Supplementary Information for

BP180 dysfunction triggers spontaneous skin inflammation in mice

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www.pnas.org/cgi/doi/10.1073/pnas.1721805115

Supporting Information Appendix (SI Appendix):

Materials and Methods

Generation of Mice with Whole Body, Skin- and Basal Keratinocyte-Specific Deletion of NC16A.

To study immunopathogenesis of BP using autoantibodies from BP patients, we previously generated a humanized *NC16A* mouse strain on the C57BL/6J background (termed *NC16A* mice or WT mice) (1). Exons 18 and 19 (red) encoding the *NC16A* domain (red) are flanked by *loxP* sites. When crossed with germline *Cre* mice (Jackson Lab #006054), Cre recombination removes the *loxP*-flanked exons 18 and 19 and maintains the remaining reading frame, resulting in mice expressing NC16A domain-truncated BP180 (this whole body deleted *NC16A* mice were termed *ΔNC16A* mice).

To generate skin-specific *ΔNC16A* mice, *NC16A* mice were crossed with UBC-Cre-ERT2 mice (Jackson Lab #008085) provided kindly by Dr. Richard Weinberg from UNC-Chapel Hill. The Tam*Cre-NC16A* mice (*ERCre*$^{+}$*NC16A*$^{fl/fl}$ mice) when treated topically with tamoxifen (Sigma, 25 μl of 10 mg/ml in 62% EtOH/sunflower oil mixture) become skin-specific *ΔNC16A* mice. To generate basal keratinocyte-specific *ΔNC16A* mice (*K14Cre/ΔNC16* or *K14Cre*$^{+}$*NC16A*$^{fl/fl}$), *NC16A* mice were crossed with *Krt-14* promoter driven *Cre* mice provided kindly from Dr. Dennis Roop at University of Colorado at Denver (Jackson Lab #004782). T and B cell-deficient mice (*Rag1*$^{-/-}$ mice, Jackson Lab) and B cell-deficient mice (B6.129S2-*Ighm*^{tm1Cgn}/J mice, Jackson Lab #002288) were crossed with *ΔNC16A* mice to generate n house respectively to generate *Rag1*$^{-/-}$*ΔNC16* mice and B cell-deficient*ΔNC16* mice (*Ighm*^{tm1Cgn}*ΔNC16*), respectively. To confirm the deletion of *NC16A*-encoding exons, tail DNA was analyzed by genomic DNA PCR. The deletion of the NC16A domain in the skin was confirmed by immunoblotting and indirect immunofluorescence using NC16-specific antibodies. To confirm that the deletion of NC16A induced by topical treatment of tamoxifen occurred only in the ear skin, DNA and protein samples from the non-treated skin and several internal organs including bone marrow, spleen and thymus were analyzed by *NC16A*-specific PCR and immunoblotting.

All the mice were bred and housed at the University of North Carolina at Chapel Hill Animal Facility. Animal care, breeding and experiments were conducted in accordance with the

Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Chapel Hill.

Human Skin Biopsy, Blood and Blister Fluid Samples.

After obtaining informed consent from patients and normal healthy individuals, 4-5 mm punch biopsies and 4-6 ml peripheral blood were taken from lesional skin of BP patients (n = 12) and control skin of healthy individuals (n = 12). All the BP patients were untreated and suffered from pruritus. The blister fluid was taken from BP and herpes zoster (n =12). Skin samples were divided into two parts. One part was fixed in formalin and then embedded in paraffin, and the other part was immediately stored at -80^{0}C together with serum and blister fluid samples. The study was approved by the local ethics committees of the Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, Xi'an, Shaanxi, China.

Detection of full-length and NC16A truncated BP180 in the skin by immunoblotting and immunofluorescence.

To detect full-length and NC16A truncated BP180 in the skin by immunoblotting, protein extracts were made from skin tissue and probed with anti-NC16A IgG purified from a patient with BP (termed anti-NC16A antibody) and a home-made rabbit antibody against mouse BP180 noncollagen 1-3 (NC1-3) domains (termed anti-NC1-3 antibody) (see Figure 1). Anti-human (from Southern Biotech) and anti-rabbit (from Cell Signaling) HRP conjugated antibodies were used for NC16A immunoblotting detection. To localize BP180 in the skin by indirect immunofluorescence, cryosections (7μm) of mouse skin were stained with anti-NC16A and anti-NC1-3 antibodies, followed by FITC-conjugated anti-human IgG and anti-rabbit IgG (Jackson ImmunoResearch), respectively.

Epidermis hyperplasia.

The thickness of the ear skin was measured with a digital caliper (Fowler). For each mouse, 4 different sites of the ear were measured and an average thickness was calculated. Ear tissues were collected and fixed in 10% formalin solution, and then embedded in paraffin and cut into slices. Sections were stained with the hematoxylin-eosin solution and photographed by a light

microscope (Zeiss). The thickness of epidermis was measured with Adobe Photoshop software; and the mean of at least 15 randomly selected sections was calculated for each group.

