FCRLA—A Resident Endoplasmic Reticulum Protein that Associates with Multiple Immunoglobulin Isotypes in B Lineage Cells

Tessa E. Blackburn, Teresa Santiago and Peter D. Burrows

Abstract FCRLA is homologous to receptors for the Fc portion of IgG (FcγR) and is located in the same region of human chromosome one, but has several unusual and unique features. It is a soluble resident ER protein retained in this organelle by unknown mechanisms involving the N-terminal domain, a disordered domain with three Cys residues in close proximity in the human protein. Unlike the FcγRs, FCRLA is not glycosylated and has no transmembrane region. FCRLA is included in this CTMI volume on IgM-binding proteins because it binds IgM in the ER, but quite surprisingly, given the isotype-restricted ligand specificity of the other FcRs, it also binds all other Ig isotypes so far tested, IgG and IgA. In the case of IgM, there is even preferential binding of the secretory and not the transmembrane form. Among B cells, FCRLA is most highly expressed in the germinal center and shows little expression in plasma cells. Based on these observations, we propose that one human FCRLA function is to stop GC B cells from secreting IgM, which would act as a decoy receptor, thus preventing the B cells from capturing antigen, processing it, and presenting the antigen-derived peptides to T follicular helper cells. Without help from these T cells, there would be limited B cell isotype switching, proliferation, and differentiation. On the other hand, FCRLA is downregulated in plasma cells, where IgM secretion is an essential function. FCRLA may also act as a chaperone involved by unknown mechanisms in the proper assembly of Ig molecules of all isotypes.

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Contents

1 Introduction—Fc Receptors and Their Relatives

Receptors on phagocytic cells for the Fc portion of IgG antibodies (FcR) were first reported more than fifty years ago (Berken and Benacerraf 1966) and have been well characterized since then. Members of this "classical" FcR family, FcγRI, FcγRII, FcγRIII, and FcRIV, are found on cells of myeloid, lymphoid, and megakaryocytic lineages (Turner and Kinet 1999; Ravetch and Bolland 2001; Kanamaru et al. 2007; Pincetic et al. 2014; Hanson and Barb 2015), where they are thought to have important regulatory roles in both cell-mediated and humoral immunities. Included among their many functions are feedback suppression of B cell responses, regulation of hypersensitivity reactions, and the induction of cellular cytotoxicity. Given these suspected essential functions of FcRs in establishing homeostasis of the adaptive immune system, it is not surprising that subversion of the normal receptor function may lead to autoimmunity and lymphoproliferative disorders.

Other immunoglobulin (Ig)-like domain-containing transmembrane FcRs include the FcμR, which binds IgM (Kubagawa et al. 2009, 2014; Wang et al. 2016); Fcα/μR, which binds IgA and IgM (Shibuya and Honda 2015) (Shibuya et al. 2000); and the polymeric Ig receptor (pIgR), which mediates transcytosis of oligomeric IgA and IgM across mucosal epithelial surfaces (Bruno et al. 2011). The neonatal FcRn that mediates perinatal transfer of Ig and maintenance of basal immunoglobulin levels in adults is related to MHC class I (Roopenian and Sun 2010; Tesar and Bjorkman 2010; Rath et al. 2013; Pyzik et al. 2015), and the FcαR (CD89), a myeloid cell receptor for IgA, is a very distant FcR relative (Ben Mkaddem et al. 2013; Aleyd et al. 2015). The *FcαR* genomic location places it in the leukocyte receptor complex on human chromosome 19q13.4, rather than within the classical FcR complex on chromosome 1q23 (see Chap. 1 by Akula and Hellman).

During the past decade, there has been an unexpected harvest of FcR-related genes from the human chromosome 1q region. Six human FcR homologs (*FCRL1-6*) were identified using a variety of approaches including protein sequence homology with conserved Ig-binding regions of the classical FcRs, diverse database analysis strategies, and by the characterization of a chromosomal translocation juxtaposing part of the *FCRL* locus with the Ig locus in a myeloma cell line [Reviewed in (Li et al. 2014)].

Like the classical Ig-binding *FcRs*, the *FCRL1-6* genes reside in the human chromosome 1q21–1q23 region and encode type I transmembrane proteins with extracellular Ig-like domains. The FCRL cytoplasmic regions contain tyrosine-based motifs, suggesting both inhibitory (ITIM) and activating (ITAM) signaling functions. Indeed, such signaling activities have been demonstrated in vitro. However, since the physiological ligands of FCRL were until recently unknown, these studies had to be performed using surrogate ligands, e.g., antibody cross-linking of FCRL and the B cell antigen receptor (BCR). Now that MHC II was identified as an FCRL6 ligand (Schreeder et al. 2010) and FCRL4 and FCRL5 were shown to be receptors for IgA and IgG, respectively (Wilson et al. 2012; Franco et al. 2013), better insight into the physiological functions of these receptors is likely to be forthcoming.

2 The Identification of FCRLA and FCRLB

During the characterization of the extended FCRL family members, we identified two additional relatives with unusual features and named them FcRX and FcRY (Davis et al. 2002; Masuda et al. 2005). Because of their independent identification by two other laboratories, FcRX was also named FREB (Fc receptor homolog expressed in B cells) and FcRL (FcR-like) (Facchetti et al. 2002; Mechetina et al. 2002), and FcRY was named FcRL1 and FREB2 (Chikaev et al. 2005; Wilson and Colonna 2005). The HUGO Gene Nomenclature Committee has adopted *FCRLA* and *FCRLB* as the approved human FcRX/FcRL/FREB and FcRY/FcRL2/FREB2 gene symbols, respectively (Maltais et al. 2006). Little is known about FCRLB because it is expressed in very few human B cells (Wilson and Colonna 2005) and because *Fcrlb* gene ablation in mice had no obvious phenotype (Masuda et al. 2010). Thus, in this chapter, we will focus mostly on FCRLA. (Based on standard nomenclature, *FCRLA* is used here to designate the human gene, *Fcrla* the mouse counterpart, and FCRLA the protein in both species.)

