



Topical ROR Inverse Agonists Suppress Inflammation in Mouse Models of Atopic Dermatitis and Acute Irritant Dermatitis

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The retinoic acid receptor-related orphan receptors ROR α and ROR γ are critical for the functions of specific subsets of T cells and innate lymphoid cells, which are key drivers of inflammatory disease in barrier tissues. Here, we investigate the anti-inflammatory potential of SR1001, a synthetic ROR α/γ inverse agonist, in mouse models of atopic dermatitis and acute irritant dermatitis. Topical treatment with SR1001 reduces epidermal and dermal features of MC903-induced atopic dermatitis-like disease and suppresses the production of type 2 cytokines and other inflammatory mediators in lesional skin. In the epidermis, SR1001 treatment blocks MC903-induced expression of TSLP and reverses impaired keratinocyte differentiation. SR1001 is also effective in alleviating acute dermatitis triggered by 12-O-tetradecanoylphorbol-13-acetate. Overall, our results suggest that ROR α/γ are important therapeutic targets for cutaneous inflammation and suggest topical usage of inhibitory ligands as an approach to treating skin diseases of inflammatory etiology.

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INTRODUCTION

Dysregulated inflammatory responses account for a large portion of skin diseases. Atopic dermatitis (AD) is the most common chronic inflammatory skin disease among children (Ong, 2014; Weidinger and Novak, 2016). The pathogenesis of AD is related to defective skin barrier and allergic inflammation mediated by T helper (Th) type 2 cell- or type 2 innate lymphoid cell (ILC2)-derived cytokines, such as IL-4, IL-5, and IL-13 (Brandt and Sivaprasad, 2011; Elias et al., 2008; Leung et al., 2004). Clinically, AD is characterized by the infiltration of CD4⁺ T cells, eosinophils, and mast cells in lesional skin, as well as an elevated serum level of IgE. Although topical glucocorticoids are the most commonly used drugs for AD treatment, their anti-inflammatory actions are often accompanied by local and systemic harmful effects, as well as rebound flare-ups (Weidinger and Novak, 2016). Therefore, identification of nonsteroid topical drugs is of clinical value for treatment of acute and chronic inflammatory skin disorders.

In addition to forming the physical permeability barrier, keratinocytes in the epidermis are actively involved in immune function via their ability to synthesize antimicrobial effectors and inflammatory mediators. Keratinocytes from AD lesions

express high levels of proinflammatory cytokines including TSLP (Ong, 2014; Soumelis et al., 2002). TSLP enhances the ability of dendritic cells to prime naïve CD4⁺ T cells, inducing their differentiation into pro-allergic Th2 cells (Nakajima et al., 2012; Soumelis et al., 2002). In addition, it facilitates the development of several innate immune cells, including ILC2s, mast cells, natural killer T cells, basophils, and eosinophils (Cianferoni and Spergel, 2014; Ziegler and Artis, 2010).

The retinoic acid receptor-related orphan receptors (RORs; ROR α , ROR β , and ROR γ) are members of the nuclear receptor superfamily (Cook et al., 2015). ROR α and ROR γ play a key role in the lineage determination of Th17 cells (Ivanov et al., 2006; Yang et al., 2008), whose malfunctions are associated with the pathology of autoimmune and inflammatory diseases (Littman and Rudensky, 2010; Patel and Kuchroo, 2015). ROR α is also required for the development of ILC2s (Wong et al., 2012), which are detected at a higher frequency in the skin lesions of AD patients (Salimi et al., 2013). In an AD mouse model, ROR α loss of function was shown to alleviate skin inflammation because of the reduced number of ILC2s (Salimi et al., 2013). This suggests that the clinical severity of AD can potentially be alleviated by pharmacological inhibition of ROR α activity.

A number of endogenous and synthetic small-molecule ligands of ROR α and ROR γ have recently been identified (Solt and Burris, 2012). The inverse agonist SR1001 selectively inhibits the transcriptional activity of ROR α and ROR γ , but not 48 other nuclear receptors (Solt et al., 2011). SR1001 binding to ROR α/γ leads to a conformational change that decreases the recruitment of transcriptional co-activators. By targeting Th17-mediated immune responses, SR1001 effectively delays the onset and reduces the clinical severity of autoimmune disease in animal models of multiple sclerosis and type I diabetes (Solt et al., 2011, 2015).

Given the regulatory functions of ROR α/γ in multiple cell types and different forms of inflammation, we examined the

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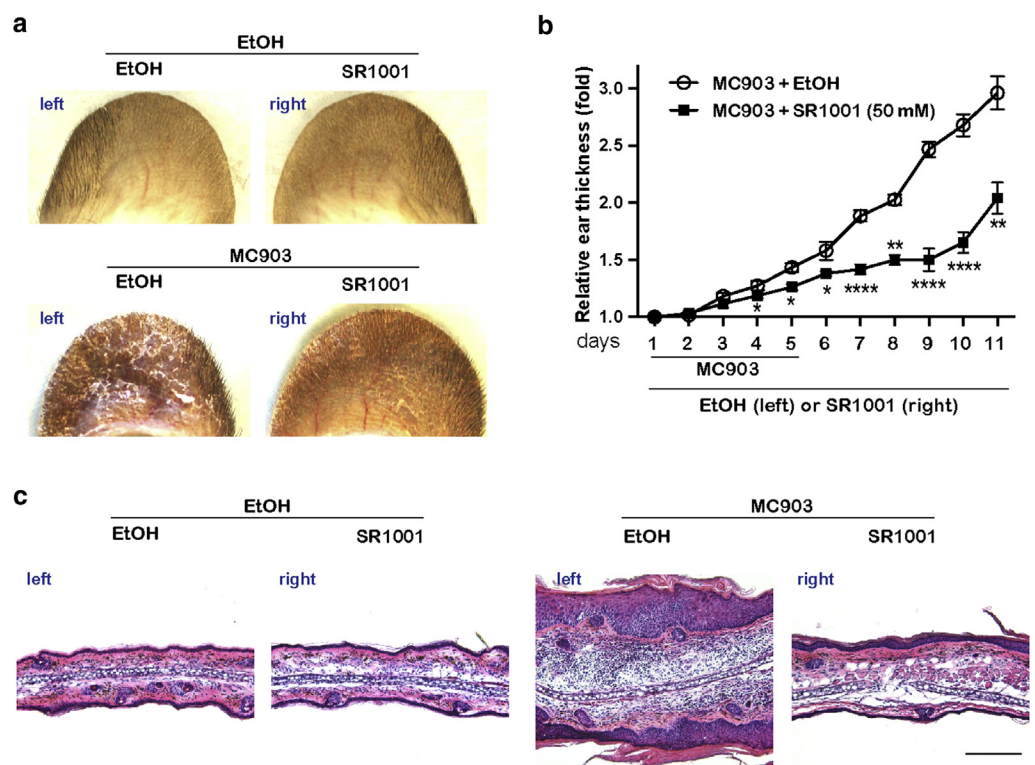
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Abbreviations: AD, atopic dermatitis; ILC, innate lymphoid cell; MKC, mouse keratinocyte; qRT-PCR, quantitative real-time reverse transcriptase-PCR; ROR, retinoic acid receptor-related orphan receptor; Th, T helper; TPA, 12-O-tetradecanoylphorbol-13-acetate

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Figure 1. SR1001 reduces MC903-induced ear thickening.

Both ears of the same mouse were topically painted with ethanol or MC903, followed by treatment with 25 μ l of 50 mmol/L SR1001 (0.6 mg) on the right ear or 100% ethanol on the left ear. (a) Representative images of mouse ears collected at day 11. (b) Mouse ear thickness. Values are normalized to the original thickness measured on day 1 and presented as mean fold $\pm$ standard error of the mean. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. $n = 14$ /group from three independent experiments. (c) Representative hematoxylin and eosin staining of frozen sections of ear tissues collected on day 11. Original magnification = $\times 4$ magnification. Scale bar = 200 μ m. EtOH, ethanol; M, mol/L.



efficacy of inhibitory ligands of ROR α/γ against two distinct models of skin diseases and their effects on keratinocyte-driven inflammatory responses. We found that topical application of SR1001 exerted strong local anti-inflammatory effects in both MC903-induced AD-like disease and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced acute irritant dermatitis in mice. These effects were likely mediated by the suppressive effect of SR1001 on the production of innate immune cell- and epidermal cell-derived inflammatory mediators. Our results suggest ROR α/γ inverse agonists as nonsteroid topical agents for the prevention and treatment of inflammatory skin disorders.

RESULTS AND DISCUSSION**ROR α/γ inverse agonists inhibit MC903-induced ear thickening**

It has been shown in mice that ROR α is required for AD-like skin inflammation induced by MC903, a low-calcemic vitamin D analog (Salimi et al., 2013). We first used this model to examine the topical effects of SR1001, an ROR α/γ inverse agonist that has been tested in several mouse models of autoimmune diseases (Solt et al., 2011, 2015). To minimize biases from individual animal variations, both ears of each mouse were challenged daily with MC903, followed by topical treatment with SR1001 (total of 0.6 mg in ethanol) on the right ear and ethanol on the left ear as vehicle control.

