Overexpression of IL-4 Alters the Homeostasis in the Skin

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IL-4 has been implicated to play an important role in the pathogenesis of many inflammatory diseases including skin diseases such as atopic dermatitis. Because it is not clear which pathologic features of atopic dermatitis are dependent on IL-4, we assessed the consequences of IL-4 overexpression in the skin, using transgenic mice overexpressing IL-4 ubiquitously. Although transgenic mice display no clinical signs of skin inflammation, IL-4 induced a wide spectrum of pathologies including an increased number of mast cells and Langerhans cells in dermis and epidermis, respectively, focal deposition of collagen and a considerably reduced adipocyte layer in the dermis as well as an increased mitotic activity of keratinocytes, reflected in acanthosis and hyperkeratosis. The increase in Langerhans cell number may be explained in part by the substantially reduced capacity by increasing the expression of MHC class II molecules.

Langerhans cell emigration from the epidermis in transgenic mice. The molecular mechanism behind this phenomenon remains to be clarified. Under in vitro culture conditions, Langerhans cells from transgenic mice undergo a maturation process similar to that of Langerhans cells from control mice, and their immunostimulatory capacity is also comparable. In contrast, transgenic Langerhans cells are superior to control Langerhans cells in their antigen-processing capacity. We conclude that the overexpression of IL-4 in the skin is, by itself, not sufficient for the induction of a full-blown atopic dermatitis phenotype, but several changes seen in the skin of transgenic mice mirror the cardinal pathologic manifestations of this disease. Keywords: adipocyte/IL-4 transgenic/keratinocyte/Langerhans cell/mast cell. J Invest Dermatol 118:767-778, 2002

The prevalence of chronic inflammatory skin diseases of atopic nature, such as chronic atopic dermatitis (AD), is still rising. In many cases, it is associated with elevated serum IgE levels, peripheral blood eosinophilia, as well as rhinoconjunctivitis and/or bronchial asthma. Genetic susceptibility, abnormal lipid synthesis with epidermal barrier dysfunction, and an altered inflammatory and immune response to irritants and allergens were suggested to contribute to AD pathogenesis (Leung, 1995; Rudikoff and Lebwohl, 1998). Acute or early lesions of AD are characterized by spongiosis and a sparse epidermal infiltrate of T lymphocytes. There is often marked dermal edema with a prominent infiltrate of inflammatory cells, particularly T lymphocytes. Mast cells are only slightly increased in number, but they are in various stages of degranulation, which indicates activation of these cells. Chronic lesions of AD exhibit hyperkeratosis, epidermal hyperplasia, minimal spongiosis, and upper dermal fibrosis. The dermal inflammatory infiltrate is composed mainly of macrophages and eosinophils, which have been found to release major basic protein (Rudikoff and Lebwohl, 1998). An increased number of not-activated mast cells, and cells belonging to the dendritic cell (DC) lineage, including dermal DC, epidermal Langerhans cells, and a distinct population of inflammatory dendritic epidermal cells expressing CD1a, CD1b, or CD36, or combinations thereof, has also been observed (Bos et al, 1986; Bieber et al, 1988; Taylor et al, 1991; Hormanseimo et al, 1994; Rudikoff and Lebwohl, 1998). Additional characteristics of DC in AD are the markedly upregulated expression of the high-affinity receptor for IgE (FcεRI) (Wollenberg et al, 1996; Klubal et al, 1997) and CD86 (Ohki et al, 1997). Influx of activated T lymphocytes into the skin lesions represents a hallmark in AD (Grewe et al, 1998) and recent results indicate a dynamic T cell-derived cytokine production in AD. In addition to the well-known Th2/Tc2 component of acute lesions (Aldis et al, 1999), chronic lesions are characterized by an Th1/Th0 cytokine pattern (Grewe et al, 1998). To what degree IL-4 contributes to the development and perpetuation of underlying pathologies remains speculative. IL-4 is a pleiotropic cytokine and is produced by CD4+ helper T cells of the Th2 subset, some CD8+ T cells, activated mast cells, basophils, and eosinophils. IL-4 affects a broad spectrum of different cell types including T cells, B cells, natural killer cells, mast cells, monocytes/macrophages, endothelial cells, fibroblasts, adipocytes, DC, Langerhans cells, and keratinocytes and regulates the immune response in a number of ways (Chomarat et al, 1998). In T cells, IL-4 directs the development of undifferentiated T cells into IL-4-producing Th2 cells (Brown and Hural, 1997; Chomarat et al, 1998). IL-4 promotes growth and increased survival in B cells, enhances their antigen-presenting capacity by increasing the expression of MHC class II molecules.
and low-affinity Fcε receptors, and Th2-derived IL-4 induces isotype switching from IgM to IgG1 and IgE production in B cells. As it has long been recognized that IL-4 plays a crucial role in directing the adaptive immune response of T and B cells and that IL-4 is a key factor in the pathogenesis of atopy, therapeutic strategies aiming to block IL-4 activity (e.g., by monoclonal antibodies, soluble receptors, or small molecular compounds) have been developed mostly to prevent asthma and other IgE-mediated diseases (Renz, 1999; Wong and Koh, 2000; Mitchell and Abbas, 2001). Conceivably, such an approach could also be useful in the treatment of AD, because IL-4 also influences cells known to be phenotypically and/or functionally altered in AD, i.e., keratinocytes, fibroblasts, endothelial cells, and Langerhans cells.

