Akt2 Affects Periodontal Inflammation via Altering the M1/M2 Ratio

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Appendix Materials & Methods:

Hematoxylin and Eosin (HE) Staining and Immunological Staining

The specimens were cut into 5-μm sections for histologic and immunostaining analysis. H&E staining was processed according to the instruction of H&E Staining Kit (G1120, Solarbio, China). Immunostaining was processed to observe inflammatory cell infiltration and macrophage polarization. Briefly, sections were rehydrated with graded ethanol series, and incubated with antigen retrieval solution (C1034, Solarbio, China) for 15 min at 95°C. To block the activity of endogenous peroxidase, sections were incubated with 3% hydrogen peroxide for 10 min at room temperature, and incubated with primary antibodies. The dilution of primary antibodies was 1:100. Appropriate secondary antibodies (ZSGB-Bio, China) were applied subsequently. After the production of brown precipitation with DAB detection kit (Sigma, USA), sections were counterstained with hematoxylin. After incubation with appropriate secondary antibodies, images were collected with microscope (Nikon, Japan) and analysis with Image Pro Plus 6.0 software. M1 and M2 macrophage were numerated by counting the number of positively stained cells. For immunofluorescence staining, appropriate secondary antibodies were applied for 1 h at room temperature and counterstained with 4,6-diamino-2-phenylindole (DAPI). Images were collected with laser scanning confocal microscope (LSCM, Zeiss, Germany).

Cell Culture, Infection/Transfection and Polarization of Macrophage

RAW 264.7 cells were cultured with high glucose Dulbecco's modified eagle medium (DMEM, Hyclone, USA), containing 10% fetal bovine serum (FBS, Biological Industries, Israel), and 50 U/mL penicillin and 50 μg/mL streptomycin (Hyclone, USA). Cells passages from 3 to 5 were used for experimental investigation.

For knocking down of Akt2, small interfering RNA (siRNA) specifically targeting to Akt2 were designed, and siNC was used as control. The transfection experiments were processed according to the protocol of the transfection reagent (PT-114-15, jetPrime, France).

For over-expression of Akt2, lentivirus carrying mouse *Akt2* expression cassette was generated and infected into RAW 264.7 cells. Over-expression *Akt2* RAW 264.7 cell line was screened with puromycin and termed as OE-Akt2. An empty lentivirus was applied as control.

The knockdown and over-expression efficiency were investigated via quantitative real-time polymerase chain reaction (qRT-PCR), immunofluorescence and western blot, and the specificities were investigated via qRT-PCR and western blot.

For M1 polarization, cells were treated with 1 μg/mL lipopolysaccharide (LPS, Sigma, USA) for 12 h before assays. For M2 polarization, cells were treated with 50 ng/mL IL-4 and 50 ng/mL IL-13 for 12 h.

The primers, mimics, and inhibitors of miR-155-3p, miR-155-5p, and mimics and inhibitors of the negative control, were purchased from Genecopoeis Inc (Guangzhou, China). RAW 264.7 cells were transfected with mimics or inhibitors according to the protocol of the transfection reagent (PT-114-15, jetPrime, France).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated as described previously (Wu et al. 2018), and the concentrations were measured with a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, USA). qRT-PCR was performed in a 7500 Real-Time PCR System (Thermo Fisher Scientific, USA) using SYBR Green reagent(Roche, Diagnostic, USA). Pro-inflammatory genes associated with M1 phenotype macrophage, including IL-1β, TNF-α, IL-6, and iNOS were detected. Arg-1 was selected as a marker for the M2 phenotype macrophage.