Measurement of total serum IgE by ELISA.

Serum samples were taken at 4, 8 and 12 weeks after birth by heart puncture followed by centrifugation at 3000 g for 10 min at 4℃. The total IgE in mouse serum was measured by using the mouse ELISA kit (Mouse IgE ELISA MAX^{TM} Deluxe, Bio-Legend).

Toluidine blue penetration assay of whole mouse embryos (E18.5).

Heterozygotes NC16A$^{+/-}$ mice were bred and E18.5 embryos were isolated from pregnant mice. Isolated embryos were dehydrated with chilled methanol, and then stained by immersing in 0.1% toluidine blue (TB) solution. TB stained embryos were then washed with PBS to remove excessive staining before pictures were taken (2, 3). Tail DNA were analyzed by PCR to identify genotype of each of the embryos after the toluidine blue penetration assay.

Evans Blue dye penetration assay.

For examining skin barrier function of adult skin, Evans Blue dye penetration assay was performed to detect barrier impairment. Mice were applied topically with Evans blue (1% in PBS, 50 ul/ear, Sigma). Two hours later mouse ear skin was collected and homogenized in formamide (Sigma). Retained amounts of Evans Blue with the skin were quantified by OD reading at 620 nm (4, 5).

FITC-conjugated albumin (BSA) permeability assay.

To assay the skin permeability for high molecular weight molecules, adult mouse ears were applied topically with FITC-conjugated BSA (0.5% in PBS, 25 ul/ear, Sigma), and 2 hours later fixed in 10% PBS-buffered formalin overnight. Fixed ear tissue was OCT embedded, sectioned and examined under a fluorescent microscope (Zeiss) to identify FITC-BAS in the skin (6).

RNA isolation, q-RT-PCR and microarray analysis.

Skin RNA was isolated from three *NC16A* (WT) and *ΔNC16A* (KO) mice, and all of them are age and sex matched. RNA extraction and purification were performed using RNeasy Fibrous

Tissue Mini Kit (Qiagen, USA). Isolated RNA from skin were used for qRT-PCR for detection of *Nc16a*, *Il4*, *Il13*, *Ifn-γ*, *Tslp*, *Il1β*, *Tnfα* (Supplemental Table S2), and for whole transcriptome microarray analysis (primer sequence for qRT-PCR are described in Supplemental Material and Method). The microarray was acquired from Agilent (Mouse Gene Expression 4x44K Microarray Kit) and performed by Microarray Core of University of North Carolina at Chapel Hill. The dataset was uploaded to NCBI GEO database and can be access by the following link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE112235.

Detection of T cells, Neutrophil, Eosinophil and Mast Cells.

The skin sample was frozen in Tissue-Tek OCT, and cryostat sections were air-dried, blocked with PBS containing 10% of normal goat serum for 5 mins and then stained with anti-CD3 (BioLegend, clone:17A2, 1:500 dilution) for T cells, anti-Iy6G (BioLegend,clone:1A8,1:1000 dilution) for neutrophils, or anti-MBP (Mayo Clinic 4 Scottsdale, 1:500 dilution) for eosinophils for 1 hour at room temperature followed by Alexa Fluor 488 Goat Anti-Rat IgG (Life Technologies; 1:1000 dilution) for 1 hour at room temperature.

To quantify the number of mast cells in the skin, ear skin sections were stained with toluidine blue (TB) (7).The mast cells of at least five randomly selected areas per section in a high-power field (magnification ×200) were counted in the upper dermis and expressed as mean ± SE.

Itch Quantification.

The frequency of scratching behavior was measured by video-taping for a 15-20 mins period. Licking of the belly and dorsal skin during grooming was disregarded and only back feet moved across face are counted. Each occurrence of back leg scratched crossed its neck, ears and nose were counted and used for statistics analysis (8). The data is expressed as # of scratching/15 min video-taping + SE.

Histamine EIA Quantification and Histamine Receptor Blockade.

Histamine levels in skin tissue and serum sample were quantified by histamine enzyme immunoassay kit (Bertin Pharma) following the procedure described by the manufacturer's instruction. WT mice at 8 weeks old were injected with 20 ug histamine on the cheek. To block histamine activity, histamine 1 receptor (H1R) antagonist (Olopatadine, 3 mg/kg) and/or

histamine 4 receptor (H4R) antagonist (JNJ7777120, 30 mg/kg) in 0.5% carboxyl methyl-cellulose were administrated orally with KO mice or WT mice 1 hour before histamine injection (9). Both antagonists were obtained from Sigma, USA. Mice were videotaped for 15 mins before and after HR blocking to quantify changes in number of scratching.

Measurement of TSLP and TSLP Blockade.

