Disruption of retinal inflammation and the development of diabetic retinopathy in mice by a CD40-derived peptide or mutation of CD40 in Müller cells

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ESM METHODS

Transgenic mice

Transgenic mice with conditional expression of WT mouse CD40 restricted to Müller cells were previously described [1]. A similar approach was used to generate transgenic mice with conditional expression of CD40 mutants. Briefly, constructs that encode mouse WT *Cd40*, mouse *Cd40* with a previously described mutation that deletes the CD40-TRAF2,3 binding sites [2] and ablates binding to TRAF2 and TRAF3 [3] (CD40 ΔTRAF2,3), or mouse CD40 with previously described point mutations in the TRAF6 binding site (QD*G*Q*A*MED) [2] that prevents binding to TRAF6 [4] (CD40 ΔTRAF6) were inserted into the *Eco* RI and *Bam* HI sites of the pTet^{os} plasmid [5]. After sequence verification, transgenes were excised by *Sal* I digestion [5]. Transgenes were microinjected into mouse oocytes (C57BL/6J; B6). Founder Tet^{OS}-*Cd40* mice (either WT or mutant *Cd40*) were identified by PCR using the primers: Tet^{OS}*Cd40* Forward: 5'-GCAACGTGCTGGTTATTGTG-3', Reverse: 5'-CCGGGACTTTAAACCACAGA-3'. The driver line consisted of transgenic mice that express tetracycline (Tet)-repressible transactivator (tTA) under the control of the *GFAP* promoter *gfa2* [6]. Homozygous Tet^{OS}-*Cd40* encoding either WT or mutant *Cd40* (responder) and heterozygous *GFAP*-tTA (driver) transgenic mice [6] (both B6) were backcrossed onto a *Cd40*$^{-/-}$ (B6) background. To confirm that mice were *Cd40*$^{-/-}$, animals were genotyped using primers that detect endogenous *Cd40* and mutant *Cd40* (neomycin cassette inserted into exon 3 resulting in lack of functional Cd40) [7]. Both lines of mice were bred and offspring identified by PCR analysis of genomic DNA. PCR primers for *Cd40* and tTA were obtained from The Jackson Laboratory (Bar Harbor, ME). Littermates that inherited only one transgene (single transgenic and non-expressing) served as controls (Trg-Ctr) for double transgenic animals (Trg-CD40; exhibiting promoter-specific expression of WT or mutant CD40).

Antibodies

Retinal sections were incubated with biotinylated anti-CD40 mAb (3/23, BioLegend, San Diego, CA; 1:50), anti-CRALBP (15356-1-AP, Proteintech Group, Rosemont, IL; 1:200), anti-vimentin (Novus Biologicals, Littleton, CO; 1:300), anti-glutamine synthetase (GTX630654, GeneTex, Irvine, CA; 1:1000), anti-Iba-1 Ab (019-19741, Wako Chemicals, Richmond, VA; 1:250), Tomato Lectin DyLight 488 (Vector Laboratories, Burlingame, CA; 1:100), anti-ICAM-1 (KAT-1, eBiosciences, San Diego, CA; 1:100), anti-TNF-α (52B83, Abcam, Cambridge, UK; 1:25), anti-CCL2 Ab (2D8, Novus Biologicals; 1:1000), anti-$P2X_7$ (AP09521PU-N, Origene, Rockville, MD; 1:100) or biotinylated anti-phospho-Tyr783 PLCγ1 (BS-3343-R, Bioss, Woburn, MA; 1:500) Abs. Abs were diluted in PBS plus 0.3% Triton X-100 and 1-5% normal goat or mouse serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Ab specificity was assessed by omitting primary Ab and, in the case of anti-CD40 mAb, by staining eyes from *Cd40*$^{-/-}$ mice.

