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Peter D. Burrows *Editors*

IgM and Its Receptors and Binding Proteins

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IgM and Its Receptors and Binding Proteins

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Preface

Among antibodies, the IgM isotype is unique in that it appears first during phylogeny, ontogeny, and the immune response. The importance of both pre-immune "natural" IgM and antigen-induced "immune" IgM antibodies in protection against infection and autoimmunity has been established through studies of mutant mice deficient in IgM secretion (Ehrenstein and Notley 2010) as well as patients with selective IgM immunodeficiency (Louis and Gupta 2014). In this *Current Topics in Microbiology and Immunology* volume entitled "IgM and Its Receptors and Binding Proteins," five groups of investigators will describe their findings in this area of research.

In Chapter "The Appearance and Diversification of Receptors for IgM During Vertebrate Evolution", Dr. Lars Hellman and Dr. Srinivas Akula describe the phylogenetic aspects of three IgM-binding receptors based on currently available genomic sequence databases: (i) polymeric immunoglobulin (Ig) receptor (pIgR) expressed on mucosal epithelial cells, (ii) Fc receptor for IgM (FcμR) on lymphocytes, and (iii) Fc receptor for IgA and IgM (Fcα/μR) on follicular dendritic cells and other cell types. Among these three receptors, the pIgR first appears during vertebrate evolution and is not found in cartilaginous fish, but in bony fish onward. The pIgR has different numbers of extracellular Ig-like domains depending on the taxonomic class: two to six in bony fish, four in amphibians, reptiles, and birds, and five in all mammals. The increase in the Ig-like domain number from four to five in mammals has been implicated to enhance the interaction of the pIgR with polymeric IgA. FcμR is suggested to appear in early reptiles and is found in all three major living (extant) groups of mammals (i.e., egg laying, marsupial, and placental mammals). Fcα/μR has only been found in mammals and is most likely the evolutionary youngest among these three IgM-binding receptors. The domain structure and possible evolutionary relationship between these three receptors and their function in immunity are also discussed.

In Chapter "Authentic IgM Fc Receptor (FcμR)", Dr. Hiromi Kubagawa and his colleagues describe several recent findings about FcμR from their own and other studies. Unlike FcRs for isotype-switched Igs, FcμR is expressed only by adaptive immune lymphocytes; B, T, and, to a lesser extent, NK cells in humans and only B

cells in mice. Conflicting reports on the expression of FcμR by non-B cells in mice are discussed along with possible explanations for such a critical discrepancy. They have shown that the configuration of IgM ligands is important in FcμR binding. FcμR-bearing cells bind pentameric IgM with a high avidity of ~10 nM and much higher (>100-fold) concentrations are required for monomeric IgM to bind FcμR. Intriguingly, their recent assessment indicates that only twofold to threefold concentration differences in FcμR binding are observed between J chain-containing pentameric and J chain-deficient hexameric IgM. This finding is thus distinct from their complement activation activities, where the IgM hexamer is ~50- to 100-fold more efficient than the IgM pentamer. In addition to the above interaction with soluble IgM, FcμR-bearing cells bind the Fc portion of IgM antibody more efficiently when it is attached to a membrane component via its Fab region on the same cell surface (*cis* interaction). This preferential *cis* engagement of FcμR led to their hypothesis that FcμR can modulate the functional activity of lymphocyte surface molecules recognized by either natural or immune IgM antibody. Several key residues in the transmembrane and cytoplasmic tail of human FcμR involved in the receptor function have been defined by mutational analyses. *Fcmr*-deficient (KO) mice have been established by several groups to define its in vivo function. B cells from these mutant mice were found to produce significantly less interleukin 10 (IL-10), an anti-inflammatory cytokine, but comparable amounts of pro-inflammatory IL-6, ex vivo upon stimulation with *Salmonella* bacteria or with ligands for Toll-like receptor 4 (TLR4), TLR7, or TLR9 as compared to those from controls. There are several significant phenotypic differences in the different *Fcmr* KO mice, and possible explanations for this are discussed.

In Chapter "FCRLA—A Resident Endoplasmic Reticulum Protein that Associates with Multiple Immunoglobulin Isotypes in B Lineage Cells", Dr. Peter Burrows, Dr. Teresa Santiago, and Ms. Tessa Blackburn describe their studies of Fc receptor-like molecule A (FCRLA), an FcR-related protein with several unusual features. Apart from its reported expression in melanocytes and melanoma cells, FCRLA is restricted in its expression to B lineage cells, in particular, germinal center (GC) B cells in humans. Biochemical and cell biological features of FCRLA have mainly been studied in human B cells, where it has been shown to be a non-glycosylated resident endoplasmic reticulum (ER) protein. The Ig isotype specificity of FCRLA is much more promiscuous than any of the other FCR molecules described in this volume, in that it associates with every isotype so far examined, IgM, IgG, and IgA. FCRLA retention in the ER is not mediated by any known protein sequence motif, e.g., KDEL at the C-terminus of other ER proteins such as BiP/GRP78, but rather by unknown mechanisms involving the structurally disordered first domain of the protein, perhaps disulfide bond formation via free Cys residues present in this domain. The most unexpected finding of their studies is that FCRLA in the GC-derived human B cell line Ramos associates with the secretory rather than the membrane form of IgM, both of which are synthesized by these cells. This specificity for IgM molecules that differ only in a short segment of the C-terminus alternately encoded by μ membrane or μ secretory exons provides tantalizing clues as to a possible function of FCRLA in preventing secretion of

"decoy" B cell receptor (BCR) molecules by antigen-responsive IgM-bearing B cells, particularly in the GC.

In Chapter "Specific IgM and Regulation of Antibody Responses", Dr. Birgitta Heyman and Dr. Anna Sörman describe the IgM antibody-mediated enhancement of humoral immune responses. In 1968, Claudia Henry and Niels Jerne reported the seminal finding that passive administration of 19S (IgM) or 7S (IgG) antibodies against sheep red blood cells (SRBC) prior to immunization of the mice with the SRBC antigen resulted in opposing immunoregulatory effects. IgM anti-SRBC antibody enhanced the subsequent immune responses to SRBC, whereas IgG anti-SRBC antibody suppressed the response (Henry and Jerne 1968). IgM-mediated feedback enhancement led to the foundation of the Ph.D. thesis studies of Dr. Heyman in the laboratory of Dr. Hans Wigzell in the 1980s. Since then, she and her colleagues have explored the molecular mechanism behind this phenomenon. Enhancement by IgM antibody is preferentially observed when mice are immunized with relatively large antigens such as erythrocytes, malaria parasites, or keyhole limpet hemocyanin. The timing is important, in that IgM antibody must be administered in close temporal relation to the antigen challenge. Moreover, antigens must be given in suboptimal doses. Complement activation, but not its lytic activity, is required for this IgM-mediated enhancement, since it is not observed in mice lacking complement receptors 1 and 2 (CR1/2), but is unaffected in mice lacking C5, a factor required for the lytic pathway. Passively administered IgM anti-SRBC antibody binds to SRBC and activates complement leading to deposition of C3d on the SRBC antigen. This IgM/SRBC/C3d complex binds to CR1/2-bearing marginal zone B cells which transport it to CR1/2-bearing follicular dendritic cells. In parallel, IgM/SRBC/C3d may cross-link CR1/2 and the BCR on B cells, thereby facilitating B cell responses. This chapter covers the nearly 35-year studies on IgM-mediated enhancement of humoral immune responses conducted by Dr. Heyman and her colleagues.

In Chapter "Role of Natural IgM Autoantibodies (IgM-NAA) and IgM Anti-Leucocyte Antibodies (IgM-ALA) in Regulating Inflammation", Dr. Peter Lobo describes the many important roles of IgM natural antibodies in regulation of inflammation. As opposed to immune IgM, the natural IgM antibodies arise spontaneously without deliberate immunization, can be produced under germfree conditions and in the absence of a thymus, and are present at high levels in human umbilical cord blood, meaning they were generated before exposure to foreign antigens. B1 and marginal zone B cells are major sources of these antibodies, which are often polyreactive and bind autoantigens as well as pathogens with low affinity but with functional consequences. They can bind neo self-antigens to prevent autoimmune disorders and inhibit the growth of microorganisms until other arms of the innate and adaptive immune system mount a protective response. These IgM antibodies can also bind to apoptotic cells to enhance their removal, least they induce an inflammatory response or autoantibody production, and can bind to live leukocytes to regulate their function. Using mice unable to produce secreted IgM, he also shows that regulatory B and T cells require IgM to control the inflammatory response. The repertoire of leukocyte-binding IgM differs in healthy and diseased

humans, which may partially explain differences in the inflammatory response after infection, ischemic injury, or organ transplantation. In this regard, natural IgM antibodies are shown to have tremendous therapeutic potential, since infusion of polyclonal IgM or DCs pre-treated ex vivo with IgM can prevent or treat over exuberant inflammatory responses in vivo.

Berlin, Germany — Hiromi Kubagawa
Birmingham, USA — Peter D. Burrows

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Contents

The Appearance and Diversification of Receptors for IgM During Vertebrate Evolution

Srinivas Akula and Lars Hellman

Abstract Three different receptors that interact with the constant domains of IgM have been identified: the polymeric immunoglobulin (Ig) receptor (PIGR), the dual receptor for IgA/IgM (FcαμR) and the IgM receptor (FcμR). All of them are related in structure and located in the same chromosomal region in mammals. The functions of the PIGRs are to transport IgM and IgA into the intestinal lumen and to saliva and tears, whereas the FcαμRs enhance uptake of immune complexes and antibody coated bacteria and viruses by B220+ B cells and phagocytes, as well as dampening the Ig response to thymus-independent antigens. The FcμRs have broad-spectrum effects on B-cell development including effects on IgM homeostasis, B-cell survival, humoral immune responses and also in autoantibody formation. The PIGR is the first of these receptors to appear during vertebrate evolution and is found in bony fish and all tetrapods but not in cartilaginous fish. The FcμR is present in all extant mammalian lineages and also in the Chinese and American alligators, suggesting its appearance with early reptiles. Currently the FcαμR has only been found in mammals and is most likely the evolutionary youngest of the three receptors. In bony fish, the PIGR has either 2, 3, 4, 5 or 6 extracellular Ig-like domains, whereas in amphibians, reptiles and birds it has 4 domains, and 5 in all mammals. The increase in domain number from 4 to 5 in mammals has been proposed to enhance the interaction with IgA. Both the FcαμRs and the FcμRs contain only one Ig domain; the domain that confers Ig binding. In both of these receptors this domain shows the highest degree of sequence similarity to domain 1 of the PIGR. All Ig domains of these three receptors are V type domains, indicating they all have the same origin although they have diversified extensively in function during vertebrate evolution by changing expression patterns and cytoplasmic signaling motifs.

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Contents

1 The Adaptive Immune System of Vertebrates

In jawed vertebrates the adaptive immune system has appeared in a stepwise manner through several major key events. The first of these was the appearance of the somatically rearranging genes for immunoglobulins (Igs) and T-cell receptors (TCRs). These seem to have occurred at the base of jawed vertebrates, around 450 million years ago. All jawed vertebrates including cartilaginous fish have mostly bona fide Igs or TCRs, whereas lamprey and hagfish, belonging to the jawless fish, the agnathans that separated from other early vertebrates during the early Cambrian Period approximately 550 million years ago, both lack classical Igs and TCRs. However, these jawless fish have other complex antigen receptors named variable lymphocyte receptors (VLRs), which functionally closely resemble IgM and TCRs, but have a completely different evolutionary origin (Hirano et al. 2011; Kasahara and Sutoh 2014). Instead of Ig-like domains they have leucine rich repeats, which are more closely related to Toll-like receptors than to Igs (Hirano et al. 2011; Kasahara and Sutoh 2014). In our minds this is one of the most striking examples of convergent evolution: Starting from a completely different set of genes but ending up with a set of molecules with very similar functions. This convergent nature extends into the three different variants of the VLRs (VLR-A, -B and -C) found in both hagfish and lamprey, which represent functional equivalents of the αβ TCRs, the Igs and the γδ TCRs, respectively (Hirano et al. 2011; Kasahara and Sutoh 2014). These VLR variants are also expressed by three distinct cell types representing equivalents of the B and T lymphocytes in jawed vertebrates (Hirano et al. 2011; Kasahara and Sutoh 2014). Furthermore, the VLR-A, -B and -Cs are encoded from three different loci, all with a high variability generating capacity (Hirano et al. 2011; Kasahara and Sutoh 2014). A recent study on the recognition of influenza virus epitopes in the mouse and lamprey also shows that the response against antigens is almost identical. Both respond against the same regions of the virus, with a relatively similar amount of Igs or VLR-Bs against the different epitopes (Altman et al. 2015). These findings indicate that the two systems are functionally very similar despite being structurally dissimilar.

In jawed vertebrates, the first Ig isotype to appear was most likely IgM as it is found in essentially the same form in all jawed vertebrates, except the coelacanths, which likely secondarily lost the gene for this Ig class (Amemiya et al. 2013;

Boudinot et al. 2014; Kaetzel 2014; Saha et al. 2014). The μ gene of IgM is also located at the 5' end of the locus adjacent to the gene segments for the variable domain of the heavy chain in almost all species studied. An exception to this rule is the zebrafish, where IgZ is located upstream of the IgM gene (Danilova et al. 2005). IgM is also generally the first isotype to be expressed by all B cells. Following the appearance of the first Igs and TCRs, the complexity of the Ig repertoire has increased gradually by gene duplications. The increase in the number of Ig isotypes has made it possible to separate effector functions and thereby increase the regulatory potential of the system. Today, mammals express up to six different Ig classes: IgM, IgD, IgG, IgE, IgA and IgO, and the total number of isotypes can sometimes exceed 15 (Zhao et al. 2009; Magadan-Mompo et al. 2013a, b; Sun et al. 2013; Akula et al. 2014; Kaetzel 2014; Estevez et al. 2016). This facilitates effector functions of the Igs such as complement activation, epithelial transfer and placental transfer, which can be regulated separately. Although we see a large difference in the number of other isotypes and Ig classes in different jawed vertebrates, they all have IgM, which is also, except in coelacanths, present in a very similar form. In fish and mammals, IgM is multimeric; where hexamers are found in fish, and pentamers in mammals (Getahun et al. 1999; Klimovich 2011). In addition, IgM is found as a membrane bound form on all B cells during early B-cell development.

2 The Appearance of Receptors Interacting with the Constant Domain of Igs

Following the appearance of bona fide Ig and TCR genes, a number of additional adaptations have later taken place to increase the roles of these antigen receptors in immunity. One such adaptation has been the appearance of a complex set of proteins interacting with the constant domains of the Igs. These molecules, named Fc receptors (FcRs), due to their interaction with the constant domain of the Igs, have a number of important functions in vertebrates including facilitating phagocytosis by opsonization, constituting key components in antibody-dependent cellular cytotoxicity as well as activating cells to release their granular content. One member of this family, the polymeric Ig receptor (PIGR), also facilitates transfer of Igs across epithelial layers; IgM and IgA in mammals and birds, IgX in amphibians and IgT/IgZ in fish (Fig. 1) (Danilova et al. 2005; Hansen et al. 2005; Zhang et al. 2010; Kaetzel 2014). This receptor therefore makes it possible to target pathogens before they have entered the tissues of the infected individual. In addition to the PIGRs, four major types of classical FcRs for IgG have been identified in mammals as well as one high-affinity receptor for IgE, one for both IgM and IgA, one for IgM and one for IgA (Figs. 1, 2, 3 and 4). All of these receptors are related in structure, where they all contain Ig-like domains. Furthermore they all, with the exception of the IgA receptor, are found on chromosome 1 in humans, indicating that they originate from one or a few common ancestors by successive gene duplications (Fig. 2).

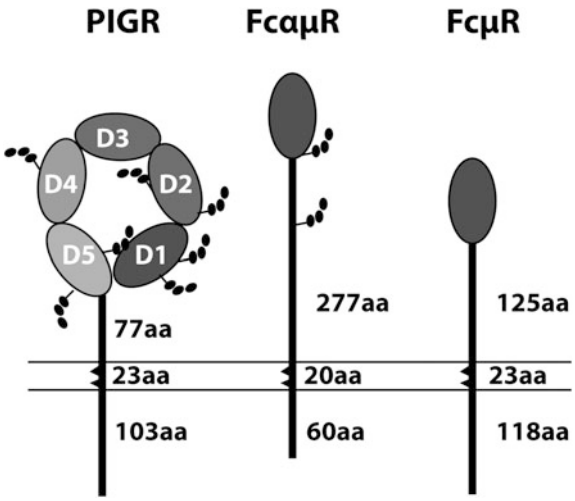


Fig. 1 A schematic presentation of the three receptors for IgM: the PIGR, the FcαμR and the FcμR. The Ig domains are depicted as ovals and potential N-linked glycosylation sites are marked with three small *connected circles*. Approximate sizes in amino acids numbers are also included for each of the three receptors. The Ig domains are generally 100–110 amino acids in size. The figure is modified from Klimovich (2011) and based on the structural information presented in Klimovich (2011), Stadtmueller et al. (2016)

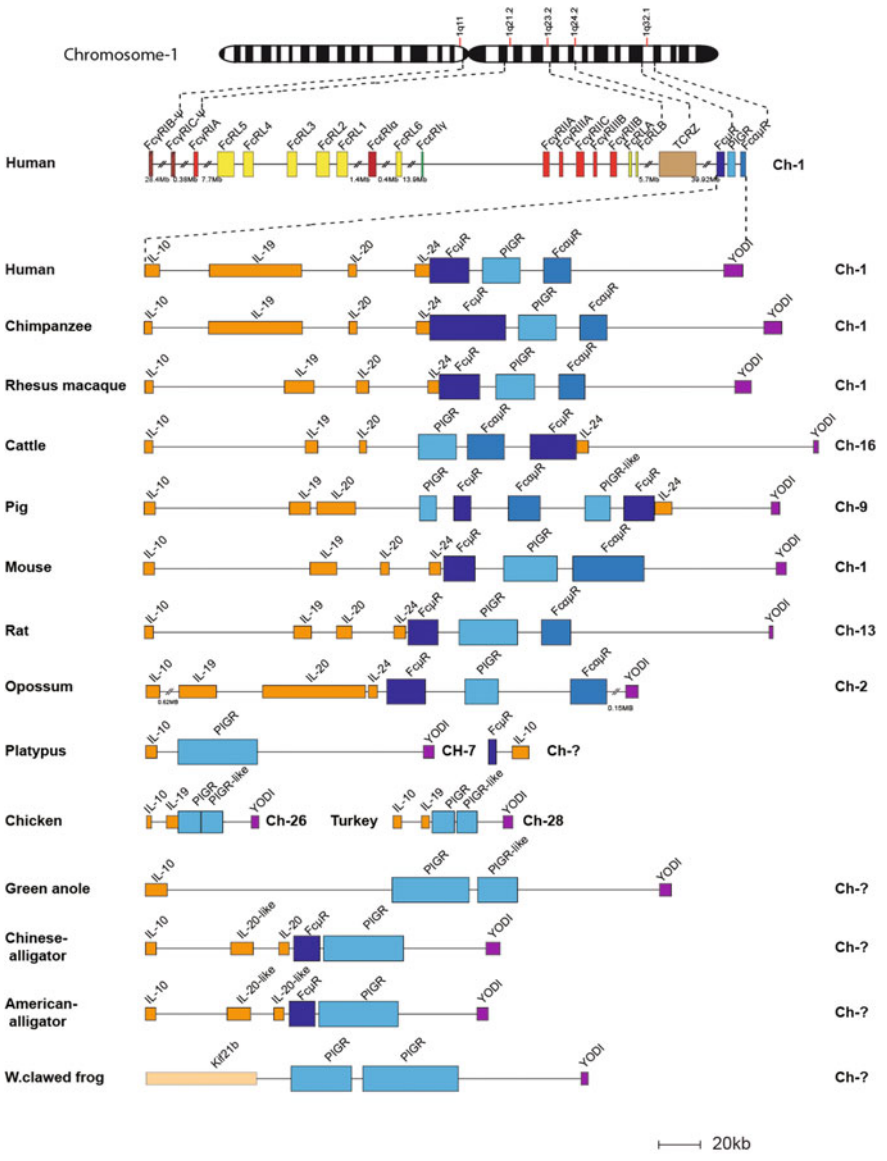
Ig domains are classified as V, C1, C2 or I domains depending on features such as the spacing of cysteine bridges and the number of beta sheets. V domains are generally found in variable regions of Igs and TCRs as well as in cluster of differentiation (CD) markers including CD2, CD4, CD80 and CD86. C1 domains are found in constant regions of Igs, TCRs and in MHC class I and II. C2 domains are found in CD2, CD4, CD80, VCAM and ICAM, and I domains are found in VCAM, ICAM, NCAM, MADCAM and numerous other diverse protein families (EMBL-EBI InterPro). All Ig domains of the FcR-like (FcRL) and classical FcRs are classed as C2 domains and the Ig domains of the PIGRs, IgM receptors (FcμRs) and IgA/IgM receptors (FcαμRs) are V type domains (Nikolaidis et al. 2005; Viertlboeck and Gobel 2011). In a phylogenetic analysis of the individual domains of these receptors, they separate into individual branches clearly separating C2 and V type domains and also individual domains within the PIGRs, FcμRs and FcαμRs (Fig. 5).

As previously described, the first Ig isotype to appear was most likely IgM. One would therefore expect the first FcRs to appear would also be the receptors for IgM. However this is only partly true. The PIGRs are found in all tetrapods and bony fish but not in cartilaginous fish (Akula et al. 2014). One of the first steps in the evolution of the classical FcRs was the appearance of the transfer receptor for transporting IgM and later IgA, IgX or IgT/IgZ over epithelial layers (Figs. 2, 3 and 4). Interestingly, one important signaling molecule for the classical FcRs, the common γ chain, also appeared with bony fish (Akula et al. 2014; Kaetzel 2014). This non Ig-domain-containing signaling subunit is a member of a small family of related molecules

including the TCR zeta chain, DAP10 and DAP12 (Weissman et al. 1988; Blank et al. 1989; Rodewald et al. 1991; Lanier 2009). The latter two proteins serve as signaling components of NK-cell receptors and as well as the related Ig-domain containing receptors (Lanier 2009). A new family of receptors, related in structure to the classical IgG and IgE receptors, was also discovered upon the completion of full genome sequences from a number of mammalian species (Davis 2007; Ehrhardt and Cooper 2011). Eight different such FcRL genes have been identified in the human genome: FcRL1-FcRL6 as well as FcRLA and FcRLB (Figs. 2 and 4). Genes closely related to these mammalian FcRL genes are also found in bony fish but not in cartilaginous fish, indicating a major step in the evolution of FcRs at the base of bony fish with the appearance of the PIGRs, the FcR γ chain and the FcRL molecules (Akula et al. 2014).

In a recent study we show that the classical receptors for IgG and IgE most likely appeared as a separate subfamily of the FcRL molecules during early mammalian evolution (Akula et al. 2014). Related genes are also found in the Western clawed frog (*Xenopus tropicalis*) and the Chinese alligator, indicating that the processes forming the subfamily of receptors that later became the classical IgG and IgE receptors may have started already during early tetrapod evolution. However, this subfamily probably did not appear as a distinct subfamily until the appearance of the mammals (Akula et al. 2014). In the Xenopus these receptors have a structure similar to the human high affinity IgG receptor, FcγRI, with three extracellular Ig domains of the C2 type. In the platypus there are both two and three domain receptors, which are similar to the human three-domain FcγRI as well as the low affinity IgG receptors FcγRII and III, which have two domains. This indicates that the development of high and low affinity receptors also took place during early mammalian evolution. Despite this knowledge, currently none of these amphibian, reptile or non-placental mammalian receptors have been studied for their isotype specificities and affinities.

In contrast to several of the receptors previously described, both the FcαμR and the FcμR seemed to appear relatively late during vertebrate evolution. The FcαμR has only been found in mammals, which indicates that this receptor appeared sometime during early mammalian evolution. A partial clone for FcαμR has been present in the platypus genome assembly but subsequently disappeared from the database, most likely due to incomplete coverage of that specific chromosomal region. However, the Ig domain encoded in this chromosomal fragment did show a high degree of homology to other mammalian FcαμRs, indicating its presence is likely in all three extant mammalian lineages (Fig. 7). The receptor for IgM, FcμR, has until very recently also only been found in mammals. However, in a recent screening of a panel of vertebrate genomes, we also found a gene for the FcμR in both the American and the Chinese alligators (Figs. 2, 4 and 8). This suggests that the receptor appeared during early amniote evolution before reptiles and mammals separated as the diapsid and synapsid lineages, possibly sometime between 320 and 360 million years ago. Interestingly, this receptor may have been secondarily lost in several reptile lineages, as it is not found in the anole lizard genome nor in any of the screened bird genomes (Fig. 2). Birds are known to have gone through massive gene losses followed by re-expansions of gene loci, as many genes found in other species are missing in birds, which may explain the lack of the FcμR gene in birds (International Chicken Genome Sequencing 2004).


