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ORIGINAL ARTICLE

Development of Atopic Dermatitis in Mice Transgenic for Human Apolipoprotein C1

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Mice with transgenic expression of human apolipoprotein C1 (APOC1) in liver and skin have strongly increased serum levels of cholesterol, triglycerides, and free fatty acids, indicative of a disturbed lipid metabolism. Importantly, these mice display a disturbed skin barrier function, evident from increased transepidermal water loss, and spontaneously develop symptoms of dermatitis including scaling, lichenification, excoriations, and pruritus. Histological analysis shows increased epidermal thickening and spongiosis in conjunction with elevated numbers of inflammatory cells (eosinophils, neutrophils, mast cells, macrophages, and CD4+ T cells) in the dermis. In addition, affected mice have increased serum levels of IgE and show abundant IgE^+ mast cells in the dermis. Partial inhibition of disease could be achieved by restoration of the skin barrier function with topical application of a lipophilic ointment. Furthermore, the development of atopic dermatitis in these mice was suppressed by corticosteroid treatment. These findings in APOC1(+/+) mice underscore the role of skin barrier integrity in the pathogenesis of atopic dermatitis.

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INTRODUCTION

Atopic dermatitis (AD) is an itchy inflammatory dermatitis with a chronic course with remissions and exacerbations. AD occurs primarily in children, but persisting cases in adulthood as well as adult late-onset AD occur (Boguniewicz *et al.*, 2006; Mohrenschlager and Ring, 2006). Usually the personal and/or family history of other atopic conditions, such as asthma and allergic rhinitis, are positive. It is estimated that about 20% of the population of the industrialized countries has an atopic constitution, and between 5 and 20% suffers at least during some period of their life from AD (Williams and Flohr, 2006). AD is mainly genetically determined with at least 20 involved genes (Hoffjan and Epplen, 2005). In addition, environmental factors are considered of importance for the development of the disease. Recent studies focus on the barrier function of the skin (Proksch *et al.*, 2006) and genetic defects of filaggrin (Palmer *et al.*, 2006).

Treatment of AD is largely confined to the application of anti-inflammatory drugs such as corticosteroids; calcineurin inhibitors are frequently prescribed as steroid sparing agents. Because such treatments show a transient effect on symptoms and do not mediate long-lasting immunosuppression of disease, there is a great need of drugs that interfere with initial triggers of the disease. The etiology of the disease is unclear, although a major part of the patients show an association with an allergic response evident from increased levels of IgE (Novak *et al.*, 2003; Leung *et al.*, 2004). The integrity of the skin barrier, in particular the composition of the stratum corneum, appears to be of great importance for the development and progression of skin lesions in patients with AD (Proksch *et al.*, 2006; Segre, 2006). The composition of the stratum corneum is dependent on lipid homeostasis and therefore it was of importance to observe that mice transgenic for human apolipoprotein C1 (APOC1) have disturbed serum levels of lipids and spontaneously develop dermatitis; at older age, mice develop severe dermatitis with moderate epidermal hyperplasia and hyper- and parakeratosis (Jong *et al.*, 1998). APOC1 is an apolipoprotein involved in lipoprotein metabolism (Jong *et al.*, 1999). In healthy individuals, the protein is predominantly expressed in liver, skin, and brain tissue with macrophages and keratinocytes as major cell types. The protein is highly conserved and a high degree of homology exists between APOC1 in mice and man. APOC1(+/+) mice have increased levels of free fatty acids, cholesterol, and triglycerides, but show complete absence of subcutaneous fat and atrophic sebaceous glands. Herein, we demonstrate that APOC1(+/+) mice display a disturbed skin barrier function and develop symptoms of AD that are sensitive to corticosteroid treatment.

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Abbreviations: AD, atopic dermatitis; APOC1, apolipoprotein C1; TCA, triamcinolone acetonide; TEWL, transepidermal water loss; TIS, three-item severity

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RESULTS

Development of dermatitis

Regardless of gender, human APOC1(+/+) transgenic mice develop signs of AD, comprising scaling, lichenification, and papules, and mild-to-severe excoriations from an age of 6 weeks onward. Figure 1 shows typical examples of mice with progressed dermatitis. In contrast, APOC1(+/−) heterozygous mice do not show such symptoms. In APOC1(+/+) mice, aspects of dermatitis are particularly evident in the upper dorsal skin, but when mice grow older also the head and eyelids may become affected. To assess the severity of dermatitis, the upper dorsal skin was evaluated employing a 3-item score by assigning 0–3 points to each of the following items: scaling, lichenification/papules, and excoriations. At an age of about 6 weeks, mice display only mild signs of scaling with a clinical severity score ranging from 0 to 2 (mild dermatitis). From this age onward the severity of the dermatitis develops gradually with increasing edema/infiltration classified as lichenification (moderate dermatitis) and excoriations. At an age of 12 weeks most of the mice have progressed to a severity score ranging from 4 to 7.

