Characterization of inflammatory cell infiltrate in human dental pulpitis

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Abstract

Introduction
To evaluate the microscopic characteristics and densities (per mm²) of tryptase⁺ mast cells, CD4⁺ T helper lymphocytes, CD45RO⁺ memory T lymphocytes, foxp3⁺ T regulatory lymphocytes, CD20⁺ B lymphocytes, CD68⁺ macrophages, and CD31⁺ blood vessels in human dental pulpitis (n = 38) and healthy pulpal tissue (n = 6).

Methodology
The pulps of 38 human teeth with a clinical diagnosis of irreversible pulpitis were removed by pulpectomy. The pulp tissue was immersed in 10% buffered formalin for evaluation using light microscopy. Tryptase, CD4, CD45RO, foxp3, CD20, CD68, and CD31 expressions were analysed using immunohistochemistry; other microscopic features, such as intensity of inflammatory infiltrate and collagen deposition, were evaluated using haematoxylin and eosin stain. Wilcoxon and Mann–Whitney tests were used for statistical analysis. The significance level was set at α = 5%.

Results
Two microscopic patterns of pulpitis were found: group 1 (G1) (n = 15) had an intense inflammatory infiltrate and mild collagen deposition; conversely, group 2 (G2) (n = 23) had a scarce inflammatory infiltrate and intense collagen deposition. The numbers of CD68⁺ macrophages (P = 0.004) and CD20⁺ B (P = 0.068) lymphocytes and the density of blood vessels (P = 0.002) were higher in G1 than in G2. However, a similar number of CD4⁺ and CD45RO⁺ T lymphocytes was found in both groups (P > 0.05). When present, tryptase⁺ mast cells were equally distributed in G1 and G2, whereas foxp3⁺ T regulatory lymphocytes were detected in 59% and 14% of the samples of G1 and G2. Controls exhibited lower numbers of foxp3, tryptase, CD4, CD45RO, CD68 and CD20 positive cells than G1 and G2.

Conclusions
Irreversible pulpitis had distinct microscopic features with important quantitative and qualitative differences in inflammatory cell infiltration.

Keywords: caries, dental pulp, dental pulpitis, immunological cells, inflammatory cells.

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Introduction
Bacteria found in dental caries are the most frequent aetiological agents of injury to the dental pulp. Inflammatory and immunologic reactions occur in response to microorganisms or their products, which penetrate into the pulp through the dentinal tubules (Bergenholtz 1981, Izumi et al. 1995, Okiji et al. 1997, Nanci 2003, Costa et al. 2009).

The inflammatory response consists of non-specific and immediate defense mechanisms, which involve vascular-exudative phenomena, such as vasodilatation and increased permeability, as well as infiltration of inflammatory cells, such as mast cells, neutrophils, and macrophages (Bergenholtz 1990, Izumi et al. 1995, Avery 2002, Abbas & Lichtman 2003). Whilst these cells play an important role in pulp defense, they also participate in the degradation of the extracellular matrix by releasing matrix metalloproteinases.

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Mast cells release several biologically active substances responsible for the modulation of inflammatory and immunologic responses, and angiogenesis (Holzer 1988, Jontell et al. 1998, Rodini et al. 2004, Freitas et al. 2007). The latter is extremely important in inflammation and repair processes, in which endothelial membrane protein CD31 represents a reliable vascular marker (Woodlin et al. 2007). Macrophages are mononuclear cells responsible for phagocytosis, antigen presentation, and immunomodulation (Jontell et al. 1998, Abbas & Lichtman 2003, Hahn & Liewehr 2007a); they also contribute to pulp repair by inducing fibroblast proliferation (Brody et al. 1992) and neovascularization (Folverini 1995).

