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Differential expression of FCRLA in naïve and activated mouse B cells

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ABSTRACT

FCRLA is an intracellular B cell protein that belongs to the FcR-like family. Using newly generated FCRLA-specific antibodies, we studied the constitutive expression pattern of mouse FCRLA and monitored changes during an immune response and following *in vitro* B cell activation. All B cell subpopulations examined expressed FCRLA. However, the level of FCRLA expression is determined by the stage of B cell differentiation. Low expression of FCRLA is characteristic of naïve follicular and marginal zone B cells. High expression was detected in a small fraction of activated B cells scattered along migratory pathways in the lymphoid tissues. FCRLA-bright cells could be subdivided into two subpopulations, with high and low/undetectable level of intracellular immunoglobulins, which phenotypically resemble either plasma or memory B cells. High expression of FCRLA in subset(s) of terminally differentiated B-cells suggests that, being an ER protein, FCRLA may participate in the regulation of immunoglobulin assembly and secretion.

1. Introduction

Antigen-induced differentiation of B cells ultimately leads to generation of antibody-secreting plasma cells (PCs) and memory B cells, both highly heterogeneous cell populations. Depending on the B cell subset activated, tissue/cellular milieu, and the nature of the antigen, the differentiation process may generate cells differing in their maturity, functional capacity and lifespan [1–4]. Immature populations of antibody-secreting cells are called plasmablasts and pre-plasma cells, whereas terminally differentiated PCs can be both short- and long-lived [4,5]. Similarly, several subpopulations of memory B cells have been described [6–9]. Because of the absence of unique markers, the relationships among the various PC and memory cell subpopulations described by different authors are not always clear-cut and are sometimes controversial. Better understanding of the late stages of B cell differentiation is important for definition of factors influencing long term immunity against pathogens as well as for understanding the genesis of immune disorders caused by autoantibodies.

Completion of the human and mouse genome sequencing projects resulted in the identification of numerous previously unknown genes, some of which are differentially expressed in B cells. One such gene, first called FCRL/FREB/FcRX and subsequently designated FCRLA, has been shown to be abundantly expressed in germinal center (GC) B cells in human tonsils [10–13]. The mature protein is composed of four domains the first three of which (D1–D3) resemble the extracellular Ig domains of FcγRI, although D1 is a highly degenerate Ig-like domain that is unlikely to assume the typical Ig domain structure. Based on protein sequence homology and genomic localization, FCRLA is related to the classical Ig-binding Fc receptors, but differs from other members of this family in several significant ways. There are no predicted N-linked glycosylation sites and, instead of a transmembrane domain, FCRLA has a unique carboxy terminal domain (D4) enriched in proline, serine and threonine residues. Most notably, human FCRLA is an intracellular rather than a plasma membrane protein. The function of FCRLA remains unclear, and the only available data come from studies of the human protein. We and others have shown that FCRLA is a resident endoplasmic reticulum (ER) protein that interacts with multiple isotypes of intracellular Igs [14,15]. Until recently FCRLA expression at the protein level has only been studied in humans. It is highly expressed in GC cells in tonsils, primarily in the proliferating centroblasts, and has been shown to be up-regulated by peripheral blood B cells after *in vitro*

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stimulation [11]. These findings suggested that the protein may have a role in antigen-activated B cells. A later study has also shown moderate expression of human FCRLA in follicular and marginal zone B cells and its absence/low level expression in plasmacytomas and CD38-positive PCs (15 and 16). In this study we extended our FCRLA expression studies to mice and have analyzed constitutive expression in conventional mice and SPF mice, as well as changes in expression following *in vitro* activation and during an *in vivo* immune response. We found that mouse naïve B cells express FCRLA at a low level. Significant up-regulation of FCRLA occurs in a small fraction of B cells ($FCRLA^{br}$) generated in response to antigenic challenge. The $FCRLA^{br}$ cells can be further divided into two subsets, one with a high level of cytoplasmic Ig and the other with either low or undetectable cytoplasmic Ig. The phenotypic features of these cells only partially overlap with the typical characteristics of PCs and memory cells. Importantly, the $FCRLA^{br}$ cells accumulate in the bone marrow of immunized mice, suggesting their possible involvement in long-term immunity.

2. Materials and methods

2.1. Mice

Conventional BALB/c mice were housed within the animal facility at the Institute of Cytology and Genetics (SB RAS, Novosibirsk, Russia). Unless otherwise stated, 8–10-wk-old female mice were used. Specific pathogen-free (SPF) female mice were purchased from the Animal Breeding Facility, Branch of Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry (Pushino, Russia). SPF CBA/J mice at 12 wk of age were used for *in vitro* mitogenic stimulation. To study FCRLA expression during an immune response *in vivo*, mice were immunized with rabbit RBC i.p. and in the rear paws, and their spleen, lymph nodes, bone marrow and PBMC were analyzed by immunohistochemical staining at 3, 7, and 14 days after immunization. All the studies were approved by the appropriate local ethical committee.

