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Complexities of targeting innate immunity to treat infection

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Innate immunity is an ancient form of host defence that is activated rapidly to enable, through a multiplicity of effector mechanisms, defence against a broad spectrum of microbial threats. From this perspective, innate immunity has desirable characteristics of a therapy against infections, and, as a consequence, the innate immune system has become a major target for the development of therapeutics to control inflammation and immune defences. Although advances in the field have come at a furious pace, and several companies are advancing the first Toll-like receptor-based drugs, there remain many unanswered questions about innate immunity and maintaining balance in the immune response. Indeed, innate immunity represents an enormously complex network of molecules, pathways and interactions, controlled by multiple positive and negative regulatory proteins, which are starting to be evaluated in more depth using systems biology approaches. However, accompanying the protective mechanisms is the production of pro-inflammatory cytokines such that, if excessive amplification of innate immunity occurs, there is the potential for such syndromes as sepsis and chronic inflammation.

Why target the innate immune system?

Innate immunity utilizes a variety of effector mechanisms (Figure 1) to prevent foreign organisms from establishing a niche in the host, operating within minutes to hours to days after exposure. The effectiveness of this system is revealed by the fact that most animals, despite exposure to hundreds of thousands of microorganisms daily, are rarely infected. Some of the earliest recognized deficiencies in the human innate immune system led to incomplete production of reactive oxygen species, complement or antimicrobial peptides. Deficiencies in any of these effectors render people susceptible to infections, proving that the human innate immune system is crucial and often sufficient for defence against infection [1,2]. The potential of the innate immune system as a therapeutic target was recognized when bacterial molecules such as lipopolysaccharide (LPS; also called endotoxin) and peptidoglycan, and their derivatives, were demonstrated to improve the outcome of model infections. More recently, the discovery of Toll-like receptors (TLRs) has revolutionized the investigation and interpretation of the immunology of microbial diseases, and introduced new prospects for therapeutic intervention [3]. There is no shortage of reviews and persuasive arguments to support targeting the innate immune system as a means to treat a wide range of health conditions [3,4].

Despite optimism in the field and a concerted effort by biotechnology and pharmaceutical companies to produce TLR-based drugs, there are relatively few publications to date to demonstrate that targeting the innate immune system can be successfully utilized to treat human maladies. It is our contention that targeting innate immunity as a potential therapeutic or adjuvant strategy has great potential, although there are significant complexities that must be considered [1,2,5]. Components of the innate immune system are redundant, rapidly evolving, complex and intimately related to the inflammatory response, adaptive immune system and other systems in the body (e.g. endocrine, circulatory, nervous). It is also worth remembering that innate immunity is designed to be self-limiting, in that, with a single stimulus, amplification of pro-inflammatory responses is followed by an anti-inflammatory dampening of this response; this Yin and Yang aspect of innate immunity reflects the complexity of this system and the large number of checks and balances in the system involving multiple positive and negative regulators. When these balances break down, some of the most serious threats to health can arise, including long-term pain, chronic inflammatory diseases, sepsis and endotoxic shock. In our opinion, the main question is no longer why or if, but how the innate immune system should be targeted in a way that is mindful of the complexity and duality of the system. Immune-based approaches that ignore these complexities and attempt to amplify or inhibit innate immunity might well be beset by unpredictable outcomes ranging from ineffectiveness to severe effects that compromise immunity or damage host tissues. We propose that therapeutic targets with more promise must be designed to modulate selectively aspects of innate immunity while maintaining a balance between positive and negative regulatory pathways of the response; this is discussed later, in the context of anti-bacterial therapeutics [6].

Innate immunity is complex: the case for a systems biology approach

The mere identification of effective modulators of immunity is a challenge in the absence of comprehensive maps of innate immunity pathways. Innate immunity is often represented as a simple cascade of events in which a single receptor–ligand interaction triggers one or two signal

