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Skin-Infiltrating CD8+ T Cells Initiate Atopic Dermatitis Lesions1

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Skin lesions in the allergic form of atopic dermatitis (AD) are induced by allergen-specific T cells that infiltrate the skin at the site of allergen exposure. Although Th2-type CD4+ T cells appear to be crucial in AD pathophysiology, little is known about the contribution of CD8+ T cells in the development of the allergic skin inflammation. In the present study, we have analyzed the respective role of CD8+ and CD4+ T cells in the development of AD skin lesions in a mouse model of allergen-induced AD. In sensitized mice, CD8+ T cells are rapidly and transiently recruited to the allergen-exposed site and initiate the inflammatory process leading to skin infiltration with eosinophils and Th1/Th2-producing cells. CD8+ T cell-depleted mice show no inflammation, demonstrating that these cells are mandatory for the development of AD. In contrast, CD4+ T cell-depleted mice develop a severe form of eczema. Furthermore, adoptive transfer of CD8+ T cells from sensitized mice into naive recipient mice leads to skin inflammation soon after allergen exposure. These data indicate that allergen-primed CD8+ T cells are required for the development of AD-like lesions in mice. The Journal of Immunology, 2007, 178: 5571–5577.

Dermatophagoides farinae (Der f) and Dermatophagoides pteronyssinus (Der p) are major aeroallergens in AD pathophysiology, as up to 75% of patients with AD have skin prick test reactivity and/or specific IgE. More importantly, epicutaneous application of house dust mite extract by atopic patch test on uninvolved skin of patients with AD elicits eczema in 30–50% of these patients (3, 4). T cells isolated from AD skin lesions and allergen patch test sites selectively respond to Der p supporting the concept that immune responses in AD skin are mediated by allergen-specific T cells and can be elicited by environmental aeroallergens (3, 5). However, the nature of T cells responsible for the skin inflammation and the mechanisms by which they induce the AD skin lesions remain unknown.

CD4+ T cells are considered pivotal in the development of eczema and skin eosinophilic inflammation by producing a mixed pattern of Th1 and Th2 cytokines including IL-4 and IL-13, which are known to induce isotype switching to IgE synthesis, as well as IL-5, which plays an important role in eosinophil development and survival (6, 7). Such hypothesis relies on indirect observations in human AD, mostly using peripheral blood CD4+ T cells and T cell clones and on histological findings showing that the majority of skin-infiltrating T cells in active AD lesions were CD4+ T cells (8). Whereas in acute AD lesions a predominance of Th2-producing cells has been observed, a shift to a Th1 cytokine pattern has been described in chronic lesions (9). Although IFN-γ has been shown to be produced in both acute and chronic skin lesions in humans (10, 11), high numbers of CD4+ and CD8+ T cells expressing IFN-γ infiltrate acute AD lesions (12). Moreover, a significant proportion of house dust mite-specific T cell clones isolated from AD lesional skin were of a CD8 phenotype (13).

Increased frequencies of both cutaneous lymphocyte-associated Ag (CLA)-positive CD4+ and CD8+ T cells producing type 2 cytokines have been detected in the blood of patients with AD (14). These circulating CLA-positive CD8+ T cells are able to induce IgE production by B cells and enhance eosinophil survival (15). Seneviratne et al. (16) recently reported the presence of Der p-specific CD8+ T cells able to produce type 1 and type 2 cytokines in the blood of patients with AD, but not in the blood of nonatopic individuals. More importantly, these authors observed a strong correlation between the level of specific CD8+ T cells and the severity of the disease. Together these studies point out to a potential role for CD8+ T cells in the development of AD lesions.

To examine the respective contribution of CD4+ and CD8+ T cells in AD pathophysiology, we investigated the allergic response of wild-type C57BL/6 mice as compared with that of CD4+ and
CD8+ T cell-deficient mice, using a mouse model of allergen (Der f)-induced skin inflammation reproducing most of the features of human AD (17). We show that effector CD8+ T cells mediate inflammation and AD-like lesions. IFN-γ-producing CD8+ T cells infiltrate the challenged skin a few hours after Der f exposure of sensitized mice and initiate a mixed Th1/Th2-type skin inflammation leading to the recruitment of mononuclear cells and eosinophils and to the development of epidermal changes typical of AD.

