1919)
1910	Paracresol & Indol	Elie Metchnikoff (RUS) (1845-1916)
1837 1863	BACTERIA OF PUTREFACTION	Theodor Schwann (GER) (1810-1882) Louis Pasteur (FRA) (1822-1895)
1884 1890	BACTERIAL POISONS	Robert Koch (GER) (1843-1910) Nicolai Gamaleïa (RUS) (1859-1949)
1888	PROTEIN TOXINS - Diphtheria - Streptococcus - Staphylococcus - Tetanus	Emile Roux (1853-1933) & Alexandre Yersin (1863-1943) (FRA) Luigi Manfredi (1861-1952) & Gaetano Traversa (ITA) Theodor von Leber (GER) (1840-1917) & J. De Christmas (DEN) (1860-1916)
1890		Knud Faber (DEN) (1862-1956) / Guido Tizzoni (1853-1932) & Giuseppina Cattani (1859-1914), Alessandro Bruschettini (1868–1932) (ITA) Louis Vaillard (1850-1935) & Hyacinthe Vincent (1862-1950) (FRA)
1892		Albert Charrin (FRA) (1856-1907)
1897		Nikolai Gamaleïa (RUS) (1859-1949) Émile van Ermengem (BEL) (1851-1932)
1892	ENDOTOXIN	Richard Pfeiffer (GER) (1858-1945)
1894	Pyrotoxina	Eugenio Centanni (ITA) (1863-1942)
1935	Antigène glucido-lipidique	André Boivin (FRA) (1895-1949) & Lydia Mesrobianu (ROU) (1908-1978)

Brieger coined the word toxin in 1888 (Brieger 1888). Putrefaction was also recognized to occur within the gut and Elie Metchnikoff (1845–1916), the 1908 Nobel Prize winner for the discovery of phagocytosis, defended the concept of autointoxication. He demonstrated that metabolites such as paracresol and indol produced by the gut bacteria could contribute to atherosclerosis, aging and even death (Metchnikoff 1910). These studies were independently confirmed some 100 years later (Wang et al. 2011; Zhu et al. 2016). Indeed, the fact that putrefaction was a microbiological process brought about by living microscopic organisms (then termed ‘infusoria’) was reported in 1837 by Theodor Schwann (1810–1882), a German medical doctor (Schwann 1837). Schwann paved the way to the final rebuttal of Louis Pasteur’s hypothesis of spontaneous generation. In 1828, the word ‘bacterium’ was coined by Christian Gottfried Ehrenberg (1795–1876). He derived the name from the Greek word *βακτηριον*, meaning ‘small stick’. Thirty-five years later, in 1863, the six species of vibrios, identified by Ehrenberg, were recognized by Louis Pasteur (1822–1895) as being the ferments of putrefaction (Pasteur 1863). The idea that poison could be released by bacteria emerged after Robert Koch discovered the bacillus of cholera (Koch 1884) and Nikolai Gamaleïa (1859–1949), a Russian microbiologist, demonstrated the presence of cholera poison within a *Vibrio cholerae* culture (Gamaleïa 1890). Presenting Gamaleïa’s communication Charles Bouchard (1837–1915), an eminent physician, mentioned that he had also observed such morbid material in the urine of patients suffering from cholera. In 1892 Gamaleïa wrote a book entitled ‘Les Poisons Bactériens’ (bacterial poisons), which was translated in English the following year. This was a comprehensive overview of the end of the 19th century knowledge of all the noxious substances elaborated by bacteria that can cause disease independently of the bacteria themselves.

Numerous experiments convincingly showed that the deleterious effects of certain pathogens were particularly due to their capacity to elaborate protein toxins. The first demonstration of an elaborated protein toxin was for a terrifying disease. Nicknamed ‘the strangling angel of children’, diphtheria, was killing thousands of young children in Europe and in the USA every year. Pierre Bretonneau (1778–1862), a famous French physician, provided the first complete description of diphtheria, hypothesizing that it was due to infectious agents. In Germany Edwin Klebs (1834–1913), an assistant of Rudolf Virchow (1821–1902), identified the causative microbe in 1883. The following year Friedrich Löffler (1852–1915), a collaborator of Robert Koch, isolated and cultured the bacterium. Soon after they were convinced that the disease was due to a toxin. The validation of the hypothesis came from the work of Emile Roux (1853–1933) and Alexandre Yersin (1863–1943) of the Institut Pasteur in Paris. Between 1888 and 1890, they published three reports illustrating that the injected filtrate of cultures of Löffler’s bacillus into guinea pigs, rabbits and pigeons would kill them (Roux and Yersin 1888). They also reported that urine taken from children shortly before their death from diphtheria could kill a guinea pig. In 1888, in Italy, Luigi Manfredi (1861–1952) and Gaetano Traversa succeeded in obtaining a toxic effect from sterile filtrates of streptococcus cultured in broths (Manfredi and Traversa 1888). That same year Theodor Karl Gustav von Leber (1840–1917) (Leber 1888) and John Carl Constant Beresford de Christmas-Dirckinck-Holmfeld (1860–1916), a physician working at the Institut Pasteur, showed that pyogenic substances were released by *Staphylococcus aureus* (de Christmas 1888). Regarding tetanus Antonio Carle (1854–1927) and Giorgio Rattone (1857–1929), both Italian, showed in 1884 that the human dis-

