Supplement

Supplemental information for Methods

2.3 Spontaneous behaviors directed toward the cheek

The mice were placed in a separate, clear plastic container in 9×9×13 cm with a small amount of bedding. A camcorder was positioned above the mice to record the behaviors of two mice at a time. Four mirrors were placed around each container, to provide a good view of the mice from all angles. The experiments were performed in a sound-proof room. The mice were placed into the container for habituation 30 mins before each two-hour period of video recording.

From each video recording the number of spontaneous wipes with the forelimb and bouts of scratching with the hindlimb, were counted [45].

2.4 Responses to mechanical and heat stimulation of the cheek

Prior to chemical challenge to the cheek, each mouse was placed daily for 30 min in a meshed chamber (4.5 x 3.5 x 5 cm). During this habituation time mechanical and thermal stimuli were periodically applied to the contralateral cheek. For the testing procedure the stimuli were first applied in order of ascending intensity and then descending order. Each stimulus was presented five times. This was followed by 5 warm and 5 noxious heat stimuli and 5 heat and 5 warm stimuli. Stimulus application began and ended with the nonpainful filament (exerting 0.23 mN with a tip of 67 μm). The stimulus duration was 1 second unless terminated earlier by the animal's withdrawal. The mechanical stimuli were presented first, followed by the warm- and then the noxious heat stimulus. The interstimulus intervals were 30 and 60s for the mechanical and heat stimuli, respectively.

A bit more about calculation

2.5 Scoring the severity of inflammation by measuring erythema, scaling and skin-fold thickness

To ensure controlled, unchanged and consistent lighting ad standardized picture acquisition to document clinical signs of inflammation a heavy black cloth eliminated interference from reflections and outside light. The camera's ISO, aperture, shutter speed and white balance were adjusted manually prior to the first picture and kept constant for all the pictures. The distance from the camera to the photographed area was 25 cm. A color picker and scale were additionally photographed with the mouse in each picture.

2.6 Ultrasound images of cheek skin, in vivo

For ultrasound image acquisition the axial and lateral resolution was 30 μm and 70 μm, respectively, with an 8.0 × 8.0 mm field of view displaying the middle of the 10 x 10 mm treated area of skin on the cheek. Parameters were set the same for all ultrasound image acquisitions. The mice were briefly anesthetized in a heated room (up to 23°C). The mice were positioned on a platform and the transducer was stabilized with a clamp and connected with an articulated arm to control the distance between the transducer and the skin surface. To ensure the quality of the image, ultrasound gel was evenly spread over the cheek skin between the skin and the transducer.

Blood flow was evaluated using doppler signals, this mode detects areas of moving matter (indicative of blood flow) and color coded these areas in the display according to the velocity of movement (35 DB = yellow, 0 DB = red). All imaging parameters were kept constant for all relevant parameters, frequency (40 MHz), power (100%), wall filter (2.5 mm/s), scan speed (2.0 mm/s), gain (20 dB), a medium velocity (5 kHz) and RF cycles (2 cycles).

2.7 Hematoxylin and Eosin (H&E) staining

After conventional paraffin embedding, the tissue was serially sectioned into 4-5 μm slices and stained as follows: hematoxylin staining for 15 min, hydrochloric acid alcohol solution for 35 s of decoloring, eosin staining for 10 min, and 90% ethanol for 40 s of decoloring. Then neutral balsam was used for mounting and the section was photographed under a digital camera DM6000 (Leica,

Germany).

For statistical comparisons, 6-7 random images of several sections for each mouse were taken to obtain a mean value. ImageJ with a color deconvolution plugin [42; 44] was used for detecting areas stained with hematoxylin (violet). In a second step, the quantification of the number of infiltrating cells per tissue sample corrected to the percentage of H&E stained tissue sample in each sample was automatically performed and detected using an adaptation to an ImageJ macro, that was designed to count cells in a designated area of the tissue sample [34]. The macro automatedly detected regions of interest by using a thresholded mask of the image and measuring the different areas. The color deconvoluting macro was used with ImageJ to separate the staining colors in each image, and the Focinator ImageJ macro was used to threshold and binarize the color-separated images, differentiate between stained and unstained regions, detect and count cells based on their shape and size and measure the differently stained areas in each picture.