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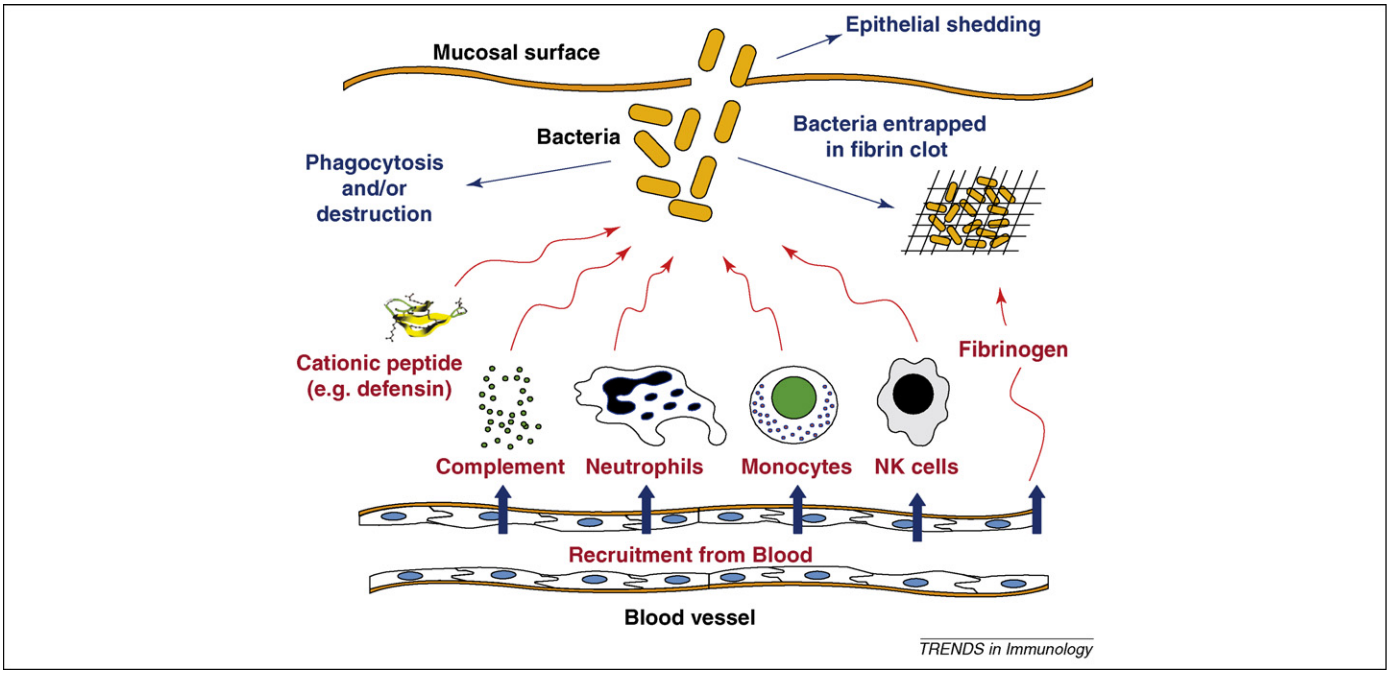

Figure 1. Major innate immune effector mechanisms involved in clearing bacteria. Several other mechanisms exist [1,2], including tissue cytokines and cells, but are not as generally applicable as those illustrated here. Recruitment from the blood is accomplished through vascular permeabilization (accomplished by histamine and other agents released by degranulation from tissue mast cells) and, for cells, chemoattraction (a function of such elements as cytokines released from tissue cells, cationic host defence peptides and complement fragments).

