Liver X Receptor Activators Display Anti-Inflammatory Activity in Irritant and Allergic Contact Dermatitis Models: Liver-X-Receptor-Specific Inhibition of Inflammation and Primary Cytokine Production

Ashley J. Fowler,*§[1] Mary Y. Sheu,*§[1] Matthias Schmuth,†‡ Jack Kao,*§ Joachim W. Fluhr,*§ Linda Rhein,** Jon L. Collins,¶ Timothy M. Willson,¶ David J. Mangelsdorf,†† Peter M. Elias,*§ and Kenneth R. Feingold*†‡

Departments of *Dermatology and †Medicine, University of California, San Francisco, and ‡Medical and §Dermatology Services, Department of Veteran Affairs Medical Center, San Francisco, California, U.S.A.; ¶Discovery Research, GlaxoSmithKline, Research Triangle Park, North Carolina, U.S.A.; **Nuclear Receptor Discovery Research, GlaxoSmithKline, Harlow, Essex, U.K.; ††Howard Hughes Medical Institute and Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas, U.S.A.

Activators of liver X receptors (LXR) stimulate epidermal differentiation and development, but inhibit keratinocyte proliferation. In this study, the anti-inflammatory effects of two oxysterols, 22(R)-hydroxycholesterol (22ROH) and 25-hydroxycholesterol (25OH), and a nonsterol activator of LXR, GW3965, were examined utilizing models of irritant and allergic contact dermatitis. Irritant dermatitis was induced by applying phorbol 12-myristate-13-acetate(TPA) to the surface of the ears of CD1 mice, followed by treatment with 22ROH, 25OH, GW3965, or vehicle alone. Whereas TPA treatment alone induced an ≈2-fold increase in ear weight and thickness, 22ROH, 25OH, or GW3965 markedly suppressed the increase (greater than 50% decrease), and to an extent comparable to that observed with 0.05% clobetasol treatment. Histology also revealed a marked decrease in TPA-induced cutaneous inflammation in oxysterol-treated animals. As topical treatment with cholesterol did not reduce the TPA-induced inflammation, and the nonsterol LXR activator (GW3965) inhibited inflammation, the anti-inflammatory effects of oxysterols cannot be ascribed to a nonspecific sterol effect. In addition, 22ROH did not reduce inflammation in LXRβ −/− or LXRαβ −/− animals, indicating that LXRβ is required for this anti-inflammatory effect. 22ROH also caused a partial reduction in ear thickness in LXRα −/− animals, however (≈50% of that observed in wild-type mice), suggesting that this receptor also mediates the anti-inflammatory effects of oxysterols. Both ear thickness and weight increased (≈1.5-fold) in the oxazolone-induced allergic dermatitis model, and 22ROH and GW3965 reduced inflammation by ≈50% and ≈30%, respectively. Finally, immunohistochemistry demonstrated an inhibition in the production of the pro-inflammatory cytokines interleukin-1α and tumor necrosis factor α in the oxysterol-treated sites from both TPA- and oxazolone-treated animals. These studies demonstrate that activators of LXR display potent anti-inflammatory activity in both irritant and allergic contact models of dermatitis, requiring the participation of both LXRα and LXRβ. LXR activators could provide a new class of therapeutic agents for the treatment of cutaneous inflammatory disorders. ***Key words: inflammation/keratinocyte/nuclear hormone receptors/oxysterols. J Invest Dermatol 120:246–255, 2003***

Nuclear hormone receptors, the largest known family of transcription factors, have been divided into four major subgroups based upon their dimerization and DNA binding properties (Mangelsdorf and Evans, 1995). The class II subfamily includes the retinoic-acid-activated receptor (RAR), thyroid hormone receptor, vitamin D receptor, peroxisome-proliferator-activated receptors (PPARs), and liver X receptor (LXR). These receptors recognize small hydrophobic compounds, such as 1,25$(OH)_2$-vitamin D, free fatty acids, retinoids, thyroid hormone, and certain oxysterols. Activation of class II receptors requires heterodimerization with RXR, which allows for optimal regulation of gene expression. In the epidermis, ligand activation of several class II nuclear hormone receptors, including RAR, PPARα, vitamin D receptor, and LXR, regulates keratinocyte proliferation and differentiation *in vitro* and *in vivo* (Bikle, 1996; Eichner *et al*, 1996; Fisher and Voorhees, 1996; Kang *et al*, 1996; Hanley *et al*, 1997, 1998; Komuves *et al*, 2000). In addition, ligands of RAR, vitamin D receptor, and PPAR display selected types of anti-inflammatory activity (Devchand *et al*, 1996; Muller and Bendtzen, 1996; Duvic *et al*, 1997; Deluca and Cantorna, 2001).

Manuscript received March 21 2002; revised September 20 2002; accepted for publication October 8 2002

[1]Both of these authors contributed equally to this paper.

Reprint requests to: Kenneth R. Feingold, M.D., Metabolism Section (111F), Department of Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94121; Email: kfngld@itsa.ucsf.edu

Abbreviations: LXR, liver X receptor; 25OH, 25-hydroxycholesterol; PPAR, peroxisome-proliferator-activated receptor; RAR, retinoic-acid-activated receptor; 22ROH, 22(R)-hydroxycholesterol; TEWL, transepidermal water loss.

Recent studies by our laboratory have shown that activators of PPARα inhibit both irritant and allergic contact dermatitis in murine skin (Sheu *et al*, 2002).

LXRs were classified initially as orphan members of the nuclear receptor superfamily, due to the unknown nature of their ligands. LXRα and LXRβ are now recognized to bind certain endogenous oxysterols, including 22(R)-hydroxycholesterol (22ROH), 24(S)-hydroxycholesterol, and 24(S),25-epoxycholesterol (Janowski *et al*, 1996; Lehmann *et al*, 1997; Peet *et al*, 1998). Activation of LXR in extracutaneous tissues regulates important steps in cholesterol, fatty acid, and bile acid metabolism (Peet *et al*, 1998; Chawla *et al*, 2001). Two genes, α and β, encode the LXR paralogs. Whereas LXRα is expressed predominately in the liver and to a lesser extent in the kidney, spleen, adrenal gland, and the small intestine (Willy *et al*, 1995), LXRβ is ubiquitously expressed (Song *et al*, 1995). Our laboratory has shown that both LXRα and LXRβ are present in cultured human keratinocytes and in fetal rat epidermis (Hanley *et al*, 1999; 2000). LXRβ is the predominate isoform in adult mouse epidermis, however (Komuves *et al*, 2002). Recently, we demonstrated that topical application of oxysterols to murine epidermis stimulates keratinocyte differentiation and inhibits proliferation (Komuves *et al*, 2002). Moreover, these effects still occurred in LXRα −/− mice, but not in LXRβ −/− animals, indicating that the stimulation of keratinocyte differentiation and the inhibition of proliferation induced by oxysterols is mediated predominately by LXRβ (Komuves *et al*, 2002). In addition, oxysterols accelerate the formation of the epidermal permeability barrier during fetal development (Hanley *et al*, 1999; 2000), and topical oxysterols improve barrier homeostasis following barrier disruption in normal mice (Komuves *et al*, 2002). Furthermore, in an animal model of epidermal hyperplasia (Komuves *et al*, 2002), oxysterol treatment largely normalized structure, barrier function, and differentiation, suggesting that LXR activators could provide a new category of therapeutic agents for cutaneous diseases that are associated with hyperproliferation and/or disordered differentiation.

Activation of several members of the class II family of nuclear hormone receptors can regulate inflammation. Oxysterols, which activate LXR, inhibit the secretion of proinflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin 1 (IL-1), by macrophages and inhibit lymphocyte activation (Ohlsson *et al*, 1996). Although these observations suggest that oxysterols could be anti-inflammatory, other studies have shown that oxysterols stimulate the secretion of IL-8 by macrophages, a proinflammatory event (Liu *et al*, 1997). Moreover, whether the effects of oxysterols on macrophages and lymphocytes are mediated by LXR or whether they occur via other pathways is unknown. The primary purpose of this study was to determine whether topical applications of oxysterols and/or GW3965, a nonsterol activator of LXR, attenuate inflammation in two distinct models of cutaneous inflammation, irritant and allergic contact dermatitis. Moreover, we addressed whether LXRα and/or LXRβ mediate this anti-inflammatory effect. The results of this study indicate that agonists of LXR, when applied topically to the skin, display receptor-mediated, via both LXRα and LXRβ, anti-inflammatory behavior.