While this report was in progress, a study was published where normal appearing skin of these mice revealed no pathology; however, a large percentage of these mice spontaneously developed a pruritic inflammatory skin disease with many of the key features of AD. We studied the structural, phenotypic, and functional properties of skin and individual skin cell populations in tg mice overexpressing IL-4 ubiquitously (Erb et al, 1994), and found that IL-4 failed to induce a spontaneous skin disease but induced a wide spectrum of pathologies including an increased number of Langerhans cells, mast cells, focal deposition of collagen, a reduced adipocyte layer, acanthosis, and hyperkeratosis.

**MATERIALS AND METHODS**

**Mice** Newborn to 42-wk-old female IL-4 tg mice (B6C3HFr/H-2R) were used in the experiments (Erb et al, 1994). The non-tg offspring served as age-matched, littermate controls. Six to twelve-wk-old female inbred mice [BALB/c (H-2b)] were obtained from Charles River Wiga GmbH (Sulzfeld, Germany).

**Reagents** RPMI 1640 medium was supplemented with 10% heat-inactivated fetal calf serum (PAA Laboratories GmbH, Linz, Austria), 25 mM HEPES, 10 μg gentamycin per ml, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 50 μM 2-ME, and 1× antibiotic–antimycotic solution (all from Gibco Life Technologies, Grand Island, NY). Unlabeled or FITC-conjugated monoclonal antibodies (MoAb) 145–2C11 and 500A2 (anti-CD3), GK1.5 and RM4–4 (anti-CD4), 53–6–72 (anti-CD8), B3B4 (anti-CD23), J11d and M1/69 (anti-CD24), 3C7 and 7D4 (anti-CD25), H2a/5 (anti-CD29), 3/23 (anti-CD40), IM7.8.1 (anti-CD44), 30F11.1 (anti-CD45), 2G2 (anti-CD5Rb), RL-1-2 (CD49d), RA3–6B2 (anti-CD45R/B220), 3E2 (anti-CD54), (MEI-14–14 (anti-CD62L), 16–10A1 (anti-CD680), GL1 (anti-CD86), and G7 (anti-CD90) were purchased from Pharmingen (San Diego, CA). The MoAb NLDC-145 (anti-DEC-205), ECCD-2 (anti-E-cadherin), F7D5 (anti-CD90), and the polyclonal antibody Ki-67 were obtained from Serotec (Oxford, U.K.), Zymed (South San Francisco, CA), Biosource (Camarillo, CA), and Novocastra (Newcastle, U.K.), respectively. The resulting cell suspension was either analyzed by flow cytometry or prepared for FACS-sorting experiments. Briefly, cultured EC were incubated with a FITC-labeled anti-DEC-205 MoAb and subsequently sorted with a FACS Vantage (Becton Dickinson, Mountain View, CA), yielding a highly viable (>98%) suspension of >95% DEC-205+ cells. Keratinocytes from control and tg mice were isolated and cultured (1×10⁶ cells per ml) in 75 cm² flasks (Costar, Cambridge, MA) at 37°C. After 3 d, nonadherent EC were harvested and dead cells were largely eliminated by density gradient centrifugation (Lymphocyte-M, Cedarlane Laboratories, Hornby, Ontario, Canada). The resulting cell suspension was either analyzed by flow cytometry or prepared for FACS-sorting experiments. Briefly, cultured EC were incubated with a FITC-labeled anti-DEC-205 MoAb and subsequently sorted with a FACS Vantage (Becton Dickinson, Mountain View, CA), yielding a highly viable (>98%) suspension of >95% DEC-205+ cells. Keratinocytes from control and tg mice were isolated and cultured (1×10⁶ cells per ml) in 24-well plates as described (Carroll et al, 1995). At selected time points, adherent and nonadherent keratinocytes were harvested from both cultures, and cell numbers and viability were assessed.

**IL-4 ELISA** EC suspensions of control and tg mice were cultured (2×10⁵ cells per ml) in 24-well plates (Costar). At selected time points, supernatants were collected and stored at −20°C until use. IL-4 concentrations were determined by ELISA (Endogen, Woburn, MA) according to the manufacturer’s instructions.

**Histology, immunocytochemistry, and ultrastructural analyzes** Ear thickness of anesthetized control and tg mice (7–15 mice per group) was measured using an engineer’s micrometer (Hahn und Kolb, Stuttgart, Germany). Alternatively, adult mice were killed and the ears and skin of the midback region were removed and cut in half. One half was embedded and snap frozen in liquid nitrogen; the other half was fixed in 4% neutral-buffered formalin, routinely processed and embedded either in paraffin or in Technovit 7100 (Kulzer, Wehrheim, Germany). Sections (3 μm) were stained using (i) Mason trichrome, for the identification of collagen fibers; (ii) 10% Giemsa solution (Merck, Darmstadt, Germany) at pH 5.5, for the identification of eosinophils and mast cells; (iii) Luna’s Biebrich red for identification of eosinophils; or (iv) immunocytochemical techniques (e.g., Ki67), and were then examined by light microscopy. Immunohistochemical staining for Ki67...
antigens was performed using the 3-Amino-9-Ethylcarbazole staining kit (Dako, Vienna, Austria) according to the manufacturer’s recommendations. Ear and back skin sections were analyzed for the presence of mast cells using Olympus BX60 (Olympus, Austria, Vienna) and analySIS, an imaging and analysis system (Soft Imaging Systems, Munich, Germany), which provided direct morphometric measurements from digitized images obtained by a video camera. The entire section areas were analyzed and the total number of mast cells per mm² of dermis was calculated.

