Review

Science review: Key inflammatory and stress pathways in critical illness – the central role of the Toll-like receptors

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Abstract

A pure reductionist approach can sometimes be used to solve an exceptionally complicated biologic problem, and sepsis is nothing if not complicated. A serious infection promptly leads to changes in many aspects of host physiology, including alterations in circulation, metabolism, renal, hepatic, and neuroendocrine function; all of these changes happen at once, and each influences one another. It is difficult to tease apart a problem of this sort, if only because the systems affected are so profoundly interactive. The key to understanding sepsis, insofar as we do understand it at present, was found in the use of genetic tools to study the very earliest events that take place at the interface of the pathogen and the host. The continued application of both forward and reverse genetic methods, in both mammals and insects, is steadily revealing the central biochemical events that occur during infection.

Keywords forward genetics, infection, innate immunity, sepsis, shock, Toll-like receptors, tumor necrosis factor

The revelation that germs cause disease did not much alter medicine at first. Many years elapsed before the first effective systemic antimicrobial agents were designed and used in human patients. Although it was an enormous advance, antimicrobial chemotherapy did not put an end to the problem of infections, or certainly to the consequences of infections. As the science of microbial pathogenesis advanced, the toxic molecules produced by bacteria and fungi that are responsible for injury to the host were elucidated one by one. Some of the toxins are proteinaceous and are apparently 'intended' as weapons. Others, however, are structural components of the microbe, and are toxic only because the host 'chooses' to view them as such.

Endotoxin was the first molecule of the latter category to be recognized, but peptidoglycan, lipoteichoic acid, various lipopeptides, and even the DNA that serves as a repository of genetic information for the microbe can provoke untoward responses in the mammalian host. As to the element of 'choice' that the host has made in viewing such molecules as toxic, a certain logic has become clear. The innate immune response is based on recognition of conserved molecules of microbial origin. The innate immune response is also intended, in the vast majority of cases in which it is invoked, to contain limited infections, caused by small inocula of microbes. The evolutionary calculation has therefore been one in which a very intense response is waged against localized infection, even though a large infection, provoking a similar response at a systemic level, may prove to be fatal.

The innate immune system is largely based on myeloid cells that are endowed with the ability to engulf and kill microbes that have invaded the interior milieu of the host. Some cells, such as the neutrophil, are particularly ferocious in their ability to destroy bacteria, but are very short lived and tend to remain within the confines of the circulatory system, exiting only when they are summoned by signals that emanate from the extravascular compartment. Other cells of the innate immune system, including the macrophage and the dendritic cell, are also active phagocytes, and are quite capable of

FADD = Fas associated death domain protein; IFN = interferon; IL = interleukin; IRAK = interleukin-1 receptor associated kinase; LBP = lipopolysaccharide-binding protein; LPS = lipopolysaccharide; NF-κB = nuclear factor-κB; RIP = receptor interacting protein; TAK = transforming growth factor-β activated kinase; TIR = Toll/IL-1/resistance; TLR = Toll-like receptor; TNF = tumor necrosis factor; TRADD = TNF-receptor associated death domain protein; TRAF = tumor necrosis factor receptor associated factor.

killing bacteria. However, they serve a dual function in that they elaborate cytokines to give warning of infection both near and far, and also contribute to the adaptive immune response, which is based on nonphagocytic lymphoid cells. Natural killer cells, which are also lymphoid, may be viewed as a rather recently emergent component of the innate immune system, and are now known to play a vital role in defense against certain viruses.

The central purpose of the present review is to discuss the molecular pathways through which the host first becomes aware of infection, and the links that exist between this afferent system and the response *per se*. The genetic methods that are being used to elucidate the key molecules involved in detecting and responding to infection are also described in some detail.

Microbial sensing in the extracellular compartment

Before microbes or any of their component molecules ever had direct contact with cells of the innate immune system, they could be detected by proteins that were retained in evolution for this express purpose. Complement, for example, can be activated and fixed on the surface of microbes via properdin, or released from leukocytes [1] or the mannan associated serine protease pathway (in which mannose binding protein interacts with a serine protease that triggers the complement cascade) [2–5]. Activation of complement can lead to destruction of bacteria and to release of bacterial molecules that can activate cell-associated components of the system for microbial sensing.

