

Development of an Antigen-independent Affinity Assay to Study the Binding of IgG to Fc Gamma Receptors

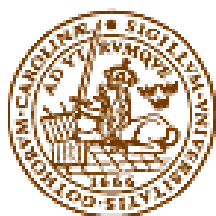
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Abstract

Fc gamma receptors (FcγRs) are membrane-bound receptors which bind the Fc fragment of antigen-bound IgGs. The binding generates cell signaling and subsequently an immunological response. In this way FcγRs are important links between the humoral and cellular parts of the immune system. Three families of FcγRs have been identified in human, FcγRI, FcγRII and FcγRIII. FcγRI has high affinity for IgG and is the only receptor capable of binding IgG in monomeric form. The other receptors have low to intermediate affinity for IgG and can only bind immune complexes. The receptors vary in IgG subclass specificity and also differ in the cellular response they give rise to upon IgG binding. The total immune response is a balance between activating and inhibiting signals.

The use of therapeutic antibodies for treatment of human diseases has grown immensely since the introduction in the 80s. Second and third generation antibodies are now entering the market. Much research is conducted on optimizing IgG regarding FcγR binding since this interaction to large extent dictates the cellular immunological response of IgG. An appropriate and reliable screening method to measure and compare the affinities of FcγRs for IgG *in vitro* would be of great assistance in the evaluation of therapeutic antibodies. In this work two such assays were developed, one based on ELISA and one on flow cytometry, both suitable for screening of approximately 24 IgGs. A number of human and murine FcγRs were expressed in soluble and cell-bound form and the apparent affinities of these for different IgGs were assayed using antigen-independent immune complexes. Receptor-specific IgG binding could be detected with both assay set-ups and discrimination between IgGs with different apparent affinity could be made.

List of abbreviations

ADCC	antibody-dependent cellular cytotoxicity
CDR	Complementarity-determining region
CH	constant heavy
CHO cells	Chinese hamster ovary cells
CL	constant light
EC50	effective concentration 50%
ECD	extra cellular domain
Fab	fragment antigen-binding
Fc	fragment crystallizable
FcγR	Fc gamma receptor
hFcγR	human Fc gamma receptor
mFcγR	murine Fc gamma receptor
FMAT	fluorometric microvolume assay technology
GPI	glycosylphosphatidylinositol
HEK cells	human embryonic kidney cells
IC	immune complex
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
NA	neutrophil antigen
NK cell	natural killer cell
SEC	size exclusion chromatography
SPR	surface plasmon resonance
TM	transmembrane
VH	variable heavy
VL	variable light

Populärvetenskaplig sammanfattning

Uppsättning av en antigenoberoende metod för att mäta bindningen mellan IgG och Fc gamma receptorer

Fc gamma receptorer (FcγR) är cellbundna receptorer som binder Fc-delen på IgG efter att antikroppen bildat immunkomplex med antigen. Interaktionen mellan FcγR och Fc leder till ett cellulärt immunsvaret. På så vis är FcγR en viktig länk mellan det antikroppsmedierade och det cellulära immunsystemet. FcγR förekommer på alla immunkompetenta celler. Hos människa finns det tre FcγR familjer med totalt sex subfamiljer, fem aktiverande och en inhiberande. Eftersom de flesta celler uttrycker flera sorters receptorer samtidigt är det totala immunologiska svaret som följer när IgG binder till FcγR summan av inhiberande och aktiverande signaler. Affiniteten för IgG såväl som preferensen för olika IgG subklasser varierar mellan de olika receptorerna.

Forskningen kring terapeutiska antikroppar och marknaden för dessa har ökat explosionsartat sedan 80-talet. Forskningsfokus har större delen av tiden varit på antikroppens bindning till antigen. På senare år har allt mer forskning bedrivits i syfte att optimera Fc-delen med avseende på FcγR-bindande egenskaper. Genom att modifiera inbindningen av IgG till FcγR och rikta den till specifika FcγR kan den immunologiska effekten av terapeutiska antikroppar påverkas. Vi har i detta arbete satt upp två screening metoder för rangordning av IgG utifrån FcγR-affinitet oberoende av antigen specificitet. FcγR uttrycktes såväl i löslig som i cellbunden form och inbindningen av immunkomplex (IgG i komplexbunden form) mättes med ELISA respektive flödescytometri.

Det var möjligt att mäta Fc-specifik inbindning av immunkomplex till FcγR med båda metoderna. Rangordningen i affinitet för olika IgG subklasser stämde överens mellan uppsättningarna såväl som med litteraturen för flertalet receptorer. Vid analys av 12 olika n-CoDeR®* antikroppar med okänd affinitet för FcγR kunde IgG med olika stark inbindning identifieras. De lösliga receptorerna var aggregerade i olika utsträckning och uttrycket av de cellbundna receptorerna varierade med receptortyp vilket förhindrade jämförelse av affinitet mellan receptorerna. Däremot kunde metoderna användas till att rangordna IgG med avseende på FcγR-affinitet för en receptor i taget. Speciellt ELISA uppsättningen kan med fördel användas för att screena ett större antal IgG och rangordna dem baserat på affinitet för en viss FcγR.

* BioInvents antikroppsbibliotek.

1 Introduction

1.1 Overview of human Fc gamma receptors in the immune system

Fc gamma receptors (FcγR) are transmembrane (TM) receptors interacting with the Fc-fragment of IgG. When IgG is part of an immune complex (IC) this interaction generates dimerization of the FcγR which in turn leads to a cellular response, most commonly antibody-dependent cellular cytotoxicity (ADCC), endocytosis, phagocytosis, antigen presentation, release of superoxides or degranulation. As IgG is the main antibody of the human immune system, FcγR are key links between the humoral and cellular parts of the immune system.

Human FcγRs are divided into three families based on sequence similarity and IgG subclass affinity; FcγRI, FcγRII and FcγRIII. These are usually further divided into six isoforms; FcγRI, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa and FcγRIIIb. Typically FcγRs consist of two Ig like extracellular domains (ECD) called D1 and D2, one TM domain and one cytosolic domain (Figure 1). FcγRI has one additional ECD and FcγRIIIb lacks the cytosolic domain. The ECDs of the FcγRs are highly homologous with > 90% sequence identity, while the cytosolic domains are highly divergent.

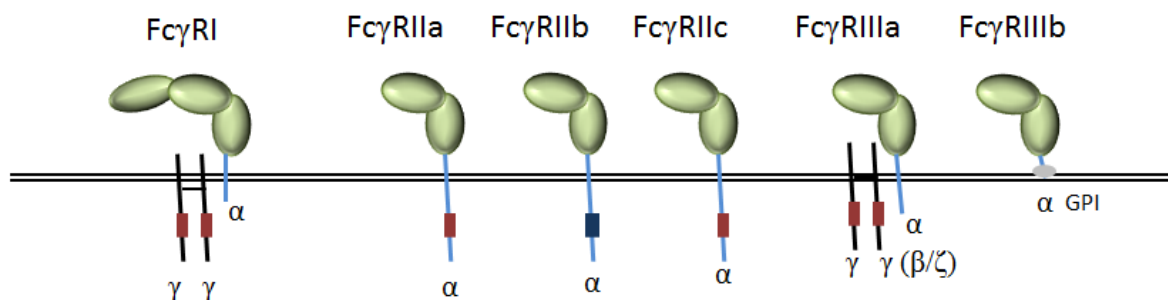







Figure 1. Schematic overview of the structure of the human FcγR and associated proteins.  ECD,  α-chain,  activating signaling sequence ITAM,  inhibiting signaling sequence ITIM,  adaptor molecules being either γ, β or ζ chains. See text for further details.

FcγRII has a signaling sequence on the cytosolic domain called immunoreceptor tyrosine-based activation motif (ITAM) for FcγRIIa and FcγRIIc and immunoreceptor tyrosine-based inhibitory motif (ITIM) for FcγRIIb (Figure 1). Since these motifs are present on the same peptide chain as the IgG binding site, FcγRII is capable of autonomous signaling. In contrast, FcγRI and FcγRIII require additional TM subunits in order to generate cell signaling. Homo dimers of γ-chains serve as signal transducing molecule in most cells except for NK cells where γ, ζ-heterodimers or ζ-homodimers sometimes play this role [1]. For FcγRIII on mast cells, β-chains can serve as assisting protein [2], [3]. These extra subunits all have an ITAM on their cytosolic domain. The γ-chain is not only important for signaling but also for receptor assembly and membrane localization [1]. Instead of a transmembrane sequence, FcγRIIIb is attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor. The signal from

FcγRIIIb is proposed to be mediated by the complement receptor 3 (CR3; CD11b/CD18) [3], [4] (not indicated in Figure 1).

FcγRs are expressed on all immune competent cells and most cells co-express activating and inhibiting receptors. It is generally accepted that NK cells, platelets and B-cells are exceptions to this as the two former only express activating receptors while the latter only express the inhibiting receptor, FcγRIIb [2] (Table 1). Some receptors could be considered as constitutively expressed while others, especially FcγRI and FcγRIII, can be up and down regulated on certain cells [5]. FcγRI is for example constitutively expressed on monocytes, macrophages and dendritic cells and can be induced on neutrophils and eosinophils by interferon-γ [2], [6]. In addition to hematopoietic cells, FcγRs are also expressed on microglial cells, mesangial cells, osteoclasts and endothelial cells. Their function on these cells has yet to be elucidated [2]. It should be noted that new findings constantly change the common belief of which receptors are expressed by which cells and what cellular responses they give rise to [1], [7].

Table1 Distribution and the main cellular response of human FcγRs [1], [8], [9].

Receptor	Distribution	Main immunologic response	Activity
FcγRI (CD64)	Macrophages, neutrophils, monocytes, dendritic cells	Antigen presentation, phagocytosis, endocytosis, ADCC	Activating
FcγRIIa (CD32a)	Macrophages, monocytes, neutrophils, eosinophils, platelets	Phagocytosis, ADCC, endocytosis, antigen presentation, cytokine release	Activating
FcγRIIb (CD32b)	B-cells, macrophages, monocytes, mastcells, neutrophils, dendritic cells, eosinophils	Down regulation of activating signals, down regulation of B-cell receptor, endocytosis*	Inhibiting
FcγRIIc (CD32c)	NK cells	Unknown	Activating
FcγRIIIa (CD16a)	NK cells, macrophages, monocytes, mastcells, T-cells	ADCC, endocytosis, cytokine release	Activating
FcγRIIIb(CD16b)	Macrophages, neutrophils, eosinophils, T-cells	Degranulation, superoxide production	Activating

* The FcγRIIb2 expressed on macrophages, in contrast to FcγRIIb1 expressed on B-cells, is capable of inducing endocytosis [1].

Many of the FcγRs are, in addition to the cell-bound form, found in a soluble form consisting of only the ECDs. Soluble receptors are formed either by alternative splicing or by proteolysis and have been shown to play a down regulatory role on pro-inflammatory responses [8]. Soluble FcγRIIIb is to a large extent released from neutrophils and soluble FcγRIIIa is derived from NK cells. Besides competing with the membrane-bound receptors for IgG, soluble FcγRs can also lead to production of inflammatory mediators by binding to complement receptors [9]. Soluble FcγRs are found to a lower extent in the plasma of patients with Systemic lupus

erythematosus, Rheumatoid arthritis and some other autoimmune diseases and could therefore be used as a diagnostic marker for such diseases. Injection of soluble FcγR could be used in therapy for these patients [10].

1.2 Human FcγR families

Even though FcγRs are structurally and sequence wise very similar, they show distinct differences in function. These are caused by the different cytosolic domains and differences in affinity for IgG [8]. The six isoforms of human FcγR exist in a number of allelic variants (Table 2), where some are associated with various inflammatory and autoimmune diseases and also affect the efficiency of immunotherapy [3], [8]. Alloforms with lower IgG affinity are associated with autoimmune disorders caused by reduced clearance of IC generating disposition of these in tissue. The ones with higher affinity on the other hand are associated with chronic inflammatory states [11].

Table 2 Human FcγR isoforms and functionally relevant allelic variants.

Human receptor	Alloforms*
FcγRI	-
FcγRIIa	H131/R131
FcγRIIb	I232/T232 b1/b2
FcγRIIc	Stop/Q13
FcγRIIIa	V158/F158
FcγRIIIb	NA1/NA2(SH)

*Numbering without signal peptide.

FcγRI

The additional ECD of FcγRI promotes it with higher affinity for IgG than the other receptors [1]. It is the only FcγR capable of binding monomeric IgG. This receptor is therefore almost always saturated with IgG *in vivo*. In order to come in close enough proximity for receptor crosslinking and subsequent cell signaling FcγRI has to bind IC.

FcγRII

FcγRII is the most widely distributed FcγR and is found in several isoforms, a, b1, b2 and c. The ECDs of FcγRIIa, b and c have 94% amino acid sequence identity but the cytosolic domains are very different. FcγRIIa and c both have an ITAM while FcγRIIb has an ITIM [6].

FcγRIIa exists in two alloforms, the high responder H131 and the low responder R131. They differ, as the names imply, in the amino acid 131 where FcγRIIa^{131H} generally has higher affinity for IgG than FcγRIIa^{131R} [12]. The FcγRIIa^{131R} alloform is associated with a higher risk

of developing Systemic lupus erythematosus and other auto immune diseases and increased susceptibility to bacterial infections [13]. FcγRIIIa is the only FcγR found on platelets where it triggers platelet aggregation [8].

FcγRIIb, being the only inhibitory receptor, plays an important role for the peripheral tolerance, regulation of pro-inflammatory and allergic reactions and modulation of B-cell development [2], [14]. Impaired FcγRIIb can lead to autoimmune diseases and reduced plasma cell apoptosis [3], [14]. Two functional polymorphisms have been identified for FcγRIIb. One is in amino acid 232 where the FcγRIIb^{I232} is more common and shows higher affinity for IgG than FcγRIIb^{T232}. The other polymorphism, b1 and b2, is a variation in the cytosolic region where the b1 form has 19 additional residues in front of the signaling motif. FcγRIIb1 is mainly expressed on B-cells while FcγRIIb2 is expressed on practically all other cells (except NK cells), foremost macrophages. FcγRIIb2 in both mouse and human is efficient in inducing endocytosis of small immune complexes while FcγRIIb1 is not [1], [2]. It has been shown that murine FcγRIIb2 can induce phagocytosis [15] and it is under debate if the human counterpart is also capable of this [14]. Crosslinking of FcγRIIb with activating FcγRs and B-cell receptor leads to down regulation of the signal from those receptors. Homo-oligomerization of FcγRIIb leads to increased cell apoptosis which is of importance for the plasma cell homeostasis and humoral tolerance [2].

FcγRIIc is proposed to be present in only 45% of the population and is expressed solely on NK cells [16]. One reason for its limited occurrence could be the allelic variation in amino acid position 13, being either a glutamine or a stop codon.

FcγRIII

FcγRIIIa is found in two functionally relevant alloforms, one high responder, FcγRIIIa^{158V}, and one low responder FcγRIIIa^{158F}. This polymorphism has been associated with a number of autoimmune diseases and is found to have an impact on the efficacy of therapeutic antibodies for the treatment of tumors. FcγRIIIa has intermediate affinity for IgG, lower than FcγRI but slightly higher than FcγRII and FcγRIIb [6].

All FcγRs are to some extent glycosylated. FcγRIIIa is the most glycosylated FcγR and it is together with FcγRIIIb the only one that is glycosylated in the IgG binding site. The degree of receptor glycosylation plays a role for the affinity of FcγRIIIa and FcγRIIIb but no such correlation has been seen for the other receptors [17], [18]. Non-glycosylated FcγRIIIa has a higher affinity for IgG than the glycosylated counterpart [19].

FcγRIIIb is found in two alloforms, NA1 and NA2. They differ in four amino acids in the so called neutrophil antigen sequence (NA) in the first ECD [4]. This leads to different glycosylation patterns and thereby also differences in IgG binding and cell response [8]. The NA1/NA2 polymorphism is proposed to influence the susceptibility to systemic autoimmune diseases [9]. FcγRIIIb NA2 has an additional polymorphism giving rise to the so called SH allo-antigen, which causes several diseases such as neonatal neutropenia [20].

This is merely a brief summary of the present knowledge of FcγR. As research constantly is being conducted, the knowledge in the fields of FcγRs and the immune system is increasing from day to day and divergent and therefore opposing data can be found in literature. The expression patterns and functions of FcγRs noted above should be taken with care. What is unknown today may be known tomorrow and what is thought to be true today may be rejected tomorrow.

1.3 IgG and therapeutic antibodies

IgG is the most frequently occurring antibody class in the human body. IgG is composed of two heavy chains and two light chains (Figure 2). The heavy chains are linked together and also to one light chain each by disulfide bridges. The light chains consist of one constant domain (CL) and one variable domain (VL) and are of κ or λ type. The heavy chains consist of three constant domains (CH) and one variable domain (VH) and are of type γ_1 , γ_2 , γ_3 or γ_4 . The differences between the heavy chain types are small and confined to the hinge region and the disulfide bridges linking the heavy chains [21]. IgG binds to antigen at the complementarity-determining regions (CDRs) on VH and VL (Figure 2). The variable region (VH + VL) is antigen specific and can vary largely between IgGs.

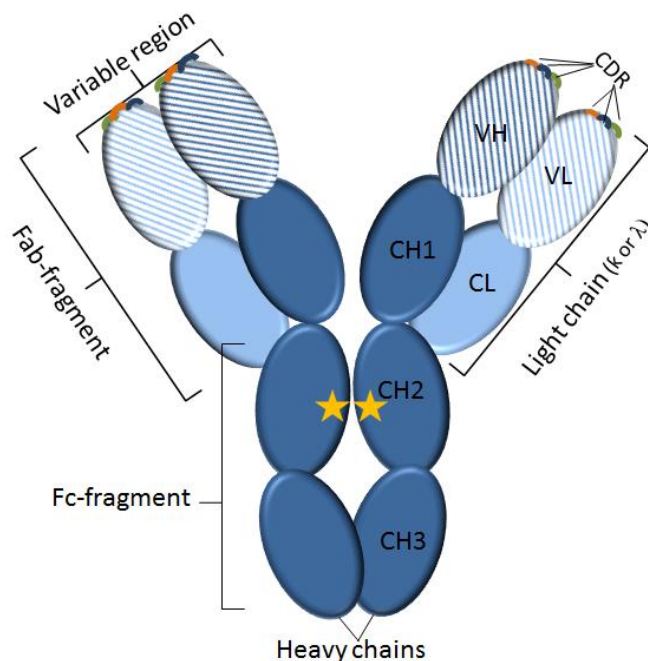


Figure 2. Schematic figure of IgG; CH- constant heavy, VH- variable heavy, CL- constant light, VL- variable light, CDR- complementarity-determining region. ★ Denotes the site of glycosylation at Asn 297.

