Expression pattern of the human FcRH/IRTA receptors in normal tissue and in B-chronic lymphocytic leukemia

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Keywords: chronic lymphocytic leukemia, Fc receptor homologs, Fc receptor-like, FcRL, immunoreceptor translocation associated, memory B cells, NK cells

Abstract

A new family of Ig domain receptors referred to as the immune receptor translocation-associated (IRTA) proteins, FcR homologs (FcRHs) or FcR-like that are expressed in lymphoid cells has been recently described. RNA expression analysis suggests that FcRH1–5/IRTA1–5 are expressed exclusively in subsets of the B-cell compartment. We generated mAbs to FcRH1–5/IRTA1–5 and examined their protein expression pattern in normal tissue and in chronic lymphocytic leukemia (CLL) cells. Our data indicated that FcRH1–5/IRTA1–5 were expressed in B-cell sub-populations; however, in some cases, the protein was not expressed in the same B-cell populations as suggested by the RNA expression analysis. FcRH1/IRTA5 was expressed throughout the B-cell lineage starting at the pro-B-cell stage but was down-regulated in plasma cells. FcRH2/IRTA4 was expressed preferentially in memory B cells. FcRH3/IRTA3 was expressed at low levels in naive, germinal center (GC) and memory B cells but was also expressed in NK cells. FcRH4/IRTA1 was expressed in a sub-population of memory B cells associated with mucosal tissue. FcRH5/IRTA2 was expressed in mature B cells and memory B cells and down-regulated in GC cells and, unlike all other B-cell-specific markers, maintained its expression in plasma cells from tonsil, spleen and bone marrow. We examined the expression of FcRH1–5/IRTA1–5 on the surface of CLL cells and found a similar pattern of expression on CLL cells as in the normal mature B cells, except for FcRH3/IRTA3 which was up-regulated in CLL.

Introduction

Recently, an interesting new family of Ig domain-containing type I membrane proteins has been described that has sequence similarity to FcRs and the cellular adhesion molecule PECAM-1 (1–3). This family was alternately named the immune receptor translocation-associated (IRTA) proteins (1), the FcR homologs (FcRHs) (3) or FcR-like (4). Members of this family have also been called IFGPs (from Ig superfamily, FcR, gp42) (5) and SPAPs (SH2 domain-containing phosphatases anchor proteins) (6). To date, six members of the FcRH/IRTA receptor family have been described: FcRH1/IRTA5, FcRH2/IRTA4, FcRH3/IRTA3, FcRH4/IRTA1, FcRH5/IRTA2 and FcRH6/IRTA6 (2, 3, 7, 8). All the FcRH/IRTAs contain some combination of canonical immunoreceptor tyrosine-based inhibitory motifs and ‘immunoreceptor tyrosine-based activation motifs-like’ signaling motifs. The activity of these signaling motifs has been demonstrated for FcRH1/IRTA5 and FcRH4/IRTA1 which can positively and negatively regulate B-cell receptor signaling, respectively (9, 10). The ligands for these receptors are unknown; however, we find that they do not bind soluble Ig with high affinity.

RNA expression data suggest that FcRH1–5/IRTA1–5 are specific for subsets of the B-cell compartment (2, 3). The FcRH1/IRTA5 and FcRH2/IRTA4 RNA expression pattern suggested that these genes would have their strongest expression in naive B cells (2). The RNA expression pattern of FcRH3/IRTA3 and FcRH5/IRTA2 suggested that they were predominately expressed in centrocytes and post-germinal center (GC) cells (2). In situ hybridization (ISH) data indicated that FcRH4/IRTA1 RNA is expressed in intra-epithelial B cells. Subsequent analysis of the protein expression pattern confirmed...
Expression of FcRH/IRTAs in normal tissue and CLL

this observation, showing that FcRH4/IRTA1 is expressed in a unique and morphologically distinct population of memory B cells localized to the epithelia of mucosa-associated lymphoid tissue (MALT) (8, 11). The observation that FcRH4/IRTA1 is expressed principally in memory B cells in epithelia of mucosal lymphoid tissue and the homology of the FcRH/IRTAs to PECAM-1 suggest a possible role for the FcRH/IRTAs in B-cell tissue tropism (8, 11).

