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Toll-like receptor 2 expression in refractory periapical lesions

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Abstract

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Aim To investigate the expression of TLR2 in refractory periapical lesions.

Methodology Refractory periapical lesion biopsies were histopathologically and clinically categorized into asymptomatic periapical granuloma ($n = 10$), symptomatic periapical granuloma ($n = 10$) or periapical cyst ($n = 10$) and prepared for immunohistochemical staining using antibodies to TLR2, CD3 and CD19 or staining with methyl green pyronin. Sections were viewed under light microscopy and the presence or absence of the target cells was correlated with the histopathological and clinical data. Additionally, TLR2 expression was quantified by counting TLR^+ cells.

Results Various mononuclear inflammatory cells in the bacteria-induced periapical lesions were reactive to TLR2 antibody, with many showing morphological similarities to lymphocytes and plasma cells. Lymphocytes were the most numerous cells in the inflammatory infiltrate. In refractory periapical granuloma, $CD3^+$ T cells were more numerous, whereas in periapical cysts, $CD19^+$ B cells were more numerous. There was a statistically significant ($P < 0.05$) higher expression of TLR2 in symptomatic periapical granuloma than asymptomatic periapical granuloma or periapical cyst.

Conclusion The presence of TLR-expressing cells in periapical granulomas and cysts provides further evidence that periapical cysts are likely to be sustained by the immune system via reaction to bacterial antigens.

Keywords: adaptive immunity, antigen-presenting cells, innate immunity, periapical cyst, periapical granuloma, Toll-like receptors.

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Introduction

Bacteria are the prime cause of periapical diseases (Kakehashi *et al.* 1965); invasion of bacteria or bacterial toxins into the periapical region from an infected root canal system leads initially to nonspecific inflammatory reactions followed by specific inflammatory reactions that include the production of antibodies, complement, cytokines and an array of inflammatory mediators targeted towards limiting the spread of infection and protecting the periapical tissues.

Periapical lesions have a diverse inflammatory and noninflammatory cellular profile that is involved in the regulation of highly complex disease processes. Numerous innate immunity cells [e.g. polymorphonuclear neutrophils (PMNs), macrophages, dendritic cells] as well as adaptive immunity cells (e.g. T and B lymphocytes, plasma cells) are present in different proportions within the granulation tissue of periapical lesions. Antigen-specific adaptive immune cells have a requirement for antigen presentation, and different antigen-presenting cells (APC) are involved in the inflammatory response. The notion of separate nonspecific innate and specific adaptive immunities has changed considerably with the recognition of receptors on various APC that discriminate between host and pathogen, known as pathogen recognition receptors (PRR) (Janeway 1989). The limited specificity of these receptors in identifying pathogen acts as a bridge between innate and adaptive immunities (Akira *et al.* 2001).

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Satisfactory root canal treatment results in healing of the majority of periapical lesions; however in some instances, certain bacteria (mainly Gram-positive bacterial species) survive the chemical and mechanical cleaning procedures leading to refractory periapical lesions (Sjögren *et al.* 1990, Molander *et al.* 1998, Sundqvist *et al.* 1998, Hancock *et al.* 2001, Pinheiro *et al.* 2003). Toll-like receptor-2 (TLR2) is a PRR expressed on the surface of various host cells and performs important functions, including recognition of a range of Gram-positive bacterial components and presenting them to appropriate immune cells, allowing the host to more efficiently combat microbial infections (Takeuchi *et al.* 1999, Iwasaki & Medzhitov 2004). It has also been shown to have a role in osteoclast formation which may be relevant in the progression of periapical pathology (Jiang *et al.* 2003).

Because Gram-positive bacterial species predominate in failed root canal treatment cases (Siqueira & Rocas 2004), it is expected that TLR2 would be expressed by a variety of immune cells present in periapical lesions and that they would play a significant role in endodontic pathogen recognition and presentation, production of various cytokines and expression of co-stimulatory molecules. The aim of this study was to demonstrate and characterize the expression of TLR2 by cells present in various refractory periapical lesions and to identify any association between the presence or absence of pain at the time of clinical presentation and TLR expression.

Materials and methods

The project received ethical approval from the University of Otago ethics committee and approval from Ngai Tahu Research consultation committee.

Sample collection

Periapical lesions removed during endodontic surgery of refractory endodontic cases were supplemented by similar samples from the histopathology archive of the University of Otago School of Dentistry Oral Pathology Diagnostic Laboratory. Histopathologically diagnosed cases of periapical granulomas and periapical cysts were selected and matched against clinical and histopathology diagnostic criteria for inclusion in the study. The histopathological diagnosis had been confirmed by two experienced specialist oral pathologists, using standard criteria (Shear & Speight 2007). Details of the patient's age, gender, relevant medical and dental history, signs and symptoms of presenting condition, clinical diagnosis, and treatment provided were recorded from the pathology request form and the clinical files. No details that could identify a patient were recorded. Only cases where the affected tooth had undergone root canal treatment and/or retreatment by endodontic postgraduate students or an endodontist prior to surgical excision were included. The patients were older than 18 years and had no medical conditions.

