Supplemental Figures

Title: Dysregulation of CXCL1 expression and neutrophil recruitment in insulin resistance and diabetes-related periodontitis in male mice

Running title: Insulin receptors and resistance in periodontitis

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Supplemental Table 1. Antibodies

Product name	Company	Cat No	condition
IRβ	Cell Signaling Technology (Beverly, MA)	#3025	WB 1:1000
IGF-1Rβ	Cell Signaling Technology	#9750	WB 1:1000
phospho(p)-IGF1R/p-IRβ	Cell Signaling Technology	#3021	WB 1:1000
p-Akt	Cell Signaling Technology	#4060	WB 1:1000
total (t)-Akt	Cell Signaling Technology	#4691	WB 1:1000
t-Erk	Cell Signaling Technology	#4695	WB 1:1000
Vimentin	Cell Signaling Technology	#5741	WB 1:1000 ICC/IF: 1:100
TLR2	Cell Signaling Technology	#13744	WB 1:1000
p-TAK1	Cell Signaling Technology	#9339	WB 1:1000
t-TAK1	Cell Signaling Technology	#5206	WB 1:1000
IκBα	Cell Signaling Technology	#4812	WB 1:1000
p-p65	Cell Signaling Technology	#3033	WB 1:1000
p65	Cell Signaling Technology	#8242	WB 1:1000
p-JNK	Cell Signaling Technology	#4668	WB 1:1000
JNK	Cell Signaling Technology	#9252	WB 1:1000
anti-rabbit IgG horseradish peroxidase (HRP)-conjugated	Cell Signaling Technology	#7074	WB 1:1000
actin HRP-conjugated	Santa Cruz Biotechnology (Santa Cruz, CA)	sc-47778	WB 1:100000
ICAM1	Santa Cruz Biotechnology	sc-8439	WB 1:200
TLR4	Santa Cruz Biotechnology	sc-293072	WB 1:200
mouse IgG HRP-conjugated	Santa Cruz Biotechnology	sc-516102	WB 1:1000
goat IgG HRP-conjugated	Santa Cruz Biotechnology	sc-2354	WB 1:1000
VCAM1	Abcam (Cambridge, MA)	ab134047	WB 1:2000
SM22α	Abcam	ab10135	WB 1:2000 ICC/IF: 1:100
wide spectrum Cytokeratin	Abcam	ab9377	WB 1:2000 ICC/IF: 1:200

NG2	Abcam	ab129051	WB 1:2000 ICC/IF: 1:180
αSMA	Abcam	ab5694	WB 1:2000 ICC/IF: 1:100
Donkey anti-rabbit IgG conjugated with Alexa488	Abcam	ab150073	ICC/IF: 1:250
Donkey anti-goat IgG conjugated with Alexa Fluor 594	Jackson ImmunoResearch Laboratories (West Grove, PA)	705-586-147	ICC/IF: 1:250
mouse CD16/32	BioLegend (San Diego, CA)	101302	FACS: 1:100
Brilliant Violet (BV) 421 mouse F4/80	BioLegend	123131	FACS: 1:100
BV 510 mouse Ly6G/Ly-6C (Gr-1)	BioLegend	108437	FACS: 1:100
BV 711 mouse CD11c	BioLegend	117349	FACS: 1:100
BV 785 mouse CD3e	BioLegend	100355	FACS: 1:100
APC/Cy7 mouse CD19	BioLegend	115529	FACS: 1:100
PE/Cy5 mouse CD45	BioLegend	103110	FACS: 1:100
PE/Cy7 mouse/human CD11b	BioLegend	101216	FACS: 1:100
APC mouse I-Ab	BioLegend	116417	FACS: 1:100

Supplemental Table.2 Reagents

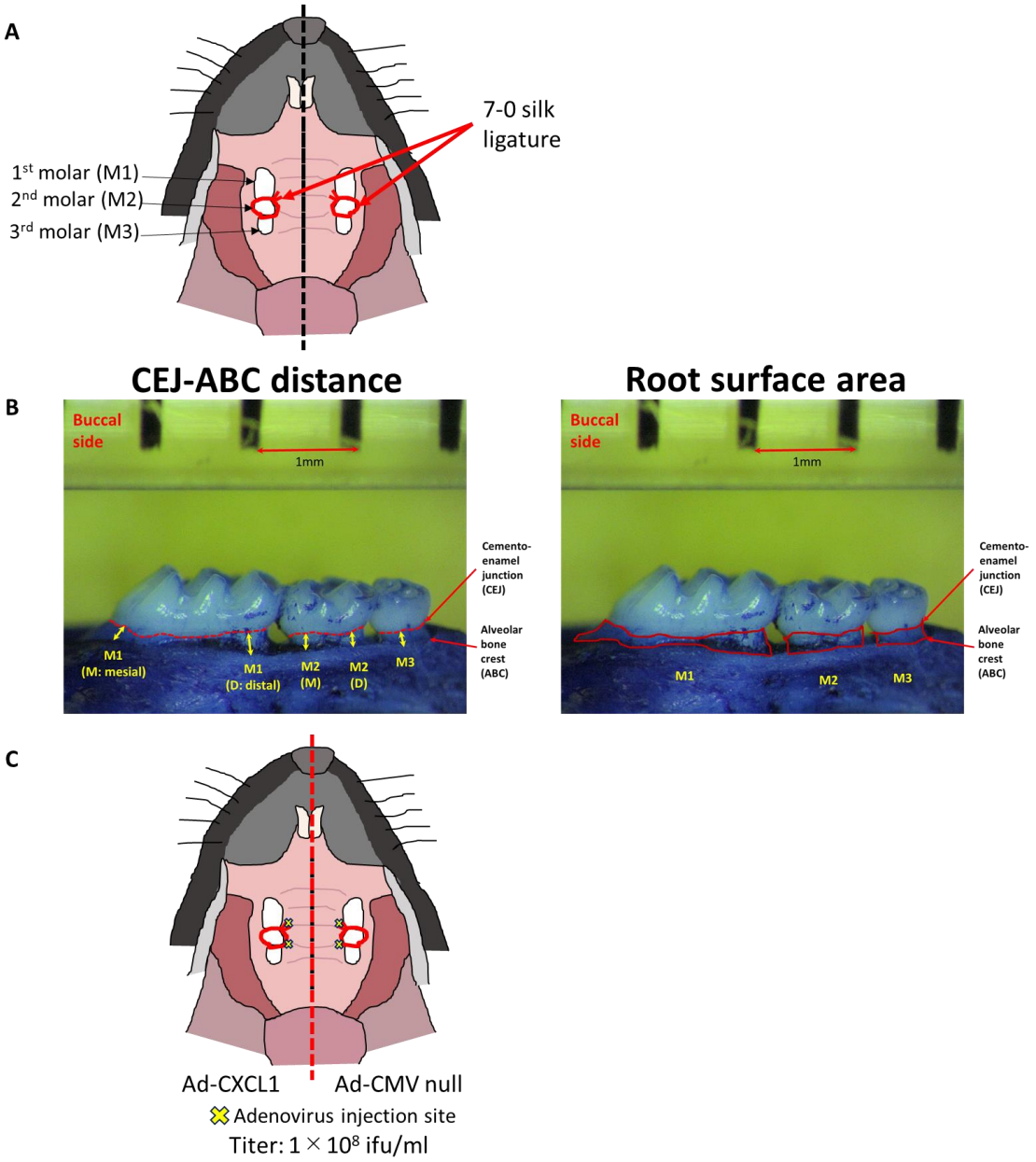
Product Name	Company	Cat No	Condition
Insulin from bovine pancreas	Sigma-Aldrich (St. Louis, MO)	I1882	1-100nM
E.coli LPS	Sigma-Aldrich	L2880	10-1000ng/ml
TRAP stain kit	Sigma-Aldrich	387A	
recombinant mouse IGF-1	R&D Systems (Minneapolis, MN)	791MG050	1-100nM
recombinant mouse TNFα	R&D Systems	410MT010	10ng/ml
Mouse CXCL1 ELISA kit	R&D Systems	MKC00B	
Human CXCL1 ELISA kit	R&D Systems	DGR00B	
Mouse IGF-1 ELISA kit	R&D Systems	MG100	
Pam3CSK4	InvivoGen (San Diego, CA)	tlrl-pms	10 or 100ng/ml
Flagellin from *Salmonella typhimurium*	Sigma-Aldrich	SRP8029	10ng/ml
ODN 1826 control (ODN 2138) Endotoxin-free (sterile)	Adipogen Life Sciences	IAX-200-201-C100	1μM
ODN 1826 Class B CpG oligonucleotide	InvivoGen	tlrl-1826	1μM
Zombie Green Fixable Viability Kit	Biolegend	423112	FACS: 1:800
Mouse insulin ELISA kit	Crystal Chem (Elk Grove Village, IL)	90080	
Mouse IGF-1 ELISA kit	Crystal Chem	80574	
BAY 11-7082	Selleckchem (Houston, TX)	S2913	5μM
SP600125	Selleckchem	S1460	10μM
wortmannin	Selleckchem	S2758	10-1000nM

