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Th2 cell fluctuation in association with reciprocal occurrence of bullous pemphigoid and psoriasis vulgaris

Editor

The coexistence of psoriasis and an autoimmune blistering disease, in particular bullous pemphigoid (BP), has been reported.[1] Early reports described that the occurrence of BP was attributed to treatments for psoriasis, such as psoralen plus ultraviolet A light (PUVA) therapy.[2] This photochemotherapy is known to shift T helper 1 (Th1) cytokine expression to T helper 2 (Th2) cells.[3] Th2 cytokine production has been found to be relevant in BP patients.[4] Here, we document a patient with psoriasis vulgaris who developed BP after PUVA therapy, focusing on the fluctuation of Th2 cells in association with the reciprocal occurrence of BP and psoriasis.

A 76-year-old man presented to our clinic with a 1-month history of pruritic blisters on the trunk and four extremities in March 2005. Prior to the development of the eruption, he had been treated for 2 years with topical steroids and PUVA therapy for psoriasis vulgaris by another clinic. On clinical examination, he had widespread tense blisters, ranging from small vesicles to large bullae, and erosions on the trunk and extremities (fig. 1a). Peripheral blood showed a leucocyte count of 15500/μL with 2% eosinophils (310/μL). Antibodies to desmoglein (Dsg) 1 and Dsg 3 were negative. A skin biopsy specimen showed a subepidermal blister and a dermal infiltrate of lymphocytes with scattered eosinophils (fig. 1b). Indirect immunofluorescence examination using human skin demonstrated IgG autoantibodies strongly bound to the basement membrane zone (> ×160) (fig. 1c). The patient's serum had specific reactivity to BP 230 and BP 180 (fig. 1d) by immunoblotting using epidermal extracts, confirming

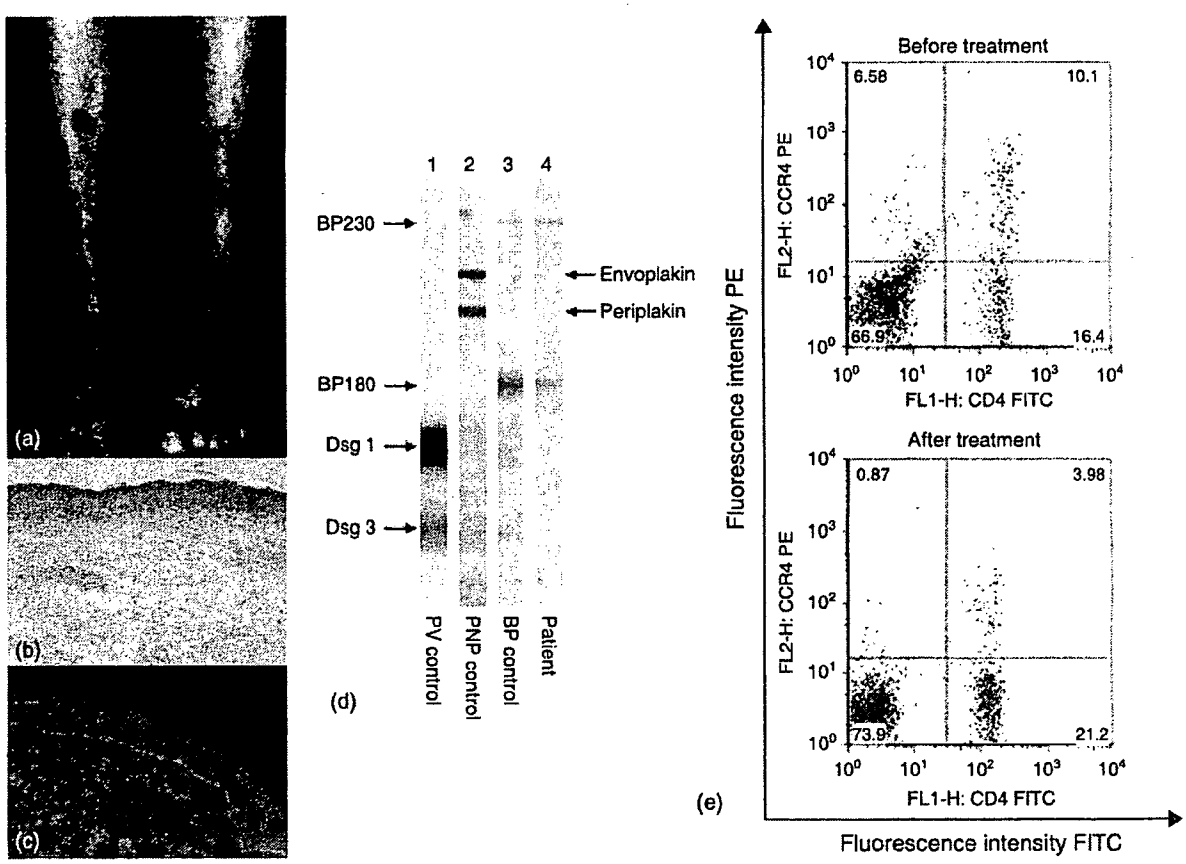

fig. 1 (a) Development of bullous eruption after PUVA therapy. (b) Histology showing a subepidermal blister formation. (c) The patient's IgG autoantibodies bound to the basement membrane zone. (d) Immunoblotting analysis. Lanes 1, 2, 3 and 4 show reactivity by control pemphigus vulgaris (PV) serum, control paraneoplastic pemphigus (PNP) serum, control BP serum and our patient's serum, respectively. (e) Flow cytometric analysis of peripheral blood mononuclear cells before and after successful treatment of BP. The $CCR4^+CD4^+$ population represents Th2 cells.

the diagnosis of BP. The patient was successfully treated with oral prednisolone, 20 mg daily.

We performed a flow cytometric analysis of the patient's peripheral blood lymphocytes before and after oral steroid therapy for BP. Before treatment, the patient had a high frequency of $CCR4^+CD4^+$ Th2 cells (10.1%) (fig. 1e), while $CXCR3^+$ $CD4^+$ Th1 cells (0.32%) were barely detected. The serum interleukin 4 (IL-4) level measured by ELISA was 9.2 pg/mL, while interferon (IFN)-γ was within normal range. Four weeks after oral prednisolone therapy, BP was remarkably improved, but psoriatic skin lesions reappeared. After successful treatment of BP, the serum IL-4 level was decreased to 5.9 pg/mL along with a decreased $CCR4^+CD4^+$ Th2 cell frequency (3.98%) (fig. 1e). Although psoriasis recurred, the percentage of $CXCR3^+CD4^+$ Th1 cells and the level of serum IFN-γ were not altered.

Psoriasis vulgaris and BP are regarded as Th1- and Th2-mediated diseases, respectively. In fact, the administration of IL-4 improves psoriasis by inducing a Th2 environment.[5] In our case, BP occurred when psoriasis was successfully treated with PUVA therapy, which is known to shift cytokine expression from Th1 to Th2.[3] Psoriasis recurred when BP was alleviated, along with normalization of Th2 deviation. This is the first report of the occurrence of psoriasis and BP reflecting Th1- and Th2-skewed conditions, respectively, in the same patient.

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Successful treatment of childhood cutaneous polyarteritis nodosa with infliximab

Editor

Polyarteritis nodosa is a necrotizing vasculitis of small and medium-size arteries. The cutaneous form of PAN (CPAN) is characterized by recurrent episodes limited to skin, muscles and joints, without visceral involvement. The clinical appearance is painful, sometimes ulcerated, inflammatory, subcutaneous nodules. The presence of fatigue, anorexia, myalgia, arthralgia or non-destructive arthritis in flares is frequent. The cause is unknown but the presence of IgM and C3 deposits in some cases and the detection of circulating immunocomplexes in others, orients towards an autoimmune pathology. In children it is a rare disease and it has been frequently related to a streptoccocal infection.

We report a 14-year-old boy diagnosed with CPAN who came to our office for control of a new flare of cutaneous lesions accompanied by mild fever, general malaise and arthralgias. To start with his condition improved following the introduction of prednisolone (0.5 mg/kg/day) and prophylactic penicillin. However, despite this treatment he continued having frequent, and sometimes severe, flares.

Laboratory examinations were normal except for the increase in ESR (80 mm/h) and an important increase in serum levels of inflammatory cytokines IL-1β and IL-6, and to a lesser extent of TNF-α (49 pg/mL vs. < 4 pg/mL in controls). Because of the clinical persistence and side-effects due to long-lasting steroid usage we decided to use humanized anti-TNF-α monoclonal antibody infliximab (Remicade®, Essex Pharma, Munich, Germany). Three 5-mg/kg infusions of infliximab at days 0, 15 and 45 were administered intravenously, achieving complete remission of the clinical picture in just 20 days (fig. 1). One month after the third infusion, the child presented a new, intense outbreak of flares that was also controlled efficiently with a single 5-mg/kg infusion. Current treatment is infliximab 2.5 mg/kg every 2 months. Treatment is being well tolerated.

The initial therapeutic indication for infliximab was rheumatoid arthritis, but more recently its use has been extended to other diseases including systemic vasculitis.

Alopecia universalis associated with impaired interleukin-4 production and low serum IgE level

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A 17-year-old female presented with alopecia universalis, which appeared following widespread eczematous dermatitis. An immunohistochemical study demonstrated that $CD8^+$ T cells infiltrated into hair follicles with satellite cell necrosis of keratinocytes. The precedence of the autosensitization dermatitis suggested that T cells concerned with the eczematous reaction also attacked hair follicles, or alternatively, that those T cells sequentially activated another population of cytotoxic T cells. We found that she had a continuously low level of serum IgE (<1 units/ml), and this abnormality urged us to investigate interleukin (IL)-4 production of T cells. Although the patient had a normal number of $CD4^+$ T cells, the production of IL-4, but not IL-10, was profoundly impaired as assessed by a reverse transcriptase polymerase chain reaction analysis, suggesting the possible relationship between the baldness and IL-4 reduction. The autoimmune hair loss might occur via activation of cytotoxic T cells and Th_1 cells as a result of a relief from IL-4 control. (J Am Acad Dermatol 2007;57:S22-5.)

Accumulating evidence has suggested that alopecia areata is a T-cell mediated, tissue-restricted autoimmune disease of the hair follicle.[1] A wide range of clinical presentations can occur, from the loss of a single patch of hair to complete loss of hair on the scalp (alopecia totalis) or over the entire body (alopecia universalis). The hair follicle is an immunologically privileged organ that is protected from the attack by cytotoxic T lymphocytes (CTLs) by decreasing major histocompatibility complex (MHC) class I expression.[2,3] In some pathologic conditions, however, attenuated down-regulation of MHC class I expression or the activation of CTLs leads to the attack on hair follicles and resultant hair loss. Here, we report a patient with alopecia universalis who exhibited such an aberrant immunological state. Our case is characterized by the precedence of a widespread eczematous eruption before the occurrence of alopecia, and the attenuation of interleukin (IL)-4 production that we noticed with an extremely low level of IgE. The possible relationship between hair loss and IL-4 was discussed.

