M2 Phenotype Macrophages Colocalize with Schwann Cells in Human Dental Pulp

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Appendix

Materials and Methods

Schwann cell isolation

Magnetic activated cell separation (MACS) was performed for Schwann cell selection (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Briefly, cells were resuspended in separation buffer (PBS containing 0.5% bovine serum albumin and 2 mM EDTA), incubated for 20 min at 4°C with MicroBeads conjugated to a monoclonal anti-p75 nerve growth factor receptor (NGFR) antibody (Miltenyi Biotec) and blocking reagent, washed with separation buffer, and then centrifuged at 300 × *g* for 5 min at 4°C. Cells were resuspended in separation buffer and applied to a MACS Column (MS type; Miltenyi Biotec) placed in the MiniMACS Separator (Miltenyi Biotec). The column was washed three times, and the retained magnetically labeled cells were flushed out with separation buffer as the NGFR-positive fraction.

Flow cytometric analysis

We performed flow cytometric analysis of healthy human samples isolated from three individuals. The healthy pulp tissue was removed, minced into small pieces (1 mm), and then incubated in Dulbecco's modified Eagle's medium containing 2 mg/ml collagenase and 5 mg/ml dispase at 37°C in a humidified atmosphere with 5% CO_2 for 80 min. The pulp tissue was completely dissociated, and

the cell suspension was wash twice with PBS and then passed through a 70-μm nylon mesh filter. We obtained 1–1.5 × 10^6 cells per individual. Isolated single cells were fixed with 4% paraformaldehyde in cold PBS, followed by washing twice with FACS buffer (2% FBS/PBS), and then blocking Fc receptors using an anti-CD16/32 antibody (Invitrogen, CA, USA). After washing, the sample was incubated with fluorescently labelled antibodies against CD68, CD86, and CD163 as listed in Appendix Table 2. Isotype control antibodies were purchased from eBiosceience (CA, USA). The labeled cells were washed twice to remove unbound antibodies and analyzed by flow cytometry (NovoCyte Flow Cytometer; ACEA Biosciences, Inc., CA, USA).

Immunofluorescence and immunohistochemistry

Immunostaining was performed as previously described (Yoshiba et al. 2018) using the primary antibodies listed in Appendix Table 2. The secondary antibodies were horseradish peroxidase (HRP)-labeled swine anti-rabbit IgG (Dako), HRP-labeled rabbit anti-mouse IgG (Dako), Alexa Fluor 488 goat anti-rabbit IgG, and Alexa Fluor 546 goat anti-mouse IgG (Invitrogen).

Quantitative RT-PCR

Total RNA was isolated from cocultures or THP-1 macrophages cultured alone for 7 days using an RNeasy Plus Micro kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Quantitative RT-PCR was carried out using a One Step SYBR PrimeScript PLUS RT-PCR kit (Takara Bio, Shiga, Japan) with the Opticon Real-Time PCR System (MJ Research, MA). The primer sets are listed in Appendix Table 3. Data were presented as the mean ± SEM (n = 3). Statistical significance between two groups was evaluated by the Student's t-test and considered significant at $P < 0.05$.

Appendix Table 1. Carious teeth examined in the present study

No. (images)	Lesion activity	Depth of cavity	Clinical symptoms
1	slowly progressing	I	-
2	slowly progressing	I	-
3	slowly progressing	II	-
4	slowly progressing	II	-
5	slowly progressing	III	-
6 (Fig. 3C)	slowly progressing	III	-
7 (Fig. 3A)	active	I	-
8	active	I	-
9	active	I	-
10	active	II	-
11	active	II	lingering sensitivity to air blowing / no episodes of spontaneous pain
12	active	III	faint sensitivity to air blowing / no episodes of spontaneous pain
13 (Fig. 3B)	active	III	faint sensitivity to air blowing / no episodes of spontaneous pain
14 (Fig. 3D)	active	III	high sensitivity to air blowing / episodes of spontaneous pain

Lesion activity: active, yellowish discolored dentin; slowly progressing, brownish discolored dentin