Materials and Methods

Animals

Female C57BL/6 mice were purchased from Charles River Breeding Laboratories. C57BL/6 mice with a mutation in the Aqf gene (MHC class IIf) and in the CD3ε cluster (CD3εf) were provided by C. Benoist and D. Mathis (Harvard Medical School, Boston, MA) and M. Malissen (Centre d’Immunologie, Marseille-Luminy, Marseille, France), respectively. All mice were reared between 2 and 3 mo of age. Groups consisted of 10 mice each. All experimental procedures were in accordance with the Comité régional d’éthique pour l’expérimentation animale guidelines on animal welfare.

Mouse model of AD

Ear mouse eczema was induced by repeated topical application on the left ear of a protein allergen solution (250 μg of protein per ear) of Der f house dust mite extract provided by P. Moengon (Stallergenes, Antony, France) once a week for 6–10 wk. Control mice were painted onto the left ear with the vehicle (70% DMSO in water) alone. Evaluation of ear thickness was done for each mouse ear before allergen application and each day after allergen application with a spring-loaded micrometer (J15; Blet). Ear swelling was calculated by subtracting the initial value from the value recorded on the corresponding day (for both allergen- and vehicle-treated mice).

Ab treatment

The rat anti-mouse CD8 mAb GK 1.5 was obtained from American Type Culture Collection (ATCC) and the rat anti-mouse CD8 mAb H35.17.2 was provided by G. Milon (Institut Pasteur, Paris, France). A β-galactosidase-specific rat IgG (clone GL113; ATCC) was used in some experiments or anti-CD8 mAbs (Tebu-Bio). Hybridomas were grown as ascites in pristane-stimulated Swiss nude mice. Mice were given i.p. injections of 20 μg of anti-CD4 mAbs or 16 μg of anti-CD8 mAbs in 200 μl of saline on days 1 and 0 of ear painting. Cell depletion was assessed in each mouse by staining for CD4 and CD8 molecules on whole draining lymph nodes (LN) as well as spleens, using FITC- or PE-labeled anti-CD4 or anti-CD8 mAbs (Tebu-Bio).

RNA extraction and RT-PCR analysis

At different intervals (24 and 48 h) after allergen exposure, ear samples were cultured using anti-CD8+ and -CD4+ mice and frozen in liquid nitrogen. The detection of RNA was conducted as described in detail elsewhere (18). In brief, total RNA was extracted using the RNAXEL kit (Eurobio Laboratories). After DNase I treatment, 1 mg of total RNA was reverse transcribed using poly(dT)12 primers and Subscript II reverse transcriptase (90 min, 37°C; Invitrogen Life Technologies). The amount of RNA to be used for detection was normalized using the housekeeping gene HPRT as reference. The cDNA obtained was amplified using different sets of primers.

For HPRT, CD4, CD8, IFN-γ the primers have been described elsewhere (19). For IL-4, the sense primer is 5′-TGGCCATTGTTAAGCAG GTC-3′ and antisense 5′-GAAAGACGAGCCAGTGTCCT-3′. For thymus- and activation-regulated chemokine (TARC) the sense primer is 5′- CAG GAA GTT GGT GAG CTG GTA ATA-3′ and the antisense primer is 5′-TGT CTT TGA GAT GAT GAA GT-3′. The amplifications were conducted with 29 cycles for HPRT and 33 cycles for the other genes (1 min at 94°C, 1 min 30 s at 60°C, 2 min at 72°C). The PCR products were analyzed on 1.5% agarose gel.

Flow cytometry, T cell isolation, and cell culture

Draining LN from vehicle- or allergen-treated mice were harvested on days 21 or 28 to prepare single-cell suspensions. CD8+ or CD4+ T cells were purified using anti-CD8 or anti-CD4 mAb-coated microbeads and positive selection columns (Miltenyi Biotec). Purity was routinely >95%.

