# Reciprocal Cytokine-Mediated Cellular Interactions in Mouse Epidermis: Promotion of γδ T-Cell Growth by IL-7 and TNFα and Inhibition of Keratinocyte Growth by γIFN

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A unique subset of γδ T cells, termed dendritic epidermal T cells (DETC), resides in symbiosis with keratinocytes in mouse epidermis. We have shown previously that interleukin 7 (IL-7) which is produced by keratinocytes, promotes growth and prevents apoptosis in DETC. To extend this observation, we examined 12 cytokines, each of which is expressed by epidermal cells at mRNA and/or protein levels, for their capacities to modulate the growth of DETC. Cytokines examined included IL-1α, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-10, tumor necrosis factor-α (TNFα), interferon-γ (IFNγ), granulocyte/macrophage-colony stimulating factor (GM-CSF), and macrophage inflammatory protein-1α (MIP-1α). When tested individually, IL-2 and IL-7 promoted maximal growth of the long-term cultured DETC line 7-17. When tested in combinations, synergistic growth-promoting effects were seen with IL-2 and IL-4 or IL-7, and with IL-7 and IL-4 or TNFα. Dose-response experiments demonstrated that TNFα, which is produced by keratinocytes, enhances IL-7–induced DETC proliferation, but inhibits IL-2–induced proliferation. The mouse keratinocyte-derived cell line Pam 212 was used to test these cytokines for their capacities to regulate keratinocyte growth. Only γIFN, which is produced by DETC, inhibited proliferation in a dose-dependent fashion. These results illustrate three reciprocal pathways by which epidermal cytokines regulate the growth of epidermal cells: 1) a paracrine mechanism by which keratinocyte-derived cytokines (e.g., IL-7 and TNFα) promote the growth of DETC, 2) an autocrine mechanism by which DETC-derived cytokines (e.g., IL-2 and IL-4) support their own growth, and 3) a reciprocal pathway in which a cytokine produced by resident epidermal leukocytes (e.g., γIFN) modulates the growth of keratinocytes. Key words: epidermal γδ T cell/keratinocyte/growth/cytokine. *J Invest Dermatol 101:543–548, 1993*

The concept of immune regulation by cytokines produced in the epidermis can be dated to the discovery that keratinocytes elaborate interleukin-1 (IL-1) [1–3]. Keratinocytes, which possess overwhelming numerical superiority in mammalian skin, are now known to produce a spectrum of cytokines, including IL-1α, IL-1β, IL-3, IL-6, IL-8, granulocyte/macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor α (TNFα) (reviewed in [4–6]). Recently, IL-7 and IL-10 have been added to this list [7,8]. In mice, keratinocytes live in symbiosis with two resident leukocyte populations: Langerhans cells and dendritic epidermal T cells (DETCs). We and others have shown that Langerhans cells, which are antigen-presenting cells of dendritic cell lineage (reviewed in [9]), are capable of expressing mRNA for and/or secreting at least three cytokines, IL-1β, IL-6, and macrophage inflammatory protein-1α(MIP-1α) [10–12]. DETCs, which are $CD3^+$, $CD4^-$, $CD8^-$, γδ T-cell receptor $(TCR)^+$, are one member of a family of γδ T cells that reside preferentially in epithelial tissues (reviewed in [13,14]). DETCs, also a source of cytokines, are known to secrete IL-2, IL-3, IL-4, and interferon-γ(IFNγ) [15–17], and recently we have observed that they also express mRNA for IL-1α, IL-6, IL-7, TNFα, TNFβ, GM-CSF, and MIP-1α.*

An important issue, then, is the role these cytokines play in regulating their own epidermal microenvironment. In fact, several keratinocyte-derived cytokines, i.e., IL-1α, GM-CSF, and TNFα, have been shown to promote the growth and/or the maturation of Langerhans cells [18–21]. Likewise, we have shown that keratinocyte-derived IL-7 serves as a growth factor for DETCs.† Moreover, IL-7 prevents apoptotic cell death that would ordinarily be induced in DETCs when exposed to exogenous corticosteroids.‡ Thus, keratinocytes support the growth and survival of these two resident epidermal leukocytes through the elaboration of relevant cytokines and growth factors. In the present study, this line of investigation was extended by examining 12 cytokines, all of which are likely to be present under normal or pathologic circumstances in mouse epi-

Manuscript received March 9, 1993; accepted for publication June 3, 1993.

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Abbreviations: DETC, dendritic epidermal T cell; DEX, dexamethasone; γIFN, interferon-γ; MIP-1α, macrophage inflammatory protein-1α.

