Current Topics in Microbiology and Immunology

Marc Daëron
Falk Nimmerjahn *Editors*

Fc Receptors

Springer

Current Topics in Microbiology and Immunology

Volume 382

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ISSN 0070-217X ISSN 2196-9965 (electronic)
ISBN 978-3-319-07910-3 ISBN 978-3-319-07911-0 (eBook)
DOI 10.1007/978-3-319-07911-0
Springer Cham Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014943921

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

High amounts of specific antibodies are produced upon antigen stimulation during adaptive immune responses. Antibody production can rapidly resume, long after a primary response, and in higher amounts, during memory responses. Altogether, antibodies of every individual recognize a wide repertoire of antigens encountered during life, including autoantigens. As a result, several grams of antibodies with multiple specificities per liter of plasma circulate throughout the body via the bloodstream. Antibodies can reach remote organs in the periphery within minutes. They are involved in a variety of biological responses in health and disease. They can both protect from infections and induce allergic, autoimmune, or other inflammatory diseases. Genetically engineered monoclonal antibodies are increasingly used in passive immunotherapy, mostly, but not exclusively in cancer. Immunoglobulins pooled from the plasma of thousands of normal donors are injected intravenously (IVIg) as an anti-inflammatory treatment in an increasing number of autoimmune diseases. Actively produced specific antibodies account for the therapeutic effects of the overwhelming majority of protective vaccines, whether prophylactic or therapeutic. How antibodies work, however, is far from being fully understood and appreciated.

Antibodies bind to specific antigens by their Fab portions with a wide range of affinities. Binding is necessary for antibodies to act on antigens. Binding, however, is not sufficient. Antibodies indeed exert little or no effect when binding to antigen only. They have no biological activities per se. Antibodies, however, *mediate* many biological activities. They are mediators rather than effectors of adaptive immunity. Biological activities mediated by antibodies require their Fc portion. The Fc portion of immunoglobulins consists of the C-terminal constant domains of the two heavy chains that are characteristic of antibody classes and subclasses. Antibody-mediated biological activities indeed depend on the class of antibodies. The reason is that the Fc portion of antibodies of different classes differentially interacts with other molecules that can induce a variety of effector functions. These molecules are of two types: soluble molecules such as components of complement, and Fc Receptors (FcRs) expressed on the membrane of various cells.

For long, the existence of FcRs has been inferred from the observed biological effects of so-called “cytophilic” antibodies. In spite of the classical opposition between cell-mediated immunity and humoral immunity, some biological properties of antibodies were indeed found to depend on cells. When binding to

antigens, antibodies called "opsonins"—literally, which prepare the food to be ingested—enabled phagocytes to internalize particulate antigen-antibody complexes. Anaphylaxis and antibody-dependent cell-mediated cytotoxicity were found to result from the release of vasoactive and cytotoxic mediators, respectively, stored in the granules of different cell types. There were "homocytotropic" antibodies, which triggered responses in homologous tissues, and "heterocytotropic" antibodies, which triggered responses in heterologous tissues; there were antibodies whose cytophilic properties were heat-labile and antibodies which were heat-stable; there were washing-resistant and washing-sensitive cell-sensitizing antibodies, all of which could trigger similar responses but under different conditions. There were also enhancing and regulatory antibodies of different IgG subclasses. Although the concept of receptors for the Fc portion of cytophilic antibodies was proposed to account for the enhanced internalization of opsonized antigens by macrophages in the 1960s (Berken and Benacerraf 1966), the term Fc Receptors was not coined until 1972 by Frixos Paraskevas to describe IgG receptors on B lymphocytes (Paraskevas et al. 1972). By being given a name, FcRs gained a material existence. They could be identified on cell membranes and they became susceptible to molecular analysis.

FcRs for the various classes of immunoglobulins were indeed identified using several means to visualize cell-bound antibodies. FcRs with a high affinity were first found on a limited number of cells by assessing the binding of radiolabeled immunoglobulins. Using this approach, IgG and IgE receptors were found on macrophages and mast cells, respectively. Homogeneous cell lines made it possible to assess FcR numbers on single cells, to measure association and dissociation constants, and thus to calculate affinity constants. These were between 10^8 (Unkeless and Eisen 1975) and 10^{10} M^{-1} (Kulczycki and Metzger 1974).

Many more receptors for the same and for other immunoglobulin isotypes were subsequently identified by assessing the binding of red cells sensitized with antibodies under the microscope. These receptors had no measurable affinity for radiolabeled monomeric immunoglobulins, but they could bind multivalent immune complexes with high avidity. Using this "rosetting" procedure, all myeloid cells and some lymphoid cells expressed FcRs, and FcRs for all five immunoglobulin classes were recognized. These findings led to the distinction of high-affinity receptors referred to as FcRI, which bind antibodies as monomers, and of low-affinity receptors referred to as FcRII, which bind antigen-antibody complexes only. FcRs were also found on parasites (Torpier et al. 1979; Vincendeau and Daëron 1989), bacteria (Langone 1982), and even virus-encoded FcRs were described on infected cells (McTaggart et al. 1978; Litwin et al. 1990; Litwin and Grose 1992).

When monoclonal antibodies were raised against FcRs, cell population analysis by flow cytometry confirmed the distinction between high- and low-affinity FcRs and their differential tissue distribution. It also revealed a further heterogeneity amongst low-affinity receptors for IgG expressed by different cell types (Unkeless et al. 1988). Low-affinity FcRs were therefore subdivided into FcγRII and FcγRIII. More recently another high-affinity receptor for IgG found in mice but not in

humans, was named FcγRIV (Nimmerjahn et al. 2005). Human FcRs identified by referenced monoclonal antibodies were given CD numbers and used as phenotypic markers of cell populations: CD16 corresponds to FcγRIII, CD32 to FcγRII, CD64 to FcγRI, CD23 to FcεRII and CD89 to FcαRI. FcεRI have no CD number.