MATERIALS AND METHODS

Animal models and tissue preparation Adult CD1 male and female mice, 6–10 wk of age, were purchased from Charles River Laboratories (Wilmington, MA) for use in this study. Phorbol 12-myristate-13-acetate (TPA) induced irritant contact dermatitis was instituted by the topical application of 10 μl 0.03% (wt/vol in acetone) TPA to both the inner and outer surface (20 μl total) of the left ears. Acetone alone (vehicle) was applied to the right ears. Forty-five minutes and 4 h after TPA application, 20 μl of test compounds, 22ROH (10 mM), 25-hydroxycholesterol (25OH; 10 mM), and GW3965 (10 mM), known LXR agonists, were applied to both surfaces of both the left and right ears (40 μl total per ear). Identical treatments were performed with 20 μl of 0.05% clobetasol (1.1 mM), a topical anti-inflammatory glucocorticoid, which served as a positive control. Cholesterol (1%, 2.5 mM), which neither binds nor activates LXR, was applied in a similar fashion as a negative control. Control animals were treated similarly with acetone alone, serving as a vehicle control. All chemical compounds were purchased from Sigma (St. Louis, MO) and were dissolved in absolute acetone (reagent grade) vehicle. GW3965 was synthesized by GlaxoSmithKline High Throughput Chemistry, as described previously (Collins *et al*, submitted).

Allergic contact dermatitis was induced by sensitization (for 2 d) on the shaved backs of CD1 female mice with 20 μl of 15% (wt/vol in acetone) oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) once a day, followed by challenge on day 7 with a single topical application of 10 μl oxazolone (2%) to the inner and outer surface of the left ears. Acetone alone (10 μl) was applied to the right ears. This challenge was followed by treatment with 22ROH (10 mM), GW3965 (10 mM), clobetasol (0.05%), or acetone at 45 min and 4 h, as described previously.

Eighteen hours after the inflammatory insult induced by either TPA or oxazolone, inflammation was assessed as the percentage increase in ear thickness and/or ear weight in the treated left ear *versus* the vehicle-treated right ear. Ear thickness was measured with a digital caliper (Mitutoyo, Tokyo, Japan), followed by a 6 mm punch biopsy to ascertain changes in ear weights. The extent of inflammation was quantitated according to the following equation: ear swelling (%)$=100\times(a-b)/b$, where a is the thickness/weight of the left (treated) ear and b is the thickness/weight of the right (untreated control) ear.

After samples were obtained for assessment of ear thickness/weight, biopsies were obtained from adjacent sites for routine histopathology (fixation in 4% freshly prepared paraformaldehyde in phosphate-buffered saline), or for immunohistochemical analysis (directly frozen in liquid nitrogen).

LXR-deficient animals Age- and sex-matched controls from the same genetic background and LXRα −/−, LXRβ −/−, and LXRα/β −/− mice, produced as described previously (Peet *et al,* 1998), were used in this study. The irritant contact dermatitis model (TPA) was performed in an identical fashion in these mice, as described above for CD1 mice. Each group of animals was divided randomly, half receiving 22ROH and the other half receiving vehicle alone, at 45 min and 4 h post TPA-induced inflammation. Ear thickness was measured at 18 h as described above, but weight determinations were not performed due to the small numbers of knockout animals available. Biopsies then were taken for hematoxylin and eosin staining and immunohistochemistry.

Epidermal function measurements Basal cutaneous permeability barrier function was determined by measuring transepidermal water loss (TEWL) with an electronic water analyzer (MEECO, Warrington, PA). The kinetics of barrier recovery were then determined after acute disruption by sequential applications of cellophane tape (Scotch tape, 3M) (TEWL $\geqslant$ 6–8 mg per cm^2 per h), at 3 and 6 h postdisruption, as described previously (Komuves *et al*, 2000). Stratum corneum integrity was defined as the number of tape strips required to produce a predetermined elevation in TEWL (Fluhr *et al*, 2001). Stratum corneum cohesion was defined as the amount of protein removed per stripping, measured with a BioRAD Assay Kit (Hercules, CA), using bovine plasma gamma globulin as the standard, as described previously (Fluhr *et al*, 2001). Changes in surface pH were measured with a flat glass electrode (Mettler-Toledo, Giessen, Germany), using a pH meter (Skin pH Meter PH 900, Courage and Khazaka, Cologne, Germany).

Immunohistochemistry Both paraffin sections (6 μm) and cryosections (5 μm) were used to detect TNF-α and IL-1α in this study. Cryosections alone were assessed in the LXR-deficient animals. Paraffin-embedded, paraformaldehyde-fixed sections were processed as described previously (Sheu *et al*, 2002), using polyclonal rabbit antimouse primary antibodies specific for IL-1α (R&D Systems, Minneapolis, MN) and polyclonal goat antimouse specific for TNF-α (Santa Cruz Biotechnology, Santa Cruz, CA). Peroxidase activity was revealed with 3,3′-diaminobenzidine substrate (Vector Laboratories, Burlingame, CA), and methyl green was used as a counterstain. The same antibodies were used for immunohistochemical localization of IL-1α and TNF-α in cryosections. After washing in Tris−HCl buffer, the sections were incubated with blocking buffer for 30 min at room temperature, followed by incubation with primary antibody (1:500) in blocking buffer for 2 h. Before incubation with the secondary antibody (1:200) for 45 min, the sections were washed in Tris−HCl buffer and incubated for 10 min with levamisole (0.05%) to inhibit endogenous alkaline phosphatase activity. Localization was achieved by the avidin−biotin peroxidase technique, using alkaline phosphatase as the chromagen (reaction time, 13 min and

45 min for IL-1α and TNF-α, respectively). The sections were rinsed with ddH_2O, dehydrated, and mounted (Cytoseal 60, Stephens Scientific, Kalamazoo, MI). Nonspecific binding of secondary IgG was not seen when the primary antibody was omitted.

Microscopy and imaging The sections were examined with a Zeiss (Axioplan 2) microscope (Jena, Germany) using bright-field optics. Digital images were captured with AxioVision software 2.05 (Carl Zeiss Vision, Munich, Germany). Photos were prepared using Adobe Illustrator (Adobe Systems, Mountain View, CA).

Statistical analyses All statistical analyses were performed using Prism 3 software (Graph Pad Software, San Diego, CA). Results were compared between multiple groups, using ANOVA, and expressed as mean ± SEM. When results between pairs were analyzed, the Student's *t* test was used.

RESULTS

Activators of LXR inhibit TPA-induced contact dermatitis We initially examined the anti-inflammatory effect of LXR activators in a TPA-induced, irritant contact dermatitis murine model. Both ear thickness (**Fig 1*A***) and ear weight (**Fig 1*B***) markedly increased (1.85-fold and 1.91-fold, respectively) after treatment with TPA. Application of vehicle (acetone) alone did not significantly affect TPA-induced inflammation (1.60-fold and 1.78-fold, for ear thickness and weight, respectively). In contrast, treatment with either 22ROH or 25OH markedly attenuated the TPA-induced increase in ear thickness and ear weight. Because the LXR activators were applied topically, high concentrations (10 mM) were used in order to ensure activation of the target cells. Treatment with 22ROH decreased both ear thickness and ear weight by 51% and 56%, respectively, *versus* the vehicle control, whereas 25OH decreased thickness and weight by 57% and 69%, respectively (**Fig 1*A, B***). These anti-inflammatory effects were comparable to those achieved by topical clobetasol, a potent glucocorticoid (ear thickness and weight were decreased by 68% and 61%, respectively). Treatment with cholesterol, however, which does not activate LXR, did not alter the extent of TPA-induced inflammation insult (**Fig 1*A, B***). Finally, because oxysterols have biologic effects other than activating LXR, we next employed a nonsterol activator of LXR, GW3965. As shown in **Fig 1**, GW3965 reduced ear thickness and ear weight by 40% and 38%, respectively, following TPA treatment. These results strongly suggest that LXR activation and not other, nonspecific effects of oxysterols account for the inhibition of TPA-induced inflammation.

