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Association of Cathepsin E Deficiency with Development of Atopic Dermatitis

Takayuki Tsukuba[*,1], Kuniaki Okamoto[*,1], Yoshiko Okamoto[2], Michiyo Yanagawa[1], Keiko Kohmura[1], Yoshiyuki Yasuda[1], Hiroshi Uchi[3], Takeshi Nakahara[3], Masutaka Furue[3], Keiko Nakayama[4], Tomoko Kadowaki[1], Kenji Yamamoto[†,1] and Keiichi I. Nakayama[4]

[1]Department of Pharmacology, Graduate School of Dental Science, Kyushu University, Higashi-ku, Fukuoka 812-8582; [2]Department of Biochemistry, Daiichi University College of Pharmaceutical Sciences Fukuoka 815-8511; [3]Department of Dermatology, Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582; and [4]Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582

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Atopic dermatitis (AD) is a pruritic inflammatory skin diseases associated with a family history of atropy. Here we show that mice lacking the endolysosomal aspartic proteinase cathepsin E spontaneously develop skin lesions similar to those of humans with AD when reared under conventional conditions but not under specific pathogen-free conditions. These mice showed the increase in the ratio of $CD4^+/CD8^+$ T cells, the strong polarization of naïve T cells to T helper 2 cells, and the systemic accumulation of IL-18 and IL-1β accompanied by a marked increase in IL-4, IL-5, and IgE. The relative rates of degradation of IL-18 and IL-1β were significantly lower in cathepsin E–deficient mice than wild-type mice. These results strongly suggest that the development of AD in cathepsin E-deficient mice is initiated by systemic accumulation of IL-18 and IL-1β, mainly due to their reduced turnover rates. In addition, the reduced expression of cathepsin E was also observed in erythrocytes of both humans with AD and the AD mouse model NC/Nga. Cathepsin E deficiency might thus be responsible for the induction of AD in humans and mice.

Key words: aspartic proteinase, atopic dermatitis, cathepsin, proteinase.

Abbreviations: AD, atopic dermatitis; *CatE*$^{-/-}$, cathepsin E–deficient; ES, embryonic stem; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; FBS, fetal bovine serum; FITC, fluorescent isothiocyanate; Ig, immunoglobulin; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; NC, NC/Nga; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PE, phycoerythrin; RT-PCR, reverse transcriptase–polymerase chain reaction; SPF, specific pathogen–free; Th, T helper; TNCB, picryl chloride (or 2,4,6-trichlorobenzene); TRITC, tetramethylrhodamine isothiocyanate.

Atopic dermatitis (AD) is a common inflammatory skin disease characterized by intermittent extreme pruritus accompanied by peripheral blood eosinophilia and, in some individuals, an increased serum concentration of immunoglobulin E (IgE) (*1*, *2*). A marked increase in the prevalence of AD over the past 3 decades has prompted efforts to characterize its pathogenesis and to develop new approaches to the therapy. Although changes in lifestyle may contribute to the recent increase in the incidence of AD, genetic factors are likely a primary determinant of its expression. Linkage studies have implicated multiple genes in the pathogenesis of AD (*3*), but the genes responsible for this condition remain to be identified definitively. Early events in an immune response for AD are primarily mediated by enhanced production of initiator cytokines for T helper (Th)2 cell responses, such as IL-18 and IL-1β, that directs the subsequent development of Th2 cells (*4*). Accumulation of such initiator cytokines can induces the subsequent production of the executioner cytokines for Th2 cell responses, such as IL-4 and IL-5, accompanied by systemic accumulation of IgE and histamine (*5*). It is assumed that systemic levels of the initiator cytokines, as well as the executioner cytokines, are controlled by the balance between production and degradation, but specific proteinase(s) responsible for the catabolism of these cytokines remains to be answered.

Cathepsin E, an endolysosomal aspartic proteinase, is predominantly expressed in immune system cells (*6*, *7*). Although it is implicated in a wide of physiological and pathological processes (*8*–*14*), a definitive demonstration of the pathophysiological roles of cathepsin E *in vivo* has been lacking, likely because of an overlap in function among various proteinases of the endolysosomal proteolytic system. More recently, we found that cathepsin E is involved in degradation of α2-macroglobulin that has the potential for capturing diverse molecules including endopeptidases, cytokines, growth factors, and hormones (*15*) and that both expression of cathepsin E mRNA and secretion of this protein in antigen presenting cells such as macrophages and dendritic cells are increased by treatment with interferon (IFN)-γ and reduced by treatment with IL-4 (unpublished data). These observations

[*]T.T. and K.O. contributed equally to this study.
[†]To whom correspondence should be addressed. Tel: +81-92-642-6337, Fax: +81-92-642-6342, E-mail: kyama@dent.kyushu-u.ac.jp