As FcRs were increasing in numbers, biochemical analysis disclosed their molecular heterogeneity. High-affinity IgE receptors first (Holowka et al. 1980), then IgG (Ernst et al. 1993) and IgA receptors (Pfefferkorn and Yeaman 1994) were found to contain several polypeptides. Most are composed of 2–3-extracellular-domain immunoglobulin-binding FcRα subunit noncovalently associated with a widely expressed, highly conserved homodimeric common subunit named FcRγ (Orloff et al. 1990) and, when expressed in mast cells or basophils, with a 4-transmembrane subunit named FcRβ whose expression is restricted to these cells (Kurosaki et al. 1992). cDNAs encoding the various FcR subunits having been cloned and expressed in different cells, their functional roles could be analyzed. FcRγ and FcRβ were found to control both the membrane expression of FcRα (Takai et al. 1994; Kinet 1999) and the ability of membrane FcRs to generate activation signals when engaged by antigen–antibody complexes. FcRγ and FcRβ were indeed shown to contain Immunoreceptor Tyrosine-based activation Motifs (ITAMs) (Reth 1989). Two single-chain low-affinity IgG receptors expressed in humans only also contained one ITAM, whereas another single-chain low-affinity IgG receptor expressed in mice and humans was found to contain an Immunoreceptor Tyrosine-based Inhibition Motif (ITIM) (Daëron et al. 1995). Other receptors triggered neither activation nor inhibition signals, but permitted a strictly controlled internalization of antibodies. The 5 extracellular domain-containing polyIg receptor enables pentameric IgM and dimeric IgA to transcytose through polarized cells (Brandtzaeg 1983), whereas the β2-microglobulin-associated MHC-I-like FcRn not only mediates the intestinal absorption of maternal IgG through the fetal gut epithelium, but protects IgG from degradation in adults (Raghavan et al. 1993; Roopenian et al. 2003).

When FcR genes were cloned, their phylogenetic relationship was established (Qiu et al. 1990) and their heterogeneity was further enriched. Capital letters were added to FcR names to designate human genes and their murine orthologs. More recently, a novel family of FcR-like (FCRL) molecules was disclosed in mice and humans, which dramatically expanded the FcR field (Ehrhardt et al. 2007). Many FCRLs still have no known ligand. Some can bind immunoglobulins. They have similar structures, similar signaling properties and similar genetic organizations as classical FcRs, but also marked differences. Genetic polymorphisms were unraveled in classical human FcRs, some of which were associated with disease, mostly autoimmune diseases, and/or with a differential efficacy of therapeutic antibodies. FcR knockout, knockin, and transgenic mice were genetically engineered that proved to be invaluable analytical tools to assess FcR function in vivo. Special efforts have been made to generate humanized mice in which murine FcRs have been more or less extensively replaced by human FcRs, sometimes with the same tissue distribution as in humans. These unique mice should be a major advance to analyze the contribution of the various FcRs to the protective and the pathogenic roles of antibodies in mouse models of human diseases. They should be also of

interest to tailor and to assess the efficacy of novel therapeutic antibodies for specific purposes. Indeed, nonhuman primates that are often viewed as the best animal models for preclinical vaccine trials, seem not to have the same FcRs as humans (Trist et al. 2014).

As knowledge on FcRs progressed, the complexity of the mechanisms by which antibodies work increased dramatically. The difficulty to understand their biological effects in health and disease and to use them as therapeutic tools increased in parallel. As a consequence, it became difficult for most scientists, including immunologists, to embrace the multifaceted and often antagonistic properties of antibodies. The aim the this issue of Current Topics in Microbiology and Immunology on FcRs was to gather in a single volume the contributions of internationally recognized FcR experts on essential novel aspects of FcR biology in physiology and pathology. To our knowledge, such a book has not been published for many years. This volume is divided into five parts, which, we believe, cover the main aspects of current knowledge on FcRs.

The Part I titled *Old and New FcRs* contains three chapters. It provides novel information on old receptors and information on novel FcRs. Hiromi Kubagawa et al. report their recent findings on human and murine FcμR. The existence of this long suspected receptor for IgM now lies on solid grounds. It also has unexpected properties that other FcRs do not have. Randall Davis et al. provide a state-of-the-art overview of the FCRL family with their known ligands, and they discuss their potential functions. Finally, Leo James describes TRIM21. This intriguing intracellular receptor with an extraordinarily high affinity for IgG and IgM has unique structural and functional properties that endow it with major protective properties, especially against viral infection.

The Part II deals with *FcR Signaling*. It also contains three chapters. Denis Thieffry et al. present their novel bioinformatic approach of FcR signaling, using high-affinity IgE receptors in mast cells as a model. They show how computational modeling can help to integrate the complexity of signaling pathways. Pierre Launay et al. focus their review on calcium channels that have long been known to be critical in FcR signaling. They discuss the role of novel channels that control intracellular calcium and how these channels are tightly regulated. Finally, Michael Huber et al. address the mechanisms by which the lipid phosphatase SHIP1 negatively regulates FcεRI signaling and how both the expression and function of SHIP1 are controlled. This hematopoietic cell-specific phosphatase is a major regulator of many signaling pathways, particularly but not only, in mast cells.