The cell cultures were performed in 96-well plates. The whole draining LN as well as isolated CD8+ or CD4+ T cells were seeded at 10⁶/well for proliferation assay. Irradiated splenocytes from naive C57BL/6 mice were used as APCs. For cytokine production (IFN-γ and IL-4) the cells were seeded at a concentration of 3 × 10⁵/well. IFN-γ and IL-4 production was titrated by ELISA using the R&D Systems Duoset kit following the manufacturer protocol. For ELISPOT assay (IFN-γ and IL-5), 96-well nitrocellulose plates (MAIP N4510; Millipore) were coated overnight at 4°C with anti-IFN-γ Ab or anti-IL-5 Ab (R46A2, BD Pharmingen) and blocked with RPMI 1640 for 1 h at 37°C. The plates were washed five times with 0.05% PBS/Tween 20 before use. Auricular LN of allergen- or vehicle-treated mice were harvested at day 3, and cell suspensions were prepared. LN cells were dispensed in the plates (10⁵/well) and incubated with 1 mg/ml Der f proteins or Der f peptide for 36 h at 37°C, 5% CO₂ in the presence of irradiated splenocytes as APCs. Der f class I peptide was also used for re-stimulation at a final concentration of 2 μM. The peptide 113–122 sequence (VGQDFQ) was determined by prediction using the Bioinformatics and Molecular Analysis Section and SYFPEITHI epitope prediction databases. After washing, plates were incubated 2 h at 37°C, 5% CO₂ with a biotinylated anti-IFN-γ or anti-IL-5 mAb (BD Pharmingen). Spot-forming cells (SFCs) were developed using avidin HRP (CliniSciences) incubated for 20 min at room temperature, and extensively washed before adding substrate (aminomethyl carbozole, Sigma-Aldrich).

For the cellular analysis of the AD-like lesions, ears were split into dorsal and ventral halves and incubated for 60 min at 37°C with 1000 U/ml collagenase IA (Sigma-Aldrich), 1000 U/ml hyaluronidase (Sigma-Aldrich), and 200 U/ml Dase N lase (Roche Diagnostics). Cells were incubated with the 2.4G2 hybridoma supernatant to block FcγR and then stained with various combinations of the following anti-mouse Abs: FITC anti-CD45 (leukocyte common Ag Ly5, clone 30-F11; BD Pharmingen), PE anti-CD8, and FITC anti-CD4. Immunofluorescence levels were analyzed on a FACScalibur flow cytometer with CellQuest software (BD Biosciences).

Histology and immunohistochemistry

Ears were fixed in a 3% formalin solution for 24 h and processed through a routine 15-h cycle to paraffin wax embedding. The 4-μm sections were cut using a microtome and mounted on Superfrost Plus slides. Sections were dried overnight at 37°C. The slides were dewaxed in Ottix baths and rehydrated in TBS/Tween 20, and incubated with various combinations of the following anti-mouse Abs: FITC anti-CD45 (leukocyte common Ag Ly5, clone 30-F11; BD Pharmingen), PE anti-CD8, and FITC anti-CD4. Immunofluorescence levels were analyzed on a FACScalibur flow cytometer with CellQuest software (BD Biosciences).

Adoptive transfer

Auxillary and inguinal LN from allergen- or vehicle-treated mice were harvested at day 28 after sensitization and used to prepare single LN cell suspensions and purified CD8+ and CD4+ T cells. A total of 12 × 10⁶ cells/200 μl of saline were i.v. transferred through the orbital vein into naive syngeneic CD3ε−/− mice. Recipient mice were challenged 1 day after cell transfer and ear swelling was measured the following days.

Results

Epicutaneous exposure to house dust mite allergen induces AD-like skin inflammation in normal mice

C57BL/6 mice were sensitized once a week for 6 consecutive weeks by skin application of a solution of Der f on the left ear and of vehicle alone on the right ear, as previously described (17). Control mice were sensitized with the vehicle alone. Twenty-four hours after the fourth allergen exposure, i.e., at day 22, Der f-treated mice developed an ear skin inflammation as manifested by increased ear thickness (Fig. 1A). Ear swelling was detectable at day 22 and increased after each subsequent allergen exposure leading to a very strong skin inflammation at day 36, i.e., 24 h after the sixth allergen exposure. Histopathological analysis of the ear skin of Der f-treated mice at day 36 revealed skin inflammatory changes characteristic of AD-like lesions (Fig. 1B), including...
Incorporation was conducted during the last 16 h of culture. Results are

auricular LNs were restimulated in vitro for 3 days with Der f. Thymidine

in vitro for 3 days with Der f. IFN-γ mice at day 35 and restimulated

and vehicle-sensitized (veh) or Der f-sensitized (Der f) mice. Total T cells, purified CD4

f-specific T cell proliferation in LNs of day-35 vehicle-treated (veh) or Der f-treated

in situ in whole ear mRNA extract from vehicle-treated (veh) or Der f-treated

AD-like lesions in normal C57BL/6 at day 36. We also tested the

thickening of the epidermis (Fig. 1B, panels 2 and 4) with apoptosis of keratinocytes (Fig. 1B, panel 5), blood vessel enlargement, infiltration of mononuclear cells, and more importantly, prominent recruitment of eosinophils (Fig. 1B, panels 3 and 6).

