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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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Running Title
Fas knockout mice develop allergic inflammation

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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 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 autoantibody (ANA and anti-SSA), and a decreased life span. To our astonishment, Balb/c Fas KO mice spontaneously develop blepharitis with not only autoimmune inflammation with deposition of autoantibody but also allergic inflammation with infiltration of eosinophil and mast cell, 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.
Introduction

Apoptosis is a physiological cell suicide mechanism essential for normal embryonic development and the 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 mAb (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 autoantibodies and an immunopathology similar to that seen in human systemic lupus erythematosus (SLE) and other rheumatic diseases (10). The incidence and severity of lymphoproliferation, autoantibody production, and histopathological manifestation of systemic autoimmunity in lpr and Fas knockout (KO) mice are considerably affected by genetic background. MRL-lpr/lpr mice exhibit severe
autoimmune disease, marked hypergammaglobulinemia and 50% mortality by five months after birth probably due to renal failure and vasculitis, although C57BL/6-\textit{lpr/lpr} and C57BL/6 Fas KO mice do not show any severe defects without accumulation of \textit{lpr} cells and generally have a life of 2 years (11-15). Thus, background gene is very important for the determination of progression of autoimmune disease. On the other hand, Fas deficiency hardly induces allergic inflammation in mice even on the autoimmune prone background.

In this study, we generated Fas-deficient mice with the Balb/c background (Balb/c Fas KO mice). As reported elsewhere, C57BL/6 Fas KO mice mildly develop systemic autoimmune disease but hardly develop allergic inflammation (14, 15). To our astonishment, Balb/c Fas KO mice, in addition to their development of severe autoimmune disease, spontaneously manifest blepharitis with not only autoimmune inflammation with deposition of autoantibody but also allergic inflammation with infiltration of eosinophil and mast cell, and show the capacity to produce hyper serum levels of IgG1 and IgE. This is the first report indicating the novel function of Fas to suppress the outbreak of allergic inflammation.
Methods

Mice
Specific pathogen-free (SPF) Balb/c mice were obtained from CLEA Japan, Inc. Fas KO mice (14) had been maintained in our laboratory. The Fas KO mice on the Balb/c background were generated by backcrossing with Balb/c mice for more than 12 generations and maintained. MRL/MpJ-lpr/lpr mice were purchased from Japan SLC, Inc. All experiments in this study were performed according to the guidelines for animal treatment at the Institute of Laboratory Animals (Kyoto University).

Histological examination
Organs were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 4 µm and stained with hematoxylin and eosin, or Toluidine blue O.

ELISA
An ELISA kit for IL-4 and IFN-γ (R&D Systems), antinuclear antibodies (ANA) and anti-SSA Ab (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
Purified splenic CD4⁺ T cells from mice by MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) (10⁵ cells in 0.2 ml/well in 96-well plates) were cultured
with immobilized anti-CD3 and anti-CD28 Abs (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 ± s.d. 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 is 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. While 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 weeks 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 of 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). Thus, the blepharitis seen with
Balb/c Fas KO mice is associated with infiltration of allergic effector cells, mast cell and eosinophil. Taken together, Balb/c Fas KO mice manifest blepharitis with allergic inflammation, which has hardly observed in MRL-\textit{lpr/lpr} and C57BL/6 Fas KO mice.

\textit{Balb/c Fas KO mice showed hyper 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-\textit{lpr/lpr} mice, respectively. Furthermore, Balb/c Fas KO mice demonstrated significantly increased serum IgE levels, more than 70-fold and 3-fold, compared to WT and MRL-\textit{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-\textit{lpr/lpr} mice were reported to produce large amounts of auto-antibodies (13). Thus we next measured serum levels of ANA and anti-SSA Ab in Balb/c WT, Balb/c Fas KO and MRL-\textit{lpr/lpr} mice (Fig. 3C). As reported elsewhere, MRL-\textit{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.

**CD4\(^+\) T cells in the spleen of Balb/c Fas KO mice do not skew to Th2 cells**

Because production of IgG1 and IgE is dependent on the balance of IL-4 and IFN-\(\gamma\), we next analyzed whether Th2 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 either Th1 cells or Th2 cells.

