Interleukins 18 and 12 Synergistically Upregulate Interferon-γ Production by Murine Dendritic Epidermal T Cells

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A unique subset of γδ T cells, termed dendritic epidermal T cells, reside in murine epidermis. It was previously reported that freshly isolated dendritic epidermal T cells and dendritic epidermal T cell lines expressed mRNA for interferon-γ. Recent studies indicated that interleukin-18, a novel cytokine which strongly induces interferon-γ production by T cells, was produced by murine keratinocytes and Langerhans cells. Interleukin-12, which is regarded as a key cytokine for Th1 type helper clone responses, has also been reported to be produced by these cells in murine skin. In this study, we demonstrated by enzyme-linked immunosorbent assay that interleukin-18 and interleukin-12 synergistically upregulated interferon-γ production by dendritic epidermal T cells in short-term cultures. This was the case in both C57/BL6 mice and BALB/C mice, although the quantity of interferon-γ produced was different in the two mouse strains. Interleukin-18 or interleukin-12 alone did not induce interferon-γ production by dendritic epidermal T cells. Interferon-γ mRNA was only weakly detected by the semiquantitative reverse transcriptase–polymerase chain reaction method in freshly isolated dendritic epidermal T cells, and the mRNA expression was much increased 12 h after stimulation with interleukin-18 and interleukin-12. We also confirmed biologic activity of interferon-γ produced by dendritic epidermal T cells by showing upregulation of major histocompatibility complex class II expression on Pam 212, murine keratinocyte cell line. Thus, this study suggests that interleukin-18 and interleukin-12 produced by keratinocytes and Langerhans cells regulate interferon-γ production by dendritic epidermal T cells and thus may play important parts in the regulation of immune responses in skin-associated lymphoid tissues.
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Adult murine epidermis contains a population of dendritic leukocytes termed dendritic epidermal T cells (DETC) (Bergstresser *et al*, 1983; Tschachler *et al*, 1983; Stingl *et al*, 1987). Critical attributes of DETC include their highly restricted T cell receptor gene utilization (Asarnow *et al*, 1988; Havran *et al*, 1989), a capacity to kill relevant skin-derived tumor targets (Kaminski *et al*, 1993a), and the ability to modulate immune responses in skin. With regard to cytokine production, freshly isolated DETC and cultured DETC cell lines have been shown to synthesize mRNA of interleukin (IL)-2, IL-1α, IL-3, IL-7, tumor necrosis factor-α and -β, granulocyte macrophage-colony stimulating factor (GM-CSF), and interferon-γ (IFN-γ) (Matsue *et al*, 1993).

IL-18, originally called IFN-γ-inducing factor, is a novel cytokine synthesized by Kupfer cells and activated macrophages (Okamura *et al* 1995). IL-18 has been found to have a variety of biologic actions, including stimulation of the proliferation of activated T cells (Okamura *et al*, 1995), enhancement of the lytic activity of T cells and natural killer cells (Okamura *et al*, 1995; Tsutsui *et al*, 1996; Dao *et al*, 1996), induction of IFN-γ, and of GM-CSF production by activated T cells (Okamura *et al*, 1995; Micallef *et al*, 1996) and promotion of Th1 type helper clone responses. Recently, murine keratinocytes and various dendritic cells, including Langerhans cells, have been shown to synthesize this cytokine (Stoll *et al*, 1997; de Saint-Vis *et al*, 1998).

IL-12 is regarded as a key cytokine for Th1 type helper clone responses, and is synthesized by activated macrophages, keratinocytes, and Langerhans cells (Lamont and Adorini, 1996). In this study, we sought to determine whether or not IL-18 and IL-12 could stimulate DETC to produce IFN-γ.

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Abbreviation: DETC, dendritic epidermal T cells.

