ITK and RLK Inhibitor PRN694 Improves Skin Disease in Two Mouse Models of Psoriasis

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The chronic and highly prevalent skin disorder psoriasis vulgaris is characterized by a hyperproliferative epidermis and aberrant immune activity. Many studies have highlighted the role of differentiated T lymphocytes in psoriasis progression. Several biologics are currently available that target proinflammatory cytokines produced by T lymphocytes, but the need for improved therapies persists. The small molecule PRN694 covalently binds ITK and RLK, two Tec kinases activated downstream of T-lymphocyte activation, both of which are up-regulated in psoriatic skin. These Tec kinases are involved in signaling cascades mediating T-lymphocyte proliferation, differentiation, and migration and proinflammatory cytokine production. In vitro analysis showed that PRN694 effectively inhibited IL-17A production from murine T helper type 17-differentiated T lymphocytes. Additionally, PRN694 effectively reduced the psoriasis-like phenotype severity and reduced epidermal proliferation and thickness in both the Rac1V12 and imiquimod mouse models of psoriasis. PRN694 also inhibited CD3ε T-cell and γδ T-cell infiltration into skin regions. Inhibition of ITK and RLK attenuated psoriasis-associated signaling pathways, indicating that PRN694 is an effective psoriasis therapeutic.


INTRODUCTION
Psoriasis vulgaris is one of the most prevalent skin disorders, affecting up to 3% of the world’s population (Schön and Boehncke, 2005). Psoriatic skin lesions typically appear as pruritic, inflamed, hyperkeratotic plaques, sometimes affecting large body surface areas (Griffiths and Barker, 2007). Microscopically, lesional psoriatic skin shows epidermal acanthosis, dysregulated epidermal differentiation, perturbed cytokine signaling, and a characteristic immune infiltrate (Nestle et al., 2009; Perera et al., 2012). In addition to the skin involvement, psoriasis is also associated with extracutaneous comorbidities such as psoriatic arthritis, cardiovascular disease, and metabolic syndrome (Carvalho et al., 2016). Activated and differentiated T lymphocytes play a key role in disease progression, largely through production of proinflammatory cytokines (Kagami et al., 2010; Lowes et al., 2008; Nickoloff and Wrone-Smith 1999). Inhibition of T-lymphocyte activity and production of cytokines, such as IL-17 and TNF-α, has been a successful approach in the treatment of psoriasis (Abrams et al., 1999; AbuHilal et al., 2016; Ellis and Krueger, 2001; Gottlieb et al., 1995, 2000; Mueller and Herrmann, 1979; Zaba et al., 2007).

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T-lymphocyte activation leads to activation of Tec kinases ITK and RLK, which mediate signaling pathways resulting in T-cell development, differentiation, proliferation, and chemotaxis (Berg et al., 2005; Readinger et al., 2009; Schwartzberg et al., 2005). ITK and RLK inhibition, therefore, would be expected to not only inhibit T-lymphocyte activation but also to inhibit proinflammatory cytokine production downstream of T-lymphocyte activation, such as IL-1, IL-2, IL-6, IL-8, IL-17, IFN-γ, and TNF-α, some of which are the targets of current biologics available in clinics (Davidovici et al., 2010; Ross et al., 2016; von Bonin et al., 2011).

The small molecule PRN694 covalently binds to and very selectively inhibits ITK and RLK kinase activity with half maximal inhibitory concentration values of 0.3 nmol/L and 1.4 nmol/L, respectively (Zhong et al., 2015). PRN694 was shown to inhibit Jurkat cell activation through blockage of NFAT1, JunB, phosphorylated IKBα, and phosphorylated Erk transcription factor nuclear translocation, as well as by inhibiting intracellular calcium flux after anti-CD25 stimulation (Zhong et al., 2015). Additionally, PRN694 inhibited T-cell receptor–induced T-cell proliferation, proinflammatory cytokine release, and T-lymphocyte migration (Cho et al., 2015; Zhong et al., 2015). PRN694 inhibited T helper type (Th) 1, Th2, or Th17 differentiation in CD4+ cells isolated from Rag2-knockout mice (Cho et al., 2015). In a colitis mouse model, PRN694 effectively inhibited CD4+ T-cell differentiation and promoted an improvement of the colitis phenotype (Cho et al., 2015), leading us to hypothesize that PRN694 may also effectively inhibit T-lymphocyte activation and differentiation contributing to psoriatic disease. In this study, we tested PRN694’s ability to inhibit IL-17 production by naïve murine CD4+ lymphocytes in vitro and whether PRN694 would improve skin disease in the recently described Rac1V12 psoriasis mouse model.
RESULTS

