On Toll-like receptors and the innate immune response in sepsis caused by Burkholderia pseudomallei (melioidosis)
Wiersinga, W.J.
Chapter 7

The role of Toll-like receptors in sepsis


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The recently discovered class of Toll-like receptors (TLRs) has emerged as the central line of defense against invading pathogens. They are the first to detect host invasion by pathogens, initiate immune responses and form the crucial link between the innate and adaptive immune systems. In general, the immune activation that follows TLR activation will be sufficient to combat the wide variety of pathogens that daily invade the human body. However, in the case of sepsis which can be defined as the disadvantageous systemic host response to infection, these TLR-mediated responses may exceed the threshold to maintain homeostasis of the immune system. This review focuses on the new insights in the pathogenesis of sepsis that is offered by the impressive amount of research that has been conducted in the TLR research field and their potential clinical implications for intensive care medicine.

The Toll-like receptor family

The innate immune system discriminates potential pathogens from self through a series of receptors that recognize conserved motifs on pathogens that are not found in higher eukaryotes. These motifs have been termed "pathogen-associated molecular patterns" or PAMPs, whereas their cognate binding partners on host cells involved in the innate immune response have been named "pattern-recognition receptors" or PPRs. Examples of PAMPs include lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria, peptidoglycan (present in most bacteria), lipoteichoic acid (in many Gram-positive bacteria) and mannans in the yeast cell wall.

The Toll family of receptors, which is conserved throughout evolution from flies to humans, has been implicated to play a central role as PPRs in the initiation of cellular innate immune responses. First discovered in the fruit fly, at present 11 human homologs of Drosophila Toll have been identified. This human receptor family has been designated Toll-like receptors or TLRs. TLRs are distinguished from other PRRs by their ability to recognize, and more significantly, discriminate between different classes of pathogens. Ligands for 9 human TLRs have been described (see Table 1). Of note, one of the TLR mysteries relates to TLR11, a receptor present in mice, but not humans, and known to recognize uropathogenic Escherichia coli. Recently, the first defined ligand for TLR11 has been described as a profilin-like protein from Toxoplasma gondii. It has to be emphasized however that the TLRs function as one system; different components of one microorganism are recognized by different TLRs. Escherichia coli for example is a Gram-negative bacterium expressing several PAMPs (peptidoglycan, LPS, flagellin en bacterial DNA), which are all recognized by different TLRs (TLR2, TLR4, TLR5 and TLR9 respectively).

TLRs: the essential link between innate and adaptive immunity

It has become clear that activation of the innate immune system is a prerequisite for the induction of the adaptive immune system. TLRs form the bridge between these two systems and play an essential role in the coordination of the adaptive immune response. TLRs control induction of T cell responses at two levels, first by induction of co-stimulatory molecules which mark the
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associated peptide as foreign and second by secretion of cytokines that are necessary to overcome the peripheral tolerance induced by regulatory T cells \(^4, 6\). In addition, TLRs are responsible for the induction of dendritic cell maturation, which is necessary to initiate adaptive immune responses. It has to be said however, that other components of the innate immune system, such as the complement system and natural killer (NK) cells, are also capable of influencing the adaptive immune system \(^7\).

