

Activation and Differentiation of Autoreactive B-1 Cells by Interleukin 10 Induce Autoimmune Hemolytic Anemia in Fas-deficient Antierthrocyte Immunoglobulin Transgenic Mice

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Abstract

The Fas (CD95) gene is among critical genetic factors in some autoimmune diseases, which are characterized by autoantibody (autoAb) productions. In mice, mutations in the Fas gene cause lymphoproliferation (*lpr*) which predominantly develops glomerulonephritis, whereas the mutations in human cause autoimmune lymphoproliferative syndrome (ALPS) characterized by autoimmune hemolytic anemia (AIHA) and thrombocytopenia. Although the mechanism of antinuclear Ab in Fas-deficient background has been well characterized, that of antierthrocyte Ab production in ALPS has been still unclear. To investigate this mechanism, we developed a mouse line by crossing the antierthrocyte antibody transgenic mice (H+L6 mice) and Fas-deficient mice. Although Fas deficiency did not break tolerance of autoreactive B-2 cells in H+L6 mice, autoreactive B-1 cells in Fas-deficient H+L6 homozygous mice became activated and differentiated into autoAb-producing cells in mesenteric lymph nodes and lamina propria of intestine, resulting in severe anemia. In addition, serum levels of interleukin (IL)-10 significantly increased in Fas^{-/-} × H+L6 homozygous mice and administration of anti-IL-10 Ab prevented exacerbation of autoAb production and AIHA. These results suggest that activation of B-1 cells is responsible for induction of AIHA in Fas-deficient condition and that IL-10 plays a critical role in terminal differentiation of B-1 cells in these mice.

Key words: autoantigen • autoantibody • B cell tolerance • autoimmune lymphoproliferative syndrome • peritoneal cavity

Introduction

Autoimmune diseases are the outcome of complicated inflammatory processes in which various cellular and humoral components are involved. In some autoimmune diseases such as myasthenia gravis, autoimmune hemolytic anemia (AIHA), and idiopathic thrombocytopenic purpura, pathogenic autoAbs produced by B cells primarily mediate the disorders (1–3). The pathogenic autoAbs directly bind to membrane-bound antigens and cause autoimmune diseases, whereas other disease-related autoAbs such as rheumatoid factor do not directly trigger clinical manifestations (4).

Fas (CD95) and Fas ligand (FasL) genes are among the critical genetic factors for autoAb production in mice and human (1, 5, 6). Fas is expressed in a variety of cell types including activated B cells and T cells, whereas FasL is only expressed in activated T cells and NK cells. Interaction of Fas and FasL induces rapid apoptosis and plays a critical role in homeostasis of peripheral lymphocytes and prevention of autoimmunity (5, 6). In mice, mutations in Fas and FasL genes cause lymphoproliferation (*lpr*) and generalized lymphoproliferative disease (*gld*), respectively (5, 6). MRL/*lpr* mice develop glomerulonephritis with lymphadenopathy and splenomegaly, caused by accumulation of CD4⁻CD8⁻ T cells and production of autoAbs to intracellular antigens (5). The production of autoAbs was blocked by the specific expression of transgenic (Tg) Fas on B cells but not on T

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cells in *lpr/lpr* mice (7, 8), suggesting that Fas deficiency in B cells is responsible for autoAb productions. Several studies using Ig Tg/*lpr* mice have shown that central tolerance to soluble form of model antigens is partially impaired in Fas deficiency and that production of disease-associated autoAbs, such as anti-DNA Ab, rheumatoid factor, and anti-Sm Ab is markedly enhanced (9–12). However, it is not clear whether production of pathogenic autoAbs against membrane-bound antigens is affected in Fas deficiency.