Epidermal sheets from ear and back skin were prepared using the ammonium-thiocyanate separation technique and either incubated with the FITC-labeled anti-MHC class II MoAb M5/114 for 60 min at 37°C or stained for ADPase activity (Elbe et al., 1989). Sheets were mounted on glass slides in PBS/glycerol (Difco, Detroit, MI), cover slipped, and viewed under a fluorescence microscope (Leitz Diaplan, Wetzlar, Germany). Observed staining patterns were documented with a Leitz Orthomate E system (Wetzlar) using 29 DIN artificial light color film (Scotch Chrome, 640-T, 3M, Milan, Italy). Labeled cells in the epidermal sheets were enumerated at 400× magnification using a rectangular grid. Eighty to a hundred fields were randomly chosen and the density of positive cells was determined and expressed as the number of cells (±SD) per mm² of skin surface. Experimental groups consisted of four mice each.

Transmission electron microscopy of single EC suspensions was performed as described (Berger et al., 1992).

Ear skin explants Ears from tg mice and controls were dissected, rinsed with 70% ethanol, air-dried, and split with forceps into dorsal (i.e., cartilage-free) and ventral halves. In some experiments, skin was separated into dermis and epidermis by means of dispase before the onset

Table I. Ear thickness in IL-4 tg mice

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>No. of mice</th>
<th>Control</th>
<th>IL-4 tg</th>
<th>% vs. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>11</td>
<td>25.3 ± 2.4</td>
<td>29.2 ± 4.7*</td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>25.8 ± 1.5</td>
<td>30.4 ± 2.3***</td>
<td>18</td>
</tr>
<tr>
<td>9</td>
<td>15</td>
<td>25.4 ± 1.3</td>
<td>28.1 ± 3.8**</td>
<td>11</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>24.3 ± 1.2</td>
<td>29.7 ± 3***</td>
<td>22</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>26 ± 1.5</td>
<td>29.1 ± 2***</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>26.1 ± 1.7</td>
<td>32.4 ± 2.6***</td>
<td>24</td>
</tr>
</tbody>
</table>

*p < 0.05; **p < 0.01; ***p < 0.001.

Table II. Mast cell accumulation in IL-4 tg skin

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>No. of mice</th>
<th>Tissue</th>
<th>Mast cells per mm² ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>2</td>
<td>ear</td>
<td>0.081 ± 0.03 0.174 ± 0.003</td>
</tr>
<tr>
<td>17</td>
<td>3</td>
<td>back skin</td>
<td>0.049 ± 0.01 0.163 ± 0.06</td>
</tr>
</tbody>
</table>
of culture (Kitano and Okada, 1983; Lenz et al., 1993). Dorsal ear halves were floated dermal side down on 2 ml of culture medium in 24 well tissue culture plates (Costar) for 3–7 d, one ear half per well (Larsen et al., 1990; Ortner et al., 1996). Depending on the duration of the culture period, they were fed every 3 d by carefully aspirating 500 μl of spent medium and adding back the same volume of fresh medium. Non-adherent migratory cells were recovered from the bottom of tissue culture wells after 3, 5, and 7 d by gentle rinsing and total cell numbers and viability were determined by counting in a hematocytometer in the presence of trypan blue to assess cell viability. Five × 10³ viable cells were placed on each reaction field of an adhesion slide (Bio-Rad, Richmond, CA) and incubated in a humidiﬁed chamber for 30 min at room temperature to allow their sedimentation. Anchoring of viable cells to the positively charged reaction ﬁeld was monitored under an inverted microscope and nonattached cells were rinsed off with PBS. Attached cells were ﬁxed with acetone for 10 min at room temperature and consecutively reacted with biotinylated anti-MHC class II-Streptavidin Texas Red/FITC-CD45 MoAb or isotype-matched control MoAb, washed, and embedded in Glycergel (Dakopatts). Data represent the mean ± SD of DC per well over four wells.

Flow cytometry For two-color analyzes, cells (3 × 10⁵ per sample) were resuspended in cold PBS/1%FCS/0.1%NaNO₃ and serially incubated with FITC-conjugated MoAb directed against selected mouse antigens and biotinylated anti-CD45 MoAb followed by SAv-PE. For the detection of E-cadherin molecules, EC were prepared as described (Tang et al., 1993). Specificity of staining was conﬁrmed using isotype-matched control MoAb. Fluorescence was measured using a FACScan ﬂow cytometer, and data were analyzed with Cell Quest software (both from Becton Dickinson). Dead cells were excluded by 7-aminoactinomycin D (Sigma) uptake.

Mixed lymphocyte reactions (MLR) T cells were prepared from mesenteric lymph nodes of BALB/c mice using Ab- and C-mediated lysis as described (Elbe et al., 1994). Briefly, cell suspensions were passed through nylon wool columns, and nonadherent cells were treated with a cocktail of the following MoAb: 3C7, 7D4, J11d.2, M5/114, IM7,
to deplete some keratinocytes and epidermal T cells. After removal of dead cells by density gradient centrifugation, resulting cells (1.5 x 10^6 cells per ml) were cultured either with or without HEL (1 mg per ml) and OVA (2 mg per ml) for 20 h in 24 well plates. Thereafter, nonadherent cells were adjusted to equal numbers of Langerhans cells as determined by flow cytometry. Antigen-pulsed and, for control purposes, unpulsed control and tg Langerhans cells (5 x 10^5 per well) were cultured either with hybridoma cells (10^4 per well) or alone in 96 well flat-bottom microtiter plates. Supernatants were harvested after 24 h and stored at -20°C until use.

