Topical Application of A Novel Immunomodulatory Peptide, RDP58, Reduces Skin Inflammation in the Phorbol Ester-Induced Dermatitis Model

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RDP58 is the first lead compound in a series of immunomodulating decapeptides discovered through activity-based screening and computer-aided, rational design. RDP58 disrupts cellular responses signaled through the Toll-like and tumor necrosis factor (TNF) receptor families and occludes important signal transduction pathways involved in inflammation, inhibiting the production of tumor necrosis factor alpha (TNFα), interferon-γ, interleukin (IL)-2, IL-6, and IL-12. These pro-inflammatory cytokines are thought to be involved in the pathogenesis of several inflammatory and autoimmune diseases, including atopic dermatitis and psoriasis. The goal of this study was to determine the ability of RDP58 to inhibit skin inflammation following exposure to the well-characterized protein kinase C activator and tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Topical application of RDP58 to the epidermis following TPA treatment resulted in the amelioration of the phorbol ester-induced irritant contact dermatitis. Substantial reductions were observed in skin thickness and tissue weight, neutrophil-mediated myeloperoxidase activity, inflammatory cytokine production, and various histopathological indicators. We also found RDP58 to be effective in reducing the compounding inflammatory damage brought on by chronic TPA exposure, and that it is capable of targeting inflammatory mediators specifically in the keratinocyte. These results demonstrate that topically applied RDP58 is an effective anti-inflammatory treatment in the phorbol ester-induced dermatitis model, and suggest that it may have therapeutic potential in a variety of immune-related cutaneous diseases.

Key words: dermatitis/keratinocytes/peptide therapeutics/skin inflammation/12-*O*-tetradecanoylphorbol-13-acetate (TPA)
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The skin plays a central role in host immunological defenses; however, the regulation of these defense mechanisms is also crucial, as inappropriate or misdirected immune activity is implicated in the pathogenesis of a large variety of acquired inflammatory skin disorders (Kupper and Fuhlbrigge, 2004). High levels of inflammatory cytokines and reactive oxygen species are proposed contributors to the pathophysiologic mechanisms associated with various inflammatory dermatoses (Elias *et al*, 1999; Trouba *et al*, 2002). It is widely recognized that the secretions of cytokines by keratinocytes in response to injury, particularly tumor necrosis factor alpha (TNFα) and interleukin (IL)1α, are key mediators of the cutaneous inflammatory response. Both the epithelial barrier cells and resident innate immune cells in the skin express receptors belonging to the Toll-like receptor (TLR) family (Takeda *et al*, 2003). The binding of TLR ligands, which include a number of pathogen-associated molecular pattern molecules, is associated with the recruitment of intracellular adaptor proteins similar to those used by IL-1R and subsequent activation of the JUN N-terminal kinase (JNK) and nuclear factor-κB (NF-κB) signaling pathways. The NF-κB signaling pathway is seen as a key link between the innate and adaptive immune systems (Medzhitov and Janeway, 1997). In the skin, NF-κB regulates the expression of numerous genes that are involved in the initiation of the inflammatory response, including adhesion molecules, chemokines and cytokines, matrix metalloproteases, nitric oxide synthase, and enzymes that control prostaglandin synthesis (Bell *et al*, 2003). Beyond the direct effects of these compounds on pathogens and abnormal cells, products of the innate immune response direct the recruitment of additional leukocytes to the site of activation. These include non-specific leukocytes, such as neutrophils and natural killer cells, as well as key components of the adaptive immune system, such as effector T cells.

We have developed an immunomodulatory peptide, RDP58, which is both an effective inhibitor of multiple pro-inflammatory cytokines (Iyer *et al*, 2002) and an upregulator of an oxidative stress-responsive enzyme, heme oxygenase-1 (HO-1) (Iyer *et al*, 1998; Cuturi *et al*, 1999). RDP58, NH_2–r–nle–nle–nle–r–nle–nle–nle–g–y–$CONH_2$, is the lead compound in a series of protease-resistant, D-isomeric decapeptides discovered through activity-based screening

Abbreviations: IL, interleukin; MPO, myeloperoxidase; NHEK, normal human epidermal keratinocytes; RDP58, rationally designed peptide 58; TNFα, tumor necrosis factor alpha; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

and computer-aided, rational design (Grassy *et al*, 1998; Iyer *et al*, 2002). Originally derived from sequences of human class I MHC molecule known to have clinically important immunomodulating effects (Cuturi *et al*, 1995; Gao *et al*, 1996; Hanaway *et al*, 1996), it has recently been discovered that RDP58 is the first of a novel class of anti-inflammatory therapeutics that disrupt formation of the TRAF6–MyD88–IRAK complex responsible for activating crucial signal transduction pathways (p38MAPK, JNK, and IKK) involved in inflammation (Lazarov *et al*, 2003).[1] Because multiple, parallel pathways are affected, RDP58 inhibits the production of several pro-inflammatory cytokines, including TNFα, interferon-γ, IL-2, IL-6, and IL-12, in both immune and epithelial cell types. These pro-inflammatory cytokines are thought to be involved in the pathogenesis of numerous inflammatory and autoimmune diseases. To date, the RDP58 peptide has demonstrated efficacy in a variety of inflammatory disease models, including UVB-induced skin injury, collagen-induced arthritis, graft-versus-host disease, allograft rejection, and inflammatory bowel disease (Buelow *et al*, 1995; Gao *et al*, 1996; Oberyszyn *et al*, 2001; Boismenu *et al*, 2002; Iyer *et al*, 2002; Murthy *et al*, 2002). The ability of RDP58 to utilize the HO-1 pathway, a stress response mechanism for protecting cells from oxidative injury, was demonstrated by the administration of peptide to mice and rats, which resulted in an increase in HO-1 expression that correlated with cardiac allograft survival (Iyer *et al*, 1998; Cuturi *et al*, 1999). These studies suggest that RDP58 may be an effective therapy for inflammation by combining anti-inflammatory properties with the ability to induce stress response pathways that mitigate oxidative damage.

In this study, we determined whether topical treatment with RDP58 would reduce cutaneous inflammation in the skin of mice following exposure to the well-characterized protein kinase C activator and tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). We also investigated the effects of RDP58 on keratinocytes, as they represent the likely targeted cell type. Our studies demonstrate that topically applied RDP58 possesses potent anti-inflammatory activity in both acute and chronic contact dermatitis models and that keratinocytes are in fact highly responsive to this immunomodulatory peptide.

