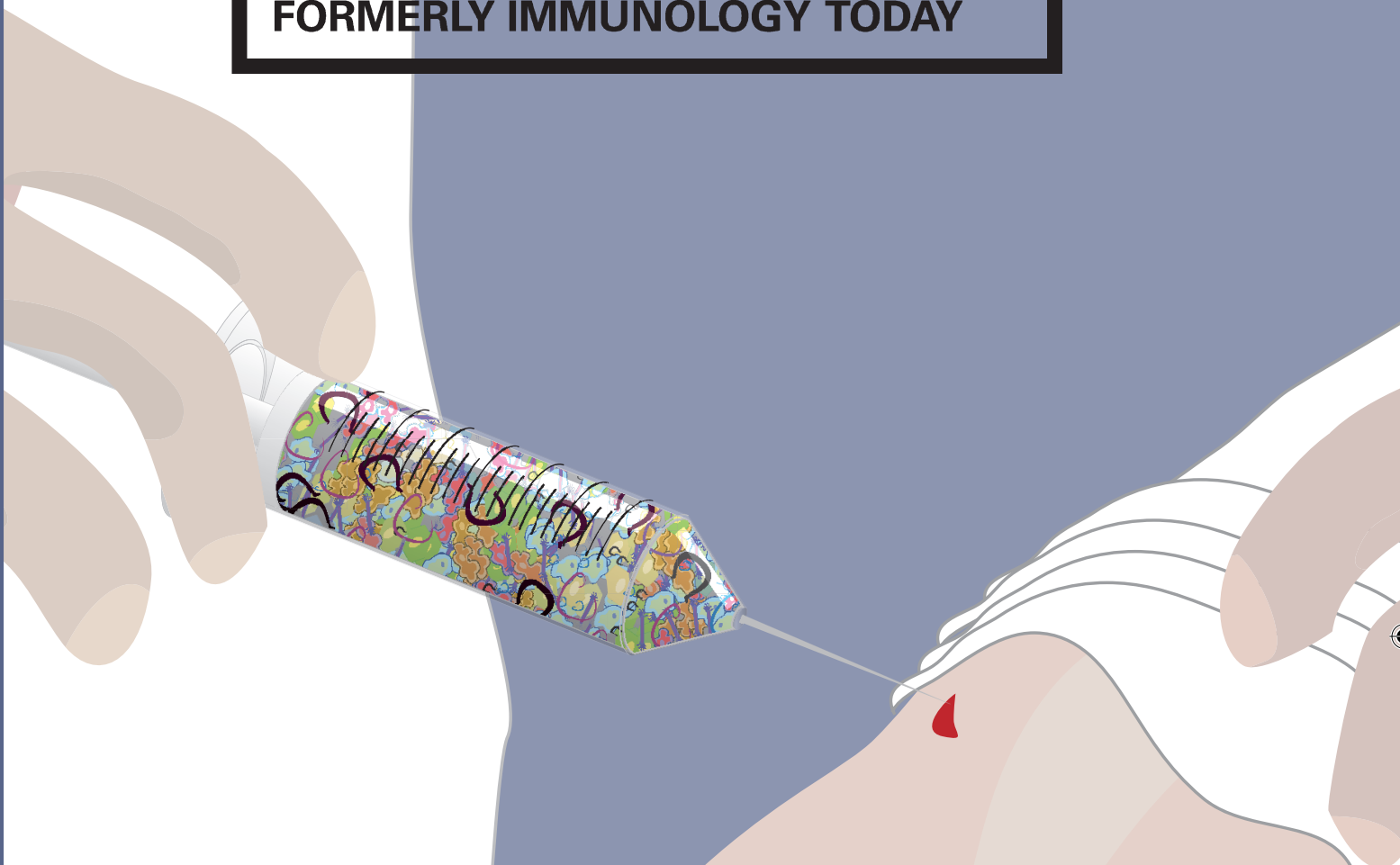




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Pathogen-derived immunomodulatory molecules: future immunotherapeutics?

