

# Supplementary Information for

## **BP180 dysfunction triggers spontaneous skin inflammation in mice**

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1 **Supporting Information Appendix (SI Appendix):**

2

3 **Materials and Methods**

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

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

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

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

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

3

4 **Human Skin Biopsy, Blood and Blister Fluid Samples.**

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

13

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

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

25

26 **Epidermis hyperplasia.**

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

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

3

#### 4 **Measurement of total serum IgE by ELISA.**

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

8

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

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

15

#### 16 **Evans Blue dye penetration assay.**

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

22

#### 23 **FITC-conjugated albumin (BSA) permeability assay.**

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

28

#### 29 **RNA isolation, q-RT-PCR and microarray analysis.**

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

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

8

### 9 **Detection of T cells, Neutrophil, Eosinophil and Mast Cells.**

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

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

19

### 20 **Itch Quantification.**

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

26

### 27 **Histamine EIA Quantification and Histamine Receptor Blockade.**

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

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

#### 6 **Measurement of TSLP and TSLP Blockade.**

7 TSLP levels in skin lysate, serum, blister fluid and supernatants of cell culture were detected and  
8 quantitated by using the mouse or human ELISA kit (R&D). To block TSLP activity in the skin,  
9 skin $\Delta$ NC16A mice with severe itch or WT mice at 8 weeks old were injected at both ears with 20  
10 ug of neutralizing TSLP antibody (Biolegend, cat# 515202) or IgG2a control antibody (in 20 ul  
11 of PBS, Santa Cruz) (10). After 1 hour post injection, WT mice were then injected with 25ng  
12 TSLP (in 20ul of PBS, R&D) at both ears. The mice were videotaped for scratching for 15 mins  
13 right before and one hour after antibody injection.

#### 15 **Immunohistology.**

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

#### 23 **Keratinocyte Culture.**

24 Primary keratinocyte isolation and culture were described before (11). Briefly, keratinocytes  
25 were isolated from the skin of TamCre-NC16A neonatal mice 48 hours after birth and maintained  
26 in E medium with 15% FBS and 50  $\mu$ M CaCl<sub>2</sub> (low Ca<sup>2+</sup> medium). E medium is in house  
27 produced and provided by Dr. Scott E Williams from UNC-Chapel Hill (11). To generated  
28  $\Delta$ NC16A keratinocyte culture, keratinocyte isolated from TamCre-NC16A mice was treated with  
29 4-Hydroxyltamoxifen (4OHT, 125 ng/ml), while the non-treated keratinocyte isolate from  
30 TamCre-NC16A was used as WT control. After 4OHT treatment,  $\Delta$ NC16A keratinocytes were  
31 passaged in regular keratinocyte culture media and NC16A deletion was confirmed by PCR. Both