2.8 Immunohistochemistry

Sections of paraffin-embedded calf skin, were cut, 4-µm thick, dewaxed in xylene, placed first in a graded ethanol series and then in deionized distilled water. Next, an antigen retrieval step was performed in boiling 10 mM citrate buffer, pH 6.0 for 20 mins [32]. The fixed tissues were stained by using the avidin-biotin peroxidase complex (ABC) technique. In the ABC system, endogenous peroxidase was quenched by incubation of the sections in 0.3% hydrogen peroxide for 10 mins at room temperature. Nonspecific binding and cross reactivity were prevented by blocking and incubating with nonimmune serum (2% goat serum albumin for 30 mins at room temperature) and then incubating with primary antibodies overnight at 4 °C at the following concentrations: (1) rat anti-CXCL10 antibody (1:200, Novus Biologicals., Littleton, US, (134013)) [32] (2) rat anti-CXCR3 antibody (1:200, Novus Biologicals., Littleton, US, RM0213-14C23) [57]; (3) rabbit anti-TNF alpha

antibody [16] (1:200, Abcam, Cambridge, UK, ab6671) [59]; (4) rabbit anti- IL1 beta antibody (1:200, Abcam, Cambridge, UK, ab9722) [58]. After washing three times with PBS, sections were treated with biotin-conjugated secondary antibody anti-rabbit for IL-1β and TNF-α, and anti-rat for CXCR3 and CXCL10 (1:500, Boster, Pleasanton, CA, USA) for 2 hrs at room temperature. Diaminobenzidine was used for the color reaction; if the IHC signals were present, the cytoplasm was stained brown. Mayer's hematoxylin was used for nuclear counterstaining. The negative control was the sole application of the secondary antibody, leading to no reaction.

2.9 Real-time quantitative PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions. The cDNA was synthesized from 100 μg RNA using Prime Script™RT Reagent Kit plus the gDNA Eraser (Takara Japan). Each cDNA sample was amplified for the target genes and β-actin as a loading control in a 15 μl reaction volume of SYBR® Premix Ex Taq™ II (Tli RNaseH Plus, Clontech).

2.10 Statistical analysis

For each type of spontaneous, site-directed behavior (number of bouts of scratching or number of wipes), differences between means were analyzed with a mixed-design analysis of variance (ANOVA), i.e**.** 3 treatment groups (ACD, ICD and control) x two sexes x 4 days of testing with repeated measures over days of testing (before and after each challenge).Behavioral Ratings derived from responses of male mice to each von Frey filament and each heat stimulus were separately analyzed with a two-way ANOVA (ICD and ACD) with repeated measures over the 4 days of testing (before and after each challenge). The effects of force and temperature on responses to the mechanical and heat stimuli, respectively, were separately analyzed with a mixed-design ANOVA, i.e. 2 groups x 4 forces or 2 temperatures x 4 days of testing with repeated measures over force or temperature and days of testing. The erythema score, scaling score, skin-fold thickness and skin thickness of different skin layers and

power doppler evaluation of ultrasound images was analyzed with a two-way ANOVA (3 treatment groups (ACD, ICD and control) x 4 days of testing with repeated measures over days of testing).

Supplemental Fig.1: Effects of ICD and ACD in eliciting spontaneous scratching and wiping behaviors in female mice. Mean bouts of spontaneous scratching (A) and mean number of wipes (B) over 2 hrs were obtained before and 24 hrs after the 1st, 2nd and 3rd challenge with SADBE in female mice that were previously sensitized to the chemical (ACD) or previously exposed only to the acetone vehicle (ICD). Control mice received only acetone before and during challenge. *$p < 0.05$, **$p < 0.01$, error bars: S.E.M. n = 12 female mice /group. ICD, irritant contact dermatitis; ACD, allergic contact dermatitis.

Supplement Table 1 Type of ANOVA and corresponding F-and p-values

	Type of ANOVA	F-value	p-value
3.1 Spontaneous itch-like scratching behavior (Fig.2 A, Supplemental Fig. 1 A)	Repeated measures three-way ANOVA 3 treatment groups (ACD, ICD and control) x 2 sexes (male and female) x 4 days of testing (before, after 1st, 2nd, 3rd challenge)		
	Treatment groups	(2,22) = 87.656	0.000*
	days of testing	(3,33) = 38.523	0.000*
	genders	(1,11) = 2.635	0.083
	Treatment groups × genders	(2,22) = 0.753	0.483
	genders × days of testing	(3,33) = 3.123	0.039*
	Treatment groups × days of testing:	(6,66) = 17.599	0.000*
	Treatment groups × genders× days of testing	(6,66) = 1.261	0.288
3.1 Spontaneous pain-like wiping Behavior (Fig.2 B, Supplemental Fig. 1 B)	Repeated measures three-way ANOVA 3 treatment groups (ACD, ICD and control) x 2 sexes (male and female) x 4 days of testing (before, after 1st, 2nd, 3rd challenge)		
	Treatment groups	(2,22) = 66.445	0.000*
	days of testing	(3,33) = 144.831	0.000*
	genders	(1,11) = 9.338	0.011*

