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

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Abstract

Fas (CD95), a member of the tumor necrosis factor receptor superfamily, mediates apoptosis-inducing signals in its expressing cells, especially in self-reactive cells. We recently reported that Fas−/− mice with a BALB/c background (BALB/c Fas−/− 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−/− 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−Thy-1+Sca-1+ cells expressed IL-18R and secreted Th2 cytokines when co-cultured with B cells or when 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−/− 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−Thy-1+Sca-1+ 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.

Keywords: allergy, Fas, IgE, IL-18, innate immunocyte

Introduction

Fas (CD95), a member of the tumor necrosis factor receptor superfamily, 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−/− mice develop systemic autoimmune disease and lymphadenopathy. In mice homozygous for Fas−/− mice, genetic background determines the incidence and severity of histopathological manifestations of systemic autoimmunity; MRL−/− mice and C57BL/6 lpr or C57BL/6 Fas−/− 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−/− mice with a BALB/c background (BALB/c Fas−/− mice) (7).

Chronic allergic inflammatory diseases are characterized by production of IL-4, IL-5, IL-9 and IL-13 from T2 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 T2 cytokines (9,
In this study, we identified a novel type 2 immune cell with a Lin-Thy-1^+Sca-1^+ phenotype in spleen of BALB/c Fas^−/− mice and found that the Lin-Thy-1^+Sca-1^+ 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-Thy-1^+Sca-1^+ cells produced T2 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-Thy-1^+Sca-1^+ cells are suggested to be maintained in homeostasis by Fas-dependent apoptosis. Then, we designated the newly identified cell as Fas-expressing natural helper cell (F-NH cell). Taken together, F-NH cells, the number of which increases greatly with a deficiency of Fas-dependent apoptosis, possibly induce hyperproduction of IgE leading to allergic inflammation.

**Methods**

**Mice**

BALB/c WT mice were purchased from CLEA Japan. Rag2^−/− mice and CD45.1 (Ly5.1) mice with the BALB/c background were kindly provided by Dr S. Sakaguchi. C57BL/6 Fas^−/− mice were maintained in our laboratory. Fas^+ mouse with the BALB/c background were generated as described previously (7). Rag2^−/−Fas^−/− mice with the BALB/c background were obtained by crossing Fas^−/− and Rag2^−/− 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 aged 8 weeks were irradiated lethally (8 Gy) and then inoculated with bone marrow cells (1 × 10^7) from sex- and age-matched BALB/c WT and Fas^−/− 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 × 10^7) from sex- and age-matched Rag2^−/−Fas^+/+ and Rag2^−/−Fas^−/− mice (CD45.2).

**Antibodies and reagents**

Phycocyanin (PE)- or biotin-conjugated mAbs specific for mouse B220 (RA3-6B2), CD3ε (145-2C11), CD11c (HL3), CD49b (DX5), Gr-1 (RB6-8C5), CD4 (GK1.5), CD8a (53–67), 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 (AS-32.1), CD44 (IM7), CD69 (H1.2F3), CD127 (B12-1), inducible co-stimulator (ICOS/RMMA-1), and Fas (Jo2). These antibodies were purchased from eBioscience, Biolegend and BD Bioscience. Fluorescein isothiocyanate (FITC)-conjugated T1/ST2 and IL-18Rx 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-aminominoacrylamine D (BD Bioscience). FACS samples were run on a FACS Calibur (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^−/− 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^−/− mice, total spleen cells were stained with PE-conjugated anti-B220 and allophycocyanin (APC)-conjugated anti-CD3, and then B220^−CD3^+ cells were sorted with a FACS Aria I (BD Bioscience). To collect B220^−CD3^+ T cells, B220^−CD3^+ cells and B220^−CD3^− cells from Fas^−/− 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-Thy-1^−Sca-1^− cells, Lin^− 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^− 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^−/− spleen (5 × 10^7 cells/well) and B cells from WT mice (5 × 10^7 cells/well) were mixed and seeded into 96-well flat-bottomed tissue culture plates in Roswell Park Memorial Institute (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 β-mercaptoethanol (50 μM) together with anti-CD40 mAb (1 μg/ml; R&D systems) plus IL-4 (0 or 50 ng/ml; R&D systems) for 7 days. For cytokine production, Lin-Thy-1^−Sca-1^− cells from Fas^−/− mice (5 × 10^6 cells/well) were stimulated with IL-2 (1000 U/ml) 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 four-time-diluted culture supernatant for 7 days. IgE and IgG1 production was measured by ELISA.
**Intracellular cytokine staining**

Lin-Thy-1-1-Sca-1-1 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 of culture, cells were treated with brefeldin A (Biolegend) and incubated for 4 h 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−/− mice and analyzed with an ELISA kit (MBL, Japan). For the measurement of IL-5 and IL-13 in the culture supernatant, Lin-Thy-1-1-Sca-1-1 cells from Fas−/− mice (5 x 10⁶ cells/well) were stimulated with IL-2 (100 μM) 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.

