Migration and Differentiation of Autoreactive B-1 Cells Induced by Activated γ/δ T Cells in Antierythrocyte Immunoglobulin Transgenic Mice

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Abstract

Using normal and transgenic (Tg) mice, we have shown that peritoneal B-1 cells are activated by administration of cytokines or lipopolysaccharide and migrate to other lymphoid organs where they differentiate into antibody-secreting cells. However, little is known about the process of B-1 cell migration and differentiation in vivo. We developed a mouse line by crossing the antierythrocyte antibody Tg mice (HL mice) with TCR-γ/δ Tg mice specific for a self-thymus leukemia (TL) antigen in the recombination activating gene (RAG)$2^{-/-}$ background. In the presence of the self-antigen, Tg γ/δ T cells increased in number and manifested activated phenotypes. Peritoneal B-1 cells in these mice migrated into mesenteric lymph nodes and differentiated into autoantibody-secreting cells, resulting in strong autoimmune hemolytic anemia. Furthermore, transfer of RAG$2^{-/-}$ × HL bone marrow or peritoneal cells into the peritoneal cavity of RAG$2^{-/-}$ × TCR-γ/δ Tg mice gave rise to donor-derived B-1 cells in mesenteric lymph nodes, and these cells produced the autoantibody. Thus, this study demonstrates that the migration of B-1 cells and differentiation into the antibody-secreting cells can be induced by noncognate T cell help and implies the possibility that γ/δ T cells may induce B-1 cell differentiation in vivo.

Key words: RAG2 deficient • γ/δ TCR transgenic • germinal center • peritoneal cavity • hemolytic anemia

Introduction

B cells are divided into two subsets, B-1 and B-2, by their distinct tissue distribution, antigen specificity, and surface markers. B-1 cells are a predominant population in the peritoneal cavity (PerC),[1] whereas they are rare in spleen and extremely few, if any, are in LNs (1, 2). B-1 cells often recognize self-antigens with low affinity and broad specificities (3, 4). In contrast, B-2 cells recognize a wide variety of antigens with high affinity and strong specificities. B-1 cells have higher surface IgM levels than B-2 cells (5). B-1 cells are also distinguished from B-2 cells by their unique surface markers such as CD5, Mac-1, and low B220 expression (6). As B-1 cells are often involved in the production of pathogenic and natural autoantibodies (3, 7–9), their generation, migration, and differentiation into antibody-producing cells are important issues in understanding autoimmune diseases and innate immunity.

We have generated transgenic (Tg) mice with H and L chains of anti-RBC antibody (HL mice [9]). As autoreactive B cells are eliminated at the immature stages in the bone marrow (BM), the number of self-reactive B cells is markedly decreased in the BM, peripheral blood, spleen, and LNs in HL mice. In contrast, the PerC of the HL mice contains a normal number of autoreactive B-1 cells. Therefore, HL mice are one of the ideal models to analyze the mechanisms of B-1 cell activation in vivo. Our previous study using HL mice has shown that peritoneal B-1 cells can be induced to produce autoantibody by T cell–independent stimulation like LPS, suggesting that bacterial infections may be responsible for B-1 cell activation (10). Indeed, over half of the HL mice bred under conventional conditions manifest severe anemia, whereas none of the HL

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[1]*Abbreviations used in this paper:* BM, bone marrow; ELISPOT, enzyme-linked immunospot; IEL, intraepithelial lymphocyte; LP, lamina propria; MLN, mesenteric LN; NF, nuclear factor; PerC, peritoneal cavity; PNA, peanut agglutinin; RAG, recombination activating gene; SPF, specific pathogen-free; Tg, transgenic; TL, thymus leukemia.

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mice bred under specific pathogen-free (SPF) conditions produce the autoantibody despite the full expansion of B-1 cells in the PerC (11). In addition, administration of some Th2 cytokines like IL-5 and IL-10 but not IL-4 can induce differentiation of autoreactive B-1 cells in HL mice (12). These studies indicate that B-1 cells in the PerC can be activated by the noncognate help of T cells, probably by the secretion of cytokines.

More recently, we have suggested that the migration of B-1 cells out of the PerC may be essential for activation and differentiation of B-1 cells (13). The alymphoplasia (*aly*) mutation of the nuclear factor (NF)-κB–inducing kinase gene (14) causes the defect of the migration of peritoneal B-1 cells into the gut-associated lymphoid tissue, especially lamina propria (LP). This migration defect is associated with the defect of IgA production in the LP. Indeed, it has been suggested that there is a link between peritoneal B cells and IgA plasma cells in the LP (15). Several lines of evidence demonstrated that half of the IgA plasma cells in the LP are derived from PerC B-1 cells. (16–18).

To test the hypothesis that migration and differentiation of B-1 cells may be regulated by noncognate T cell help, we have developed a mouse line by crossing the HL mice with TCR-γ/δ Tg mice on the recombination activating gene (RAG)$2^{-/-}$ background, in which all T cells recognize a self-thymus leukemia (TL) antigen. In these mice, the γ/δ T cells became activated in the presence of the self-antigen. In addition, B-1 cells migrated into mesenteric LNs (MLNs) and differentiated into autoantibody-producing cells, resulting in severe autoimmune hemolytic anemia in the HL mice. Thus, this study demonstrates that the migration of B-1 cells and differentiation into antibody-producing cells can be induced by a T cell help that is not through so-called cognate interaction, and implies the possibility that γ/δ T cells may induce B-1 cell differentiation in vivo. In addition, these results clearly support the hypothesis that migration from the PerC is required for the terminal differentiation of B-1 cells.

Materials and Methods

Tg Mice. Tg mouse lines carrying either the H or L chain gene for the anti-RBC antibody (4C8 antibody) were established and double-Tg (HL) mice with H and L chain transgenes were generated as described previously (9). RAG2-deficient mice (19) and Tg mice carrying productively rearranged Vγ2/Vδ5 TCR genes that recognize a TL antigen of H-2^b haplotype (KN6 Tg mice [20]) were crossed to obtain RAG2-deficient KN6 Tg heterozygous mice. By mating these mice with H and L chain Tg mice, we finally obtained RAG2-deficient KN6 Tg × HL mice. The mice were maintained under SPF conditions in our animal facility. The H-2 haplotype was determined by flow cytometry with peripheral blood cells. In this study, mice were analyzed at 8–14 wk of age.

Preparation of Single Cell Suspensions. LP lymphocytes and intraepithelial lymphocytes (IELs) of the small intestine were isolated by the method described previously (21, 22). BM cells were prepared by flashing femur bones with a staining buffer (PBS containing 2% FCS and 0.05% sodium azide). Peritoneal washout cells were prepared by flushing the PerC with 10 ml of the staining buffer.

