

Cyclosporin A inhibits 12-O-tetradecanoyl-phorbol-13-acetate-induced cutaneous inflammation in severe combined immunodeficient mice that lack functional lymphocytes

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Summary

A single application of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to mouse skin results in an acute inflammatory response, with an influx of neutrophils and lymphocytes, epidermal hyperplasia and abnormal keratinocyte differentiation. This response is significantly inhibited by topical cyclosporin A (CyA). Although CyA is known to inhibit T-cell activation, the role of T cells in TPA-induced cutaneous inflammation is not well understood. In this study, we have used severe combined immunodeficient (SCID) mice, which carry a spontaneous mutation resulting in the absence of functional T and B lymphocytes, to examine whether lymphocytes are required for the TPA response in mouse skin and whether CyA inhibits the TPA response in SCID mice. A significant increase in epidermal and deep dermal inflammation was observed in both SCID and CB-17 mice 24 h after a single application of TPA (10 nmol) compared with vehicle ($P < 0.05$, $n = 5–7$). Simultaneous application of CyA (1.7 μmol) plus TPA resulted in a significant reduction in epidermal and deep inflammation at 24 h compared with TPA alone in SCID and CB-17 mice ($P < 0.05$, $n = 7$). In contrast to hairless mice, a variable increase in epidermal thickness was observed in both SCID and CB-17 mice after treatment with TPA at 24 and 72 h, which was not significantly affected by CyA. These data indicate that TPA-induced inflammation in mouse skin does not depend on lymphocytes. In addition, the inhibition of TPA-induced epidermal and deep dermal inflammation by CyA in SCID mouse skin suggests that CyA exerts effects on cutaneous inflammation in mice in the absence of functioning T cells.

Systemic cyclosporin A (CyA) is an effective treatment for psoriasis^{1,2} as well as other inflammatory dermatoses.^{3,4} CyA blocks the activation and proliferation of T cells through inhibition of T cell receptor-mediated interleukin 2 (IL-2) induction.⁵ CyA binds to intracellular receptors, cyclophilin members of the immunophilin family. The drug-immunophilin complex binds to and inhibits the Ca^{2+} and calmodulin-dependent phosphatase calcineurin.⁶ This leads to an increase in the phosphorylation state of calcineurin substrates such as the cytoplasmic subunit of nuclear factor of activated T cells (NF-AT). NF-AT is a transcription factor essential for IL-2 gene transcription and, in its phosphorylated state, cannot undergo translocation to the nucleus.⁷ Thus, by inhibiting calcineurin, CyA blocks IL-2 production, which is required for T-cell activation.

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The evidence indicates that CyA exerts effects in skin that are independent of its action on T cells. CyA induces hair growth both *in vivo* and *in vitro*,^{8,9} indicating a direct epidermal effect. CyA inhibits the proliferation of keratinocytes and fibroblasts in culture¹⁰ at concentrations (1–10 $\mu\text{g}/\text{mL}$) that have been found in psoriatic plaques after systemic administration of CyA. However, CyA inhibits keratinocyte growth only under serum-free conditions, possibly related to higher cell-associated concentrations of lipophilic CyA. Thus, whether CyA exerts an effect on keratinocyte proliferation *in vivo* remains controversial. CyA also inhibits antigen presentation by murine and human Langerhans cells¹¹ and inhibits neutrophil chemotaxis in psoriatic patients.¹² Thus, although CyA exerts profound effects on lymphocytes, its precise mechanism of action in skin and in psoriatic plaques, in particular, is not completely understood.

The application of a single dose of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to mouse skin results in