transduction pathways, activating certain key transcription factors and ultimately resulting in a defined downstream response. This simplistic view, however, is fundamentally misleading. Innate immunity-stimulating ligands, for example, often bind to multiple receptors; an excellent example of this is peptidoglycan, and fragments thereof, which are known to bind to TLR2, Nod proteins and peptidoglycan recognition proteins [2,7]. Similarly, although the innate response to LPS is typically depicted as utilizing the TLR4 to nuclear factor κB (NF-κB) pathway, there are in fact multiple pathways employed in LPS responses, including the obligate involvement of the p38 and extracellular signal-regulated kinases Erk1 and Erk2 mitogen-activated protein kinase pathways, the phosphatidylinositol 3-kinase (PI 3-kinase) pathway and molecules from other known signalling pathways, such as the oncoprotein kinase Tpl2, Bruton's tyrosine kinase (Btk), the Rho GTPase Rac and NF-κB-interacting kinase (NIK) [8,9]. This complexity further extends to the level of transcription factors; NF-κB alone involves five subunits acting as homo- or heterodimers with several positive or negative regulatory influences [10], and numerous other transcription factors, including activator protein-1 (AP1), interferon (IFN) regulatory factor-1 (IRF1), IRF3, early growth response factor-1 (EGR1), CCAAT-enhancer binding protein (CEBP), cyclic AMP-responsive element-binding protein (CREB), serum response factor (SRF) and the Ets oncogene family member Elk1, are also activated downstream of these LPS-responsive pathways. Several hundreds of downstream genes are up- or down-regulated in response to these transcription factors [11–15], yet we understand the function of only a modest subset of these. The broad specificity of innate immunity, and the range of context-dependent responses displayed to specific stimuli, hint at the enormous complexity of the innate immune interaction network (Figure 2; Table 1). Although a comprehensive map of this network has yet to be publicly released, the Institute for Systems Biology (ISB) has described an in-house interaction database comprising 5200 biomolecules and 17 600 interactions [11], and Oda and Kitano [12] have published a map of the TLR and interleukin (IL)-1 receptor signalling networks containing a total of 652 biomolecules and 444 interactions. A more complete network, including alternative receptors and downstream pathways, will undoubtedly be far more complex.

Thus, the safe design and application of molecules that productively influence (modulate) innate immunity should ideally involve an attempt at understanding the impact of these molecules on all aspects of innate immunity; to enable this, we propose that there is a need to understand the systems biology of the innate immune network. Systems biology is the comprehensive analysis of a pathway, process, organelle, cell or organism. It integrates multiple experimental methods, firstly to identify the biomolecular interaction network underlying a system of interest and to then quantify the dynamic behaviour of network components in response to a specific stimulus or perturbation. In an iterative process, experimental data are examined in the context of a network, typically using bioinformatics techniques, and a mathematical model and/or hypothesis explaining the behaviour of the system can be proposed and experiments designed to refine the model further.

Microarray-based interrogation of the transcriptional impact of innate immune stimulation has been used to analyse the response to microbes, TLR agonists and host defence peptides [11,13–15]. Through clustering and mapping of quantitative data to existing interaction networks, trends not apparent in the microarray data alone have been identified. For example, Calvano *et al.* [13] identified a set of LPS-responsive genes in leukocytes, and, by mapping