**Reverse transcription-polymerase chain reaction**

Lin-Thy-1-1-Sca-1-1 cells from Fas−/− mice were stimulated with IL-2 (100 μM) and IL-18 (100 ng/ml) for 24 h. Total RNAs were prepared using NucleoSpin RNA XS (TAKARA), and reverse transcription-polymerase chain reaction (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′-GCACTCCTTCTCGACTCCAGC-3′ for IL-6; and forward, 5′-GCACCACCAATGGGGCATCA-3′, and reverse, 5′-TGCCCTGCCATGGTGTTGC-3′ for IL-9.

**Statistical analysis**

Data are expressed as the mean ± SD 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−/− mice develop blepharitis with allergic inflammation in association with hyperproduction of IgE**

Recently, we found that BALB/c Fas−/− mice with hyperproduction of IgE in vivo, with a significantly elevated level of IgE and IgG1 in the serum (Fig. 1A), as well as in MRL-lpr/lpr mice or C57BL/6 Fas−/− mice (7). Thus, hyper-IgE production preceded the allergic inflammation in BALB/c Fas−/− mice.

To clarify whether the high serum levels of IgE and IgG1 in Fas−/− 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−/− mice (Ly5.2) into lethally irradiated congeneric recipient WT mice (Ly5.1). At 20 weeks after the transplantation, serum concentrations of IgE and IgG1 were significantly higher in mice receiving Fas−/− bone marrow cells than in control chimera mice receiving WT bone marrow cells, indicating that hematopoietic cells from Fas−/− mice contributed to the hyperproduction of IgE and IgG1 (Fig. 1B). When total spleen cells from WT and Fas−/− mice were cultured with anti-CD40 mAb plus IL-4, Fas−/− cells produced much higher levels of IgE and IgG1 than WT cells (Fig. 1C). However, splenic B cells (B220+CD3−) cells from WT and Fas−/− mice produced comparable levels of IgE and IgG1 during the culture (Fig. 1D). These results indicated that Fas−/− hematopoietic cells, but not Fas−/− B cells, are responsible for hyperproduction of IgE and IgG1 in vivo.

**B220+CD3− cells from Fas−/− mice enhance IgE production from WT B cells.**

BALB/c Fas−/− 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−/− mice enhanced IgE production by B cells, Fas−/− splenic cells were fractionated into B220+CD3− cells (B cells), B220−CD3− cells (T cells), B220+CD3− cells (double-negative cells: DN cells) and B220+CD3− cells (double-positive cells: DP cells) (Fig. 2A). Fas−/− DP cells include abnormal T cells (ipr cells) that can survive owing to a deficiency of Fas-mediated apoptosis (4, 26, 27). Isolated T, DN and DP cells from Fas−/− 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. DN cells were able to significantly enhance IgE production by WT B cells (Fig. 2B). In contrast, DN cells from C57BL/6 Fas−/− mice did not enhance IgE production from WT B cells (Supplementary Figure 1, available at International Immunology Online).

To confirm that the Fas−/− DN cells enhancing IgE production were neither B nor T cells, we established BALB/c Rag2−/−Fas−/− mice completely lacking T and B cells (28). Rag2−/−Fas−/− or Rag2−/−Fas−/− 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−/−Fas−/− mice had a significantly greater effect (Fig. 2C), revealing that non-T/non-B DN cells in Fas−/− 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−/−Fas−/− or Rag2−/−Fas−/− mice (donor). Bone marrow cells from Rag2−/−Fas−/− or Rag2−/−Fas−/− 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 Fig. 2).
The serum levels of IgE were higher in chimera mice between WT (recipient) and Rag2−/−Fas−/− mice than control chimera mice between WT and Rag2−/−Fas+/+ mice, indicating that non-T/non-B cells from Fas−/− mice are responsible for in vivo hyperproduction of IgE in the mixed-bone marrow chimera mice. All the results suggest that non-T/non-B DN cells are responsible for hyperproduction of IgE in BALB/c Fas−/− 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−/− mice. Flow cytometry analysis showed the proportions of these cells in spleen to be comparable between Fas−/− and WT mice (Supplementary Figure 3, available at International Immunology Online).