ITK and RLK transcription is up-regulated in lesional human psoriatic skin

Initially, we performed an in silico analysis of the expression levels of the PRN694 inhibitory targets ITK and RLK in publicly available datasets of mRNA expression from full-thickness skin biopsy samples of psoriatic lesional, nonlesional, or normal control skin (Gene Expression Omnibus accession ID: GSE13355). We found that the mRNA expression levels of both ITK and RLK were increased in lesional psoriatic skin compared with nonlesional psoriatic or healthy skin (Figure 1a and b).

PRN694 inhibits IL-17 production by naïve mouse CD4⁺ splenocytes polarized to Th17 differentiation in vitro

To determine the effectiveness of PRN694 in inhibiting proinflammatory cytokines in differentiated T cells, naïve mouse CD4⁺ splenocytes were isolated and differentiated under Th17-influencing conditions in the presence of various concentrations of PRN694. After 96 hours, cells were re-stimulated with phorbol 12-myristate 13-acetate/ionomycin, and intracellular IL-17 production was quantified by FACS analysis. PRN694 potently inhibited IL-17 production with a half maximal inhibitory concentration value of 4.3 ± 2.1 nmol/L (Figure 1c). To corroborate these findings, further studies were conducted in which soluble IL-17 was measured via Alphascreen ELISA (Perkin Elmer, San Jose, CA). Re-stimulation of Th17-differentiated cells treated with varying concentrations of PRN694 resulted in inhibition of soluble IL-17 with a half maximal inhibitory concentration of 3.9 ± 2.7 nmol/L (Figure 1c).

PRN694 significantly improves psoriatic skin and reduces epidermal hyperplasia in vivo

Next, we evaluated the effects of inhibiting ITK and RLK in the Rac1V12 psoriasis mouse model, which presents a skin phenotype closely resembling human psoriasis most pronounced in ears, feet, and tail (Winge et al., 2016). Intraperitoneal doses of 10 mg/kg PRN694, or vehicle control, were administered daily to Rac1V12 mice for 40 days beginning at 7 days old (n = 6). A clinical score for assessing phenotype severity was developed and was scored by three blinded observers (n = 3) on a scale of 0–3, with 3 representing a severe phenotype. Ears were evaluated for scaling and edema, feet were evaluated for erythema and edema, and tails evaluated for edema, scaling, erythema, and areas of skin lesion or wounding. Mice treated with PRN694 presented a less severe skin phenotype than vehicle-treated mice (Figure 2a), and PRN694-treated mice had, on average, less severe evaluation scores than the vehicle control group (Figure 2b).

PRN694 treatment produced a normalization of the pronounced psoriatic hyperplasia characteristic of lesional Rac1V12 mouse skin (Figure 3a), resulting in significant reductions in epidermal thickness (Figure 3b) and epidermal area (Figure 3c). Reduced epidermal proliferation in tail tissue of PRN694-treated mice was shown by Ki67 immunofluorescence staining (Figure 3d), with significant reductions in proliferation of both basal and suprabasal epidermis (Figure 3e).

Up-regulation of STAT3 signaling is closely linked to activated keratinocytes and immune cells in psoriasis (Andrés et al., 2013; Sano et al., 2005) and has been shown to contribute to T-cell pathogenicity (Durant et al., 2010). Immunofluorescence staining for phosphorylated STAT3 (Figure 3f) showed that PRN694-driven ITK/RLK inhibition led to decreased phosphorylated STAT3 not only in cells present in dermal regions of tail histological sections but also in basal and suprabasal epidermis (Figure 3g).