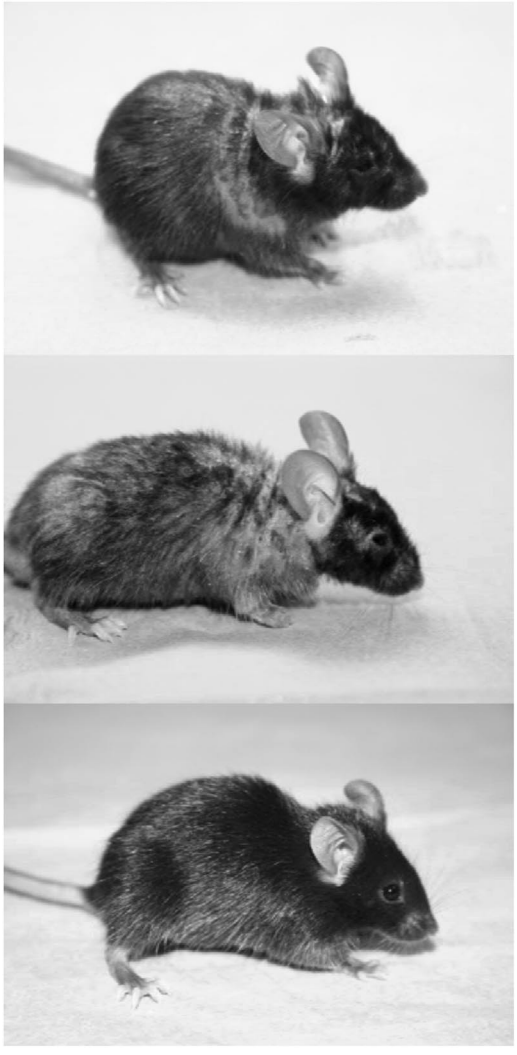

Figure 1. Appearance of atopic dermatitis in APOC1(+/+) mice. AD in APOC1(+/+) mice is associated with scaling, papules, lichenification, and excoriations. Upper panel, 12-week-old APOC1(+/+) mouse; middle panel, 18-week-old APOC1(+/+) mouse; lower panel, 12-week-old APOC1(+/−) control mouse.

Involvement of inflammatory cells

To gain more insight into the underlying mechanism of dermatitis in these mice, skin sections from the upper dorsal area of APOC1(+/+) mice were evaluated with regard to hyperplasia and the presence of inflammatory cells. Figure 2a shows that dermatitis is associated with thickening of epidermis and dermis. In addition, more than 90% of the mice showed mild-to-moderate spongiosis from an age of 6 weeks onward (Figure 2a, insert). At an age of 6 weeks, various inflammatory cell types are increased in number in APOC1(+/+) mice as compared to APOC1(+/−) mice. This was demonstrated for eosinophils, mast cells, CD4+ T cells, and macrophages. Figure 2 shows the increased involvement of neutrophils and eosinophils (Figure 2b), mast cells (Figure 2c), CD4+ T cells (Figure 2g), and CD11b+ macrophages (Figure 2h) in the skin of 12-week-old APOC1(+/+) mice, as compared to age-matched APOC1(+/−) mice (Figure 2e–f and j–k). Results in wild-type mice were similar to those in APOC1(+/−) mice (data not shown). Increased numbers of eosinophils are already found at early age when they outnumber neutrophils (Figure 3). Numbers of mast cells are increased at young age, and these numbers further increase during disease progression (Figure 4a); part of the mast cells show degranulation (Figure 2c, arrow). Because this may be due to cross-linking of IgE receptors, we evaluated the involvement of IgE. At an age of 8 weeks, IgE serum levels in APOC1(+/+) mice were on average $0.77 \pm 0.93\,\mu g\,ml^{-1}$ and comparable to the levels in age-matched APOC1(+/−) or wild-type mice. As shown in Figure 4b, serum levels of IgE increase in APOC1(+/+) mice in parallel with disease progression. At an age of 12 weeks, serum levels of IgE in APOC1(+/+) mice were $7.05 \pm 4.00\,\mu g\,ml^{-1}$ and significantly higher than those in APOC1(+/−) mice ($2.09 \pm 1.89\,\mu g\,ml^{-1}$; $P<0.01$) or wild-type mice ($1.00 \pm 0.91\,\mu g\,ml^{-1}$; $P<0.01$). From an age of 12 weeks onward mast cells in the dermis become IgE-positive (Figure 2i). A gradual increase in numbers of IgE^+ mast cells correlated with the appearance of IgE in serum (Figure 4c). As shown in Figure 5, epidermal hyperplasia is already found at an early age; disease progression is associated with increased thickening of the epidermis.

