Introduction

Evidence has accumulated over the last decade, documenting that levels of certain inflammatory cytokines, most notably TNF and IL-1β, as well as the cytokine-inducible isoform of nitric oxide synthase (iNOS), are increased in hearts of patients with heart failure regardless of etiology, although the proximal events leading to their expression are unknown. Noting that expression of a human homologue of Drosophila Toll, a proximal innate immunity transmembrane signaling protein in the fly, now termed human Toll-like receptor 4 (hTLR4), appeared to be relatively high in the heart, we examined TLR4 mRNA and protein abundance in isolated cellular constituents of cardiac muscle and in normal and abnormal murine, rat, and human myocardium. TLR4 expression levels in cardiac myocytes and in coronary microvascular endothelial cells could be enhanced by either LPS or IL-1β, an effect inhibited by the oxygen radical scavenger PDTC. Transfection of a constitutively active TLR4 construct, CD4/hTLR4, resulted in activation of a nuclear factor-κB reporter construct, but not of an AP-1 or an iNOS reporter construct, in cardiac myocytes. In normal murine, rat, and human myocardium, TLR4 expression was diffuse, and presumably cytoplasmic, in cardiac myocytes. However, in remodeling murine myocardium remote from sites of ischemic injury and in heart tissue from patients with idiopathic dilated cardiomyopathy, focal areas of intense TLR4 staining were observed in juxtaposed regions of 2 or more adjacent myocytes; this staining was not observed in control myocardium. Increased expression and signaling by TLR4, and perhaps other Toll homologues, may contribute to the activation of innate immunity in injured myocardium.

Toll4 (TLR4) expression in cardiac myocytes in normal and failing myocardium

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Expression of innate immune response proteins, including IL-1β, TNF, and the cytokine-inducible isoform of nitric oxide synthase (iNOS), have been documented in the hearts of humans and experimental animals with heart failure regardless of etiology, although the proximal events leading to their expression are unknown. Noting that expression of a human homologue of Drosophila Toll, a proximal innate immunity transmembrane signaling protein in the fly, now termed human Toll-like receptor 4 (hTLR4), appeared to be relatively high in the heart, we examined TLR4 mRNA and protein abundance in isolated cellular constituents of cardiac muscle and in normal and abnormal murine, rat, and human myocardium. TLR4 expression levels in cardiac myocytes and in coronary microvascular endothelial cells could be enhanced by either LPS or IL-1β, an effect inhibited by the oxygen radical scavenger PDTC. Transfection of a constitutively active TLR4 construct, CD4/hTLR4, resulted in activation of a nuclear factor-κB reporter construct, but not of an AP-1 or an iNOS reporter construct, in cardiac myocytes. In normal murine, rat, and human myocardium, TLR4 expression was diffuse, and presumably cytoplasmic, in cardiac myocytes. However, in remodeling murine myocardium remote from sites of ischemic injury and in heart tissue from patients with idiopathic dilated cardiomyopathy, focal areas of intense TLR4 staining were observed in juxtaposed regions of 2 or more adjacent myocytes; this staining was not observed in control myocardium. Increased expression and signaling by TLR4, and perhaps other Toll homologues, may contribute to the activation of innate immunity in injured myocardium.

vation of the adaptive immune response (11). Also, transfection of a constitutively active form of hTLR4 into Jurkat cells was shown to induce activation of nuclear factor-κB (NF-κB) (13).

The cytoplasmic TIR domain of Toll exhibits a high degree of homology with that of the mammalian receptor of IL-1 (IL-1R) and the IL-1R accessory protein (IL-1RAcP). Muzio et al. (14) and Medzhitov et al. (15) have shown recently that hTLR4 signaling occurs in a manner similar to that mediated by IL-1 (16). After recruiting the adapter protein MyD88 (similar to Drosophila tube) and interleukin receptor–associated kinase (IRAK), homologous to Drosophila pelle, the protein kinase NF-κB–inducing kinase (NIK) is activated through TNF coronary receptor–activated factor-6 (TRAF6). NIK subsequently activates an IκB kinase that results in phosphorylation of IκB (Drosophila cactus), thereby promoting NF-κB (Drosophila dorsal), translocation to the nucleus, and gene transcription. Indeed, a number of proteins involved in host defense with TIR domains and IRAK/pelle–like serine/threonine innate immunity kinases (SIIKs) have now been identified in plants, invertebrates, and vertebrates, attesting to their utility in roughly a billion years of evolution (17, 18).