Hematoxylin and eosin stained sections from the same TPA-treated mice are shown in **Fig 2**. TPA application alone (**Fig 2*B***) produced a marked increase in ear thickness and an abundance of inflammatory cells infiltrating the epidermis and dermis (**Fig 2*A*** *vs* **2*B***). In contrast, 22ROH treatment markedly reduced both ear thickness and the extent of the inflammatory infiltrate in the epidermis and dermis (**Fig 2*B*** *vs* **2*C***). Again, the reduction in inflammation was similar to that seen with clobetasol treatment (**Fig 2*C*** *vs* **2*D***). These results further demonstrate the anti-inflammatory properties of oxysterols in the TPA-irritant contact dermatitis model.

Stratum corneum function remains normal in LXRα, LXRβ, and LXRα/β knockout mice Previous studies with LXRβ −/− and LXRα/β −/− mice demonstrated a slight decrease in the expression of epidermal differentiation markers, with thinning of the normal epidermis, whereas such changes did not occur in LXRα −/− mice (Komuves *et al*, 2002). We therefore next determined whether the modest morphologic alterations that occur in animals deficient in LXRβ result in functional abnormalities. In all three strains of LXR-deficient mice (LXRα, LXRβ, and LXRα/β −/−), basal TEWL and the kinetics of barrier recovery following acute barrier disruption were similar to rates observed in wild-type littermates (data not shown). In addition, stratum corneum cohesion and integrity

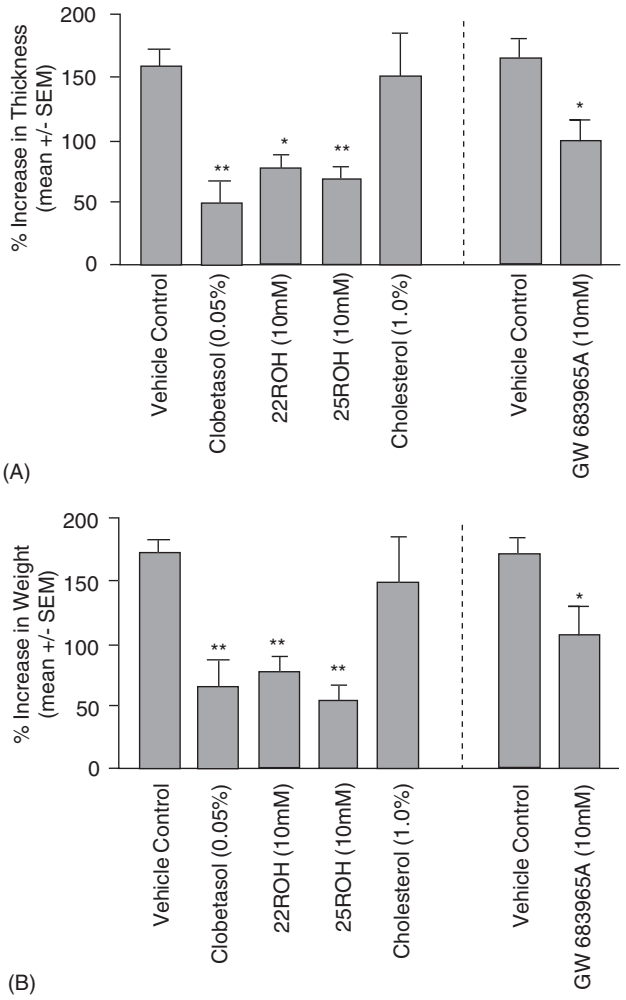

Figure 1. Activators of LXR reduce TPA-induced inflammation. Mice were treated with 10 μl of TPA (0.03%) to the inner and outer surfaces of the left ears. The mice were treated topically on both ears 45 min and 4 h after TPA application with either clobetasol (0.05%), the LXR ligands 22ROH (10 mM) and 25OH (10 mM), cholesterol (1.0%), or GW3965 (10 mM) (a nonsterol activator of LXR). A separate vehicle control is shown for the GW3965 experiment because the experiment was carried out at a different time. At 18 h post TPA application, ear thickness and ear weight (6 mm punch) were measured. Treatment with LXR activators or clobetasol results in a marked reduction in ear thickness (*A*) and ear weight (*B*) compared to treatment with vehicle alone. Cholesterol, which does not activate LXR, has no significant effect. Results are shown as the mean percentage increase in ear thickness and weight (mean ± SEM; $n = 4–8$; $^{*}p < 0.01$, $^{**}p < 0.001$). Statistical significance was determined using ANOVA for multiple groups and a Student's *t* test for two groups.

were similar in LXR-deficient mice and control mice (data not shown). Lastly, surface pH was not altered by the absence of either LXRα or LXRβ (data not shown). Thus, LXR deficiency does not produce significant functional cutaneous abnormalities.

The anti-inflammatory effects of oxysterols are mediated by LXR In order to determine definitively whether the anti-inflammatory effects of oxysterols are mediated by LXR, we next compared the anti-inflammatory effects of oxysterols on TPA-induced inflammation in LXRα −/−, LXRβ −/−, and LXRα/β −/− *versus* wild-type mice. Treatment with TPA increased ear thickness to a similar extent in LXRα- and LXRβ-deficient mice compared to wild-type mice. Yet, TPA-induced ear thickness was blunted in the LXRα/β −/− double knockout mice