Detection of Anti-RBC Autoantibody Production. The amounts of autoantibodies on erythrocytes were measured by flow cytometry with FITC-goat anti–mouse IgM antibody (ICN Biomedicals/Cappel) as described previously (9). Mean fluorescence intensity was measured by FACSCalibur™ (Becton Dickinson).

Enzyme-linked Immunospot Assay. The number of antibody-producing cells in HL mice was determined by enzyme-linked immunospot (ELISPOT) assay with goat anti–mouse IgM antibody (Cappel) as described previously with slight modification (23). We used alkaline phosphatase–goat anti–mouse IgM antibody (Southern Biotechnology Associates, Inc.).

Flow Cytometry. The following monoclonal antibodies were used: FITC-conjugated antibodies anti–TCR-α/β (H57-597) and anti-Vγ2 TCR (UC3-10A6); PE-conjugated antibodies anti-CD45R/B220 (RA3-6B2), anti-CD11b (Mac-1 α chain, M1/70), anti–TCR-γ/δ (GL3), anti-CD5 (53-7.3), anti-CD43 (S7), anti-CD44 (IM7), and anti-CD69 (H1.2F3); and CyChrome–conjugated antibody anti-CD45R/B220. These antibodies were purchased from BD PharMingen. FITC-F(ab′)$_2$ fragment of goat anti–mouse IgM was from Cappel. PE-streptavidin was from Dako. Antiidiotype antibody (S54) against the transgene-encoded anti-RBC antibody (4C8) was biotinylated according to the directions provided by the manufacturer (Pierce Chemical Co.). Flow cytometric analysis was performed as described (24) by a FACSCalibur™ with CELLQuest™ software v3.1 (Becton Dickinson). After excluding dead cells by propidium iodide gating, cells present in the lymphocyte gate defined by forward and side light scatters were analyzed.

Cytoplasmic Staining. Single cell suspensions from MLNs, PerC, and LP of small intestine were mounted on glass slides by cytocentrifuge preparation (Cytospin 3; Shandon Inc.). They were fixed in methanol for 15 min, and blocked with 5% heat-inactivated normal goat serum. Slides were incubated with FITC-F(ab′)$_2$ fragment of goat anti–mouse IgM (Cappel) for 30 min at room temperature. After the final wash, the slides were mounted and examined under a confocal fluorescence microscope.

Adoptive Transfer. A total of 1.5×10^7 BM cells or 7×10^6 PerC cells from RAG2-deficient HL mice were injected intraperitoneally into RAG$2^{-/-}$ recipient mice with or without KN6 Tg. After 6 wk, the mice were killed, and lymphocytes in various lymphoid organs were analyzed by flow cytometry and cytoplasmic staining. Autoantibody levels on erythrocytes were measured by flow cytometry.

Immunohistological Analysis. Sections of MLN were fixed in formalin and stained with hematoxylin and eosin by standard methods. Fluorescence immunohistology was performed on frozen sections. Sections of 6 μm were cut from tissue blocks and mounted onto glass slides. The sections were air dried for 30 min, fixed in 4% paraformaldehyde for 15 min, and blocked with 5% heat-inactivated normal goat serum. The sections were stained with biotin–anti-mouse IgM antibody (Cappel), followed by FITC–anti–peanut agglutinin (PNA; Vector Laboratories) or anti-Vγ2 TCR (BD PharMingen) antibody and Texas red–streptavidin (Life Technologies). After the final wash, the slides were mounted and examined under a confocal fluorescence microscope.

ELISA. Amounts of IL-4, IL-5, IL-6, and IL-10 in the serum from mice 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.

Results

Activated Tg γ/δ T Cells Induce Autoantibody Production and Anemia in HL × RAG2$^{-/-}$ Mice. RAG2$^{-/-}$ × KN6 Tg mice have neither conventional T cells nor B cells, but have Tg γ/δ T cells that recognize an autologous TL antigen encoded by one of the MHC class I genes designated 27^{b} (25). The MHC class I gene 27^{b} is mapped on the MHC TL region in C57BL/6 strain (H-2^{b}) but not in BALB/c (H-2^{d}). The TL antigen is mainly expressed on thymocytes, splenic T and B cells, PerC Mac-1^{+}, and CD5^{+} cells. Although Tg γ/δ T cells are mostly deleted in RAG2$^{+/-}$ mice with the H-2^{b} haplotype as described previously (20), higher numbers of Tg γ/δ T cells were identified in RAG2$^{-/-}$ mice (Fig. 1 A), probably because they escaped clonal deletion more easily in the absence of endogenous lymphocytes expressing the TL antigen. The percentages of the Tg γ/δ T cells increased dramatically in MLNs and PerC in RAG2$^{-/-}$ mice with the H-2^{b} haplotype. However, the total cell number in PerC ($0.8 \pm 0.2 \times 10^5$) of these mice was somewhat decreased compared with that of RAG2$^{+/-}$ mice ($1.5 \pm 0.5 \times 10^5$), probably because the continuous interaction with Mac-1^{+} cells, which express relatively large amounts of the TL antigen (25), resulted in rapid apoptosis of activated Tg γ/δ T cells. In contrast, the total cell number in MLNs ($23.8 \pm 6.1 \times 10^5$) was increased in RAG2$^{-/-}$ compared with that in RAG2$^{+/-}$ mice ($5.6 \pm 1.1 \times 10^5$). Thus, the size of the Tg γ/δ T cell compartment in MLNs is clearly enlarged in RAG2$^{-/-}$ mice with the H-2^{b} haplotype.

To see the status of Tg γ/δ T cells in RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^{b} haplotype, we analyzed cells in spleen, MLNs, PerC, and IELs by flow cytometry with anti-Vγ2 antibody and either anti–TCR-γ/δ, anti-CD44, or anti-CD69 antibody. Tissue distribution of γ/δ T cells in these mice was indistinguishable from that of RAG2$^{-/-}$ × TCR-γ/δ Tg mice with the H-2^{b} haplotype (data not shown). As shown in Fig. 1 B, Tg γ/δ T cells in the H-2^{b} haplotype mice downregulated TCR expression slightly, whereas their cell sizes were enlarged as assessed by forward scatter, and their expression levels of CD44 and CD69, activation markers of peripheral T cells (26–28), were enhanced compared with Tg γ/δ T cells in the H-2^{d} haplotype mice. These results indicate that the majority of Tg γ/δ T cells in the MLNs and PerC are activated in RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^{b} haplotype.