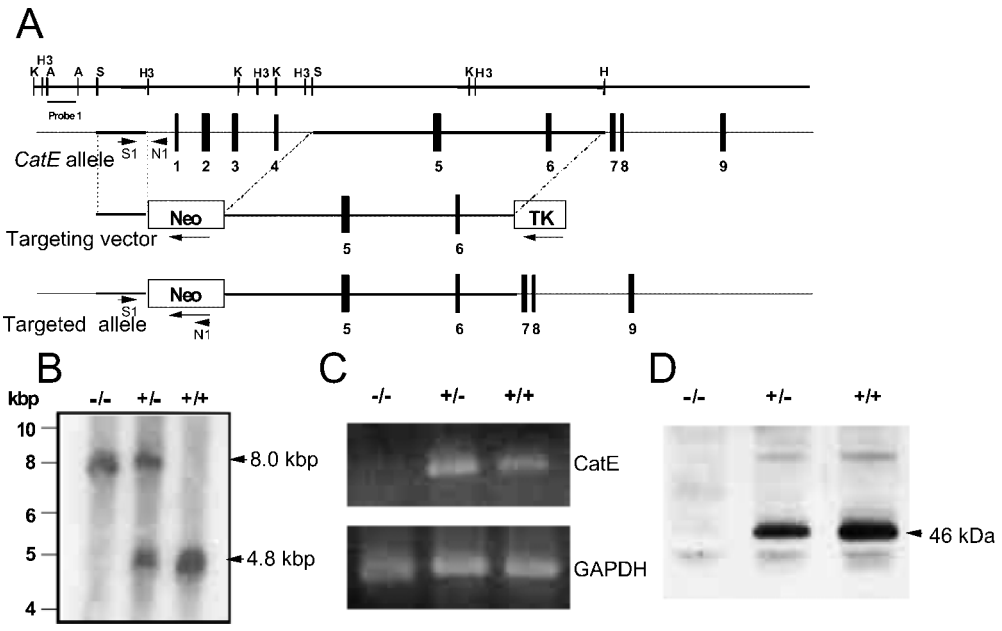

Fig. 1. **Targeted disruption of the mouse cathepsin E gene.** (A) Structures of the targeting vector (pCTSE-KO) of the mouse *CatE* locus and of the mutant allele resulting from homologous recombination. Exons are depicted by filled boxes. A genomic fragment used as a probe for Southern blot analysis (probe 1) is indicated. The box labelled Neo represents a PGK-neo-poly(A) cassette, comprising the phosphoglycerate kinase gene promotor linked to the neomycin transferase gene and poly(A), with the arrow indicating its orientation; that labelled TK represents a PGK-TK-poly(A) cassette, comprising the phosphoglycerate kinase gene promoter linked to the thymidine kinase gene of herpes simplex virus and poly(A). Restriction enzyme sites: A, *Ava*II, H, *Hpa*I; H3, *Hin*dIII; K, *Kpn*I; S, *Stu*I. (B) Southern blot analysis of genomic DNA. Genomic DNA extracted from the tail of mice of the indicated *CatE* genotypes (–/–, +/–, or +/+) was digested with *Kpn*I and subjected to hybridaization with the probe. The positions of the 8.0- and 4.8-kbp hybridising fragments derived from the mutant and wild-type *CatE* alleles, respectively, are indicated. (C) RT-PCR analysis of the cathepsin E (upper panel) and glyceraldehyde-3-phosphoate dehydrogenase (lower panel) genes in mouse spleens of the indicated genotypes. (D)Western blot analysis of spleen extracts from mice of the indicated genotypes with antibodies to cathepsin E. The position of the 46-kDa immunoreactive protein is indicated.

strongly indicate that cathepsin E contributes to immune responses. To assess the specific role of cathepsin E *in vivo*, we generated mice deficient in this enzyme (*CatE*$^{-/-}$ mice) by homologous recombination. Here we report that *CatE*$^{-/-}$ mice spontaneously developed skin lesions similar to those characteristic of AD in humans when reared under standard laboratory (conventional) conditions, but not when maintained under specific pathogen-free (SPF) conditions. We demonstrate that this effect is mediated by systemic accumulation of both initiator and executioner cytokines for Th2 cell responses accompanied by enhanced production of IgE. We also observe the reduced expression of cathepsin E both in humans with AD and in a mouse model NC/Nga(NC) of AD, suggesting that cathepsin E deficiency might be responsible for the induction of AD in humans and mice. We thus indicate that *CatE*$^{-/-}$ mice may constitute a new model of AD that should both provide insight into the pathogenesis of this condition and facilitate the development of new treatments.

EXPERIMENTAL PROCEDURES

Generation of CatE$^{-/-}$ *Mice*—Genomic DNA corresponding to the *CatE* locus was isolated from a 129/Sv mouse genomic library (Stratagene). The targeting vector, pCTSE-KO, was constructed by replacing a 3.1-kbp fragment containing exons 1 to 4 with a PGK-neo-poly(A) cassette. The targeting vector contained 1.2- and 7.0-kbp regions of homology 5′ and 3′ of the neomycin resistant marker, respectively. A PGK-TK-poly(A) cassette was ligated at the 3′ end of the insert. The maintenance, transfection, and selection of embryonic stem (ES) cells were performed as described previously (*16*). G418-resistant colonies were screened by Southern blot analysis with the 1,075-bp *Ava*II probe (Fig. 1a). The expected sizes of hybridizing fragments by *Kpn*I digetion of genomic DNA are 4.8 and 8.0 kbp for the wild-type and mutant *CatE*$^{-/-}$ alleles, respectively. The mutant ES cells were microinjected into C57BL/6 blastocysts, and the resulting male chimeras were mated with female C57BL/6 mice. The germ-line transmission of the mutated allele was confirmed by Southern blot analysis as described above. Heterozygous offspring were intercrossed to produce homozygous mutant animals. All mice were screened by Southern blot analysis and by polymeratse chain reaction (PCR) with probes able to distinguish wild-type, heterozygous and homozygous mutant mice (S1: 5′-AGGGTGGGGTTGATGGTAAG-3′; W1: 5′-TGAAAATGAGGGTGTTGAGGT-3′; N1: 5′-TGGCTGCTATTGGGCGAAGTG-3′). For reverse transcriptase (RT)-PCR analysis, total RNA was extracted using Trizol reagent (Life Technologies), and the first strand cDNA was synthesized using ReveTra Ace (TOYOBO, Osaka). For PCR, the cDNA was amplified by *Taq* DNA polymerase (Takara Biomedicals, Tokyo). A thermal cycle of 94°C for 1 min, 55°C for 1 min and 72°C for 30 sec was performed

using a program of 35 cycles for cathepsin E and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The amplified products were separated by electrophoresis on a 2% agarose gel and detected. Primers used for mouse cathepsin E were 5′-TGAACCCCTCATCAACTACCT-3′ and 5′-CACTGCATATTCTCCATCAAT-3′, and for GAPDH were 5′-ATGTCGTGGACTCTACTGGC-3′ and 5′-TGACCTTGCCCACAGCCTTG-3′. All animal experiments were carried out according to the guideline for Animal Experiments, Kyushu University. Unless indicated otherwise, all mice were maintained in an SPF animal facility at the Kyushu University Station for Collaborative Research.

Experimental Contact Dermatitis—Experimental contact dermatitis was induced essentially according to the protocol recommended by Japan Charles River (Yokohama). In brief, 5-week-old mice were immunized by painting the abdominal skin with 50 μl of 5% picryl chloride (2,4,6-trichlorobenzene, TNCB) (Tokyo Kasei Kogyo, Tokyo) in ethanol/acetone (4:1, v/v). After 1 week, 150 μl of 5% TNCB was administered epicutaneously on the back of each animal, without shaving, every 3.5 days. The severity of dermatitis at the indicated times after the first epicutaneous treatment was scored as the sum of individual scores for each of five signs (itching, erythema-hemorrhage, edema, excoriation-erosion, scaling-dryness). NC mice were purchased from Japan Charles River (Yokohama).

Determination of Serum Ig and Cytokine Concentrations—The serum concentrations of immunoglobulin (Ig)G1, IgG2, IgM, IgE, interleukin (IL)-18, and IL-1β were determined by enzyme-linked immunosorbent assays using kits from various commercial sources (IgG1, IgG2, and IgM from Bethyl, IgE from Shibayagi, IL-18 from Medical & Biological Lab., and IL-1β from Biosource Int.).

Cell Culture and Cytokine Detection—Spleen cells from 8- and 12-week-old mice were suspended in NH4Cl lysis buffer to induce lysis of red cells and then cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. The spleen cells were stimulated with phorbol myristate acetate (50 ng/ml) and ionomycin (500 ng/ml) for 42 h. The concentrations of cytokines in the culture supernatants were determined by enzyme-linked immunosorbent assays (Bio Source International).