Because human AD lesions are infiltrated by Th1/Th2 cells, we examined the presence of CD4+ and CD8+ T cells and the expression of IL-4, IL-5, and IFN-γ in the skin of allergen-induced AD-like lesions in normal C57BL/6 at day 36. We also tested the expression of CCL17 (TARC), a chemokine associated with the

homing of T lymphocytes in AD lesions in both humans and mice (20, 21). PCR analysis demonstrated that mRNA encoding for these molecules were either not found (CD8, IFN-γ, IL-4, and CCL17) or expressed at very low levels (CD4) in the skin of vehicle-treated mice. In contrast, mRNA for CD8, IFN-γ, IL-4, and CCL17 were induced in the skin of Der f-treated mice, and in CD4 transcripts only moderately increased compared with vehicle-treated skin (Fig. 1C).

We next analyzed the allergen-specific immune response generated at day 35 in auricular LNs, draining the allergen-challenged ear. As shown in Fig. 1D, LN cells from Der f-treated mice vigorously proliferated in response to in vitro restimulation with allergen-pulsed syngeneic cells. Purified CD8+ T cells gave higher proliferation rates than CD4+ T cells, suggesting that both allergen-specific CD8+ and CD4+ T cells had been primed. Moreover, the Der f-specific immune response in total draining LN cells was associated with the production of high levels of IFN-γ and IL-5, whereas no IL-4 could be detected (Fig. 1E). Finally, Der f-treated mice exhibited high serum IgE levels compared with vehicle-treated mice (Fig. 1F).

Although not shown in this experiment, similar histological, molecular, and immunological data were obtained in the skin and LNs of mice at days 22 and 29, i.e., after the fourth and fifth allergen exposure, respectively. Thus, Der f skin sensitization induced an AD-like disease associated with a mixed Th1/Th2-type dermatitis, specific CD4+ and CD8+ T cells and high serum IgE levels.

CD8+ T cells mediate AD-like skin lesions

These results showed that development of AD-like lesions in C57BL/6 mice was associated with prominent up-regulation of CD8 mRNA suggestive of an infiltration of the skin by CD8+ T cells. To study the respective role of CD4+ and CD8+ T cells in our model, we next analyzed the development of AD-like inflammation in C57BL/6 mice and in mice acutely depleted in CD8+ or CD4+ T cells by in vivo treatment with mAbs specific for CD8 and CD4, respectively. Ab treatment was achieved twice a week, before and on the same day as Der f painting, and yielded to a ~95% depletion of the respective T cell subset (data not shown). As shown in Fig. 2A, normal undepleted mice, as well as mice treated by a rat IgG isotype control (data not shown), developed a skin inflammation 24 h after the fourth topical allergen exposure (day 22). CD4+ T cell-depleted mice developed a quicker and stronger response than undepleted mice because skin inflammation was already significant after the second allergen painting (day 8) and increased to a severe dermatitis after the fourth Der f application (day 22). This result suggests that CD4+ T cells do not behave as effector cells but rather contain cells endowed with down-regulatory properties. More importantly, no skin inflammation was induced in CD8+ T cell-deficient mice, demonstrating that CD8+ T cells are necessary for the development of AD-like skin inflammation.

Analysis of the allergen-specific immune response in draining LNs at day 22 was done by IFN-γ ELISPOT assay, using in vitro restimulation of T cells by a H-2b-restricted, class I Der f peptide. Fig. 2B shows that sensitized mice displayed a mean of 80 SFCs/106 LN cells. Der f-specific T cells were found in the CD8+ T cell subset because depletion of CD8+ T cells, but not of CD4+ T cells, abrogated the IFN-γ response. That CD8+ T cells were the major IFN-γ-producing cells in our model was confirmed by IFN-γ ELISPOT experiments using purified CD8+ and CD4+ T cells recovered from draining LNs of day-22 sensitized mice (Fig. 2C).
Analysis of CD8⁺ and CD4⁺ T cell recruitment in allergen-treated ear skin was done by FACS analysis of skin cell suspensions at day 22 (i.e., 24 h after the fourth Der f application) (Fig. 2, D and E). In undepleted mice, treated with the vehicle alone, most of the T cells infiltrating the skin were CD4⁺⁺ T cells (5% of total CD45⁺ cells), whereas CD8⁺ T cells represented only 0.5–1% of CD45⁺ cells (Fig. 2D, upper left panel). In undepleted, Der f-treated mice, the proportion of CD4⁺⁺ T cells in the skin was similar to that found in vehicle-treated mice. In contrast, CD8⁺ T cells represented up to 5% of infiltrating cells, indicating that allergen exposure was responsible for the preferential recruitment of CD8⁺ T cells (Fig. 2D, upper right panel).

