

# Toll-like receptor 4-dependent pathways as sensors of endogenous "danger" signals. New evidences and potential therapeutic targets

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**EL RECEPTOR TIPO TOLL 4 COMO SENSOR DE SEÑALES ENDÓGENAS DE "PELIGRO". NUEVAS EVIDENCIAS Y DIANAS TERAPÉUTICAS POTENCIALES**

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## RESUMEN

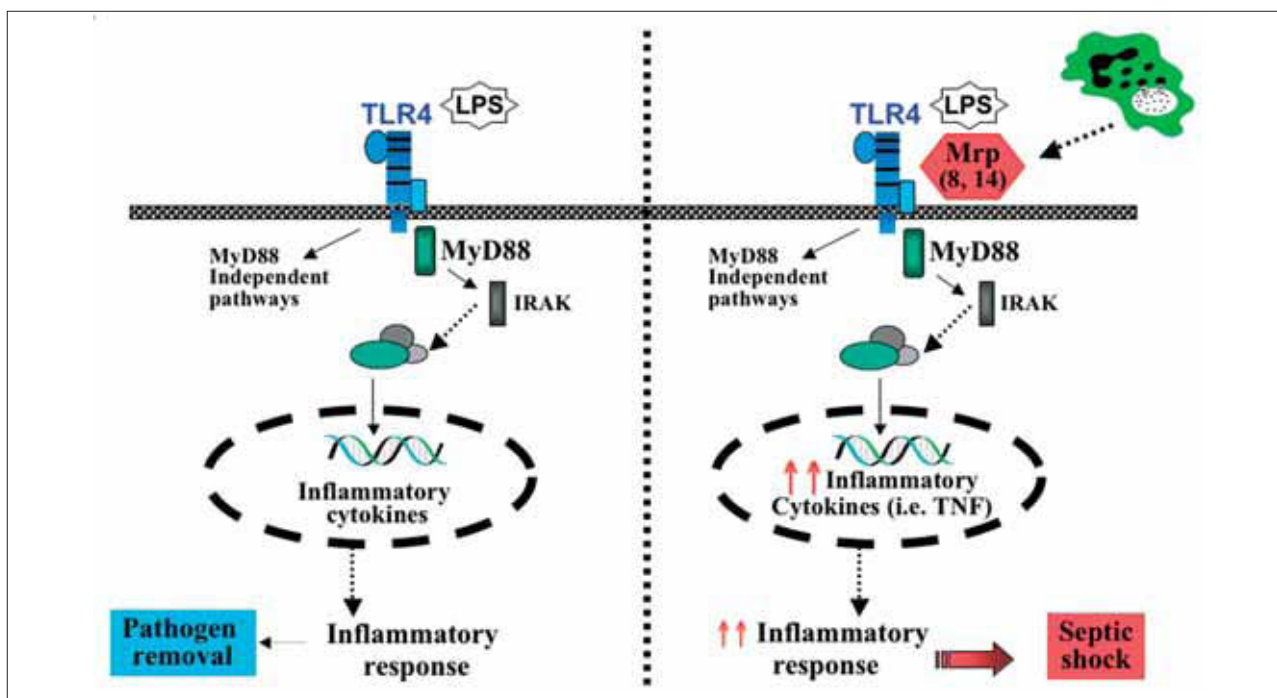
Los receptores de mamíferos homólogos de Toll (TLRs) son una familia de proteínas implicadas en el reconocimiento de patrones moleculares asociados a patógenos, que activan tanto la respuesta inmunológica innata como la adaptativa. TLR4 es el miembro de la familia que une el producto bacteriano lipopolisacárido (LPS). Hay evidencias de que los TLR son capaces de reconocer moléculas endógenas, aunque es un tema en debate. Dos artículos recientemente publicados destacan tanto el papel de la señalización mediada por TLR4 en el reconocimiento de señales endógenas de "peligro", como su implicación en patogénesis. Vogl y colaboradores describen una conexión entre TLR4 y el choque séptico a través de las proteínas de origen mieloide de la familia de proteínas S100 que unen calcio (Mrp) 8 y 14, dos moléculas proinflamatorias secretadas durante la activación fagocitaria. Apetoh y colaboradores describen un nuevo mecanismo por el cual las células tumorales que mueren por efecto de la quimioterapia liberan la proteína HMGB1, que es a su vez reconocida por TLR4, lo que induce una respuesta inmune necesaria para el éxito de la terapia antitumoral. Estos interesantes estudios no solo aumentan nuestro conocimiento de la biología de los TLR, sino que apoyan el modelo de "peligro", pues en ambos artículos se identifican moléculas "propias", producidas después del daño tisular o de la muerte inducida por quimioterapia, que actúan como señales de alarma activando el sistema inmune a través de cascadas de señalización mediadas por TLR4. Mas aun, estos artículos identifican nuevas dianas para el desarrollo de terapias frente al choque endotóxico y para la mejora de la eficacia de tratamientos antitumorales.