**Statistical analysis** Two-tailed t test was used to evaluate the significance of experimental *versus* control groups.

**RESULTS**

**IL-4 tg EC produce IL-4** To test whether functional protein is produced in tg epidermis, we examined the supernatants of EC cultures for the presence of IL-4 by ELISA. As shown in Fig 1, supernatants from cultures derived from tg mice contained at all time points elevated amounts of IL-4 relative to cultures derived from control mice.

**IL-4 alters the skin architecture** Tg mouse skin appeared dry with signs of scaling but exhibited no macroscopic signs of skin disease. Light microscopic examination of ear sections (Figs 2A, B) and ear measurements (Table I) revealed a significantly thickened skin in tg mice compared with controls. This was partly due to acanthosis and hyperkeratosis and partly to deposition of collagenous material in the superficial and deep dermis as demonstrated by trichrome staining (Figs 2A, B). Giemsa-stained sections revealed an increase in mast cell numbers in the ear and back skin dermis of tg mice by a factor of approximately two (Figs 2C, D, Table II). In contrast to control mice, considerable numbers of mast cells in the tg skin were located between fat cells and in the muscle layer. The observation that the tg dermis had less and smaller sized adipocytes than control dermis was striking (Figs 3A, B). In some tg mice the dermal fat tissue disappeared completely (Fig 3C). (Immuno)histochemistry of the tg dermis showed no evidence of acute inflammation (no eosinophil, T cell, macrophage and neutrophil infiltration) (data not shown). Two approaches were chosen to study the hyperplastic epidermal morphogenesis. First, proliferating keratinocytes were identified by immunohistochemistry. Although Ki-67" nuclei were observed in the basal skin layer of both control and tg mice (Figs 4A, B), the proportion of Ki-67" cells was greater in tg mice, indicating accelerated proliferation of epidermal keratinocytes. Scattered Ki-67" cells were also identified in the suprabasal layers of the tg epidermis (data not shown). In a second series of experiments, we compared the yields of viable cells in keratinocyte cultures from control and tg mice at selected time points. Over the entire observation period of 10 d, we found a 2–3-fold increase of keratinocyte cell numbers in cultures isolated from tg mice (Fig 4C). Thus, we conclude that IL-4 causes keratinocyte hyperproliferation, induces mast cell accumulation and dermal collagen deposition, and leads to a loss of fat tissue in the dermis.

**Impaired emigration of IL-4 tg Langerhans cells from ear skin explants** IL-4 has been described as a factor enhancing the yield of bone marrow-derived DC in vitro (Lutz et al, 2000). We investigated if and how IL-4 influences the numbers of epidermal Langerhans cells in our tg mice. Epidermal sheets from the ears of control and tg mice were prepared and the distribution and numbers of Langerhans cells were compared. Immunophenotypic and quantitative analyzes revealed that the epidermis from tg mice contained 2–3-fold higher numbers of dendritic MHC class II" cells than controls (Fig 5A–C). Notably the tg epidermis contained clusters with cells that stained much more brightly with an anti-MHC class II MoAb than Langerhans cells from control animals (Fig 5B, inset). By using ADPase, a well-recognized marker for dendritic cells, the numbers of dendritic, ADPase" cells in the epidermis of tg mice were increased 2–3 times as compared with

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**Figure 5. Accumulation of Langerhans cells in the IL-4 tg epidermis.** Ear epidermal sheets from control (A, D) and IL-4 tg mice (B, E) were either exposed to the FITC-labeled anti-MHC class II MoAb M5/114 (A, B) or stained for membrane ADPase activity (D, E). The epidermis from tg mice (black columns) contained 2–3-fold higher numbers of dendritic MHC class II/ADPase" cells than controls (hatched columns). It is notable that the tg epidermis contained several clusters with Langerhans cells that stained much more brightly with the anti-MHC class II MoAb (inset), and that tg Langerhans cells show stronger ADPase activity than control Langerhans cells (D, E). p < 0.001 vs. control (G), p < 0.01 vs. control (F). Scale bars: (B) 50 µm; (inset) 25 µm; (E) 20 µm.

RA3–3A1 for 30 min at 4°C. Subsequently, cells were incubated with Low-tox-M rabbit C′ (Cedarlane) for 45 min at 37°C; 97%–99% of these cells were CD3" as determined by flow cytometry. To purify CD4" or CD8" T cells, MoAb 3.168.81 or GK1.5 plus 2B6.2D8 were added to the Ab cocktail. T cells were > 97% CD44low, CD45RBhigh CD62Lhigh as determined by flow cytometry (data not shown). Control and IL-4 tg EC were irradiated (X-ray, 15 Gray, 1.5 Gray per min, Philips RT 305, Philips, Vienna, Austria) and cultured in 96 well round-bottom microtiter plates (5 x 10^4 per well). After 3 d, purified lymph node T cells (2 x 10^5 per well) were added to the cultures. In other experiments, cultured, FACS-sorted Langerhans cells were incubated with either allogeneic CD4" (>98%) or CD8" (>99%) lymph node T cells, or alone in 96 well round bottom culture plates (Costar) at 37°C. At the indicated time points, 37 KBq (3H)-TdR was added to each well for 10–12 h. Thereafter, cells were harvested and (3H)-TdR incorporation was measured in a liquid scintillation counter (Packard Instruments, Meriden, CT). Data are expressed as mean cpm ± SD of triplicate unless indicated otherwise.