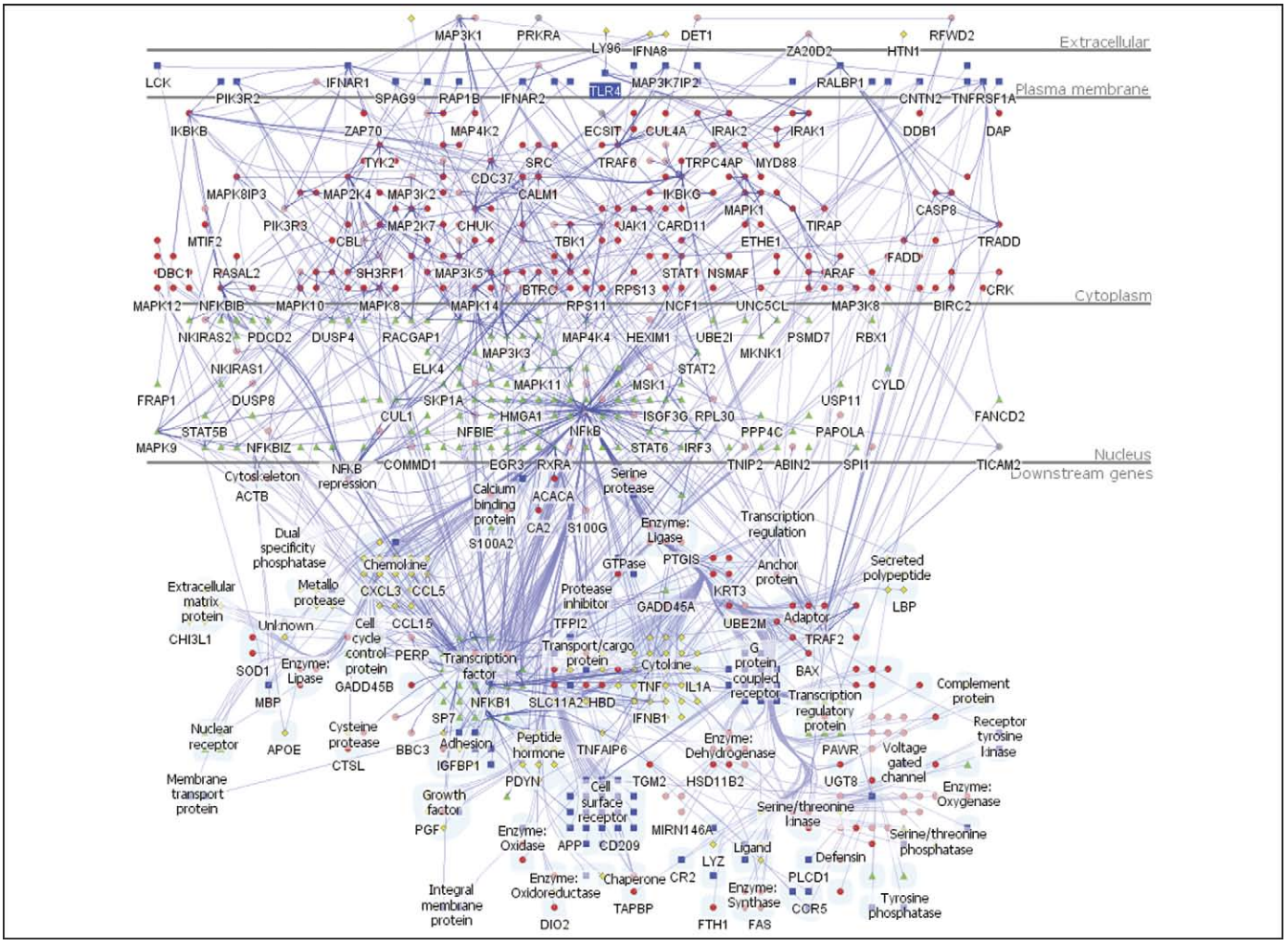

Figure 2. The TLR4–MAPK–NF-κB interaction network, containing 772 nodes (biomolecules) and 1387 edges (interactions involving complex formation, transcriptional responses or biochemical modification such as phosphorylation involved in signal transduction). This illustrates the complexity in what is only a small subset of pathways that comprise the innate immune system. The network is presented as a cross-sectional view of the cell, with the nodes in the 'Downstream genes' region representing genes activated by transcription factors in response to particular stimuli. Genes in this region are further grouped according to function. Nodes are shaped and coloured according to subcellular localization – yellow diamonds (extracellular), blue squares (plasma membrane) red circles (cytoplasm), green triangles (nuclear) and grey circles (localization unknown). Graphic generated using Cytoscape and the Cerebral [47] Cytoscape plugin. Abbreviation: NFkB, NF-κB.

these to a commercially available interaction database, compartmentalized the innate responses to LPS into multiple functional modules. Other *in silico* analyses by Gilchrist *et al.* [11] led to the identification of activating transcription factor-3 (ATF3) as a negative regulator of certain pro-inflammatory genes, a prediction that was confirmed experimentally. These two early studies make a compelling argument for the use of more global approaches to understand innate immunity. Acquisition of such information will certainly expedite identification and development of therapeutics that manipulate this process, and, based on connectivity to the pro-inflammatory aspects of the response, systems biology might enable the investigator to select targets with minimal clinical side effects.

Table 1. Selected innate immunity-related bioinformatics resources

Resource	URL	Description
Pathguide	http://www.pathguide.org	Comprehensive listing of interaction, network and pathway resources, both commercial and public
Cytoscape	http://www.cytoscape.org	Open-source tools for the visualization and analysis of biological networks
ImmPort	http://www.immport.org	Provides a list of 4274 immunity-involved human genes
SepticShock.org	http://www.septicshock.org	Provides a list of 1580 immunity-involved genes and other systems biology software resources
Immune Response *In Silico*	http://share.gene.com/clark.iris.2004/iris/iris.html	Provides a list of 1562 immunity-involved human genes

Effective targeting of innate immunity to treat bacterial infections

We have reached a crucial time when the inexorable rise of antibiotic resistance, combined with a steady decline in the rate of discovery of new antibiotics, is severely threatening our healthcare system [16]. Thus, there is no doubt about the need for, and potential benefit of, conceptually novel therapeutic strategies against microbial infections. Because innate immunity is activated rapidly, involves a broad spectrum of diverse mechanisms (discouraging resistance development) and promotes the normal activation of an adaptive immune response (as a precautionary measure for recurrent or progressive infections), it has many of the characteristics of an ideal antimicrobial therapeutic. The selective boosting of the innate immune response (increasing infection-fighting mechanisms while suppressing harmful inflammatory responses) represents a conceptually advantageous approach towards the development of a new generation of antimicrobials.