* Matsue H, Cruz PD Jr, Bergstresser PR, Takashima A: Profiles of cytokine mRNA expressed by dendritic epidermal T cells in mice. *J Invest Dermatol 101:537–542, 1993 (this issue)*.

† Matsue H, Bergstresser PR, Takashima A: Keratinocyte-derived IL-7 serves as a growth factor for dendritic epidermal T cells (manuscript submitted).

‡ Matsue H, McDowall A, Bergstresser PR, Takashima A: Keratinocyte-derived IL-7 rescues dendritic epidermal T cells from apoptosis (manuscript submitted).

0022-202X/93/$06.00 

**Table I.** Cytokine mRNA Expression by Mouse Epidermal Cell Subpopulations

| Cytokine | EC[a] | KC Line[b] | Thy-1$^+$EC[c] | DETC Line[d] | Ia$^+$EC[e] |
|---|---|---|---|---|---|
| IL-1$\alpha$ | + | + | + | + | |
| IL-1$\beta$ | + | | | − | + |
| IL-2 | − | − | + | + | |
| IL-3 | + | + | | + | |
| IL-4 | ±[f] | − | − | + | |
| IL-5 | + | + | | | |
| IL-6 | + | + | | + | |
| IL-7 | + | + | | + | |
| TNF$\alpha$ | + | + | | + | |
| TNF$\beta$ | + | + | | + | |
| IFN$\gamma$ | ± | − | + | + | |
| GM-CSF | + | + | | + | + |
| MIP-1$\alpha$ | + | | | + | + |
| IL-8 [53] | | | | | |
| IL-10 [8] | | | | | |

[a] Epidermal sheet preparation or epidermal cell suspension.
[b] Pam 212 keratinocyte line.
[c] FACS-purified Thy-1$^+$ epidermal cells (DETC).
[d] Short or long-term DETC lines.
[e] FACS-purified Ia$^+$ epidermal cells (Langerhans cells).
[f] Faint signal.

dermis, for their potential to modulate the growth of both DETCs and keratinocytes.

## MATERIALS AND METHODS

**Cells** The long-term cultured DETC line 7-17 was used for these experiments. This line was first established from AKR mice by culturing fluorescence-activated cell sorter (FACS)-purified DETCs with 2$\mu$g/ml Con A and 5 U/ml IL-2 [22]. 7-17 DETCs have been maintained by repeated restimulation with 1 $\mu$g/ml Con A (every 1-3 weeks) and by feeding with human rIL-2 (R&D Systems, Minneapolis, MN). The culture medium has been RPMI 1640, supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 1% nonessential amino acids, 10 $\mu$M sodium pyruvate, 20 $\mu$M L-glutamine, 1% penicillin/streptomycin, and 50 $\mu$M 2-mercaptoethanol (complete RPMI) [15,23]. At the time of use, cells were harvested by a 3-min incubation with 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM HEPES in Hanks' balanced salt solution. 7-17 DETCs have retained the phenotype of DETCs *in situ*. They are Thy-1$^+$, CD3$^+$, $\alpha\beta$ TCR$^-$, $\gamma\delta$ TCR$^+$, and V$\gamma$3/V$\delta$1$^+$, as judged by FACS analyses using the following monoclonal antibodies (MoAb): anti-Thy-1.1 (Becton Dickinson, Mountainview, CA), anti-CD3$\epsilon$ (kindly provided by Dr. J. Allison, University of California, Berkeley, CA), anti-$\alpha\beta$ TCR (Pharmingen, San Diego, CA), anti-$\gamma\delta$ TCR (Pharmingen), anti-V$\gamma$3 (Pharmingen), and 17D1, an antibody that recognizes the V$\gamma$3/V$\delta$1 TCR (kindly provided by Dr. R. E. Tigelaar, Yale University). 7-17 DETCs have been used extensively by several investigators, and their other characteristics are described elsewhere [16,24,25].

BALB/c mouse keratinocyte-derived Pam 212 cells [26] were maintained as described previously [27]. Briefly, cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with the same additives used to supplement RPMI1640 (complete DMEM).

**Cytokines** Mouse rIL-1$\alpha$, rIL-3, rIL-4, rIL-8, rTNF$\alpha$, rGM-CSF, and rINF$\gamma$, and human rIL-8 were purchased from Genzyme (Cambridge, MA); mouse rIL-6, rIL-7, and rMIP-1$\alpha$, and human rIL-2 were from R&D Systems; and mouse rIL-10 were from Bachem Bioscience, (Philadelphia, PA).