TSLP levels in skin lysate, serum, blister fluid and supernatants of cell culture were detected and quantitated by using the mouse or human ELISA kit (R&D). To block TSLP activity in the skin, skin*ΔNC16A* mice with severe itch or WT mice at 8 weeks old were injected at both ears with 20 ug of neutralizing TSLP antibody (Biolegend, cat# 515202) or IgG2a control antibody (in 20 ul of PBS, Santa Cruz) (10). After 1 hour post injection, WT mice were then injected with 25ng TSLP (in 20ul of PBS, R&D) at both ears. The mice were videotaped for scratching for 15 mins right before and one hour after antibody injection.

Immunohistology.

Paraffin embedded sections (7 um) of fixed mouse and human skin were cut, and heat-induced epitope retrieval was performed in a 0.05% sodium citrate buffer (pH 6.0). The slides were incubated with rabbit anti-mouse TSLP (ab188766, Abcam, 1:50 dilution) or rabbit anti-human TSLP (ab47943, Abcam, 1:100 dilution) at 4^0C overnight and then washed with PBS twice. A HRP-labeled secondary antibody (svooo2, Boster, 1:500 dilution) was used with a DAB chromogen.

Keratinocyte Culture.

Primary keratinocyte isolation and culture were described before (11). Briefly, keratinocytes were isolated from the skin of Tam*Cre-NC16A* neonatal mice 48 hours after birth and maintained in E medium with 15% FBS and 50 μM CaCl2 (low Ca2+ medium). E medium is in house produced and provided by Dr. Scott E Williams from UNC-Chapel Hill (11). To generated *ΔNC16A* keratinocyte culture, keratinocyte isolated from Tam*Cre-NC16A* mice was treated with 4-Hydroxyltamoxifen (4OHT, 125 ng/ml), while the non-treated keratinocyte isolate from Tam*Cre-NC16A* was used as WT control. After 4OHT treatment, *ΔNC16A* keratinocytes were passaged in regular keratinocyte culture media and *NC16A* deletion was confirmed by PCR. Both

WT and *ΔNC16A* keratinocytes were stimulated with mouse TNFα (0-10 ng/ml in PBS). Supernatants were collected 12 hrs and 24 hrs after TNFα stimulation.

Statistical Analysis. The data were expressed as mean ± SE and were analyzed using the Student's *t*-test. A *p* value less than 0.05 was considered significant.