The immune response is a more specific defense mechanism that becomes active after the inflammatory response. Immune reactions are characterized by antigenic processing, presentation, and recognition (Avery 2002, Abbas & Lichtman 2003). In the dental pulp, antigen-presenting cells (APCs), which express major histocompatibility complex class II (MHC class II) molecules, capture antigens and present peptide fragments to antigen-specific helper T lymphocytes in lymphonodes. Activated T helper lymphocytes (CD4+ T helper lymphocytes) rapidly multiply, create many clones, differentiate into memory T cells (CD45RO+ memory T lymphocytes) (Jontell et al. 1998), and, through blood or lymphatic streams (Pimenta et al. 2003), migrate to the pulp tissue, where native APCs start phagocytosis and present the antigens directly to these T cells. Once activated, CD4+ T helper lymphocytes trigger an effective immune response to any new entrance of the aggressor in the pulp. According to the cytokine production profile, CD4+/CD45RO+ T lymphocytes may stimulate the migration and activation of macrophages, other CD4+ T helper lymphocytes, and CD20+ B lymphocytes (Jontell et al. 1998, Abbas & Lichtman 2003), which produce antibodies against specific antigens (Abbas & Lichtman 2003).

Subsets of T lymphocytes, known as T regulatory (Treg) cells, play an important role in regulating the immune and inflammatory responses through secretion of anti-inflammatory cytokines such as interleukin-10 and transforming growth factor β (TGF-β) (Abbas & Lichtman 2003, Akbar et al. 2003, Chen et al. 2003). These cells are characterized by the constitutive expression of a transmembrane protein, the alpha chain of the receptor for interleukin-2 (CD25), the cytotoxic T lymphocyte antigen-4 (CTLA-4), and the forkhead transcription factor (Foxp3) (Akbar et al. 2003, Chen et al. 2003, Zheng & Rudensky 2007). Of these, foxp3 expression may be the most specific marker of Treg cells. (Chen et al. 2003, Zheng & Rudensky 2007).

Despite the high frequency of dental pulpsitis in endodontic clinics, few studies have described its inflammatory and immunologic profiles (Bergenholtz 1981, Izumi et al. 1995, Jontell et al. 1998). Therefore, new studies should investigate the pathogenesis of pulpitis so that more efficient therapies may be developed.

To understand the pathogenesis and microscopic characteristics of human dental pulpsitis better, this study investigated the presence of several immune inflammatory cells (mast cells, T helper lymphocytes, memory T lymphocytes, regulatory T lymphocytes, B lymphocytes, and macrophages).

**Materials and methods**

**Dental pulp samples**

This study was approved by the ethics committee of the Federal University of Goiás, Brazil, and informed consent was obtained from all patients.

Thirty-eight human teeth were randomly selected (19 maxillary incisors and 19 maxillary molars, whose pulps were removed from their palatal roots) with a clinical diagnosis of inflamed pulp from patients (mean age, 29.8 ± 13; 20 men) treated at the Dental Emergency Service of the School of Dentistry, Federal University of Goiás, Brazil. These patients had no history of systemic diseases or had consumed any medicaments in the last 3 months. The clinical diagnosis of irreversible pulpsitis was based on the following criteria: spontaneous symptomatology: positive response to pulp sensibility test (PST; tetrafluoroethane spray); absence of pulp exposure associated with dental caries. The teeth were not associated with periodontal or periapical diseases.

After the clinical diagnosis of irreversible pulpsitis, the patients were referred to a specialist for root canal treatment. The first step was pulpectomy, according to the following guidelines: anaesthesia, tooth isolation, antisepsis of the operative field with 1% sodium hypochlorite, and access cavity preparation on the occlusal surface of the tooth using a diamond-tipped spherical bur at high speed and under water-air cooling. The working length was set 1 mm from the occlusal surface of the tooth.
apex according to periapical radiographs. A small diameter K-file was introduced between the root canal wall and the pulp tissue to create space and release the tissue, and then the tissue was carefully excised with a Hedström file. After pulpectomy, root canal preparation and filling were performed at the same appointment.

In addition, healthy dental pulps (control) (n = 6) were collected from human permanent erupted teeth (without clinical dentine caries) extracted for orthodontic reasons.

The pulp tissues were immersed in 10% buffered formalin for further evaluation using light microscopy in the Oral Pathology Laboratory of the Federal University of Goiás, Brazil.

**Light microscopy**

All the pulp tissue specimens were fixed in 10% buffered formalin (pH 7.4) and embedded in paraffin blocks. Microscopic features, including intensity of the inflammatory infiltrate, collagen deposition, calcification, and necrotic areas, were evaluated using a 5-μm section of each sample stained with haematoxylin and eosin (HE).