Western Blot

The protocol of protein separation and western blot were according to the previously published work (Wu et al. 2018). The expressions of associated proteins were visualized with enhanced chemiluminescence reagent (SW2020, Solarbio, China). The expression of total(*t*)-P38 (Affinity BioScience, USA), phosphorylated(*p*)-P38 (Affinity BioScience, USA), total(*t*)-Erk1/2 (Affinity BioScience, USA), phosphorylated(*p*)-Erk1/2 (Affinity BioScience, USA), total(*t*)-JNK1/2 (Affinity BioScience, USA), phosphorylated(*p*)-JNK1/2 (Affinity BioScience, USA), total(*t*)-Akt2 (Affinity BioScience, USA) , phosphorylated(*p*)-Akt2 (Affinity BioScience, USA), and Arginase-1 (Arg-1; Santa Cruz, USA), total(*t*)-c-Jun (CST, USA), phosphorylated(*p*)-c-Jun (CST, USA), DET1 (Zenbio, China) were detected, and GAPDH (Affinity BioScience, USA) served as the endogenous control.

CCT128930 (an Akt2 inhibitor) and JNK-IN-7 (a JNK1/2 inhibitor) were selected for rescue studies.

Flow Cytometry and Immunofluorescence

For flow cytometry assays, CD86 (Cat:105011, BioLegend, USA) and CD163 (Cat: 155307, BioLegend, USA) antibodies were used to characterize the M1 and M2 polarization of macrophage, respectively. Meanwhile, APC Rat IgG2a, κ isotype (BioLegend, USA) and PE Rat IgG2a, κ (BioLegend, USA) were used as isotype control to analyze the polarization of macrophages. Labeling was quantified with a BD Accuri C6 Flow Cytometer (USA).

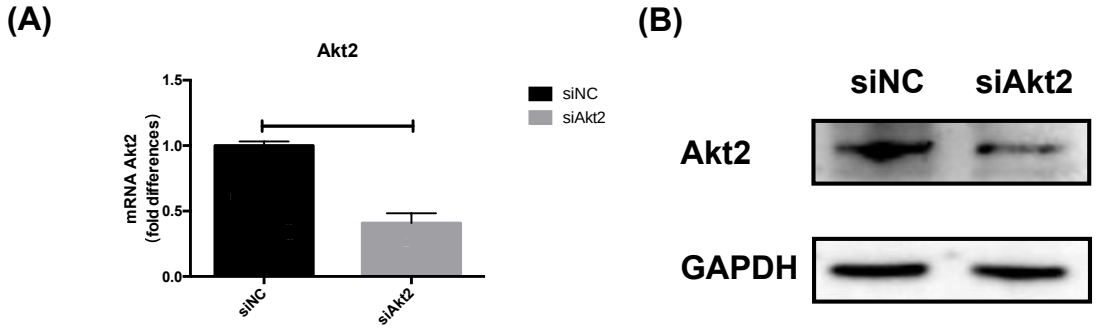
For immunofluorescence assays, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 5% bovine serum albumin (BSA, Sigma, USA) for 1 h. Cells were incubated with anti-CD86 primary antibody for the characterization of M1 phenotype macrophages, anti-CD163 (Abcam, UK) antibody for the characterization of M2 phenotype macrophage at 4°C overnight. After incubating cells with secondary antibodies for 1 h at room temperature and counterstained with 4,6-diamino-2-phenylindole (DAPI), images were collected with laser scanning confocal microscope (LSCM, Zeiss, Germany). The dilution of CD86 and CD163 antibodies for immunofluorescence were 1:200.