Antibodies and FACS Analysis—Freshly isolated spleen cells of *CatE*$^{-/-}$ and wild-type mice at 14 weeks of age were stained with fluorescent isothiocyanate (FITC)- and phycoerythrin-conjugated (PE) monoclonal antibodies specific for CD4 (YTS191.1) and CD8 (KT15). Stained cell populations were analyzed with a FACScan flow cytometer (Beckman-Coulter).

Preparation of Erythrocyte Ghosts—The preparation of human or mouse erythrocytes were performed as described previously (*17*). Briefly, the freshly heparin containing peripheral blood was well washed with phosphate buffered saline (pH 7.4), and were lysed with 10 mM sodium phosphate buffer (pH 8.0), and then centrifuged at 15,000 rpm for 20 min. The pellets were washed with the same buffer three or four time, and centrifuged until the erythrocyte membrane being completely white. The final samples were solubilized with Triton X-100 to a final concentration of 0.05%.

Aspartic Proteinase Assay—Cathepsin E activity was determined with acid-denatured hemoglobin as a substrate at pH 3.5 (*17*). Data are expressed as units per milligram of protein, where 1 U is defined as the amount of enzyme that results in an increase in absorbance at 660 nm equivalent to 1 μg of tyrosine in 1 min. Each symbol represents cells from an individual human or mouse, with mean values indicated by the horizontal bars.

Determination of Cytokine Turnover Rate—Mice were deprived of food for 16 h and then injected intraperitoneally with 2.5 μCi of [^{14}C]leucine (>300 mCi/mmol, DuPont-New England Nuclear). Food was restored after an additional 4 h. Three days after the initial injection, the fasting regimen was repeated and the animals were injected with 7.5 μCi of [^{3}H]leucine (>140 Ci/mmol, DuPont-New England Nuclear). The mice were killed 4 h after this second injection and the blood were collected. Serum prepared from the blood by centrifugation was mixed with Pansorbin (Calbiochem) to prevent nonspecific binding to IgG–protein A complexes. After centrifugation, the supernatant was incubated with antibodies specific for IL-18 (Medical & Biological Lab.), for IL-1β (Genzyme Teche), or for albumin (control) (ICN Biomedicals). The resulting immune complexes were precipitated with Protein A-Sepharose beads (Amersham Pharmacia Biotech), which were then washed and the associated ^{14}C and ^{3}H radioactivity was measured with a liquid scintillation counter (Beckman, LS-6001C).

Statistical Analysis—The statistical significance of differences between mean values was assessed by Student's *t*-test. *P* values of <0.05 were considered statistically significant.

RESULTS

Generation of Cathepsin E–Deficient Mice—The genomic locus of the mouse cathepsin E gene includes nine exons that span ~16 kbp. To generate cathepsin E knockout mice, we designed a targeting construct to delete the genomic region encompassing exon 1 including the translation initiation site, to exon 4 (Fig. 1A); exon 3 encodes one of two active site motifs, a consensus *N*-linked oligosaccharide binding site, and a cysteine residue required for dimmer formation. The targeting vector contained 1.2- and 7.0-kbp regions of homology located 5′ and 3′, respectively, relative to the neomycin-resistant marker as well as a PGK-TK-poly(A) cassette at the 3′ end of the insert. Embryonic stem (ES) cells were transfected with the targeting vector, recombinant clones were selected and injected into C57BL/6 mouse blastocysts, and chimeric males that exhibited germ-line transmission of the mutated allele were obtained. Heterozygotes were mated to produce homozygous mutant mice. Southern blot (Fig. 1B), RT-PCR (Fig. 1C), and immunoblot (Fig. 1D) analyses demonstrated a complete absence of cathepsin E DNA, mRNA and protein, respectively, in the homozygous mutant animals. Genotyping of offspring from crosses of heterozygous mice revealed a frequency of ~25% *CatE*$^{-/-}$ animals, consistent with the expected Mendelian frequency. *CatE*$^{-/-}$ mice were fertile and exhibited normal breeding behavior. The *CatE*$^{-/-}$ mice showed no obvious phenotypes when raised under SPF conditions, and no gross abnormalities clearly related to genotype

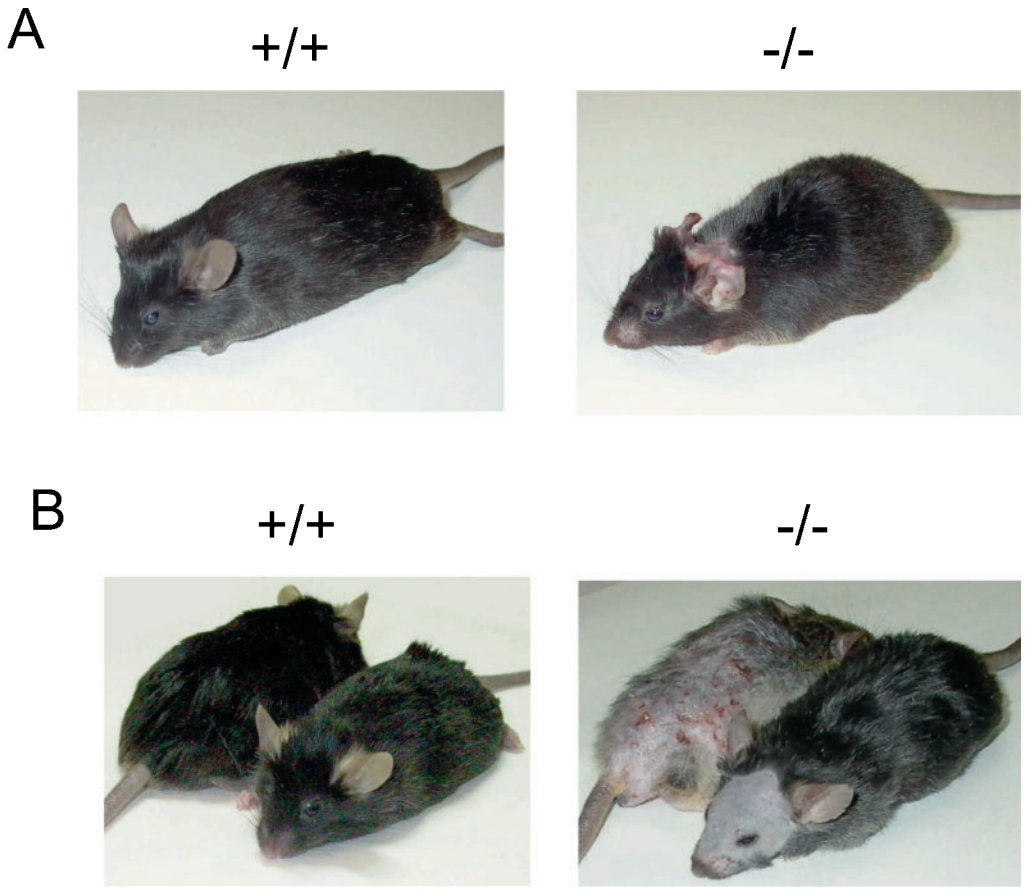

Fig. 2. **Characteristics of *CatE*$^{-/-}$ mice that had been maintained under conventional conditions.** (A) Gross appearance of *CatE*$^{-/-}$ mice at 14 weeks of age compared with that of wild-type littermates. (B) Gross appearance of *CatE*$^{-/-}$ mice at 50 weeks of age compared with that of wild-type littermates.

were noted at any age. Terminal body weight or the weights of brain, kidney, adrenals, thymus, heart, liver, and spleen, did not differ among genotypes.

