Expression of the immunoregulatory molecule FcRH4 defines a distinctive tissue-based population of memory B cells

Götz R.A. Ehrhardt,1,7 Joyce T. Hsu,1 Lanier Gartland,1,7 Chuen-Miin Leu,1 Shuangyin Zhang,1 Randall S. Davis,1,2,3,5 and Max D. Cooper1,3,4,5,6,7

1Division of Clinical and Developmental Immunology, 2Division of Hematology/Oncology, 3Department of Medicine, 4Department of Pediatrics, 5Department of Microbiology, 6Department of Pathology, and 7Howard Hughes Medical Institute, University of Alabama at Birmingham, Birmingham, AL 35294

The FcRH4 transmembrane molecule, a member of the Fc receptor homologue family, can potently inhibit B cell receptor (BCR) signaling. We show that cell surface expression of this immunoregulatory molecule is restricted to a subpopulation of memory B cells, most of which lack the classical CD27 marker for memory B cells in humans. The FcRH4+ and FcRH4− memory B cells have undergone comparable levels of immunoglobulin isotype switching and somatic hypermutation, while neither subpopulation expresses the transcription factors involved in plasma cell differentiation. The FcRH4+ memory cells are morphologically distinctive large lymphocytes that express the CD69, CD80, and CD86 cell activation markers. They are also shown to be poised to secrete high levels of immunoglobulins in response to stimulation with T cell cytokines, but they fail to proliferate in response either to BCR ligation or Staphylococcus aureus stimulation. A heightened expression of the CCR1 and CCR5 chemokine receptors may facilitate their preferential localization in lymphoid tissues near epithelial surfaces. Cell surface FcRH4 expression thus marks a unique population of memory B cells with distinctive morphology, functional capabilities, and tissue localization.

B lineage cells are generated in the bone marrow throughout life in humans (1, 2). After the functional rearrangement of heavy and light chain immunoglobulin genes during the progenitor (pro-B) and precursor (pre-B) cell stages in differentiation, naive B cells selected for nonself reactivity migrate from bone marrow to colonize the peripheral lymphoid tissues, including the spleen, lymph nodes, tonsils, intestinal Peyer’s patches, and appendix (for reviews see references 3, 4). Within the secondary lymphoid tissues, naive B cells bearing cell surface IgM and IgD receptors are activated through antigen stimulation and T cell help to form germinal centers, wherein they undergo proliferation, immunoglobulin class switching, and variable region somatic hypermutation to produce higher affinity antibodies (5). On their departure from germinal centers, B cells may undergo differentiation into immunoglobulin-secreting plasma cells or become memory B cells. Germinial center B cells in humans express the TNF receptor family member CD27. The expression of CD27 may also persist after cells leave the germinal center to serve as a practical marker for memory B cells (6, 7). Upon interaction of CD27 with its CD70 ligand, recruitment of TRAF2 and TRAF5 to the CD27 intracellular domain leads to the activation of JNK and NF-κB (8, 9). The signals transduced by CD27 on memory B cells enhance plasma cell differentiation (10, 11).

The generation of memory B cells is an important component of the adaptive immune response. Antibodies made by antigen-reactivated memory B cells are predominantly of a class-switched isotype, and their somatically mutated variable regions reflect their selection for higher antigen affinity (12, 13). Following antigen stimulation, memory B cells may enter the cell cycle 20–30 h sooner than naive B cells (14), and their subsequent differentiation into antibody-secreting plasma cells leads to higher levels of specific antibodies after secondary antigenic challenge.

Human B cells have been shown to differentially express five members of a recently
identified family of immunoglobulin domain–containing transmembrane molecules (15–17). All of these Fc receptor relatives possess activating and/or inhibitory motifs in their cytoplasmic domains and thus have immunomodulatory potential. Although they are variously referred to as Fc receptor homologues (FcRHs) (15); immunoglobulin superfamily, FcR, gp42 (17); and immunoglobulin superfamily receptor translocation-associated (IRTA) (16), for simplicity we use the provisional FcRH nomenclature here. Previous studies suggest that FcRH4 is preferentially expressed by memory B cells (16, 18, 19). Functional analysis of its immunoreceptor tyrosine-based inhibitory motif–containing intracellular domain indicates that, when tyrosine phosphorylated, FcRH4 has potent inhibitory potential for B cell receptor (BCR)-mediated signaling through the recruitment of protein tyrosine phosphatases SHP-1 and/or SHP-2 (18). The present study defines the cells that bear FcRH4 as a novel subpopulation of memory B cells with distinctive morphology, function, and tissue localization, characteristics that distinguish them from the previously identified CD27+ memory B cells.