MC903/ethanol-painted left ears developed erythema and edema gradually and appeared dry and crusted at the end of treatment (see Supplementary Figure S1a online). Such pathological signs were not observed in MC903/SR1001-treated right ears in the same animals (Figure 1a). The average thickness of MC903-treated left ears increased to 3

times their original size on day 11. Between days 4 and 11, the magnitude of ear thickening was significantly reduced by SR1001 treatment (Figure 1b). Histological analysis showed a marked expansion of epidermis and dermis in MC903-treated skin with a massive cellular infiltration in the dermis. In contrast, topical SR1001 significantly attenuated the severity of these inflammatory features (Figure 1c). The effect of SR1001 on ear swelling was detected from day 6, when a lower dose (0.12 mg) was applied to MC903-treated skin (see Supplementary Figure S1a).

As an alternative regimen, SR1001 was applied onto mouse ears after the completion of 5-day MC903 treatment. In this condition, SR1001 treatment elicited a significant suppressive effect on ear thickening from day 10 on (see Supplementary Figure S1b). The anti-inflammatory potential of SR1001 was also tested in another model of chronic skin inflammation, induced by multiple topical application of TPA (Stanley et al., 1991). Ear thickness continued to increase and reached 4.7 times the original size on day 9 in the TPA/ethanol-treated control group; however, the magnitude of ear thickening was markedly reduced by SR1001 treatment (see Supplementary Figure S1c). Overall, these results indicate the potential of SR1001 in the prevention and treatment of AD-like chronic inflammatory diseases.

Although SR1001 and the ROR α selective synthetic ligand SR3335 (Kumar et al., 2011) have similar affinities for ROR α (Solt and Burris, 2012), SR3335 exerted a similar but somewhat delayed effect, day 9 being the earliest time point showing a significant reduction in skin thickening (see Supplementary Figure S2a online). Between day 8 and day 11, the magnitude of ear thickening in the MC903/ethanol-treated left side was significantly smaller in the SR3335-

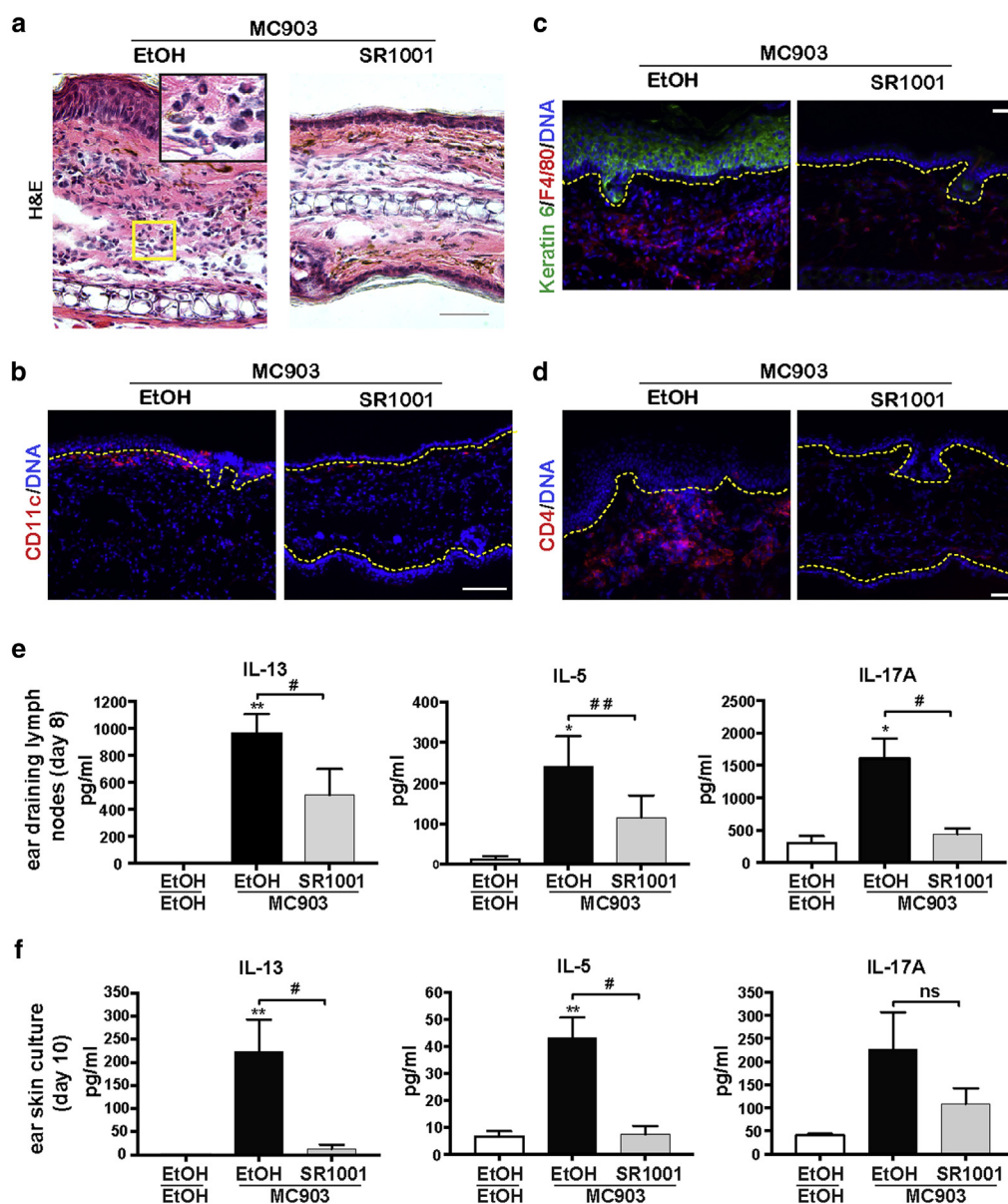


Figure 2. SR1001 suppresses immune cell responses to MC903 challenge.

(a–d) Mouse ears were challenged with MC903, as described in Figure 1. (a) Representative H&E images showing eosinophils in the dermis (inset). (b–d) Representative images of immunostaining with antibodies against (b) CD11c, (c) keratin 6 and F4/80, or (d) CD4. DNA was counterstained with Hoechst. Scale bars = 50 μ m in a, c, and d or 100 μ m in b. (e–f) Single cell suspension was prepared using (e) skin-draining lymph nodes and (f) skin tissue collected from both sides at day 8 and day 10, respectively. Cells were re-stimulated with 12-*O*-tetradecanoylphorbol-13-acetate/ionomycin for 24 hours. Cytokines in culture supernatants were analyzed by ELISA. Values are presented as mean $\pm$ standard error of the mean. $n = 3$ –5/group. * $P < 0.05$, ** $P < 0.01$. Difference in cytokine production was also analyzed between the two ears of MC903-treated mice. # $P < 0.05$, ## $P < 0.01$. EtOH, ethanol; H&E, hematoxylin and eosin; ns, not significant.

treated group than in the SR1001-treated group (see [Supplementary Figure S2b](#)), indicating a bigger systemic effect of SR3335. The partition coefficient, or log P , represents a physical property that measures the lipophilicity and membrane permeability of a given small molecule ([Grunbauer et al., 1979](#); [Lipinski et al., 2001](#)). Based on the log P values, SR3335 (+0.167) is about 8-fold more lipophilic than SR1001 (−0.794) (see [Supplementary Figure S3](#) online) and, thus, may have a better ability to penetrate the skin barrier and cause systemic effects during topical application.

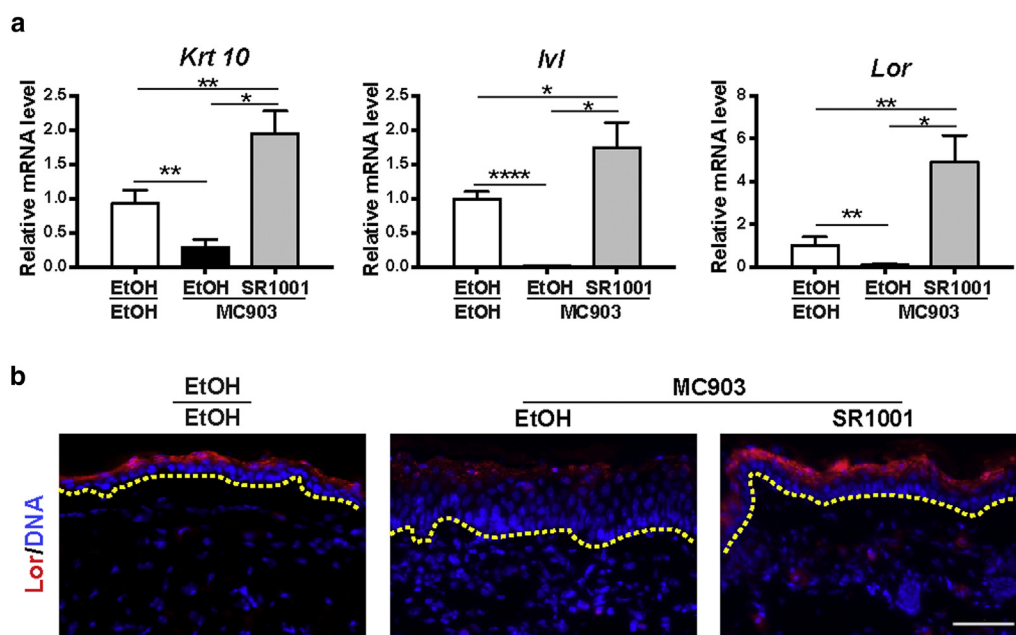
It has been reported that ILC2 numbers increase in MC903-treated ear skin tissue and draining lymph nodes and that depletion of these cells attenuates AD-like skin inflammation in this model ([Kim et al., 2013](#); [Salimi et al., 2013](#)). Bone marrow chimeras involving *Rora*^{sg/sg} mice have shown a role for ILC2-specific ROR α in the development of MC903-induced AD-like disease ([Salimi et al., 2013](#)). Hence, SR1001 and SR3335 may exert their anti-inflammatory effects by inhibiting ROR α activities in ILC2 cells.

