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An Atopic Dermatitis-Like Skin Disease with Hyper-IgE–Emia Develops in Mice Carrying a Spontaneous Recessive Point Mutation in the *Traf3ip2* (*Act1/CIKS*) Gene

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Spontaneous mutant mice that showed high levels of serum IgE and an atopic dermatitis (AD)-like skin disease were found in a colony of the KOR inbred strain that was derived from Japanese wild mice. No segregation was observed between hyper-IgE–emia and dermatitis in (BALB/c × KOR mutant) N₂ mice, suggesting that the mutation can be attributed to a single recessive locus, which we designated *adjm* (atopic dermatitis from Japanese mice). All four *adjm* congenic strains in different genetic backgrounds showed both hyper-IgE–emia and dermatitis, although the disease severity varied among strains. Linkage analysis using (BALB/c × KOR-*adjm/adjm*) N₂ mice restricted the potential *adjm* locus to the 940 kb between *D10Stm216* and *D10Stm238* on chromosome 10. Sequence analysis of genes located in this region revealed that the gene AI429613, which encodes the mouse homologue of the human TNFR-associated factor 3-interacting protein 2 (TRAF3IP2) protein (formerly known as NF-κB activator 1/connection to IκB kinase and stress-activated protein kinase/Jun kinase), carried a single point mutation leading to the substitution of a stop codon for glutamine at amino acid position 214. TRAF3IP2 has been shown to function as an adaptor protein in signaling pathways mediated by the TNFR superfamily members CD40 and B cell-activating factor in epithelial cells and B cells as well as in the IL-17–mediated signaling pathway. Our results suggest that malfunction of the TRAF3IP2 protein causes hyper-IgE–emia through the CD40- and B cell-activating factor-mediated pathway in B cells and causes skin inflammation through the IL-17–mediated pathway. This study demonstrates that the TRAF3IP2 protein plays an important role in AD and suggests the protein as a therapeutic target to treat AD. *The Journal of Immunology*, 2010, 185: 000–000.

The term atopic dermatitis (AD) was first coined by Wise and Sulzberger (1) to describe “confusing types of localized and generalized lichenification, generalized neurodermatitis or a manifestation of atopy.” Today, AD (or atopic eczema) is recognized as a strongly heritable disease characterized by complex symptoms, including chronically relapsing, extreme pruritus and eczematous skin disease, both of which are frequently

associated with IgE hyperresponsiveness to environmental allergens (2–4). The rapid increase in the prevalence of AD during the past three decades has sparked an intense effort to elucidate the underlying pathogenesis of the disease and has led to the use of radical treatments for the disorder (3, 5).

The causative factors for AD generally fall into two categories: environmental and genetic. In the environmental category, the involvement of allergens, such as house dust, mites, and air pollution, has been suggested strongly by epidemiological studies (6). Conversely, linkage studies of atopic and nonatopic phenotypes also strongly suggest that genetic factors linked to several different candidate regions on chromosomes are involved (see Ref. 7 and references therein). Uncertainty as to the specific genetic factors that contribute to AD exists due to the difficulty of performing linkage analyses on multigenic diseases in humans, largely because of the effects of the different genetic backgrounds. Furthermore, appropriate animal models for human AD are lacking.

Recently, two promising mouse models for human AD were described by Matsuda et al. (8) and Natori et al. (9). These models are represented by the inbred strains NC/Nga (NC) and Naruto Research Institute Otsuka Atrichia (NOA). The NC strain was established in 1957 by Kondo et al. (10, 11). NC mice spontaneously suffer severe dermatitis in the presence of nonspecific allergens. Morbid NC mice show AD symptoms, including itching, erythema, hemorrhage, edema, crust, drying, and excoriation/erosion hyperplasia of the epidermis of the face, neck, and/or back, which are exacerbated by aging. Furthermore, NC mice display some of the characteristic histopathological features of AD, such as macrophage and eosinophil invasion of the dermis, increased numbers and activation of mast cells and lymphocytes, reduction of

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Abbreviations used in this paper: *Act1*, NF-κB activator 1; AD, atopic dermatitis; *adjm*, atopic dermatitis from Japanese mice; BAFF, B cell-activating factor; CD40L, CD40 ligand; *CIKS*, connection to IκB kinase and stress-activated protein kinase/Jun kinase; F, female; HIES, hyper-IgE syndrome; M, male; NC, NC/Nga; NOA, Naruto Research Institute Otsuka Atrichia; TRAF, TNFR-associated factor; *Traf3ip2*, TRAF3-interacting protein 2.

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ceramide (12), the appearance of activated mast cells, and CD4⁺ T cells in the lesion. These lines of evidence suggest that the symptoms displayed by NC mice are clinically, pathologically, and immunologically similar to those of human AD. Quantitative traits linkage analysis suggests that NC mice carry several genetic determinants of dermatitis, and the major determinant is located on murine chromosome 9 (13).

Like the NC mouse, the NOA mouse also shows ulcerative skin lesions with an accumulation of mast cells and increased serum IgE levels. Linkage analysis has demonstrated that the major gene responsible for dermatitis in the NOA mouse is located in the middle of chromosome 14. The incidence of disease in these animals clearly differs according to parental strain, and the mode of inheritance is autosomal recessive with incomplete penetrance. Statistical analyses have shown that the critical region is in the vicinity of *D14Mit236* and *D14Mit160* (9). Furthermore, two modifier genes have been reported as candidate loci, one in the middle of chromosome 7 and the other in the telomeric region of chromosome 13. These loci correspond to regions of synteny in human chromosomes where linkages to asthma, atopy, or related phenotypes (e.g., serum IgE levels) have been documented (14). However, no mouse strains have been shown to develop dermatitis due to alterations in a single gene thus far.

We have identified a new mouse model for human AD, the phenotype of which is controlled by a recessive mutation. This mutation was successively isolated by positional cloning and found to be a nonsense mutation in the TNFR-associated factor 3-interacting protein 2 (*Traf3ip2*) locus on mouse chromosome 10. The TRAF3IP2 protein, an adapter molecule, was cloned using a functional genetic screen based on its ability to activate NF- κ B (15). The molecule also was cloned simultaneously using a yeast two-hybrid screen. In that report, the molecule was named connection to I κ B kinase and stress-activated protein kinase/Jun kinase (CIKS) based on its interaction with I κ B kinase- γ (16). The TRAF3IP2 protein does not possess any identified enzymatic domains but does contain a helix-loop-helix at its N terminus, a coiled-coil at its C terminus (15), and two putative TNFR-associated factor (TRAF) binding sites (17), suggesting that it functions through protein-protein interactions in signaling pathways. Qian et al. (18) showed that TRAF3IP2 plays a critical role in the CD40-mediated signaling pathway in epithelial cells in vitro. Additionally, targeted disruption of the *Traf3ip2* locus in mice causes a dramatic increase in the number of peripheral B cells, culminating in lymphadenopathy, splenomegaly, hypergammaglobulinemia, and autoantibody production. Detailed analyses have indicated that TRAF3IP2 negatively regulates CD40- and B cell-activating factor (BAFF)-mediated signaling and suggest that the TRAF3IP2-mediated attenuation of this pathway plays an important role in the homeostasis of B cells (19). Conversely, TRAF3IP2 functions as a positive regulator in the IL-17-dependent signaling pathway associated with autoimmunity and inflammatory diseases (20). This antipathetic nature of TRAF3IP2 in signaling pathways has been suggested to be a result of the differential usage of protein-protein interacting domains in the TRAF3IP2 molecule (21).

