THE HOST DEFENSE OF INSECTS: A PARADIGM FOR INNATE IMMUNITY

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by

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Strasbourg, France.

I grew up in Luxembourg after the Second World War. My father (Figure 1) was a high school teacher, who spent his spare time collecting and describing insects in various settings, particularly around brooks and ponds in the countryside. From early age on, I accompanied him, and by and by participated in the identification of various insect species. This led me, with his obvious help, to publish at the age of 17 my first paper on the waterbug (Heteroptera) in Luxembourg. This period, which I dearly remember to this day, generated in me a fascination for insects, which represent the most important group of the present day fauna. It is believed that they make up some 80% of all extant animal species. They play a considerable role in human health by transmitting microbial pathogens that put close to one third of the human population at risk and yearly kill tens of thousands. They also have a strong impact on the economy: on the positive side through pollination, for instance, and on the negative side through the destruction of crops. It is estimated that one third of human crops are destroyed annually by insect pests world-wide.

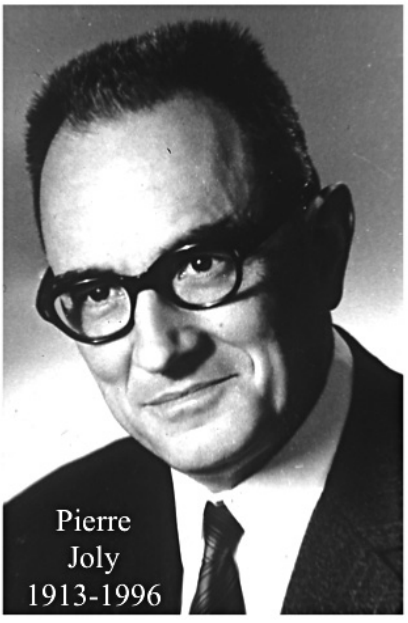
After completing high school in Luxembourg, I left for Strasbourg University where I studied Life Sciences, in particular Zoology and Physiology. After graduation, I was fortunate to be accepted for Ph.D. studies by Professor Pierre Joly, the Chair of the Laboratory of General Biology at the Institute of Zoology (Figure 1). The Joly laboratory was the only group doing experimental research on insect models in Strasbourg. Their studies focused on the endocrine and neuroendocrine control of insect development and reproduction. The laboratory also had a particular interest in phase differentiation in grasshoppers *(Locusta migratoria),* which represented a major plague in Northern and Western Africa at that time. Professor Joly proposed to me the study of antimicrobial defences in grasshoppers. In fact, as he explained, his laboratory had over decades transplanted endocrine organs and even whole brains from one insect to another without ever taking antiseptic precautions, but they had never observed the appearance of opportunistic infections. To Professor Joly, this could only be explained if efficient antimicrobial defences existed in these insects.

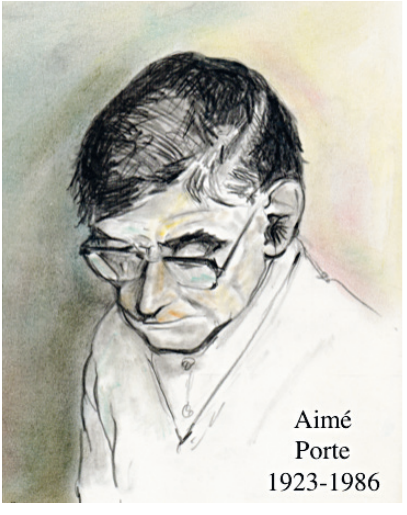
I was very excited by this project. A survey of the existing literature rapidly indicated that very little information was available on antimicrobial reactions

in insects. At the end of the 19th century, Eli Metchnikoff had discovered phagocytosis in starfish larvae and established its role in water-flea antimicrobial defences ("cellular immunity") (Metchnikoff, 1884). Further, interesting studies by André Paillot and Rudolf Glaser in the early 20th century had pointed to the appearance of inducible antimicrobial activities in caterpillars following a microbial challenge ("humoral immunity") (Glaser, 1918; Paillot, 1919, 1933). Episodic investigations in a variety of insect species had analysed melanisation, tissue repair and other defence aspects (reviewed in Jehle, 2009), but in essence no clear-cut picture of these defences had emerged when I started my thesis work.

In the mid-sixties, the Joly laboratory was not equipped for biochemical studies and we relied on experimental biology, classical histology and electron microscopy to address this research topic. My initial investigations confirmed that phagocytosis was an essential arm of grasshopper antimicrobial defences. Injections of low doses of microbes (we used *Bacillus thuringiensis* during the first years of the project) induced a significant protection against subsequent administrations of higher and even lethal doses. I could correlate this induced protection with a strongly upregulated production of blood cells, namely of phagocytes. At that time, I was not able to detect any specificity in this mechanism of induction. The sometimes massive changes in the hemograms (blood cell counts) following experimental infections raised the question of the post-embryonic origin of blood cells in these insects. Hemopoiesis was not well understood in insects at that time, but through the combination of experimental biology (namely severe bleeding) and histology/ultrastructural studies, I eventually identified a well-organised hemopoietic tissue in the vicinity of the dorsal blood vessel in the abdomens of both larval and adult grasshoppers. Dr Aimé Porte (Figure 1), an exceptional cell biologist with a strong medical background, was my direct supervisor during this period and our thorough ultrastructural analysis of the grasshopper hemopoietic tissue revealed some unexpected similarities with hemopoiesis in mammals (Hoffmann et al., 1968, Hoffmann et al., 1970; Hoffmann 1973).

To establish the functional significance of this newly identified juxtacardiac hemopoietic tissue, I went on to submit it to selective X-ray treatment. The results were spectacular and were to orient the studies of the laboratory for many years to come. For one, grasshoppers which had their hemopoietic tissue selectively subjected to X-ray treatment rapidly succumbed to septicemia by opportunistic microbes; sham irradiated grasshoppers did not show a similar phenotype. This result underlined the crucial role of hemopoiesis in antimicrobial defences, namely through the massive production of phagocytes. A second, totally unexpected result was the observation that the endocrine control of moulting was upset. In short, grasshopper larvae undergo five cycles of moulting: it was understood that these cycles were dependent on a gland, referred to as prothoracic gland, and that this gland released the moulting hormone ecdysone (*ecdysis* meaning moult in Greek) at a precise moment within each larval instar (referred to as a "critical period"). When the X-ray treatment of the hemopoietic tissue was performed before this





critical period within any instar, the following moults were blocked; if the treatment was performed after the critical period, the next moults was not blocked but subsequent moults were still suppressed. The moulting hormone ecdysone, a 27-carbon polar steroid, had been isolated and characterized by Professor Peter Karlson in Germany from the butterfly *Bombyx* (Butenandt and Karlson, 1954; Karlson et al., 1965). Professor Joly was particularly interested in the results of the X-ray treatment. He arranged for me to spend some time in the Karlson laboratory to relate our observations with the studies on the synthesis, blood transport and metabolism of ecdysone which were being undertaken by Professor Karlson and Dr Jan Koolman at the Institute of Chemical Physiology of the University of Marburg – a 4 hour drive from Strasbourg. Meanwhile, he suggested, the antimicrobial defence studies in grasshoppers should be continued in Strasbourg by my first doctoral student (who actually was my wife Danièle whom I had met in the Joly laboratory some time before). For years, our laboratory carried the official CNRS denomination "Endocrinology and Immunology of Insects" and was renamed only in 1994

"Immune Response and Development in Insects". I will not recount here the fruitful years that we spent with our colleagues from Marburg investigating the biosynthesis, metabolism, and mode of action of the steroid hormone ecdysone in grasshoppers. These studies became a hallmark of our laboratory for many years, during which we collaborated with the Department of Organic Chemistry in Strasbourg, and specifically with Professor Guy Ourisson and Dr Luu Bang. Thanks mainly to the enthusiastic involvement of Marie Lagueux and Charles Hetru, the "Ecdysone years" allowed our group to mature in the fields of biochemistry and analytical chemistry, to invest in appropriate equipment and to recruit talented scientists. Eventually, we were in excellent condition when we concentrated on the immune studies in flies from the mid-80s on.

