Review Article

Role of toll-like receptors in respiratory diseases

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Abstract

There has been growing interest in the last 10 years in the study of innate immunity, in particular because of the possible role that toll-like receptors (TLR) may play in the pathogenesis of some respiratory diseases including, asthma, chronic obstructive pulmonary disease, and infections. TLR are a family of type I transmembrane proteins, responsible for recognizing molecular patterns associated with pathogens (PAMP, pathogen-associated molecular patterns), and expressed by a broad spectrum of infectious agents. This recognition leads to a quick production of cytokines and chemokines which provides a long-lasting adaptive response to the pathogen. At present, it is considered that it is currently considered that the administration of drugs which modulate the activity of these receptors upwards or downwards may represent major therapeutic progress for handling these diseases. The aim of this review is to describe the different TLR, define their possible role in the pathogenesis of the main respiratory diseases and finally, speculate over the therapeutic possibilities which their modulation, agonist or antagonist, offers as possible therapeutic targets.

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Introduction

The immune system consists of various key lines of defence.1 Innate (natural or unspecific) immunity, which lacks specificity and always present and ready to go into immediate action without the need for a latent period to trigger a response. Acquired (adaptive or specific) immunity, also known as immunulatory response, is much more complex than unspecific immunity and is characterised by its adaptability to the antigen, specificity and memory. This acquired immunity identifies specific pathogen peptides presented by antigen-presenting cells, which, in turn, activate a cellular and humoral

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immunitary response mediated by T (cellular) and B (humoral) cells. An efficient immunitary response will depend on the interaction between innate and acquired immune systems. Up to now, both immunitary responses have been characterised separately. In the field of immunology, focus has centred mainly on the knowledge of acquired immunity, nevertheless, the natural immune system in mammals is yet to be adequately characterised.

Activation of the innate immunitary system constitutes a crucial step for the development of specific acquired immunity against antigens. The primary response to pathogens in the innate immunitary system is mediated by pattern recognition receptors (PRR) which recognise pathogen-associated molecular patterns (PAMP) present in a wide array of microorganisms. The most important PRR are toll-like receptors (TLR) which selectively recognise a large number of varied and complex PAMP, characteristic molecules of microorganisms such as lipopolysaccharides, flagellin, mannos or nucleic acids from virus and bacteria. Once the PRR, and in particular the TLR, recognise these microorganism-specific molecules, an innate immune response is triggered which activates the production of inflammatory mediators such as a large number of interleukins (IL), interferons (IFN) and tumour necrosis factor-alpha (TNF-α).

Functions and Types of Toll-like Receptors

Towards the end of the 1990s, the toll receptor was identified as one of the key receptors involved in the defence (innate immune response) of Drosophila against fungal infections. One year later, it was shown that a homologous mammal toll receptor, TLR4, would induce inflammatory gene expression. To date, approximately 15 TLR have been indentified in mammals. In humans, only 10 functional TLR have been described.

TLR are type I transmembrane proteins that include multiple copies of leucine-rich repeats (LRR) in the extracellular domain and an intracellular signaling domain shared by toll receptors and IL-1 receptors called TIR (toll/interleukin-1 receptor) (Figure 1). This TIR domain has the ability to bind and activate distinct molecules, among them MyD88 (myeloid differentiation factor 88), the Toll/IL-1R domain-containing adaptor protein (TIRAP), Toll/IL-1R domain-containing adaptor inducing IFN- beta (TRIF), the TRIF-related adaptor molecule (TRAM), Interleukin-1 receptor-associated kinase (IRAK), tumour necrosis factor (TNF), and TNF receptor-associated factor 6 (TRAF6); all necessary to activate different pathways such as mitogen-activated protein kinases (MAP), signal transducers and activators of transcription (STAT) and the nuclear factor-kappa B (NF-κB) pathway.