**PALABRAS CLAVE:** Receptores tipo Toll /Proteínas Mrp/ Choque séptico/ HMGB1/ Inmunidad frente a tumores/ Terapia anticáncer.

## ABSTRACT

Toll-like receptors (TLRs) are a family of proteins involved in the recognition of pathogen-associated molecular motifs that activate both innate and adaptive immune responses. TLR4 is the member of the family that binds to bacterially produced lipopolysaccharide (LPS). Although still on debate, increasing evidences show that TLRs are also able to recognize endogenous molecules. Two recent reports have highlighted the role of TLR4 signaling pathways as sensors of endogenous 'danger' signals and their association to pathogenesis. Vogl and colleagues describe a connection between TLR4 and septic shock through the calcium-binding S100 family of myeloid-related proteins (Mrp) 8 and 14, two proinflammatory molecules secreted by phagocytes upon activation. Apetoh and colleagues describe a new pathway whereby tumor cells dying after chemotherapy release the high-mobility group box 1 protein (HMGB1), which is recognized by TLR4 and induces an immune response that is required for the success of therapy. These interesting studies not only increase our understanding of the biology of TLR4, but also support the 'danger' model since both reports identify 'self' molecules, produced after tissue damage or chemotherapy-induced cell death, that act as 'alarm' signals activating the immune response through TLR4-dependent pathways. Moreover, these articles identify new targets for developing novel therapeutic treatments for septic shock and for the improvement of the efficacy of anticancer drugs.

**KEY WORDS:** Toll-like receptors/ Mrp proteins/ Septic shock/ HMGB1/ Antitumor immunity/ Cancer therapies.



**Figure 1.** Mrp cooperates with LPS in the overstimulation of TLR4 leading to septic shock. Bacterial LPS is a strong activator of various immune responses. TLR4 engagement leads to activation of MyD88-dependent and -independent pathways and subsequent expression of inflammatory cytokines that ultimately lead to solve the infection (left panel). Vogl and colleagues have identified the phagocyte-derived proteins Mrp8 and Mrp14 as endogenous TLR4 ligands that cooperate with LPS in the massive production of cytokines leading to sepsis and lethal endotoxic shock in mice (right panel).

## TOLL-LIKE RECEPTORS (TLRS)

TLRs are pattern recognition receptors that activate the early host response to microbial components, and generate signals leading to initiate adaptive immune responses<sup>(1,2)</sup>. It was initially thought that TLRs help the immune system to discriminate 'self' from 'infectious non-self' molecules, however growing evidences show that TLRs can sense host-derived molecules under certain circumstances, i.e. endogenous damage or 'danger' signals originated at the site of inflammation. However, this is still a controversial issue due to possible LPS contamination of reagents used in some published data.

