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Complement C3 Exacerbates Imiquimod-Induced Skin Inflammation and Psoriasiform Dermatitis

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TO THE EDITOR

The complement system is pivotal in protection against pathogens, but it also plays important roles in bridging innate and adaptive immune responses (Scott and Botto, 2016) and in modulating local and systemic inflammation (Markiewski and Lambris, 2007). Activation of complement occurs through three different pathways (classical, alternative, and lectin), converges at C3 cleavage, and culminates in the formation of the membrane attack complex. The anaphylatoxic fragments, C3a and C5a, generated during the proteolytic cascade, recruit immune cells that can promote the removal of debris and pathogens, but they can also cause tissue damage (Markiewski and Lambris, 2007).

The main source of complement is the liver. However, locally produced complement, particularly C3, can modulate inflammation in a variety of organs. There is also evidence that complement components are produced not only by immune cells such as

macrophages and dendritic cells but also by nonimmune cells, which can contribute to local complement synthesis. In the skin, keratinocytes are a potential source of C3 (Pasch et al., 2000). A role for C3 in the skin has been identified in UV-induced immune tolerance (Hammerberg et al., 1998) and in the protection against T-cell-mediated inflammation (Purwar et al., 2011). Furthermore, locally produced C3 contributes to the inflammatory responses accompanying wound healing (Rafail et al., 2014), an effect mediated mainly through C5a/C5aR interactions. The latter also play an important role in influencing the skin microbiome (Chehoud et al., 2013). In keeping with these observations in animal models, complement has been shown to have a role in the pathogenesis of many human skin diseases, including psoriasis (Kotnik, 2011).

Psoriasis is an inflammatory skin disease characterized by epidermal hyperplasia, infiltration of immune cells, and secretion of inflammatory

cytokines (Tortola et al., 2012). The anaphylatoxic fragments, C3a and C5a, have been found in corneal scale extracts from psoriatic lesions (Takematsu et al., 1986). The C5a from these lesions has been shown to chemoattract monocyte-derived dendritic cells (Mrowietz et al., 2001), indicating that complement may contribute to the inflammatory process in this disease. In the inducible AP-1-dependent psoriasis-like mouse model, the S100A8-S100A9 complex that promotes skin inflammation has been shown to up-regulate C3 expression (Schonthaler et al., 2013). Psoriasis-like dermatitis can be induced by topical application of the toll-like receptor 7 agonist imiquimod (IMQ) (van der Fits et al., 2009) in the form of Aldara cream (3M Pharmaceuticals, St Paul, MN) (Walter et al., 2013). Here, we explore the role of complement in IMQ-mediated psoriasiform dermatitis.

We first tested whether cutaneous IMQ treatment induces local C3 synthesis (experimental methods are provided as [Supplementary Materials](#) online) and found a progressive increase in C3 mRNA in the skin with repeated IMQ applications (Figure 1a, and see [Supplementary Figure S1a](#) online). Immunohistochemistry showed



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Abbreviations: IMQ, imiquimod; WT, wild type

