Targeted Disruption of MAIL, a Nuclear IκB Protein, Leads to Severe Atopic Dermatitis-like Disease*

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MAIL (molecule-possessing ankyrin repeats induced by lipopolysaccharide) is a nuclear IκB protein that is also termed interleukin-1-inducible nuclear ankyrin repeat protein or inhibitor of nuclear factor κB (IκB) ɣ. In this study, we generated Mail−/− mice to investigate the roles of MAIL in whole organisms. Mail−/− mice grew normally until 4–8 weeks after birth, when they began to develop lesions in the skin of the periorcular region, face, and neck. MAIL mRNA and protein were constitutively expressed in the skin of wild type controls, especially in the keratinocytes. Serum IgE was higher in Mail−/− mice than in normal. Histopathological analysis indicated that the Mail−/− skin lesions appeared to be atopic dermatitis (AD) eczema with inflammatory cell infiltration. In addition, markedly elevated expression of some chemokines such as thymus and activation-regulated chemokine was detected in the Mail−/− skin lesions, similar to that observed in the skin of patients with AD. In Mail−/− mice, MAIL-deficient keratinocytes might be activated to produce chemokines and induce intraepidermal filtration of inflammatory cells, resulting in the onset of the AD-like disease. These findings suggest that MAIL is an essential molecule for homeostatic regulation of skin immunity. The Mail−/− mouse is a valuable new animal model for research on AD.

Atopic dermatitis (AD) is a common, chronic, inflammatory, pruritic skin disease that occurs in patients with an individual or family history of atopy, and it is frequently associated with elevated serum IgE levels (1). Of the major clinical features of AD, pruritus and chronic or relapsing eczematous lesions with typical shape and distribution are essential for the diagnosis. AD afflicts 10–20% of children worldwide and its pathogenesis is still poorly understood. As is true for other allergic diseases, AD has a strong genetic component, but few candidate genes have been identified. Recent results indicate that immunological mechanisms play a key role in the development of AD (1, 2).

In addition to the presence of a mononuclear cellular infiltrate that consists mainly of CD4+ T cells, the development of AD has been shown to involve eosinophils, mast cells, and inflammatory cytokines and chemokines (2, 3).

The members of the nuclear factor κB (NF-κB) family of transcription factors are known to regulate the expression of various genes that are associated with cell growth, cell survival, differentiation, development, and immune and inflammatory responses (4, 5). Multiple stimuli, such as cytokines, microbiological components, stress, chemical reagents, and ultraviolet radiation, can lead to the activation of NF-κB; these stimuli induce the phosphorylation and subsequent degradation of inhibitor of NF-κB (IκB) proteins in the cytoplasm via intracellular signaling pathways, thereby releasing NF-κB proteins for translocation to the nucleus to function as transcription factors. Several studies have demonstrated that NF-κB or components of the system such as IκB kinase appear to be involved in epidermal development and homeostasis (6, 7).

Furthermore, dysregulation of NF-κB has been suggested to play important roles in the development of various skin pathologies, including proliferative disorders, and immune diseases (7–11).

We identified a nuclear IκB protein, which we named MAIL for molecule possessing ankyrin repeats induced by lipopolysaccharide, also termed interleukin (IL)-1-inducible nuclear ankyrin repeat protein or IκBγ, in mice (12–16). The expression level of MAIL mRNA is below the limits of detection constitutively but increases dramatically and rapidly after stimulation by lipopolysaccharide or proinflammatory cytokines, such as IL-1 and tumor necrosis factor-α (16). The MAIL protein enhances or suppresses transcription of NF-κB target genes in a gene- or cell type-specific manner. For example, ectopically expressed MAIL enhanced production of IL-6 in fibroblasts but inhibited NF-κB in macrophages (12, 13, 17). However, the precise roles of MAIL, especially in vivo, remain to be elucidated.

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The abbreviations used are: AD, atopic dermatitis; IL, interleukin; IκB, inhibitor of NF-κB; KC, keratinocyte; LC, Langerhans cell; MAIL, molecule possessing ankyrin repeats induced by lipopolysaccharide; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; RANTES, regulated on activation T cell expressed and secreted; RT, reverse transcriptase; TARC, thymus and activation-regulated chemokine; MHC, major histocompatibility complex.
Here, we inactivated MAIL in mice by targeted gene disruption to investigate the roles of MAIL in immune and inflammatory responses. MAIL-deficient mice developed an AD-like disease accompanied by typical cellular infiltrates and chemokine expression patterns in the skin similar to those of patients with AD, providing a possible new mouse model for the study of this common allergic disease.

