Functional characterization of the high affinity IgG receptor

-making heads and tails of FcγRI-

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CONTENTS

Chapter 1	Introduction: Functional characteristics of the high affinity IgG receptor, FcγRI	9
	Scope of thesis	24
Chapter 2	Cytokine induced immune complex binding to the high affinity IgG receptor, FcγRI, in the presence of monomeric IgG	33
Chapter 3	Filamin A stabilizes FcγRI surface expression and prevents its lysosomal routing	53
Chapter 4	Protein 4.1G binds to a unique motif within the FcγRI cytoplasmic tail	73
Chapter 5	Colocalization of 4.1G and actin during FcγRI induced phagocytic cup formation	87
Chapter 6	General discussion	103
	Nederlandse samenvatting	113
	Dankwoord	121
	Curriculum Vitae	125
	Publications	127
	Abbreviations	129
	Color section	131

Chapter 1

Introduction: Functional characteristics of the high affinity IgG receptor, FcγRI

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ABSTRACT

IgG Fc-receptors are important mediators of immunity and play a key role during antibody based immunotherapy. Within the leukocyte IgG receptor family, only FcγRI is capable of IgG binding with high affinity. FcγRI exists as a complex of a ligand binding α-chain and an FcR γ-chain. The receptors' α-chain can, furthermore, elicit several functions independent of the ITAM bearing FcR γ-chain. Functional implications of high affinity IgG binding and mechanisms underlying FcR γ-chain independent signaling remain unclear to this day. Here we provide an overview of past literature on FcγRI and address the implications of recently described interactions between cytosolic proteins and the FcγRI α-chain, as well as cytokine enhanced FcγRI immune complex binding. Furthermore, an analysis of potential polymorphisms within the *FCGR1A* gene is provided.

INTRODUCTION

Antibodies are key players in immunity. The specificity and potent immune-activating properties of antibodies, furthermore, are exploited during monoclonal antibody (mAb) immunotherapy. Fc receptors have been shown to play a significant role in clearance of tumor cells during antibody therapy[1]. All 28 mAb currently accepted for clinical use by the United States FDA are of the IgG isotype, making IgG antibody interacting receptors a major field of interest.

FcγRI is a unique member within the Fcγ receptor family as it represents the only Fcγ receptor which can bind IgG with high affinity. This property initially sparked significant interest of the Fc receptor field. Over the years however, attention shifted to other Fc receptors as the high affinity IgG binding was postulated to result in the receptors' saturation by serum IgG possibly hampering binding of opsonized pathogens or tumor cells[2,3]. As a result, the popularity of FcγRI has waned. In the last few years however, the number of publications addressing FcγRI increased profoundly. Here, we give an overview of current knowledge on FcγRI, including recent studies on interactions with cytosolic proteins and the role of FcγRI in immunity and antibody based therapies. We analyzed putative SNPs within the *FCGRIA* gene and discuss their potential impact on FcγRI functioning based on a structural model of the receptor.

THE Fc RECEPTOR FAMILY

Antibodies may exert their effects either directly by neutralizing pathogens or toxins, or indirectly by activating the immune system via complement or Fc receptors. Antibodies can bind to membrane bound Fc receptors that serve as an adaptor between leukocytes and opsonized pathogens, allowing ingestion and killing of pathogens by leukocytes. Opsonization of pathogens or tumor cells causes immobilization of multiple Ig molecules, leading to Fc receptor crosslinking. This may result in phagocytosis, antigen presentation, the production of ROS and cytokines, and in the case of opsonized tumor cells, antibody dependent cellular cytotoxicity (ADCC)[4].

There are five Ig classes (IgM, IgA, IgD, IgG, IgE), and most of these classes can bind to their own respective Fc receptor class. For instance, IgA can bind to FcαRI, IgE binds to FcεRI and FcεRII, Fcα/μR can bind both IgM and IgA, and IgD receptors have been described[5]. IgG represents the most abundant Ig isotype in serum, and can interact with the neonatal Fc receptor (FcRn) and the leukocyte Fcγ receptors. FcRn is widely expressed including on syncytiotrophoblasts, which form the barrier between fetus and mother, vascular endothelium and leukocytes from the myeloid lineage[6]. FcRn is an MHC-I like molecule involved in the transfer

of maternal IgG to neonates, prolongation of IgG half life by preventing its breakdown by the vascular endothelium and has been suggested to play a role during phagocytosis[6,7].

The Fcγ receptor family in humans is comprised of the activating receptors, FcγRI, FcγRIIA, FcγRIIC and FcγRIIIA, and an inhibitory receptor, FcγRIIB[2,3,8-10]. FcγRI and FcγRIIA are expressed on most myeloid cells including monocytes, macrophages, and DC. In addition, granulocytes express FcγRI upon activation, and FcγRIIA can also be expressed on platelets and B cells. IFNγ is a potent stimulator of FcγRI expression on all of the aforementioned cell types via the IFNγ response region in the FcγRI promoter[11]. FcγRIIIA is primarily expressed on B cells, NK cells, DCs and a subset of monocytes. FcγRIIB is also expressed on these cell types with the exception of NK cells. FcγRIIC is thought to be the product of an unequal crossover event between the *FCGR2A* and *FCGR2B* genes. NK cells and CD14 positive monocytes and/or neutrophils have been shown to express mRNA for this receptor and FcγRIIC protein expression was confirmed on NK cells[12]. FcγRIIIB codes for a GPI-anchored receptor, which is only expressed by neutrophils. Coclustering with FcγRIIA has been implicated in signal initiation by FcγRIIIB [13].Our insights into the role of Fc receptors have benefited largely from studies in knock out mouse models. In mice, FcγRI has similar high affinity binding properties as its human ortholog. Although its extracellular structure is comparable, there are considerable differences in intracellulair domains between mice and man (only 19% sequence similarity[14]). Mice lack the activating form of FcγRII but do express FcγRIV, which shows 63% sequence similarity with human FcγRIII (a comprehensive comparison of mouse and human Fc receptors was reviewed by Nimmerjahn and Ravetch [9]). Importantly, murine FcγRI and FcγRIV preferentially bind mIgG2a, where in contrast to FcγRI, FcγRIV binds this isotype with moderate affinity[15]. Mouse models have clearly underlined the role of various Fc receptors in normal immunity, in various disease models and antibody therapy. Notably, based on sequence data and the existence of FcγRIV, considerable differences exist between murine and human Fc receptor families.

THE FcγRI COMPLEX

FcγRI consists of a ligand binding α-chain and an ITAM bearing FcR γ-chain. For the human α-chain, there are three genes (*FCGR1A-C*)[16,17] located on chromosome 1 reported to encode at least 6 transcripts[18]. Due to the cassette like structure of the *FCGR1A* gene (each Ig domain is encoded by a separate exon) it was previously thought that splice variants could encode for putative low affinity (FcγRIb2) or soluble receptors (FcγRIb1, FcγRIc). However, it has been shown that the transcript coding for a low affinity receptor, FcγRIa2 is not expressed on the plasma membrane of transfectants[19]. It would seem that the bona fide high affinity IgG receptor is encoded by the FcγRIa1 transcript.

Most FcγR are highly homologous in their extracellular domains. The extracellular domains of FcγRII and FcγRIII consist of two Ig like domains. FcγRI is unique in its membrane proximal third Ig domain (EC3). Up to date, neither the crystal structure of FcγRI, nor a complete model including EC3 has been reported. Applying the latest modeling servers, we were able to generate a complete putative model which is further discussed below.

FcγRI-IgG INTERACTION

It has been suggested that both EC2 and EC3 contribute to FcγRIs high affinity[20]. This finding was based on observations made with chimaeric receptors where FcγRIs EC domains were replaced with EC domains of FcγRII. Studies on murine FcγRI suggested a similar model[21]. Notably, FcγRIs EC3 of is critical for its high affinity but cannot convey this property to a low affinity receptor such as FcγRII.

Comparing amino acid sequences and models of FcγRI with FcγRII and FcγRIII clearly suggests a binding site for IgG in the first two EC domains. The trypthophan residues which are important for the binding of proline 239 of IgG are shared by all Fcγ receptors, including FcγRI. Furthermore, EC2 of FcγRII and III contains a hydrophobic pocket important for IgG binding [22]. It seems that this pocket is more hydrophobic in FcγRI, thus contributing to the higher affinity of the receptor which coincides with the finding that EC2 is needed for a high affinity receptor[20]. Given the homology between the various Fcγ receptor members in EC1 and EC2, and the suggested structure of these domains of FcγRI it would be hard to envisage EC3 directly contributing to the IgG bindingsite (see also Figure 2C).

FcγRI DOWNSTREAM SIGNALING

As most activating Fc receptors (the only exception being human FcγRIIa), FcγRI is largely dependent on the FcR γ-chain for signaling. The FcR γ-chain contains a classic ITAM motif which is a key signaling element. Signal initiation occurs after crosslinking of the receptor complex, leading to ITAM phosphorylation by Src kinases such as hck and lyn[23], and subsequent recruitment of Syk[24]. The Syk kinase, normally in an auto-inhibited state, is activated upon interaction with its two tandem SH2 domains to phosphorylated ITAM motifs[25]. Interestingly, although Syk is critical for phagocytosis via Fcγ receptors, actin polymerization still occurs upon receptor crosslinking in Syk deficient macrophages, suggesting an additional role for Src kinases in Fc receptor mediated phagocytosis[26]. Indeed, Src Kinases are known to activate WASP, a protein involved in ARP2/3 activation which is important for actin polymerization[27].

Several proteins, including Vav, PLCγ, PI3K and SLP76 can interact with Syk to initiate further downstream signaling leading to cytoskeletal changes, ROS and cytokine production (for a

schematic overview of FcγRI signaling see Figure 1). The guanine nucleotide exchange factor Vav, was shown not to be involved during FcγR mediated phagocytosis by bonemarrow derived macrophages, although phagocytosis was dependent on the GTPase Rac[28]. SLP-76 is needed for FcγR mediated ROS production in neutrophils and is phosphorylated upon FcγRI crosslinking in macrophages[29,30]. However, SLP-76 deficient macrophages showed no signs of altered phagocytosis or ROS production[29,31]. Most studies used immunecomplexes rather of monoclonal Ab to crosslink Fc receptors, therefore it is not possible to draw conclusions on FcγRI specific signaling. It should be noted however, that FcγRI is expressed on mouse macrophages but not, or only in low levels, on mouse neutrophils. Transmembrane adaptor proteins (TRAPs) LAT and LAT2 (also known as NTAL or LAB) can be phosphorylated by Syk and Src kinase Lyn,[32,33] and have been shown to be critically involved in myeloid ITAM signaling[34-36]. These TRAPs can recruit Grb2, PLCγ1, Sos1, Gab1 and c-CBL leading to further signal propagation. It has been suggested that FcγRI, but not FcγRIIa crosslinking leads to phosphorylation of LAT2 in THP-1 cells. Other studies have also shown differential signaling between FcγRI and FcγRIIa[37], although both receptors signal in a Syk dependent manner[38,39]. The basis for the observed differential signaling between various Fcγ receptors remains unknown, but could be due to differences in ITAM motifs (FcγRIIA has a non cannonical ITAM motif). Most studies rely either on general Fcγ stimulating immune complexes or use cell lines that do not express Fc receptor at a similar level, complicating qualitative differentiation between Fc receptor signaling. Alternatively, differential signaling between Fc receptors may be mediated by the ligand binding α-chains.

INTERACTORS OF THE FcγRI LIGAND BINDING α-CHAIN

Van Vugt et al were the first to describe a role for FcγRI ITAM independent effector functions. Using transfectants expressing an ITAM mutated γ-chain (referred to as NOTAM) and cytoplasmic truncations of the α-chain the authors described a region in the α-chain that triggered antigen presentation[40]. Other studies documented abrogated IL-6, but not IL-1β induction and decreased phagocytosis by a cytosolically truncated FcγRI, and suggested FcγRI α-chain to enhance γ-chain signaling[41,42]. Based on these findings, our laboratory performed yeast two-hybrid screens with the FcγRIa cytosolic tail (FcγRI-CY) and documented the interaction of FcγRI-CY with cytosolic proteins periplakin, 4.1G and filamin A [43-45].

Filamin A is known for its actin filament chelating properties. It comprises a flexible homodimer of two 280 kDa subunits each containing an actin bindin domain at its N-terminus. Using filamin-deficient cell lines and siRNA knockdown, we found filamin A to be necessary for stable FcγRI surface expression[44]. FcγRI crosslinking abolished FcγRI-filamin colocalization in monocytes, in agreement with previous studies[46]. We found furthermore that, in the absence of filamin A, FcγRI relocated to a lysosomal compartment suggesting filamin to either

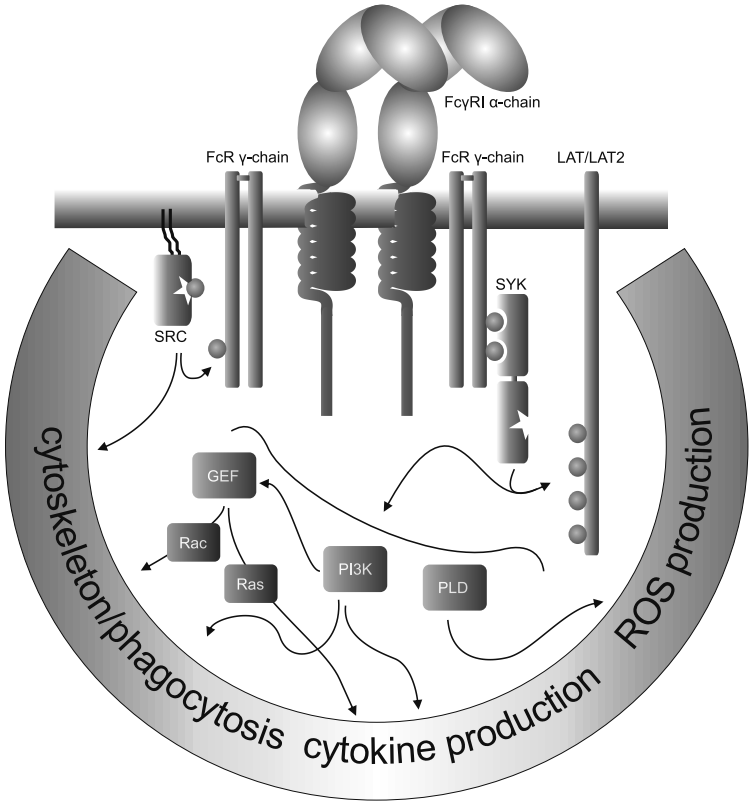

Figure 1. Signaling dowstream of FcγRI. Shown are several key players and downstream signaling effects of FcγRI. Crosslinking of the α-chain by multivalent ligand causes activation of Src kinases (SRC) such as Hck, Fgr and Lyn. These kinases phosphorylate the ITAM motifs within the FcR γ-chain. Phosphorlyated ITAMs form a docking site for the tandem SH2-domain of the kinase Syk. Conformational changes in Syk lead to activation of its kinase domain. Syk is important for phagocytosis, but not for initial actin remodeling, suggesting additional roles for Src kinases in this process. Syk is capable of phosphorylating TRAPs such as LAT and/or LAT2. These serve as docking sites for Grb2, Sos1, Gab1 and c-Cbl (not shown). PI3-K can potentially be activated via this complex, although it can also directly bind to Syk. PI3-K activity leads to generation of position D3 phosphorylated inositol phosphate lipids thus recruiting pleckstrin homology domain containing proteins such as PKB (not shown) and GEFs.GEFs play an important role in downstream signaling and mediate cytoskeletal rearrangement and activation of the MAPK pathways leading to cytokine production. PLD was shown to be specifically activated upon FcγRI crosslinking leading to ROS production.

prevent internalization by tethering FcγRI to the cortical actin cytoskeleton or shielding off domains in the α-chain needed for internalization and subsequent antigen presentation.

Protein 4.1G is a member of the band 4.1 family, which contain a Four-point-one, Ezrin, Radixin, Moesin (FERM) domain, a Spectrin Actin Binding domain (SABD) and a C Terminal Domain (CTD), each interspaced by three unique regions[47-49]. 4.1G was found to interact with FcγRI-CY in a membrane proximal domain, which appears to be mainly shared by G-coupled

receptors[45]. The best characterized member of the 4.1 family is 4.1R, which plays a central role in organization of erythrocyte membrane skeleton[48]. Moreover, SABD domains from 4.1R and 4.1G proteins show localization at cell-cell contacts and were suggested to be involved in organization of spectrin-actin complexes in neuronal cells[50]. 4.1G has been implicated to play a role in membrane stabilization of Glutamate receptor subunits GluA1 and GluA4[51,52]. Studies in mice deficient in 4.1G and significantly knocked down 4.1N, however, did not support an essential role for these 4.1 proteins in GluR functioning[53]. Two studies reported a role for 4.1 proteins in the regulation of T cell signaling. Ralston et al documented interactions between 4.1G and PTA-1 and suggested a role for 4.1G during the formation of the immunological synapse[54]. In a recent study using 4.1R knockout mice, Kang et al showed 4.1R to negatively regulate LAT phosphorylation, thereby inhibiting TCR signaling. The role of 4.1G during FcγRI signaling, however, needs to be further elucidated.

Periplakin was initially described as a constituent of desmosomes and the cornified envelope of epidermal keratinocytes[55,56]. It consists of an N terminal plakin domain allowing membrane localization, a coiled-coil rod domain for homodimerization or dimerization with envoplakin and a C-terminal linker subdomain which binds to intermediate filaments[57,58]. We previously documented the interaction between FcγRI-CY and a stretch within the periplakin linker domain[14,43]. Notably, periplakin has also been described to interact with the μ-opoid receptor and the melanin concentrating hormone receptor-1 (MCH-1) which, similarly to FcγRI, adopt an alpha-helical structure containing a stretch of three hydrophobic amino acids followed by two basic amino acids within the periplakin binding domain[59,60]. Inhibition of association of endogenous periplakin with FcγRI using small periplakin derived peptides increased FcγRI ligand binding in murine IIA1.6 cells[14,43]. Endogenous periplakin may therefore decrease FcγRI ligand binding. It has been speculated that decreased ligand binding could facilitate replacement of prebound monomeric IgG by immune-complexes. In a recent study, however, we documented cellular activation by cytokines to enhance immune complex binding, with marginally affected binding to monomeric IgG[61]. Cytokine enhanced ligand binding lead to binding of immune complexes despite FcγRI saturation by monomeric IgG. In the models employed in the latter study, cytokine enhanced binding appeared periplakin independent. Periplakin may well function as a signaling adaptor, as it has been documented to bind to PKB. The PI3K-PKB signaling axis is crucial for efficient Fc receptor mediated phagocytosis[62]. Studies using periplakin deficient mice[63], crossed with human FcγRI transgenic mice, may shed new light on the biological role of periplakin modulated FcγRI ligand binding and signaling.

Overall, the identification of proteins interacting with the FcγRI α-chain has added a new layer of complexity in the functioning of this receptor. Although filamin prevents default FcγRI α-chain degradation and as such may contribute to γ-chain independent antigen presentation, the physiological importance and molecular mechanism of FcγRI α-chain signaling is still unclear.

ROLE OF FcγRI IN IMMUNITY

The role of FcγRI in immunity remains unclear. Knockout mice documented a critical role for FcγRI in models of *Bordetella pertussis* clearance and arthus reaction[64-66]. These studies, furthermore, suggested a role for FcγRI in DC antigen presentation, in an *in vivo* model of heamolytic anemia, and during mIgG2a induced anaphylaxis and antigen induced arthritis. These models however, also support a considerable redundancy with other FcγR, such as FcγRIII and FcγRIV[65] which may be partially attributable to the use of mIgG2a, and overlapping isotype specificity between murine FcγRI and FcγRIV.

Human FcγRI deficiency in four related individuals did not appear to lead to overt increased susceptibility to infections[67,68]. It remains unclear how FcγRI contributes to clearance of preformed immune complexes. The high affinity nature of FcγRI has been postulated to hamper interaction with immune complexes under serum conditions[2]. Notably, the high IgG content in serum (7-16 g/l for adults) is theoretically sufficient for near saturation of low affinity Fcγ receptors by serum IgG (Table 1). Several hypotheses have been proposed to explain FcγRIs' role in immunity: I) due to its high affinity, FcγRI is 'pre-armed' with IgG and functions as a type of scavenger receptor, either analogous to the high affinity receptor for IgE, FcεRI, or as a sampling receptor of extracellular antigens, II) local inflammation leads to production of IFNγ causing *de novo* synthesis of free FcγRI, or III) reorganization of the receptor on the membrane and/or conformational changes leads to preferred binding to multimeric IgG i.e. immune complexes (inside out signaling). Although there is no direct evidence supporting the first hypothesis in humans, macrophages may well encounter antigens or pathogens that have not been in contact with IgG yet. FcγRI is known to internalize from the plasma membrane upon binding of monomeric IgG and could as such sample a constant source of extracellular antigens[69]. Indeed, non-crosslinking FcγRI specific m22-F(ab') fragments induce anti m22 idiotype titers in FcγRI transgenic, but not in wildtype mice [70]. Cytokine enhanced upregulation of FcγRI is well documented and leads to significant increases in receptor expression levels, often in concert with downregulation of negative regulators such as FcγRIIB[71-73]. Inside out regulation is a relatively new phenomenon with respect to Fc receptors. In analogy with integrins, it has been shown for FcγRIIA and FcαRI that cytokine stimulation leads to a fast (typically within 15-30 minutes) increase in ligand binding independent of changes in receptor expression levels[74-76]. As for FcγRI, it was shown previously that receptor affinity can be changed by associated proteins[14,43,77] and we have observed cytokine stimulation to induce more efficient competition between immune complexes and pre-bound monomeric IgG for FcγRI[61]. Mouse macrophages were found incapable of inside out signaling, and mice may well depend more on the moderate affinity IgG receptor FcγRIV, which does not bind monomeric IgG [9,15]. Thus, in humans, FcγRI inside out regulation may allow the high affinity receptor to contribute to immune complex binding and uptake, despite saturation by serum IgG.

Table 1. Fc receptors are nearly saturated with ligand at serum IgG levels.

	IgG1		IgG2		IgG3		IgG4	
	Mw (kDa)	146	Mw (kDa)	146	Mw (kDa)	170	Mw (kDa)	146
	serum g/L	5.9	serum g/L	3.4	serum g/L	0.6	serum g/L	0.2
	serum mol/L	4.04E-05	serum mol/L	2.33E-05	serum mol/L	3.53E-06	serum mol/L	1.37E-06
Fc receptor	Kd	%occupied	Kd	%occupied	Kd	%occupied	Kd	%occupied
FcγRI	1.54E-08	100.0		100.0	1.64E-08	100	2.94E-08	97.9
FcγRIIa H131	1.92E-07	99.5	2.22E-06	91.3	1.12E-06	76	5.88E-06	18.9
FcγRIIa R131	2.86E-07	99.3	1.00E-05	70.0	1.1E-06	76	4.76E-06	22.3
FcγRIIIa F158	8.55E-07	97.9	3.33E-05	41.1	1.3E-07	96	5.00E-06	21.5
FcγRIIIa V158	4.83E-07	98.8	1.43E-05	62.0	1.02E-07	97	4.00E-06	25.5

The percentage of occupied receptors is equal to the ligand concentration divided by the sum of the ligand concentration and the dissociation constant (Kd). The Kd was calculated from the association constants reported by Bruhns et al[102]. FcγRI was found not to bind to IgG2.

ROLE OF FcγRI DURING IMMUNOTHERAPY

Whereas work in FcR γ-chain knockout mice (which do not express activating Fc receptors) provided information on the role of Fc receptors in general, single, double and triple knockouts for the individual Fc receptors in mice have been instrumental in determining the role of individual receptor in immunotherapy models[9]. Using knockout mice, Bevaart *et al*. showed in the well established B16F10/TA99 melanoma model that antibody treatment depended critically on FcγRI[78]. Contribution of FcγRI was shown in the therapeutic efficacy of CD20 mAb in a lymphoma model and of TA99 mAb in a liver metastases model [79,80]. These studies, however, utilized mIgG2a mAb, an isotype which interacts with high affinity with mouse and human FcγRI. A study using human IgG1 mAb against a malaria antigen showed treatment against the parasite to be completely dependent on transgenic expression of FcγRI[81].

Furthermore, it was shown that treatment with bispecific antibodies (BsAb) targeting FcγRI and *Candida Albicans* protected against infection and induced potent anti-*C. Albicans* immunity in FcγRI transgenic, but not in wildtype mice[82]. In a study using FcγRI and B cell lymphoma targeted BsAb it was shown that BsAb treatment with G-CSF was highly effective in B cell lymphoma eradication and induced long term T-cell immunity in FcγRI transgenic mice[83].

The use of BsAb has shown the potential of FcγRI targeted immunotherapy in humans. Several clinical trials employed chemically crosslinked H22 F(Ab'), a humanized form of anti-FcγRI mAb M22, to tumor specific F(ab') such as those derived from murine mAb Ki-4 (anti-CD30)[84], murine mAb 520C9 (anti-HER2/neu)[85], and F(ab') derived from humanized H425 (anti-EGFR)[86]. MDX-H210, a H22 F(ab') x 520C9 F(ab') was studied in several trials most of which included the use of cytokines such as G-CSF, GM-CSF and IFNγ. The rationale was not only to obtain increased numbers of circulating PMN but also to induce FcγRI expression on these effector cells.

The BsAb were capable of 'arming' neutrophils and monocytes *in vivo,* and a promising trial treating prostate cancer patients with MDX-H210 in combination with GM-CSF reported improvement in pain scores in 58% of patients and decreased PSA velocity in 83% of patients[87,88]. In contrast, a phase I study using MDX-H120 in combination with IFNγ did not report objective responses[89,90]. Similarly, a trial using MDX-H210 in combination with G-CSF did not induced clear clinical improvements in breast cancer patients[91]. However, the single doses of MDX-H210 given during this dose escalation trial might well be inadequate since the half-life of BsAb are generally short (5-17 hours depending on the dose). A promising phase 1 trial combining H22 with an anti CD30 reported 1 complete remission, 3 partial remissions and 4 patients with stable disease out of a total panel of 10 patients with refractory Hodgkin Lymphoma[84].

Overall, FcγRI directed BsAb were relatively successful, given the fact that early studies were hampered due to toxicities[86,87], difficulties in generating true heterodimeric BsAb when employing chemical crosslinking, and BsAb short half-lives. However, more modern expression platforms allow the formation of recombinant BsAb such as a recombinant H22scFv and Ki4 scFv BsAb which may well overcome these problems[92]. Furthermore, the limitation of short serum half live of bispecific molecules may be effectively overcome by the use of portable minipumps, as shown in trials with bispecific T cell engager (BiTe) antibodies[93].

FcγRI AS A TARGET FOR IMMUNOTHERAPY

Next to functioning as a potential effector molecule in antibody immunotherapy, FcγRI might also represent a target for immunotherapy. Several studies document the use of toxins coupled to FcγRI specific mAb during acute myeloid leukaemia (AML) and chronic inflammatory diseases (reviewed by Thepen et al [94]). Macrophages and dendritic cells might represent potential therapeutic targets during chronic inflammation, as they are not only capable of inflicting direct tissue damage but also influence the inflammatory response by antigen presentation and the release of soluble mediators such as TNFα.

Local application of humanized anti-FcγRI mAb H22 coupled to Ricin A (H22-RiA), a plant derived toxin, was shown to be effective in resolving cutaneous inflammation in a transgenic mouse model[95]. In *in vitro* experiments, the immunotoxin was most effective against cells expressing high FcγRI surface levels. Furthermore, in a study investigating the use of H22-RiA during rheumatoid arthritis, the immunotoxin efficiently reduced macrophages from synovial fluid from RA patients, while only moderately affecting peripheral blood monocytes[96]. The *in vivo* effectiveness of H22-RiA arthritis treatment was shown in a transgenic rat model[97]. These studies support the potential of FcγRI targeted immunotherapy for treatment of chronic inflammation.

Next to treating chronic inflammation, FcγRI targeted immunotherapy has been investigated as a treatment for AML. AML blasts are known to express FcγRI, and H22-RiA was

shown to inhibit growth of FcγRI expressing cell lines both *in vitro* and *in vivo*[98]. Recombinant anti-FcγRI scFv fused to *Pseudomonas* Endotoxin A showed promising results in inducing apoptosis in primary AML cells[99].

Overall, these studies suggest FcγRI as a suitable target for immunotherapy. However, as these studies rely on the use of chemically linked immunotoxins they are hampered by potential non specific toxicity. Furthermore, non human toxins might well encounter problems with repeated use because of potential immunogenicity. Development of recombinant immunotoxins such as the recently described H22 scFv fused to granzyme B may represent an important improvement towards clinical application of FcγRI targeted immunotoxins[100].

FcγRI POLYMORPHISMS

Research on Fc receptor polymorphisms identified a role for Fc receptors during antibody immunotherapy[101]. The finding that a single amino acid difference, F158V, in FcγRIIIA can affect not only the IgG affinity of the receptor and possibly surface expression[102-104], but also the clinical outcome of rituximab (RTX) treatment, supports a role for Fc receptor mediated effector mechanisms during antibody therapy[105]. Similar studies have been published for FcγRIIA, where the FcγRIIA-H131 allotype was found to have a higher affinity for human IgG1, and homozygosity for this allele was reported to be associated with response rates during RTX treatment[106]. Furthermore, FcγRIIA-R131 and FcγRIIIA- F158 alleles were found associated with autoimmune diseases such as SLE and rheumatoid arthritis, and the occurrence of bacterial infections[107-110].

Up to date no polymorphisms have been described for FcγRI that alter receptor affinity or function[111,112]. FcγRI deficiency does not appear to increase susceptibility to infections or auto-immune diseases[67,68,68]. Although this suggests that FcγRI could play a redundant role in immunity, a role during mAb based immunotherapy is unclear.

We set out to find possible polymorphisms that could alter receptor function or expression using the online ENSEMBL database. Although ENSEMBL reports 181 candidate SNPS in a 10kb region containing the *FCGR1A* gene, it is unclear whether all of these SNPs are true polymorphisms, or possibly represent sequence differences between the highly homologous *FCGR1A*, *FCGR1B* and *FCGR1C* genes. By aligning the three genes and the described SNPS we identified 57 candidate SNPs that do not seem to be the result of single base pair differences between *FCGR1* genes. Of note, these candidate SNPs may still represent sequencing artifacts, and polymorphisms that did not meet our selection criteria may still be true SNPs. Only three of the 57 candidate SNPs are nonsynonymous, i.e. encode for an aminoacid difference (rs7531523, rs12078005, rs1050208), and are shown in Figure 2A. In addition to the stopcodon previously described by van de Winkel et al[67], a mutation resulting in a stopcodon was identified in EC3 (rs1338887, Q224*). Frequency data for the potential SNPs were not

available with the exception of the previously named stopcodon for which 2 out of 60 (~3%) European individuals were found to be heterozygous.

To gain a better insight in the possible impact of potential SNPs on receptor function, we adopted an *in silico* approach to predict the overall structure of FcγRI (Figure 2B-D). Using the latest application for protein structure prediction[113], we were able to model all three EC domains with acceptable C-scores (C-scores 0.74 for EC1 and 2 and -0.25 for EC3, where a higher C-score suggests higher prediction confidence and typically ranges between -5 and 2). The overall structure of EC1 and EC2 appeared similar to predictions made by Sonderman et al[22]. Assembly of the extracellular domains was performed using the HADDOCK server[114]. (Figure 2B). Of these predictions, prediction 1 and 3 seem the most reliable, based on their low HADDOCK combined energy score. However, prediction 3 would seem unlikely as immune complex binding could suffer from steric hindrance as the expected binding of IgG would cause the IgG Fab arms to extend laterally, rather than upwards and away from the membrane.

We found a potential SNP in EC1 (rs7531523, coding for V39I). It would seem unlikely that V39 contributes to IgG binding, as in all predicted models it is largely buried within the structure of EC1 and does not locate within the expected IgG binding site (Figure 2C and 2D). However, alignment of FcγRI, FcγRIIA, FcγRIIB and FcγRIII showed V39 to be highly conserved within the Fcγ receptor family implying possible necessity of this amino acid in FcR structure and/or function (data not shown).

It is difficult to predict the consequences of the I301M change (rs12078005) in the transmembrane region (TM) as both residues are hydrophobic. However, the TM of FcγRI has been shown to play a role in surface expression[115]. Structural predictions made by I-TASSER for the transmembrane domain and intracellular tail were less reliable (C-scores of -3.49 and -2.06, respectively), complicating interpretations on functional consequences of the potential SNPs located in these domains. The I338T mutation (rs1050208) in the intracellular tail appears to be located outside the expected binding sites for protein 4.1G and periplakin (Figure 2A and 2D). Further analyses are needed to unravel whether the potential SNPs reported here affect receptor functioning.

In recent years, gene copy number variations (CNV) have gained more interest in the Fc receptor field (reviewed by Fanciulli et al [116]). CNV are known to greatly affect gene expression and analysis along the whole genome revealed significant enrichment for genes related to the immune system. CNV of *FCGR3B* and *FCGR2C* have already been linked to increased susceptibility of auto-immunediseases[12,117]. Low copy number of *FCGR3B* is associated with an increased risk of glomerulonephritis and presence of *FCGR2C* open reading frame has been shown to be associated with idiopathic thrombocytopenic purpura. CNV for the *FCGRIA* gene have not yet been reported.

Specific sequencing of the FCGR1A gene and CNV analysis during autoimmunity or mAb based immunotherapy might shed more light on the function of this receptor in immunity.