In this report, we show that the AD-like phenotype in AD from Japanese mice (*adjm*) is caused by the presence of a truncated form of the TRAF3IP2 molecule and that the severity of the phenotype is controlled by the genetic background of the mouse strains used. The use of these mouse strains (e.g., KOR, C57BL/6, BALB/c, AKR/J, and A/J; see *Materials and Methods*) to study the dual functions of TRAF3IP2 should help to elucidate the mechanisms underlying the two major symptoms of human AD, especially hyper-IgE-emia, and should facilitate exploration of the question of why human AD is multigenic.

Materials and Methods

Mice

KOR mice were derived from Japanese wild mice (*Mus musculus molossinus*) (11). KOR-*adjm/adjm* and C57BL/6-, BALB/c-, AKR/J-, and A/J-*adjm* congenic strains were maintained in-house at the Saitama Cancer Center. All of the mice were housed under specific pathogen-free conditions ($22 \pm 2^\circ\text{C}$, $50 \pm 10\%$ controlled humidity, and a 12 h/12 h light/dark schedule). A regular laboratory diet (F-2; Funabashi Farm, Chiba, Japan) and water were provided ad libitum. All of the animal experiments were approved by the Committees for Guidelines and Regulation of Animal Experiments of the Saitama Cancer Center.

Pathological study

As a prefixative, tissues were immersed twice in 4% paraformaldehyde and irradiated using a microwave oven for 30 s at 550 W. Tissues were then post-fixed in the same fixative for 4 h on ice. Fixed tissues were dehydrated in methanol, embedded in polyester wax (Sekisui Medical, Tokyo, Japan), and sectioned at 3 μm . Sections were dewaxed, stained with H&E (Wako Pure Chemicals, Tokyo, Japan) or with Masson's trichrome solution (Muto Pure Chemicals, Tokyo, Japan), and mounted on a coverslip for photomicrography.

Total serum IgE measurement

Blood was collected from the retro-orbital plexus of mice under ether anesthesia and immediately heparinized. Plasma samples were obtained by centrifugation and stored at -20°C until use. The total serum IgE level was measured using a mouse IgE measurement kit (Mouse IgE EIA Kit) according to the manufacturer's instructions (Yamasa Shoyu, Tokyo, Japan). Briefly, the test serum (100 μl of a 1:24 dilution) was incubated in a microplate precoated with anti-mouse IgE mAbs for 30 min at room temperature. After being washed, the wells were further incubated with HRP-labeled anti-mouse IgE for 30 min at room temperature, followed by washing and incubation with the colorimetric substrate for 30 min. The reaction was stopped by the addition of 100 μl per well of a 2.0 N HCl solution, and the OD was measured at 450 nm. Mouse IgE isotype standard solutions were used to construct a standard curve (10–500 ng/ml). Total serum IgE levels were calculated from the standard curve.

Linkage and haplotype analyses

Genome-wide simple sequence length polymorphism tests were performed using *Mit* microsatellite marker pairs (ResGen, Huntsville, AL) and a PCR amplification kit (Takara Bio, Shiga, Japan). Approximately 20 ng genomic DNA was added to the final 20- μl reaction mixture. The thermocycling program consisted of one initial denaturation at 94°C for 1 min and 30 cycles of 94°C , 55°C , and 72°C for 1 min each. PCR-amplified products were separated in a 4% agarose gel (NuSieve:Agarose, 1:3; Cambrex Bio Science Rockland, Rockland, ME) in Tris-borate-EDTA buffer and visualized by UV light after being stained with ethidium bromide.

Mutation analysis

The nucleotide sequences of the genes (i.e., *Traf3ip2*, *Fyn*, *ENSMUSG-00000064311*, *Q9D6T1*, and *Lama4*) were obtained from the Ensembl Genome Browser (www.ensembl.org), and PCR primers were designed for the amplification of each exon. To screen for mutations between KOR and KOR-*adjm*, total RNA was isolated from 5-wk-old mouse brain and spleen using TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. cDNA was generated with the Omniscript RT Kit (Qiagen, Hilden, Germany) using 1 μg of DNase-pretreated total RNA. The entire genomic region of the *Traf3ip2* gene was amplified by long and accurate PCR (Takara Bio) to produce overlapping PCR products from both strains as well as from other inbred strains (C57BL/6J, A/J, and BALB/c) as controls. The sequences of the primers used, including the sequencing primers, are shown in Table II. PCR products were gel-purified, sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and analyzed on a 3100 Genetic Analyzer (Applied Biosystems). Genomic DNA from KOR, KOR-*adjm/+*, KOR-*adjm/adjm*, C57BL/6-*adjm/+*, C57BL/6-*adjm/adjm*, BALB/c, BALB/c-*adjm/+*, and BALB/c-*adjm/adjm* animals also was amplified (forward primer, A1429613int1F; reverse primer, A1429613int2R; see Table II) by PCR. PCR products were digested with PstI, separated in a 1% agarose gel, and stained with ethidium bromide.

RT-PCR

Total RNA was isolated from 5-week-old BALB/c-*+/+* and BALB/c-*adjm/adjm* mouse spleen using TRIzol (Invitrogen) and a TRIzol Plus Purification Kit (Invitrogen) following the manufacturer's protocol.

cDNA was generated with the ThermoScript RT-PCR System (Invitrogen) using 0.1 μ g of DNase-pretreated total RNA. The cDNA was amplified for 30 cycles (98°C for 20 s and 68°C for 4 min) using KOD FX (TOYOBO, Osaka, Japan). Primer sets for amplification of a 272-bp product used primers (AI429613ex1F and AI429613ex2R; Table II) located at exons 1 and 2. The products were subjected to agarose gel electrophoresis. cDNA integrity was confirmed by *Gapdh* (22).

Quantitative RT-PCR

Total RNA was isolated from 5-wk-old BALB/c-+/+ and BALB/c-*adjm/adjm* mouse skin, spleen, and brain using TRIzol and a TRIzol Plus Purification Kit following the manufacturer's protocol. Reverse transcription and quantitative PCR were carried out using a SuperScript VILO cDNA Synthesis Kit (Invitrogen) and a QuantiTect SYBR Green PCR Kit (Qiagen) according to the manufacturer's protocol. *Gapdh* was used as an endogenous control. Primers for mouse *Traf3ip2* (Mm_AI429613_1) and *Gapdh* (Mm_Gapdh_3) were purchased from Qiagen. All of the samples were analyzed in triplicate using the ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems). Data were analyzed using the Relative Quantification Software of the Applied Biosystems 7500 Fast, with expression levels in C57BL/6-+/+ spleen assigned an arbitrary value of 1.

Western blotting

Proteins were isolated from murine spleen using T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL) according to the manufacturer's protocol. Proteins (5 μ g) were fractionated by 10% SDS-PAGE and transferred onto a Hybond-P polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, U.K.). A rabbit polyclonal Ab raised against TRAF3IP2 (anti-Act1 [H-300]) was obtained commercially (Santa Cruz Biotechnology, Santa Cruz, CA) and has been characterized previously (23, 24). The Ab was used at a dilution of 1:400. The membrane was subsequently stripped and blotted with an anti- β -actin mAb (1:10,000; Sigma-Aldrich, St. Louis, MO) to control for protein loading. HRP-conjugated secondary Abs were used at a dilution of 1:40,000; blots were developed using an ECL Advance Western blotting Detection Kit (GE Healthcare).