To assess whether the presence of activated Tg γ/δ T cells enhances autoantibody production and anemia in HL mice, we examined the amount of the autoantibody bound to erythrocytes and the incidence of anemia in the presence or absence of the H-2^{b} haplotype. We identified large amounts of the autoantibody bound to erythrocytes in RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^{b} haplotype, whereas we could not detect significant amounts of the autoantibody in other control mice that do not have activated Tg γ/δ T cells in MLNs (Fig. 2 A). Similarly, induction of anemia was observed only in RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^{b} haplotype (Fig. 2 B). These results indicate that the presence of activated γ/δ T cells is critical for induction of the autoantibody production and anemic phenotype in HL mice.

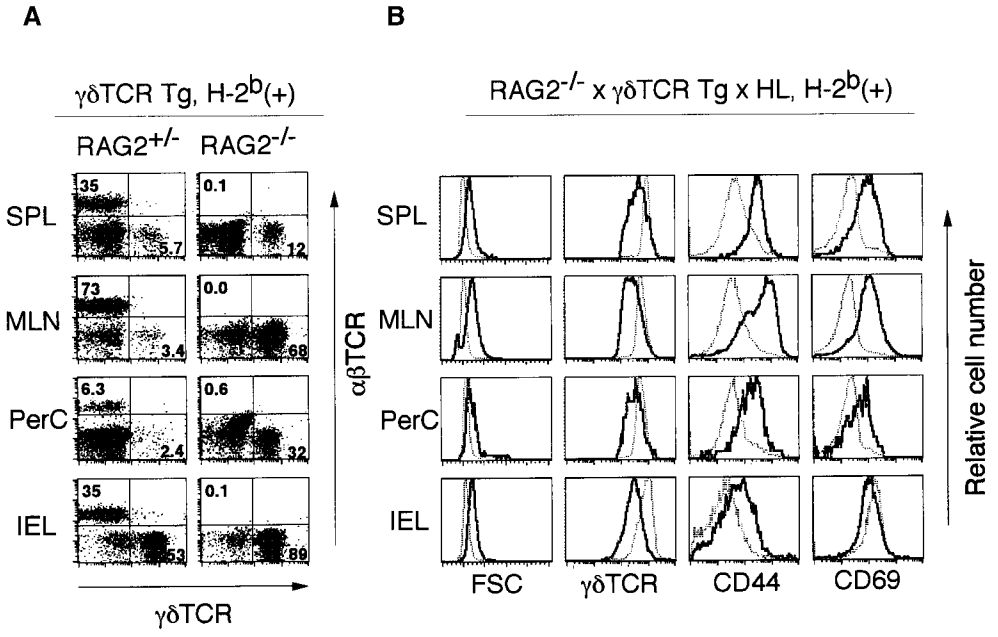

Figure 1. The expansion and activation of Tg γ/δ T cells. (A) Expansion of Tg γ/δ T cells in RAG2-deficient mice with the H-2^{b} haplotype. The cells were isolated from spleen (SPL), MLNs, PerC, and IELs of γ/δ Tg mice with the H-2^{b} haplotype in RAG2$^{+/-}$ (left) or RAG2$^{-/-}$ (right) background and stained with FITC–anti–TCR-α/β and PE–anti- TCR-γ/δ antibodies. Percentages of α/β T and γ/δ T cells are shown. The data are representative of at least three mice. (B) Activation of γ/δ T cells in RAG2-deficient TCR-γ/δ Tg × HL mice with the H-2^{b} haplotype. Cells were isolated from spleen (SPL), MLNs, PerC, and IELs of RAG2$^{-/-}$ × KN6 Tg × HL mice with (solid lines) or without H-2^{b} haplotype (dotted lines) and stained with FITC–anti-Vγ2 TCR antibody and either PE–anti–TCR-γ/δ, anti-CD44, or anti-CD69 antibody. Data are the staining profiles of Vγ2^{+} cells.

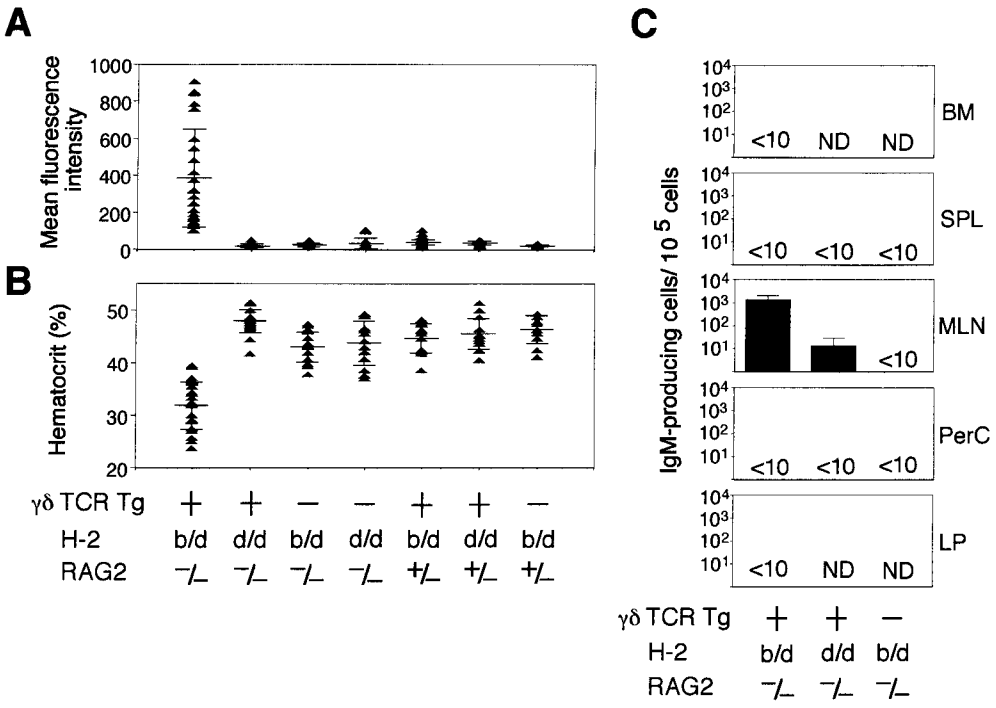

Figure 2. Induction of autoantibody production and anemia in RAG2-deficient TCR-γ/δ Tg × HL mice with the H-2^b haplotype. (A) Amounts of anti-RBC autoantibody bound to circulating RBCs. Peripheral blood RBCs were incubated with FITC-anti–mouse IgM antibody and analyzed by flow cytometry. Filled triangles indicate the mean fluorescence intensity of individual mice. Horizontal long bars and short bars indicate the mean and SD of each group, respectively. (B) Hematocrit values of peripheral blood. Filled triangles indicate the hematocrit value of individual mice. Horizontal long bars and short bars indicate the mean and SD of each group, respectively. (C) Numbers of IgM-producing cells in BM, spleen (SPL), MLNs, PerC, and LP were measured by ELISPOT assay. The mean and SD of IgM-producing cells per 10^5 cells were calculated from at least three mice. ND, not done.