MATERIALS AND METHODS

Animals and reagents C57BL/6 and BALB/C female mice were obtained from Japan SLC (Hamamatsu, Japan) and were maintained under specific-pathogen-free conditions in our animal facilities. Mice at the age of 8–12 wk were used in this study. Recombinant mouse IL-18 and IL-12 were purchased from Pepro Tech EC Ltd. (London, U.K.) and were diluted and stored according to manufacturer's guidelines. The following monoclonal antibodies were used: goat anti-hamster IgG (Cappel, Aurora, OH), anti-mouse CD3ε chain (clone 145-2C11), fluorescein isothiocyanate (FITC) anti-mouse Thy-1.2 (clone 30-H12), biotin-anti-mouse CD45 (clone 30-F11), FITC-anti-mouse T cell receptor αβ (clone H57–597), FITC-anti-mouse T cell receptor Vγ3 (clone 536), FITC-anti-I-A^d (clone AMS-32.1) and streptavidin-FITC (PharMingen, San Diego, CA). FITC-hamster IgG (Cedarlane Lab., Hornby, Canada), FITC-rat IgG2b (clone R35-38), FITC-mouse IgG2b (clone 49.2), and biotin-rat IgG2b (clone R35–38, PharMingen) were used as isotype controls.

Cell preparation Murine DETC were purified using the panning method as previously described (Koyama *et al*, 1990) with slight modifications, and the purification of DETC calculated from the first several

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experiments was over 95%. Briefly, murine truncal skin was treated with dispase (3000 U per ml, Godo Shusei, Tokyo, Japan) for 8 h for C57/BL6 mice and for 3 h for BALB/C mice at 37°C. Epidermis was separated from dermis and incubated with 0.025% DNase (Sigma, St Louis, MO) for 20 min at room temperature. Epidermal cell suspension was obtained by gently mixing and pipetting epidermal sheets. The epidermal cell suspension was then treated with anti-mouse CD3ε monoclonal antibody (1 : 50) for 1 h on ice. The cells were incubated in plates which were already coated with goat anti-hamster IgG (1:200) for 1 h at 4°C. After washing out floating cells, adherent cells were collected and used as freshly isolated DETC. Pam 212, murine keratinocyte cell line, were kindly provided by Nishioka K (Department of Dermatology, Tokyo Medical and Dental University School of Medicine, Japan).

Cell culture Freshly isolated DETC (2×10^5 cells per well) were incubated in 96-well flat-bottom plates (Becton Dickinson Labware, Franklin Lakes, NJ) in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics.

Flow cytometry The surface phenotype of freshly isolated DETC was identified using various monoclonal antibodies on a FACScan flow cytometer (Becton Dickinson). Briefly, freshly isolated DETC were incubated with antibodies (1:100) for 30 min at 4°C, and then washed with phosphate-buffered saline containing 1% fetal bovine serum three times. Second antibodies (1:100) were added as necessary and DETC were incubated for 30 min at 4°C, and then washed three times. Samples were analyzed using FACScalibur. Propidium iodide (Becton Dickinson, San Jose, CA) was added in order to exclude dead cells.

Measurement of IFN-γ concentration IFN-γ concentrations in culture media of DETC were measured by enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN). The minimum detectable dose of mouse IFN-γ was 2 pg per ml in this assay.

Analysis of expression of IFN-γ mRNA in DETC Cytoplasmic mRNA was isolated from 1×10^6 freshly purified and cultured DETC using QuickPrep Micro mRNA Purification Kits (Pharmacia Biotech, Uppsala, Sweden). cDNA was synthesized from mRNA using First-Strand cDNA Synthesis Kits (Pharmacia Biotech). The oligonucleotide primers that specifically amplify murine IFN-γ and G3PDH transcripts were obtained from Clontech (Palo Alto, CA). Two microliter aliquots of the cDNA reaction products were polymerase chain reaction (PCR) amplified in 50 μl reactions containing the following: 5 μl of 10 × PCR buffer, each dNTP of 0.04 mM, 1 μl of each primer, 2.5 mM $MgCl_2$, and 0.5 μl of recombinant Taq DNA polymerase enzyme (Toyobo, Osaka, Japan). The reaction consisted of 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. The final extension was at 72°C for 5 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining.

