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Immunohistochemical Study of Phenotypes of Dendritic Cells in Dental Pulps from Non-Carious and Carious Teeth

Melissa A. Harmon
Virginia Commonwealth University

IMMUNOHISTOCHEMICAL STUDY OF PULPS FROM NON-CARIOUS AND

CARIOUS TEETH

A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science in Dentistry at Virginia Commonwealth University.

by

MELISSA ANN HARMON
BA Biology, University of Missouri-Kansas City, 2001
DDS, University of Missouri-Kansas City, 2001

Director: KARAN J. REPLOGLE
CHAIR, DEPARTMENT OF ENDODONTICS

Virginia Commonwealth University
Richmond, Virginia
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Acknowledgement

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Abstract

IMMUNOHISTOCHEMICAL STUDY OF PHENOTYPES OF DENDRITIC CELLS IN

DENTAL PULPS FROM NON-CARIOUS AND CARIOUS TEETH

By Melissa A. Harmon, DDS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of
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Major Director: Karan J. Replogle, DDS, MS
Chair, Department of Endodontics

Mature dendritic cells (DCs) in inflamed tissues may promote inflammation but the

status of DCs in pulpitis is not known. We hypothesized that DC maturation would

correlate with carious lesion depth and that CD4+ cells would be found in association with

mature DCs. Pulps were collected from teeth exhibiting: (I) no caries (n=9), (II) shallow

dentinal caries (n=5), and (III) deep caries (n=9). Pulpal tissues were cryo-sectioned and

positive cells were examined with immunohistochemistry, Mature DCs (CD83+) were

almost exclusively restricted to pulps from deep caries. Furthermore, CD209+ DCs in deep

caries were elevated over other groups and CD209+ cells about doubled the CD83+ cells suggesting that immature DCs had accumulated and were available for terminal maturation. CD4+ cells were found associated with both mature DCs and macrophages in pulps from deep caries suggesting that T cells may be a source of pro-inflammatory cytokines at this inflamed site. This document was created in Microsoft Word 2000.

Introduction

Healthy dental pulp is equipped with antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages (1, 2). APCs are important because they function as a bridge between innate and adaptive immunity by presenting antigens to T cells. DCs are the most potent T cell stimulators and are critical for priming naïve T cells (3). Immature DCs, which are vigorously endocytic, reside in peripheral tissues including normal dental pulp along the subodontoblastic layer. These DCs are thought to provide immune-surveillance in normal tissues and serve as sentinels for the immune system (2, 4). When infection and tissue damage occur, immature DCs take up antigens in the context of inflammatory signals, which promotes DC maturation (5). During maturation, DCs down-regulate endocytic receptors and CD14 expression while up-regulating T cell co-stimulatory molecules such as CD80, CD86 to become potent APCs (6).

The DC populations in healthy human dental pulp are identified by their dendritic morphology and the expression of various dermal DC markers. Most pulpal DCs (87%) express HLA-DR, and FXIIIa (human coagulation factor 13a, a dermal DC marker) and macrophage markers (CD14, CD68) (7). A small portion of these DCs (13%) that express HLA-DR and FXIIIa, lack CD68 and are thought to have the same potential as dermal DCs (FXIIIa+, CD14-) and function as conventional immature DCs. A small group of pulpal

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DCs, located mainly in the odontoblast/predentin region, are thought to be capable of migrating to regional lymph nodes to present antigens to naïve T cells (8). Later studies found a correlation between the severity of caries and an increased number of FXIIIa+ cells (4) and HLA-DR+ cells (9). Furthermore, they reported that these cells were co-localizing with memory T cells in clusters, suggesting that antigen presentation by monocytoid phenotype DCs takes place in these pulpal DC-T cells clusters. However the maturity of these DCs in inflamed pulp has not been established.

DC-SIGN (CD209) is a 44kD DC-specific ICAM-3 receptor, which is expressed by immature and mature DCs but not by monocytes or macrophages. It binds HIV envelop glycoprotein and stabilizes the initial DC-resting T cell interaction during primary immune responses (10). It is found on dermal DCs in skin and is expressed abundantly in lamina propria beneath the mucosa. CD209 expression increases as DCs mature but fully mature DCs have decreased CD209 expression while expressing abundant levels of CD83 (11).