MLN Is the Main Site of the Autoantibody Production in RAG2$^{-/-}$ × KN6 Tg × HL Mice with the H-2^b Haplotype. To see where the autoantibody is produced, we measured the number of IgM-producing cells in BM, spleen, MLNs, PerC, and LP of RAG2$^{-/-}$ × KN6 Tg × HL mice in the absence or presence of the H-2^b haplotype by the ELISPOT assay. We identified a large number of IgM-secreting cells in the MLNs but not in the BM, spleen, PerC, or LP of RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^b haplotype (Fig. 2 C). In contrast, a small number of IgM-producing cells was found in MLNs and only few numbers of IgM-producing cells were observed in the other organs of RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^d haplotype (Fig. 2 C). The results of the ELISPOT assay were further confirmed by cytoplasmic IgM staining of cytospin preparations. The majority of IgM$^+$ cells in the MLNs of RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^b haplotype had typical plasma cell morphology (Fig. 3, top panels). We also confirmed that PerC and LP of the small intestine contained only a small number of plasma cells in these mice under the SPF conditions (Fig. 3, middle and bottom panels). Taken together, these results indicate that the autoantibody production predominantly occurred in the MLNs but much less in the BM, spleen, PerC, and LP in RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^b haplotype.

Decrease of B-1 Cells in PerC and Increase of B220$^-$IgM$^+$ Cells in MLNs of RAG2$^{-/-}$ × KN6 Tg × HL Mice with the H-2^b Haplotype. As normal PerC B1 cells have been shown to migrate to MLNs and to differentiate into antibody-producing cells (13), we examined whether Tg B-1 cells migrate from the PerC and differentiate into the autoantibody-producing cells in the MLNs of RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^b haplotype. As RAG2 deficiency completely blocks the development of endogenous B cells, all IgM$^+$ cells in RAG2$^{-/-}$ × HL mice are Tg B cells with the autoreactivity. Regardless of the H-2 haplotype, RAG2$^{-/-}$ × KN6 Tg × HL mice contained some B220$^+$IgM$^+$ cells in BM (Fig. 4 A), in agreement with the previous report of HL mice (9). Although in the spleen, MLNs, and LP, very few B220$^+$IgM$^+$ cells could be detected, PerC contained a normal number of B220$^+$ IgM$^+$Mac-1$^+$ cells in RAG2$^{-/-}$ × KN6 Tg × HL mice without the H-2^b haplotype (Fig. 4 A, and data not shown). Similarly, a normal number of B-1 cells was observed in the PerC of RAG2$^{-/-}$ × HL mice under SPF conditions (data not shown). In contrast, PerC B-1 cells were drastically reduced in RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^b haplotype (Fig. 4 A). Importantly, MLNs of these mice contained a markedly increased number of IgM$^+$ cells, especially B220$^-$IgM$^+$ large lymphocytes, which are most likely plasma cells (Fig. 3). In addition, these B cells expressed Mac-1, CD5, CD43, and CD44 antigens (Fig. 4 B), suggesting that they might be derived from PerC B-1 cells (6). The absence of B-2 cells in the periphery and the drastic decrease of B-1 cells in PerC with concomitant appearance of IgM-secreting cells in MLNs strongly suggest that the autoreactive PerC B-1 cells may have migrated to MLNs and differentiated into plasma cells in RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^b haplotype in parallel with the observation in normal B1 cells (13).

Interaction between Activated Tg γ/δ T Cells and Autoantibody-producing B Cells in the MLNs of HL Mice. To clarify how autoreactive Tg B-1 cells interact with activated Tg γ/δ T cells and differentiate into autoantibody-producing cells in MLNs, we performed immunohistological analysis.

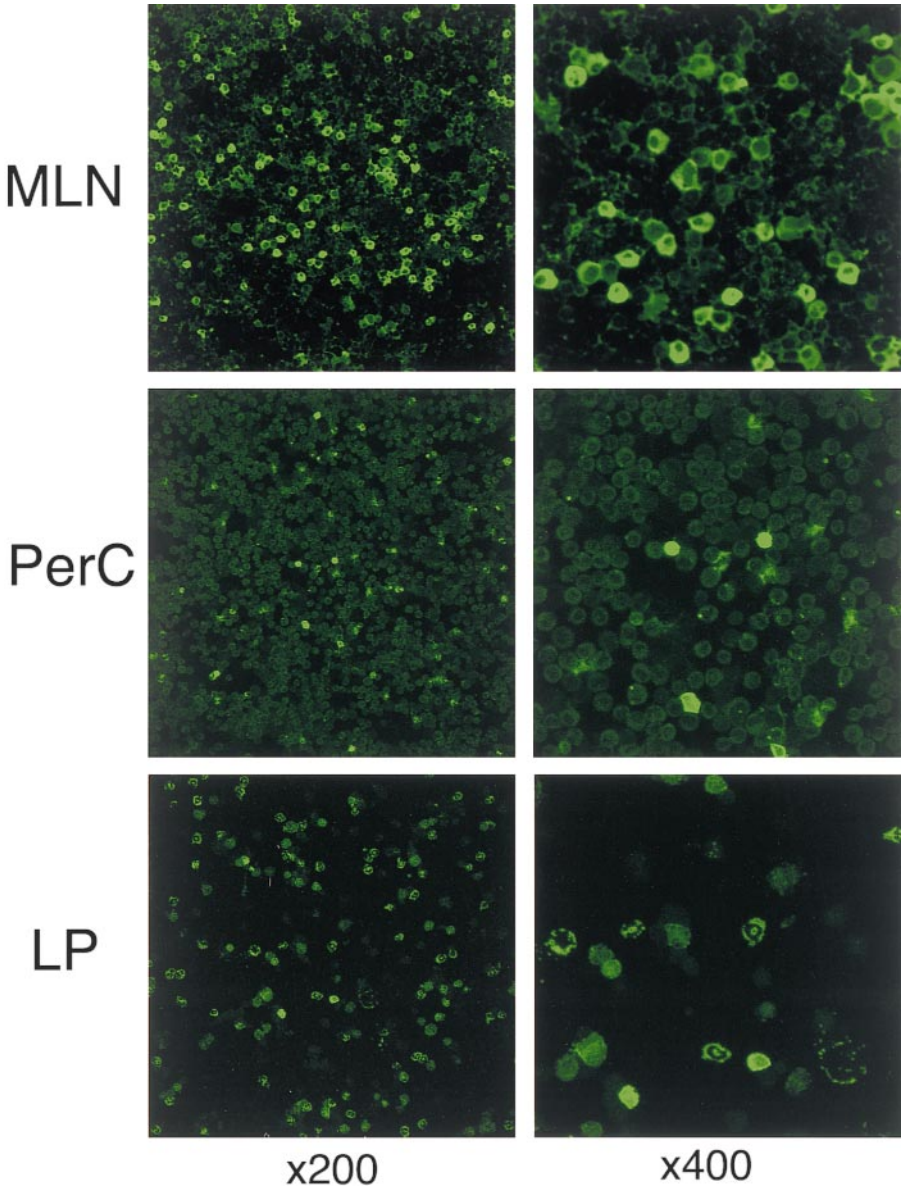

Figure 3. Plasma cells exist mainly in MLNs in RAG2$^{-/-}$ × TCR-γ/δ Tg × HL mice with the H-2^{b} haplotype. Cells were isolated from MLNs, PerC, and LP of RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^{b} haplotype, and then 2 × 10^{5} cells of each were fixed and stained with FITC-anti–mouse IgM antibody. Preparations were examined and photographed by a fluorescence microscope.