Although ROR γ ⁺ group 3 ILCs (ILC3s) were also detected in the skin-draining lymph nodes upon MC903 application, knockout of *Rorc* (the ROR γ gene) did not prevent AD-like skin inflammation ([Kim et al., 2013](#)). However, we observed that the ROR γ -specific inverse agonist SR1555 ([Solt et al., 2012](#)) suppressed MC903-induced ear swelling on day 7 and onward (see [Supplementary Figure S2c](#)). SR1555 has a much higher log P value (+5.196) than SR1001 (−0.794) and SR3335 (+0.167), suggesting a substantially higher lipophilicity and potential for skin penetration (see [Supplementary Figure S3](#)). Further analysis of SR1555 action against ROR γ in vivo will help show the mechanisms underlying its anti-inflammatory effects in the AD model.

SR1001 inhibits MC903-induced AD-like skin inflammation

Next, we performed histological analysis and immunostaining to further evaluate the anti-inflammatory effects of SR1001. A number of cell types were found to infiltrate the dermis of MC903-treated ear skin, including eosinophils

Figure 3. SR1001 modulates the expression of keratinocyte differentiation markers in MC903-treated mouse skin. Mouse ears were treated with EtOH or MC903, as described in Figure 1. Ear samples were collected at day 7. (a) The mRNA expression of indicated differentiation markers was measured by quantitative real-time reverse transcriptase–PCR and normalized to *36B4*. Values from MC903-treated ears are normalized to EtOH-treated control ears (set as 1), and presented as mean fold $\pm$ standard error of the mean. $n = 4$ /group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (b) Representative images of immunostaining of frozen ear tissue sections with an antibody against loricrin. DNA was counterstained with Hoechst. Scale bar = 50 μ m. EtOH, ethanol; IvI, involucrin; Krt, keratin; Lor, loricrin.



(Figure 2a), dendritic cells (Figure 2b), macrophages (Figure 2c), and CD4⁺ T cells (Figure 2d). The hyper-proliferative epidermis had test results positive for keratin 6, a marker of keratinocytes in inflamed epidermis (Figure 2c). In the presence of SR1001, epidermal expression of keratin 6 and dermal infiltration were significantly attenuated (Figure 2a–d). In addition, SR1001 broadly attenuated MC903-induced expression of cytokines (*Il1b*, *Il6*), chemokines (*Cxcl1*, *Cxcl2*, *Cxcl10*), and other inflammatory mediators (*Ptgs2*) (see Supplementary Figure S4a online).

MC903 application under our regimen induced a systemic increase in serum IgE levels at day 12 (see Supplementary Figure S4b). Consistent with the involvement of Th2 responses in AD-like skin disease, cells isolated from the ear-draining lymph nodes and the skin of MC903-challenged mice produced high levels IL-13 and IL-5 in culture; these responses were markedly reduced with cells derived from SR1001-treated skin (Figure 2e and f). Similarly, MC903 application also triggered a modest increase in lymph node cells that could produce IL-17A; this response was also suppressed by SR1001 treatment (Figure 2e and f). In addition, quantitative real-time reverse transcriptase–PCR (qRT-PCR) analysis showed an increase in *Il4* expression and a decrease in *Ilfng* expression in MC903-challenged skin (see Supplementary Figure S4c). SR1001 treatment counteracted the induction of *Il4* but not the reduction of *Ilfng* (see Supplementary Figure S4c). Together, these results show that topical application of the ROR α/γ inverse agonist SR1001 exerts potent anti-inflammatory effects, including the suppression of Th2 and Th17 cytokine production, in an MC903-dependent AD model.

It has been shown that MC903 can trigger AD-like skin inflammation, including Th2 cytokine production, in *Rag1*^{−/−} mice, indicating that T and B cells are dispensable in this model (Kim et al., 2013; Li et al., 2006). Consistently, our FACS analysis did not show an increase in the number of Th2 (IL-4⁺) or Th17 (IL17A⁺) cells among CD3⁺CD4⁺ T cells in

the ear-draining lymph nodes at day 8 after MC903 treatment (see Supplementary Figure S4d). This suggests that the high levels of IL-13/IL-5 and IL-17A cytokine production from MC903-challenged mice (Figure 2e and f) arise mainly from innate sources, such as ILCs, rather than CD4⁺ T cells (Figure 2d). Therefore, the anti-inflammatory effects of SR1001 are likely mediated, at least in part, by directly suppressing ILC2 development and function. SR1001 has been shown to inhibit Th17 polarization without altering *Foxp3* expression (Solt et al., 2011). Further in vitro studies are needed to prove the ILC2-directed effects of SR1001 and other ROR α ligands.

Impaired keratinocyte differentiation in MC903-treated skin is reversed by SR1001

Along with its proinflammatory action, MC903 greatly reduced epidermal expression of keratin 10, involucrin, and loricrin, keratinocyte markers of different stages of differentiation (Figure 3a). The expression of these markers was restored by SR1001 to levels even higher than those in ethanol-treated control ears (Figure 3a). The effect of SR1001 on loricrin expression, related to epidermal barrier function, was confirmed at the protein level by immunostaining (Figure 3b). Restored epidermal barrier may contribute to the reduced number of dermal infiltrates in SR1001-treated ears.

Our previous study showed that ROR α was highly expressed in human keratinocytes and played a role in promoting their differentiation via the activation of FOXN1 (Dai et al., 2013). In mice, ROR α transcripts are highly expressed in the skin, and homozygous ROR α ^{sg/sg} mutant mice exhibit sparse pelage and a slow rate of hair regrowth after shaving (Hamilton et al., 1996). These observations raise the possibility that ROR α inhibitors, particularly when topically applied, suppress keratinocyte differentiation and result in epidermal barrier disruption. No such adverse effects have so far been observed in SR1001-treated mice. SR1001 rather restored keratinocyte differentiation and epidermal