Development of AD-Like Skin Lesions in CatE$^{-/-}$ *Mice*—Transfer of *CatE*$^{-/-}$ mice to conventional conditions (in air-filtered conventional room, 21 ± 2°C, 55 ± 15% humidity) resulted in the spontaneously development of AD-like skin lesions (Fig. 2), indicating that such lesions are induced by environmental factors such as bacterial infection. Consistent with this finding, when samples from the skin lesions of conventional *CatE*$^{-/-}$ mice at 14 weeks of age were inoculated onto salt egg yolk agar

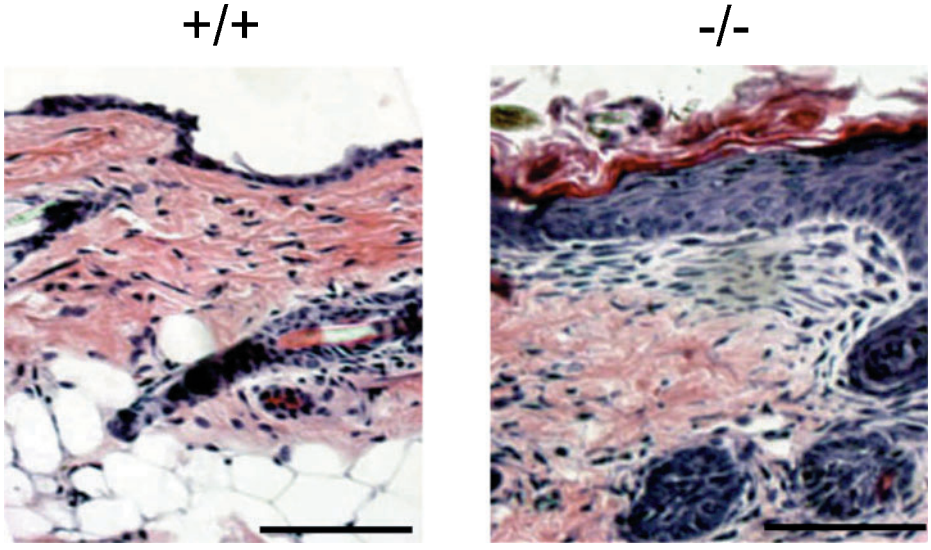

Fig. 3. **Histological analysis of skin lesions of *CatE*$^{-/-}$ mice at 14 weeks of age.** Skin sections of wild-type and *CatE*$^{-/-}$ mice were stained with hematoxylin-eosin. Scale bars, 100 μm.

base Nissui (EY agar, Nissui Pharmaceutical, Tokyo) and incubated at 37°C, bacterial colonies were grown and identified *Staphylococcus aureus* as a preferential bacterium (data not shown). Various grades of such lesions were apparent on the face, neck, ears, nose, and dorsal skin, depending on the age and the duration of exposure to conventional conditions. These skin lesions of *CatE*$^{-/-}$ mice at 50 weeks of age were more severe than those at 14 weeks of age (Fig. 2B). These animals exhibited additional clinical signs, including itching, erythema, crust formation, erosion, alopecia, and hyperplasia of the epidermis, that were also similar to the symptoms of humans with AD. For animals raised under conventional conditions from birth, the AD-like skin lesions were first apparent at ~10 weeks, and the time required for the induction of dermatitis tended to decrease with age at the time of transfer to conventional conditions. Only a small percentage of *CatE*$^{+/-}$ mice showed the AD-like skin lesions under the same conditions. The epidermis of *CatE*$^{-/-}$ mice was thickened by hyperplasia, and a large number of eosinophils, macrophages, lymphocytes, and mast cells were present in the dermis (Fig. 3). In contrast, no substantial abnormalities were detected in age-matched *CatE*$^{+/+}$ (Fig. 3) or in *CatE*$^{-/-}$ mice raised under SPF conditions (data not shown). The number of eosinophils in peripheral blood was markedly increased in *CatE*$^{-/-}$ mice raised under conventional conditions compared with those in wild-type mice or in *CatE*$^{-/-}$ mice raised under SPF conditions (Table 1). There were no significant differences in the numbers of lymphocytes, neutrophils, erythrocytes, and platelets between control and *CatE*$^{-/-}$ mice maintained under conventional conditions. In addition, biochemical analysis of peripheral blood revealed that there were no significant differences in all of the parameters tested between age-matched *CatE*$^{+/+}$ and *CatE*$^{-/-}$ mice raised under conventional conditions (Table 2).

Increased Production of IgE and Th2 Cytokines in CatE$^{-/-}$ *Mice*—We next examined the role of Th2 cell-mediated responses in the development of AD-like skin lesions in 9- to 14-week-old *CatE*$^{-/-}$ mice raised under conventional conditions from birth. Although the serum concentrations of IgG1, IgG2, and IgM did not differ between *CatE*$^{-/-}$ and wild-type mice, that of IgE was ~10-times higher in *CatE*$^{-/-}$ mice than in wild-type animals (Fig. 4). The serum concentration of IgE in *CatE*$^{+/-}$ mice raised under conventional conditions was also about three times that of wild-type mice. Under SPF conditions, *CatE*$^{-/-}$ mice did not exhibit an increased serum IgE concentration at ages up to 12 months (data not shown).