**Balb/c Fas KO mice develop severe autoimmune inflammation and allergic inflammation**

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 lymphocytes infiltration was found principally around peribronchial areas. On the other hand, severe massive lymphocytes infiltrated around vessels and not around peribronchial areas in Balb/c Fas KO mice (Fig. 5A). In liver, while 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 develop much severer cell infiltration into lung and liver than MRL-\textit{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 were supposed to be comparable to lupus bands of human SLE, in eyelids of Balb/c Fas KO mice but not of BALB/c WT mice (Supplementary Figure 2). Thus, inflammation with deposition of autoantibody was induced in eyelid 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 level 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 Figure 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 hyper production of IgE. One mechanism is overproduction of Th2 cytokines including IL-4 from proliferating CD4+ T cells biased toward Th2 (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 to produce a large amount of IL-4 in or around the germinal center where the Ig 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 our found allergic inflammation, 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 of eosinophil and mast cell 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 eyelid induces itch and scratching behavior, which may induce IL-18 expression leading to expression of IgE (23) and infiltration of eosinophil 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 more IgG1 and IgE production by B cells than those from Balb/c WT mice, while 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 our found unique mouse model, Balb/c Fas KO mice, we hope to underline the mechanisms of the novel allergic inflammation in the near future.
**Abbreviations**

1, ANA: antinuclear antibodies;

2, FasL: Fas ligand;

3, HIES: hyper-IgE syndrome;

4, KO: knockout;

5, SLE: systemic lupus erythematosus;

6. WT: wild-type.
Funding

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Disclosures

The authors have no financial conflicts of interest.

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Figure Legends

**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.

**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 (×4), 100 µm (×20)

**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-<i>lpr/lpr</i> 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-<i>lpr/lpr</i> mice at 20 weeks.
Fig. 4.  Balb/c Fas KO T cells were not Th2-prone.

Total splenic CD4⁺ T cells from WT and BALB/c Fas KO mice were cultured with immobilized anti-CD3 and anti-CD28 (each 1 µg/ml for coating) for 2 days. Concentrations of IL-4 (A) and IFN-γ (B) in supernatants were measured by ELISA. Each symbol represents a single mouse. *; p<0.01, and **; P<0.005.

Fig. 5. Balb/c Fas KO mice developed severe autoimmune diseases.

HE staining of lung (A) and liver (B) sections from 20-week-old Balb/c WT, Balb/c Fas KO, and MRL-1pr/lpr mice. Scale bar, 500 µm (×4), 100 µm (×20)
Figure 1

A

Survival rate (%)

Weeks (n=9)

B

Incidence of blepharitis (%)

Weeks (n=23)

C

Image of a mouse.
Figure 2

A
Balb/c WT  Balb/c Fas KO

X4

X20

B
Balb/c WT  Balb/c Fas KO

X4

X20
Figure 3

A

IgG1 (mg/ml)

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IgE (µg/ml)

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B

IgG1 (mg/ml)

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IgE (µg/ml)

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C

Anti-SSA [IgG+IgM+IgA] (x 10^6 U/ml)

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ANA [IgG+IgM+IgA] (mg/ml)

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Figure 5

A

Balb/c WT  MRL / lpr  Balb/c Fas KO

X4

X20

B

Balb/c WT  MRL / lpr  Balb/c Fas KO

X4

X20
**Supplementary Figure Legend**

**Supplementary Figure 1.** Balb/c Fas KO mice developed allergic blepharitis with infiltration of eosinophil. 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. Arrows and arrowheads indicate neutrophil and eosinophil, respectively. Scale bar, 50 µm.

**Supplementary Figure 2.** Confocal microscopic examination of IgG deposit in the eyelid of Balb/c WT and Balb/c Fas KO mice. Frozen sections of eyelid (8 µm thick) freshly isolated from 40-week-old Balb/c WT and Balb/c Fas KO mice were fixed by acetone, incubated with Biotin-anti-mouse IgG (Biolegend), and then stained with streptavidin-FITC. The sections were examined under a confocal microscope Zeiss LSM 510 (Carl Zeiss). Images obtained by transmitted light were indicated by white (upper half of figures), and IgG was indicated by green. Scale bar, 50 µm.
Supplementary Figure 1

Eosinophil ↔ Neutrophil

Scale bar 50µm