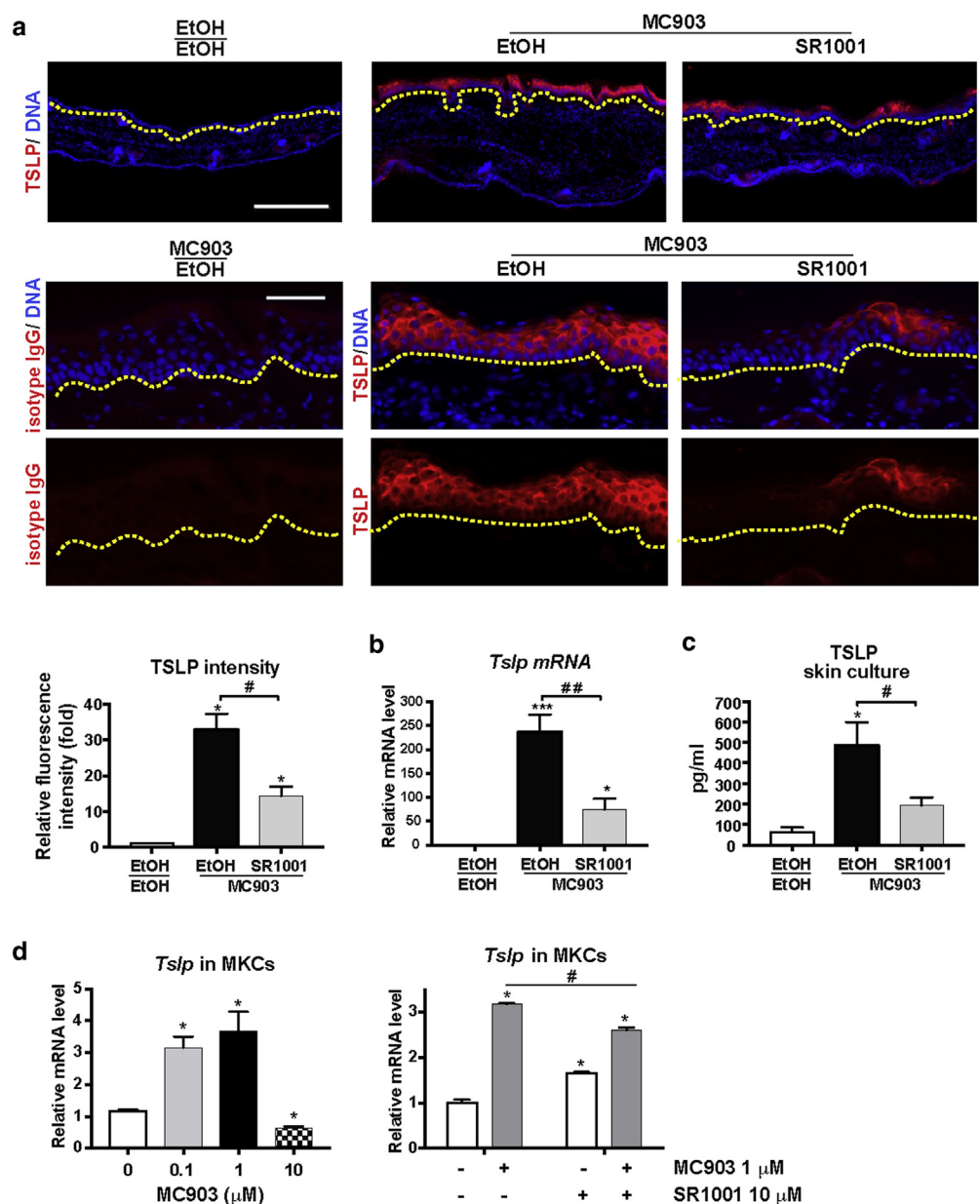


Figure 4. SR1001 suppresses MC903-induced expression of TSLP in the skin. (a–c) Mouse ears were painted with EtOH or MC903, as described in Figure 1. Ear samples were collected at day 7. (a) Representative images of immunostaining with the TSLP antibody or the isotype control IgG. Scale bars = 200 μ m (original magnification $\times 4$) or 50 μ m (original magnification $\times 20$). Fluorescence intensity of TSLP was quantified on three fields/section with ImageJ software (National Institutes of Health, Bethesda, MD). Combined values from MC903-treated ears are normalized to control ears and are presented as mean $\pm$ standard error of the mean ($n = 3$ /group). (b) *Tslp* mRNA expression was measured by quantitative real-time reverse transcriptase–PCR. (c) TSLP production from ear skin cultures was measured by ELISA, as described in Figure 2f. (d) Reverse transcriptase–PCR analysis of *Tslp* mRNA in primary MKCs stimulated by MC903 (20 hours). EtOH, ethanol; M, mol/L; MKC, mouse keratinocyte.

homeostasis in MC903-challenged skin. Further elucidation of ROR α/γ functions in keratinocytes will help identify the cellular targets of ROR α/γ inhibitors that are crucial for their pharmacological mechanism of action.

SR1001 reduces epidermal TSLP expression in MC903-treated skin

MC903-induced AD-like disease relies on keratinocyte expression of TSLP (Li et al., 2006, 2009). Immunofluorescence staining confirmed that TSLP expression was strongly induced in the suprabasal layers of the epidermis after 7 days of exposure to MC903 (Figure 4a). The intensity of TSLP staining and *Tslp* mRNA was markedly reduced in the skin of SR1001-treated ears (Figure 4a and b, and see Supplementary Figure S5 online). ELISA of supernatants of cultured whole skin confirmed the suppressive effect of SR1001 on TSLP production (Figure 4c). These results suggest that the

anti-inflammatory activity of SR1001 is related, at least in part, to reduction in the proinflammatory cytokine TSLP.

In vitro studies were performed to directly assess the effect of SR1001 on *Tslp* expression in primary mouse keratinocytes (MKCs). As shown in Figure 4d, *Tslp* mRNA increased significantly by MC903 at concentrations of 0.1 and 1.0 μ mol/L. In contrast to its robust in vivo effect (Figure 4a–c), SR1001 at 10 μ mol/L moderately suppressed *Tslp* expression in MC903-treated MKCs (Figure 4d). This discrepancy may be attributable to differences in the kinetics and concentrations of cell exposure to MC903 and SR1001 in vitro and in vivo. Furthermore, non-epithelial cells, such as stromal cells, may contribute to TSLP production in vivo; the effects of SR1001 on TSLP expression in vivo may reflect a combined action of SR1001 on multiple cell types in the skin.

A multitude of nuclear receptors play a role in TSLP gene transcription in keratinocytes. MC903-induced expression of

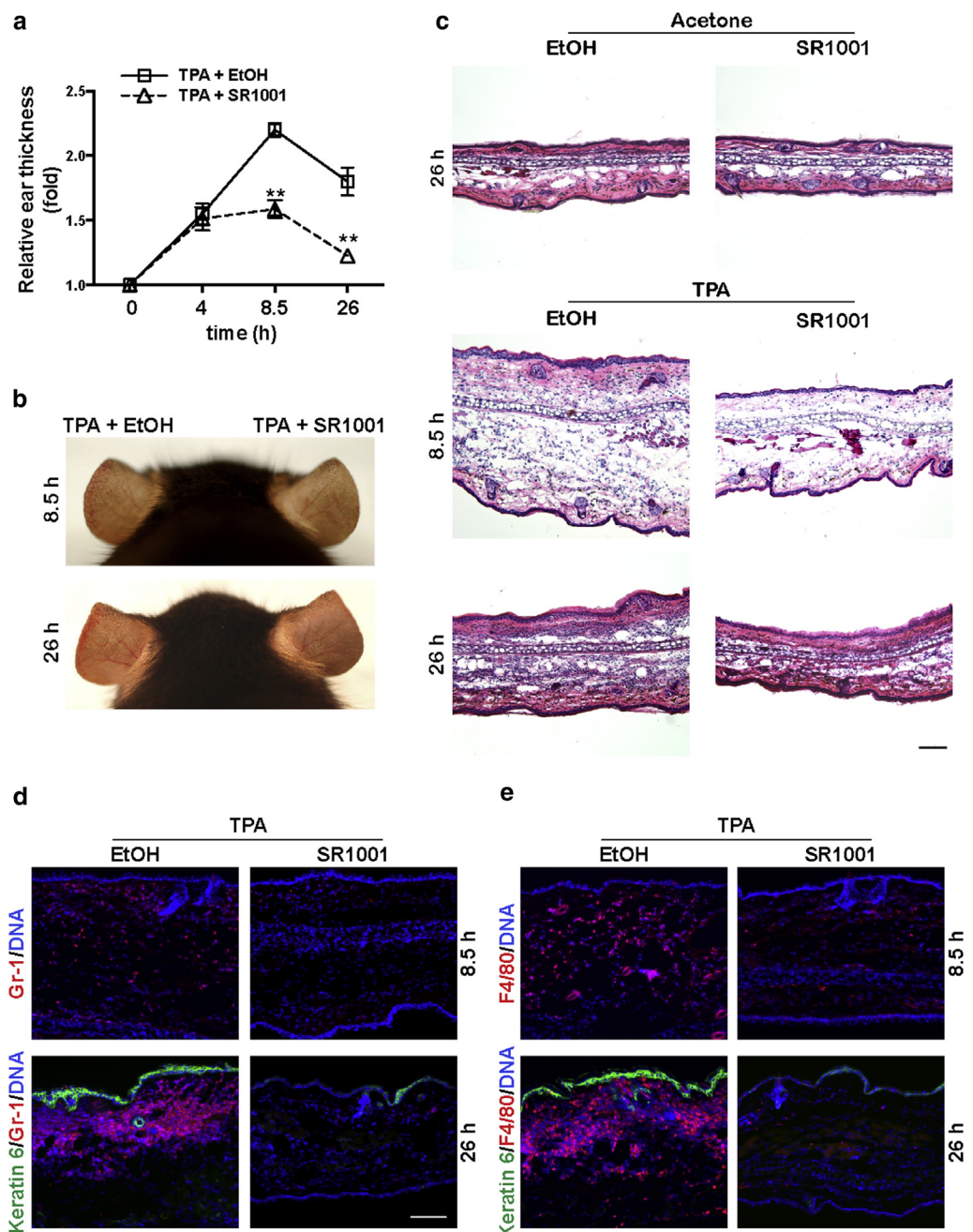
Figure 5. SR1001 inhibits TPA-induced ear swelling and skin inflammation.

Both ears of each mouse were topically challenged with TPA, followed by treatment with 25 μ L of 50 mmol/L SR1001 (right) or ethanol (left) at 1 hour and 4 hours.

(a) Mouse ear thickness. Values are normalized to the original size measured before TPA application and are presented as mean fold $\pm$ standard error of the mean. $**P < 0.01$. $n = 8$ –10/group from at least three experiments.

(b) Representative images of mouse ears at 8.5 hours and 26 hours. (c) Representative images of hematoxylin and eosin staining of mouse ear tissue sections.

(d–e) Representative immunostaining of frozen ear tissue sections with antibodies against (d) keratin 6 plus Gr-1 or (e) F4/80. DNA was counterstained with Hoechst. Scale bars = 100 μ m. EtOH, ethanol; h, hour; M, mol/L; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.



TSLP has been shown to rely on the activation of the vitamin D receptor (Li et al., 2006). In addition, there is evidence suggesting a role for RXRs (RXR α and RXR β), RAR α , and PPAR in modulating keratinocyte expression of TSLP (Lee et al., 2008; Li et al., 2006). It remains to be clarified whether ROR α and/or ROR γ can directly regulate TSLP gene transcription in keratinocytes and other cell types, such as mast cells and basophils (Sokol et al., 2008; Soumelis and Liu, 2004).