In the case of immunoblots, membranes were probed with antibody to PLCγ1 (5690S; 1:4000), phospho-Tyr783 PLCγ1 (2821S; 1:2000), Src (2108S; 1:3000), phospho-Tyr416 Src (6943S; 1:2000), IκBα (4814P; 1:1000), phospho-Ser32 IκBα (2859S; 1:1000), JNK (9252S; 1:2000), phospho-Thr183/Tyr185 JNK (4668S; 1:3000), ERK1/2 (4695T; 1:5000), phospho-Thr202/Tyr204 ERK1/2 (4370T; 1:3000), p38 MAPK (8690T; 1:3000), phospho-Thr180/Tyr182 p38 MAPK (4511T; 1:2000), TRAF2 (4712T; 1:2000), TRAF6 (8028T; 1:2000; all from Cell Signaling Technologies, Danvers, MA) or actin (sc47778; 1:10000; Santa Cruz Biotechnologies, Santa Cruz, CA).

RNAi-mediated silencing

SMARTPool siRNAs (ON-TARGETplus) for human TRAF2, TRAF6, PLCγ1 and non-targeting siRNA were obtained from Horizon Discovery (Cambridge, UK). RNAi mediated silencing of Src was reported previously [8, 9]. Cells were transfected with siRNA (50 nM) using TransIT-X2 Dynamic Delivery System (Mirus, Madison, WI).

Flow cytometry

Human Müller cells were incubated with anti-CD40 (5C3), anti-ICAM-1 (555511, both BD Biosciences) or isotype control mAbs. Retinal cell suspensions from mice were obtained by digestion with papain (15 IU/ml)/DNAse (15 μg/ml; Worthington Biochemicals, Freehold, NJ) as described [10]. Cells were incubated with anti-CD40 (124605; BioLegend), anti-CD29 (HM β1-1, BD Biosciences, San Jose, CA) or isotype control antibodies. Flow cytometry data were acquired using an LSR II and running FACSDiva software. Expression of CD40 was analyzed on $CD29^+$ (Müller) cells.

Measurement of extracellular ATP

The ecto-ATPase inhibitor β, γ-methylene-ATP (300 μmol/l; Sigma-Aldrich, St Louis, MO) was added 15 min prior to stimulation with CD154. Extracellular ATP was quantified using an ATP bioluminescence assay kit and an ATP standard curve (Sigma-Aldrich) [11].

References

[1] Portillo J-AC, Lopez Corcino Y, Miao Y, et al. (2017) CD40 in retinal Müller cells induces $P2X_7$-dependent cytokine expression in macrophages/microglia in diabetic mice and development of early experimental diabetic retinopathy in mice. Diabetes 66: 483-493

[2] Subauste CS, Andrade RM, Wessendarp M (2007) CD40-TRAF6 and autophagy-dependent anti-microbial activity in macrophages. Autophagy 3: 245-248

[3] Hsing Y, Hostager BS, Bishop GA (1997) Characterization of CD40 signaling determinants regulating nuclear factor-kappa B activation in B lymphocytes. J Immunol 159: 4898-4906

[4] Jalukar SV, Hostager BS, Bishop GA (2000) Characterization of the roles of TNF receptor-associated factor 6 in CD40-mediated B lymphocyte effector functions. J Immunol 164: 623-630

[5] Sarao R, Dumont DJ (1998) Conditional transgene expression in endothelial cells. Transgenic Res 7: 421-427

[6] Lin W, Kemper A, McCarthy KD, et al. (2004) Interferon-γ induced medulloblastoma in the developing cerebellum. J Neurosci 24: 10074-10083

[7] Kawabe T, Naka T, Yoshida K, et al. (1994) The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. Immunity 1(3): 167-178

[8] Muniz-Feliciano L, Van Grol J, Portillo J-AC, et al. (2013) *Toxoplasma gondii*-induced activation of EGFR prevents autophagy protein-mediated killing of the parasite. PLoS Pathog 9: e1003809

[9] Portillo JC, Muniz-Feliciano L, Lopez Corcino Y, et al. (2017) *Toxoplasma gondii* induces FAK-Src-STAT3 signaling during infection of host cells that prevents parasite targeting by autophagy. PLoS Pathog 13(10): e1006671.