I-A^d expression on Pam 212 Pam 212 (1×10^3) was incubated in 96-well flat-bottom plates (Becton Dickinson Labware) for 72 h with 24 h culture medium of DETC with 100 ng IL-18 per ml plus 10 ng IL-12 per ml or medium only. Anti-mouse IFN-γ (20 ng per ml, clone XMG1.2, PharMingen) was added to neutralize IFN-γ produced by DETC. After 72 h, cells were collected and I-A^d expression on the surface was analyzed using FACScalibur as described above. Mean fluorescent intensity (MFI) was determined for I-A^d and isotype control, and the following formula was used to calculate the percentage of positivity of I-A^d in cultured Pam 212.

Percentage modulation =

$$[(\text{MFI of I–A}^d - \text{MFI of isotype control})/\text{MFI of isotype control}] \times 100\ (\%)$$

Statistics Differences between mean values were analyzed with the Student's t test, and $p < 0.05$ was considered statistically significant.

RESULTS

Isolation of DETC from C57/BL6 mice and BALB/C mice Six age-matched mice were used in each experiment. The number of DETC obtained per mouse were $(2.03 \pm 0.71) \times 10^5$ for C57/BL6 mice and $(1.17 \pm 0.47) \times 10^5$ for BALB/C mice. Because more DETC were isolated from C57/BL6 mice than from BALB/C mice, C57/BL6 mice were mainly used and "DETC" in this study are from C57/BL6 mice unless indicated otherwise.

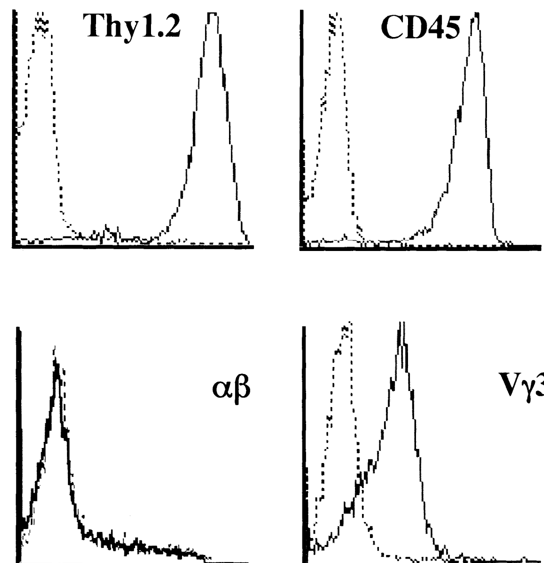

Figure 1. Characterization of purified DETC. Freshly isolated DETC were stained with FITC-anti-mouse Thy-1.2, biotin–anti-mouse CD45$^+$ streptavidin–FITC, FITC–anti-mouse T cell receptor αβ, and FITC-anti-mouse T cell receptor Vγ3 (*solid lines*). Isotype-matched nonreactive monoclonal antibody were used as negative controls (*dotted lines*).

Characterization of purified DETC The expression of Thy-1.2, CD45, T cell receptor αβ and T cell receptor Vγ3 of freshly isolated DETC was examined. Most DETC were Thy-1.2$^+$, CD45$^+$, T cell receptor αβ$^-$ and T cell receptor Vγ3$^+$ (**Fig 1**), which was consistent with previous reports (Asarnow *et al*, 1988; Steiner *et al*, 1988; Havran *et al*, 1989).

Synergy between IL-18 and IL-12 for IFN-γ production by DETC at 24 h IFN-γ concentrations in 24 h culture media of DETC were measured using the enzyme-linked immunosorbent assay method. After stimulation with IL-18 (0.1, 1, 10, 100 ng per ml) or IL-12 (0.1, 1, 10, 100 ng per ml) alone, culture media contained no detectable IFN-γ (**Fig 2**). When IL-18 and IL-12 were added together, IFN-γ was detected in 24 h culture media of DETC. As shown in **Fig 2**, IL-18 and IL-12 synergistically upregulated IFN-γ production by DETC. With 100 ng IL-18 per ml, the addition of 1–100 ng IL-12 per ml to the culture medium significantly enhanced IFN-γ production by DETC. With 10 ng IL-12 per ml, the addition of 100 ng IL-18 per ml significantly enhanced IFN-γ production by DETC. The most effective doses were found to be 100 ng IL-18 per ml and 10 ng IL-12 per ml.