MLNs of C57BL/6 mice have primary and secondary follicles which contain PNA^{+} germinal centers, and plasma cells are found in the paracortex of LNs (Fig. 5, top panels). In contrast, PNA^{+} germinal centers were not detected in follicles of MLNs of RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^{b} haplotype, although we could identify small follicles. In these mice, Tg γ/δ T cells were accumulated in the paracortex area of B cell follicles, whereas IgM^{+} plasma cells were found near Tg γ/δ T cells (Fig. 5, bottom right panel). MLNs of RAG2$^{-/-}$ × KN6 Tg × HL mice without the H-2^{b} haplotype did not contain IgM^{+} cells (data not shown). These results indicate that activated Tg γ/δ T cells helped activation and terminal differentiation of autoreactive B-1 cells in vivo without germinal center formation.

To examine which cytokines are involved in B-1 cell activation and terminal differentiation in RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^{b} haplotype, serum concentrations of IL-4, IL-5, IL-6, and IL-10 were assessed by ELISA. The serum level of IL-10 but not IL-4, IL-5, and IL-6 significantly increased in RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^{b} haplotype compared with the mice without the H-2^{b} haplotype (Fig. 6). These results suggest that IL-10 may play a critical role in B-1 cell activation and terminal differentiation in those mice with the H-2^{b} haplotype as described previously in LPS-treated RAG2$^{-/-}$ × HL mice (29).

BM and PerC Cells from RAG2$^{-/-}$ × HL Mice Generate B-1 Cells in PerC and Plasma Cells in MLNs of RAG2$^{-/-}$ × KN6 Tg Mice with the H-2^{b} Haplotype. To confirm whether autoreactive Tg B-1 cells in PerC can migrate into MLNs and differentiate into antibody-producing cells in the presence of activated Tg γ/δ T cells, we transferred BM cells from RAG2$^{-/-}$ × HL mice into the PerC of RAG2$^{-/-}$ × KN6 Tg mice with or without the H-2^{b} haplotype and an-

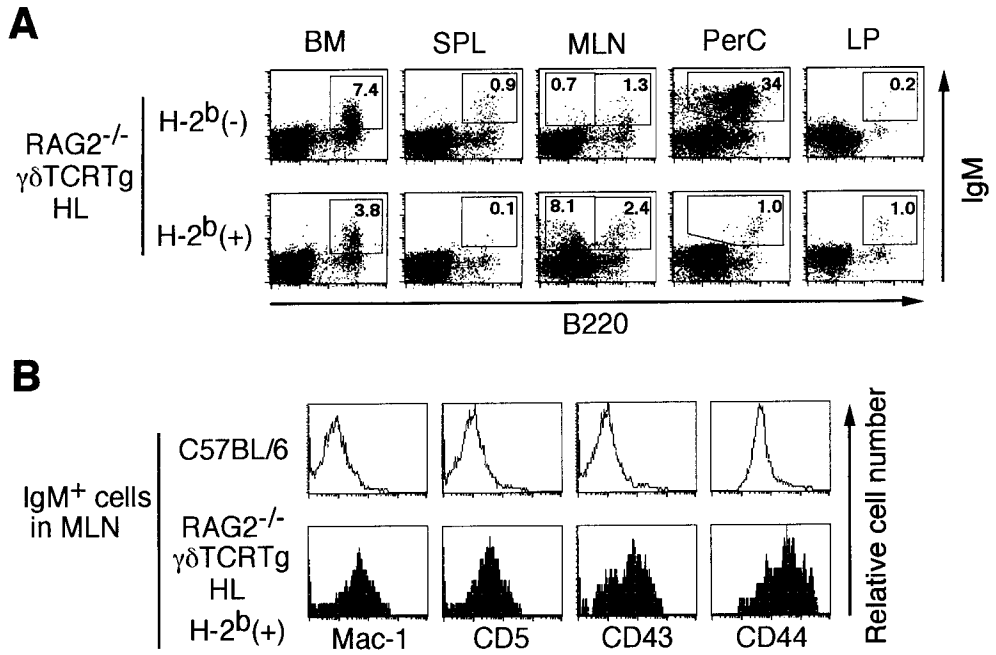

Figure 4. Localization of autoreactive B cells in RAG2$^{-/-}$ TCR-γ/δ Tg × HL mice with the H-2^b haplotype. (A) Flow cytometric analysis of BM, spleen (SPL), MLNs, PerC, and LP of RAG2$^{-/-}$ × TCR-γ/δ Tg × HL mice with (bottom) or without the H-2^b haplotype (top). Cells were stained with FITC-anti–mouse IgM and Cy-Chrome–anti-B220. The percentage of the cells for a given phenotype in viable lymphocyte gate is shown. (B) Cells from MLNs were stained with FITC-anti–mouse IgM antibody and either PE–anti–Mac-1, anti-CD5, anti-CD43, or anti-CD44 antibody. Control stainings using MLN cells of C57BL/6 mice are shown. Data are the staining profiles of IgM$^+$ cells.

alyzed lymphoid tissues 6 wk after the transfer. Flow cytometric analyses of PerC cells revealed that a large number of IgM$^+$ cells could be detected in recipient RAG2$^{-/-}$ × KN6 Tg mice regardless of the H-2 haplotype (Fig. 7). In addition, the majority of PerC IgM$^+$ cells expressed the Mac-1 antigen but not CD5 (data not shown), suggesting that they belong to the B-1b cell subset (30). In contrast, MLNs of RAG2$^{-/-}$ × KN6 Tg mice with the H-2^b haplotype but not those without the H-2^b haplotype contained IgM$^+$ cells, the majority of which also express Mac-1. It is worth noting that MLNs also contained a large number of Mac-1 strongly positive macrophages when activated Tg

