Fas deficiency in mice with the Balb/c background induces blepharitis with allergic inflammation and hyper-IgE production in conjunction with severe autoimmune disease

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Abstract
Fas (CD95) is a cell surface death receptor belonging to the tumor necrosis factor receptor superfamily, which mediates apoptosis-inducing signaling when activated by Fas ligand or its agonistic antibody. lpr mice with a loss of apoptosis-inducing function mutation in the Fas gene develop systemic autoimmune disease and lymphadenopathy but not allergic inflammation. In the case of Fas mutations including lpr and knockout (KO), background genes determine the incidence and severity of lymphadenopathy and histopathological manifestation of systemic autoimmunity: MRL-lpr/lpr mice and C57BL/6-lpr/lpr or C57BL/6 Fas KO mice develop severe and minimum disease, respectively. We generated Fas KO mice with the Balb/c background that show severer autoimmune phenotypes than MRL-lpr/lpr mice, such as critical infiltration of mononuclear cells into lung, liver and spleen, elevated serum levels of auto-antibodies and a decreased life span. To our astonishment, Balb/c Fas KO mice spontaneously develop blepharitis with not only autoimmune inflammation with deposition of auto-antibody but also allergic inflammation with infiltration by eosinophils and mast cells and show the capacity to strongly increase serum level of IgE and IgG1 along with their aging. Thus, Fas expression regulates development of not only autoimmune disease but also allergic inflammation.

Keywords: allergy, auto-antibody, CD95, eyelid dermatitis, lpr

Introduction
Apoptosis is a physiological cell suicide mechanism essential for normal embryonic development and maintenance of homeostasis. Depression of apoptosis causes cancer, autoimmune diseases and viral infective diseases, whereas an excess of apoptosis generates neurodegenerative diseases, immunodeficiency diseases and hepatopathy (1). Fas (CD95/Apo-1) is a cell surface receptor belonging to the tumor necrosis factor receptor superfamily, which introduces apoptosis-inducing signals into cells via ligation with Fas ligand (FasL) or agonistic anti-Fas monoclonal antibody (2–5). Fas expression was observed in various tissues including thymus, spleen, heart, lung, liver and ovary, and high levels of Fas are expressed on T and B lymphocytes especially when they are activated (6–8). The Fas/FasL system has been clarified to be the principal component of the peripheral immune system to eliminate autoreactive cells by analyses using loss-of-function mutants of Fas and FasL.

Mice with loss-of-function mutations in Fas (lpr) (7) or FasL (gld) (9) progressively increase accumulation of B and T cells, in particular unusual CD3+ B220+ CD4+ CD8− T cells, called lpr cells. They develop systemic autoimmune disease with the production of auto-antibodies and an immunopathology similar to that seen in human systemic lupus erythematosus (SLE) and other rheumatic diseases (10). The incidence and severity of lymphoproliferation, auto-antibody production and histopathological manifestation of systemic autoimmunity in
Splenic CD4+ T cells from mice were purified using 288 Fas knockout mice develop allergic inflammation

IL-4 and IFN-γ

After incubation, supernatants were harvested and tested for (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml).

IgG1 were measured by ELISA as described previously (16).

Automation, Inc.) were used. Serum levels of mouse IgE and clear antibodies (ANA) and anti-SSA antibody (Diagnostic

An ELISA kit for IL-4 and IFN-γ (R&D Systems), antinuclear antibodies (ANA) and anti-SSA antibody (Diagnostic Automation, Inc.) were used. Serum levels of mouse IgE and IgG1 were measured by ELISA as described previously (16).

In vitro stimulation of CD4+ T cells

Splenic CD4+ T cells from mice were purified using MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) (10^5 cells in 0.2 ml/well in 96-well plates) and were cultured with immobilized anti-CD3 and anti-CD28 antibodies (each 1 µg/ml for coating) for 2 days in RPMI 1640 supplemented with 10% fetal bovine serum, 2-ME (50 µM), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml). After incubation, supernatants were harvested and tested for IL-4 and IFN-γ by ELISA.

Statistical analysis

Data are given as the mean ± SD. Student’s t-test and regression analysis were used. P values < 0.05 were considered statistically significant.

