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Innate immunity: an overview

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Abstract

Though sometimes portrayed as "new," the science of innate immunity made its start more than 100 years ago. Recent progress has reflected the application of new methods to old problems. In particular, genetic dissection of innate immune pathways has been pursued with great success in model organisms. This has opened the way to an understanding of innate immune sensing. The effector arm of innate immunity has also been tackled, largely though the use of biochemical methods.
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1. Introduction

1.1. Innate immunity: the science and its historical context

Within this chapter, two aspects of innate immunity are discussed: the *afferent* (or *sensing*) arm, and the *efferent* (or *effector*) arm. The former field deals with how we (and all multicellular organisms) perceive infection; the latter field with how we eradicate infection. Each arm of innate immunity may further be divided into *cellular* and *humoral* components. All four topics will be discussed in turn, but to begin with, it is useful to consider afferent and efferent approaches, and their separate historical roots. Efferent and afferent systems were investigated independently, by workers who used entirely different tools.

1.2. Early approaches to sensing

As to the afferent arm, the modern field of innate immunity, with its intense concern over how microbes are sensed, is very much linked to the older field microbial pathogenesis. When Pasteur and Koch independently propounded the germ theory of disease, they declared that microbes caused all infections. The implicit corollary to this decree was the fact that however complex the observable events in an infection might be, they must ultimately be traceable to specific molecular components of microbes. It did not take long for investigators to focus their attention on the microbial "poisons" that gave infections their nefarious character. In the fullness of time, it became clear that the innate immune system itself is what makes microbes poisonous, for in its attempt to combat infection, the host may harm its own tissues and undermine its own survival.

Microbial poisons had been approached in the pre-microbial era. Von Haller and later Magendie (for reviews, see Rietschel and Westphal (1999), Beutler and Rietschel (2003)) had investigated the toxic properties of putrefied organic material (extracts of rotten meat or fish) in the hope of understanding why putrescence had such severe systemic effects when it occurred in vivo, as in a gangrenous limb. These earliest workers did not attempt to purify a toxin, but established its existence. Later, "sepsin," (Von Bergmann, 1868, 1872) was isolated as a molecular derivative of the remnants of beer fermentation, and so may have been derived from yeast, bacteria, or plant residue. It was purportedly crystallized, and was found to be toxic both to dogs and frogs, causing intestinal hemorrhage and fever. Still later (Panum, 1874), the so called "putrid poison" was isolated from infected tissues: a substance that was alcohol insoluble, heat stable, and purified through a series of purification steps that would likely have retained what we now call "endotoxin," or lipopolysaccharide (LPS).

Because of its stability, the ease with which it could be purified, and the potency of its biological effects, endotoxin offered an important inroad into the decipherment of innate immune sensing, as described below. It was Pfeiffer who gave endotoxin its name (Pfeiffer, 1892). Working in the post-microbial era, and starting with pure cultures of *Vibrio cholerae*, he noted that even animals immunized against the bacteria would succumb to its inoculation, though no viable organisms could be retrieved from their bodies afterward. He found that a heat-stable derivative of the microbes could induce fever and shock in guinea pigs, and this principle,

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he reasoned, was responsible for the symptoms observed during an authentic infection. Several decades were to pass before LPS was chemically characterized (Nikaido, 1962; Osborn, 1963; Raetz and Whitfield, 2002), and before it was realized that LPS was a surface component (Osborn et al., 1974; Nikaido et al., 1966) of virtually all Gram-negative (but not Gram-positive) bacteria.

The story of LPS was to some degree mirrored in the later determination that many other components of microbes were toxic to the host. Among these were peptidoglycan, lipoteichoic acid, DNA (which is unmethylated in microbes and therefore structurally distinguishable from host DNA), and dsRNA (produced in abundance by many viruses; even DNA viruses, which commonly produce bi-directional transcripts). Moreover, the toxicity of LPS was seen to be only one of its biological properties. In small doses, LPS exerted a "cross protective" effect, rendering animals resistant to subsequent challenge with virulent pathogens. LPS also had a strong adjuvant effect, and it had long been known that many microbes (most notably mycobacteria) would help to generate a strong response to a co-injected protein antigen (Condie et al., 1955b).

1.2.1. Early approaches to killing

As to the effector arm of innate immunity, Hunter first recognized leukocytes at the site of inflammation in 1774 (Silverstein, 1979). However, the observations of Metchnikoff, who witnessed the engulfment of particulate dyes and fungal spores by "wandering cells" in invertebrates in 1882 and announced his cellular theory of immunity in 1884 (Metchnikov, 1884), must be taken as a starting point in the functional analysis of innate immune cells. By 1890, Massart and Bordet had shown that injured cells release chemical substances that attract phagocytes, and by 1917, time-lapse cinematography had given a more tangible reality to the phenomenon of chemotaxis.

As Metchnikoff made his observations, the science of adaptive immunity had begun to flourish independently, and there was initially much controversy between "cellularists" and "humoralists" as to which system of host defense was the most important in vertebrates, and particularly in humans. To some extent, the conflict was resolved in 1903 by the finding that antibodies fulfill an opsonizing function (Wright et al., 1903), and when Ehrlich and Metchnikoff shared the Nobel Prize in Physiology and Medicine in 1908, the cooperative interactions between innate and adaptive immunity were emphasized by the Royal Swedish Academy (Mörner, 1908). Nonetheless, for a time, the science of innate immunity was eclipsed by the science of adaptive immunity, in part because cell-based defenses were much harder to analyze. Only in 1966, with the demonstration (Holmes et al., 1966) that a discrete mutational defect of effector function could cause a severe immunodeficiency disease, was a genetic foothold gained for the study of innate immunity, and only with the development of modern genomic tools was it possible to understand the central sensing mechanism by which innate immune cells "see" the world.

All the while, there were those who felt that neither cellular nor humeral immune components per se were of prime importance to the inflammatory response. For example, the "rubor" of inflammation was ascribed to a primary vascular effect, promoting the leakage of humeral components into the tissue (Cohnheim, 1873). The idea has some justification, and in light of modern knowledge, it is clear that there is much interplay between leukocytes and the vascular wall. But all this merely begs the question as to just what innate immunity is: where it starts and where it ends.

1.3. The limits of innate immunity

All metazoan organisms have evolved complex immune defense systems, used to repel invasive microbes that would parasitize or kill them. These immune systems are remarkably effective insofar as severe or sustained infections are quite rare. They are imperfect in that serious infections sometimes do occur, and also, in that immune responses may sometimes injure the host. Innate immunity is the most universal, the most rapidly acting, and by some appraisals, the most important type of immunity. Most organisms survive through innate immune mechanisms alone; only in vertebrates have alternative systems for pathogen recognition and elimination, collectively called adaptive immunity, been evolved.

As a topic for investigation, innate immunity is enormously broad, and it is sometimes difficult to decide where the innate immune system ends and the rest of the host begins. In part, this is because innate immune mechanisms are dynamic on an evolutionary time scale. The host population is shaped by the selective pressures that microbes impose, and survives as best it can. On occasion, proteins that clearly evolved to fulfill a task unrelated to host defense are co-opted to kill pathogens. To cite one example of this, it is well known that a coding variant of the human β-globin chain gives rise to HbS (sickle hemoglobin). The mutation responsible for HbS has achieved a high frequency in some human populations because of the selective pressure imposed by *Plasmodium falciparum*: a virulent pathogen that began to infect humans only a few thousand years ago. HbS provides heterozygotes with dominant resistance to *P. falciparum*, because infected erythrocytes sickle and are removed by the spleen. Is hemoglobin, at least as it is modified by this mutation, a component of the innate immune system? Perhaps, but it is not a core element of the innate immune system, and not utilized for any immune purpose by most organisms.