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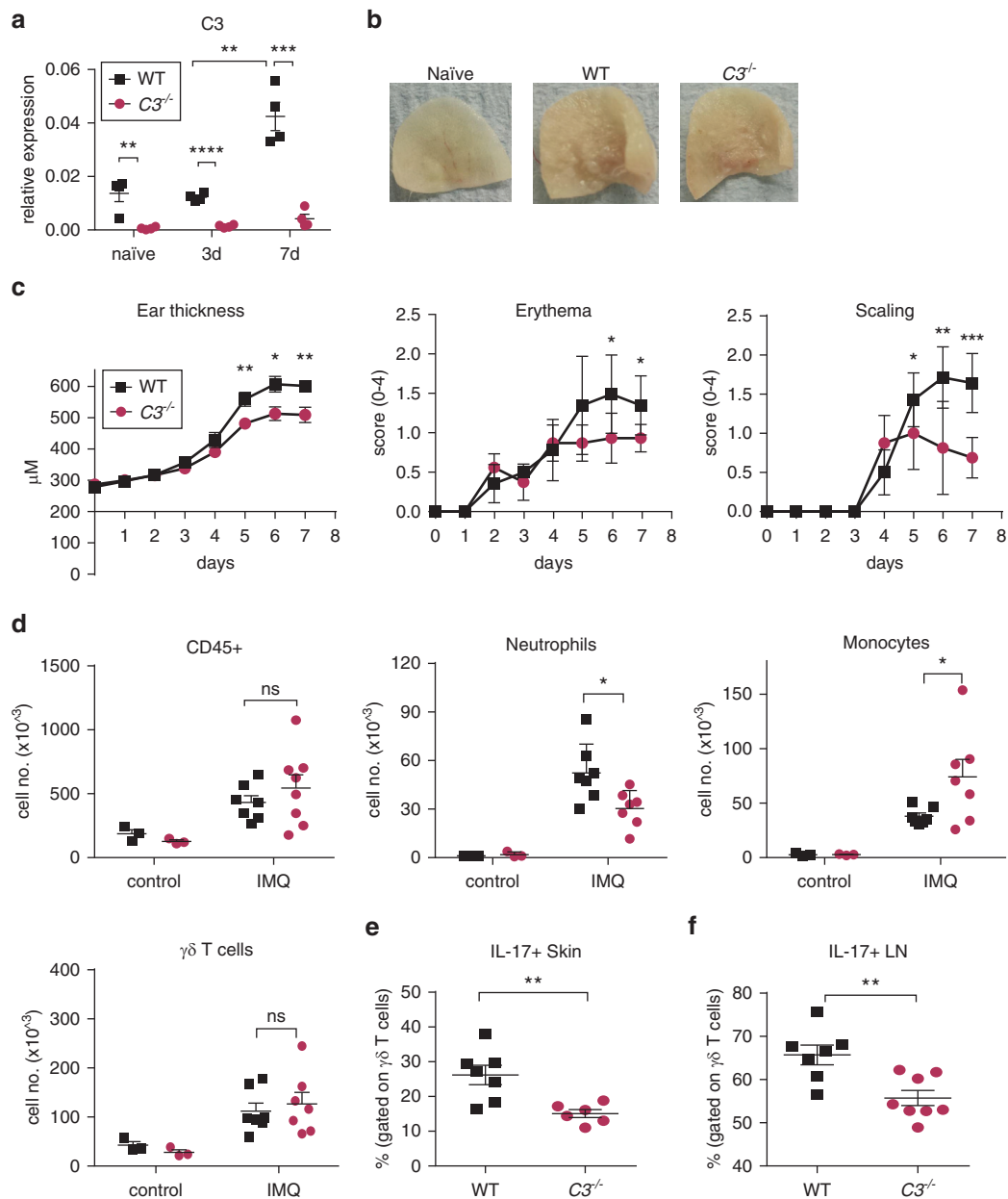


Figure 1. IMQ-induced skin inflammation and psoriasisform dermatitis is impaired in C3-deficient mice. IMQ was applied for 7 days to the ear of BALB/c WT (black square) and BALB/c.C3^{-/-} (red circle) mice. **(a)** C3 expression in whole skin quantified by quantitative real-time PCR 24 hours after the last application (n = 4). Naïve indicates untreated mice, mean ± standard error of the mean relative to cyclophilin, unpaired *t* test. **(b)** Representative pictures of IMQ-treated ears at day 7. **(c)** Ear thickness (mean ± standard error of the mean) and clinical scores (mean ± standard deviation) for erythema and scaling. Representative experiment of two, Mann-Whitney test. **(d)** Number of CD45⁺ cells, neutrophils (Ly-6G⁺), monocytes (CD11b⁺Ly-6C⁺Ly-6G⁻), and $\gamma\delta$ T cells (CD4⁻CD3⁺ $\gamma\delta$ TCR⁺) at day 7. **(e-f)** Proportion of IL-17⁺ $\gamma\delta$ T cells from **(e)** skin and **(f)** LN after in vitro restimulation. Mean ± standard error of the mean, unpaired *t* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. d, day; IMQ, imiquimod; LN, lymph node; M, meter; no., number; WT, wild type.