The Part III entitled *FcR Biology* deals with various FcR functions, mostly, but not exclusively under physiological conditions. It contains six chapters. Marc Daëron first discusses how FcRs function as adaptive immunoreceptors (with an adaptive specificity, structure and signaling) that trigger adaptive biological responses with an extensive combinatorial functional diversity, depending on the functional repertoire of FcR-expressing cells selected by antibodies. Pauline Rudd et al. provide a comprehensive overview of the role that glycosylation plays in FcR functions. The glycosylation of antibodies is well known to determine their

binding to FcRs. That of FcRs is much less known. The interplay between carbohydrate–carbohydrate and carbohydrate–protein interactions, in ligands and receptors, opens a novel field of investigation. Birgitta Heyman discusses how, depending on the antigen and on the antibody class, antigen–antibody complexes can exert potent adjuvant effects or, on the contrary, suppress antibody responses. Understanding the mechanisms behind these versatile effects is essential for antibody-based immunotherapy. Renato Monteiro et al. review the anti-inflammatory properties of IgA and IgA receptors. IgA indeed uses several receptors and several mechanisms to regulate inflammatory processes generated during immune responses and the resulting tissue damage observed in autoimmune and inflammatory diseases. Jeffrey Ravetch et al. discuss how humanized mice can be used to assess the role of IgG-FcγR interactions in the in vivo effects of therapeutic antibodies used in the clinic. They focus on such a mouse in which all the FcγRs have been deleted and replaced by human FcγRs with a human tissue distribution. Finally, Sally Ward et al. review the mechanisms used by FcRn to protect IgG from lysosomal degradation through recycling and transcytosis, to deliver antibodies across cellular barriers to sites of pathogen encounter, to maintain and regulate renal filtration and to present antigen. These multifaceted functions open new FcRn-targeted therapies.

The Part IV specifically deals with *FcRs and Disease*. It contains three chapters. Robert Kimberly et al. address the issue of FcR polymorphism in human diseases. They review the single nucleotide polymorphisms, as well as the copy number variations in classical FcRs, including FcRn, but also in FCRLs, and they discuss their roles in infectious and inflammatory diseases associated wit these genetic variations. René Toes et al. focus on the roles of autoantibody–FcR interactions in rheumatoid arthritis. Specifically, they show how anti-citrullinated protein antibodies determine joint damage through the interplay between activating an inhibitory receptors. Finally, Mark Hogarth et al. provide a comparative analysis of human and non-human primate FcγRs, in viral infection. They focus on the polymorphism of macaque FcγRs and HIV infection and discuss how faithful the macaque model is for designing safe and efficient HIV vaccine strategies.

The Part V bears on *FcRs and Therapeutic Antibodies*. It contains three chapters. Mark Cragg et al. first discuss how ITIM-containing inhibitory FcγRIIB, that were shown to decrease the efficacy of therapeutic antibodies such as Rituximab or Trastuzumab can, on the contrary, enhance that of antibodies against members of the TNF Receptor superfamily such as anti-CD40 antibodies. Jantine Bakema and Marjolein van Egmond review the mechanisms involved in FcR-dependent passive immunotherapy of cancer. They specifically focus on therapeutic antibodies of the IgA class, instead of the anti-tumor IgG antibodies that are commonly used. Finally, Falk Nimmerjahn et al. review the anti-inflammatory activity of normal IgG and, specifically, the role of IgG glycosylation in this property. They discuss how IgG sialylation critically determines the therapeutic effects of IVIg in several models of autoimmune diseases by affecting both innate and adaptive immune responses through several receptors and mechanisms.

We hope this volume will interest scientists and clinicians, immunologists and non-immunologists, who are willing to know more about FcRs and to master better antibodies for therapeutic purposes. We also wish they will share with us the pleasure we had to put these chapters together when they read them.

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Contents

Part I
Old and New FcRs

The Old but New IgM Fc Receptor (FcμR)

Hiromi Kubagawa, Yoshiki Kubagawa, Dewitt Jones, Tahseen H. Nasti, Mark R. Walter and Kazuhito Honjo

Abstract IgM is the first Ig isotype to appear during phylogeny, ontogeny and the immune response. The importance of both pre-immune "natural" and antigen-induced "immune" IgM antibodies in immune responses to pathogens and self-antigens has been established by studies of mutant mice deficient in IgM secretion. Effector proteins interacting with the Fc portion of IgM, such as complement and complement receptors, have thus far been proposed, but fail to fully account for the IgM-mediated immune protection and regulation of immune responses. Particularly, the role of the Fc receptor for IgM (FcμR) in such effector functions has not been explored until recently. We have identified an authentic FcμR in humans using a functional cloning strategy and subsequently in mice by RT-PCR and describe here its salient features and the immunological consequences of FcμR deficiency in mice. Since the FcμR we cloned was identical to Toso or Fas inhibitory molecule 3 (FAIM3), there have been spirited debates regarding the real function of FcμR/Toso/FAIM3 and we will also comment on this topic.

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M. Daëron and F. Nimmerjahn (eds.), *Fc Receptors*, Current Topics in Microbiology and Immunology 382, DOI: 10.1007/978-3-319-07911-0_1,

Contents

1 Introduction

Antibody has dual binding activities: to antigen via its amino terminal variable regions and to effector molecules such as Fc receptors (FcRs) via its carboxyl terminal constant regions. FcRs are expressed by many different cell types in the immune system, and their interaction with antibody can initiate a broad spectrum of effector functions that are important in host defense. These functions include phagocytosis of antibody-coated microbes, lysosomal degradation of endocytosed immune complexes, antibody-dependent cell-mediated cytotoxicity, secretion of cytokine and chemokines, release of potent inflammatory mediators, enhancement of antigen presentation, and regulation of antibody production by B lymphocytes, and plasma cell survival. These diverse regulatory roles depend upon the antibody isotype and cellular distribution of the corresponding FcR. FcRs for IgG (FcγRI to FcγRIV), IgE (FcεRI) or IgA (FcαR) have been extensively characterized at both protein and genetic levels (see other chapters in this volume; Refs. (Ravetch and Kinet 1991; Daëron 1997; Monteiro and Van De Winkel 2003; Nimmerjahn et al. 2005)). IgM is the first antibody isotype to appear during phylogeny, ontogeny and immune responses, and the existence of an FcR for IgM (FcμR) on various cell types (B, T, NK cells, macrophages (Mφs), and granulocytes) has been suggested for decades with conflicting results (see Refs in (Kubagawa et al. 2009)). Thus, it has long been a puzzle why the gene encoding an FcμR has defied identification. In this article, we will describe the identification of an authentic FcμR, its genetic and biochemical features, and the cellular distribution and function of FcμR in both humans and mice.