Time-course measurement of IFN-γ production by DETC Time course measurements of IFN-γ levels were performed both at the most effective doses of IL-18 and IL-12, and with no cytokines (**Fig 3**). The IFN-γ concentration in the culture media was increased after 12 h of incubation with IL-18 (100 ng per ml) and IL-12 (10 ng per ml). In the culture media of DETC cultured for 6 h with or without cytokines, there was no detectable IFN-γ. IFN-γ concentrations induced by IL-18 plus IL-12 reached a plateau after 36 h of incubation.

The levels of IFN-γ mRNA expression in fresh, 6 h and 12 h cultured DETC IFN-γ mRNA was barely detectable in freshly isolated DETC or DETC stimulated with IL-18 (100 ng per ml) and IL-12 (10 ng per ml) for 6 h. The level of IFN-γ mRNA expression was increased at 12 h after stimulation with IL-18 and IL-12 (**Fig 4**). Six hour and 12 h cultured DETC without cytokines contained as little IFN-γ mRNA as fresh DETC (data not shown).

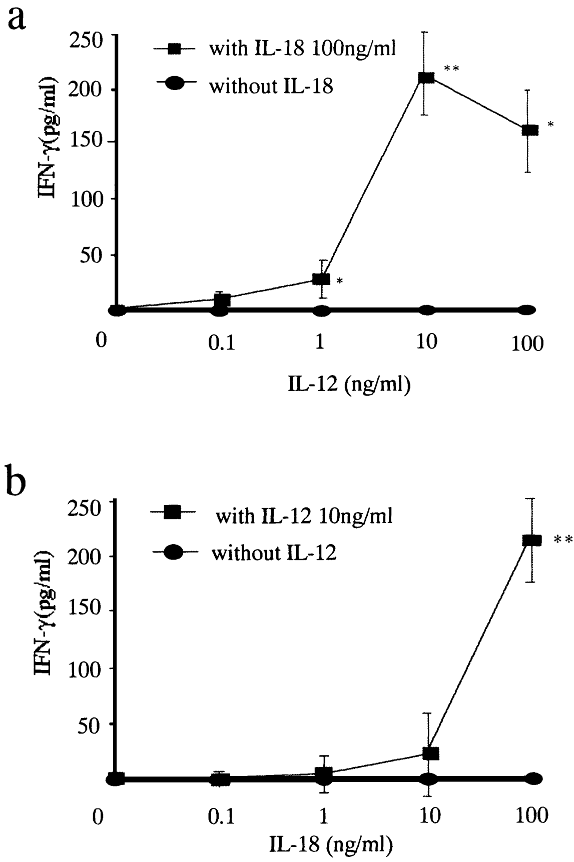

Figure 2. IL-18 and IL-12 synergistically upregulated IFN-γ production by DETC. DETC (2×10^5) was cultured in 96 well flat-bottom plates with IL-12 in the presence or absence of IL-18 (100 ng per ml) (*a*) and with IL-18 in the presence or absence of IL-12 (10 ng per ml) (*b*). Data represent mean ± SD of triplicate samples. *$p < 0.05$, **$p < 0.01$.