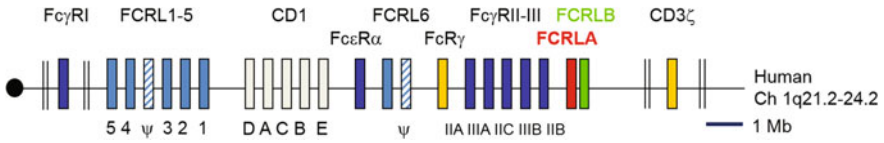

Fig. 1 *FCRLA* genomic landscape. *FCRLA* and *FCRLB* are located in the 1q21.2–1q24.2 interval on chromosome 1; *FCRLB* is located ∼7 kb telomeric of *FCRLA*. This region is enriched for FCR and FCR-related genes, including the classic *FcγRI-III* genes, the ligand-binding *FcεRα* chain of FcεRI, *FCL1*-5, *FCER1G*, the gene encoding the FcR γ chain, a signaling component in FcεRI, activating FCγRs, and other immune system receptors. Also located in this region are *CD3ζ*, which encodes an essential signaling component in the TCR complex, and the *CD1* genes, which encode MHCI-related proteins involved in presenting lipid and glycolipid antigens to NKT cells

3 The *FCRLA* Genome Landscape

FCRLA is located in the q23.3 region of chromosome 1, a region enriched for genes encoding FCR-type molecules (Fig. 1). These include all of the FcγRs, the ligand-binding chain of the high-affinity FcεR (FcεRI), *FCER1G*, the gene encoding the Fcγ chain, a signaling component in FcεRI, activating FCγRs, and several other immune system receptors, FCRL1-6, FCRLA, and FCRLB. Other genes of immunological interest in the vicinity encode CD1A-E, non-classical MHC1 molecules that present lipid antigens to NKT cells, and the CD3ζ signaling chain of the TCR complex. Interestingly, genes encoding IgM-binding receptors, Fcαμ, Fcμ, and the polymeric Ig receptor, are clustered together in a region ∼45 MB telomeric of *FCRLA*.

4 Features of the FCRLA Protein

The predicted sequence of FCRLA has features in common with previously identified FcR and FCRL, as well as several features that are unique (Figs. 2 and 3). FCRLA has a predicted molecular weight of 35.849 kDa and a predicted signal sequence at the N terminus. (Unlike the signal sequence of all other FCR-related family members including FCRLB, the FRLA signal sequence in humans and mice is not encoded by two exons S1 and a 21 or 36 bp S2 mini-exon.) Next in the protein structure is the D1 domain, which is predicted to be disordered (see below), and then two Ig-like domains (D2–D3). FCRLA does not have a transmembrane region and, instead, terminates with a mucin-like domain (D4, see below). Our studies indicate that the signal sequence is sufficient to drive translocation of FCRLA into the endoplasmic reticulum (ER) lumen of B lineage cells (Santiago et al. 2011). The FCRLA D1 ancestor may have been an Ig-like domain, since the D1 encoded by the closely linked gene *FCRLB* is an authentic Ig domain (Chikaev et al. 2005; Masuda et al. 2005; Wilson and Colonna 2005). However, the contemporary 47 amino acid long D1 is shorter than a typical Ig-like domain (∼90 aa),

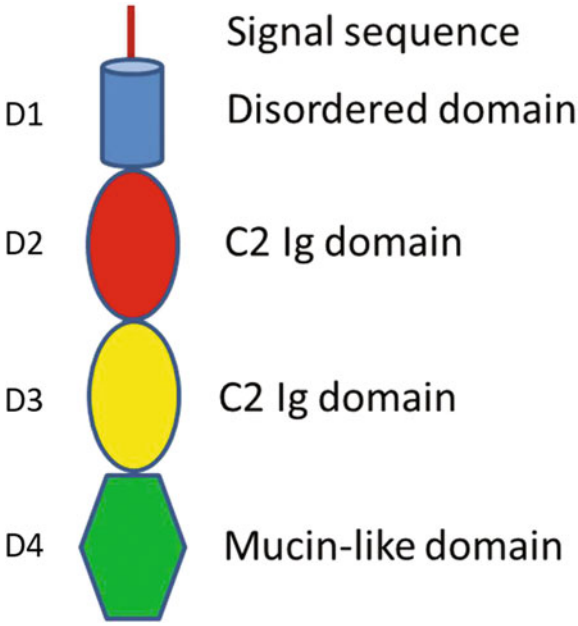

Fig. 2 Model of FCRLA protein structure. The signal peptide and features of the four domains are depicted. D1 is predicted to be disordered, D2 and D3 are authentic C2 Ig-like domains, and D4 is a bipartite domain with an N-terminal mucin-like region, rich in proline, serine, and threonine residues, and a C-terminal region predicted to form an α-helix

is not composed of predicted β sheets, and lacks a properly positioned second cysteine residue that would typically form an intrachain disulfide bond to stabilize an Ig-like domain fold. Instead, there are two closely spaced cysteines (separated by ten residues) in human and mouse FCRLA near the D1 amino terminus. The human FCRLA D1 contains an additional cysteine located between these two conserved residues, making three or two cysteine residues in the human and mouse protein, respectively, potentially available for disulfide bond formation with other proteins or to form inter- or intramolecular bonds with itself.