The mannose binding lectin in plasma engages mannosyl residues that are typically found on fungi and bacteria. It is not alone, however, and other soluble proteins engage in direct contact with invasive pathogens to elicit an immune response. In mammals, at least one soluble form of peptidoglycan recognition protein is also known to bind tightly to bacteria [6,7] and may contribute to bacterial recognition, although a role in defense has yet to be established experimentally. In 1990, it was shown that lipopolysaccharide (LPS) from Gram-negative bacteria is tightly engaged by a plasma protein called LPS-binding protein (LBP) [8]. LBP conveys LPS to the surface of the cell, where it is transferred to CD14 [9], a leucine rich protein that is anchored to the lipid bilayer by a glycophosphatidylinositol group. CD14 can also exist as a soluble protein and, in this case also, it can convey LPS to the cell surface. It is an important part of the host LPS recognition pathway [10].

Hence, before a microbe has ever had direct contact with a mammalian cell, it is able to induce chemical reactions that reveal its presence. However, it is the cellular response to microbes that creates the septic syndrome, and a handful of specific receptors alert the host to the presence of an infection.

The effector limb of the response

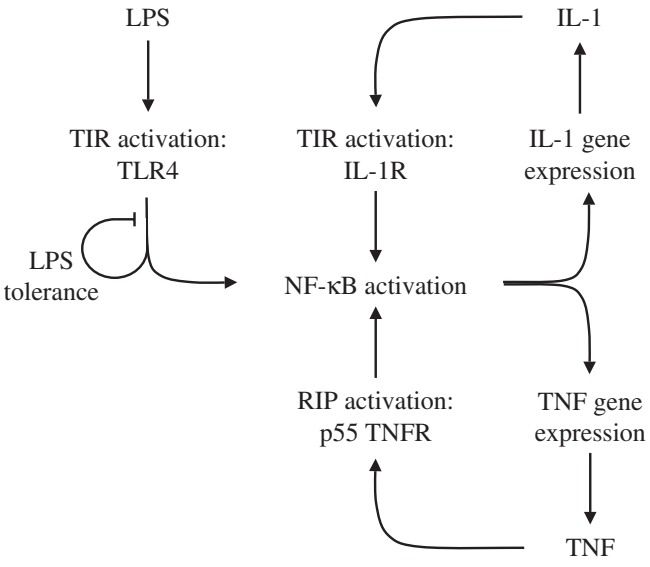
During the decade of the 1980s, one of the key discoveries in sepsis research concerned the role played by soluble mediators of monocyte/macrophage origin in shock pathogenesis. Tumor necrosis factor (TNF), purified from mouse macrophages as an endotoxin induced mediator [11,12], was found to be one of the most abundant macrophage secretory products and is produced within a very short period of time after LPS administration [13]. Blocking TNF by passive immunization was shown to render animals partially resistant to the lethal effect of LPS [14]. Furthermore, TNF could evoke many of the pathologic changes caused by endotoxin and other microbial inducers [15]. These observations suggested that TNF might act as one of a collection of endogenous mediators of endotoxicity.

TNF was known to synergize with IFN-γ in many of its effects (i.e. in its cytolytic effect on certain cultured tumor lines [16]). Moreover, IFN-γ greatly augments the production of TNF induced by endotoxin *in vivo* or *in vitro* [17]. In subsequent studies, knockout of the TNF p55 receptor [18] and knockout of the IFN-γ receptor [19] produced marked resistance to the lethal effect of LPS. It is likely that both TNF and IFN-γ, which signal through very different pathways, contribute to the development of shock in sepsis.

A third inflammatory cytokine, IL-1, has also been implicated in the pathogenesis of inflammation and shock [20,21] and appears to synergize with TNF, diminishing the 50% lethal dose of the latter [22]. Although it lacks the toxicity of either TNF or IFN-γ when administered by itself, IL-1 does mimic TNF in a number of ways. For example, it suppresses the expression of certain anabolic enzymes [23] and induces production of proteases and prostaglandins [24] that contribute to inflammation on a local level. The IL-1 receptor has Toll/IL-1/resistance (TIR) domains similar to the Toll-like receptors that are primarily responsible for transducing microbial signals. On this basis, it might be assumed that the IL-1 receptor participates in an amplification loop (Fig. 1).