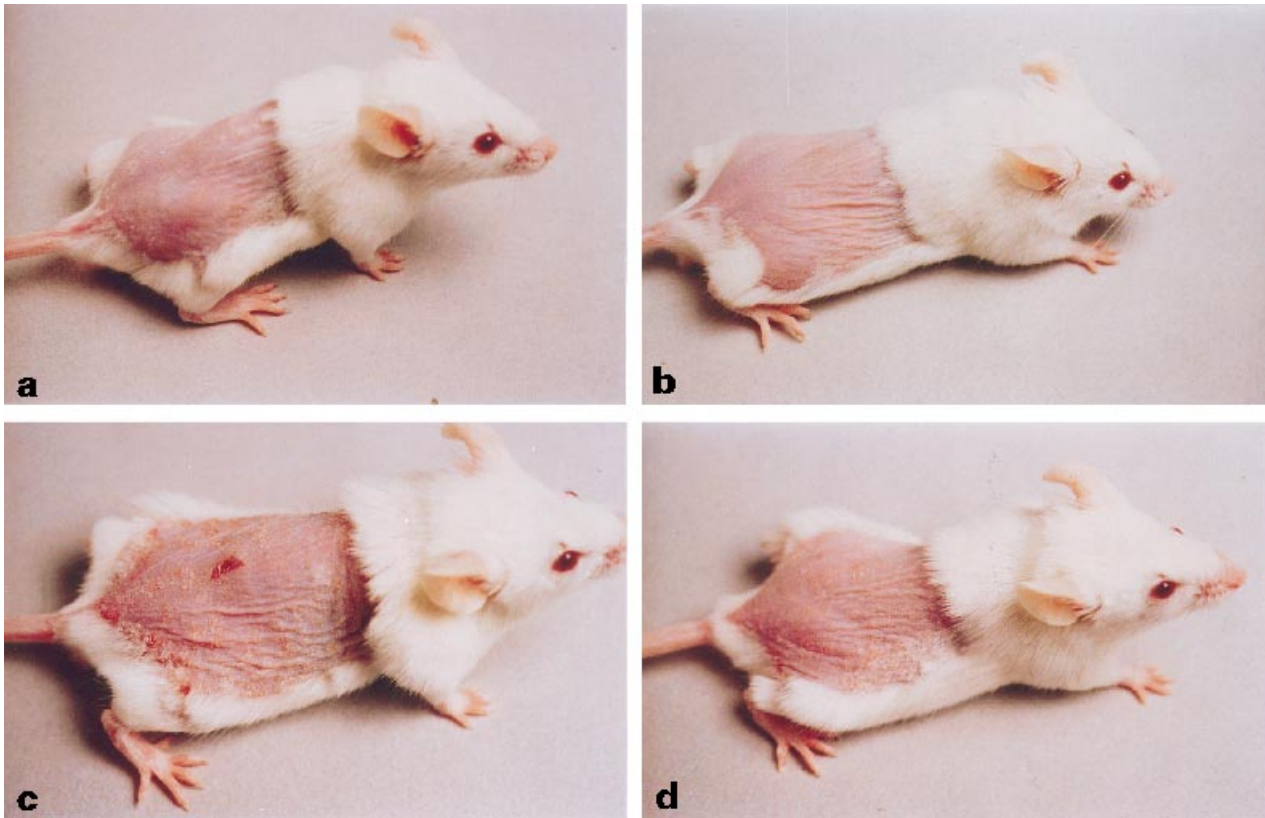


Figure 1. 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) induces redness and scaling in severe combined immunodeficient and CB-17 mouse skin that is inhibited by topical cyclosporin A (CyA). SCID mice (A and B) and CB-17 control mice (C and D) 72 h after the application of TPA (10 nmol) (A,C) and TPA (10 nmol) plus CyA (1.7 mmol) (B,D).

a wide range of responses, including epidermal hyperplasia, induction of ornithine decarboxylase (a marker of cell growth), induction of epidermal transglutaminase (a marker of terminal differentiation), alteration of keratin expression, increased release of arachidonic acid and prostaglandin (PG) E_2 , increased IL-1 α and IL-1 β and leucocyte infiltration.¹³⁻¹⁹ In this study, we have used severe combined immunodeficient (SCID) mice, which lack functional T and B lymphocytes but have normal monocytes, macrophages, neutrophils, megakaryocytes and erythrocytes,²⁰ to examine directly whether functional T and B lymphocytes are necessary for the phorbol ester response in mouse skin. It was also determined whether CyA inhibits the TPA response in SCID mice, in the absence of functioning T and B lymphocytes.

Materials and methods

Mice

Homozygous CB-17/lcrTac-SCID/DF (SCID) mice and

CB-17 control mice were purchased from Taconic (Germantown, NY, U.S.A.). Mice were derived from pathogen-free breeder stocks and were maintained in a pathogen-free animal facility at the University of Michigan in rooms vented with filtered air. Mice were housed in microisolator cages containing sterilized food and water. Investigators wore gowns, masks and sterile gloves when handling the mice.