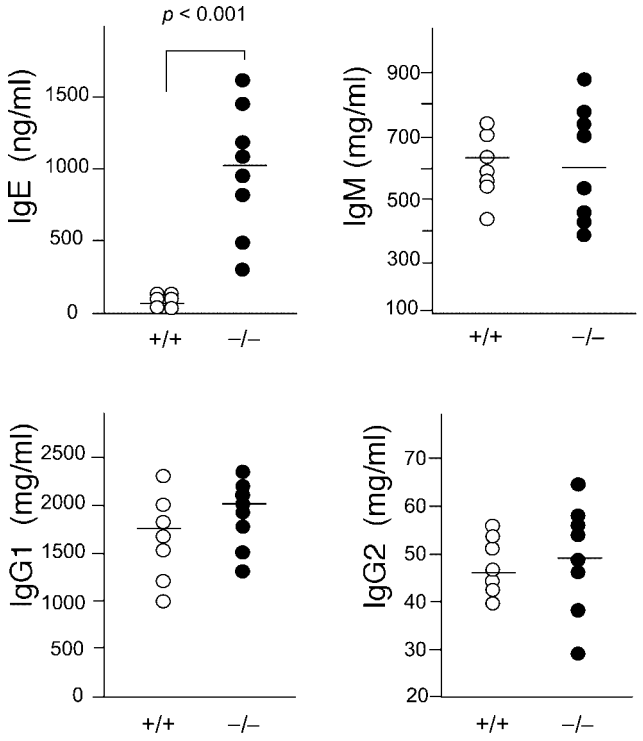

Fig. 4. **Serum Ig concentrations of *CatE*$^{-/-}$ and wild-type mice at 14 weeks of age raised under conventional conditions.** Each symbol represents an individual animal, with mean values indicated by horizontal bars.

Similar to humans with AD, the ratio of CD4^{+}/CD8^{+} T cells among splenic lymphocytes in *CatE*$^{-/-}$ mice raised under conventional conditions was markedly increased compared with that in wild-type mice (Fig. 5A). The local cytokine profile is an important determinant in the AD

Table 1. **Quantitation of blood cell types in wild-type and *CatE*$^{-/-}$ mice.**

Genotype	Conditions	*n*	Neutrophils (10^{-2} ml^{-1})	Lymphocytes (10^{-2} ml^{-1})	Eosinophils (10^{-2} ml^{-1})	Basophi ls (10^{-2} ml^{-1})	Monocytes (10^{-2} ml^{-1})	Erythrocytes (10^{-4} ml^{-1})	Platelets (10^{-4} ml^{-1})
CatE$^{+/+}$	SPF	4	2.3 ± 0.9	28.1 ± 10.8	0.5 ± 0.4	0	1.9 ± 0.9	824 ± 69	72.8 ± 9.8
	Convent.	7	3.8 ± 2.1	18.1 ± 14.1	0.3 ± 0.2[a]	0	2.7 ± 1.4	768 ± 100	70.4 ± 15.1
CatE$^{-/-}$	SPF	5	4.0 ± 2.2	30.1 ± 15.1	0.4 ± 0.3[b]	0	1.7 ± 1.5[c]	810 ± 79	74.9 ± 9.3
	Convent.	10	5.6 ± 2.2	20.7 ± 6.7	1.2 ± 0.6[a,b]	0	4.1 ± 2.5[c]	771 ± 137	64.1 ± 16.2

Heparinized peripheral blood was analyzed with an automatic white blood cell analyzer. Data are means ± SD for the indicated number of animals (*n*). [a]$p < 0.001$, for the difference of eosinophils between *CatE*$^{+/+}$ and *CatE*$^{-/-}$ conventional mice; [b]$p < 0.001$, for the difference of eosinophils between SPF and conventional *CatE*$^{-/-}$ mice; [c]$p < 0.05$, for the difference of monocytes between SPF and conventional *CatE*$^{-/-}$ mice.

Table 2. **Biochemical analysis of blood of wild-type and *CatE*$^{-/-}$ mice under conventinal conditions.**

Genotype	*n*	Hemoglobin (g/dl)	Hematocrit (%)	MCV (fl^{-1})	MCH (pg)	MCHC (g/ml)	Total protein (g/dl)	GOT (IU/liter)	GPT (IU/liter)	BUN (mg/dl)	Creatinin (mg/dl)
CatE$^{+/+}$	5	12.3 ± 0.9	43.3 ± 10.8	56.4 ± 1.5	16.1 ± 0.7	28.4 ± 0.5	4.8 ± 0.3	38.4 ± 4.5	19 ± 6.4	18.2 ± 4.4	0.3 ± 0.04
CatE$^{-/-}$	9	11.8 ± 2.0	41.7 ± 6.3	54.8 ± 1.0	15.5 ± 0.5	27.6 ± 1.1	4.6 ± 0.4	35.0 ± 4.2	20 ± 5.0	17.7 ± 4.4	0.3 ± 0.09

Heparinized peripheral blood was analyzed with an automatic blood analyzer. Data are means ± SD for the indicated number of animals (*n*).

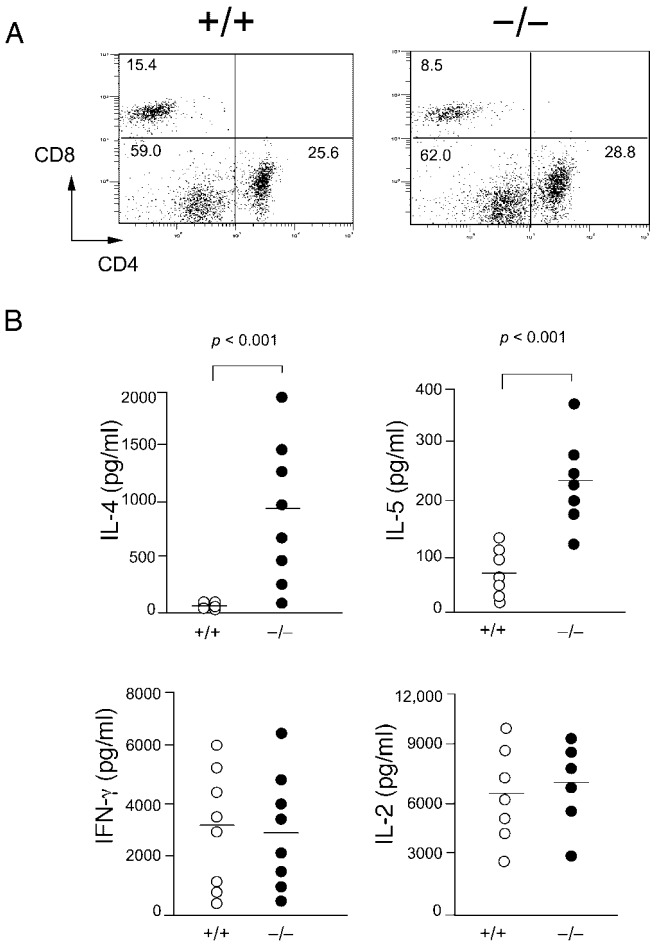

Fig. 5. **Immunological characterization of *CatE*$^{-/-}$ mice compared with the wild-type littermates.** (A) Percentages of CD4$^+$ and CD8$^+$ T cells in spleen cells of *CatE*$^{-/-}$ and wild-type mice at 14 weeks of age. Freshly isolated spleen cells were stained with FITC- and PE-conjugated monoclonal antibodies specific for CD4 and CD8, respectively, and the percentages of CD4$^+$ and CD8$^+$ T cells were determined by flow cytometry. Data are from representative animals. (B) Cytokine production by spleen cells isolated from *CatE*$^{-/-}$ and wild-type mice at 14 weeks of age. Spleen cells were stimulated with phorbol myristate and ionomycin for 42 h, after which the concentrations of IL-4, IL-5, IFN-γ, and IL-2 in the culture supernatants were determined. Each symbol represents cells from an individual animal, with mean values indicated by the horizontal bars.