RESULTS
Production of anti-FcRH4 monoclonal antibodies
Hybridoma clones producing monoclonal anti-FcRH4 antibodies were generated by immunizing mice with recombinant protein corresponding to the extracellular domain of FcRH4 and fusion of lymph node B cells with a non–immunoglobulin-producing myeloma cell line. The 2A6 hybridoma clone was selected on the basis of ELISA and Western blot assays demonstrating FcRH4 specificity for its antibody product. To verify the anti-FcRH4 reactivity of the 2A6 monoclonal antibody with cell surface FcRH4 molecules, A2O-IIA1.6 cells were transiently transfected with a construct in which GFP was fused c-terminally to FcRH4 and stained with biotinylated F(ab\(^2\))\(_2\) fragments of this anti-FcRH4 antibody plus streptavidin coupled to phycoerythrin. FcRH4 specificity of the 2A6 antibody was indicated by selective staining of FcRH4-transfected cells (Fig. 1A) and by immunoprecipitation analysis of recombinant FcRH4 proteins (Fig. 1B). Cell surface immunofluorescence analysis of B cell lines also indicated that the 2A6 antibody does not react with B cell lines that express FcRH1, -2, -3, and/or -5 (Table I) (20, 21).

FcRH4 expression is restricted to a subpopulation of memory B cells
Evidence indicating that memory B cells may preferentially express FcRH4 messenger RNA (mRNA) was presented in an earlier study (18). When immunofluorescence analysis was performed, a discrete subpopulation of the CD19+ tonsillar B cells (9.49 ± 4.95% SEM, n = 24) was found to express cell surface FcRH4 (Fig. 2A). When the IgD and CD38 markers were used to divide tonsillar B cells into naïve (IgD+CD38−), pregerminal center (IgD+/CD38+), germinal center (IgD−/CD38+), plasma cell (IgD−/CD38−), and memory B cell subpopulations (IgD−/CD38−) (Table I).

Table I. Immunofluorescence analysis of FcRH4 expression by CD19+ B cells in different tissues and B lineage cell lines

| Tissue/cell lines | Tumor cell types | FcRH4+ B cells (no. of samples) | % |
|-------------------|------------------|-------------------------------|---|
| Bone marrow       | Acute lymphoblastic leukemia (pro-B cell line) | <0.1 |
| Peripheral blood  | Acute lymphoblastic leukemia (pro-B/pre-B cell line) | <0.1 |
| Spleen            | Acute lymphoblastic leukemia (pro-B cell line) | <0.1 |
| Nalm16            | Burkitt’s lymphoma (EBV+) | <0.1 |
| Daudi             | Burkitt’s lymphoma (EBV+) | <0.1 |
| Raji               | Burkitt’s lymphoma (EBV+) | <0.1 |
| Namalwa           | Burkitt’s lymphoma (EBV+) | <0.1 |
| BJAB               | Burkitt’s lymphoma (EBV−) | <0.1 |
| Ramos             | Burkitt’s lymphoma (EBV−) | <0.1 |
| WSU-1             | Diffuse large B cell lymphoma | <0.1 |
| SUDHL-6           | Diffuse large B cell lymphoma | <0.1 |
| NCI-H929          | Multiple myeloma     | 84 |
| RPMI-8226         | Multiple myeloma     | 24 |
| U226              | Multiple myeloma     | 10 |