The core elements of the innate immune system were, for the most part, fixed in the tree of life hundreds of millions (in some cases billions) of years ago, and hence, are broadly represented. While it is sometimes considered that innate immunity is "primitive" or "crude" compared to adaptive immunity, the opposite is true: in fact, the innate immune

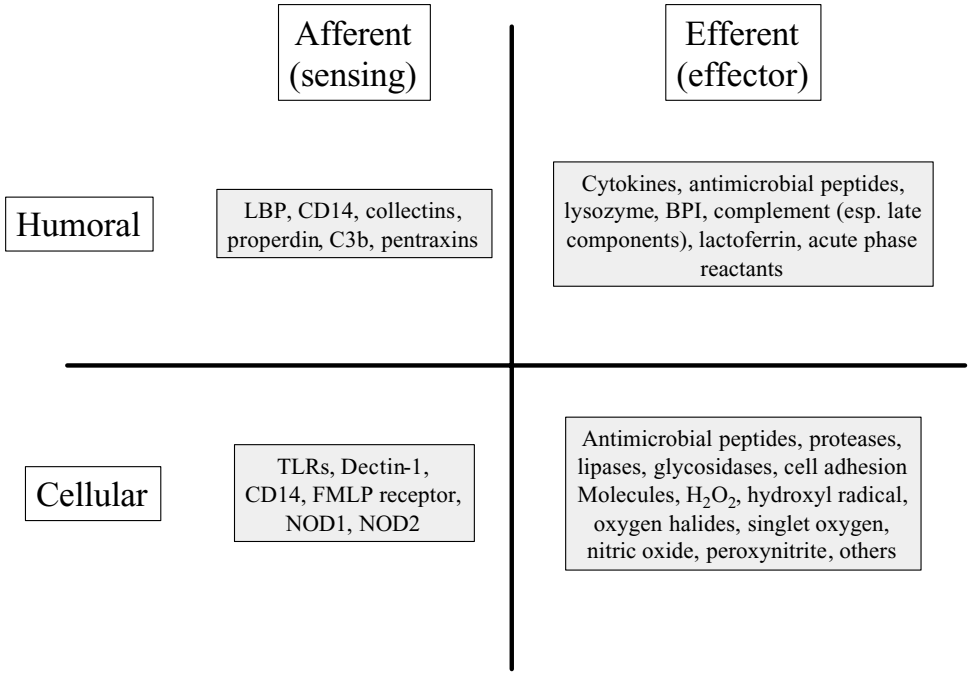

Fig. 1. The afferent and efferent arms of the innate immune system each have cellular and humoral components. A partial list.

system has been refined for a longer period of time than the adaptive immune system, and is more perfect in almost every way.

1.3.1. The core elements of an immune system, and their essential functions

It is widely considered that any "true" immune system, however advanced or primitive, must be capable of doing three things:

1. Recognition of a diverse array of pathogens.
2. Killing these pathogens once they are recognized.
3. Sparing tissues of the host (i.e. there must be self-tolerance).

The innate immune system solved these problems long ago, and vestiges of the original innate immune "battle plan" may be seen in all advanced life forms today. The adaptive immune system, arising as it did in the context of a functioning innate immune system, managed to achieve the same ends, but did so in a very different way. The adaptive immune system was something of a luxury, since a high level of protection was already provided by the innate system that preceded it. The early events through which adaptive immunity became permanently engrafted within the vertebrate line, ultimately intermeshing with the innate immune system and complementing it, are among the greatest mysteries with which biology must contend.

The principal components of the innate immune system include cellular and humoral elements, each of which is endowed with afferent and efferent arms as discussed above (Fig. 1).

2. The cellular components of innate immunity

In vertebrates, innate immunity is largely dependent upon myeloid cells: professional immunocytes that engulf and destroy pathogens. In large part, these cells have stand-alone capabilities: neutrophils are capable of killing bacteria in vitro, for example. But they have evolved to function best in conjunction with cells and proteins of the adaptive immune system. For example, antibodies produced by lymphoid cells of the adaptive immune system opsonize bacteria for destruction by myeloid cells.

Myeloid cells include *mononuclear phagocytes* and *polymorphonuclear phagocytes*. The mononuclear phagocytes are the *macrophages*, derived from blood *monocytes*, and the closely related *dendritic cells*, also of monocyte origin, which are highly efficient at presenting antigens to T cells of the adaptive immune system. Macrophages are distributed throughout the body of the host, in some cases (e.g. heart, brain, lung, and liver) lying within the parenchyma of major organs. Should an infectious inoculum be introduced by any route, a macrophage will rarely be far away from the invasive organism. Macrophages are not a uniform population of cells; rather they are morphologically diverse, encompassing the spindle-shaped tissue histiocyte, the flattened Küpffer cell of the hepatic sinusoids, and the stellate microglial cell of the central nervous system. Osteoclasts, too, are a form of macrophage (or at least, are derived from blood monocytes). Dendritic cells also assume a variety of guises (e.g. the Langerhans cell of the skin; the plasmacytoid dendritic cell of the spleen). They exist as a minority population among the mononuclear phagocytes.

Macrophages are capable of engulfing and killing microbes, but perhaps their most important functions are supervisory. Through the elaboration of chemotactic cytokines, they recruit other myeloid cells, and in particular, polymorphonuclear phagocytes, to the site of infection. Macrophages—and even more so dendritic cells—also initiate the adaptive immune response to most pathogens by presenting antigen to CD4+ T cells via class II MHC antigen.

The polymorphonuclear phagocytes (which include *neutrophils*, *basophils*, and *eosinophils*) are of key importance in the containment of infection. Neutrophils, in particular, are specialized killers, endowed with a broad array of weapons with which to destroy their microbial prey (see below). They are short-lived cells, surviving in the circulation for only about 6 h before undergoing apoptosis, whether or not they have encountered microbes to destroy. About 5000 of them are found per mm^3 of blood under normal circumstances, though their numbers may be elevated 5–10-fold under conditions of severe infection. Eosinophils and basophils are more immediately concerned with the production of mediators that shape the inflammatory milieu, and are responsive to cytokines elaborated by the adaptive immune system. These cells are ordinarily much rarer than neutrophils, but eosinophils, in particular, can be produced in great numbers in the course of parasitic infections, or as a result of allergic adaptive immune responses. *Mast cells*, tissue-dwelling descendants of the myeloid line, are cast in a similar role, and like eosinophils, are important mediators of allergic responses.

The innate immune system evolved long before the adaptive immune system, and in many respects, the older system supports the function of the newer one. Hence, to a greater extent than the converse, cells of the adaptive immune system are dependent upon myeloid elements. The elimination of even one subset of innate immune effector cells (for example, neutrophils) may be sufficient to cause a profound immunodeficiency state, more severe than that observed as a result of lymphoid aplasia. Without the vital antigen-presenting function of innate immune cells, and without the production of cytokines of innate immune origin (including IL-12, CD40L, IL-1, type I interferons, and TNF), adaptive immune responses are ineffectual. In this respect, while adaptive and innate immunity work hand in hand, adaptive immunity is subordinate to innate immunity.

As in the vertebrates, phagocytic cells of invertebrate organisms help to eliminate pathogens. Far less is known about the killing potential of the amoeboid phagocytes of invertebrates. The hemocytes of *Drosophila*, the coelomocytes of tunicates, and the "phagocytes" originally observed by Metchnikoff in the larvae of echinoderms are examples of these cells, and it is assumed that each plays a role analogous to the cells of mammals. However, the type of immune deficiency that results from mutations that ablate these cell populations or impair their function has in no case been characterized; rather, vertebrate systems have been far better studied.

2.1. Cell-based innate immune sensing

The general strategy of innate immune detection is one in which a limited number of receptors are dedicated to the recognition of microbial molecules that are conserved across broad taxa (Kimbrell and Beutler, 2001). The target molecules are indispensable components of the microbes, for which reason they are not readily altered by mutation and selection. The innate immune receptors must detect pathogen molecules within the microenvironment of the infectious inoculum, so as to permit interdiction of the infection before microbes proliferate, disseminate, and overwhelm the host. At least for the most part, the receptors must be indifferent to molecules of host origin (the basis of innate immune discrimination between self and non-self).

During the recent past, the term "pattern recognition receptors" (PRRs) has been applied to denote those host molecules that recognize microbial infection, and "pathogen-associated microbial patterns" (PAMPs) has been applied to denote the structural features of microbes that are recognized (Janeway, C.A. Jr., 1989). On the one hand, the concept that the host innate immune system recognizes features that are unique to microbes is inarguable. On the other hand, the concept of PRRs and PAMPs is both vague and in some measure incorrect.