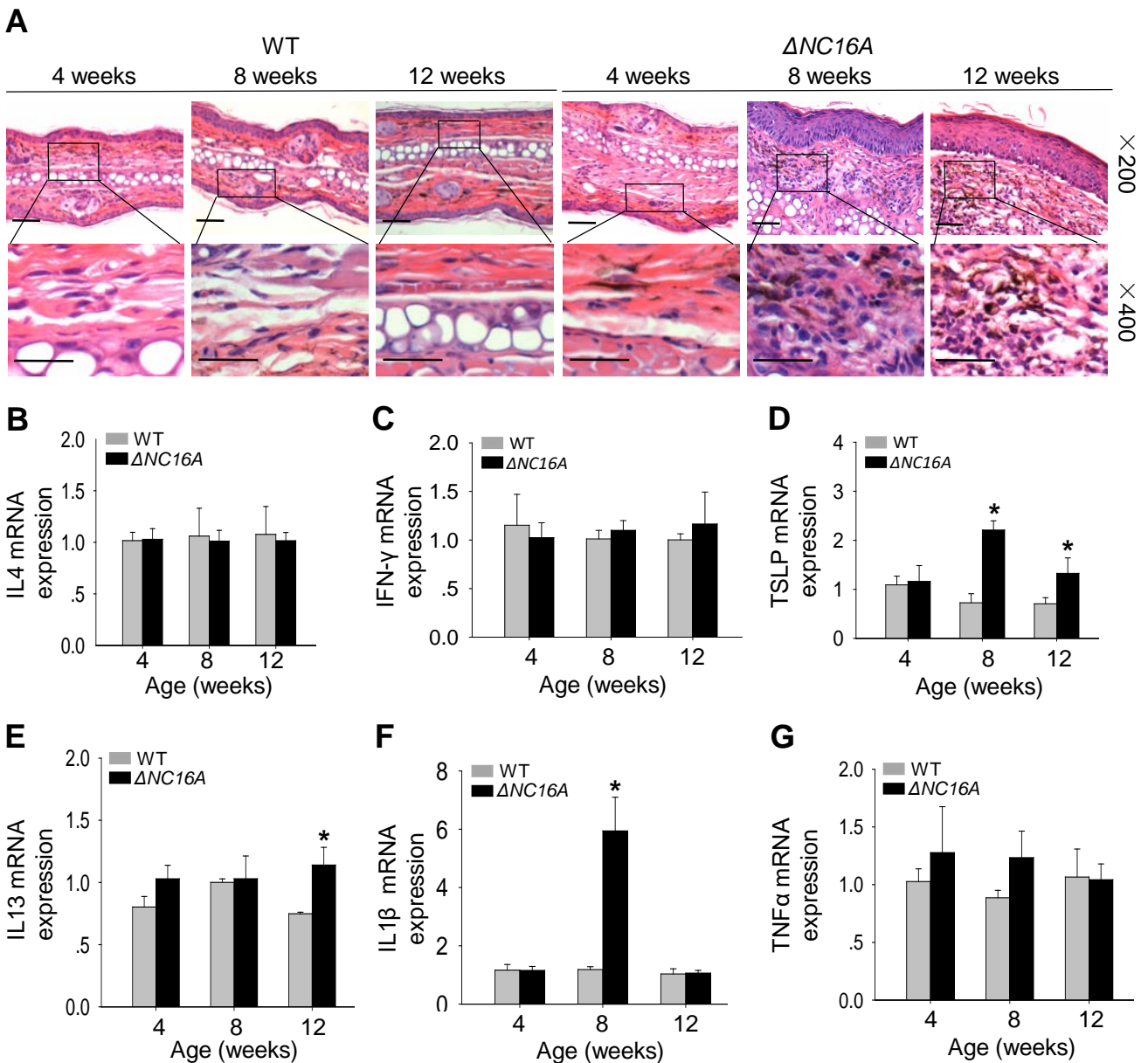
1 WT and  $\Delta NC16A$  keratinocytes were stimulated with mouse TNF $\alpha$  (0-10 ng/ml in PBS).  
2 Supernatants were collected 12 hrs and 24 hrs after TNF $\alpha$  stimulation.