IgGs could be glycosylated both on the Fab- and Fc-fragments. The only glycosylation of IgG known to affect the FcγR binding is that on asparagine 297 on the Fc-fragment (Figure 2) [22]. The most common glycosylation structure on the Fc-fragment of human IgGs is biantennary (Figure 3) [23]. Two important factors for the therapeutic effect of IgGs are the sialic acid residues in the terminus of the sugar moieties and fucose in the core [19], [23], [24]. In

general, non-glycosylated Fc has lower affinity for FcγR than glycosylated [12] and a high degree of sialylation or fucosylation decreases the affinity for FcγRs [24]. The glycan chain is proposed to not make direct contact with the receptor. The effect on affinity caused by the glycosylation of IgGs is due to changes in the structure and conformation of the Fc region upon glycosylation [14]. The degree of glycosylation depends on the organism, cell type, cell status etc. Differences in affinity caused by glycosylation have been shown to have clinical effects; increased glycosylation of IgG for instance is associated with autoimmune diseases such as Rheumatoid arthritis [1], [19], [25]. Regarding therapeutic antibodies it is therefore important to be aware of that production conditions such as cell line, temperature and medium may affect the glycosylation pattern, and that the pattern can vary between batches.

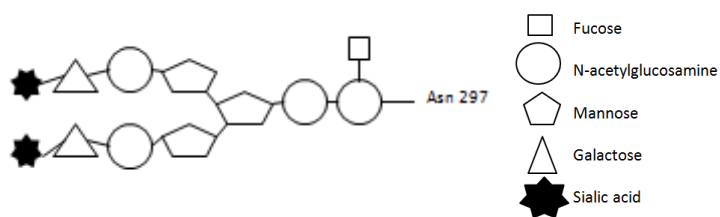


Figure 3. Example of biantennary glycosylation of IgG on Asn 297.

The main function of therapeutic antibodies is exerted by the ability to target and bind a specific antigen and thereby neutralizing it. In the field of therapeutic antibody research most emphasis has been set on the variable regions and the antigen binding properties of IgG. Immunological effector functions of therapeutic antibodies such as ADCC are determined by the Fc-fragment and mainly by IgG binding to FcγRs. Thereby IgGs specific for the same antigen can give rise to different immunological responses. The last couple of years more and more research has focused on the FcγR binding properties of IgG with the aim to obtain antibodies with enhanced or decreased cellular response [1], [14], [26], [27]. Two strategies used to optimize the effector function of therapeutic IgGs are to engineer the Fc-fragment and to modify the glycosylation pattern [2], [28], [29].

Most therapeutic antibodies are of subclass 1 as FcγRs have highest affinity for IgG1 resulting in an efficient cellular response. In addition IgG1 has a longer half-life in circulation than the other IgG subclasses. Therapeutic antibodies not intended to trigger FcγR mediated immune responses are often of subclass 2 or 4 as the affinity of these for FcγRs is low [27]. The first monoclonal antibodies approved by FDA (Food and Drug Administration) for cancer therapy were rituximab (Rituxan®, Genentech) in 1997 and trastuzumab (Herceptin®, Genentech) in 1998. Both are known to mediate part of the immunological efficiency by binding to FcγRs [21]. Today more than 20 monoclonal antibodies are approved for cancer therapy, over two dozen are in late clinical trials and hundreds are in early clinical trials [26].

1.4 Structure of FcγR and interaction with IgG

The IgG Fc-fragment binds to the FcγR in a non-symmetrical manner, prohibiting binding of more than one receptor per IgG [30], [31]. Both the receptor and the Fc-fragment undergo a slight conformational change upon binding. Without IgG bound, D1 and D2 of the receptor are bent at 50-55° to each other. When IgG binds the structure opens up, increasing this angle. In addition, the symmetry of the Fc-fragment on IgG is disrupted [32] (Figure 4). The exact binding site for different IgGs to the FcγRs varies between the receptor isoforms and allotypes, which is one reason for differences in affinity and specificity. There is a common set of residues which take part in all IgG/FcγR binding. These interaction sites on IgG have been mapped to the CH2 domain, close to the hinge region between the Fc and Fab-fragments [25] and to some parts of the CH3 domain. On the receptors, the sites of interaction are localized on the D1 domain [12] while D2 is proposed to play a more stabilizing role [1].

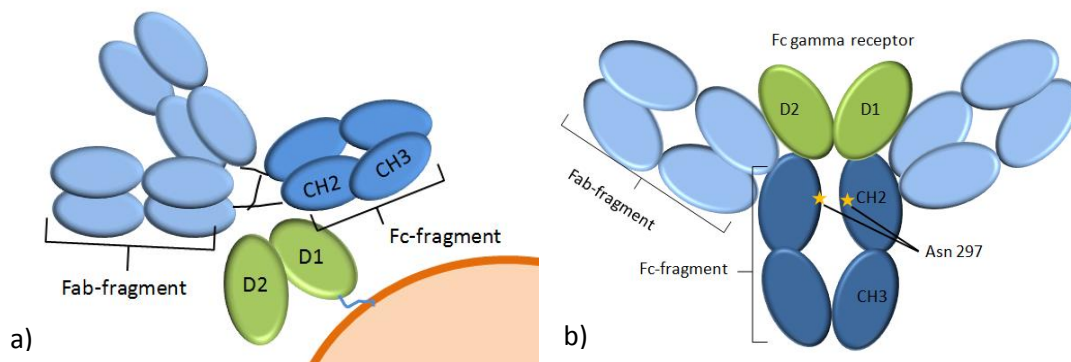


Figure 4. a) Schematic figure of IgG interacting with cell-bound FcγRII or FcγRIII. Residues on the CH2 domain of IgG close to the hinge region of the IgG and some parts of the CH3 domain interact with D1 of the receptor. b) figure a turned 90°, showing only IgG and FcγR. ★ Asn297, the site of Fc glycosylation on IgG known to affect IgG/FcγR interaction. Drawn after inspiration from [33].

1.5 Affinities of human and murine FcγRs

Many *in vivo* experiments are conducted in mice. The murine immune system has been extensively studied and has been found to be very similar to the human counterpart. Mice, like humans, have a number of FcγR families: mFcγRI, mFcγRII, mFcγRIII and mFcγRIV. These all have orthologues in human (Table 3). The sequences and structures of the human and murine FcγRs are very similar. Mice also have one high-affinity and one inhibiting receptor. The high affinity receptor, mFcγRI, is homologous to human FcγRI. mFcγRII is inhibitory and corresponds to hFcγRIIb. mFcγRIII is orthologous to hFcγRIIIa and is activating with low affinity. mFcγRIV is proposed to be the murine counterpart to hFcγRIII [5]. The cellular response is, however, not entirely the same for the human and murine orthologues.

All human FcγRs have highest affinity for hIgG1 and/or hIgG3 (Table 3). These two are the most inflammatory human IgG subclasses. hFcγRI has the highest absolute affinity for hIgG1,

hIgG3 and hIgG4 while hFcγRIIa has the highest absolute affinity for hIgG2. In fact hFcγRIIa^{131H} is the only receptor which binds hIgG2 with significant affinity. hFcγRIIb/c are the receptors with lowest affinity for IgG [34].

The murine FcγRs bind mIgG2a and mIgG2b the strongest and these IgGs are the main inflammatory ones in mice. mIgG2a corresponds to hIgG1. All mFcγRs can, in addition to mIgG1, mIgG2a and mIgG2b, bind hIgG1 and hIgG3. No murine receptor has been shown to bind mIgG3. The human FcγRs are capable of binding mIgG2a. In addition, hFcγRII and hFcγRIII bind mIgG2b [6]. The affinity of hFcγRs for hIgG is generally lower than the affinity of mFcγRs for mIgG [2].

Table 3 Approximate hIgG1 affinity and hIgG preference of hFcγRs [34]. Murine orthologues are also listed. Crossed over IgG indicate that the receptors are not found to bind that subclass. In absolute numbers FcγRI has the highest affinity for all IgGs except IgG2 for which FcγRIIa^{131H} has higher affinity. See text for further details.

Human receptor	hIgG1 affinity (Kd)	hIgG preference	Murine orthologue
hFcγRI	10 ⁻⁸⁻⁹ M	IgG1=IgG3>Ig4 IgG2	mFcγRI
hFcγRIIa	10 ⁻⁶ M	IgG1>IgG3>IgG2>IgG4* IgG1>IgG3>IgG4>IgG2**	mFcγRIII
hFcγRIIb	10 ⁻⁶ M	IgG1=IgG3>IgG4>>IgG2	mFcγRII
hFcγRIIc	10 ⁻⁶ M	Not determined	
hFcγRIIIa	10 ⁻⁶⁻⁷ M	IgG3>IgG1>>IgG4>IgG2	mFcγRIV
hFcγRIIIb	10 ⁻⁶ M	IgG3>IgG1 IgG2, IgG4	

*Preference of FcγRIIa^{131H}, ** preference of FcγRIIa^{131R}

1.6 Assaying FcγR/IgG affinity

Affinity measurements are to a large extent affected by the experimental set-up. For example one study reports higher affinity of soluble FcγRIIa for IgG1 than soluble FcγRIIb and FcγRIIIa but when measuring on cell-bound receptors FcγRIIIa was found to show higher affinity for IgG1 than both FcγRIIa and FcγRIIb [1]. Due to variations in experimental methods and conditions, affinity data found in literature differs significantly [5], [18], [21], [34].

As FcγRII and FcγRIII bind IgG weakly, it is not straight forward to measure the affinity. Binding of IC to FcγR is stronger than the monomeric interaction due to avidity and IC are therefore often used when studying low-affinity FcγRs. Binding of IgG to FcγR has been assayed using many different methods from “simple” heterogeneous methods like centrifugation, dialysis and affinity chromatography to more complicated homogenous “mix and read” methods such as isothermal titration calorimetry and AlphaScreen™. For a long

time, radioactive assay systems were used due to the high sensitivity. Nowadays fluorescent methods have become more frequent to avoid the hazard of handling radioactive compounds. Examples of such methods for affinity measurements are flow cytometry, fluorometric microvolume assay technology (FMAT), fluorescence polarization and fluorescence linked immunosorbent assay. The sensitivity of these varies with the fluorophore used. Additional non-radioactive techniques span from optical assays like surface plasmon resonance (SPR) and total internal reflection fluorescence to enzymatic assays like enzyme linked immunosorbent assay (ELISA) and those based on energy transfer like fluorescence resonance energy transfer [35]. As the number of available techniques for affinity determination is numerous only those used in this study will be discussed below.

ELISA

ELISA is a classic routine method to detect and measure antibody binding. This is the most common method used in antibody-based pharmaceutical research and is also a frequent method in diagnostics.

ELISA has many benefits: Simple, fast, requires low amount of sample, relatively cheap, fairly high throughput and can be conducted without having to label the analyte. However, the method also has its drawbacks. There is a great risk of introducing artifacts in the structure when immobilizing a protein directly on a surface, which consequently may affect affinity. In ELISA, an apparent affinity constant is determined rather than a true one as the antibody and analyte are in different phases and hence equilibrium on the surface is not obtained [36]. Kinetic constants can be estimated using ELISA but this is rather complicated and not very reliable [37]. A way to circumvent the problems associated with direct immobilization of the analyte is to apply sandwich ELISA. In this method the plate is coated with a capture antibody to which the analyte binds. Sandwich ELISA is associated with the same benefits as direct ELISA and also suffers from the drawback that true affinity constants are not obtained. The sensitivity of ELISA depends on the method of detection and signal amplification and on the general assay setup.

Flow cytometry

Flow cytometry utilizes the fluorescing characteristics of individual cells and labeling of cells with fluorophores. Cells pass by a laser beam one by one (Figure 5). The cell morphology and size can be analyzed by detecting the forward and sideward scattering of light generated. Incubating the cells with differently labeled ligands or antibodies and utilizing lasers of diverse wavelengths enables simultaneous detection of several ligands or antibodies [35]. Flow cytometry is one of few methods available to measure affinity constants for membrane proteins in their “true” environment integrated in the cell membrane, but kinetic measurements are laborious [37]. The sensitivity depends on the general set-up of the experiment as well as the fluorophore used and detector sensitivity. The throughput of flow cytometry can be fairly high, but not as high as with ELISA and the amount of cells required is relatively large. Hence, the technique is not optimal for large screening experiments.

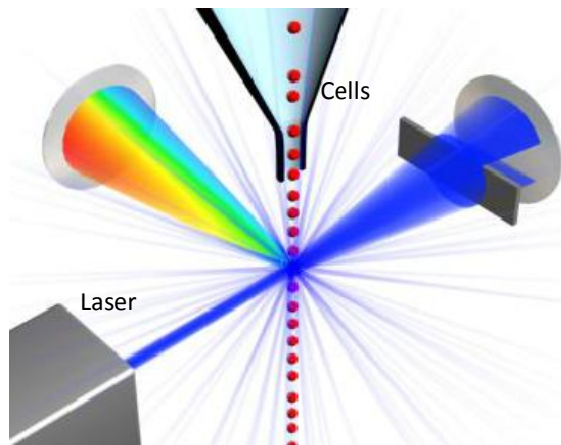


Figure 5. In flow cytometry cells pass a laser beam one by one and the forward and sideward scatter is detected. Ligands bound to the cells can be detected by staining with fluorophore-conjugated antibodies. (Figure from www.invitrogen.com)

1.7 This project

As noted above engineering of the Fc-fragment of IgG can be an effective way to design antibodies with desired immunological functions as the binding of the Fc-fragment to FcγR plays a key role for the cellular response. In the development of therapeutic antibodies an assay for measuring Fc-FcγR affinities would be of value. The assay should preferably have a relatively high throughput to enable screening of IgG early in the antibody discovery process.

The aim of this work was to set up an antigen-independent affinity assay to study the Fc/FcγR interaction in order to confirm expected binding and/or identify antibodies with deviating affinity for FcγRs. The assay could e.g. be used for quality testing of produced IgGs enabling exclusion or reproduction of atypical antibodies prior to use in time-/resource-consuming *in vitro* and *in vivo* testing. Results from the assay could also facilitate interpretation of data from functional assays and provide information which may increase the understanding of the biological function of the antibody. The assay should allow screening of at least 24 IgGs in parallel.

An optimal assay is sensitive, specific, reproducible, robust, appropriate, cheap, simple and fast. All of these requirements are difficult to achieve at the same time. For all methods it is, however, crucial that the experimental set-up is appropriately designed and that correct control experiments are conducted. In this work the methods of choice were ELISA and flow cytometry as these are common and validated methods, and to some extent complementary. Furthermore the required equipment was easily accessible. Soluble and cell-bound forms of a number of FcγRs were cloned and expressed. Antigen-independent ICs were generated and optimized in order to assay the low and intermediate affinity receptors. Several experimental set-ups were tested and a number of control experiments performed to validate the chosen parameters. Two set-ups, one ELISA based and one flow cytometry based, were selected for screening of IgG.

2 Material and methods

Cultivation of cells

Adherent Chinese hamster ovary cells (CHO-K1) from ATCC were cultured in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Gibco) at 37°C, 5% CO₂. Suspension adapted human embryonic kidney cells (HEK-293 EBNA) from ATCC were cultured in FreeStyle 293 Expression Medium (Invitrogen) at 37°C, 8% CO₂ and 150 rpm.

Antibodies

Antibodies used in affinity assays and for IC generation are listed in Appendix A. Some n-CoDeR[®] antibodies contained the N297Q mutation giving IgGs without Fc glycosylation which reduces the affinity for FcγRs. A few antibodies were deglycosylated using PNGase F (Sigma) according to manufacturer's recommendations in order to reduce the Fc/receptor affinity.

Generation of immune complexes

ICs were prepared by thoroughly mixing F(ab')₂ anti-human F(ab')₂ or F(ab')₂ anti-human λ light chain with IgG in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄) pH 7.4 at different F(ab')₂:IgG ratios (w:w). Pre-labeled ICs were generated in the same manner but by mixing HRP- or FITC- conjugated F(ab')₂ anti-human F(ab')₂ with IgG. Samples were incubated 1 h at 37°C or 2-6 h at room temperature and thereafter stored at 4°C.

Cloning

DNA coding for the full length form and ECD of hFcγRI, hFcγRIIIa^{131H}, hFcγRIIIa^{158F}, hFcγRIIIa^{158V}, hFcγRIIIb-01 (hFcγRIIIbNA1), hFcγRIIIb-02 (hFcγRIIIbNA2), mFcγRI, mFcγRIII and hFceRg1 (human gamma chain) were synthesized by GeneArt[®] (see Appendix B for corresponding protein sequences).

All restriction enzymes and buffers as well as DNA polymerase were purchased from Roche Diagnostics GmbH. DNA concentration was measured using a NanoDrop (Thermo Fisher Scientific Inc.).

Two vectors designed by BioInvent were used for cloning and expression; one for the soluble receptors and one for the full length receptors and human gamma chain. Both vectors coded for origin of replication in *E. coli*, ampicillin resistance, the CMV promoter (for transcription in mammalian cells), a multiple cloning site and a poly A signal (for adenylation of mRNA). The vector for the soluble receptors also coded for a C-terminal mouse IgG3Fc tag. GeneArt constructs and cloning vectors were digested with appropriate restriction enzymes. Cleaved DNA was mixed with loading dye (0.25% bromophenolblue, 0.25% xylene cyanol FF, 30 % glycerol in H₂O) at a v:v ratio of 6:1, and run together with a 1 kb plus DNA ladder (Invitrogen) on 0.75% agarose gels containing ethidium bromide. Fragments corresponding to expected size were extracted from the gel using QIAquick gel extraction kit (QIAGEN) according to manufacturer's recommendations. Purified vectors were dephosphorylated

using Shrimp Alkaline Phosphatase (Roche) according to manufacturer's recommendations. Purified fragments and vectors were ligated 2 h at room temperature with T4 DNA ligase (Invitrogen) with a vector:insert ratio of 1:3 (mol:mol). Ligation products were transformed into chemically competent *E. coli* TOP10 cells by heat shock. In short; ligation products were added to competent cells and incubated on ice 20 min, at 42°C 90 s, on ice 5 min. Thereafter SOC medium (Invitrogen) was added and the sample was incubated 1 h at 200 rpm and 37°C for phenotypic expression before plating on LA plates containing ampicillin (100 µg/ml).

Insertion and transformation of correct DNA fragment was confirmed with colony PCR and DNA sequencing. Colonies were picked and used as template for PCR. Colony PCR was conducted using the Expand Long template PCR system polymerase with conditions as follows; initial denaturation 94°C 2 min, denaturation 94°C 30 s, annealing 52°C 30 s, elongation 68°C 1 min, final elongation 68°C 5 min, 30 cycles were run. Primers flanking the regions of interest were used. PCR products were mixed with loading dye and run on 1% agarose gel containing ethidium bromide as described above in order to analyze size of plasmid insert. Plasmids with insert of expected size were purified using QIAprep Spin miniprep (Qiagen) according to manufacturer's recommendations and sequenced to verify correct sequences prior to transfection.

Sequencing based on the Sanger dideoxy termination method was conducted using BigDye Terminator mix v3.1 and ABI PRISM 3100GeneticAnalyzer (Applied Biosystems) according to manufacturer's recommendations. Data was analyzed in Accelrys gene (Accelrys Software Inc.). A few plasmids were sequenced by GATC Biotech AG.