Results

RDP58 inhibits TPA-induced cutaneous inflammation We first assessed the anti-inflammatory activity of RDP58 in the TPA model of acute irritant contact dermatitis. Increased skin thickening is often the first hallmark of skin irritation and local inflammation. This parameter is indicative of a number of processes that occur during skin inflammation, including increased vascular permeability, edema and swelling within the dermis, and proliferation of the epidermal keratinocytes. Skin thickness was measured in the dorsal skin prior to and 18 h following treatments using digital calipers, and results

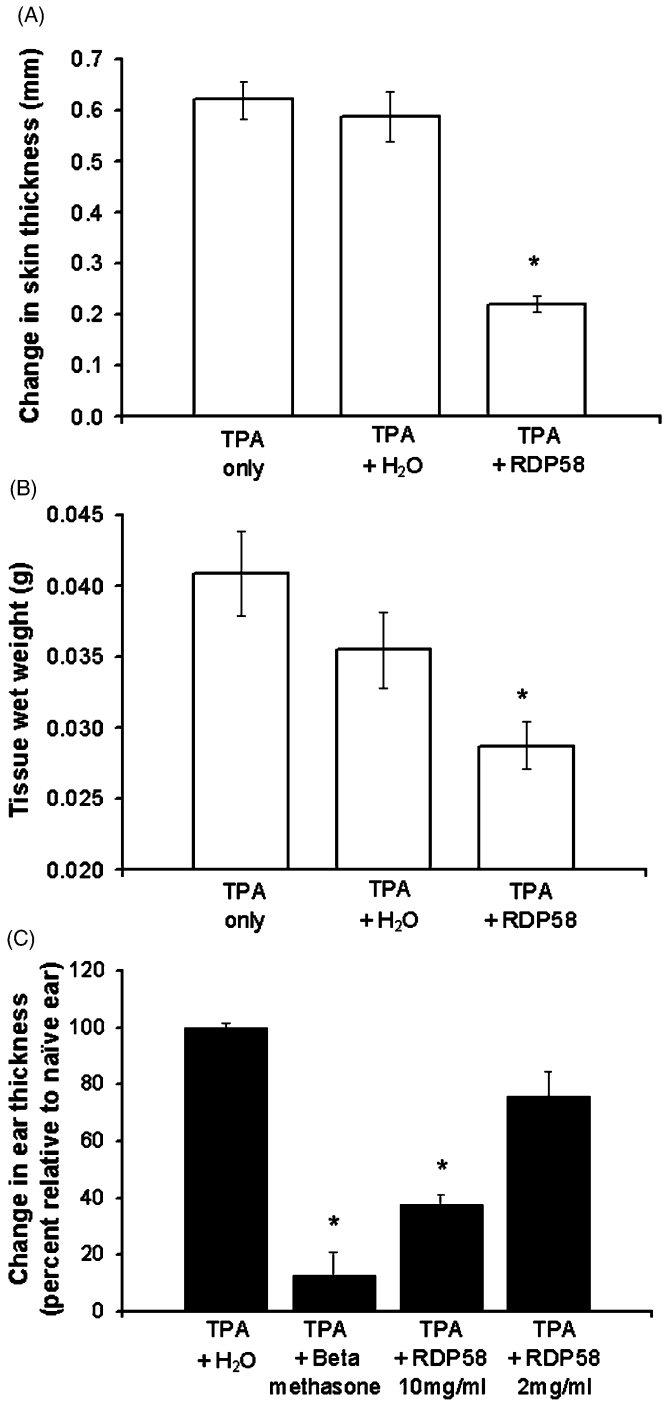

Figure 1
Rationally designed peptide 58 (RDP58) reduces 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin and ear swelling. Skin thickness (*A*, *C*) and weight (*B*) are markedly increased in TPA-induced irritant dermatitis. Topical treatment with RDP58 (10 and 2 mg per mL), or a positive control corticosteroid, 0.05% betamethasone, 30 min following TPA application reduces the increases in dorsal skin thickness (*A*) and weight (*B*) as well as ear thickening (*C*), whereas water-vehicle alone has no effect. Thickness measurements were made using digital calipers and expressed as the difference between 0 and 18 h measurements in dorsal skin thickness or as the percent change in thickness relative to naïve, untreated ear tissue. Skin biopsies were collected from the dorsal skin for weight measurements. Data are presented as mean $\pm$ SEM from to to three experiments consisting of three to five animals per group in each experiment. Statistical significance was determined using Student's unpaired *t* test; $^*p < 0.05$.

[1]Lazarov M, Welihinda A, Muhr E, Mo L, Buelow R, Fong T: RDP58, a novel anti-inflammatory Peptide, Inhibits Multiple Intracellular Messenger Pathways and transcription factors to block TNFα, IFNγ, and IL12 production. In Research Forum—America, Gastroenterology Association (abstr)

were graphed as the difference in skin thickness expressed in mm^2. Exposure of the dorsal epidermis of FVB/N mice to TPA resulted in marked increases in both skin thickness (Fig 1*A*) and tissue weight (Fig 1*B*). Topical application of acetone or RDP58 alone did not alter the skin thickness significantly. Treatment with RDP58 30 min after TPA application, however, significantly inhibited the phorbol ester-induced increase in both skin thickness (65% decrease; $p < 0.0001$) and weight (30% decrease; $p < 0.01$), indicating a therapeutic effect of this peptide (Fig 1*A*, *B*).

The ability of RDP58 to reduce cutaneous inflammation as related to a change in skin thickening was also observed in the ear (Fig 1*C*). The experimental design used with ear applications allowed for normalization between treated and untreated ears from the same animal from which a mean change in thickness relative to naïve tissue was established in each treatment group ($n = 5$). Treatment with RDP58 resulted in an approximately 60% decrease in TPA induced ear thickening ($p < 0.0001$), which was comparable with the potent topical corticosteroid betamethasone. The ability of RDP58 to reduce ear thickness was diminished at a lower 2 mg per mL concentration (Fig 1*C*), indicating a specific concentration dependence of this effect.

We next examined hematoxylin and eosin (H&E)-stained sections of the ears from TPA-treated animals. As shown in Fig 2, TPA application results in a marked increase in ear thickness, with clear evidence of edema, epidermal hyperplasia, and large numbers of inflammatory cells infiltrating the dermis (*A vs B*). RDP58 treatment reduced ear thickness and the associated pathological indicators to an extent comparable with betamethasone (Fig 2*C*, *D*). These results provide further evidence that RDP58 ameliorates TPA-induced contact dermatitis, directly illustrating its effects within the target tissue.