3

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

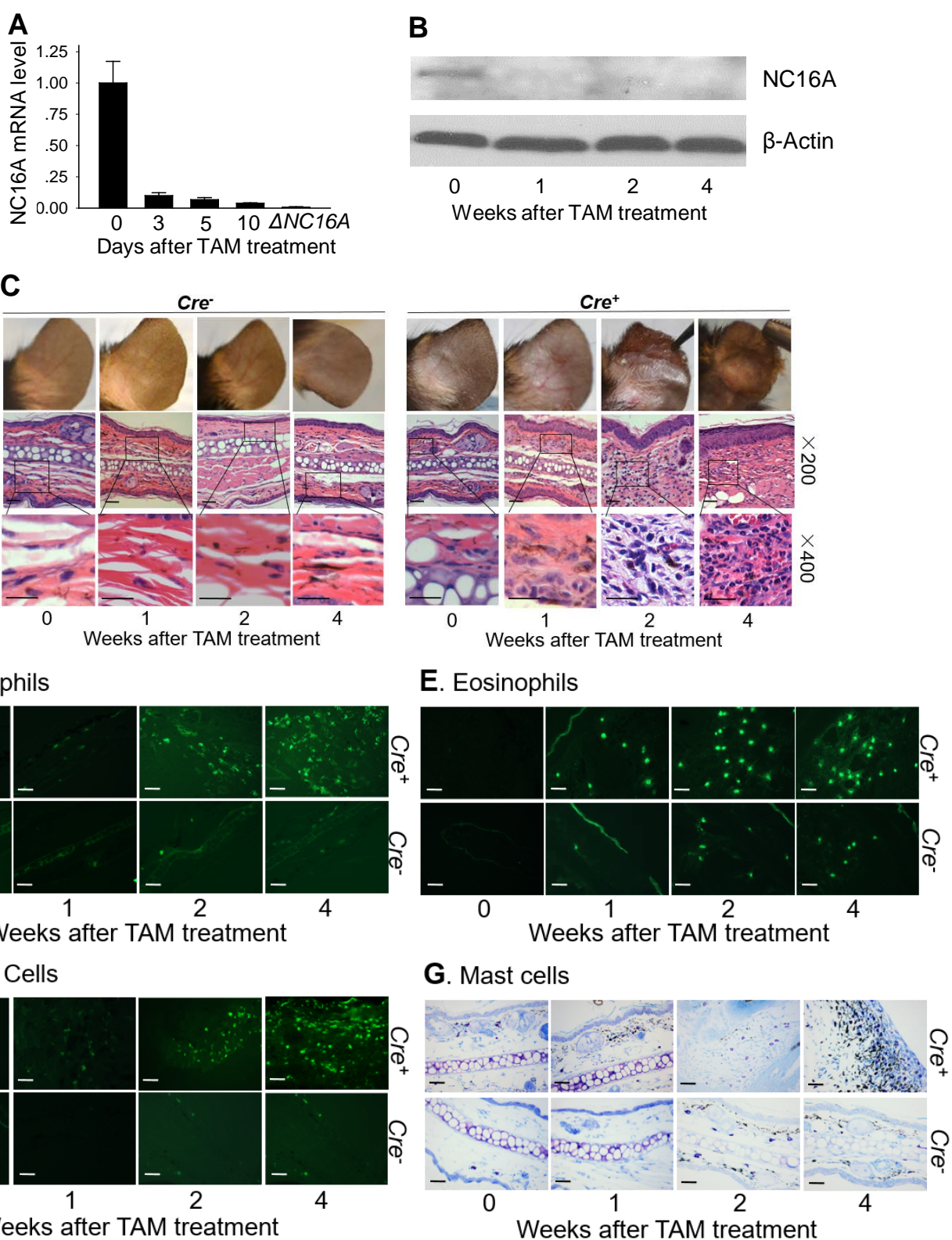
