

## **Identification of a novel type 2 innate immunocyte with ability to enhance IgE production**

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### **Running Title**

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## Abstract

Fas (CD95), a member of the TNF receptor super family, mediates apoptosis-inducing signals in its expressing cells, especially in self-reactive cells. We recently reported that Fas<sup>-/-</sup> mice with a BALB/c background (BALB/c Fas<sup>-/-</sup> mice) developed blepharitis with allergic inflammation that was accompanied by hyper IgE production. Here, we found a novel type of immunocyte in the spleen of BALB/c Fas<sup>-/-</sup> mice, which enhanced the production of IgE by B cells in the presence of IL-4 and CD40 signaling *in vitro*. The immunocyte did not express lineage markers, but expressed Thy-1 and Sca-1 just like recently identified type 2 innate lymphoid cells, such as natural helper (NH) cells and nuocytes. However, they did not express c-Kit, IL-7R and IL-33R (T1/ST2), important markers of type 2 innate lymphoid cells. Instead, our identified Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells expressed IL-18R, and secreted Th2 cytokines when co-cultured with B cells or stimulated with IL-18 and IL-2. Moreover, we found essentially the same type of cells in BALB/c wild-type mice as in BALB/c Fas<sup>-/-</sup> mice, which enhanced IgE production in contact with B cells *in vitro*. These cells from BALB/c wild-type mice expressed Fas, and were sensitive to Fas-mediated apoptosis. Collectively, the newly identified Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cell, which we designated a F-NH cell (Fas-expressing natural helper cell), is a novel type 2 innate immunocyte with activity to enhance IgE production from B cells with the help of IL-4 and CD40 signaling. F-NH cells may play an important role in the development of chronic allergic inflammation.

## Introduction

Fas (CD95), a member of the TNF receptor super family, plays an important role in mediating apoptosis-inducing signaling to maintain lymphocyte homeostasis, and to eliminate self-reactive cells (1-3). Mice with a loss of function mutation in Fas (*lpr*) and Fas<sup>-/-</sup> mice develop systemic autoimmune disease and lymphadenopathy. In mice homozygous for Fas-*lpr* and Fas<sup>-/-</sup>, genetic background determines the incidence and severity of histopathological manifestations of systemic autoimmunity; MRL-*lpr* mice and C57BL/6-*lpr* or -Fas<sup>-/-</sup> mice spontaneously develop severe and minimal autoimmune diseases, respectively (4-6). Furthermore, we recently reported a novel phenotype showing blepharitis with allergic inflammation in association with extremely high serum levels of IgE and IgG1 in Fas<sup>-/-</sup> mice with a BALB/c background (BALB/c Fas<sup>-/-</sup> mice) (7).

Chronic allergic inflammatory diseases are characterized by production of IL-4, IL-5, IL-9 and IL-13 from Th2 cells, production of IgE from B cells and recruitment of effector cells to sites of tissue inflammation (8). However, it has been also reported that other cells, such as follicular T cells, basophils and mast cells, are important sources of Th2 cytokines (9,10). In addition, type 2 innate lymphoid cells, including natural helper (NH) cells, nuocytes, innate type2 helper (Ih2) cells and multipotent progenitor type2 (MMP<sup>type2</sup>) cells, were also found to secrete Th2 cytokines when stimulated with IL-25 and IL-33, members of the IL-17

and IL-1 families, respectively (11-15). IL-33 is important for innate type mucosal immunity in the lung as well as gut, and relevant for airway inflammation (16-18).

IL-18, another member of the IL-1 family, is also known to be involved in Th2 responses. Although IL-18 induces production of IFN- $\gamma$  by Th1 cells and NK cells, especially in combination with IL-12 (19,20), IL-18 can increase IgE production by its administration into mice (21,22). In the presence of IL-3, furthermore, IL-18 causes basophils and mast cells to express high levels of IL-4, IL-13 and histamine (23). IL-18 also stimulates Th2 responses in both T cells and NKT cells when functions alone or together with IL-2 (24).

In this study, we identified a novel type 2 immunocyte with a Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> phenotype in spleen of BALB/c Fas<sup>-/-</sup> mice and found that the Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells enhanced IgE production from B cells *in vitro* in the presence of anti-CD40 mAb and IL-4. These cells were different from the previously reported type 2 innate lymphoid cells in terms of surface marker expression and sensitivity to IL-18. Our Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells produced Th2 cytokines during the culture with B cells or by the stimulation with IL-2 plus IL-18. Furthermore, similar cells in terms of both phenotype and function were also found in BALB/c wild-type (WT) mice in small numbers. Since they expressed Fas, the number and function of the Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells are suggested to be maintained in homeostasis by Fas-dependent apoptosis. Then, we designated the newly identified cell a F-NH cell (Fas-expressing natural helper cell). Taken together, F-NH cells, the number of which

increases greatly with a deficiency of Fas-dependent apoptosis, possibly induce hyper-production of IgE leading to allergic inflammation.

## Methods

### *Mice*

BALB/c WT mice were purchased from CLEA Japan. RAG2<sup>-/-</sup> mice and CD45.1 (Ly5.1) mice with the BALB/c background were kindly provided by Dr. S. Sakaguchi. C57BL/6 Fas<sup>-/-</sup> mice (4) were maintained in our laboratory. Fas<sup>-/-</sup> mice with the BALB/c background were generated as described previously (7). RAG2<sup>-/-</sup>Fas<sup>-/-</sup> mice with the BALB/c background were obtained by crossing Fas<sup>-/-</sup> and RAG2<sup>-/-</sup> mice. Mice were maintained in Specific Pathogen-Free conditions. All experiments in this study were performed according to the guidelines for animal treatment at the Institute of Laboratory Animals (Kyoto University).

For bone marrow transplantation, CD45.1 (Ly5.1) mice at 8 weeks old were irradiated lethally (8 Gy) and then inoculated with bone marrow cells ( $1 \times 10^7$ ) from sex- and age-matched BALB/c WT and Fas<sup>-/-</sup> mice (CD45.2). For the generation of mixed-bone marrow chimera, CD45.1 mice were irradiated at a sub-lethal dose (6 Gy) and reconstituted with bone marrow cells ( $1 \times 10^7$ ) from sex- and age-matched RAG2<sup>-/-</sup> Fas<sup>+/+</sup> and RAG2<sup>-/-</sup>Fas<sup>-/-</sup> mice (CD45.2).

### *Antibodies and reagents*

Phycoerythrin (PE)- or biotin-conjugated mAbs specific for mouse B220 (RA3-6B2), CD3 $\epsilon$

(145-2C11), CD11c (HL3), CD49b (DX5), Gr-1 (RB6-8C5), CD4 (GK1.5), CD8 $\alpha$  (53-6.7), CD11b (M1/70), and Siglec-F (E50-2440) were used as lineage markers. Other mAbs used in this study were specific for mouse Thy-1 (30-H12), Sca-1 (E13-161), c-Kit (2B8), CD25 (PC61), MHC class II (ASM-32.1), CD44 (IM7), CD69 (H1.2F3), CD127 (B12-1), ICOS (RMMA-1), and Fas (Jo2). These antibodies were purchased from eBioscience, Biolegend and BD Bioscience. FITC-conjugated T1/ST2 and IL-18R $\alpha$  were purchased from MD bioscience and R&D systems, respectively. FITC-conjugated streptavidin, purchased from Biolegend, was used to reveal the staining with biotinylated mAbs. Dead cells were removed by TO-PRO-3 iodide (Invitrogen) or 7-aminoactinomycin D (BD Bioscience). FACS samples were run on a FACS callibur (BD Biosciences) or FACS canto II (BD Biosciences), and analyzed using FlowJo software (version 7.6.1, Tree Star).

#### *Detection of serum levels of IgE and IgG1*

Serum samples were collected from 20-week-old WT and Fas<sup>-/-</sup> mice or recipient mice 20 weeks after bone marrow transplantation. The levels of IgE and IgG1 in sera were measured by ELISA as described previously (25).

#### *Cell preparation and culture*

For the preparation of B cells from WT and Fas<sup>-/-</sup> mice, total spleen cells were stained with

PE-conjugated anti-B220 and APC-conjugated anti-CD3, and then B220<sup>+</sup>CD3<sup>-</sup> cells were sorted with a FACS Aria I (BD Bioscience). To collect B220<sup>-</sup>CD3<sup>+</sup> T cells, B220<sup>+</sup>CD3<sup>+</sup> cells and B220<sup>-</sup>CD3<sup>-</sup> cells from Fas<sup>-/-</sup> mice, spleen cells were stained with PE-conjugated anti-B220 and APC-conjugated anti-CD3 and then sorted on a FACS Aria I. To purify Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells, Lin<sup>-</sup> cells were enriched by negative selection on an AutoMACS (Miltenyi Biotec) with PE-conjugated lineage markers and anti-PE magnetic beads (Miltenyi Biotec). Subsequently, enriched Lin<sup>-</sup> cells were stained with mAbs for Thy-1 and Sca-1, and sorted on a FACS Aria I. B cells from WT mice were enriched using the AutoMACS with magnetic beads conjugated with a mAb for B220 (Miltenyi Biotec).

Various cells from Fas<sup>-/-</sup> spleen ( $5 \times 10^4$  cells/well) and B cells from WT mice ( $5 \times 10^4$  cells/well) were mixed and seeded into 96-well flat bottomed tissue culture plates in RPMI-1640 medium (sigma) supplemented with 10% (vol/vol) fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine (2 mM) and  $\beta$ -mercaptoethanol (50  $\mu$ M) together with anti-CD40 mAb (1  $\mu$ g/ml; R&D systems) plus IL-4 (0 or 50 ng/ml; R&D systems) for 7 days. For cytokine production, Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice ( $5 \times 10^4$  cells/well) were stimulated with IL-2 (100 pM) and IL-18 (100 ng/ml) for 7 days. To evaluate the cytokine activity, WT B cells were stimulated with anti-CD40 mAb plus IL-4 in the presence of the 4-times diluted culture supernatant for 7 days. IgE and IgG1 production was measured by ELISA.



### *Intracellular cytokine staining*

Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells were co-cultured with WT B cells with anti-CD40 mAb (1 µg/ml) plus IL-4 (0 or 50 ng/ml). After 3 days culture, cells were treated with brefeldin A (Biolegend), and incubated for 4h at 37°C. Cells were stained with FITC-conjugated anti-B220 for 30 min. After that, the sample was fixed and permeabilized by PermWash Buffer (BD Biosciences). PE-conjugated cytokine antibodies were added and incubated for 30 min at room temperature. FACS Analysis was performed using FACS cant II. All antibodies to cytokines were obtained from Biolegend and eBioscience.

### *Detection of cytokines by ELISA*

For the measurement of IL-18 in serum, serum samples were collected from 20-week-old WT and Fas<sup>-/-</sup> mice, and analyzed with an ELISA kit (MBL). For the measurement of IL-5 and IL-13 in the culture supernatant, Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice (5×10<sup>4</sup> cells/well) were stimulated with IL-2 (100 pM) and IL-18 (100 ng/ml) for 7 days. Recombinant mouse IL-2 and IL-18 were purchased from R&D systems and MBL, respectively. After that, the supernatant was collected and measured by ELISA (R&D system). ELISAs were performed according to the manufacturer's directions.