PD98059	Cell Signaling Technology	9900	1-20μM
puromycin	InvivoGen	ant-pr-1	1mg/ml

Supplemental Table.3 Primers

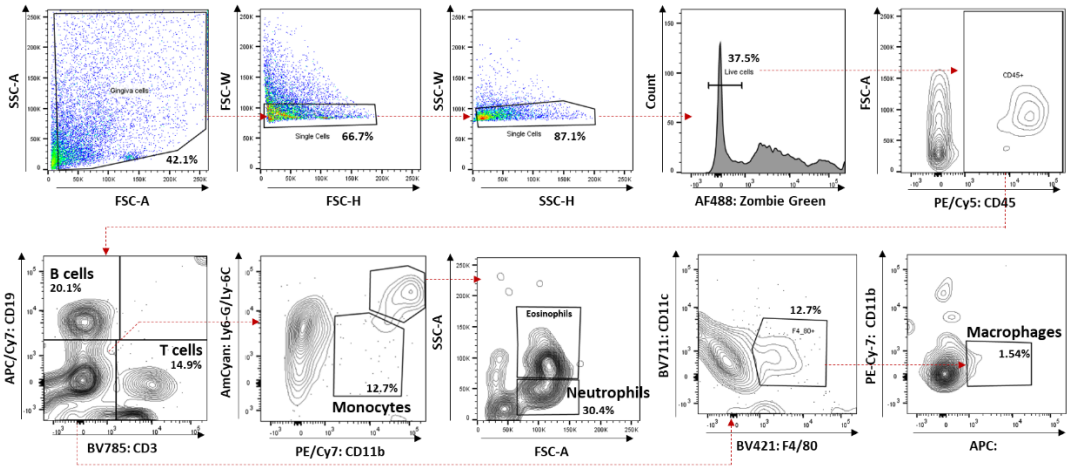
Gene name	Forward	Reverse
Eubacterial 16S	ACTCCTACGGGAGGCAGCAGT	ATTACCGCGGCTGCTGGC
mCxcl1	TGCACCCAAACCGAAGTCAT	TTGTCAGAAGCCAGCGTTCAC
mCxcl2	CCAGACAGAAGTCATAGCCA	TTAGCCTTGCCTTTGTTCAG
mMcp1	GTCCCTGTCATGCTTCTGG	GCTCTCCAGCCTACTCATTG
mTnfα	GCCACCACGCTCTTCTGTCT	GTCTGGGCCATAGAACTGAT
mIL-1β	TCCCGTGGACCTTCCAGGATGAG	TCGGAGCCTGTAGTGCAGTTGTC
mIL-17a	AACACTGAGGCCAAGGACTT	ACCCACCAGCATCTTCTCG
mRankl	TGTACTTTCGAGCGCAGATG	AGGCTTGTTTCATCCTCCTG
mOpg	AGCAGGAGTGCAACCGCACC	TTCCAGCTTGCACCACGCCG
mCxcl15	TGTTCACAGGTGACTGCTCC	AGCCCATAGTGGAGTGGGAT
mMPO	TTTGACAGCCTGCACGATGA	GTCCCCTGCCAGAAAACAAG
mCD11b	ATGGACGCTGATGGCAATACC	TCCCCATTCACGTCTCCCA
m18S rRNA	GCTTAATTTGACTCAACACGGGA	AGCTATCAATCTGTCAATCCTGTC
hCXCL1	GCGCCCAAACCGAAGTCATA	ATGGGGGATGCAGGATTGAG
h18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Supplemental Fig.1 (related to Methods)