Disturbed skin barrier function as an underlying cause of dermatitis in APOC1(+/+) mice

APOC1(+/+) mice show a disturbed lipid metabolism, lack of subcutaneous fat, and atrophic sebaceous glands, and it might therefore be that this condition affects the quality of the skin barrier. We verified the integrity of the skin barrier by determining transepidermal water loss (TEWL).

Figure 6 shows the results of measurements in individual mice at an age of 12 weeks. Normal TEWL values in wild-type mice or in APOC1(+/−) mice range from 10 to

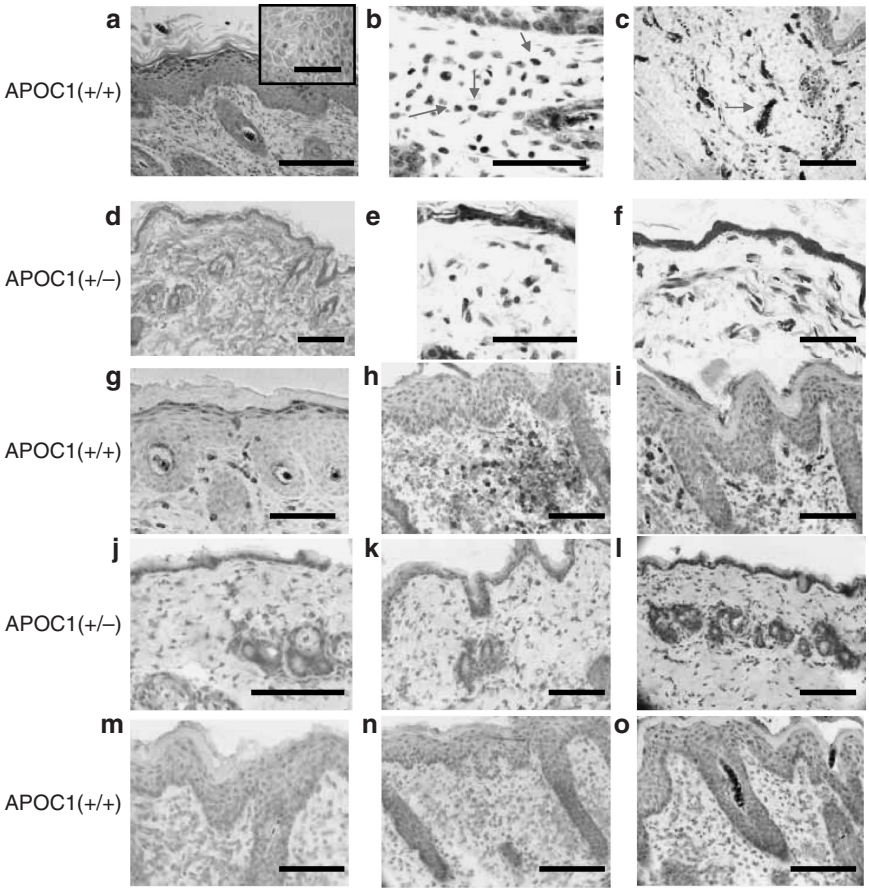

Figure 2. Histopathology of AD. Mice were killed at an age of 12 weeks and skin sections were evaluated with respect to epidermal hyperplasia (**a** and **d**, hematoxylin/eosin staining), the presence of eosinophils (arrows) and neutrophils (**b** and **e**; LUNA staining), mast cells (**c** and **f**; toluidin blue staining), CD4+ T cells (**g** and **j**), $CD11b^+$ cells (**h** and **k**), and IgE^+ cells (**i** and **l**). (**a–c** and **g–l**) The results for APOC1(+/+) mice, whereas (**d–f** and **j–l**) show the staining of tissues from APOC1(+/−) controls. (**m–o**) The isotype controls for CD4, CD11b, and IgE, respectively. The insert of (**a**) shows an example of spongiosis at an age of 12 weeks. The arrow in (**c**) indicates degranulation of mast cells. Bar = 50 μm in (**b**, **c**, **e**, and **f**) and the insert of (**a**); bar = 100 μm in all other panels.

$15\,g\,h^{-1}\,m^{-2}$. However, APOC1(+/+) mice at an age of 12 weeks have a four-fold higher TEWL. At an age of 3 weeks, APOC1(+/+) mice already show increased TEWL of $17.2 \pm 1.9\,g\,h^{-1}\,m^{-2}$ as compared to a value of $11.7 \pm 2.4\,g\,h^{-1}\,m^{-2}$ in heterozygous littermates of the same age ($P < 0.0005$). Subsequently, a further increase develops gradually (data not shown). Because ointments may have a beneficial effect in the treatment of AD patients, we were interested whether basic creams frequently used in clinical practice would have an influence on the TEWL. We compared 20% petrolatum in cetomacrogolus cream as a lipophilic ointment with a hydrophilic moisturizing cream that is comparable to the cream used for the formulation of triamcinolone acetonide (TCA; see also Figure 8). As shown in Figure 6a, mice subjected to 3 weeks of treatment with 20% petrolatum showed diminished loss of barrier function; the moisturizing hydrophilic cream was ineffective in this respect. In a separate experiment, 3 weeks of treatment with 20% petrolatum diminished TEWL and this effect was associated with a decreased severity of dermatitis according to the three-item severity score (TIS; Figure 6b).