To date, several members of the TLR family have been identified, 11 in humans and 13 in mouse<sup>(1)</sup>. TLRs can sense a broad range of microbial pathogen signatures, including surface components and nucleic acids. TLR4, the first mammalian homologue of *Drosophila* Toll proteins identified in humans, has generated much interest since it is the long-sought receptor for lipopolysaccharide (LPS), a glycolipid component from the outer membrane of Gram-negative bacteria. At the membrane of immune cells, LPS recognition is complex and involves TLR4, in conjunction with CD14 and myeloid differentiation protein 2 (MD2). Binding of

LPS triggers a signaling cascade that begins with myeloid differentiation factor (MyD) 88 translocation to the cell membrane, where it activates interleukin-1 receptor-associated kinase-1 (IRAK-1) (Fig. 1, left panel). Subsequent activation of nuclear factor  $\kappa$ B transcription factor (NF- $\kappa$ B) promotes the expression of inflammatory genes, i.e. tumor necrosis factor (TNF)- $\alpha$ , IL-1, and IL6. Alternative pathways may occur through the induction of MyD88-independent signaling cascades leading to the activation of interferon regulatory factor 3 and subsequent expression of IFN-inducible genes.

Evidences from genetic, clinical and basic science studies support the role of TLR in pathogenesis. TLR function has been involved in several diseases including sepsis, atherosclerosis, immunodeficiencies, asthma, and diabetes<sup>(3,4)</sup>, a reason why TLR-dependent pathways are considered relevant pharmacological targets for the treatment of different pathological conditions.

## TLR4 AND SEPTIC SHOCK:

### A CONNECTION THROUGH Mrp8-Mrp14

Sepsis, and its most severe form, septic shock are characterized by a systemic inflammatory response that can

lead to multiple organ failure, and continue to be a major cause of morbidity and mortality in critically ill patients. Gram-negative bacteria cause more than half of the cases of sepsis. In general, infection can be controlled with antibiotics, while the systemic inflammatory response is difficult to manage using existing treatment methods. Unfortunately, various failed attempts to control sepsis have kept expectations concerning its treatment rather low.

Since LPS has been associated with initiation of inflammatory responses leading to septic shock, and TLRs play a central role in the inflammatory response during bacterial infection, TLR-dependent pathways have attracted a lot of attention as possible targets for controlling the course of sepsis. In general, LPS activates TLR4-dependent signal pathways leading to the activation of an inflammatory response that eliminate bacteria. However, under certain circumstances, overstimulation of TLR signaling can result in a systemic inflammatory response, progressing to hypotension, shock, organ failure and even death. Studies in mice models and human TLR polymorphisms provide evidences for a role of TLR in sepsis<sup>(4)</sup>. The lack of TLR4 signaling, i.e. MyD88 and IRAK4, provides protection against sepsis in animal models. In humans, polymorphisms in TLR4 have been related to susceptibility to Gram-negative infections and septic shock. A sequence polymorphism affecting the extracellular domain of TLR4 is associated with LPS response deficiency and with a reduced susceptibility to cardiovascular disease. Regarding the TLR4 (Asp299Gly) polymorphism, found in 8-10% of Caucasians, data supporting the association of TLR4 mutation and LPS signaling deficiency is still controversial. Polymorphisms in TLR4 signal molecules such as IRAK-4 are also associated with impaired responses to bacterial infection. Now reporting in *Nature Medicine*, Vogl and colleagues<sup>(5)</sup> describe a link between TLR4 and septic shock through endogenous proteins released during phagocyte activation (Fig. 1, right panel).

The calcium-binding S100 family of myeloid-related proteins (Mrp) 8 and Mrp14 are cytosolic molecules released after activation of neutrophils and monocytes that have been associated to inflammatory diseases<sup>(6)</sup>. To address the extracellular function of these proteins, Vogl et al. performed gene-expression analysis of mouse bone marrow cells from Mrp14<sup>-/-</sup> mice. The study revealed that Mrp14 deficiency impaired the expression of several LPS-induced genes, including the proinflammatory cytokine TNF- $\alpha$ , as compared to wild-type cells. Chromatin immunoprecipitation experiments suggested a role of Mrp14 in the regulation of TNF- $\alpha$  expression, as judged by reduced binding of NF- $\kappa$ B factors p50 and p65 to the TNF- promoter in Mrp14<sup>-/-</sup> cells. Moreover, addition of recombinant Mrp8 alone, or a Mrp8-