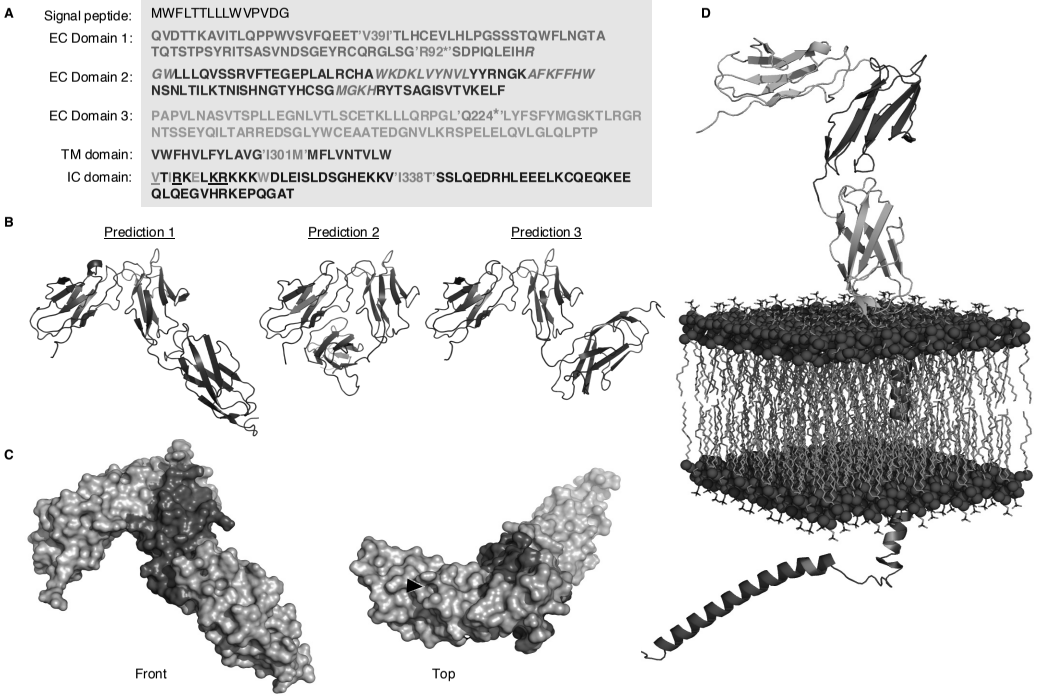

Figure 2. FcγRIA structure and variations. A) Amino acid sequence divided in different domains. The five non-synonymous SNPs are indicated in red (V39I: rs7531523, R92*: mutation described by van de Winkel et al[67], Q224*: rs1338887, I301M: rs12078005, I338T: rs1050208, stars denote stopcodon), the probable surface residues that facilitate IgG-binding (lightblue) and the interaction sites with 4.1G (underlined) and periplakin (green)[22,43,45]. B) Cartoon representations of predicted extracellular FcγRI structure. EC1-EC2 and EC3 were separately predicted using I-TASSER[113], and subsequently assembled and refined by docking algorithms from the HADDOCK server[114]. The three most likely models are shown. C) Surface of the extracellular domains of prediction 1 as seen from front and top. IgG-binding domain is marked in blue and V39 in EC1 is in red and marked by the arrowhead D) Interpretation of FcγRI within a plasma membrane to illustrate the interaction and variation sites. Structure prediction 1 was used for this model. the color codes are the same as in (A), 4.1G bindingsite is shown in magenta. All images were created in PyMol. (See color section for a full-color version)

CONCLUSION

Despite intense study, the biological role of FcγRI remains unclear. Nevertheless, given the potent cytotoxic effects and its capability to efficiently mediate antigen presentation, FcγRI may well play a role in mAb based immunotherapy. Recent data from our laboratory suggests inside out signalling to possibly overcome some of the competition by serum IgG during binding of opsonized targets. This may open up new avenues of FcγRI mediated immunotherapy, especially since the documentation of aglycosylated IgG binding selectively to FcγRI[118]. Otherwise, bispecific antibodies may well be used to circumvent Fc receptor ligand binding sites. One of the goals of mAb based immunotherapy should be to induce long term memory against tumor or pathogen derived antigens. FcγRI may be capable of mediating such effects[83]. Indeed, recent work documented anti HER2/neu mAb immunotherapy induced tumor directed CD8 T cell responses[119]. In general, therapeutic mAb are administered at high dose; therefore, it should be investigated whether circulating monocytes become armed with therapeutic antibodies during mAb therapy. Finally, our computational studies suggest at least seven putative polymorphisms to exist in the *FCGR1A* gene. Experimental studies on these polymorphisms and/or CNV focused may provide clues on the exact role of FcγRI in immunity and antibody therapy.

SCOPE OF THESIS

This thesis focuses on human FcγRI, a high affinity receptor for antibodies of the IgG isotype. FcγRI exists as a complex of a ligand binding α-chain and the ITAM containing FcR γ-chain. Like other leukocyte FcR, most downstream functions are mediated via the ITAM signaling motif. However, several biological functions of FcγRI, including antigen presentation and endocytosis, appear ITAM independent, supporting a role in signaling for the α-chain. Furthermore, high affinity IgG binding by FcγRI is unique within the FcγR family. This property may hamper binding of immune complexes due to IgG saturation under serum conditions, thus making the biological role of this receptor in immunity unclear. In addition, insight in the functioning of FcγRI may be relevant for antibody therapeutic strategies. In this thesis functional implications of proteins interacting with the 'head' (Chapter 2) and the 'tail' of FcγRI (Chapter 3-5) were studied. Chapter 2 documents the effects of cytokine stimulation on monomeric and multivalent IgG binding to the class I IgG receptor. We observed cytokine stimulation to enhance binding of multivalent IgG, allowing association of immune complexes to IgG saturated FcγRI. In chapter 3, we address the functional implications of the association between FcγRI and the actin binding protein, filamin A. Filamin A interaction was found to stabilize FcγRI surface expression, thereby preventing default routing of the receptor to lysosomal compartments. The interaction between protein 4.1G and FcγRI is characterized in Chapter 4. Alanine scans and alignments with other 4.1G binding proteins suggested a membrane proximal 4.1G bindingsite on the FcγRI α-chain. In Chapter 5, the functional consequences of 4.1G and FcγRI interaction are addressed. Confocal laser scanning microscopy studies revealed a potential role for 4.1G during ingestion of large particles by macrophages. A summarizing discussion is provided in Chapter 6.

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Chapter 2

Cytokine induced immune complex binding to FcγRI in the presence of monomeric IgG

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ABSTRACT

FcγRI is the sole high affinity IgG receptor on leukocytes. Its role in immunity and clearance of opsonized particles has been challenged as the receptor function may well be hindered by serum IgG. Here, we document immune complex binding by FcγRI to be readily enhanced by cytokine stimulation, whereas binding of monomeric IgG to be only modestly increased. Enhanced immune complex binding was independent of FcγRI surface expression levels. FcγRI, saturated with pre-bound IgG, was found capable of effective immune complex binding upon cytokine stimulation. Cytokine enhanced binding was observed across a variety of immune complexes, including huIgG3 or mIgG2a opsonized RBCs, rituximab or ofatumumab opsonized B cell lymphoma and cetuximab opsonized glioblastoma cells. This study contributes to our understanding how FcγRI can participate in clearance of opsonized particles despite saturation by monomeric IgG.

INTRODUCTION

Fc receptors are receptors for immunoglobulins and play a central role in immunity. On human leukocytes, a myriad of Fcγ receptors are expressed; among these receptors, FcγRI is the only known high affinity receptor for IgG[1]. FcγRI is constitutively expressed on monocytes, macrophages and myeloid dendritic cells. IFNγ can enhance surface expression of FcγRI on these cells, and FcγRI expression can be induced on granulocytes by IFNγ or G-CSF stimulation. In contrast, cytokines such as IL-4, IL-10 and TGF-β downregulate activating Fc receptors, including FcγRI, and enhance expression of inhibitory receptor FcγRIIb[2,3]. *In vitro,* IFNγ treatment leads to increased Fcγ receptor induced cytokine production[4].

In vivo, the role of FcγRI in immunity remains unclear as, due to its high affinity, FcγRI is believed to be saturated with monomeric IgG. This has led to the concept that pre-bound monomeric IgG prevents participation of FcγRI in clearing immune complexes by extravasated effector cells[5]. Nevertheless, several *in vivo* studies documented a role for FcγRI, varying from a contribution during inflammation and autoimmune reactions [6-8], or during mAb based immunotherapy in melanoma and B cell lymphoma models[9,10], to a malaria model in which transgenic expression of human FcγRI was central for effective mAb treatment[11]. Furthermore, FcγRI can induce potent pro-inflammatory signaling compared to FcγRIIa[12], and can efficiently mediate both MHC-II antigen presentation and cross-presentation[13-16].

It remains unclear how FcγRI contributes to immune complex clearance in the presence of high IgG levels. For FcαRI[17,18] and FcγRIIa[19,20] it has been shown that, cytokine stimulation can increase ligand binding of these receptors. This appears analogous to inside out regulation described for integrins. Many integrins are expressed in a low-affinity binding state which can be transformed to high affinity form upon cellular activation[21]. We hypothesized that inside out regulation could contribute to immune complex binding to FcγR occupied by IgG. Indeed, several studies support intracellular proteins interacting with FcγRI may affect ligand binding[22,23].

In this study, we investigated the effect of cytokine stimulation on FcγRI ligand binding. Stimulation of FcγRI expressing Ba/F3 cells and primary monocytes resulted in increased binding of immune complexes, whereas binding of monomeric IgG was only moderately enhanced. Upon cellular activation, FcγRI could readily bind immune complexes despite pre-engaged monomeric IgG. Taken together, these data might explain how FcγRI supports leukocyte interaction with immune complexes.

MATERIALS & METHODS

Antibodies and Reagents

Anti FcγRI-A647 was from Biolegend (clone 10.1). Unlabeled 10.1 mAb and mouse IgG1/ IgG2a isotype was from Ebioscience and BD pharmingen, respectively. Anti human Glycophorin A mouse IgG2a hybridoma supernatant was described previously[24], anti RhD human IgG3 and IgG1 (clone BRAD3 and BIRMA D6 respectively) were from IBGRL. Human IgG1 anti RhD clones LHM76/58 and ESD-1 were from Alba Bioscience. Unlabeled, polyclonal human IgG3 an IgG1 was purchased from Sigma. Okadaic acid, LY294002 and U0126 were purchased from Alexis biochemicals. IV.3 mAb were isolated from hybridoma supernatant. IV.3 Fab fragments were made by Fusion Antibodies.

Cell lines and monocyte isolation

Ba/F3 cells were cultured in RPMI1640 medium (Gibco) supplemented with 10% fetal calf serum, penicillin/streptomycin and mouse IL-3, as described in[25]. A1207 and Daudi were kept in RPMI1640 medium supplemented with 10% fetal calf serum, penicillin/streptomycin.

The retroviral vector pMX FcγRI IRES GFP was described previously[26]. Amphotropic viral particles produced in HEK293T cells were used to transduce Ba/F3 cells. After transduction, Ba/F3-FcγRI cells were sorted on a FACS Aria (BD) on GFP expression and further subcloned by limited dilution. All experiments where subclones are shown, results were repeated with a polyclonal line and/or with at least 2 subclones.

Primary monocytes were from healthy donors. MACS isolated monocytes were obtained from PBMCs using CD14-beads (Miltenyi).

Red blood cells (RBC) were from ficoll separated blood. RBCs were kept in Alsever buffer (Sigma) for a maximum of 1 week at 4°.

Monomeric IgG binding

Ba/F3-FcγRI cells were stimulated as described in the "Facs based rosette assay" paragraph. Next, cells were washed and incubated with various amounts of mouse IgG2a or huIgG3. After 1 hr incubation on ice, cells were washed and stained with goat F(ab')2 anti mouse IgG or –anti human IgG –PE (Southern Biotech). After washing, cells were fixed with 1%PFA in PBS and analysed on a FACS Canto II.

FACS based EA rosette assay's

FACS based EA rosette assay was adapted from Beekman et al[27]. Briefly, Ba/F3-FcγRI cells were starved overnight in RPMI1640 containing 1% FCS (RPMI 1%FCS). The next day, cells were stimulated for various time points with 30 ng/ml recombinant mouse IL-3 (Peprotech) in RPMI 1%FCS or in RPMI 1% FCS alone (medium control). RBC were fluorescently labeled with PKH26 (Sigma) according to manufacturer's protocol, opsonized on ice with mIgG2a anti glycophorin or huIgG3 anti Rhesus D and subsequently washed and resuspended in RPMI EDTA (RPMI1640, 1%FCS, 10mM EDTA). In a 96 wells plate, $1x10^5$ Ba/F3 cells/well were combined with $1x10^6$ RBC/well in RPMI EDTA and incubated for 1 hour at 4° on a shaker. Cells were washed once with cold PBS and resuspended in cold PBS containing 1%PFA. Rosetting was analysed on a FACS Canto II (BD). It was shown previously that bovine IgG does not bind to human FcγRI[28].

Blocking mAb 10.1 or isotype control was added at 20 µg/ml to Ba/F3-FcγRI cells prior to rosette assay and were kept present during assay at 10 µg/ml.

Rosette assays using Ig coupled to tosyl activated Dynabeads was adapted from Bracke et al.[29]. Briefly, human IgG1 was coupled to tosyl activated Dynabeads-M450 (Invitrogen) as described by the manufacturer. IgG coupled dynabeads were then added to Ba/F3-FcγRI cells in RPMI EDTA in a ratio of 3.5 beads/cell. Dynabeads were found sufficiently autofluorescent in the PE channel to discriminate between Ba/F3 cells, beads and Ba/F3 cell bound beads. Rosettes were visualized on a LSM710 confocal microscope (Zeiss) equipped with transmission photomultiplier tube for DIC imaging. Pinhole opening was set to maximum.

For competition assays with monomeric ligand, Ba/F3-FcγRI cells were incubated with various concentrations of polyclonal huIgG3 on ice (Sigma). Unbound IgG was removed by washing Ba/F3-FcγRI cells before EA rosette assay. For inhibition expertiments, Ba/F3- FcγRI cells were treated with for 30 min with DMSO, 20µM LY294002, 20µM U-0126 or 1µM okadaic acid (OA) and stimulated with IL-3 in the presence of the inhibitor. Subsequently, cells were washed and subjected to EA rosette assay.

For measuring binding to tumor cells, tumor cell lines were fluorescently labeled with Lavacell (Activemotif), an amino reactive dye, and subsequently opsonized with 0.25 µg/ml rituximab (Roche) or ofatumumab (Genmab/GSK) for Daudi or cetuximab (Merck) for A1207. After washing, the opsonized cells were incubated with Ba/F3-FcγRI for 1 hour at 4°C on a shaker. Cells were washed once with cold PBS and fixed in PBS containing 1%PFA. Binding was measured on a FACS Canto II and visualized on a LSM710 confocal microscope (Zeiss) equipped with transmission photomultiplier tube for DIC imaging. Pinhole opening was set to maximum.

Binding assay with primary monocytes

For inhibition of inside out signaling, monocytes were isolated as described in "*Cell lines and monocyte isolation*" paragraph. After overnight incubation with 400U/ml IFNγ, monocytes

were labeled with CFSE or Celltracker violet (invitrogen) and treated with okadaic acid for 30min at 37°. Monocytes were plated out at $1x10^5$ cells per well in RPMI EDTA containing 5µg/ml IV.3 Fabs. After 10 min incubation on ice, $1x10^6$ opsonized RBC were added. Cells were allowed to bind for 1 hour at at 4°C on a shaker. After fixation in 1% PFA, binding was analysed on a FACS Canto II.

For short term stimulation with interleukins, PBMC were isolated using ficoll. Cells were allowed to rest for 1 hour in 1%FCS RPMI at 37° C. Next, PBMC were stimulated with either IL-4 (500U/ml), IL-6 (200U/ml) or TNFα and IFNγ (resp 500 and 400 U/ml) for 1 hour in 1%FCS RPMI at 37°C. Subsequently, PBMC were allowed to bind to PKH26 labeled, opsonized erythrocytes for 1 hour at 4° C in a 96 wells V-bottom plate. After incubation, the plate was spun down and CD14-FITC in PBS with 5%NMS was added to each well. The amount of monocyte binding to RBC was scored as the percentage of CD14, PKH26 double positive events.

Statistical analysis

Data analysis was performed using MS Excel and GraphPad Prism. Sigmoidal dose-response curves were used to fit rosette bindingcurves data. Fitted data was tested using F-test (comparing curves between treatments). Blocking experiments were tested using unpaired stutent t-test. Error bars depict standard deviations

RESULTS

IL-3 stimulation does not affect surface expression of FcγRI on Ba/F3-FcγRI cells

To determine whether FcγRI is regulated inside out, we used the IL-3 dependent Ba/F3 model. Parental Ba/F3 cells do not express any endogenous FcγR receptors but do express the FcRγ chain (Ho et al.[30] and supplemental Figure 1). One hour IL-3 stimulation of an FcγRI transduced Ba/F3 subclone (Ba/F3-FcγRI) did not affect FcγRI surface expression levels (Figure 1A). Similar results were obtained in a polyclonal line (data not shown). These findings allowed us to perform ligand binding studies independent of changes in receptor surface expression.

Monomeric IgG binding is a unique characteristic of FcγRI. IL-3 treated Ba/F3-FcγRI cells showed a modest, albeit significant, increased binding to mouse IgG2a (mIgG2a), human IgG1 and human IgG3 (huIgG3, see Figures 1B, 1C and 1D, respectively). These data demonstrate that short term stimulation with IL-3 mildly affects monomeric IgG binding in the absence of effects on receptor surface expression levels.

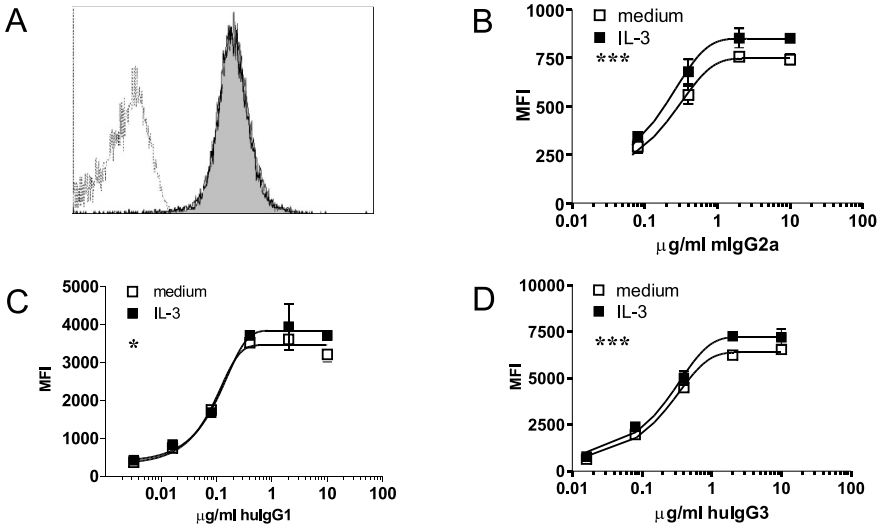

Figure 1. IL-3 stimulation has no effect on FcγRI surface expression and slightly increases monomeric IgG binding. Ba/F3 expressing cells were starved overnight and subsequently restimulated with IL-3 for one hour. A) FcγRI surface expression on a starved (open histogram) and restimulated (closed histogram) subclone of FcγRI transduced Ba/F3 cells. Dotted line represents isotype control. B,C and D) Binding of monomeric mouse IgG2a (B), human IgG1 (C) or human IgG3 (D) to FcγRI expressing Ba/F3 cells. Open squares represent starved cells, filled squares represent IL-3 restimulated Ba/F3 cells. Similar results were seen in 3 independent experiments. Datapoints represent means of at least 4 replicates. *:P<0.05, ***: P<0.001

Inside out regulation of FcγRI enhances immune complex binding

To investigate whether treatment of Ba/F3-FcγRI cells with IL-3 altered immune complex (IC) binding we used a FACS based EA rosette assay. This method was previously shown to correlate with EA rosette counting by light miscroscopy[31]. An example of a FACS based EA rosette assay and gating strategy is shown in supplemental figure 2.

Upon stimulation with IL-3, Ba/F3-FcγRI cells exhibited increased binding to mIgG2a-IC compared to control cells, as detected by higher percentages of Ba/F3-FcγRI:RBC clusters (Figure 2A). Enhanced IC binding was observed as early as 15 minutes after stimulation (Figure 2C), reaching maximum levels by 1 hour. Blocking with FcγRI-specific mAb 10.1 demonstrated basal binding and IL-3 enhanced binding to be FcγRI dependent (Figure 2B).

Similar to mIgG2a-IC, Ba/F3-FcγRI cells showed higher level binding of huIgG3-IC upon IL-3 stimulation (Figure 2D). Furthermore, IL-3 stimulation led to marked increased binding of two or more opsonized RBC to Ba/F3-FcγRI (Figure 2E). Binding to huIgG3-IC was FcγRI dependent as determined by receptor blocking with FcγRI mAb 10.1 (data not shown).

We tested several IgG1 RhD clones (BIRMA D6, ESD-1 and LHM76/58) in the rosette assay. Despite considerable opsonization, IgG1 opsonized RBC failed to bind to Ba/F3-FcγRI or

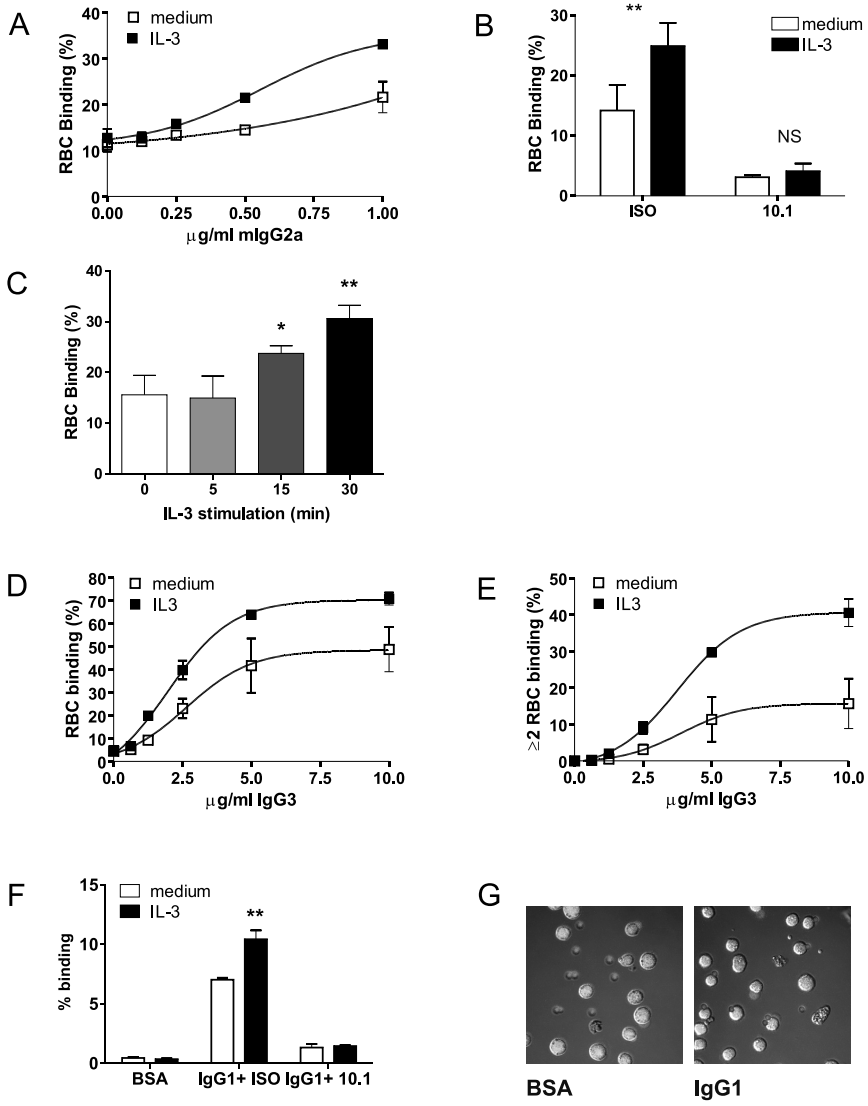

Figure 2. Stimulation of primary monocytes and Ba/F3-FcγRI cells results in enhanced IC binding. Percentage of Ba/F3-FcγRI cells binding RBC opsonized with IgG2a (A, B and C) or human IgG3 (D and E). Open squares represent starved cells, filled squares represent starved Ba/F3-FcγRI cells stimulated with IL-3 for one hour. B) EA rosette assay in the presence of FcγRI specific mAb 10.1 (10.1) or isotype control (iso). **: P<0.01; NS: not significant, t-test. C) Effect of duration of IL-3 stimulus on IC binding. *:P<0.05; **:P<0.01, t-test. D) Binding of 1 or more RBC to Ba/F3 cells. E) Binding of 2 or more RBC to Ba/F3 cells. Datapoints represent the means of at least 3 replicates. Similar results were obtained in 3 independent experiments. ***: P<0.001. F) Binding of Ba/F3-FcγRI cells to BSA or IgG1 coupled beads in the presence of 10.1 mAb (10.1) or isotype control (ISO). Similar results were obtained in 3 independent experiments. **:P<0.01, t-test. G) Rosetting of BSA and IgG1 coupled beads with Ba/F3-FcγRI cells visualized with fluorescence microscopy. Beads are detected in the red channel, Ba/F3-FcγRI in the green channel. (See color section for a full-color version)

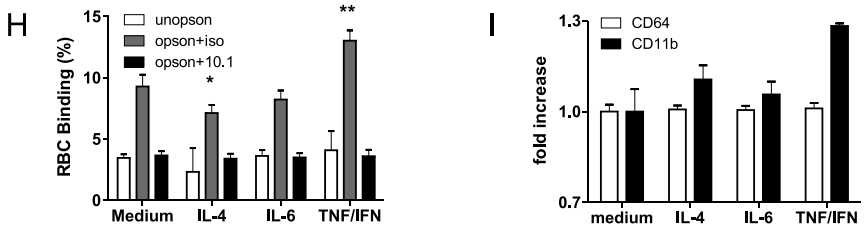

Figure 2. (continued) H) Binding of unopsonized (unopson) and opsonized (opson) erythrocytes to primary monocytes in the presence of 10.1mAb (10.1) or isotype control (iso). Data represent means of triplicates measured from one donor. Similar results were seen in 3 out-of -4 donors. *: P<0.05; ** P<0.01. I) FcγRI (CD64) and CD11b expression on CD14+ gated monocytes. Shown is the fold increase in surface expression relative to unstimulated (medium) cells. Data represent mean from triplicates.

primary monocytes (data not shown). This is in concordance with previous reports[32]. It was suggested that in contrast to IgG1, the longer hinge region of IgG3 is capable to overcome the forces between the negatively charged RBC and effector cell. Therefore, we used IgG1 coupled beads (huIgG1-IC) to evaluate cytokine enhanced binding of huIgG1-IC to Ba/F3-FcγRI. IgG1 coupled beads bound to Ba/F3-FcγRI cells whereas BSA coupled beads did not. IL-3 stimulation enhanced only binding of IgG1 coated beads and binding was FcγRI dependent as blocking mAb 10.1 decreased binding to near background (Figure 2F). The formation of rosettes with IgG1 coupled beads and Ba/F3-FcγRI cells could clearly be observed with fluorescence microscopy (Figure 2G).

We next assessed whether FcγRI was similarly regulated in primary monocytes, and tested the effect of several proinflammatory cytokines on IC binding. Stimulation with TNFα and IFNγ, a known macrophage stimulus[33], enhanced IC binding by primary monocytes. IC binding was inhibited by Ab 10.1, but not isotype control, supporting the involvement of FcγRI (Figure 2H and supplemental figure 3A). Under these conditions, monocytes were positive for surface IgG. Upon stimulation, only slightly increased surface IgG levels were detected suggesting that TNFα and IFNγ enhanced IC binding to monocytes was not due to a decrease of pre-bound IgG to FcγRI after stimulation (supplemental figure 3B). Furthermore, stimulation did not alter FcγRI surface expression levels but often (2 out-of-4 donors) coincided with increased CD11b expression levels (Figure 2I and supplemental figure 3B). The cytokines IL-6 and IL-4 did not enhance binding of IC to FcγRI.

Taken together, these data suggested cytokine stimulation to increase binding of multivalent ligands (IC) to FcγRI.

Cytokine stimulation enhances FcγRI binding to opsonized tumor cells

To date, rituximab (RTX) is one of the most successful monoclonal antibodies used in the clinic[34]. Using RTX in combination with a B cell lymphoma line (Daudi) we studied binding of

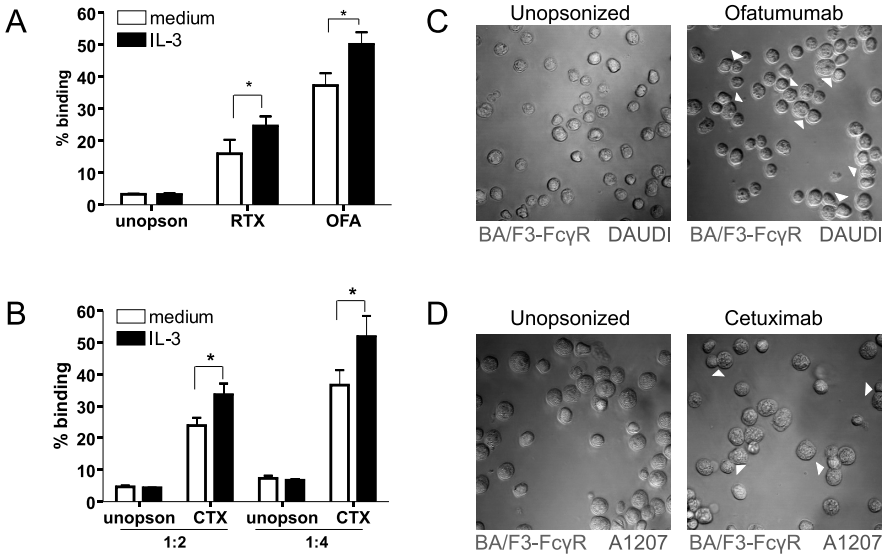

Figure 3. Cellular activation leads to enhanced FcγRI mediated binding of opsonized tumor celllines. A) Percentage of Ba/F3- FcγRI cells binding to Daudi cells opsonized with Rituximab (RTX), Ofatumumab (OFA) or unopsonized (unopson) Daudi cells. E/T ratio was 1/ 2. B) Percentage of Ba/F3- FcγRI cells binding to Cetuximab (CTX) or unopsonized (unopson) A1207. E/T ratio was either 1/2 or 1/4. Data represent the means of at least 3 replicates. FcγRI blocking mAb 10.1 was used to investigate FcγRI dependent binding (not shown) *: P<0.05. Experiment was repeated three times yielding similar results. C&D) Binding of IL-3 stimulated Ba/F3-FcγRI to Ofatumumab opsonized Daudi cells (C) or Cetuximab opsonized A1207 cells (D) determined with fluorescence microscopy. Ba/F3-FcγRI cells are shown in green, Daudi and A1207 cells in red. Arrowheads denote contacts between Ba/F3-FcγRI and tumor cells. (See color section for a full-color version)

Ba/F3-FcγRI to multivalent ligands. IL-3 stimulation led to increased binding of RTX opsonized Daudi cells to Ba/F3-FcγRI (Figure 3A). IL-3 induced similar increased binding of Ba/F3-FcγRI to Daudi cells opsonized with ofatumumab, a newly developed human CD20 mAb (Figure 3A). Binding was FcγRI dependent as evidenced by blocking with mAb 10.1 (supplemental figure 4). To investigate whether this effect was limited to B-cell lymphoma and CD20 mAb, we tested the human glioblastoma cell line A1207. IL-3 stimulation increased cetuximab opsonized A1207 binding to Ba/F3-FcγRI (Figure 3B). Binding of Ba/F3-FcγRI to opsonized Daudi or A1207 was confirmed using fluorescence microscopy (Figure 3C, and D, respectively).

FcγRI inside out regulation is inhibited by the phosphatase inhibitor, okadaic acid

IL-3 stimulation of Ba/F3 cells leads to activation of PI-3K-PKB and Raf/MEK/ERK pathways. It has been shown that FcαRI inside out regulation in Ba/F3 cells is dependent on PI-3K and PP2a[35,36]. We investigated whether similar signaling pathways underlie FcγRI inside-out regulation. In our model, both a PI3-Kinase inhibitor (LY294002)[37] and a MEK1/2 inhibitor

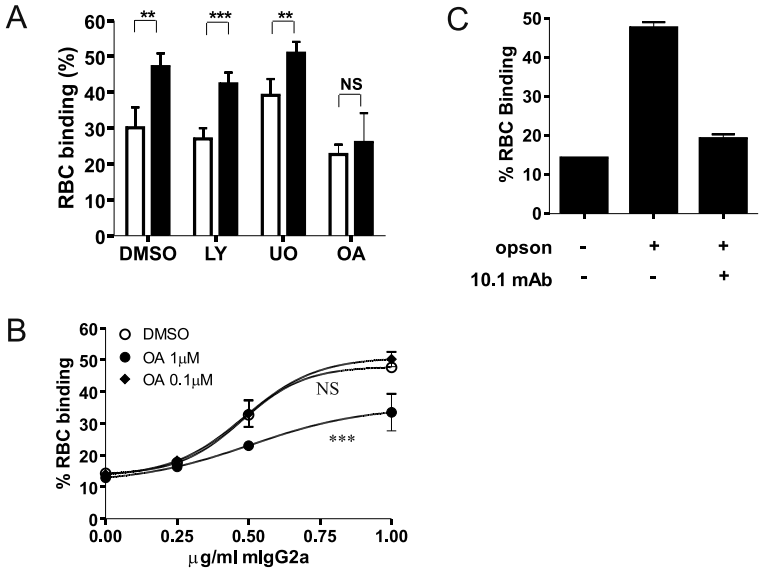

Figure 4. Okadaic acid inhibits cytokine enhanced FcγRI IC binding on primary monocytes and Ba/F3-FcγRI cells. A) Ba/F3-FcγRI cells were treated with DMSO, LY294002 (LY), U-0126 (U0) or okadaic acid (OA) and stimulated with IL-3. IC Binding was tested using mIgG2a opsonized RBC. Data represent means of at least 4 replicates. Similar results were obtained in 3 independent experiments. **: P<0.01; ***: P<0.001; NS: not significant. B) IFNγ stimulated monocyte binding to mIgG2a opsonized RBC after okadaic acid treatment. *** P<0.001, comparing 1μM OA treatment with DMSO fitted curve; NS: not significant, comparing 0.1μM OA treatment with DMSO treated fitted curve. C) Effect of FcγRI blocking mAb 10.1 binding of mIgG2a opsonized RBC to FcγRI. Similar results were seen in 3 independent experiments.

(U0126)[38] did not affect IL-3 enhanced binding to mIgG2a-IC (Figure 4A). Okadaic acid (OA, a PP2a inhibitor[39]) completely inhibited the IL-3 increased binding.

To investigate whether FcγRI was similarly regulated on primary monocytes, we stimulated monocytes overnight with IFNγ prior to OA treatment. OA efficiently inhibited binding of opsonized RBC to monocytes in a dose dependent manner (Figure 4B). Importantly, treatment of OA did not affect FcγRI expression levels (supplemental figure 5). In these assays, binding of IC to FcγRII was prevented by the addition of FcγRII specific IV.3 Fab prior to adding IC. FcγRI dependent binding of opsonized RBC was confirmed with mAb 10.1 (Figure 4C).