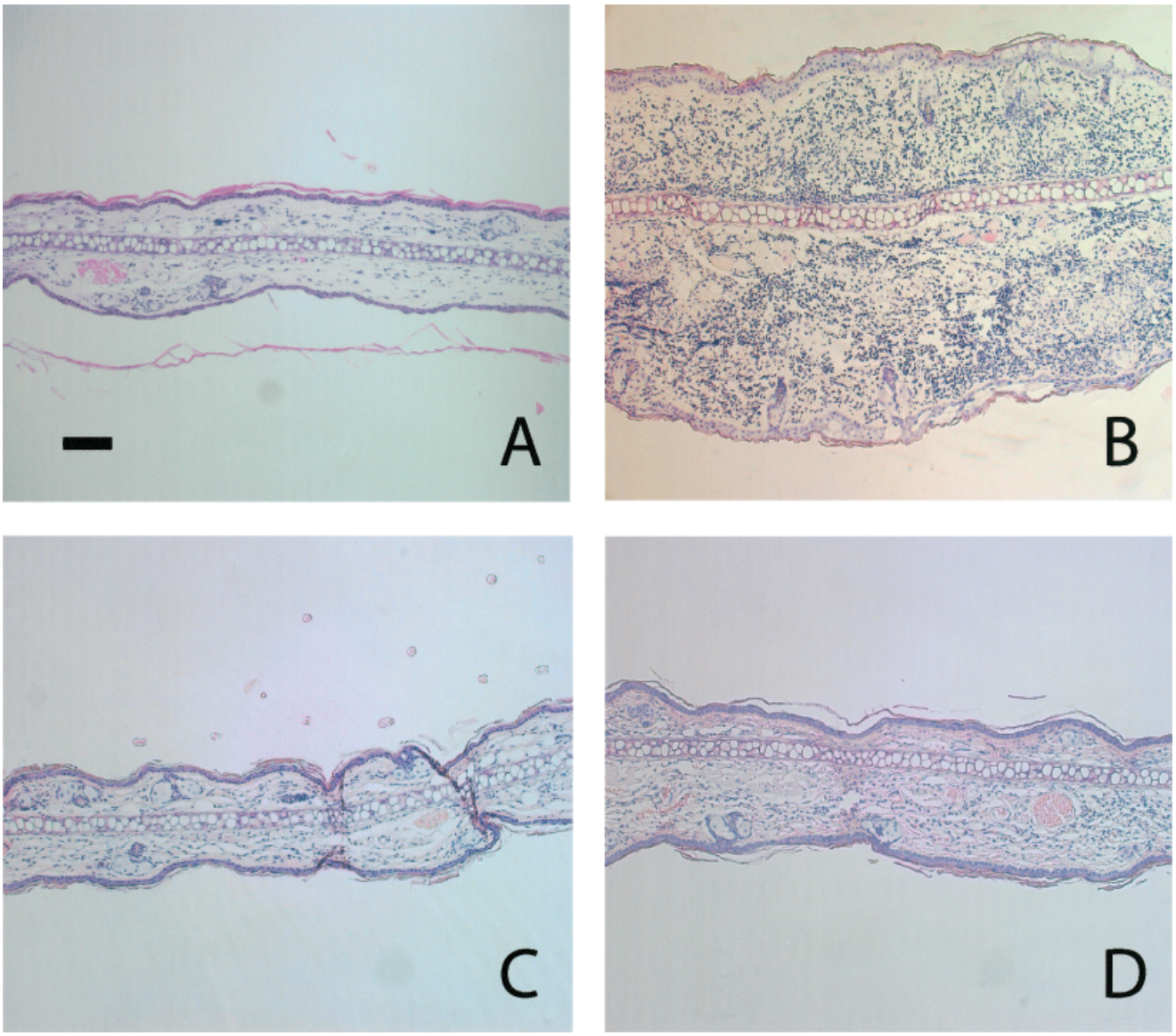

Figure 2. Inflammation induced by TPA is reduced by topical application of LXR activators. Ten microliters of TPA (0.03%) was applied to the left ears (both surfaces) of mice. Forty-five minutes and 4 h after TPA application, the animals were treated topically with activators of LXR, clobetasol, or vehicle (acetone). The animals were sacrificed at 18 h after TPA application and biopsies of the ears were obtained. The specimens were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin. A marked increase in ear swelling and inflammatory cells was found in the vehicle-treated ear (*B*). Treatment with 22ROH (*C*) reduces ear thickness and the degree of inflammatory infiltrate, similar to clobetasol treatment (*D*). A normal untreated mouse ear is provided for comparison (*A*). Sections above are representative findings from three animals per group. *Scale bar*: 50 μm.

(not shown). Treatment with 22ROH following TPA resulted in a similar reduction in ear thickness in the LXR wild-type control mice as seen in the CD1 mice (**Fig 1** *vs* **Fig 3**). In contrast, 22ROH treatment did not significantly reduce ear thickness in the LXRβ −/− or LXRα/β −/− animals (**Fig 3**). Clobetasol treatment resulted in a reduction in ear thickness in LXRβ −/− mice, however, to a degree virtually identical to that seen in wild-type mice (LXRβ −/− 29% decrease *versus* wild-type 34%, not significant, $n = 3$–4). These results indicate that the failure of 22ROH to inhibit TPA-induced inflammation in LXRβ −/− animals is not due to a general impairment of these animals to elicit an anti-inflammatory reaction. Finally, whereas treatment with 22ROH partially reduced the ear thickness in the LXRα −/− animals, the extent of anti-inflammatory effects was less than those observed in the wild-type animals (32% *vs* 58%, respectively). These findings indicate that the anti-inflammatory effects produced by oxysterol treatment are inhibited by LXR, and primarily require LXRβ, but that LXRα is also of importance.

Oxysterols reduce oxazolone-induced allergic contact dermatitis We next examined the effects of the LXR agonist 22ROH in a model of allergic contact dermatitis. **Fig 4(*A*), (*B*)** demonstrates the marked increase in both ear thickness and weight induced by topical oxazolone applications to sensitized animals (≈1.5-fold). Treatment with 22ROH reduced the oxazolone-induced inflammation, as shown by both decreased ear thickness (**Fig 4*A***) and decreased ear weight (**Fig 4*B***) (58% and 55%, respectively). Moreover, treatment with a nonsterol activator of LXR, GW3965, also significantly decreased ear thickness and ear weight, by 28% and 26%, respectively. Clobetasol (0.05%) treatment produced a stronger anti-inflammatory effect than that observed with the LXR activators at the concentrations employed here (over a 90% reduction in ear

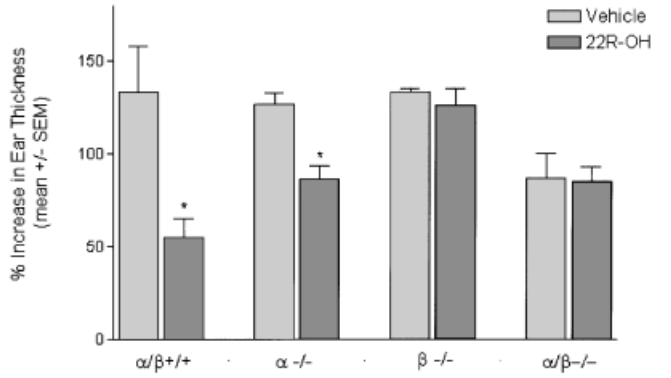

Figure 3. 22ROH does not reduce TPA-induced ear inflammation in LXRβ −/− and LXRα/β −/− mice and 22ROH partially reduces inflammation in LXRα −/− mice. Ear swelling with TPA was induced in the LXR-deficient animals as described for the CD1 animals. Each set of knockout animals was treated with vehicle or 22ROH and ear thickness was measured 18 h after TPA application. Ear thickness is markedly reduced after topical treatment with 22ROH (10 mM) in wild-type animals. 22ROH does not reduce the swelling in LXRβ −/− or LXRα/β −/− animals. 22ROH has a partial effect in the LXRα −/− mice, as ear swelling is somewhat diminished. Results are shown as the reduction (percentage) in ear thickness observed following treatment with 22ROH *versus* vehicle control. Data represent means ± SEM; $n = 4$–8, $p < 0.01$. Statistical analysis was performed using Student's *t* test.

thickness and weight). Finally, hematoxylin and eosin stained sections demonstrated a marked increase in ear thickness with an abundance of inflammatory cells in both epidermis and dermis in oxazolone-treated animals, changes that were markedly reduced by 22ROH treatment (**Fig 5**). Thus, oxysterols reduce inflammation in a murine model of allergic contact dermatitis.

LXR activators inhibit TNF-α and IL-1α generation in wild-type but not in LXRβ-deficient mice Keratinocytes generate the primary cytokines TNF-α and IL-1α in response to a variety of forms of cutaneous injury, leading to a downstream inflammatory response (Piguet, 1993; Murphy *et al*, 2000). Previous studies by our laboratory have shown that both epidermal TNF-α and IL-1α immunoreactivity increase following either TPA- or oxazolone-induced inflammation (Sheu *et al*, 2002). Using a similar immunohistochemical approach, we next examined whether 22ROH treatment decreased the production of TNF-α and IL-1α in response to TPA or oxazolone challenge. **Figs 6** and **7** demonstrate a comparable decrease in TNF-α and IL-1α immunostaining in both the epidermis and dermis of 22ROH- and clobetasol-treated animals in both the irritant and allergic contact dermatitis models. These findings provide further evidence that treatment with oxysterols inhibits these inflammatory responses.

To further elucidate the importance of LXRβ in the anti-inflammatory effects produced by oxysterols, we next examined whether treatment with 22ROH would decrease the production of TNF-α and IL-1α in animals lacking LXRβ, using immunohistochemical techniques. **Fig 8(*A*), (*B*)** demonstrates a decrease in the staining of TNF-α and IL-1α, respectively, in the epidermis and dermis of wild-type animals after treatment with 22ROH in the irritant contact dermatitis model. A decrease in immunostaining was not evident after 22ROH treatment in TPA-challenged LXRβ −/− animals, however. These findings provide further evidence that oxysterol activators reduce inflammation by an LXRβ-specific mechanism.