Secondary structure predictions suggest that D1 contains disordered regions and is probably largely unfolded (https://genesilico.pl/meta2), and we have shown that D1 is involved in ER retention of FCRLA (Santiago et al. 2011) (see below). D2 and D3 have a high degree of interspecies protein sequence identity and are authentic C2-type Ig-like domains. (Ig-like domains can be classified according to the numbers of antiparallel β strands that comprise the two β sheets of the domain such as β sheet I: ABED strands and β sheet II: CFG strands (Bork et al. 1994; Halaby and Mornon 1998). The C1-type is the classical Ig-like domain found exclusively in molecules involved in the immune system. In the C2-type, strand D is deleted and replaced by strand C', which is directly connected to strand E). D4 is a bipartite domain consisting of an N-terminal mucin-like region, rich in proline, serine, and threonine residues, and a C-terminal region predicted to form an α-helix. Although mucin-like, D4 is not O-glycosylated and, notably, FCRLA also has no

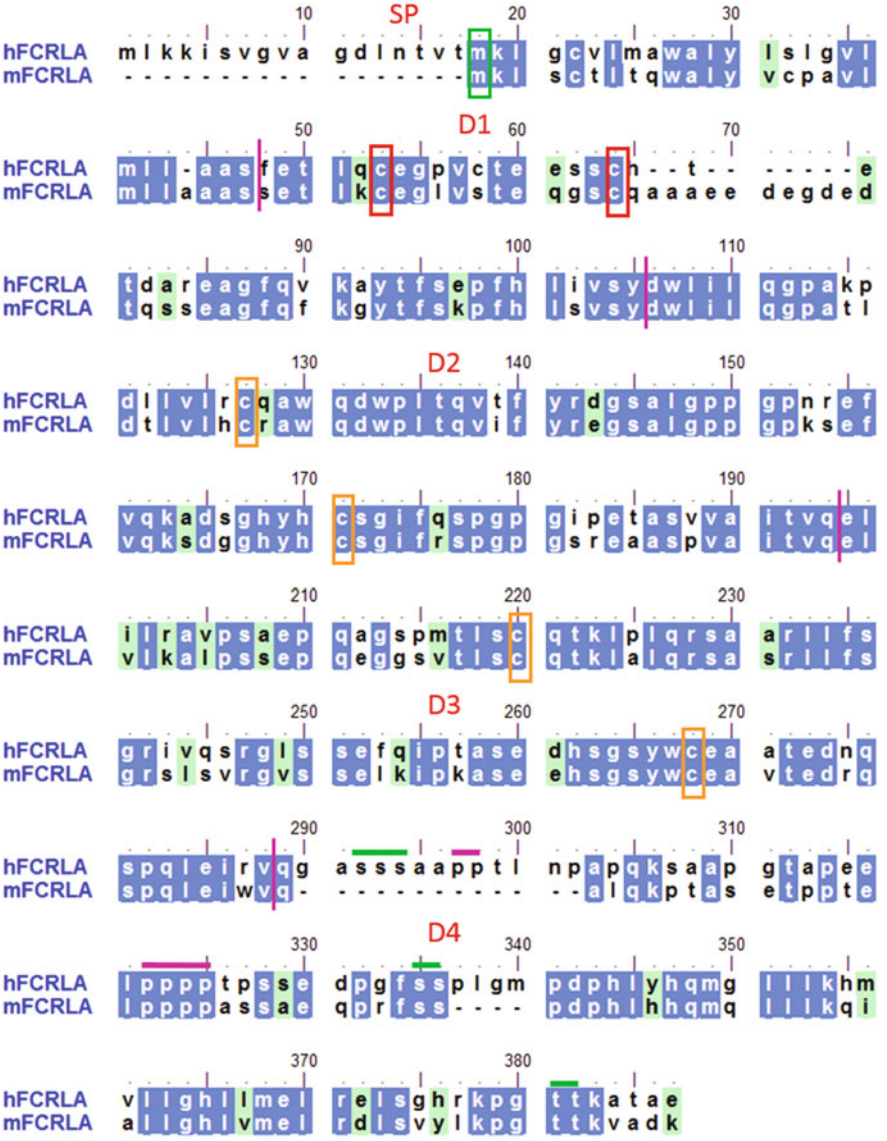

Fig. 3 Alignment of human and mouse FCRLA protein sequences. Amino acid sequence identity is indicated in *blue*, and amino acids with similar physiochemical features, i.e., charged, polar, and hydrophobic, are in *light green*, absent sequence by a *dot*. Domain boundaries are indicated by a *vertical purple bar*. The predicted ATG start codon is in a *green box*; the conserved D1 Cys residues are in *red*; the Ig-like domain Cys residues in D2 and D3 involved in the intrachain disulfide bond are in *orange*; stretches of two or more Pro and Ser residues in D4 are indicated by *purple* and *green overbars*, respectively. Note the absence of a KDEL endoplasmic reticulum retention motif in the C terminus of FCRLA in either human or mouse

N-linked glycosylation sites; thus, unlike most FCR and FCRL proteins, it is not a glycoprotein. FCRLA is not secreted or expressed on the plasma membrane, in keeping with the absence of a predicted transmembrane region or a glycosylphosphatidylinositol (GPI) linkage signature (Davis et al. 2002; Facchetti et al. 2002; Mechetina et al. 2002). Our studies indicate that FCRLA is a soluble protein retained in the ER, i.e., it is not a type II transmembrane protein anchored in the ER membrane by an uncleaved signal sequence (Santiago et al. 2011) (see below). The KDEL sequence motif (single-letter amino acid code) found on the C terminus of many soluble resident ER proteins is perhaps the best-characterized ER retention signal (Munro and Pelham 1987). However, the C-terminal amino acid sequences of the human (ATAE) and mouse (VADK) proteins do not correspond to this motif; thus, FCRLA must be retained in the ER by other mechanisms involving D1.

