SUPPLEMENT

Update on toll-like receptor-directed therapies for human disease

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Innate responses to microbes are mediated in large part by toll-like receptors (TLRs), which recognise a diverse range of molecules produced by viruses, bacteria and fungi. Great effort has been directed towards translating this knowledge into the development of new therapies for a wide spectrum of diseases, including infectious, malignant, autoimmune and allergic diseases. This review will provide a brief update on completed, ongoing and planned clinical trials of TLR ligand-based therapies for the treatment of diseases in humans.

Understanding of the molecular basis for innate immune recognition of microbial pathogens has increased dramatically in the last decade. Epithelial cells, mononuclear and polymorphonuclear immunocytes, and many other cell types express preformed pattern recognition receptors (PRRs) that identify potential pathogens.^{1–3} These receptors interact with microbial components often referred to as pathogen-associated molecular patterns (PAMPs). As originally defined, PAMPs were thought to meet certain criteria including: (1) their expression by microbes but not mammalian cells, (2) conservation of structure across a wide range of pathogens, and (3) the capacity to stimulate innate immunity. However, while the term PAMP has been accepted into the vocabulary of immunology, PAMPs are not unique to pathogens, as they are also produced by microbes that do not cause disease. Toll-like receptors (TLRs) have been shown to be important PRRs for the detection of PAMPs and host protection from microbial infections. Nonetheless, while TLR ligands are not readily generated by mammalian cells under physiological conditions, it appears that under conditions of tissue inflammation, oxidative stress and/or necrosis, molecules with TLR stimulatory activities (heat shock proteins, modified RNA and DNA species, and potentially others) are generated and released by dying cells.^{2–4}

At least 11 mammalian TLRs have been identified,^{2–5} although not all are expressed in all species. TLR2 interacts with peptidoglycan, a cell wall constituent of essentially all bacterial species, with the exception of *Chlamydia* and mycoplasma. TLR2 also interacts with additional molecules produced by Gram-positive bacteria, mycobacteria and fungi. TLR3 responds to double-stranded RNA and may also be responsive to select species of single-stranded RNA. TLR4 recognises most species of lipopolysaccharide (LPS). TLR5 is activated by bacterial flagellin. TLR7 (mouse and human) and TLR8 (human) respond to synthetic imidazoquinolones, as well as several single-stranded viral RNA sequences. TLR9 is activated by DNA sequences that are rare in mammalian genomes but common in the genetic material of bacteria, fungi and DNA viruses. TLR11 (mice) recognises an undefined PAMP associated with uropathogenic bacteria and a profilin-like molecule produced by *Toxoplasma gondii*. Certain TLRs (TLR1, TLR2 and TLR6) are also recruited into the phagosome where they heterodimerise with other TLRs.² Such interactions between different TLRs may serve to expand the range of

PAMPs recognised by this family of proteins and therefore, the number of microbes to which the innate immune system can respond.

Signalling pathways activated by TLR ligands lead to NF- κ B and MAPK activation, cytokine gene transcription (eg, IL6, IL10 and IL12), and co-stimulatory molecule expression (eg, CD40 and B7).^{2–6} Interestingly, despite common use of several signalling molecules, qualitative differences exist in the cellular responses elicited by ligands for different TLRs. It is believed that collateral signalling pathways are responsible for this diversity. For example, molecular studies have found that TIRAP, TRAM and TRAF participate in signalling through only a limited number of TLRs.² Moreover, ligands for some TLRs (TLR3, TLR4, TLR7 and TLR9) induce type 1 IFN production in responsive cells by upregulating expression of interferon regulatory factors, while ligands for TLR2 and potentially other TLRs do not.^{2–7} Furthermore, unlike all other TLRs investigated, MyD88 does not participate in signalling through TLR3.^{2–5} While further discussion is beyond the scope of this review, it is also worth noting that in addition to TLRs, which recognise PAMPs at the cell surface or within lysosomes and endosomes, additional cytoplasmic PRRs exist for the detection of PAMPs, including members of the NOD-LLR and CARD helicase families of proteins.^{2–5}