APOC1(+/+) mice display increased pruritus

As indicated above, APOC1(+/+) mice develop increased numbers of IgE^+ mast cells in the dermis during progression of disease. Therefore, individual APOC1(+/+) mice were monitored with regard to scratching behavior employing a system based on movements of unique frequency between 18 and 25 Hz. Mice were housed individually in a cage fitted on a platform and monitored overnight for a period of 12 hours. Both the number of scratch events and the duration of scratching were monitored. According to this system, APOC1(+/−) heterozygous littermates, which do not develop clinical symptoms of dermatitis, show a baseline scratch frequency ranging between 5 and 25 scratches per hour. APOC1(+/+) mice show an increased frequency of scratching (and duration; not shown) at an age of 9 weeks and a further increase while disease progresses (Figure 7).

Sensitivity of dermatitis in APOC1(+/+) mice toward treatment with corticosteroids

To evaluate whether the development of dermatitis in APOC1(+/+) mice was sensitive to treatment with

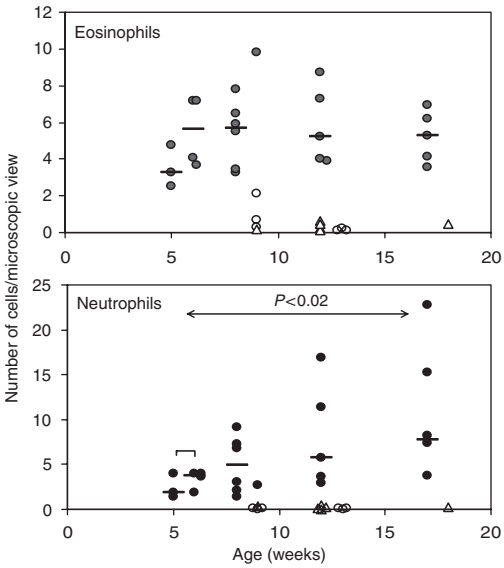

Figure 3. Dynamics of inflammatory cells in APOC1(+/+) mice. Mice were killed at different ages starting at an age of 6 weeks, and tissue sections were evaluated with respect to the number of eosinophils and neutrophils. Individual APOC1(+/+) mice are indicated with filled circles; results of APOC1(+/−) and wild-type mice are indicated as triangles and open circles, respectively. For each individual mouse, three non-serial sections were evaluated (10 sequential microscopic views per section). Each symbol represents an individual mouse with the mean number of cells per microscopic view at a ×1000 magnification. For statistical analysis data obtained in 5- and 6-week-old mice were combined; the same was done for 8- and 9-week-old mice. The Kruskal–Wallis test showed significant differences for eosinophils ($P<0.0005$) and neutrophils ($P<0.00005$). At all time points, both cell types were significantly increased as compared to wild-type mice or APOC1(+/−) mice (Mann–Whitney *U*-test, $P<0.01$).