RDP58 reduces neutrophil activation and pro-inflammatory cytokine production *in vivo* To assess the efficacy of RDP58 at the cell and molecular level, we next looked at

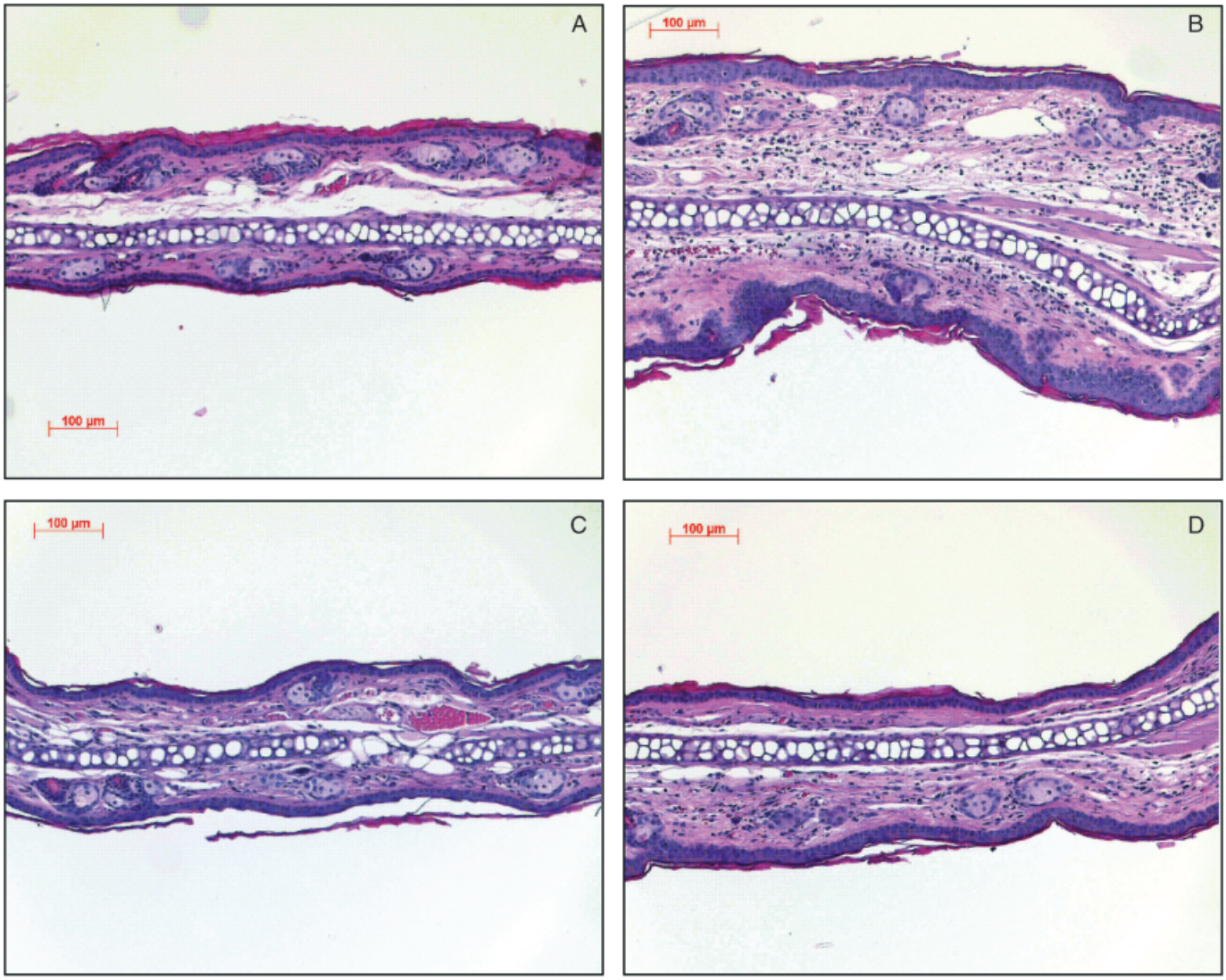

Figure 2
Rationally designed peptide 58 (RDP58) reduces histopathology associated with skin inflammation. Hematoxylin- and eosin-stained sections of normal mouse ear (*A*), 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced irritant dermatitis (*B*), 0.05% betamethasone (*C*), and 10 mg per mL RDP58 treated (*D*). TPA treatment induced a marked increase in ear thickness accompanied by the characteristic pathological features of edema, epidermal hyperplasia, and inflammatory cell infiltration (*B*). RDP58 treatment reduces ear thickness and the associated pathological indicators to an extent comparable with betamethasone (*C vs D*). Sections shown are representative of observations from five animals in each group.

prominent inflammatory mediators in the skin. The TPA-induced acute inflammatory response in skin is associated with an increase in the infiltration of activated neutrophils into the dermis. These activated neutrophils produce myeloperoxidase (MPO), which can be quantitated as a measure of the magnitude of neutrophil activation (Bradley *et al*, 1982). Topical application of TPA to the dorsal skin of FVB/N mice substantially increased the amount of MPO detected within the skin at 18 h (Fig 3*A*). Compared with vehicle alone, treatment with 10 mg per mL RDP58 30 min after TPA exposure significantly decreased the dermal MPO activity detected at 18 h (50% decrease; $p < 0.05$). We also observed significant reductions in MPO activity levels with RDP58 treatment in the ears of mice exposed to TPA ($p < 0.0001$) (Fig 3*B*). The effectiveness of RDP58 was concentration dependent and comparable to betamethasone at the 25 mg per mL concentration. A negative control peptide, which lacks anti-inflammatory activity, had no protective effect, ruling out generalized peptide-inhibiting consequences.

Another important mechanism for cutaneous inflammation is the secretion of cytokines, such as IL-1 and IL-6, by keratinocytes in response to injury (Kupper *et al*, 1989; Kupper and Groves, 1995). A reduction in the secretion of these two primary cytokines would be expected to result in a decrease in cutaneous inflammation. As shown in Fig 4, TPA treatment results in an increase in IL-1β and IL-6 protein levels in ear biopsy homogenates. Treatment with TPA plus RDP58 reduced both IL-1β and IL-6 cytokine levels significantly (50%–60% decrease; $p < 0.001$). The negative control peptide was not effective in reducing the levels of these cytokines. Thus, RDP58 may reduce the levels of activated cellular infiltrates and local secretion of cytokines, thereby reducing cutaneous inflammation.