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CASE REPORT

A 17-year-old Japanese female was referred to our department in March, 1995 because of a 1-month history of an exudative, itchy, eczematous eruption on the earlobes, external meatus, and palpebral regions with facial edema. On admission, her eruption spread to all four extremities, forming itchy red papules. A biopsy specimen from the lesion demonstrated an upper dermal infiltrate of lymphocytes, some of which invaded the epidermis with epidermal spongiosis and vesicles. A tentative diagnosis of contact dermatitis and subsequent autosensitization dermatitis was made, although results of patch test to all the five topical drugs and cosmetics that she had used were negative. On blood examination, she had a normal leukocyte count but eosinophilia (17%; 1,343/μl). Other blood cell counts and routine chemistry were all normal, except for an extremely low level of IgE (<1 units/ml; normal, 30-250 units/ml). She was treated with oral prednisolone (15 mg/day for 2 weeks) and topical steroid ointments, followed by oral ketotifen (2 mg/day for 2 weeks).

The patient's eruption completely improved following the aforementioned therapies, but in the interim the hair on her scalp began to fall out. Over the next year, she lost all of the hair on her scalp, nose, axillae, and pubic area, and was diagnosed as having alopecia univerisalis (Fig 1, *A*). A biopsy

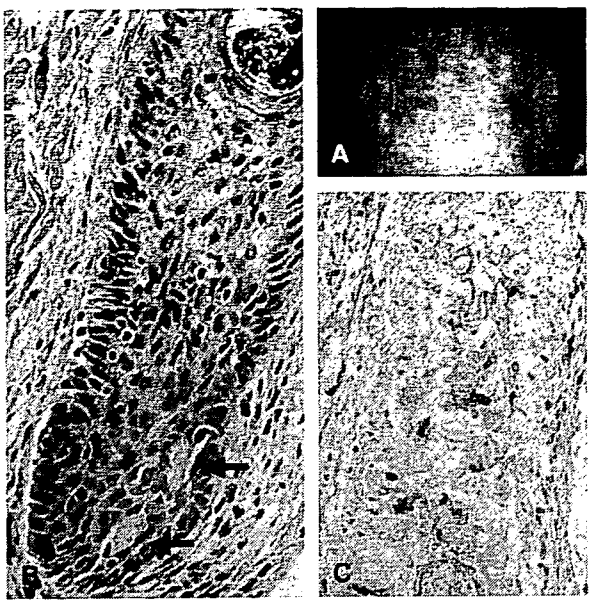

Fig 1. Clinical appearance and histologic pictures. **A**, Complete baldness of the scalp in March, 1996. **B**, Histologic picture showing a hair follicle into which lymphocytes infiltrate with satellite necrosis of keratinocytes (*arrows*). **C**, Immunohistochemical staining for CD8, showing infiltration of $CD8^+$ T cells into a destructed hair follicle. (**B**, Hematoxylin–eosin stain; **C**, avidin-biotin stain; **B** and **C**, original magnification: ×100.)

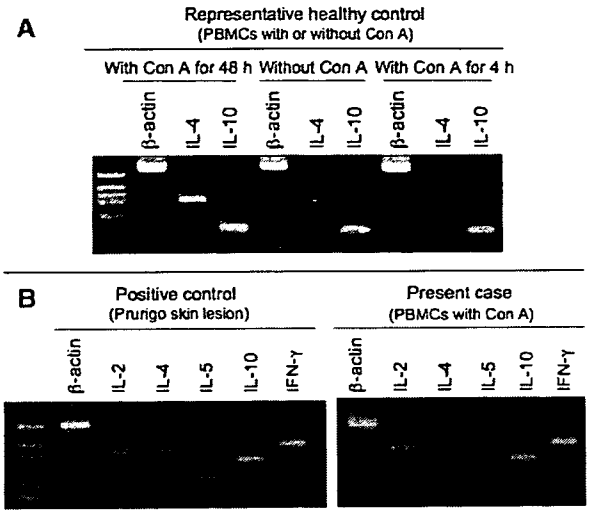

Fig 2. Cytokine mRNA expression pattern of the patient's lymphocytes. **A**, Normal individual's PBMCs, which were freshly isolated (without Con A) or cultured with 5 μg/ml Con A for 4 or 48 hours, were subjected to RT-PCR. **B**, The patient's PBMCs were cultured for 48 hours and the expression of various cytokines were tested. As a positive control, a biopsy specimen from a skin lesion of prurigo subacuta was used because of its expression of all the cytokines tested.[5] Total RNA was prepared from these samples, and a RT-PCR analysis was performed as described previously.[5,6] The sizes of predicted base pairs were 661 for β-actin, 458 for IL-2, 456 for IL-4, 291 for IL-5, 352 for IL-10, and 501 for IFNγ.

specimen from her scalp disclosed lymphocytic infiltration into hair follicles (Fig 1, *B*). Immunohistochemically, the infiltrating cells were mostly $CD8^+/CD3^+$ T cells with a smaller number of $CD4^+$ T cells (Fig 1, *C*). Repeated examinations of her peripheral blood between 1995 and 1997 showed that her eosinophil counts fluctuated from 234/μl to 1,652/μl (normal, <400/μl), but it was enigmatic that the level of IgE was under detection level (<1 units/ml) at any of five times that she was examined.

The patient's alopecia was treated with topical steroid cream or lotion, but no therapeutic response was observed during the next 6 months. For her own personal reasons, she was lost to follow-up. According to her mother, however, her hair began to regrow 2 years later, and she completely recovered from alopecia by the time she reached the age of 20. Currently, at the age of 28 years, she has no recurrence and is in good health.

Cytokine expression in PBMCs

Because IgE production is controlled by IL-4,[4] we investigated the production and expression of IL-4 by the patient's T cells. A flow cytometric analysis of her peripheral blood mononuclear cells (PBMCs) in March, 1996 contained the following populations: CD3, 63%; CD4, 40%; CD8, 26%; CD7, 82%; CD20, 10%; CD56, 29%; and HLA-DR, 14%; indicating normal T cell (normal CD4/CD8 ratio) and B cell percentages and a slightly high natural killer cell percentage. When cultured in 96-well plates for 72 hours and tested in ^{3}H-thymidine incorporation, the patient's PBMCs proliferated well in response to concanavalin A (Con A) at 5 μg/ml (112,319 ± 2,761 cpm *vs* 1,841 ± 167 cpm of non-addition control). To see the production of IL-2 and IL-4, PBMCs were cultured (2×10^6 cells/well of 1.2 ml medium) in duplicate with Con A at 5 μg/ml for 72 hours, and the concentration of IL-4 and IL-2 in the supernatants was measured by enzyme-linked immunosorbent assay. Although IL-2 was produced at a concentration of 22.6 units/ml, IL-4 was under detection level (<31 pg/ml).Because this undetectable level of IL-4 was the same as the level in normal healthy subjects, we investigated mRNA expression of IL-4 along with other cytokines in the following study.

PBMCs from the patient and healthy subjects were cultured with Con A at 5 μg/ml for 48 hours and subjected to a reverse transcriptase polymerase chain reaction analysis, as described previously.[5,6] As a positive control, we chose a skin lesion of

subacute prurigo, because the specimen expressed mRNA for all the cytokines tested.[5] As represented by Fig 2, *A*, freshly isolated PBMCs (without Con A) from a normal control expressed mRNA for IL-4 as well as another Th_2 cytokine, IL-10. When cultured with Con A for 48 hours, the level of IL-4 expression was increased (with Con A for 48 hours). We examined the expression of IL-4 in 10 healthy controls and found that all the healthy subjects had positive IL-4 expression as reported previously.[5,6] In the patient's PBMCs, however, while interferon-γ (IFNγ), IL-2, and IL-10 were positively expressed, no signal for IL-4 or IL-5 was detected (Fig 2, *B*). Although IL-5 message was also undetectable by this RT-PCR in PBMCs from normal individuals and even atopic patients, the absence of expression of IL-4 was definitely abnormal. Thus, the ability of T cells to produce IL-4 was impaired in this patient.

DISCUSSION

We diagnosed this patient as having alopecia universalis because of hair loss over the entire body, histologic and immunohistochemical infiltration of $CD8^+$ T cells into the hair follicles, and the completely curable nature of the disease. The patient's alopecia was preceded by autosensitization dermatitis. Although the precise mechanism underlying these two sequential events remains unclear, T cells concerned with the eczematous reaction also might attack hair follicles. Alternatively, those T cells might sequentially activate another population of T cells cytotoxic to the hair follicle. In any case, the precedence of dermatitis implies that the patient's alopecia was caused by transiently-disordered T cell immunity.

Although our patient had a normal number of $CD4^+$ T cells, their IL-4 production was impaired as assessed by RT-PCR analysis. The reduced production of IL-4 is strongly supported by the markedly low level of serum IgE. IL-4 is one of the cytokines produced by Th_2 cells. However, the patient had eosinophilia, which is induced by IL-5 overproduction,[4] and her PBMCs expressed mRNA for IL-10 at a comparable level to a healthy subject. Thus, among Th_2 cytokines, IL-4 seems to be preferentially downmodulated, and this IL-4 decrease might be closely associated with the patient's baldness. The transcription factors GATA-3 and Stat6 play a central role in regulating Th_2 cell differentiation.[7] It is possible that the signaling pathway involving these molecules was transiently-disordered in this patient.

Alopecia areata is regarded as a T-cell mediated, tissue-restricted autoimmune disease of the hair follicle. The predominant expression of Th_1 cytokines has been reported in this hair disorder,[8,9] although it is not necessarily consistent among studies.[10] Because Th_1 cytokines stimulate $CD8^+$ CTLs, Th_1 dominancy is in accordance with immunological hair loss. Two possibilities may be put forward on the pathogenesis of this patient's alopecia. First, because IL-4 suppresses the function of Th_1 cells and CTLs,[9] the decreased production of IL-4 may allow the CTL and its supporter Th_1 cells to attack hair follicles, as histologically and immunohistochemically seen in our case. In addition, the relative increase of IFNγ to IL-4 may augment the expression of MHC class I molecules, which are the target of CTLs.[11] Second, it is possible that IL-4 directly turns the hair cycle from telogen to anagen. Dermal papilla cells control the hair follicle growth through their elaboration of mitogenic factors and extracellular matrix components. With regard to cytokines capable of regulating hair growth, tumor necrosis factor—α, epidermal cell growth factor, IL-1α, IL-1β and transforming growth factor β1 inhibit hair growth by downregulating dermal papilla cells,[12] while there has been no convincing report on hair growth-promoting cytokines. Only one study showed that IL-4 induces apoptosis in cultured human follicular keratinocytes, but not in dermal papilla cells.[13]

Taken together, these observations make it likely that in our patient the autoimmune hair loss occurred via activation of CTLs and Th_1 cells as a result of a relief from IL-4 control. Although we could not monitor chronological changes in the production/expression of T-cell cytokines throughout the clinical course, the spontaneous and complete recovery and the absence of recurrence provide a speculation that her T-cell immunological condition had been gradually normalized over the course of a couple of years. Our patient suggests that IL-4 plays an important role for hair growth in a certain condition.