	Treatment groups × genders	(2,22) = 1.668	0.212
	genders × days of testing	(3,33) = 3.384	0.030*
	Treatment groups × days of testing:	(6,66) = 28.884	0.000*
	Treatment groups × genders× days of testing	(6,66) = 1.261	0.288
3.2 Behavioral responses to mechanical stimuli (Fig. 3 A)	Repeated measures three-way ANOVA 2 treatment groups (ACD and ICD) x 4 forces (0.23mN, 2mN, 10mN and 20 mN) x 4 days of testing (before, after 1st, 2nd, 3rd challenge)		
	Treatment groups	(1,10) = 86.076	0.000*
	days of testing	(3,30) = 48.992	0.000*
	force	(3,30) = 517.916	0.000*
	Treatment groups × force	(3,30) = 8.269	0.000*
	force × days of testing	(9,90) = 8.639	0.000*
	Treatment groups × days of testing:	(3,30) = 38.652	0.000*
	Treatment groups × force× days of testing	(9,90) = 3.516	0.001*
3.3 Behavioral responses to heat (Fig. 3 B)	Repeated measures three-way ANOVA 2 treatment groups (ACD and ICD) x 2 forces (38°C and 52°C) x 4 days of testing (before, after 1st, 2nd, 3rd challenge)		
	Treatment groups	(1,10) = 12.064	0.006*
	days of testing	(3,30) = 16.878	0.000*
	heat	(1,10) = 386.221	0.000*
	Treatment groups × heat	(1,10) = 10.038	0.010*
	heat × days of testing	(3,30) = 5.288	0.005*
	Treatment groups × days of testing:	(3,30) = 3.964	0.017*
	Treatment groups × heat× days of testing	(3,30) = 0.801	0.503
3.4 Clinical assessment of skin reactions -Skin thickness (Fig. 4 A)	Repeated measures two-way ANOVA 3 treatment groups (ACD, ICD and control) x 4 days of testing (before, after 1st, 2nd, 3rd challenge)		
	Treatment groups	(2,22) = 15.622	0.002*
	days of testing	(3,33) = 24.129	<0.0001*
	Treatment groups × days of testing:	(6,66) = 30.143	<0.0001*
3.4 Clinical assessment of skin reactions - Erythema (Fig. 4 B)	Repeated measures two-way ANOVA 3 treatment groups (ACD, ICD and control) x 4 days of testing (before, after 1st, 2nd, 3rd challenge)		

	Treatment groups	(2,22) = 770.830	0.002*
	days of testing	(3,33) = 358.679	<0.0001*
	Treatment groups × days of testing:	(6,66) = 220.218	<0.0001*
3.4 Clinical assessment of skin reactions - Scaling (Fig. 4 B)	Repeated measures two-way ANOVA 3 treatment groups (ACD, ICD and control) x 4 days of testing (before, after 1st, 2nd, 3rd challenge)		
	Treatment groups	(2,22) = 60.257	0.002*
	days of testing	(3,33) = 51.000	<0.0001*
	Treatment groups × days of testing:	(6,66) = 29.894	<0.0001*
3.5 Ultrasound images– area of blood flow (Fig. 5 D)	two-way ANOVA 3 treatment groups (ACD, ICD and control) x 4 days of testing (before, after 1st, 2nd, 3rd challenge)		
	Treatment groups	(2,84) = 17.607	0.002*
	days of testing	(3,84) = 8.118	<0.0001*
	Treatment groups × days of testing:	(6,84) = 2.167	0.054
3.5 Skin thickness of calf skin (Supplemental Fig. 2 A)	Repeated measures two-way ANOVA 3 treatment groups (ACD, ICD and control) x 2 days of testing (before, and after 2nd challenge)		
	Treatment groups	(2, 38) = 207.8	<0.0001*
	days of testing	(1, 38) = 622.2	<0.0001*
	Treatment groups × days of testing:	(2, 38) = 222.6	<0.0001*
3.5 Ultrasound images of skin layers – skin thickness (Supplemental Fig. 2 B)	two-way ANOVA 3 treatment groups (ACD, ICD and control) x 4 days of testing (before, after 1st, 2nd, 3rd challenge)		
	Treatment groups	(2, 125) = 189.4	<0.0001*
	days of testing	(3, 125) = 56.72	<0.0001*
	Treatment groups × days of testing:	(6, 125) = 24.03	<0.0001*
3.5 Ultrasound images of skin layers – Stratum Corneum (Supplemental Fig.	two-way ANOVA 3 treatment groups (ACD, ICD and control) x 4 days of testing (before, after 1st, 2nd, 3rd challenge)		