The present study was undertaken to examine the phenotypes of conventional markers for immature (CD209+) and mature DCs (CD83+) in inflamed pulp from carious teeth with lesions of increasing depth. We reasoned that DC maturation would correlate with increasing lesion depth and that co-localization of mature DCs and CD4+ cells would further support the concept that DCs present antigens to T cells in inflamed dental pulp.

Materials and Methods

Teeth collection

Extracted teeth were obtained from the Oral Surgery Clinic of the Dental School and were exempted from the University Institutional Review Board. The extracted teeth were stored in saline before being cracked open longitudinally with a pair of pliers. The pulpal tissues were removed and frozen with O.C.T compound (Tissue-Tek®, Fisher Scientific) within 3 hours after extraction. The depth of each carious lesion was determined by an explorer and the discoloration. The specimens were classified as follows: (I) non-carious intact teeth; (II) shallow dentinal caries group: teeth with caries at least 2 mm away from the pulp chamber; and (III) deep caries group: teeth with caries within 2 mm from the pulp chamber. Frozen samples were kept in -80°C before cryosectioning.

The pulp samples were serially sectioned at 7 μm in thickness using a Leica (Jung Frigocut 2800E) cryostat and air-dried. Two sections were collected on each silanized treated slide. Sections were then fixed in acetone and stored at -80°C before immunohistochemical staining.

Immunohsitochemistry

Every 10th slide was subjected to Hematoxylin and Eosin staining to identify the sections containing inflammatory cells in each pulp. Endogenous peroxidase activity was eliminated by treating the sections with 3% H_2O_2 in methanol for 30 min. Nonspecific binding on each section was blocked by use of 2.5% normal horse serum (Vector Laboratories, Burlingame, CA) for 20 min. The sections were then labeled in duplicate with appropriate dilutions of CD83 (HB15A, 1:400), CD209 (AZND1, 1:40) (Beckman Coulter, Miami, FL) and CD14 (RPA-M1, 1:200, Zymed laboratories, San Francisco, CA) monoclonal antibodies for 1 h. After washing, the sections were treated with peroxidase conjugated anti-mouse Ig polymer (ImmPress®, Vector Laboratories, Burlingame, CA). A chromagen substrate, 3, 3'-diaminobenzidine (DAB, Zymed Laboratories), was used to visualize peroxidase in tissue sections according to the manufacturer's instructions. Sections were then counter-stained with Meyer's hematoxylin (Sigma, St. Louis, MO), dehydrated and mounted. Sections reacting with mouse primary antibody isotype control (Zymed Laboratories) were included as negative controls and sections from human tonsil tissue were used as positive controls for the primary antibodies.

Double labeling

The co-localization of APCs and CD4+ T cells were performed using a double immunohistochemical method. The monocytes/macrophages or pulpal (immature) DCs were labeled with CD14 while mature DCs were labeled with CD83 as described above. The second antibody (CD4, RPA-T4, 1:200, Zymed Laboratories) was added after the first

substrate reaction (DAB-Ni, Vector Laboratories) according to the manufacturer's instruction. CD4+ cells were visualized with the ImmPress and the second substrate, NovaRED® (Vector Laboratories). The sections were then dehydrated and cover-slipped with Poly-mount (Polysciences, Warrington, PA).

Counting method

Cell quantification was performed in a representative section of each double stained specimen. The counting method was modified from a previous published method described by Okiji et al (12). The number of positively-stained cells were counted in three selected fields using a 10 mm x 10 mm ocular grid under a magnification of 100 x. The three fields in the inflammatory site were randomly selected in the area where CD83+ cells were located and the total count in three fields of each antibody was recorded.

Statistical analysis

The numbers of positive cells for each phenotype were log transformed to improve the fit to normal distribution and analyzed using a using a repeated-measures mixed-model of ANOVA. Post hoc comparisons were used to determine the differences in three carious groups in each marker. The back-transformed least square means for each experimental condition were calculated. The significance level accepted was $p < 0.0163$ corrected for multiple comparisons.