SR1001 suppresses inflammation in a mouse model of acute irritant dermatitis

We next aimed to determine whether the anti-inflammatory effects of SR1001 extended to other forms of cutaneous

inflammation. Topical TPA has been used as an experimental means to induce acute, self-resolving skin inflammation (Young et al., 1983). Similarly to the MC903 experiment, we subjected both ears of mice to single TPA application, followed by topical treatment with SR1001 on the right ear and vehicle on the left ear. The thickness of left ears increased rapidly and reached the maximum at 8.5 hours after TPA application (Figure 5a). This process was accompanied by signs of vasodilation and erythema around the edge of pinnae (Figure 5b). The magnitude of ear swelling and erythema was significantly reduced by SR1001 treatment (Figure 5a and b), with an effect comparable to that achieved by clobetasol propionate (0.0025%), a potent glucocorticoid (see Supplementary Figure S6 online). Although ROR α -specific

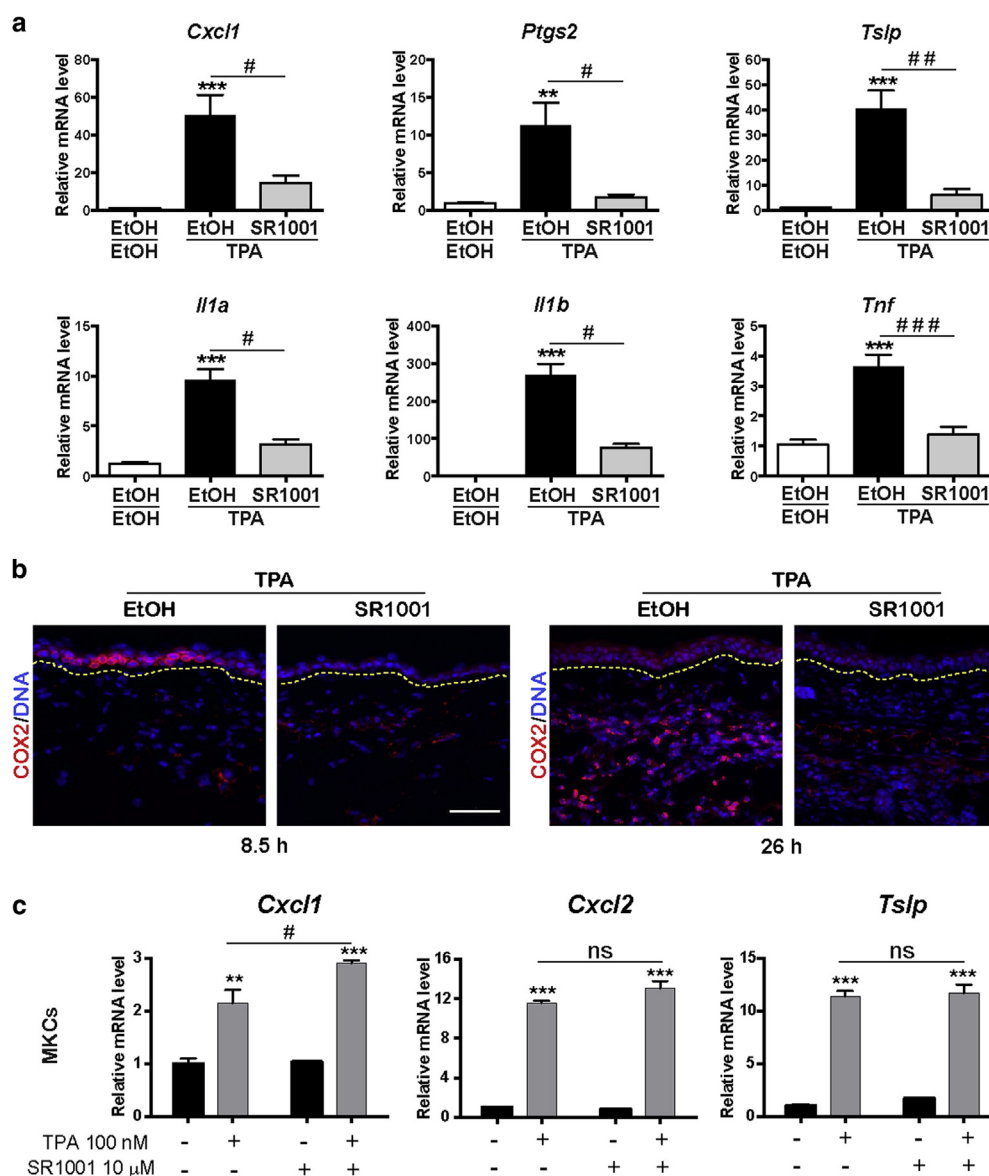


Figure 6. SR1001 inhibits the expression of pro-inflammatory factors induced by TPA in the skin. (a

–b) Both ears of each mouse were topically painted with TPA and treated with EtOH (left) or 50 mmol/L of SR1001 (right). (a) RNA was isolated from ear tissues at 8.5 hours after TPA application. The mRNA expression of indicated inflammatory factors was measured by quantitative real-time reverse transcriptase–PCR. Values from TPA-treated ears are normalized to EtOH-treated ears (set as 1), and presented as mean fold $\pm$ standard error of the mean. $n = 4$ –5/group. ** $P < 0.01$, *** $P < 0.001$. Difference in gene expression between the two ears of TPA-treated mice was also analyzed. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.

(b) Representative immunostaining of frozen sections of mouse ear skin samples with the antibody against cyclooxygenase-2. DNA was counterstained with Hoechst. Scale bar = 50 μ m. (c) Primary MKCs were pretreated with 10 μ mol/L of SR1001 or ethanol for 1 hour, followed by a 4-hour treatment with 100 nmol/L of TPA. The mRNA expression of indicated cytokines was analyzed by quantitative real-time reverse transcriptase–PCR. Values are shown as mean-fold over control $\pm$ standard error of the mean. $n = 3$ /treatment. ** $P < 0.01$, *** $P < 0.001$. Difference in gene expression was also analyzed within TPA group between ethanol- and SR1001-treated cells. # $P < 0.05$. COX2, cyclooxygenase 2; EtOH, ethanol; h, hour; M, mol/L; MKC, mouse keratinocyte; ns, not significant; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

SR3335 significantly reduced TPA-induced ear swelling at 26 hours after TPA treatment, the ROR γ -specific SR1555 showed such a trend without significance (see [Supplementary Figure S6](#)).

Histological analysis showed an extensive dermal expansion in TPA-treated control ears around 8.5 hours with cellular infiltrates ([Figure 5c](#)), which contained Gr-1⁺ neutrophils and F4/80⁺ macrophages ([Figure 5d and e](#)). Dermal infiltration became more intense and epidermal hyperproliferation detectable at 26 hours after TPA challenge ([Figure 5c–e](#)). The severity of dermal infiltration and epidermal hyperproliferation was significantly lower in SR1001-treated ears ([Figure 5c–e](#)). Consistently, SR1001 markedly attenuated TPA-induced expression of inflammatory mediators including TSLP (encoded by *Tslp*) and cyclooxygenase-2 (encoded by *Ptgs2*) in the epidermal and dermal compartments ([Figure 6a and b](#)). However, TPA induction of *Tslp* and chemokine genes (*Cxcl1* and *Cxcl2*) was not blocked by SR1001 treatment in cultured keratinocytes

([Figure 6c](#)). Therefore, the anti-inflammatory effect of SR1001 on TPA-induced acute dermatitis likely reflects its action on keratinocyte-independent innate immune responses. Different mechanisms appear to drive MC903- and TPA-induced inflammation and account for the anti-inflammatory actions of SR1001 in these distinct models.

CONCLUSION

Here, we show that topical application of ROR α/γ inverse agonists produces beneficial effects in mouse models of AD and acute irritant dermatitis. In addition, SR1001 attenuates the expression of the epidermal-derived inflammatory mediator TSLP, which is critical for the onset of allergic skin inflammation. By targeting pro-allergic molecular processes in both hematopoietic-derived and epithelial cells (i.e., ILC2 development/function and epidermal TSLP expression, respectively), the inverse agonists of ROR α/γ may represent a new class of nonsteroidal anti-inflammatory compounds for the treatment of skin inflammatory diseases including AD.

MATERIALS AND METHODS

Reagents

SR1001, MC903 (calcipotriol hydrate), TPA, and ionomycin were purchased from Sigma (Saint Louis, MO).

Skin inflammation models

Female C57BL/6J mice of 8–10 weeks were obtained from Jackson Labs (Bar Harbor, ME) and housed in a pathogen-free environment. All animal studies were conducted under Institutional Animal Care and Use Committee-approved protocols. In the AD model, both ears were painted with 2.5 nmol of MC903 in 25 μ l ethanol. Five hours later, right ears were topically treated with 25 μ l of SR1001 (50 mmol/L or 10 mmol/L) and left ears with ethanol as vehicle control. In the control group, ethanol was applied to replace MC903 as the stimulant. After 5 consecutive days, ears continued to receive SR1001 or ethanol daily without MC903. Ear thickness was measured daily with a digital caliper (Mitutoyo Corp., Tokyo, Japan). At the end of the experiment, half of the ear was snap frozen into liquid nitrogen for RNA isolation and the other half was embedded in optimal cutting temperature compound to prepare frozen sections.