TLR LIGANDS AS INFECTIOUS DISEASE VACCINE ADJUVANTS

Studies in mice and other animal species have established that TLR ligands can profoundly influence antigen-specific immunity.^{6–10} Thus, their clinical potential as vaccine adjuvants has been considered by a number of laboratories. In general, TLR ligands have been shown to be excellent vaccine adjuvants in animal studies, promoting development of robust antigen-specific humoral and cellular responses, including cytotoxic T lymphocyte (CTL) responses.^{6–11–13} However, as vaccination is routine for all citizens of developed countries, adjuvants must have a high margin of safety to obtain government approval and many promising candidates have failed to meet this litmus test. Currently, alum is the only adjuvant approved for use in

Abbreviations: CTL, cytotoxic T lymphocyte; MPL, monophosphoryl lipid; ODN, oligodeoxynucleotide; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; SLE, systemic lupus erythematosus; TLRs, toll-like receptors

vaccines used in the USA. To address the need for additional and potentially superior vaccine adjuvants, a number of TLR agonists are currently being tested in early and late phase clinical trials, and several have demonstrated excellent safety and efficacy profiles.^{14–15} Compared to alum, this new generation of adjuvants appears superior in many respects, particularly with regards to their capacity to induce CTL responses.

While LPS is fairly toxic in humans, monophosphoryl lipid (MPL), another TLR4 ligand with a greatly improved safety profile, has recently won approval for use in Argentina and the European Union, as an adjuvant incorporated into hepatitis B vaccines.¹⁵ These MPL-containing vaccines were found to induce protective antibody titres against hepatitis B in almost all subjects with two injections, while the standard hepatitis B–alum vaccine required three injections to elicit protective antibody levels in the majority of recipients.^{16–17} In addition to MPL, CpG oligodeoxynucleotide (CpG ODN; TLR9 agonist) is being developed as an adjuvant for use in hepatitis B vaccines. Results from phase 1–3 clinical trials have shown CpG ODN to be a safe and highly effective adjuvant, even in patients known to have poor responses to alum-containing hepatitis B vaccines (ie, the elderly).^{18–19} In addition, to TLR4 and TLR9 agonists, ligands for TLR3, TLR5 and TLR7/8 are currently being investigated in phase 1 and 2 human vaccine trials.^{14–15} Microbial targets being actively pursued in these preclinical studies and human trials include hepatitis, human papilloma virus, anthrax, influenza and HIV. If proven safe, vaccine development is an arena in which TLR agonists are likely to have a great deal of clinical utility.

TLR LIGAND-BASED THERAPIES FOR ALLERGIC DISEASES

The hygiene hypothesis proposes that microbial exposures protect against the genesis of allergic diseases.^{20–23} TLR ligands are produced by microbes and can profoundly influence the allergic phenotype.^{9–10–24–26} Therefore, a great deal of attention has been paid to their use for the treatment of allergic diseases. As endotoxin (TLR4) exposures during early life are suggested to protect children from atopy,^{27–28} MPL (TLR4) was a logical candidate for this purpose. Preclinical studies conducted in our laboratory and others have found that CpG ODN-based therapies are highly effective in murine allergic disease models.^{14–23} Therefore, TLR9-directed therapies are also being developed for the treatment of allergic diseases.

Pollinex Quattro, a cocktail of MPL and several allergens, is currently marketed in Europe for use as an ultra-short course of immunotherapy. A series of four Pollinex Quattro injections was shown to induce clinical improvements in patients with allergic rhinitis during the pollen season.^{29–31} With CpG ODN, several immunotherapeutic approaches are being evaluated in clinical trials. Following the traditional vaccination paradigm, CpG ODN has been delivered as an adjuvant mixed with allergen.¹⁵ A more sophisticated therapeutic, Tolamba (CpG ODN conjugated Amb a 1; ragweed allergen), has also been developed. In preclinical studies, CpG ODN allergen conjugates were shown to be several times more immunogenic than allergen mixed with CpG ODN, presumably due to adjuvant/allergen co-localisation within antigen-presenting cells.^{32–33} Moreover, CpG–allergen conjugates have proven to be far less allergenic than allergen/CpG cocktails, due to the steric/electrostatic interference created by CpG conjugation, which prevents preformed IgE from binding to allergen.^{25–32–33} Tolamba has now been studied in phase 1–3 clinical trials. In these trials, a series of six Tolamba injections was shown to be well tolerated and protective against ragweed-induced symptoms for at least 2 years.^{26–34–35} A third strategy being investigated in ongoing clinical trials is the use of allergen-free CpG ODN