The intensity of the associated inflammatory infiltrate (absent, mild or intense) was determined for each dental pulp specimen. After analysing 10 representative microscopic high-power fields (400x magnification) using a graticule (Carl Zeiss, Göttingen, Germany), specimens were classified according to inflammation: a) no inflammation, when most fields (>7) had no inflammatory cells; b) mild inflammation, when most fields (>7) had <35% of the graticule space filled by inflammatory cells; c) intense inflammation, when most fields (>7) had more than 35% of the graticule space filled by inflammatory cells.

Collagen deposition was characterized by eosinophilic area with reduced cellularity and blood vessels density or even acellular eosinophilic region lacking blood vessels. The collagen deposition was classified as mild, when most fields (>7) had <35% of the graticule space filled by collagen and intense, when most fields (>7) had more than 35% of the graticule space filled by collagen.

The calcification was scored as absent, mild, and intense, and the necrotic areas were considered as present or absent.

**Immunohistochemistry**

Sections of 3 μm were deparaffinized and dehydrated. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide. The sections were submitted to antigen retrieval (Table 1). Sections were then blocked by incubation with 3% normal goat serum for 20 min. Mast cells, T helper lymphocytes, memory T lymphocytes, regulatory T lymphocytes, B lymphocytes, macrophages, and blood vessels were detected using anti-tryptase, anti-CD4, anti-CD45RO, anti-foxp3, anti-CD20, anti-CD68, and anti-CD31 antibodies, respectively (Table 1). The slides were then incubated with the primary antibodies for 18 h at 4 °C. After washing in TBS, the sections were treated with the Novolink System (Novocastra, Newcastle, UK) or the LSAB®+ system, HRP Peroxidase Kit (Dako, Carpinteria, CA, USA) (Table 1), and then incubated in 3,3’-diaminobenzidine (DAB) (Dako) for 2–5 min. Finally, the sections were stained with Mayer’s haematoxylin and covered. Samples of tonsil tissue were used as positive controls for all markers. Negative controls were obtained by omitting primary antibodies and using 1% PBS-BSA and non-immune rabbit (X0902, Dako) or mouse (X501-1, Dako) serum.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Antibodies and protocols of immunohistochemical reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies® (clone)</td>
<td>Dilution</td>
</tr>
<tr>
<td>Anti-Tryptase® (AA1)</td>
<td>1 : 2000</td>
</tr>
<tr>
<td>Anti-CD4® (4B12)</td>
<td>1 : 100</td>
</tr>
<tr>
<td>Anti-CD45RO® (UCHL1)</td>
<td>1 : 300</td>
</tr>
<tr>
<td>Anti-foxp3® (236A/E7)</td>
<td>1 : 400</td>
</tr>
<tr>
<td>Anti-CD20® (L26)</td>
<td>1 : 2000</td>
</tr>
<tr>
<td>Anti-CD68® (KP1)</td>
<td>1 : 1000</td>
</tr>
<tr>
<td>Anti-CD31® (1A10)</td>
<td>1 : 200</td>
</tr>
</tbody>
</table>

*All antibodies were mouse monoclonal.

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Histochemistry
Mast cells density was also evaluated using toluidine blue (TB) (Heaney et al. 1997). Sections of 5 μm were deparaffinized and hydrated with water. TB staining was performed with a 1% TB solution diluted in phosphate buffer (pH 4–6) for 45 s. After rinsing in phosphate buffer for 1 min, sections were quickly dehydrated through 70%, 96% ethanol and acetone p.a. to xylene and mounted in synthetic resin.