Figure 1. Specificity analysis of the 2A6 anti-FcRH4 monoclonal antibody. (A) A20-IIA1.6 B cells lacking the FcγRIIb receptor were transiently transfected with expression constructs encoding a FcRH4-GFP fusion protein (filled histogram) or GFP-only control constructs (open histogram). Cells were stained with biotinylated F(ab\(^2\))-fragments of anti-FcRH4 antibodies and streptavidin-PE. Analysis gates were set on the GFP (filled histogram) or GFP-only control constructs (open histogram). (B) Lysates from 293T cells transiently transfected with expression constructs encoding a FcRH4-GFP fusion protein were stained with biotinylated F(ab\(^2\))/H9253 or GFP-only control constructs (open histogram). Cells transduced with expression constructs encoding a FcRH4-GFP fusion protein were divided into CD38− (24) and CD38+ subpopulations (8).
analysis of the IgD−/CD38− memory B cell compartment suggested that the FcRH4+ cells are larger than the FcRH4− cells (Fig. 2 D), and this was true for FcRH4+ IgDlo cells as well (not depicted). Microscopic examination of the isolated subpopulations of memory B cells confirmed the relatively large size of the FcRH4+ cells and indicated that they have distinctive morphological features, which include the possession of prominent nucleoli and an extensive cytoplasm that is rich in mitochondria. Rough endoplasmic reticulum was not abundant in either lymphocyte type (Fig. 2 F).

FcRH4-bearing cells have hypermutated immunoglobulin variable regions

Defining features of memory B cells include the expression of switched immunoglobulin isotypes and variable region somatic mutations. Analysis of the immunoglobulin isotypes expressed by FcRH4+ memory B cells indicated that the majority of these cells express IgG (63.17 ± 14.92% SEM, n = 4), followed by cells expressing IgA (27.68 ± 2.46% SEM, n = 4) or IgM (11.05 ± 5.56% SEM, n = 4). To evaluate the mutational status of the immunoglobulin V H genes in FcRH4+ and FcRH4− memory B cells, we amplified V H13 gene family regions via RT-PCR of FACS-purified cells. Sequence analysis of these V H region transcripts indicated that the FcRH4+ and FcRH4− subpopulations of tonsillar IgD−/CD38− B cells have comparable somatic mutation frequencies (mean 4.35 ± 2.43% SEM, n = 26 vs. 4.5 ± 3.29% SEM, n = 25). Of the V H13 genes analyzed, only one sequence in the FcRH4+ population was found to be devoid of mutation. In both subpopulations, the R/S values were greater for the CDR than for the framework (FR) region (R/S CDR = 2.41 and R/S FR = 1.48 for FcRH4+ cells versus R/S CDR = 2.47 and R/S FR = 1.59 for FcRH4− cells), suggesting that both types of memory B cells undergo the affinity selection process in germinal centers. The combined results of this analysis indicate that both FcRH4+ and FcRH4− cells in the IgD−/CD38− tonsillar compartment are bona fide memory B cells.

FcRH4-bearing cells have a distinctive activated phenotype

Because the FcRH4+ cells were found primarily in the IgD−/CD38− memory B cell compartment, we examined their expression of cell surface markers commonly associated with memory B cells. Expression levels for the IL-2 receptor α chain (CD25) were found to be low to undetectable. Like their FcRH4− counterparts, the FcRH4+ cells were positive for CD20, CD21, CD23, CD32, CD40, CD44, CD69, CD80, CD84, and CD86 but were negative for the CD138 plasma cell marker. Most of these cell surface markers were expressed at slightly higher levels on FcRH4+ cells, but this may not equate with higher receptor density given their relatively large size. The much higher level of CD20 on the larger FcRH4+ cells nevertheless represents a notable difference. Conversely, the FcRH4+ cells express lower levels of the complement receptor 2 (CD21) than their FcRH4− counterparts (Fig. 3).

Figure 2. Analysis of FcRH4 expression on B cell subpopulations in human tonsils. (A) CD19-purified tonsilar B cells were stained with anti-CD38, anti-IgD, and anti-FcRH4. A subpopulation of ~10% were stained positively for cell surface FcRH4 (M2 gate). M1 gate is the entire population of CD19 positive tonsilar B cells. (B) Analysis of the entire population of tonsilar B cells (M1 gate) and (C) FcRH4+ tonsilar B cells (M2 gate) for expression of CD38 and IgD. (D and E) CD19-purified tonsillar B cells were stained for CD38, IgD, CD27, and FcRH4. The gate was set on the CD38−/IgD− memory B cell population and analyzed for forward scatter (FSC) versus FcRH4 and CD27 versus FcRH4 expression, respectively. Note the increased forward light scattering of the FcRH4+ population (D) and the discordant FcRH4 and CD27 expression (E). (F) Wright-Giemsa staining and electron microscopy of FcRH4+ (top) and FcRH4− (bottom) memory B cells reveals their striking morphological differences. Bar, 2 μm.