5 FCRLA—Phylogeny and Disease Association

Homologs of the pIgR, the FcR γ signaling chain, and several FCRL genes first appear in bony, but not cartilaginous fish. On the other hand, a gene encoding FCRLA has thus far only been found in mammals, in all extant genera including dolphins, whose putative FCRLA protein is 79% identical to the human FCRLA (information concerning mammalian FCRLA orthologs can be found at https://www.ncbi.nlm.nih.gov/gene/?Term=ortholog_gene_84824[group]). Mammals have complex and highly developed germinal centers (GCs), the site of highest human FCRLA expression and perhaps where it performs one of its major functions (see below), which may account for the relatively recent phylogenetic appearance of *FCRLA*.

FCRLA and *FCRLB* are part of a large group of 3274 genes that are differentially expressed in abdominal aortic aneurysm tissue compared to non-aneurysmal controls (Nischan et al. 2009). Genome-wide association studies have linked FCRLA to systemic lupus erythematosus and to the response to the hepatitis B vaccine (Davila et al. 2010; Bentham et al. 2015; Kim et al. 2016). FCRLB has been linked to IgA nephropathy (Zhou et al. 2013). However, the functional relevance to these associations remains unknown since none of them has been verified experimentally, e.g., using in vitro disease models or by introducing the suspect single nucleotide polymorphism (SNP) into mice. Moreover, *FCRLA* is relatively closely linked to the classical Fc receptor genes. If it is in linkage disequilibrium with these genes, the SNPs in *FCRLA* may be carrier polymorphisms.

6 FCRLA—Expression Pattern and Regulation

Based on immunohistochemical analysis of human tonsils, FCRLA was initially described as preferentially expressed in the proliferating GC centroblasts (Facchetti et al. 2002; Mechetina et al. 2002). As different human lymphoid tissues have been analyzed, the observation that human FCRLA is very highly enriched in GCs has been confirmed (Masir et al. 2004). However, human FCRLA is also expressed in splenic marginal zone B and, to a lesser extent in mantle zone tonsillar B cells (Masir et al. 2004), results that are in accord with our initial mRNA analysis (Davis et al. 2002). We have used flow cytometry and IgD, CD19, and CD38 mAb as a more discriminating assay to detect FCRLA expression and intensity by human tonsillar B cell subsets. We found that, indeed, FCRLA is most highly expressed in $IgD^{+}CD38^{+}$ pre-GC and $IgD^{-}CD38^{+}$ GC cells, but also in naïve ($IgD^{+}CD38^{-}$) and memory ($IgD^{-}CD38^{-}$) B cells (Santiago et al. 2011). Intriguingly, its expression was lowest in plasma cells, a point that we will discuss later. Apart from its B cell-restricted expression, FCRLA is expressed in human melanoma cells and normal melanocytes (Inozume et al. 2005). Its function in the melanocyte lineage is unknown, but FCRLA deficiency has no effect on pigmentation in mice. Interestingly, FCRLA has been defined as a tumor antigen since IgG serum antibodies from some melanoma patients react with FCRLA and an FCRLA-dendritic cell vaccine protects against a B cell lymphoma in mice (Inozume et al. 2005, 2007).

The regulation of *FCRLA* expression has not been extensively examined. Facchetti et al. analyzed the response of human blood B cells stimulated with Protein A-bearing *Staphylococcus aureus* (SA) in the presence or absence of IL-2, IL-3, IL-4, IL-6, IL-10, or IL-12 (Facchetti et al. 2002). These investigators reported that freshly isolated blood B cells do not express FCRLA but that there was significant induction following culture with SA. This induction could be partially inhibited by IL-4, and there was a nearly complete inhibition with the above cocktail of cytokines. On the contrary, using a fluorochrome-conjugated FCRLA mAb, we could see clear intracellular staining of most, but not all, $CD19^{+}$ human peripheral blood B cells by flow cytometry (Fig. 4a). This result was confirmed by RT-PCR of FACS-sorted blood B cells (Santiago et al. 2011).

No reports characterizing the transcriptional regulation of *FCRLA* expression have been published. Computational analysis of the 200 bp region upstream of the *FCRLA* transcription start site, as defined by the 5′ UTR of the longest reported *FCRLA* cDNA, suggests several candidate transcription factors such as PAX5, XBP1, STAT1, and IRF1 and the transcriptional repressor YY1 (unpublished). Experimentally, E2A has been shown by chromatin immunoprecipitation to bind to the *Fcrla* promoter region in anti-CD40 plus IL-4-activated mouse B cells, a treatment that upregulates *Fcrla* mRNA levels (Wohner et al. 2016).