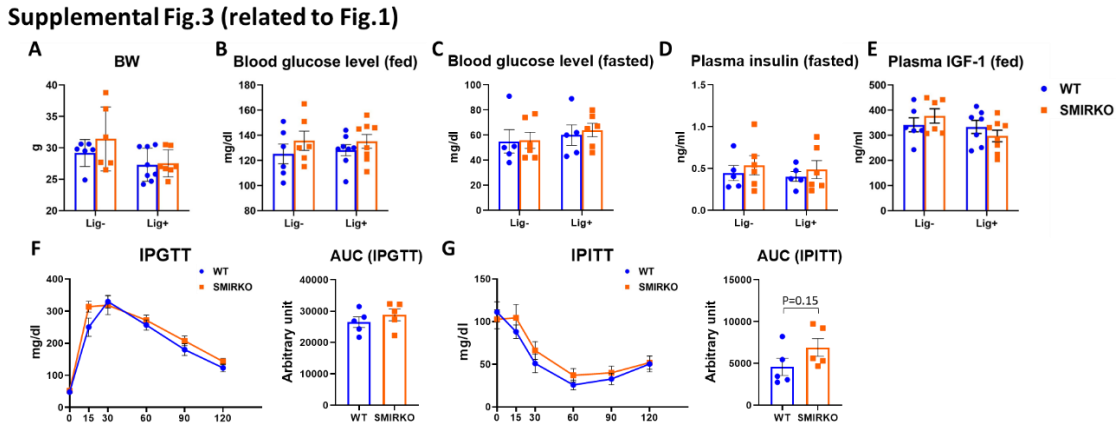
Supplemental Fig.1 Induction of periodontitis, assessment of bone loss and intragingival adenovirus injection in mice. A: A schema of silk-ligation around maxilla 2nd molar in mice. B: Measurement method of alveolar bone loss as previously reported (21). C: A schema of intragingival adenorvirus injection in mice.

Supplemental Fig.2 (related to Methods)



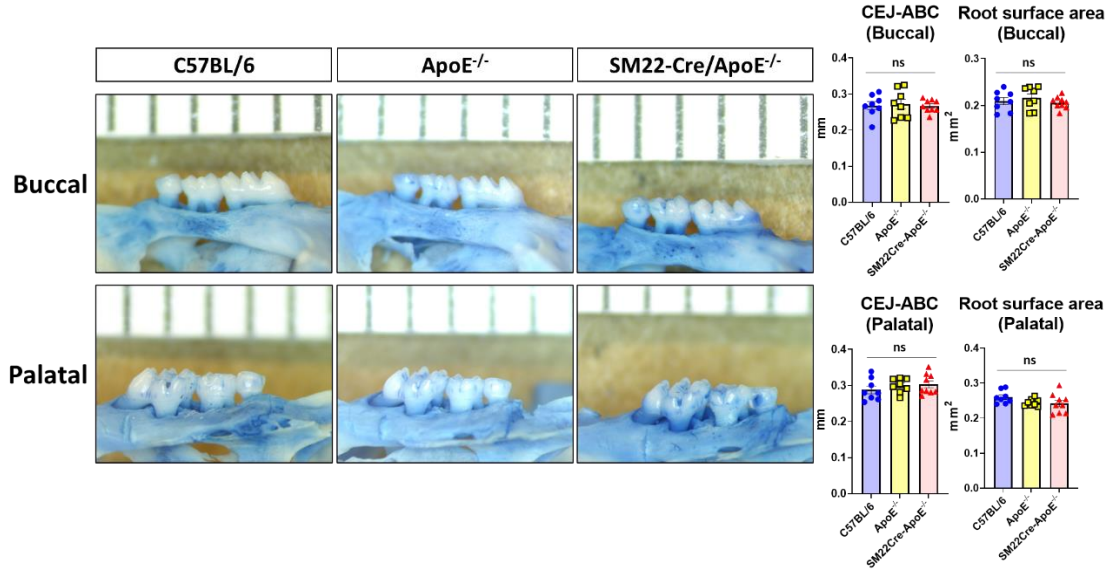
Supplemental Fig.2 A FACS gating strategy.

Gingival cells were used in the figure as an example.



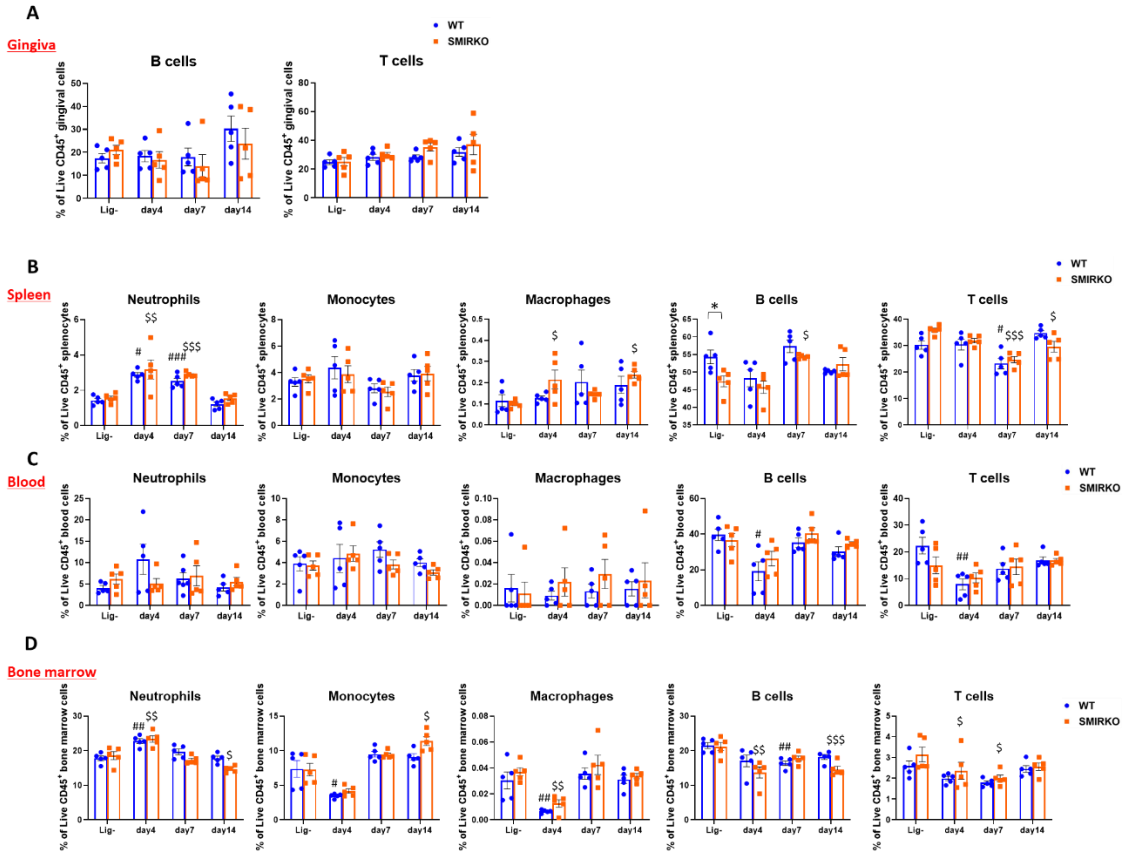
Supplemental Fig.3 Basal characteristics of WT and SMIRKO mice. A: Body weight (N=6-8), B: Blood glucose levels in fed state (N=6-8), C: Blood glucose levels in fasted state (N=5-6), D: Plasma insulin concentration in fasted state (N=5-6), E: Plasma IGF-1 levels in fed state (N=6-8), F: IPGTT (N=5) and G: IPITT (N=5) in WT and SMIRKO mice. Blue circle: WT, orange square: SMIRKO.