Chromosome-1
1q11
1q21.2
1q23.2
1q24.2
1q32.1
Human
Ch-1
TCRZ
FcμR
PIGR
Chimpanzee
Rhesus macaque
Cattle
Ch-16
Pig
Ch-9
Mouse
Rat
Ch-13
Opossum
Ch-2
Platypus
CH-7
Ch-?
Chicken
Ch-26
Turkey
Ch-28
Green anole
Chinese-alligator
American-alligator
W.clawed frog
IL-10
IL-19
IL-20
IL-24
YODI
PIGR-like
IL-20-like
Klf21b
20kb

◂**Fig. 2** Fc receptor genes from a panel of selected tetrapods focusing on the genes for the three IgM receptors, the PIGR, the FcαμR and the FcμR. The regions of the major FcR gene loci in humans are shown above the figure as a reference. Each *horizontal line* corresponds to a chromosome on which different FcR genes are located. Genes are color coded. The Fc receptor-like (FcRL) genes are shown in *yellow* except the FcRLA and B that are in *light green*, classical IgG receptors in *red* (pseudogenes in *striped red*), IgE receptor gamma chain in *dark green*, the TCR zeta chain in *light brown*, the IgM receptor in *dark blue*, the PIGR and the IgA/IgM receptor in other shades of *blue*. A number of bordering genes have also been included as reference genes for the chromosomal region of interest. The enlarged region containing the three receptors binding IgM are bordered at one side by a number of cytokine genes including IL-10, IL-19, IL-20 and IL-24. In cattle and pigs an inversion has occurred resulting in the movement of the IL-24 gene to the other end of the region encoding the IgM receptors. A rearrangement involving the region containing the cytokine genes has also occurred in the Western clawed frog genome

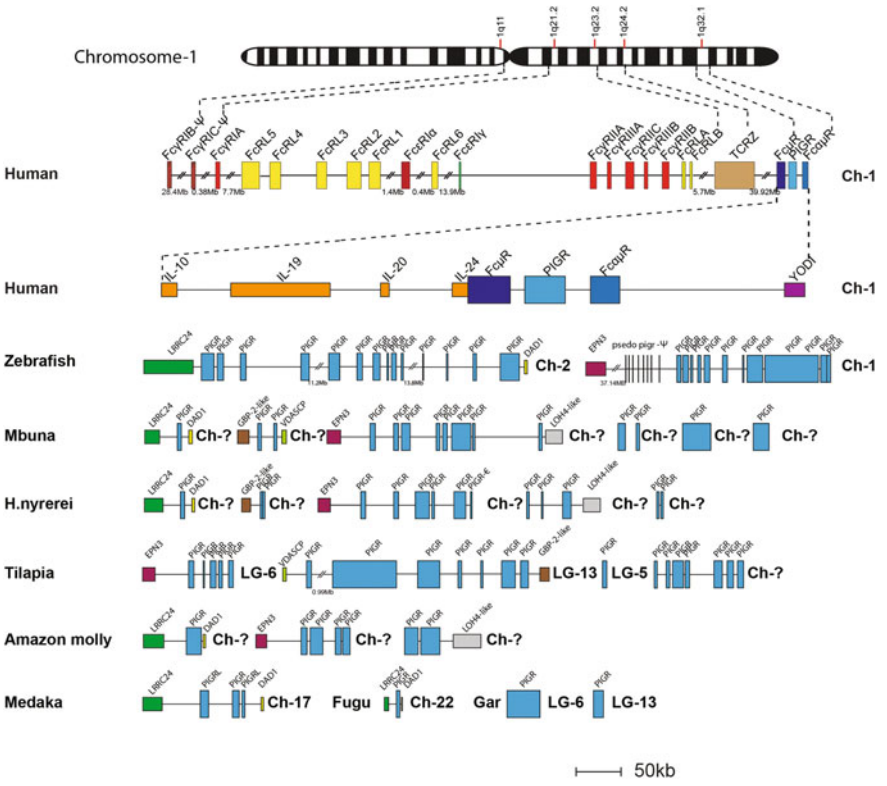
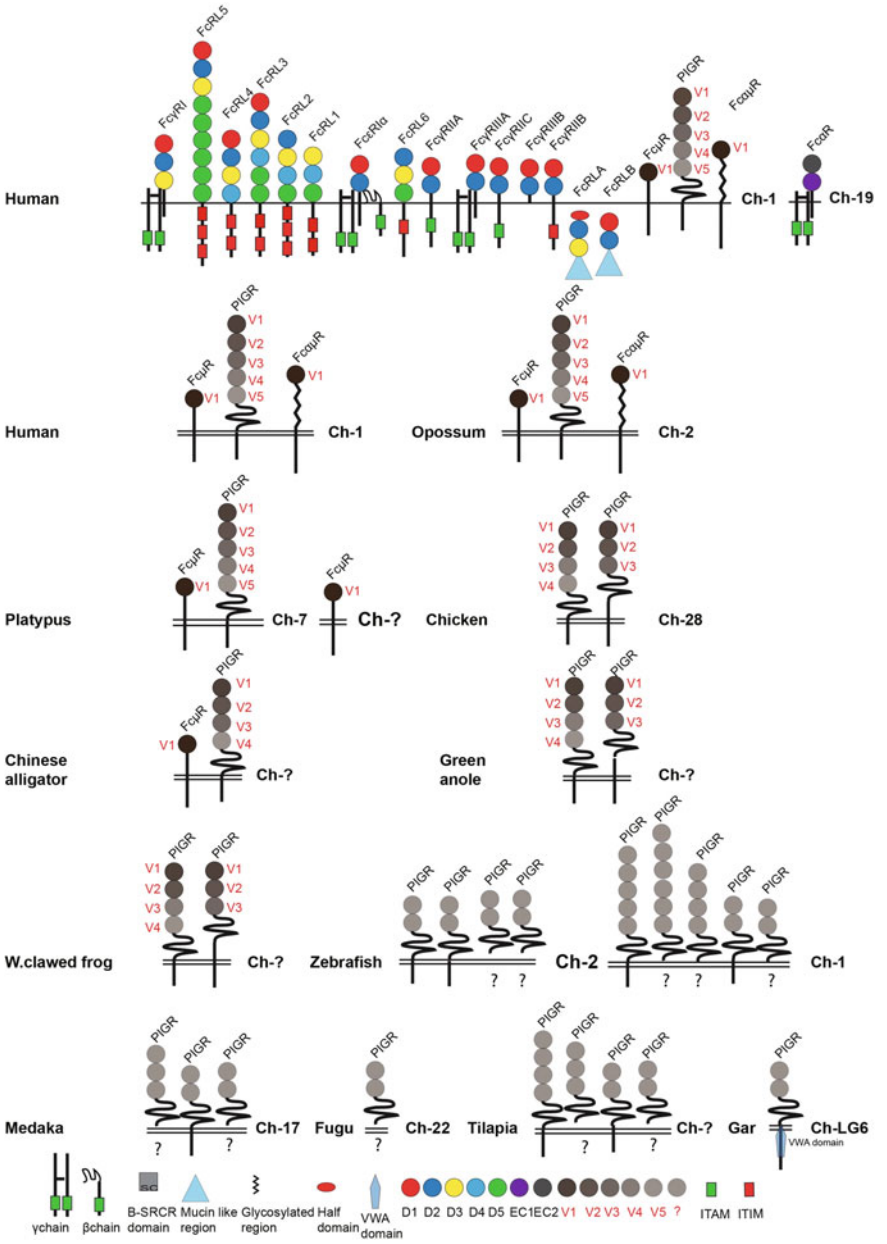


Fig. 3 The PIGR gene loci in a panel of fish genomes. The regions of the major FcR gene loci in humans are shown above the figure as a reference. Each *horizontal line* corresponds to a chromosome on which different FcR genes are located. Genes are color coded as in Fig. 2 with the PIGR genes in *light blue*


Human
FcγRI
FcRL5
FcRL4
FcRL3
FcRL2
FcRL1
FcεRIα
FcRL6
FcγRIIA
FcγRIIIA
FcγRIIC
FcγRIIIB
FcγRIIB
FcRLA
FcRLB
FcμR
PIGR
V1
V2
V3
V4
V5
FcαμR
Ch-1
FcαR
Ch-19
Human
Ch-1
Opossum
Ch-2
Platypus
Ch-7
Ch-?
Chicken
Ch-28
Chinese alligator
Ch-?
Green anole
Ch-?
W.clawed frog
Ch-?
Zebrafish
Ch-2
Ch-1
Medaka
Ch-17
Fugu
Ch-22
Tilapia
Ch-?
Gar
Ch-LG6
VWA domain
ychain
βchain
B-SRCR domain
Mucin like region
Glycosylated region
Half domain
VWA domain
D1 D2 D3 D4 D5 EC1EC2 V1 V2 V3 V4 V5 ?
ITAM
ITIM

◂**Fig. 4** A summary figure of domain structures and signaling motifs of the various vertebrate Fc receptors. The Ig-like domains are depicted as *filled circles* with color-coding according to the similarities in sequences based on phylogenetic analyses (Fayngerts et al. 2007; Guselnikov et al. 2008) and Fig. 5. The domain type D1, D2, D3, D4 and D5 show a relatively conserved pattern in most tetrapods and have therefore been color-coded in *red*, *dark blue*, *yellow*, *light blue* and *green*. A phylogenetic analysis of individual domains of a panel of different FcR is presented in Fig. 5. The color-coding here is based on the result from Fig. 5. The extracellular regions, the transmembrane regions and cytoplasmic tails are not to scale in order to show the positions of potential signaling motifs like immuno-tyrosine activation motifs (ITAMs) (*green boxes*) and immuno-tyrosine inhibitory motifs (ITIMs) (*red boxes*), which regulate the biological function the FcRs. Some of the intracellular proteins contain C-terminal mucin-like regions, which are depicted as *blue triangles*. The PIGR domains are depicted in different shades of *gray*, with domain 1 in *darker gray*, and domain 5 and unassigned domains of fish PIGRs as the lightest shade of *grey*

The relatively late appearance of the specific receptor for IgM is interesting from an evolutionary perspective. IgM is the first Ig isotype to appear but the specific IgM receptor did not enter the scene until relatively late. The only two receptors that show a later appearance are the dual FcαμR and the specific IgA receptor. The latter is only found in placental mammals, where in humans is the only FcR that is located on another chromosome than the other classical FcRs, and is found together with the NK-cell receptors in a gene cluster on chromosome 19 (Bakema and van Egmond 2011; Akula et al. 2014).

The three receptors interacting with IgM are all related in structure; they contain at least one Ig domain of the V type (Fig. 1). However, other parts of the proteins are very different. By analyzing the relatedness between the Ig-like domains of the PIGR, the FcαμR and the FcμR, it is evident they form a separate subfamily in a phylogenetic tree (Fig. 5). They clearly separate from the domains of the IgG, IgE and IgA specific receptors that all contain C2 type domains, which also confirms that the domains of the three different receptors for IgM, with their V type domains, are closely related in structure (Fig. 5) (Nikolaidis et al. 2005). In the following separate sections, we will discuss the structures, functions and possible evolutionary origins of the three different receptors for IgM in more detail.

3 The Polymeric Ig Receptor—PIGR

In mammals, the PIGR is responsible for the transfer of IgM and IgA antibodies from body fluids into secretions including tears, saliva, breast milk as well as into the intestinal lumen by the mucus epithelium and ducts of excretory glands. The antibodies bind at the baso-lateral side of the epithelial cells and the antibody receptor complexes are then transported, by transcytosis, to the apical side of the cells in vesicles. At the apical side proteases cleave the PIGR, leaving the extracellular part in complex with IgM or IgA. This part that stays firmly attached to IgM or IgA is termed the secretory component (SC), which has a protective role by limiting proteolytic degradation of IgM or IgA by bacterial and intestinal proteases

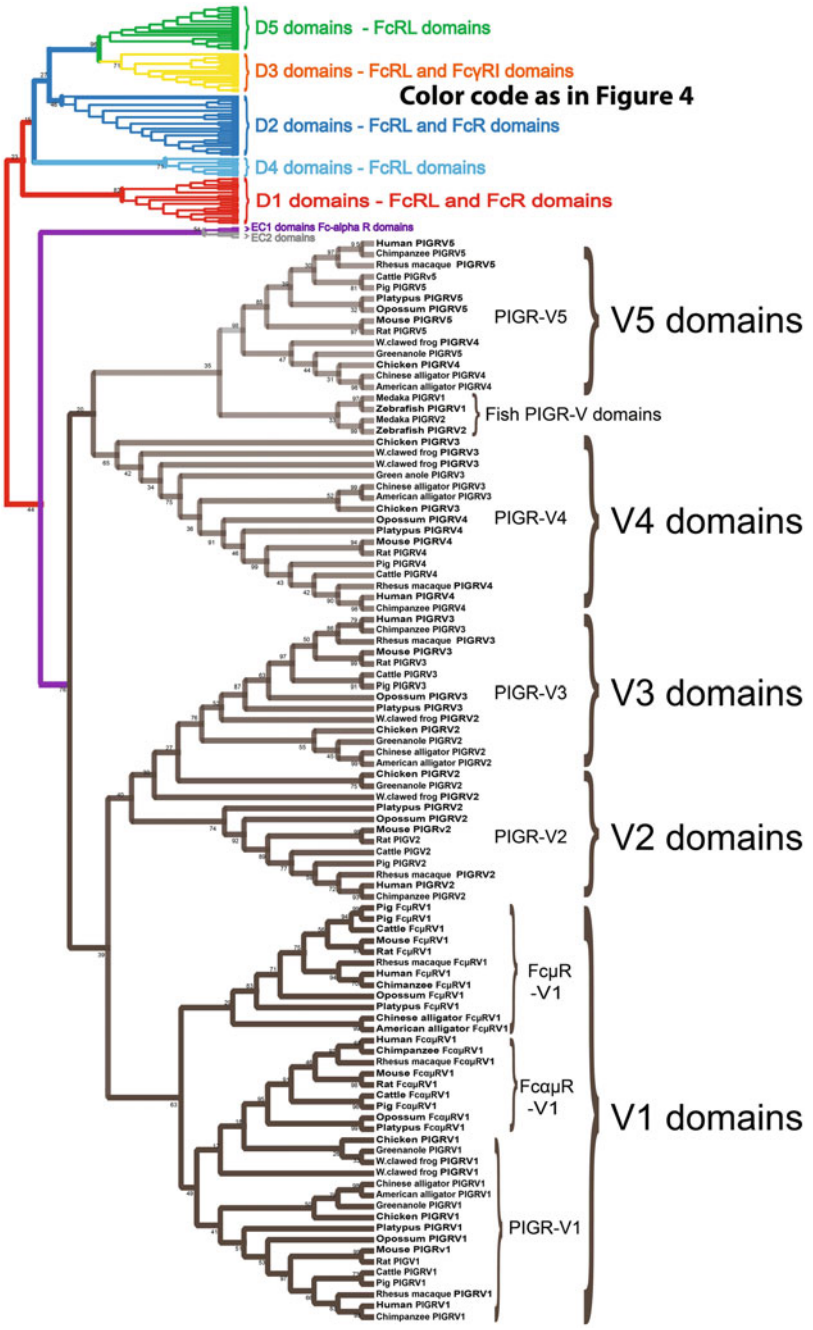


Fig. 5 A phylogenetic tree of individual domains of a panel of different Fc receptor sequences from a number of different vertebrates analyzed for their sequence relatedness. Protein domains were identified with the SMART software (Letunic et al. 2012). The rooted maximum likelihood tree was constructed by using MEGA5.2 software (Tamura et al. 2011). Bootstrap analysis was performed with 500 replicates. Bootstrap values are indicated in the phylogenetic tree. The individual domain types are color-coded as in Fig. 4

(Kaetzel 2005). The PIGR is highly glycosylated, containing 22% carbohydrate by weight although these carbohydrates do not seem to directly influence ligand binding (Sletten et al. 1975; Bakos et al. 1991). This receptor is expressed at its highest levels in the small and large intestines but is also present in the kidneys, pancreas, lungs and endometrium (Krajci et al. 1989; Klimovich 2011).

In all mammals studied, covering all three extant mammalian lineages; monotremes, marsupials and placental mammals, the PIGR has five Ig-like domains each approximately 100–110 amino acids in length. Structural studies show that they most closely resemble Ig variable domains, so called V type domains, where short hinge-like regions are found between domains 1 and 2, and domains 3 and 4 (Pumphrey 1986; Hamburger et al. 2004). Amphibians, reptiles and birds have four domain PIGRs and teleost fish have even more variable numbers of Ig domains in PIGRs. Interestingly, most tetrapods have one or at the most two copies of the PIGR gene, whereas the numbers in bony fish sometimes exceed thirty (Figs. 2 and 3). The fact that all mammals have five domain PIGRs and all reptiles and amphibians have only four domains, indicates that the duplication of the second domain resulting in five domain PIGRs occurred during early mammalian evolution, possibly 200–250 million years ago (Fig. 4). One step in this process has been an internal duplication involving domains 2 and 3, leading to an exon containing two domains, which is in contrast to all other Ig domains within various PIGR genes, which are encoded by a separate exon. This duplication did not appear or was later reverted in the chicken genome (Wieland et al. 2004). The second and third domains appear to be of importance for efficient transport of dimeric IgA (Norderhaug et al. 1999). The reasons for the very large number of PIGR genes in fish as well as the difference in the number of domains within fish PIGR genes are not yet known. In any case, it is strikingly different from the tetrapod scenario and may indicate that PIGRs have more than one function in fish. Previously, fish have only been considered to express two and three domain PIGRs, however, a re-screening of their updated genomes showed that they also have members with four, five and even six Ig domains, although the majority of the consist of two or three domains (Figs. 3 and 4). In the zebrafish databases there are now 34 PIGR sequences of which 27 have two domains, 3 have three domains, 1 has four domains, 2 have five domains and 1 has six domains. Interestingly, many of the fish PIGRs also seem to lack transmembrane regions, which may mean they are found as soluble forms with potentially other functions than epithelial transport (Fig. 4). This is somewhat precautionary, as the possibility of other forms of membrane anchoring has not been studied.

The PIGRs of placental mammals are remarkably well conserved in all regions except the linker region between domain 5 and the membrane anchoring hydrophobic region (Fig. 6). Furthermore, parts of the cytoplasmic region are conserved in all tetrapods from amphibians to mammals (Fig. 6). Conserved tyrosines of the cytoplasmic tail are important for the internalization process; the first step in the transport of the receptor from the baso-lateral to the apical side of the cell (Okamoto et al. 1992). The phosphorylation of serine 664 (marked by green star, Fig. 6) is also important for the translocation into the endosomes and their

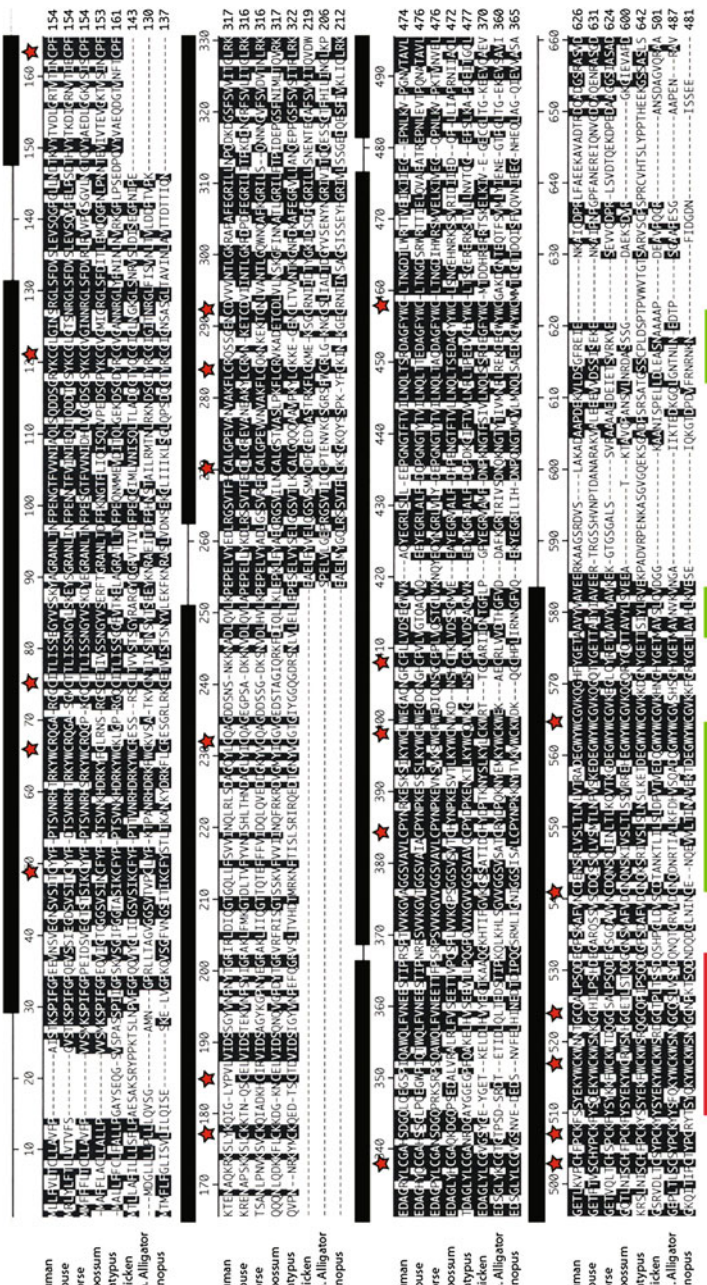


Fig. 6 A sequence alignment of a panel of PIGRs. Conserved residues are shown within *black boxes*. The Ig domain, the hydrophobic transmembrane region and the conserved potential signaling motifs in the cytoplasmic region are marked by a *thick black line*, a *red line* and *green lines*, respectively. Cysteine residues involved in intra-domain cysteine bridges are marked by *red stars*. The conserved phosphorylated cytoplasmic serine 664 is marked by a *green star*

subsequent transfer to the apical side of the cell, which is dependent on microtubule dynamics (Casanova et al. 1990; Hunziker et al. 1991). Mutation of this residue into an alanine markedly slows down the translocation of the complex within the cell, and conversely mutation into a negatively charged aspartic acid, resembling a negatively charged phosphorylated serine, enhances the rate of translocation (Casanova et al. 1990).

Studies on the binding properties of PIGRs in different species show that the first domain, domain 1, is essential and sufficient for the binding of IgA and IgM (Norderhaug et al. 1999; Kaetzel 2005; Klimovich 2011). However, this binding affinity increases 20-fold by adding additional domains, suggesting that although domain 1 is essential, other domains are of importance at least in some species (Zikan and Bennett 1973; Klimovich 2011). A recent study of the structure of the human PIGR shows an almost closed ring or triangular structure of the five domain PIGR when not bound to IgA or IgM. In this orientation, domain 1 directly interacts with domains 2, 4 and 5 to form this triangular structure and upon ligand binding this opens up into a more extended structure (Stadtmueller et al. 2016). Analysis of a two domain fish PIGR by the same lab shows that the fish PIGR forms an extended structure even in the absence of direct contact with Igs (Stadtmueller et al. 2016). Interestingly, the addition of domain 2 in mammalian PIGRs seems to facilitate the triangular shape and also to stabilize the complex between the SC and IgA (Stadtmueller et al. 2016). There is also a marked difference in ligand specificity between PIGRs from different species. In primates, PIGRs bind both IgA and IgM, whereas rodent (mouse, rat and rabbit) PIGRs only bind IgA and not IgM (Brandtzaeg and Johansen 2001). The PIGR Ig domains are of the V type and three complementarity determining regions (CDRs) can also, similar to Ig V regions, be identified in the PIGR domain 1, which are important for the interactions between the PIGR and IgA and IgM (Coyne et al. 1994). During a domain swapping experiment between human and rabbit PIGRs, the exchange of CDR2 from the rabbit into the human PIGR results in a loss of affinity for IgM, indicating this region is of major importance for the interaction with IgM (Roe et al. 1999). The increase in domain numbers improves IgA binding and the duplication of the exon for domain 2 has been implicated in this increased IgA affinity (Norderhaug et al. 1999). The binding of IgA and IgM is dependent on the presence of the J chain of both of these isotypes (Ferkol et al. 1995). However, the CH4 domain of IgM also appears to be essential for binding of IgM (Ferkol et al. 1995). It has been proposed that the driving force in the evolution of the PIGR from two domains to four and five domain PIGRs has been interactions with the commensal microbiota (Kaetzel 2014).

The number of PIGR genes in the different teloeost fish is remarkable (Fig. 3). Currently, 34 PIGR genes have been identified in the zebrafish genome and several other fish have more than 5 or even 10 such genes. One exception is the gar, where only one PIGR gene has been observed. The gar represents an early branch of bony fish, which may indicate that the massive expansion in many fish species has occurred after the diversification of the bony fish. One major question that remains

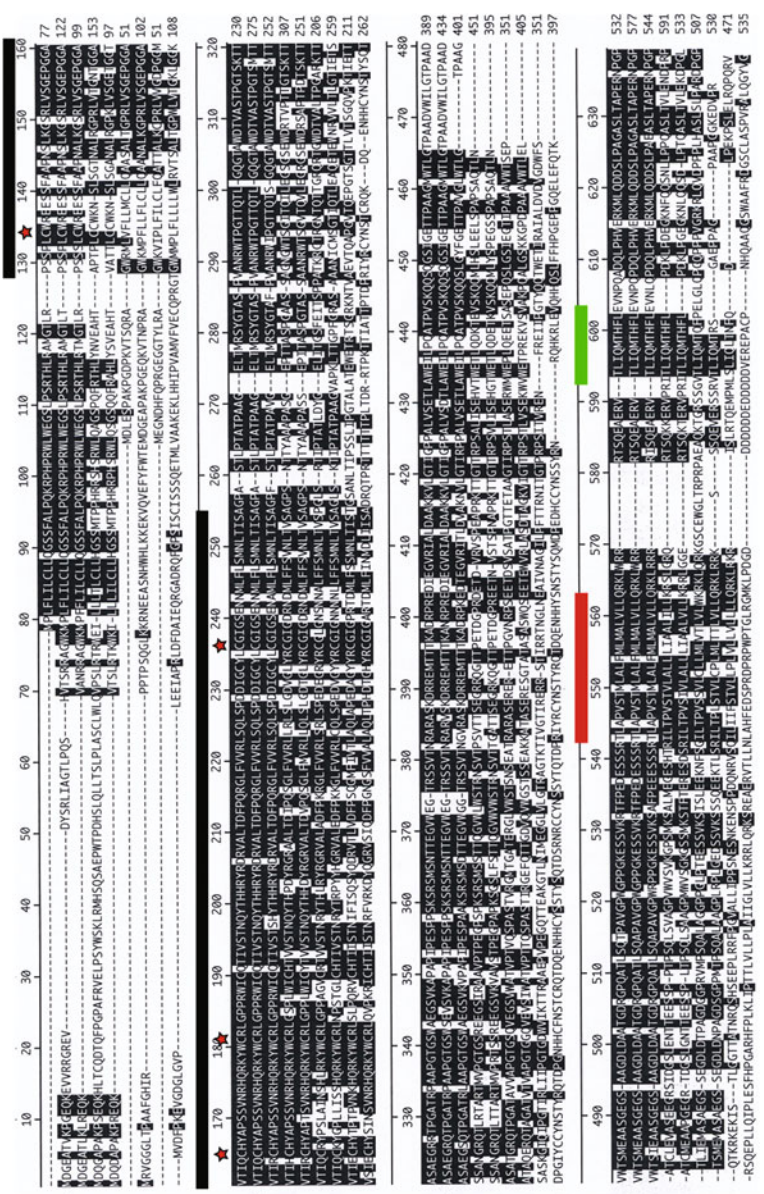


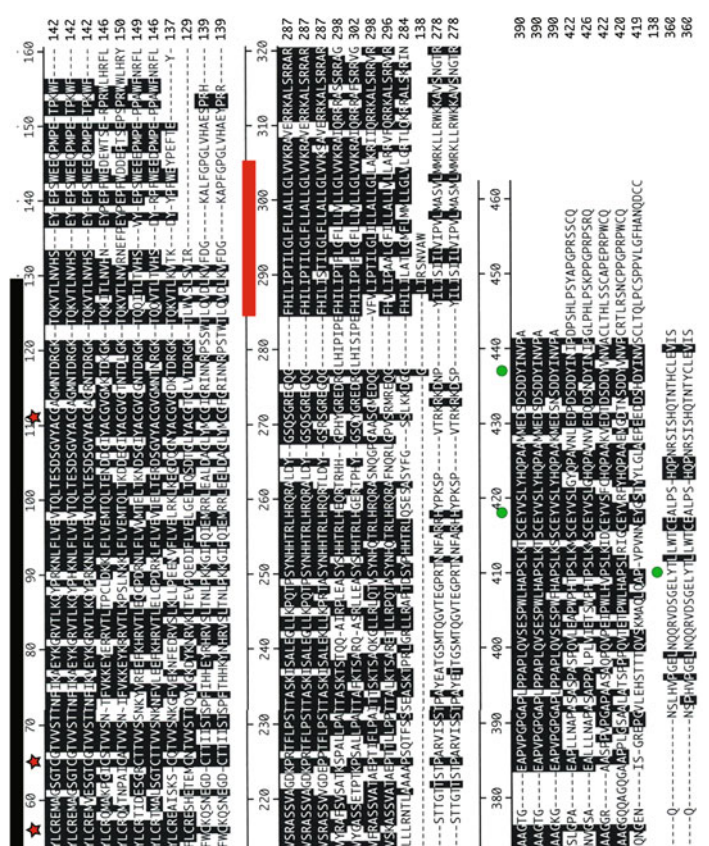
Fig. 7 A sequence alignment of a panel of FcαμRs. Conserved residues are shown within *black boxes*. The Ig domain, the hydrophobic transmembrane region and the conserved potential signaling motifs in the cytoplasmic region are marked by a *thick black line*, a *red line* and *green lines*, respectively. Cysteine residues involved in intra-domain cysteine bridges are marked by *red stars*. The platypus sequence is probably only partly correct. Only the Ig domain shows significant homology to the other FcαμR sequences, and the remaining parts of the protein is likely to be wrongly annotated in the database

to be addressed here are the functions of all of these PIGRs in bony fish. In our minds it is unlikely that they are all involved in epithelial transport.

4 The FcαμR

The dual receptor for IgA and IgM is a 55 kD protein and is involved in endocytosis of IgM coated microparticles and bacteria (Shibuya et al. 2000). The mature FcαμR is a remarkably stable homodimeric glycoprotein with an M_r of 115–135 kD (Kikuno et al. 2007). Among the hematopoietic cells it is expressed primarily on follicular dendritic cells (FDC) in both mice and humans (Kikuno et al. 2007). It is also expressed by certain cell populations in the liver, kidneys, small and large intestines, testes and placenta (Shibuya et al. 2000; Sakamoto et al. 2001; Kikuno et al. 2007). Interestingly the expression level is highest in the kidney, indicating a potent physiological role not only in hematopoietic cells (Sakamoto et al. 2001). In contrast to the PIGR, this receptor has only one Ig-like domain, which is most closely related to domain 1 of the PIGR in tetrapods (Shibuya et al. 2000). The binding affinity of this domain is approximately ten times stronger for IgM than for IgA, 2.9×10^9 M^{-1} and 3×10^8 M^{-1}, respectively, but it clearly binds both isotypes indicating that it is a bona fide dual receptor (Shibuya et al. 2000; Klimovich 2011). In contrast to PIGR, the binding does not appear to be dependent on the J chain as monomeric IgA can also bind, at least to the mouse receptor (Shibuya et al. 2000). Furthermore, as IgA and IgM seem to compete for binding to the mouse receptor, it indicates a common site for their interaction (Yoo et al. 2011). In addition to the N terminal Ig domain this receptor has a relatively long, approximately 277 amino acids (in humans), extracellular mucin-like domain of unknown function (Shimizu et al. 2001). The transmembrane portion of the human receptor is 20 amino acids long with no charged residues (Figs. 1 and 7) and the 61 amino acid cytoplasmic region has no archetypal ITIMs or ITAMs, which are found in the classical mammalian IgG and IgE receptors (Shimizu et al. 2001). A di-leucine motif in the cytoplasmic region of the mouse receptor appears to be involved in the internalization process (Shibuya et al. 2000). However, this motif is not conserved in any of the other species listed (Fig. 7) including the human receptor, indicating that other motifs are of importance for this receptor in other species (Shibuya et al. 2000). Instead, there is another motif that is relatively well conserved between all placental mammals studied and also in the opossum, a marsupial (Fig. 7). This conserved sequence is located approximately 15 amino acids in from the membrane and has the consensus sequence V/I-T/S-L-I-Q-M-T-H-F-L-E/D. This motif may be of major importance for intracellular signaling processes (Fig. 7), although this needs to be studied and verified experimentally.