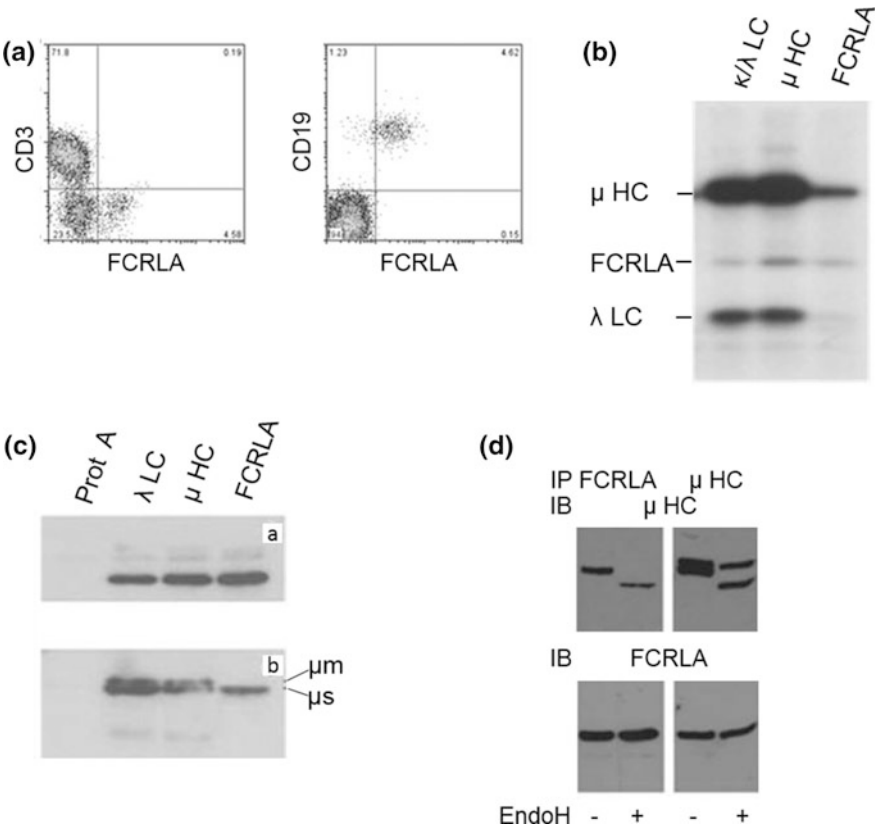

Fig. 4 FCRLA expression by normal B cells and its preferential association with μs in the Ramos B cell line. **a** Human blood mononuclear cells were stained for cell surface CD3 to identify T cells (*left panel*) and CD19 to identify B cells (*right panel*) and then fixed, permeabilized, and stained of intracellular FCRLA. Most B cells express FCRLA at readily detectable levels, but T cells are negative, as expected based on previous analysis of *FCRLA* mRNA. **b** Ramos B cells (μλ) were metabolically labeled and NP-40 cell lysates were immunoprecipitated with the indicated antibodies. The samples were analyzed by SDS-PAGE under reducing conditions. The positions of the μ HC, FCRLA, and λ LC are indicated. **c** Ramos cell lysates were immunoprecipitated with the indicated antibodies or Protein A-coupled beads alone, resolved by SDS-PAGE under reducing conditions and then analyzed by Western Blotting. The blot in **a** was probed with FCRLA antibody and in **b** with anti-μ. Note the two closely migrating bands in **b** corresponding to μm and μs in the anti-μ and anti-λ lanes. Only the lower μs band is immunoprecipitated with the FCRLA antibody. **d** Ramos cell lysates were immunoprecipitated (IP) with the indicated antibodies and then treated with endoglycosidase H (EndoH) (+) or mock treated (−). Samples were resolved by SDS-PAGE under reducing conditions and then analyzed by Western Blotting. The top blot was probed (IB) with anti-μ then stripped and probed with FCRLA antibody (*bottom*). EndoH treatment allows for very clear resolution of the μm and μs bands, confirming the preferential association of FCRLA with μs

7 FCRLA is a Soluble Resident ER Protein

The predicted sequence of FCRLA suggested it was a cytoplasmic protein. We and others have confirmed that predication and also show that it is a resident, soluble ER protein. Its location in this organelle raises intriguing questions about FCRLA function. Our studies (Santiago et al. 2011) have shown that (1) FCRLA cannot be detected on the cell surface of live B cells by immunofluorescence microscopy, even using directly conjugate mAb, but when cells are fixed and permeabilized it is readily detected in the intracellularly. (2) Confocal microscopic imaging showed clear co-localization of FCRLA and ER marker proteins, calreticulin and calnexin, but not with the intermediate compartment protein, p58, or the Golgi complex protein giantin in BJAB B cells and *FCRLA*-transfected HeLa carcinoma cells. (3) FCRLA was totally resistant to digestion in protease protection experiments using isolated ER vesicles. This indicates that FCRLA is present within the ER and not, e.g., attached on the outside of the ER or partially protruding through the ER membrane. (4) Extraction with carbonate buffer pH11 retains the general integrity of the ER membrane but opens the ER vesicles, thus allowing the release of soluble but not integral ER membrane proteins. When this type of analysis was applied to FCRLA, the results indicated that FCRLA is a soluble ER protein and not trans-membrane associated, e.g., by an uncleaved signal sequence.

8 FCRLA is Retained in the ER via its N-terminal Disordered Domain

As described in Sect. 5, FCRLA lacks any known ER retention signal, and thus, as a first approach to defining the mechanism for its retention, we constructed domain deletion expression vectors, transfected them into a fibroblast cell line, (293T) and then analyzed for the presence of FCRLA in both cell lysates and their culture supernatants by Western Blot (Santiago et al. 2011). As expected, the wild-type FCRLA (FCRLA-WT) was only detectable in cell lysates; however, when D1 (see Fig. 2) was deleted, the truncated protein (FCRLA-ΔD1) was found in both cell lysates and supernatants. The secreted FCRLA-ΔD1 showed evidence of extensive O-linked glycosylation, most likely on the multiple Ser/Thr residues located in the mucin-like region of D4. Since O-glycosylation occurs in the Golgi, these results are consistent with our additional data, indicating that FCRLA-WT is a resident ER protein that does not enter the secretory pathway and thus never transits to the Golgi. The exact FCRLA retention mechanism is currently unknown, although it does not require B cell-specific proteins since these mutation experiments were done in a fibroblast cell line, and FCRLA-transfected HeLa cells also showed ER localization by confocal microscopy. Since D1 is predicted to contain disordered

regions and be largely unfolded, it may interact with one of the ER chaperones, e.g., BiP. An additional possibility is that FCRLA is disulfide bonded to another resident ER protein via one of the D1 Cys residues.