In the acute irritant dermatitis model, both ears were challenged with 2 μ g of TPA. At 1 and 4 hours after TPA application, right ears were treated with 25 μ l of 50 mmol/L SR1001 and left ears with ethanol.

Histology and immunofluorescence

For histological analysis, frozen sections were fixed with 10% formalin and stained with hematoxylin and eosin (Protocols, Kalamazoo, MI). For immunostaining, frozen sections (8 μ mol/L) were fixed with 4% paraformaldehyde/phosphate buffered saline, permeabilized with 0.1% Nonidet P-40/phosphate buffered saline, and incubated with the primary antibodies at 4 °C overnight. Slides were then incubated with Alexa 488- or Alexa 594-conjugated secondary antibodies (Invitrogen, Grand Island, NY) plus Hoechst (Invitrogen) for DNA detection. Fluorescence microscopy was carried with Nikon TE300 inverted fluorescence microscope (Nikon, Melville, NY). A list of primary antibodies for immunostaining is provided in the [Supplementary Materials and Methods](#) online.

qRT-PCR

Total RNA from ear tissues were isolated with Trizol (Ambion—Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions, followed by further purification with RNeasy Mini kit (Qiagen, Hilden, Germany). For mRNA analysis, 100 μ g of total RNA was analyzed by qRT-PCR with Kappa SYBR FAST One-Step qRT-PCR kit (Kappa Biosystems, Wilmington, MA) on the Light Cycler 480 Real Time PCR instrument (Roche Applied Science, Indianapolis, IN). Each sample was tested in duplicate, and results were normalized with the expression of the housekeeping 36 β 4 gene. The list of gene-specific primers for qRT-PCR is provided in [Supplementary Table S1](#).

ELISA of cells isolated from ear draining lymph nodes and skin tissues

Ear draining lymph nodes were dissected at day 8 after MC903 application, according to the protocol described by [Mac-Daniel et al. \(2016\)](#). Ear skin tissues were collected at day 10. Single cell suspension from lymph nodes and skin tissues were plated at a density of 5×10^5 cells/well in 96-well plates or 7×10^5 cells/well in 48-well plates, respectively. After cells were re-stimulated with 50 ng/ml TPA and 1 μ mol/L of ionomycin for 24 hours, culture media were collected for

cytokine production measured by ELISA, using commercial kits from eBioscience (Thermo Fisher Scientific, Madison, WI).

In vitro study using primary mouse keratinocytes

Primary MKCs were isolated from the skin of pups (postnatal days 3–4) of wild-type C57BL/6 mice and cultured in the CnT 07S medium from CELLnTEC Advanced Cell Systems (Bern, Switzerland). MKCs were stimulated with different concentrations of MC903 for 20 hours or 100 nmol/L of TPA for 4 hours, and harvested for total RNA isolation and qRT-PCR analysis.

Statistics

All statistical evaluations were carried out using Prism 7 (GraphPad Software, La Jolla, CA). Paired two-tailed Student *t* test was used to compare the statistical difference of ear thickness or gene expression between the two ears of the same animal. *P*-values for two data sets between different animals were obtained with unpaired two-tailed Student *t* test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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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.2017.07.819>.

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SUPPLEMENTARY INFORMATION

Topical ROR inverse agonists suppress inflammation in mouse models of atopic dermatitis and acute irritant dermatitis

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Supplementary Figure S1

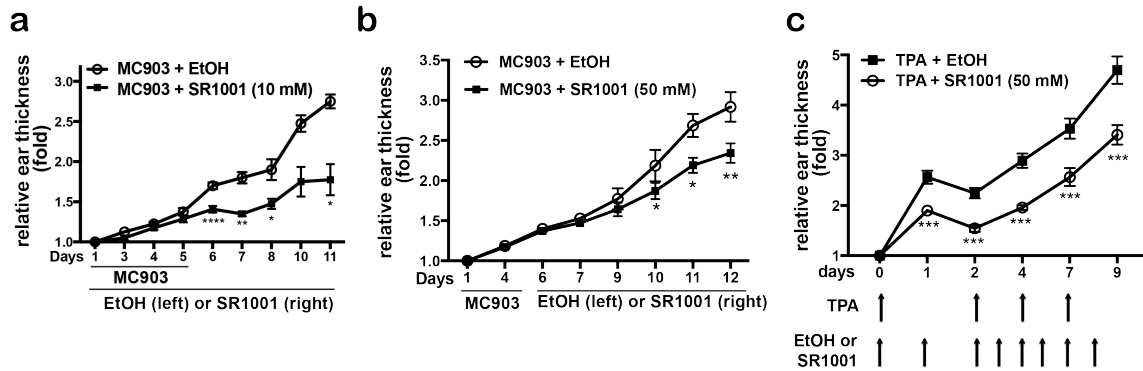


Figure S1. SR1001 suppresses ear thickening in chronic dermatitis mouse models. Mouse ear thickness was measured following different treatments. **(a)** Both ears were topically painted with MC903 for 5 days. From day 1, right ears were daily treated with 25 μ l of 10 mM of SR1001 (0.12 mg) and left ears were treated with EtOH. **(b)** Both ears were topically painted with MC903 for 5 days. Starting from day 6, right ears were treated with 25 μ l of 50 mM SR1001 (0.6 mg) daily, and left ears were treated with EtOH. **(c)** Ears from two groups of mice were topically challenged with TPA (2 μ g per ear) on day 0, day 2, day 4, and day 7, with one group treated with SR1001 and other with EtOH at indicated time points. Thickness of each ear is normalized to its original size measured on day 1, and presented as mean fold $\pm$ S.E.M., *, $p < 0.05$, **, $p < 0.01$, ****, $p < 0.0001$, $N=5-7$ /group.

Supplementary Figure S2

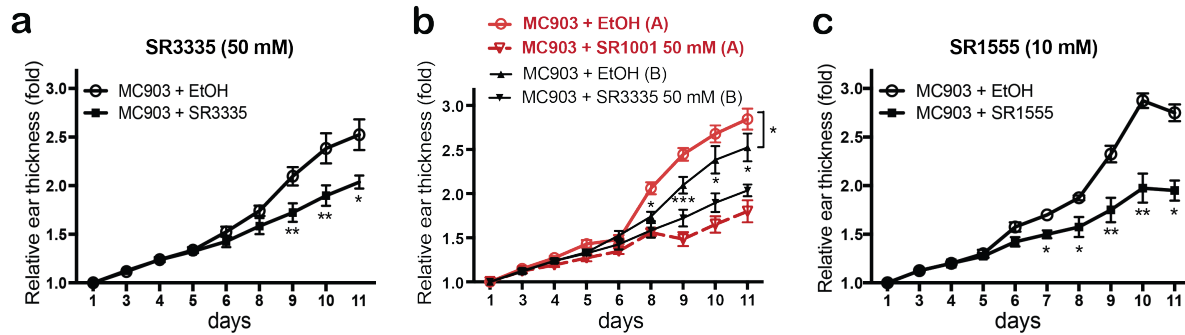
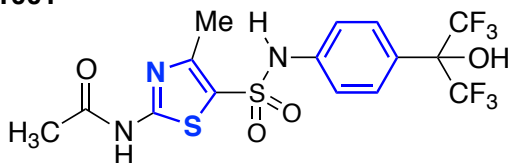


Figure S2. Effects of ROR α and ROR γ specific inverse agonists on MC903-induced ear thickening. Both ears were topically painted with MC903 for 5 days. Left ears were treated once daily with 25 μ l of EtOH and right ears were treated with 50 mM of the ROR α specific inverse agonist SR3335 (0.51 mg) **[a]** or 10 mM of the ROR γ selective inverse agonist SR1555 (0.11 mg) **[c]**. Mouse ear thickness was measured daily. Values are normalized to the original thickness measured on day 1 (set as 1), and presented as mean fold $\pm$ S.E.M., *, $p < 0.05$, **, $p < 0.01$, ****, $p < 0.0001$, $N=4-7/\text{group}$. **(b)** Overlay of ear thickness curves between SR3335-treated group B as shown in Figure S2a and SR1001-treated group A as shown in Figure 1b.

Supplementary Figure S3

Calculated Log-P Values of SR Compounds Based on Rekker's Hydrophobic Constants For Organic Functional Groups and Fragments.