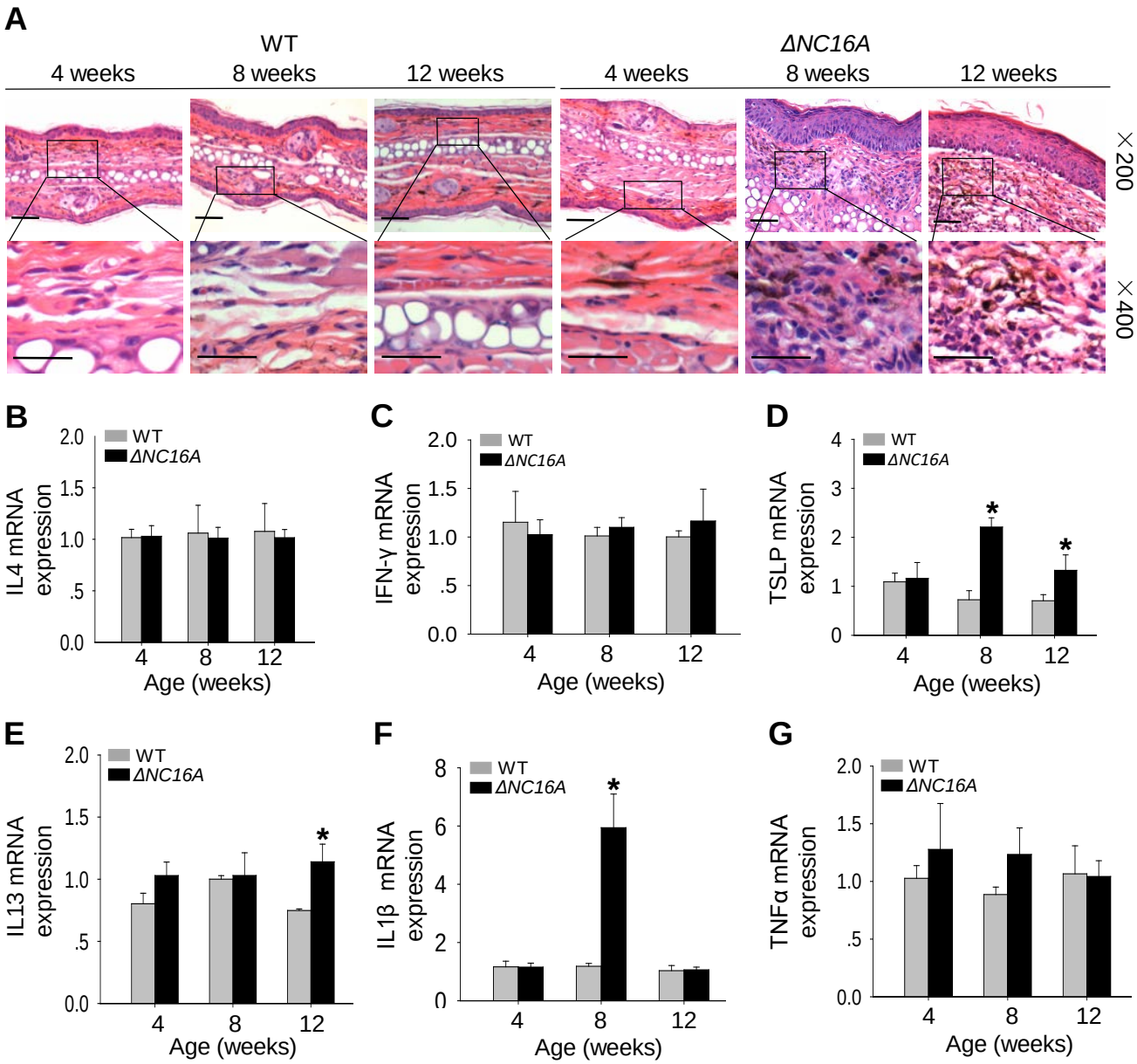

Fig. S1. Skin inflammation and Quantification of cytokines at mRNA levels in *ΔNC16A* mice. H/E of ear skin revealed minor skin inflammation at 8 weeks old and minor epidermal/dermal separation at 12 weeks old in *ΔNC16A* mice compared to WT mice. Original magnification, x200. (scale bar = 50 μm). Ear skin showed that *ΔNC16A* mice started to have significantly increased infiltrating cells starting at 8 weeks old. Original magnification, x400. (scale bar = 50 μm). mRNA levels of AD-related cytokines in the skin of *NC16A* (WT) and *ΔNC16A* mice at 4, 8, and 12 weeks old were analyzed by quantitative RT-PCR (qRT-PCR). *ΔNC16A* mice showed significantly increased levels of TSLP (*D*), IL-13 (*E*) and IL-1β (*F*) compared to WT, while there were no difference between *ΔNC16A* (KO) and WT mice in IL-4 (*B*), IFN-γ (*C*), and TNFα (*G*). (*$p < 0.05$, Student's *t*-test, $n = 5$/group).