In humans, mutations in Fas gene cause autoimmune lymphoproliferative syndrome (ALPS) (13). ALPS patients uniquely develop AIHA, idiopathic thrombocytopenic purpura, and autoimmune neutropenia, in addition to the common symptoms to MRL/*lpr* mice (13). Furthermore, ALPS patients but not MRL/*lpr* mice show increased numbers of CD5⁺ B cells and elevated levels of serum IL-10 (13–15). These results suggest that CD5⁺ B cells and IL-10 may play a role in production of pathogenic autoAbs to membrane-bound self-antigens.

We have generated several Tg mouse lines (H×L and H+L) in which almost all B cells have specificity for a membrane-bound antigen on self-RBC and cause AIHA (2). As autoreactive B cells are eliminated at the immature stages in the bone marrow, the number of mature B cells is markedly decreased in the periphery of these mice. In contrast, their peritoneal cavity (PerC) contains autoreactive B-1 cells, that can be activated to produce autoAb by IL-10, inducing AIHA (16, 17).

To assess whether production of pathogenic autoAb against membrane-bound self-antigens is affected in Fas deficiency, we crossed Fas-deficient mice and H+L6 mice. Autoreactive B-1 cells in Fas-deficient H+L6 homozygous mice were activated to induce severe anemia. In addition, serum levels of IL-10 significantly increased in these mice and administration of anti-IL-10 Ab blocked exacerbation of autoAb production and anemia. These results suggest that activation of B-1 cells, triggered by IL-10, is responsible for induction of AIHA in Fas-deficient condition.

Materials and Methods

Tg Mice. We generated several lines of the anti-RBC mAb (4C8 mAb) Tg (H+L) mice which carried tandem joined H and L chain transgenes (18). By mating H+L6 heterozygous mice and Fas-deficient mice (19), we obtained Fas-deficient H+L6 homozygous mice. The genotype was determined by PCR of tail DNA. The PCR primers were described previously (18, 19). The homozygosity of the transgene was screened by Southern analysis. The mice were maintained under conventional conditions in our animal facility and were analyzed at 8–12 wk of age.

Detection of Anti-RBC AutoAb Production. The amounts of autoAbs on RBCs were measured as described previously (20).

Preparation of Single Cell Suspensions. Isolation of lamina propria (LP) lymphocytes from the small intestine and preparation of cells from bone marrow, mesenteric lymph nodes (MLNs), and PerC were done as described previously (20).

Flow Cytometry. Flow cytometric analysis was performed by a FACSCalibur™ with CELLQuest™ software version 3.1 (Bec-

ton Dickinson) as described previously (20). After excluding dead cells by propidium iodide gating, cells present in the lymphocyte gate defined by forward and side light scatters were analyzed.

Enzyme-linked Immunospot Assay and Cytoplasmic Staining. Enzyme-linked immunospot (ELISPOT) assay and cytoplasmic staining were performed on freshly isolated cells from lymphoid organs as described previously (20).

Cytokine ELISA. The levels of serum IL-4, IL-5, IL-6, and IL-10 were assessed using the mouse IL-4-, IL-5-, IL-6-, and IL-10-ELISA systems (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Administration of Anti-Mouse IL-10 Ab. Either rat anti-mouse IL-10 mAb (100 µg/injection; Genzyme) or control rat IgG (100 µg/injection; BD PharMingen) was injected intraperitoneally into 4-wk-old Fas-deficient H+L6 homozygous mice weekly for 4 wk.

Results

Fas Deficiency Markedly Enhances AutoAb Production and Anemia in H+L6 Homozygous Mice. To assess how autoimmunity for membrane-bound autoantigens develops in Fas deficiency, we crossed H+L6 mice with Fas^{-/-} mice and compared the phenotypes of H+L6 heterozygous or homozygous mice. In H+L6 mice, the size of Tg B-1 cell compartment in PerC is larger in homozygous than heterozygous as described previously (18). As bacterial infection and/or normal bacterial flora can induce autoAb production of B-1 cells in H×L mice (2) and conventional conditions represent the situations that ALPS patients are exposed in daily life, we bred H+L6 mice under conventional conditions.