In spite of strenuous efforts we were not able in the 70s and 80s to get hold of any significant inducible antimicrobial substances in challenged grasshoppers, beyond that of lysozyme. Professor Hans Boman, from Stockholm, was a member of the Ph.D. defence committee of Danièle Hoffmann (1978) and he proposed that she join his laboratory for a postdoctoral period. Right at that time, Boman and his associates were about to identify the inducible antimicrobial peptide cecropin from challenged pupae of the moth *Hyalophora cecropia* (Steiner et al, 1981). Danièle joined the Boman laboratory in 1979 to work with Dan Hultmark on another lepidopteran insect, *Galleria mellonella* (D. Hoffmann et al, 1981). Once Danièle was back from Stockholm, she developed a severe allergy to the dust present on the wings (elytra) of grasshoppers. There were no real perspectives that we could use *Locusta* for genetic studies and we decided that we would shift our studies on insect antimicrobial defences to dipteran species and abandon grasshoppers. Our objectives were to eventually turn to the genetically tractable *Drosophila melanogaster* model. In the early 80s the methodologies available to us still prevented the direct characterisation of inducible antimicrobial substances from small organisms, like fruit flies. We therefore chose the large fly *Phormia terranovae*, which could be mass-raised in our laboratory and which provided ample amounts of blood for biochemical analysis. The project was to subsequently characterise inducible antimicrobial substances in the fruit fly through homology cloning based on the peptide sequences from the molecules identified in large flies. In retrospect, this was an excellent decision as we now know that grasshoppers do not rely for their antimicrobial defences on the massive secretion of antibacterial peptides into their blood, in contrast to flies, as we shall see below. By that time, the number of persons in our laboratory working on antimicrobial defences had significantly increased. In addition to Danièle, we now had Jean-Luc Dimarcq, Daniel Zachary and Jean-Marc Reichhart working on this topic. The members working on the endocrine aspects (steroids, neurohormones) included Marie Lagueux, Christine Kappler, Charles Hetru, Marie Meister and Maurice Charlet.

Our efforts directed at identifying inducible antimicrobial peptides in *Phormia* eventually led in the late 80s to the characterisation of a glycine-

rich 82-residue polypeptide which we named Diptericin (from Diptera, two-winged insects) (Dimarcq et al, 1988). It had taken 100 ml of blood from challenged larvae to generate this sequence, indeed a formidable task, which the spectacular progress of physico-chemical methods in the following years has fortunately made obsolete. We cloned the corresponding gene and followed its expression pattern in flies challenged by an injection of a mix of bacteria (Reichhart et al, 1989). Activity spectra of purified Diptericin showed that it was particularly active against Gram-negative bacteria. Importantly for our future studies, we were able to clone a *Diptericin* homologue in *Drosophila* in 1990 (Wicker et al, 1990). From 1990 on, we felt confident that we were able to directly identify inducible antimicrobial substances from challenged fruit flies (Figure 2), in spite of their small size. Through the intense work of Charles Hetru, Jean-Luc Dimarcq, Philippe Bulet and others in the group,

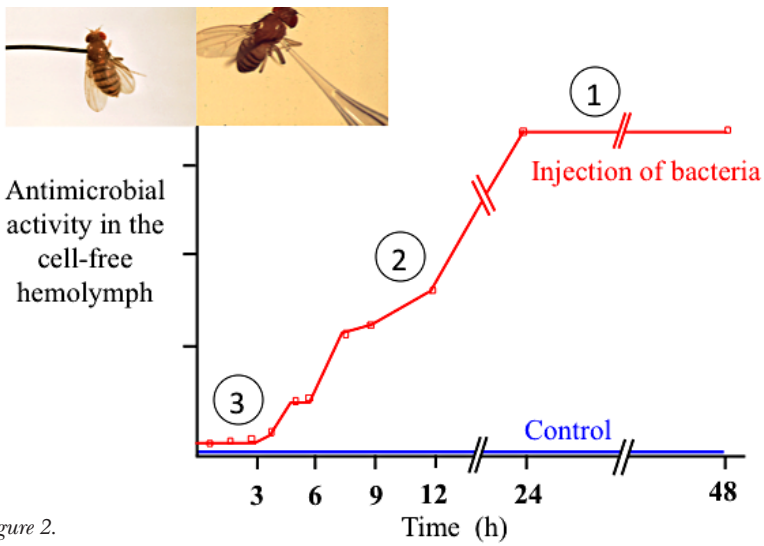


Figure 2.

we actually identified several antimicrobial peptides (Figure 3), namely the disulphide-bridged Defensin (Dimarcq et al. 1994), whereas our colleagues in the Boman laboratory cloned genes encoding *Drosophila* homologues of the linear polypeptides Cecropins and Attacins, which they had initially identified in the *Cecropia* moth (Kylsten et al. 1990, Åsling et al. 1995). Altogether, there was now evidence that the fruit fly fat body, an equivalent of the mammalian liver, produces several families of potent antibacterial peptides, with distinct and sometimes overlapping activity spectra against either Gram-positive or Gram-negative bacteria. The corresponding genes are transcribed rapidly after microbial challenge and after translation of the corresponding mRNAs, the prepropeptides are matured and the mature peptides are secreted into the blood of the fly at remarkably high concentrations, where they oppose invading microorganisms via membrane-disruptive mechanisms (the mode of action is reviewed in Shai, 2002).

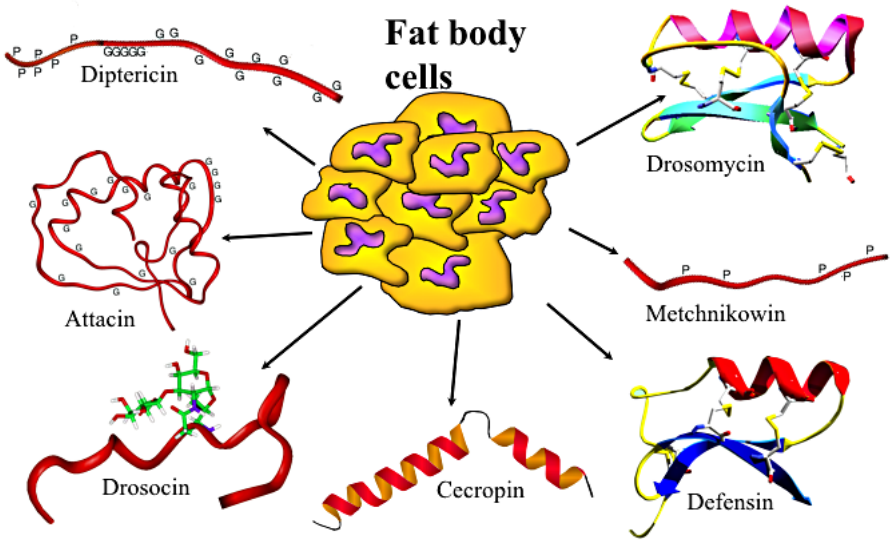


Figure 3.

As mentioned above (Figure 2), a paramount feature of the antimicrobial peptides of *Drosophila* is the rapid inducibility of their expression following challenge. This rapid induction of a potent antimicrobial activity illustrated immediately to us the potentials of this experimentally easily amenable system for a molecular genetic analysis of the immune induction of the antimicrobial defences in *Drosophila*. Further, as described below, once the genes encoding some of the antimicrobial peptides were cloned, it became apparent that their promoters contained sequences similar to mammalian NF-κB binding sites in immune response genes (Sen and Baltimore, 1986). I had read recently papers from Professor Charles Janeway at Yale University (Janeway, 1989) and from Professor Alan Ezekowitz (Sastry et al., 1991) at Harvard and, in June 1992, I visited their laboratories and presented our data on the inducibility of antimicrobial peptides during the host defence of *Drosophila* and the presence of NF-κB binding sites in the promoter of the *Diptericin* gene. At that time we knew that these sites were mandatory for the immune-inducibility of this gene (see below). Both Janeway and Ezekowitz felt attracted by the fly model, and we decided that we would embark on a collaboration, with the aim of understanding and comparing sensing and signalling in mice and flies during infections. In 1993, Charlie Janeway, Shunji Natori from Tokyo University (working on the fly model *Sarcophaga peregrina*) and I organised a conference at Versailles near Paris on the topic of innate immunity (Figure 4). In retrospect, this may have been the first international meeting on innate immunity, a term still not in universal use at that time. We decided with Charlie Janeway, Shunji Natori and Alan Ezekowitz, after the Versailles meeting to submit a formal grant application to the Human Frontiers in Science Programme (HFSP), whose General Office is based in Strasbourg. Fotis Kafatos, who had just been named Director General of the European Molecular Biology Laboratory at Heidelberg and had started

Figure 4.

working on antiparasitic reactions in mosquitoes, also joined our collaborative project. On behalf of our five laboratories, I submitted the project to HFSP. Much to our delight it was accepted in 1995 and generously funded. For four years, the five groups met regularly to exchange and discuss results and ideas. It is through these meetings that I became acutely aware of the problems raised by the interactions between innate immunity and adaptive immunity in mammals (*Drosophila* lacks adaptive immunity) and that our colleagues followed the developments of our studies on activation of immune defences in challenged flies. After termination of this grant in 1998, Alan Ezekowitz coordinated a follow-up project of our groups, which was funded by the NIH. A common article in *Science*, on the Phylogenetic Perspectives of Innate Immunity, published in 1999, is a testimony to our fruitful and happy interactions during these pioneering years (Hoffmann et al., 1999).