**EXPERIMENTAL PROCEDURES**

*Generation ofMail*−/−*Mice*—The targeting vector was designed to replace the five exons (4–8) that encode ankyrin repeats I and II with a PGK-neo cassette and to insert a nonsense mutation just downstream of the start codon of the MAIL protein (Fig. 1A). Correctly targeted AB2.2 ES-cell (129 strain) clones were identified by Southern blot analysis (Fig. 1B) of EcoRI-digested genomic DNA using a probe that detected a 10- or 6-kb band for the wild type or mutant locus, respectively. Single integration was confirmed using a probe corresponding to the sequence of the neomycin-resistant gene. Two ES cell clones were injected into C57BL/6 murine blastocysts to generate chimeric mice. Both clones were germ line-transmitted in the chimeric mice. The sequence of the neomycin-resistant gene. Two ES cell clones were detected a 10- or 6-kb band for the wild type or mutant locus, respectively for the wild type allele and a 430-bp fragment for the mutant allele.

*Mail*−/−*mice were always compared with wild type and heterozygous littermates. All animal experiments were approved by the ethical committees at Iwate University and Hokkaido University. Histology, Enzyme Histochemistry, and Immunohistochemistry—

Skin biopsy samples from *Mail*−/−and *Mail*+/+ mice were fixed in 10% neutral buffered formalin or Bouin’s solution. The fixed tissues were embedded in paraffin, and 4-μm sections were cut. Representative paraffin sections of each skin sample were stained with hematoxylin and eosin or with toluidine blue for detection of mast cells. For detection of the start codon of the MAIL protein (Fig. 1C), PCR-based genotyping of embryos from a heterozygous mating using primers a, b, and c, as shown in A. M. indicates molecular size markers.

**Serum IgE Measurement**—Serum samples were prepared from *Mail*−/−and control heterozygous and wild type littermates. The concentration of IgE was quantitatively determined against a standard curve (clone 27-74; BD Pharmingen, San Diego, CA) in a fluorometric sandwich enzyme-linked immunosorbent assay employing monoclonal anti-mouse IgG as the capture antibody (clone R35-72; BD Pharmingen), biotin-conjugated anti-IgE as the detection antibody (clone R35-118, BD Pharmingen), and streptavidin-conjugated α-galactosidase for detection (Invitrogen).

**Reverse Transcriptase (RT)-PCR**—Total RNA was prepared from tissue samples using TRIzol reagent (Invitrogen). The cDNA was synthesized from RNA templates (3 μg) using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s directions. Conventional RT-PCR was performed for detection of MAIL, thymus and activation-regulated chemokine (TARC), eosinophil, and CCR3 mRNAs. The PCR amplifications were performed using 34 cycles with the following primers: MAIL, 5′-CGCGATCCAGCTGGAAGCGCCACAT-CC-3′ and 5′-GGCTGTTCCGCTGATTGC-3′; TARC, 5′-CAGGAACTTGGTGAGGCCTG-3′ and 5′-GGCTGTTCCGCTGATTGC-3′; and 5′-TTTCTTTCGCCTCTTCACCAGGTTTCTCC-3′; and primer c, 5′-TACGCCACCTGAAAGGCAC-3′. These primers generate a 520-bp fragment for the wild type allele and a 430-bp fragment for the mutant allele.

**In Vivo Eosinophil and Mast Cell counts**—Eosinophil and mast cells in the skin sections were quantified using兔 anti-mouse IgE (clone R35-118, BD Pharmingen) and streptavidin-conjugated rabbit polyclonal antibody against MAIL (rabbit polyclonal antibody, Caltag, Burlingame, CA) at 8 μg/ml in PBS containing 0.05% Tween 20 (Wako, Osaka, Japan) for 3 h. For negative controls, PBS or IgE was used instead for the primary antibody.
were generated using gene-targeting techniques (Fig. 1). The designated as the LC-rich fraction. fraction, whereas the bound (enriched for I-Aα) cells were separated by washing under a magnetic field. The un-bound cells (depleted of I-Aα) were designated as the KC-rich fraction, whereas the bound (enriched for I-Aα) cells cell fraction was designated as the LC-rich fraction.