These data suggest that protein phosphatases play a role during FcγRI inside out regulation.

Cytokine stimulation enhances binding of immunecomplexes to saturated FcγRI

Inside out regulation of FcγRI in the Ba/F3 model mainly affected binding to multivalent ligands (immune complexes) rather than monomeric IgG. We hypothesized that this shift could contribute to immunecomplex binding of FcγRI already saturated by monomeric ligands.

By saturating Ba/F3-FcγRI with monomeric IgG3 after stimulation, we studied whether opsonized RBC were able to bind to IgG occupied FcγRI. In unstimulated cells, pre-incubation with as little as 5 µg/ml monomeric IgG3 blocked RBC binding to Ba/F3-FcγRI cells to near background levels. Stimulation with IL-3, however, lead Ba/F3-FcγRI to bind opsonized RBC effectively (Figure 5A, B). Binding was lower compared to unoccupied FcγRI but quickly reached plateau, which was maintained at saturating levels of monomeric IgG3 (20µg/ml). At this concentration FcγRI was saturated by IgG3 as evidenced by surface IgG staining (Figure 5C).

These data document that cytokine stimulation can lead to FcγRI IC binding despite saturation of the receptor with monomeric IgG.

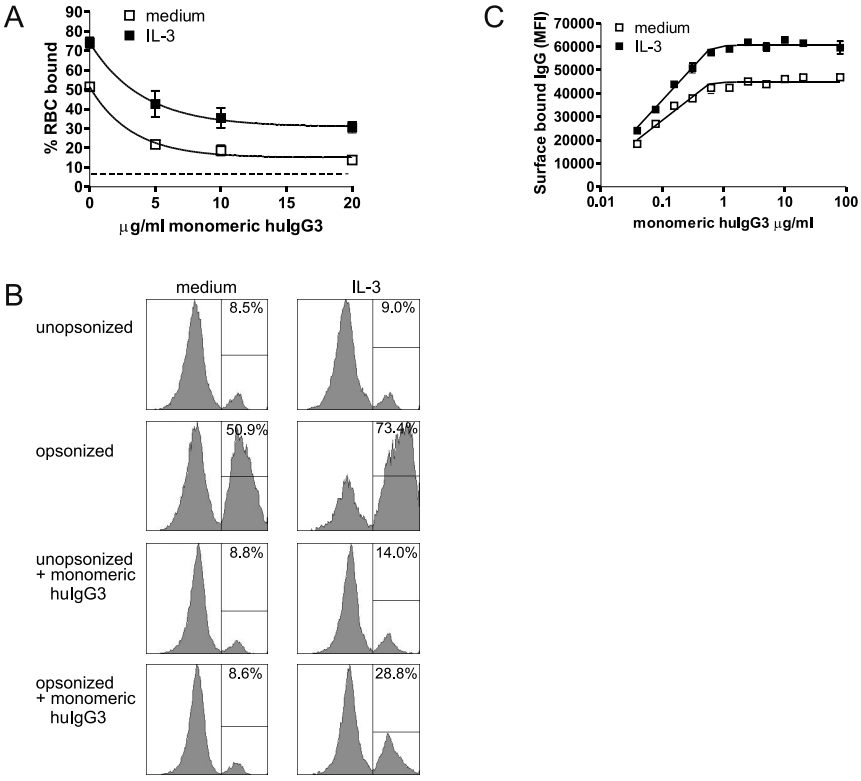

Figure 5. Effect of cellular activation on immune complexes binding to saturated FcγRI. A) Binding of Ba/F3-FcγRI to huIgG3 opsonized RBC (Y-axis) after incubating Ba/F3-FcγRI with varying amounts of monomeric huIgG3 (X-axis). Dotted line represents binding of Ba/F3-FcγRI to unopsonized RBC. B) Percentage of Ba/F3-FcγRI binding to RBC. One representative sample of triplicates is shown. C) Amounts of cell bound Ba/F3-FcγRI IgG3 after incubation with varying amounts of monomeric human IgG3 as detected by flowcytometry. Experiments were repeated 3 times yielding similar results.

DISCUSSION

The role of FcγRI in immunity remains unclear as it is unknown how a high affinity Fcγ receptor can bind immunecomplexes. Extravasating monocytes originate from an IgG rich milieu and it is believed that, due to the high affinity of FcγRI, this receptor may well be saturated with monomeric IgG. Only after *de novo* synthesis, FcγRI has been postulated to contribute to clearance of IgG opsonized particles. Here, we show FcγRI ligand binding can be regulated inside out (Figure 2, 3 and 4) and that this regulation impacts IC binding to a saturated receptor (Figure 5). Inside out regulation represents a fast process as IL-3 enhanced binding could be detected within 15 min post stimulation (Figure 2C). Cytokine enhanced IC binding was observed with different IC including tumor cell lines opsonized with IgG1 therapeutic mAbs, as well as mouse IgG2a and human IgG3 opsonized RBCs and human IgG1 coupled beads (Figures 2 and 3). Increased binding induced by cytokines was not associated with differences in receptor surface expression levels. The presence of EDTA in our binding assays makes it unlikely that integrin interactions are involved during cytokine enhanced IC binding. Furthermore, we did not observe increased binding to unopsonized cells upon stimulation.

Increased binding of ligand upon cellular activation was particularly striking when multivalent ligands (ICs) were used (Figure 3). Monovalent ligand showed a small increase in binding (Figure 1) upon stimulation. This could be due to small aggregates in our IgG preparations as IgG dimers formed with mouse anti human κ light-F(ab')2 showed a small increase in binding upon stimulation (data not shown). Since increase in binding was most apparent using multivalent ligands, we speculate that the main mechanism involved in regulating IC binding is likely due to alterations in receptor mobility and/or receptor clustering. Photobleach experiments with an FcαRI-YFP fusion protein suggested increased mobility of the receptor upon IL-3 stimulation in Ba/F3 cells (Honing et al, submitted). On the other hand, dimerization of FcγRI has been previously suggested[40] and FcγRI has been shown to associate with plasma membrane microdomains[41]. Indeed, FcγRI binding capacity could be altered upon cholesterol depletion. A model where lipid raft localization and subsequent clustering affects ligand binding has also been suggested for FcγRIIA[42]. It is important to note that the inside out regulation described here is probably different from the active "trapping" of IC by FcγR as suggested by Dale et al[43] as our binding experiments were performed at 4° C. Cytokine enhanced ligand binding was capable of inducing IC binding despite the presence of pre-bound IgG to FcγRI. It is possible that the affinity of FcγRI might differ between antigen bound (in IC) and antigen unbound IgG (pre-bound IgG to the receptor). However, it seems likely that such differences would be antigen dependent.

Several mouse models have shown involvement of FcγRI during immunotherapy. We found that ligand binding by mouse FcγRI is at least differently regulated compared to human FcγRI. Ligand binding to IC by mouse bone marrow derived DCs and bone marrow derived macrophages was not affected by cytokine stimulation or okadaic acid treatment (supplemental

figure 5). Thus, mouse FcγRI is not capable of inside out regulation or it is dependent on different stimuli. Mouse and human FcγRI both bind IgGs with high affinity, however, they show only 19% sequence similarity with the intracellular, c-terminal tail.

Previously, dephosphorylation of FcαRI was shown to regulate ligand binding and dependent on protein phosphatase PP2A[44]. Unlike FcαRI however, serine phosphorylation of the cytosolic tail of FcγRI α-chain is unlikely to play a role during inside out regulation, since a truncated receptor lacking all serines was found to respond to cytokine stimulation (data not shown). Strikingly, PI-3K inhibition did not affect cytokine enhanced IC binding to FcγR,I suggesting disctinct mechanisms underlying FcγRI regulation compared to FcαRI.

In earlier reports, we published the interaction between the cytosolic tail of FcγRI and periplakin[45,46] to affect receptor-ligand binding. Ba/F3-FcγRI cells did not show periplakin dependent inside-out regulation, as truncation of FcγRI and overexpression of c-terminal periplakin did not affect cytokine enhanced ligand binding (data not shown). This could be due to low expression levels of endogenous periplakin in Ba/F3 cells.

Overall, our data suggest that FcγRI saturated with monomeric IgG is capable of binding multivalent immunecomplexes. This finding contributes to the Fcγ receptor field as it suggests that FcγRI can participate in clearance of IgG opsonized particles despite saturation with monomeric ligand. Furthermore, it opens the field for targeted immunotherapy to this receptor. FcγRI targeted immunotherapy can be achieved via bispecific antibodies or using aglycosylated IgGs binding selectively to FcγRI [47]. Studies using a chimaeric receptor consisting of CEA specific scFv fused to the cytosolic tail of FcγRI, have demonstrated the potent cytotoxic effects mediated through this receptor[48]. Direct targeting of FcγRI by expressing scFv-H22 on tumor cell lines showed similar results[49].

Our data provides new insights of the role of FcγRI in biology although the precise mechanism underlying FcγRI inside out regulation needs to be elucidated. Furthermore, our finding that FcγRI can contribute to immunecomplex binding warrants further research in to the efficacy and role of FcγRI during immunotherapy.

ACKNOWLEDGEMENTS

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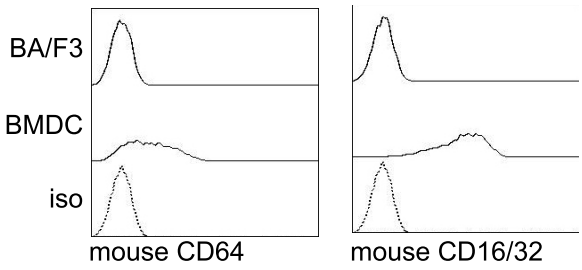
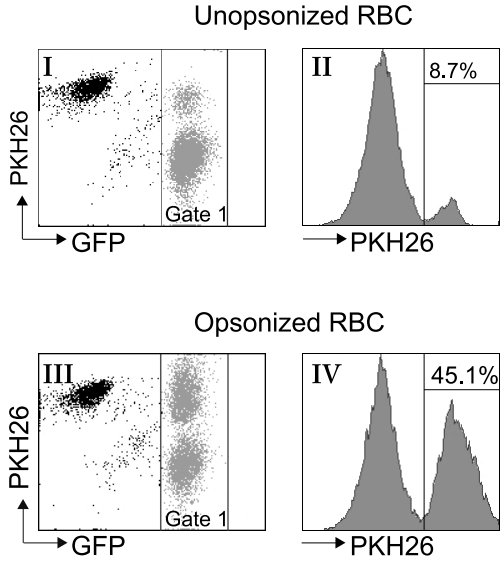
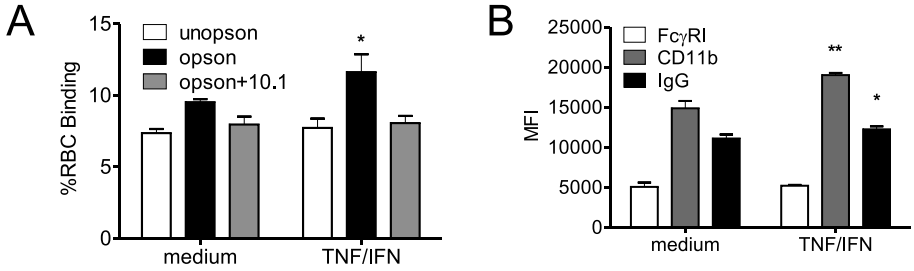


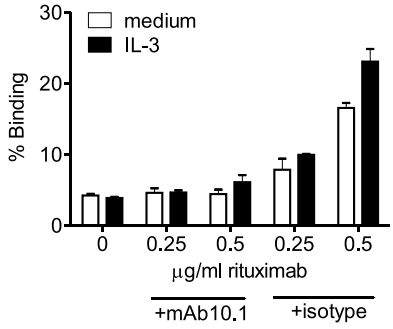
Supplemental Figure 1. Ba/F3 cells do not express endogenous FcγR-I, -II or -III. Histograms of Ba/F3 cells stained with anti mouse FcγRI (left panel) or anti mouse FcγRII/III (right panel). Bone marrow derived DCs were used as positive control (BMDC). Experiment was repeated twice, yielding similar results.



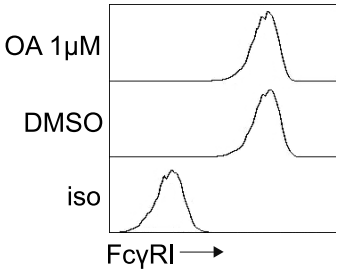
Supplemental Figure 2. Binding of RBC by Ba/F3-FcγRI cells scored by flowcytometry. PKH26 labeled RBC (FL-2 positive) can be readily distinguished from GFP (FL-1 positive) expressing Ba/F3-FcγRI cells. RBC bound to Ba/F3- FcγRI cells are detected as double positive events. The percentage of RBC binding is defined as the relative amount of Ba/F3-FcγRI cells bound to RBC. Panel I) dotplot showing unopsonized, PKH26 labeled RBC, GFP positive Ba/F3-FcγRI cells and double positive RBC bound Ba/F3-FcγRI cells. Panel II) histogram from events in gate 1 shown in panel I and sub-gate showing the percentage of RBC binding. Panel III) Similar to panel I, using huIgG3 opsonized RBC. Panel IV) histogram from events in gate 1 shown in panel III.



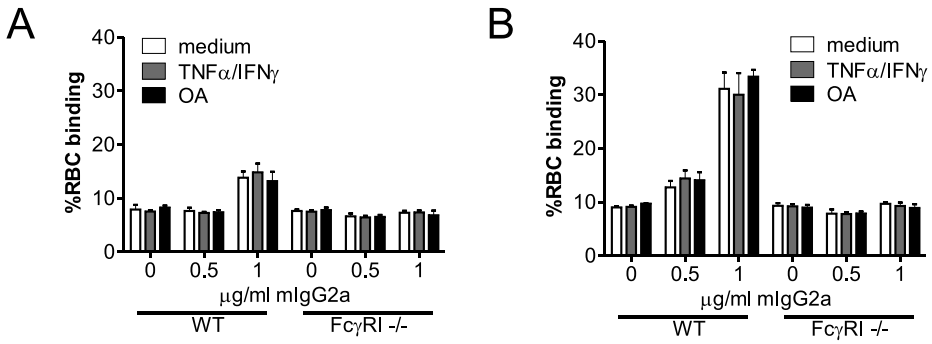
Supplemental Figure 3. Surface levels of IgG on primary monocytes after cytokine stimulation. A) Freshly isolated PBMC (different donor as shown in figure 2H and 2I) were stimulated with TNF-α with IFNγ (TNF/IFN) or medium and FcγR binding to CD14+ monocytes was evaluated using huIgG3 opsonized RBCs. mAb 10.1 was used to evaluate FcγRI specific binding.* $p<0.05$ vs medium control. B) In the same experiment, surface IgG levels and FcγRI and CD11b surface expression was measured on CD14+ monocytes with flowcytometry. * $p<0.05$; ** $p<0.01$ vs medium control.



Supplemental Figure 4. FcγRI dependent binding of rituximab opsonized daudi cells to Ba/F3-FcγRI cells. Starved Ba/F3-FcγRI cells were stimulated with IL-3 or not and allowed to bind to rituximab opsonized daudi cells in the presence of FcγRI blocking mAb 10.1 or isotype control. The relative amount of Ba/F3-FcγRI binding daudi cells is shown. Experiment was repeated three times, yielding similar results.



Supplemental Figure 5. Similar FcγRI surface expression after okadaic acid treatment of monocytes. Primary monocytes were stimulated overnight with IFNγ and treated the next day with okadaic acid or DMSO. Shown are representative histograms of anti FcγRI or isotype (iso) stained monocytes.



Supplemental figure 6. Inside out regulation of FcγRI differs between mouse and man. Bone marrow derived DCs (BMDCs, see panel A) or bone marrow derived macrophages (BMMs, see panel B) from wildtype (WT) were used in an EA rosette assay with mIgG2a opsonized RBCs. BMDCs and BMMs were treated with TNFα and IFNγ (TNFα/IFNγ) or 1μM okadaic acid (OA) for 1 hour. Binding of mIgG2a opsonized erythrocytes was mouse FcγRI dependent as BMDCs and BMMs from FcγRI deficient (FcγRI-/-) mice did not show appreciable binding. Experiment was repeated twice, yielding similar results.

Chapter 3

Filamin A stabilizes FcγRI surface expression and prevents its lysosomal routing

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ABSTRACT

Filamin A, or actin-binding protein 280, is a ubiquitously expressed cytosolic protein that interacts with intracellular domains of multiple receptors to control their subcellular distribution, and signaling capacity. Here, we document interaction between FcγRI, a high affinity IgG receptor, and filamin A by yeast two-hybrid techniques and co-immunoprecipitation. Both proteins co-localized at the plasma membrane in monocytes, but dissociate upon FcγRI triggering. The filamin-deficient cell line M2, and a filamin-reconstituted M2 subclone (A7), were used to further study FcγRI-filamin interactions. FcγRI transfection in A7 cells with filamin resulted in high plasma membrane expression levels. In filamin-deficient M2 cells and in filamin RNA interference studies, FcγRI surface expression was consistently reduced. FcγRI localized to LAMP-1 positive vesicles in absence of filamin as shown by confocal microscopy indicative for lysosomal localisation. Mouse IgG2a capture experiments suggested a transient membrane expression of FcγRI before being transported to the lysosomes. These data support a pivotal role for filamin in FcγRI surface expression via retention of FcγRI from a default lysosomal pathway.

INTRODUCTION

Immune cells interact with antibody-antigen complexes through a variety of Fc receptors (FcR) [1]. The class I IgG receptor (FcγRI) is constitutively expressed on monocytes, macrophages and dendritic cells. FcγRI is a high affinity receptor for IgG and exists as a multimeric complex comprised of a ligand-binding α chain and the FcR γ-chain [2-4]. Its in vivo role is illustrated by FcγRI$^{-/-}$ mice that exhibit impaired antibody-dependent cellular processes such as bacterial clearance, phagocytosis, antigen presentation, and cytokine production [5, 6]. For signaling, FcγRI relies both on the FcR γ-chain and the cytosolic domain of its α-chain (FcγRI-CY). FcγRI-CY facilitates MHC class II antigen presentation without active FcR γ-chain signaling [7], whereas deletion of FcγRI-CY retarded kinetics of endocytosis and phagocytosis, and abrogated FcγRI-triggered interleukin-6 secretion [8]. Unlike the FcR γ-chain, FcγRI-CY does not contain ITAM or other tyrosine-containing signaling motifs.

Identification of interacting partners of FcγRI-CY may aid to decipher signaling routes that control FcγRI function. We recently described an interaction between FcγRI-CY and periplakin that affects FcγRI-ligand binding, and downstream effector functions [9, 10]. Previously, actin binding protein 280, or Filamin A (filamin), was shown to co-immunoprecipitate with FcγRI [11]. Filamin represents a homo-dimer composed of 280 kD subunits that organizes actin filaments into orthogonal networks (reviewed in [12, 13]. Here, we identified filamin in yeast-two-hybrid screens using FcγRI-CY, and functionally characterized this interaction. Studies with a naturally filamin-deficient cell line (M2 cells), and a filamin-reconstituted subclone (A7 cells) indicated filamin to be crucial for cell morphology and locomotion, as well as subcellular localization and signaling of various receptors [14-21]. To address the role of filamin for FcγRI biology, the sub-cellular distribution of FcγRI and filamin was studied in human monocytes. Stable FcγRI transfectants were generated in filamin-deficient M2 cells, and its filamin-reconstituted subclone A7 to assess the biological role of FcγRI-filamin interaction.

MATERIALS & METHODS

Constructs and chemicals

FcγRI (GenBankAccession Number L03418) was subcloned from pcDNA3 [22] (Invitrogen, Paisley, UK) containing neomycin resistance to pcDNA3.1 with zeocin resistance. The murine FcR γ-chain was HindIII/XbaI cloned into pCB7 containing hygromycin resistance [22]. The TCR α-chain of pMX-TCRα-chain-IRES-GFP [23], kindly provided by Dr. S.B. Ebeling (Dep. of Hematology, University Medical Center Utrecht, The Netherlands) was removed by BamHI/NotI digestion and replaced by FcγRI or FcαRI subcloned from pCAV [24]. PCR reagents for cloning were from Perkin-Elmer (Nieuwerkerk a/d IJssel, The Netherlands) except for oligo-nucleotide

primers (Isogen Bioscience, Maarssen, the Netherlands). All constructs were verified by dideoxy sequencing using BigDye Terminators (Applied Biosystems, Warrington, United Kingdom), and analyzed on an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems). Chemicals were from Sigma Aldrich (Steinheim, Germany), unless stated otherwise.

Yeast two-hybrid screens

A MATCHMAKER human bone marrow cDNA library from Clontech (Palo Alto, CA) was screened with FcγRI-CY as described in [22]. Protein interactions were assessed by growth of transfected yeast cells on selective media lacking leucine, tryptophane and histidine (-LTH), and a filter-lift β-galactosidase assay.

Cell culture, transfection, and Bafilomycin A1 treatment

Human peripheral blood monocytes were isolated from healthy volunteers. Mononuclear cells were isolated from ficoll gradients, and cultured with Iscove's Modified Dulbecco's Medium containing L-glutamine (Gibco BRL, Gaithersburg, MD) supplemented with 10 % FCS, penicillin and streptomycin. After ~3 hours at 37°C, non-adherent cells were removed, and adherent cells were incubated overnight with 300 IU/ml IFN-γ (IFN-γ1b, Boehringer Ingelheim, Biberach, Germany). Human melanoma cells selected for filamin deficiency (M2) and its filamin reconstituted subclone (A7) were cultured as described [14]. Cells were transfected with fugene (Roche, Mannheim, Germany) according to the manufacturer's instructions. Stable FcγRI-transfected cells were selected with zeocin (500 μg/ml). Inhibition of lysosomal maturation was accomplished by incubating cells with 100 nM Bafilomycin A1 in medium for various timepoints. Carrier control represents DMSO 500-fold diluted in medium. U937 cells were cultured in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% FCS, 100 unit/ml penicillin (GibcoBRL, NY) and 100μg/ml streptomycin (GibcoBRL, NY).

Immuno-precipitation

For FcγRI immunoprecipitation, U937 cells were stimulated with IFN-γ overnight. Cells were then lysed in cold NP40 buffer, 1% NP40 in phospate buffered saline solution with Complete EDTA-free protein inhibitor cocktail (Roche). Lysates were incubated overnight with either isotype control- (Sigma, St. Louis, MO) or m22 antibodies (Medarex Europe) coupled to protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, beads were washed in NP40 buffer, boiled in sample buffer and subjected to western blotting.

RNA interference

U937 cells were transfected with siGenome SMARTpool targeting human filamin A or with non-targeting siControl smartpool (Dharmacon, Lafayette, CO) using Amaxa nucleofection (Amaxa Inc, Gaithersburg, MD). Cells were cultured in RPMI 1640 10% FCS and stimulated overnight with IFN-γ after 48 hours. 72 hours post transfection, when filamin knock-down was maximal, the cells were stained with mAb anti-CD64 M22-FITC (Medarex, Annandale, NJ) and analysed on a FACSCalibur™ (BD Biosciences). Filamin protein levels were assessed by westernblotting using mAb1680 anti-human filamin A (Chemicon Int, Temecula, CA) followed by Goat anti mouse IgG-HRP (Jackson Immunoresearch, Suffolk, UK). For flow cytometric analysis of filamin protein levels, cells were fixed with 4% paraformaldehyde and stained in PBS containing 0.1% BSA, 0.1% saponin, 5% goat serum and 5% rabbit serum using mAb1680 and goat anti mouse IgG-RPE (Southern Biotechnology, Birmingham, AL).

Capture experiments using mIgG2a-FITC

M2 or A7 stabile transfectants were incubated with 2 μg/ml Mouse IgG2a-FITC (Dakocytomation, Glostrup, Denmark) in RPMI 1640 10% FCS at 37° C or at 4° C. After 6 hours, cells were washed with PBS and trypsinized. Subsequently, cells were split and washed 3 times with either normal RPMI 2% FCS or with RPMI 2% FCS adjusted to pH 2.5 (acidic wash)[25]. Cells were then analysed using a FACSCalibur™ (BD Biosciences).

Immuno-fluorescence

Monocytes were isolated as described above, resuspended in PBS with 2 mM EDTA and 0.1% bovine serum albumin, washed in medium, and adhered to poly-L-lysine-coated object glasses. For co-stainings with filamin, cells were fixed in methanol at -20°C, washed extensively, and permeabilized in PBS containing 0.1% saponin, 0.2% bovine serum albumin (BSA), 5% normal goat serum, and 5% normal rabbit serum. FcγRI was stained with 10 μg/ml FITC-conjugated humanized anti-FcγRI mAb H22 (Medarex, Annandale, NJ). Filamin was stained by mIgG1 anti-filamin (Chemicon, Temecula, CA), and goat anti-mIgG1-alexa555 conjugates (Molecular Probes, Leiden, The Netherlands). For internalization experiments, adhered cells were incubated in medium with 10 μg/ml FITC-conjugated H22 or mIgG2a for various timepoints at 37°C, fixed with methanol, and stained for filamin. Melanoma cells were grown on coverslips, fixed in PBS with 3% paraformaldehyde (or methanol when filamin was co-stained), and stained for FcγRI as described above, or with H22 $F(ab')_2$ followed by FITC-conjugated goat $F(ab')_2$ anti-human-κ-light chain (Southern Biotech, Birmingham, AL). Endoplasmatic reticulum was indicated by rabbit anti-calreticulin, cis-golgi by anti-GM130, and trans-golgi by anti-p230; endosomal and lysosomal compartments were indicated with

anti-EEA1, anti-CD63 and anti-CD107a (all mIgG1 unless indicated otherwise; BD Biosciences, San Diego, CA). Secondary detection was with goat anti-mIgG1-alexa555 conjugates (Molecular Probes), or goat anti-rabbit CY3 conjugates (Jackson ImmunoResearch, West Grove, PA). For Transferrin internalization, cells were incubated with 40 µg/ml Transferrin-conjugated to alexa555 (Molecular Probes) at 4°C, washed and incubated at 37°C in medium for 15 min, fixed and processed for immuno-fluorescence as above. Slides were examined with a 63x planapo objective on a Leitz DMIRB fluorescence microscope (Leica, Voorburg, The Netherlands) interfaced with a Leica TCS4D confocal laser microscope (Leica). Colocalization was quantified with Image J (http://rsb.info.nih.gov/ij/) using identical settings for each experiment (minimal pixel threshold 50, ratio 50%). Images from total cells were assessed for pixels that were positive both in the green (FcγRI) and red channel (subcellular markers, or filamin) and the total number of pixels in the green channel (FcγRI). Percentage FcγRI colocalization with subcellular marker/filamin = colocalized pixels between FcγRI and a specific marker / total FcγRI pixels × 100%.

Flow cytometry

M2 and A7 cells were detached 48 hours post-transfection, and stained in PBS containing 0.1% BSA, 2 mM EDTA, and 10% normal mouse serum. Cells were washed, and surface FcγRI, or total FcγRI was detected with monoclonal antibody (mAb) 10.1-FITC (Serotec, Oxford, UK) in the absence or presence of 0.1% saponin, respectively, at 4°C. Cells were washed and analyzed with a FACSCalibur™ (BD Biosciences). Surface expression was scored positive when it exceeded three times background staining of untransfected cells. When FcγRI expression was compared with FcαRI expression, FcγRI and FcαRI were stained in 40 µl with 20 µg/ml mIgG1 clone 10.1 and A59, respectively, followed by 10 µg/ml goat anti-mouse phycoerythrin (PE) conjugated (Jackson ImmunoResearch).

RT-PCR

RNA was extracted from cells with Qiagen RNeasy midi columns (Qiagen, Hilden, Germany), and reverse transcribed with oligo-dT primers of a GeneAmp RNA PCR kit (Applied Biosystems). FcγRI was amplified by 33 cycles as described in ref [22]. Actin was amplified by 25 cycles with 5'-gtggggcgcccccaggcaccag-3' and 5'-ctccttaatgtcacgcacgatttc-3' under standard conditions for polymerase chain reactions (PCR).

Western blot

1 x 10^5 cells were lysed in reducing Laemmli sample buffer, and proteins separated with SDS-PAGE (12% gel). Proteins were transferred to nylon membranes, and stained for FcR γ-chain

(Upstate, Lake Placid, NY) that was detected by goat anti-rabbit conjugated to horse radish peroxidase (Pierce, Rockford, IL). Enhanced chemiluminescence and Biomax™ films were from Amersham Biosciences, Buckinghamshire, UK.

RESULTS

Filamin interacts directly with cytoplasmic domains of multiple receptors, and can profoundly affect their function [13]. We found filamin (a schematic representation of filamin is shown in Fig. 1A) to interact with FcγRI using yeast two-hybrid screens on a bone marrow cDNA library. Co-transfection of the filamin-containing cDNA with empty bait plasmids did not allow yeast cells to grow on selective media (data not shown). Importantly, we confirmed this interaction by co-immunoprecipitating filamin via FcγRI from IFN-γ stimulated U937 cells expressing both proteins endogenously (Fig. 1B). We next analyzed the subcellular localization of FcγRI and filamin in primary IFN-γ stimulated monocytes (Fig. 1C). A significant portion of FcγRI and filamin co-localized at the plasma membrane when cells were fixed and stained with the CD64 mAb H22.

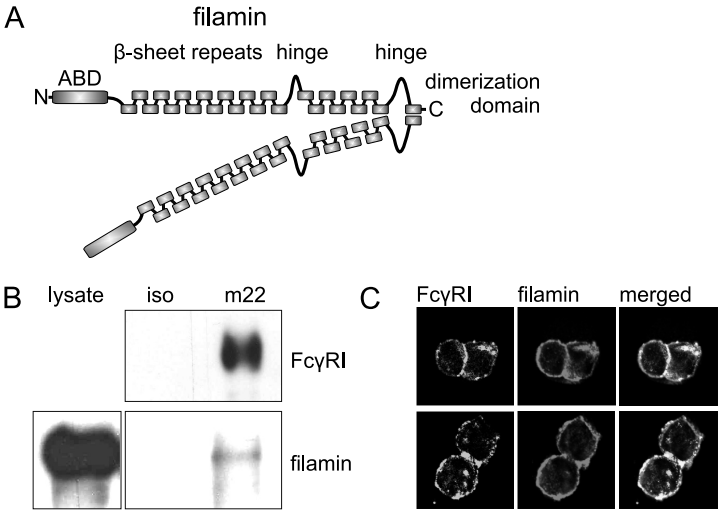

Figure 1. FcγRI interaction with filamin in yeast cells. (A) Schematic representation of filamin. ABD, actin binding domain. (B) Co-immunoprecipitation of filamin and FcγRI. U937 cells were lysed after overnight stimulation with IFN-γ. Subsequently, immunoprecipitation was performed using an anti-FcγRI antibody (m22) or isotype control (iso). Western blotting was used to detect immunoprecipitated FcγRI (top panel) and co-immunoprecipitated filamin (lower panel). Experiments were performed thrice, all yielding similar data. (C) Subcellular localization of FcγRI and filamin in monocytes. Cells were stimulated overnight with 300 U/ml IFN-γ. Cells were adhered to glass slides, fixed in methanol, and FcγRI was stained with CD64 mAb H22 conjugated to FITC. Filamin was stained red. Green, red and merged pictures of two stainings are shown. Co-localization is indicated in yellow. (See color section for a full-color version)

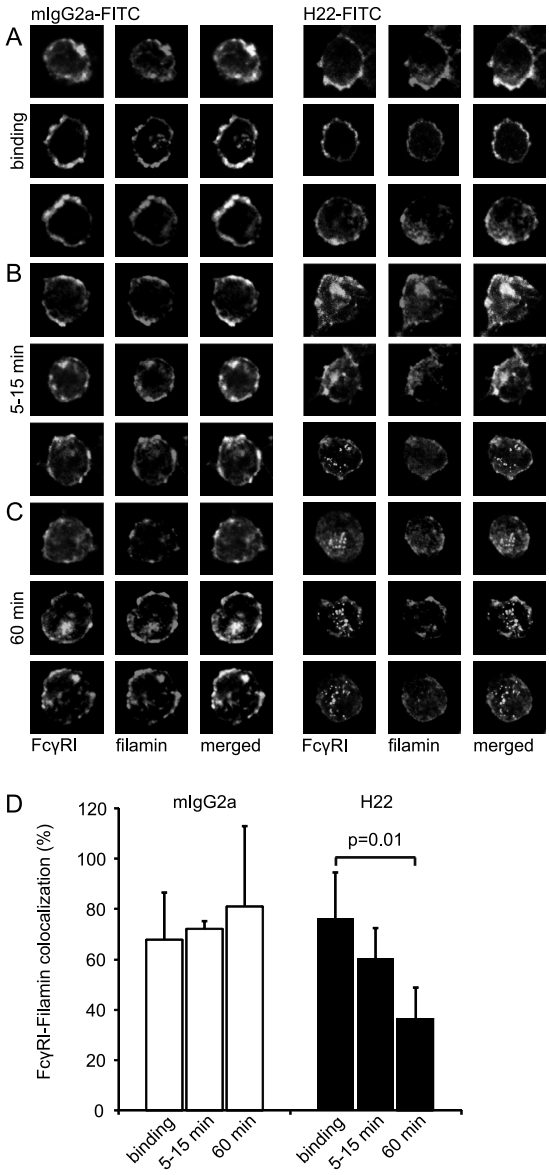

Figure 2. Subcellular localization of FcγRI and filamin in IFN-γ stimulated monocytes. (A) Monocytes were stimulated overnight with 300 U/ml IFN-γ. Cells were incubated with monomeric mIgG2a or mAb H22 (both FITC-conjugated) for 60 min at 4°C. Cells were then fixed, and stained for filamin. Three representative cells are shown. (B+C) FcγRI ligands were added at 37°C at t=0, and remained present throughout the experiment. Cells were fixed at different time points (B: 5-15 min, C: 60 min, three representative examples shown). Filamin was stained red. Experiments were repeated thrice, all yielding similar data. (D) The amount of FcγRI/filamin colocalization was quantified using Image J (see Material and Methods section), and the percentage of FcγRI/filamin colocalization was expressed as function of time (n=4, Students T test). 100% co-localization was determined by incubating H22-FITC or mIgG2A for 60 min. at 4°C. Errorbars indicate standard deviation. (See color section for a full-color version)

When monocytes were incubated with monomeric ligand (mIgG2a-FITC), or cross-linking FcγRI mAb (H22-FITC) that can trigger FcγRI internalization [26, 27], extensive co-localization was observed at the plasma membrane, and on mIgG2a-positive intracellular compartments (Fig 2A-C, left panels). Some filamin still co-localizes intracellularly with FcγRI at early timepoints (5-15 min) after H22-FITC incubation (Fig. 2A and B, right panels). However, H22-FITC induced drastic FcγRI-filamin dissociation at later timepoints (Fig. 2A-C, right panels and D). Together, these results suggest that FcγRI and filamin predominantly interact at the plasma membrane, and to a minor extent at an intracellular compartment.