Padraic G. Fallon¹ and Antonio Alcamí^{2,3}

¹ School of Biochemistry and Immunology, Trinity College Dublin, Dublin 2, Ireland

² Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, 28049 Madrid, Spain

³ Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, UK, CB2 2QQ

The identification of molecules from various pathogens that modulate innate and/or adaptive immunity is a dynamic and rapidly developing area of research. These immunomodulatory molecules (IM) have been optimized during pathogen–host co-evolution, and have a potential application as novel immunotherapeutics. In this review, we illustrate the use of pathogen IM that have been produced as recombinant proteins, with different modes of modulatory activity, and discuss their potential to modulate undesirable immune responses in human diseases.

Introduction

Some of the major human diseases are caused by malfunctions in the immune response. The immune system did not evolve to cause these diseases but developed primarily to control bacterial, viral, fungal and parasitic infections. In modern societies, there is a greater prevalence of certain diseases, for example asthma [1], that are associated with dysregulated immunity. One hypothesis that explains the increase in these immune-mediated diseases in developed countries is the effect of the immune system functioning in environments with reduced infectious diseases [2]. Thus, an active area of research is the investigation of pathogen modulation of immune processes that overlap with protective or exacerbating inflammatory responses associated with other diseases. Ultimately, understanding the mechanisms that pathogens have evolved to manipulate immunity might result in new therapies for inflammatory diseases.

The use of pathogens as therapeutics is well established through the exposure of people to live or attenuated pathogens as vaccines for infectious disease. An extension of this strategy is using the potentially desirable immune-modulating effects of pathogen infection for treating unrelated inflammatory diseases. In fact, patients with inflammatory bowel diseases or allergic rhinitis are being deliberately infected with parasitic worms to evaluate their therapeutic use [3,4]. Instead of infecting people with pathogens, with the inevitable risk of side effects, a more-rational approach is to identify the immunomodulatory molecules (IM) that

selectively mimic the desirable effects of infection as a novel therapeutic approach.

In immunology, the archetypical example of pathogen–host adaptation is the delineation of distinct and overlapping functions of the innate-immunity receptors, and their downstream signalling pathways, in selectively recognizing pathogens [5]. The specificity of Toll-like receptor (TLR) recognition of pathogen-associated molecular patterns (PAMPs) from various pathogens is remarkable, as illustrated in bacteria (lipopolysaccharide through TLR4, or CpG DNA through TLR9), viruses (double-stranded RNA through TLR3), fungi (Zymosan through TLR2 or TLR6) and parasites (*Toxoplasma gondii* profilin-like protein through TLR11, or *Plasmodium* species' hemozoin through TLR9). Indeed molecules from the pathogens, for example, bacterial CpG or the Th2 cytokine-inducing glycans from parasitic worms, constitute some of the most powerful natural activators of innate-adaptive immunity, and are potential adjuvants. Here, we will not address PAMPs in immune modulation because these have been described extensively previously [5]. Although immunomodulatory activities have been identified in pathogens, in this review, we will only address pathogen IM that have been produced as recombinant proteins, and use selected examples of molecules with different mechanisms of modulation.

Which pathogen to source an IM?

A PubMed search (<http://www.ncbi.nlm.nih.gov/>) would indicate that, in descending order, there are more recombinant IM characterized in viruses, bacteria and parasites, and few in fungi. This ranking is somewhat arbitrary, as the number of IM discovered to date in different pathogens is owing to a multiplicity of factors, including the medical importance of the disease caused by the pathogen (this might influence the amount of research funding and number of scientists active in the area), the size of the pathogen genome, the ease of laboratory studies of the pathogen, and the availability of appropriate animal models. Nevertheless, it demonstrates that different pathogens would have evolved various immune-evasion strategies involving selecting a range of IM with distinct or overlapping modes of modulatory activity. Although there are potent

Corresponding author: Fallon, P.G. (pfallon@tcd.ie).