### *RT-PCR*

Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice were stimulated with IL-2 (100 pM) and IL-18 (100 ng/ml) for 24 h. Total RNAs were prepared using NucleoSpin RNA XS (TAKARA), and RT-PCR was carried out using a ThermoScript RT-PCR system (Invitrogen) according to the manufacturer's instructions. Primers used in this study were as follows: forward, 5'-GGTGACAACCACGGCCTTCCC-3', and reverse, 5'-GCCACTCCTTCTGTGACTCCAGC-3' for IL-6; and forward, 5'-GCAGCACCA CATGGG GCATCA-3', and reverse, 5'- TGCCTGCCATGGTCTGGTTGC-3' for IL-9.

### *Statistical analysis*

Data are expressed as the mean  $\pm$  s.d. in each sample. Statistical significance was determined by the two-tailed Student's *t*-test. All experiments were performed two or three times and representative results are shown.

## Results

### ***BALB/c Fas<sup>-/-</sup> mice develop blepharitis with allergic inflammation in association with hyper-production of IgE.***

Recently, we found that BALB/c Fas<sup>-/-</sup> mice developed allergic inflammation around the eyelid, allergic blepharitis, which has not been observed in Fas<sup>-/-</sup> mice with the C57BL/6 background (C57BL/6 Fas<sup>-/-</sup> mice) as well as MRL-*lpr/lpr* and BALB/c WT mice (7). In that study, a significant elevation of serum IgE and IgG1 levels detected at as early as 6 weeks of age and allergic blepharitis became obvious from 15 weeks with about 85% of the mice suffering from severe allergic blepharitis at age 35 weeks. Consistent with the previous results, serum levels of IgE and IgG1 were remarkably higher in BALB/c Fas<sup>-/-</sup> mice than WT mice at 20 weeks of age (Fig. 1A), as well as in MRL-*lpr/lpr* mice or C57BL/6 Fas<sup>-/-</sup> mice (7). Thus, hyper IgE production preceded the allergic inflammation in BALB/c Fas<sup>-/-</sup> mice.

To clarify whether the high serum levels of IgE and IgG1 in Fas<sup>-/-</sup> mice were due to functions of Fas-deficient hematopoietic cells, we generated bone marrow chimera mice by transferring bone marrow cells from either WT or Fas<sup>-/-</sup> mice (Ly5.2) into lethally irradiated congenic recipient WT mice (Ly5.1). At 20 weeks after the transplantation, serum concentrations of IgE and IgG1 were significantly higher in mice receiving Fas<sup>-/-</sup> bone

marrow cells than in control chimera mice receiving WT bone marrow cells, indicating that hematopoietic cells from Fas<sup>-/-</sup> mice contributed to the hyper-production of IgE and IgG1 (Fig. 1B). When total spleen cells from WT and Fas<sup>-/-</sup> mice were cultured with anti-CD40 mAb plus IL-4, Fas<sup>-/-</sup> cells produced much higher levels of IgE and IgG1 than WT cells (Fig. 1C). However, splenic B cells (B220<sup>+</sup>CD3<sup>-</sup> cells) from WT and Fas<sup>-/-</sup> mice produced comparable levels of IgE and IgG1 during the culture (Fig. 1D). These results indicated that Fas<sup>-/-</sup> hematopoietic cells, but not Fas<sup>-/-</sup> B cells, are responsible for hyper-production of IgE and IgG1 *in vivo*.

***B220<sup>-</sup>CD3<sup>-</sup> cells from Fas<sup>-/-</sup> mice enhance IgE production from WT B cells.***

BALB/c Fas<sup>-/-</sup> mice and their spleen cells were shown to be able to produce much larger amounts of IgE and IgG1 *in vivo* and *in vitro*, respectively. To seek what type of splenic cells from Fas<sup>-/-</sup> mice enhanced IgE production by B cells, Fas<sup>-/-</sup> splenic cells were fractionated into B220<sup>+</sup>CD3<sup>-</sup> cells (B cells), B220<sup>-</sup>CD3<sup>+</sup> cells (T cells), B220<sup>-</sup>CD3<sup>-</sup> cells (double-negative cells: DN cells) and B220<sup>+</sup>CD3<sup>+</sup> cells (double-positive cells: DP cells) (Fig. 2A). Fas<sup>-/-</sup> DP cells include abnormal T cells (*lpr* cells) that can survive owing to a deficiency of Fas-mediated apoptosis (4,26,27). Isolated T, DN and DP cells from Fas<sup>-/-</sup> spleen were co-cultured with WT B cells in the presence of anti-CD40 mAb plus IL-4 for 7 days, and the amounts of secreted

IgE in the culture medium were quantitated. In the result, DN cells were able to significantly enhance IgE production by WT B cells (Fig. 2B). In contrast, DN cells from C57BL/6 Fas<sup>-/-</sup> mice did not enhance IgE production from WT B cells (Supplementary Figure 1 is available at International Immunology Online).

To confirm that the Fas<sup>-/-</sup> DN cells enhancing IgE production were neither B nor T cells, we established BALB/c RAG2<sup>-/-</sup>Fas<sup>-/-</sup> mice completely lacking T and B cells (28). RAG2<sup>-/-</sup>Fas<sup>+/+</sup> or RAG2<sup>-/-</sup>Fas<sup>-/-</sup> splenic cells were co-cultured with WT B cells in the presence of anti-CD40 mAb plus IL-4. Although both splenic cells enhanced IgE production from WT B cells, those from RAG2<sup>-/-</sup>Fas<sup>-/-</sup> mice had a significantly greater effect (Fig. 2C), revealing that non-T/non-B DN cells in Fas<sup>-/-</sup> mice can enhance IgE production from B cells with the help of IL-4 and CD40 signaling *in vitro*.

We also generated mixed-bone marrow chimera mice between WT mice (recipient) and RAG2<sup>-/-</sup>Fas<sup>+/+</sup> or RAG2<sup>-/-</sup>Fas<sup>-/-</sup> mice (donor). Bone marrow cells from RAG2<sup>-/-</sup>Fas<sup>+/+</sup> or RAG2<sup>-/-</sup>Fas<sup>-/-</sup> mice were transferred to sub-lethally irradiated (6 Gy) WT recipient mice (Ly5.1), and serum concentrations of IgE were quantitated 20 weeks later (Supplementary Figure 2A and B are available at *International Immunology* Online). The serum levels of IgE were higher in chimera mice between WT (recipient) and RAG2<sup>-/-</sup>Fas<sup>-/-</sup> (donor) mice than control chimera mice between WT and RAG2<sup>-/-</sup>Fas<sup>+/+</sup> mice, indicating that non-T/non-B cells from Fas<sup>-/-</sup> mice are responsible for *in vivo* hyper production of IgE in the mixed-bone

marrow chimera mice. All the results suggest that non-T/non-B DN cells are responsible for hyper production of IgE in BALB/c Fas<sup>-/-</sup> mice *in vivo*.

Prior to identifying the cells responsible for augmenting spontaneous IgE and IgG1 production, we compared the proportion of NK cells, dendritic cells, macrophages, and granulocytes among DN cells between WT and Fas<sup>-/-</sup> mice. Flow cytometry analysis showed the proportions of these cells in spleen to be comparable between Fas<sup>-/-</sup> and WT mice (Supplementary Figure 3 is available at *International Immunology Online*).

***Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> spleen enhance IgE production by B cells.***

Recently, type 2 innate lymphoid cells, such as NH cells, nuocytes, Ih2 cells and MMP<sup>type2</sup> cells were identified to be able to produce Th2 cytokines such as IL-5 and IL-13 (11-15).

These type 2 innate lymphoid cells contribute to protection against helminth infection, and are considered to be involved in allergic diseases such as atopy and asthma (29). However, it has not been clarified whether these innate cells enhance IgE production by direct interaction with B cells. Type 2 innate lymphoid cells were reported not to express lineage markers, but to express hematopoietic stem cell markers, such as Thy-1, c-Kit and Sca-1 (30,31), although the expression levels of these markers varied among individual cell types. Therefore, we then analyzed whether the numbers of these type 2 innate lymphoid cells increased in Fas<sup>-/-</sup> splenic

cells and these cells contributed to enhance IgE production. By flow cytometry analysis, we found that the number of Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells increased about 5-times more in spleens of Fas<sup>-/-</sup> mice than those of WT mice (Fig. 3A). In addition, the number of the Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells increased about 3-times more in spleens of RAG2<sup>-/-</sup>Fas<sup>-/-</sup> mice than those of RAG2<sup>-/-</sup>Fas<sup>+/+</sup> mice (data not shown).

To confirm that Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells actually enhance IgE production, we purified them from Fas<sup>-/-</sup> mice, and co-cultured them with WT B cells in the presence of anti-CD40 mAb plus IL-4 for 7 days. Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice were revealed to markedly enhance IgE production (Fig. 3B). In addition, they also enhanced IgE and IgG1 production by Fas<sup>-/-</sup> B cells as well as WT B cells (Fig. 3C). In contrast, neither Lin<sup>-</sup>Thy-1<sup>-</sup>Sca-1<sup>-</sup> cells nor Lin<sup>-</sup>Thy-1<sup>-</sup>Sca-1<sup>+</sup> cells enhanced IgE production (Supplementary Figure 4A is available at *International Immunology* Online).

Next, we examined whether cell-to-cell contact between B cells and Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells was necessary to promote IgE production using the Transwell culture system. When Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells and B cells were separated by a Transwell filter, Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells were incapable of enhancing IgE production (Supplementary Figure 4B is available at *International Immunology* Online). When Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells were co-cultured with WT B cells, they produced Th2 cytokines, such as IL-5 and IL-13, which were detected by intracellular cytokine staining (Supplementary Figure 4C is available at *International*

*Immunology* Online). All the results indicate that Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> spleen possess activity to effectively enhance IgE production from B cells through direct cell-to-cell interaction between Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells and B cells.

***Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice are different from type 2 innate lymphoid cells.***

We performed flow cytometry analysis to examine whether our identified Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice express important surface markers of type 2 innate lymphoid cells (Fig. 4). Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice expressed neither c-Kit nor T1/ST2 (IL-33R). A part of Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice expressed CD44 and CD69, known as activation markers, and CD25 (IL-2R $\alpha$ ) and MHC class II. Interestingly, CD127 (IL-7R) and ICOS, specific markers of some sorts of type 2 innate lymphoid cells, were not expressed on Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice. Notably, we found that Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice expressed IL-18R $\alpha$  and responded to IL-18 (Fig. 4 and 5), whereas NH cells did not respond to IL-18 (K. Moro and S. Koyasu, personal information). These results suggest that the expression pattern of cell surface markers on Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells conforms to none of the pattern for type 2 innate lymphoid cells (See Discussion). Taken together, Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice reveal several different features from previously reported type 2 innate lymphoid cells.



***Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells expand and produce cytokines in response to IL-2 plus IL-18.***

To investigate the activation mechanisms of the Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice, we focused on the function of IL-18, because we found that serum levels of IL-18 were significantly higher in Fas<sup>-/-</sup> mice than WT mice at 20 weeks of age (Fig. 5A), and that the Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells expressed IL-18R $\alpha$  as shown in Figure 4. When Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells were cultured in the presence of IL-2 plus IL-18, they proliferated vigorously within 3 days up to nearly 10-fold the starting number (Fig. 5B), but they did not survive in the absence of these exogenous cytokines. After cultivation with IL-2 and IL-18, the expression pattern of lineage markers was not changed (Fig. 5C), suggesting that the Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells do not differentiate into other types of hematopoietic cells after stimulation with IL-2 plus IL-18.

During the culture for 7 days, Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells secreted significantly large amounts of IL-5 and IL-13 into their culture supernatants only in the presence of IL-2 plus IL-18, but not IL-2 alone (Fig. 5D). Furthermore, expression levels of IL-6 and IL-9 mRNA in Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells significantly increased when cultured with IL-2 plus IL-18 for 24 h (Fig. 5E), although there was no sign of IL-4 production (data not shown). We then compared the expression levels of Th2 cytokines mRNA between Th2 cells and Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells

by real-time quantitative RT-PCR (qRT-PCR). Expression levels of IL-5, IL-6, IL-9 and IL-13 in Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells were almost comparable to, but a little lower than, those in Th2 cells (Supplementary Figure 5 is available at *International Immunology* Online). Taken together, it may be feasible that Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells have the potential to produce cytokines supporting IgE production by B cells.

***Soluble factors from Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells stimulated with IL-2 plus IL-18 enhance IgE production from B cells.***

We then examined whether soluble factors derived from the proliferating Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells by IL-2 plus IL-18 enhance IgE and IgG1 production from WT B cells in concert with anti-CD40 mAb plus IL-4 for 7 days. In fact, B cells cultured with the culture supernatant derived from Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells stimulated with IL-2 plus IL-18 produced significantly higher levels of IgE and IgG1, compared to those cultured with IL-2 alone or without IL-2 and IL-18 (Supplementary Figure 6A is available at *International Immunology* Online). Of note, IgE production by B cells was not enhanced by direct stimulation with IL-2 and IL-18 in the presence of anti-CD40 mAb plus IL-4 (Supplementary Figure 6B is available at *International Immunology* Online). These results indicate that Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells can not only proliferate but also release soluble factors in response to the stimulation with IL-2 plus

IL-18 to augment B cell IgE production, even in the absence of cognate interactions with B cells.

***Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells in WT spleen can also enhance IgE production from B cells***

Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells were also found in spleen of WT mice, though at much lower numbers than in Fas<sup>-/-</sup> spleen as shown in Figure 3A. Because Fas is important to the removal of self-reactive and activated immunocytes by apoptosis (4,26,27), we hypothesized that Fas may play a role to eliminate moderate number of Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells under steady state conditions. As expected, flow cytometry analysis confirmed the expression of Fas on the Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells in WT spleen (Fig. 6A). To examine whether WT Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells are sensitive to Fas-dependent apoptosis, we stimulated the cells with agonistic anti-Fas mAb *in vitro*. In the result, cell death was induced in about 80 % of WT Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells after stimulation with anti-Fas mAb, and apoptosis-specific chromosomal fragmentation and chromosomal condensation were induced (Supplementary Figure 7 is available at *International Immunology* Online). Taken together, the Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells would be constantly but moderately removed by Fas-dependent apoptosis in WT mice. Like the Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells in Fas<sup>-/-</sup> mice, Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells in WT mice were shown to express IL-18R $\alpha$  (Fig. 6A). Expression patterns of other surface markers in WT

Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells (Supplementary Figure 8 is available at *International Immunology* Online) were similar to those of Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice (Figure 4). The ability of WT Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells to enhance IgE production was also examined by culturing with WT B cells in the presence of IL-4 plus anti-CD40 mAb for 7 days (Fig. 6B). Compared with the Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice, those from WT mice manifested moderately lower activity to enhance IgE production, but the enhancing activity of WT Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells was significant. Thus, Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from WT mice as well as Fas<sup>-/-</sup> mice can enhance IgE production from B cells in the presence of IL-4 plus anti-CD40 mAb. Taken together, Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells in WT BALB/c mice would belong to the same cell lineage as Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells found in Fas<sup>-/-</sup> BALB/c mice.

## Discussion

Here, we found a novel type of Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells in BALB/c Fas<sup>-/-</sup> and WT mice, which enhanced IgE production by B cells when co-cultured with B cells in the presence of anti-CD40 mAb plus IL-4 *in vitro*. To our knowledge, our found Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells appeared similar to previously reported type 2 innate lymphoid cells, such as NH cells, nuocytes, MMP<sup>type2</sup> cells and Ih2 cells (11-15), but there exist some differences. First, the tissue distribution is different between our identified cells and type 2 innate lymphoid cells. Type 2 innate lymphoid cells were found in mesenteric lymph nodes (MLN) or FALC (fat-associated lymphoid cluster) mainly around the gut (11-15), while the Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells existed in the spleen. Second, some of the surface markers expressed on these two types of cells are different (11-15) (Fig. 4). While Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells highly express Sca-1, Thy-1 and CD69, Ih2 cells and MMP<sup>type2</sup> cells do not express Sca-1, and Thy-1 and CD69, respectively. Although expression levels of Thy-1 and Sca-1 on Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells are similar to those on NH cells and nuocytes, expression levels of c-Kit, T1/ST2, CD127 and ICOS are significantly different: it has been reported that NH cells are c-Kit<sup>+</sup>T1/ST2<sup>+</sup>CD127<sup>+</sup> and nuocytes are c-Kit<sup>+/-</sup>T1/ST2<sup>+/-</sup>CD127<sup>+</sup> ICOS<sup>+</sup> (11-15), while Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from BALB/c Fas<sup>-/-</sup> spleen are c-Kit<sup>-</sup>T1/ST2<sup>+/-</sup>CD127<sup>-</sup> ICOS<sup>-</sup>. Thus, Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice do not show completely the same surface markers as previously reported type 2

innate lymphoid cells. Third, our identified Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells produce IL-5, IL-6, IL-9 and IL-13 in response to stimulation with IL-18 plus IL-2. We found that Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells express IL-18R $\alpha$ , and can respond to IL-18, whereas type 2 innate lymphoid cells were reported to respond to IL-33 and IL-25 (11-15). Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells do not belong to the cell types responsive to IL-18, such as T cells, NKT cells, basophils and mast cells, which have been shown to secrete IL-4 when stimulated with IL-18 together with IL-2 or IL-3 (23,32). Based on these distinguishable features, we concluded that the newly identified Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells in BALB/c mice are a novel type of immunocyte. We designated them F-NH cells (Fas-expressing natural helper cells), because previously reported NH cells do not express Fas (K. Moro and S. Koyasu, personal information).

F-NH cells were capable of encouraging B cells to produce IgE *in vitro* in two ways, one was dependent on direct cell-to-cell interaction and the other was mediated through the secretion of soluble factors. In both cases, exogenous IL-4 is essential for class-switch recombination (33,34), while F-NH cells were unable to produce IL-4. In this respect, F-NH cells may not be primarily responsible for evoking allergic inflammation. On the other hand, we recently found another type of cell, which can produce a large amount of IL-4 in Fas<sup>-/-</sup> mice (S. Futatsugi-Yumikura *et al.*, unpublished data). Therefore, we anticipate that such IL-4-producing cells act synergistically on B cells leading to the hyper-production of IgE in Fas<sup>-/-</sup> mice.

Spleen cells from RAG2<sup>-/-</sup> Fas<sup>+/+</sup> mice and F-NH cells from WT spleen could also enhance IgE production from B cells, but they seemed to be less active than spleen cells from RAG2<sup>-/-</sup>Fas<sup>-/-</sup> mice and F-NH cells from Fas<sup>-/-</sup> spleen, respectively. We speculate that F-NH cells from RAG2<sup>-/-</sup> Fas<sup>+/+</sup> and WT mice possess comparably weak activity, because they tend to be eliminated by Fas-mediated apoptosis through the interaction of Fas-expressing F-NH cells (Fig. 6A) and FasL-expressing activated B cells (35). Thus, the number and activity of F-NH cells to enhance IgE production might be tightly regulated by Fas system. Although WT F-NH cells are quite few in number *in vivo*, they can enhance IgE production when co-cultured with equal numbers of B cells *in vitro*. WT F-NH cells may be able to induce hyper-production of IgE and allergic diseases under certain conditions such as inhibition of apoptosis in F-NH cells and/or excessive growth of F-NH cells.

While F-NH cells were mainly found in spleen, the number of other Type 2 innate lymphoid cells, such as NH cells, nuocytes, MMP<sup>type2</sup> cells and Ih2 cells, has been shown to be very few in spleen (11-15). This may be the reason why other types of Type 2 innate lymphoid cells were not found in the fraction of F-NH cells in BALB/c mice. In C57BL/6 mice, other type of Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells than N-FH cells were found in spleen, and the number of Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells in C57BL/6 Fas<sup>-/-</sup> mice were higher than that in BALB/c Fas<sup>-/-</sup> mice (Supplementary Figure 9A is available at *International Immunology* Online). We anticipate that most of the Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from C57BL/6 mice are different from

F-NH cells found in BALB/c mice, because 1) expression patterns of IL-18R $\alpha$ , CD25 and CD44 are different, 2) C57BL/6 WT Lin-Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells do not express Fas (Supplementary Figure 9B is available at *International Immunology* Online), and 3) DN cells from C57BL/6 Fas<sup>-/-</sup> mice containing Lin-Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells did not enhance IgG1 and IgE production from B cells (Supplementary Figure 1 is available at *International Immunology* Online). Thus, the generation of F-NH cells seems to be co-related well with the outbreak of hyper IgE production-associated allergic inflammation.

There are two different types of allergic inflammation, immediate-type and chronic inflammation (36). The immediate-type allergy is induced within minutes after exposure to allergen such as in pollinosis. On the other hand, chronic allergy such as asthma and atopic dermatitis can persist for days to years. It has been generally considered that allergen-specific IgE is involved in immediate-type allergy, and chronic allergic inflammation is mediated by T cells (36). However, IgE was reported to also contribute to development of chronic allergic inflammation under certain conditions independent of T cells (37). In addition, high serum IgE levels in atopic patients could simply reflect the increased expression of Th2 cytokines often observed in allergic conditions. In addition, treatment with humanized anti-IgE Ab was reported to reduce severity of symptoms not only in allergic rhinitis but also in asthma (38,39). These reports indicated that suppression of IgE production is important to improve chronic allergic disorders. Because our newly identified F-NH cells play a role in enhancing IgE



production *in vitro*, they may contribute to several types of allergic diseases including not only allergic blepharitis but also other allergies such as atopic dermatitis and asthma. By DNA array analysis, we have identified several candidate molecules expressed specifically on F-NH cells. We are now trying to generate specific mAbs for these molecules. It will be important to clarify whether depletion or blockage of these cells by treatment with these mAbs is effective to improve allergic diseases.

It has been unclear whether allergic diseases are developed by the functions of F-NH cells. We are now conducting further studies to elucidate the role of F-NH cells in the outbreak of allergic diseases with hyper-production of IgE, which may lead to novel strategies to improve allergic disease.

