Toll-like Receptors. III. Biological Significance and Impact for Human Medicine

(apoptosis / autoimmunity / defensins / dendritic cells / immunostimulation)

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Abstract. The ability of the innate immune system to recognize and respond to microbial components has been largely attributed to the family of TLRs. They are able to discriminate among distinct molecular patterns associated with microbial components. Recognition of microbial products by TLRs results in induction of innate immunity mechanisms as well in development of antigen-specific adaptive immune responses. Some of TLR ligands start to be used to enhance immune defence mechanisms in fighting infections or malignancies. On the contrary, others were shown to be involved in immunopathogenesis of autoimmune disorders such as SLE.

Invertebrates do not possess adaptive immune mechanisms; the only way to survive an infection is the activation of innate immune mechanisms by pattern recognition receptor pathways that recognize molecular patterns (PAMPs) characteristic for particular groups of microorganisms. Among the most important pattern recognition receptors (PRRs) are Toll-like receptors (TLRs). It was shown that this system has also been widely used by vertebrates.

When encountered with a microbial invasion, activation of PRRs results in production of **antimicrobial peptides**. The latest data demonstrate that both animals and plants possess potent, broad-spectrum antimicrobial peptides, which they use to fend off a wide range of microorganisms, including bacteria, fungi, viruses and protozoa. Mammals produce antimicrobial peptides such as β-defensins in several kinds of epithelial cells residing in the respiratory and gastrointestinal mucosa and skin. These peptides disrupt the bacterial membrane, leading to pathogen cell death. Paneth cells in the base of the small intestine crypts secrete microbicidal β-defensins in response to bacterial challenge or LPS stimulation (Ayabe et al., 2002). In the upper respiratory tract, the expression of ß-defensins in human tonsillar tissue correlates well with the expression of TLR2 and TLR4 (Claeys et al., 2003). Labelling of TLR2 in normal human airways revealed TLR2 expression throughout the epithelium, with an apparently higher level of expression on non-columnar basal epithelial cells.

Two bacterial PAMPs, the outer membrane protein A from *K. pneumoniae* and flagellin, which signal via TLR2 and TLR5, respectively, directly stimulate human NK cells. NK cells have been shown to constitutively express β-defensins, and outer protein A and flagellin rapidly induce their release (Chalifour et al., 2004). These data demonstrate for the first time that NK cells directly recognize and respond to pathogen components via TLRs and also evidence defensins as a novel and direct cytotoxic pathway involved in NK cell-mediated protection against microorganisms.

Altogether, these data indicate that mammalian antimicrobial peptides are produced in response to various microbial stimuli at the epithelial surface, the front line of defence between the pathogen and its host, thereby inducing direct killing of the pathogens; this production is mediated by TLRs.

Apoptosis represents another way how the spread of pathogens can be limited by localizing their death at the site of their invasion. The first evidence of pathogen-induced cell death came more than 10 years ago. Invasive strains of *Shigella flexneri* induced programmed cell death in macrophages in *lamina propria* of intestinal villi (Zychlinsky et al., 1992). Of known TLR ligands, LPS was found among the first to mediate apoptosis of endothelial cells by a Fas-associated death domain (FADD) protein and caspase 8-dependent pathway. Further experiments identified a role for MyD88 and IRAK-1 in inducing LPS pro-apoptotic signalling (Choi et al., 1998; Bannerman et al., 2002). Triacylated bacterial lipoproteins, apart from inducing cytokine secretion, were identified to initiate apoptosis in human monocytes and in epithelial cell lines. Other TLR2 ligands inducing the apoptotic pathway dependent on MyD88 and FADD include synthetic diacylated lipopeptide MALP-2, lipoproteins from *Mycoplasma fermentans* and a 19 kDa protein isolated

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Abbreviations: CFA – complete Freund's adjuvant, DCs – dendritic cells, IFN – interferon, LPS – lipopolysaccharide, MHC – major histocompatibility complex, PAMPs – pathogen-associated molecular patterns, PRRs - pattern recognition receptors, SLE – systemic lupus erythematosus, TLR(s) – Toll-like receptor(s).

from the cell wall of *Mycobacterium tuberculosis*. These results indicate that caspase activation is an innate immune response to microbial pathogens (Lopez et al., 2003; Into et al., 2004). Imiquimod, a TLR7 ligand, when topically applied is used for the treatment of both external and perianal genital warts as well as for the therapy of benign and malignant epithelial lesions. One of the mechanisms involved in the action of imiquimod is induction of apoptosis in human epithelial cell lines and keratinocytes. Imiquimod also possesses considerable direct pro-apoptotic activity against tumour cells both *in vitro* and *in vivo*. The apoptotic process is presumably, at least in part, mediated through bcl-2-dependent release of mitochondrial cytochrome c and subsequent activation of caspase-9. These findings suggest that the mode of action of imiquimod to eliminate virus-infected, dysplastic or neoplastic epithelial cells may also include the induction of apoptotic processes (Meyer et al., 2003; Schon et al., 2004).