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Table 1. Examples of IM from various pathogens classified by mechanism of modulation^a

IM	Species	Modulatory activity (therapeutic efficacy)	Refs
Protease inhibitor			
Serp-1	Myxoma virus	Inhibits inflammation (in Phase II trials on patients with acute coronary syndrome)	[77]
Complement inhibitor			
VCP	Vaccinia virus	Blocks complement activation (reduces transplant rejection and CNS damage in animal models)	[15]
CHIPS	<i>Staphylococcus aureus</i>	Binds to C5a and formylated peptides	[20]
SCIN	<i>Staphylococcus aureus</i>	Binds to C3 convertases	[19]
Cytokine and chemokine homologues			
vIL-10	Epstein-Barr virus	IL-10 homologue (suppression inflammation in various animal models)	[21]
vMIP-II	Human herpesvirus 8	Chemokine homologue (inhibits chemokine-mediated responses)	[40]
C-18	<i>Toxoplasma gondii</i>	CCR5 ligand (blocks HIV infection of human cells <i>in vitro</i>)	[34]
pTSP, pMMP	<i>Plasmodium</i> species	Converts latent TGF- β to active form	[30]
CKBPs			
M-T7	Myxoma virus	Binds to CC, CXC and C chemokines (prevents atherosclerotic plaque formation)	[48,49]
35 kDa	Vaccinia virus, myxoma virus, cowpox virus	Binds to CC chemokines and inhibits chemokine-receptor interactions (prevents transplant vasculopathy and airway inflammation)	[48,49]
M3	Murine gammaherpesvirus 68	Broad spectrum chemokine inhibitor, blocks interaction of chemokines with specific receptors and GAGs (inhibits transplant vasculopathy, atherosclerotic plaque formation and skin inflammation)	[48,49]
SmCKBP	<i>Schistosoma mansoni</i>	Inhibits CC, CXC and C chemokines and binds to GAGs (suppresses acute inflammation in mice)	[60]
Cell signalling			
A52R	Vaccinia virus	Inhibits TLR activation of NF- κ B (blocks inflammation in mice)	[65,67]
YopJ	<i>Yersinia</i> species	Blocks MAPK and NF- κ B activation	[70,71]

^aAbbreviations: pTSP, *Plasmodium thrombospondin*-like molecule; pMMP, *Plasmodium* matrix metalloproteinase.

immune-modulator extracts in fungi, including complement modulation by *Cryptococcus neoformans* polysaccharide capsule [6], few fungal recombinant proteins with IM activity have been described. It is not our intention to address all pathogen IM in this review; we include selected examples of IM from viruses, bacteria or parasites, grouped by modulatory activity (Table 1).

Serp-1 – a viral IM in clinical trials

Myxoma virus, a poxvirus of rabbits, encodes a secreted serine-protease inhibitor (serpin) termed Serp-1. Serp-1 could be the first pathogen IM available to patients. Serpins constitute up to 10% of plasma proteins and regulate numerous pathways including coagulation, fibrinolysis, complement activation and inflammation [7]. Serp-1 inhibits a variety of human proteases, and its anti-inflammatory potential has been tested extensively in animal models. Serp-1 blocks atherosclerotic-plaque growth in models of arterial trauma, being efficient when administered at a single picogram-to-nanogram dose or systemically by intravenous injection [8,9]. Serp-1 also has beneficial effects in aortic and heart-transplant models, and a model of antigen-induced arthritis [10–12]. Studies of Serp-1 have established that viral IM can be effective at low doses, and have the capacity to reduce the magnitude of the initial pro-inflammatory stimuli and the subsequent chronic inflammatory response that causes immunopathology after physical trauma. Serp-1 is currently undergoing a Phase II clinical trial (Viron Therapeutics Inc.) on patients with acute coronary syndrome. Although a serpin is also secreted from the parasitic filarial nematode *Brugia malayi* [13], the recombinant protein does not have inhibitory activity [14].

IM inhibitors of the complement cascade

Several different pathogens have developed mechanisms for blocking the complement system. Poxviruses and herpesviruses have acquired complement-regulatory proteins from their hosts to control the early activation of the complement at the infection site [15]. The secreted vaccinia complement-control protein (VCP) and the orthologue encoded by cowpox virus, known as inflammatory modulatory protein, have anti-inflammatory properties. VCP prolongs survival after heart transplants, a situation in which complement responses mediate hyperacute rejection and limit the potential for xenotransplantation [16]. VCP also shows an effect in animal models of injury to the central nervous system [17].