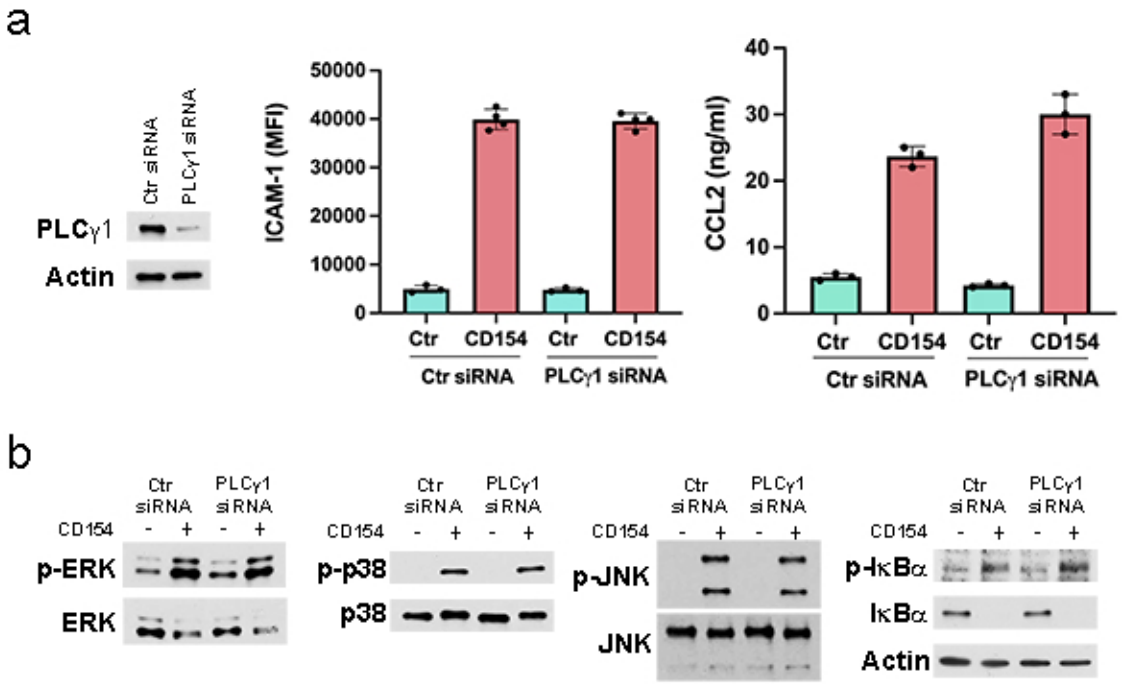
[10] Portillo J-AC, Okenka G, Kern TS, Subauste CS (2009) Identification of primary retinal cells and ex vivo identification of pro-inflammatory molecules in retinal cells using flow cytometry. Mol Vis 15: 1383-1389

[11] Blum AE, Joseph SM, Przybylski RJ, Dubyak GR (2008) Rho-family GTPases modulate Ca(2+)-dependent ATP release from astrocytes. Am J Physiol Cell Physiol 295(1): C231-241.