(22, 23) (Fig. 2 B), the FcRH4−bearing cells were found primarily in the IgD−/CD38− memory B cell fraction (Fig. 2 C), although a small subset of tonsillar FcRH4+ cells expressed low levels of IgD (1.13 ± 1.26% SEM, n = 20). One third of the IgD−/CD38− memory B cells were FcRH4+ (35 ± 10.65% SEM, n = 11), and most of these did not express CD27 (72.1 ± 8.3% SEM, n = 10). Likewise, analysis of the FcRH4+/IgDlo subset indicated that most were CD27-negative (77.1 ± 11.9% SEM, n = 10) (Fig. 2 E). Conversely, memory B cells lacking FcRH4 expression were overwhelmingly CD27-positive. Light scatter
Although often used as a plasma cell marker, CD138 is not found on all plasma cells (24). To verify that the FcRH4 memory B cells have not yet begun to undergo plasma cell differentiation, we analyzed mRNA levels of transcription factors that promote plasma cell differentiation, namely BLIMP-1 (25), the spliced isoform of XBP-1 (26) and IRF4 (27), in the FcRH4+ and FcRH4- memory cells, germinal center cells, and plasma cells. BCL-6 levels were also assessed. Transcripts of BLIMP-1, XBP-1, and IRF4 were barely detectable in either FcRH4+ or FcRH4- memory B cells or in the germinal center B cells, whereas a prominent signal was observed for these transcripts in plasma cells (Fig. 4 A). BCL-6 mRNA was detected in germinal center cells and at lower levels in FcRH4- memory cells. These findings indicate that the FcRH4+ memory B cells have a distinctive nonplasma cell phenotype.

**Tissue distribution and chemokine receptor profile of FcRH4+ versus FcRH4- memory B cells**

FcRH4 mRNA analysis (18) and the aforementioned demonstration that FcRH4 is expressed on a subpopulation of the memory B cells suggest stringent regulatory control of FcRH4 expression. Although cell surface FcRH4 is detectable on ~10% of the tonsillar B cells, FcRH4+ B cells were rarely detected in bone marrow, spleen, and blood samples from healthy individuals. In contrast, CD27+ B cells were relatively abundant among B cell populations in the tonsils (53.0 ± 12.7% SEM, n = 11), blood (30.8 ± 16.2% SEM, n = 12), and spleen as expected (6, 7, 22). This highly selective pattern of FcRH4 expression was also reflected by the fact that almost all of the B lineage cell lines that were analyzed were negative for FcRH4 expression. The notable exceptions were multiple myeloma cell lines, three of which were found to express variable levels of FcRH4 mRNA and protein (Table I and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050879/DC1).
CCR7 expression has been used to distinguish memory T cells (CCR7+) from effector T cells (CCR7−) (28), and the chemokine receptors expressed by these T cell subpopulations may influence their tissue localization preferences. We therefore surveyed the chemokine receptor expression profiles for the FcRH4+ and FcRH4− subpopulations of memory B cells. While differences were not seen for most chemokine receptors, including CCR7 and CXCR4, mRNA levels for CCR1 and CCR5 were strongly up-regulated in FcRH4+ cells in comparison with the FcRH4− memory B cells (Fig. 4 B). These findings suggest that localized production of the chemokine ligands for these two chemokine receptors may influence the tissue localization pattern of FcRH4+ memory B cells.

**FcRH4+ and FcRH4− memory B cell responses to different activating stimuli**

Whereas BCR ligation induces the activation and proliferation of memory B cells (29), the intracellular domain of FcRH4 has been shown to have a profound inhibitory effect on BCR-mediated signaling (18). To evaluate the responsiveness of FcRH4+ versus FcRH4− cells to BCR-mediated stimulation, the purified subpopulations of memory B cells were stimulated with either intact anti-immunoglobulin antibodies or their F(ab′)2 fragments. The two subpopulations were also analyzed for responsiveness to the polyclonal activator *Staphylococcus aureus* Cowan strain (SAC) and the cytokines IL-2, IL-10, and CD40L. The assessment of thymidine incorporation after 40 h in culture indicated that FcRH4+ cells respond well to both BCR ligation and cytokine stimulation (Fig. 5 A). In contrast, FcRH4+ cells responded to cytokine stimulation but not BCR ligation. Moreover, SAC treatment barely elicited a detectable response of the FcRH4+ cells (Fig. 5 A), and treatment with SAC did not affect the activation response to IL-2, IL-10, or CD40L (unpublished data).