The TNF receptors are of two types. The p55 TNF receptor, which is responsible for most of the toxicity of TNF, has a death domain and as such is involved in activation of the caspase cascade by means of intermediate transducers such as receptor interacting protein (RIP) [25], TNF-receptor associated death domain protein (TRADD) [26] and, indirectly, Fas associated death domain protein (FADD) [27]. In addition, it activates nuclear factor-κB (NF-κB), a central transcriptional activator of many proinflammatory genes [18,26,28]. The p75 TNF receptor signals by way of TNF receptor associated factor (TRAF) family members [29], and also activates NF-κB, but is less involved in the cytolytic activity of TNF and in its toxic action [30]. The IFN-γ receptor activates a rather different collection of genes through interaction with Janus kinase (JAK) family tyrosine kinases, which in turn phosphorylate members of the STAT (signal transducer and activator of transcription) family of transcription factors [31].

Figure 1



The relationship between Toll-like receptor (TLR)4, tumor necrosis factor (TNF), and IL-1. Nuclear factor-κB (NF-κB) produced in response to signals traversing TLR4 (or other TLRs) stimulates the transcription of inflammatory cytokine genes, including TNF and IL-1. The cytokines that are produced, in turn, trigger receptors that also activate NF-κB, via Toll/IL-1/resistance (TIR) domain containing receptors (in the case of IL-1) or TNF-family receptors (in the case of TNF). Hence, there is the potential for an amplification loop, dampened by feedback inhibition of the TLR4 pathway (endotoxin tolerance). IL-1R, IL-1 receptor; LPS, lipopolysaccharide; RIP, receptor interacting protein; TNFR, TNF receptor.

The downstream effects of these cytokines are exceedingly complicated. Together, however, they act to alter coagulation, induce fever, and cause hypotension and tissue injury, as is widely observed in septic shock. In part, they do so by triggering the release of many secondary cytokines, including IL-6, and numerous chemokines, which may further contribute to the septic syndrome.

It should be emphasized that the systemic effect of these cytokines are quite different from their local effects, which are well tolerated by the organism as a whole. The 'intended' function of TNF, acting at short range, probably entails recruitment and activation of neutrophils, 'walling off' of an infectious focus, and local upregulation of antigen presenting molecules that contribute to the development of an adaptive immune response. Similarly, IFN-γ favors a T-helper-1 response by the adaptive immune system, in which granuloma formation and intense activation of macrophages prevail. Hence, it may contribute to the more tardive adaptive defense against pathogens that have been temporarily contained by the innate immune response.

The principal receptors that engender a local or systemic inflammatory response

Awareness of the chief effectors of innate immunity provided end-points to follow in identifying the initial events that transpire during infection. For the most part, TNF production was taken as a biologically relevant marker of infection, and was used to identify the receptors that sense host invasion and transduce the essential signal across the membrane of the effector cells.

A remarkable new family of receptors, named 'Toll-like receptors' (TLRs) in view of their relationship to the prototypic protein Toll in *Drosophila,* have recently been identified as sensors that are believed to directly engage and recognize microbial LPS [32–34], lipopeptides and peptidoglycan [35,36], DNA [37], flagellin [38], double-stranded RNA [39], and probably other conserved determinants as well. The TLRs are exceptionally powerful exponents of the response to infection, and without them the mammalian innate immune system would be largely blind to the presence of infectious organisms.

The TLRs are single spanning transmembrane proteins that are coupled to a wide array of signaling molecules within the cell. All of the TLRs (of which 10 are recognized in humans) have a conserved TIR motif in the cytoplasmic domain and, as far as is known, require MyD88 (a cytoplasmic TIR-bearing protein) for signal transduction, at least in some measure [40–42].

The best studied TLR is TLR4, the mammalian LPS receptor. Its function was elucidated by positional cloning [32,43], through which it was proved that endotoxin resistant mice bear mutations in the *Tlr4* gene and that these mutations prevent LPS sensing. LPS sensing also depends on a small protein known as MD-2, which is required for TLR4 surface expression and signaling [44], and on at least one other molecule, encoded by a locus termed *Lps2*. This latter locus has recently been identified through forward genetic studies [45].

Sepsis in flies: Toll and *Drosophila*

Although insects are dramatically different from mammals in terms of gross anatomy and physiology, similarities at the cellular level are compelling. Lacking an adaptive immune system altogether, they do possess an innate immune system, which was well entrenched in the last common ancestor of insects and mammals as much as 800 million years ago. Insects are remarkably resistant to septic injury and depend largely on the production of antimicrobial peptides for defense. These peptides are induced at the transcriptional level by NF-κB like proteins. In *Drosophila*, Dif and Relish are the NF-κB homologs that induce the synthesis of antimicrobial peptides such as diptericin and drosomycin.