Preparation of Primary Mouse Embryonic Fibroblast Cells—Primary mouse embryonic fibroblast cells were prepared from embryos at 11.5 days postcoitum as previously described (20). The head was removed, and the remainder of the embryo was cut into small pieces and trypsinized. The cells were rinsed in medium and plated onto one 60-mm plate/embryo. The embryonic fibroblasts were expanded in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

Separation of Keratinocytes (KCs) and Langerhans Cells (LCs)—Epidermal cells were prepared from mouse skin using a previously described technique (21). KCs and LCs were separated using immunomagnetic beads. The epidermal cells were treated with fluorescein isothiocyanate-conjugated anti-I-Aα (an MHC class II antigen), washed, exposed to anti-fluorescein isothiocyanate antibody-coated magnetic microbeads (Miltenyi Biotech, Berg. Gladback, Germany), and separated into two fractions by washing under a magnetic field. The unbound cells (depleted of I-Aα cells) were designated as the KC-rich fraction, whereas the bound (enriched for I-Aα cells) cell fraction was designated as the LC-rich fraction.

RESULTS

Gross Observations of Mail+/− Mice—MAIL mutant mice were generated using gene-targeting techniques (Fig. 1). The ratio of wild type (+/+): heterozygous (+/−): homozygous (−/−) mutant mice born from heterozygous intercrosses was 106:179:8 (1:1.7:0.1), indicating that ~90% of Mail+/− embryos died in utero. The Mail+/− mice that survived to birth grew normally and were of normal appearance until 4–8 weeks after birth; to the extent examined, no evident morphological or behavioral abnormalities were observed.

After 4–8 weeks, the Mail+/− mice began to show a slight thickening and scaling of the periorcular skin and a marked swelling of the eyelid (Fig. 2A). Subsequently, the Mail+/− mice developed alopecia of the periorcular skin (Fig. 2B), which extended progressively to the skin of the face, neck, and parts of the ventral trunk (Fig. 2C). Thickening of the skin also became more severe. The eruptions were highly pruritic, based on our observations of the behavior of the mice, which used their forelegs to scratch their faces. In contrast, the heterozygote and wild type mice appeared normal (Fig. 2D). E, bar graphs showing the levels of serum IgE in control (Mail+/− or −/−) and Mail+/− mice. The values presented are the means ± S.E.

Histopathology of the Skin of Mail+/− Mice—Skin samples from wild type mice (Fig. 3, A, D, and J) and from young (Fig. 3, B, E, and K) and aged (Fig. 3, C, F, G, H, I, and L) Mail+/− mice were examined histologically. The two young Mail+/− mice (40 and 70 days old) examined had acute eczema that was characterized by mononuclear epidermal and dermal infiltrates, acanthosis, and slight hyperkeratosis in the facial skin (Fig. 3, B and E). The inflammatory infiltrates were restricted to the epidermis and superficial dermis. A small number of neutrophils were seen in the dermis, in addition to the mononuclear cell infiltration. Eosinophils were rarely observed. The number of mast cells in the skin of the Mail+/− mice was not significantly different from that of the normal mice (Fig. 3, J and K). No ulceration was seen on the skin surface.

The three aged Mail+/− mice examined (162, 174, and 189 days old) had AD-like severe chronic eczema in the facial skin (Fig. 3, C and F). The affected tissues showed mononuclear epidermal infiltration similar to that seen in the young Mail+/−
mice; however, acanthosis, parakeratosis, and hyperkeratosis were more prominent in the aged mice. In addition to the mononuclear cells, the infiltrate in the older animals included lymphoid cells, melanophages, an increased number of neutrophils, and numerous mast cells (Fig. 3L) and eosinophils. Because of the excessive cell infiltration throughout the skin, the skin adnexa and cutaneous muscle layer were destroyed. Although partial spongiosis associated with the mononuclear epidermal infiltration was observed, vesiculation was not noted. The affected skin of the older mice had partial ulceration and scabbing on the skin surface.

Immunohistochemistry for MHC class II, B220, CD4, and CD8, and histchemistry for acid phosphatase of the skin of the aged Mail+/− mice revealed that the mononuclear epidermal infiltrate consisted of MHC class II-positive cells with a dendritic shape that were probably LCs and CD4- and CD8-positive T cells (Fig. 3, G–I). B220-positive B cells and acid phosphatase-positive macrophages, as well as CD4 and CD8 cells, were found in the inflamed dermis and subcutaneous tissue, but not in the epidermis, in contrast to the LCs and T cells.