ease could be transmitted to rabbits. Additionally in 1884, in Göttingen (Germany), Arthur Nicolaïer (1862–1942) isolated the causative bacterium (*Plectridium* (*Clostridium*) *tetani*). In 1890 the Danish Knud Faber (1862–1956), professor of internal medicine at the University of Copenhagen (Faber 1890), came to the conclusion that the lethal effects were due to a toxin. This conclusion was reached the same year by different groups. In Bologna (Italy) Prof. Guido Tizzoni (1853–1932) published his observation with Giuseppina Cattani (1859–1914) (Tizzoni and Cattani 1890), while Alessandro Bruschetti (1868–1932), from the same team, published on his own (Bruschetti 1890). In France Louis Vaillard (1850–1935), who has just created the very first research laboratory of the French Defense Health Service, published together with Hyacinthe Vincent (1862–1950), a military physician, the experimental results that filtered cultures of the tetanus bacillus could induce the disease and kill mice, guinea pigs and rabbits (Vaillard and Vincent 1890). Furthermore, in 1890 Albert Charrin (1856–1907) published that the pyocyanic bacillus (*Pseudomonas aeruginosa*) was able to release substances acting on the nervous system (Charrin and Gley 1890). To mention a final discovery, that of *Clostridium botulinum*, which started in 1820 when Justinus Kerner (1786–1862) investigated the lethal food poisoning due to the so-called sausage poison (Kerner 1820). Giving rise to the bacterium’s name, the Greek word for sausage is *botulus*. The discovery concluded with the elucidation of its etiology in 1895 by the Belgian microbiologist Émile van Ermengem (1851–1932). He showed that porcelain filtration yielded a sterile product that had the same toxic effect on mice, rabbits, cats and guinea pigs than the unfiltered maceration (van Ermengem 1897).

Of course it is difficult to assess the quality of all the preparations used for these demonstrations. It is most probable that many of the preparations were contaminated by endotoxins, which are among the most powerful toxins. This was doubtlessly the case of the filtered culture of pyocyanic bacillus (*P. aeruginosa*), which was shown to induce fever in rabbits in the absence of live bacteria (Charrin and Ruffer 1889). Also, Gamaleïa claimed that there were two specific poisons of cholera: a thermoresistant one leading to death without diarrhea, and a thermosensitive one that induces diarrhea. One can speculate that he was dealing with the endotoxin and the cholera toxin (Gamaleïa 1890, 1892). The word endotoxin was first coined by Richard Pfeiffer (1858–1945), a German physician and bacteriologist, who thought that the toxic properties of bacteria were due to a factor released by lysed bacteria (Pfeiffer 1892, Rietschel and Cavaillon 2003). It was later recognized that the factor was the main constituent of outer membrane of Gram-negative bacteria. The lipopolysaccharidic constitution of endotoxins was first demonstrated in Bucharest in 1935 by Lydia Mesrobian (1908–1978), who worked under the supervision of André Boivin (1895–1949) (Boivin and Mesrobian 1935). If one excludes the metabolites, it was the first instance that a non-protein molecule was recognized as an antigen and a toxin. Initially called ‘antigène glucido-lipidique’, it was later renamed as lipopolysaccharide or LPS.

PROTEIN TOXINS AND SEROTHERAPY

In 1890 Emil von Behring (1854–1917) and Shibasaburo Kitasato (1853–1931) showed that immune sera from guinea pigs, rabbits, sheep and goat were protective against diphtheria and tetanus (Behring and Kitasato 1890). The use of serotherapy was initiated in children in 1891, and in 1894 the first published clinical trial that was performed on 220 children suffering from