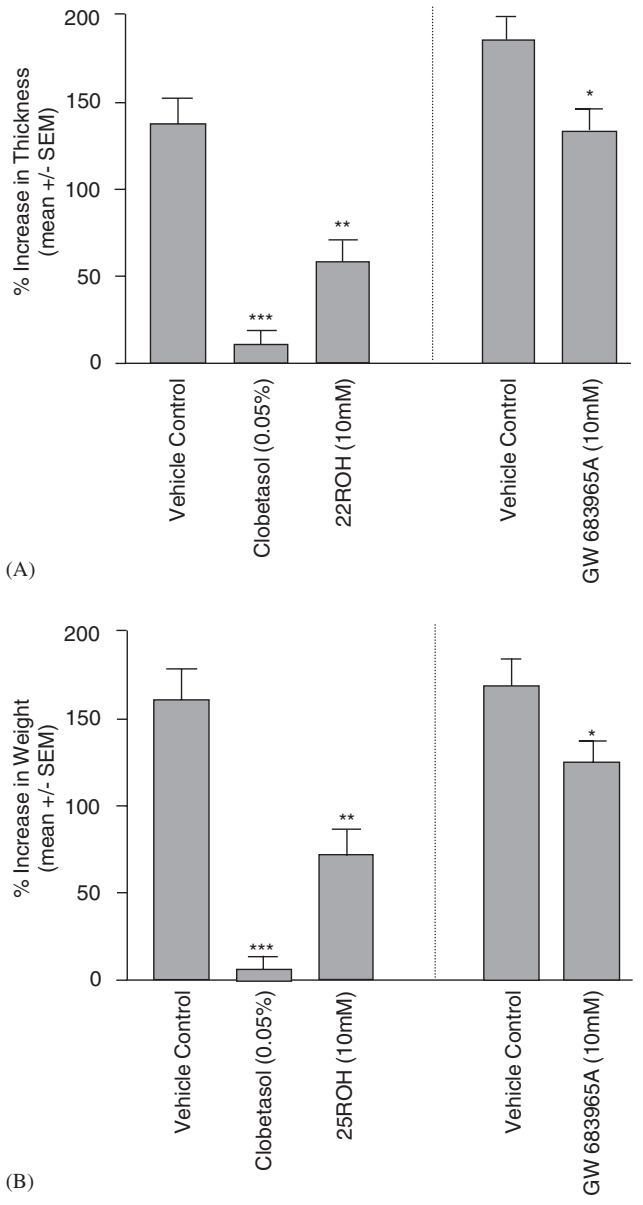

Figure 4. Activators of LXR reduce inflammation in an allergic contact dermatitis model. Animals were sensitized on shaved backs with 2% oxazolone (day 0), followed by application of 15% oxazolone (day 7) to the left ear. The mice were treated topically on both ears 45 min and 4 h after oxazolone application with either 22ROH (10 mM), clobetasol (0.05%), GW3965 (a nonsterol LXR activator) (10 mM), or vehicle (acetone). At 18 h post oxazolone application, ear thickness and ear weight (6 mm punch) were measured. Ear thickness (*A*) and ear weight (*B*) are markedly increased in oxazolone-induced allergic contact dermatitis. 22ROH (10 mM) and GW3965 (10 mM) treatment reduces oxazolone-induced ear thickness (*A*) and weight (*B*). Clobetasol (0.05%) treatment nearly eliminates the swelling. Results are shown as the mean percentage increase in ear thickness and weight (mean ± SEM; $n = 5$–7, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Statistical significance was determined using ANOVA and Student's *t* test.

DISCUSSION

This study is the first definitive demonstration that activators of LXR possess anti-inflammatory properties. In both TPA-induced inflammation, a model of irritant contact dermatitis, and oxazolone-induced allergic contact dermatitis, both oxysterol activation of LXR and GW3965, a nonsterol activator of LXR, reduce

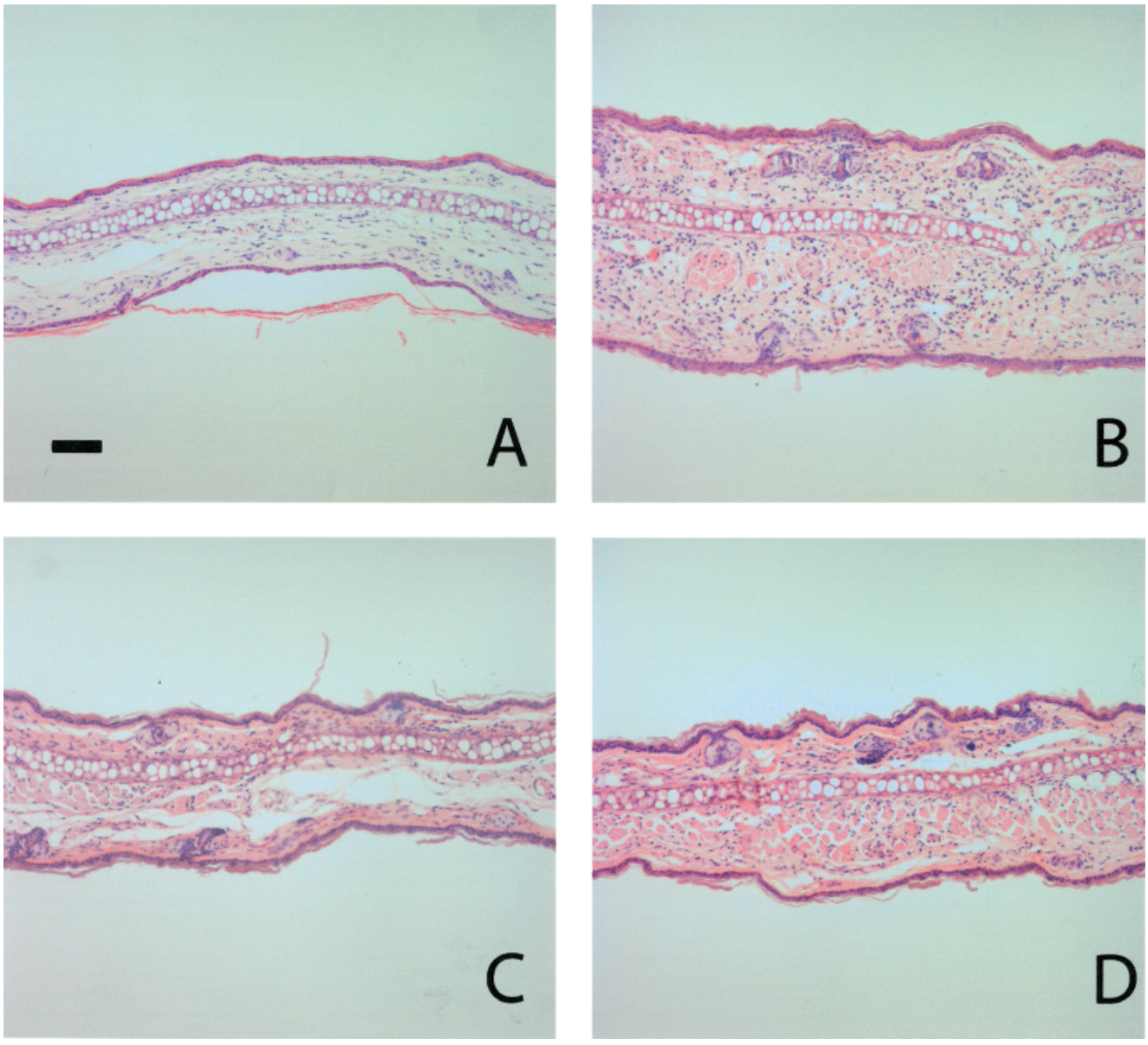

Figure 5. LXR activators decrease oxazolone-induced inflammation. Allergic contact dermatitis was induced by primary sensitization on day 0 with 2% oxazolone (20 μl) to the shaved backs of mice, followed by application of 15% oxazolone (10 μl) to the left ear on day 7. Forty-five minutes and 4 h after TPA application, the animals were treated topically with either clobetasol (0.05%), 22ROH (an oxysterol), or vehicle (acetone). The animals were sacrificed at 18 h after TPA application and biopsies of the ears were obtained. The specimens were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin. Topical oxazolone application results in an increase in ear thickness and the amount of inflammatory cells (*B*). Treatment with 22ROH (*D*) and clobetasol (*C*) reduces ear thickness and the number of inflammatory cells. A normal untreated mouse ear (*A*) is provided for comparison. Sections above are representative findings from three animals per group. *Scale bar*: 50 μm.

cutaneous inflammation. Thus, two different types of LXR activators are able to decrease inflammation in two different, *in vivo* models of cutaneous inflammation. Oxysterols were sometimes more effective in inhibiting inflammation than the nonsterol LXR activator GW3965 in the topical inflammation assays. A likely reason for this difference in potency is that the ability of oxysterols to permeate an intact stratum corneum and activate keratinocytes is greater than for the nonsterol LXR activator GW3965. In contrast, cholesterol, a sterol that does not activate LXR, exhibited no anti-inflammatory activity in these models. Moreover, we showed that in the TPA-irritant contact dermatitis model, the anti-inflammatory properties of the LXR activators are of a magnitude similar to that seen with clobetasol, a potent topical glucocorticoid. In the oxazolone allergic contact dermatitis model, however, the anti-inflammatory properties of glucocorticoids exceed those seen with the LXR activators at the doses employed here. As allergic contact dermatitis requires an adaptive immune response involving T lymphocytes, glucocorticoids could inhibit these pathways in the immune system more effectively than can LXR agonists, which could account for the greater anti-inflammatory activity in the allergic contact dermatitis model (Leung *et al*, 1999; Weston and Bruckner, 2000). Yet, because glucocorticoids can exhibit adverse side-effects, their usefulness as anti-inflammatory agents is limited (Ashwell *et al*, 2000). In fact, whereas exogenous glucocorticoids impair cutaneous permeability barrier homeostasis, as well as stratum corneum integrity/cohesion,[2] we have shown that LXR activators instead improve stratum corneum barrier function (Komuves *et al*, 2002). Thus, LXR activators could display more discrete

[2]Kao JS, Fluhr JW, Man M *et al* (Abstr.)