Analysis of mutant flies that are incapable of responding to various pathogens has greatly enlightened our understanding

of the biochemical pathways that are activated in insects following infection. A large volume of work has led to the conclusion that Toll (a plasma membrane protein that is important in fly embryogenesis) serves in the adult organism as a sensor of infection by fungi [46] or Gram-positive bacteria [47,48], which make their presence known by activating a proteolytic cascade that leads to a generation of protein ligand called Spätzle. When Spätzle binds to Toll, it triggers activation of *Drosophila* MyD88, as well as proteins called Tube and Pelle (a homolog of the mammalian IL-1 receptor associated kinase [IRAK]), which in turn signal the activation of Dif (a homolog of NF-κB) and then the production of antimicrobial peptides.

Flies possess a separate signaling pathway, known as the imd (immunodeficiency) pathway, which is activated by a separate receptor – a member of the peptidoglycan recognition protein family of proteins in the fly. *Imd* itself is a homolog of the mammalian protein RIP, and the *imd* signaling pathway overall resembles the TNF signaling pathway of mammals very closely [49]. Although flies do not have a TNF homolog, they have retained the same ancestral signaling pathway that was inherited by mammals. Although many details remain to be uncovered, the innate immune response in insects appears very similar in many of its details to that of mammals. It is therefore possible to draw certain conclusions about what might occur in mammals from what does occur in flies, and *vice versa*.

Strikingly, the mammalian equivalents of the *Drosophila* Toll and *imd* pathways have become linked in tandem, through the remarkably versatile cytokine TNF, which has no counterpart in flies (Fig. 2). Hence, where two pathways are used for microbial sensing in the dipteran scheme (Toll for detecting Gram-positive and fungal pathogens, and *imd* for detecting Gram-negative pathogens), the same two pathways are used sequentially in mammals: first for detecting microbes and then for amplifying the response to microbes. The situation is similar to that portrayed for the IL-1 amplification loop in Fig. 1, in which the TIR domain of the IL-1 receptor signals the presence of an endogenous protein (IL-1) rather than a microbial molecule.

Lipopolysaccharide resistance in mammals and the discovery of Toll-like receptor-4 function

Since 1965, a single mutation was found to abolish LPS sensing in mice of the C3H/HeJ strain, but to leave most aspects of immune function intact [50,51]. Animals that could not sense LPS were more susceptible to infection by several different types of Gram-negative organisms [52–56]. It was therefore evident that endotoxin sensing, whatever its risks, is beneficial overall and necessary to protect the host from a small inoculum of Gram-negative bacteria.

Genetic mapping studies suggested that this mutation, said to affect the *Lps* locus, was present on chromosome 4 [57]. It was not related to LBP and CD14 (mentioned above as proteins that convey LPS to the surface of responding cells). Because CD14 has no cytoplasmic domain, it is incapable of actually transducing the LPS signal. It was widely assumed that the LPS signal transducer must cross the membrane, and it was believed that this transducer might be encoded by the gene that was altered in the C3H/HeJ mouse.

Positional cloning work, in which the position of the mutation was confined to a narrow chromosomal interval by classical mapping methods and all sequences within this interval were examined for mutations, revealed that the lesion altered the structure of a mammalian homolog of Toll – TLR4 [32]. Hence, one (and only one) of the murine homologs of Toll confers protection against Gram-negative bacteria, whereas Toll itself confers protection against fungal and Gram-positive infection in flies.