## **Abbreviations**

- 1, DN: double negative
- 2, DP: double positive
- 3, F-NH: Fas-expressing natural helper
- 4, Ih2: innate type2 helper
- 5, Lin: lineage
- 6, NH: natural helper
- 7, MMP<sup>type2</sup>: multipotent progenitor type2
- 8, qRT-PCR: real-time quantitative RT-PCR
- 9, WT: wild-type.

## **Supplementary data**

Supplementary data are available at *International Immunology* Online.

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The authors have no financial conflicts of interest.

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## References

- 1 Nagata, S. 1997. Apoptosis by Death Factor. *Cell* 88:355.
- 2 Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S.-I., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* 66:233.
- 3 Yonehara, S. 2002. Death receptor Fas and autoimmune disease: from the original generation to therapeutic application of agonistic anti-Fas monoclonal antibody. *Cytokine Growth Factor Rev* 13:393.
- 4 Senju, S., Negishi, I., Motoyama, N., Wang, F., Nakayama, K.-i., Nakayama, K., Lucas, P. J., Hatakeyama, S., Zhang, Q., Yonehara, S., and Loh, D. Y. 1996. Functional significance of the Fas molecule in naive lymphocytes. *Int Immunol* 8:423.
- 5 Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A., and Nagata, S. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 356:314.
- 6 Vidal, S., Kono, D. H., and Theofilopoulos, A. N. 1998. Loci predisposing to autoimmunity in MRL-Fas lpr and C57BL/6-Faslpr mice. *J Clin Invest* 101:696.
- 7 Takahashi, S., Futatsugi-Yumikura, S., Fukuoka, A., Yoshimoto, T., Nakanishi, K., and Yonehara, S. Fas-deficiency in mice with the Balb/c background induces blepharitis with allergic inflammation and hyper IgE production in conjunction with severe autoimmune disease. *Int Immunol* in press.
- 8 Yazdanbakhsh, M., van den Biggelaar, A., and Maizels, R. M. 2001. Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease. *Trends Immunol* 22:372.
- 9 Gessner, A., Mohrs, K., and Mohrs, M. 2005. Mast Cells, Basophils, and Eosinophils Acquire Constitutive IL-4 and IL-13 Transcripts during Lineage Differentiation That Are Sufficient for Rapid Cytokine Production. *J Immunol* 174:1063.
- 10 Reinhardt, R. L., Liang, H.-E., and Locksley, R. M. 2009. Cytokine-secreting

- follicular T cells shape the antibody repertoire. *Nat Immunol* 10:385.
- 11 Koyasu, S. and Moro, K. 2011. Type 2 innate immune responses and the natural helper cell. *Immunology* 132:475.
  - 12 Moro, K., Yamada, T., Tanabe, M., Takeuchi, T., Ikawa, T., Kawamoto, H., Furusawa, J.-i., Ohtani, M., Fujii, H., and Koyasu, S. 2010. Innate production of TH2 cytokines by adipose tissue-associated c-Kit+Sca-1+ lymphoid cells. *Nature* 463:540.
  - 13 Neill, D. R., Wong, S. H., Bellosi, A., Flynn, R. J., Daly, M., Langford, T. K. A., Bucks, C., Kane, C. M., Fallon, P. G., Pannell, R., Jolin, H. E., and McKenzie, A. N. J. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464:1367.
  - 14 Price, A. E., Liang, H.-E., Sullivan, B. M., Reinhardt, R. L., Eisley, C. J., Erle, D. J., and Locksley, R. M. 2010. Systemically dispersed innate IL-13–expressing cells in type 2 immunity. *Proc Natl Acad Sci U S A* 107:11489.
  - 15 Saenz, S. A., Siracusa, M. C., Perrigoue, J. G., Spencer, S. P., Urban Jr, J. F., Tocker, J. E., Budelsky, A. L., Kleinschek, M. A., Kastelein, R. A., Kambayashi, T., Bhandoola, A., and Artis, D. 2010. IL25 elicits a multipotent progenitor cell population that promotes TH2 cytokine responses. *Nature* 464:1362.
  - 16 Schmitz, J., Owyang, A., Oldham, E., Song, Y., Murphy, E., McClanahan, T. K., Zurawski, G., Moshrefi, M., Qin, J., Li, X., Gorman, D. M., Bazan, J. F., and Kastelein, R. A. 2005. IL-33, an Interleukin-1-like Cytokine that Signals via the IL-1 Receptor-Related Protein ST2 and Induces T Helper Type 2-Associated Cytokines. *Immunity* 23:479.
  - 17 Humphreys, N. E., Xu, D., Hepworth, M. R., Liew, F. Y., and Grencis, R. K. 2008. IL-33, a Potent Inducer of Adaptive Immunity to Intestinal Nematodes. *J Immunol* 180:2443.
  - 18 Yasuda, K., Muto, T., Kawagoe, T., Matsumoto, M., Sasaki, Y., Matsushita, K., Taki, Y., Futatsugi-Yumikura, S., Tsutsui, H., Ishii, K. J., Yoshimoto, T., Akira, S., and Nakanishi, K. 2012. Contribution of IL-33–activated type II innate lymphoid cells to pulmonary eosinophilia in intestinal nematode-infected mice. *Proc Natl Acad Sci U S A* 109:3451.

- 19 Kohno, K., Kataoka, J., Ohtsuki, T., Suemoto, Y., Okamoto, I., Usui, M., Ikeda, M., and Kurimoto, M. 1997. IFN-gamma-inducing factor (IGIF) is a costimulatory factor on the activation of Th1 but not Th2 cells and exerts its effect independently of IL-12. *J Immunol* 158:1541.
- 20 Gu, Y., Kuida, K., Tsutsui, H., Ku, G., Hsiao, K., Fleming, M. A., Hayashi, N., Higashino, K., Okamura, H., Nakanishi, K., Kurimoto, M., Tanimoto, T., Flavell, R. A., Sato, V., Harding, M. W., Livingston, D. J., and Su, M. S. S. 1997. Activation of Interferon- $\gamma$  Inducing Factor Mediated by Interleukin-1 $\beta$  Converting Enzyme. *Science* 275:206.
- 21 Yoshimoto, T., Mizutani, H., Tsutsui, H., Noben-Trauth, N., Yamanaka, K.-i., Tanaka, M., Izumi, S., Okamura, H., Paul, W. E., and Nakanishi, K. 2000. IL-18 induction of IgE: dependence on CD4<sup>+</sup> T cells, IL-4 and STAT6. *Nat Immunol* 1:132.
- 22 Wild, J. S., Sigounas, A., Sur, N., Siddiqui, M. S., Alam, R., Kurimoto, M., and Sur, S. 2000. IFN- $\gamma$ -Inducing Factor (IL-18) Increases Allergic Sensitization, Serum IgE, Th2 Cytokines, and Airway Eosinophilia in a Mouse Model of Allergic Asthma. *J Immunol* 164:2701.
- 23 Yoshimoto, T., Tsutsui, H., Tominaga, K., Hoshino, K., Okamura, H., Akira, S., Paul, W. E., and Nakanishi, K. 1999. IL-18, although antiallergic when administered with IL-12, stimulates IL-4 and histamine release by basophils. *Proc Natl Acad Sci U S A* 96:13962.
- 24 Nakanishi, K., Yoshimoto, T., Tsutsui, H., and Okamura, H. 2001. INTERLEUKIN-18 REGULATES BOTH TH1 AND TH2 RESPONSES. *Annu Rev Immunol* 19:423.
- 25 Yoshimoto, T., Bendelac, A., Hu-Li, J., and Paul, W. E. 1995. Defective IgE production by SJL mice is linked to the absence of CD4<sup>+</sup>, NK1.1<sup>+</sup> T cells that promptly produce interleukin 4. *Proc Natl Acad Sci U S A* 92:11931.
- 26 Adachi, M., Suematsu, S., Suda, T., Watanabe, D., Fukuyama, H., Ogasawara, J., Tanaka, T., Yoshida, N., and Nagata, S. 1996. Enhanced and accelerated lymphoproliferation in Fas-null mice. *Proc Natl Acad Sci U S A* 93:2131.
- 27 Krueger, A., Fas, S. C., Baumann, S., and Krammer, P. H. 2003. The role of CD95 in the regulation of peripheral T-cell apoptosis. *Immunol Rev* 193:58.

- 28 Shinkai, Y., Rathbun, G., Lam, K. P., Oltz, E. M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A. M., and Alt, F. W. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68:855.
- 29 Halim, Timotheus Y. F., Krauß, Ramona H., Sun, Ann C., and Takei, F. 2012. Lung Natural Helper Cells Are a Critical Source of Th2 Cell-Type Cytokines in Protease Allergen-Induced Airway Inflammation. *Immunity* 36:451.
- 30 Wilson, A., Laurenti, E., Oser, G., van der Wath, R. C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C. F., Eshkind, L., Bockamp, E., Lió, P., MacDonald, H. R., and Trumpp, A. 2008. Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. *Cell* 135:1118.
- 31 Wright, D. E., Wagers, A. J., Gulati, A. P., Johnson, F. L., and Weissman, I. L. 2001. Physiological Migration of Hematopoietic Stem and Progenitor Cells. *Science* 294:1933.
- 32 Yoshimoto, T., Min, B., Sugimoto, T., Hayashi, N., Ishikawa, Y., Sasaki, Y., Hata, H., Takeda, K., Okumura, K., Van Kaer, L., Paul, W. E., and Nakanishi, K. 2003. Nonredundant Roles for CD1d-restricted Natural Killer T Cells and Conventional CD4<sup>+</sup> T Cells in the Induction of Immunoglobulin E Antibodies in Response to Interleukin 18 Treatment of Mice. *J Exp Med* 197:997.
- 33 Bergstedt-Lindqvist, S., Moon, H.-B., Persson, U., Möller, G., Heusser, C., and Severinson, E. 1988. Interleukin 4 instructs uncommitted B lymphocytes to switch to IgG1 and IgE. *Eur J Immunol* 18:1073.
- 34 Coffman, R. L., Seymour, B. W. P., Lebman, D. A., Hiraki, D. D., Christiansen, J. A., Shrader, B., Cherwinski, H. M., Savelkoul, H. F. J., Finkelman, F. D., Bond, M. W., and Mosmann, T. R. 1988. The Role of Helper T Cell Products in Mouse B Cell Differentiation and Isotype Regulation. *Immunol Rev* 102:5.
- 35 Hahne, M., Renno, T., Schroeter, M., Irmeler, M., French, L., Bornand, T., MacDonald, H. R., and Tschopp, J. 1996. Activated B cells express functional Fas ligand. *Eur J Immunol* 26:721.
- 36 Gaga, M., Frew, A. J., Varney, V. A., and Kay, A. B. 1991. Eosinophil activation and

T lymphocyte infiltration in allergen-induced late phase skin reactions and classical delayed-type hypersensitivity. *J Immunol* 147:816.