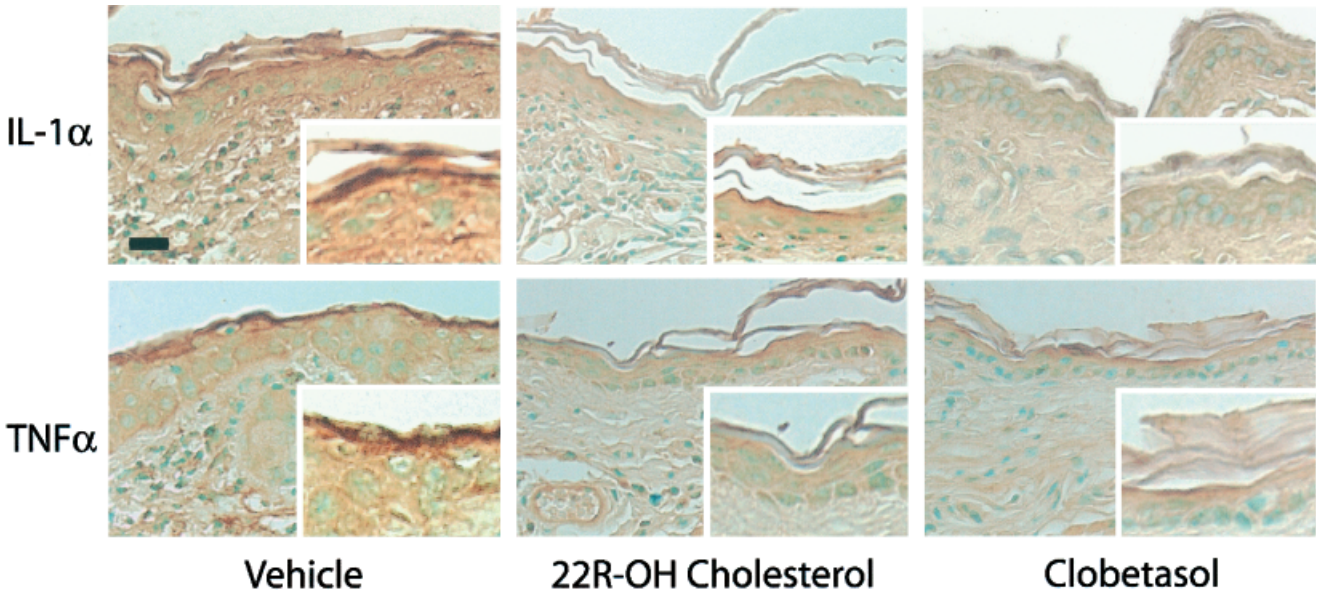

Figure 6. Oxysterols reduce expression of IL-1α and TNF-α in TPA-induced model of irritant contact dermatitis. Ear samples were obtained 18 h after TPA-induced contact dermatitis. The mice were treated 45 min and 4 h after TPA application as indicated. Paraformaldehyde-fixed sections were stained with rabbit polyclonal anti-IL-1α and polyclonal goat anti-TNF-α antibodies. IL-1α and TNF-α immunoreactivity was visualized by biotinylated secondary antibodies in combination with the 3,3-diaminobenzidine technique (brown color) and counterstained with methyl green. Immunohistochemical staining demonstrates a more pronounced expression of proinflammatory cytokines IL-1α and TNF-α in the epidermis and dermis following treatment with TPA and vehicle alone. After treatment with 22ROH (10 mM) or clobetasol (0.05%) following TPA application, IL-1α and TNF-α staining is reduced. *Insert* shows higher magnification. Sections above are representative findings from three animals per group. *Scale bar*: 10 μm.

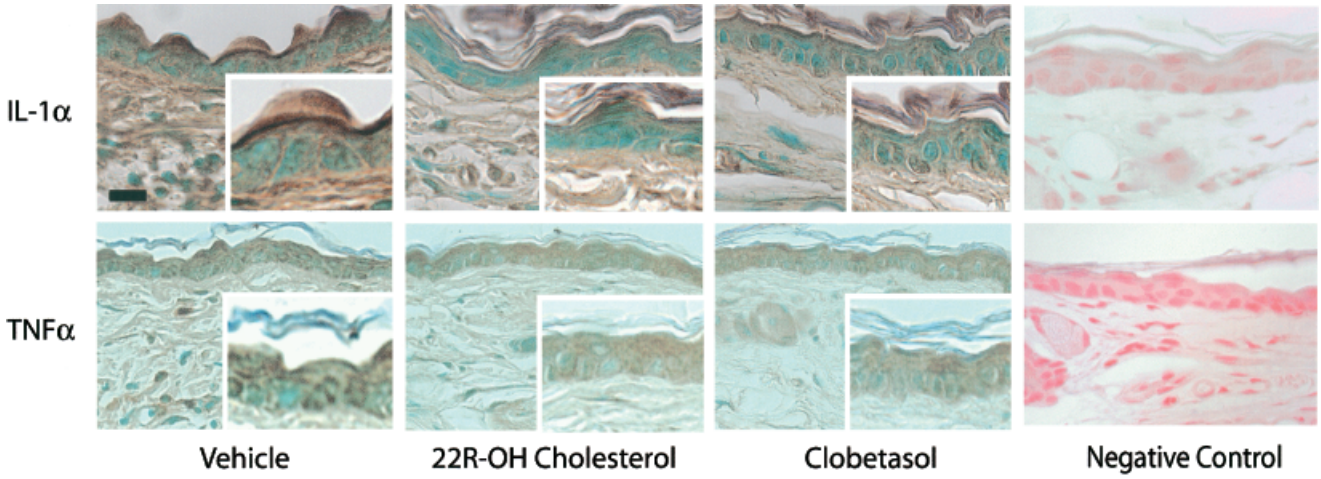

Figure 7. Oxysterols reduce expression of IL-1α and TNF-α in oxazolone-induced model of allergic contact dermatitis. Ear samples were obtained 18 h after oxazolone-induced allergic contact dermatitis to sensitized mice. The mice were treated 45 min and 4 h after oxazolone application as indicated. Paraformaldehyde-fixed sections were stained with rabbit polyclonal anti-IL-1α and polyclonal goat anti-TNF-α antibodies. IL-1α and TNF-α immunoreactivity was visualized by biotinylated secondary antibodies in combination with the 3,3-diaminobenzidine technique (brown color) and counterstained with methyl green. Immunohistochemical staining demonstrates an increase in IL-1α and TNF-α in the epidermis and dermis of oxazolone-treated animals after treatment with vehicle (acetone) alone. After treatment with 22ROH (10 mM) or clobetasol (0.05%), IL-1α and TNF-α staining is reduced. Note the negative control (counterstained with nuclear fast red) provided for comparison. *Insert* shows higher magnification. Sections above are representative findings from three animals per group. *Scale bar*: 10 μm.

and less global effects than glucocorticoids, suggesting that they can be useful for the treatment of inflammatory skin disorders.