Quantitative and qualitative analysis
The numbers of tryptase+ mast cells, CD4+ T helper lymphocytes, CD45RO+ memory T lymphocytes, foxp3+ T regulatory lymphocytes, CD20+ B lymphocytes, and CD68+ macrophages in the human dental pulps were calculated using the graticule. All CD31+ blood vessels with a vascular lumen were counted, even if they were very small (microvessels). All immune inflammatory cells and blood vessels were counted in 5–10 representative and consecutive microscopic high-power fields (400× magnification); at this magnification, each field of the graticule had an area of 0.0961 mm². The location and distribution of cells and blood vessels were also analysed. For all analysis, one slide with two representative sections for each sample was evaluated. Descriptive analyses were expressed as mean ± standard deviation (SD) of n observations per mm². The Wilcoxon test was used to compare all cell populations, and the non-parametric Mann–Whitney test for comparisons between experimental groups. A P-value of <0.05 was considered to be statistically significant.

Results
The microscopic features of the samples analysed revealed two distinct patterns of pulpitis: dental pulp with intense inflammatory infiltrate and mild collagen deposition (n = 15) (Group 1 – G1) (Fig. 1a–c), and dental pulp with scarce inflammatory infiltrate and intense collagen deposition (d–f).

Figure 1 Dental pulp with intense inflammatory infiltrate and mild collagen deposition (a–c) and dental pulp with scarce inflammatory infiltrate and intense collagen deposition (d–f). Preserved, dilated and congested blood vessels (b and e – arrow), and calcifications (c and f – arrow). HE, Original Magnification: a,d, ×100; b,c,e,f, ×400.
intense collagen deposition \( (n = 23) \) (Group 2 – G2) (Fig. 1d–f). No samples had necrotic areas, 90% had areas of calcification, and all had preserved blood vessels (Figs 1 and 2). The main microscopic features of the samples are summarized in Table 2. Both pulpitis groups: Group 1 (dental pulp with intense inflammatory infiltrate and mild collagen deposition) and Group 2 (dental pulp with scarce inflammatory infiltrate and intense collagen deposition) had a similar mean age G1 \( (31.2 \pm 12.8 \text{ years}) \) and G2 \( (28.8 \pm 15.1 \text{ years}) \).

Although two distinct patterns of pulpitis were found using microscopy, both G1 and G2 specimens had the same clinical characteristics (spontaneous symptomatology; positive response to pulp sensibility test; absence of pulp exposure associated with dental caries).

The immune inflammatory cells evaluated were diffusely distributed throughout the pulp tissue in all samples \( (n = 38) \). Tryptase\(^+\) mast cells (Fig. 3a) were found in 24\% of the samples at a low density \( \text{mean} = 6.6 \text{ cells mm}^{-2} \). We did not identify mast cells in these samples using TB staining. Similarly, foxp3\(^+\) T regulatory lymphocytes (Fig. 3b) were found in 34\% of the samples at densities ranging from 1.1–27.1 cells mm\(^{-2}\) \( \text{mean} = 10.49 \text{ cells mm}^{-2} \). The tryptase\(^+\) mast cells and foxp3\(^+\) T regulatory lymphocytes were absent in control samples.

Results also showed that, in all samples, there were lower densities of CD4\(^+\)T helper lymphocytes (Fig. 3c) and CD45RO\(^+\) memory T lymphocytes (Fig. 3d) than of CD68\(^+\) macrophages (Fig. 3e) and CD20\(^+\) B lymphocytes (Fig. 3f) \( (\text{Wilcoxon}, P < 0.05 \text{ for all comparisons}) \).

The number of CD68\(^+\) macrophages per mm\(^2\) was significantly higher in G1 than in G2 \( (\text{Mann–Whitney}, P = 0.004) \) (Fig. 4). Similarly, the density of CD20\(^+\) B lymphocytes was higher in G1 than in G2, although this difference was not statistically significant \( (\text{Mann–Whitney}, P = 0.068) \). A similar number of CD4\(^+\) and CD45RO\(^+\) T lymphocytes was found in both G1 and G2 \( (\text{Mann–Whitney}, P > 0.05) \) (Fig. 4). The control group showed significantly lower numbers of CD68\(^+\) macrophages, CD20\(^+\) B lymphocytes, CD4\(^+\) T helper lymphocytes, and CD45RO\(^+\) memory T lymphocytes when compared to the G1 and G2 groups.