First, we examined the amount of the autoAb bound to circulating RBCs by flow cytometry and the incidence of anemia by measuring hematocrit (Ht) values of the peripheral blood (Fig. 1). Neither the level of the autoAb nor the Ht values was changed between Fas^{+/-} and Fas^{-/-} H+L6 heterozygous mice. In contrast, a small amount of the autoAb was detected in Fas^{+/-} × H+L6 homozygous mice and their Ht values were slightly reduced compared with Fas^{+/-} × H+L6 heterozygous mice. More importantly, in Fas^{-/-} × H+L6 homozygous mice, the amount of the autoAb was markedly increased and Ht values were drastically reduced. These results indicate that Fas deficiency enhances autoAb production and causes severe anemia in only H+L6 homozygous mice.

Localization of Autoreactive B Cells and AutoAb-producing Cells in H+L6 Homozygous Mice Carrying Fas Deficiency. As H+L6 homozygous mice contain a larger population of autoreactive B-1 cells than H+L6 heterozygous mice, it is possible that B-1 cells may be involved in the autoAb production in these mice. To examine the localization of autoreactive B cells, we stained cells in bone marrow, spleen, MLNs, PerC, and LP of the gut with the anti-IgM and anti-idiotypic (S54) Abs and analyzed them by flow cytometry. As both anti-IgM and anti-idiotypic Abs stain determinants on the same Ig molecule, the Tg B cells which express both Tg IgH and IgL on their surface were stained tight diagonal as described previously (10, 18, 21). Thus,

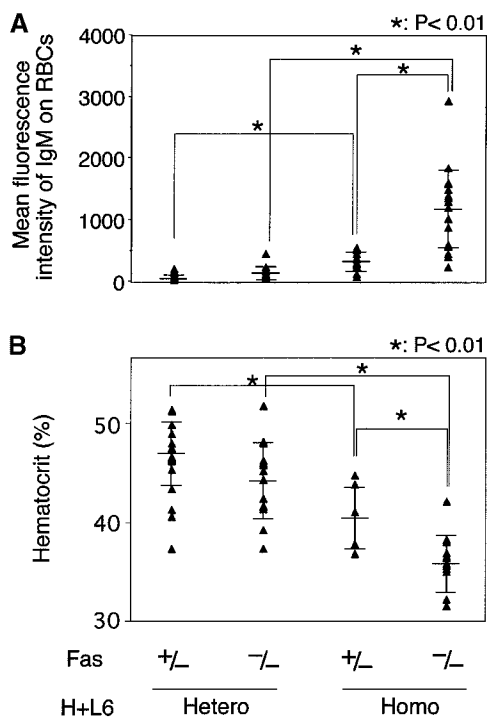


Figure 1. Marked enhancement of autoAb production and anemia in H+L6 homozygous mice by Fas deficiency. (A) Amounts of anti-RBC autoAb bound to circulating RBCs. Peripheral blood RBCs were incubated with FITC-anti-mouse IgM antibody and analyzed by flow cytometry. Closed triangles indicate the mean fluorescence intensity of IgM on RBCs in individual mice. Horizontal long and short bars indicate the mean and SD of each group, respectively. (B) Hematocrit values of peripheral blood. Closed triangles indicate the hematocrit value of individual mice. Horizontal bars are as described in A. The Student's *t* test for unpaired data was used to compare the values between indicated groups.

this allows cleaner recognition and enumeration of truly autoreactive B cells.