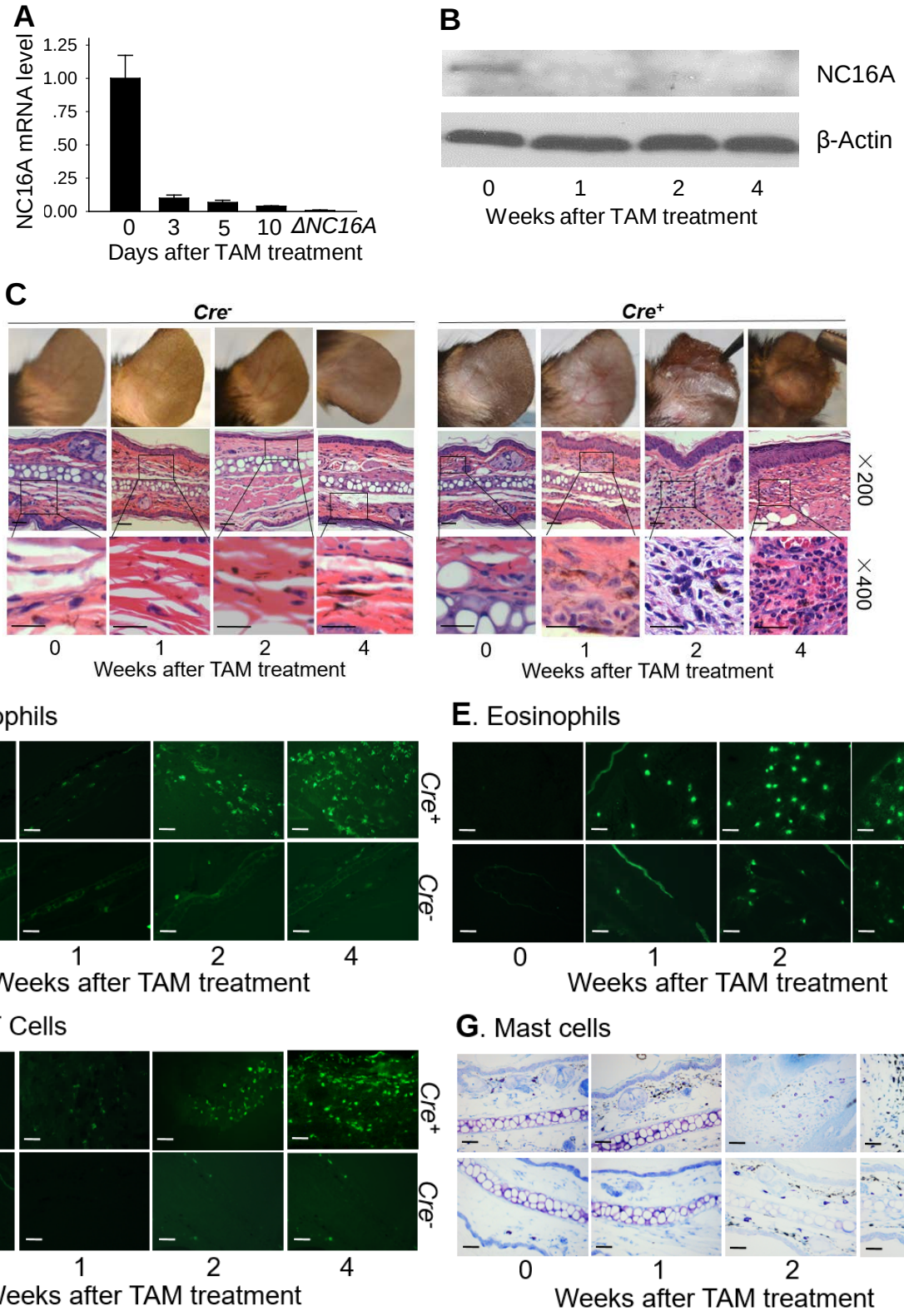

Figure S2. Generation of skin-specific ΔNC16A (skinΔNC16A) mice. The ears of *ERCre*$^{+}$*NC16A*$^{fl/fl}$ mice were topically treated with tamoxifen to induce Skin*ΔNC16A*, while *ERCre*$^{-}$*NC16A*$^{fl/fl}$ mice treated with tamoxifen were using as control mice. (*A*) Quantitative RT-PCR showed drastically reduction of full-length BP180 mRNA. mRNA from whole body *ΔNC16A* mice was used as control. (*B*) Immunoblotting of ear skin protein extract confirmed deletion of *NC16A* domain of BP180 in skin *ΔNC16A* mice using anti-NC16A antibody. (*C*) The ears of skinΔNC16A mice started showing skin lesions clinically and histologically at (Original magnification, x200. scale bar = 50 μm) and increased immune cells infiltration (Original magnification, x400. scale bar = 50 μm) 2 weeks after tamoxifen treatment compared to control mice (*Cre*$^{-}$). Immune staining of ear skin showed that skin*ΔNC16A* mice have significantly increased infiltrating neutrophil (*D*), eosinophil (*E*) and CD3 positive T cells (*F*) starting at 1-2 weeks post tamoxifen treatment. Toluidine blue (TB) staining indicated that there was a significant increase in mast cells skin*ΔNC16A* mice starting at 1 week post tamoxifen treatment (*G*). Original magnification, x200 (scale bar = 50 μm). *n* = 8/group.