Whereas these and previous *in vitro* studies suggest that oxysterols possess anti-inflammatory properties (Ohlsson *et al*, 1996), oxysterols display biologic effects beyond the activation of LXR. For example, oxysterols inhibit the proteolytic conversion of SREBP to an active transcription factor, which accounts for the ability of oxysterols to inhibit cholesterol synthesis and to reduce LDL receptor levels (Brown and Goldstein, 1997). Yet, in this study we demonstrate that a nonsterol activator of LXR, GW3965, also displays anti-inflammatory properties, further increasing the likelihood that the cutaneous anti-inflammatory effects of oxysterols are mediated via LXR. Alternatively, the oxysterol compounds may regulate non-LXR pathways, such as the inhibition of SREBP signaling. To demonstrate definitively the importance of LXR, we carried

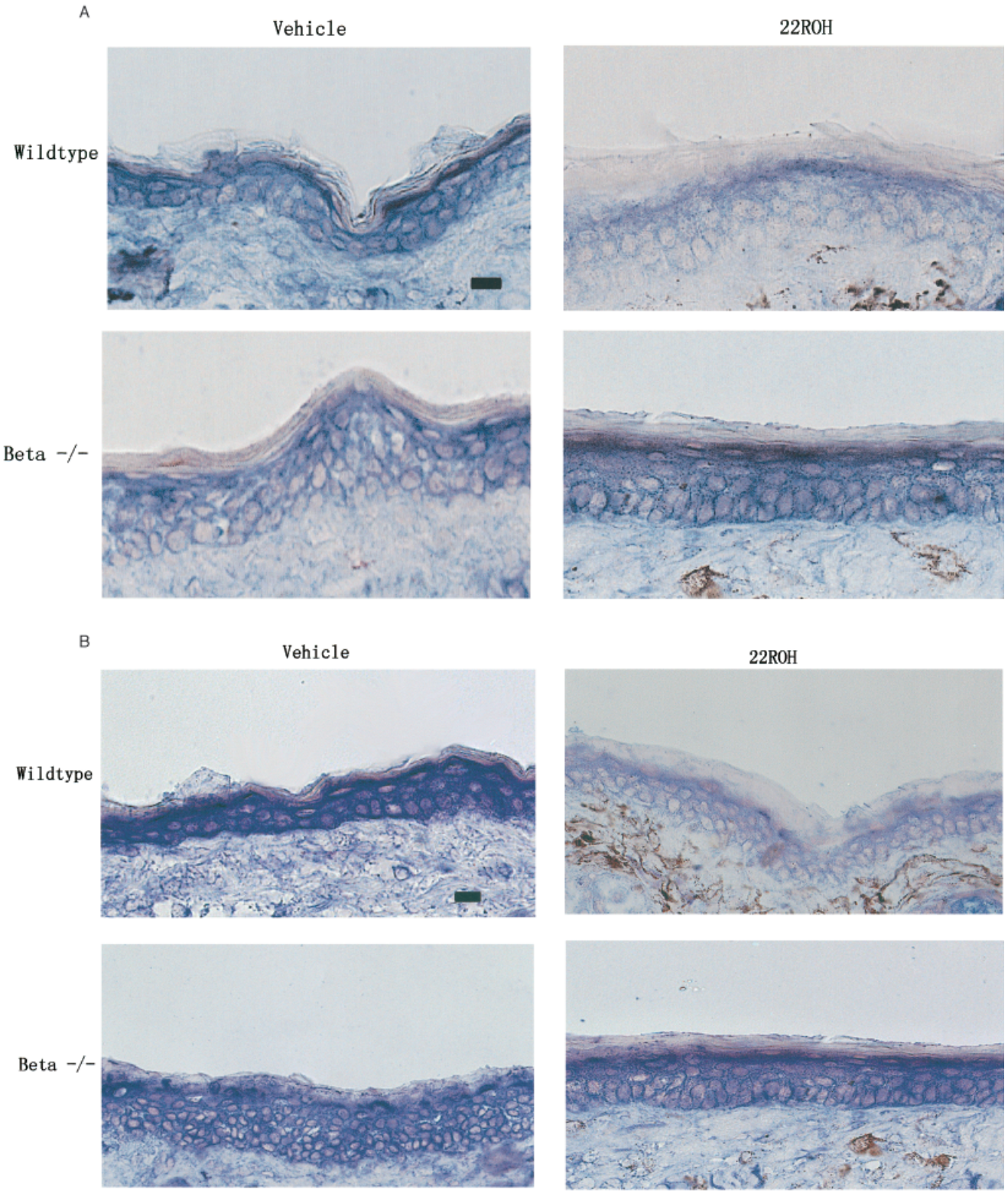

Figure 8. Oxysterols do not reduce IL-1α and TNF-α expression in LXRβ −/− animals following TPA-induced inflammation. Wild-type and LXRβ-deficient mice were treated with TPA followed by 22ROH and vehicle (acetone) as described for the wild-type animals. Ear biopsies were taken 18 h after TPA application and cryosections were obtained. The samples were stained with rabbit and goat polyclonal antibodies to TNF-α (*A*) and IL-1α (*B*), respectively. Bound antibodies were detected with secondary biotin-conjugated antibodies in combination with alkaline phosphatase (purple color). No counterstaining was performed. The reduction in IL-1α and TNF-α expression induced following treatment with 22ROH compared to vehicle treatment in the wild-type animals is not observed in the LXRβ −/− animals. Treatment with 22ROH does not reduce IL-1α and TNF-α staining compared to vehicle in the LXRβ −/− mice. *Scale bar*: 10 μm.

out studies using LXR-deficient animals. Oxysterols did not inhibit inflammation in either LXRβ −/− or LXRα/β −/− mice, indicating that the anti-inflammatory properties of oxysterols on cutaneous inflammation require LXRβ, and that LXRα alone cannot compensate for an absence of LXRβ.

In the LXRα-deficient mice the situation is more complex, as oxysterols are able to decrease inflammation but the anti-inflammatory effects are reduced in comparison to wild-type mice (≈50% reduction *versus* wild-type mice). In previous studies using northern blotting we did not detect LXRα in mouse epidermis (Komuves *et al*, 2002). In both cultured human keratinocytes and fetal rat epidermis, however, LXRα is easily observed (Hanley *et al*, 1999; 2000). It is possible that LXRα is present in small quantities in mouse epidermis, and that it plays a separate role in the anti-inflammatory properties of oxysterols, which cannot be compensated for by the presence of LXRβ. Alternatively, LXRα could be present in inflammatory cells, dermal cells, or other cells that contribute to the cutaneous inflammatory response and oxysterols may reduce inflammation by activating LXRα in these cells.

The secretion of TNF-α and IL-1α by keratinocytes in response to injury mediates the cutaneous inflammatory response (Piguet, 1993; Murphy *et al*, 2000). In this study, we demonstrate that activators of LXR inhibit the secretion of TNF-α and IL-1α in both the irritant and allergic models of contact dermatitis. Oxysterols did not reduce cytokine generation in LXRβ-deficient mice. Together, these results indicate that LXR activation inhibits cytokine secretion, at least in part, accounting for the anti-inflammatory properties observed in these studies.

Ligands of other class II nuclear hormone receptors, such as PPAR and the vitamin D receptor, have been shown to have anti-inflammatory properties (Devchand *et al*, 1996; Muller and Bendtzen, 1996; Duvic *et al*, 1997; Staels *et al*, 1998; Delerive *et al*, 1999; 2000; Deluca and Cantorna, 2001). The expression of many genes required for the inflammatory response are regulated by nuclear factor κB (NF-κB) activation (Ghosh *et al*, 1998), and both the PPARs and the vitamin D receptor have been shown to inhibit NF-κB, thereby reducing inflammation (Yu *et al*, 1995; Delerive *et al*, 2001). In vascular smooth muscle cells, oxysterols inhibit lipopolysaccharide-induced activation of NF-κB (Ares *et al*, 1995). Whether the effects of LXR activation on cutaneous inflammation are mediated by alterations in NF-κB and/or other pathways, however, remains to be determined.

Previous studies have demonstrated that LXR activators stimulate epidermal differentiation, improve permeability barrier homeostasis, and inhibit epidermal proliferation (Komuves *et al*, 1998; 2002; Hanley *et al*, 2000). In this study we demonstrate that LXR activators also possess anti-inflammatory properties. A number of cutaneous disease states, such as psoriasis, are characterized by hyperproliferation, inflammation, decreased differentiation, and impaired permeability barrier homeostasis (Christophers and Mrowietz, 1999). Given the profile of activity of LXR activators, it is possible that compounds that activate LXR will be useful in the treatment of psoriasis and other inflammatory dermatoses. Additionally, if further studies demonstrate that LXR activators also reduce inflammation in extracutaneous tissues, these compounds could be useful in the treatment of a wide variety of other inflammatory disorders.