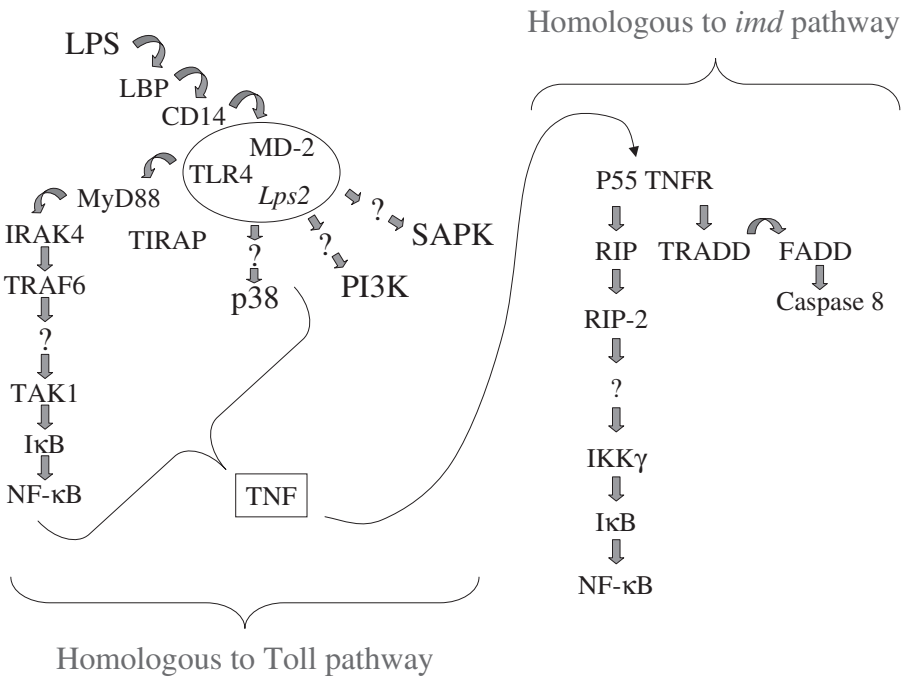
Later work, based on transfection studies and gene knockouts, revealed that other TLRs detect other microbial products. For example, TLR2 is capable of detecting bacterial lipopeptides and peptidoglycan [35,36]. TLR5 is capable of detecting flagellin [38] and TLR9 is capable of detecting unmethylated DNA from microbial cells [37]. Collectively, the TLRs are required for all responses to microbes and 'light the fire' of septic shock. Where endotoxin is concerned, the entire lethal signal is transduced through a few nanograms of TLR4 protein on macrophages throughout the body of a mouse (and perhaps shock may be initiated via a few micrograms of TLR4 protein in a human).

Reverse genetics and forward genetics: further analysis of signaling pathways in sepsis

Two fundamental approaches have guided discovery in the innate immune field (Fig. 3). 'Forward genetics' is an approach that begins with phenotype and leads to the gene. Equivalent to the classical genetic method practiced by Mendel, Morgan, Bridges, and their heirs, forward genetics may be applied in many different genetic model organisms. In flies, and in mice, it has led to the elucidation of the key proteins of the innate immune response.

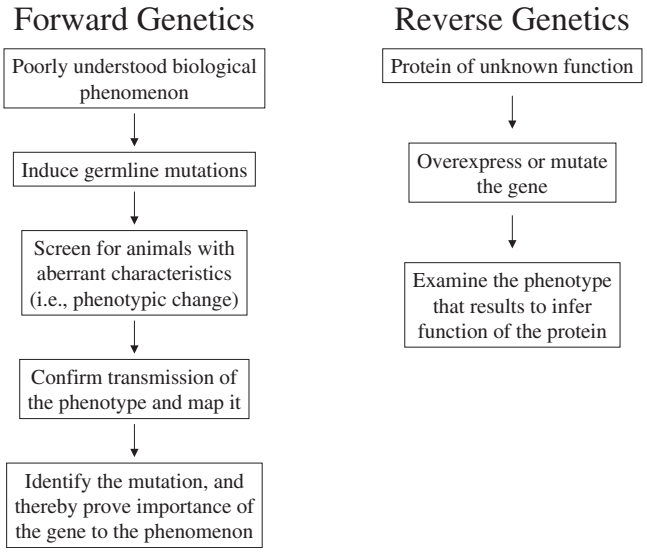
'Reverse genetics' is an approach that ends with phenotype. More precisely, the function of a protein is deduced, where such function was previously unknown. Reverse genetic methods include the over-expression of a gene in mammalian cells or in whole organisms, or alternatively the deletion of the gene in question, so that a deficiency state can be studied. Reverse genetic methods have been used to study the mammalian TLRs, and have led to the general conclusion that each TLR has a separate and nonoverlapping function in microbial sensing. However, several of the TLRs currently remain orphans. None appears to be involved in development, and in *Drosophila*, among the nine 'Tolls' (paralogs of the original Toll protein) that are known to exist, only Toll itself has an immune function.

Figure 2



The Toll-like receptor (TLR) and tumor necrosis factor (TNF) pathways, represented as Toll and *imd* pathways in *Drosophila*, are arranged in tandem in the mammalian host, and are connected by the cytokine TNF (which does not exist in flies). Each pathway has the effect of activating nuclear factor-κB (NF-κB), although the second (TNF receptor) limb of the pathway may affect different cells than the first (TLR) limb. FADD, Fas associated death domain protein; IRAK, interleukin-1 receptor associated kinase; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide; PI3K, phosphatidylinositol-3 kinase; RIP, receptor interacting protein; SAPK, stress activated protein kinase; TAK, transforming growth factor-β activated kinase; TNFR, TNF receptor; TRADD, TNF-receptor associated death domain protein; TRAF, tumor necrosis factor receptor associated factor.