Treatment of mice and assessment of skin histology

Experimental protocols were approved by the University of Michigan Committee on Use and Care of Animals. Mice were used for experiments at between 6 and 12 weeks of age. The backs of the mice were shaved with electric clippers and treated with a depilatory cream (Nair; Carter Products, New York, NY, U.S.A.). Only mice that displayed no evidence of hair regrowth after 3 days (i.e. hair was in the resting stage of the cycle) were used for experimentation. A single dose of TPA (Sigma Chemical Co., St Louis, MO, U.S.A.) or TPA

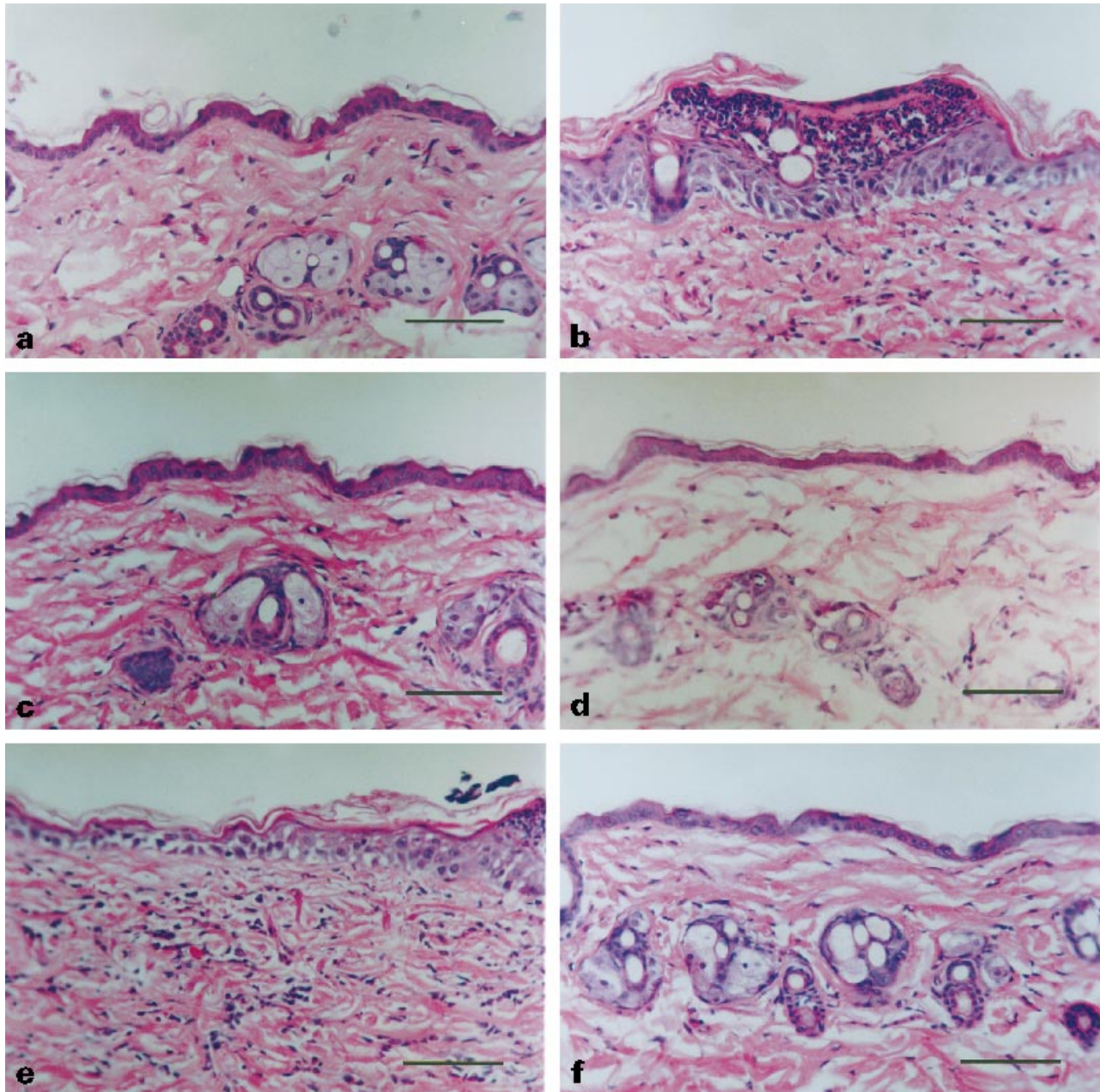


Figure 2. Topical cyclosporin A (CyA) inhibits 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced acute epidermal and dermal inflammation in severe combined immunodeficient (SCID) and CB-17 mice. SCID mice (A–C) and CB-17 control mouse skin (D–F) were treated with vehicle (A,D), TPA (10 nmol) (B,E) or TPA (10 nmol) plus CyA (1.7 mmol) (C,F), and biopsies were performed 24 h later. Scale bar = 50 μ m.

plus CyA (a kind gift from Sandoz Pharmaceutical, Basle, Switzerland) dissolved in 200 μ L of acetone or vehicle was applied using a micropipette. Mice were assessed 24 or 72 h after treatment for erythema and scaling, and 4 mm punch biopsies were taken from the treated skin. Histological assessments were made by an investigator who was unaware of the treatment