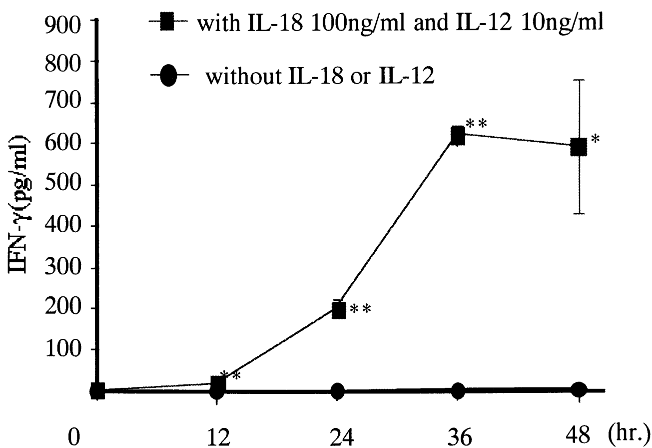

Figure 3. IFN-γ concentrations in the culture media were increased after 12 h of incubation with IL-18 and IL-12. DETC (2×10^5) was cultured in 96-well flat-bottom plates with 100 ng IL-18 per ml plus 10 ng IL-12 per ml, or with no cytokines. IFN-γ concentrations were assayed in the culture media at 12, 24, 36, and 48 h after stimulation. Data represent mean ± SD of triplicate samples. *$p < 0.05$, **$p < 0.01$.

Comparison between IFN-γ production by 24 h cultured DETC from C57/BL6 mice and BALB/C mice DETC from BALB/C mice also synthesized IFN-γ in response to IL-18 (100 ng per ml) and IL-12 (10 ng per ml), although the concentration was relatively low ($p < 0.05$) (**Fig 5**).

I-A^d expression on Pam 212 Pam 212 expressed I-A^d antigen during culture (percentage modulation = 11.9). Addition of 24 h culture medium of DETC with IL-18 (100 ng per ml) and IL-12 (10 ng per ml) increased I-A^d expression on Pam 212 ($p < 0.01$) (**Fig 6**). Anti-mouse IFN-γ (20 ng per ml) significantly neutralized this effect ($p < 0.05$), suggesting that IFN-γ in culture medium of DETC upregulated I-A^d expression on Pam 212.

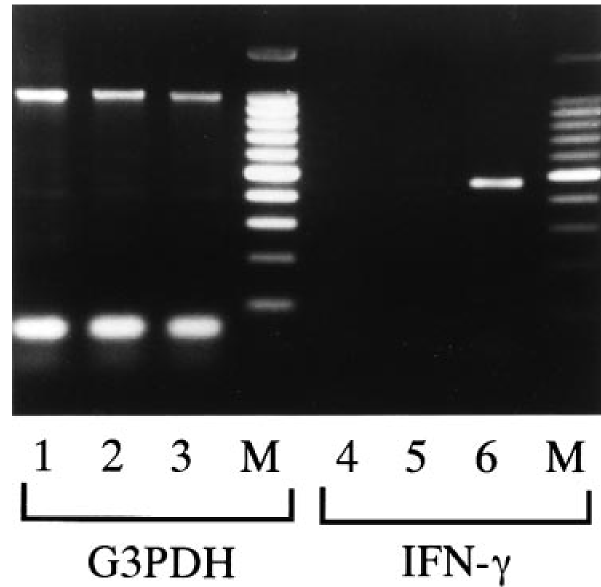

Figure 4. The level of IFN-γ mRNA expression was increased at 12 h after stimulation. Cytoplasmic mRNA was isolated from 1×10^6 freshly isolated DETC and from DETC cultured for 6 or 12 h with 100 ng IL-18 per ml plus 10 ng IL-12 per ml. G3PDH and IFN-γ mRNA expression was examined by reverse transcriptase-PCR. *Lanes 1–3*, G3PDH mRNA; *lanes 4–6*, IFN-γ mRNA; M, size marker. *Lanes 1* and *4* are from freshly isolated DETC, *lanes 2* and *5* are from 6 h cultured DETC, and *lanes 3* and *6* are from 12 h cultured DETC.