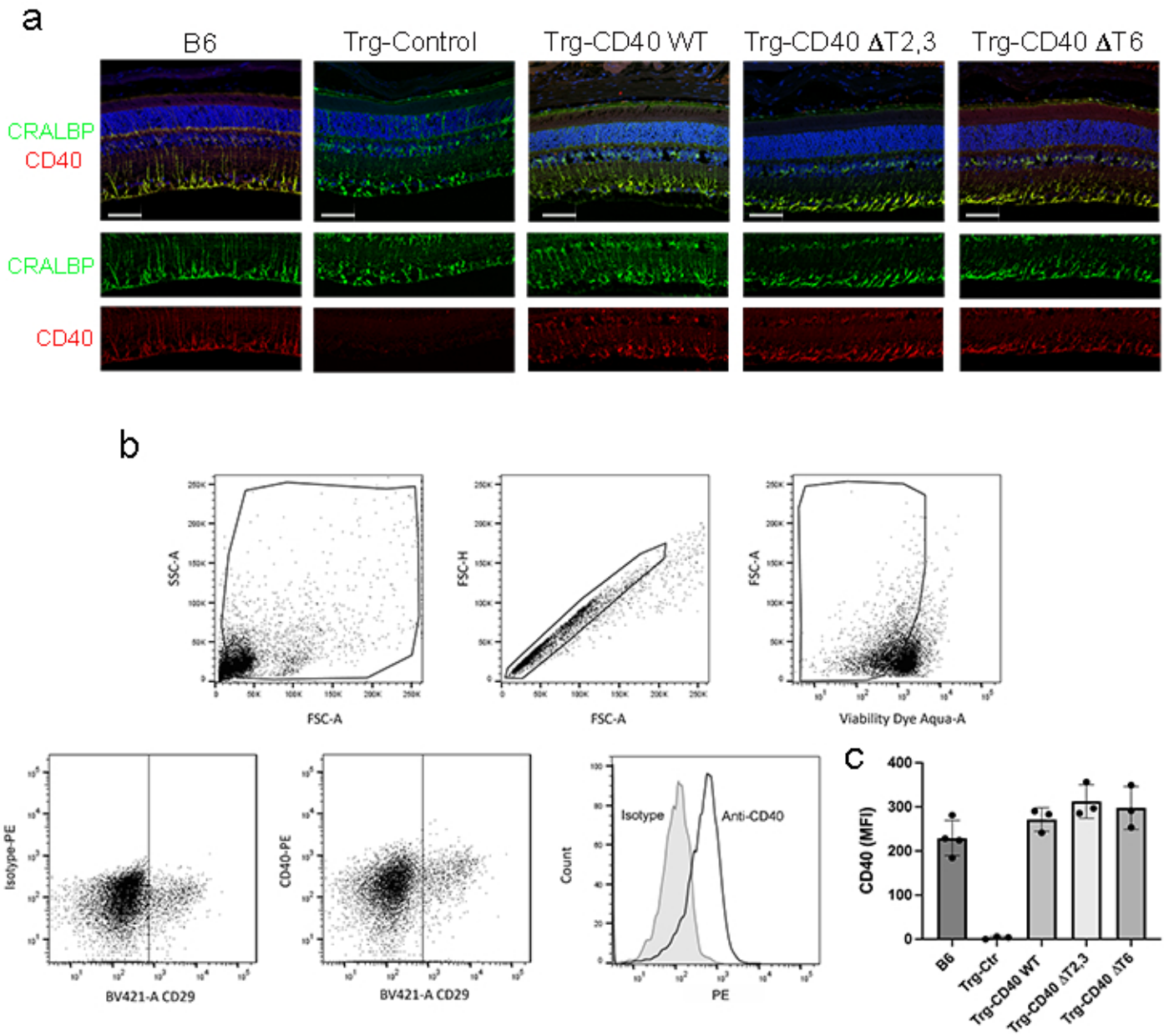
ESM Table 1. Average weight, blood glucose and HbA_{1c} in control and diabetic mice