The human pathogen *Staphylococcus aureus* is an example of a bacterium that modulates the complement pathway through the secretion of various IM with different modes of action [18]. Recently, a staphylococcal IM has been described that blocks all complement pathways (lectin, classical and alternative), through interactions between staphylococcal complement inhibitor (SCIN) and surface-bound C3 convertases [19]. Another IM from the same bacterium, called chemotaxis inhibitory protein of *S. aureus* (CHIPS), binds to the complement chemoattractant protein C5a, and also to bacterial formylated peptides, and inhibits neutrophil chemotaxis [20]. These bacterial proteins have a high potential for anti-inflammatory therapy but their activity in animal models has not been reported to date.

Cytokine homologues

Epstein-Barr virus encodes an interleukin (IL)-10 homologue (vIL-10) [21], which, because of the potent suppressive activity of IL-10 [22], is a promising

therapeutic. vIL-10 exhibits immune-inhibitory properties [suppression of T-helper (Th)1 responses and monocyte activation] but has lost the immunostimulatory activity (activation of dendritic and T cells) of the host homologue. vIL-10 has been tested extensively, showing a beneficial effect in animal models of arthritis, allograft transplantation, osteolysis, glomerulonephritis, diabetes, uveoretinitis, venous thrombosis and sepsis [23]. The other viral cytokines tested as potential therapeutics are the chemokine homologues encoded by *Molluscum contagiosum* virus (MC148) and human herpesvirus 8 (vMIP-II). Both chemokine homologues prolong cardiac-allograft survival in mice [24]. vMIP-II also improves the recovery after cerebral ischemia and spinal-cord injury [25,26].

Homologues of migration-inhibitory factor (MIF) have been described in various parasitic worms [27,28], with one of the two MIFs in *B. malayi* shown to be bio-active *in vivo* [27]. To date, there is limited data on the functionality of these parasite-worm-derived MIF homologues in modulating unrelated immune responses.

The anti-inflammatory cytokine transforming growth factor (TGF)- β has been usurped by several pathogens; for example, *B. malayi* encodes two genes with sequence homology to human TGF- β . The product of the *B. malayi* TGF- β gene *tgh-2* is functional, as it binds to mammalian TGF- β receptors, and might have immunomodulatory activity [29]. *Plasmodium* upregulates TGF- β levels in infected hosts by activating latent TGF- β into the bio-active form by secreting two potential IM: a homologue of human thrombospondin-1 and a metalloproteinase [30].

Although it is possible to envisage using a pathogen IM that stimulates TGF- β to suppress systemic or local inflammation, a major concern is the induction of tissue damage by the potent fibrogenic activity of TGF- β . This is in contrast to vIL-10, where the viral protein has the suppressive therapeutic effects of human IL-10, but lacks its undesirable immunostimulatory activities. Analyzing the structure–function difference between vIL-10 and mammalian IL-10 identified a single amino acid, an isoleucine at position 87, that determines the immunostimulatory activity [31]. A mutant human IL-10, an alanine replacing the isoleucine, is suppressive and not stimulatory. The example of vIL-10 reinforces the potential insights into immune function and regulation that can be achieved by dissecting the biological activity of pathogen IM.

As part of the immune-evasion strategies of the parasitic protozoan *T. gondii*, parasite antigens induce a state of ‘paralysis’ in dendritic cells through a CC-chemokine-receptor CCR5-dependent mechanism [32,33]. One of the *T. gondii* IM that mediate this modulation of dendritic cells is C-18, an 18-kDa cyclophilin, which binds to CCR5 [34]. As CCR5 is a co-receptor for HIV-1, C-18 could be a potential therapy for HIV. Indeed, C-18 binds to CCR5 on human cells and thereby inhibits HIV-1 infection of T cells and macrophages [35]. The virus-encoded chemokines (vMIP-1 and vMIP-II) can also block HIV infections [36].

C-18 is a noteworthy example of the progression from basic research on the pathogen modulation of immunity to applied studies for developing the IM as a therapy for a different disease. This pathway includes identifying novel

modulatory activities by the pathogen, isolating crude pathogen extracts showing bioactivity, cloning the functional recombinant IM, confirming its activity, and elucidating the mode of action. The potential application of the IM for other inflammatory conditions requires further investigations. The current structure–function studies elucidating the specificity of binding between C-18 and CCR5 to improve the activity of C-18 as a therapy for HIV [37] are an example of the rational design required to optimize the therapeutic activity of the molecule. However, the use of exploiting C-18–CCR5 interactions as a therapeutic for HIV might be confounded by the recent demonstration of an important protective role for CCR5 during West Nile virus infection [38]. Could the therapeutic use of an IM to treat an inflammatory condition inadvertently result in increased susceptibility to infection with an unrelated pathogen, or, indeed, the pathogen from which the IM was derived? Although we can only speculate on the answer to this question, it is a plausible scenario in light of the re-activation of tuberculosis in a small percentage of rheumatoid arthritis patients treated with anti-tumour necrosis factor (TNF) therapies [39].