Figure 3



Practical application of forward and reverse genetics.

In practice, where mice are concerned, forward genetics depends on the use of powerful mutagens such as *N*-ethyl-*N*-nitrosourea, which can induce germ-line mutations at a high frequency. In order to determine which genes are required for a robust innate immune response, the investigator maintains close surveillance over innate immunity, measuring independent parts of the innate immune response or the global ability to defend against a specific type of infection. Mice with an abnormal phenotype are isolated, transmissibility is demonstrated, and the mutation responsible is mapped using a genome-wide panel of markers. Ultimately, if mapping is performed to sufficiently high resolution, then the mutation that is responsible can be identified, and the gene of note cloned.

As mentioned above, the function of the mammalian TLRs was first indicated by a forward genetic approach applied to a spontaneous mutation: the positional cloning of *Lps* and its identification as *Tlr4*. Since that time, at least one new mutation that abolishes much of the endotoxin response has been identified. This mutation has been designated *Lps2* [45] (Fig. 2). Increasingly, it appears that mammalian microbial sensors may be quite complex, involving the participation of numerous proteins. That some of the molecular components of these receptors may be shared is not excluded.

What proteins remain?

The canonical signaling pathway used by the TLRs is one in which MyD88 is recruited following the engagement of a microbial ligand, with the subsequent recruitment of IRAK4 [58], through a death domain interaction. IRAK4, in a manner not yet understood, recruits TRAF6 to the activation complex. This, in turn, leads to the activation of the transforming growth factor-β activated kinase (TAK)1, and then to the phosphorylation of IκB, with its disassociation from NF-κB. NF-κB activates many of the genes that are induced by LPS. However, it is known that other events transpire as well. Numerous kinases within the cell (including the mitogen activated protein kinases [59], stress activated protein kinases [60], p38 kinases [61], and phosphatidylinositol 3-kinase [62]) are activated by LPS. MyD88 is not absolutely required for the full spectrum of cellular events that follow induction by LPS [41]. MyD88 deficient mice still show phosphorylation of p38 mitogen activated protein kinase, for example, which occurs in normal mice as well, and which depends on events that are presently not well understood. There are numerous gaps in the signaling pathway. The junction between TRAF6 activation and TAK1 activation is not a solid one, or is it clear that TAK1 directly phosphorylates IκB. There is certainly room for other proteins, both outside the cell and within it. Forward genetic analysis perhaps offers the best approach for finding some of these participants, although it has not yet enjoyed the tremendous success in mammals that it has in *Drosophila* – a model organism with several advantages from the genetic point of view.

Conclusion

The dramatic advances that have occurred within the past 4 years in our understanding of how cytokine storm is elicited during severe infection offer a good deal of promise. Although success in treating sepsis with individual cytokine inhibitors has not been achieved to date, there is reason to think that a more global strategy of inhibition might possibly succeed in quelling the systemic response that we know as sepsis. If it is accepted that the innate immune system arose primarily to contain local challenges of microbes (surely the most common type of insult that occurs in nature), then it might be granted that the system is somewhat inept at the containment of overwhelming infection. It might also be granted that antibiotics are a useful tool for destroying bacteria that have escaped the primary focus of infection. If both of these proposals were accepted, then it would appear logical to block the innate immune response systemically while maintaining bacteriostasis with antibiotics in an effort to treat the patient.

Several molecules already present attractive targets in this regard. MyD88 and IRAK4 are very broadly involved in signal transduction, and regardless of the type of microbe that is involved global dampening of the LPS signal might be achieved by drugs that inhibit transduction through these molecules [63]. Already, forward genetic data suggest that other 'choke points' may also exist, insofar as some mutations appear to forbid signaling in a relatively broad way (Hoebe K *et al*., in preparation).

Not only infectious diseases but perhaps autoimmune diseases also may be approached by blockade of TLR pathways. Autoimmune exacerbations sometimes occur in the setting of infection, and it is possible that the primary genetic lesions in autoimmunity involve components of the same system that induces cytokine production in the course of an infection. Here, too, forward genetic methods may have an important role to play.

Competing interests

None declared.

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