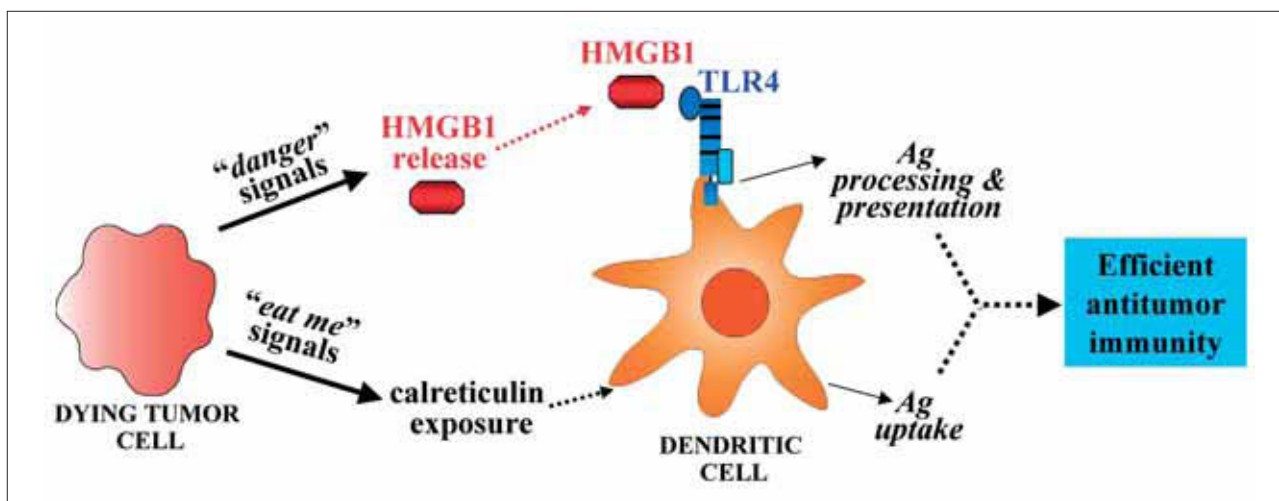
Mrp14 complex, partially rescued LPS-induced TNF- $\alpha$  expression, and co-treatment with LPS doubled the effect. The results of Vogl et al. suggest that Mrp8-Mrp14 and LPS have additive effects on the expression of inflammation-related genes, and that Mrp8 seems to be the active component while Mrp14 modulates its actions. Whether LPS needs to bind directly to the Mrp8-Mrp14 complex or not remains to be studied.

Next, Vogl et al. studied Mrp-induced signaling cascades. The authors observed that Mrp8 and LPS shared signaling routes in mouse bone marrow cells and human monocytes. Additionally, Mrp8 activated molecular effectors of TLR4 signaling, namely MyD88 translocation from the cytosol to the plasma membrane and hyperphosphorylation and activation of IRAK1, in human alveolar epithelial cells. Mouse phagocytes expressing a non-functional TLR4 mutant did not respond to Mrp or LPS activation. HEK293 cells transfected with TLR4, CD14 and MD2 showed Mrp8-induced NF- $\kappa$ B activation. Furthermore, the authors demonstrated direct and specific binding of Mrp8 to the TLR4-MD2 complex by surface plasmon resonance studies *in vitro*. These results demonstrate that Mrp8 is an endogenous ligand for TLR4, and suggest that is not a ligand for the receptor for advanced glycation end products (RAGE), as seen with other S100 proteins<sup>(6)</sup>.

The biological contribution of the Mrp8-Mrp14 complex to exacerbated inflammation during sepsis was evaluated in two mouse models, namely LPS-induced shock and *E. coli*-induced abdominal sepsis models. Vogl and colleagues observed that mice lacking Mrp8-Mrp14 complexes survived significantly longer than wild-type mice after LPS injection. Moreover, lethality was restored after intravenous administration of Mrp8 and the Mrp8-Mrp14 complex, thus arguing for a crucial role of Mrp8-Mrp14 complexes in the pathogenesis of endotoxic shock in mice.

The results of Vogl et al. demonstrate that TLR4 can sense endogenous molecules released during phagocyte activation. The relevance of endogenous ligands of TLRs is still controversial, since some authors argue that traces of LPS or lipopeptide present in recombinant reagents could explain many of the published data, i.e. heat-shock protein (HSP) 70-mediated LPS signaling. Aware of this possibility, the authors claim that they have followed a strict control to make sure that the Mrp preparations were endotoxin-free.