- 37 Mukai, K., Matsuoka, K., Taya, C., Suzuki, H., Yokozeki, H., Nishioka, K., Hirokawa, K., Etori, M., Yamashita, M., Kubota, T., Minegishi, Y., Yonekawa, H., and Karasuyama, H. 2005. Basophils Play a Critical Role in the Development of IgE-Mediated Chronic Allergic Inflammation Independently of T Cells and Mast Cells. *Immunity* 23:191.
- 38 Holgate, S. T., Chuchalin, A. G., Hébert, J., Lötvall, J., Persson, G. B., Chung, K. F., Bousquet, J., Kerstjens, H. A., Fox, H., Thirlwell, J., and Della Cioppa, G. 2004. Efficacy and safety of a recombinant anti-immunoglobulin E antibody (omalizumab) in severe allergic asthma. *Clin Exp Allergy* 34:632.
- 39 Holgate, S., Casale, T., Wenzel, S., Bousquet, J., Deniz, Y., and Reisner, C. 2005. The anti-inflammatory effects of omalizumab confirm the central role of IgE in allergic inflammation. *J Allergy Clin Immunol* 115:459.



## Figure Legends

### **Fig. 1. BALB/c Fas<sup>-/-</sup> mice can produce significantly larger amounts of IgE and IgG1**

**than WT mice. (A)** Serum IgG1 and IgE levels in 20-week-old WT and Fas<sup>-/-</sup> mice were

measured by ELISA. Ten mice were analyzed in each group (n=10). \* $p < 0.05$  and \*\* $p < 0.01$ ,

Student *t* test. **(B)** Bone marrow chimera mice were generated by transferring bone marrow

cells from CD45.2<sup>+</sup> Fas<sup>-/-</sup> mice into CD45.1<sup>+</sup> WT recipient mice irradiated at 8 Gy (Fas<sup>-/-</sup>).

Control CD45.1<sup>+</sup> recipient mice received bone marrow cells from CD45.2<sup>+</sup> WT mice (WT).

The ratio between CD45.2<sup>+</sup> and CD45.1<sup>+</sup> cells in peripheral blood was more than 95 % in the

chimera mice 20 weeks after the transfer. Serum levels of IgE and IgG1 were measured by

ELISA at 20 weeks after the transfer (n=5). \* $p < 0.05$  and \*\* $p < 0.01$ , Student *t* test. **(C)** Spleen

cells were isolated from WT and Fas<sup>-/-</sup> mice, and cultured with anti-CD40 mAb (1  $\mu$ g/ml) plus

IL-4 (0 or 50 ng/ml) for 7 days. The amounts of IgE and IgG1 in culture supernatants were

measured by ELISA. \* $p < 0.05$ , Student *t* test. **(D)** B220<sup>+</sup>CD3<sup>-</sup> B cells were purified from

spleen of WT and Fas<sup>-/-</sup> mice by cell sorter, and cultured with anti-CD40 mAb plus IL-4 for 7

days. The amounts of IgE and IgG1 in the culture supernatants were measured by ELISA. NS,

not significant.

Error bars denote  $\pm$ s.d.

**Fig. 2. B220<sup>-</sup>CD3<sup>-</sup> (DN) cells from Fas<sup>-/-</sup> mice enhance IgE production by WT B cells. (A)**

Splenic cells from 20-week-old BALB/c WT and Fas<sup>-/-</sup> mice were stained for B220 and CD3, and analyzed by flow cytometer. Values indicated in the figure were percentages of B220<sup>-</sup>CD3<sup>+</sup> cells (T cells), B220<sup>+</sup>CD3<sup>+</sup> cells (DP cells), B220<sup>-</sup>CD3<sup>-</sup> cells (DN cells) and B220<sup>+</sup>CD3<sup>-</sup> cells (B cells). (B) B cells isolated from WT mice by AutoMACS were cultured with purified T cells, DP cells, or DN cells from Fas<sup>-/-</sup> mice in the presence of anti-CD40 mAb plus IL-4 for 7 days, and the amounts of IgE in culture supernatants were measured by ELISA. (C) WT B cells were cultured with spleen cells from RAG2<sup>-/-</sup> Fas<sup>+/+</sup> or RAG2<sup>-/-</sup> Fas<sup>-/-</sup> mice in the presence of anti-CD40 mAb plus IL-4 for 7 days, and the amounts of IgE in culture supernatants were measured by ELISA.

\* $p < 0.05$  and \*\* $p < 0.01$ , Student  $t$  test. Error bars denote  $\pm$ s.d.

**Fig. 3. The number of Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells in spleen of Fas<sup>-/-</sup> mice can enhance IgE**

**production from B cells. (A)** The number of Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells was compared between

20-week-old WT and Fas<sup>-/-</sup> mice by flow cytometry after the gating of lineage-negative cells.

Three mice in each group were analyzed and representative results are shown. (B) WT B cells

were cultured with or without Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice in the presence of

anti-CD40 mAb plus IL-4 for 7 days, and the amounts of IgE in culture supernatants were

measured by ELISA. (C) WT or Fas<sup>-/-</sup> B cells were co-cultured with Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup>

(L<sup>-</sup>T<sup>+</sup>S<sup>+</sup>) cells from Fas<sup>-/-</sup> mice in the presence of anti-CD40 mAb plus IL-4 for 7 days, and the amounts of IgE and IgG1 in culture supernatants were measured by ELISA.

\**p*<0.05, Student *t* test. Error bars denote ±s.d.

**Fig. 4. Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice express different surface markers from**

**type 2 innate lymphoid cells.** Flow cytometry analysis for the indicated cell surface

molecules was carried out in spleen Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice at 18-20 weeks of age. Shaded areas represent respective isotype-controls. Analyses were repeated 3 times with different mice, and representative results are shown.

**Fig. 5. Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells produce Th2 cytokines by the stimulation with IL-2 plus**

**IL-18. (A)** Serum IL-18 levels in WT and Fas<sup>-/-</sup> mice were determined by ELISA at 20 weeks

of age (n=9). \**p*<0.05, Student *t* test. **(B)** Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice were

cultured in the presence or absence of IL-18 plus IL-2 for 3 days, and then cell numbers were

enumerated. \*\**p*<0.01, Student *t* test. **(C)** Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice stimulated

with IL-2 plus IL-18 for 3 days as in (B) were analyzed for the expression of Thy-1 and Sca-1

by flow cytometer. **(D)** Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells were cultured alone or with IL-2 and/or IL-18,

and the amounts of IL-5 and IL-13 secreted into the culture supernatants were measured by

ELISA. **(E)** After the stimulation with IL-2 plus IL-18 for 24 h, expression levels of IL-6 and

IL-9 mRNAs in Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells were analyzed by RT-PCR.

**Fig. 6. Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from WT mice also enhance IgE production by B cells. (A)**

Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from spleen of WT mice were analyzed for expression of Fas and IL-18R $\alpha$  by flow cytometer. Shaded areas represent isotype-controls. Analyses were repeated 3 times, and representative results were shown. **(B)** B cells isolated from WT mice were cultured with Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from WT mice with anti-CD40 mAb plus IL-4 for 7 days, and the amounts of IgE in culture supernatants were measured by ELISA. \* $p < 0.05$ , Student  $t$  test. Error bars denote  $\pm$ s.d.