diphtheria reported a 77% cure rate. This new effective approach was rapidly implemented worldwide, and the 30.75% mortality rate reported in Boston between 1890 and 1894 declined to 12.61% between 1895 and 1897. In Paris 1639 deaths were reported in 1890, 411 deaths were reported in 1894 and only 274 deaths were reported in 1897 from diphtheria (McCollom 1898). In France Emile Roux, who has discovered the diphtheria toxin with Alexandre Yersin, introduced the use of horses to prepare the immune sera. In 1894 with Louis Martin (1864–1946), his colleague of Institut Pasteur, and Dr Auguste Chaillou (1866–1915), from Necker Hospital, he published the treatment of 300 children with a 50% decreased in mortality. These results achieved on a devastating disease, which was killing thousands of young children, had a great impact. The Parisian newspaper 'Le petit Journal' published a picture of Emile Roux saving a child on its front page (24 September 1894). The French daily newspaper 'Le Figaro' also echoed this success and launched for a call for donations. The newspaper gathered more than a million francs, which were more than welcomed by the research institute. The donations varied from a few francs donated by modest people to 20 000 francs donated by the Baron Edmond de Rothschild, and 50 000 donated by the city of Paris. Sarah Bernardt, a star actress, also raised funds for the Institut Pasteur. She offered a hundred seats up for auction, at the theater de la Renaissance, for the 1894 première of Gismonda, a four act melodrama by Victorien Sardou. In recognition of the discovery of the protective immunity provided by serotherapy, Emil von Behring was awarded, the first Nobel prize in medicine or physiology in 1901. In his Nobel lecture Behring paid tribute to his colleagues Löffler and Roux. Between 1901 and 1932 Emile Roux received 115 nominations for the Nobel Prize, but they were in vain. In 1895 Theobald Smith (1859–1934) was serving as professor of comparative pathology at Harvard University when he was nominated as the director of the Massachusetts State Antitoxin and Vaccine Laboratory. Under Smith's administration, diphtheria antitoxin production increased from 1700 to 33 000 doses within 4 years. Smith estimated that during the first 7 years of production of the antitoxin in the state laboratory, 10 000 lives were saved thanks to its use. Smith undertook many practical and theoretical studies on the production of diphtheria antitoxins. He was one of the first to demonstrate that killed cultures of disease causing organisms produce immunity and that a mixture of diphtheria toxin and antitoxin confers immunity (Smith 1909).

Behring demonstrated the efficacy of serotherapy for both diphtheria and tetanus (Behring and Kitasato 1890). Edmond Nocard (1850–1903), a veterinarian, former assistant to Louis Pasteur, and a professor at the veterinary school of Maison-Alfort, provided his colleagues with 7000 vials of the anti-tetanus antiserum to protect horses, donkeys, bulls, rams and pigs. He concluded that the preventive use of anti-tetanus serum was completely effective (Nocard 1897). When the First World War started in 1914, the Institut Pasteur had 300 horses and was preparing 80 000 vials of antisera a month. In 1918 there were 1462 horses, allowing for the preparation of 600 000 vials per month.

PROTEIN TOXINS AND VACCINES

Due to the noxious effects on animals of injected protein toxins, the preparation of the antisera was a tricky process. Paul Ehrlich (1854–1915) developed the standardized method needed to guarantee reproducible titers with high toxin-neutralizing activity in the antiserum (Ehrlich, Kossel and Wassermann 1894). In 1908 Ehrlich, the father of humoral immunity, shared with Elie

Metchnikoff the Nobel Prize for his discovery of the side-chain theory of antigen–antibody interaction. A few years earlier, using ricin toxin and tetanus toxin, Ehrlich made a major discovery (Silverstein 1996): the transfer of immune protection from mother to offspring (Ehrlich 1892; Ehrlich and Hübener 1894). For his proof, he injected female mice with a toxin, and mated them with naïve male mice. He then showed that the progeny had antitoxin antibodies. Additionally, when he did the opposite experiment (i.e. injected male mated with naïve female mice) there were no toxin-specific antibodies in the newborn mice. After having demonstrated that during pregnancy, antibodies are transmitted from the mother to the fetus, he went further. He showed that suckling mice of an immunized female conserved the marked protective immunity, while there was a rapid decline of antibodies if placed with naïve mothers. He demonstrated that breastfeeding contributes to protective immunity. Continuing, Ehrlich investigated pregnant goats that he immunized with tetanus toxin and demonstrated the presence of protective antibodies in their milk collected after delivery (Brieger and Ehrlich 1892). He was able to titrate the levels of antibodies and show the consequences of booster injections (Brieger and Ehrlich 1893). Attempts to purify anti-diphtheria toxin antibodies from both the serum and the milk were later reported (Ehrlich and Wassermann 1894).

There were efforts to find a simpler way to vaccinate using a diphtheria toxin with attenuated toxicity. Alexander Thomas Glenny (1882–1965), who was the head of the immunology department of the Wellcome Physiological Research Laboratories in London, proposed in 1923 to use a formalin-treated toxin that he called 'toxoid' (Glenny and Hopkins 1923). However, the treatment still required a co-injection of antitoxin into the guinea pig. Later, he discovered the greatly enhanced immunizing power of the alum-precipitated toxoid (Glenny and Barr 1931). More importantly, 2 years earlier, while investigating the induction of immunity to diphtheria toxin, Glenny demonstrated the concept of primary and secondary responses to immunization. He established that the rapid immune response to the secondary stimulus offered a striking contrast to the small and gradual response to the primary stimulus (Glenny and Südmersen 1921). On his side, Gaston Ramon (1886–1963) coined the concept of anatoxin. Ramon was a veterinarian, hired by Roux to prepare the horse antisera at the Institut Pasteur. He showed that the treatment of the diphtheria toxin with formalin and heat treatment made an immunizing molecule that had lost its toxicity, but retained its antigenicity in guinea pigs and horses (Ramon 1923). The first vaccine against diphtheria was invented and became very popular. Ramon made another more important discovery. He observed that the antibody titers varied among the immunized horses. He observed that those who had the highest levels of antibodies had a small abscess at the site of injection. On purpose, he instigated such events with either pus or various other irritating substances. His preferred one was tapioca, although later on he also studied lanolin. He concluded from his experiments that there was a need to involve an inflammatory reaction at the site of injection of the antigen to enhance the immune response. He had discovered the adjuvant. Between 1930 and 1953, Ramon was nominated 155 times for the Nobel Prize, but it was never awarded to him. To this day, he is still regarded as the champion in this category, just in front of Roux!