Therapeutics to modify innate immunity, such as interferons, colony-stimulating factors, adjuvants, activated protein C (Xigris) and imiquimod (a TLR7 agonist), are already being marketed, although not for the treatment of bacterial infections. Others that have particular promise include TLR agonists and other agents that stimulate TLR-activated pathways (e.g. viable organisms), cationic host defence peptides, and colony-stimulating factors, growth factors, chemokines and regulators of cell death that enable the directed expansion and recruitment of effector cells that would normally be activated by the innate immune system [17,18].

TLR agonists are showing substantial promise as immune modulators. Most TLR agonists (especially ligands of TLR7 and TLR9) on the market or in the late stages of development are vaccine adjuvants or are directed against cancers, viruses or asthma [3,4,19]. Such agonists include the peptidoglycan subunit muramyl dipeptide, LPS-derived monophosphyl lipid A (MPL), fungal cell wall β-glucans and a wide range of synthetic agonists of TLRs [19,20]. Similarly, species-specific CpG oligonucleotides, representing a bacterial signature DNA sequence, function through TLR9 to enhance host resistance to bacterial (and viral) infections. CpGs have demonstrated efficacy in experimental infection models against a range of pathogens and in models of intra-abdominal polymicrobial sepsis [21], although, generally speaking, they need to be applied before the onset of infection for effective 'priming' of innate immunity. It was recently reported that the TLR7 agonist imiquimod enhanced local innate immunity and decreased the duration of infection of *Chlamydia trachomatis* in a murine model of female genital tract infection [22]. Furthermore, it is now accepted that the endogenous microflora possess immune modulating capabilities, and probiotics are widely used as a way to stimulate the mucosal immune system [23–25], being arguably the most ancient method of stimulating immunity. Lactic acid bacteria have been successfully used in defence against a wide range of conditions, including *Helicobacter pylori* infections, cancer and inflammatory bowel diseases [26]. Probiotics have been further shown to assist in maintaining homeostasis of female genitourinary tract and oral cavity health [27]. However, it should be noted that the presence of TLRs does not always confer protection against infection; TLR3, a pattern recognition receptor proposed to be crucial to innate immunity to certain viruses, actually promotes entry of West Nile virus into the brain, and lethal encephalitis [28], and contributes to a detrimental inflammatory response and reduced survival to influenza [29].

A class of anti-infective modulators that function independently of TLRs are cationic host defence ('antimicrobial') peptides. Some of these host defence peptides have direct antimicrobial activity, although others seem to have more profound broad immunomodulatory roles [30]. They represent ancient components of innate immunity, as judged by the impact on susceptibility to infection of their depletion in certain human syndromes, such as specific granule deficiency and morbus Kostmann disease, and in transgenic mice and *Drosophila* [31]. When utilized therapeutically, they can prevent endotoxaemia, in addition to resolving bacterial infections in rodents, and thus serve as templates for the synthesis of small immunomodulatory peptides that have great promise to treat infectious diseases. For example, a 13-amino acid synthetic peptide without direct antimicrobial activity was recently developed which protected against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) by acting on the innate immune system [32]. Depletion experiments indicated that monocytes and macrophages, but not neutrophils or lymphocytes, were crucial to protection, and in infected animals the peptides induced increases in the monocyte chemokine monocyte chemoattractant protein-1 (MCP-1) and the anti-inflammatory cytokine IL-10, while decreasing the levels of pro-inflammatory cytokines tumour necrosis factor α (TNF-α) and IL-6. *In vitro* experiments presented a consistent picture, while also expanding on the mechanism of action, and detailed microarray experiments are enabling a systems biology analysis.