Figure 1

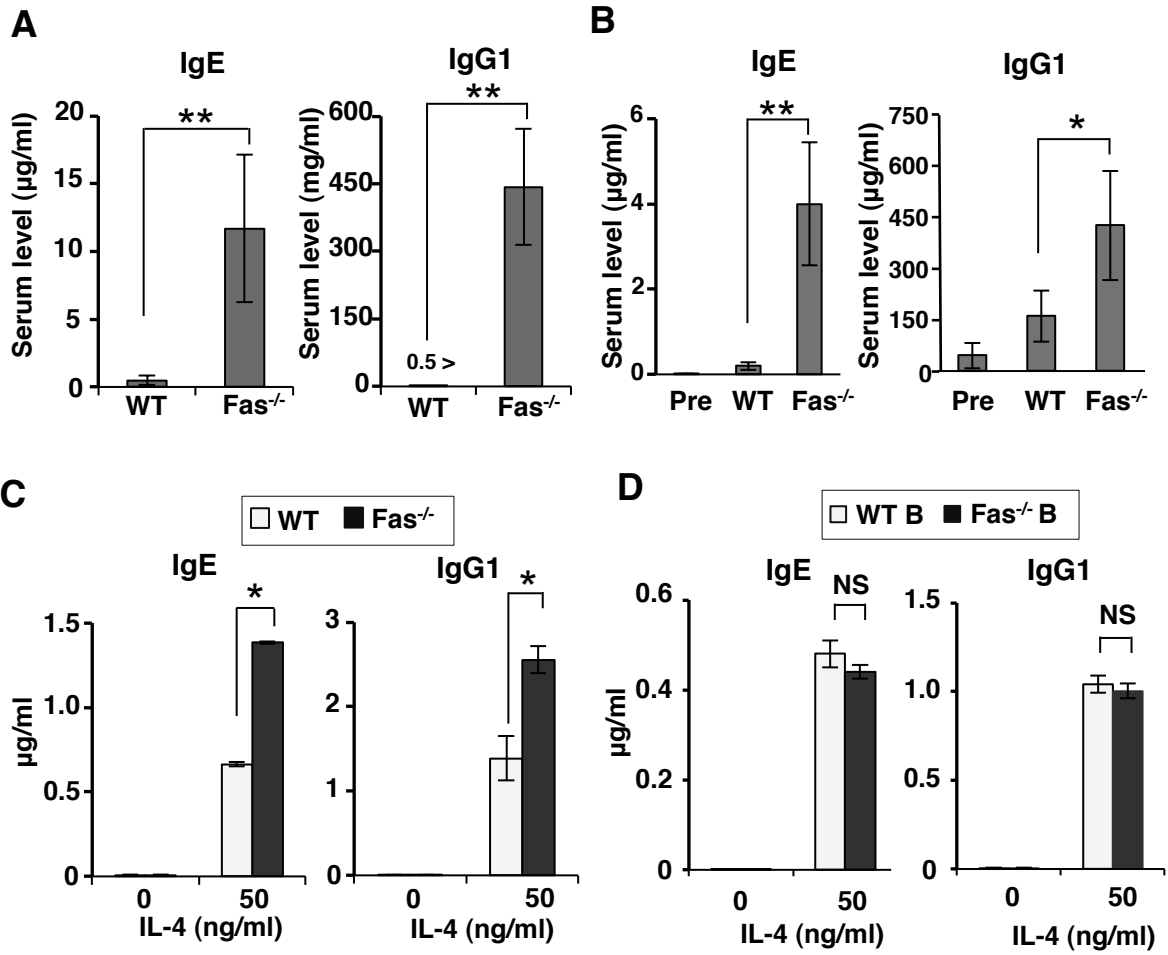
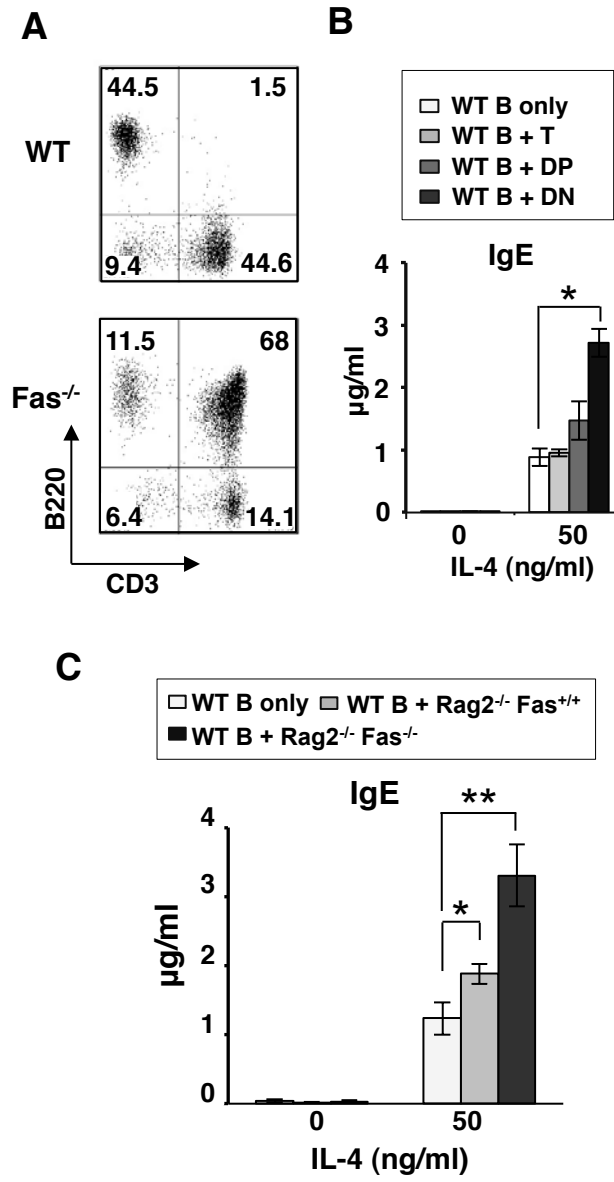
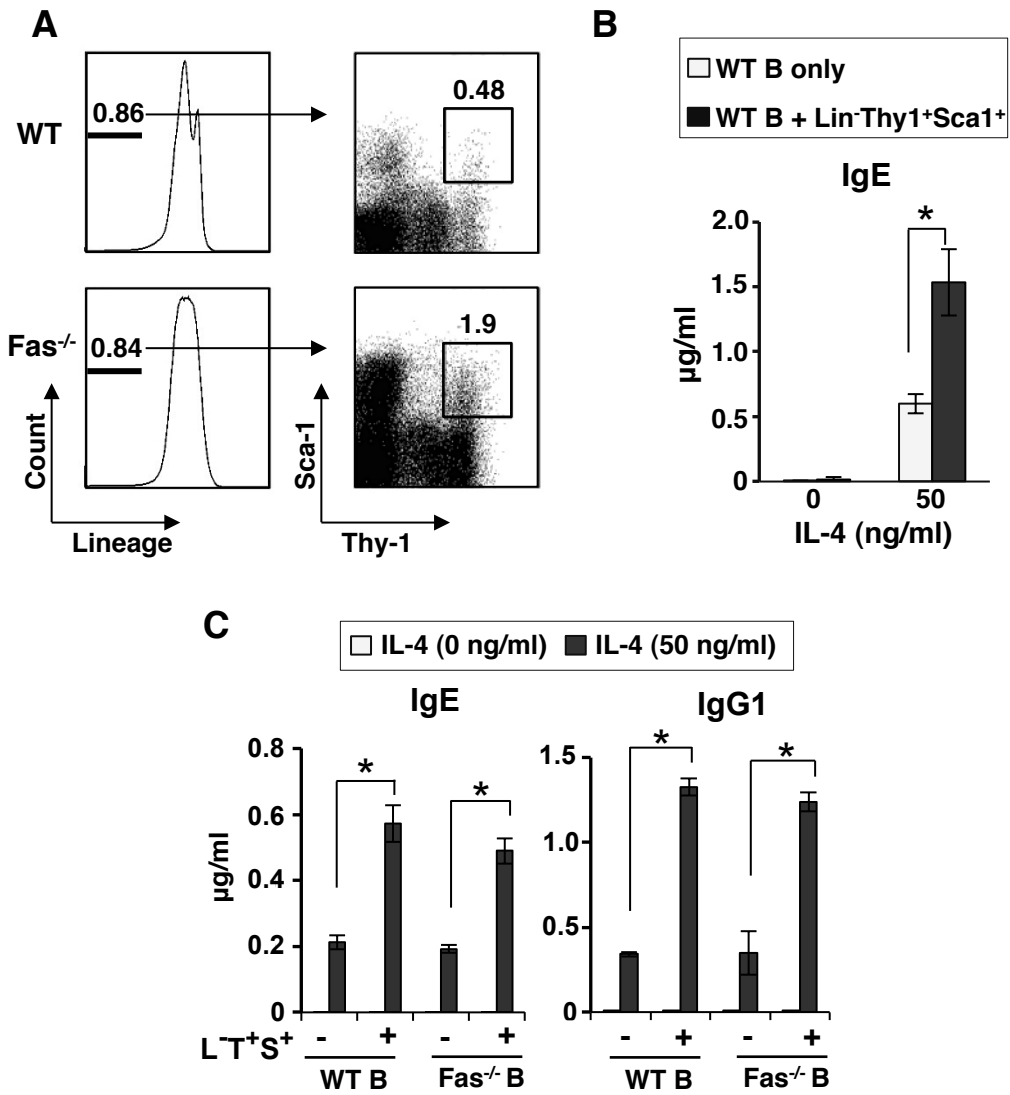


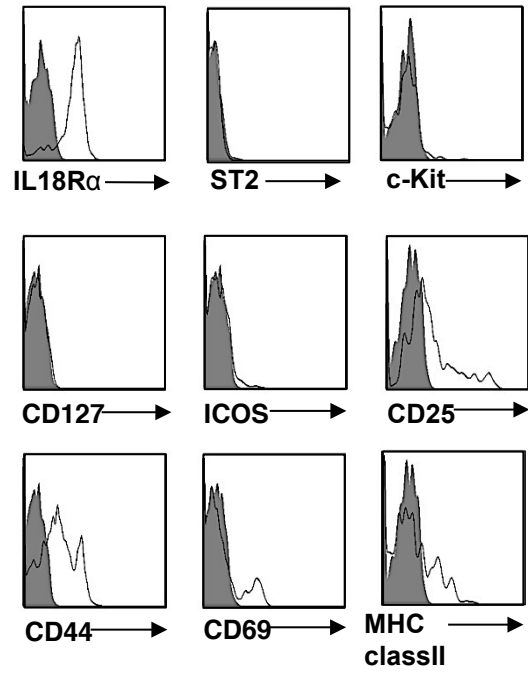
Figure 2



**Figure 3**



**Figure 4**





**Figure 5**

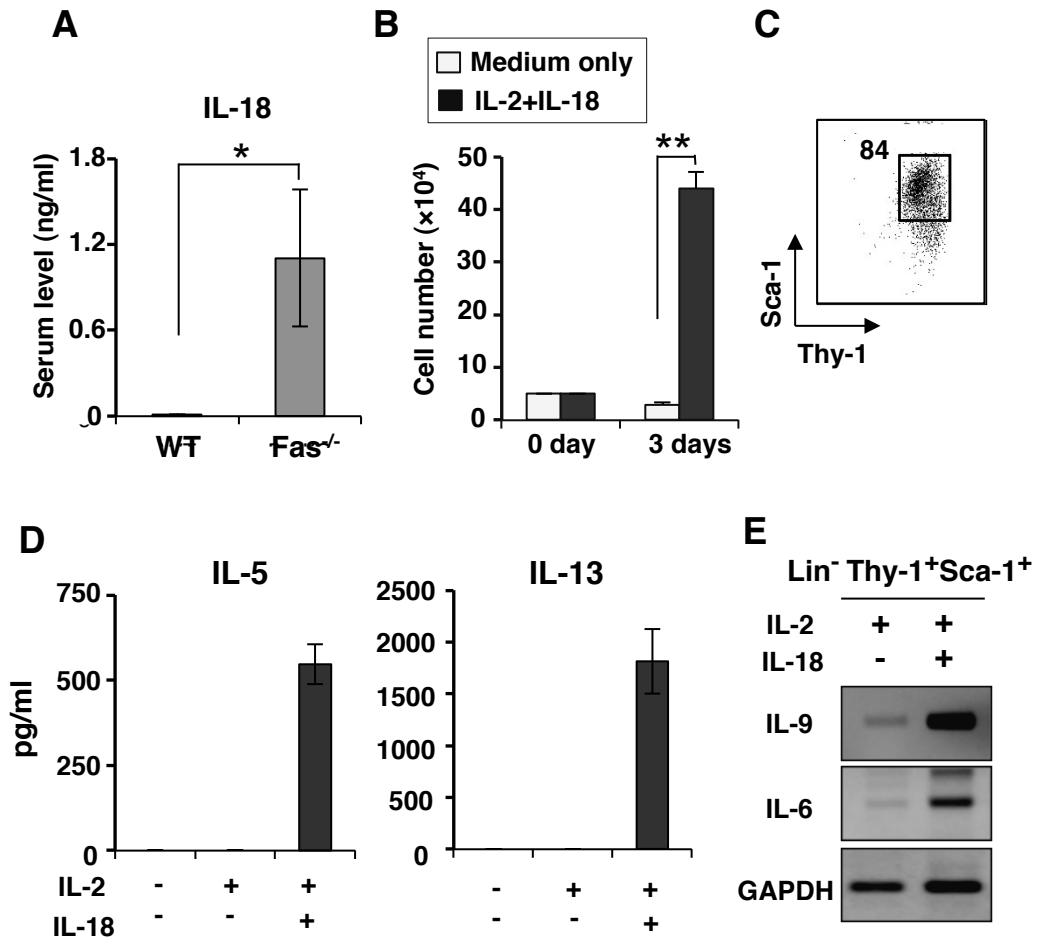
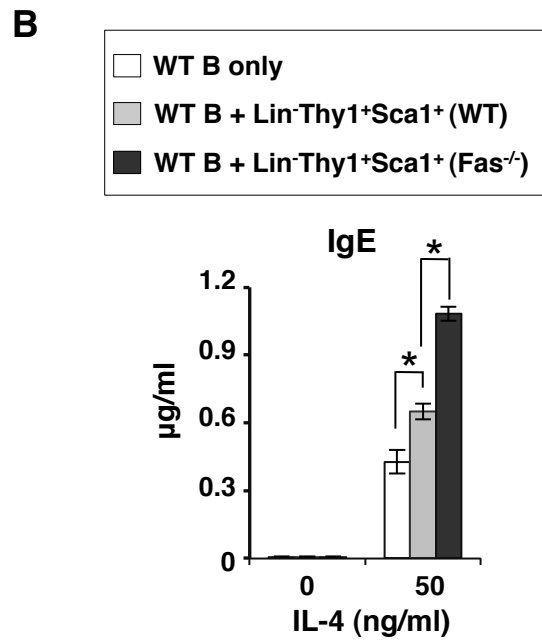
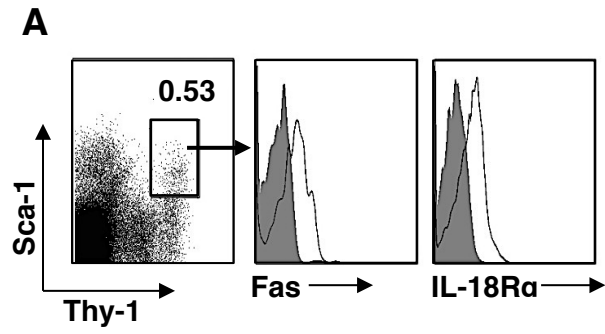


Figure 6



## Supplementary Methods

### *Generation of T helper type 2 (Th2 cells)*

Naïve splenic CD4<sup>+</sup>CD62L<sup>high</sup> T cells from BALB/c WT mice were sorted by FACS Aria II, and cultured with human IL-2 (100 U/ml; PEPROTEC), IL-4 (10 ng/ml; R&D systems), anti-CD28 mAb (1 µg/ml; BioLegend) and anti-IFN-γ (10 µg/ml) in the 48 well plates coated with anti-CD3ε mAb (1 µg/ml; BioLegend) as described previously (Nakahira, M. and Nakanishi, K. 2011. Requirement of GATA-binding protein 3 for Il13 gene expression in IL-18-stimulated Th1 cells. *Int Immunol* 23:761). Then, cells were treated with Ficol-Raque<sup>TM</sup> PLUS (GE Healthcare) to obtain live cells. After that, cells were re-stimulated with anti-CD3ε mAb and human IL-2 for 24h.