PRN694 inhibits T lymphocytes, reduces systemic TNF-α, and shows trends of reduced proinflammatory cytokines in Rac1V12 mice

Immunofluorescence staining of mouse tail histological sections for CD3, a marker for naïve T lymphocytes (Smith-Garvin et al., 2009), showed significant reduction of CD3⁺ cells in PRN694-treated Rac1V12 mice (Figure 4a and b). The
The Rac1V12 mouse model was previously noted to have increased populations of cells expressing RORγ (Winge et al., 2016). To see if PRN694 not only reduced CD3⁺ cell infiltrate but similarly reduced Th17 differentiated T lymphocytes, tail histological sections were also immunostained for RORγ, as a transcriptional regulator of Th17 differentiated lymphocytes (Solt et al., 2011). PRN694 treated Rac1V12 mice had a significant reduction of RORγ⁺ cells in mice treated with PRN694 (Figure 4c and d). Finally, we assessed Th1 (IFN-γ), Th17 (IL-17a, IL-23a), and Th2 (IL-4, IL-13) cytokines in the spleens of Rac1V12 mice treated for a shorter duration (5 days). We noted a trend of reduced Th17, but not Th1 or Th2 transcripts (see Supplementary Figure S1a online).

To assess differences in systemic cytokine production, plasma collected from Rac1V12 mice after 40 days of PRN694 or vehicle control treatment presented a decrease of TNF-α by Luminex assay (Affymetrix, Santa Clara, CA) (Figure 4e). Other Th1 or Th17 cytokines that showed a (nonsignificant) trend of reduced expression were GCSF/CSF3, VEGF, IFNG, and IL-4 (see Supplementary Figure S1b). Luminex mean fluorescence intensity values were undetectable for IL-17A.

To access effects of PRN694 on non-T immune cells, we assessed expression of monocyte/macrophage markers CD68 and F4/80 by immunofluorescence microscopy. We found no differences in CD68 expression between PRN694 and vehicle-treated mice; however, increased numbers of F4/80⁺ cells were noted in PRN694-treated mice (see Supplementary Figure S2 online).

PRN694 reduces psoriasiform phenotypes in the IMQ mouse model

With encouraging improvement of the psoriasiform phenotypes in the Rac1V12 mouse model, we next assessed the effects of PRN694 in the IMQ mouse model, a common model with T-cell and IL-17/-23 axis dependency (Cai et al., 2011; Flutter and Nestle, 2013; Pantelyushin et al., 2012; van der Fits et al., 2009). A 2 × 2-cm back region of C57BL/6j (B6) mice was shaved 1 day before the experimental start, and PRN694 at 10
mg/kg or vehicle control was administered 2 hours before topical Aldara (5% IMQ; 3M Health Care Limited, Loughborough, England) application (Figure 5a). IMQ application to B6 mice yielded epidermal thickening and psoriasiform dermatitis, as reported by Swindell et al. (2017) (Figure 5b). Although topical IMQ resulted in dehydration and decreased mouse weight (Swindell et al., 2017), PRN694 treatment before IMQ application resulted in a trend toward recovering weight faster than vehicle control mice (Figure 5b). With PRN694 treatment, B6 mouse spleens tended to be heavier than vehicle-treated IMQ B6 mice (Figure 5c). After 5 days of dosing, B6 mouse backs were photographed and clinically scored. PRN694-treated mice presented less erythema and scaling on mouse backs were photographed and clinically scored. (Figures 5f and g). To assess inhibition of T lymphocytes, we immunostained skin sections in IMQ-treated skin and noted a significant reduction of CD3+ cells in PRN694-treated mice (Figures 5h and i). Proliferative activity of the epidermis was reduced, as assessed by cell-cycling marker Ki67, in cells in proximity to the basement membrane zone with PRN694 treatment (Figure 5j and k). ITK is noted to play a role in the development of γδ T cells (Felices et al., 2009; Qi et al., 2009), and application of IMQ onto δ T-cell receptor-γ- mice results in reduced psoriasiform phenotypes (Cai et al., 2011). Immunochemistry staining for the δ T-cell receptor showed reduced numbers of positively staining γδ T cells in epidermal regions of IMQ-treated back skin after PRN694 treatment (Figure 5l and m).

**DISCUSSION**

The critical role that T lymphocytes play in promoting hyperplasia and altered differentiation of psoriatic epidermis is well known (Griffiths and Barker, 2007; Nestle et al., 2009; Perera et al., 2012). Inhibition of T lymphocytes or their proinflammatory cytokines has been shown to be a successful and well-used method for alleviating psoriasis (Abrams et al., 1999; Ellis and Krueger, 2001; Gottlieb et al., 1995, 2000; Mueller and Herrmann, 1979; Ross et al., 2016). Despite recent advances, there remains a need for improved psoriasis therapies (Kimball et al., 2015). Most existing therapies target systemic cytokines or their receptors. In contrast, this study evaluated a small molecule ITK and RLK inhibitor of T lymphocyte activation, proliferation, differentiation, and migration. The Rac1V12 mouse model (Winge et al., 2016) was ideal for studying PRN694 therapy because of its clinical, histological, and biologic phenotype very closely resembling human psoriasis. The Rac1V12 mouse model expresses a keratin 14-driven constitutively active mutant of the small GTPase Rac1 in basal keratinocytes and displays marked psoriasiform
Inhibition of ITK and RLK Improves Psoriatic Phenotype in Two Murine Models