As suggested by Ramon, after his successful diphtheria anatoxin preparation, the same experimental approach was developed to prepare the tetanus anatoxin. In 1925 Pierre Descombey (1895–1930), a young pasteurian who passed away when he was only 35, successfully immunized horses and guinea pigs

(Descombey 1925). The following year after A. Lafaille Ramon's colleague tested the safety of the injection on himself, Ramon tested the tetanus anatoxin on a hundred humans and obtained strong neutralizing activity of their sera after three injections (Ramon and Zoeller 1925). They also reported that the vaccine could be delivered with other vaccines, such as the typhoid vaccine, without any alteration of the response. Remarkably, the father of the cellular innate immunity, Elie Metchnikoff investigated the toxicity of tetanus toxin in different animal species and their capacity to produce an antitoxin activity (Metchnikoff 1897). He reported that the toxin was devoid of any toxicity in beetles and scorpions, and as long as the animals were maintained below 30°C fishes, frogs, axolotls and tortoises all failed to elaborate any antitoxin activity. In contrast, in hens, guinea pigs and crocodiles (maintained at 32°C–37°C) he could obtain a neutralizing response against the toxin.

Metchnikoff had also compared the sensitivity of different animal species to *Vibrio cholera*. He found that guinea pigs were more sensitive than rabbits, which were more sensitive than mice, and that pigeons and hens were insensitive (Metchnikoff, Roux and Taurelli-Salimbeni 1896). In addition, he demonstrated that the toxicity was due to a toxin and that the antitoxin immunity was protective. For his demonstration he placed live bacteria in a bag preventing the bacteria's dispersal, and then he implanted the bag into the peritoneal cavity of guinea pigs. Most of them died, thus illustrating that a diffusible product was responsible of the poor outcome. When he placed dead bacteria or culture medium in the bag, the animals survived. Then, he selected the few animals that survived the bags containing the live bacteria, and re-injected them with a lethal dose of *V. cholerae*. Not only were the animals protected, but they were able to resist further injections of up to 16 lethal doses. Afterwards, Metchnikoff prepared the cholera toxin and successfully immunized guinea pigs, rabbits, goats and horses demonstrating that their sera displayed a protective activity. It is admirable that the father of cellular immunity made key experiments demonstrating the importance of humoral immunity against bacterial toxins.

To end this section linking protein toxins and the birth of vaccination, let us mention that the relationship continues to this day and that recently, toxins have been proposed as a tool to favor antigen delivery. This has been done particularly in the case of *Bordetella pertussis* adenylate cyclase toxin hemolysin (Sebo et al. 1995; Dadaglio et al. 2014; Sebo, Osicka and Masin 2014).

PROTEIN TOXINS AND IMMUNE CELL DEATH

Protein toxins have the capacity to kill various types of target cells. We will only focus on their toxicity toward immune cells. The first report appeared in 1963, when James Hirsch (Hirsch, Bernheimer and Weissmann 1963) demonstrated that streptolysin O (SLO) and streptolysin S induce a rapid and extensive lysis of cytoplasmic granules of rabbit neutrophils. Similarly, in rabbit macrophages, a lysis of cytoplasmic granules is followed by alterations in the cytoplasm and the membrane. Dorothea Zucker-Franklin (1929–2015), a well-recognized expert in electron microscopy, published detailed pictures of the process. She added eosinophils among the target cells on which streptolysin O induces the rupture of the cellular membrane that yields an osmotic lysis (Zucker-Franklin 1965). Robert Fauve (1930–1995) extended the observation to murine macrophages on which streptolysin O induces extensive cell damage and their death (Fauve et al. 1966). Toxins can induce different types of cell death. For

example, staphylococcal toxins induce necroptosis (Kitur et al. 2015), as does pneumolysin on infiltrating macrophages (Gilley et al. 2016). Apoptosis was reported to occur upon the action of diphtheria toxin (Chang et al. 1989). Similarly, *B. pertussis* adenylate cyclase hemolysin induces apoptosis of macrophages (Khelef, Zychlinsky and Guiso 1993), and staphylococcal alpha toxin provokes apoptosis of T-lymphocytes (Jonas et al. 1994). Pyroptosis, a cell-death mechanism, which follows the activation of the inflammasome was initially reported for the anthrax lethal toxin (Muehlbauer et al. 2007; Wickliffe, Leppla and Moayeri 2008), but may be observed with other bacteria such as *Salmonella* (Fink, Bergsbaken and Cookson 2008).