### Real-time quantitative RT-PCR (qRT-PCR)

Total RNA was isolated by NucleoSpin RNA XS (TAKARA) according to manufacturer's instructions. Reverse transcription (RT) reactions were carried out with ReverTra Ace qPCR RT Kit (TOYOBO) according to manufacturer's instructions. RT products were analyzed by StepOne real-time PCR system (Applied Biosystems). Primers used in this study were as follows: forward, 5'-GAAGTGTGGCGAGGAGAGAC-3', and reverse, 5'-GCACAGTTTTGTGGGGTTTT-3' for IL-5; 5'-GGTGACAACCACGGCCTTCCC-3', and reverse, 5'-GCCACTCCTTCTGTGACTCCAGC-3' for IL-6; forward, 5'-CAGCTCCCTGGTTCTCTCAC-3', and reverse, 5'-CCACACTCCATACCATGCTG-3' for IL-13; and forward, 5'-GCAGCACCA CATGGG GCATCA-3', and reverse, 5'-TGCCTGCCATGGTCTGGTTGC-3' for IL-9.

### *Induction of Fas-mediated apoptosis*

Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from WT BALB/c mice were incubated with IL-2 and 100 ng/ml agonistic anti-Fas antibodies (RK-8; MBL) for 12h as described previously (Nishimura, Y., Hirabayashi, Y., Matsuzaki, Y., Musette, P., Ishii, A., Nakauchi, H., Inoue, T, and Yonehara, S. 1997. In vivo analysis of Fas antigen-mediated apoptosis: effects of agonistic anti-mouse Fas mAb on thymus, spleen and liver. *Int Immunol*, 9:307-316). After that, 1 µg/ml Propidium iodide (PI) was added into media directly to detect fragmented nuclei of apoptotic cells. Cells were observed by fluorescence microscope (BioZero; KEYENCE) with a 20 × objective and count PI<sup>+</sup> cells number.

## Supplementary Figure Legends

**Supplementary Figure 1. DN cells from C57BL/6 Fas<sup>-/-</sup> mice could not enhance IgE production.** B cells and DN cells were isolated from C57BL/6 WT and Fas<sup>-/-</sup> mice, respectively. B cells were cultured with DN cells with anti-CD40 mAb plus IL-4 for 7 days, and the amounts of IgE in the culture supernatants were measured by ELISA.

**Supplementary Figure 2. Analysis of mixed bone marrow chimera mice between WT and RAG<sup>-/-</sup>Fas<sup>-/-</sup> mice.** (A) Bone marrow cells from RAG2<sup>-/-</sup>Fas<sup>+/+</sup> or RAG<sup>-/-</sup>Fas<sup>-/-</sup> mice (CD45.2) were transferred into irradiated (6Gy) WT recipient mice (CD45.1). The population of CD45.1<sup>+</sup> and CD45.2<sup>+</sup> cells in peripheral blood was analyzed at 8 weeks after the transfer. (B) Serum levels of IgE were measured before (pre) or at 20 weeks after the transfer (n=3).

**Supplementary Figure 3. Flow cytometry analysis of DN cells.** B220<sup>-</sup>CD3<sup>-</sup> DN cells from WT and Fas<sup>-/-</sup> mice were analyzed by flow cytometer. Values represent percentages of myeloid cells as CD11c<sup>-int</sup>CD11b<sup>+</sup> (upper panels), dendritic cells as CD11c<sup>+</sup>CD11b<sup>+</sup> (upper panels) and granulocytes as Gr-1<sup>+</sup>CD11b<sup>+</sup> (middle panels) by gating of B220<sup>-</sup>CD3<sup>-</sup> cells. The percentage of NK cells was indicated as Dx5<sup>+</sup>B220<sup>-</sup>CD3<sup>-</sup> (lower panels) in bulk spleen cells.

**Supplementary Figure 4. Cell-to-cell contact is necessary for Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice to enhance IgE production by B cells.** (A) Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells, Thy-1<sup>+</sup>Sca-1<sup>-</sup> cells, Thy-1<sup>-</sup>Sca-1<sup>+</sup> cells or Thy-1<sup>-</sup>Sca-1<sup>-</sup> cells among Lin<sup>-</sup> splenic cells from Fas<sup>-/-</sup> mice were co-cultured with WT B cells with anti-CD40 mAb plus IL-4 for 7 days, and the amounts of IgE in culture supernatants were measured by ELISA. (B) Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells and WT B cells were cultured in the upper and lower chambers of the Transwell culture, respectively, with anti-CD40 mAb plus IL-4 for 7 days. As a positive control, Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells and B cells were cultured together in the lower chamber. The amounts of IgE in culture supernatants were measured by ELISA. (C) Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells were co-cultured with B cells with anti-CD40 mAb plus IL-4 for 3 days, and then stained for B220, IL-5 and IL-13. Shaded areas represent isotype-controls.

**Supplementary Figure 5. Comparison of the expression levels of Th2 cytokines mRNA between Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells and Th2 cells by Real-time qRT-PCR.** RNA was isolated from Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells and Th2 cells after stimulation with IL-2 plus IL-18 and re-stimulation with anti-CD3ε mAb and IL-2, respectively. Real-time qRT-PCR was performed using specific primers for IL-5, IL-6, IL-9 and IL-13.

**Supplementary Figure 6. Factors secreted by Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells enhance IgE and IgG1 production.** (A) Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from Fas<sup>-/-</sup> mice were cultured with the indicated cytokines for 7 days, and culture supernatants were collected. B cells isolated from WT mice were cultured with these supernatants in the presence of anti-CD40 mAb plus IL-4 for 7 days, and the amounts of IgE and IgG1 in culture supernatants were measured by ELISA. \**p*<0.05 and \*\**p*<0.01, Student *t* test. Error bars denote ±s.d. (B) WT B cells were cultured with the indicated combinations of IL-2, IL-18 and IL-4 for 7 days, and the amounts of IgE in culture supernatants were measured by ELISA.

**Supplementary Figure 7. Induction of Fas-mediated apoptosis in WT Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells.** Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from WT BALB/C mice were stimulated or not stimulated with anti-Fas agonistic mAb RK-8 (100 ng/ml) in the presence of IL-2 plus IL-18 for 12h. Then, cells were stained with PI. (A) Number of PI-positive dead cells and PI-negative viable cells were quantified, and % of dead cells was indicated. (B) Cells were stained with PI and analyzed under a fluorescence microscope. Scale bar, 50 μm.

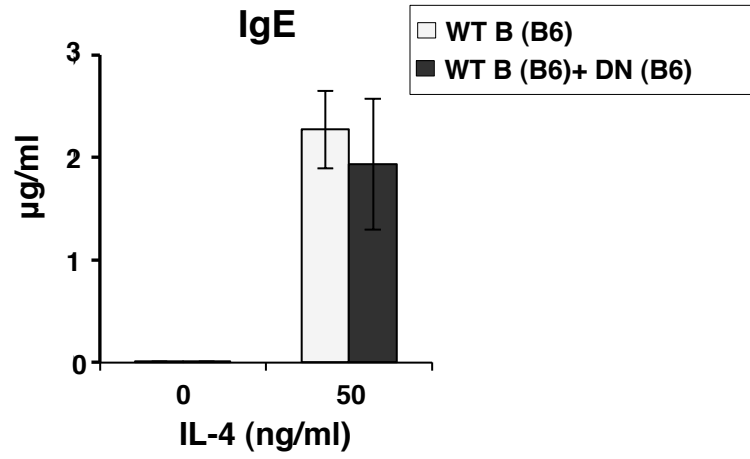
**Supplementary Figure 8. Flow cytometry analysis of WT Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells.**

Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from spleen of WT BALB/c mice were analyzed for expression of the indicated surface markers by flow cytometer. Shaded areas represent isotype-controls.

**Supplementary Figure 9. Flow cytometry analysis of Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from**

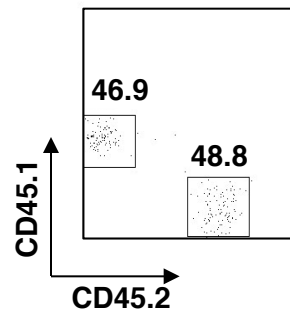
**C57BL/6 WT and Fas<sup>-/-</sup> mice.** (A) Lin<sup>-</sup> spleen cells from C57BL/6 WT and Fas<sup>-/-</sup> mice were analyzed by flow cytometer. Values represent percentages of Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells. (B) Flow cytometry analysis for the indicated cell surface molecules was carried out in spleen Lin<sup>-</sup>Thy-1<sup>+</sup>Sca-1<sup>+</sup> cells from 18-week old C57BL/6 WT and Fas<sup>-/-</sup> mice. Shaded areas represent respective isotype-controls.