Cytokine and chemokine receptors and binding proteins

The expression of secreted proteins that bind to cytokines is an immune-modulation strategy found almost exclusively in poxviruses, with a few examples described in herpesviruses [36,40,41]. Some of these proteins have sequence similarity to the cytokine-binding domain of host cytokine receptors, suggesting that they have been acquired from the host by horizontal gene transfer. These include secreted receptors for TNF, IL-1 β or interferon (IFN)- γ , or the IL-18 binding protein (BP) that resembles the host IL-18BP unrelated to membrane IL-18 receptors. The secretion of soluble versions of cytokine receptors is a strategy used by the host immune system to limit inflammatory responses, and the virus has also incorporated this strategy. However, viral receptors have often unique properties that could enhance their immunomodulatory properties. For example, in contrast to the host soluble receptors, poxvirus IFN- γ and TNF receptors are multimeric and might block the multimeric ligands more efficiently [42,43]. The IFN- α / β BP and IL-18BP from poxviruses are retained locally by interacting with the cell surface, probably enhancing their immunomodulatory activity [40]. Studies on viral cytokine receptors have highlighted physiological functions of cytokines: vaccinia viruses encoding the IL-1 β receptor demonstrated a key role of IL-1 β in controlling fever during poxvirus infections [44], and the viral homologue of CD30 has uncovered a role for CD30 in Th1 inflammatory responses [45].

A second class of viral cytokine inhibitors does not share sequence similarity to host receptors, and includes secreted binding proteins for type I IFN encoded by poxviruses or the colony-stimulating factor 1 binding protein in herpesviruses [40,41]. Yabapoxviruses encode a TNF binding protein (BP) (encoded by the *2L* gene) distinct from mammalian TNF receptors and related to MHC class I molecules that bind to TNF with high affinity and block their activity. The TNFBP interacts with human TNF in a way different to that of human TNF receptors and might

provide an alternative therapeutic strategy for inhibiting TNF [46,47]. An expanding group of these proteins comprises secreted viral chemokine (CK)BPs (vCKBPs) identified in poxviruses and herpesviruses [48,49]. The host chemokine receptors are seven-transmembrane proteins that, in contrast to other cytokine receptors, cannot be secreted after proteolytic cleavage, and, thus, this strategy of immune modulation is exclusive to viruses and parasites. With the exception of the vCKBP encoded by human cytomegalovirus [50], vCKBPs have the peculiarity of binding a broad range of chemokines. The structure of the murine gammaherpesvirus 68 vCKBP M3 complexed to chemokines illustrates how these viral proteins generate a binding domain that mimics the interaction of chemokine receptors with chemokines [51]. There is no sequence similarity among different CKBPs from viruses or from *Schistosoma mansoni* (see later in this section), suggesting that they have evolved independently. The absence of human soluble chemokine receptors, together with the crucial role that chemokines have in the immune response, make vCKBPs attractive therapeutic reagents, which has been demonstrated in animal models of inflammatory diseases. The myxoma virus vCKBP M-T7 reduced macrophage and T-cell invasion, and atherosclerotic-plaque growth at sites of vascular injury after either transplant or balloon angioplasty intervention [52,53]. The therapeutic potential of the 35-kDa poxvirus vCKBP has been shown in models of chronic transplant vasculopathy and airway inflammation [53,54].

The murine gammaherpesvirus 68 vCKBP M3 immunomodulatory activity has been demonstrated in models of aortic allograft vasculopathy and skin inflammation [53,55]. The generation of transgenic mice expressing the vCKBP M3 for delivering the viral chemokine inhibitor represents an elegant alternative approach to injecting purified recombinant protein, and was used to demonstrate the ability of M3 to block intimal hyperplasia in response to arterial injury [56]. The inhibition of inflammatory conditions in transgenic mice expressing the viral protein M3 constitutes a proof-of-principle for the potential therapeutic use of pathogen IM. Moreover, it also demonstrates a role of chemokines in these inflammatory processes.