Let me turn back to the period of the Versailles meeting. We had by that time, as already mentioned, found the presence of NF-κB binding sites in the *Diptericin* promoter (Reichhart et al., 1992) and shown by site-directed mutagenesis that they were mandatory for inducibility of this gene by microbial challenge (Kappler et al., 1993; Meister et al., 1994). In parallel, Ylva Engström in Stockholm and her colleagues, had obtained similar results for the *Cecropin* gene in flies (Engström et al., 1993) (of note, the presence of NF-κB binding sites had been first reported in the *Attacin* gene of *Hyalophora cecropia* by Ingrid Faye (Sun et al., 1991). NF-κB was known to be a transcriptional activator responsible for the challenge-induced expression of many immune and stress proteins in mammals (Sen and Baltimore, 1986), and the *Drosophila* genome also contained at least one member of this family, namely the *Dorsal* gene (Steward, 1987) (Figure 5). In *Drosophila,* the groundbreaking work of Christiane Nüsslein-Volhard (Nüsslein-Volhard et al., 1980) had shown by unbiased mutagenesis experiments that the *Dorsal* gene was involved in dorso-ventral patterning in the early embryo (Figure 6). Further mutagenesis screens by her laboratory and that of Trudi Schupbach identified a cascade of genes that direct the nuclear translocation of the Dorsal protein, which subsequently controls the expression of developmental genes (reviewed in Belvin and Anderson, 1996). Kathryn Anderson worked out the

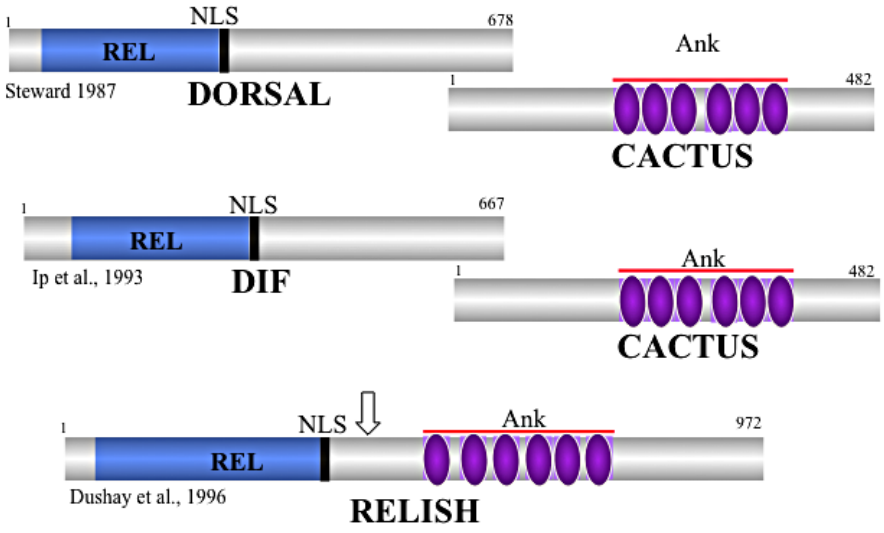


Figure 5.

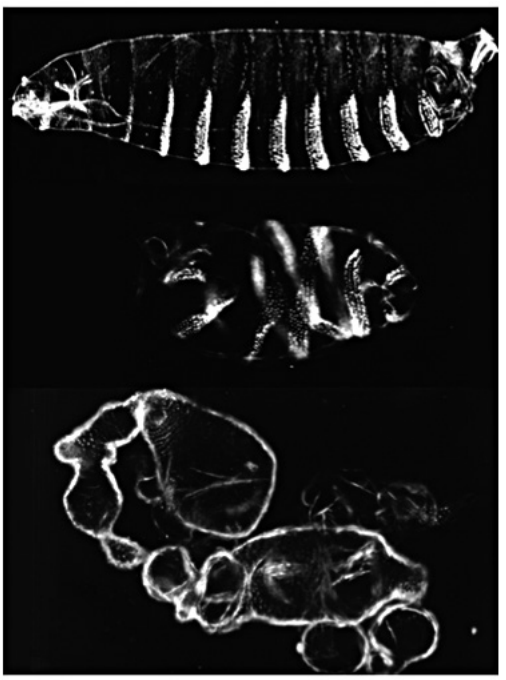


Figure 6.

role of a key gene in this signalling pathway, namely *Toll* (Anderson et al., 1985a, 1985b), which had originally been isolated in the zygotic screens that won the Nobel Prize for Christiane Nüsslein-Volhard and Eric Wieschaus. This cascade involves several extracellular serine proteases and culminates in the cleavage of the cysteine-knot polypeptide Spätzle. Cleaved Spätzle then activates the type I transmembrane receptor Toll and triggers an intracellular series of events which result in the phosphorylation of the inhibitor protein

Cactus, inducing its dissociation from Dorsal (also reviewed in Belvin and Anderson, 1996). Although these genes had initially been characterized by Nüsslein-Volhard and her colleagues because of their maternally expressed phenotypes, we confirmed that they were also expressed in larvae and adults, including males, and notably, that their expression was upregulated by microbial challenge (Reichhart et al., 1993). We also showed, using appropriate antibodies in collaboration with Ruth Steward, that an immune challenge induces the nuclear translocation of the Dorsal protein in fat body cells (Reichhart et al., 1993; Lemaitre et al., 1995a). Further, we found that a microbial challenge induced the appearance in these cells of a protein complex binding to the NF-κB sites of the *Diptericin* gene, that this complex was competed off by excess probe and supershifted by an antibody specific to Dorsal (Reichhart et al, 1993; Kappler et al., 1993; Georgel et al., 1993).

For a short moment, I believed that we were close to solving the problem of the control of antimicrobial gene expression. But then, to my utter dismay, we found that in loss-of-function mutants for *Dorsal*, the *Diptericin* gene was induced like in wild-type flies (Reichhart et al., 1993; see also Lemaitre et al., 1995b). We were of course aware at that time of the report by the Levine and Engström laboratories of the existence of a second NF-κB family member, namely DIF (for Dorsal related immunity factor) (Ip et al., 1993), but *Dif* mutants would only be generated years later (Meng et al., 1999; Rutschmann et al., 2000a). Several mutants with potentially abnormal defence reactions were available in the community at that time, namely *mbn-2* (Gateff, 1978) and *Black cells* (Rizki et al., 1980), which was considered to have a block in the phenol-oxidase cascade that normally leads to the formation of melanin and is activated by injury. Bruno Lemaitre, a *Drosophila* geneticist, who had joined our group in the fall of 1992, shortly after his Ph.D. defence, and who was to become a driving force in the *Toll* saga in the laboratory, analysed the expression of *Diptericin* in *Black cells* mutants and observed that the induction of this antibacterial peptide gene was blocked. The *Black cells* mutation had been created by ethyl methane sulphonate (EMS) mutagenesis by Grell in 1969. I had shared our preliminary and unpublished results on the effect of the *Black Cells* mutation with Professor Michael Levine when I visited his group in San Diego in 1993. Given that the role of proteolytic cascades in immune defences had been highlighted by the studies of Professor Sadaaki Iwanaga at Fukuoka University on immune defences in the horse-shoe crab (reviewed in Iwanaga, 2002), and that the *Black Cells* mutation was considered to affect such a cascade, it was tempting to speculate on the potential similarities in the induction of the immune responses in the two systems. The Levine laboratory had had access to a *Black Cells* mutant line, and they informed us that they could not reproduce our results on the lack of inducibility of the antibacterial peptide with that line. Given that both groups were certain of their data, the only valid explanation that came to our minds was that our EMS-mutated line carried a second-site recessive mutation. This mutation would be responsible for the failure of *Diptericin* induction and be unrelated to the phenol-oxidase cascade, which had led to the initial isolation of the

mutant strain. Bruno Lemaitre, Elisabeth Kromer and Marie Meister set out to isolate the mutant gene and found that it mapped one centiMorgan away from the *Black Cells* locus (Lemaitre et al., 1995b). We called this mutation *immune deficiency* (imd). This first allele was a weak hypomorph. Null alleles of the *shadok* class of alleles that would be generated some years later by Dominique Ferrandon display a much stronger sensitivity to immune challenges (Gottar et al., 2002). The *imd* locus resides in a genetically poorly characterised region. Its identification entailed considerable mapping efforts, with the generation of an overlapping set of deletions. The region corresponding to the relevant deletions was sequenced in 1999 by our collaborators of Exelixis Inc (San Francisco), as the *Drosophila* genome sequence was not yet available. The identification of the correct gene in the 30 kilobases region was facilitated by the detection of three mutations in the most likely candidate gene in the three alleles of *imd* that were available at this time. With Silvia Naitza, Philippe Georgel and other colleagues from our laboratory and from Exelixis Inc. we found that *imd* encoded a death domain protein that shared significant similarities with that of mammalian RIP (TNF-receptor interacting protein) (Georgel et al., 2001).

In 1995, then, we understood that the immune induction of the antibacterial peptide Diptericin was dependent on the *imd* gene and independent of the Dorsal member of the NF-κB family. Given the biochemical competence of the laboratory, we considered the possibility of purifying the proteins of the complex bound to the NF-κB response elements of the *Diptericin* gene. For this, Christine Kappler and Emma Langley spent more than a year to produce massive quantities of spinner cultures of LPS-treated cells and to purify the nuclear protein extracts by affinity chromatography with multiple NF-κB binding nucleotide sequences. As a result of these efforts, a faint band of a size in the range of the Dorsal (or DIF) protein, and an additional one of higher molecular weight (with hindsight, this might have corresponded to the Relish NF-κB family member, see below) were detected by gel electrophoresis in the pooled protein extracts. These samples were further analysed by mass spectrometry after an "in-gel trypsic digestion" by Andrej Shevchenko from the group of Mathias Mann at the EMBL in Heidelberg. Unfortunately, the quantities of the purified proteins were below the resolution limits of mass spectrometry at that time and in the absence of the genome sequence of *Drosophila*, the small fragments of protein that were sequenced were of no help.