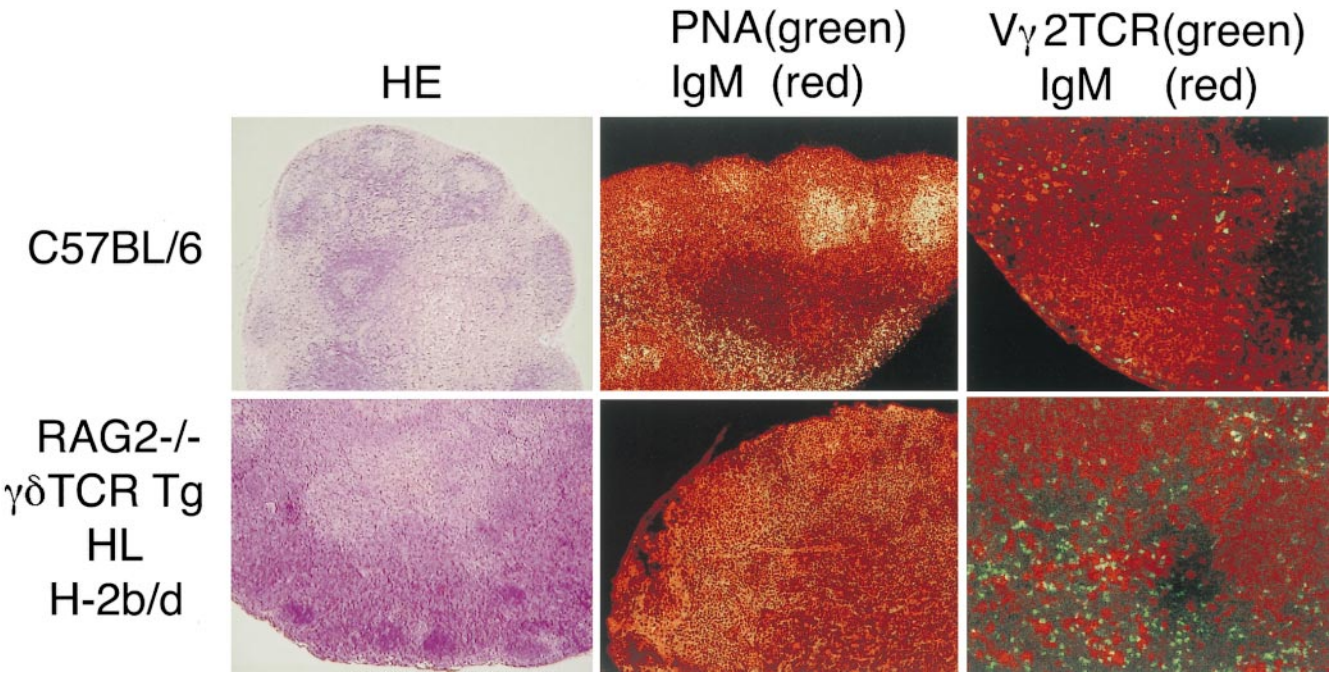

Figure 5. Immunohistological analysis of MLNs from RAG2$^{-/-}$ × TCR-γ/δ Tg × HL mice with the H-2^b haplotype. The sections of MLNs from C57BL/6 (top) and RAG2$^{-/-}$ × TCR-γ/δ Tg × HL mice with H-2^b haplotype (bottom) were fixed in formalin and stained with hematoxylin and eosin (HE) by standard methods (left). Frozen sections were stained with biotin-anti–mouse IgM antibody followed by FITC–anti-PNA (green) and Texas red–streptavidin (red) (middle); biotin-anti–mouse IgM antibody followed by FITC–anti-Vγ2 TCR (green) and Texas red–streptavidin (red) (right). Preparations were examined by a confocal fluorescence microscope. Original magnifications: (left) ×50; (middle) ×100; (right) ×150.

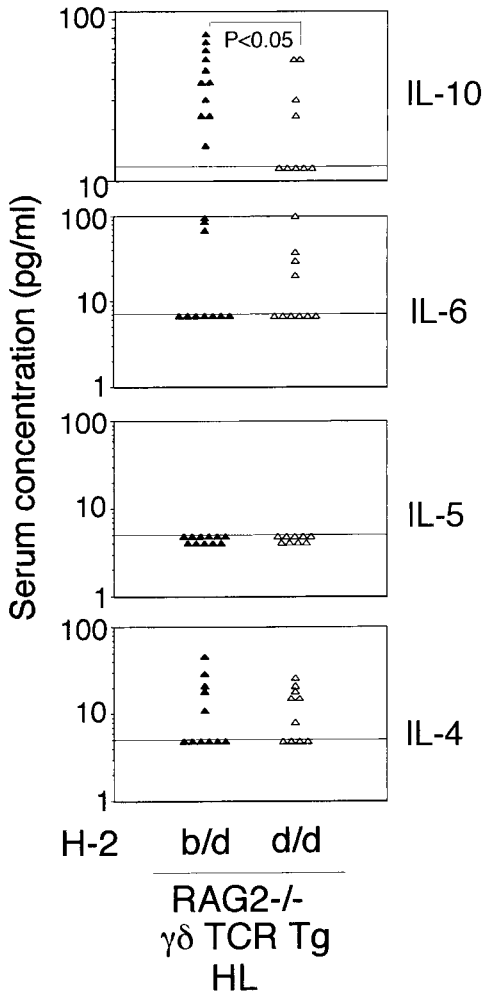

Figure 6. Serum IL-10 but not IL-4, IL-5, and IL-6 levels increased in RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^b haplotype. Amounts of IL-4, IL-5, IL-6, and IL-10 in the serum from RAG2$^{-/-}$ × KN6 Tg × HL mice with the H-2^b haplotype (▲) and without the H-2b haplotype (△) were assessed by ELISA. 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 two groups.

γ/δ T cells were present. Moreover, we identified a significant amount of the autoantibody bound to erythrocytes only in RAG2$^{-/-}$ × KN6 Tg mice with the H-2^b haplotype. Spleen and BM of recipient mice contained few IgM$^+$ B cells. Taken together, these results suggest that BM cells from RAG2$^{-/-}$ × HL mice can generate B-1b cells in PerC and that activated γ/δ T cells can induce these B-1b cells to migrate to MLNs and to differentiate into autoantibody-producing cells.

In another set of transfer experiments, we further confirmed that the autoantibody production occurred in MLNs by cytoplasmic IgM staining. Although PerC IgM$^+$ cells were stained only on the cell surface, the majority of MLN IgM$^+$ cells had typical plasma cell morphology in RAG2$^{-/-}$ × KN6 Tg mice with the H-2^b haplotype which were transferred with Tg BM cells (Fig. 8). Furthermore, we confirmed that transfer of Tg PerC cells also generated B-1 cells in PerC and plasma cells in MLNs of RAG2$^{-/-}$ × KN6 Tg mice with the H-2^b haplotype (data not shown, and Fig. 8). Taken together, these results suggest that BM or PerC cells from RAG2$^{-/-}$ × HL mice can give rise to B-1 cells in PerC and that activated γ/δ T cells can induce these B-1 cells to migrate to MLNs and to differentiate into autoantibody-producing cells.