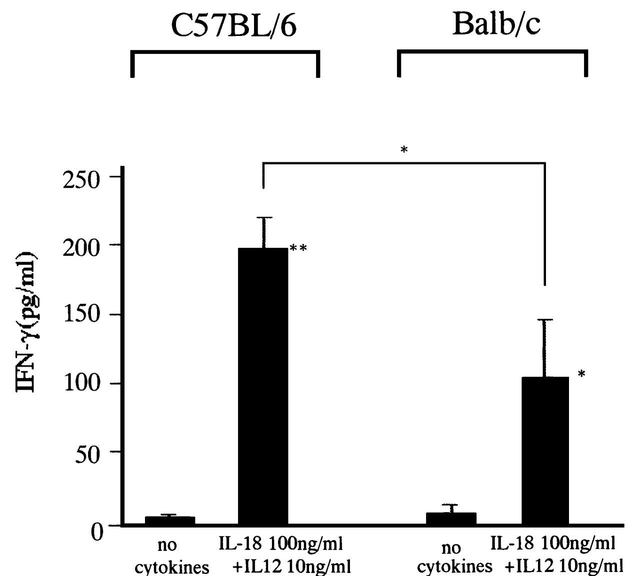

Figure 5. DETC from C57/BL6 mice and from BALB/C mice produced IFN-γ at different levels. DETC (2×10^5) from C57/BL6 mice and BALB/C mice was cultured in 96-well flat-bottom plates for 24 h with 100 ng IL-18 per ml plus 10 ng IL-12 per ml or without cytokines. Data represent mean ± SD of triplicate samples. *$p < 0.05$, **$p < 0.01$.

DISCUSSION

The results of the experiments in this study demonstrated that: (i) IL-18 and IL-12 synergistically enhanced IFN-γ production by DETC; (ii) the level of IFN-γ mRNA expression was barely

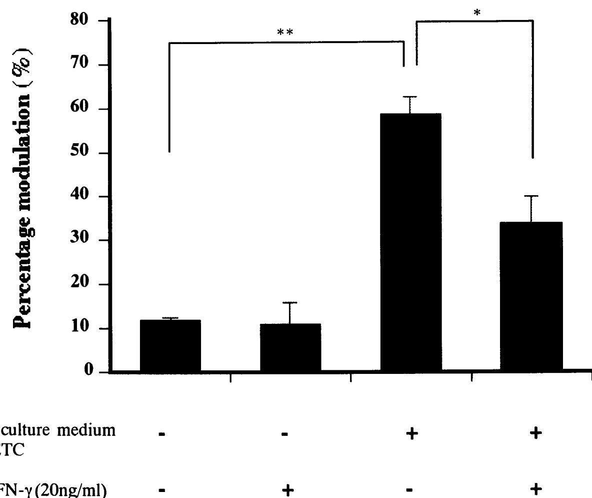

Figure 6. Culture medium of DETC upregulated I-A^d expression on Pam212. Pam 212 (1×10^3) was incubated for 72 h with 24 h culture medium of DETC with 100 ng IL-18 per ml plus 10 ng IL-12 per ml or medium only. Anti-mouse IFN-γ (20 ng per ml) was added to neutralize IFN-γ produced by DETC. Data represent mean ± SD of triplicate samples. *$p < 0.05$, **$p < 0.01$.

detectable in fresh DETC and was enhanced in DETC cultured for 12 h with IL-18 and IL-12; (iii) increased levels of the IFN-γ polypeptide began to increase at 12 h after stimulation with IL-18 and IL-12, although the polypeptide was not detectable in 6 h cultured DETC; (iv) IL-18 and IL-12 upregulated IFN-γ production by DETC from both C57/BL6 mice and BALB/C mice, although the quantity of IFN-γ produced by the two mouse strains differed; and (v) IFN-γ produced by DETC upregulated I-A^d expression on Pam 212. We also confirmed by reverse transcriptase–PCR that murine Langerhans cells purified by the panning method (Koyama *et al*, 1990) and Pam 212, murine keratinocyte cell line, did not produce IFN-γ with IL-18 and IL-12 (data not shown), suggesting that IFN-γ detected in this experiment was produced exclusively by DETC.