The dysregulation of the expression of the FcRH/IRTAs has been linked to several diseases. FcRH1–5/IRTA1–5 are located adjacent to each other on chromosomal bands 1q21–q22 and FcRH6/IRTA6 is located nearby on 1q23. 1q21–q23 is frequently the site of chromosomal abnormalities in non-Hodgkin’s lymphoma and multiple myeloma. Further, FcRH5/IRTA2 RNA expression is dysregulated in cell lines with 1q21 abnormalities (1, 2, 12). However, it is not known if this dysregulation plays any role in the transformation of B cells. 1q21–q23 has been implicated in susceptibility to autoimmune disease and recent work shows that a single nucleotide polymorphism in the promoter of FcRH3/IRTA3 which results in the up-regulation of FcRH3/IRTA3 transcription is associated with predisposition to several autoimmune diseases (13).

To further explore the biology of these receptors and their potential use as targets for therapeutic antibodies, we generated antibodies specific to individual FcRH1–5/IRTA1–5 proteins and mapped their protein expression pattern by FACS and by immunohistochemistry (IHC) in tonsil, spleen and bone marrow. In several instances, the protein expression pattern was different than the RNA expression pattern but was generally restricted to the B-cell compartment. The signaling potential and expression of the FcRH/IRTAs in specific B-cell sub-populations suggest that these receptors could play a significant role in the development and regulation of the human immune system. In addition, we found that the pattern of FcRH/IRTA expression in chronic lymphocytic leukemia (CLL) cells was similar to that in normal tissue.

Methods

Generation of FcRH/IRTA stable cell lines

SVT2 mouse fibroblast cell lines stably expressing the tagged and untagged FcRH/IRTA were generated for screening antibodies. The FcRH/IRTA cDNAs were PCR amplified from a human spleen cell library (a gift from James Lee, Genentech, South San Francisco, CA, USA) and TA cloned into pCR4 (Invitrogen, Carlsbad, CA, USA). To make the untagged expression construct, the open reading frames (ORFs) were cloned into the mammalian expression vector pCMV.PD.nbe (a gift from Amy Shen, Genentech). N-terminal-tagged expression constructs were made by amplification of the FcRH/IRTA ORFs without the signal sequence and ligation of the PCR product into the pMSCVneo (Clontech, Palo Alto, CA, USA) vector containing the gD tag and signal sequence, MGGAARLGAVILFVVIVGHGVRGKYALADASLKMADPNRFR-GKDLPVLQDLL. For each FcRH/IRTA, two stable cell lines were established for use in FACS screening of the mAbs and to determine cross-reactivity among the FcRH/IRTA. The gD-tagged and untagged expression vectors were transfected into SVT2 cells by the standard Lipofectamine 2000 (Invitrogen) protocol. The gD-tagged transfectants were put under 0.5 mg ml⁻¹ Geneticin (Invitrogen) selection for 1 week and then single-cell FACS sorted with gD-tag-specific mAb (gD:952, Genentech) to acquire the highest expressing clone as determined by flow cytometry with anti-gD. The untagged transfectants were put under 5.0 μg ml⁻¹ puromycin (Calbiochem, La Jolla, CA, USA) selection until visible colonies grew out. RNA from each colony was isolated by the standard Trizol® (Invitrogen) protocol and screened by TaqMan® (ABI, Foster City, CA, USA) to determine the highest producing clone.

Generation of mAbs to the FcRH/IRTAs

Protein for immunization of mice was generated by transient transfection of vectors that express the His-tagged extracellular domains (ECDs) of the FcRH/IRTAs into CHO cells. The proteins were purified from the transfected cell supernatants on nickel columns and the identity of the protein was confirmed by N-terminal sequencing.