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Primary cutaneous anaplastic large cell lymphoma with fatal leukemic outcome in association with CLA and CCR4-negative conversion

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A 70-year-old Japanese male presented with a 1-year history of skin tumors, which were diagnosed as primary cutaneous anaplastic large cell lymphoma (ALCL) because of the $CD3^{low+}$, $CD4^+$, $CD25^+$, $CD30^+$, $CD45RO^+$, $CD71^+$, HLA-DR^+, $CD8^-$, $CD56^-$, and NPM/ALK^- phenotype and monoclonal T-cell receptor-rearranged property of tumor cells as well as the absence of systemic involvement. At this time, the tumor cell was positive for cutaneous lymphocyte–associated antigen (CLA) and TH_2 chemokine receptor CCR4. The eruption had repeatedly appeared and spontaneously regressed or regressed by virtue of several therapeutic modalities, including radiotherapy, interferon-α and chemotherapy, until the tumor cell invaded the gastric mucosa and spread to the peripheral blood 5 years later. Upon progression to the fatal leukemic change, the skin lesions inversely disappeared. Flow cytometric monitoring of the phenotype of peripheral blood and skin-infiltrating lymphocytes disclosed that the expression of CLA and CCR4 on the tumor cells was converted from positive to negative in association with the leukemic change. The altered expression of skin-homing receptors might change its clinical behavior. (J Am Acad Dermatol 2007;57:S92-6.)

Primary cutaneous anaplastic large cell lymphoma (ALCL) is one of the distinct types of lymphoma and belongs to the group of primary cutaneous $CD30^+$ lymphoproliferative disorders, which encompass lymphomatoid papulosis to this type of lymphoma.[1] In addition to CD30, the tumor cells bear CD25, CD71, human leukocyte antigen (HLA)–DR, and mostly CD3 and CD4. A number of cytologic properties of the tumor cell have been found, such as possession of cytotoxic molecules,[2] TH_2 type of cytokine production pattern,[3] and the expression of fascin.[4] In contrast to nodal ALCL, primary cutaneous ALCL lacks the expression of t(2;5)-associated p80 NPM/ALK fusion protein.[5-8]

The prognosis of primary cutaneous ALCL is usually favorable, as even spontaneous regression occurs frequently.[9] Patients commonly show good therapeutic responses to radiotherapy, surgical excision, biologic response modifiers, and chemotherapy. Skin lesions may be localized or, less commonly, multifocal. Multifocal primary cutaneous ALCL tends to relapse after systemic chemotherapy and is generally considered more prone to progress to extracutaneous involvement than the localized type.[10] Extracutaneous dissemination occurs in approximately 10% of patients, and mainly involves the regional lymph nodes.[9] A fatal outcome as a result of systemic involvement has been rarely reported.[11,12] We report a case of primary cutaneous ALCL exhibiting a leukemic change. This progression was documented to be associated with the negative conversion of cutaneous lymphocyte–associated antigen (CLA)[13] and TH_2 chemokine receptor CCR4[14] on the tumor cell.

CASE REPORT

A 70-year-old Japanese male was referred to us in December 2000 for further evaluation of a 1-year history of skin tumors. The eruption first appeared as a single tumor on the right aspect of the back and subsequently extended as multiple tumors or nodules to the lumbar area. His past and family histories were unremarkable. On the initial examination, several tumors/nodules were present on the right lower back (Fig 1, *A*). He was otherwise healthy, and neither lymphadenopathy nor hepatosplenomegaly

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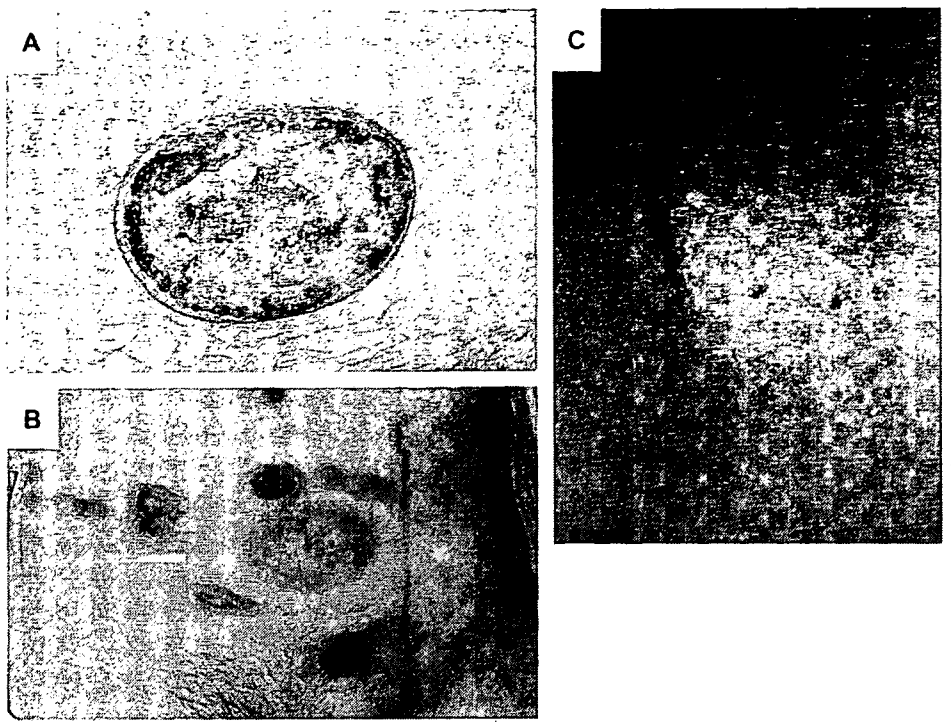

Fig 1. Clinical pictures. Initial presentation in December 2000 (**A**), regressing and reappearing tumors in March 2004 (**B**), and remarkable left axillar lymph node swelling in April 2005 (**C**).

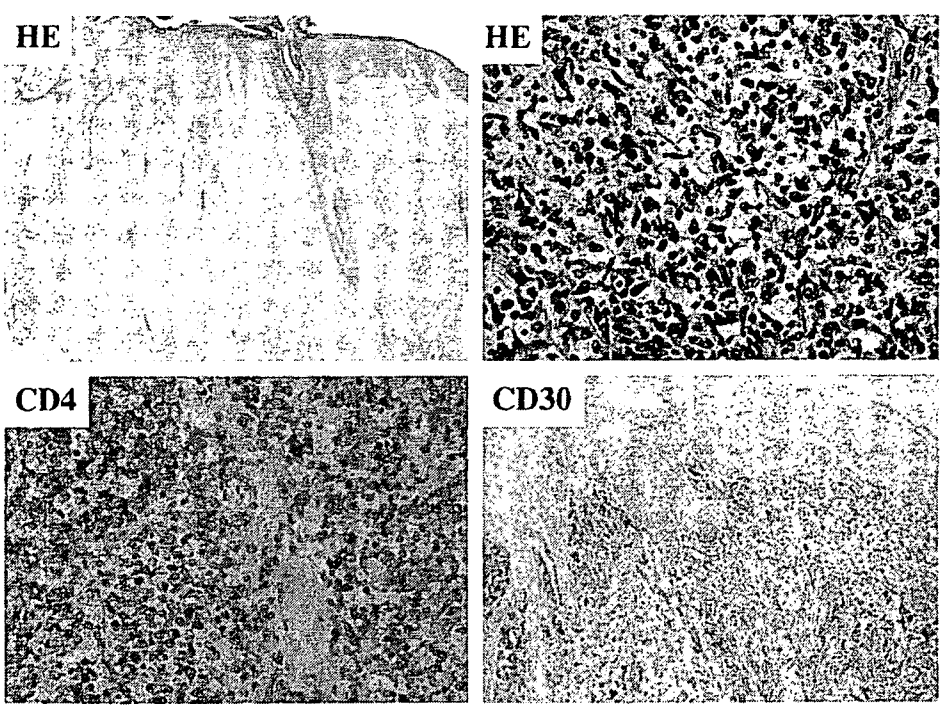

Fig 2. Histologic pictures of skin tumor. Low (*upper left*) and high (*upper right*) power views of skin tumor. Immunohistochemical staining for CD4 (*lower left*) and CD30 (*lower right*). (Hematoxylin–eosin stain; original magnification: *upper left*, ×40; *upper right*, ×200.)

was noted. Complete blood cell counts, blood chemistry and soluble interlekin-2 receptor were all within normal limits, and anti-human T-lymphotropic virus-1 antibody was negative. A biopsy specimen from a tumor disclosed massive dermal and subcutaneous infiltration of large atypical lymphoid cells that

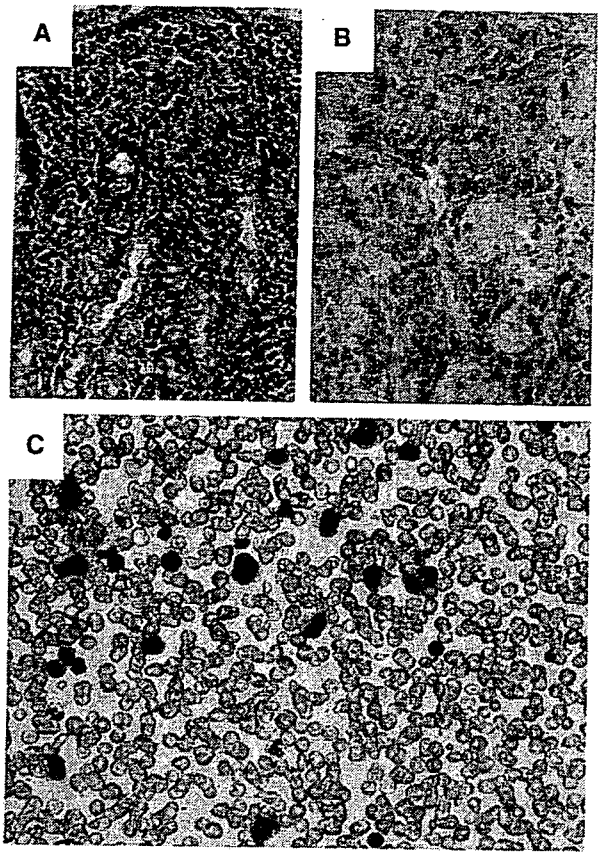

Fig 3. Gastric involvement and leukemic change. Histologic (**A**), CD30-immunohistochemical (**B**), and blood film (**C**) all taken in May 2005. (**A** and **B**, Hematoxylin–eosin stain; **C**, May–Giemsa stain; original magnifications: **A**, ×100; **B**, ×200; and **C**, ×400.)

contained large pale nuclei with prominent nucleoli (Fig 2, top *left* and *right*). Occasional Reed–Sternberg cells were also observed.