**Antigen-processing assays** The processing activity of control and tg Langerhans cells (H-2b/k) was measured using MHC class II-restricted T cell hybridomas (C10.9, HEL-specific, I-Ak-restricted, E8, OVA-specific, I-Eb-restricted). The activation of hybridoma cells was determined by measuring IL-2 production by ELISA (Endogen). Briefly, freshly prepared control and tg EC were treated with anti-Thy-1.2 MoAb for 30 min at 4°C, followed by Low-Tox-M-rabbit C′ for 40 min at 37°C.
cells in these mice, i.e., (i) Langerhans cell proliferation in situ, (ii) increased influx of Langerhans cell progenitors from the blood into the skin, (iii) a decreased eflux of Langerhans cells from the epidermis, and (iv) reduced apoptosis of Langerhans cells. By electron microscopy, we found that tg Langerhans cells, similar to controls, displayed an indented nucleus and contained typical Birbeck granula. Regular cell organelles such as Golgi, endoplasmic reticulum, and mitochondria were well developed (data not shown). The screening of numerous sections revealed no mitotic Langerhans cells in control and tg skin, suggesting that the enhanced Langerhans cell density in the tg mouse epidermis is not due to in situ proliferation. To test whether IL-4 overexpression causes an abnormal influx of Langerhans cells progenitors from the blood to the skin, epidermal sheets from newborn tg and control mice were assessed for the presence of Langerhans cells. Surprisingly, we found less ADPase+ cells in the newborn tg epidermis compared with controls. Fourteen days postpartum the numbers of ADPase+ cells in the tg epidermis and in the controls were similar and later increased in tg mice (data not shown). These data imply that IL-4, either directly or indirectly, partially inhibits the entry of Langerhans cell precursors into the epidermis. The epidermal skin explant model was used to test the hypothesis that tg mice have a deficiency in the Langerhans cell mobilization. This model allows for the examination of Langerhans cell migration from the epidermis to the dermal lymphatics and may reflect, in part, their in vivo migratory capacity (Larsen et al., 1990). We found that over 3 d of culture, the relative decrease of Langerhans cells in the epidermis was greater in control than in tg mice (Figs 6A, B, G). In control mice, migrating DC accumulated in a characteristic string-like pattern (i.e., cords) in dermal lymphatic vessels (Figs 6C, E), whereas cords were reduced in tg mice, and if present, contained only few MHC class II+ cells (Figs 6D, F). At later time points (days 5–7) cords were also formed, though considerably less abundant than in controls (data not shown). Emigration was measured by counting the numbers of Langerhans cells/DC present in the culture medium. After 3 d, the numbers of emigrated MHC class II+, CD45+ Langerhans cells/DC from the skin of control mice was 4–5-fold higher than from tg skin (Fig 6H). Even after 5 d of culture, Langerhans cells/DC numbers present in the wells with tg skin explants were lower when compared with numbers obtained from control skin explants (data not shown). To test whether tg Langerhans cells per se are able to leave the epidermis, epidermal sheets were cultured and the emigration capacity of Langerhans cells was tested. After 3 d, the number of emigrated MHC class II+, CD45+ Langerhans cells from the epidermis of tg mice was 2–3-fold higher than from control epidermis (numbers of cells per ear half: tg: 2.6 × 10^4 ± 0.4; control: 0.7 × 10^4 ± 0.1). In accordance with this was the observation that the cultured epidermis from both tg and control mice was almost negative for MHC class II+ cells. In conclusion, these data suggest that tg Langerhans cells, similar to control Langerhans cells, are able to leave the epidermis as such but, in contrast to control Langerhans cells, they are less capable of passing the epidermal basement membrane in intact skin and entering the lymphatic vessels. Alternatively, an IL-4-induced product (e.g., IL-4 activated mast cells) could possibly inhibit Langerhans cell migration from the epidermis in IL-4 tg mice. Cytokines produced by EC in situ are thought to play a major role in Langerhans cell survival and migration. To investigate whether differences in the cytokine milieu between control and tg epidermis could be responsible for the failure of tg Langerhans cells to migrate to the dermis, we compared the cytokine production in EC cultures derived from control and tg mice. Surprisingly, we found that the levels of cytokines that are known to promote Langerhans cell emigration, survival, and maturation (e.g., TNF-α, GM-CSF, IL-1) (Koch et al., 1990; Wang et al., 1997; Stoitzner et al., 1999) were higher in the supernatants of tg EC than in those from control mice (Fig 7). Taken together our data suggest that the reduced emigration of epidermal Langerhans cells in skin explants may be a result of a cytokine imbalance in the skin of tg mice. Whether

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**Figure 6. Impaired migration of IL-4 tg Langerhans cells out of skin explants.** Dorsal ear skin from control (A, C, E) and IL-4 tg (B, D, F) mice was floated on medium and cultured in 24 well plates. After 3 d, epidermal (A, B) and dermal (C–F) sheets were prepared and stained with the FITC-labeled anti-MHC class II MoAb M5/114. Considerably more Langerhans cells could be detected in tg epidermis compared with controls (A, B, G, tg = black columns, control = hatched columns). MHC class II+ cells in epidermal sheets were counted and expressed as the mean ± SD. In the dermis, migrating DC formed cords within the dermal lymphatics of control mice (C, E). In contrast, these cords were hardly detectable in tg mice (D, F). After 3 d, nonadherent cells were harvested from the bottom of wells and the total cell number was determined by counting with a hematocytometer. The proportion of DC was calculated by examining anti-FITC-CD45, biotinylated MHC class II-TR-SA-stained adhesion slides. Results represent the mean ± SD of DC per well over four wells (H, tg = black column, control = hatched column). The number of emigrated DC from the skin of control mice was 4–5-fold higher than from tg skin. p < 0.001 vs. controls (G, H). Scale bars: (B, F) 50 μm; (D) 100 μm.