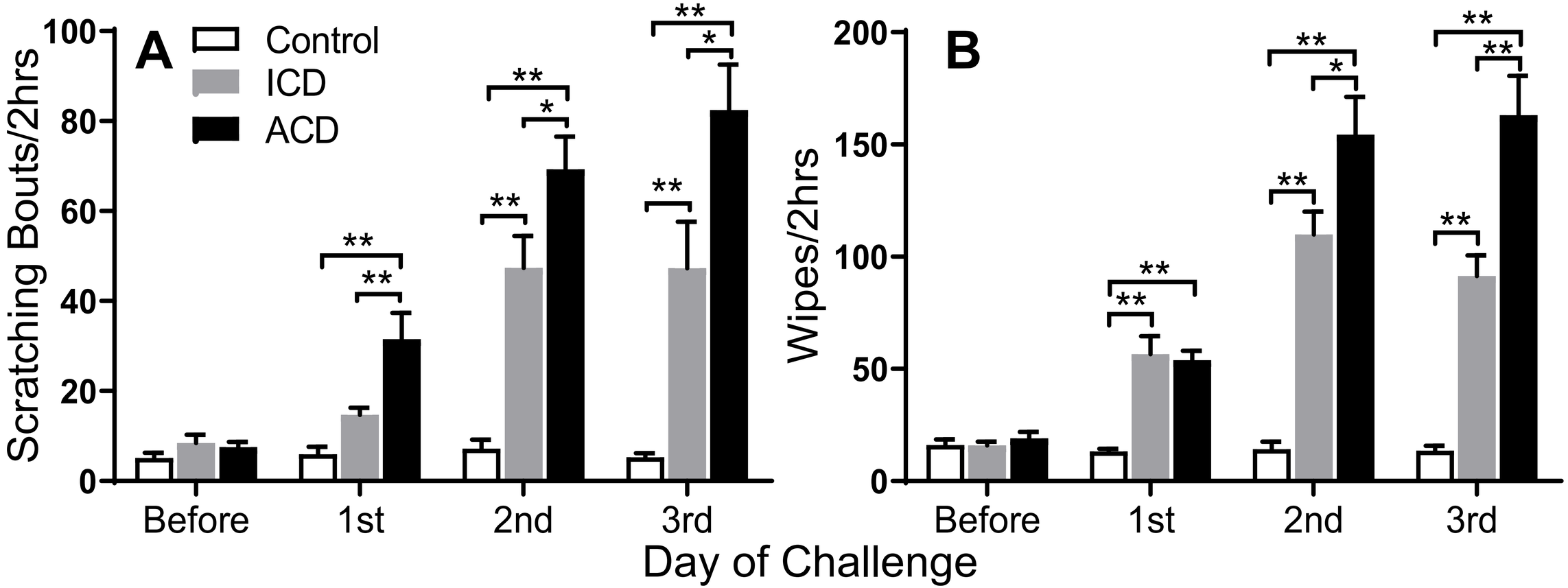
2 C)			
	Treatment groups	(2, 125) = 51.13	<0.0001*
	days of testing	(3, 125) = 18.47	<0.0001*
	Treatment groups × days of testing:	(6, 125) = 10.33	<0.0001*
3.5 Ultrasound images of skin layers – epidermis (Supplemental Fig. 2 D)	two-way ANOVA 3 treatment groups (ACD, ICD and control) x 4 days of testing (before, after 1st, 2nd, 3rd challenge)		
	Treatment groups	(2, 125) = 20.55	<0.0001*
	days of testing	(3, 125) = 6.039	0.0007*
	Treatment groups × days of testing:	(6, 125) = 1.589	0.1557
3.5 Ultrasound images of skin layers – Dermis (Supplemental Fig. 2 E)	two-way ANOVA 3 treatment groups (ACD, ICD and control) x 4 days of testing (before, after 1st, 2nd, 3rd challenge)		
	Treatment groups	(2, 125) = 83.77	<0.0001*
	days of testing	(3, 125) = 21.76	<0.0001*
	Treatment groups × days of testing:	(6, 125) = 10.57	<0.0001*
3.5 Ultrasound images of skin layers – Hypodermis (Supplemental Fig. 2 F)	two-way ANOVA 3 treatment groups (ACD, ICD and control) x 4 days of testing (before, after 1st, 2nd, 3rd challenge)		
	Treatment groups	(2, 125) = 64.93	<0.0001*
	days of testing	(3, 125) = 20.07	<0.0001*
	Treatment groups × days of testing:	(6, 125) = 8.003	<0.0001*
3.6 Histological analyses of calf skin (Fig. 6 A and B)	one-way ANOVA 3 treatment groups (ACD, ICD and control)		
	Percentage of stained area	(2,75) = 46.930	<0.0001*
	Number of cells	(2,75) = 81.362	<0.0001*
3.7 Protein expression (Fig. 7 A, B, C and D)	one-way ANOVA 3 treatment groups (ACD, ICD and control)		