In summary, this study demonstrates conclusively that compounds that activate LXR reduce inflammation in animal models of irritant and allergic contact dermatitis by a receptor-mediated process that involves suppression of cytokine production.

David J. Mangelsdorf, M.D., is funded by the Howard Hughes Medical Institute and the Robert A. Welch Foundation. This work was supported by NIH grants HD 29706, AR 29706, and PO 039448, and by the Veterans Affairs Research Funding.

REFERENCES

Ares MP, Kallin B, Eriksson P, Nilsson J: Oxidized LDL induces transcription factor activator protein-1 but inhibits activation of nuclear factor-κB in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 10:1584–1590, 1995

Ashwell JD, Lu FW, Vacchio MS: Glucocorticoids in T-cell development and function. *Annu Rev Immunol* 18:309–345, 2000

Bikle DD: 1,25 OH_2D_3-modulated calcium induced keratinocyte differentiation. *J Invest Dermatol Symp Proc The* 1:22–27, 1996

Brown MS, Goldstein JL: The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 89:331–340, 1997

Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ: Nuclear receptors and lipid physiology: opening the X-files. *Science* 294:1866–1870, 2001

Christophers E, Mrowietz U: Epidermis: disorders of persistent inflammation, cell kinetics, and differentiation. In: Freedberg IM, *et al*: eds. *Fitzpatrick's Dermatology in General Medicine.* New York: raw–Hill, 1999: pp 509–512

Collins JL, Fivush AM, Watson MA, *et al*: Identification of a nonsteroidal liver receptor agonist through parallel array synthesis of tertiary amines. *J Med Chem* 45:1963–1966, 2002

Delerive P, De Bosscher K, Besnard S, *et al*: Peroxisome proliferator-activated receptor α negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-κB and AP-1. *J Biol Chem* 274:32048–32054, 1999

Delerive P, Gervois P, Fruchart JC, Staels B: Induction of IκBα expression as a mechanism contributing to the anti-inflammatory activities of peroxisome proliferator-activated receptor-α activators. *J Biol Chem* 275:36703–36707, 2000

Delerive P, Fruchart JC, Staels B: Peroxisome proliferator-activated receptors in inflammation control. *J Endocrinol* 169:453–459, 2001

Deluca HF, Cantorna MT: Vitamin D: its role and uses in immunology. *FASEB J* 15:2579–2585, 2001

Devchand PR, Keller H, Peters JM, Vazquez M, Gonzalez FJ, Wahli W: The PPAR-α leukotriene B4 pathway to inflammation control. *Nature* 384:39–43, 1996

Duvic M, Nagpal S, Asano AT, Chandraratna RA: Molecular mechanisms of tazarotene action in psoriasis. *J Am Acad Dermatol* 37:S18–S24, 1997

Eichner R, Gendimenico GJ, Kahn M, *et al*: Effects of long-term retinoic acid treatment on epidermal differentiation *in vivo*, specific modification in the program of terminal differentiation. *Br J Dermatol* 135:687–695, 1996

Fisher GJ, Voorhees JJ: Molecular mechanisms of retinoid actions in skin. *FASEB J* 10:1002–1012, 1996

Fluhr JW, Kao J, Jain M, Ahn SK, Feingold KR, Elias PM: Generation of free fatty acids from phospholipids regulates stratum corneum acidification and integrity. *J Invest Dermatol* 117:44–51, 2001

Ghosh S, May MJ, Kopp EB: NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. *Ann Rev Immunol* 16:225–260, 1998

Hanley K, Jiang Y, Crumrine D, *et al*: Activators of the nuclear hormone receptors PPAR-α and FXR accelerate the development of the fetal epidermal barrier. *J Clin Invest* 100:705–712, 1997

Hanley K, Jiang Y, He SS, *et al*: Keratinocyte differentiation is stimulated by activators of the nuclear hormone receptor PPAR-α. *J Invest Dermatol* 110:368–375, 1998

Hanley K, Komuves LG, Bass NM, *et al*: Fetal epidermal differentiation and barrier development *in vivo* is accelerated by nuclear hormone receptor activators. *J Invest Dermatol* 113:788–795, 1999

Hanley K, *et al*: Oxysterols induce differentiation in human keratinocytes and increase AP-1-dependent involucrin transcription. *J Invest Dermatol* 114:545–553, 2000

Janowski BA, Willy PJ, Rama-Devi T, Falck JR, Mangelsdorf DJ: An oxysterol signaling pathway mediated by the nuclear receptor LXRα. *Nature* 383:728–731, 1996

Kang S, Li XY, Voorhees JJ: Pharmacology and molecular action of retinoids and vitamin D in skin. *J Invest Dermatol Symp Proc The* 1:15–21, 1996

Komuves LG, Hanley K, Jiang Y, Elias PM, Williams ML, Feingold KR: Ligands and activators of nuclear hormone receptors regulate epidermal differentiation during fetal rat skin development. *J Invest Dermatol* 111:429–433, 1998

Komuves LG, Hanley K, Man MQ, Elias PM, Williams ML, Feingold KR: Keratinocyte differentiation in hyperprolferative epidermis. Topical application of PPARα restores tissue homeostasis. *J Invest Dermatol* 115:361–367, 2000

Komuves LG, Schmuth M, Fowler AJ, *et al*: Oxysterol stimulation of epidermal differentiation is mediated by liver-X-receptor-α in murine epidermis. *J Invest Dermatol* 118:25–34, 2002

Lehmann JM, Kliewer SA, Moore LB, *et al*: Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *J Biol Chem* 272: 3137–3140, 1997

Leung DY, Diaz LA, DeLeo V, Soter NA: Allergic and immunologic skin disorders. *JAMA* 278:1914–1923, 1997

Liu Y, Hulten LM, Wiklund O: Macrophages isolated from human atherosclerotic plaques produce IL-8. *Arteriocler Thromb Vasc Biol* 2:317–323, 1997

Mangelsdorf DJ, Evans RM: The RXR heterodimers and orphan receptors. *Cell* 83:841–850, 1995

Mangelsdorf DJ, Thummel C, Beato M, *et al*: The nuclear receptor superfamily: the second decade. *Cell* 83:835–839, 1995

Muller K, Bendtzen K: 1,25-Dihydroxyvitamin D3 as a natural regulator of human immune functions. *J Invest Dermatol Symp Proc The* 1:68–71, 1996

Murphy JE, Robert C, Kupper TS: Interleukin-1 and cutaneous inflammation: a crucial link between innate and acquired immunity. *J Invest Dermatol* 114:602–608, 2000

Ohlsson BG, Englund MC, Karlsson AL, *et al*: Oxidized low density lipoprotein inhibits lipopolysaccharide-induced binding of nuclear factor-κB to DNA and the subsequent expression of tumor necrosis factor-α and interleukin-1β in macrophages. *J Clin Invest* 98:78–89, 1996

Peet DJ, Turley SD, Ma W, Janowski BA, Lobaccaro JM, Hammer RE, Mangelsdorf DJ: Cholesterol and bile acid metabolism are impaired in mice lacking nuclear oxysterol receptor LXRα. *Cell* 93:693–704, 1998

Piguet PF: TNF and the pathology of the skin. *Res Immunol* 144:320–326, 1993

Sheu MY, Fowler AJ, Kao JK, *et al*: Topical PPAR-α activators reduce inflammation in irritant and allergic contact dermatitis models. *J Invest Dermatol* 118:94–101, 2002

Song C, Hiipakka RA, Kokonitis JM, Liao S: Ubiquitous receptor structures, immunocytochemical localization, and modulation of gene activation by receptors for retinoic acids and thyroid hormones. *Ann NY Acad Sci* 761:38–49, 1995

Staels B, Koenig W, Habib A, *et al*: Activation of human aortic smooth-muscle cells is inhibited by PPARα but not by PPARγ activators. *Nature* 393: 790–793, 1998

Weston WL, Bruckner A: Allergic contact dermatitis. *Pediatr Clin North Am* 47: 897–907, Vii 2000

Willy PJ, Umensono K, Ong ES, Evans RM, Heyman RA, Mangelsdorf DJ: LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev* 9:1033–1045, 1995

Yu XP, Bellido T, Manolagas SC: Down-regulation of NF-κB protein levels in activated human lymphocytes by 1,25-dihydroxyvitamin D3. *Proc Natl Acad Sci USA* 92:10990–10994, 1995