Similarly, the TLR7 agonist imiquimod, an approved drug for genital warts and basal cell carcinoma, also promotes an inflammatory response and the expression of chemokines such as CXCL10 [also known as interferon-inducible protein of 10 kDa (IP-10)], CXCL11 [also known as interferon-inducible T cell α chemoattractant (ITAC)], CC chemokine ligand (CCL) 8 (also known as MCP-2), CCL3 [also known as macrophage inhibitory protein-1α (MIP-1α)], CCL4 (also known as MIP-1β), CCL5 [also known as regulated on activation, normal T cell-expressed and -secreted cytokine (RANTES)] and CXCL12 [also known as stromal cell-derived factor-1 (SDF1)] to chemoattract immune cells to kill cancerous cells [33]. Enhanced recruitment of leukocytes was also recently demonstrated in $CCR4^{-/-}$ mice, which are resistant to LPS-challenge and infection [34]. Similar to the peptides, the exact mechanism of defence in $CCR4^{-/-}$ mice (beyond enhanced cellular recruitment) is unknown. Of interest, TLR signalling and activation of NF-κB was suppressed in $CCR4^{-/-}$ macrophages, yet the cells produced elevated levels of anti-inflammatory and regulatory cytokines and chemokines that the authors suggest is the result of the stimulation of both the p38 and the c-Jun N-terminal protein

kinase (JNK) mitogen-activated protein kinase (MAPK) pathways. Similarly, we have shown that host defence peptides activate p38, and Erk1 and Erk2, and suppress LPS-induced translocation of NF-κB in monocytes and macrophages, diminishing the transcription and release of pro-inflammatory mediators yet enabling the transcription of chemokines and negative regulatory molecules of the TLR pathway [14,31,32]. Chemokines not only attract subsets of leukocytes, but might also exert immunomodulatory effects of their own. For example, CXCL10 and CXCL4 [also known as platelet factor 4 (PF4)] inversely control the expression of transcription factors t-bet and GATA-3, and thereby regulate the expression of T helper (Th) 1 and Th2 polarizing cytokines [35]. These studies indicate that non-TLR-mediated responses through chemokines and accessory signalling pathways (Erk1 and Erk2, and JNK) are potential therapeutic targets to boost innate immunity effectively [9], and, indeed, some chemokines have been demonstrated to exert therapeutic activity against animal models of infection (e.g. Ref. [36]).

As attributed to Janeway [48], 'innate immunity instructs adaptive immunity', in that innate immunity can function as a preparation for the transition to antigen-specific immunity, although there must be mechanisms to limit this transition because it does not always occur. Contrary to commonly held beliefs, the interplay between innate and adaptive immunity might not be entirely dependent on TLR activation but involve other agents of innate immunity. This is apparent in a report by Gavin *et al.* [37], which demonstrates robust antibody responses to challenges *in vivo* in the absence of TLR signalling.

Unfortunately, with few exceptions, the action of these anti-infective, immune-boosting agents is only partially understood, leading to the potential for adverse events such as death (observed with cytokine therapy) and extreme reactions (e.g. sepsis and cytokine storms) [38,39]. Indeed, in North America each year, sepsis afflicts more than 750 000 individuals, causing as many as 215 000 deaths, with repetitive attempts to suppress the immune and inflammatory responses enjoying limited success [38]. However, TLR antagonists have recently been shown to be effective anti-sepsis agents – for example, eritoran (a TLR4 antagonist [40]) and a monoclonal antibody against TLR2 [41]. In addition, TLR2-antisense therapy protected against the detrimental effects of inflammation induced by ischaemia and reperfusion injury [42]. The concern with these agents is that they block components of the immune response that are certainly required to ward off subsequent infection and to overcome the septic state. Although monotherapies such as anti-TNF-α therapy have been largely ineffective at controlling sepsis, they have had substantial success in treating rheumatic disease, proving the systems biology concept that a single molecular target can have a significant impact on inflammation and immunity, depending on the context in which it is used (i.e. the other influences affecting the innate immune network). It should be noted that such therapies can be associated with side effects. In particular, patients on anti-TNF-α therapy became more susceptible to bacterial infections [43], again illustrating the complexity of the innate immune system and dangers associated with an unbalanced immune response.