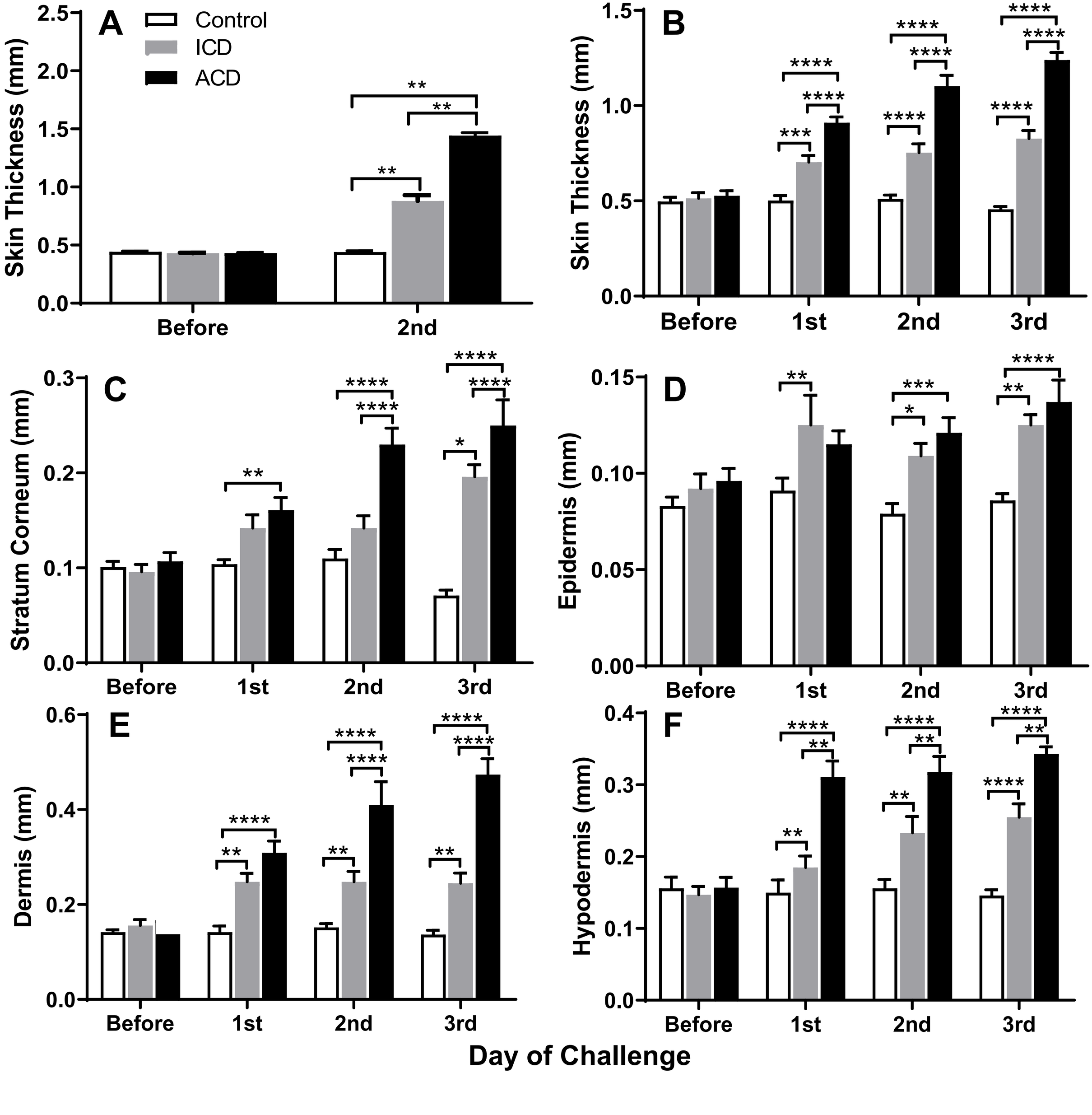
	IL-1β	(2,45) = 5.829	0.006*
	TNF-α	(2,52) = 13.346	<0.0001*
	CXCR3	(2,34) = 43.069	<0.0001*
	CXCL10:	(2,56) = 19.087	<0.0001*
3.8 mRNA expression (Fig. 8 A, B, C and D)	one-way ANOVA 3 treatment groups (ACD, ICD and control)		
	IL-1β	(2,10) = 53.757	<0.0001*
	TNF-α	(2,10) = 10.475	0.004*
	CXCR3	(2,11) = 12.380	0.002*
	CXCL10:	(2,11) = 140.183	<0.0001*