that the C3 expression was predominantly in the dermis (see [Supplementary Figure S1b](#)). In vitro experiments with isolated dermal stromal cells showed that these cells can produce C3 upon stimulation with inflammatory cytokines known to be induced in IMQ-treated skin but not upon direct challenge with a toll-like receptor 7 agonist (see [Supplementary Figure S1c](#)). To test whether C3

contributes to the psoriatic-like lesions induced by IMQ, we then treated wild-type (WT) and C3^{-/-} mice for 7 consecutive days. The treatment resulted in skin thickening, scaling, and erythema ([Figure 1b](#)). However, mice lacking C3 displayed less skin inflammation compared with WT mice ([Figure 1c](#)). Consistent with the reduced skin response in C3^{-/-} mice, we found significantly fewer infiltrating

neutrophils, but slightly more monocytes, and no difference in the number of resident $\gamma\delta$ T cells compared with WT animals ([Figure 1d](#)). IL-17 secretion by $\gamma\delta$ T cells plays a key role in the IMQ-induced psoriasis model ([van der Fits et al., 2009](#)), and our data confirmed that IL-17 secretion was mainly restricted to these cells. The frequency of IL-17-positive $\gamma\delta$ T cells in both skin and draining lymph nodes was

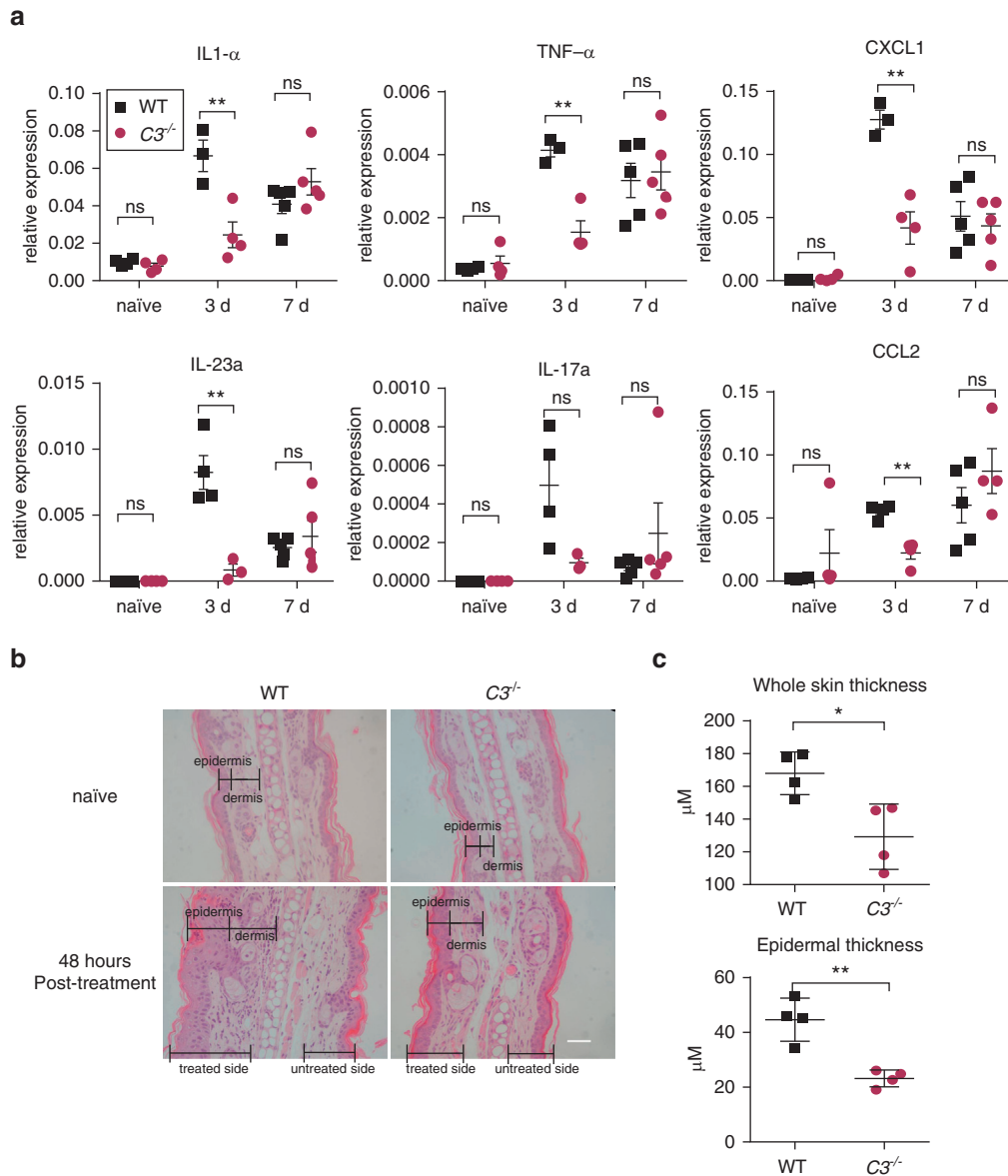


Figure 2. Reduced early proinflammatory response and skin thickening in IMQ-treated C3-deficient mice. (a) IMQ was applied for 3 or 7 days to the ear of BALB/c WT (black square) and BALB/c. $C3^{-/-}$ (red circle) mice. Gene expression was analyzed in whole skin 24 hours after the last IMQ exposure ($n = 3-5$ /group). Naïve indicates untreated mice, mean $\pm$ standard error of the mean relative to the control gene cyclophilin (SYBR Green assay, Life Technologies, Waltham, MA) or GAPDH (Tagman assay ThermoFisher, Waltham, MA) levels. (b) Representative images showing hematoxylin and eosin staining of cross-sectional ear skin from BALB/c and BALB/c. $C3^{-/-}$ mice untreated and 48 hours after the third IMQ application. The epidermis/dermis boundaries are indicated. Scale bars = 50 μm . (c) Quantification of whole-skin and epidermal thickness calculated using Image J software (National Institutes of Health, Bethesda, MD). Each symbol represents an individual mouse ($n = 4$). Mean $\pm$ standard error of the mean; unpaired t test. * $P < 0.05$, ** $P < 0.01$. d, day; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IMQ, imiquimod; M, meter; ns, not significant; TNF, tumor necrosis factor; WT, wild type.

significantly lower in the absence of C3 (Figure 1e and f). All animals were handled in accordance with institutional guidelines, and the UK Home Office approved the procedures.

We next evaluated whether C3 contributes to the local inflammatory response. To this end, we analyzed the gene expression of a selected number of cytokines/chemokines known to be induced in the skin by IMQ treatment (Di Meglio et al., 2014; Walter et al., 2013).

At the peak of clinical inflammation on day 7, the $C3^{-/-}$ mice did not show any obvious differences in inflammatory gene expression (Figure 2a). However, at the onset of the clinical pathology, day 3, when the cytokine/chemokine gene response peaks (Di Meglio et al., 2014), C3-deficient mice had a markedly reduced response, suggesting that C3 modulates the inflammatory gene induction that precedes clinical manifestations. To substantiate this, we carried

out a time-course analysis of skin gene expression after 3 days of IMQ treatment. This showed that in the absence of C3, the resolution of the IMQ-triggered inflammation was faster compared with WT mice. Twenty-four hours after the last application, C3-deficient mice had significantly reduced levels of all genes analyzed, namely those for IL-1 α , TNF- α , IL-17a, IL-23a, CXCL1, and CCL2 (see Supplementary Figure S2a and Supplementary Table S1 online).

Consistent with this, thickening of the skin assessed by histology was significantly reduced in the C3^{-/-} mice (Figure 2b and c). The altered skin pathology in the IMQ-treated C3-deficient mice appeared to affect mainly the epidermis. Untreated skin of WT and C3^{-/-} mice was histologically indistinguishable.