Ten Balb/c mice (Charles River Laboratories, Hollister, CA, USA) or 20 Xenomice™ (Abgenix, Fremont, CA, USA) were hyperimmunized with the recombinant His-tagged ECD protein in Ribi adjuvant (Ribi Immunococh Research, Inc., Hamilton, MO, USA). B cells from mice demonstrating high antibody titers against the immunogen by direct ELISA, and specific binding to SVT2 mouse fibroblast cells stably expressing the FcRH/IRTA of interest by FACS, were fused with mouse myeloma cells (X63.Ag8.653; American Type Culture Collection, Rockville, MD, USA) as previously described (14, 15). After 10–12 days, the supernatants were harvested and screened for antibody production and binding by direct ELISA and FACS. Positive clones, showing the highest immunobinding after the second round of subcloning by limiting dilution, were expanded and cultured for further characterization, including FcRH1–5/IRTA1–5 specificity and cross-reactivity. The supernatants harvested from each hybridoma lineage were purified by affinity chromatography (Pharmacia fast protein liquid chromatography; Pharmacia, Uppsala, Sweden) as previously described (14). The purified antibody preparations were then sterile filtered (0.2-μm pore size; Nalgene, Rochester, NY, USA) and stored at 4°C in PBS. The antibodies used in these studies are anti-FcRh1/IRTA5 1F9 (human IgG4), anti-FcRh1/IRTA5 2A10 (human IgG2), anti-FcRh2/IRTA4 7G7 (mouse IgG1), anti-FcRh3/IRTA3 6F2 (human IgG2), anti-FcRh4/IRTA1 1A3 (mouse IgG2a) and anti-FcRh5/IRTA2 7D11 (mouse IgG2b).

Flow cytometry

All the antibodies for flow cytometry were obtained from BD Biosciences (San Jose, CA, USA) except anti-CD14–APC which was obtained from Caltag Laboratories (Burlingame, CA, USA). Cells (10⁶ cells in 100 μl volume) were first incubated with 1 μg of each anti-CD16, anti-CD32, anti-CD64 antibodies and 10 μg each of human and mouse gamma globulin (Jackson ImmunoResearch, Laboratories, West Grove, PA, USA) to block non-specific binding, and then incubated with optimal concentrations of mAbs for 30 min in the dark at 4°C. When biotinylated antibodies were used, streptavidin–PE or streptavidin–APC (Jackson ImmunoResearch...
Laboratories) were then added according to manufacturer’s instructions. Flow cytometry was performed on a FACScalibur (BD Biosciences). Forward scatter and side scatter signals were recorded in linear mode and fluorescence signals in logarithmic mode. Dead cells and debris were gated out using scatter properties of the cells. Data were analyzed using CellQuest Pro software (BD Biosciences) and FlowJo (Tree Star Inc.).

Mononuclear cell preparation

Human blood samples were collected from healthy individuals through the Genentech research blood program; bone marrow was obtained from AliCells (Berkeley, CA, USA) and prepared by standard density centrifugation over lymphocyte separation medium (LSM) medium (ICN/Cappel, Aurora, OH, USA). Tonsils and spleens were obtained through Bio-Options (Fullerton, CA, USA), minced, digested with 1 mg ml⁻¹ collagenase and 0.1 U ml⁻¹ deoxyribonuclease (US Biological, Swampscott, MA, USA) in RPMI-1640 at 37°C for 20 min and put through a 30-µm cell strainer (BD Biosciences) to achieve the single-cell suspension and then prepared by the standard density centrifugation over LSM medium.

Specific cell population identification

From PBMCs, B cells were first isolated with CD20 MicroBeads and LS MACS columns (Miltenyi Biotec, Auburn, CA, USA) and then identified by staining and gating on the CD20-APC-positive populations; the purity of the B cells was >96%. The T-cell, NK-cell and monocyte populations were identified by staining the negative fraction of CD20 MACS isolate with anti-CD3–APC, anti-CD56–APC and anti-CD14–APC, respectively, and gating on the positive population. Blood granulocytes were isolated by first treating human blood (1:1) with 3% Dextran 500 (Amersham Bioscience, Piscataway, NJ, USA) in PBS for 30 min at room temperature to remove the majority of the RBCs, and then collecting the pellet from the standard density centrifugation over LSM medium. Staining and gating on the CD15-APC-positive population further identified the granulocyte population.