A novel chemokine-binding domain has been recently identified in the cytokine response modifier (Crm)B protein of variola (smallpox) virus, and has been designated the smallpox virus-encoded chemokine receptor (SECRET) domain [57]. Interestingly, the SECRET domain binds to a narrow set of chemokines, some of which are involved in mucosal and skin inflammation, and is part of the virus-encoded TNF receptors CrmB and CrmD, expressed by some of the most virulent poxviruses (variola virus, monkeypox virus and ectromelia virus). Soluble versions of human TNF receptors are already used clinically to inhibit harmful inflammatory responses, and their therapeutic value has been demonstrated [58]. The finding that poxviruses have added a chemokine-binding domain to their soluble TNF receptors suggests that the addition of this chemokine-inhibitory domain enhances the anti-inflammatory properties of soluble TNF receptors. As TNF has potent pleiotropic activity, it is not unexpected that a

range of IM from various bacteria and viruses have been described that modulate TNF activity [59].

The first non-viral CKBP was detected in the human trematode parasite *S. mansoni*. smCKBP is secreted from the eggs of *S. mansoni*, and no other parasite life-cycle stage, and was also detected in eggs from *S. haematobium* and *S. japonicum*, the two other major schistosome species that infect humans [60]. smCKBP binds selectively to certain members of the chemokine sub-families and to the glycosaminoglycan heparan. Recombinant smCKBP suppressed inflammation induced in a mouse contact-hypersensitivity model and blocked CXCL8-induced pulmonary inflammation [60].

Since the discovery of chemokines, the development of small-molecule antagonists or antibodies that inhibit chemokine–receptor interactions has generated great interest [61,62]. Several small-molecule inhibitors targeting specific chemokine–receptor pairs are in Phase II or Phase III clinical trials. The chemokine system is complex, with ~50 ligands and 20 receptors, and we are still learning the therapeutic benefit of targeting specific chemokine–receptor interactions. The apparent redundancy of the chemokine system makes the broad chemokine specificity of CKBPs encoded by pathogens an attractive option for modulating the system. Various pathogens opt for proteins that inhibit several chemokines, and we might learn from these strategies which combination of chemokine inhibitors provides a therapeutic advantage for treating inflammatory diseases.

IM modulation of intracellular signalling

Intracellular pathogens can release IM directly within the cell, where they can modulate various cellular processes, including signalling pathways [63]. Any pathogen IM that can modulate cell signalling could have a particular application as a therapeutic in inflammatory diseases [64]. One IM that modulates signalling is A52, a protein encoded by vaccinia virus [65]. A52 is a multifunctional IM that blocks TLR-induced nuclear factor (NF)- κ B activation and also activates TLR-induced IL-10 [66]. The ability of A52 to inhibit TLR signalling has been tested as an anti-inflammatory, with a peptide from A52 reducing disease in a mouse model of bacterial-induced ear inflammation [67].

Whereas viruses can readily modulate intracellular processes, extracellular pathogens must develop mechanisms of getting the IM into the cell (Figure 1). Bacteria have developed multiple novel mechanisms for translocating IM into the host-cell cytosol [68]. One example is the type III secretion system (TTSS) used by several pathogenic Gram-negative bacteria, which facilitates the translocation of a range of virulence proteins, including modulators of eukaryotic signalling, directly into the cytosol of host cells [68]. For example, pathogenic *Yersinia* species use the TTSS to deliver *Yersinia* outer proteins (Yops) with various IM into the cell [69]. YopJ suppresses pro-inflammatory responses by inhibiting mitogen-activated protein kinase (MAPK) and NF- κ B pathways, through the acetylation of MAPKK6 or de-ubiquitinating NF- κ B [70,71].