Results

Healthy and Shallow caries groups

Labeling of DCs (CD209+ cells) and monocytes/macrophages with a few pulpal DCs (CD14+ cells) are shown in Fig. 1. The insert shows CD209 positive cells at a higher magnification and the irregular shape characteristic of DCs is apparent. Adjacent sections from healthy and shallow caries were also labeled with anti-CD83 but reactive cells were rarely found. The same distribution of APCs was observed in both the healthy and the shallow caries groups and the data are summarized in Table 1. The number of CD14+ cells was about double the number of CD209+ cells but there were no differences in the frequency of CD209+ and CD14+ cells between healthy and shallow caries groups. Nevertheless, the repeated-measures ANOVA indicated that there was a significant difference depending upon the caries type and the cell surface markers ($p < 0.0001$). We attribute this significant difference to the marked increase in both the CD209 and CD14 positive cells in the deep caries group as well as the appearance of significant numbers of CD83+ cells in the deep caries group.

Deep caries group

Significantly higher numbers of CD209+ cells (p=0.0036) as well as CD14+ cells (p=0.0042) were observed in deep caries when compared with the healthy caries group.

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The number of CD14+ cells in the deep caries group was also significantly higher than the count in shallow caries (p=0.0044) but the increase of CD209+ cells in deep caries did not reach the p<0.0163 required to conclude significance. Of particular importance was the increased number of CD83+ mature DCs in the deep caries group (*p*s<0.0001). Nevertheless, the CD209+ cells about doubled the CD83+ cells in deep caries suggesting that a large store of immature DCs had accumulated and were available for terminal maturation. Note that the CD83+ cells were localized near the severe inflammatory sites but were rare in the area of the abscess (Figs. 2A and 2a). In contrast, many CD14+ cells were distributed close to the abscess area (Figs. 3B and 3b). Moreover, CD4+ T cells could be found closely associated with CD83+ cells (Fig. 3A) and some CD4+ cells could be found associated with CD14+ cells as illustrated in Fig. 3B. Interestingly, some CD4+ cells formed clusters around the APCs.

Table 1. Relationship between the Number and Type of APCs in Dental Pulp and the Severity of Dental Caries

Marker	Caries type	Counts/3 fields	
		LSM[a]	95% CI
CD83	Healthy	0.2	0.0[b] -0.6 [c]
	Shallow	0	0.0-0.4
	Deep	21.3 [d]	13.8-32.9
CD209	Healthy	17.5	10.5-28.8
	Shallow	23.5	13.1-42.0
	Deep	49.1 [e]	31.3-77.0
CD14	Healthy	57.2	35.3-93.5
	Shallow	51.5	28.5-92.7
	Deep	153.2 [f]	99.9-234.8

a : least square mean of total cell counts in three selected ocular grids under 100 x magnification.

b : lower 95% confidence interval (CI)

c ; upper 95% confidence interval (CI)

d ;deep caries group exhibited a significantly higher count of CD83 than shallow and healthy groups (ps<0.0001).

e: deep caries group exhibited a significantly higher count of CD209 than healthy group (p=0.0036).

f: deep caries group exhibited a significantly higher count of CD14 than healthy group (p=0.0042) and shallow caries group (p=0.0044).

Figure 1: Healthy Pulp. The presence of CD 209+ and CD14+ cells in healthy pulp. Immunohistochemical labeling was performed with anti-CD209 (A) or anti-CD14 (B) specific momoclonal antibodies. This primary labeling was followed with peroxidase conjugated anti-mouse Ig polymer (ImmPress®) and 3, 3'-diaminobenzidine (DAB) chromagen. The bar size is 50 μ. The insert (a) is the enlargement of the designated area.
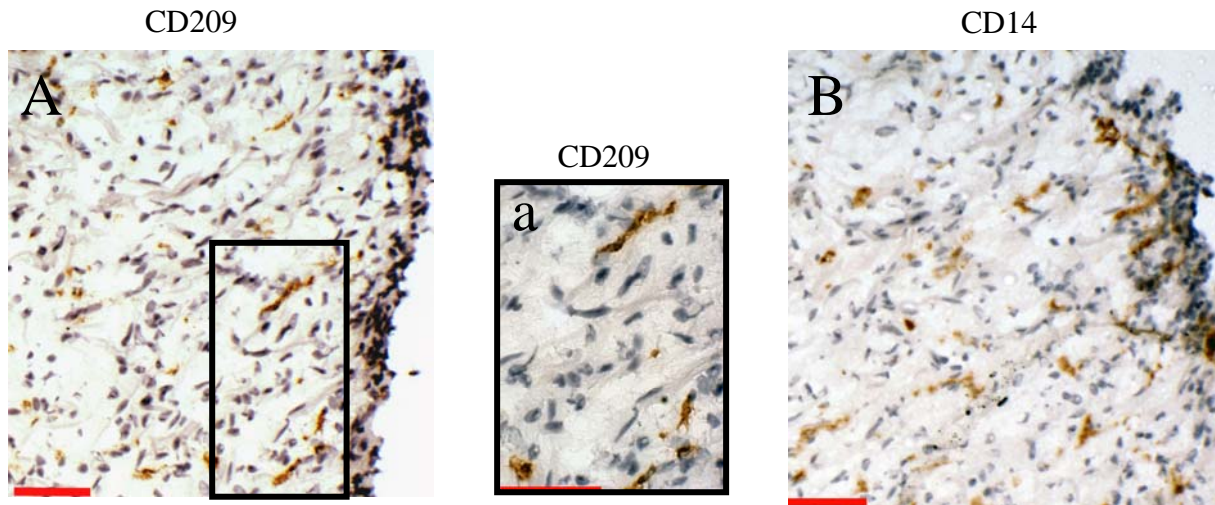