Here, we examine the expression of TLR4 in human and rodent heart. Constitutive expression of this vertebrate Toll homologue was found in normal cardiac muscle, almost exclusively within cardiac myocytes. Dissociation of heart tissue, followed by isolation and primary culture of cardiac myocytes and coronary microvascular endothelial cells (CMEMs), was found to result in robust TLR4 expression in both cell types. Interestingly, in tissue sections from hearts of humans with cardiomyopathies and of rodents with experimental cardiac dysfunction, myocyte TLR4 expression becomes more focal and intense.

Methods

Chemicals. Human recombinant IL-1β was purchased from Calbiochem-Novabiochem Corp. (San Diego, California, USA); murine IFN-γ (rmIFN-γ) was purchased from Life Technologies Inc. (Gaithersburg, Maryland, USA). All other chemicals, including LPS (Escherichia coli B05:55), were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) unless noted otherwise.

Cell isolation and culture. Neonatal rat ventricular myocytes (NRVMs) were isolated from 1-day-old Sprague-Dawley pups as described previously (19). Cells subsequently underwent 2 preplatings to minimize nonmyocyte contamination to less than 5% of the enriched myocyte population (17) and cultured in DMEM containing 10% FCS (Life Technologies Inc.). After 48 hours, the medium was changed to DMEM/F12 phenol red–free medium (Life Technologies Inc.) containing 1% insulin, transferrin, and selenium media supplement (ITS; Sigma Chemical Co.) with antibiotics; treatments were instituted 12 hours later.

Figure 1
Alignment of the hTLR4 and rTLR4 sequences. A full-length rTLR4 cDNA was sequenced by RACE PCR from a rat cDNA library using the hTLR4 sequence to design primers. The full-length rTLR4 contains 3,385 bp with an open reading frame of 2,508 bp, encoding a protein of 836 amino acids. The hTLR4 and rTLR4 sequences exhibited an overall 84% homology in DNA sequences (top, rTLR4; bottom, hTLR4; alignment by an algorithm: http://vega.igh.cnrs.fr; : = amino acid identity; = conservative substitution).
Calcium-tolerant adult rat ventricular myocytes (ARVMs) were isolated from adult male Sprague-Dawley rats (225–275 g) as described previously (20). Primary myocyte cultures were plated on laminin-coated (1 μg/cm²) dishes and maintained at 37°C in 5% CO₂. All experiments described were initiated within 24 hours of cell isolation.

CMECs from adult rat hearts were isolated as described previously (21). These primary isolates have been documented to contain more than 90% endothelial cells, with a phenotype at low passage number consistent with their microvascular origin, as described previously (21). After reaching confluency, CMECs were serum starved for 12 hours before treatment.

Myocardial infarction in mice. C57BL6/C129 mice, 8–12 weeks old and 25–30 g, underwent coronary artery ligation for the production of myocardial infarction. The surgical procedure has been described in detail elsewhere (22). Because direct viewing of the left anterior descending artery is suboptimal in mice, paleness and enlargement of the left ventricle after suture placement suggested the presence of an infarction. In sham-operated animals, the suture was placed but not ligated. Antibiotics were not given during the procedure, but no apparent infection had developed by the time of autopsy. All mice were housed under identical conditions and given food and water ad libitum. The Standing Committee on Animal Research from our institution approved the animal study protocol.

Cloning of the rat Toll4 cDNA. Total RNA was extracted from rat heart as described later here. Double-stranded cDNA was synthesized by the Marathon cDNA Amplification kit (CLONTECH Laboratories Inc., Palo Alto, California, USA) according to the manufacturer’s protocol and was ligated to the Marathon cDNA adapter. To clone rat Toll4 (rTLR4), PCR primers were designed based on the hTLR4 sequence (11), corresponding to bp’s 2,256–2,280 and 2,676–2,697. Based on the subsequent sequences that were generated, additional oligonucleotides were selected as primers in combination with API for the rapid amplification of 3’ and 5’ cDNA ends (3’-RACE and 5’-RACE; CLONTECH Laboratories Inc.), and the entire sequence was cloned using repetitive nested primers according to the manufacturer’s instructions.