DETC are a type of γδ T cells, which are a minor subset of T cells, but are the major T cell type in epithelial tissues (Brenner *et al,* 1988; Raulet, 1989). Although γδ T cells, including DETC, have been shown to synthesize IFN-γ in response to various stimuli (Dieli *et al*, 1997a; Duhindan *et al*, 1997), there has been no direct evidence of the relationship between IFN-γ production by γδ T cells and IL-18, a novel cytokine inducing IFN-γ production by activated T cells (Okamura *et al*, 1995; Micallef *et al*, 1996). This study showed that IL-18, in association with IL-12, potently upregulates IFN-γ production by γδ T cells. Despite the fact that IL-18 has been found to promote GM-CSF production by activated T cells (Micallef *et al*, 1996) and that mRNA for GM-CSF was detected in cultured DETC lines (Matsue *et al*, 1993), the culture media of DETC in this study did not contain detectable GM-CSF (data not shown).

Recently, murine keratinocytes and various dendritic cells, including Langerhans cells, have been shown to synthesize IL-18 (Stoll *et al*, 1997; de Saint-Vis *et al*, 1998). Stoll *et al* (1997) showed that murine keratinocytes were induced to upregulate IL-18 mRNA and protein synthesis by contact allergens but not irritants. DETC were reported to be activated in contact sensitivity (Kaminski *et al*, 1993b) and Dieli *et al* (1997b) reported that T cell receptor Vγ3(+) cells accumulated after skin sensitization in the lymph nodes and at the antigen challenge site. These facts suggest that DETC play an important part in contact sensitivity, although whether they enhance or downregulate the reaction is still controversial (Sullivan *et al*, 1986; Welsh and Kripke, 1990; Dieli *et al*, 1997b). This study strongly suggests that in contact sensitivity, DETC respond to IL-18 and IL-12 synthesized by neighboring keratinocytes and produce IFN-γ, which is a key cytokine in murine contact sensitivity (Grabbe and Schwarz, 1998).

The synergy between IL-18 and IL-12 may be based on the fact that IL-12 upregulates IL-18 receptor expression on T cells (Ahn *et al*, 1997). In contrast, there has been no report suggesting that IL-18 has any effect on the expression of IL-12 receptor on T cells. Considering the fact that neither IL-18 nor IL-12 alone upregulated IFN-γ production by DETC, it is possible to speculate that there may be little or no expression of IL-18 receptor on fresh DETC and that IL-12 may upregulate its expression. In this study, IFN-γ mRNA expression was upregulated only after 12 h of culture with IL-18 and IL-12. As cytokine mRNA expression is generally upregulated about 6 h after stimulation, it may be necessary first for IL-12 to upregulate the expression of IL-18 receptor on DETC, resulting in DETC responsiveness to IL-18. This may explain the relatively long time required to increase the expression of IFN-γ mRNA.

DETC from BALB/C mice produced less IFN-γ than those from C57/BL6 mice in this study ($p < 0.05$). It is generally thought that BALB/C mice are susceptible to infection with *Leishmania major* and that Th2 type responses are predominant (Sadick *et al*, 1986; Guler *et al*, 1996). It is possible that the reduced production of IFN-γ in response to IL-18 and IL-12 in BALB/C mice compared with C57/BL6 mice is one of the mechanisms underlying these strain differences.

This study also demonstrated that IFN-γ produced by DETC upregulated major histocompatibility complex class II expression on Pam 212, corresponding to previous reports (Nishioka *et al*; 1987; Aiba and Tagami, 1987). Keratinocytes can express major histocompatibility complex class II antigens in many skin disorders with prominent lymphocytic infiltrate (Auböck *et al*, 1986) and major histocompatibility complex class II$^+$ keratinocytes *in vitro* induced proliferation of alloreactive CD4$^+$ T cell lines (Nickoloff *et al*, 1986). Considering these facts, IFN-γ produced by DETC may play some immunologic functions in skin through up-regulation of major histocompatibility complex class II antigens on keratinocytes.

IFN-γ has antiviral, antiproliferative, immunoregulatory, and proinflammatory activities. It seems quite reasonable that murine epidermis, which is adjacent to the outer world and the first defense against bacterial and viral infections, contains resident T cells producing IFN-γ. This study suggests that IL-18 and IL-12 produced by neighboring keratinocytes and Langerhans cells regulate DETC function and play important parts in skin-associated lymphoid tissues.

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