Of interest, the capacity of some protein toxins, such as SLO, to contribute to reversible permeabilization of cell membranes was harnessed to allow the delivery of antisense oligonucleotides (Barry, Gesek and Friedman 1993) or proteins (Walev et al. 2001). An oxidized non-hemolytic form of SLO was considered to reduce or modulate collagen levels in murine models of scleroderma (Mamber et al. 2004). Another therapeutic use of SLO was proposed to address *Rhodococcus equi* pneumonia in foals as an adjunct therapy with antibiotics (Horohov et al. 2011).

TOXINS AND FEVER

Many toxins display the capacity to activate the inflammasome (Greaney, Leppla and Moayeri 2015). Inflammasomes are molecular platforms aimed to sense danger. They activate through an autoproteolytic cleavage and dimerization of the interleukin-1 converting enzyme (caspase 1). Caspase 1 cleaves the 30 kDa inactive precursor forms of interleukin-1 β (IL-1 β) and IL-18 into their active 17.5 kDa active mature forms (Martinon, Burns and Tschoop 2002). We have recently detailed the major historical milestones that emerged from the first report that bacterial products induce fever (Cavaillon 2018). Briefly, Albert Charrin and Armand Ruffer (Charrin and Ruffer 1889) reported that filtrates of bacterial cultures could induce fever, and Eugenio Centanni named the responsible bacterial poison 'pyrotoxina' (Centanni 1894). Endotoxin (Bennet and Beeson 1950), streptococcal exotoxin (Watson 1960) and staphylococcal exotoxin (Clark and Borison 1963) were identified among the bacterial entities able to induce a fever when injected into rabbits. The pyrogenicity of the toxins was recognized with new names that were given to the erythrogenic toxin, also known as the scarlet fever toxin and the streptococcal pyrogenic exotoxin (Kim and Watson 1970). The identification of the host mediator responsible for the initiation of a fever, called the endogenous pyrogen, was made in 1953 (Bennett and Beeson 1953). Because it was recognized that many biological activities were shared by the same molecule but reported under different names (endogenous pyrogen, osteoclast activating factor, catabolin, hemopoietin 1 and lymphocyte activating factor), it was decided to name it interleukin-1 (IL-1) (Mizel and Farrar 1979). Of note, IL-6 is the very last cytokine induced by IL-1 that initiates a fever within the brain (Chai et al. 1996). Later on endotoxin (Gery, Gershon and Waksman 1972), toxin shock syndrome toxin-1 (Ikejima et al. 1984), streptococcal pyrogenic exotoxin A (Hackett and Stevens 1992) and others were reported to induce the production of IL-1. In fact, there are two molecules respectively called IL-1 α and IL-1 β . IL-1 α can be expressed on the cell surface or remain intracellular with an intracrine property acting directly within the nucleus. In contrast, once cleaved by caspase 1, mature IL-1 β is released following signals delivered by ATP via its P2 $\times$ 7 receptor. Remarkably, the streptococcal pyrogenic exotoxin B displays enzymatic

properties that cleave the IL-1 β precursor (Kapur et al. 1993; LaRock et al. 2016). More recently, it was reported that pore-forming toxins such as *Aeromonas aerolysin* activate caspase-1 (Gurcel et al. 2006). Many virulence factors/toxins delivered intracellularly can inactivate Rho GTPases, resulting in the activation of the pyrin inflammasome. This process was reported for the *C. difficile* TcdB toxin (Xu et al. 2014), the *B. cenocepacia* effector TecA (Aubert et al. 2016) and the *Yersinia* effectors YopE and YopT. In contrast, *Yersinia* effector YopM blocks the activation of pyrin (Chung et al. 2016). The contribution of these effectors to bacterial virulence and modulation of the host's response has been established by using bacteria deficient for one or the other virulence factor.

TOXINS AND IMMUNOSTIMULATION

Deciphering the interactions of toxins with immune cells has led to important discoveries. One was the capacity of microbial toxins to induce lymphocyte proliferation. The capacity of plant lectins to induce mitosis within lymphocytes was reported in 1960 using phytohemagglutinin A (PHA) (Nowell 1960). In 1964 it was reported for the first time that a microbial toxin, streptolysin S, could activate human lymphocytes and inducing mitosis like PHA does (Hirschhorn et al. 1964). The following year, endotoxins were also shown to display mitogenic properties (Oppenheim and Perry 1965), mainly favoring the proliferation of B-lymphocytes. It took a few more years to add new protein toxins to the list of mitogens. In 1971, streptolysin O was shown to induce the stimulation of rabbit lymphocytes (Abe 1971). A few years later, in 1973, two teams described the capacity of Scarlet fever toxin/streptococcal erythrogenic (pyrogenic) exotoxins (SPE) to induce the proliferation of rabbit, rat, guinea pig and human lymphocytes (Hrčbalová and Pospíšil 1973; Naucliel 1973). Then Watson's group confirmed that this property was shared by all three exotoxins: SPE A, SPE B and SPE C (Barsumian, Schlievert and Watson 1978). A major achievement was accomplished by Philippa Marrack and John Kappler (Kappler et al. 1989; Marrack and Kappler 1990) when they deciphered the mechanism of action of some of the exotoxins, which they called superantigens. They demonstrated that these exotoxins can bridge antigen-presenting cells and T-lymphocytes by binding to both the MHC class II antigen-presenting glycoproteins on macrophages or dendritic cells and the V β chain of the T-cell receptor (TCR). A recent study revealed that staphylococcal enterotoxin B (SEB) also binds to CD28, favoring the cross-talk between antigen-presenting cells and T-lymphocytes, further amplifying the inflammatory cytokine gene expression (Levy et al. 2016). Preventing the costimulatory axis CD28/CD86 was shown to be protective in a SEB lethal challenge. The critical role of superantigens has been clearly established in the severity of a disease. For example, staphylococcal superantigens have been shown to play a central role in a rabbit model of *S. aureus* infection (Salgado-Pabon et al. 2013).