Production of ECD

Soluble forms of FcγRs with a C-terminal mIgG3Fc tag were produced by transient transfection of suspension adapted HEK-293 cells with plasmids coding for the ECDs using a DNA concentration of 1 µg/ml. In short; one day prior to transfection cells were seeded at a density of $1.1 \cdot 10^6$ cells/ml in FreeStyle 293 Expression Medium. On the day of transfection the density was $2.1 \cdot 10^6$ cells/ml. Four hours after transfection the volume was doubled by adding Freestyle medium supplemented with 20 g/l tryptone. Cells expressing murine ECDs and human FcγRI were cultivated 24 h at 37°C, 8% CO₂ and 150 rpm and thereafter at 32°C another 7-9 days before harvest. Cells expressing the other ECDs were cultivated at 37°C, 8% CO₂ and 150 rpm for 6 days before harvest. ECDs were purified from the supernatant by proteinA affinity chromatography using MabSelect resin (GE-Healthcare). ECDs were eluted in elution buffer (PBS-glycine 100 mM pH 2.8) and neutralized to pH 5.5 with NaP 0.8 M in PBS pH 8.6 or dialyzed to PBS pH 7.4 or citric acid buffer pH 5.5 (25 mM). Protein concentration was measured using a NanoDrop.

SDS-PAGE

SDS-PAGE was conducted on purified samples both in non-reduced and reduced state on a gradient gel, NuPAGE4-12% Bis-Tris gel (Invitrogen), with SeeBlue Plus 2 pre-stained (Invitrogen) as standard. 3 µg sample was diluted to a final volume of 15 µl in LDS NuPAGE

sample buffer (Invitrogen), incubated 10 min at 70°C and loaded on the gel. Reducing agent was added to samples to be reduced prior to heating. The gel was run for 60 min at 150 V in MOPS (Invitrogen), stained in coomassie stain (Simply Blue SafeStain, Invitrogen) and de-stained in H₂O according to manufacturer's recommendations.

SEC analysis

To analyze the quality of the purified ECDs and the aggregation state of the ICs generated Size Exclusion Chromatography (SEC) was conducted. Samples were applied on a Dionex MabPac SEC-1 column 4x300 mm on an Ultimate 3000 RS DIONEX system with UV detection. 5 mM K₂HPO₄, 0.4 M NaCl pH 6.5 was used as running buffer at a flow rate of 0.3 ml/min. A mixture of 1 mg/ml of thyroglobin (~660 kDa), IgG (~150 kDa), ovalbumin (44 kDa) and benzamidin (120 Da) was used as standard. Data was analyzed in the Chromeleon 6.8 software (Thermo Fisher Scientific Inc.).

Generation of semi-stable FcγR expressing CHO-K1 cells

One day before transfection cells were seeded at a density of 0.1-0.2*10⁶ cells/well in 6-well plates (Corning, surface area 9.5 cm²). Plasmids coding for full length FcγR, gamma chain and puromycin resistance, respectively, were co-transfected into CHO-K1 cells with the aim to incorporate the plasmid DNA in the nucleus. Lipofectamin 2000 (Invitrogen) was used as transfection reagent with a reagent:DNA ratio of 1.4:1 (v:w) and a total amount of DNA of 4 µg/well. In short; plasmids and lipofectamin were separately incubated in opti-MEM (Invitrogen) 5 min at room temperature. The lipofectamin mixture was added to the DNA mixture followed by incubation 20 min at room temperature. Directly before transfection the media on the cells was changed to DMEM/F12 without FBS. The DNA/lipofectamin solution was carefully added to the cells drop-wise under constant agitation. After 4 h incubation at 37°C the media was replaced with fresh DMEM/F12 + 10% FBS. Antibiotic pressure was applied 24 or 48 h after transfection by changing to DMEM/F12 + 10% FBS with 10 µg/ml puromycin (InvivoGen). One well/plate was used as control and was only transfected with FcγR and gamma chain plasmids, i.e. lacking puromycin resistance. Transfection efficiency was measured after 48 h using flow cytometry as described below. If cells showed receptor expression cultivation under antibiotic pressure was continued until the cells in the control well were dead. The expression of receptor was measured again using flow cytometry. Polyclonal cultures showing expression were seeded at a density of 0.5 cells/well in 96-well plates (Corning) in DMEM/F12 + 10% FBS with 10 µg/ml puromycin and 10 mM HEPES (Gibco) and maintained at 37°C, 5% CO₂ until individual colonies were visible, approximately 7-14 days. Some of these colonies were picked for FMAT analysis (see below) and those showing positive signal were expanded and again analyzed using flow cytometry. Cell banks for storage at -80°C were generated of the clones showing best receptor expression in the final flow cytometry analysis. These cells were later used in the affinity assay set-up.

FMAT and Flow cytometry analysis

FMAT analysis

Samples of colonies appearing after seeding in 96-well plates (see above) were transferred to 96-well FMAT plates (PE Biosystems) and incubated at 37°C, 5% CO₂ another 2-3 days. 12 h prior to analysis the media was removed and 40 µl/well of DMEM/F12 without serum was added followed by addition of 30 µl/well of a mixture containing mouse anti-receptor-specific antibody (see appendix A) diluted 1:30 and APC-conjugated anti-mouse antibody diluted 1:1200 in DPBS pH 7.4 (Dulbecco's PBS, Invitrogen) with 60 mM HEPES. Analysis was conducted in FMAT™ (8200 Cellular detection system, Applied Biosystems) and data analyzed using 8200 Analysis software (Applied Biosystems).

Flow cytometry analysis- protein expression

Cells were detached using cell dissociation buffer (Hanks, Invitrogen), washed once in FACS buffer (DPBS + 0.5% bovine serum albumin), resuspended in 100 µl buffer and incubated 30 min on ice in darkness with 10 µl pre-labeled receptor-specific antibody or isotype control. After incubation the cells were again washed twice in FACS buffer, resuspended in 200 µl buffer and data collected using a FACSCalibur flowcytometer (Becton Dickinson). Data was analyzed in BD CellQuest™ Pro Software (BD Bioscience).

Flow cytometry analysis- affinity measurements

Semi-stable CHO-K1 cells expressing human FcγRI, FcγRIIa^{131H}, FcγRIIIa^{158V}, FcγRIIIa^{158F}, FcγRIIIb-01, FcγRIIIb-02 and murine FcγRI were generated in this work. Cells expressing FcγRIIb and murine FcγRIV had previously been generated by BioInvent.

ICs were diluted in FACS buffer in 11 steps (25000-0.5 ng/ml IgG) in duplicate. Monomeric IgGs were diluted in the same way (5000-0.3 ng/ml). FcγR-expressing cells were detached using cell dissociation buffer, washed once in FACS buffer and transferred to 96-well round bottom plates at a density of 0.1-0.2*10⁶ cells/well. Cells were spun down and the supernatant was discarded. Cells were resuspended in 20 µl/well of diluted IC, except for cells expressing the high affinity receptor FcγRI which were resuspended in monomeric IgG, incubated 1 h on ice and thereafter washed twice in FACS buffer. Cells were resuspended in 20 µl/well of APC-conjugated anti-human IgG and incubated 30 min on ice in darkness. Cells were again washed twice in FACS buffer, resuspended in 100 µl/well and data collected using FACSCalibur. Results were analyzed using the FlowJo 7.6 software (Tree Star Inc.). Dose response curves were generated of the mean fluorescent signals.

ELISA

Most of the soluble receptors used for ELISA experiments were produced in this work as described above. The ECDs of human FcγRIIa^{131R} and FcγRIIb had previously been produced by BioInvent. As the quality and yield of the ECD of human FcγRI produced in this work was poor purchased soluble FcγRI (R&D Systems) was used for ELISA assays. Incubation was made at room temperature and with agitation at 500 rpm unless otherwise stated. 96-well

plates (LUMITRAC 600, Greiner Bio-one GmbH) were used in all experiments. All samples were analyzed in duplicates.

Affinity measurement- direct coating of IgG

IgG was diluted in two-fold serial dilutions in coating buffer (100 mM carbonate buffer pH 9.5) and coated in 11 concentrations (100000-98 ng/ml) at 4°C overnight. Plates were washed three times manually in PBS pH 7.4 and thereafter blocked with 50 µl/well PBS pH 7.4 containing 0.45 % fish gelatin (Sigma). After 1 h incubation plates were washed as before and 50 µl/well receptor (0.25, 0.5, 1, 2, 4 µg/ml) diluted in PBS pH 7.4 + 0.45% fish gelatin was added and incubated 1.5 h. Plates were washed as before and bound receptor was detected by incubation 1 h with 50 µl/well of HRP-conjugated F(ab')₂ goat anti-mouse Fcγ diluted 1:10000 in PBS pH 7.4 + 0.45% fish gelatin. Thereafter plates were washed manually three times with PBS and two times with 20 mM Tris-HCl + 1 mM MgCl₂, pH 9.8. 50 µl/well substrate (PIERCE, Super signal ELISA pico Maximum) diluted 1:20 in 20 mM Tris-HCl + 1 mM MgCl₂, pH 9.8 was added and luminescence read in Wallac Victor²™ multi label counter (Perkin Elmer™) after 10 min incubation in darkness. Coating density was studied by incubation with PBS pH 7.4 + 0.45% fish gelatin instead of receptor and applying HRP-conjugated F(ab')₂ goat anti-human Fc.

Affinity measurement- direct coating of IC

ELISA with direct coating of IC was conducted in the same manner as with direct coating of IgG with the exception that IC was coated instead of IgG.

Affinity measurement- direct coating of F(ab')₂

F(ab')₂ anti-human F(ab')₂ was coated at 30 µg/ml in coating buffer, 50 µl/well, overnight at 4°C. The plates were washed, blocked and washed as described for direct coating of IgG. IgG was diluted in 11 steps in two-fold serial dilutions (30000-29 ng/ml) in PBS + 0.45% fish gelatin. 50 µl/well of diluted IgG was transferred to the ELISA plate and incubated 1.5 h. Addition of receptor and detection was conducted as described for direct coating of IgG.

Affinity measurement- direct coating of receptor

Receptors were coated at 5 different concentrations (0.25, 0.5, 1, 2, 4 µg/ml) in coating buffer, 50 µl/well, overnight at 4°C. Plates were washed, blocked and washed as described for direct coating of IgG. ICs were diluted in 11 steps (30000-0.5 ng/ml) in PBS + 0.45% fish gelatin in a dilution plate. For measurements with the high affinity receptor FcγRI monomeric IgG was diluted in 11 steps (5000-0.5 ng/ml) in PBS + 0.45% fish gelatin. 50 µl/well of diluted IC/monomeric IgG was transferred to the ELISA plate and incubated 1.5 h. Plates were washed three times in PBS followed by 1 h incubation with biotin-conjugated anti-human F(ab')₂ diluted 1:5000 in PBS + 0.45 % fish gelatin, 50 µl/well. After yet another wash step 50 µl/well of HRP-conjugated streptavidin (Dako) diluted 1:10000 in PBS + 0.45 % fish gelatin was added and incubated 1 h. Detection was thereafter conducted as described for direct coating of IgG.

Quality control- direct coating of receptor

To verify the quality of purified receptors plates were coated with 50 µl/well of receptor diluted to 0.5, 1 and 2 µg/ml in coating buffer and incubated overnight at 4°C. After washing and blocking as described for direct coating of IgG plates were incubated 1 h with 50 µl/well of receptor-specific primary antibody (2 µg/ml) (see appendix A) diluted in PBS + 0.45% fish gelatin. Plates were washed and 50 µl/well of HRP-conjugated secondary antibody diluted 1:10000 in PBS + 0.45% fish gelatin was added followed by 1 h incubation. Coating density was detected with HRP-conjugated anti-mouse Fcy. Detection was conducted as described for direct coating of IgG

Affinity measurement- sandwich ELISA

To reduce the risk of the affinity being disturbed by artifacts introduced when immobilizing the receptor or IgG onto the plates a sandwich ELISA was set up by coating plates with F(ab')₂ goat anti-mouse Fcy at a concentration of 6 µg/ml overnight. Plates were thereafter washed twice in PBS and soluble receptor loaded at a concentration of 2 µg/ml. ELISA was thereafter conducted as described for *affinity measurements-direct coating of receptor* with the exceptions that biotin-conjugated anti-human F(ab')₂ was diluted 1:1000, HRP-conjugated streptavidin was diluted 1:5000, the substrate was diluted 1:5 in PBS and the final wash was conducted in only PBS, not Tris-HCl.

Affinity measurement- Cell ELISA

FcyR-expressing cells were detached using cell dissociation buffer and seeded in 96-well plates (White micro assay plate, Greiner Bio-One) at 15000 cells/well in DMEM/F12 + 10% FBS with 10 µg/ml puromycin followed by incubation 24 h at 37°C, 5% CO₂. Plates were washed with 100 µl/well PBS containing 1% BSA. 50 µl/well of IC diluted in 11 steps in three-fold serial dilutions (20000-0.3 ng/ml IgG) in DMEM/F12 + 10 % FBS + 50 mM Hepes (Invitrogen) was added and the plates were incubated 1.5 h at room temperature. The plates were washed and thereafter incubated 1 h with 50 µl/well HRP-conjugated F(ab')₂ goat anti-human F(ab')₂ diluted 1:10000 in PBS + 10% FBS. Detection was thereafter conducted as described for direct coating of IgG.

Calculations and affinity comparison

Dose response curves from sandwich ELISA and flow cytometry affinity measurements were compared by visual means. Curves reaching a plateau were normalized with respect to their maximum signal generating curves with a common maximum of 1. EC50 values were derived, when possible, using a five-parameter nonlinear curve-fitting program (XLfit, Guildford, Surrey, UK). Dose response curves were generated from the mean of duplicates. For trastuzumab a mean EC50 was calculated based on the EC50 values derived from two or more different experiments. For n-CoDeR[®] antibodies the EC50 values were based on one experiment.

3 Results

FcγRs were produced in soluble and cell-bound form. Antigen-independent ICs of human IgG were generated and affinity assays were set up to study the binding of ICs to FcγRs.

3.1 Soluble FcγRs

The aim was to generate hFcγRI, hFcγRIIIa^{131H}, hFcγRIIIa^{158V}, hFcγRIIIa^{158F}, hFcγRIIIb-01, hFcγRIIIb-02, mFcγRI, mFcγRII, mFcγRIII and mFcγRIV in soluble form. To obtain this the ECDs of the receptors were expressed with a C-terminal mouse IgG3 Fc tag by transient transfection of suspension adapted HEK293-EBNA cells. The Fc tag enabled purification with ProteinA affinity chromatography and capture of the receptors on ELISA plates using anti-tag F(ab')₂-fragments. The yield varied much amongst the receptors and between batches ranging from 1 to 25 µg/ml culture medium.

SDS-PAGE analysis showed that the samples were > 90% pure and that the Fc tag induced dimerization of the receptors as expected (Figure 6). Murine receptors and human FcγRI turned out to be difficult to express and therefore expression at 32°C instead of 37°C was tested. Expression at lower temperature might bring about less stress on the cells, promoting correct translation and folding. Despite lowering the temperature murine FcγRIII was still not possible to express and the yield of human FcγRI was very low.

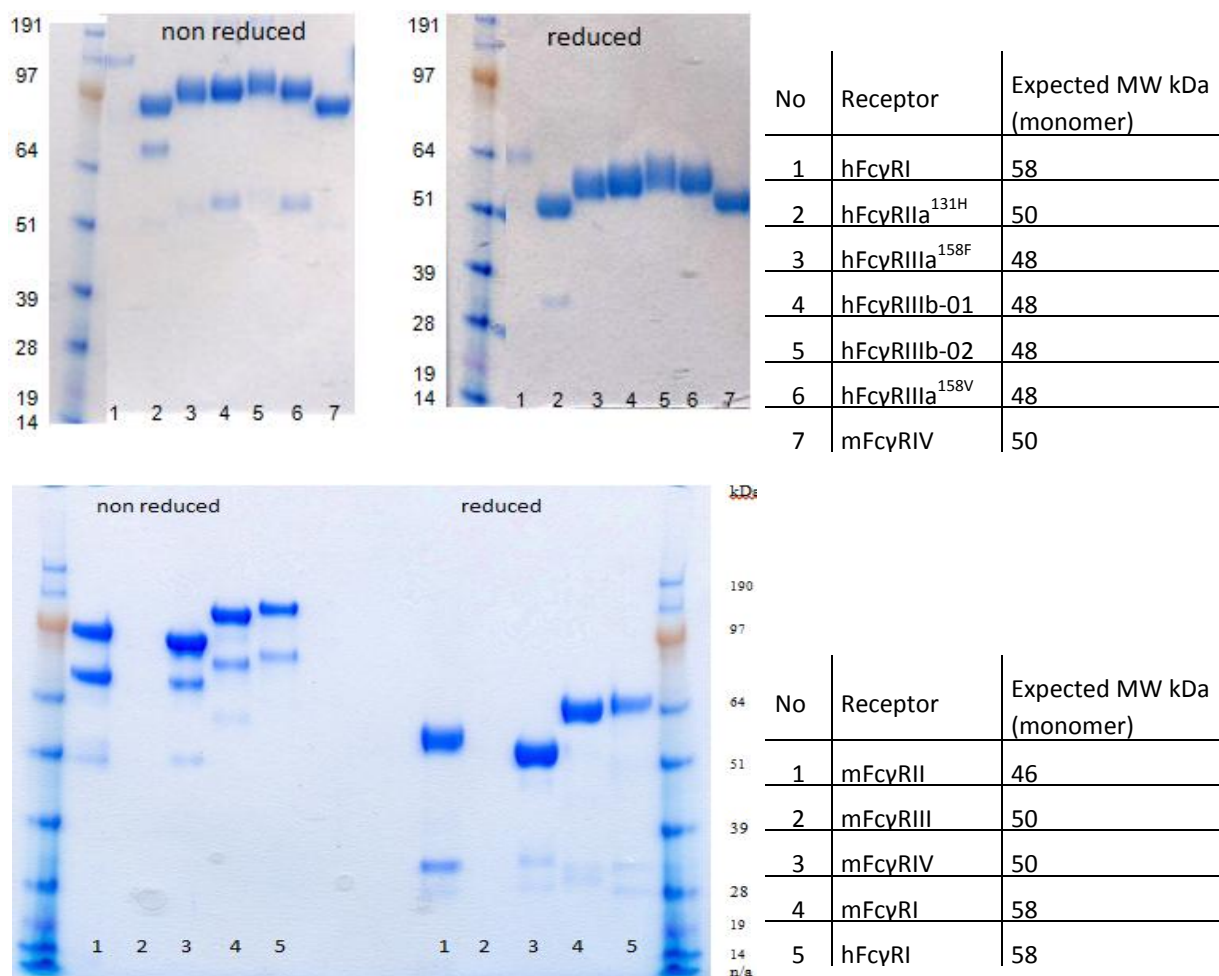


Figure 6. SDS-PAGE of purified soluble FcγRs in non-reduced and reduced form as indicated. The Fc tag promotes dimerization of the receptors giving duplication of the apparent MW when comparing reduced and non-reduced gels. Bands are visible at expected size. hFcγRIII is highly glycosylated resulting in broad bands. The Fc tag was most likely cleaved off from a fraction of the receptors in some of the samples giving rise to a band at approximately 50 and 25 kDa for non-reduced and reduced samples respectively. The identity of the fragment appearing approximately 30 kDa below the top band for the non-reduced samples on the lower gel is unknown.