Avoiding the cytokine storm and sepsis

Historically, immune-based therapies (e.g. IL-2 and IFN-α) have been plagued by unwanted side effects, thereby limiting their use to situations in which the benefit outweighs the potential harm [18]. Safe, efficacious drug targets that boost the innate immune response must fail to induce, or ideally dampen, the toxic arm of the innate immune response (inflammation) to reduce side effects, while maintaining immunostimulatory power. Because the normal inflammatory response to infection is primed by bacterial signature molecules (TLR ligands), one major concern for immune-boosting therapeutics is whether they will amplify the infection-induced inflammatory response, leading to a cytokine storm or sepsis, thus producing unfavourable outcomes. Based on the current successes (or failures) of immune-based drugs, effective immune-boosting therapeutics with limited side effects might share certain general properties (Table 2). These agents could either actively suppress pro-inflammatory responses, as demonstrated for host defence peptides, or mediate a more modest activation of immunity that is not accompanied by considerable induction of pro-inflammatory cytokines, as observed for CpG oligodeoxynucleotides [21] and other

Table 2. Properties of TLR-based therapeutics that safely boost immunity

Property	Anti-infective effect	Anti-inflammatory considerations
Immunostimulatory	Boost immunity	Should favour low-affinity agonists or negative allosteric modulators of TLRs
Multifunctional	Effective against multiple infectious agents; enhanced potency at reduced concentrations	Might promote anti-inflammatory processes
Target modulators of immunity	Precise control over elements of the immune and inflammatory response	Avoid modulators that promote expression of TLRs or endogenous TLR agonists; target modulators with an automatic negative feedback loop; avoid targeting conserved or ubiquitously expressed molecules or domains
Promote expression of chemokines	Alter the nature of the response by promoting infiltration and activation of particular subsets of leukocytes	Promote expression of anti-inflammatory agents (cytokines or negative regulators of TLR signalling) or administer with natural anti-inflammatory compounds (e.g. probiotics)
Ensure transition to adaptive immunity when needed	Engage cells and regulators of the adaptive immune system to promote humoral and cellular immunity	Promote expression of Th1 over Th2 cytokines

TLR agonists, such as MPL [19]. The important lesson from such molecules is that protection against infections can be achieved without a traditional inflammatory response and the attendant risks.

TLR-based therapies in development for the treatment of asthma and allergy, or as vaccine adjuvants, should provide insight into the mechanisms underlying these effects, and particularly how toxicity can be avoided. Factors such as the relatively short life span of peptides, the expression of particular TLRs on a limited number of cells, and/or low toxicity towards eukaryotic cells might also reduce nonspecific and prolonged systemic effects. Along the same lines, immune-based targets that positively regulate their own expression or that of TLRs or endogenous TLR agonists should be avoided. Similarly, it is important to examine, in the context of the microbes (or their TLR-interacting signatures), the impact of interaction with cells of prospective therapeutic agents because there is always a potential that such agents will reinforce microbe-induced inflammation. Conversely, therapeutics that also antagonize specific molecules in the negative feedback loop of TLR signalling [e.g. TNF-α inhibitory protein 3 (TNFAIP3; also known as A20)] are of considerable interest, and might prove useful in the treatment of infection and inflammatory diseases [9,44], as might medicinal anti-inflammatory remedies such as astilbin (which induces IL-10) [45] and epigallocatechin gallate (which induces monocyte apoptosis) [46], in addition to natural remedies, such as *Echinacea*, or viable probiotic bacteria.

Conclusions and future studies

There is no doubt that the boosting of innate immunity offers great potential but must be approached with caution. Thus, it is a fertile area for future investigations. To realize the potential implicit in targeting innate immunity, it is imperative to understand this process in considerably more detail. Research areas that warrant more attention are those that will shed light on the complex, redundant and finely balanced nature of the innate immune system. Such areas will include systems biology and translational approaches to deciphering innate immunity, and particularly TLR biology, deciphering the impact of modulators regulating inflammation, and of regulatory molecules balancing inflammatory responses, and deciphering the complex mechanisms of key human genetic mutations that alter disease susceptibility. This information is imperative to enable the orderly and safe development of effective therapies and therapeutic targets addressing the innate immune system, and to enable researchers to foresee and avoid the potential side effects associated with different therapies.

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