In CD4⁺⁺ T cell-depleted and Der f-treated mice, which developed severe skin inflammation, CD8⁺ T cells accounted for up to 10–20% of infiltrating cells, whereas <0.5% of CD4⁺⁺ T cells were found (Fig. 2D, lower left panel). Finally, in CD8⁺⁺ T cell-depleted and Der f-treated mice, in which no skin inflammation could be obtained, no CD8⁺⁺ T cell infiltration was observed and CD4⁺⁺ T cells represented 5% of skin CD45⁺ cells (Fig. 2D, lower right panel). Fig. 2D shows that infiltration of CD8⁺⁺ T cells in the challenged skin is a transient phenomenon because an increased number of CD8⁺⁺ T cells are observed at 24 h but not at 48 h after Der f painting. At that time the percentage of CD8⁺⁺ T cells represent only 1.5% of CD45⁺ skin cells. Of note, development of the skin inflammation is also associated with infiltration of CD4⁺⁺ T cells, which occurs however later than the CD8⁺⁺ T cell recruitment, and is observed in both Der-f and vehicle-challenged mice. Indeed, CD4⁺⁺ T cells represent up to 6% of CD45⁺ skin cells at 48 h but only 2% at 24 h postchallenge (Fig. 2E). Immunohistochemical analysis of CD8⁺⁺ T cells in the skin of CD4⁺⁺ T cell-depleted mice confirmed FACS data showing that Der f exposure at day 21 resulted in a marked infiltration of the skin by CD8⁺⁺ T cells, which peaked at 24 h (day 22) (Fig. 2F). At that time, CD8⁺⁺ T cells were observed in the dermis and more importantly in the epidermis, whereas at 48 h only dermal CD8⁺⁺ T cells could be found.

Taken together these data indicate that the magnitude of the Der f-induced eczema is positively correlated to the number of CD8⁺⁺ T cells infiltrating the skin.

Mice deficient in CD4⁺⁺ T cells develop severe AD-like skin lesions and enhanced priming of allergen-specific CD8⁺⁺ T cells

To confirm that allergen-induced CD8⁺⁺ T cells can induce AD-like inflammation in the absence of CD4⁺⁺ T cells, we tested whether AD-like disease could be induced by repeated Der f sensitization in Aβ gene °/° (MHC class II °/°) mice, genetically deficient in MHC class II-restricted CD4⁺⁺ T cells. Similarly to anti-CD4 mAb-treated B6 mice (Fig. 2A), MHC class II °/° mice developed a quicker and more intense skin inflammation as compared with normal C57BL/6 mice (Fig. 3A). Skin inflammation was already significantly up-regulated after the second allergen
FIGURE 3. MHC class II-deficient (II °/°) mice develop severe AD-like inflammation. A, Allergen-induced skin inflammation was analyzed in wild-type C57BL/6 and MHC class II-deficient mice sensitized with a Der f solution on the left ear, once a week for five consecutive weeks. MHC class II-deficient mice treated with the vehicle alone (C) were included as controls. Results are expressed as the mean ear swelling (in micrometers) at 24 h after each sensitization, using five mice per group ± SD. Results are representative of three independent experiments. B, Histological analysis of the ear skin of Der f-sensitized C57BL/6 mice and MHC class II-deficient mice (II °/°) at day 22 (i.e., 24 h after the fourth sensitization). HES staining at a magnification of ×200 is shown. C, RT-PCR analysis of CD8, IFN-γ, IL-4, and CCL17 transcripts in ears of Der f-treated C57BL/6 (BL/6) and MHC class II-deficient mice (II °/°) at days 22 and 23. Control includes mRNA expression in the ears of vehicle-treated (veh) MHC class II-deficient mice at day 22. D, Der f-specific proliferative responses in total auricular LN cells from Der f-sensitized (●) and vehicle-sensitized (□) C57BL/6 and MHC class II-deficient mice at day 21 after in vitro restimulation with Der f. Thymidine was added for the last 16 h of culture. Results are expressed as total cpm supress (×10⁻³). **, p < 0.01. E, Der f-specific IFN-γ-producing cells were determined by ELISPOT assay in LN cells from day 21 Der f-sensitized (●) or vehicle-sensitized (□) C57BL/6 and MHC class II-deficient (II °/°) mice, after overnight restimulation with Der f. Results are expressed as the number of SFCs per 10⁶ LN cells. Results in D and E are presented as the mean ± SD in culture triplicate. F, Titration of serum IgE (nanograms per milliliter) in either Der f-sensitized (●) or vehicle-sensitized (□) C57BL/6 and MHC class II-deficient mice at day 36 by ELISA. Results are presented as the mean (three animals ± SD).