monotherapy to treat asthma and other atopic diseases but patient outcome data are not currently available.¹⁵ Although highly effective for the treatment of symptoms, the majority of therapies currently used to treat allergic diseases fail to reverse the underlying allergic hypersensitivities that fuel them. On the other hand, traditional immunotherapy can extinguish allergic hypersensitivities in about half of treated patients but requires several years and many injections to be effective.³⁶ Therefore, if proven safe and effective, TLR agonist therapies would have tremendous potential for the treatment of allergic diseases and the underlying allergic hypersensitivities that fuel them.

TLR AND CANCER

Immunological tolerance is one of several factors that allow malignant cells to flourish within their host.^{37–39} Therefore, TLR agonists have been intensively studied as vaccine adjuvants and antigen-independent immunostimulants for cancer therapy. Many preclinical studies have found TLR agonist therapies to be efficacious in the clearance of tumours and they are currently being evaluated in a number of clinical trials with patients with cancer.^{15–40–42} In some trials, TLR ligands have been used as adjuvants for antigen-specific vaccination (CpG ODN), while others have used TLR ligands as monotherapy (CpG ODN and imiquimod) or in conjunction with traditional cancer therapeutics (CpG ODN).^{15–42} In general, the immunological aim of these approaches has been to boost Th1 immunity, increase anti-tumour CTL numbers and/or to inhibit T regulatory cell activity within the tumour and its draining lymph nodes.^{37–39}

In one study, a melanoma cancer vaccine composed of a relevant antigen, CpG ODN, and incomplete Freund's Adjuvant increased antigen-specific CD8 T cell numbers in patients around 10-fold compared to a vaccine composed of antigen plus incomplete Freund's Adjuvant alone.⁴³ However, clinical improvement was not seen in these patients. Another CpG ODN-based vaccine targeting a different melanoma antigen induced a partial response in a subset of patients with advanced melanoma after seven or more immunisations (published as abstract only).⁴² CpG-containing vaccines are also being developed for other malignancies and additional clinical trial results are anticipated in the near future. To date, despite promising results in preclinical animal models and phase 1–2 trials in patients with end-stage cancer, TLR-adjuvant containing vaccines have yet to induce documented remissions.

One older study found that oral imiquimod (a TLR7 agonist) monotherapy induced systemic IFN α release but did not increase tumoricidal activity in cancer patients.⁴⁴ However, as topical monotherapy, imiquimod has established clinical efficacy in the treatment of various cutaneous premalignant and malignant skin diseases including human papilloma viral infections and basal cell carcinomas, and has been given approval for their treatment.^{15–45–47} As monotherapy, intraleisional CpG ODN injection was found to induce tumour regression in one out of five treated melanoma patients, while non-injected tumours in this patient continued to grow (published as abstract only).⁴² In another study, as therapy for basal cell carcinomas, four out of five treated patients demonstrated regression of CpG ODN-injected tumours (published as abstract only).⁴² In an additional phase 1/2 clinical trial, 28 patients with advanced cutaneous T cell lymphoma who failed traditional chemotherapies were given CpG ODN at escalating doses by weekly subcutaneous injection (published as abstract only).⁴² Three of these patients had complete and six had partial responses to CpG ODN injections. Moreover, there was little toxicity beyond local reactions at the CpG ODN injection site and flu-like symptoms. To date, monotherapy with TLR 7 or 9 ligands has been well tolerated and preliminary evidence suggests this treatment strategy could prove an

efficacious alternative to conventional therapies for select malignancies.