TLR1, 2, 4, 5, 6 and 10 are expressed on the cellular surface and migrate to phagosomes (a membrane-bound vacuole in a phagocyte formed by the inward folding of the cell membrane and phagocytised material) after activation on recognising the ligand. TLR3, 7, 8 and 9 are expressed in intracellular compartments, mainly in endosomes and the endoplasmic reticulum. Each TLR recognises a group of characteristic molecules. TLR expressed in the cellular membrane recognise molecules such as the lipopolysaccharides of gram-negative bacteria (TLR2 associated with TLR1 or TLR6), the lipopolysaccharides of gram-negative bacteria (TLR4) and the flagellin of bacterial flagella (TLR5). Infections from RNA virus and from respiratory syncytial virus and influenza are the major causes of inflammation of the airways. TLR expressed in intracellular compartments (TLR3, 7, 8 and 9) recognise nucleic acids of these viruses and thus detect the intracellular infection (Table 1 and Figure 2).

The recognition of the ligands by TLR, present in dendritic and macrophage cells, leads to the rapid production of cytokines and chemokines that indicate the presence of the pathogen. This response initiates a rapid recruitment of cells from the immune system to the site of the infection and activates them, launching an immediate response against the pathogen. Signals originated by the TLR promote the expression of adherence molecules in epithelial cells as well as in circulating haematopoietic cells.

Among the antigen-presenting cells, those which process and present the antigen fused to the molecules of the main class II histocompatibility complex, are monocytes/macrophages, dendritic cells and B cells. These cells, in particular the dendritic cells, form the interface between both types of responses (innate and acquired). TLR ligands cause these cells to mature and become activated.
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligands</th>
<th>Localisation</th>
<th>Characteristics</th>
<th>Expression</th>
<th>Regulation</th>
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<tbody>
<tr>
<td>TLR1, TLR2, TLR6 and TLR10</td>
<td>Lipoproteins (bacteria), glycoprophosphatidinositol (Trypanosoma cruzi), lipoparinobinomanan (Mycobacterium tuberculosis), purins (Neisseria meningitidis, Klebsielapneumoniae), zimosen (fungi), phenol-soluble modulin (Staphylococcus), macrophage-activating lipopeptide-2 (for TLR2/6)</td>
<td>Cellular surface</td>
<td>TLR1: shares 68% identical aminoacid sequences with TLR6, and 48% with TLR10</td>
<td>TLR1: monocytes, macrophages, dendritic cells, leucocytes, polymorphonuclears, B, T and cytotytic cells</td>
<td>TLR1: IL-6, and by elevated concentrations of IFN-αβ, IL-10 and TNF-α</td>
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<td>TLR2: IL-6 and TNF-α, IL-1β and IL-10&lt;sup&gt;21-30&lt;/sup&gt;</td>
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<td>TLR6: IFN-α and IL-1β</td>
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<td>TLR3</td>
<td>Bacterial DNA and viral double-chain RNA</td>
<td>Intracellular compartment&lt;sup&gt;24&lt;/sup&gt;</td>
<td>Associated with TLR5, TLR7 and TLR8, each with 26% identical aminoacid sequences</td>
<td>Placenta and pancreas&lt;sup&gt;26&lt;/sup&gt;, Dendritic cells, T cells and cytotytic cells</td>
<td>TLR10: IFN-α, IL-1β, IL-6, IL-10 and TNF-α&lt;sup&gt;27-30&lt;/sup&gt;</td>
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<td>TLR4</td>
<td>Lipopolysaccharide (gram-negative bacteria), taxol (antitumour agent in humans) and F protein (syncytial virus)</td>
<td>Cellular surface</td>
<td>Associated with TLR1 and TLR6, each with 25% identical aminoacid sequences</td>
<td>In the spleen and peripheral blood leucocytes (B cells, dendritic cells, monocytes, macrophages, granulocytes and T cells&lt;sup&gt;25-26&lt;/sup&gt;)</td>
<td>IFN-α and IL-1β</td>
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<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td>Cellular surface</td>
<td>Associated with TLR3 in 26% identical aminoacid sequences</td>
<td>Ovary, prostate, peripheral blood leucocytes&lt;sup&gt;26&lt;/sup&gt;</td>
<td>IL-6, IL-10, TNF-α and IFN-α&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td>TLR7, TLR8</td>
<td>Guanosine and uridine from single chain RNA (TLR8), Luxoridine (a guanosine analogue) TLR7, Imidazoquinolin (antiviral compound)&lt;sup&gt;19-27&lt;/sup&gt; Bacterial DNA and viral RNA</td>
<td>Intracellular compartment</td>
<td>TLR7: associated with TLR8 and TLR9 in 43% and 36% aminoacid sequences, respectively</td>
<td>TLR7: lung, placenta, spleen, lymph nodes and tonsils&lt;sup&gt;27-30&lt;/sup&gt;</td>
<td>TLR7: IL-6, IFN-α and IL-1β</td>
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<td>TLR8: IL-1β, IL-6, IL-10 and TNF-α</td>
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<td>Expression enhanced by exposure to IFN-α</td>
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Table 1: Toll-like receptors (TLR) in humans
antigen-presenting cells as they induce the expression of costimulator molecules (such as CD40, CD80 and CD86), necessary for the activation of T lymphocytes. Many TLR-induced IL guide the differentiation of T cells into helper T lymphocytes (CD4+) or cytotoxic lymphocytes (CD8+). T helper-1 lymphocytes (stimulated by IL-12) produce IL-2, IFN-γ and TNF, and control useful cellular immunity reactions against infection by microorganisms of intracellular growth. T helper-2 lymphocytes (stimulated by IL-4) produce IL-4, IL-5 and IL-6 and collaborate in humoral immunity reactions, fundamental in neutralising toxins and infections by germs of extracellular growth.