The capacity of exotoxins and superantigens to activate immune cells was further illustrated by their capacity to induce the release of inflammatory cytokines. The first report was published in 1981 demonstrating that staphylococcal enterotoxin A (SEA), SEB, SEC, and SED were able to induce the production of gamma-interferon (IFN γ) by human peripheral blood mononuclear cells (PBMC) (Langford et al. 1981). The following year we showed that streptococcal pyrogenic exotoxin A (SPEA) induced the release of IFN γ by murine splenocytes (Cavaillon et al. 1982). In fact, SPEA, as any other exotoxin, induces a wide panel of

inflammatory cytokines by both murine and human PBMC, including IL-1 α , IL-1 β , IL-3, IL-6, IL-8, tumor necrosis factor (TNF) and lymphotoxin- α (Lt α) (Muller-Alouf et al. 1992, 1994). SPEA also induces the release of anti-inflammatory cytokines such as IL-4, IL-10 and IL-1 receptor antagonist (IL-1Ra) (Muller-Alouf et al. 1996). A similar capacity to induce the main inflammatory cytokines such as IL-1, TNF and IFN γ has been reported for other exotoxins, such as the toxic shock syndrome toxin 1 a major contributor of the dreadful staphylococcal toxic shock syndrome (Jupin et al. 1988; Stich et al. 2010). Furthermore, the capacity of superantigens to induce the release of chemokines perpetuates the inflammatory reaction (Svedova et al. 2016).

Endotoxins share with protein toxins the capacity to induce the release of inflammatory cytokines. However, the lowest concentration needed, the cytokine profiles and the kinetics are quite different (Muller-Alouf et al. 1994). Indeed, LPSs are the most potent toxins that induce cytokine production. They act through the receptor complex CD14/Toll-like receptor-4 (TLR4)/myeloid differentiation protein 2 (MD2), favoring cytokines amplification loops, cascades of cytokines and inflammatory mediators productions (Cavaillon 2018).

In an elegant demonstration, Sriskandan, Evans and Cohen (1996) established that SPEA also induces a cascade of cytokine production. IL-12 contributes to the SPEA-induced production of IFN γ , which in turn contributes to the production of Lt α . Most importantly, this group reported the presence of circulating Lt α in patients with streptococcal toxic-shock syndrome (Sriskandan, Moyes and Cohen 1996). This observation illustrates the concept of cytokine storm that characterizes sepsis patients with either Gram-negative or Gram-positive infections (Cavaillon, Müller-Alouf and Alouf 1997; Cavaillon et al. 2003; Faulkner et al. 2005; Spaulding et al. 2013). This overzealous production of inflammatory cytokines induced by bacterial exotoxins, endotoxins and other pathogen-associated molecular patterns is responsible for organ failure and eventually death (Tisoncik et al. 2012). Unfortunately, all therapeutic approaches aimed at neutralizing inflammatory cytokines, which have been extremely beneficial in murine models of severe bacterial infection, have failed in humans. This illustrates the limitations of the murine model to study such severe infections (Stortz et al. 2017). This discrepancy is due to the great resilience of mice to most infectious agents and their high resistance to protein toxins and endotoxins (Warren et al. 2010). Of note, the cytokine storm also involves anti-inflammatory cytokines (Annane, Bellissant and Cavaillon 2005), and both exacerbated productions are concomitant (Cavaillon et al. 2001). The concept of 'compensatory anti-inflammatory response syndrome' has emerged as a consequence of the excessive anti-inflammatory reaction that is associated with an altered immune status of circulating cells and a possible increased susceptibility of patients to nosocomial infection (Adib-Conquy and Cavaillon 2009). Although new approaches to save patients have been proposed by boosting the immune status (Hotchkiss, Monneret and Payen 2013), we have raised our concerns about such an approach since it does not take into account the on-going inflammatory process occurring in different organs of septic patients (Cavaillon, Eisen and Annane 2014).