SEC analysis revealed that the receptors were recovered in a more or less aggregated state. The aggregation varied between receptors and between batches (Figure 7). SEC analysis of human FcγRI showed that the quality of this receptor was poor (see Appendix C). Due to this and the low yield the FcγRI preparation was not used for affinity measurements. Instead a purchased form of FcγRI was used. For chromatograms from SEC analysis of soluble FcγRs used in antibody screening (section 3.5) see Appendix C.

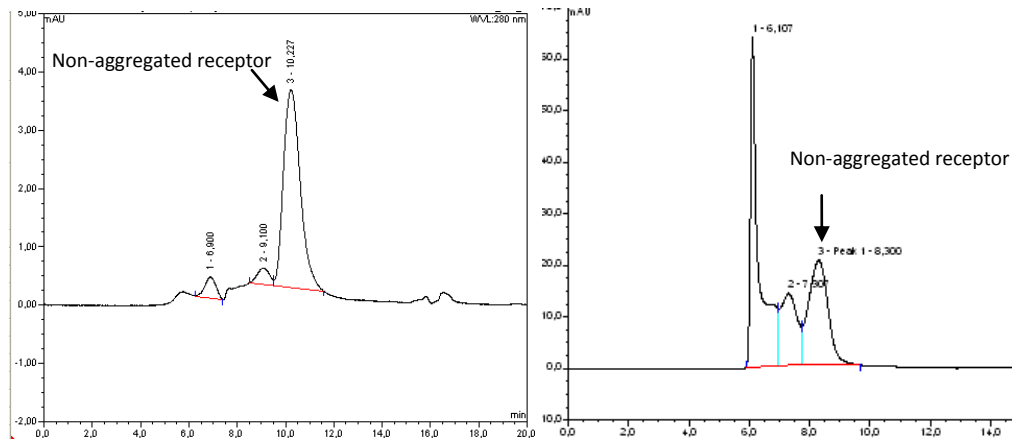


Figure 7. SEC chromatograms of two different batches of FcγRIIIb-01 exemplifying how the degree of aggregation of the receptors varied between batches. Peak corresponding to non-aggregated receptor as noted. The percentage non-aggregated receptor was 88 and 34% for the left and right sample, respectively.

The receptors were not stable at 4°C. To reduce the risk of working with aggregated receptors the samples were frozen at -20°C directly after purification and thawed right before affinity measurements. Freezing had only minor effect on the affinity of the receptors and enriched aggregation was not observed (data not shown).

3.2 Cell-bound FcγRs

As the FcγRs are membrane-bound, semi-stable cell lines expressing the receptors were generated. The aim in this study was to generate cells expressing hFcγRI, hFcγRIIIa^{131H}, hFcγRIIIa^{158V}, hFcγRIIIa^{158F}, hFcγRIIIb-01, hFcγRIIIb-02, mFcγRI and mFcγRIII. For correct function, expression and localization in the membrane all receptors but human FcγRII require simultaneous expression of the γ-chain. Therefore plasmids coding for the receptor, the γ-chain and puromycin resistance, respectively, were co-transfected into adherent CHO-K1 cells. Generation of semi-stable cell lines expressing the receptors intended was successful except for mFcγRIII. The degree of expression varied from receptor to receptor (Figure 8).

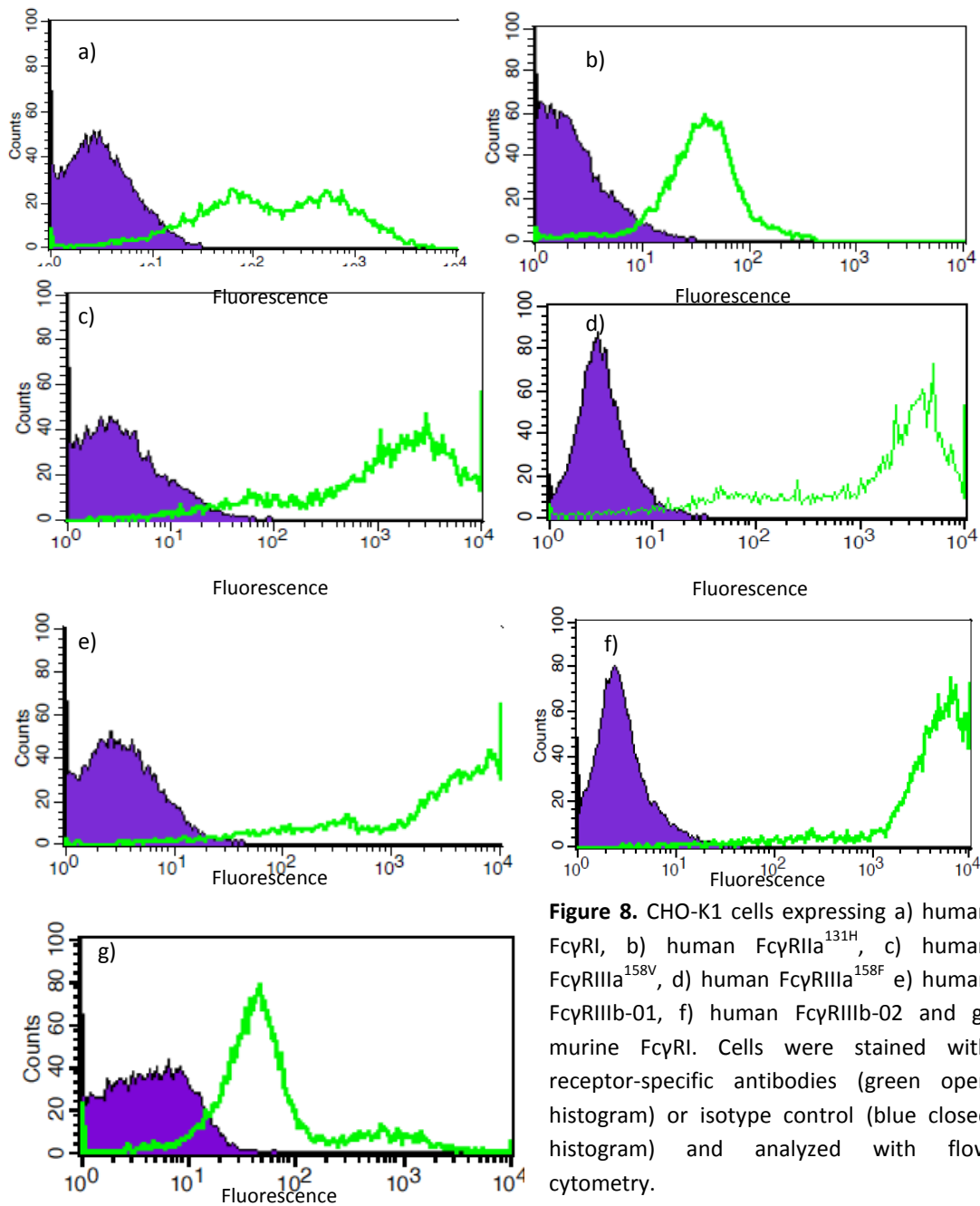


Figure 8. CHO-K1 cells expressing a) human FcγRI, b) human FcγRIIa^{131H}, c) human FcγRIIIa^{158V}, d) human FcγRIIIa^{158F} e) human FcγRIIIb-01, f) human FcγRIIIb-02 and g) murine FcγRI. Cells were stained with receptor-specific antibodies (green open histogram) or isotype control (blue closed histogram) and analyzed with flow cytometry.

3.3 IC generation

Since the affinity of FcγRII and FcγRIII for IgG is low, IgG had to be in a complex-bound form in order to detect binding. Antigen-independent ICs were generated by incubating F(ab')₂ anti-human-F(ab')₂ with IgG (Figure 10b). Different ratios were tested to elucidate the optimum for affinity measurements. SEC analysis of the formed complexes showed that increasing the F(ab')₂:IgG ratio generated increasing degree of aggregation (Figure 9a). This result was verified using ELISA where FcγR showed higher affinity for ICs with higher F(ab')₂:IgG ratio, indicating that more of the IgG was in complex-bound form (Figure 9b). The

same experiment was conducted with five IgGs with different antigen-specificity, the same pattern was seen for all IgGs (results not shown).

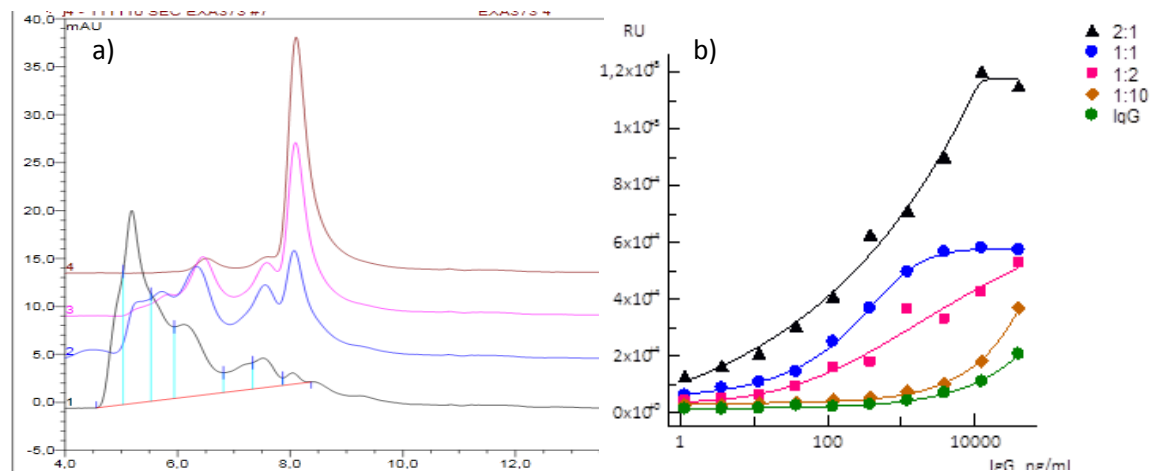


Figure 9. a) Overlay of chromatograms from SEC analysis of ICs of trastuzumab with different $F(ab')_2$:IgG ratios (w:w) showing that the degree of IgG in complex-bound form increased with increasing $F(ab')_2$:IgG ratio. Ratios are; (—) 2:1, (—) 1:1, (—) 1:2, (—) 1:10. b) The affinity of $Fc\gamma RIIa$ for ICs with different $F(ab')_2$:IgG ratio increased with increasing ratio when measured using ELISA.

Based on SEC analysis a $F(ab')_2$:IgG ratio (w:w) of 2:1 was used in affinity measurements since the main part of IgG was in a complex-bound state at that ratio (Figure 10a).

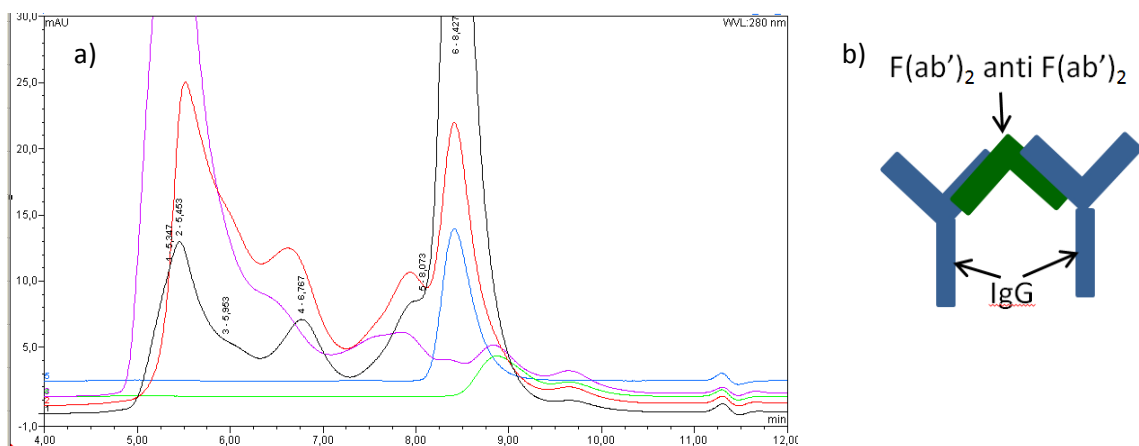


Figure 10. a) Overlay of SEC chromatograms of trastuzumab, $F(ab')_2$ and ICs generated with different $F(ab')_2$:trastuzumab ratios (w:w) show that $F(ab')_2$ and IgG form complexes to largest extent at ratio 2:1. Samples are: (—) $F(ab')_2$, (—) trastuzumab, (—) ratio 1:2, (—) ratio 1:1, (—) ratio 2:1. b) Schematic figure of the IC.

ICs of antibodies with the same variable region but different constant regions gave rise to similar aggregation patterns (Figure 11) indicating that the complex formation was IgG subclass independent.

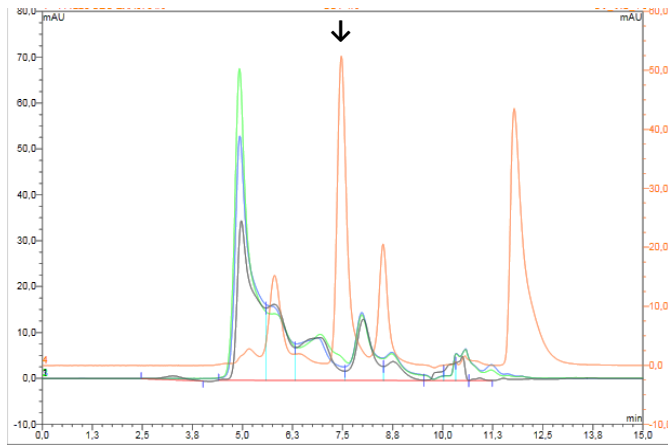


Figure 11. SEC analysis of IC of IgG with the same variable region but different constant region show that the complexes are of similar aggregation status. Samples are: (—) IgG1, (—) IgG2, (—) IgG4 and (—) standard (↓) 150 kDa.

In order to do a fair comparison of the affinities of FcγRs for different IgGs ICs with similar aggregation pattern were required since the IC size proved to affect the affinity (Figure 9b). IgG with κ light chain gave more complex formation of IgG than IgG with λ light chain at the same F(ab')₂:IgG ratio, prohibiting comparison of affinity of FcγR for IgGs with different light chains (Figure 12a). Even after increasing the F(ab')₂:IgG ratio to 10:1 no more than 50% of the IgG was obtained in complex state. By using F(ab')₂ anti-λ light chain for complex formation of λ light chain IgG more of the IgG was found in complex-bound form (Figure 12b). The ICs of n-CoDeR[®] antibodies used in this work (all λ light chain) generated in this way showed similar aggregation pattern as the ones of the commercial antibodies used in this study (all κ light chain) generated with F(ab')₂-anti human F(ab')₂. Thereby comparison of the affinity of FcγR for different IgGs, regardless of light chain, was feasible.

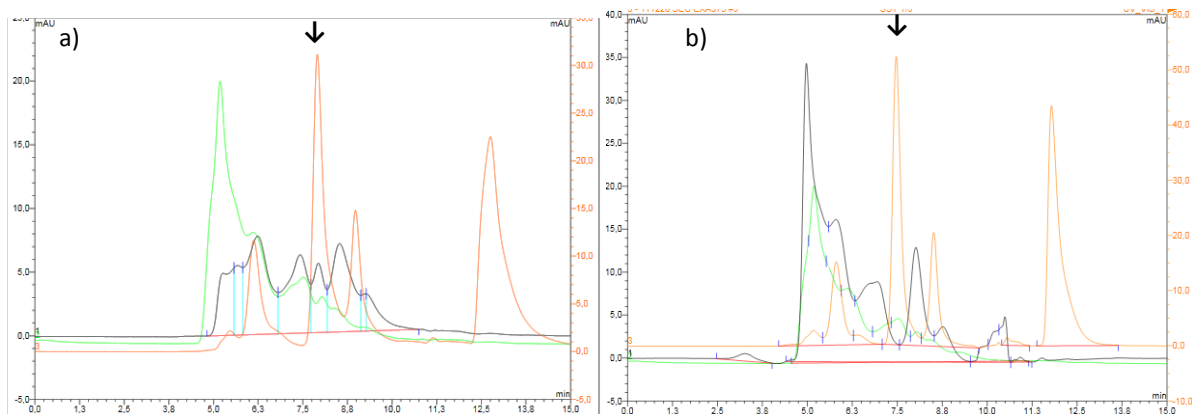


Figure 12. SEC chromatograms of ICs as overlay with (—) standard. a) Comparison of trastuzumab:F(ab')₂ (—) to n-CoDeR[®] x IgG1:F(ab')₂. (—) λ light chain antibodies generate less and smaller immune complexes with the F(ab')₂-fragment compared to κ light chain antibodies. b) Comparison of trastuzumab:F(ab')₂ (—) to n-CoDeR[®] x IgG1:F(ab')₂ anti-λ light chain (—). In this case the ICs show similar aggregation patterns. (↓) 150 kDa

The fraction larger than 150 kDa, i.e. IgG in complex-bound form, varied approximately 5-10% between different IC preparations. Complexes formed after approximately two hours

were stable over time when stored at 4°C. Freezing and thawing the complexes did not affect the aggregation pattern (results not shown).

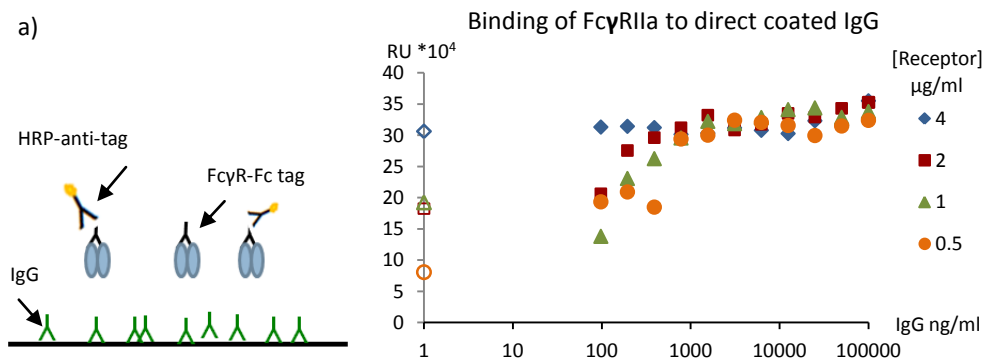
In order to reduce the number of washing steps in ELISA and flow cytometry, attempts were made to generate pre-labeled ICs by incubating IgG with labeled F(ab')₂-fragments. These complexes did, however, not show good aggregation in SEC analysis. In addition initial ELISA and flow cytometry measurements with these ICs generated inconsistent results (data not shown). This was possibly due to the label being a steric hindrance for IC formation. Therefore this strategy was abandoned.