Semiquantitative analysis of cytokines

The major cytokines present in Th1, Th2, and Th17 cells were analyzed semiquantitatively at the in-house microarray technology office of the Tokyo Metropolitan Institute of Medical Science using cytokine Ab arrays for 97 cytokines (Mouse Cytokine Antibody Array G Series 6; RayBiotech, Norcross, GA) following the manufacturer's protocol.

Th17 cell counting

Affected and nonaffected skin surrounding the eyes was removed, minced into pieces ~3 mm in width, incubated with DMEM/10% FBS/2 mg/ml dispase II at 4°C for 18 h, and then washed with PBS. Epidermis was separated from dermis and minced as finely as possible; it was then incubated with DMEM/10% FBS/1 mg/ml collagenase type III at 37°C for 1 h and filtered through a 70- μ m nylon mesh sheet to yield a single-cell suspension.

Spleen cells were resuspended in DMEM/10% FBS and filtered through a 70- μ m nylon mesh sheet to prepare single-cell suspensions.

The single-cell suspensions were treated with hypotonic buffer to induce hemolysis; they were then treated with anti-CD16/32 mAb (BD Pharmingen, San Diego, CA) and normal rat serum to prevent nonspecific binding and stained with FITC-conjugated anti-CD4 (L3T4) mAb (BD Pharmingen). CD4⁺ cells were trapped on anti-FITC microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) by MACS. The enriched CD4⁺ cells were treated with BD Cytotfix/Cytoperm solution (BD Biosciences, San Jose, CA) and stained with PE-conjugated anti-IL-17A (TC11-18H10) mAb (BD Pharmingen); 10⁵ cells in each CD4⁺ T cell suspension were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA).

Results

Isolation and establishment of mutant strains with dermatitis

As stated above, KOR is an inbred strain established from Japanese wild mice (*M. musculus molossinus*) (11). During the maintenance of the strain, we discovered two littermates (marked by an arrow in Fig. 1A) that developed severe dermatitis. This dermatitis became apparent at 5 wk of age, was localized mainly to the face (including the ears), and became progressively more severe. When two pairs

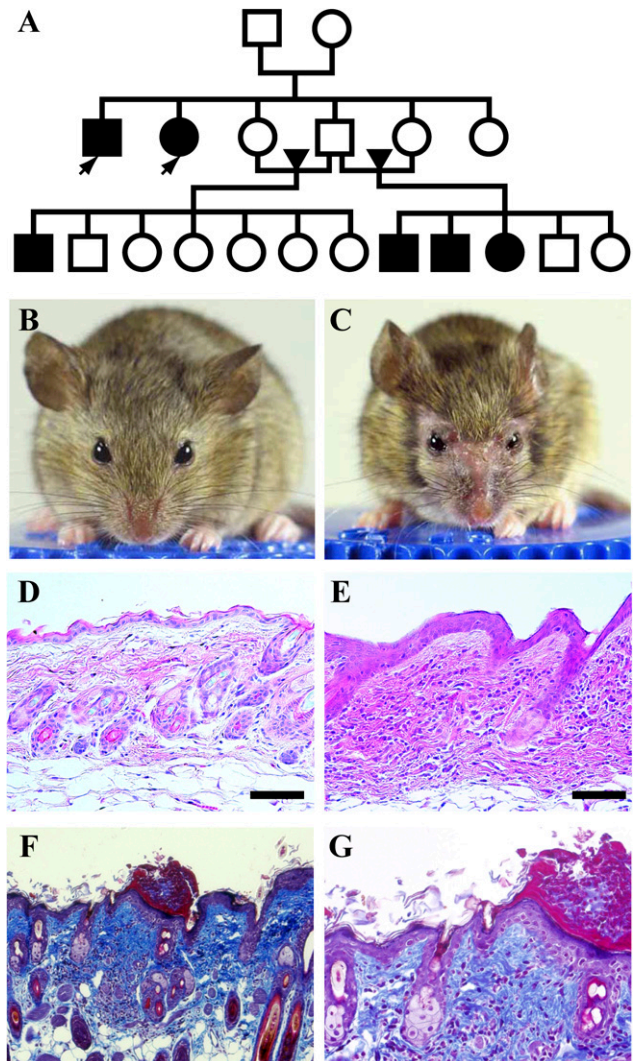


FIGURE 1. Phenotype segregation in the KOR colony. A, A pedigree of the KOR strain in which *adjm* mutant mice were first discovered (shown by arrows). Squares and circles represent males and females, respectively. Closed and open symbols represent affected and nonaffected individuals, respectively. B and C, Phenotypic characterization of the *adjm* mutant. Appearance of a healthy KOR-*adjm*/+ mouse (B) and a KOR-*adjm*/*adjm* mouse (C) after disease onset. D–G, Skin histology of a KOR-*adjm*/+ mouse (D, F) and a KOR-*adjm*/*adjm* mouse (E, G). Specimens were stained with H&E (D, E) and Masson's trichrome solution (F, G). Scale bar, 100 μ m. Original magnification $\times 20$.

of nonaffected mice from the same litter were crossed, 4 of the 12 offspring in the next generation developed dermatitis (Fig. 1A), indicating that the mutation was recessive. Using the founder pair (marked with arrows in Fig. 1A), we established a mutant strain that naturally develops dermatitis. This strain has been designated KOR-*adjm*/*adjm*. To maintain the KOR-*adjm*/*adjm* strain, nonaffected littermates are crossed randomly (e.g., pairs shown by closed arrowheads in Fig. 1A); heterozygosity is confirmed by the appearance of affected individual(s) in the next generation. The heterozygous pairs are used for strain maintenance.

Phenotype analysis

Histological examination of skin specimens from KOR-*adjm*/*adjm* mice revealed that the affected skin had a thickened epidermis and showed a massive infiltration of inflammatory cells, including eosinophils (Fig. 1B–G). Scratching of the face and ears with the hind legs, most likely due to itching, was observed frequently in all

of the mice analyzed (data not shown). Furthermore, the levels of serum IgE in these animals spontaneously increased with age and reached >10 ng/ml by 11 wk of age (Fig. 2A). The hyper-IgE-emia showed a sexual disparity, with female mice displaying serum IgE levels two times higher than those of male mice until 10 wk of age (Fig. 2B). Importantly, except for the short life span of these animals, all of the phenotypes observed in the mutant mice resemble those seen in patients with AD. Indeed, treatment of the affected skin with an ointment containing Tacrolimus (FK506; Astellas Pharma, Tokyo, Japan) ameliorated the dermatitis in the mutant mice, similar to what has been observed in AD patients (Y. Matsushima, unpublished observations).

The life span of *KOR-adjm/adjm* mice was much shorter than that of *KOR-+/+* or *KOR-adjm/+* mice. The $t_{1/2}$ spans were estimated to be 15.1 wk in homozygous male mutant mice and 11.6 wk in homozygous female mutant mice, whereas there was no difference in the $t_{1/2}$ spans of wild-type and heterozygous mice (Fig. 3). Female sterility also was found in all of the homozygous mutant mice (Fig. 3). The underlying mechanism of early death and female sterility in *KOR-adjm/adjm* mice remains unclear, although infection is suggested to be a major cause of death because ulcerative yellow pustules were sometimes seen in the forelimbs of severely affected individuals (data not shown). The pustules may have been caused by infection with *Staphylococcus aureus*, which is an indigenous bacterium in the lesional skin of AD patients.