Northern and Western blots. Total cellular RNA was isolated by a modification of the acid guanidinium/thiocyanate phenol/chloroform extraction method with Trizol LS (Life Technologies Inc.) according to the manufacturer’s protocol (23). RNA was size fractionated on 1.0% agarose, transferred to a nylon membrane, and ultraviolet cross-linked. A 555-bp rat Toll cDNA was radiolabeled by a random-primed labeling kit (Life Technologies Inc.) with [α-32P]dCTP and was hybridized with QuickHyb solution (Stratagene, La Jolla, California, USA) at 68°C for 2 hours. Membranes were washed at room temperature twice for 15 minutes with 2·SSC and 0.1% SDS and at 60°C for 30 minutes with 0.1× SSC and 0.1% SDS. Normalization of RNA for equal loading was carried out by rehybridizing with GAPDH cDNA probe. Autoradiograms were scanned using NIH Image version 1.61 (National Institutes of Health, Bethesda, Maryland, USA).

For Western blots, cells were harvested in an RIPA lysis buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease in PBS. After sonication, cell extracts were boiled for 5 minutes with 2× SSC and 0.1% SDS and at 60°C for 30 minutes with 0.1× SSC and 0.1% SDS. Normalization of RNA for equal loading was carried out by rehybridizing with GAPDH cDNA probe. Autoradiograms were scanned using NIH Image version 1.61 (National Institutes of Health, Bethesda, Maryland, USA).
Figure 3
Time course of rTLR4 expression in response to LPS. Primary cultures of confluent CMECs (a) and NRVMs (b) were exposed to 10 µg/mL LPS for the periods indicated. Cumulative data from 5 independent experiments for both cell types are shown (*P < 0.05). Data have been normalized to GAPDH mRNA content. The rTLR4 mRNA content at 24 hours was defined as 1 arbitrary unit.
less than 0.05. Statistical analyses were carried out using StatView statistics program (Abacus Concepts Inc., Berkeley, California, USA).

Results
Cloning of the full-length rat Toll4 cDNA. Based on the hTLR4 sequence (11), the full-length rTLR4 cDNA was sequenced using RACE PCR from a rat heart cDNA library. The full-length cDNA contains 3,385 bp with an open reading frame of 2,508 bp, encoding a protein of 836 amino acids. The rTLR4 sequences exhibited an 84% homology with the hTLR4 DNA sequences and no similarities with TLR1-3 and 5. The extracellular domain has an identity of 57% or, taking conservative substitutions into account, a similarity of 72% with the amino acid sequence of hTLR4. As with Drosophila Toll (23–25), rTLR4 has an extracellular leucine-rich repeat domain with cysteine-rich flanking sequences; the tertiary structure is presumed to describe a horseshoe-like pattern analogous to the ribonuclease inhibitor (reviewed in ref. 26). The COOH-terminal TIR domain is highly conserved, with an identity of 84% and similarity of 91% to the primary structure of hTLR4 (overall primary structure identity with hTLR1 is 39%; with hTLR2, 37%; with hTLR3, 38%; and with hTLR5, 39%). The alignment of human and rat TLR4s is shown in Figure 1.

TLR4 expression in rodent heart. In their original description of the human homologue of Drosophila Toll, Medzhitov et al. (11) noted that hToll4 expression was relatively abundant in cardiac muscle, as well as in lung, peripheral blood leukocytes, and in spleen. Therefore, TLR4 mRNA expression was examined in fresh isolates of ARVMs and confluent primary cultures of NRVMs and CMECs. A 3.4-kb band consistent with the rTLR4 transcript was readily detectable by Northern blot in both adult and neonatal rat myocyte cultures, whereas an approximately 5-fold higher basal level of Toll mRNA abundance was present in the CMEC preparation (Figure 2).

Regulation of TLR4 expression by LPS, IL-1β, and IFN-γ. The innate immune system is known to recognize pathogen-associated molecular patterns (PAMPs) such as LPS. Therefore, we determined whether rTLR4 expression is regulated by LPS (10 ng/mL) in endothelial cells and cardiac myocytes. Confluent, serum-starved primary cultures of NRVMs and CMECs both exhibited significant increases in rTLR4 mRNA abundance after 24 hours, but not at 12 hours (Figure 3b). Similar results were observed at 100 ng/mL LPS (data not shown). To investigate further the role of cytokines that play key effector roles in the innate immune response, primary cultures of neonatal myocytes and endothelial cells were treated with either 4 U/mL or 40 U/mL rhIL-1β or 50 U/mL rmIFN-γ for 24 hours. As shown in Figure 4, endothelial cells showed about a 2-fold increase in rTLR4 mRNA after IL-1β and IFN-γ, and neonatal myocytes, about a 5-fold higher level of Toll mRNA abundance was present in the CMEC preparation (Figure 2).