3.4 Affinity assays

ELISA and flow cytometry were the methods chosen for assay set up as both are cheap, fairly simple with relatively high throughput and the equipment was available.

ELISA

Different ELISA set-ups were tested in order to design an antigen-independent affinity assay (Figure 13). Direct coating of IgG and detection of bound receptor with HRP-conjugated anti-tag F(ab')₂-fragment resulted in high background signals (13a). Similar results were obtained with direct coating of F(ab')₂ anti-human F(ab')₂ onto which IgG was captured and detection of bound receptor (data not shown). Direct coating of IC and detection of bound receptor with an HRP conjugated anti-tag antibody resulted in high unspecific binding (13b). Direct coating of receptor and detection of bound IC using a HRP-conjugated F(ab')₂ anti-human F(ab')₂ (13c) gave large variation in signals. Coating of F(ab')₂ anti-tag on which the receptor was captured and detection of bound IC using HRP-conjugated F(ab')₂ anti-human F(ab')₂ generated small variations between replicates and low background signals (13d). This sandwich ELISA set-up was therefore selected for further studies. The risk of introducing artifacts in the protein structure upon coating to the ELISA plate was reduced in the sandwich ELISA set-up. It could be argued that this design bears more resemblance to IC binding to a cell-bound receptor than when the receptor is coated directly to the plate.



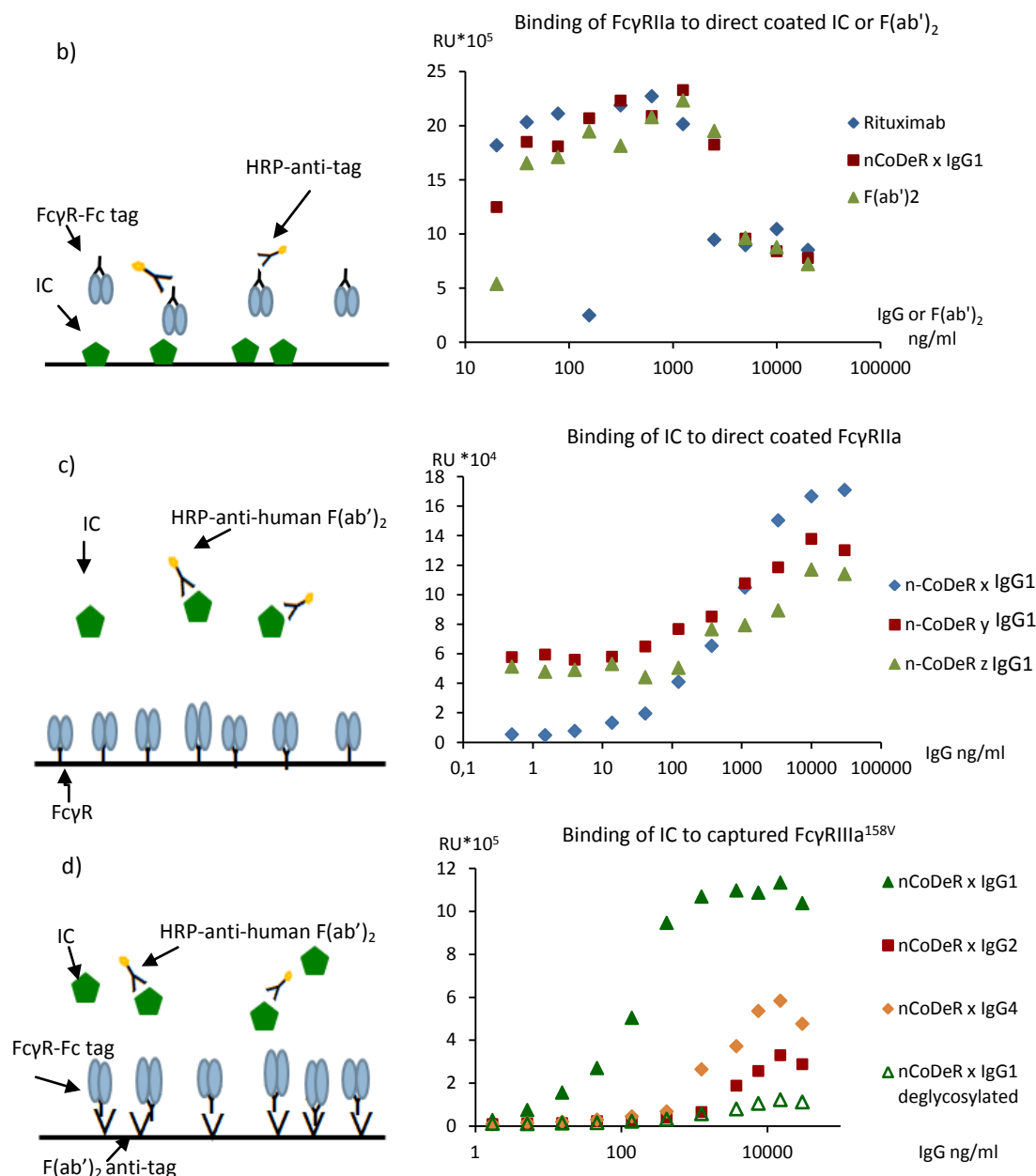
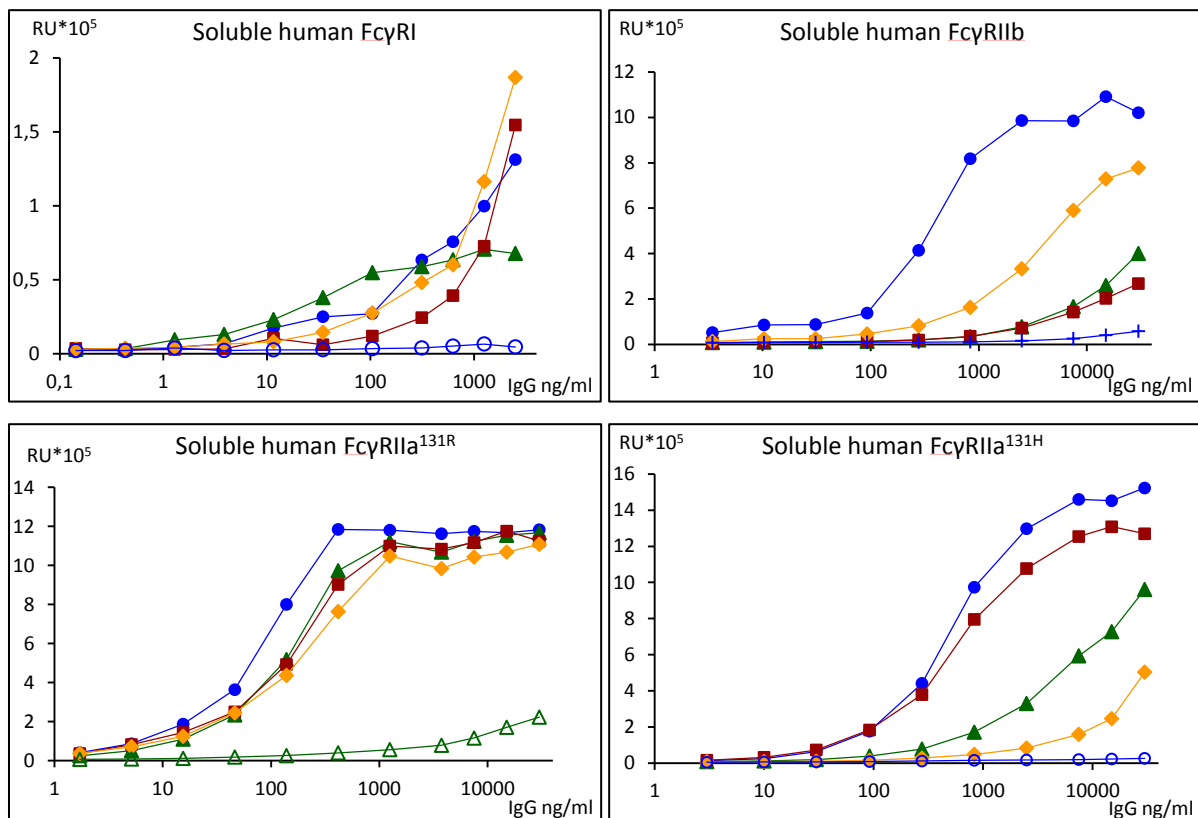


Figure 13. ELISA set-ups tested in this work; to the left a schematic figure of the set-up and to the right an example of ELISA results. a) Direct coating of monomeric IgG (rituximab) and detection of bound receptor with HRP-conjugated anti-tag F(ab') $_2$ -fragment, empty figures to the left in the graph indicate background signals, i.e. signals obtained without coated IgG. b) Direct coating of IC and detection of bound receptor with an HRP-conjugated anti-tag antibody. c) Direct coating of receptor and detection of bound IC using a HRP-conjugated F(ab') $_2$ anti-human F(ab') $_2$. d) Coating of F(ab') $_2$ anti-tag to which the receptor was captured and detection of bound IC using HRP conjugated F(ab') $_2$ anti-human F(ab') $_2$. See text for further details.

With the sandwich ELISA set-up it was possible to detect binding of IC formed with glycosylated IgG and significantly decreased or no binding was observed when ICs formed with deglycosylated IgG or IgG with the N297Q mutation were used, indicating that the binding was Fc-specific. The high-affinity receptor, Fc γ RI, bound monomeric IgG while the low-affinity receptors did not (Figure 14). It was also possible to detect variations in affinity for ICs with different degree of aggregation, i.e. IC size. This underlined the importance of having ICs with similar size in order to be able to ascribe dissimilarities in affinity to

differences in Fc binding. A receptor-typical IgG subclass preference could be detected for all receptors with a subclass affinity ranking as noted in Table 4. All human receptors, except FcγRIIa^{131H} and FcγRIIb, showed highest apparent affinity for IgG1 and lowest apparent affinity for IgG2. FcγRIIa^{131H} bound IgG2 the strongest and very low apparent affinity for IgG4. FcγRIIb showed higher apparent affinity for IgG4 than for IgG1 and IgG2. FcγRIIa^{131R} showed similar affinity for all IgG subclasses. FcγRIIIa^{158V/F} appeared to bind IgG4 stronger than IgG2, but with low affinity for both. As already noted affinity measurements with soluble FcγRI were conducted with a purchased form of the receptor. The purchased receptor lacked affinity tag and was therefore directly coated to the ELISA plate, increasing the variation in results. FcγRI showed highest apparent affinity for IgG1 and lower affinity for IgG4 and IgG2. The maximal signal was not the same for all FcγR/IC combinations. The cause of this was not elucidated. Normalization of the curves facilitated affinity comparison, but since not all curves reached a plateau normalization could not be conducted in the same way for all curves hampering a fair comparison of affinities.



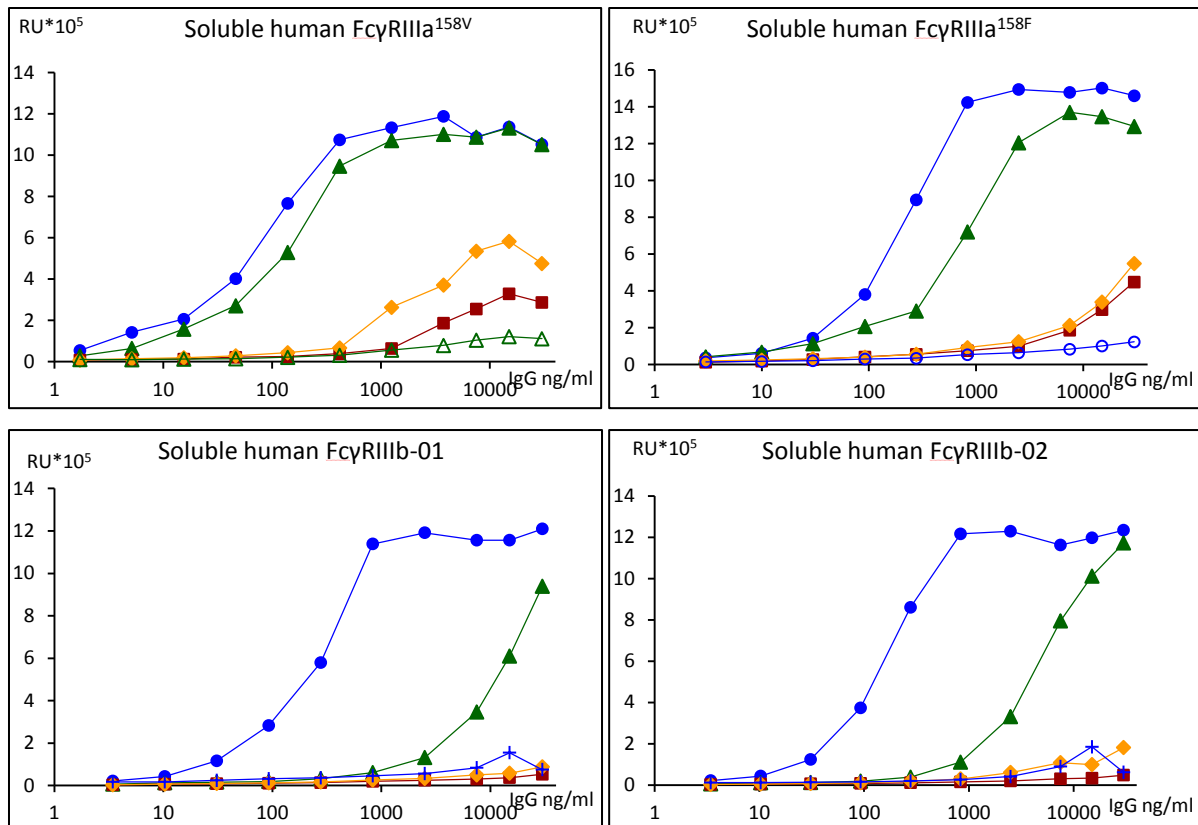


Figure 14. Apparent affinities of soluble human Fc γ R for IC/IgG measured with the sandwich ELISA set-up. All receptors show Fc-specific binding and a receptor-typical IgG subclass preference. The ICs were titrated from 30 μ g/ml and added to the low-affinity receptors, i.e. all receptors except Fc γ RI. Monomeric IgG was titrated from 5 μ g/ml and added to the high-affinity receptor, Fc γ RI. (●) trastuzumab, (▲) n-CoDeR[®] x IgG1, (■) n-CoDeR[®] x IgG2, (◆) n-CoDeR[®] x IgG4, (○) trastuzumab deglycosylated, (Δ) n-CoDeR[®] x deglycosylated, (+) monoeric trastuzumab when applied to the low affinity receptors. Graphs are representative examples of several measurements See text for further details.

The aggregation state of the different receptors varied and since this affected the IgG binding limited conclusions should be drawn regarding the differences in affinity for IgG between Fc γ Rs. It could, however, be observed that Fc γ RI showed higher affinity than the other receptors and that Fc γ RIIIb and Fc γ RIIb generally exhibited lower affinity than Fc γ RIIIa. If the higher affinity observed for Fc γ RIIIa^{158V} compared to Fc γ RIIIa^{158F} was due to the polymorphism or due to differences in receptor aggregation was not possible to elucidate. For the same reasons no definite conclusions could be drawn regarding differences in affinity caused by polymorphism of Fc γ RIIIb and Fc γ RIIIa. Rankings of the IgG subclass preference could, however, be compared in between the receptors (Table 4).

Affinity of IgG for some of the murine Fc γ Rs was also tested in the sandwich ELISA set-up. Binding of monomeric IgG to mFc γ RI and binding of ICs to mFc γ RIV was analyzed. No affinity for deglycosylated IgG was seen for any of the murine receptors. mFc γ RI showed higher apparent affinity for n-CoDeR[®] x IgG4 than for trastuzumab while mFc γ RIV appeared to bind trastuzumab stronger than the n-CoDeR[®] x antibodies (Figure 15).

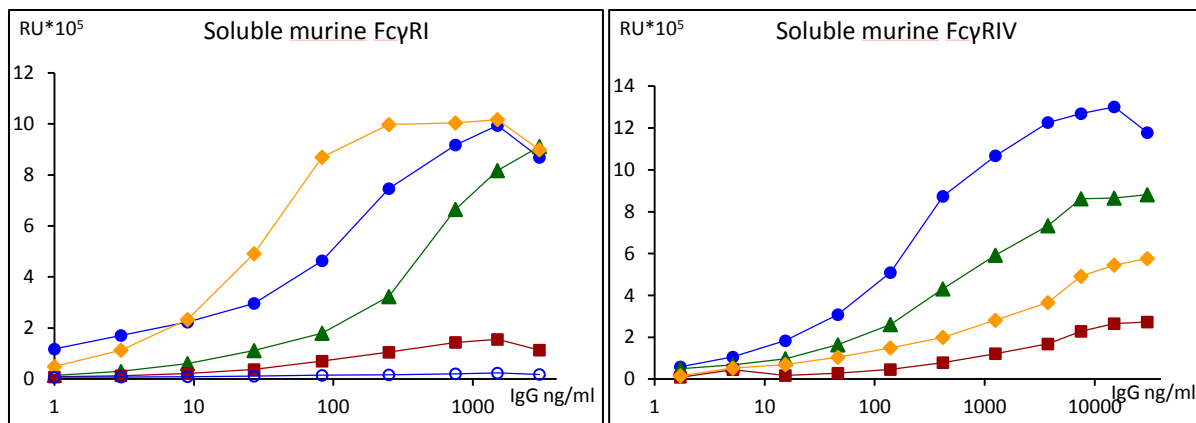


Figure 15. Affinity of mFcγRI and mFcγRIV for IgG and IC respectively, measured with the sandwich ELISA set-up. Both receptors show a Fc-specific affinity for IC and receptor-typical IgG subclass preferences. (●) trastuzumab, (▲) n-CoDeR® x IgG1, (■) n-CoDeR® x IgG2, (◆) n-CoDeR® x IgG4, (○) trastuzumab deglycosylated. Graphs are representative examples of several measurements. See text for further details.

Cell ELISA

To ascertain that the affinities of the soluble receptors seen in sandwich ELISA reflected the affinity of cell-bound FcγRs cell ELISA was tested. However antibodies with the N297Q mutation, not supposed to bind FcγR, showed binding to the receptors in cell ELISA (Figure 16). Optimization of this assay set-up was terminated since it could not be concluded that the binding was Fc-specific.