Genetic analyses

A preliminary linkage analysis using (BALB/c $\times$ *KOR-adjm/adjm*) N_2 mice suggested that the mutation was controlled by a single recessive locus on mouse chromosome 10 (data not shown). No segregation was observed between the two prominent phenotypes, dermatitis and hyper-IgE-emia. We established four *adjm* congenic

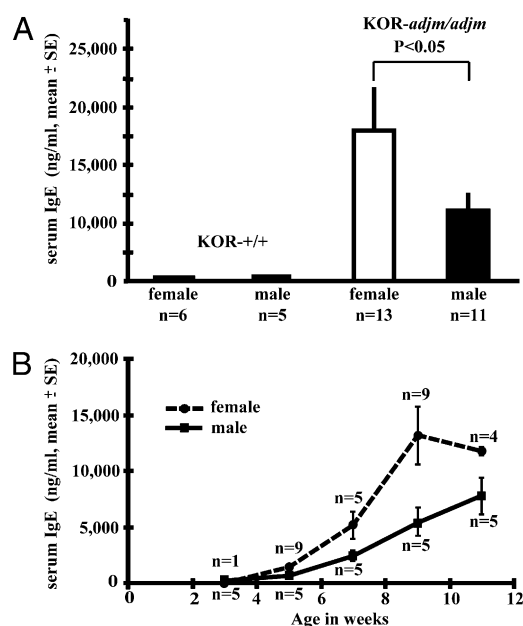


FIGURE 2. IgE levels in *adjm* mutant mice. **A**, Comparison of serum IgE levels in *KOR* and *KOR-adjm/adjm* mice at 10–12 wk of age. Plasma from both female and male wild-type *KOR* mice contained <500 ng/ml IgE, whereas the plasma from mutant mice contained 1.1×10^4 to 1.8×10^4 ng/ml IgE. In the mutant animals, there were significant differences in the IgE levels of males and females ($p < 0.05$); the serum IgE level of female mice was twice that of male mice. **B**, Age-dependent increase in serum IgE level. The increase began at 5 wk of age, and the IgE level reached $>1 \times 10^4$ ng/ml by the age of 11 wk. The IgE levels in female mutant mice were twice as high as those in male mice.

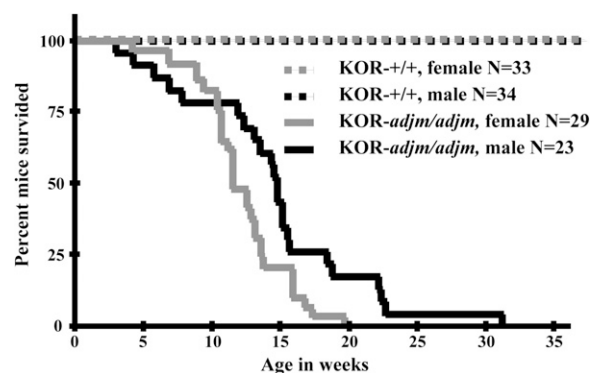


FIGURE 3. Comparison of survival curves of *KOR-+/+* and *KOR-adjm/adjm* mice. The survival rate of *KOR-adjm/adjm* mice (solid lines) differed significantly from that of control *KOR-+/+* mice (dotted lines) for both females and males.

strains in different genetic backgrounds (C57BL/6, BALB/c, AKR/J, and A/J). Both dermatitis and hyper-IgE-emia were transmitted stably to the progeny of the congenic strains, although the severity of the dermatitis and the levels of serum IgE varied depending on the genetic background (Fig. 4). The original *KOR-adjm/adjm* strain had the most severe phenotypes, the mutation on congenic strains with a propensity for Th2 responses (i.e., BALB/c, AKR/J, and A/J) showed less severe phenotypes, and the mutation backcrossed into a Th1-skewed strain (C57BL/6J) showed the least severe phenotype. Interestingly, the female sterility that was observed in the original *KOR* strain was not observed in any of the congenic strains (data not shown). These results suggest that the *adjm* locus is responsible for the dermatitis and hyper-IgE-emia in the mutant mice and that additional loci may influence the *adjm* locus as modifiers.

To identify the gene responsible for the *adjm* phenotype, 1170 (BALB/c $\times$ *KOR-adjm/adjm*) N_2 segregants were generated. Of these, 140 developed dermatitis by 8 wk of age. The frequency of the N_2 segregants with dermatitis was lower than expected, likely because we did not account for animals with a late onset of dermatitis. Linkage analysis mapped the *adjm* locus near the *D10mit53* locus. Further analysis with newly identified micro-

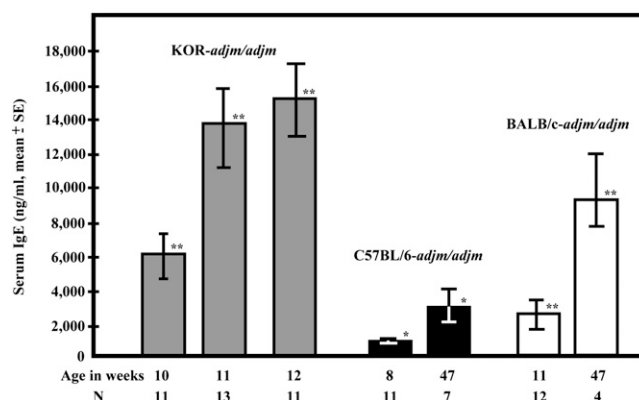


FIGURE 4. The effect of genetic background on serum IgE level. Serum IgE levels were compared among *KOR-*, *C57BL/6-*, and *BALB/c-adjm/adjm* congenic strains. The serum IgE level of *KOR-adjm/adjm* mice at 12 wk of age was especially high (1.5×10^4 ng/ml), whereas the levels in *C57BL/6* and *BALB/c* congenic strains were low. At 47 wk of age, the serum IgE levels of *C57BL/6-* and *BALB/c-adjm/adjm* mice were 0.3×10^4 and 1×10^4 ng/ml, respectively. The clinical skin condition (data not shown) of the *BALB/c-adjm/adjm* mice (a Th2-skewed strain) was significant in comparison with that of the *C57BL/6-adjm/adjm* mice (a Th1-skewed strain), which corresponded with their serum IgE levels.

satellite markers (Table I) in 86 other mice obtained during the development of the *adjm* congenic strain in the BALB/c background further restricted the locus to the 940 kb between *D10Stm216* and *D10Stm238* (Fig. 5A). In this region, five genes were identified in the European Molecular Biology Laboratory database (Fig. 5A). Two of the five genes appear to be the most likely candidates for the *adjm* mutation, because both have been shown to be involved in signal transduction in the immune system; one gene encodes for a Src family kinase (*Fyn*), and the other gene (*Traf3ip2*, formerly *AI429613*) encodes a homologue of human CIKS (16)/NF- κ B activator 1 (*Act1*) (15).

Identification of the *adjm* candidate gene

A mutation survey comparing mutants and their isogenic littermates revealed that the mutant mice (but not their control littermates) carried a C $\rightarrow$ T transition in the second exon of the murine *Traf3ip2* gene (DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank accession numbers AB238206–AB238207; www.ncbi.nlm.nih.gov; Fig. 5B), whereas the sequence of the *Fyn* gene in both the mutant mice and the controls was identical. The presence of this alteration was confirmed by PstI digestion of PCR-amplified genomic DNA from *adjm* homozygotes and heterozygotes (Fig. 5C). The transition changed amino acid 214 from glutamine (CAG) to a stop codon (TAG) (Fig. 5B), and this results in a C-terminal truncation of the TRAF3IP2 protein. The truncation removes the second TRAF binding site (EEERPA) and the N-terminal coiled-coil domain (21) (Fig. 5D). These lines of evidence suggest that the *Traf3ip2* gene is a candidate gene for the *adjm* locus.