controls (Fig 5D–F), implying that most, if not all, MHC class II+ cells in the tg mouse epidermis are indeed Langerhans cells. Similar to our finding in the ear epidermis, the numbers of MHC class II+/ADPase+ cells with dendritic morphology were increased in the abdominal epidermis from tg mice (data not shown). Several possible hypotheses exist to explain the accumulation of Langerhans cells and their impaired migration in the epidermis of IL-4 tg mice.
cytokines other than those investigated are also changed cannot be definitively excluded at this point. We are currently performing studies to determine which other cytokines and chemokines are induced in IL-4 EC in situ.

The overexpression of IL-4 affects the Langerhans cell phenotype in situ To test whether IL-4 and the changed epidermal cytokine milieu affects the expression of cell surface molecules on Langerhans cells, we analyzed the phenotype of freshly isolated tg and control Langerhans cells. Flow cytometry corroborated the immunohistochemistry results that 2–3-fold more CD45+, MHC class II+ Langerhans cells are present in the IL-4 tg epidermis as compared with controls (Fig 8A). Furthermore, in contrast to control Langerhans cells, we found a slight upregulation of MHC class II, e-cadherin, CD54, and CD86 on a subpopulation of tg Langerhans cells (Fig 8A, and data not shown). Molecules like FcγR, integrins (CD11b, CD11c, CD29), CD24, CD44, and F4/80 were similarly expressed on the surface of control and tg Langerhans cells (Fig 8A, and data not shown). Like control Langerhans cells, freshly prepared tg Langerhans cells failed to express CD25, CD23, and CD80 (data not shown). In summary, these data demonstrate that IL-4 only slightly changes the phenotypic profile of Langerhans cells in situ.

IL-4 tg Langerhans cells are equally potent stimulators of naïve allogeneic T cells but superior to control Langerhans cells in their ability to process native protein antigens To determine whether tg Langerhans cells are able to mature in culture, EC were isolated, incubated for 3 d, enriched for Langerhans cells by density gradient centrifugation and analyzed by flow cytometry. Both control and tg Langerhans cells exhibited a similar increase in the cell surface expression of CD29, CD44, CD40, CD54, CD80, and CD86 as well as a similar decrease in the expression of FcγR, e-cadherin, CD24, and F4/80 molecules. Tg Langerhans cells, however, expressed considerably less DEC-205 and more CD49d than control Langerhans cells. Most Tg Langerhans cells failed to express CD25 (Fig 8B, and data not shown). To clarify whether such phenotypic differences influence the functional capacity of Langerhans cells, control Langerhans cells were compared with tg Langerhans cells in different antigen presentation assays. We used the allogeneic MLR to investigate the capacity of cultured EC/Langerhans cells to induce primary T cell responses. When EC were used as stimulators in these assays, the proliferative T cell response induced by tg EC was approximately 2-fold higher than with control EC [tg: 70.812 cpm; control: 39.680 cpm (n = 3)]. When equal numbers of Langerhans cells were compared in the allogeneic MLR, however, both the magnitude and the kinetics of the proliferative response induced by tg Langerhans cells compared with those induced by control Langerhans cells, with a peak occurring on days 4 and 5 for CD8+ and CD4+ T cells, respectively (Fig 9A). We next determined whether Langerhans cells that remain in the epidermis of skin explants over 5 d of culture are viable and functional. Therefore, at the end of the culture period, the epidermis was separated from the dermis by trypsin digestion and the Langerhans cell numbers were determined. When equal numbers of such control and tg Langerhans cells were used as stimulators in MLR assays, the proliferative T cell response induced by tg Langerhans cells was comparable with that evoked by control Langerhans cells. Not only the magnitude but also the kinetics of this response were comparable, with a peak of the proliferation observed on days 3–4 (data not shown). These findings show that tg Langerhans cells, although surrounded by a different cytokine milieu and control Langerhans cells, are comparable stimulators in the MLR. To further analyze whether the altered Langerhans cell phenotype in tg mice influences their ability to process and present native protein antigen, we performed antigen-processing assays. We found that the presentation of HEL (Fig 9B) and OVA (Fig 9C) protein to the respective MHC-class I-limited T cell hybridomas was substantially higher in tg than in control Langerhans cells. In conclusion, these data show that tg Langerhans cells, similar to

Figure 7. Cytokine pattern of IL-4 tg EC culture supernatants. EC isolated from control (hatched columns) and tg (black columns) mice were cultured for the indicated time points. Supernatants were screened for the presence of TNF-α, GM-CSF, and IL-1α by ELISA. Data are expressed in pg per ml as mean concentration ± SD of triplicates. p < 0.01 (24 h), p < 0.05 (48 h) (A); p < 0.05 (24 h), p < 0.01 (48 h) (B); differences in (C) are not statistically significant.
control Langerhans cells, mature into potent stimulators of naïve, allogeneic T cells and that freshly isolated tg Langerhans cells are two times more efficient than control Langerhans cells in taking up, processing, and presenting native protein antigens.