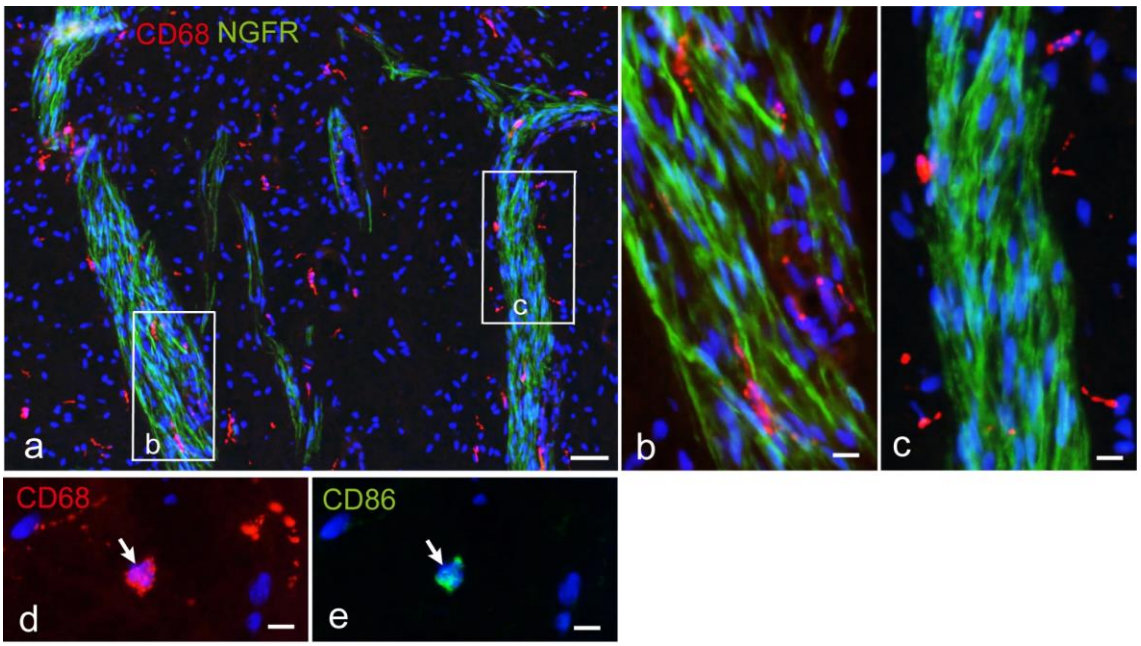
Depth of cavity: grade I, less than one-third of the dentin; grade II, from one-to two-thirds; grade III, more than two-thirds

Appendix Table 2. Antibodies used in the present study

Antibody	Isotype	Clone	Manufacturer	Fluorochrome
CD68	Mouse IgG1	KP1	Dako	
CD86	Rabbit IgG	Polyclonal	Bioss	
CD163	Mouse IgG1	10D6	Leica Biosystems	
CD163	Mouse IgG1	EDHu-1	Serotec	
CD138	Mouse IgG1	MI15	Dako	
HLA-class II	Mouse IgG1	CR3/43	Dako	
NGFR	Rabbit IgG	EP1039Y	abcam	
NGFR	Mouse IgG1	REA648	Miltenyi Biotec	PE
S100	Rabbit IgG	Polyclonal	Dako	
S100	Mouse IgG2a	4B3	abcam	
MBP	Rabbit IgG	Polyclonal	proteintech	
BDNF	Rabbit IgG	EPR1292	abcam	
CD68	Mouse IgG2a	Y1/82A	BioLegend	FITC (FACS)
CD86	Mouse IgG1	BU63	BioLegend	APC (FACS)
CD163	Mouse IgG1	GHI/61	BioLegend	PE (FACS)

Appendix Table 3. Primer sequences for quantitative RT-PCR analysis

Gene	*Primer*
CD68	sense: 5′-GCTACATGGCGGTGGAGTACAA-3′
	antisense: 5′-ATGATGAGAGGCAGCAAGATGG-3′
CD163	sense: 5′-CCAGTCCCAAACACTGTCCT-3′
	antisense: 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′
β -actin	sense: 5′-GATTCCTATGTGGGCGACGA-3′
	antisense: 5′-GTGGTGCCAGATTTTCTCCA-3′



Appendix Figure. Double immunofluorescence staining of CD68 (pan-macrophage marker) and nerve growth factor receptor (NGFR) (a–c) or CD86 (M1 phenotype marker) (d, e) in healthy dental pulp. (b) and (c) are closer views of the boxed area in (a). White arrows in (d, e) indicate M1 phenotype macrophage. Nuclei were counterstained with DAPI (blue). Scale bars: 50 μm (a), 10 μm (b–e).