Activation of dendritic cells (DCs) via TLR3 and TLR4 induces type I IFN secretion by means of induction of the transcription factor IRF-3. After binding to its receptors, it activates STAT transcription factors and induces T_H-1 chemokine genes, promotes dendritic cell maturation and initiates antiviral responses (Luft et al., 1998; Toshchakov et al., 2002). Moreover, type I IFNs potently enhance the primary antibody response stimulating the production of all subclasses of IgG and induce long-term antibody production and immunological memory (Decker et al., 2005). The adjuvant activities of CFA (complete Freund's adjuvant) were abolished in IFN-α/β receptor-deficient mice. This indicates the potent adjuvant activity of type I IFNs and their important role in linking innate and adaptive immunity (Le Bon et al., 2001; Decker et al., 2005).

Myeloid DC (mDCs) and plasmacytoid DC (pDCs) express different TLRs and display different cytokine secretion profiles in response to pathogenic stimuli. Various stimuli, including LPS, induce IL-12 production from mDCs. In contrast, upon viral infection and CpG DNA stimulation, pDCs preferentially produce IFN-α (Cella et al., 1999; Siegal et al., 1999). Recent data also showed that mDCs produced IL-12 in response to TLR7 ligands, while pDCs secreted IFN-α. Altogether these data indicate that not only TLR expression, but also the type of dendritic cell subset determines distinct cytokine response patterns (Klinman, 2004). Deregulation of type I IFN production may result in serious immunological disorders, e.g. patients suffering from SLE display elevated levels of INF-α (Blanco et al., 2001; Baechler et al., 2004; Liew et al., 2005) (Table 1).

The activation of TLRs by various microbial components induces activation of adaptive immunity. Since DCs interact with T and B cells, mainly they provide the signals for activation of adaptive immunity. Located at the periphery, immature DCs exhibit high endocytic potential, which is required for the uptake of microbial antigens. Prior to antigen encounter immature DCs express a broad spectrum of TLRs. The expression of individual TLRs changes as they mature. In the course of maturation, expression of TLR1, 2, 4, 5 decreases (Visintin et al., 2001); in contrast, TLR3 is expressed in mature DCs only (Muzio et al., 2000). The maturation process of DCs is elicited through a broad variety of TLR ligands; these include peptidoglycan, lipoteichoid acids, various lipoproteins, LPS, the cell wall skeleton of *Mycobacteria* and CpG DNA (Hemmi et al., 2000; Hertz et al., 2001; Kaisho et al. 2001; Michelsen et al., 2001). In humans, all TLRs except for TLR7 and TLR9 are expressed in mMDCs, while TLR7 and TLR9 are almost exclusively expressed in pDCs (Jarrossay et al., 2001; Klinman, 2004). As a result, TLR-mediated activation of DCs induces production of proinflammatory cytokines and enhances expression of co-stimulatory molecules such as CD80/CD86 on the surface of dendritic cells (Akira et al., 2001). Once matured, DCs migrate from periphery to the regional lymph nodes and loose their endocytic capacity. In lymph nodes, DCs present microorganism-derived peptides bound in the groove of MHC class II molecules to naïve T cells, thereby initiating an antigen-specific adaptive immune response (Bucová, 2002; Klinman, 2004).

Initiation of the adaptive immune response is also under the control of regulatory T cells (T_R – $CD4^+CD25^+$), whose function is to prevent the activation of autoreactive T cells (Lan et al. 2005). According

Table 1. Diseases with a contribution of TLR signalling (modified from Liew et al., 2005)

Disease	TLR	Mechanisms
SLE	TLR4	DNA-Ab complexes activate B cells and dendritic cells
DM1A	TLR2, 3, 4, 9	TLR ligands increase cytotoxic and inflammatory immunity
Cardiomyopathy	TLR2, 3, 4, 9	TLR ligands promote dendritic cell function by presenting heart antigens
Atherosclerosis	TLR4	TLR signals trigger pro-inflammatory responses
Asthma	TLR4	LPS induces an inflammatory response to inhaled antigens

DM1A – autoimmune diabetes mellitus

to the recent data TLRs also participate in immune induction that is independent of co-stimulation. In this case the induction of a Toll pathway abolished the suppressive effect of T_R cells, enabling the activation of adaptive immune responses. IL-6, whose secretion is induced in DCs by microbial stimulation of TLRs, was partially responsible for overcoming the suppressive action of T_R cells (Pasare and Medzhitov, 2003).