It is not "molecular patterns" per se that are recognized in most instances. There is usually no question of multivalence, or event a need for the presentation of repeating units. Rather it is *molecules*—specific, individual molecules—that are recognized by the host. In the sense that these molecules were recognized many years ago through classic investigation of microbial pathogenesis, and then structurally defined through modern techniques of chemical analysis, the use of the term "PAMP" seems somewhat atavistic. Rather, it is best to refer to the molecules involved in a direct and unambiguous fashion. Moreover, it is not pathogens only, but all microbes that are sensed. For these reasons, the terms PRR and PAMP will not be used further, but a discussion of the molecules in question is certainly worthwhile. What are they?

2.1.1. Microbial activators of the innate immune response

Where mammals are concerned, the molecules that are sensed by the innate immune system were identified as a result of classical work in the field of microbial pathogenesis. Probably the best characterized of these molecules is bacterial "endotoxin," or lipopolysaccharide (LPS), the principal biological effects of which were first reported by Pfeiffer more than 100 years ago (Pfeiffer, 1892), and the exact chemical structure of the toxic lipid A moiety of an endotoxin molecule was determined during the 1980s, whereupon an active molecule was synthesized (Galanos et al., 1985). LPS has occupied a prominent place in infectious disease research for many decades, because of its clear relationship to the development of septic shock, a clinical problem that is responsible for hundreds of thousands of deaths each

year. By prompting the systemic release of cytokines such as TNF, IL-1, and IL-6 (Beutler, 2001), LPS causes fever, hypotension, inadequate tissue perfusion, metabolic acidosis, and organ failure. At the same time, it is recognized that LPS perception is required for the effective containment of at least some Gram-negative infections at an early stage following inoculation (O'Brien et al., 1980; Rosenstreich et al., 1982; Agace et al., 1992). Hence, the mechanism of LPS sensing has immense practical importance.

The key question, then, came to focus on the receptor for LPS (and what the receptors for inducers with related effects, such as peptidoglycan, unmethylated DNA, dsRNA, and lipoteichoic acid) might be. Positional cloning studies, aimed at elucidating the mechanism of LPS resistance in C3H/HeJ mice, revealed that LPS is recognized by TLR4 (Poltorak et al., 1998b; Poltorak et al., 1998a). This discovery was foreshadowed by a remarkable observation in *Drosophila*, which demonstrated that fruit flies require Toll—the namesake of the family—to sense infection by fungi (Lemaitre et al., 1996). The fact that a single family of receptors could subserve microbial recognition in such divergent species as *Drosophila melanogaster* and *Mus musculus* was remarkable, and suggested that there might be much more to the story. In a short time, it emerged that there was.

TLR4 is one of eleven TLR paralogs identified to date in mammals, ten of which have been published (Chuang and Ulevitch, 2000; Du et al., 2000; Chuang and Ulevitch, 2001). The determination that TLR4 recognizes LPS immediately suggested that other molecules of microbial origin might signal via other paralogs of the TLR family. This has turned out to be the case, and to a large extent, TLRs are responsible for microbial sensing in mammals (Fig. 2).

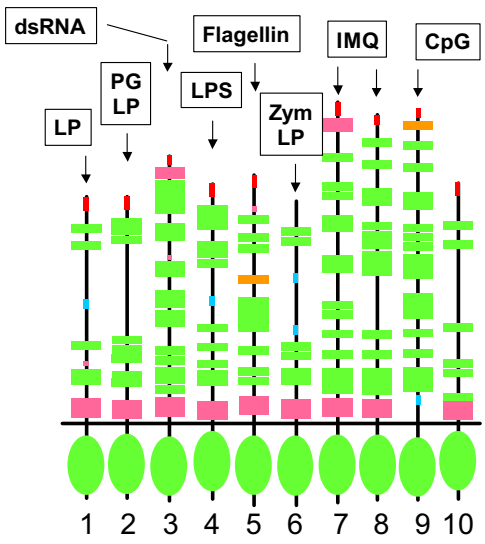

Fig. 2. The Toll-like receptors that act as sensors of the innate immune system, and the ligands that they are known to recognize. LP, lipopeptide; PG, peptidoglycan; Zym, zymosan; LPS, lipopolysaccharide; CpG, DNA with unmethylated CpG motifs; IMQ, imiquimod. Rectangles represent leucine-rich repeats. Ovals represent TIR domains.

Humans express ten TLRs, enumerated 1 through 10. Mice do not express TLR10, but do express TLRs 1 through 9, and have two additional paralogs (11 and 12) that are not represented in humans. Each TLR is thought to confer the ability to recognize a discrete set of ligands: perhaps only a single ligand (as in the case of TLR4) or several ligands (peptidoglycan, bacterial lipopeptides, and lipoteichoic acid among others in the case of TLR2). Some of the TLRs form heteromeric complexes with others. For example, TLRs 1 and 6 each form complexes with TLR2, and in so doing, broaden the repertoire of microbial stimuli that signal via TLR2. Others seem to work entirely on their own. Not only bacteria and fungi, but protozoa (Campos et al., 2001) and viruses (Hoebe et al., 2003a; Alexopoulou et al., 2001) are recognized by the TLRs. In the case of viruses, nucleic acids seem to be of prime importance in triggering a response.

Other cell-associated molecules have been mentioned as microbial sensors. For example, the f-methionyl-leucyl-phenylalanyl (fMLP) receptor on neutrophils, a multi-spanning G-protein receptor, is probably important as an inducer of chemotaxis during infection, and fMLP deficiency has been shown to make mice more susceptible to *Listeria monocytogenes* infection (Gao et al., 1999). In addition, intracellular proteins with motif similarity (though not primary structural homology) to defensive proteins of plants have been suggested to serve a role as intracytoplasmic microbial sensors (Girardin et al., 2003a,b; Girardin et al., 2003). Among these are NOD1, NOD2, and several other proteins.

Since each microbe might be expected to have an innate immune "signature," based on its content of LPS, LTA, peptidoglycan, unmethylated DNA, and other inducing molecules, and their accessibility to innate immune cells of the host, it has been guessed that the innate immune system might be attuned to generate an optimal response for any given infectious stimulus. To some degree this is undoubtedly true, but it should be noted that the concept has its limits. The notion that a given infectious organism might actually be diagnosed based on the gene induction events that it elicits has not yet been borne out, and in fact, there is degeneracy in the innate immune response, to the extent that even such disparate invaders as Gram-negative bacteria and viruses can trigger the production of identical signaling molecules, yielding effects that are formally similar. For example, as described below, it has emerged that the LPS signaling pathway is very much a shared pathway, in that parts of it are utilized in the response to both bacterial and viral infections (Hoebe et al., 2003a; Yamamoto et al., 2003).

2.1.2. The signaling adapters that serve TLR responses

Each TLR is endowed with a TIR (Toll/IL-1 receptor/resistance motif) domain: an ancient protein fold that has been associated with disease resistance since a time before the divergence of plants and animals. A total of five cytoplasmic adapter proteins are presently known to be available to carry signals from the TLRs into the cyto-