This was obviously a delicate moment in our efforts. Fortunately, a breakthrough then occurred in the laboratory with the discovery of the antifungal peptide Drosomycin. Let me briefly recount how this came about. The biochemists in the group had so far isolated antibacterial peptides on the basis of growth inhibition assays, a classical procedure used by the Boman laboratory to isolate cecropins and attacins in the early 80s. In 1992 we decided with Jean-Luc Dimarcq, Philippe Bulet and Charles Hetru to engage in a massive experiment of comparing chromatographic profiles from several thousands of individually challenged flies with those of as many naive flies,

independently of their potential antimicrobial effects. One objective was to view the immune response in *Drosophila* beyond the simple induction of antimicrobial peptides, and to isolate other types of immune response polypeptides. In pooled extracts of these flies, we noted the appearance, following challenge, of a major absorption peak. Upon sequencing of the peptide contained within this peak, Jean-Luc Dimarcq, Philippe Bulet and Charles Hetru identified a 5-kDa molecule with four disulphide bridges. The inducible peptide was inactive, however, against all the bacterial strains available to us. It was not an inhibitor of circulating proteases either– an idea which we had favoured at a given moment, given the structure of the peptide. At a session on antimicrobial peptides at the 1993 meeting of the Federation of European Biochemical Societies in Stockholm, to which Hans Boman had invited me, I heard the presentation by Professor Willem Broeckaert from Leuwen University in Belgium on plant antimicrobial peptides and was struck by the similarity between one of the peptides from *Raphanus sativus* (Rs-Antifungal Peptide 1) and our novel 5-kDa inducible peptide from challenged fruit flies. We rapidly started collaboration between our two laboratories and in 1994 we showed that the fly peptide was potently antifungal against some filamentous fungi, hence the name "Drosomycin" proposed in our joint paper (Fehlbaum et al., 1994).

At this point, things began to come together. The *Drosomycin* gene was rapidly cloned by Jean-Marc Reichhart and Lydia Michaut. Bruno Lemaitre then added *Drosomycin* to the set of probes for RNA blots to study the expression of this and the other inducible antimicrobial peptide genes in wild-type and mutant fly lines. The data which he generated were compelling: the *Drosomycin* gene was perfectly inducible by immune challenge (bacterial mix) in *imd* mutants, but was clearly NOT induced in Toll pathway mutants by the same challenge. Particularly striking were the results obtained with *Cactus*-deficient flies, in which the *Drosomycin* gene was strongly expressed in the absence of infection, but not the *Diptericin* gene (Figure 7). Bruno Lemaitre went on to probe the immune induction of the characterised antimicrobial

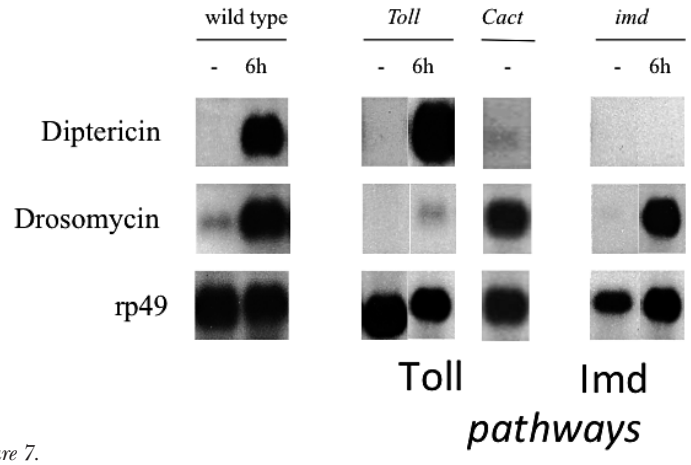


Figure 7.

peptide genes in all the available mutants affecting the dorsoventral regulatory cascade. These studies were published in *Cell* in 1996 (Lemaitre et al., 1996). They demonstrated that: (1) essential components of the dorsoventral pathway, namely the *Spätzle/Toll/Cactus* gene cassette, control expression of the gene encoding the antifungal peptide Drosomycin; (2) two distinct pathways control the expression of the antimicrobial peptide genes (Toll controlling *Drosomycin,* IMD driving *Diptericin* and *Drosocin* – possibly a combined action of both pathways on *Cecropin*, *Attacin*, and *Defensin* expression); (3) the induction of all antimicrobial peptide genes is impaired in double mutant flies for the *Toll* and *imd* genes, which excludes the existence of additional pathways for this particular aspect of the immune defence; (4) mutations which affect the synthesis of antimicrobial peptide genes dramatically lower the resistance of flies to infection. More specifically to this point: the collaboration with the Broeckaert laboratory had led to the introduction of fungal strains into our laboratory (up to that stage, our studies had focused on antibacterial defences). Bruno Lemaitre developed comparative survival tests to bacterial and fungal infections in mutant backgrounds and showed that the Toll pathway essentially protects against fungal infections, (and, as later shown by Dominique Ferrandon and Julien Royet, also against some Gram-positive bacterial infections; Michel et al., 2001; Rutschmann et al., 2002), whereas the *imd* gene is mostly involved in fighting Gram-negative infections (Figure 8) (Lemaitre et al., 19956b; see also below, Lemaitre et al., 1997)

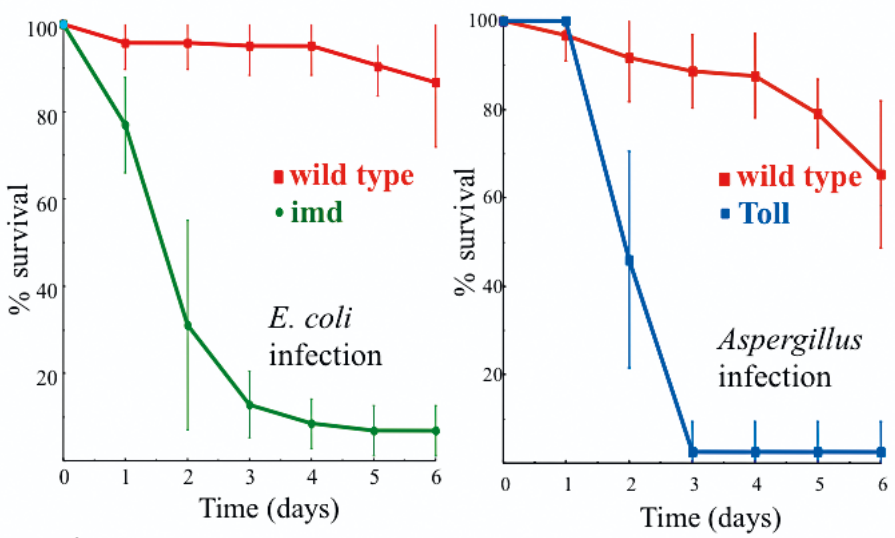


Figure 8.

Studies later performed with microarray analysis (De Gregorio et al., 2001; Irving et al., 2001; De Gregorio et al., 2002; Boutros et al., 2002) indicated that the Toll pathway and the IMD pathway (which had been largely characterised by that time, see below) each control the expression of hundreds of genes, with some overlap (De Gregorio et al., 2002).

The results summarised above were obtained in an ebullient context where several laboratories were searching for the receptors of innate immune cells that would be capable of recognising conserved microbial cell wall components ("Pattern Recognition Receptors", Janeway, 1989) and, in response, activate NF-κB to direct the synthesis of immune-response genes. In the case of mammals, it was proposed that this would not only lead to the expression of innate defence genes but would also stimulate adaptive immune responses (Janeway, 1989). It had been known for some time that in mammals, lectin-like molecules (Fraser et al., 1998) and scavenger receptors (Krieger, 1997) were able to recognise the molecular patterns that adorn bacteria, but the link between recognition and signalling had remained elusive. The Toll transmembrane receptor, initially cloned in the context of the dorsoventral patterning system (Hashimoto et al., 1988), contains an extracellular leucine-rich repeat domain, evocative of that of the LPS-binding protein CD14 (GPI-anchored and hence incapable of signalling to NF-κB) (Wright et al.,1990). Its intracytoplasmic domain is highly similar to the intracellular signalling domain of the IL-1 receptor (referred to as TIR domain, for Toll-Interleukin Receptor), an established activator of NF-κB (Gay and Keith, 1991; Schneider et al., 1991; Rosetto et al., 1995). I first presented our data on the immune function of the Toll pathway in flies at our regular HFSP meeting held in June 1996 in Annisquam and hosted by Charlie Janeway. I vividly remember the interest and receptiveness they generated with our colleagues. Although our laboratory in Strasbourg never worked directly on mammalian models, we were delighted to observe over the following years the relevance that our work on innate immunity in *Drosophila* took across the phylogenetic spectrum. To this day, we remain in close contact with the community working on mammalian models.