Expression of Genes of AD-associated Chemokines in the Skin of Mail+/− Mice—Several chemokines, such as TARC and eotaxin, are expressed at high levels in skin affected by AD and lead to infiltration of inflammatory cells (2, 22–24). To determine whether these chemokine genes were expressed in the skin of 6-week-old Mail+/− mice, we performed RT-PCR (Fig. 4A). Expression of the TARC gene was not detectable in the skin of wild type and heterozygote mice but was strongly expressed in the skin of Mail+/− mice. In contrast, the levels of expression of the genes for eotaxin and its receptor CCR3 in the skin of young Mail+/− mice did not differ from those in wild type mice (data not shown). As Mail+/− mice aged and the skin lesions became more severe, eotaxin and CCR3 gene expression also increased (Fig. 4B); however, this might have been attributable to a response to persistent scratching.

Expression of MAIL mRNA and Protein in the Facial Skin of Normal Mice—As the lack of MAIL expression gave rise to AD-like lesions, we ascertained whether MAIL was expressed in the skin of normal wild type mice. RT-PCR showed that MAIL mRNA was strongly expressed in the facial skin and less intensely, yet substantially, in the skin of the trunk of normal mice (Fig. 5A). Proliferation of KCs and infiltration of LCs was observed on histopathological examination (above) and might represent primary pathological changes in the Mail+− skin lesions. Consequently, we performed RT-PCR on KC- and LC-rich fractions of normal skin cells and found that MAIL mRNA was expressed predominantly in the isolated KCs as compared with the LC-rich fraction (Fig. 5B). To confirm the localization of MAIL protein in the skin, we performed immunocytochemistry using a specific antibody against MAIL. Positive immunoreaction for MAIL was observed in the KCs located in and around the neck of the hair follicles. The magnification was ×300.

Discussion

In this study, we generated MAIL-deficient mice and investigated their phenotype, with particular attention to the skin. The main findings were: 1) MAIL is constitutively expressed in KCs in normal wild type skin; 2) the lack of MAIL expression in the skin led to severe dermatitis, which appeared to be AD on histopathological examination; and 3) skin cells of Mail−/− mice express markedly elevated levels of chemokines associated with AD. Yamamoto et al. (25) recently reported the generation of MAIL-deficient mice.
of IxβC-deficient mice independently of our group. The authors reported that the mice developed AD-like skin lesions; however, the description of the pathophysiological analysis was very limited. In the current study, we characterized the AD-like lesions in Mail−/− mice pathologically and biochemically and investigated the relationship between the expression of MAIL and the development of skin lesions in detail.

The characteristics of Mail−/− mice, including the skin abnormalities, scratching of the face (indicating pruritus), and the elevated serum IgE concentration, indicated that these mice might be affected with allergic dermatitis. In the facial skin of normal wild type mice, where the dermatitis was most severe in the MAIL-deficient mice, MAIL mRNA and protein were strongly expressed in KCs. It is well documented that KCs play an important role in the immunopathogenesis of skin diseases such as AD (2). For instance, KCs proliferate and release chemokines in AD, attracting various leukocytes into the skin (26). Thus, the current results raise the possibility that the dermatitis in Mail−/− mice might be attributable to the deficiency of MAIL in the KCs of the skin. It should be mentioned that MAIL was originally identified as an inducible molecule in immunocompetent organs and cells (12–15, 17); this is the first report demonstrating that MAIL is constitutively expressed in some types of cell in the skin and that it participates in the homeostatic regulation of skin immunity.

The skin lesions were initially observed around the eyes. The histological analysis demonstrated both blepharitis and conjunctivitis in the Mail−/− mice. In the IxβC-deficient mice generated by Yamamoto et al. (25), pathological changes observed in the conjunctiva were similar to those seen in our Mail−/− mice. Because MAIL-immunoreactive KCs were detected in the palpebral skin, but not the conjunctiva, of normal wild type mice, the conjunctivitis in the Mail−/− mice might be elicited by an inflammatory expansion of the primary blepharitis lesions.

The eczema in the Mail−/− mice was characterized by epidermal and dermal infiltrations of inflammatory cells, acanthosis, and hyperkeratosis. Although eczema and cellular infiltrations were restricted in the early phase (1–2 months after birth), an extremely large number of inflammatory cells, including lymphoid cells, macrophages, granulocytes, and mast cells, infiltrated into the whole skin during the late phase (more than 5 months after birth). These pathological findings are consistent with those for human AD (1, 2). To diagnose allergic dermatitis, discrimination between AD and allergic contact dermatitis is required. Because the eczema in Mail−/− mice occurred in a region protected by hair and expanded nonfocally and bilaterally, it seems unlikely that specific contact agents were involved in their generation. Taken together, the present results suggest that the skin lesions observed in Mail−/− mice appear to be AD-like eczema rather than allergic contact dermatitis.