Micro-CT Examination

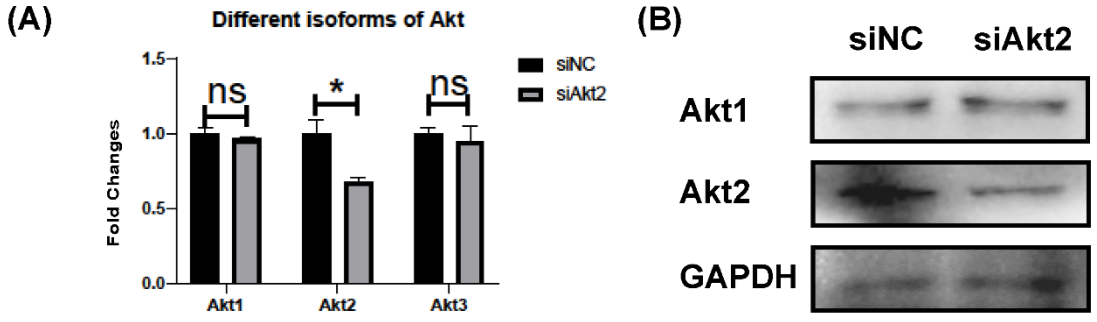
The fixed mice maxillae were scanned with an animal micro-CT scanner (SCANCO MEDICAL, Switzerland). 3D reconstruction and X-Ray were performed to visualize the absorption of alveolar bones. Bone volume to tissue volume ratio (BV/TV) was calculated to quantitatively analyze the bone loss after ligation.

Appendix Table 1: Primer pairs used in qRT-PCR analysis

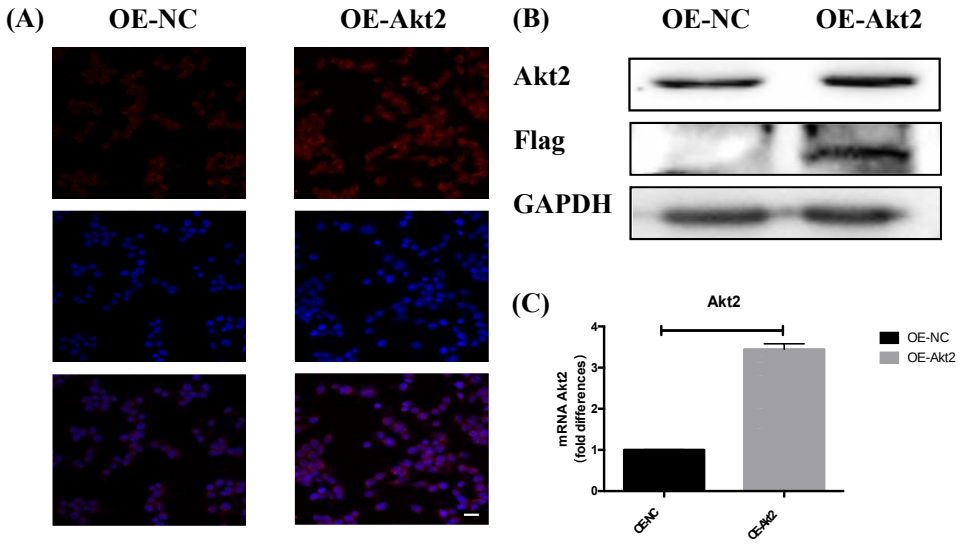
Gene	Forward primer (5'-3')	Reverse primer (5'-3')
IL-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
TNF-α	CCTGTAGCCCACGTCGTAG	GGGAGTAGACAAGGTACAACCC
iNOS	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
Arg-1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
Akt2	GAGGTCCCAGTGATGCGAAG	TCATATCGGTCTGGGGGTGT
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA



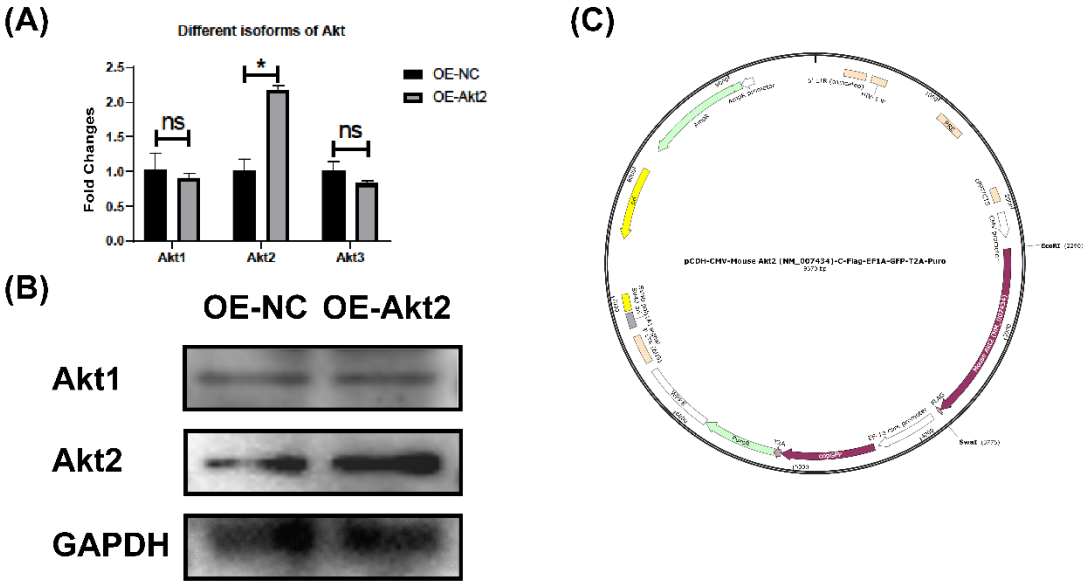
Appendix Fig. 1: Akt2 knock-down efficiency was examined via qRT-PCR (A) and western blot (B). $p<0.05$ represents a significant difference between the indicated groups.



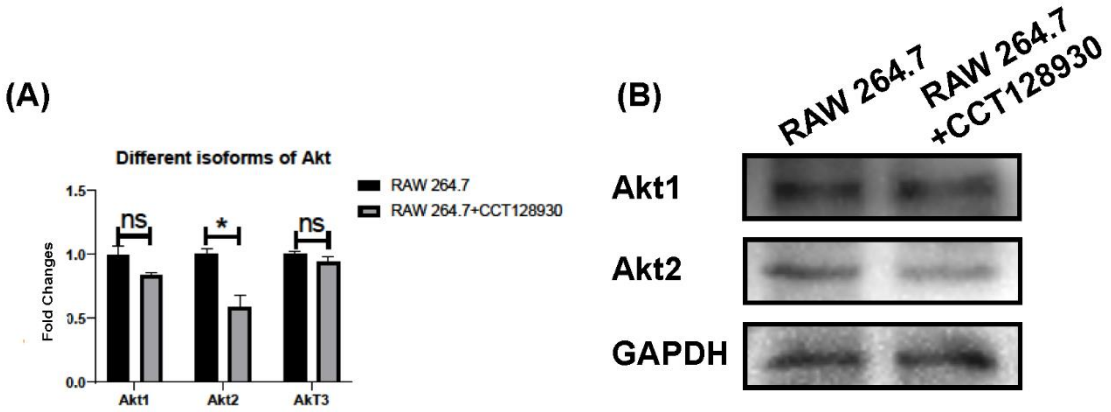
Appendix Fig. 2: The specificity of siAkt2 was examined via qRT-PCR (A) and western blot (B). $p<0.05$ represents a significant difference between the indicated groups.