Because memory B cells have been shown to differentiate into immunoglobulin-secreting cells in response to in vitro treatment with the T cell derived cytokines IL-2, IL-4, and IL-10, or following CD40 ligation by CD40L (10, 30), we measured the levels of immunoglobulin secretion by purified FcRH4+ and FcRH4− memory B cells after stimulation with cytokines and CD40L. Analysis of culture supernatants after 4 d of stimulation indicated that the FcRH4+ cells secreted more immunoglobulin than FcRH4− cells in response to IL-2 and IL-10 or to IL-2, IL-10, and CD40L (Fig. 5 B). ELISPOT assays further indicated that the increased levels of immunoglobulin secretion resulted from increased numbers of immunoglobulin-secreting cells (Fig. 5 C). In keeping with their memory status, the majority of both FcHR4+ and FcRH4− subpopulations of B cells secreted IgG antibodies (~60%), followed by subpopulations of lesser abundance that produced IgM or IgA.

We also examined the possibility that the FcRH4− memory B cells could be induced to express FcRH4 as an intermediate step in memory B cell differentiation. In these experiments, the FcRH4− cells were labeled with a succimidyl ester of carboxyfluorescein diacetate (CFSE), a fluorescent dye that is equally distributed between daughter cells. As anticipated, the stimulation of FcRH4− memory B cells with IL-2, IL-10, and CD40L led to their proliferation, but this response was not accompanied by the expression of FcRH4 within the 48-h interval of observation.

**DISCUSSION**

The present experiments define two distinctive subpopulations of memory B cells in humans that are distinguishable...
on the basis of whether or not they express the transmembrane immunoregulatory FcRH4 molecule. The FcRH4-bearing cells that we characterized are confined largely within the IgD+/CD38− memory B cell population in the tonsils, although a minor subset of the FcRH4+ cells expressed low levels of IgD. Although FcRH4-bearing B cells comprise ~10% of the tonsillar B cells, they are rarely detectable in the circulation and were also rarely seen in the bone marrow or spleen. This unusual distribution pattern has been noted in another study that employed a different monoclonal anti-FcRH4 antibody to assess the tissue distribution of FcRH4-bearing cells (Polson, A., personal communication). FcRH4+ cells have been detected by immunohistochemical analysis in tonsils, Peyer’s patches, lymph nodes, and in inflamed tissues from patients with Hashimoto’s thyroiditis, Sjögren’s sylodenitis, and Helicobacter pylori–associated gastritis (19). We conclude from these observations that the FcRH4-bearing B cells represent a specialized tissue-based subpopulation of memory B cells.

The unusual nature of the FcRH4-bearing subpopulation of memory B cells is emphasized by the lack of FcRH4 expression by any of 10 leukemia or lymphoma cell lines of varying phenotypes and differentiation stages. Interestingly, the only exceptions were three multiple myeloma cell lines whose FcRH4 mRNA and protein expression levels were variable. In this regard, FcRH4 was originally identified as a translocated gene in a myeloma cell line (16), although a role for this gene in myeloma development has not been established. This finding may also suggest that FcRH4 expression persists during the earliest states of plasma cell differentiation by FcRH4-bearing memory B cells.

Although CD27 is currently an accepted marker for memory B cells in humans (6, 7), we found that most FcRH4-bearing cells do not express this cell surface molecule. This finding appears to differ from the conclusion reached in two reports that describe coexpression of CD27 and FcRH4/IRTA1 (19, 31). Whereas the immunohistochemical analysis of FcRH4+ cells in tonsils with atypical marginal zone hyperplasia indicated that these FcRH4+ cells do not express CD27, concomitant FcRH4 and CD27 expression was noted for B cells in normal tonsillar tissue (32). However, the frequency of single versus dual FcRH4 and CD27 expression was not evaluated in this report. An earlier study which reported CD27 and FcRH4 coexpression employed polyclonal rabbit antibodies directed against a cytoplasmic FcRH4 epitope, therefore requiring fixation and permeabilization for flow immunocytometric analysis of the cells (19). The resultant low intensity staining for FcRH4 expression prevented an unambiguous discrimination of FcRH4-positive cells, and this may account for the discrepancy between results obtained with the polyclonal and monoclonal antibodies.