Results

Balb/c Fas KO mice develop blepharitis with allergic inflammation

The onset and severity of the autoimmune disease in Fas KO mice are greatly affected by their genetic background. To analyze the phenotype of Fas KO mice with the Balb/c background, C57BL/6 Fas KO mice were backcrossed with Balb/c mice for more than 12 generations. When bred on the Balb/c background, Fas KO mice were born at the expected Mendelian ratio with normal litter sizes and were fertile (data not shown). Balb/c Fas KO mice had massive lymphadenopathy and splenomegaly like C57BL/6 Fas KO or MRL-lpr/lpr mice. Although all of the examined wild-type (WT) control mice survived 25 weeks after birth, Balb/c Fas KO mice started to die at as early as 12 weeks of age and showed 50 and 80% mortality by 25 and 40 weeks, respectively (Fig. 1A). These phenotypes are similar to those of MRL-lpr/lpr mice (13). Balb/c Fas KO mice, however, developed eyelid dermatitis/blepharitis about 20–30 weeks after birth and the blepharitis worsened with aging (Fig. 1B and C).

A histological analysis of eyelids from WT and Balb/c Fas KO mice showed that Balb/c Fas KO mice developed skin thickening and massive infiltration by hematopoietic cells including lymphocytes, neutrophils and eosinophils (Fig. 2A) and toluidine blue-positive mast cells (Fig. 2B). More elaborate analysis of eyelids indicated that neutrophils and eosinophils mainly invaded the dermis and the region of blepharitis, respectively (Supplementary Figure 1, available at International Immunology Online). Thus, the blepharitis seen with Balb/c Fas KO mice is associated with infiltration of allergic effector cells, mast cells and eosinophils. Taken together, Balb/c Fas KO mice manifest blepharitis with allergic inflammation, which has been hardly observed in MRL-lpr/lpr and C57BL/6 Fas KO mice.

Balb/c Fas KO mice showed very high serum levels of IgG1 and IgE

We next examined whether the blepharitis in Balb/c Fas KO mice is accompanied with elevated serum levels of IgE and IgG1. Balb/c Fas KO mice produced significant amounts of IgG1, about 200-fold and 10-fold more than Balb/c WT and MRL-lpr/lpr mice, respectively (Fig. 3A). Then, we measured serum IgG1 and IgE levels with chronological age. Balb/Fas KO mice started to produce high serum concentrations of IgE and IgG1 as early as 6 weeks after birth and levels significantly increased thereafter (Fig. 3B). These results indicate that the deficiency of Fas resulted in spontaneous hyper allergic reactions with production of large amounts of IgE and IgG1 in the Balb/c genetic background.
Fas-deficient MRL-\(lpr/lpr\) mice were reported to produce large amounts of auto-antibodies\(^{(13)}\). Thus, we next measured serum levels of ANA and anti-SSA antibody in Balb/c WT, Balb/c Fas KO and MRL-\(lpr/lpr\) mice (Fig. 3C). As reported elsewhere, MRL-\(lpr/lpr\) mice produced large amounts of anti-SSA and ANA, and, as expected, Balb/c Fas KO mice produced somewhat larger amounts of anti-SSA and ANA.

**Fig. 1.** Balb/c Fas KO mice developed eyelid dermatitis. (A) Survival curve of Balb/c Fas KO mice. (B) Incidence rate curve of eyelid dermatitis in Fas KO mice. Onset of eyelid dermatitis was evaluated by phenotypic alterations on a naked eye. (C) Representative images of eyelid dermatitis from Fas KO mice at 40 weeks of age.

Fas knockout mice develop allergic inflammation

Because production of IgG1 and IgE is dependent on the balance of IL-4 and IFN-\(\gamma\), we next analyzed whether T\(_{h2}\) cytokine production (IL-4) was enhanced in Balb/c Fas KO mice. Splenic CD4\(^+\) T cells from Balb/c WT or Balb/c Fas KO mice were cultured with anti-CD3 and anti-CD28 antibodies for 2 days, and then expression levels of IL-4 and IFN-\(\gamma\) in the culture supernatant were quantified by ELISA. Levels of both IL-4 and IFN-\(\gamma\) in the culture supernatant were significantly higher in splenic CD4\(^+\) T cells from Balb/c Fas KO mice than those from Balb/c WT mice (Fig. 4A and B). Thus, CD4\(^+\) T cells of Balb/c Fas KO mice are partly activated but do not skew to either T\(_{h1}\) cells or T\(_{h2}\) cells.