The sequencing of the *Drosophila* genome revealed in 2000 that this species has nine genes encoding Toll receptors (Adams et al., 2000). Jean-Luc Imler in our group investigated the potential roles of the eight other Toll receptors and of DmelMyD88 in the control of antimicrobial peptide gene expression (Tauszig et al., 2000; Tauszig-Delamasure et al., 2002). In spite of an in-depth analysis, we and others have been unable so far to demonstrate that, with the marked exception of Toll, any of the other Tolls is involved in an NF-κB driven expression of these peptides during systemic infections (Ooi et al., 2002; Yagi et al., 2010; Narbonne-Reveau et al., 2011; Akhouayri et al., 2011).

In spite of the progress described above, the question remained as to how Toll is activated during infection, all the more so when it became clear that the Toll pathway responds both to fungal and Gram-positive bacterial infection (see above). The spectacular results on the identities of the ligands activating the various mammalian Toll-like Receptors (reviewed in Kawai and Akira, 2011) continued to bring up the questions in meetings whether a similar situation might not prevail, or at least occur, in insects. In 1999, Elena Levashina, Emma Langley, Jean-Marc Reichhart and colleagues, when analysing loss-of-function mutants for a gene encoding an inhibitor of serine proteases, the serpin Necrotic, found that a proteolytic cascade leads to the

cleavage of Spätzle in the blood of adult flies and can activate Toll (Levashina et al., 1999). This cascade is different from that which cleaves Spätzle during embryogenesis, as already noted by Bruno Lemaitre (Lemaitre et al., 1996). Biochemical analysis by Nick Gay (Cambridge) and Jean-Luc Imler in the laboratory further showed that proteolytic processing of Spätzle allows its binding with high affinity to the Toll extracellular domain, thus triggering the intracellular signalling cascade (Weber et al., 2003). It is of interest here to mention that Spätzle is a member of a family of neurotrophin-like proteins that play essential roles in the development of the nervous system (Parker et al., 2001; Zhu et al., 2008). Of the six members of this family (now referred to as DNT- *Drosophila* neurotrophins) only one member, *i.e.*, Spätzle, has conclusively been shown to date to activate an immune response – in addition to its developmental role. Why evolution has selected one Toll member out of a family of nine, and one Spätzle/DNT out of a family of six to play an immune function, is one of the intriguing questions in this field.

Experiments performed by Bruno Lemaitre in 1997 had indicated that the *Drosophila* innate immune system is able to discriminate between various classes of invading microbes (Lemaitre et al., 1997). Expression data of various antimicrobial peptide genes in response to various microbial challenges indeed showed that the IMD pathway is strongly induced by Gram-negative bacteria and Gram-positive bacilli (which contain a peptidoglycan in their envelope which is distinct from that of other Gram-positive bacteria, see below). In contrast, the Toll pathway is stimulated preferentially by fungi and Gram-positive bacteria, and to a lesser extent by Gram-negative bacteria. These findings suggested the existence of receptors able to discriminate between these various classes of microorganisms. When we finally addressed this question around 2000, the genome of *Drosophila* had been sequenced and many candidate genes attracted interest. However, the answers to the questions regarding the identities of the receptors for microbial ligands with potential to activate NF-κB, came from unbiased genetic approaches, which had been initiated in our laboratory by Dominique Ferrandon in 1995 on the second chromosome and by Louisa Wu and Kathryn Anderson (Wu et al., 2001) on the third chromosome. In essence, the Ferrandon screen in Strasbourg was initially based on large-scale EMS mutagenesis using transgenic flies expressing distinct reporter genes as read-outs for each pathway (Jung et al., 2001). These screens and later, additional ones performed by the Lemaitre (by then at Gif-sur-Yvette) (X chromosome) and Schneider (Stanford) (P-element insertions) laboratories identified several genes in the IMD pathway, as described below. The Ferrandon screen as based on the use of a dual reporter system, also allowed identifying genes involved in the Toll pathway, for instance the only known *Dif* point mutants (Rutschmann et al., 2000a). By applying the unbiased reporter screening approach on the first chromosome, Julien Royet isolated in 2001 the first mutant fly line in which the Toll pathway was not activated by Gram-positive bacteria, but was still responsive to fungal infection (Michel et al., 2001). Upon cloning, the mutated gene turned out to be Peptidoglycan Recognition Protein-SA, a member of

a protein family previously characterised in Lepidoptera (Kang et al. 1998; Ochiai et al., 1999). Genomic data mining and expression profile studies by Dan Hultmark, Håkan Steiner and colleagues later established that *Drosophila* encodes 13 members of the PGRP family of proteins (Werner et al., 2000). They are either circulating, intracellular or transmembrane proteins (Figure 9) and have in common a domain (called PGRP homology domain) that is derived from an evolutionary ancient amidase enzyme, already present in some bacteriophages. Remarkably, the amidase function is conserved in 7 out of the 13 fly PGRPs whereas the others have lost their catalytic function and today serve as recognition PGRPs (reviewed in Royet and Dziarski, 2007). Shortly after the identification of PGRP-SA, Dominique Ferrandon and Julien Royet demonstrated that one of the transmembrane PGRP family members, that is PGRP-LC, is required for activation of the IMD pathway and resistance to Gram-negative bacteria (Gottar et al., 2002). The role of PGRP-LC was independently established, at the same time, in the laboratories of Kathryn Anderson (Choe et al., 2002) and Alan Ezekowitz (Rämet et al., 2002). Biochemical and structural studies performed by several groups subsequently established that the PGRP homology domain has a well-defined groove to which peptidoglycan binds for enzymatic cleavage, or for recognition (Chang et al., 2004; Chang et al., 2006; Lim et al., 2006). Importantly, depending on the PGRP member, the groove can selectively bind a Lysine-type peptidoglycan which is predominant in most Gram-positive bacteria or alternatively, a diaminopimelic acid form of peptidoglycan which is typical for Gram-negative bacteria (these amino acids are in position 3 of the stem peptides linking the two glycan chains of peptidoglycan (Leulier et al., 2003; Kaneko al., 2004; see also the reviews of Royet and Dziarski, 2007 and Royet et al., 2011 for PGRPs in general).

The detection of microorganisms is, however, not restricted to the PGRP

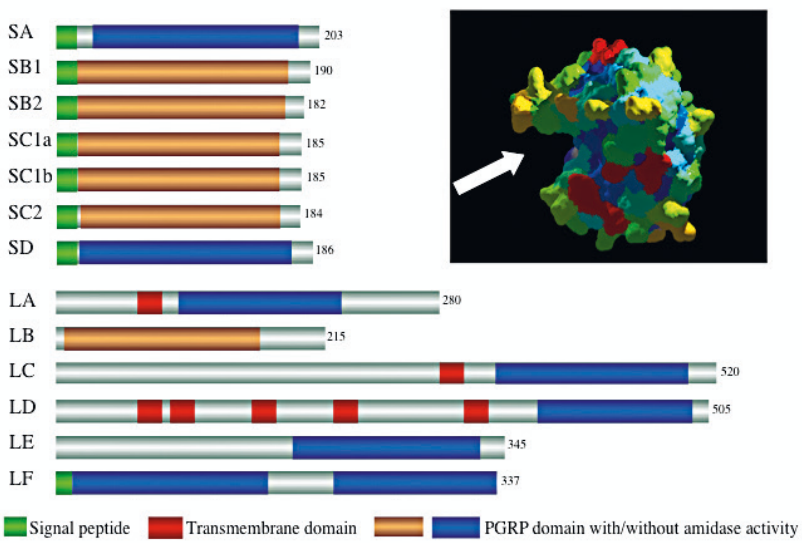


Figure 9.

family and involves a second family, the GNBP/ßGRP family. Dominique Ferrandon in the laboratory demonstrated that a circulating protein that interacts with fungal ß-(1,3)-glucan triggers the proteolytic cascade leading to the cleavage of Spätzle and the activation of the Toll pathway (Figure 10) (Gottar et al., 2006; Mishima et al., 2009). This protein, GNBP3, is a member of a small family of proteins initially characterised in the silkworm Bombyx mori. The first member was reported to bind Gram-negative bacteria, hence the name GNBP for Gram-negative binding protein (Lee et al., 1996). The GNBP3 orthologue of *B.mori* had originally been identified in the Ashida laboratory for its ability to bind to ß-(1,3)-glucans and trigger the phenol-oxidase cascade (Ochiai et al., 1988; see also Ochiai et al., 2000). Of note, another Drosophila member of the family, GNBP1, appears to function as a coreceptor of PGRP-SA for the detection of Lys-type peptidoglycan Gram-positive bacteria, both in Diptera and Coleoptera (Gobert et al., 2003; Filipe et al., 2005; Wang et al., 2006a; Park et al., 2007).