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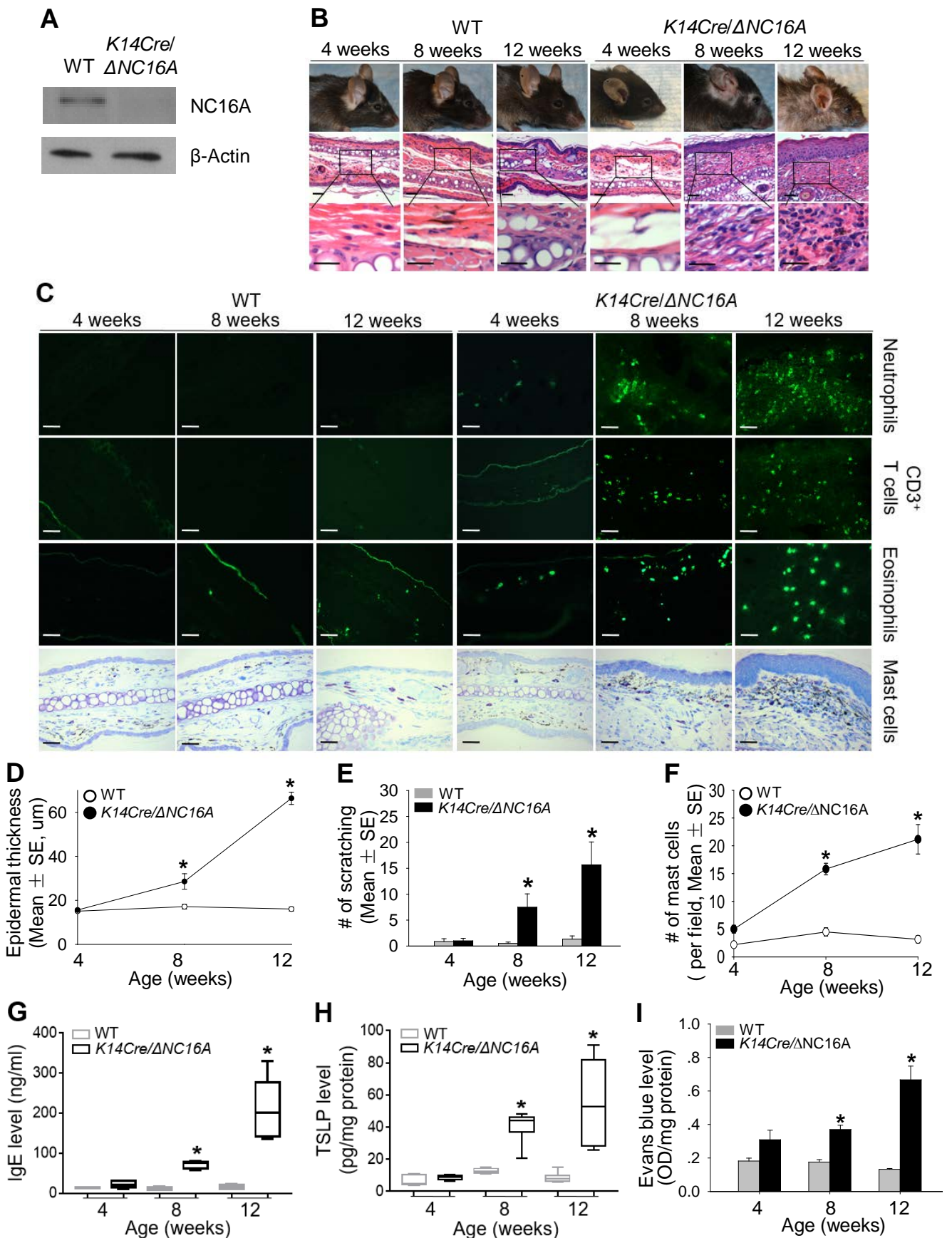