**DISCUSSION**

Because IL-4 is produced in AD skin (Kapsenberg et al, 1992; Hamid et al, 1994), the possibility has been raised that this cytokine plays an important role in the pathogenesis of this disease. Therefore, we have examined IL-4 tg mice to study the consequences of IL-4 overexpression for the skin. Tg mice had no signs of spontaneous skin disease, but displayed histopathologic changes involving both the epidermis and the dermis, including epidermal hyperplasia, hyperkeratosis, an increase of Langerhans cells, an upregulation of specific surface markers on a subpopulation of Langerhans cells, proliferation of fibroblasts, an accumulation of collagen and mast cells in the dermis, and a reduction of skin adipocytes.

Among all pathologic changes induced by IL-4 transgene expression, its influence on adipocytes is of particular interest. Results demonstrate that overexpression of IL-4 leads to a dramatic reduction of fat cells (both in number and in cell size). To our knowledge this is the first report to describe such effects of IL-4 in vivo. In hematopoietic cells, IL-4 is the prototypical activator of Stat-6 (Nelms et al, 1999). It has only been shown recently that IL-4 signals through Stat-6 in preadipocytes and induces tyrosine phosphorylation of other proteins in mature adipocytes (Deng et al, 2000); however, the molecular mechanism leading to fat reduction in IL-4 tg mice is currently unclear. In AD patients such a phenomenon has not been described yet, but it is conceivable that the extra infraorbital eyelid fold (Leung et al, 1993) may be a consequence of reduced fat cells.

A prominent finding in chronic AD lesions is fibrosis of the upper dermis (Leung, 1995). Similarly, we found in IL-4 tg skin a subepidermal or upper dermal fibrosis characterized by fibroblast accumulation stretching along regions beneath the epidermis. Histochemical staining revealed a meshwork of collagen in these areas. Other signs of fibrosis, e.g., hydroxyproline levels, have not yet been determined in these mice. Furthermore, we cannot exclude the possibility that IL-4 induced the skin to secrete another pro-fibrotic factor, such as TGF-β (Rose and Leskovsek, 1998), which in turn might be responsible for the dermal fibrosis in these mice; however, we consider this possibility rather unlikely because we found no infiltrating T lymphocytes in the tg skin and evidence that IL-4 indeed plays an important role in the etiology of skin fibrosis comes from several observations. The development of skin fibrosis in tight-skin mice, an experimental mouse model of heritable systemic fibrosis that bears some similarities to human scleroderma, was abrogated by (i) the administration of neutralizing anti-IL-4 antibodies (Ong et al, 1998) and (ii) IL-4−/−, Stat6−/−, IL-4Rα−/− mutation (Ong et al, 1999; McGaha et al, 2001).
Figure 8. Phenotypic characteristics of freshly isolated and cultured IL-4 tg Langerhans cells. Freshly prepared EC were stained and analyzed by flow cytometry (A). Three day-cultured, nonadherent EC were enriched for Langerhans cells by density gradient, washed, stained, and analyzed (B). In all experiments, stained cells were gated for viable cells and 10,000 events per sample were acquired. Dot plots represent results of 12 (A) or three (B) independent experiments.
Furthermore, it has been shown that IL-4 stimulates collagen gene expression in fibroblasts (Gillery et al., 1992). In our mice fibroblasts themselves produce IL-4 and most likely induce collagen accumulation in an autocrine fashion.

Another remarkable feature was that the overexpression of IL-4 in the skin leads to changes in keratinocyte proliferation. This is supported by the observation that in the epidermis of IL-4 tg mice the mitotic index was increased by approximately 2-4 times when compared with control mice. The proliferating cells were located in the basal layer and, to a lesser extent, at a suprabasal layer. Keratinocytes are known to be activated by IL-4, because it has been shown to stimulate human keratinocyte proliferation in vitro (Junghans et al., 1996; Yang et al., 1996). Therefore, we conclude that IL-4 released by EC in our mice is an autocrine stimulator of keratinocytes that evokes their proliferation, resulting in epidermal hyperplasia. In addition, our findings confirm that the expressed IL-4 is biologically active in vivo. These results were different to those of a most recent study of tg mice were IL-4 was specifically expressed in the epidermis (Chan et al., 2001). Whereas normal appearing skin in these mice revealed essentially no pathology, only the chronic inflammatory lesions demonstrated hyperkeratosis and hyperproliferation (Chan et al., 2001). The reasons for these differences are not known but it is conceivable that due to the ubiquitous expression of IL-4 in our model, its effects are more severe.

In terms of the composition of the cellular infiltrate in the IL-4 tg skin, mast cells and Langerhans cells were the most abundant cell types, whereas T lymphocytes and eosinophils were sparse or absent and macrophage numbers were not increased. These data are in contrast to findings in IL-4 tg mice expressing IL-4 in the epidermis (Chan et al., 2001). Similar to our study they do not find cellular infiltration with T cells and eosinophils in normal appearing skin; however, they found these cells in early and chronic inflammatory lesions. The reasons for these differences are not clear. It is conceivable that in our model IL-4-induced chemokines (e.g., eotaxin) are produced in many tissues, not only skin, thus abolishing the gradient required for selective recruitment of eosinophils and T cells. Furthermore, our tg mice, in contrast to those of Chan et al. (2001), have reduced peripherial T cell densities and in addition to that many of the residual T cells exhibit the CD44highMel-14low phenotype (Erb et al., 1994) that might alter their skin-homing capabilities. Another explanation may be that IL-4 in our mouse model failed to induce VCAM-1 expression on venular endothelial cells and, thus, T lymphocytes and eosinophils were not able to migrate to the skin. Indeed, preliminary immunohistochemical stainings of IL-4 tg skin showed no VCAM-1 expression (F. Koszik, unpublished observation).