In summary, we demonstrate that C3 is involved in the development and resolution of the psoriasiform skin inflammation induced by short-term treatment with IMQ. The proinflammatory effect of C3 is likely to be mediated by several mechanisms. In the absence of C3 the expression of psoriasis-relevant genes in the skin was impaired, neutrophil infiltration into the inflamed site was decreased, and IL-17 production by $\gamma\delta$ T cells in the skin and the draining lymph nodes was reduced. Taken together, these data support a proinflammatory role of C3 during psoriasis-like skin inflammation.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <http://dx.doi.org/10.1016/j.jid.2016.11.011>.

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Integrin- β 4—TNS4—Focal Adhesion Kinase Signaling Mediates Keratinocyte Proliferation in Human Skin

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TO THE EDITOR

TNS4, also known as CTEN, is a cytoplasmic scaffold protein containing Src

homology 2 and phosphotyrosine-binding domains (Lo, 2014). TNS4 localizes to focal adhesions involved in

integrin-mediated signaling, which plays an important role in cell adhesion, migration, and proliferation in a context-dependent manner (Duperret and Ridky, 2013). Although TNS4 was initially identified as a tumor suppressor, accumulating evidence suggests that TNS4 promotes tumorigenesis in many cancers (Lo, 2014). Although TNS4 is proposed as a prognostic

Abbreviations: ERK, extracellular signal-regulated kinases; GFP, green fluorescent protein; FAK, focal adhesion kinase; ITGB, integrin β ; NHEK, normal human epidermal keratinocyte; siRNA, small interfering RNA

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Supplementary information online

Title: Complement C3 exacerbates imiquimod-induced skin inflammation and psoriasiform dermatitis

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Materials and Methods

Mice

Mice deficient in complement component C3 ($C3^{-/-}$) (Wessels et al., 1995) or C1q ($C1qa^{-/-}$) (Botto et al. 1998) were backcrossed onto the BALB/c background for more than 10 generations. BALB/c or C57BL/6 wild type (WT) mice were purchased from Charles River (UK). Experimental mice were 8-12 weeks of age, strain-, sex- and age-matched. All animals were handled in accordance with the institutional guidelines and the UK Home Office approved the procedures. The experiments with animals were conducted following the ARRIVE guidelines.

IMQ treatment

The Aldara® cream (3M Pharmaceutical - 5% Imiquimod) was applied for 7 consecutive days on the ventral side of the right ear. Skin thickening was assessed daily by measuring ear thickness using a micrometer (Mitutoyo, Japan). Erythema and scaling were measured blindly using a score ranging from 0 to 4 (0 = absence of clinical signs, 4 = severe phenotype). Mice were sacrificed after 7 topical applications. Treated ears were collected, digested as described below and cells stained for surface markers. Contralateral untreated ears were also collected and used as control.

Skin digestion

The ventral side of the ears were split mechanically before being minced and transferred to digestion buffer consisting of PBS, 0.13 Wünsch units/mL of Liberase TM Research grade (Roche), 25 µl/ml of DNase I (Roche) for 90 minutes at 37°C with gentle shaking. After the enzymatic digestion, skin fragments were processed using a gentle MACS homogeniser (Milteny) and the cell suspension was filtered through a 70 µm cell strainer (BD). Cells were washed, resuspended in flow cytometry buffer (PBS/1%BSA) before staining.

Flow Cytometry

Cells were stained using standard protocols in the presence of saturating anti-FcγRII/III (2.4G2). The following antibodies were used: CD45 (30-F11), Ly-6c (HK1.4), CD11b (M1/70), all from e-Bioscience; TCRγδ (GL3), Ly-6G (1A8) CD45 (30-F11), CD90.2 (53-2.1) all from Biolegend; CD3e (500A2), CD4 (RM4-5), IL-17A (TC11-18H10), IFNγ (XMG1.2), Ly-6A/E (E13-161.7) CD45.2 (104) all from BD Pharmingen. Flow cytometry was performed with a BD FACSVerser (BD 40 Biosciences, CA, USA). Data were analysed using FlowJo software, version 7.6.5 (TreeStar Inc, Ashland, OR, USA).