### Table 1. Toll-like receptor ligands in infectious diseases

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Pathogen/PAMP</th>
<th>Origin of ligand</th>
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<tbody>
<tr>
<td>TLR1 (with TLR2)</td>
<td>Triacyl lipopeptides</td>
<td>(Myco)bacteria</td>
</tr>
<tr>
<td></td>
<td>Soluble factors</td>
<td>Neisseria meningitides</td>
</tr>
<tr>
<td>TLR2</td>
<td>Lipoproteins</td>
<td>Various pathogens</td>
</tr>
<tr>
<td></td>
<td>Peptidoglycan</td>
<td>Gram-positive bacteria</td>
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<tr>
<td></td>
<td>Lipoteichoic acid</td>
<td>Gram-positive bacteria</td>
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<tr>
<td></td>
<td>Modulin</td>
<td>S. epidermidis</td>
</tr>
<tr>
<td></td>
<td>Atypical LPS</td>
<td>Leptospira, P. gingivalis</td>
</tr>
<tr>
<td></td>
<td>Porins</td>
<td>Neisseria, H. influenzae</td>
</tr>
<tr>
<td></td>
<td>AraLAM</td>
<td>Mycobacteria</td>
</tr>
<tr>
<td></td>
<td>19 kD antigen</td>
<td>M. tuberculosis</td>
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<tr>
<td></td>
<td>STF</td>
<td>M. tuberculosis</td>
</tr>
<tr>
<td></td>
<td>Zymosan</td>
<td>Fungi</td>
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<tr>
<td>TLR3</td>
<td>ds RNA</td>
<td>Viruses</td>
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<tr>
<td>TLR4</td>
<td>LPS</td>
<td>Gram-negative bacteria</td>
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<td></td>
<td>Fusion protein</td>
<td>Respiratory syncytial virus</td>
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<td></td>
<td>Taxol</td>
<td>Plants</td>
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<tr>
<td></td>
<td>Hyaluronic acid</td>
<td>Host</td>
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<tr>
<td></td>
<td>Fibrinogen</td>
<td>Host</td>
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<td></td>
<td>HSP70*</td>
<td>Host</td>
</tr>
<tr>
<td></td>
<td>HSP60*</td>
<td>Chlamydia pneumonia</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td>Various bacteria</td>
</tr>
<tr>
<td>TLR6 (with TLR2)</td>
<td>Diacyl lipopeptides</td>
<td>Mycoplasma</td>
</tr>
<tr>
<td></td>
<td>Lipoteichoic acid</td>
<td>Gram-positive bacteria</td>
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<tr>
<td></td>
<td>Zymosan</td>
<td>Fungi</td>
</tr>
<tr>
<td>TLR7</td>
<td>ss RNA</td>
<td>Viruses</td>
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<tr>
<td></td>
<td>Imidazoquinoline</td>
<td>Synthetic compound</td>
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<td>TLR8</td>
<td>ss RNA</td>
<td>Viruses</td>
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<tr>
<td></td>
<td>Imidazoquinoline</td>
<td>Synthetic compound</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG DNA</td>
<td>Bacteria and viruses</td>
</tr>
<tr>
<td>TLR10</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>TLR11</td>
<td>Largely unknown</td>
<td>Uropathogenic bacteria</td>
</tr>
<tr>
<td></td>
<td>Profilin-like protein</td>
<td>Toxoplasma gondii</td>
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</tbody>
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* heat shock proteins, or stress proteins, are present in all cells and function as transport proteins within the cell. They are released under stressful conditions such as heat, cold or hypoxemia. When they are expressed at the cell surface, they play a role as signaling proteins in the recognition of diseased cell by the immune system. They are endogenous ligands of TLRs.
**TLR signaling**

The discovery that TLR4 is the long sought after LPS receptor was a major breakthrough in immunology. However, TLR4 showed to be not the only protein important in the recognition of LPS (see Figure). First, LPS binds to LPS-binding-protein (LBP), which transfers LPS to CD14. CD14 is expressed on the outer membrane of monocytes, some granulocytes and activated B-lymphocytes. MD-2, another TLR4-associated protein, is required to activate the CD14-MD-2-TLR4–complex (see figure 1). Binding of LPS to CD14 leads to the association of CD14 with MD-2 and TLR4. So far, TLR4 is the only known TLR that requires an extra protein next to the ligand to be activated. After stimulation of the TLR, the adapter molecule myeloid differentiation primary-response protein 88 (MyD88) is recruited. MyD88 associates with IL-1R-associated kinase (IRAK) 4. This results in the phosphorylation of IRAK-1 which forms a complex with TNF-receptor-associated-factor (TRAF) 6. This then interacts with another preformed complex (consisting of TAK1, TAB1 and TAB2 or TAB3) which leads to the activation of the inhibitor-of-nuclear-factor-κB (IKK) complex. The IKK complex phosphorylates the IκBs. Subsequent release of nuclear-factor-κB (NF-κB) results in the transcription of a whole range of inflammatory genes. In addition, it was recently shown that the transcription factor IRF-5, which forms a complex with MyD88 and TRAF6, also functions as a general signal transducer that mediates MyD88-dependent gene induction of proinflammatory cytokines.