Knock out experiments in mice show that the absence of the FcαμR does not affect the titers of IgG and IgM in sera nor the T-cell dependent antibody responses (Honda et al. 2009). However, a marked increase in IgG3 levels to thymus independent antigens is observed as well as an IgG3 germinal center dependent memory


Human
Chimpanzee
Macaque
Mouse
Rat
Cow
Pig
Opossum
Platypus
Ch. Alligator
Am. Alligator

◂**Fig. 8** A sequence alignment of a panel of FcμRs. Conserved residues are shown within *black boxes*. The Ig domain, the hydrophobic transmembrane region and the conserved potential signaling motifs in the cytoplasmic region are marked by a *thick black line*, a *red line* and *green lines*, respectively. Cysteine residues involved in intra-domain cysteine bridges are marked by *red stars*. The platypus FcμR sequence is only partial, only containing a signal sequence and the Ig domain. The three Tyr residues conserved in all mammalian sequences are marked by *green dots* (Tyr 315, 366 and 385). A Tyr, possible corresponding to Tyr 366 and/or 385, in the alligator sequences is also marked by a *green dot*

response (Honda et al. 2009). The number of germinal center B cells increases significantly after injection with thymus independent antigens but not with T-dependent antigens. These effects of FcαμR are dependent on complement and complement receptors, as blocking monoclonal antibodies to complement receptors 1 and 2 results in an almost complete loss of these responses (Honda et al. 2009). Concluding, this receptor seems to be involved in the internalization of antigen in B cells, which is most likely for presentation on MHC molecules, but also as a negative regulator of IgG3 responses to thymus independent antigens.

5 The FcμR

The receptor specific for IgM was the last of these receptors to be identified. Indications for the presence of such a receptor has been apparent for many years but it was not until very recently that it was finally identified through expression cloning into mammalian cells by the lab of Hiromi Kubagawa (Kubagawa et al. 2009, 2014). FcμR is a 60 kD transmembrane protein expressed by human B, T and NK cells and binds pentameric IgM with a very high avidity. In mice it is primarily expressed by B cells and not at significant levels on T and NK cells (Shima et al. 2010; Honjo et al. 2012; Ouchida et al. 2012; Honjo et al. 2014). However, there are some controversies regarding the expression pattern of this receptor as independent groups have provided varying results when using different monoclonals or when analysing mRNA levels by qPCR or Northern blotting, which explains why there is still slight doubt over the exact tissue distribution of this receptor (see Chapter of Kubagawa et al.) (Wang et al. 2016). The FcμR is also internalized upon ligand binding, which complicates the analysis by when using monoclonals analyzing its surface expression (Wang et al. 2016). After translation the primary polypeptide is only 41 kD, hence a large fraction of the molecular weight is attributed to carbohydrate residues. Despite this, no N-linked carbohydrate addition sites (N-X-T/S) are found in the human or the mouse sequences (Figs. 1 and 8). Recently several O–linked glycosylation sites have been identified in the linker region by point mutational analyses, showing at least the majority of these carbohydrates are O-linked (Kubagawa et al. 2009; Vire et al. 2011; Kubagawa et al. 2014). The isoelectric point also is markedly different when comparing the 41 kD

polypeptide chain to the 60 kD glycosylated form. The change is from a predicted pI 9.9 to 5, indicating a high percentage of negatively charged sialic acid residues in the carbohydrate chains (Kubagawa et al. 2014). FcμR is also the only receptor that exclusively binds IgM and interestingly, the CDR1 loop of the receptor is considerably shorter than the corresponding region in both the PIGR and the FcαμR; five amino acids compared to nine. Furthermore, the receptor lacks an Arg residue in this region, which is predicted to interact with IgA, suggesting that this difference is responsible for the monospecificity of the FcμR (Kubagawa et al. 2009, 2014).

This receptor is present in all three extant mammalian lineages and it was also recently identified in both the American and the Chinese alligator genomes, indicating that it appeared relatively early in tetrapod evolution. However, as this receptor has not been found in any of the amphibian genomes, it implies that it appeared in an early reptile, which became the ancestor to both reptiles and mammals. The FcμR is involved in a number of important steps in B-cell development including IgM homeostasis, B-cell survival, humoral immune responses and in autoantibody formation (Honjo et al. 2012; Ouchida et al. 2012; Choi et al. 2013; Honjo et al. 2014). Despite these observations, quite contradicting results have come from three independent knockout mice that have been generated (Nguyen et al. 2011; Honjo et al. 2012; Ouchida et al. 2012; Choi et al. 2013; Kubagawa et al. 2014; Wang et al. 2016). Two recent reviews have discussed the effects by functionally inactivating the FcμR and the discrepancies between the three different knockout mouse strains, therefore only a short list of some of the key findings described from studies will be given below. (Kubagawa et al. 2014; Wang et al. 2016). All three knockouts show alterations in B-cell populations, although with varying effects between the different mice. They also all show dysregulated humoral immune responses, impaired B-cell proliferation after ligation of surface Ig, and an increase in autoantibody production. Interestingly, mice lacking secretory IgM, through a deletion of the genomic region encoding the secretory terminal region of the CH4 exon, results in a mouse that only expresses cell surface bound IgM and not secretory IgM, showing a very similar phenotype to the FcμR knockout mice (Kubagawa et al. 2014; Wang et al. 2016). Although there are clear differences between the knockout models, the consensus is that the FcμR has major effects on B-cell differentiation and Ig homeostasis (Kubagawa et al. 2014; Wang et al. 2016). The FcμR also appears to be the only receptor among the FcRs directly involved in IgM homeostasis (Honjo et al. 2012, 2014).

The signaling from the FcμR does not involve classical ITAMs or ITIMs. However, there are several conserved tyrosines and serines in its cytoplasmic tail (Fig. 8) (Kubagawa et al. 2014; Wang et al. 2016). Three tyrosines are conserved within the cytoplasmic tail of all eutherian mammalian FcμRs but this number drops to one tyrosine when alligators are also included (Fig. 8). Four serines are conserved between all mammals and two additional serines are almost fully conserved. However, similarly to the tyrosines, a drop is seen when including alligators, resulting in only two fully conserved residues (Fig. 8). A recently identified Ig tail tyrosine (ITT) phosphorylation motif Glu/Asp X_{6-7}-Asp-Tyr-X-Asn, which is present in membrane bound IgG and IgE, is also found in the C-terminal end of

mammalian FcμRs but not in the corresponding region in the alligator sequences (Fig. 8) (Wang et al. 2016). In Igs this ITT motif is involved in triggering and activating switched memory B cells (Wang et al. 2016). Only one of the motifs is conserved between all species analyzed and has the sequence N-I/V-Y-S-A-C-P-R (Fig. 8, marked with a *green box*). A quite extensive mutational analysis has been performed on the trans-membrane and cytoplasmic tail of the human FcμR. The membrane proximal Tyr (Tyr-315) that is found in the motif marked in green in Fig. 8 has been shown to be of importance for the anti-apoptotic effect of the FcμR. This mutation has almost the same effect on the anti-apoptosis as deleting almost the entire intracellular domain (Honjo et al. 2015). Mutations of the His residue in the transmembrane region and the two membrane distal Tyr residues 366 (Tyr to Phe) and 385 (Tyr to Phe) also affects the anti-apoptosis however to a lesser extent. The two latter mutations also showed a pronounced effect on internalization of the receptor indicating a prominent role of these two Tyr residues in receptor-mediated endocytosis (Honjo et al. 2015). These three Tyr residues are marked by green dots in Fig. 8. In this figure a Tyr possible corresponding to the Tyr 366 and/or 385 in the alligator sequences is also marked by a green dot.

6 Concluding Remarks

Following the appearance of Igs in early jawed vertebrates there has been a parallel increase in the complexity of molecules interacting with these antigen specific molecules during vertebrate evolution. The first molecules to interact with Igs have probably been components of the complement system. All of the essential components of the classical complement system have been identified in a number of cartilaginous fish (Goshima et al. 2016). These components seem to absent in jawless fish, indicating the appearance of this branch of the complement system with the jawed vertebrates. The second additions were most likely the PIGRs, the FcR common γ chain and the FcRL molecules. This was later followed by an increase in complexity of the FcRL molecules in tetrapods, resulting in the appearance of the classical receptors for IgG and IgE in mammals (Akula et al. 2014). Although all of the evidence indicates that IgM is the evolutionary oldest of the different Ig isotypes, two of the three receptors for IgM, FcμR and FcαμR, appeared relatively late during vertebrate evolution. The FcμR may have appeared sometime during early reptile evolution whereas the FcαμR probably appeared during early mammalian evolution. Due to the high sequence similarity in the first Ig-like domain of all three IgM receptors, the most likely scenario is that they have appeared by successive gene duplication events involving domain 1 of the PIGR. The origins of the other parts of both the FcμR and the FcαμRs are still a mystery, as no closely related sequences can be found in either the mouse or human genomes. Following these duplications, these three receptors have diversified quite extensively in function by changing expression patterns and cytoplasmic signaling motifs. In tetrapods, the PIGR primarily functions as a transport receptor for IgM and IgA (IgX in amphibians). The situation in fish is still not fully

known. However, the large complexity of PIGRs in some bony fish, such as the zebrafish, is exciting and needs further detailed analyses to obtain a more complete picture of their roles in fish immunity. What is also somewhat surprising is the complete absence of known FcRs in cartilaginous fish. The obvious question is how have they solved the problems of Ig mediated antigen uptake by phagocytic and antigen presenting cells as well as transport of Ig over epithelial layers and potential triggering of granule release by hematopoietic cells, which is attributed to FcRs in mammals? To our knowledge no good candidates for such molecules have been identified yet. Additionally, the complex role of the FcμR and FcαμR in regulating B-cell responses is a fascinating research area. Originating from a PIGR, with primarily a transport function, they have gained important roles in thymus-independent B-cell responses, B-cell homeostasis and autoantibody formation. In the near future, new insights from the studies of the different knockout animals will most likely shed even more light on these intricate regulatory mechanisms by these three receptors.

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Authentic IgM Fc Receptor (FcμR)

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Abstract Since the *bona fide* Fc receptor for IgM antibody (FcμR) was identified eight years ago, much progress has been made in defining its biochemical nature, cellular distribution, and effector function. However, there are clearly conflicting results, especially about the cellular distribution and function of murine FcμR. In this short article, we will discuss recent findings from us and other investigators along with our interpretations and comments that may help to resolve the existing puzzles and should open new avenues of investigation.

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1 Introduction

It has been established from studies of mutant mice deficient in IgM secretion that both preimmune "natural" IgM and antigen-induced "immune" IgM are important in responses to pathogens and self-antigens (Ehrenstein and Notley 2010). Effector proteins interacting with the Fc portion of IgM, such as complement and its receptors, have so far mainly been elucidated in the context of IgM-mediated immune protection and regulation (Heyman 2000) (see also Chap. 4 by Anna Sörman and Birgitta Heyman). The role of IgM Fc receptor (FcμR) in such effector functions has just begun to be explored, since the *FCMR* was identified in 2009 (Kubagawa et al. 2009). In this chapter, recent findings about the FcμR in both humans and mice are reviewed, along with emphasis on its significance and the discrepancies among different reports especially in murine studies. Several review articles on FcμR and its ligand IgM have already been published elsewhere, and the authors recommend them for further information on both of these topics (Baumgarth 2016; Ehrenstein and Notley 2010; Klimovich 2011; Kubagawa et al. 2014a, c; Panda and Ding 2015; Wang et al. 2016).

2 Lymphocyte-Restricted Expression of FcμR

The cellular distribution of FcμR in mice is still not completely resolved. In our studies, FcμR expression at *the level of transcription* is only detectable in B-lineage cells and not in other cell types. This was investigated by reverse transcriptase polymerase chain reaction (RT-PCR) using RNAs from the following tissue/cell samples. (i) Splenocytes or liver tissues of mice deficient for recombination-activating gene 1 (*Rag1*), which are devoid of B and T cells but contain abundant granulocytes and macrophages and (ii) CD19-positive B or CD19-negative non-B cells and Gr1- or Ly6G-positive myeloid cells enriched twice to high purity by fluorescence-activated cell sorting (FACS) from wild-type (WT) mouse bone marrow (BM) and spleen (Honjo et al. 2012a, 2013). By contrast, Choi et al. (2013) using a similar RT-PCR analysis of FACS sorted cells found clear expression of FcμR by the splenic Gr1-positive cell population in addition to B cells, but curiously, total BM cells, which should contain more abundant Gr1-positive myeloid cells than B cells, did not express significant levels of FcμR. The B cell-restricted expression pattern of FcμR is also documented in two large-scale expression databases of immune cells: Immunological Genome Project (https://www.immgen.org) and Reference Database of Immune Cells (http://refdic.rcai.riken.jp/welcome.cgi). Intriguingly, however, another study has recently reported that by single-cell RNA sequencing along with complex algorithmic assessments and its functional annotation, FcμR is suggested as one of the critical regulators of Th17 pathogenicity in myelin oligodendrocyte glycoprotein (MOG)-induced autoimmune encephalomyelitis (EAE) (Gaublomme et al. 2015). In our studies using the T cell

transfer colitis model in *Rag1*$^{-/-}$ mice, however, none of the sorted T cell subpopulations with the phenotype of IL-17^{+}, IFNγ^{+}, or IL-17^{+}/IFNγ^{+} express FcμR transcripts as determined by gene array analysis (Zimmermann et al. unpublished). In this regard, transcriptome analysis of even homogenously isolated cell populations (e.g., Th17 cells) is a mixed snapshot of asynchronously propagated, metabolically heterogeneous cell populations. On the other hand, single-cell RNA sequence analysis along with multiple algorithmic assessments may distinguish distinct states of cells within such populations. Even based on single-cell RNA sequencing data, however, it is difficult to distinguish whether such a small subpopulation of Th17 cells may indeed express full-length FcμR transcripts or may passively acquire them through exosomes or membrane vesicles from FcμR-bearing B cells via trogocytosis (Gyorgy et al. 2011). It will also be difficult to prove formally that such a minor population of Th17 cells expresses functional FcμR at low levels on their cell surface and plays a major regulatory role in the pathogenesis of MOG-induced EAE. The experimental data leading to this idea were based on the ex vivo data of Th17-polarizing cells from *Fcmr*-deficient (KO) and WT mice. This issue will be further discussed in the section of *Fcmr* KO mice (Sect. 4).

With respect to *the cell surface expression* of FcμR using receptor-specific monoclonal antibodies (mAbs) and IgM-ligand binding, we and others have found that the expression of FcμR is restricted to adaptive immune lymphocytes: both B and T cells and, to a lesser extent, natural killer (NK) cells in humans (Kubagawa et al. 2009; Murakami et al. 2012) and only B cells in mice (Honjo et al. 2012a, 2013; Lapke et al. 2015; Ouchida et al. 2012; Shima et al. 2010). [NK cells are the only known non-adaptive immune cell in humans to express FcμR, but this cell is now thought to have both adaptive and innate immune cell features (Vivier et al. 2011)]. This lymphocyte-restricted expression suggests a distinct function of FcμR compared to FcRs for switched Ig isotypes (e.g., FcγRs, FcεRI, and FcαR), which are also expressed by various innate immune cells. The species difference in cellular distribution of FcμR indicates that the results from murine FcμR studies may not necessarily reflect the human situation. Given the fact that IgM is the first Ig isotype appearing during phylogeny, ontogeny, and immune responses and that IgM is considered as a first line of defense against infection, the lymphocyte-restricted expression pattern is somewhat unexpected and may have some functional significance. In this regard, it is also noteworthy that unlike the phylogenetically broad distribution of IgM from jawed vertebrates (i.e., cartilaginous fish) onward, analysis of currently existing genomic sequence databases indicates that the IgM FcR first appears in early reptiles and is found in all three major living (extant) groups of mammals (i.e., egg laying, marsupial and placental mammals) (Akula et al. 2014) (also see Chap. 1 by Srinivas Akula and Lars Hellman).

Contrary to the B cell-restricted expression pattern in mice described in the above-mentioned studies, other groups using a rat mAb (B68 clone) against mouse Toso, an original name of FcμR (Hitoshi et al. 1998; Kubagawa et al. 2015), reported the weak but "functional relevant expression" of FcμR by myeloid cells, dendritic cells (DCs), and T cells (Brenner et al. 2014; Lang et al. 2013a, b; Nguyen

et al. 2011). Strangely, in their analyses, Ly6G-positive BM myeloid cells were weakly positive for B68 mAb staining, whereas Ly6G-negative cells, which should contain abundant B-lineage cells, were completely negative, suggesting a non-optimal assessment with this mAb (Lang et al. 2013a, b). In fact, Lapke et al. (2015) have recently demonstrated that the expression of Toso/FcμR in mice is restricted to B cells using the same B68 and additional A96 mAbs, consistent with the results from our analysis using a panel of five different mAbs (MM1, MM2, MM3, MM4, and MM6 clones) (Honjo et al. 2012a, 2013). Notably, most studies dealing with the predicted function of FcμR in granulocytes, monocyte/macrophages, and DCs were based on comparative analysis in adoptive transfer experiments of WT *versus Fcmr* KO BM cells (Brenner et al. 2014; Lang et al. 2013a, b, 2015) and not on any actual convincing data of the cell surface FcμR expression by non-B cells, hence the use of the phrase "functional relevant expression." Apart from this, there is another concern regarding the specificity or cross-reactivity of several commercially available polyclonal or monoclonal reagents raised against synthetic peptides of both human and mouse FcμRs, which might account for the reported expression of Toso/FcμR by non-hematopoietic cell types such as pancreatic ß cells (Dharmadhikari et al. 2012).

The lymphocyte-restricted expression pattern of FcμR is thus distinct from the expression of other IgM-binding receptors. Polymeric Ig receptor (pIgR) is predominantly expressed by mucosal epithelial cells (Kaetzel 2005), and FcR for IgA and IgM (Fcα/μR) is expressed by follicular dendritic cells and other cell types including Paneth cells in small intestinal crypts, the proximal tubular epithelial cells in kidneys, and the serous acini and small epithelial cells of salivary glands (Kikuno et al. 2007).

Several types of stimulations or conditions have been shown to modulate cell surface expression of FcμR. Upon antigen receptor ligation with antibodies or phorbol myristate acetate stimulation, FcμR expression in humans is up-regulated on B cells but is down-modulated on T cells, suggesting differential regulation of FcμR expression during B cell and T cell activation (Kubagawa et al. 2009; Nakamura et al. 1993; Sanders et al. 1987). Stimulation of T and NK cells with IL-2 in vitro also down-regulates FcμR expression in a STAT5-dependent manner (Murakami et al. 2012). In diseases, the enhanced expression of FcμR is a hallmark of chronic lymphocytic leukemia (CLL) B cells, as first demonstrated many years ago by rosette formation with IgM-coated erythrocytes (Ferrarini et al. 1977; Pichler and Knapp 1977), followed by IgM-ligand binding (Ohno et al. 1990; Sanders et al. 1987), gene expression (Catera et al. 2008; Pallasch et al. 2008; Proto-Siqueira et al. 2008; Rosenwald et al. 2001; Wang et al. 2004), and receptor-specific mAbs (Li et al. 2011; Vire et al. 2011). Intriguingly, surface FcμR levels are also significantly elevated in the non-CLL B cells and T cells in CLL patients (Li et al. 2011). Immunotherapies targeting for the FcμR have been designed for CLL cells. One is an immunotoxin-coupled IgM Fc (Cμ2-Cμ4) (Vire et al. 2014), and another is chimeric antigen receptor-modified T cells using a single-chain fragment (scFv)-containing the variable regions of an anti-FcμR mAb (6B10) (Faitschuk et al. 2016). In both cases, patient CLL B cells appear to be

selectively eliminated in vitro without affecting the non-leukemic B and T cells. In patients with selective IgM immunodeficiency, cell surface FcμR levels on a particular blood B cell subset with a marginal zone (MZ) phenotype ($IgM^+/IgD^+/CD27^+$) are significantly diminished as compared to age-matched controls, but the molecular basis for this reduction remains unclear (Gupta et al. 2016).

3 Unique Ligand-Binding Activity

3.1 Fcμ-Specificity, Ligand-Binding Avidity, and Glycosylation

After identifying the FcμR cDNA from human B-lineage cell-derived cDNA libraries by a functional cloning strategy (i.e., IgM-ligand binding), cell lines stably expressing FcμR have mainly been used to investigate their ligand-binding specificity. The FcμR-bearing cells clearly bind IgM in a dose-dependent manner, but not other Ig isotypes (i.e., IgG1-4, IgA1-2, IgD, or IgE) (Kubagawa et al. 2009). The inability of FcμR to bind polymeric IgA clearly indicates that FcμR is distinct from pIgR and Fcα/μR, both of which bind IgM and polymeric IgA and are clustered within the *FCMR* locus on chromosome 1q32.2. [Recent domain swapping analysis has revealed that unlike pIgR, Fcα/μR can bind J chain-deficient IgM hexamers (Yoo et al. 2011); hence, all three IgM-binding receptors are different in terms of ligand-binding specificity]. Binding of IgM by FcμR is mediated by its Fc5μ fragments, consisting mostly of Cμ3/Cμ4 domains, but not by Fabμ fragments, thereby confirming its IgM Fc-binding specificity (Kubagawa et al. 2009; Murakami et al. 2012). Recent domain swapping analysis reveals the Cμ4 as the target of FcμR (Lloyd et al. 2017). By Scatchard plot analysis assuming a 1:1 stoichiometry of FcμR to IgM ligand, FcμR binds IgM pentamers with a strikingly high avidity of $\sim$10 nM. This in turn suggests that serum IgM, the concentration of which is $\sim$1 μM, constitutively binds FcμR on the surface of B, T, and NK cells and explains why detection of cell surface FcμR is enhanced by preculture of cells, especially T cells, in IgM-free media (Kubagawa et al. 2009; Nakamura et al. 1993). In this regard, Vire et al. (2011) found that FcμR on CLL B cells was rapidly internalized upon IgM binding and shuttled to the lysosomes for degradation. The configuration of IgM is also important for FcμR binding, as higher concentrations (>100-fold) are required for binding of monomeric IgM to the $FcμR^+$ cells than pentameric IgM. In addition to IgM pentamer, J chain-deficient IgM hexamers are also present in normal sera, albeit at unknown concentrations, but the complement activation activity of the IgM hexamers is 50- to 100-fold higher than IgM pentamers (Randall et al. 1992; Wiersma et al. 1998). Intriguingly, our preliminary findings show that the dissociation constant (K_D) of a recombinant IgM hexamer for FcμR is only twofold to threefold higher than that of IgM pentamers (Fig. 1).

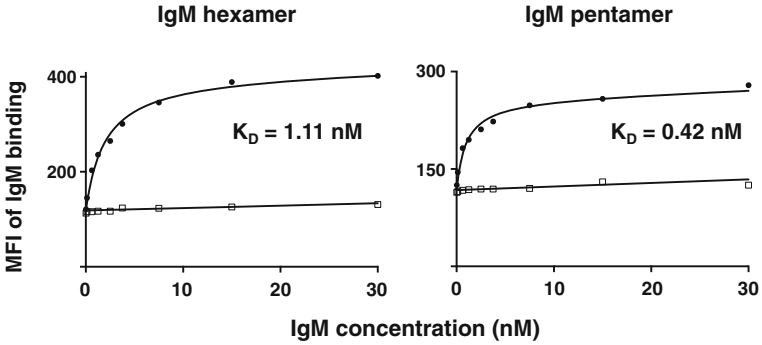

Fig. 1 Binding of hexameric and pentameric IgM to FcμR. An equal mixture of murine thymoma line BW5147 stably expressing both FcμR and green fluorescent protein (GFP) (•), and WT ($Fc\mu R^-/GFP^-$) control cell line (□) was incubated with various concentrations (25 pM–30 nM) of recombinant hexameric (*left* IgM-αTp) or pentameric (*right* rIgM) human IgM ligands, the preparation and purity of which were described elsewhere (Yoo et al. 2011) and were confirmed prior to use in the present studies. The bound IgM was assessed by addition of phycoerythrin-labeled goat antibodies specific for human μ heavy chain, followed by flow cytometry. The mean fluorescent intensity (MFI) of IgM binding at each concentration was plotted, and the K_D was calculated by nonlinear regression analysis using GraphPad Prism software. Two independent experiments yielded similar K_D values (nM), and one of them is shown

FcμR does not have N-linked glycosylation motifs (NxS/T; x indicates any amino acid) in the extracellular region, consistent with our previous biochemical characterization of the IgM-binding protein (Ohno et al. 1990). Since the core peptide of FcμR is predicted to have a M_r of ∼41 kDa and the FcμR expressed on B and T cells has a M_r of ∼60 kDa, one-third of the M_r of the mature FcμR is thus made up of O-linked glycans. Potential glycosylation sites were determined by mutagenesis experiments: Thr residues at positions of 161, 164, 165, 181, 182, and 185 and Ser at 178 and 179 (Vire et al. 2011). Removal of sialic acids from $Fc\mu R^+$ cells by neuraminidase treatment slightly enhanced IgM-ligand binding, suggesting that desialylated FcμR has better ligand-binding activity (Kubagawa et al. 2009). Notably, Colucci et al. have recently shown that natural IgM, which is rich in terminal sialic acid residues, is internalized by T cells in humans and inhibits T cell responses such as anti-CD3/anti-CD28 mAbs- or PHA-mediated proliferation ex vivo and expression of pro-inflammatory cytokine genes. In contrast, desialylated natural IgM is not internalized and has poor inhibitory activity (Colucci et al. 2015). It remains to be elucidated how carbohydrate moieties of FcμR and its IgM ligands affect their interaction, as has been well documented in the case of FcγRs and IgG ligands (Pincetic et al. 2014; Schwab and Nimmerjahn 2013). In this regard, it has recently been reported that IgM binds FcμR and is internalized irrespective of its glycosylation (Lloyd et al. 2017).

3.2 *Cis Engagement*

In addition to the above findings of ligand binding in solution, a unique ligand-binding property of FcμR was found in the assay system using cell surface-attached IgM such as an agonistic IgM anti-Fas mAb (CH11 clone). Apoptosis-prone human Jurkat cells stably expressing FcμR were shown to be protected from Fas-/CD95-mediated apoptosis when ligated with the IgM anti-Fas mAb (Hitoshi et al. 1998), but not when ligated with an agonistic IgG anti-Fas mAb or Fas ligand (Honjo et al. 2012b; Kubagawa et al. 2009; Murakami et al. 2012). Notably, co-ligation of FcμR and Fas with the corresponding IgG mAbs plus a common secondary reagent [e.g., $F(ab')_2$ fragments of anti-mouse γ Ab] had no inhibitory effects on the IgG anti-Fas mAb-induced apoptosis (Kubagawa et al. 2009). This suggests that the anti-apoptotic activity of FcμR depends on usage of the IgM anti-Fas mAb, and not on physical proximity of two receptors by artificial co-ligation as observed in immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptors such as FcγRIIb (Ravetch and Lanier 2000) and paired Ig-like receptor of inhibitory isoform (PIR-B) (Bléry et al. 1998).

To determine how FcμR protects from apoptosis induced by IgM anti-Fas mAb, we added various concentrations of soluble IgM or its immune complexes as inhibitors into the apoptosis assays. The results are summarized in a cartoon fashion in Fig. 2. Addition of IgM anti-Fas mAb at 10 ng/ml induced robust apoptosis of FcμR-negative WT control, but not of FcμR-positive Jurkat cells.

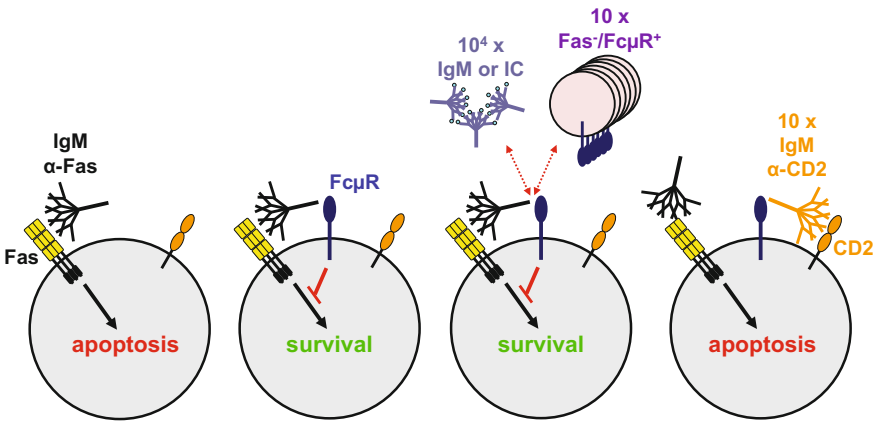

Fig. 2 Cis engagement of FcμR. Ligation of Fas/CD95 death receptor trimer (*yellow*) with agonistic IgM anti-Fas mAb (*black broom shape*) induces apoptosis in WT Jurkat cells (1st *gray circle*), but not in FcμR (*blue tennis racket shape*)-positive Jurkat cells (2nd). This FcμR-mediated protection is not blocked by addition of 10^4 molar excess of IgM or its immune complexes (*pale blue broom*) or tenfold excess of FcμR-positive, Fas-negative cells (pale purple small circles), suggesting an efficient *cis* interaction of IgM Fas mAb and FcμR on the same cell surface, but not a *trans* interaction between neighboring cells (3rd). Addition of tenfold excess of IgM mAb (*orange broom*) reactive with CD2 (*orange peanut-shell shape*) on Jurkat cells can efficiently block the interaction of IgM Fas mAb and FcμR, resulting in apoptosis (4th)

This FcμR-mediated protection was found to occur in *cis,* but not in *trans,* interactions of the Fc portion of IgM anti-Fas mAb with FcμR, because addition of excessive FcμR-positive/Fas-negative cells did not diminish the protection (Honjo et al. 2015). Addition of more than 10,000 molar excess of IgM Ab or its immune complexes (e.g., 1-21 IgM anti-α1-3 dextran mAb/α1-3 dextran) was required for partial, but significant, blockade of the *cis* interaction of the Fc portion of IgM anti-Fas mAb with FcμR. This suggests that the soluble IgM immune complexes are not potent competitors in the FcμR-mediated protection from apoptosis. When the IgM mAb reactive with CD2 (C373 clone) (Weiss and Stobo 1984) on the surface of Jurkat cells was employed as a potential competitor for the interaction of IgM Fas mAb with FcμR, a tenfold excess of IgM anti-CD2 mAb was sufficient to efficiently block the above *cis* interaction, thereby permitting the $FcμR^+$ cells to undergo apoptosis. [In particular, same results were also obtained with an IgM anti-TCR mAb (C305 clone)]. Collectively, these findings show that although FcμR binds soluble IgM pentamers at a high avidity of $\sim$10 nM, FcμR binds more efficiently to the Fc portion of IgM antibody when it is attached to a membrane component via its Fab region on the same cell surface. The preferential *cis* engagement of FcμR is thus distinct from the *trans* engagement of FcγRIIb, an inhibitory FcγR, in death receptor-mediated apoptosis. The interaction of agonistic IgG mAbs against death receptors, including Fas/CD95, with FcγRIIb is essential for the death receptor-mediated apoptosis and occurs in *trans,* but not in *cis* (Bando et al. 2002; Li and Ravetch 2011; Xu et al. 2003). The *cis* engagement of FcμR in turn implies that FcμR can modulate the functional activity of lymphocyte surface receptors or proteins recognized by either natural or immune IgM antibody.