The final sample size from the pool comprised 10 each of asymptomatic periapical granuloma (Group 1), symptomatic periapical granuloma (Group 2) and periapical cyst (Group 3). Two specimens of periapical fibrous scar tissue and one dentigerous cyst associated with an unerupted mandibular third molar were also included as experimental negative controls (Group 4). Additionally, inflamed gingival tissues were used as a positive control for TLR staining (Mori *et al.* 2003), whereas human tonsil tissue was used as a positive control for CD3 and CD19 immunostaining. An archival sample of hyperplastic gingival tissue with predominant plasma cells was utilized as positive sample for methyl green pyronin (MGP) staining.

Sample preparation

The excised periapical lesions were fixed in 10% formalin and processed routinely. A series of 5-μm sections from three different areas of each tissue sample were cut for all samples in groups 1, 2 and 3. The first section of each series was stained with haematoxylin and eosin, the second section was used for immunohistochemical staining for expression of TLR2, the third and fourth sections were used for immunohistochemical staining with CD3 and CD 19 antibodies to identify T lymphocytes and B lymphocytes, respectively, and the final section was stained with MGP to identify plasma cells.

Immunohistochemistry

TLR2

Pilot studies were undertaken to establish the optimum dilution of the antibody as well as confirming concentration and application times for the chemicals used during the immunohistochemistry (IHC) procedure. Briefly, sections were picked up onto a slide, deparaffinized in xylene, dehydrated in graded alcohol, washed and then heat treated in sodium citrate buffer (pH 7.0), for 10 min at 80 °C, to unmask antigens. The sections

were cooled and washed in phosphate-buffered saline (PBS, pH 7.2). Endogenous peroxidase activity was quenched by incubating the sections in a solution of 3% H_2O_2 in methanol for 15 min. Specimens were washed with PBS and incubated in blocking agent (foetal calf serum, 25 μL per 4 mL of normal saline) for 15 min. Sections were incubated with mouse anti-human TLR2 monoclonal primary antibodies (sc-21759; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1 : 50 dilution for 30 min at 25 °C. The sections were then washed in PBS and incubated with secondary antibodies using the LSAB2-HRP link system (DakoCytomation, Carpinteria, CA, USA).

The manufacturer's instructions were followed for the sequential incubation and durations for the exposure to the secondary antibodies. After washing with PBS, the sections were incubated with diaminobenzidine substrate kit (Dako, Carpinteria, CA, USA) that resulted in a brown-coloured precipitate at the antigen–antibody binding sites. Finally, all the sections were washed in deionized water, dehydrated and mounted on individual slides. Positive (inflamed gingival tissue) and negative controls (periapical scar or dentigerous cyst) as well as experimental sections with no primary antibody were included in all immunohistochemical runs.

CD3 and CD19 antibodies

Immunohistochemistry staining with antibodies CD3 and CD19 was performed using an automatic immunostainer (Benchmark XT; Ventana Medical Systems Inc., Tucson, AZ, USA). The preparation of the sections for immunostaining was similar to the TLR2 antibody technique. The dilutions used for polyclonal rabbit antihuman CD3 and monoclonal mouse antihuman CD19 primary antibodies (Dako) were 1 : 20 and 1 : 100, respectively. The duration of incubation was 2 h for both the primary antibodies. The secondary antibody system used by the automatic immunostainer was polymer-based Universal HRP multimer (ultraView™; Ventana Medical systems Inc.). Finally, the sections were incubated with universal alkaline phosphatase red detection chromogen kit (ultraView™; Ventana Medical systems Inc.), washed in deionized water, dehydrated and mounted. Positive (human tonsils) and negative controls (no primary antibody) were included in all immunohistochemical runs.

Methyl green pyronin staining

Experimental and positive control (hyperplastic gingival tissue) sections were stained with MGP (MGP) stain. The section was picked up onto a slide, deparaffinized in xylene, dehydrated in graded alcohol, washed in acetate buffer (pH 4.8) and dipped into staining solution for 25 min. The section was then washed with acetate buffer, dried with blotting paper, dehydrated and mounted.

Qualitative analysis

All the stained sections were viewed under a light microscope (Leica CTR5000; Leica Microsystems, Wetzlar, Germany) under magnifications up to 1000×. The presence or absence of cells expressing TLR2, CD3 and CD19 was correlated with the histopathological and clinical data. A cell was counted as positive when it demonstrated distinct brown (for TLR2) or red (for CD3 and CD19) surface staining. A MGP-stained cell was deemed positive with blue-green nuclear staining and red cytoplasmic staining.

Quantitative analysis

All the TLR2-stained slides from Groups 1, 2 and 3 were included in the quantitative analysis. Four representative sites in each sample were photographed at 400× magnification and captured with a software system (CS3, version 10.0.1; Adobe photoshop, San Jose, CA, USA and Leica FireCam, version 1.5; Leica Microsystems, Cambridge, UK). A calibrated grid was then digitally superimposed over the photograph to identify an area of 250 μm^2 in each site (total 1 mm^2 area). The total number of cells present and those stained positively with TLR2 were counted manually for all four representative sites. Expression of TLR2 was reported as the percentage of TLR^+ cells of all cells. Mann–Whitney *U* tests were performed to measure any statistical significance in expression of TLR2 amongst the groups using SPSS statistic software (version 17.0; SPSS Inc., Chicago, IL, USA). Values of $P < 0.05$ were considered statistically significant.