conditions. The degree of epidermal, upper and deep dermal inflammation was assessed using an ordinal semiquantitative five-point scale in half-unit increments, in which 0 indicated the absence of inflammation and 4 indicated the maximum degree of inflammation, as described previously.²¹ Epidermal thickness, measured as the distance from the bottom of the stratum corneum to

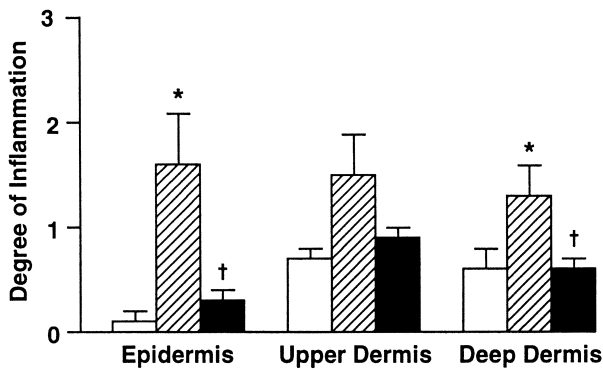


Figure 3. Inhibition of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced epidermal and deep dermal inflammation by cyclosporin A (CyA) in severe combined immunodeficient mice. Dorsal mouse skin was treated with TPA, TPA plus CyA or vehicle, and biopsies were performed 24 h later. The degree of epidermal, upper dermal and deep dermal inflammation was assessed on a semiquantitative scale, as described in Materials and methods. □, vehicle ($n = 5$); hatched bar, TPA, 10 nmol ($n = 7$); ■, TPA, 10 nmol + CyA, 1.7 mmol ($n = 7$). Data points represent mean \pm SEM. * $P < 0.05$ vs. vehicle; † $P < 0.05$ vs. TPA alone.

the basement membrane in the interfollicular epidermis, was determined directly from the mean of five measurements using a calibrated micrometre scale, under light microscopy at $\times 100$ magnification.

Statistical analysis

For measurements of epidermal thickness, epidermal, upper dermal and deep dermal inflammation, comparisons