3.3 Modulatory Effect of FcμR by Cis Engagement

The physiological relevance of the *cis* engagement of FcμR may be related to the unique features of IgM antibody, especially natural IgM antibody. Serum levels of IgM in mice raised in germfree conditions are similar to those in mice maintained under conventional or specific pathogen-free housing conditions (Hashimoto et al. 1978; Haury et al. 1997; Thurnheer et al. 2003). Two-thirds of the newly generated B cells in BM react with self-antigens such as double- or single-stranded DNA, insulin, and lipopolysaccharide (Wardemann et al. 2003). In our assessments, one-fourth of the IgM secreted from Epstein–Barr virus-transformed B cell lines derived from neonatal B cells reacts with lymphocyte surface components (unpublished observation). IgM anti-lymphocyte antibodies are often present in individuals with autoimmune diseases or chronic viral infections and recognize many different surface antigens (e.g., CD45, CD175/Tn, CD3ε, CD4, chemokine receptors, sphingo-sine-1-phosphate receptor 1), and some of those antibodies regulate T cell-mediated inflammatory responses in vitro (Cappione et al. 2004; Daniel et al. 1989; Koren et al. 1992; Liao et al. 2009; Lobo et al. 2008; Muller et al. 1994;

Silvestris et al. 1989; Warren et al. 1988; Winfield et al. 1997) (see also Chap. 5 by Peter Lobo). It is thus quite possible that these IgM antibodies reactive with lymphocyte surface components engage FcμR in a *cis* interaction on the shared membrane surface, thereby modulating the functional activity of lymphocyte surface antigens or receptors by FcμR (see Fig. 4).

To explore this possibility, we compared Ca^{2+} mobilization upon ligation of lymphocyte surface antigen alone with co-ligation of lymphocyte surface antigen plus FcμR (Honjo et al. 2015). In the *first* experiment, an equal mixture of WT (i.e., FcμR-negative/GFP-negative) and FcμR-positive/GFP-positive Jurkat cells was preloaded with Ca^{2+} dye and then simultaneously stimulated by IgM anti-CD2 mAb. As shown in Fig. 3a, the rise in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) occurred significantly faster in $FcμR^+$ cells than in $FcμR^-$ WT cells when ligated with the anti-CD2 mAb. In contrast, the ionomycin-induced $[Ca^{2+}]_i$ increase occurred at the same time in both cell types. These findings suggest that the co-ligation of CD2 and FcμR induces a more rapid increase in $[Ca^{2+}]_i$, presumably release from the intracellular store, than does the ligation of CD2 alone. In the *second* experiment, Ca^{2+} mobilization by freshly prepared blood B cells was assessed following stimulation with a mitogenic IgM anti-κ mAb (END-5C1 clone) in the presence of IgG2bκ anti-FcμR mAb with blocking (HM7 clone) or non-blocking (HM3 clone) activity for IgM-ligand binding (Kubagawa et al. 2014b). IgM anti-κ mAb-induced Ca^{2+} mobilization was the same in the absence or presence of FcμR non-blocking mAb. In contrast, the FcμR-blocking mAb diminished the IgM anti-κ mAb-induced Ca^{2+} mobilization of blood B cells (Fig. 3b), suggesting that FcμR provides a stimulatory signals upon B cell receptor (BCR) cross-linkage with IgM mAbs. Collectively, FcμR expressed on B, T, and NK cells may thus have a potential to modulate the function of target antigens or receptors when they are recognized by natural or immune IgM antibodies, on the same cell surface (see the model shown in Fig. 4).

3.4 *Key Residues in the Transmembrane and Cytoplasmic Tail for FcμR Function*

In the case of pairs of activating and inhibitory receptors with highly homologous ectodomains, such as FcRs, killer cell Ig-Like receptors (KIRs), and PIRs, a general receptor structural rule has become appreciated. Namely, when the ligand-binding α chain has a short cytoplasmic tail with no signal-transmitting potential, then it contains a charged residue in the transmembrane segment that facilitates non-covalent association with another transmembrane protein containing immunoreceptor tyrosine-based activation motifs (ITAMs). This association allows for the transmission of activating signals to cells, as seen with FcγRI, FcγRIII, FcαR, FcεRI, KIR2DS or KIR3DS, and PIR-A (Blank et al. 1989; Clevers et al. 1988; Ernst et al. 1993; Kubagawa et al. 1999; Morton et al. 1995; Ravetch 1994).

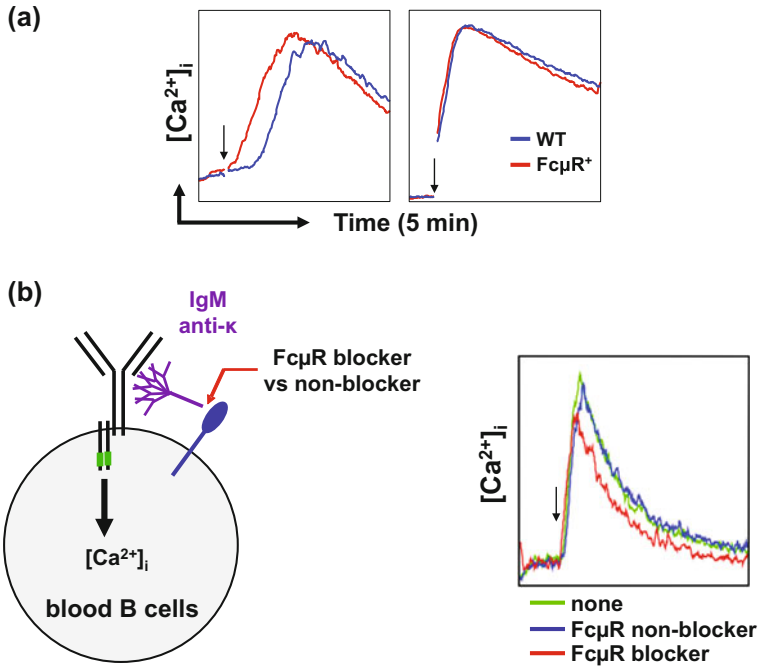

Fig. 3 Ca^{2+} mobilization by IgM mAbs against CD2 or Igκ. **a** An equal mixture of WT ($Fc\mu R^{-}$/GFP^{-}; *blue lines*) and $Fc\mu R^{+}$/GFP^{+} (*red lines*) Jurkat cells preloaded with the Ca^{2+} dye Indo-1/AM was stimulated with IgM anti-CD2 (373 clone) mAb at 10 μg/ml (*left*) or by 1 μM ionomycin (*right*) at the time points indicated by *arrows*. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was assessed by the 405/485 nm fluorescence ratio in each viable cell population using an LSR II flow cytometer. Note that the $[Ca^{2+}]_i$ rise occurs faster in co-ligation of CD2 and FcμR than ligation of CD2 alone and that the ionomycin-induced $[Ca^{2+}]_i$ rise occurs at the same time in both cell types. **b** The experimental design is depicted in a cartoon fashion (*left*). Fluo-4-loaded blood B cells were treated with IgM anti-human Igκ mAb (END-5C1; 10 μg/ml) in the absence (*green line*) or presence of IgG2b, FcμR-blocking (HM7 clone; *red line*) mAb, or FcμR-non-blocking (HM3 clone; *blue line*) mAb (100 μg/ml) at the time point indicated by an *arrow*. The $[Ca^{2+}]_i$ levels were assessed by fluorescence intensity during a 5-min period. Note that FcμR provides a stimulatory signal upon BCR cross-linkage with IgM mAbs

By contrast, when the ligand-binding α chain has a long cytoplasmic tail, then it contains a conventional hydrophobic transmembrane segment and ITIMs in the cytoplasmic tail (Vély and Vivier 1997). Upon phosphorylation of ITIMs after receptor ligation, the ITIM recruits either the polyphosphate inositol 5-phosphatase or the tyrosine phosphatases 1 and 2 to attenuate signaling, as seen with FcγRIIb, KIR2DL or KIR3DL, and PIR-B.

Unlike this general consensus, FcμR is unusual in having both features (Fig. 5): a charged His residue (H^{253}) in the transmembrane segment and a long cytoplasmic tail containing three conserved Tyr (Y^{315}, Y^{366}, Y^{385}) and five conserved Ser (S^{283}, S^{299}, S^{359}, S^{368}, S^{382}) residues, when comparing seven different species (Kubagawa et al.

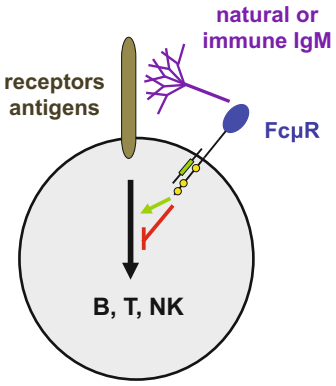

Fig. 4 Working hypothesis for FcμR function in humans. FcμR expressed on the plasma membrane of lymphocytes (B, T, and NK cells) consists of two subunits. The α chain (*blue badminton racket shape*) has ligand-binding activity via its Ig-like domain (head part) and signal-transducing ability via its conserved Tyr (three small *yellow circles*) and Ser (not shown) residues in the cytoplasmic tail. The other subunit termed the adaptor is non-covalently associated with the α chain and contains an ITAM (*green square*) signaling motif. (The molecular identity of this adaptor is presently unknown.) FcμR can either negatively or positively modulate the functional activity of lymphocyte surface protein/receptors (*brown sausage shape*) recognized by natural or immune IgM (*purple broom shape*) through the *cis* engagement

2009). The C-terminal Tyr matches the recently described Ig-tail tyrosine (ITT) motif (DYxN) (Engels et al. 2009; Engels and Wienands 2011), but the other two Tyr residues do not correspond to an ITAM ($D/Ex_2Yx_2L/Ix_{6\text{-}8}Yx_2V/I$), ITIM ($I/VxYx_2L/V$), or switch motif ($TxYx_2V/I$). Collectively, these characteristics suggest a dual signaling ability of FcμR: one from a potential as yet unidentified adaptor protein non-covalently associating with the FcμR via the H^{253} residue and the other from its own Tyr and/or Ser residues in the cytoplasmic tail (see Figs. 4 and 5).

To explore whether the aforementioned amino acid residues in FcμR are responsible for the receptor function, we made human FcμR cDNA constructs with point mutations (H253F, Y315F, Y366F, or Y385F) or a deletion of most of the cytoplasmic tail (A281–A390; ΔCy) and expressed them in Jurkat T cells. The results (Honjo et al. 2015) are summarized as follows (see Fig. 5). (i) Although non-mutated and mutated FcμR-bearing cells expressed comparable levels of cell surface FcμR as judged by receptor-specific mAbs, IgM-binding activity was significantly increased in the ΔCy mutant. [The ΔCy mutant lacks most of the cytoplasmic tail but includes an eight post-transmembrane basic amino acid-rich region (K^{273}–K^{280})]. Our subsequent data suggested that this enhancement was likely due to the formation of oligomeric FcμR due to its presumably mobile nature within the plasma membrane, rather than to the inside-out regulation of FcμR ligand binding by its cytoplasmic tail as seen in adhesion molecules (Kinashi 2005). (ii) The His^{253} residue was found to be important in the anchoring of FcμR in the plasma membrane. When examining the fate of IgM bound to FcμR by immunofluorescence microscopy, enhanced cap formation was clearly observed with the H253F mutant

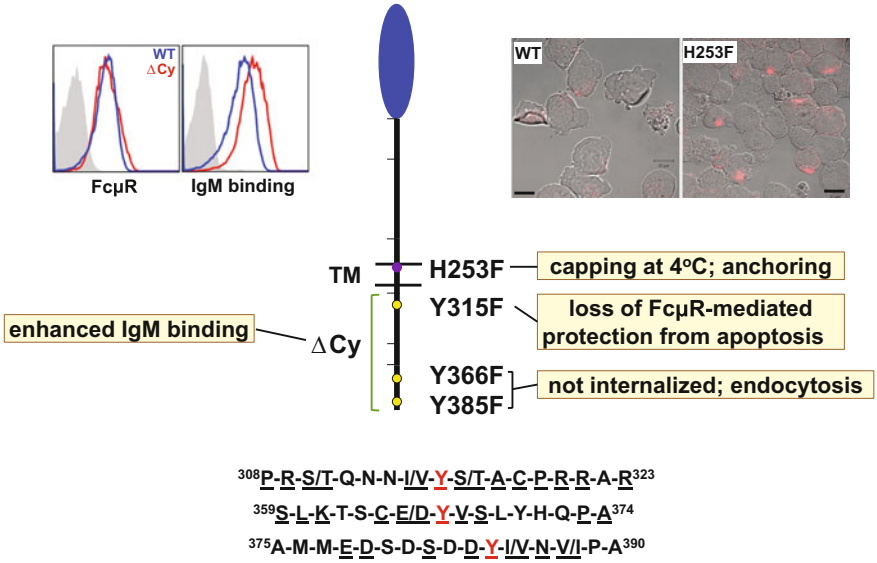

Fig. 5 Summary of FcμR mutational analysis. The human FcμR cDNA encodes a type I transmembrane protein that consists of a single V-set Ig-like domain (*blue oval shape*), an additional extracellular region (stalk) with no known domain structure, a transmembrane (TM) segment (between two *thick lines*) containing a charged His residue (*purple circle*), and a relatively long cytoplasmic tail containing three conserved Tyr residues (*yellow circles*). Point mutations are indicated, and the extent of the deletion of the cytoplasmic tail is shown by the green bracket. *Hatch marks* indicate exon boundaries in the *FCMR* gene. In representative flow cytometric profiles (*upper left*), cells stably expressing FcμR WT (*blue*) and ΔCy (*red*) were stained with biotin-labeled anti-FcμR (*open*) or isotype-matched control (*shaded*) mAb for cell surface expression of FcμR (*left panel*) and with biotin-IgM (*open*) or PBS (*shaded*) for ligand binding (*right panel*). Because profiles with control mAb or PBS were the same between FcμR WT and ΔCy cells, only one shaded profile is shown. Note the enhanced IgM binding by FcμR ΔCy cells as compared to FcμR WT cells, despite their equivalent levels of surface FcμR. In representative epi-fluorescence microscopic images (*upper right*), the FcμR WT (*left*) and H253F cells (*right*) were incubated with Alexa Fluor 555-IgM (without NaN_3) on ice, washed, and cytocentrifuged. Fluorescence images were combined with phase contrast cell images (scale bars = 10 μm). Altered phenotypes observed in mutant FcμR cells or potential function of the indicated residues are shown in the *yellow-filled boxes*. The unique sequences around three conserved Tyr residues are also shown with *underlines* indicating conserved amino acid residues

even at 4 °C as compared to the cells expressing non-mutant or other mutant FcμRs (except the ΔCy mutant), which exhibited a more broadly localized staining pattern. Notably, unlike other multi-chain FcRs, the FcμR H253F mutant was expressed on the surface of Jurkat cells without a potentially associated membrane protein. (iii) Consistent with the findings of Vire et al. (2011), the two C-terminal conserved Tyr residues were involved in receptor-mediated endocytosis. (iv) The FcμR-mediated protection from IgM anti-Fas mAb-induced apoptosis was significantly diminished in the Y315F and ΔCy mutants, as the frequency of apoptotic cells in these mutants was indistinguishable from those in $FcμR^-$ control cells.

This is of interest, given the unique sequence around the Y^{315} residue: ^{308}**P·R·S/T·**Q·N·N·**I/V·Y·S/T·A·C·P·R·R·**A· **R**^{323} (bold type indicates conserved amino acids). This does not match any known Tyr-based signaling motif. The mechanism of Toso-/FcμR-mediated protection from apoptosis was suggested to result from potentiation of the cellular FLIP [FADD-like IL-1ß-converting enzyme (FLICE)-like inhibitory protein], a master anti-apoptotic regulator (Hitoshi et al. 1998), or alternatively by prevention of internalization of Fas, an important step for apoptosis signaling (Vignaux et al. 1995; Yamauchi et al. 1996), owing to simultaneous cross-linkage of both Fas and FcμR with IgM Fas mAb (Murakami et al. 2012).

In addition to the mutational analysis, our previous findings indicated that ligation of FcμR with preformed IgM immune complexes induced the phosphorylation of both Tyr and Ser residues of the receptor. Intriguingly, the phosphorylated FcμR migrated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis faster than the unphosphorylated form, unlike most proteins, which usually run slower when phosphorylated (Kubagawa et al. 2009). It remains unclear whether the phosphorylation causes a global structural change of FcμR leading to increased mobility as seen, e.g., in CD45 on activated myeloid cells (Buzzi et al. 1992) or if proteolytic cleavage occurs in the cytoplasmic tail of FcμR after receptor ligation as observed in FcγRIIa on platelets (Gardiner et al. 2008). Ligation of FcμR on NK cells with IgM immune complexes was shown to lead to the phosphorylation of PLCγ2 and ERK1/2 (Murakami et al. 2012).

4 FcμR Deficiency in Mice

Fcmr KO mice have been independently generated by three different laboratories [K. H. Lee (Hannover Medical School, Hannover, Germany); H. Ohno (RIKEN, Yokohama, Japan); and T. W. Mak (Princes Margaret Cancer Center/University Health Network, Toronto, Canada)] and have been characterized by five different groups of investigators (Brenner et al. 2014; Choi et al. 2013; Honjo et al. 2014; Honjo et al. 2012a; Lang et al. 2013a; Nguyen et al. 2011; Ouchida et al. 2012). Clear differences in the reported phenotypes exist among these mice that are well summarized in a recent review article (Wang et al. 2016). While the basis for these differences requires further investigation, they could in part be attributed to: (i) different strategies for gene targeting (i.e., deletion of exon 4-7, exon 2-4 *versus* exon 2-8 and/or the absence *versus* presence of the *Neo* gene in the mouse genome); (ii) embryonic stem (ES) cells of C57BL/6 *versus* 129 origin as well as the extent of the 129 mouse-origin DNA around the *Fcmr* gene remaining after backcrossing onto C57BL/6. [In this regard, the region closely flanking the targeted gene, called the passenger genome, remains of donor origin and typically contains mutations called passenger mutations. Annotating these passenger mutations to the reported genetically modified congenic mice generated using 129-ES cells revealed that nearly all these mice possess multiple passenger mutations potentially influencing

the phenotypic outcome (Vanden Berghe et al. 2015)]; (iii) investigators' ideas regarding the cellular distribution of FcμR/Toso in B cells *versus* myeloid and T cells and its function as an IgM Fc-binding protein *versus* an anti-apoptotic protein; and/or (iv) other factors (e.g., age of the mice, environments including intestinal microbiota or reagents used). Nevertheless, the abnormal phenotypes commonly observed in *Fcmr* KO mice are as follows: (i) alterations in B-lineage cell subpopulations; (ii) dysregulation of humoral immune responses; and (iii) predisposition to autoantibody production (Choi et al. 2013; Honjo et al. 2012a; Ouchida et al. 2012). Notably, many of the abnormalities seen in *Fcmr* KO mice mirror those observed in mice deficient in IgM secretion, suggesting that FcμR is a critical sensor of secreted IgM. In *Fcmr* KO mice on autoimmune backgrounds, FcμR was shown to play important regulatory roles in: (i) the autoantibody production; (ii) the differentiation of MZ B cells into plasma cells; and (iii) the formation of Mott cells, aberrant plasma cells with intracytoplasmic Ig inclusion bodies (Honjo et al. 2014).

Apart from these mutant mice, the laboratory of N. Baumgarth has recently generated a mouse strain in which the second stalk region exon (exon 4) of *Fcmr* is flanked by loxP sites allowing the B cell-specific deletion of *Fcmr* by crossing with *Cre-Cd19* transgenic mice (Nguyen et al. 2017). Several interesting findings were obtained by comparative analysis between mice with such a B cell-specific FcμR deletion and control mice. FcμR directly interacts with membrane-bound IgM BCR in the *trans*-Golgi network of BM immature B cells, thereby regulating the surface expression of BCR and eventually resulting in limiting tonic BCR signaling. B cell-specific FcμR deficiency results in dysregulated spontaneous activation and differentiation of B-1 and B-2 cells and development of a lympho-proliferative disorder. This suggests that FcμR constrains BCR expression to regulate the fundamental homeostasis and biology of B cells. [It is worth noting, however, that deletion of exon 4 could still allow production of soluble form of FcμR by the mutant B cells via a reading frame shift in exon 5 (TM) (see hatch marks in Fig. 5). Such a soluble FcμR could have unexpected consequences on B cell function. In fact, we have identified another splice variant in CLL patients that results from the direct splicing of exon 4 to exon 6 (1st cytoplasmic), skipping exon 5. This splice event results in a reading frame shift in exon 6 and generates a novel 70-amino acid hydrophilic, carboxyl-terminal tail and the resultant soluble FcμR protein is clearly elevated in many patient's sera as determined by enzyme-linked immunosorbent assays (Li et al. 2011)]. Contrary to the above FcμR-mediated suppression of BCR signaling, FcμR has also been shown to enhance survival of mature B cells upon BCR cross-linkage ex vivo with $F(ab')_{2\gamma}$ anti-μ antibodies by activation of the non-canonical NF-κB pathway, but not upon CD40 ligation or LPS stimulation (Ouchida et al. 2015). Thus, FcμR may have a potential to transmit both positive and negative signals to cells.

Another difference in *Fcmr* KO mice between T. W. Mak and H. Ohno is that splenic B cells from our (the latter) *Fcmr* KO mice produce significantly less IL-10, but comparable amounts of IL-6, ex vivo upon stimulation with *Salmonella* bacteria or with ligands for Toll-like receptor 4 (TLR4), TLR7, or TLR9 (Fig. 6). Since B cell-derived IL-10 has been implicated as an important negative regulator of

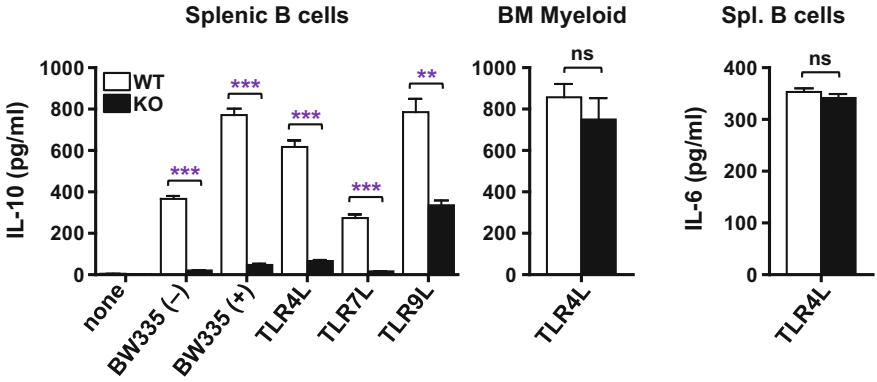

Fig. 6 Diminished IL-10 production by FcμR-deficient B cells. Splenic B (*left* and *right*) and bone marrow myeloid (*middle*) cells (4×10^5 cells) from sex and age-matched *Fcmr*-deficient (*black* columns) or littermate control (*white* columns) mice were cultured for 2 days in the absence (none) or presence of heat-killed, non-opsonized (−) or serum-opsonized (+) BW335, an LT2 strain of *Salmonella enterica* serovar Typhimurium (4×10^5 cfu), or the indicated TLR ligands (TLRL): LPS (10 μg/ml) for TLR4, Gardiquimod (1 μg/ml) for TLR7, and ODN1826 (2 μM) for TLR9. The concentration (pg/ml) of IL-10 (*left* and *middle*) and IL-6 (*right*) in the culture supernatants was assessed by ELISA in triplicate. The symbols ** and *** and "ns" indicate $p < 0.01$, $p < 0.001$, and "not significant," respectively, as assessed by Student's *t* test. *Note* (i) diminished production of IL-10, but not IL-6, by FcμR-deficient B cells and (ii) comparable production of IL-10 by marrow myeloid cells from both groups of mice. Identical results were obtained seven times for IL-10 and twice for IL-6, and representative experiments are shown

MOG-induced EAE (Fillatreau 2015), we thought that our *Fcmr* KO mice would be more *susceptible* than WT to MOG-induced EAE. However, the results reported by Brenner et al. (2014) suggest that this is not the case. Their *Fcmr/Toso* KO mice are *resistant* to MOG-induced EAE, because their *Fcmr/Toso* KO DCs are immature and tolerogenic and weak stimulators of inflammatory T cell responses. *Fcmr/Toso* KO Th17-polarizing cells secrete significantly less IL-17 and IL-10 than WT control mice (Gaublomme et al. 2015). Intriguingly, passive administration of a recombinant, soluble human FcμR–IgG fusion protein ameliorates MOG-induced EAE in WT mice (Brenner et al. 2014). The basis for this discrepancy (susceptible *versus* resistant) remains unclear at the moment, because we have never examined the susceptibility of our *Fcmr* KO mice to MOG-induced EAE and Brenner et al. have never assessed IL-10 production by B cells in their *Fcmr/Toso* KO mice. A side-by-side analysis of these two different strains of *Fcmr/Toso* KO mice would facilitate the resolution of these conflicting results, and it is highly likely that this discrepancy results from different strategies for gene targeting (deletion of exon 2-4 without *Neo* for ours *versus* deletion of exon 2-8 with remaining of *Neo* in the mouse genome for Brenner et al.).

5 Epilogue

FcRs for switched Igs are expressed by many different cell types, including myeloid cells, and are considered to be central mediators coupling innate and adaptive immune responses. Rewardingly, much of the knowledge gained from studies of these FcRs has been translated into clinical fields. On the other hand, the long elusive IgM FcR was finally identified eight years ago by functional cloning. However, since the cloned FcμR cDNA was identical to the cDNA-encoding TOSO or Fas apoptosis inhibitory molecule 3 (FAIM3), which was also previously identified by functional cloning as a potent inhibitor for Fas-mediated apoptosis, there have been spirited debates regarding the real function of this receptor, Fcμ-binding *versus* anti-apoptotic activity. Notably, there is now a general consensus that TOSO/FAIM3 is an authentic Fcμ-binding protein and not a Fas inhibitory protein per se (Kubagawa et al. 2015). Several interesting findings about the FcμR have recently been reported: FcμR binds more efficiently to the Fc portion of IgM antibody when it is attached to a membrane component via its Fab region on the same cell surface (i.e., *cis* interaction) than to the Fc portion of IgM in solution (*trans* interaction). FcμR directly interacts with membrane-bound IgM BCR in the *trans*-Golgi network of BM immature B cells, thereby regulating the surface expression of IgM BCR and eventually resulting in limiting tonic BCR signaling. FcμR can also regulate the differentiation of MZ B and B1 cells. By contrast, FcμR may enhance survival of mature B cells upon BCR cross-linkage via activation of the non-canonical NF-κB pathway. Immunotherapy targeting the FcμR is now designed for CLL cells. Many conflicting results still exist, but we hope that this short article may help to resolve these existing puzzles and will open new avenues of investigation.

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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.)