SR1001



Chemical Formula: $C_{15}H_{13}F_6N_3O_4S_2$

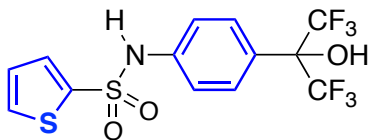
Exact Mass: 477.0252

Molecular Weight: 477.4018

Organic Fragments	Rekker Constant f	Total
2 CH ₃ Aliphatic Carbons	+0.702	+1.404
1 3° Aliphatic Carbon	+0.235	+0.235
1 Phenyl Ring (–C ₆ H ₄ –)	+1.688	+1.688
2 CF ₃	+0.757	+1.514
1 Thiazole Ring	+0.420	+0.420
1 Amide (–C=O–NH–)	–1.970	–1.970
1 Alcoholic OH	–1.491	–1.491
1 "NH" from (RSO ₂ NH–Ar)	–0.964	–0.964
1 Sulfone from "–SO ₂ –R"	–1.63	–1.63

Estimated Log-P **–0.794**

SR3335



Chemical Formula: $C_{13}H_9F_6NO_3S_2$

Exact Mass: 404.9928

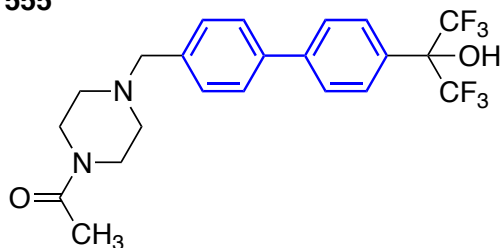
Molecular Weight: 405.3359

Organic Fragments	Rekker Constant f	Total
1 3° Aliphatic Carbon	+0.235	+0.235
1 Phenyl Ring (–C ₆ H ₄ –)	+1.688	+1.688
2 CF ₃	+0.757	+1.514
3 Aromatic C–H's	+0.235	+0.705
1 Aromatic "S"	+0.110	+0.110
1 Alcoholic OH	–1.491	–1.491
1 "NH" from (RSO ₂ NH–Ar)	–0.964	–0.964
1 Sulfone from "–SO ₂ –R"	–1.63	–1.63

Estimated Log-P **+0.167**

Supplementary Figure S3 (continued)

SR1555



Chemical Formula: $C_{22}H_{22}F_6N_2O_2$

Exact Mass: 460.1585

Molecular Weight: 460.4127

Organic Fragments	Rekker Constant f	Total
1 CH ₃ Aliphatic Carbon	+0.702	+0.702
5 2° Aliphatic Carbons	+0.530	+2.65
1 3° Aliphatic Carbon	+0.235	+0.235
2 Phenyl Rings (–C ₆ H ₄ –)	+1.688	+3.376
2 CF ₃	+0.757	+1.514
1 3° Amine “N”	+0.18	+0.18
1 Amide (–C=O–N–)	–1.970	–1.970
1 Alcoholic OH	–1.491	–1.491
Estimated Log-P		+5.196

Supplementary Figure S4

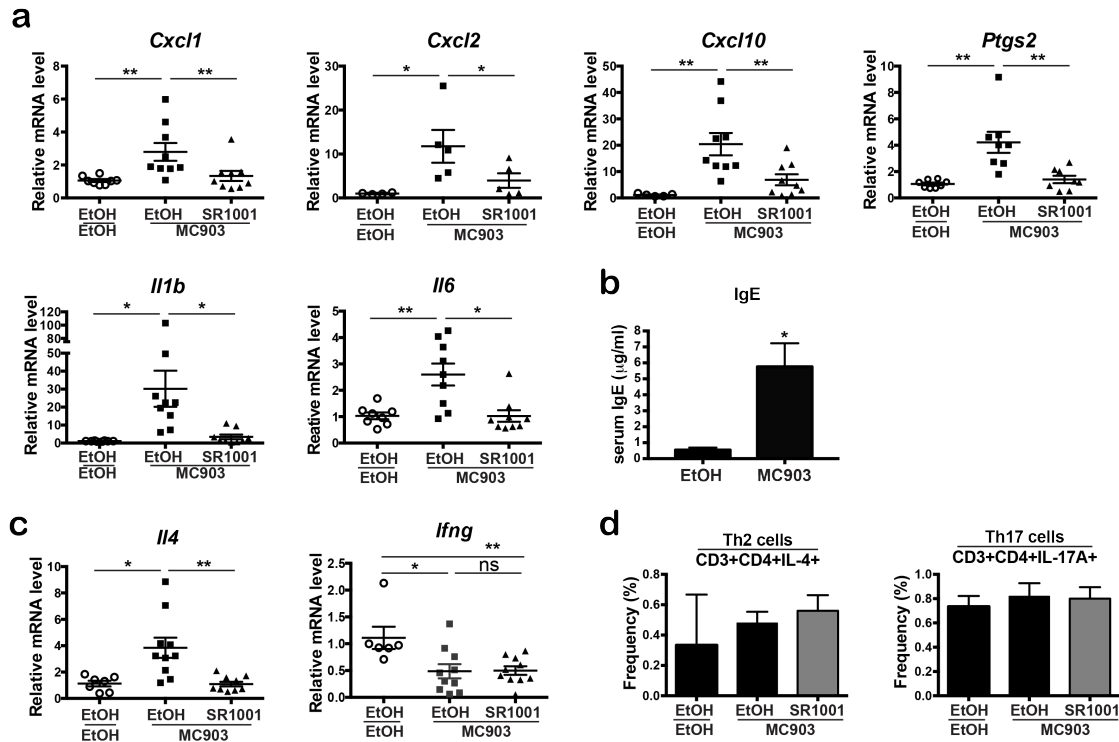


Figure S4. SR1001 modulates the expression of inflammatory cytokines induced by MC903.

(a) Quantitative RT-PCR analysis of mRNA expression of indicated inflammatory factors in ear tissues. Mouse ears were treated with MC903, followed by treatment with EtOH on the left ear and 50 mM of SR1001 on the right ear, as described in Figure 1. RNA samples were isolated from the ear tissues collected on day 11. The mRNA level of each gene was normalized to 36B4. Values from MC903-treated ears are normalized to ethanol-treated control ears (set as 1), and presented as mean $\pm$ S.E.M. (N=8-10/group) from two independent experiments, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. **(b)** ELISA of serum IgE levels from mice with ears painted with EtOH or MC903 daily for 5 days. Blood was collected on day 12. **(c)** Quantitative RT-PCR analysis of mRNA expression of indicated inflammatory factors in ear tissues, as described in (a). **(d)** Ear-draining lymph nodes were collected at day 8 to make single cell suspensions, as described in Figure 2e. Cells were re-stimulated with PMA/ionomycin for 4 h and stained with fluorescence-conjugated antibodies against cell surface markers (CD3 and CD4) and intracellular cytokines (IL-4 and IL-17A). Percentages of total cells (Frequencies) of Th2 and Th17 cells were analyzed by flow cytometry. Values are presented as mean $\pm$ S.E.M. (N=3-5/group).

Supplementary Figure S5

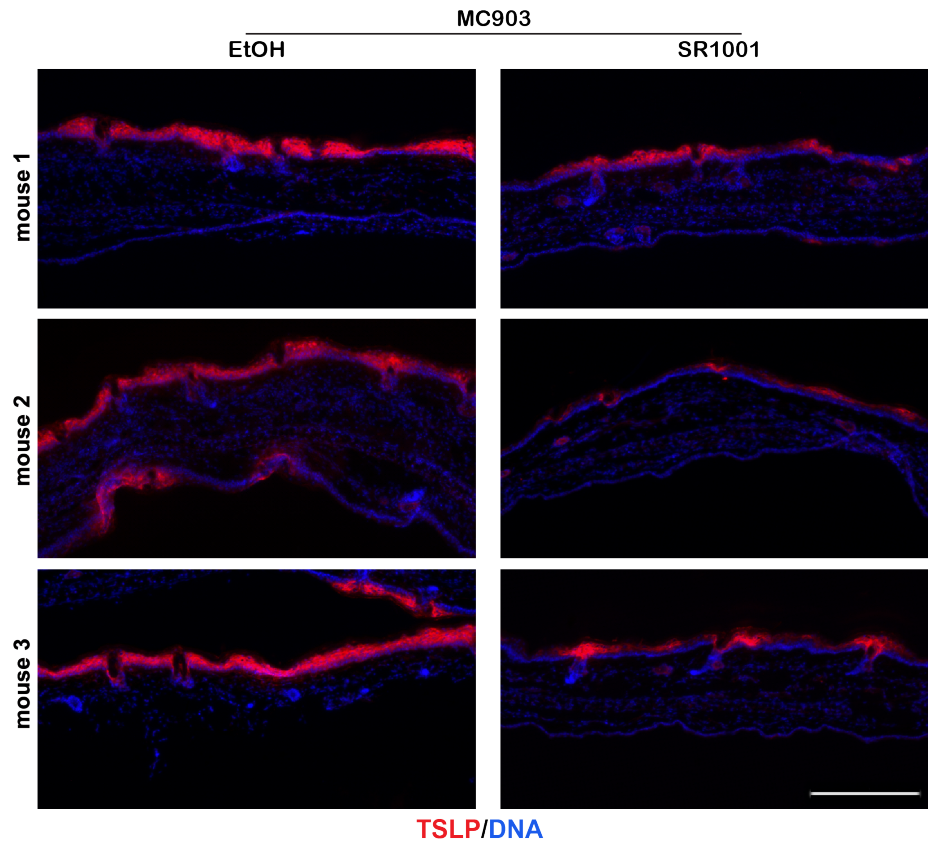


Figure S5. SR1001 reduces MC903-induced TSLP expression in the epidermis. Mouse ears were treated with MC903, followed by treatment with ethanol on the left ear and 50 mM of SR1001 on the right ear. Ear samples were collected on day 7 after MC903 application for preparation of frozen sections. Data are representative immunostaining images of frozen sections of ear samples from 3 mice, stained with the antibody against TSLP. DNA was counterstained with Hoechst. Images were viewed with 4x magnification. Scale bar = 200 μ m.

