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Trigeminal Nociceptors Express TLR-4 and CD14: a Mechanism for Pain due to Infection

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Abstract

Although certain bacterial species appear to be risk factors for pain due to odontogenic infections, comparatively little is known about the potential mechanisms mediating this effect. In this study, we tested the hypothesis that trigeminal nociceptive neurons express the TLR4 or CD14 receptors, thus enabling sensory neurons to detect and respond to tissue levels of bacterial substances such as lipopolysaccharide (LPS). Immunohistochemical analyses of human and rat trigeminal neurons demonstrated that a capsaicin-sensitive subclass of nociceptors (defined by expression of TRPV1, a capsaicin receptor) expresses both TLR4 and CD14. Moreover, human dental pulp collected from patients with caries lesions demonstrated co-localization of TLR4 and CD14, with markers of peripheral sensory neurons. Collectively, these studies indicate that the capsaicin-sensitive subclass of trigeminal nociceptors expresses TLR4 and CD14. These results indicate that pain due to bacterial infections may result, in part, from direct activation of nociceptors by bacterial products such as LPS.

Keywords

pain; infection; TRPV1; TLR4; CD14; bacteria; LPS

INTRODUCTION

Considerable research has focused on mechanisms of immune surveillance during the development of pulpal infections and apical periodontitis (Jontell et al., 1998; Yoshiba et al., 1998; Yoshiba et al., 2003). Toll-4 (TLR4) and CD14 function as pattern recognition receptors for innate immune detection of tissue levels of bacterial-derived factors such as lipopolysacharide (Fitzgerald et al., 2004; Miller et al., 2005). Both TLR4 and CD14 have been identified on dendritic and other cell types in dental pulp (Okiji et al., 1997; Sakurai et al., 1999). These receptors coordinate the development of apical periodontitis in response to bacterial infection of dental pulp, since mice with genetic deletion of TLR4 have significantly reduced development of periradicular osteolytic lesions (Hou et al., 2000). Interestingly, humans with a TLR4 polymorphism are at risk for chronic marginal periodontitis (Schroder et al., 2005); the effect of TLR4 polymorphisms on apical periodontitis remains unknown.

Odontogenic infections may lead to severe inflammatory pain, and certain bacteria found in necrotic root canal systems, but not all bacteria, are associated with this clinical symptom (Hashioka et al., 1992; Siqueira and Rocas, 2003; Siqueira et al., 2004). Despite these correlational studies, the mechanisms for bacterially induced odontogenic pain remain unknown. It is possible that bacteria or bacterial by-products might indirectly activate nociceptors by evoking the release of certain soluble factors (*e.g.*, prostanoids, cytokines, etc.)

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from cells in the infected tissue that activate nociceptors *via* a paracrine action. However, it is also possible that bacteria or bacterial by-products might directly activate nociceptors. The objective of this study was to address this alternative, direct hypothesis by determining whether the capsaicin-sensitive subclass of nociceptors, defined by the expression of the transient receptor potential receptor vanilloid subtype 1 (TRPV1) (Caterina et al., 1997), co-expresses TLR4 and CD14. We investigated this hypothesis in human and rat trigeminal neurons and in human dental pulp samples.

METHODS

All clinical studies were approved by the UTHSCSA IRB, and patients provided written informed consent. Two sources of human tissue were used in this study. Human trigeminal ganglia (TG) were provided by the National Disease Research Interchange (Philadelphia, PA, USA). *Post mortem* samples of TG were collected from two subjects (one was a 77-year-old male who had died of cardiac arrest associated with congestive heart failure, and the other was a 70-year-old female who had died of cardiac arrest; the TG of both subjects were collected within 7 hrs of death). In the clinical study, patients who satisfied the criteria of a normal tooth scheduled for extraction, and reported a short-lasting response to a thermal test, no spontaneous pain, no caries or restoration, and no periradicular radiolucency were selected; all teeth had fully developed roots. For the purposes of this study, a clinical diagnosis of irreversible pulpitis required a prolonged response to thermal stimulation, caries invading the pulp chamber, and no periradicular radiolucency. Following extraction, the tooth was split in half, and the dental pulp was carefully removed.