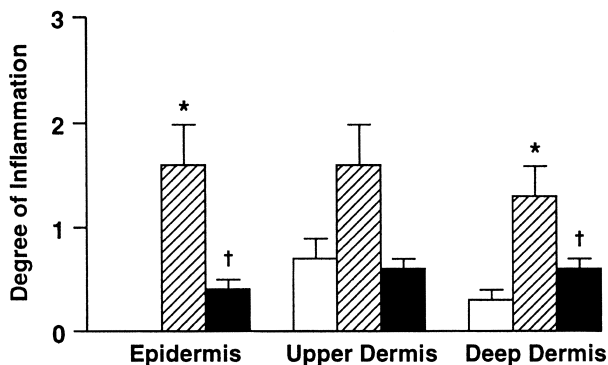


Figure 4. Inhibition of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced epidermal and deep dermal inflammation by cyclosporin A (CyA) in CB-17 mice. Dorsal mouse skin was treated with TPA, TPA plus CyA or vehicle, and biopsies were performed 24 h later. The degree of epidermal, upper dermal and deep dermal inflammation was assessed on a semiquantitative scale, as described in Materials and methods. □, vehicle ($n = 5$); hatched bar, TPA, 10 nmol ($n = 7$); ■, TPA, 10 nmol + CyA, 1.7 mmol ($n = 7$). Data points represent mean \pm SEM. * $P < 0.05$ vs. vehicle; † $P < 0.05$ vs. TPA alone.

among treatment groups were performed with one-way ANOVA and Fisher's least significant difference test.

Results

Topical treatment with TPA caused redness and scaling to similar extents in SCID and CB-17 control mouse skin at 72 h (Fig. 1). The degree of redness and scaling in both SCID and CB-17 mice was typically less in mice treated with TPA (10 nmol) plus CyA (1.7 μ mol) compared with TPA (10 nmol) alone (Fig. 1).

TPA (10 nmol) treatment induced an influx of inflammatory cells into the epidermis and dermis as well as spongiosis and dermal oedema 24 h after application in both SCID and CB-17 control mice (Fig. 2B,E). The inflammatory cells consisted predominantly of neutrophils, which formed microabscesses (Fig. 2B), and the inflammatory reaction appeared to be similar in SCID and CB-17 mice (Fig. 2B,E). Analysis of semiquantitative histological scores showed that the treatment of mouse skin with TPA resulted in a significant increase in epidermal and deep dermal inflammation in both SCID and CB-17 mice 24 h after application ($P < 0.05$, $n = 5-7$; Figs 3 and 4). CyA (1.7 mmol), applied simultaneously with TPA (10 nmol), almost completely abrogated the TPA-induced influx of inflammatory cells into the epidermis and dermis and the TPA-induced spongiosis and dermal oedema 24 h after application in both SCID and CB-17 mice (Fig. 2C,F). Analysis of the semiquantitative histological scores showed that CyA resulted in significant reductions in epidermal and deep dermal inflammation, compared with the effects of TPA alone, at 24 h in SCID and CB-17 mice ($P < 0.05$, $n = 7$, Figs 3 and 4).

By 72 h after treatment, TPA-induced epidermal, upper dermal and deep dermal inflammation had subsided back towards control values (semiquantitative scores: 0.8 ± 0.1 , 1.0 ± 0.1 and 0.8 ± 0.1 , $n = 8$, respectively, for SCID mice; and 0.7 ± 0.2 , 1.3 ± 0.3 and 0.8 ± 0.1 , $n = 8$, respectively, for CB-17 mice). Simultaneous application of CyA and TPA resulted in no significant effect on epidermal and dermal inflammation in SCID and CB-17 mice compared with TPA alone at 72 h.

A single application of TPA (10 nmol) resulted in a variable increase in epidermal thickness in SCID and CB-17 mice at 24 and 72 h (Fig. 2 and Table 1). Overall, TPA induced a significant increase in epidermal thickness, compared with vehicle, only in SCID mouse skin at 24 h (Table 1); no significant increase in epidermal thickness in response to TPA was observed in CB-17 mice at 24 or 72 h, compared with vehicle (Fig. 2 and Table 1). Epidermal thickness was not significantly

Treatment	Epidermal thickness ($\mu\text{mol/L}$)			
	24 h		72 h	
	CB-17 mice	SCID mice	CB-17 mice	SCID mice
Vehicle	18.5 \pm 1.7 ^a	14.1 \pm 0.6 ^a	ND	ND
TPA (10 nmol)	24.0 \pm 0.8 ^b	22.7 \pm 2.3 ^{b*}	26.7 \pm 3.2 ^c	25.6 \pm 2.0 ^{c**}
TPA (10 nmol) plus CyA (1.7 μmol)	21.3 \pm 2.1 ^b	22.3 \pm 1.8 ^b	29.0 \pm 4.2 ^c	24.4 \pm 4.4 ^c

Compounds were applied to the dorsal skin of mice as indicated, and epidermal thickness was assessed in skin biopsies taken 24 h or 72 h later, as described in Materials and methods. Data points represent mean \pm SEM for ^a $n=5$ mice/group, ^b $n=7$ mice/group, ^c $n=8$ mice/group. * $P<0.05$ compared with vehicle. ** $P<0.05$ compared with vehicle at 24 h ($F_{0.067}$).

affected by simultaneous application of CyA (Fig. 2 and Table 1).