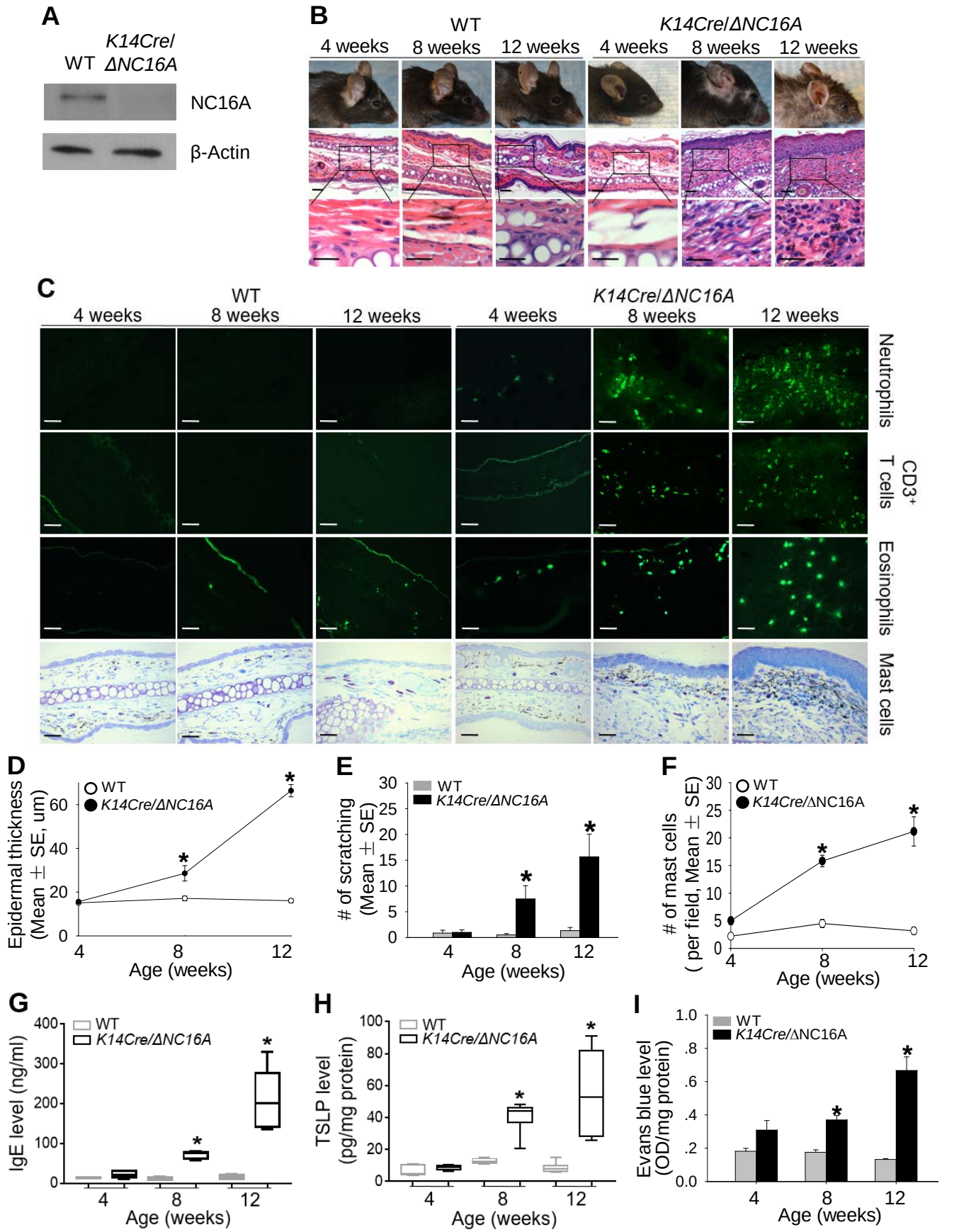

Fig. S3. Basal keratinocyte-specific *ΔNC16A (K14Cre/ΔNC16A)* mice exhibit AD-like skin inflammation. (*A*) Deletion of NC16A in *K14Cre/ΔNC16A* mice was confirmed by IB using anti-NC16A antibody. (*B-C*) As compared to WT control, *K14Cre/ΔNC16A* mice start showing clinical skin lesions, increased epidermal thickness by routine histology examination and increased infiltrating neutrophils, $CD3^+$ T cells, eosinophils and mast cells by indirect immunofluorescence and toluidine blue staining staring at 8 weeks old (scale bar = 50 μm). *K14Cre/ΔNC16A* mice also exhibited a significant increase of epidermis thickness (*D*), scratching (*E*), mast cell infiltration (*F*), IgE (*G*), TSLP in leisonal skin (*H*) and impaired barrier function(*I*).). (*$p< 0.05$, Student's *t*-test, *n* = 8/group, graph *D*, *E*,*F* and *I* show mean ± SE).