Supplemental Fig.4 (related to Fig.2)



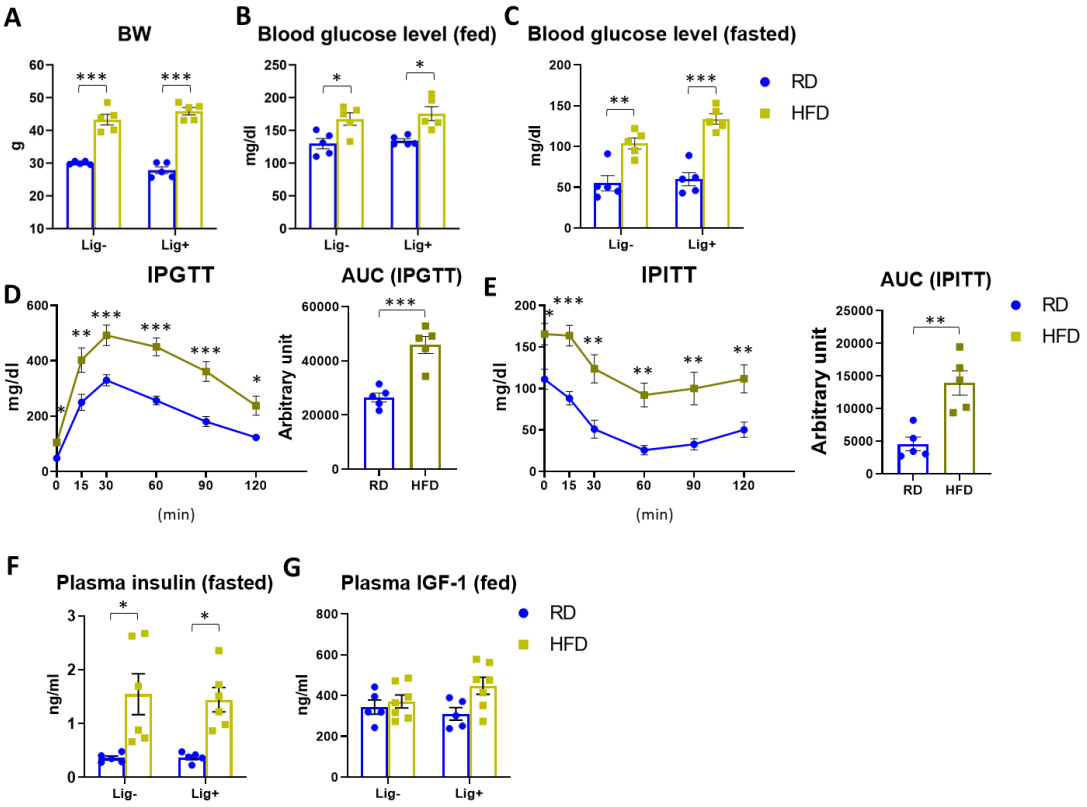
Supplemental Fig.4 Confirmation of Cre recombinase overexpression on the progression of experimental periodontitis in mice. C57BL/6 (N=7), ApoE-/- (N=8) and SM22Cre-ApoE-/- (N=8) mice were ligatured at 14 weeks old and sacrificed 2 weeks later. The bone loss assessment was performed. ns: no significance.

Supplemental Fig.5 (related to Fig.2)



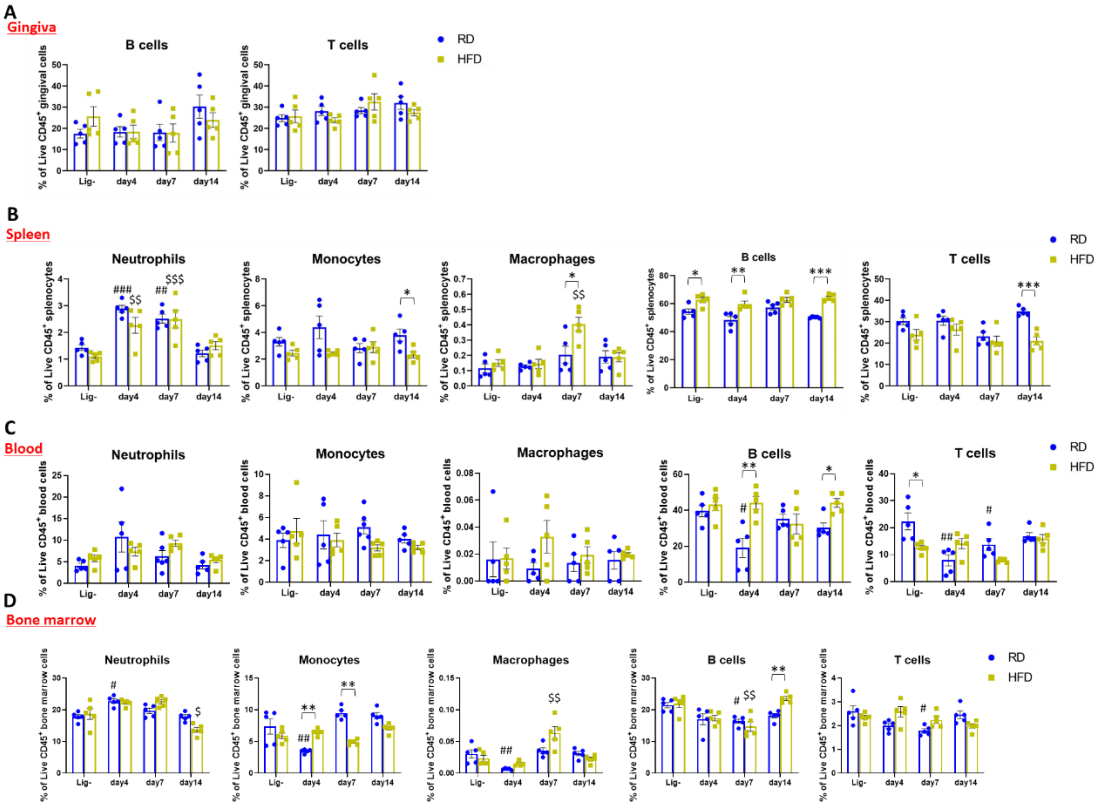
Supplemental Fig.5 FACS analyses in gingiva, spleen, blood and bone marrow of WT and SMIRKO mice. A: B and T cell populations in the gingiva (N=5), B: immune cell populations in the spleen (N=5), C: blood (N=5) and D; bone marrow of WT and SMIRKO mice after ligation (N=5). #p<0.05, ###<0.001 vs WT without ligature. $p<0.05, $$p<0.01, $$$p<0.001 vs SMIRKO without ligature. Blue circle: WT, orange square: SMIRKO.