TOXINS AND ANTI-CANCER IMMUNOTHERAPY

The relationship between toxins and cancer immunotherapy appeared by the end of the 19th century. In 1891 William Coley, a

surgeon working at the New York Cancer hospital, started treating inoperable sarcoma patients with erysipelas (*Streptococcus pyogenes*). In 1893 he published his first cases, reporting a cure rate of 41% (Coley 1893). He suspected that the bacteria were releasing a toxic substance, and admixed them with those of *Bacillus prodigiosus* (*Serratia marcescens*). This toxic crude material was then used to treat 140 patients. A few patients had partial remission, but in 16 cases there was a complete disappearance of tumors (Coley 1898). The fact that bacterial products can non-specifically boost the immune system to fight cancer has also led to the use of BCG (Villasor, Fetalino and Ramirez 1963), which is still employed as a treatment of bladder cancer (Sylvester, van der and Lamm 2002). Interestingly, the use of both BCG and endotoxin injected in mice led to the discovery of TNF, the active endogenous molecule with anti-tumoral activity (Carswell et al. 1975).

Another approach emerged in 1976, when researchers from the Boston University School of Medicine (Moolten, Zajdel and Cooperband 1976) constructed hybrid molecules made with anti-tumor antibodies coupled to either diphtheria toxin or to ricin toxin to kill tumor cells. In the early 1980s, a few groups published similar approaches (Krolick et al. 1980; Masuho and Hara 1980; Ross et al. 1980). The word 'immunotoxin' was coined in 1981 to name these chimeric molecules aimed to kill tumor cells, including T- or B-cell leukemia (Blythman et al. 1981; Krolick et al. 1982). Another construct made with the diphtheria toxin receptor-binding domain and the enzyme active and translocating domain coupled with interleukin-2 was designed to target and kill T-cell lymphoma expressing the IL-2 receptor (IL-2R) (Williams et al. 1987). This is now a drug (Denileukin difitox) that was approved by FDA in 1999 to treat patients with late stage cutaneous T-cell lymphoma. Unfortunately, the drug was discontinued in 2014, as announced by the FDA, because of a shortage of the chimeric molecule. A similar target was considered in 1984 by Iran Pastan and his group at NIH (Bethesda) built an immunotoxin made with anti-IL-2R antibodies and *Pseudomonas* exotoxin (FitzGerald et al. 1984). Since then, Pastan never stopped improving his immunotoxins and their productions (Chaudhary et al. 1990; Brinkmann et al. 1993; Lorberbourn-Galski et al. 1988, 1989; Beers et al. 2000). The LMB2 molecule, which has been tested in a clinical study, is a fusion molecule made with the Fv portion of an anti-CD25 (α -chain of IL-2R) antibody and a 38 kDa truncated *Pseudomonas* exotoxin (PE38), designed to reduce immunogenicity. Combined with other anti-cancer drugs, it led to complete remission in 60% of the tested patients with T-cell leukemia (Kreitman et al. 2016). A similar approach is underway to kill tumor cells that express CD22. BL22 (Moxetumomab pasudotox) is a recombinant protein composed of the variable region of a monoclonal antibody that binds to CD22 bound to PE38. It appears as a promising agent in patients with hairy cell leukemia (Kreitman and Pastan 2011). Many other attempts to kill cancer cells have been performed with immunotoxin associating the native or truncated *Pseudomonas* exotoxin and antibodies against transferrin receptor, epidermal growth factor receptor (Pirker et al. 1985; Chandramohan et al. 2013) or tumor antigens (P-glycoprotein, B3 or mesothelin) (FitzGerald et al. 1987; Brinkmann, Lee and Pastan 1993; Hassan et al. 2013). Other constructs with cytokines (IL-2, IL-4, IL-13 and GM-CSF) and the native or truncated *Pseudomonas* exotoxin have also displayed promising properties (Lorberbourn-Galski et al. 1989; Debinski et al. 1995; Kreitman, Puri and Pastan 1995; Kreitman and Pastan 1997). A large number of clinical trials have been performed or are underway to treat different types of

leukemia, lymphoma and other types of cancers (Alewine, Hassan and Pastan 2015).

A large panel of antibodies has been used to create new immunotoxins with the aim to eliminate T-lymphocytes (CD3, CD5, CD25, and CTLA-4), with expected applications in clinical settings such as transplantation and in graft versus host disease (Kernan et al. 1984; Cavazzana-Calvo et al. 1990; Knechtle 2001; Palmisano et al. 2004). Elimination of regulatory T-cells (Treg) has been proposed with toxins linked to an anti-IL-2 receptor, an anti-chemokine receptor (CCR4) or IL-2 (Litzinger et al. 2007; Powell et al. 2008; Zhou et al. 2009; Wang et al. 2016), with the goal to boost vaccines or to boost anti-tumor therapies.