There are other types of lymphocytes, such as T helper-17 and T regulators which are important in the development and control of immunitary response.

As mentioned earlier, 15 types of TLR have been identified in mammals,6,7 13 of them, from TLR1 to TLR13, in humans and mice. Other equivalent forms have been isolated in other mammalian species; nevertheless, some TLR found in humans are not present in other mammals and, conversely, other mammals may express TLR that have not been isolated in humans. For example, TLR11, 12 and 13 are only expressed in mice. For the moment only 10 functional TLR have been described in humans.10,11,24,25 This circumstance conditions or limits the use of experimental animals as innate immunity models extrapolable to the human species. The principal characteristics of human TLR are shown in Table 1.

### Toll-like Receptors in Asthma and Allergic Diseases

Allergic diseases like asthma, rhinitis and atopic dermatitis constitute a group of high prevalence processes.39-42 Susceptibility to their development is influenced by multiple genetic and environmental factors.41-43 There is a variety of conclusive evidence that indicates that exposure to microbial products in infancy plays an important role in the post-natal maturity of the immune system. It has been hypothesized that the rise of prevalence of allergic diseases in industrialised countries over the last 20 years could be related to the reduction of microbial load in those geographic areas. This
hypothesis, known as the “hygiene hypothesis”, is based on the observation that in developed countries there is an inverse relation between the increase in allergic diseases and the reduction of microbial exposure in the first stages of life, which leads to a defect in the organism’s immune regulators. The discovery of TLR and their actions provides an immunological basis for the study of the hygiene hypothesis.

Today, there are still many unanswered questions about the aetiology, pathogenesis and phenotypes of asthma. Why, for example, are there 2 well differentiated types of asthma: allergic or extrinsic and non-allergic or intrinsic? Allergic asthma is characterised by affecting in a large part children and young adults, by having a personal or family history of allergy (rhinitis, atopic dermatitis), having elevated total and specific immunoglobulin E and its pathogenic mechanism is owed in part to a type I hypersensitivity (immediate). In contrast, non-allergic asthma predominates in adults, there is rarely a personal or family history of allergy and immunoglobulin E is within reference values. Similarly, questions arise on the role played by the different inflammatory cells implicated in its pathogenesis. Take, for example, the universal prominence of eosinophil in the different phenotypes of asthma and its implication in actual clinical practice; the lack of an adequate response to the inflammatory activity of glucocorticoids in some patients (refractory asthma); the important role that the neutrophil appears to perform in some types of asthma or asthmatic situations like occupational asthma, virus-induced exacerbations and life-threatening sudden-onset asthma. It is believed that these inflammatory cells, combined with innate immunity, perform a relevant differentiated role in asthma’s pathogenesis. This is more than a simple academic question: given its inflammatory heterogeneity, what is known today as asthmatic disease, may be a syndrome rather than a homogenous process, which, as a result, may have practical repercussions for possible specific treatments in the future.