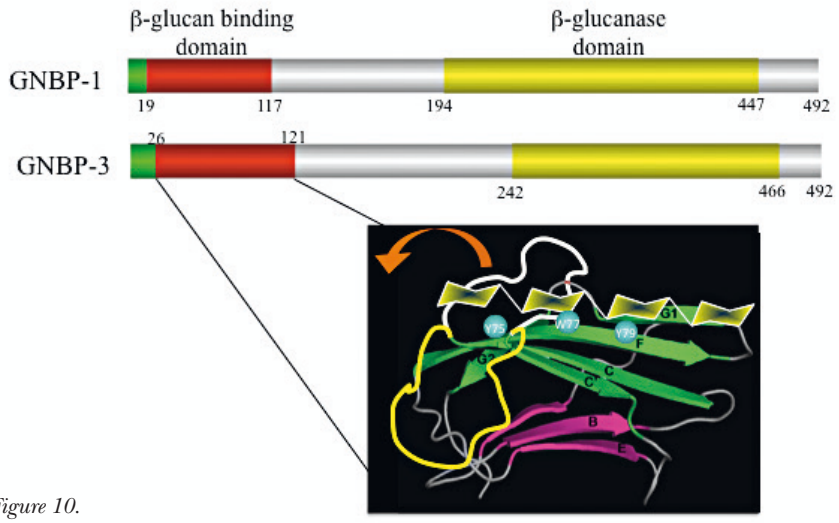


Figure 10.

Figure 11 summarizes our view as it had emerged in the early 2000s. Two essential microbial inducers, *i.e.* Lys-PGN and ß-Glucan trigger the Toll pathway by activating an upstream proteolytic cascade upon recognition by PGRPs or GNBPs respectively. In addition, as shown by Dominique Ferrandon and Jean-Marc Reichhart, proteases secreted by invading entomopathogenic fungi, or bacteria, interact with a dedicated circulating serine-protease (dubbed *Persephone* by Petros Ligoxygakis, see Ligoxygakis et al., 2002) which also feeds into the proteolytic cascade upstream of Spätzle and Toll (Gottar et al., 2006; El Chamy et al., 2008). Thus the systemic immune response of *Drosophila* is not only activated by "pattern recognitions receptors" (Janeway, 1989) but also by sensing the catalytic activity of microbial virulence factors. The IMD pathway, in turn, is activated via a direct

interaction of DAP-PGN with the transmembrane PGRP-LC. In this case, no evidence exists for a circulating amplification cascade, as opposed to the interaction described above for Toll activation. Studies in several groups have identified most of the players of the proteolytic cascades upstream of Spätzle. The ultimate protease, termed Spätzle Processing Enzyme (SPE), was identified in 2006 by Won-Jae Lee and colleagues (Jang et al., 2006). The molecular mechanisms that lead from binding of ß-glucans to GNBP-3 or of Lys-peptidoglycan to PGRP-SA, to activation of the upstream serine proteases are still under investigation.

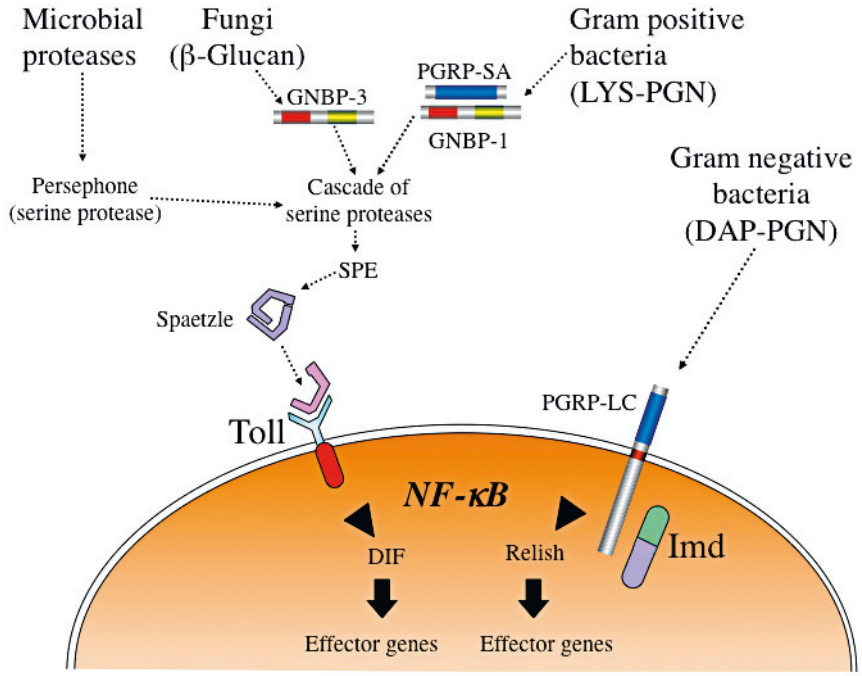


Figure 11.

We and others have devoted many efforts to decipher the intracellular pathways which lead to the expression of antimicrobial peptide genes downstream of either the Toll or the PGRP-LC transmembrane proteins. A simplified picture is presented for both pathways in Figures 12 and 13. Basically, both pathways direct activation of dormant cytoplasmic NF-κB family members. These are in the case of the Toll pathway the Dorsal or DIF proteins in larvae or the sole DIF protein in adults (Meng et al., 1999; Manfruelli et al. 1999; Rutschman et al., 2000a). Both proteins are retained in the cytoplasm by their interactions with the ankyrin-repeat inhibitor protein Cactus. Phosphorylation of Cactus leads to the dissociation from either Dorsal and/or DIF and to subsequent degradation of the inhibitor by the proteasome. Upon the ensuing nuclear translocation, Dorsal or DIF control the expression of hundreds of immune response genes, predominantly but not exclusively, in fat body cells. Prominent among these is the antifungal

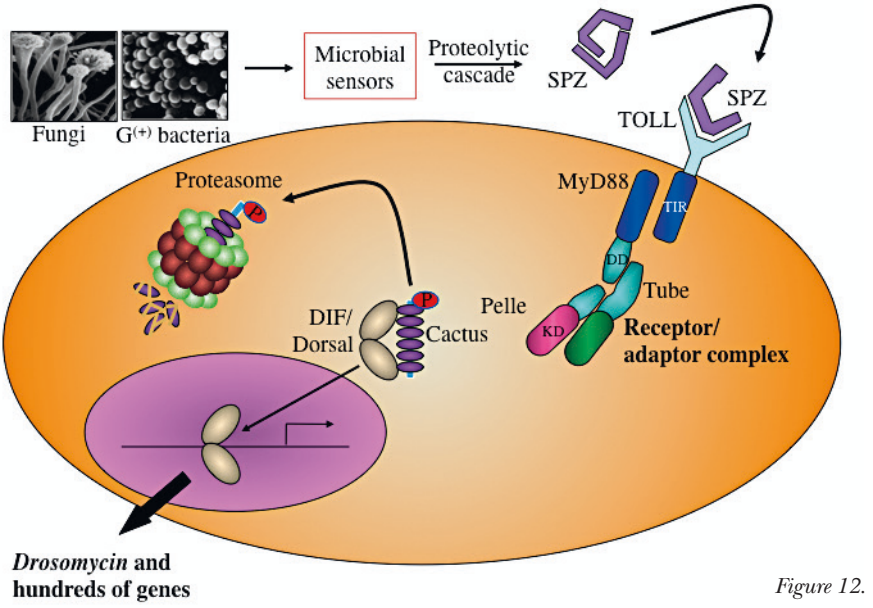


Figure 12.

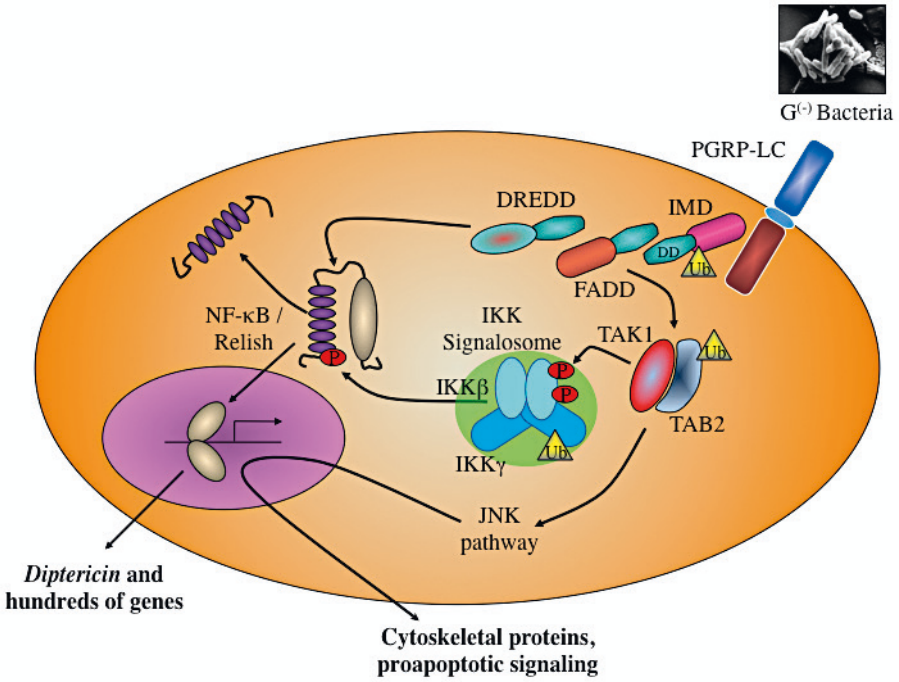


Figure 13.