When present, mast cells were equally distributed in G1 and G2. However, foxp3\(^+\) cells were found in 59\% and 14\% of the samples of G1 \( (n = 10) \) and G2 \( (n = 3) \). Findings also revealed a higher number of foxp3\(^+\) T regulatory lymphocytes in G1 \( (\text{mean} = 13.7 \text{ cells mm}^{-2}) \) than in G2 \( (\text{mean} = 6.2 \text{ cells mm}^{-2}) \).

The number of CD31\(^+\) blood vessels was significantly higher in G1 than in G2 \( (\text{Mann–Whitney}, P = 0.002) \) (Fig. 2). There were no significant differences between the pulpitis \( (G1 \text{ mean} = 190.00 \text{ blood vessel mm}^{-2} \) and G2 mean = 120.2 blood vessel mm\(^{-2}\) and control groups \( (132.5 \text{ blood vessel mm}^{-2}\)).

### Table 2 Microscopic characterization of human dental pulpitis \( (n = 38) \)

<table>
<thead>
<tr>
<th>Microscopic feature</th>
<th>Number of cases</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>Inflammatory infiltrate</td>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Intense</td>
<td>15</td>
</tr>
<tr>
<td>Collagen deposition</td>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Intense</td>
<td>23</td>
</tr>
<tr>
<td>Calcification</td>
<td>Absent</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Intense</td>
<td>18</td>
</tr>
<tr>
<td>Necrotic areas</td>
<td>Absent</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>0</td>
</tr>
<tr>
<td>Density of CD31(^+) blood vessels (mean: 144 blood vessels mm(^{-2}))</td>
<td>Low (≤144)</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>High (&gt;144)</td>
<td>15</td>
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</tbody>
</table>

Bruno et al. Immunological aspects of dental pulpitis

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Discussion

The microscopic evaluation of pulpitis revealed different patterns of response: pulps with intense inflammatory infiltrate and mild collagen deposition, and pulps with scarce inflammatory infiltrate and intense collagen deposition. However, these two distinct response patterns seen microscopically were not clinically confirmed, an indication of the difficulties of establishing associations between histopathological and clinical events, a finding corroborated by other studies (Seltzer et al. 1963, Närhi 2003). The correlation between symptoms and histopathological changes in pulpitis seems to be poor and the determination of the type and extent of the inflammatory changes on the basis of symptoms is inaccurate (Seltzer et al. 1963). It is important to consider that inflammatory response and subsequent repair process in dental pulps may be influenced by several factors such as type and duration of infection (Hahn & Liewehr 2007a,b), traumatic dental injury (Bruno et al. 2009), age-related changes (Tranasi et al. 2009), and others.

The presence of mast cells in the inflamed pulp, especially during the chronic phase of inflammation, has been reported (Farnoush 1984, Walsh et al. 1995). Mast cells do not seem to contribute only to the early vascular or specific immune responses in the initial dental pulp pathosis, but are also involved in chronic phases of pulp inflammation, such as in pulp polyps (Freitas et al. 2007).

In relation to the staining techniques for mast cells, both toluidine blue (TB) and immunohistochemistry identification techniques reliably identify mast cells granules; although recent studies (Batista et al. 2005, Oliveira-Neto et al. 2007) showed that the immunohistochemistry method is more specific than metachromatic staining by TB. In accordance, TB was not useful for identification of mast cells in this study.

Most inflamed pulps had no mast cells, and when present, their number was small, which is in agreement with previous studies that reported small populations or
no mast cells in non-inflamed pulps, as well as in early pulpitis (Farnoush 1984, Freitas et al. 2007). This reduced population of mast cells in pulpitis may interfere with the pulpal defense mechanism, because they are immunoregulatory cells that release vasoactive amines, enzymes, and cytokines, such as tumoral necrosis factor α (TNF-α). Conversely, the absence of mast cells might be interpreted as a form of modulation of the inflammatory response of the pulp, which minimizes increases in tissue pressure and prevents tissue necrosis. Massive mast cell degranulation in pulps would result in a significant increase in inflammatory cell recruitment and vasodilatation, and, consequently, high intrapulpal pressure and extensive tissue destruction (Walsh 2003).