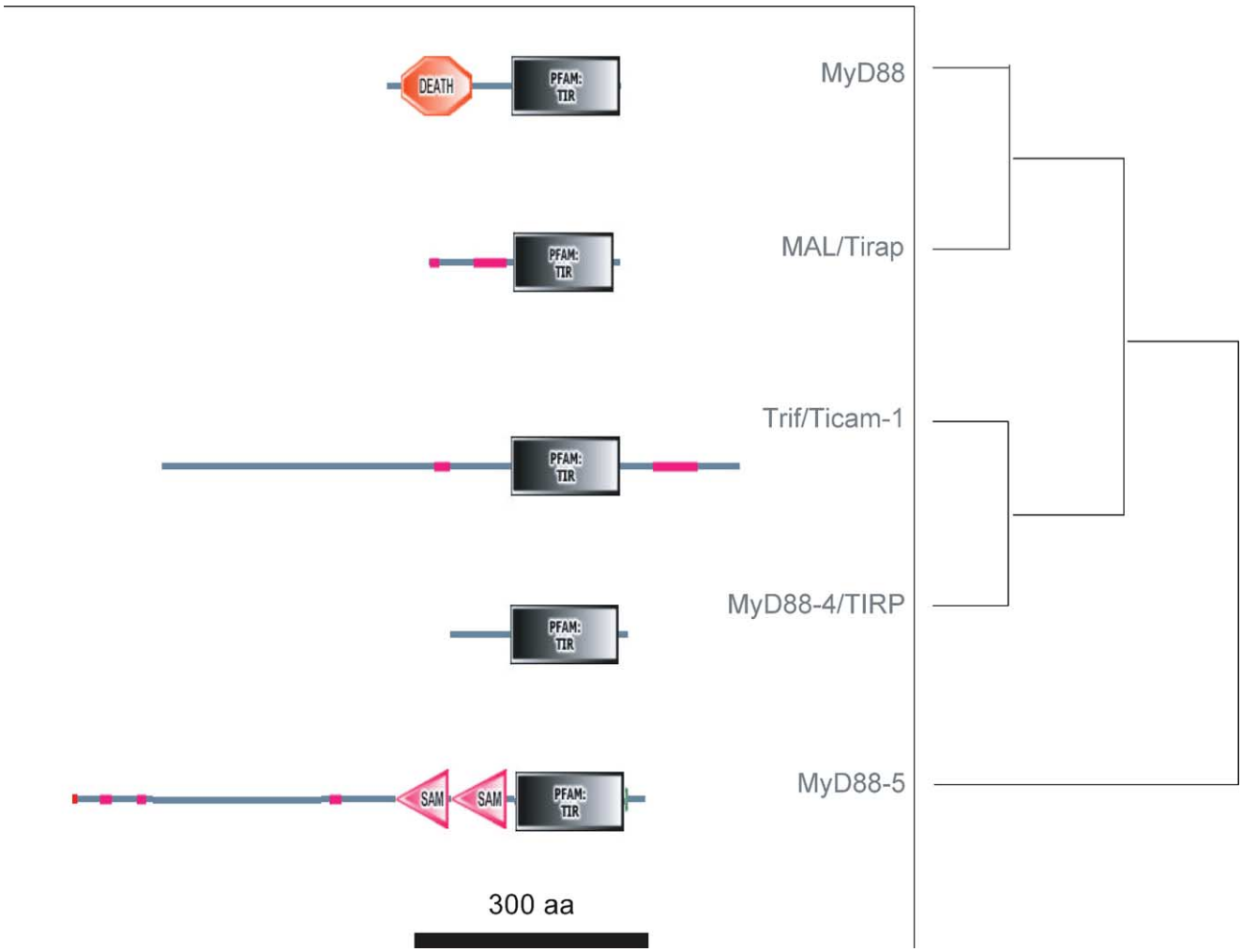

Fig. 3. Schematic illustration of the five TIR adapter proteins presently known to exist in mammals. SAM, sterile alpha motif (a putative protein interaction motif). Death, death domain. TIR domains are aligned; scale shown in inset.

plasm. These adapters, known as MyD88, MAL (or Tirap), TRIF (or Ticam-1), MyD88-4 (sometimes called TIRP or TRAM), and MyD88-5 (Fig. 3). These proteins are believed to engage the TLRs in heterotypic (TIR:TIR) interactions, and to activate other proteins in a signaling cascade that leads to activation of the cell. The "core" signaling pathway, homologous to the Toll signaling pathway of *Drosophila*, involves the recruitment of IRAK-4 by MyD88. IRAK-4 phosphorylates IRAK-1 and IRAK-2. Although endowed with kinase activity, these latter proteins may serve as scaffolds. TRAF-6 is then recruited to the complex, leading to the activation of TAK-1, and the phosphorylation of IKK-γ within the signalosome. The activated signalosome, in turn, causes the phosphorylation and degradation of IB, leading to nuclear translocation of NF-κB and the transcriptional activation of many cytokine genes.

This pathway serves most (but not all) of the mammalian TLRs. The dsRNA receptor TLR3, for example, is entirely MyD88-independent. Moreover, the LPS receptor TLR4 is largely MyD88-independent. In both of these pathways, TRIF substitutes for MyD88 to initiate a separate series of events, leading to the activation of IRF-3 and other intermediates, causing both upregulation of the costimulatory response to antigen and the production of interferon-β, which in turn exerts many effects of its own through the STAT pathway (Fig. 4) (Hoebe et al., 2003b; Yamamoto et al., 2003). TLR2 (the receptor for peptidoglycan and lipopeptides) and TLR4 utilize MAL as well as MyD88 for signaling (Yamamoto et al., 2002; Horng et al., 2002). And there is evidence that in some cells, TLR4 uses a fourth adapter as well (in addition to MyD88, MAL, and TRIF) (Hoebe et al., 2003a).

2.1.3. The evolution of the adapters and TLRs, and their interaction

It may be supposed that most ligand/receptor/transducer systems evolve "from the inside out." A transducer, such as a TIR domain protein, exists in the first instance as a cytoplasmic protein, participating in a specific enzymatic pathway (and TIR domains continue to exist solely in this

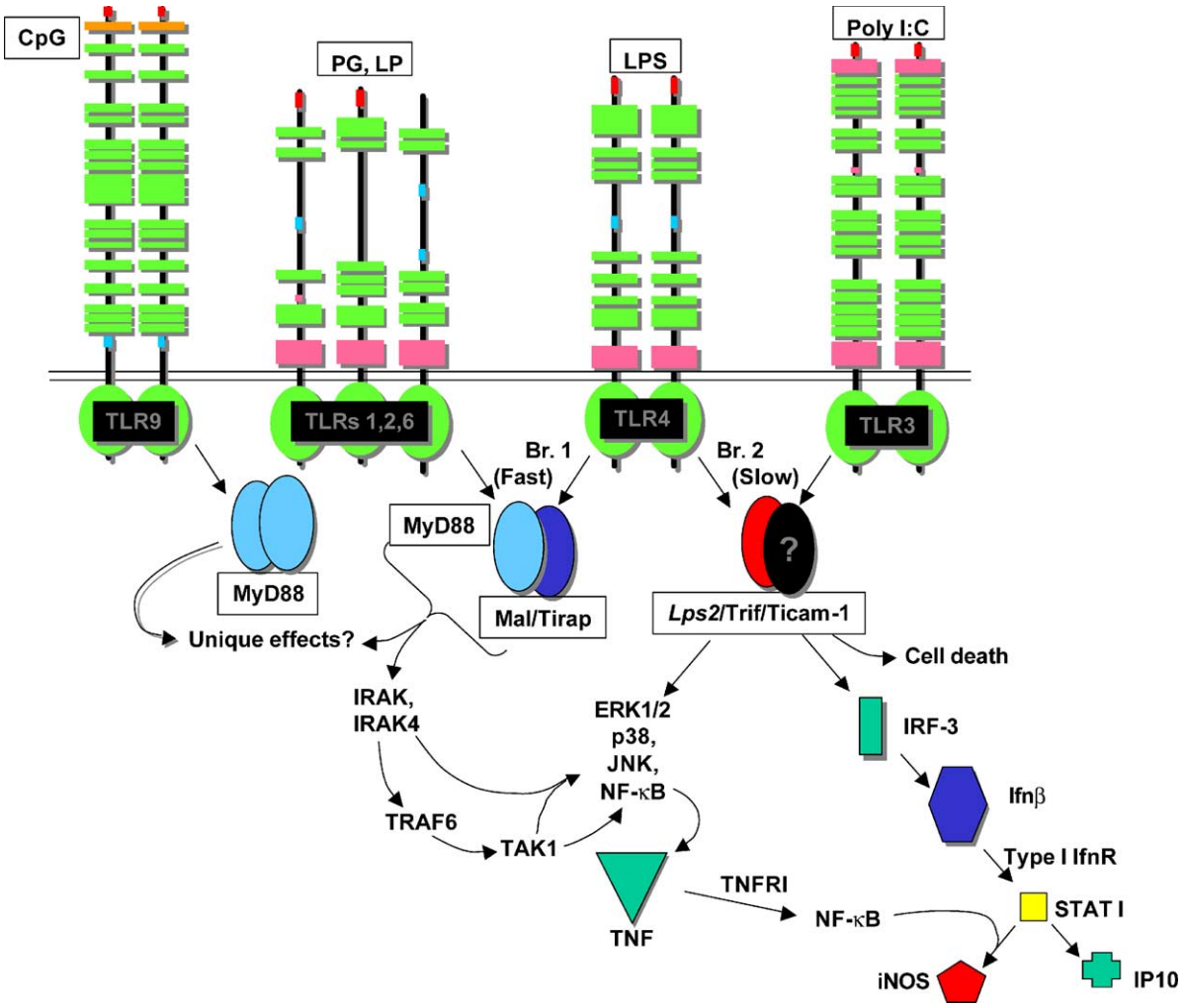

Fig. 4. Present understanding of mammalian TIR signaling pathways. Some adapters (?) are believed to exist, but have not yet been identified. Both NF-κB and STAT pathways are activated by different TLRs, sometimes with synergistic effects.