Next, we studied FcγRI in a filamin-deficient cell system, and stably transfected filamin-deficient M2 cells, and its filamin-reconstituted subclone A7 with the α-chain of FcγRI (FcγRI) [14]. From three independent FcγRI transfections, 16 zeocin-resistant A7 clones, and 12 M2 clones were selected. FcγRI surface expression in A7 cells was observed in ~50% of subclones (8-out-of 16 clones, Fig. 3A). Surprisingly, FcγRI cell surface expression in 12 subcloned M2 cells was extremely low or even undetectable, albeit that six-out-of seven M2 transfectants expressed FcγRI at the transcript level (Fig. 3B). This indicated filamin to be an important determinant of FcγRI surface expression (percentage FcγRI positive A7 clones were compared with M2 clones, Fisher's exact test p=0.0083). Next, we assessed FcγRI expression at the subcellular level in A7 and M2 cells by confocal microscopy (Fig. 3C). In the presence of filamin, FcγRI surface expression was evident, although some intracellular staining could be detected. In M2 cells lacking filamin, FcγRI-specific staining predominantly localized intracellularly, and was more difficult to detect suggesting lower total FcγRI protein levels (Fig. 3C and 7A). M2 cells were confirmed to be filamin-negative by Western blot and immuno-fluorescence analyses (data not shown). FcγRI co-localized with filamin at the plasma membrane, and to some extent at intracellular vesicles in A7 cells (data not shown; n=3). Knock down of filamin using siRNA in U937 cells lead to a decreased cell surface expression of endogenous expressed FcγRI (Fig 3D, left panel. Mann-Whitney test p=0.004). Filamin knock down was confirmed by flowcytometry (Fig 3D, right panel) and westernblot (data not shown).

To assess whether the defective plasma membrane expression in M2 cells was selective for FcγRI, we included the receptor for IgA, FcαRI or CD89, as a control in transient transfections of M2 cells and A7 cells with FcγRI. Both receptors were expressed from vectors that co-expressed enhanced green fluorescent protein (GFP). Surface expression levels of FcγRI, and FcαRI were comparable upon transfection in cells with filamin (Fig. 4A, n=3). Transfection efficiencies were comparable as indicated by GFP signals. Cells without filamin, however, displayed impaired FcγRI surface levels, whereas FcαRI surface levels were unaffected. Control staining was performed on mock-GFP transfected cells with isotype controls (Fig. 4A) and untransfected cells (data not shown, n=3). Similar differences were apparent at days three and four post-transfection (data not shown, n=2).

In mice, in vivo surface expression of many FcR, including FcγRI, has been found to (partly) rely on the associated FcR γ-chain [3, 4, 28]. Therefore, to address whether the FcR γ-chain was

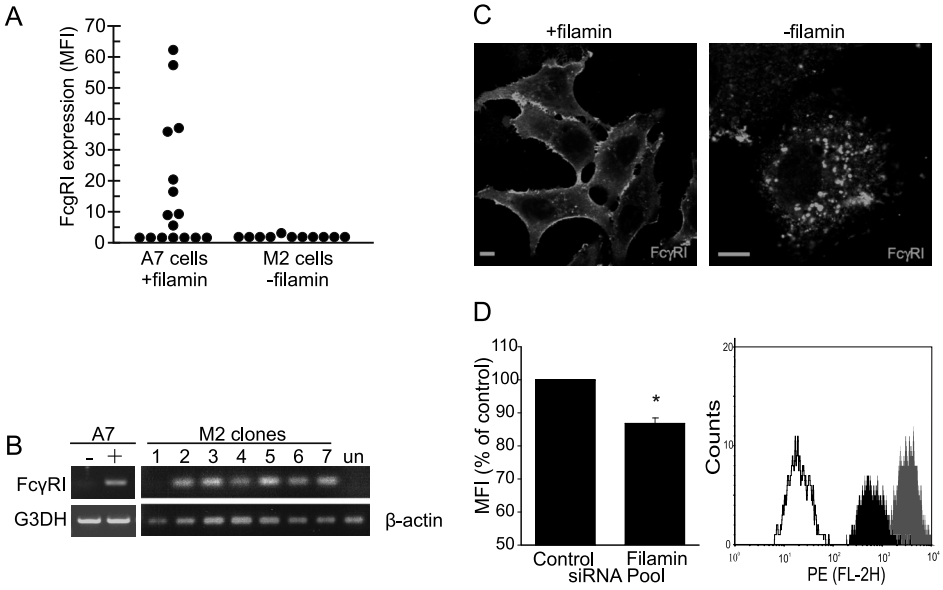

Figure 3. FcγRI surface expression on cells with or without filamin. (A) Stable zeocin-resistant clones were randomly selected from three independent transfection experiments in cells with or without filamin. FcγRI expression was detected by CD64 mAb 10.1-FITC, and assessed by flow cytometry. Mean fluoresence intensities (MFI) are indicated. (B) RNA was extracted from two A7 clones (left lane negative (-), and right lane positive (+) for FcγRI surface expression) and seven M2 clones, and RT-PCR for FcγRI was performed. (C) Subcellular localization of FcγRI in filamin-deficient and filamin-reconstituted cells. FcγRI was stained by monoclonal 10.1-FITC after paraformaldehyde fixation (4) in filamin expressing clones (left panel) and filamin-deficient M2 cells (right panel). Bar marks 20 µm. (D) Relative FcγRI surface expression after filamin knockdown in U937 cells using siRNA (non targeting control pool versus filamin targeting pool) was assessed by flowcytometry (left panel, * p=0.004, Mann-whitney test, n=3). Errorbars indicate standard deviation. Filamin knockdown was confirmed by westernblot (not shown) and flowcytometry (right panel, one representative experiment). Open histogram depicts background of secondary antibody, grey histogram depicts filamin staining in cells transfected with control pool and black histogram shows cells transfected with filamin targeting pool. (See color section for a full-color version)

capable of rescuing the impaired surface expression in this system, it was transiently co-transfected in three FcγRI-transfected M2 and A7 subcloned cell lines. No appreciable differences were observed for surface FcγRI upon mock or FcR γ-chain transfection (Fig. 4B, n=2). FcR γ-chain transfections were confirmed by Western blot, and one representative sample is shown in figure 4C.

Next, we set out to identify the intracellular compartment in which FcγRI resides in filamin-deficient cells by immuno-fluorescence and electron microscopy (Fig. 5). We tested a panel of antibodies recognizing compartments that are involved in afferent plasma membrane pathways such as Calreticulin (Fig. 5A) for the endoplasmatic reticulum (ER), and p230 (Fig. 5B) and GM-130 (data not shown) for Golgi. Endosomal compartments were analyzed by Transferrin-uptake (Fig. 5C), and mAb stainings for early endosomal antigen-1 (EEA-1; Fig.

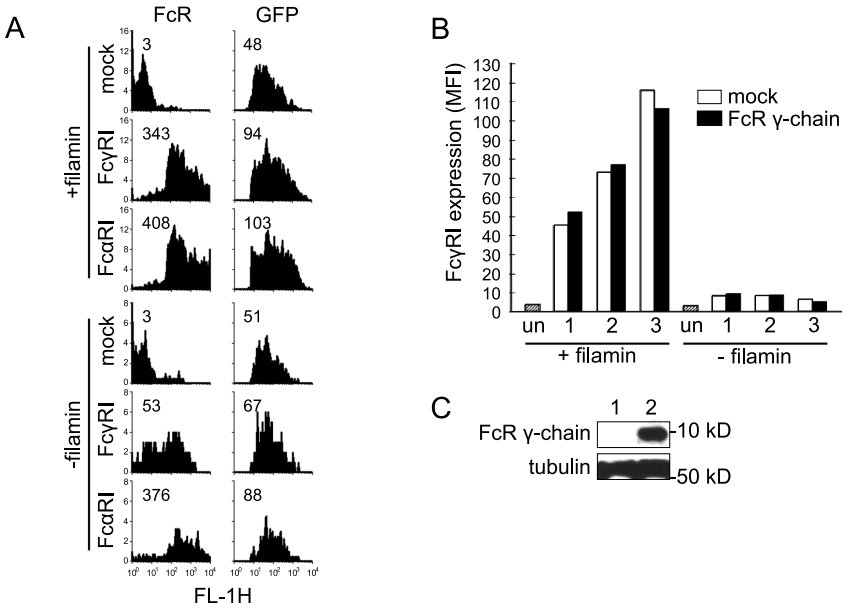

Figure 4. (A) Transient transfection of M2 and A7 cells with FcγRI and FcαRI cDNA. Cells were transfected with FcγRI, and FcαRI expressed from plasmids that co-expressed enhanced GFP (GFP). FcγRI and FcαRI were stained with mAb M22, and A59, respectively, followed by incubation with PE-labeled anti-mouse IgG, and flow cytometry. Gates for viable GFP-positive cells were applied, and cells were assessed for FcR and GFP expression 48 hours post-transfection. Histograms of GFP-gated cells stained for FcγRI, and FcαRI are shown in the left column, associated GFP levels are shown on the right. Cells were transfected with the original vector that co-expressed TCR α-chain and GFP to check for staining specificity. The mock cells shown in this figure were transfected with the original vector that co-expressed TCR α-chain and GFP and stained with antibodies against FcγRI. However, essentially the same results were obtained when these cells were stained with antibodies against FcαRI (data not shown). MFI are shown in the top-left corner of each histogram. Three experiments were performed, yielding essentially identical results. (B) Effect of FcR γ-chain transfection on FcγRI surface expression. Three filamin-positive and filamin-negative lines that expressed FcγRI transcripts were transfected with FcR γ-chain (black bars) or mock plasmid (white bars). Hatched bars represent untransfected parental cells (un). Cells were stained for surface FcγRI by mAb 10.1-FITC, and assessed by flow cytometry (MFI are indicated). Two experiments yielded similar data. (C) Western blot for FcR γ-chain, and tubulin after transfection of mock (lane 1) or FcR γ-chain (lane 2) plasmids in M2 cells. Transfected A7 cells showed similar levels of FcR γ-chain. Tubulin was used as loading control.

5D), lysosomal integral membrane protein-1 (CD63; Fig. 5E), and lysosomal-associated membrane protein LAMP-1/CD107a (Fig. 5F). We did not observe a clearly defined compartment in which FcγRI accumulated using these markers, suggestive for a transient passage through the compartments tested. Most co-localization between FcγRI and a subcellular marker was observed for the lysosomal marker LAMP-1 as indicated by quantification of colocalized signals of cells from three different experiments (Fig. 5G). Treatment of cells with nocodazole, which disrupts microtubuli and disperses cellular organelles, reduced some co-localization of FcγRI and LAMP-1 (data not shown, n=2). In A7 cells, intracellular FcγRI could be found

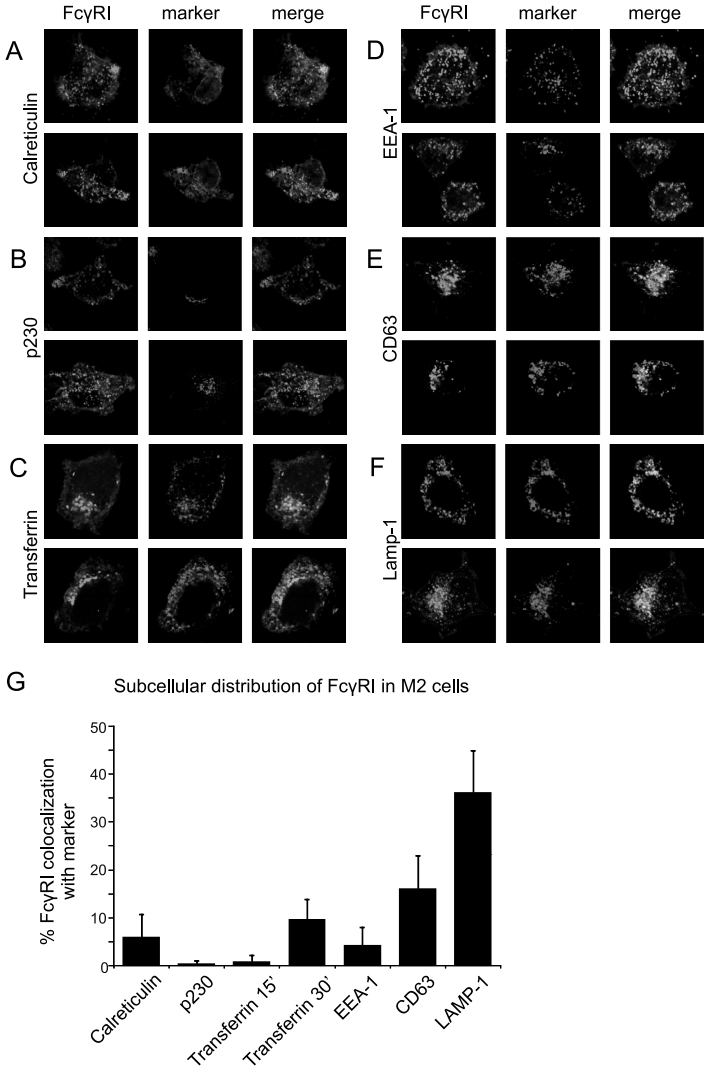

Figure 5. Intracellular location of FcγRI in cells without filamin. M2 cells were stained for markers involved in plasma membrane afferent pathways, and the endosomal/lysosomal pathway. FcγRI was stained in green (H22-FITC), all other markers in red. Panel (A) was stained for Calreticulin, (B) for p230 (Golgi), (C) for Transferrin uptake, (D) for EEA-1, (E) for CD63, (F) for LAMP-1/CD107a. (G) Percentage co-localization with subcellular markers was calculated using Image J as described in Materials and Methods. Cells from three independent experiments were quantified. The asterisk indicates statistical significance between LAMP-1 and other markers (Student's T test, $p<0,05$). (See color section for a full-color version)

in similar compartments as for cells without filamin, although A7 cells displayed plasma membrane accumulation (data not shown).

To test whether in the absence of filamin, FcγRI is indeed transported to the plasma membrane before entry into a lysosomal pathway, we performed mIgG2a-FITC capture

experiments. M2 and A7 transfectants were incubated with mIgG2a-FITC at 37° (Fig 6A. and C) to allow binding and endocytosis or at 4° (Fig. 6B and D) to allow only binding to FcγRI. M2 and A7 transfectants became FITC positive after incubation with mIgG2a-FITC. Washing cells with low pH removed surface bound IgG (Fig. 6B and C) but only partly abrogated FITC signal when cells were incubated at 37°, suggesting endocytosed mIgG2a-FITC in both M2 (Fig. 6A and C) and A7 (Fig. 6 A) transfectants. Uptake of mIgG-FITC appeared dependent on FcγRI as the untransfected parental M2 cells did not obtain FITC signal. In combination with the co-lozalization experiments these data suggested filamin to stabilize FcγRI at the plasma membrane, and to prevent entry of FcγRI into a lysosomal pathway.

Total FcγRI protein levels in M2 and A7 clones were assessed by flow cytometry upon cell permeabilization with saponin (Fig. 7A). FcγRI protein levels were significantly reduced in the absence of filamin. To demonstrate that these cells produced significant levels of FcγRI, and that the low FcγRI amounts were a consequence of lysosomal degradation, we incubated cells with Bafilomycin A1. Bafilomycin A1 inhibits the vacuolar-type H+-ATPase that regulates endosomal pH, and hence prevents intracellular degradation of endocytosed cargo and receptors (35,37). FcγRI levels in cells without filamin increased profoundly upon incubation

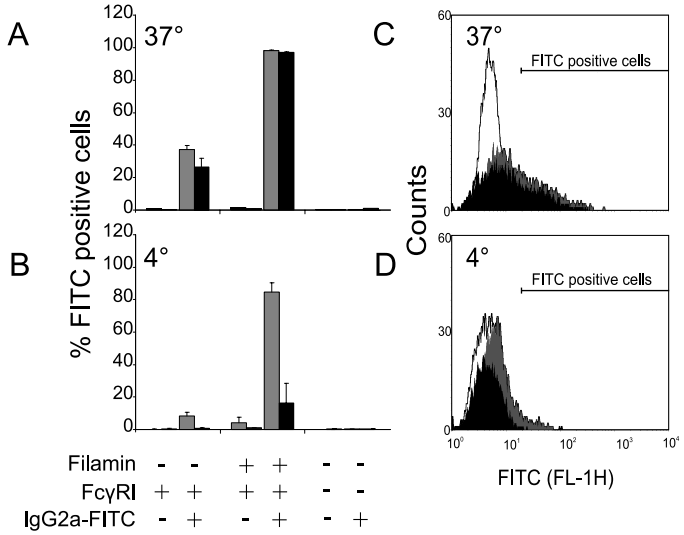

Figure 6. Mouse IgG2a uptake by M2 and A7 cells expressing FcγRI. Stabile clones of M2 (M2 FcγRI), A7 (A7 FcγRI) and parental M2 cells (M2) were incubated with or without mouse IgG2a-FITC for 6 hours. After incubation at 37° (A) or at 4° C (B), cells were washed at either normal pH (grey bars) or at pH 2.5 (black bars) to remove surface bound IgG2a. Cells were analysed by flowcytometry. Bargraphs show the percentage of FITC positive cells defined by regions drawn in panels C and D. Bars represent the mean of three experiments. Error bars indicate standard deviation. (C+D) Histograms from one representative experiment of M2 cells expressing FcγRI incubated with mouse IgG2a-FITC at 37° C (C) or at 4° C (D). Open histograms represent cells without mIgG2a, grey histograms depict cells incubated with mIgG2a and washed at normal pH, black histograms represent cells washed at pH 2.5.

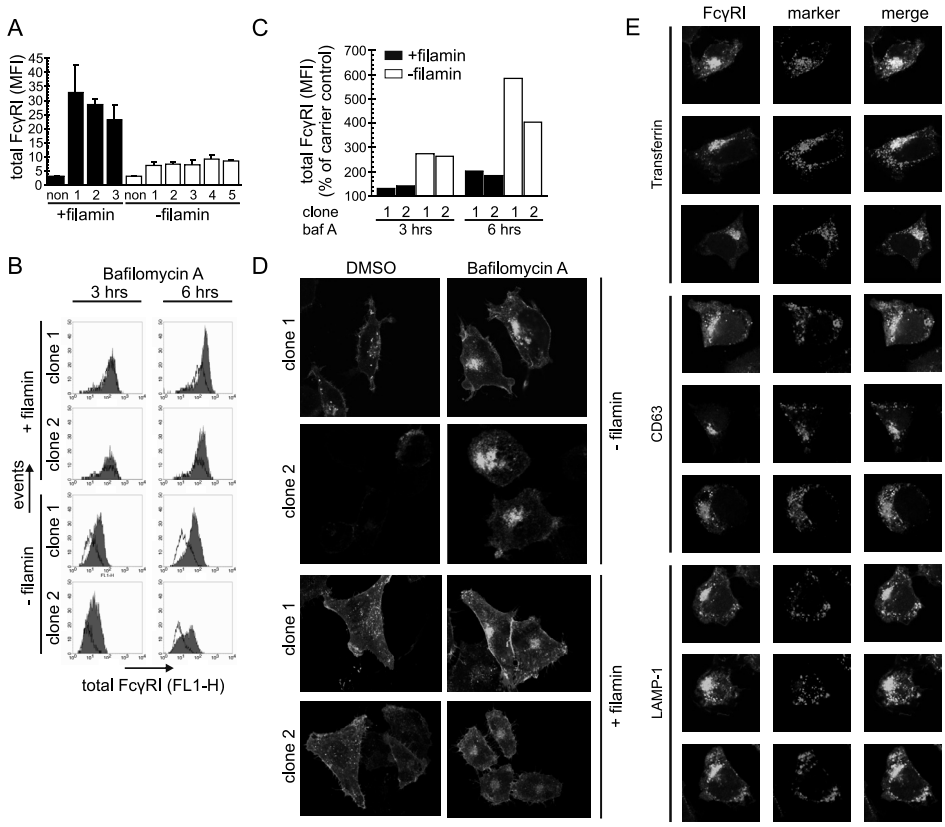

Figure 7. Filamin prevents lysosomal degradation of FcγRI. (A) Filamin-positive and negative lines that expressed FcγRI transcripts were stained for total FcγRI in the presence of 0.1% saponin. Black bars represent A7 clones, white bars M2 clones, un untransfected cells. Numbers represent independent clones of FcγRI transfectants. Average mean values of four independent experiments are shown, error bars indicate standard error, of the mean. (B) Two FcγRI-expressing clones with or without filamin were incubated with Bafilomycin A1 (100 nM) for three or six hours. Total FcγRI was assessed as in A. Black-lined open histograms represent total FcγRI after incubation in carrier control (DMSO 500-fold diluted); grey-filled histograms represent total FcγRI after Bafilomycin A1 treatment. Representative data of three independent experiments are shown. (C) Relative increase of total FcγRI after incubation of cells with Bafilomycin A1. Two FcγRI-transfected cell lines with (black bars) or without (white bars) filamin were compared. For each cell line, total FcγRI levels after DMSO incubation was set at 100%. (D) FcγRI transfected M2 and A7 cells were assessed by confocal microscopy after 2 hour incubation with Bafilomycin A1 or DMSO. FcγRI was stained by mAb 10.1 FITC-conjugated. (E) Intracellular location of FcγRI in cells without filamin after Bafilomycin A1 treatment. M2 cells were stained for markers involved in endosomal (Transferrin uptake) and lysosomal (CD63, LAMP-1) pathways. FcγRI was stained in green (H22-FITC), all other markers in red. (See color section for a full-color version)

with Bafilomycin A1 (Fig. 7B+C, n=3), but not with the proteasomal inhibitor lactacystin (data not shown). After six hours, FcγRI levels almost reached those of A7 cells incubated with carrier control. Some accumulation of FcγRI after six hours was observed for FcγRI in A7 cells, suggesting limited lysosomal FcγRI degradion in the presence of filamin. No changes

were observed for FcγRI surface levels in the presence of Bafilomycin A1, suggesting FcγRI internalization not to be affected (data not shown, refs 35,37). Bafilomycin A1 clearly elevated intracellular staining of FcγRI in cells without filamin (Fig. 7D). Upon Bafilomycin A1 treatment A7 cells showed an increase of intracellular staining of FcγRI as well (Fig. 7D) which coincided with flowcytometric analysis of total protein levels (Fig. 7C). Interestingly, Bafilomycin A1 treatment caused FcγRI to accumulate predominantly in the endosomal compartment (Fig. 7E, transferrin staining), although some colocalization was also seen with lysosomal markers CD63 and LAMP-1.

DISCUSSION

In order to identify protein effectors of FcγRI, we performed yeast two-hybrid screens and identified filamin, an actin binding protein. This interaction was confirmed in co-immunoprecipation experiments with endogenously expressed FcγRI and filamin (Fig. 1B). We found filamin to partially co-localize with FcγRI in primary monocytes after fixation (staining with mAb recognizing FcγRI, independently of bound ligand), or by addition of monomeric IgG, and an FcγRI-cross-linking mAb at 37°C prior to fixation (Fig. 1C and fig. 2). Most co-localization was observed at the plasma membrane where filamin may act to stabilize FcγRI surface expression by tethering FcγRI to actin, and preventing FcγRI to internalize, as was suggested by our studies in filamin-deficient cells. As monomeric IgG induces FcγRI internalization into a recycling route that prevents its lysosomal degradation [29], intracellular FcγRI-filamin interactions upon monomeric IgG incubation may contribute to its surface expression by retaining FcγRI in a recycling pathway, similar as described for calcitonin receptor-filamin interactions [19]. Although our data suggest that IgG-occupied FcγRI can interact with filamin (Fig. 2), a previous report indicated filamin to preferentially interact with ligand-free FcγRI [11]. As suggested by these authors, effective IgG-induced dissociation of FcγRI-filamin might require larger IgG complexes that may coincide with induction of phagocytosis [11].

We observed FcγRI surface expression *in vitro* to depend on filamin, shown in a transfection model of cells that differed by filamin expression and by RNA interference (Fig. 3-5). Routing of FcγRI towards the plasma membrane in filamin-negative cells appeared normal: FcγRI did not accumulate in compartments that are involved in afferent plasma membrane transport such as ER and Golgi, its expression was insensitive to co-expressed FcR γ-chain, and some surface expression was observed in transient transfection experiments. Moreover, capture experiments suggested transitory FcγRI surface expression (Fig. 6) in the absence of filamin. However, a significant proportion of FcγRI localization was confined to (pre)lysosomal compartments in M2 cells as apparent from confocal studies (in the absence of crosslinking ligand, Fig. 5). FcγRI protein levels were also highly sensitive to Bafilomycin A1 in cells without filamin (Fig. 6). Interestingly, Bafilomycin A1 treatment of M2 cells caused FcγRI to accumu-

late predominantly in the endosomal compartment (Fig 7E). This coincides with previous publications showing that acidification is critical for fusion of yeast vacuoles [30, 31]. Capture experiments and colocalization studies suggested that FcγRI does not accumulate on the cell surface, but transiently pass through this compartment to be finally degraded in lysosomal structures. FcγRI is unique amongst multi-subunit FcR, and harbors intracellular residues that facilitate MHC class II presentation after immune complex triggering [7]. This pathway may be inhibited by filamin activity in resting immune cells to facilitate FcγRI surface expression, and prevent undesired degradation and presentation of antigens (a model is presented in Fig. 8).

Previous studies have demonstrated *in vitro* surface expression of FcγRI, in contrast to FcγRIII and FcεRI, to be independent of co-expressed FcR γ-chain [32, 33]. FcγRI lacks ER retention motifs present in the α-chains of FcγRIII and FcεRI that are masked by the FcR γ-chain to allow surface expression [33-35]. This was supported by the inability of the FcR γ-chain to rescue FcγRI surface expression in our experiments (Fig. 4). *In vivo*, surface expression of FcγRI, FcγRIII and FcεRI is reduced in FcR γ-chain deficient mice, albeit that FcγRI is detectable at ~20% of wild type levels [3-5]. This may indicate that in these cells the remaining FcγRI is stabilized by filamin, or that co-expressed FcR γ-chain modulates filamin activity *in vivo*.

We recently reported periplakin to interact with the membrane-proximal domain of FcγRI under similar conditions as described here for filamin, albeit that periplakin and FcγRI did not colocalize on intracellular vesicles [10, 22]. Although it remains unclear how periplakin and filamin interact in FcγRI functioning, the present data may suggest these proteins to affect separate FcγRI functions. Blockade of FcγRI-periplakin interaction by overexpressed C-terminal periplakin or blocking peptides modulated FcγRI ligand binding capacity and

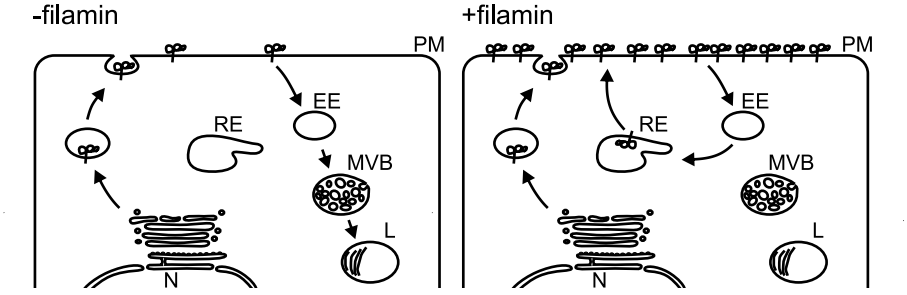

Figure 8. Model of FcγRI-filamin interactions. In the absence of filamin (left panel), FcγRI is capable of reaching the plasma membrane after synthesis, but is internalized rapidly, and routed by default towards lysosomes where it becomes degraded. In the presence of filamin (right panel), FcγRI is routed towards the plasma membrane after synthesis in the ER. Filamin interactions with FcγRI stabilize plasma membrane localization of FcγRI by reducing internalization of ligand-free FcγRI. Internalized FcγRI may be sequestered into a recycling pathway by filamin to further support plasma membrane expression, and prevent unwanted lysosomal degradation of receptor and cargo. EE early endosome, L lysosome, MVB multivesicular body, N nucleus, PM plasma membrane, RE recycling endosome.

downstream effector functions but not surface expression [10, 22]. Both proteins can also act as cytoskeletal associated scaffold for signal transducers [12, 36, 37], suggesting that periplakin and filamin may coordinate different signaling pathways. The data presented here point at a vital role for filamin in FcγRI biology by stabilizing surface expression, and retention of FcγRI from a default lysosomal pathway that mediates FcγRI degradation.

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Chapter 4

Protein 4.1G binds to a unique motif within the FcγRI cytoplasmic tail

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ABSTRACT

The C-terminal domain of protein 4.1G was identified to interact with the cytosolic tail of the high affinity IgG receptor, FcγRI, in yeast two-hybrid screens. Proteins of the 4.1 family have previously been found to mediate receptor/cytoskeleton interactions. In the study presented here, we show an alternatively spliced 4.1G product to be associated with increased FcγRI binding in yeast two-hybrid assays, and to be selectively enriched in most immune cells at the transcript level. In addition, a detailed analysis of the 4.1G 'docking site' within FcγRI is provided by examining FcγRI-CY-truncated, and alanine-substituted mutants. These pointed to an FcγRI-membrane-proximal core motif of HxxBxxxBB (H represents hydrophobic residues, B basic residues, x any residue), followed by hydrophobic and (potentially) negatively charged residues to be central for interaction with protein 4.1G.

INTRODUCTION

Fc receptors (FcR) mediate interactions between immune cells and immunoglobulins (Ig) [1, 2]. Multiple FcR exist as heterologous complexes of ligand binding α-chains, and promiscuous subunits that carry intracellular tyrosine-based activation motifs (ITAM). With the exception of class II FcγR members, the α-chains of multi-chain FcR bear no recognized signaling motifs in their cytoplasmic domains. However, recent studies demonstrated the cytoplasmic domain of FcγRI to be important for protein interactions that modulate receptor function [3, 4]).

FcγRI (CD64) represents a high affinity IgG receptor that is constitutively expressed by myeloid cells. The 72 kDa type I glycoprotein can effectively capture small immune complexes, and facilitates presentation of antigens in the context of MHC class I, and II [5-8]. The FcγRI cytosolic domain (FcγRI-CY) of approximately 65 residues interacts with cytoskeleton-associated proteins like periplakin and filamin A (filamin, or ABP-280) [3, 9]. The role of these proteins as scaffold for FcγRI signaling complexes is not well understood, but may be profound as truncation of FcγRI-CY prevented FcR γ-chain independent antigen presentation, IL-6 production, and lead to altered kinetics of endocytosis and phagocytosis [6, 10].

Here we studied interactions between FcγRI-CY and splice-forms of protein 4.1G, which were identified to bind FcγRI-CY in yeast two-hybrid screens. Protein 4.1 family members have been shown to functionally modulate multiple receptors [11-13]. The C-terminus of protein 4.1G interacted with FcγRI-CY, and was found selectively spliced in immune cells at the transcript level. Truncated and alanine-substituted mutant FcγRI-CY were assessed for interaction with 'immuno-enriched' 4.1G.

MATERIALS & METHODS

DNA constructs, antibodies and chemicals

cDNA encoding the cytosolic tail of human FcγRI (and mutants), FcγRIIa, FcγRIIIa, FcαRI, FcεRI and murine (m) FcγRI were expressed from pGBT9 [3, 4]. Recombinant proteins were expressed from pGEX-2T (Amersham Biosciences, Amersham, UK) containing the original GST tag in case of FcγRI, or containing a replaced His-tag (6xhistidine) in case of 4.1G-CTDΔ14 (starts at bp 2121-2402 coupled to 2652-3062, GenBank Accession Number NM_001431) [4]. Integrity of cDNA generated by PCR was verified by sequence analysis using BigDye Terminator mix (Applied Biosystems, Warrington, United Kingdom). Western blots were performed with anti-GST goat polyclonal serum (Amersham), rabbit serum against FcγRI-CY (a kind gift of Dr. S. Tridandapani, Department of Internal Medicine, Ohio State University, Columbus, OH), and monoclonal antibody against 6xHis-Gly (Invitrogen, Leek, The Netherlands). Detection was performed by using horse radish peroxidase-conjugated mAb (Pierce, Rockford, IL; and Dako,

Glostrup, Denmark). Chemicals were from Sigma (Steinheim, Germany) unless indicated otherwise.

Yeast two-hybrid

A human bone marrow cDNA library in pACT-2 was screened for FcγRI-CY interacting proteins in yeast strain YGH1 according to Clontech protocols (Clontech, Palo Alto, CA). Protein interactions were assessed by growth of colonies on histidine depleted media, and β-galactosidase activity using replica filter assays as described in [3]. Quantification of FcγRI interaction with protein 4.1G cDNA clones in yeast cells were performed in a liquid β-galactosidase assay using ONPG as substrate (protocol described in Clontech's Yeast Protocols Handbook).

RT-PCR

Transcripts of 4.1G were amplified from oligo-dT primed cDNA by 30 cycles (30 sec 95°C, 30 sec 62°C, 30 sec 72°C) using forward primers (Isogen, Maarssen, The Netherlands) that recognized a 4.1G-specific region in the first part of exon 14 (5'-accaccgagtgaccgagggc-3') and reversed primers that hybridized adjacent to 4.1G stopcodon (5'-cagatgtgatggtttgtgcgg-3'). Monocytes were cultured towards immature dendritic cells (DC, day 5), and were matured for two days as described [14, 15]. Interferon γ (IFN-γ) stimulations were overnight with 300 U/ml (IFN-γ1b, Boehringer Ingelheim, Biberach, Germany). The presence of cDNA in samples was verified by GAPDH PCR. AmpliTaq Gold and PCR reagents were from Perkin-Elmer (Nieuwerkerk a/d IJssel, The Netherlands). PCR products were extracted from agarose and sequenced.