9 FCRLA Associates with Multiple Ig Isotypes in the ER

Our studies using human B cell lines as well as primary tonsillar B cells have demonstrated that FCRLA specifically interacts with IgM, IgG, and IgA (Figs. 4b and 5) (Santiago et al. 2011) and results are confirmed for IgM and IgG by Wilson et al. in B cell lines (Wilson et al. 2010) [IgD and IgE have not been tested for FCRLA binding].

The ability of a single Fc receptor to bind three Ig isotypes is unprecedented, although the cytosolic tripartite motif-containing protein 21 (TRIM21) is known to bind the three major Ig isotypes (see below). The pIgR binds polymeric IgM and IgA, but via a common ligand J chain. The Fcα/μR (Fcα/μR) binds Fc regions of both IgM and IgA via its single Ig-like domain, and details of these interactions have been fairly well characterized. The Ig-like domain of Fcα/μR has three CDR3-like loops that contribute to binding of its IgA and IgM ligands (Yang et al. 2013). On the ligand side, an exposed PLAF loop in the Cα2/Cα3 interdomain region and a homologous motif (PNRV) in Cμ4 are required for Fcα/μR binding (Ghumra et al. 2009). Whether there is some other short structural motif in Cμ, Cα, and Cγ that mediates binding to FCRLA remains to be determined. [In fact, none of the laboratories working in this field has yet formally demonstrated that FCRLA interacts with the Fc region of Ig].

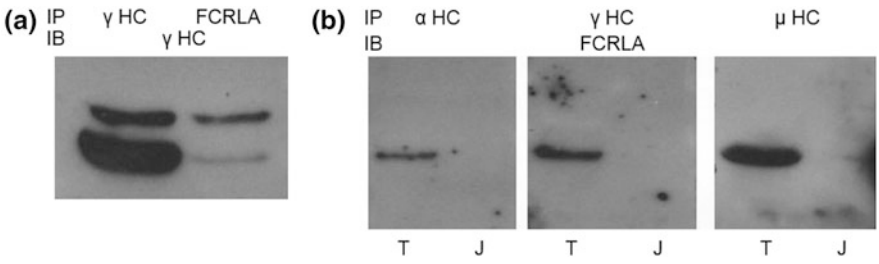

Fig. 5 FCRLA associates with IgG in the IM9 B cell line and with IgM, IgG, and IgA in tonsillar B cells. **a** NP-40 cell lysates of the IM9 (γκ) B cell line were immunoprecipitated (IP) with anti-γ HC or anti-FCRLA antibodies, resolved by SDS-PAGE under reducing conditions and then analyzed by Western Blotting (IB). As can be seen in the *left panel*, IM9 synthesizes mainly the secretory form of IgG (γs, lower band). In contrast to the situation with the Ramos cells (Fig. 4c and d), FCRLA does not show preferential association with γs, if anything there is preferential association with γm (*upper band*). **b** NP-40 cell lysates of tonsil (T) and Jurkat (J) cells as a negative control were immunoprecipitated with anti-α HC, anti-γ HC or anti-μ HC antibodies, resolved by SDS-PAGE under reducing conditions and then analyzed by Western Blotting with FCRLA antibody

One finding that would argue against the existence of such a common FCRLA recognition motif is our totally unexpected discovery that, at least in the Ramos Burkitt's lymphoma B cell line (μλ), FCRLA preferentially associates with the secretory form of IgM synthesized by these cells (Fig. 4c and d). During their development, B cells can differentially regulate transport of the membrane (μm) and secretory (μs) IgM heavy chain (HC) at the post-translational level. For example, many B cell lines synthesize μm and μs HC in similar amounts and assemble with Ig light chains (LCs) into $\mu_{m2}LC_2$ and $\mu_{s2}LC_2$ complexes. The μm-BCR is allowed to leave the ER, whereas the μs complexes are degraded (Brooks et al. 1983; King and Corley 1989). The rationale for this differential regulation is clear, since the release of a soluble decoy BCR would impede antigen recognition by the transmembrane BCR; however, its mechanism has not been well understood. We propose that FCRLA is a likely candidate responsible for the differential retention of the secretory IgM in B cells. We first noticed this phenomenon when we performed immunoprecipitation of Ramos cell lysates (NP-40 detergent) with FCRLA-, μ HC-, or λ LC-specific antibodies, followed by SDS-PAGE and Western Blotting with antibodies of the same specificity. As expected, immunoprecipitation with the anti-μ or anti-λ antibodies brought down both μ_m and μ_s, as well as FCRLA (Fig. 4c), since it is associated with the complete IgM molecule in the ER. In striking contrast, anti-FCRLA immunoprecipitated only μ_s HC and FCRLA. Although μ_m and μ_s HC can be distinguished by SDS-PAGE under these conditions, their resolution is not optimal. To unambiguously confirm this unexpected finding, we treated the immunoprecipitated material with endoglycosidase H (EndoH), an enzyme that removes high-mannose N-linked oligosaccharides from glycoproteins, allowing for very clear separation of μ_m and the ER-retained μ_s. Again, FCRLA antibody co-immunoprecipitated only the lower μ_s band, confirming that FCRLA can preferentially associate with the secreted, but not the membrane-bound form of IgM in the Ramos B cell line (Fig. 4d).