CD209

CD14

CD209

Figure 2: Deep Caries. The presence of CD 83+ or CD14+ cells in pulp beneath a deep carious lesion. Adjacent sections from a dental pulp with localized abscess (*) beneath a deep caries were labeled immunohistochemically with anti-CD83 (A) or anti-CD14 (B). The bar size is 50 μ. The inserts (a, b) are the enlargement of the designated areas respectively.

CD83
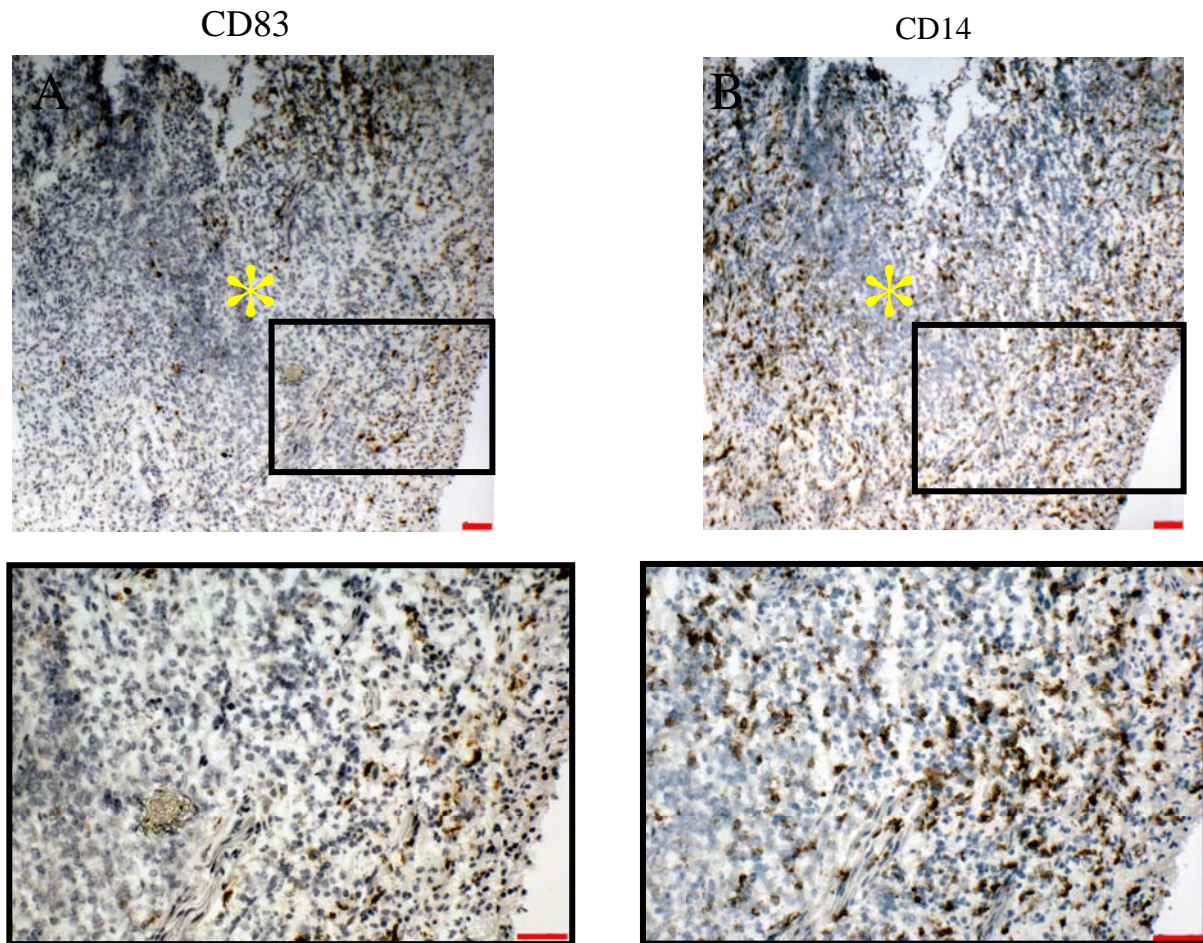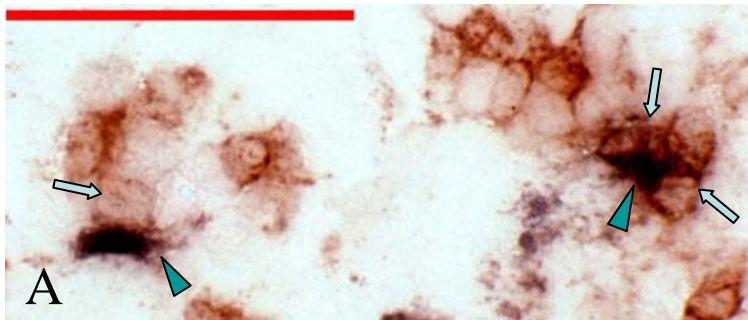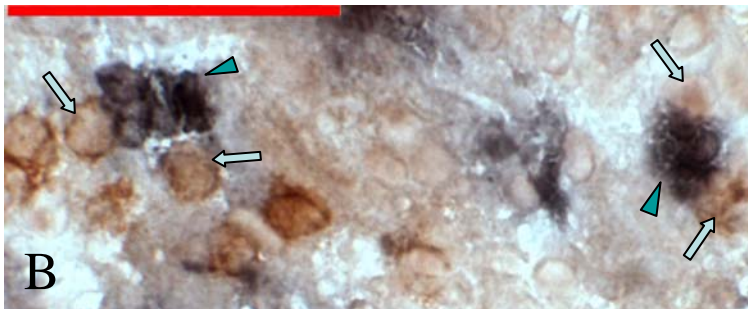
CD14

Figure 3. Double labeling of CD83+/CD4+ cells or CD14+/CD4+ cells in pulp beneath a deep carious lesion. The mature DCs were labeled with anti-CD83 (A, arrowhead) and monocytes/macrophages plus pulpal (immature) DCs were labled with CD14 (B, arrowhead). CD4+ cells (arrows) was localized near CD83+ cells or CD14+ cells sometimes in clusters. The bar size is 50 μ.

CD83-CD4

CD14-CD4

Discussion

Our results are the first to demonstrate immunohistochemically the presence of mature DCs (CD83+) in inflamed dental pulps beneath deep caries. Mature DCs are conventionally localized in draining lymph nodes. However, recent studies localized CD83+ DCs in chronically inflamed skin and periodontal tissue (13-15) and these mature DCs are thought to present antigens at the inflamed sites (16-18). The close proximity of CD83+ cells and CD4+ cells observed in our study further supports the concept that local antigen presentation can take place in inflamed pulp. The significance of local antigen presentation by mature DCs in peripheral tissues is not well-understood but mature DCs and activated T cells produce chemokines which are thought to help recruit effector leukocytes, including memory T cells, to the inflamed tissues (4, 15, 19-22). Thus, interaction of DCs and T cells in pulpitis likely promotes the production of chemokines and pro-inflammatory cytokines that will sustain local inflammation. A recent study reported that a subgroup of mouse pulpal DCs with elevated CD86, another mature DC marker, migrated to regional lymph nodes in response to injury (23). We reason that immature pulpal DCs mature upon caries insult and that some will migrate to the draining lymph nodes while others mature locally and engage T cells at the site of inflammation. Nevertheless, the number of CD209+ cells in deep caries was markedly elevated over other groups and the CD209+ cell number was about double that of mature CD83+ DCs in deep

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caries suggesting that a large store of immature DCs had accumulated in deep lesions and were available for terminal maturation.