The animal study was approved by the UTHSCSA IACUC and conformed to NIH guidelines. Male Sprague-Dawley rats (200-250 g) were housed for 1 wk in micro-isolator cages with food and water available *ad libitum*. Rats were killed by decapitation, and their TG were promptly removed and fresh-frozen at −80°C in OCT compound.

The tissue samples were fixed (4% formaldehyde, 4°C × 1 hr), washed (PBS, 3-5 min), sectioned (20 μm), permeabilized (0.2% Triton-X-100, 22°C × 1 hr), blocked (10% goat serum, 3X, 10 min), and incubated with primary antibody overnight. To evaluate co-localization, we performed a double immunohistochemical analysis in which sections were incubated with guinea pig primary antibody against TRPV1 (1:3000, Neuromics)**(AQ)** or N52 (1:15,000 Sigma), with either anti-rabbit TLR4 (Santa Cruz Biotech, 1:200 for TG and 1:800 for dental pulp) or anti-rabbit CD14 (Santa Cruz Biotech, 1:200 for TG and 1:800 for dental pulp) overnight at 4°C; results were detected by an anti-guinea-pig-conjugated AlexaFluor488 (green) or anti-rabbit-conjugated AlexaFluor594 (red, 1:300 each, Molecular Probes; 1 hr incubation followed by PBS wash 4 × 5 min). Images were collected by means of a confocal microscope at the UTHSCSA core facility. Control experiments with blocking peptides, or exclusion of primary antisera, abolished the staining in both human and rat tissues.

RESULTS

Human trigeminal sensory neurons express the TLR4 receptor (Fig. 1, panels A-I). Co-localization studies indicated that TLR4 was expressed on the capsaicin-sensitive subclass of nociceptors, as indicated by co-localization of TLR4 with TRPV1 (Figs. 1A-1C; white arrows). Not all neuronal profiles expressed TLR4 with TRPV1; neurons that expressed one of these markers, but not both, are indicated by yellow arrows. Human TG neurons were also found to express TLR4 on a broader population of sensory neurons that expressed a neurofilament found in myelinated afferent fibers (Figs. 1D-1F, N52 antisera). A similar pattern of expression was seen in human TG neurons from the other human TG (data not shown). To verify that the expression of TLR4 on human TG neurons was not due to potential confounding factors

(*e.g.*, time *post mortem*, age, concurrent disease), we next evaluated the expression pattern of TLR4 on rat TG neurons. TLR4 was found on the capsaicin-sensitive subclass of rat TG neurons (Fig. 1, panels G-I). Similar results were seen for TLR4 co-localization with N52 in rat TG neurons (data not shown).

Human trigeminal sensory neurons also express the CD14 receptor (Fig. 1, panels J-O). Human trigeminal (TG) neurons were found to expressed CD14 on the capsaicin-sensitive subclass of nociceptors, as indicated by co-localization of TLR4 with TRPV1 (Figs. 1J-1L; white arrows). The co-localization of CD14 with TRPV1 did not occur in all human TG neurons; neurons that expressed one of these markers, but not both, are indicated by yellow arrows. A similar pattern of expression was seen in the human TG from the other subject. To verify that the expression of CD14 on human TG neurons was not due to potential confounding factors, we next evaluated the expression pattern of CD14 on rat TG neurons. CD14 was found on the capsaicin-sensitive subclass of rat TG neurons (Fig 1., panels M-O). Similar results were seen for TLR4 co-localization with N52 in rat TG neurons (data not shown).

