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 RORα and RORγ 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 RORα/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 RORα/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.

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; RORα, RORβ, and RORγ) are members of the nuclear receptor superfamily (Cook et al., 2015). RORα and RORγ 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). RORα 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, RORα 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 RORα activity.

A number of endogenous and synthetic small-molecule ligands of RORα and RORγ have recently been identified (Solt and Burris, 2012). The inverse agonist SR1001 selectively inhibits the transcriptional activity of RORα and RORγ, but not 48 other nuclear receptors (Solt et al., 2011). SR1001 binding to RORα/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 1 diabetes (Solt et al., 2011, 2015).

Given the regulatory functions of RORα/γ in multiple cell types and different forms of inflammation, we examined the
efficacy of inhibitory ligands of RORα/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 RORα/γ inverse agonists as nonsteroid topical agents for the prevention and treatment of inflammatory skin disorders.

RESULTS AND DISCUSSION
RORα/γ inverse agonists inhibit MC903-induced ear thickening

It has been shown in mice that RORα 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 RORα/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 RORα selective synthetic ligand SR3335 (Kumar et al., 2011) have similar affinities for RORα (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-
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 Rorc<sup>−/−</sup> mice have shown a role for ILC2-specific RORα 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 RORα activities in ILC2 cells.

Although RORγ<sup>+</sup> group 3 ILCs (ILC3s) were also detected in the skin-draining lymph nodes upon MC903 application, knockout of Rorc (the RORγ gene) did not prevent AD-like skin inflammation (Kim et al., 2013). However, we observed that the RORγ-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 RORγ 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...
Effects of ROR Inverse Agonists in Mouse Dermatitis Models

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 ± 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 μm. EtOH, ethanol; IvI, involucrin; Krt, keratin; Lor, loricrin.

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 RORα/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 RORα ligands.

(Feature 2a), dendritic cells (Figure 2b), macrophages (Figure 2c), and CD4+ T cells (Figure 2d). The hyperproliferative 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 (Pigs2) (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 responses were markedly reduced with cells derived from mice produced high levels IL-13 and IL-5 in culture; these

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homeostasis in MC903-challenged skin. Further elucidation of RORα/γ functions in keratinocytes will help identify the cellular targets of RORα/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 μmol/L. In contrast to its robust in vivo effect (Figure 4a–c), SR1001 at 10 μmol/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
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 (RXRα and RXRβ), RARα, and PPAR in modulating keratinocyte expression of TSLP (Lee et al., 2008; Li et al., 2006). It remains to be clarified whether RORα and/or RORγ 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 RORα-specific
SR3335 significantly reduced TPA-induced ear swelling at 26 hours after TPA treatment, the RORγ-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). 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 (Cxe1l and Cxe12) 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 RORα/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 RORα/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/6 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 μl ethanol. Five hours later, right ears were topically treated with 25 μl 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 μg of TPA. At 1 and 4 hours after TPA application, right ears were treated with 25 μl 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 μm) were meabilized with 0.1% Nonidet P-40/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 μg 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 36B4 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 × 10^6 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 μmol/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 075 medium from CELInTEC 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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