peptide Drosomycin and a significant number of small (3-9kDa) peptides (Uttenweiler-Joseph et al., 1998), the functions of which have eluded analysis so far. The molecular mechanisms linking the binding of Spätzle to the phosphorylation of Cactus warrant further investigation. Recent studies have proposed that the Spätzle-Toll complex is internalised into endocytic

vesicles (Huang et al., 2010; Lund et al., 2010). The kinase Pelle, which is present in the Toll-receptor-adaptor complex together with MyD88 and the death domain protein Tube, has similarities to mammalian IRAKs (Towb et al., 2009). Pelle does not appear to act as the Cactus-kinase (Grosshans et al., 1994), and this kinase still remains to be identified, in spite of many efforts in the recent past by several laboratories. In the case of the IMD pathway, the NF-κB family member controlling immune gene expression is called Relish. This large-sized protein, identified by Dan Hultmark in 1996, carries at its C-terminus, the inhibitory functions of Cactus (ankyrin repeats) (Dushay et al., 1996). Activation of Relish consequently occurs via a proteolytic cleavage roughly in the middle of the protein (Stöven et al., 2000; Leulier et al., 2000; Stöven et al., 2003; Ertürk-Hasdemir et al., 2009). Cleaved Relish translocates into the nucleus to control expression of antimicrobial peptides. As for the Toll intracellular signalling cascade, the IMD pathway is as yet not completely understood in mechanistic terms. Activation of the PGRP-LC receptor (which comes in three distinct splice isoforms, Werner et al., 2000) by binding to DAP-Peptidoglycan (Leulier et al., 2003) leads to the association of a protein complex involving the adaptor protein IMD (Choe et al., 2005; Kaneko et al., 2006), the caspase-8 homologue DREDD, the homologue of mammalian FADD (Naitza et al., 2002; Leulier et al., 2002) and the inhibitor of Apoptosis DIAP2 (Kleino et al., 2005, Gesellchen et al., 2005). This complex activates the MAP-3 kinase TAK1 (associated with the non catalytic protein TAB 2) via an as yet undefined mechanism (Vidal et al., 2001; Silverman et al., 2003) that in turn phosphorylates both IRD5 (Lu et al., 2001), a fly homologue of mammalian IKKβ (Silverman et al., 2000) and the JUN-kinase pathway (Silverman et al., 2003). The IKK beta homologue IRD5 in turn associates with a homologue of mammalian IKKγ (Kenny) (Rutschman et al., 2000b) leading to phosphorylation of Relish (Silverman et al., 2000). Relish is thought to be cleaved by the Caspase DREDD, followed by the nuclear translocation of the N-terminal, phosphorylated part, and participates in the control of gene expression, and namely of the genes encoding Diptericin and Drosocin (Leulier et al., 2000; Stöven et al., 2000; Stöven et al., 2003; Ertürk-Hasdemir et al., 2009). As is the case for the Toll pathway, the IMD cascade controls the expression not only of antimicrobial peptides, but of many hundreds of other immune genes the functions of which remain mostly poorly understood in the context of the antimicrobial defences. Some evidence suggests that DIAP2 (in conjunction with its associated proteins Ue1va and Ubc13) functions as an E3 ligase that K63-ubiquitinates IMD, the TAB2 protein (associated with TAK1) and the IKKγ homologue Kenny. It is plausible that polyubiquitin chains bring many of the members of this signalling cascade into close proximity, as suggested recently by Neal Silverman (Paquette et al., 2010 and references therein). With Hidehiro Fukuyama in the laboratory, we have recently engaged on a functional analysis of the interactome of the IMD pathway proteins and detected protein-protein interactions between the eleven canonical members of the pathway described so far and a total of more than 300 proteins. Functional characterisation of the

newly-identified genes is currently under way, but RNAi knockdown of many of them affects IMD signalling. Significantly, half of the proteins yielding a phenotype under these conditions are conserved between flies and mammals (Fukuyama et al. in preparation).

CONCLUDING REMARKS AND PERSPECTIVES

Innate immunity was a relatively neglected field of research twenty years ago. Insects were considered by most immunologists at that time to be too primitive and distant from mammals to represent an interesting model. Indeed, the second half of the 20th century was a time of ground-breaking discoveries on the roles of lymphocytes, the generation of very large repertoires of antigen receptors, clonal expansion and memory cells. Nevertheless, invertebrates, including the extremely large class of insects, represent around 95% of all living species on earth today, and they apparently cope well with invading microbes by solely relying on innate immunity. A primary incentive to start our studies was to unravel the mechanisms of this remarkable resistance. It was the physico-chemical identification of the molecules responsible for the "humoral immunity" observed by Glaser and Paillot, which gave us an opening into the field, once these methods had been introduced into our laboratory. The initial identification of the linear antimicrobial peptide cecropin by Hans Boman in butterflies, and our subsequent characterisation of disulphide-bridged inducible peptides in fruit flies, were essential steps that provided us with an easily amenable system to analyse the upstream mechanisms of this defence. It is now understood that all multicellular organisms (animals as well as plants) produce antimicrobial peptides for their defences. Most of these molecules are small-sized, cationic, and membrane-active. Although they show a great diversity during evolution, some are remarkably similar between groups. To give just one example, Mihai Netea and colleagues (Simon et al., 2008) have recently identified an antimicrobial peptide in human skin, which is structurally so close to *Drosophila* Drosomycin that they named it Human Drosomycin Like peptide. Whether this similarity reflects convergent evolution or a common ancestry remains an open question. Antimicrobial peptides are mostly expressed in the various zoological groups on barrier epithelia and in blood cells. In recent insect groups (holometabolous orders) they are in addition secreted into the blood stream to oppose microbes that have succeeded in breaching epithelial barriers (Tzou et al., 2000). We do not yet know how widespread this "systemic response" is among the various zoological groups, in addition to the common "epithelial response".

Antimicrobial peptide genes are mostly expressed under the control of the transcriptional activator NF-κB, which has by now been found in nearly every animal group (an exception is C. *elegans*, which appears to have lost a number of pathways). Strikingly, the intracellular signalling cascades that lead to the activation of NF-κB during immune responses show marked similarities throughout evolution. These similarities are not only structural,

but also functional, as illustrated by the comparison between *Drosophila* and mice. Obviously, much of our information on the gene products in these cascades still relies on data mining of recently sequenced genomes, but as more and more experimental data become available, they tend to confirm the assumption of a high degree of functional conservation in the NF-κB activating cascades during evolution (see for example recent studies on the sea anemone *Nematostella vectensis* (Wolenski et al., 2011) (Figure 14).

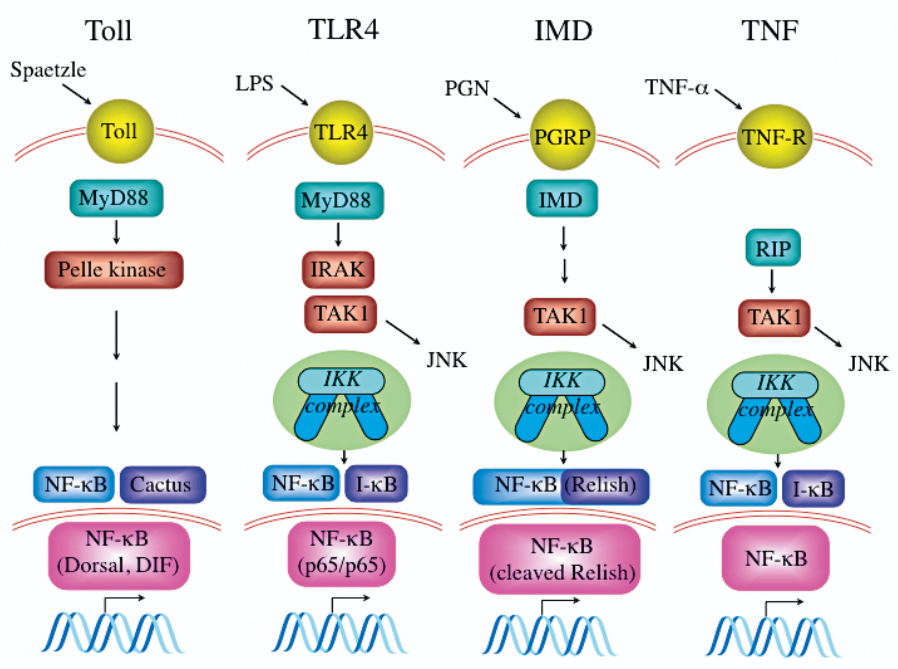


Figure 14.