The origin of these immature/mature DCs in inflamed pulps is yet to be determined. They could be derived from circulating immature DCs or monocytes as well as residential pulpal DCs. Large numbers of immature DCs can be generated from monocytes in vitro (24) and we recently demonstrated that *S. mutans* is a potent inducer of rapid DC maturation (25). We reason that circulating monocytes can extravasate into infected/inflamed pulpal tissues, where they are transformed into immature DCs upon carious antigen stimulation, and they mature to become CD83+ cells locally. Pulpal DCs may also go through a similar maturation process to become CD83+ cells. Further studies are warranted to identify the cellular sources of mature DCs in inflamed pulp. A significant increase of mature DC in tissues beneath deep caries can not only be caused by a rapid maturation of DCs locally but also can be due to prohibition of their migration. Inflammatory mediators, cytokines (e.g., IL-1 and TNF-α) and vasoactive intestinal peptide (VIP) in pulpitis promote DC maturation (26, 27). Neuropeptides such as calcitonin gene-related peptide (CGRP) and VIP can inhibit the migration of mature DCs to the regional lymph nodes (28).

Our immunohistochemical study revealed that small numbers of CD209+ and CD14+ cells were present in both normal and shallow caries groups. Dendritic-like cells labeled with CD14 and CD209 in normal and shallow caries groups correspond with the pulpal DCs reported by others (4, 12, 29). The lack of statistical difference of the counts of positively labeled cells between the normal and shallow caries group may be due to the low grade of

pulpal irritation beneath shallow caries that failed to elicit an obvious cellular response (30). A significant increase of CD209+ cells in irreversibly inflamed pulpal tissues beneath deep caries is consistent with previous studies demonstrating an increasing of HLA-DR+ pulpal DCs as well as FXIIIa+ pulpal DCs respectively with the advance of caries (4, 19). The inability to detect a significant difference in the number of CD209+ cells between shallow and deep caries likely relates to increased variability in the shallow caries group (Table 1). The increased variability suggests that DC numbers are likely increasing in some samples and it should be appreciated that CD209 is expressed on both immature and mature DCs. Furthermore, high expression of CD209 is associated with Th2 polarization (14, 31). An increased expression of CD209 in irreversibly inflamed pulpal tissues beneath deep caries corresponds with a mixed Th1/Th2 cellular profile change from Th1 with shallow caries. Interestingly, B cells and plasma cells are common in deep caries (1, 32) and this would be consistent with a Th2 profile.

The number of CD14+ cells (which would include monocytes, macrophages and CD14+ pulpal DCs) beneath deep caries was significantly increased compared with healthy and shallow caries groups. This result is in agreement with previous studies (1, 19). Although our data did not allow for comparison of counts between CD14+ and CD209+ cells within each group, Izumi et al. observed a higher number of macrophages than DCs in all stages of caries invasion (19). Interestingly, CD14+ cells were evenly scattered in and around the abscess area while CD83+ cells were localized outside the abscess. The localization of CD14+ cells near abscesses may reflect the scavenging activity of macrophages. Although

we do not have proof of antigen presentation by macrophages in this study, the close proximity of CD14+ cells and CD4+ T cells supports the notion.

In conclusion, we demonstrated the presence of mature DCs in severely inflamed pulp beneath deep caries. Close proximity of CD4+ cells and DCs as well as macrophages support the concept that both DCs and macrophages can function as APC in inflamed pulp. The immunological implications of mature DCs in chronically inflamed tissues may shed insight to the pathogenesis of pulpitis with future applications in vital pulp therapy.

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VITA

Melissa A. Harmon was born in 1976 in Chico, CA. She obtained a BA in Biology and her DDS degree from the University of Missouri in Kansas City in 2001. She completed an Advanced Education in General Dentistry program at Fort Lewis, WA in 2002. She served three years in the United States Army and currently resides in Rio Rancho, NM.