Phenotypic Analysis of Immunocompetent Cells in Healthy Human Dental Pulp

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Abstract

Introduction: Like other tissues in the body, the human dental pulp is equipped with a network of immune cells that can be mobilized against pathogens when they invade the tooth. Very little data, mostly obtained with classic histologic methods, have reported their quantities and relative percentages. The objective of this study was to characterize and precisely quantify immunocompetent cells in healthy human dental pulp by using fluorescence-activated cell sorting, together with identifying specific cell subsets in the leukocyte ($CD45^+$) cells. **Methods:** Healthy human third molars were collected from 42 young patients. Dental pulps were separated from the hard tissues and prepared for flow cytometry or immunostaining analyses. **Results:** $CD45^+$ cells represented 0.94% ± 0.65% of cells obtained from the enzymatic digestion of whole dental pulps (n = 34). $CD16^+CD14^+$ granulocytes/neutrophils (50.01% ± 9.08%, n = 7) were found to represent the major subpopulation in $CD45^+$ cells followed by $CD3^+$ T lymphocytes (32.58% ± 11%, n = 17), $CD14^+$ monocytes (8.93% ± 5.8%, n = 7), and HLA-DR^{high} $Lin1^-$ dendritic cells (4.51% ± 1.12%, n = 7). Minor subpopulations included $CD3^-CD56^+$ natural killer cells (2.63% ± 1.15%, n = 7) and $CD19^+$ B lymphocytes (1.65% ± 0.89%, n = 17). We further identified cells harboring a phenotype compatible with Foxp3/CD25-expressing regulatory T lymphocytes ($CD45^+CD3^+CD4^+CD127^{low}$). Fluorescence-activated cell sorting analysis and confocal microscopy also revealed expression of HO-1 in HLA-DR^+ cells. **Conclusions:** For the first time, this study identifies and precisely quantifies the relative proportion of immunocompetent cells potentially involved in tissue homeostasis of healthy human dental pulp. *(J Endod 2015;41:621–627)*

Key Words

Fluorescence-activated cell sorting, healthy dental pulp, immune cells, immunosurveillance

Human dental pulp is a highly dynamic tissue equipped with a network of resident immunocompetent cells that are believed to play a major role in the maintenance of tissue homeostasis. Injuries such as dental caries, trauma, operative procedures, and periodontal diseases are susceptible to break this balance. Dental caries result from a complex process in which the composition of the oral bacterial biofilm evolves as the penetration of microorganisms through the enamel, dentin, and pulp occurs (1). Bacteria and their toxins present in dentinal tubules trigger several important cellular and molecular changes within the pulpal tissues. Like any other injured tissue in the body, dental pulp is able to mount innate adaptive immune responses intended to fight infection (2, 3). During bacterial penetration through the dentin, inflammatory mediators can be released by acidic degradation of the carious dentin and produced by resident pulp cells such as odontoblasts, pulpal fibroblasts, stem cells, endothelial cells, and/or tissue-resident immune cells (1, 4). Because of their peripheral localization, odontoblasts are considered the first line of cell defense of the pulp tissue. They express a variety of pathogen recognition receptors and chemokines and have been proposed to trigger the initial inflammatory and immune pulp response in cooperation with antigen-presenting dendritic cells (DCs) (5, 6). DCs, macrophages, T lymphocytes, natural killer (NK) cells, and B lymphocytes have been identified in inflamed human dental pulp based on histologic analysis (7, 8).

Immune cells like DCs and T lymphocytes are also known to be resident in healthy pulp where they play a role in immunosurveillance. Immunosurveillance is a term used to describe the processes by which cells of the immune system look for and recognize foreign pathogens, such as bacteria and viruses, in the body. Immunosurveillance is well documented for skin, mucosa, lung, blood, and brain tissues. To our knowledge, precise characterization and quantification of immunocompetent cells in healthy human pulp have not been reported (2, 3). Fluorescence-activated cell sorting (FACS) is widely used to identify and characterize specific cell subsets in cellular suspensions obtained from tissues and is a great help in the determination of cell percentages in the tissues. Thus, FACS gives the opportunity to identify small cell populations with a set of different markers within the whole dental pulp tissue. Precise quantification, relative repartition among the leucocyte population, and detection of specific immunoregulatory cells will lead to a better understanding of the pulp initial capacity to mount efficient immune responses and regulate them. This knowledge is important to better

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0099-2399/$ - see front matter

http://dx.doi.org/10.1016/j.joen.2015.01.005

understand the physiopathology of the pulp during bacterial aggression. Using FACS analysis, our study aimed to characterize and precisely quantify immunocompetent cells and identify specific subsets among the leukocytes ($CD45^+$ cells) present in healthy human dental pulp. Furthermore, the detection of subsets involved in immunoregulation should lead to the discovery of targets for future therapeutic strategies.

Materials and Methods

Collection of Pulp Samples

Tissue samples were obtained from 42 healthy patients between the ages of 11 and 25 years who were elected to have extractions of healthy third molars under general anesthesia for orthodontic reasons. Teeth were collected with patients' (or the parents of patients) informed consent. The surgeries were performed at the Clinic of Stomatology of Nantes under approved guidelines. Impacted or embedded teeth that were cut during removal were excluded. Freshly extracted molars were taken directly from the surgical site and placed into sterile phosphate buffered saline (PBS) (pH = 7.4). Attached soft tissue was removed from the root surface by sterile scalpel blades. They were then cracked open, and the pulp tissue was gently removed with cotton pliers and placed in a Petri dish containing 1 mL PBS.