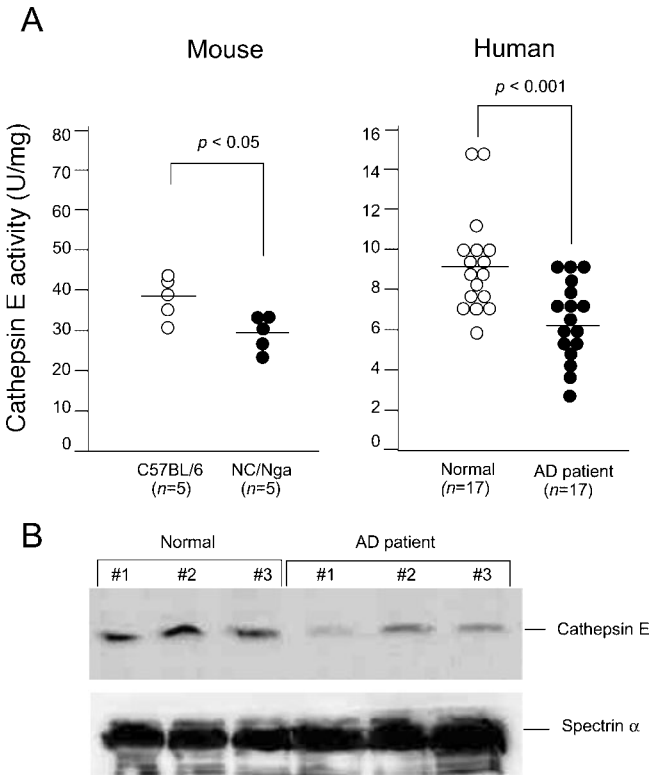

Fig. 6. **Cathepsin E activity and expression in erythrocyte ghosts derived from individuals with AD or healthy control subjects or from NC and control mice.** (A) Cathepsin E activity in erythrocyte ghosts derived from AD patients or control subjects or from NC or C57BL/6 mice raised under conventional conditions. All patients with AD gave informed consent to this study, which was approved by our institutional board. Cathepsin E activity was determined with acid-denatured hemoglobin as a substrate at pH 3.5 Each symbol represents cells from an individual human or mouse, with mean values indicated by the horizontal bars. (B) Immunoblot analysis of erythrocyte ghosts (100 μg of protein) from three healthy volunteers and three AD patients with antibodies specific for cathepsin E or spectrin α as control.

skin lesion. In AD patients, most Th lymphocytes are of the Th2 phenotype and secrete Th2 cytokines such as IL-4 and IL-5 (*18*). IL-4 acts on B cells to induce IgE synthesis and this effect is prompted by IL-5 (*19*). IL-4 also induces a polarization of antigen-stimulated CD4$^+$ T cells into the Th2 phenotype (*20*). IL-5 is critical for eosinophilic inflammation in allergic diseases (*21*). In contrast, the Th1 cytokines IFN-γ and IL-2 play an important role in cell-mediated immunity and chronic inflammation and promote the development of Th1 cells (*21*). We thus investigated cytokine production by spleen cells isolated from *CatE*$^{-/-}$ and age-matched *CatE*$^{+/+}$ mice raised under conventional conditions. The amounts of IL-4 and IL-5 in the supernatants of spleen cell cultures stimulated with phorbol myristate and ionomycin were about sixfold and threefold greater, respectively, for cells derived from *CatE*$^{-/-}$ mice than for those from wild-type animals (Fig. 5B). In contrast, the amounts of the IFN-γ and IL-2 produced by the cells from the two types of mice were similar. These results indicate that cathepsin E deficiency results in a marked polarizing of naïve CD4$^+$ T cells into the Th2 phenotype and that increased concentrations of IL-4 and IL-5 are likely responsible for the increased production of IgE and the consequent development of AD-like skin lesions in *CatE*$^{-/-}$ mice maintained under conventional conditions.

Cathepsin E Levels in AD Patients and NC Mice—To determine whether cathepsin E production is also impaired in AD patients and NC mice, we prepared hemoglobin-free erythrocyte "ghosts" by subjecting washed cells isolated from fresh blood to hypotonic lysis (*17*). Cathepsin E is the only aspartic proteinase, and is present in the membrane-associated form in human (*17*) and mouse erythrocytes (*22*). The abundance of cathepsin E in erythrocytes is believed to reflect its levels in other hemopoietic cells including granulocytes and lymphocytes. The level of cathepsin E activity in erythrocyte

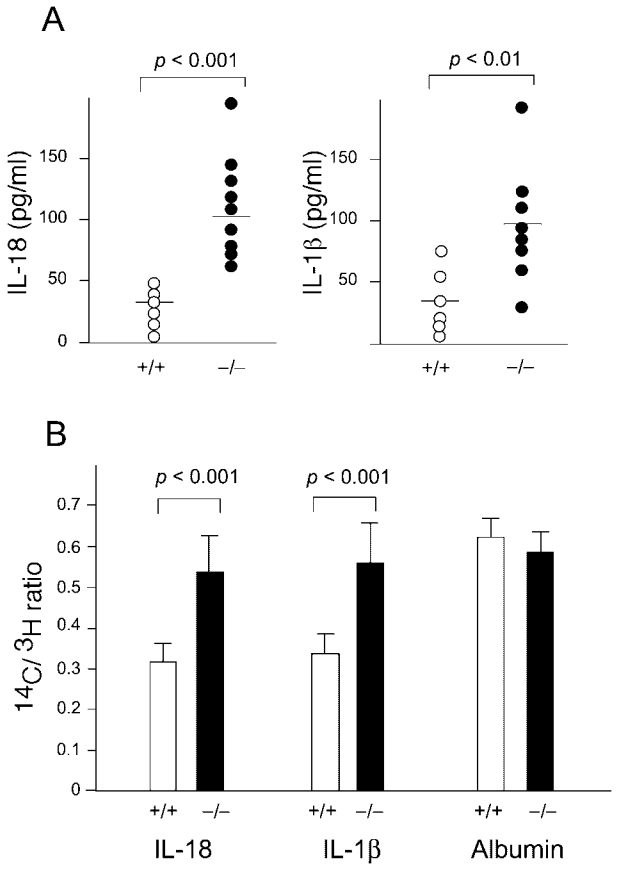

Fig. 7. **Concentrations and turnover rates of IL-18 and IL-1β in the serum of *CatE*$^{-/-}$ mice raised under conventional conditions.** (A) Serum concentrations of IL-18 and IL-1β in 14-week-old *CatE*$^{+/+}$, *Cat*$^{+/-}$ and *CatE*$^{-/-}$ mice raised conventional conditions. Each symbol represents an individual animal, with mean values indicated by horizontal bars. (B) Relative turnover rates of IL-18, IL-1β, and albumin in the serum of *CatE*$^{-/-}$ and wild-type mice as reflected in the $^{14}C/^{3}H$ ratio. Data are means ± SD of values from 6 animals of each genotype.