Results

The age range of the patients from which the refractory periapical lesions ($n = 32$) were collected was 18–81 years with 14 samples from women. Pain, swelling and/or draining sinus characterized the clinical presentation of all symptomatic periapical granulomas and most cysts (7 of 10 lesions), whereas all the asymptomatic periapical granulomas, three of the periapical cysts and both the periapical scar tissues were asymptomatic.

Qualitative analysis

Light microscopy

Refractory periapical granuloma lesions stained with H&E showed typical fibrous tissue containing cells with morphological similarities to lymphocytes, plasma cells, macrophages, fibroblasts and endothelial cells. PMNs were noted in abundance within symptomatic periapical granulomas, mainly towards the centre of the lesion away from the surrounding fibrous capsule. In numerous regions, PMNs could be seen in the blood vessels, in close proximity to the endothelial cells (margination) and in the process of migrating out between endothelial cells into the extravascular space. Periapical cysts were characterized by a lumen lined by nonkeratinizing stratified squamous epithelium with various degrees of inflammatory cell infiltrate in the adjacent connective tissue. Neither the periapical fibrous tissue scars nor the dentigerous cyst demonstrated the presence of inflammatory cells.

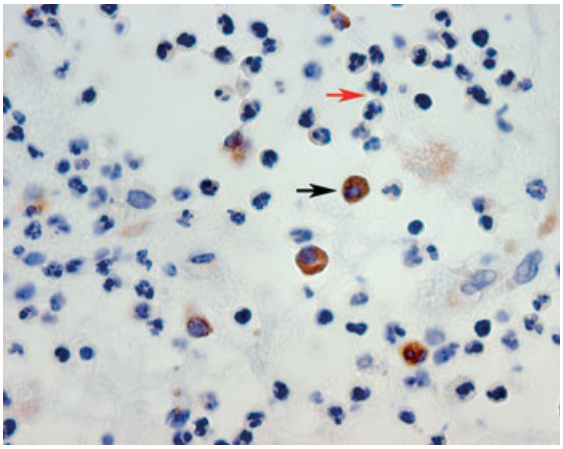

Figure 1 Cell surface expression of TLR2 on some mononuclear cells (black arrow) in a section of a symptomatic periapical granuloma. Note that multilobed polymorphonuclear neutrophils did not react to TLR2 antibody (red arrow) (TLR2 immunostaining, ×1000).

Immunohistochemistry

Various mononuclear inflammatory cells in the periapical lesions (Groups 1, 2, & 3) were reactive to TLR2 antibody (Fig. 1). The positive control (inflamed gingival tissue) demonstrated cells expressing TLR2, whereas negative controls did not show any positive staining (Fig. 2).

Many of the mononuclear cells that reacted positive to TLR2 immunostaining showed morphological similarities to lymphocytes and plasma cells (Fig. 3). Lymphocytes were the most numerous cells in the inflammatory infiltrate. PMNs, endothelial cells and fibroblasts did not react to any types of staining tested. In some periapical lesions, foamy macrophages demonstrated positive cytoplasmic staining with TLR2 antibody (Fig. 4).

In order to gain some insight into the phenotypic characterization of the inflammatory cells expressing TLR2, adjacent tissue sections were stained either with CD3 and CD19 antibodies to determine the presence of T or B cells or with MGP to demonstrate the presence of cells with morphological similarities to plasma cells.

It was observed that in the refractory periapical granuloma samples (Groups 1 & 2), $CD3^+$ T cells were more numerous than $CD19^+$ B cells (Fig. 5), whereas in periapical cysts (Group 3), more lesions showed higher expression of $CD19^+$ B cells than $CD3^+$ T cells (Fig. 6). Comparison of adjacent sections of periapical granulomas and cysts revealed that $TLR2^+$ cells and $CD3^+$ T and $CD19^+$ B cells were abundant in the same microenvironment (Fig. 7). In addition, many $TLR2^+$ cells demonstrated morphological similarities to plasma

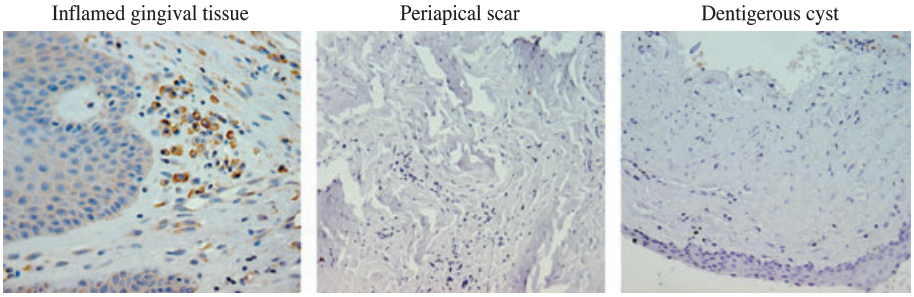

Figure 2 Expression of TLR2 by cells in the positive control; inflamed gingival tissue and no TLR2 expression in negative controls; periapical scar or dentigerous cyst (×400).