PDTC inhibits upregulation of rTLR4 expression in vitro in response to IL-1β or LPS. CMECs and NRVMs were exposed to LPS or cytokines in the presence or absence of PDTC as indicated. This Western blot is representative of 3 independent experiments and illustrates a 96-kDa band consistent with TLR4. CMECs were treated for 36 hours, and NRVMs for 48 hours, with LPS, cytokines, or the NF-κB inhibitor PDTC, as indicated.

NF-κB and the regulation of TLR4 expression. To investigate the role of NF-κB activity in the regulation of rTLR4 expression, CMEC and NRVM primary cultures were treated with 10 and 100 μmol PDTC, respectively, and the TLR4 mRNA and protein content was measured after 24 hours. The Northern blots shown are representative of 4 independent experiments.
to 48 hours of treatment. In serum-starved confluent endothelial and cardiac myocyte cultures, the increase in TLR4 mRNA by either LPS or IL-1β described in Figure 4 could be suppressed by the reactive oxygen intermediate (ROI) scavenger PDTC in a dose-dependent manner. Moreover, in the presence of PDTC alone, particularly at 100 μM, TLR4 mRNA levels were depressed significantly (Figure 5). Similarly, TLR4 protein levels were downregulated by PDTC in endothelial cells after 24 hours and in cardiac myocytes after 48 hours (Figure 5).

As IL-1β and LPS have been shown to activate NF-κB and induce iNOS in endothelial cells and cardiac myocytes, we also verified that iNOS-dependent production of NOx was decreased by PDTC. Activation of iNOS was monitored by measuring NOx accumulation in medium conditioned by either LPS or IL-1β-treated microvascular endothelial cells or cardiac myocytes. As expected, PDTC suppressed iNOS-dependent NOx expression in response to both LPS and IL-1β.

To determine whether this effect of the ROI scavenger PDTC on rTLR4 was due to its ability to block the activation of NF-κB induced by IL-1β or LPS, electrophoretic mobility shift assays (EMSAs) were performed using nuclear extracts from microvascular endothelial cell and neonatal cardiac myocyte cultures. As shown in Figure 6, a and b, PDTC prevented retar-
The specific cytokines and cytokine receptors most intensively studied to date with regard to the pathophysiology of heart failure, specifically TNF, IL-1β, IL-6, chemokines,
and IFN-γ, along with iNOS, are now recognized as being principal effector proteins of innate immunity, a phylogenetically ancient and essential component of the immune response. Unlike adaptive immunity, for which specific antigen receptors are generated by somatic hypermutation and selection “in the field,” innate immune systems use hardwired germ-line-encoded proteins that act as pattern recognition receptors (PRRs), which recognize largely invariant PAMPs (reviewed in refs. 5–7 and 18).

Considerable data now exist linking innate immunity effector proteins to the pathophysiology of myocardial dysfunction in heart failure. For example, both serum and intramyocardial levels of IL-1β and TNF-α are activated in patients with myocardial dysfunction resulting from several disease processes, and both cytokines have been shown to have direct cardiodepressant effects (reviewed in refs. 2, 4, and 25). TNF is produced by cardiac myocytes in response to increased strain in vitro and in failing cardiac muscle in vivo (26), and genetically engineered mice that were designed to overexpress TNF selectively in cardiac myocytes exhibited progressive cardiac hypertrophy and, ultimately, progressive chamber dilation and heart failure (28, 29). Also, increased expression of iNOS has also been demonstrated in experimental animals and in humans with heart failure due to various etiologies (30–33). Finally, in addition to the effector proteins of innate immunity implicated in the pathophysiology of heart failure already noted here, it has been demonstrated recently that several innate immunity PRRs, including pentraxin 3 (34) and a number of complement components, including C3 and C9 (35, 36) and possibly others, are synthesized in the heart. Indeed, as demonstrated by McGeer and colleagues, myocardial levels of C3 and C9, after ischemia followed by reperfusion, exceed levels of production of these complement components in the liver in a rabbit model (35, 36).

We have demonstrated here that TLR4 is expressed in nonprofessional immunocyte cell types: cardiac myocytes and microvascular endothelial cells. In agreement with these findings, it has been recently published

![Figure 9](image-url)
that human dermal endothelial cells do express TLR4 (37). TLR4 mRNA abundance in vitro could be enhanced by LPS and the cytokines IL-1β and IFN-γ. This is not surprising, as PAMPs such as LPS, and innate immunity effector cytokines such as IL-1β, are known to amplify expression of a number of innate immunity cytokines and signaling proteins. Furthermore, a “basal” level NF-κB activity, at least under the in vitro conditions we used here, was necessary for maintenance of TLR4 expression because PDTC could reproducibly suppress TLR4 mRNA and protein abundance in both cell types, even in the absence of IL-1 or LPS. Unlike the diffuse, homogeneous immunohistochemical staining for TLR4 in normal cardiac muscle, myocytes in vitro exhibited intense circumferential staining, as did myocytes within inflammatory infiltrates in areas of ischemic injury. Finally, in sections obtained from both rodent and human hearts undergoing progressive remodeling and dilatation that were remote from areas of injury or discrete inflammatory infiltrates, intense focal staining for TLR4 was observed in portions of membranes of 2 or more juxtaposed cells.