ghosts derived from individuals with AD was only 70% of the value for healthy controls (Fig. 6A). To our surprise, in >50% of AD patients, the level of cathepsin E activity was less than the lowest value for control individuals. These results were confirmed by immunoblot analysis (Fig. 6B). To demonstrate specificity of this observation for AD, we also determined cathepsin E levels in erythrocytes from patients with psoriatic skin diseases (2 patients) and allergic conjunctivitis (3 patients). The results indicated that cathepsin E levels in patients with these diseases are not significantly different from those of healthy donors, suggesting the specificity of the present observation. In addition, cathepsin E levels in erythrocyte ghosts derived from NC mice raised under conventionsal conditions was significantly reduced as compared to those from control mice (Fig. 6A). NC mice are known to spontaneously develop AD-like skin lesions in the presence of nonspecified allergens (*23*, *24*), although the genetic factors underlying this condition remain unknown. Our observation thus implicates a defect in the production of cathepsin E in the development of AD-like skin lesions in both humans and mice.

Accumulation and Reduced Turnover of IL-18 and IL-1β in CatE$^{-/-}$ *Mice*—Bacterial infection has been shown to influence the induction of AD through activation of Toll-like receptors and subsequent IL-18 secretion (*12*, *25*). Administration of IL-18 to normal mice is known to induce the production of IgE by B cells and that of IL-4 and IL-13 by basophils, mast cells, and CD4^{+} T cells (*26*, *27*). Together with IL-4, IL-18 induces a Th2-dominant state in NC mice (*23*) and IL-18 transgenic mice develop skin diseases at about 6 months of age even under SPF conditions (*4*). Importantly, the serum concentration of IL-18 is significantly increased both in patients with AD (*4*, *28–30*) and in NC mice (*24*, *28*, *31*). These observations thus indicate that the accumulation of IL-18 results in increases in the serum concentrations of IgE, IL-4, and IL-13 and ultimately develops a wide range of AD-like skin lesions (*4*, *27*). Meanwhile, overproduction of IL-1β has also been shown to accelerate the development of skin alterations initiated by abnormal accumulation of IL-18 (*4*). Thus, IL-18 and IL-1β likely function as initiator cytokines that contribute to the development of AD-like skin lesions through induction of the consequent expression of Th2 cytokines.

The serum concentrations of IL-18 and IL-1β were found to increase *CatE*$^{-/-}$ mice than *CatE*$^{+/+}$ mice reared under conventional conditions (Fig. 7A). To investigate the mechanism responsible for the accumulation of these cytokines in the serum of *CatE*$^{-/-}$ mice, we examined their turnover by a double isotope technique. The $^{14}C/^{3}H$ ratio, which is inversely related to turnover rate, for both IL-18 and IL-1β was apparently increased by a factor of 1.72 and 1.71, respectively, in *CatE*$^{-/-}$ mice compared with the values for wild-type animals (Fig. 7B). The $^{14}C/^{3}H$ ratios for both cytokines in *CatE*$^{-/-}$ raised under SPF conditions did not differ from those in *CatE*$^{-/-}$ mice raised under conventional conditions, indicating that the rate of degradation of these cytokines is reduced as a direct result of cathepsin E deficiency.

Sensitivity to Hapten-Induced Experimental Contact Dermatitis in CatE$^{-/-}$ *Mice*—To further characterize *CatE*$^{-/-}$ mice, we examined the susceptibility of these animals to contact dermatitis by the use of the hapten-induced experimental contact dermatitis model system. *CatE*$^{-/-}$ mice, as well as the wild-type littermates and NC mice, raised under conventional conditions were treated with TNCB to induce the contact dermatitis. The clinical signs of dermatitis, including itching, erythema-hemorrhage, edema, excoriation-erosion, and scaling-dryness of the dorsal skin, were evaluated according to a four-point scale of severity. Total severity scores for *CatE*$^{-/-}$ mice increased in a time-dependent manner, achieving the maximum value (15 points) at 5 weeks after the first epicutaneous administration of TNCB (Fig. 8A). The rate of development and the severity of contact dermatitis were markedly greater in *CatE*$^{-/-}$ mice than NC mice, indicating that the former animals are more sensitive to hapten-induced contact dermatitis than are the latter. Wild-type mice did not develop substantial signs of contact dermatitis under the sam conditions. Histological analysis of the dorsal skin revealed that, although no significant pathological signs were apparent at 3.5 days after the first epicutaneous administration of TNCB in *CatE*$^{-/-}$ mice (6.5 weeks of age), marked changes, including abscesses with