Supplemental Fig.6 (related to Fig.3)



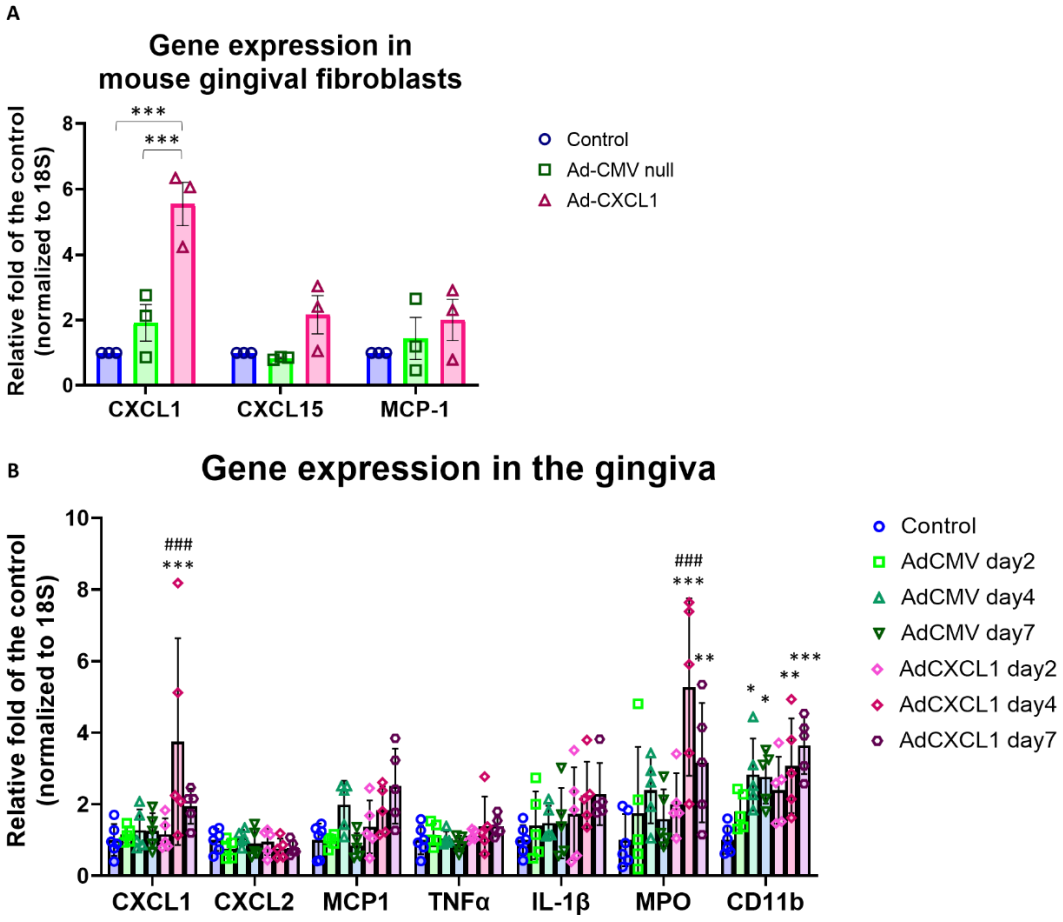
Supplemental Fig.6 Physiological parameters of RD and HFD-fed mice. A: Body weight (N=5), B: blood glucose levels in fed state (N=5), and C: in fasted state (N=5). D: IPGTT (N=5), E: IPITT (N=5). F: Plasma insulin concentrations in fasted state (N=5-6) and G: plasma IGF-1 levels in fed state (N=5-7). *p<0.05, **p<0.01, ***p<0.001. Blue circle: RD-fed, yellow square: HFD-fed.

Supplemental Fig.7 (related to Fig.3)