form in many plants). Its immobilization at the cell surface occurs subsequently; the capture of an activating ligand occurs last. The ligand/receptor relationship need not remain static, of course. The appropriation of new ligands is a common phenomenon in evolution, as is the appropriation of adapters. Both occur through a process of gene duplication, as outlined in the case of the TNF ligand/receptor family (Bazzoni and Beutler, 1995; Bazzoni and Beutler, 1996). While the TLR ligands are supplied by microbes, adapter exchange has clearly occurred in the TLR family. The unique association between MAL and TLRs 2 and 4, and the unique association of TRIF with TLRs 3 and 4, provide examples of this.

A drastic modification of TLR structure appears to have occurred as an isolated event near the dawn of vertebrate evolution, whereby a novel "TLR" was created. This modified TLR was bereft of leucine-rich repeats that form the bulk of most TLR ectodomains; rather, it had Ig motifs. This was the basis of the IL-1R/IL-18R family of receptors. These receptors produce a signal that is similar to those emitted by the TLRs, but engage endogenous cytokine mediators that are made in response to TLR signaling: IL-1 and IL-18. Other members of the receptor family include SIGIRR and ST2: receptors with uncharacterized ligands. These receptors act as a conduit between cells of the innate immune system and the adaptive immune system, and also help to propagate the infectious signal beyond the bounds of the primary responder cells.

2.1.4. The chicken-and-egg paradox of Drosophila immunity-and-development

It is to be noted that Toll, the namesake of the TLR family, is involved in immunity but none of the other eight Toll paralogs represented in flies have an immune function, all being concerned with development instead. Toll itself has a developmental function as well. By contrast, none of the mammalian TLRs have developmental functions. Which came first? The immune function or the developmental function? On the one hand, it might be argued that development must be the primordial function, since there can be no im-

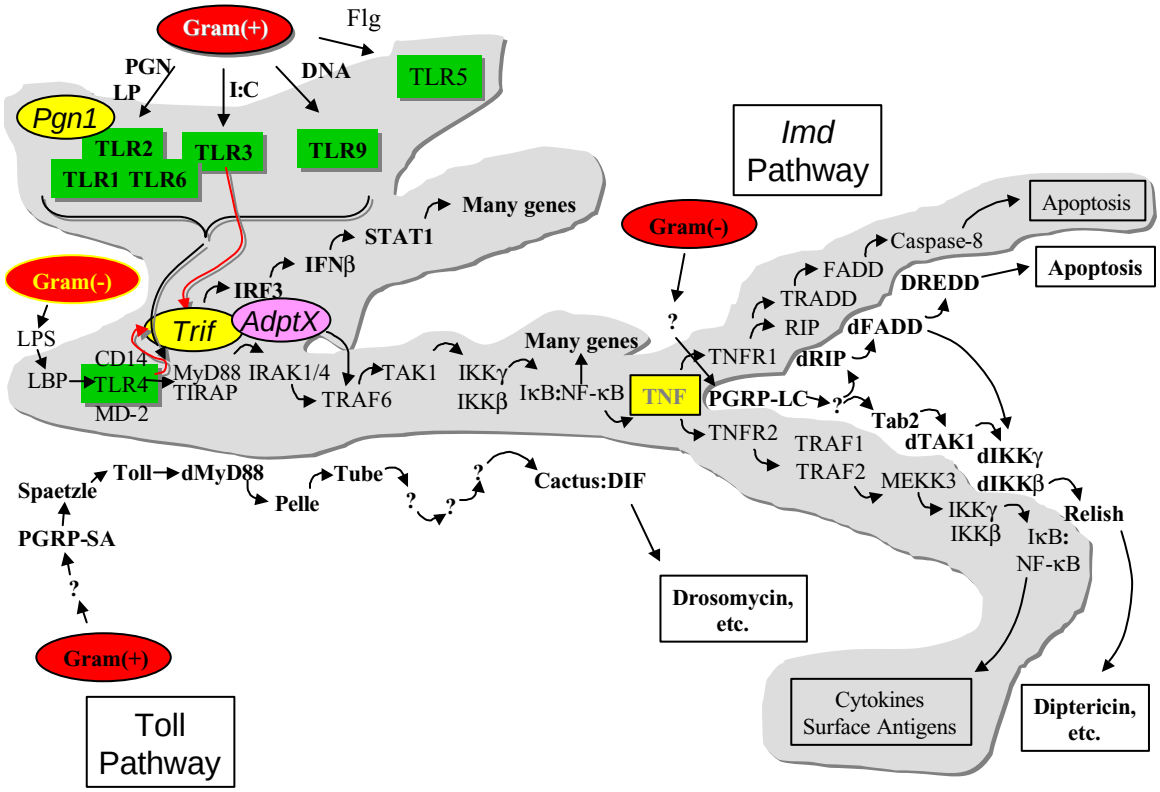

Fig. 5. Comparative biochemistry of mammalian and dipteran pathways for innate immune response. Many true homologs exist, though the mammalian pathway is unitary (shaded area), with equivalents of IMD and Toll pathways in the fly joined by the cytokine TNF. Yellow color indicates mammalian participants that have been identified to date by forward genetic screening of ENU mutant mice. TLRs are shown in green. Adapter X is an inferred adapter, believed to exist on the basis of work with spontaneous mutants and knockout mice.

mune system absent development. However, the ancestral function of the TIR domain was almost certainly immunologic, given that TIR domains serve an immune function even in plants. Therefore, it seems most probable that the domain was co-opted for use in development by selected organisms, yet tends to retain its defensive function in most instances.

2.1.5. The primary mammalian innate immune response pathway is mirrored but split in flies

The progenitor of mammals and flies may have had two pathways that served responses to different types of microbe, which became fused via TNF to form a “single” pathway in mammals. Alternatively, a unitary primordial pathway may have split into two pathways (Toll and Imd) in flies (Fig. 5). At any rate, sufficient homology is evident to say that the immune responses of these very different organisms share a common ancestry, and to declare that TIR domains, death domains, and several kinases have had defensive functions for a very long time. In mammals, adaptive immunity sprang from the innate immune system that preceded it, and in addition, many accessory cytokine pathways other than TNF (most of them dependent on very different types of signaling) seem to connect the innate immune response to diverse tissues of the host. So far as we know, there are no counterparts of these accessory pathways in flies.

2.2. Cell-based effector mechanisms

It is not enough for the host to sense microbes. It must kill microbes as well, or remain a mute witness to its own demise. A fearsome array of chemical weapons for killing has been evolved for this purpose. The distinction between afferent and efferent are blurred, since particularly in the mammalian host, cytokines are produced by immune cells and act on other immune cells, but have no direct effect on the microbes themselves. Further, many of the systemic effects of cytokine mediators are aimed at attracting other cells, diverting blood flow to the affected tissue so as to favor the influx of cells capable of killing microbes, and antibodies capable of agglutinating them.

The autotoxic character of the inflammatory response to infection has been the subject of much inquiry, and falls in the category of *sepsis* research. Perhaps the major point to be made about sepsis, the systemic consequences of a severe infection, is that the same mechanisms that contain a small infection can, if generalized, threaten the survival of

the host. Nonetheless, evolution has calculated that it is best that these mechanisms be maintained.