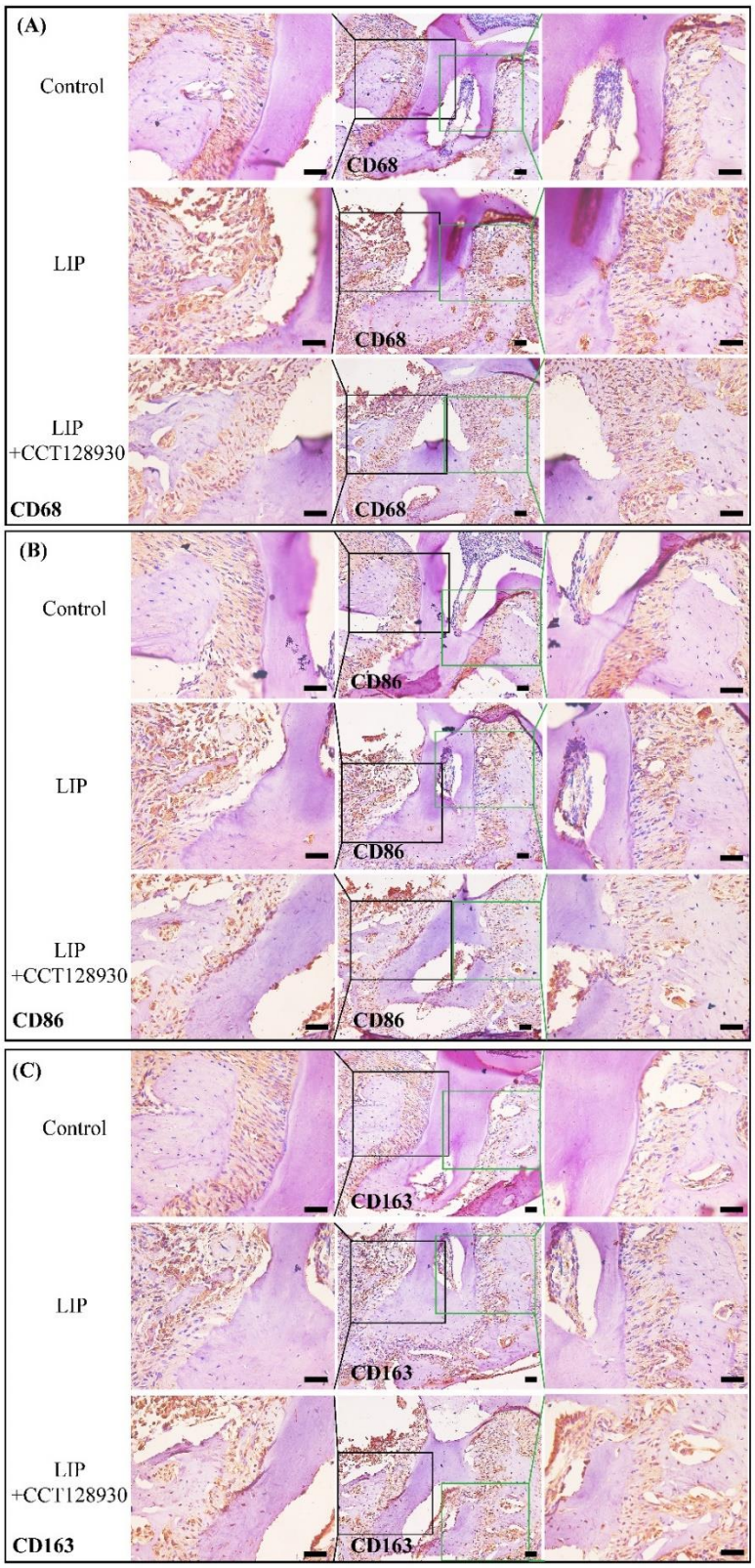
Appendix Fig. 3: Akt2 over-expression efficiency was examined via immunofluorescence (A), western blot (B) and qRT-PCR (C). $p<0.05$ represents significant difference between the indicated groups. Scale bar = 20 μm.