To examine the effect of the *adjm* mutation on *Traf3ip2* RNA expression, a series of primer sets were used to amplify the 5' and 3' regions of cDNA fragments (Table II). RT-PCR analysis of splenic RNA resulted in a single band from wild-type, *adjm*+/+, and *adjm/adjm* mice. However, the *Traf3ip2* transcript level appeared to be reduced in *adjm* mutants relative to that of *GAPDH* controls (Fig. 6A). From a quantitative analysis of band intensities, we estimated that *Traf3ip2* transcripts in *adjm*+/+ and *adjm/adjm* mice are ~40 and 10% as abundant as those in wild-type mice, respectively.

To confirm the results described above, we carried out quantitative RT-PCR using total RNA isolated from 5-wk-old BALB/c+/+ and BALB/c-*adjm/adjm* mouse skin, spleen, and brain. The relative amounts of *Traf3ip2* transcripts in skin, spleen, and brain from BALB/c-*adjm/adjm* mice were ~40% as abundant as those in wild-type mice (Fig. 6B). Therefore, the relative amounts of *Traf3ip2* transcripts found in BALB/c-*adjm/adjm* by RT-PCR, as mentioned above, are greatly underestimated.

The *adjm* mice showed prominent defects in TRAF3IP2 protein expression in multiple tissues. Western blot analysis of protein

extracts from spleens of wild-type mice using a TRAF3IP2-specific Ab yielded bands of the expected size (~72 kDa). However, protein extracts from spleens of *adjm/adjm* mice showed no specific Ab labeling in the positions expected for TRAF3IP2 (Fig. 6C).

Relationship between IL-17 expression and seriousness of dermatitis

Recently, a new lineage of CD4 Th cells that produced IL-17, the Th17 lineage, was identified (20, 21). IL-17 is a proinflammatory cytokine that upregulates the expression of inflammatory genes in fibroblasts, epithelial cells, and some other cell types. IL-17 levels are elevated in patients with allergic and autoimmune diseases (see Ref. 21 and references therein). To examine the relationship between IL-17 expression and seriousness of dermatitis, we estimated the relative amounts of eight major cytokines in the serum of C57BL/6-+/+ and C57BL/6-*adjm/adjm* mice with mild or severe dermatitis by Ab microarray. Of these eight cytokines, we found that IL-17 was expressed strongly in the serum of affected female mice (C57BL/6-*adjm/adjm*) but not in that of affected male mice (Fig. 7A).

We then attempted to examine the numbers of Th17 cells in the lesional skin of severely affected mice. However, no Th17 cells were found (data not shown), suggesting that the population of Th17 cells in the skin is very low (<0.01% in CD4⁺ T cells). When the spleen was examined, twice as many Th17 cells were found in the spleens of severely affected homozygous female mice compared with unaffected female mice, although little difference was found in Th17 cells of affected male mice (Fig. 7B). This observation is consistent with the results concerning the relative amounts of IL-17 in affected and nonaffected mice (Fig. 7A). We do not have conclusive evidence that shows whether the differences in Th17 cell number and IL-17 levels in female mice are specific to the C57BL/6 strain or common to other genetic backgrounds. However, our preliminary results suggest that the differences are strain specific, because the relative levels of IL-17 in C57BL/6 were 1.2 times those of BALB/c mice (data not shown). This point will be addressed in future experiments.

Discussion

Two mouse strains, NC and NOA, have been reported as possible models of human AD. Although the phenotypes of both strains are controlled by polygenic traits, the major genetic determinant is located on chromosome 9 (*derm1* locus) in NC (13) and on chromosome 14 in NOA (9). NOA also possesses two additional modifier genes on chromosomes 7 and 13 (14). The phenotype in KOR-*adjm/adjm*, dermatitis and hyper-IgE-emia, is controlled by a single locus on chromosome 10 (Fig. 5). Because the mutant gene in the KOR-*adjm/adjm* strain differs from those in the

Table I. *adjm* genotyping primer information

Microsatellite Marker	Ensembl Position	Primer Sequence	
		Forward (5'–3')	Reverse (5'–3')
<i>D10Stm200</i>	38311451–38311488	ATATATTGGTCTTGGACTTG	CTTGAACATAGTGGACATACT
<i>D10Stm206</i>	38595050–38595087	CTGGCTGTCTCGGAACAC	CCAGGAAAAGGGGATAAAGCT
<i>D10Stm216</i>	38876849–38876898	CTTACCCTGTGTGCTCTCAA	TATCCTGATCCTTATGCCGC
<i>D10Stm225</i>	39087215–39087258	GCCTTATCTCCACAGGGGAA	GACAAGCAACACACACGCGC
<i>D10Stm238</i>	39380779–39380818	TTTCCTTCTTGCTACCTTTG	ATTCTTGGACTTTCCATTCA
<i>D10Stm242</i>	39529556–39529611	GTCAAGCCTGACAACCTGAG	GCTGGAGAAGACTGTGAGAT
<i>D10Stm265</i>	40061077–40061124	TACACAGTGAGTCTCAGGCT	ACAGATGGATGGACACACAC
<i>D10Stm275</i>	40361432–40361473	TCTTAGGTTAAATTGGCTTTCTGAC	TGACTATTGCTGGCCTATCA
<i>D10Stm287</i>	40615191–40615236	CCTCTGGGGGACATGGCAA	TGGTGCCCTGGTCCCATCTCT
<i>D10Stm296</i>	40834322–40834367	CCTGAGATTCTCCTGATGG	CAATGACCCACACTTAGAA
<i>D10Stm313</i>	41234034–41234077	ACAAATAATATCCAGTCGATACAGG	GCTCTCCATTGTGAATTTTC