From bone marrow mononuclear cells (BM-MNCs), CD19+ B cells were first isolated with CD19 MicroBeads and LS MACS columns (Miltenyi Biotec) and then stained with a marker combination of anti-CD34–PE, anti-CD19–PerCP–Cy5.5, anti-CD27–FITC, anti-κ light chain–FITC or anti-λ light chain–FITC or anti-κ light chain–FITC or anti-λ light chain–FITC, pro-B cells were identified as CD34+/CD19+/

CD27–/κ and λ light chain, while pre-B cells were identified as CD34+/CD19+/CD27–/κ and λ light chain. The negative fraction of CD19 MicroBeads selection was also collected and stained with a marker combination of anti-CD34–PE, anti-CD19–PerCP–Cy5.5 and anti-CD20–FITC. The stem cell/B-cell progenitors were identified as CD34+/CD19+/CD20–. Plasma cells were isolated with CD138 MicroBeads and LS MACS columns (Miltenyi Biotec) from BM-MNCs, and further identified by gating on the CD38-PerCP-Cy5.5-high and CD138-PE-positive population.

From tonsil or spleen mononuclear cells (MNCs), CD19+ B cells were first isolated with CD19 MicroBeads and LS MACS columns (Miltenyi Biotec) and then stained with a marker combination of anti-IgD–PE and anti-CD38–PerCP–Cy5.5.

Naive B cells were identified as CD38–/IgD+, memory B cells were identified as CD38–/IgD–, GC cells were identified as CD38+/IgD– and plasma cells were identified as CD38++/IgD–. Plasma cells were also isolated directly with CD138 MicroBeads and LS MACS columns (Miltenyi Biotec) from tonsil or spleen MNCs, and further identified by staining and gating on the CD38-PerCP-Cy5.5-high and CD138-PE-positive population.

For the experiments involving bone marrow or spleen, the analysis was performed at least twice with different donors. For the experiments involving tonsil and peripheral blood, the expression pattern was assessed in at least five different donors. Except where noted, we did not see any variation in expression pattern of the FcRH/IRTA between donors.

Peripheral blood samples were obtained from CLL patients obtained after Institutional Review Board review and patient consent in collaborations with John Hainsworth of the Sarah Cannon Research Institute and David Irwin of the Alta Bates Summit Medical Center. Patient PBMCs were isolated by gradient centrifugation over LSM medium and then stained with biotinylated antibody followed by staining with anti-CD5–PE, anti-CD19–PerCP–Cy5.5 and anti-CD20–FITC antibodies and streptavidin–APC.

IHC

Frozen and formalin-fixed paraffin-embedded (FFPE) lymphoid tissues (tonsil, spleen, lymph nodes, Peyer’s patches) from the Genentech Human Tissue Bank were sectioned at 5 µm. Immunochemical staining for FcRH2/IRTA4 was performed on frozen sections using biotinylated horse anti-mouse IgG and avidin-biotin peroxidase (Vector Laboratories) with diaminobenzidine (DAB) as chromogen. For immunofluorescent detection of FcRH2/IRTA4, frozen tissue sections were stained with mAb 7G7 and rabbit anti-IgD (DakoCytomation), followed by a mixture of donkey anti-mouse Cy3 and anti-rabbit Cy2 conjugates (Jackson Immunoresearch Laboratories). FcRH4/IRTA1 was detected in FFPE sections using biotinyl-tyramide (Perkin Elmer) and DAB. Negative control slides were stained with isotype-matched mouse IgG (Pharmingen) or rabbit IgG (Jackson Immunoresearch Laboratories) at equivalent concentrations. Microscope slides were visualized on Olympus and Nikon microscopes, equipped fluorescein and rhodamine filter sets and with CCD cameras (Diagnostic Instruments).