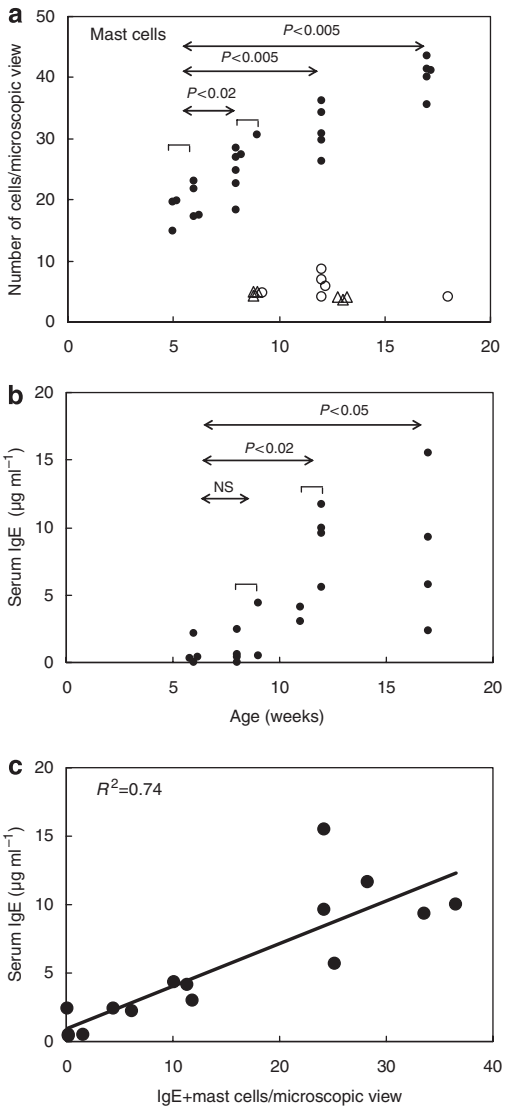

Figure 4. AD in APOC1(+/+) mice: mast cells and IgE. Mice were killed at different ages starting at an age of 6 weeks, and tissue sections were evaluated with respect to the number of (**a**) mast cells or (**c**) IgE^+ cells. Individual APOC1(+/+) mice are indicated with filled circles. Heterozygous APOC1(+/−) mice (triangles) or −/− wild-type mice (open circles), which do not develop AD, are included as controls. (**b**) IgE levels were tested by ELISA as described in Materials and Methods. For each individual mouse, three non-serial sections were evaluated (10 sequential microscopic views per section). Each symbol represents an individual mouse with the mean number of cells per microscopic view at a ×400 magnification. (**c**) Number of IgE^+ cells was determined and for each individual mouse it was plotted against the corresponding serum value. For statistical analysis, data obtained in 5- and 6-week-old mice were combined; the same was done for 8- and 9-week-old mice. The Kruskal–Wallis test showed significant differences for mast cells ($P<0.00001$) and for IgE ($P<0.01$). At all time points, numbers of mast cells were significantly increased (Mann–Whitney *U*-test, $P<0.01$), as compared to wild-type mice or APOC1(+/−) mice. Differences as compared to week 5/6 are indicated in the figure.

corticosteroids, mice were treated for a period of 3 weeks by daily topical application of 0.1% TCA or a hydrophilic moisturizing cream (Lanette cream, see Materials and Methods). As shown in Figure 8a, TCA treatment inhibited epidermal hyperplasia and caused rather a thinning of the epidermis, a well-known adverse effect associated with prolonged exposure to corticosteroids. We also evaluated numbers of inflammatory cells and found that treatment with TCA diminished numbers of granulocytes, $CD11b^+$ macrophages, and CD4+ T cells (Figure 8b); numbers of mast cells were not different from mice left untreated or from mice treated with the moisturizing cream.

DISCUSSION

Mice with transgenic overexpression of human APOC1 develop a skin disease that is characterized by a variety of features resembling AD. As reported earlier by Jong *et al.* (1998), the epidermis of APOC1(+/+) mice shows an altered lipid composition as compared to wild-type mice, with decreased levels of triglycerides, wax diesters, and lanosterol, increased levels of free fatty acids and free cholesterol, and no differences in ceramides and polar lipids. Possibly, local overexpression of APOC1 also prevents the production of sebum. It is likely that these factors are responsible for a disturbed skin barrier function, and this was substantiated by demonstrating increased TEWL at an age of 3 weeks immediately after weaning. Although it is tempting to speculate that a change in housing conditions is

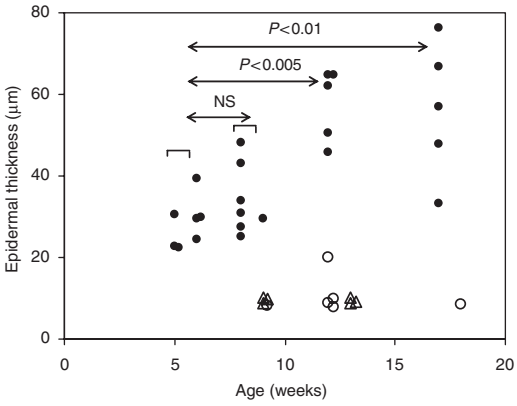

Figure 5. Increased epidermal thickening during progression of dermatitis in APOC1(+/+) mice. Heterozygous APOC1(+/−) mice (triangles) or −/− wild-type mice (open circles), which do not develop AD, are included as controls. For each individual mouse, three non-serial sections were evaluated and for each section the epidermal thickness was determined (10 measurements with an interval of 250 µm). For each individual mouse, the average thickness of 30 measurements is shown. For statistical analysis, data obtained in 5- and 6-week-old mice were combined; the same was done for 8- and 9-week-old mice. The Kruskal–Wallis test showed a significant difference between the groups ($P<0.00005$). At all time points, epidermal thickness was significantly increased (Mann–Whitney *U*-test, $P<0.01$) as compared to wild-type mice or APOC1(+/−) mice. Differences as compared to week 5/6 are indicated in the figure.