Immunohistochemically, the tumor cells were positive for CD3, CD4, CD25, CD30, CD45RO, CD71, and HLA-DR, but negative for CD8 and CD56 (Fig 2, bottom *left* and *right*). The fusion protein p80 NPM/ALK as a result of t(2,5), ordinarily observed in nodal ALCL, was negative, as assessed by immunohistochemical staining with anti-ALK monoclonal antibody.[5] A Southern blot analysis with restriction enzyme BamHI or EcRV showed monoclonal rearrangement of T-cell receptor (TCR) $C\beta1$. Roentgenographic and computed tomographic examinations showed no abnormality in the internal organs. Based on these findings, we diagnosed the skin lesions as primary cutaneous ALCL.

Over the course of the next 4 years, we treated the patient intermittently with electron beam radiation (30 Gy per tumor), or systemic or intralesional interferon-γ (IFN-γ) injections (200 Japanese reference units daily). The tumors disappeared with the treatments or sometimes regressed spontaneously, while new lesions reappeared on the lower back, buttocks, and lower abdomen (Fig 1, *B*). In March 2004, because of deterioration of the tumorous eruption, we treated the patient with combination of etoposide (25-75 mg daily intermittently for 1-2 weeks) and prednisolone (20 mg daily), followed by CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone) therapy. This treatment resulted in partial response, and his skin lesions were controlled by prednisolone alone (5-10 mg daily) for the next 6 months.

In April 2005, many tumors appeared on the trunk and inguinal to scrotal areas with lymphadenopathy, especially in the left axilla (Fig 1, *C*). He was treated with elecron beam radiation (30 Gy), prednisolone (20 mg daily), and topical injections with IFN-γ (200 Japan reference units daily). Although the skin lesions were markedly improved as only sequelae remained, the hemoglobin level was subsequently reduced from 11.6 to 7.2 mg/dl. Concomitantly, he had a high temperature and occult bleeding of stool. An endoscopic biopsy specimen from an erosive elevated lesion of the stomach revealed massive infiltration of $CD30^+$ large atypical cells in the submucosal area (Fig 3, *A* and *B*). Along with the disappearance of skin eruption and the involvement of stomach, the blood leukocyte count was elevated to 52,500/μl with 71% atypical lymphoid cells (Fig 3, *C*) in May 2005. Following methylprednisolone pulse therapy (500 mg daily for 3 days), THP-COP (therarubicin, cyclophosphamide, vincristine, and prednisolone) therapy was performed. Although his leukocyte count and percentage of his atypical lymphoid cells decreased, his respiratory function declined. After the development of disseminated intrtavascular coagulation, he died in June 2005. Autopsy was not performed.

Phenotypic alterations in skin-infiltrating and circulating tumor cells

Throughout the clinical course, we monitored the phenotype of tumor cells in the skin and peripheral blood. Skin tumor–infiltrating lymphocytes were isolated as described previously[15] and analyzed by flow cytometry in December 2000 and March 2004, with virtually the same results. The tumor cells expressed $CD3^{low}$, CD4, CD25, CD30, HLA-DR, CLA, CCR4, and CTLA-4 (Fig 4, *left*), but not CD7 or CD20. Thus, the tumor cells bore TH_2 or regulatory T-cell markers CCR4 and CTLA-4[16] as well as the usual ALCL markers, and it is notable that they expressed skin-homing receptor CLA.

The peripheral lymphocyte counts from December 2000 to April 2005 were within normal range,

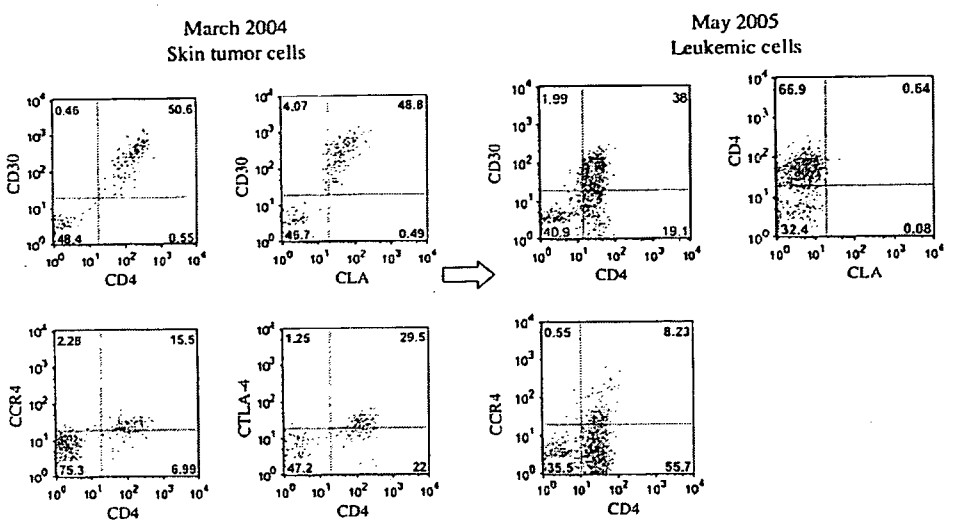

Fig 4. Flow cytometry of skin tumor-infiltrating cells and peripheral blood lymphocytes. Tumor-infiltrating cells were isolated by teasing in March 2004 (non-leukemic state, *left*). Peripheral blood mononuclear cells were taken in May 2005 (leukemic change, *right*). They were double-stained with the indicated monoclonal antibodies.

and the phenotypic analysis performed four times during this period showed the presence of normal populations of lymphocytes. However, in May 2005, when the leukocyte count was elevated to 52,500/μl, approximately 40% of lymphocytes were $CD4^+$, $CD30^+$, and $HLA\text{-}DR^+$. It was noteworthy that the circulating tumor cells clearly lost CLA, CCR4 (Fig 4, *right*), and CD3 (data not shown). Therefore, the expression of CLA and CCR4 was converted from positive to negative along with the leukemic change and disappearance of skin lesions.

DISCUSSION

The present case of primary cutaneous ALCL is highly characterized by the leukemic change at its terminal stage. The original primary cutaneous nature was confirmed not only by the absence of lymph node and internal organ involvements that lasted for the first 4 years, but also by the negative immunohistochemistry of p80 NPM/ALK which is usually expressed in nodal but not primary cutaneous ALCL.[6-8]

Patients with primary cutaneous ALCL of poor prognosis or fatal outcome have been rarely reported,[11,12] and there has been no report of the leukemic change. To our best knowledge, this is the first reported case of primary cutaneous ALCL documenting dissemination of malignant cells to the peripheral blood. The concomitant invasion of tumor cells into the gastric mucosal tissue is also particularly remarkable.

Upon development of the leukemic change, we found the negative conversion of CLA expression on the tumor cells, since the skin-infiltrating tumor cells in March 2004 was positive for CLA and the leukemic cells in May 2005 was negative for this skin-homing molecule.[13] Moreover, the expression of CCR4, a TH_2 chemokine receptor that is associated with skin infiltration of lymphoma cells,[14,15] was also decreased in the leukemic cells with this conversion. Simultaneously, skin tumors disappeared and gastric lesions occurred, presumably as a result of the loss of CLA and CCR4, supporting the crucial role of these molecules for the selectivity of tumor invasion sites. Our case demonstrated that the change of homing receptor expression could dramatically alter the clinical behavior of the tumor cell.

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ORIGINAL ARTICLE

Cutaneous Hypersensitivities to Hapten Are Controlled by IFN-γ-Upregulated Keratinocyte Th1 Chemokines and IFN-γ-Downregulated Langerhans Cell Th2 Chemokines

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There are immediate, late-phase, and delayed-type reactions to exogenous agents. In IFN-γ-knockout (IFN-$\gamma^{-/-}$) and wild-type B6 mice, we examined the response to picryl chloride (PCl) for assessing delayed-type reactions, and the responses to repeatedly challenged FITC for immediate and late-phase reactions. The delayed-type hypersensitivity was depressed in IFN-$\gamma^{-/-}$ mice, and the immediate and late-phase reactions were enhanced in IFN-$\gamma^{-/-}$ mice. As skin-infiltrating lymphocytes were scarce at the PCl-challenged site of IFN-$\gamma^{-/-}$ mice, we investigated chemokine production by keratinocytes and Langerhans cells (LCs). A real-time PCR analysis demonstrated that Th1 chemokines (CXCL9 and CXCL10) and Th2 chemokines (CCL17 and CCL22) were derived mainly from keratinocytes and LCs, respectively. Challenge with PCl or FITC augmented keratinocyte expression of Th1 chemokines in wild-type but not in IFN-$\gamma^{-/-}$ mice, and Th2 chemokine production by LCs was induced by repeated FITC in IFN-$\gamma^{-/-}$ mice. Finally, transfer of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled draining lymph node cells from hapten-sensitized B6 mice or lymph node cells from sensitized green fluorescent protein (GFP) mice to naive IFN-$\gamma^{-/-}$ mice revealed less infiltration of $CFSE^+$ or GFP^+ lymphocytes at the challenged site. Our study suggests that one of the crucial actions of IFN-γ is upregulation of keratinocyte production of Th1 chemokines and downregulation of LC production of Th2 chemokines.

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INTRODUCTION

IFN-γ is one of the critical cytokines involved in cutaneous hypersensitivity responses as well as in systemic immune reactions. First, as a representative Th1 cytokine, IFN-γ supports $CD8^+$ effector T cells that evoke contact hypersensitivity (CHS) response (Bour *et al.*, 1995; Xu *et al.*, 1996), graft-versus-host reaction (Blazar *et al.*, 1998), and tumor immunity (Seo and Tokura, 1999). Second, IFN-γ upregulates the immunological functions of keratinocytes, including expression of CD54 (Griffiths *et al.*, 1990), production of proinflammatory cytokines such as IL-1α and tumor necrosis factor (TNF)-α (Pastore *et al.*, 1998), production of chemokines such as IL-8, CCL5/RANTES (regulated upon activation, normal T-cell expressed and secreted) (Li *et al.*, 1996), and Th1- and Th2-associated chemokines (Sebastiani *et al.*, 2002). Finally, IFN-γ modulates the various functions of Langerhans cells (LCs), including expression of major histocompatibility complex class II and co-stimulatory molecules (Salgado *et al.*, 1999) and production of cytokines and chemokines (Matsue *et al.*, 1992).

Although knowledge regarding the *in vitro* effects of IFN-γ on skin immunocompetent cells has been thus accumulating, one can realize that the *in vivo* actions of IFN-γ have not extensively been studied. For example, as a Th1 cytokine, systemic IFN-γ skews Th balance to Th1, and the treatment of Th2 disorders is improved by IFN-γ. However, patients with atopic dermatitis are not necessarily alleviated by IFN-γ (Stevens *et al.*, 1998). In another example, IFN-γ promotes the *in vitro* production of Th2 chemokines by keratinocytes (Sebastiani *et al.*, 2002), but it remains unknown as to whether this seemingly ambivalent phenomenon really occurs in *in vivo* settings. To resolve these issues, *in vivo* studies using IFN-γ-deficient mice are required.