Results

Generation of antibodies to FcRH/IRTA family members

We generated cDNA clones of the six FcRH/IRTA family members by PCR amplification from a human spleen cDNA library. The only difference we found between the published sequences and our clones was an extra exon in FcRH6/IRTA6 that resulted in a predicted ORF with a longer cytoplasmic region than the previously described sequence. We generated mAbs to the ECDs of FcRH1/IRTA5, FcRH2/IRTA4, FcRH3/IRTA3, FcRH4/IRTA1 and FcRH5/IRTA2. Since some of the Ig domains have amino acid identities of >80% between family members, it was probable that some of the antibodies would cross-react with more than one family member.
Therefore, we tested the antibodies for specificity by ELISA and by FACS of stable cell lines expressing single gD-tagged FcRH/IRTA family members. We were able to obtain for use in this study at least one FACS reactive, specific antibody for FcRH1–5/IRTA1–5 (Fig. 1).

Expression of FcRH/IRTAs in normal tissue

Previous RNA and protein expression data suggested that FcRH1/IRTA5 is specific for the B-cell compartment (2, 3, 9). Our analysis of the surface protein expression pattern with mAbs specific for FcRH1/IRTA5 confirms these observations (Fig. 2A). FcRH1/IRTA5 was expressed in pro-B and pre-B cells, although at a much lower level than in naive and memory B cells, but was not expressed in CD19−/CD34+ stem cells (Fig. 2C). Expression of FcRH1/IRTA5 was expressed in most of the mature B-cell populations, including CD20+/CD27− peripheral blood naive B cells, CD20+/CD27+ peripheral blood memory B cells, IgD+CD38− tonsil and spleen naive B cells and IgD−CD38− tonsil and spleen memory B cells (Fig. 2, spleen data not shown). However, FcRH1/IRTA5 was down-regulated or absent from IgD−CD38+ GC cells (Fig. 2B). We found very low-level expression of FcRH1/IRTA5 on plasma cells from two of four tonsil samples we examined but did not detect any FcRH1/IRTA5 expression on plasma cells from bone marrow (data not shown).

In peripheral blood, FcRh2/IRTA4 expression was confined to a subset of the CD20+ cells population (Fig. 3A). In tonsil and spleen, FcRh2/IRTA4 is only expressed at high levels in the CD20+ IgD−CD38− population (Fig. 3B, spleen data not shown). This population consists mostly of memory B cells. Within the B-cell compartment, CD27 is a marker of memory B cells as defined by hypermutated Ig genes (16). In peripheral blood, FcRh2/IRTA4 is expressed only in CD20+CD27+ cells (Fig. 3C). Frozen tonsil sections stained with anti-FcRHI/IRTA4 antibody 7G7 showed staining only in the intra- and sub-epithelial areas adjacent to the mantle zone (Fig. 3D and E). Co-staining of tonsil and spleen sections with anti-FcRh2/IRTA4 (7G7) and anti-IgD antibodies showed very little overlap in the expression of IgD and FcRh2/IRTA4 (Fig. 3E and F) consistent with our FACS data indicating that FcRh2/IRTA4 is a marker of memory B cells. We found no expression of FcRh2/IRTA4 in pre-B cells, pro-B cells or plasma cells from bone marrow. However, in four of six cases, we found low-level expression of FcRh2/IRTA4 in CD138+CD38++ plasma cells from tonsil (data not shown).

FcRh3/IRTA3 was the only FcRH/IRTA we examined that had expression outside the B-cell compartment. In blood, FcRh3/IRTA3 was expressed in CD56+ lymphocytes, indicating that FcRh3/IRTA3 is expressed in NK cells (Fig. 4A). The FACS data suggest that FcRh3/IRTA3 surface expression is very low, if present at all, on naive, memory cells and GC B cells, and it is absent from pro-B cells, pre-B cells or plasma cells from bone marrow (Fig. 4, data not shown). The fluorescence signal for FcRh3/IRTA3 is very weak in B cells; however, real-time PCR data indicate that the RNA for FcRh3/IRTA3 is expressed in B cells, suggesting that FcRh3/IRTA3 is expressed in B cells but at a low level on the surface (data not shown). Mouse FcRh3/IRTA3 RNA is expressed in marginal zone B cells (17), so we examined the expression of FcRh3/IRTA3 on the equivalent human population CD27+ IgD+B cells (18, 19) as compared with the CD27+ IgD− memory B cells in spleen and peripheral blood but found no difference in FcRh3/IRTA3 expression (data not shown).