In summary, Vogl and colleagues demonstrate that the neutrophil and monocyte cytoplasmic proteins Mrp8 and Mrp14 are TLR4 ligands. Furthermore, the 'self' molecules Mrp8 and Mrp14 cooperate with a 'non-self' molecule, LPS, to overstimulate TLR4-dependent signaling pathways



**Figure 2.** Efficient immune response against tumors requires both "eat me" and "danger" signals. It was previously described that calreticulin, acting as an "eat me" signal generated by dying cells, promotes antigen uptake. Apetoh et al. identify the endogenous protein HMGB1 released by chemotherapy-induced cell death as a 'danger' signal that binds to TLR4 present in dendritic cells and promotes processing and presentation of tumor antigens. The authors propose that both "eat me" and "danger" signals are necessary to induce efficient antitumor immunity.

leading to an exacerbated inflammatory response that terminates in lethal septic shock in mice. Mrp proteins, found to be upregulated in many inflammatory disorders<sup>(6)</sup> and involved in the pathogenesis of endotoxic shock upstream of TNF- $\alpha$  activation<sup>(5)</sup>, may be promising targets for the therapeutic interventions of sepsis and other undesirable inflammatory processes. Questions remain regarding the relevance of these proteins in the initiation of human sepsis.

### TLR4-HMGB1 ROLE IN THE IMMUNE RESPONSE AGAINST TUMORS

Most current cancer therapies are supposed to eliminate tumor cells through an apoptotic pathway. Even though it was commonly assumed that programmed cell death does not stimulate immune responses, some evidences suggest that apoptosis activates adaptive responses during pathogen infection. The groups led by Guido Kroemer and Laurence Zitvogel have previously shown that tumor cell death induced by some cytotoxic agents can also induce antitumor immune responses<sup>(7)</sup>. Furthermore, exposure of calreticulin, formerly described as an 'eat me' signal from apoptotic cells<sup>(8)</sup>, is critical in immunogenic cancer cell death<sup>(9)</sup>. Now reporting in *Nature Medicine*, Guido Kroemer, Laurence Zitvogel and colleagues<sup>(10)</sup> describe a new pathway by which tumor cells dying after chemotherapy treatment secrete 'danger' signals such as the high-mobility group box 1 protein (HMGB1). This protein is recognized by TLR4 and induces an immune response required for the efficacy of therapy (Fig. 2).

TLRs are known to bind to damage-associated endogenous proteins such as HSP60, fibronectin etc, and the recently reported non-histone chromatin-binding nuclear constituent HMGB1<sup>(11)</sup>. In order to study whether TLRs are involved in the immune response against tumors, Apetoh et al. inoculated mice lacking different TLRs with doxorubicin-treated or oxaliplatin-treated dying thymoma, sarcoma, or colon cancer cells. The authors observed that only TLR4-deficient mice were defective in T cell priming after re-stimulation with tumor antigens. Moreover, dendritic cells expressing TLR4 were required for the immune response to dying tumor cells. By using immunoprecipitation assays, the authors showed that HMGB1 is secreted by dying tumor cells and acts as a 'danger' signal that initiates the immune response by interacting with and activating TLR4 expressed on dendritic cells, which are selectively involved in the cross-priming of anti-tumor T lymphocytes *in vivo*. Moreover, HMGB1 seems to be essential for the immune response against tumors, since pre-incubation of tumor cells with a specific small interfering RNA, or neutralization of HMGB1 with antibodies inhibited the activation of dendritic cells by dying tumor cells. Upon HMGB1 recognition, TLR4 activates MyD88-dependent signaling pathways, as the observed response to exposure to dying tumor cells was similar in dendritic cells from MyD88- and TLR4-deficient mice.

Next, the authors evaluated the putative role of the HMGB1-TLR4-MyD88 pathway in the efficacy of anticancer drugs in TLR4<sup>-/-</sup> and MyD88<sup>-/-</sup> mice. Apetoh et al. found that the effectiveness of chemotherapy or local radiotherapy



was compromised in TLR4- and MyD88-deficient mice with established tumors, since therapies were not as efficient at slowing tumor growth or increasing survival as in wild-type mice.