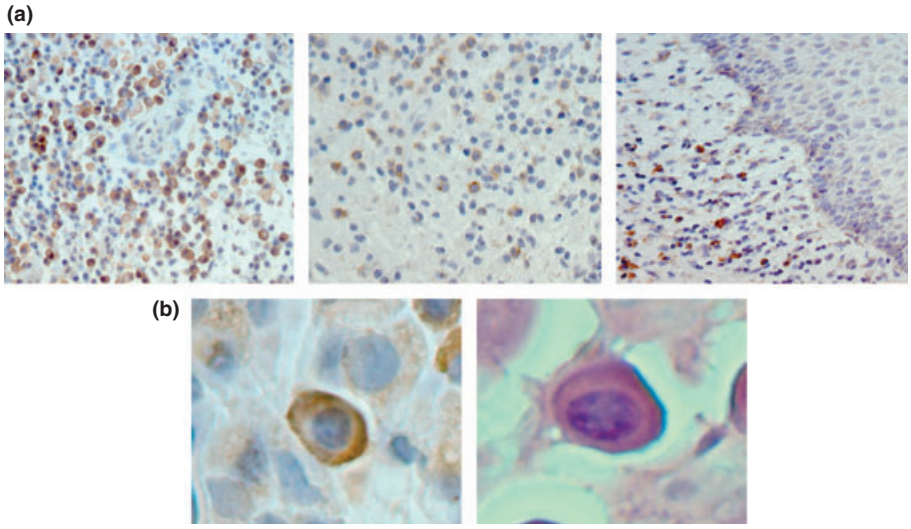

Figure 3 (a) Representative sections from a symptomatic periapical granuloma (left and centre) and periapical cyst (right) with numerous mononuclear cells demonstrating TLR2 expression (TLR2 immunostaining, ×400) (b) Some of the $TLR2^+$ mononuclear cells show morphological similarities to either lymphocytes (left) or plasma cells (right) (TLR2 and H & E staining ×1000).

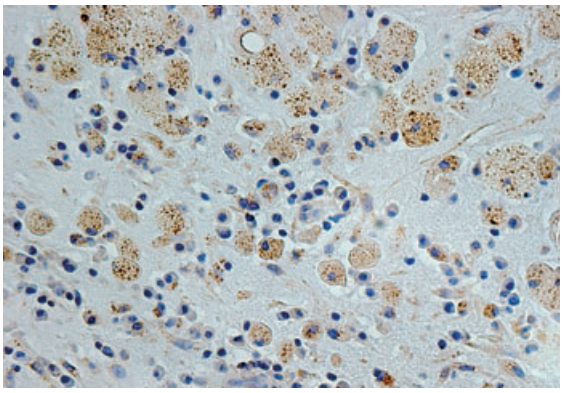

Figure 4 Foamy macrophages in a symptomatic periapical granuloma showing intracellular staining of TLR2, possibly due to phagocytosis of apoptotic or necrotic cell debris from cells that had expressed TLR2 during life (TLR2 immunostaining, ×400).

cell-like cells (MGP^+ cells) indicating the possibility of TLR2 expression on the cell surface of plasma cells. In contrast, there were numerous areas within a periapical granuloma or cyst where there was no association of $TLR2^+$ cells with $CD3^+$, $CD19^+$ or MGP^+ cells.

Quantitative analysis

TLR2 expression was demonstrated in all periapical lesions examined except in Group 4 (periapical scar) and three samples of Group 1 (Table 1). ANOVA test revealed a statistically significant ($P < 0.05$) difference in the number of cells expressing TLR2 between Groups 1, 2 and 3. A nonparametric test for assessing two independent samples (Mann–Whitney *U* test) showed statistically significant ($P < 0.05$) higher expression of TLR2 in Group 2 (symptomatic periapical granuloma) than Groups 1 (asymptomatic periapical granuloma,) and 3 (periapical cyst).

Discussion

Numerous studies have characterized the expression of TLR2 by different circulating leucocytes and inflammatory cells present in various tissues, though not periapical tissues. TLR2-mediated cellular responses have been reported in PMNs (Hayashi *et al.* 2003, Nilsen *et al.* 2004), mast cells (McCurdy *et al.* 2003), monocytes and macrophages (Underhill *et al.* 1999, Nilsen *et al.* 2004), T cells (Komai-Koma *et al.* 2004), T_r cells (Liu *et al.* 2006) and B cells (Vasilevsky *et al.* 2008). Additionally, experiments on murine dental pulp demonstrated expression of TLR2 by fibroblasts, odontoblasts and dendritic cells (DC) when stimulated with appropriate ligand (Staquet *et al.* 2008, Keller *et al.* 2010).