We did not detect any expression of FcRh4/IRTA1 in peripheral blood by FACS with the exception of the CD14+ monocyte.
population where we observed a very small but reproducible shift (Fig. 5A). This shift is probably an artifact because real-time reverse transcription–PCR analysis suggests that monocytes do not express FcRH4/IRTA1 RNA (data not shown). We did not detect FcRH4/IRTA1 in pre-B cells, pro-B cells or plasma cells from bone marrow (data not shown). However, in tonsil, a subset of the CD20+ IgD−/C255 CD38− B-cell population showed strong expression of FcRH4/IRTA1 (Fig. 5B). Gating on the FcRH4/IRTA1+ cells and looking at the expression of IgD showed that all the FcRH4/IRTA1+ cells were IgD−. This population was much smaller or undetectable in spleen (Fig. 5C). IHC with anti-FcRH4/IRTA1 antibody 1A3 showed that numerous FcRH4/IRTA1-positive cells are localized to the follicular margin and epithelium of tonsils and Peyer’s patches but greatly reduced in spleen and lymph node (Fig. 5D).

Our FACS analysis supports the previous work suggesting that FcRH5/IRTA2 RNA is only expressed in B cells. We detected surface protein expression of FcRH5/IRTA2 at low levels on pre-B cells (data not shown) and it reached full expression on naive and memory B cells from the blood, tonsil and spleen and at lower or undetectable levels on GC cells (Fig. 6A–C). Unlike the other FcRH/IRTAs, we found significant expression of FcRH5/IRTA2 on the surface of plasma cells from tonsil, spleen and bone marrow (Fig. 6D).

Expression of FcRHs in CLL

We tested the expression of the B-cell-specific FcRH1–5/IRTA1–5 in normal CD5+ naive and memory B cells and in CLL cells. PBMCs isolated from patients diagnosed with CLL were stained with anti-CD19–PerCP–Cy5.5 and anti-CD5–PE and the cells gated on the double-positive population and tested for the expression of the FcRH1/IRTA1. The percentage of the lymphocyte gate that was CD5+ CD19+ was >18% in all the
samples tested and >50% in 19 of the 22 samples examined. The normal CD5+ and CD5− B cells showed the same expression pattern of FcRH2/IRTA4, including the increased expression of FcRH2/IRTA4 in the CD27+ (memory B cell) population (Fig. 7A). In general, the expression pattern of the FcRH/IRTA genes in CLL was consistent with the expression pattern of the FcRH/IRTA genes in normal CD5+ B cells (Fig. 7B). However, comparing the means of the expression levels between CLL and normal B cells (Welch’s t-test) suggests that FcRH3/IRTA3 is up-regulated in CLL (P = 0.005). FcRH1/IRTA5 and FcHR5/IRTA2 were expressed in almost all of the 15 samples. FcRH2/IRTA4 was expressed in 15 of the 20 samples, FcRH3/IRTA3 was expressed at a low level in 14 of the 20 samples and FcRH4/IRTA1 was expressed at a low level in only 1 of the 20 samples.

Discussion

The FcRH/IRTA gene family members are Ig superfamily type I membrane proteins that have homology to FcyRs and PECAM-1 and have both positive and negative signaling potential (1–3, 7). Analysis of the RNA expression pattern of FcRH1–5/IRTA1–5 indicated that these genes were expressed
only in the B-cell compartment (1-3). These observations suggest a role for the FcRH/IRTAs in immune modulation and/or development.

Although the amino acid similarity between the FcRH/IRTA family members and their similarity to the FcRs provide useful clues to the origin and function of these proteins, it also complicates the study of the function of individual genes because of the difficulty of making specific antibodies. We developed mAbs specific for FcRH1/IRTA5, FcRH2/IRTA4, FcRH3/IRTA3, FcRH4/IRTA1 and FcRH5/IRTA2 proteins and analyzed their expression pattern by flow cytometry and, where possible, by IHC in both normal tissue and CLL cells.