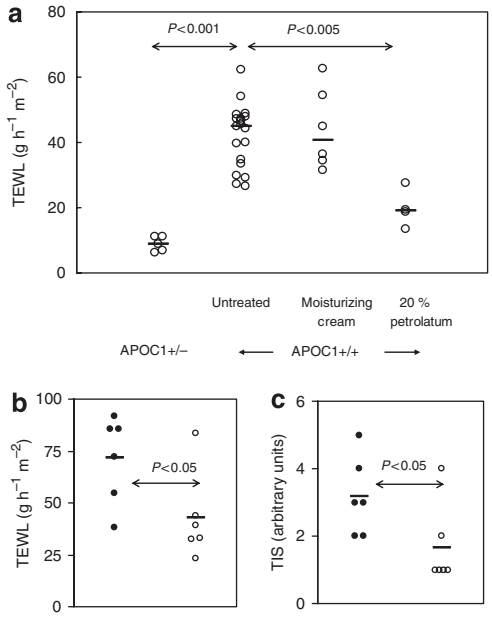

Figure 6. Increased TEWL in APOC1(+/+) mice: effect of lipophilic ointment. (**a**) TEWL was evaluated in the upper dorsal area of 12-week-old APOC1(+/+) mice left untreated or after treatment by topical application (daily, 3 weeks) with 20% petrolatum in cetomacrogolus cream or a moisturizing cream; APOC1(+/−) mice were included as controls. In a separate experiment, 9-week-old APOC1(+/+) mice were left untreated (filled circles) or treated for a period of 3 weeks with 20% petrolatum in cetomacrogolus cream (open circles), and evaluated with respect to TEWL (**b**) and the severity of dermatitis according to the TIS score (**c**).

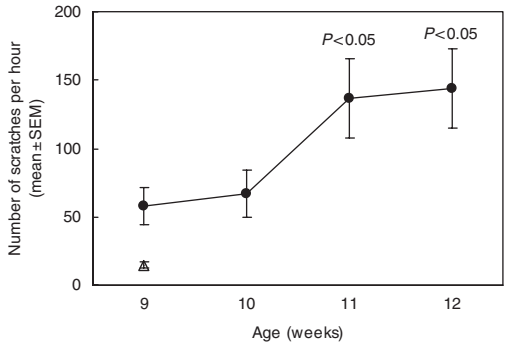

Figure 7. The development of AD in APOC1(+/+) mice is associated with pruritus. Mice ($n=8$) were included at an age of 9 weeks and individually monitored at weekly intervals with respect to their scratching behavior, as described in Materials and Methods. Results are expressed as group means ± SEM. The triangle indicates the mean number of scratches per hour in 9-week-old heterozygous APOC1(+/−)mice. Changes in scratch frequency were evaluated with the Wilcoxon signed ranks test. *P*-values show significant differences as compared to the values at ages of 9 and 10 weeks.

responsible for the subsequent onset of dermatitis, this could not be attributed to an influence of temperature and humidity inasmuch as these factors remained constant. Because specific pathogen-free APOC1(+/+) mice that are not subject to a change in housing conditions show similar development of dermatitis, it is more likely that loss of integrity of the skin barrier facilitates exposure of innate immune cells to environmental antigens (e.g. commensal skin microorganisms) and the subsequent development of an inflammatory response. APOC1(+/+) mice already show at an early age that TEWL, inflammatory cells, and epidermal hyperplasia are increased, and therefore it is difficult to conclude with certainty which event represents the initial trigger of the disease. Topical treatment with a lipophilic ointment improved several aspects of dermatitis, most likely by partial restoration of the skin barrier function and by preventing exposure to environmental antigens. Similarly, beneficial effects of emollients have been observed in patients (Loden, 2005).

The inflammatory response in APOC1(+/+) mice shows similarity to the response that takes place after epicutaneous sensitization with ovalbumin, where AD-like disease is characterized by increased numbers of eosinophils, mast cells, preferential infiltration by CD4+ T cells, spongiosis, and comparably increased levels of serum IgE (Wang *et al.*, 2007). In addition, epicutaneous exposure to ovalbumin was associated with local upregulation of mRNA encoding IL-4, IL-5, and IFN-γ (Spergel *et al.*, 1998), with an essential role for IL-13 in the induction of a Th2 response (Herrick *et al.*, 2003). Recent interest has also implicated thymic stromal lymphoprotein—a cytokine produced by keratinocytes and responsible for local activation of skin dendritic cells—in the pathogenesis of AD and the development of Th2 cells (Yoo *et al.*, 2005). Therefore, studies focused on the local expression of Th2-promoting factors in the skin of APOC1(+/+) mice may help to gain insight into the pathogenesis of the disease in this particular model. In this