Discussion

In this study, we investigated whether Tg B-1 cells expressing anti-RBC autoantibody can be activated by a T cell help that is not based on the shared antigen specificity in vivo. We crossed anti-RBC antibody Tg mice with RAG2$^{-/-}$ × KN6 Tg mice carrying the H-2^b haplotype. KN6 Tg γ/δ T cells are either activated or tolerized by the TL antigen associated with the H-2^b locus. However, in the RAG2 deficiency, a large number of activated Tg γ/δ T cells escape tolerance and expand in MLNs (Fig. 1). In this system, we have shown that the anti-RBC antibody level in the serum was markedly elevated, resulting in reduction of the hematocrit value (Fig. 2). In addition, the numbers of PerC B-1 cells were drastically reduced with concomitant increase of IgM$^+$ cells in MLNs (Fig. 4). IgM$^+$ cells in MLNs are actually antibody-secreting cells (Fig. 2 C) and show plasma cell morphology (Fig. 3). These antibody-producing cells were found in MLNs without forming germinal centers (Fig. 5). Serum IL-10 level was elevated in these mice (Fig. 6). These results suggest a scenario in which Tg PerC B-1 cells migrate to MLNs and differentiate into antibody-producing plasma cells in the presence of activated γ/δ T cells even with different antigen specificity. This hypothesis gained further support by the lymphocyte transfer experiment of RAG2$^{-/-}$ × HL BM or PerC cells into the PerC of RAG2$^{-/-}$ × KN6 Tg mice. Only when Tg γ/δ T cells are activated by the presence of the H-2^b haplotype do donor-derived B-1 cells migrate to MLNs and produce the autoantibody (Figs. 7 and 8).

The results indicating that Tg B-1 cells can be activated and induced to migrate to MLNs by a T cell help that is not through so-called cognate interaction agree with our previous reports that LPS, IL-5, or IL-10 administration can activate Tg B-1 cells in the PerC, giving rise to the autoantibody production and autoimmune hemolytic anemia in HL mice (10, 12). As LPS cannot directly stimulate T cells, macrophages are likely to be another source of cytokines and chemokines for migration and activation of PerC B-1 cells as proposed previously (29). In this study, we have also shown that activated γ/δ T cells not only themselves accumulate in MLNs but also induce accumulation of macrophages in MLNs (Fig. 7), suggesting that activated T cells may secrete some chemokines to induce migration of macrophages (31–33). Macrophages, in turn, may communicate with activated T cells by cytokines (34, 35). As B cells express TL antigen, there is a possibility that γ/δ T cells directly interact with Tg B-1 cells and can give "semicognate" help. However, we could not detect typical germinal centers in MLNs (Fig. 5), indicating that Tg B-1 cells

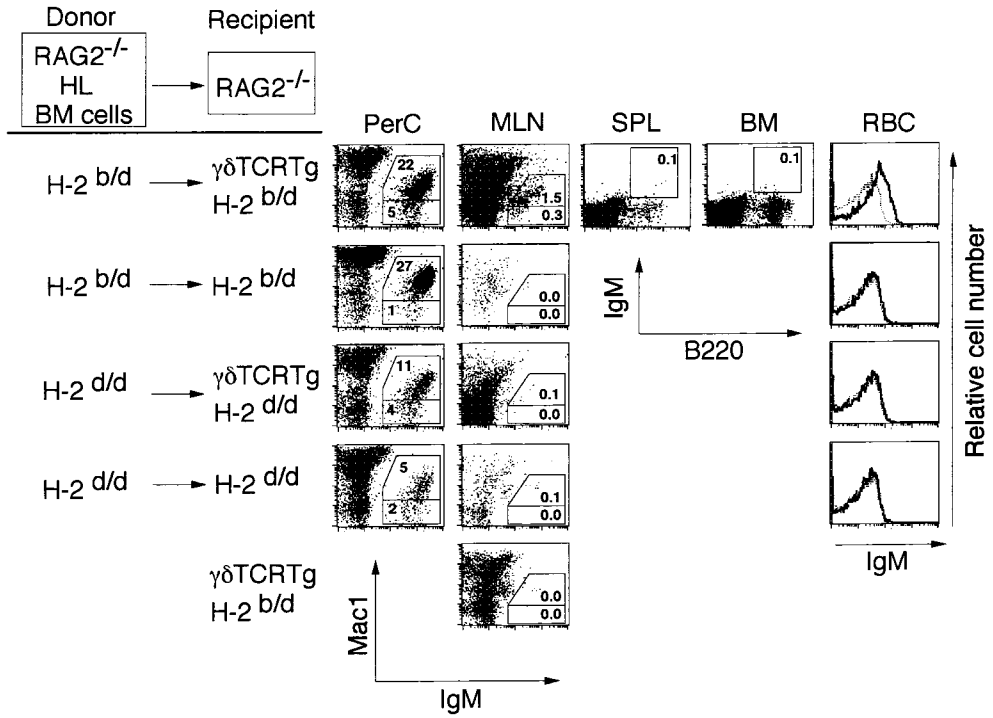

Figure 7. Transfer of BM cells from RAG2-deficient HL mice into RAG2$^{-/-}$ × TCR-γ/δ Tg mice generates B-1 cells in PerC and MLNs, and induces autoantibody production. BM cells from RAG2-deficient HL mice were injected into the PerC of RAG2$^{-/-}$ × TCR-γ/δ Tg mice with the same H-2 haplotype. After 6 wk, cells from BM, spleen (SPL), MLNs, and PerC were stained with FITC-anti–mouse IgM and PE–anti–Mac-1 or Cy-Chrome–anti-B220 antibodies. The percentage of the cells for a given phenotype in viable lymphocyte gate is shown. Amounts of anti-RBC autoantibody bound to circulating RBCs were measured by flow cytometry. Data are the staining profiles of RBCs of each mouse (solid lines). Control stainings using RBCs of RAG2$^{-/-}$ × TCR-γ/δ Tg mice without transfer are shown (dotted lines).