Figure 4. PRN694 reduces CD3+ cells present in dermal regions proximal to the epidermis. (a) Immunofluorescence stains of CD3 and integrin-α6, as a basement membrane zone marker, in PRN694- or vehicle control-treated mouse tail sections. (b) Quantified CD3+ cells per 20x microscopic field view. (c) Immunofluorescence stains of RORγ and integrin-α6. (d) Quantification of RORγ+ cells per 20x field view. (e) Mouse serum analyzed via Luminex assay for TNF-α normalized to wild-type mouse serum. (a, c) Scale bar = 100 μm. (b, d, e) Mean ± standard error of the mean, n = 6, one-tailed t test. RU, relative units.

hyperplasia, a characteristic immune cell infiltrate, and a clear dependence on T cells to produce the disease phenotype, because crossing the Rac1V12 strain with T-cell-deficient NOD/SCID background eliminated the psoriatic phenotype (Winge et al., 2016). For these reasons, we hypothesized that inhibition of T-cell function would result in alleviation of the psoriasis-like phenotype of Rac1V12 mice.

The ears, feet, and tail are the areas that most prominently display signs of psoriasiform dermatitis, including scaling, erythema, and edema. These are also the areas where the mice frequently scratch or bite themselves, worsening the phenotype through Koebnerization (Weiss et al., 2002). With 40 days of intraperitoneal injections of PRN694, we found that inhibition of ITK and RLK led to clinical reduction of erythema and scaling. There was reduction in scored tail skin lesions, which may indicate reduction in scratching. Histologically, there was a reduction of epidermal hyperplasia and reduction of skin infiltration of CD3+ and RORγ+ cells after PRN694 treatment, indicative of inhibition of T-cell activation after ITK and RLK inhibition.

There was also a marked decrease in psoriasis-associated STAT3 activation in both dermal and epidermal cells of these Rac1V12 mice after PRN694 treatment. Inhibition of STAT3 activation in Rac1V12 mouse epidermis suggests that PRN694 disrupts an epidermal-immune positive feedback loop, which in turn drives the psoriatic phenotype in Rac1V12 mice. With much improvement seen with PRN694 treatment of Rac1V12 mice, it should be noted that complete resolution of abnormalities was not achieved. This may be indicative of ITK/RLK independent signaling cascades continuing to be active and contributing to changes seen in the Rac1V12 mice.

Serum TNF-α levels were decreased in PRN694-treated Rac1V12 mice. TNF-α is known to play a key role in psoriasis, and anti-TNFα therapy effectively reduces psoriatic skin lesions (Fantuzzi et al., 2008). Not only is TNF-α noted to be elevated in psoriatic lesional skin (Ettehadi et al., 1994), but severity of Psoriasis Area and Severity Index scores have been shown to correlate with serum TNF-α levels (Mussi et al., 1997). TNF-α also correlates with psoriatic arthritis and is found in high concentrations in joint synovium (Partsch et al., 1997). These observations, together with PRN694’s reduction of foot and tail edema, suggest inhibition of ITK and RLK, and its downstream reduction of systemic TNF-α suggests that PRN694 could affect systemic psoriasis disease complications such as joint disease, but further investigation is necessary.