In addition, the acquired immune response in asthma is well characterised and involves the activation of T helper-2 lymphocytes by the allergen, with the consequent eosinophilic inflammation of the airways. In this way, the activated eosinophils then secrete cytotoxic granules (main basic protein, eosinophil cationic protein), which induce the bronchial hyperresponse and consequent symptoms in the patient. In the case of the non-eosinophilic asthma subtype (and neutrophilic), where the symptoms and bronchial hyperresponse persist in spite of the absence of eosinophils, the pathogenic mechanism is not well characterised. However, there are arguments to implicate neutrophils and IL-8 in its pathogenesis; it would be a kind of preprogrammed response by the natural immune system that has been conserved throughout evolution.

Some recent studies have led to interesting expectations by observing that in neutrophilic asthma there is an increase in the expression TLR2, TLR4, CD14 and surfactant protein-A (SP-A), and that the activation of TLR, by an allergen for example, generates a cascade of signals driven by the activation and nuclear translocation of NF-κB, that results in a cytokine-mediated inflammatory response (TNF-α, IL-8 e IL-1α). Just like in allergic asthma, non-allergic asthma exhibits an increase of eosinophils and T helper-2 cells in the bronchial mucus, but it develops in the absence of a family or personal history of atopy. Among the aetiopathological factors of non-eosinophilic asthma, there is speculation that unidentified allergens, autoimmunity or bacterial or viral infection are involved. Non-allergic asthma is often associated with viral (rhinovirus, coronavirus) or previous bacterial infections, which could mean that TLR may also be implicated in this other clinical form of asthma.

The development of drugs that act on TLR is centred on the use of agonist and antagonist ligands. Agonists are molecules that bind with TLR and create a response in the cell, while antagonists inhibit the binding of natural agonist ligands and, consequently, do not cause any response. The possible role of diverse TLR agonists as adjuvants in vaccines, antimicrobial treatments and anti-allergy treatments and cancer is currently under evaluation, along with structural analogues of agonists that bind to the receptor but do not induce any signal in the cell. As far as the treatment of asthma and allergic rhinitis, the administration of CpG oligodeoxynucleotides (cytosine guanine dinucleotide), a drug with TLR agonist capability, has been effective in the prevention and reversal of (antigen-induced) bronchial eosinophil inflammation in animal experimentation models. CpG are detected by the TLR9 in the B lymphocytes in dendritic cells, whose ligand activates multiple signals in cascade in responsive cells such as T helper-1 lymphocytes.

TLR7 and 8 in intracellular compartments, mainly in the endosomes, may confer greater susceptibility to the development of diseases such as asthma, rhinitis and atopic dermatitis. It has recently been observed that imiquimod, a new synthetic TLR7 ligand often used in dermatology reduces inflammation, bronchial hyperresponse, concentrations of total immunoglobulin E in blood serum and cytokines in bronchio-alveolar lavage, and attenuates the expression of the transforming growth factor-beta (TGF-β1) in the remodelled airways, in murine models of acute allergic asthma.

However, in spite of the interesting advances in knowledge of the functions of TLR and their potential usefulness in the treatment of inflammatory diseases, it must be taken into account that a number of inflammatory cells express them, among these epithelial cells, T and B cells, mastocytes and eosinophils. This conditions the prediction of the concrete response of TLR to an agonist or antagonist. In addition, it is likely that the microbial products that generate this response contain diverse ligands for various types of TLR, making it even more difficult to predict the possibility of a favourable clinical response.