In addition to the approaches just described, TLR agonists have been found to be effective adjunct therapies for several malignancies when delivered together with standard chemotherapeutics. In one clinical trial of 111 previously untreated patients with stage IIIB non-small cell lung cancer, the response rate for chemotherapy (taxane and platinum) plus CpG ODN was 38% compared to 19% for patients receiving chemotherapy alone (published as abstract only).⁴² In addition, the 1 year survival rate was 50% versus 33% for patients treated with chemotherapy alone. TLR ligand therapy delivered in combination with traditional chemotherapeutics has also been found to improve clinical outcome measures of patients with other malignancies.^{15 42} In addition to chemotherapeutics, CpG ODN is being tested as an adjunct therapy with radiation and monoclonal antibody for the treatment of malignancies but clinical efficacy data are not yet available.^{48 49} While clinical trial experience with TLR agonists as adjunctive cancer therapeutics is limited, preliminary outcome data suggest that their use may lead to improved patient outcomes and, importantly, that they add little toxicity to that induced by the primary intervention.

TLR ANTAGONIST THERAPY FOR AUTOIMMUNE DISEASES AND SEPSIS

The danger of using TLR agonists in humans lies in their potential to induce predictable and/or idiosyncratic side effects. One case study has already found that treatment with the TLR7 agonist, imiquimod, led to the aggravation and spread of psoriatic plaques in study subjects.⁵⁰ Moreover, a growing body of evidence suggests that endogenously produced TLR ligands have a role in the pathogenesis of autoimmune diseases.⁵¹ As examples, TLR3 ligands have been identified in the synovial fluid of patients with rheumatoid arthritis⁵² and TLR9 ligands have been found in the immune complexes that develop with systemic lupus erythematosus (SLE).⁵³ Such observations raise concerns that pharmacological doses of TLR agonists could precipitate autoimmunity. These considerations have also prompted interest in the potential of TLR antagonists, as therapeutic agents for autoimmune diseases. In preclinical studies, TLR9 specific inhibitory ODNs have already been reported to suppress autoimmunity in mouse models of SLE.^{15 53} Moreover, serum IFN α is a biomarker of disease activity for patients with SLE and TLR9 antagonistic ODNs are able to block IFN α production by normal human peripheral blood mononuclear cells.^{54 55} Based on these and other preclinical results, there is reason to suggest TLR9-specific inhibitory ODNs could ameliorate autoimmune disease activity and clinical trials are planned, but to date, none have been initiated.

Septic shock is caused by overwhelming and potentially lethal TLR stimulation in the setting of bacterial infection. Therefore, TLR antagonists have also been studied as potential therapies for this condition.¹⁵ Two TLR4 antagonists (E5564 and TAK-242) have already been shown to be effective in the prevention and treatment of LPS-induced shock in animal models.^{56 57} Moreover, both were found safe in phase 1 and 2 clinical trials.^{15 58} Finally, in a clinical trial with E5564, a single dose of the TLR4 antagonist protected healthy adults from the physiological changes associated with LPS challenge, in a dose-dependent manner.⁵⁸ Ongoing phase 3 clinical trials will determine the effectiveness of TLR4 antagonists in the treatment of patients with endotoxaemia.

CONCLUSIONS

In the last decade, it has become apparent that TLRs play a key role in immunological detection and innate responsiveness to microbes. This knowledge has led to a great deal of research

effort directed towards developing TLR agonists and antagonists as therapeutics for the prevention and treatment of diseases. In relatively small clinical trials, TLR agonists have been used as adjuvants for vaccines aimed at preventing infections, extinguishing allergic hypersensitivities and clearing malignant cells. Moreover, TLR agonists have been investigated as monotherapies and adjunct therapies for the treatment of patients with infectious, allergic and malignant diseases. Finally, TLR antagonists are being considered in preclinical studies and clinical trials as therapeutics for autoimmune diseases and sepsis. In general, favourable safety and preliminary efficacy outcome data have come from these clinical trials. Nonetheless, pharmacological manipulation of TLR signalling could have unintended consequences that may only come to light when large numbers of patients have been treated with TLR agonists and antagonists. Additional clinical experience with TLR ligand-based immunotherapeutics acquired during the next decade should establish their relative clinical safety and efficacy for the prevention and treatment of disease in humans.

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