Samples of human dental pulp were next examined (Fig. 2). Normal control dental pulp had very low levels of TLR4 and CD14 in coronal areas (Figs. 2A-2B), with detectable levels of sensory neurons, as indicated by N52 staining (Fig. 2C). Samples of inflamed coronal dental pulp collected from teeth with caries lesions demonstrated two sources of TLR immunoreactivity (Figs. 2D-2F). One cellular source consisted of rounded or stellate cells, and the second source was fibers that co-expressed N52 immunoreactivity. A similar pattern was observed for CD14 staining, with cariously inflamed coronal pulp demonstrating two sources of CD14: rounded or stellate cells, and fibers that expressed N52 staining (Figs. 2G-2I). Since inflamed pulp is associated with immune cell infiltration, it is possible that the immunoreactivity was confounded by cells capable of binding the secondary antibodies (Pulver et al., 1977;Haug et al., 2001). Therefore, control studies were conducted where the primary antibodies were excluded, and only the secondary antibody, containing AlexaFluor488 (Panel 2J) or AlexaFluor594 (Panel 2K), was processed in inflamed human pulp. Although the background was more prominent, it is important to note that there were no morphologically distinct structures in these control sections; instead, the structures observed in the prior panels were found only in sections exposed to the primary antibody.

DISCUSSION

The present findings indicate that trigeminal afferent neurons express the TLR4 and CD14 receptor complex. Thus, afferent neurons innervating infected tissue have the receptors required for the direct detection of bacterially derived by-products, such as LPS. The existence of this receptor complex on trigeminal neurons is likely to have considerable physiologic significance, since neuropeptides are released from capsaicin-sensitive pulpal fibers (Bowles et al., 2003; Hargreaves et al., 2003), have been demonstrated to exert important immunomodulatory functions (Pascual, 2004; Steinman, 2004), and appear to regulate the rate of pulpal necrosis due to bacterial infection (Byers and Taylor, 1993). Thus, LPS activation of TLR4/CD14 may trigger intracellular signaling cascades, leading to peripheral neuropeptide exocytosis and central nociceptive neurotransmission.

Although several TLR4 signaling pathways are well-recognized (Takeda and Akira, 2004), a newly reported TLR4 signaling pathway is *via* PKC epsilon (Aksoy et al., 2004). This is of particular interest in pain research, because PKC epsilon is expressed in nociceptors, where it leads to activation of these sensory neurons (Numazaki et al., 2002; Vellani et al., 2004). Thus, the finding that capsaicin-sensitive nociceptors express TLR4/CD14 is consistent with the hypothesis that LPS might activate these neurons *via* the TLR4/CD14 complex, and this might involve the PKC epsilon signaling pathway. Accordingly, these findings might have

considerable significance in mechanisms of severe or prolonged inflammatory pain associated with bacterial infections (Aley et al., 2000). In addition, there is evidence that other bacterial by-products can activate CD14, including lipoteichoic acid, a substance derived from Gram-positive organisms (Ginsburg, 2002). Thus, the finding that the TLR4/CD14 complex is expressed on afferent trigeminal neurons may have physiologic implications for understanding mechanisms of odontogenic pain due to infections.

Given these findings, it is reasonable to speculate why marginal periodontitis, a process associated with LPS (Muthukuru et al., 2005), is not generally reported as painful. It is possible that certain environmental conditions may inhibit LPS stimulation of nociceptors. Possible hypotheses might include the following: (1) Pathogens in marginal periodontitis might inactivate TLR4/CD14 *via* released peptidases that are known to cleave the receptors (Deschner et al., 2003); (2) chronic LPS may induce a down-regulation in TLR4 expression, and, in fact, a nine-fold reduction in TLR4 has been reported in marginal periodontitis (Wang et al., 2001; Muthukuru et al., 2005); and (3) nociceptor innervation or function might differ in differing target tissues, such as dental pulp *vs.* periodontal pockets.