Adoptive transfer of CD8⁺ but not CD4⁺ T cells from Der f-sensitized mice can induce AD-like skin lesions

To confirm the dominant role of CD8⁺ T cells in the pathophysiology of Der f-induced eczema, CD8⁺ T cells (or CD4⁺ T cells) purified from the LNs of vehicle- or Der f-treated mice at day 21 were i.v. transferred into naive T cell-deficient CD3 °/° recipient mice, which were ear challenged with Der f 24 h later. Results are expressed as ear swelling at day 6 postchallenge.

exposure and further augmented to a severe dermatitis after the fourth Der f application (day 21). Histological analysis of the ears of MHC class II °/° mice at day 22 showed enhanced thickening of the epidermis and increased skin infiltration by mononuclear cells, compared with C57BL/6 mice (Fig. 3B). Increased skin inflammation in MHC class II °/° mice correlated with a rapid up-regulation of CD8, IFN-γ, and IL-4 transcripts in the allergen-exposed ear as well as appearance of CCL17 mRNA, at 24 h after Der f exposure (Fig. 3C).

Analysis of the Der f-specific immune responses at day 21 in draining auricular LNs revealed that MHC class II °/° mice exhibited increased Der f-specific T cells as assessed by proliferative responses (Fig. 3D) and frequency of IFN-γ-producing cells (Fig. 3E). However, the number of IL-5-producing LN cells was similar in MHC class II °/° mice to that observed in wild-type C57BL/6 mice and ranged from 30 to 80 IL-5 SFCs/10⁶ LN cells (data not shown). IgE was undetectable in the serum of Der f-sensitized mice and ranged from 30 to 80 IL-5 SFCs/10⁶ LN cells (data not shown). IgE was undetectable in the serum of Der f-sensitized mice at day 22. Control includes mRNA expression in the ears of vehicle-treated (veh) MHC class II-deficient mice at day 22. D, Der f-specific proliferative responses in total auricular LN cells from Der f-sensitized (●) and vehicle-sensitized (□) C57BL/6 and MHC class II-deficient mice at day 21 after in vitro restimulation with Der f. Thymidine was added for the last 16 h of culture. Results are expressed as total cpm supress (×10⁻³). **, p < 0.01. E, Der f-specific IFN-γ-producing cells were determined by ELISPOT assay in LN cells from day 21 Der f-sensitized (●) or vehicle-sensitized (□) C57BL/6 and MHC class II-deficient (II °/°) mice, after overnight restimulation with Der f. Results are expressed as the number of SFCs per 10⁶ LN cells. Results in D and E are presented as the mean ± SD in culture triplicate. F, Titration of serum IgE (nanograms per milliliter) in either Der f-sensitized (●) or vehicle-sensitized (□) C57BL/6 and MHC class II-deficient mice at day 36 by ELISA. Results are presented as the mean (three animals ± SD).
that were challenged with Der f 1 day after transfer. As shown in the Fig. 4, mice reconstituted with CD8+ T cells from Der f-treated mice developed a severe skin inflammation starting 24 h after Der f challenge and peaking at day 6 postchallenge. In contrast, no skin inflammation was observed upon Der f challenge in CD3εεεεεε recipients adoptively transferred with purified CD4+ T cells. These data provide evidence that CD8+ T cells are the pathogenic effector T cells responsible for the onset of Der f-induced mouse AD.