**Proliferation Assays** Cell proliferation was determined as described previously [15,23,27]. Briefly, 7-17 DETCs were pre-activated with Con A (1 $\mu$g/ml) for 18 h, washed extensively, and then cultured for 1-3 d in the absence of added IL-2. These cells were then harvested with EDTA, plated ($3 \times 10^4$ cells/well) in 96 round-bottom well-plates (Corning, Corning, NY), and cultured for up to 5 d in the presence of each cytokine or cytokine combination. Cells were pulsed with 1 $\mu$Ci/well of $^3$H-thymidine (New England Nuclear, Boston, MA) during the last 16 h, and harvested using an automated cell harvester (PHD, Cambridge, MA). Pam 212 keratinocytes isolated from semi-confluent cultures were plated in 96 flat-bottom well-plates, cultured, and pulsed as above. These adherent cells required a brief treatment with trypsin prior to harvesting, as described previously [27]. All the data were analyzed for statistical significance by t test for independent samples using Kwikstat 3.3 (TexaSoft).

**Dexamethasone Treatment of DETCs** DETC apoptosis was induced with dexamethasone (DEX) as described previously.‡ Briefly, 7-17 DETCs ($1 \times 10^5$ cells/well) in a resting state (activated with Con A 9-14 d previously) were cultured in complete RPMI in 24-well plates, in the presence or absence of DEX ($1 \times 10^{-7}$ M). After 20 h, cells were harvested with EDTA, and cell viability was assessed by trypan blue exclusion; at least 400 cells were counted per sample.

## RESULTS

**IL-2 and IL-7 are Major Growth Factors for DETCs** As described in the *Introduction*, each of the epidermal cell subpopulations (i.e., keratinocytes, Langerhans cells, and DETCs) elaborates a distinct profile of cytokines. Results from our reverse-transcription-polymerase chain reaction analyses are summarized in Table I, which lists cytokine mRNAs expressed by freshly isolated epidermal cells, Pam 212 keratinocytes, FACS-purified DETCs, short- and long-term DETC lines, and FACS-purified Langerhans cells. The term "epidermal cytokines" will be used to identify all of these cytokines as a group (IL-1$\alpha$, IL-1$\beta$, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-10, IFN$\gamma$, TNF$\alpha$, GM-CSF, and MIP-1$\alpha$).

In the first set of experiments, each epidermal cytokine was tested individually for its capacity to modulate DETC proliferation. Representative results from three independent experiments are shown

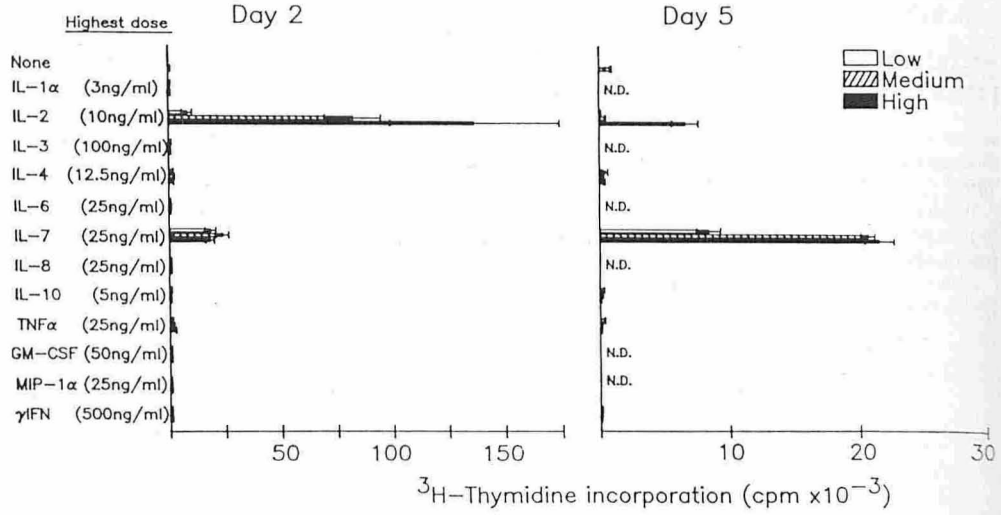


**Figure 1.** Promotion of DETC proliferation by epidermal cytokines. 7-17 DETCs were stimulated with Con A and tested 2 d later for proliferative responsiveness to selected cytokines, at three different concentrations: high (shown in *parentheses*), medium (five times dilution), and low (25 times dilution). Data shown are the means ± SEM (n = 3) for $^3$H-thymidine incorporation harvested on day 2 *(left)* or day 5 *(right)*.