Immunohistochemical demonstration of TLR2 cell surface antigen in human periapical tissue has not been previously been reported. In the present study,

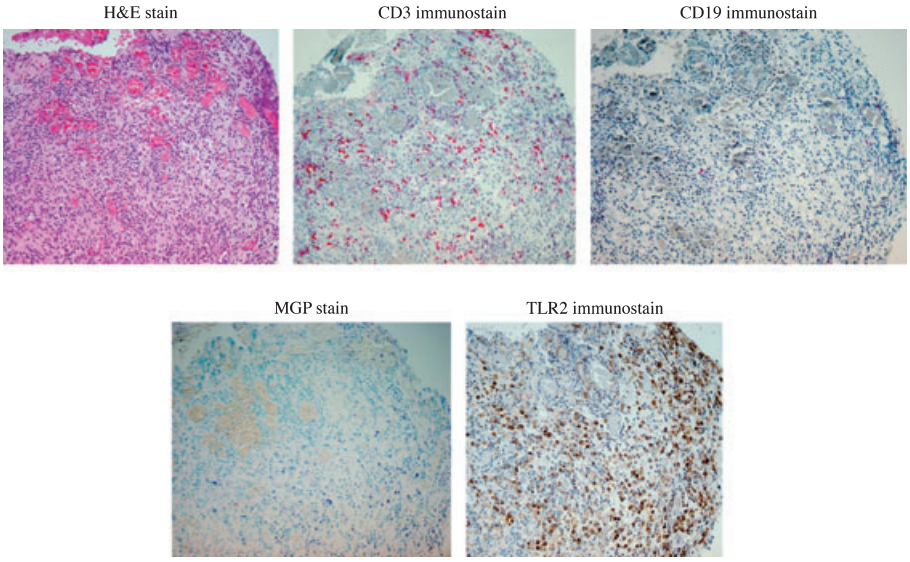

Figure 5 Representative serial sections of a symptomatic periapical granuloma showing higher numbers of $CD3^+$ T cells than $CD19^+$ B cells associated with the expression of TLR2. Also note that a number of TLR^+ positive sites do not have $CD3^+$ T cells, $CD19^+$ B cells or MGP^+ cells associated with them (×100).

immunohistochemical examination of human refractory periapical granulomas and cysts showed expression of TLR2 by various chronic inflammatory cells, which is in accordance with the infectious origin of periapical diseases. Polymorphonuclear neutrophils, fibroblasts and endothelial cells did not express TLR2,

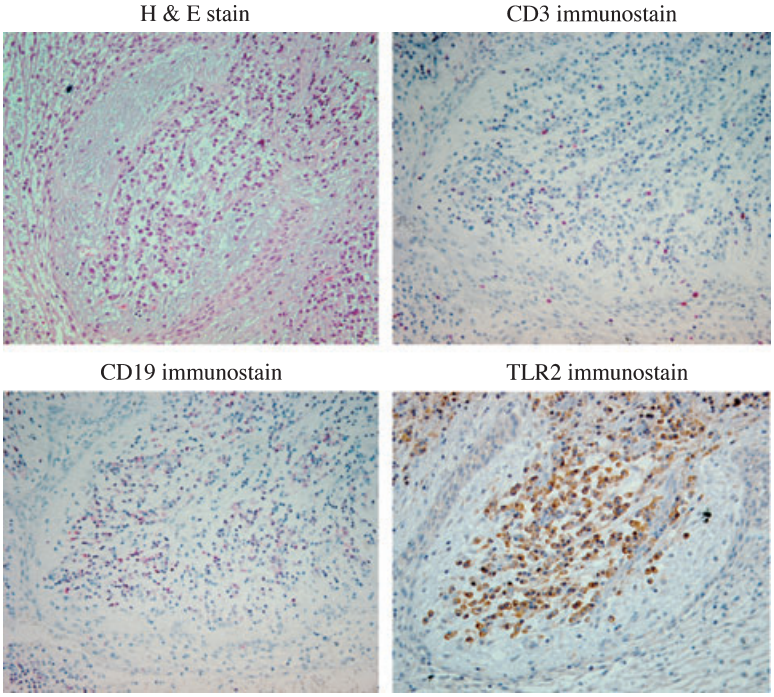

Figure 6 Serial sections from a periapical cyst demonstrating greater numbers of $CD19^+$ B cells than $CD3^+$ T cells associated with $TLR2^+$ mononuclear cells (×200).

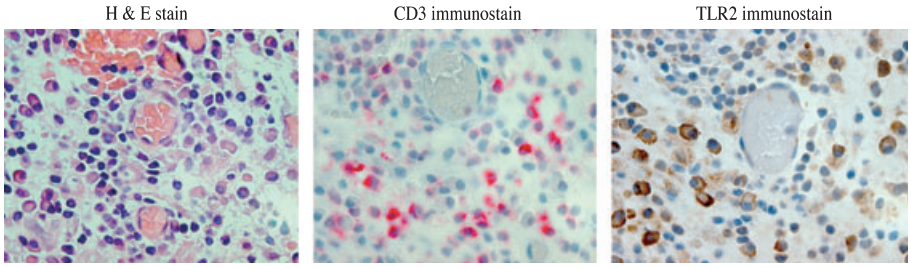

Figure 7 Representative adjacent sections of an asymptomatic periapical granuloma showing the association of CD3 T cells and $TLR2^+$ mononuclear cells in the vicinity of a blood vessel (×1000).