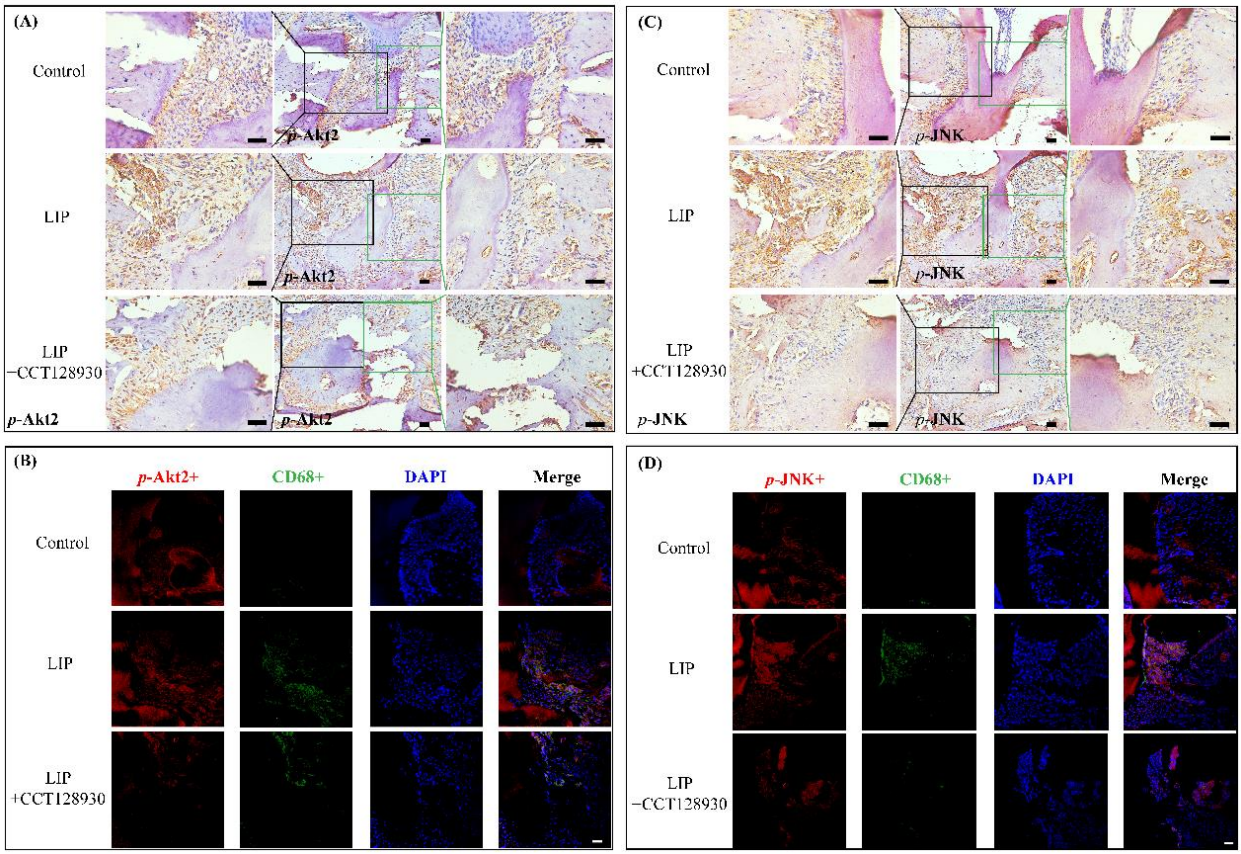
Appendix Fig. 4: The specificity of Akt2 over-expression was examined via qRT-PCR (A) and western blot (B). $p<0.05$ represents significant difference between the indicated groups. (C) The map of the vector containing the *Akt2* expression cassette.



Appendix Fig. 5: The specificity of CCT128930 was examined via qRT-PCR (A) and western blot (B). $p<0.05$ represents significant difference between the indicated groups.



Appendix Fig. 6: Immunohistochemistry staining of CD68 (A), CD86 (B), and CD 163 (C) in control, LIP and LIP+CCT128930 groups. The scale bar is 100 μm.



Appendix Fig. 7: Immunohistochemistry staining of *p*-Akt2 (A) and *p*-JNK (C) in control, LIP and LIP+CCT128930 groups. The scale bar is 100 μm. Immunofluorescence double staining of *p*-Akt2+/CD68+ (B) and *p*-JNK+/CD68+ (D) in control, LIP, and LIP+CCT128930 groups. The scale bar is 50 μm and the magnification of B and D is 200 ×.