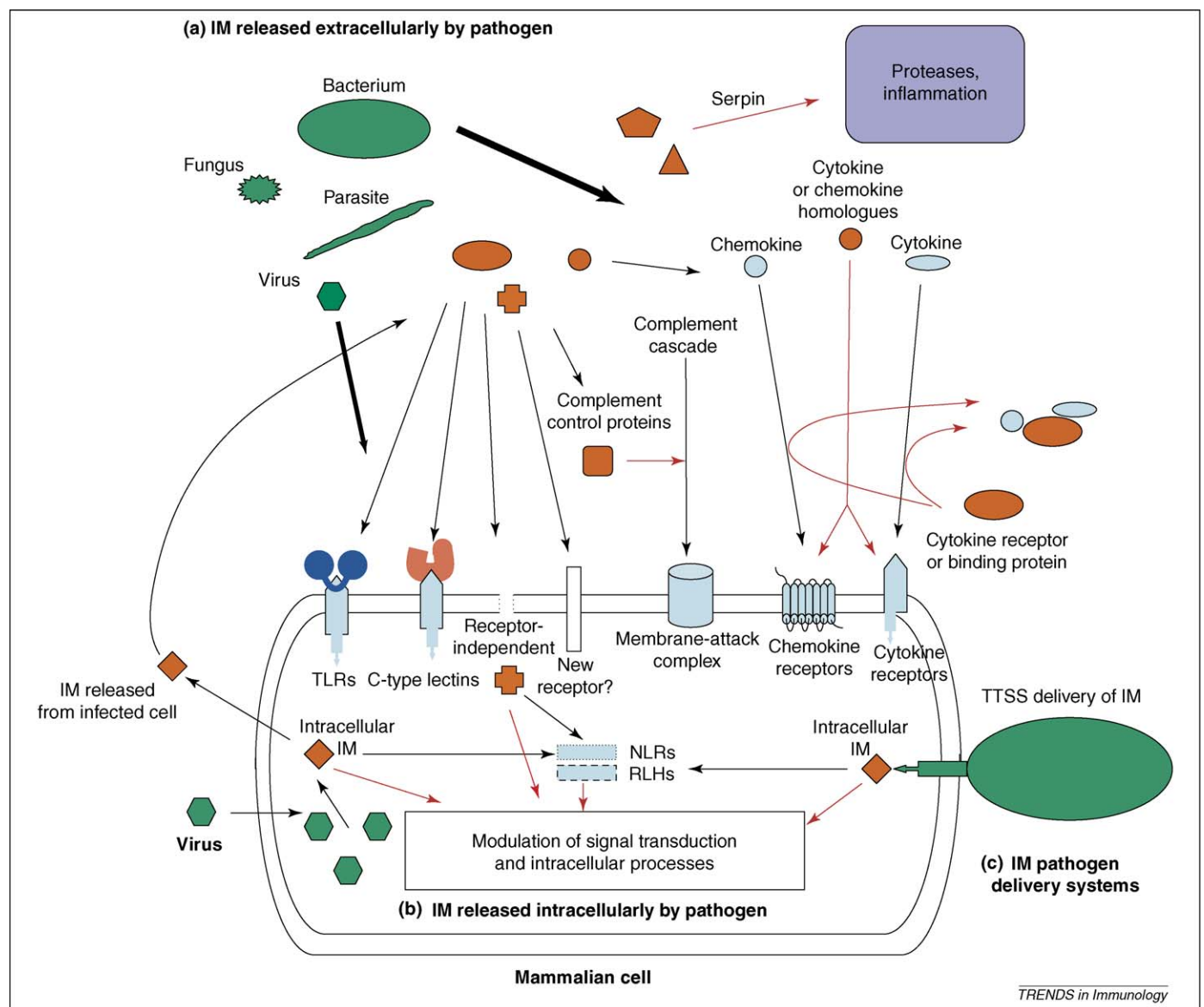


Figure 1. Examples of potential extracellular or intracellular mechanisms of modulation by IM from viruses, bacteria, parasites or fungi. The pathogens can directly activate TLRs, C-type lectins or other receptors, and induce signalling resulting in the activation of innate responses. **(a)** Pathogens will release IM that can modulate immunity by altering immune processes outside the cells, or through the released IM directly interacting with cells through various receptor-dependent or -independent mechanisms. Secreted IM might inhibit proteases (through serpins) and the inflammatory response, or block the complement cascade and the formation of the membrane-attack complex by interacting with components of the complement system (through complement control proteins). Some viruses and parasites encode homologues of cytokines or chemokines that bind to cellular receptors inducing or blocking signalling pathways. Viruses might encode secreted versions of cytokine receptors that neutralize the activity of cytokines and chemokines. **(b)** Pathogens that reside within the cell might release the IM intracellularly where cell processes can be modulated, for example, interactions with NLRs or RLHs. Alternatively, the IM is released from the infected cell and is functional extracellularly. IM might interact with cells through receptors on the surface or use receptor-independent mechanisms. **(c)** Pathogens can use delivery systems to get the IM into the cells; shown is the example of TTSS used by bacteria. Pathogens are indicated in green. Cellular proteins are represented in blue. IM encoded by pathogens are in red. Red arrows indicate inhibition of immune pathways. Abbreviations: IM, immunomodulatory molecule; NLR, NOD-like receptor; RLH, RIG-like helicases; TLR, toll-like receptor; TTSS, type III secretion system.

General discussion

Throughout evolution, pathogens have learned the molecular mechanisms crucial in immunity, and have optimized strategies to counteract immune pathways. A better understanding of how pathogens modulate the immune response should provide insights into the mechanisms of immunity and new strategies for immune modulation. As discussed here, we can use this information to design new therapeutic strategies to modulate immunopathological reactions that cause human diseases. Furthermore, in some instances, we might be able to use the IM encoded by pathogens as therapeutic reagents,

particularly in those cases for which no human homologues have been identified.

It is likely that the pathogen-derived IM induce antibody responses that might neutralize their activity and/or compromise their half-life, limiting their therapeutic value for chronic, but not acute, disease conditions. However, all human or non-human proteins currently licensed as human therapeutics show some degree of immunogenicity in patients [72]. Thus, several strategies that are being investigated to reduce the immunogenicity of therapeutic proteins, including attachment of polyethylene glycol (PEGylation), epitope removal, humanization and