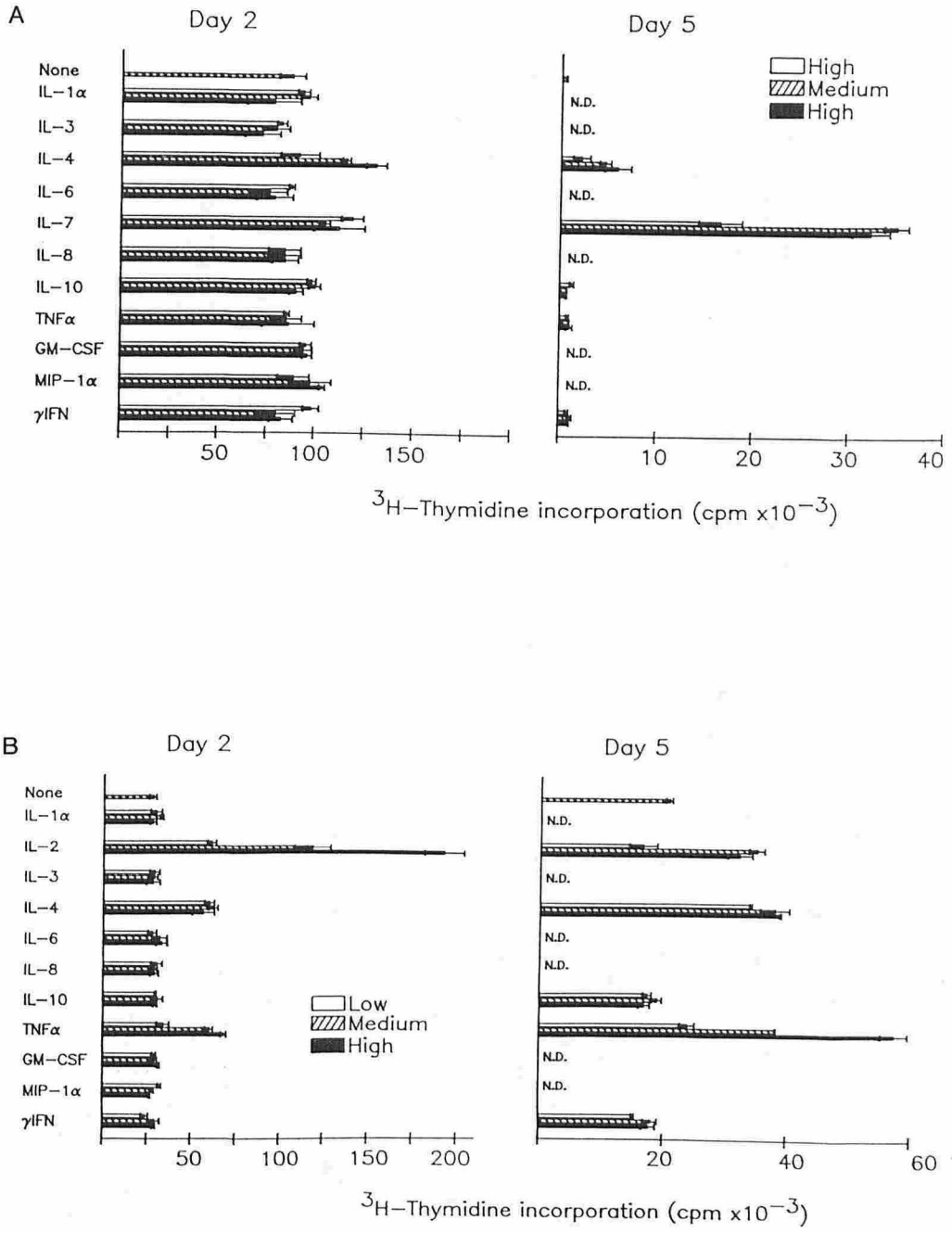


**Figure 2.** Synergistic effects of epidermal cytokines on DETC proliferation. 7-17 DETCs stimulated with Con A 2 d previously were cultured in the presence of rIL-2 (2 ng/ml) *(A)* or rIL-7 (5 ng/ml) *(B)*. Other cytokines were also added at three different concentrations (see Fig 1) to these cultures. Data shown are the means ± SEM (n = 3) for $^3$H-thymidine incorporation harvested on day 2 *(left)* or day 5 *(right)*.