2.2. Northern blot analysis

The expression of FCRLA mRNA in mouse tissues was examined by Northern blot analysis. And *Eco*RI–*Not*I fragment of the mouse cDNA clone ms73c01 (I.M.A.G.E. clone 617184) was used as a probe. Poly(A)+ RNA from tissues of BALB/c mice was isolated from total RNA on oligo-dT cellulose (Sigma–Aldrich, St. Louis, MO, USA), fractionated (5 μg/line) on 1% agarose gels and vacuum blotted onto a Zeta-Probe nylon membrane (Bio-Rad Laboratories, Hercules, CA, USA). The blot was hybridized with either ^{32}P-labeled *Fcrla* or β-*actin* probes under high stringency conditions following the Bio-Rad recommendations.

2.3. RT-PCR

The mouse T cell line EL4, B cell lines A20 and M12, macrophage cell line J774, pro-B cell line L1210, melanoma B16, and plasmacytomas NS1 and NS0 were maintained in RPMI 1640 supplemented with 50 μg/ml gentamicin, 2 mM L-glutamine, and 10% FBS. Total RNA extracted from the cell lines was reverse transcribed with SuperScript II RNase H reverse transcriptase (*Gibco-BRL*, Grand Island, NY, USA) according to the manufacturer's recommendations. The following gene-specific primer pair was used in the RT-PCR analysis of *Fcrla* expression: forward, 5′-ATGTCTGCCCTGCTGTGCTCC-3′ and reverse, 5′-GACCAGATGACCGAGGAGAGC-3′. The samples underwent denaturation at 94 °C for 3 min followed by 30 cycles of amplification (94 °C for 30 s, 68 °C for 30 s, 72 °C for 1 min). A positive control (the *Fcrla* plasmid) and a negative control containing all the reagents except cDNA were included in every PCR analysis. The cDNA samples were additionally checked by applying RT-PCR analysis to β-actin. Oligonucleotides used as primers for PCR amplification of a mouse β-actin fragment were 5′-CGCGAGAAGATGACCCAGATC-3′ and 5′-TTGCGATCCACATCTGCTGG-3′.

2.4. Rabbit antiserum

Recombinant mouse FCRLA protein was generated using an *Escherichia coli* expression system. The pT7-ABPb and pT7-TZZb expression vectors were generously provided by Dr. S. Stahl (The Royal Institute of Technology, Stockholm, Sweden). The FCRLA fragment lacking the predicted leader peptide and the fourth domain was expressed as a part of the ABP- or TZZ-fused proteins. FCRLA–TZZ, which contains an *Staphylococcus aureus* protein A-derived Ig binding motif, was affinity purified using rabbit IgG coupled to Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden). A rabbit was immunized with the purified protein (three injections 200 μg each in complete Freund's adjuvant, Sigma–Aldrich). The immunoglobulin fraction of antiserum was prepared by precipitation with $(NH_4)_2SO_4$ (40% saturation).

2.5. Western blotting

Cells (5×10^4 per sample) were lysed for 5 min in a loading SDS buffer at 100 °C and subjected to reducing 12% SDS–PAGE. After electrophoresis, the separated proteins were transferred to nitrocellulose membrane Hybond-C (Amersham Biosciences, Piscataway, NJ, USA). The membrane was immuno-stained using FCRLA-specific Ab and HRP conjugated anti-rabbit IgG.

2.6. Transfections

293T cells were transiently transfected with the pCI-neo-*Fcrla* plasmid DNA using Unifectin 56 (IBCH, Moscow, Russia) according to the manufacturer's protocol. After 72 h, the cells and supernatants of transfectants were harvested and used for analysis of FCRLA expression as described above.

2.7. Intracellular staining and confocal microscopy

Transient transfections of COS-7 cells were performed with DEAE-dextran (Mr = 500,000; Amersham-Pharmacia Plc, Bucks, UK). Cells were harvested 60 h after the transfection, fixed with 2% PFA for 15 min on ice, and permeabilized in 0.2% saponin containing 1% FBS for 5 min at room temperature. Then cells were incubated with FCRLA-specific rabbit Ab and labelled with secondary anti-rabbit Alexa Fluor 647 conjugate (Molecular Probes, Eugene, OR, USA) and Alexa Fluor 488-conjugated cholera toxin B. Stained and washed cells were mounted onto microscope cover slips. The fluorescent signals were recorded and visualized with Olympus FLUOView500 laser scanning confocal microscope (Olympus, Hamburg, Germany). Images were further processed by ImageJ software (Wayne Rasband, NIH, Bethesda, MD, USA).

To analyze intracellular localization of FCRLA, 293T cells were grown on coverslips and transiently transfected with pCI-neo-FCRLA. Forty-eight hours after the transfection, double immunofluorescent staining with Abs against FCRLA and either p58K (Abcam, Cambridge, UK) to label Golgi or calnexin (BD Transduction Laboratories, Lexington, KY, USA) to label ER was performed. Confocal microscopic analysis was performed using an LSM 510 microscope (Carl Zeiss Inc, Jena, Germany).