A significant number of IgM⁺S54⁺ cells, expressing Mac-1 (data not shown), were observed in PerC of H+L6 homozygous mice, and their compartment was much larger in H+L6 homozygous than in heterozygous mice (Fig. 2 A). Importantly, although IgM⁺S54⁺ cells were slightly increased in PerC of Fas^{-/-} × H+L6 heterozygous mice compared with the Fas^{+/-} mice, the IgM⁺S54⁺ cells were significantly reduced in Fas^{-/-} × H+L6 homozygous mice compared with the Fas^{+/-} mice. Neither bone marrow or spleen contained distinct IgM⁺S54⁺ cells in H+L6 homozygous mice (Fig. 2 B), because of clonal deletion of autoreactive B-2 cells as described previously (18). In contrast, both MLNs and LP in Fas^{-/-} × H+L6 homozygous mice had a small but significant number of IgM⁺S54⁺ cells. Taken together, the absence of B-2 cells in the periphery and the decrease of B-1 cells in PerC with concomitant appearance of IgM⁺S54⁺ cells in MLNs and LP are in parallel with the observation we described previously (20). Thus, these results strongly suggest that the autoreactive B-1 cells in PerC may have migrated to MLNs and LP and differentiated into plasma cells in Fas^{-/-} × H+L6 homozygous mice. Staining of IgM⁺S54⁺ cells in MLNs and LP differs