Marafioti and colleagues have identified a subpopulation of memory B cells in the interfollicular T cell rich areas of tonsillar and lymph node tissue sections on the basis of their stellate or dendritic morphology (31) that may or may not belong to the tissue-based subpopulation of memory B cells that we describe here. The interfollicular B lymphocytes that they describe share certain characteristics with the FcRH4-bearing memory B cells, including the absence of CD27, relatively large cell size, and variable region somatic hypermutation, albeit at higher levels. However, other characteristics of the FcRH4-bearing cells, including the expression of CD21, CD23, and the CD80 and CD86 activation markers, are not shared. The large FcRH4+ and interfollicular B lymphocytes therefore could represent different subsets of memory B cells, or their apparent phenotypic differences may reflect differences in cell marker discrimination via the flow immunocytometric and immunohistochemical methods employed for their characterization.

Higher levels of CCR1 and CCR5 mRNA accompany the expression of FcRH4 as another discriminating feature of the FcRH4+ and FcRH4− memory B cells. These chemokine receptors recognize an overlapping set of ligands that include Mip-1α, MIP-1β, and RANTES (for review see reference 33). Although these chemokines are commonly viewed as T cell–targeting factors, CCR1 and CCR5 expression and chemotactic responses to their ligands have also been reported for B cells (34, 35). This pattern of chemokine receptor expression suggests that FcRH4+ memory B cells and T cells could be attracted by the same chemokine gradients, thereby facilitating T cell–dependent activation of the FcRH4+ cells. The preferential expression of these two chemokine receptors could also contribute to the lymphoepithelial tissue localization of FcRH4+ cells, because epithelial cells and their stromal cell neighbors release these chemokines in inflammatory responses (36).

In addition to their different phenotypic features and tissue distribution, the FcRH4+ and FcRH4− memory B cells have different functional characteristics. The FcRH4+ memory cells produce much higher levels of secreted antibodies than FcRH4− memory cells when stimulated in vitro with T cell–derived cytokines. Together with their increased cell size and activated state, this finding indicates that the FcRH4+ memory B cells are primed to undergo terminal plasma cell differentiation. Nevertheless, these cells do not express increased levels of the transcription factors that promote plasma cell differentiation, including the spliced isoform of XBP-1, BLIMP-1, and IRF4. The possibility that expression of FcRH4 marks a common intermediate step between memory B cell and plasma cell differentiation is not supported by analysis of cell surface FcRH4 expression following stimulation of FcRH4− cells with cytokine combinations that induce plasma cell differentiation. Our findings are thus more consistent with the idea that FcRH4+ and FcRH4− memory B cells represent different sublineages of memory B cells.

Notably, the FcRH4− memory B cells undergo proliferation both in response to cytokines that simulate T–dependent stimulation and to direct BCR cross-linking. In contrast, their FcRH4+ counterparts are preferentially
responsive to T-dependent stimulation; their unresponsiveness to BCR ligation could be attributable to the relatively low levels of CD21 expression, because engagement of this complement receptor has been shown to lower the threshold requirement for BCR-mediated activation (37). Another attractive possibility is that FcRH4 inhibits the proliferative response to BCR ligation. Irrespective of the mechanisms involved, our data indicate that FcRH4+ cells are poised to undergo plasma cell differentiation in response to T-dependent stimulation, whereas FcRH4− memory B cells can be activated equally well by T-dependent and T-independent stimulation to mount an immediate proliferative response.

FcRH4 is structurally and functionally related to FcyRIIB (15, 18), the low affinity receptor for IgG antibodies that is expressed on memory B cells. Studies in animal models implicate FcγRIIB dysfunction in a number of pathological situations, including enhanced antibody responses (38), enhanced IgG- and IgE-induced anaphylactic reactions (39), hypersensitivity to collagen-induced arthritis (40, 41), and development of spontaneous systemic lupus erythematosus (42). Although no FcRH4 orthologue is found in mice (43), three lines of evidence implicate FcRH4 as a potentially important regulator of memory B cell activation and proliferation in humans: (1) biochemical analysis has established the potent inhibitory potential of the intracellular domain of FcRH4 (18); (2) FcRH4-bearing memory B cells fail to proliferate in response to BCR ligation; and (3) IRAK1/FcRH4 was identified as a translocation-associated gene in a multiple myeloma cell line (16), wherein the signal peptide coding region and the 5′ end of FcRH4 were juxtaposed next to the constant region of the immunoglobulin α heavy chain, thereby effectively creating a knockout FcRH4 allele. Collectively, these observations raise the intriguing possibility that function-loss FcRH4 mutations or failures in mechanisms that control FcRH4 expression may contribute to B cell–mediated immunopathology.