An interesting finding from the present study is that TLR4/CD14 are not exclusively expressed on the capsaicin-sensitive (*i.e.*, TRPV1-positive) subclass of nociceptors. N52 is a monoclonal antibody that binds to neurofilament H, a protein normally expressed in myelinated neurons. Thus, the present results indicate that TLR4 and CD14 are expressed on TRPV1-positive neurons (primarily a non-myelinated population), as well as on myelinated neurons. Although myelinated nociceptors are positive for N52 (Lawson et al., 1997; Lawson, 2002), and recent research indicates that some forms of peripheral injury induce small-diameter neurons to express NF-H (and hence bind N52) (Hammond et al., 2004), the present results do not discount the possibility that N52-labeled trigeminal afferent fibers include a subpopulation of non-nociceptive trigeminal afferent neurons that also express TLR4/CD14.

The major findings of this study are that human TG neurons, including the capsaicin-sensitive subclass of nociceptors, express both TLR4 and CD14. The findings in the human TG samples cannot be attributed to potential issues such as a delay prior to *post mortem* collection, concurrent age, or disease process, since we observed a similar pattern on TLR4 and CD14 expression in rapidly isolated and fixed rat TG neurons. Further, sensory neurons in inflamed dental pulp expressed both TLR4 and CD14. Based upon these findings, we conclude that afferent neurons, including a major class of nociceptors, may be able to detect bacterial by-products such as LPS. Thus, one possible mechanism for odontogenic pain associated with bacterial infection is the activation of nociceptors *via* direct activation of the TLR4/CD14 complex expressed on sensory neurons.

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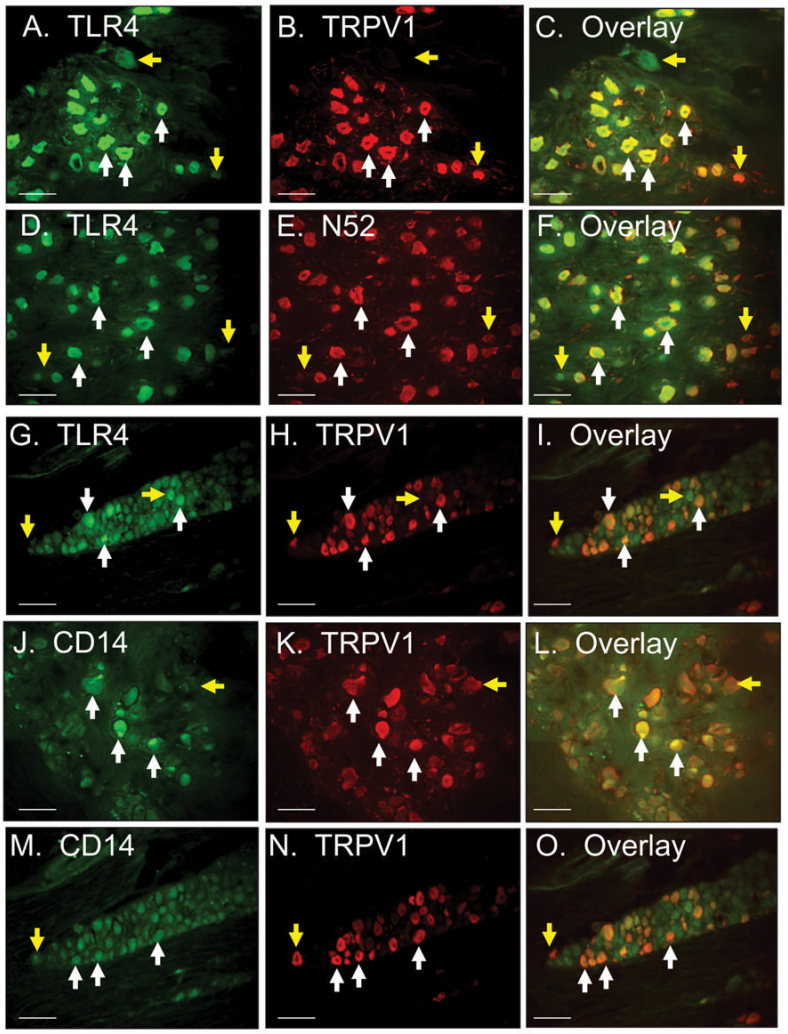

Figure 1.