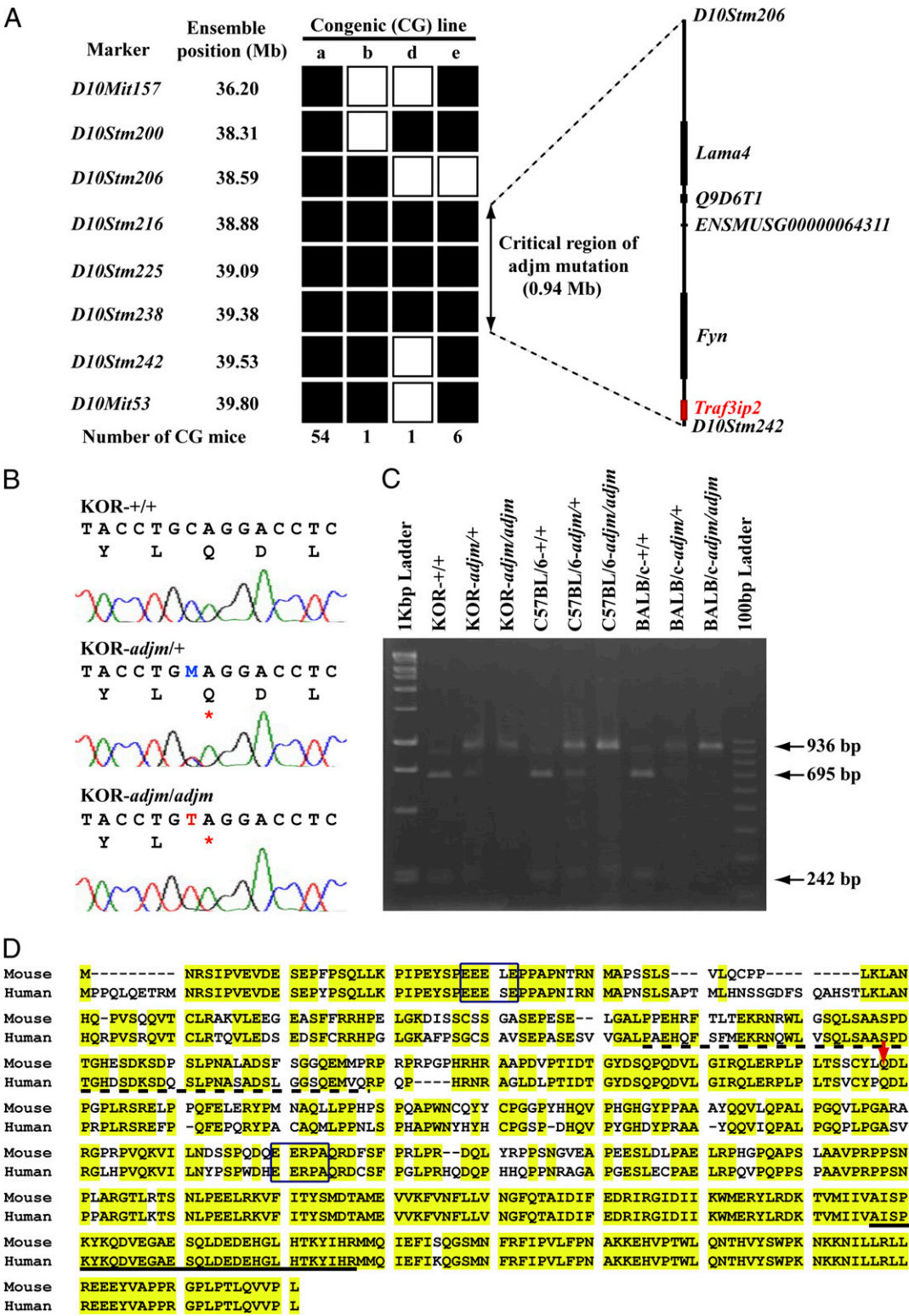


FIGURE 5. Positional cloning of the *adjm* mutation. **A**, Fine congenic map and physical map near the *adjm* locus. Open and closed squares represent homozygous and heterozygous KOR-specific loci. Names and orders of genes located in the critical region of the *adjm* mutation were obtained from Ensembl. **B**, Comparison of KOR, KOR-*adjm*/+, and KOR-*adjm*/*adjm* *Traf3ip2* genomic sequences. A C → T transition was detected in KOR-*adjm*/+ and KOR-*adjm*/*adjm* mice that is not present in the founder KOR mice. The mutation occurred in codon 214 in the second exon of *Traf3ip2* (Q → Stop). The mutation sites are shown in red. **C**, The *adjm* mutation disrupts a PstI restriction site (CTGCAG) in the *Traf3ip2* gene. The digestion of amplicons from wild-type mice produces bands at 694 and 242 bp. However, *adjm*/*adjm* mice are homozygous for the disruption of the PstI site and thus yield only a single 936-bp band, whereas *adjm*/+ mice are heterozygous for the mutation, as demonstrated by the two banding patterns superimposed on one another. **D**, Comparison of the mouse and human TRAF3IP2 protein sequences. The amino acid sequences in open boxes or underlined with broken or solid lines are the TRAF binding sites, helix-loop-helix domain, and coiled-coil domain, respectively.

Table II. Mouse Traf3ip2 PCR and sequencing primer information

Primer	Transcript Position	Ensembl Position	Sequence (5'–3')
AI429613ex1F	304–331	39481301–39481328	CCACTTACCTGGGACTGGAGAGGGCGAG
AI429613int1F	—	39493870–39493895	GCACGTGATCTATCTTCTGAGACTGGC
AI429613ex2R	558–576	39494059–39494077	CACCTGGAGCACAGACAAGC
AI429613ex2F	1109–1135	39494610–39494636	GAACCTTGAGAGGTATCCAATGAACGCC
AI429613int2R	—	39494780–39494805	TCTCTGGACGTGGCAGTGTGTGTGAG
AI429613ex3R	1315–1342	39502691–39502718	CAGGATGACCTTCTGCACAGGGCGTGGG
AI429613ex9F	1943–1960	39522447–39522464	GAGCATGTGCCGACCTGG
AI429613ex9R	2163–2190	39522667–39522667	GGACTCTGTAGAAGAGGTGTTCAGGGC
AI429613ex9R2	2787–2813	39523291–39523317	GCTTAGTCAGAGGTACACTCAGGAGTG

strains mentioned above, it is evident that KOR-*adjm/adjm* is a new model for human AD. This conclusion also is supported by the results of linkage analysis of NC, which revealed no close genetic association between dermatitis and hyper-IgE-emia. Conversely, a close genetic association was found in the congenic KOR-*adjm/adjm* strains described in this paper (Fig. 4). Animals homozygous for the *adjm* mutation showed distinct sex differences in hyper-IgE-emia (Fig. 2), life span, the amount of serum IL-17, and the splenic Th17 cell population (Fig. 7). These lines of experimental evidence suggest the existence of modifier gene(s) for the *adjm* locus that are controlled by sex hormones. We consider this issue very interesting and important with respect to discovery of the molecular regulatory mechanisms of the *adjm* gene and signaling pathways (e.g., the IL-17/IL-17R signaling pathway [see Ref. 21 and references therein]) by hormone(s).

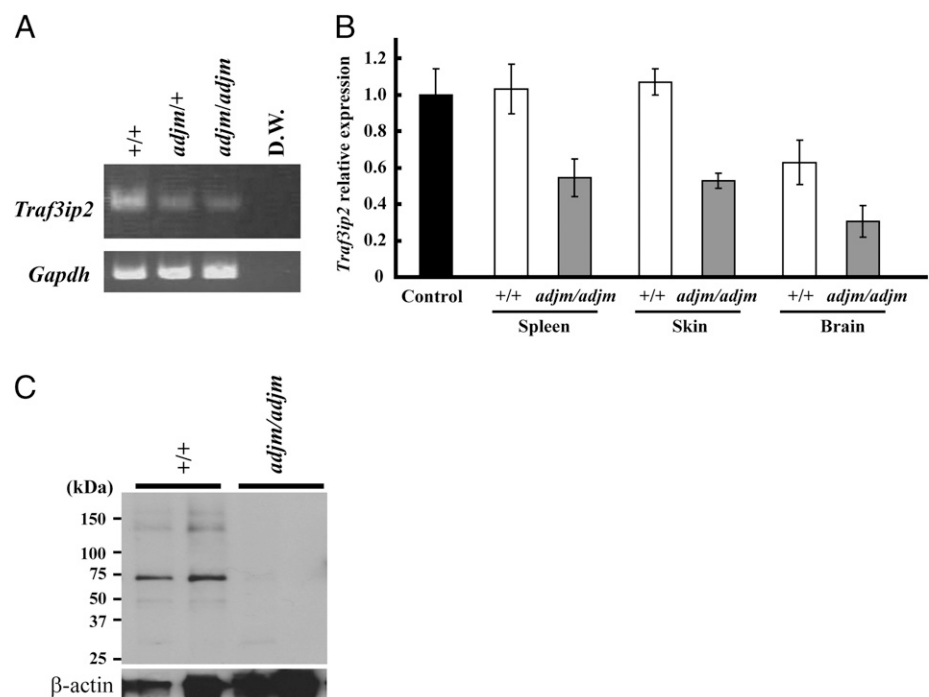
We propose that the mutation detected in *Traf3ip2* (*Act1/CIKS*) is responsible for the phenotype observed in KOR-*adjm/adjm*. TRAF3IP2 is associated with and activates I κ B kinase and stimulates both the NF- κ B and the JNK signaling pathways (15). TRAF3IP2 does not possess any enzymatic domains, but it contains a helix-loop-helix at its N terminus and a coiled-coil at its C terminus, along with two putative TRAF binding sites, EEESE (residues 38–42) and EERPA (residues 333–337). Importantly, the *adjm* mutation deletes the C-terminal TRAF binding site and the coiled-coil region (17, 21). Previous studies demonstrate that