**Fig. 2.** Balb/c Fas KO mice developed blepharitis with allergic inflammation. (A) HE staining in Balb/c WT and Balb/c Fas KO mice eyelid skin sections. Forty-week-old Balb/c WT and Balb/c Fas KO mice were analyzed. (B) Toluidine blue staining of eyelid skin sections from 40-week-old Balb/c WT and Balb/c Fas KO mice. Scale bar, 500 µm (x4), 100 µm (x20).

CD4\(^+\) T cells in the spleen of Balb/c Fas KO mice do not skew to T\(_{h2}\) cells

Histological analysis of Balb/c Fas KO mice showed a severer autoimmune disease phenotype than MRL-\(lpr/lpr\) mice. In the lungs of MRL-\(lpr/lpr\) mice, moderate lymphocyte infiltration was found principally around peribronchial areas. On the
other hand, severe and massive infiltration by lymphocytes occurred around vessels and not around peribronchial areas in Balb/c Fas KO mice (Fig. 5A). In liver, although MRL-lpr/lpr mice did not develop severe inflammation with lymphocyte infiltration, we could detect severe infiltration with lymphocytes and plasma cells around Glisson's capsule and bile ducts only in Balb/c Fas KO mice (Fig. 5B). These severe phenotypes of autoimmune disease in lung, liver and other tissues (salivary gland and intestine) were observed at 20–25 weeks after birth and aggravated with aging. Thus, Balb/c Fas KO mice developed much severer cell infiltration into lung and liver than MRL-lpr/lpr mice.

We then analyzed whether autoimmune reaction occurred in eyelids of Balb/c Fas KO mice developing blepharitis with allergic inflammation. Confocal microscopical analysis revealed the presence of deposits of IgG along the dermoepidermal junction, which are supposed to be comparable to lupus bands of human SLE, in eyelids of Balb/c

![Fig. 3. Balb/c Fas KO mice produced large amounts of serum IgG1/IgE. (A) Serum concentrations of IgG1 and IgE in WT, Balb/c Fas KO and MRL-lpr/lpr mice at 20 weeks of age were measured by ELISA. Each symbol represents a single mouse. ***P < 0.001, **P < 0.005 and *P < 0.01. (B) Chronological serum IgG1 and IgE levels in WT and Balb/c Fas KO mice. (C) Serum auto-antibodies in Balb/c WT, Balb/c Fas KO and MRL-lpr/lpr mice at 20 weeks.](https://academic.oup.com/intimm/article-abstract/25/5/287/667611)
Fas KO mice but not of Balb/c WT mice (Supplementary Figure 2, available at International Immunology Online). Thus, inflammation with deposition of auto-antibody was induced in eyelids of Balb/c Fas KO mice, where allergic inflammation with infiltration of eosinophil and mast cell was also induced. Taken together, Balb/c Fas KO mice develop autoimmune disease as well as allergic disease and produced abnormally high levels of IgE, suggesting the influence of genetic background on the development of both autoimmune disease and allergic disease.

Discussion

Fas, which belongs to the tumor necrosis factor superfamily, was reported to have important roles in the maintenance of immune regulation. Mice of the MRL strain carrying mutations in the Fas (lpr) or FasL (gld) gene exhibit lymphadenopathy and severe autoimmune disease, SLE. As shown in Fig. 5, Balb/c Fas KO mice showed intensive invasion of mononuclear cells into liver and lung, and severer tissue damage than MRL-lpr/lpr mice. In this regard, the Balb/c Fas KO mouse is relevant to the study of severe autoimmune diseases with marked tissue damage. On the other hand, we found a significant difference between Balb/c Fas KO and MRL-lpr/lpr mice; blepharitis with allergic inflammation developed in Balb/c Fas KO mice with aging, while we have never observed blepharitis in MRL-lpr/lpr, C57BL/6 Fas KO and Balb/c WT mice. We also found dramatically higher levels of serum IgE and IgG1 in Balb/c Fas KO mice than the other mice (Fig. 3). By 20 weeks, half of Balb/c Fas KO mice presented with a high serum IgE level (>5 µg/ml) (Fig. 3). Because a high serum IgE level was reported to play an important role in the induction of both local and systemic allergic reactions in vivo (17), the dramatically high serum IgE level might be responsible for the outbreak of blepharitis in Balb/c Fas KO mice.