Accumulations of mast cells have been associated with several cutaneous diseases, including atopic diseases and psoriasis, while their role is still obscure. Th cells cannot differentiate into IL-4-producing Th2 subtype without an initial pulse of exogenous IL-4. As mast cells have been shown to contain and secrete IL-4 (Horsmannheimo et al., 1994), they could provide the initial amount of IL-4 needed by the Th2 cells in AD. In accordance with this, evidence exists that IL-4 sustains and promotes the growth and development of murine mast cells in vitro (Hamaguchi et al., 1987). Therefore, the finding that IL-4−/− mice have normal numbers of dermal mast cells was surprising (Hart et al., 2000); however, these mast cells are dysfunctional with respect to expression of c-kit, the receptor for stem cell factor, and degranulation upon UVB-irradiation (Hart et al., 2000), implying that in the absence of IL-4 mast cells cannot differentiate. Our observation that mast cells are increased in IL-4 tg mice suggests that IL-4 is also a growth-promoting cytokine for these cells in vivo and supports a previous observation in IL-4 tg mice expressing IL-4 in the epidermis (Chan et al., 2001).

One of the most striking features in the skin of IL-4 tg mice was an increase in the numbers of Langerhans cells in the epidermis. Our data suggest that this accumulation of Langerhans cells was not due to their in situ proliferation, but rather due to their impaired efflux from the epidermis to the dermis. It remains to be investigated whether this is a direct or indirect effect of IL-4. Keratinocytes express IL-4R (Junghans et al., 1996; Wery-Zennaro et al., 1999) and, thus, can respond to IL-4. Therefore, it is conceivable that IL-4-activated tg keratinocytes release mediators that may affect the migration of tg Langerhans cells. Langerhans cell migration is initiated and regulated by inflammatory mediators such as TNF-α (Cumberbatch et al., 1994; Kondo et al., 1995; Wang et al., 1997); however, recent results from skin organ cultures revealed that high concentrations (5000 U per ml) of TNF-α can partially inhibit Langerhans cell migration (Stoitzner et al., 1999). In analogy to these findings higher amounts of TNF-α produced by tg EC may be sufficient to hinder tg Langerhans cell emigration out of the skin. Alternatively, IL-4 itself may be responsible for the decreased efflux of Langerhans cells, as it has been shown with human Langerhans cells that IL-4 can inhibit their migratory activity by downregulating the expression of TNF-RII (Takayama et al., 1999).

Whether a similar mechanism and/or other receptors play a role in...
our mouse model remains to be investigated. Evidence that IL-4 prevents Langerhans cell emigration in vivo comes from a study using contact hypersensitivity (Blümel et al., 1999). When Blümel et al. employed the same type of mice as used in this study, they found enhanced epidermal IL-6 gene expression and reduced numbers of MHC class II+/CD80+ cells in the ear-draining lymph nodes of tg mice after sensitization with oxazolone compared with controls, and concluded that this might be due to a decreased migration of epidermal-derived antigen-presenting cells resulting in a reduced antigen presentation in the draining lymph nodes. Furthermore, they found a significantly increased TNF-α and MIP-2 gene expression in the epidermis of hapten-treated IL-4 tg but not in sensitized control mice. On the basis of these findings they assumed that the reduced migration of epidermal Langerhans cells towards the skin-draining lymph nodes is a result of a cytokine imbalance in the skin of IL-4 tg mice, which is in line with our data.

It is possible that not only the reduced capacity of tg Langerhans cells to emigrate out of the skin, but also their enhanced longevity may be responsible for the increased numbers of these cells in the epidermis. It has previously been shown that keratinocyte-derived cytokines (e.g., GM-CSF, IL-1, TNF-α) promote survival and maturation of Langerhans cells (Witter-Pack et al., 1987; Heufler et al., 1988; Koch et al., 1995). Now it was observed that the spontaneous release of IL-4 from these cytokines was increased in supernatants of unstimulated tg EC compared with supernatants from control EC and could, thus, prolong survival of Langerhans cells. Indeed, our preliminary data indicate that GM-CSF extends survival of FACStagged tg Langerhans cells in vitro compared with control Langerhans cells (A. Elbe-Bürger, unpublished observation). In this regard also keratinocytes from AD patients have an enhanced GM-CSF production and increased numbers of Langerhans cells (Pastore et al., 1997), a picture that is similar to our mice. Functionally, we found that the tg Langerhans cells were equally potent stimulators of the allogeneic MLR as control Langerhans cells, whereas tg Langerhans cells were more efficient in their capacity to present a soluble protein antigen than control Langerhans cells, which might be a consequence of augmented antigen uptake. Indeed, it has been shown only recently that GM-CSF either alone or in combination with IL-4 augments the rate of pinocytosis by DC (Lutz et al., 1996), a mechanism that might also be operative in tg Langerhans cells.

Our findings identify IL-4 as a potent multifunctional cytokine at the skin site that is capable of evolving numerous inflammatory processes ranging from epidermal hyperplasia to fibrotic responses and suggest that IL-4 is not only a key cytokine in the development of AD but seems to play an important role for the perpetuation of pathologic changes in chronic skin conditions, including chronic AD. Thus, IL-4 tg mice are a unique model to further study certain aspects of AD pathogenesis, and suggest that IL-4 antagonists may ameliorate some key features of AD.

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