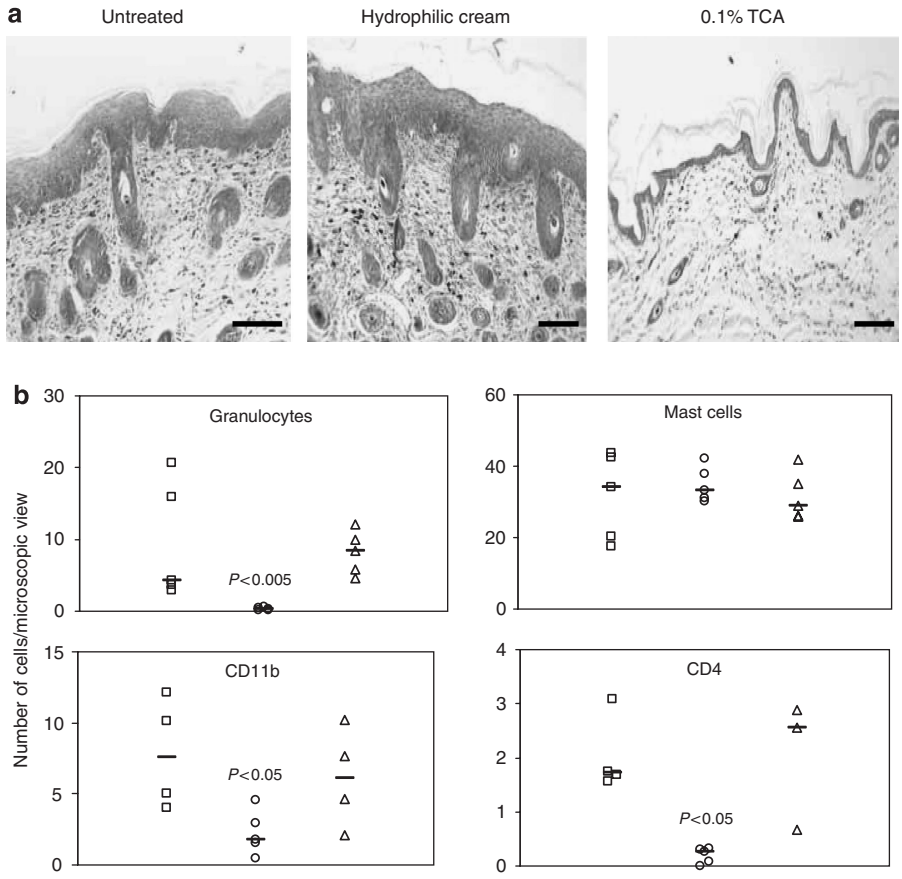

Figure 8. Inhibition of epidermal hyperplasia and inflammation by TCA. APOC1(+/+) mice, 9 weeks of age, were included and left untreated or subjected to daily topical treatment with 0.1% TCA or moisturizing cream. Treatment resulted in significant (**a**) inhibition of epidermal thickening and (**b**) inhibition of infiltration with inflammatory cells (□ untreated, ○ 0.1% TCA, △ moisturizing cream). Bar = 100 μm.

respect, the possibility that overexpression of APOC1 has a modulating effect on innate immunity and the development of a Th2 type of inflammatory response needs further investigation.

Progression of disease in APOC1(+/+) mice occurred in conjunction with the development of pruritus, in particular from an age of 9 weeks onward when mice developed increased levels of IgE in serum and IgE^+ mast cells in the dermis. This suggests that pruritus increases particularly by activation of mast cells once they have bound IgE on their surface. Because these events occur later than infiltration by inflammatory cells and epidermal thickening, we consider scratching as a consequence rather than a primary cause of dermatitis in this model.

APOC1(+/+) mice share many features with AD in humans; however, there are several differences as well. First of all, the stratum corneum of patients has decreased ceramide levels and this was not observed in APOC1(+/+) mice (Jong *et al.*, 1998). Secondly, increased plasma levels of APOC1, cholesterol, triglycerides, and free fatty acids are not typical for AD. It remains to be established whether AD in patients is associated with the upregulation of APOC1 in the skin. The involvement of neutrophils is also different from the clinical situation; this may occur in the APOC1(+/+) mouse because pruritus was left untreated and this condition could promote an inflammatory response, for example, to microorganisms associated with the skin. On the other hand, many relevant aspects (elevated eosinophils, IgE, CD4+ T cells, macrophages, mast cells, spongiosis, and pruritus) are involved and several of these aspects were sensitive to TCA. Preliminary data indicate that dermatitis in this model is also sensitive to topical treatment with fluticasone proprionate or tacrolimus (Oranje *et al.*, submitted for publication), and to oral treatment with dexamethasone. These findings substantiate an important role of T cells and macrophages and the usefulness of the APOC1 model to evaluate underlying mechanisms in AD.