tolerization, could also be used for pathogen IM. Similar to any new bio-therapeutic of human origin, the limitation of the potential immunogenicity of pathogen products should be evaluated in each case.

The IM encoded by pathogens have been optimized to work in the context of infection. IM that function intracellularly during infection can be released into the cytosol by a virus, or introduced by the pathogen itself, for example, YopJ is delivered by bacterial TTSS. Therefore, a possible drawback for the potential therapeutic use of any intracellular IM is difficulty in translocating the IM through the cell membrane for its delivery to the cytosol. A range of strategies that are already used for intracellular delivery of therapeutics, such as the Tat peptide from the protein-transduction domain from HIV-1 [73], could be used for IM described here. Indeed, adding polyarginine cell-transducing sequence to the C-terminus of a peptide from the viral IM A52 resulted in cell internalization of the IM peptide, with the polyarginine-A52 peptide inhibiting inflammatory responses *in vitro* and, more significantly, *in vivo* [67].

The development of pathogen IM as therapeutics is in its infancy. There are many immunomodulatory activities encoded by pathogens that have not been identified. In other cases, for which the activity has been described, we do not know the IM involved or the molecular mechanism that causes immune modulation. Although we focus here on recombinant proteins, it must be stressed that some of the most potent modulatory molecules from pathogens contain glycans or lipids; synthetic forms of these non-protein IM could also be developed as therapeutics. In this article, we have restricted our analysis of IM to those derived from conventional pathogens. However, other pathogens are also a source of potential IM; for example, IM have been described in the saliva of parasitic ticks, including a CKBP that binds to CXCL8 [74]. A broader approach is to also consider non-pathogenic microbes as a reservoir of potential IM, such as an immunomodulatory activity of molecules from symbiotic gut bacteria [75], or from cyanobacteria (blue-green algae) [76]. We should not be reductionist in the search for therapeutic IM from microbes, and look for opportunities to develop therapeutics from molecules in 'bugs' that are friend or foe.

Concluding remarks

The genomes of pathogens are repositories of information on the host immune system that has accumulated over millions of years of co-evolution of pathogens with their hosts. Identifying and characterizing IM from various pathogens is an expanding area of research that should offer a unique opportunity to uncover a large collection of natural modulators of inflammation with the potential for use as novel immunotherapeutics to treat immune-mediated human diseases.

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