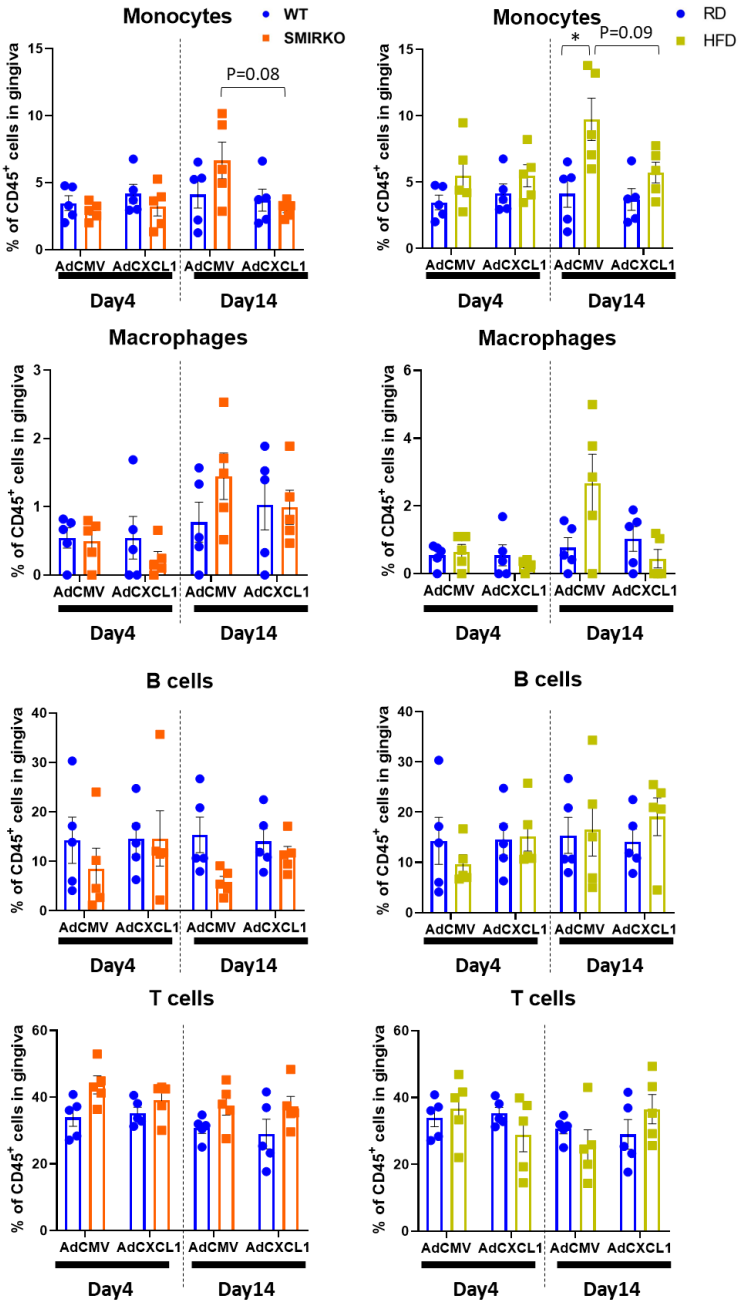
Supplemental Fig.7 FACS analyses in gingiva, spleen, blood and bone marrow of RD and HFD-fed mice. A: B and T cell populations in the gingiva (N=5), B: immune cell populations in the spleen (N=5), C: blood (N=5) and D; bone marrow of RD and HFD-fed mice after ligation (N=5). *p<0.05, **p<0.01, ***p<0.001. #p<0.05, ##p<0.01, ###<0.001 vs RD-fed without ligature. $p<0.05, $$p<0.01, $$$p<0.001 vs HFD-fed without ligature. Blue circle: RD-fed, yellow square: HFD-fed.

Supplemental Fig.8 (related to Fig.4)



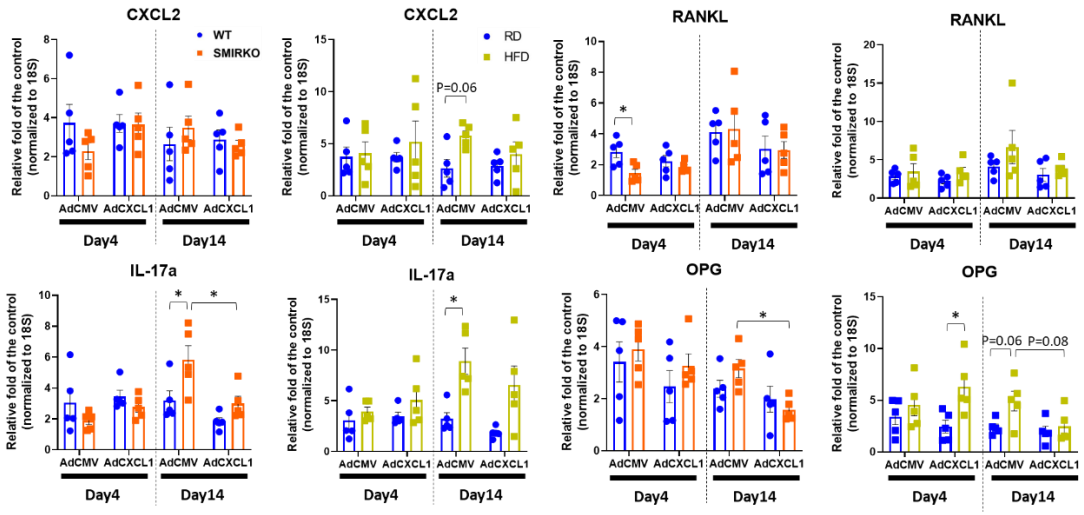
Supplemental Fig.8 Adenoviral CXCL1 overexpression in the GFs and mice gingiva. A: Gene expressions in GFs of WT mice with or without adenoviral infection after 6 hours (N=3). B: Time course of gene expressions in the gingiva after intragingival adenoviral injection with CMV or mCXCL1 (N=5). *p<0.05, **p<0.01, ***p<0.001 vs control (non-injected gingiva). ##p<0.01, ###p<0.001 vs 4 days after AdCMV injection.

Supplemental Fig.9 (related to Fig.4)



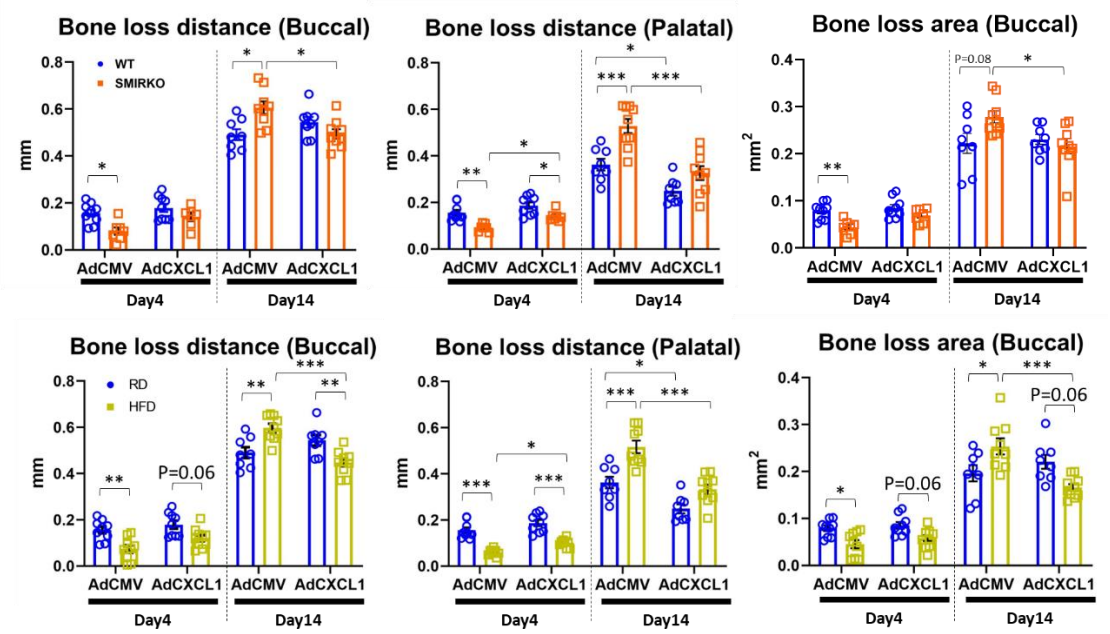
Supplemental Fig.9 Immune cell populations in the gingiva of WT (RD-fed), SMIRKO and HFD-fed mice following intra-gingival adenoviral injection (N=5). *p<0.05. Blue circle: WT (RD-fed), orange square: SMIRKO, yellow square: HFD-fed.