In vitro stimulation and intracellular staining

Single cell suspensions from draining (dLN) or treated skin harvested at day 7 were plated in a U-bottom 96 well plate at 4×10^5 cells/well in a final volume of 200 µl of RPMI 1640 (Gibco), 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were stimulated *in vitro* with PMA (25ng/mL) (Sigma) and Ionomycin (Sigma)

(1 µg/ml) in the presence of Golgi stop (monensin-BD). After 4 hours cells were harvested, permeabilised/fixed with Cytofix/CytoPerm Kit (BD), according to manufacturer's instructions, and stained for intracellular IL-17A.

Gene expression analysis and histology after IMQ treatment

Mice were treated with 5 mg of Aldara® cream on the ventral side of the right ear for 3 or 7 consecutive days and the skin was analysed at different time points after the last treatment. Ear skin tissue was: i) processed for histology and stained with haematoxylin and eosin (H&E); ii) embedded in OCT (Tissue-Tek) and frozen immediately on dry ice; iii) stored in RNA later (Sigma Aldrich) at -20°C. C3 immunofluorescence: 8 µM frozen sections were fixed in acetone, blocked with 10% normal goat serum and stained with a FITC-labelled goat anti-mouse C3 antibody (Cappel/ICN, dilution of 1:100). Samples were analysed using a Leica SP5 confocal microscope. Gene expression analysis: RNA was extracted from whole skin using RNeasy mini kit (Qiagen), after homogenisation with TissueLyserII (Qiagen). RNA was quantified by absorbance spectroscopy (Nanodrop) and cDNA synthesized by reverse-transcriptase reaction (Bio-Rad). qRNA analysis was performed using the Real-time PCR Detection System and Power SYBR Green (ThermoFisher) or TaqMan system (ThermoFisher) (supplementary table S1). The data are expressed as relative expression to the Cyclophilin or the GAPDH housekeeping gene.

Isolation of dermal stromal cells and C3 secretion

Primary dermal stromal cells were isolated from C57BL/6 mice. 1x1 cm of shaved skin was excised, minced and digested in 10 ml of digestion buffer: DMEM/F12 media (Gibco), 0.13 Wünsch units/mL of Liberase TM Research grade (Roche) and 1x antibiotic/antimycotic (Life Technologies). After 2 hour incubation at 37°C, the skin fragments were washed, resuspended in 15% FBS DMEM/F12 medium and the cells transferred to culture flasks. The medium was changed every 3-4 days and the tissue fragments were removed after 1 week. When cells reached 70% to 80% confluence, the adherent cells were expanded into larger flasks. The cells were phenotyped by assessing the expression of CD11b, CD45.1, CD71, Sca-1 (Ly-6A/E), CD90.2 and PDGFR-α (CD140a) by flow cytometry. Cells were stimulated with IL-1β (100 ng/ml) or TNF-α (100 ng/ml) or IL-36 (100 ng/ml) or R848 (doses ranging from 0.1 to 1 mg/ml) for 24hrs. C3 levels were measured using as previously described (Ruseva et al., 2013).

Statistical Analysis

Comparison between two groups was performed using an unpaired two-tailed Student's *t* test. Non-parametric data were assessed using Mann-Whitney test. Results were expressed as mean ± SEM. *P* values less than 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA).

Table S1

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
C3a	CAAACAGATGTCCCTGACCA	TGCTTTTGAGTTTGGCATGA
S100a9	GGAGCGCAGCATAACCACCAT C	GCCATCAGCATCATACACTCCTC A
TNF- α	AGGGATGAGAAGTTCCCAAAT G	CACTTGGTGGTTTGCTACGAC
IL1- α	TTGGTTAAATGACCTGCAACA	GAGCGCTCACGAACAGTTG
CXCL1	ACTCAAGAATGGTCGCGAG	GTGCCATCAGAGCAGTCTG
CCL2	CCCAATGAGTAGGCTGGAGA	CCTTAGGGCAGATGCAGTTT
Cyclophilin	CAAATGCTGGACCAAACACAA	CCATCCAGCCATTCAGTCTTG
Gene	Taqman primer	
IL-17a	Mm00439618_m1	
IL-23	Mm00518984_m1	
GADPH	Mm.304088	