TOXIN AND TRANSGENIC MICE

Another mean to eliminate immune cells has been achieved with the construction of transgenic mice, which express either the diphtheria toxin or its receptor. Their genes are incorporated with tissue- or cell-specific genes. The first description of such transgenic animal was reported in 1987 with mice expressing the gene of the A chain of diphtheria toxin either fused with the elastase I gene expressed in the pancreas or with γ 2-cristallin expressed in lens nuclear fiber cells (Breitman et al. 1987; Palmiter et al. 1987). The A chain of diphtheria toxin inhibits protein biosynthesis by inactivating elongation factor EF2 in the presence of NAD⁺. If the transgenesis is achieved with the diphtheria toxin receptor, then conditionally mediated and targeted cell ablation is possible after injection of the toxin; the toxin is non-toxic for normal mice (Saito et al. 2001). Either method allows for the generation of mice devoid of macrophages (Lang and Bishop 1993; Duffield et al. 2005), NK cells (Arase et al. 1999), dendritic cells (Jung et al. 2002) or Treg (Feuerer et al. 2009). More recent approaches have used conditional genetic tools. Mice expressing the Cre recombinase in tissues or in specific cells are crossed with mice expressing the A chain of diphtheria toxin or diphtheria toxin receptor under control of a loxP-flanked stop cassette. The loxP sites being recognized by the Cre enzyme leads to the recombination and transference of specific toxin sensitivity to cells or tissue (Brockschneider et al. 2004). Such an approach was used to allow efficient ablation of B-lymphocytes or T-lymphocytes after exposure to diphtheria toxin (Buch et al. 2005).

PROTEIN TOXINS AND ALLERGY

Protein toxins are also linked to the discovery of a very peculiar immune response, called anaphylaxis (from Greek *αντα* (against) and *πρωλαξισ* (protection)), a deleterious response instead of a protective one. In 1901 Paul Portier (1866–1962) and Charles Richet (1850–1935) embarked on the Princess Alice II, the yacht of Prince Albert of Monaco. On board they studied the physalia, a marine animal close to jellyfish that produces the poison called hypnotoxin. After an initial injection into a dog, there was no particular effect, but after a second injection 2 to 3 weeks later resulted in the death of the animal (Portier and Richet 1902). Back in France they reproduced the same experiment with extracts from tentacles of sea anemones. While the first injection had no effect, the second injection caused the animals to present with the classic symptoms of shock: itching, dyspnea, vomiting and hypotension leading to their death within half an hour. Richet later showed that this immediate hypersensitivity, called anaphylactic shock, was transferable by injecting the

serum of a sensitized animal to a naive animal. For his discovery, Paul Richet was awarded the Nobel Prize in 1913. It was not until 1966 with the discovery by Kimishige Ishizaka of IgE, a class of particular immunoglobulins, to understand this phenomenon. These IgE antibodies bind to specific receptors on the surface of blood basophils and tissue mast cells. Once present on the surface of these cells, they will interact with the antigen during a subsequent exposure. This results in the degranulation of basophils and mast cells and the release of extremely inflammatory mediators, including histamine that is associated with allergy.

This raises the question of why Nature would have maintained an IgE-dependent response in most placental mammalian species, often considered as an immunological maladaptive reaction, through evolution. The initial hypothesis implicated the resistance to helminth infections, which are associated with an IgE-mediated immune defense. The 'toxin hypothesis' proposed in 1991 by Margie Profet (Profet 1991) is an alternative. She argued that allergy is designed to be a mechanism of defense against toxins from plants, insects, reptiles, marine animals and bacteria. She explained that the process results in the release of chemicals by mast cells, which cause vomiting, diarrhea, coughing, sneezing, scratching and tear formation, which may rapidly expel toxins, and slow the rate of circulation of toxins to target organs by decreasing blood pressure. Accordingly, an allergic reaction would be a defense mechanism that allows the sensitized host to respond immediately to and neutralize/avoid, noxious substances that might be indicative of a potentially life-threatening situations. However, it remains unclear how toxins, which Profet considers often behaving as hapten, could favor a Th2 response and the release of IL-4 or IL-13 to promote the switch to IgE production in B-lymphocytes. Profet's 'toxin hypothesis' was largely ignored by the scientific community until supporting experimental evidence emerged with the work of Stephen Galli's team (Marichal et al. 2013). They demonstrated the beneficial role of IgE in host defense against honeybee venom. Mice injected with a sublethal dose of honeybee venom develop a type II immune response producing a venom-specific IgE. When rechallenged with a potentially lethal amount of honeybee venom, they survived. In contrast, mice lacking the functional specific IgE or deficient for one or the other chain of the Fcε receptor type I were not protected in this model. The authors concluded that the evidence shows that an IgE-dependent immune responses against venom can enhance survival in mice. This conclusion supports the hypothesis that IgE, which also contributes to allergic disorders, has an important function in the protection of the host against noxious substances such as toxins.

CONCLUSION

The research on toxins is closely linked to the birth of immunology, serotherapy, vaccinology and immunotherapy. Toxin research has also helped to decipher some of the mechanisms of the immune response against pathogens. Toxinology is also associated with the investigation aimed to understand the mechanisms of immunopathology. It is fascinating to see how the research on toxins at the end of the 19th century promoted the understanding of humoral immunity and paved the way to the discovery of antibodies. Toxinology's link to immunology continues to this day. Toxinology may help to decipher why the allergic IgE-mediated response, which for a long time have been considered an immunological error, may in fact have been conserved

through evolution to help the host deal with environmental toxins.

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