MATERIALS AND METHODS

Monoclonal and polyclonal antibodies. Monoclonal anti-FcRH4 antibodies were generated by hyperimmunizing mice with baculo virus–derived recombinant protein corresponding to the extracellular domain of FcRH4 and fusion of lymph node cells with the non–immunoglobulin–derived recombinant protein corresponding to Ag8–653 myeloma variant. 34 out of 120 hybridoma clones produced ELISA-reactive antibodies against recombinant FcRH4 extracellular domain. Three of these produced antibodies that reacted with a subpopulation of tonsillar B cells. Clone 2A6 antibodies were tested for FcRH4 specificity via FACS, immunoprecipitation, and Western blotting analyses performed as described previously (44). F(ab′)2 fragments of the 2A6 antibodies generated by pepsin digest according to the manufacturer’s instructions (Pierce Biotechnology) were coupled to biotin (Pierce Biotechnology) or generated by pepsin digest according to the manufacturer’s instructions (Pierce Biotechnology) or generated by pepsin digest according to the manufacturer’s instructions (Pierce Biotechnology).

Blood, tonsil, spleen, and bone marrow tissue samples were obtained from the human tissue procurement service of the University of Alabama at Birmingham with Institutional Review Board approval and informed consent according to the declaration of Helsinki. Single cell suspensions were generated by tissue mincing, filtration through 70 μm wire mesh, and cell centrifugation on a ficoll-hypaque gradient. Tonsillar B cells of >99% purity were obtained by using anti-CD19 coupled to magnetic beads (Miltenyi Biotec) followed by magnetic separation. After labeling with the indicated monoclonal antibody combinations, the cells were analyzed by using a FACS Calibur instrument (BD Biosciences). For microscopic analysis of the FcRH4+ and FcRH4− populations, the cells were stained with anti-IgD, anti-CD38, and anti-FcRH4 antibodies before isolation of FcRH4+ and FcRH4− cells using a MoFlow FACS sorter (DAKO Cytomation). The cells were spun onto glass slides, stained with Wright-GIEMSA stain (Sigma-Aldrich) and examined by light microscopy (magnification, 400). For electron microscopy (magnification ×10,000), the cells were fixed in 2% glutaraldehyde, followed by incubation in 1% osmium and dehydration in a graded alcohol series before embedding in spur resin. After staining, the sections were treated with 2% uranyl acetate and Reynolds’s lead citrate. For proliferation and immunoglobulin secretion assays, tonsillar B cells were obtained from the memory B cell subpopulations. Because RP2 transcripts were found to be the most reliable transcripts for normalization based on cell numbers, all quantitative RT-PCR reactions were performed using the Pharmingen GeneTACs information system (IMGT) (http://imgt.cines.fr) (45).

Sequence analysis of V\textsubscript{H} genes. First-strand complementary DNA (cDNA) was generated by performing random primed RT-PCR on CD19+ memory B cells after FACS-sorting into IgD+/CD38−/FcRH4+ and IgD−/CD38+/FcRH4+ subpopulations. For the first round of PCR amplification with a high fidelity PCR polymerase (Invitrogen), a primer mix was used that recognizes all V\textsubscript{H} gene family members and J\textsubscript{H} gene family members (12). The second round of PCR amplification employed specific primers in conjunction with primers recognizing all six J\textsubscript{H} gene family members. PCR products were cloned into pBluescript for sequence analysis of the 264 nucleotides encompassing the framework 1, 2, and 3 regions as well as the CDR1 and CDR2 regions of the V\textsubscript{H}3 genes. Sequence analysis was performed using the international ImMunoGeneTics information system (IMGT) (46).