Discussion
In this study, we demonstrate that CD8+ T cells represent the dominant effector cell responsible for allergen-induced skin inflammation. The AD-like dermatitis that developed after repeated exposure to the aeroallergen Der f displays all the major histological, molecular, and immunological features of human allergic AD. Histological changes include dermal thickening, vasodilatation, infiltration by eosinophils and mononuclear cells including CD4+ and CD8+ T cells, and epidermal thickening with presence of several apoptotic cells. Molecular changes in the skin consist of up-regulation of transcripts for Th1-type (IFN-γ) and Th2-type (IL-4) cytokines and for the chemokine CCL17, involved in the recruitment of type 2-producing cells (22). In lymphoid organs, Der f-primed T cells are present within both the CD4+ and CD8+ T cell subsets and produce high levels of IFN-γ and IL-5. Finally, Der f-primed mice have high levels of circulating IgE. Therefore, the Der f-induced AD mouse model is suitable for the study of AD pathophysiology.

Our data clearly demonstrate that CD8+ T cells represent the effector cells that mediate skin inflammation. Our finding is supported by several lines of evidence. First, repeated in vivo skin sensitization with the allergen Der f is able to prime a vigorous CD8+ T cell response in skin draining LNs and to recruit CD8+ T cells in skin. Second, in vivo Ab depletion of CD8+, but not CD4+ T cells, in wild-type mice completely abrogates the skin inflammation, indicating that CD8+ T cells are required for the skin inflammatory process. Third, CD8+ T cell-deficient MHC class II εεεεεεεεεε mice develop severe AD lesions. Finally, primed CD8+ T cells from LNs of allergen-sensitized mice can adoptively transfer AD lesions upon allergen challenge in naive recipients.

The observation that CD4+ T cell-depleted mice develop an early and exacerbated inflammation indicates that CD8+ T cells can be primed and developed into effector cells without the need for CD4+ T cell help. Moreover, several observations indicate that CD4+ T cells may comprise a subset of regulatory cells controlling the skin inflammatory process by regulating the intensity of the CD8+ T cell response. Indeed, mice with a CD4+ T cell defect (including both anti-CD4 mAb-treated C57BL/6 and MHC class II εεεεεεεεεε mice) exhibit a more rapid appearance of AD lesions. This response is associated with a higher CD8+ T cell response in draining LNs and a higher number of CD8+ T cells recruited into the skin. Although the subset of CD4+ T cells endowed with regulatory properties has not been tested, our findings are reminiscent to previous studies in AD patients, indicating that IL-10-producing CD4+ regulatory T cells control the magnitude of AD skin inflammation (23).

CD8+ T cell recruitment and activation in AD-like lesions is a rapid but transient phenomenon occurring in the hours following allergen exposure. This outcome was demonstrated by kinetic studies of the phenotype of skin-infiltrating T cells using flow cytometry, immunohistochemistry, and RT-PCR analysis. Because CD8+ T cells are the major IFN-γ producers in our model, the up-regulation of IFN-γ mRNA, which paralleled CD8+ T cell infiltration, is thought to reflect CD8+ T cell activation. CD8+ T cells are found in the epidermises at 24 h postallergen exposure (both in wild-type and MHC class II εεεεεεεεεε mice) and are no longer detectable at 48 h. Conversely, CD4+ T cells are recruited only at 48 h of challenge, i.e., subsequent to skin infiltration by CD8+ T cells. These observations suggest that, once recruited into skin, allergen-primed CD8+ T cells require a narrow window of time for exerting their effector function that initiates the subsequent skin inflammatory process. Persistence of these CD8+ effector T cells in skin is not necessary for the skin inflammation to proceed probably because other cells involved in AD pathophysiology, especially eosinophils (24) have been recruited in the meantime. Our data are reminiscent to previous studies in human AD showing that, although CD4+ T cells predominated in AD skin lesions, T cells infiltrating the epidermises were almost exclusively CD8+ T cells (12). Along these lines, studies in mouse models of AD reported that allergen challenge is associated with epidermal recruitment of CD8+ T cells (25), which is dramatically enhanced by coexposure to the superantigen staphylococcal enterotoxin B (26). However, only a few CD8+ T cells, compared with CD4+ T cells, were detected in the skin in these studies probably because of the time at which T cell infiltration was analyzed. It may be proposed that this peculiar transient kinetics of CD8+ T cell recruitment and activation in the allergen-exposed skin may explain why previous studies have underestimated the effector role of CD8+ T cells in AD.