Contact hypersensitivity belongs to the delayed-type hypersensitivity (DTH), peaks at 24–48 hours after challenge, and involves Th1/Tc1 cells (Akiba *et al.*, 2002), cutaneous dendritic cells (epidermal LCs and dermal dendritic cells)

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Abbreviations: B6, C57BL/6J; CFSE, carboxyfluorescein diacetate succinimidyl ester; CHS, contact hypersensitivity; C_t, cycle threshold; DTH, delayed-type hypersensitivity; EC, epidermal cell; GFP, green fluorescent protein; IFN-$\gamma^{-/-}$, IFN-γ-knockout; LC, Langerhans cell; PCl, picryl chloride

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serving as antigen-presenting cells (Kissenpfennig and Malissen, 2006), and keratinocytes producing IL-1α, TNF-α, and GM-CSF (Heufler *et al.*, 1998) stimulatory for LCs (Sugita *et al.*, 2007). However, when a given contactant is prone to stimulate Th2 cells, certain responses earlier than DTH take place and include immediate and late-phase reactions. The immediate type occurs at 15 minutes to 1 hour and is induced by IgE and mast cells (Kitagaki *et al.*, 1995), and the late-phase reaction peaks at 4–8 hours and is mediated by Th2 cells and eosinophils (Dearman and Kimber, 2000; Ying *et al.*, 2002).

Chemokines derived from epidermal cells (ECs) are one of the recent topics in CHS responses. Keratinocytes secrete Th1 chemokines, CXCL9/Mig and CXCL10/IP-10, and Th2 chemokines, CCL17/TARC and CCL22/MDC (Sebastiani *et al.*, 2002). IFN-γ stimulates keratinocytes to produce both Th1 and Th2 chemokines (Kakinuma *et al.*, 2002; Sebastiani *et al.*, 2002). The stimulatory ability of IFN-γ for Th1 chemokines appears to be reasonable, as a Th1-polarized local response may be induced by IFN-γ. However, its ability to produce Th2 chemokines needs some sophisticated idea to harmoniously construct sequential events. In contrast, LCs are capable of producing Th2 and Th1 chemokines *in vitro*, including CCL17, CCL22, CXCL10, CXCL9, and CXCL11/I-TAC with Th2 chemokine dominancy. IFN-γ is mostly suppressive for the LC expression of Th2 chemokines, CCL17 and CCL22 (Fujita *et al.*, 2005). Thus, IFN-γ can modulate both keratinocytes and LCs in their chemokine production and in the resultant occurrence of CHS.

In this study, we addressed the role of IFN-γ for the immediate, late-phase, and delayed-type cutaneous hypersensitivities by using IFN-γ-knockout (IFN-$\gamma^{-/-}$) mice. As our preliminary study showed the extent of skin infiltration of lymphocytes was different between the wild-type and IFN-$\gamma^{-/-}$ mice, we focused on the effects of IFN-γ on the chemokine production by keratinocytes and LCs. Results suggest that Th1 and Th2 chemokines are produced mainly by keratinocytes and LCs, respectively, and IFN-γ upmodulates the production of Th1 chemokines by keratinocytes but downmodulates the production of Th2 chemokines by LCs, leading to the reduction of cutaneous DTH response and the enhancement of the early responses.

RESULTS

Reduced CHS response to 2,4,6-trinitrochlorobenzene (picryl chloride) in IFN-$\gamma^{-/-}$ mice

Initially, we tested the degree of ordinary CHS response in IFN-$\gamma^{-/-}$ mice along with wild-type C57BL/6J (B6) mice. Mice were sensitized and challenged with picryl chloride (PCl), and their ear swelling responses were measured 24 hours after challenge. A significant degree of ear swelling response was observed in B6 but not in IFN-$\gamma^{-/-}$ mice, as compared to the negative control mice challenged without sensitization (Figure 1a). CHS responses to PCl were monitored at 1, 4, and 24 hours after challenge. IFN-$\gamma^{-/-}$ mice did not exhibit a substantial swelling response throughout the time course (Figure 1b). The ear swelling response of B6 mice was reduced at 48 hours (7.0×10^{-3} cm) compared to that at 24 hours. No significant difference was observed in the ear swelling of IFN-$\gamma^{-/-}$ mice between 24 and 48 hours (data not shown).

Histologically, the intensity of inflammatory cell infiltrate and dermal edema at the PCl-challenged site was lower in IFN-$\gamma^{-/-}$ mice than in B6 mice (Figure 2a). When $CD3^+$ T cells in the dermis and subcutaneous tissue were enumerated, T cells scarcely infiltrated in IFN-$\gamma^{-/-}$ mice (Figure 2b). Thus, IFN-$\gamma^{-/-}$ mice lacked a capacity to develop cutaneous DTH, and this inability was associated with the absence of skin-infiltrating T cells.

Elevated immediate and late-phase reactions to repeatedly challenged FITC in IFN-$\gamma^{-/-}$ mice

To evaluate whether IFN-$\gamma^{-/-}$ mice can develop early-phase cutaneous reactions, we used the repeated hapten challenge system, a known method to induce a Th2-mediated early skin response (Kitagaki *et al.*, 1997), with the use of Th2-skewing hapten FITC (Dearman and Kimber, 2000). FITC was painted one, three, or six times on the ears of B6 and IFN-$\gamma^{-/-}$ mice that were sensitized with this hapten. The single challenge with FITC did not elicit any ear swelling response at 1, 4, or

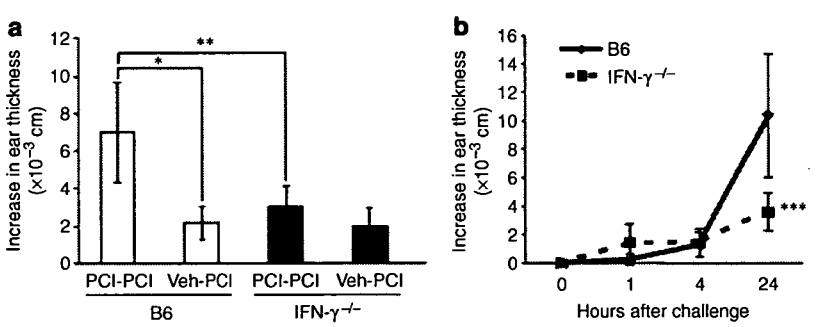

Figure 1. CHS to PCl in B6 and IFN-$\gamma^{-/-}$ mice. (**a**) B6 and IFN-$\gamma^{-/-}$ mice were sensitized with 5% PCl on the shaved abdomen and challenged with 0.5% PCl on the ears (PCl-PCl). Control mice were painted with vehicle alone and challenged with PCl (Veh-PCl). Ear thickness swelling was measured 24 hours later. (**b**) The time course of ear swelling responses was monitored after elicitation with PCl in B6 and IFN-$\gamma^{-/-}$ mice. Data are expressed as the mean ± SD of six mice. *$P = 0.0016$, **$P = 0.00029$, and ***$P = 0.0060$.

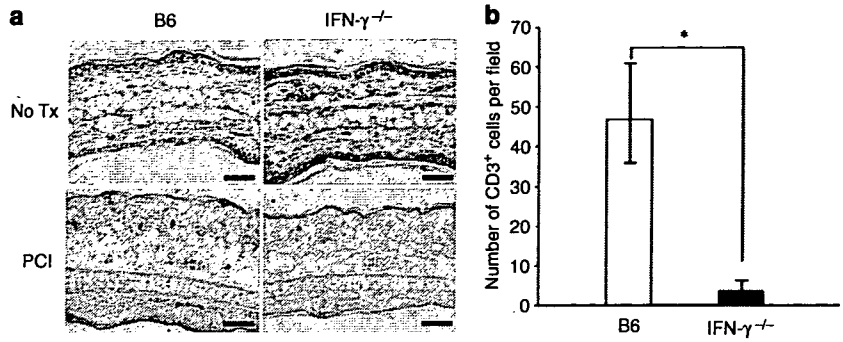

Figure 2. Histology and numbers of CD3$^+$ T cells in PCI-challenged skin of B6 and IFN-γ$^{-/-}$ mice. (**a**) B6 and IFN-γ$^{-/-}$ mice were sensitized with 5% PCl and challenged with 0.5% PCl. The challenged ears, along with no-treated ones (no Tx), were stained with hematoxylin and eosin. (**b**) Cryostat sections of the challenged ears were immunohistochemically stained with anti-CD3 mAb, and CD3$^+$ cells were enumerated in original magnification × 500 field. Data are expressed as the mean ± SD of five mice. *P = 0.00018. Bar = 80 μm.

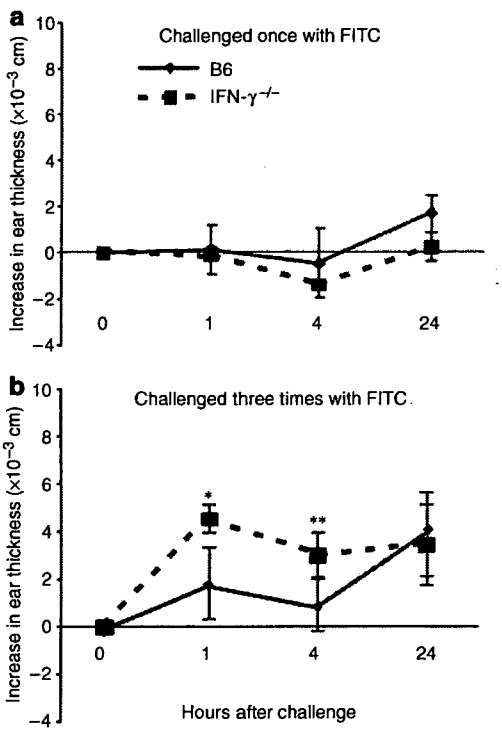

Figure 3. Time course of ear swelling responses of B6 and IFN-γ$^{-/-}$ mice receiving single-time or three time-elicitation with FITC. B6 and IFN-γ$^{-/-}$ mice were sensitized with 1% FITC on the abdomen and challenged with 0.5% FITC once or thrice (3- to 4-day intervals) on the ears. One, four, and 24 hours after the final challenge, the ear swelling responses were measured. Data are expressed as the mean ± SD of five mice. *P = 0.0048, **P = 0.0060.

24 hours after challenge in either strain of mice (Figure 3a). When challenged three times, significantly higher swelling responses were found at 1 and 4 hours in IFN-γ$^{-/-}$ mice than in B6 mice (Figure 3b). There was no difference between the two strains at 24 hours. The six-time challenge induced similar but slightly higher levels of these reactions (data not shown). The results suggested that IFN-γ$^{-/-}$ mice are prone to develop immediate and late-phase cutaneous reactions instead of DTH, suggesting a Th2-skewed immunological state.