The data presented here extend previous observations on the protein expression pattern of FcRH1/IRTA5 and FcRH4/IRTA1. Our data for the expression of FcRH1/IRTA5 in normal tissue are in agreement with the published data, with the exception that we see low-level expression in pro-B cells and pre-B cells, whereas Leu et al. (9) only detect expression in pre-B cells. This could just be due to a greater sensitivity of our antibody or FACS procedure. The anti-FcRH1/IRTA5 mAb described here provides a direct method to identify or isolate by FACS or IHC this unique subpopulation of memory B cells.

The published ISH data for FcRH2/IRTA4 RNA expression show mostly mantle zone expression (2), whereas we found that the FcRH2/IRTA4 protein was expressed in the adjacent sub-epithelial area in tonsil or the marginal zone in spleen by IHC (Fig. 3D-F) as would be expected from our FACS data showing that FcRH2/IRTA4 was mostly expressed in memory B cells. The reasons for these differences are not clear, but could be due to RNA expression levels not necessarily reflecting the level of protein expression because of post-transcriptional regulation. As with FcRH1/IRTA5, we observed some expression of FcRH2/IRTA4 on plasma cells isolated from tonsil, but not from bone marrow, suggesting that their expression is lost as the plasma cells mature (20).

We found that FcRH3/IRTA3 and FcRH5/IRTA2 were expressed on both naive and memory B cells, whereas the RNAs were mostly expressed in post-GC cells of the marginal zone, although weak expression of the RNAs of these receptors was seen in the mantle zone (2). Thus, while the apparent level of RNA expression of FcRH3/IRTA3 and FcRH5/IRTA2 differed from our protein expression analysis, the overall pattern of

### Fig. 4.

**Expression pattern of FcRH3/IRTA3.** (A) FcRH3/IRTA3 expression in peripheral blood. The experiment was the same as in Fig. 2(A) except the cells were stained with a biotinylated isotype control (gray histogram) and the biotinylated anti-FcRH3/IRTA3 antibody 6F2 (black line) and streptavidin–PE. (B) FcRH3/IRTA3 expression in tonsil B cells. The experiment was the same as in Fig. 2(B) except the cells were stained with a biotinylated isotype control (gray histogram) and the biotinylated anti-FcRH3/IRTA3 antibody 6F2 (black line) and streptavidin–APC. (C) FcRH3/IRTA3 staining of spleen B cells. Experiment was the same as in (B) only with cells isolated from spleen.
expression was consistent with our finding of FcRH3/IRTA3 and FcRH5/IRTA2 expression in naive and memory B cells. In addition, we found that FcRH3/IRTA3 was expressed in NK cells. FcRH5/IRTA2 is the only FcRH/IRTA that is expressed in all populations of plasma cells. This is interesting because most previously described B-cell-specific surface proteins are down-regulated or absent on plasma cells. Additionally, these data suggest that FcRH5/IRTA2 could make an excellent marker for plasma cells.

The specific expression pattern and signaling potential of the FcRH/IRTAs suggest that they have an interesting role in immune function and the elucidation of the pattern of surface expression of the FcRH/IRTAs is an important first step in discovering their function. As previously noted (8), the tissue-specific expression pattern of FcRH4/IRTA1 suggests that it could be involved in the localization of memory B cells. Unlike FcRH4/IRTA1, none of the other FcRH/IRTAs examined showed preferential expression in a particular tissue type. Therefore, the expression patterns of FcRH1/IRTA5, FcRH2/IRTA4, FcRH3/IRTA3 and FcRH5/IRTA2 are more consistent with a role in B-cell development or regulation rather than localization or migration. Identification of the ligands of the FcRH/IRTAs would help determine the biological function of these receptors. The sequence similarity of the FcRH/IRTAs to the FcγRs suggests that they could bind Ig Fc regions or other Ig domain-containing proteins. We tested the binding of all the human Ig isotypes to FcRH/IRTA-expressing cell lines by FACS and found that the FcRH/IRTAs do not bind soluble Ig with one exception; FcRH5/IRTA2 bound to a sample containing a mixture of IgG isotypes (Fig. S1, available at International...
Immunology Online). However, the individual IgG isotypes did not bind FcRH5/IRTA2 (Fig. S1B, available at International Immunology Online) and none of the Ig samples we tested bound the FcRH/IRTAs by Bio-Layer Interferometry (data not shown). It is unclear why this mixture of IgG isotypes bound FcRH5/IRTA2 cell line; we tested all the Ig samples for aggregates by size exclusion chromatography, and although ~10% of the mixed IgG sample was aggregated, other IgG samples with similar or greater aggregation levels did not bind any to the FcRH/IRTAs. These data suggest that monomer Ig is not a high-affinity ligand for the FcRH/IRTAs but it does not rule out low-affinity interactions or binding to Ig complexes as a possible activity of the FcRH/IRTAs. Identification of the ligands for the FcRH/IRTAs with the expression data presented here should provide a clearer understanding of the function of these interesting molecules.