Strain		Duration (m)	n	Weight (g)	Glucose (mmol/l)	HbA1c (mmol/mol)	HbA1c (%)
B6	ND	2	23	28.1 ± 0.5	7.51 ± 1.8	1	1.9 ± 0.2
	DM	2	43	26.3 ± 0.7	25 ± 0.8	57	7.4 ± 0.4
Trg-Ctr	ND	2	17	27.0 ± 0.4	7.2 ± 0.2	1	2.0 ± 0.2
	DM	2	26	24.4 ± 0.4	25.1 ± 0.9	53	7.0 ± 0.3
Trg-CD40 WT	ND	2	19	28.1 ± 0.6	7.3 ± 1	2	2.3 ± 0.3
	DM	2	34	23.8 ± 0.4	25.7 ± 0.7	54	7.1 ± 0.3
Trg-CD40 ΔT2,3	ND	2	12	28.0 ± 0.4	7.3 ± 0.2	1	2.0 ± 0.2
	DM	2	18	24.4 ± 0.7	25.5 ± 0.8	55	7.2 ± 0.3
Trg-CD40 ΔT6	ND	2	8	29.1 ± 1.1	7.4 ± 0.3	1	1.9 ± 0.2
	DM	2	8	23.4 ± 0.7	26.1 ± 0.8	55	7.2 ± 0.3
B6	ND	8	6	36 ± 2.4	7.7 ± 0.5	16	3.6 ± 0.1
	DM	8	8	27 ± 1.3	27.5 ± 1..4	100	11.3 ± 0.9
Trg-Ctr	ND	8	6	34.6 ± 2.5	7.6 ± 0.4	15	3.5 ± 0.1
	DM	8	6	26 ± 0.5	27.4 ± 1.5	98	11.1 ± 0.7
Trg-CD40 WT	ND	8	6	35 ± 2.3	7.5 ± 0.6	15	3.5 ± 0.1
	DM	8	8	25.5 ± 0.5	27.0 ± 2.0	104	11.7 ± 1
Trg-CD40 ΔT2,3	ND	8	6	34.6 ± 2.5	7.2 ± 0.4	15	3.5 ± 0.1
	DM	8	7	25 ± 2.7	27.9 ± 1.5	107	11.9 ± 0.9