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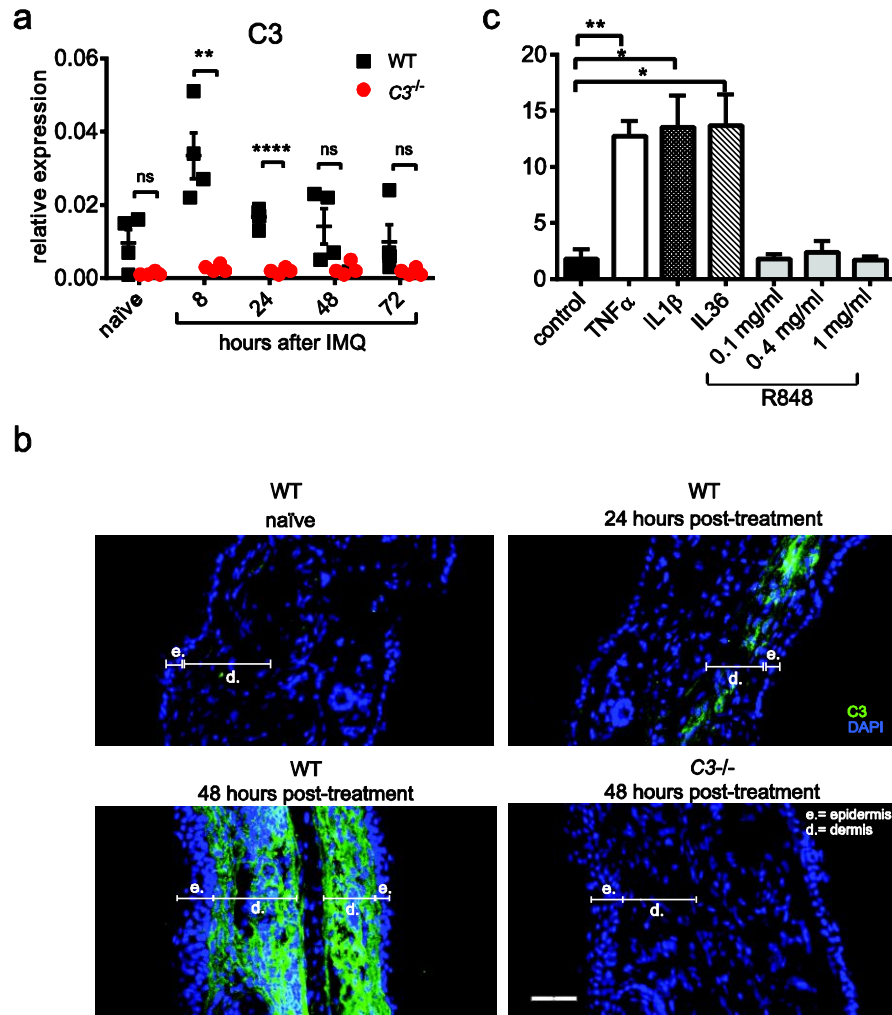


Figure S1. Cutaneous IMQ exposure promotes local C3 production.

IMQ was applied topically for 3 consecutive days to the ventral ear of BALB/c WT and BALB/c. $C3^{-/-}$ mice and (a) expression of C3 in whole ear skin was quantified by qRT-PCR at the indicated times after the last application ($n=4$). Untreated mice were used as controls. Data are expressed as mean $\pm$ SEM relative to cyclophilin. Results shown are representative of two independent experiments ($n=3-4$ /group). p values by unpaired t test; ns=non-significant; ** $p < 0.01$; **** $p < 0.0001$. (b) Representative images showing cross-sections of naïve and IMQ-treated skin stained with C3 (green) 24 and 48 hrs after the last IMQ treatment. Nuclei (blue) identified with DAPI. Scale bar represents 50 μ M. (c) C3 secretion by dermal stromal cells following *in vitro* stimulation with the cytokines indicated or R848 (ranging from 0.1 to 1 mg/ml) for 24 hours. C3 measured by ELISA. Data are expressed as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$ (unpaired t test).