Discussion

TPA induction of epidermal, upper dermal and deep dermal inflammation to an equivalent extent in SCID and CB-17 control mice indicates that these effects are not dependent on lymphocytes. The reduction in TPA-induced epidermal and deep dermal inflammation at 24 h by CyA in SCID mice also indicates that CyA can exert effects on inflammatory responses in mouse skin in the absence of functioning T cells. These results are consistent with the previously observed ability of topical CyA to inhibit TPA-induced epidermal hyperplasia, leucocyte infiltration and keratinocyte-derived transglutaminase and ornithine decarboxylase activity in mouse skin.^{22,23}

The mechanisms of CyA inhibition of TPA-induced inflammation in SCID mouse skin remain to be elucidated. Even though TPA activates protein kinase C (PKC) in mouse skin, CyA does not block TPA-induced PKC activation or down-regulation.²³ One possible mechanism is that CyA binds to cyclophilin A within keratinocytes²⁴ and modulates PKC signal transduction downstream of PKC activation, resulting in the inhibition of the TPA-induced release of arachidonic acid, PGE₂, IL-1 α and IL-1 β .²³ Previous studies have indicated the importance of PGE₂ and IL-1 α in the cutaneous response to TPA in mouse skin.^{16,19,25} The inhibition of TPA-induced, keratinocyte-derived, transglutaminase activity and ornithine decarboxylase activity by CyA²³ is also consistent with CyA exerting a direct effect on epidermal keratinocytes. Alternatively, CyA may modulate the recruitment of inflammatory

cells into the skin by, for example, inhibiting the ability of inflammatory cells, including neutrophils, to respond to the epidermal inflammatory signals induced by TPA or by inhibiting the induction of endothelial adhesion molecules.

Overall, epidermal hyperplasia in response to 10 nmol of TPA appeared to be less marked in SCID and CB-17 control mice than in hairless mice.²³ The reason for these differences is unknown, but different strains of mice vary in their response to TPA.²⁶ Also, variation in the follicular response to hair removal may be relevant to the TPA response.²⁷ Nevertheless, epidermal hyperplasia 72 h after the application of TPA was observed in SCID mouse skin in certain experiments, which suggests that lymphocytes may not be required for this process. In contrast to its effects in hairless mouse skin,²³ CyA did not significantly reduce TPA-induced epidermal hyperplasia in CB-17 control mice. This finding may have resulted from the relatively small induction of epidermal hyperplasia by TPA observed in CB-17 control mice, compared with hairless skin.

The data presented above are consistent with the concept that some of the therapeutic effects of CyA in inflammatory skin disease are mediated through T-cell-independent mechanisms. Thus, although the inhibition of T-cell activation by CyA is likely to be an important therapeutic mechanism of action in psoriasis, CyA may also exert direct effects on keratinocytes. Although this hypothesis requires further investigation, CyA is known to inhibit the release of inflammatory mediators from mast cells,²⁸ and it is possible that CyA inhibits the release of keratinocyte-derived inflammatory mediators, such as arachidonic acid, prostaglandins, leukotrienes and

Table 1 Epidermal thickness after a single treatment of dorsal SCID and CB-17 control mouse skin with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and TPA plus cyclosporin A

cytokines, which are known to be increased in psoriatic plaques.^{29–31} Consistent with this hypothesis, Kojima *et al.*³² observed a marked reduction in GRO- α mRNA levels within psoriatic plaques in response to CyA before detectable clinical improvement. Topical CyA is ineffective in psoriasis, probably because of inadequate absorption, as intralesional CyA induces local clearance.³³ However, preliminary studies with FK506 suggest that topical therapy is effective in atopic eczema.³⁴ Taken together, these studies suggest that keratinocyte immunophilins may be playing a part in the therapeutic action of CyA and FK506 in inflammatory skin disease.

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