2 Human FcμR

2.1 Functional Cloning, Predicted Structure, and Biochemical Nature of FcμR

In 1986 during her analysis of B cell activation antigen, Sheila Sanders, then a post-doctoral fellow in the laboratory of Max Cooper, serendipitously identified a single chain polypeptide of ~60 kDa by mouse IgM monoclonal antibodies (mAbs) irrespective of their antigen binding specificities that was expressed on the surface of human blood B cells following activation with phorbol myristate acetate (PMA) (Sanders et al. 1987). The ~60 kDa IgM binding protein was also detectable on freshly isolated chronic lymphocytic leukemia (CLL) B cells (see Sect. 2.7) and on the PMA-activated human pre-B cell line 697 (Sanders et al. 1987; Ohno et al. 1990). The IgM-binding by PMA-activated normal blood B or 697 pre-B cells as well as by CLL B cells could be demonstrated by flow cytometry using highly purified IgM preparations. Several attempts over the years to obtain protein sequence of this molecule to assist its molecular cloning met with failures. It was not until 2009 that we were able to identify the gene encoding the IgM binding protein. Two different cDNA libraries were constructed from CLL B cells and the PMA-activated 697 pre-B cell line and ligated into a retroviral expression vector before transfection into a packaging cell line and transduction into a mouse T cell line BW5147, which lacks IgM binding. The resultant IgM-binding transduced cells were initially present at very low frequency, but could be enriched by magnetic and fluorescence-activated cell sorting (FACS) and were finally subcloned by limiting dilution. Nucleotide sequence analyses of the ~2 kb insert cDNAs responsible for IgM binding in these single cell-derived subclones defined an identical 1,173-bp open reading frame in both cDNA libraries (Kubagawa et al. 2009).

The human FcμR cDNA encodes a 390-aa type I transmembrane protein (17-aa signal peptide, 234-aa extracellular region, 21-aa transmembrane segment, and 118-aa cytoplasmic tail) (Fig. 1a). The amino terminal half of the extracellular region contains a single V-set Ig-like domain with homology to two other IgM-binding receptors (the polymeric Ig receptor (pIgR) and the FcR for IgA and IgM (Fcα/μR)), but the remaining extracellular region has no identifiable domain features, designated the "stalk" region in this article. The core peptide is predicted to have an M_r of ~41 kDa and an isoelectric point (pI) of ~9.9. There are no N-linked glycosylation motifs (NxS/T; single aa letter code and x indicating any aa) in the extracellular region, consistent with results of our previous biochemical characterization of the IgM binding protein (Sanders et al. 1987; Ohno et al. 1990). By using both receptor-specific mAbs and IgM ligands, the surface FcμR expressed on FcμR cDNA-transduced cells, PMA-activated 697 pre-B cells, CLL B cells and blood mononuclear cells has an M_r of ~60 kDa on SDS-PAGE under both reducing and non-reducing conditions, albeit with a more intense signal under