in Fig 1. To begin, 7-17 DETCs showed minimal $^3$H-thymidine incorporation on days 2 and 5, in the absence of added growth factors (Fig 1). This baseline proliferation was clearly upregulated by added IL-2, with significant activity detected even at the lowest concentration (0.4 ng/ml) and increasing progressively, in a dose-dependent fashion. IL-7 (1–25 ng/ml) also exhibited marked growth-promoting activity. Importantly, IL-2 and IL-7 promoted DETC growth with different kinetics. IL-2–induced proliferation occurred rapidly, with substantial $^3$H-thymidine incorporation on day 2 and less by day 5. By contrast, IL-7 responses were modest on day 2 but remained at that level even on day 5 (Fig 1). Both IL-4 and TNFα showed modest, but statistically significant ($p < 0.01$), activities on day 2: 900 ± 50 cpm (baseline), 2400 ± 200 cpm (12.5 ng/ml IL-4), and 2800 ± 40 cpm (25 ng/ml TNFα). All other cytokines failed to promote or to inhibit DETC proliferation under the conditions employed for these experiments.

**TNFα Augments IL-7–Induced DETC Proliferation** In the next set of experiments, epidermal cytokines were tested in several combinations. In two independent experiments, both IL-4 and IL-7 consistently augmented IL-2–induced proliferation of DETCs on day 5 (Fig 2*A*). These effects were synergistic rather than additive ($p < 0.05$); the magnitudes of proliferation were 510 ± 130 cpm (IL-2), 370 ± 60 cpm (IL-4), 21,000 ± 500 cpm (IL-7) for individual cytokines, and 5,900 ± 1,400 cpm (IL-2 plus IL-4), and 35,000 ± 1,300 cpm (IL-2 plus IL-7) in combinations.

When tested in combination with IL-7, three cytokines (IL-2, IL-4, and TNFα) also showed a synergistic augmentation ($p < 0.01$) of proliferative responses on both day 2 and day 5 (Fig 2*B*). For example, the magnitudes of proliferation on day 5 were 20,000 ± 1,600 cpm (IL-7), 460 ± 50 cpm (IL-2), 370 ± 60 cpm (IL-4), 240 ± 40 cpm (TNFα) for the individual cytokines, and 32,000 ± 2,000 cpm (IL-7 plus IL-2), 39,000 ± 120 cpm (IL-7 plus IL-4), and

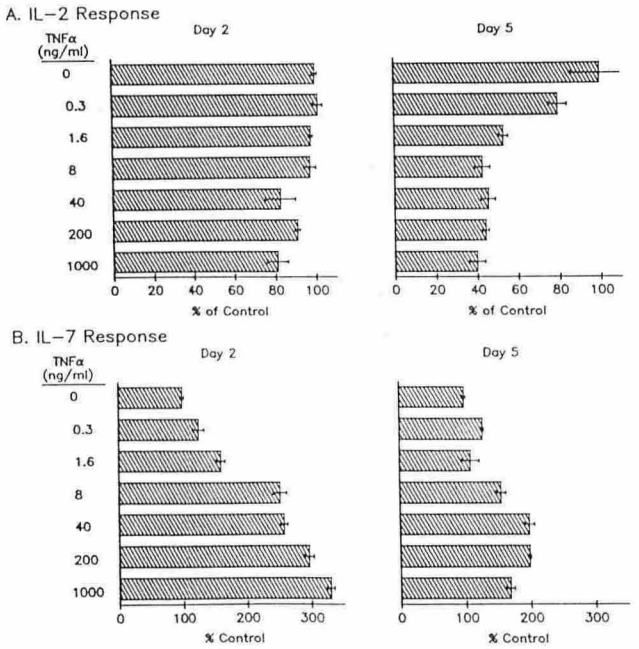


**Figure 3.** TNFα amplifies IL-7-induced DETC proliferation. 7-17 DETCs stimulated with Con A 3 d previously were cultured in the presence of rIL-2 (2 ng/ml) *(A)* or rIL-7 (5 ng/ml) *(B)*. rTNFα was also added to these cultures at the indicated concentrations. Data shown are the means ± SEM (n = 3) for the percent of control (in the absence of TNFα) on day 2 *(left)* or day 5 *(right)*.