Finally, we now know that Toll transmembrane receptors are present through evolution from Sponges to Mammals. Tolls are typically associations between extracellular leucine-rich recognition/interaction domains and intracellular TIR domains which often associate with adaptor proteins to signal to immune responsive transactivators (and namely to NF-κB). These domains of Toll show structural variabilities, in particular as regards the extracellular domains, which can have variable numbers and positions of cysteine clusters. Phylogenetic trees illustrate the high degree of complexity in the evolution of the Toll family (Roach et al., 2005). We also have to be aware that in invertebrates at least, Tolls can have developmental as well as defence roles. Whether this duality evolved very early in life history and when/why it was lost, is one of the challenging questions.

Drosophila is generally credited with having significantly contributed to a renewed interest in innate immunity and to our present view of a high conservation of its molecular and cellular mechanisms throughout evolution. Using a metaphor of which Hans Boman was fond, we may ask here as a

final reflexion, which are today the Golden Apples of the Hesperides in the orchard of the studies on fruit fly immune defences?

A first obvious apple relates to the study of antiviral defences. *Drosophila* is under the continuous threat of viral infections and several defence mechanisms have been unravelled over the last decade. Paramount among these is RNA interference (Galiana-Arnoux et al., 2006; Wang et al., 2006b; van Rij et al. 2006), but viral infections also induce the expression of genes encoding polypeptides with poorly understood roles in opposing the development of viruses (see *e.g.,* Deddouche et al., 2008). Viral infections have also been shown to induce cytokine productions which lead to some gene reprogramming via the conserved JAK-STAT pathway (Dostert et al., 2005). A possible role in the fight against viruses of the Toll and IMD pathways discussed above in the context of antibacterial and antifungal defences, has been proposed. Further investigations are required to substantiate this involvement and to define the levels at which genes of these pathways could play a role in the control of the viral load (reviewed in Imler and Hoffmann, 2012; see also references therein).

A second apple in the orchard is the rapidly evolving field of epithelial immune defences in *Drosophila*, and namely of the gut and tracheal epithelia. Significant progress has been made recently in this field and it is now understood that the IMD, and not the Toll, pathway mediates the induction of antimicrobial peptide expression in epithelia (Ferrandon et al., 1998, Tzou et al., 2000; Liehl et al., 2006; Ryu et al., 2006, Nehme et al., 2007). However, its activation is finely regulated at multiple levels by negative feedback loops, especially in the intestinal epithelium so as to tolerate commensal microbiota (Ryu et al., 2008; Lhocine et al., 2008, Ragab et al., 2011). Of note, antimicrobial peptide-mediated responses are complemented in the gut by a potent reactive oxygen species response generated by the dual oxidase enzyme (Ha et al., 2005).

A third apple is the deciphering of *Drosophila* defence reactions in a non-infectious context. As already pointed out in this text, a series of instances have been reported in which the IMD pathway (and probably also the Toll pathway) are activated by endogenous ligands. Our information on the endogenous inducers and their receptors is almost non-existent. There are potential parallels here with mammalian responses to so-called "danger signals" (Gallucci and Matzinger, 2001) and this field of research is bound to attract much interest in the future. Possibly some, if not many, of the genes induced by the IMD and Toll pathways, and whose roles we fail to understand in the present "anti-infectious context", are functionally related to responses to endogenous ligands. It remains to be established whether some of these target genes might be involved in another facet of host defence that has been referred to initially as tolerance by phytopathologists (Schneider and Ayres, 2008) (endurance, homeostasis). Briefly, this relates to the ability of the organism to withstand and repair damage inflicted either by microbial virulence factors or by the host's own immune response. This concept is well illustrated by the proliferation of intestinal stem cells that compensates the

loss of apoptotic enterocytes and thus maintains the homeostasis of the intestinal epithelium (Cronin et al., 2009; Jiang et al., 2009; Buchon et al., 2009).

Insects are the largest group of extant species, as outlined at the beginning of this presentation. Insect immunity can of course not be restricted to that of *Drosophila*. At the present time, studies on immune defences are performed on several species representative of various orders such as Hymenoptera, Coleoptera, and Lepidoptera. Of particular medical interest are investigations on disease transmitting insects such as mosquitoes. To give but one example, the antiparasitic reactions of the vector insect *Anopheles* towards *Plasmodium* species, have been the focus of intense research in a dozen of groups over the last 20 years. Cellular and molecular analyses of these reactions have shed significant insights into the mechanisms that the mosquito uses to oppose invading parasites, and have namely unravelled the roles of complement-like proteins (thioester containing proteins, TEPs) in parasite killing – a process not paralleled in *Drosophila* in which the role of TEPs is still not well understood (Levashina et al., 2001; Blandin et al., 2004; Blandin et al., 2009). Mosquitoes also transmit viruses of great impact on human health, and here the investigations show marked parallels with the antiviral reactions of fruit flies. In particular, RNA interference and inducible responses regulated by the JAK/STAT pathways appear to be shared assets of antiviral host defences in flies and mosquitoes (Fragkoudis et al. 2009).

In ending this presentation, I would simply like to remind the reader that it was not intended to be a classical review, but rather a narrative of our work on innate immunity put into a historical and societal context. Many aspects of *Drosophila* antimicrobial defences are therefore understandably not covered here (for detailed reviews, see e.g. Lemaitre and Hoffmann, 2007; Ferrandon et al., 2007; Aggarwal and Silverman, 2008).

My final message will be to young scientists who feel interested in the studies presented here and who ponder whether or not to engage in this field: our investigations so far have really only touched the tip of the iceberg of invertebrate immunity and many important discoveries lie ahead for the next generation. The methodologies for this type of research have evolved beyond what I could ever have dreamt of, and our current understanding of the evolution of the innate immune system warrants that the results that will be obtained with insect studies will be one way or another relevant throughout the whole phylogenetic spectrum, including humans (Figure 15).

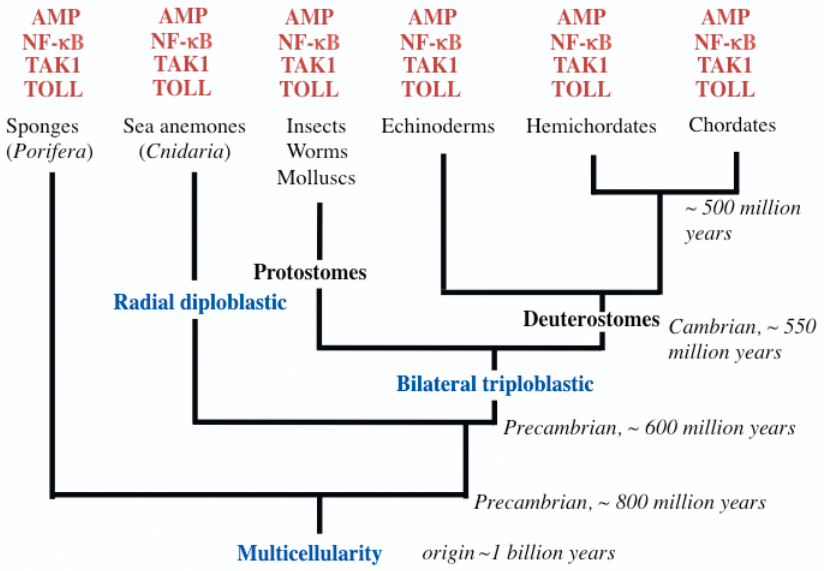


Figure 15.

ACKNOWLEDGMENTS

It goes without saying that the scientific achievements of our laboratory over the many years, as recounted above, are to be credited to a long list of collaborators of high intellectual and human calibre. Many in this list are now Distinguished Class Professors and Directors of Scientific Departments or heads of well-recognised groups in the field, in this and in other countries. I express my deep admiration and warm gratitude to all of them, and in particular to Marie Lagueux, Charles Hetru, Jean-Marc Reichhart, Jean-Luc Dimarcq (Institut Hospitalo-Universitaire, Strasbourg), Marie Meister (Zoological Museum, Strasbourg), Philippe Bulet (Grenoble), Bruno Lemaitre (EPFL, Lausanne), Elena Levashina (Strasbourg/MPI Berlin), Jean-Luc Imler, Dominique Ferrandon, Julien Royet (IBDM Marseille) and Christine Kappler, for their exceptional contributions to our common endeavour. I am further indebted to many colleagues from other institutions in various countries and would like to make a special mention here of Alan Ezekowitz, who has accepted to do a critical reading of the present text. I warmly remember very constructive discussions with Charlie Janeway, Fotis Kafatos and Shunji Natori. Since the early 2000s, we have had a close interaction with Bruce Beutler and Shizuo Akira on the phylogeny of antiviral defences. I would like to warmly thank Charles Hetru, Dominique Ferrandon, Jean-Luc Imler, Jean-Marc Reichhart, Elena Levashina, Jean-Luc Dimarcq and Christine Kappler for their dedicated and constructive help in the preparation of this text and its illustrations. I would also like to acknowledge the significant contributions of many colleagues from other laboratories worldwide to the field of insect immunity, and in particular that of the colleagues

of the Boman group in Stockholm. Our studies were supported by the French National Research Agency CNRS, the University of Strasbourg, the Human Science Frontiers Program, the European Union, the US National Institutes of Health, the Fondation pour la Recherche Médicale and the companies Rhone-Poulenc, Exelixis and EntoMed.

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