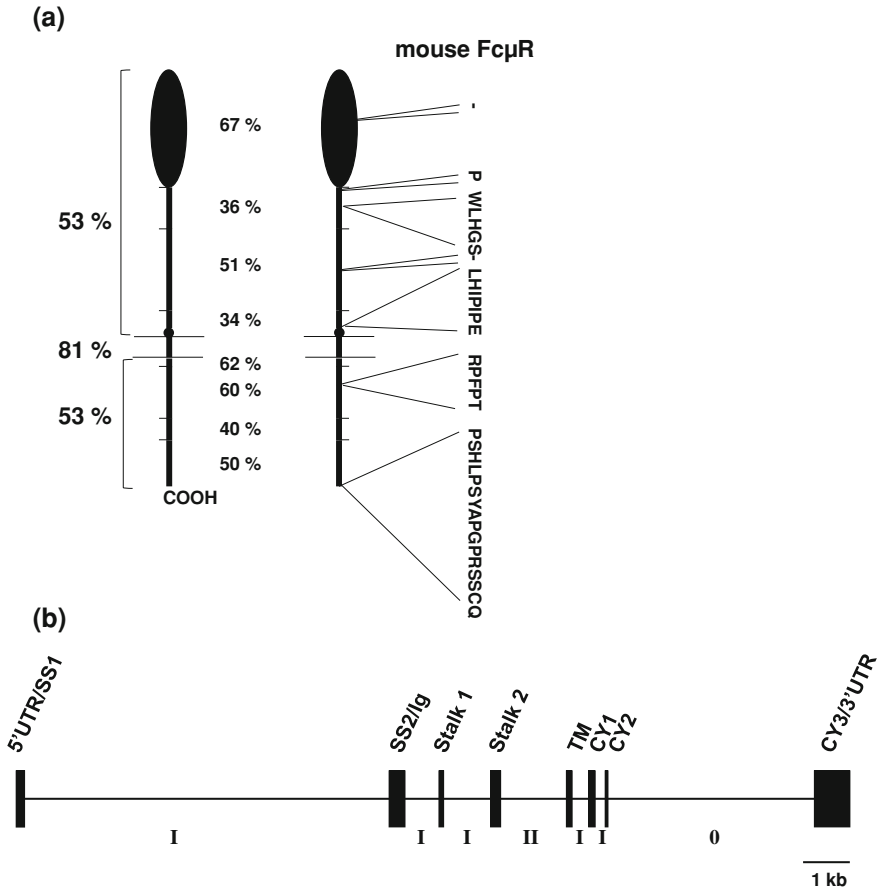

Fig. 1 *Schematic representation of FcμR.* **a** *Homology between human and mouse FcμR proteins. Left* FcμR is depicted as a badminton-like shape: amino terminal Ig-like domain (*black closed oval shape*), stalk region (above the *top line*), transmembrane (between the *two lines*) and cytoplasmic tail (below the *bottom line*). Hatch marks indicate exon boundaries and the small closed circle in the transmembrane region indicates a charged His residue. Numbers indicate the aa identity in the indicated regions between human and mouse receptors. *Right* The sites of additional aa or gaps in the mouse FcμR are shown by the single aa letter code or *dashes* (-), respectively. **b** *Schematic representation of the FCMR gene.* The exon (*black closed boxes*) organization of *FCMR* is drawn to the scale indicated, along with intron phases ("phase 0" indicating between codons; "phase I" between the first and second nucleotide of a codon; "phase II" between the second and third nucleotide). Exons encoding particular regions of the receptor are denoted as follows: the 5′ untranslated (5′UT), the signal peptide (SS1 and 2), the Ig-like domain (Ig), the uncharacteristic extracellular (stalk 1 and 2), the transmembrane (TM), the cytoplasmic (CY1–3), and the 3′ untranslated (3′UT) regions

reducing conditions, and into a spot with a pI of ∼5 on two-dimensional PAGE analysis, suggesting that one third of the M_r of the mature FcμR is made up of carbohydrate moiety containing many sialic acids (Kubagawa et al. 2009).

Removal of sialic acid by neuraminidase treatment of FcμR$^+$ cells slightly enhanced IgM binding, suggesting a role of sialic acid in this interaction.

Unlike our earlier observation that the IgM-binding protein on PMA-activated 697 pre-B cells could be attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) linkage (Ohno et al. 1990), the structure predicted by the FcμR cDNA is of a transmembrane protein. (Notably, Tetsuya Nakamura had previously noticed that the ~60 kDa IgM-binding protein on blood T cells, unlike PMA-activated 697 pre-B cells, was resistant to GPI-specific phospholipase C (GPI-PLC) (Nakamura et al. 1993)). We thus reexamined this issue using a highly purified GPI-PLC. After GPI-PLC treatment, the surface level of FcμR on both the BW5147 transductants and PMA-activated 697 pre-B cell line was unchanged, whereas the expression of the GPI-anchored Thy-1 or CD73 was reduced by ~65 %, indicating that FcμR is an genuine transmembrane protein, consistent with the predicted structure encoded by the FcμR cDNA (Kubagawa et al. 2009). We also searched for a cDNA encoding a potential GPI-linked form of FcμR but failed to identify it. However, the experimental data not relying on GPI-PLC in previous studies by Tatsuharu Ohno (Ohno et al. 1990) could not be ignored because the results were unambiguous and thus worthy of reconsideration. Namely, when cell surface-iodinated, PMA-activated 697 pre-B cells were incubated at 37 °C even without GPI-PLC, significant amounts of the ~60 kDa IgM-binding protein were released into the medium. Furthermore, the ~60 kDa IgM-binding protein was clearly precipitated by IgM-coupled beads from the culture supernatants of metabolically labeled, PMA-activated 697 pre-B cells. For direct comparison of FcμR in the cell lysates and supernatants, NP-40 detergent was also added into the supernatants to obtain equivalent conditions in SDS-PAGE analysis of immunoprecipitated materials (Ohno et al. 1990). Thus, it is quite conceivable that FcμR is released as small vesicles or exosomes from the plasma membrane upon certain types of activation. The physiological relevance of FcμR-containing exosomes, as well as of the soluble form of FcμR of ~40 kDa described in Sect. 2.7, might be to deliver IgM/antigen complexes to other cell types or to remotely modulate IgM-mediated immune regulation as a decoy receptor.

2.2 *Exon Organization of* FCMR

FCMR is a single copy gene located on chromosome 1q32.2, adjacent to two genes encoding other IgM-binding receptors, *PIGR* expressed on mucosal epithelium and *FCAMR* expressed on follicular dendritic cells (FDCs), separated by ~40 Mb from the cluster of genes encoding FcγRs, FcεRI, and FcR-like molecules on 1q21 to 1q23 (Kubagawa et al. 2009). *FCMR* spans ~17.6 kb and is composed of eight exons (Fig. 1b). Unlike the FcRs and most of their relatives, which as a conserved feature have a signal peptide encoded by two separate exons, the second of which is either a 21-bp or 36-bp "mini-exon," *FCMR* lacks this feature as do the *PIGR*

and *FCAMR* genes (Kikuno et al. 2007; Davis et al. 2001) The intron to exon length proportion of *FCMR* is ~8, suggesting that it belongs to the class of genes with relatively short introns, based on the fact that the average intron/exon ratio is ~26 with a median of ~11 (Castillo-Davis et al. 2002). Given the fact that the transcription process in eukaryotes is slow (~20 nucleotide transcribed per second) and biologically expensive (at least two ATP molecules per nucleotide), transcription of genes with short introns is thus less costly than those with long introns, which are particularly common in mammals (Ucker and Yamamoto 1984). Intriguingly, highly expressed genes have substantially shorter introns than genes expressed at low levels. In this regard, natural selection appears to favor short introns in highly expressed genes to minimize the cost of transcription and other molecular processes, such as splicing. Selection favoring short introns is particularly strong for genes that have to be expressed at high levels at short notice, such as stress-induced proteins. The *FCMR*, whose product is selectively expressed on adaptive immune cells, may thus belong to the intermediate response family of genes.