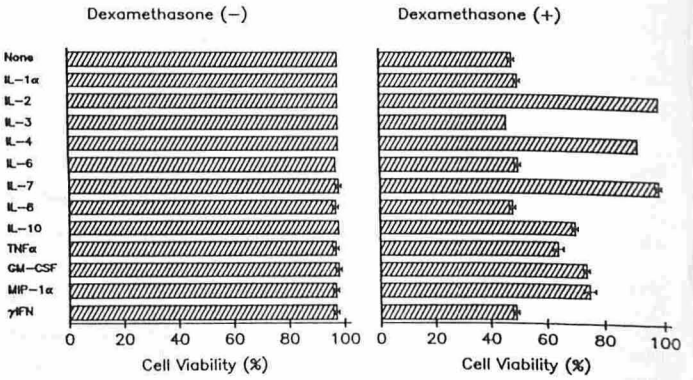


**Figure 4.** Effects of epidermal cytokines on DETC survival. 7-17 DETCs stimulated with Con A 10 d previously were cultured for 20 h in 96-well plates ($1 \times 10^5$ cells/well) in the presence *(right)* or absence *(left)* of DEX ($1 \times 10^{-7}$ M). Cytokines were tested at the "medium" concentrations listed in Fig 1. Data shown are the means ± SEM (n = 2) of cell viabilities as determined by trypan blue exclusion.

58,000 ± 2,000 (IL-7 plus TNFα) in combinations. These results illustrate both autocrine and paracrine mechanisms by which DETC proliferation is promoted by DETC-derived cytokines (IL-2 and IL-4) and by keratinocyte-derived cytokines (IL-7 and TNFα), respectively.

To study the regulatory effect of TNFα more closely, its dose response was examined in combination with IL-2 or IL-7. Surprisingly, TNFα was found to inhibit IL-2–induced DETC proliferation (Fig 3*A*). The IL-2–induced response on day 5 (8,400 ± 1,200 cpm) was inhibited 60% with TNFα, with the effective TNFα concentration in the range between 1.6 and 8 ng/ml. By marked contrast, TNFα augmented IL-7–induced DETC proliferation (Fig 3*B*), an effect that was most prominent on day 2. The IL-7 response (6,600 ± 1,200 cpm) was amplified up to threefold by added TNFα, with this activity occurring at concentrations between 8 and 200 ng/ml. Importantly, proliferative responses to TNFα alone were negligible (< 1000 cpm at each data point). Thus, we conclude that TNFα augments IL-7–induced DETC proliferation but inhibits IL-2–induced proliferation.

**IL-2, IL-4, and IL-7 Prevent Dexamethasone-Induced DETC Death** We have observed previously that 7-17 DETCs undergo apoptosis in response to *in vitro* treatment with DEX.‡ Cellular changes have included 1) reduced cell viability (trypan blue exclusion), 2) DNA fragmentation (DNA laddering in electrophoresis), 3) a requirement for macromolecule biosynthesis (inhibition by cycloheximide or actinomycin D), and 4) chromatin condensation and loss of microvilli (electron microscopy), all of which typify cells that are undergoing apoptosis (reviewed in [28]). When IL-7 was added simultaneously with the DEX, apoptosis did not occur.‡ It was, therefore, of particular interest to determine whether DETC apoptosis can be induced or prevented by other epidermal cytokines. First, none of the cytokines induced significant cell death in 7-17 DETCs in the absence of DEX in any of the three independent experiments (Fig 4, *left*). When the same cytokines were tested for their ability to prevent DEX-induced cell death, three cytokines, IL-2, IL-4, and IL-7, consistently showed significant activities ($p < 0.05$); cell viabilities were 98% (no DEX), 47% (DEX alone), 98% (DEX plus IL-2), 91% (DEX plus IL-4), and 98% (DEX plus IL-7) (Fig 4, *right panel*). Relatively modest effects were also observed, but only occasionally, with several other cytokines (i.e., IL-10, TNFα, GM-CSF, and MIP-1α).

**γIFN Inhibits the Growth of Pam 212 Keratinocytes** Having confirmed the biologic activities of epidermal cytokines on DETC proliferation and apoptosis, additional experiments were conducted to determine whether these same cytokines would modulate the function of other epidermal cells. In the absence of stable lines of Langerhans cells, and recognizing the comprehensive studies of Steinman and his colleagues [19–21,29], we chose to limit this examination to the effects of these epidermal cytokines on keratinocyte growth. Pam 212 keratinocytes were tested for their proliferative responses to added cytokines with two protocols: 1) relatively large numbers of cells, with harvesting on day 2 (Fig 5*A*), and 2) fewer cells, with harvesting on day 5 (Fig 5*B*). In both protocols, $^3$H-thymidine incorporation was measured with cells in the exponential growth phase, as indicated with arrows in the left panel of Fig 5. Pam 212 cell growth was clearly inhibited by added IFNγ, under both protocols ($p < 0.05$ at 20 ng/ml, and $p < 0.01$ at 100 ng/ml IFNγ). Maximal activity (40–60% inhibition) was achieved at a IFNγ concentration of 100 ng/ml. All other cytokines failed to promote or to inhibit the growth of this keratinocyte line in two independent experiments.