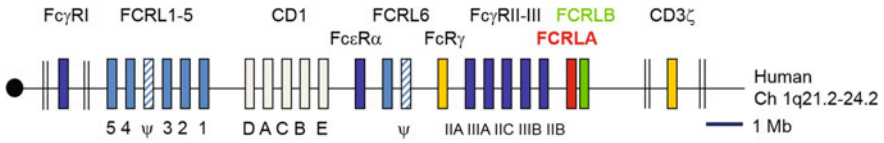

Fig. 1 *FCRLA* genomic landscape. *FCRLA* and *FCRLB* are located in the 1q21.2–1q24.2 interval on chromosome 1; *FCRLB* is located ~7 kb telomeric of *FCRLA*. This region is enriched for FCR and FCR-related genes, including the classic *FcγRI-III* genes, the ligand-binding *FcεRα* chain of FcεRI, *FCL1*-5, *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),

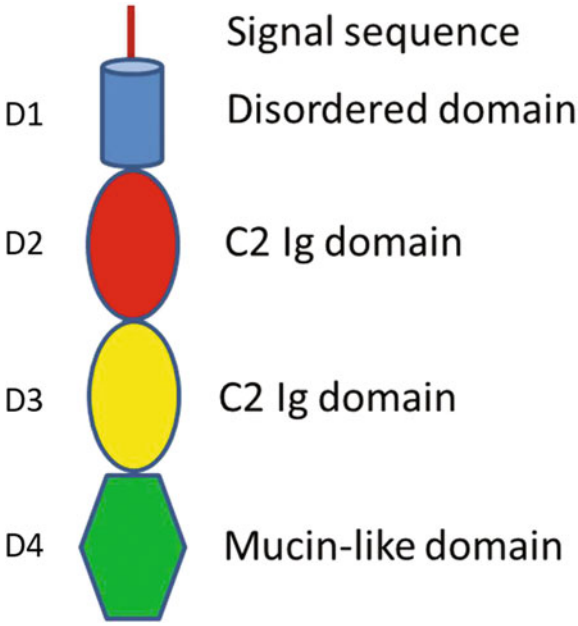

Fig. 2 Model of FCRLA protein structure. The signal peptide and features of the four domains are depicted. D1 is predicted to be disordered, D2 and D3 are authentic C2 Ig-like domains, and D4 is a bipartite domain with an N-terminal mucin-like region, rich in proline, serine, and threonine residues,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

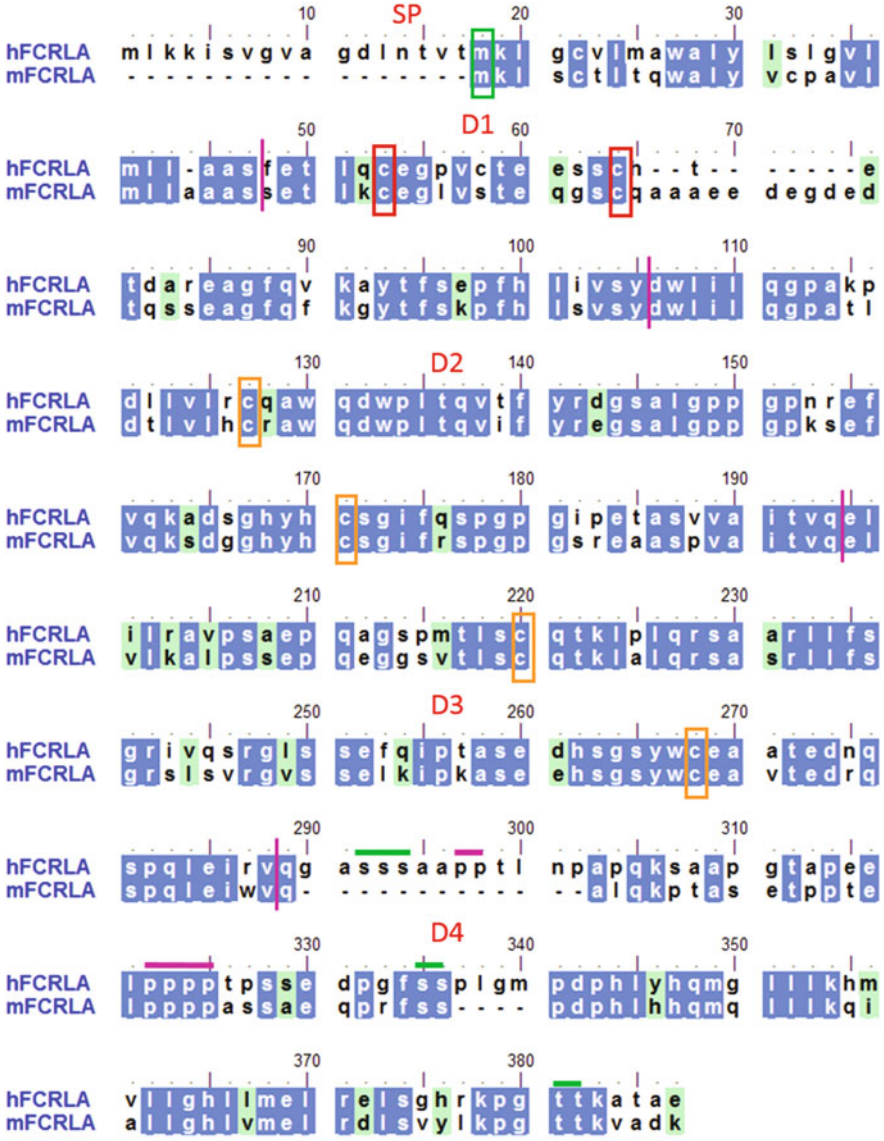

Fig. 3 Alignment of human and mouse FCRLA protein sequences. Amino acid sequence identity is indicated in *blue*, and amino acids with similar physiochemical features, i.e., charged, polar, and hydrophobic, are in *light green*, absent sequence by a *dot*. Domain boundaries are indicated by a *vertical 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).

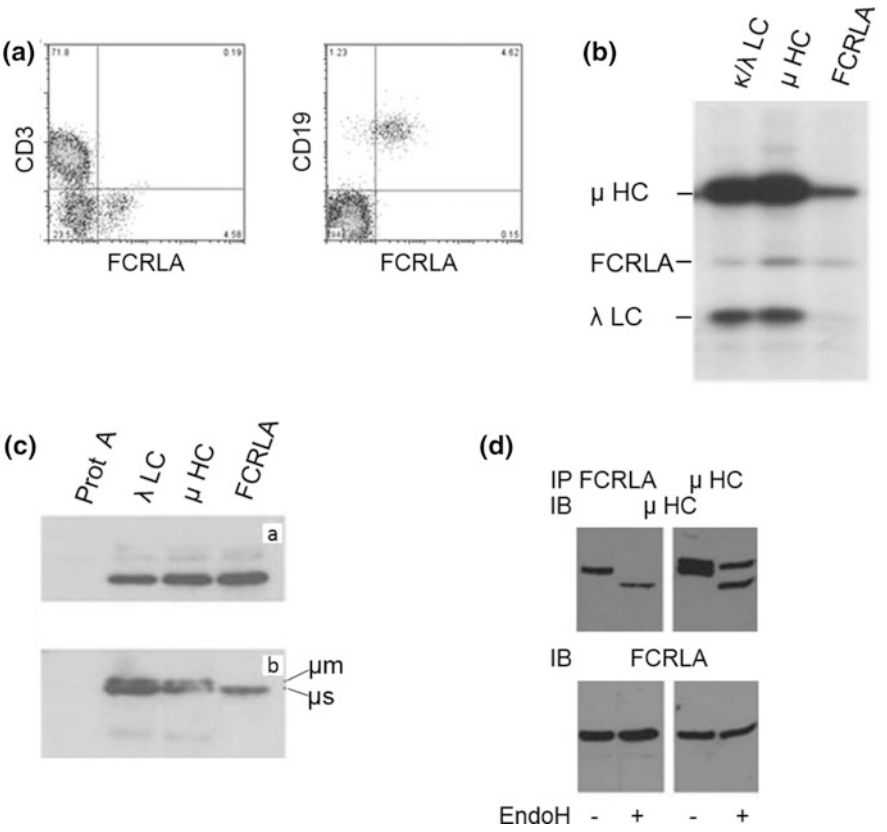

Fig. 4 FCRLA expression by normal B cells and its preferential association with μs in the Ramos B cell line. **a** Human blood mononuclear cells were stained for cell surface CD3 to identify T cells (*left panel*) and CD19 to identify B cells (*right panel*) and then fixed, permeabilized, and stained 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 transmembrane 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].

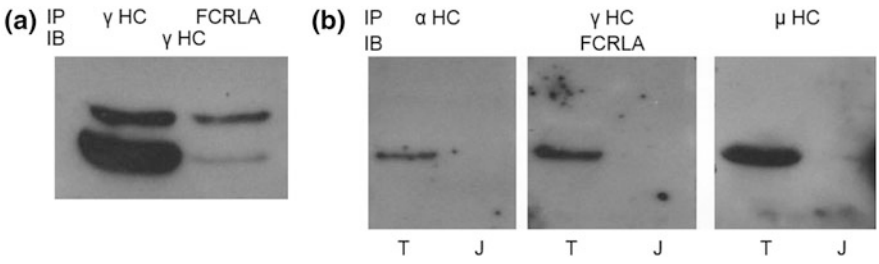

Fig. 5 FCRLA associates with IgG in the IM9 B cell line and with IgM, IgG, and IgA in tonsillar B cells. **a** NP-40 cell lysates of the IM9 (γκ) B cell line were immunoprecipitated (IP) with anti-γ HC or anti-FCRLA antibodies, resolved by SDS-PAGE under reducing conditions and then analyzed by Western 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

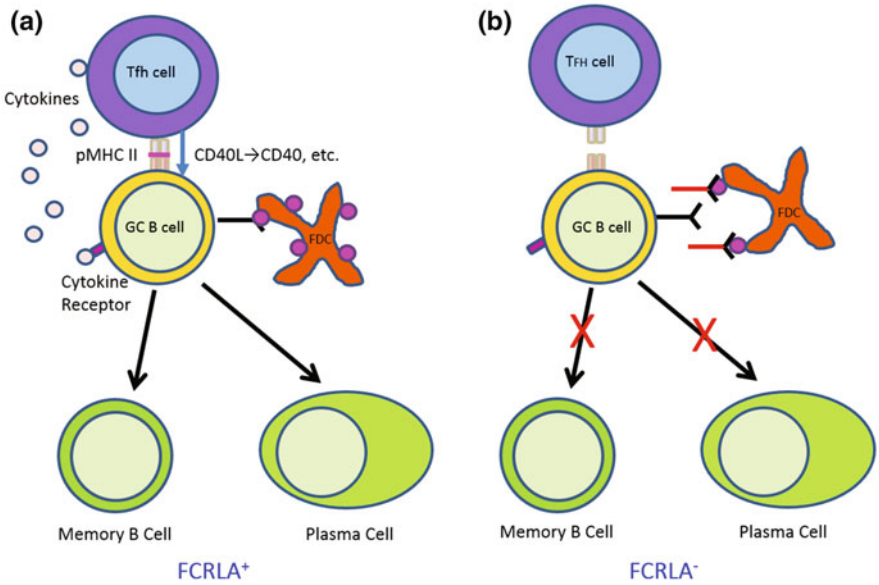

Fig. 6 Model for the function of FCRLA in the germinal center. **a** Germinal center (GC) B cells undergo somatic hypermutation in the GC dark zone, and then, cells with a high-affinity B cell receptor (BCR) are positively selected in the GC light zone, where follicular dendritic cells display antigen 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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Specific IgM and Regulation of Antibody Responses

Anna Sörman and Birgitta Heyman

Abstract Specific IgM, administered together with the antigen it recognizes, enhances primary antibody responses, formation of germinal centers, and priming for secondary antibody responses. The response to all epitopes on the antigen to which IgM binds is usually enhanced. IgM preferentially enhances responses to large antigens such as erythrocytes, malaria parasites, and keyhole limpet hemocyanine. In order for an effect to be seen, antigens must be administered in suboptimal concentrations and in close temporal relationship to the IgM. Enhancement is dependent on the ability of IgM to activate complement, but the lytic pathway is not required. Enhancement does not take place in mice lacking complement receptors 1 and 2 (CR1/2) suggesting that the role of IgM is to generate C3 split products, i.e., the ligands for CR1/2. In mice, these receptors are expressed on follicular dendritic cells (FDCs) and B cells. Optimal IgM-mediated enhancement requires that both cell types express CR1/2, but intermediate enhancement is seen when only FDCs express the receptors and low enhancement when only B cells express them. These observations imply that IgM-mediated enhancement works through several, non-mutually exclusive, pathways. Marginal zone B cells can transport IgM-antigen-complement complexes, bound to CR1/2, from the marginal zone and deposit them onto FDCs. In addition, co-crosslinking of the BCR and the CR2/CD19/CD81 co-receptor complex may enhance signaling to specific B cells, a mechanism likely to be involved in induction of early extrafollicular antibody responses.

Contents

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1 Introduction

When antibodies are passively administered together with their specific antigen, they can dramatically change the antibody response towards the antigen. The response can be completely suppressed or enhanced by several hundred-fold. This phenomenon is called antibody-mediated feedback regulation. In experimental situations, the regulating antibodies are usually given intravenously within a few hours of the antigen, but feedback regulation also works in a more natural setting with endogenously produced antibodies as regulators. In early studies, reviewed by Uhr and Möller (1968), the source of the regulating antibodies was usually serum from immunized animals, and therefore the effect of individual antibody classes could not be determined. With the arrival of antibody separation techniques and the hybridoma technology, the immunoregulatory effects of different antibody isotypes has been extensively investigated [reviewed in (Heyman 2000; Hjelm et al. 2006; Sörman et al. 2014)]. In studies of antibody-mediated feedback regulation, both antibodies and antigen are administered in physiological salt solutions, i.e., without adjuvants. Although there are exceptions, the regulatory effects are generally antigen but not epitope-specific. This suggests that the response to the entire antigen, captured by the antibody, is affected, regardless of to which epitope the regulating antibody binds.

1.1 IgG-Mediated Feedback Suppression

IgG, administered together with erythrocytes, can completely suppress the antibody response. This is utilized in the clinic to prevent Rh-negative women carrying Rh-positive fetuses from becoming immunized against fetal erythrocytes acquired via transplacental hemorrhage. Maternal IgG crosses the placenta and can damage the erythrocytes of the fetus or newborn. A small dose of preformed IgG anti-Rh, given to the mother during pregnancy or immediately after delivery, prevents hemolytic disease of the newborn (Clarke et al. 1963; Bowman 1988). The mechanism behind IgG-mediated suppression is still not understood. One possibility is that IgG masks the antigen and prevents naïve B cells from binding to it. Another, not mutually exclusive, possibility is that erythrocytes covered with IgG will be rapidly eliminated from the circulation and therefore be unable to stimulate an immune response. Complement activation is not required for IgG-mediated suppression (Heyman et al. 1988b; Bergström and Heyman 2015) and IgG suppresses equally well in the absence of all known Fc-receptors for IgG (Karlsson et al. 1999, 2001; Bernardo et al. 2015; Bergström and Heyman 2015).

1.2 IgG-Mediated Feedback Enhancement

When IgG is administered with soluble protein antigens, it will enhance antibody responses. In fact, the same monoclonal IgG anti-TNP which suppresses responses to SRBC-TNP can enhance responses to KLH-TNP (Enriquez-Rincon and Klaus 1984; Wiersma et al. 1989), illustrating the important role of the type of antigen. Enhancement by the murine subclasses IgG1, IgG2a, and IgG2b requires Fc-receptors for IgG (Wernersson et al. 1999) whereas IgG3 largely operates via complement (Diaz de Ståhl et al. 2003; Zhang et al. 2014). The most likely mechanism for enhancement by the Fc-receptor-dependent subclasses is increased uptake of IgG-antigen by dendritic cells, followed by increased T helper cell induction (Getahun et al. 2004; de Jong et al. 2006; Hamano et al. 2000) whereas IgG3 probably acts by increasing the delivery of antigen to B cell follicles and FDCs (Zhang et al. 2014).

1.3 IgE-Mediated Feedback Enhancement

IgE, administered with soluble protein antigens, will enhance antibody and T helper cell responses (Getahun et al. 2005). This process requires the low affinity receptor

for IgE, CD23, and the receptor must be expressed on B cells. The mechanism appears to be that IgE-antigen is captured by recirculating $CD23^+$ B cells which rapidly transport the antigen to B cell follicles (Hjelm 2008). In the spleen, $CD11c^+$ dendritic cells somehow acquire the antigen and present it to T cells which subsequently help B cells to produce antibodies (Henningsson et al. 2011).

1.4 IgM-Mediated Feedback Enhancement

In 1968, Niels Jerne and Claudia Henry published a paper where they dissected the opposing immunoregulatory effects of IgG and IgM on antibody responses to sheep erythrocytes (SRBC) (Henry and Jerne 1968). SRBC-specific IgG (then denoted 7S antigen receptors), SRBC-specific IgM (19S antigen receptors) or a mixture of the two antibodies were administered intravenously to mice. Within one hour, the mice received an intravenous dose of SRBC and a few days later the active IgM-response was measured as hemolytic plaque-forming cells per spleen. With this assay, single plasma cells secreting IgM anti-SRBC can be measured: one hemolytic plaque represents one plasma cell (Jerne and Nordin 1963). Comparisons between groups given SRBC alone and groups given IgM prior to SRBC showed that IgM enhanced the response, provided suboptimal doses of antigen were used. In contrast, IgG suppressed more than 99% of the response and, interestingly, a mixture of IgG and IgM had an intermediate effect.

Thus, by separating antibodies into IgM and IgG instead of using whole serum, Henry and Jerne found that different isotypes could have different regulatory effects (Henry and Jerne 1968). Their paper was the start of modern studies of antibody feedback regulation and was also the foundation for B.H.'s Ph.D. studies in Hans Wigzell's laboratory in Uppsala in the 1980s. At that time, Niels Jerne's network theory, based on the notion that antibodies and B cell receptors (BCRs) also constitute antigens, was very much discussed (Jerne 1974). The epitopes of the antigen-binding regions of a certain antibody or BCR is defined as its idiotype. Since our antibody repertoire can recognize all antigens in the universe, an idiotype will be recognized by so called anti-idiotypic antibodies. In the 1970s and 1980s such idiotype/anti-idiotype interactions were thought to be of major importance in regulation of the immune response, and we set out to investigate whether IgM-mediated enhancement could be explained by network regulation. Whereas another laboratory reported that an anti-SRBC response could be generated in mice given IgM anti-SRBC without antigen (Forni et al. 1980), we failed to find evidence of anti-idiotypic regulation by IgM (Heyman et al. 1982).

Today, the most favored explanation for how specific IgM can feedback-enhance antibody responses is that IgM, binding to an antigen, rapidly activates complement

thus forming an IgM-antigen-complement complex. This complex can bind to complement receptors 1 and 2 (CR1/2) which are expressed on B cells and FDCs and are known to play an important role in the generation of robust antibody responses [reviewed in (Sörman et al. 2014)]. Binding of immune complexes to these receptors can positively influence the antibody response in at least three different ways, and the relative importance of these is currently not understood. First, co-crosslinking of CR2 and BCR lowers the threshold for B cell activation and an immune complex could serve as the crosslinker (Carter et al. 1988). Second, marginal zone (MZ) B cells shuttle between the MZ and the B cell follicles (Cinamon et al. 2008; Arnon et al. 2013) and because they express high levels of CR1/2, they can transport complement-opsonized immune complexes into the follicle (Youd et al. 2002; Ferguson et al. 2004; Cinamon et al. 2008). Third, the deposition of immune complexes onto FDCs is most likely facilitated by their expression of CR1/2.

Below we will review the experimental observations leading to the hypothesis that antigen-specific IgM enhances antibody responses by activating complement and forming IgM-antigen-complement complexes which bind to CR1/2.

2 Basic Parameters of IgM-Mediated Enhancement

2.1 Antigens

IgM has generally been reported to enhance responses to large antigens such as erythrocytes (Henry and Jerne 1968; Dennert 1971; Wason 1973; Schrader 1973; Heyman et al. 1982; Whited Collisson et al. 1983; Heyman et al. 1985), malaria parasites (Harte et al. 1983), keyhole limpet hemocyanine (KLH) (Ding et al. 2013) and haptens coupled to KLH (Enriquez-Rincon and Klaus 1984; Coulie and Van Snick 1985; Youd et al. 2002) but occasionally IgM enhances responses to small proteins such as ovalbumin (OVA) (Whited Collisson et al. 1983). Notably, also human IgM enhances antibody responses as discovered during studies of Rhesus prophylaxis. Here, IgG anti-Rh suppressed and IgM anti-Rh enhanced the Rhesus-specific antibody responses (Clarke et al. 1963). IgM can only enhance responses to suboptimal doses of antigen (Henry and Jerne 1968; Powell et al. 1982; Lehner et al. 1983).

2.2 The IgM Molecule and Mode of Administration

Not only polyclonal, but also monoclonal IgM antibodies (Heyman et al. 1982; Powell et al. 1982; Harte et al. 1983; Coulie and Van Snick 1985; Heyman et al. 1988a; Youd et al. 2002) can enhance antibody responses. This finding is difficult to

reconcile with idiotypic network regulation because a monoclonal IgM antibody cannot be expected to bind to more than a few BCRs and stimulation of only a few B cells would go unnoticed. A wide range of IgM concentrations are able to enhance (Dennert 1971; Heyman et al. 1982) but too high concentrations may lead to suppression (Pearlman 1967; Möller and Wigzell 1965), probably owing to epitope masking. IgM can have dual effects also in other situations. IgM is generally administered within 2 hours of the antigen but delaying IgM-administration until 1–2 days after antigen may result in suppression instead of enhancement (Wason 1973). Moreover, IgM that enhances in vivo can have a suppressive effect in vitro (Schrader 1973) and to our knowledge there are no reports showing that IgM-mediated enhancement works in vitro.

The structural requirements on the IgM molecule for ability to enhance has been scarcely studied. As will be discussed in detail in Sect. 4.1, monoclonal as well as polyclonal IgM with a point mutation in the constant part of the μ heavy chain leading to inability to bind C1q, is unable to feedback enhance antibody responses (Heyman et al. 1988a; Ding et al. 2013). Likewise, monomeric IgM, which cannot activate complement, is unable to enhance (Youd et al. 2002). Hexameric IgM is a more efficient complement activator than pentameric IgM (Davis et al. 1988), but feedback regulation by hexameric IgM has not been studied.

2.3 *Primary Antibody Responses*

The vast majority of studies demonstrating IgM-mediated enhancement have analyzed primary IgM responses (Henry and Jerne 1968; Dennert 1971; Wason 1973; Schrader 1973; Heyman et al. 1982; Whited Collisson et al. 1983; Heyman and Wigzell 1985) using Jerne's direct hemolytic plaque-forming cell assay (Jerne and Nordin 1963). However, IgM enhances also primary IgG responses measured either as indirect plaque-forming cells or serum IgG (Heyman et al. 1982; Heyman and Wigzell 1985; Applequist et al. 2000; Rutemark et al. 2012; Ding et al. 2013). All IgG subclasses (Heyman et al. 1985) as well as IgE (Strannegård and Belin 1971) can be enhanced, and the IgG levels remain high during several months (Heyman and Wigzell 1985).

The magnitude of the responses to SRBC and KLH administered together with specific IgM, is similar to that seen with a 10-fold higher dose of antigen alone although the responses to the higher doses of antigen alone peak earlier (Henry and Jerne 1968; Youd et al. 2002).

2.4 *Priming for Memory Responses*

Mice primed with IgM + antigen and boosted with antigen alone, have an enhanced secondary response (Heyman and Wigzell 1985; Youd et al. 2002). This was most

clearly demonstrated in adoptive transfer experiments where spleen cells from the primed mice were transferred to naïve recipients which were "boosted" with antigen (Heyman and Wigzell 1985). Boosting the same mice that had been primed, sometimes concealed the enhanced memory owing to feedback suppression mediated by the higher levels of endogenous IgG anti-SRBC induced in IgM + SRBC-primed mice (Heyman and Wigzell 1985).

2.5 Avidity of the Enhanced Response

The avidity of the response after administration of IgM and antigen has been reported to be either unchanged (Whited Collisson et al. 1984) or enhanced (Corley et al. 2005). In an experimental system where primed mice were challenged with IgM-immune complexes, it was suggested that feedback regulation by endogenous IgM drove affinity maturation (Zhang et al. 2013). When the injected IgM, forming the immune complexes, had a low affinity the endogenous affinity maturation could proceed. When high affinity IgM was injected, the affinity maturation was impaired. These observations are probably due to IgM antibodies masking the antigen that had bound to FDCs.

2.6 Germinal Center Responses

Specific IgM administered together with KLH or SRBC, promotes the formation of germinal centers (Ferguson et al. 2004; Ding et al. 2013). Since germinal centers are crucial for development of memory B cells and longlived plasma cells and for affinity maturation, these results agree well with the observations that these parameters are all enhanced by IgM (Heyman and Wigzell 1985; Youd et al. 2002; Ferguson et al. 2004; Corley et al. 2005).

2.7 Specificity of the Enhanced Antibody Response

IgM-mediated enhancement is antigen specific, but usually IgM specific for one epitope will lead to enhancement also of responses to other epitopes present on the same antigen particle (Henry and Jerne 1968; Heyman et al. 1982; Coulie and Van Snick 1985; Wason 1973; Whited Collisson et al. 1983; Heyman et al. 1988a; Ding

et al. 2013). For example, mice immunized with IgM anti-SRBC together with SRBC conjugated to OVA (SRBC-OVA) will have an enhanced antibody response both to SRBC and OVA (Ding et al. 2013). Similarly to the observations that monoclonal IgM antibodies are efficient enhancers, non-epitope-specific enhancement is hard to reconcile with network regulation and suggest involvement of Fc-mediated functions.

2.8 T Cells and IgM-Mediated Enhancement

IgM cannot enhance responses to erythrocytes in mice lacking T cells and thus cannot compensate for T cell help (Whited Collisson et al. 1983; Powell et al. 1982; Lehner et al. 1983; Coutinho and Forni 1981). Very few studies have addressed the question of whether IgM can enhance T cell responses in parallel with antibody responses. In a report using malaria-specific monoclonal IgM, an enhanced induction of T helper cells was seen (Harte et al. 1983). On the other hand, IgM had no effect on proliferation of OVA-specific T cells in mice immunized with IgM anti-SRBC + OVA-SRBC although the antibody response was efficiently enhanced (Ding et al. 2013). To resolve this question, further experiments are required.

3 Complement in Antibody Responses to Uncomplexed Antigen

A still quite unknown function of complement is to facilitate humoral immune responses [reviewed in (Sörman et al. 2014; Carroll 2008)]. This was first demonstrated by the poor antibody responses to SRBC in mice depleted of C3 by treatment with cobra venom factor (Pepys 1974). Subsequently, it was shown that animals and humans lacking C1, C2, C3, C4, or the complement receptors 1 and 2 (CR1/2 or CD35/CD21) have severely impaired antibody responses. The main ligands for CR1/2 are split products of factor C3 of the complement cascade and the phenotype of mice lacking C1, C3, or C4 closely resembles that of mice lacking CR1/2. Therefore, it is generally assumed that the impaired antibody responses seen in C1-, C3-, or C4-deficient animals are explained by their failure to generate ligands for CR1/2. In other words, the effects of these complement factors on antibody responses would all be mediated via CR1/2. In mice, these two receptors are splice variants from the same gene, Cr2, and are expressed on B cells and FDCs. Recent data suggest that FDCs primarily express CR1 while B cells primarily express CR2 (Donius et al. 2013).

4 Complement in Antibody Responses to IgM-Antigen Complexes

4.1 Complement Activation by IgM

IgM is a very efficient activator of the classical pathway and one single IgM molecule can induce lysis of an erythrocyte (Borsos and Rapp 1965). IgM in solution does not bind C1 and it is thought that binding to a multivalent antigen is required to induce the conformation changes that expose the C1 binding sites (Feinstein and Munn 1969; Czajkowsky and Shao 2009).

Regarding antibody-mediated feedback regulation, it is particularly interesting that lack of C1q is associated with severe defects in antibody production against SRBC (Cutler et al. 1998; Rutemark et al. 2011). C1q is required for activation of the classical, but not the alternative or lectin, pathways. In the late 1980s, Marc Shulman generated a series of mutant monoclonal IgM antibodies, one of which (Mutant13) had lost its ability to bind C1q and to initiate hemolysis owing to a point mutation in the μ heavy chain (Shulman et al. 1987). We were interested to find out whether the ability of IgM to enhance antibody responses was related to its ability to activate complement. Therefore, mice were immunized mice with TNP-specific Mutant13 or wild-type IgM together with SRBC-TNP or with antigen alone. The results showed that only wildtype IgM was able to enhance antibody responses (Heyman et al. 1988a). Recently, these findings were confirmed using non-complement activating polyclonal IgM obtained from Cμ13 knock-in mice, which have the same point mutation as Mutant13 (see below) (Ding et al. 2013) (Fig. 1). The importance of complement for IgM-mediated enhancement is further supported by the observations that IgM cannot enhance in C3-depleted mice

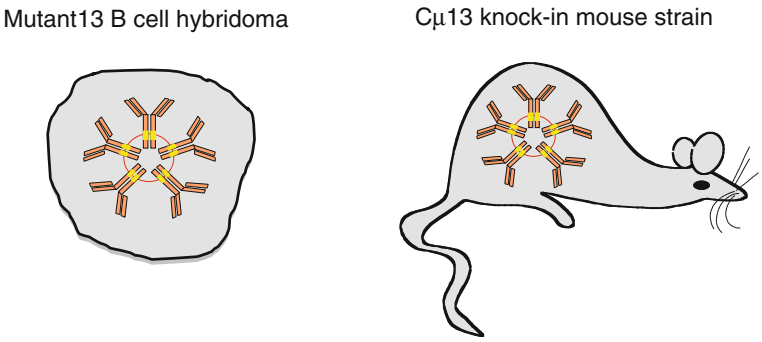


Fig. 1 Mutant IgM from B cell hybridomas and from a knock-in mouse strain. The monoclonal IgM anti-TNP, produced by the B cell hybridoma Mutant13, has a serine - > proline mutation in position 436 of the IgM heavy chain leading to inability to bind C1q and to induce hemolysis (Shulman et al. 1987). The Cμ13 knock-in mouse strain has the same mutation in its genome and all IgM antibodies produced by these mice are unable to bind C1q and to activate the classical pathway (Rutemark et al. 2011)

(Heyman et al. 1988a) and that monomeric IgM, which cannot activate complement, lost its enhancing capacity (Youd et al. 2002). The involvement of complement raises the question whether IgM-mediated lysis of erythrocytes may render them more immunogenic and that this is the mechanism behind the enhancing effect. Two experimental findings argue against this idea. First, response to the protein KLH, which cannot be lysed, is enhanced by IgM (Ding et al. 2013; Youd et al. 2002; Ferguson et al. 2004). Second, IgM enhances responses to SRBC in AKR mice which lack C5 and thereby the lytic pathway (Heyman et al. 1988a).