Next to this so-called MyD88-dependent pathway, a MyD88-independent route, exists which is used only by TLR4 and TLR3. The MyD88-independent route will result in the delayed activation of NF-κB and the production of interferon-β. Furthermore, it is now known that different TLRs...
use different adaptor molecules. This explains why various TLRs lead to different patterns of gene expression. Next to MyD88, the adapter molecules TIR-domain-containing-adaptor-protein (TIRAP), TIR-domain-containing-adaptor-protein-inducing-IFN-β (TRIF) and TRIF-related-adaptor-molecule (TRAM) are identified. TIRAP is essential for MyD88-dependent signaling through TLR2 and TLR4. TRIF is essential for the TLR3 and TLR4-mediated activation of the MyD88-independent pathway. TRAM is involved in TLR4 mediated MyD88-independent/TRIF-dependent signaling pathways.

At first sight, the TLR signaling pathway seems overwhelming by complexity. However, it is fascinating to see that the innate immune signals through a channel of relatively low complexity: only 11 TLRs, 4 adapter molecules and a couple of protein kinases are required for the recognition and response to a whole universe of often complex microbial molecules. After this narrow strait there are again thousands of different possible host responses. Beutler has called this the “hourglass” shape of the innate immune response.

**Regulation of TLR signaling**

In order to prevent strong uncontrolled inflammatory reactions tight regulation of the TLR signaling pathway is mandatory. Generally spoken, an encounter of the immune system with pathogens will result in the upregulation of a whole spectrum of different TLRs. For instance, in patients with sepsis caused by the Gram-negative bacterium *Burkholderia pseudomallei* increased expression of TLR1, TLR2 and TLR4 on the cell surface of circulating monocytes and granulocytes is seen, together with increased TLR1, TLR2, TLR4, TLR5 and TLR10 mRNA levels in blood cells (our unpublished data). However, the exact consequences of these enhanced TLR expression profiles for host defense remain to be established. Furthermore, it has been shown that certain cytokines play essential roles in TLR regulation. Besides LPS, inflammatory cytokines, such as IL-2, IL-15, IL-1β, IFNγ and TNFα, are able to induce TLR2 gene expression in mouse macrophages. Interestingly, this did not hold true for TLR4 gene expression.

Negative regulation of the TLR signaling pathway is essential (see Figure and Table 2). In the cytoplasm, IRAK-M inhibits the dissociation of the IRAK1-IRAK4 complex from the receptor, suppressor-of-cytokines-signaling-1 (SOCS1) probably directly inhibits IRAK1 and a short form of MyD88 (MyD88s) blocks the association of IRAK4 with MyD88. On the cell membrane, other members of the TIR-superfamily, such as Single-immunoglobulin-IL-1R-related-molecule (SIGIRR) and ST2, also negatively modulate TLR signaling. More specifically, ST2 is an inhibitor of TLR2, TLR4 and TLR9 signaling. Lastly, the TLR-like molecule RP105, which surface expression is dependent on the co-expression of the MD-2 homolog MD-1, interacts directly with the TLR4 signaling complex, inhibiting its ability to bind a microbial ligand.

Other mechanisms by which TLR signaling can be controlled include the reduction of TLR expression by TLR degradation or inhibition by anti-inflammatory cytokines. Furthermore, it has become clear that TLRs can function as death receptors; this TLR-induced apoptosis may be important in the control of a dysregulated TLR response.
ARE THE TLRs CENTRAL IN THE HOST DEFENSE AGAINST SEPSIS?