In addition to assessing PRN694 in the Rac1V12 psoriasis model, we validated our findings in the IMQ model, a long-standing mouse psoriasiform dermatitis model used in over 200 studies. The IMQ model has been shown to have a T-cell contribution to the psoriasiform dermatitis phenotype and is driven by the IL-17/-23 axis central for Th17-mediated pathology (Flutter and Nestle, 2013). This model exhibits variation with regard to sex and mouse strain used (Swindell et al., 2017). We chose female B6 mice for our studies, which according to studies conducted by Swindell et al. exhibited heightened epidermal thickening after IMQ treatment and, of the mouse strains studied, had RNA transcriptional signatures in response to IMQ most closely corresponding to psoriasis, involving the Th17/23 axis. Considering ITK’s role for Th17 differentiation and the trend of down-regulated Th17, but not Th1 or Th2 transcripts in Rac1V12 spleens, we chose to confirm PRN694’s inhibitory role on the IL-17/-23 axis in the IMQ model on the B6 background with these RNA signatures reported by Swindell et al. in mind. Our results established that PRN694 perturbed this axis in psoriatic signaling and reduced the extent of psoriasiform dermatitis phenotypes...
Figure 5. PRN694 reduces psoriasiform phenotypes in the IMQ mouse model. (a) Timeline of IMQ studies. (b) Weight change from day 0. (c) Spleen weights. (d) Representative photos of mouse backs. (e) Clinical scores (scale = 0–3, 3 = severe phenotype). (f) Hematoxylin and eosin-stained skin tissue sections. (g) Quantified interfollicular epidermal thickness. (h) IF of CD3 and integrin-α6. (i) CD3+ cells per 10× field view. (j) IF of Ki67 and integrin-α6. (k) Percentage of Ki67+ epidermal cells in basal and suprabasal regions. (l) IF of γδ T cells and integrin-α6. (m) γδ+ cells per millimeter of epidermis. (d) Ruler marks indicate millimeters. (f, h, j, k) Scale bar = 100 μm. (c, e, g, i, k, m) Mean ± standard error of the mean; vehicle and PRN694 groups, n = 5; no Aldara reference, n = 3; one-tailed t test, *P < 0.05, **P < 0.01. D, day; IF, immunofluorescence; IP, intraperitoneal; IMQ, imiquimod; n.s., not significant.
exhibited in the IMQ mouse model, further indicating that PRN694 is a potential therapy for psoriasis.

In summary, this study shows that PRN694 improves both the immune and epidermal phenotype of two murine psoriasis models and indicates that inhibition of T-effector cell function through ITK/RLK is a potential pharmaceutical therapy for psoriasis.

MATERIALS AND METHODS
Antibodies used in this study are listed in the Supplementary Materials online (see Supplementary Table S1 online).

Microarray analysis
Whole-skin punch biopsy samples were taken from 64 healthy individuals and 58 psoriasis patients from involved and noninvolved sites (see Nair et al., 2009 for further method details and statement that participating patients gave informed consent under local institutional review board-approved protocols). RNA samples were hybridized to the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix), and resulting data were processed using the robust multichip average method, where robust multichip average values are adjusted (on the log scale) to account for batch and sex effects. Expression data can be found on the public National Center for Biotechnology Information Gene Expression Omnibus database (accession number GSE13355).

Th17 polarization assays
Naïve CD4+ T cells from Balb/C mice were isolated using a CD4+ T-cell isolation kit (StemCells, Inc., Newark, CA). Isolated cells were plated at 1 × 10^6 cells/ml per well in 12-well plates and activated by plate-bound anti-CD3 (5.0 µg/ml) (BD Biosciences, Heidelberg, Germany) for 96 hours in the presence of Th17-polarizing conditions: 2 µg/ml anti-CD28, 5 ng/ml TGF-β, 30 ng/ml IL-6, 10 µg/ml anti-IL-4, 10 µg/ml anti-IFN-γ, and 5 µg/ml anti-IL-2 (R&D Systems, Minneapolis, MN, and BD Biosciences). For flow cytometry analysis, cells were washed and re-stimulated with brefeldin for 4 hours. Cells were washed and stained with anti-CD4–FITC followed by anti-IL-17–AF647 and acquired at 15,000 CD4+ T cells. For IL-17 Alpha-FITC and acquired at 15,000 CD4+ T cells. For IL-17 Alpha-FITC, field view micrographs were pieced together using Adobe Photoshop CS4. Pixel values were calibrated to micrometer values, then epidermal areas on histological sections were quantified in binary histological images, generated by manual selection of the epidermis. For both Rac1V12 and IMQ studies, epidermal thickness was quantified by averaging line measurements of the epidermis, excluding the stratum corneum in ImageJ software (National Institutes of Health, Bethesda, MD). Cell count quantifications were conducted using the cell counter plug-in feature in ImageJ. For γδ T-cell quantifications, positive cell counts were divided by basement membrane length of general interfollicular regions measured by a manual line trace in ImageJ.