The peak of IFN-γ production in the challenged skin is maximal at 24 h and decreases rapidly after 48 h. This is in contrast with human studies on atopy patch test that showed an early and transient up-regulation of IL-4 at 24 h followed by a switch toward IFN-γ production, which appeared at 48 h only (27). Although this discrepancy may reflect subtle differences between mouse and human disease models, it could be hypothesized that the previous studies have missed the first wave of allergen-specific T cells infiltrating the challenged skin and transiently producing IFN-γ. Ongoing studies testing for the presence of type 1 and type 2 cytokines in atopy patch tests after 6 and 12 h postchallenge may help to better understand the early events involved in the induction of the AD skin inflammation.

The fate of skin-infiltrating CD8+ T cells is not known precisely yet. It is possible that the recruited CD8+ T cells rapidly leave the skin through the lymphatic vessels shortly after having delivered their effector function. Alternatively, CD8+ T cells may die by activation-induced cell death after allergen-induced T cell activation. In this respect, Akdis et al. (8) demonstrated that apoptosis of circulating memory/effector T cells is a constant feature of AD skin inflammation that involves Fas-Fas ligand (FasL) interaction. The fact that activation-induced cell death is mostly confined to Th1 cells, whereas Th2 cells are resistant to apoptosis, could explain why a Th2-skewed immune response is consistently described in patients with AD (8). Finally it is tempting to speculate that CD4+ T cells endowed with down-regulatory functions are responsible for the rapid clearance of CD8+ T cells, inasmuch as recruitment of CD4+ T cell observed at 48 h, but not at 24 h, paralleled the disappearance of CD8+ T cells from the allergen-exposed skin. The mechanisms by which CD4+ T cells may contribute to the clearance of Der f-primed CD8+ T cells remain, however, unknown.

How CD8+ T cells induce the AD skin pathology? Epidermal abnormalities including epidermal spongiosis and vesicle formation are pathological hallmarks of AD (22). Apoptosis of keratinocytes caused by skin-infiltrating, FasL-expressing T cells plays a key role in spongiosis formation. Studies of Akdis et al. (8) in human AD indicate that T cell-derived IFN-γ is responsible for up-regulation of Fas by keratinocytes, which become susceptible to apoptosis through Fas-FasL interaction. Moreover, perforin and
granzyme B are cytotoxic molecules produced by activated cytotoxic CD8+ T cells, which are strongly expressed in AD lesional skin (28). Our data showing a correlation between the infiltration of IFN-γ-producing CD8+ T cells in the epidermis, the appearance of apoptotic cells, and the development of the skin inflammation are in line with our reported findings and suggest that, similarly to their mode of action in allergic contact dermatitis to hapten (29), allergen-specific CD8+ T cells could exert their pathogenic function via cytotoxicity against allergen-presenting keratinocytes.

Thus, the following scheme could be proposed to explain the pathogenic role of CD8+ T cells in induction of AD-like skin inflammation in allergen-exposed mice. First, CLA-positive allergen-specific CD8+ T cells, primed in skin draining LNs, are recruited into the epidermis through interactions with E-selektins and P-selectins expressed on endothelial cells. Second, specific CD8+ T cells are activated by allergen-presenting epidermal cells resulting in both cytotoxic activity and activation of skin-resident cells. Thirdly, amplification of the inflammatory reaction occurs through production of cytokines and chemokines by skin cells, such as CCL17/TARC and CCL22/macroage-derived chemokine, promoting the subsequent recruitment of leukocytes, including CD4+ T cells and eosinophils (22). Further studies will have to define more precisely the mechanisms responsible for the initial CD8+ T cell recruitment in the skin.

In summary, we have identified a critical role for CD8+ T cells in allergen-induced mouse AD and have shown that CD8+ T cells are required for the full development of the allergic skin inflammation. Our data are in line with recent studies reporting on the effector role of CD8+ T cells in allergic asthma (30, 31) and strongly support the idea that the CD4+ Th2 dogma in allergic diseases should be revisited (7). However, although the results presented come from a representative mouse model, studies in humans are required to confirm the hypothesis that CD8+ T cells are involved in the development of AD skin lesions.

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