2.2.1. Sequential generation of superoxide and H_2O_2; then hypochlorous acid, singlet oxygen, hydroxyl radicals, and peroxynitrite

Within the phagosome of neutrophils and macrophages, reactive oxygen intermediates are produced to kill microbes in a series of reactions initiated by NADPH oxidase, an enzyme complex made of four separate subunits and active in neutrophils as well as mononuclear phagocytes. In neutrophils, the enzyme is classically induced by zymosan (a TLR2 agonist); other microbial stimuli also trigger its activation, believed to proceed through phosphorylation of the regulatory subunit p47. The p91 subunit, a cytochrome, generates superoxide (O_2^-), a free radical, from the common form of molecular oxygen, O_2.

$$2O_2 + NADPH \rightarrow 2O_2^{\bullet -} + NADP + H^+$$

The superoxide anion is not itself a reactive oxidant, but is a substrate for the production of other reactive oxygen species, by way of the production of H_2O_2. In turn, H_2O_2 is an abundant substrate for the generation of more violent oxidants (Fig. 6).

Superoxide is converted to hydrogen peroxide through a reaction catalyzed by superoxide dismutase. This is the key reaction that permits the subsequent generation of reactive oxygen species:

$$2H^+ + 2O_2^{\bullet -} = H_2O_2 + O_2$$

Among the products that are generated from H_2O_2 are the reactive halides, including HOCl (hypochlorous acid; equivalent to household bleach), which is produced from H_2O_2 through the action of myeloperoxidase:

$$Cl^- + H_2O_2 + H^+ = HOCl + H_2O$$

The reactive halides are powerfully microbicidal, and stand at the center of the neutrophil's armamentarium. Not only do they act directly to kill microbes, but they generate other metabolites that are capable of doing so. Hypochlorite (OCl^-) can generate singlet oxygen ($^1O_2$), a high-energy form of oxygen which is strongly reactive with carbon:carbon double bonds:

$$OCl^- + H_2O_2 = {}^1O_2 + Cl^- + H_2O$$

It can also react with superoxide to yield the extremely reactive hydroxyl radical:

$$O_2^{\bullet -} + HOCl = O_2^{\bullet -} + OH^{\bullet} + Cl^-$$

The hydroxyl radical ($OH^{\bullet}$) can be produced independently from H_2O_2 and superoxide, via the Fenton reaction, which is catalyzed by iron:

$$O_2^{\bullet -} + H_2O_2 = OH^{\bullet} + OH^- + O_2$$

Reactive nitrogen species are also produced though a spontaneous reaction, using superoxide as a substrate:

$$NO^{\bullet} + O_2^{\bullet -} = ONOO^-$$

Peroxynitrite, the product of the reaction, undergoes a secondary reaction to produce a nitrating agent, the identity of which is still unknown.

It has also been reported that ozone (O_3), generated from singlet oxygen and water through a catalytic mechanism requiring immunoglobulins is involved in the microbicidal process(Wentworth Jr. et al., 2002, 2003). Since many immunoglobulin folds (including all those present in antibodies) are believed to catalyze this reaction, it is not possible to adduce mutational evidence of its contribution to defense.

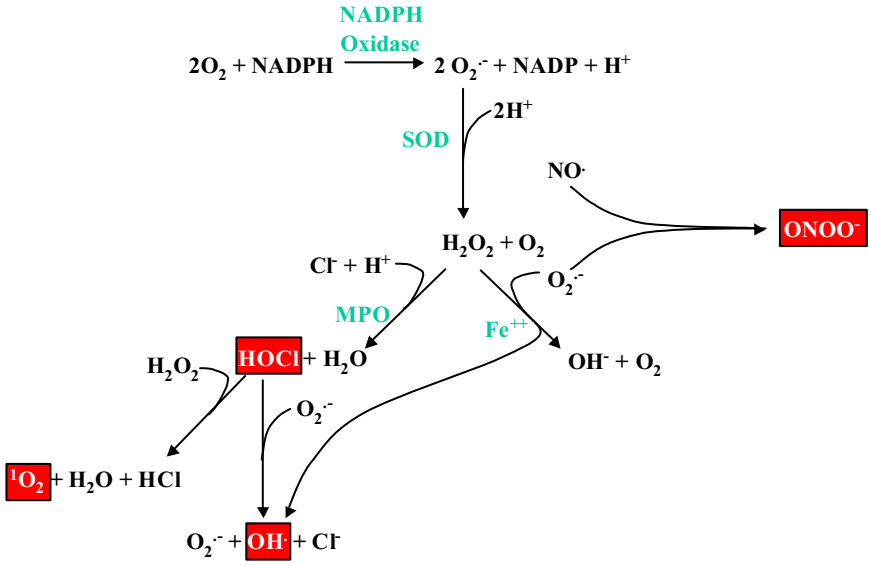

Fig. 6. The generation of microbicidal molecules by enzymatic machinery of the mammalian neutrophil. The same pathways are represented in macrophages. Enzymes are shown in green; red boxes indicate species that are toxic to microbes.

Hydroxyl radicals, singlet oxygen, oxygen halides, hydrogen peroxide, and the unknown nitrating agent produced from peroxynitrite all kill microbial targets by reacting with diverse molecular targets within the latter, including lipids, proteins, and nucleic acids. The host itself is not impervious to these molecules and they may cause substantial injury to healthy tissues. Hence, the innate immune system is imperfect in its specificity, but on balance, the weaponry that has evolved is certainly beneficial. In humans and in other mammals, mutations affecting the generation of reactive oxygen species (i.e. mutations of the components of NADPH oxidase, or myeloperoxidase) create severe immunodeficiencies, despite the presumed integrity of innate immune signaling adaptive immune responses. This fact in itself bespeaks the importance of innate immunity to mammalian host defense.

2.2.2. *Adhesion, diapedesis, and chemotaxis*

Before microbes can be engulfed and destroyed, leukocytes in the periphery must be able to reach them. The process is a complex one, and mutational data indicate that it is extremely important: severe immunodeficiencies result from a failure of leukocyte adhesion, diapedesis, and chemotaxis (Fig. 7).