Given their central role in the recognition of microbes, it is rational to hypothesize that TLRs play a central role in sepsis pathogenicity. Indeed, animals lacking the gene encoding TLR4 do not develop septic shock in response to LPS. Although LPS is the best studied and probably most important mediator of sepsis, peptidoglycan, lipoteichoic acid, bacterial CpG motifs and flagella are other important microbial products implicated in the pathogenesis of sepsis. All these PAMPs signal through different TLRs. As a result, the relationship between TLR expression and human sepsis may be complex.

In recent years TLR2 has been recognized as the Gram-positive TLR because of its ability to sense major Gram-positive cell wall components such as peptidoglycan and lipoteichoic acid, whereas TLR4 – the LPS receptor – is seen as the Gram-negative TLR. However, as more knowledge about the precise role TLRs in different bacteria becomes available, this concept has to be modified. For instance, Streptococcus pneumoniae is sensed by the innate immune system not only through TLR2 which recognizes lipoteichoic acid and peptidoglycan, but also through TLR4 with recognizes pneumolysin. It was recently showed that TLR2 is indispensable for alveolar macrophage responsiveness toward S. pneumoniae. However in the same study, TLR2 gene-deficient mice intranasally
inoculated with non-lethal to lethal doses of *S. pneumoniae* displayed only a modestly reduced inflammatory response in their lungs and showed an unaltered antibacterial defense and survival in comparison with wild-type mice 17. These data suggest that the function of TLR2 is limited in the innate immune response to *S. pneumoniae*. Clearly, other PRRs play an important role.

It seems to be obvious that TLR4 has an important role in Gram-negative infections. As mentioned, TLR4 deficient mice do not develop septic shock after administration of high doses of LPS 16. Furthermore, TLR4 deficiency resulted in diminished clearance of *H. influenzae* and *K. pneumoniae* in a mouse model of pneumonia 18, 19, suggesting that the recognition of LPS by TLR4 contributes to an effective immune response during these infections. However, not all studies show the importance of TLR4 signaling in Gram-negative infections. When mice lacking the *TLR4* gene were inoculated with the Gram-negative bacterium *B. pseudomallei* in a mice model of severe sepsis no differences are observed in terms of inflammatory response (cytokine production, histological organ damage) or outcome (survival) when compared to normal wild type mice (our own unpublished data). In the same model, TLR2 mutant mice show a clear survival advantage over wildtype mice. To make things more complicated, responsiveness of TLR2 to LPS has also been described 20. In this respect, it is interesting that pretreatment with bacterial lipoprotein, a TLR2 ligand, protected otherwise highly susceptible TLR4-deficient C3H/HeJ mice from *S. typhimurium* induced Gram-negative sepsis via enhanced bacteria clearance 21.

The relationship between TLR expression and human sepsis may be complex, as is suggested by some recent studies in septic patients 22, 23. Increased cellsurface (neutrophils and monocytes) expression and increased mRNA levels of both TLR2 and TLR4 are seen in septic patients 22, 23. No association with functional outcome could be determined 22. Interestingly, in one study increased levels of TLR2 mRNA were seen in both Gram-positive and Gram-negative sepsis, whereas TLR4 mRNA was only increased in Gram-positive sepsis 22.

NEW TLR MEDIATED PLAYERS IN THE SEPSIS ARENA

Some recently discovered mediators of sepsis are directly involved in TLR signaling, all of which are regarded as promising new therapeutic targets.