Other fundamental questions regarding the evolution of *FCMR* include: (i) do FcμR and IgM genes coevolve during evolution? If so, *Fcmr* should appear in jawed, but not jawless, vertebrates, i.e., from cartilaginous fish onwards. (ii) Is FcμR structurally conserved? (iii) Which cell types express FcμR? In mammals like humans and mice, do adaptive immune cells exclusively express FcμR (see below)? In this regard, the recent bioinformatics analysis revealed that both FcμR and Fcα/μR were suggested to appear during early mammalian evolution (Akula et al. 2014).

2.3 IgM-Binding (FcμR) Versus Anti-apoptotic (FAIM3/Toso) Functions

When we analyzed our FcμR cDNA sequence using the basic local alignment search technique (BLAST) database, to our surprise, it was identical to that of the previously identified human Fas apoptosis inhibitory molecule 3 (FAIM3), except for one nucleotide difference at a position reported as a synonymous single nucleotide polymorphism. FAIM3 was also identified in a similar cDNA library-based retroviral functional assay as a potent inhibitor of Fas/CD95-induced apoptosis and was originally designated as Toso after a Japanese liquor enjoyed on New Year's Day to celebrate long life and eternal youth (Hitoshi et al. 1998). However, apoptosis in this functional assay was induced by ligation of Fas with an agonistic IgM mAb (CH11 clone), raising the concern that the CH11 mAb bound the Fas receptor via its variable Fabμ region and also to FAIM3/Toso via its constant Fcμ region.

To reconcile the conflicting FcμR functions, IgM Fc binding (FcμR) versus inhibition of Fas apoptosis (FAIM3/Toso), we first examined the $FcμR^+$ BW5147 cells for their Ig-binding specificity. The $FcμR^+$ cells clearly boud IgM, but not other Ig isotype (IgG1-4, IgA1,2, IgD, or IgE), in a dose-dependent manner and the IgM binding was mediated by its Fc5μ fragments consisting mostly of Cμ3/Cμ4 domains, but not by Fabμ fragments, thereby confirming its IgM Fc-binding

activity. FcμR binds IgM pentamers with a strikingly high avidity of ~10 nM as determined by Scatchard plot analysis with the assumption of a 1:1 stoichiometry of FcμR to IgM ligand (Kubagawa et al. 2009). Next, to determine if the FcμR has anti-apoptotic activity, we first repeated the experiment of Hitoshi et al. (1998) and introduced the FcμR cDNA into an apoptosis-prone human T cell line Jurkat. Ligation of Fas with the CH11 IgM mAb induced robust apoptotic cells in the control cells, but not in the FcμR$^+$ cells (Fig. 2), consistent with the reported anti-apoptotic activity of FAIM3/Toso. However, ligation of Fas with an agonistic IgG3 mAb (2R2), which should have the same biological effect as the CH11 mAb, or with the recombinant Fas ligand induced apoptosis in both FcμR$^+$ and control cells, indicating that FAIM3/Toso is not an anti-apoptotic protein (Kubagawa et al. 2009; Honjo et al. 2012a). Since FcμR$^+$ Jurkat cells used in these experiments bound IgM and were reactive with receptor-specific mAbs, whereas control Jurkat cells were not, these results taken together clearly demonstrated that the FAIM3/Toso is an authentic IgM Fc binding protein. This conclusion was recently confirmed by others (Vire et al. 2011; Murakami et al. 2012). Similar results with agonistic IgM versus IgG3 anti-Fas mAb were also observed with Epstein Barr virus-transformed B cell lines expressing both endogenous FcμR and Fas on their cell surface. Thus, the correct functional designation of this gene product should be FcμR and not FAIM3 or Toso.

2.4 *Comparison of the Ig-Like Domain Among FcμR, pIgR, and Fcα/μR*

The molecular mechanisms responsible for FcμR binding specificity will likely require crystal structure analysis of FcμR and the IgM/FcμR complex. Fortunately, the crystal structure of domain 1 (D1) of pIgR, which shares ~31 % aa sequence identity with the Ig-like domain of FcμR, has been solved and suggests the Ig-like domain of FcμR shares the same overall β-sandwich fold observed for the pIgR D1 (Hamburger et al. 2004). Sequence alignments and structural analysis suggest the FcμR Ig-like domain Cys residues (C^{37} and C^{104}) and (C^{49} and C^{58}) pair to form intra-chain disulfide bonds, as observed for pIgR. The Ig-like domain of FcμR also forms a salt bridge, between R^{75} and D^{98}, which is conserved in other Ig domains, including pIgR. However, notably missing from FcμR is the invariant Trp, which is a Leu (L^{48}) in FcμR. Several additional residues are conserved in pIgR and Fcα/μR sequences but not in FcμR (Fig. 3, shown in purple). The greatest difference between FcμR and the other two receptor is in the CDR1 regions. The CDR1 of the pIgR, and Fcα/μR, consists of nine amino acids (PPTSVNRHT for human pIgR). In contrast, the corresponding CDR1 region of FcμR consists of only five amino acids (PEMHV for human FcμR). Furthermore, an Arg found in CDR1 of pIgR, which is solvent exposed and thought to directly interact with polymeric IgA (Hamburger et al. 2004), has been replaced by a noncharged aa residue (M^{42} in humans or L^{42} in