deletion of the C-terminal region (residues 316–573) abolishes TRAF3IP2-induced NF- κ B activation (15). Therefore, it is likely that the *adjm* mutation leads to the loss of TRAF3IP2 function, although the truncated protein is produced (Fig. 5D).

Together with recent findings examining the dual function of TRAF3IP2 (21), the results of this study also explain why a tight genetic association exists between hyper-IgE-emia and dermatitis. The forced expression of TRAF3IP2 in 293T cells and HeLa cells has been reported to activate both NF- κ B and JNK (18). Endogenous TRAF3IP2 was recruited to CD40, a member of the TNFR superfamily, in human epithelial cells upon stimulation with CD40 ligand (CD40L). Furthermore, transfection of *Traf3ip2* into TRAF3IP2-negative epithelial cells rendered them sensitive to CD40L-induced NF- κ B activation (17, 18, 25, 26). Despite these results, the recent generation of TRAF3IP2-null mice shows that TRAF3IP2 functions as a negative regulator rather than an activator in CD40- and BAFF-mediated signaling (18, 19, 21). Therefore, the truncation of TRAF3IP2 may cause a malfunction of this adaptor protein, which results in the hyper-IgE-emia observed in our mutant mouse.

IL-17 plays an important role in promoting tissue inflammation in host defenses against infection and in autoimmune diseases. IL-17 was first discovered as the founding member of the IL-17 cytokine family and has been reported to regulate the expression of proinflammatory cytokines, chemokines, and matrix

FIGURE 6. *Traf3ip2* gene and protein expression in wild-type (+/+), *adjm/+*, and *adjm/adjm* mice. A, RT-PCR analysis of *Traf3ip2* expression in the spleen of +/+, *adjm/+*, and *adjm/adjm* mice using primers (AI429613ex1F and AI429613ex2R; Table II) located at exons 1 and 2 (top panel). cDNA integrity was confirmed with a GAPDH control band (bottom panel). B, Relative levels of *Traf3ip2* mRNA in the spleen, skin, and brain of 5-wk-old BALB/c-+/+ (white bars) and BALB/c-*adjm/adjm* (gray bars) mice. *Traf3ip2* mRNA expression was detected by quantitative RT-PCR; C57BL/6-+/+ spleen mRNA was used as a control. Values shown are relative expression levels of triplicate samples (means and SDs) ($n = 3$). C, Western blot analysis of TRAF3IP2 protein expression in the spleen of +/+ and *adjm/adjm* mice. C57BL/6 (left lane) and BALB/c background (right lane) spleens were probed with anti-Act1 Ab (top panel). The same blot was reprobed with anti- β -actin Ab to confirm the concentration of the charged samples.



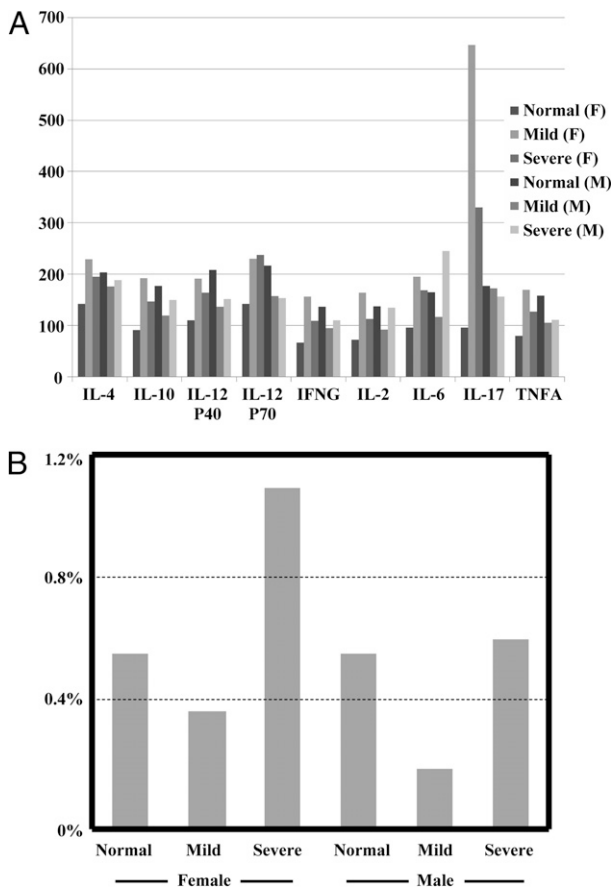


FIGURE 7. Relative levels of eight serum cytokines and comparison of splenic Th17 cell populations in C57BL/6-+/+ and C57BL/6-*adim/adim* mice. **A**, Relative amounts of eight serum cytokines that are major determinants for the Th lineage were measured by protein microarray. Normal denotes C57BL/6-+/+ mice without dermatitis, whereas mild and severe denote C57BL/6-*adim/adim* mice with mild and severe dermatitis, respectively. Normal (F and M) and mild (M), $n = 2$; mild (F) and severe (F and M), $n = 3$. The relative amounts of each cytokine were estimated by comparison of the fluorescence signal to that of reference arrays (2-, 4-, and 8-fold dilutions). **B**, Percentage of Th17 cells in splenic CD4⁺ T cells. Spleen cells were isolated from C57BL/6-+/+ mice without dermatitis and from C57BL/6-*adim/adim* mice with mild and severe dermatitis. After being stained with anti-CD4 mAb and anti-IL-17 mAb, 10,000 cells from each CD4⁺ T cell-enriched fraction were subjected to flow cytometry. F, female; M, male.

metalloproteinases (27, 28). Previous work has demonstrated that IL-17 activates the NF- κ B and MAPK pathways and requires TRAF6 to induce IL-6 (20, 26, 27). Additionally, Chang et al. (27) have demonstrated that the intracellular region of the IL-17R family proteins shares sequence homology with the Toll/IL-1R domain and with *Traf3ip2*. Furthermore, TRAF3IP2 and IL-17R directly associate, likely via a homotypic interaction. In fibroblasts, TRAF3IP2 deficiency abrogates IL-17-induced cytokine and chemokine expression as well as the induction of C/EBP β , C/EBP δ , and I κ B ζ . Yamamoto et al. (29) reported that mice deficient for I κ B ζ , a member of the I κ B family involved in the regulation of NF- κ B signaling, develop AD-like skin lesions with acanthosis and lichenoid changes by 10 wk of age. Together with the results of the current study and those of the study on *Traf3ip*-null mice (19), this observation suggests that dysregulation of the NF- κ B signaling pathway leads to the development of AD-like skin disease in mice. The KOR-*adim/adim* and congenic mice established in the current study therefore will be useful animal models of human AD.