Augmentation of IL-4 expression by repeated FITC challenge in immune lymph nodes of IFN-γ$^{-/-}$ mice

To confirm the preponderant expression of Th2 cytokines in IFN-γ$^{-/-}$ mice, B6 and IFN-γ$^{-/-}$ mice were sensitized with PCl or FITC, and challenged once with PCl or six times with FITC, respectively. Cervical lymph nodes were taken from these mice 24 hours after the last challenge, and IFN-γ and IL-4 expressions were examined by real-time PCR. B6 mice had IFN-γ expression at a higher level by single challenge with PCl than by repeated challenge with FITC, whereas IFN-γ$^{-/-}$ mice naturally exhibited no IFN-γ expression (Figure 4). As for IL-4, the repeated FITC challenge enhanced its expression in both strains of mice as compared to PCl challenge, but the level of enhancement by FITC was greatly higher in IFN-γ$^{-/-}$ mice than in B6 mice. These data indicated preferential activation of Th2 cells in IFN-γ$^{-/-}$ mice after repeated FITC challenge.

Infiltration of Th2 cells and presence of IgE and IL-4 at repeatedly FITC-challenged skin of IFN-γ$^{-/-}$ mice

B6 and IFN-γ$^{-/-}$ mice were sensitized and challenged six times with FITC, and their ears were histologically examined. Both types of mice exhibited an inflammatory infiltrate mainly in the dermis (Figure 5a). Some lymphocytes infiltrated into the epidermis with spongiosis. Immunohistochemically, IgE and IL-4 were present in IFN-γ$^{-/-}$ mice presumably in association with mast cells and T cells, respectively (Figure 5b).

Epidermal cell suspensions, including epidermotropic lymphocytes as well as ECs, were prepared from the challenged skin and subjected to real-time PCR analysis for two chemokine receptors. CXCR3 is expressed mostly on Th1 cells, and CCR4 is preferentially on Th2 cells (Sebastiani *et al.*, 2002). Whereas CXCR3 was markedly expressed by FITC challenge in B6 but not in IFN-γ$^{-/-}$ mice, CCR4 expression was dramatically increased by repeated FITC painting in IFN-γ$^{-/-}$ but not in B6 mice (Figure 6). Taken together with the presence of IL-4 in IFN-γ$^{-/-}$ mice challenged with FITC, this finding provides a possibility that IFN-γ deficiency allows Th2 cells to infiltrate into the skin, leading to the occurrence of immediate and late-phase reactions.

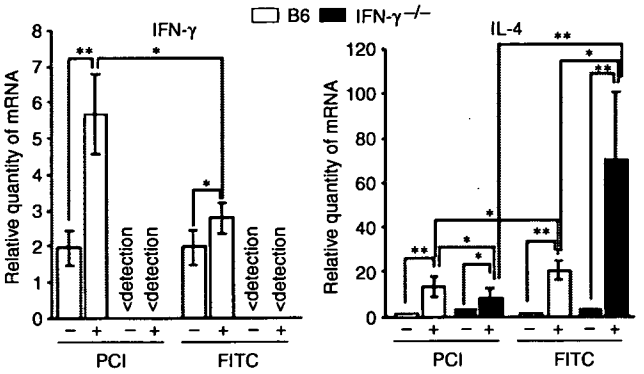

Figure 4. Real-time PCR analysis of mRNA expression for cytokines in cervical lymph node cells. B6 and IFN-$\gamma^{-/-}$ mice were sensitized with 5% PCl and challenged once with 0.5% PCl, or sensitized with 1% FITC and challenged six times with 0.5% FITC (twice a week for 3 weeks). Single-cell suspensions of immune cervical lymph node cells were prepared and subjected to real-time PCR analysis for IFN-γ and IL-4. Data are expressed as the mean ± SD of four mice. $*P<0.05$, $**P<0.005$.

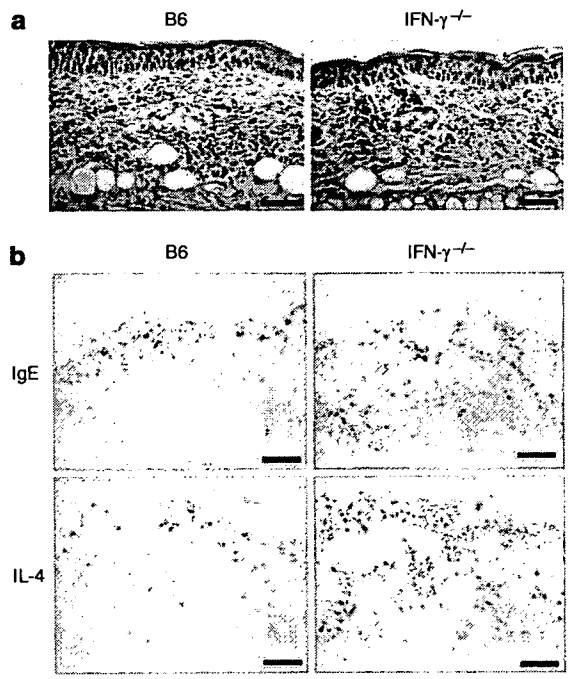

Figure 5. Histology and immunohistochemical stainings for IgE and IL-4 in B6 and IFN-$\gamma^{-/-}$ mice receiving six times repeated elicitation with FITC. (a) B6 and IFN-$\gamma^{-/-}$ mice were sensitized with 1% FITC and challenged with 0.5% FITC repeatedly six times (twice a week). The challenged ears were stained with hematoxylin and eosin. **(b)** Cryostat sections of the challenged ears were immunohistochemically stained with anti-IgE or anti-IL-4 mAb. Bar = 50 μm.

Reduced CHS response to PCl in IFN-$\gamma^{-/-}$ mice receiving adoptive transfer

Draining lymph node cells (5×10^6 cells) from wild-type B6 mice that were sensitized with PCl 5 days before were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and transferred to naive B6 or IFN-$\gamma^{-/-}$ mice.

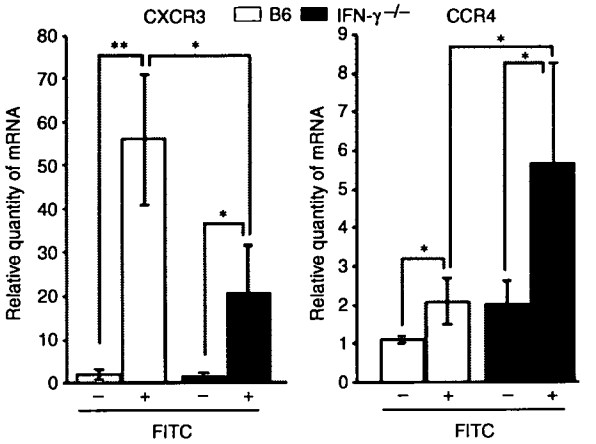

Figure 6. Real-time PCR analysis of mRNA expression for chemokine receptors in skin-infiltrating cells. B6 and IFN-$\gamma^{-/-}$ mice were sensitized with 5% PCl and challenged once with 0.5% PCl, or sensitized with 1% FITC and challenged six times with 0.5% FITC (twice a week for 3 weeks). Single-cell suspensions of epidermal and dermal cells were prepared and subjected to real-time PCR analysis for CXCR3 and CCR4. Data are expressed as the mean ± SD of four mice. $*P<0.05$, $**P<0.005$.

Immediately after transfer, mice were challenged with PCl on their ears. Although B6 mice transferred with sensitized lymph node cells exhibited a significantly high ear swelling response, IFN-$\gamma^{-/-}$ mice failed to respond to the challenge (Figure 7a). This result clearly indicated impairment of the efferent phase of CHS in IFN-$\gamma^{-/-}$ mice. When the ears of mice receiving CFSE-labeled lymph node cells were histologically examined 24 hours after challenge, the number of CFSE-positive cells in the ears of IFN-$\gamma^{-/-}$ mice was significantly lower than that of B6 mice (Figure 7b).

To further confirm this paucity of infiltration, draining lymph node cells (5×10^6 cells) from green fluorescent protein (GFP) mice sensitized with DNFB 5 days before were transferred to naive B6 or IFN-$\gamma^{-/-}$ mice. The recipients were immediately challenged with DNFB. B6 but not IFN-$\gamma^{-/-}$ mice had a high ear swelling response (Figure 7c). Again, histological examination at 24 hours after challenge showed less infiltration of GFP-positive lymphocytes in IFN-$\gamma^{-/-}$ mice (Figure 7d).

To test the participation of IFN-γ in the afferent limb, immune lymph node cells from B6 or IFN-$\gamma^{-/-}$ mice sensitized with PCl were transferred to naive B6 mice, and the recipient mice were challenged with PCl. The CHS response of recipients receiving sensitized lymph node cells of IFN-$\gamma^{-/-}$ mice was lower than that of mice receiving B6 lymph node cells (Figure 7e). This suggests that IFN-γ is involved in both induction and elicitation of CHS.

Reduction of Th1 chemokine production by keratinocytes in IFN-$\gamma^{-/-}$ mice and enhancement of Th2 chemokine production by LCs in IFN-$\gamma^{-/-}$ mice

Epidermal cell suspensions were obtained from the ears of B6 and IFN-$\gamma^{-/-}$ mice that were challenged once with PCl or six times with FITC. Crude ECs were analyzed for EC-derived chemokines. Both challenges with PCl and FITC elevated the