## DISCUSSION

The reported experiments tested 12 different epidermal cytokines for their potential to modulate the growth of DETCs and keratinocytes. The most striking observations were that IL-7 and TNFα, both of which are produced by keratinocytes [7,30], promote the growth of DETCs, in a synergistic fashion. IL-7, which was first characterized as a bone marrow stroma cell–derived pro- and pre-B-cell growth factor [31,32], has also been demonstrated to serve as a growth factor for early lymphoid cells of both B- and T-cell lineages (reviewed in [33]). In addition to mature T cells, IL-7 also promotes the proliferation of thymocytes [34–36] and in our earlier report, we observed that IL-7 also promotes the growth of DETC.† Recently, it has become evident that TNFα has the capacity to promote the growth of early thymocytes, especially when com-

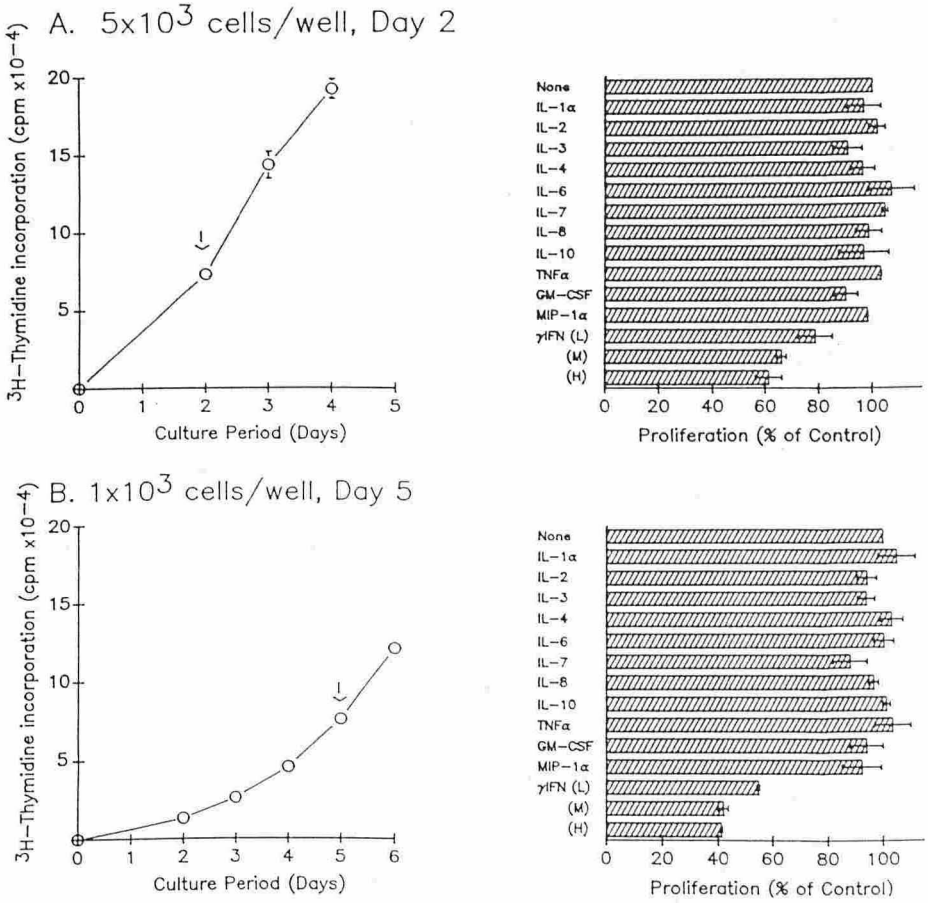


**Figure 5.** Effects of epidermal cytokines on Pam 212 keratinocyte proliferation. Pam 212 keratinocytes were plated in 96 flat-bottom-well plates at $5 \times 10^3$ cells/well *(A)* or $1 \times 10^3$ cells/well *(B)*. Cytokines were tested at "medium" concentrations listed in Fig 1, except that IFN$\gamma$ was added at three different concentrations: 500 ng/ml (H), 100 ng/ml (M), and 20 ng/ml (L). Data shown are the kinetics of proliferation (mean ± SEM, n = 3) in the absence of cytokines *(left)* and the $^3$H-thymidine incorporation (mean ± SEM, n = 3) in the presence of cytokines on day 2 *(A)* or day 5 *(B)*.