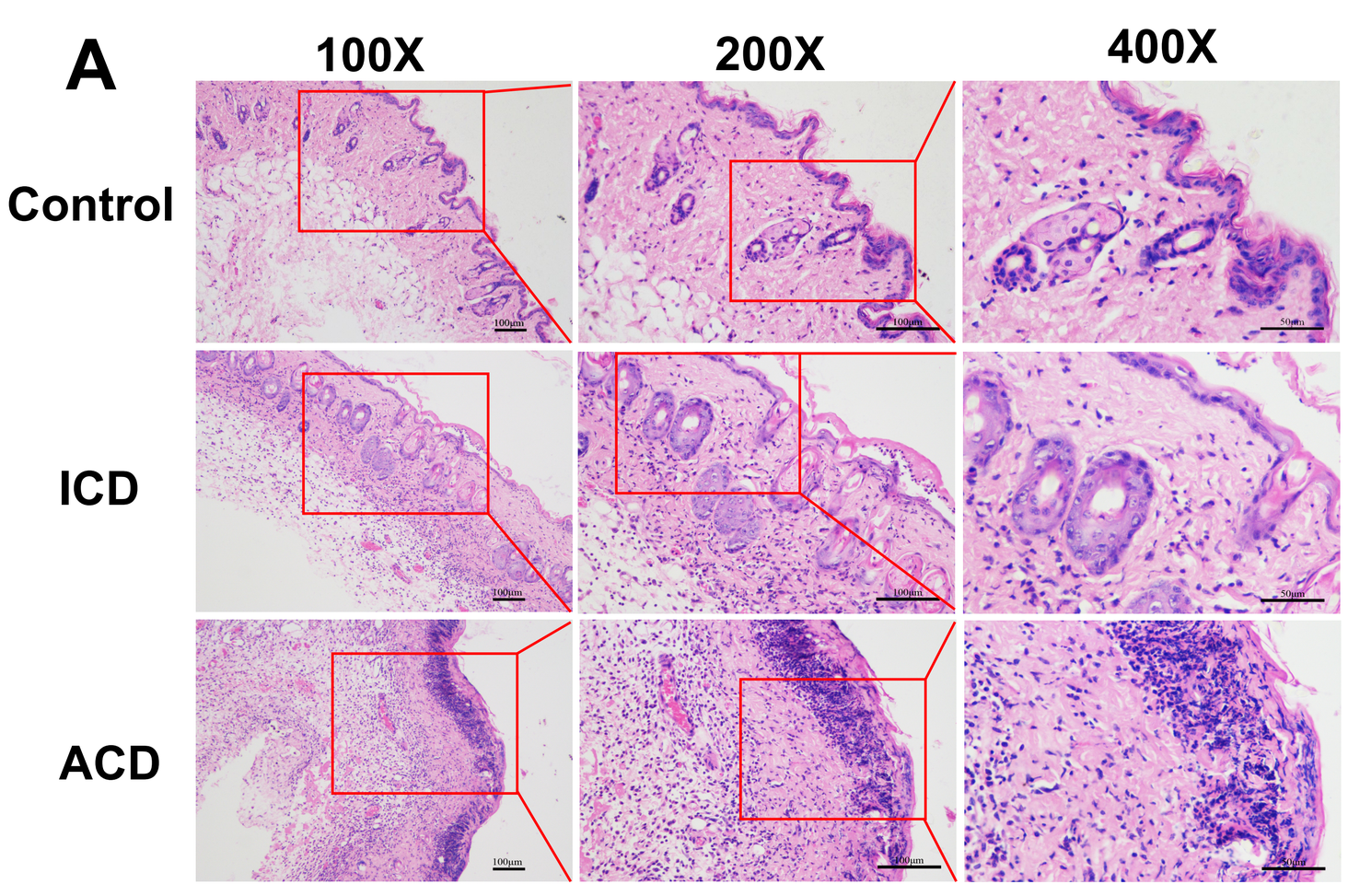
Supplemental Fig. 2:. The Effects of ICD and ACD on skin-fold thickness as measured by micrometer for the calf and by ultrasound for the cheek. The mean thickness of a fold of calf skin (A) was obtained before, and 24 hrs after the 2nd challenge with SADBE (ACD or ICD) or acetone vehicle (control). $^{**}p < 0.01$, error bars: SEM, n = 16 male mice /group. Measurements obtained from ultrasound images at the cheek were used to estimate the overall mean thickness (B), and the thickness of component layers including stratum corneum(C), epidermis (D), dermis (E) and hypodermis (F). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$, error bars: S.E.M., n = 10-12 male mice/ group. ICD, irritant contact dermatitis; ACD, allergic contact dermatitis.

Supplemental Fig. 3: Histological differences between ICD and ACD in skin of the calf. (A) Exemplary histological images with H&E staining for magnifications of ×100, ×200, and ×400. The red square inset in a given row indicates the region magnified in the image in the next column to the right. (B) Mean numbers of infiltrating cells from all H&E stained histological sections of calf skin for control, ICD, ACD groups obtained after the 2nd challenge. $^{*}p<0.05$, $^{**}p<0.01$, $^{****}p<0.0001$, error bars: S.E.M. n = 4 male mice. ICD, irritant contact dermatitis; ACD, allergic contact dermatitis.

Supplemental Fig. 4: Effects of ACD and ICD mRNA expression levels of IL-1β (A), TNF-α (B), CXCR3 (C) and CXCL10 (D) relative to β-actin in calf skin. Tissue samples were obtained 24 hrs after the 2nd SADBE treatment. Data are presented as $2^{-\Delta\Delta}$CT values. $^{*}p < 0.05$, $^{**}p < 0.01$, error bars: S.E.M. n = 3-5 male mice/group. ICD, irritant contact dermatitis; ACD, allergic contact dermatitis.