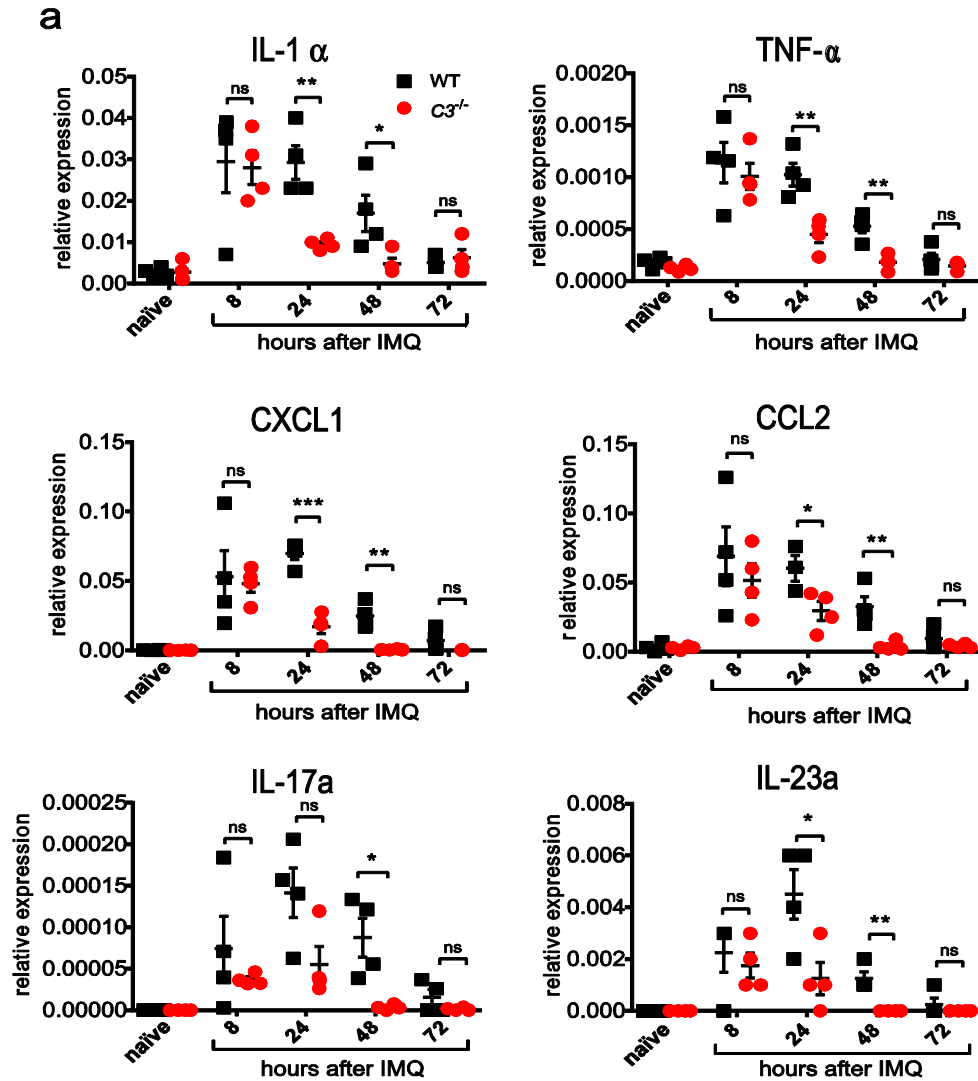


Figure S2. C3 deficiency accelerates the resolution of the inflammation triggered by IMQ.

IMQ was applied for 3 days to BALB/c WT and BALB/c.C3^{-/-} mice. (a) Expression of psoriasis-relevant genes quantified by qRT-PCR at the indicated times after the 3rd application (n=4). Gene expression is relative to cyclophilin (SYBR Green assay) or GAPDH (Taqman assay) levels. Each symbol represents an individual mouse (n=4). Data shown as mean $\pm$ SEM; unpaired *t* test; ns=non-significant; **p* < 0.05, ***p* < 0.01; ****p* < 0.001. Naïve=untreated mice. Results representative of two independent experiments.