Co-immunoprecipitation of recombinant proteins

GST-FcγRI and His-tagged 4.1G-CTDΔ14 were purified under non-denaturing conditions as described in [3]. Tosyl-activated M-280 Dynabeads (Dynal, Oslo, Norway) were covalently attached to anti-GST antibody according to the manufacturers' instructions. Beads were incubated overnight with ~1 µg of GST-FcγRI or GST, and ~1 µg of His-4.1G in 500 µl RIPA buffer (150 mM NaCl, 100 mM Tris HCl pH 8.3, 0.1% SDS, 0.5% DOC, 1% TritonX-100). Beads were washed three times in RIPA, boiled in Laemmli sample buffer, and analyzed by SDS-PAGE and Western blotting.

Sequence alignments

Sequences of 4.1G CTD interacting proteins were obtained from Entrez Nucleotide at www.ncbi.nlm.gov/. GenBank Accession Numbers: PTA-1: NM_006566, D2/D3 dopamine receptors:

NM_000795/NM_000796, A1 adenosine receptor: AF042079, Nuclear mitotic apparatus protein (NuMa; Z11583) and FK506 binding protein 13 (FKBP13, XM_215196). GluR-1/GluR-D AMPA receptors were found in the Swiss-Prot database, accession P19493/P19490. Secondary structures were predicted with PHD [16], FASTA [17], SSPro [18], 3D-PSSM [19], and JPRED [20]. Sequences were edited using BioEdit software available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html.

RESULTS

FcγRI and 4.1G interact in yeast two-hybrid screens

The cytosolic domain of FcγRI, and the FcR γ-chain both trigger cellular functions [6, 10]. Little is known, however, about protein effectors of the FcγRI cytosolic tail, albeit that recent studies indicate protein interactions with FcγRI-CY to modulate receptor function [3]. We, therefore, searched for novel FcγRI-CY interacting proteins by yeast two-hybrid screens.

The C-terminal domain (CTD) of 4.1G was found to interact with FcγRI-CY in two screens of human bone marrow cDNA. Three-out-of six identified cDNA had different inserts, and β-galactosidase assays are shown in Fig. 1A and B. One cDNA (IV.140) displayed reduced interaction with FcγRI as it showed no blue staining after filterlift and reduced β-galactosidase activity. Protein 4.1 members have three conserved regions (FERM, spectrin-actin binding domain (SABD), CTD) that are each preceded by unique sequences (Fig. 1C). The topology of FcγRI-CY interacting 4.1G cDNA is indicated in Fig. 1C. The smallest cDNA (IV.53) encompassed the complete C-terminal domain of 4.1G, and started at the end of exon 14. Four cDNA (I.58, IV.32, IV.36, IV.58; or 4.1GΔ14) started at the beginning of exon 14, however, carried in-frame deletions of the second half of exon 14 which was cleaved at site caag↓guag (a conserved splice sequence is underlined). The largest cDNA (IV.140 or 4.1GΔSABD) started at bp 1782, but lacked exons 12 and 13 (SABD) resulting in a protein with 43 additional N-terminal residues compared to 4.1GΔ14, and a complete exon 14. The exon structure of the 4.1G mRNA, and spliced fragments are schematically depicted in Fig. 1D (gene EPB41L2, GeneID: 2037, www.ncbi.nlm.nih.gov/entrez). These data suggested splice variants of 4.1G to selectively interact with FcγRI.

A 4.1G splice variant is predominantly expressed in immune cells

RT-PCR with 4.1G-specific primers that encompassed the 249 nt deletion fragment of exon 14 were used to discriminate between spliced 4.1G transcripts. Most immune cells tested showed expression of the 4.1GΔ14 splice-form, although DC, as well as MCF7 cells (human epithelial cell line), COS7 cells (monkey fibroblastic cell line), and IIA1.6 cells (mouse B/macrophage like cell line), were found positive for the full-length PCR fragment (Fig. 2). Incubation

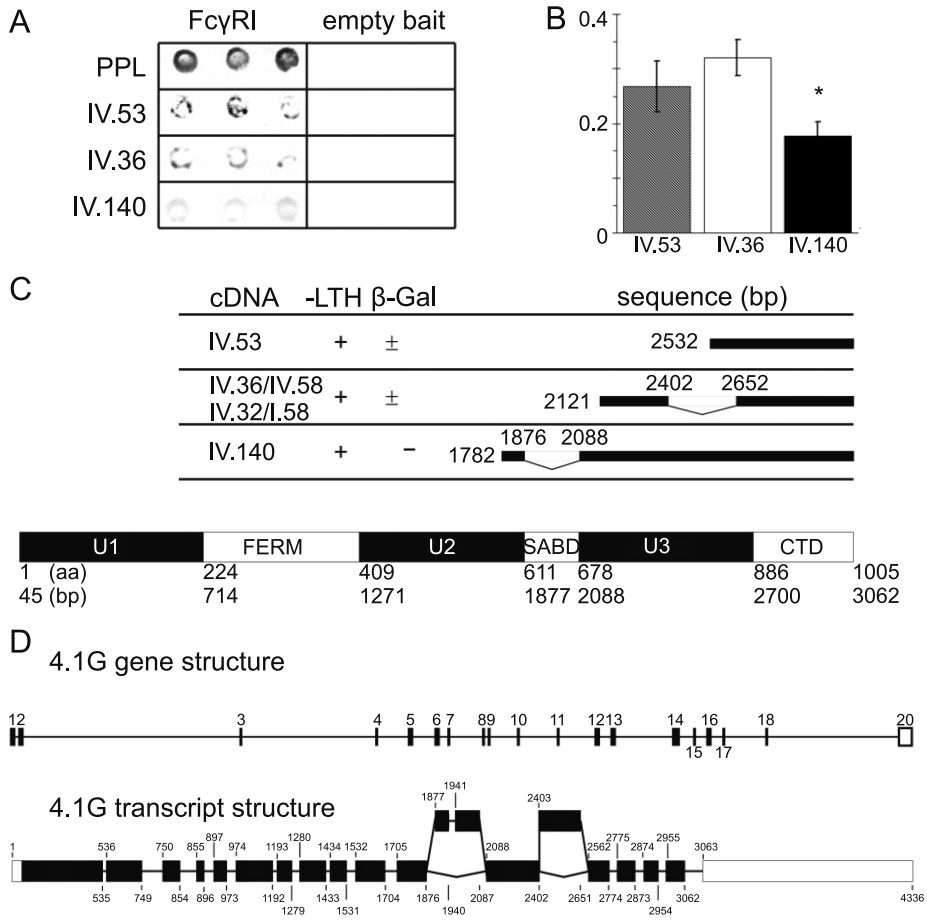

Figure 1. Interaction of FcγRI with protein 4.1G splice variants. (A) Yeast colonies were assessed for growth on selective media (-LTH) and β-galactosidase activity upon transfection of FcγRI (or empty bait vector) and 4.1G cDNA in triplicate. Periplakin cDNA was used as positive control [3], n=3). (B) Quantification of interaction strength for yeast clones IV.53, IV.36 and IV.140. Nine colonies per transfection were selected and tested for β-galactosidase activity in a liquid β-galactosidase assay using ONPG as substrate (protocol described in Clontech's Yeast Protocols Handbook). Data were averaged, error bars indicate standard deviation (n=2). (C) Domain structure of full length 4.1G, and schematic presentation of six isolated cDNA. Growth on medium lacking LTH, and β-galactosidase activity (β-GAL, in hours before prominent blue staining was observed) are indicated. Spliced regions are marked by a white gap, and a cusped line. White boxes indicate conserved domains, whereas black boxes mark 4.1G unique domains (U1, U2, U3). Domain boundaries are indicated in amino acids, or base pairs (counted according to Genbank Accession Number NM_001431). (D) Graphic view of 4.1G exon structure on genomic and transcript level. The different cDNA obtained by yeast two-hybrid assays were transposed on the exon structure of the 4.1G mRNA. Numbers above and below the sequence indicate the starting, and ending bp of associated exons, respectively. 4.1G spliced regions found in yeast two-hybrid sceens are indicated. Black boxes represent coding sequences, white boxes untranslated regions. (See color section for a full-color version)

with IFN-γ (16 h), DC maturation, or co-transfection with FcγRI or the C-terminus of periplakin -another FcγRI-CY associating protein [3]- did not alter the transcript profile. Sequence analyses of the two transcripts confirmed the 249 nt deletion that was also observed in the 4.1G cDNA identified in yeast two-hybrid screens (data not shown).

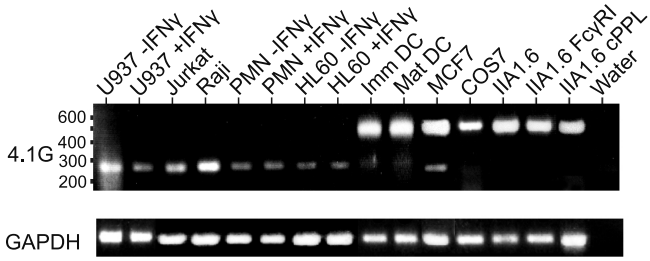

Figure 2. Splicing of 4.1G transcripts in immune cells. 4.1G specific primers were designed that encompassed the 249 nt deleted region observed in 4.1G clones from yeast two-hybrid screens, and used to amplify cDNA derived from a panel of cells. IFN-γ stimulations of cells were performed overnight with 300 U/ml. Monocyte-derived immature dendritic cells (Imm DC, day 5) were cultured, and matured (Mat DC) as described in Material and Methods. FcγRI (IIA1.6 FcγRI) or C-terminal periplakin (IIA1.6 C-PPL)-transfected IIA1.6 cells were also tested. Products of 516 bp were expected for transcripts containing exon 14, whereas 267 bp products were expected in case of 4.1GΔ14-like transcripts. GAPDH was amplified as positive control.

Recombinant FcγRI and 4.1G interact

To further assess the capacity of these proteins to interact, we expressed GST-tagged FcγRI-CY and His-tagged 4.1G clone I.58 (both with a predicted size of 35 kDa) in E.coli and performed immuno-precipitations with purified protein preparations. As indicated in the left panel of Fig. 3, anti-GST coated beads pulled down GST-FcγRI (lane 1) and GST (lane 2). The presence of FcγRI in lane 1 was confirmed by using an FcγRI-specific rabbit antiserum. Co-precipitation of His-4.1G was selectively observed with FcγRI-absorbed beads (right panel, Fig. 3). The His-tagged protein migrated slightly slower than FcγRI.

4.1G binds to the membrane proximal region of the FcγRI cytoplasmic tail

Cytosolic domains of activating FcR, and truncated or alanine-substituted FcγRI mutants were used to better characterize the 4.1G interaction sites in yeast two-hybrid binding assays. Growth of yeast colonies on selective media (-LTH) was permitted after co-transformation of 4.1GΔ14 with FcγRI, and FcεRI (Fig. 4A). Only human FcγRI induced sufficient levels of β-galactosidase. However, no interactions were observed for FcγRIIA, FcγRIIIA, FcαRI, or mFcγRI. Various N and C-terminal truncated mutants of FcγRI-CY were tested for interaction with 4.1G-CTDΔ14. Minimal binding requirements for 4.1G interaction were located within FcγRI-CY residues 312-332 as indicated by β-galactosidase assays (Fig. 4B). The importance of the

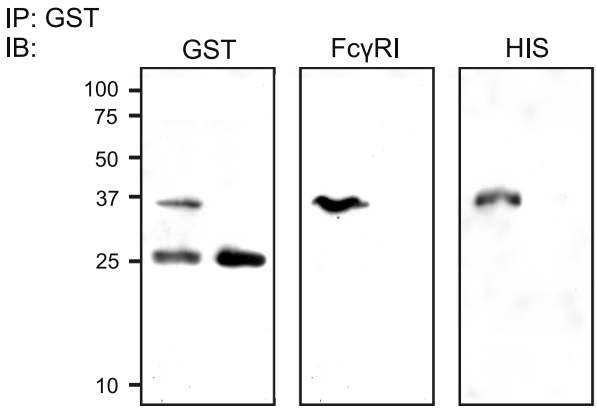

Figure 3. Co-immunoprecipitation of recombinant GST-FcγRI and His-4.1G-CTDΔ14. Tosyl-activated beads were coated with goat polyclonal GST antibodies, and incubated overnight with GST-FcγRI (lane 1) or GST alone (lane 2), and His-4.1G-CTDΔ14 in RIPA buffer. Western blots of these fractions were stained with antibodies recognizing GST (left panel), FcγRI-CY (middle panel), or His-tag (right panel). One-out-of three representative experiments is shown.

FcγRI membrane-proximal region was further illustrated in experiments in which we replaced single residues of FcγRI-CY (311-325) for alanine (Fig. 4C). Most alanine-substitutions abrogated interaction with 4.1G. Replacement of E316, R319, K321 and D324, however, resulted in increased β-galactosidase activity. Notably, FcγRI and FcεRI displayed considerable sequence similarity in their membrane-proximal domains (Fig. 4D).

4.1G interacting proteins share sequence similarities

To assess whether there are motifs within FcγRI that are shared with other 4.1G-CTD binding proteins, we compared the primary sequence of FcγRI with other proteins that interact with 4.1G-CTD. These include transmembrane proteins such as platelet and T cell activation antigen 1 (PTA-1/CD226/DNAM-1) [21], D2 and D3 dopamine receptors (D2DR, D3DR) [22], A1 adenosine receptor (A1AR) [23], GluR-1 [24] and GluR-D AMPA receptors [25]. Nuclear mitotic apparatus protein (NuMa) [26] and FK506-binding protein 13 (FKBP13) [27] are cytosolic proteins that bind 4.1G. The membrane-proximal domains of these receptors showed significant sequence similarity, and indicated a role for stretches of basic residues situated beneath the membrane (Fig. 5A). Cytosolic proteins FKBP13 and NuMa did not contain the large basic regions, but displayed somewhat similar patterns. The membrane-proximal region of FcγRI-CY is predicted to adopt an alpha-helix, followed by loops and extended configurations (Fig. 5B). Similar structures were predicted for membrane-proximal regions of other 4.1G binding proteins using PHD predictions [16] (Fig. 5C), and also for PTA-1 after submission to 3D-PSSM [19] (result not shown). A schematic representation of FcγRI-residues 310-327 as alpha-helical wheel is displayed in Fig. 5D.

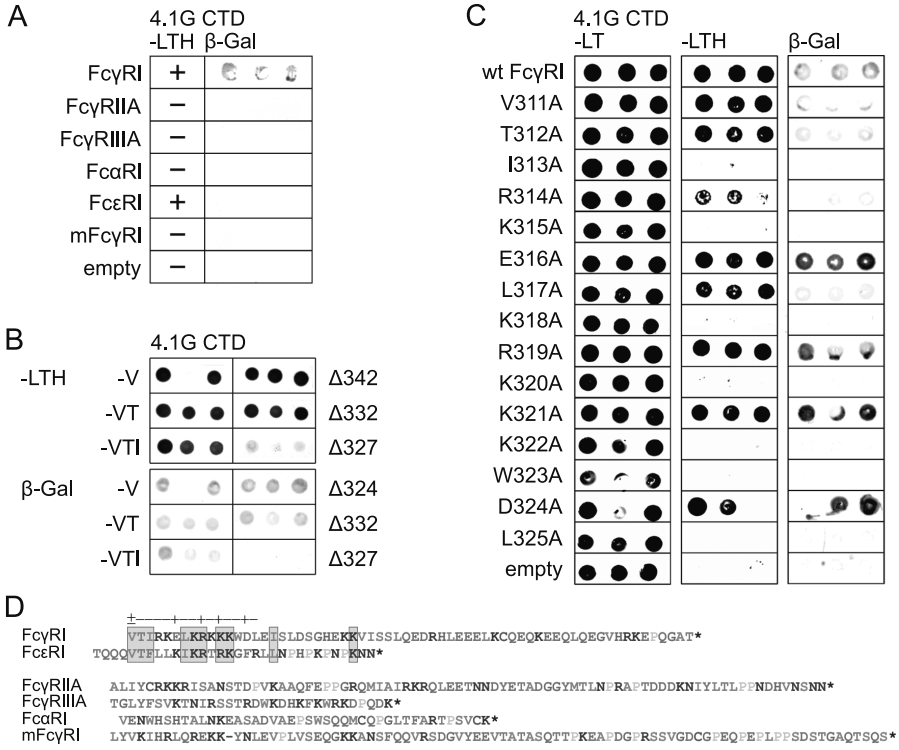

Figure 4. Identification of protein 4.1G interaction sites in FcR cytoplasmic domains. Protein interactions were assessed after transfer of three yeast colonies to media without leucine, tryptophane (-LT) and histidine (-LTH), and a β-galactosidase (β-GAL) activity assay. (A) Interactions between a panel of activating FcR α-chain cytosolic domains, and 4.1GΔ14. mFcγRI denotes murine FcγRI. (B) Interactions between truncated FcγRI-CY mutants, and 4.1G CTDΔ14. Numbers refer to the final C-terminal residue present in the FcγRI-truncated molecules. -V, -VT, -VTI constructs lack the first one, two, or three N-terminal residues of FcγRI-CY. (C) Interaction of alanine-substituted FcγRI mutants with 4.1G CTDΔ14. The single substitutions of FcγRI's membrane-proximal region (311-325) were denoted as V311A (Valine at position 311 for Alanine, T312A Threonine for Alanine at 312, etc). (D) Cytosolic domains of FcR. Similar regions within FcγRI and FcεRI are boxed in grey. (See color section for a full-color version)

DISCUSSION

FcγRI cross-linking initiates signaling cascades that culminate in cellular functions. Although the cytosolic domains of most multi-subunit FcR are considered 'inert', interactions of FcγRI-CY with cytoskeletal-associated proteins such as periplakin and filamin A have been reported [3, 9]. We now report the binding of FcγRI to protein 4.1G, a globular multi-domain protein that may act as signaling scaffold, and tethers to the actin cytoskeleton. We found the FcγRI-CY membrane-proximal domain to interact with a conserved CTD within 4.1G in yeast two-hybrid screens.

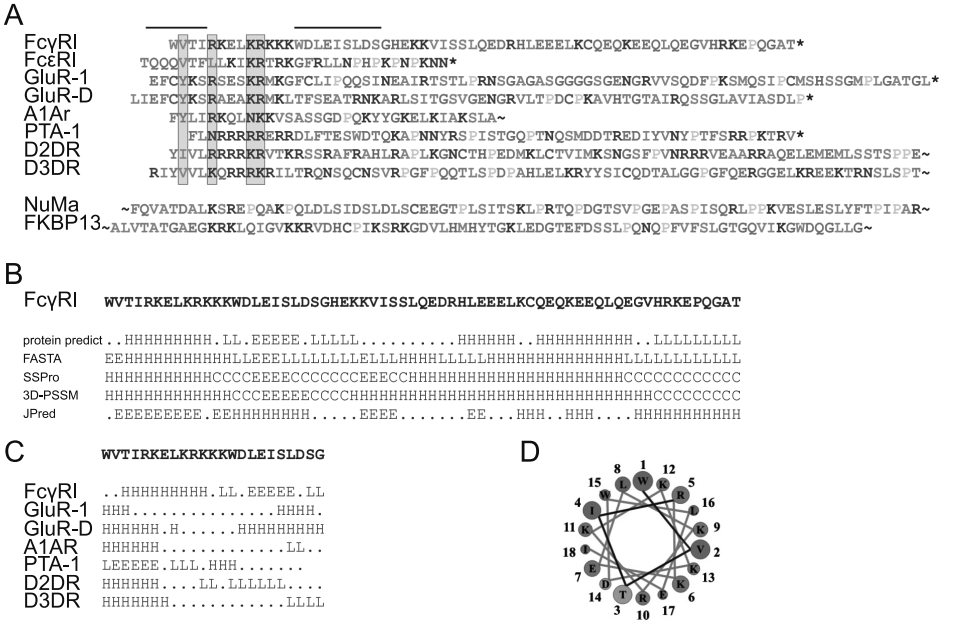

Figure 5. Sequence analysis of 4.1G interacting proteins. (A) Primary sequence alignment of 4.1G CTD binding proteins. Cytosolic domains of 4.1G-CTD-interacting proteins were predicted using PHDhtm prediction software [16], and similar regions were aligned. Transmembrane receptors are shown in the upper alignment (interacting loops are indicated for seven membrane-spanning receptors (dopamine receptors, A1AR)), and cytosolic proteins (not full sequences) in the lower alignment. Grey boxes mark similar residues within transmembrane receptors, allowing one mismatch. Regions of hydrophobic and (potentially) negatively charged residues are indicated by black lines. Amino acids in green: hydrophobic/aromatic, blue: basic/hydrophilic, red: acidic, yellow: aliphatic. (B) Secondary structures of FcγRI-CY were predicted after web submission of FcγRI (TM region and CY) to PredictProtein, FASTA, SSPro, 3D-PSSM and JPred. H indicates a predicted alpha-helix structure, E:extended β-sheet, and C/L: coil/loop. Dots mark unreliable predictions. (C) Secondary structures of membrane-proximal domains of 4.1G-interacting protein. Regions were predicted with PredictProtein, and had cut-off accuracies of 82% (PHD prediction, Sub_sec). (D) Schematic presentation of an alpha-helical wheel for FcγRI residues 310-327. (See color section for a full-color version)

The FcγRI 'docking-site' within 4.1G resided within residues 830-1005 (Fig. 1). The somewhat larger splice variant 4.1GΔ14 lacked residues 787-869, but interacted efficiently suggesting 4.1G residues 870-1005 to contain FcγRI-interacting capacity. Another spliced message (4.1GΔSABD) contained 43 additional N-terminal residues, and a complete exon 14 compared to 4.1GΔ14. This variant demonstrated a reduced capacity to interact with FcγRI-CY. This suggested the N-terminal 43 residues, or the spliced region of exon 14 (or both) to carry 'inhibitory potential' for FcγRI-CY binding. Notably, the better FcγRI-interacting 4.1GΔ14 related transcripts were found enriched in most immune cells, suggesting alternative splicing of 4.1G to possibly regulate FcγRI interaction (Fig. 2). 4.1GΔ14 was, furthermore, confirmed to interact with FcγRI-CY by co-immunoprecipitation studies (Fig. 3). Tissue-specific splicing is commonly observed for 4.1 proteins, and can be regulated by selective receptor-ligand inter-

actions, or phorbol esters, such as TPA [21, 28]. In Jurkat cells, TPA-induced upregulation of PTA-1 expression has been associated with selective expression of a 4.3 kb 4.1G splice-form (21). We, however, did not observe differences after stimulation with IFN-γ or upon DC maturation.

Our data indicated both FcγRI and FcεRI (albeit with reduced efficacy) to interact with 4.1G-CTDΔ14 in yeast cells, and demonstrated a critical role for FcγRI membrane-proximal residues in this interaction (Fig. 4). Sequence alignments of 4.1G binding domains of multiple proteins indicated stretches of hydrophobic residues (sometimes containing serines, or threonines), followed by a basic cluster, and a less defined region of hydrophobic and (potentially) negative residues (S,T,D,E) to be important for interactions with 4.1G. Most of these proteins are predicted to enter the cytosol as alpha helices, but then to loop, and/or extend. Consensus residues (HxxBxxxBB, H represents hydrophobic residues, B basic residues (K/R), x any residue) within FcγRI and other 4.1G CTD-interacting proteins were found in the predicted FcγRI alpha-helical structure. These aligments suggested a stretch of basic residues at one side of the helix (that have expected turns every 3.6 residues) to be a critical sequence pattern for 4.1G CTD interaction. We, recently, also reported periplakin to interact with the FcγRI-CY membrane-proximal region, suggesting these proteins to possibly compete for FcγRI-CY [4].

Besides FcγRI and FcεRI, FcεRIIa and Fcα/μM bear membrane-proximal basic clusters, and to some extent FcγRI-similar adjacent sequences, and may interact with 4.1G for signaling [29, 30]. Studies of dopamine receptors [22], A1AR [23], AMPA receptors [24, 25], parathyroid hormone receptor [31] and metabotropic glutamate receptor mGlu1α [32] indicated a prominent role for 4.1G interactions in stabilization of surface expression, or signal transduction (PTA-1 [21], A1AR and mGlu1α [32, 33]). Other FERM domain-containing proteins have been shown implicated in formation of the immunological synapse by segregation of CD43 from the TCR area [34] and in B cell receptor induced lipid raft dynamics [35]. The impact of 4.1G interaction for FcγRI biology, however, remains to be determined.

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Chapter 5

Colocalization of 4.1G and actin during FcγRI induced phagocytic cup formation

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ABSTRACT

FcγRI plays an important role in protection against bacterial infection. Downstream effector functions of this receptor are mostly dependent on ITAM mediated signaling by the associated FcR γ-chain. However, the cytosolic tail of the ligand binding α-chain (FcγRI-CY) has been shown to play a role during endocytosis, antigen presentation and phagocytosis. We recently described an interaction between cytoskeletal protein 4.1G and FcγRI-CY. Various protein 4.1G splicevariants are known to be expressed in cells and a high molecular weight form of 4.1G was found to be differentially expressed upon differentiation from monocytes to dendritic cells or macrophages. In the present study, no role for 4.1G was found in any of the aforementioned functions of FcγRI-CY. 4.1G, however was found to colocalize with actin at phagocytic cups during FcγRI mediated phagocytosis. The functional relevance of the interaction between 4.1G and FcγRI is unclear, which may well be attributable to redundancy within the 4.1G family.

INTRODUCTION

Phagocytosis by macrophages plays an important role during tissue remodeling and the clearing of self and non-self antigens[1]. Macrophages express numerous phagocytic receptors, including the IgG receptors FcγRI, FcγRIIa, FcγRIIb and FcγRIIIa[2]. FcγRI (CD64) is the only known high affinity Fcγ receptor and is important in protection against bacterial infection[3]. Similar to FcγRIIIa, it associates with the ITAM bearing FcR γ-chain which plays a central role in signaling during phagocytosis[4]. Upon receptor crosslinking, the ITAMs of the FcR γ-chain are phosphorylated by Src family kinases, leading to binding and activation of Syk[5]. Subsequently, Syk phosphorylates several downstream signaling molecules that induce cytokine and ROS production and actin polymerization. Actin polymerization by the Arp2/3 complex is crucial for phagocytic cup formation[6]. Phosphatidylinositol-phosphate (PtdInsP) lipids located at the inner leaflet of the plasma membrane are important signaling intermediates during phagocytosis as PtdIns(4,5)P_2 (PIP_2) further stimulates the Arp2/3 complex via WASP[7].

Besides FcR γ-chain signaling, the cytosolic tail of the ligand binding alpha chain of FcγRI (FcγRI-CY) has been shown to play a role during endocytosis, antigen presentation and IL-6 production[8,9]. FcγRI-CY was suggested to enhance γ-chain phosphorylation and truncation of the cytosolic tail inhibited FcγRI mediated phagocytosis[10]. Furthermore, recent studies show differential signaling between FcγRI and FcγRIIa[11].

We recently documented an interaction between 4.1G and FcγRI-CY[12]. A splice variant of 4.1G which lacks a part of exon 14 (4.1GΔ14) was found to selectively interact with the membrane proximal part of FcγRI-CY. However, the functional relevance of this interaction is unclear. 4.1G belongs to the band 4.1 protein family which includes 4.1R, 4.1N and 4.1B. Similar to 4.1G, these proteins consists of several domains including the Four point one-Ezrin-Radixin-Moeisin domain (FERM), Spectrin/Actin Binding Domain (SABD) and a C- Terminal domain (CTD). These domains are interspaced by the unique regions U1, U2 and U3[13-15]. The FERM domains are highly conserved within this family and can bind to PIP_2[16]. The SABD of 4.1G and 4.1R have been shown to associate with F-actin. The C-terminal part of 4.1G can interact with other receptors including dopamine receptors D2DR and D3DR, GluR-AMPA receptors subunits GluA-1 and GluA4, A1-adenosine receptor and the adhesion molecule PTA-1. 4.1G has been suggested to play a role in stabilization of surface expression and/or signaling of these receptors[17-22]. Like the FERM and SABD, the C-terminal domain is highly conserved between mouse and man (93% sequence identity).

We set out to study the functional role of the interaction of 4.1G and FcγRI-CY using colocalization studies and functional assays including overexpression, 4.1G knockdown and 4.1G knockout dendritic cells and macrophages. Differential expression of 4.1G variants were confirmed in myeloid cells and cell lines. 4.1G deficiency, knockdown or overexpression of the C-terminus of 4.1G did not affect FcγRI cell surface expression levels. Furthermore, we did not observe a role for 4.1G during endocytosis or FcγRI mediated antigen presentation.

During FcγRI mediated phagocytosis, however, 4.1G colocalized with actin in the phagocytic cups of THP-1 cells and primary monocytes supporting a potential role of 4.1G during FcγRI mediated phagocytosis.

MATERIAL AND METHODS

Constructs, Antibodies and Reagents

Affinity purified, polyclonal rabbit anti c-terminal 4.1G was generated by Eurogentec by immunizing rabbits with the last c-terminal aminoacids of 4.1G: CVVVHKETELAEEGED. The additional n-terminal cysteine was added for coupling purposes. Anti FcγRI–Alexa647 (clone 10.1) was purchased from Biolegend. FcγRIa in pcDNA3 and murine FcR-γ chain in pNUT were described previously. The cDNA clone CTD4.1GΔ14, as described in Beekman et al.[23] (basepairs 2121-3062 with basepairs 2402-2652 spliced out, genbank accession NM_001431), was amplified from pACT-II and subsequently cloned with BamHI and XbaI to pcDNA3.1 His C (Invitrogen). GFP-CTD4.1GΔ14 was generated by inserting the BamHI-XbaI fragment from the pcDNA3.1 His C vector in the pEGFP-C1 vector (Clontech). FcγRIa in pMX ires GFP was described elsewhere[24]. Integrity of cDNA was verified by sequence analysis using Big Dye Terminator mix (Applied biosystems). Anti CD32 mAb (IV.3) and anti CD16 mAb (3G8) were purified from hybridoma culture supernatant. IV.3 Fab and 3G8 F(ab)2 were generated by Fusion antibodies and Genmab, respectively. Anti glycophorin mIgG2a was described previously[25]. Human anti RhD IgG3 (BRAD3) was from IBGRL.

Cells

The B cell lymphoma line IIA1.6 was transfected with human FcγRIa in pcDNA3 and murine FcR-γ chain in pNUT which contains a methotrexate resistance cassette. A stabile cell line expressing both FcγRIa and FcR-γ chain was generated by culturing the cells in the presence of methotrexate post transfection (described in van Vugt et al[26]). These cells were then transiently transfected with GFP only or GFP-4.1GΔ14 using AMAXA nucleofection, Kit V, program T-020. IIA1.6 and THP-1 cells were cultured in RPMI1640 containing penicillin, streptomycin and 10% FCS.

Monocytes from healthy donors were isolated using CD14 magnetic beads (Miltenyi) and cultured for 9 days in RPMI1640 with 10% human serum supplemented with 100 ng/ml M-CSF (Peprotech). To generate monocyte derived DCs (MDC), monocytes were cultured for 4 days in the presence of 500 U/ml GM-CSF and 800 U/ml IL-4 (Peprotech). Where indicated, 4 day old MDC or monocytes were stimulated overnight with 400U/ml IFNγ (Boehringer Ingelheim).

MDM and BMDC from 4.1G deficient mice

4.1G deficient mice were described previously[27]. BMDCs and BMDM were cultured as previously described[28]. Briefly, bone marrow was flushed from femur and tibia from wildtype or 4.1G knockout mice and cultured for 7 days in the presence of 5 ng/ml GM-CSF. At the start of culture, bone marrow cells plated on retronectin coated plates were transduced with human FcγRI using culture supernatant from phoenix eco cells stably expressing the FcγRI IRES GFP construct. At day 1 and 2, transduction was repeated by replacing half of the culture medium with fresh phoenix eco supernatant. Adherent cells were used as BMDM and nonadherent cells as BMDC. Experiments were approved by local animal ethical committee.

Immuno-fluorescence

Macrophages were grown on coverslips and fixed using 3.7% paraformaldehyde. Next, cells were permeabilized with blockbuffer (PBS containing 0.1% BSA , 5% normal goat serum and 0.1% saponin). FcγRI was stained with 10.1-Alexa647, 4.1G was stained with rabbit anti-4.1G. Secondary antibodies were mouse anti rabbit IgG-CY5 or goat anti rabbit IgG-Cy3 (Jackson Immunoresearch). F-actin was stained using Phalloidin-FITC. Cells were visualized on a Zeiss LSM710 equipped with transmission-PMT for DIC imaging.

Endocytosis/phagocytosis

For endocytosis experiments, macrophages were grown on coverslips and were washed in PBS and incubated with 10.1-Alexa647 on ice. After washing in PBS, cells were incubated with goat F(ab)2-anti mouse IgG (Southern Biotech) on ice and incubated at 37° C for the indicated timepoints. Internalization was stopped by washing cells with icecold PBS and subsequent fixation with cold 3.7%PFA in PBS. Next, 4.1G was stained as indicated above.

For phagocytosis experiments, IFNγ stimulated THP-1 cells were allowed to bind IgG3 opsonized erythrocytes at 4° C. in the presence of IV.3 Fab. After 30 min of incubation at 37° C, cells were washed with cold PBS and adhered to Poly-l-lysine coated coverslips. Cells were then fixed and stained with anti 4.1G, phalloidin-FITC and anti FcγRI. IFNγ stimulated MDM's (grown on coverslips) were allowed to bind IgG3 opsonized erythrocytes in the presence of IV.3 Fab and 3G8 $F(ab)_2$. Next, cells were placed on 37° C for 30 min. and were subsequently washed with cold PBS prior to fixation.

Phagocytosis with mouse BMM were performed using pHrodo (Invitrogen) labeled *Staph. epidermis*. Labeled bacteria were opsonized with human serum, washed and added to 7 day old BMM in suspension in the presence of 2.4G2 $F(ab)_2$ at an E/T ratio of 1/10. Bacteria were allowed to bind to BMM at 4° C for one hour. Next, cells were placed at 37° C on a shaker for indicated time points. Cells were then washed in ice cold PBS and fixed in 1% paraformalde-

hyde. Phagocytosis was measured by counting the percentage of GFP positive cells that had phagocytosed one or more pHRodo positive bacteria on a Zeiss LSM710.

RESULTS

4.1G is differentially expressed in monocytic and monocyte derived cells.

We previously described expression of 4.1G in several FcγRI expressing myeloid derived primary cells and cell lines at the mRNA level. To study expression at the protein level, we developed a polyclonal antiserum directed at the last 15 amino acids of the C-terminus of 4.1G. On western blots, this antibody detected several bands in lysates of monocyte derived macrophages (MDM) and the monocytic cell line THP-1 (Figure 1A). Consistent with previous reports[19,29], a high molecular weight band around 160 kDa was detected.