Table 1 Percentage of cells expressing TLR2 in refractory periapical lesions

	Group 1 Asymptomatic periapical granuloma	Group 2 Symptomatic periapical granuloma	Group 3 Periapical Cyst
Mean (%) ± standard deviation	11.93 ± 8.53	25.37 ± 8.48	19.99 ± 4.59

nor did any cells present in periapical fibrous scar tissue or noninfected dentigerous cyst observations that correlate with the absence of bacterial antigens and inflammatory cells in these lesions.

In agreement with previous studies, it was observed that $CD3^+$ T lymphocytes were the predominate lymphocytes in all the periapical granulomas (Torabinejad & Kettering 1985, Yu & Stashenko 1987, Lukic *et al.* 1990, Suzuki *et al.* 2001, Liapatas *et al.* 2003). B lymphocytes and plasma cells have been observed in higher frequencies in advanced periapical lesions (Akamine *et al.* 1994, Kawashima & Stashenko 1999, Walker *et al.* 2000), and in the present study, there was a greater expression of $CD19^+$ B lymphocytes in periapical cysts. Macrophages with abundant foamy cytoplasm containing $TLR2^+$ material were seen in some periapical granulomas (Fig. 4). It was thought that this material was debris from $TLR2^+$ cells that had undergone apoptosis or necrosis and subsequent phagocytosis. In the light of this observation and the knowledge that macrophages produce nitric oxide and reactive oxygen species (ROS) which are implicated in the induction and progression of inflammatory periapical lesions (Lin *et al.* 2007), it is of interest that TLR 2 may have a role in ROS production by macrophages after stimulation with bacteria commonly found in root canals (Marcato *et al.* 2008), the association between macrophages, TLR and periapical pathology should be explored further.

The demonstration of T and B lymphocytes as well as plasma cell-like cells in the lesions helped characterize the expression of TLR2 by various cells. In the periapical lesions examined, some of the $TLR2^+$ cells had morphological similarities to lymphocytes and were observed in $CD3^+$ and $CD19^+$ cell-rich areas (Fig. 5) and in subepithelial regions of cysts (Fig. 6) indicating probable communication between antigen recognition and effector cells as well as possible involvement in exaggeration of the proliferative potential of epithelial cells present in the periapical cysts and some granulomas. This is in broad agreement with other experiments demonstrating TLR activity on T and B cells (Komai-Koma *et al.* 2004, Pasare & Medzhitov 2005, Liu *et al.* 2006, Vasilevsky *et al.* 2008.

It was interesting to note the presence of TLR2 on the surface of plasma cell-like cells. Several studies have mentioned high numbers of plasma cells and various classes of immunoglobulins in periapical lesions (Torabinejad *et al.* 1981, Stern *et al.* 1982, Cymerman *et al.* 1984, Nilsen *et al.* 1984, Torabinejad & Kettering 1985, Babal *et al.* 1987, Takahashi *et al.* 1996, Liapatas *et al.* 2003, Colic *et al.* 2009). So far, TLR2 has not been demonstrated on human or animal plasma cell surfaces. It is not clear why there was TLR2 expression on plasma cell-like cells in the present study; however, it may be because of stimulated $CD4^+$ T cells acquiring a plasma cell-like morphology over time (Page *et al.* 2004) or that plasma cells differentiated from memory B cells retained their surface TLR expression (Tangye *et al.* 2003).

Comparison of serial sections revealed that many of the mononuclear cells that were positive to TLR2 immunostaining were $CD3^-$, $CD19^-$ and MGP^-, indicating that these cells could possibly include dendritic cell subsets or macrophages. It is acknowledged however that identification of the types of cells expressing TLR2 in the present study is not definitive and that

further more specific cell marking is required to determine cell phenotypes.

It has been suggested that periapical true cysts are self-sustaining and require a surgical approach for elimination (Nair *et al.* 1996). On the contrary, Lin *et al.* (2009) proposed that growth requirements of periapical true cysts are similar to periapical pocket cysts and are controlled by inflammatory mediators present in the subepithelial region. Removal of the inflammatory stimulation (e.g. bacterial antigen) would result in regression of the true cyst by the process of apoptosis. Until now, no study has been able to satisfactorily describe the mechanism behind the growth of a periapical true cyst. Based on the demonstration of TLR2 expression in the periapical cysts in the current study, and the existing knowledge of various regulatory mechanisms of TLRs (Kawai & Akira 2006, Lang & Mansell 2007), it can be speculated that impaired negative regulatory pathways of TLRs may lead to an over-expression of TLRs by various immunity cells present in the periapical cyst. The resulting sustained subepithelial inflammatory reactions and the pooling of surrounding cytokines may lead to the continuous proliferation of epithelium and growth of the cyst in the absence or presence of continuous antigenic stimulus from the root canal.