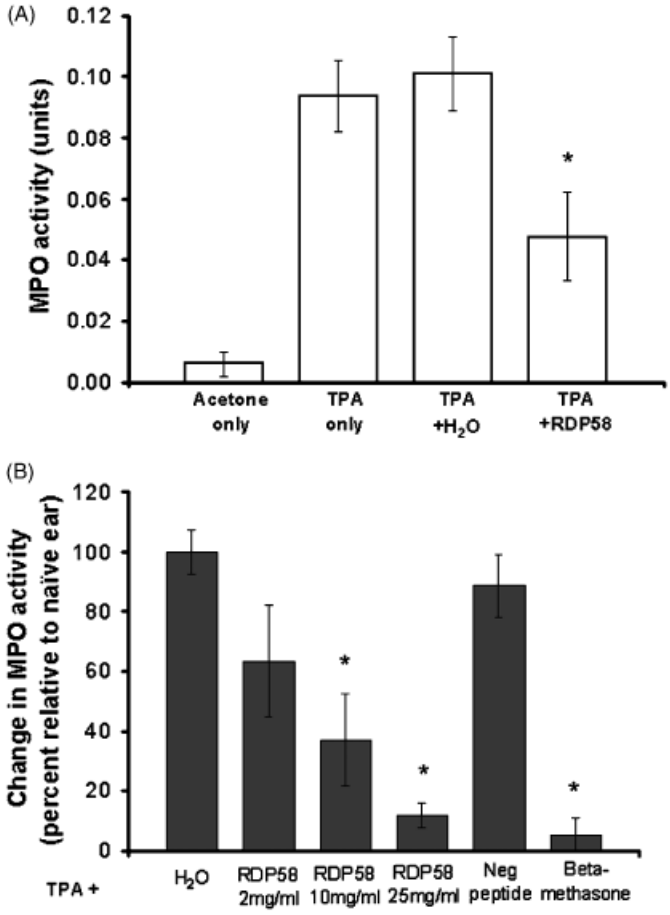

Figure 3
Inhibition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced myeloperoxidase (MPO) activity by rationally designed peptide 58 (RDP58). Neutrophil activation levels were determined by MPO activity assay in dorsal skin (*A*) and ear (*B*) tissue homogenates of mice following TPA-induced contact dermatitis. Exposure to TPA resulted in a marked increase in cutaneous MPO levels in the dorsal skin as compared with acetone-vehicle alone (*A*). Treatment with 10 mg per mL RDP58 30 min after TPA application significantly decreased cutaneous MPO levels compared to vehicle treatment controls. Elevated levels of MPO activity were also observed in the ears of TPA-induced mice (*B*). RDP58 treatment reduced the level of MPO activity in a concentration-dependent manner and was comparable to a positive control steroid, 0.05% betamethasone, at the highest concentration used. A negative control peptide, which lacks anti-inflammatory activity, did not reduce TPA-induced MPO activity. Data are presented as mean ± SEM from at least 3 experiments consisting of three to five animals per group in each experiment. Values represent units of enzymatic activity in the dorsal skin and as percent change in MPO activity relative to naïve tissues for the ear homogenates. Statistical significance was determined using ANOVA with a *post hoc* Dunnett's multiple comparison test; $^{*}p < 0.05$.

Inhibition of pro-inflammatory mediators in keratinocytes by RDP58 Since the topical application of RDP58 on the skin comes into contact mainly with the epidermis, we also investigated the cytokine-inhibitory effect of the drug specifically on keratinocytes. Primary normal human epidermal keratinocytes (NHEK) were treated with one of three different stimuli thought to mediate specific aspects of cutaneous inflammation, including TNFα, IL-1β, or FBS, in the presence or absence of RDP58 (50 μM). The production of IL-1α and IL-6 in the supernatants was subsequently evaluated by ELISA. The production of IL-1α by the NHEK cells was elevated in all stimulation groups including the unstimulated controls. This reflects the constitutive expression of the IL-1α isoform; however, RDP58 was able to inhibit the production of IL-1α in these cells under all conditions by approximately 80%–90% (Fig 5*A*). The production of IL-6 by the NHEK cells was specifically increased upon stimulation. RDP58 was able to inhibit the induced IL-6 production mediated by each of the three different stimuli in NHEK cells (Fig 5*B*). This confirms that RDP58 is capable of mediating pro-inflammatory cytokine production stimulated by different signals specifically in the keratinocyte.

Since mechanism of action studies for RDP58 demonstrated an ability to reduce inflammation by targeting pathways that lead to the activation of the transcription factors AP-1 and NF-κB (Lazarov *et al*, 2003), we decided to further evaluate whether RDP58 treatment decreased the activity of these transcription factors in keratinocytes. NHEK cells were transiently transfected with luciferase-reporter constructs, stimulated with different inflammatory stimuli in the presence or absence of RDP58, and then assayed for luciferase activity, which served as an indicator of the direct activation of the respective transcription factor. The transcriptional activity of both NF-κB and AP-1 transcription factors was moderately induced with FBS and IL-1β stimulations (Fig 5*C*). The activation of the reporter constructs was much more robust with the TNFα stimulation, indicating a 3.1-fold induction of AP-1 activity and a 10.7-fold induc-

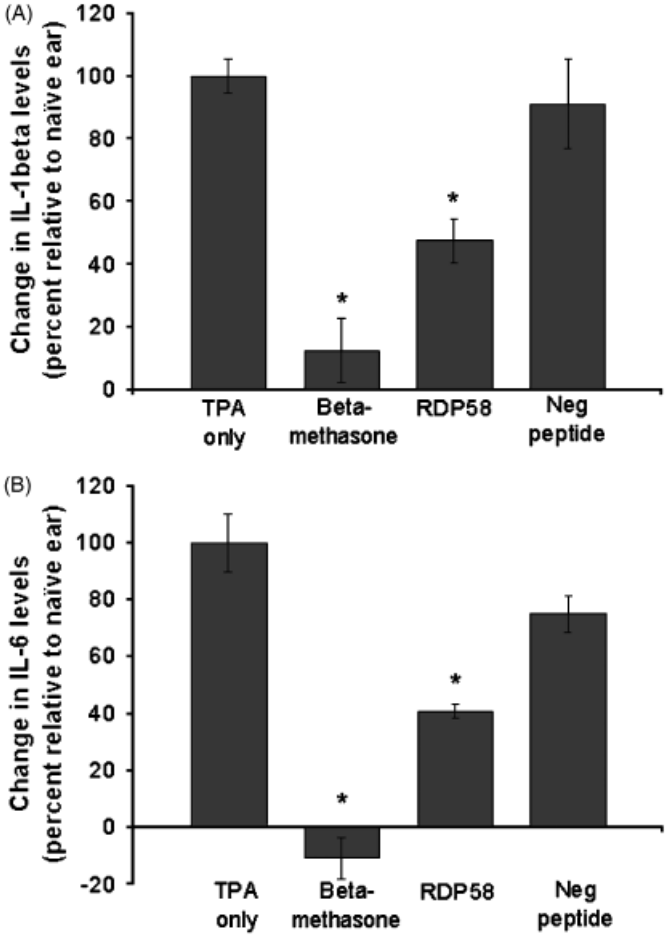

Figure 4
Inhibition of intrerleukin (IL)-1 and IL-6 production by rationally designed peptide 58 (RDP58) in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced irritant dermatitis. Ear punch biopsies were taken 18 h following the induction of TPA-induced contact dermatitis, and tissue homogenates were examined for cytokine production using ELISA. Cytokine levels were calculated as pg per mg total protein and then expressed as percent change relative to naïve ear homogenates. Exposure to TPA resulted in a marked increase in cutaneous levels of both IL-1 (*A*) and IL-6 (*B*). Treatment with 10 mg per mL RDP58 30 min after TPA application significantly decreased the production of these cytokines. The positive control steroid, 0.05% betamethasone, effectively reduced both IL-1 and IL6 to near-background levels and below that achieved for RDP58 at the 10 mg per mL concentration. A negative control peptide, which lacks anti-inflammatory activity, did not significantly reduce TPA-induced production of IL-1 and IL-6. Data are presented as mean $\pm$ SEM from to to three experiments consisting of three to five animals per group in each experiment. Statistical significance was determined using ANOVA with a *post hoc* Dunnett's multiple comparison test; $^{*}p < 0.05$.