Quantitative RT-PCR analysis. To prevent contamination with non–B cells, tonsillar B cells were stained with an anti-CD20 monoclonal antibody before subpopulation sorting and mRNA harvesting by using the RNAeasy Kit (QIAGEN). Random primed cDNA corresponding to 5,000 cells/reaction was used as a template. Whenever possible, oligonucleotides were designed to overlap exon–intron borders to avoid amplification of genomic DNA. Primer sequences are described in Fig. S2 (available at http://www.jem.org/cgi/content/full/jem.20050879/DC1). To eliminate contaminating genomic DNA, RNA preparations were treated with RNase-free DNase (QIAGEN). Quantitative RT-PCR was performed using SYBR-Green PCR Master Mix (Applied Biosystems) on a 7900HT Sequence Detection System (Applied Biosystems). Because the widely used transcripts of GAPDH and β-actin can be subject to regulation, thus making them less suitable for normalization (46), we analyzed transcripts for the large subunit of 18S rRNA, which is expressed at a constant level across different cell types. Transcripts were normalized to the product of 18S rRNA transcription and GAPDH transcription using the cycle threshold (C\textsubscript{T}) of the transcript with the lowest expression as the calibrator for each individual cell line. To determine the expression of V\textsubscript{H}3 genes, we used the following primer pair: 5′-GTCTCGTCTGGTCTGCTTCCGC-3′ and 5′-CGGGTTCTAGCCTCTGCTTCTC-3′. The C\textsubscript{T} values for each transcript were analyzed using the comparative C\textsubscript{T} method (46). The expression level of V\textsubscript{H}3 genes in FcRH4+ tonsillar B cells was normalized to the expression level of the V\textsubscript{H}3 genes in FcRH4− tonsillar B cells.

Cell proliferation and immunoglobulin secretion assays. Cells plated in triplicate at a density of 15,000 per round-bottom well in 96-well plates were cultured for 40 h with 2 μg/mL intact anti-immunoglobulin, 1.33 μg/mL F(ab′)2 anti-immunoglobulin, 60 ng/mL IL-2, 200 ng/mL IL-10, 2 μg/mL CD40L (R&D Systems), and/or 0.001% SAC (Sigma-Aldrich) before addition of 1 μCi [3H] thymidine for an additional 10 h. Cells were harvested and the amount of [3H] thymidine incorporation was measured using a beta-counter. The amount of [3H] thymidine incorporation was normalized to the total number of cells in each well. The expression level of V\textsubscript{H}3 genes in FcRH4+ tonsillar B cells was normalized to the expression level of the V\textsubscript{H}3 genes in FcRH4− tonsillar B cells.

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we were harvested with a Basix’96 Harvester (Skatron Instruments) and thymi-
dincorporation measured with a Wallac liquid scintillation counter.

For immunoglobulin secretion analysis, cells (15,000/well) were plated in
triplicate in a volume of 150 μL and incubated with the different stimuli for
4 d before supernatant collection and addition to ELISA plates coated with
a mixture of mouse anti-human IgA, IgM, and IgG (2 μg/mL). After
overnight incubation at 4°C, plates were washed and a secondary horseradish
peroxidase (HRP)-labeled goat anti-human immunoglobulin (Jackson Im-
munoresearch) antibody was added for 1 h at room temperature. The plates
were washed again and HRP-substrate added for 30 min before microplate
reader analysis at 405 nm. For ELISPOT assays, FcRγ+ and FcRγ− cells were
cultured as described above for 4 d. The cultured cells were then washed in
HANKS balanced salt solution, plated on Multiscree filter plates (Millipore)
coated with monoclonal anti-IgG, anti-IgA, or anti-IgM antibodies, and
incubated at 37°C for an additional 6 h. After incubation, the plates were
washed three times, then incubated with goat anti-human immunoglo-
bulin HRP-labeled antibodies for 1 h, and the assay developed using AEC
(Moss, Inc.) as substrate. Immunoglobulin-producing cells were counted using
an Immunospot Analyses instrument (Cellular Technology LTD).

Online supplemental material. Fig. S1 shows the oligonucleotide se-
quences used as primers for quantitative PCR analysis of the transcription
factor and chemokine receptors of FcRγ+ and FcRγ− memory B cells. Fig. S2
depicts FcRγ4 expression by several multiple myelomas as a mechanism for enhanced
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