To investigate the clinical relevance of these findings, the authors performed a study in 280 breast-cancer patients. They observed that the *TLR4* (Asp299Gly) polymorphism not only reduced the interaction between TLR4 and HMGB1, but also blocked presentation of tumor antigens by dendritic cells to cytotoxic T cells. In addition, the time to metastasis in patients with non-metastatic breast cancer who had been treated with anthracyclines after surgery because of lymph node involvement was also analyzed. The authors observed that a specific mutation of TLR4, Asp299Gly, compromised the effectiveness of chemotherapy in breast cancer, as the frequency of metastasis was higher in the TLR4-mutant carriers than in patients carrying the normal *TLR4* allele. Whether this clinical outcome could be overcome with TLR4 ligands remains to be studied.

In summary, these interesting and clinically relevant findings suggest that cytotoxic cancer agents not only kill tumor cells but that chemotherapy-induced cell death also activate the host's immune system contributing to the success of therapy, at least for some protocols used to treat breast cancer that include anthracyclines. Based on these and previous results, the authors propose that an optimal therapeutic outcome requires the immunoadjuvant effect of chemotherapy-induced tumor cell death, and emission of both 'eat me' and 'danger' signals by dying tumor cells is necessary for efficient immune responses against tumors (Fig. 2). Alarm or 'danger' signals produced by injured cells, i.e. HMGB1, but not by healthy cells or by cells undergoing normal physiological death, activate TLR4-mediated immune responses. As to the clinical relevance of these remarkable results, they open new avenues to improve the immunogenicity of current chemotherapy procedures. Questions remain regarding whether immunogenetic factors also operate and determine the clinical outcome in other tumors and different therapeutic agents.

## TLR AND THE 'DANGER' MODEL

Two theories have been proposed to explain the activation of the immune system. In the 'infectious non-self' or 'stranger' model proposed by Charles Janeway in the late 80s, the immune cells might recognize pathogen-associated pattern molecules. Years later, this model found support in the discovery of TLRs, which act as pattern recognition receptors that detect 'infectious-non self' molecules conserved in pathogens. However the model was not able to explain old

issues, i.e. autoimmune diseases. Then, Polly Matzinger proposed the 'danger' model, in which the immune system might be activated by alarm or 'danger' signals from injured tissues upon exposure to pathogens, toxins, mechanical damage, etc.<sup>(12)</sup>. The model has been supported by the identification of uric acid as a potential 'danger' signal that activates an immune response to dying cells<sup>(13)</sup>. The description of two new 'self' proteins as TLR4 ligands, now reported in *Nature Medicine*<sup>(5,10)</sup>, provides new evidences in favor of the danger model, since TLRs can sense both exogenous and endogenous molecules. Host-derived molecules produced or modified by injured or chemotherapy-induced dying cells acting as 'danger' signals, i.e. HMGB1, are not produced by healthy cells or by cells undergoing physiological death. Moreover, endogenous molecules released after tissue damage could activate the innate immune response through TLRs, even in the absence of pathogens, or cooperate with 'infectious non-self' molecules, i.e. Mrp8-Mrp14 and LPS, to initiate an exaggerated inflammatory response leading to an inflammatory disease. Then, the proposed 'danger' mechanism would complement the 'infectious non-self' model, and might help to explain the molecular basis of some inflammatory diseases, autoimmunity, atherosclerosis, etc.

## CONCLUDING REMARKS

These recent reports from Vogl et al. and Apetoh et al. help to understand the molecular mechanisms underlying septic shock and tumor immune responses, and also support the 'danger' model. A common player, TLR4 and its adaptor molecules, can detect host-derived proteins acting as 'danger' signals leading to sepsis and to an efficient immune response against tumors. Additional questions remain about the recognition of the endogenous ligands Mrp8-Mrp14 and HMGB1 by TLR4, but their utilization is likely to develop new therapies for several pathologies such as inflammatory disorders and autoimmune diseases.

## DISCLOSURES

The author declares no financial conflict of interest.

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