4.1G is known to be extensively spliced[22]. Incubation with c-terminal 4.1G peptide during staining suggested the extra bands detected by our polyclonal antiserum were not the result of aspecific staining, with the possible exception of two bands around 37 and 45 kDa (Figure 1B). The pattern of anti 4.1G reactive bands differed between cell-lines and was altered during differentiation as the ~160 kDa band in MDM was similarly detected in monocyte derived dendritic cells (MDC) but not in monocytes (Figure 1C). Consistent with previous work, IFNγ stimulation did not alter 4.1G expression profiles[30]. These data support the expression profile of different 4.1G forms to change upon differentiation of monocytic cells.

4.1G colocalizes with F-actin in the phagocytic cup but is not critical for FcγRI mediated phagocytosis of bacteria.

During the uptake of large particles, the actin cytoskeleton plays a crucial role in the formation of phagocytic cups[31] and 4.1G has been suggested to bind actin through its SABD[32]. 4.1G was previously described to localize at the cell membrane in transfected CHO cells[22], presumably due to PIP_2 binding at the inner leaflet of the plasma membrane via the 4.1G FERM domain. We studied whether 4.1G was similarly localized in MDM using confocal microscopy. 4.1G was found to localize at the cell membrane, especially near contact points between the cell and coverslip or in ringlike structures just below the cell membrane (Figure 2A and 2D). 4.1G staining was specific in both cases as signals were absent in the presence of 4.1G blocking peptide (Figure 2A, right panel and data not shown). We sometimes observed nuclear staining in MDM by our polyclonal 4.1G antiserum (Figure 2C). In the presence of 4.1G blocking peptide, no nuclear signal was observed, suggesting specific staining, which is in concordance with previous publications[33].

To investigate whether 4.1G plays a role during FcγRI mediated phagocytosis, we allowed THP-1 and MDM to bind and phagocytose IgG opsonized erythrocytes. Clear phagocytic cup

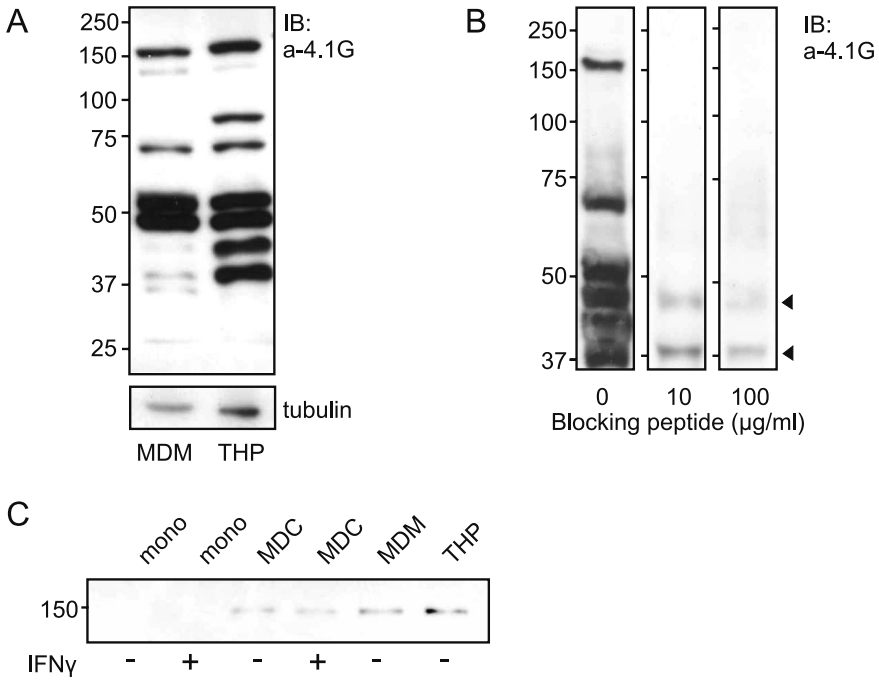

Figure 1. Differential expression of protein 4.1G upon differentiation towards monocyte derived dendritic cells (mDC) and monocyte derived macrophages (MDM). A) 4.1G expression in monocyte derived macrophages and THP-1 cells (THP) detected with anti 4.1G antiserum on western blot. Tubulin is included as a loading control. B) Blocking of anti 4.1G polyclonal antiserum with a peptide comprising the last 15 c-terminal amino acids of 4.1G. Two different concentrations of peptide were added to the buffer containing the anti-4.1G polyclonal antiserum and used to stain blotted lysates of THP-1 cells. Arrow heads mark potential aspecific bands. C) Expression of the ~160kDa band was examined in lysates from monocytes (mono), monocyte derived DCs (MDC) and MDM. Where indicated, monocytes and DCs were stimulated overnight with IFNγ. Data are representative of two experiments.

formation could be seen after 30 minutes incubation with opsonized erythrocytes. We found FcγRI to sporadically colocalize with 4.1G in THP-1 and MDM (Figure 2B, top panels and Figure 2D). Most notable was the colocalization of 4.1G with filamentous actin at phagocytic cups (Figure 2B lower panels and Figure 2E). To quantify the level of colocalization, we calculated Manders overlap coefficient in either entire THP-1 cells or only at phagocytic cups. A significant increase in overlap coefficient of 4.1G and F-actin was observed at phagocytic cups (Figure 2C). A similar trend was observed in MDM (data not shown). Initial experiments with transduced 4.1G deficient and wildtype BMM however, suggested FcγRI mediated phagocytosis of *Staph. epidermis* not to be dependent on 4.1G (Figure 2F). Under these conditions, phagocytosis was at least partially dependent on human FcγRI as phagocytosis was inhibited in the presence of FcγRI blocking mAb 10.1.

Overall, these data document 4.1G localization at phagocytic cups, but support 4.1G not be crucial for FcγRI mediated phagocytosis of *Staph. epidermis*.

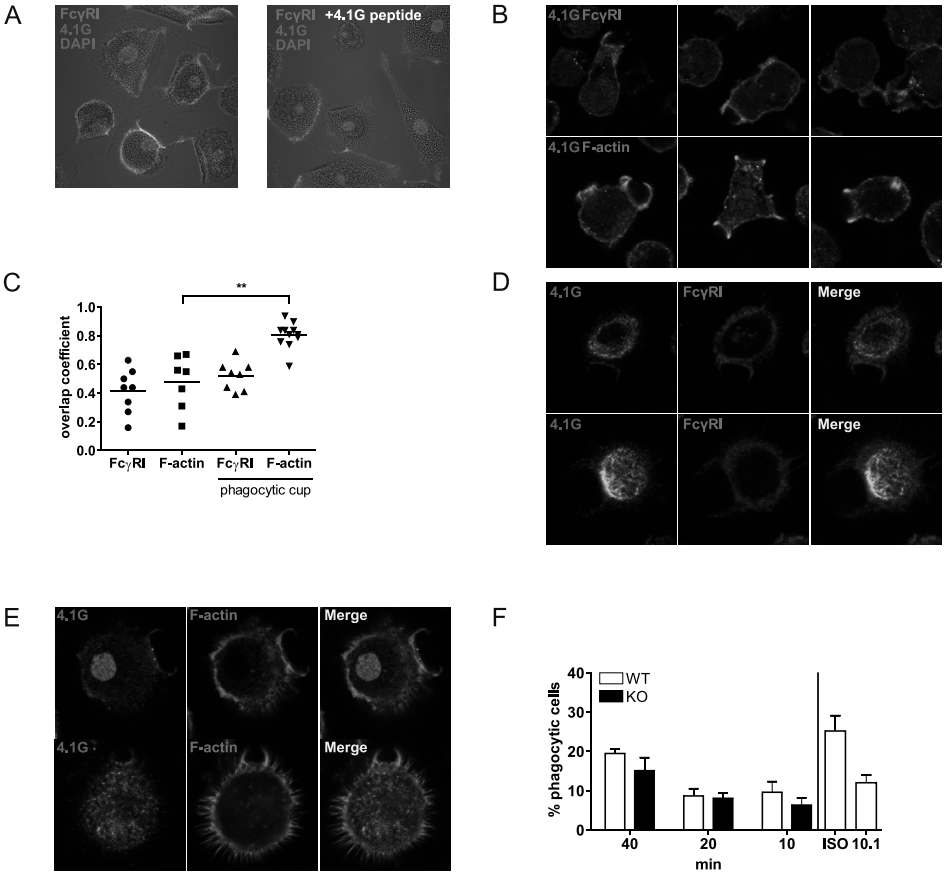

Figure 2. 4.1G localization and function during FcγRI mediated phagocytosis. A) Monocyte derived macrophages stained for FcγRI (red channel) and 4.1G (green channel) in the absence (left panel) or presence of C terminal 4.1G peptide (right panel) as observed by confocal microscopy. A merged image is presented showing FcγRI and 4.1G staining and nuclei by DAPI staining. One-of-three independent stainings is shown. B) 4.1G (green channel) localization compared to FcγRI (red channel, top panels) and f-actin (red channel, lower panels) in THP-1 phagocytosing IgG3 opsonized erythrocytes in the presence of anti FcγRII (clone IV.3) F(ab) fragments. Erythrocytes were lysed during the staining procedure. C) Quantification of co-localization between 4.1G and FcγRI and 4.1G and F-actin (Manders overlap coefficient) calculated for entire THP-1 cells or in phagocytic cups only. **: P<0.01, student t-test. D and E) IFNγ treated MDM were allowed to phagocytose IgG3 opsonized erythrocytes in the presence of IV.3 F(ab) fragments and anti FcγRIII (clone 3G8) F(ab)2 fragments. Cells were fixed and stained for 4.1G and FcγRI (D) and 4.1G and F-actin (E). Experiment was repeated twice, one representative experiment is shown. F) Phagocytosis of *Staph. epidermis* by wildtype (WT) or 4.1G deficient (KO) BMM. BMM were allowed to phagocytose bacteria for the indicated timepoints. In the same experiment, WT BMM were allowed to phagocytose bacteria for 40 min in the presence of FcγRI blocking mAb 10.1 or isotype control. N=1, mean and standard deviation of triplicates are shown. (See color section for a full-color version)

4.1G localization is unaltered upon FcγRI endocytosis.

FcγRI endocytosis can occur in the absence of FcR γ-chain ITAM signaling. To investigate whether 4.1G plays a role during this process, we studied 4.1G localization upon FcγRI cross-linking. While FcγRI gradually internalized over time, 4.1G localization remained stable at all time points (Figure 3). The lack of colocalization during FcγRI endocytosis suggests 4.1G not to play a direct role during receptor internalization within a time frame of 15 to 60 minutes.

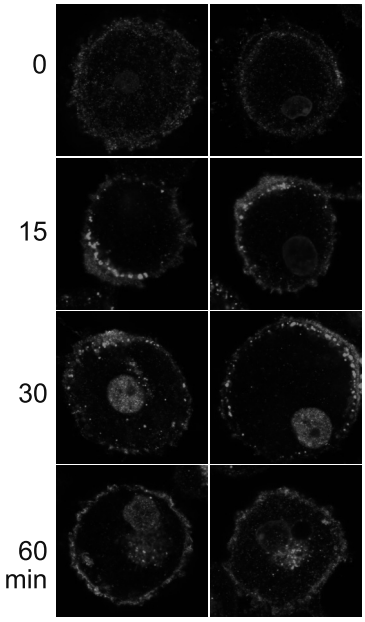

Figure 3. 4.1G localization upon FcγRI endocytosis in monocyte derived macrophages. FcγRI was crosslinked with mAb 10.1 and goat anti mouse IgG and allowed to internalize for the indicated time periods. FcγRI expression is shown in red, 4.1G in green and DAPI in blue. Representative results of two independent experiments are shown. (See color section for a full-color version)

4.1G does not alter FcγRI surface expression levels on THP-1 and IIA1.6 cells and mouse primary BMDC.

4.1G has been reported to interact with dopamine receptors, adenosine receptors and the GluR AMPA receptor subunits GluA1 and GluA4 and to stabilize surface expression levels of these molecules. To investigate whether FcγRI surface expression was regulated by 4.1G, we first overexpressed a GFP-4.1GΔ14 fusion construct in IIA1.6 cells stably expressing exogenous FcγRI and FcR γ-chain. Despite reaching high protein expression levels, we did not observe differences in FcγRI surface expression levels between GFP and GFP-4.1GΔ14 transfected IIA1.6 cells (Figure 4A). Over-expression of c-terminal periplakin, another FcγRI-CY associating cytosolic protein was previously shown to affect IgG binding by the receptor[34]. In contrast, overexpression of GFP-4.1GΔ14 did not affect binding of IgG opsonized RBC to FcγRI (Figure 4B). Similar to IIA1.6 cells, siRNA mediated knockdown of 4.1G in THP-1 cells did not affect FcγRI surface expression levels (Figure 4E). A modest decrease in FcγRI

surface expression levels was observed after overnight IFNγ stimulation. In THP-1 cells, siRNA mediated knockdown only affected the 160 kDa band (Figure 4C and D). To exclude that other potential 4.1G splice variants could stabilize FcγRI surface expression, we transduced bonemarrow derived DC's from either wildtype (wt) or 4.1G knockout (4.1GKO) mice with an FcγRI IRES GFP construct. Although transduction efficiency was slightly lower compared to wt, we did not observe decreased FcγRI surface expression levels in 4.1GKO DCs (Figure 4F). These data suggest 4.1G not to impact FcγRI surface expression levels.

DISCUSSION

Leukocyte IgG receptors are potent mediators of phagocytosis. FcγRI can efficiently mediate phagocytosis and we recently showed the capacity of FcγRI to bind immune complexes despite saturation by serum IgG (chapter 2). 4.1G was shown to be expressed at the protein level in several myeloid cell types and differentiation of monocytes to MDM or mDC lead to differential protein expression patterns, suggestive of alternative splicing (Figure 1).

The actin binding properties of the 4.1G SABD prompted us to investigate a role for 4.1G during actin remodeling[35]. Upon ingestion of large particles, 4.1G clearly co-localized with actin at phagocytic cups (Figure 2) and a trend towards FcγRI and 4.1G co-localization was observed. PIP_2 is enriched in phagocytic cups[36] and it has been shown that PIP_2 binding by the FERM domain can regulate association of membrane proteins with the 4.1RCTD [37]. A similar mechanism might exist for FcγRI and 4.1G. Phagocytosis of ~1 μm particles, such as bacteria, are mainly actin independent [38,39] which might explain why 4.1G deficient macrophages displayed no gross abnormalities during FcγRI mediated phagocytosis of bacteria. The role of 4.1G during FcγRI mediated phagocytosis may be more apparent during uptake of larger particles such as opsonized tumour cells.

It has previously been shown that the FcγRI-CY mediates effector functions such as endocytosis and antigen presentation independent of a functional FcR-γ chain. 4.1G did not co-localize with FcγRI in resting macrophages or during internalization of FcγRI (Figure 3) within a 15 to 60 minutes timeframe. FcγRI mediated uptake of OVA-immune complexes occurs via endocytosis and we found GFP-CTD4.1GΔ14 overexpression not to affect FcγRI mediated OVA antigen presentation in IIA1.6 cells (data not shown, n=3). In experiments using wildtype and 4.1G deficient BMDCs, antigen presentation was found to be mainly dependent on FcγRI transduction efficiency rather than 4.1G expression (data not shown). Overall, this suggests 4.1G not to play a direct role during FcγRI-CY mediated endocytosis or subsequent antigen presentation.

4.1G stabilizes surface expression levels of dopamine receptors D2 and D3, A1AR and AMPA receptors subunits[18,19,21,40]. For FcγRI it has been documented that interaction with the

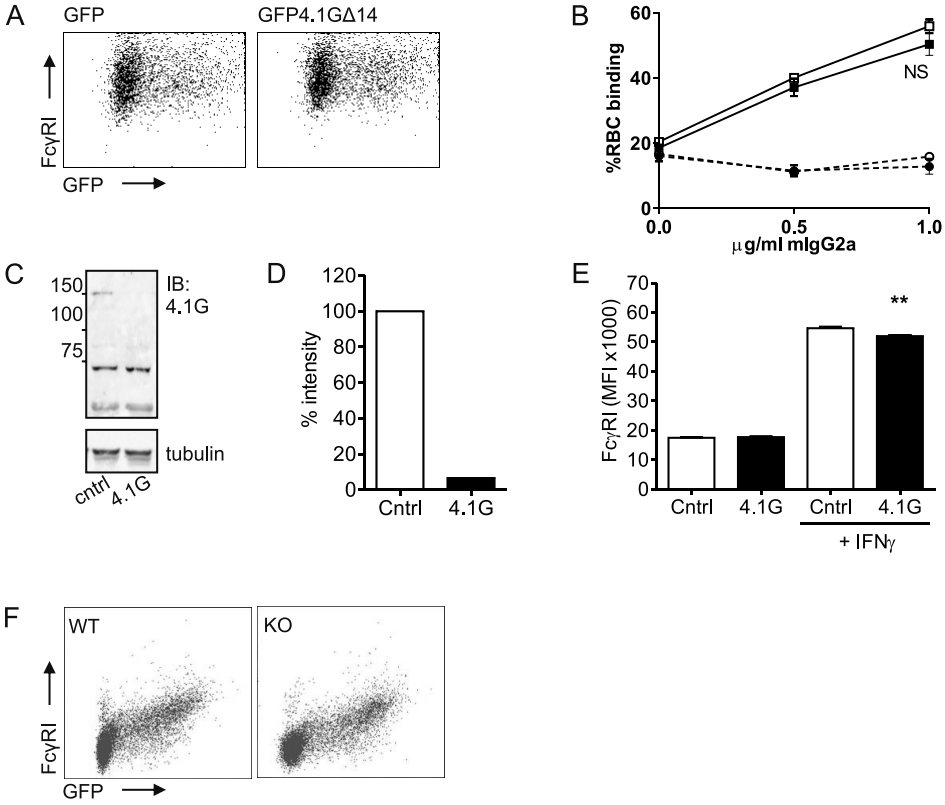

Figure 4. 4.1G does not affect cell FcγRI surface expression levels or ligand binding. A) FcγRI cell surface expression in GFP (left panel) or GFP4.1GΔ14 (right panel) transfected IIA1.6 expressing exogenous FcγRI and FcR γ-chain. B) GFP (open squares) or GFP4.1GΔ14 (closed squares) transfected IIA1.6 –FcγRI (continuous line) were used in a FACS based EA rosette assay. Erythrocytes were opsonized with different concentrations of mIgG2a anti glycophorin A mAb. Transfected IIA1.6 cells expressing FcαRI (dotted line) were used as negative control. NS: P>0.05, students t test. C) siRNA mediated knockdown of 4.1G in THP-1 cells. THP-1 cells were transfected with control (cntrl) or 4.1G targeting siRNA pools. 4.1G expression was evaluated 72hrs later by westernblot and Odyssey Imaging system. Tubulin was used as loading control. D) Relative intensity of ~160kDa band of 4.1G as shown in C, corrected for tubulin. E) Surface expression of FcγRI in unstimulated or overnight IFNγ treated THP-1 cells 72 hrs after transfection with control (cntrl) or 4.1G targeting pool. F) Expression of transduced FcγRI IRES GFP in wildtype (WT) or 4.1G deficient (KO) BMDC. Experiments were repeated at least two times, yielding similar results.

cytosolic protein Filamin A is needed for receptor surface expression[41]. No role however, was found for 4.1G in FcγRI surface stabilization in three independent models (Figure 4).

Periplakin binds FcγRI-CY membrane proximally, near the bindingsite of 4.1G and it has been suggested that disruption of the interaction between periplakin and FcγRI-CY in IIA1.6 cells affects receptor IgG binding. Despite some overlap of binding sites, GFP-CTD4.1GΔ14 overexpression did not affect ligand binding in a similar model, suggesting periplakin and

4.1G may simultaneously interact with FcγRI-CY (Figure 4B), or that interaction between these proteins and FcγRI-CY is differentially regulated.

Co-localization studies allude to a potential role for 4.1G during the phagocytosis of large particles. However, we found no role of 4.1G during FcγRI endocytosis, regulation of ligand binding and surface expression stabilization. Redundancy within the 4.1 family is not unlikely, as considerably homology exists within the 4.1 family. Further studies using FcγRI transgenic mice on a 4.1G knockout and/or 4.1G-4.1R double knockout background may well shed further light on the functional relevance of the interaction between FcγRI and 4.1G.

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Chapter 6

General discussion

SUMMARY

Antibodies are variable in their specificity and their effector mechanisms. Binding of multiple antibodies to their specific antigen on, for instance pathogens or tumor cells, leads to the formation of immune complexes. Fc-receptors can bind antibodies and can thereby respond to these immune complexes, endowing the potent innate immune system with the versatility of the adaptive immune system. IgG is the most abundant type of antibody in serum and its respective Fc-γ receptor family broaden IgG functions by triggering cytokine release, antibody dependent cytotoxicity, phagocytosis and antigen presentation and therefore play critical roles in immunity, auto-immunity and mAb based immunotherapy. Within the leukocyte Fc-γ receptor family, only one high affinity receptor is known: FcγRI[1]. Despite the description of its first cDNA clone more than two decades ago[2], its function in biology remains unclear. Unlike low affinity Fc receptors, FcγRI is thought to be consistently saturated by serum IgG, thereby preventing immune complex binding. Furthermore, the ligand binding α-chain of FcγRI has been described to mediate functions independent of the associated ITAM signaling FcR γ-chain. In this thesis we investigated whether ligand binding to the extracellular 'head' of FcγRI can be regulated, and how this affects immune complex binding (Chapter 2). In Chapters 3 to 5 we describe protein interactions to the cytosolic 'tail' of the FcγRI α-chain (FcγRI-CY) and functional consequences of these interactions.

In Chapter 2, we investigated whether cytokine stimulation can lead to altered FcγRI ligand binding. We found ligand binding to be increased upon cytokine stimulation, independent of changes in surface expression levels. Cytokine stimulation mainly impacted binding to immune complexes, rather of monomeric IgG. We found that FcγRI saturated with monomeric IgG was able to bind immune complexes upon cytokine stimulation, thereby providing insight in the way FcγRI may contribute to immunity.

In Chapter 3, we described the interaction of FcγRI with actin binding protein filamin A. It was found that in the absence of filamin, FcγRI was routed from the plasma-membrane to the lysosomal pathway leading to its breakdown. Co-localization studies with monocytes suggested interaction of FcγRI with filamin at the plasma-membrane, which was lost upon receptor crosslinking. Thus, upon FcγRI crosslinking by immune complexes, filamin interaction with FcγRI is lost, and the receptor with immune complex is targeted to the lysosome.

Chapter 4 documents the interaction between FcγRI and cytosolic protein 4.1G. Similar to periplakin, 4.1G binds membrane proximal to the cytosolic tail of the FcγRI α-chain. A specific splice variant, that lacks part of exon 14 (4.1GΔ14), was found to interact preferentially with FcγRI. Alignment of FcγRI and several other 4.1G interacting proteins suggested the presence of a consensus sequence of basic residues for 4.1G binding. The membrane proximal residues of the cytosolic tail of FcγRI were predicted to form an α-helix, with the 4.1G binding consensus sequence located on one side of the helix.

Functional consequences of the 4.1G- FcγRI interaction were studied in chapter 5. A high molecular weight form of 4.1G was found expressed upon differentiation from monocytes to dendritic cells (DC) or macrophages. Co-localization of 4.1G and actin was observed during phagocytic cup formation upon FcγRI induced phagocytosis. 4.1G showed minimal colocalization with FcγRI at rest, or during endocytosis. We speculated that there might be a role for 4.1G during the uptake of larger particles, and a possible redundancy with other 4.1 family members.

FcγRI AND THE CYTOSKELETON

All three cysotolic proteins that have been described to interact with FcγRI are associated with several members of the cytoskeleton. FcγRI-CY can interact with periplakin[3] which has been described to interact with actin and intermediate filaments[4,5]. 4.1G is known to bind spectrin and actin, filamin A is known to crosslink actin fibers into orthogonal networks[6-8]. For most of these proteins, cytoskeletal binding appears to be a regulated event, thus, not all FγRI associated proteins are necessarily attached to the cytoskeleton simultaneously. Recent work relying on PIP5K deficient macrophages underline the complexities of the actin cytoskeleton during binding and phagocytosis of IgG opsonized particles. While FcγR clustering and subsequent particle binding requires actin depolymerization, actin polymerization is needed for particle ingestion[9]. The cytoskeleton is furthermore intimately involved in the formation and regulation of membrane rafts in which FcγRI was shown to reside[10,11]. Thus, although the precise role of potential cytoskeleton thethering by the FcγRI associated proteins is not known, it is clear that the cytoskeleton is involved in multiple Fc receptor mediated functions.

FUNCTIONAL IMPACT OF FcγRI INSIDE OUT REGULATION

In Chapter two we show that, analogous to the regulation of ligand binding by integrins and FcγRIIA and FcαRI, cytokine stimulation can increase IgG binding by FcγRI independent of changes in surface expression levels. The high affinity IgG binding property of FcγRI has made its role in immunity controversial. Binding and subsequent uptake of immune complexes appears to be central to Fc receptor mediated immunity and it is unclear how FcγRI contributes to this, as the receptor may mostly be saturated by serum IgG. We found that cytokine enhanced IgG binding allowed efficient interaction of FcγRI with immune complexes despite receptor saturation by monomeric IgG. Upon cytokine stimulation, immune complexes are likely to compete with monomeric IgG for the binding site of FcγRI as structural and sequence alignment with other Fcγ receptors suggests a single binding site per receptor (Chapter 1 and Sondermann et al.[12]). In Ba/F3 cells, IL-3 rapidly enhanced immune complex binding.

This is consistent with other reports on downstream IL-3 receptor effects in these cells[13]. In experiments with monocytes, we found that one hour IFNγ and TNFα stimulation was sufficient for overnight upregulation of FcγRI surface levels (data not shown). FcγRI molecules generated *de novo* are free of IgG and can therefore directly contribute to immune complex binding. In FcγRI mediated immunity, initial cytokine enhanced immune complex binding might therefore represent a "first line of defence".

MECHANISM OF INSIDE OUT REGULATION: CYTOKINE RECEPTOR PROXIMAL SIGNALING

The mechanisms underlying FcγRI inside out regulation remain unclear. Similar to FcαRI, okadaic acid is capable of inhibiting cytokine enhanced ligand binding. Okadaic acid is a potent inhibitor of PP2A, but is also known to inhibit other protein phosphatases especially at higher concentrations[14]. Given the fact that, unlike FcαRI, FcγRI does not associate with PP2A[15] and okadaic acid mediated inhibition only at higher concentrations, we suspect other protein phosphatases could perhaps be involved. Serine residues in the FcγRI-CY have been shown to be phosphorylated in resting state[16]. However, IL-3 enhanced ligand binding was unperturbed in truncation mutants of FcγRI-CY lacking all serines (unpublished data). In contrast to FcαRI, inside out regulation of FcγRI was found to be independent of PI3K activity which suggests different cytokine receptor proximal signalling pathways to be involved. Furthermore, inhibition of MEK1/2 did not affect IL-3 enhanced FcγRI ligand binding (Chapter 2). The downstream signalling from the IL-3 receptor involves activation of multiple pathways including signalling components such as Ras, Jak, Lyn and Syk. IL-3 receptor is furthermore capable of activating the GTPase Rap1 which is known to be involved in integrin inside out regulation[17,18]. Further study is needed to unravel the cytokine receptor proximal signalling underlying FcγRI inside out regulation.

FcγRI PROXIMAL MECHANISMS OF INSIDE OUT REGULATION

Regulation of ligand binding by integrins is suggested to occur through conformational changes and receptor clustering. Conformational changes can be imparted by segregation of the integrins' α- and β-chains, while clustering in focal adhesion points allow signalling and thethering to the cytoskeleton[19,20]. Inside out regulation of FcγRIIA and FcαRI results in the appearance of new extracellular epitopes on these molecules suggesting a conformational change upon activation (Kanters *et al*[21]. and Bakema *et al,* unpublished results) and a similar mechanism may well exist during FcγRI inside out regulation. Binding of monomeric IgG was modestly increased upon cytokine stimulation. This could suggest conformational changes of FcγRI dur-

ing inside out activation, as FcγRI and IgG are expected to bind in 1:1 stoichiometry[22]. However, this increase may well be the result of aggregated IgG, as in most solutions a proportion of IgG is known to dimerize. FcγRI clustering has been suggested earlier[23] and preliminary studies in our laboratory using Cy3 and Cy5 labeled IgG suggested increased FRET efficiency upon IL-3 stimulation (data not shown). Receptor clustering may well be regulated by aggregation of specialized membrane rafts. FcγRI has been shown to localize to GM-1 enriched membrane rafts in IFNγ stimulated monocytes[24]. Cholesterol depletion appears to affect ligand binding by FcγRI in IIA1.6 and HL60 cells, as well as in a recently described Ba/F3 model (Beekman et al[25], Chapter 2 and unpublished results). For FcγRIIA, association with lipid microdomains has been suggested to regulate ligand binding[26] and similar mechanisms have been described for integrin clustering[27,28]. Finally, it should be noted that changes in avidity (clustering of receptors) do not preclude changes in affinity (conformational change of the receptor) and vice versa. This is illustrated by the observation that cytokine stimulation not only induces formation of new epitopes on FcαRI, but also affects receptor mobility in the plasma membrane. Thus, although preliminary results suggest clustering to underlie regulation FcγRI ligand binding, inside out regulation may well rely on both receptor clustering and conformational changes. A hypothetical model of FcγRI inside out regulation is depicted in Figure 1.

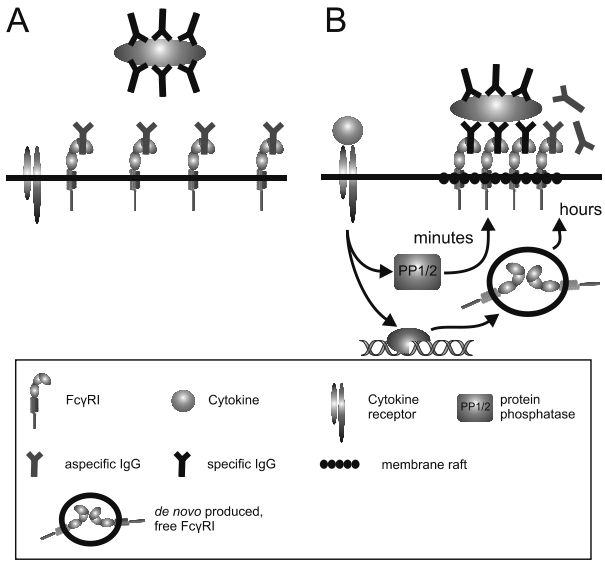

Figure 1. Inside out regulation of FcγRI allows binding of immune complexes despite serum IgG receptor saturation. A) In unstimulated cells, FcγRI can not contribute to immune complex binding due to saturation of receptors with a-specific IgG from serum. B) During inflammation, cytokine stimulation may induce FcγRI clustering in membrane rafts within minutes. Protein phosphatases are likely to underlie receptor inside out regulation. Clustering could facilitate competition of immune complexes over monomeric IgG for receptor binding. On a longer timescale, cytokine stimulation induces *de novo* protein expression of free FcγRI molecules which can further contribute to complex binding.

DYNAMICS OF FcγRI-CY INTERACTIONS

4.1G and periplakin are both capable of binding the membrane proximal part of FcγRI-CY and have been suggested to compete for binding[29]. The cytosolic tail is predicted to enter the cytosol as an α-helix and residues important for periplakin and 4.1G binding appear to locate mainly on opposite sides of the helix. The binding site for filamin on FcγRI-CY remains to be elucidated. In contrast to c-terminal periplakin, and the FERM containing protein talin that regulates integrin ligand binding, overexpression of c-terminal 4.1G did not affect ligand binding[30]. Unlike filamin A, periplakin and 4.1G did not appear to affect FcγRI surface expression levels. This suggests either that I) 4.1G, periplakin and filamin are all capable of binding FcγRI-CY simultaneously or II) binding to FcγRI by the cytosolic proteins is differentially regulated in time and/or space. We favor the second hypothesis as simultaneous binding of all three high molecular weight proteins to the reasonably short cytoplasmic tail of FcγRI would seem unlikely. Although at least for periplakin and filamin elongated structures are predicted, it is hard to envisage binding of all three molecules to such a relatively small surface area.

A second argument relies on co-localization studies, and although co-localization is not directly indicative of protein-protein interactions, it is at least a prerequisite. A hypothetical model based on these observations is shown in Figure 2. Without crosslinking, FcγRI exhibits minimal co-localization with 4.1G. In contrast, clear co-localization between periplakin and FcγRI, and between filamin and FcγRI, has been previously described in monocytes[31]. Upon

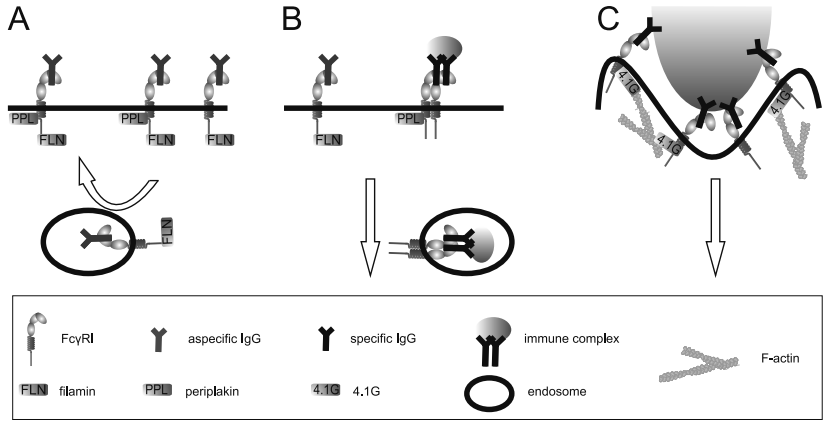

Figure 2. Model of FcγRI and its cytosolic binding partners. A) At rest, periplakin (PPL) and filamin (FLN) associate with te cytosolic tail of FcγRI. Monomeric IgG binding induces receptor recycling during which filamin and FcγRI continue to interact. Upon binding and ingestion of small immunecomplexes (B), FcγRI – filamin association is lost leading to exposure of acidic clusters (not shown) and lysosomal routing of receptor for subsequent antigen presentation. C) Phagocytosis of large particles requiring actin remodelling induces FcγRI-4.1G-actin complex formation allowing stabilization of the phagocytic cup. Meanwhile, loss of filamin induces fusion of lysosomes with the phagosome for phagosomal maturation. Arrows indicated receptor recycling (A) or lysosomal targeting (B and C). F-actin: filamentous actin.

receptor crosslinking, however, co-localization of these proteins is lost. Thus, immune complex binding, either mediated by inside out regulation or by binding of particles to prebound IgG, triggers receptor crosslinking and dissociation of periplakin-FcγRI-CY and filamin-FcγRI-CY interactions. During the uptake of large particles, 4.1G was found to co-localize with actin. 4.1G might stabilize the formation of the phagosome by binding FcγRI-CY by its c-terminal domain and actin through the spectrin actin binding domain. Filamin dissociation leads to routing towards the lysomal compartment for ligand degradation and subsequent antigen presentation. Lysosomal routing could be mediated via a cluster of acidic residues present in FcγRI-CY[32]. Notably, DEC205, a c-type lectin receptor expressed on dendritic cells, contains a similar acidic cluster which has been implicated in receptor mediated antigen presentation[33].