tion of NF-κB activity. Remarkably, RDP58 was able to inhibit the transcriptional activity of both NF-κB and AP-1 induced after all stimulation conditions to a level well below the unstimulated background (Fig 5*C*). This is consistent with the findings in other leukocyte and epithelial cell types (Lazarov *et al*, 2003). The ability of RDP58 to inhibit the activation of NF-κB and AP-1, which are critical for the transcriptional regulation of a number of pro-inflammatory cytokines, in keratinocytes suggests that it may effectively modulate this important participant in cutaneous immune responses.

RDP58 effectively suppresses prolonged inflammation induced by repeated TPA application As a second *in vivo* measure of the anti-inflammatory activity of RDP58, the peptide was administered topically in three different dosing schedules in a mouse model of chronic skin inflammation induced by repeated exposure to phorbol ester. The skin inflammation in this model is persistent and has been useful in assessing whether topically applied compounds are able to resolve an existing inflammatory lesion (Stanley *et al*, 1991). A 25 mg per mL solution of RDP58 markedly reduced the edema, epidermal hyperplasia, and cell infiltration in this model (Fig 6). Staining with the CD3 cell surface marker revealed a major T cell component to the infiltrate population, which was also reduced by RDP58 and betamethasone treatments. Consistent with the histopathology, RDP58 reduced the level of MPO activity in the inflamed skin with reductions of 40%, 50%, and 70%, respectively, measured in the one-, three-, or five-treatment dosing schedules (Fig 7). These findings support the ability of RDP58 to resolve an existing, persistent inflammatory lesion with a single application and even more effectively with multiple topical applications to a level comparable with corticosteroid treatment.

Discussion

This study provides evidence that RDP58, when applied topically, has anti-inflammatory activity in the skin. In animal models of both acute and chronic irritant contact dermatitis, we showed that topical treatment with RDP58 markedly reduces cutaneous inflammation. This was supported by observed reductions in skin thickness and weight, amelioration of several histopathological indicators, and direct measurement of decreased pro-inflammatory cytokines and neutrophil activation. We also were able to demonstrate the ability of RDP58 to specifically act on keratinocytes, with reductions in cytokine expression and transcription factor activation, which supports the previously described mechanism of action.

By the nature of its mechanism of action, RDP58 has broad immunomodulatory activity and may prove beneficial for several different inflammatory disorders. The limited bioavailability of RDP58, however, makes it best suited for clinical indications that can be treated with topical therapeutics. Although the skin is an easily accessible tissue for topical administration, its barrier functions pose enormous challenges for most therapeutics. The biophysical properties relating to size and charge of the RDP58 peptide are not optimal for skin penetration. In this proof of concept study, we do not use formulations to facilitate delivery; however, the mouse skin barrier is relatively easy to penetrate and we may also be taking advantage of a disrupted lipid barrier caused by the acetone in the TPA applied prior to treatment. We are currently testing formulations with skin penetration-enhancing properties to optimize the stability and delivery of the RDP58 peptide.

It is widely recognized that the secretions of cytokines by keratinocytes in response to injury are key mediators of the cutaneous inflammatory response (Piguet, 1993; Murphy *et al*, 2000). In this study, we demonstrated that topical treatment with RDP58 inhibits the secretion of IL-1 and IL-6