**Toll-like Receptors in Infections**

Around a third of the world population is infected by *Mycobacterium tuberculosis*, but little more than 10% of those infected (immunocompetent) will develop the disease. The immunological mechanisms that distinguish which of these people will develop tuberculosis remain unknown. Genetic variations in the TLR of these individuals have been observed. Among these, TLR8 polymorphisms increase susceptibility to pulmonary tuberculosis and the expression of TLR8 in macrophages is increased after inoculation with the tuberculosis vaccine, BCG (bacilo Calmette-Guerin).

Diverse pathogens, chemical agents, avian H5N1 influenza and severe acute respiratory syndrome have been identified as causing acute respiratory distress syndrome. Studies performed on an experimental model (rat) of acute lung injury (ALI) induced by acid aspiration and inactivated influenza A H5N1 virus have shown that the mutation of TLR4 confers a natural resistance to acute pulmonary damage. Phospholipid oxidation and cytokine production by macrophages via TLR4-TRIF was identified as the cause of ALI. The TLR4 mutation in rats demonstrated a natural resistance to acute pulmonary damage induced by ALI, and TLR4-TRIF-TRAF6 is a key to controlling the severity of ALI. Other studies in which rats were exposed to hyperoxic conditions attributedTLR3 with a relevant role in the development of acute respiratory distress, and indicated that its absence during the hyperoxy would confer a protective effect and that said effect was associated with the induction of proapoptotic factors such as caspase-8, caspase-9, PTEN and Bid. It has been affirmed that the caspase-8/Bid pathway has an important role in the induction of signals associated with hyperoxic pulmonary injury and cellular death both in vivo and in vitro. The genetic absence or presence, therefore, of monoclonal TLR3 antagonist antibodies appears to attenuate onset as well as amplification of hyperoxia-induced pulmonary injury and favour its later resolution. This could...
suggest that innate immunity and TLR have a relevant role in the pathogenesis of acute pulmonary injury.78

The antiviral action of nucleic acid-base agonists for the activation of TLR has been evaluated in influenza virus infection. It has been shown that TLR3 expressed in dendritic cells, respiratory epithelia and macrophages perform a central role in the mediation of innate immunity inflammatory response against viral infections. Influenza viruses may inhibit the host’s ability to produce IFN and suppress the antiviral defence mechanisms of the immune system. It has been evidenced that the intranasal administration in rats of poly-ICLC (polyribinosin-polyribocytidylic acid) and of liposome-encapsulated poly-ICLC, IFN-inducing TLR3 agonist molecules and cytolytic lymphocytes confer a high degree of protection against lethal influenza A H5N1 virus. The protective effect lasted 3 weeks for the liposome-encapsulated poly-ICLC and 2 weeks for the poly-ICLC. Similarly, prior treatment in mice with Cpg-oligonucleotides (TLR9 agonist) conferred complete protection against influenza A virus.79

Toll-like Receptors in Chronic Obstructive Pulmonary Disease (COPD)

To understand diseases like emphysema it is necessary to know the mechanisms that allow the lung to maintain its structural integrity against constant environmental aggressions such as tobacco.80 Emphysema is characterised by the gradual loss of elasticity of the lung and the irreversible enlargement of the air spaces (insufflations and entrapment), generally in the last decades of life and in relation to exposure to cigarette smoke. Although smoking cigarettes is a determinant risk factor associated with the development of COPD, only 10 to 20% of serious smokers develop it.81,82 This suggests the influence of other conditions in the development of pulmonary emphysema related to the host itself.