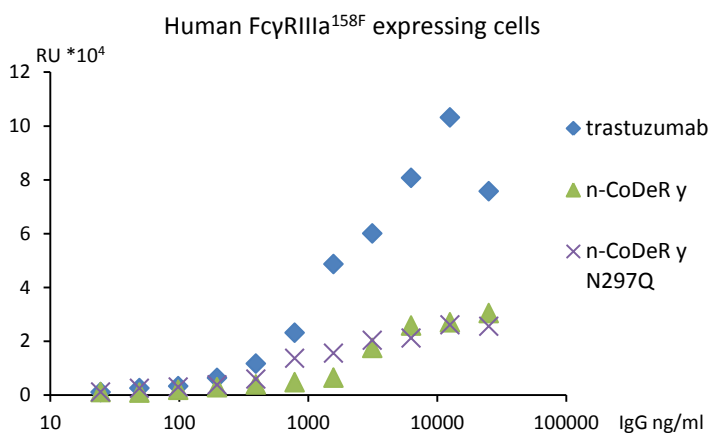
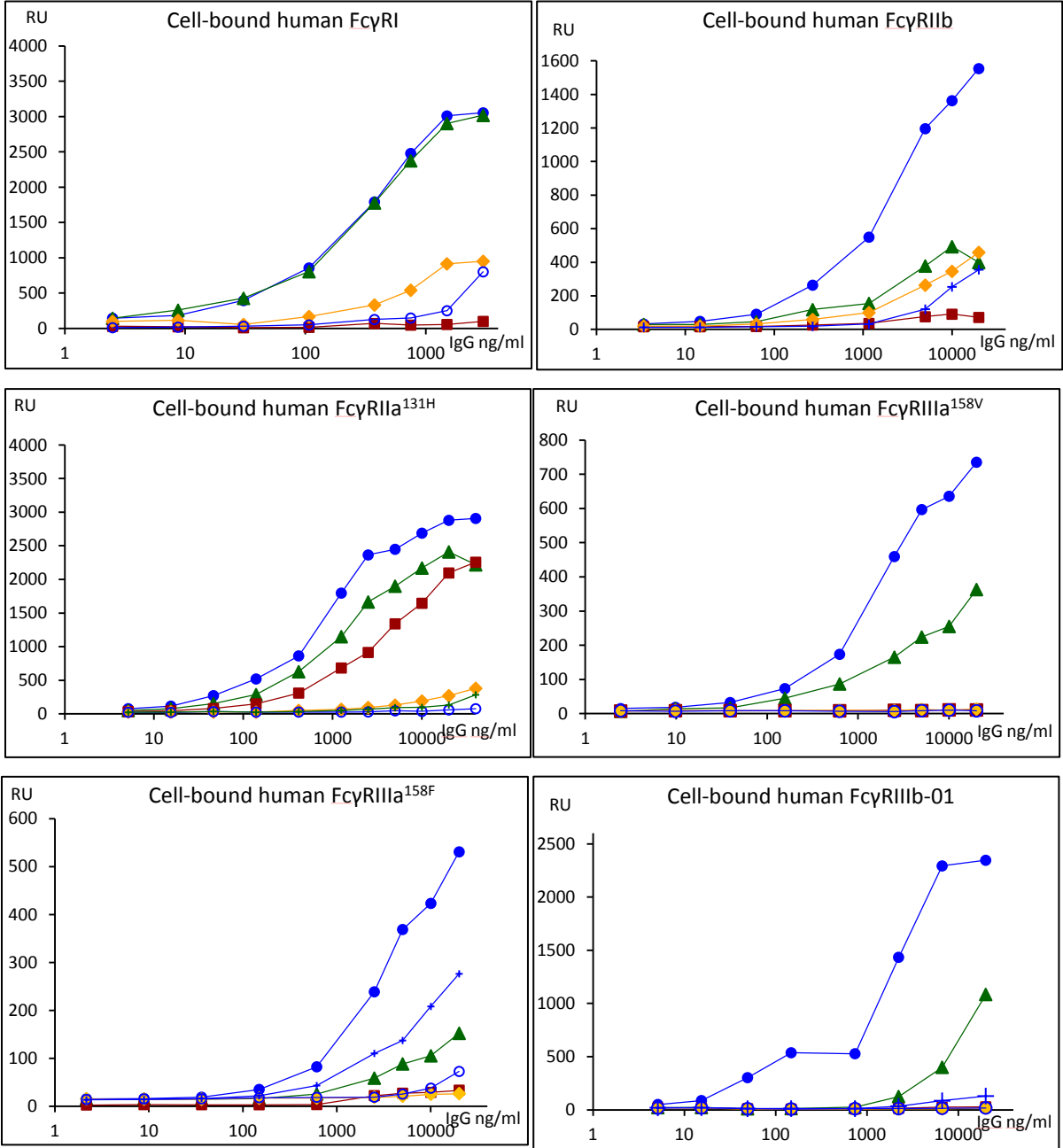


Figure 16. Human FcγRIIIa^{158F} expressing cells showed affinity for ICs of trastuzumab and n-CoDeR® y IgG1 but also for n-CoDeR® y IgG1 with the N297Q mutation, which is not supposed to bind FcγRs.

Flow cytometry

Another approach to elucidate if the affinities detected in the sandwich ELISA correlated with the affinities of cell-bound FcγRs was to set up an assay based on flow cytometry. Affinity of low-affinity receptors for ICs could be detected and binding of monomeric IgG to the high-affinity receptor. A receptor-typical IgG subclass preference was observed (Table 4). The binding of IC of deglycosylated IgG was low (Figure 17). Binding of IC of IgG subclasses for which the receptors had very low affinity could not be detected. Taking this into account the ranking observed was well in accordance with that found in literature [6], [34]. There were some signals observed for deglycosylated IgG. This could be caused by incomplete

deglycosylation. Binding of monomeric IgG to the low-affinity receptors could be detected in some cases, probably due to IgG forming complexes with the detection antibody. Untransfected CHO-K1 cells did not show affinity for any ICs (results not shown). The maximal signal was in most cases higher for trastuzumab than for the n-CoDeR® antibodies (Figure 17). Since the expression of FcγRs varied from cell line to cell line direct affinity comparison between receptors could not be performed. However, an IgG subclass preference ranking could be established for each receptor and this ranking could be compared in between receptors (Table 4).



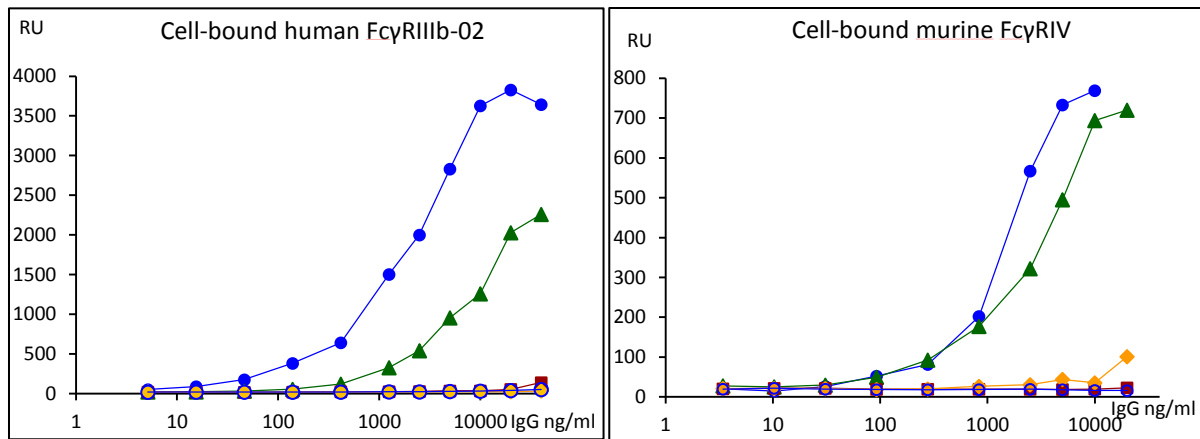


Figure 17. Apparent affinity of Fc γ R expressing cells for IC and/or monomeric IgG detected using flow cytometry. All receptors show Fc-specific affinity for IC and a Fc γ R typical IgG subclass preference. Monomeric IgG was titrated from 5 μ g/ml and added to the high-affinity receptor, Fc γ RI, IC were titrated from 30 μ g/ml and added to the other receptors. (●) trastuzumab, (▲) n-CoDeR[®] x IgG1, (■) n-CoDeR[®] x IgG2, (◆) n-CoDeR[®] x IgG4, (○) trastuzumab deglycosylated, (+) monomeric trastuzumab when applied to the low-affinity Fc γ Rs, (+) monomeric n-CoDeR[®] x IgG1 when applied to the low-affinity Fc γ Rs. Graphs are representative examples of several measurements See text for further details.

Comparison of sandwich ELISA and flow cytometry results and literature

The IgG affinity ranking obtained with the two assay set-ups was the same except for Fc γ RIIa^{131H} and Fc γ RIIb (Table 4). Cell-bound Fc γ RIIa^{131H} showed similar apparent affinity for IgG1 and IgG2 in contrast to the higher affinity for IgG2 observed in ELISA. Cells expressing Fc γ RIIb bound IgG1 and IgG4 with similar affinity, to be compared with the higher apparent affinity for IgG4 than IgG1 seen in ELISA. The ranking determined based on flow cytometry results correlated better with rankings found in literature while the ranking based on results from the ELISA set-up deviated for Fc γ RIIa^{131H} and Fc γ RIIb [6], [34]. The reason for these differences between the methods was most likely due to the aggregation of the soluble receptors used in ELISA. It could be assumed that the rankings based on flow cytometry results are more biologically relevant as the receptors were still cell-associated and not partly aggregated as in the sandwich ELISA. It has been reported that Fc γ Rs expressed by HEK and CHO cells have different glycosylation patterns and that this might affect the Fc γ RIIIa affinity for IgG [18]. It is known that the glycosylation pattern of Fc γ Rs differs between cells in the body [38] and the effect on affinity caused by the variation in glycosylation pattern between HEK and CHO cells was probably negligible in this assay format. Therefore this aspect was overlooked in the context of this study. The glycosylation of IgG, on the other hand, has larger effect on the affinity for Fc γ Rs. This factor was not analyzed in this work and could be a reason for unexpected inconsistencies regarding affinity rankings.

Table 4. Ranking of apparent IgG affinity for human and murine receptors as determined with ELISA and flow cytometry in this work. ≥, > and >> denotes 1-2, 2-5 and 5-10 times higher affinity respectively. >>> denotes more than 10 times higher affinity. Crossed over IgG denotes that no significant binding was detected. Signs between IgG and crossed over IgG indicate difference to background. A ranking from literature is included for comparison.

Receptor	ELISA	Flow cytometry	According to literature [34]*
hFcγRI	IgG1>IgG4>IgG2	IgG1>IgG4>>IgG2	IgG1>IgG4, IgG2
hFcγRIIIa ^{131H}	IgG2>IgG1>IgG4	IgG1>IgG2>>>IgG4	IgG1>IgG2>IgG4
hFcγRIIIa ^{131R}	IgG1=IgG2=IgG4	Not tested	IgG1>>IgG2=IgG4
hFcγRIIb	IgG4>IgG1>IgG2	IgG1=IgG4>>IgG2	IgG1=IgG4>IgG2
hFcγRIIIa ^{158V}	IgG1>>IgG4>IgG2	IgG1>>>IgG2, IgG4	IgG1>>IgG4>IgG2
hFcγRIIIa ^{158F}	IgG1>>IgG4=IgG2	IgG1>IgG2, IgG4	IgG1>>IgG4>IgG2
hFcγRIIIb-01	IgG1>>IgG2, IgG4	IgG1>>>IgG4, IgG2	IgG1, IgG2, IgG4
hFcγRIIIb-02	IgG1>>>IgG4>IgG2	IgG1>>>IgG2, IgG4	IgG1, IgG2, IgG4
mFcγRI	IgG4>IgG1>IgG2	Not tested	Unknown
mFcγRIV	IgG1>IgG4>IgG2	IgG1>IgG4>IgG2	Unknown

*Based on SPR measurements; Similar ranking was found in other literature references based on different types of methods.

The ELISA set-up appeared to be more sensitive than the flow cytometry set-up. The affinity for IgG2 and IgG4, for instance, was negligible for cell-bound FcγRIIIa and no binding of IgG4 to cell-bound FcγRIIIa^{131H} could be detected in contrast to the ELISA set-up where affinity of soluble FcγRIIIa for IgG2 and IgG4 and of soluble FcγRIIIa^{131H} for IgG4 was observed. Affinity rankings of murine FcγRs for human IgG subclasses were not found in the literature and therefore the ranking observed in this study could not be compared to any literature data. It is, however, known that all murine receptors bind human IgG1 [6], which was also seen for the soluble receptors used in this work (results shown for soluble mFcγRI and mFcγRIV, (Figure 15) and for cell-bound mFcγRIV (Figure 17)).

The main advantages of the flow cytometry based assay were that the receptors were cell-bound and in this way more reflected an *in vivo* state and that the assay required less reagents than the ELISA. However, the reproducibility was lower in the flow cytometry assay, which could be caused by the receptors being more or less affected by detachment of the cells. In addition the histogram peaks were in some cases not uniform giving another source of uncertainty. The flow cytometry assay required large amounts of cells, the throughput was intermediate and affinities could not be compared between receptors as the degree of expression varied from cell line to cell line. Affinities could, in theory, be compared between FcγRs by normalizing the signals to receptor expression, however, in practice this is difficult to perform. The main advantages of the ELISA set-up were the high throughput, good reproducibility and repeatability, high sensitivity and that all reagents could be prepared beforehand and kept in the freezer. The largest drawback of this set-up was the aggregated

receptors prohibiting comparison of affinity for the same IgG between receptors. Both assays suffered from the uncertainty of the size of IC having to be fairly equal for all tested antibodies to allow comparison of affinities against a specific receptor.

EC50 values for trastuzumab were determined from dose response curves for soluble and cell-bound FcγRs (Table 5). The EC50 values determined from the flow cytometry based assay were generally higher than those determined based on ELISA results, indicating lower affinity in the former set-up. This underlined that measured affinities highly depend on the selected method and the design of the assay set-up. The EC50 values calculated in this work from the ELISA results were in line with those found in literature derived from a similar ELISA set-up [28], but they indicated approximately 100-1000 times higher affinity than values determined from SPR experiments with monomeric IgG [34]. Binding of IC to FcγR detected using ELISA and flow cytometry is highly influenced by avidity. The higher apparent affinity determined based on ELISA measurements are therefore expected but pinpoints that the experimental set-up affects the results obtained.

Table 5. Mean EC50 values for trastuzumab determined from ELISA or flow cytometry experiments with 95% confidence interval. Literature values, included for comparison, for an IgG1 using a sandwich ELISA set-up similar to the one used in this study [28].

Receptor	EC50 (ng/ml) trastuzumab	EC50 (ng/ml) trastuzumab	EC50 (ng/ml) IgG1 according to literature
	flow cytometry	ELISA	ELISA
hFcγRI	200±70	-	8
hFcγRIIa ^{131H}	5000 ±400	860 ±250	1500
hFcγRIIa ^{131R}	-	190 ±40	-
hFcγRIIb	16000 ±1900	1400±200	6000
hFcγRIIIa ^{158V}	7200±2700	240±60	410
hFcγRIIIa ^{158F}	-	480±30	3000
hFcγRIIIb-01	-	2600±200	-
hFcγRIIIb-02	9200±500	6900±900	-

Ranking the receptors based on apparent affinity for trastuzumab did not give the same order with the ELISA results and the flow cytometry results (Table 6). The ranking of the receptors depends to some extent on the specific IgG1 tested and thus comparison with literature is not always fair but in general IgG1 is proposed to bind FcγRIIIa with a higher affinity than FcγRIIa. FcγRIIb and FcγRIIIb are expected to bind IgG1 with similar affinity, lower than the other receptors [28], [34]. Even though the deviations from the expected ranking are small they are large enough to prohibit a reliable ranking of receptor affinity for a specific IgG. However, the assays can be used to compare IgG binding in relative terms to determine if one IgG binds better than the other to one receptor at the time (provided that the ICs are of similar aggregation).

Table 6. Ranking of human FcγR affinity for human IgG1 according to the ELISA and flow cytometry set-ups in this work (using trastuzumab) and a SPR set-up from literature [34].

Study	Order of receptor affinity
Flow cytometry*	FcγRI > FcγRIIa ^{131H} > FcγRIIIa > FcγRIIIb-02 > FcγRIIb
ELISA**	FcγRIIa ^{131R} > FcγRIIIa > FcγRIIa ^{131H} > FcγRIIb > FcγRIIIb
SPR	FcγRI > FcγRIIa ^{131H} > FcγRIIa ^{131R} > FcγRIIIa > FcγRIIIb > FcγRIIb

* EC50 for FcγRII^{131R} was not determined, ** EC50 for FcγRI was not determined.

3.5 Antibody screening

The overall goal of the project was to design an antigen-independent affinity assay for screening of antibodies. To test the sandwich ELISA and flow cytometry based set-ups, developed in this study, the apparent affinity of human FcγRI, FcγRIIa^{131H}, FcγRIIb, FcγRIIIa^{158F} and FcγRIIIb-02 for 12 different n-CoDeR[®] IgG1 antibodies, x and z1-11, was analyzed. The ICs generated with the different antibodies showed similar aggregation pattern in SEC analysis (see Appendix D for chromatograms). IgG with different apparent affinity could be observed (Appendix E). As noted above the maximal signal was not the same for all curves, meaning that normalization of the curves for comparison of affinity was performed (not shown). Not all curves reached a plateau giving some uncertainty and bias in the affinity ranking. In general, the apparent affinity was relatively high for n-CoDeR[®] z8, z9 and z10 and relatively low for n-CoDeR[®] z3, z5 and z6. The ranking of IgG affinity was not the same for all FcγRs indicating receptor-specific affinity. n-CoDeR[®] z2 was a clear example of this. FcγRI showed relatively high apparent affinity for n-CoDeR[®] z2 while FcγRIIIa^{158F} showed intermediate apparent affinity and the other receptors relatively low apparent affinity. Another example was the order in affinity of FcγRIIb and FcγRIIIa^{158F} for n-CoDeR[®] z4 and z8 where the former receptor showed higher apparent affinity for n-CoDeR[®] z8 than z4 while the affinity of FcγRIIIa^{158F} appeared to be in the opposite order (Figure 18).