Previously, several mechanisms were reported to induce very high production of IgE. One mechanism is overproduction of T_{H2} cytokines including IL-4 from proliferating CD4+ T cells biased toward T_{H2} (18, 19). We, however, showed that splenic CD4+ T cells from Balb/c Fas KO mice were not Th2-prone, although spleen CD4+ T cells from Balb/c Fas KO mice can produce a higher amount of IL-4 than those from Balb/c WT mice (Fig. 4). It is necessary to analyze whether there exist CD4+ T cells that produce a large amount of IL-4 in or around the germinal center where the immunoglobulin class switch is induced. Another mechanism to overproduce IgE is the effect of IL-18, an IL-1-like cytokine, in a CD4+ T cell-, IL-4- and STAT6-dependent fashion (16). Preliminary results showed that IL-18 is highly produced in sera of Balb/c Fas KO mice (A. Fukuoka et al., unpublished data), suggesting that IL-18 is involved in the overproduction of IgE in Balb/c Fas KO mice. We then suppose that the allergic inflammation that we found spontaneously developed in Balb/c Fas KO mice, is classified as previously reported...
innate-type allergy, which can be developed even without specific allergen (16, 20).

We observed lupus bands in the eyelids of Balb/c Fas KO mice, indicating that autoimmune inflammation was induced in eyelids of Balb/c Fas KO mice as previously found in eyelids of experimental autoimmune models (21, 22). On the other hand, allergic inflammation with infiltration by eosinophils and mast cells was also induced in eyelids of Balb/c Fas KO mice, but such allergic inflammation was not observed in previously reported autoimmune blepharitis. One possibility we suppose is that autoimmune inflammation in eyelids induces itch and scratching behavior, which may induce IL-18 expression leading to expression of IgE (23) and infiltration by eosinophils followed by blepharitis with allergic inflammation in Balb/c Fas KO mice.

Recent studies have clarified that dominant negative mutations in STAT3 are a major cause of a classical hyper-IgE syndrome (HIES) (24), which is a complex primary immunodeficiency disorder. HIES is associated with severe dermatitis with recurrent staphylococcal infections in the skin, chronic eczema, boils, cyst-forming pneumonias, elevated levels of serum IgE, retained primary dentition and bone abnormalities (25–27). In Balb/c Fas KO mice, however, STAT3 is normal, and immunodeficiency has not been observed, suggesting that overproduction of IgE in Balb/c Fas KO mice is not related with HIES.

To clarify the mechanism producing the very large amounts of IgE in serum of Balb/c Fas KO mice, we examined the activity of a variety of spleen mononuclear cells to enhance IgG1 and IgE production by B cells in vitro in the presence of IL-4 and CD40 signaling. Preliminary data indicated that total spleen mononuclear cells from Balb/c Fas KO mice have the potential to enhance more than 10-fold the IgG1 and IgE production by B cells than those from Balb/c WT mice, whereas isolated spleen B cells from Balb/c Fas KO mice have no such activity (A. Fukuoka et al., unpublished data). These preliminary results suggest that spleen cells other than B cells play an important role in IgE production by B cells. We are now examining what type of spleen cells in Balb/c Fas KO mice is responsible for enhancing IgE production by B cells with the help of IL-4 and CD40 signaling.

By analyzing this unique mouse model that we have found Balb/c Fas KO mice, we hope to underline the mechanisms of the novel allergic inflammation in the near future.

Supplementary data
Supplementary data are available at International Immunology Online.

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