It has been shown that in the absence of TLR-induced inflammatory cytokines, DC maturation and migration to the lymph nodes is not sufficient for T-cell activation *in vivo*. Moreover, TLR-induced signal is required for memory $CD4^+$ T-cell differentiation (Pasare and Medzhitov, 2004). Experiments using MyD88-deficient mice demonstrated a crucial role of TLRs in the induction of adaptive immune response. When immunized with CFA mixed with antigen, these mice displayed defective production of IFN-γ and antigen-specific IgG2a. In addition, they showed a profound defect in the activation of antigen-specific T_H-1 but not T_H-2 immune responses (Kaisho et al., 2002). Moreover, the T_H-1 immune response to intracellular protozoan parasite was severely abrogated, suggesting that exposure to a T_H-1 microbial stimulus developed a pure T_H-2 response (Jankovic et al., 2002). It seems that MyD88 plays a critical function in determining pathogen-induced polarization of $CD4^+$ T cells towards the T_H-1 type of immune response.

The induction of T_H-1 versus T_H-2 immune response is dependent on distinct types of DCs (Rissoan et al., 1999; Kadowaki et al., 2001). However, it seems that it is the microbial microenvironment that plays a crucial role in the ability of DCs to steer the T_H-cell polarization. For example, the activation of TLR4 by LPS or TLR9 by CpG DNA motifs induces IL-12 secretion, thereby shifting T_H-cell differentiation toward the T_H-1 type. Even exposure of different types of LPS may have profound effects: whereas LPS from *Escherichia coli* (a TLR4 agonist) induces the T_H-1 type response, LPS from *Porphylomonas gingivalis* (a TLR2 ligand) evokes the T_H-2 type response. This differential outcome is attributed to the ability of *E. coli* LPS, but not *P. gingivalis* LPS, to trigger production of IL-12 from DCs. Many other microbial components have been identified to elicit adjuvant immunostimulatory activity. The cell-wall skeletal fraction isolated from the *Mycobacterium bovis* BCG strain (BCG-CWS), similarly to CFA, exerts a potent immunostimulatory activity. Several clinical trials showed its effect as an adjuvant for gastric and lung cancer immunotherapy (Matsumoto et al., 2001). TLR2 and TLR4 have been reported to recognize the components of BCG-CWS (Tsuji et al., 2000). The immunopotentiating activity of neisserial porins, the major outer membrane proteins of the pathogenic *Neisseriaceae,* is mediated by its ability to stimulate B cells and up-regulate the surface expression of CD86. This ability is dependent on MyD88 and TLR2 expression, as demonstrated by a lack of response by B cells from the MyD88 or TLR2 knock-out mice to porins (Massari et al., 2002; Fisette et al. 2003).

CpG DNA, a ligand recognized by TLR9, is another molecule that has been proved to elicit adjuvant activity. CpG motifs directly activate B cells, monocytes, macrophages, pDCs and NK cells, which secrete IL-12p70 and up-regulate the expression of co-stimulatory molecules such as CD80, CD86, CD40 and class II MHC. IL-12p70 is the bioactive form of IL-12 and it is a strong T_H-1 response-polarizing agent (Krieg 1999, 2000; Askew et al., 2000; Klinman, 2004). CpG motifs are capable to redirect the isotype production of B cells to "T_H-1 like" immunoglobulin isotypes. The promotion of class-switching is critically dependent upon TLR9 and MyD88 (Lin et al., 2004). This provides an explanation for excellent activity of CpG oligodeoxynucleotides as humoral vaccine adjuvants.