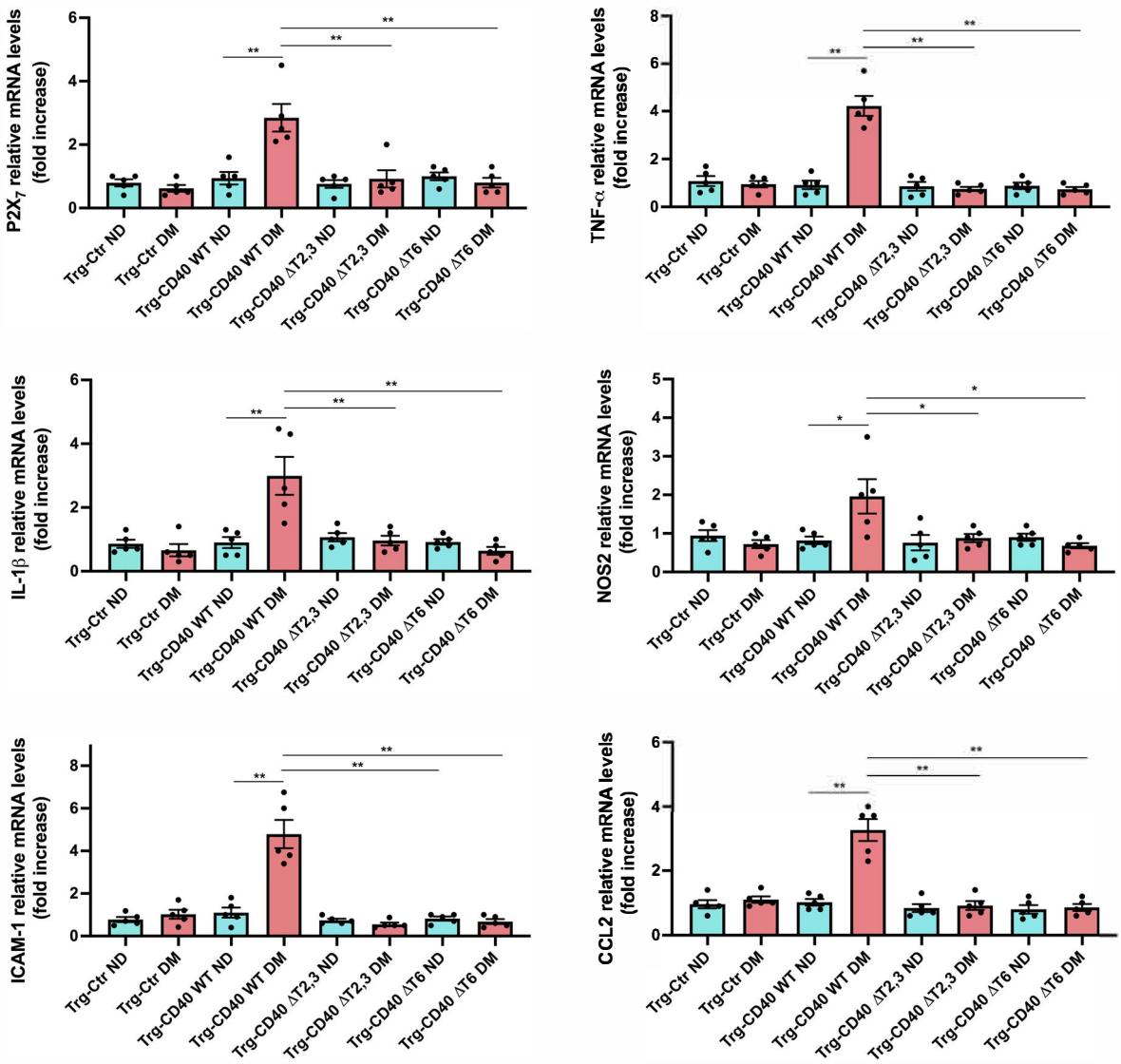
ND, Non-diabetic; DM, Diabetic



ESM Fig. 1. PLCγ1 is not required for upregulation ICAM-1 and CCL2 in Müller cells as well as for activation of signaling molecules that drive expression of these pro-inflammatory molecules. Human Müller cells were transfected with control or PLCγ1 siRNA. *a*, Immunoblot confirmed knockdown of PLCγ1. Müller cells were stimulated with or without CD154. ICAM-1 expression was examined by flow cytometry while CCL2 production was examined by ELISA (both at 24 h). *b*, Phosphorylation of ERK1/2, JNK, p38 MAPK and NFκB were examined at 30 min. Representative results of 2-3 different experiments.



ESM Fig. 2. Transgenic mice with expression of CD40 WT or CD40 with mutations in the TRAF binding sites. *a*, CD40 expression in Müller cells (CRALBP+) from B6, single transgenic (Trg-Ctr) or double transgenic mice that express CD40 WT (Trg-CD40 WT), CD40 ΔT2,3 (Trg- CD40 ΔT2,3) or CD40 ΔT6 (Trg-CD40 ΔT6). Scale bar, 50 μm. *b*, CD40 expression on Müller cells (CD29+) isolated from the retina as assessed by flow cytometry. Dot plot show gating strategy. CD40 was analyzed on live cells (did not stain with Aqua LIVE/DEAD kit) that were CD29+ (Müller cells). Dot plots and histogram shows representative CD40 expression in a B6 mouse. c, Graph bars shows mean + SEM of CD40 mean fluorescence intensity (3-4 mice per group).



ESM Figure 3. Expression of CD40 ΔT6 in Müller cells from diabetic mice prevents upregulation of *P2X7*, *Tnf-α*, *Il-1β*, *Nos2*, *Icam-1* and *CCL2* mRNA in the retina. At 2 m of diabetes, mRNA levels were assessed by real time quantitative PCR using 18S rRNA as internal control. One non-diabetic B6 mouse was given an arbitrary value of 1 and data are expressed as fold-increase compared to this animal. Horizontal bars represent mean +/- SEM. 5 animals per group. * $P < 0.05$, ** $P < 0.01$ by ANOVA.