In the pathophysiological development of human AD, several chemokines and cytokines that are secreted by the skin KCs and endothelial cells facilitate the infiltration of inflammatory cells into the skin lesions (2, 22–24, 27). For example, TARC and eotaxin attract Th2 lymphocytes and eosinophils, respectively, into lesions (2, 22). The present results demonstrate that TARC was markedly expressed in the skin tissues of Mail−/− mice as compared with those of wild type mice. In addition, expression of the genes for eotaxin and its receptor CCR3 were also higher in the severe skin lesions than in normal wild type skin. The up-regulation of chemokines supports the hypothesis that the skin lesions of Mail−/− mice are AD-like eczema. Furthermore, the expression levels of two chemokines that are associated with inflammatory diseases, interferon-γ-inducible protein 10 and RANTES (24, 27, 28), were also markedly increased in Mail−/− mouse embryonic fibroblast cells stimulated by typical inflammatory stimulants such as IL-1β and lipopolysaccharide (data not shown). Thus, MAIL plays suppressive roles in the production of chemokines both in the presence and in the absence of inflammatory stimulants. The precise molecular mechanisms underlying the promotion of chemokine production remain unclear. In light of a report by Yamazaki et al. (13) demonstrating that MAIL associates with the p65/p50 heterodimers and p50/p50 homodimers of NF-κB, which are able to activate transcription of some chemokine genes (23, 29), and suppresses transcriptional activities of NF-κB (13), it is possible that MAIL deficiency leads to accelerated chemokine production through overactivation (or desuppression) of NF-κB.

Other murine models for AD have been reported previously. For example, mice deficient in RelB, a member of the NF-κB family, were clinically normal until 4–10 weeks after birth, at which point thickening of the skin and hair loss developed (10). In addition, the mRNA levels of chemokines such as eotaxin and CCR3 were elevated in the skin of RelB−/− mice, and the serum IgE concentration was increased (10, 30). Because no direct association between MAIL and RelB has been reported, the mechanism for the development of the skin lesions in the Mail−/− mice might differ from that in the RelB−/− mice. In another AD model, NC/Nga mice developed an eczematous condition with increased serum IgE from 8 weeks of age (31). TARC, eotaxin, and CCR3 were expressed in the skin of NC/Nga mice (22). However, there are important differences between the existing murine models of AD and Mail−/− mice. In NC/Nga mice, the dominant lesions are observed in the dorsal skin (22, 31), and the gene(s) responsible has not been identified. Although Mail−/− mice developed AD-like eczema when kept under specific pathogen-free conditions, the NC/Nga mice have been reported to develop an eczematous condition when kept in conventional housing but not when kept under specific pathogen-free conditions. The current data support the contention that Mail−/− mice might provide a useful model for the study of the pathogenesis of AD from a new viewpoint.

Genome-wide linkage studies have been performed to identify susceptibility loci for AD (1). Lee et al. (32) demonstrated that AD in humans is linked to chromosome 3q21. Interestingly, this locus is near the region that encodes the Mail gene (15). Thus, Mail might represent a susceptibility gene for AD.

These results suggest that the eczema of Mail−/− mice might be an AD-like disease, which is probably elicited by MAIL deficiency and the subsequent up-regulation of chemokines such as TARC and eotaxin in skin KCs. We hypothesize that the pathophysiology of the AD in Mail−/− mice proceeds as follows. KCs activated (or desuppressed) by the MAIL deficiency proliferate and produce chemokines such as TARC and eotaxin, resulting in intraepidermal infiltration of LCs and CD4+ T cells. These infiltrating cells elicit primary inflammatory responses that are restricted to the epidermis and superflcial dermis during the early phase. Cytokines derived from CD4+ T cells sensitized by LCs then bring about the secondary infiltration and proliferation of B cells and mast cells into the dermis. The mast cells secrete several chemical mediators, such as histamine and serotonin, causing facial pruritus that elicits persistent scratching in the affected mice. This scratching results in progressive tissue destruction and consequently exacerbates the pathology. Further comprehensive studies, including proteomic and transcriptomic studies, will provide information about the molecular events underlying the development of eczematous dermatitis in Mail−/− mice.

In conclusion, we generated Mail−/− mice by targeted disruption of the Mail gene. These mice were affected with an
AD-like skin disease. We believe that these mice are a suitable model for research on severe dermatitis such as AD.

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