Lin-Thy-1−Sca-1+ cells from Fas−/− spleen enhance IgE production by B cells

Recently, type 2 innate lymphoid cells, such as NH cells, nuocytes, Ih2 cells and MMPpop cells were identified to be able to produce T2 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−/− splenic cells and these cells contributed to enhance IgE production. By flow cytometry analysis, we found that the number of Lin-Thy-1−Sca-1+ cells increased about five times more in spleens of Fas−/− mice than those of WT mice (Fig. 3A). In addition, the number of Lin-Thy-1−Sca-1+ cells increased about three times more in spleens of Rag2−/−Fas−/− mice than those of Rag2−/−Fas+/− mice (data not shown).

To confirm that Lin-Thy-1−Sca-1+ cells actually enhance IgE production, we purified them from Fas−/− mice and co-cultured them with WT B cells in the presence of 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 ±SD.

Fig. 1. BALB/c Fas−/− 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−/− mice were measured by ELISA. Ten mice were analyzed in each group (n = 10). **P < 0.01, Student’s t-test. (B) Bone marrow chimera mice were generated by transferring bone marrow cells from CD45.2+ Fas−/− mice into CD45.1+ WT recipient mice irradiated at 8 Gy (Fas−/−). Control CD45.1+ recipient mice received bone marrow cells from CD45.2+ WT mice (WT). The ratio between CD45.2+ and CD45.1+ 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’s t-test. (C) Spleen cells were isolated from WT and Fas−/− mice and cultured with anti-CD40 mAb (1 μg/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’s t-test. (D) B220−CD3+ B cells were purified from spleen of WT and Fas−/− 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 ±SD.

2A and B, available at International Immunology Online. The serum levels of IgE were higher in chimera mice between WT (recipient) and Rag2−/−Fas−/− (donor) mice than control chimera mice between WT and Rag2−/−Fas+/+ mice, indicating that non-T/non-B cells from Fas−/− mice are responsible for in vivo hyperproduction of IgE in the mixed-bone marrow chimera mice. All the results suggest that non-T/non-B DN cells are responsible for hyperproduction of IgE in BALB/c Fas−/− mice in vivo.

Lin-Thy-1−Sca-1+ cells from Fas−/− spleen enhance IgE production by B cells

Recently, type 2 innate lymphoid cells, such as NH cells, nuocytes, Ih2 cells and MMPpop cells were identified to be able to produce T2 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−/− splenic cells and these cells contributed to enhance IgE production. By flow cytometry analysis, we found that the number of Lin-Thy-1−Sca-1+ cells increased about five times more in spleens of Fas−/− mice than those of WT mice (Fig. 3A). In addition, the number of Lin-Thy-1−Sca-1+ cells increased about three times more in spleens of Rag2−/−Fas−/− mice than those of Rag2−/−Fas+/+ mice (data not shown).

To confirm that Lin-Thy-1−Sca-1+ cells actually enhance IgE production, we purified them from Fas−/− mice and co-cultured them with WT B cells in the presence of anti-CD40 mAb plus
IL-4 for 7 days. Lin-Thy-1⁻Sca-1⁺ cells from Fas⁻/⁻ mice were revealed to markedly enhance IgE production (Fig. 3B).

In addition, they enhanced IgE and IgG1 production by Fas⁻/⁻ B cells and WT B cells (Fig. 3C). In contrast, neither Lin-Thy-1⁻Sca-1⁺ cells nor Lin-Thy-1⁻Sca-1⁻ cells enhanced IgE production (Supplementary Figure 4A, available at International Immunology Online).

Next, we examined whether cell-to-cell contact between B cells and Lin-Thy-1⁻Sca-1⁺ cells was necessary to promote IgE production using the Transwell culture system. When Lin-Thy-1⁻Sca-1⁺ cells and B cells were separated by a Transwell filter, Lin-Thy-1⁻Sca-1⁺ cells were incapable of enhancing IgE production (Supplementary Figure 4B, available at International Immunology Online). When Lin-Thy-1⁻Sca-1⁺ cells were co-cultured with WT B cells, they produced T_2 cytokines, such as IL-5 and IL-13, which were detected by intracellular cytokine staining (Supplementary Figure 4C, available at International Immunology Online). All the results indicate that Lin-Thy-1⁻Sca-1⁺ cells from Fas⁻/⁻ spleen possess the ability to effectively enhance IgE production from B cells through direct cell-to-cell interaction between Lin-Thy-1⁻Sca-1⁺ cells and B cells.