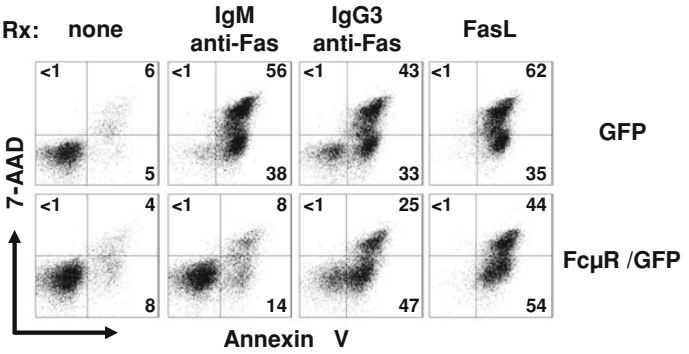

Fig. 2 *Role of FcμR in Fas-mediated apoptosis in Jurkat T cells*. Jurkat cells transduced with the bicistronic construct containing both human FcμR and GFP cDNA (FcμR/GFP) or only GFP (GFP) as a control were cultured at 37 °C for 20 h without (none) or with agonistic mouse anti-human Fas mAb of IgMκ (CH11; 10 ng/ml) or IgG3k isotype (2R2; 1 μg/ml) or with a recombinant human Fas ligand (FasL; 10 ng/ml). Cells were stained with 7-aminoactinomycin D (7-AAD) and allophycocyanin-labeled annexin V to identify early (annexin V^+/7-AAD^-) and late (annexin V^+/7-AAD^+) apoptotic and dead (annexin V^-/7-AAD^+) cells by flow cytometric analysis. Numbers indicate percentages of cells. Note the resistance of the FcμR/GFP transductant to Fas-mediated apoptosis by IgM mAb, but not by IgG3 mAb or FasL

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                    20        30        40                  50        60
                    ..|....|....|....|....|....|     ....|....|....|....|....|...
Hu FcμR             VSGALRILPEVKVEGELGGSVTIKCPLP----EMHVRIYLCREMAGSGTCGTVVSTTNFI
Mo FcμR             ......V....QLNV.W...II.E....----QL...M....Q..KP.I.S....N.-.V
Hu pIgR             ---KSP.FGPEE.NSVE.N..S.T.YY.PTSVNR.T.K.W..QG-AR.G.I.LI.SEGYV
Mo pIgR             ---KSP.FGPQE.SSIE.D..S.T.YY.DTSVNR.T.K.W..QG-A..M.T.LI.SNGYL
Hu Fcα/μR           ---PNSLKGSRL.S..P..A...Q.HYAPSSVNR.Q.K.W..LGPPRWI.Q.I...NQYT
Mo Fcα/μR           ---TNALRGPRL.T.NT..A...H.HYAPSSVNR.Q.K.W..LGSPLWI.H.....NQYT

                    70        80        90        100       110       120
                    .|....|....|....|....|....|....|....|....|....|....|....
Hu FcμR             KAEYKGRVTLKQYPRKNLFLVEVTQLTESDSGVYACGAGMNTDRGKTQKVTLNVHS
Mo FcμR             .K..ER....TPCLD.K.....M.....N.D.I....V..K..K.....I.....N
Hu pIgR             SSK.A..AN.TNF.ENGT.V.NIA..SQD...R.K..L.I.SR-.LSFD.S.E.--
Mo pIgR             SK..S..AN.INF.EN.T.VINIE...QD.T.S.K..L.TSNR-.LSFD.S.E.--
Hu Fcα/μR           HHR.RD..A.TDF.QRG..V.RLS..SPD.I.C.L...I.SENN-MLFLSMN.TI--
Mo Fcα/μR           HPD.R..AA.TDI.QSG..V.RLLR.SLG.V.L.R...I.DRN.-MLFFS.N.T.--
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Fig. 3 *Amino acid sequence alignment of IgM-binding receptors*. The Ig-binding domains of FcμR, pIgR and Fcα/μR of human and mouse origin are aligned to each other. The numbers indicate the aa position from the first Met residue of human FcμR (NP_005440). Amino acid identity is indicated by *dots* (·) and gaps by *dashes* (-). Residues conserved in all three receptors and in pIgR and Fcα/μR are highlighted in *yellow* and *purple*, respectively. Accession numbers of these sequences are: mouse FcμR (NP_081252); pIgR of human (P01833) and mouse (070570); Fcα/μR of human (AAL51154) and mouse (NP_659209)

mice) in FcμR. These molecular differences are consistent with the stringent specificity of FcμR for IgM compared with the promiscuous binding of pIgR and Fcα/μR to polymeric IgA and IgM.