Tissue Preparation

Pulp tissues were minced into small pieces (0.5 mm^3) using sterile scalpel blades, transferred into a 50-mL sterile tube, and centrifuged at 1500 rpm for 10 minutes. The supernatant was removed, and the tissue pellet was digested in 5 mL collagenase D (Roche, Meylan, France) and then incubated at 37° for 45 minutes. The pulp tissue was completely dissociated in 30 to 45 minutes. EDTA (10 mmol/L) was added for the last 5 minutes to stop the enzymatic digestion. The cell suspension was washed twice with 50 mL sterile PBS, passed through a 100-μm nylon mesh filter, and resuspended in 2 mL PBS supplemented with 2 mmol/L EDTA and 2% fetal calf serum (FCS). We routinely obtained 1 to 7 million cells per patient, which were subsequently used for antibody staining.

Flow Cytometry

For cell surface staining, suspensions of single pulp cells were distributed into 96-well V-bottomed plates, washed, and incubated in PBS/2% FCS/0.2% azide. According to the manufacturer's recommendations, cells were incubated on ice for 30 minutes with anti-CD45 PE-Cy7 (clone HI-30, mouse immunoglobulin [Ig] G1) and monoclonal conjugated antibodies (mAbs [Table 1]) or isotype controls. The following combinations were used to identify pulp immunocompetent cells: B lymphocytes: CD45/CD19; T lymphocytes: CD45/CD3/CD4/CD8; NK cells: CD45/CD3/CD56; DCs: CD45/HLA-DR/Lin1; and monocytes, granulocytes, and macrophages: CD45/CD14/CD16. Single-cell suspensions were washed twice with PBS/0.2% FCS/0.1% azide before fixation with 2% paraformaldehyde and then maintained in the dark at 2°C–8°C until further use. Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of other patients by Ficoll-Hypaque density gradient centrifugation (Amersham, Les Ulis, France) in order to be used as positive controls. Flow cytometry analysis was performed by using the BD FACS Canto Flow Cytometer (Becton Dickinson, San Jose, CA). Results were analyzed using FlowJo analysis software (Tree Star, Ashland, OR). Primary gates based on physical parameters (forward and side light scatter) and fixable viability dye eFluor450 (eBioscience, Paris, France) were used in all experiments to exclude dead cells or debris.

For intracellular staining, cells were distributed into 96-well V-bottomed plates, washed, and incubated in PBS 0.5% saponin for 20 minutes on ice. After 3 washes in PBS 0.1% saponin, cells were incubated for 30 minutes on ice according to the manufacturer's recommendations with anti-CD45 PE-Cy7 (clone HI-30, mouse IgG1) and mAbs (Table 1) or isotype controls. The following mAb combinations were used: DCs–heme oxygenase-1 (HO-1): CD45/HLA-DR, regulatory T lymphocytes (Tregs) CD45/CD3/CD4/CD127/Foxp3, and CD45/CD3/CD127/CD4/CD25. After 2 washes with PBS/0.2% FCS/0.1% saponin and 1 wash with PBS/2% FCS/0.2% azide, cells were fixed with 2% paraformaldehyde and analyzed as previously described.

Immunohistology and Confocal Image Analysis

For immunohistology, third molar dental pulps from healthy donors were frozen and cryosectioned (10 μm). Tissue sections were fixed with acetone and saturated using PBS/4% bovine serum albumin/10% goat serum. Slides were then permeabilized with 0.5% saponin and stained with anti–HO-1 primary mAb (Stressgen, San Diego, CA). Slides were then incubated with biotinylated conjugated mAb diluted at 1/500. A second incubation was performed with streptavidin 568 conjugated biot mAb diluted 1/1000. Cells were also stained with anti-HLA-DR FITC mAb (BD Biosciences, Le Pont de Claix, France). Matching isotype control mouse antibodies were included. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Slides were mounted in ProLong AntiFade reagent (Molecular Probes, Saint-Aubin, France) and analyzed with a Leica confocal microscope (Leica Microsystems, Heidelberg, Germany) and Leica TCS NT software.

Results

For the first time, we propose a precise quantification of leukocytes within the healthy human dental pup. Cellular suspensions were obtained

TABLE 1. Monoclonal Antibodies Used in This Study

Antibodies	Fluorochrome	Isotype	Clone	Manufacturer
CD45	PE-Cy7	Mouse IgG1	HI-30	BD Biosciences
CD19	PE	Mouse IgG1	HIB19	BD Biosciences
CD3	APC-Cy7	Mouse IgG1	SK7	BD Biosciences
CD4	PerCP-Cy5.5	Mouse IgG1	L200	BD Biosciences
CD8	FITC	Mouse IgG1	HIT8a	BD Biosciences
HLA-DR	V500	Mouse IgG2a	G46-6	BD Biosciences
Lin1	FITC	Mouse IgG2b	NCAM16.2	BD Biosciences
CD14	PE	Mouse IgG2a	M5E2	BD Biosciences
CD16	FITC	Mouse IgG1	3G8	BD Biosciences
HO-1	FITC	Mouse IgG1	HO-1-1	Abcam, Cambridge, UK
CD127	PE	Mouse IgG1	HIL-7R-m21	BD Biosciences
CD25	FITC	Mouse IgG1	M-A251	BD Biosciences
Foxp3	APC	Rat Ig2a	PCH101	eBioscience

Ig, immunoglobulin.