bined with other cytokines (e.g., IL-2 and IL-7) [37–39]. Thus, our results suggest that DETCs, which are also derived from thymic precursors and express the identical TCR (i.e., V$\gamma$3/V$\delta$1) to that found on day 16 thymocytes [40,41], resemble early thymocytes in their growth-factor requirements as well. Thus, these findings illustrate paracrine mechanisms by which cytokines (e.g., IL-7 and TNF$\alpha$) produced by neighboring keratinocytes promote the growth of epidermal resident $\gamma\delta$ T cells.

IL-2 also induced marked proliferation of 7-17 DETCs, corroborating our previous observations with DETCs freshly procured from skin [23]. On the other hand, IL-4 synergized with IL-2 or IL-7 to enhance DETC proliferation substantially. We and others have shown that DETCs are capable of producing these two cytokines [15,17], and our recent reverse transcription–polymerase chain reaction analyses demonstrated that mRNA for IL-2 and IL-4 are expressed exclusively by DETCs among the epidermal cell subpopulations.* Thus, these pathways through IL-2 and IL-4 most likely represent relevant autocrine mechanisms.

Our observation that IL-1$\alpha$ and IL-10 failed to modulate DETC growth was unexpected, because these two cytokines have also been considered to regulate T-cell growth in other systems. IL-1 primarily serves as a co-stimulatory factor in T-cell activation processes, by upregulating mitogen/antigen-induced IL-2 production and IL-2 receptor expression [42,43]. Therefore, our assays, in which proliferative responses of pre-activated DETCs were measured, may not be ideal to detect possible effects of IL-1. IL-10, which is also produced by keratinocytes [8], has been shown to modulate thymocyte/mature T-cell proliferation (reviewed in [44]). Again, our assays may not be suitable for detecting IL-10 effects, because this cytokine appears to act primarily on antigen-presenting cells by downregulating their function to activate T cells [45] or to affect cytokine production by a particular T-cell subset [46]. Because DETC proliferation in these assays is independent of antigen-presenting cells, it will be necessary to examine the possible roles played by IL-1 and IL-10 in assay systems in which DETC growth may rely on antigen-presenting cells.

We also sought to determine proliferative responses of keratinocytes to epidermal cytokines. Among the cytokines tested, IL-1, IL-6, and TNF$\alpha$ have been reported to upregulate keratinocyte growth: 1) IL-1 has been implicated as being responsible for epidermal hyperplasia in inflammatory skin disorders [47]; 2) IL-6, which also promotes the growth of human keratinocytes, is overexpressed in psoriatic skin, again suggesting its role in epidermal hyperplasia [48]; and 3) TNF$\alpha$ promotes the growth of human papillomavirus-infected keratinocytes in an autocrine mechanism [49]. In other systems, however, the growth of human keratinocytes was not affected by added IL-1 or IL-6 [50]. Our inability to detect a growth-promoting potential for these three cytokines most likely reflects the fact that each of these factors is produced endogenously (at least at mRNA levels) by the Pam 212 keratinocytes used in our study, leading to the possibility that their effects were already operative (Table I) [1,2]. This assumption is supported by the unambiguous effect of IFN$\gamma$, which is produced by DETCs but not by Pam 212 cells [16].* In fact, this pronounced inhibition of cell growth, attributable to IFN$\gamma$, has been observed in other systems [51,52]. Thus, these observations illustrate at least one pathway by which cytokines produced by a resident epidermal leukocyte regulate the growth of keratinocytes.

In summary, we have demonstrated that epidermal cell subpopulations in mouse skin may affect each other through the elaboration of relevant cytokines. Three distinct mechanisms were illustrated: 1) a paracrine mechanism in which keratinocyte-derived cytokines (e.g., IL-7 and TNF$\alpha$) promoted the growth of DETCs; 2) an autocrine mechanism in which DETC-derived cytokines (e.g., IL-2

and IL-4) supported their own growth; and 3) a reciprocal paracrine mechanism in which a cytokine produced by a resident leukocyte modulated the growth of keratinocytes.

*We thank Dale Edelbaum for his technical assistance and Mrs. Betty Janes for her superb secretarial assistance.*

*This work was supported by NIH grants R01-AR35068, R01-AR40042 and R01-AR41150, by a grant from the Dermatology Foundation, and by a research grant from L. E. Simon Co., Ltd. (Tokyo).*

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