Many genes have been implicated in susceptibility to emphysema, specifically those which regulate alpha-1-antitrypsin,83 the macrophage elastase enzyme,84 Klotho,85 surfactant D,86 mesosomal epoxide hydrolase87 and nuclear factor erythroid 2-related factor 2 (Nrf2).88 Oxidative/antioxidative or protease/antiprotease imbalances may be the cause of alterations observed in the aforementioned genes.89 The precise molecular mechanism provided to maintain oxidative and protease balance is still unknown. Studies in rats have shown that TLR4 expression in structural cells of the lung is necessary to maintain its normal architecture and halt oxidative stress. In the absence of TLR4, endothelial cells express elevated concentrations of Nox3, intracellular oxidants derived from the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, capable of generating an inflammatory response that influences the development of COPD. As a result, it is believed that TLR4 acts as a suppressor of endogenous Nox3 activity in the lung, and that its presence allows pulmonary integrity to be maintained through the modulation of the said oxidative system.90

TLR2 is a receptor that performs a crucial role in innate and acquired immunity. For this reason, molecules with TLR2-agonist activity may open a new preventive and therapeutic strategy in allergic and chronic obstructive respiratory diseases in humans.90-93 As already mentioned, the expression of TLR2 and TLR4 on CD14+ monocytes has been observed to diminish significantly in patients with stable COPD and healthy smokers,94 suggesting that perhaps the innate immune response is depressed in those patients. The activation of alveolar macrophages and epithelial alveolar cells via TLR4-NF-κB, endotoxin receptor, expressed and present in type II pulmonary epithelial cells, may also perform a role in the inflammation of the airways of COPD through the production and activation of pro-inflammatory mediators such as IL-8.95,96 Furthermore, it has been affirmed that acute exposure to cigarette smoke (2 cigarettes, twice a day for 3 days) induces acute inflammation in rat lungs, and this is TLR4/MyD88 and IL-1R1/MyD88 signalling dependent.97 Smoking during pregnancy has also been described as attenuating TLR-mediated immune responses, which probably increases the risk in the child of developing allergies and asthma.98 The expression on human mastocytes and coding variation of TLR6 is also thought to perform a relevant role in the pathogenesis of COPD and asthma.99,100

Toll-like Receptors and Other Respiratory Diseases

Respiratory epithelial cells fulfill an important role in the defence mechanisms and inflammatory responses of the host. In current treatment of bronchial inflammatory diseases, inhaled glucocorticoids are usually the drugs of choice. They effectively reduce the production of inflammatory mediators such as cytokines and chemokines, essential molecules for the host’s defence responses. The effect of glucocorticoids on TLR expression on respiratory epithelial cells has been studied recently. It has been found that TLR2 expression is increased by the synergic action derived from the combination of TNF-α, IFN-γ and glucocorticoids (dexamethasone). This effect may be related to glucocorticoid receptors since the action of the dexamethasone is abolished by RU-486, an antagonist of these receptors. This provides glucocorticoids with another novel and beneficial function in addition to their well known anti-inflammatory capability.100

In cystic fibrosis, the affected airways represent an environment potentially rich in TLR agonists. The chronic inflammatory phenotype evident in the epithelial cells of the airways also performs an important role in TLR activities. A significantly greater rise in TLR5 expression has been affirmed in patients with this disease.101,102 TLR modulation is currently being evaluated as a possible therapeutic target in the treatment of the disease.103

In another, no less important, field of pneumology studies in mice have shown that low volume mechanical ventilation in healthy lungs induces a TLR4 dependent inflammatory response (without altering the integral structure of the lung), significantly increasing endogenous TLR4 ligands in the broncho-alveolar lavage and the relative expression of TLR4 and TLR2 messenger RNA in lung tissue. This circumstance may open expectations for improved knowledge of the possible changes in the immune response experienced by ventilated patients.105

In short, many experimental studies agree that TLR have an important role in defence mechanisms and immunoregulation. Evidence indicates that possible alterations in said receptors could intervene in the inflammatory pathogenesis of diverse respiratory diseases, particularly asthma, COPD and infections. A predictable greater knowledge of the molecules which empower (agonist) or inhibit (antagonist) them could increase the therapeutic arsenal against these diseases in the future. A variety of clinical trials are currently underway that, in their different stages of development, are exploring their effectiveness and safety. Let us hope that in the future these positive expectations will be confirmed and a new pharmacological treatment will be available to us.

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