**Fig. S1. Skin inflammation and Quantification of cytokines at mRNA levels in  $\Delta NC16A$  mice.** H/E of ear skin revealed minor skin inflammation at 8 weeks old and minor epidermal/dermal separation at 12 weeks old in  $\Delta NC16A$  mice compared to WT mice. Original magnification, x200. (scale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





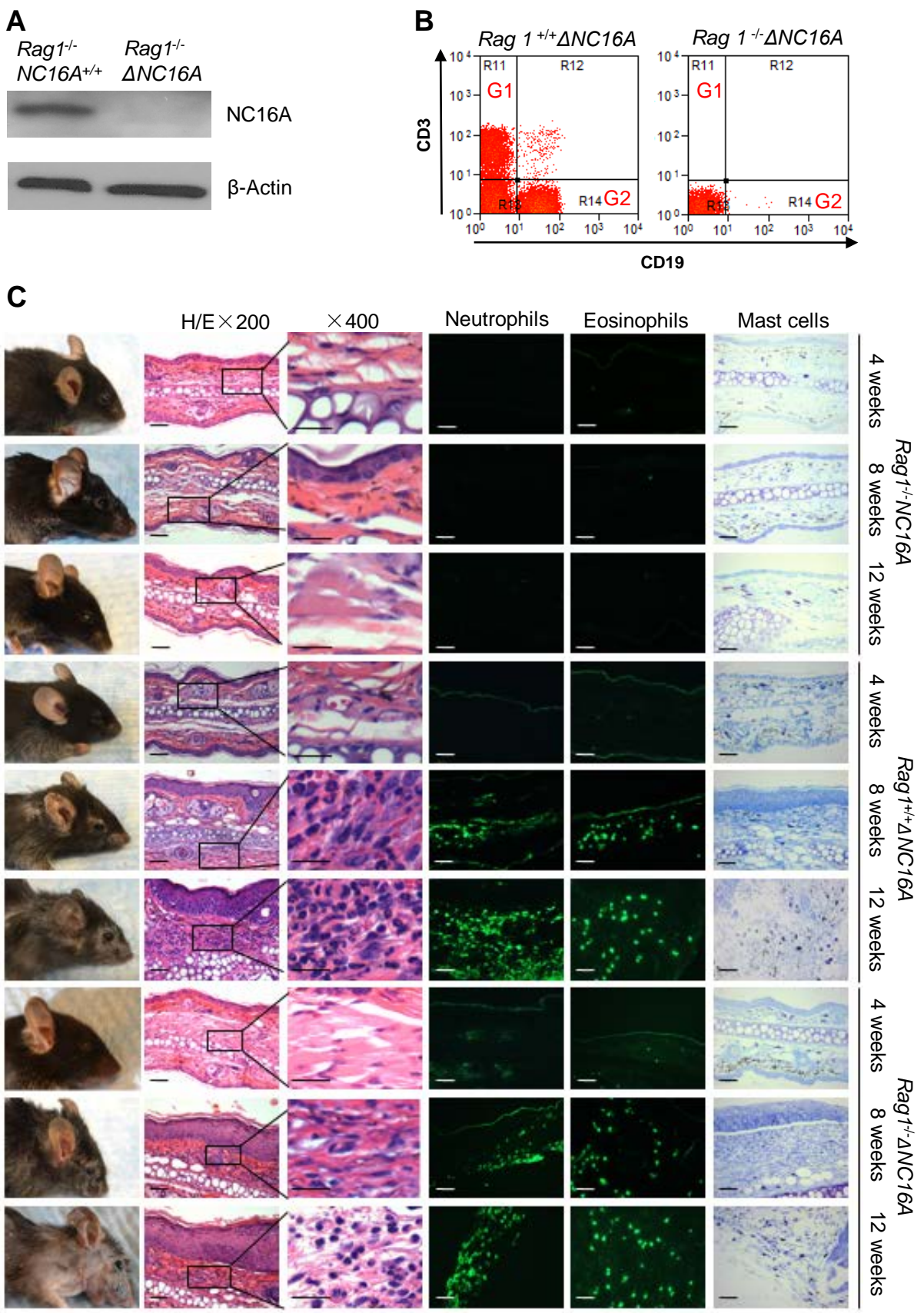
**Figure S2. Generation of skin-specific  $\Delta NC16A$  (skin $\Delta NC16A$ ) mice.** The ears of  $ERCre^+NC16A^{fl/fl}$  mice were topically treated with tamoxifen to induce Skin $\Delta NC16A$ , while  $ERCre^-NC16A^{fl/fl}$  mice treated with tamoxifen were used as control mice. (A) Quantitative RT-PCR showed drastic reduction of full-length BP180 mRNA. mRNA from whole body  $\Delta NC16A$  mice was used as control. (B) Immunoblotting of ear skin protein extract confirmed deletion of NC16A domain of BP180 in skin  $\Delta NC16A$  mice using anti-NC16A antibody. (C) The ears of skin $\Delta NC16A$  mice started showing skin lesions clinically and histologically at (Original magnification,  $\times 200$ , scale bar = 50  $\mu m$ ) and increased immune cells infiltration (Original magnification,  $\times 400$ , scale bar = 50  $\mu m$ ) 2 weeks after tamoxifen treatment compared to control mice ( $Cre^-$ ). Immune staining of ear skin showed that skin $\Delta NC16A$  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 $\Delta NC16A$  mice starting at 1 week post tamoxifen treatment (G). Original magnification,  $\times 200$  (scale bar = 50  $\mu m$ ).  $n = 8$ /group.