This preferential association of FCRLA with the secretory form of IgM is intriguing and quite unexpected since μ_m and μ_s HC are identical for the first $\sim$556 amino acids and then diverge, with membrane and secretory C-termini encoded by separate exons. The μ_s HC C terminus is only 20 amino acids in length but contains a preterminal Cys involved in interchain disulfide bond formation to form the IgM pentamer. This Cys could conceivably form an S–S bond with one of the Cys residues in D1 of FCRLA, and this in turn could account for the fact that we observed no such preferential association with the γs HC in the IM9 (γκ) cell line (Fig. 5a), since it lacks this Cys residue. Both $\alpha1_s$ and $\alpha2_s$ HC have this Cys, but technical issues have prevented clear resolution of the α_s and α_m chains by SDS-PAGE, which would help resolve this important issue. Thus, the possibility remains that FCRLA may utilize different or multiple mechanisms to associate with IgM/IgA and IgG.

The binding of FCRLA to γ and α HCs appears to occur independently of any additional proteins since they both appeared as independent hits in a yeast two-hybrid screen in which full-length FCRLA was used as bait and a spleen cDNA library as prey (Tim Wilson, personal communication). No μ HC was identified by

this approach, but since plasma cells likely contributed most of the Ig transcripts in the library, the absence of μ HC may reflect the relative abundance of IgM versus IgG and IgA plasma cells. Surprisingly, κ and λ LCs made up 50 and 17%, respectively, of the Ig hits in this screen, suggesting that FCRLA can interact directly with both HC and LC. This very intriguing possibility needs confirmation by an independent approach since the LCs may be partially unfolded or not folded properly in the reducing environment of the yeast cytosol. If FCRLA is an Ig chaperone (see final section), it may preferentially bind to such molecules. The binding of FCRLA to HCs, on the other hand, has been observed in both yeast two-hybrid experiments and by immunoprecipitation of the endogenous complexes from B cell lysates.

10 TRIM21—One Other Intracellular Fc Receptor that Binds Multiple Ig Isotypes

Tripartite motif-containing 21 (TRIM21), also known as E3 ubiquitin-protein ligase TRIM21 and a member of the large TRIM family, is uniquely involved in intracellular destruction of antibody-bound viruses, particularly non-enveloped viruses (Mallery et al. 2010; Foss et al. 2015). The antibodies in this case are non-neutralizing and thus do not prevent viral entry into the target cell. Once inside the cell, TRIM21 recognizes the antibody portion of the virus/antibody complex and targets the pathogen for elimination via the ubiquitin/proteasome pathway, a mechanism termed antibody-dependent intracellular neutralization. The antibody is also degraded during this process, but TRIM21 survives. Intriguingly, TRIM21 can interact with the Fc region of multiple Ig isotypes, IgM, IgG, and IgA. This interaction is mediated by a PRYSPRY domain in the C terminus of TRIM21.

In addition to destroying viruses before they have time to replicate, TRIM21 acts to trigger a cytosolic danger signal. Antibodies are not normally present in the cytosol and function as danger-associated molecular patterns recognized by TRIM21. This recognition leads to activation of an inflammatory response and induction of an antiviral state, further protecting the host cell. Recent studies have suggested that TRIM21 can also inhibit seeded tau aggregation (McEwan et al. 2017). Cytoplasmic aggregation of the microtubule-associated protein tau is a common feature of Alzheimer's and some other neurodegenerative diseases. Transcellular transfer of tau misfolding is thought to be the major mechanism of spreading tau aggregates in the brain. Experimentally administered tau antibodies enter cells as a complex with tau seeds and are recognized by TRIM21. The tau seeds are then neutralized, similar to the fate of antibody–virus complexes.

Despite their common ability to recognize multiple Ig isotypes, there are major differences between TRIM21 and FCRLA, including: (1) structural features—TRIM21 is not an Ig domain protein but a multidomain protein that includes an N-terminal RING domain with E3 ubiquitin ligase activity; (2) the PRYSPRY

domain—FCRLA does not contain one; (3) expression pattern—TRIM21 is expressed in nearly all cells while FCRLA is restricted to a subset of B cells and melanocytes; and (4) intracellular location—TRIM21 is in the cytosol and FCRLA is in the ER.

11 FCRLA Function—Facts and Speculations

11.1 Facts

Human FCRLA is a protein resident in the ER, where it can bind IgM, IgG, or IgA. No functional studies have been done with mouse FCRLA. An *Fcrla* knockout mouse has been made but had no apparent phenotype (Wilson et al. 2012); thus, there may be redundancy in the system. FCRLB might seem an obvious candidate to assume FCRLA function in the *Fcrla* knockout; however, in humans at least, FCRLB is expressed in only very rare cells in the tonsil germinal centers, unlike FCRLA which is expressed by a significant fraction of GC B cells, and moreover, such $FCRLB^{+}$ cells are FCRLA-negative (Wilson and Colonna 2005). No expression studies of FCRLB have been done in the mouse, but by RT-PCR, we noted very low *Fcrlb* transcript levels (Masuda et al. 2005), so FCRLB seems an unlikely substitute for FCRLA in the knockout situation.