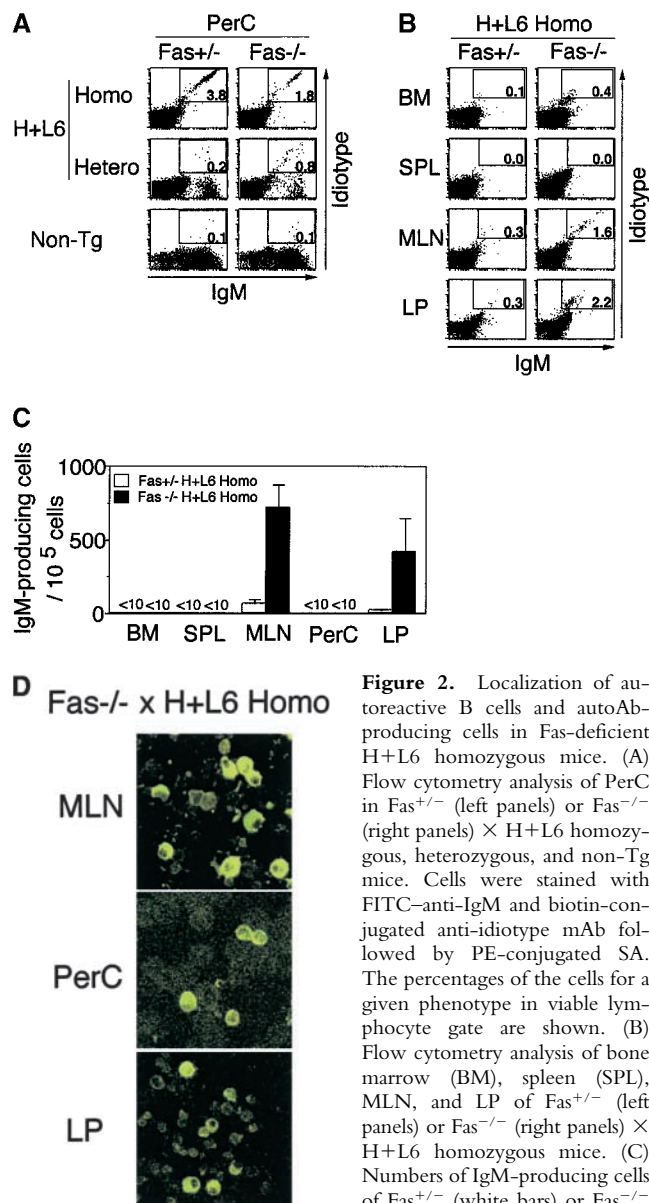


Figure 2. Localization of autoreactive B cells and autoAb-producing cells in Fas-deficient H+L6 homozygous mice. (A) Flow cytometry analysis of PerC in Fas^{+/-} (left panels) or Fas^{-/-} (right panels) × H+L6 homozygous, heterozygous, and non-Tg mice. Cells were stained with FITC-anti-IgM and biotin-conjugated anti-idiotypic mAb followed by PE-conjugated SA. The percentages of the cells for a given phenotype in viable lymphocyte gate are shown. (B) Flow cytometry analysis of bone marrow (BM), spleen (SPL), MLN, and LP of Fas^{+/-} (left panels) or Fas^{-/-} (right panels) × H+L6 homozygous mice. (C) Numbers of IgM-producing cells of Fas^{+/-} (white bars) or Fas^{-/-} (black bars) × H+L6 homozygous mice in BM, SPL, MLN, PerC, and LP were measured by ELISPOT assay. The mean and SD of IgM-producing cells per 10⁵ cells was calculated from at least three mice. (D) Cells were isolated from MLN, PerC, and LP of Fas^{-/-} × H+L6 homozygous mice, and then 2 × 10⁵ cells of each were fixed, and stained with FITC-anti-mouse IgM antibody. Preparations were examined and photographed by fluorescence microscope. Original magnifications: each panel ×400.

from that in PerC. This is probably because of lower surface Ig expression on Ig-secreting cells in MLNs and LP.

To show where the autoAb is produced in Fas^{-/-} × H+L6 homozygous mice, we measured the numbers of autoAb-producing cells in the bone marrow, spleen, MLNs, PerC, and LP (Fig. 2 C). As H+L6 homozygosity strongly blocks the rearrangement of endogenous Ig genes, almost all IgM⁺ cells in these mice express Tg IgM (18). Therefore, we performed ELISPOT assay using anti-IgM Ab. Although only small numbers of IgM-producing cells were observed in Fas^{+/-} × H+L6 homozygous mice, we

detected large numbers of Ab-producing cells in MLNs and LP, but not in other organs, of Fas^{-/-} × H+L6 homozygous mice.

As we found a difference in the distribution between surface Ig expressing cells and IgM-producing cells, we further confirmed staining pattern and morphology by cytoplasmic IgM staining. Almost all IgM⁺ cells in PerC of Fas^{-/-} × H+L6 homozygous mice showed surface staining pattern of IgM (Fig. 2 D). In contrast, the majority of IgM⁺ cells in MLNs and LP had typical plasma cell morphology. These IgM⁺ plasma cells probably have lost some of surface Ig expression. Taken together, these results suggest that the autoreactive PerC B-1 cells may migrate and differentiate in MLNs and LP. They are also consistent with our previous observation that the autoAb production predominantly occurred in MLNs in recombination activating gene (RAG)2^{-/-} × H×L mice with activated $\gamma\delta$ T cells (20).

Serum Levels of IL-10 But Not IL-4, IL-5, and IL-6 Are Significantly Elevated in Fas-deficient H+L6 Homozygous Mice. To examine which cytokine is involved in B-1 cell migration and terminal differentiation in Fas^{-/-} × H+L6

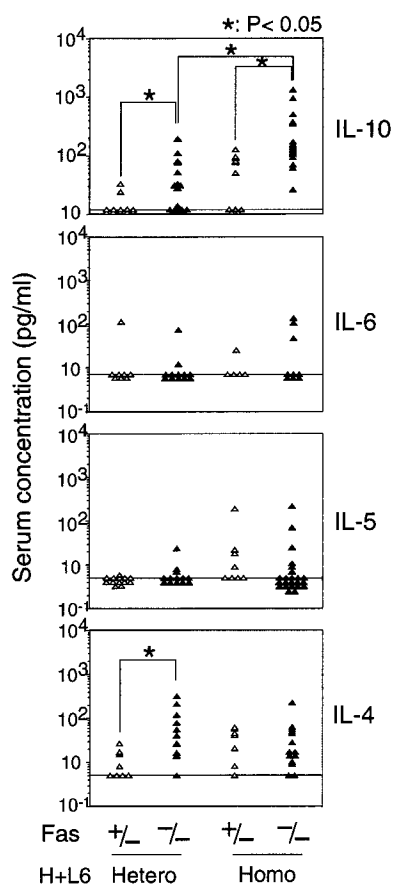


Figure 3. The serum levels of IL-10, but not IL-4, IL-5, and IL-6 were elevated in Fas-deficient H+L6 homozygous mice. Amounts of IL-4, IL-5, IL-6, and IL-10 in the serum were assessed in H+L6 heterozygous and homozygous mice with (closed triangles) or without (open triangles) Fas-deficient background by ELISA. Horizontal long bars indicate the minimum detectable levels of each cytokine. The Student's *t* test for unpaired data was used to compare the values between indicated groups.