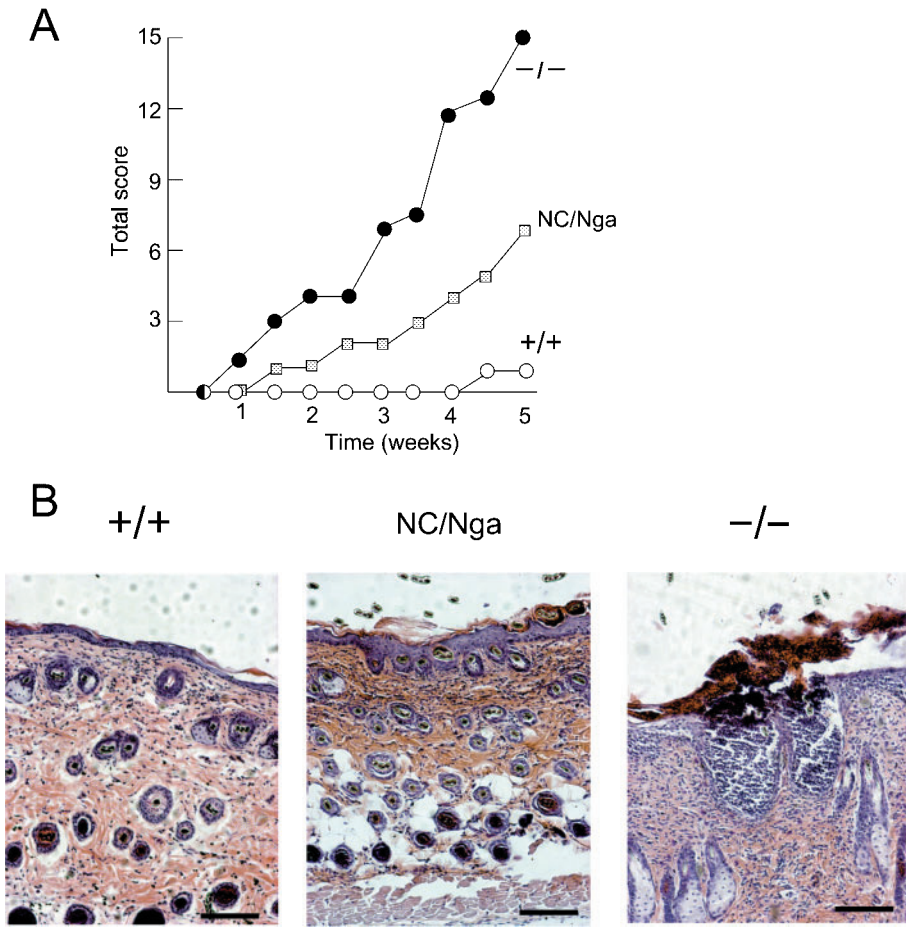

Fig. 8. **Comparison of the susceptibilities to hapten-induced experimental contact dermatitis between *CatE*$^{-/-}$ mice and NC mice or wild-type mice.** (A) Severity of the clinical signs of dermatitis. Five-week-old *CatE*$^{-/-}$, wild-type, or NC mice raised under conventional conditions were immunized by painting the abdominal skin with TNCB and, beginning 1 week later, subjected to epicutaneous administration of TNCB on the back every 3.5 days. The severity of dermatitis was determined as the sum of individual scores (0, none; 1, mild; 2, moderate; 3, severe) for each of five signs (itching, erythema-hemorrhage, edema, excoriation-erosion, scaling-dryness) as described. Data are from representative animals. (B) Histopathologic analysis of skin lesions. Sections of the dorsal skin of the three different types of mice were examined by hematoxylin-eosin staining 5 weeks after the onset of epicutaneous administration of TNCB. Scale bars, 100 μm.

severe cellular infiltration that were covered with a crust or scab, thickening of the epidermis due to hyperplasia, prominent hyperkeratosis, and increased numbers of eosinophils, macrophages, and lymphocytes, were observed after 5 weeks (Fig. 8B). Staining with acidic toluidine blue (pH 4.0) also revealed substantial infiltration of mast cells (data not shown). Histopathologic analysis also revealed that the lesions developed by NC mice were less severe than were those apparent in *CatE*$^{-/-}$ mice; abscesses with cellular infiltration were thus not evident in the NC mice even 5 weeks after the first epicutaneous treatment. Wild-type mice showed no substantial histological changes in response to TNCB. The results indicate that *CatE*$^{-/-}$ mice are more susceptible to contact dermatitis than NC and wild-type mice.

DISCUSSION

This study provides the first evidence that deficiency of cathepsin E is associated with the development of AD-like inflammatory skin diseases in mice. *CatE*$^{-/-}$ mice spontaneously developed AD-like skin lesions when reared under conventional conditions, but not when maintained under SPF conditions. This condition appeared to be induced at least in part by bacterial infection. In fact, the preferential colonization of *Staphylococcus aureus* was observed in samples from the skin lesions of conventional *CatE*$^{-/-}$ mice. Therefore, it is more likely that the bacterial infections stimulate secretion of the initiator cytokines for Th2 cell responses via Toll-like receptors that recognize foreign antigens, thereby inducing AD-like skin lesions in these mice. Consistent with this notion, we demonstrated the systemic accumulation of both IL-18 and IL-1β in *CatE*$^{-/-}$ mice raised under conventional conditions. IL-18 is originally identified as a potent inflammatory cytokine able to induce IFN-γ production in T cells and natural killer cells in collaboration with IL-12 (*5*). However, in the absence of IL-12, IL-18 has been shown to exhibit allergy-promoting effects such as induction both of the development of naïve T cells into Th2 cells and of the production of IL-4, IL-13, and histamine by basophils and mast cells (*5*). Therefore, it is considered that the accumulation of IL-18 alone induces the increase in the serum concentrations of IgE, IL-4, and IL-13 (*27*). IL-1β is also known to accelerate the development of skin alterations initiated by accumulation of IL-18 (*4*). The AD-like skin lesions in *CatE*$^{-/-}$ mice raised under conventional conditions are thus likely initiated by the accumulation of IL-18 and IL-1β and the consequent stimulation of production of the executioner cytokines, such as IL-4 and IL-5, accompanied by an increased serum concentration of IgE.

The precise mechanism by which cathepsin E deficiency is associated with the development of AD remains to be answered, but a more plausible explanation is that cathepsin E plays a crucial role in the normal metabolic

turnover of IL-18 and IL-1β. Only minute amounts of IL-18 have been detected in both intracellular and extracellular compartments as a result of the interaction between dendritic cells and alloreactive T cells (*33*), suggesting that most of the secreted cytokine is taken up by the responder T cells or the antigen presenting cells. Alternatively, IL-18 and IL-1β may be degraded by cathepsin E secreted by antigen presenting cells. It is interesting to note that cathepsin E in macrophages is rapidly activated and secreted into extracellular spaces upon stimulation with IFN-γ and/or LPS, suggesting its involvement in extracellular degradation of these cytokines (data not shown). Given that cathepsin E is abundant in hematopoietic cells, including lymphocytes and antigen presenting cells (*6*, *7*), it is more likely that this enzyme is responsible for intra- and/or extracellular degradation of these initiator cytokines.

To our surprise, cathepsin E levels in erythrocytes from patients with AD were significantly lower than those from control healthy donors and those from patients both with control inflammatory skin diseases such as psoriasis and with other atopic diseases such as allergic conjunctivitis were not significantly different from those from control healthy donors, indicating the specificity of the observation for AD. No significant change in the level of cathepsin E in erythrocyte membranes is not observed during maturation of erythrocytes (*22*). It is thus unlikely that low levels of cathepsin E in erythrocytes from patients with AD are due to prematuration of erythrocytes. Our demonstration of a deficiency of cathepsin E not only in humans with AD but also in the NC mouse model of this disorder further validates the *CatE*$^{-/-}$ mouse raised under conventional conditions as an animal model of AD. Further studies with this model should both provide new insight into the pathogenesis of AD and facilitate the development of new therapeutic approaches to this condition.

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