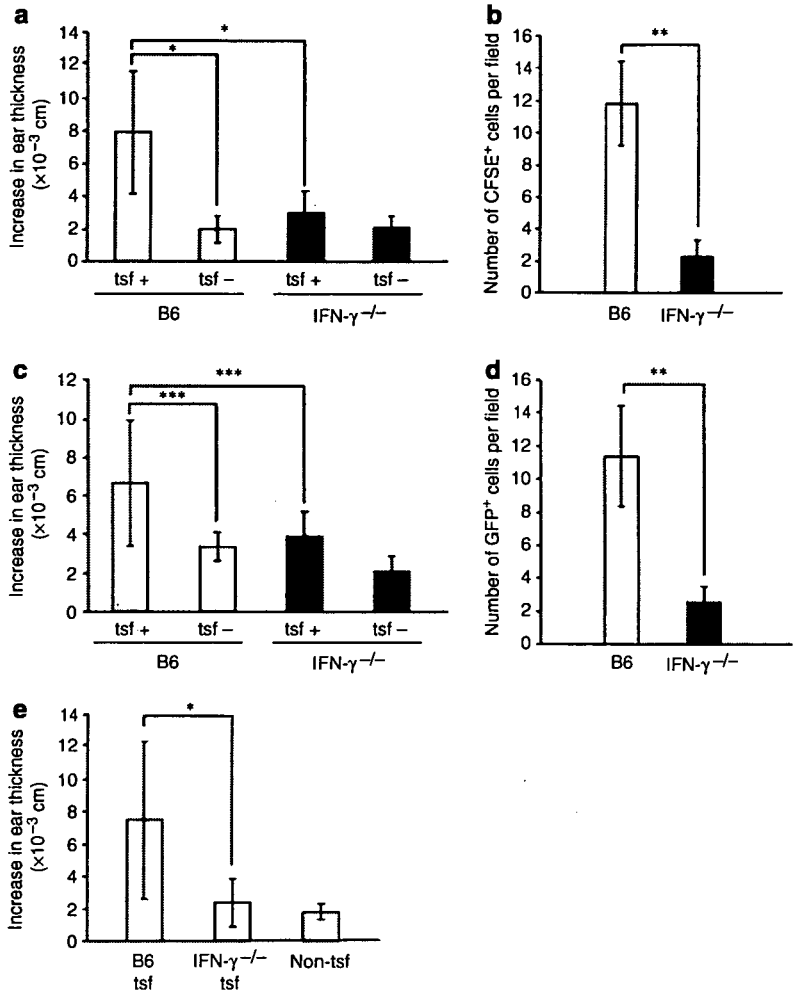

Figure 7. Adoptive transfer of CHS using immune lymph node cells labeled with CFSE and those from GFP mice. (**a**) Axillary, bradial, and inguinal lymph node cells (5×10^6 cells) of B6 mice that were sensitized 5% PCl on the shaved abdomen 5 days before were labeled with CFSE and transferred to naive B6 or IFN-$\gamma^{-/-}$ mice. Immediately after transfer (tsf), mice were challenged with 10 μl of 0.5% PCl to both sides of ears (tsf +). Non-transferred B6 and IFN-$\gamma^{-/-}$ mice were described as tsf−. The increment of ear swelling was measured at 24 hours. (**b**) Ears of B6 and IFN-$\gamma^{-/-}$ mice receiving transfer of CFSE-labeled immune lymph node cells from PCl-sensitized mice were taken 24 hours after transfer and subjected to the histological study. The number of CFSE-positive cells in the dermis was counted in six different original magnification × 200 power fields and represented by the mean. (**c**) Immune lymph node cells were prepared from GFP mice sensitized with 0.5% DNFB and transferred to naive B6 or IFN-$\gamma^{-/-}$ mice. Mice were challenged with 10 μl of 0.3% DNFB to both sides of ears (tsf +). Non-transferred mice were represented as tsf−. The increment of ear swelling was measured at 24 hours. (**d**) Ears of B6 and IFN-$\gamma^{-/-}$ mice receiving transfer of immune lymph node cells (5×10^6 cells) from DNFB-sensitized GFP mice were taken 24 hours after transfer and subjected to the histological study. The number of GFP-positive cells in the dermis was counted in six different original magnification × 200 power fields and represented by the mean. (**e**) Lymph node cells were prepared from B6 or IFN-$\gamma^{-/-}$ mice sensitized with 5% PCl and transferred to naive B6 mice. The recipient mice were challenged with 10 μl of 0.5% PCl (B6, IFN-$\gamma^{-/-}$). The non-transferred mice represent the group of mice challenged alone. The ear thickness change was measured at 24 hours. Data are expressed as the mean ± SD of four mice. *$P<0.001$, **$P<1.0 \times 10^{-6}$, and ***$P<0.05$.

expression of Th1 chemokine CXCL9 and CXCL10 in B6 mice, whereas such an augmentation was not found in IFN-$\gamma^{-/-}$ mice (Figure 8, left). As for Th2 chemokines, PCl challenge induced the expression of CCL17 and CCL22 in both B6 and IFN-$\gamma^{-/-}$ mice. Repeated FITC challenge augmented the expression of Th2 chemokines CCL17 and CCL22 in IFN-$\gamma^{-/-}$, but FITC augmentation for CCL17 and CCL22 was minimal in B6 mice. It was reported that CCL27/CTACK-CCR10 signaling is an important regulator for skin immune inflammation (Homey *et al.*, 2002). Therefore, CCL27 expression was also monitored, but there was no substantial difference between challenged and non-treated ears, or between B6 and IFN-$\gamma^{-/-}$ mice. Only marginal increment was observed in IFN-$\gamma^{-/-}$ mice that were challenged six times with FITC compared to non-treated IFN-$\gamma^{-/-}$ mice (relative quantitative mRNA: 3.1 vs 1). Accordingly, no enhanced expression of CCR10, a ligand for CCL27, was found in PCl- or FITC-challenged skin of B6 or IFN-$\gamma^{-/-}$ mice.

When CD11c$^+$ cells, namely LCs in the epidermis, were depleted from crude EC suspensions, and again subjected to real-time PCR analysis, signals of Th2 chemokines

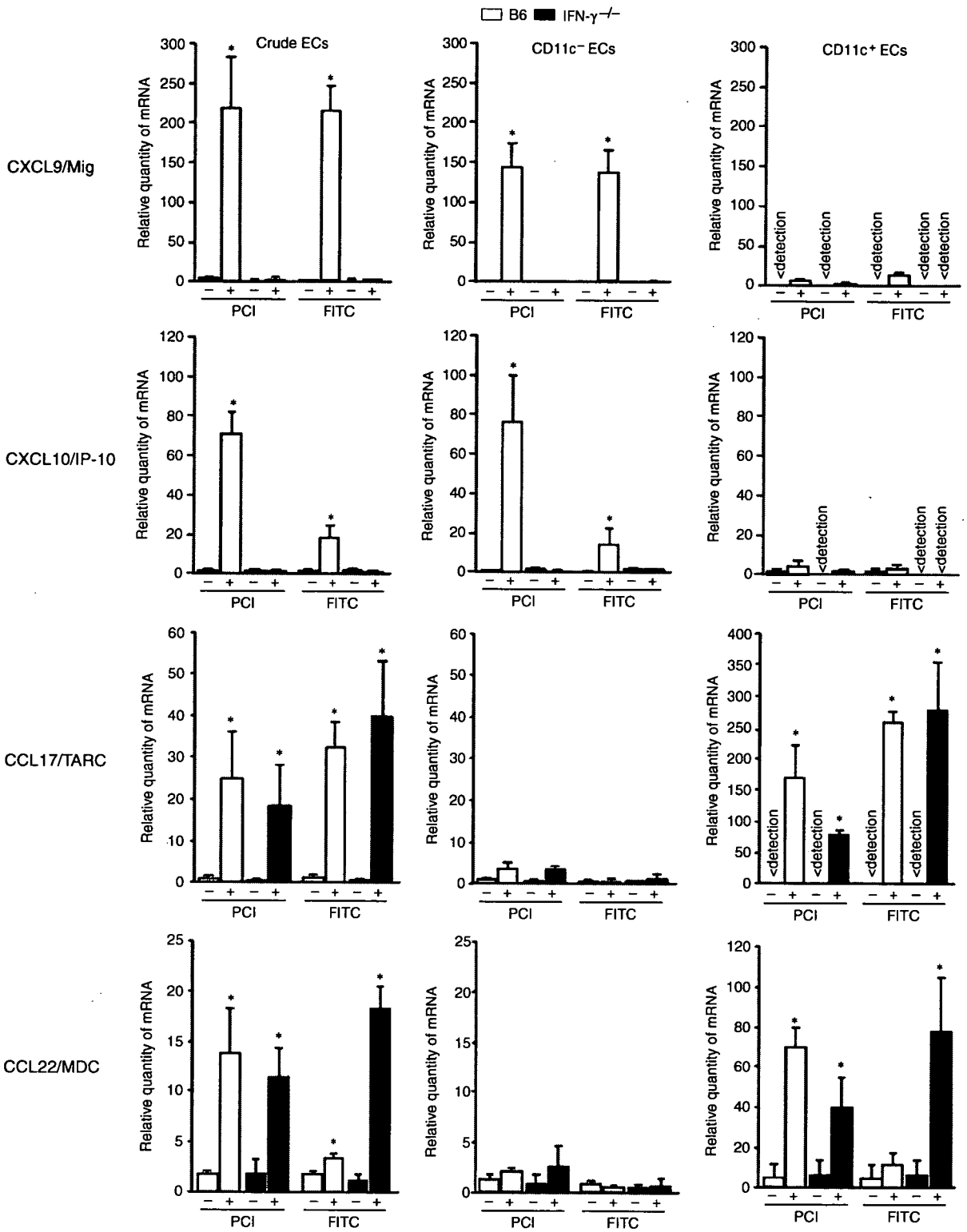

Figure 8. Real-time PCR analysis of mRNA expression for chemokines in ECs. B6 and IFN-$\gamma^{-/-}$ mice were sensitized with PCl and challenged once with PCl, or sensitized with FITC and challenged six times with FITC (twice a week for 3 weeks). Single-cell suspensions of epidermal cells were prepared from the challenged ears. Unfractionated (crude ECs), CD11c$^+$ cell-depleted (CD11c$^-$), and CD11c$^+$-purified ECs were subjected to real-time PCR analysis for Th1 chemokines (CXCL9 and CXCL10) and Th2 chemokines (CCL17 and CCL22). The expression of mRNA is represented as fold increase ($2^{-\Delta\Delta C_t}$), where $\Delta\Delta C_t = [\Delta C_t(\text{sample})] - [\Delta C_t(\text{B6 CD11c}^- \text{ ECs without treatment})]$, and $\Delta C_t = [C_t(\text{sample})] - [C_t(\beta\text{-actin})]$. Data are expressed as the mean + SD of four mice. *$P<0.03$, compared between treated B6 mice or IFN-$\gamma^{-/-}$ mice, and no treatment B6 mice.

disappeared, but those of Th1 chemokines remained (Figure 8, middle). Therefore, it was considered that Th1 chemokines are produced by keratinocytes, whereas Th2 chemokines are derived mainly from LCs.

The expression of Th2 chemokines, but not Th1 chemokines, in LCs was confirmed with $CD11c^+$ cell-purified ECs. LCs from FITC or even PCl-challenged mice expressed Th2 chemokines but not Th1 chemokines (Figure 8, right). The results indicated that IFN-γ is required for keratinocyte expression of Th1 chemokines, but suppressive for LC expression of Th2 chemokines. IFN-$\gamma^{-/-}$ mice had the decreased ability of keratinocytes to produce Th1 chemokines even by PCl challenge, but retained the ability of LCs to produce Th2 chemokines particularly upon repeated FITC challenge.

DISCUSSION

In this study, we chose two distinct experimental systems to discriminate Th1 and Th2 cutaneous hypersensitivity reactions. The ordinary delayed-type CHS to PCl was used as a Th1-mediated response, and the immediate or late-phase reaction to repeatedly challenged FITC was used as a Th2-mediated response. We investigated the differences between the wild-type and IFN-$\gamma^{-/-}$ mice in these two cutaneous hypersensitivity systems. IFN-$\gamma^{-/-}$ mice were low responders in DTH to PCl, but high responders in the immediate and late-phase responses to FITC. The Th1/Th2 dichotomy-related immunological status was confirmed by the IFN-γ expression in PCl-challenged wild-type mice and the IL-4 expression in FITC-challenged IFN-$\gamma^{-/-}$ mice.

The depression of DTH to PCl and the enhancement of immediate and late-phase responses to FITC in IFN-$\gamma^{-/-}$ mice are not particularly surprising, because the Th2-preponderant immunological balance in these mice is easily expected as a result of a relief from suppression of Th2 cells by IFN-γ. In addition, participation of IFN-γ in both afferent and efferent limbs duly results in depressed DTH. However, as inflammatory cells did not infiltrate in the PCl-challenged ears of IFN-$\gamma^{-/-}$ mice, the observed downmodulation of cutaneous DTH could not be simply attributable to the systemic immunological shift to Th2 cells or to the impaired antigen-presenting function of cutaneous dendritic cells, but the failure in infiltration of T cells to the skin might be causative. Conversely, the extent of inflammatory cell infiltrate in FITC-challenged IFN-$\gamma^{-/-}$ mice was high. Therefore, chemokines produced by ECs appeared to be critical for determining the magnitude of these cutaneous responses.