homozygous mice, serum concentrations of IL-4, IL-5, IL-6, and IL-10 were assessed by ELISA. The levels of IL-10 but not IL-4, IL-5, and IL-6 were significantly elevated in Fas^{-/-} × H+L6 homozygous mice compared with the other control mice (Fig. 3). Thus, these results suggest that the elevation of IL-10 may play a critical role in autoreactive B-1 cell migration and terminal differentiation in Fas^{-/-} × H+L6 homozygous mice, as described previously (17, 20). In addition, although the levels of IL-10 in Fas^{-/-} non-Tg mice were not increased (data not shown), the levels of IL-10 in Fas^{-/-} × H+L6 heterozygous mice were slightly but significantly increased when compared with Fas^{+/-} control mice. Therefore, the increase of serum IL-10 concentration closely correlated with the migration and terminal differentiation of autoreactive B-1 cells in Fas deficiency.

Administration of Anti-IL-10 Ab Prevents Exacerbation of Anemia in Fas-deficient H+L6 Homozygous Mice. To test whether the elevated IL-10 levels play an essential role in autoAb production in H+L6 homozygous mice, we injected the anti-IL-10 mAb into Fas-deficient H+L6 homozygous mice weekly for 4 wk. Injection of anti-IL-10 Ab blocked reduction of Ht values in Fas^{-/-} × H+L6 homozygous mice compared with control rat IgG injection (Table I). IL-10 neutralization also suppressed increase in the amount of anti-RBC Ab and the numbers of autoAb-producing cells in the MLN of Fas^{-/-} × H+L6 homozygous mice. These results clearly demonstrate that the elevation of serum IL-10 is responsible for the enhancement of autoAb production in Fas^{-/-} × H+L6 homozygous mice.

Discussion

In this study, we found marked autoAb production for RBC membrane Ag in only H+L6 homozygous but not in heterozygous mice under Fas-deficient background. Despite the same Ag specificity, higher levels of surface Tg

Table I. Administration of Anti-IL-10 mAb Blocks Exacerbation of Anemia in Fas-deficient H+L6 Homozygous Mice

Treatments	Hematocrit values (%)	Amounts of the autoAb bound to RBCs (mean fluorescence intensity)	Ig-producing cells (per 10 ⁵ cells)
Anti-IL-10	37.5 ± 2.5 ^a	494.4 ± 364.2 ^b	235.8 ± 173.1 ^b
Control Ig	31.5 ± 0.5	1,419.2 ± 497.2	660.6 ± 240.2

Either anti-IL-10 mAb or control rat IgG (100 μ g/injection) was injected weekly into Fas-deficient H+L6 homozygous mice. 4 wk after the first injection, hematocrit values (%) were measured. Amounts of the autoAb bound to RBCs were assessed as mean fluorescence intensity of FITC-conjugated anti-IgM Ab bound to RBCs. Numbers of anti-RBC Ab-producing cells in MLN were measured by ELISPOT assay using anti-IgM Ab. Values represent the mean \pm SD of five mice in each experimental group. Paired *t* test values calculated between anti-IL-10 and control rat IgG.

^a*P* < 0.01.