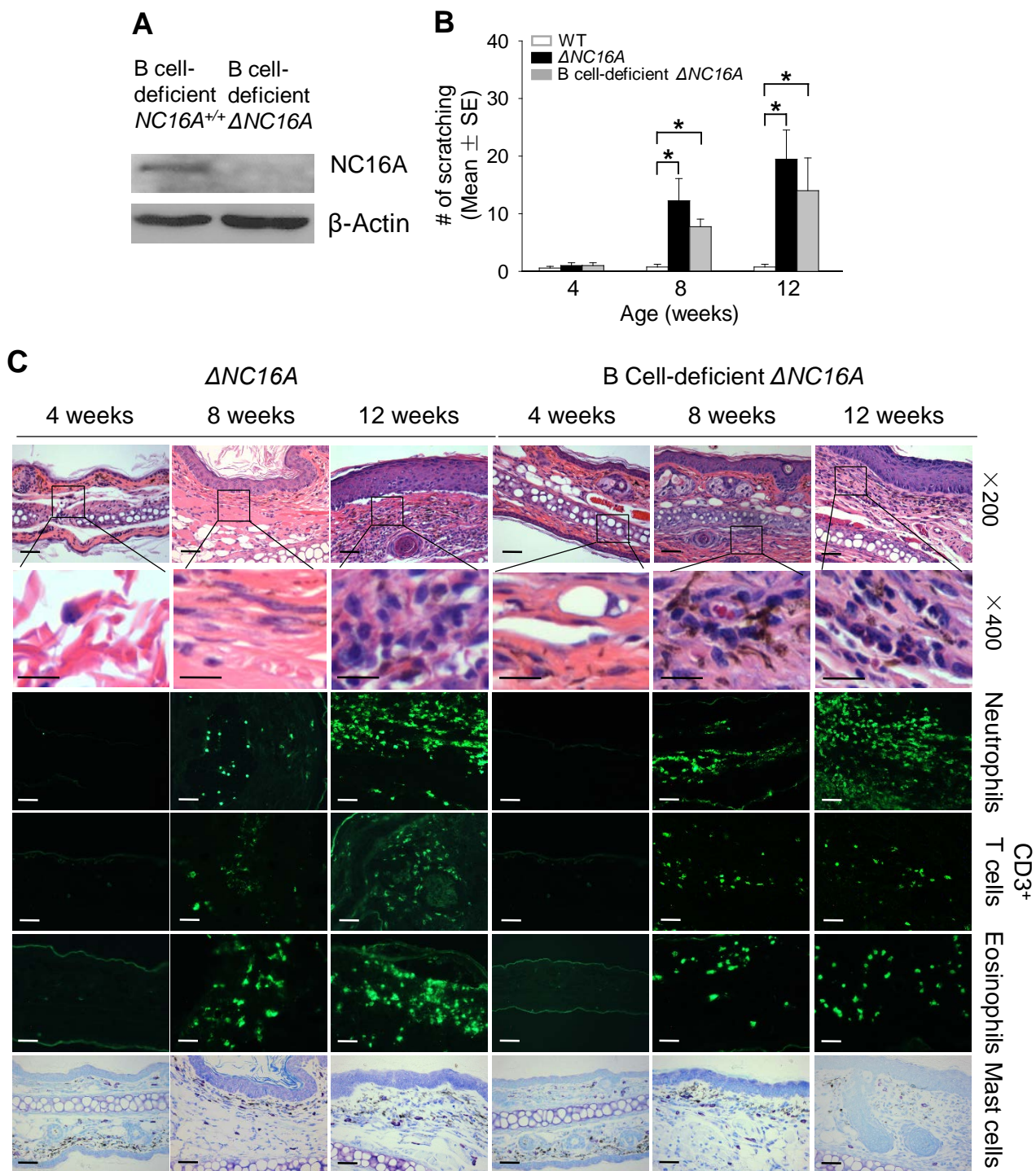
**Fig. S3. Basal keratinocyte-specific  $\Delta$ NC16A (*K14Cre/delta*NC16A) mice exhibit AD-like skin inflammation.**

(A) Deletion of NC16A in *K14Cre/delta*NC16A mice was confirmed by IB using anti-NC16A antibody. (B-C) As compared to WT control, *K14Cre/delta*NC16A mice start showing clinical skin lesions, increased epidermal thickness by routine histology examination and increased infiltrating neutrophils, CD3<sup>+</sup> T cells, eosinophils and mast cells by indirect immunofluorescence and toluidine blue staining starting at 8 weeks old (scale bar = 50  $\mu$ m). *K14Cre/delta*NC16A mice also exhibited a significant increase of epidermis thickness (D), scratching (E), mast cell infiltration (F), IgE (G), TSLP in lesional 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  $\pm$  SE).