Evaluation of the expression patterns of TLR4 and CD14 in trigeminal sensory neurons. White arrows depict examples of neurons expressing both markers for each row of three images, and yellow arrows depict examples of neurons that express one but not both markers. Human trigeminal neurons were evaluated for co-localization of TLR4 (Panels **A,D**), CD14 (Panel **J**), with a marker for the capsaicin-sensitive subclass of nociceptors (TRPV1, Panels **B,C** for TLR4 and Panels **K,L** for CD14), or a marker of myelinated sensory neurons (N52, Panels **E,F**). Rat trigeminal neurons were evaluated for co-localization of TLR4 with TRPV1 (Panels **G-I**) and CD14 with TRPV1 (Panels **M-O**). The addition of blocking peptide or the deletion of primary or secondary antisera produced a complete loss of signal in both the human and rat tissues. Scale bar is 100 μm for Panels A-F and J-L, and 200 μm for Panels G-I and M-O.

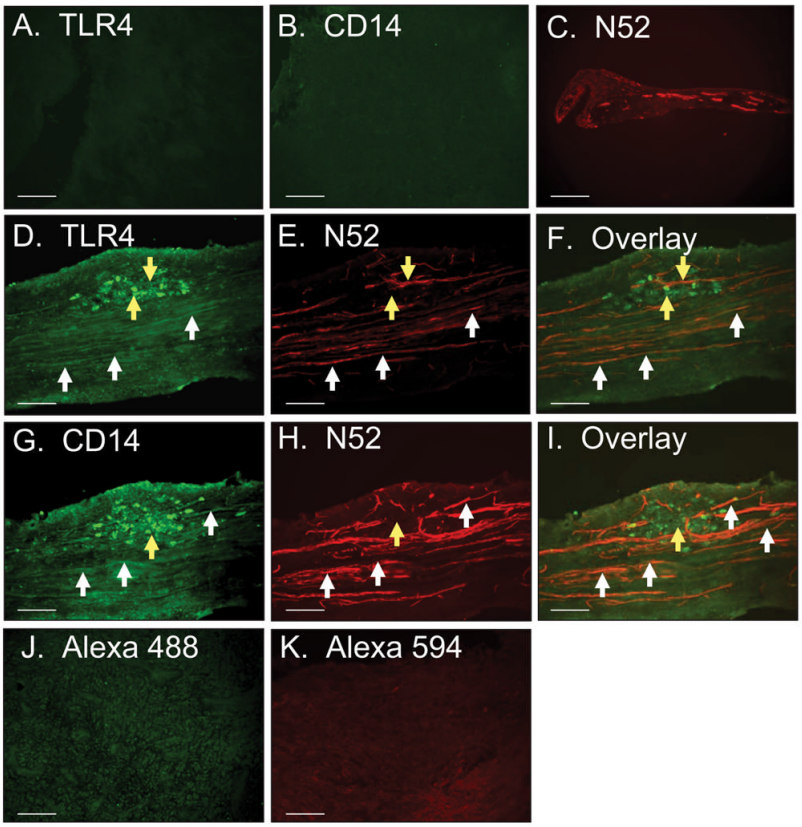

Figure 2.

Evaluation of the expression patterns of TLR4 and CD14 in human coronal dental pulp. Normal control dental pulp was examined for expression of TLR4 (Panel **A**), CD14 (Panel **B**), and a marker of myelinated neurons (N52, Panel **C**). Inflamed dental pulp (defined as teeth with a response to thermal stimulation but**(AQ)** deep caries lesions) had two populations of TLR4 and CD14, with TLR4 (Panel **D**) or CD14 (Panel **G**) observed in round or stellate cells or in fibers coursing through coronal pulp. At least some of these fibers expressed N52 (TLR4, Panels **E,F**; CD14, Panels **H,I**). Exposure of inflamed pulp to only the secondary antibodies produced low background signal (Panels **J,K**). Scale bar is 100 μm.