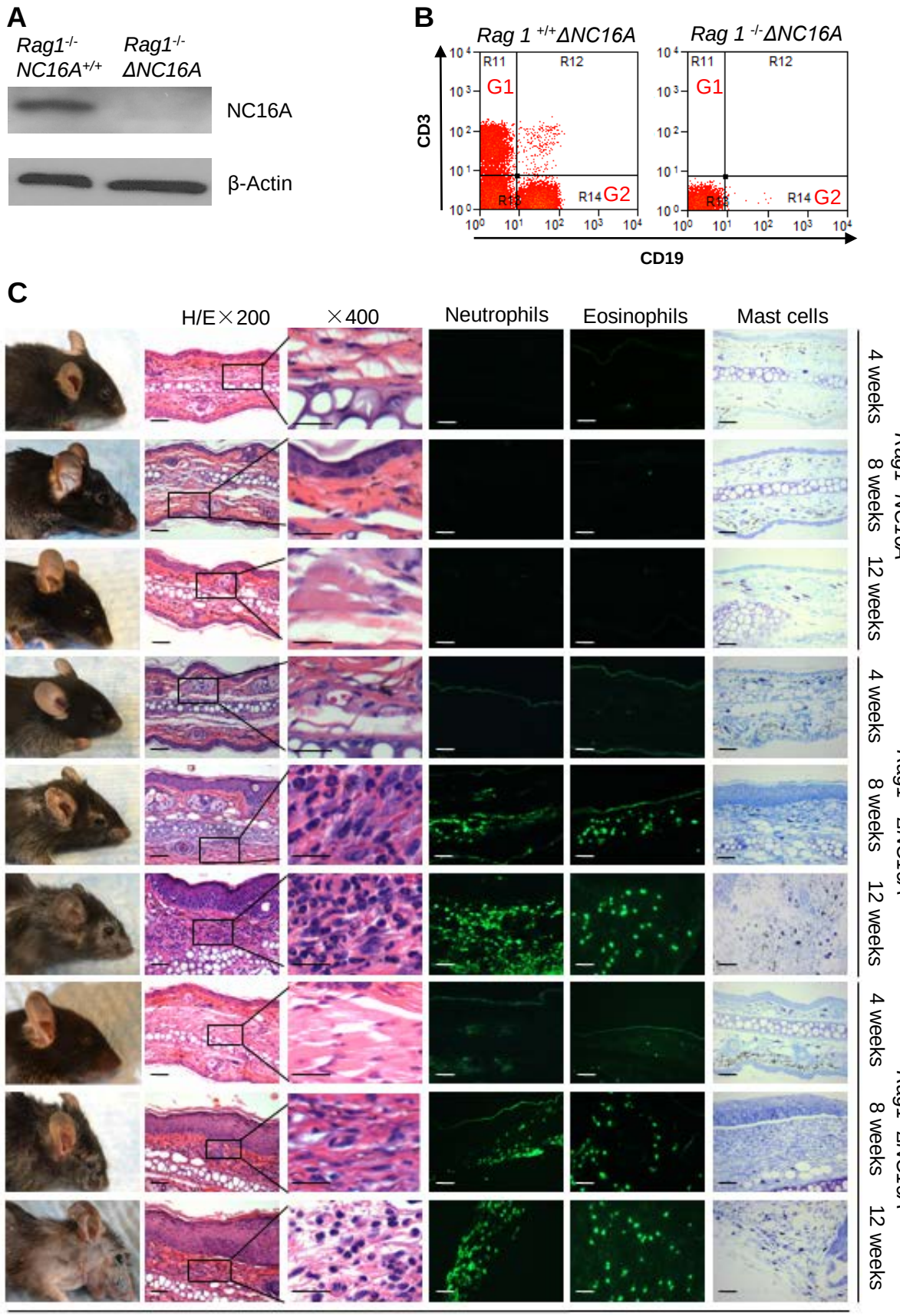

Fig. S4. *ΔNC16A* mice deficient of both T and B cells *(Rag1$^{-/-}$ΔNC16A)* develop similar AD-like skin inflammation as whole body *ΔNC16A* mice. *ΔNC16A* mice were crossed with *Rag1$^{-/-}$* mice to generate *Rag1$^{-/-}$ΔNC16A* mice. (*A*) Immunoblotting of ear skin protein extract using anti-NC16A antibody confirmed NC16A deletion in *Rag1$^{-/-}$ΔNC16A* mice. (*B*) Flow cytometry confirmed that CD3^{+}T cell (G1) and CD19^{+}B cell (G2) were absent in *Rag1$^{-/-}$ΔNC16A* mice. (*C*) Similar to *ΔNC16A* mice, *Rag1$^{-/-}$ΔNC16A* mice developed inflammatory skin lesion by clinical examination, epidermal hyperplasia and immune cell infiltration by H/E of ear skin, and increased infiltration of neutrophils, eosinophils and mast cells by indirect IF and toluidine blue staining starting at 8 weeks old compared to *Rag1$^{-/-}$NC16A* mice. *n* = 4-6 mice/group. (scale bar = 50 μm).