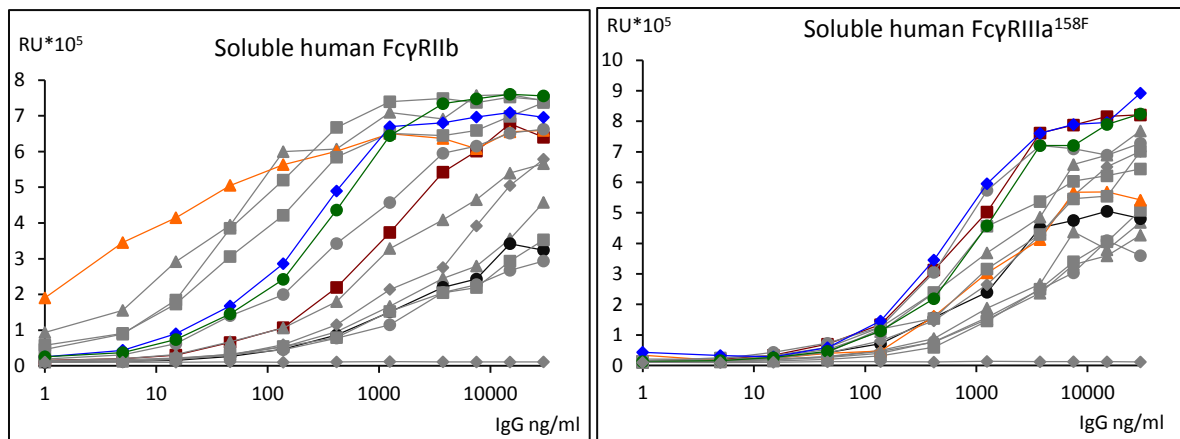


Figure 18. Receptor-specific affinities were detected with both assay set-ups developed in this project. Soluble FcγRIIb bound n-CoDeR[®] z8 with a higher apparent affinity than n-CoDeR[®] z4 while the order of apparent affinity of FcγRIIIa^{158F} for the same antibodies is the other way around. Cell-bound FcγRI showed relatively high apparent affinity for n-CoDeR[®] z2 for which soluble FcγRIIIa^{158F} showed intermediate apparent affinity and soluble FcγRIIb showed relatively low apparent affinity. (●) rituximab, (◆) trastuzumab, (●) n-CoDeR[®] z2, (■) n-CoDeR[®] z4, (▲) n-CoDeR[®] z8. Gray figures denote other n-CoDeR[®] antibodies (see appendix E).

The ranking correlated well between the two assays for FcγRI, FcγRIIb and FcγRIIIa^{158F}, but not for FcγRIIb-02 and FcγRIIIa^{131H}. IgG with relatively high or low apparent affinity for the different receptors could be identified. The EC₅₀ values determined from ELISA and flow cytometry results were within the same range (results not shown), in contrast to the lower values determined for trastuzumab in ELISA compared to flow cytometry (Table 5).

4 Concluding remarks and future prospects

IgGs are important molecules both for our health and in economic terms. Vast amounts of money are annually spent on research, development, production and distribution of therapeutic antibodies. Different strategies are used to increase the efficiency of therapeutic antibodies. As the Fc-fragment stands for most of the anti-inflammatory activity and ADCC effect of a therapeutic antibody, optimizing the Fc-fragment regarding binding properties plays a large role for the efficiency of the antibody. One often uses inter-species cross-reactive antibodies for *in vivo* studies in animals prior to clinical tests. It is of value to gain knowledge in variations in affinity for FcγRs from different species before entering *in vivo* and clinical studies. In this study, two antigen-independent affinity assays were set up, one based on ELISA and one on flow cytometry. The study focused on the affinity of mouse and human FcγRs for human IgG. By expressing soluble FcγRs and/or generating semi-stable cell

lines expressing FcγRs of other types the assay could be adopted to include any other species. It is very difficult to model *in vivo* situations with *in vitro* systems. The total cellular response upon IgG binding to FcγRs depends on a vast number of parameters such as level of expression, ratios between activating and inhibiting FcγRs, cell-type and general status of the individual. This complexity, and lack of correlation between species, has to be taken into account when designing *in vitro* and *in vivo* experiments and when interpreting the biological relevance of the results.

The two affinity assays designed in this work could both, with ease, be used to screen more than 24 IgGs and rank them based on the affinity for FcγRs in an antigen-independent manner. The rankings observed in this study were in general in accordance with the consensus of rankings found in literature. Differences in IgG ranking between the ELISA and flow cytometry assays were limited to a few receptors as noted in table 4. The assay set-ups can in the current state only be used to obtain relative affinities and comparisons are restricted to one receptor at the time. This is, however, sufficient in order to confirm expected binding and identify IgGs with atypical binding to FcγRs. The results obtained from the affinity measurements can be used to facilitate the interpretation of results from functional assays, *in vitro* and *in vivo*, as well as being a quality control for IgGs produced at different times or under different conditions. Despite the high similarity in amino acid sequence between the different n-CoDeR[®] antibodies used in this work differences in apparent affinity for FcγRs could be detected. Observed differences in affinity may be caused by variations in amino acid sequence, IgG glycoprofile or aggregation state of the IC. Screening of antibodies against FcγRs ought to be combined with analysis of the IgG glycoprofile and the aggregation state of the IC to deduce possible correlations between these variables and the receptor affinity.

Optimizing the FcγR binding of an IgG has been shown to be a possible way to enhance the desired effect of a therapeutic antibody. Several examples exist where this has been proven successful *in vitro* [27]. There is though, neither *in vitro* nor *in vivo*, any guarantee that a higher affinity for the activating receptors leads to increased cellular response or that a higher affinity for the inhibiting receptor leads to decreased cellular response [16]. The total immunological response, *in vivo*, is a sum of many factors and FcγR binding is just one factor affecting the final response. Results from the affinity assay might be used to indicate mode of action of an antibody. If for example one antibody has a high ADCC effect, a concomitant high affinity for FcγRIIIa could indicate that the ADCC is caused by IgG binding to FcγRIIIa.

A possible continuation of the assay development would be to set up an assay based on SPR, as such assays have been found to be complementary to flow cytometry and ELISA [39] and also gives kinetic data. Using SPR the uncertainty with the aggregation state of the ICs can be circumvented since binding of monomeric IgG to the low-affinity receptors can be measured. Obtaining high throughput using SPR is not as straight forward as for ELISA, but SPR could be a way to verify ELISA or flow cytometry results for a few selected clones.

Optimization of expression of soluble receptor as well as purification and formulation would be of value in order to obtain less aggregated receptors. Purification based on affinity for IgG could be a way to enrich functional receptors and thereby obtain a better starting material.

5 Acknowledgments

This master thesis project has been a journey where I have encountered many people, methods and not to forget many receptors. Luckily I have been provided with good guides during this journey. Starting with Björn Frendeus guiding me in to BioInvent and Mikael Matsson who dared to take on the main responsibility to Kerstin Mårtensson, Annika Sundberg and Anna Särnefält who have followed me through all the project meetings and all ups and downs. Many other persons have helped and supported me during the time at BioInvent. For this I am truly thankful. With the risk of someone feeling forgotten I want to say a special thanks to Eva Erlandsson, Sidonie Karlsson, Eva Nyblom and Torbjörn Schiött and of course all other members of SES, KOMB and KOCP.

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Appendix A: Antibodies and antibody fragments used in this study

Antibody	Host/Format	Supplier	Immunogen	Application
PE-anti-human CD64	Mouse/IgG	BD Bioscience #558592	Human FcγRI	Flow cytometry
PE-isotype mouse IgG2b	Mouse/IgG	BD Bioscience #555058	Dansyl	Flow cytometry isotype control
APC-anti-human CD32	Mouse/IgG	BD Bioscience #559769	Human FcγRII	Flow cytometry and FMAT
APC-isotype mouse IgG1k	Mouse/IgG	BD Bioscience #555749	Dansyl	Flow cytometry isotype control
PE-Cy5-anti-human CD16	Mouse/IgG	BD Bioscience #555408	Human FcγRIII	Flow cytometry
PE-Cy5-isotype mouse IgG2b	Mouse/IgG	BD Bioscience #555744	Dansyl	Flow cytometry isotype control
anti-human CD64	Mouse/IgG	BD Bioscience #555525	Human FcγRI	FMAT and ELISA
anti-human CD32	Mouse/IgG	AbD Serotec #MCA 1075xz	Human FcγRII	FMAT and ELISA
anti-human CD16	Mouse/IgG	BD Bioscience #550383	Human FcγRIII	FMAT and ELISA
Alexa fluor 647 anti-mouse CD64	Mouse/IgG	BD Bioscience #558539	Mouse FcγRI	Flow cytometry, FMAT and ELISA
Alexa fluor 647 isotype mouse IgG1 k	Mouse/IgG	BD Bioscience #557714	Unknown	Flow cytometry isotype control
PE-anti-mouse FcγRII/III	Rat/IgG	R&D Systems #FAB1460P	Mouse FcγRII/III	Flow cytometry
PE-isotype rat IgG2b	Rat/IgG	R&D Systems #ICO13P	Unknown	Flow cytometry isotype control
APC-anti-human IgG	Goat/IgG	Jackson ImmunoResearch #109136088	Human IgG	Flow cytometry
APC-anti-mouse IgG	Goat/F(ab') ₂	Jackson ImmunoResearch #115136146	Mouse IgG	FMAT
HRP-anti-human F(ab') ₂	Goat/F(ab') ₂	Jackson ImmunoResearch #109036006	Human F(ab') ₂	ELISA and Immune complex
HRP-anti-mouse IgG	Donkey/IgG	Jackson ImmunoResearch #715035151	Mouse IgG	ELISA
HRP-anti-rat IgG	Donkey/IgG	Jackson ImmunoResearch #712036153	Rat IgG	ELISA
Anti-mouse CD32/CD16	Rat/IgG	R&D Systems #MAB1460	Mouse FcγRII/III	FMAT and ELISA
Anti-mouse Fcγ	Goat/F(ab') ₂	Jackson ImmunoResearch #115006071	Mouse Fcγ	ELISA
HRP-anti-mouse Fcγ 3	Goat/F(ab') ₂	Jackson ImmunoResearch #115035209	Mouse Fcγ	ELISA
Biotin-SP-anti-human F(ab') ₂	Goat/F(ab') ₂	Jackson ImmunoResearch #109066097	Human F(ab') ₂	ELISA
Anti-human F(ab') ₂	Goat/F(ab') ₂	Jackson ImmunoResearch #109006006	Human F(ab') ₂	Immune complex
Anti-human λ light chain	Goat/F(ab') ₂	AbD Serotec #STAR182	Human λ light chain	Immune complex
FITC-anti-human F(ab') ₂	Goat/F(ab') ₂	Jackson ImmunoResearch #109096097	Human F(ab') ₂	Immune complex
Avastin (bevacizumab)	Humanized/IgG	Roche	VEGF-A	Affinity assay
Herceptin (trastuzumab)	Humanized/IgG	Roche	HER2	Affinity assay
Mabthera (rituximab)	Human-Mouse/IgG	Roche	Human-CD20	Affinity assay
n-CoDeR® x	Human/IgG1, IgG2, and IgG4	BioInvent International	x	Affinity assay
n-CoDeR® y	Human/IgG1 and IgG1-N297Q	BioInvent International	y	Affinity assay
n-CoDeR® z 1-11	Human/IgG1	BioInvent International	z	Affinity assay

Appendix B: Amino acid sequences of FcγRs

Table B1. Amino acid sequences of human receptors and the human gamma chain used in this study and Uniprot accession number of corresponding protein. Sequences were verified by DNA sequencing at BioInvent. Bold residues denote the residues differing between the full length form and the ECD. Residues underlined in parenthesis indicate functionally relevant polymorphisms.

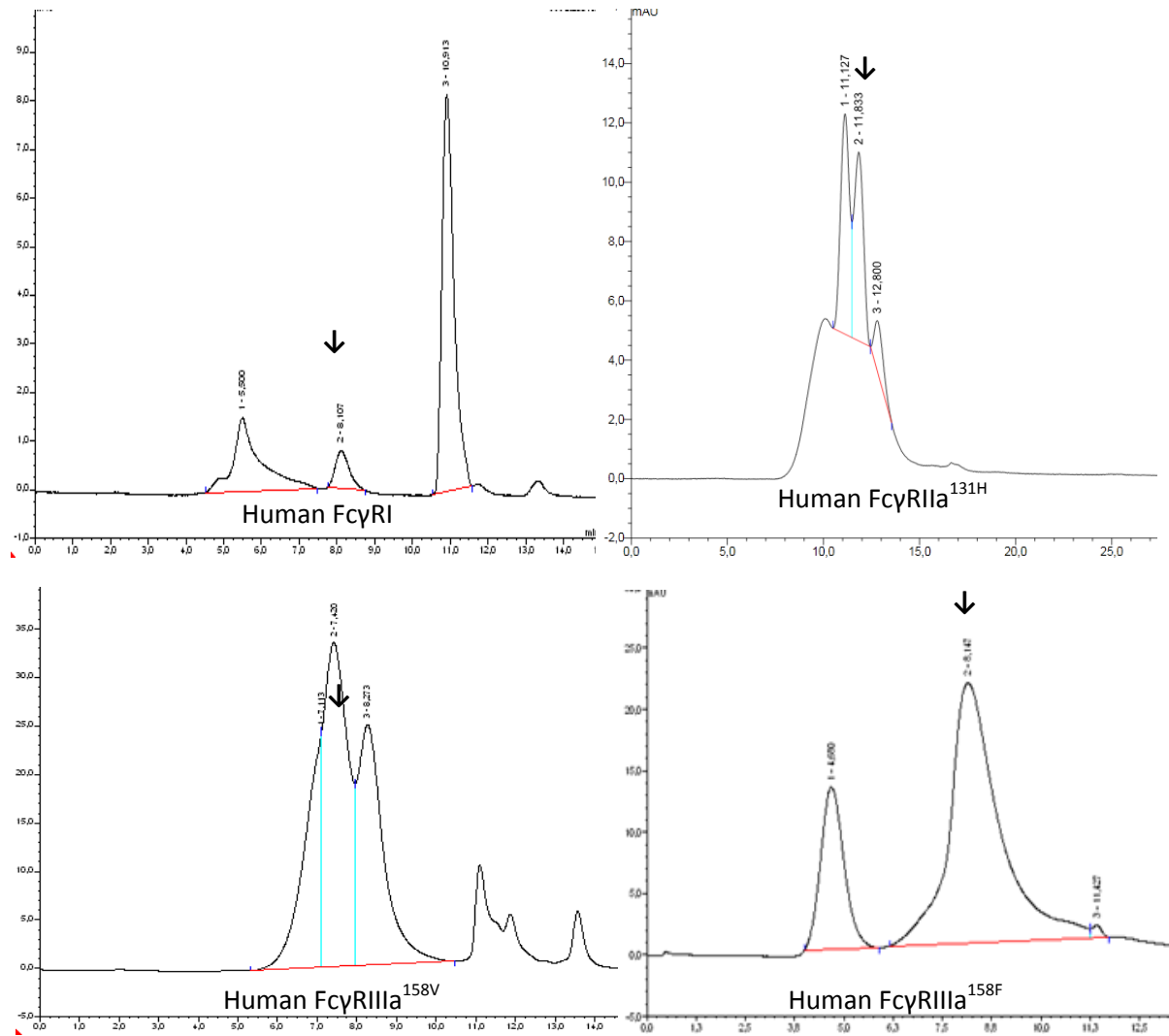
Protein	Uniprot accession	Comment	Amino acid sequence
Human FcγRI	P12314		MWFLTLLLVVVDGQVDTTKAVITLQPPWVSVFQEETVTLHCEVLHLP GSSSTQWFLNGTATQTSTPSYRITSASVNDSGEYRCQRGLSGRSDPIQLEI HRGWLLQVSSRVFTEGEPLALRCHAWKDKLVYVNVLYRNGKAFKFFHW NSNLTKTNISHNGTYHCSGMGKHRYTSAGISVTVKELFPAPVLNASVTSP LLEGNLVTLSCKETLLLQRPGLQLYFSFYMGSKTLRGRNTSSEYQILTARRED SGLYWCEAATEDGNVLRKRSPELELQVLG LQLPTPVWFHVLVFLYLA VGIMFL VNTVLWVTIRKELRKKKWDLEISLDSGHEKKVISSLQEDRHLEELKQCE QKEEQIQEGVHRKEPQGAT
Human FcγRIIa-131H/R	P12318	FcγRIIa ^{131R} was available as purified protein and semi-stable cell line at the start of this study	METQMSQNVCPRNWLLQPLTVLLLASADSQAAAPPKAVLKLEPPWIN VLQEDSVTLTCCGARSPESDSIQWFHNGNLIPTHTQPSYRFKANNNDSGE YTCQTGQTSLSDPVHLTVLSEWLVLQTPHLEFQEGETIMLRCHSWKDKPL VKVTFQNGKSKQFS(H/R)LDPTFSIPQANHSHSGDYHCTGNIGYTLFSSK PVTITVQVPSMGSSSPMGII VAVVIATAVA AAVA AVVALIYCRKKRISANS TDPVKA QFEP GRQMIAIRKRQLEETNNDYETADGGYMTLNPRAPT DDKNIYLTLPNDHVSNN
Human FcγRIIb-232I/T	P31994	FcγRIIb ^{232I} was available as purified protein and semi-stable cell line at the start of this study	MGILSFLPVLATESDWADCKSPQPWGHMLLWTAFLVFLAPVAGTPAAPPK AVLKLEPQWINVLQEDSVTLTCRGTHSPESDSIQWFHNGNLIPTHTQPSYR FKANNNDSGEYTCQTGQTSLSDPVHLTVLSEWLVLQTPHLEFQEGETIVLR CHSWKDKPLVKVTFQNGKSKKFSRSDPNFSIPQANHSHSGDYHCTGNIG YTYLSSKPVTTITVQAPSSSPMGII VAVVTG(I/T) AVAA IAAVVALIYCRKKR ISALPGY PECREMGETLPEK PANPTNPDEADK VG AENTIT YSLLMHPDAL EEDDQ NR I
Human FcγRIIIa-158V/F	P08637		MWQLLLPTALLLVSAGMRTEDLPKAVVFLPQWYRVLEKDSVTLKCG AYSPEDNSTQWFHNESLISSQASSYFIDAATVDDSGEYRCQTNLSTLSDPV QLEVHIGWLLQAPRWVFKEDPIHLRCHSWKNTALHKVTYLQNGKGRK YFHHNSDFYIPKATLKDSGSYFCRGL(V/F)GSKNVSSETVNITITQ GLAVSTI SSFFPPGYQVSFCLVMVLLFAVD TGLYFSVKT NIR SSTRD WKD HKFKWR KDPQDK
Human FcγRIIIb-NA1/NA2	O75015	In this study denoted FcγRIIIb-01/02	MWQLLLPTALLLVSAGMRTEDLPKAVVFLPQWY(R/S)VLEKDSVTLKC QGAYSPEDNSTQWFH(N/S)ENLISSQASSYFIDAATV(D/N)DSGEYRCQT NLSTLSDPVQLEVH(V/I)GWLLQAPRWVFKEDPIHLRCHSWKNTALHK VTYLQNGKDRKYFHHNSDFHIPKATLKDSGSYFCRGLVGSKNVSSETVNITI TQ GLAVSTISSFPPGYQVSFCLVMVLLFAVD TGLYFSVKTNI
Human gamma chain	P30273		MIPAVVLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTLLYCRLKIQRKAAI TSYEKSDGVYTLSTRNQETYETLKHEKPPQ