Small numbers of foxp3+ T regulatory lymphocytes were found in most samples, a highly important finding because this appears to be the first study to detect these cells in inflamed pulp tissue. Interestingly, high amounts of foxp3+ T regulatory lymphocytes were found in pulps with intense inflammatory infiltrate (G1), which may indicate that they affect immunity control (Lehner 2008) and may prevent exacerbated inflammation and rapid necrosis of pulpal tissue. Also, foxp3+ T regulatory lymphocytes may suppress T cells (Lehner 2008) and, consequently, reduce their number, which was suggested by the lower numbers of both CD4+ and CD45RO+ T lymphocytes than of CD68+ macrophages and CD20+ B lymphocytes in all the samples analysed. Nevertheless, the role that T regulatory lymphocytes play in the pathogenesis of pulpitis should be explored further.

The greater number of B than of T lymphocytes in pulpitis suggests a humoral response mediated by antibodies. After the activation of T cells by APCs, T cells secrete cytokines and differentiate into several effectors: CD4+ T helper lymphocytes, CD45RO+ memory T lymphocytes, foxp3+ T regulatory lymphocytes, and CD8+ cytotoxic lymphocytes. The nature of the responses of these effector cells depends on the dose, affinity, and nature of the antigen, as well as on the type and concentration of cytokines in the tissue. According to current knowledge, an increase in the dose of antigenic peptide leads to changes in the CD4+ T helper lymphocytes response pattern of cytokine production (Hahn & Liewehr 2007b). CD4+ Th1 cells produce interleukin 2 (IL-2) and interferon-gamma (IFN-γ) predominantly, which play a role in the recruitment and activation of macrophages, whereas Th2 cells produce IL-4, IL-5, and IL-6, which stimulate B lymphocytes proliferation, differentiation, and activation (Jontell et al. 1998, Abbas & Lichtman 2003). In teeth with deep caries, there is a predominance of Th2-type response pattern, which leads to an increase in the number of B lymphocytes in the pulp (Jontell et al. 1998, Hahn & Liewehr 2007b).

There are large numbers of CD68+ macrophages in pulpitis; in fact, they are the most prevalent immune cells in the tissue (Jontell et al. 1998) and their number is likely to increase with the progression of caries (Hahn & Liewehr 2007b). The presence of these cells in deep caries may be associated with the expression of chemokine ligands, such as monocyte chemotactic protein-1 (CCL2/MCP-1), and macrophage inflammatory protein 3-alpha (CCL20/MIP-3α) (Nakanishi et al. 2005, Hahn & Liewehr 2007b). Also, macrophages play an important role in tissue homeostasis and repair after inflammation, as they stimulate fibroblast proliferation by releasing basic fibroblast growth factor (FGFb) (Brody et al. 1992, Hahn & Liewehr 2007b), as well as in revascularization, because they release vascular endothelial growth factor (VEGF) (Polverini 1995, Hahn & Liewehr 2007b, Mattuella et al. 2007a,b). FGFb leads to an increase in collagen fibre production, which was also confirmed in this study.
because collagen deposition was found at varying degrees in both groups.

The blood vessels of all the samples examined were preserved. The highest density of CD31+ blood vessels was seen in pulps with large numbers of inflammatory cells (G1), as well as increased numbers of macrophages, cells that are responsible for the production of VEGF, a potent factor that induces angiogenesis and vascular permeability (Hahn & Liewehr 2007b). The increased number of vessels may contribute to the reparative response of the pulp-dentine complex because of the augmented transport of inflammatory cells, nutrients, and oxygen to the site of inflammation (Polverini 1995, Woodfin et al. 2007).

Recent advances in immunology have disclosed the enormous complexity of the immune regulatory system. The dental pulp is equipped to mount an innate and adaptive immune response to caries, which involves several types of cells and inflammatory mediators (Hahn & Liewehr 2007a,b). Knowledge of the pathogenesis of pulp disease may help to develop more efficient therapies.

**Conclusion**

Irreversible dental pulpitis revealed distinct microscopic features with important quantitative and qualitative differences in inflammatory cell infiltration. Further experimental studies should be conducted to determine the clinical and microbiological factors involved and how they affect the inflammatory response outcome and consequently the pulpal prognosis.

**Acknowledgements**

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