Among ECs, keratinocytes and LCs are the essential sources of chemokines (Sebastiani *et al.*, 2002). $CXCR3^+$ Th1 cells infiltrated in the PCl-challenged B6 mice, whereas $CCR4^+$ Th2 cells were seen in the FITC-challenged IFN-$\gamma^{-/-}$ mice. Our real-time PCR analysis of EC-derived chemokines revealed that Th1 chemokines, CXCL9 and CXCL10, are produced by keratinocytes, and Th2 chemokines, CCL17 and CCL22, are mainly exposed by LCs. In IFN-$\gamma^{-/-}$ mice, the Th1 chemokines were not expressed by PCl challenge, whereas the expression of Th2 chemokines retained when challenged with PCl or FITC. Therefore, it is likely that IFN-γ is necessary for the production of Th1 chemokines by keratinocytes, but unnecessary or rather suppressive for the elaboration of Th2 chemokines by LCs. It is considered that the lack of development of cutaneous DTH to PCl in IFN-$\gamma^{-/-}$ mice stems from the inability of keratinocytes to produce Th1 chemokines. In accordance with this finding, our transfer study using immune lymph node cells obtained from sensitized B6 mice and labeled with CFSE and those from sensitized GFP mice demonstrated low infiltration of transferred lymphocytes in IFN-$\gamma^{-/-}$ mice.

The role of IFN-γ for the production of CXCL9 and CXCL10 by keratinocytes has been reported (Albanesi *et al.*, 2000b; Mahalingam *et al.*, 2001; Sebastiani *et al.*, 2002). CCL17 and CCL22 have also been reported to be released from keratinocytes (Sebastiani *et al.*, 2002), and a combination of IFN-γ and TNF-α is one of the most effective stimuli for keratinocytes to produce CCL17 and CCL22 (Kobayashi *et al.*, 2004). However, this study demonstrated that keratinocytes are not the main source of these Th2 chemokines, but that LCs are the producers. In accordance with this notion, cultured normal human keratinocytes cannot produce CCL17 even under stimulation with IFN-γ and TNF-α (Tsuda *et al.*, 2003). It has been reported that LCs produce or express various chemokines, including CCL17, CCL22, CXCL11, and CCL5 (Fujita *et al.*, 2004, 2005). Cytokines such as TNF-α, GM-CSF, IL-1β, and IL-4 stimulate LCs to produce CCL17 and CCL22 (Soumelis *et al.*, 2002). It is notable that IFN-γ is not a requirement for LCs to produce CCL17 and CCL22 but rather suppressive for their production (Xiao *et al.*, 2003). In IFN-$\gamma^{-/-}$ mice, therefore, Th2 cells are prone to infiltrate in the dermis, and the late-phase reaction can occur as a result of successful migration of Th2 cells to the skin. In this context, it is an interesting issue that the infiltrating lymphocytes showed epidermotropism possibly because of Th2 chemokine production by LCs. The production of the chemokines by LCs may support the recent concept that LCs are rather regulatory for the development of CHS (Kaplan *et al.*, 2005; Kissenpfennig *et al.*, 2005).

Interferon-γ, as well as IL-12 (Wolf *et al.*, 1994), is the key cytokine for Th1 responses. Furthermore, recent accumulating evidence has suggested the important role of IL-17 for CHS (Nakae *et al.*, 2002; He *et al.*, 2006). As IL-17 stimulates keratinocytes to release certain cytokines and chemokines with synergistic or additive effects when used together in IFN-γ (Albanesi *et al.*, 2000a), our results in IFN-$\gamma^{-/-}$ mice possibly stem from the relief of the synergistic effects. Our study suggests that IFN-γ has a mandatory role for conditioning the skin milieu by modulating Th1 and Th2 chemokines.

MATERIALS AND METHODS

Animals and chemicals

Eight to 10-week-old female B6 mice were purchased from Japan SLC (Hamamatsu, Japan) and Kyudo Co. (Tosu, Japan). B6 and IFN-$\gamma^{-/-}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME), and GFP mice were obtained from Japan SLC. These mice were maintained in the Laboratory Animal Research Center in University of Occupational and Environmental Health under specific pathogen-free conditions. Female mice were used in this study. All animal experiments were performed according to the guidelines for the care

and use of animals approved by our university. PCl was purchased from Tokyo Kasei (Tokyo, Japan), FITC from Sigma Chemical Co. (St Louis, MO), and DNFB from Nakalai Tesque (Kyoto, Japan).

Sensitization and elicitation of CHS

For induction of CHS to PCl, mice were painted on the clipped abdomen with 50 μl of 5% PCl in ethanol/acetone (3:1). Five days after sensitization, mice were challenged by painting both sides of each ear with 20 μl of 0.5% PCl in ethanol/acetone (3:1). The ear thickness of mice was measured using dial thickness gauge (Ozaki Co., Tokyo, Japan) before and 24 hours after challenge. Ear swelling was calculated as (ear thickness after challenge)−(ear thickness before challenge).

For induction of CHS to FITC, mice were painted on the clipped abdomen with 200 μl of 1% FITC in acetone/di-*n*-butylphthalate (1:1). Five days after sensitization, mice were challenged by painting both sides of each ear with 20 μl of 0.5% FITC in acetone/di-*n*-butylphthalate (1:1) twice a week for 3 weeks. The ear thickness of mice was measured using dial thickness gauge before, 0, 1, 4, and 24 hours after challenge.

For induction of CHS to DNFB, mice were painted on the clipped abdomen with 50 μl of 0.5% DNFB in acetone/olive oil (4:1). The ears were challenged with 20 μl of 0.3% DNFB in acetone/olive oil (4:1).

Preparation of ECs, CD11c$^+$ cell-depleted ECs, purified CD11c$^+$ ECs, and lymph node cells

Ears were obtained from mice 24 hours after the last challenge with PCl or FITC, and epidermal and dermal skin was obtained by removing cartilage and subcutaneous tissues. After incubation for 1 hour at 37 °C in a 0.2% solution of trypsin in PBS, the epidermis was separated from the dermis, and ECs were dispersed in PBS supplemented with 10% fetal calf serum (Gibco, Carlsbad, CA) by rubbing the separated epidermal sheets (Tokura *et al.*, 1994). The cells were filtered through a cell strainer and washed twice in PBS. In some experiments, CD11c$^+$ ECs were purified with automagnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). After ECs were incubated with RPMI 1640 supplemented with 10% fetal calf serum for 30 minutes, ECs were first reacted with CD11c microbeads (120-000-322; Miltenyi Biotec) for 30 minutes and selected for CD11c$^+$ ECs, representing LCs. The purity of CD11c$^+$ cells was 70–80% (Sugita *et al.*, 2007). After removing CD11c$^+$ cells, CD11c$^-$ ECs were obtained and CD11c$^+$ cells were less than 0.1%. Among CD11c-positive fractionated cells from ECs, more than 99% of CD11c$^+$ cells were Langerin (eBioscience, San Diego, CA) positive (data not shown).

B6 and IFN-γ$^{-/-}$ mice were sensitized with 5% PCl and challenged once with 0.5% PCl, or sensitized with 1% FITC and challenged six times with 0.5% FITC (twice a week for 3 weeks). Single-cell suspensions of immune cervical lymph node cells were prepared from the treated mice.

Histological assessment

Twenty-four hours after challenge with PCl and FITC, mice were killed by cervical dislocation and ears were amputated. The ears were fixed with formalin and embedded in paraffin. Two- to three-micrometer- thick sections were cut and stained with hematoxylin and eosin. An indirect immunohistochemical staining was performed. The first antibodies included anti-CD3 (clone KT3, rat IgG2a; Chemicon, Billerica, MA), anti-IgE (clone 23G3, rat IgG1κ; eBioscience), and IFN-γ (clone XMG1.2, rat IgG1κ; eBioscience) and mAbs. A goat anti-rat IgG antibody conjugated with horseradish peroxidase was used as the second antibody, and reaction was visualized with diaminobenzidine.

Adoptive transfer

B6 mice were sensitized with 50 μl of 5% PCl in ethanol/acetone (3:1) on the clipped abdomen. Five days after sensitization, the axillary, bradial, and inguinal lymph nodes were removed, single-cell suspensions were prepared and labeled with 6.5 μM CFSE (Molecular Probes, Eugene, OR). The cells resuspended in RPMI containing 0.2% fetal calf serum were injected intravenously to naive B6 mice or naive IFN-γ$^{-/-}$ mice (5×10^6 cells per mouse). Immediately after the cell transfer, the ears were challenged with 20 μl of 0.5% PCl in ethanol/acetone (3:1), and the increase in ear swelling was measured 24 hours later. In parallel, ears of transferred B6 and IFN-γ$^{-/-}$ mice were histologically examined 24 hours after cell transfer. CFSE-positive cells in about 7 μm ear section of each mouse were counted.

In another transfer study, GFP mice were sensitized with 50 μl of 0.5% DNFB in acetone/olive oil (4:1). Five days later, the immune lymph node cells were prepared as above and injected intravenously to naive B6 mice or naive IFN-γ$^{-/-}$ mice (5×10^6 cells per mouse). The ears were challenged with 20 μl of 0.3% DNFB in acetone/olive oil (4:1). The ear swelling response, histology, and GFP-positive cells were examined as above.

Real-time quantitative PCR

Total cellular RNA was extracted with an RNA extraction kit (Promega, Madison, WI) from freshly prepared skin samples. RNA was then reverse-transcribed and amplified by random hexamer in single-tube assay using the TaqMan Reverse Transcription Reagants (Applied Biosystems, Foster City, CA) with gene-specific sense and antisense primers and a detection probe labeled on the 5′-end with the reporter dye 6-FAM. Primers and probes were obtained from TaqMan Gene Expression Assays Inventories (Applied Biosystems). Using an ABI Prism 7000 Sequence Detection Systems (Applied Biosystems), duplicate samples were reverse-transcribed and amplified under the following consecutive steps: 2 minutes at 50 °C, 10 minutes at 95 °C, followed by 50 amplification cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Sequence-specific amplification was detected as an increased fluorescent signal of 6-FAM exceeding the threshold limit during the amplification cycle. Quantification of gene-specific message levels was determined by comparing fluorescence intensity from unknown RNA samples to the fluorescence intensity of standard curve generated from control mRNA levels. Amplification of the gene for mouse β-actin was performed on all samples to control interspecimen variations in RNA amounts. The result for each gene was normalized to the quantity of mouse β-actin detected in the sample. Levels of gene-specific message were graphed as normalized message units as determined from standard curve.

Statistical analysis

Data were analyzed using an unpaired two-tailed *t*-test. $P<0.05$ was considered to be significant.