**Fig. S4.  $\Delta$ NC16A mice deficient of both T and B cells (*Rag1*<sup>-/-</sup> $\Delta$ NC16A) develop similar AD-like skin inflammation as whole body  $\Delta$ NC16A mice.**  $\Delta$ NC16A mice were crossed with *Rag1*<sup>-/-</sup> mice to generate *Rag1*<sup>-/-</sup> $\Delta$ NC16A mice. (A) Immunoblotting of ear skin protein extract using anti-NC16A antibody confirmed NC16A deletion in *Rag1*<sup>-/-</sup> $\Delta$ NC16A mice. (B) Flow cytometry confirmed that CD3<sup>+</sup>T cell (G1) and CD19<sup>+</sup>B cell (G2) were absent in *Rag1*<sup>-/-</sup> $\Delta$ NC16A mice. (C) Similar to  $\Delta$ NC16A mice, *Rag1*<sup>-/-</sup> $\Delta$ NC16A 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*<sup>-/-</sup>*NC16A* mice. *n* = 4-6 mice/group. (scale bar = 50  $\mu$ m).



**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<sup>+</sup> 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  $\Delta NC16A$  mice.** Skin RNA was isolated from three age- and sex-matched *NC16A* (WT) and  $\Delta NC16A$  (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	<i>Saa3</i>	32.70	0.057
A_55_P1998471	<i>S100a9</i>	17.89	0.005
A_55_P2156697	<i>Il17a</i>	11.99	0.004
A_55_P1990032	<i>Cxcl5</i>	9.02	0.053
AGI_MM_OLIGO_A_51_P217463	<i>Cxcl2</i>	8.11	0.063
AGI_MM_OLIGO_A_51_P509573	<i>Ccl4</i>	7.00	0.0622
A_55_P2128144	<i>Il19</i>	5.40	0.083
A_55_P2103249	<i>Cxcr1</i>	4.93	0.0672
AGI_MM_OLIGO_A_51_P363187	<i>Cxcl1</i>	4.58	0.0249
A_55_P2070869	<i>Lcn2</i>	4.34	0.00386
A_55_P1962209	<i>Cxcr6</i>	2.81	0.0342
A_66_P132710	<i>Il20</i>	2.23	0.0213
A_55_P2147712	<i>Ctla4</i>	2.05	0.0086
A_66_P109708	<i>Il1f6</i>	2.03	0.0641
A_55_P2138386	<i>Il5</i>	0.57	0.028

**Table S2. Primer sequences for qRT-PCR.** Primers sequences of *Il4*, *Il13*, *Ifn- $\gamma$* , *Tsp*, *Il1 $\beta$* , *Tnfa*.

Gene	Forward primer sequence(5'-3')	Reverse primer sequence(5'-3')
<i>Il4</i>	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
<i>Il13</i>	CCTGGCTCTTGCTTGCCTT	GGTCTTGTGTGATGTTGCTCA
<i>Ifn-<math>\gamma</math></i>	GAAAATCCTGCAGAGCCAGATT	TGATGGCCTGATTGTCTTTCAA
<i>Tsp</i>	GACTGTGAGAGCAAGCCAGCT	CTCCGGGCAAATGTTTGTC
<i>Tnfa</i>	GACCCTCACACTCAGATCATCTTCT	CCTCCACTTGGTTTGCT
<i>Il1<math>\beta</math></i>	AGTTGACGGACCCCAAAAGAT	GTGCAGTTGTCTAATGGGAACGT



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