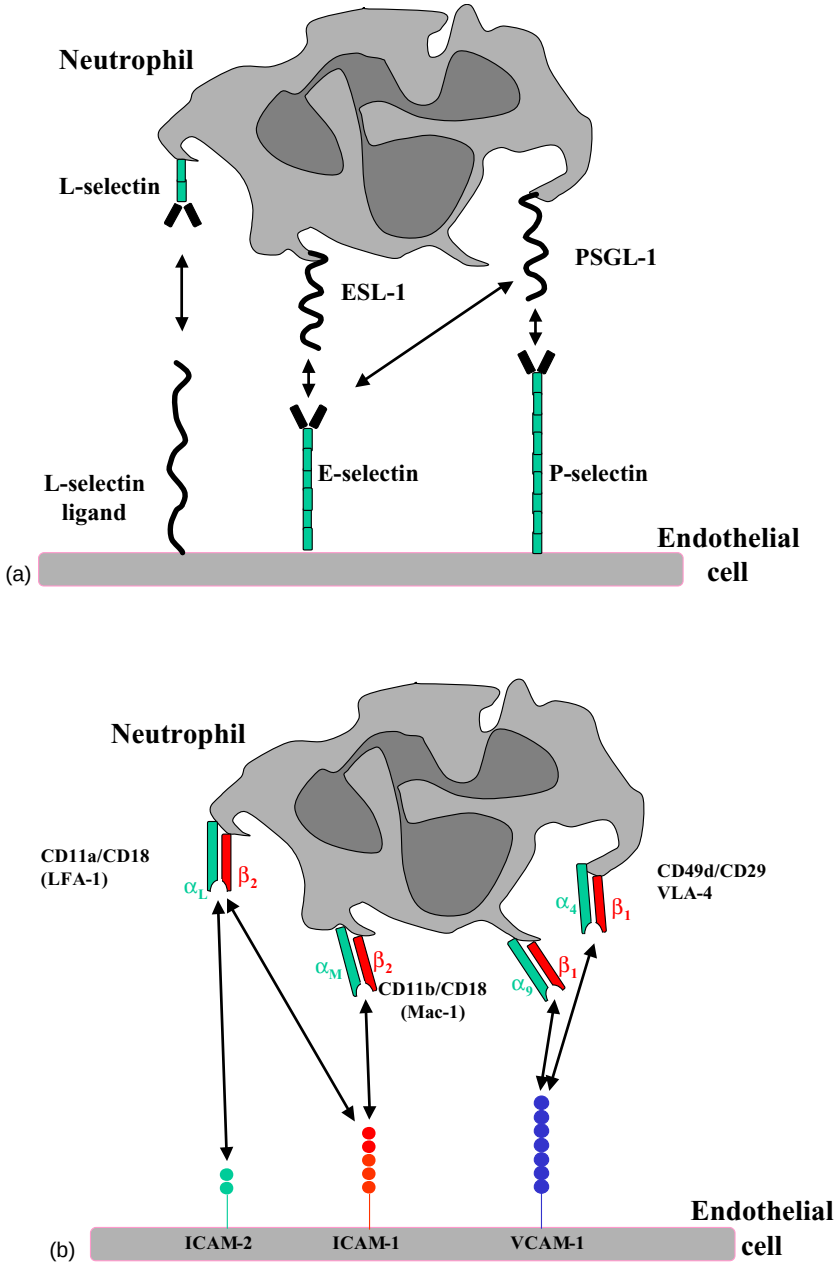

Fig. 7. Essential interactions between circulating polymorphonuclear leukocytes and vascular endothelial cells that permit effective egress of the former to sites of infection. (a) Interactions between the selectins and their target ligands permit initial contact to occur. (b) Interactions between the VCAMs and integrins permit tight association and diapedesis.

Within the immediate environment of a focal infection, chemotactic mediators (especially proteins of the chemokines family) are released, along with cytokines such as IL-1 and TNF, and autacoid substances such as histamine, bradykinin, and other small molecules. The autacoids cause localized vasodilation, increasing the volume of blood that traverses the zone of infection, but simultaneously slowing the velocity of flow within the microvasculature; especially in postcapillary venules.

Under normal circumstances, leukocytes (especially polymorphonuclear leukocytes) roll along the surface of vessels at a far slower velocity than the flow of blood. Rolling is maintained by contact between vascular selectin molecules and integrins on the leukocyte surface. Under conditions of inflammation, rolling becomes slower still, and eventually, firm adhesion between the leukocyte and the vessel wall occurs as a prelude to diapedesis, the process by which the leukocyte crosses the vascular barrier.

TNF and IL-1 act regionally to alter the transcriptional profile of vascular endothelial cells, upregulating the expression of vascular adhesion molecules such as E-selectin, which increases the percentage of rolling leukocytes and makes the rolling slower, and VCAM-1, which is involved in later stages of adhesion. Leukocyte integrins of the CD18 complex (CD11a/CD18, or LFA-1 and CD11b/CD18, or Mac-1) permit firm adhesion to the vascular surface.

Chemokines, such as IL-8, stimulate leukocyte diapedesis and chemotaxis in the extravascular space. Diapedesis itself is a complex process that depends upon several molecules, including platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD31), intercellular adhesion molecule-1 (ICAM-1, CD54; a ligand for LFA-1 and Mac-1), CD-11a/CD18 (also known as LFA-1), integrin-associated protein (IAP; CD47), and VLA-4 ("Very Late Antigen-4," CD49d/CD29; aka 4ß1 integrin: a ligand for VCAM-1), and VE-cadherin.

Mutations that disrupt CD18 expression result in leukocyte adhesion deficiency (LAD), a severe form of immunodeficiency. Other mutations affecting neutrophils migration (e.g., "lazy leukocyte syndrome") are less characterized, and redundancy in systems for chemotaxis and diapedesis may make it more difficult to identify them.

It must also be said that many other proteins are undoubtedly required for effective host defense, including the proteolytic enzymes, lipases, and glycohydrolases that dispose of microbes once they have been killed. In a sense, we have hardly scratched the surface in our efforts to find all of the requisite components of the effector arm of cellular innate immunity.

3. The humoral components of innate immunity

Cells are not required for all innate immune reactions. Proteins and perhaps other molecules are sufficient to kill microbes that have not yet been engulfed by cells. As with cell-based innate immunity, however, "humoral" innate immunity may be divided into afferent and effector components. The molecules that sense microbes are not necessarily the same as those that kill them. A great deal of cooperation between innate and adaptive immune systems is evident as well, in that antibodies may mark microbes for destruction by complement. Yet even without adaptive immunity, there is no doubt that innate immune proteins provide a measure of protection.

3.1. Sensing microbes in the extracellular compartment

Direct (and specific) contact between microbes and molecules of the host is accomplished by many different molecules. In mammals, the mannose-binding protein (Super and Ezekowitz, 1992) recognizes terminal mannosyl residues present on the surface of numerous microbes, and can activate the complement cascade via the MASP pathway (Matsushita and Fujita, 1992; Matsushita and Fujita, 1995; Terai et al., 1997; Matsushita et al., 1998; Thiel et al., 1997). Other members of the mannose-binding protein family, globally known as "collectins," may recognize other microbial determinants and thereby permit early recognition of pathogens. Lipopolysaccharide binding protein (LBP) and CD14, in its shed form, also serve to recognize microbes in the extracellular compartment and have a means of making their presence known. The C-reactive protein, and other members of the "pentraxin" family, also bind microbes directly, presumably through a lectin-like interaction, to uncertain ends.

3.2. Humoral effector mechanisms

While the plasma proteins just discussed do not have any means of killing microbes, other extracellular proteins are capable of doing so. Complement, lysozyme, lactoferrin, and antimicrobial peptides are among the proteins most commonly cited in this regard. Lysozyme, famously discovered by Alexander Fleming as a component of his own nasal secretions, is found in other secreted fluids as well and destroys the cell wall of Gram-negative and Gram-positive bacteria through an enzymatic mechanism. Lactoferrin alters the motility of certain bacteria, such as *Pseudomonas aeruginosa*, and their ability to form biofilms (Singh et al., 2002), and may help to cause their elimination. Bactericidal permeability increasing (BPI) factor, a distant homolog of LBP, binds LPS, neutralizing its TLR4-activating potential (Gazzano-Santoro et al., 1992), and also destroys certain Gram-negative microbes (Elsbach and Weiss, 1993). Complement proteins and antimicrobial peptides (which are mostly intracellular and constitutively expressed in mammals but extracellular and inducible in invertebrates) are very clearly designed for defense, and mutational evidence substantiates their importance in innate immune function.

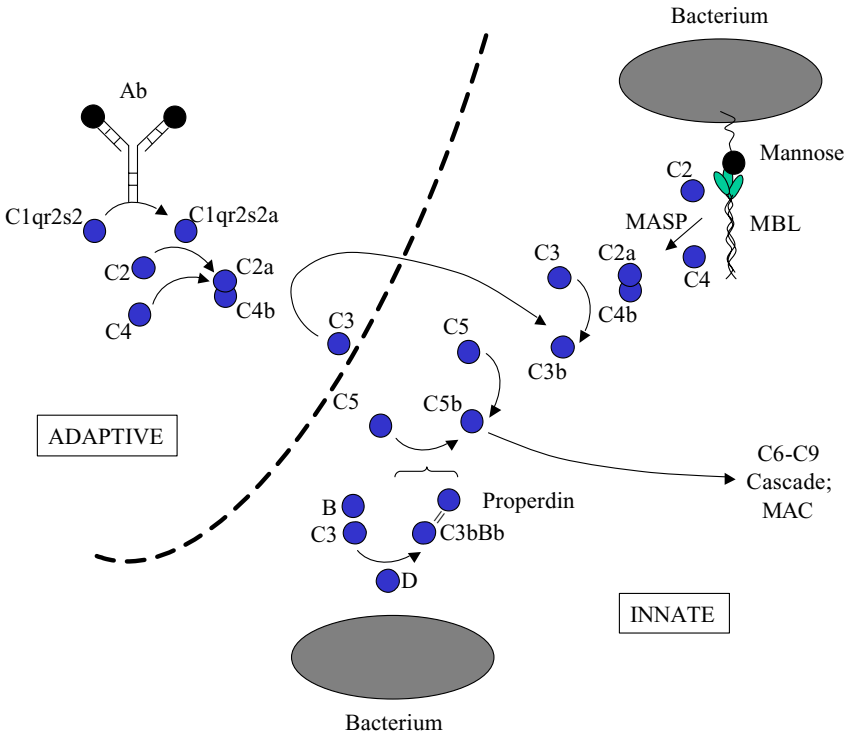

Fig. 8. Essentials of the complement cascade, which bridges innate and adaptive responses. Complement can be activated by the classical (antibody-mediated) pathway, the properdin pathway, and the MASP pathway. In all instances, the critical event is the generation of C3b, which acts as an opsonin and also leads to distal components of the cascade, yielding the membrane attack complex (MAC).