**Lin-Thy-1⁻Sca-1⁺ cells from Fas⁻/⁻ mice are different from type-2 innate lymphoid cells**

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

**Lin-Thy-1⁻Sca-1⁺ cells expand and produce cytokines in response to IL-2 plus IL-18**

To investigate the activation mechanisms of the Lin-Thy-1⁻Sca-1⁺ cells from Fas⁻/⁻ mice, we focused on the function of IL-18 because we found that serum levels of IL-18 were significantly higher in Fas⁻/⁻ mice than WT mice at 20 weeks of age (Fig. 5A) and that the Lin-Thy-1⁻Sca-1⁺ cells expressed IL-18Rα as shown in Fig. 4. When Lin-Thy-1⁻Sca-1⁺ 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-Thy-1⁻Sca-1⁺ 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-Thy-1⁻Sca-1⁺ 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-Thy-1⁻Sca-1⁺ 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 mRNA for T_2 cytokines between T_2 cells and Lin-Thy-1⁻Sca-1⁺ cells by real-time quantitative RT-PCR. Expression levels of IL-5, IL-6, IL-9 and IL-13 in Lin-Thy-1⁻Sca-1⁺ cells...
were almost comparable with, but a little lower than, those in Th2 cells (Supplementary Figure 5, available at International Immunology Online). Taken together, it may be feasible that Lin−Thy-1+Sca-1+ cells have the potential to produce cytokines supporting IgE production by B cells.

Soluble factors from Lin-Thy-1+Sca-1+ 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-Thy-1+Sca-1+ 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-Thy-1+Sca-1+ cells stimulated with IL-2 plus IL-18 produced significantly higher levels of IgE and IgG1 compared with those cultured with IL-2 alone or without IL-2 and IL-18 (Supplementary Figure 6A, 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, available at International Immunology Online). These results indicate that Lin-Thy-1+Sca-1+ 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-Thy-1+Sca-1+ cells in WT spleen can also enhance IgE production from B cells

Lin-Thy-1+Sca-1+ cells were also found in spleen of WT mice, though at much lower numbers than in Fas−/− spleen as shown in Fig. 3A. Because Fas is important to the removal of self-reactive and activated immunocytes by apoptosis...
Fig. 4. LinThy-1+Sca-1+ cells from Fas−/− 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 LinThy-1+Sca-1+ cells from Fas−/− mice at 18–20 weeks of age. Shaded areas represent respective isotype controls. Analyses were repeated three times with different mice, and representative results are shown.

As expected, flow cytometry analysis confirmed the expression of Fas on the LinThy-1+Sca-1+ cells under steady-state conditions. We hypothesized that Fas may play a role to eliminate moderate numbers of LinThy-1+Sca-1+ cells under steady-state conditions. However, the flow cytometry analysis revealed that Fas expression was significantly lower on LinThy-1+Sca-1+ cells than on Fas−/− mice, which would be consistent with a role for Fas in the elimination of LinThy-1+Sca-1+ cells in the absence of anti-CD40 mAb plus IL-4. To our knowledge, our results indicate that LinThy-1+Sca-1+ cells are eliminated by Fas-dependent apoptosis and are similar to previously reported type 2 innate lymphoid cells, such as NH cells, nuocytes, MMP12+ cells and IL2+ cells, 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 are found in mesenteric lymph nodes or fat-associated lymphoid clusters mainly around the gut (11–15), whereas LinThy-1+Sca-1+ cells exist in the spleen. Second, some of the surface markers expressed on these two types of cells are different (11–15) (Fig. 4). While LinThy-1+Sca-1+ cells highly express Sca-1, Thy-1 and CD69, Lin2+ and MMP12+ cells do not express Sca-1, and Thy-1 and CD69, respectively. Although expression levels of Thy-1 and Sca-1 on LinThy-1+Sca-1+ 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+T1/ST2−CD127+ and nuocytes are c-KIt−T1/ST2−CD127−ICOS− (11–15), while LinThy-1+Sca-1+ cells from BALB/c Fas−/− mice express different surface markers as previously reported type 2 innate lymphoid cells. Thus, LinThy-1+Sca-1+ cells from Fas−/− mice do not show completely the same surface markers as previously reported type 2 innate lymphoid cells. Third, our identified LinThy-1+Sca-1+ cells produce IL-5, IL-6, IL-9 and IL-13 in response to stimulation with IL-18 plus IL-2. We found that LinThy-1+Sca-1+ cells express IL-18Rx and can respond to IL-18, whereas type 2 innate lymphoid cells were reported to respond to IL-33 and IL-25 (11–15). LinThy-1+Sca-1+ cells do not belong to the cell types responsive to IL-18, such as T cells, NK 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). On the basis of these distinguishable features, we concluded that the newly identified LinThy-1+Sca-1+ cells in BALB/c mice are a novel type of immunocyte. We designated them F-NH cells because previously reported NH cells do not express Fas (K. Moro and S. Koyasu, personal communication).