CONCLUDING REMARKS

Interactions to the head and tail of FcγRI direct the biological functions of this receptor. Immune complex binding represents an important characteristic of Fc receptors in immunity. Inside out regulation may allow FcγRI to contribute to the uptake of immune complexes. Proteins such as filamin A, which interact with the cytosolic tail of FcγRI, can profoundly impact receptor function and further studies on the biological role of the 4.1G and FcγRI interaction is therefore needed. In depth knowledge of these molecular mechanisms may contribute to our understanding of the biological role of FcγRI in immunity. Insight in this role and knowledge on the involvement of FcγRI during antibody therapy may well lead to improved treatment for patients.

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Nederlandse samenvatting voor niet ingewijden

INLEIDING

Antilichamen zijn eiwitten die het lichaam aanmaakt ter bescherming tegen pathogenen, zoals bacteriën en virussen. Deze antilichamen, ook wel immunoglobulines of Ig genoemd, bestaan uit een variabel deel, het Fab gedeelte, dat aan een bacterie of virus bindt, en een constant deel, het Fc gedeelte. Tijdens de aanmaak van een antilichaam kunnen delen in het Fab gedeelte door het lichaam aangepast worden. Als gevolg hiervan kan er een grote diversiteit aan antilichamen gegenereerd worden en kunnen verschillende antilichamen verschillende bacteriën of virussen herkennen.

Het Fc gedeelte van het antilichaam fungeert als een signaal voor het immuunsysteem. Cellen van het immuunsysteem kunnen binden aan het Fc gedeelte van het antilichaam met behulp van zogenaamde Fc receptoren, die op het oppervlakte van de cel aanwezig zijn (zie Figuur 1A). Via antilichamen kan de cel de bacterie of het virus herkennen en vervolgens "opeten", dit wordt ook wel fagocytose genoemd (*fago-* komt van het grieks 'eten'). Meerdere antilichamen kunnen aan hetzelfde pathogeen binden en een zogeheten immuuncomplex vormen. Belangrijk hierbij is, dat er meerdere Fc staarten aanwezig zijn, die de Fc receptoren op de immuuncel dichtbij elkaar brengen. De cel krijgt alleen dan een signaal dat het kan beginnen met het immuuncomplex op te eten. Hierdoor wordt voorkomen dat vrije antilichamen, die niet deel uitmaken van een immuuncomplex, de cellen activeren (Figuur 1B).

Een ontwikkeling in kankeronderzoek van de laatste 20 jaar is de zogenaamde antilichaam- of immunotherapie. Hierbij krijgt een kankerpatiënt antilichamen die tumor-specifiek zijn. Bij deze vorm van therapie is gebleken dat Fc receptoren een belangrijke rol spelen en het immuunsysteem helpen de tumor als "lichaamsvreemd" te herkennen en op te ruimen.

De Fc receptoren worden ingedeeld op basis van welk type Fc deel er gebonden kan worden. Het eiwit, dat gebonden wordt, wordt het ligand van de Fc receptor genoemd. Zo kunnen bijvoorbeeld Fc-ε receptoren IgE (immunoglobuline met Fc deel type "E") binden en kunnen Fc-γ receptoren IgG (immunoglobuline met Fc deel type "G"), binden. IgG is het meest voorkomende type antilichaam in het bloed en wordt het meest gebruikt bij de huidige antilichaamtherapieën. Om deze redenen wordt er veel onderzoek gedaan naar Fc-γ receptoren.

In dit proefschrift hebben we naar een specifieke Fc receptor gekeken, de humane FcγRI. De rol van deze receptor in het immuunsysteem is onduidelijk, omdat het extracellulaire gedeelte, in tegenstelling tot dat van andere Fc-γ receptoren, met hoge affiniteit aan IgG bindt. Bloed bevat veel IgG en er is waarschijnlijk altijd vrij IgG aan de receptor gebonden. Het dogma bestaat dan ook dat deze receptor daardoor niet meer kan binden aan complexen

van pathogeen-antilichaam of tumorcel-antilichaam en een beperkte rol heeft in de Fc receptor-gemedieerde immuniteit.

Ook de manier waarop FcγRI signalen aan de cel geeft, lijkt te verschillen met de andere Fc-γ receptoren. De meeste Fc-γ receptoren zijn compleet afhankelijk van een apart eiwit, dat de signalen van de Fc receptor doorgeeft en, verwarrend genoeg, FcR γ-keten heet. Daarentegen kan het intracellulaire gedeelte van FcγRI zelf ook een aantal signalen doorgeven. Deze signalen lijken een rol te spelen bij de opname van kleine immuuncomplexen en bij een secundaire reactie, zogenaamde antigeen-presentatie, waardoor andere delen van immuunsysteem geactiveerd kunnen worden om verder te helpen bij de bestrijding van het pathogeen.

DOEL VAN HET ONDERZOEK BESCHREVEN IN DIT PROEFSCHRIFT

Door onderzoek naar FcγRI te doen, kunnen we meer leren over de rol van deze receptor in de bescherming van ons lichaam tegen pathogenen en tijdens antilichaamtherapie tegen tumoren. Daarom kan dit onderzoek mogelijk bijdragen aan de verbetering van immunotherapie in de strijd tegen kanker.

Recent onderzoek heeft laten zien, dat de Fc receptor- ligand binding van *binnen* in de cel gereguleerd kan worden, als gevolg van een signaal van *buiten* de cel. Deze signalen worden bijvoorbeeld afgegeven tijdens een ontsteking. Dit fenomeen wordt ook wel *inside-out* regulatie genoemd en is alleen beschreven bij twee andere typen Fc receptoren.

We hebben onderzocht of de IgG binding aan het extracelullaire gedeelte, de 'kop', van FcγRI ook gereguleerd kan worden en of dit de receptor in staat stelt om immuuncomplexen te binden, ondanks dat er al vrij IgG aan de receptor gebonden is. De resultaten van dit onderzoek staan beschreven in hoofdstuk 2.

Om verdere functies van het intracellulaire gedeelte, de 'staart', van FcγRI te onderzoeken, is gekeken naar interacties tussen de FcγRI staart en eiwitten in de cel. De resultaten van deze studies zijn beschreven in hoofdstuk 3, 4 en 5.

HOOFDSTUK 2

De rol van FcγRI in de immuniteit van de mens is onduidelijk. Het verwijderen van immuuncomplexen, in de vorm van IgG-pathogeen complexen of IgG-tumorcel complexen, is een belangrijke functie van Fc receptoren. FcγRI heeft, binnen de Fcγ receptor familie, de unieke eigenschap om sterk aan IgG te binden. Dit heeft als gevolg dat deze receptor zeer waarschijnlijk altijd bezet is met vrij IgG uit het bloed. Dit maakt het binden van IgG in immuuncomplexen onmogelijk en daardoor ook Fc receptor- gemedieerde fagocytose. Men

denkt dat voornamelijk Fcγ receptoren met een lage affiniteit, zoals FcγRIIIA, deze rol vervullen. Toch is uit studies met genetisch gemodificeerde muizen, die de FcγRI niet meer hebben, gebleken dat een hoge affiniteits Fcγ receptor wel degelijk kan bijdragen aan bescherming tegen bacteriën en tijdens immunotherapie bij tumoren.

In hoofdstuk 2 laten we zien, dat cytokines de IgG binding van FcγRI kunnen beïnvloeden, een proces wat ook wel inside-out regulatie heet en eerder bij andere Fc receptoren gezien is. Cytokines zijn eiwitten, die uitgescheiden worden door cellen om over langere afstanden met elkaar te communiceren. Tijdens een bacteriële infectie worden plaatselijk cytokines uitgescheiden om cellen van het immuunsysteem te activeren en naar de specifieke locatie toe te leiden.

Om IgG binding door FcγRI tijdens cytokine-stimulatie te bestuderen hebben we BAF3 cellen, die normaal geen Fc receptoren hebben, voorzien van humaan FcγRI. Deze cellen zijn eenvoudig te stimuleren met het cytokine interleukine 3 (IL-3). Als de cellen kort (15-60 minuten) gestimuleerd worden met IL-3, zien we een veel effectievere binding van immuuncomplexen aan FcγRI dan bij ongestimuleerde cellen. Echter, dezelfde stimulatie beïnvloedt nauwelijks de binding van FcγRI aan vrij IgG. Belangrijk is het gegeven dat de hoeveelheid Fc receptoren op het celoppervlak niet lijkt te veranderen na de cytokine stimulatie.

Als we deze cellen na stimulatie, eerst vrij IgG laten binden en vervolgens in contact laten komen met immuun complexen, dan blijkt dat in tegenstelling tot cellen die niet gestimuleerd zijn, immuuncomplexen plotseling wel aan de receptoren kunnen binden.

Ditzelfde blijkt te werken met bepaalde immuuncellen (monocyten) direct geïsoleerd uit humaan bloed. Stimulatie van monocyten zorgt dat deze cellen immuuncomplexen kunnen binden, ondanks het feit, dat FcγRI op deze cellen in eerste instantie bezet is met vrij IgG uit het bloed. De cytokine geïnduceerde binding van immuuncomplexen lijkt volledig afhankelijk te zijn van FcγRI en niet van andere Fcγ receptoren met een lage affiniteit, die ook op het cel oppervlak van deze monocyten aanwezig zijn.

Het precieze mechanisme, hoe de IgG binding van FcγRI gereguleerd wordt, is nog niet duidelijk. De experimentele data suggereren tot nu toe, dat er op het oppervlak van de cel na cytokine stimulatie clusters van receptoren gevormd worden. Zo'n cluster zou mogelijk makkelijker immuuncomplexen binden dan losse receptoren op het oppervlak (zie ook Figuur 1 van hoofdstuk 5).

Deze studie laat zien dat, in tegenstelling tot eerdere ideeën, FcγRI wel degelijk in staat is om immuuncomplexen te binden, zelfs als de receptor al eerder in contact is geweest met vrij IgG. Dit zou kunnen verklaren hoe FcγRI bijdraagt aan de IgG-gemedieerde immuniteit.

HOOFDSTUK 3

Het is bekend, dat FcγRI zonder de geassocieerde Fc-γ keten, antigeen-presentatie kan induceren. Antigeen-presentatie is een essentieel onderdeel van de immuniteit, omdat het het immuunsysteem in staat stelt flexibel te reageren tegen lichaamsvreemde pathogenen en, omdat het immunologisch "geheugen" kan induceren. Dit betekent dat het immuunsysteem beter beschermd is als het pathogeen voor een tweede keer het lichaam besmet. Het immunologisch geheugen ligt zo ook ten grondslag aan de effectiviteit van vaccinaties. Voor antigeen-presentatie is afbraak van immuuncomplexen in de cel nodig. Dit gebeurt in een zogeheten lysosoom, een deel van de cel, dat gespecialiseerd is in het afbreken van eiwitten.

In hoofdstuk 3 hebben we de interactie beschreven tussen de intracellulaire staart van FcγRI en het intracellulaire eiwit Filamin A. Op basis van fluorescentie microscopie, waarbij we de verschillende eiwitten kunnen aankleuren met fluorescente markers, blijkt dat in monocyten FcγRI en Filamin A allebei op dezelfde plek in de cel zitten. FcγRI zit aan de oppervlakte van de cel en Filamin A lijkt zich onder het oppervlak vlakbij FcγRI te bevinden. Echter, als FcγRI immuuncomplexen bindt, blijkt dat na verloop van tijd FcγRI de cel in verdwijnt, terwijl Filamin A onder het oppervlak blijft. Dit suggereert dat er op dat moment ook geen interactie meer bestaat tussen FcγRI en Filamin A.

Als cellen zonder Filamin A voorzien worden van FcγRI, dan lijkt het dat FcγRI helemaal niet op de cel oppervlakte aanwezig is, maar binnen in de cel blijft. Hier wordt de receptor afgebroken in een lysosoom. We kunnen aantonen, dat in afwezigheid van Filamin A, FcγRI wel degelijk normaal op het celoppervlak komt, maar daar direct weer van verdwijnt richting lysosoom. Uit deze data kunnen we concluderen, dat Filamin A zorgt voor een stabiele aanwezigheid van FcγRI op het celoppervlak, waardoor binding aan immuuncomplexen mogelijk is. Na het binden van een immuuncomplex aan FcγRI, wordt de interactie met Filamin A verbroken en worden de receptor en het immuuncomplex getransporteerd naar het lysosoom. Afbraak van het immuuncomplex maakt het mogelijk voor de cel antigenen te presenteren. Op deze manier zou FcγRI een rol kunnen spelen in antigeen-presentatie en immuunreacties kunnen verbreden en versterken.

HOOFDSTUK 4

In hoofdstuk 4 is de interactie beschreven tussen de staart van FcγRI en het intracellulaire eiwit 4.1G. We hebben voor het verhelderen van deze interactie gebruik gemaakt van het DNA van FcγRI . Het DNA codeert voor een ketting aan aminozuren, die de bouwstenen zijn van een eiwit, in dit geval FcγRI. Door veranderingen, mutaties, in het DNA van FcγRI te maken, hebben wij verkorte stukken van de staart van de receptor verkregen, zogeheten truncaties. Het blijkt dat een groot deel van de intracellulaire staart van de receptor niet nodig is voor de

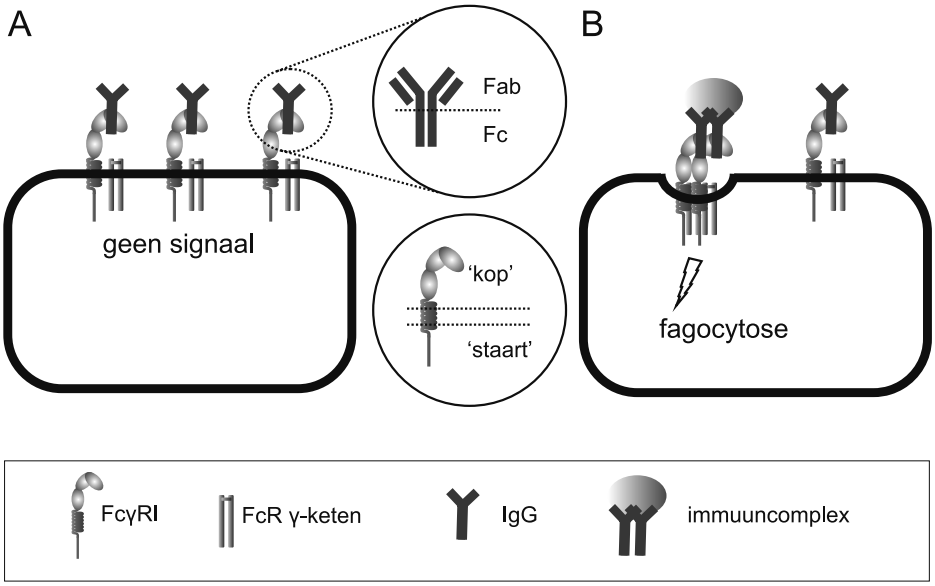
binding van 4.1G. Door zeer kleine veranderingen te maken, blijken een aantal aminozuren het meest belangrijk te zijn voor de binding van 4.1G aan de staart van FcγRI. Verder hebben we de aminozuursequentie van de bindingsplaats van FcγRI aan 4.1G vergeleken met de bindingsplaats van andere eiwitten, waar 4.1G aan kan binden. Er lijkt een vergelijkbare sequentie te bestaan voor de bindingsplaats van 4.1G aan verschillende eiwitten, waaronder FcγRI. Dit suggereert dat er één specifiek gedeelte is in de staart van FcγRI, dat aan 4.1G kan binden.

HOOFDSTUK 5

Dit hoofdstuk is een vervolg op hoofdstuk 4. In hoofdstuk 5 hebben we gekeken naar de mogelijke functie van de interactie tussen FcγRI en 4.1G. 4.1G is opgebouwd uit een aantal eiwit domeinen. Deze domeinen komen ook voor in andere 4.1 eiwitten zoals in 4.1R. Zo bezitten beide eiwitten een domein dat actine kan binden. Actine is een deel van het cytoskelet (waar cyto- "van de cel" betekent, dus letterlijk het skelet van de cel) en is belangrijk bij het bewegen en bij behoud en verandering van de vorm van de cel. FcγRI kan de fagocytose van immuuncomplexen induceren. Bij fagocytose van grote immuuncomplexen worden actine kettingen gebruikt, zodat de cel zich om het immuuncomplex heen kan vervormen, totdat het immuuncomplex helemaal is opgenomen.

We hebben met behulp van fluorescentie microscopie gekeken naar de locatie van FcγRI en 4.1G tijdens het opnemen van grote immuuncomplexen. Normaal lijken FcγRI en 4.1G niet bij elkaar in de buurt te zitten. FcγRI bevindt zich, zoals verwacht, aan het oppervlak, en 4.1G meer binnen in de cel. Wanneer de cel echter grote immuuncomplexen opneemt, verschijnt 4.1G aan de rand waar de cel zich om het immuuncomplex heen beweegt en waar ook veel actine aanwezig is. Door cellen van muizen, die geen 4.1G hebben, te voorzien van humaan FcγRI, lijkt het echter alsof 4.1G geen rol speelt bij het opnemen van kleinere immuuncomplexen zoals bacteriën. Dit klopt met onze observatie dat het opnemen van kleine immuuncomplexen de locatie van 4.1G niet doet veranderen. Verder is gebleken dat 4.1G geen rol lijkt te spelen bij stabilisatie van FcγRI op het cel oppervlak, zoals wij dat eerder wel konden aantonen voor Filamin A, en dat 4.1G de binding van de receptor aan IgG niet verandert.

Deze studie suggereert, dat 4.1G een rol zou kunnen spelen tijdens het opnemen van grote immuuncomplexen, zoals IgG gebonden tumorcellen, door FcγRI. Echter, het formele bewijs hiervoor ontbreekt nog.



Figuur 1. IgG binding en fagocytose door FcγRI. A) Schematische weergave (niet op schaal) van een immuuncel met FcγRI op het oppervlak. Te zien is de extracellulaire 'kop' en de intracellulaire 'staart'. Ook de geassocieerde FcR γ-keten is aangegeven. Vrij IgG kan de receptor binden maar initieert geen fagocytose. Een meer gedetaillieerde weergave van IgG geeft het Fab en het Fc gedeelte van het IgG eiwit aan. B) Wanneer een immuuncomplex met meerdere IgG eiwitten aan FcγRI bindt, initiëren de bijeengebrachte receptoren fagocytose.

CONCLUSIE

De studies in dit proefschrift zorgen voor een stap voorwaarts in ons begrip hoe FcγRI zou kunnen helpen in de IgG gemedieerde immuunrespons. Belangrijk is de bevinding dat FcγRI, na cytokine stimulatie, immuuncomplexen kan binden. Dit is een belangrijke voorwaarde, waaraan FcγRI moet voldoen, wil het kunnen bijdragen aan bijvoorbeeld het opruimen van bacteriën en het verwijderen van tumorcellen tijdens antilichaamtherapie. Of FcγRI daar daadwerkelijk een rol in speelt, is nu de volgende stap.

De verschillende eiwitten die aan de intracellulaire staart binden laten ons zien welke complexe mechanismen aan de functie van de deze receptor ten grondslag liggen. Hoewel dit niet direct bestudeerd is, lijken in ieder geval op basis van functionele proeven en fluorescentie microscopie, niet alle intracellulaire eiwitten tegelijk aan de staart van FcγRI te binden. Zo kan men concluderen dat 4.1G en Filamin A waarschijnlijk niet tegelijkertijd binden aan de staart. We verwachten, dat Filamin A de receptor staart los laat na het binden van immuuncomplexen aan de receptor. Dit is in tegenstelling tot 4.1G, dat juist meer een rol lijkt te spelen ná binding van het immuuncomplex. Toekomstig onderzoek zou de rol van

4.1G moeten bekijken tijdens het opnemen van IgG gebonden tumorcellen en naar verdere mechanismen hoe FcγRI antigeen-presentatie kan induceren.

Omdat muis FcγRI veel verschilt van humaan FcγRI, voornamelijk in de staart, is het verstandig om in toekomstig onderzoek zoveel mogelijk humane cellen te gebruiken. Hoewel bij deze experimenten de omstandigheden moeilijker te controleren zijn dan bij studies met muizen, zijn er, natuurlijk binnen ethische grenzen, wel degelijk mogelijkheden om dit te doen. Het is bijvoorbeeld gebleken dat bij mensen mutaties in andere Fc-γ receptoren voorkomen en dat deze mutaties een effect hebben op de uitkomst tijdens de behandeling van tumoren met antilichaamtherapie. Op dezelfde manier zou men kunnen kijken naar het bestaan en eventueel effect van mutaties van FcγRI tijdens antilichaamtherapie.

Deze onderzoeken kunnen leiden tot een beter begrip welke rol FcγRI speelt in het immuunsysteem en zouden nieuwe inzichten kunnen geven ter verbetering van antilichaamtherapie.

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Dit is het laatste lange verhaal van dit proefschrift. Als je dit boekje van voren af aan bent gaan met lezen en je bent zojuist bij deze passage aangekomen, bedankt en houd nog even vol. Als je vanaf hier begonnen ben met lezen, dan ook welgemeend bedankt en lees alsjeblieft nog even verder.

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Ten eerste de piepels van Utrecht: Joost (aka J-man), Jorinde, Michaël, Selena, Nicoline, Yonathan, Madelon, Jeroen, Matthijs, Kristan (ook trouwe reagentia leverancier), Frank (aka fix-it-Frank), Eefje, Robbert (dr SNP en de man van de vette 3D structuren in hoofdstuk 1, wanneer komt het volgende paper?), Tamara, Jan Willem en Sanne. JW bedankt dat je wilt paranimfen en voor de dikke trips naar Egypte en Lissabon. Liever geen FFTBM tijdens de verdediging ok? Marleen en Saskia van de IBB, bedankt voor de lol, feestjes en het koken tijdens mijn studie!

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Lieve Mam en Pap, jullie dachten natuurlijk dat er niets terecht kwam van mijn promotie met al die verhalen over mislukte proeven. Misschien dat er een wat scheef beeld van mijn onderzoek is ontstaan. Sorry daarvoor. Bedankt voor jullie steun, vertrouwen en goede adviezen. Pap, dit boekje is van jou, schrijf er gerust in (sterker nog, dat moet).

Marieke en Maarten, bedankt voor jullie aerobed, steun, lol, kennis van mooie dingen en de openbaring, die San Francisco heet. We hebben nog geen San Fran lied?!

Lieve Suze, dat een labnerd zoals ik, zo'n mooie en lieve vriendin kon vinden. Wauw. Bedankt voor je begrip als ik laat moest werken en voor het me naar (je) huis sturen. Maar het meeste bedankt voor je begrip bij mijn rare post-doc plannen.

Je bent bijzonder en ik hou van je.

Als je vanaf hier begonnen bent met lezen dan heb je een belangrijk deel overgeslagen.

OK band, op de 1.

Curriculum Vitae

Cees E. van der Poel werd op 24 september 1980 geboren in De Bilt. Na het behalen van zijn VWO diploma op de Werkplaats Kindergemeenschap te Bilthoven, volgde hij de studie Medische Biologie op de Universiteit Utrecht. Na zijn afstuderen in 2005 was hij een jaar werkzaam als junior scientist bij Bioceros te Utrecht. Hij begon in 2006 als assistent in opleiding bij de groep immunotherapie, afdeling Immunologie, Universitair Medisch Centrum Utrecht, onder leiding van Dr. Jeanette Leusen en Prof.dr. Jan van de Winkel. Het resultaat van dit onderzoek staat beschreven in dit proefschrift.

List of Publications

Cytokine induced immune complex binding to the high affinity IgG receptor, FcγRI, in the presence of monomeric IgG.
CE van der Poel, RA Karssemeijer, P Boross, JA van der Linden, M Blokland, JGJ van de Winkel, JHW Leusen.
Blood. 2010 Aug 30.

Filamin A stabilizes FcγRI surface expression and prevents its lysosomal routing.
JM Beekman *, **CE van der Poel** *, JA van der Linden, DL van den Berg, PV van den Berghe, JGJ van de Winkel, JHW Leusen.
J Immunol. 2008 Mar 15;180(6):3938-45

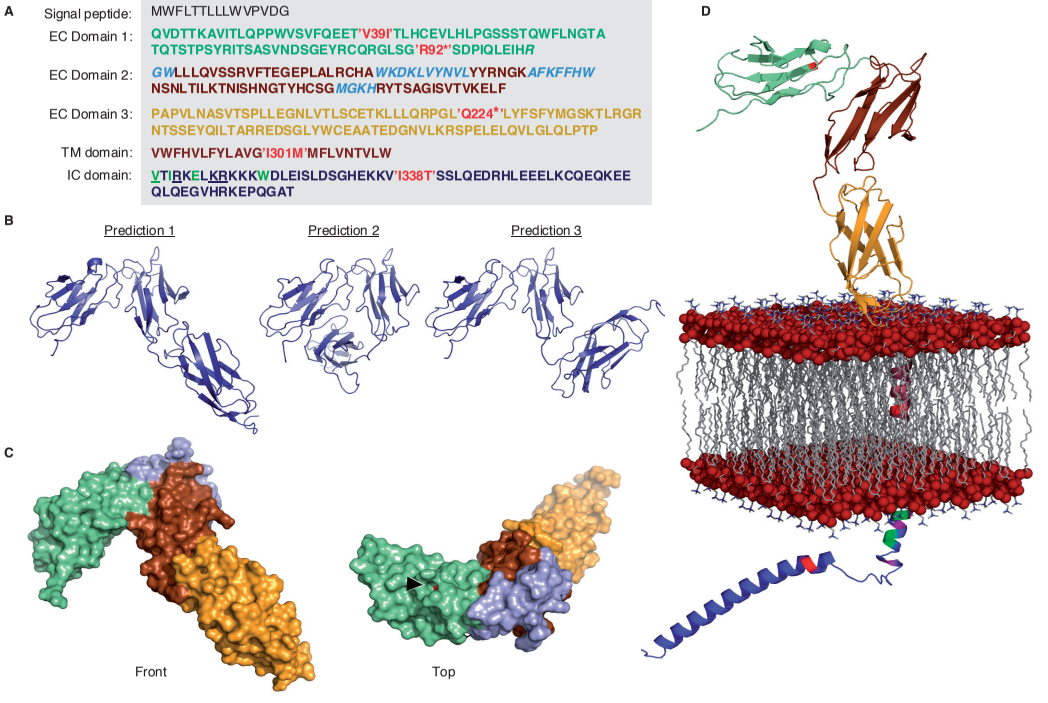
Protein 4.1G binds to a unique motif within the FcγRI cytoplasmic tail.
JM Beekman *, JE Bakema*, **CE van der Poel**, JA van der Linden, JGJ van de Winkel, JHW Leusen.
Mol Immunol. 2008 Apr;45(7):2069-75.

Fc receptors.
P Boross, **CE van der Poel**, JGJ Van de Winkel, JHW Leusen
Encyclopaedia of Life Sciences. July, 2008

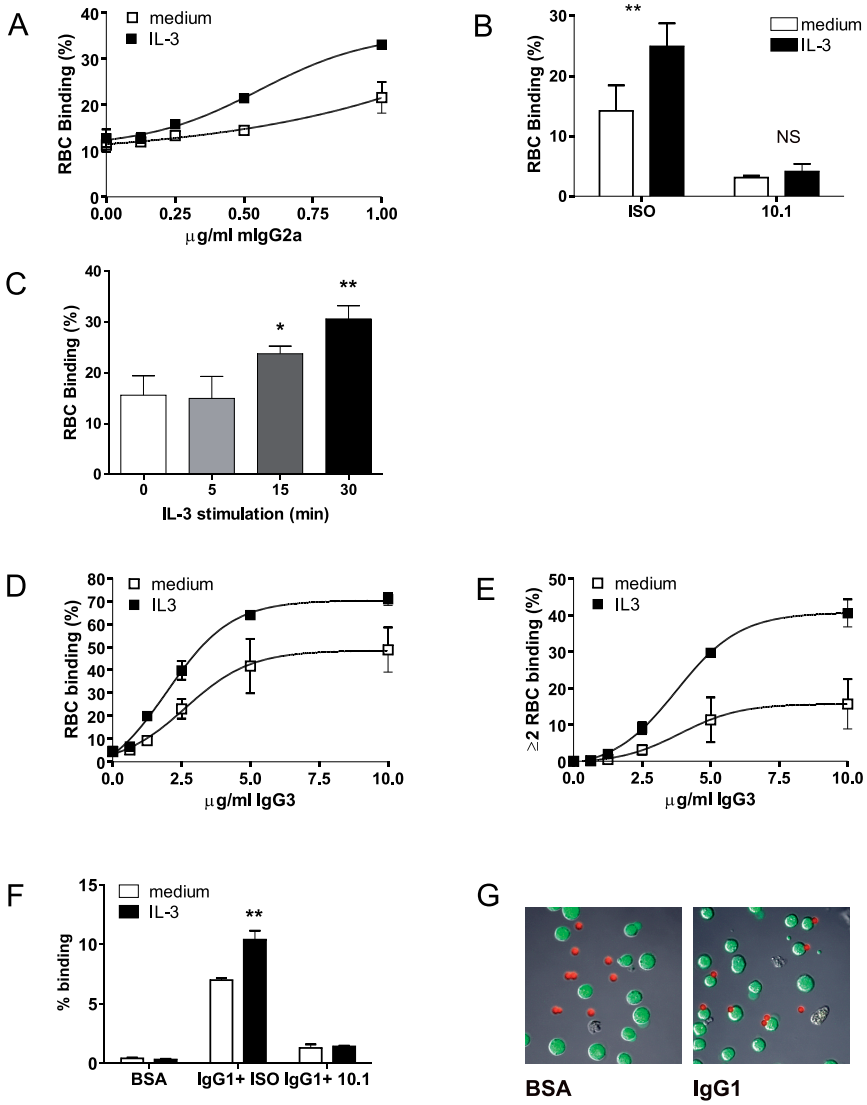
*Both authors contributed equally to these manuscripts

Abbreviations

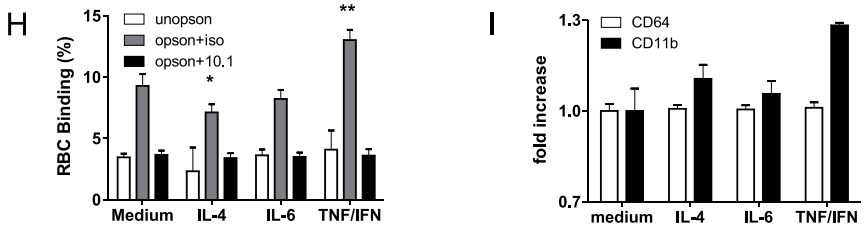
AML	Acute Myeloid Leukemia
ADCC	Antibody Dependent Cytotoxicity
cDNA	complementary DNA
DC	Dendritic Cell
EC	Extracellular domain
Fab	Fragment, antigen binding
Fc	Fragment, crystalizable
FcγR	Fc receptor for IgG
FcγRI-CY	intracellular, cytosolic tail of FcγRIa
FERM	Four point one, Ezrin, Radixin, Moesin domain
Fln A	Filamin A
GFP	Green Fluorescent Protein
IRES	Internal Ribosomal Entry Site
ITAM	Immunereceptor Tyrosine-based Activation Motif
KO	Knock-out
LAMP-1	Lysosomal-Associated Membrane Protein 1
-LTH	medium without leucine, tryptophane and histidine
mAb	monoclonal Antibody
MFI	Mean Fluorescence Intensity
MVB	Multi Vesicular Body
NOTAM	mutated, signalling incompetent ITAM
PPL	periplakin
ROS	Reactive Oxygen Species
SABD	Spectrin Actin Binding Domain
scFv	Single-chain Variable Fragment
SNP	Single Nucleotide Polymorphism
TM	Transmembrane region
TRAP	Transmembrane Adaptor Protein
un	untransfected
WT	wildtype
β-gal	β-galactosidase



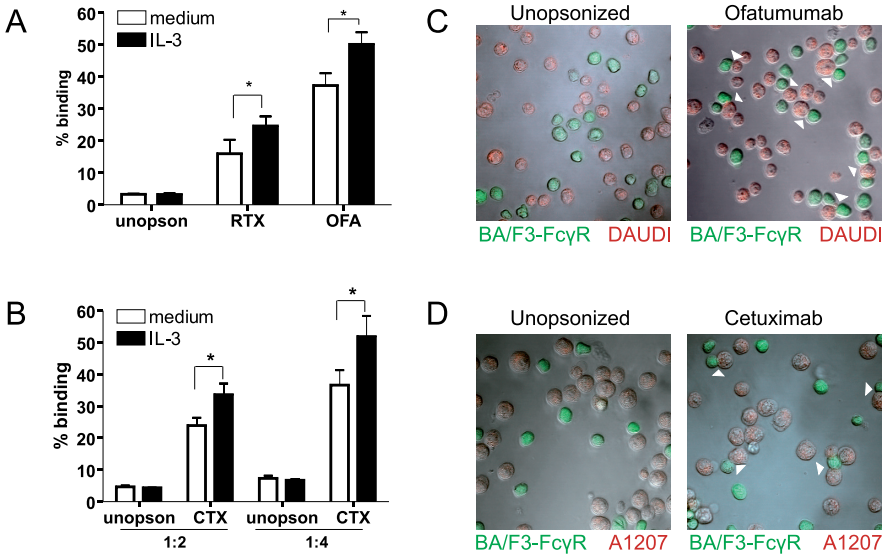
Chapter 1. Figure 2. FcγRIA structure and variations. A) Amino acid sequence divided in different domains. The five non-synonymous SNPs are indicated in red (V39I: rs7531523, R92*: mutation described by van de Winkel et al[67], Q224*: rs1338887, I301M: rs12078005, I338T: rs1050208, stars denote stopcodon), the probable surface residues that facilitate IgG-binding (lightblue) and the interaction sites with 4.1G (underlined) and periplakin (green)[22,43,45]. B) Cartoon representations of predicted extracellular FcγRI structure. EC1-EC2 and EC3 were separately predicted using I-TASSER[113], and subsequently assembled and refined by docking algorithms from the HADDOCK server[114]. The three most likely models are shown. C) Surface of the extracellular domains of prediction 1 as seen from front and top. IgG-binding domain is marked in blue and V39 in EC1 is in red and marked by the arrowhead D) Interpretation of FcγRI within a plasma membrane to illustrate the interaction and variation sites. Structure prediction 1 was used for this model. the color codes are the same as in (A), 4.1G bindingsite is shown in magenta. All images were created in PyMol.