A
Control
ICD
ACD
Scratching Bouts/2hrs
100
80
60
40
20
0
**
**
**
*
**
*
**
**
Before
1st
2nd
3rd
B
Wipes/2hrs
200
150
100
50
0
**
**
**
*
**
**
**
Before
1st
2nd
3rd
Day of Challenge


A
Control
ICD
ACD
Skin Thickness (mm)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
Before
2nd
B
Skin Thickness (mm)
1.5
1.0
0.5
0.0
***
****
****
****
****
****
****
****
****
Before
1st
2nd
3rd
C
Stratum Corneum (mm)
0.3
0.2
0.1
0.0
**
****
****
*
****
****
Before
1st
2nd
3rd
D
Epidermis (mm)
0.15
0.10
0.05
0.00
**
*
***
**
****
Before
1st
2nd
3rd
E
Dermis (mm)
0.6
0.4
0.2
0.0
**
****
**
****
**
****
Before
1st
2nd
3rd
F
Hypodermis (mm)
0.4
0.3
0.2
0.1
0.0
**
****
**
**
****
**
****
Before
1st
2nd
3rd
Day of Challenge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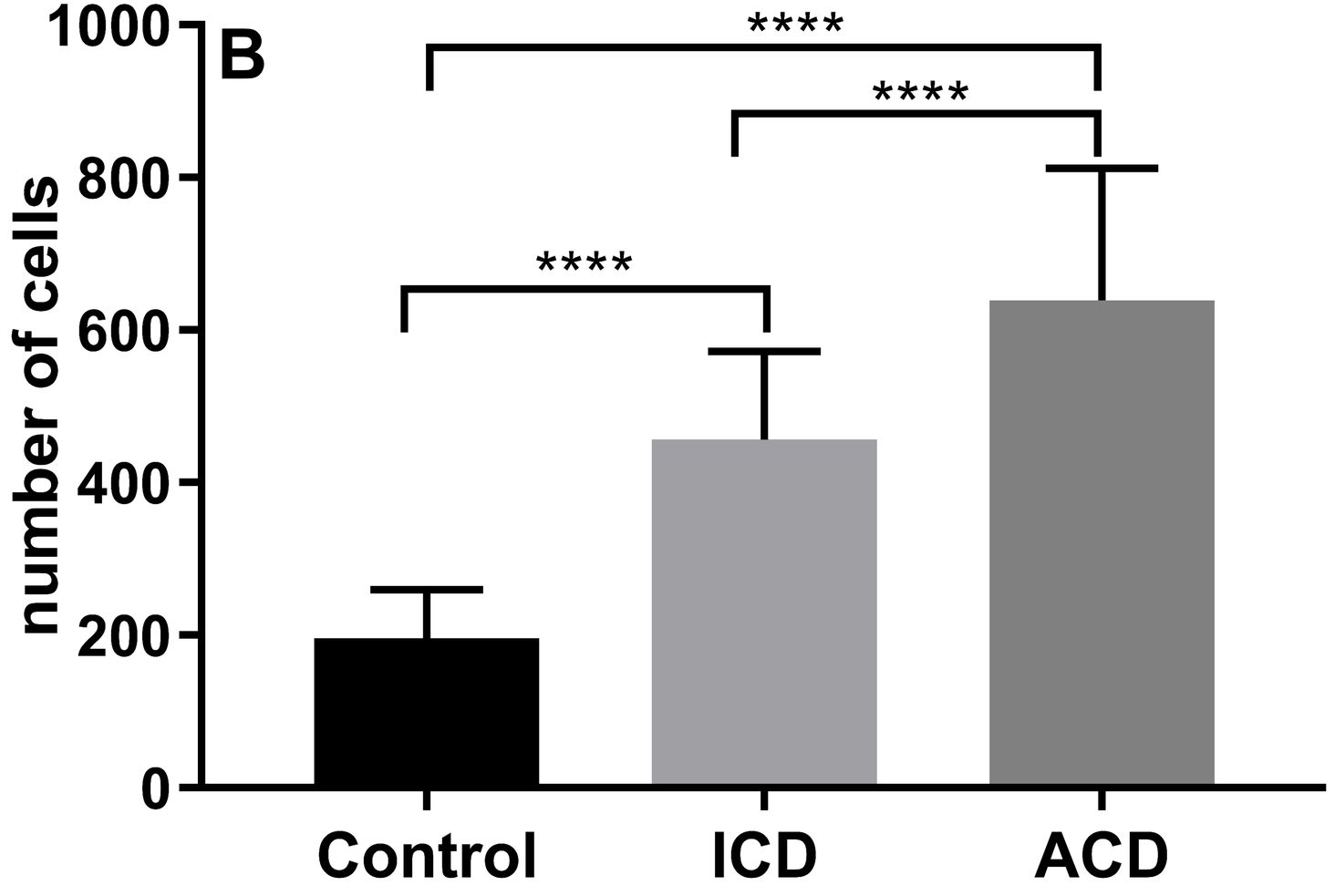
200X
400X
Control
ICD
ACD
100μm
50μm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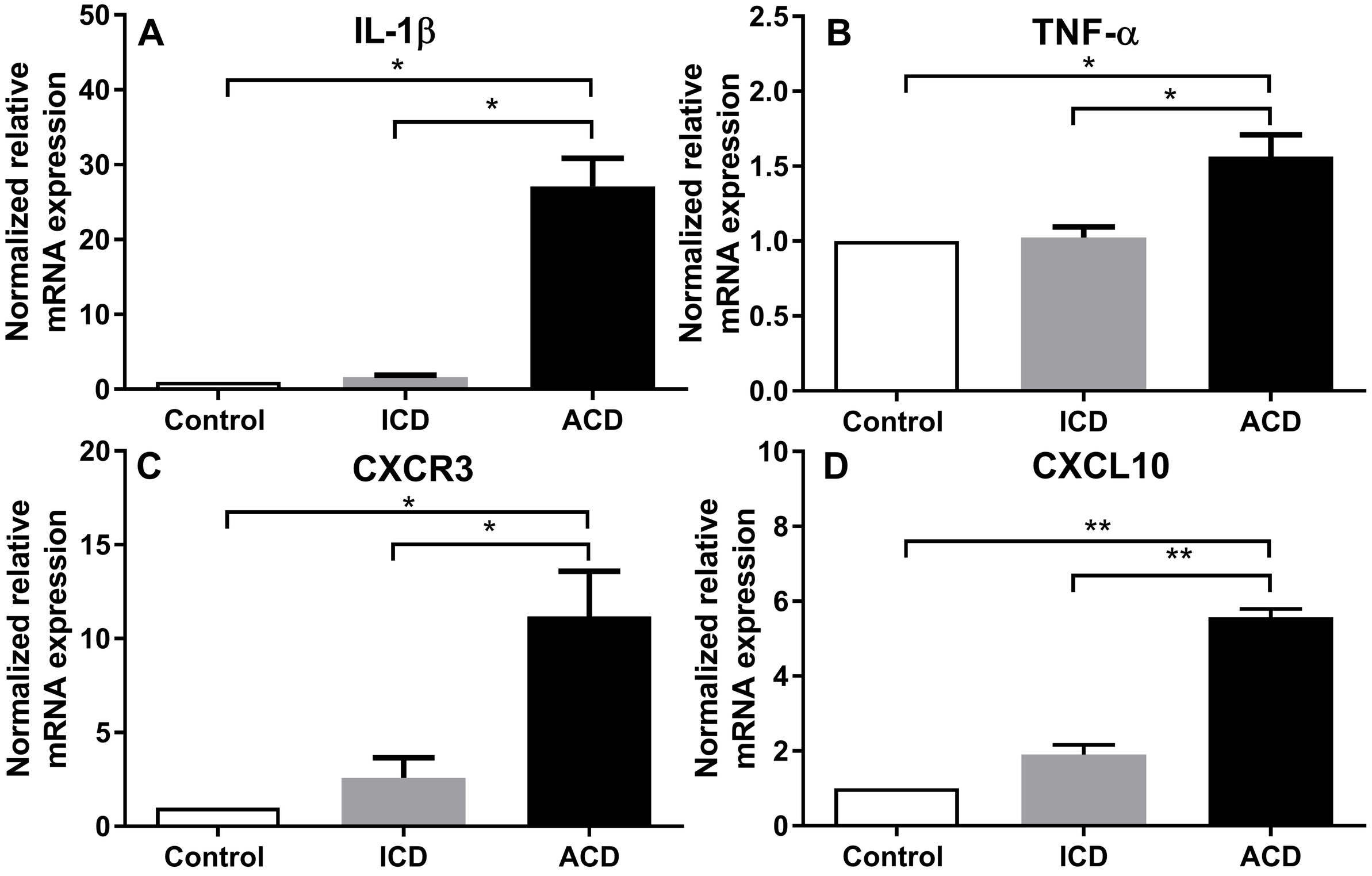
800
600
400
200
0
****
****
****
Control
ICD
ACD


A
IL-1β
Normalized relative mRNA expression
50
40
30
20
10
0
*
*
Control
ICD
ACD
B
TNF-α
Normalized relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Control
ICD
ACD
C
CXCR3
Normalized relative mRNA expression
20
15
10
5
0
*
*
Control
ICD
ACD
D
CXCL10
Normalized relative mRNA expression
10
8
6
4
2
0
**
**
Control
ICD
ACD