# Supplementary Figure 1

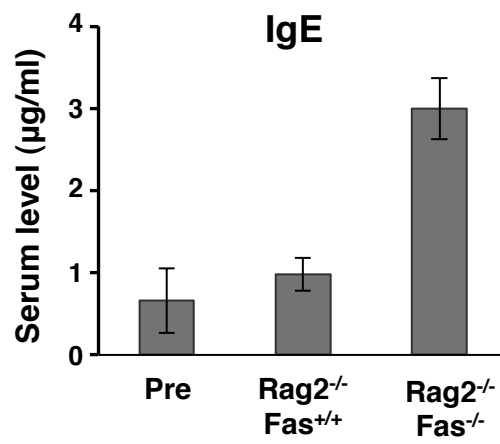


# Supplemental Figure 2

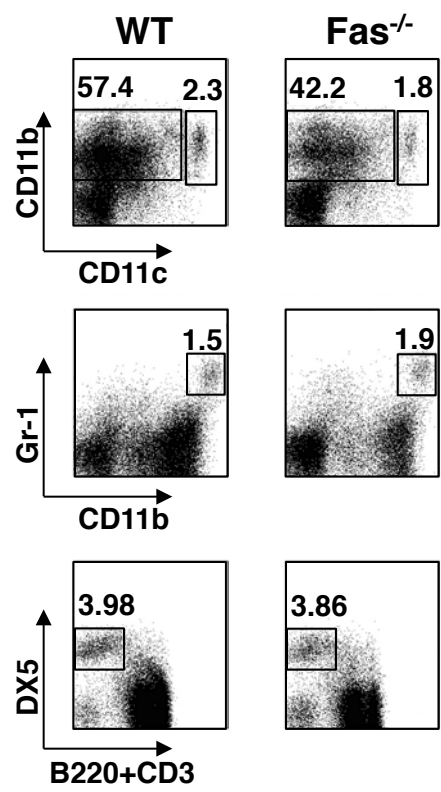
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**B**



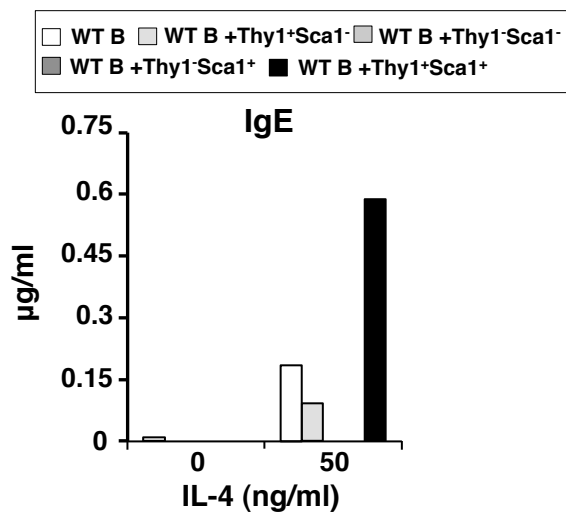
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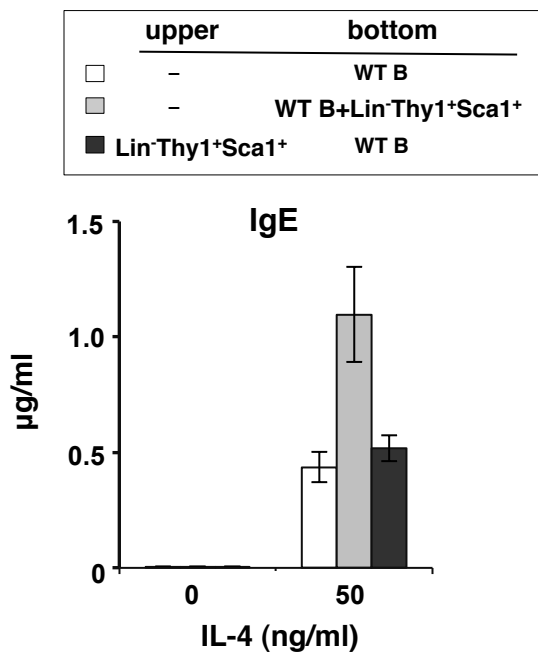


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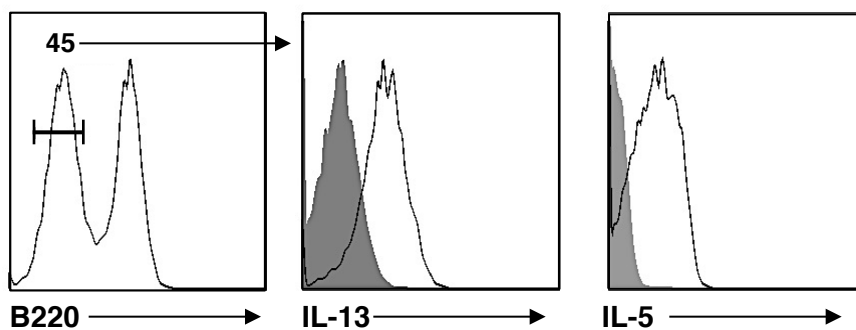
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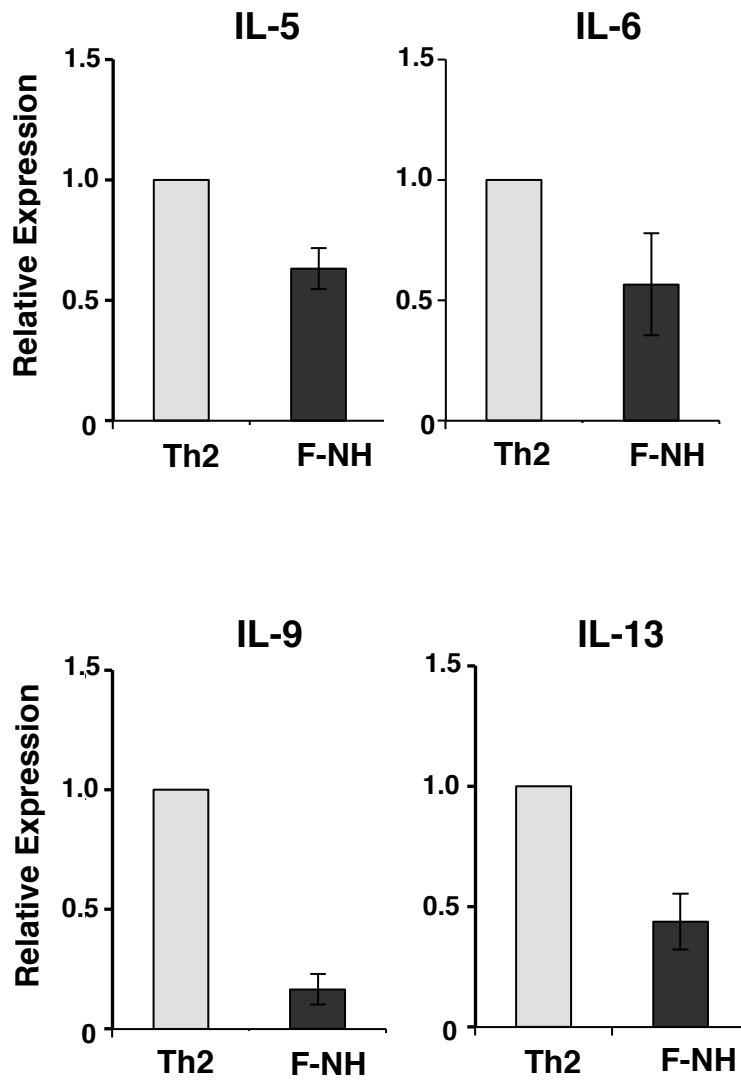
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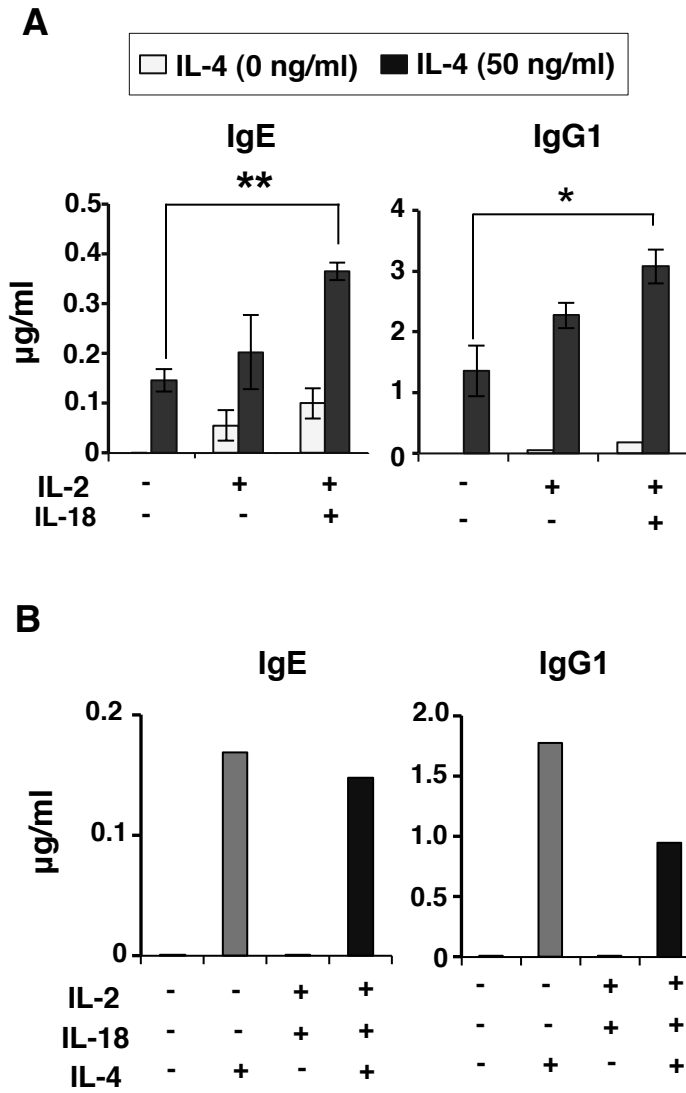
**C**



## Supplementary Figure 5

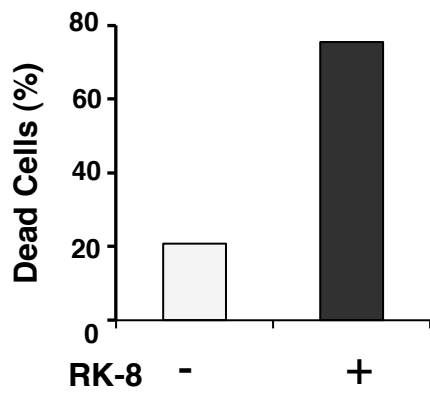


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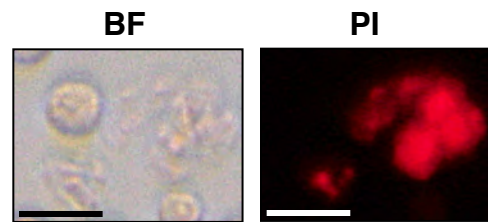


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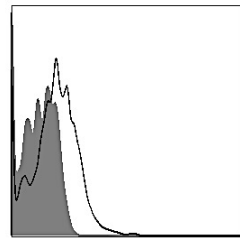
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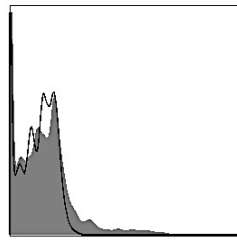
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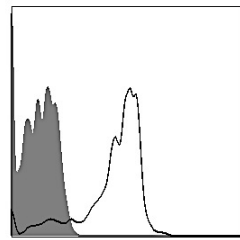
# Supplementary Figure 8



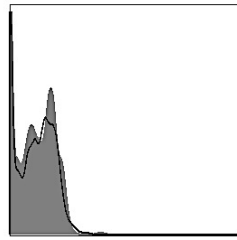
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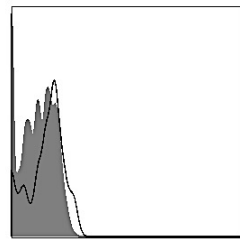
**CD69**



**CD44**



**c-kit**



**CD127**

# Supplementary Figure 9