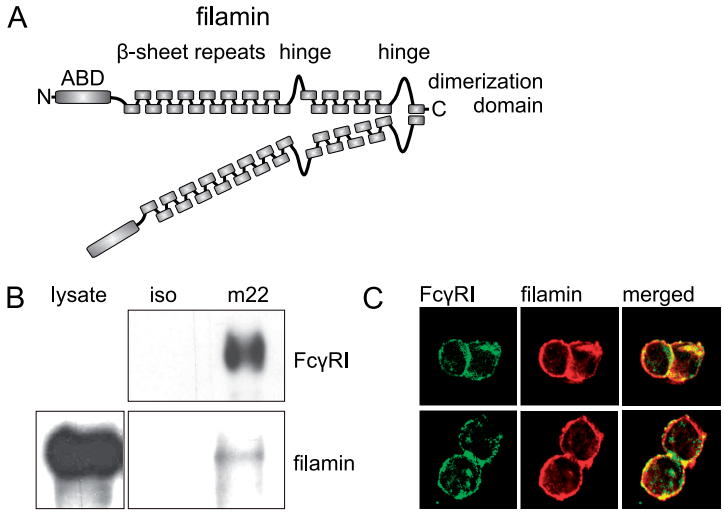
Chapter 2. Figure 2. Stimulation of primary monocytes and Ba/F3-FcγRI cells results in enhanced IC binding. Percentage of Ba/F3-FcγRI cells binding RBC opsonized with IgG2a (A, B and C) or human IgG3 (D and E). Open squares represent starved cells, filled squares represent starved Ba/F3-FcγRI cells stimulated with IL-3 for one hour. B) EA rosette assay in the presence of FcγRI specific mAb 10.1 (10.1) or isotype control (iso). **: P<0.01; NS: not significant, t-test. C) Effect of duration of IL-3 stimulus on IC binding. *:P<0.05; **:P<0.01, t-test. D) Binding of 1 or more RBC to Ba/F3 cells. E) Binding of 2 or more RBC to Ba/F3 cells. Datapoints represent the means of at least 3 replicates. Similar results were obtained in 3 independent experiments. ***: P<0.001. F) Binding of Ba/F3-FcγRI cells to BSA or IgG1 coupled beads in the presence of 10.1 mAb (10.1) or isotype control (ISO). Similar results were obtained in 3 independent experiments. **:P<0.01, t-test. G) Rosetting of BSA and IgG1 coupled beads with Ba/F3-FcγRI cells visualized with fluorescence microscopy. Beads are detected in the red channel, Ba/F3-FcγRI in the green channel.



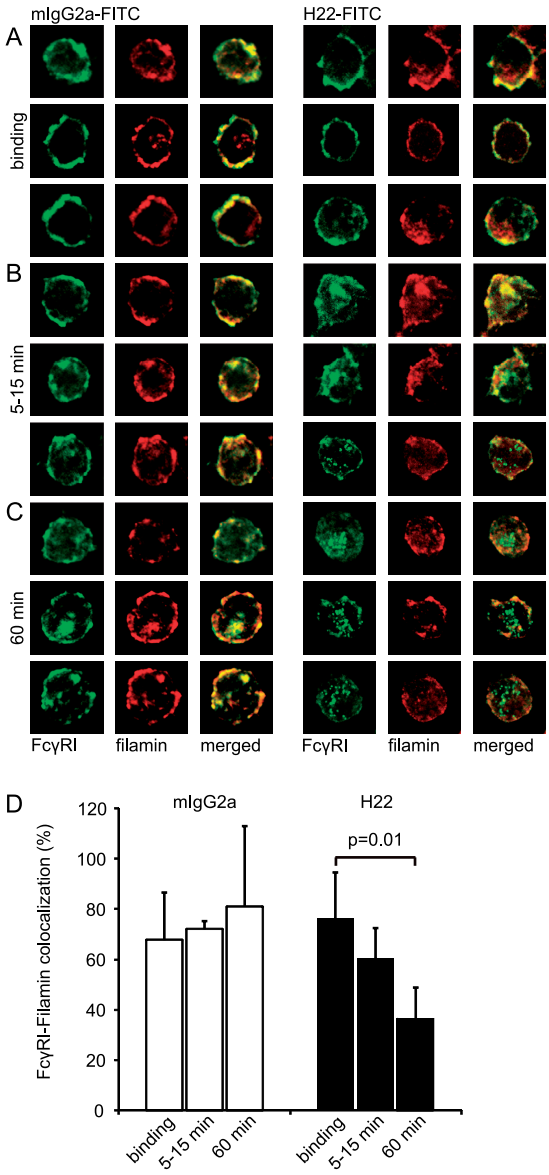
Chapter 2. Figure 2. (continued) H) Binding of unopsonized (unopson) and opsonized (opson) erythrocytes to primary monocytes in the presence of 10.1mAb (10.1) or isotype control (iso). Data represent means of triplicates measured from one donor. Similar results were seen in 3 out-of -4 donors. *: P<0.05; ** P<0.01. I) FcγRI (CD64) and CD11b expression on CD14+ gated monocytes. Shown is the fold increase in surface expression relative to unstimulated (medium) cells. Data represent mean from triplicates.



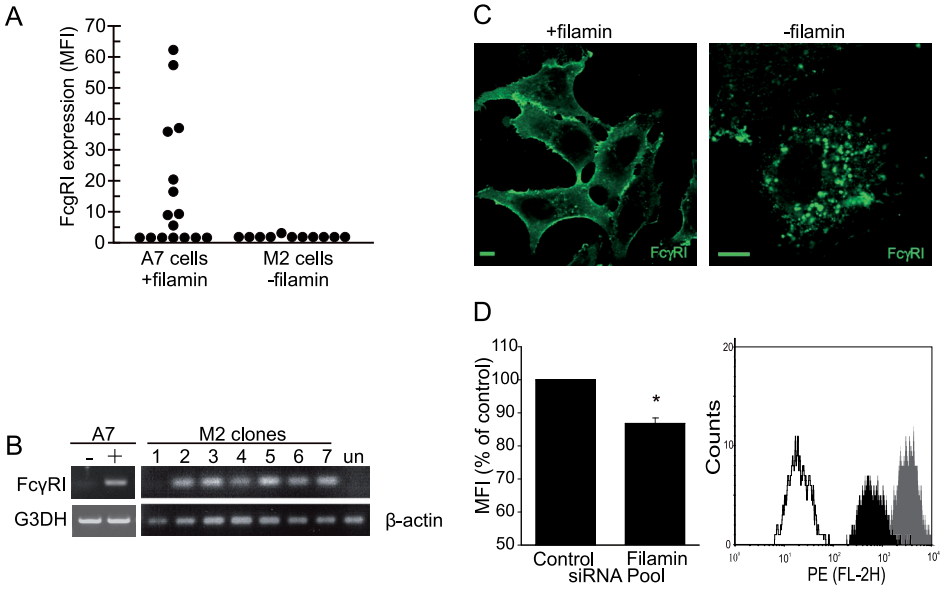
Chapter 2. Figure 3. Cellular activation leads to enhanced FcγRI mediated binding of opsonized tumor celllines. A) Percentage of Ba/F3- FcγRI cells binding to Daudi cells opsonized with Rituximab (RTX), Ofatumumab (OFA) or unopsonized (unopson) Daudi cells. E/T ratio was 1/ 2. B) Percentage of Ba/F3-FcγRI cells binding to Cetuximab (CTX) or unopsonized (unopson) A1207. E/T ratio was either 1/2 or 1/4. Data represent the means of at least 3 replicates. FcγRI blocking mAb 10.1 was used to investigate FcγRI dependent binding (not shown) *: P<0.05. Experiment was repeated three times yielding similar results. C&D) Binding of IL-3 stimulated Ba/F3-FcγRI to Ofatumumab opsonized Daudi cells (C) or Cetuximab opsonized A1207 cells (D) determined with fluorescence microscopy. Ba/F3-FcγRI cells are shown in green, Daudi and A1207 cells in red. Arrowheads denote contacts between Ba/F3-FcγRI and tumor cells.



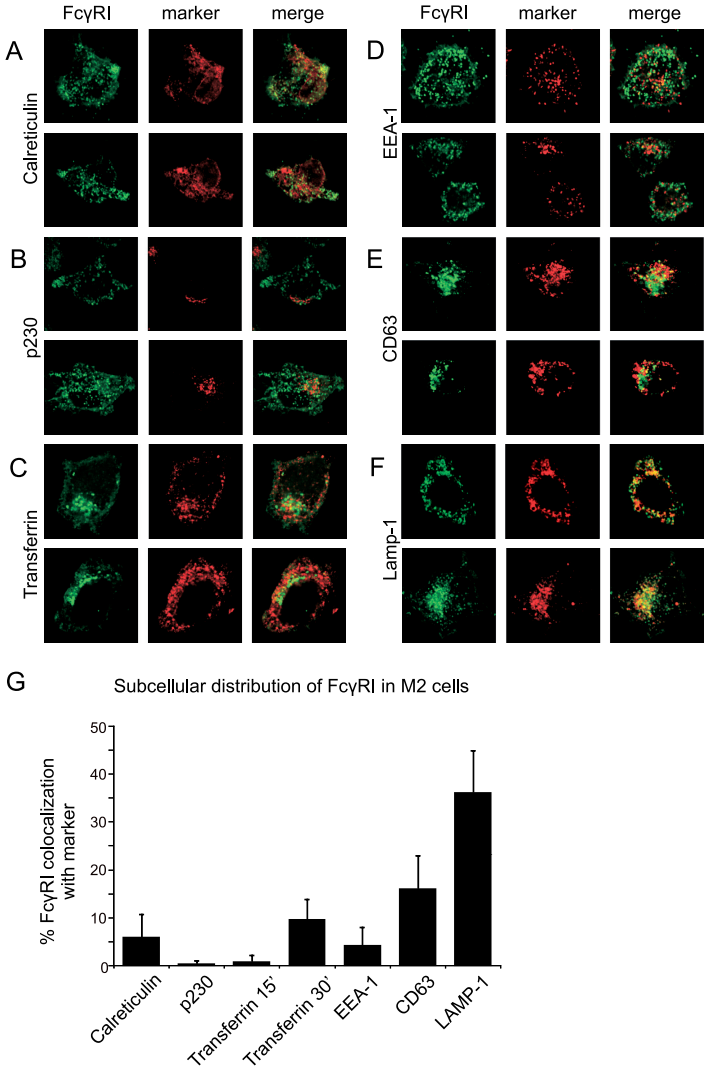
Chapter 3. Figure 1. FcγRI interaction with filamin in yeast cells. (A) Schematic representation of filamin. ABD, actin binding domain. (B) Co-immunoprecipitation of filamin and FcγRI. U937 cells were lysed after overnight stimulation with IFN-γ. Subsequently, immunoprecipitation was performed using an anti-FcγRI antibody (m22) or isotype control (iso). Western blotting was used to detect immunoprecipitated FcγRI (top panel) and co-immunoprecipitated filamin (lower panel). Experiments were performed thrice, all yielding similar data. (C) Subcellular localization of FcγRI and filamin in monocytes. Cells were stimulated overnight with 300 U/ml IFN-γ. Cells were adhered to glass slides, fixed in methanol, and FcγRI was stained with CD64 mAb H22 conjugated to FITC. Filamin was stained red. Green, red and merged pictures of two stainings are shown. Co-localization is indicated in yellow.



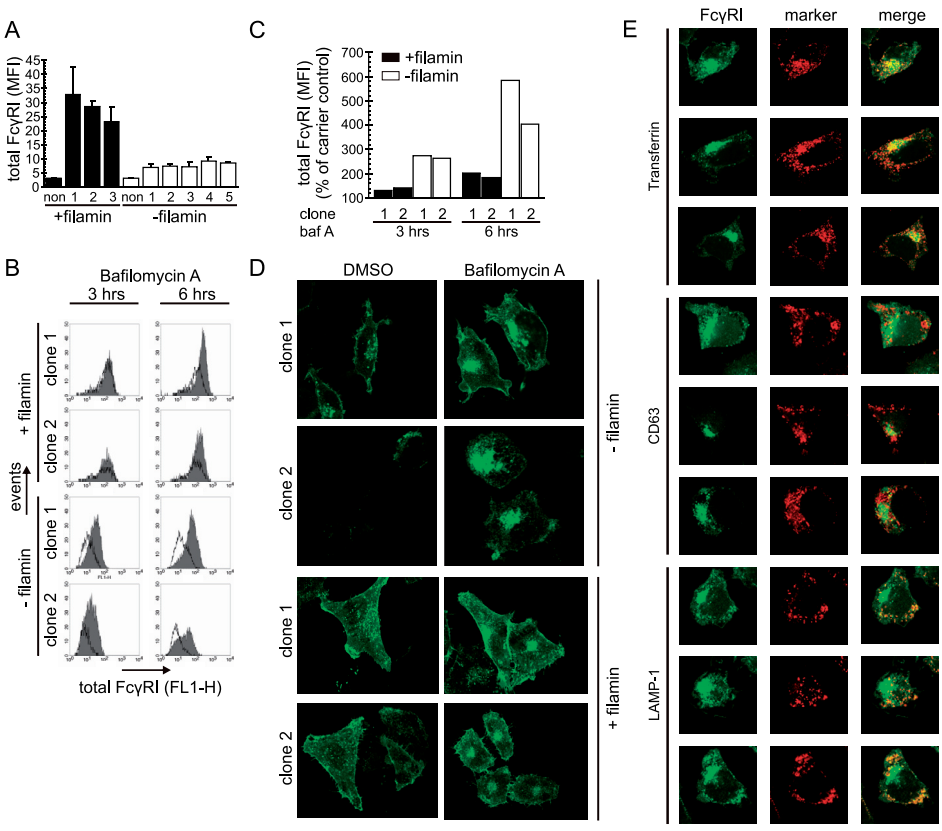
Chapter 3. Figure 2. Subcellular localization of FcγRI and filamin in IFN-γ stimulated monocytes. (A) Monocytes were stimulated overnight with 300 U/ml IFN-γ. Cells were incubated with monomeric mIgG2a or mAb H22 (both FITC-conjugated) for 60 min at 4°C. Cells were then fixed, and stained for filamin. Three representative cells are shown. (B+C) FcγRI ligands were added at 37°C at t=0, and remained present throughout the experiment. Cells were fixed at different time points (B: 5-15 min, C: 60 min, three representative examples shown). Filamin was stained red. Experiments were repeated thrice, all yielding similar data. (D) The amount of FcγRI/filamin colocalization was quantified using Image J (see Material and Methods section), and the percentage of FcγRI/filamin colocalization was expressed as function of time (n=4, Students T test). 100% co-localization was determined by incubating H22-FITC or mIgG2A for 60 min. at 4°C. Errorbars indicate standard deviation.



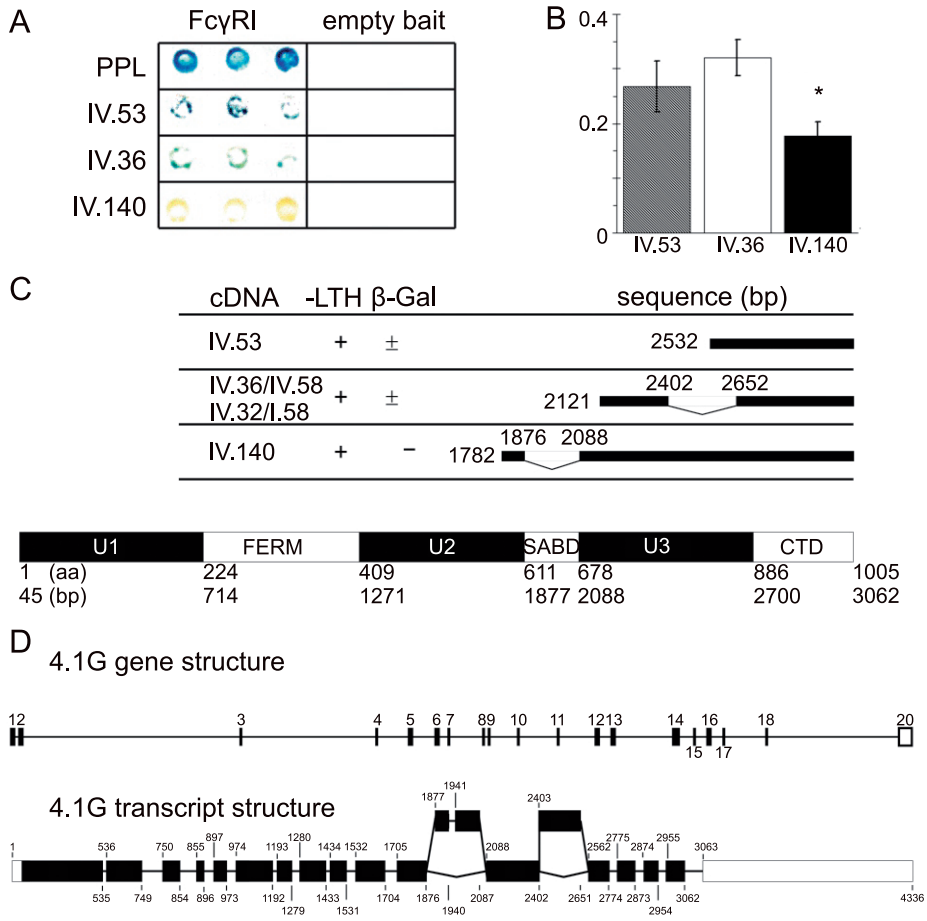
Chapter 3. Figure 3. FcγRI surface expression on cells with or without filamin. (A) Stable zeocin-resistant clones were randomly selected from three independent transfection experiments in cells with or without filamin. FcγRI expression was detected by CD64 mAb 10.1-FITC, and assessed by flow cytometry. Mean fluoresence intensities (MFI) are indicated. (B) RNA was extracted from two A7 clones (left lane negative (-), and right lane positive (+) for FcγRI surface expression) and seven M2 clones, and RT-PCR for FcγRI was performed. (C) Subcellular localization of FcγRI in filamin-deficient and filamin-reconstituted cells. FcγRI was stained by monoclonal 10.1-FITC after paraformaldehyde fixation (4) in filamin expressing clones (left panel) and filamin-deficient M2 cells (right panel). Bar marks 20 μm. (D) Relative FcγRI surface expression after filamin knockdown in U937 cells using siRNA (non targeting control pool versus filamin targeting pool) was assessed by flowcytometry (left panel, * p=0.004, Mann-whitney test, n=3). Errorbars indicate standard deviation. Filamin knockdown was confirmed by westernblot (not shown) and flowcytometry (right panel, one representative experiment). Open histogram depicts background of secondary antibody, grey histogram depicts filamin staining in cells transfected with control pool and black histogram shows cells transfected with filamin targeting pool.



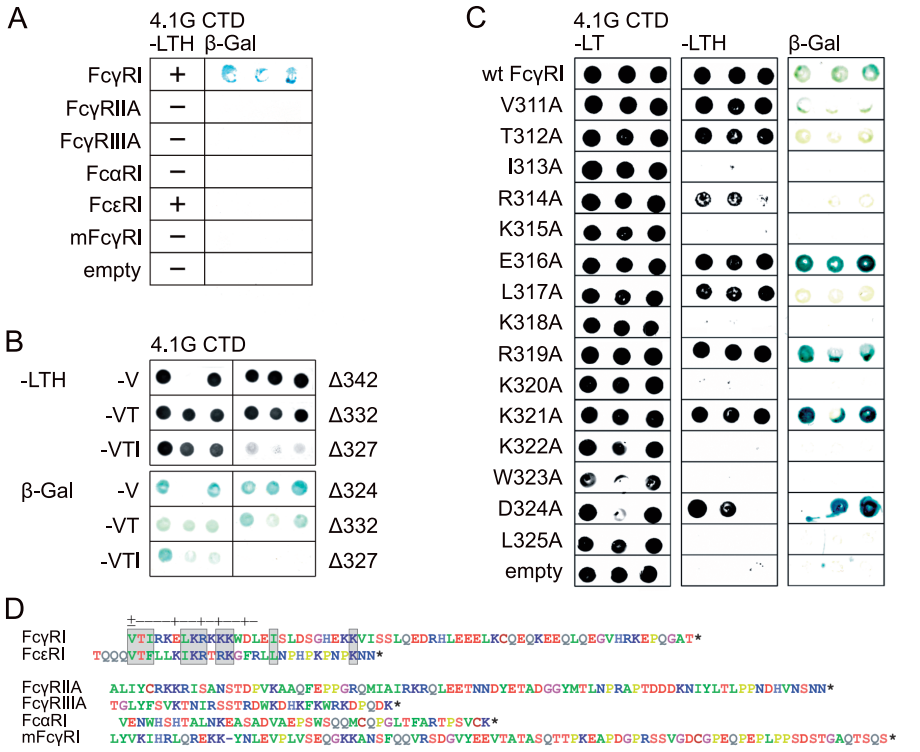
Chapter 3. Figure 5. Intracellular location of FcγRI in cells without filamin. M2 cells were stained for markers involved in plasma membrane afferent pathways, and the endosomal/lysosomal pathway. FcγRI was stained in green (H22-FITC), all other markers in red. Panel (A) was stained for Calreticulin, (B) for p230 (Golgi), (C) for Transferrin uptake, (D) for EEA-1, (E) for CD63, (F) for LAMP-1/CD107a. (G) Percentage co-localization with subcellular markers was calculated using Image J as described in Materials and Methods. Cells from three independent experiments were quantified. The asterisk indicates statistical significance between LAMP-1 and other markers (Student's T test, $p<0,05$).



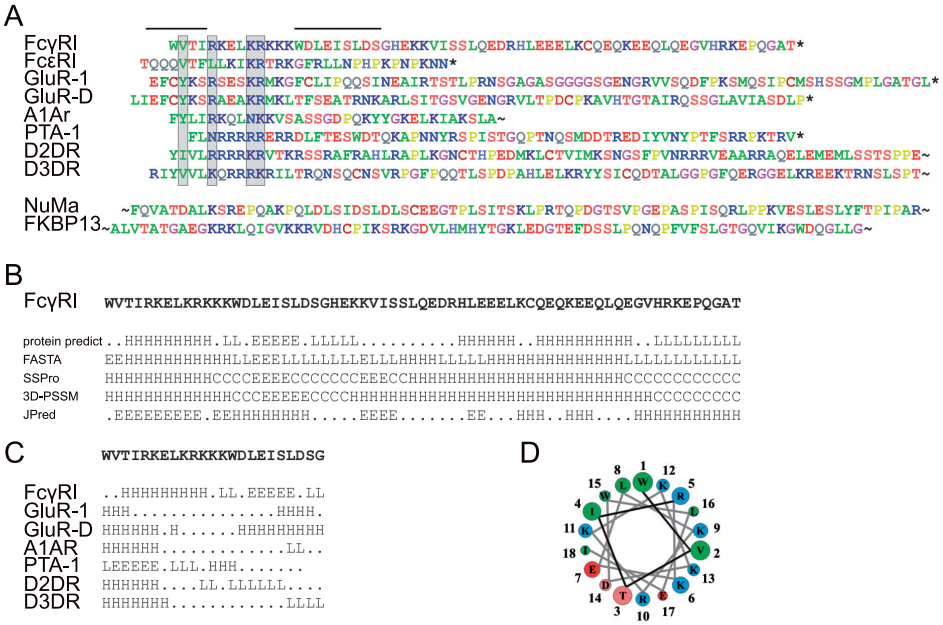
Chapter 3. Figure 7. Filamin prevents lysosomal degradation of FcγRI. (A) Filamin-positive and negative lines that expressed FcγRI transcripts were stained for total FcγRI in the presence of 0.1% saponin. Black bars represent A7 clones, white bars M2 clones, un untransfected cells. Numbers represent independent clones of FcγRI transfectants. Average mean values of four independent experiments are shown, error bars indicate standard error, of the mean. (B) Two FcγRI-expressing clones with or without filamin were incubated with Bafilomycin A1 (100 nM) for three or six hours. Total FcγRI was assessed as in A. Black-lined open histograms represent total FcγRI after incubation in carrier control (DMSO 500-fold diluted); grey-filled histograms represent total FcγRI after Bafilomycin A1 treatment. Representative data of three independent experiments are shown. (C) Relative increase of total FcγRI after incubation of cells with Bafilomycin A1. Two FcγRI-transfected cell lines with (black bars) or without (white bars) filamin were compared. For each cell line, total FcγRI levels after DMSO incubation was set at 100%. (D) FcγRI transfected M2 and A7 cells were assessed by confocal microscopy after 2 hour incubation with Bafilomycin A1 or DMSO. FcγRI was stained by mAb 10.1 FITC-conjugated. (E) Intracellular location of FcγRI in cells without filamin after Bafilomycin A1 treatment. M2 cells were stained for markers involved in endosomal (Transferrin uptake) and lysosomal (CD63, LAMP-1) pathways. FcγRI was stained in green (H22-FITC), all other markers in red.



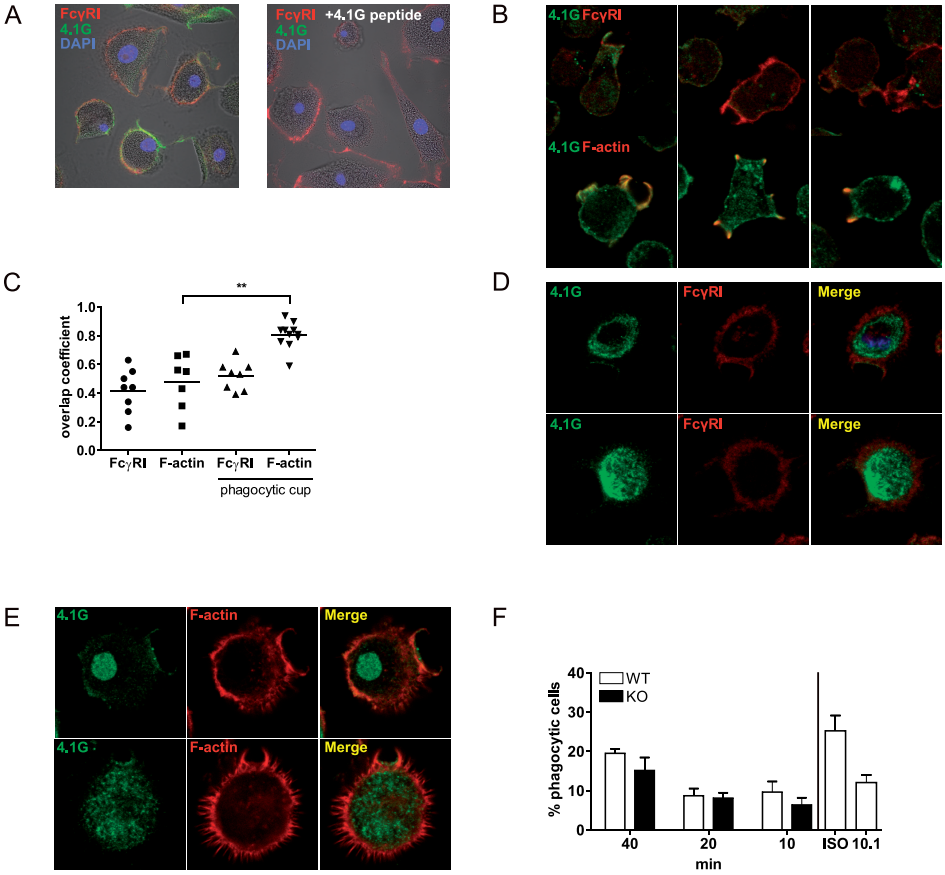
Chapter 4. Figure 1. Interaction of FcγRI with protein 4.1G splice variants. (A) Yeast colonies were assessed for growth on selective media (-LTH) and β-galactosidase activity upon transfection of FcγRI (or empty bait vector) and 4.1G cDNA in triplicate. Periplakin cDNA was used as positive control [3], n=3). (B) Quantification of interaction strength for yeast clones IV.53, IV.36 and IV.140. Nine colonies per transfection were selected and tested for β-galactosidase activity in a liquid β-galactosidase assay using ONPG as substrate (protocol described in Clontech's Yeast Protocols Handbook). Data were averaged, error bars indicate standard deviation (n=2). (C) Domain structure of full length 4.1G, and schematic presentation of six isolated cDNA. Growth on medium lacking LTH, and β-galactosidase activity (β-GAL, in hours before prominent blue staining was observed) are indicated. Spliced regions are marked by a white gap, and a cusped line. White boxes indicate conserved domains, whereas black boxes mark 4.1G unique domains (U1, U2, U3). Domain boundaries are indicated in amino acids, or base pairs (counted according to Genbank Accession Number NM_001431). (D) Graphic view of 4.1G exon structure on genomic and transcript level. The different cDNA obtained by yeast two-hybrid assays were transposed on the exon structure of the 4.1G mRNA. Numbers above and below the sequence indicate the starting, and ending bp of associated exons, respectively. 4.1G spliced regions found in yeast two-hybrid sceens are indicated. Black boxes represent coding sequences, white boxes untranslated regions.



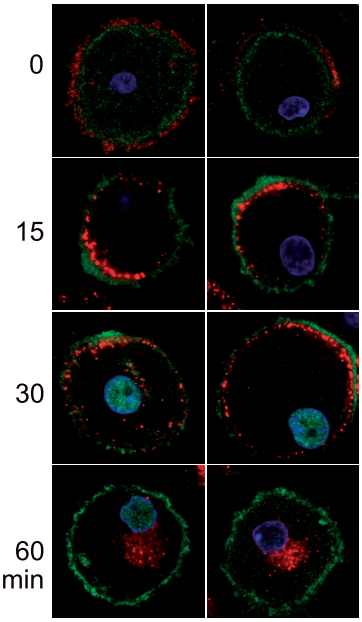
Chapter 4. Figure 4. Identification of protein 4.1G interaction sites in FcR cytoplasmic domains. Protein interactions were assessed after transfer of three yeast colonies to media without leucine, tryptophane (-LT) and histidine (-LTH), and a β-galactosidase (β-GAL) activity assay. (A) Interactions between a panel of activating FcR α-chain cytosolic domains, and 4.1GΔ14. mFcγRI denotes murine FcγRI. (B) Interactions between truncated FcγRI-CY mutants, and 4.1G CTDΔ14. Numbers refer to the final C-terminal residue present in the FcγRI-truncated molecules. -V, -VT, -VTI constructs lack the first one, two, or three N-terminal residues of FcγRI-CY. (C) Interaction of alanine-substituted FcγRI mutants with 4.1G CTDΔ14. The single substitutions of FcγRI's membrane-proximal region (311-325) were denoted as V311A (Valine at position 311 for Alanine, T312A Threonine for Alanine at 312, etc). (D) Cytosolic domains of FcR. Similar regions within FcγRI and FcεRI are boxed in grey.



Chapter 4. Figure 5. Sequence analysis of 4.1G interacting proteins. (A) Primary sequence alignment of 4.1G CTD binding proteins. Cytosolic domains of 4.1G-CTD-interacting proteins were predicted using PHDhtm prediction software [16], and similar regions were aligned. Transmembrane receptors are shown in the upper alignment (interacting loops are indicated for seven membrane-spanning receptors (dopamine receptors, A1AR)), and cytosolic proteins (not full sequences) in the lower alignment. Grey boxes mark similar residues within transmembrane receptors, allowing one mismatch. Regions of hydrophobic and (potentially) negatively charged residues are indicated by black lines. Amino acids in green: hydrophobic/aromatic, blue: basic/hydrophilic, red: acidic, yellow: aliphatic. (B) Secondary structures of FcγRI-CY were predicted after web submission of FcγRI (TM region and CY) to PredictProtein, FASTA, SSPro, 3D-PSSM and JPred. H indicates a predicted alpha-helix structure, E:extended β-sheet, and C/L: coil/loop. Dots mark unreliable predictions. (C) Secondary structures of membrane-proximal domains of 4.1G-interacting protein. Regions were predicted with PredictProtein, and had cut-off accuracies of 82% (PHD prediction, Sub_sec). (D) Schematic presentation of an alpha-helical wheel for FcγRI residues 310-327.



Chapter 5. Figure 2. 4.1G localization and function during FcγRI mediated phagocytosis. A) Monocyte derived macrophages stained for FcγRI (red channel) and 4.1G (green channel) in the absence (left panel) or presence of C terminal 4.1G peptide (right panel) as observed by confocal microscopy. A merged image is presented showing FcγRI and 4.1G staining and nuclei by DAPI staining. One-of-three independent stainings is shown. B) 4.1G (green channel) localization compared to FcγRI (red channel, top panels) and f-actin (red channel, lower panels) in THP-1 phagocytosing IgG3 opsonized erythrocytes in the presence of anti FcγRII (clone IV.3) F(ab) fragments. Erythrocytes were lysed during the staining procedure. C) Quantification of co-localization between 4.1G and FcγRI and 4.1G and F-actin (Manders overlap coefficient) calculated for entire THP-1 cells or in phagocytic cups only. **: P<0.01, student t-test. D and E) IFNγ treated MDM were allowed to phagocytose IgG3 opsonized erythrocytes in the presence of IV.3 F(ab) fragments and anti FcγRIII (clone 3G8) F(ab)2 fragments. Cells were fixed and stained for 4.1G and FcγRI (D) and 4.1G and F-actin (E). Experiment was repeated twice, one representative experiment is shown. F) Phagocytosis of *Staph. epidermis* by wildtype (WT) or 4.1G deficient (KO) BMM. BMM were allowed to phagocytose bacteria for the indicated timepoints. In the same experiment, WT BMM were allowed to phagocytose bacteria for 40 min in the presence of FcγRI blocking mAb 10.1 or isotype control. N=1, mean and standard deviation of triplicates are shown.



Chapter 5. Figure 3. 4.1G localization upon FcγRI endocytosis in monocyte derived macrophages. FcγRI was crosslinked with mAb 10.1 and goat anti mouse IgG and allowed to internalize for the indicated time periods. FcγRI expression is shown in red, 4.1G in green and DAPI in blue. Representative results of two independent experiments are shown.