MATERIALS AND METHODS

Mice

Human APOC1(+/+) transgenic mice were generated as described previously (Jong *et al.*, 1998) employing breeding couples of female APOC1(+/−) and male APOC1(+/+) mice. From two transgenic lines, line 11/1 was selected because it showed the highest expression of APOC1 with the highest plasma lipid levels. Heterozygous APOC1(+/−) mice were derived from the same breeding stock. Wild-type C57BL/6 mice were derived from a colony, housed under the same conditions. Animals were housed in

individually ventilated cages until an age of 6 weeks and subsequently maintained under clean conventional conditions. These housing conditions were comparable in terms of temperature (21°C) and humidity (55%). All of these studies were performed with the approval of the Animal Welfare Committee and in compliance with Dutch governmental regulations on animal experimentation.

TIS score and thickening of the skin

Mice were monitored regarding the progression of dermatitis with the use of a TIS score adapted from Wolkerstorfer *et al.* (1999) comprising the following items: scaling, papules and lichenification, excoriations. Each of the items were graded from 0 (normal) to 3 (severe), and included in a TIS scale ranging from 0 to 9. A TIS of 7 and above is considered unacceptable for reasons of animal welfare and accordingly mice were killed if such a clinical condition developed.

At different time points mice were evaluated with respect to the thickness of a fold of skin in the upper dorsal area, employing a caliper (Mitutoyo, Veenendaal, The Netherlands).

Treatment

Where indicated mice were treated for a period of 3 weeks by daily topical application of approximately 70 mg of 0.1% TCA (Pharmachemie, Haarlem, The Netherlands; composition: 0.1% TCA, 15% cetomacrogol wax, 20% Cetiol V, 4% sorbitol, 0.2% sorbic acid, 60.8% water). Control groups were treated with 20% petrolatum in cetomacrogolus cream (Fagron Pharmaceuticals BV, Nieuwerkerk a/d IJssel, The Netherlands; composition: 20% petrolatum, 16% Cetiol V, 12% cetomacrogol emulsifier; 3.2% sorbitol, 0.16% sorbic acid, 48.6% water) or moisturizing cream (Lanette cream, Pharmachemie; composition: 20% Cetiol V, 15% cetostearylalcohol emulgator B, 4% sorbitol, 0.15% sorbic acid, 60.9% water).

Transepidermal water loss

TEWL was measured by placing a 12-mm detection probe of a skin evaporative water recorder (Tewameter® TM 300, Courage &, Khazaka, Cologne, Germany) on the upper dorsal skin area of each individual mouse. Measurements were performed at 55% humidity and at a temperature of 21°C. Results were recorded when TEWL readings were stabilized, that is approximately 1 minute after the probe had been placed on the skin. If mice were subjected to topical treatment, TEWL measurements were performed 24 hours after treatment.

Histology

Skin tissue derived from the upper dorsal area was fixated with formaldehyde and embedded in paraffin. For the measurement of epidermal thickness, 5 μm sections were stained with hematoxylin-eosin-saffron and the thickness of the epidermis was determined via microscopic field analysis using a metric ocular (Zeiss, Sliedrecht, The Netherlands). For each individual mouse, three non-sequential sections were evaluated and for each section the epidermal thickness was determined by 10 sequential measurements with intervals of 250 μm. Numbers of neutrophils and eosinophils were evaluated by differential counting after LUNA staining at a ×1000 magnification. Results are expressed as numbers of cells per microscopic view of 50 μm^2. Mast cells were evaluated after staining of tissue sections with toluidin blue, at a magnification of ×400 (microscopic view of 190 μm^2). CD4+ T cells, IgE^+ cells, and macrophages were determined by immunostaining of cryopreserved tissues with rat anti-mouse CD4 (dilution 1:100; BD Biosciences, San Diego, CA), followed by incubation with biotinylated goat anti-rat Ig (dilution 1:200, BD Biosciences), biotin-labeled rat anti-mouse IgE (dilution 1:800; BD Biosciences), and biotin-labeled rat anti-mouse CD11b (dilution 1:1000; BD Biosciences), respectively. After incubation, biotinylated antibodies were detected by incubation with streptavidin-HRP (dilution 1:500; Vector Laboratories, Burlingame) using 3-amino-9-ethylcarbozole (Sigma, St Louis, MO) as a substrate. Corresponding rat Ig2a and IgG2b isotype controls (BD Biosciences) were used to verify for background staining. Cells visualized by immunostaining were counted at a ×400 magnification (microscopic view of 190 μm^2).

ELISA

Levels of mouse IgE in serum were determined by ELISA (BD OptEIA kit, BD Biosciences). Samples were tested at 1:100 and 1:1000 dilutions. Results are expressed in $\mu g\,ml^{-1}$.

Statistical analysis

All statistical analyses were performed using the statistical software program SPSS 11.5 for Windows (SPSS Inc., Chicago, IL). Differences between groups were evaluated using the Kruskal–Wallis test, followed by the Mann–Whitney *U*-test. Changes within individual mice were evaluated with the Wilcoxon signed ranks test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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