3.2.1. Complement

A set of proteolytic enzymes, collectively known as "complement," can be activated by three separate pathways (classical, properdin, and MASP), each of which is ultimately triggered by the presence of microbes in the interior milieu (Fig. 8). The classical pathway is activated by antibodies, which may combine with microbes to which the host has previously been exposed. The alternate (properdin) pathway is dependent upon a plasma glycoprotein, properdin, which is endowed with a series of thrombospondin-like repeats, and a set of cofactors (factors B, D, H, and I), which directly recognizes microbes, guided by interaction between factor B and the microbial surface. The MASP pathway is activated by mannose-binding protein engagement of its microbial target.

The effects of activation of the complement cascade, by any of these three routes, includes: (1) the activation of C3b, which binds to microbes and opsonizes them for phagocytosis; (2) the generation of C5a, which is inflammatory and chemotactic; (3) the activation of C5 through C9, the latter component of which forms a ring-shaped assembly of protein subunits (the 'membrane attack complex") that can lyse Gram-negative bacteria, and also inactivate viruses.

3.2.2. Antimicrobial peptides

In *Drosophila*, a total of 20 antimicrobial peptide genes, falling within seven distinct families, have now been identified (see review by J. Hoffmann, in press). These peptides are largely adapted for inducible production, and for secretion into the hemolymph. In mammals, on the other hand, there are only two major families of antimicrobial peptides: defensins and cathelicidins, which are expressed in neutrophils and in epithelia, respectively. Defensins (Ganz and Lehrer, 1998; Lehrer and Ganz, 2002b) consist of three subfamilies (alpha-, beta- and theta-defensins). Cathelicidins (Lehrer and Ganz, 2002a), are defined by a highly conserved propeptide, linked to a great variety of C-terminal sequences (that are often but not always clipped off by a protease). These C-terminal sequences resist classification (but have been designated as protegrins, cyclic dodecapeptide, indolicidin, LL-37, to name a few examples). In many instances, cathelicidins are unique to a single mammal or a group of closely related mammals. Constitutive production is the rule and for the most part, the peptides are not found in the blood.

Much controversy attends the mechanism by which antimicrobial peptides kill microbes. It is clear that they disrupt biological membranes, and that they are very specific for microbial cells, but the reasons for this are unclear. Also unexplained is the fact that microbes seem to remain sensitive to the lytic effect of antimicrobial peptides for the most part, while they are quite capable of developing defenses against most microbially derived or synthetic antibiotics.

3.2.3. The acute phase response

Most infections trigger many secondary effects throughout the host. These include the development of fever, a fall

in plasma iron, and the elaboration of proteins such as haptoglobin, fibrinogen, and serum amyloid A protein, each of which may abet the immune response in one way or another. Some of these proteins, collectively termed "acute phase reactants," have rather well defined defensive functions. For example, LBP binds LPS and assists in its further detection; C-reactive protein, a pentraxin, binds to the capsule of streptococci and may signal their presence.

The acute phase response is dependent upon cytokines, which elicit responses in tissues that may be far removed from the site of infection (the brain, where fever is concerned, or the liver, where the synthesis of acute-phase reactants takes place). In selected instances, the acute phase response has been shown to be helpful to the host; e.g. the fact that a rise in temperature helps the poikilothermic reptile *Dipsosaurus dorsalis* to survive infection (Vaughn et al., 1974; Bernheim et al., 1978; Bernheim and Kluger, 1976), or the classical use of fever as a clinical tool in the treatment of *Treponema pallidum* infection in humans. The synthesis of fibrinogen may be helpful in "walling off" infectious foci, or may be related to the fact that trauma and prolonged bleeding frequently accompanies a severe infection.

4. The bridge to adaptive immunity

Lymphocytes came into being approximately 550 million years ago, and are represented in all vertebrates save the jawless fishes. The roots of adaptive immunity are buried deep in the soil of the innate immune system that preceded it. So much is evident from the fact that antigen presentation occurs via specialized molecules, the class I and class II MHC antigens, that are found on innate immune cells. Indeed, the class II antigens are found chiefly on macrophages and dendritic cells, and for more than three decades, it has been known that macrophages are required for an adaptive immune response to occur (Hoffmann and Dutton, 1971; Dutton et al., 1970; Hartmann et al., 1970; Mishell et al., 1970; Mishell and Dutton, 1966).

These cells are also endowed with antigens for "copresentation." It is not enough to present antigens to the T cell receptor via MHC antigens to ignite an adaptive immune response. Rather, other stimuli, provided by the upregulation of surface molecules such as CD80 (B7.1), CD86 (B7.2) and CD40 are required for the adaptive response to proceed, as a result of interaction between these molecules and specific activating receptors present on T cells (CD28 and CTLA, for example). Mutational deletion of CD80 and CD86 abolishes the adaptive immune response (Borriello et al., 1997), and quiescence of these proteins is believed to impede the response.

Upregulation of copresentation antigens is believed to account for the adjuvant effect of microbes, first noticed many decades ago (Lewis and Loomis, 1924; Freund et al., 1937). It was first reported that LPS acts as an adjuvant nearly 50 years ago (Condie et al., 1955a), and since that time, innumerable publications have shown that other microbial products have adjuvant effects as well. The *Lps* locus was shown in 1976 to be essential for the adjuvant effect of LPS (Skidmore et al., 1976). In light of the more recent revelation that *Lps* encodes TLR4 (Poltorak et al., 1998a), one or more of the adapter proteins that carry the TLR4 signal into the cytoplasm must account for adjuvanticity.

It appears that neither MyD88 nor Tirap are required for this role, despite early suggestions that each was responsible. Rather, Trif serves this function, and does so through a pathway that may conveniently be split from that which leads to cytokine production (Hoebe et al., 2003b). Hence, the possibility of creating an adjuvant effect without the need for the inflammatory effects that normally attend adjuvanticity may at least be entertained.

5. Conclusions: some words of advice for travelers in the realm of innate immunity

As in many areas of science that have undergone a rapid expansion, there is much that is true—and also much that is not. How may one parse the published data, forming opinions as to which is which?

For those with an experimental bent: do not be beguiled by weak methods! In this, the golden age of genomic inquiry, there is no excuse for claims that are not substantiated by germline mutations, and many misinterpretations have arisen from simple in vitro transfection-based data. Phenotype driven research reigns supreme in the study of innate immunity. It has broken open the field (insofar has it *has* been broken open), and it still has far to go. As in any unexplored domain of biology, it is best to start with a phenotype and ask "why?" or to ask "why not?" and attempt to create a phenotype de novo. It is less satisfactory to start with a protein and ask its function. While a successful outcome may result from this approach, the advance will most often be less impressive.

For those who love science in general and evolution in particular: do not imagine that every detail of the immune response in *Drosophila* is mirrored by a comparable detail in mammals. There is no mammalian Spätzle, for example, and there is no *Drosophila* TNF. The mammalian TLRs do not, so far as we know, have a developmental role. Evolutionary arguments can suggest experiments, but cannot provide answers in and of themselves, and a good deal of the innate immune system has been lost, gained and re-wired since we and flies were one and the same.

For those who are clinicians: do not be deceived by the lure of curing sepsis through application of a single drug, or by blocking a single mediator. There are few areas of clinical science that have been more abused than the sepsis field. Here, false claims have been proffered, alongside false hope, and even cytokines that exist only in name. One cannot raise the dead, and this is not likely to change

soon, despite the hyperbole that attends each incremental advance in our understanding. The day will come when true immunological lesions can be identified in sepsis, and bypassed so that sepsis can in some instances be prevented. Great strides have been made on the basis of hard facts: for example, the identification of germs as the source of infection. Other strides will doubtless follow, but only commensurate with the facts that are produced by ardent and sincere investigation.

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