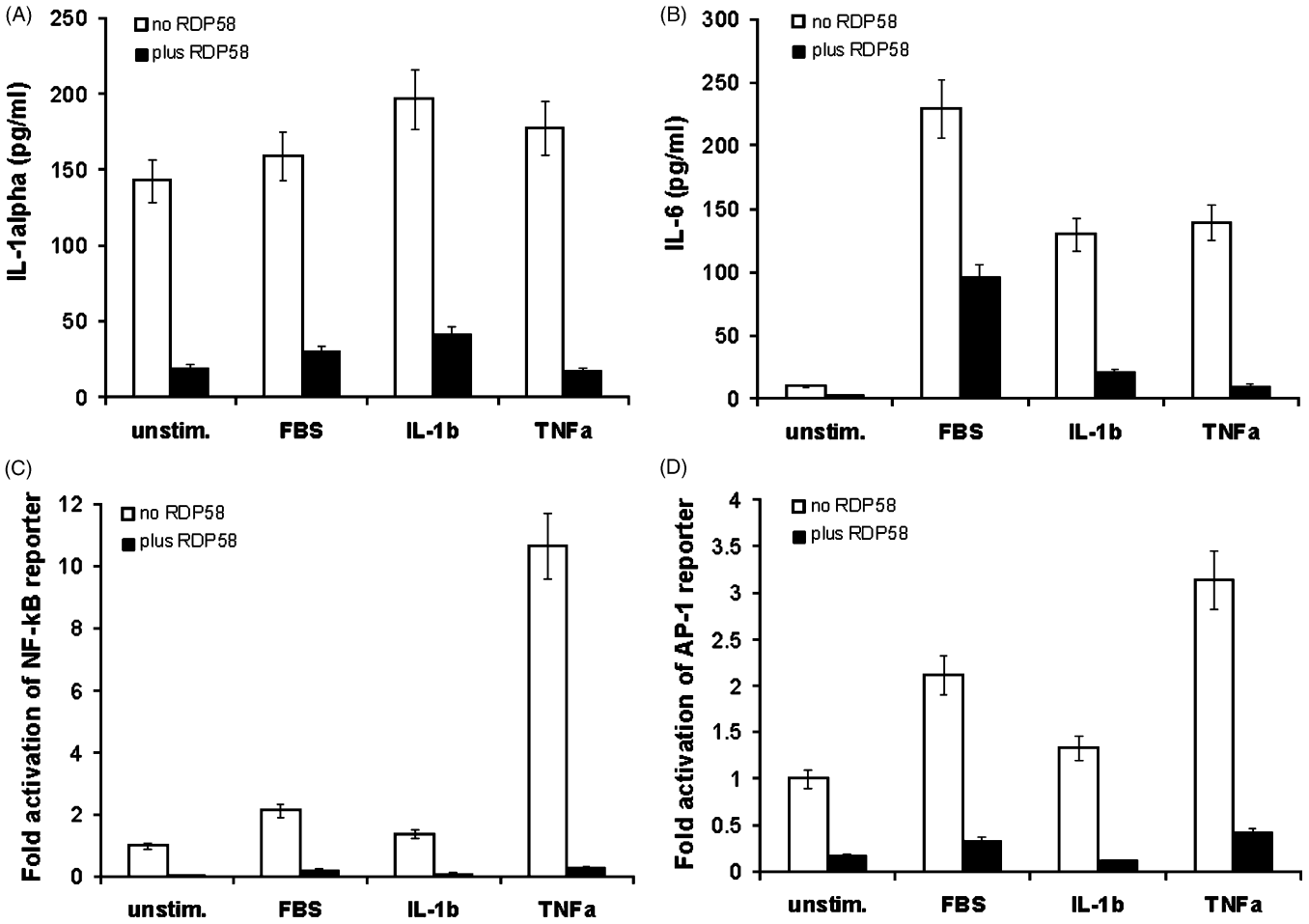

Figure 5
Inhibition of pro-inflammatory mediators in primary keratinocytes by rationally designed peptide 58 (RDP58). Primary normal human epidermal kerationocytes (NHEK) cells were stimulated with either fetal bovine serum, interleukin (IL)-1β, or tumor necrosis factor alpha in the presence or absence of 50 μM RDP58. The production of IL-1α (*A*) and IL-6 (*B*) in the supernatant was then evaluated by ELISA. IL-1α levels were markedly elevated under all stimulation conditions. The background concentration observed in unstimulated cells suggests a high constitutive expression level in the keratinocyte. RDP58 treatment substantially reduced levels of IL-1α produced in all stimulation groups (*A*). IL-6 was specifically induced by each stimulus, but in all cases, treatment with RDP58 reduced cytokine production (*B*). The effect of RDP58 on the transcriptional activity of nuclear factor-κB (NF-κB) (*C*) and AP-1 (*D*) was evaluated by transiently transfecting NHEK cells with luciferase-reporter constructs. The cells were then stimulated in the presence or absence of RDP58 and the luciferase activity was calculated as the fold activation relative to unstimulated and untreated cells. Although activation levels varied between stimuli, in all cases, RDP58 treatment was able to substantially reduce both NF-κB and AP-1 activation to levels below background.

in the acute irritant contact dermatitis model of inflammation. It is likely that this inhibition of cytokine secretion at least partially accounts the reduction in inflammation observed in these studies. RDP58 was also shown to reduce these cytokines in cultured keratinocytes. The transcription of cytokines, such as IL-1, IL-6, and TNFα, and many of the effectors of cytokine action, such as vascular cell adhesion molecule-1, and cyclo-oxygenase-2, are regulated by NF-κB (Baeuerle and Baltimore, 1996; Ghosh *et al*, 1998). RDP58 is known to interfere with NF-κB activation by disrupting upstream signaling events and demonstrated as much in this study with keratinocytes. In addition, other mechanisms could contribute to the anti-inflammatory properties of RDP58. Specifically, RDP58 negatively interferes with AP-1 transcription pathways and this transcription factor is known to have important roles in the inflammatory process (Karin *et al*, 1993). Furthermore, RDP58 induces heme oxygenase-1, which would reduce oxidative stress that would also be anti-inflammatory (Fuchs *et al*, 2001). Thus, there are a number of different mechanisms that could contribute to the anti-inflammatory response of RDP58 treatment.

The magnitude of the inhibition of cutaneous inflammation induced by treatment with RDP58 was similar to that observed after topical treatment with a potent corticosteroid, betamethasone. It is well recognized that, whereas steroids have potent anti-inflammatory effects, they can also induce systemic side-effects, including immune suppression that may limit their clinical usefulness (Ashwell *et al*, 2000). Due to the limited bioavailability of RDP58, it is likely that the anti-inflammatory effects will be more localized and less global than the effects of steroids, which could be beneficial in the long-term treatment of inflammatory conditions localized to the skin. Several specific cytokine and chemokine inhibitors are also currently in development as therapeutics for a number of inflammatory and autoimmune diseases. These therapies, however, focus mainly on the inhibition of one specific cytokine or chemokine. Conversely,