Asymptomatic periapical inflammatory lesions may or may not have bacteria within the extraradicular tissues. However, bacteria present in the root canal system can sustain the disease process by invasion of their antigenic by-products into periapical tissue via the apical foramen (Stashenko *et al.* 1998). PRR sense the presence of different bacterial antigens, and accordingly, increased bacterial load, as in symptomatic cases, should lead to an increased expression of TLR2. In the present study, this was correlated with statistically significant differences in the proportion of $TLR2^+$ cells that were observed between various periapical lesions (Table 1). Clinically asymptomatic refractory chronic periapical granulomas showed significantly lower numbers of $TLR2^+$ cells than lesions with clinical symptoms. Further, it was noted that in periapical cysts (seven of 10 patients with cysts presented with pain), there was a significantly higher expression of $TLR2^+$ cells than in asymptomatic chronic periapical granulomas. A quantitative measurement of TLR2 expression in symptomatic and asymptomatic cases may be potentially utilized as a surrogate for antigen or bacterial detection in various periapical lesions as a means of determining the microbial activity associated with the lesion.

Conclusion

The study demonstrates the presence of $TLR2^+$ cells in periapical granulomas and cysts and provides further evidence that periapical cysts are likely to be sustained by the immune system via reaction to bacterial antigens.

References

Akamine A, Anan H, Hamachi T, Maeda K (1994) A histochemical study of the behavior of macrophages during experimental apical periodontitis in rats. *Journal of Endodontics* **20**, 474–8.

Akira S, Takeda K, Kaisho T (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature Immunology* **2**, 675–80.

Babal P, Soler P, Brozman M, Jakubovsky J, Beyly M, Basset F (1987) In situ characterization of cells in periapical granuloma by monoclonal antibodies. *Oral Surgery, Oral Medicine, and Oral Pathology* **64**, 348–52.

Colic M, Gazivoda D, Vucevic D, Vasilijic S, Rudolf R, Lukic A (2009) Proinflammatory and immunoregulatory mechanisms in periapical lesions. *Molecular Immunology* **47**, 101–13.

Cymerman JJ, Cymerman DH, Walters J, Nevins AJ (1984) Human T lymphocyte subpopulations in chronic periapical lesions. *Journal of Endodontics* **10**, 9–11.

Hancock HH, Sigurdsson A, Trope M, Moiseiwitsch J (2001) Bacteria isolated after unsuccessful endodontic treatment in a North American population. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology & Endodontics* **91**, 579–86.

Hayashi F, Means TK, Luster AD (2003) Toll-like receptors stimulate human neutrophil function. *Blood* **102**, 2660–9.

Iwasaki A, Medzhitov R (2004) Toll-like receptor control of the adaptive immune responses. *Nature Immunology* **5**, 987–95.

Janeway CA Jr (1989) Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor Symposia on Quantitative Biology* **54**, 1–13.

Jiang J, Zuo J, Hurst IR, Holliday LS (2003) The synergistic effect of peptidoglycan and lipopolysaccharide on osteoclast formation. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology & Endodontics* **96**, 738–43.

Kakehashi S, Stanley HR, Fitzgerald RJ (1965) The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. *Oral Surgery, Oral Medicine, and Oral Pathology* **20**, 340–9.

Kawai T, Akira S (2006) TLR signaling. *Cell Death & Differentiation* **13**, 816–25.

Kawashima N, Stashenko P (1999) Expression of bone-resorptive and regulatory cytokines in murine periapical inflammation. *Archives of Oral Biology* **44**, 55–66.

Keller JF, Currouel F, Colomb E *et al.* (2010) Toll-like receptor 2 activation by lipoteichoic acid induce differential

production of proinflammatory cytokine in human odontoblasts, dental pulp fibroblasts and immature dendritic cells. *Immunobiology* **215**, 53–9.

Komai-Koma M, Jones L, Ogg GS, Xu D, Liew FY (2004) TLR2 is expressed on activated T cells as a costimulatory receptor. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 3029–34.

Lang T, Mansell A (2007) The negative regulation of Toll-like receptor and associated pathways. *Immunology & Cell Biology* **85**, 425–34.

Liapatas S, Nakou M, Rontogianni D (2003) Inflammatory infiltrate of chronic periradicular lesions: an immunohistochemical study. *International Endodontic Journal* **36**, 464–71.

Lin SK, Kok SH, Lin LD *et al.* (2007) Nitric oxide promotes the progression of periapical lesion via inducing macrophage and osteoblast apoptosis. *Oral Microbiology & Immunology* **22**, 24–9.

Lin LM, Ricucci D, Lin J, Rosenburg PA (2009) Nonsurgical root canal therapy of large cyst-like inflammatory periapical lesions and inflammatory apical cysts. *Journal of Endodontics* **35**, 607–15.

Liu H, Komai-Koma M, Xu D, Liew FY (2006) Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ regulatory T cells. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 7048–53.

Lukic A, Arsenijevic N, Vujanic G, Ramic Z (1990) Quantitative analysis of the immunocompetent cells in periapical granuloma: correlation with the histological characteristics of the lesions. *Journal of Endodontics* **16**, 119–22.

Marcato LG, Ferlini AP, Freire Bonfim RC *et al.* (2008) The role of Toll-like receptors 2 and 4 on reactive oxygen species and nitric oxide production by macrophage cells stimulated with root canal pathogens. *Oral Microbiology & Immunology* **23**, 353–9.