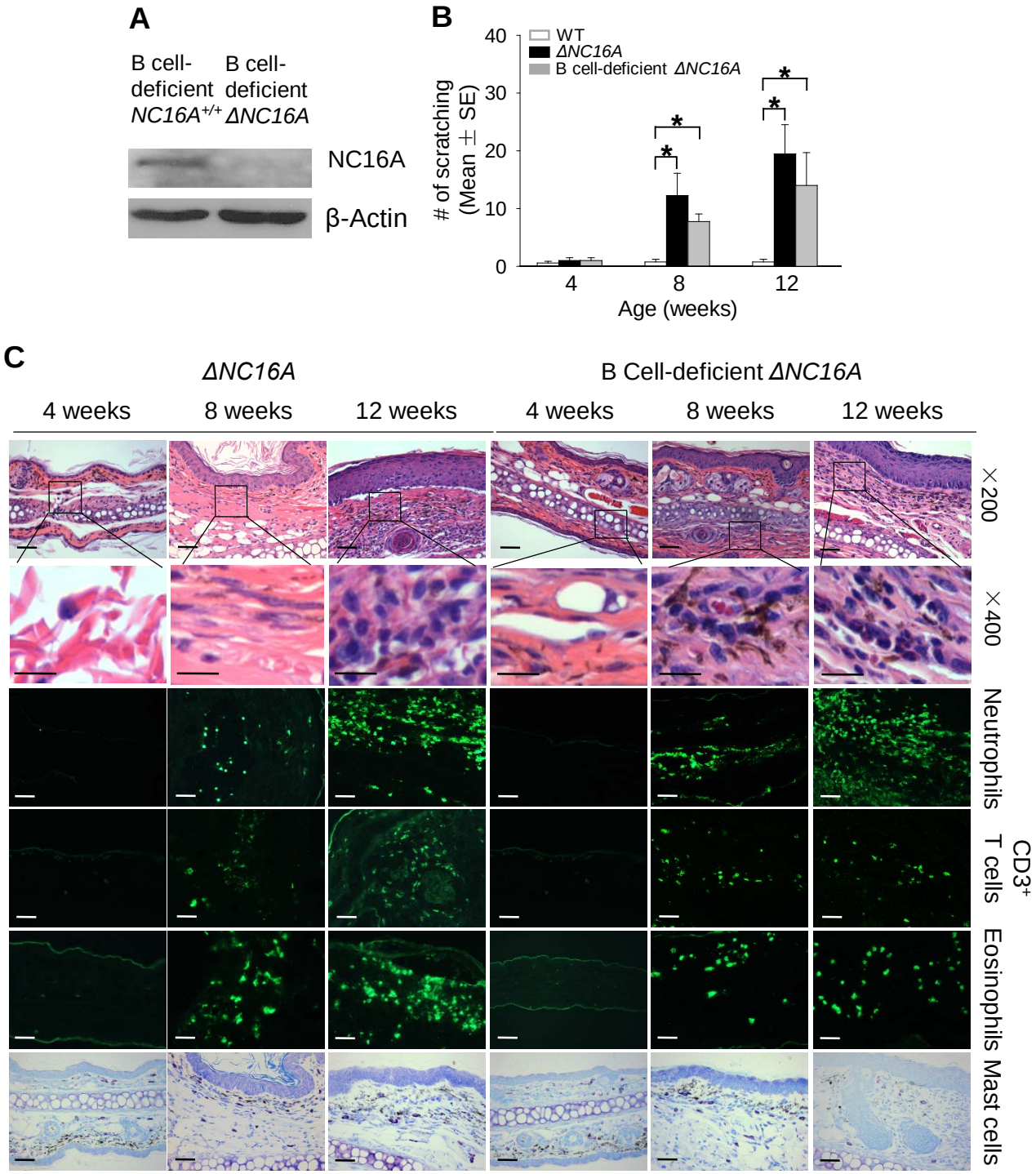

Fig S5. B cell-deficient *ΔNC16A* mice develop AD-like skin inflammation. *ΔNC16A* mice were crossed with B cell-deficient mice to generate B cell-deficient *ΔNC16A* mice. (*A*) NC16A deletion in B cell-deficient *ΔNC16A* mice was confirmed by immunoblotting using anti-NC16A antibody. (*B*) B cell-deficient *ΔNC16A* mice exhibited a similar degree of itch as *ΔNC16A* mice. (*C*) B cell-deficient *ΔNC16A* mice and *ΔNC16A* mice showed the same degree of increased epidermal thickness by H/E (Original magnification, x200 and 400), infiltration of neutrophils, $CD3^+$ T cells, and eosinophils by indirect IF, increased mast cells by toluidine blue staining. (scale bar = 50 μm), (*$p < 0.05$, Student's *t*-test, $n = 5$/group, graph *B* show mean ± SE).

Table S1. Increased expression of proinflammatory cytokines in *ΔNC16A* mice. Skin RNA was isolated from three age- and sex-matched *NC16A* (WT) and *ΔNC16A* (KO) mice (8 weeks old) and were used for whole transcriptome microarray analysis. Increased expression of proinflammatory cytokines and chemokines associated with dermatitis are listed.

Agilent Microarray ID	Name of Genes in dataset	Exp Fold Change	*P* value
A_55_P1953169	*Saa3*	32.70	0.057
A_55_P1998471	*S100a9*	17.89	0.005
A_55_P2156697	*Il17a*	11.99	0.004
A_55_P1990032	*Cxcl5*	9.02	0.053
AGI_MM_OLIGO_A_51_P217463	*Cxcl2*	8.11	0.063
AGI_MM_OLIGO_A_51_P509573	*Ccl4*	7.00	0.0622
A_55_P2128144	*Il19*	5.40	0.083
A_55_P2103249	*Cxcr1*	4.93	0.0672
AGI_MM_OLIGO_A_51_P363187	*Cxcl1*	4.58	0.0249
A_55_P2070869	*Lcn2*	4.34	0.00386
A_55_P1962209	*Cxcr6*	2.81	0.0342
A_66_P132710	*Il20*	2.23	0.0213
A_55_P2147712	*Ctla4*	2.05	0.0086
A_66_P109708	*Il1f6*	2.03	0.0641
A_55_P2138386	*Il5*	0.57	0.028

Table S2. Primer sequences for qRT-PCR. Primers sequences of *Il4*, *Il13*, *Ifn-γ*, *Tslp*, *Il1β*, *Tnfα.*

Gene	Forward primer sequence(5'-3')	Reverse primer sequence(5'-3')
Il4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
Il13	CCTGGCTCTTGCTTGCCTT	GGTCTTGTGTGATGTTGCTCA
Ifn-γ	GAAAATCCTGCAGAGCCAGATT	TGATGGCCTGATTGTCTTTCAA
Tslp	GACTGTGAGAGCAAGCCAGCT	CTCCGGGCAAATGTTTGTC
Tnfα	GACCCTCACACTCAGATCATCTTCT	CCTCCACTTGGTTTGCT
Il1β	AGTTGACGGACCCCAAAAGAT	GTGCAGTTGTCTAATGGGAACGT

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