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, xenogenous 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−/− 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 hyperproduction of IgE in Fas−/− mice.

Spleen cells from Rag2−/−Fas−/− mice and F-NH cells from WT spleen could also enhance IgE production from B cells, as shown in Fig. 5A. Taken together, Lin-Thy-1+Sca-1+ cells in WT BALB/c mice would belong to the same cell lineage as Lin-Thy-1+Sca-1+ cells found in Fas−/− BALB/c mice.

Discussion

Here, we found a novel type of Lin-Thy-1+Sca-1+ cells in BALB/c Fas−/− 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 findings indicate that Lin-Thy-1+Sca-1+ cells are eliminated by Fas-dependent apoptosis and are similar to previously reported type 2 innate lymphoid cells, such as NH cells, nuocytes, MMP12+ cells and IL2+ 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 are found in mesenteric lymph nodes or fat-associated lymphoid clusters mainly around the gut (11–15), whereas Lin-Thy-1+Sca-1+ cells exist in the spleen. Second, some of the surface markers expressed on these two types of cells are different (11–15) (Fig. 4). While Lin-Thy-1+Sca-1+ cells highly express Sca-1, Thy-1 and CD69, IL2+ cells and MMP12+ cells do not express Sca-1, and Thy-1 and CD69, respectively. Although expression levels of Thy-1 and Sca-1 on Lin-Thy-1+Sca-1+ 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+T1/ST2−CD127+ and nuocytes are c-KIt−T1/ST2−CD127−ICOS− (11–15), while Lin-Thy-1+Sca-1+ cells from BALB/c Fas−/− spleen are c-KIt+T1/ST2−CD127−ICOS+. Thus, Lin-Thy-1+Sca-1+ cells from Fas−/− mice do not show completely the same surface markers as previously reported type 2 innate lymphoid cells. Third, our identified Lin-Thy-1+Sca-1+ 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-Thy-1+Sca-1+ cells express IL-18Rx 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-Thy-1+Sca-1+ cells do not belong to the cell types responsive to IL-18, such as T cells, NK 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). On the basis of these distinguishable features, we concluded that the newly identified Lin-Thy-1+Sca-1+ cells in BALB/c mice are a novel type of immunocyte. We designated them F-NH cells because previously reported NH cells do not express Fas (K. Moro and S. Koyasu, personal communication).
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 ability of F-NH cells to enhance IgE production might be tightly regulated by the 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 hyperproduction 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>pro</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 types of Lin-Thy-1<sup>−</sup>Sca-1<sup>+</sup> cells than F-NH cells were found in spleen, and the number of Lin-Thy-1<sup>−</sup>Sca-1<sup>+</sup> cells in C57BL/6 Fas<sup>−/−</sup> mice was higher than that in BALB/c Fas<sup>−/−</sup> mice (Supplementary Fig. 9A, available at International Immunology Online). We anticipate that most of the Lin-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 (i) expression patterns of IL-18R<sub>α</sub>, CD25 and CD44 are different, (ii) C57BL/6 WT Lin-Thy-1<sup>−</sup>Sca-1<sup>−</sup> cells do not express Fas (Supplementary Fig. 9B, available at International Immunology Online) and (iii) 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, available at International Immunology Online). Thus, the generation of F-NH cells seems to be co-related well with the outbreak of allergic inflammation associated with hyper-IgE.

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 T_{2} 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.

Fig. 6. Lin-Thy-1{superscript}−Sca-1{superscript}− cells from WT mice also enhance IgE production by B cells. (A) Lin-Thy-1{superscript}−Sca-1{superscript}− cells from spleen of WT mice were analyzed for expression of Fas and IL-18Ra by flow cytometry. Shaded areas represent isotype controls. Analyses were repeated three times, and representative results were shown. (B) B cells isolated from WT mice were cultured with Lin-Thy-1{superscript}−Sca-1{superscript}− 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’s t-test. Error bars denote ± SD.

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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 hyperproduction of IgE, which may lead to novel strategies to improve allergic disease.

Supplementary data

Supplementary data are available at International Immunology Online.

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