^b*P* < 0.02.

immunoglobulin expression of H+L6 homozygous mice results in stronger clonal deletion of autoreactive B-2 cells from periphery compared with heterozygous mice. At the same time, the size of autoreactive B-1 cell compartment in PerC is larger in homozygous than in heterozygous mice (18). Thus, our results suggest that B-1 cells are responsible for autoAb production in Fas-deficient background. Indeed, in Fas^{-/-} × H+L6 homozygous mice, autoreactive B-1 cells can efficiently migrate and differentiate into plasma cells (Fig. 2, A–D). In contrast, in H+L6 heterozygous mice, Fas deficiency did not alter the size of the B-1 cell population (data not shown, and Fig. 2 A).

Because Fas deficiency enhances migration and differentiation but not expansion of autoreactive B-1 cells, secondary genetic factors responsible for expansion of B-1 cells may be required for induction of AIHA. Indeed, genetic background plays a critical role for autoAb production and autoimmune diseases in *lpr* mice and activation of B-1 cells in PerC (22, 23). In human, additional genetic factors are also suggested to be involved in lymphoproliferation and autoimmunity in ALPS (24).

Although we have shown that the serum levels of IL-10 were significantly elevated in Fas^{-/-} × H+L6 homozygous mice, we could not identify a particular cell population as the main source of IL-10 *in vivo*. Our preliminary experiments of intracellular cytokine staining suggested that not only B-1 cells but also aberrant T cells and macrophages in PerC of H+L6 homozygous mice can produce significant amount of IL-10 by *ex vivo* stimulation (unpublished data). Indeed, in ALPS patients, aberrant CD4⁺CD8⁻ T cells with activated phenotypes accumulate in peripheral lymphoid organs and can produce large amounts of IL-10 (15). B-1 cells can also produce IL-10 (25, 26). In addition, we previously showed that both activated T cells and macrophages are involved in increased serum levels of IL-10 and in migration and differentiation of peritoneal B-1 cells (17, 20). Thus, aberrant T cells, autoreactive B-1 cells, and macrophages may cooperate in elevation of IL-10, resulting in activation of autoreactive B-1 cells in Fas-deficient condition.

How IL-10 induces activation of autoreactive B-1 cells is not yet clear. *In vivo* role of IL-10 in murine B cell function is limited (27). However, several previous studies suggest that IL-10 is important for B-1 cell expansion and activation (23, 28, 29). We have also shown that IL-10 elevation induced by LPS administration or IL-10 administration itself can induce autoAb production and anemia in H×L mice (16, 17). However, we could not detect direct effects of IL-10 for proliferation or differentiation of autoreactive B-1 cells (17). Furthermore, we did not find anti-apoptotic effect for B-1 cells in PerC against RBC injection in Fas^{-/-} × H+L6 homozygous mice (data not shown). On the other hand, Fas-deficient background markedly enhances migration and differentiation of autoreactive B-1 cells to not only MLNs but also LP in H+L6 homozygous mice, whereas autoreactive B-1 cells in RAG2^{-/-} × H×L mice with activated $\gamma\delta$ T cells predom-

inantly migrate into MLNs but not LP (20). Thus, Fas deficiency may help autoreactive B-1 cells to terminally differentiate and migrate into LP, escaping from rapid apoptosis. Recently, Balabanian et al. have shown that IL-10 increases the effects of stromal cell-derived factor-1 on the proliferation and survival of B-1 cells in PerC and that IL-10 is chemokinetic for peritoneal B-1 cells, increasing their random mobility (30). Thus, these effects of IL-10 may play a role in indirect activation of autoreactive B-1 cells of Fas^{-/-} × H+L6 homozygous mice.

In summary, we have shown that autoreactive B-1 cells in PerC migrate into MLNs and LP and differentiate into Ab-producing cells with Fas-deficient background. Elevated IL-10 levels are involved in the activation of autoreactive B-1 cells. These results are in parallel with the observation that ALPS patients with AIHA manifest increased numbers of CD5⁺ B cells and elevated serum levels of IL-10, implying that blocking of IL-10 signals may become a therapeutic approach to autoimmune diseases in ALPS patients.

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