The complement dependence probably explains why enhancement is generally limited to large antigens such as erythrocytes, malaria parasites, and KLH which are large enough to allow IgM to bind with all five arms and assume the conformation change required for C1 binding (Feinstein and Munn 1969; Czajkowsky and Shao 2009).

4.2 Complement Receptors 1 and 2, CR1/2, in Antibody Responses to IgM-Antigen Complexes

The complement dependence of IgM-mediated enhancement led us to investigate whether CR1/2 were involved in this feedback circle. At that time, there were no CR1/2 knockout mice ($Cr2^{-/-}$) available, but Taroh Kinoshita had developed a monoclonal antibody, 7G6, which efficiently blocked the ligand-binding sites of both CR1 and CR2 (Kinoshita et al. 1988). Initially, we pretreated mice with 7G6 and then immunized them with IgM anti-SRBC + SRBC + horse erythrocytes (HRBC), or with SRBC + HRBC alone. HRBC was intended as a specificity control, establishing that IgM enhanced only the SRBC response. To our surprise, all mice treated with 7G6 had extremely low antibody responses both to SRBC and HRBC. The conclusion was that CR1/2 were required for all types of antibody responses, and not only for those enhanced by IgM, and led to the first publication of the dramatic role of CR1/2 for antibody responses in vivo (Heyman et al. 1990). Subsequently, other laboratories generated $Cr2^{-/-}$ mice and confirmed the importance of CR1/2 in antibody responses (Molina et al. 1996; Ahearn et al. 1996). Using such mice, we were able to show that IgM could not enhance in the absence of CR1/2 (Applequist et al. 2000; Rutemark et al. 2012) whereas IgE- and IgG2a-mediated enhancement, used as positive controls, remained intact (Applequist et al. 2000). Interestingly, enhancement by murine IgG3 is also dependent on CR1/2 (Diaz de Ståhl et al. 2003; Zhang et al. 2014).

As mentioned above, CR1/2 are expressed on B cells and FDCs in mice. Studies in bone marrow chimeras between CR1/2 knockout and wild-type mice showed that optimal IgM-mediated enhancement required that both B cells and FDCs expressed CR1/2 (Rutemark et al. 2012). However, less pronounced enhancement was also seen when only FDCs or only B cells expressed the receptors (Rutemark et al. 2012) (Fig. 2).

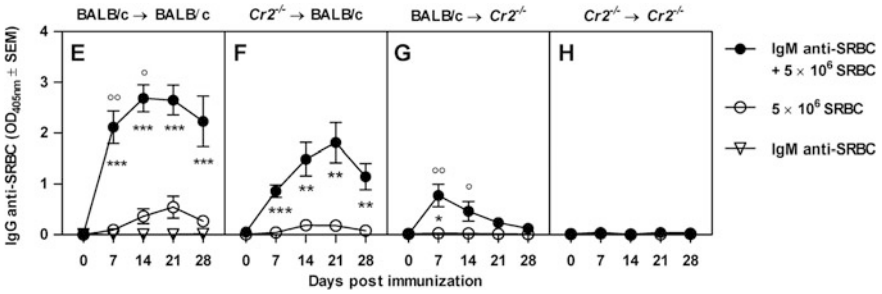


Fig. 2 CR1/2 on B cells and FDCs are required for optimal antibody responses to IgM-SRBC complexes. BALB/c and $Cr2^{-/-}$ mice were irradiated and reconstituted with either BALB/c or $Cr2^{-/-}$ bone marrow. Six weeks later they were immunized as indicated and screened for IgG anti-SRBC in serum. Two statistical comparisons were made, both using Student's *t*-test. First, comparisons between the responses in mice immunized with SRBC alone versus IgM + SRBC (to determine whether IgM enhanced antibody responses significantly); * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Second, comparisons between the responses between various chimeras immunized with IgM + SRBC (to determine whether $CR1/2^+$ B cells contributed significantly to the antibody response to IgM + SRBC in mice with $CR1/2^+$ FDCs (E vs. F) and $CR1/2^-$ FDCs (G vs. H); ° = $p < 0.05$; °° = $p < 0.01$; °°° = $p < 0.001$. Non-significant differences are not indicated. Adapted from (Rutemark et al. 2012)

4.3 *Cμ13 Knock-in Mice with a Point Mutation in the IgM Heavy Chain Abolishing C1q-Binding*

The observation that lack of C1q of the classical pathway leads to impaired primary antibody responses seems paradoxical for two reasons: (i) also alternative and lectin pathway activation would generate the C3 fragments which are the ligands for CR1/2, and (ii) the classical pathway is activated by antibodies binding to their antigens and, in naïve mice, very little specific antibodies would be present at the time of immunization. In 1998, it was found that mice lacking secretory IgM had impaired antibody responses and that the responses could be restored by transfer of non-immune IgM from normal mouse serum (Ehrenstein et al. 1998). This led to a possible explanation to the paradox described above, suggesting that natural IgM, present in naïve mice, would bind antigen with low affinity, activate complement and facilitate an early primary response in the same way as specific IgM does during feedback-enhancement. Once specific IgM is produced, classical IgM-mediated enhancement would ensue thus further potentiating the response.

During B.H.'s sabbatical with Michael Carroll, starting in 2001, we decided to test this hypothesis and generated knock-in mice (Cμ13), carrying the same point mutation in the μ heavy chain as the Mutant13 IgM, known to be unable to activate complement and to enhance antibody responses (Shulman et al. 1987; Heyman et al. 1988a). As a consequence of the mutation, all IgM antibodies produced by these mice, regardless of specificity, are unable to bind C1q. A.S. (neé Bergman) and Christian Rutemark, both junior Ph.D. students in B.H.'s lab at the time,

immunized Cμ13 and wild-type animals with KLH or SRBC and compared their antibody responses. In the majority of the experiments, no significant differences between responses in wild-type and Cμ13 mice were seen (Rutemark et al. 2011). Occasionally, the antibody responses in Cμ13 mice were slightly reduced but not nearly to levels as low as those seen in CR1/2 knockout mice (Rutemark et al. 2011). Thus, it appeared that the ability of natural IgM to activate complement did not explain the requirement for classical pathway activation in primary antibody responses. Possibly, the antigen doses that were tested were too high or other C1q-activating substances may play a role. Nevertheless, this was an unexpected result which prompted further investigations described below Sects. (4.4 and 4.6).

4.4 FcμR (Toso/Faim3) and IgM-Mediated Enhancement

In 2012, two research groups published that mice lacking the Fc-receptor for IgM, FcμR (Toso/Faim3), had impaired antibody responses to suboptimal doses of antigen (Ouchida et al. 2012; Honjo et al. 2012). This opened the possibility that FcμR could be involved in IgM-mediated feedback enhancement. The conclusion that IgM-mediated enhancement depends on the ability of IgM to activate complement was based on the loss of enhancing capacity by monoclonal or polyclonal IgM with the same point mutation in the μ heavy chain (Heyman et al. 1988a; Ding et al. 2013) and on the loss of enhancing capacity by monomeric IgM (Youd et al. 2002). Hypothetically, the IgM mutation could also have affected FcμR binding and monomeric IgM may not bind to FcμR. In collaboration with Ji-Yang Wang's laboratory, we addressed this question and found that IgM from Cμ13 knock-in and wildtype mice bound equally well to cells expressing FcμR (Ding et al. 2013). Thus, since IgM from Cμ13 mice was unable to enhance antibody responses and to activate complement but bound well to FcμR (Ding et al. 2013), the results strongly suggest that complement activation, but not FcμR binding, is required for induction of enhancement.

4.5 Other IgM-Binding Receptors and IgM-Mediated Enhancement

Not only FcμR (Toso/Faim3), but also poly-IgR (pIgR) (Johansen et al. 2000), Fcα-/μR (Ohno et al. 1990; Shibuya et al. 2000), and CD22 (Adachi et al. 2012) are known to bind IgM. However, their involvement in IgM-mediated enhancement has not been studied.

4.6 Specific IgM from Wildtype but not Cμ13 Mice, Causes Rapid Deposition of C3 on SRBC in Vivo

Possible caveats when testing the complement activation by Mutant13 and IgM from Cμ13 mice are that tests are performed in vitro and that guinea pig complement, instead of mouse complement, is used. To compare physiological complement activation by wild-type and Cμ13 IgM in vivo, SRBC-specific IgM of either type was administered intravenously to mice which 30 min later were given SRBC. In blood obtained as early as one minute after the last injection, large amounts of C3 fragments were deposited on SRBC in mice given wild-type but not Cμ13 IgM (Ding et al. 2013). Thus, IgM binds to intravenously administered antigens and activates the classical pathway within seconds, leading to heavy deposition of C3 fragments on the antigen. It is easy to envisage that the complement-opsonized SRBC seen in mice given IgM and SRBC (Ding et al. 2013; Sörman et al. 2014) will bind to CR1/2.

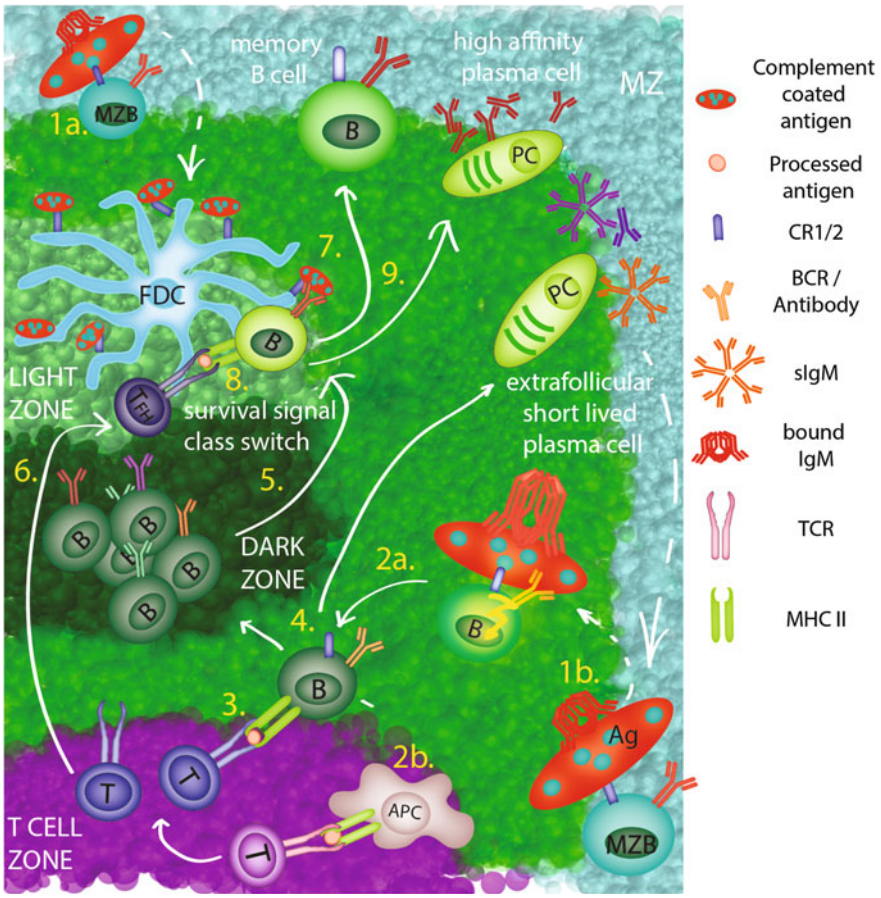
5 Transport of IgM-Antigen Complexes to Splenic B Cell Follicles

Early studies reported a correlation between the degree of IgM-mediated enhancement of the antibody response to SRBC and how much ^{51}Cr-labeled SRBC was trapped in the spleen (Dennert et al. 1971; Dennert 1971). More recently, Richard Corley's laboratory, using monoclonal IgM anti-NP, found that pentameric, but not monomeric IgM in complex with NP-BSA caused localization of antigen on FDCs in splenic B cell follicles (Youd et al. 2002). In mice lacking CR1/2 or C3, the IgM-antigen complexes were trapped in the MZ and did not move further into follicles. The same pentameric, but not monomeric (non-complement-activating) IgM, enhanced antibody responses to NP-KLH. Enhancement against NP-BSA was not investigated, or at least not reported. The same laboratory later reported that the cells responsible for transport of IgM-NP-BSA complexes from the MZ into the follicle were MZ B cells (Ferguson et al. 2004). Subsequently, Cinamon et al. showed that MZ B cells shuttle between the MZ and the follicle and deliver TNP-Ficoll to FDCs (Cinamon et al. 2008). Intravital imaging of MZ B cells demonstrated that as much as 20% of the cells exchanged compartment every hour (Arnon et al. 2013). Another study where virus-like particles (VLP) were used as antigens, showed that VLP-dimers required specific IgM for transport into follicles, whereas larger VLPs only required natural IgM (Link et al. 2012). In analogy to the studies above, follicular localization generally required CR1/2, C3, and C1q (Link et al. 2012). Thus, although only one study directly correlated IgM-mediated enhancement of antibody responses to antigen localization to the spleen (Dennert 1971), the other studies described above are highly compatible with such a scenario.

6 Summary and Concluding Discussion

The molecular mechanisms behind the onset of an antibody response are complicated and not yet fully understood. A current model, based on recent reviews (Victora et al. 2010; Vinuesa et al. 2010; Chan and Brink 2012; Heesters et al. 2014), is presented in Fig. 3. The question of major interest for the present discussion is how specific IgM can interfere with these processes and cause the enhancement of primary IgM and IgG responses, germinal center formation and induction of memory responses described above.

A central finding is that IgM must be able to activate complement in order for enhancement to be initiated (Heyman et al. 1988a; Youd et al. 2002; Ding et al. 2013). Studies in mice immunized with IgM anti-SRBC + SRBC show that the SRBC in circulating blood are covered by C3 fragments already 10 s after immunization (Sörman et al. 2014). The role of complement in IgM-mediated



◂**Fig. 3** Schematic overview over generation of antibody responses in the spleen. (*1a*, *b*) Antigen enters the splenic B cell follicles. Small antigens can enter via conduits (Nolte et al. 2003) whereas larger antigens, e.g. KLH, bind to MZ B cells via complement receptors (Ferguson et al. 2004) (*1a*, *b*). These cells shuttle between the MZ and the follicle and deposit antigen on FDCs (Cinamon et al. 2008) (*1a*). (*2a*) In the follicle, antigen is recognized by naïve follicular B cells which migrate towards the T cell zone after antigen encounter. (*2b*) T cells are simultaneously activated by antigen-presenting cells displaying peptides on their MHC-II. (*3*) A subgroup of the activated T cells upregulate CXCR5 and down-regulate CCR7, causing them to migrate towards the T-B-cell border where they meet and activate specific B cells. (*4*) Some of the activated B cells differentiate into short-lived extrafollicular plasma cells, mainly producing IgM (MacLennan et al. 2003). The majority of the activated B cells proliferate and form the dark zone of the germinal center. (*5*) In the dark zone, B cells undergo somatic hypermutation and then migrate to the light zone. (*6*) Some of the activated T cells are further triggered by the B cells to upregulate CXCR5 and differentiate into T_{FH} cells and move towards the light zone. (*7*) Here, B cells meet FDCs that display intact antigens on their dendrites (Heesters et al. 2013). (*8*) High affinity B cells capture the antigen, process and display it on MHC-II to a limiting number of T_{FH} cells, which provide the B cells with survival signals ensuring that B cells with the highest affinity survive (Schwickert et al. 2011; Shulman et al. 2013; Gitlin et al. 2014). The high affinity B cells subsequently undergo class-switch recombination. (*9*) Some B cells differentiate into memory B cells or high affinity longlived plasma cells which exit the follicles. Others return to the dark zone for another round of hypermutation. As detailed in the text, experimental observations suggest that specific IgM, through its ability to deposit C3 fragments onto the antigens, can interfere in the generation of antibody responses at several levels: (*1a*, *b*) Transport of IgM-antigen-complement complexes by $CR1/2^+$ MZ B cells into the follicle. (*2a*) Co-crosslinking of BCR and CR2/CD19/CD81 by IgM-antigen-complement complexes leading to facilitated B cell signaling and/or increased B cell activation simply owing to increased levels of antigen. (*7*) capture of antigen on FDCs for presentation to B cells during the affinity maturation process

enhancement seems to be to opsonize antigen for binding to CR1/2 rather than to increase the immunogenicity of the antigen through hemolysis: IgM enhances in mice lacking C5, a factor required for the lytic pathway (Heyman et al. 1988b) but not in mice lacking CR1/2 (Applequist et al. 2000; Rutemark et al. 2012).

CR1/2 are expressed on B cells and FDCs in mice. The only study which to our knowledge has addressed the question of which of these cells must express CR1/2 in order for IgM to be able to enhance antibody responses, was done in bone marrow chimeras with SRBC as the antigen and measured IgG responses (Rutemark et al. 2012). Expression of CR1/2 on both FDCs and B cells were required for optimal enhancement by IgM. However, expression on FDCs alone resulted in an intermediate enhancement and expression on B cells alone resulted in a weak enhancement (Rutemark et al. 2012) (Fig. 2).

Starting with B cells, at least three mechanisms have been described through which they, via CR1/2, could hypothetically increase antibody responses. In vitro, they can take up and present complement-opsonized antigens to T cells (Thornton et al. 1996; Boackle et al. 1997) and they can co-crosslink the CR2/CD19/CD81 complex and the BCR, lowering the threshold for B cell signaling (Carter et al. 1988; Matsumoto et al. 1993; Dempsey et al. 1996). In vivo, MZ B cells can transport complement-opsonized antigens into the B cell follicles (Youd et al. 2002; Ferguson et al. 2004; Cinamon et al. 2008; Link et al. 2012; Arnon et al. 2013). To

date, there is no evidence that antigen presentation to CD4 T cells via increased uptake of IgM-immune complexes by B cells via CR1/2 plays a significant role in vivo but it is noteworthy that the influence of IgM on activation of the T follicular helper cell subset has not been selectively investigated. However, IgM does not enhance activation and proliferation of adoptively transferred transgenic antigen-specific CD4 T cells although the antibody responses were enhanced in the same animal (Ding et al. 2013). Similarly, studies of the role of CR1/2 for in vivo T cell responses to uncomplexed antigen did not reveal a role for these receptors (Gustavsson et al. 1995; Da Costa et al. 1999; Carlsson et al. 2009). Moreover, mice lacking CR1/2, or mice where the receptors were blocked, have poor antibody responses to T cell independent antigens (Thyphronitis et al. 1991; Wiersma et al. 1991; Carlsson et al. 2009). Since such antigens do not need to be processed and presented to T cells in order to induce antibody responses, the observations are hard to reconcile with an in vivo role for CR1/2 in antigen presentation to T cells. This reasoning leaves antigen transport by MZ B cells and facilitated B cell signaling as two non-mutually exclusive mechanisms through which B cells can be involved in IgM-mediated enhancement. The increased availability of antigen as a result of MZ B cell-mediated transport of IgM-complement-opsonized antigens into the follicle could lead to increased deposition of antigen on FDCs (Fig. 31a, 7). It could also lead to increased activation of specific follicular B cells in general and/or to increased B cell signaling caused by complement-opsonized antigens co-crosslinking the BCR and the CR2/CD19/CD81 co-receptor complex (Fig. 31b, 2a). The relative importance of B cell-mediated antigen transport versus B cell signaling is presently unknown. However, since the early IgM responses induced by specific IgM probably represent an extrafollicular response, neither transport of antigen into follicles nor binding to FDCs would be required. Therefore, it seems likely that in this situation co-crosslinking of BCR and CR2/CD19/CD81 plays a significant role.

Not only B cells but also CR1/2$^+$ FDCs are important for optimal IgM-mediated enhancement, and judging from the only direct experiment testing their relative roles, FDCs are the most important cells (Rutemark et al. 2012) (Fig. 2). Complement-opsonized antigen, transported into follicles either via MZ B cells or via other pathways, is likely to be captured by FDCs and presented to B cells competing for antigen after their hypermutation processes (Fig. 31a, 7).

In conclusion, specific IgM must be able to activate complement in order to enhance antibody responses and ligation of CR1/2 on both B cells and FDCs are involved. The ability of specific IgM to enhance antibody responses is likely to play a physiological role in optimizing antibody responses. Since also natural IgM and FcμR influence antibody responses, the relative roles of these components and those of specific IgM and complement is an interesting subject for future research.

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Role of Natural IgM Autoantibodies (IgM-NAA) and IgM Anti-Leukocyte Antibodies (IgM-ALA) in Regulating Inflammation

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Abstract Natural IgM autoantibodies (IgM-NAA) are rapidly produced to inhibit pathogens and abrogate inflammation mediated by invading microorganisms and host neoantigens. IgM-NAA achieve this difficult task by being polyreactive with low binding affinity but with high avidity, characteristics that allow these antibodies to bind antigenic determinants shared by pathogens and neoantigens. Hence the same clones of natural IgM can bind and mask host neoantigens as well as inhibit microorganisms. In addition, IgM-NAA regulate the inflammatory response via mechanisms involving binding of IgM to apoptotic cells to enhance their removal and binding of IgM to live leukocytes to regulate their function. Secondly, we review how natural IgM prevents autoimmune disorders arising from pathogenic IgG autoantibodies as well as by autoreactive B and T cells that have escaped tolerance mechanisms. Thirdly, using IgM knockout mice, we show that regulatory B and T cells require IgM to effectively regulate inflammation mediated by innate, adaptive and autoimmune mechanisms. It is therefore not surprising why the host positively selects such autoreactive B1 cells that generate protective IgM-NAA, which are also evolutionarily conserved. Fourthly, we show that IgM anti-leukocyte autoantibodies (IgM-ALA) levels and their repertoire can vary in normal humans and disease states and this variation may partly explain the observed differences in the inflammatory response after infection, ischemic injury or after a transplant. Finally we also show how protective IgM-NAA can be rendered pathogenic under non-physiological conditions. IgM-NAA have therapeutic potential. Polyclonal IgM infusions can be used to abrogate ongoing inflammation. Additionally, inflammation arising after ischemic kidney injury, e.g., during high-risk elective cardiac surgery or after allograft transplantation, can be prevented by pre-emptively infusing polyclonal IgM, or DC pretreated ex vivo with IgM, or by increasing in vivo IgM with a vaccine approach. Cell therapy with IgM pretreated cells, is appealing as less IgM will be required.

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1 Introduction

Nature, by creating polyreactive pentavalent natural IgM autoantibodies (IgM-NAA), has accomplished a difficult task of protecting the host from both diverse foreign pathogens and from diverse self-neoantigens that are constantly being generated. As a result, the adaptive immune system has time to mount a highly specific immune response to foreign antigens and, in addition, such a mechanism lessens the burden on the host to maintain diverse B cell clones producing highly specific IgG autoantibodies which have the potential of causing autoimmune disease owing to their high affinity binding. Secondly, these IgM-NAA have taken over another task of subduing an excessive inflammatory response induced by both foreign and self-neoantigens. Again, we will show how the low binding affinity of IgM-NAA to live leukocytes, together with their inability to fully activate complement at body temperature (37 °C), has helped these antibodies to regulate these inflammatory cells without causing cell damage within the host. It is therefore not surprising why these IgM-NAA antibodies, which first arose in cartilaginous fish, have been conserved during evolution (reviewed in Dooley and Flajnik 2006) and why IgM-NAA make up about 70–80% of circulating IgM (Baumgarth et al. 1999; Thurnheer et al. 2003). Additionally, natural IgM is also evolutionarily functionally conserved among mammalian species, as human IgM has the same effect as murine IgM on murine cells in vitro or when used in vivo in

mice (Robey et al. 2002; Zhang et al. 2008; Lobo et al. 2015). The presence of polyreactive IgM BCR on these IgM-NAA producing B cell clones has enabled them to be rapidly activated by a foreign or an auto-neoantigen for deploying protective IgM antibodies.

Recently, B1 cells have also been shown to exist in humans. Human B1 cells, unlike murine B1 cells, are CD20+ CD43+ and CD27+. Like murine B1 cells, human B1 cells can spontaneously secrete antibody and such cells represent about 50% of umbilical cord B cells and 15–20% of circulating adult B cells, and these cells are the predominant source of human IgM-NAA (Griffin et al. 2011). CD5 is not a specific marker of human B1 cells as both B1 and B2 cells express this marker. Similarly, about 20% of CD43+ CD27+ B cells have characteristics of B2 derived pre-plasmablasts and hence CD43 and CD27 are also not reliable markers of human B1 cells (Covens et al. 2013; Tangye 2013). Human IgM-NAA are also polyreactive and bind similar autoantigens as in mice, including oxidized neodeterminants and leukocyte receptors (Chen et al. 1998; Lobo et al. 2008a; Chou et al. 2009; Lobo et al. 2015).

An important characteristic of IgM-NAA is their low binding affinity (Zhou et al. 2007). The IgM BCR expressed on B1 cells may also have a low binding affinity and this latter characteristic may be involved in preventing autoreactive B1 cells from being deleted or undergoing negative selection. In fact, several studies would indicate that autoreactive B1 cells are positively selected and this process requires both the autoantigen and the relevant BCR (Hayakawa et al. 1999; Martin and Kearney 2000; Cancro and Kearney 2004; Tian et al. 2006). The need to positively select B1 cells secreting IgM-NAA would indicate that these antibodies have an important physiological role. During life, the repertoire of IgM-NAA is shaped by T-independent antigen activation (Martin and Kearney 2002; Kretschmer et al. 2003).

IgM-NAA have been shown to have specificity for certain common epitopes present on phylogenetically conserved self-antigens. As a result, diverse IgM secreting clones with different specificities have been identified. These include IgM secreting clones with specificity for leukocyte receptors (IgM-ALA) (Lobo et al. 2008a, 2015), the Fc domain of IgG (rheumatoid factor) (Casali et al. 1987; Hardy et al. 1987), complement components (Rieben et al. 1999), collagen, thyroglobulin, intracellular constituents such as cytoskeletal proteins, cytosolic enzymes, dsDNA or nucleosomes, neutrophil cytoplasmic enzymes (ANCA) (Avrameas 1991; Vittecoq et al. 1999) as well as oxidized neodeterminants (e.g., phosphorylcholine (PC)) that are exposed when lipids are oxidized or cells undergo apoptosis (Baumgarth 2011; Gronwall et al. 2012). While some IgM-ALA have monoreactivity e.g., to some cytokines, most are polyreactive with each polyreactive IgM-NAA clone having a selective binding profile. For example, IgM anti-PC NAA will bind to ABO blood type antigens, endotoxins and oxidized neodeterminants on apoptotic cells but this autoantibody has no binding reactivity to nuclear antigens or to IgG (Baxendale et al. 2008). Conversely, IgM anti-dsDNA will bind to cytoskeletal proteins but will not bind to PC. Additionally, these IgM-NAA, by virtue of being polyreactive, also cross-react with pathogen-expressed molecules,

for example phosphorylcholine (PC) on *Streptococcus pneumoniae* and other antigens expressed by various viruses and parasites (Baxendale et al. 2008; Baumgarth 2011; Gronwall et al. 2012) Hence, it has been suggested that these natural IgM antibodies are protective, serving as a first line of defense against infections and protecting the host from pathogen-mediated apoptotic cells and oxidized neodeterminants which can induce pathogenic IgG autoantibodies (Baumgarth 2011; Gronwall et al. 2012). Additionally, polyreactive IgM-NAA have been shown to bind to idiotypic determinants on self-reactive IgG, thus providing another mechanism to protect the host from high affinity binding IgG autoantibodies that are potentially pathogenic (Adib et al. 1990; Avrameas 1991).