According to the latest studies small antiviral compounds of the imidazoquinoline family (imiquimod, resiquimod, loxoribine, bropirimine) possess very strong immunostimulatory activities exerted by the TLR7/TLR8 pathway. Imiquimod treatment inhibited lesion development and/or virus shedding in guinea pigs inoculated with herpes simplex virus 1 (HSV-1), HSV-2 or virus isolates resistant to acyclovir (Miller et al., 1999). Clinical trials using subcutaneous administration of imiquimod alone demonstrated significant reduction of genital HSV recurrences (Harrison et al., 2001). Imiquimod was the first immune-response modifier (IRM) molecule to be licensed (Aldara, imiquimod 5% cream) for the treatment of external anogenital warts (Miller et al., 2002). Further experiments using the guinea pig model of genital herpes showed that resiquimod, an analogue of imiquimod, gave promising results in controlling recurrent HSV infection (Bernstein et al., 2001). Resiquimod stimulates specific cells of the innate immune system to produce cytokines (in particular IFN-α, IFN-γ, IL-12 and TNF) that initiate and drive the development of the T_H-1 acquired immune response against HSV-infected cells. Thus resiquimod shows a promise as a new treatment option for genital herpes infection (Miller et al., 2002). Loxoribine, a guanine ribonucleotide derivative, acts as synthetic adjuvant in anti-tumour responses. As with imiquimod and resiquimod, loxoribine activates cells of the innate immune system selectively via TLR7/MyD88-dependent signalling pathway (Heil et al., 2003). *In vitro* experiments showed that loxoribine enhanced the cytotoxic activity of fludarabine on B-cell chronic lymphocytic leukaemia cells (Tosi et al., 1997). Loxoribine was used in a double-blind randomized phase I study evaluating the safety, pharmacokinetics, and immunologic effects in patients with an advanced cancer. It proved to be safe at doses up to 10 mg/kg and produced modest immunologic effects. However, fur-

ther testing is necessary to evaluate the drug's efficacy (Agarwala et al., 2000). Several clinical trials demonstrated the anticancer activity of bropirimine, an oral immunomodulator, in the treatment of transitional cell carcinoma *in situ* (CIS) in both the bladder and upper urinary tract. An activity has also been documented in patients after prior therapy with BCG (Sarosdy et al., 1998). These studies show that bropirimine, an orally administered drug that can be self-administered to outpatients with more acceptable local toxicity compared to BCG, could be an effective first-line therapy in patients with CIS of the urinary bladder (Witjes et al., 1999). Bropirimine therapy was also evaluated in a murine prostate cancer model, where impeding of tumour growth was achieved by inhibiting bcl-2 expression and significant depression of TNF expression (Shaw et al., 1998). Studies designed to elucidate the mechanism of action suggest that bropirimine is likely to have direct anti-tumour activity rather than cytokine-mediated anti-tumour effect (Tei et al., 2002).

Taken together, TLRs recognize a great variety of pathogen-derived as well as synthetic compounds, which may provide deeper insight into the molecular mechanisms of how they exert their activity.

Conclusions

Since the discovery of TLRs a couple years ago, significant progress has been made in understanding the mechanisms of innate immune recognition. It is a generally accepted fact today that the innate immune system detects the invasion of microorganisms in first line via the TLRs that recognize structurally conserved motifs of pathogens and subsequently triggers defence mechanisms. TLRs also play a crucial role in orchestrating the adaptive immune response. However, there are still many questions that need to be answered. It is very likely that more PRRs, including TLRs, will be discovered in the near future (Martinon and Tschopp, 2005) and identification of their ligands will represent a challenge for the scientific community. Moreover, it is not clear whether recognition of ligands of some TLRs involves a direct or some not yet known indirect mechanism. As it is obvious from the data presented in our review articles many questions also remain open with regard to TLR signalling pathways: much effort will be necessary to clarify how the activation of individual TLRs causes differential gene expression and biological responses. Apart from activation of the common MyD88 signalling pathway, the response specificity of each TLR is attributed to the signalling cascades specific for each of the TLRs. These TLR-specific signalling pathways have not yet been completely elucidated. Finally, since there is an established connection between TLRs and activation of adaptive immunity mechanisms, it is probable that TLRs could be involved in some disorders. Indeed, it was shown that TLR9 had been involved in the pathogenesis of SLE. These data establish a critical link between the innate and adaptive immune systems in the development of systemic autoimmune disease and explain the preponderance of autoantibodies reactive with nucleic acid-protein particles. The unique features of this dual-engagement pathway could facilitate the development of therapies that specifically target autoreactive B cells. This strongly suggests that autoimmune disorders may be induced by the cross-talk between innate and adaptive immune response mechanisms. Further studies will be necessary to understand the complexity of cooperation between innate and adaptive immune systems.

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