The absence of TRAF3IP2 results in a selective defect in IL-17-induced activation of the NF- κ B pathway. These results indicate that TRAF3IP2 is a membrane-proximal adaptor of the IL-17R and that it has an essential role in the induction of inflammatory genes. Thus, this study not only reveals an immediate signaling mechanism downstream of an IL-17 family receptor for the first time but also has implications for the therapeutic treatment of various immune diseases.

Th17 cells are a novel subset of CD4⁺ T cells that are regulated by TGF- β , IL-6, and IL-23. Th17 cells have been shown to be important in inflammation and in the control of certain bacteria. After stimulation with IL-17, the recruitment of TRAF3IP2 to IL-17R requires the IL-17R conserved cytoplasmic SEFIR (similar expression to fibroblast growth factor genes and IL-17Rs) domain and is followed by the recruitment of the TGF- β -activated kinase 1 and an E3 ubiquitin ligase (TRAF6), both of which mediate the downstream activation of NF- κ B. IL-17-induced expression of inflammation-related genes was abolished in TRAF3IP2-deficient primary astroglial and gut epithelial cells, and this reduction was associated with less severe inflammatory disease in vivo in both autoimmune encephalomyelitis and dextran sodium sulfate-induced colitis (20). Therefore, our mutant mice provide an excellent tool for detailed analysis of the function of the N-terminal TRAF3IP2 protein in these pathways because they express a truncated protein.

The adapter molecule TRAF3IP2 regulates autoimmunity through both T and B cell-mediated immune responses. The coordinated regulation of T and B cell-mediated immune responses plays a critical role in the control and modulation of autoimmune diseases. Whereas the TRAF3IP2 molecule is an important negative regulator of B cell-mediated humoral immune responses through its role in CD40L and BAFF signaling (19), recent studies have shown that TRAF3IP2 is also a key positive regulator of the IL-17 signaling pathway and that it is critical for Th17-mediated autoimmune and inflammatory responses. The dual functions of TRAF3IP2 are evident in TRAF3IP2-deficient mice that display B cell-mediated autoimmune phenotypes, including a dramatic increase in peripheral B cells, lymphadenopathy and splenomegaly, hypergammaglobulinemia, and Sjögren's disease in association with lupus nephritis (19, 21), but show resistance to Th17-dependent experimental autoimmune encephalomyelitis and colitis (20, 21, 30). Such seemingly opposite functions of TRAF3IP2 in the CD40 or BAFFR and the IL-17R signaling pathways are orchestrated by the different domains of TRAF3IP2. Whereas TRAF3IP2 interacts with the IL-17R through the C-terminal SEFIR domain in TRAF3IP2, TRAF3IP2 is recruited to CD40 and BAFFR indirectly by TRAF3 binding to the TRAF binding site in TRAF3IP2 (17, 21). Such a delicate regulatory mechanism may provide a common vehicle that promotes the balance between host defense to pathogens and tolerance to self (21).

Conflicting evidence has been reported regarding the association between IL-17 production and AD exacerbation. Two reports suggest that Th17 cells exacerbate dermatitis because increased numbers of Th17 cells were found in the peripheral blood and acute lesional skin of AD patients (31). Kawakami et al. (32) also support this idea with their study of NK cell function in AD patients (23). In contrast, two reports suggest that low IL-17 expression or reduced responses to IL-17 in the skin cause AD-like symptoms (24, 33). To resolve these conflicting lines of evidence, we determined the number of Th17 cells in affected skin regions of the faces of homozygous *adim* mice. No Th17 cells were observed in the skin (data not shown), although these cells were found in the spleens of severely affected homozygous female mice at twice the number as in nonaffected female mice (Fig. 7B). Similarly, IL-17 protein levels in the peripheral blood of affected female mice were

3.3- to 6.5-fold higher than those of unaffected mice. Conversely, few differences were observed between affected and unaffected male mice (Fig. 7). This is consistent with the finding that female mice were affected more severely than male mice by hyper-IgE-emia and dermatitis (Figs. 2–4). On the basis of our results with female mice, it is suggested that reduced IL-17 responses cause AD. However, it remains unknown why no correlation was found between IL-17 expression and features of AD in male mice.

Act1/TRAFF3IP2-deficient mice showed a drastic increase of peripheral B cells and plasma cells, together with lymphadenopathy and splenomegaly. Serum levels of IgG and IgE in these animals were >10-fold higher than those of normal mice (19). These phenotypes occur mainly as a result of TRAF3IP2 deficiency in B cells, because B cell-specific TRAF3IP2-deficient mice showed similar, although less severe, phenotypes. It is therefore probable that the hyper-IgE-emia observed in KOR-*adjml/adjm* mice and congenic *adjml/adjm* mice is due to increased CD40- and BAFFR-mediated B cell stimulation and Ig class switching in the absence of functional TRAF3IP2. Importantly, lymphadenopathy and splenomegaly were observed in all of the mutant mice generated in this study.

Hyper-IgE syndrome (HIES; i.e., Job's syndrome) is an autosomal dominant syndrome that is often associated with dermatitis. Recently, close association of this syndrome with mutations at the STAT3 locus (34–36) has been demonstrated. These reports showed that HIES phenotypes can cause a specific deficit in the generation of Th17 cells and IL-17 production (35, 36). Although it is interesting that separate mutations affecting IL-17 signaling result in a nearly identical AD phenotype and hyper-IgE-emia, the two mutants show opposite phenotypes with respect to the generation of Th17 cells and IL-17 production. With respect to IL-17R signaling (21), it is reasonable that both mutants show nearly identical phenotypes, because both mutations attenuate downstream IL-17/IL-17R signaling. HIES mutants suffer an attenuation due to the insufficiency of IL-17, whereas the *adjm* mutation also causes attenuation through the dysfunction of the IL-17R signaling complex brought about by the presence of deficient TRAF3IP2 protein.

Although we assume that TRAF3IP2-null mice in the BALB/c background develop less severe dermatitis than KOR-*adjml/adjm* mice (TRAF3IP2-null mice have been shown to develop skin inflammation with epidermal hyperplasia and T cell infiltration), detailed studies of dermatitis development in this mouse strain have not been reported. However, we found that the AD-like skin disease that developed in BALB/c-*adjml/adjm* mice was less severe than that which occurred in KOR-*adjml/adjm* mice. Although, on the basis of the phenotypes of *adjm* mutant and TRAF3IP2-null mice, there is no doubt that a deficiency in functional TRAF3IP2 is responsible for the development of dermatitis in these strains, the underlying mechanism remains to be clarified.

Because, as observed in BAFF transgenic mice (37–39), TRAF3IP2-null mice have been shown to produce autoantibodies against DNA and histones (19), it is possible that autoantibodies against some components of the skin trigger dermatitis. Expression of TRAF3IP2 is not restricted to B cells but is ubiquitous. It is therefore also possible that the deficiency of functional TRAF3IP2 leads to dysregulated homeostasis of skin cells, such as epithelial cells and fibroblasts. Indeed, TRAF3IP2-deficient embryonic fibroblasts demonstrated enhanced NF- κ B activation when stimulated via ectopically expressed CD40 or BAFFR. It has been suggested that TRAF3IP2 in non-B cells may play a role in the signaling events mediated by other members of the TNFR superfamily, especially the subsets of signaling proteins that use TRAFs.

If this is the case, then B cell-specific TRAF3IP2-deficient mice may not develop dermatitis. However, studies of the development of dermatitis in such mice have not been reported.

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