11.2 Speculations

Based on its high-level expression in human germinal centers, we propose a model for FCRLA function depicted in Fig. 6. B cell activation requires interaction of cognate antigen with the BCR complex composed of two components in mature naïve B cells, antigen-specific transmembrane IgM, and the Igα/β signal transduction chains. It would be clearly advantageous for the B cell, which synthesizes both membrane and secretory forms of IgM, to retain the secretory form, which would behave as a soluble decoy BCR. Based on our studies of the Ramos GC-derived B cell line, we suggest that FCRLA performs this function, which takes on added importance in the GC. There, B cells responding to T-dependent antigen undergo massive expansion and somatic hypermutation in the GC dark zone. They then move into the light zone where they attempt to scavenge antigen displayed on the surface of follicular dendritic cells (FDCs) (Fig. 6a) (De Silva and Klein 2015; Corcoran and Tarlinton 2016; DeFranco 2016; Zhang et al. 2016; Spillane and Tolar 2017). The antigen is internalized and processed, and the peptides are loaded into MHC II and presented to T follicular helper (Tfh) cells, which provide help for isotype switching, proliferation, and differentiation. This is a competitive process, B cells with higher affinity BCRs get more antigens, and therefore, more T cells help. In this case, it

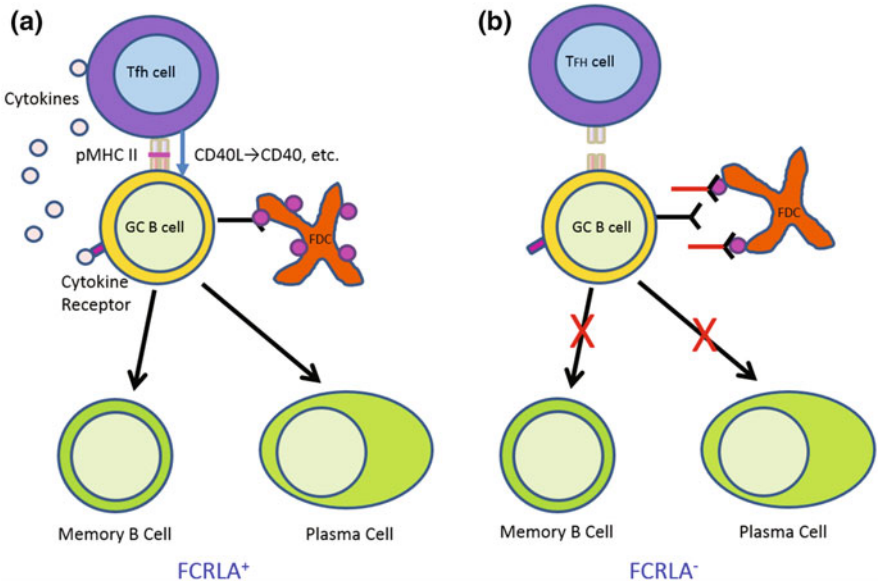

Fig. 6 Model for the function of FCRLA in the germinal center. **a** Germinal center (GC) B cells undergo somatic hypermutation in the GC dark zone, and then, cells with a high-affinity B cell receptor (BCR) are positively selected in the GC light zone, where follicular dendritic cells display antigen on their cell surface. B cells collect this antigen, process it, and present it to T follicular helper cells (Tfh), which provide help for isotype switching, proliferation, and differentiation via soluble cytokines and direct cell interactions, e.g., via CD40L/CD40. **b** In the absence of FCRLA, the GC B cells would secrete soluble antibody (depicted with a red HC constant region), which would compete for binding and uptake of antigen by the BCR. The GC B cells would thus receive limited help from the TFH cells, and the production of high-affinity, isotype-switched B cells and plasma cells would be impaired

would be particularly important for the B cell not to secrete IgM, since it could eliminate Tfh help and impair selection for high-affinity isotype-switched antibodies. Given all this, it is surprising that the *Fcrla*$^{-/-}$ mice had no obvious phenotype, e.g., inability to produce high-affinity antibodies (Wilson et al. 2012). We believe that this is due to a species difference between mice and men. Human FCRLA has an important structural difference; i.e., an extra Cys in the D1 domain that we have proposed may be covalently attached to the preterminal Cys in the μ_m HC. There are also notable differences in the FCRLA expression pattern; highest levels of the human protein are found in the GC, whereas this is not the case in mice, where FCRLA is rather uniformly expressed in most B cells and is in fact downregulated in GC B cells (Wilson et al. 2012). Consistent with this model, FCRL expression is downregulated in plasmablast/plasma cells, where high rate Ig secretion is essential and FCRLA would be detrimental to this process.

To end on a less speculative note, the ER-restricted location of FCRLA and its ability to associate with the multimeric Ig proteins in B cells are reminiscent of

features of molecular chaperones, which are defined as proteins that interact with and aid in the folding or assembly of another protein without being part of the final structure (Kim et al. 2013). Many such ER chaperones have been identified and functionally characterized, including BiP/GRP78, GRP94/gp96, GRP170/ORP150, GRP58/ERp57, PDI, ERp72, calnexin, calreticulin, EDEM, and Herp (Hebert and Molinari 2007; Ni and Lee 2007). BiP/GRP78 is perhaps the closest functional analog of FCRLA. It is part of the ER quality control system and binds to many proteins, the most relevant here being the Ig HC. BiP binds to the partially unfolded CH1 domain and retains the HC in the ER until this interaction is disrupted by binding of LC to the HC, forming a complete Ig molecule that can enter the secretory pathway (Haas 1991; Gething 1999; Lee et al. 1999). The results of the yeast two-hybrid analysis described in Sect. 10 are consistent with such a chaperone function. Detailed study of the precise sites of interaction of FCRLA with Ig HC or LC is needed to better define its role in the physiological environment of the ER. Knockdown/knockout studies in cell lines such as Ramos, where FCRLA specifically binds to the μs HC, followed by analysis of IgM secretion would also be informative. Finally, an unbiased proteomics approach of FCRLA-associated proteins in B cells and melanocytes/melanoma cells would reveal if the nature of any other binding partners.

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