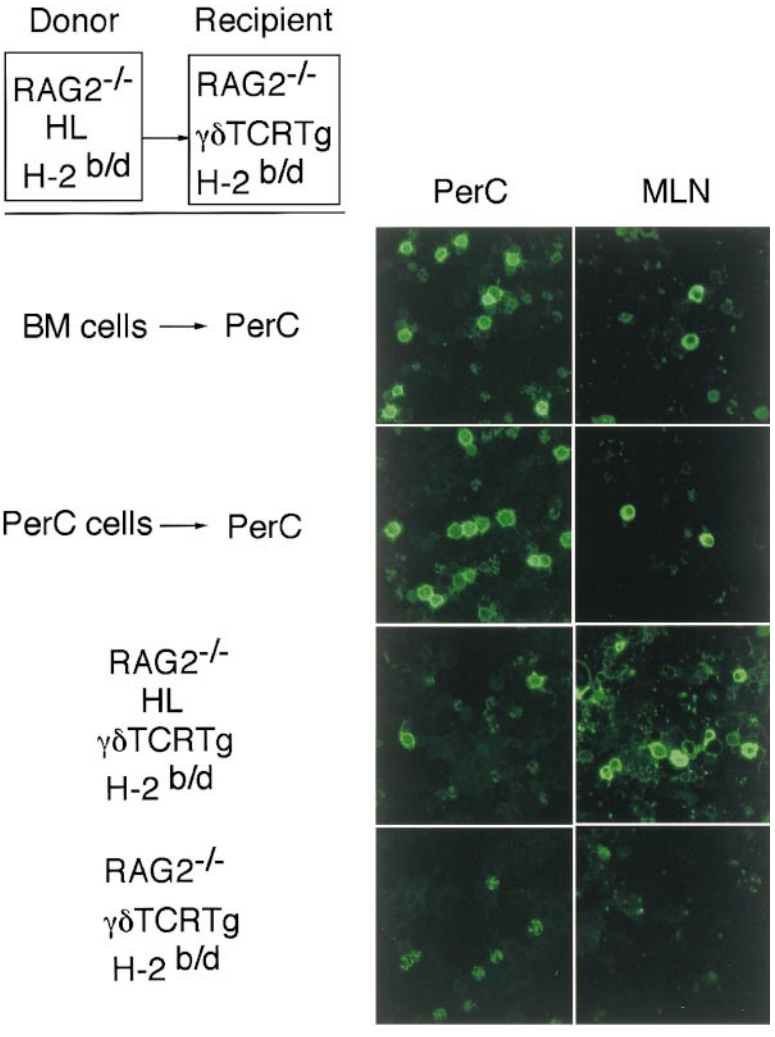

Figure 8. Transfer of BM or PerC cells from RAG2-deficient HL mice into RAG2$^{-/-}$ × TCR-γ/δ Tg mice generates plasma cells in MLNs but not PerC. BM or PerC cells from RAG2-deficient HL mice were injected into PerC of RAG2$^{-/-}$ × TCR-γ/δ Tg mice with the same H-2 haplotype. After 6 wk, cells in MLNs and PerC were isolated, and then 2 × 10^5 cells of each were fixed and stained with FITC-anti–mouse IgM antibody. Preparations were examined and photographed by a fluorescence microscope. Original magnification: ×400. Control stainings using MLN and PerC cells of RAG2$^{-/-}$ × KN6 Tg mice (H-2^{b+}) with or without HL Tg are shown.

did not differentiate into plasma cells by cognate help of T cells. In addition, the serum IL-10 levels were significantly increased in these mice. Taken together, these results suggest that both activated T cells and macrophages may be involved in activation, migration, and differentiation of peritoneal B-1 cells in a noncognate manner.

γ/δ T cells have unique features in contrast to α/β T cells (36, 37). γ/δ T cells develop earlier in ontogeny than α/β T cells. γ/δ T cells expressing specific Vγ chain show unique tissue distribution such as in the epidermal and mucosal epithelia in the adult mouse. Some γ/δ T cells develop mainly from fetal progenitors, a common feature with B-1 cells. γ/δ T cells recognize nonpeptide antigens like pyrophosphate derivatives, as well as polypeptide antigens like nonclassical class I molecules and heat shock proteins (38–41). Thus, it has been suggested that γ/δ T cells are involved in the primary and innate host defense mechanisms. As B-1 cells are also involved in innate immunity (5, 42), γ/δ T cells may cooperate with B-1 cells in such an evolutionally ancient host defense system.

Recently, we showed that normal peritoneal B-1 cells migrate to MLNs and LP to differentiate into antibody-producing cells. The alymphoplasia mutation in NF-κB–inducing kinase gene causes severe defects in migration and differentiation of peritoneal B-1 cells (13). This suggests that PerC B-1 cells respond to chemokine for their migration and that NF-κB is involved in the signal transduction process of chemokine receptors. Unlike the findings of previous reports that antibody-secreting cells can be found in PerC (10, 12, 23, 29), our recent study clearly indicates that peritoneal B-1 cells have to migrate to either LNs or LP to differentiate (13). B-1 cell differentiation into plasma cells depends on chemokines that stimulate migration of PerC B-1 cells into LP of the gut or MLNs where B-1 cells become plasma cells probably by stimulation with cytokines. LP of the gut and MLNs are preferred targets of migration probably because of the anatomical localization. This study confirms and extends the latter finding. Interestingly, B-1 cells in RAG2$^{-/-}$ × KN6 Tg × HL mice carrying the H-2^{b} haplotype predominantly migrate into MLNs but not into the LP. MLN is also the preferred site of HL B-1 cell migration under conventional conditions (9). HL Tg B cells might be deleted during migration to LP of the gut because they have to go into blood circulation.

We have identified B220^{-}IgM^{+} cells as the major population of IgM^{+} cells in the MLNs of RAG2$^{-/-}$ × KN6 Tg × HL mice in the presence of the H-2^{b} haplotype. As the majority of IgM^{+} cells in the MLNs in these mice show plasma cell morphology (Fig. 3) and a significant fraction (10^{-1}) secretes IgM (Figs. 2 C and 4 A), we assume that B220^{-}IgM^{+} cells are most likely plasma cells because the presence of B220^{-}IgA^{+} plasma cells in LP of the gut in various mouse strains (21). As almost all B-2 cells are deleted in the periphery and only PerC B-1 cells survived in this system, these IgM^{+}B220^{-} plasma cells are most likely derived from PerC B-1 cells. In general agreement with this conclusion, when BM and PerC cells were transferred into the PerC of RAG2$^{-/-}$ × KN6 Tg mice, we found B-1 cells in PerC and plasma cells in MLNs (Figs. 7 and 8).

In summary, we have shown that autoreactive B-1 cells in PerC migrate into MLNs and differentiate into antibody-producing cells by a T cell help that is not through so-called cognate interaction in HL Tg mice. This help involves other type of cells, probably macrophages, for providing various chemokines and cytokines. This result agrees with and explains our previous findings that HL Tg PerC B-1 cells can be activated by cytokines (IL-5 and IL-10) or LPS, and that normal PerC B-1 cells have to migrate to MLNs or LP of the gut in order to differentiate into antibody-producing cells.

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