**Triggering Receptor Expressed on Myeloid cells-1 (TREM-1)**

TREM-1 amplifies the TLR-mediated inflammatory response to microbial products 24. TREM-1, which signals through the adapter protein DAP12, is strongly and specifically expressed on monocytes and neutrophils from patients with sepsis 24. In human endotoxemia, monocytes display a gradual up-regulation of TREM-1, whereas granulocyte TREM-1 expression was high at baseline and immediately down-regulated upon LPS exposure along with an increase in soluble TREM-1 25. Elevated concentrations of soluble TREM-1 in bronchoalveolar-lavage fluid can indicate ventilator-associated pneumonia in patients receiving mechanical ventilation 26, and high concentrations in plasma can indicate infection in patients with systemic inflammatory response syndrome 27. Excitingly, blockade of TREM-1 protected mice against LPS-induced shock,
as well as microbial sepsis caused by live *Escherichia coli* or cecal ligation and puncture (CLP) \(^{24}\). In addition, a synthetic peptide mimicking a short highly conserved domain of sTREM-1 protected septic animals from LPS hyper-responsiveness and death \(^{28}\).

Intriguingly, although TREM-1 signals through the adapter protein DAP12 \(^{24}\), a recent study showed that DAP12-deficient mice have – contrary to what would be expected - enhanced TLR responses *in vitro*, as indicated by an enhanced production of pro-inflammatory cytokines by DAP12-deficient macrophages in response to TLR agonists *in vitro* and *in vivo*, as indicated by an increased susceptibility to endotoxic shock \(^{29}\). Thus, perhaps certain DAP12-associated receptors function as negative regulators of TLR responses.

**Macrophage Migration Inhibitory Factor (MIF)**

In recent years MIF has emerged as a pivotal regulator of innate immunity that has been implicated in sepsis pathogenesis \(^{30, 31}\). MIF regulates innate immune responses through modulation of TLR4 \(^{30}\): when MIF-deficient mice were challenged with LPS they showed a defective response as a direct result of decreased TLR4 expression \(^{30}\). In patients, MIF levels correlate with fatal outcome in sepsis \(^{32}\). MIF-directed therapies might offer a new treatment opportunity for sepsis. Inhibition of MIF activity with neutralizing anti-MIF antibodies protected mice from septic shock \(^{31}\). Furthermore, a specific small molecule inhibitor of MIF, named ISO-1, partially protects mice from sepsis induced by endotoxin or CLP \(^{33}\).

**High-Mobility Group Box 1 protein (HMGB-1)**

HMGB-1 is recognized as a cytokine and functions as a late mediator of sepsis and is elevated in the majority of septic patients \(^{34, 35}\). It is secreted by activated immune cells and, along with the receptor for advanced glycation end products (RAGE), interacts with TLR2 and TLR4, which may provide an explanation for the ability of HMGB-1 to generate inflammatory responses that are similar to those initiated by LPS \(^{36}\). LPS stimulation was found to mediate the release of HMGB-1 from macrophages at a considerably later stage than the release of the pro-inflammatory cytokines TNF\(\alpha\) and IL-1 \(^{35}\). Administration of HMGB-1 itself was lethal to mice, whereas the administration of antibodies to HMGB-1 diminished endotoxin lethality \(^{35}\).

**TLR POLYMORPHISMS IN SEPSIS**

Recent phenotype-genotype studies showed that TLR polymorphisms can alter both the susceptibility to and the clinical course of infectious diseases. Mutations in TLR encoding genes are not uncommon. For instance, the reported incidence of the TLR4 Asp299Gly polymorphism lies between 6 to 11% in the Caucasian population \(^{37, 38}\).