Supplementary Figure S6

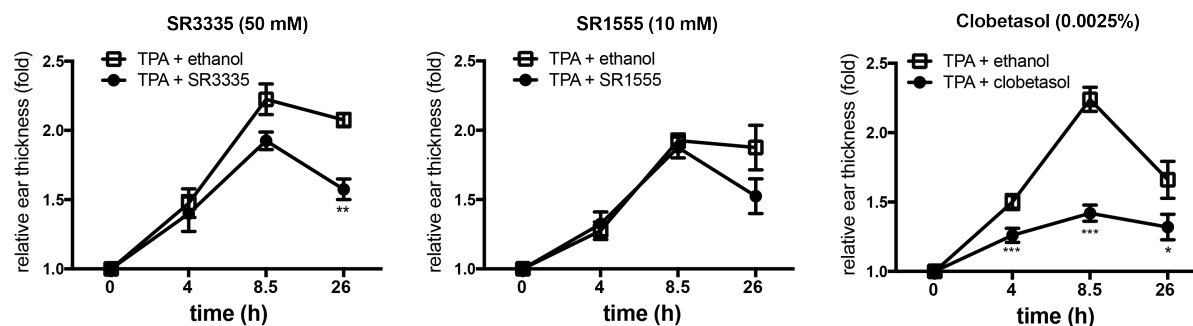


Figure S6. Inverse agonists of ROR α and ROR γ have differential efficacy in suppressing TPA-induced ear thickening. Both ears of each mouse were topically challenged with TPA. Left ears were topically treated with 25 μ l of vehicles at 1 h and 4 h after TPA application, and right ears were treated with 25 μ l of 50 mM SR1001 (0.60 mg), 10 mM SR1555 (0.11 mg), or 0.0025% calcipotriol propionate. Ear thickness was measured at indicated time points after TPA application. Values are normalized to the original size measured before TPA application (set as 1), and presented as mean fold $\pm$ S.E.M., *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, $N = 4/\text{group}$.

Supplementary Materials and Methods

Reagents

Clobetasol propionate was purchased from Sigma (Saint Louis, MO). SR3335 and SR1555 were purchased from Cayman Chemical (Ann Arbor, MI).

TPA-induced chronic skin inflammation model

In this chronic skin inflammation model (Stanley et al., 1991), mice were divided into two groups. Ears of both groups were topically painted with 2 μ g (1 μ g on each surface) of TPA on day 0, day 2, day 4, and day 7. SR1001 (50 mM) was applied on the right ears daily in the treatment group, whereas ethanol was used in the control group. Ear thickness was measured at the time points indicated in supplementary Fig. S1c.

Primary antibodies used for immunostaining

CD4 (ab25475, Abcam, Cambridge, MA), CD11c (ab33483, Abcam), TSLP (AF555, R&D Systems, Minneapolis, MN), keratin 6 (PRB-169P, BioLegend, Dedham, MA), Ly-6G and Ly-6C (Gr-1) [553125, BD Pharmingen], F4/80-biotin (MCA497, AbD Serotec, Bio-Rad, Hercules, CA), COX2 (aa 584-598, Cayman Chemical, Ann Arbor, MI), loricrin (PRB-145P, BioLegend), normal goat IgG (sc-2028, Santa Cruz Biotechnologies Inc., Santa Cruz, CA).

ELISA of serum IgE levels

Two groups of mice were topically painted with EtOH or MC903 (2.5 nmols per ear) on both ears once per day for 5 days. Peripheral blood was collected on day 12. Serum IgE levels were measured by ELISA, using a commercial kit from eBioscience (Thermo Fisher Scientific, Madison, WI).

Flow cytometry with cells isolated from ear draining lymph nodes

Ear draining lymph nodes were dissected at day 8 after MC903 application, according to the protocol described in (Mac-Daniel et al., 2016). Single cell suspensions prepared from ear-draining lymph nodes were re-stimulated with 50 ng/ml TPA + 1 μ M ionomycin for 1 h. After addition of 1x protein transport inhibitor (eBioscience), cells were further stimulated with TPA/ionomycin for additional 3 h. Cells were stained with Percp-Cy5.5–CD3 (45-0031) and APC-CD4 (17-0042) antibodies following the protocol for staining of cell surface targets (eBioscience). After fixation and permeabilization, cells were stained with FITC-IL-4 (11-7042) and PE-IL-17A (12-7177) antibodies, following the staining protocol for intracellular antigens (eBioscience). All fluorescence-conjugated antibodies were purchased from eBioscience, and diluted according to manufacturers' instructions. Samples were analyzed on a FACSCanto flow cytometer (BD Biosciences) using FlowJo software.

Calculation of Log-P values

The “*Partition Coefficient*”, or Log-P, represents a physical property that measures the lipophilicity and membrane permeability of a given small molecule. The more positive of a given Log-P value is, the more lipophilic of the respective small molecule should be and the better it can diffuse through membranes. Overall, Log-P is a key component of Lipinski's Rule of Five for drug design and development, and a critical molecular property associated with elements of ADME (Absorption, Distribution, Metabolism, and Excretion) in pharmacokinetics and pharmacodynamics (Kubinyi, 1995, Lipinski et al., 2001)

While Log-P values can be obtained experimentally, they are often estimated through methods of calculations. There are various accepted methods (Chou and Jurs, 1979, Leo et al., 1975, Pleiss and Grunewald, 1983), and the Rekker's hydrophobic parameter (Grunbauer et al.,

1979) (f values) is among the most accurate and frequently employed ones (the others being Leo-Hansch constants (Leo et al., 1975) and π -values (Kubinyi, 1995). This simple and additive method strives to predict the lipophilicity of a small molecule through tallying lipophilicity of each functional group and fragment contained in the molecule. Lipophilicity numbers of organic functional groups and fragments are assigned theoretically via QSAR (quantitative structure-activity relationships employing statistical methods) and based on fundamental understanding of chemical bonding and polarity (See tables below for some values) [Lemke et al. 2012]. The more positive of an assigned value is, the more lipophilic of the respective functional group or fragment is assumed to be.

Fragment	Rekker constant f	Leo constant f	π_{benzene}
H	0.175	0.23	0.00
CH ₃	0.702	0.89	0.56
>CH ₂	0.530	0.66	
>CH-	0.235	0.43	
C ₆ H ₅	1.886	1.90	1.96
C ₆ H ₄	1.688		
C ₆ H ₃	1.431		

Fragment	Rekker constant f		Leo constant f		π_{benzene}
	Aliph.	Arom.	Aliph.	Arom.	
F	-0.462	0.399	-0.38	0.37	0.14
Cl	0.061	0.922	0.06	0.94	0.71
Br	0.270	1.131	0.20	1.09	0.86
I	0.587	1.448	0.60	1.35	1.12
OH	-1.491	-0.343	-1.64	-0.40	-0.67
-O-	-1.581	-0.433	-1.81	-0.57	
COOH	-0.954	-0.093	-1.09	-0.03	-0.32
NH ₂	-1.428	-0.854	-1.54	-1.00	-1.23
NH	-1.825	-0.964	-2.11	-1.03	
NO ₂	-0.939	-0.078	-1.26	-0.02	-0.28
CONH ₂	-1.970	-1.109	-2.18	-1.26	-1.49
>C=O	-1.703	-0.842	-1.90	-0.32	
CF ₃	0.757	1.331			0.88
C≡N	-1.066	-0.205	-1.28	-0.34	-0.57
SH	0.000	0.620			0.39
-S-	-0.510	0.110	-0.79	0.03	

Online Tables Abstracted From: Dr. Hugo Kubinyi at www.kubinyi.de

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Supplementary Table S1: Primer sequences of mouse genes for RT-PCR

GENES	FORWARD	REVERSE
<i>Il4</i>	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
<i>Ifng</i>	ATCTGGAGGAACTGGCAAAA	TCTGGCTCTGCAGGATTTTC
<i>Il1b</i>	GGCAGGCAGTATCACTCATT	GAGGATGGGCTCTTCTTCAAA
<i>Ptgs2</i>	CCCCCACAGTCAAAGACACT	GGTTCTCAGGGATGTGAGGA
<i>Il6</i>	CCAGAAACCGCTATGAAGTTCC	TTGTCACCAGCATCAGTCCC
<i>Cxcl1</i>	GCCAATGAGCTGCGCTGT	CCTTCAAGCTCTGGATGTTCTTG
<i>Cxcl2</i>	ATCCAGAGCTTGAGTGTGACGC	AAGGCAAACCTTTTGGACCGCC
<i>Cxcl10</i>	GAATCCGGAATCTAAGACCATCAA	GTGCGTGGCTTCACTCCAGT
<i>Krt10</i>	GACAACCTGACAATGCCAACG	GGTCAGCTCATCCAGTACCC
<i>Ivl</i>	AAAGCTTCAAGGGAACAGCA	CGGTTCTCCAATTCGTGTTT
<i>Lor</i>	GAGACAACAGAGCTGGAAGA	TCTGCAGAACAGGATACACC
<i>Il1a</i>	TCCAGGGCAGAGAGGGAGT	GGAACCTTTGGCCATCTTGATTT
<i>Tnf</i>	GCCTCCCTCTCATCAGTTCTAT	CACTGGTGGTTTGCTACGA
<i>Tslp</i>	GCTACCCTGAAACTGAGAGAAA	TCTGGAGATTGCATGAAGGAATA