Supplemental Fig.10 (related to Fig.4)



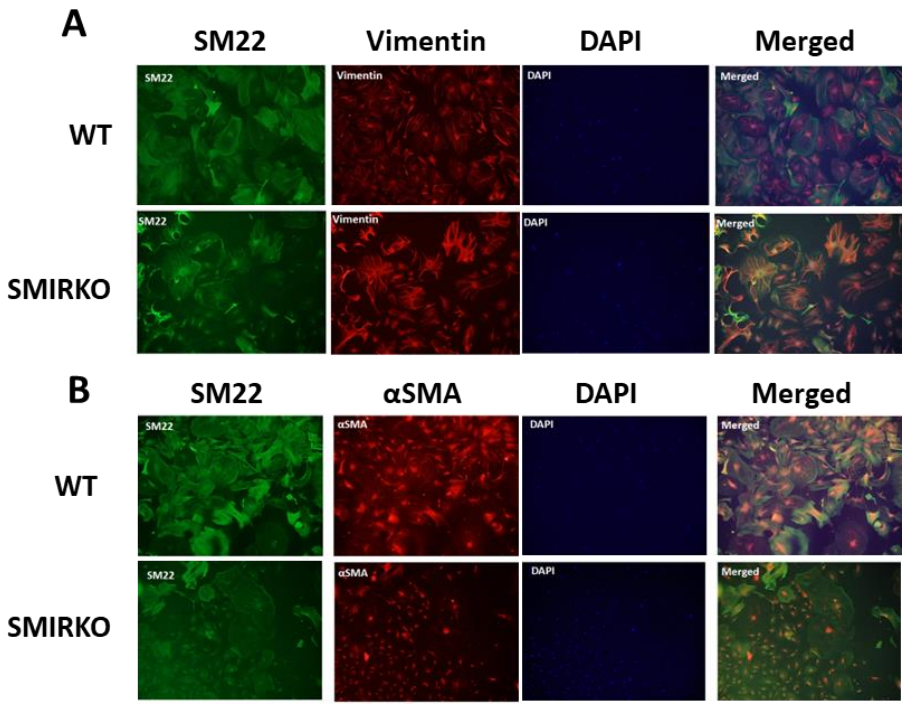
Supplemental Fig.10 Gene expressions in the gingiva of WT (RD-fed), SMIRKO and HFD-fed mice following intragingival adenoviral injection (N=5). *p<0.05. Blue circle: WT (RD-fed), orange square: SMIRKO, yellow square: HFD-fed.

Supplemental Fig.11 (related to Fig.4)



Supplemental Fig.11 Net bone loss assessment in ligatured-WT (RD-fed), SMIRKO and HFD-fed mice with intragingival adenoviral injection of AdCMV or AdCXCL1 (N=7-10). *p<0.05; **p<0.01; ***p<0.001.

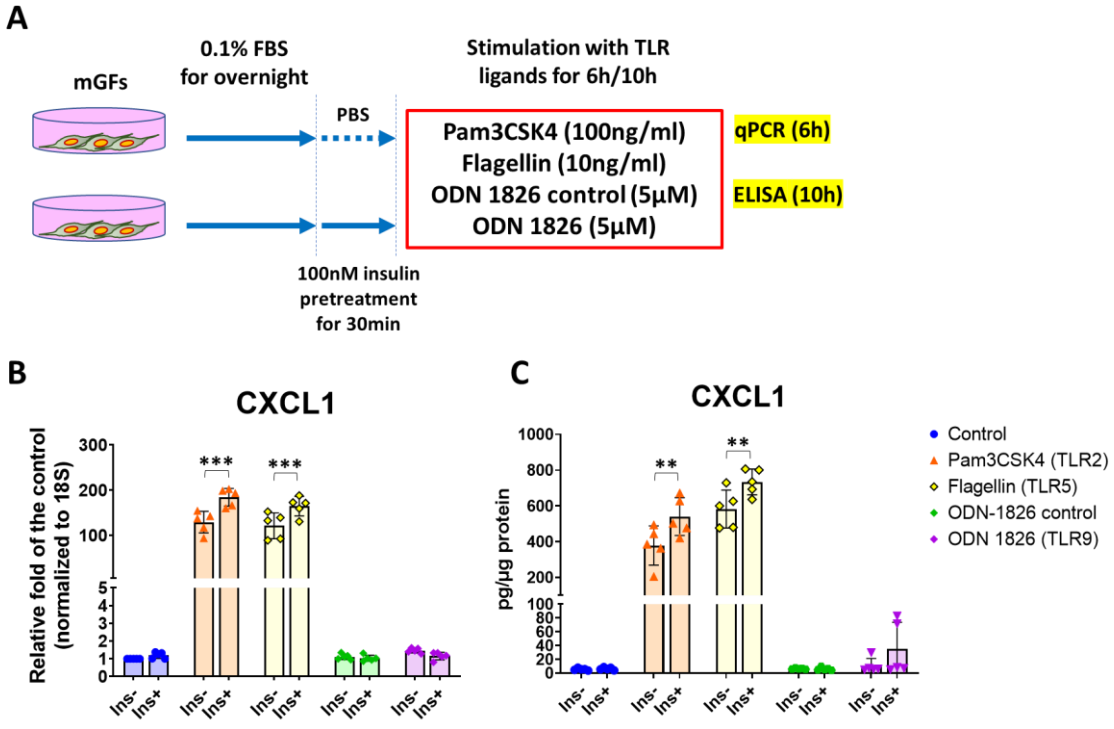
Supplemental Fig.12 (related to Fig.5)



Supplemental Fig.12 Immunofluorescence stain in mouse GFs

A-B: Immunofluorescence stain with SM22α and Vimentin or αSMA in mouse GFs.