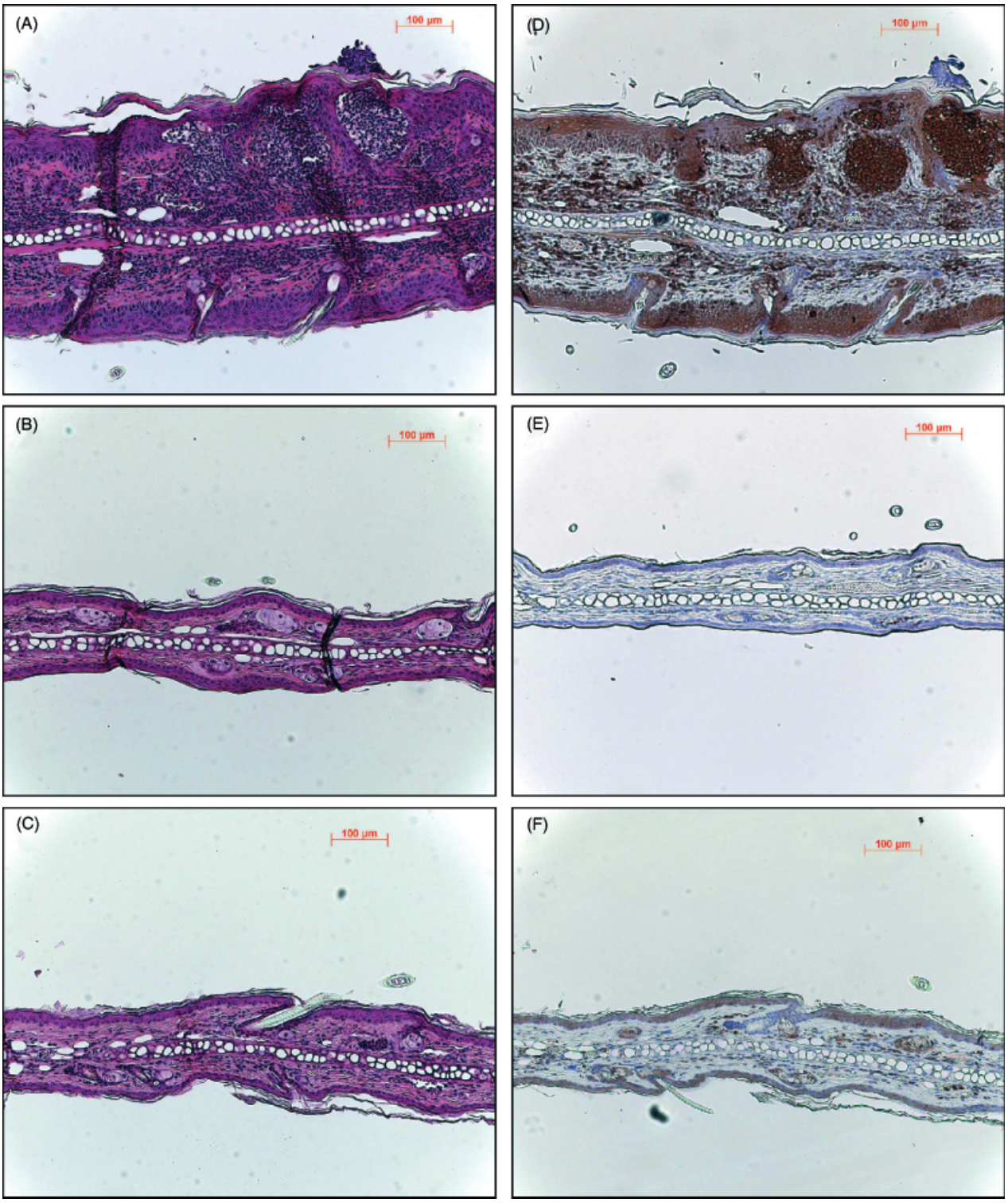

Figure 6
Rationally designed peptide 58 (RDP58) ameliorates the compounding inflammatory damage brought on by chronic 12-*O*-tetradecanoylphorbol-13-acetate (TPA) exposure. Female CD-1 mice were treated with 0.01% TPA in acetone applied to the inner and outer surfaces of the ear every other day for 5 d. RDP58 (25 mg per mL) or betamethasone (0.05%) was applied topically to the right ear after 30 min following each TPA application. Skin punch biopsies from control and treated ears of each mouse were harvested for histological analysis 18–20 h following the final TPA treatment. Hematoxylin- and eosin-stained sections of chronic TPA-induced contact dermatitis reveal marked increases in ear thickness accompanied by severe edema, epidermal hyperplasia, and inflammatory cell infiltration (*A*). RDP58 treatment (*C*) reduces ear thickness and the associated pathological indicators to an extent comparable with betamethasone (*B*). Immunohistochemical staining with the CD3 cell-surface marker indicated a substantial infiltration of T-cells after the 5 d of TPA application (*D*). With betamethasone (*E*) and RDP58 (*F*) treatment CD3 staining is reduced. Sections shown are representative of observations from four animals in each group.

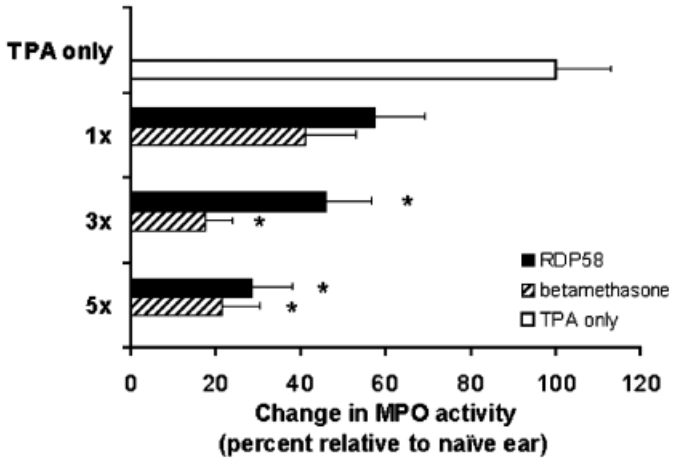

Figure 7
Rationally designed peptide 58 (RDP58) reduces myeloperoxidase (MPO) levels induced by chronic 12-*O*-tetradecanoylphorbol-13-acetate (TPA) exposure. Neutrophil activation levels were determined by MPO activity assay in ear tissue homogenates from mice following chronic TPA-induced contact dermatitis. Chronic exposure to TPA, every other day for 5 d, resulted in a marked increase in cutaneous MPO levels in the ear. The value for this group was set as a 100% change in MPO activity relative to naïve ear tissue. Treatment with RDP58 (25 mg per mL in water) or betamethasone (0.05%) was carried out according to one of the following schedules: (1) a single treatment following the fifth and final TPA application (1 $\times$); (2) three treatments beginning with the third TPA application (3 $\times$); or (3) following each of the five TPA applications (5 $\times$). All three treatment courses with RDP58 reduced cutaneous MPO levels induced by the chronic TPA exposure to an extent comparable to the positive control steroid, betamethasone. Data are presented as mean $\pm$ SEM from two experiments with three to five animals per group in each experiment. Values represented as percent change in MPO activity relative to naïve ear tissue. Statistical significance was determined using ANOVA with a *post hoc* Dunnett's multiple comparison test; $^{*}p<0.05$.

since so many cytokines are involved in these inflammatory and autoimmune diseases, drugs such as RDP58 that inhibit multiple cytokines pathways may prove to be more useful.

In summary, this study demonstrates that topical application of RDP58 has an anti-inflammatory action in both chronic and acute irritant contact dermatitis. Coupled with the previously described effects of RDP58 in the UVB-induced skin inflammation model makes it likely that this platform therapeutic will be useful in the treatment of a variety of cutaneous disorders.

Materials and Methods

Experimental animals and reagents Six-to 8-wk-old female FVB/N mice (Harlan, Hollister, California) and CD-1 mice (Charles Rivers, Wilmington, Massachusetts) were maintained in the animal facility at Sangstat Medical Corporation. Animals were housed under conventional conditions with laboratory chow and water accessible *ad libitum*, and maintained for at least 1 wk prior to inclusion in experiments. All animal procedures were conducted in compliance with the *NIH Guide for the Care and Use of Laboratory Animals* and approved by the Sangstat Medical Corporation IACUC. All chemicals were purchased from Sigma (St Louis, Missouri) unless otherwise noted. Peptides (RDP58: NH_2-r-nle-nle-nle-r-nle-nle-nle-g-y-$CONH_2$, negative control peptide: NH_2–nle–nle–nle–r–r–nle–nle–r–g–r–$CONH_2$) were synthesized using a solution-phase process, purified by HPLC and shown to be of equal or greater than $\geqslant$98% purity by analytical HPLC (UCB Bioproducts, Brussels, Belgium). Betamethasone dipropriate cream (USP, 0.05%, Fougera Melville, New York), a synthetic adrenocorticosteroid, was used as a positive treatment control.