2 Natural Autoantibodies and B1 Cells

In the last 40 years, much has been learned about natural autoantibodies (NAA) of different isotypes (Steele and Cunningham 1978; Dighiero et al. 1983; Hardy and Hayakawa 1986; Casali et al. 1987; Hardy et al. 1987; Nakamura et al. 1988; Kantor and Herzenberg 1993; Kasaian and Casali 1993; Mouthon et al. 1996; Clarke and Arnold 1998; Rieben et al. 1999). The term "natural antibodies" has been used to describe these Igs, as high levels of these autoantibodies are present in the umbilical cord, i.e., before foreign antigen exposure, and secondly because such antibodies can be produced under germfree conditions and in the absence of the thymus (Avrameas 1991). The full repertoire of IgM-NAA develops by early childhood. In mice, NAA are predominantly produced by the CD5+ B1 cells while marginal zone splenic B (MZB) cells contribute the remainder. These B1 cells differ from B2 cells in that they spontaneously produce IgM, IgA and IgG3 autoantibodies (Sidman et al. 1986; Solvason et al. 1991; Griffin et al. 2011) independently of T helper cells, and exhibit an enhanced response to innate immune signals such as TLR agonist (Murakami et al. 1994; Nisitani et al. 1995; Ha et al. 2006; Yang et al. 2007). Additionally, autoantibody producing B1 cells are positively selected for their self-reactivity thus implying that NAA are conserved by design (Hayakawa et al. 1999; Martin and Kearney 2000; Cancro and Kearney 2004; Tian et al. 2006). Furthermore, the finding that IgM-NAA comprise the majority of circulating IgM underscores their importance (Baumgarth et al. 1999; Thurnheer et al. 2003). Cross-sectional studies in humans and rodents would indicate that IgM-NAA decrease with age (Adib et al. 1990; Love et al. 2000; Simell et al. 2008; Griffin et al. 2011) or lose their effectiveness with age (Nicoletti et al. 1993), except for one report where follow-up of 5 healthy individuals for 25 years revealed no change in IgM-NAA levels (Lacroix-Desmazes et al. 1999). However, IgG NAA can increase (Nagele et al. 2013) but do not decrease with age (Lacroix-Desmazes et al. 1995; Bachi et al. 2013).

Natural IgM-NAA should not be confused with immune IgM that is produced several days after exposure to foreign antigens or pathogens and, in general, is antigen-specific and produced by B2 cells that require antigen binding to BCR and

additional T helper cells to generate antigen-specific antibodies. Production of immune IgM is limited as antigen-activated B2 cells migrate to B cell follicles, where with help from follicular T helper cells, these cells undergo class switch recombination (CSR) and somatic hypermutation (SHM). B2 cells differentiate into long-lived memory B cells and plasma cells that generate IgG antibodies with high binding affinity. B2 cells are distinct from B1 cells in many respects and they are derived from different progenitors (Sidman et al. 1986; Solvason et al. 1991; Montecino-Rodriguez et al. 2006). Furthermore, during an immune response, B1 cells have intrinsic mechanisms to actively inhibit CSR and SHM. In this regard, there are mechanisms to actively prevent B1 cells from entering B cell lymphoid follicles and B1 cells actively maintain low levels of activation induced deaminase (AID), which induces SHM and CSR, and through this mechanism B1 cells inhibit production of high affinity, IgG anti-self Ab, which may be pathogenic (Ishida et al. 2006; Matejuk et al. 2009).

3 Physiological Role of IgM-NAA

There are several in-depth reviews on the physiological functions of IgM-NAA (Ehrenstein and Notley 2010; Baumgarth 2011; Gronwall et al. 2012; Kaveri et al. 2012; Lobo 2016). Briefly, functions that have been attributed to IgM-NAA have included the following: (i) Providing the first line of defense against pathogens while the adaptive immune system, i.e., B2 and T cells, is being deployed to mediate a more specific and effective immune response that is longlasting and has memory. (ii) Inhibiting IgG autoantibody production and inflammatory responses by clearing apoptotic cells and binding to oxidized neodeterminants as well as by blocking pathogenic IgG autoantibodies via anti-idiotypic mechanisms (iii) Inhibiting inflammation by binding of IgM-NAA to receptors on live leukocytes, i.e., via IgM anti-leukocyte autoantibodies (IgM-ALA) and by binding of IgM anti-PC to phosphorylcholine (PC) expressed by apoptotic cells and (iv) Inhibiting expansion of B1 cells and enhancing antigen presentation to B2 and helper T cells in splenic lymphoid follicles.

The above observations indicate that IgM-NAA protect the host from invading organisms and more importantly maintain several homeostatic mechanisms primarily aimed at preventing autoimmunity and over exuberant inflammation, which can have detrimental effects on the host. Table 1 summarizes some of the physiological and pathological concepts outlined above. Several observations indicate that infective and other inflammatory states increase all IgM-NAA subsets, especially IgM anti-PC to clear the increased production of apoptotic cells that could trigger autoimmunity and secondly increase IgM-ALA to subdue excess inflammation that can be detrimental to the host (reviewed in Gronwall et al. 2012 and next section). Based on the preceding observations, one could predict that a

Table 1 Physiological function of non-pathogenic natural IgM autoantibodies

Protection from microorganisms	– Binds to bacteria and enhances phagocytosis (req. C1q, Fcα/μR) – Inhibits HIV by blocking entry and inactivating cells
Prevent autoimmunity	– Blocks anti-self IgG Ab (anti-idiotypic) IgM masks neoantigen – Binds to apoptotic cells (PC), nuclear and cytoplasmic debris, and enhances phagocytosis (req. C1q, MBP)
IgM-ALA abrogates inflammation	– Inhibits serum complement – Decreases production of TNF-α, IL-17, IFNγ – Regulates DC, T cells, and enhances T regs – Binds to CD40, CD86, CD4, CD3, and TcR – Downregulates NFκB and ZAP-70 phosphorylation – Blocks chemokine receptors
B cell homeostasis	IgM regulates B1 cell expansion via FcμR

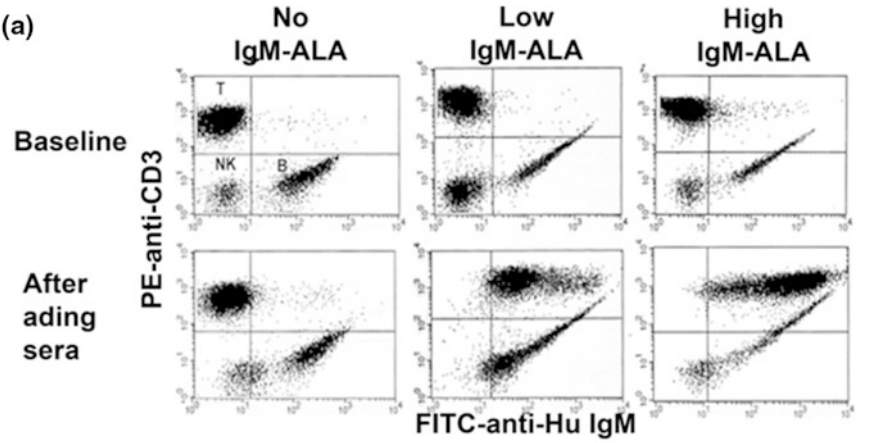
decrease in IgM-NAA, as can occur in aging (Nicoletti et al. 1993; Love et al. 2000; Simell et al. 2008; Griffin et al. 2011), could predispose to increased autoimmunity and increased morbidity and mortality from an excess inflammatory response. In this review, we will focus on the role of IgM-ALA in inhibiting inflammation.

4 Physiologic Role of IgM-ALA in Regulating Inflammation

Initial observations demonstrating that IgM can bind to receptors on live autologous and allogeneic leukocytes were made in 1970 (Terasaki et al. 1970). The role of IgM-ALA in inflammation was recognized when several investigators showed that the level of these antibodies, e.g., IgM anti-PC and IgM-ALA, increased with diverse infections and inflammatory states (reviewed in Lobo et al. 2008a). However, it was unclear whether high levels of IgM-ALA were pathogenic or not. The idea that IgM-ALA may have anti-inflammatory function and may be protective came from observations in allograft recipients, where patients with high levels of IgM-ALA were found to have significantly less rejections after kidney

Fig. 1 *High levels of serum IgM-ALA in transplant recipients are associated with better kidney allograft survival and decreased alloantibody levels*. **a** Dot plots labeled "baseline" depict IgM staining on B lymphocytes, but not on T cells, in the absence of sera. The *lower panels* (after adding sera) depict differences in the level of IgM bound to donor B and T lymphocytes (IgM-ALA) after addition of pre-transplant serum from different ESRD patients. **b** Alloantibody titer in ESRD pre-transplant sera measured by cytotoxicity is correlated to presence or absence of IgM-ALA present in the same pre-transplant sera. **c** Data depicting the difference in percentage of acute rejections and graft loss comparing high IgM-ALA versus the no and low IgM-ALA groups. *MCF* indicates increase in mean channel fluorescence of anti-IgM staining to T cells after addition of serum. Figure and legend reproduced with permission from Transplantation. 1981;32(3):233–7 copyright 1981 Wolters Kluwer Health Inc. (panel **b**) and J Clin Immunol. 2010;30(1):31–6. Copyright 2010 Springer Science + Business Media LLC. (panels **a**, **c**) ▶

(a)



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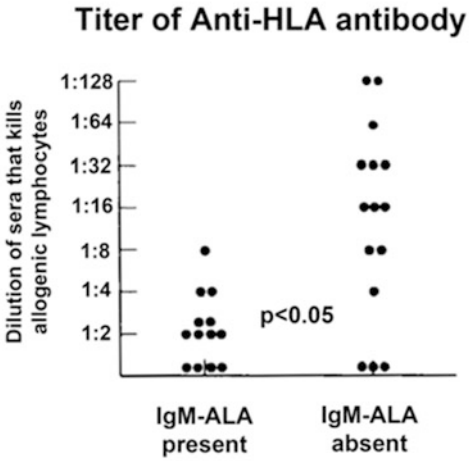


(c)

	No IgM-ALA (MCF<20)	Low IgM-ALA (MCF 21-200)	High IgM-ALA (MCF>200)
# of Patients	65	22	21
%Acute Rejections Requiring treatment	32	32	*9.5
%Graft loss at 1 yr	20	9.1	*0

*p<0.05 comparing High IgM vs. Low and No IgM

transplantation and developed less alloantibodies after a sensitizing event with alloantigens (Fig. 1) (Lobo 1981; Lobo et al. 2008a). We therefore hypothesized that the increase in natural IgM-ALA during inflammatory states provided a mechanism to regulate leukocyte function and prevent excess inflammation that may be detrimental to the host. Two characteristics, i.e., their low affinity binding to different leukocyte receptors including co-stimulatory molecules and chemokine receptors (Lobo et al. 2008a, 2015) and secondly, their inability to lyse leukocytes at body temperature, despite the presence of complement, allowed us to develop this hypothesis (Winfield et al. 1975; Lobo 1981; Lobo et al. 2008a). We postulated that the low affinity binding to leukocyte receptors modulated their function without causing cell lysis or apoptosis.

4.1 B Cell Clones Obtained from Human Umbilical Cord Produce IgM-ALA that Exhibit Leukocyte Receptor Specificity—Binding of IgM to Leukocytes Was not Mediated by FcμR

It became necessary to determine if IgM-ALA exhibited leukocyte receptor specificity, especially since these antibodies are polyreactive and could non-specifically bind to carbohydrate or other moieties and hence cross-react with several leukocyte receptors. This issue was evaluated by isolating B cell clones from human umbilical cord blood. We observed that >90% of umbilical cord B cells were IgM secreting but, surprisingly, only 10% of these IgM clones had IgM-ALA binding activity when examined by flow cytometry on a cell mixture of B (Daudi), T (Jurkat, Sup T1) and macrophage (U937) human cell lines. These observations therefore indicated that binding of IgM to receptors on human B cells and macrophages occurred independently of FcμR, which is not expressed by human macrophages (Kubagawa et al. 2009). Secondly, we observed that IgM-ALA had receptor specificity, especially since some of the IgM-ALA monoclonal antibodies only bound to receptors expressed by all leukocytes while other IgM monoclonals bound to receptors expressed by either T cells (SupT1, Jurkat) or macrophages (U937) or B cells (Daudi) (Fig. 2a) (Lobo et al. 2008a). The latter is exemplified by a T cell-specific human monoclonal IgM, which immunoprecipitated CD4 from cell lysates and bound to recombinant soluble CD4 (Figs. 2b, 3a).

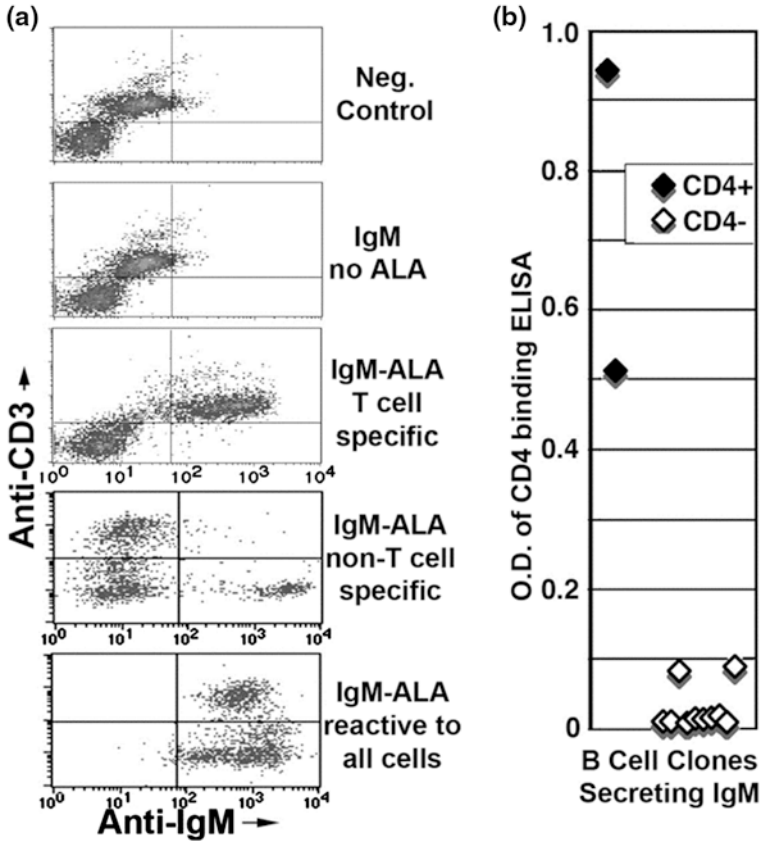


Fig. 2 *Presence of IgM-ALA in supernatants from umbilical cord B cells.* **a** and **b**, IgM-ALA reactivity in IgM-containing supernatants from B cell clones activated with EBV. IgM-ALA reactivity was detectable in 8 of 79 supernatants. **a** Supernatants were interacted with cells containing a mixture of human cancer cell lines, i.e., Jurkat (T cells), SupT-1(T cells), Daudi (B cells), and U937 (monocyte). Daudi B cells in the cell mixture were initially pre-treated with un-labelled anti-IgM and washed to block intrinsically expressed IgM. Note that the supernatants have IgM-ALA that is specific for either T or non-T cells or all four cell lines. Subsequent studies revealed that IgM anti-non-T cell had binding reactivity to only U937 cells and not to Daudi cells. **b** Data on IgM anti-CD4 reactivity using an ELISA technique. Note that only 2/79 IgM-containing supernatants had IgM anti-CD4 reactivity and both these supernatants also had IgM that bound to leukocytes. Figure and legend reproduced with permission from J Immunol. 2008 Feb 1;180 (3):1780–91. Copyright 2008. The American Association of Immunologists, Inc

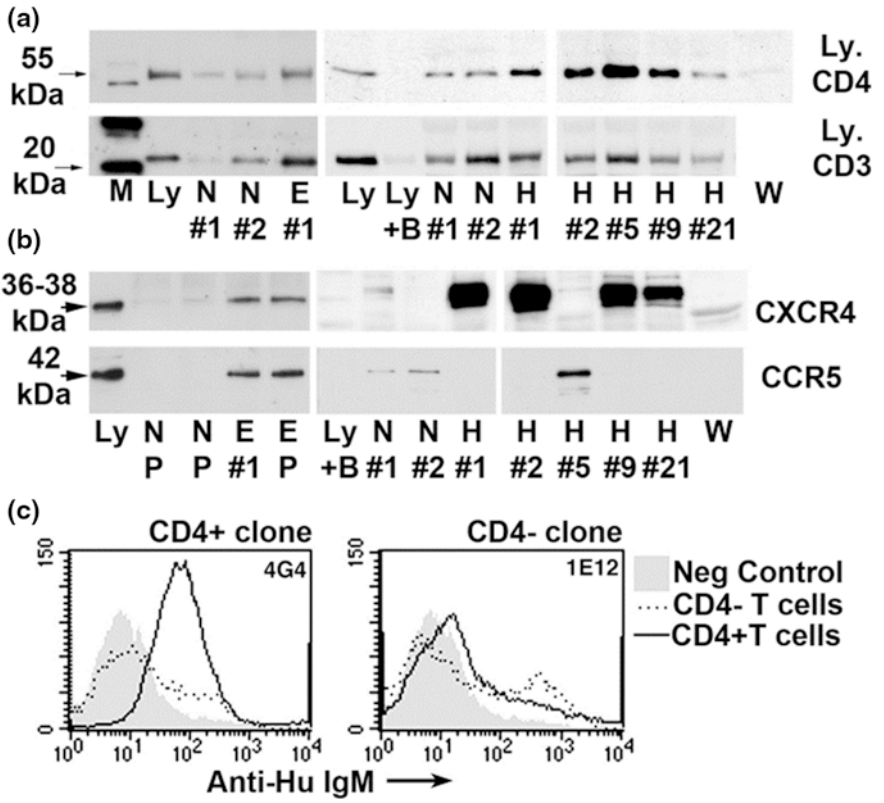
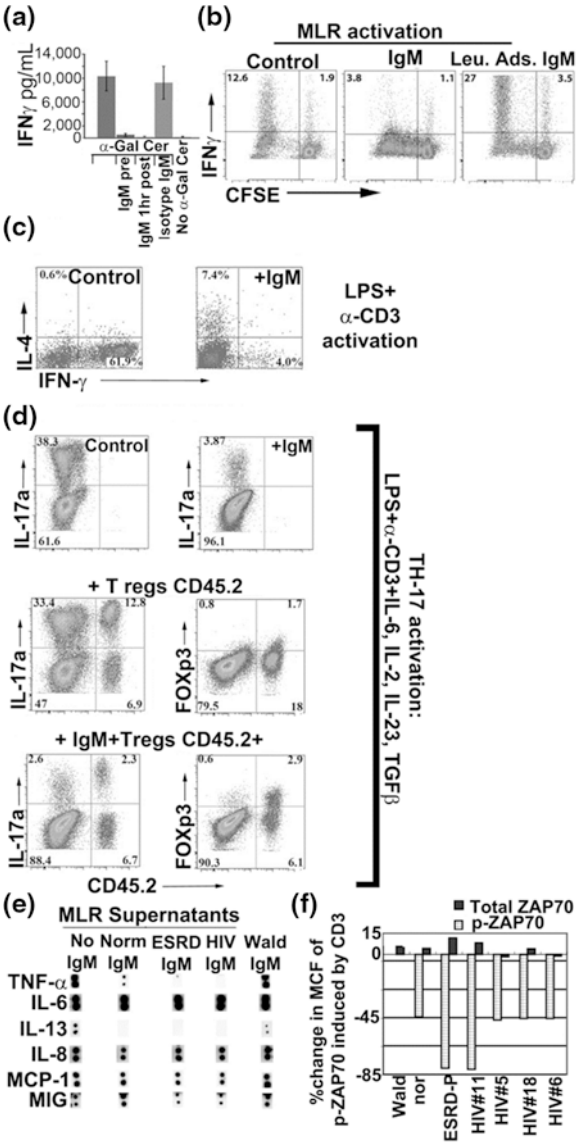


Fig. 3 *Immunoprecipitation experiments to show binding of human polyclonal IgM to CD3*, *CD4*, *CCR5*, *and CXCR4*. **a**, **b** Identical quantities of individual (labeled no. 1, 2, etc.) or pooled (labeled P) IgM from normal (labeled N), HIV (labeled H), ESRD (labeled E), or Waldenstrom (labeled W) were used to immunoprecipitate leukocyte receptors from equal amounts of whole cell lysates or recombinant soluble CD4. As controls, Western blots were performed with cell lysates in the absence of agarose beads (to control for binding of primary Ab to leukocyte receptor and to determine receptor size (labeled Ly). In another control, agarose beads without IgM were added to lysate to determine whether the leukocyte receptor nonspecifically bound to the bead (B plus Ly). Note that severalfold more receptors were immunoprecipitated by ESRD and HIV IgM when compared with normal IgM. **c** Significantly increased binding of IgM from B cell clone 4G4 to CD4+ T cells when compared with CD4− T cells (MCF 71.6 vs. 16.6). Clone 4G4 secreted IgM with anti-CD4 reactivity. Note that no increased binding was observed on CD4+ T cells using a B cell clone (IE12) secreting IgM without anti-CD4 reactivity (MCF 22.5 vs. 22.3). Figure and legend reproduced with permission from J Immunol. 2008 Feb 1;180(3):1780–91. Copyright 2008. The American Association of Immunologists, Inc

4.2 *IgM-ALA from Different Human Sera Differ in Their Repertoire for Receptor Binding. IgM Regulates Human T Effector Cells and DC Without Affecting Tregs or Chemokine Production*

This was studied by using polyclonal IgM purified from sera of normal controls, HIV patients and patients with end stage renal disease (ESRD) (Lobo et al. 2008a). IgM in these studies was purified by size exclusion chromatography, as ammonium chloride precipitation affected IgM-ALA binding [see method details in Lobo et al. (2008a, 2015)]. In these studies we used purified polyclonal IgM to immunoprecipitate CD3, CD4, CCR5 and CXCR4 from lysates of cell lines and showed that the repertoire of IgM-ALA was different among individuals, especially patients, as exemplified with HIV patients (see Fig. 3). This finding demonstrating differences in the repertoires of IgM-ALA may in part explain the different clinical manifestations of inflammation among different individuals. Prior exposure to different infective agents or foreign antigens may provide a possible explanation for the observed differences in the repertoire of IgM-ALA among different individuals (Adib et al. 1990).

Addition of polyclonal human IgM to cultures of human peripheral blood mononuclear cells (PBMC) differentially inhibited co-stimulatory receptor upregulation, cytokine production, and proliferation of T cells (Lobo et al. 2008a). This was not observed with Waldenstrom's IgM lacking IgM-ALA. IgM obtained from both normal individuals and patients downregulated expression of CD4, CD2 and CD86 but not CD8 and CD28 on blood PBMC activated with alloantigens (MLR) (Lobo et al. 2008a). Additionally, physiological doses of polyclonal IgM obtained from either normal individuals or patients inhibited production of the same set of cytokines, i.e., TNF-α, IL13, and IL-2 but not IL-6 and chemokines when human PBMC were activated by alloantigens (Fig. 4e and Lobo et al. 2008a). Other investigators using a monoclonal IgM-ALA with TCR reactivity have shown that natural IgM can inhibit IL-2 production and T cell proliferation by binding to the TCR (Marchalonis et al. 1994; Robey et al. 2002). Similarly, we have shown that IgM can inhibit Zap-70 phosphorylation and T cell proliferation induced by CD3 ligation as well as T cell proliferation induced by alloantigens (Fig. 4f and Lobo et al. 2008a). Importantly, IgM inhibited T cell proliferation in the mixed leukocyte reaction (MLR) without altering Treg levels. Finally, we show that IgM can inhibit chemokine-induced chemotaxis of activated PBMC by binding to the receptor and blocking chemokine binding. However, we also show that IgM does not inhibit chemokine production (Fig. 4e) (Lobo et al. 2008a). In these studies, patient IgM had a more inhibitory effect when compared to normal IgM and these functional differences between normal and patient IgM may be explained by differences in their IgM-NAA levels and repertoire, especially since we used identical quantities of purified IgM in these in vitro studies (Fig. 3) (Lobo et al. 2008a). Other investigators have also shown that polyclonal human IgM can inhibit proliferation of human T cells (Robey et al. 2002; Vassilev et al. 2006).



In summary, we show that the quantity and repertoire of IgM-ALA varies in different individuals especially in disease. Additionally, we show that polyclonal IgM, in physiological doses, inhibits human T effector cell activation and proliferation and that, in addition, IgM regulates production of certain cytokines by binding to co-stimulatory molecules (CD4, CD3, TCR) and inhibiting Zap-70 phosphorylation. We also show that IgM does not inhibit Tregs. IgM-ALA do not

◂**Fig. 4** *Polyclonal IgM inhibits IFN-γ production and T cell proliferation and differentiation into TH1 and TH17 cells of murine splenic cells and specific pro-inflammatory cytokines from human leukocytes activated with alloantigens.* **a** Supernatant IFN-γ in 48 h culture media of splenic cells activated with a-gal-ceramide which specifically activates NKT-1 cells. IgM was added either 0.5 h before activation (IgM pre) or one hour post activation. **b**, **c**, **d** CFSE labeled WT-B6 splenocytes (2.5×10^5 in 0.5 ml media) were activated either in a one way MLR (using 7.5×0^5 BALB/c irradiated splenocytes) or LPS (350 ng) and soluble anti-CD3. Cells were cultured for 4 to 5 days. IgM (10–15 μg) was added at the initiation of culture unless otherwise indicated. In (**d**), the effect of Tregs was evaluated by co-culturing 2.5×10^5 CD45.1 WT-B6 splenic leukocytes, containing 1.8% CD4+ Foxp3+ cells, with 0.5×10^5 sorted CD45.2 WT-B6 Tregs (76% Foxp3+) under cytokine conditions favoring TH-17 differentiation. **e** Pooled human normal, ESRD, and HIV IgM but not Waldenstrom IgM significantly inhibit the increase in TNF-α and IL-13 but not that of IL-6, IL-8, MIG, and MCP-1 produced in response to alloantigen activation of T cells. Supernatants were obtained from day 5 MLR cultures stimulated in the presence or absence of pooled IgM (15 μg/ml), added on day 0. **f** Pooled human IgM inhibits anti-CD3 mediated Zap-70 phosphorylation of human T cells. IgM was added 30 min before anti-CD3/28 and cells were cultured overnight before quantitation by flow cytometry. Figures and legend reproduced with permission from J Immunol. 2012 Feb 15;188(4):1675–85 (Panels A to D) and J Immunol 2008 Feb1; 180(3):1780–91 (panel **e**, **f**). Copyright 2008 and 2012. The American Association of Immunologists, Inc

appear to affect the production of chemokines by leukocytes, but interferes with their action by binding to chemokine receptors.

4.3 The Function of Murine T Effector Cells, DC and NKT Cells but not Tregs Is Regulated by Binding of Polyclonal IgM to Specific Co-Stimulatory Receptors

Using murine splenic leukocytes, we observed a severalfold increased binding of IgM-ALA to live granulocytes, DC and B cells when compared to T cells and this binding occurred despite pronase digesting cells to remove FcμR. Furthermore, we showed that IgM could immunoprecipitate several different leukocyte receptors, thus indicating that the IgM bound to more than one receptor expressed on the cell membrane (Fig. 5d) (Lobo et al. 2012). However, IgM binding to all leukocytes was enhanced when cells were activated (see Fig. 5a). We therefore investigated if IgM had an inhibitory effect on the function of T cells, DC and NKT cells by binding to receptors, e.g., antigen presenting receptors and co-stimulatory receptors that are upregulated during activation.

The functional effect of physiological doses of IgM on murine T cells was examined in vitro studies using splenic cells. We showed that murine polyclonal IgM inhibited naïve T cells from differentiating into TH1 and Th17 cells (Fig. 4b, c, d), even when IgM was added 48 h after activation (Lobo et al. 2012). This inhibitory effect on T cells was independent of DC as the same inhibitory effect was noted when T cells were activated with insoluble anti-CD3/28. IgM, in addition, inhibits differentiation of Foxp3+ Tregs into TH17 cells. This is exemplified in

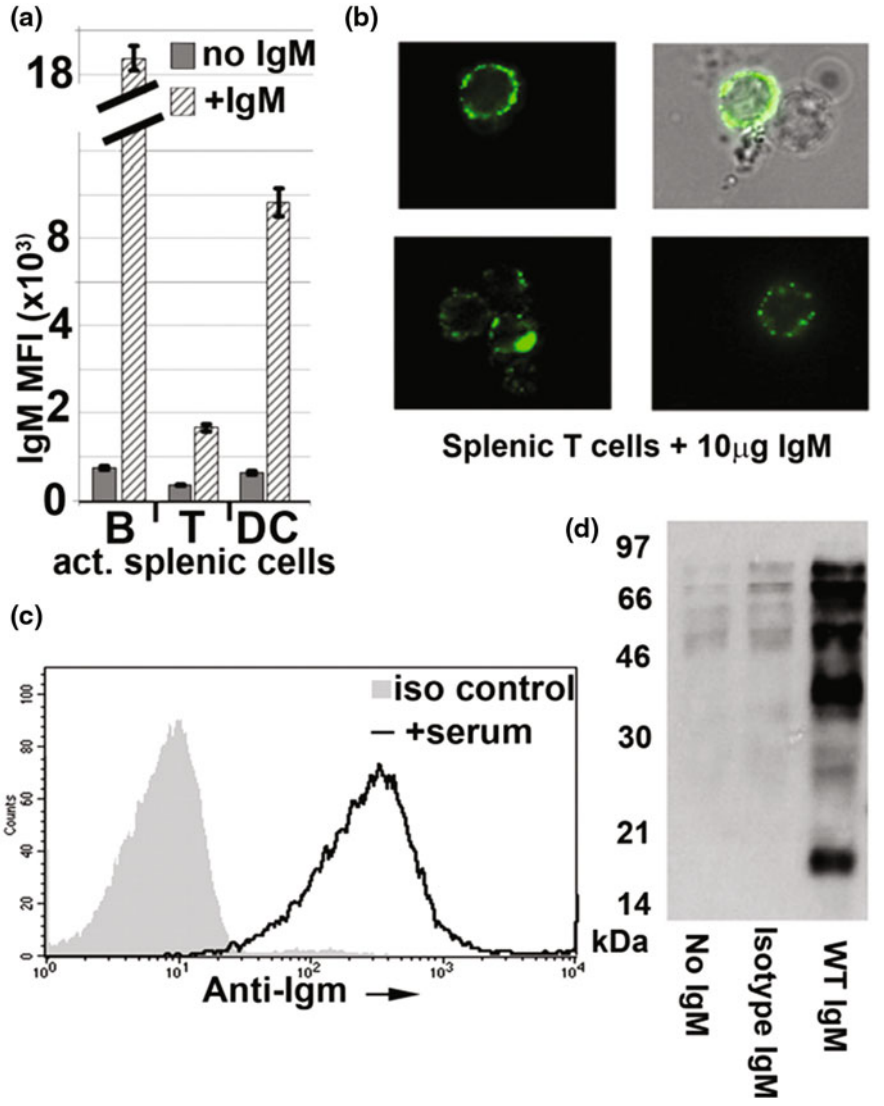


Fig. 4d where physiological doses of IgM significantly inhibited sorted Foxp3 + (CD45.2+) Tregs from losing Foxp3 expression and differentiating into TH17 effectors when co-cultured with splenic cells under cytokine conditions favoring TH17 differentiation (Lobo et al. 2012).