Luminex analysis
Mouse plasma was submitted to the Human Immune Monitoring Center at Stanford University for 38-plex Luminex assay analysis, as previously described (Winge et al., 2016). Pixel values were calibrated to micrometer values, then epidermal areas on histological sections were quantified in binary histological images, generated by manual selection of the epidermis. For both Rac1V12 and IMQ studies, epidermal thickness was quantified by averaging line measurements of the epidermis, excluding the stratum corneum in ImageJ software (National Institutes of Health, Bethesda, MD). Cell count quantifications were conducted using the cell counter plug-in feature in ImageJ. For γδ T-cell quantifications, positive cell counts were divided by basement membrane length of general interfollicular regions measured by a manual line trace in ImageJ.

In vivo studies
All mouse experiments were conducted in accordance with Administrative Panel on Laboratory Animal Care protocols at Stanford University. Rac1V12 transgenic mice, described by Winge et al., 2016, were treated with 10 mg/kg PRN694 (n = 6) or vehicle control in 5% ethanol, 95% Captex 355 (n = 6) (Principia Biopharma, South San Francisco, CA; Abitec, Janesville, WI), which was administered intraperitoneally into the Rac1V12 mice starting at age 7 days every 24 hours for 40 days. IMQ mouse model experiments were conducted similarly to those of Swindell et al., 2017. Female C57BL/6 J (B6) mice (stock no. 000651; The Jackson Laboratory) were treated with 10 mg/kg PRN694 (n = 5) or vehicle control (n = 5) intraperitoneally once daily 2 hours before 62.5 mg IMQ (5% Aldara; 3M Health Care Limited, Loughborough, England) for 5 days and then euthanized for experiments on the sixth day. Mice were weighed daily and provided with warm saline subcutaneously as needed per calculated weight loss from day 0 of the study.

Clinical evaluation using clinical photographs
High-resolution photographs (Nikon D90, Nikkor 18-to-200—mm lens; Nikon, Tokyo, Japan; Galaxy Note4; Samsung Electronics, Suwon, South Korea) were taken at the end of the experiment while mice were under isoflurane anesthesia. Photographs were scaled to a millimeter ruler for size comparability and clinical scoring. For Rac1V12 studies, ears, feet, and tails were scored for separate criteria such as scaling, erythema, edema, or percentage wounded area on a scale of 0–3 in increments of 0.5. Scoring was done by blinded researchers (n = 3). Scores indicating the following: 0 = normal, 1 = mild, 2 = moderate, or 3 = severe. For IMQ studies, back skin was scored for erythema and scaling.

Histology and quantification
Tissues were embedded in Optimal Cutting Temperature compound (Tissue-Tek, Sakura Finetec, Torrance, CA), cut into 7-µm sections, and then fixed in 100% methanol for 10 minutes at −20°C. Rehydration was performed in 1 × Tris-buffered saline for 5 minutes at room temperature before staining. For histological stains, tissue sections were stained with Modified Mayer’s Hematoxylin (American MasterTech, Lodi, CA) and Eosin Y solution (Sigma-Aldrich, Steinheim, Germany). For immunofluorescence stains, tissue sections were blocked with 10% normal goat serum (Vector Laboratories, Burlingame, CA) or 10% normal donkey serum (Vector Laboratories) for 1–3 hours at room temperature. Primary antibodies (see Supplementary Table S1) were incubated overnight at 4°C. Fluorophore-conjugated secondary antibodies targeting primary antibody host species were diluted 1:400 in 1:5,000 Hoechst 33342 stain (Life Technologies, Grand Island, NY) in 1 × Tris-buffered saline and incubated for 1 hour at room temperature. For epidermal area and thickness quantifications of Rac1V12, field view micrographs were pieced together using Adobe Photoshop CS4. Pixel values were calibrated to micrometer values, then epidermal areas on histological sections were quantified in binary histological images, generated by manual selection of the epidermis. For both Rac1V12 and IMQ studies, epidermal thickness was quantified by averaging line measurements of the epidermis, excluding the stratum corneum in ImageJ software (National Institutes of Health, Bethesda, MD). Cell count quantifications were conducted using the cell counter plug-in feature in ImageJ. For γδ T-cell quantifications, positive cell counts were divided by basement membrane length of general interfollicular regions measured by a manual line trace in ImageJ.
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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2017.10.029.

REFERENCES