Treatments and tissue preparation Acute irritant contact dermatitis was induced in groups of five female FVB/N mice by a single topical application of 10 μL of 0.03% (wt/vol in acetone) TPA on the inner and outer surfaces of both ears (Sheu *et al*, 2002). RDP58, dissolved in water at concentrations ranging from 2 to 25 mg per mL, was applied to the right ear at 30 min following inflammatory insult with TPA in one group of animals. In a separate set of animals, acetone alone was applied to both ears as a vehicle control. A total of 20 μL of test compound or vehicle was applied to each surface of the right and left ears, respectively. As additional controls, 0.05% betamethasone was used as a positive control and a similar peptide lacking anti-inflammatory activity was used as a negative control. At 18 h, when TPA-induced inflammation is maximal, animals were euthanized by CO_2 asphyxiation and ear thickness was measured with a digital caliper (VWR, West Chester, Pennsylvania). The difference in thickness between treated right ear and control TPA left ear of each mouse was calculated as a percent decrease, and then normalized to the difference relative to naïve ear thickness. This was immediately followed by 5 mm ear punch biopsies being taken for weight determinations, biochemical analysis, and histology. Alternatively, TPA treatment was conducted by painting 5 μg of TPA in 200 μL acetone on the dorsal skin of mice. Briefly, the back of each mouse was shaved 2 d before each experiment, and RDP58 was applied topically to the shaved area of the dorsal skin 30 min after application of the TPA/acetone solution. Skin thickness was measured before TPA application and 18 h after using digital calipers on a fold of skin in the center of the shaved area. The change in dorsal skin thickness was calculated from the difference between these timepoints. A total of six 5mm biopsy punches were taken from an approximately 2 cm $\times$ 2 cm shaved treatment area for biochemical analyses and histology. The tissue weight was calculated from the sum of four of these biopsy punches prior to freezing.

A chronic persistent skin inflammation was induced in the ears of CD-1 mice (five per group) by the repeated treatment of phorbol ester using the procedure of Stanley *et al* (1991). TPA (10 μL, 0.01% in acetone) was applied to ears on days 0, 2, 4, 6, and 8. RDP58 in water was applied 30 min after TPA according to one of the following treatment schedules: (1) a single treatment following the fifth and final TPA application, (2) three treatments beginning with the third TPA application, or (3) following each of the five TPA applications. 18 h after the last application, ear punch biopsies were removed and either snap-frozen for later analysis of myeloperoxidase content or fixed in 10% buffered formalin (Fisher Scientific, Hampton, New Hampshire) for histological evaluation.

Histology For histological assessment of cutaneous inflammation, biopsies from control and treated ears of mice in each treatment group were collected and fixed in 10% neutral-buffered formalin (Fisher Scientific). The preparation of tissues by embedding in paraffin, sectioning, and staining was carried out by QualTek Molecular Laboratories (Santa Barbara, California). A series of ear cross sections were prepared for the evaluation of general cutaneous inflammation parameters using H&E stain. The identification of T cell-specific infiltration in the chronic inflammation studies utilized immunohistochemistry with the CD3 cell-surface marker. Subsequent analysis of stained tissue sections was carried out in a blinded fashion by trained investigators. All sections were examined using standard bright-field optics (Zeiss, Axioskop 40 Zeiss, Thornwood, New York).

MPO activity assay MPO activity can be used as a quantitative measure of the extent of infiltration of polymorphonuclear neutrophils into the inflamed tissue (Bradley *et al*, 1982). Following treatment, as described above, 5 mm skin punches collected from either the dorsal skin or ear of each mouse were homogenized in 1 mL of T-PER protein extraction reagent (Pierce, Rockford, Illinois)

containing a protease inhibitor cocktail (Sigma) using the FastPrep System (Qbiogene, Carlsbad, California). Homogenates were then centrifuged at 16,000 × *g* for 30 min, and MPO activity was measured by adding 100 μL of supernatant to 200 μL of an assay reaction mixture containing 0.5% hexadecyltrimethylammonium bromide (in 50 mM potassium phosphate, pH 6.4), 0.165 mg per mL *o*-dianisidine dihydrochloride, and 0.0015 % H_2O_2. After a 30-min incubation period at room temperature, absorbance at 450 nm was measured. Absolute MPO activity was determined based on the generation of a standard curve.

Cytokine expression analysis Cytokine protein levels for IL-1β and IL-6 were determined in the ear biopsy homogenates, prepared as described above for the MPO assay, using ELISA kits following the manufacturer's instructions (BioSource, Camarillo, California). Absorbances were measured at 450 nm with a Microplate Reader (Molecular Devices, Sunnyvale, California). Total protein was determined using the BCA assay (Pierce). Cytokine content was calculated as pg per mg total protein, and then expressed as percent change relative to naïve tissue.

Keratinocytes Primary NHEK (Clonetics, San Diego, California) were cultured in keratinocyte cell medium (Clonetics) supplemented with KGM2 Bullet Kit (Clonetics). Cell density was not allowed to exceed 70%. RDP58 was dissolved to make a 1 mg per mL stock solution in double-processed tissue culture grade water (Sigma). The RDP58 stock solution was stored at −20°C until use. The primary NHEK cells were plated at 60% confluency and treated with the following stimuli: 50 ng per mL TNFα (Peprotech, Rocky Hill, New Jersey), 50 ng per mL IL-1β (Peprotech), or 5% FBS (ATCC, Manassas, Virginia), in the presence or absence of RDP58 (50 μM).

Cell culture supernatants were assayed by ELISA for human IL-1α and IL-6 using the corresponding DuoSet kits (R&D Systems, Minneapolis, Minnesota) according to the manufacturer's instructions. The absorbance was measure at 450nm with a Microplate Reader (Molecular Devices). For transient transfection assays using luciferase reporter constructs, NHEK cells were transfected in six-well plates at 50% confluency with a total of 4 μg DNA per well using Fugene6 (Roche, Indianapolis, Indiana) according to the manufacturer's instructions. The luciferase reporter plasmids pNF-κB-Luc and pAP1-Luc were obtained from Stratagene (La Jolla, California). 20–24 h after transfection, cells were subcultured and stimulated in black 96-well plates (ViewPlate, Perkin-Elmer, Boston, Massachusetts). Cell culture supernatants were harvested 20–24 h after stimulation, and the luciferase activity in the cell lysates was measured using Steady-Glo Luciferase Assay (Promega, Madison, Wisconsin) according to the manufacturer's instructions. Luminescence was read on a Victor2 Wallac luminometer (Perkin-Elmer).

Statistical analysis Data are presented as mean values ± SEM. When comparisons were made between two groups statistical significances were determined using Student's unpaired *t* test. When comparisons were made between multiple groups, statistical significances were determined using ANOVA, with *post hoc* comparisons by Dunnett's Multiple Comparison Test. A p value less than 0.05 was considered significant. All statistical analyses were performed using GraphPad Prism version 3.03 for Windows (GraphPad Software, San Diego, California).

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