It was first shown that TLR4 mutations (Asp299Gly and Thr399Ile) are associated with endotoxin hyporesponsiveness in humans \(^{39}\). Subsequently, it was reported that polymorphisms in TLR4 could predispose people to develop septic shock with Gram-negative microorganisms \(^{37, 40}\). Most likely, the increased susceptibility to Gram-negative sepsis is caused by a diminished and thus inadequate response to LPS. Further associations have been found between the TLR2 Arg753Gln
polymorphism and increased susceptibility to sepsis caused by Staphylococcus aureus. On the other hand, in a series of 1047 patients with culture proven meningococcal disease, the TLR4 Asp299Gly polymorphism was – contrary to the hypothesis – not associated with host susceptibility or severity of disease. In a cohort of 252 critically ill patients, it was shown that single nucleotide polymorphisms (SNPs) in CD14 and TLR2 are associated with increased prevalence of sepsis, but not with altered prevalence of septic shock or decreased 28-day survival. In this study, as was expected, CD14 SNPs were associated with Gram-negative infections and TLR2 SNPs with Gram-positive infections. Interestingly, when these results are taken together, it could well be that certain SNPs in TLRs may alter recognition and clearance of bacteria, but they do not seem to change the outcome of patients with sepsis. In addition, one has to bear in mind the importance of ethnic differences in genetic variations; for example in a recent Japanese cohort of 197 critically ill patients and 214 healthy controls not one single participant carried a TLR4 polymorphism and no association was found between CD14 polymorphism and sepsis.

New studies that investigate the role of TLR polymorphisms in sepsis are underway. SNP analyses can serve as both an important research tool to further elucidate the complex pathogenicity of sepsis and as a clinical instrument to predict the clinical course of ICU patients and to ultimately individualize treatment.

THE TOLL-LIKE RECEPTORS AS A NEW TREATMENT TARGET IN SEPSIS

Manipulation of TLR pathways has great therapeutic potential: novel TLR immune-regulatory drugs are being developed to treat a wide range of conditions, such as infectious diseases, asthma, inflammatory bowel disease and cancer. In the case of sepsis, one could think of TLR antagonists, TLR signaling pathway inhibitors or stimulators of the negative regulators of the TLR pathway as new treatment targets.

Some examples. A recent mouse study in sepsis showed a marked reduction in the sepsis related mortality after selective blockage of TLR2 after inoculation with Gram-positive bacteria. Furthermore, studies in animal models have demonstrated the utility of anti-CD14 monoclonal antibody therapy in septic shock and these agents are currently being evaluated in clinical phase-2 trials. Another strategy could involve TLR9. TLR9 recognizes CpG DNA, a specific pattern of nucleotides that is common in bacteria and viruses, but uncommon in humans. By using synthetic CpG sequences an innate and adaptive immune response could be generated involving cytotoxic T cells and disease-specific antibodies. In a mouse model of severe Gram-negative sepsis, CpG treatment one hour before inoculation with R. pseudomallei offered protection due to the rapid induction of proinflammatory cytokines.

The treatment goal of TLR agents in sepsis should be to normalize and not to completely abolish the dysregulated and harmful inflammatory response. Maintaining a balance between host-defense functions and potentially harmful effects (e.g. tissue destruction and the induction of autoimmune disease) will be of vital importance in the development of TLR therapeutics.
CONCLUSION

The discovery of TLRs has been of enormous importance in both the field of microbiology and immunology and has shown that the innate immune system is not aspecific. TLRs form the crucial link between the innate and adaptive immune response. Surprisingly, these very complex immune responses are initiated by this family of only 11 different receptors. The first human studies on TLR expression in sepsis and experiments with mice lacking TLR genes have provided us new insights in the pathogenesis of sepsis and have underlined the importance of TLRs as the crucial first line of defense against microorganisms. Taken together, severe sepsis can probably be seen as the clinical manifestation of a TLR mediated dysregulation of the immune response to invasive pathogens. Despite this significant progress in our understanding of the sepsis enigma many of the complex immune reactions during sepsis are still a mystery. These outstanding questions on pathogenesis can be summarized by the fact that we need to know how dysregulation of the TLR system precisely results in clinical syndromes such as sepsis. In the end, it comes down to the question whether all this newly gained knowledge will help to improve the care of septic patients. Hopefully, in the not to distant future the TLR genotypic profiling of patients will help clinicians to make better treatment decisions. Most importantly, unraveling the role of TLRs in sepsis will provide new highly selective treatment targets in sepsis.

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