Since there are <1.5% DC in murine spleens, we used 7–8 day cultured murine bone marrow DC (BMDC) to investigate the functional effects of IgM on DC. We showed that polyclonal murine IgM, but not IgM pre-adsorbed with activated splenic leukocytes, bound to recombinant soluble CD40, CD86 and PD1 but not

◂**Fig. 5** *Polyclonal murine WT IgM binds to membrane receptors on leukocytes*. **a** Polyclonal IgM has severalfold increased binding to LPS-activated murine splenic B cells and dendritic cells (DC). Splenic leukocytes activated for 48 h were incubated with purified mouse IgM at 4 °C and evaluated for IgM binding using IgG anti-IgM (clone 11/41). Isotype monoclonal IgM with reactivity to KLH did not bind to activated leukocytes (data not shown). IgM binding to B cells was evaluated by blocking intrinsically expressed IgM with unlabeled IgG anti-IgM (clone 11/41). **b** depicts immunofluorescence microscopy images of IgM binding to cell membranes of splenic T lymphocytes. **c** compares binding of IgM and isotype IgM on CD3+WT B6 pronase-pretreated splenic leukocytes. Spleen cells were pronase digested to remove cell surface proteins including FcμR and to show that IgM-ALA can bind to other non-FcμR receptors on cell membranes. **d** depicts a representative example of a Western blot from two separate experiments demonstrating immunoprecipitation by WT polyclonal IgM of biotinylated membrane proteins from the murine macrophage cell line J77. In this experiment, WT polyclonal IgM is compared with an equal amount of isotype IgM that has no binding activity to leukocytes using flow cytometry. Figure and legend reproduced with permission from J Immunol 2015 Dec 1;195 (11), 5215–26 (panel **a**) J Immunol. 2012 Feb 15;188(4):1675–85 (Panels **b**–**d**). Copyright 2012 and 2015. The American Association of Immunologists, Inc

PDL-1, CD40L and CD80 indicating therefore that IgM-ALA has binding specificity to certain DC receptors (Lobo et al. 2015), just as we observed with human T cell receptors where IgM bound to CD4, CD3 and CD2 but not to CD8 (Lobo et al. 2008a). Additionally, IgM inhibited LPS-induced CD40 upregulation, but not upregulation of CD86, PDL1 and MHCII by BMDC and downregulated basal expression of PD1 on BMDC. IgM also downregulated LPS-induced p65NF-κB activation (Lobo et al. 2015) but not activation induced by LPS+ anti-CD40 (agonistic Ab), thus indicating that IgM can inhibit p65NF-kB upregulation mediated by TLR4 activation, but not when both TLR4 and CD40 are activated (Lobo et al. 2015). Interestingly, IgM inhibited TLR4 activation by a mechanism that did not involve inhibition of LPS binding to cell receptors (Lobo et al. 2015). There was however no decrease in IL12 production or increase in IL10 production when LPS-activated BMDC were pretreated with IgM, indicating therefore that LPS-induced production of IL12 and IL10 are not dependent on p65NF-κB (Lobo et al. 2015), In in vivo studies (described in the next section), we show that IgM pretreatment of LPS-activated BMDC switches these activated BMDC to a regulatory phenotype, which can inhibit innate inflammation induced by reperfusing kidneys following renal ischemia.

We next tested the effect of polyclonal IgM on Type1 NKT cell function. For these in vitro studies, we used α-gal-ceramide, a glycolipid that is taken up by DC and presented via the CD1d MHC Class I-like molecule to Type 1 NKT cells. Only Type 1 NKT, but not T effector cells, will secrete IFN-γ after the invariant TCR on Type 1 NKT recognizes α-gal-ceramide presented by CD1d. In these studies, physiological doses of IgM inhibited α-gal-ceramide induced IFN-γ production of splenic leukocytes, even when IgM was introduced 1 h after α-gal-ceramide (Fig. 4a) (Lobo et al. 2012). We have not yet defined the mechanism for the

Table 2 In-vitro effects of IgM-ALA on human and murine leukocytes

	T cells	NKT-1 cells	BMDC
Cell receptor binding	CD4, CD3, TcR, downregulates CD4, CD2; inhibits HIV entry	TcR, CD4	Binds CD86, CD40, PD-1
Intracellular signaling	Inhibits ZAP-70 activation		Inhibits LPS-induced NFκB activation
Pro-inflammatory mediators	Inhibits production of IFNγ, IL-17, TNFα, IL-2 but not IL-6, MCP-1	Inhibits production of IFNγ, not IL-4	No effect on IL-12 or IL-10
Anti-inflammatory mediators	Enhances production of IL-4, enhances T regs		Switches BMDC to regulatory phenotype (PD-1, IL-10 dependent)
Proliferation	Inhibits T cell proliferation (alloantigen and anti-CD3/28)		

inhibitory effect of IgM on Type 1 NKT function, but it is possible that IgM directly inhibits NKT-1 cells or DC presentation of α-gal-ceramide.

In summary (see Table 2), these in vitro studies indicate that IgM-ALA regulate leukocyte function by binding and downregulating certain leukocyte receptors (e.g., CD4 and CD2 on T cells, CD40 and CD86 on DC) and inducing regulatory DC function, possibly by downregulating NFκB. Physiological doses of IgM regulate leukocyte activation, proliferation and chemotaxis to attenuate excess inflammation (Fig. 4 and Lobo et al. 2008a). There are marked individual variations in the repertoire of IgM-ALA, with specificity for different leukocyte receptors, especially in disease states, and this could potentially explain the differences in the vigor and character of inflammatory responses in different individuals exposed to the same inciting agent. Additionally, there are differences in total levels of IgM-NAA or IgM-ALA as we observed in transplant recipients (Fig. 1) and this could also influence the inflammatory response. Finally, IgM-ALA, by binding to leukocyte receptors and inhibiting cell activation, can provide another mechanism to limit viral entry into cells and replication as we have shown with the HIV-1 (Lobo et al. 2008b).

4.4 Innate Immune Inflammatory Response in Renal Ischemia Reperfusion Injury (IRI) Is Inhibited by IgM-ALA

We used an in vivo murine model of renal IRI to test the inhibitory effects of IgM-ALA on DC and NKT-1 cells (Li et al. 2007). Renal vessels to both kidneys

are completely occluded with clamps for 26 or 32 min to induce either mild or severe ischemic renal tubular injury. After unclamping the blood vessels, the extent of renal injury or decrease in renal function is evaluated at 24 h after reperfusion by quantitating plasma creatinine, which increases as this substance is normally only removed by the kidneys. The initial ischemic injury in this model is insufficient to impair renal function, but it is the innate inflammatory response to products released (after reperfusion) by ischemic renal cells (e.g., DAMPS and glycolipids) that significantly worsen kidney injury, which leads to loss of function. DAMPS and glycolipids released by ischemic renal cells are taken up by DC and in the splenic marginal zone, DC present glycolipids in the context of CD1d to activate NKT cells, which rapidly release IFN-γ to activate innate effector cells, especially granulocytes, macrophages, and NK cells (Li et al. 2007). Chemokines released by ischemic cells enhance extravasation of activated innate effectors from the blood-stream into the kidney interstitium, where these effector inflammatory cells cause further renal tubular injury with loss of kidney function and an increase in plasma creatinine. This acute loss in kidney function is referred to as acute kidney injury (AKI).

Two approaches were employed to test the protective role of IgM in suppressing this renal ischemia-induced innate inflammatory response. First, we performed renal ischemia in B6/S4-IgMko mice (referred to as IgM KO) that lack circulating IgM but have normal levels of other immunoglobulins. Their normal functioning B cells express membrane IgM BCR but are unable to secrete IgM and these mice have normal or increased levels of Tregs, Bregs and IL10. We demonstrated that these mice are very sensitive to renal ischemia and develop AKI with mild ischemia (26 min clamp time), which is insufficient to cause AKI in their WT counterparts (Lobo et al. 2012). Administering 240 μg dose of polyclonal IgM intravenously, to achieve plasma levels similar to that in their WT counterparts, protected these IgM KO mice from developing AKI with mild ischemia, thus indicating that their sensitivity to ischemia resulted from a lack of circulating IgM (Lobo et al. 2012).

A single dose (150 μg) of purified polyclonal IgM was also administered intravenously to wild type C57BL6 (WT-B6) mice to increase levels of baseline circulating IgM by about 30–50%. In this second approach, increasing plasma IgM levels protected WT-B6 from severe renal ischemia (32 min clamp time) (Lobo et al. 2012). This protection was mediated by IgM-ALA, as administering similar quantity of polyclonal IgM pre-adsorbed with activated splenic leukocytes to remove IgM-ALA, failed to protect these WT-B6 mice from severe renal IRI (Lobo et al. 2012).

In these studies, physiological doses of polyclonal IgM mediated protection by decreasing the ischemia-induced innate inflammatory response as we observed a very minimal inflammatory response with no or minimal tubular injury in the protected kidneys. This protective effect of IgM-ALA could be mediated through several mechanisms including regulation of NKT and DC and maintaining or enhancing Tregs, which also mediates protection in this model of innate inflam-mation (Kinsey et al. 2009).

4.5 *Ex Vivo Induced Regulatory DC Are Protective in Renal Ischemia. Regulatory DC Require Tregs, B Cells, Circulating IgM and IL10 to Mediate in vivo Protection*

Since IgM-ALA bound to co-stimulatory receptors and downregulated CD40 and NFκB and had severalfold increased binding to splenic DC, when compared to T cells, we investigated the role of IgM-ALA in regulating DC in this model. In these studies, we used 7–8-day cultures of BMDC, which were activated ex vivo for 48 h with LPS with or without polyclonal IgM. After washing these activated BMDC, 0.5×10^6 BMDC were intravenously infused into mice 24 h before performing renal ischemia. We showed that IgM + LPS pretreated BMDC, but not LPS pretreated BMDC, protected mice from AKI by inhibiting the ischemia-induced inflammatory response that worsens kidney injury (Lobo et al. 2015). Importantly, IgM + LPS-activated BMDC protected kidneys only when IgM was present during the 48 h BMDC culture and not when IgM was added to BMDC at the end of the 48 h LPS activation, indicating therefore that regulation of BMDC by IgM is an active process requiring both NF-κB and CD40 downregulation induced by IgM (Lobo et al. 2015). Preventing downregulation of NFκB and CD40 by adding the agonistic anti-CD40 antibody to LPS + IgM during the 48 h BMDC activation negated the protective effect. These studies would indicate that NF-κB and CD40 downregulation are required to switch activated BMDC to a regulatory phenotype (Lobo et al. 2015). It is possible that binding of IgM to CD40 induces this regulatory phenotype.

In these studies, we needed to exclude the role of IgM anti-PC in mediating this anti-inflammatory effect, especially since there are 20–30% apoptotic cells in the ex vivo culture of IgM + LPS pretreated BMDC (Chen et al. 2009). Such a possibility seemed unlikely, as in prior studies mice were administered large numbers of apoptotic cells (2.5×10^7 thymocytes) to induce regulatory activity of in vivo antigen presenting cells (APC) (Chen et al. 2009), while in our studies, only 0.5×10^6 BMDC were used (Lobo et al. 2015). However, to exclude this possibility, we increased apoptosis in the ex vivo pretreated BMDC to >80% by subjecting activated LPS + IgM pretreated BMDC to UV irradiation. Such UV-irradiated apoptotic LPS + IgM pretreated BMDC failed to protect mice from ischemia-induced AKI, thus excluding the role of apoptotic cell/IgM complexes in inducing protection. These studies clearly demonstrated that IgM-ALA mediated protection by switching ex vivo LPS-activated BMDC to a regulatory phenotype. Regulatory BMDC required IL10 but not IDO (indoleamine 2,3-dioxygenase) as IgM + LPS pretreatment of *Il10* ko BMDC, but not *IDO* ko BMDC, failed to protect mice from developing AKI after renal ischemia (Lobo et al. 2015) In further studies, we also show that injected regulatory BMDC require the presence of other in vivo suppressive mechanisms such as circulating IgM, IL10, Tregs and B cells to mediate protection (Lobo et al. 2015).

In summary, both the in vitro and in vivo studies indicate that IgM-ALA inhibits the ischemia-induced innate inflammatory response by several mechanisms,

including switching activated DC to a regulatory phenotype, inhibiting NKT cell IFN-γ production and inhibiting chemotaxis of leukocytes by binding to chemokine receptors. However, IgM-ALA require the presence of other in vivo suppressive mechanisms such as IL10, Tregs and B cells to effectively inhibit the ischemia-induced inflammatory response. Conversely, these other in vivo suppressive mechanisms such as Tregs and Bregs also require IgM-NAA to effectively inhibit this innate inflammatory response as *Igm* ko mice that lack secretory IgM were sensitive to mild ischemia despite having normal levels of Tregs and Bregs (Lobo et al. 2015).

4.6 Inflammation Mediated by Adaptive Immune Mechanisms in Allograft Transplantation Is Inhibited by Polyclonal IgM

In these studies we used two approaches to test if IgM could inhibit allograft rejection. These studies were prompted by several prior observations including our clinical observations (Fig. 3) and the in vitro studies demonstrating that IgM (a) inhibited alloantigen-activated T cell proliferation and differentiation into Th1 and Th17 independently of DC (Fig. 4) and (b) induced regulatory function in DC (Lobo et al. 2012). First, cardiac transplants were performed intra-abdominally in *Igm* KO mice using B6-bm12 donor hearts, which are minimally incompatible at the MHC class II locus (Ia) with the recipient. In this transplant model, cardiac rejection occurs at >2 months in WT recipients as there is a mild chronic form of cellular rejection and a vasculopathy that is initiated by a T cell-mediated inflammatory process. However, in *Igm* KO recipients, graft loss occurred significantly earlier, i.e., at 2–3 weeks (Lobo et al. 2012). Additionally, there were considerably more TH17 cells infiltrating the cardiac allograft in the *Igm* KO recipients, despite no significant difference in infiltrating Tregs between the groups (Lobo et al. 2012). The observed histological findings on T cells mirror the in vitro studies, where IgM inhibited naïve T cells and Foxp3+ T cells from differentiating into TH17 cells without affecting levels of Tregs (Fig. 4d).

In the second approach, circulating levels of IgM in WT-B6 mice were increased by intravenous IgM injections to determine if higher IgM levels inhibited the severe and rapid rejection that occurs by day 5 in the setting of fully MHC-incompatible donor hearts (i.e., from BALB/c donors) (Lobo et al. 2012). 175 μg IgM was administered 24 h *after* ascertaining that cardiac surgery was successful, and the dose of IgM was repeated on days 3 and 5. Histological evaluation on day 6 clearly demonstrated that IgM markedly inhibited the severe inflammation in the cardiac allograft induced by rejection. This lack of leukocyte infiltration in the cardiac parenchyma of IgM-treated recipients was also associated with no or minimal CXCL1+ leukocytes and with no or minimal fragmentation of capillaries, as identified by the endothelial cell marker CD31 (Lobo et al. 2012).

In summary, these studies indicate that physiological doses (175 μg) of polyclonal IgM can abrogate inflammatory responses mediated by an adaptive immune mechanism. Potential mechanisms include (a) a direct inhibitory effect of IgM-ALA on T effector cells, but not Tregs. In vitro studies have shown that IgM can bind and down-modulate CD3/TCR and certain specific co-stimulatory receptors such as CD4 and CD2 but not CD8, and these mechanisms could be involved in inhibiting T effector cell proliferation and production of certain specific cytokines (e.g., TNF, IFN-γ, IL17, but not IL6, and chemokines) as well as in inhibiting their differentiation into TH1 and TH17 pro-inflammatory cells (b) by binding of IgM to CD40 and switching activated DC to a regulatory phenotype with downregulation of CD40 and p65NF-κB and (c) by inhibiting chemotaxis. It is highly unlikely that IgM anti-PC could have a significant role in inhibiting allograft rejection in our studies as we used small doses of polyclonal IgM (175 μg) and did not infuse large numbers of apoptotic cells (Chen et al. 2009).

4.7 Autoimmune-Mediated Insulitis in NOD Mice Is Inhibited by Polyclonal IgM

Insulitis in the NOD mouse is primarily mediated by autoimmune T cells but there is data to indicate that B cells are also involved (Kendall et al. 2004; Xiu et al. 2008; Ryan et al. 2010). Adoptively transferring CD3+ T cells from diabetic NOD mice can rapidly (<2 weeks) induce diabetes mellitus (DM) in young 4–5-week-old non-diabetic NOD mice thus indicating that T effectors are primarily involved in islet cell injury in this model. Because our in vitro studies demonstrated that polyclonal IgM inhibited T cell proliferation and differentiation into TH1 and TH17 cells (Fig. 4b, c, d), we performed studies to determine whether IgM could inhibit autoimmune insulitis that results in islet cell destruction and DM in NOD mice (Chhabra et al. 2012). Around 4–5 weeks after birth, these NOD mice develop a silent and non-destructive inflammatory process characterized by leukocyte infiltration of the perivascular and periductal regions in the pancreas as well as the peripheral islet regions and consisting of a heterogeneous mixture of CD4 and CD8 T cells, B cells, macrophages and DC (peri-insulitis). At 8–12 weeks of age, the immune infiltrate enters the islet areas and induces beta cell destruction (insulitis) and significant destruction first becomes evident around 12–13 weeks of age with mice exhibiting overt diabetes (DM).

In these studies, the effect of increasing IgM levels on development of DM was studied (Chhabra et al. 2012). At 5 or 11 weeks of age, NOD mice were administered bi-weekly intraperitoneal polyclonal IgM (50 μg/dose) and IgM was discontinued when mice were 18 weeks old. At 25 weeks of age, 0% of mice ($n = 30$) treated with IgM beginning at 5 weeks developed DM while 80% of control mice ($n = 30$) developed DM. Importantly, only 20% of pre-diabetic mice ($n = 20$) treated with IgM beginning at 11 weeks of age developed DM at 25 weeks of age.

At 18–25 weeks of age the pancreas revealed no or minimal insulitis in NOD mice treated with IgM beginning at 5 weeks of age. Other investigators using monoclonal polyreactive natural IgM in the neonatal period have also obtained similar results (Andersson et al. 1991, 1994). Importantly, despite discontinuing IgM at 18 weeks of age, the majority (73%) of NOD mice were protected from developing insulitis even when evaluated at 28 weeks of age, thus indicating that the anti-inflammatory effect of IgM also involves the induction of other regulatory mechanisms.

In summary, the beneficial effects of polyclonal IgM in inhibiting autoimmune insulitis could be mediated via several mechanisms including inhibition of autoimmune T effectors, blocking IgG autoantibodies via anti-idiotypic mechanisms and by inhibiting the B cells that produce them. Additionally, IgM, by switching activated DC to a regulatory phenotype and maintaining Tregs could enhance this protective effect.

5 Pathogenic Effects of IgM-NAA Under Non-physiological Conditions

In this section we will show how protective IgM-NAA can, under non-physiological conditions, become pathogenic and induce inflammation.

1.1 *Binding of IgM-NAA at cold temperatures can induce these antibodies to become pathogenic*: This is best exemplified in human kidney transplant recipients having high IgM-ALA and IgM anti-endothelial cell antibody (IgM-AEA) levels at the time of the kidney transplant. These recipients have a high incidence of delayed kidney graft function (DGF) (Lobo et al. 1984; Sturgill et al. 1984). DGF occurs when, after vascular anastomosis, warm blood is allowed to flow into a cold kidney. At cold kidney temperatures, binding of IgM-AEA to glomerular endothelial cells causes complement-induced glomerular endothelial cell injury. We show that this self-limiting injury can be prevented by warming the kidney prior to re-instituting blood flow. Such observations highlight the nature of IgM-NAA, i.e., their potential for complement mediated cytotoxicity under non-physiological cold conditions (Terasaki et al. 1970; Winfield et al. 1975; Lobo 1981).

1.2 *Binding of natural IgM to unmasked neoantigens can induce these antibodies to become pathogenic.* This is best exemplified by unmasking of the ubiquitous neoantigen "non-muscle myosin heavy chain type IIA and C (NMM)" after acute ischemia to the small bowel, skeletal muscle (hind limb) and heart in mice. About 1–2% of IgM-NAA B-1 cell clones in mice secrete IgM anti-NMM, probably to protect against NMM derived from infectious organisms (Zhang et al. 2004; Betapudi 2014). Injury in this murine model is predominantly mediated during

reperfusion by innate inflammation triggered by IgM binding to the unmasked NMM neoantigen and activation of complement (Austen et al. 2004; Zhang et al. 2004, 2006, 2008). Hence *Rag1* KO mice, which lack IgM-NAA, are normally protected from ischemia to the small bowel or hind limb but succumb to ischemic injury after infusion of polyclonal IgM or monoclonal IgM anti-NMM (Zhang et al. 2004, 2006). Additionally, in this murine model, one can observe binding of IgM and complement to NMM expressed by ischemic epithelial cells in the bowel or striated muscle cells in the hind limb (Austen et al. 2004; Zhang et al. 2004).

Interestingly, IgM anti-NMM mediated innate inflammation is not observed after renal ischemia, even though endothelial cells in murine glomeruli and peritubular capillaries express NMM (Arrondel et al. 2002; Renner et al. 2010). It is possible that NMM in the peritubular capillaries or in the tubules is not unmasked after ischemia. After renal IRI, one can detect increased IgM binding to glomeruli but not to the extensive network of NMM-containing capillaries that surround the outer medullary renal tubules where most of the ischemia-induced kidney injury occurs (Renner et al. 2010). Additionally, depleting B1 cells did not protect tubules from renal injury, but decreased glomerular injury, thus indicating that the tubular injury seen after renal ischemia is not mediated by natural IgM and complement (Renner et al. 2010). Other studies would indicate that the inflammatory response after renal ischemia is mediated by innate immune cellular mechanisms involving NK and NKT cells, which are activated by products (DAMPS) released by ischemic tubules (Li et al. 2007). Hence, unlike small bowel or hind limb ischemia, *Rag1* KO mice and *Igm* KO mice without secretory IgM are not protected from renal ischemia (Park et al. 2002; Burne-Taney et al. 2005; Lobo et al. 2012; Gigliotti et al. 2013; Lobo et al. 2015).

1.3 *Pathogenesis mediated by non-physiologic expansion of specific IgM-NAA clones*. This is best exemplified by hepatitis C-induced expansion of certain B1 cell clones that specifically secrete IgM-NAA that binds to self IgG, i.e., rheumatoid factor (RhF). Excess RhF production predisposes to formation of large circulating IgM/IgG complexes, referred to as cryoglobulins as these complexes precipitate ex vivo in the cold, which cause thrombosis of small blood vessels, especially in the kidney glomeruli and skin (Charles and Dustin 2009; Gorevic 2012). Patients are treated by plasmapheresis to remove cryoglobulins and agents to deplete B cells. There is no good explanation as to why expansion of RhF-secreting B1 cell clones is commonly seen after chronic hepatitis C infection and, in addition, we do not understand the normal physiological role of RhF, even though RhF was the first IgM-NAA to be discovered.

6 Conclusion

Figures 6 and 7 summarize our concepts regarding the inter-relationship between pathogens and natural antibodies. In both murine models and humans, the evidence shows that these polyreactive and low affinity binding IgM-NAA function under physiological conditions to (i) provide a first line of defense against invading microorganisms, (ii) protect the host from autoimmune inflammation mediated by autoimmune B2 and T cells that have escaped tolerance mechanisms, (iii) protect the host from endogenous oxidized neodeterminants and other neoantigens that are unmasked during tissue damage, and (iv) regulate excess inflammation mediated by both innate and adaptive immune mechanisms. The full repertoire of IgM-NAA develops during the first few years of life, but their levels and repertoire differ among healthy individuals, as well as in disease, and could contribute to the varying inflammatory response as, e.g., after an infection or alloantigen exposure. We hypothesize that infections maintain high protective levels of IgM-NAA, especially IgM-ALA and anti-PC (reviewed in Adib et al. 1990; Kearney 2000; Lobo et al. 2008a) and this could explain the significantly low incidence of autoimmune disorders such as SLE or sarcoidosis in rural parts of Africa, where malaria and other infections are endemic (Greenwood 1968; Lobo 1972; Jacyk 1984; Symmons 1995). Further support for this concept comes from a murine model of SLE, where malaria infection or purified IgM from malaria infected mice protected NZB mice from SLE-induced renal failure and death (Greenwood et al. 1970; Hentati et al. 1994). IgM-NAA have an important role in regulating inflammation even though there are other suppressive mechanisms (e.g., Tregs, Bregs, IL10, TGFβ). We show that IgM-NAA require Tregs, B cells and IL10 to effectively regulate inflammation (Lobo et al. 2015). Conversely, our studies and that of others, using mice deficient

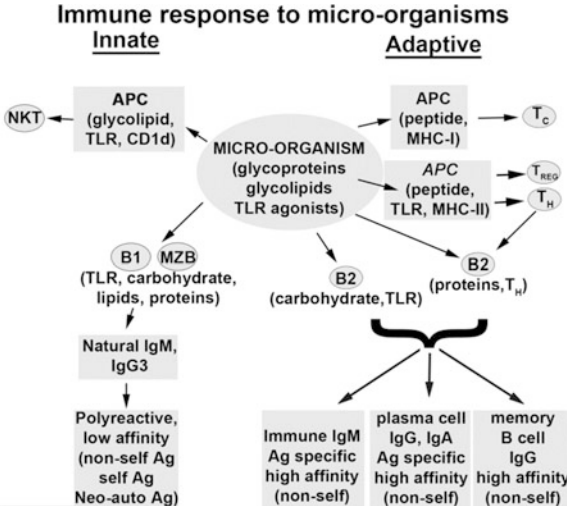


Fig. 6 Immune response to microorganisms

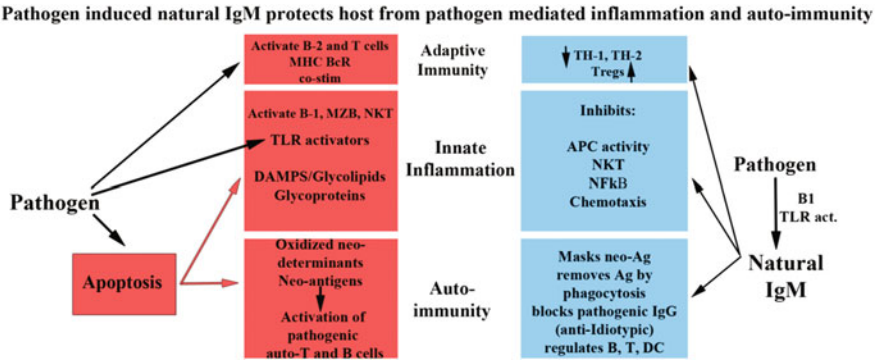


Fig. 7 Pathogen-induced natural IgM protects the host from pathogen-mediated inflammation and autoimmunity

in IgM secretion, would also indicate that Tregs and Bregs also require IgM-NAA to effectively control inflammation (Boes et al. 2000; Ehrenstein et al. 2000; Lobo et al. 2012). Understanding how diverse infectious agents increase IgM-ALA would help with development of a vaccine to increase IgM-NAA. We need to also determine if prolonged high IgM-NAA levels can induce excess immunosuppression that may be detrimental to the host. Cell therapy, especially with IgM pretreated DC, could provide an alternative approach requiring minimal quantities of IgM to prevent ischemic acute renal failure (e.g.,in high-risk patients undergoing cardiac surgery) or delayed graft function after renal transplantation (Lobo et al. 2015).

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