These data, however, beg the question that if there is no evidence of infection in the hearts of animals or patients with cardiac dysfunction, what is driving Toll expression and signaling under these conditions? Unlike the rapid clarification to date of the intracellular signaling pathways used by Toll, the ligand for Toll activation in vertebrates is unknown. In the Drosophila embryo, the Toll ligand is Spätzle, a cysteine-knot superfamily member that is generated from an inactive precursor by a serine protease cascade that includes Nudel, Snake, and Easter (8). While Spätzle remains essential for innate immunity–mediated Toll signaling in the adult fly, the cleavage of pro-Spätzle is mediated by a different sequence of proteases (reviewed in ref. 38). Although currently unknown, it is likely that this protease is also activated by a serine protease cascade (perhaps analogous to the complement or clotting cascades) and will likely have been highly conserved, like Toll, throughout evolution (17). Finally, there are now 2 reports that TLR2, which is highly expressed in peripheral blood leukocytes, spleen, and bone marrow, acts as a receptor for LPS (39, 40). TLR2 “sensing” of LPS was also dependent on soluble LPS-binding protein, and the presence of CD14, along with TLR2, markedly enhanced the sensitivity of cells expressing both proteins to LPS.

This interaction with CD14 is now known not to be unique to TLR2. A more recent report identifying the gene responsible for LPS resistance in 2 inbred strains of mice (C3H/HeJ and C57BL/10ScCr) was found to be TLR4 (41, 42). C3H/HeJ mice have a single point mutation in the cytosolic TIR domain, resulting in a proline→histidine substitution, whereas C57BL/10ScCr mice express no TLR4 mRNA. The most economical explanation using the information available as of this writing is that TLR4 is required for LPS-mediated hypotension, and that the LPS-CD14 interaction accentuates this response. However, although we could show NF-κB activation by the constitutively active CD4/TLR4 construct, we have not seen increases in iNOS expression whose promoter contains several functional NF-κB binding sites or of AP-1 activation, although both are activated by LPS in cardiac myocytes as in other cell types, implicating a TLR4-independent pathway for LPS signaling in these cells.

In the absence of evidence for infection, there is another explanation for the involvement of Tolls and perhaps other innate immunity PRRs and effector proteins in injured tissues. Over the past 2 years, a number of reports have revealed the role of innate immunity pattern recognition receptors and effector proteins in facilitating engulfment of apoptotic cells by both professional phagocytes and parenchymal cells (43, 44). Perhaps best understood is the role of class B scavenger receptors, such as CD36 and the homologous Drosophila protein croquemort (“catcher of death”) (45). Recently, Masaki and colleagues (46) have demonstrated that class A scavenger receptors, such as the lectin-like OxLDL receptor/(LOX-1) on endothelial cells, also recognizes apoptotic cells. This is presumably due to interactions with anionic phospholipids (e.g., phosphatidylserine), prompting engulfment of dying cells by these “amateur” phagocytes.

Perhaps the most intriguing recent observation linking innate immunity and apoptosis was the discovery that CD14, a leucine-rich repeat PRR attached to the plasma membrane of a number of cell types by a GPI tether, participates in recognition and engulfment of apoptotic cells (43). An antibody directed against CD14 was sufficient to inhibit engulfment of apoptotic lymphoma cells by macrophages, whereas transfection of COS cells with a CD14 construct mediated docking and engulfment of apoptotic cells by COS cells (albeit not as efficiently as in a professional phagocyte). These authors also noted that although LPS-initiated CD14 signaling in phagocytes is well known to initiate multiple proinflammatory signals, this did not occur with CD14’s interaction with apoptotic cells (44).

In summary, the vertebrate Toll homologue TLR4 is abundantly expressed in the heart. Although the role of Toll-mediated signaling in the injured or remodeling heart remains to be defined, the evidence reported suggests that TLR4 expression and signaling could be participating in a response to tissue injury.

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