McCurdy JD, Olynych TJ, Maher LH, Marshall JS (2003) Cutting edge: distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. *Journal of Immunology* **170**, 1625–9.

Molander A, Reit C, Dahlen G, Kvist T (1998) Microbiological status of root-filled teeth with apical periodontitis. *International Endodontic Journal* **31**, 1–7.

Mori Y, Yoshimura A, Ukai T, Lien E, Espevik T, Hara Y (2003) Immunohistochemical localization of Toll-like receptors 2 and 4 in gingival tissue from patients with periodontitis. *Oral Microbiology & Immunology* **18**, 54–8.

Nair PN, Pajarola G, Schroeder HE (1996) Types and incidence of human periapical lesions obtained with extracted teeth. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology & Endodontics* **81**, 93–102.

Nilsen R, Johannessen AC, Skaug N, Matre R (1984) In situ characterization of mononuclear cells in human dental periapical inflammatory lesions using monoclonal antibodies. *Oral Surgery, Oral Medicine, and Oral Pathology* **58**, 160–5.

Nilsen N, Nonstad U, Khan N *et al.* (2004) Lipopolysaccharide and double-stranded RNA up-regulate toll-like receptor 2 independently of myeloid differentiation factor 88. *The Journal of Biological Chemistry* **279**, 39727–35.

Page G, Sattler A, Kersten S, Thiel A, Radbruch A, Miossec P (2004) Plasma cell-like morphology of Th1-cytokine-producing cells associated with the loss of CD3 expression. *American Journal of Pathology* **164**, 409–17.

Pasare C, Medzhitov R (2005) Control of B-cell responses by Toll-like receptors. *Nature* **438**, 364–8.

Pinheiro ET, Gomes BPFA, Ferraz CCR, Sousa ELR, Teixeira FB, Souza-Filho FJ (2003) Microorganisms from canals of root-filled teeth with periapical lesions. *International Endodontic Journal* **36**, 1–11.

Shear M, Speight P (2007) *Cysts of the oral and maxillofacial regions*, 4th edn. Oxford: Blackwell Munksgaard, pp. 1–2.

Siqueira JF Jr, Rocas IN (2004) Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology & Endodontics* **97**, 85–94.

Sjögren U, Hagglund B, Sundqvist G, Wing K (1990) Factors affecting the long-term results of endodontic treatment. *Journal of Endodontics* **16**, 498–504.

Staquet MJ, Durand SH, Colomb E *et al.* (2008) Different roles of odontoblasts and fibroblasts in immunity. *Journal of Dental Research* **87**, 256–61.

Stashenko P, Teles R, D'Souza R (1998) Periapical inflammatory responses and their modulation. *Critical Reviews in Oral Biology & Medicine* **9**, 498–521.

Stern MH, Dreizen S, Mackler BF, Levy BM (1982) Isolation and characterization of inflammatory cells from the human periapical granuloma. *Journal of Dental Research* **61**, 1408–12.

Sundqvist G, Figdor D, Persson S, Sjögren U (1998) Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology & Endodontics* **85**, 86–93.

Suzuki T, Kumamoto H, Ooya K, Motegi K (2001) Immunohistochemical analysis of CD1a-labeled Langerhans cells in human dental periapical inflammatory lesions – correlation with inflammatory cells and epithelial cells. *Oral Diseases* **7**, 336–43.

Takahashi K, MacDonald DG, Kinane DF (1996) Analysis of immunoglobulin-synthesizing cells in human dental periapical lesions by in situ hybridization and immunohistochemistry. *Journal of Oral Pathology & Medicine* **25**, 331–5.

Takeuchi O, Hoshino K, Kawai T *et al.* (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11**, 443–51.

Tangye SG, Avery DT, Deenick EK, Hodgkin PD (2003) Intrinsic differences in the proliferation of naive and memory human B cells as a mechanism for enhanced secondary immune responses. *Journal of Immunology* **170**, 686–94.

Torabinejad M, Kettering J, Bakland LK (1981) Localization of IgE immunoglobulin in human dental periapical lesions by the peroxidase-antiperoxidase method. *Archives of Oral Biology* **26**, 677–81.

Torabinejad M, Kettering JD (1985) Identification and relative concentration of B and T lymphocytes in human chronic periapical lesions. *Journal of Endodontics* **11**, 122–5.

Underhill DM, Ozinsky A, Hajjar AM *et al.* (1999) The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature* **401**, 811–5.

Vasilevsky S, Chattopadhyay G, Colino J *et al.* (2008) B and CD4+ T-cell expression of TLR2 is critical for optimal induction of a T-cell-dependent humoral immune response to intact Streptococcus pneumoniae. *European Journal of Immunology* **38**, 3316–26.

Walker KF, Lappin DF, Takahashi K, Hope J, Macdonald DG, Kinane DF (2000) Cytokine expression in periapical granulation tissue as assessed by immunohistochemistry. *European Journal of Oral Sciences* **108**, 195–201.

Yu SM, Stashenko P (1987) Identification of inflammatory cells in developing rat periapical lesions. *Journal of Endodontics* **13**, 535–40.