2.5 Conserved Ser and Tyr Residues in the FcμR Cytoplasmic Tail

Unlike many paired receptors having a similar extracellular region but transmitting opposite signal potentials, such as FcγRs, NK cell receptors, and paired Ig-like receptors (Kubagawa et al. 1997), FcμR is unique in that a charged His residue (H^{253}) exists in the predicted transmembrane region and the cytoplasmic tail is relatively long (118 aa) and contains conserved residues, three Tyr and five Ser, when compared with FcμR from six different species (Fig. 4; Ref. (Kubagawa et al. 2009)). This suggests that FcμR may have a dual signaling capacity: one from a potential adaptor protein noncovalently associating with FcμR via the H^{253} residue, similar to the association of FcR common γ chain with FcγRI, and the other from its own Tyr and/or Ser residues in the cytoplasmic tail. In our previous studies, an $\sim$40 kDa membrane protein (p40) was often co-precipitated with the 60 kDa ligand-binding chain of FcμR but it remains unclear whether p40 represents another membrane protein non-covalently associated with FcμR or an unglycosylated form of FcμR (Kubagawa et al. 2009). The carboxyl terminal Tyr matches the recently described Ig tail tyrosine (ITT) motif (DYxN) in IgG and IgE isotypes (Engels et al. 2009), but the other two do not correspond to an ITAM ($D/Ex_2Yx_2L/Ix_{6\text{-}8}Yx_2L/I$), ITIM ($I/VxYx_2L/V$) or switch motif ($TxYx_2V/I$). Ligation of Fc$\mu$R with preformed IgM immune complexes induced the phosphorylation of both Tyr and Ser residues of the receptor (Kubagawa et al. 2009). Intriguingly, phosphorylated FcμR migrated on SDS-PAGE faster than the unphosphorylated form, unlike the findings that most proteins usually ran slower when phosphorylated. This observation suggests either that phosphorylation may cause a global structural change of FcμR leading to increased mobility as seen in CD45 on PMA-activated myeloid cells (Buzzi et al. 1992) or that proteolytic cleavage may occur in the cytoplasmic tail of FcμR after receptor ligation as observed in FcγRIIa on platelets (Gardiner et al. 2008). Upon IgM binding, FcμR was rapidly internalized, and this activity was mediated by the two carboxyl terminal Tyr residues, as determined by mutation analysis (Vire et al. 2011). Ligation of FcμR on NK cells with IgM immune complexes was shown to induce phosphorylation of PLCγ and Erk1/2 (Murakami et al. 2012).

2.6 Cellular Distribution

Given the fact that IgM is the first antibody isotype to appear during phylogeny, ontogeny, and immune responses and is the first line of defense against pathogens, it seemed reasonable to assume that FcμR would have a broad cellular distribution. Results from earlier studies with rosette formation using IgM-coated erythrocytes also suggested the existence of an FcμR on various cell types (B, T, NK, and phagocytic cells) in humans and rodents. Contrary to this assumption, however,

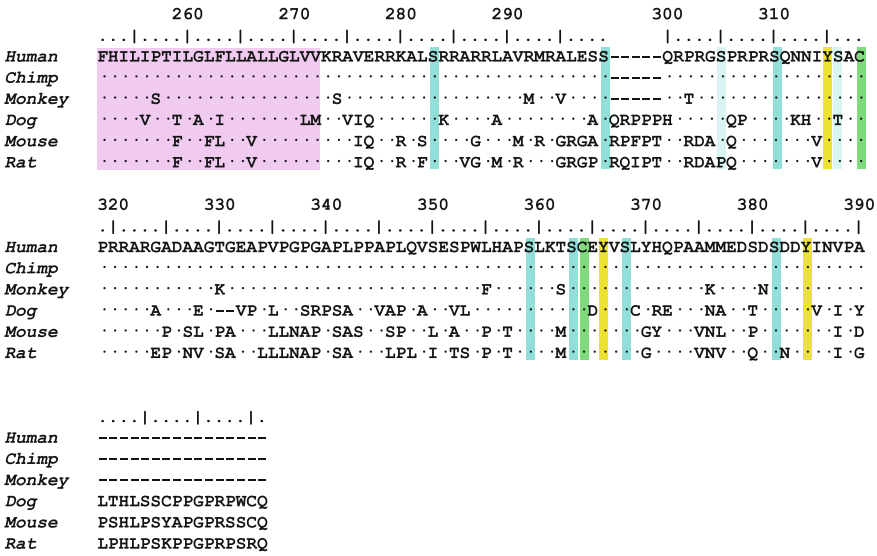

Fig. 4 *Amino acid sequence alignment of the transmembrane and cytoplasmic regions of FcμRs*. The transmembrane and cytoplasmic regions of FcμR from six mammalian species are aligned to each other. Amino acid identity is indicated by *dots* (·) and gaps by *dashes* (-). The predicted transmembrane region is colored in pink. Conserved Tyr, Ser and Cys residues are also highlighted in *yellow*, *dark or light blue*, and *green*, respectively. *Light blue* indicate conservation of Ser residues in five species. The numbers indicate the aa position from the first Met residue of human FcμR. Accession numbers of these sequences are: chimpanzee (chimp; XP_001165341), monkey (XP_001084243), dog (XP_547385), and rat (Q5M871)

current studies using FcμR-specific, RT-PCR primers and mAbs, have shown that FcμR is predominantly expressed by adaptive immune cells, both B and T lymphocytes and, to a lesser extent, NK cells, but not by other hematopoietic cells (i.e., myeloid and erythroid cells and platelets) (Kubagawa et al. 2009). (NK cells are the only known exception for FcμR expression by nonadaptive immune cells, but are now thought to have features of both adaptive and innate immune cells (Vivier et al. 2011)). Treatment of myeloid cells with various stimuli including PMA/ionomycin, LPS, mitogens and several cytokines did not induce the cell surface expression of FcμR. Thus, FcμR is the only FcR constitutively expressed on T cells of all cell types (i.e., αβ T, γδ T, CD4 T, CD8 T, Treg) which are generally negative for the expression of other FcRs. For B cells, FcμR is the only IgM-binding receptor expressed; Fcα/μR was initially thought to be expressed by B cells (Shibuya et al. 2000), but our subsequent analysis revealed that the major cell type expressing Fcα/μR is the FDC in both humans and mice (Kikuno et al. 2007). The restriction of FcμR expression to adaptive immune cells is remarkable, because FcR for the switched Ig isotypes (FcγRs, FcεRI, and FcαR) are expressed by various hematopoietic cells, including phagocytes, and are thought to be central mediators that couple innate and adaptive immune responses (Ravetch and Kinet 1991; Daëron 1997; Monteiro and Van De Winkel 2003; Nimmerjahn et al. 2005). The physiological relevance of such