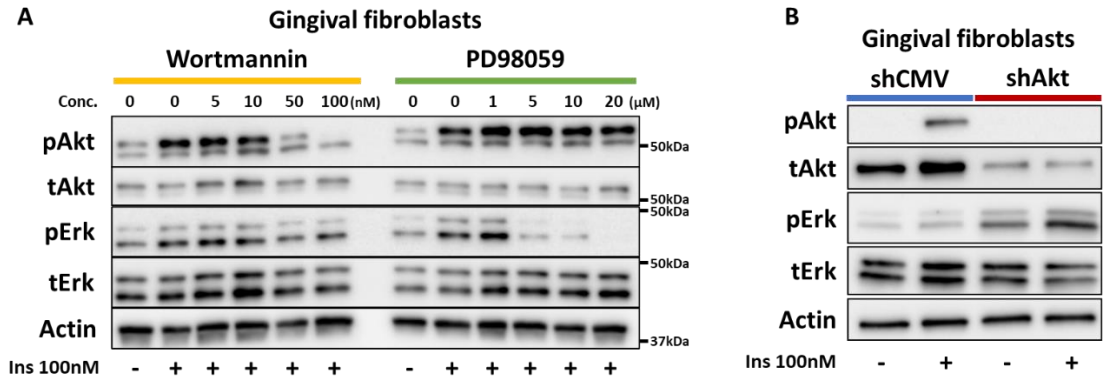
Supplemental Fig.13 (related to Fig.6)



Supplemental Fig.13 Confirmation of CXCL1 expression in mouse GFs stimulated with TLR2, 5 and 9 ligands

A: A schema of in vitro study. B: CXCL1 mRNA (N=5) and C: protein expression in GFs stimulated with 100ng/ml Pam3CSK4 (TLR2 ligand), 10ng/ml flagellin from *S.t.* (TLR5 ligand), 1μM ODN 1826 control and 1μM ODN 1826 (TLR9 ligand) with or without 100nM insulin pretreatment for 30 minutes. **p<0.01; ***p<0.001.

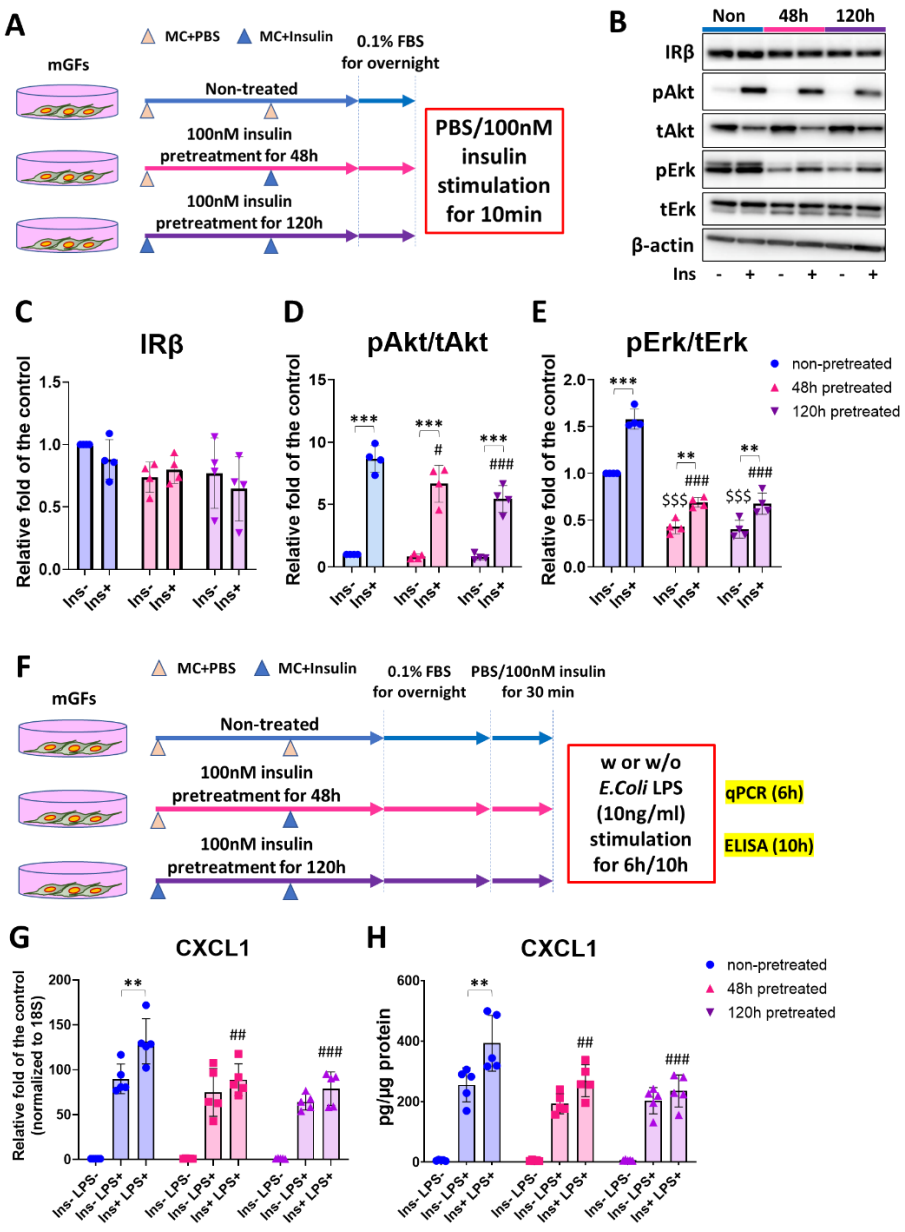
Supplemental Fig.14 (related to Fig.6)



Supplemental Fig.14

A: Determination of effective concentration of wortmannin and PD98059 in murine GFs, and B: confirmation of Akt1 knockdown by lentiviral transfection with shRNA in murine GFs.

Supplemental Fig.15 (related to Fig.6)



Supplemental Fig.15 LPS-stimulated CXCL expression in mouse GFs with hyperinsulinemia-induced insulin resistance

A. A schema of the cellular study. B-E. Insulin signaling in mouse GFs treated with insulin for 0, 48 and 120 hours before 100nM insulin stimulation (N=4). F. A schema of in vitro study. G-H. LPS-induced CXCL1 gene and protein expression in mouse GFs with or without insulin 100nM under hyperinsulinemia-induced insulin resistance (N=5). **p<0.01; ***p<0.001. $$$p<0.001 vs non-treated GFs without insulin (D-E) and without insulin and LPS (G-H). ##p<0.01,

###p<0.001 vs non-treated GFs with 100nM insulin (D-E) and with insulin and LPS (G-H).