Possibly, the most important aspect of determining the normal expression pattern of the FcRH/IRTAs is to lay a foundation for their potential use in the clinic as targets for treatment or diagnosis of disease. Increased FcRH3/IRTA3...
RNA expression is associated with autoimmune disease (13) and the observation of expression of FcRH3/IRTA3 surface protein on NK cells and mature B cells in addition to the availability of an FcRH3/IRTA3-specific antibody should further the investigation of this interesting finding. We tested the expression of FcRH1–5/IRTA1–5 in CLL cells to see if any of the FcRHs could be potential targets for antibody therapy for CLL. In general, the expression pattern of the FcRH/IRTAs in CLL reflected what we observed in the normal peripheral CD5+ B cells (Fig. 7). We found that almost all the CLL samples examined expressed FcRH1/IRTA5 and FcRH5/IRTA2. Since both of these proteins are specific for B cells, they are good potential targets for antibody-based therapies for CLL. As expected from its expression pattern in normal B cells, FcRH4/IRTA1 showed very little expression. It was expressed in only one of the CLL samples and that was at a low level (Fig. 7). FcRH3/IRTA3 was also expressed at a low level as expected; however, comparing the means of the expression levels between CLL and normal B cells suggests that FcRH3/IRTA3 is up-regulated in CLL. Given the low level of FcRH3/IRTA3 expression, it is not clear if this has any clinical utility, but may turn out to have interesting biological implications.

FcRH2/IRTA4 expression is limited to the CD27+ population of B cells in peripheral blood (Fig. 3C) and CD27 expression correlates with somatic hypermutation of V-region genes in B cells (16) including CD5+ B cells (21), indicating that FcRH2/IRTA4+ cells are hypermutated. Approximately 50% of CLLs have mutated V\textsubscript{H} genes (22–24) and one would expect that a similar percentage of CLLs to be FcRH2/IRTA4+. We found that 15 of the 20 (75%) CLLs expressed for FcRH2/IRTA4 and 9 of the 20 (45%) had expression of FcRH2/IRTA4 by FACS that was $>50$ mean fluorescent intensity (Fig. 6B), suggesting the possibility that FcRH2/IRTA4 is a marker for mutated V\textsubscript{H} genes in CLL. This observation could be useful because hypermutation is linked with a more indolent course of disease in CLL (22, 23). In summary, FcRH1/IRTA5 and FcRH5/IRTA2 appear to be good targets for antibody therapy in CLL and FcRH2/IRTA4 is a possible marker for indolent CLL.

**Supplementary data**

Supplementary data are available at International Immunology Online.
Acknowledgements

We thank Dan Eaton, Fred deSauvage and Flavio Martin for helpful discussions and guidance; Sothy Yi for cloning assistance; Lino Gonzalez and Irene Tom for Bio-Layer Interferometry testing; Mike Elliott for chromatography help and John Hainsworth and David Irwin for CLL sample collection.

Abbreviations

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<td>APC</td>
<td>allophycocyanin</td>
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<td>BM-MNC</td>
<td>bone marrow mononuclear cell</td>
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<td>CLL</td>
<td>chronic lymphocytic leukemia</td>
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<td>diaminobenzidine</td>
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<td>ECD</td>
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References