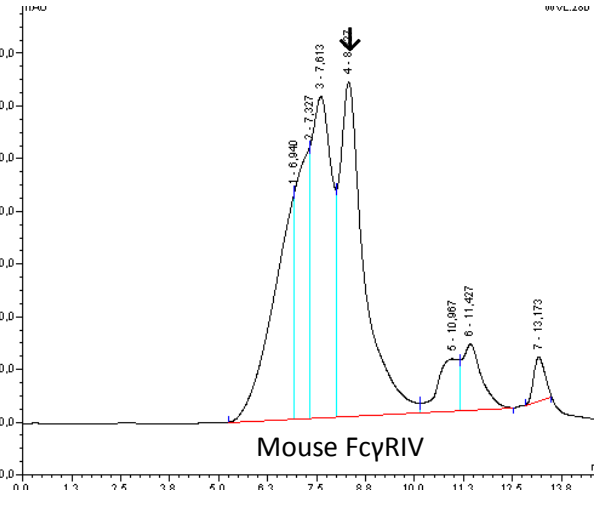
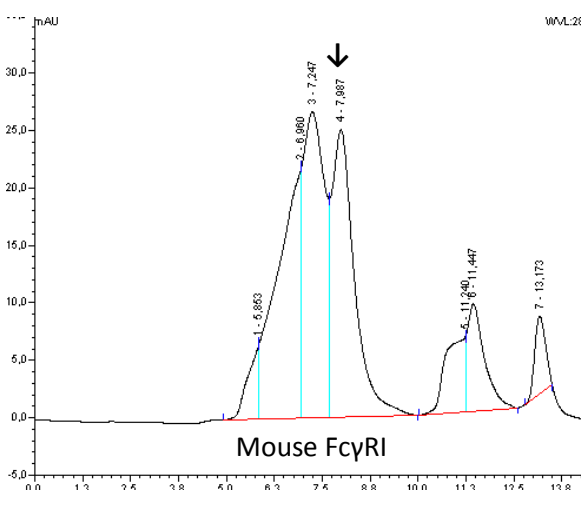
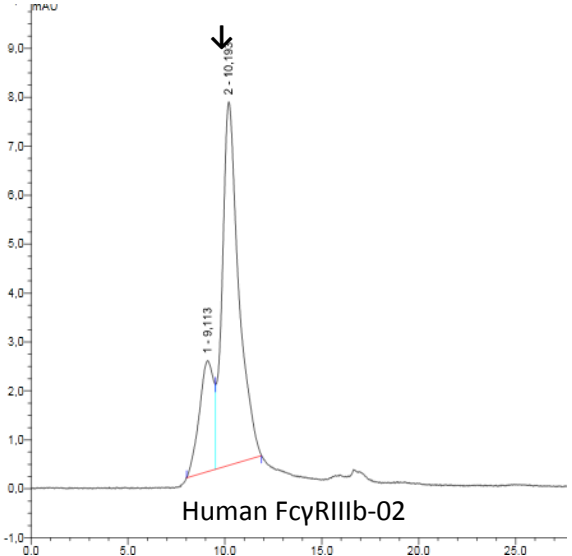
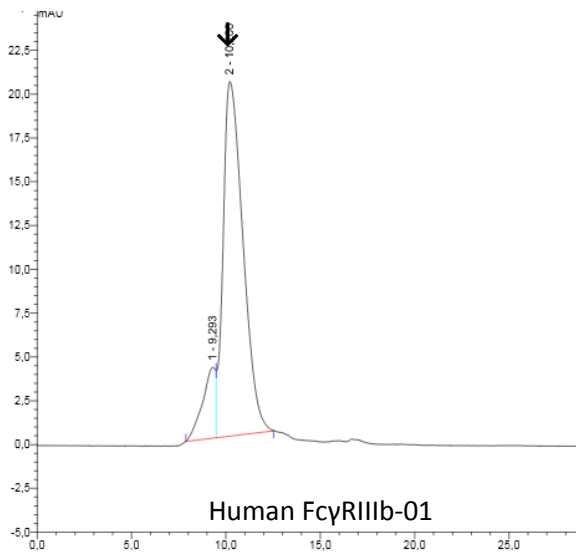
Table B2. Amino acid sequences of murine receptors and the murine gamma chain used in this study and Uniprot accession number of corresponding protein. Sequences were verified by DNA sequencing at BioInvent. Bold residues denote the residues differing between the full length form and the ECD.

Protein	Uniprot accession	Comment	Amino acid sequence
Murine FcγRI	P26151		MILTSFGDDMWLLTLLLVVPVGGVEVNATKAVITLQPPW VSIFQKENVTLWCEGPHLPGDSSTQWFINGTAVQISTPSYSI PEASFQDSGEYRCQIGSSMPSDPVQLQIHNDWLLQASRR VLTEGEPLALRCHGWKNKLVYNVVFYRNGKSFQFSSDSEVA ILKTNLSHSGIYHCSGTGRHRYTSAGVSITVKELFITPVLRASV SSPFPEGSLVTLNCETNLLLQRPGLQLHFSFYVGSKILEYRNTS SEYHIARAEREDAGFYWCEVATEDSSVLKRSPELELQVL GPQ SSAPVWFHILFYLSVGIMFSLNTVLYVKIHRLQREKKYNLEV PLVSEQGKKANSFQQVRSDDGVYEEVTATASQTTTPKEAPD GPRSSVGDGCGPEQPEPLPPSDSTGAQTSQS
Murine FcγRIII	Q7TMW9		MFQNAHSGSQWLLPPLTILLFAFADRQSAALPKAVVKLDP PWIQVLKEDMVTLMCEGTHNPGNSSTQWFHNWSSIRSQ VQSSYTFKATVNDSGEYRCQMEQTRLSDPVDLGVISDWLLL QTPQRFLEGETITLRCPSWRNKLLNRISFFHNEKSVRYHHY KSNFSIPKANHSHSGDYCKGSLGSTQHQS KPVTTITVQDPAT TSSISLVWHHTAFSLVMCLLFAVD TGLYFYVRRNLQTPRDY WRKLSIRKHKQAPQDK
Murine FcγRII	P08101	Was available as plasmid and semi-stable cell line at the start of this study.	MESNWTVHVFSRTLCHMILLWTAVLNLAAGTHDLPKAVVK LEPPWIQVLKEDVTLTCEGTHNPGNSSTQWFHNWSSIRSQ VQASYTFKATVNDSGEYRCQMEQTRLSDPVDLGVISDWLLL QTPQLVLEGETITLRCHSWRNKLLNRISFFHNEKSVRYHHY SSNFSIPKANHSHSGDYCKGSLGRTQHQS KTVTTITVQGPKS SRSLP VLTIVA AVTGI AVA AIV IILVSLVYLK KQVPALGPNP DHREMGETLPEEVGEYRQPSGGSPVSPGPPSGLEPTSSS PYNPPDLEEA AKTEA ENTITYSLLKHPEALDEETEHDYQNH I
Murine FcγRIV	Q8R2R4	Was available as plasmid and semi-stable cell line at the start of this study.	MWQLLLPTALVLTAFSGIQAGLQKAVVNLDPKWVRVLEED SVTLRCQGTFSPEDNSIKWFHNESLIPHQDANYVIQSARVK DSGMYRCQTALSTISDPVQLEVHMGWLLLQTTKWLFQEG DPIHLRCHSWQNRPVKVTYSQNGKGGKKYFHENSELLIPKA THNDSGSYFCRGLIGHNNKSSASFRISLGDPGSPSMFPPWH QITFCLLIGLLFAIDTVLYFSVRRGLQSPVADYE EPKIQWSKE PQDK
Murine gamma chain	P20491	Was available as plasmid at the start of this study.	MISAVILFLLLVEQAAAALGEPQLCYILDAVLFYGLVLTLLYCR LKIQRKAAIASREKADAVYTGLNTRSQETYETLKHEKPPQ

Appendix C: SEC chromatograms of soluble FcγR

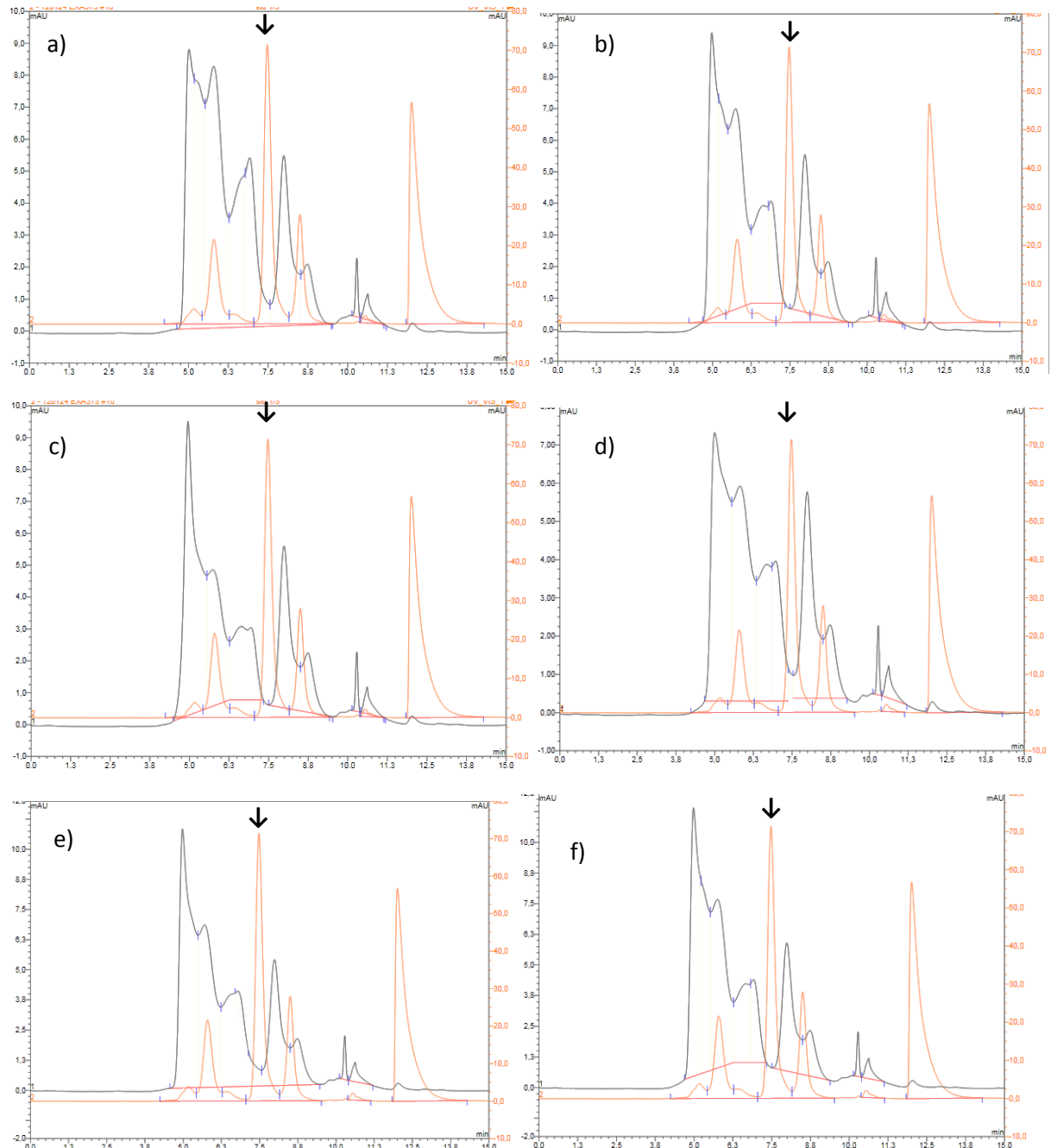
SEC chromatograms of soluble FcγR produced in this work and used in the affinity assays, including FcγRI not used for affinity assays. ↓ indicating peaks corresponding to non-aggregated receptor. The fraction of non-aggregated ECD corresponds to approximately 20, 42, 88, 80, 50, 77, 40 and 47% for hFcγRI, hFcγRIIa^{131H}, hFcγRIIIa^{158V}, hFcγRIIIa^{158F}, hFcγRIIIb-01, hFcγRIIIb-02, mFcγRI and mFcγRIV respectively. hFcγRIIa^{131R} and hFcγRIIb were produced previously and therefore not included.

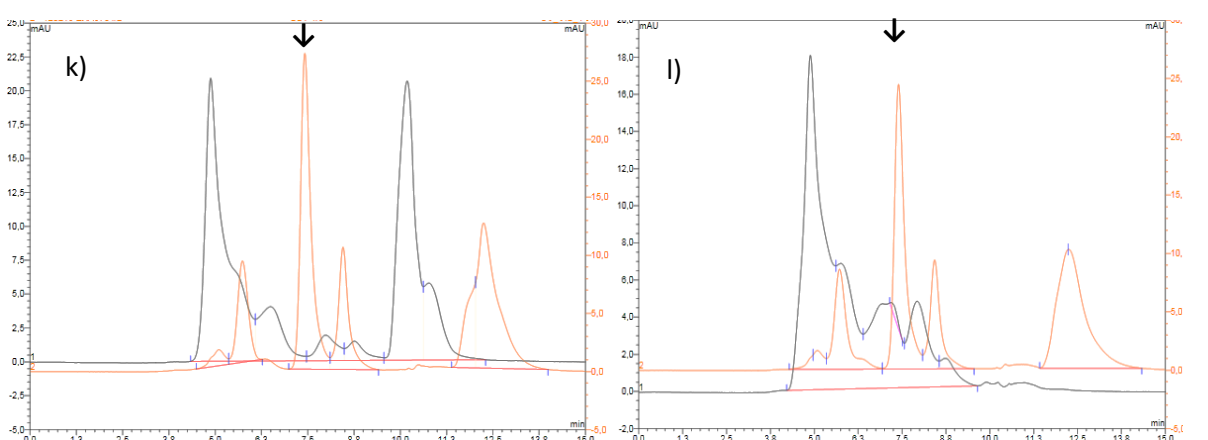
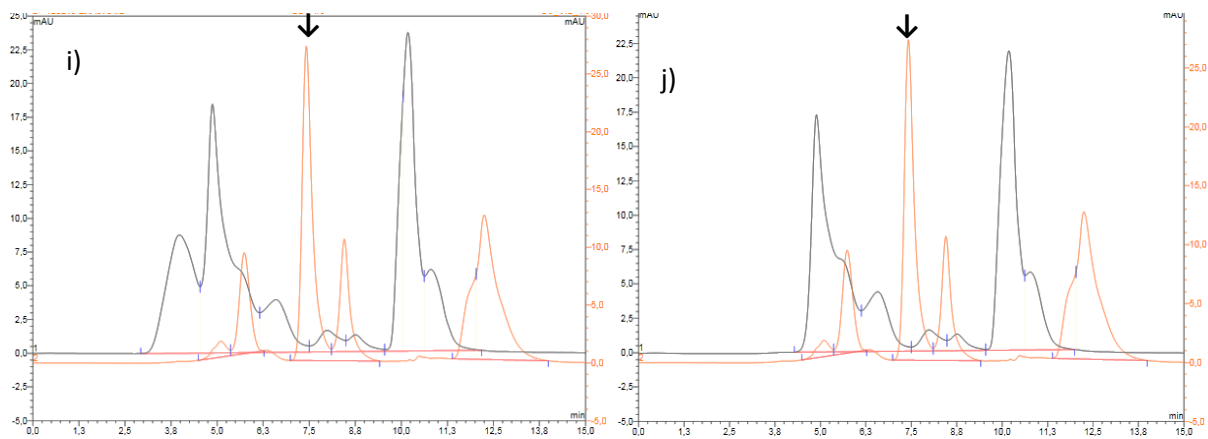
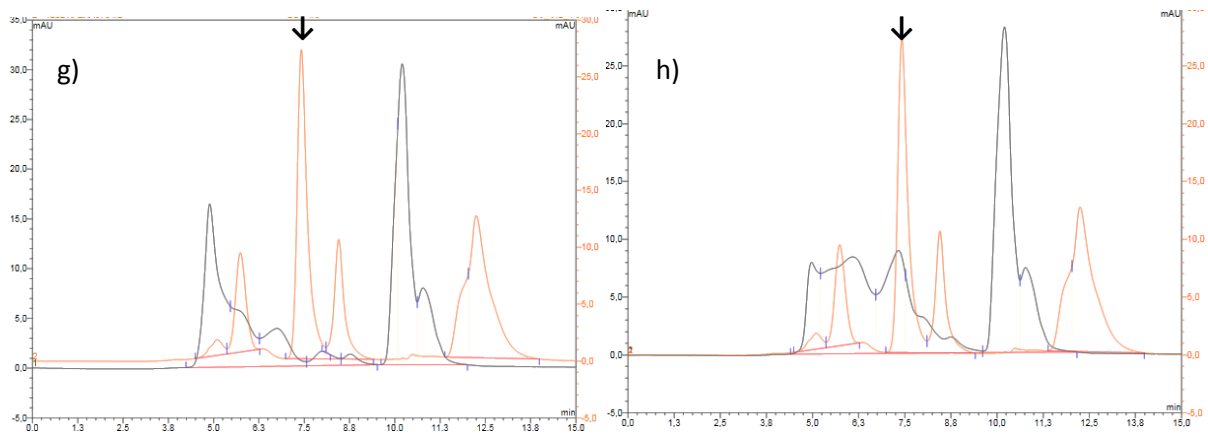




Appendix D: SEC Chromatograms of n-CoDeR® ICs

SEC chromatograms of ICs of (—) n-CoDeR® antibodies used in affinity assays as overlay with (—) standard (↓)150 kDa; a) n-CoDeR® z1, b) n-CoDeR® z2, c) n-CoDeR® z3, d) n-CoDeR® z4, e) n-CoDeR® z5, f) n-CoDeR® z6, g) n-CoDeR® z7, h) n-CoDeR® z8, i) n-CoDeR® z9, j) n-CoDeR® z10, k) n-CoDeR® z11, l) n-CoDeR® xIgG1. Peaks at approximately 10 min are buffer associated and do not relate to the ICs.





Appendix E: Antibody screening

Graphs showing affinity measurements of human soluble and cell-bound FcγRs for n-CoDeR[®] z1-11, nCoDeR[®] x, trastuzumab and rituximab in the sandwich ELISA set-up and flow cytometry based assay. Fc-specific and receptor-specific affinities could be detected for all FcγRs. The ranking of the IgG affinity correlated well between the two assay set-ups for FcγRI, FcγRIIb, FcγRIIIa^{158F} but not for FcγRIIIa^{131H} and FcγRIIIb-02. The high affinity receptor, FcγRI was incubated with monomeric IgG titrated from 5 μg/ml. The other receptors were incubated with IC titrated from 30 μg/ml in the ELISA set-up and from 25 μg/ml in the flow cytometry set-up. Samples are; (●) rituximab, (◆) trastuzumab, (▲) n-CoDeR[®] z1, (●) n-CoDeR[®] z2, (◆) n-CoDeR[®] z3, (■) n-CoDeR[®] z4, (□) n-CoDeR[®] z5, (○) n-CoDeR[®] z6, (▲) n-CoDeR[®] z7, (▲) n-CoDeR[®] z8, (■) n-CoDeR[®] z9, (●) n-CoDeR[®] z10, (■) n-CoDeR[®] z11, (▲) n-CoDeR[®] x, (◆) deglycosylated trastuzumab. In graphs to the right all antibodies except rituximab, trastuzumab, n-CoDeR[®] z2, n-CoDeR[®] z4 and n-CoDeR[®] z8 are colored gray.

